ABSTRACT

Title: **ECOLOGY AND MOLECULAR EPIDEMIOLOGY OF AVIAN AND SWINE INFLUENZA A VIRUSES IN GUATEMALA**

Ana Silvia Gonzalez Reiche, Ph.D., 2015

Directed By: **Professor Dr. Daniel R. Perez, Department of Veterinary Medicine**

The greatest diversity of Influenza A viruses (IAV) is found in waterfowl species from numerous geographic locations. In addition, multiple IAV are, and continue to be, perpetuated in swine populations around the globe. Due to the zoonotic potential of IAV, and to respond more effectively to potential agricultural and public health threats, there is a need to increase surveillance in avian and swine hosts in understudied geographical regions. In Latin America, avian influenza surveillance has been scarce, localized only to places where outbreaks in poultry have occurred. Similarly, active swine influenza surveillance was implemented only after the emergence of the 2009 pandemic strain (pH1N1). The main objective of the project presented here was to investigate the circulation of IAV in wild birds and pigs in Guatemala. Over 2200 birds were sampled during six consecutive migration seasons from 2007 to 2013 in different locations. Virus prevalence detected by rRT-PCR in positive species ranged from 5.2% to 38%. Preliminary data indicates temporal variation of IAV prevalence in migratory waterfowl. Eighty-three viruses were recovered with 22 different subtype combinations. Through

phylogenetic inferences and the analysis of virus genotypes and gene constellations of 60 fully sequenced genomes, we provide a detailed description of the genetic structure of avian IAV circulating in Guatemala. Our results suggest that the virus diversity in this location is sourced from multiple migration flyways from North America. Overlap of these flyways, in a natural geographical bottleneck such as the Neotropics, may contribute to the patterns of extensive genetic reassortment observed at a continental scale.

In addition, the results from two nationwide multistage random surveys in pigs demonstrated circulation of swine influenza in commercial and peridomestic herds in Guatemala. Herd prevalence of IAV was 36.3% in 2010 and 34.6% in 2011. Viruses of the H1N1 and H3N2 subtypes and antibodies against viruses of distinct genetic lineages of these subtypes were detected. Our results indicate that human-animal contact likely plays a role in the IAV epidemiology in local swine populations. The findings from this research constitute the most abundant data on the ecology and epidemiology of animal influenza currently available for Central America.

ECOLOGY AND MOLECULAR EPIDEMIOLOGY OF AVIAN AND SWINE INFLUENZA A VIRUSES IN GUATEMALA

by

Ana Silvia Gonzalez Reiche

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Advisory Committee:

Professor Daniel R. Perez, Chair
Professor Jeffrey DeStefano
Associate Professor Najib El-Sayed
Assistant Professor George Belov
Dr. John Patton

Dedication

To my grandmothers, Berta y Magdalena, for setting the example and show me to not limit my self, and encourage self-cultivation as part of pursuing freedom and happiness.

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List of Abbreviations

AIV	avian influenza virus
BIC	Bayesian information criterion
CPE	cytophathic effect
ECE	embryonated chicken eggs
ELISA	enzyme linked immunosorbent assay
GTR	general time reversible
HA	hemagglutinin
HI assay	hemmaglutination inhibition assay
HKY	Hasegawa-Kishino-Yaho
HPAI	highly pathogenic avian influenza
IAV	Influenza A virus
IRD	influenza research database
LPAI	low pathogenicity avian influenza
MDCK	Madin Darby canine kidney
ML	maximum likelihood
NA	neuraminidase
PPU	pig production unit
rRT-PCR	real-time reverse-transcriptase polymerase chain reaction
TN93	Tajima-Nei 93
TVM	transversion model
UVG	Universidad del Valle de Guatemala

Chapter 1. Introduction

1.1. Literature review: Influenza A virus

1.1.1. Classification

Influenza A viruses (IAV) belong to the virus family *Orthomyxoviridae*, that comprises 6 genera: Influenzavirus A, B and C, Isavirus, Quaranjavirus and Thogotovirus (1). All members of this family have negative-sense, single-strand RNA viruses with a segmented genome (between six to eight segments depending on the virus genera) (2).

IAVs are further classified into subtypes based on the antigenic properties of their surface proteins Hemagglutinin (HA) and Neuraminidase (NA) with amino acid sequence divergences of ≥30% among different subtypes (3, 4). IAVs belonging to 16 HA and 9 NA different subtypes have been isolated from birds and other animal hosts (5). More recently, two novel subtypes, H17N10 and H18N11 were detected in bats (6, 7). The nomenclature of the influenzavirus genera is based according to their type, the host species origin, the geographical location, a sample or isolate identification number, the year when the sample was obtained and, in the case of IAVs, the HA and NA subtypes (3). For example, A/Victoria/361/2011 (H3N2) would be a human influenza A virus isolated in Victoria, Australia in 2011 of the H3N2 subtype, and A/mallard/Gurjev/263/82 (H14N5) would be a virus strain recovered from a mallard duck in Gurjev, Rusia in 1982 of the H14N5 subtype.

1.1.2. Virus structure

IAVs are enveloped viruses enclosed in a host-derived lipid membrane. Virus particles vary in morphology and can be spherical and/or filamentous. Spherical particles are typically 100 nm in diameter and filamentous particles may be up to 300 nm in length. The lipid membrane displays the two surface proteins spikes, HA and NA (8). Underneath the lipid membrane, the virion is constituted of matrix protein (M1) and the viral ribonucleoprotein complexes (vRNPs) in which each viral RNA segment is looped and wrapped around multiple units of nucleoprotein (NP) in a rod-like structure (9). The end of each one vRNP is bound to a single polymerase complex comprised of polymerase basic 1 and 2 (PB1, PB2) and polymerase acid (PA) protein subunits. vRNPs incorporation into virions seem to follow a selective packaging mechanism during virus assembly that ensures that most virions obtain one set of all 8 gene segments (10) (explained below). The exact arrangement of the vRNPs inside the virus particle is still a controversial subject. The most recent model based on electron microscopy studies suggests that the distribution in space inside the virion of individual vRNPs may vary, where some will have the polymerase-binding ends oriented towards the budding tip of the budding virions, and others will be inversely oriented at the opposite side at the bottom of the budding virus (11).

1.1.3. Genome structure

The genome of IAV viruses is ~13.6 kb, fragmented into eight negative-sense single stranded RNA segments (8). The viral RNA segments are conventionally numbered in order of their decreasing length (Table 1). The linear coding sequence of

each RNA segment is flanked by segment-specific untranslated regions (UTR) of variable length. The terminal ends of the UTR of all gene segments share conserved sequences partially complementary between the 5' and 3' ends. These regions are important for the formation of the vRNP complex and packaging of RNA segments into virions during virus assembly, and also as regulatory *cis*-elements for vRNA, mRNA and cRNA synthesis during virus replication (8). The genome codes for 11 to 12 proteins; segments 1, 4, 5 and 6 encode one protein per segment and the remaining segments produce two proteins each from different open reading frames (ORF) using multiple mechanisms. Segment 2, encodes for PB1 and a second protein, PB1-F2 from the overlapping +1 ORF (12). Segment 3, encodes for PA and PA-X through frameshifting from the PA ORF (13). Segments 7 (matrix protein and M2) and 8 (non-structural protein 1 and nuclear export protein) express their secondary products by using an alternative splicing mechanism (14). In addition, other viral protein products have been recently discovered, although their functions remain to be further elucidated; such proteins include PB1-N40, PA-N155, PA-N182, M42 and NS3 (13, 15-19). The role of the most-well studied proteins is explained in the following section.

1.1.4. Viral proteins

IAVs encode for non-structural and structural proteins. As explained above, the vRNP subunits are constituted by the viral RNA, the nucleoprotein (NP) and the polymerases PB2, PB1 and PA. The PB2 is an mRNA cap-binidng protein, the PB1 has the RNA-dependent RNA polymerase activity, and PA is an endonuclease (20).

Table 1.1. Influenza A virus RNA segments and proteins

Segment	Encoded genes	Length (nt)	Protein size (aa)	Name and protein function
1	PB2	2341	759	Polymerase basic 2. Subunit of the polymerase complex with cap-binding activity.
2	PB1	2341	757	Polymerase basic 1. Subunit of the polymerase complex with RNA-dependent polymerase activity.
	PB1-F2		87	PB1-F2, antiapoptotic factor, regulator of polymerase activity.
3	PA	2233	716	Polymerase acid. Subunit of the polymerase complex with endonuclease activity.
	PA-X			PA-X. PA-endonuclease activity with C-terminal "X" domain that regulates host-immune response
4	HA	1728-1778	539-550	Hemagglutinin. Type-III transmembrane glycoprotein. Receptor recognition, mediates fusion of virus membrane into infected cells. Surface antigen.
5	NP	1565	498	Nucleoprotein. RNA binding activity. Interacts with vRNA to form RNP complexes.
6	NA	1453-1470	454-468	Neuraminidase. Sialic-acid destroying enzyme activity to release virus particles. Surface antigen.
7	M1	1027	252	Matrix protein 1. Oligomeric protein viral capsid underneath viral envelope. Determinant of virus morphology, involved in packaging of RNPs and virus assembly.
	M2		97	M2, ion-channel for dissociation of viral particles. Involved in virus assembly. Inhibitor of autophagy.
8	NS1	890	230	Non-structural protein 1. RNA-binding domain. Antagonist of host-immune response (Interferon, and interferon signaling). Suppressor of gene expression in nucleus.
	NEP		121	Nuclear export protein. Regulates production of viral RNA species (vRNA, cRNA and mRNA). Participates in virus budding.

The HA is a Type-I transmembrane glycoprotein that recognizes sialic acid (SA) residues on the surface of susceptible cells and mediates fusion of the viral and cell membranes during infection. The HA is a determinant of the species host range and tissue tropisms as will be discussed later. The HA protein is a homotrimer, in which each subunit is synthesized as a single polypeptide from the single ORF of segment 4. Each of these chains is subsequently cleaved into two chains, HA1 and HA2. In the trimer structure, each monomer consists of one HA1 chain covalently attached to one HA2 chain by a disulfide bond; the monomers are associated via noncovalent interactions to form the trimeric protein. The tridimensional trimer displays a globular head that contains the receptor-binding site (HA1) and a stalk (HA2). The HA1 and most of the HA2 constitute the extracellular domains, whereas the membrane-anchoring region and the cytoplasmic tail reside in the C terminus of the HA2. The HA protein is further modified by glycosylation of multiple asparagine residues (21). The glycosylation of the HA protein is thought to be important in modulating receptor binding, fusion activity, antigenicity and replication (and transmissibility) of the virus (22-24).

The NA is a type II integral membrane protein with sialic-acid destroying enzymatic activity that cleaves the surface sialic-acid receptors to release new viral particles after budding from the cell (8). The NA protein is a homotetramer with each subunit arranged in a circular mushroom-like conformation. The head is displayed towards the outer surface of the lipid membrane and contains the active site in the center; the stalk is attached to the membrane via N-terminal transmembrane domain and a cytoplasmic tail (25). The length of the stalk can vary between different NA subtypes and virus strains. The length of the NA stalk has been shown to modulate replication and

virulence in different hosts (26-28). Like the HA, the NA is glycosylated in specific asparagine residues, although the role of glycosylation in NA is less understood but it has been implicated in stability, antigenicity, replication and virulence (29, 30). The HA and NA are the major antigenic determinants of virus and their plasticity leads to evasion of the host immune pressure, a process known as antigenic drift (discussed in section 1.1.6).

The matrix protein M1 constitutes the viral capsid protein, and it is largely implicated in virus morphology (filamentous or spherical particles). In a virus particle, M1 is in contact with the enclosed vRNPs, through interactions with NP. Similar interactions occur during vRNP packaging and with the cytoplasmic tails of the surface proteins (HA and NA) for its recruitment to the cell surface during virus assembly.

The ion-channel M2 protein is a tetrameric integral membrane protein, with multiple functions during the replication cycle. During its transition in the endosome, the M2 unidirectional proton pump activity leads to acidification of the virus interior, a step necessary for dissociation of M1 from the vRNPs and release of the latter into the cytoplasm. The M2 protein participates in virus assembly and budding through interactions between its cytoplasmic tail and M1 and HA (31-33). In addition, the M2 protein acts as an antagonist of apoptosis, blocking fusion between autophagosomes and lysosomes, counteracting the host-immune response during infection (34).

Non-structural proteins include the NS1, found during infection in the nucleus and the cytoplasm where it exerts its anti-interferon activity via multiple mechanisms (reviewed in (35)). The NS1 protein contains an RNA binding domain at the first 73 amino acids of the N-terminal, which interacts with RIG-I like receptors (RLR) to suppress the interferon activation cascade, including the induction of interferon

stimulated genes (ISG). NS1 is also known to directly inhibit the activity of multiple ISGs, by the same mechanisms of RNA sequestration. The NS1 also inhibits host mRNA processing in the nucleus through binding of host transcription factor PSF (reviewed in (35)). The nuclear export protein NS2/NEP mediates export of RNPs together with M1 via interaction with nuclear export machinery, and blocking nuclear localization signals contained in the vRNP complexes. In addition NEP is a regulating factor of viral, complementary and mRNA synthesis during virus replication, and also participates in later stages of the replication cycle during the budding process (36).

The viral protein PB1-F2 is an 87 amino acid, pro-apoptotic viral factor that stimulates release of cytochrome c from the mitochondria, which ultimately leads to cell death (12). However PB1-F2 is expressed as a truncated product or not expressed at all in some virus strains. Human influenza viruses evolve into strains with truncated PB1-F2 open reading frames. PB1-F2 pro-apoptotic activity is controversial and appears to be strain specific, due to its role in regulation of polymerase activity (37-41). The last and most recently described protein of IAV is PA-X, of 61 amino acids. It contains the PA endonuclease activity and a C-terminal domain that is thought to be involved in modulation of host immune response, through repression of cellular gene expression (13). With the exception of the viral proteins that are involved in specific interactions with host immune responses and other factors that are not necessary during viral replication, additional functions and interactions of viral proteins are explained in the next section.

1.1.5. Replication cycle

When influenza viruses enter the respiratory or gastrointestinal tract of susceptible hosts, their surface proteins can recognize and bind specific SA residues displayed in the

surface of susceptible cells. Sialic acids are acid sugars with a nine-carbon skeleton that are ubiquitously distributed in vertebrates. The most common SA is N-acetylneuraminic acid (Neu5Ac) (42). The recognition of SA by the receptor-binding site of the viral HA depends on the configuration of the linkage of the SA to the adjacent galactose. IAVs can recognize the α-2,3 and α-2,6 conformations of Neu5Ac distributed along the mucosa of the intestinal and respiratory tract of birds and mammals. The distribution of α-2,3 and α-2,6 SA vary among different hosts and tissues, being an important determinant of cell, tissue and hosts tropism for the virus (43, 44). As an example, avian influenza viruses have preference for α-2,3 SA present in the gastrointestinal mucosa, whereas mammalian adapted viruses recognize α-2,6 SA mainly present in the upper respiratory tract (45-47). In addition to epithelial cells, other susceptible cells may include immune cells such as alveolar macrophages and dendritic cells recruited during infection; however the specific SA or other lectin receptors that may confer susceptibility to infection of these types of cells remains unknown (48, 49).

HA attachment to the SA triggers fusion of the virus and cell membranes, mostly via clathrin-mediated endocytosis [although other entry mechanisms have been described (50)]. Upon internalization, a low pH-mechanism mediates conformational change in the HA molecule (cleavage into HA1 and HA2 subunits) that exposes the fusion peptide, making it accessible for fusion of the viral and endosomal membranes, necessary for the release of the viral RNPs into the cytoplasm. During this step the stability of the glycosylated HA molecule appears to determine the pH of fusion and whether uncoating will occur in the early or late endosomes. The internal acidification of the virus during this process, necessary to disrupt molecular interactions of viral structural components, is

mediated by M2's proton-pump activity (8). Once the vRNPs are released into the cytoplasm, nuclear localization signals present in protein components of the vRNP complex interact with the cellular machinery for nuclear import. As an exception for RNA viruses, viral replication of IAV occurs in the nucleus, where cellular replication, transcription and splicing components and mechanisms are exploited by the viral polymerase complex for the production of different viral RNA species (vRNA, cRNA and mRNA). Viral mRNAs are transported to the cytoplasm for translation into viral proteins. Newly synthesized RNP proteins are transported back into the nucleus to promote viral replication, and assembly of new vRNP complexes with each gene segment. The NS2/NEP in conjunction with M1 protein interacts with nuclear localization signals, masking them and allowing the export of vRNPs from the nucleus to the cytoplasm to initiate virus assembly. Timing of protein expression and accumulation of protein products determines initiation of virus assembly during later stages of the viral replication cycle. Virus assembly is triggered by a combination of aggregation of processed (multimerized and glycosylated) structural and surface proteins (M2, HA and NA) in the cell membrane, and protein-protein interactions of surface components among themselves and M1. In addition, oligomerization of M1 triggered by interactions with lipids from the cell membrane ensure multilevel interactions between viral proteins during this process (51). Selective packaging of all eight viral RNAs segments occurs via segment-specific signals at the 3’ and 5’ ends for 1) bundling of each RNA segment with each other in a full set of 8 gene segments and 2) incorporation of gene segments into the budding virus (52). The vRNPs are thought to interact with M1 protein for its recruitment to the cell membrane. Oligomerization of M1 forms a curvature in the membrane

required for viral egress and the budding of IAV occurs in the apical side of the infected epithelial cells. Once the viral particle is completely formed, it will remain attached to SA molecules on the cell surface. The SA destroying activity of the NA is obligatory for the release of readily infectious virus to the extracellular space to complete the final step of the virus replication cycle. After release of infectious viruses, the surface HA is cleaved by mucosal trypsin-like proteases making it readily available to recognize cellular receptors for the next round of infection (8).

The multiple barriers that IAV encounters during its replication, i.e. compatibility of cellular components with viral proteins in each step of the replication cycle, determine the success or failure of an infection. Consequently, virus-host interactions have shaped the evolution of IAV within its multiple hosts.

1.1.6. Evolution of Influenza A Virus

Antigenic drift, antigenic shift, reassortment and recombination in IAV

Two major mechanisms are known to drive IAV evolution (53), particularly at the level of surface gene segments. There is stronger selective pressure on the surface gene segments in comparison to internal gene segments due to their constant exposure to the host humoral response. This interaction imposes a bottleneck for selection of escape variants, where accumulation of point mutations occurs at antigenic sites, a process known as antigenic drift. The second mechanism is antigenic shift resulting from reassortment of gene segments from two or more IAV strains, resulting in variants with alternative HA and NA combinations. Horizontal gene transfer through this mechanism has conferred an evolutionary advantage to IAV viruses not only to counteract the effect of deleterious mutations in specific gene segments, but also to allow the viruses to

conquer newer niches for replication (by increasing the virus tissue tropism or host range). Reassortment is the underlying mechanism that allows the emergence of novel influenza strains from avian and swine origin in the human population (54). Among other mechanisms of evolution, less known for IAV, is genetic recombination. There is evidence that non-homologous recombination occurs, but it has only been observed with the HA segments of H7 subtype viruses, resulting in strains of increased pathogenicity in poultry (55, 56).

Gene flow, adaptation and evolution of Influenza A virus

As an RNA virus, IAVs evolve quickly under a spectrum of intrinsic (nature of RNA genomes) and extrinsic (host and environmental) selective pressures. The RdRp of IAV is a low fidelity enzyme with no proofreading activity (estimated error rate of $7.63x10^{-5}$) (57), therefore, IAV viruses like other RNA viruses, subsist at the limit of an error threshold (i.e. functional RNA genomes can still be produced despite the accumulation of mutations). It is commonly accepted that IAV exists as a population of genetically diverse variants during infection (58-60). These variants are represented by dominant populations selected for improved fitness under particular conditions. Changes in conditions (i.e. during infection of new hosts or tissues), can quickly change the composition of viral populations (as other variants are selected and become dominant). Evolution of RNA viruses through this mechanism can occur very quickly, and the plasticity of this process is reflected in the diversity of IAVs (multiple subtypes and genetic lineages) and their ability to infect and adapt to a variety of host species in nature (60). The main drivers of evolution of IAV are 1) the ability of the virus to establish infection in a particular host (within-host adaptations), 2) the ability of the virus to

transmit and perpetuate in a host population (adaptations for between-hosts transmission) and 3) host behavior, as spatial and temporal distribution of susceptible species has shaped evolution of IAV viruses according to their hosts and geographic location, the last one particularly noticeable in avian hosts.

Influenza A viruses are known to infect multiple vertebrate hosts, including birds and mammals (see section 1.1.7). The existence of host-specific genetic lineages represents the within-host adaptations of IAV throughout prolonged periods of time. However, IAV from different hosts are still capable of crossing the species barrier and adapting to new hosts. An IAV needs to overcome two host-specific barriers: 1) To be able to infect and initiate a productive infection in the new host, which will depend on a number of within-host selective pressures that include receptor specificity, compatibility of the viral proteins and the host-cell machinery, and the ability of the host to counterattack the infection. The second barrier requires the virus to acquire sustainable transmissibility within members of the new hosts species. Host behavior and environmental conditions may allow contact between species in sufficient numbers for the virus to be introduced to new hosts, and to persist and spread over multiple transmission events (61). Such selective pressures combined with the plasticity of the IAV genome, have resulted in the diversity of strains that co-circulate in animal and human populations, each one contributing to a larger pool of genetic variants as IAVs continue to evolve.

Parallel virus-host evolution, influenced by host behavior and physical separation of ecological niches, has led to established host-specific lineages. For example, avian influenza viruses share a common ancestry that distinguishes them from human or other

mammalian viruses and their respective lineages (specifically, human, canine, equine and swine lineages). Phylogenetic analysis of IAV diversity, demonstrates that all IAV derive from a genetically common ancestor of avian origin (62, 63), consistent with the higher diversity of subtypes (16 HA and 9 NA) that has been found in these hosts. The origin of IAV HA and NA subtypes has been traced back to around 1000 years ago (58, 63, 64). In contrast, the origin of the genetic diversity of the internal genes, with the exemption of NS-B lineage, has been dated to less than 200 years (58, 65). A recent study, employing more refined phylogenetic inference under a host-specific local clock model (with the assumption that viruses evolve at different rates in different host species), suggest that a spill over event and spread of viruses from an outbreak in horses into domestic birds, may have played a major role in shaping the evolution of contemporary influenza viruses late in the 19th century, explaining the recent evolutionary history of the internal gene segments (64). Global replacement of gene constellations through periodic sweeps has been proposed to explain the patterns of evolution, and persistence of IAV throughout the years. Similar patterns of gene replacement are observed in seasonal human influenza on a global scale and within specific geographic lineages of avian influenza (66-69).

In addition to host-specific genetic lineages, geographic separation of host species in nature has resulted in the diversification of IAV of avian origin into distinguishable lineages associated to specific geographic locations, including the Eurasian, North American, and more recently described the South American, and potentially Oceania and Antarctica lineages (53, 70-72). The frequency of intercontinental or inter-lineage exchange of viruses may depend on spatial and temporal overlap of two or more continental migration routes such that birds following a particular migration route are in

contact with other bird populations harboring viruses from a separate genetic lineage (73). Recent surveillance studies in Alaska and Iceland document co-circulation of viruses from two different genetic lineages (North American and Eurasian) in ducks and shorebirds (74). Through phylogenetic analysis, limited numbers of reassortant genomes have been found and limited inter-lineage reassortment has been documented (75-78). It remains to be further explored whether functional compatibility of viral gene segments from different lineages impact reassortment in nature.

1.1.7. Influenza A viruses in animal hosts

From the three genera of influenza viruses (Type A, B and C), type A influenza viruses are recognized for their wide host range and their ability to cross interspecies barriers and, eventually infect humans (79-81). Other hosts of IAVs include natural avian hosts, mainly *Anseriformes* (ducks, geese, and swans) and *Charadriiformes* (gulls, terns, and shorebirds). Non-natural avian hosts include poultry species (chickens, quails and turkeys). Among the known mammalian hosts, are humans, pigs, carnivores (dogs, minks, civet, ferrets, raccoons, felines), marine mammals (whales, dolphins, seals), with accumulating evidence of some fruit bat species (5-7, 53). In avian hosts influenza A infection is predominantly an asymptomatic infection of the gastrointestinal tract in the majority of its natural reservoirs (waterfowl). Depending on the virus strain and host species (i.e. domestic birds), infection may occur via the respiratory tract and become systemic with involvement of the nervous system (53). In mammalian hosts, IAV is transmitted through direct contact or aerosols and respiratory droplets between hosts, and the infection is primarily respiratory. The spectrum of disease and pathogenicity in mammals may vary between hosts and virus strains; some strains may spread to lower

tissues in the respiratory tract, such as bronchi and lungs and also become systemic (5). Wild birds and pigs have been recognized as two of the most important animal reservoirs. These hosts are the main focus of this work and their roles in IAV transmission are described in the following sections.

1.1.8. Influenza A viruses in wild birds

Influenza A viruses have been isolated from over a 100 species of wild birds, from 12 different orders. Despite the diversity of reported avian hosts, the majority of records and isolates come from species of the orders *Anseriformes* and *Charadriiformes* (79, 82). Avian species from both orders are broadly distributed around the world. Many of their member species are known to perform seasonal migrations and travel across long distances between different geographical areas and countries every year. The nature of this behavior has been linked to the role of these species to allow the movement and geographical spread of AIVs viruses around the world (82, 83). Specific bird species vary across geographical locations, but IAVs are able to infect a broad spectrum of genera (43, 45).

Influenza A viruses that infect birds can be classified as low pathogenicity (LPAI) and high pathogenicity avian influenza (HPAI) viruses based on the severity of disease signs in gallinaceous birds. The high pathogenicity phenotype is associated with the insertion of a polybasic amino acid sequence at the cleavage site of the HA molecule (84). The presence of a polybasic-motif in the HA (e.g. PQRESRRKK/GLF) exposes the cleavage site to other ubiquitously-expressed proteases in the intracellular and extracellular environments, expanding the cell tropism of the virus and making it more likely to produce a systemic infection (85). The pathogenicity of a particular virus can be

tested in the laboratory, in viruses that grow in cell culture in the absence of trypsin (a protease that cleaves and activates the HA), and through the intravenous pathogenicity index (IVPI) in chickens (86). In this test a HPAI virus is classified as such if it has an IVPI greater than 1.2. Viruses of the H5 and H7 subtypes are known to mutate and generate HPAI strains in nature (79). Disease caused by HPAI viruses involves acute infection of the respiratory tract and can be generalized with mortality rates ranging from 75% to 100% (87, 88). HPAI viruses cause important economic losses in the poultry industry due to high susceptibility of domestic bird species.

Low pathogenicity viruses preferentially infect the epithelial cells of the intestinal tract in birds. Field and experimental evidence suggest that under certain conditions, or perhaps virus strains, infection in wild birds with LPAI may have a physiological impact in their fitness and behavior during migration, with changes in their returning rates, feeding rate and body weight, the length of traveling distances and reproductive success (89). However clinical signs of disease are rarely seen in wild infected birds, possibly as a result of co-adaptation between the virus and its natural avian hosts (79). High virus titers are produced and the virus is excreted in high concentrations in the feces. Excreted viruses persist in the water maintaining the transmission cycle within susceptible bird species. The fecal-oral route is thought to be the primary mode of transmission; while respiratory secretions as well as fecal-cloacal transmission have been proposed as alternative routes (90-92).

Circulation of LPAI in wild bird populations varies seasonally and temporally in relation to the behavior of its multiple avian hosts. Most of the available information of LPAI prevalence in nature derives from extensive surveillance studies at the temperate

zones in North America and Europe for more than 30 years (53, 93, 94). In nature, ducks from different species share aquatic habitats where they are exposed to multiple LPAI viruses, mixed infections and co-circulation of multiple subtypes are common in these habitats, although variations in prevalence of LPAI and subtypes may exist in individual bird species. In general, prevalence of LPAI in migrating ducks tends to peak late in the summer to early autumn in the breeding grounds (82, 95, 96) most likely due to the presence of an immunologically naïve population of juvenile birds (83). Prevalence decreases later in fall, before migration begins. Depending on the species, migration distances may vary, but the majority of birds will make multiple stops before arriving to their wintering grounds. This behavior is important for LPAI transmission, as infected birds from one population congregate with other species at these stop-over habitats, where mixing and new infections of LPAI may occur, and new viruses may be carried to other places as birds continue to migrate (91). Prevalence of LPAI at the wintering grounds tends to be low in comparison to peak prevalence at the breeding grounds (97, 98). Surveillance data at the wintering grounds in the south suggest that the relative proportion of individual subtypes co-circulating at a time seems to be subjected to seasonal patterns associated with the geographical location, host species and host biology (99, 100).

In terms of the host biology, experimental infection in mallards and other duck species show that antibody immune response to LPAI infection in serum is weak and transient, re-infection with homosubtypic viruses seem to be limited and infection with heterosubtypic viruses may be prevented in some cases (101, 102). Higher susceptibility of juvenile birds to infection, observed by higher prevalence of IAV in this age group in

nature (94, 97, 103), supports that pre-existing immunity may limit re-infection in adult ducks. The duration of the antibody response in serum has been estimated to last less than a year (104), and it has been hypothesized that environmental stressors associated with a long-distance migration may compromise the immunological status of ducks, making them susceptible to multiple infections throughout their lives (105). Population immunity may play a role in determining the prevalence of different subtypes co-circulating in flocks at a time (106). In this context, the role of mucosal immunity in modulating infection in ducks remains unknown.

IAVs of the H13 and H16 subtypes appear confined to Charadiiriformes (82), (107, 108), specifically in gull populations, although viruses from other subtypes have been isolated from these and other shorebirds (including H1-7, H10-12) (93, 109). Waders play an important role in perpetuating certain subtypes of LPAI in nature (H1 and H12) with distinct seasonal patterns. Gulls and shorebird species are of interest for performing long-distance intercontinental migrations, and playing a potential role in spreading viruses to other geographic locations (67, 75, 110, 111). LPAI viruses have been isolated from wild geese, swans, rails, petrels and cormorants. IAV isolations have occurred also from Passeriformes and Columbiformes but prevalences in these birds are lower than those observed in dabbling ducks and shorebirds (112-114), and their role in LPAI epidemiology remains to be established, as evidence of sustained transmission in these populations is lacking (115, 116).

Zoonotic potential of avian influenza viruses

Transmission of circulation of IAV in avian reservoirs to mammalian hosts is very limited in nature due to host-restriction factors. Although it is not clear what may trigger

a transmission event during exposure, wholly avian viruses may infect humans, without prior reassorment with other mammalian strains (5). Viruses of avian origin have contributed genes for the generation of multiple pandemic strains throughout history, such as the 1918 H1N1 in which many of its gene segments had ancestors of avian origin (117-119). Subsequent pandemic strains are thought to be descendants of the 1918 H1N1 virus that reassorted with other avian viruses: the H2N2 in 1957, contained novel HA, NA and PB1 gene segments of avian origin, and the pandemic strain from 1968 was a reassortant of the H2N2 virus that acquired novel avian-origin H3 and PB1 gene segments. The 1977 pandemic was caused by re-emergence of the human 1957 H1N1 virus, likely the result of unintended release from a laboratory. Lastly, the most recent 2009 pandemic virus originated from a reassortant swine virus that acquired the PB2 and PA genes from an avian source (54).

In addition, other viruses from different subtypes are known to have caused infection in humans, including the H5, H6, H7, H9 and H10 with different levels of disease severity (120). The most notable of these is the H5N1 virus that became highly pathogenic after its introduction to domestic poultry, causing devastating outbreaks in Asia and other parts of the world since 1997. The same virus has been the cause of 667 human cases and 393 deaths since 2003 (as of July 27, 2014 (121)). Although these viruses have been able to cross the species barrier, they have failed to adapt to the human hosts and become transmissible between mammals; however their ability to infect human hosts increases their pandemic potential, representing a constant threat to public health (120).

Many outbreaks of LPAI and HPAI that have occurred in poultry have been traced to viruses that spilled over from wild birds (122-127). Outbreaks of influenza in poultry are thought to be a risk factor for transmission of avian strains into humans, probably as a result of increased exposure to infected hosts. Although several molecular changes have been proposed as determinants of virus transmission from birds to humans, the factors that are required for complete mammalian adaptation including acquisition of airborne transmissibility are still poorly understood (128). The recognition of the role of avian-viruses in the evolution of pandemic influenza, and the multiple spillover events from birds to humans observed in recent decades highlights the importance of reinforcing surveillance in avian hosts in nature. In order to minimize the risk of zoonotic transmission to domestic animals and humans it is necessary to monitor virus diversity and better understand the global patterns of virus transmission and evolution in nature (120, 129).

1.1.9. Influenza A viruses in pigs

Reports of an outbreak of respiratory disease in pigs in 1892 have been proposed as the first indication of influenza in pigs, coinciding with an epidemic in humans; historical references do not provide evidence of prior circulation of influenza viruses in pigs (130). Influenza A infection in pigs was later reported in 1918, by clinical observations in herds in the U.S, Hungary and China, during the same period when the Spanish influenza pandemic emerged in humans (131). Although viruses from pigs were not recovered at that time, analysis of the first swine influenza virus isolated in 1930, revealed similar pathogenicity in animal models (132), and later studies linked the origin of the H1N1 swine virus to a common ancestor from which the pandemic of 1918 also

diverged (133, 134). Through phylogenetic and seroarcheological analyses, it has been established that the causative agent was a reassortant human H1N1 that acquired a N1 gene of avian origin (119, 135, 136). It is speculated that this virus was introduced to pigs from humans, and persisted in the pig population establishing a genetically distinct lineage, currently known as the classical swine H1N1 lineage (137). This lineage persisted until the 1990s as a single H1N1 lineage in pigs in North America. In 1998 an outbreak in pigs in North Carolina was reported where the etiologic agent was confirmed to be a H3N2 virus, a novel reassortant of a human H3N2 virus and the H1N1 swine virus (138). At the same time other outbreaks were reported in Texas, Minnesota and Iowa, caused by H3N2 viruses, which were characterized as reassortants of other H3N2 viruses, the H1N1 swine virus and a virus of avian origin. This "triple reassortant" gene constellation of human (HA, NA, PB1), swine (NS, NP, and M) and avian (PB2 and PA) origin, resulted in stable viruses that quickly spread and became endemic among swine populations in North America (139). Co-circulation of H1N1 and H3N2 viruses in the US and Canada has resulted in emergence of multiple viruses (H1N1, H1N2 and H3N2) that have evolved independently through mutation and antigenic drift from their parental viruses into distinct genetic and antigenic lineages. As a result, swine influenza H1 viruses are now classified based on the genetic and antigenic properties of the HA molecule into alpha, beta, gamma and delta genetic clusters, and similarly the H3 viruses have been classified in four (I – IV) genetic clusters (139-142).

Serological evidence suggested that the classical swine H1N1 lineage also circulated in Northern Europe between the 1940s and 1950s. After a 20-year period of apparent absence the swine influenza from classical swine H1N1 lineage emerged in

northern Italy in 1976, most likely introduced from the US at that time (131). The virus spread to other countries in subsequent years and became endemic in many European countries (143). In addition to the classical swine lineage, a human-like H1N1 circulated in pigs in Europe between 1938 and 1940 (144, 145). Other human-like viruses were isolated from pigs after the Russian pandemic of H1N1 in 1977, and avian-like H1N1 viruses, genetically and antigenically different from the classical swine H1N1 lineage, have circulated in Europe since 1979 (Kuntz-Simon & Madec, 2009). The avian-like viruses have been the predominant viruses, but introduction of human-like H3N2 swine viruses from Asia has resulted in co-circulation of reassortant strains with different genetic and antigenic properties (146-148). In Asia, infections in pigs with human-like viruses of the H3N2 subtype have been documented since the 1970s (149) and similarly in Europe and North America, introduction of North American and Eurasian swine viruses has provided a pool of genes for reassortment and generation of multiple strains that currently circulate in these areas (150-152). In addition to the subtypes H1N1, H1N2 and H3N2, other virus subtypes detected in pigs include the H4N6 in Canada, the H2N3 in pig farms in the US, and a number of H9N2 avian-like reassortant viruses in Asia; however the circulation of these virus subtypes has been limited to sporadic outbreaks in those regions (153-156). Historical evidence of circulation of swine viruses in pigs is limited in other geographical regions. Retrospective phylogenetic analyses have shown that viruses from humans have been introduced to pigs around the world in multiple occasions in the last century (131, 157).

In 2009, zoonotic transmission of a triple reassortant virus from pigs, resulted in the first influenza pandemic of the 21st century (158, 159). This virus was a reassortant of

a triple reassortant virus of North American origin from the gamma cluster that acquired the NA and M segments from a Eurasian avian-like H1N1 swine lineage, and was the first evidence of introduction of Eurasian origin genes into swine of the North American viruses. The virus was speculated to have emerged and circulate silently in swine populations before crossing into humans. After the rapid spread and establishment of the pandemic lineage in the human population, multiple independent introductions of the viruses back into pigs have been documented around the globe (160). Circulation of the pandemic lineage in the swine populations has increased the genetic pool of the viruses available for generation of novel reassortants in pigs (161, 162). Some of these reassortant viruses still hold the potential to transmit back to humans, such as the H3N2 variant that contains the M segment from pandemic H1N1 (163).

Influenza A virus infection in pigs

Influenza A viruses in pigs can cause an acute respiratory infection with clinical presentation similar to human infections (164). Infected pigs may present a combination of signs including fever, anorexia, inactivity and respiratory signs (including shortness of breath, sneezing, coughing, nasal discharge and conjunctivitis) (132). The incubation period is 1 to 3 days and the infection may last up to 7 days when animals start to recover after disease onset. Virus shedding can be detected up to 29 days. Depending on the virus strain and the immunological status of infected pigs, infections vary in severity, and may progress to pneumonia with infiltration of lymphocytes, that resolves with clearance of infection (137).

Influenza infection in pigs is considered a herd disease due to its rapid spread once introduced into a farm. It is characterized for its high morbidity (up to 100%), but

low mortality (<1%). Infection of pigs with influenza viruses is considered a risk factor for concomitant bacterial or viral infections; consequently swine influenza viruses are part of the porcine respiratory disease complex (PRDC) with other respiratory pathogens including the porcine reproductive and respiratory syndrome (PRRSV), type 2 porcine circovirus (PCV-2), *Pasterella multocida*, *Mycoplasma hyopneumoniae* and *Actinobacilus pleuroneumoniae* (137). Thus, influenza infection in pigs can have a negative impact in the development and desirable weight gain in production animals, representing a significant economic burden to the pig industry.

Incidence and patterns of infection of influenza in pigs are associated with the nature of pig production systems. Risk factors for the introduction of influenza into a herd include movement of animals from infected to susceptible herds, and contact with other infected species such as birds or humans. Once introduced, the virus can spread through direct contact and aerosol or respiratory droplets between animals. Confinement and overcrowding of animals, environmental conditions (e.g. cold weather) and some husbandry practices can propitiate virus spread (165-167). Influenza virus infections can be maintained in herds though multiple cycles of the production system with the periodical exposure of immunologically naïve piglets. After becoming endemic, the viruses may circulate as subclinical infections, becoming difficult to detect, unless active surveillance to detect IAV is conducted (131).

As described in the previous section, IAV of avian and human origin can infect pigs. It has been hypothesized that the presence and distribution of both α-2,3 and α-2,6 SA virus receptors in the respiratory mucosa of these animals provide favorable conditions for reassortment of viruses with different receptor specificities during co-

infection. Therefore the pig has been proposed to be an intermediate host or "mixing vessel" for reassortment between avian (α-2,3 binding) and mammalian (α-2,6 binding) viruses (168). However studies using differential glycan staining, and determining the binding preference and localized replication of avian and mammalian viruses in these animals showed that the distribution patterns of both type of receptors were similar to those of the human respiratory tract (169-171). These later observations have failed to support the "pig as a mixing vessel" hypothesis; suggesting that similar to pigs, humans may be equally likely to be infected with avian viruses and serve as hosts for reassortment (172, 173).

It is unquestionable that viruses from human and avian origin have contributed to the diversity of IAVs in pigs around the world. The spectrum of genetically and antigenically different viruses that have evolved in pigs represents a constant challenge for the development of successful control measures and strategies for outbreak prevention.

Control and prevention of influenza A in pigs

Depending on their transmissibility, some viruses may spread rapidly once introduced into a herd, making it difficult to control. Among the control strategies are isolation of animals to prevent contact between uninfected animals. This includes separating sources of water, food, etc. and disinfection of areas and equipment to prevent further spread. If the virus becomes persistent in a farm, depopulation and disinfection of the affected farms is the only way to completely eliminate the disease (131).

Vaccination against influenza viruses has been established as a practice to prevent outbreaks of influenza in pigs in many countries, especially those with larger production

systems (137). Vaccination against swine influenza has mostly relied on the use of inactivated viruses, as single or multivalent adjuvanted preparations that include strains from different genetic clusters that may represent the diversity of circulating viruses in a given location (174, 175).

In a farm, sows are vaccinated to stimulate passive transfer of neutralizing antibodies to piglets. Inactivated vaccines provide limited protection against heterologous infection, and while they might prevent disease, they may not prevent infection and further virus transmission in a herd (137). In addition, the correlates of protection against heterologous viruses and broadly neutralizing responses are not fully understood. To address these issues, recently there has been more emphasis in the development of Live Attenuated Influenza Vaccines (LAIV) that better mimic the natural infection and stimulate humoral and cellular responses (176, 177). Other experimentally tested vaccine candidates are based on alternative strategies such as vector and DNA vaccines (175).

Regardless of the vaccination strategy, the main objective of vaccination in a population is to decrease virus circulation and disease. Little is known about the diversity of viruses, and of genetic and antigenic lineages that circulate in areas of the world where surveillance for swine influenza has not been systematically performed. In these areas, implementation of vaccination should be based on baseline information on locally circulating strains in order to maximize vaccine efficacy (174). To this end, characterization of swine influenza viruses is one of the components of this work as explained in the following section.

1.2. Rationale for this work

Latin America represents an understudied region for influenza viruses in animal hosts, including wild birds and pigs. There is little information on which viruses circulate and how the interaction between animal reservoirs and humans is shaping their evolution. In general, animal raising and handling practices in Latin America resemble those from other developing countries (such as Asian countries) that are believed to be associated with an increased risk of exposure to zoonotic influenza viruses. Despite this fact, the circulation of IAV in animal reservoirs in Latin America is still largely unknown. Central America is located in an intermediate point between bird migration routes. Over the past 20 years there have been two major poultry outbreaks related to avian influenza in the region. After a major outbreak in 1994 that started in Mexico and later spread to neighboring countries, the H5N2 subtype is now endemic in Mexico, Guatemala and El Salvador. The 2012 outbreak caused by a H7N3 strain in Mexico, resulted in significant economic losses for one of the biggest egg and poultry producers from Latin America. The circulation of this strain in Mexico raises concern about further spread across the region. The appearance of the HPAI H5N1 virus in Asia, its unprecedented devastating impact in poultry and the pandemic potential of this strain evidenced by hundreds of human cases, were the starting points of many surveillance efforts worldwide. As part of these efforts, surveillance for animal influenza started in Guatemala in 2007, aiming to confirm the presence of avian influenza viruses (IAV), and serve as a platform for the early detection of the introduction of HPAIV strains in wild birds (Chapter 2). Phylogenetic comparisons were made at a regional level, accompanied by an extensive literature search for avian influenza viruses in Latin America in order to understand the

current knowledge of AIV in Latin America, and to analyze phylogenetic relationships among publicly available viral genomes (Chapter 3). Our initial findings, justified continuation of sampling of waterbirds in Guatemala with focus on the duck species *Anas discors*, to confirm its role as a host for IAV in Central America. Seasonal patterns in the circulation of IAV viruses in this host in multiple migration seasons were analyzed (Chapter 4). Phylogenetic analysis and characterization of full genomes and gene constellations were done, with emphasis in genes that were found to be closely related and potential precursors of the HPAI H7N3 outbreak in Mexico. Viruses of the H14 subtype were found in the western hemisphere for the first time in 2010, and we detected this subtype in Guatemala, in 2011. Our findings provide evidence of a potential outbreak of H14 viruses, after its introduction in the wintering grounds in Guatemala. The last part of this work focuses on the characterization of IAV circulating in the swine population in Guatemala (Chapter 5) a work that was started following the appearance of the pandemic H1N1 strain in 2009.

This work presents the results obtained for these studies, recommendations and a proposed strategy to further characterize the circulation of influenza of avian and swine origin in Guatemala (Chapter 6). The work was performed to address four main research objectives described below.

1.3. Research objectives

Specific aim 1. To detect and identify influenza viruses circulating in wild aquatic birds from Guatemala.

- To detect, isolate and identify influenza viruses in different wild bird species from the Pacific coast of Guatemala.
- To establish the subtype diversity and analyze year-to-year variation of influenza viruses circulating in wild aquatic bird species during the winter migration in Guatemala.

Specific aim 2. To analyze phylogenetic relationships of avian influenza viruses in wild birds in Latin America.

- To perform an extensive literature review for avian influenza in all countries from Latin America (Central America, South America and the Caribbean).
- To perform phylogenetic analysis at the regional level to detect reassortment events between co-circulating genetic lineages.

Specific aim 3. To establish the origin and genomic diversity of influenza viruses circulating in wild aquatic birds from Guatemala.

- To characterize virus genome constellations and genetic diversity of sequenced viruses.

- To obtain full-length genome sequences of representative virus isolates and infer the phylogenetic relationships with other influenza viruses circulating across the American continent.

Specific aim 4. To identify and characterize the influenza viruses that circulates in the swine population from different production systems in Guatemala.

- To detect, isolate and identify influenza viruses circulating in pigs in Guatemala.
- To detect and identify serological responses to different influenza virus subtypes and genetic clusters in pig production units in Guatemala.
- To characterize the origin of influenza viruses isolated from pigs from Guatemala and identify genetic traits associated with transmission and pathogenesis in swine and human hosts.

Chapter 2. Influenza A viruses from wild birds in Guatemala belong to the North American lineage

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2.1. Abstract

The role wild bird species play in the transmission and ecology of AIV is well established; however, there are significant gaps in our understanding of the worldwide distribution of these viruses, specifically about the prevalence and/or significance of AIV in Central and South America. As part of an assessment of the ecology of AIV in Guatemala, we conducted active surveillance in wild birds on the Pacific and Atlantic coasts. Cloacal and tracheal swab samples taken from resident and migratory wild birds were collected from February 2007 to January 2010. A total of 1913 samples were collected and virus was detected by real time RT-PCR (rRT-PCR) in 28 swab samples from ducks (*Anas discors*). Virus isolation was attempted for all positive samples, and 15 isolates were obtained from the migratory duck species Blue-winged teal. The subtypes identified included H7N9, H11N2, H3N8, H5N3, H8N4, and H5N4. Phylogenetic analysis of the viral sequences revealed that AIV isolates are highly similar to viruses from the North American lineage suggesting that bird migration dictates the ecology of these viruses in the Guatemalan bird population.

2.2. Introduction

The role of wild birds in the transmission of AIVs has become highly significant with the introduction and spread of Highly Pathogenic Avian Influenza Viruses (HPAIV) of the H5N1 subtype into different countries in Asia, Europe, and Africa (178-181). It is generally accepted that aquatic wild birds are the primary reservoirs of AIVs as evidenced by the fact that most of the different possible combinations of HA and NA subtypes (e.g. H4N2) have been found in these animals (83, 181). AIVs have been isolated from over 100 species of wild birds belonging to 12 different orders, mainly Anseriformes and Charadriiformes (79, 82). The virus has also been reported at low prevalence in small terrestrial birds (e.g. Passerines) ranging from 0.9% to 6.6% (114, 182, 183) and it has been proposed that such species can act as bridges between the wild aquatic and domestic birds because they co-exist with both ecosystems (184-189). Several surveillance studies have provided insight into the evolution of AIVs and its relationship with wild bird behavior (70, 93, 94, 190). Information regarding intercontinental exchange of viruses and genetic reassortment between Eurasian and North American viruses has been reported (65, 75, 191); however, little is known about the exchange of genetic material between viruses in the Americas, particularly between the Northern and Southern hemispheres. In Central America, the presence of AIV was confirmed with the isolation of the low pathogenic avian influenza virus (LPAIV) of the H5N2 subtype from poultry in 2000 in Guatemala and 2001 in El Salvador. Genetic characterization of the H5N2 isolates revealed that the virus was most likely introduced from Mexico (192). Vaccination against H5N2 in Guatemala has been used as the primary control strategy (193). The circulation of other AIV subtypes of poultry in

Guatemala, and elsewhere in Central America, has not been reported, although it must be noted that there has been limited surveillance.

Guatemala is located on a geographic bottleneck (the Central American Isthmus) that funnels millions of migrating birds from several North American flyways (Mississippi, Pacific and Atlantic American) through a narrow area. The tropical habitats of Central America constitute a terrestrial bridge between North and South America for well over 120 species of migratory birds (194). The forests and wetlands of tropical areas provide shelter and stopover habitats for several species of terrestrial and aquatic migratory birds (195, 196). As it has been hypothesized, these sites could be important for AIV transmission and reassortment between different bird species and from different migration flyways (83). To date, there is very little information regarding the circulation and ecology of AIV in Central America and only recently information about AIV in wild birds from South America has been reported (70, 125, 197, 198). Thus the role of specific bird species in the spread of AIVs throughout these regions remains unclear (91, 199, 200).

In this study we conducted surveillance of AIV in wild birds in several sites along the Pacific and Atlantic coasts of Guatemala. Resident and migratory wild birds associated with aquatic habitats were sampled to detect the presence of AIV. The aim of the study was to provide an initial assessment of the presence and ecology of AIV in Guatemala and serve as a platform for the early detection of the introduction of HPAIV strains (e.g. H5N1) from wild birds.

2.3. Materials and methods

2.3.1. Ethics Statement.

Collection of bird samples were approved by the Institutional Animal Use and Care Committee of the Universidad del Valle de Guatemala and reviewed and approved by the Institutional Animal Use and Care Committee of the University of Maryland, College Park under protocol number R-08-10. Sampling of hunter-killed birds was exempt of animal use and care regulations.

We conducted AIV surveillance in different sites of Guatemala in the Atlantic and the Pacific coast. For all sampling activities official permits were approved by the Center for Conservation Studies (CECON) and the National Council of Protected Areas (CONAP). The Ministry of Agriculture of Guatemala (MAGA) approved the study.

2.3.2. Sample collection

Samples were collected by trained veterinarians and technicians from the Center of Health Studies of University del Valle de Guatemala (CHS-UVG). Tracheal and cloacal swab specimens were obtained from hunter-killed ducks and mist net captured birds. Captures with mist nets and sampling were conducted at the study sites of Monterrico, Machacas del Mar and Puerto Barrios every 5-6 months, from October 2007 to July 2009. For mist-net captured birds, cloacal and tracheal swabs were collected from all animals, except for small birds in which case only cloacal swabs were collected. In addition to mist net captures, sport hunters were contacted for sampling of aquatic birds immediately after hunting. Samples from killed birds were obtained during the migratory seasons of 2007, 2008, and 2009 in Monterrico, El Pumpo and Pasaco villages. Prior to

specimen collection, trained ornithologists and technicians identified birds by species, sex, and age (adult, juvenile, hatch year or after hatch year). After specimen collection, swabs were placed into 1-1.5 mL of freshly prepared viral transport medium (VTM, Medium 199 with Hanks balanced salt solution, 2 mM L-glutamine, 0.5% bovine serum albumin, 0.35 g/liter sodium bicarbonate) with antibiotics and antimycotics (2 x 10^6 IU/L Penicillin, 200 mg/L Streptomycin, 2 x 10^6 IU/L Polimyxin B, 250 mg/L Gentamycin, 0.5 x 10^6 IU/L Nistatin, 60 mg/L Ofloxacine, and 0.2 g/L Sulphamethoxazol) (201). Specimens were transported to the laboratory on ice, frozen in liquid nitrogen or on dry ice in double sealed plastic bags depending on availability and estimated time of delivery to the laboratory. Samples were then stored at -70ºC until processed.

2.3.3. Type A influenza virus detection by rRT-PCR

For tracheal swabs, RNA was extracted from 200 µL of supernatant with QIAamp viral RNA kit (QIAGEN, Valencia, CA). Extracted RNA was eluted from the QIAgen columns to a final volume of 100 µL of elution buffer and stored at -70ºC. For cloacal specimens, RNA was extracted from 250 µL of supernatant with Trizol LS reagent (Invitrogen, Carlsbad, CA) (202). Extracted RNA was then resuspended in 100 µL of DEPC treated water, and stored at -70ºC until tested for molecular detection of influenza viral RNA (vRNA).

Prior to sample testing, a formerly reported rRT-PCR assay for Type A influenza virus detection (203) was optimized at CHS-UVG. For standardization, influenza type A viral RNA was extracted from a clinical sample provided by Dr. A. Estevez (Laboratory of Respiratory Diseases, IEIP, CHS-UVG) and was used as positive control to determine the detection limits of the assay. Transcribed RNA from cloned matrix (M) protein

(A/Guinea/fowl/HK/99/H9N2) in pDP2002 was also used as positive control (204). Plasmid DNA was transcribed with the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, WI) in accordance with manufacturer's instructions. Clinical sample RNA and transcribed RNA were quantitated by spectrophotometer (GenSpecI, Naka Instruments, Dalian, China).

All rRT-PCR reactions with matrix gene specific primers and probe were carried out using the QuantiTect Probe RT-PCR Kit (QIAGEN, Hilden, Germany) and the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). For a 25 µL reaction mixture the following conditions were used: 12.5 µL of kit-supplied 2X RT-PCR master mix, 10 pmol of each primer, 0.3 µM probe, 0.25 µL of kit-supplied enzyme mix, 6.5U RNase inhibitor and 8 µL of RNA template. Thermal cycling conditions comprised one cycle of reverse transcription at 50ºC for 30 min and 94ºC for 15 min, followed by 45 cycles of denaturation at 94ºC for 1s and a combined annealing and extension at 60ºC for 27s. Fluorescence signal was obtained at the end of each cycle after the annealing/extension step. After amplification, quantitation data were analyzed with the 7300 System SDS Software v1.4.0 (Applied Biosystems). Positive control RNA was calibrated to a Ct value between 25 and 35 for the diagnostic purposes of the assay(202).

Eight µL of RNA samples extracted from tracheal and cloacal swabs were analyzed by rRT-PCR for Type A influenza virus detection. For each rRT-PCR run, duplicates of calibrated positive control RNA and a water non-template control (NTC) were included. Extracted RNA from cloacal material was analyzed in duplicate and samples with Ct value between 20 and 35 were considered positive. For tracheal swabs, extracted RNA was tested in a single reaction and samples with a Ct value between 20

and 40 were considered presumptive positive and were re-tested for their confirmation. All samples that tested positive for the rRT-PCR assay were processed for viral isolation and molecular characterization.

2.3.4. Virus isolation and genetic characterization

For viral isolation the swab supernatant of each specimen was filtered and BHI (brain heart infusion media) supplemented with antibiotics and antimycotics was added to a volume of 1.5 mL. A 200µL volume of this mixture was then inoculated into the allantoic cavity of three, 9-day-old SPF embryonated chicken eggs (ECE) per sample. Following incubation at 37°C for 72 hours, allantoic fluid was collected and tested for the presence of virus by HA assay and by Flu Detect (Synbiotics, San Diego, CA). After three blind passages in ECE, samples without virus growth were considered negative for the presence of viable virus.

Viral RNA was extracted from 200 µL of allantoic fluid with RNeasy Mini kit (Qiagen), according to manufacturer instructions. Extracted RNA was eluted in 40 µL of RNase-free water. After cDNA preparation, full-length PCR amplification of the influenza virus segments was performed followed by direct sequencing with the BigDye terminator kit (Applied Biosystems) on ABI 3100 Avant Genetic Analyzer (Applied Biosystems) or ABI 3500 Genetic Analyzer (205). Segments that could not be sequenced from PCR products were cloned into pCR 2.1 vector using a TA cloning kit (Invitrogen) and were sequenced using vector based M13 primers. At least two sequencing reactions were prepared for each gene. Partial and full-length sequences were acquired from overlapping partial sequences obtained with forward and reverse primers. Nucleotide sequences are assigned Genbank accession numbers CY067667 to CY067682

and CY096621 to CY096724. RNA extracts from original swab samples negative for virus isolation, were subjected to direct sequencing by the same method described above, in attempt to identify other virus subtypes.

For each genome segment of each virus isolate, BLAST searches at the nucleotide level were initially performed to identify the most closely related viruses. Full-length genome segments from North American and selected Eurasian and South American viruses available at the Influenza Research database (www.fludb.org) were then obtained to be included in the phylogenetic analysis. Sequences of each segment were initially aligned by Clustal V (Lasergene v.8.1.5., DNAStar, Madison, WI) and percent identities were calculated. Sequences from representative isolates were selected and aligned with ClustalW (Lasergene). Rooted phylogenetic trees were generated by Neighbor-Joining method with 1000 bootstrap replicates using PAUP 4.0b10 (Sinauer Associates, Inc., Sunderland, MA).

2.4. Results

From February 2007 to January 2010, AIV surveillance was conducted in five locations in Guatemala, two sites on the Atlantic coast within the state of Izabal (villages of Puerto Barrios and Machacas del Mar), and three sites on the Pacific coast wetlands, in the states of Santa Rosa (villages of Monterrico and El Pumpo) and Jutiapa (village of Pasaco) (Table 2.1, Figure 2.1). 1913 tracheal and cloacal swabs were collected from 969 birds from 78 different species, representing 22 different families and 11 different orders (Table S1). 50.4% (489/969) were resident and 49.6% (480/969) were migratory bird species. Samples were produced from sport-hunter killed aquatic birds or from shorebird species of the orders Anseriformes (n=239), Gruiformes (n=4), and Pelecaniformes (n=1). The remaining samples (n=725) were collected from live captured terrestrial birds that were under study as part of West Nile virus surveillance (Morales-Betoulle, unpublished data).

Table 2.1. Sites for AIV surveillance in wild birds, Guatemala, 2007-2010.

Location	State	Site	Latitude (N)	Longitude (W)	Collection method*	Season
Atlantic Coast	Izabal	Machacas del Mar	15°45'48.10"	88°31'48.60"	A	2007-2009
		Puerto Barrios	15°43'0.00"	88°35'60.00"	A	2007-2009
Pacific Coast	Santa Rosa	Monterrico	13°53'39.00"	90°28'48.00"	A / K	2007-2010
		El Pumpo	13°53'51.80"	90°29'33.20"	K	2007-2010
	Jutiapa	Pasaco	13°53'8.60"	90°11'45.30"	K	2009

*A = Active surveillance (mist nets, live captured birds), K = Hunter-killed

Figure 2.1. Location of sample collection sites in the Atlantic (1 and 2) and the Pacific (3, 4 and 5) coasts of Guatemala.
Geographical coordinates of surveillance sites are provided in Table 2.1

The RNA extracts from collected samples were tested by rRT-PCR assay for the detection of type A influenza virus (203). From these, 2 tracheal and 30 cloacal swabs tested positive (Table 2.2) corresponding to 28 dabbling ducks (Blue-winged Teal), and 2 resident terrestrial birds: a Golden-fronted woodpecker (Piciformes) and a Brown-crested flycatcher (Passeriformes). In 2ducks, both cloacal and traqueal swabs were positive for AIV. The presence of AIV was detected every year of the study. The overall percentage of rRT-PCR positive samples (tracheal and cloacal swabs) was 1.67%.

To determine factors associated with detection of AIV in wild bird samples, rRT-PCR results were analyzed by habitat (aquatic vs. terrestrial). Detection of AIV was significantly higher in aquatic birds (11.2%) when compared to terrestrial birds (0.3%) ($p<0.0001$). For the aquatic birds, positive results were obtained for the Blue-winged Teals, thus comparisons for this species were done by specimen's age (juvenile vs. adult), sex (female vs. male).AIV detection rate was similar for both juvenile (12.5%, n=27) and adults (12.8%, n=129), and for females (14.9%, n=87) and males (10.1%, n=129). No significant differences were observed between these categories.

In Blue-winged teals, the percentage of rRT-PCR positive samples varied between sampling seasons, 10.0% (2006-07), 7.3% (2007-08), 5.2% (2008-09) and26.6% (2009-10). The number of sampled aquatic birds ranged between 61 to 96 birds per season, with the exception of the 2006-07 season in which only 10 samples were obtained. The proportion of positive samples detected in the 2009-10 season was significantly higher ($p<0.0009$) compared to the previous seasons. To investigate if AIV prevalence varied during the migratory season, we compared AIV rRT-PCR detection frequencies at the beginning and the end of the seasons. The proportion of AIV positives obtained from October through December (Southern migration) compared from January to March (Northern migration) was 16.7% and 9.7%, respectively; however, the difference was not statistically significant.

