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Development and Application of Polyclonal Antibody Based Proximity Ligation Assays in Detecting Antigenic Variants of Influenza A Viruses

Brigitte Elizabeth Martin

Development and application of polyclonal antibody based proximity ligation assays in detecting antigenic variants of influenza A viruses

By

Brigitte Elizabeth Martin

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By

Brigitte Elizabeth Martin

Approved:

_____

Xiu-Feng (Henry) Wan
(Major Professor)

_____

Larry Hanson
(Committee Member/Graduate Coordinator)

_____

Jeffrey B. Eells
(Committee Member)

_____

G. Todd Pharr
(Committee Member)

_____

Kent H. Hoblet
Dean
College of Veterinary Medicine

Name: Brigitte Elizabeth Martin

Date of Degree: May 5, 2017

Institution: Mississippi State University

Major Field: Veterinary Medical Science

Major Professor: Dr. Xiu-Feng (Henry) Wan

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Pages in Study 178

Candidate for Degree of Doctor of Philosophy

Influenza A virus (IAV) is a zoonotic pathogen which consists of a large genetically and antigenically diverse viral population. Swine IAVs not only cause disease outbreaks among swine, but can also be transmitted to humans, causing sporadic infections and even pandemic outbreaks apart from human seasonal IAV. Antigenic variant identification is fundamental for an effective vaccination program. Red blood cell based immunological tests have been used to identify antigenic variants among circulating IAV strains. Because these assays require viral isolation, they are time consuming and labor intensive. Thus only limited numbers of virus isolates are subjected to antigenic characterization in influenza surveillance studies and much of this important information is lost. In this project, a novel polyclonal antibody based proximity ligation assays (polyPLA) was developed and validated to characterize IAV antigenic variants directly using clinical samples. The application of this method with clinical samples from influenza surveillance had aided in the understanding of the antigenic evolution of IAV in human and swine populations.

DEDICATION

To my mother and father who have always supported me and have been my rock.

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CHAPTER I

INTRODUCTION

Influenza viruses and diseases

Currently, there are four genera of influenza viruses that belong to the family *Orthomyxoviridae*: Influenza virus A, B, C, and D (IAV, IBV, ICV, and IDV, respectively). IAV and IBV have genomes that consists of 8 negative-sense, RNA segments that encode at least 11 proteins: RNA polymerase subunit 2 (PB2), RNA polymerase subunit 1 (PB1 and PB1-F2), RNA polymerase unit (PA), hemaggluinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins (M1 and M2), and nonstructural proteins (NS1 and NS2). ICV and IDV have 7 negative-sense, RNA segments that encode at least 9 proteins. IAV is a zoonotic pathogen which consists of a large genetically and antigenically diverse viral population that, to date has subtypes from 18 HA and 11 NA.

The influenza virus is a perpetual threat to public health. Seasonal influenza infections are associated with 30,000 deaths a year in the United States (1); additionally, influenza epidemics, every few years, increase the annual mortality level to 40,000 to 45,000 deaths. Unpredictably, global pandemics of influenza occur, infecting 20% to 40% of the population in a single year, dramatically raising death rates above normal levels. The influenza vaccine is the most viable option in counteracting and reducing the impact of influenza outbreaks(2). Since the antigenicity of influenza virus population is

continuously changing, new strains can potentially escape the immunity of previously exposed hosts(3, 4). Therefore, the vaccine strains need to be updated almost annually to obtain antigenic matches between the vaccine strain and the strain potentially causing future outbreaks(5, 6).

Host range and zoonosis of influenza A viruses

While aquatic waterfowl are the major natural reservoir for IAV, swine can serve as a host for both human and avian origin IAV(7). Avian- and human-origin IAVs typically preferentially bind to receptor saccharides containing terminal α2,3-linked sialic acid-galactose (SA2,3Gal) or α2,6-linked sialic acid-galactose (SA2,6Gal), respectively(8, 9). Swine tracheal epithelium expresses both SA2,3Gal and SA2,6Gal receptors(10), and swine are therefore proposed as the intermediate host for avian IAV adaptation and as a "mixing vessel" for generating novel viruses by reassortment between avian-origin and human-origin IAVs(11-13). Subtypes which have been isolated from global swine populations include: H1, H2, H3, H4, H5, H7, and H9(14).