Virus isolation was attempted from positive rRT-PCR samples, in 9-day-old SPF ECE; 15 viruses were obtained from either the first (9 viruses), second (5 viruses) and third passage (1 virus). After 3 blind passages, no viable viruses were detected in the remaining samples. To identify the subtype of rRT-PCR positives from which viruses

could not be isolated, direct sequencing was attempted in cDNA generated from initial RNA extracts; however, no other subtypes could be identified by this method. The percentage of recovered viruses from rRT-PCR positive samples by isolation in ECE was 46.9%. The viruses were isolated only from duck samples of the species *Anas discors* (Blue-winged Teal) obtained from Santa Rosa (2008, 2010) and Jutiapa (2009). After PCR amplification and sequencing of cDNA, the viruses were classified as H7N9 (n=2), H11N2 (n=3), H3N8 (n=1), H8N4 (n=5), H5N3 (n=2), H5N4 (n=2) representing 5 HA and 5 NA different subtypes (Table 2.3). The two H5N4 isolates were found to contain also N3 NA consensus gene sequences, suggesting a mixed infection in these two samples. Sequencing results of complete virus genomes indicated that these viruses encode for the 11 protein genes known for IAV, including the 87-90 amino acid protein PB1-F2. The H7 and H5 viruses carry the typical low pathogenic cleavage sites (PENPKTRGLF and PQRETRGLF respectively)(206). The overall percentage of AIV detection in Blue-winged Teals (n=234) based on virus isolation was 6.41%. No rRT-PCR positives or virus isolates were obtained from other aquatic bird species including the northern shoveler (*Anas clypeata*, n=2), ring-neck duck (*Aythya collaris*, n=2) and black-bellied whistling duck (*Dendrocygna autumnalis*, n=1).

Genetic similarity among the internal gene segments of the Guatemalan isolates ranged from 72.5% for the non-structural gene (NS) to 100% for the matrix (M) gene. For phylogenetic analysis, identical sequences were excluded and only one representative of each sequence was used. Sequence comparison by BLAST searches and phylogenetic analysis revealed that surface glycoprotein genes of the Guatemalan isolates share sequence identity and cluster with segments of AIV strains isolated from waterfowl of

North American (Figure 2.2 to 2.8). The HA and NA gene segments were found to be closely related to strains isolated along the Mississippi (H3N8 subtype) and Pacific American flyways (other subtypes). As expected, the internal gene segments were also phylogenetically related to North America strains. A single isolate (H3N8) carry a NS gene segment corresponding to allele B, whereas the rest carry an allele A (Figure 2.6, 2.7 and 2.8).

The Guatemalan isolates were classified among distinct clades of the North America lineage. Clade identification was supported by bootstrap values of >70% (207). 8 clades were identified for segment M, 9 for segments PB2, PB1 and NP, and 13 clades were identified for PA and NS (including alleles A and B) segments. An identification number from 1 to "n" (n = 8, 9 or 13) was assigned to each clade of each gene segment to allow inference regarding genetic similarities and reassortment events that may have occurred within a given viral subtype. Based on this analysis, 9 unique genome constellations were identified among the 15 isolates (Figure 2.9), although only on the H8N4 viruses (n=5) there was evidence of 4 different gene constellations, indicative of reassortment events. The H5N3 viruses (n=2) belonged to the same constellation except for the NP gene. For the other strains – H7N9, H11N2, and H5N4 – each subtype contained its own constellation of segments. These results suggest multiple introductions and reassortment of AIVs in wild birds in Guatemala.

Table 2.2. Distribution by order and species of wild bird samples collected in Santa Rosa, Jutiapa, and Izabal, 2007-2010

Order	Species	Common name	Status	Sampled birds*	rRT-PCR positives (%)
Anseriformes	*Anas clypeata*	Northern shoveler	M	2	
	A. discors	Blue-winged Teal	M	234	28 (12%)
	Aythya collaris	Red-neck Duck	M	2	
	Dendrocygna autumnalis	Black-bellied Whistling Duck	R	1	
Ciconiiformes	*Butorides virescens*	Green Heron	R	1	
Columbiformes	*Columba sp.*	Pigeon	R	1	
	C. livia	Feral Pigeon	R	1	
	Columbina inca	Inca Dove	R	3	
	C. minuta	Plain-Breasted Ground-Dove	R	7	
	C. passerina	Common Ground-Dove	R	1	
	C. talpacoti	Ruddy Ground-Dove	R	63	
	Leptotila plumbeiceps	Grey-Headed Dove	R	4	
	L. verreauxi	White-Tipped Dove	R	5	
	Patagioenas cayennensis	Pale-Vented Pigeon	R	2	
	Zenaida asiatica	White-Winged Dove	R	1	
	Zenaida macroura	Mourning Dove	R	6	
Coraciiformes	*Ceryle torquata*	Ringed Kingfisher	R	1	
	Eumomota superciliosa	Turquoise-Browed Motmot	R	6	
Cuculiformes	*Crotophaga sulcirostris*	Groove-Billed Ani	R	29	
	Piaya cayana	Squirrel Cuckoo	R	2	
Gruiformes	*Gallinula chloropus*	Common Moorhen	M	2	
	Laterallus ruber	Ruddy Crake	R	2	
Passeriformes	*Calocitta formosa*	White-Throated Magpie-Jay	R	2	
	Pachyramphus aglaiae	Rose-Throated Becard	R	5	
	Euphonia hirundinacea	Yellow-Throated Euphonia	R	1	
	Oryzoborus funereus	Thick-Billed Seedfinch	R	9	
	Passerina ciris	Painted Bunting	M	2	
	P. cyanea	Indigo Bunting	M	4	
	Piranga rubra	Summer Tanager	M	3	
	Ramphocelus passerinii	Scarlet-Rumped Tanager	R	2	
	Saltator atriceps	Black-Headed Saltator	R	1	
	S. coerulescens	Greyish Saltator	R	1	
	Sporophila aurita	Variable Seedeater	R	3	
	S. torqueola	White-Collared Seedeater	R	30	
	Thraupis abbas	Yellow-Winghed Tanager	R	1	
	T. episcopus	Blue-Grey Tanager	R	2	
	Volatinia jacarina	Blue-Black Grassquit	R	3	
	Dives dives	Melodius Backbird	R	7	
	Icterus galbula	Baltimore Oriole	M	8	
	I. gularis	Altamira Oriole	R	4	
	I. pectoralis	Spot-Breasted Oriole	R	1	

Order	Species	Common name	Status	Sampled birds*	rRT-PCR positives (%)
	I. spurius	Orchard Oriole	M	1	
	Psarocolius montezuma	Montezuma Oropendola	R	1	
	Quiscalus mexicanus	Great-Tailed Grackle	R	45	
	Scaphidura oryzivora	Giant Cowbird	R	5	
	Dumetella carolinensis	Grey Catbird	M	55	
	Catharus ustulatus	Swainson's Thrush	M	46	
	Hylocichla mustelina	Wood Thrush	M	2	
	Turdus grayi	Clay-Colored Thrush	R	154	
	Dendroica magnolia	Magnolia Warbler	M	2	
	D. petechia	Yellow Warbler	M	14	
	Icteria virens	Yellow-Breasted Chat	M	17	
	Seiurus aurocapillus	Ovenbird	M	5	
	S. noveboracensis	Northern Waterthrush	M	70	
	Setophaga ruticilla	American Redstart	M	1	
	Sphyrapicus varius	Yellos-Bellied Sapsucker	M	1	
	Campylorhynchus rufinucha	Rufous-Naped Wren	R	3	
	C. zonatus	Band-Backed Wren	R	2	
	Thryothorus maculipectus	Spot-Breasted Wren	R	4	
	T. pleurostictus	Banded Wren	R	1	
	Contopus cinereus	Tropical Pewee	R	1	
	Elaenia flavogaster	Yellos-Bellied Elaenia	R	1	
	Empidonax sp.	Flycatcher	M	1	
	E. trailli	Willow Flycatcher	M	3	
	Mionectes oleaginus assimilis	Ochre-Bellied Flycatcher	R	1	
	Myiarchus crinitus	Great Crested Flycatcher	M	1	
	M. nuttingi	Nutting's Flycatcher	R	2	
	M. tuberculifer	Dusky-Capped Flycatcher	R	1	
	M. tyrannulus	Brown-Crested Flycatcher	R	4	1 (25%)
	Myiozetetes similis	Social Flycatcher	R	4	
	Pitangus sulphuratus	Great Kiskadee	R	14	
	Tyrannus melancholicus	Tropical Kingbird	R	6	
	Vireo griseus	White-Eyed Vireo	M	2	
	V. olivaceus	Red-Eyed Vireo	M	2	
Psittaciformes	*Aratinga astec*	Aztec Parakeet	R	2	
Pelecaniformes	*Phalacrocorax auritus*	Double-crested Cormoran	R	1	
Piciformes	*Melanerpes aurifrons*	Golden-Fronted Woodpecker	R	23	1 (4.3%)
Strigiformes	*Glaucidium brasilianum*	Ferruginous Pygmy-Owl	R	6	
TOTAL				969	30 (3.1%)

*Cloacal and tracheal swab samples were taken from each individual, with few exceptions depending on the size of the specimens, when the bird was too small only cloacal swab was taken. M: Migratory, R: Resident.

Table 2.3. Positive species for influenza type A by rRT-PCR and viral isolates obtained in this study.

Season	Location	Species	# Birds sampled	Positives (%)		Virus subtypes
				rRT-PCR*	VI	
2006-2007	Santa Rosa	*Anas discors*	10	1(10)	-	N/D
2007-2008	Santa Rosa	*Anas discors*	96	7(7.3)	2(2.1)	H7N9
2008-2009	Santa Rosa	*Anas discors*	61	3(4.9)	-	N/D
	Izabal	*Melanerpes aurifrons*	21	1(4.8)	-	N/D
		Myiarchus tyrannulus	1	1(100)	-	N/D
2009-2010	Jutiapa	*Anas discors*	20	4(20)	3(1.5)	H11N2
	Santa Rosa	*Anas discors*	47	13(27.7)	10(21.3)	H8N4 (5), H5N3 (2), H5N4 (2), H3N8 (1)
Total				30	15(1.6)	6

*Percentage of positive samples obtained by real-time RT-PCR (rRT-PCR) and Virus Isolation (VI) based on the total number of sampled birds.
N/D: Non-Determined

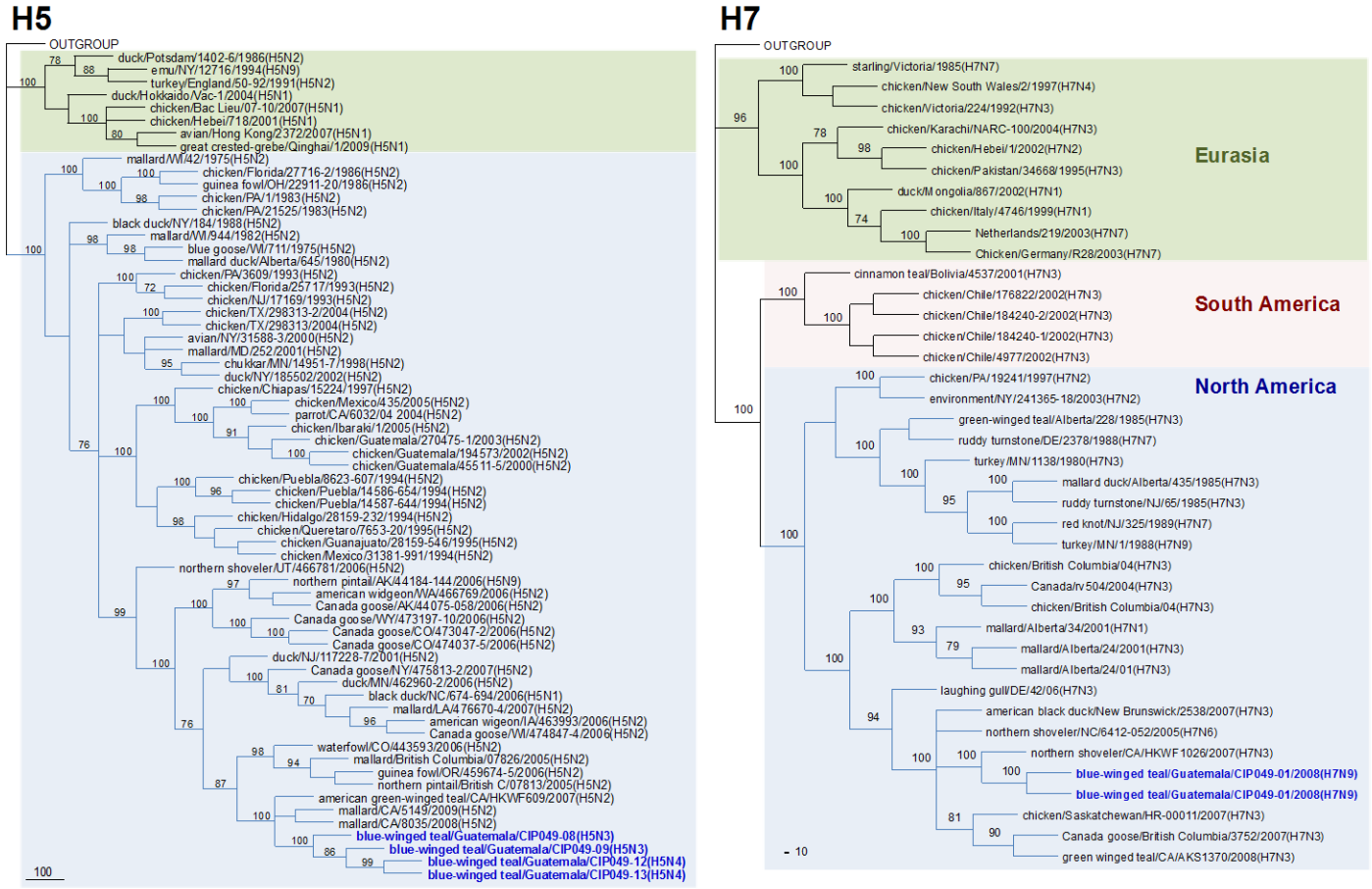

Figure 2.2. Phylogenetic trees for H5 and H7 HA genes.
Trees were generated in PAUP 4.0b10 using the Neighbor-Joining method with 1000 bootstrap replicates (bootstrap values above 70% are shown).

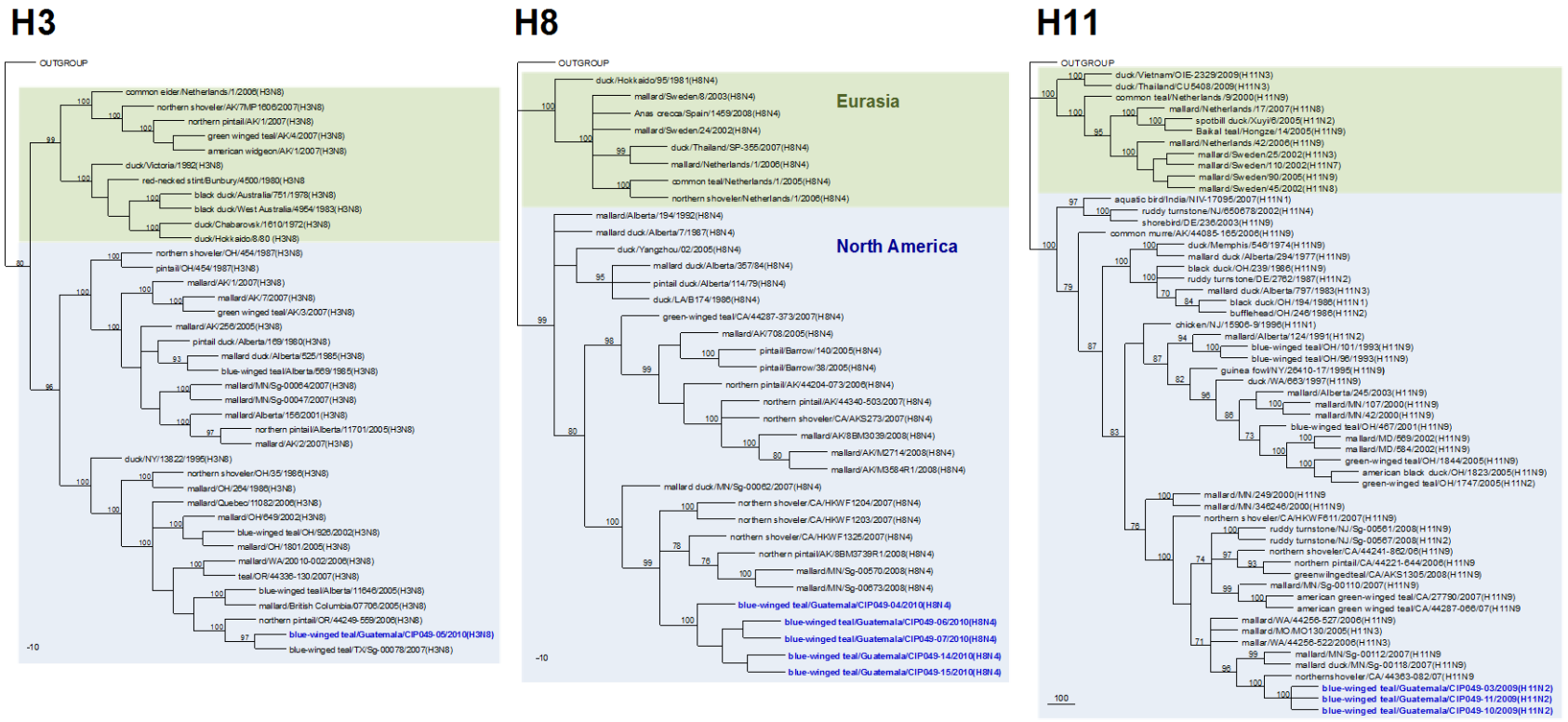

Figure 2.3. Phylogenetic trees for H3, H8 and H11 HA genes.
Neighbor-Joining inference with 1000 bootstrap replicates (bootstrap values above 70% are shown).

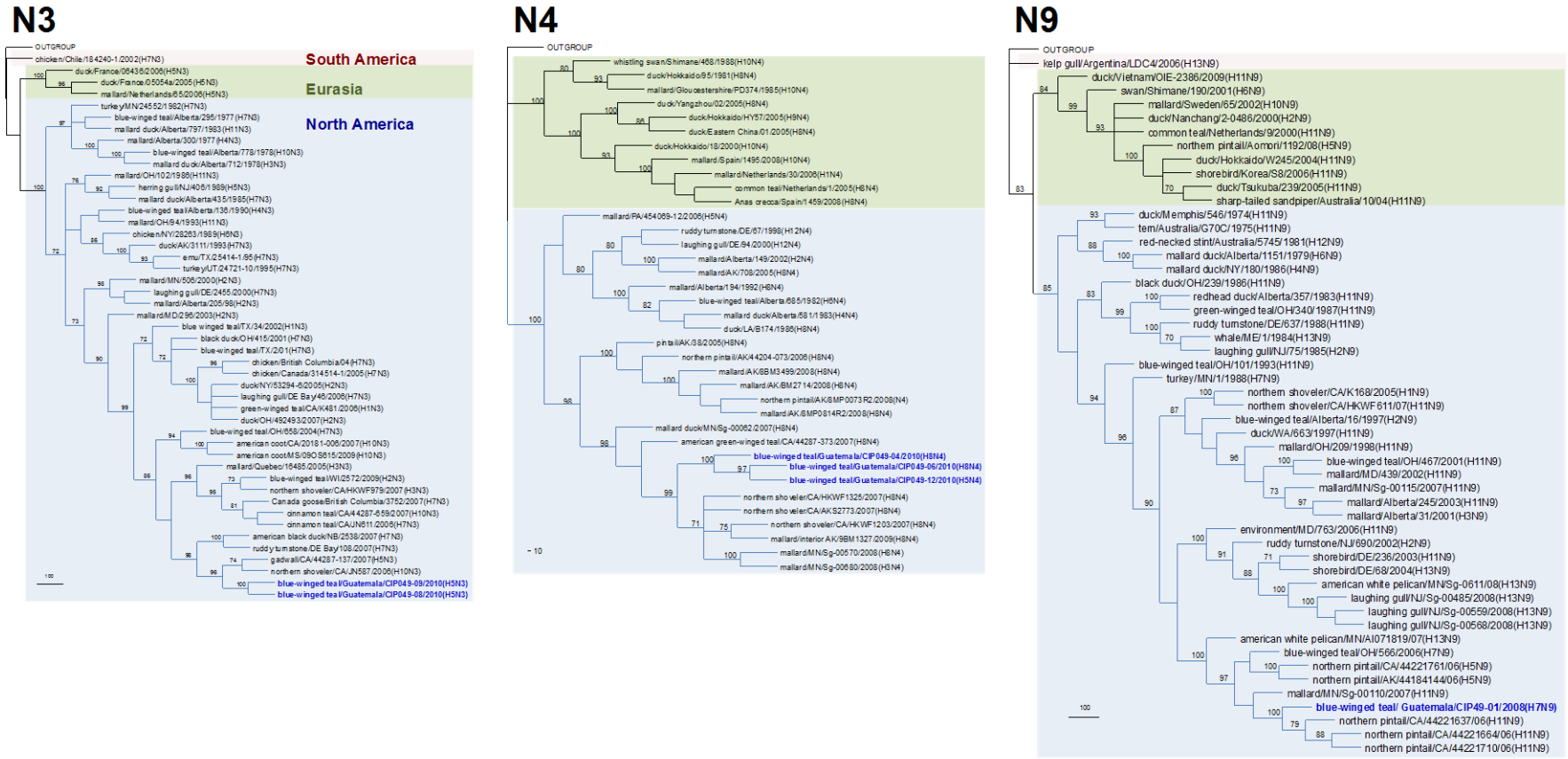

Figure 2.4. Phylogenetic trees for N3, N4 and N9 NA genes.
Neighbor-Joining inference with 1000 bootstrap replicates (bootstrap values above 70% are shown).

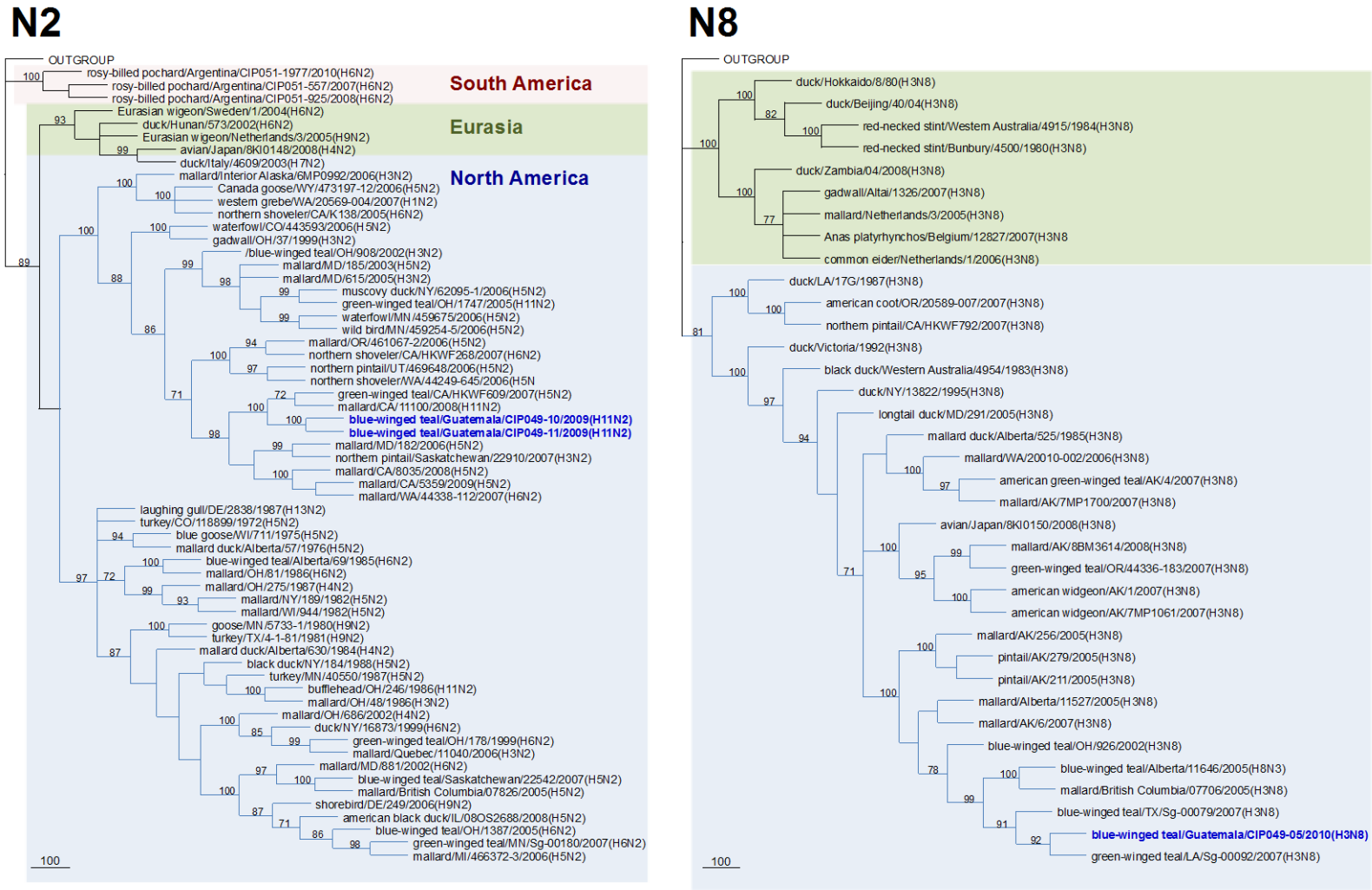

Figure 2.5. Phylogenetic trees for N2 and N8 NA genes.
Neighbor-Joining inference with 1000 bootstrap replicates (bootstrap values above 70% are shown).

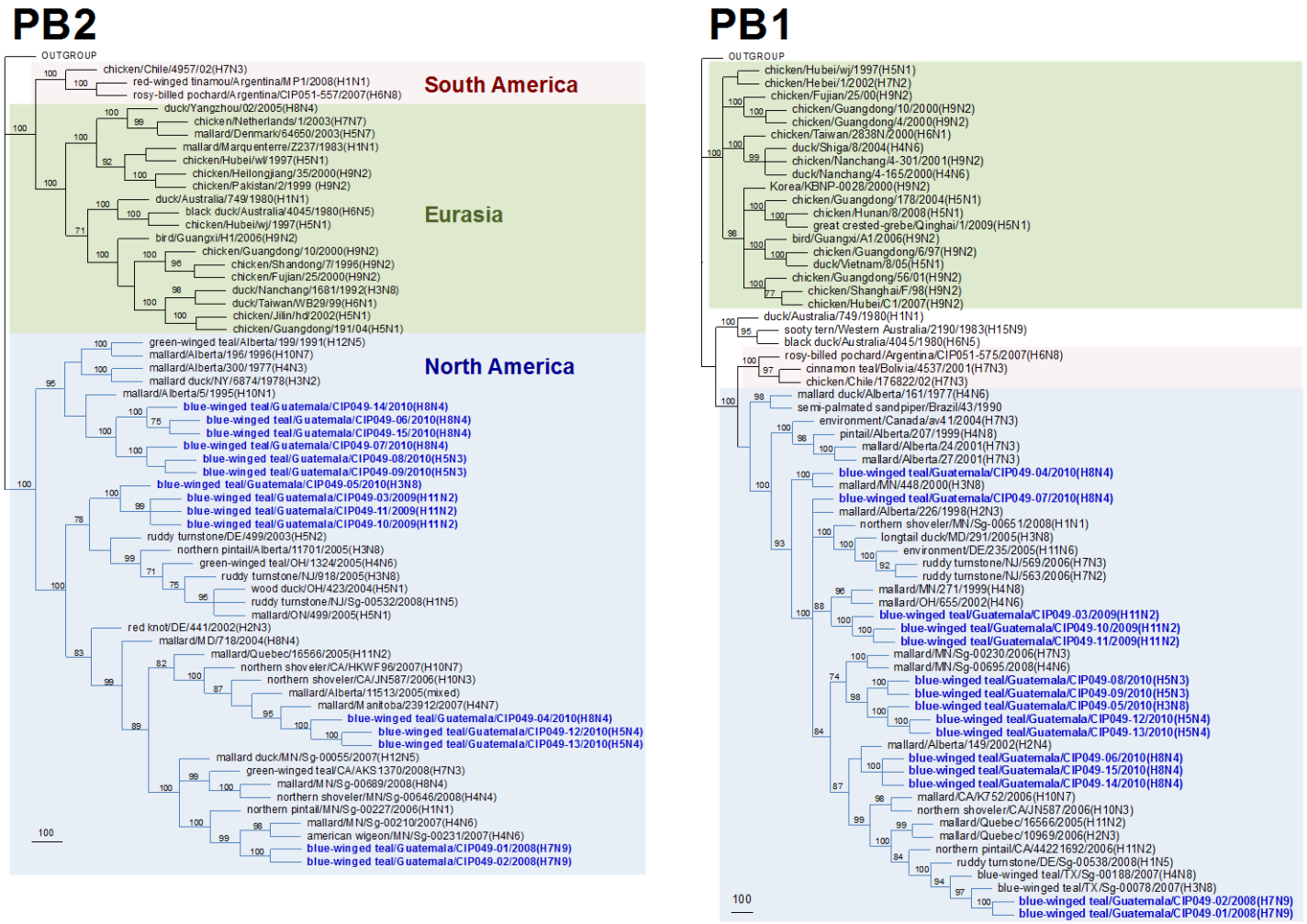

Figure 2.6. Phylogenetic trees for internal gene segments PB2 and PB1.
Neighbor-Joining inference with 1000 bootstrap replicates (bootstrap values above 70% are shown).

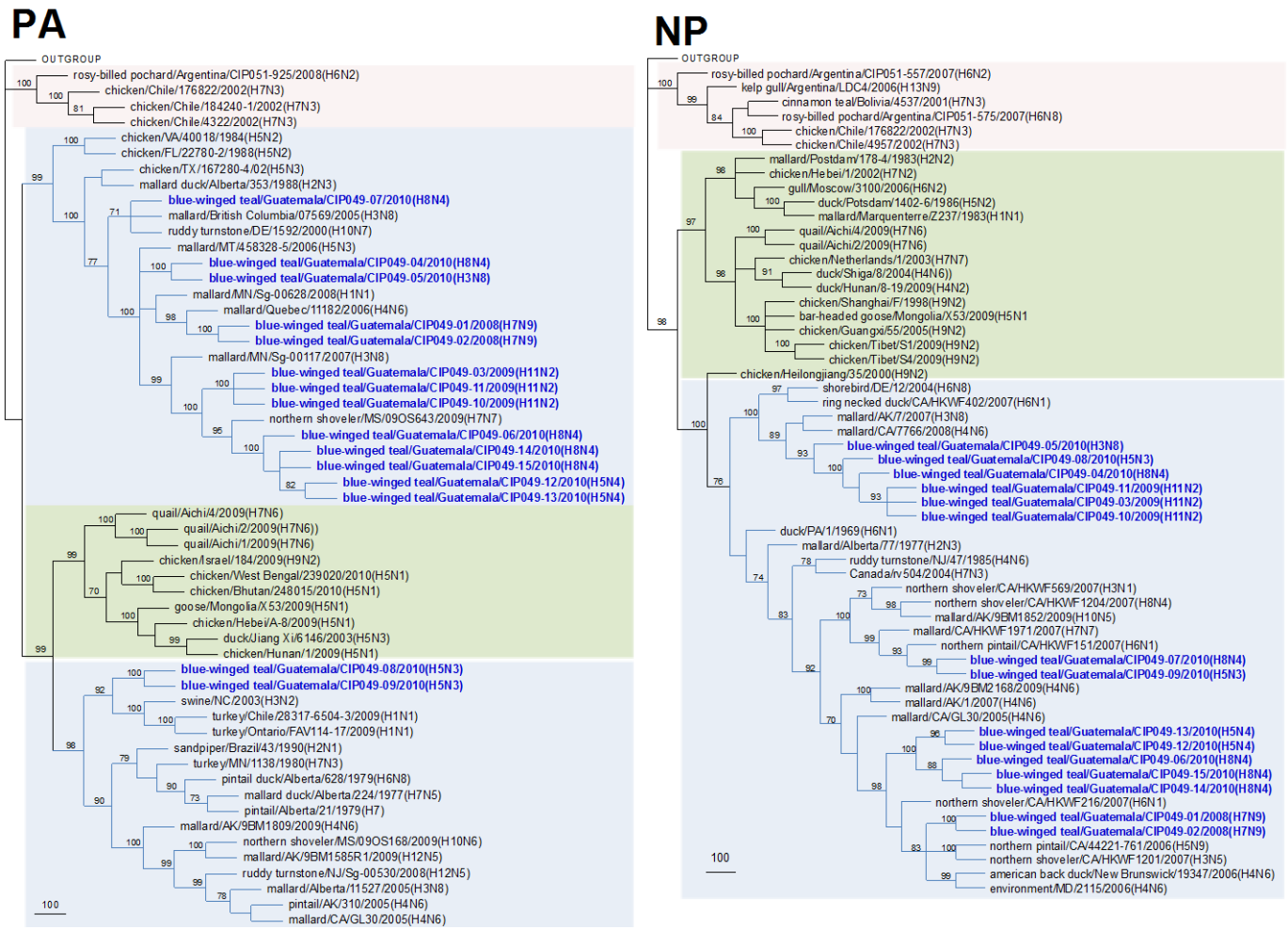

Figure 2.7. Phylogenetic trees for internal gene segments PA and NP
Neighbor-Joining inference with 1000 bootstrap replicates (bootstrap values above 70% are shown).

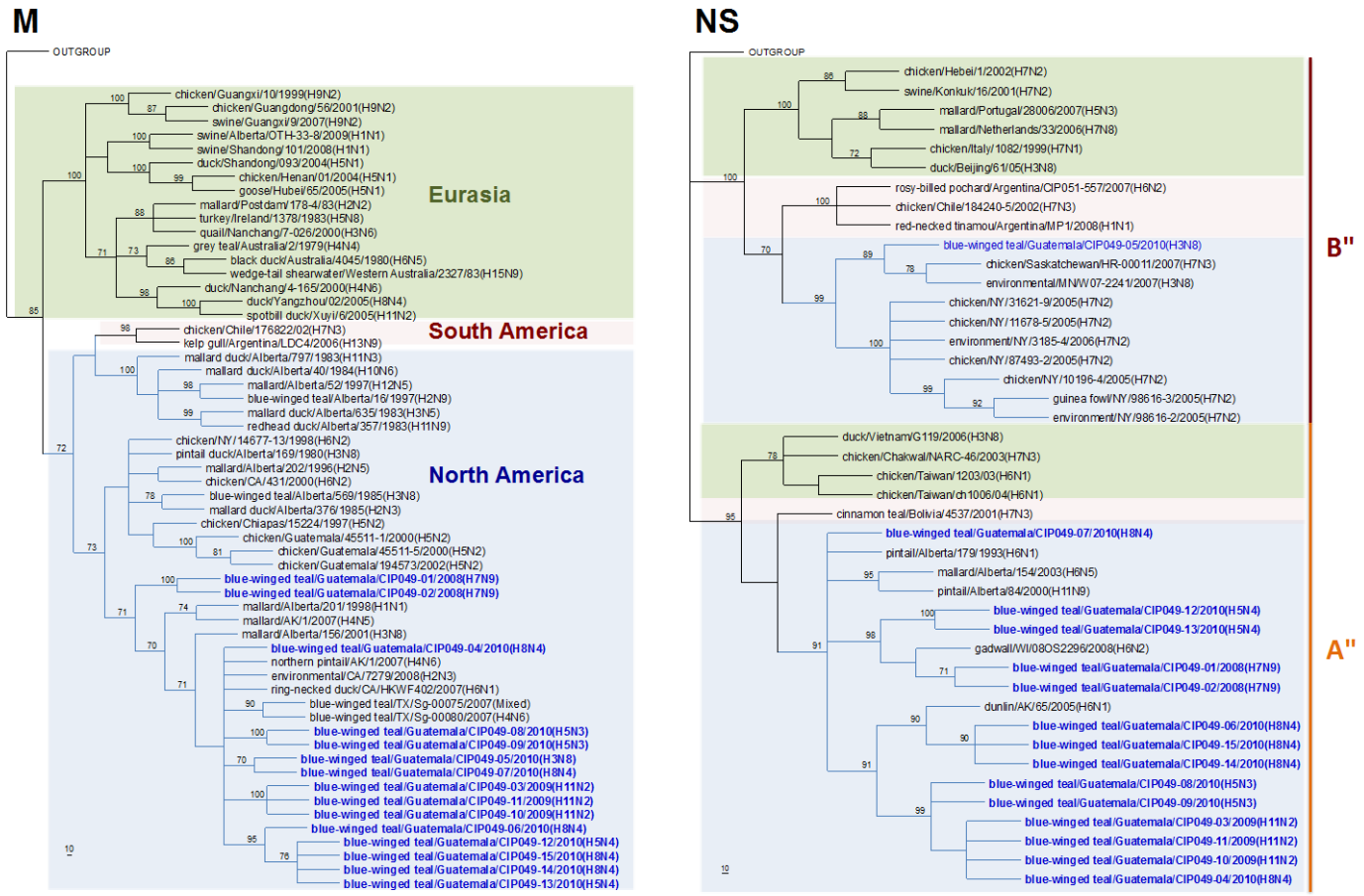

Figure 2.8. Phylogenetic trees for internal gene segments M and NS.
Neighbor-Joining inference with 1000 bootstrap replicates (bootstrap values above 70% are shown).

Subtype	Virus	Percent similarity to reference isolate					
		PB2	PB1	PA	NP	M	NS
H7N9	CIP049-01*	-	-	-	-	-	-
	CIP049-02	99.6	99.7	99.5	99.2	100	99.9
H11N2	CIP049-03	95.0	95.7	97.3	93.1	97.6	96.6
	CIP049-10	95.0	95.4	97.3	93.1	97.6	96.6
	CIP049-11	95.0	95.3	97.3	93.1	97.6	96.6
H8N4	CIP049-04	97.0	94.9	97.4	93.2	97.3	96.4
	CIP049-06	92.4	97.2	97.2	96.4	97.6	96.5
	CIP049-07	92.4	96.8	96	94.3	97.5	97.5
	CIP049-14	92.5	97.2	97.2	96.4	97.6	96.6
	CIP049-15	92.4	97.2	97.2	96.4	97.6	96.4
H3N8	CIP049-05	95.0	96.8	97.3	92.4	97.6	72.5
H5N3	CIP049-08	92.3	97.2	88.6	93.2	96.9	96.7
	CIP049-09	92.2	97.3	88.6	94.1	97.1	97.5
H5N4	CIP049-12	96.8	96.8	97.1	96.5	97.6	98.9
	CIP049-13	96.8	96.8	97.1	96.5	97.5	98.9
Total number of clades		4	6	6	4	2	6

Clades:	1	2	3	4	5	6	7	8	9	10	11	12	13

Figure 2.9. Genome constellations of AIVs obtained from wild birds in Guatemala.
Nucleotide percent similarities are shown. The different colors represent different clades supported by bootstrap values >70%. *Isolate CIP049-01 was used as reference to estimate sequence percent similarities.

2.5. Discussion

In Central America, the ecology of AIVs is not well understood. In order to collect surveillance data from this understudied region, several variables were considered to evaluate the presence/absence, diversity, and seasonality of AIVs. Such variables included type of sample(s), target species and study sites. Based on these variables, hunter-harvested waterfowl and terrestrial bird species that were under surveillance for other zoonotic diseases were chosen as target population. Sampling hunter-harvested waterfowl is a convenient method to collect bird samples during the migration season. In addition, sampling of terrestrial birds associated with coastal and aquatic habitats may provide more insights into the role of these species as vectors between aquatic and poultry species.

In this study, AIV was detected by rRT-PCR in tracheal and cloacal swabs from migratory ducks from all the study sites where samples were collected. The majority of rRT-PCR positive samples, and consequently all virus isolates, were obtained from the wild duck species *Anas discors* (Blue-winged teal) sampled in wintering seasons (from late October to early March). The hypothesis that dabbling ducks play an important role in maintaining AIV transmission in nature by feeding on the water surface is supported by surveillance studies in wild ducks and environmental sampling (53, 91). In our case, the overall proportion of rRT-PCR positive birds obtained in this study is in agreement with findings reported for other geographical regions. Specifically, we found rRT-PCR positive samples in 11.2% of waterfowl. The reported prevalence of LPAI in sampled waterfowl ranges between 0.03 to 22.2% (93, 208, 209). In the case of waterfowl, this prevalence value tends to be higher after breeding in temperate zones and peaks between

late summer and early fall before migration occurs (82). Our results are in agreement with other studies, particularly for Blue-winged teals, reported prevalence estimates ranges from 6.6% to 10.9% in temperate zones (96) and from 4.2% up to 22% in wintering grounds in North America(97, 98, 210). In our study, the proportion of AIV Blue-winged teals detected in the early months of the migratory season was apparently higher than the proportion of positives at the end of the season. Although the difference was not statistically significant this finding could support the previous observations that the AIV prevalence decreases during migration (93). However, approximately 10% of the ducks were infected close to the end of the migration period, suggesting that these birds could still carry a significant amount of AIV prior to returning to the temperate zones. Our findings are further supported by other studies in wintering areas in the United States, where late winter infection in this particular species at relatively high prevalence (>10%) has been observed (98).

When the prevalence values for AIV in Blue-winged teals were compared by age groups (juvenile or adult), no significant differences were observed, which is in contrast to what has been reported elsewhere where juvenile birds tend to harbor higher prevalence of infection than adults (93, 97, 211). This discrepancy may be explained by the limited sample size of our study (n=27 for juvenile and n=129 for adult teals), which may have resulted in low power in the statistical analyses. The Blue-winged teal is a dabbling duck species that performs a long-distance migration to Central America, the Caribbean, and some areas of South America. It is one of the first species to migrate south and one of the last to return to the north (212, 213). In Guatemala, the blue-wing teal is one of the most abundant of the 16 Anseriformes species reported, with daily

counts as high as 8,000 individuals during the last months of the wintering season (214). Their early migration to the south together with other behavioral and ecological factors may influence the role of blue winged teals as reservoirs for AIV (215, 216). The impact that long-distance traveling may have on their immunological status (216) may also contribute to explain the fact that adults in this study were found infected in a similar proportion to juvenile birds. This could be important as this may increase the number of available reservoirs for virus infection at the migration sites.

An increase in virus detection was observed in the 2009-10 sampling season, in which one sampling site was added (Jutiapa). However, the majority of positive samples came from the site in Santa Rosa, where no significant changes occurred in the number of collected samples compared to the previous years. As it has been described previously, the increase in virus detection/prevalence could be related to a seasonal pattern followed by some influenza viruses (93). Only long-term surveillance together with the implementation of more systematic sampling methodologies will provide more and better surveillance data to support this finding.

In this study, a wide diversity of virus subtypes was observed in Blue-winged teals in3 out of the 4 sampling seasons. Interestingly, even though the AIV subtypes isolated in Guatemala have been isolated in North America with the same HA/NA combinations, most of them have been isolated only sporadically or at low frequencies. Some of these subtypes, such as the H5N4 has been reported only once (93). Here the most detected subtype combination was H8N4 (5 out of 15 isolates). In contrast, other subtypes more prevalent in North America, such as H4 and H6, were not detected in our study (215). It is important to note that the number of sampled species, seasonal variation

as well as the adaptation of the viruses to different environmental conditions may influence the diversity and prevalence of isolated subtypes. Despite the limited number of sampled birds and isolates obtained, the fact that these "low prevalent" subtypes for North America were most frequently detected in Guatemala warns of the possibility that stopover habitats could function as repositories for maintenance of subtypes and genetic diversity. Our findings are supported by other studies at wintering areas in Texas, where the subtype diversity was mainly represented by non-frequently occurring subtypes, including the H8 (98).

Both H5 and H7 subtypes were isolated during this study. Although H5N1 and H7N3 subtypes are of most interest for their association with emergence of HP strains (207), other combinations such as the ones isolated here (e.g. H5N3) have been related to outbreaks of LP AIV in turkey farms and other poultry species(217). Further characterization of the pathogenicity of the viruses in chickens and other avian models in the laboratory could help address the significance and potential impact on poultry population of the circulation of H5 and H7 subtypes in the region.

As revealed by phylogenetic analysis the Guatemalan isolates are more closely related to recent isolates from the Mississippi and Pacific American flyways. Although there is limited sequence data and information of AIV viruses circulating in wild bird populations of adjacent territories (Mexico and other Central and South American countries), this observation bolster the possibility that the viruses are being introduced or more likely in constant exchange by migratory birds coming from the North.

For the internal genes (PB2, PB1, PA, NP and NS) the nucleotide sequences exhibited higher diversity as evidenced by the number of genome constellations. This

observation suggests that there have been multiple AIV introductions into the coastal sites in Guatemala. In addition, the finding of two H5 strains with similar HA genes but with different NA subtypes (N3 and N4), represents potential evidence for reassortment between viruses at the site of sample collection. These observations are consistent with other studies where frequent reassortment has been found to occur between viruses recovered from the same sites (207) over several years (65, 218), and supports the idea of independent reassortment between gene segments and continuous virus introduction and exchange by wild birds.

Recently, several AIV isolates from North and South American countries including Mexico, Argentina, Chile, and Bolivia have been described (70, 125, 197, 219) . In addition, there is evidence that South American viruses constitute a genetic subgroup distinct from other influenza viruses (55). The occurrence and frequency of reassortment between these two lineages or genetic groups and/or the exchange of virus between North and South American territories remains unknown. The high frequency of detection and genetic diversity reflects multiple AIV introductions from numerous waterfowl populations from North America occurring each year. Virus exchange between migration flyways at wintering grounds could result in virus reassortment upon bird's arrival to temperate zones. In the case of Blue-winged teals, it is not entirely clear whether conspecific populations breed in the tropics, as it has been observed that some small groups of ducks do not return north after the fall migration (220, 221). As competent reservoirs, these conspecific populations could play an important role in not only introducing AIV subtypes into the tropics, but also transmitting and perpetuating them

among local bird populations during the non-migration seasons. Further sampling during the non-migration season is essential to confirm this hypothesis.

In addition to AIV detection and isolation from wild aquatic birds, two positive samples were obtained from a single flycatcher and a woodpecker, both of which are non-migratory species. However, virus presence in these samples could not be confirmed by virus isolation or direct sequencing of cDNA. The significance of AIV RNA detection in two non-aquatic resident species needs further investigation, including virological and serological surveillance in these species could provide more insights on the importance of these birds as reservoirs of AIV.

The tropical wetlands and forest of Guatemala are regions with great diversity of avian species (222). The impact of AIV circulation in a high species diversity ecosystem such as the neotropics needs further study. Only prolonged research of influenza viruses in Central America and other South American territories will provide insight into the seasonality, molecular evolution and exchange of genetic material between South and North American viruses carried by avian hosts. Moreover, the study sites are located near rural communities with scarce resources where the habitants often depend on poultry farming and live in close contact with their domestic and free-ranged animals, as well as wild animals. Summed to this, cultural background and limited resources hinder the establishment of adequate biosecurity practices. In this context, considering the geographic spread of HPAI H5N1 (223), and the frequency of outbreaks of H5 and H7 (LPAI and HPAI) viruses in different regions worldwide (55, 91, 224-233), the presence of H7 and H5 viruses in wild birds crossing into Central America represents a threat to domestic fowl that cannot be ignored.

In summary, 15 isolates of LPAI from 6 different subtypes including H5 and H7 were recovered from wild aquatic birds in Guatemala. Most of the isolated subtypes constitute a group of viruses that have been sporadically found in other geographical regions. Phylogenetic analysis suggests that these viruses are genetically similar to North American strains. Our findings provide the first description of LPAI isolated from wild birds in Central America, and provide clear evidence of frequent introduction and exchange of AIV in the Neotropical ecosystems by migratory birds. These findings highlight the importance of continued surveillance efforts of AIV not only in wild but also domestic birds in the central and southern western hemisphere.

2.6. Acknownledgements

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Chapter 3. Where do avian influenza A viruses meet in the Americas?

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3.1. Abstract

Avian influenza virus (AIV) surveillance has been scarce in most countries of Latin America and the Caribbean. Historically, surveillance efforts of avian influenza in Central and South America have been localized in places where outbreaks in poultry have occurred. Since the emergence of the H5N1 subtype in Asia, active surveillance in wild birds has increased in a number of Latin American countries including Barbados, Guatemala, Argentina, Brazil, Mexico and Peru. A broad diversity of virus subtypes has been detected; however, nucleotide sequence data is still limited in comparison to other regions of the world. Here we review the current knowledge of AIV in Latin America, including phylogenetic relationships among publicly available viral genomes. Overall AIV reports are sparse across the region and the co-circulation of two distinct genetic lineages is puzzling. Phylogenetic analysis reflects bias in time and location where sampling has been conducted. Increased surveillance is needed to address the major determinants for AIV ecology, evolution and transmission in the region.

3.2. Introduction

In North America, particularly in the United States and Canada, AIV surveillance programs were developed in the 1970's and are currently well established. A large number of samples is collected every year and significant nucleotide sequence data is available. In contrast, in Latin America and the Caribbean[1], AIV surveillance has been scarce, localized mainly in places where outbreaks in commercial poultry have occurred.

The earliest report of AIV in Latin America and the Caribbean dates from 1970s from Brazil (234-237) where the presence of virus was detected in resident and migratory birds, and in domestic fowl, by the hemmaglutination inhibition (HI) assay. Later, the virus was detected in wild ducks (*Dendrocygna viduata*, white-faced whistling duck) and in exotic birds, and isolated from samples collected between 1997 and 1998 from resident and migratory birds (238, 239). There is no virus subtype or sequence data associated with these reports. The limited number of AIV sequences available from Brazil comes from a large-scale sequence analysis study, from which sample collection methodologies are not described (218).

In 1994, in Mexico, a strain of the H5N2 subtype of low pathogenicity avian influenza (LPAI) emerged in poultry farms (240). The virus evolved into a highly pathogenic avian influenza (HPAI) strain, which has been controlled mostly by the use of vaccination. Although to date there is no evidence of circulation of the HPAI H5N2 strain in Mexico, the LPAI H5N2 strain continues to circulate, which ultimately resulted in spill

[1] In this article, the United Nations definitions for the sub-regions of Latin America and the Caribbean were used, in which Latin America comprises all countries of Central America (from Mexico to Panama) and South America (from Colombia to Argentina).

over neighboring Central American countries including Guatemala and El Salvador (192).

In 2002, an outbreak of HPAI H7N3 in poultry occurred in Chile. The same virus subtype isolated from a species of wild duck (*Anas cyanoptera*, Cinnamon Teal) in Bolivia provided evidence that the outbreak strain had originated from a LPAI virus in wild birds and transmitted to poultry before acquiring the HPAI phenotype (55) and that the virus contained genetically divergent sequences from avian viruses from the North American lineage (125, 197).

The emergence of HPAI H5N1 subtype in Asia and its unprecedented geographic spread triggered the interest of countries in Latin America and the Caribbean into programs aimed at early detection of the virus. Such mechanisms were set in motion at the Latin America and the Caribbean Hemispheric Conference on Avian Influenza that took place in Brasilia, Brazil from November 30 to December 2, 2005. In some countries, this initial interest expanded into more active surveillance efforts. For example, a two year surveillance study in Barbados (241) yield the presence of a H4N3 subtype virus that was isolated from Blue-Winged Teal (*Anas discors*). More recently, two active surveillance studies were initiated in Guatemala and Argentina, aimed at detecting and identifying circulating AIVs in wild birds. In these two countries, sample collection included hunter-killed and captured wild birds (70, 242). The first virus isolate reported from Argentina provided evidence of a potential unique phylogenetic sub-lineage in South America. This finding was later confirmed as more isolates were obtained from other subtypes and bird species (190, 243). In contrast, the viruses identified in Guatemala, Central America were found to be closely related to viruses from the North

American Lineage. Additionally, virus isolates from Peru (198, 244) were also reported to be more closely related to the North American lineage. Additional reports of AIV from wild birds in Chile and Brazil have been published, however the molecular characteristics of those viruses have not been described (233). Overall these studies have provided evidence of AIV circulation in understudied regions. A high diversity virus subtypes from multiple bird species has been described, however, the amount of sequence data is still limited and does not reflect the number of reports. To date the geographical extension of the North American and the South American sub-lineages is unknown as well as the extent of exchange of genetic material between them.

In this manuscript we conducted a review of AIV reports from Latin America. Available sequence data, virus and host species diversity per geographic location and time were analyzed. In addition, we performed phylogenetic analysis of AIV isolated in Latin America with two specific aims: 1) to provide an updated phylogeny of the viruses circulating in Latin America and 2) to detect reassortment events between sub-lineages within available data. As additional nucleotide sequence data is generated, these studies are necessary to better understand and assess the risk of AIV for the region, and define geographic areas, bird species and sampling times that could provide crucial information on the evolutionary history and ecology of AIVs in the Americas.

3.3. Materials and methods

A literature review for AIV in Latin America was performed from indexed sources available online. Reported virus subtypes, accessible sequence data, and host species diversity were summarized per geographic location (country) and year of sample collection. The Influenza Research Database (IRD) was used to perform nucleotide searches (245). The data set included sequences from Mexico, Guatemala, El Salvador, Barbados, Peru, Brazil, Bolivia, Chile, and Argentina. Duplicate or identical sequences (100% identity) were removed from the dataset and one representative for each virus subtype, location, date and species was selected when multiple sequences were available from one single location and year. Sets of coding region sequences were downloaded and used for phylogenetic analysis. Sequences were aligned using MUSCLE with default parameters (246). The best-fit nucleotide substitution models were selected individually for each gene by jModeltest (247, 248) GTR+Γ (PB2, PA, M genes) or GTR+Γ+I (PB1 and NP genes) and TVM+I+G (NS gene). Phylogenetic trees were then constructed using the Bayesian inference method with MrBayes v.3.2 (249) run for 3.0 to 6.0 x 10^4 generations, sampled every 50 generations.

Two alternative methods for phylogenetic inference were tested to confirm tree topologies: Distance and Maximum Likelihood. Rooted phylogenetic trees were generated with the neighbor-joining method with Maximum Likelihood (ML) distance estimator, and 100 bootstrap replicates using PAUP 4.0b10 (Swofford, D. L. 2003, Sinauer Associates, Inc., Sunderland, MA) and Maximum Likelihood method and 100 bootstrap replicates using RAxML v 7.3.0 (250) under the GTR+Γ (PB2, PA, M) and GTRCAT (PB1, NP, NS) models of nucleotide substitution.

3.4. Results

In order to determine the extent of AIV surveillance in Latin America, we performed PubMed and Google searches using the words “avian influenza”, “surveillance”, “wild birds”, “Latin America”, “virus isolation”, “South America”, “Central America”. These searches resulted in 24 references, which are detailed below. Circulation of AIV has been found in poultry and wild birds (Figure 3.1). 14 reports contained information on specific virus subtypes, and only 8 were linked to nucleotide sequence data available on GenBank (and other related influenza virus databases) (Table 3.1). The reported hemagglutinin (HA) subtypes included H1 to H11 and H13. The neuraminidase (NA) subtypes included N1 to N5, N8, and N9 (Table 3.2). Virus isolates have been recovered from multiple bird species mostly from waterfowl species from at the orders (Anseriformes, Charadriiformes, Pelecaniformes,) and poultry (Galliformes) (Table 3.3). In addition, virus isolation and/or virus genome detection by molecular techniques has been reported in terrestrial bird species from the orders Tinamiformes, Piciformes, Apodiformes and Passeriformes in Argentina, Guatemala and Peru (190, 242, 251).

Based on sequence data entries for “North America” (excluding USA and Canada) and “South America” on IRD, records for 196 virus strains were found (up to March 2012). Thirty-nine virus sequences were removed from further analysis because they represented duplicate sequences of the same viruses. The overall proportions of virus strains from domestic and wild birds were 80.9% and 19.1% respectively (n=157). From these, only 28 (22%) were complete genomes (sequences from all 8 gene segments available). The remaining strains were represented by one to seven segment sequences.

Sequences from the surface genes (segments 4 and 6) were the most prevalent among the database followed by sequences from the matrix (M) gene (segment 7). Eighty-two percent of the strains were from Central and North America (Barbados, El Salvador, Guatemala, Mexico) and the remaining 18% from South America (Argentina, Bolivia, Brazil, Chile and Peru). The number and origin of the set of sequences selected for phylogenetic analysis is shown in Table 3.4. For the purpose of this manuscript, only sequences from the internal genes were analyzed. The high diversity of subtypes reported for the HA and NA genes makes it harder to analyze relationships among all available sequences together and data has been published regarding their phylogenetic relationships between the North and South American lineages (70, 125, 190, 242, 243).

Maximum taxon representation (i.e. major number of strains and geographic locations) was obtained for the M and the nonstructural (NS) gene segments. The generated phylogenetic trees are shown in Figure 3.2. In all phylogenetic trees in this article, the North American lineage is represented by the viruses from Guatemala and Mexico as the phylogenetic relationships among these viruses within the North American lineage have been previously established (242, 252). In the present study, all viruses derived from the H5N2 outbreak from Mexico, Guatemala and El Salvador clustered together, separated from the viruses found in wild birds in similar geographic locations. As reported, the virus from Barbados (253), and one sequence from Peru (251), clustered together with the North American Lineage. As expected, the viruses from South America clustered together in a separate sub-lineage. Interestingly, as it was previously reported (125), the M gene of A/Cinnamon teal/Bolivia/4537/2001(H7N3) clustered among the North American lineage; however, the rest of the sequences clustered among the South

American sub-lineage. Likewise, sequence analysis from the same virus subtype linked to the outbreak in poultry from Chile, A/Chicken/Chile/184240-5/2002 revealed that the M and PB2 segments were derived from a North American-lineage virus whereas the rest of the genome belonged to the South American lineage. This argument, however, does not apply to sequences from the same year and location related to the same outbreak (Figure 3.2 to 3.4). This analysis suggests that reassortment may have occurred at the time of the outbreak with another co-circulating virus. Further evidence for the circulation of viruses with mixed North and South American lineage genomes is depicted by A/semi-palmated sandpiper/Brazil/43/1990 (H2N1), whose PB2 and PA gene segments clearly belong to the North American lineage but its M gene segment is closely related to the South American lineage. The phylogenetic analysis of the NS segment reflects well separated clades for both the North and South American lineages, as well as for type of birds (wild and domestic), with no apparent exchange of NS segments between viruses from different lineages. A similar argument can be made about other internal gene segments (PB1, NP) in which no evidence of exchange of these genes was observed between lineages. Overall, topologies for phylogenetic relationships among taxa were consistent when results were compared with those from Maximum likelihood and Neighbor Joining (data not shown).

Figure 3.1. Countries of Latin America and the Caribbean from where Avian Influenza has been reported.

Mexico, Guatemala, El Salvador, Barbados, Peru, Brazil, Bolivia, Chile and Argentina. In dark grey are shown the countries where AIV has been reported from both poultry and wild birds, and in light grey, countries where AIV has been reported only from wild birds. The insert map of the western hemisphere shows a schematic representation of the migratory route of the Blue-Winged Teal, breeding areas are marked in light gray and wintering grounds are marked in dark gray.

Table 3.1. Virus subtypes and published references for Avian Influenza in Latin America and the Caribbean per year of sample collection and country.

Country	Year	Virus subtype	nt sequences available	Reference
Argentina	2007	H13N9	Yes	(70)
	2009	H1N1	Yes	(190)
	2007-2010	H6N2, H6N8, H9N2	Yes	(243, 254)
Barbados	2004	H4N3	Yes	(241)
Brazil	1971	N/A		(234)
	1977	N/A		(235)
	1982	N/A		(236, 237)
	1990	H2N1	Yes	(218)
	2005	N/A		(238)
	2007	H3		(255)
	2011	N/A		(239)
Chile	2003	H7N3	Yes	(55, 125, 256)
	2008	H5N9, H13N2		(233)
	2009	H1N1	Yes	(257)
Colombia	2002-2005	H9N2		(192)
El Salvador	2001	H5N2	Yes	(240)
Guatemala	2007-2010	H7N9, H11N2, H8N4, H5N2, H5N3, H5N4, H3N8	Yes	(240, 242)
Mexico	2006	H7N3		(219)
	1994-1998	H5N2	Yes	(240)
	2007-2009	H5		(258)
Peru	2008	H3N8, H4N5, H10N9, H13N2		(198)
	2006-2007	H7N3, H2N9		(244)

Table 3.2. Hemmaglutinin (HA) and Neuraminidase (NA) subtypes reported from Latin America and the Caribbean.

NA	HA																	Total NA
	?	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
?	1					9	1	1		1								13
1		2	1			1												4
2					1	106	3			2		3		2				116
3					2	2		18										20
4						2			5									7
5					5													5
6																		
7																		
8				3			2											5
9								2			3			1				6
Total HA	1	2	1	3	6	120	6	21	5	2	3	3		3				176

*Number estimates based on published reports (table 1) and nucleotide sequence data available on IRD.

South America (Argentina, Brazil, Chile, Peru)

Central America (El Salvador, Guatemala, Mexico)

Caribbean (Barbados)

Central and South America

Table 3.3. Avian influenza detections and isolations in Latin America and the Caribbean.

Order	Species	Common name	Country	Reference
Anseriformes	*Anas bahamensis*	White-cheeked pintail	Peru	(198)
	Anas clypeata	Northern shoveler	Mexico	(258)
	Anas cyanoptera	Cinnamon teal	Mexico, Peru	(198, 219)
	Anas discors	Blue-Winged Teal	Barbados, Guatemala	(241, 242)
	Aythya americana	Redhead	Mexico	(258)
	Dendrocygna viduata	White-faced Whistling Duck	Brazil	(239)
	Haematopus palliates	American oystercatcher	Peru	(198)
	Netta peposaca	Rosy-billed Pochard	Argentina	(243, 254)
	Unknown	wild bird	Brazil	(233)
	Unknown	wild duck	Peru	(244)
Charadriiformes	*Arenaria interpres*	Ruddy turnstone	Peru	(198)
	Calidris pusilla	Semipalmated sandpiper	Brazil	(218)
	Larus dominicanus	Kelp gull	Argentina, Peru	(70, 198)
	Numenious phaeopus	Whimbrel	Peru	(198)
	Unknown	Gull	Chile	(233)
Pelecaniformes	*Pelicanus occidentalis thagus*	Peruvian pelican	Peru	(198)
Galliformes	*Gallus gallus*	Chicken	Mexico, El Salvador, Guatemala, Chile, Colombia	(192, 240, 259)
	Meleagris gallopavo	Turkey	Chile	(257, 259)
Tinamiformes	*Rhynchotus rufescens*	Red-winged tinamous	Argentina	(190)
Passeriformes	*Campylorhamphus pucherani*	Greater Scythebill	Peru	(251)
	Elaenia mesoleuca	Olivaceous Elaenia	Brazil	(238)
	Myiarchus tyrannulus	Brown-Crested Flycatcher	Guatemala	(242)
	Vireo olivaceous	Red-eyed Vireo	Brazil	(238)
Piciformes	*Melanerpes aurifrons*	Golden-Fronted Woodpecker	Guatemala	(242)

Table 3.4. Number of nucleotide sequences per geographic location included in the phylogenetic analysis.

Country	Host	Segment					
		PB2	PB1	PA	NP	M	NS
Argentina	Wild birds	7	6	7	7	7	7
Barbados	Wild birds	-	-	-	-	1	1
Bolivia	Wild birds	1	1	1	1	1	1
Brazil	Wild birds	1	1	1	1	1	1
Chile	Poultry	4	4	5	5	8	9
El Salvador	Poultry	1	1	1	1	1	1
Guatemala	Wild birds and Poultry	17	17	14	14	14	15
Mexico	Poultry	22	31	23	21	24	25
Peru	Wild birds	-	-	-	-	1	-
TOTAL		53	61	52	50	58	60

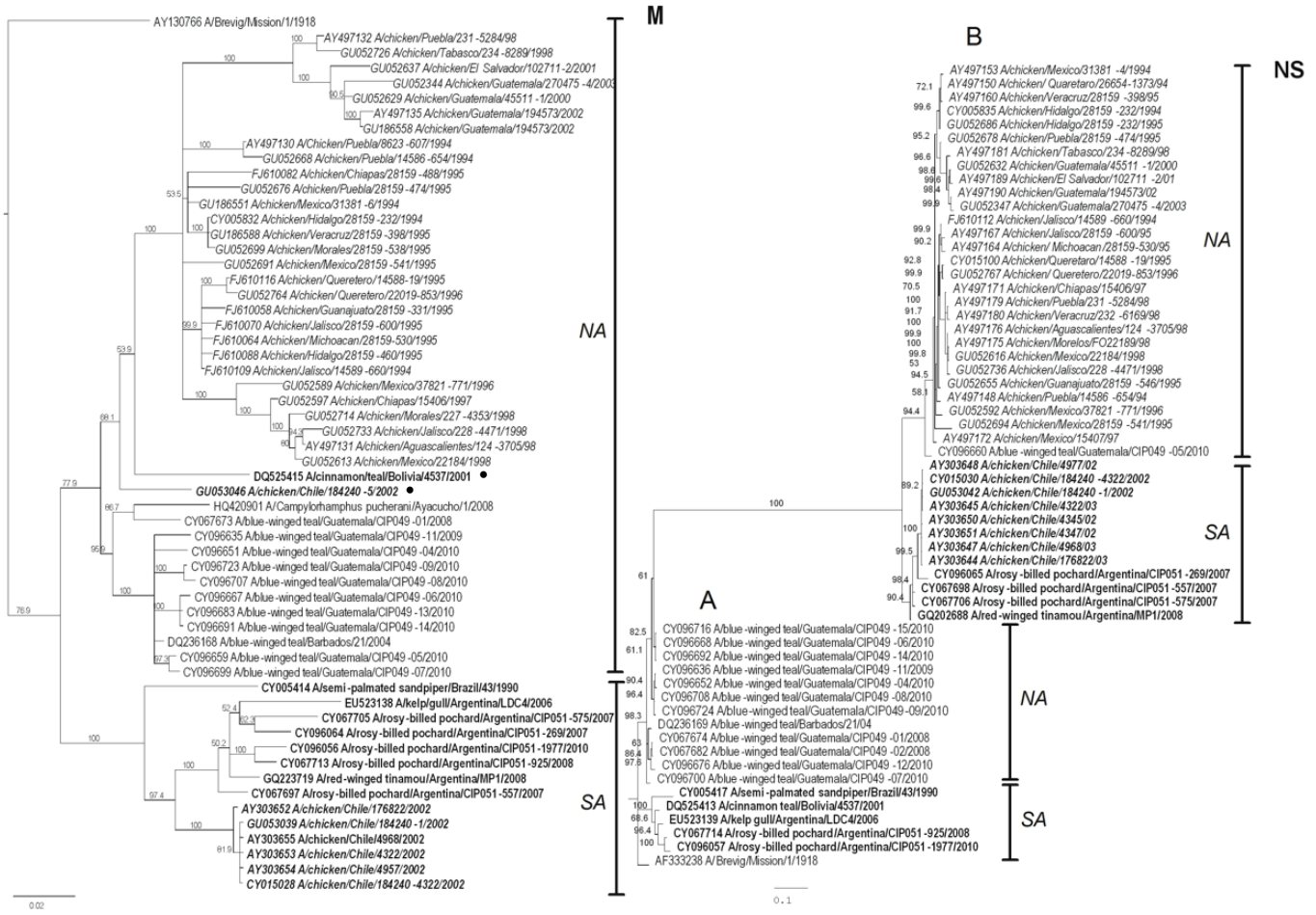

Figure 3.2. Bayesian phylogenetic trees for Matrix (M) and nonstructural (NS) gene segments of AIV sequences reported for Latin America and the Caribbean.

Percentage posterior probabilities are shown. Viruses from South America appear in bold, and from domestic birds appear in italics. Viruses from one geographic location that cluster in the opposite expected lineage are marked with •. Scale bar depicted represents number of substitutions per site.

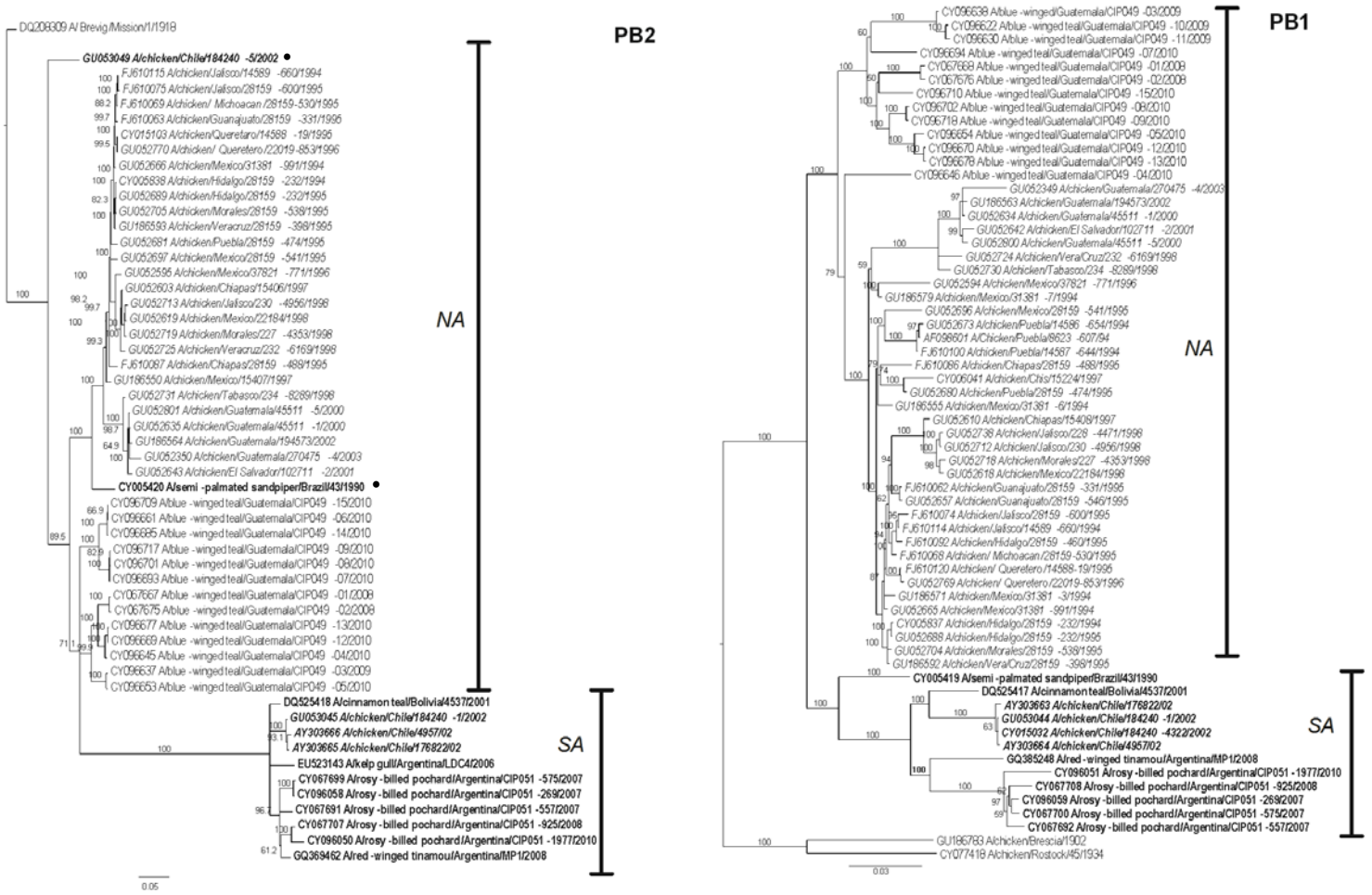

Figure 3.3. Bayesian phylogenetic trees for PB2 and PB1 internal gene segments of AIV sequences reported for Latin America.
Percentage posterior probabilities are shown. Viruses from South America appear in bold, and from domestic birds appear in italics. Viruses from one geographic location that cluster in the opposite expected lineage are marked with •. Scale bar depicted represents number of substitutions per site.

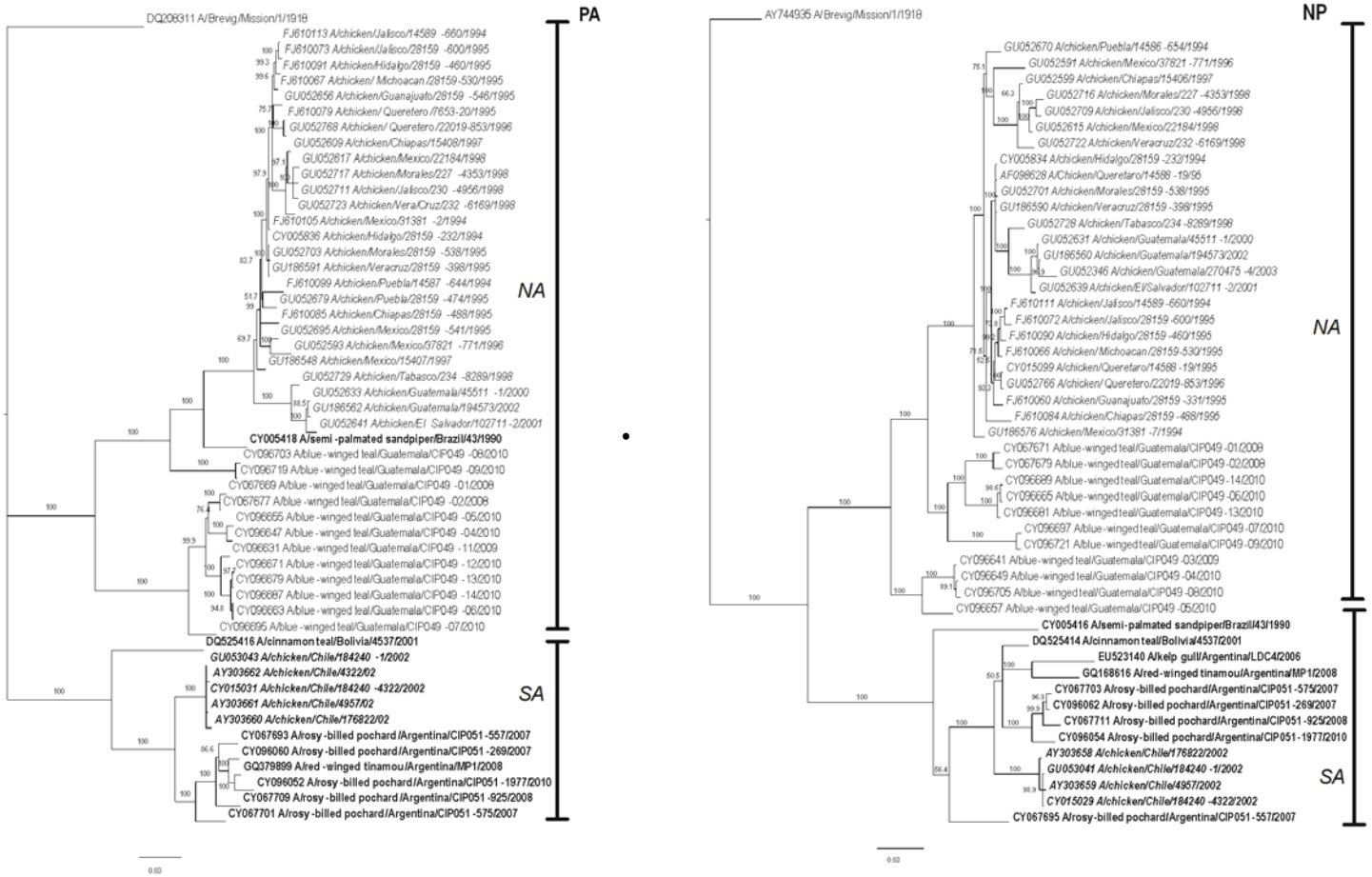

Figure 3.4. Bayesian phylogenetic trees for PA and NP internal gene segments of AIV sequences reported for Latin America.
Percentage posterior probabilities are shown. Viruses from South America appear in bold, and from domestic birds appear in italics. Viruses from one geographic location that cluster in the opposite expected lineage are marked with •. Scale bar depicted represents number of substitutions per site.

3.5. Discussion

The ecology and natural history of AIV in Latin America and the Caribbean is poorly understood (83, 91, 190). The emergence of the HPAI H5N1 in Asia led to a better recognition of the importance of AIV surveillance in the region. Plans have been put in place by animal diagnostic laboratories for the early detection of AIV in commercial poultry with an emphasis on the potential introduction of HPAI H5N1 in the region. However, efforts aimed at better defining AIV circulation in Latin America and the Caribbean continues to be limited. Lack of adequate resources, local infrastructure and more urgent animal health priorities, have somehow conspired against the establishment of long term AIV surveillance programs in the region. Under this context, it is perhaps important to note that countries that initiated long-term AIV surveillance programs have yield significant and valuable information; however, more is needed from these and other countries to better understand the ecology of AIV in this part of the world. Here we reviewed the current knowledge of AIV in Latin America and the Caribbean, and summarized available data by geographic location, bird species, and virus subtypes. Figure 3.1 summarizes the distribution of AIV reports with a list of countries conducting surveillance studies that has been increasing over time. With limited available information on the precise geographic location of sampling collection from all countries we recognize that considering an entire country on Figure 3.1, may over represent the scale of existing studies. There are still major gaps in the tropical region between Central and South American countries from where no information is available.

3.5.1. Avian influenza in poultry and wild birds.

The H5N2 and the H7N3 outbreaks in Mexico and Chile represent two examples of virus introduction from migratory waterfowl into poultry. As described by Horimoto and collaborators, the Mexican H5N2 lineage evolved from a common ancestor: a virus from the North American lineage, introduced into poultry from migratory waterfowl (252). The subsequent spread and evolution of this virus into other areas of Mexico and Central America, as a result of a spill-over event, vaccination or other events facilitated the establishment of different sub-lineages in the region (240). Besides the two poultry outbreaks in Mexico and Chile, the majority of virus isolates and virus subtypes reported for Latin America and the Caribbean, have been recovered from wild duck species, particularly Blue-Winged Teal, cinnamon teal, and other *Anas* species. The Blue-Winged Teal is likely as a potential key host for virus dispersal given its long distance migration between North and South America (83, 91). As evidenced by the studies from Central America, the viruses from Blue-Winged Teals from Guatemala comprise a wide array of genome constellations, suggesting multiple events of introduction or exchange between North and Central America from different bird migration pathways. Additional evidence from Barbados and Peru, suggest that bird migration seems to play a major role for virus spread across the American continent (198, 242, 253). Alternatively, differences in species diversity and distribution of bird populations have been suggested as possible determinants for the emergence of an independent genetic lineage in South America (70, 190).

In the context of AIV ecology in Latin America and the Caribbean, particular attention has been given to waterfowl species. However the role of terrestrial species in

transmission of AIV in different locations around the world is still controversial. In Latin America, only one virus isolate has been recovered from a wild terrestrial bird species (190) and virus RNA has been detected in a few other specimens (238, 242, 251). In the US, terrestrial birds such from the order Passeriformes have been shown to harbor relatively high rates of AIV infection (114). Moreover, the introduction and spread of West Nile virus across Central America and the Caribbean has shown that small migratory terrestrial birds may play an important role on spread of infectious diseases into new populations in the region (199, 260, 261). Under this perspective, the role of terrestrial resident and migratory bird species in AIV dispersal needs further study.

3.5.2. Phylogenetic relationships

Evidence of reassortment between viruses from the North and South American lineages has been detected for both the surface and internal genes of isolated viruses (190, 243). Reassortment occurs at low frequencies between the Eurasian and the North American lineage viruses (65, 75, 191). In South America, more data is needed in order to estimate the frequencies of such reassortment events. Based on the summarized data, subtype diversity seems to be as high as in other regions of the world. Both H7 and H5 viruses have been detected in wild bird populations (197, 242, 258). It is well known that reassortment is a major mechanism for influenza viruses to maintain genetic diversity and the existence of a separate genetic lineage provides a larger genetic pool for potential reassortment and generation of novel subtypes in the wild.

The overall topology and relationships among different viruses in the phylogenetic analysis is in agreement with results reported by the corresponding studies. However, this analysis probably reflects bias in time, location and bird populations from

which samples have been collected. This creates a scenario with unbalanced data from only a few locations and, most likely, poor taxon representation to perform a more complete analysis of the evolutionary relationships among these viruses at a continental scale. The extent of the interaction between bird species and the spread of virus lineages from both ends of the American continent is difficult to address with current available data. Underlined by the precedent of two LPAI and HPAI outbreaks in poultry, surveillance efforts in Latin America need to be expanded to include previously unstudied localities. Coordinated efforts with those countries with established capacity, would be of great benefit to better address which bird species, geographic locations as well as seasons for sampling collection could be more important to reveal the extent of reassortment between lineages and better understand the evolutionary history of AIV viruses in the Americas.

Chapter 4. Prevalence and diversity of low pathogenicity avian influenza viruses in wild ducks at the wintering grounds in Guatemala, 2010-2013

Co-authors: M. L. Muller, A.L. Ramirez, L. Ortiz, M. Angel, C. Cordon-Rosales, D. R. Perez. (Manuscript in preparation)

4.1. Abstract

Waterfowl species are known to harbor the greatest diversity of low pathogenicity influenza A virus (LPAIV) subtypes, and are recognized as their main natural reservoir. In Guatemala there is evidence of circulation of LPAIV from the North American lineage; however the bird species contributing to viral diversity during the winter migration in Central America are unknown. In this study, samples from hunter-killed waterfowl were collected at the Pacific coast of Guatemala during three winter migration seasons between 2010 and 2013. A total of 1250 birds of 22 different species were sampled. LPAIV prevalence detected by rRT-PCR was 38.2%, 23.5% and 24.7% in the 2010-11, 2011-12, and 2012-13 seasons respectively. Analysis of LPAIV prevalence over time indicated a decreasing trend in monthly prevalence during a migration season. Sixty-eight viruses were isolated and 9 HA and 7 NA subtypes were identified in 19 subtype combinations. In 2012-13 the most prevalent subtype was H14, a subtype identified for the first time in the western hemisphere in 2010. The majority of positive samples and viral isolates were obtained from the blue-winged teal (*Anas discors*). Phylogenetic analysis revealed viruses from different genetic clades, including reassortant stains with genes from the Eurasian lineage. The relatively high abundance of blue-winged teals in

comparison to other duck species, and the diversity of viruses found in these birds make them a candidate species for targeted LPAIV surveillance in the Neotropics.

4.2. Introduction

Despite the increased awareness of the need to expand global efforts for surveillance of influenza A virus (IAV) in its natural reservoirs, there are still significant knowledge gaps. This is particularly the case with respect to the viruses that circulate in Central and South America [Chapter 3, (262)]. In 2012 an IAV outbreak caused by a HPAI H7N3 strain of wild bird origin in Mexico resulted in significant economic losses for one of the biggest egg and poultry producers of Latin America (127, 257, 263), raising concerns about further virus spreading across the region and serving as a reminder of the risk of IAV spillover from wild bird populations into domestic birds.

In North America, mallards (*Anas platyrhynchos*) and northern shovelers (*Anas clypeata*) are two of the duck species with the highest prevalence of IAV (99, 103, 210, 264). In addition, the blue-winged teals have been observed to harbor high diversity of virus subtypes at the wintering grounds, in particular in locations across the Mississippi and Central migration flyways (97, 98, 100, 210, 265). Studies at the wintering grounds in North America and Europe suggest that viral prevalence in waterfowl species tend to be low in comparison to the levels observed at the breeding grounds. The number of virus infections decreases over time in the population during an annual migration cycle that concludes with the northern migration, as birds return from the wintering grounds (94, 95, 99).

The evolution of IAV reflects geographical trends regarding the distribution and behavior of the reservoir species (53). The two more clearly defined phylogeographic lineages are the North American and Eurasian lineages (67, 83, 266). The co-circulation of multiple gene variants and the extensive reassortment that occurs within a single

lineage demonstrates that IAV evolution is highly dynamic (218), illustrating the complexities of studying the genetic structure of virus populations from specific locations at any given time.

Little information is available on the dynamics of influenza circulation in the southern subtropical regions of the Americas. In Guatemala, there is evidence of circulation of IAV from the North American lineage in wild waterfowl (Chapter 2 (242)); but the main bird species that contribute to viral diversity during and between migration seasons remains poorly characterized. Temporal variation of IAV in Central America has not been studied due to the limited number of samples obtained in the past. Convergence of multiple flyways into a reduced geographical area, distinguishes the wintering grounds of Central America from those in North America (267). In Europe, IAV surveillance in wild birds in a location where multiple flyways overlap, provided evidence of increased gene flow between host populations from different geographical regions, resulting in high diversity of locally circulating viruses (268). Similarly, congregation of bird populations from multiple migration flyways into a geographical bottleneck in Central America may provide unique niches for virus reassortment during the winter migration contributing to explain the patterns of extensive reassortments observed in viruses from the North (67, 269, 270).

To better understand the virus diversity at the wintering grounds in Central America, samples from hunter-killed waterfowl were collected at the Pacific coast of Guatemala during three consecutive winter migrations between 2010 and 2013. The patterns of IAV prevalence observed during the winter migration for blue-winged teal, the most abundantly sampled bird species, are described. Detailed description of virus

diversity and the genetic structure of the virus population from Guatemala are provided. Phylogenetic analysis, genotype classification and analysis of genetic constellations were conducted in order to compare the viruses found to those from other geographical regions with overlapping migration flyways.

4.3. Materials and Methods

4.3.1. Ethics statement

Collection of samples from wild birds was approved by the Institutional Animal Use and Care Committee from Universidad del Valle de Guatemala. Permits for sampling different bird species at the different sampling sites were obtained from the Center for Conservation Studies (CECON) and the National Council of Protected Areas (CONAP). Sampling of hunter-killed birds was exempt from animal use and care regulations from the Institutional Animal Use and Care Committee of the University of Maryland.

4.3.2. Sample collection

Samples were collected from hunter-killed ducks during the winter migration season from 2010 to 2012 in the villages of el Pumpo in the department of Santa Rosa, Pasaco in the department of Jutiapa and in La Gomera in the department of Escuintla. Tracheal and cloacal swab samples were collected from birds as previously described (Chapter 2).

4.3.3. Virus detection, isolation and molecular characterization

All samples were tested for the presence of IAV RNA by rRT-PCR (Spackman, et al 2002 (203)). The details of the methods for RNA extraction, molecular testing and virus isolation are described in Chapter 2. Only IAV positive samples by rRT-PCR were tested for virus isolation.

The subtype of all viral isolates was initially identified by partial sequencing of the HA and NA genes with universal primers (205, 271). One representative of each virus

subtype (i.e. one of each different HA and NA combinations) from each sampling point was chosen for full-length genome sequencing. The full-genome sequence of the one virus isolate from 2010 was obtained by direct sequencing with the BigDye terminator kit on a ABI 3500 Genetic Analyzer also described in Chapter 2. For the viruses from 2011 to 2013, complete genome sequences from 45 viruses were obtained by pyrosequencing in a 454 GS Junior system (272). For 30 of the 45 sequenced genomes, an initial multi-segment amplification of IAV genes (M-RTPCR) (273) was performed in order to increase the number of virus specific reads. Sequencing reads were processed for shotgun run with the Junior Newbler software (v.2.7 and 3.0, Roche, Branford, CT). GS De Novo Assembler (v.3.0) was used to assemble initial contigs. BLAST searches with the initial contigs were performed to identify closest matches. Reference genomes were then downloaded from NCBI Genbank database and IAV sequence reads were re-assembled using the GS Reference Mapper software (v.3.0). Contig assembly was refined with multiple rounds of reference mapping with default parameters to obtain final consensus sequences for each virus genome.

4.3.4. Virus prevalence

Prevalence of IAV was estimated for different bird species as the number of rRT-PCR positives divided by the total number of individuals tested by rRT-PCR. Differences in prevalence between waterfowl species, sample collection sites, and seasons (2010-11, 2011-12 and 2012-13) were analyzed with χ^2 test at the 95% confidence level; only species with n>5 per sampling point were included in this analysis in GraphPad Prism v.6.0 (La Jolla, CA, www.graphpad.com). In addition, changes in prevalence over time were analyzed by univariate logistic regression for only those species and seasons with at

least 10 observations of the least expected outcome (IAV positive) for the independent variable (collection date) (274). For all analyses, a two-sided alternative hypothesis was assumed with p-values <0.05 considered significant. Logistic regression was done using the Real Statistics Resource Pack software (Release 3.2.1) for Excel (275).

In order to estimate viral diversity, the Simpson diversity index was calculated based on the number of different subtype combinations (276). Confidence intervals were estimated according to Grundmann et al, 2001 (277). Viral diversity was compared between seasons (2011-12 vs 2012-13) using the Sørensen-Dice coefficient (278, 279), given by $CC=n_{1,1}/(n_{1,0}+n_{0,1})$, where $n_{1,1}$ is the number of subtypes present in both years, $n_{1,0}$ and $n_{0,1}$ are the number of unique subtypes observed in the first and the second season respectively . A CC = 1 describes identical communities, with an increased level of differences as CC approaches to zero.

4.3.5. Phylogenetic analysis, gene constellations and genetic differentiation

Phylogenetic analysis was conducted for all internal genes. Background virus strains with full-genome sequence data, collected between 2000 and 2013 from waterfowl species from different intercontinental genetic lineages available at the Influenza Research Database (245), were included in the analysis. Sequences were selected as follows: For the North American strains, one virus per month/year/state/country (Canada, Mexico, USA) was selected (n=250). For strains of Eurasian origin (Europe, Africa, Asia, Oceania) one virus per month/year/country was selected (n=81). Due to the limited number of sequences available for Central and South America, all virus isolates available for Guatemala (n=15) and South America (n=12) were included. Coding sequences were aligned with MUSCLE in MEGA 6.0 (280). The best-fit model of nucleotide substitution

was determined for each gene by the Bayesian Information Criterion (BIC). Final phylogenetic trees were constructed using Maximum-Likelihood (ML) inference with the nucleotide substitution models, General Time reversible, with Gamma distribution and invariant sites GTR+Γ+I (PB2, PB1, PA, NP, NS), or only with gamma distribution and uniform rates GTR+Γ (NS). Robustness of tree topologies was assessed with 100 neighbor-joining bootstrap replicates.

For the surface genes, phylogenies were inferred for the H14 (one of the most abundant HA subtypes) and the N3 (the most abundant NA subtype). For the H14 subtype, a phylogenetic tree was inferred using the minimum evolution method under the best-fit model of nucleotide substitution Tajima-Nei with Gamma distribution (TN93+G) determined by the BIC criterion. In addition to the viruses from Guatemala, all other available sequences of the H14 subtype from North America and Europe were included and a few background H4 viruses. For the N3 gene additional sequences from virus isolates between 2008 and 2013 from North America were included and a maximum likelihood tree was inferred with the best-fit model of nucleotide substitution Hasegawa-Kishino-Yaho with Gamma distribution, HKY+G. Robustness of tree topology was assessed with 1000 neighbor-joining bootstrap replicates and tree topology was confirmed with a second inference with the distance method of Maximun Composite Likelihood and assessed with 1000 bootstrap replicates.

In order to study virus diversity the genotype of the viruses from Guatemala was determined with the FluGenome tool (281). To study within lineage diversity, the lineages where the viruses from Guatemala were found were subdivided in clades. A clade was defined as a group of viruses within a particular lineage supported by boostrap

values of >70% and that shared >96% identity with each other. Clades were defined for all internal genes; each clade was identified according to the segment lineage (determined by FluGenome tool) and numbered from 1 to n. For the surface genes (HA and NA) searches with full-length nucleotide sequences were performed in BLAST to identify the top 10 hits. In addition, pairwise distances were computed for each set of isolates of the same subtype in order to measure within subtype variability and identify potentially distinct variants. For this purpose, isolates with <95% identity between each other were identified as different variants. The information of variants for the surface genes was included in the analysis of gene constellations by digital genotyping (282). Gene constellations were defined as unique gene combinations at the level of clades; colors were assigned to different genes and used to build segment identity matrices.

To compare similarity of the viruses from Guatemala with those from different migration flyways, the level of genetic differentiation among virus populations from different geographic locations was estimated. The FST values were calculated using DnaSP v.5 (283). For this analysis, the most abundant subtype of the surface genes was used. Nucleotide sequences of the N3 gene of viruses from the US and Canada between 2010 and 2012, were downloaded from IRD. The sequences were classified according to the migration flyways based on the geographical state or province of isolation (http://www.flyways.us/). Due to the number of sequences available of the N3 subtype between 2010 and 2012 from North America, only comparisons between the Pacific (n=49) and the Mississippi (n=50) flyways were possible. The level of genetic differentiation between populations was compared by $\chi 2$ test, at the 95% level of confidence.

4.4. Results

4.4.1. Influenza A prevalence

Paired cloacal and tracheal swab samples were collected from 1250 birds at three localities between October 2010 and February 2011, and between November and January in 2011-12 and in 2012-13. Eight species were sampled in 2010-11, 16 in 2011-12, and 10 in 2012-13 (Table 4.1). Most of the sampled birds were waterfowl species, in particular blue-winged teals, followed by northern shovelers and green-winged teals, all of which were sampled in all three seasons. A small number of samples were obtained from other bird families including doves, coots, and shorebirds (Table 4.2).

Table 4.1 Study sites and distribution of sampled birds per season, Guatemala 2010-2013

Season	Department	Location	Latitude	Longitude	Sampled Birds
2010-2011	Escuintla	La Gomera	13° 56' 39.40"N	91° 6' 46.09"W	98
	Jutiapa	Pasaco, Finca la Danta	13°53'8.60"N	90°11'45.30"W	4
Total					102
2011-2012	Escuintla	La Gomera	13° 56' 39.40"N	91° 6' 46.09"W	359
	Jutiapa	Pasaco, Finca la Danta	13°53'8.60"N	90°11'45.30"W	191
Total					550
2012-2013	Escuintla	La Gomera	13° 56' 39.40"N	91° 6' 46.09"W	276
		La Gomera (Salinas)	13° 55' 47.28"N	91° 6' 56.808"W	246
	Santa Rosa	El Pumpo	13°53'51.80"N	90°29'33.20"W	76
Total					598
Total					**1250**

IAV was detected in 39 (38.2%) birds in 2010-11, in 129 (23.5%) birds in 2011-12 and 148 (24.7%) birds in 2012-13. Prevalence estimates per species are shown in Table 4.2. Among duck species, the higher prevalence was observed in northern shovelers (35.2%), followed by blue-winged teals (24.7%) and green-winged teals (11.1%) (p=0.0168, χ^2=8.173, df=2). Two white-winged doves tested positive for IAV. Analyses of prevalence for different bird species between migration seasons were performed only for ducks, as the majority of positive samples were from these species. Only those species with n>5 per sampling point were included, specifically the blue-winged teal and the northern shoveler.

For blue-winged teals, prevalence was significantly different between collection sites in 2011-12 (p=0.0324, χ^2=4.577, df=1), but not in 2012-13. For the northern shoveler, the number of sampled birds was smaller, and IAV prevalence was not significantly different between sampling sites in 2011-12 or 2012-13 (Fisher test). Samples from hunted-waterfowl were consistently obtained from the department of Escuintla through all sampling seasons and in greater proportion than the other locations. In order to remove possible confounding factors due to variations between sampling sites, only data from Escuintla was used to analyze differences in prevalence between host species and sampling seasons; and to analyze temporal variations of IAV detection in blue-winged teals.

In the 2011-12 season, the prevalence of IAV in northern shovelers (42%, n=64) was significantly higher than in blue-winged teals (19%, n=272). Similarly, in the subsequent season (2012-13) a higher prevalence in northern shovelers was observed (38% vs 25%), but this difference was not significant. For the blue-winged teal,

prevalence was compared between seasons and a higher prevalence was observed in 2010-11 ($p=0.0117$, $\chi2=8.901$, $df=2$). Univariate logistic regression was used to analyze variation of IAV detection over time (by date and month) in blue-winged teals for 2011-12 (n=272) and 2012-13 (n=484). In 2011-12, a decrease in IAV prevalence towards the end of the migration season was observed (Figure 4.1). This change in prevalence over time was significant when results were analyzed by collection date as continuous variable ($p=1.94\times10\text{-}5$, $\chi2=18.2$), or by month as categorical variable ($p=1.95\times10\text{-}6$, $\chi2=22.6$). Data from blue-winged teals in January 2013 was available only for one of the two sampling sites of Escuintla. Data from both collection sites was combined for the regression analysis; the changes in prevalence by collection date or month observed during in 2012-13 were not significant.

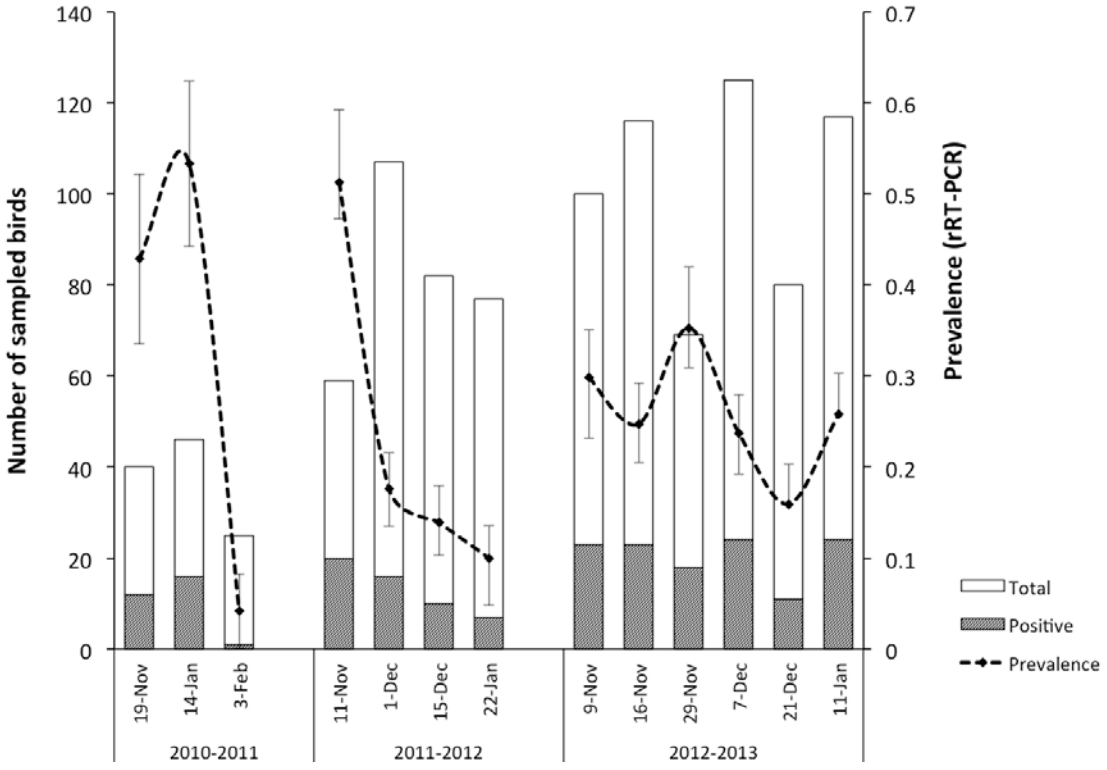

Figure 4.1. Temporal distribution of IAV prevalence in blue-winged teals in Guatemala, 2010-2013

Table 4.2. Percentage prevalence for waterfowl species sampled during the wintering migration seasons in Guatemala, 2010-2013

Family	Species (Common name)	2010-2011		2011-2012		2012-2013		Total	
		Positive (%)	Total	Positive (%)	Total	Positive (%)	Total	Positive (%)	Total
Anatidae	*Anas acuta* (Northern Pintail)			0(0)	2	0(0)	3	0(0)	5
	Anas americana (American Wigeon)					2(28.6)	7	2(28.6)	7
	Anas clypeata (Northern Shoveler)	1(25)	4	28(37.8)	74	8(29.6)	27	37(35.2)	105
	Anas crecca (Green-winged Teal)	0(0)	1	1(6.3)	16	1(100)	1	2(11.1)	18
	Anas discors (Blue-winged Teal)	29(35.4)	82	99(22.8)	434	135(24.5)	550	263(24.7)	1066
	Anas sp. (Dabbling duck)	4(57.1)	7	(0)				4(57.1)	7
	Aythya affinis (Lesser Scaup)			0(0)	1	0(0)	1	0(0)	2
	Cairina moschata (Muscovy Duck)			0(0)	2			0(0)	2
	Dendrocygna autumnalis (Black-bellied Whistling-Duck)	1(50)	2	(0)				1(50)	2
	Dendrocygna bicolor (Fulvous Whistling-Duck)	2(100)	2	(0)				2(100)	2
	Oxyura jamaicensis (Ruddy Duck)			0(0)	1	0(0)	1	0(0)	2
Ciconiidae	*Mycteria americana* (Wood Stork)			0(0)	1			0(0)	1
Columbidae	*Columba flavirostris* (Red-billed Pigeon)			0(0)	1			0(0)	1
	Streptopelia decaocta (Eurasian Collared-Dove)					0(0)	2	0(0)	2
	Zenaida asiatica (White-winged Dove)			0(0)	8	2(40)	5	2(15.4)	13
	Zenaida macroura (Mourning Dove)			0(0)	2			0(0)	2
Pelecanidae	*Pelecanus erythrorhynchos* (American White Pelican)			0(0)	1			0(0)	1
Phalacrocoracidae	*Phalacrocorax brasilianus* (Neotropic Cormorant)			0(0)	1			0(0)	1
Rallidae	*Fulica americana* (American Coot)			1(25)	4			1(25)	4
Scolopacidae	*Catoptrophorus semipalmatus* (Willet)	1(50)	2					1(50)	2
	Limnodromus scolopaceus (Long-billed Dowitcher)			(0)	1			0(0)	1
Threskiornithidae	*Eudocimus albus* (White Ibis)	1(50)	2	(0)	1	0(0)	1	1(25)	4
Total		**39(38.2)**	**102**	**129(23.5)**	**550**	**148(24.7)**	**598**	**316(25.3)**	**1250**

4.4.2. Virus isolation and viral subtypes

Sixty-eight viruses were isolated from 316 rRT-PCR positive samples with an isolation rate of 21.5%. The majority of isolates were obtained from blue-winged teals (n=61), and the remaining isolates were obtained from the northern shoveler (n=5), the green winged teal (n=1) and the American widgeon (n=1) (Table 4.3). The percentage of IAV isolation per species was 5.7% for blue-winged teals, 4.7% for northern shovelers. A total of 19 subtype combinations were found, from 9 different HA subtypes and 7 different NA subtypes. Only one virus isolate, of the H5N3 subtype was obtained in the 2010-11 season. In the 2011-12 season, 34 isolates were obtained and the most prevalent subtype combinations were H3N2 (n=10, 29%) and H1N3 (7, 20%). In the season of 2012-13, 33 isolates were obtained and the most prevalent subtype was H14N3 (9, 27%), followed by H4N2 (7, 21%) and H3N2 (5, 15%). From the HA subtypes found, in 2011-12 the most prevalent were the H3 (n=14, 41%), the H1 (7, 20%) and the H4 (5, 15%), and in 2012-13, the H14 (12, 36%), H3 and H4 (8, 23% both). The most frequent NA subtypes were N3 (17, 50%) and N2 (12, 35%) in 2011-12; both subtypes were also the most frequently isolated during 2012-13 (n_{N3}=13, 39%, and n_{N2}=12, 36%). H5 virus isolates were obtained during all three seasons (one isolate per season) and two H7 isolates were obtained in the second and third seasons respectively. All H5 and H7 viruses were determined to be of low pathogenicity, by analysis of the monobasic amino acid sequence of the HA cleavage site.

Table 4.3. Subtypes of IAV isolated during the wintering season in Guatemala, 2010-2013

Species	November			December			January		Total
	2010	2011	2012	2010	2011	2012	2012	2013	
Anas americana (American wigeon)	-	-	-	-	-	H4N2	-	-	1
Anas clypeata (Northern shoveler)	-	H1N3	-	-	H11N3	H4N2	H7N3	H3N8	5
Anas crecca (Green-winged teal)	-	-	-	-	-	-	-	H7N9	1
Anas discors (Blue-winged teal)	H5N3	H1N3 (3) H3N2 (10) H3N8 (2) H4N2 (2) H4N3 (2) H4N8	H3N2 (4) H3N3 H3N8 H4N2 (4) H4N6 H11N3 H11N9 H14N3 (7) H14N5	-	H14N6 H1N3 (3) H3N3 (2) H5N3 H11N3 H12N5 H14N3 (2)	H2N3 H3N2 H4N2	-	H14N3 (2) H14N4 (2) H5N3	61
Total	**1**	**21**	**21**	**-**	**12**	**5**	**1**	**7**	**68**

Similar numbers of isolates were obtained during the 2011-12 and 2012-13 seasons. H1 and H12 subtypes were only detected during 2011-12, and the H2 subtype was only detected in 2012-13, with the remaining six HA subtypes identified both seasons. The N9 and N4 subtypes were only found during the 2012-13 season; the remaining 5 NA subtypes (N2, N3, N5, N6, N8) were found during both seasons. From the 19 subtype combinations found, only seven were observed during both seasons. The estimated indexes of diversity were 0.860(0.794,0.925) and 0.843(0.776,0.910) for 2011 and 2012, respectively. The diversity index indicated high diversity of subtypes during both seasons; based on the subtype combinations, the composition of the virus populations between the two seasons was different as indicated by the Sørensen-Dice coefficient (CC = 0.269).

Variation of subtype diversity over time is shown in Figure 4.2. The most prevalent subtypes H3 and H4, N3 and N2 were found in at least two months of each season. The N3 subtype was the only NA subtype detected in 2010 and observed during all months in the following seasons. The highest diversity of virus subtypes was detected in the months during peak prevalence as detected by rRT-PCR. All other subtypes, with the exception of the H14 (see below), were found sporadically or only during one month within a season. H5 viruses were isolated in November in 2010, December in 2012, and in January in 2013, whereas the H7 viruses were only detected in January, in the 2011-12 and 2012-13 seasons. There is no evidence of circulation of H14 viruses in the western hemisphere prior to 2010. In Guatemala three H14 viruses were recovered during the 2011-2012 season (100), and during the 2012-13 season the H14 was the most prevalent subtype, detected in November (8 of 21 isolates) and January (4 of 7 isolates).

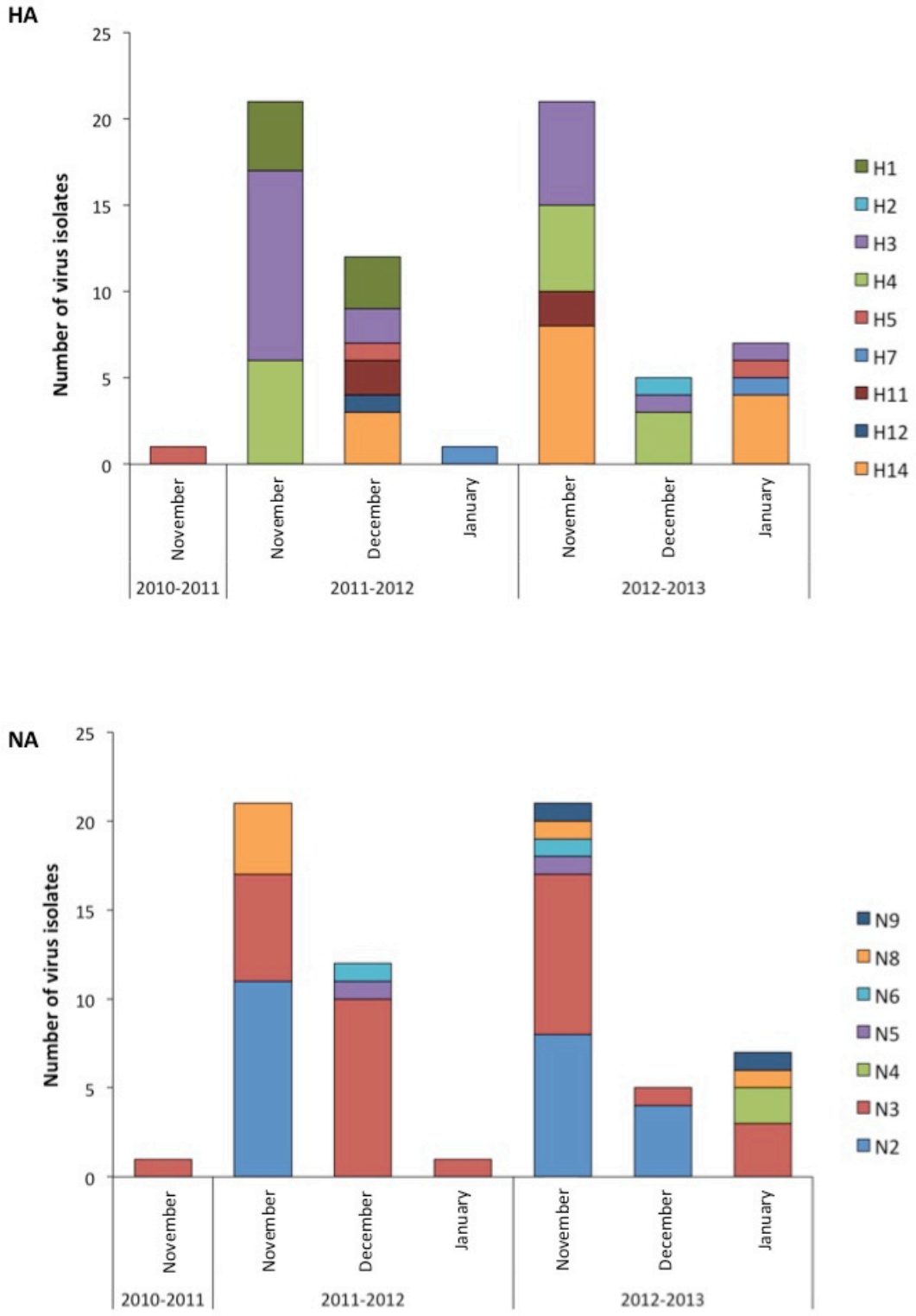

Figure 4.2. Subtypes by month of the viruses isolated from wintering waterfowl in Guatemala between 2010-2013

Mixed infections were detected on six occasions (i.e. virus isolation samples having more than one variant of an internal or surface gene segment detected by deep sequencing). In three samples, two variants of one or two internal gene segments were detected (with <95% shared identity at the nucleotide level), being PA (isolate identifier H105-15, subtype H14N3), NP and NS (H102-09, H3N2), and PB2 and NS (H109-03, H3N8), respectively. Two viruses with two NA subtypes were found (H102-23, H3N2/N8 and H110-14, H14N5/N3), and one sample with two variants of PB2, PA, HA and NA (H114-76, H7/H11, N3/N9).

4.4.3. Phylogenetic analysis, gene constellations and gene flow

Phylogenetic inference by the maximum likelihood method of the internal genes, including sequences from other geographic locations, revealed that for the most part the viruses are closely related to other contemporary viral strains from North America. For each of the internal genes it was observed that the virus isolates from Guatemala are from different clades within the North American lineage, including some of the same clades of the viruses previously isolated in Guatemala. For the PB2 gene (Figure 4.3), viruses from 7 different clades were observed, for the PB1 gene 5 clades (Figure 4.4) and for the PA, gene variants from 9 different clades were found (Figure 4.5), where two viruses (H109-14 and H114-76) clustered together within the Eurasian lineage with other viruses recently isolated in the US. For the NP gene, viruses from 5 clades within the North American lineage were observed (Figure 4.6); in addition, three isolates recovered in 2012 (H112-31, H112-60 and H114-14) clustered with viruses isolated in or prior to 2007 in the US, in a separate basal lineage (distinct to the major North American lineage). For

the M gene, 5 clades were found (Figure 4.7) including three viruses isolated in 2011 (H102-46, H103-05, H104-39) closely related to viruses isolated mainly between 2000 and 2007 in the US. For the NS, viruses from both alleles (A and B) were found (Figure 4.8). Viruses from at least 4 clades were found for the allele A, including the same basal lineage (distinct to the other lineages) observed for NP. Viruses from 1 clade were found for NS allele B. For all the genes, no reassortants with viruses from the South American lineage were found.

For the surface genes, the top matches on BLAST searches are contemporary viruses (from 2009 to 2013) from North America from different geographic locations; most of them from the Pacific and the Mississippi flyways, and some from locations across the Atlantic and Central flyways (Appendices section, Table 0.2). Some viruses were found to have more divergent surface genes, explained as follows: For two of the H3 viruses (H112-60 and H114-14), the top 10 matches included few isolates from 2010 and 2009, 2007 and one from 1987 (Table 4.4). These two isolates shared 99.8% identity with each other, but only 80% with the other H3 genes from Guatemala. Similarly for the H4 subtype, the closest match for one isolate (H112-31) included a virus isolated the subsequent year in Louisiana and one quail H4N2 isolate from California in 2012. The rest of the matches included isolates from or prior to 2007, and two viruses from Canada from 1977 and 1990 respectively (Table 4.4). The H4 HA shared only 83% identity with the other H4 HAs from Guatemala. Definition of different gene alleles with <95 % identity resulted in two gene variants for the H3 subtype, three for the H4, and two for the H5; for the rest of the subtypes (H1, H2, H7, H11, H12 and H14) only one variant was observed with shared % identities between 95% and 100%.

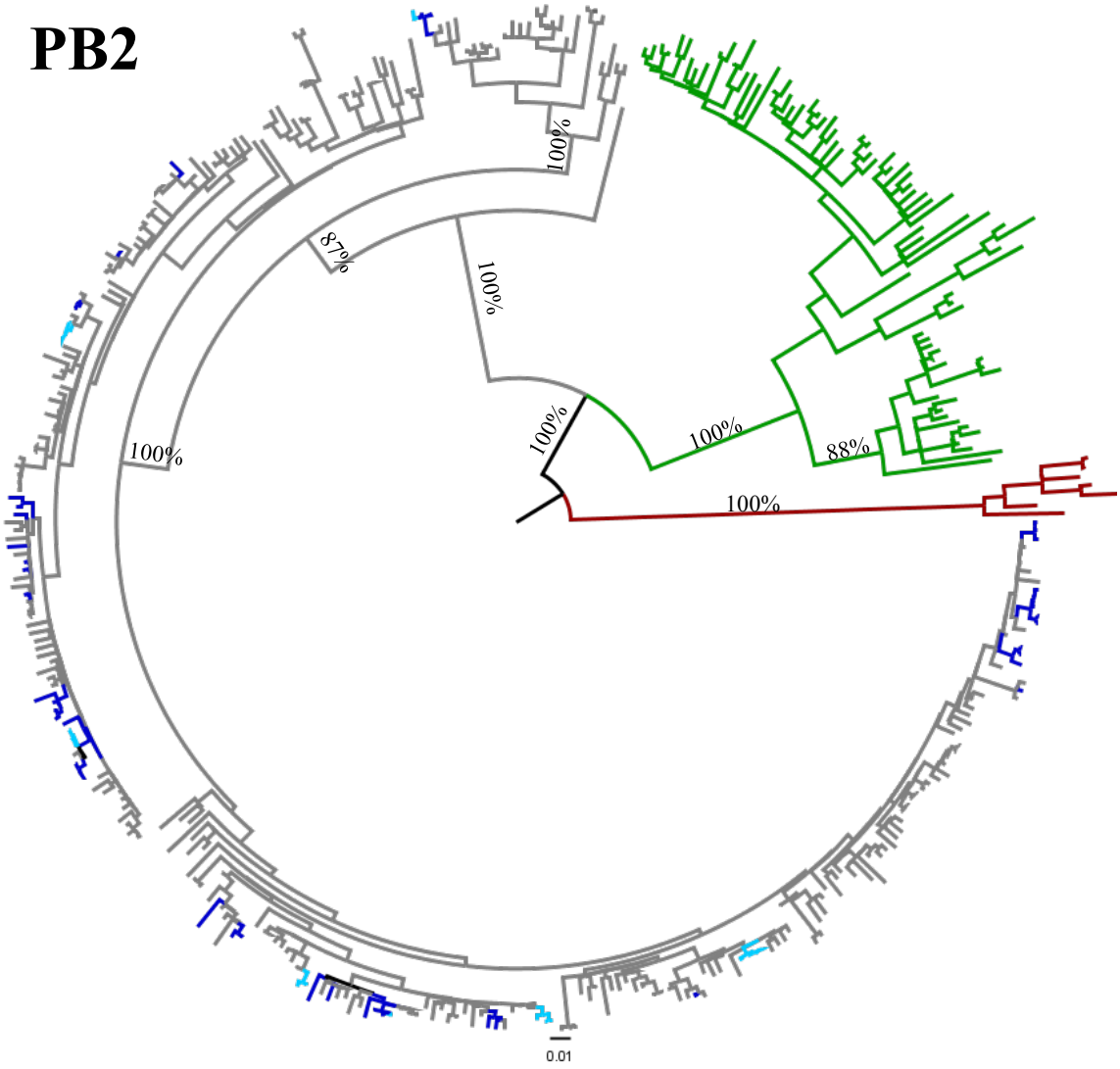

Figure 4.3 Maximum Likelihood Phylogenetic inference for the Polymerase Basic 2 (PB2) gene of LPAIV from Guatemala and other LPAIV from the North American, Eurasian and South American lineages between 2000 and 2013.
The Eurasian lineage is shown in green, the South American lineage is shown in red, and the North American lineage is shown in grey. The viruses from Guatemala isolated are shown in light blue (2008-2010) and dark blue (2010-2013). Bootstrap support values >70% are shown for lineages and main clades. Bar-scale at the bottom of the tree represents number of nucleotide substitution per site.

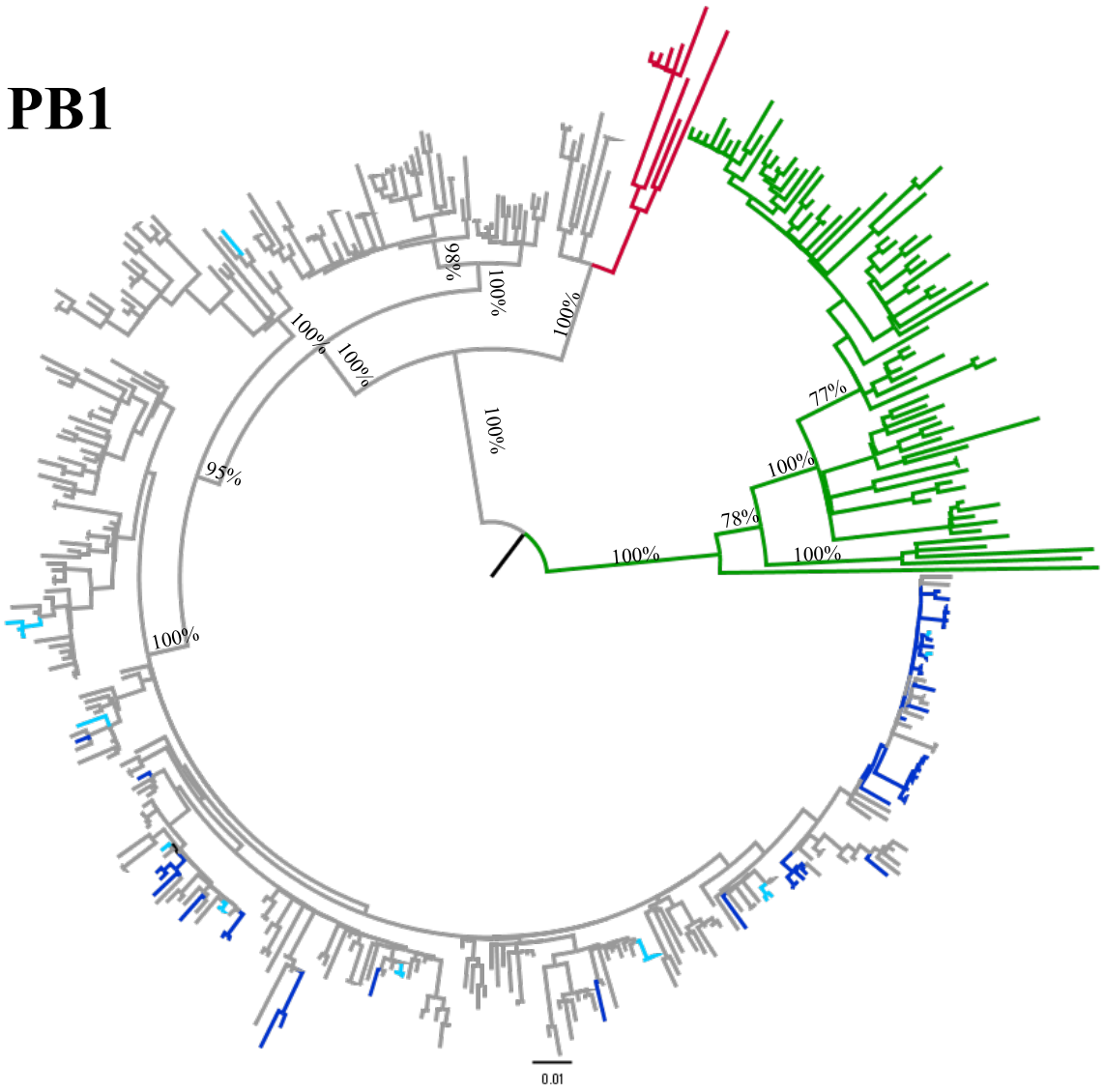

Figure 4.4. Maximum Likelihood Phylogenetic inference for the Polymerase Basic 1 (PB1) gene of LPAIV from Guatemala and other LPAIV from the North American, Eurasian and South American lineages between 2000 and 2013.
The Eurasian lineage is shown in green, the South American lineage is shown in red, and the North American lineage is shown in grey. The viruses from Guatemala isolated are shown in light blue (2008-2010) and dark blue (2010-2013). Bootstrap support values >70% are shown for lineages and main clades. Bar-scale at the bottom of the tree represents number of nucleotide substitution per site.