Humans have been reportedly infected with swine IAV since 2005(15), and such viruses are referred to as "variants". Many cases of variant IAV have been traced back to swine exposure at agricultural fairs within the United States(16-19); with the most notorious of these viruses being the 2009 H1N1 pandemic(20-22). As of 2011, there have been 364 reported human disease cases caused by H3N2 variant (H3N2v) IAV in 14 states(15, 19, 23); many of the infected people reported direct or indirect contact with swine at agricultural fairs and only rarely was human-to-human transmission of the virus thought to have occured(24, 25).

Also, swine have been shown to be susceptible to human influenza virus strains(26). In the past five decades, genomic analyses suggested at least 20 introductions of IAVs from humans to swine, the majority being human seasonal subtype H3N2 viruses(27). Human-to-swine transmission, also referred to as reverse zoonosis, of influenza A(H1N1)pdm09 virus was found in swine approximately one month after the virus was detected in humans(28). The A(H1N1)pdm09 virus co-circulated with endemic swine influenza virus, including triple-reassortant H3N2, human-origin H1N2 (H1 δ1), and classical H1N1 (H1 γ) swine influenza viruses(29), resulting in reassortment events(30-33). (H3N2)v swine influenza viruses with matrix gene segments from A(H1N1)pdm09 virus were isolated in humans in 2011(25).

Evolution of Influenza A viruses

Mutations, reassortment, and recombination

The RNA polymerase of IAV lacks a proofreading mechanism, leading to a mutation rate that ranges from 0.4×10^{-3} to 2.0×10^{-6} per nucleotide per year, depending on strain and gene (34-38). The rapid evolution of the surface glycoproteins, HA and NA, leads to antigenic drift (39). Genetic and antigenic diversity is also accomplished through mixed infection of two or more viruses within the same host and the generation novel viruses by reassortment of gene segments, which can result in antigenic shift. Recombination, rare in IAV, can also occur in a mixed infection in which the polymerase switches templates, creating a novel mosaic structure(40). Novel antigenic variants enable influenza viruses to cause epidemics in humans on almost a yearly basis, with potentially serious medical consequences very young and elderly patients (41).

Viral quasispecies Selection and fitness

Infection within a host is not limited to one identical viral genome; the proliferating population of viruses is closely related, yet is not identical(42-45). The viral quasispecies of IAV within a host is due to the rapid virus diversification because of the error-prone genome replication and are these quasispecies are subject to continuous competition (44, 46). Some mutations can be positively selected in order for a virus to escape from host antibody neutralization or to replicate more efficiently, leading to virus variants, which could become predominant in the population (47). Population-level fitness has also been shown to be increased by cooperative interactions between variants within a quasispecies(48-52).

Influenza vaccination

Influenza vaccination in humans

Vaccination is the primary approach for the control and treatment of human seasonal influenza outbreaks(2). Antigenic variant identification and antigenic match between vaccine strain and epidemic strain is the key to a successful vaccination program(35). The global influenza surveillance network coordinated by the World Health Organization(53) characterizes antigenic properties of influenza viruses using the golden standard antigenic characterization method of hemagglutination inhibition assay (HI) and sequencing of HA1 domain(54, 55). Antigenic, genetic, and epidemiological data are examined to make recommendations for candidate vaccine strains. Data collection also aids policy makers in understanding the risk factors for severe disease, variation of influenza severity from season to season and its relationship to virus types or subtypes, the burden of disease related to influenza, and other factors critical to public health

decision-making(53). One major limitation for implementing changes to the influenza vaccine is the time restriction on current virus strain assessment. Recommendations are based on predicting the future impact of circulating viruses, most notably, emerging antigenic variants, before full epidemiological significance is known(36).

Accuracy in deciding vaccine composition is important because typically this must be completed almost a full year before the peak of seasonal influenza activity(5). Worldwide epidemiological surveillance is necessary in order to monitor IAV vaccine effectiveness; this is accomplished by the isolation and characterization of currently circulating viruses. Additionally, the early detection of IAV infection by reliable laboratory results can prevent spread of disease and assist with optimal treatment decisions.