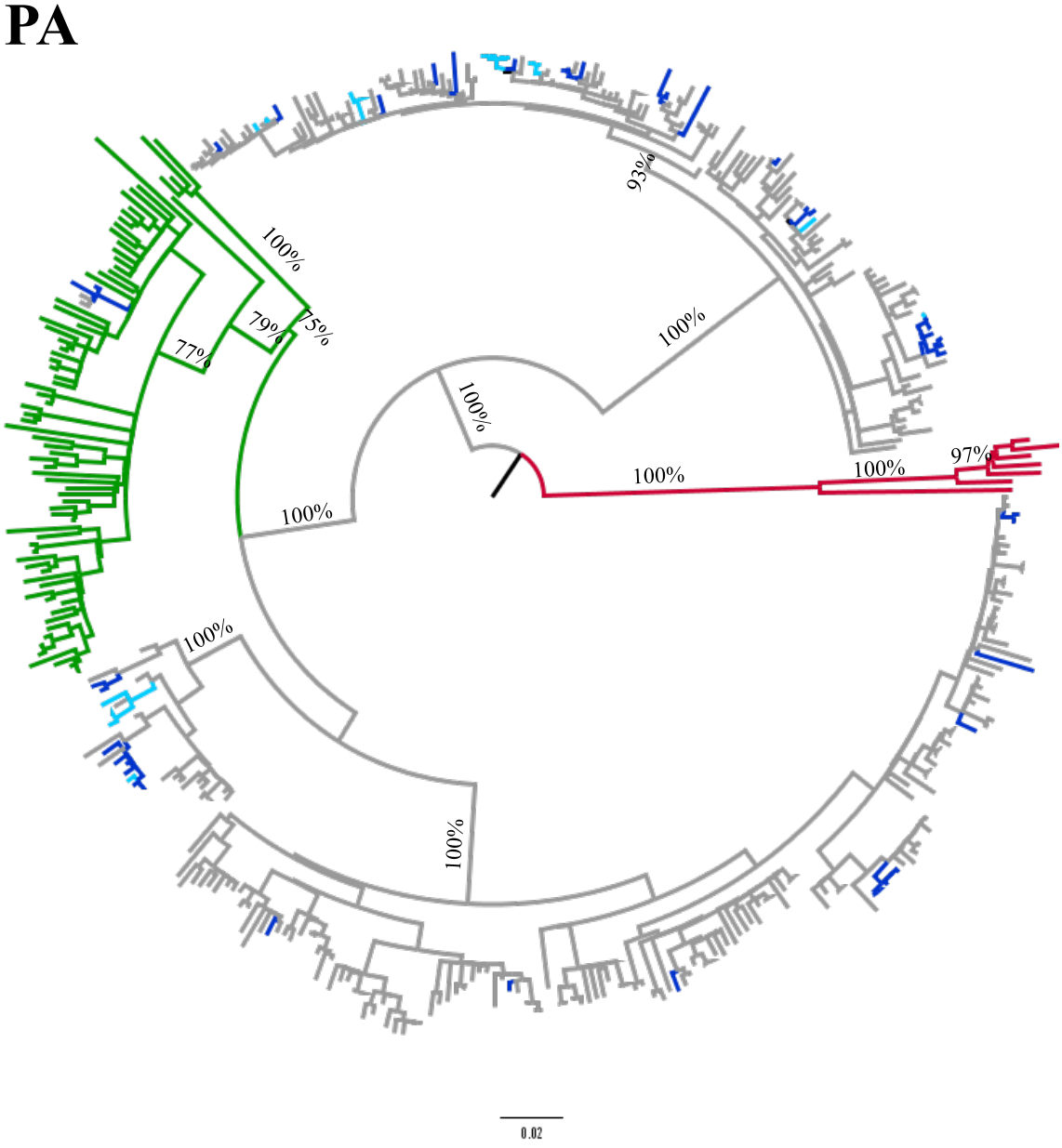

Figure 4.5. Maximum Likelihood Phylogenetic inference for the Polymerase Acid (PA) gene of LPAIV from Guatemala and other LPAIV from the North American, Eurasian and South American lineages between 2000 and 2013.
Polymerase acid (PA) gene. The Eurasian lineage is shown in green, the South American lineage is shown in Red, and the North American lineage is shown in grey. The viruses from Guatemala isolated are shown in light blue (2008-2010) and dark blue (2010-2013). Bootstrap support values >70% are shown for lineages and main clades. Bar-scale at the bottom of the tree represents number of nucleotide substitution per site.

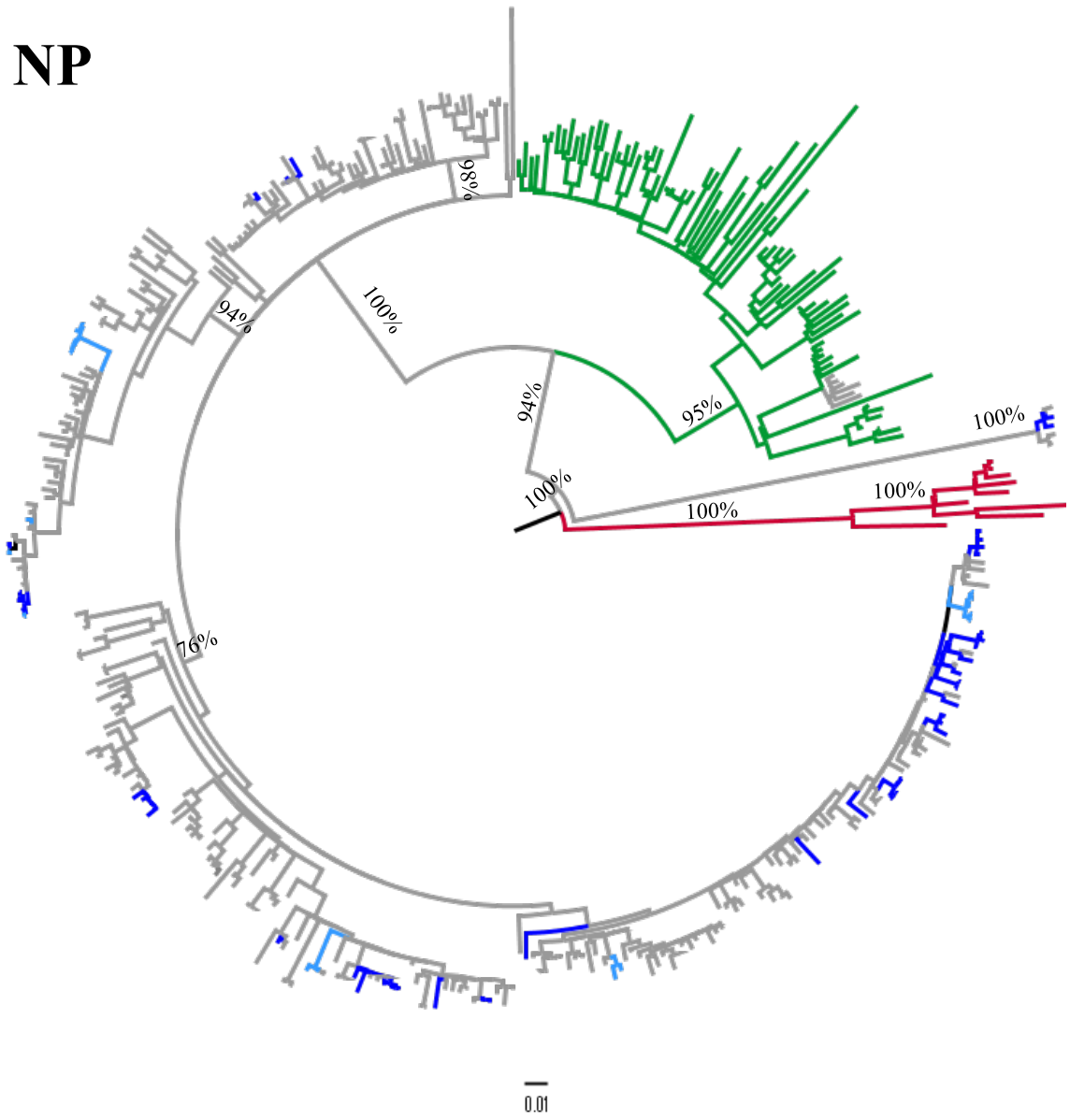

Figure 4.6. Maximum Likelihood Phylogenetic inference for the Nucleoprotein (NP) gene of LPAIV from Guatemala and other LPAIV from the North American, Eurasian and South American lineages between 2000 and 2013.
The Eurasian lineage is shown in green, the South American lineage is shown in Red, and the North American lineage is shown in grey. The viruses from Guatemala isolated are shown in light blue (2008-2010) and dark blue (2010-2013). Bootstrap support values >70% are shown for lineages and main clades. Bar-scale at the bottom of the tree represents number of nucleotide substitution per site.

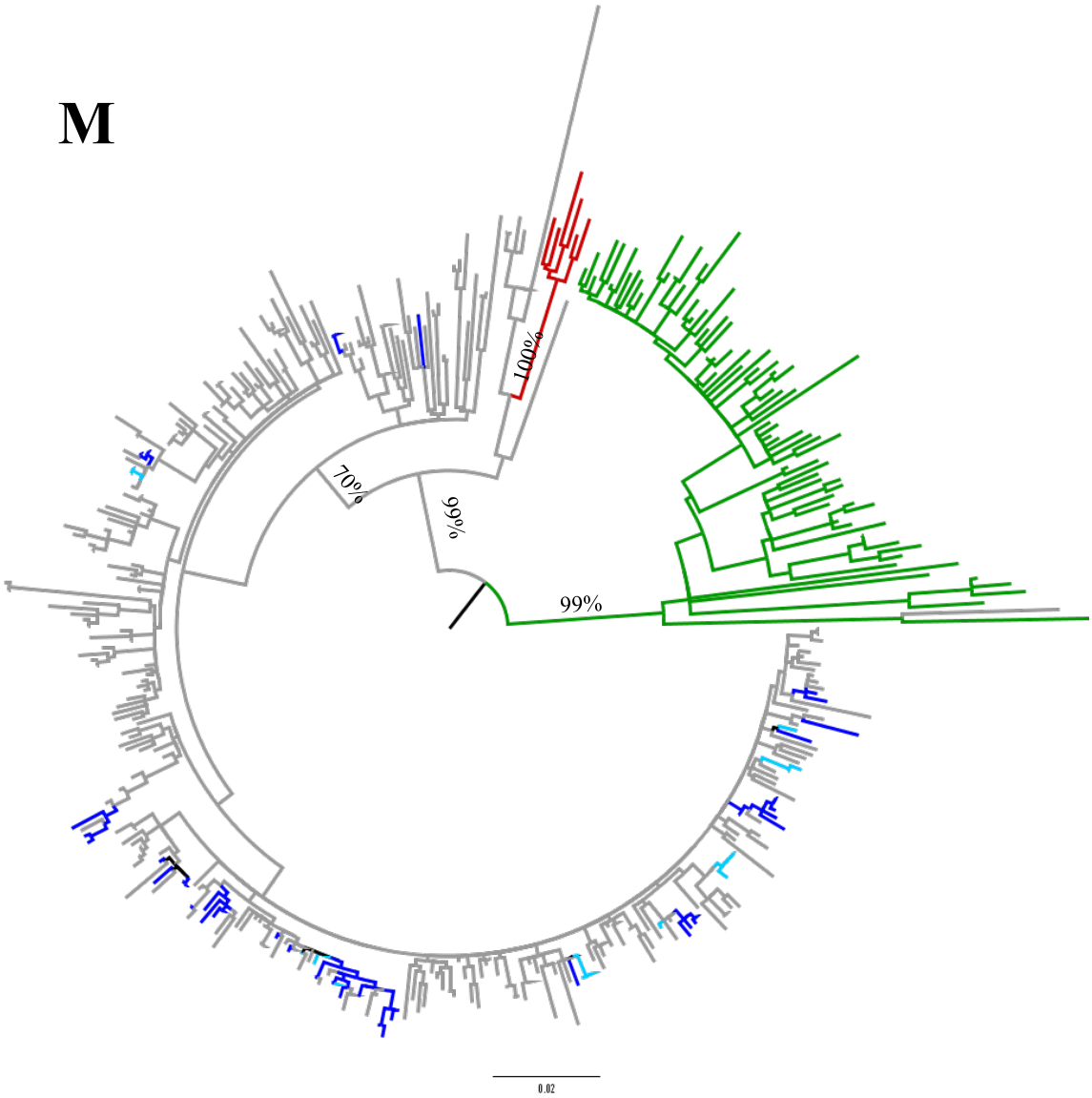

Figure 4.7. Maximum Likelihood Phylogenetic inference for the Matrix protein (M1) gene of LPAIV from Guatemala and other LPAIV from the North American, Eurasian and South American lineages between 2000 and 2013.

The Eurasian lineage is shown in green, the South American lineage is shown in Red, and the North American lineage is shown in grey. The viruses from Guatemala isolated are shown in light blue (2008-2010) and dark blue (2010-2013). Bootstrap support values >70% are shown for lineages and main clades. Bar-scale at the bottom of the tree represents number of nucleotide substitution per site.

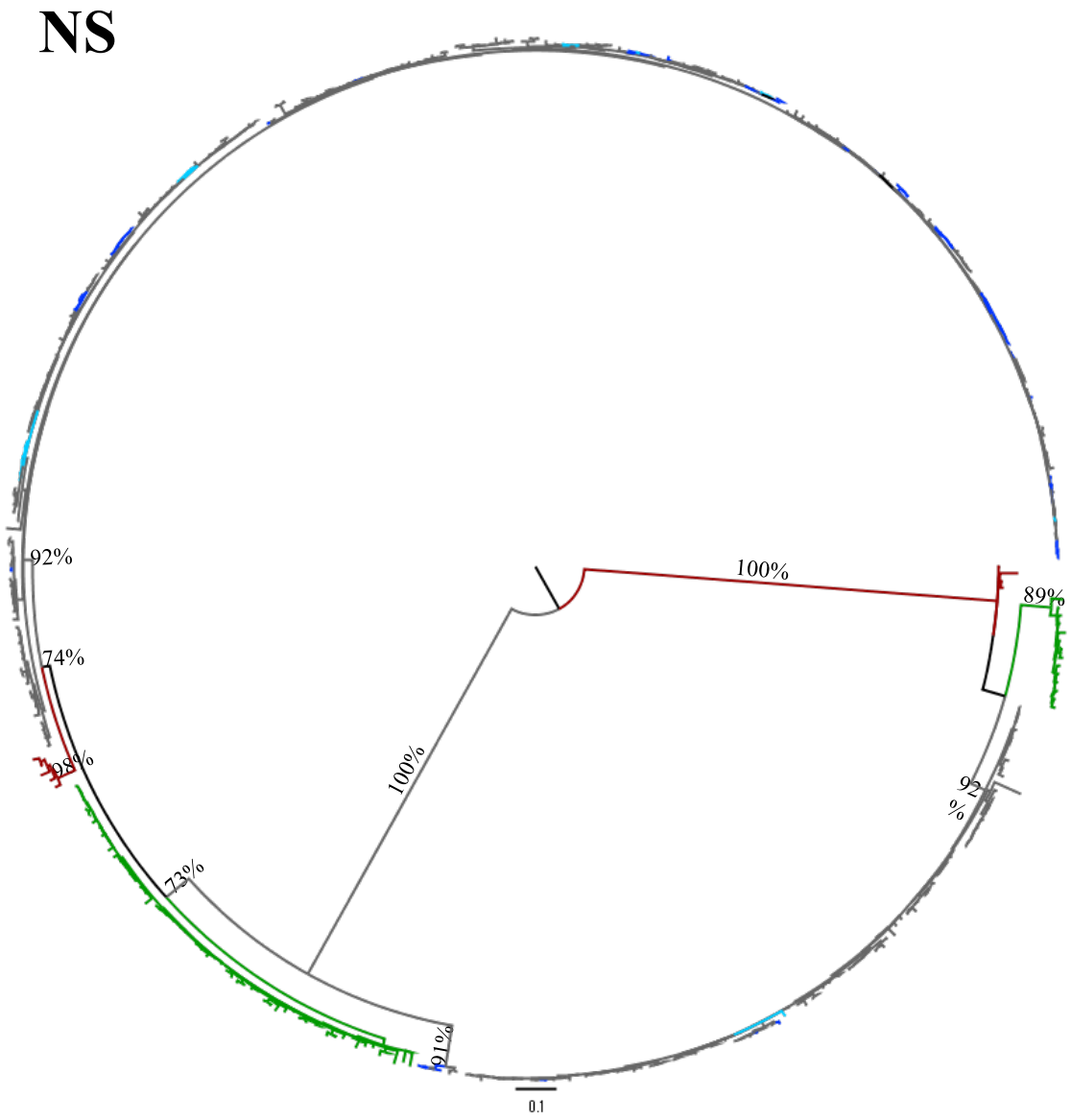

Figure 4.8. Maximum Likelihood Phylogenetic inference for the Non-structural 1 (NS1) gene of LPAIV from Guatemala and other LPAIV from the North American, Eurasian and South American lineages between 2000 and 2013.
The Eurasian lineage is shown in green, the South American lineage is shown in Red, and the North American lineage is shown in grey. The viruses from Guatemala isolated are shown in light blue (2008-2010) and dark blue (2010-2013). Bootstrap support values >70% are shown for lineages and main clades. Bar-scale at the bottom of the tree represents number of nucleotide substitution per site.

Table 4.4. Top 10 BLAST hits of the most divergent HA genes of IAV isolated in Guatemala in wintering waterfowl between 2012 and 2013

GenBank Accession|Virus name	% identity		
	H112-60	H114-14	H112-31
H3			
JN864063.1|A/Anas strepera/Arizona/A00271503/2009(H3N8)	99.0	98.8	
CY076165.1|A/American coot/Oregon/20589-007/2007(H3N8)	98.6	98.5	
CY097718.1|A/mallard/Mississippi/386/2010(H3N8)	98.5	98.4	
CY097726.1|A/mallard/Mississippi/390/2010(H3N8)	98.5	98.4	
CY097702.1|A/mallard/Mississippi/354/2010(H3N8)	98.5	98.3	
CY032720.1|A/northern pintail/California/HKWF792/2007(H3N8)	98.4	98.3	
CY097710.1|A/mallard/Mississippi/360/2010(H3N8)	98.4	98.3	
CY097678.1|A/American green-winged teal/Mississippi/285/2010(H3N8)	98.3	98.2	
CY076013.1|A/cinnamon teal/California/44287-325/2007(H3N8)	98.1	98.0	
EU871828.1|A/duck/LA/17G/1987(H3N8)	94.7	94.7	
H4			
KJ413565.1|A/blue-winged teal/LA/AI13-1334/2013(H4N2)			98.2
CY122499.1|A/environment/California/NWRC183970-18/2006(H4N2)			98.1
CY122503.1|A/environment/New Mexico/NWRC184137-06/2006(H4N2)			98.0
CY053821.1|A/pekin duck/California/P30/2006(H4N2)			97.5
CY122536.1|A/environment/Utah/NWRC186341-12/2007(H4N2)			97.4
KM244114.1|A/turkey/Minnesota/16423-3/2014(H4N2)			97.4
CY122546.1|A/environment/Colorado/NWRC186600-24/2007(H4N2)			97.4
KF986862.1|A/quail/California/D113023808/2012(H4N2)			96.4
CY005968.1|A/mallard duck/ALB/291/1977(H4N1)			86.9
CY005963.1|A/blue-winged teal/ALB/103/1990(H4N5)			86.8

For the NA genes, the top 10 BLAST hits for most of the viruses included recent virus isolates from North America, from different locations in the US and Canada (Appendices section, Table 0.3). Similarly to the HA genes, it was found that the top 10 BLAST hits for the H4N2 of virus H112-31 included the same isolate from Louisiana, and another H4N2 isolate of a turkey from Minnesota from 2014, with the rest of the top hits including isolates from 1978 to 2010 with apparent low frequency of detection in the last decades in the US (Table 4.5). The N2 of virus H112-31 shared only 87% identity with the other N2 genes from Guatemala. Among the N3, the top 10 BLAST hits for

three isolates, from the 2010-2011 (H098-32), and from the 2011-2012 season (H104-69 and H105-05), included viruses isolated during a poultry outbreak of HPAI H7N3 in Mexico in 2012. For the N6, the top BLAST hits were viruses from Canada isolated between 2006 and 2008. For the N8, the NA of the two viruses with the most divergent H3 genes (H112-60 and H114-14) had as top matches viruses from the US isolated between 2007 and 2010, but also included one isolate from Louisiana from 1987. The two N8 genes shared 98.7% identity with each other, but only 78% with the other N8 viruses isolated in Guatemala, which were similar to more recent isolates from North America (Table 4.5). Based on pairwise distances, two gene variants of N2 sharing 87% identity where identified. For the N3, three gene variants were identified with 93 to 94% identity between groups, two variants for the N5 (94% identity between groups), and three for N8, with 78 and 93% shared identity between groups. For the rest of the NA subtypes (N4, N6, and N9) only one variant was detected with >97% identity within variant groups.

From a total of 50 virus genomes (including mixed infections), 31 unique genotypes were identified (Table 4.6) and 49 gene constellations. Genes from 1 lineage were identified for the PB2 and PB2 genes, and from 3 lineages (H, E and D) for the PA. Most NP genes were H lineage, with the exception of three strains that couldn't be assigned to any particular lineage and were therefore designated as "unknown" or U. For the M gene, all genes were from lineage E and for the NS, three lineages were identified including 1D, E and 2B. For the HA genes, the 9 lineages corresponding the 9 HA subtypes were assigned, but the two most divergent H3 and one H4 couldn't be assigned to any specific lineage within the H3 and H4 groups respectively; therefore the viruses

were assigned to an "unknown" lineage and were named 3U and 4U accordingly. In addition, the HA of the H14 viruses from North America share <90% identity with the HA of the H14 viruses originally described in the eastern hemisphere (272, 284, 285). According to the FluGenome 90% identity as the cutoff value for lineage definition, the H14 genes were classified in an "unknown" lineage within this subtype and named 14U. For the NA, genes were assigned to 7 lineages, with the exception of the two most divergent N2 and one N8 genes that were assigned to "unknown" lineages designated as "U". Analysis of gene constellations by digital genotyping for each year is shown in Figure 4.9 and Figure 4.10 for the 2011-12 and 2012-13 seasons respectively. This analysis revealed great variability between gene constellations within and between migration seasons and allowed identification of common gene clades and gene combinations for each year. Even when the number of gene constellations, were almost as many as the number of individual viruses observed, some isolates were found to share partial gene combinations of specific lineages. For example, a common gene combination was PB1 (F-1), M (E-1) and NS (1D-1) during both seasons. In addition, in 2010-11 viruses from the PB2 clade C-1 and PA clade H-2 were more prevalent whereas in 2012-13 the PB2 C-2 clade and PA E-1 clade were more abundant; the PA E-1 clade was found almost exclusively in combination with the PB2 C-2 lineage. In fact for the PA gene, the H lineage was more prevalent in 2011-12 and the E lineage was more prevalent in 2012-13 (Table 4.6). For the M and NS genes, during both years the M (E-1) clade and the NS (1D-1) clade were most prevalent. Viruses from other clades were observed both years, but these clades were different each year, being the M clades E-3, E-4 and E-5 and NS clade 1D-2 for 2011-12 and the M clade E-2, and the NS clades 1D-2, 2B and E for 2012-

13. For the NP gene only one clade was different between years: the H-5 clade was not observed in 2012-13 and instead NP genes from an unidentified lineage were observed. The gene constellation of the viruses with HA and NA from unassigned lineages were almost unique to these viruses and included the NP variant with unassigned lineage.

The phylogenetic analysis of the H14 viruses indicates that the viruses from Guatemala from 2011 were closely related to viruses later isolated in North America in 2012 (Mississippi and Arkansas) and were the closest ancestors of the viruses found in Guatemala during the winter in 2011-2012 and in Texas during the spring of 2013 (Figure 4.11). Phylogenetic analysis of the N3 gene suggested a close relationship among three of the viruses from Guatemala [one from 2010 (H098-32) and two from 2011 (H104-69 and H105-05)] and the NA genes of the HPAI H7N3 strain isolated from poultry in Mexico in 2012 (Figure 4.12). Comparison of genetic differentiation between the N3 gene of the viruses from Guatemala and those from the Pacific and Mississippi flyways revealed that the virus populations from these localities are not significantly different from each other. The FST values were 0.163 between Guatemala and the Mississippi flyway, 0.214 between the Guatemala and the Pacific Flyway and 0.116 between the Mississippi and the Pacific flyways. The FST between the virus populations from Guatemala from 2011-12 and 2012-13 was 0.292 (Table 4.7). Comparisons between migration flyways by year were not possible given the small number of sequences available for some years.

Table 4.5. Top 10 BLAST hits for the most divergent NA genes of LPAIV isolated in Guatemala between 2011 and 2013

	% identity							
GenBank Accession|Virus name	**H112-31**	**H098-32**	**H103-05**	**H104-69**	**H105-05**	**H110-27**	**H112-60**	**H114-14**
N2								
KJ413567.1|A/blue-winged teal/LA/AI13-1334/2013(H4N2)	98.7							
KM244116.1|A/turkey/Minnesota/16423-3/2014(H4N2)	98.4							
CY097754.1|A/mallard/Mississippi/407/2010(mixed)	98.0							
CY053823.1|A/pekin duck/California/P30/2006(H4N2)	97.5							
CY004785.1|A/mallard duck/ALB/354/1978(H4N2)	91.7							
CY014560.1|A/mallard duck/Alberta/205/1978(H6N2)	91.6							
CY004045.1|A/pintail duck/ALB/367/1978(H6N2)	91.6							
CY004001.1|A/mallard duck/ALB/250/1978(H6N2)	91.6							
CY004050.1|A/blue-winged teal/ALB/651/1978(H6N2)	91.6							
CY004041.1|A/pintail duck/ALB/133/1978(H6N2)	91.6							
N3								
CY132991.1|A/American green-winged teal/Illinois/10OS3343/2010(H2N3)		98.5		97.8	97.9			
CY097336.1|A/mallard/Wisconsin/2560/2009(H2N3)		98.3	99.2					
CY096996.1|A/blue-winged teal/Wisconsin/2572/2009(H2N3)		98.2	99.1					
CY097376.1|A/mallard/Wisconsin/2785/2009(H2N3)		98.1	99.0					
CY039598.1|A/northern shoveler/California/HKWF2031/2008(H7N3)		97.9	98.2	97.4	97.5			
CY097320.1|A/mallard/Wisconsin/4236/2009(mixed)		97.8	98.5	97.3	97.3			
CY039542.1|A/green winged teal/California/AKS1370/2008(H7N3)		97.7	98.1	97.2	97.3			
CY125730.1|A/Mexico/InDRE7218/2012(H7N3)		97.7		99.0	97.9			
CY032730.1|A/northern shoveler/California/HKWF979/2007(H3N3)		97.7	98.1	97.1	97.2			
EU500862.1|A/chicken/SK/HR-00011/2007(H7N3)		97.6	97.9	96.9	97.0			
KJ413442.1|A/blue-winged teal/TX/AI12-614/2012(H10N3)				99.4	98.2			
CY166180.1|A/mallard/Mississippi/12OS445/2012(H10N3)				99.3	98.1			
JX317626.1|A/chicken/Jalisco/CPA1/2012(H7N3)				99.2	98.0			

GenBank Accession|Virus name	% identity		
N6			
CY103120.1|A/mallard/Alberta/246/2006(H4N6)	98.5		
CY103128.1|A/mallard/Alberta/254/2006(H4N6)	98.5		
CY103213.1|A/mallard/Alberta/106/2007(mixed)	98.5		
CY103238.1|A/mallard/Alberta/114/2007(H4N6)	98.5		
CY103317.1|A/mallard/Alberta/156/2007(mixed)	98.3		
CY103326.1|A/mallard/Alberta/160/2007(H4N6)	98.4		
CY103342.1|A/northern pintail/Alberta/265/2007(H4N6)	98.4		
CY103469.1|A/mallard/Alberta/121/2008(H4N6)	98.5		
CY103477.1|A/mallard/Alberta/270/2008(H4N6)	98.5		
CY140477.1|A/mallard/Minnesota/Sg-00931/2008(H4N6)	98.3		
N8			
CY076167.1|A/American coot/Oregon/20589-007/2007(H3N8)		98.3	99.0
CY097704.1|A/mallard/Mississippi/354/2010(H3N8)		98.1	98.7
CY032722.1|A/northern pintail/California/HKWF792/2007(H3N8)		98.1	98.7
CY097728.1|A/mallard/Mississippi/390/2010(H3N8)		98.1	98.7
CY097712.1|A/mallard/Mississippi/360/2010(H3N8)		98.0	98.6
CY097720.1|A/mallard/Mississippi/386/2010(H3N8)		98.0	98.6
CY097129.1|A/mallard/Ohio/2039/2009(mixed)		97.9	98.6
CY076015.1|A/cinnamon teal/California/44287-325/2007(H3N8)		97.8	98.5
CY097680.1|A/Am. green-winged teal/Mississippi/285/2010(H3N8)		97.8	98.5
EU871830.1|A/duck/LA/17G/1987(H3N8)		94.6	95.3

(Table 4.5. Cont.)

Table 4.6. Genotypes and gene constellations of sequenced IAV from Guatemala isolated from wintering waterfowl (2010-2013)

		Genetic clade							
Virus ID	**Genotype**	**PB2**	**PB1**	**PA**	**HA**	**NP**	**NA**	**M**	**NS**
H098-32	CFH5CH3AE1D	C-1	F-1	H-2	5C-1	H-3	3A-1	E-1	1D-1
H101-25	CFE4AH2DE1D	C-4	F-1	H-2	4A-1	H-1	8A-1	E-1	1D-1
H101-29	CFH3CH8AE1D	C-1	F-4	E-2	3C	H-4	2D	E-4	1D-1
H102-05	CFH4AH2DE1D	C-1	F-1	H-2	4A-1	H-3	2D	E-1	1D-1
H102-09a*	CFH3CH2DE2B	C-7	F-1	H-3	3C	H-3	2D	E-1	1D-1
H102-09b*	CFH3CH2DE1D	C-7	F-1	H-3	3C	H-3	2D	E-1	2B
H102-10	CFH4AH8AE1D	C-1	F-1	H-2	4A-2	H-5	8A-1	E-1	1D-1
H102-23	CFE3CH2DE1D	C-7	F-1	E-4	3C	H-5	2D	E-4	1D-1
H102-23	CFE3CH8AE1D	C-7	F-1	E-4	3C	H-5	8A-1	E-4	1D-1
H102-32	CFE3CH2DE1D	C-4	F-1	E-5	3C	H-3	2D	E-1	1D-1
H102-46	CFE1DH3AE1D	C-1	F-2	E-5	1D	H-4	3A-3	E-3	1D-1
H103-05	CFH1DH3AE1D	C-6	F-1	H-3	1D	H-1	3A-1	E-5	1D-1
H103-20	CFH3CH2DE1D	C-1	F-2	H-3	3C	H-1	2D	E-1	1D-1
H103-37	CFH4AH3AE1D	C-2	F-2	H-2	4A-2	H-3	3A-2	E-1	1D-1
H104-38	CFH1DH3AE1D	C-1	F-1	H-2	1D	H-3	3A-3	E-3	1D-1
H104-69	CFH11CH3AE1D	C-2	F-2	H-2	3C	H-1	3A-2	E-1	1D-1
H104-93	CFE3CH3AE1D	C-1	F-1	E-2	12A	H-2	5D-1	E-1	1D-1
H104-99	CFH12AH5DE1D	C-1	F-1	H-2	11C	H-1	3A-1	E-1	1D-1
H105-01	CFH1DH3AE1D	C-2	F-1	H-2	1D	H-3	3A-2	E-1	1D-1
H105-04	CFH11CH3AE1D	C-4	F-5	H-1	11C	H-1	3A-3	E-1	1D-1
H105-05	CFH5CH3AE1D	C-1	F-2	H-2	5C-2	H-1	3A-1	E-1	1D-1
H105-08	CFH3CH3AE1D	C-1	F-2	H-1	3C	H-5	3A-3	E-1	1D-1
H105-15a*	CFE14UH3AE1D	C-4	F-1	E-2	14U	H-5	3A-2	E-1	1D-1
H105-15b*	CFE14UH3AE1D	C-4	F-1	E-2	14U	H-5	3A-3	E-1	1D-1
H105-31	CFH14UH3AE1D	C-4	F-1	H-2	14U	H-2	3A-3	E-1	1D-1
H106-62	CFH14UH6AE1D	C-6	F-1	H-1	14U	H-5	6A	E-1	1D-1
H107-88	CFE7FH3AE1D	C-6	F-1	E-2	7F	H-1	3A-2	E-1	1D-3
H108-02	CFE14UH3AE1D	C-2	F-1	E-1	14U	H-1	3A-1	E-1	1D-1
H108-39	CFE4AH2DE1D	C-1	F-1	E-4	4A-2	H-3	2D	E-1	1D-1
H108-45	CFE3CH2DE1D	C-1	F-1	E-2	3C	H-1	2D	E-1	1D-1
H109-06	CFH4AH2DE1D	C-5	F-3	H-1	4A-1	H-2	2D	E-1	1D-2
H109-14	CFD3CH3AE1D	C-2	F-2	D	3C	H-1	3A-2	E-1	1D-1
H109-15	CFE3CH2DE2B	C-1	F-1	E-2	3C	H-1	2D	E-1	2B
H109-38a*	CFE3CH8AE1D	C-2	F-1	E-2	3C	H-2	8A-2	E-2	1D-2
H109-38b*	CFE3CH8AE1D	C-4	F-1	E-2	3C	H-2	8A-2	E-2	1D-1
H109-49	CFE14UH3AE1D	C-2	F-1	E-1	14U	H-1	3A-1	E-1	1D-2
H110-04a*	CFH14UH5DE1D	C-1	F-1	H-2	14U	H-1	3A-1	E-2	1D-1
H110-04b*	CFH14UH3AE1D	C-1	F-1	H-2	14U	H-1	5D-2	E-2	1D-2
H110-27	CFE4AH6AE1D	C-4	F-2	E-1	4A-1	H-1	6A	E-1	1D-1
H110-31	CFH14UH3AE1D	C-2	F-1	H-1	14U	H-3	3A-1	E-1	1D-1
H110-37	CFH11CH3AE1D	C-1	F-1	H-1	11C	H-4	3A-1	E-1	1D-1
H111-98	CFna2HH3AE1D	C-4	F-1	na	2H	H-1	3A-2	E-1	1D-1
H112-31	CFE4UU2UEE	C-2	F-3	E-1	4U	U	2U	E-2	E
H112-60	CFE3UU2UEE	C-2	F-3	E-1	3U	U	8U	E-2	E
H113-07	CFE14UH3AE1D	C-2	F-1	E-1	14U	H-1	3A-1	E-1	1D-2
H113-08	CFE14UH4AE1D	C-2	F-1	E-1	14U	H-1	4A	E-1	1D-2
H113-53	CFH5CH3AE1D	C-2	F-1	H-1	5C-1	H-1	3A-1	E-1	1D-1
H113-76	CFE14UH4AE1D	C-2	F-1	E-1	14U	H-1	1A	E-1	1D-2
H114-14	CFE3UU8UEE	C-2	F-3	E-1	3U	U	8U	E-2	E
H114-76a*	CFE11CH3AE2B	C-1	F-1	D	11C	H-1	9A	E-2	2B
H114-76b*	CFD7FH9AE2B	C-1	F-1	E-3	7F	H-1	3A-1	E-2	2B

* Mixed infections; the gene constellations in mixed infections cannot be assigned individually to each of the co-infecting viruses, the order in which their genes appear listed in the table are only with the purpose of representing mixed genotypes. Duplicated genotypes appear shaded in grey.

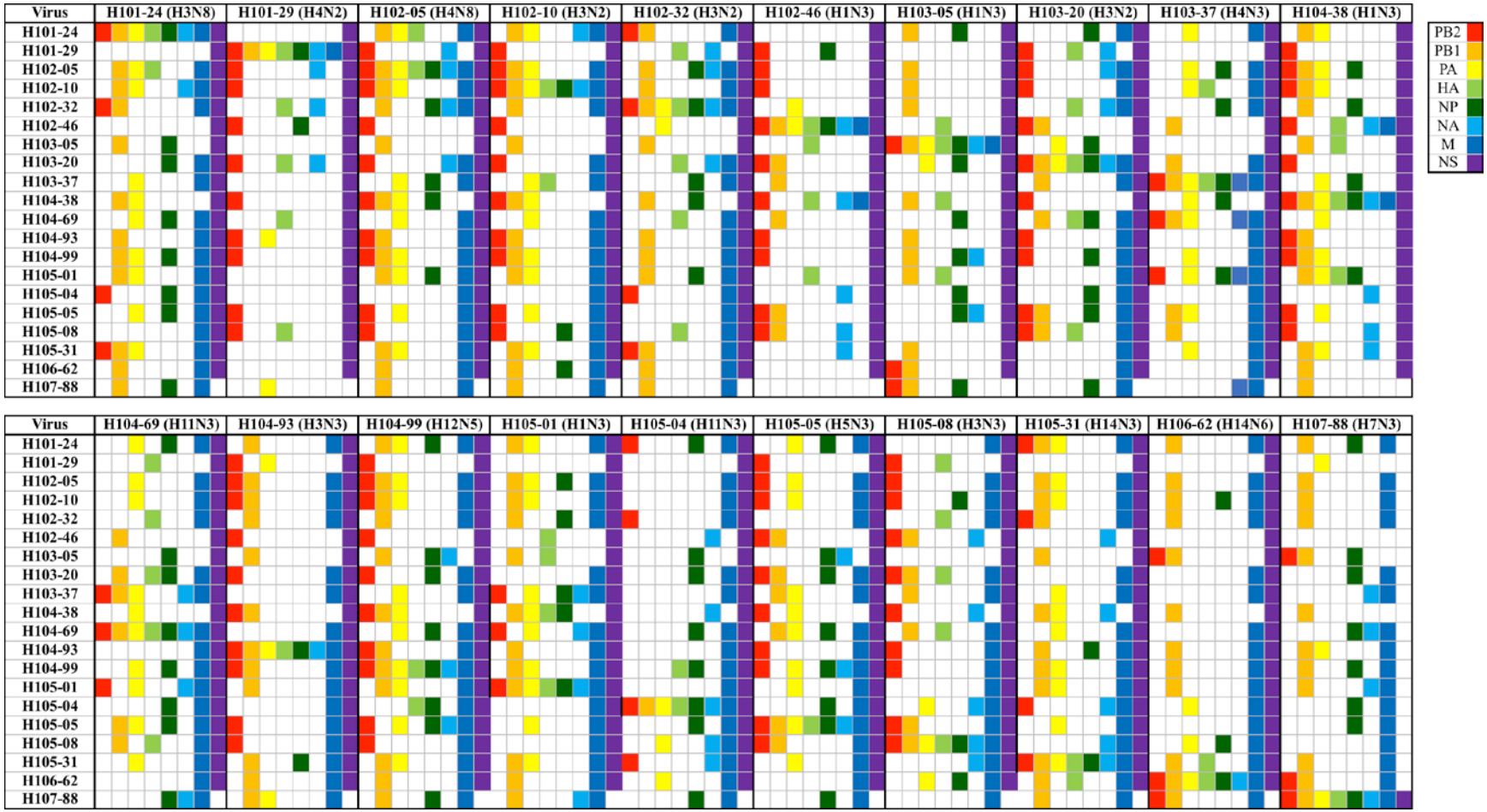

Figure 4.9. Digital genotyping of AIV isolated from wintering waterfowl in Guatemala during the 2011-12 season.
Virus genomes are represented in a segment identity matrix based on the genetic constellations derived from the clades (with >96% ID and >70% bootstrap clade support) identified by phylogenetic analysis.

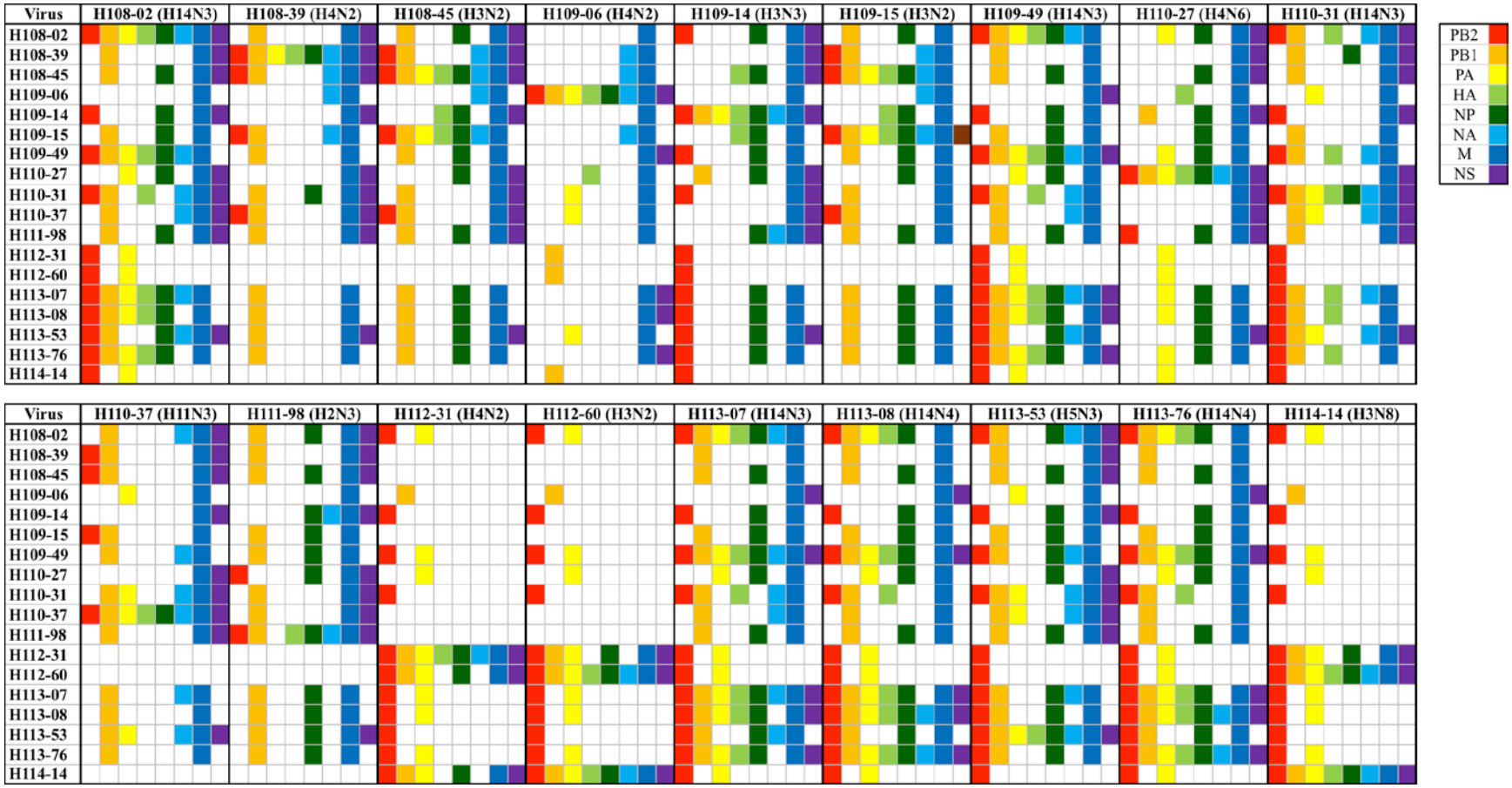

Figure 4.10. Digital genotyping of AIV isolated from wintering waterfowl in Guatemala during the 2012-13 season.
Virus genomes are represented in a segment identity matrix based on the genetic constellations derived from the clades (with >96% ID and >70% bootstrap clade support) identified by phylogenetic analysis.

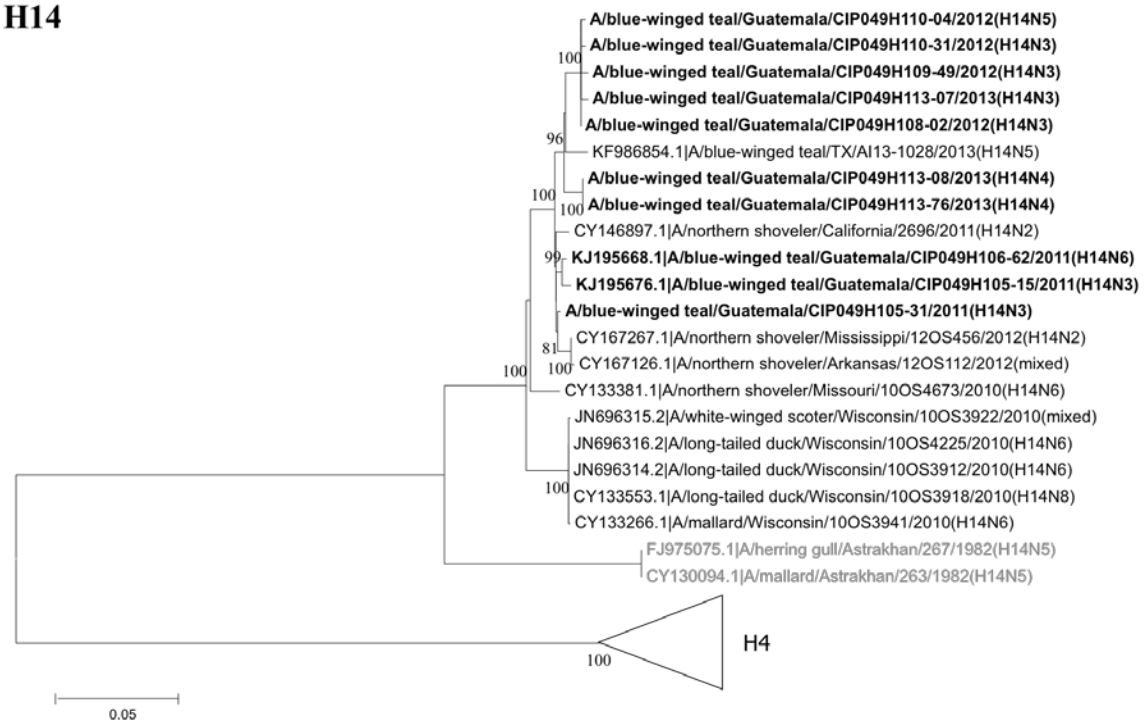

Figure 4.11. Evolutionary relationships inferred by the Minimum Evolution method of the HA genes of H14 IAV from Guatemala

Viruses from Guatemala are shown in bold. Bootstrap support values >70% are shown. Scale-bar in the bottom left indicates number of nucleotide substitutions per site.

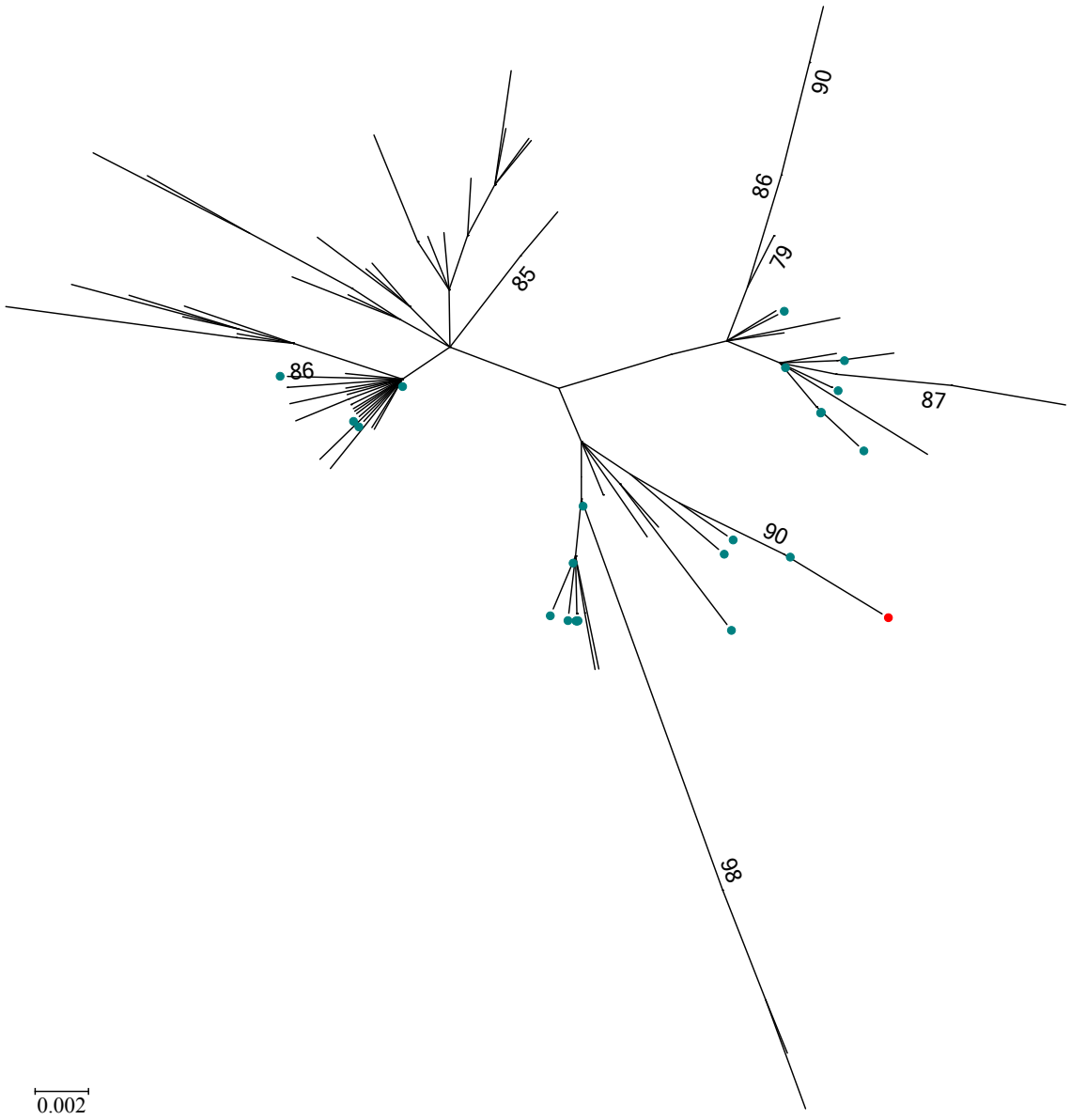

Figure 4.12. Maximum Likelihood phylogenetic inference for the NA gene of the N3 viruses from Guatemala and North America isolated between 2008 and 2013.
Viruses from Guatemala are indicated with green circles, the N3 of a chicken derived HPAI H7N3 virus from Mexico in 2012 is indicated with a red circle. Bootstrap support values >70% are shown. Scale-bar in the bottom left indicates number of nucleotide substitutions per site.

Table 4.7. Genetic differentiation index between the virus populations from Guatemala, and the locations across the Mississippi and Pacific flyways in North America.

Population 1	Population 2	Genetic differentiation			
		Fst	χ^2	P-value	df
Location					
Guatemala[1]	Mississippi flyway[2]	0.1633	73	0.348	69
Guatemala[1]	Pacific flyway[3]	0.2140	72	0.4115	70
Mississippi flyway[2]	Pacific flyway[3]	0.1167	99	0.3965	96
Year					
Guatemala, 2011	Guatemala, 2012	0.2917	22	0.3405	20

[1] N3 neuraminidase nucleotide sequences of the virus isolates from Guatemala obtained during the winter migration between 2010 and 2013

[2] N3 neuraminidase nucleotide sequences available in the IRD of virus isolates obtained between 2010 and 2012 in locations across the Mississippi flyway (United States: Arkansas, Illinois, Louisiana, Minnesota, Mississippi, Missouri, Ohio, Wisconsin; Canada: Saskatchewan)

[3] N3 neuraminidase nucleotide sequences available in the IRD of virus isolates obtained between 2010 and 2012 in locations across the Pacific Flyway (United States: Alaska and California).

4.5. Discussion

Sampling of hunter-harvested waterfowl in Guatemala revealed relatively a high detection rate of IAV at wintering grounds. Prevalence estimates based on rRT-PCR for northern shovelers and blue-winged teals are higher than reported in wintering populations in other geographical regions (97, 99). Overall prevalence estimates based on virus isolation for blue-winged teals (5.7%) and northern shovelers (4.7%) are comparable with studies from wintering ducks from California and Louisiana (99, 100, 210). A higher prevalence based on rRT-PCR is not surprising, as quantitative rRT-PCR is known to be more sensitive than virus isolation techniques. Selection of the strains that are able to replicate in ECE during virus isolation may also explain the differences in prevalence estimates obtained with each method. It is important to note that virus prevalence estimates should be interpreted and compared to other studies in the context of the testing methodologies. An increase in rRT-PCR virus prevalence compared to previous years was observed previously in 2010 in Guatemala (Chapter 2). The reasons of the high prevalence observed in 2010 and during the present study are unknown; the high diversity of co-circulating subtypes and subtype combinations detected may help to explain this observation. A high diversity of AIV subtypes was observed in wintering grounds in California and Texas (97, 99, 265), with comparable virus isolation rates.

During the 2010-11 and 2011-12 seasons a decreasing trend in prevalence was observed towards the end of the migration, supporting observations from previous years in Guatemala and similar to patterns of prevalence observed in wintering grounds in California and Texas (97, 99, 265). This decrease is likely explained by the accumulation of population immunity (as the number of seroconverted birds increases) resulting in a

reduced number of susceptible individuals, not only to circulating subtypes but to other viruses from related genetic clades (286). During 2012-13 the pattern of IAV detection over time was different and the prevalence of IAV in January two-times higher in comparison to previous years. A plausible explanation could be the introduction and a potential outbreak of H14, the most prevalent HA subtype detected during that season. As mentioned above, the H14 is presumably a novel LPAI subtype to the western hemisphere. In January 2013, 4 of the 5 virus isolates obtained from blue-winged teals that month were from the H14 subtype. Current reports indicate that isolation of H14 viruses has been sporadic in North America (287, 288). It is not clear if the H14 virus persisted in wintering grounds in Guatemala after its first detection in 2011, or if it was re-introduced during the following season. Phylogenetic analysis confirmed the genetic relationships observed in previous studies, and the tree topology with additional H14 sequences from 2012-2013 suggest that the viruses from Guatemala were similar to and closest ancestors of the viruses isolated in different locations across the Mississippi flyway in the following seasons (2012 and 2013) (272, 287).

Whether outbreaks of H14 in waterfowl have occurred in other locations in North America is unknown. Competitive exclusion between different viruses in a population already infected with multiple subtypes during peak prevalence periods at the breeding grounds may impede a higher level of transmission of newly introduced subtype of distinct genetic lineages such as the H14 (65, 75). It has been hypothesized that the wintering grounds may serve as repositories to maintain subtype diversity as ducks may arrive susceptible to subtypes that they were not exposed in the breeding grounds (98, 242). A possible explanation to the high levels of H14 observed in Guatemala could be

that after the first introduction of H14 in 2011, these viruses persisted in (yet unknown) local reservoirs between migration seasons, spreading upon the arrival of a new susceptible population in the subsequent season. Maintenance and contribution to virus diversity by resident duck populations have been demonstrated in studies in California (289, 290) and amplification of locally circulating subtypes upon arrival of migrants to a particular location may result in epizootic events (291). Either of these possibilities is supported by isolation of other rare subtypes and subtype combinations at the wintering grounds in Texas and Guatemala (98, 100). Experimental infections with North American strains of H14 (272) indicate that these viruses are very similar in their phenotype to other LPAIV subtypes already circulating in the region. In the long-term the H14 viruses will likely continue to circulate in the western hemisphere, providing a unique an opportunity to study virus movement and spread between host species and geographical across the American continent. In addition to the H14, other prevalent subtypes found in this study include the H3 and the H4, both of which are commonly isolated in North America (93). However, these subtypes were found in non-commonly isolated combinations such as H1N3 (the second most prevalent subtype in 2011-12), H3N3 and the H4N3. The H6 subtype, also prevalent in North America (65, 99), was not found in this study.

Mixed infections included viruses from the H3, H14 and H7/H11 subtypes. Detection of mixed infections provides evidence of reassortment at the wintering grounds. Whether these subtypes are more likely than others to reassort is unknown, and the lack of detection of mixed infection involving other subtypes may be just a result of their high relative abundance of the H3, H14 and H11 circulating at that moment. The

frequency of mixed infections has been usually measured by the percentage of virus isolates from a single sample containing more than one HA and NA subtype (99, 292, 293). However the presence of more than one gene variant of any of the internal gene segments should equally be considered a mixed infection. Such argument implies that frequencies of co-infection in the natural host have perhaps been underestimated in the past. Deep sequencing data provides detailed information of all variants of specific genes present in a single sample, making possible to resolve mixed infections (294). Incorporation of next-generation sequencing technologies into wild bird surveillance would help to provide better estimates of co-infections and reassortment events in nature allowing comparisons between different geographical locations. It must be noted that virus isolation may have an impact in the detection of mixed infections (295). Despite evidence of mixed infections in this study, the real number of co-infections may still be underestimated.

Phylogenetic analysis of the internal gene segments and the N3 NA surface gene segment, as well as BLAST searches of HA gene segments, indicates that birds from at least two migration flyways (Pacific and Mississippi) may be equally contributing to the diversity of viruses circulating in Guatemala. This observation is supported by similar levels of genetic differentiation estimated among the three populations (Pacific, Mississippi and Guatemala). The high mutation rates of the IAV RNA replication process, make it difficult to infer gene flow rates among populations from genetic differentiation estimates (296). Comparison of gene polymorphisms in other subtypes, and internal genes, between virus populations from multiple migration flyways and years, may help trace gene movements at higher resolution. The number of viruses from Central

America is still limited in comparison to the large number of viral genomes from North America. More accurate estimates of gene flow at a large scale are only possible as data accumulates not only from Guatemala but also from other locations in Central America.

The high diversity of viruses that circulate in ducks wintering in Guatemala, estimated by the diversity index, is also reflected in the number of different genotypes and gene constellations observed. At least 60% of the viruses differed from each other in at least one gene at the level of genotype and 96% of the viruses were unique at the level of gene constellations. The diversity of genotypes has been observed to be higher in areas where migrations flyways overlap and duck populations congregate (74, 78, 297, 298). Our results support the hypothesis that congregations of birds from different flyways at a natural geographical bottleneck in Central America, may increase opportunities for virus reassortment, contributing to the extensive gene flow between bird populations from different migration flyways observed in North America (67, 269, 270). Thus, Guatemala represents a "hotspot" for IAV surveillance, previously underappreciated until this study.

In the literature, many approaches have been undertaken to study the diversity and genetic composition of viral populations at different geographic locations. However, there is a lack of standardized methodologies that not only describe the genetic diversity of the viruses circulating in specific populations, but also allow comparisons among geographic locations. Phylogenetic analysis provides information of the genetic relationships among virus populations from different geographic locations. However, such analysis is based on individual gene sequences and makes it difficult to compare entire viral genomes. In this study, we combined the characterization of virus genotypes with publicly available tools (FluGenome) for virus genotyping. Thus, I was able to

compare my findings to other studies using similar approaches (298, 299). This analysis showed that the genotypes that circulate in Guatemala are similar to those that circulate in other locations at the other end of the Atlantic and the Pacific flyways. Virus genotyping with FluGenome, however, does not provide information of genetic variant diversity within a single lineage. The assignment of genetic clades and the use of digital genotyping helped to visualize the genetic structure of the virus population, illustrating the contribution of genetic shift to the genetic diversity observed at the wintering grounds in Guatemala. The results from this analysis indicate that certain combinations of internal genes were more likely to be detected within a season, and that the main gene combinations varied between different years. In addition, digital genotyping allowed detection of potential reassortant strains that emerged during the period of sample collection. This was observed by changes in gene constellations in viruses from the same subtypes towards the end of the migration season, for example H14N4 viruses were only observed at the end of the migration season, with similar combination of internal genes harbored by the H14N3 viruses at the beginning of the season.

One interesting finding was the detection of rarely observed variants of H4, H3 and N2 and N8 genes. The viruses had similar genotypes and gene constellations from each other, but different from the majority of the other viruses. Two of their internal genes, NP and NS, belonged to the same lineage of a non-contemporary virus detected in Louisiana in 2013, A/blue-winged teal/Louisiana/AI12-1334/2013(H4N2) (300). For the other internal genes unique clades were observed. The lack of sequences of recent viruses with similar genetic makeup, and the relatively long branch lengths observed for the viruses in Guatemala within these clades suggest that these viruses may have been

circulating without being detected. These viruses may have been present either in a lower proportion to those from other contemporary lineages, or in locations where no surveillance is currently taking place, making it difficult to assess their origin. Interestingly, those rare variants were not observed at the beginning of the migration season, but only from December 2012 and January 2013 in Guatemala, which were presumably the same viruses observed during the spring of 2013 in the US.

In addition, to generate information of locally circulating viruses, avian influenza surveillance in Guatemala, as in many countries, was started to serve as a platform for the early detection of HPAI strains, including Eurasian-origin H5N1 viruses (Explained in Chapter 2). In this study PA genes from Eurasian lineage were detected in viruses from the H3N3 and H3N8 subtypes. The phylogenetic analysis indicates that this could be a separate introduction than of the H14 strain, as the PA genes are related to viruses detected recently in North America, distinct from the H14 strains. Detection of genes from the Eurasian lineage (in addition to the detection of the H14 subtype) provides evidence of long distance gene migration across the American Continent and that virus spread of Eurasian strains by migratory birds to Central America is plausible and could happen over the course of a few annual migration cycles. In the current study no reassortants strains with the South American lineage were detected. The frequency of this event and geographic locations where it is more likely to occur remains to be elucidated. Distribution of host species and the geographical barriers have been hypothesized to explain the limited reassortment between the North and South American lineages (300). However, as previously mentioned, the gap of surveillance in countries from Latin America is still significant.

Another finding from this study is the detection of N3 genes closely related to the HPAI H7N3 strain that caused a poultry outbreak in Mexico in 2012; this was also observed for some of the internal genes of the viruses from Guatemala, when the viruses from chickens were included in the analysis (data not shown). In our study, the H7 subtype was only found at the end of the migration season. A higher probability of detection of H7 subtype at the end of the wintering season and early during the spring is supported by observations in the blue-winged teal (100), suggesting that risk of spillover to poultry of viruses from this subtype could be subjected to seasonal patterns of LPAIV circulation in wild birds. The link between viruses from wild-bird origin and the HPAI H7N3 from Mexico highlights the importance of increasing surveillance at both ends of the migration flyways, to better understand virus migration patterns between geographic locations through out the year.

Among other species that tested positive for IAV were two white-winged doves. The number of samples collected from Columbiformes in this study was limited and no isolates were obtained from the rRT-PCR positive samples. There is natural and experimental evidence that doves and pigeons are susceptible to infection with IAV, including the H7N9 subtype from Asia (115). Although, their role in IAV transmission remains unclear, association with habitats where waterfowl species are abundant, such as wetlands, may increase the probability of exposure to IAV in terrestrial birds (discussed in Chapter 2). Nonetheless, the number of natural infections observed has been limited, suggesting that these species may solely act as incidental hosts.

In summary, we detected a wide diversity of virus subtypes and genomic constellations in ducks during the wintering season in Guatemala in this study. The

diversity of circulating viruses seems to vary between years and overall virus prevalence seems to decrease at the end of the migration season at the studied locations. Detection of rare gene variants indicates silent circulation of viruses in unknown reservoirs or ecological niches; these observations underscore the importance of expanding surveillance to additional geographic locations to capture the diversity of circulating strains across the American Continent. Our findings are supported by previous observations at the wintering grounds in Guatemala and other locations. A potential epizootic of the H14 subtype in blue-winged teals, supported by high levels of its detection during the 2012-13 season, provides further evidence that the wintering grounds in Central America may serve as places where virus variants with limited circulation may be amplified upon arrival of yearly migrants. Detection of reassortant strains with genes from the Eurasian lineage provides evidence of long distance movement of viruses from different genetic lineages and migration flyways between North and Central America. To our knowledge, the genomes reported in this study constitute the greatest collection of LPAIV from Central America. Surveillance in other species, and other locations in Central America, characterization of viruses that circulate in resident bird populations (during and after the winter migration), and incorporation of more systematic sampling methodologies, (including the use of geo-transmitters), are needed to better understand the ecology of IAV in this region. Although additional bird species need to be investigated, the high relative abundance of the blue-winged teals in comparison to other duck species in Central America, the particular behavior of this long-distance migrant (83, 300, 301), and the diversity of viruses found in these ducks makes them a candidate species for targeted IAV surveillance in the Neotropics.

4.6. Acknowledgements

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Chapter 5. Swine influenza surveillance in Guatemala

Co-authors: Ramirez, A.L., Müller, M.L., Orellana, D., Sosa, S. Paniagua, J., Ola, P., Hernandez, J., Perez, D.R. (Manuscript in preparation).

5.1. Abstract

In 2009 the emergence of the pandemic H1N1 (pH1N1) strain of influenza A virus (IAV) of swine origin highlighted the need of increasing surveillance of IAV in pigs on a global scale. In Central America, Guatemala is the country with the largest pork production and the circulation of IAV in the swine population has not been investigated in detail. The main objective of this study was to determine the presence of IAV in the swine population in Guatemala. Two nation-wide multistage random surveys for IAV were conducted during October in 2010 and from June to August in 2011. Nasal swabs and blood samples were collected from commercial farms and smallholder production systems. Samples were collected from 154 herds in 2010 (n=500) and 126 herds in 2011 (n=489). Herd prevalence for IAV detected by rRT-PCR was 36.3 and 34.6% for 2010 and 2011 respectively. Herd sero-prevalence based on ELISA was 19.5 and 5.6% for 2010 and 2011 respectively. Antibodies against H1N1 and H3N2 subtypes to viruses from different genetic clusters of swine origin (including pH1N1) were detected by hemagglutination inhibition assay. Three fully pH1N1 and one fully H3N2 seasonal human-like viruses were isolated. Results from the present study suggest that different IAV circulate in pigs in Guatemala and that human-animal contact may play an important role in the introduction of novel strains into the local swine population.

5.2. Introduction

The emergence of pandemic H1N1 (pH1N1) influenza A virus (IAV) of swine origin in 2009, highlighted the need of increasing surveillance for influenza virus in pigs on a global scale (302). Introduction of swine IAV into humans as well as reverse zoonotic transmission has been observed on multiple occasions in different geographical regions. As a result, interspecies transmission events of IAV viruses between human and animal hosts are now being recognized to be important in the generation of novel reassortant strains with the potential to spread among humans and swine populations (130, 160, 303).

In Latin America, very limited information is available on circulating strains of swine influenza and its epidemiology remains poorly understood (304). In general, animal raising and handling practices in many Latin-American countries resemble those from other regions (such as Asia) that are believed to be associated with an increased risk of exposure to zoonotic influenza viruses (305). In Central America, Guatemala is the country with the largest swine industry (estimated population size > 2.7 million (306)). Although large-scale commercial closed farming systems exist, the majority of pig production units (PPU) are peridomestic – in household backyards or open smallholdings – without specialized animal handling practices. A characteristic of these farming systems is that pigs are raised in open spaces or kept in contact with other domestic animals (307, 308). In Guatemala, there are no recommendations or regulations for vaccination against swine influenza, and little is known about the genetic and antigenic properties of circulating viruses. Circulation of swine H1N1 and H3N2 in the country has been documented by serological surveys in the past (309); however, only a limited

number of strains were used in this study which may have resulted in limited detection of the antigenic diversity. To our knowledge virus isolation from animal samples has not been attempted; consequently, the genetic makeup of circulating viruses remains unknown. In addition to the genetic diversity of virus strains, the distribution of IAV in the swine population and its year-to-year variations have not been investigated. In this study, two multistage cross-sectional surveys were performed in pigs in Guatemala to determine circulation of swine influenza subtypes including the 2009 pandemic H1N1 strain. Viral infection and exposure were investigated using molecular and serological testing. Information on the type of PPU, geographical location and animal characteristics were collected to investigate different risk exposure variables.

5.3. Materials and methods

5.3.1. Study design

Two nation-wide cross-sectional surveys were done in 2010 (October) and 2011 (June-August) to investigate the circulation of IAV in pigs, including the 2009 pandemic H1N1 strain. A sample size per year of n=500, was estimated using 5% prevalence at a 95% confidence level (estimated population of ~650,000 individuals) (Winepiscope 2.0 (310)). A multistage-random design was used. The number of pigs and pig production units (PPU) were chosen proportionally by department (administrative subdivisions in Guatemala) and type of farming system (farms and smallholdings), according to the country's distribution of the pig population. A form was designed to collect different exposure variables at the farm level (type of farm, geographic location) and at the animal level (animal health status, age and sex).

5.3.2. Sample collection

Field veterinarian epidemiologists from the Guatemala Ministry of Agriculture conducted recruitment and identification of participating farms and smallholdings, data collection, and sampling. Samples were collected from pigs with preference given to animals with signs of respiratory disease or when a foci of respiratory disease (defined as ≥ 10% of susceptible pigs in a PPU showing respiratory signs) was observed in the PPU at the time of sampling. For virus detection, nasal swabs were collected using sterile Dacron swabs and kept in tubes containing 3 mL of viral transport medium (VTM, Medium 199 with Hanks balanced salt solution, 2 mM L-glutamine, 0.5% bovine serum albumin, 0.35 g/liter sodium bicarbonate) with antibiotics and antimycotics (2×106 IU/L

Penicillin, 200 mg/L Streptomycin, 2×106 IU/L Polimyxin B, 250 mg/L Gentamycin, 0.5×106 IU/L Nistatin, 60 mg/L Ofloxacine, and 0.2 g/L Sulphamethoxazol). Additionally, for antibody detection, 2 mL of blood were collected from the orbital sinuous veins using 5 or 10 mL Vacutainer (Becton, Dickinson and Co., Franklin Lakes, NJ) collection tubes, and a 22 or 18 x 1 ½ gauge needle. Samples were preserved at 4°C and submitted between 1 to 4 days to the laboratory of Universidad del Valle de Guatemala for testing.

5.3.3. Virus detection and isolation

Viral RNA was extracted from 50 µL of nasal swab supernatant with the MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions and eluted in a final volume of 50 µL. Influenza virus A RNA was detected by rRT-PCR with Matrix specific primers according to published protocols (203). Two rRT-PCR assays were validated at the UVG laboratory using the One-Step RT-PCR and the Quantitec QuantiTect Probe RT-PCR Kits (QIAGEN, Hilden, Germany) in the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Influenza A Matrix rRT-PCR positive samples were tested for N1 subtype with recommended specific primers for pandemic H1N1 (311). Positive controls for Matrix and N1 RNA detection included RNA extracts from inactivated virus (provided by the National Veterinary Services Laboratories, USDA, Ames, Iowa) or *in-vitro* transcribed RNA from DNA plasmid coding for the corresponding gene segment. The positive control was calibrated to a Ct value between 25 and 35 for the diagnostic purposes of the assay. Samples with Ct values between 20 and 40 were considered positive and re-tested for confirmation.

All rRT-PCR positive samples were tested for virus isolation in MDCK cells. Briefly, 1 mL of a 1:2 dilution of clarified nasal swab supernatant was inoculated in 1-well of a 6 well plate containing approximately 3×10^5 MDCK cells. Samples were incubated for adsorption for 1 hour at 35°C in a 5% CO_2 atmosphere, after which the swab supernatant was removed and replaced with 3 mL of freshly prepared OptiMEM media (Life technologies, Grand Island, NY) with 1 μg/mL TPCK trypsin (SIGMA, St. Louis, MO) and antibiotics and antimycotics (SIGMA). Samples were kept at 35°C in a 5% CO_2 atmosphere, and supplemented with TPCK trypsin (1 μg/mL) and tested for virus presence every 3 days by HA assay. Samples were observed for presence of cytophatic effect (CPE) up to 10 days post inoculation. Presence of influenza A virus was confirmed in samples where CPE was observed and the samples tested positive by HA and by the rapid strip test Flu Detect (Synbiotics, San Diego, CA). For samples negative for CPE and HA assay blind passages were done at 5 dpi with 1 mL of clarified tissue culture supernatant, and treated as described above. Up to three blind passages were done per sample to test for viable virus in all samples.

5.3.4. Genome sequencing and molecular characterization

Virus isolates were identified by direct sequencing of HA and NA genes with Influenza A universal primers (205) and comparison with BLAST (312) as previously described (Chapter 2). Full genome sequences were obtained for all virus isolates (Genbank accessions KJ175112 to KJ175143). Full-length nucleotide sequences of all gene segments of all virus isolates were compared to other contemporary H1N1 and H3N2 strains by BLASTN (NCBI) to obtain the closest match for each segment. Pandemic H1N1 and human H3N2 sequences available for the region of Central America

and the Caribbean were selected for sequence comparison. Protein alignments were performed against selected sequences in order to identify point mutations relative to the closest relative viruses of human origin. A second set of alignments was performed using HA and NA protein sequences of the representative swine isolates and reference strains of swine and human origin to identify sequence variation at the antigenic sites. In addition, the Sequence Feature Variant Types tool of the Influenza Research Database (313) was used to identify genetic markers present in the pig isolates associated with increased replication, virulence or pathogenicity in mammalian hosts.

For the surface genes, nucleotide sequences of human H3N2 (from 2007 to 2013) and pandemic H1N1 viruses (from 2009 to 2013) from Central America and the Caribbean were downloaded from the EpiFlu database of the Global Initiative on Sharing All Influenza Data (GISAID, http://platform.gisaid.org). As a reference, vaccine strains of H3N2 (from 2007 to 2013) and pandemic H1N1 were included. Phylogenetic analyses were performed using MEGA software version 6.0 (280). Sequences were manually trimmed and final coding sequences were aligned with MUSCLE for codons. After alignment, identical sequences were removed and taxon datasets were subsampled in order to reduce sampling bias, by keeping 1 of every 5 sequences with equal genetic distance (as determined by the number of differences) when samples from the same location and year had a distance of d=0 between each other. Final phylogenetic trees were constructed using Maximum-Likelihood (ML) inference with the best-fit model of nucleotide substitution determined by the BIC criterion, Hasegawa-Kishino-Yano (HKY) + Γ. Robustness of tree topologies was assessed with 100 neighbor-joining bootstrap replicates. In addition, a global phylogeny including background sequences from other

geographical regions (Africa, Asia, Europe, North and South America, and Oceania) was inferred for the human H3 HA with viruses between 2010 and 2012. The tree was inferred by ML, under the GTR+GAMMA model of nucleotide substitution in RAxML (250). Tree topology was confirmed by Bayesian analysis using MrBayes v.3.2.2 (249) under the GTR+GAMMA model. Tree parameters are available upon request. Convergence was reached after 8×10^6 generations. Final sequences included in each tree are listed in appendices section (Table 0.4).

5.3.5. Serological testing

Serum samples were tested for antibodies against Influenza A with the commercially available kit, IDEXX ELISA Influenza A Ab (IDEXX, Westbrook, ME). According to manufacturer's instructions, a 1:10 dilution of each pig sample was tested. The cut off value was validated and adjusted from 0.50 to 0.56 (314). ELISA positive samples were tested by Hemagglutination inhibition assay (HI) against a selected panel of swine and human H1 and H3 viruses from different genetic clusters. HI assay was performed with standard protocols (315). Briefly, one volume of pig sera was treated with 3 volumes of RDE by incubating at 37°C overnight. After inactivation of RDE at 56°C for 30 minutes, 0.85% saline was added to obtain a 1:10 dilution of the original sample. 25 µl aliquots of 2-fold serially diluted serum samples were incubated for 30 min at RT with 25 µl of virus containing 8 HA units of the corresponding virus strains. The assay was read after adding 50 µl of 0.5% turkey red blood cells and incubating at RT for 30-40 min. Seropositive and seronegative ferret sera were used as positive and negative controls respectively. A serum control for each sample (sample without virus) was also tested to check for unspecific binding. HI titers were recorded as the reciprocal of the

last dilution of antiserum that completely inhibited the hemagglutination of the virus dilution containing 8 HA units. Samples with an HI titer ≤ 1:40 were considered negative for antibodies against the tested viruses. Samples were considered positive to the antigen with the highest inhibition titer, and exposure to multiple viruses was considered positive when the inhibition titer was the same for more than one reference antigen and when positive to multiple subtypes.

5.3.6. Epidemiological analysis

Pig production units were considered positive when at least one animal tested positive by rRT-PCR. Herd prevalence values were estimated as the total of positive units (farms and villages) over the total of sampled units. For herd prevalence estimates, PPU were defined as follows: for commercial farms, each farm was considered an individual PPU, and the total number of sampled farms was used to estimate prevalence per state. Pigs in smallholdings are commonly kept in open spaces and in proximity or shared with other smallholdings within the same village. Therefore prevalence in smallholdings was calculated at the village level (1 village = 1 PPU), and the total number of sampled villages was used to obtain national prevalence estimates. Prevalence values were computed with 95% confidence.

In addition, prevalence values were compared by χ^2 test and Fisher's test ($\alpha<0.05$) when assumptions were met, to establish differences at the herd level among geographical regions (central, north, east, south, west) and types of farms (commercial farm or small holding). Prevalence at the animal level was estimated as the total number of influenza A positive rRT-PCR samples over the total of collected samples per year, and for a subset of observations with complete data, differences between animal health

status (in 2010 and 2011), age and sex (data available only in 2011) were analyzed. Serological prevalence values were estimated similarly based on ELISA results at the herd and animal level and analyzed by different statistical comparisons as explained above.

To analyze the association of collected variables with virological and serological detection of IAV, prevalence risk ratios (PRR) were estimated at the herd level for type of farm and geographical region, and at the animal level for health status, age and sex. The use of PRR was chosen over prevalence odds ratio (POR), as POR is more sensitive to variations in disease prevalence, with stronger deviation from the null hypothesis and less precise than PRR estimates in cross-sectional studies (316, 317).

5.4. Results

5.4.1. Sample collection

Samples were collected from 154 herds in 2010 (500 pigs) and 129 herds in 2011 (499 pigs). In 2010, 54% sampled PPU were commercial farms (n=83, 330 pigs) and the rest were smallholdings (46%, n=71, 170 pigs). In 2011, 53 commercial farms were sampled (41%, 231 pigs), and 73 smallholdings (56%, 258 pigs). In addition, samples were submitted from 1 abattoir (4 pigs), and from 2 farms that were not identified as commercial or smallholdings (5 and 1 pigs respectively). Samples from the abattoir and the unidentified farms were tested in the laboratory, but they were excluded from all statistical analyses. At the time of sampling in 2010, 92% (n=460) of pigs were reported with respiratory signs, 5.8% (n=29) with no signs, and 2.2% (n=11) as "unknown" for health status. In 2011, only a total of 14.1% (n=69) of pigs were reported with respiratory signs, 70.6% (n=345) with no signs, and 15.3% (n=75) as "unknown". Information on animal sex and age was collected only in 2011. A summary of the sample distribution by collected exposure variables is shown in Table 5.1.

5.4.2. Virus prevalence and seroprevalence

Similar herd prevalences for IAV detected by rRT-PCR were obtained in both years. Herd prevalence was 36.4% in 2010 and 34.9% in 2011 (Table 5.2), with no significant difference between these years. Circulation of influenza A virus in pigs was detected all over the country in both years of the study, with the exception of two departments in 2010. Virus prevalence varied among different departments and

geographical regions, and the distribution of positive PPU varied between the two years of the study (Table 5.3).

Table 5.1. Characteristics of pigs sampled for IAV in Guatemala, 2010-2011

Variable	Year				Total	
	2010		2011			
	n	(%)	n	(%)	n	(%)
Sex						
Female	N/D	-	192	(38)	192	(19)
Male	N/D	-	262	(53)	262	(26)
Unknown	500	(100)	45	(9)	545	(55)
Age						
Pup	N/D	-	66	(13)	66	(7)
Weanling	N/D	-	9	(2)	9	(1)
Juvenile	N/D	-	337	(68)	337	(34)
Adult	N/D	-	76	(15)	76	(8)
Unknown	500	(100)	11	(2)	511	(51)
Type of PPU						
Farm	330	(66)	231	(47)	561	(57)
Smallholding	170	(34)	258	(53)	428	(43)
Health Status						
Sick	460	(92)	69	(14)	529	(53)
Healthy	29	(6)	345	(71)	374	(38)
Unknown	11	(2)	75	(15)	86	(9)
Geographical region						
Central	51	(10)	51	(10)	102	(10)
North	112	(22)	111	(22)	223	(22)
East	101	(20)	101	(20)	202	(20)
South	109	(22)	109	(22)	218	(22)
West	127	(25)	127	(25)	254	(25)

N/D: Not determined.

Herds from different departments were grouped by relative geographic location (central, north, east, south and west); herd prevalence was not significantly different among regions in 2010, although borderline associations were observed with states from the east and west (Table 5.4). In contrast, in 2011 the distribution of positive herds varied significantly ($p = 0.0012$, $\chi^2 = 17.99$, df = 4), with significant associations with the north and south, and borderline association with western departments [$PRR_{95\%} = 1.2$ (0.7, 2)].

In 2010 a higher prevalence was observed in commercial farms (43.4% of herds positive) when compared to smallholdings (28.1%). This difference was not significant, but a borderline association [$PRR_{95\%}$ = 1.5 (0.98, 2.4)] was observed. In 2011 a similar prevalence was observed in smallholdings (35.6%) and commercial farms (34.0%) with no significant difference.

At the animal level no significant difference in virus prevalence was observed between sex or age (Table 5.5). However a borderline association with IAV detection was observed in juvenile pigs [$PRR_{95\%}$ = 1.2 (0.7, 2.2)]. Data available for health status was analyzed independently for each year (data not shown), and combined for both years. A higher prevalence in sick animals (16.5%) was observed in 2010 in comparison to healthy individuals (10.3%). In 2011 similar prevalence was observed in healthy (11%) and sick animals (9%). When both years were combined, sick animals had a higher prevalence (16%) than healthy animals (11%), with no significant difference. The estimated *PRR* suggested an association between IAV detection and sick animals (Table 5.5).

Table 5.2. Prevalence and seroprevalence of IAV in pig herds in Guatemala, 2010-2011

Year	Prevalence (rRT-PCR)			Seroprevalence (ELISA)		
	Number of PPU	Positive PPU	% Positive PPU (95% CI)	Number of PPU	Positive PPU	% Positive PPU (95% CI)
2010	154	56	36.4(28.7, 44.0)	154	30	19.5(13.2, 25.7)
2011	126	44	34.9(26.6, 43.2)	125	7	5.6(1.6, 9.6)

Table 5.3. Prevalence of IAV detected by rRT-PCR and geographic distribution of PPU and pigs tested in 2010 and 2011

Department	2010				2011			
	No. PPU	**No. pigs**	**Positive PPU (%)**	**Positive pigs (%)**	**No. PPU**	**No. pigs**	**Positive PPU (%)**	**Positive pigs (%)**
North								
Alta Verapaz	7	24	3(42.9)	4(16.7)	7	24	1(14.3)	1(4.2)
Peten	10	27	3(30.0)	3(11.1)	10	27	3(30.0)	3(11.1)
Total	**17**	**51**	**6(35.3)**	**7(13.7)**	**17**	**51**	**4(23.5)**	**4(7.8)**
Central								
Baja Verapaz	2	13	2(100)	4(30.8)	2	13	2(100)	2(15.4)
Chimaltenango	7	32	1(14.3)	1(3.1)	4	27	1(25.0)	1(3.7)
Guatemala	11	38	4(36.4)	4(10.5)	7	33	6(85.7)	6(18.2)
Sacatepequez	9	29	0 -	0 -	6	29	3(50.0)	3(10.3)
Total	**29**	**112**	**7(24.1)**	**9(8.0)**	**19**	**102**	**12(63.2)**	**12(11.8)**
East								
Chiquimula	11	21	2(18.2)	2(9.5)	16	21	0 -	0 -
El Progreso	4	13	2(50.0)	2(15.4)	3	13	0 -	0 -
Izabal	5	10	2(40.0)	2(20.0)	3	10	2(66.7)	2(20.0)
Jalapa	2	10	1(50.0)	1(10.0)	1	10	1(100)	3(30.0)
Jutiapa	10	34	4(40.0)	4(11.8)	11	34	1(9.1)	1(2.9)
Zacapa	7	13	6(85.7)	9(69.2)	7	13	3(42.9)	3(23.1)
Total	**39**	**101**	**17(43.6)**	**20(19.8)**	**41**	**101**	**7(17.1)**	**9(8.9)**
South								
Escuintla	8	36	2(25.0)	3(8.3)	5	36	3(60.0)	5(13.9)
Retalhuleu	6	17	1(16.7)	1(5.9)	3	17	0 -	0 -
Santa Rosa	4	27	1(25)	3(11.1)	4	27	4(100)	4(14.8)
Suchitepequez	8	29	3(37.5)	4(13.8)	4	28	4(100)	5 (17.9)
Total	**26**	**109**	**7(26.9)**	**11(10.1)**	**16**	**108**	**11(68.8)**	**14(13.0)**
West								
Huehuetenango	10	53	3(30.0)	6(11.3)	8	53	5(62.5)	8(15.1)
Quetzaltenango	7	16	2(28.6)	3(18.8)	7	16	1(14.3)	1(6.3)
Quiche	8	17	5(62.5)	10(58.8)	3	17	1(33.3)	2(11.8)
San Marcos	5	15	0 -	0 -	9	15	2(22.2)	4(26.7)
Solola	4	12	4(100)	9(75.0)	3	12	1(33.3)	1(8.3)
Totonicapán	9	14	5(55.6)	5(35.7)	3	14	3(100)	3(21.4)
Total	**43**	**127**	**19(44.2)**	**33(26.0)**	**33**	**127**	**13(39.4)**	**19(15.0)**
Total	**154**	**500**	**56(36.4)**	**80(16.0)**	**126**	**489**	**47(37.3)**	**58(11.9)**

With respect to the detection of antibodies against IAV, herd seroprevalence values of 19.5 and 5.6% were observed for 2010 and 2011 respectively (Table 5.6). Prevalence was lower in 2011. However, the composition of the sample between years was different in terms of animal health status: in 2010 the majority of sampled pigs had respiratory signs (92%), whereas in 2011 only 15% had signs. Due to the potential differences in susceptibility and prior-exposure to IAV of these two groups, seroprevalences between years were not statistically compared. Distribution of seropositive PPU by department varied between different years ranging between 0% and 100% seropositive PPU in both years (Table 5.6). When compared by geographical region, herd seroprevalence varied among different areas in 2010 ($p = 0.0005$, $\chi^2 = 19.8$, df = 4), with significant association with the east and south (Table 5.7). The number of seropositive herds in 2011 was too low to perform statistical comparisons, but the majority of positive herds were located in the west and central areas.

At the animal level, comparisons of seropositive pigs were made by sex and age, but for this analysis only data from 2011 was available. No significant difference was observed between male and female animals. The number of seropositive pigs was too low for statistical comparisons; the higher proportion of positive samples was found in adult pigs (Table 5.8).

5.4.3. Hemagglutination inhibition assay

Antigenic responses against H1N1 and H3N2 subtypes from different genetic and antigenic clusters (Table 5.9) were detected in ELISA positive samples. In both years, antibodies against the pandemic H1N1 strain were found in the majority of samples. Additionally in 2010 antibodies against swine viruses from the H1N1 subtype (including

alpha, and gamma clusters) were detected; antibodies against the H1 beta cluster were only found in one sample with mixed exposures (positive to more than one virus). All samples were negative when tested against the H1 delta antigen. For the H3 subtype, the majority of samples tested positive against H3 (cluster III) followed by H3 (cluster IV). A number of mixed exposures between the pandemic H1N1 virus and other H1 or H3 viruses of swine origin were detected. In 2011 only 7 samples tested positive by ELISA, three of which were positive to the pandemic strain and two that had mixed exposures with the pandemic H1N1 and the H3 (IV) subtype. All samples from both years tested negative against a human-like H3N2 isolate obtained in 2010 [A/Guatemala/CIP049-IP040078/2010(H3N2)] (see below). The subtype specific response of 8 ELISA positive samples from 2010 and of 2 from 2011 could not be identified with tested antigens.

Exposure to different subtypes varied between types of PPU: exposure to H1 viruses of swine origin was detected in farms, whereas in smallholdings only exposure to pandemic H1 was found. With respect to the H3 subtype, exposure to antigens from clusters III and IV was detected in farms in 2010 as single or multiple exposures. In smallholdings exposure to H3 viruses was only found in samples with co-exposure to pandemic H1. The samples that couldn't be identified were from both farms (n=6) and smallholdings (n=4).

Table 5.4 Prevalence risk ratios of farm exposure factors associated with IAV detection in sampled PPU in Guatemala, 2010-2011

Variable	2010				2011			
	IAV positive (%)	IAV negative (%)	PRR* (95% CI)	*p*, χ^2, *df*	IAV positive (%)	IAV negative (%)	PRR (95% CI)	*p*, χ^2, *df*
Type of PPU								
Smallholding	20(35.7)	51(52)	Reference	0.0506, 3.8, 1	26(59.1)	47(57.3)	Reference	0.8475, 0.04, 1
Farm	36(64.3)	47(48)	**1.5(1,2.4)**		18(40.9)	35(42.7)	1(0.6,1.6)	
Geographical region								
Central	6(10.7)	11(11.2)	1(0.5,1.9)	0.2977, 4.9, 4	4(9.1)	13(15.9)	0.6(0.3,1.6)	**0.0012, 18, 4**
North	7(12.5)	22(22.4)	0.6(0.3,1.2)		10(22.7)	9(11)	**1.7(1,2.8)**	
East	17(30.4)	22(22.4)	**1.3(0.8,2)**		7(15.9)	34(41.5)	0.4(0.2,0.8)	
South	7(12.5)	19(19.4)	0.7(0.4,1.4)		10(22.7)	6(7.3)	**2(1.3,3.2)**	
West	19(33.9)	24(24.5)	**1.3(0.9,2)**		13(29.5)	20(24.4)	**1.2(0.7,2)**	

*Significant associations (lower limit of 95% CI <1) and p values (p<0.05) are shown in bold

Table 5.5. Prevalence risk ratios of animal exposure factors associated with IAV detection in sampled pigs in Guatemala, 2010-2011

Variable	IAV positive (%)	IAV negative (%)	PRR (95% CI)*	*p, χ2, df**
Sex				
Female	23(44.2)	167(42.5)	Reference	0.8119, 0.06, 1
Male	29(55.8)	226(57.5)	0.9(0.6,1.6)	
Age				
Pup	8(14.5)	58(13.7)	1.1(0.5,2.1)	0.7605, 1.2, 3
Weanling	1(1.8)	8(1.9)	1(0.1,6.2)	
Juvenile	40(72.7)	287(67.8)	**1.2(0.7,2.2)**	
Adult	6(10.9)	70(16.5)	0.6(0.3,1.5)	
Health status				
Healthy	42(33.9)	332(42.6)	Reference	0.0662, 3.4, 1
Sick	82(66.1)	447(57.4)	**1.4(1,2)**	

*Significant associations (lower limit of 95% CI <1) and *p* values ($p<0.05$) are shown in bold

5.4.1. Virus isolation and molecular characterization

In 2010, 4 viruses were isolated from rRT-PCR positive samples. CT-values from rRT-PCR positive samples ranged between 17 and 41 (mean 36.5, median 37.7, stdev = 4.5), 10 samples had CT-values <30 and 11 samples had CT-values between 30 and 35. The CT-values of the samples from which the viruses were isolated were between 27 and 35. Full-length genome sequences were obtained and the viruses were identified as H1N1 (3 isolates) and H3N2 (one isolate) subtypes based on >95% homology in BLAST searches. Viruses were identified as A/swine/Guatemala/CIP049-IP070165/2010 (H1N1), A/swine/Guatemala/CIP049-IP070166/2010 (H1N1), A/swine/Guatemala/CIP049-IP070167/2010 (H1N1) and A/swine/Guatemala/CIP049-IP040078/2010 (H3N2). All gene segments of the three H1N1 viruses shared >98% sequence identity with each other, and clustered with the pandemic lineage. The H3N2 (here after 040078-H3N2) virus was identified as a fully human-like strain. No reassortant viruses were detected and in 2011 no virus isolates were obtained.

Table 5.6. Seroprevalence of IAV detected by ELISA and geographic distribution of PPU and pigs tested in 2010 and 2011

Department	2010				2011			
	No. PPU	**No. pigs**	**Positive PPU (%)**	**Positive pigs (%)**	**No. PPU**	**No. pigs**	**Positive PPU (%)**	**Positive pigs (%)**
North								
Alta Verapaz	7	24	1(14.3)	1(4.2)	7	24	0-	0-
Peten	10	27	3(30.0)	5(18.5)	10	27	2(20.0)	2(7.4)
Total	**17**	**51**	**4(23.5)**	**6(11.8)**	**17**	**51**	**2(11.8)**	**2(3.9)**
Central								
Baja Verapaz	2	13	0(-)	0(-)	2	13	0(-)	0(-)
Chimaltenango	7	32	0(-)	0(-)	4	27	0(-)	0(-)
Guatemala	10	38	0(-)	0(-)	7	33	0(-)	0(-)
Sacatepequez	9	29	1(11.1)	1(3.4)	6	29	0(-)	0(-)
Total	**28**	**112**	**1(3.6)**	**1(0.9)**	**19**	**102**	**0(-)**	**0(-)**
East								
Chiquimula	11	21	3(27.3)	5(23.8)	16	21	1(6.3)	1(4.8)
El Progreso	4	13	1(25.0)	1(7.7)	3	13	0(-)	0(-)
Izabal	5	10	0(-)	0(-)	3	10	0(-)	0(-)
Jalapa	2	10	0(-)	0(-)	1	10	0(-)	0(-)
Jutiapa	10	34	3(30.0)	13(38.2)	11	34	0(-)	0(-)
Zacapa	7	13	2(28.6)	3(23.1)	7	13	0(-)	0(-)
Total	**39**	**101**	**9(23.1)**	**22(21.8)**	**41**	**101**	**1(2.4)**	**1(1.0)**
South								
Escuintla	8	36	4(50.0)	6(16.7)	5	36	0(-)	0(-)
Retalhuleu	6	17	1(16.7)	1(5.9)	3	17	0(-)	0(-)
Santa Rosa	4	27	4(100)	7(25.9)	4	27	0(-)	0(-)
Suchitepequez	8	29	3(37.5)	6(20.7)	4	28	0(-)	0(-)
Total	**26**	**109**	**12(46.2)**	**20(18.3)**	**16**	**108**	**0(-)**	**0(-)**
West								
Huehuetenango	11	53	2(18.2)	2(3.8)	7	53	0(-)	0(-)
Quetzaltenango	7	16	0(-)	0(-)	7	16	0(-)	0(-)
Quiche	8	17	2(25.0)	2(11.8)	3	17	0(-)	0(-)
San Marcos	5	15	0(-)	0(-)	9	15	2(22.2)	3(20.0)
Solola	4	12	0(-)	0(-)	3	12	1(33.3)	1(8.3)
Totonicapán	9	14	0(-)	0(-)	3	14	0(-)	0(-)
Total	**44**	**127**	**4(9.1)**	**4(3.1)**	**32**	**127**	**3(9.4)**	**4(3.1)**
Total	**154**	**500**	**30(19.5)**	**53(10.6)**	**125**	**489**	**6(4.80)**	**7(1.4)**

Table 5.7. Prevalence risk ratios of farm exposure factors associated with IAV seropositivity in sampled PPU in Guatemala, 2010-2011

Variable	2010				2011			
	IAV positive (%)	IAV negative (%)	PRR (95% CI)	*p, χ2, df*	IAV positive (%)	IAV negative (%)	PRR (95% CI)	*p**
Geographical region								
Central	4(13.3)	13(10.5)	1.2(0.5,3.1)	**0.0005, 19.8, 4**	2(33.3)	15(12.3)	**3.3(0.6,16.5)**	
North	1(3.3)	27(21.8)	0.2(0,1.1)		0(0)	21(17.2)	0.4(0,6.5)	
East	9(30)	30(24.2)	**1.3(0.6,2.5)**		1(16.7)	40(32.8)	0.4(0.1,3.5)	
South	12(40)	14(11.3)	**3.3(1.8,6)**		0(0)	17(13.9)	0.5(0,8.1)	
West	4(13.3)	40(32.3)	0.4(0.1,1)		3(50)	29(23.8)	**3(0.6,14.1)**	
Type of PPU								
Smallholding	8(26.7)	63(50.8)	Reference	**0.0173, 5.7, 1**	4(66.7)	69(58)	Reference	1.00
Farm	22(73.3)	61(49.2)	**2.4(1.1,5)**		2(33.3)	50(42)	0.7(0.1,3.7)	

* P value for Fisher's exact test, comparisons by geographical region in 2011 were not done due to the low number of seropositive pigs
Significant associations (lower limit of 95% CI <1) and p values (p<0.05) are shown in bold

Table 5.8. Prevalence risk ratios of animal exposure factors associated with IAV seropositivity in sampled pigs in Guatemala, 2011

Variable	IAV positive (%)	IAV negative (%)	PRR (95% CI)	*p**
Sex				
Female	4(57.1)	189(42.3)	-	0.47
Male	3(42.9)	258(57.7)	0.6(0.1,2.4)	
Age				
Pup	1(14.3)	65(13.5)	1.1(0.1,8.7)	
Weanling	0(0)	9(1.9)	3.2(0.2,52.3)	
Juvenile	3(42.9)	334(69.4)	0.3(0.1,1.5)	
Adult	3(42.9)	73(15.2)	**4.1(0.9,17.8)**	

*p-value for Fisher's exact test. Significant associations (lower limit of 95% CI <1) and p values ($p<0.05$) are shown in bold

Virus isolates were analyzed for the presence of mutations in the protein sequence in comparison to other viruses circulating in the region (Mexico, Central America and the Caribbean) between 2009 and 2011. A number of mutations in the pandemic-like isolates were identified in different genes, including PB2, PA, HA, NA, M2 and NS1 (Table 5.10). Two synonymous mutations found in the HA (V251L and R222K) were to residues that are more frequent in swine-origin viruses of the H1 subtype. This was also the case for one of the mutations identified in PA (E688G) and two of the mutations identified in NA (G298A and a mixed base E462D). A specific role for these residues has not been described; the HA R222K mutation is present in the antigenic site Ca1, while the others are mapped to experimentally determined epitopes reported in the IRD. For the HA protein five glycosylation sites were predicted at positions 28, 40, 104, 294 and 304. In addition, protein alignments of the HA1 and NA sequence were performed against reference pandemic strains and swine viruses from different H1 genetic clusters (318). In comparison to reference pandemic strains and in contrast with the reference swine viruses, no other mutations were found at the antigenic sites (with the exception of the

R222K mutation) (Figure 5.1 and Figure 5.2). The presence of phenotypic markers associated with transmissibility and pathogenesis in mammalian hosts showed that the pH1N1 isolates contain the prototypic motifs described for the pandemic viruses (Table 5.11).

For the human-like H3N2 virus three mutations were identified when compared to human H3N2 viruses circulating in the region between 2009 and 2011, one in PB1 and two in HA (Table 5.10). The PB1 mutation N213D is located in the nuclear localization signal. For the HA one mutation was observed in the signal peptide (A16T) and one in the HA1 extracellular domain. The mutations are contained between experimentally determined epitopes, but a specific role for the amino acids at the positions has not been described. The H3N2 virus codifies for a truncated PB1-F2 protein of 53 amino acids. A total of seven glycosylation sites were predicted for the H3 protein at positions 24, 38, 79, 149, 181, 301, 499. Protein alignments of HA1 (H3) showed two mutations in the antigenic site C of 040078-H3N2 when compared to seasonal human vaccine strains (Figure 5.3A). Differences in the antigenic sites were observed in comparison to other swine viruses (Figure 5.3B). For the N2 no mutations were observed when compared to human strains (Figure 5.4) and only one mutation was found when compared to swine viruses (Figure 5.5). Analysis of phenotypic markers showed that the H3N2 isolate is a well-adapted human isolate that contains several of the described determinants of mammalian and human transmission, including markers for high transmissibility in humans (Table 5.12). No markers related to increased infectivity or replication that have been described for swine respiratory epithelial cells were found. The M2 protein of 040078-H3N2 contains amino acid 31N, associated with resistance to amantadine.

Table 5.9. Antigenic responses detected by hemagglutination inhibition assay detected in pig sera, Guatemala, 2010-2011

		2010			2011		
Virus	**Reference strain**	**Farm(%)**	**Smallholding(%)**	**Total(%)**	**Farm(%)**	**Smallholding(%)**	**Total(%)**
H1 pandemic	A/Mexico/4108/2009	9(20.5)	1(11.1)	10(18.9)	2(66.7)	1(25)	3(42.9)
H1N1 α only	A/swine/Minnesota/02053/2008 (α)	5(11.4)		5(9.4)			
H1N1 β only	A/swine/Nebraska/02013/2008 (β)						
H1N1 γ only	A/swine/Missouri/02060/2008 (γ)	2(4.5)		2(3.8)			
H1N2 δ only	A/swine/Iowa/02039/2008 (δ)						
swH3N2 (III) only	A/swine/Wisconsin/14094/99	9(20.5)		9(17)			
swH3 (IV)	A/turkey/Ohio/313053/2004 or A/swine/Illinois/A01201606/2011	6(13.6)		6(11.3)			
huH3 only	A/swine/Guatemala/IP-04-0078/2008						
H1 pandemic, H1 α			1(11.1)	1(1.9)			
H1 pandemic, H1 β		1(2.3)		1(1.9)			
H1 pandemic, H1 γ		1(2.3)	2(22.2)	3(5.7)			
H1 pandemic, swH3 (III)			1(11.1)	1(1.9)			
H1 pandemic, swH3 (IV)		2(4.5)		2(3.8)		2(50)	2(28.6)
H1 γ, H3 (III)		1(2.3)		1(1.9)			
swH1		2(4.5)	1(11.1)	3(5.7)			
swH1, H3 (III)		1(2.3)		1(1.9)			
U		5(11.4)	3(33.3)	8(15.1)	1(33.3)	1(25)	2(28.6)
Total		**44**	**9**	**53**	**3**	**4**	**7**

*number of HI positives from ELISA positive samples.

In the phylogenetic analysis the pH1N1 isolates the viruses cluster with other contemporary pandemic viruses that circulated in Guatemala and other neighboring countries (Belize, El Salvador and Mexico) during the same year. The topology of the HA tree contained multiple clusters. The HA of the three viruses from Guatemala was found in separate clusters from early pH1N1 isolates and other swine pH1N1 viruses from other countries, indicating an independent introduction event from humans (Figure 5.6). In contrast, the NA clustered with early isolates and other pH1N1 viruses from pigs, indicating a less divergent NA in viruses circulating across the region (Figure 5.7). The relatively short branch lengths for the HA and the NA of the swine isolates indicate a recent introduction into pigs, without extensive adaptation.

For the H3N2 virus, the HA and NA clustered with contemporary viruses that circulated in humans in Guatemala between 2010 and 2012. The HA clustered with other human viruses 2010 to 2012 that circulated in Central America, Mexico and the Caribbean but in a separate cluster from the most recent vaccine lineages (Figure 5.8). The same was not observed for the N2, which clustered within the lineage of the current vaccine (Figure 5.9). The existence of a separate HA clade was confirmed by generating a tree that included additional H3N2 viruses from previous vaccine lineages since 2000 (data not shown). In addition, to confirm if the circulation of viruses from this clade was specific to Central America, background viruses from other geographical regions (North and South America, Africa, Asia, Europe and Oceania) between 2010 and 2012 were included to construct a global maximum likelihood phylogenetic inference. In this tree the swine isolate from Guatemala still clustered in a separate clade from the vaccine lineages (A/Perth/16/2009, A/Victoria/361/2011 and A/Texas/50/2012), with viruses

from other geographical regions, indicating that circulation of viruses from this clade was not exclusive to Central America. However, the majority of sequences reported between those years from Central America, and to a lesser extent the viruses from South America, were contained in the same clade with the swine virus from Guatemala. This observation suggests a more extensive circulation of viruses in the region during those years. Co-circulation of a genetically distinct clade of H3N2 viruses has not been described in the literature, and the most recently published H3N2 phylogenies include viruses up to 2010. The topology of the global inference was further confirmed by Bayesian analysis, obtaining similar results (Appendices section, Figure 0.1 and Figure 0.2).

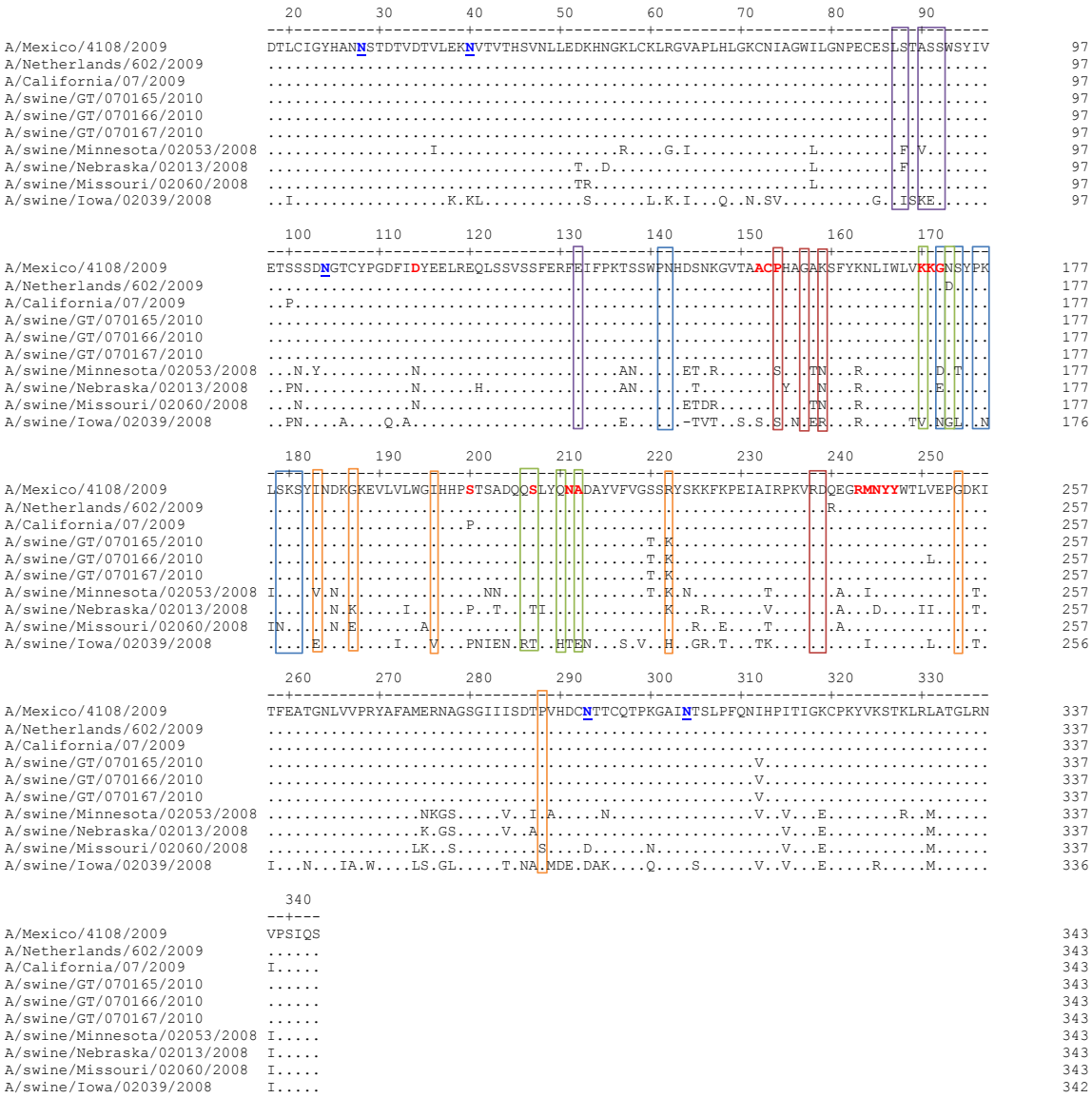

Figure 5.1. Protein alignment of the HA1 region of pH1N1 isolates, with human and swine viruses
Antigenic sites are shown in colored boxes A (red), B (green), C (purple), D (orange), E (blue). The N residues of predicted glycosylation sites for 040078-H3N2 are marked in blue in the consensus sequence. Receptor binding sites are marked in red in the consensus sequence.

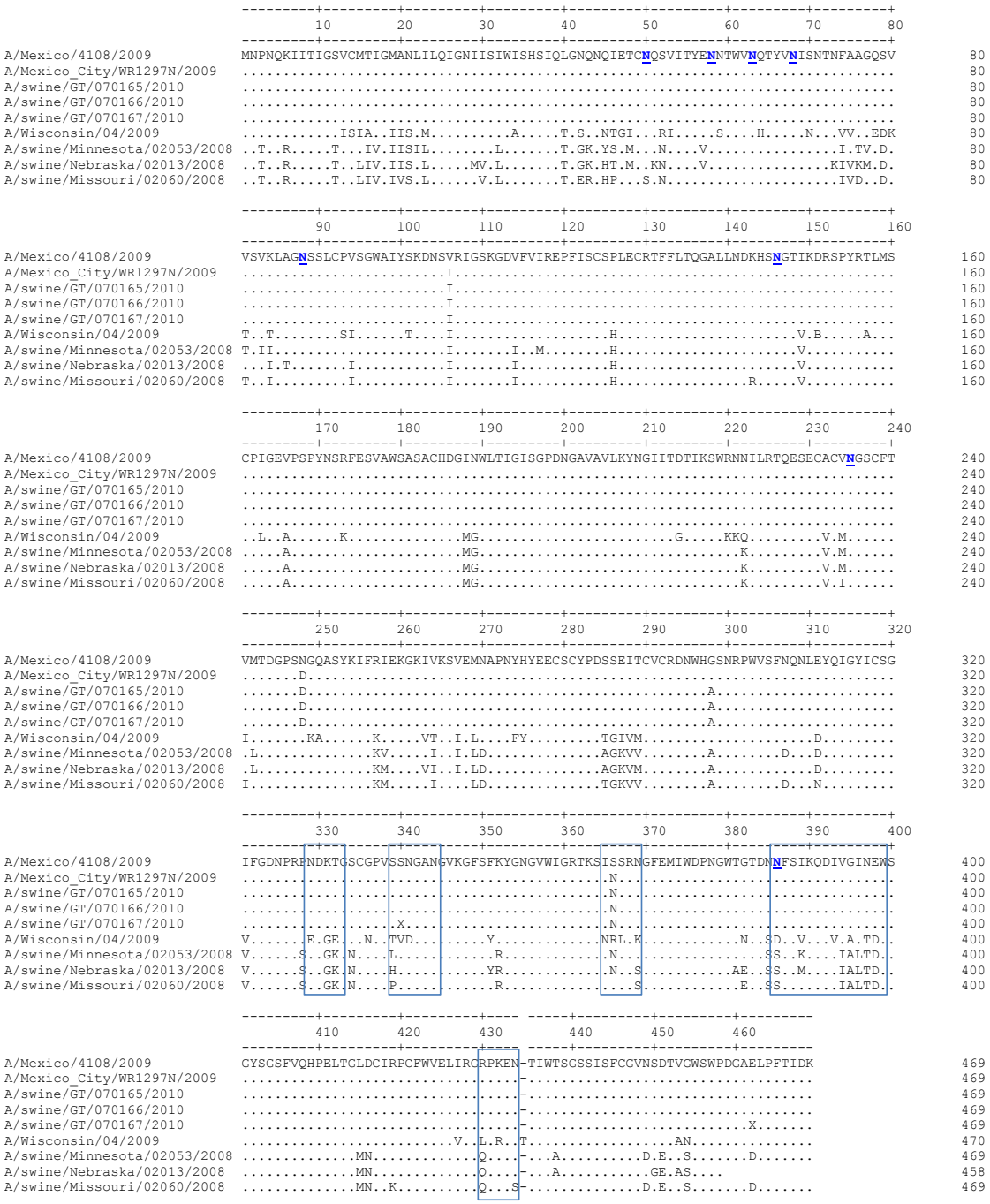

Figure 5.2. Alignment of NA amino acid sequences of pH1N1 isolates, with human and swine viruses Antigenic sites are shown in blue boxes. The N residues of predicted glycosylation sites for A/sw/GT/CIP049-040078/2010 are marked in blue.

Table 5.10. Mutations in swine isolates from Guatemala in comparison to co-circulating viruses between 2009-2011

Subtype	Gene	Mutation	Type of mutation	Isolate	% in database (pandemic strains)	% in database (swine strains)	Location
H1N1							
	PB2	Q288R	non-synonymous (polar to basic)	all	0.04	0.09	
		Xaa (S688F)	non synonymous (polar to aromatic)	070167	0.04	N/D	
	PA	Xaa (L589F)	non-synonymous (nonpolar to aromatic)	070165	0.02	N/D	PB1 binding region
		Xaa (S600F)	non synonymous (polar to aromatic)	070167	0.02	N/D	PB1 binding region
		E688G	non-synonymous (acidic to polar)	all	0.22	20.6	PB1 binding region
		Xaa (A689P)	synonymous	070167	N/D	N/D	PB1 binding region
	HA	V251L	synonymous	070166	0.1	53.0	HA1 extracellular domain, beta strand
		R222K	synonymous	all	3.7	73.0	HA1 extracellular domain, beta strand. Antigenic site Ca1
	NA	G298A	synonymous	all	0.01	58.9	catalytic domain
		Xaa (S340F)	non synonymous (polar to aromatic)	070167	0.46	0.52	catalytic domain
		Xaa (E462D)	synonymous	070167	0.01	60.6	catalytic domain
	M1		no				
	M2	T43I	non synonymous (polar to nonpolar)	all	0.2	0.55	transmembrane domain
	NS1	V60F	non-synonymous (nonpolar to aromatic)	all	N/D	N/D	NS1 alpha helix of RNA binding domain
H3N2							
	PB1	N213D	non synonymous (polar to acidic)	0400078	0.13	0.3	nuclear localization motif
	PB1-F2	1 - 53	truncated peptide (53 aa)	0400078	13.39	0.0	
	HA	A16T	non synonymous (nonpolar to polar)	0400078	1.11	0.2	Signal peptide
		Xaa (G234R) (218 HA1)	non synonymous (nonpolar to basic)	0400078	0.16	0.0	HA1 extracelular domain

Table 5.11. Phenotypic markers associated with transmission and pathogenicity found in all pH1N1 swine isolates from Guatemala

Protein	Amino Acid	Reference*	Comments
PB2	271A	PMID: 20181719	The residue 271 contributes to enhanced polymerase activity and viral growth in mammalian hosts. In this study the T271A mutation showed enhanced virus growth compared to that of the WT in mammalian cells in vitro.
	360Y	PMID: 18768983	The study was done on PR8/H5N1 6:2 reassortant wherein NS gene was derived from PR8(Cambridge) strain and remaining internal genes from PR8(UW) strain). The Tyr residue at position 360 of PR8(UW) PB2 confers high efficiency of vaccine seed virus growth in MDCK cells. Glu at position 55 of NS1 mediates growth enhancement of viruses in MDCK cells.
	627E	PMID: 19119420	The K627E mutation reduces transmission of human influenza viruses in the guinea pig, presumably because of reduced replicative ability in the upper respiratory tract.
	590S, 591R	PMID: 19995968	SR polymorphism enhances replication of virus in host cells. Adaptive mutation in 2009 swine origin virus enhances polymerase activity in human hosts and compensates for E627.
PA	85I, 186S, 336M	PMID:21561908	These residues are responsible for the enhanced polymerase activity in mammalian cells.
	57R, 62I, 65S, 85I, 86M, 91V, 100V, 114E, 186S	PMID:23283952	Multiple residues on PA of avian-origin pH1N1 influenza viruses suppress host cell protein synthesis during infection, allowing for preferential production of viral proteins.
	409N	PMID: 17073083	S409N enhances transmission in humans
	245K	PMID: 7966557	Mutations at these positions are capable of suppressing the temperature-sensitivity phenotype of the virus.
NP	184K	PMID: 19475480	A change from alanine to a lysine at residue 184 of NP results in increased replication and pathogenicity of the viruses in chickens.
	16G	PMID: 18058063	Wild-type A/PR/8 is highly pathogenic in mice, whereas the PR/8 with D16G is less lethal confirming that a single mutation in the N terminus of NP of the human PR/8 virus ignificantly decreases the pathogenicity of the virus in mice. Similarly, introduction the human-like G16D substitution into the NP of highly pathogenic A/Vietnam/1203/04 (H5N1) virus decreases lethality in mice.

	34G	PMID: 12620793	Multiple loci confer ts phenotype to the vaccine strains from master donor virus (MDV) A/AA/6/60 used in FluMist: PB1 (K391E and E581G), PB2 (N265S), and NP (D34G). The PB1 (A661T) also contributes to the ts phenotype.
	214R, 217R, 253I	PMID:24335312	Residues 214, 217, and 253 of Aichi NP play a critical role in influenza virus morphology, possibly through interaction with the M1 layer during virus budding
NA	R226G	PMID: 20522774	Introducing mutation R194G can compensate for the effects of H274Y mutation in NA (H274Y decreases surface expression of NA as well as reduces viral fitness) and thereby restore its activity. Therefore oseltamivir resistance is enabled by these permissive compensatory mutations that allow for subsequent H274Y mutations in the virus.
	469C	PMID: 11533192	The C-terminal Lys at this position is critical for virulence and its ability to replicate in the mouse. It supports plasminogen-binding activity which is critical for WSN virus neurotropism.
	146, 469K	PMID: 11533192	The presence of a C-terminal Lys 453 and the lack of glycosylation at position 130 (146 in N2 numbering) are required for binding of the NA to plasminogen.
M1	30N, 142A, 207N, 209T	PMID:23209789	Determinants of virus morphology (pH1N1 spherical). Single replacement of either one of the residue in M1 protein reduces overall viral production as well as growth kinetics.
M2	31N	PMID: 22132146	31N confers resistance to Adamantane
NS1	149A	PMID: 16971424	Residue 149 is crucial for the difference in virulence between GS/GD/1/96 and GS/GD/2/96 strains in chickens. Recombinant virus with Ala149 can antagonize the induction of interferon levels in chicken embryo fibroblasts (CEFs), but a recombinants with Val149 are not capable of the same effect.
	92D	PMID: 12195436, 23042343	This residue of NS1 is responsible for H5N1 viruses' resistance to the interferons and TNF-alpha and depends on the substitution of glutamic acid for aspartic acid at position 92 of the NS1 molecule. This has been disputed by subsequent research
	42S	PMID: 18032512	This residue (P42S) is critical for the pathogenicity of H5N1 influenza viruses in mammalian hosts and plays an important role in decreasing the antiviral immune response of the host cell.
	103F, 106M	PMID: 17522219	Changing any one of the two residues, either L103 to F or I106 to M, in the HK97 NS1A protein caused increase in the rate of virus replication. HK97 NS1A protein is intrinsically defective in binding CPSF30.
	227..230 (PEQK)	PMID: 18334632	The avian and equine strains always have ESEV at the C-terminal region and human almost never have that. In contrast, majority of the human signatures have RSKV sequence; the remaining have avian signatures and are known to be of avian origin.