Influenza vaccination in domestic swine

Vaccination is commonly used in the United States for IAV control in swine. Swine vaccines typically, available as licensed commercial products, consist of culture-derived virions in crude allantoic fluid from SPF chicken eggs, which are then chemically inactivated, and formulated into a mineral oil emulsion vaccine(56). Current swine influenza vaccines are strain specific, consisting of two or more H1 and H3 isolates, and fail to induce cross-protection against genetic and antigenic virus variants(57). Unlike human vaccines, this process avoids costly purification steps for enrichment of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA)(58), but booster vaccinations are necessary in order to achieve and maintain protective levels of systemic hemagglutination-inhibiting antibodies(59).

Because vaccines are produced locally and autogenously, each geographic region differs in vaccine strain, exact adjuvant formulation, and antigen dose. Vaccines that are antigenically inaccurate for circulating viruses can lead to vaccine-induced immune pressure, causing antigenic drift(60). The generation of novel escape mutants gradually replace circulating virus strains(61, 62). Such antigenic drift events in swine populations have been documented(57, 63, 64).

Influenza vaccination in domestic poultry

In domestic poultry, influenza vaccines are not only used for the prevention of disease and death, but for the prevention of infection or reduction of viral replication in respiratory and digestive tracts to limit viral spread to uninfected flocks(65-68). Currently, vaccines are available against H5 or H7 in live IAV vaccines, that are only available for chickens, or inactivated avian IAV vaccines, that are available for a wider range of species, including chickens, turkeys, ducks, geese, and zoo birds(67).

Antigen diversity of IAVs in different hosts

Antigenic diversity of IAVs in domestic swine

Swine may play a role in the reassortment and adaptation of novel zoonotic viruses; it is possible that a novel virus with pandemic potential could cause a spill-over event into the human population(11, 69, 70). Influenza surveillance in swine is seen as important for the protection of public health and pandemic prevention. Swine IAVs not only cause outbreaks among swine, but can also be transmitted to humans, causing sporadic infections and even pandemic outbreaks apart from human seasonal IAV. Domestic swine are thought to be the intermediate host for avian origin influenza A

viruses for their adaptation before these viruses can infect humans(71-74). Both avian-like H1N1 and H3N2 viruses have been isolated from domestic swine(74-76); avian origin influenza A viruses of subtypes H1 to H13 have been shown to infect and replicate in swine under experimental conditions(74), as well as direct avian H2N3, H3N1, and H4N6 to swine transmission of influenza in nature(12, 57, 77-84).

Influenza surveillance studies in domestic swine from 2009 to 2012 identified the co-circulation of H1N1, H1N2, and H3N2 IAVs, which are genetically and antigenically diverse, with different amounts of cross-reactivity in serological assays(80-83). The H1 subtypes form four genetic clusters: swH1α (classic H1N1), swH1ß (reassortant H1N1-like), swH1γ (H1N2-like), and swH1δ (human-like H1); swH1γ are further divided into subclusters swH1γ and swH1γ2; swH1δ are further divided into subclusters swH1δ1 (human-like H1N2) and swH1δ2 (human-like H1N1) (20). Additionally, the 2009 H1N1 (A(H1N1)pdm09) virus emerged from swH1γ, a classic H1N1 virus, but has evolved into a distinct genetic and antigenic lineage after being introduced back to the swine population(77). There are four genetic clusters of H3N2 subtype IAV present in the United States swine population: clusters I-IV(7, 78, 79); cluster IV is currently predominant in domestic swine, and can be further divided into two antigenic clusters: H3N2-α and H3N2-ß(80). Antigenic characterization studies demonstrate that these genetically diverse H1 and H3 viruses are antigenically distinct, showing different amounts of cross-reactivity in serologic assays(85, 86).

Antigenic diversity of IAVs in feral swine

Migratory waterfowl are the major natural reservoir of IAVs(7, 87) but avian origin influenza A viruses do not typically transmit directly from avian to humans. Feral

swine, *Sus scrofa*, in the United States, are domestic swine that escaped from commercial operations or were intentionally released, descendants of Eurasian wild boar introduced for hunting purposes, or hybrids of the two(88). The feral swine have an estimated population size of 5 million across 35 states, which is ever-increasing. Although the same species as domestic swine, feral swine are free ranging, with capabilities to come in contact with wild waterfowl, domestic swine, poultry, and humans, making feral swine a potential vector for the bi-directional transmission of influenza viruses.

H1N1 and H3N2 IAVs have been recovered from feral swine, and a recent serological surveillance in feral swine revealed 9.15% IAV seropositive samples from 31 states(80). Feral swine have opportunities to encounter wild waterfowl by frequenting the same bodies of water, feeding in the same areas, and preying or scavenging on wild waterfowl, which can provide potential for IAV transmission from wild birds to feral swine. Because feral swine are highly mobile, they can also have opportunities to come into contact with IAVs from infected domestic swine, poultry, and even humans via contaminated fomites or aerosol dispersal(89).

Antigenic diversity of IAVs in domestic poultry

Domestic poultry can be infected with nonpathogenic, low pathogenic, and highly pathogenic IAV. There are a variety of HA subtypes that are less virulent and do not cause death in experimental infection. In contrast, H5 and H7 subtypes are highly virulent and cause 100% death in experimental infection. The emergence of antigenic variants in domestic poultry has occurred after escape from immune responses generated by vaccination(90).

Antigenic diversity of IAVs in wild birds

IAVs have been recovered from at least 105 wild bird species of 26 different families(91). Migratory waterfowl, such as the birds in the orders *Anseriformes* (e.g. ducks, geese, swans) and *Charadriiformes* (e.g. gulls, terns, and waders), are considered the major natural reservoirs of IAVs(7). Sixteen IAV HA (H1–H16) and NA (N1-N9) subtypes have been recovered from migratory waterfowl. The prevalence of IAV infection is up to 30% among wild birds(7), and virus transmission typically occurs via exposure to virus shed in the feces of infected animals(92, 93). It has been conceptually proposed that antigenic evolution in migratory waterfowl could be static(94); for example, this theory is supported by recent studies indicating a lack of antigenic diversity among H3 and H7 IAVs in migratory waterfowl in North America(95, 96).

Influenza vaccine strain selection and challenges

Current WHO strain selection

Human seasonal vaccines need to be updated almost every year due to frequent antigenic drift events in the epidemic viruses. On the other hand, recommendations of influenza vaccine strains are based on the prediction of circulating viruses, most notably, possibly emerging antigenic variants, before full epidemiological significance is known(97). One major challenge for selecting an influenza vaccine strain is the time restriction that at least six months are required for vaccine manufacture before the next influenza season begins. For example, for the Northern Hemisphere, the vaccine strain for the fall season has to be determined early spring (usually on Feb 15 each year). Thus, to create a successful influenza vaccination program, a rapid antigenic variant identification is essential to warrant essential efficacy against emerging variants(98, 99).

Since 1993, antigenic and biochemical differences in the HA proteins of influenza viruses have been noted after passage in both Madin-Darby canine kidney (MDCK) cell and eggs(100). There are altered patterns of agglutination for influenza viruses propagated in eggs and after growth in cell culture spiked with non-immune horse serum in comparison to the original isolate(98, 100-108). Additionally, sequence analysis has shown that changes in the H3 HA residues occur in the antigenic sites (residue 137 (antigenic site A), 156 (B), 186 (B), 248 (D), 276 (C))(101). Both monoclonal and polyclonal antisera can detect a difference in the antigenicity between HAs before and after passage in eggs(100). The altered viruses will negate the goal of choosing vaccine viruses which are likely to be circulating during the impending influenza seasons. Alternatively, vaccine effectiveness, in the 2012-13 influenza season, was affected by egg-adapted mutations in the A/Victoria/361/2011 vaccine strain (IVR-165)(109).

Antigenic profiles

Reference ferret sera are used to determine antigenic properties of currently circulating viruses for influenza surveillance and vaccine strain selection(97, 109-114). Variant viruses are identified to have ≥8-fold reduction of cross-reactivity to the ferret sera as compared to that of the homologous vaccine virus. Vaccines are also evaluated with human post-vaccination sera for cross-reactivity with circulating variants; a reduction of ≥50% in geometric mean titers significantly indicates low vaccine efficiency(115). In a comparison between ferret and human sera, it has been shown that ferret sera does not always represent vaccine-induced responses in humans(111, 115-117). One such example of vaccine mismatch occurred in the 2014-2015 Northern Hemisphere H3N2 vaccine virus with the circulating variants(116).

H3 antigenic differences in 2014 with H3N2 subclades 3C.2a and 3C.3a

In the 2014-2015 influenza season, two H3N2 subclades mutated independently and rapid spread of the novel antigenic mutations occurred due to a lack of immune protection. Previously, in the HA of genetic clade 3C.1, phenylalanine (F) was at residue 159 and asparagine (N) was at residue 225. Both subclades mutated residue 225 first to aspartic acid (D), followed by mutation of residue 159 to serine (S) or tyrosine (Y) for clades 3C.3a or 3C.2a, respectively.