*PMID PubMed identification number

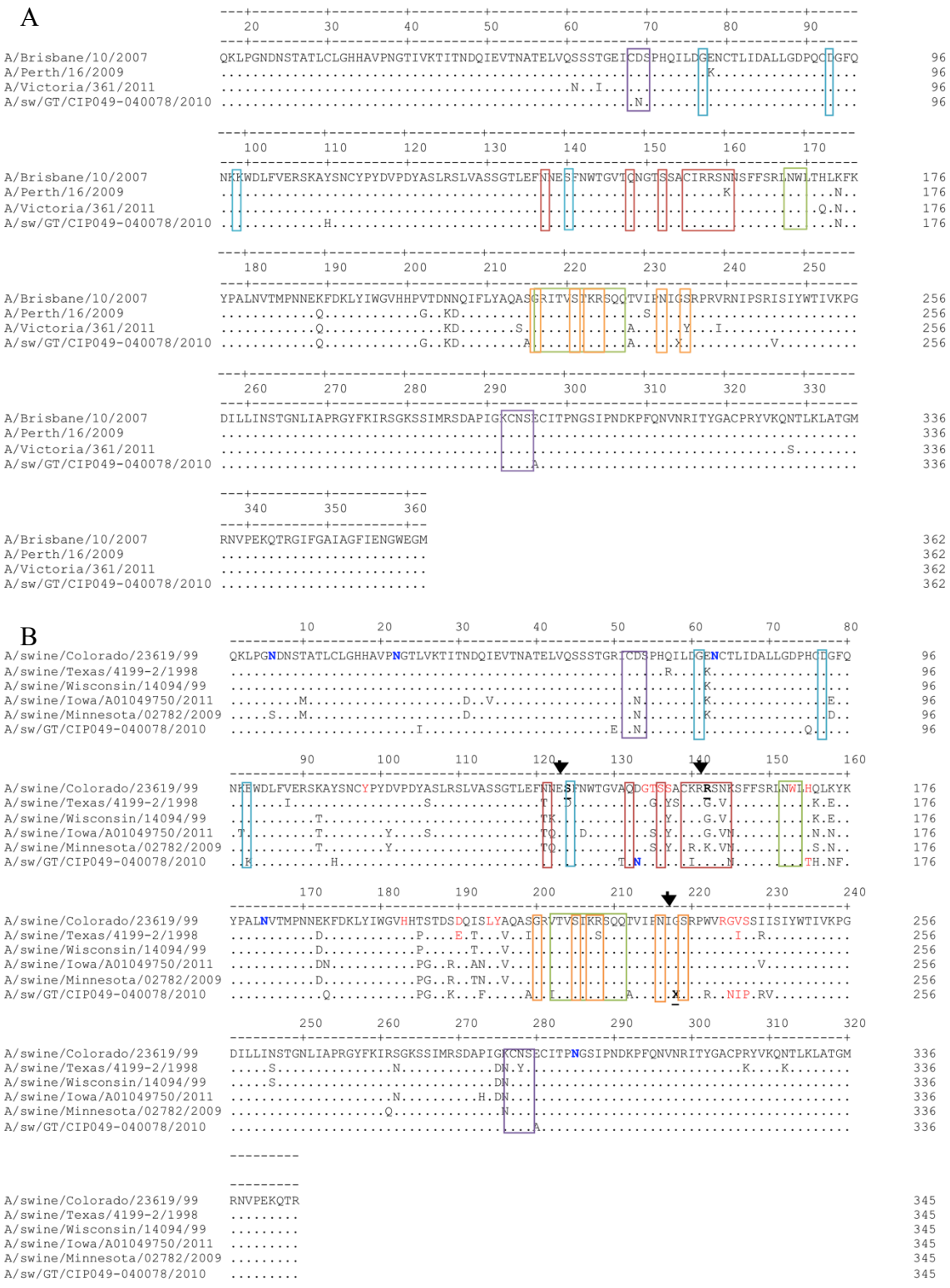

Figure 5.3. Alignment of the HA1 region for 040078-H3N2 with A) human and B) swine IAV viruses Antigenic sites are shown in colored boxes A (red), B (green), C (purple), D (orange), E (blue). The N residues of predicted glycosylation sites for A/swine/Guatemala/CIP049-040078/2010 (H3N2) are marked in blue in the consensus sequence. Receptor binding sites are marked in red in the reference sequence.

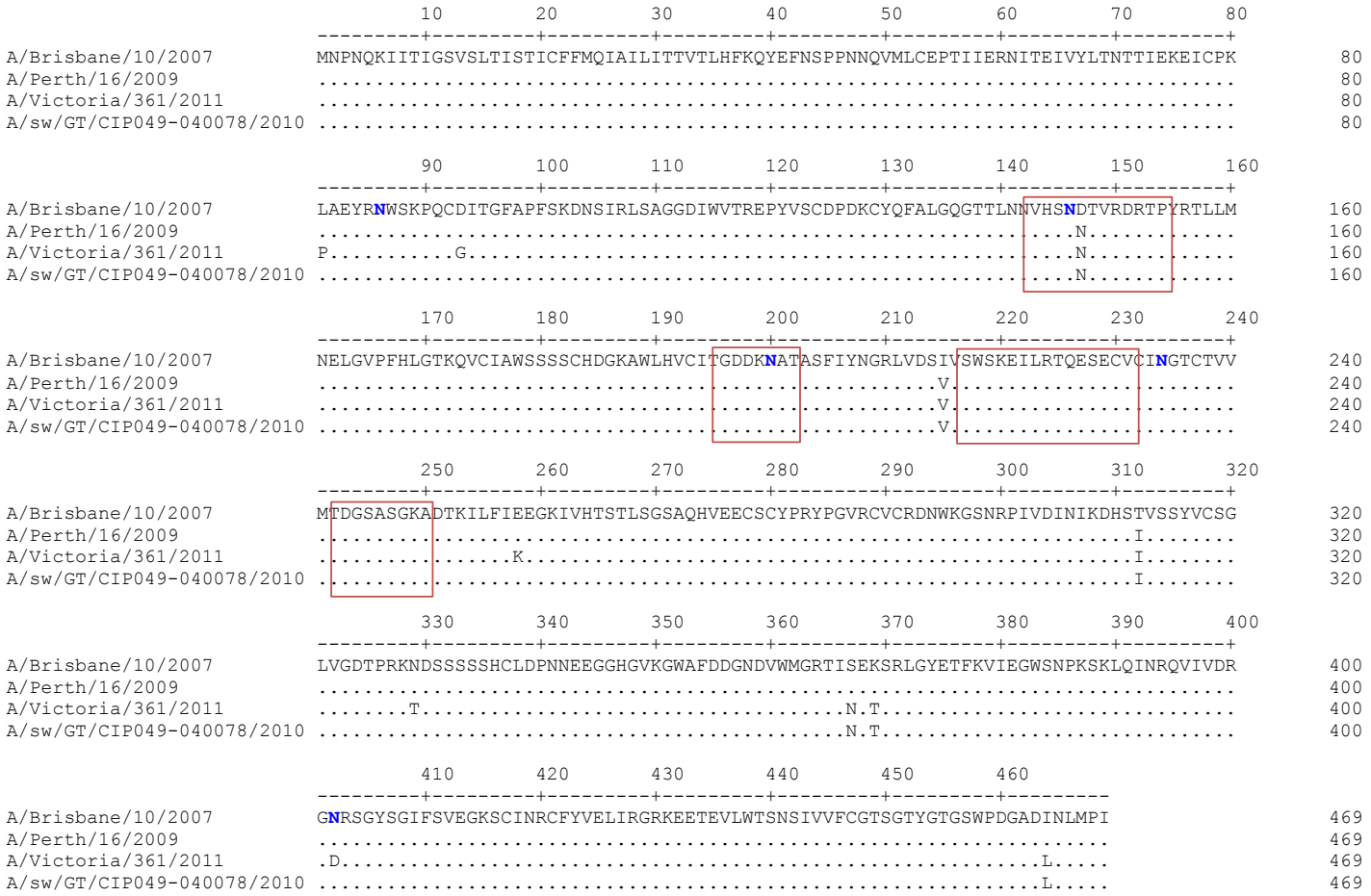

Figure 5.4. Alignment of the NA a minoacid sequences of 040078-H3N2 and contemporary human seasonal IAV viruses
Antigenic sites are shown in red boxes. The N residues of predicted glycosylation sites for A/sw/GT/CIP049-040078/2010 are marked in blue

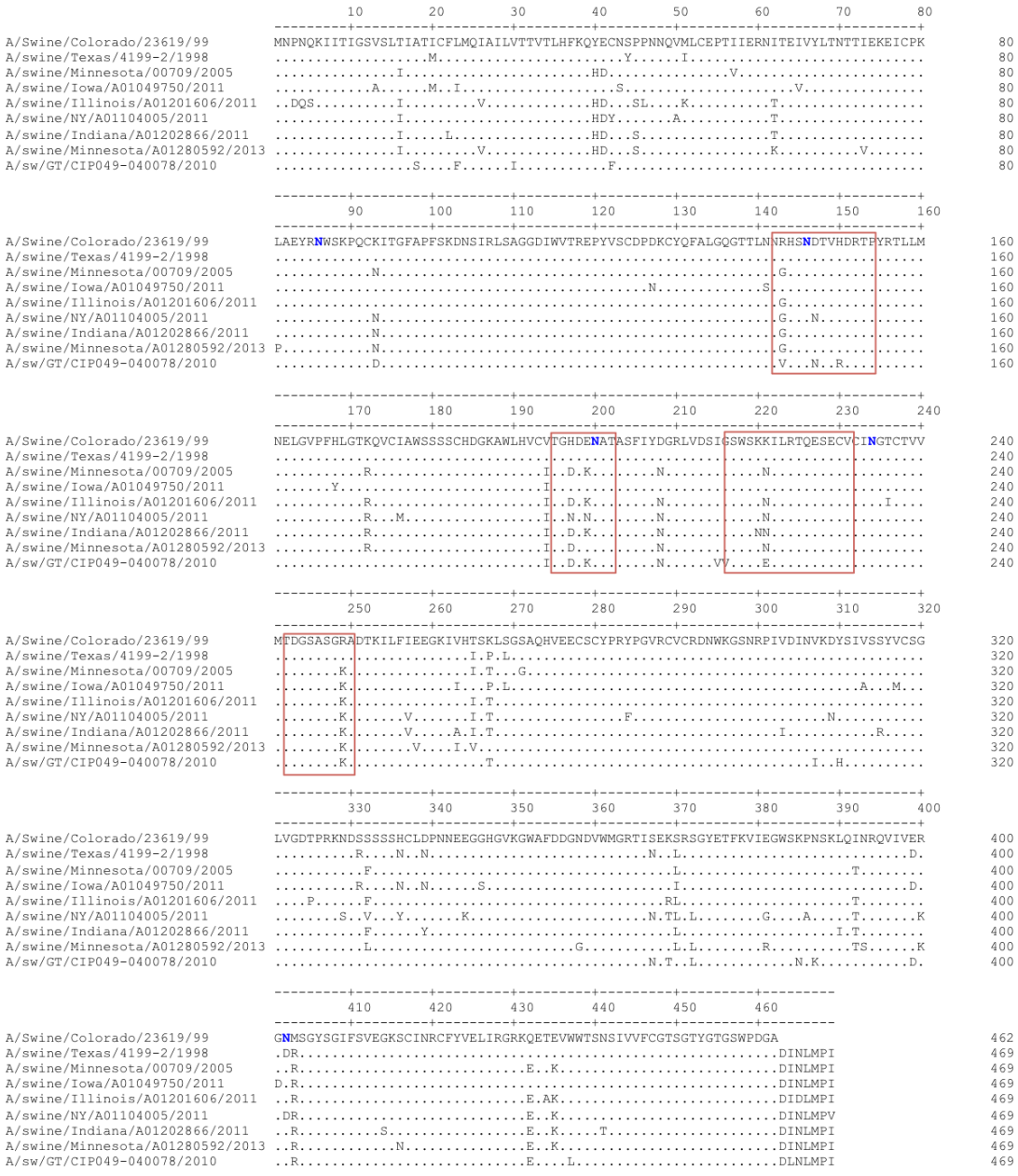

Figure 5.5. Alignment of the NA amino acid sequences of 040078-H3N2 and swine IAV viruses
Antigenic sites are shown in red boxes. The N residues of predicted glycosylation sites for A/sw/GT/CIP049-040078/2010 are marked in blue

Table 5.12. Phenotypic markers associated with transmission and pathogenicity found in A/swine/Guatemala/CIP049-040078/2010(H3N2)

Protein	Amino Acid	Reference	Comments
PB2	199S	PMID: 17073083, PMID: 10769072	The presence of an A199S substitution enhances viral transmission to humans
	271A	PMID: 20181719	The mutation T271A contributes to enhanced polymerase activity and viral growth in mammalian cells in vitro.
	360Y	PMID: 18768983	The Tyr residue at position 360 of PR8(UW) PB2 confers high efficiency of vaccine seed virus growth in MDCK cells.
	627K	PMID: 11546875	The E627K substitution in avian isolates enhances replication and pathogenicity in mammals at a lower temperature in the upper respiratory tract.
	627K	PMID: 17073083, PMID: 10769072	The presence of an E627K substitution enhances virulence
	661T	PMID: 18315849, PMID: 10769072	The presence of an A661T substitution enhances viral transmission to humans
	667I	PMID: 10769072	The presence of an V667I substitution enhances viral transmission to humans
	702R	PMID: 18315849, PMID: 11546875, PMID: 19211790	The presence of an K702R substitution enhances viral transmission to humans
PB1	-	-	No markers of virulence or pathogenicity associated with PB1 found in 040078
PB1-F2	66N	PMID: 21084483	The S66N substitution results in reduced virulence thought reduced inhibition of the early interferon response.
PA	409N	PMID: 17073083	S409N enhances transmission in humans
	245K	PMID: 7966557	Mutations at these positions are capable of suppressing the temperature-sensitivity phenotype of the virus.
HA	138A	PMID: 22353399, 18329747	A serine at amino acid 138 is essential but not solely sufficient for a high infectivity phenotype in primary swine respiratory epithelial cells (SRECs).
	124S	PMID: 22353399	G124D exhibits high infectivity phenotype in swine respiratory epithelial cells (SRECs); preferentially binds polylactosamine glycans.
	142R	PMID: 22353399	G142E exhibits high infectivity phenotype in swine respiratory epithelial cells (SRECs).

	218G/R	PMID: 20702632	The G218W (HA1) as well as the T156N(HA2) mutation enhance replication and virulence in vivo in mouse model.
	218G/R	PMID: 18983930	The single mutation Gly218Glu causes high pathogenicity in mice at late passage 10 and also causes enhanced binding abilities to ?2,3 and ?2,6 sialic acid-linked receptors.
NP	184K	PMID: 19475480	A change from alanine to a lysine at residue 184 of NP results in increased replication and pathogenicity of the viruses in chickens.
	214K, 217S, 253I	PMID:24335312	Residues 214, 217, and 253 of Aichi NP play a critical role in influenza virus morphology, possibly through interaction with the M1 layer during virus budding. A/Aichi/2/68 214K, 217S, 253I (filamentous)
NA	-	-	No markers of virulence, pathogenicity or neuraminidase inhibitors resistance were observed in NA of 040078
M1	-	-	No markers determinants of virulence associated with M1 found in 040078
M2	16G	PMID: 22132146	A16G substitution leads to enhanced transmission in humans
	31N	PMID: 22132146	31N confers resistance to Adamantane
	55F	PMID: 22132146	C55F substitution leads to enhanced transmission in humans
	11I, 14E, 16G, 18R, 20N	PMID: 15777646	WSN strain possesses Ile, Glu, Gly, Arg and Asn, determinant of host range specificity.
	31N	PMID: 15673732	It is shown that single-amino-acid substitution at this position within the transmembrane domain of M2 produces amantadine resistance in Influenza viruses
NS1	149A	PMID: 16971424	Recombinant GS/GD/1/96 virus with Ala149 can antagonize the induction of interferon levels in chicken embryo fibroblasts (CEFs), recombinants with Val149 are not capable of the same effect.
	106M	PMID: 17522219	Changing any one of the two residues, either L103 to F or I106 to M, in the HK97 NS1A protein caused increase in the rate of virus replication. HK97 NS1A protein is intrinsically defective in binding CPSF30.
	227..230 (RSKV)	PMID: 18334632	Majority of the human strains have the signature RSKV sequence; the remaining have avian signatures ESEV and are known to be of avian origin.

*PMID PubMed identification number

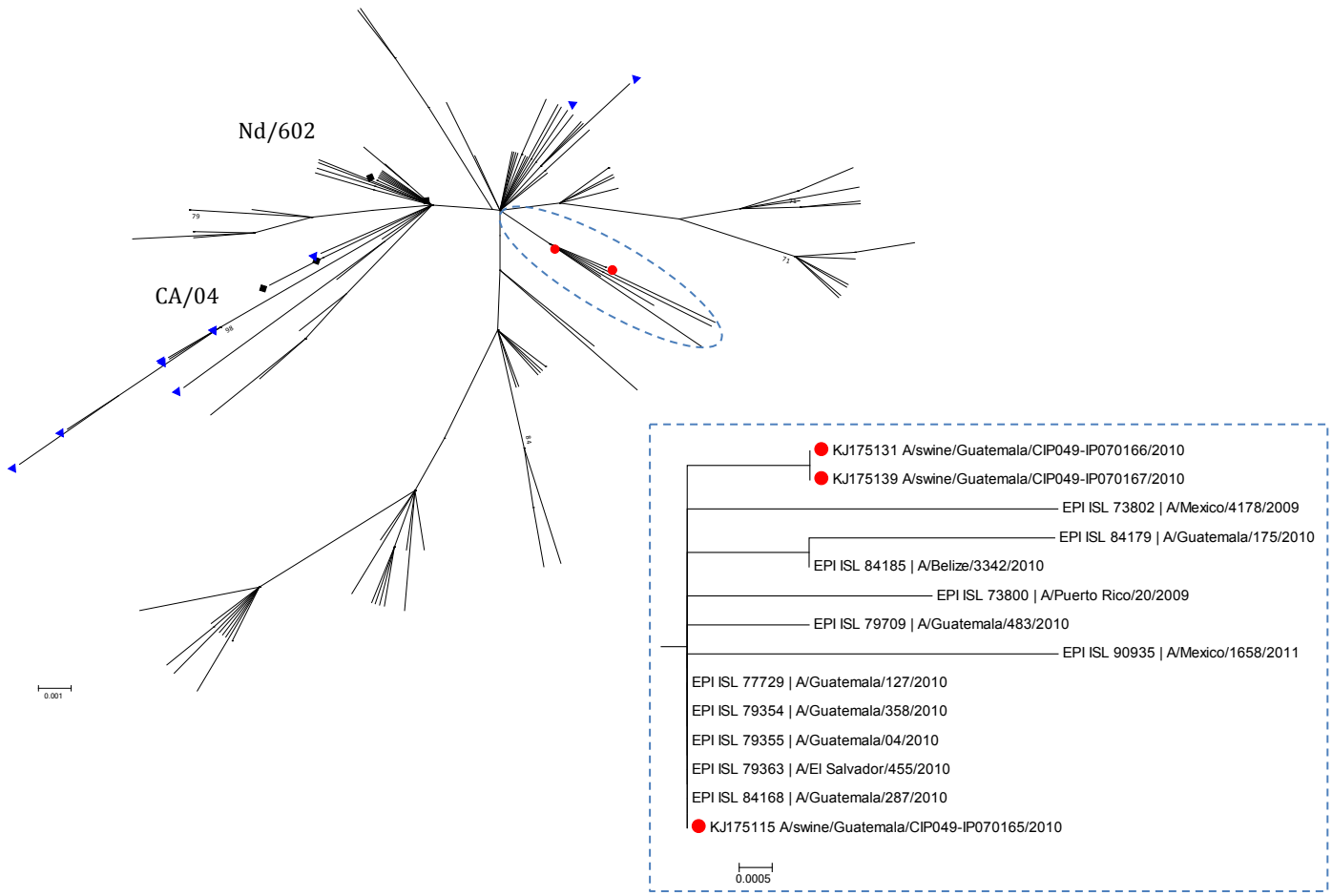

Figure 5.6 Phylogenetic inference for the Hemagglutinin gene of pH1N1 viruses from Central America and the Caribbean (2009-2013).
Maximum Likelihood phylogenetic inference using the best-fit model Hasegawa-Kishino-Yano (HKY) + Γ model of nucleotide substitution. Neighbor-Joining bootstrap support values ≥70% are shown. Reference strains, A/California/04/2009 (CA/04) and A/Netherlands/602/2009 (Nd/602) were included and are marked with black squares. The swine isolate from Guatemala is marked with a red circle. Swine isolates from other countries (Cuba, Mexico and Costa Rica) are marked with blue triangles. The cluster delineated by a circle is shown on the right.

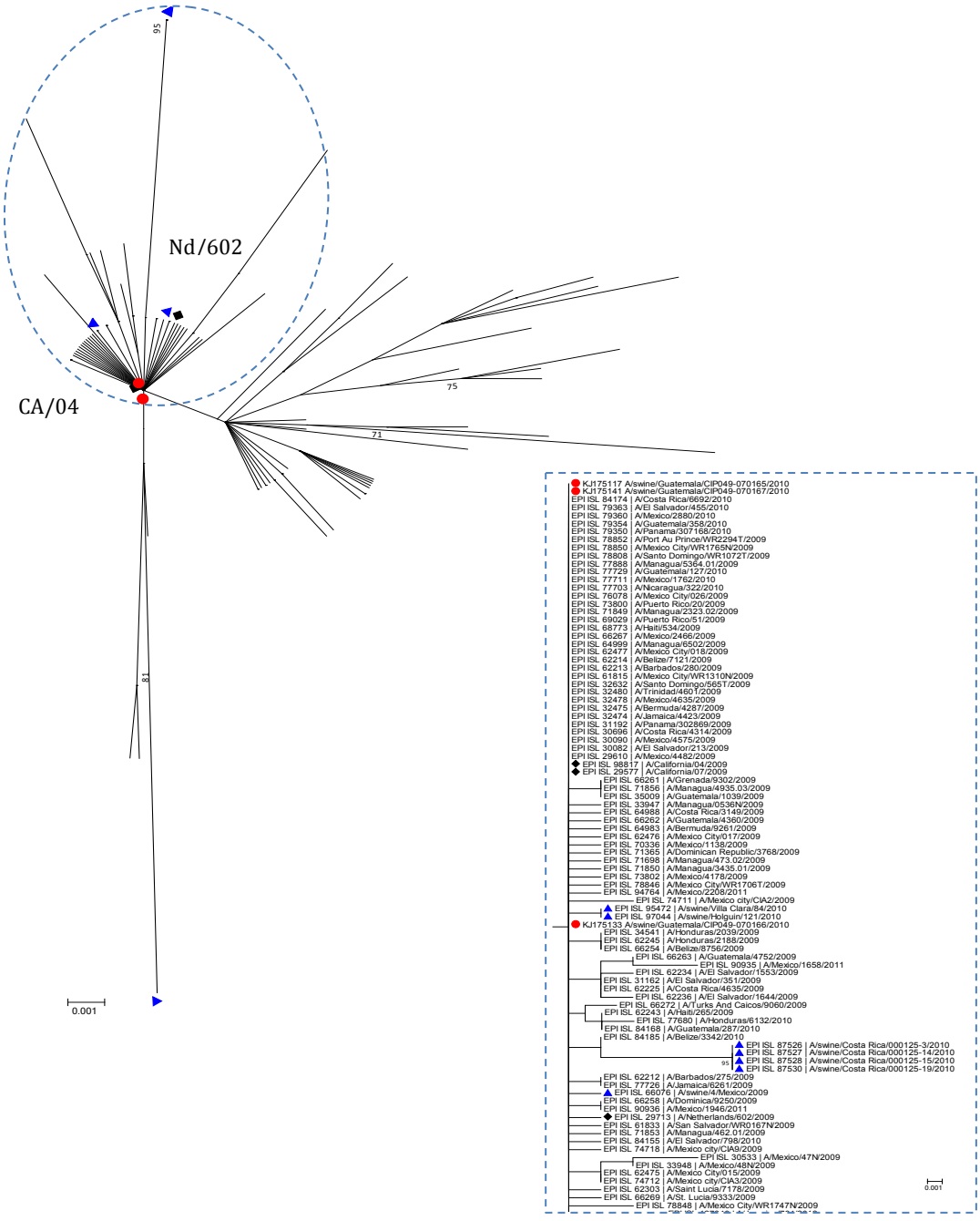

Figure 5.7 Phylogenetic inference for the N1 Neuraminidase gene of pH1N1 viruses from Central America and the Caribbean (2009-2013)

Maximum Likelihood phylogenetic inference using the best-fit model Hasegawa-Kishino-Yano (HKY) + Γ model of nucleotide substitution. Neighbor-Joining bootstrap support values ≥70% are shown. Reference strains, A/California/04/2009 (CA/04) and A/Netherlands/602/2009 (Nd/602) were included and are marked with black squares. The swine isolate from Guatemala is marked with a red circle. Swine isolates from other countries (Cuba, Mexico and Costa Rica) are marked with blue triangles. The cluster delineated by a circle is shown on the right.

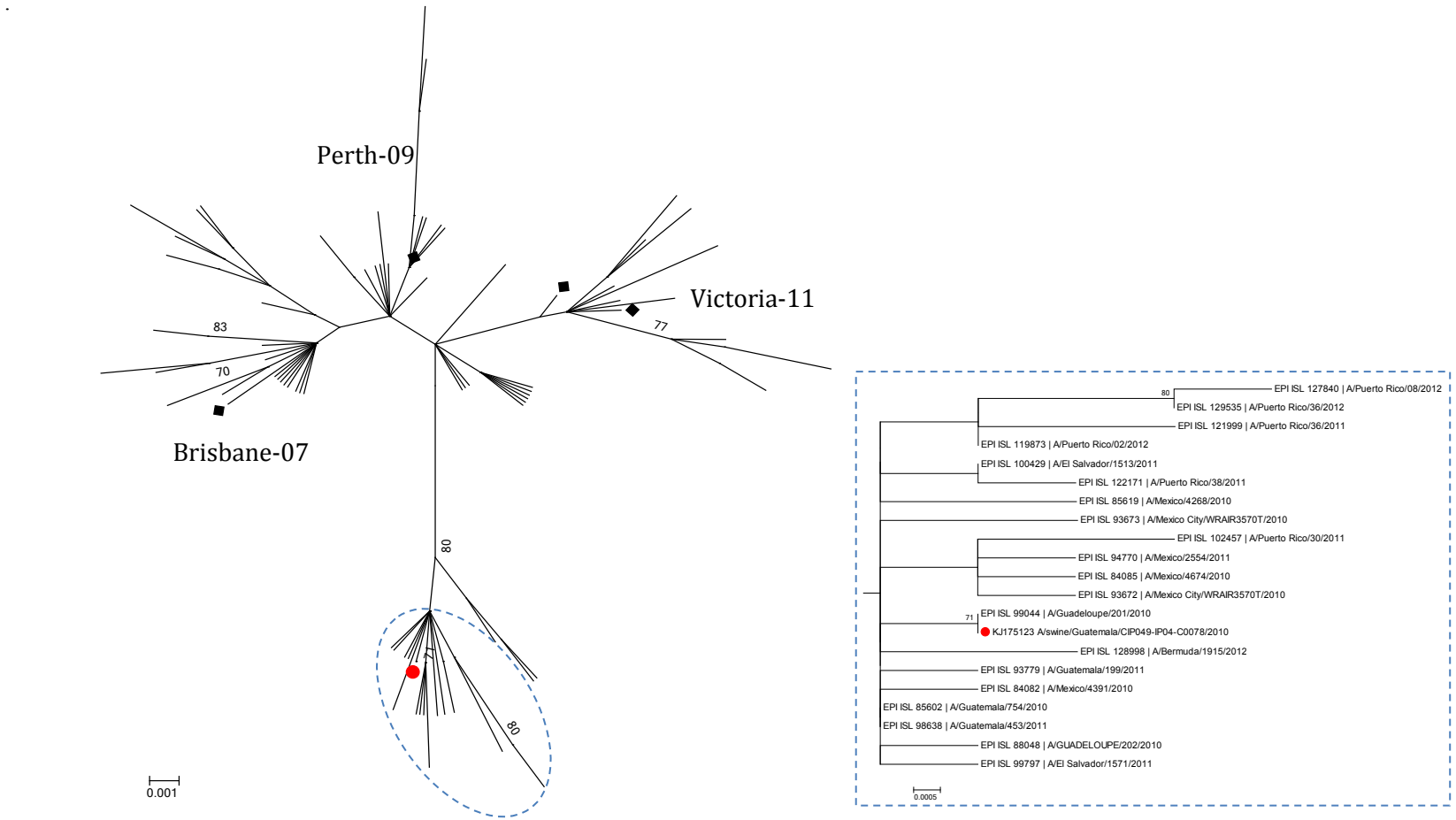

Figure 5.8 Phylogenetic inference for the Hemagglutinin gene of H3N2 viruses from Central America and the Caribbean (2007-2013)
Maximum Likelihood phylogenetic inference using the best-fit model Hasegawa-Kishino-Yano (HKY) + Γ model of nucleotide substitution. Neighbor-Joining bootstrap support values ≥70% are shown. Reference vaccine strains were included to define lineages, and are marked with black squares. The swine isolate from Guatemala is marked with a red circle. The cluster delineated by a circle is shown on the right.

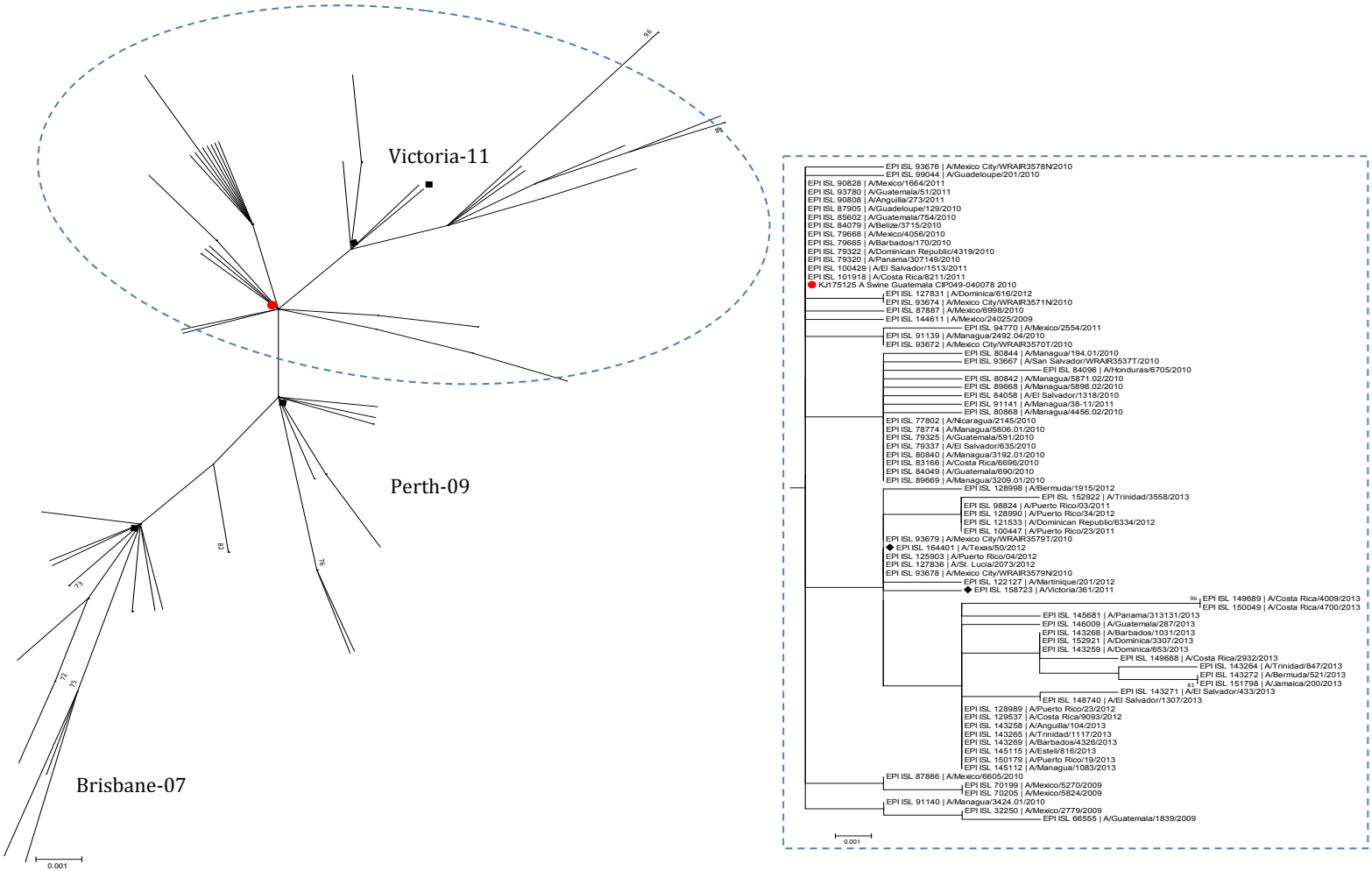

Figure 5.9 Phylogenetic inference for the Neuraminidase gene of H3N2 viruses from Central America and the Caribbean (2007-2013)
Maximum Likelihood phylogenetic inference using the best-fit model Hasegawa-Kishino-Yano (HKY) + Γ model of nucleotide substitution. Neighbor-Joining bootstrap support values ≥70% are shown. Reference vaccine strains were included to define lineages, and are marked with black squares. The swine isolate from Guatemala is marked with a red circle. The cluster delineated by a circle is shown on the right.

5.5. Discussion

The results of this study confirm that IAV circulated widely in PPU in Guatemala between 2010 and 2011. Detection by rRT-PCR yielded 14% of positive samples over the two-year period, with more than 30% positive herds each year. Reports from other regions about herd prevalence based on virus detection from cross-sectional studies are limited. In 2010 a similar study from Cuba reported 31.3% of farms positive to pH1N1, and 32.8% of tested pigs, positive to influenza A detected by rRT-PCR (319). In Costa Rica 2 out of 25 sampled PPU (8%) were positive for IAV by the same method during the same year (320). In a study from Mexico, the herd prevalence was not described but a positivity rate of 16.6% of tested samples was reported (321). From studies in other geographical regions, the percentage of positive herds or positive samples ranges between 4% to more than 90% (165, 322-324). This variability suggests differences in the dynamics of IAV infection in different pig populations and geographical regions, although differences in study designs, sampled populations and laboratory-testing methods (rRT-PCR, virus isolation) have to be considered when making these comparisons.

Cross-sectional studies to estimate seroprevalence have been conducted more broadly, usually targeting sows or adult animals; the percentage of seropositive herds on these studies are also variable, but herd prevalences of >50% are commonly reported (166, 325-329). In Guatemala the percentage of seropositive herds (19.5% and 5.6%) were estimated from data on all the sampled population, without making distinction by age, which may explain the relatively low seroprevalence found. The lower prevalence

found in 2011 compared to 2010, may indicate variations of IAV infection in pigs throughout the year. Samples from 2010 were taken in October and samples from 2011 were taken from June to August. The environmental factors that influence IAV transmission in the tropical countries like Guatemala are not well defined; in humans the proportion of IAV cases usually reaches a peak during the rainy season (at the middle of the year) and IAV transmission seem to be associated with specific humidity (330-333). Whether IAV prevalence in pigs is also influenced by environmental conditions, or is just a result of the nature of the annual pig production cycle (a greater proportion of pigs would have been exposed in later months of the year) is unclear; longitudinal studies are necessary to further address these questions.

With respect to the geographical distribution of positive herds, the borderline association between detection of influenza infection and herds located in the east and the west observed in 2010 is further supported by the identification of two clusters of positive farms detected by spatial analysis using geographical coordinates from the sampled farms (334). In 2010, these clusters were found in the west and east of the country. Similarly, the association observed in 2011 between positive PPU and their location in the southeast departments of the country, was also supported by a major cluster identified in this region by spatial analysis. The Ministry of Agriculture operates within five defined work zones based on these geographical regions (central, north, east, south, west), and our results indicate that prevalence risk ratio may help to predict the areas that are at greater risk of influenza infection when integrated as part of national IAV surveillance plan.

Differences in IAV prevalence between types of PPU also varied between years. Although these differences were not significant, patterns of IAV infection in commercial

farms and smallholdings may be driven by different exposure factors, such as differences in infrastructure and animal handling practices associated with each type of system.

In 2010 the majority of samples were collected from pigs reported with signs of respiratory disease, whereas in 2011 the majority of sampled pigs were reported as "healthy". The heterogeneity between samples from both years may be a confounding factor and comparisons at the animal level between years were not done. Data from both years were combined, and an association between IAV virus detection and sick pigs was found. However, this association was weak, and the difference between groups (sick or healthy) was not significant. Considering that the percentage of positive samples found in 2011 (mostly from healthy animals) was not different from 2010, observation of respiratory signs of disease pigs may not be a good indicator of IAV circulation in PPU from Guatemala. In addition, the occurrence of subclinical infections may explain the relatively high number of positive "healthy" animals observed in 2011.

Borderline associations were found between detection of IAV and juvenile pigs, and between detection of seropositive animals IAV and adult pigs. The results of these analyses are limited as only data from 2011 was available (when only 7 samples tested positive for IAV antibodies). However, a higher risk of IAV infection in juvenile pigs, and a higher seroprevalence in adult animals (in particular sows) are in agreement with other studies (166, 329). In order to confirm these observations, additional studies designed to study particular associations in targeted populations will have to be performed.

Evidence of exposure to IAV viruses of swine origin was found by HI assay. Exposure to a higher diversity of viruses of swine origin was observed in commercial

farms and in 2010, including viruses from the H1 alpha cluster and viruses from the H3 subtype. Exposure to different type of viruses found in farms and smallholdings may reflect differences in the epidemiology of IAV in these populations. Our results indicate that smallholdings may be at higher risk of exposure to viruses of human origin, whereas commercial farms may be at higher risk of exposure to swine derived strains associated with animal movement.

Exposure to the pH1N1 virus was found in the majority of positive animals, regardless of the type of PPU. Since the emergence of the pH1N1 in humans, introduction of pH1N1 viruses into pigs has been documented in many countries in Latin America, including Argentina, Brazil, Colombia, Costa Rica, Cuba and Mexico (319, 320, 328, 335-338). In Guatemala, there is no information about the viruses that circulated in pigs prior to the emergence of the pandemic in 2009. Although reverse zoonotic transmission from humans is the most likely explanation, with the available information, it remains unknown whether other swine viruses with similar antigenic properties to the pH1N1 were already circulating in the region.

Exposures to more than one virus were detected in a number of samples suggesting co-circulation of different viruses at the same time in positive PPU. In general the HI titers against the tested antigens were relatively low (geometric mean titers, GMT, ranged between 80 and 269, data not shown), which may indicate cross-reactive rather than strain specific responses. Summed to this, the subtype specific response of 10 ELISA positive samples could not be identified by HI assay with the panel of tested antigens, indicating that other viruses with different antigenicity may be circulating in pigs in Guatemala. Inclusion of more recent strains in the diagnostics panel as well as

continued characterization of local strains is needed to improve current diagnostics methods.

In 2010, from all samples positive for IAV matrix rRT-PCR, only 10 tested positive for the presence of pH1N1 with a second rRT-PCR targeting the N1 gene. In 2011 all samples tested negative for N1 by the same test, but serological exposure to pH1N1 was detected in both years. The samples from which the pH1N1 viruses were isolated were negative when they were initially tested by the N1 specific rRT-PCR. Comparison of viral sequences revealed a mismatch with the sequences of the N1-R330 3' primer (5'-ggaaccgattcttaTactgttgtc-3'), which may have prevented primer extension during the amplification reaction. Results based on subtype specific assays need to be interpreted cautiously when there is little information on the viruses circulating in a specific region (e.g. Guatemala), as they may provide an underestimated prevalence of different subtypes. The surface genes are subjected to stronger evolutionary pressures, making difficult the design of primers and probes that are specific enough to distinguish between genetic lineages of one virus subtype but that still capture all variants within the same genetic lineage, such as the pH1N1 lineage. Therefore, to better capture the virus diversity of circulating viruses in future studies in Guatemala, we recommend the use of a IAV specific assay directed to a higher conserved gene (such as the matrix gene-based assay) followed by direct sequencing with next generation sequencing (when available) and molecular characterization of virus isolates. Until sufficient information of locally circulating viruses is generated, the application of subtype specific assays developed for viruses in other geographical regions will remain limited.

In this study 4 virus isolates were obtained from 139 samples tested (rRT-PCR positive). For avian influenza, low rates of viral isolation from samples with CT-values >30 have been reported (268). In our study the majority of rRT-PCR positive samples (115/139) had CT-values above 35, which may explain the limited recovery of viral isolates from nasal swabs. The H3N2 virus was isolated from a sample with CT-value of 27.5 and the three pH1N1 viruses were isolated from second and third cell passages of samples with 32<CT-value>35. Pandemic viruses are known to replicate well in tissue culture systems. Their high replication efficiency above other mammalian viruses (339, 340) may explain the fact that these viruses could be recovered from samples with a low cDNA copy number. In future studies, virus isolation may be improved by follow up of identified foci of respiratory disease over time with laboratory confirmation and collection of different types of samples (e.g. lung biopsies collected from pigs at slaughter houses, or death sick animals).

With respect to the molecular characterization of viral isolates, *in-silico* analysis of genetic markers for adaptation of influenza viruses to human and swine hosts suggest that the H3N2 and the pH1N1 viruses were recent introductions in pigs at the time of sampling. Alignments of the HA amino acid sequence of isolate 040078-H3N2 with other seasonal human H3N2 viruses revealed little differences in the antigenic sites between these viruses. Comparison of 040078-H3N2 isolate with other contemporary H3 viruses of swine origin suggest that although pigs in Guatemala were found to be exposed to H3 viruses of swine origin (in particular from clusters III and IV), they could have little cross protection to a virus like the 040078-H3N2. All pigs from the same farm and department from which the virus was recovered in 2010 tested negative by rRT-PCR, and no

seropositive pigs for the H3 subtype were found. Evidence of circulation of a similar virus in 2011 was not detected, suggesting that spread of the human-like H3N2 in pigs may have been limited. Experimental evaluation of the transmissibility of the 040078-H3N2 virus in pigs could help to better assess the risk of establishment and spread of seasonal human-like H3N2 viruses in pigs in Guatemala.

Phylogenetic analysis of the 040078-H3N2 HA revealed that a distinct lineage of seasonal H3N2 viruses co-circulated with those from current human vaccine-lineage in Central America and other countries between 2010 and 2012. When sequences of more recent viruses (from 2013 and 2014) from Central America were incorporated into the phylogenetic analysis the majority of viruses isolated after 2012 clustered within the vaccine lineage suggesting lineage displacement of the other lineage in the region. However, it should be noted that the amount of sequences available of seasonal human IAV for Central America is very limited, making it difficult to interpret the significance of our results. Our findings underscore the importance of generating local information from animal and human viruses to guide local vaccine recommendations more accurately.

The analysis of phenotypic markers and protein alignments of surface genes of the pH1N1 isolates demonstrates little variation from other pH1N1 strains from humans. In comparison to other pH1N1-like viruses recovered from pigs in other countries in Latin America, the viruses from Guatemala were more similar to the viruses of human origin. This indicates that the pH1N1 isolates found in pigs in Guatemala were a recent introduction from humans; however, the presence of amino acid residues that are more prevalent in pigs revealed undergoing adaptation of these viruses in the swine hosts. In addition, virological and serological detection of pH1N1 virus in the present study

demonstrate extensive circulation of the pandemic H1N1 lineage in pigs from different farms (commercial and smallholdings) in different departments since 2010. Although no reassortant viruses were found as part of our study, introduction of the pH1N1 pigs in Guatemala may represent the establishment of a novel genetic lineage with the potential to reassort with co-circulating viruses in this population. Only long-term surveillance will help to characterize the pool of genetic and antigenic different viruses that are contributing to the epidemiology of IAV in pigs in Guatemala.

In summary, our results provide evidence that different influenza A viruses (H1N1 and H3N2) circulate in the swine population of Guatemala in different types of PPU. Circulation of "human-like" strains in pigs suggests that human-animal contact may play an important role in the introduction of novel strains into the swine population in Guatemala. Differences in prevalence and seroprevalence, and distribution of positive PPU between different years, suggest seasonal variation in the circulation influenza along the year that warrants further investigation with longitudinal studies in affected PPU. This study is the first in Guatemala analyzing IAV prevalence and its distribution in swine farms. Further studies are needed that address the risk factors associated with IAV infection in different types of PPU in Guatemala. Continued sampling is needed to increase the number of locally isolated strains. Surveillance for IAV in different species in Guatemala, and other countries in Central America, is important to improve our understanding of the ecology of animal influenza in the Neotropics.

5.6. Acknowledgments

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Chapter 6. Conclusions and future prospects

6.1. Conclusions

6.1.1. Surveillance of Influenza A virus in wild birds in Guatemala

The study of influenza A viruses in wild birds in Guatemala since 2007, has resulted in the most abundant data related to this subject available for Central America to date. Despite this fact, given the limitations in our study (which includes only a small number of sampled birds), our results are biased towards hunter-targeted species that are present during the winter migration at the sampled locations. Phylogenetic analysis of the viruses obtained during the initial years of the study demonstrated a close relationship with virus strains from North American wild waterfowl, and revealed high frequency of detection of viruses that are rarely found at the breeding grounds in North America. Continued surveillance from 2010 to 2013 provided additional data that allowed preliminary analysis of temporal variations in virus prevalence during the periods when reservoirs are highly abundant. The results of this analysis support a model of low virus prevalence towards the end of the migration cycle observed in other geographical locations. However, with the current amount of data derived from only a limited period of time, no definitive conclusions of the patterns of IAV transmission can be made. To this end, only intense systematic year round surveillance focused on specific bird populations would provide a better understanding of the seasonality of influenza transmission at the wintering grounds in Central America.

The detection of high levels of the H14 subtype during the migration season of 2012-13 suggests amplification and increased transmission of locally circulating subtypes

at wintering locations upon the arrival of migrants. Isolation of other subtypes and subtype combinations rarely seen at the breeding grounds during our study and in studies conducted in other geographical locations support this conclusion. In addition, the diversity of viruses found in blue-winged teals and their high relative abundance at the sampled locations make this a candidate species for targeted LPAIV surveillance in the Neotropics. However the contribution of resident bird populations and species remains unknown. The relative abundance of different species in Guatemala may be an influencing factor for the role of individual bird species to serve as IAV reservoirs. Implementation of additional methodologies to study multiple bird populations (including migrant and resident species) could help to address these questions.

The literature revision and phylogenetic analysis of all sequence data on avian influenza available for Latin America and the Caribbean highlighted a significant gap in surveillance in the region, and a lack of understanding of the ecology of the viruses circulating in the southern hemisphere. Detection of distantly related gene variants in Guatemala provide evidence that current surveillance efforts across the region may not be adequately capturing the total diversity of viruses that circulate in natural hosts. Detection of reassortant viruses with genes from Eurasian origin in Guatemala confirms that long-distance migration of viruses is plausible between two remote geographical locations.

The diverse virus subtypes and genome constellations observed for the internal and surface genes suggest that birds from multiple migration routes are sourcing the viruses that circulate during winter in Guatemala. These observations provide additional evidence for the hypothesis that stopover habitats with overlapping flyways may serve as important places for virus transmission, by linking host populations from different

flyways through genetic reassortment and contributing to shape the evolution of the viruses at a continental scale. We propose that the existence of a natural geographical bottleneck in Central America provides such a unique niche for the birds migrating from North America, but we recognize that only continued surveillance over prolonged periods of time and in additional adjacent locations would help to confirm this hypothesis.

6.1.2. Surveillance of Influenza A virus in swine in Guatemala

The findings from IAV surveillance in swine populations in Guatemala between 2010 and 2011 provide evidence of circulation of different IAV in the two main types of animal production systems. Sample collection was conducted during different seasons in 2010 and 2011 (dry and rainy season, respectively). Differences in prevalence and seroprevalence, and distribution of positive PPU between different years, may suggest seasonal variation in the circulation of IAV in pigs throughout the year. Nevertheless our results may be influenced by the confounding factors related to the limitations in our study, such as the variability in the demographics of sampled populations between both years, and the quality of the information recovered from the sampled farms. We recognize such factors should be taken into account to interpret the similarities and discrepancies in levels of viral exposure observed between the two years. However, the variations observed warrant further investigation with longitudinal studies to define swine IAV seasonality in Guatemala.

Circulation of "human-like" strains in pigs suggests that human-animal contact may play an important role in the introduction of novel strains into the swine population in Guatemala. Although no reassortant viruses were found as part of our study, introduction of the pH1N1 and a H3N2 strain of human-origin in pigs in Guatemala could

mean the establishment of novel genetic lineages with the potential to reassort with co-circulating viruses in this population, as observed in other geographic locations. Only long-term surveillance will help to characterize the pool of genetic and antigenic variants that are contributing to the epidemiology of IAV in pigs in Guatemala.

6.2. Future prospects and general recommendations

6.2.1. Surveillance of Influenza A virus in wild birds in Guatemala

Sampling of hunted birds is a cost effective strategy that allows capturing information about circulating IAV subtypes on an annual basis. Such a strategy could be applied in other countries from the region that, like Guatemala, have limited resources to investigate the ecology of zoonotic diseases. This does not mean that other sampling methodologies cannot be applied as sampling of hunted-killed birds doubtfully guaranties capturing the total diversity of viruses circulating in a particular region.

Detection of viruses genetically related to HPAI strains detected in recent poultry outbreaks in intermediary locations between North and Central America, justifies continued surveillance at both ends of the migration flyways. We anticipate that our results will help to increase awareness at the regional level for the need to characterize strains from the southern hemisphere.

In Guatemala, work still needs to be done to define the 1) patterns of viral circulation, 2) important host species and in particular, 3) how virus diversity is maintained between migration seasons. Systematic methodologies that integrate live bird captures and environmental sampling in other habitats and bird species, coupled with serological studies in wild bird populations, could help to answer those questions. In

addition, collaboration with other groups working in other locations across the same migration flyways, would allow better comparisons of virus diversity among different bird species, locations and seasons. Finally, increased laboratory capability for diagnosis in Guatemala could help to speed up the process of virus characterization.

With the use of deep sequencing data, detection of low-frequency variants in isolates of wild bird origin may help to provide critical information to better understand which genetic determinants are required for transmission of IAV to poultry. In addition, deep-sequencing data from field isolates could be used to assess the contribution of genetic drift and selection of gene variants to the evolution of IAV in its natural host. Sequencing of additional viruses isolates could help to identify more events of interlineage reassortment (including the South American lineage), and would contribute to characterizing the patterns of virus reassortment during one migration season. In this context, standardization of the methods to characterize the genetic structure of the virus population and gene constellations could simplify the way to visualize virus diversity at a given location and time. At the same time, this would facilitate direct comparison between virus populations from different locations.

6.2.2. Surveillance of Influenza A virus in swine in Guatemala

In order to better understand the epidemiology of influenza in swine populations in Guatemala, further studies are needed to analyze the risk factors associated with IAV infection in different types of PPU. In 2012 a pilot study was conducted to investigate in greater detail influenza circulation in two geographic locations within Guatemala, distinguished by differences in their climate and ecosystems. The results from this study suggested that the presence of domestic waterfowl on the farms represents a risk factor

for influenza infection in pigs (341). The implementation of longitudinal studies is needed in order to define the seasonal patterns of IAV infection in swine farms. Current efforts from our group in Guatemala are focused on the development of longitudinal studies at the human-animal interface.

Continued surveillance for IAV in different species in Guatemala is important to improve our understanding of the ecology of animal influenza in tropical regions. The ultimate goal of influenza surveillance in animal and human hosts is to characterize local virus populations and their antigenic properties, in order to guide local vaccine recommendations. The establishment of sustainable long-term active IAV surveillance in pigs in Guatemala is hampered by limited resources, which are usually directed to the study of other pathogens of higher priority in the swine production sector. Our experience in IAV surveillance in pigs in Guatemala demonstrates that cooperation between governmental and academic institutions, supported by local and external funding, can help to optimize resources to study locally circulating pathogens in developing countries. Participation of the governmental sector is critical to increase awareness of swine producers, and increasing accessibility to farms for sample collections, whereas participation of the academic sector helps to provide technical advice and training for adequate sample collection and scientifically sound interpretation of the results. We advocate for such collaborations to continue, as this could increase the capability of the country to respond in the event of future animal and public health emergencies.

APPENDICES

Table 0.1. Viruses isolated from wintering waterfowl in Guatemala between 2010 and 2013

Sample identifier	Strain Name	Collection Date
H006-02	A/blue-winged teal/Guatemala/CIP049-01/2008(H7N9)	7-Feb-08
H008-41	A/blue-winged teal/Guatemala/CIP049-02/2008(H7N9)	5-Mar-08
H094-10	A/blue-winged teal/Guatemala/CIP049-10/2009(H11N2)	11-Nov-09
H094-11	A/blue-winged teal/Guatemala/CIP049-11/2009(H11N2)	11-Nov-09
H094-15	A/blue-winged teal/Guatemala/CIP049-03/2009(H11N2)	11-Nov-09
H096-04	A/blue-winged teal/Guatemala/CIP049-04/2010(H8N4)	31-Jan-10
H096-05	A/blue-winged teal/Guatemala/CIP049-05/2010(H3N8)	31-Jan-10
H096-07	A/blue-winged teal/Guatemala/CIP049-06/2010(H8N4)	31-Jan-10
H096-14	A/blue-winged teal/Guatemala/CIP049-12/2010(H5N4)	31-Jan-10
H096-20	A/blue-winged teal/Guatemala/CIP049-13/2010(H5N4)	31-Jan-10
H096-31	A/blue-winged teal/Guatemala/CIP049-14/2010(H8N4)	31-Jan-10
H096-34	A/blue-winged teal/Guatemala/CIP049-07/2010(H8N4)	31-Jan-10
H096-39	A/blue-winged teal/Guatemala/CIP049-08/2010(H5N3)	31-Jan-10
H096-41	A/blue-winged teal/Guatemala/CIP049-15/2010(H8N4)	31-Jan-10
H096-48	A/blue-winged teal/Guatemala/CIP049-09/2010(H5N3)	31-Jan-10
H098-32	A/blue-winged teal/Guatemala/CIP049H098-32/2010 (H5N3)	19-Nov-10
H101-24	A/blue-winged teal/Guatemala/CIP049H101-24/2011 (H3N8)	5-Nov-11
H101-29	A/blue-winged teal/Guatemala/CIP049H101-29/2011 (H4N2)	5-Nov-11
H101-36	A/blue-winged teal/Guatemala/CIP049H101-36/2011 (H4N8)	5-Nov-11
H102-05	A/blue-winged teal/Guatemala/CIP049H102-05/2011 (H4N2)	11-Nov-11
H102-08	A/blue-winged teal/Guatemala/CIP049H102-08/2011 (H1N3)	11-Nov-11
H102-09	A/blue-winged teal/Guatemala/CIP049H102-09/2011 (H3N2)	11-Nov-11
H102-10	A/blue-winged teal/Guatemala/CIP049H102-10/2011 (H4N8)	11-Nov-11
H102-11	A/blue-winged teal/Guatemala/CIP049H102-11/2011 (H3N2)	11-Nov-11
H102-18	A/blue-winged teal/Guatemala/CIP049H102-18/2011 (H1N3)	11-Nov-11
H102-19	A/blue-winged teal/Guatemala/CIP049H102-19/2011 (H3N2)	11-Nov-11
H102-23	A/blue-winged teal/Guatemala/CIP049H102-23/2011 (H3N2)	11-Nov-11
H102-25	A/blue-winged teal/Guatemala/CIP049H102-25/2011 (H3N2)	11-Nov-11
H102-29	A/blue-winged teal/Guatemala/CIP049H102-29/2011 (H3N2)	11-Nov-11
H102-32	A/blue-winged teal/Guatemala/CIP049H102-32/2011 (H3N8)	11-Nov-11
H102-46	A/blue-winged teal/Guatemala/CIP049H102-46/2011 (H1N3)	11-Nov-11
H103-05	A/northern shoveler/Guatemala/CIP049H103-05/2011 (H1N3)	25-Nov-11
H103-13	A/blue-winged teal/Guatemala/CIP049H103-13/2011 (H3N2)	25-Nov-11
H103-20	A/blue-winged teal/Guatemala/CIP049H103-20/2011 (H3N2)	25-Nov-11
H103-27	A/blue-winged teal/Guatemala/CIP049H103-27/2011 (H3N2)	25-Nov-11
H103-30	A/blue-winged teal/Guatemala/CIP049H103-30/2011 (H4N3)	25-Nov-11
H103-37	A/blue-winged teal/Guatemala/CIP049H103-37/2011 (H4N3)	25-Nov-11
H104-38	A/blue-winged teal/Guatemala/CIP049H104-38/2011 (H1N3)	1-Dec-11
H104-69	A/northern shoveler/Guatemala/CIP049H104-69/2011 (H11N3)	1-Dec-11
H104-93	A/blue-winged teal/Guatemala/CIP049H104-93/2011 (H3N3)	1-Dec-11
H104-99	A/blue-winged teal/Guatemala/CIP049H104-99/2011 (H12N5)	1-Dec-11
H105-01	A/blue-winged teal/Guatemala/CIP049H105-01/2011 (H1N3)	8-Dec-11
H105-04	A/blue-winged teal/Guatemala/CIP049H105-04/2011 (H11N3)	8-Dec-11
H105-05	A/blue-winged teal/Guatemala/CIP049H105-05/2011 (H5N3)	8-Dec-11
H105-08	A/blue-winged teal/Guatemala/CIP049H105-08/2011 (H3N3)	8-Dec-11
H105-15	A/blue-winged teal/Guatemala/CIP049H105-15/2011 (H14N3)	8-Dec-11
H105-31	A/blue-winged teal/Guatemala/CIP049H105-31/2011 (H14N3)	8-Dec-11

Sample identifier	Strain Name	Collection Date
H105-32	A/blue-winged teal/Guatemala/CIP049H105-32/2011 (H1N3)	8-Dec-11
H106-62	A/blue-winged teal/Guatemala/CIP049H106-62/2011 (H14N6)	15-Dec-11
H107-88	A/northern shoveler/Guatemala/CIP049H107-88/2012 (H7N3)	22-Jan-12
H108-02	A/blue-winged teal/Guatemala/CIP049H108-02/2012 (H14N3)	9-Nov-12
H108-04	A/blue-winged teal/Guatemala/CIP049H108-04/2012 (H14N3)	9-Nov-12
H108-11	A/blue-winged teal/Guatemala/CIP049H108-11/2012 (H14N3)	9-Nov-12
H108-39	A/blue-winged teal/Guatemala/CIP049HT108-39/2012 (H4N2)	9-Nov-12
H108-45	A/blue-winged teal/Guatemala/CIP049H108-45/2012 (H3N2)	9-Nov-12
H108-53	A/blue-winged teal/Guatemala/CIP049H108-53/2012 (H4N2)	9-Nov-12
H108-67	A/blue-winged teal/Guatemala/CIP049H108-67/2012 (H3N2)	9-Nov-12
H109-06	A/blue-winged teal/Guatemala/CIP049H109-06/2012 (H4N2)	16-Nov-12
H109-14	A/blue-winged teal/Guatemala/CIP049H109-14/2012 (H3N3)	16-Nov-12
H109-15	A/blue-winged teal/Guatemala/CIP049H109-15/2012 (H3N2)	16-Nov-12
H109-28	A/blue-winged teal/Guatemala/CIP049H109-28/2012 (H3N2)	16-Nov-12
H109-38	A/blue-winged teal/Guatemala/CIP049H109-38/2012 (H3N8)	16-Nov-12
H109-49	A/blue-winged teal/Guatemala/CIP049H109-49/2012 (H14N3)	16-Nov-12
H109-67	A/blue-winged teal/Guatemala/CIP049H109-67/2012 (H14N3)	16-Nov-12
H109-76	A/blue-winged teal/Guatemala/CIP049H109-76/2012 (H14N3)	16-Nov-12
H109-86	A/blue-winged teal/Guatemala/CIP049H109-86/2012 (H4N2)	16-Nov-12
H110-04	A/blue-winged teal/Guatemala/CIP049H110-04/2012 (H14N5)	29-Nov-12
H110-27	A/blue-winged teal/Guatemala/CIP049H110-27/2012 (H4N6)	29-Nov-12
H110-31	A/blue-winged teal/Guatemala/CIP049H110-31/2012 (H14N3)	29-Nov-12
H110-36	A/blue-winged teal/Guatemala/CIP049H110-36/2012 (H11N9)	29-Nov-12
H110-37	A/blue-winged teal/Guatemala/CIP049H110-37/2012 (H11N3)	29-Nov-12
H111-98	A/blue-winged teal/Guatemala/CIP049H111-98/2012 (H2N3)	7-Dec-12
H112-03	A/American wigeon/Guatemala/CIP049H112-03/2012 (H4N2)	21-Dec-12
H112-31	A/blue-winged teal/Guatemala/CIP049H112-31/2012 (H4N2)	21-Dec-12
H112-60	A/blue-winged teal/Guatemala/CIP049H112-60/2012 (H3N2)	21-Dec-12
H112-79	A/northern shoveler/Guatemala/CIP049HT112-79/2012 (H4N2)	21-Dec-12
H113-07	A/blue-winged teal/Guatemala/CIP049H113-07/2013 (H14N3)	11-Jan-13
H113-08	A/blue-winged teal/Guatemala/CIP049H113-08/2013 (H14N4)	11-Jan-13
H113-53	A/blue-winged teal/Guatemala/CIP049H113-53/2013 (H5N3)	11-Jan-13
H113-74	A/blue-winged teal/Guatemala/CIP049H113-74/2013 (H14N3)	11-Jan-13
H113-76	A/blue-winged teal/Guatemala/CIP049H113-76/2013 (H14N4)	11-Jan-13
H114-14	A/northern shoveler/Guatemala/CIP049HT114-14/2013 (H3N8)	19-Jan-13
H114-76	A/green-winged teal/Guatemala/CIP049H114-76/2013 (H7N9)	19-Jan-13

Table 0.2. Top ten BLAST hits for the HA genes of the viruses isolated from wintering waterfowl in Guatemala, 2010-2013.

H1	% Identity			
Accession|Virus name	H102-46	H104-38	H105-01	H103-05
CY167159.1|A/mallard/Arkansas/12OS179/2012(H1N1)	99.2	99.3	99.1	99.0
CY177037.1|A/mallard/California/3937/2012(H1N1)	99.2	99.2	99.0	99.0
CY133290.1|A/mallard/Mississippi/10OS4593/2010(H1N2)	99.1	99.1	99.0	99.0
CY133713.1|A/mallard/Mississippi/11OS34/2011(H1N3)	99.1	99.1	99.0	99.0
CY133801.1|A/mallard/Missouri/10MO0253/2010(H1N2)	99.1	99.1	99.0	99.0
CY120651.1|A/northern pintail/California/3452/2010(H1N1)	99.1	99.1	99.0	98.9
CY132338.1|A/mallard/Ohio/11OS2203/2011(mixed)	99.1	99.1	99.0	
CY133480.1|A/northern shoveler/Illinois/10OS4360/2010(mixed)	99.0	99.1	98.9	99.0
CY166306.1|A/mallard/Illinois/11OS5582/2011(H1N1)	99.0	99.1	98.9	98.9
CY166282.1|A/mallard/Illinois/11OS5564/2011(H1N8)	99.0	99.0	98.9	98.8
CY186648.1|A/blue-winged teal/Illinois/12OS4788/2012(H1N1)				99.1

H2	% ID
Accession|Virus name	H111-98
CY186989.1|A/northern shoveler/Ohio/12OS5647/2012(H2N3)	99.4
CY157278.1|A/mallard/California/3046/2012(H2N3)	98.4
CY157318.1|A/mallard/California/3118/2012(H2N3)	98.3
KJ568185.1|A/blue-winged teal/Ohio/13OS2104/2013(H2N3)	97.9
CY039707.1|A/env/California/7313/2008(H2N3)	97.0
CY039692.1|A/env/California/7286/2008(H2N3)	97.0
CY039684.1|A/env/California/7279/2008(H2N3)	97.0
CY039715.1|A/env/California/7335/2008(H2N3)	96.9
CY039700.1|A/env/California/7303/2008(H2N3)	96.9
JF327327.1|A/duck/OH/492493/2007(H2N3)	96.8

H3	% Identity												
Accession|Virus name	H101-24	H102-09	H104-93	H108-45	H109-15	H109-38	H102-23	H105-08	H102-32	H103-20	H109-14	H112-60	H114-14
CY186879.1|A/blue-winged teal/Ohio/12OS2229/2012(H3N6)	98.9	99.1	99.0	98.8	98.6	99.4	99.1	98.8	98.9	99.4	99.1		
CY166234.1|A/mallard/Wisconsin/11OS4081/2011(H3N8)	98.8	99.0	99.0	98.7	98.6	99.0	99.2	99.0	98.8	99.1	98.9		
CY187119.1|A/mallard/Wisconsin/12OS4331/2012(H3N8)	98.8	99.0	98.7	99.7	98.5	99.1	99.0	98.7	98.8	99.1	98.8		
CY141025.1|A/mallard/Arkansas/AI09-5900/2009(H3N8)	98.7	98.8	98.6	98.7	98.3	98.9	98.8	98.6	98.7	99.0	98.7		
CY142626.1|A/American green-winged teal/Interior Alaska/9BM6410R0/2009(H3N8)	98.3	98.5	98.3	98.4	98.1	98.6	98.5	98.3	98.3	98.7	98.4		
CY142938.1|A/northern pintail/Interior Alaska/9BM7836R0/2009(H3N8)	98.3	98.5	98.3	98.4	98.1	98.6	98.5	98.3	98.3	98.7	98.4		
CY143322.1|A/northern pintail/Interior Alaska/9BM12647R0/2009(H3N8)	98.3	98.5	98.3	98.4	98.1	98.6	98.5	98.3	98.3	98.7	98.4		
CY143331.1|A/mallard/Interior Alaska/9BM12694R1/2009(mixed)	98.3	98.5	98.3	98.4	98.1	98.6	98.5	98.3	98.3	98.7	98.4		
CY140860.1|A/blue-winged Teal/North Dakota/AI09-3642/2009(H3N1)	98.3	98.5	98.3	98.4									
CY096656.1|A/blue-winged teal/Guatemala/CIP049-05/2010(H3N8)	97.6	97.7	97.5	97.7	97.4	97.9							
CY143146.1|A/mallard/Interior Alaska/9BM12285R0/2009(H3N8)					98.0	98.5	98.4	98.2	98.3	98.7	98.4		
CY142810.1|A/northern pintail/Interior Alaska/9BM11487R0/2009(H3N8)							98.5	98.3					
CY143066.1|A/northern pintail/Interior Alaska/9BM10819R0/2009(H3N8)									98.3	98.7	98.4		
JN864063.1|A/Anas strepera/Arizona/A00271503/2009(H3N8)												99.0	98.8
CY076165.1|A/American coot/Oregon/20589-007/2007(H3N8)												98.6	98.5
CY097718.1|A/mallard/Mississippi/386/2010(H3N8)												98.5	98.4
CY097726.1|A/mallard/Mississippi/390/2010(H3N8)												98.5	98.4
CY097702.1|A/mallard/Mississippi/354/2010(H3N8)												98.5	98.3
CY032720.1|A/northern pintail/California/HKWF792/2007(H3N8)												98.4	98.3
CY097710.1|A/mallard/Mississippi/360/2010(H3N8)												98.4	98.3
CY097678.1|A/American green-winged teal/Mississippi/285/2010(H3N8)												98.3	98.2
CY076013.1|A/cinnamon teal/California/44287-325/2007(H3N8)												98.1	98.0
EU871828.1|A/duck/LA/17G/1987(H3N8)												94.7	94.7

H4	% Identity							
Accession|Virus name	H101-29	H102-05	H110-27	H102-10	H103-37	H108-39	H109-06	H112-31
CY166979.1|A/blue-winged teal/Missouri/11OS2641/2011(H4N8)	99.5							
CY133769.1|A/blue-winged teal/Missouri/10MO030/2010(H4N6)	99.4							
CY166477.1|A/blue-winged teal/Wisconsin/11OS2657/2011(H4N6)	99.4							
CY128966.1|A/American black duck/New Brunswick/03553/2009(H4N6)	99.4							
CY128974.1|A/American black duck/New Brunswick/03511/2009(H4N6)	99.4							
CY128990.1|A/American black duck/New Brunswick/03552/2009(H4N6)	99.4							
CY166485.1|A/northern pintail/Wisconsin/11OS3295/2011(H4N2)	99.4							
CY166557.1|A/northern shoveler/Wisconsin/11OS3013/2011(H4N8)	99.4							
CY166971.1|A/blue-winged teal/Missouri/11OS2640/2011(H4N8)	99.4							
CY128540.1|A/American black duck/New Brunswick/03559/2009(H4N6)	99.3							
CY166768.1|A/American green-winged teal/Mississippi/11OS5869/2011(H4N6)		99.4	99.1					
CY186496.1|A/mallard/Ohio/12OS1418/2012(mixed)		99.4	99.5					
CY132133.1|A/mallard/Ohio/10OS1319/2010(H4N6)		99.3	98.8					
CY166696.1|A/mallard/Wisconsin/11OS4309/2011(H4N6)		99.2	98.9					
CY166704.1|A/mallard/Wisconsin/11OS4310/2011(H4N6)		99.2	98.9					
CY186716.1|A/mallard/Illinois/12OS4808/2012(H4N8)		99.1	98.8					
CY139539.1|A/mallard/New Brunswick/00992/2010(mixed)		99.1	98.7					
CY139522.1|A/American black duck/New Brunswick/00988/2010(mixed)		99.1						
CY139531.1|A/American black duck/New Brunswick/00991/2010(H4N6)		99.1						
KF534792.1|A/Anas discors/New Mexico/A00327695/2007(H4N6)		98.8						
CY132393.1|A/mallard/Ohio/11OS2083/2011(H4N8)			98.8					
CY132384.1|A/mallard/Ohio/11OS2072/2011(mixed)			98.8					
CY132517.1|A/mallard/Ohio/11OS2085/2011(H4N6)			98.8					
CY097026.1|A/blue-winged teal/Wisconsin/2741/2009(H4N6)				99.4	99.2	99.1		
CY096970.1|A/bufflehead/Illinois/4016/2009(H4N8)				99.3	99.2			
CY132901.1|A/blue-winged teal/Illinois/10OS1561/2010(H4N6)				99.3	99.2			
CY132909.1|A/blue-winged teal/Illinois/10OS1563/2010(H4N6)				99.3	99.2			
KJ567929.1|A/American green-winged teal/Ohio/13OS1864/2013(H4N8)				99.3	99.1			
KJ568169.1|A/mallard/Ohio/13OS1989/2013(H4N8)				99.3	99.1			

H4	% Identity				
CY141001.1|A/blue-winged teal/Louisiana/AI09-5291/2009(H4N6)	99.2	99.1			
CY140769.1|A/mallard/Minnesota/AI09-3047/2009(mixed)	99.2	99.1			
KJ567977.1|A/blue-winged teal/Ohio/13OS2016/2013(H4N6)	99.2	99.0			
KJ568241.1|A/blue-winged teal/Iowa/13OS2367/2013(H4N8)	99.2	99.0			
CY166378.1|A/mallard/Illinois/11OS4311/2011(H4N2)			99.6		
CY167028.1|A/northern pintail/Illinois/11OS4756/2011(mixed)			99.4		
CY166832.1|A/American green-winged teal/Mississippi/11OS5903/2011(H4N6)			99.2		
CY132917.1|A/American green-winged teal/Illinois/10OS1598/2010(H4N8)			99.2		
CY133577.1|A/mallard/Iowa/10OS2420/2010(H4N6)			99.2		
CY138006.1|A/blue-winged teal/Nova Scotia/00296/2010(mixed)			99.2		
CY132790.1|A/American green-winged teal/Iowa/10OS2467/2010(mixed)			99.1		
CY138504.1|A/blue-winged teal/Nova Scotia/01008/2010(H4N6)			99.1		
CY138512.1|A/blue-winged teal/Nova Scotia/01009/2010(H4N6)			99.1		
CY139319.1|A/mallard/New Brunswick/00854/2010(H4N6)				99.2	
CY133069.1|A/mallard/Wisconsin/10OS2659/2010(H4N2)				98.7	
CY186951.1|A/blue-winged teal/Ohio/12OS3128/2012(H4N6)				98.6	
CY133432.1|A/mallard/Iowa/10OS2685/2010(H4N2)				98.5	
CY157486.1|A/mallard/California/3471/2012(H4N6)				98.5	
CY167020.1|A/northern shoveler/Illinois/11OS4707/2011(H4N6)				98.5	
CY177255.1|A/mallard/California/1398/2013(H4N6)				98.5	
CY177175.1|A/mallard/California/1369/2013(H4N6)				98.4	
CY187168.1|A/blue winged-teal/Wisconsin/12OS4224/2012(H4N8)				98.4	
CY103135.1|A/mallard/Alberta/256/2006(mixed)				98.1	
KJ413565.1|A/blue-winged teal/LA/AI13-1334/2013(H4N2)					98.2
CY122499.1|A/environment/California/NWRC183970-18/2006(H4N2)					98.1
CY122503.1|A/environment/New Mexico/NWRC184137-06/2006(H4N2)					98.0
CY053821.1|A/pekin duck/California/P30/2006(H4N2)					97.5
CY122536.1|A/environment/Utah/NWRC186341-12/2007(H4N2)					97.4
KM244114.1|A/turkey/Minnesota/16423-3/2014(H4N2)					97.4
CY122546.1|A/environment/Colorado/NWRC186600-24/2007(H4N2)					97.4
KF986862.1|A/quail/California/D113023808/2012(H4N2)					96.4
CY005968.1|A/mallard duck/ALB/291/1977(H4N1)					86.9
CY005963.1|A/blue-winged teal/ALB/103/1990(H4N5)					86.8

H5	% Identity		
Accession|Virus name	H098-32	H113-53	H105-05
CY033444.1|A/American green-winged teal/California/HKWF609/2007(H5N2)	98.6	97.1	
CY096704.1|A/blue-winged teal/Guatemala/CIP049-08/2010(H5N3)	98.3	96.8	
CY094181.1|A/mallard/California/8834/2008(H5N9)	98.3	96.7	
GQ923253.1|A/avian/Missouri/466554-6/2006(H5N2)	98.3	96.7	
CY096720.1|A/blue-winged teal/Guatemala/CIP049-09/2010(H5N3)	98.3	96.7	
CY167175.1|A/American green-winged teal/Mississippi/12OS191/2012(H5N3)	98.2	96.7	
CY133153.1|A/American green-winged teal/Wisconsin/10OS3127/2010(H5N2)	98.2	96.6	
CY133785.1|A/mallard/Missouri/10MO084/2010(H5N9)	98.1		
CY096680.1|A/blue-winged teal/Guatemala/CIP049-13/2010(H5N4)	98.0	96.5	
CY096672.1|A/blue-winged teal/Guatemala/CIP049-12/2010(H5N4)	97.9	96.4	
CY133909.1|A/northern shoveler/California/2815/2011(H5N2)		96.6	
CY132485.1|A/mallard/Ohio/11OS2156/2011(H5N2)			99.6
CY132565.1|A/mallard/Ohio/11OS1961/2011(H5N2)			99.5
CY132461.1|A/mallard/Ohio/11OS2239/2011(H5N2)			99.5
CY132469.1|A/mallard/Ohio/11OS2119/2011(H5N2)			99.5
CY132477.1|A/mallard/Ohio/11OS2006/2011(H5N2)			99.5
CY166637.1|A/American green-winged teal/Wisconsin/11OS3431/2011(H5N2)			99.5
CY166645.1|A/American green-winged teal/Wisconsin/11OS3432/2011(H5N2)			99.4
CY132453.1|A/mallard/Ohio/11OS2229/2011(H5N2)			99.4
CY186667.1|A/mallard/Illinois/12OS5080/2012(H5N1)			99.3
CY187005.1|A/mallard/Ohio/12OS2218/2012(H5N1)			99.3

H7	% Identity	
Accession|Virus name	H107-88	H114-76
CY097317.1|A/mallard/Wisconsin/4236/2009(mixed)	98.5	
CY132821.1|A/American green-winged teal/Mississippi/11OS98/2011(mixed)	98.3	
CY133177.1|A/northern shoveler/Wisconsin/10OS3226/2010(H7N3)	98.2	
CY133323.1|A/mallard/Wisconsin/10OS3232/2010(mixed)	98.2	
CY133777.1|A/mallard/Missouri/10MO053/2010(H7N4)	98.2	
CY097622.1|A/mallard/Missouri/220/2009(H7N3)	98.1	
CY079412.1|A/northern shoveler/Mississippi/09OS643/2009(H7N7)	98.0	
CY079308.1|A/American green-winged teal/Mississippi/09OS046/2009(H7N7)	98.0	
CY132501.1|A/mallard/Ohio/11OS2033/2011(H7N8)	98.0	
CY132509.1|A/mallard/Ohio/11OS2010/2011(H7N8)	98.0	
KJ527614.1|A/blue-winged teal/Ohio/13OS1770/2013(H7N3)		98.8
KJ527622.1|A/American green-winged teal/Ohio/13OS1769/2013(H7N2)		98.8
CY177045.1|A/mallard/California/3956/2012(H7N3)		98.5
CY133669.1|A/American green-winged teal/Mississippi/11OS250/2011(H7N3)		98.3
CY167232.1|A/American green-winged teal/Mississippi/12OS397/2012(H7N7)		98.2
CY167248.1|A/northern pintail/Mississippi/12OS420/2012(mixed)		98.2
CY167208.1|A/northern shoveler/Mississippi/12OS347/2012(H7N7)		98.2
CY167224.1|A/northern shoveler/Mississippi/12OS362/2012(H7N7)		98.2
CY167143.1|A/northern shoveler/Arkansas/12OS158/2012(H7N1)		98.1
CY185945.1|A/blue-winged teal/Louisiana/AI11-2911/2011(H7N3)		98.1

H11	% Identity			
Accession|Virus name	H104-69	H105-04	H110-37	H114-76
CY134407.1|A/cinnamon teal/California/225/2011(H11N9)	99.0	99.2	98.7	98.4
CY186903.1|A/blue-winged teal/Ohio/12OS2152/2012(H11N9)	98.7		98.0	97.7
CY078050.1|A/mallard/Minnesota/Sg-00118/2007(H11N9)	98.6	98.8	98.1	97.8
CY078130.1|A/mallard/Minnesota/Sg-00177/2007(H11N9)	98.6	98.8	98.1	97.8
CY097566.1|A/mallard/Illinois/4179/2009(H11N9)	98.5	98.6		
CY097066.1|A/common goldeneye/Iowa/3192/2009(H11N9)	98.5	98.8	98.2	98.0
CY078154.1|A/mallard/Minnesota/Sg-00198/2007(H11N9)	98.5	98.7		
CY133505.1|A/mallard/Illinois/10OS4130/2010(H11N2)	98.4	98.6		
CY132859.1|A/mallard/Illinois/10OS3249/2010(H11N2)	98.4			
KF542875.1|A/Anas acuta/New Mexico/A00629381/2008(H11N9)	98.2	98.5	97.8	97.6
CY141009.1|A/mallard/Arkansas/AI09-5663/2009(H11N9)		98.7	98.1	98.0
CY133117.1|A/bufflehead/Wisconsin/10OS3214/2010(mixed)		98.6	98.0	97.8
CY134173.1|A/mallard/California/2571P/2011(H11N2)			98.0	97.7
CY134182.1|A/mallard/California/2571V/2011(mixed)			98.0	97.7

H12	% ID
Accession|Virus name	H104-99
CY033700.|A/mallard duck/Minnesota/Sg-00055/2007(H12N5)	98.3
CY064065.2|A/mallard/Minnesota/Sg-00055/2007(H12N5)	98.3
CY097646.1|A/mallard/Ohio/1688/2009(H12N5)	98.1
CY122508.1|A/environment/Nebraska/NWRC184928-24/2007(H12N5)	98.6
CY130372.1|A/mallard/Interior Alaska/10BM02111R0/2010(H12N5)	99.4
CY132850.1|A/mallard/Illinois/10OS3248/2010(mixed)	98.8
CY134279.1|A/mallard/California/2595P/2011(H12N5)	98.6
CY134287.1|A/mallard/California/2595V/2011(H12N5)	98.6
CY186871.1|A/blue-winged teal/Ohio/12OS2140/2012(H12N5)	98.8
KM244060.1|A/guinea fowl/Massachusetts/14075-3/2013(H12N5)	98.4

H14	% Identity							
Accession|Virus name	H105-31	H109-49	H110-31	H113-07	H113-08	H113-76	H108-02	H110-04
CY167267.1|A/northern shoveler/Mississippi/12OS456/2012(H14N2)	99.4	98.0	98.1	98.0	98.3	98.3	98.3	98.1
KJ195668.1|A/blue-winged teal/Guatemala/CIP049H106-62/2011(H14N6)	99.3	98.1	98.3	98.1	98.3	98.3	98.4	98.3
CY146897.1|A/northern shoveler/California/2696/2011(H14N2)	99.3	98.0	98.1	98.0	98.3	98.3	98.3	98.1
CY167126.1|A/northern shoveler/Arkansas/12OS112/2012(mixed)	99.2	97.9	98.0	97.9	98.1	98.1	98.2	98.0
KJ195676.1|A/blue-winged teal/Guatemala/CIP049H105-15/2011(H14N3)	99.2	98.1	98.1	98.0	98.2	98.2	98.3	98.1
KF986854.1|A/blue-winged teal/TX/AI13-1028/2013(H14N5)	98.4	98.2	98.3	98.2	98.3	98.3	98.5	98.3
CY133381.1|A/northern shoveler/Missouri/10OS4673/2010(H14N6)	97.8	96.6	96.7	96.6	96.9	96.9	96.9	96.7
JN696316.2|A/long-tailed duck/Wisconsin/10OS4225/2010(H14N6)	97.1	96.0	96.1	95.9	96.2	96.2	96.2	96.1
JN696315.2|A/white-winged scoter/Wisconsin/10OS3922/2010(mixed)	97.1	95.9	96.0	95.9	96.1	96.1	96.2	96.0
JN696314.2|A/long-tailed duck/Wisconsin/10OS3912/2010(H14N6)	97.1	95.9	96.0	95.9	96.1	96.1	96.2	
CY133553.1|A/long-tailed duck/Wisconsin/10OS3918/2010(H14N8)								96.1

Table 0.3. Top ten BLAST hits for the NA genes of the viruses isolated from wintering waterfowl in Guatemala, 2010-2013.

N2	% Identity										
Accession|Virus name	H101-29	H102-09	H103-20	H108-39	H108-45	H109-06	H102-05	H102-23	H102-32	H109-15	H112-31
CY167251.1|A/northern pintail/Mississippi/12OS420/2012(mixed)	99.4	99.5	99.4	99.1	99.0	99.1	99.3		99.3	98.8	
CY132640.1|A/mallard/Illinois/10OS3245/2010(mixed)	99.3	99.5	99.2	99.0	98.8		99.2	99.2		98.8	
CY133155.1|A/American green-winged teal/Wisconsin/10OS3127/2010(H5N2)	99.3	99.4	99.4	99.0	99.1	99.2				98.7	
CY140995.1|A/blue-winged teal/Louisiana/AI09-5234/2009(H11N2)	99.2	99.4	99.3	99.0	98.9	99.0	99.5	99.4	99.4	99.0	
CY132824.1|A/American green-winged teal/Mississippi/11OS98/2011(mixed)	99.2	99.4	99.3	99.0	98.9	99.0					
CY167269.1|A/northern shoveler/Mississippi/12OS456/2012(H14N2)	99.2	99.3	99.2	98.9	98.8				99.2		
CY096626.1|A/blue-winged teal/Guatemala/CIP049-10/2009(H11N2)	99.1	99.3	99.2	98.8	98.8	98.7	99.3	99.1	99.2	98.8	
CY096642.1|A/blue-winged teal/Guatemala/CIP049-03/2009(H11N2)	99.1	99.3	99.2	98.8		98.7	99.3	99.1	99.2	98.8	
CY096634.1|A/blue-winged teal/Guatemala/CIP049-11/2009(H11N2)	98.9	99.1	99.1	98.8		98.7	99.2	99.1	99.2	98.8	
CY033446.1|A/American green-winged teal/California/HKWF609/2007(H5N2)	98.8	99.0				98.4	98.8	98.7	98.8	98.4	
CY120589.1|A/mallard/California/2396/2010(H5N2)			99.2	98.9	98.8					98.8	
CY177031.1|A/northern shoveler/California/3769/2012(H6N2)					99.4	99.5					
CY177055.1|A/bufflehead/California/4935/2012(H11N2)					99.2	99.2					
CY166260.1|A/northern pintail/Wisconsin/11OS4111/2011(H6N2)							99.5	99.4	99.4		
CY133803.1|A/mallard/Missouri/10MO0253/2010(H1N2)							99.5	99.4	99.4		
CY133458.1|A/American wigeon/Iowa/10OS2748/2010(H2N2)							99.2	99.2	99.2	98.8	
CY132183.1|A/mallard/Ohio/11OS2213/2011(H11N2)								99.2			
KJ413567.1|A/blue-winged teal/LA/AI13-1334/2013(H4N2)											98.7
KM244116.1|A/turkey/Minnesota/16423-3/2014(H4N2)											98.4
CY097754.1|A/mallard/Mississippi/407/2010(mixed)											98.0
CY053823.1|A/pekin duck/California/P30/2006(H4N2)											97.5
CY004785.1|A/mallard duck/ALB/354/1978(H4N2)											91.7
CY014560.1|A/mallard duck/Alberta/205/1978(H6N2)											91.6
CY004045.1|A/pintail duck/ALB/367/1978(H6N2)											91.6
CY004001.1|A/mallard duck/ALB/250/1978(H6N2)											91.6
CY004050.1|A/blue-winged teal/ALB/651/1978(H6N2)											91.6
CY004041.1|A/pintail duck/ALB/133/1978(H6N2)											91.6

N3	% Identity											
Accession|Virus name	H098-32	H103-05	H104-69	H105-05	H109-49	H110-04	H110-31	H110-37	H113-07	H113-53	H114-76	H108-02
CY132991.1|A/American green-winged teal/Illinois/10OS3343/2010(H2N3)	98.5		97.8	97.9								
CY097336.1|A/mallard/Wisconsin/2560/2009(H2N3)	98.3	99.2			98.9	98.7	99.0	98.7	98.7	98.7	98.8	98.7
CY096996.1|A/blue-winged teal/Wisconsin/2572/2009(H2N3)	98.2	99.1										98.7
CY097376.1|A/mallard/Wisconsin/2785/2009(H2N3)	98.1	99.0										
CY039598.1|A/northern shoveler/California/HKWF2031/2008(H7N3)	97.9	98.2	97.4	97.5	98.2	98.1	98.3	98.1	98.1	98.1	98.1	
CY097320.1|A/mallard/Wisconsin/4236/2009(mixed)	97.8	98.5	97.3	97.3	99.2	99.0	99.2	99.0	99.0	98.8	99.0	99.0
CY039542.1|A/green winged teal/California/AKS1370/2008(H7N3)	97.7	98.1	97.2	97.3								
CY125730.1|A/Mexico/InDRE7218/2012(H7N3)	97.7		99.0	97.9								
CY032730.1|A/northern shoveler/California/HKWF979/2007(H3N3)	97.7	98.1	97.1	97.2								
EU500862.1|A/chicken/SK/HR-00011/2007(H7N3)	97.6	97.9	96.9	97.0								
KJ413442.1|A/blue-winged teal/TX/AI12-614/2012(H10N3)			99.4	98.2								
CY166180.1|A/mallard/Mississippi/12OS445/2012(H10N3)			99.3	98.1								
JX317626.1|A/chicken/Jalisco/CPA1/2012(H7N3)			99.2	98.0								
CY141019.1|A/mallard/Arkansas/AI09-5761/2009(H2N3)		98.8										
CY134313.1|A/American wigeon/California/2930/2011(H10N3)		98.4			99.2	99.1	99.3	99.1	99.1	98.9	99.0	99.0
KJ413543.1|A/blue-winged teal/TX/AI13-1009/2013(H11N3)					99.7	99.5	99.4	99.5	99.5	99.4	99.3	99.5
CY167194.1|A/American green-winged teal/Mississippi/12OS311/2012(H5N3)					99.6	99.4	99.7	99.4	99.4	99.2	99.4	99.4
KJ527616.1|A/blue-winged teal/Ohio/13OS1770/2013(H7N3)					99.5	99.4	99.6	99.4	99.4	99.2	99.3	99.3
CY177047.1|A/mallard/California/3956/2012(H7N3)					99.4	99.3	99.5	99.3	99.3	99.1	99.2	99.2
CY097624.1|A/mallard/Missouri/220/2009(H7N3)					99.0	98.9	99.1	98.9	98.9	98.7	98.8	98.9
KJ568347.1|A/blue-winged teal/Iowa/13OS2316/2013(H7N3)					99.0	98.9	99.1	98.9	98.9	98.7	98.8	98.8

N3 (cont)	% Identity										
Accession|Virus name	H102-46	H104-38	H105-08	H105-31	H103-37	H104-93	H105-01	H107-88	H111-98	H109-14	H105-04
CY167177.1|A/American green-winged teal/Mississippi/12OS191/2012(H5N3)	99.7	99.8	99.7	99.6							
CY157019.1|A/mallard/California/3435/2012(H2N3)	99.6	99.7	99.6	99.4							
CY157152.1|A/mallard/California/2837/2012(H2N3)	99.6	99.7	99.6	99.4							
CY157248.1|A/mallard/California/2997/2012(H2N3)	99.6	99.7	99.6	99.4							
CY157424.1|A/mallard/California/3310/2012(H2N3)	99.6	99.7	99.6	99.4							
CY157176.1|A/mallard/California/2840/2012(H2N3)	99.5	99.6	99.5	99.4							
CY157408.1|A/mallard/California/3223/2012(H2N3)	99.5	99.6	99.5	99.4							
CY157448.1|A/mallard/California/3381/2012(H2N3)	99.5	99.6	99.5	99.4							
CY157472.1|A/mallard/California/3430/2012(H2N3)	99.5	99.6	99.5	99.4							
CY157577.1|A/mallard/California/3078/2012(H2N3)	99.5	99.6	99.5	99.4							
KJ195678.1|A/blue-winged teal/Guatemala/CIP049H105-15/2011(H14N3)					99.8	99.9	99.8	98.8	99.3	98.4	
CY167186.1|A/northern shoveler/Mississippi/12OS231/2012(mixed)					99.5	99.6	99.6	98.8	99.2		
CY133418.1|A/northern shoveler/Missouri/10OS4750/2010(H7N3)					99.4	99.4	99.4	98.5	99.2	98.5	
CY185947.1|A/blue-winged teal/Louisiana/AI11-2911/2011(H7N3)					99.4	99.4	99.4	98.5	99.0		
CY186742.1|A/blue-winged teal/Illinois/12OS2951/2012(H11N3)					99.4	99.4	99.4	98.4			
CY097736.1|A/northern shoveler/Mississippi/397/2010(H1N3)					99.3	99.4	99.4	98.5	99.1	98.6	
CY133391.1|A/northern shoveler/Missouri/10OS4700/2010(mixed)					99.3	99.4	99.4	98.5	99.1	98.6	
CY133410.1|A/gadwall/Missouri/10OS4731/2010(H7N3)					99.3	99.4	99.4	98.5	99.1	98.6	
CY186991.1|A/northern shoveler/Ohio/12OS5647/2012(H2N3)					99.2	99.2	99.2	98.3	99.8		
CY133039|A/American green-winged teal/Illinois/10OS4014/2010(H7N3)					99.2						
CY133039.1|A/American green-winged teal/Illinois/10OS4014/2010(H7N3)						99.2	99.2	98.5	99.0		
CY133731.1|A/northern shoveler/Mississippi/11OS289/2011(H7N3)									98.8		
CY130390.1|A/northern pintail/Interior Alaska/10BM02539R0/2010(H7N3)										99.1	
CY094575.1|A/green-winged teal/California/1841/2009(H7N3)										99.0	
CY120565.1|A/mallard/California/1438/2010(H2N3)										98.9	
CY094463.1|A/green-winged teal/California/11275/2008(H7N3)										98.8	
CY039582.1|A/northern shoveler/California/HKWF1026/2007(H7N3)										98.2	

N3 (cont)	% Identity
CY134329.1|A/mallard/California/2961/2011(H2N3)	99.4
CY135723.1|A/mallard/Interior Alaska/10BM05347R0/2010(H7N3)	99.4
CY141533.1|A/mallard/Interior Alaska/10BM07066R0/2010(H7N3)	99.4
CY141688.1|A/northern pintail/Interior Alaska/10BM08586R0/2010(H7N3)	99.4
CY141776.1|A/mallard/Interior Alaska/10BM09563R0/2010(H7N3)	99.4
CY141800.1|A/mallard/Interior Alaska/10BM09818R0/2010(H7N3)	99.4
CY141808.1|A/northern pintail/Interior Alaska/10BM09832R0/2010(H7N3)	99.4
CY141816.1|A/northern pintail/Interior Alaska/10BM10166R0/2010(H7N3)	99.4
CY141824.1|A/mallard/Interior Alaska/10BM10324R0/2010(H7N3)	99.4
CY141984.1|A/mallard/Interior Alaska/10BM12534R0/2010(H7N3)	99.4

N4	% Identity	
Accession|Virus name	H113-08	H113-76
CY132783.1|A/northern shoveler/Illinois/10OS3632/2010(mixed)	99.0	99.0
CY166164.1|A/mallard/Wisconsin/11OS4489/2011(H8N4)	98.6	98.6
CY166941.1|A/blue-winged teal/Missouri/11OS2563/2011(H12N4)	98.6	98.6
CY166128.1|A/northern shoveler/Wisconsin/11OS4631/2011(mixed)	98.5	98.5
CY167014.1|A/mallard/Illinois/11OS4421/2011(mixed)	98.3	98.3
CY096666.1|A/blue-winged teal/Guatemala/CIP049-06/2010(H8N4)	97.9	97.9
CY096682.1|A/blue-winged teal/Guatemala/CIP049-13/2010(H5N4)	97.9	97.9
CY096674.1|A/blue-winged teal/Guatemala/CIP049-12/2010(H5N4)	97.8	97.8
CY096714.1|A/blue-winged teal/Guatemala/CIP049-15/2010(H8N4)	97.8	97.8
CY096650.1|A/blue-winged teal/Guatemala/CIP049-04/2010(H8N4)	97.7	97.7

N5	% Identity	
Accession|Virus name	H104-99	H110-04
CY130226.1|A/northern pintail/Interior Alaska/10BM02561R0/2010(H12N5)	99.5	
CY143847.1|A/northern pintail/Interior Alaska/10BM10556R0/2010(mixed)	99.5	
CY130374.1|A/mallard/Interior Alaska/10BM02111R0/2010(H12N5)	99.4	
KM244062.1|A/guinea fowl/Massachusetts/14075-3/2013(H12N5)	99.0	
CY096952.1|A/northern shoveler/Illinois/3767/2009(mixed)	98.8	
CY097640.1|A/northern pintail/Missouri/319/2009(H12N5)	98.8	
CY120557.1|A/mallard/California/1390/2010(H7N5)	98.5	
CY064067.2|A/mallard/Minnesota/Sg-00055/2007(H12N5)	98.3	
CY134281.1|A/mallard/California/2595P/2011(H12N5)	98.3	
CY004340.1|A/pintail/Alberta/49/2003(H12N5)	97.8	
CY166212.1|A/mallard/Wisconsin/11OS3577/2011(H6N5)		99.0
CY166631.1|A/American green-winged teal/Wisconsin/11OS3425/2011(H12N5)		99.0
CY186849.1|A/American green-winged teal/Ohio/12OS2128/2012(H6N5)		98.6
CY186559.1|A/American green-winged teal/Ohio/12OS2256/2012(H6N5)		98.6
CY140376.1|A/green-winged teal/Minnesota/Sg-00820/2008(H4N5)		98.5
CY079181.1|A/northern pintail/Interior Alaska/8BM3736/2008(H12N5)		98.4
CY094119.1|A/mallard/California/6524/2008(H12N5)		98.4
CY077184.1|A/mallard/Alberta/162/2007(H12N5)		98.3
CY186873.1|A/blue-winged teal/Ohio/12OS2140/2012(H12N5)		98.3
CY079390.1|A/northern shoveler/Mississippi/09OS025/2009(H12N5)		98.3

N6	% ID
Accession|Virus name	H110-27
CY103120.1|A/mallard/Alberta/246/2006(H4N6)	98.5
CY103128.1|A/mallard/Alberta/254/2006(H4N6)	98.5
CY103213.1|A/mallard/Alberta/106/2007(mixed)	98.5
CY103238.1|A/mallard/Alberta/114/2007(H4N6)	98.5
CY103317.1|A/mallard/Alberta/156/2007(mixed)	98.3
CY103326.1|A/mallard/Alberta/160/2007(H4N6)	98.4
CY103342.1|A/northern pintail/Alberta/265/2007(H4N6)	98.4
CY103469.1|A/mallard/Alberta/121/2008(H4N6)	98.5
CY103477.1|A/mallard/Alberta/270/2008(H4N6)	98.5
CY140477.1|A/mallard/Minnesota/Sg-00931/2008(H4N6)	98.3

N8	% Identity					
Accession|Virus name	**H101-24**	**H102-10**	**H102-23**	**H109-38**	**H112-60**	**H114-14**
CY133571.1|A/blue-winged teal/Iowa/10OS2411/2010(H3N8)	99.3					
CY133763.1|A/blue-winged teal/Missouri/10MO021/2010(H3N8)	99.2					
CY166559.1|A/northern shoveler/Wisconsin/11OS3013/2011(H4N8)	98.9					
CY166957.1|A/blue-winged teal/Missouri/11OS2585/2011(H4N8)	98.9					
CY132919.1|A/American green-winged teal/Illinois/10OS1598/2010(H4N8)	98.9					
CY140838.1|A/blue-winged Teal/Texas/AI09-3464/2009(H4N8)	98.8					
CY140593.1|A/mallard/Minnesota/Sg-01042/2008(H6N8)	98.3					
CY132412.1|A/mallard/Ohio/11OS1982/2011(mixed)	98.1					
CY128477.1|A/mallard/Nova Scotia/02445/2007(mixed)	98.1					
CY128760.1|A/American black duck/New Brunswick/02375/2007(H4N8)	98.1					
CY132951.1|A/mallard/Wisconsin/10OS2773/2010(H3N8)		99.5				
CY132959.1|A/wood duck/Wisconsin/10OS2778/2010(H3N8)		99.5				
CY166284.1|A/mallard/Illinois/11OS5564/2011(H1N8)		99.5				
CY133325.1|A/mallard/Wisconsin/10OS3232/2010(mixed)		99.3				
CY187015.1|A/gadwall/Ohio/12OS2222/2012(H3N8)		99.3				
CY187023.1|A/American black duck/Ohio/12OS5510/2012(H3N8)		99.3				
KJ567907.1|A/blue-winged teal/Iowa/13OS2346/2013(H3N8)		99.1				
KJ568275.1|A/blue-winged teal/Iowa/13OS2345/2013(H3N8)		99.1				
CY139405.1|A/mallard/New Brunswick/00913/2010(mixed)		99.0				
KJ568123.1|A/American green-winged teal/Wisconsin/13OS3465/2013(H6N8)		99.0				
CY132029.1|A/mallard/Ohio/11OS2201/2011(mixed)			99.1			
CY132041.1|A/mallard/Ohio/11OS2208/2011(mixed)			99.1			
CY132332.1|A/mallard/Ohio/11OS2153/2011(H3N8)			99.0			
CY140771.1|A/mallard/Minnesota/AI09-3047/2009(mixed)			99.0			
CY166973.1|A/blue-winged teal/Missouri/11OS2640/2011(H4N8)			98.9			
CY132015.1|A/mallard/Ohio/11OS2138/2011(mixed)			98.9			
CY186758.1|A/blue-winged teal/Missouri/12OS2519/2012(H4N8)			98.7			
KJ527632.1|A/blue-winged teal/Ohio/13OS1759/2013(H4N8)			98.4			
KJ568115.1|A/American green-winged teal/Ohio/13OS2084/2013(H6N8)			98.0			
KJ568307.1|A/American green-winged teal/Ohio/13OS2086/2013(H6N8)			98.0			
CY073722.1|A/surface water/Minnesota/W07-2241/2007(H3N8)				98.9		
KJ568179.1|A/blue-winged teal/Ohio/13OS2018/2013(H3N8)				98.6		
KJ568267.1|A/blue-winged teal/Ohio/13OS3827/2013(H3N8)				98.6		

N8	% Identity		
KJ568363.1|A/American green-winged teal/Ohio/13OS2108/2013(H3N8)	98.6		
KJ568403.1|A/wood duck/Ohio/13OS3300/2013(H3N8)	98.6		
KJ568099.1|A/American green-winged teal/Ohio/13OS2106/2013(H3N8)	98.6		
KJ567899.1|A/blue-winged teal/Iowa/13OS2368/2013(H4N8)	98.4		
KJ568027.1|A/blue-winged teal/Ohio/13OS1783/2013(H4N8)	98.4		
KJ568155.1|A/blue-winged teal/Iowa/13OS2349/2013(H4N8)	98.4		
KJ568195.1|A/American green-winged teal/Ohio/13OS1775/2013(H4N8)	98.4		
CY076167.1|A/American coot/Oregon/20589-007/2007(H3N8)		98.3	99.0
CY097704.1|A/mallard/Mississippi/354/2010(H3N8)		98.1	98.7
CY032722.1|A/northern pintail/California/HKWF792/2007(H3N8)		98.1	98.7
CY097728.1|A/mallard/Mississippi/390/2010(H3N8)		98.1	98.7
CY097712.1|A/mallard/Mississippi/360/2010(H3N8)		98.0	98.6
CY097720.1|A/mallard/Mississippi/386/2010(H3N8)		98.0	98.6
CY097129.1|A/mallard/Ohio/2039/2009(mixed)		97.9	98.6
CY076015.1|A/cinnamon teal/California/44287-325/2007(H3N8)		97.8	98.5
CY097680.1|A/American green-winged teal/Mississippi/285/2010(H3N8)		97.8	98.5
EU871830.1|A/duck/LA/17G/1987(H3N8)		94.6	95.3

N9 Accession|Virus name	% ID H114-76
CY133483.1|A/northern shoveler/Illinois/10OS4360/2010(mixed)	98.9
CY133643.1|A/American green-winged teal/Mississippi/11OS90/2011(H11N9)	98.9
CY133651.1|A/northern shoverl/Mississippi/11OS145/2011(H7N9)	98.8
CY167013.1|A/mallard/Illinois/11OS4421/2011(mixed)	98.8
CY167120.1|A/mallard/Arkansas/12OS061/2012(H1N9)	98.8
CY133695.1|A/American green-winged teal/Mississippi/11OS257/2011(H11N9)	98.7
CY133063.1|A/mallard/Wisconsin/10OS2667/2010(H11N9)	98.7
CY133787.1|A/mallard/Missouri/10MO084/2010(H5N9)	98.7
CY166372.1|A/blue-winged teal/Illinois/11OS4176/2011(H11N9)	98.7
CY167202.1|A/northern shoveler/Mississippi/12OS346/2012(H11N9)	98.6

Table 0.4 Sequences used for phylogenetic analysis of the H3N2 swine influenza A viruses retrieved from GISAID

We acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu™ Database that were used as background data for generating the phylogenetic trees of the virus isolated from Guatemala. The list is detailed below.

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
H3						
EPI325849	HA	Dominican Republic	2010-Jun-29	A/Santo Domingo/WRAIR3514T/2010		Other Database Import
EPI325801	HA	Mexico	2010-Jan-26	A/Mexico City/WRAIR1752T/2010		Other Database Import
EPI315772	HA	Nicaragua	2010-Jun-07	A/Managua/3209.01/2010		Other Database Import
EPI315224	HA	Nicaragua	2010-Jun-01	A/Managua/1155.01/2010		Other Database Import
EPI463954	HA	Mexico	2008-Dec-01	A/Mexico/24014/2008		Other Database Import
EPI463946	HA	Mexico	2008-Dec-01	A/Mexico/24013/2008		Other Database Import
EPI463938	HA	Mexico	2008-Dec-02	A/Mexico/24012/2008		Other Database Import
EPI463917	HA	Mexico	2008-Nov-01	A/Mexico/24011/2008		Other Database Import
EPI463901	HA	Mexico	2008-Nov-01	A/Mexico/24009/2008		Other Database Import
EPI463887	HA	Mexico	2009-Apr-01	A/Mexico/24008/2009		Other Database Import
EPI463833	HA	Mexico	2008-Nov-01	A/Mexico/24003/2008		Other Database Import
EPI277421	HA	Nicaragua	2010-Jun-10	A/Managua/2867.01/2010		Other Database Import
EPI178230	HA	Nicaragua	2007-Jun-05	A/Managua/4348.01/2007		Other Database Import
EPI176959	HA	Australia	2007-Jan-01	A/Brisbane/10/2007		Other Database Import
EPI173816	HA	Nicaragua	2007-Aug-03	A/Managua/4902.01/2007		Other Database Import
EPI154444	HA	Nicaragua	2007-Jun-27	A/Managua/25/2007		Other Database Import
EPI154122	HA	Nicaragua	2007-Jun-12	A/Managua/33/2007		Other Database Import
EPI154098	HA	Nicaragua	2007-Jun-23	A/Managua/34/2007		Other Database Import
EPI154074	HA	Nicaragua	2007-Jun-22	A/Managua/29/2007		Other Database Import
EPI154034	HA	Nicaragua	2007-Jun-22	A/Managua/20/2007		Other Database Import
EPI309767	HA	Guadeloupe	2010-Nov-30	A/GUADELOUPE/202/2010	Centers for Disease Control and Prevention	WHO Collaborating Centre for Reference and Research on Influenza
EPI309389	HA	Mexico	2010-Nov-04	A/Mexico/6998/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI301119	HA	Mexico	2010-Aug-13	A/Mexico/4268/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI295227	HA	Mexico	2010-Aug-31	A/Mexico/4674/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
EPI295208	HA	Mexico	2010-Aug-27	A/Mexico/4391/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI243572	HA	Mexico	2009-Aug-07	A/Mexico/5216/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI459841	HA	El Salvador	2013-Mar-18	A/El Salvador/433/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI347486	HA	El Salvador	2011-Sep-21	A/El Salvador/1513/2011	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI295104	HA	El Salvador	2010-Aug-31	A/El Salvador/1103/2010	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI279985	HA	El Salvador	2010-Aug-06	A/El Salvador/1060/2010	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI278812	HA	El Salvador	2010-Jun-08	A/El Salvador/636/2010	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI484551	HA	Costa Rica	2013-Jul-23	A/Costa Rica/4009/2013	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI397057	HA	Costa Rica	2012-Jul-17	A/Costa Rica/9093/2012	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI349761	HA	Costa Rica	2011-Nov-11	A/Costa Rica/8211/2011	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI211317	HA	Costa Rica	2009-Jun-05	A/Costa Rica/5179/2009	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI395233	HA	Dominican Republic	2012-Jun-04	A/Dominican Republic/6524/2012	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI279996	HA	Dominican Republic	2010-Aug-03	A/Dominican Republic/4389/2010	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI254592	HA	Dominican Republic	2009-Dec-04	A/Dominican Republic/3743/2009	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI211266	HA	Dominican Republic	2009-May-25	A/Dominican Republic/988/2009	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI378216	HA	Puerto Rico	2011-Dec-07	A/Puerto Rico/38/2011	Centers for Disease Control and Prevention	Centers for Disease Control and Prevention

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
EPI377477	HA	Puerto Rico	2011-Nov-20	A/Puerto Rico/36/2011	Centers for Disease Control and Prevention	Centers for Disease Control and Prevention
EPI513286	HA	Australia	2011-Oct-24	A/Victoria/361/2011	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention
EPI211334	HA	Australia	2009-Jan-01	A/Perth/16/2009	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention
EPI468141	HA	Guatemala	2013-Jul-09	A/Guatemala/287/2013	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI301071	HA	Guatemala	2010-Oct-05	A/Guatemala/754/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI295095	HA	Guatemala	2010-Sep-02	A/Guatemala/690/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI211337	HA	Guatemala	2009-Jun-15	A/Guatemala/1913/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI193980	HA	Guatemala	2009-Jul-09	A/Guatemala/29/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI193924	HA	Guatemala	2009-Jun-03	A/Guatemala/1066/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI232463	HA	Haiti	2009-Jul-01	A/Haiti/66/2009	Laboratoire National de Sante Publique	Centers for Disease Control and Prevention
EPI193974	HA	Honduras	2009-Jun-30	A/Honduras/2243/2009	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI185779	HA	Honduras	2009-May-05	A/Honduras/56/2009	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI465947	HA	Nicaragua	2013-May-07	A/Esteli/816/2013	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI396164	HA	Nicaragua	2012-Jul-10	A/Managua/726.03/2012	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI497653	HA	Trinidad and Tobago	2013-Oct-27	A/Trinidad/3558/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459823	HA	Trinidad and Tobago	2013-Mar-19	A/Trinidad/1117/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459805	HA	Dominica	2013-Jan-21	A/Dominica/653/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459802	HA	Anguilla	2013-Jan-10	A/Anguilla/104/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI394812	HA	Bermuda	2012-Jun-13	A/Bermuda/1915/2012	Caribbean Epidemiology Center	Centers for Disease Control and Prevention

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
EPI326251	HA	Anguilla	2011-Jan-24	A/Anguilla/273/2011	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI232523	HA	Cayman Islands	2009-Oct-22	A/Cayman Islands/9266/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI185785	HA	Jamaica	2009-Apr-28	A/Jamaica/2970/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI537015	HA	United States	2012-Apr-15	A/Texas/50/2012	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention
EPI397054	HA	Puerto Rico	2012-Jun-27	A/Puerto Rico/36/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI394790	HA	Puerto Rico	2012-Jun-26	A/Puerto Rico/34/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI394787	HA	Puerto Rico	2012-Jun-14	A/Puerto Rico/23/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI391271	HA	Puerto Rico	2012-Jun-11	A/Puerto Rico/08/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI387763	HA	Puerto Rico	2012-Jun-03	A/Puerto Rico/04/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI371957	HA	Puerto Rico	2012-Mar-02	A/Puerto Rico/02/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI371811	HA	Puerto Rico	2012-Feb-12	A/Puerto Rico/03/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI353489	HA	Puerto Rico	2011-Nov-26	A/Puerto Rico/30/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI347536	HA	Puerto Rico	2011-Oct-20	A/Puerto Rico/23/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI295256	HA	Puerto Rico	2010-Sep-24	A/Puerto Rico/01/2010	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI193918	HA	Puerto Rico	2009-Jun-02	A/Puerto Rico/46/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI193906	HA	Puerto Rico	2009-May-26	A/Puerto Rico/25/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI185802	HA	Puerto Rico	2009-Feb-09	A/Puerto Rico/18/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI185949	HA	Trinidad and Tobago	2009-Jan-01	A/Trinidad/2940/2009		Centers for Disease Control and Prevention
EPI172432	HA	Costa Rica	2008-Jun-19	A/Costa Rica/7172/2008		Centers for Disease Control and Prevention
EPI163130	HA	Guatemala	2008-May-19	A/Guatemala/494/2008		Centers for Disease Control and Prevention

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
EPI162156	HA	Nicaragua	2007-Jan-01	A/Managua/2760.01/2007		Centers for Disease Control and Prevention
EPI162130	HA	Mexico	2008-Jan-17	A/Mexico/499/2008		Centers for Disease Control and Prevention
EPI157966	HA	Mexico	2007-Oct-18	A/Mexico/3701/2007		Centers for Disease Control and Prevention
EPI162124	HA	Mexico	2007-Oct-18	A/Mexico/3701/2007		Centers for Disease Control and Prevention
EPI157956	HA	Martinique	2007-Feb-09	A/Martinique/12/2007		Centers for Disease Control and Prevention
EPI162123	HA	Martinique	2007-Feb-09	A/Martinique/12/2007		Centers for Disease Control and Prevention
EPI157670	HA	Honduras	2007-Oct-11	A/Honduras/6453/2007		Centers for Disease Control and Prevention
EPI162072	HA	Honduras	2007-Oct-11	A/Honduras/6453/2007		Centers for Disease Control and Prevention
EPI157664	HA	Honduras	2007-Oct-05	A/Honduras/6374/2007		Centers for Disease Control and Prevention
EPI162066	HA	Honduras	2007-Oct-05	A/Honduras/6374/2007		Centers for Disease Control and Prevention
EPI157579	HA	El Salvador	2007-Sep-03	A/El Salvador/579/2007		Centers for Disease Control and Prevention
EPI161999	HA	El Salvador	2007-Sep-03	A/El Salvador/579/2007		Centers for Disease Control and Prevention
EPI157551	HA	Costa Rica	2007-May-25	A/Costa Rica/4082/2007		Centers for Disease Control and Prevention
EPI161990	HA	Costa Rica	2007-May-25	A/Costa Rica/4082/2007		Centers for Disease Control and Prevention
EPI155856	HA	Dominica	2007-Feb-25	A/Dominican Republic/2896/2007		Centers for Disease Control and Prevention
EPI155503	HA	Costa Rica	2007-Jan-05	A/Costa Rica/176/2007		Centers for Disease Control and Prevention
EPI155501	HA	Costa Rica	2006-Nov-09	A/Costa Rica/7322/2006		Centers for Disease Control and Prevention
EPI155203	HA	Honduras	2006-Oct-01	A/Honduras/1922/2006		Centers for Disease Control and Prevention
EPI342275	HA	Guadeloupe	2010-Nov-30	A/Guadeloupe/201/2010	National Influenza Center French Guiana and French Indies	Centers for Disease Control and Prevention
EPI326288	HA	Guatemala	2011-May-25	A/Guatemala/199/2011	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
EPI326291	HA	Guatemala	2011-Mar-02	A/Guatemala/51/2011	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI340921	HA	Guatemala	2011-Sep-02	A/Guatemala/453/2011	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI342193	HA	Guatemala	2010-Jul-13	A/Guatemala/591/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI346446	HA	El Salvador	2011-Oct-03	A/El Salvador/1571/2011	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI331240	HA	Mexico	2011-Apr-08	A/Mexico/2554/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI325913	HA	Mexico	2010-Dec-22	A/Mexico City/WRAIR3570T/2010		Other Database Import
EPI325921	HA	Mexico	2010-Dec-22	A/Mexico City/WRAIR3570T/2010		Other Database Import
EPI336107	HA	Mexico	2008-Jan-14	A/Mexico/UASLP-012/2008		Other Database Import
EPI336112	HA	Mexico	2008-Jan-10	A/Mexico/UASLP-011/2008		Other Database Import
N2						
EPI154036	NA	Nicaragua	2007-Jun-22	A/Managua/20/2007		Other Database Import
EPI154068	NA	Nicaragua	2007-Jun-21	A/Managua/16/2007		Other Database Import
EPI154100	NA	Nicaragua	2007-Jun-23	A/Managua/34/2007		Other Database Import
EPI154140	NA	Nicaragua	2007-Jun-11	A/Managua/28/2007		Other Database Import
EPI154446	NA	Nicaragua	2007-Jun-27	A/Managua/25/2007		Other Database Import
EPI155502	NA	Costa Rica	2007-Jan-05	A/Costa Rica/176/2007		Centers for Disease Control and Prevention
EPI155855	NA	Dominica	2007-Feb-25	A/Dominican Republic/2896/2007		Centers for Disease Control and Prevention
EPI155857	NA	Dominica	2007-Feb-25	A/Dominica/2898/2007		Centers for Disease Control and Prevention
EPI155990	NA	Mexico	2007-May-03	A/Mexico/2222/2007		Centers for Disease Control and Prevention
EPI156038	NA	Panama	2007-May-22	A/Panama/0475/2007		Centers for Disease Control and Prevention
EPI156056	NA	El Salvador	2007-Aug-09	A/El Salvador/428/2007		Centers for Disease Control and Prevention
EPI156058	NA	El Salvador	2007-Aug-15	A/El Salvador/507/2007		Centers for Disease Control and Prevention
EPI157580	NA	El Salvador	2007-Sep-03	A/El Salvador/579/2007		Centers for Disease Control and Prevention
EPI161998	NA	El Salvador	2007-Sep-03	A/El Salvador/579/2007		Centers for Disease Control and Prevention
EPI157586	NA	El Salvador	2007-Sep-06	A/El Salvador/590/2007		Centers for Disease Control and Prevention

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EPI162004	NA	El Salvador	2007-Sep-06	A/El Salvador/590/2007		Centers for Disease Control and Prevention
EPI157632	NA	Guatemala	2007-Aug-18	A/Guatemala/7562/2007		Centers for Disease Control and Prevention
EPI162057	NA	Guatemala	2007-Aug-18	A/Guatemala/7562/2007		Centers for Disease Control and Prevention
EPI157659	NA	Honduras	2007-Aug-26	A/Honduras/5560/2007		Centers for Disease Control and Prevention
EPI162060	NA	Honduras	2007-Aug-26	A/Honduras/5560/2007		Centers for Disease Control and Prevention
EPI157663	NA	Honduras	2007-Oct-27	A/Honduras/602/2007		Centers for Disease Control and Prevention
EPI162063	NA	Honduras	2007-Oct-27	A/Honduras/602/2007		Centers for Disease Control and Prevention
EPI157665	NA	Honduras	2007-Oct-05	A/Honduras/6374/2007		Centers for Disease Control and Prevention
EPI162065	NA	Honduras	2007-Oct-05	A/Honduras/6374/2007		Centers for Disease Control and Prevention
EPI157671	NA	Honduras	2007-Oct-11	A/Honduras/6453/2007		Centers for Disease Control and Prevention
EPI162071	NA	Honduras	2007-Oct-11	A/Honduras/6453/2007		Centers for Disease Control and Prevention
EPI157957	NA	Martinique	2007-Feb-09	A/Martinique/12/2007		Centers for Disease Control and Prevention
EPI162122	NA	Martinique	2007-Feb-09	A/Martinique/12/2007		Centers for Disease Control and Prevention
EPI160188	NA	Nicaragua	2007-Jun-21	A/Managua/17/2007		Other Database Import
EPI162129	NA	Mexico	2008-Jan-17	A/Mexico/499/2008		Centers for Disease Control and Prevention
EPI162165	NA	Nicaragua	2007-Jun-13	A/Managua/68.01/2007		Centers for Disease Control and Prevention
EPI163131	NA	Guatemala	2008-May-19	A/Guatemala/494/2008		Centers for Disease Control and Prevention
EPI163133	NA	Guatemala	2008-May-28	A/Guatemala/545/2008		Centers for Disease Control and Prevention
EPI163228	NA	Honduras	2007-Nov-18	A/Honduras/1038/2007		Centers for Disease Control and Prevention
EPI172433	NA	Costa Rica	2008-Jun-19	A/Costa Rica/7172/2008		Centers for Disease Control and Prevention
EPI173818	NA	Nicaragua	2007-Aug-03	A/Managua/4902.01/2007		Other Database Import
EPI176127	NA	Nicaragua	2007-Jun-24	A/Managua/1507.01/2007		Other Database Import
EPI176961	NA	Australia	2007-Jan-01	A/Brisbane/10/2007		Other Database Import
EPI178232	NA	Nicaragua	2007-Jun-05	A/Managua/4348.01/2007		Other Database Import
EPI185947	NA	Trinidad and Tobago	2009-Jan-01	A/Trinidad/2940/2009		Centers for Disease

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						Control and Prevention
EPI277439	NA	Nicaragua	2010-Jun-08	A/Managua/5806.01/2010		Other Database Import
EPI463840	NA	Mexico	2008-Nov-01	A/Mexico/24003/2008		Other Database Import
EPI463895	NA	Mexico	2009-Apr-01	A/Mexico/24008/2009		Other Database Import
EPI463940	NA	Mexico	2008-Dec-02	A/Mexico/24012/2008		Other Database Import
EPI463956	NA	Mexico	2008-Dec-01	A/Mexico/24014/2008		Other Database Import
EPI464030	NA	Mexico	2009-Apr-27	A/Mexico/24025/2009		Other Database Import
EPI285244	NA	Nicaragua	2010-Jun-05	A/Managua/3192.01/2010		Other Database Import
EPI285260	NA	Nicaragua	2010-Jun-08	A/Managua/5871.02/2010		Other Database Import
EPI285276	NA	Nicaragua	2010-Jun-08	A/Managua/194.01/2010		Other Database Import
EPI285468	NA	Nicaragua	2010-Jun-06	A/Managua/4456.02/2010		Other Database Import
EPI295198	NA	Belize	2010-Jan-01	A/Belize/3715/2010		Centers for Disease Control and Prevention
EPI315766	NA	Nicaragua	2010-Jun-06	A/Managua/5898.02/2010		Other Database Import
EPI315774	NA	Nicaragua	2010-Jun-07	A/Managua/3209.01/2010		Other Database Import
EPI320630	NA	Nicaragua	2010-Jul-02	A/Managua/2492.04/2010		Other Database Import
EPI320638	NA	Nicaragua	2010-Jun-30	A/Managua/3424.01/2010		Other Database Import
EPI320646	NA	Nicaragua	2011-Jan-28	A/Managua/38-11/2011		Other Database Import
EPI325803	NA	Mexico	2010-Jan-26	A/Mexico City/WRAIR1752T/2010		Other Database Import
EPI325875	NA	El Salvador	2010-Aug-11	A/San Salvador/WRAIR3537T/2010		Other Database Import
EPI325915	NA	Mexico	2010-Dec-22	A/Mexico City/WRAIR3570T/2010		Other Database Import
EPI325931	NA	Mexico	2010-Nov-22	A/Mexico City/WRAIR3571N/2010		Other Database Import
EPI325947	NA	Mexico	2010-Dec-08	A/Mexico City/WRAIR3578N/2010		Other Database Import
EPI325963	NA	Mexico	2010-Dec-09	A/Mexico City/WRAIR3579N/2010		Other Database Import
EPI325971	NA	Mexico	2010-Dec-09	A/Mexico City/WRAIR3579T/2010		Other Database Import
EPI336098	NA	Mexico	2008-Jan-21	A/Mexico/UASLP-013/2008		Other Database Import
EPI185801	NA	Puerto Rico	2009-Feb-09	A/Puerto Rico/18/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI193907	NA	Puerto Rico	2009-May-26	A/Puerto Rico/25/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI193919	NA	Puerto Rico	2009-Jun-02	A/Puerto Rico/46/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI295252	NA	Puerto Rico	2010-Sep-17	A/Puerto Rico/02/2010	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI346467	NA	Puerto Rico	2011-Aug-25	A/Puerto Rico/03/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI347535	NA	Puerto Rico	2011-Oct-20	A/Puerto Rico/23/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI387762	NA	Puerto Rico	2012-Jun-03	A/Puerto Rico/04/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI394786	NA	Puerto Rico	2012-Jun-14	A/Puerto Rico/23/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
EPI394789	NA	Puerto Rico	2012-Jun-26	A/Puerto Rico/34/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI486427	NA	Puerto Rico	2013-Sep-15	A/Puerto Rico/19/2013	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI537014	NA	United States	2012-Apr-15	A/Texas/50/2012	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention
EPI309437	NA	Guadeloupe	2010-Oct-20	A/Guadeloupe/129/2010	National Influenza Center French Guiana and French Indies	Centers for Disease Control and Prevention
EPI342268	NA	Guadeloupe	2010-Nov-30	A/Guadeloupe/200/2010	National Influenza Center French Guiana and French Indies	Centers for Disease Control and Prevention
EPI342274	NA	Guadeloupe	2010-Nov-30	A/Guadeloupe/201/2010	National Influenza Center French Guiana and French Indies	Centers for Disease Control and Prevention
EPI378089	NA	Martinique	2012-May-07	A/Martinique/201/2012	National Influenza Center French Guiana and French Indies	Centers for Disease Control and Prevention
EPI279978	NA	Barbados	2010-Aug-11	A/Barbados/170/2010	Public Health Laboratory	Centers for Disease Control and Prevention
EPI185783	NA	Jamaica	2009-Apr-28	A/Jamaica/2970/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI193943	NA	Anguilla	2009-Jun-10	A/Anguilla/4711/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI319647	NA	Anguilla	2011-Jan-24	A/Anguilla/273/2011	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI391244	NA	Dominica	2012-Mar-08	A/Dominica/616/2012	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI391258	NA	Saint Lucia	2012-May-24	A/St. Lucia/2073/2012	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI394899	NA	Bermuda	2012-Jun-13	A/Bermuda/1915/2012	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459801	NA	Anguilla	2013-Jan-10	A/Anguilla/104/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459804	NA	Dominica	2013-Jan-21	A/Dominica/653/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459819	NA	Trinidad and Tobago	2013-Feb-28	A/Trinidad/847/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459822	NA	Trinidad and Tobago	2013-Mar-19	A/Trinidad/1117/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459831	NA	Barbados	2013-Jan-12	A/Barbados/1031/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459834	NA	Barbados	2013-Feb-13	A/Barbados/4326/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459843	NA	Bermuda	2013-Jan-28	A/Bermuda/521/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI498066	NA	Dominica	2013-Oct-07	A/Dominica/3307/2013	Caribbean Epidemiology Center	Centers for Disease

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						Control and Prevention Centers for Disease
EPI498070	NA	Trinidad and Tobago	2013-Oct-27	A/Trinidad/3558/2013	Caribbean Epidemiology Center	Control and Prevention Centers for Disease
EPI278759	NA	Panama	2010-Jun-08	A/Panama/307149/2010	Instituto Conmemorativo Gorgas de Estudios de la Salud	Control and Prevention Centers for Disease
EPI467431	NA	Panama	2013-Jun-17	A/Panama/313131/2013	Instituto Conmemorativo Gorgas de Estudios de la Salud	Control and Prevention Centers for Disease
EPI274473	NA	Nicaragua	2010-Jun-02	A/Nicaragua/2145/2010	Laboratorio de Virologia, Direccion de Microbiologia	Control and Prevention Centers for Disease
EPI466924	NA	Nicaragua	2013-Jun-20	A/Managua/1083/2013	Laboratorio de Virologia, Direccion de Microbiologia	Control and Prevention Centers for Disease
EPI465946	NA	Nicaragua	2013-May-07	A/Esteli/816/2013	Laboratorio de Virologia, Direccion de Microbiologia	Control and Prevention Centers for Disease
EPI492693	NA	Jamaica	2013-Oct-21	A/Jamaica/200/2013	University of the West Indies	Control and Prevention Centers for Disease
EPI185777	NA	Honduras	2009-May-05	A/Honduras/56/2009	Laboratorio Nacional de Virologia	Control and Prevention Centers for Disease
EPI185780	NA	Honduras	2009-May-06	A/Honduras/639/2009	Laboratorio Nacional de Virologia	Control and Prevention Centers for Disease
EPI193898	NA	Honduras	2009-May-14	A/Honduras/105/2009	Laboratorio Nacional de Virologia	Control and Prevention Centers for Disease
EPI193975	NA	Honduras	2009-Jun-30	A/Honduras/2243/2009	Laboratorio Nacional de Virologia	Control and Prevention Centers for Disease
EPI211350	NA	Honduras	2009-Jul-13	A/Honduras/2426/2009	Laboratorio Nacional de Virologia	Control and Prevention Centers for Disease
EPI274452	NA	Honduras	2010-Apr-13	A/Honduras/6065/2010	Laboratorio Nacional de Virologia	Control and Prevention Centers for Disease
EPI295258	NA	Honduras	2010-Aug-17	A/Honduras/6705/2010	Laboratorio Nacional de Virologia	Control and Prevention Centers for Disease
EPI232462	NA	Haiti	2009-Jul-01	A/Haiti/66/2009	Laboratoire National de Sante Publique	Control and Prevention Centers for Disease
EPI193925	NA	Guatemala	2009-Jun-03	A/Guatemala/1066/2009	Laboratorio Nacional De Salud Guatemala	Control and Prevention Centers for Disease
EPI193981	NA	Guatemala	2009-Jul-09	A/Guatemala/29/2009	Laboratorio Nacional De Salud Guatemala	Control and Prevention Centers for Disease
EPI211338	NA	Guatemala	2009-Jun-15	A/Guatemala/1913/2009	Laboratorio Nacional De Salud Guatemala	Control and Prevention Centers for Disease
EPI232440	NA	Guatemala	2009-Jun-14	A/Guatemala/1839/2009	Laboratorio Nacional De Salud Guatemala	Control and Prevention Centers for Disease
EPI278774	NA	Guatemala	2010-Jul-13	A/Guatemala/591/2010	Laboratorio Nacional De Salud Guatemala	Control and Prevention Centers for Disease
EPI295094	NA	Guatemala	2010-Sep-02	A/Guatemala/690/2010	Laboratorio Nacional De Salud Guatemala	Control and Prevention Centers for Disease
EPI301070	NA	Guatemala	2010-Oct-05	A/Guatemala/754/2010	Laboratorio Nacional De Salud	Control and Prevention Centers for Disease

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					Guatemala	Control and Prevention
					Laboratorio Nacional De Salud	Centers for Disease
EPI326290	NA	Guatemala	2011-Mar-02	A/Guatemala/51/2011	Guatemala	Control and Prevention
					Laboratorio Nacional De Salud	Centers for Disease
EPI468140	NA	Guatemala	2013-Jul-09	A/Guatemala/287/2013	Guatemala	Control and Prevention
					WHO Collaborating Centre for	
					Reference and Research on	Centers for Disease
EPI211335	NA	Australia	2009-Jan-01	A/Perth/16/2009	Influenza	Control and Prevention
					WHO Collaborating Centre for	
					Reference and Research on	Centers for Disease
EPI513285	NA	Australia	2011-Oct-24	A/Victoria/361/2011	Influenza	Control and Prevention
					Laboratorio de Investigacion /	
					Centro de Educacion Medica y	
					Amistad Dominico Japones	Centers for Disease
EPI211267	NA	Dominican Republic	2009-May-25	A/Dominican Republic/988/2009	(CEMADOJA)	Control and Prevention
					Laboratorio de Investigacion /	
					Centro de Educacion Medica y	
					Amistad Dominico Japones	Centers for Disease
EPI254585	NA	Dominican Republic	2009-Nov-12	A/Dominican Republic/3668/2009	(CEMADOJA)	Control and Prevention
					Laboratorio de Investigacion /	
					Centro de Educacion Medica y	
					Amistad Dominico Japones	Centers for Disease
EPI278765	NA	Dominican Republic	2010-Jul-20	A/Dominican Republic/4319/2010	(CEMADOJA)	Control and Prevention
					Laboratorio de Investigacion /	
					Centro de Educacion Medica y	
					Amistad Dominico Japones	Centers for Disease
EPI376514	NA	Dominican Republic	2012-Apr-13	A/Dominican Republic/6334/2012	(CEMADOJA)	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI211318	NA	Costa Rica	2009-Jun-05	A/Costa Rica/5179/2009	Influenza	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI232480	NA	Costa Rica	2009-Jul-24	A/Costa Rica/5023/2009	Influenza	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI291555	NA	Costa Rica	2010-Jul-16	A/Costa Rica/6696/2010	Influenza	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI349760	NA	Costa Rica	2011-Nov-11	A/Costa Rica/8211/2011	Influenza	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI397143	NA	Costa Rica	2012-Jul-17	A/Costa Rica/9093/2012	Influenza	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI484548	NA	Costa Rica	2013-Jul-12	A/Costa Rica/2932/2013	Influenza	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI484732	NA	Costa Rica	2013-Jul-23	A/Costa Rica/4009/2013	Influenza	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI485799	NA	Costa Rica	2013-Jul-26	A/Costa Rica/4700/2013	Influenza	Control and Prevention
EPI278809	NA	El Salvador	2010-Jun-28	A/El Salvador/635/2010	Contiguo a Hospital Rosales	Centers for Disease

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						Control and Prevention
EPI295135	NA	El Salvador	2010-Aug-24	A/El Salvador/1318/2010	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI347485	NA	El Salvador	2011-Sep-21	A/El Salvador/1513/2011	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI459840	NA	El Salvador	2013-Mar-18	A/El Salvador/433/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI477436	NA	El Salvador	2013-Jul-04	A/El Salvador/1307/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI185798	NA	Mexico	2009-Mar-11	A/Mexico/2779/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI243562	NA	Mexico	2009-Sep-15	A/Mexico/5270/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI243568	NA	Mexico	2009-Sep-05	A/Mexico/7880/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI270306	NA	Mexico	2009-Aug-17	A/Mexico/5824/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI279987	NA	Mexico	2010-Aug-13	A/Mexico/4056/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI309386	NA	Mexico	2010-Oct-11	A/Mexico/6605/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI334632	NA	Mexico	2010-Nov-04	A/Mexico/6998/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI319722	NA	Mexico	2011-Feb-18	A/Mexico/1664/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI331239	NA	Mexico	2011-Apr-08	A/Mexico/2554/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
H1						
EPI230497	HA	Mexico	2009-Apr-01	A/swine/4/Mexico/2009		Other Database Import
EPI277889	HA	Mexico	2009-Sep-23	A/Mexico City/WR1675T/2009		Other Database Import
EPI277881	HA	Mexico	2009-Oct-05	A/Mexico City/WR1673N/2009		Other Database Import
EPI277873	HA	Mexico	2009-Sep-23	A/Mexico City/WR1668T/2009		Other Database Import
EPI277665	HA	Mexico	2009-Oct-22	A/Mexico City/WR1090N/2009		Other Database Import
EPI277657	HA	Dominican Republic	2009-Jun-10	A/Santo Domingo/WR1072T/2009		Other Database Import
EPI275081	HA	Nicaragua	2009-Jul-21	A/Managua/5364.01/2009		Other Database Import
EPI267000	HA	Mexico	2009-May-02	A/Mexico City/026/2009		Other Database Import
EPI266992	HA	Mexico	2009-May-01	A/Mexico City/025/2009		Other Database Import
EPI266952	HA	Mexico	2009-Apr-30	A/Mexico City/020/2009		Other Database Import
EPI256738	HA	Mexico	2009-May-16	A/Mexico city/CIA9/2009		Other Database Import
EPI249582	HA	Nicaragua	2009-Aug-18	A/Managua/2323.02/2009		Other Database Import
EPI249092	HA	Nicaragua	2009-Aug-15	A/Managua/3275.01/2009		Other Database Import
EPI249068	HA	Nicaragua	2009-Jun-16	A/Managua/2330.02/2009		Other Database Import

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EPI249036	HA	Nicaragua	2009-Aug-18	A/Managua/164.01/2009		Other Database Import
EPI237350	HA	Nicaragua	2009-Jun-01	A/Managua/4467.05/2009		Other Database Import
EPI217610	HA	Mexico	2009-May-10	A/Mexico City/018/2009		Other Database Import
EPI217594	HA	Mexico	2009-May-09	A/Mexico City/015/2009		Other Database Import
EPI217578	HA	Mexico	2009-May-09	A/Mexico City/012/2009		Other Database Import
EPI217570	HA	Mexico	2009-May-09	A/Mexico City/011/2009		Other Database Import
EPI217546	HA	Mexico	2009-May-10	A/Mexico City/008/2009		Other Database Import
EPI217490	HA	Mexico	2009-May-07	A/Mexico City/001/2009		Other Database Import
EPI215462	HA	Dominican Republic	2009-Jun-25	A/Santo Domingo/WR1068N/2009		Other Database Import
EPI215454	HA	Dominican Republic	2009-Jun-30	A/Santo Domingo/WR1059N/2009		Other Database Import
EPI215446	HA	Dominican Republic	2009-Jul-02	A/Santo Domingo/WR1058N/2009		Other Database Import
EPI215438	HA	Dominican Republic	2009-Jul-02	A/Santo Domingo/WR1057N/2009		Other Database Import
EPI215398	HA	El Salvador	2009-Jun-09	A/San Salvador/WR0167N/2009		Other Database Import
EPI215390	HA	El Salvador	2009-Jun-12	A/San Salvador/0169T/2009		Other Database Import
EPI215334	HA	Mexico	2009-Sep-10	A/Mexico City/WR1311T/2009		Other Database Import
EPI215326	HA	Mexico	2009-Sep-09	A/Mexico City/WR1310N/2009		Other Database Import
EPI215310	HA	Mexico	2009-Sep-14	A/Mexico City/WR1307N/2009		Other Database Import
EPI215302	HA	Mexico	2009-Sep-02	A/Mexico City/WR1306N/2009		Other Database Import
EPI215294	HA	Mexico	2009-Sep-12	A/Mexico City/WR1301N/2009		Other Database Import
EPI215286	HA	Mexico	2009-Sep-03	A/Mexico City/WR1297N/2009		Other Database Import
EPI215278	HA	Mexico	2009-Jun-29	A/Mexico City/WR1100N/2009		Other Database Import
EPI215270	HA	Mexico	2009-Jun-19	A/Mexico City/WR1087T/2009		Other Database Import
EPI190863	HA	Mexico	2009-Apr-25	A/Mexico/48N/2009		Other Database Import
EPI190855	HA	Nicaragua	2009-Jun-26	A/Managua/0536N/2009		Other Database Import
EPI186657	HA	Dominican Republic	2009-May-26	A/Santo Domingo/565T/2009		Other Database Import
EPI186244	HA	Dominican Republic	2009-May-24	A/Santo Domingo/572N/2009		Other Database Import
EPI180755	HA	Mexico	2009-Apr-25	A/Mexico/47N/2009		Other Database Import
EPI179001	HA	Mexico	2009-Jan-01	A/Mexico/InDRE4114/2009		Other Database Import
EPI273882	HA	Mexico	2009-Dec-18	A/Mexico/5569/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI273879	HA	Mexico	2010-Mar-01	A/Mexico/1762/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI273925	HA	Mexico	2009-Oct-18	A/Mexico/4178/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI243933	HA	Mexico	2009-Oct-26	A/Mexico/1138/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI243905	HA	Mexico	2009-Nov-18	A/Mexico/476/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI232961	HA	Mexico	2009-Mar-12	A/Mexico/2964/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI231329	HA	Mexico	2009-Jul-10	A/Mexico/2466/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention

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EPI231326	HA	Mexico	2009-Jun-30	A/Mexico/2445/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI179102	HA	Mexico	2009-Apr-20	A/Mexico/4593/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI179080	HA	Mexico	2009-Apr-03	A/Mexico/3955/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI177338	HA	Mexico	2009-Apr-03	A/Mexico/4108/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI176587	HA	Mexico	2009-Apr-14	A/Mexico/4482/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI183131	HA	El Salvador	2009-May-13	A/El Salvador/351/2009	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI179074	HA	El Salvador	2009-Apr-26	A/El Salvador/213/2009	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI227650	HA	Costa Rica	2009-Jul-13	A/Costa Rica/3149/2009	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI216778	HA	Costa Rica	2009-May-16	A/Costa Rica/6321/2009	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI216767	HA	Costa Rica	2009-Jul-17	A/Costa Rica/3896/2009	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI181744	HA	Costa Rica	2009-Apr-28	A/Costa Rica/4314/2009	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI247263	HA	Dominican Republic	2009-Dec-15	A/Dominican Republic/3768/2009	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI183311	HA	Netherlands	2009-Jan-01	A/Netherlands/602/2009	Erasmus University of Rotterdam	Centers for Disease Control and Prevention
EPI231317	HA	Guatemala	2009-Oct-26	A/Guatemala/4752/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI231315	HA	Guatemala	2009-Sep-15	A/Guatemala/4360/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI216851	HA	Guatemala	2009-Aug-03	A/Guatemala/3798/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI216848	HA	Guatemala	2009-Jul-14	A/Guatemala/3185/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI194155	HA	Guatemala	2009-Jun-02	A/Guatemala/1039/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI239674	HA	Haiti	2009-Oct-19	A/Haiti/534/2009	Laboratoire National de Sante Publique	Centers for Disease Control and Prevention
EPI216857	HA	Haiti	2009-Aug-12	A/Haiti/265/2009	Laboratoire National de Sante Publique	Centers for Disease Control and Prevention
EPI273771	HA	Honduras	2010-May-19	A/Honduras/6132/2010	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention

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EPI216863	HA	Honduras	2009-Jun-25	A/Honduras/2188/2009	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI191948	HA	Honduras	2009-Jun-17	A/Honduras/2039/2009	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI273920	HA	Jamaica	2009-Jul-07	A/Jamaica/6261/2009	University of the West Indies	Centers for Disease Control and Prevention
EPI273844	HA	Nicaragua	2010-Apr-01	A/Nicaragua/322/2010	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI227690	HA	Nicaragua	2009-Aug-25	A/Managua/6502/2009	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI227684	HA	Nicaragua	2009-Jul-27	A/Managua/46601/2009	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI211485	HA	Panama	2009-Jun-18	A/Panama/4252/2009	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI183241	HA	Panama	2009-May-11	A/Panama/302869/2009	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI231353	HA	Turks and Caicos Islands	2009-Oct-13	A/Turks And Caicos/9060/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231350	HA	Trinidad and Tobago	2009-Oct-22	A/Trinidad/9184/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231341	HA	Saint Lucia	2009-Sep-25	A/St. Lucia/9333/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231312	HA	Grenada	2009-Oct-23	A/Grenada/9302/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231305	HA	Dominican Republic	2009-Oct-23	A/Dominica/9250/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231291	HA	Belize	2009-Oct-08	A/Belize/8756/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI217091	HA	Saint Lucia	2009-Jul-31	A/Saint Lucia/7178/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI217088	HA	Saint Kitts and Nevis, Federation of	2009-Jul-29	A/Saint Kitts/7274/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI216730	HA	Belize	2009-Jul-28	A/Belize/7121/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI211402	HA	Barbados	2009-Sep-07	A/Barbados/7992/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI220980	HA	British Virgin Islands	2009-Jun-15	A/British Virgin Islands/5437/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI186269	HA	Bermuda	2009-Jun-03	A/Bermuda/4287/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI186266	HA	Jamaica	2009-Jun-06	A/Jamaica/4423/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI216727	HA	Barbados	2009-Sep-10	A/Barbados/280/2009	Public Health Laboratory	Centers for Disease Control and Prevention

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EPI216724	HA	Barbados	2009-Sep-04	A/Barbados/275/2009	Public Health Laboratory	Centers for Disease Control and Prevention
EPI189129	HA	Martinique	2009-Jun-20	A/Martinique/15/2009	National Influenza Center French Guiana and French Indies	Centers for Disease Control and Prevention
EPI273751	HA	Puerto Rico	2009-Dec-18	A/Puerto Rico/20/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI240403	HA	Puerto Rico	2009-Dec-06	A/Puerto Rico/51/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI217082	HA	Puerto Rico	2009-Aug-07	A/Puerto Rico/50/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI273937	HA	Guatemala	2010-Jan-01	A/Guatemala/127/2010		Centers for Disease Control and Prevention
EPI186300	HA	Trinidad and Tobago	2009-Jan-01	A/Trinidad/4601/2009		Centers for Disease Control and Prevention
EPI185355	HA	Costa Rica	2009-May-02	A/Costa Rica/4857/2009		Centers for Disease Control and Prevention
EPI338314	HA	Cuba	2010-Nov-01	A/swine/La Habana/130/2010		Other Database Import
EPI338288	HA	Cuba	2010-Nov-01	A/swine/Pinar del Rio/3/2010		Other Database Import
EPI335977	HA	Cuba	2010-Nov-01	A/swine/Villa Clara/84/2010		Other Database Import
EPI307826	HA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-20/2010		Other Database Import
EPI307823	HA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-19/2010		Other Database Import
EPI307820	HA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-16/2010		Other Database Import
EPI307817	HA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-15/2010		Other Database Import
EPI307814	HA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-14/2010		Other Database Import
EPI307811	HA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-3/2010		Other Database Import
EPI341574	HA	United States	2009-Jan-01	A/California/04/2009		Other Database Import
EPI278009	HA	Haiti	2009-Nov-13	A/Port Au Prince/WR2294T/2009		Other Database Import
EPI277993	HA	Mexico	2009-Nov-19	A/Mexico City/WR1765N/2009		Other Database Import
EPI277977	HA	Mexico	2009-Nov-09	A/Mexico City/WR1747N/2009		Other Database Import
EPI277969	HA	Mexico	2009-Oct-09	A/Mexico City/WR1708T/2009		Other Database Import
EPI277961	HA	Mexico	2009-Sep-18	A/Mexico City/WR1706T/2009		Other Database Import
EPI277937	HA	Mexico	2009-Sep-15	A/Mexico City/WR1697T/2009		Other Database Import
EPI277929	HA	Mexico	2009-Sep-17	A/Mexico City/WR1696T/2009		Other Database Import
EPI277921	HA	Mexico	2009-Oct-01	A/Mexico City/WR1695N/2009		Other Database Import
EPI277905	HA	Mexico	2009-Sep-23	A/Mexico City/WR1687T/2009		Other Database Import
EPI509496	HA	Mexico	2013-Mar-01	A/Mexico/01/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI508443	HA	Mexico	2013-Aug-31	A/Mexico/2733/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI504791	HA	Mexico	2013-Dec-27	A/Mexico/06/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI503827	HA	Mexico	2013-Oct-21	A/Mexico/3280/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention

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EPI467233	HA	Mexico	2013-Jul-13	A/Mexico/2410/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI391298	HA	Mexico	2012-Feb-13	A/Mexico/5698/2012	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI355516	HA	Mexico	2011-Dec-12	A/Mexico/3752/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI353408	HA	Mexico	2011-Dec-28	A/Mexico/52/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI353405	HA	Mexico	2011-Dec-14	A/Mexico/3720/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI353402	HA	Mexico	2012-Jan-08	A/Mexico/210/2012	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI331210	HA	Mexico	2011-Mar-15	A/Mexico/2208/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI330992	HA	Mexico	2011-Mar-22	A/Mexico/1946/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI320045	HA	Mexico	2011-Feb-22	A/Mexico/1658/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI278878	HA	Mexico	2010-Apr-07	A/Mexico/2880/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI534020	HA	El Salvador	2013-Nov-04	A/El Salvador/2260/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI508446	HA	El Salvador	2013-Nov-22	A/El Salvador/2360/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI484974	HA	El Salvador	2013-Jul-05	A/El Salvador/1396/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI380014	HA	El Salvador	2012-Apr-17	A/El Salvador/530/2012	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI295399	HA	El Salvador	2010-Jul-13	A/El Salvador/798/2010	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI278888	HA	El Salvador	2010-May-26	A/El Salvador/455/2010	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI482166	HA	Costa Rica	2013-May-30	A/Costa Rica/5790/2013	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI482164	HA	Costa Rica	2013-Jun-18	A/Costa Rica/8288/2013	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI397472	HA	Costa Rica	2012-Jun-24	A/Costa Rica/6288/2012	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI376365	HA	Costa Rica	2012-Jan-17	A/Costa Rica/4023/2012	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI350003	HA	Costa Rica	2011-Oct-11	A/Costa Rica/8107/2011	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI348171	HA	Costa Rica	2011-Oct-30	A/Costa Rica/6796/2011	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention

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EPI295456	HA	Costa Rica	2010-Jul-16	A/Costa Rica/6692/2010	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI295453	HA	Costa Rica	2010-May-07	A/Costa Rica/5529/2010	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI295450	HA	Costa Rica	2010-May-06	A/Costa Rica/5458/2010	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI477445	HA	Dominican Republic	2013-Jun-15	A/Dominican Republic/7626/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI484726	HA	Dominican Republic	2013-Jun-05	A/Dominican Republic/7548/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI467203	HA	Dominican Republic	2013-Jun-12	A/Dominican Republic/7603/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI467200	HA	Dominican Republic	2013-Jun-10	A/Dominican Republic/7703/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI459759	HA	Dominican Republic	2013-Apr-01	A/Dominican Republic/7238/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI457503	HA	Dominican Republic	2013-May-01	A/Dominican Republic/7293/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI457456	HA	Dominican Republic	2013-May-01	A/Dominican Republic/7291/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI396139	HA	Dominican Republic	2012-May-28	A/Dominican Republic/6493/2012	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI395238	HA	Dominican Republic	2012-Jun-08	A/Dominican Republic/6551/2012	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI376381	HA	Dominican Republic	2012-Feb-21	A/Dominican Republic/6188/2012	Laboratorio de Investigacion / Centro de Educacion Medica y	Centers for Disease Control and Prevention

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EPI335804	HA	Dominican Republic	2011-Jul-18	A/Dominican Republic/5542/2011	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI320014	HA	Dominican Republic	2011-Apr-11	A/Dominican Republic/5145/2011	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI320011	HA	Dominican Republic	2011-Mar-28	A/Dominican Republic/5059/2011	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI362991	HA	Guatemala	2012-Feb-12	A/Guatemala/18/2012	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI295471	HA	Guatemala	2010-Sep-17	A/Guatemala/175/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI295438	HA	Guatemala	2010-Aug-27	A/Guatemala/287/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI280305	HA	Guatemala	2010-Jun-03	A/Guatemala/483/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI278869	HA	Guatemala	2010-Jul-19	A/Guatemala/598/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI278866	HA	Guatemala	2010-Jun-12	A/Guatemala/04/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI278863	HA	Guatemala	2010-Apr-28	A/Guatemala/358/2010	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI394852	HA	Honduras	2012-Jun-26	A/Honduras/9746/2012	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI394737	HA	Honduras	2012-Jun-14	A/Honduras/9720/2012	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI497702	HA	Jamaica	2013-Oct-30	A/Jamaica/198/2013	University of the West Indies	Centers for Disease Control and Prevention
EPI391296	HA	Jamaica	2012-Feb-13	A/Jamaica/764/2012	University of the West Indies	Centers for Disease Control and Prevention
EPI465462	HA	Nicaragua	2013-Jun-14	A/Managua/30074.01/2013	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI465282	HA	Nicaragua	2013-Apr-14	A/Managua/601/2013	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI465279	HA	Nicaragua	2013-Apr-20	A/Managua/687/2013	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI346507	HA	Nicaragua	2011-Oct-10	A/Managua/748/2011	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention

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EPI468142	HA	Panama	2013-Jun-13	A/Panama/313106/2013	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI467381	HA	Panama	2013-Jun-25	A/Panama/313202/2013	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI394749	HA	Panama	2012-Jun-05	A/Panama/310521/2012	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI335822	HA	Panama	2011-Jun-10	A/Panama/309335/2011	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI278854	HA	Panama	2010-Jun-14	A/Panama/307207/2010	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI278852	HA	Panama	2010-Jun-07	A/Panama/307168/2010	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI497726	HA	British Virgin Islands	2013-Oct-01	A/British Virgin Islands/3138/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI497720	HA	Barbados	2013-Sep-19	A/Barbados/3024/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI497714	HA	Saint Vincent and the Grenadines	2013-Oct-08	A/St. Vincent and Grenadines/3292/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI497711	HA	Belize	2013-Sep-23	A/Belize/3161/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI497649	HA	Trinidad and Tobago	2013-Oct-24	A/Trinidad/3568/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI465708	HA	Haiti	2013-Jun-02	A/Haiti/1792/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI465472	HA	Haiti	2013-Jun-20	A/Haiti/2036/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI465403	HA	Haiti	2013-Jun-18	A/Haiti/2030/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI465088	HA	Haiti	2013-Jun-02	A/Haiti/1790/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI460142	HA	Trinidad and Tobago	2013-Mar-01	A/Trinidad/979/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459774	HA	Trinidad and Tobago	2013-Mar-13	A/Trinidad/982/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI394847	HA	Belize	2012-Jan-01	A/Belize/334/2012	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI335837	HA	Trinidad and Tobago	2011-Jul-08	A/Trinidad/1648/2011	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI320155	HA	Turks and Caicos Islands	2011-Jan-25	A/Turks and Caicos/211/2011	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI320005	HA	Cayman Islands	2011-Jan-14	A/Cayman Islands/284/2011	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI319999	HA	Barbados	2011-Jan-12	A/Barbados/104/2011	Caribbean Epidemiology Center	Centers for Disease Control and Prevention

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EPI295492	HA	Belize	2010-Aug-11	A/Belize/3342/2010	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI516535	HA	United States	2009-Apr-09	A/California/07/2009	Naval Health Research Center	Centers for Disease Control and Prevention
EPI509486	HA	Puerto Rico	2013-Dec-16	A/Puerto Rico/21/2013	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI486392	HA	Puerto Rico	2013-Oct-01	A/Puerto Rico/18/2013	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI465269	HA	Puerto Rico	2013-Jun-14	A/Puerto Rico/05/2013	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI465070	HA	Puerto Rico	2013-Jun-14	A/Puerto Rico/01/2013	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI397629	HA	Puerto Rico	2012-Jul-03	A/Puerto Rico/43/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI396142	HA	Puerto Rico	2012-Jun-29	A/Puerto Rico/39/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI391293	HA	Puerto Rico	2012-Jun-07	A/Puerto Rico/06/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI391282	HA	Puerto Rico	2012-May-18	A/Puerto Rico/05/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI368690	HA	Puerto Rico	2012-Feb-14	A/Puerto Rico/01/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI366314	HA	Puerto Rico	2011-Aug-18	A/Puerto Rico/25/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI366308	HA	Puerto Rico	2011-May-31	A/Puerto Rico/05/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI349362	HA	Puerto Rico	2011-Nov-21	A/Puerto Rico/8233/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI347556	HA	Puerto Rico	2011-Oct-22	A/Puerto Rico/21/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI346510	HA	Puerto Rico	2011-Aug-03	A/Puerto Rico/04/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI341961	HA	Puerto Rico	2011-Jun-24	A/Puerto Rico/02/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI316427	HA	Puerto Rico	2011-Feb-04	A/Puerto Rico/01/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
				N1		
EPI338308	NA	Cuba	2010-Nov-01	A/swine/Holguin/121/2010		Other Database Import
EPI338290	NA	Cuba	2010-Nov-01	A/swine/Pinar del Rio/3/2010		Other Database Import
EPI335975	NA	Cuba	2010-Nov-01	A/swine/Villa Clara/84/2010		Other Database Import
EPI307824	NA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-19/2010		Other Database Import
EPI307818	NA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-15/2010		Other Database Import

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EPI307815	NA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-14/2010		Other Database Import
EPI307812	NA	Costa Rica	2010-Nov-01	A/swine/Costa Rica/000125-3/2010		Other Database Import
EPI230499	NA	Mexico	2009-Apr-01	A/swine/4/Mexico/2009		Other Database Import
EPI492854	NA	Jamaica	2013-Oct-30	A/Jamaica/198/2013	University of the West Indies	Centers for Disease Control and Prevention
EPI486391	NA	Puerto Rico	2013-Oct-01	A/Puerto Rico/18/2013	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI485767	NA	Costa Rica	2013-Jun-10	A/Costa Rica/7069/2013	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI484973	NA	El Salvador	2013-Jul-05	A/El Salvador/1396/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI484981	NA	Costa Rica	2013-May-30	A/Costa Rica/5790/2013	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI484963	NA	Costa Rica	2013-Jun-18	A/Costa Rica/8288/2013	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI477444	NA	Dominican Republic	2013-Jun-15	A/Dominican Republic/7626/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI477421	NA	Dominican Republic	2013-Jun-05	A/Dominican Republic/7548/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI467410	NA	Panama	2013-Jun-13	A/Panama/313106/2013	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI467380	NA	Panama	2013-Jun-25	A/Panama/313202/2013	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI467232	NA	Mexico	2013-Jul-13	A/Mexico/2410/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI467199	NA	Dominican Republic	2013-Jun-10	A/Dominican Republic/7703/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI465474	NA	Haiti	2013-Jun-02	A/Haiti/1792/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI465471	NA	Haiti	2013-Jun-20	A/Haiti/2036/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI465463	NA	Nicaragua	2013-Jun-19	A/Matagalpa/1076/2013	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI465461	NA	Nicaragua	2013-Jun-14	A/Managua/30074.01/2013	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI465402	NA	Haiti	2013-Jun-18	A/Haiti/2030/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI465278	NA	Nicaragua	2013-Apr-20	A/Managua/687/2013	Laboratorio de Virologia,	Centers for Disease

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					Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI465087	NA	Haiti	2013-Jun-02	A/Haiti/1790/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI465069	NA	Puerto Rico	2013-Jun-14	A/Puerto Rico/01/2013	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI460141	NA	Trinidad and Tobago	2013-Mar-01	A/Trinidad/979/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI459773	NA	Trinidad and Tobago	2013-Mar-13	A/Trinidad/982/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI457455	NA	Dominican Republic	2013-May-01	A/Dominican Republic/7291/2013	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI398006	NA	Puerto Rico	2012-Jul-03	A/Puerto Rico/43/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI397471	NA	Costa Rica	2012-Jun-24	A/Costa Rica/6288/2012	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI396138	NA	Dominican Republic	2012-May-28	A/Dominican Republic/6493/2012	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI395237	NA	Dominican Republic	2012-Jun-08	A/Dominican Republic/6551/2012	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI394846	NA	Belize	2012-Jan-01	A/Belize/334/2012	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI394751	NA	Panama	2012-Jun-26	A/Panama/310630/2012	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI394748	NA	Panama	2012-Jun-05	A/Panama/310521/2012	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI394739	NA	Honduras	2012-Jun-26	A/Honduras/9746/2012	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI394851	NA	Honduras	2012-Jun-14	A/Honduras/9720/2012	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI392906	NA	Puerto Rico	2012-Jun-12	A/Puerto Rico/20/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI391295	NA	Jamaica	2012-Feb-13	A/Jamaica/764/2012	University of the West Indies	Centers for Disease Control and Prevention
EPI391292	NA	Puerto Rico	2012-Jun-07	A/Puerto Rico/06/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI378077	NA	El Salvador	2012-Apr-17	A/El Salvador/530/2012	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI376380	NA	Dominican Republic	2012-Feb-21	A/Dominican Republic/6188/2012	Laboratorio de Investigacion /	Centers for Disease

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					Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI376364	NA	Costa Rica	2012-Jan-17	A/Costa Rica/4023/2012	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI368448	NA	Puerto Rico	2011-Dec-29	A/Puerto Rico/33/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI368446	NA	Puerto Rico	2012-Feb-14	A/Puerto Rico/01/2012	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI366313	NA	Puerto Rico	2011-Aug-18	A/Puerto Rico/25/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI366310	NA	Puerto Rico	2011-Jul-19	A/Puerto Rico/09/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI366307	NA	Puerto Rico	2011-May-31	A/Puerto Rico/05/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI366298	NA	Mexico	2012-Jan-19	A/Mexico/689/2012	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI366289	NA	Mexico	2012-Jan-21	A/Mexico/1818/2012	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI366286	NA	Mexico	2012-Jan-26	A/Mexico/1474/2012	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI362990	NA	Guatemala	2012-Feb-12	A/Guatemala/18/2012	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI355515	NA	Mexico	2011-Dec-12	A/Mexico/3752/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI353442	NA	Mexico	2012-Jan-02	A/Mexico/254/2012	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI353436	NA	Mexico	2011-Dec-12	A/Mexico/3723/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI353410	NA	Mexico	2011-Dec-30	A/Mexico/56/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI353404	NA	Mexico	2011-Dec-14	A/Mexico/3720/2011	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI353401	NA	Mexico	2012-Jan-08	A/Mexico/210/2012	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI350002	NA	Costa Rica	2011-Oct-11	A/Costa Rica/8107/2011	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI349361	NA	Puerto Rico	2011-Nov-21	A/Puerto Rico/8233/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI348170	NA	Costa Rica	2011-Oct-30	A/Costa Rica/6796/2011	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI347555	NA	Puerto Rico	2011-Oct-22	A/Puerto Rico/21/2011	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI346509	NA	Puerto Rico	2011-Aug-03	A/Puerto Rico/04/2011	Puerto Rico Department of	Centers for Disease

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					Health	Control and Prevention
					Laboratorio de Virologia,	Centers for Disease
EPI346506	NA	Nicaragua	2011-Oct-10	A/Managua/748/2011	Direccion de Microbiologia	Control and Prevention
EPI341572	NA	United States	2009-Jan-01	A/California/04/2009		Other Database Import
						Centers for Disease
EPI335836	NA	Trinidad and Tobago	2011-Jul-08	A/Trinidad/1648/2011	Caribbean Epidemiology Center	Control and Prevention
					Instituto Conmemorativo Gorgas	Centers for Disease
EPI335821	NA	Panama	2011-Jun-10	A/Panama/309335/2011	de Estudios de la Salud	Control and Prevention
					Laboratorio de Investigacion /	
					Centro de Educacion Medica y	
					Amistad Dominico Japones	Centers for Disease
EPI335803	NA	Dominican Republic	2011-Jul-18	A/Dominican Republic/5542/2011	(CEMADOJA)	Control and Prevention
					Laboratorio de Virus	Centers for Disease
EPI331209	NA	Mexico	2011-Mar-15	A/Mexico/2208/2011	Respiratorio	Control and Prevention
						Centers for Disease
EPI320154	NA	Turks and Caicos Islands	2011-Jan-25	A/Turks and Caicos/211/2011	Caribbean Epidemiology Center	Control and Prevention
					Laboratorio de Virus	Centers for Disease
EPI320047	NA	Mexico	2011-Mar-22	A/Mexico/1946/2011	Respiratorio	Control and Prevention
					Laboratorio de Virus	Centers for Disease
EPI320044	NA	Mexico	2011-Feb-22	A/Mexico/1658/2011	Respiratorio	Control and Prevention
					Laboratorio de Investigacion /	
					Centro de Educacion Medica y	
					Amistad Dominico Japones	Centers for Disease
EPI320010	NA	Dominican Republic	2011-Mar-28	A/Dominican Republic/5059/2011	(CEMADOJA)	Control and Prevention
						Centers for Disease
EPI320004	NA	Cayman Islands	2011-Jan-14	A/Cayman Islands/284/2011	Caribbean Epidemiology Center	Control and Prevention
						Centers for Disease
EPI319998	NA	Barbados	2011-Jan-12	A/Barbados/104/2011	Caribbean Epidemiology Center	Control and Prevention
						Centers for Disease
EPI295491	NA	Belize	2010-Aug-11	A/Belize/3342/2010	Caribbean Epidemiology Center	Control and Prevention
					Laboratorio Nacional de	Centers for Disease
EPI295455	NA	Costa Rica	2010-Jul-16	A/Costa Rica/6692/2010	Influenza	Control and Prevention
					Laboratorio Nacional De Salud	Centers for Disease
EPI295437	NA	Guatemala	2010-Aug-27	A/Guatemala/287/2010	Guatemala	Control and Prevention
						Centers for Disease
EPI295398	NA	El Salvador	2010-Jul-13	A/El Salvador/798/2010	Contiguo a Hospital Rosales	Control and Prevention
						Centers for Disease
EPI278887	NA	El Salvador	2010-May-26	A/El Salvador/455/2010	Contiguo a Hospital Rosales	Control and Prevention
					Laboratorio de Virus	Centers for Disease
EPI278877	NA	Mexico	2010-Apr-07	A/Mexico/2880/2010	Respiratorio	Control and Prevention
					Laboratorio Nacional De Salud	Centers for Disease
EPI278862	NA	Guatemala	2010-Apr-28	A/Guatemala/358/2010	Guatemala	Control and Prevention
					Instituto Conmemorativo Gorgas	Centers for Disease
EPI278851	NA	Panama	2010-Jun-07	A/Panama/307168/2010	de Estudios de la Salud	Control and Prevention

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EPI278011	NA	Haiti	2009-Nov-13	A/Port Au Prince/WR2294T/2009		Other Database Import
EPI277995	NA	Mexico	2009-Nov-19	A/Mexico City/WR1765N/2009		Other Database Import
EPI277979	NA	Mexico	2009-Nov-09	A/Mexico City/WR1747N/2009		Other Database Import
EPI277963	NA	Mexico	2009-Sep-18	A/Mexico City/WR1706T/2009		Other Database Import
EPI277659	NA	Dominican Republic	2009-Jun-10	A/Santo Domingo/WR1072T/2009		Other Database Import
EPI275083	NA	Nicaragua	2009-Jul-21	A/Managua/5364.01/2009		Other Database Import
EPI273936	NA	Guatemala	2010-Jan-01	A/Guatemala/127/2010		Centers for Disease Control and Prevention
EPI273919	NA	Jamaica	2009-Jul-07	A/Jamaica/6261/2009	University of the West Indies	Centers for Disease Control and Prevention
EPI273878	NA	Mexico	2010-Mar-01	A/Mexico/1762/2010	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI273843	NA	Nicaragua	2010-Apr-01	A/Nicaragua/322/2010	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI273770	NA	Honduras	2010-May-19	A/Honduras/6132/2010	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI267002	NA	Mexico	2009-May-02	A/Mexico City/026/2009		Other Database Import
EPI256740	NA	Mexico	2009-May-16	A/Mexico city/CIA9/2009		Other Database Import
EPI256704	NA	Mexico	2009-Jul-06	A/Mexico city/CIA3/2009		Other Database Import
EPI256697	NA	Mexico	2009-Jul-06	A/Mexico city/CIA2/2009		Other Database Import
EPI273924	NA	Mexico	2009-Oct-18	A/Mexico/4178/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI254050	NA	Puerto Rico	2009-Dec-18	A/Puerto Rico/20/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI249640	NA	Nicaragua	2009-Jun-15	A/Managua/4935.03/2009		Other Database Import
EPI249616	NA	Nicaragua	2009-Jun-26	A/Managua/462.01/2009		Other Database Import
EPI249592	NA	Nicaragua	2009-Aug-18	A/Managua/3435.01/2009		Other Database Import
EPI249584	NA	Nicaragua	2009-Aug-18	A/Managua/2323.02/2009		Other Database Import
EPI249118	NA	Nicaragua	2009-Aug-15	A/Managua/473.02/2009		Other Database Import
EPI247262	NA	Dominican Republic	2009-Dec-15	A/Dominican Republic/3768/2009	Laboratorio de Investigacion / Centro de Educacion Medica y Amistad Dominico Japones (CEMADOJA)	Centers for Disease Control and Prevention
EPI243932	NA	Mexico	2009-Oct-26	A/Mexico/1138/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI240402	NA	Puerto Rico	2009-Dec-06	A/Puerto Rico/51/2009	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI239673	NA	Haiti	2009-Oct-19	A/Haiti/534/2009	Laboratoire National de Sante Publique	Centers for Disease Control and Prevention
EPI231352	NA	Turks and Caicos Islands	2009-Oct-13	A/Turks And Caicos/9060/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231349	NA	Trinidad and Tobago	2009-Oct-22	A/Trinidad/9184/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention

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EPI231340	NA	Saint Lucia	2009-Sep-25	A/St. Lucia/9333/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231328	NA	Mexico	2009-Jul-10	A/Mexico/2466/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI347040	NA	Guatemala	2009-Oct-26	A/Guatemala/4752/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI231314	NA	Guatemala	2009-Sep-15	A/Guatemala/4360/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI231311	NA	Grenada	2009-Oct-23	A/Grenada/9302/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231304	NA	Dominican Republic	2009-Oct-23	A/Dominica/9250/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI231290	NA	Belize	2009-Oct-08	A/Belize/8756/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI227689	NA	Nicaragua	2009-Aug-25	A/Managua/6502/2009	Laboratorio de Virologia, Direccion de Microbiologia	Centers for Disease Control and Prevention
EPI227649	NA	Costa Rica	2009-Jul-13	A/Costa Rica/3149/2009	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI227627	NA	Bermuda	2009-Oct-23	A/Bermuda/9261/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI217612	NA	Mexico	2009-May-10	A/Mexico City/018/2009		Other Database Import
EPI217604	NA	Mexico	2009-May-09	A/Mexico City/017/2009		Other Database Import
EPI217596	NA	Mexico	2009-May-09	A/Mexico City/015/2009		Other Database Import
EPI217524	NA	Mexico	2009-May-08	A/Mexico City/005/2009		Other Database Import
EPI217090	NA	Saint Lucia	2009-Jul-31	A/Saint Lucia/7178/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI216862	NA	Honduras	2009-Jun-25	A/Honduras/2188/2009	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI216856	NA	Haiti	2009-Aug-12	A/Haiti/265/2009	Laboratoire National de Sante Publique	Centers for Disease Control and Prevention
EPI216836	NA	El Salvador	2009-Jul-16	A/El Salvador/1644/2009	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI216821	NA	El Salvador	2009-Jul-14	A/El Salvador/1553/2009	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI216774	NA	Costa Rica	2009-Jul-22	A/Costa Rica/4635/2009	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI216729	NA	Belize	2009-Jul-28	A/Belize/7121/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI216726	NA	Barbados	2009-Sep-10	A/Barbados/280/2009	Public Health Laboratory	Centers for Disease Control and Prevention
EPI216723	NA	Barbados	2009-Sep-04	A/Barbados/275/2009	Public Health Laboratory	Centers for Disease Control and Prevention
EPI215400	NA	El Salvador	2009-Jun-09	A/San Salvador/WR0167N/2009		Other Database Import
EPI215328	NA	Mexico	2009-Sep-09	A/Mexico City/WR1310N/2009		Other Database Import

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
EPI194156	NA	Guatemala	2009-Jun-02	A/Guatemala/1039/2009	Laboratorio Nacional De Salud Guatemala	Centers for Disease Control and Prevention
EPI191949	NA	Honduras	2009-Jun-17	A/Honduras/2039/2009	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention
EPI190865	NA	Mexico	2009-Apr-25	A/Mexico/48N/2009		Other Database Import
EPI190857	NA	Nicaragua	2009-Jun-26	A/Managua/0536N/2009		Other Database Import
EPI186659	NA	Dominican Republic	2009-May-26	A/Santo Domingo/565T/2009		Other Database Import
EPI186301	NA	Trinidad and Tobago	2009-Jan-01	A/Trinidad/4601/2009		Centers for Disease Control and Prevention
EPI186277	NA	Mexico	2009-Apr-20	A/Mexico/4635/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI186267	NA	Bermuda	2009-Jun-03	A/Bermuda/4287/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI186265	NA	Jamaica	2009-Jun-06	A/Jamaica/4423/2009	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI183240	NA	Panama	2009-May-11	A/Panama/302869/2009	Instituto Conmemorativo Gorgas de Estudios de la Salud	Centers for Disease Control and Prevention
EPI183130	NA	El Salvador	2009-May-13	A/El Salvador/351/2009	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI181742	NA	Costa Rica	2009-Apr-28	A/Costa Rica/4314/2009	Laboratorio Nacional de Influenza	Centers for Disease Control and Prevention
EPI180757	NA	Mexico	2009-Apr-25	A/Mexico/47N/2009		Other Database Import
EPI179101	NA	Mexico	2009-Apr-20	A/Mexico/4575/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI179076	NA	El Salvador	2009-Apr-26	A/El Salvador/213/2009	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI177291	NA	Netherlands	2009-Jan-01	A/Netherlands/602/2009		Erasmus University of Rotterdam
EPI176588	NA	Mexico	2009-Apr-14	A/Mexico/4482/2009	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI221062	NA	United States	2009-Apr-09	A/California/07/2009	Naval Health Research Center	Centers for Disease Control and Prevention
EPI508440	NA	Puerto Rico	2013-Dec-16	A/Puerto Rico/21/2013	Puerto Rico Department of Health	Centers for Disease Control and Prevention
EPI497648	NA	Trinidad and Tobago	2013-Oct-24	A/Trinidad/3568/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI497710	NA	Belize	2013-Sep-23	A/Belize/3161/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI497713	NA	Saint Vincent and the Grenadines	2013-Oct-08	A/St. Vincent and Grenadines/3292/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI497716	NA	Saint Vincent and the Grenadines	2013-Oct-07	A/St. Vincent and Grenadines/3291/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention
EPI497719	NA	Barbados	2013-Sep-19	A/Barbados/3024/2013	Caribbean Epidemiology Center	Centers for Disease Control and Prevention

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab
EPI504751	NA	El Salvador	2013-Nov-28	A/El Salvador/2388/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI534019	NA	El Salvador	2013-Nov-04	A/El Salvador/2260/2013	Contiguo a Hospital Rosales	Centers for Disease Control and Prevention
EPI503826	NA	Mexico	2013-Oct-21	A/Mexico/3280/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI504790	NA	Mexico	2013-Dec-27	A/Mexico/06/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI508442	NA	Mexico	2013-Aug-31	A/Mexico/2733/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention
EPI508466	NA	Mexico	2013-Oct-08	A/Mexico/3093/2013	Laboratorio de Virus Respiratorio	Centers for Disease Control and Prevention

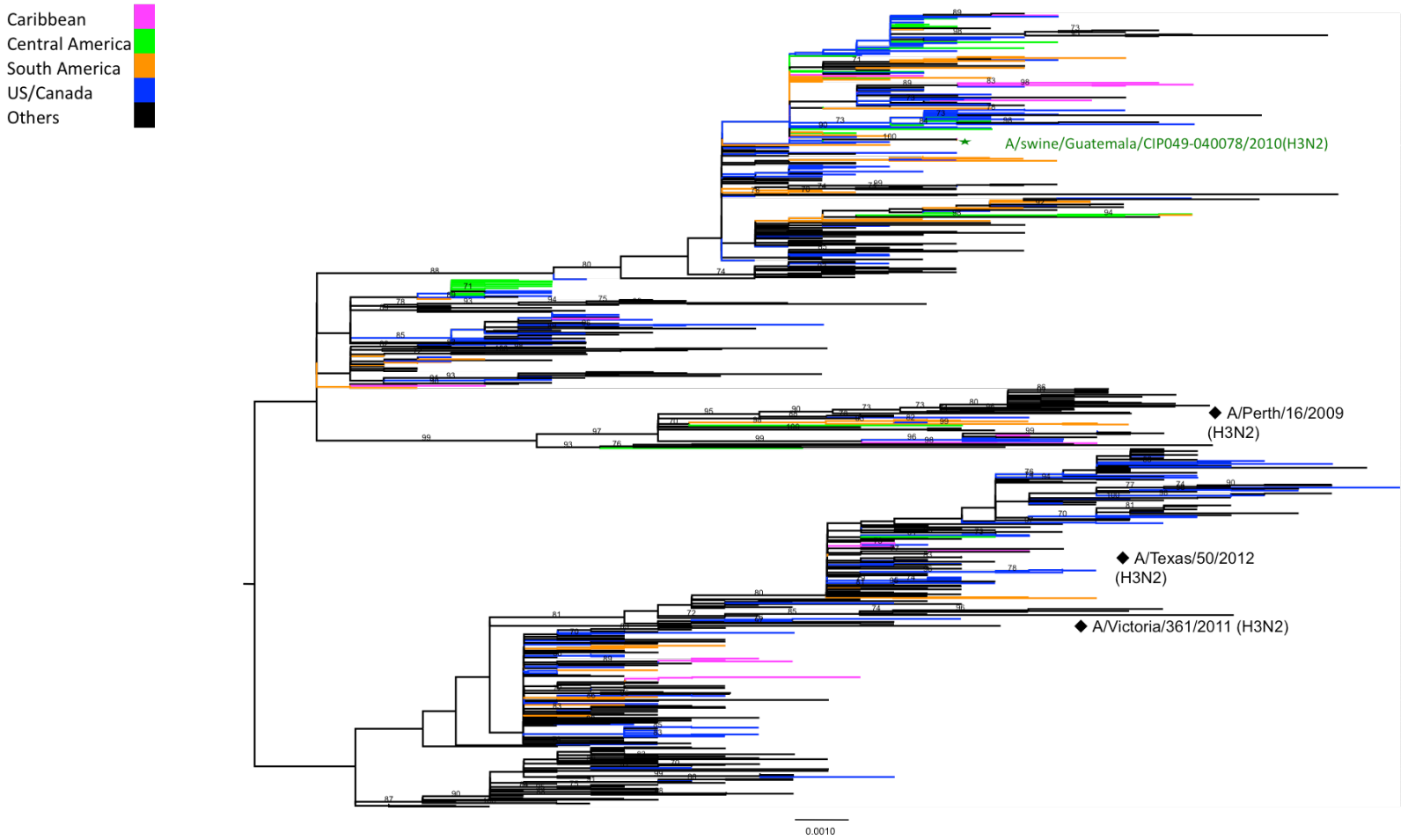

Figure 0.1. Maximum likelihood inference of the phylogenetic relationships between global human and human-like H3N2 viruses, 2010-2012.
The virus of Guatemala is shown in green, and the name of the vaccine strains is indicated in black. Bootstrap support values are shown. Scale-bar depicts number of substitutions per site.

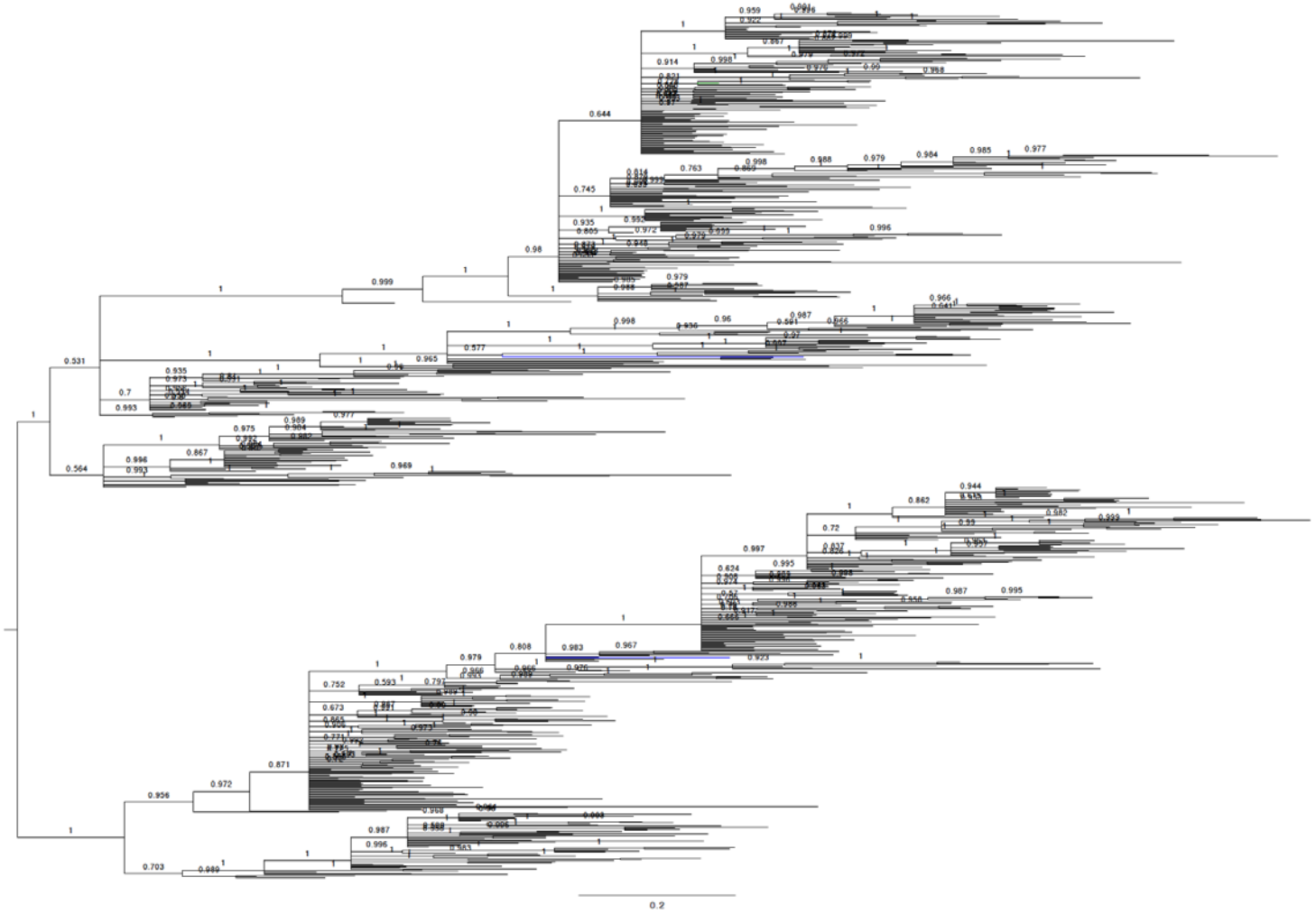

Figure 0.2 Bayesian phylogenetic inference of the relationships between global human and human-like H3N2 viruses, 2010-2012.
Posterior probabilities are shown. Scale-bar depicts number of substitutions per site.

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