Low or undetectable hemagglutination activity was common, particularly for 3C.2a, which caused antigenic characterization of the novel viruses to be more difficult (118). Ferret sera from cell-propagated 3C.3a and 3C.2a inhibited most circulating viruses, so the WHO concluded that 3C.3a and 3C.2a were antigenically related. The majority of the viruses analyzed were antigenically related to 3C.3a and A/Switzerland/9715293/2013-like virus (3C.3a) was chosen as the 2015-2016 vaccine virus(118, 119).

The following season, 2015-2016, the majority of viruses analyzed were 3C.2a with 3C.3a co-circulating but at a lower proportion(118). Like the previous year, antigenic characterization was technically challenging due to low or undetectable hemagglutination activity. Reference sera for 3C.2a were able to inhibit the majority of viruses from both 3C.3a and 3C.3b; A/Hong Kong/4801-like virus (3C.2a) was chosen as the 2016-2017 vaccine virus(118).

Technology

Diagnosis

Clinical signs and symptoms of influenza can include: fever, muscle aches, headache, lethargy, dry cough, sore throat, nasal congestion, and possible runny

nose(120). Influenza diagnosis based on clinical signs and symptoms alone is difficult because they are similar to other pathogens. There are several diagnostic tests available for the detection of influenza virus, including viral culture, serology, rapid antigen testing reverse transcription polymerase chain reaction (RT-PCR), immunofluorescence assays, and rapid molecular assays. Sensitivity and specificity of these tests depend on type of test used, time from onset of illness until sample collection, type of sample collected, sample handling, and test performance.

Serological assays

Routinely, immunological tests, such as HI (86) assays and microneutralization (121) assays, have been relied upon to identify antigenic variants among the circulating strains(122). The HI assay is an experiment to measure how a test influenza antigen and a reference antigen (e.g. a current vaccine strain) match through the immunological reaction between the test antigen and the reference antiserum. This reference antiserum is usually generated in ferrets using the reference antigen. However, the data from HI assays are notoriously noisy, and HI experiments are affected by many factors. For example, red blood cells (RBCs) used from different species, which could have different sialic acid receptors, can produce varied results(122). Both HI and MN require viral isolation, thus are labor intensive and for highly pathogenic viruses, a BSL-3 facility is required. The data are subject to interpretations and the HI assays are difficult to automate and standardize.

More importantly, mutations of the receptor binding site in HA(4) (antigenic drift) are causing human seasonal H1N1(100, 123) and H3N2 influenza A viruses(124) to lose the ability to bind to RBCs. For example, the mutations at residues 193, 196, 197, and 225 in

the human epidemic H1N1 influenza A viruses in 1988 or later caused the loss of their abilities to agglutinate chicken RBCs due to four amino acid changes(125). For H3N2 viruses, the Gly190Asp substitution has been correlated to the loss of the ability to agglutinate chicken erythrocytes(122, 124, 126-130). Since 2000, human seasonal H1N1 and H3N2 influenza A viruses have been losing their binding abilities to turkey RBCs(122, 131). This may be attributed to a reduced affinity for sialic acid-linked receptors (particularly α2-6-linked receptors), which are at lower levels on chicken and turkey RBCs compared to levels on guinea pig RBCs(122, 131). In addition, heterogeneous populations which contain minor antigenic variants cannot be detected with HI assays(132).

In addition, both HI and MN require viruses to be isolated and propagated from the original clinical samples. The virus isolation process is not only time-consuming but also can change the antigenic properties of the viruses in the patients due to the laboratory adaptation in cells or embryonated eggs(121, 133-135). Furthermore, some viruses cannot even be recovered from the specimen. The virus isolation and propagation may require a high-level biocontainment, e.g. for highly pathogenic influenza A viruses. Thus, there is a critical need for a viral isolation-free and RBC independent assay to assess antigenic changes in influenza viruses in order to further expand influenza surveillance.

Antigen detection

When a patient has an influenza like illness (ILI), it is important that throat, nasal and nasopharyngeal secretions or tracheal aspirate and washings are properly collected, stored, and transported for laboratory detection of influenza virus. Laboratory confirmation can be completed with direct antigen detection, virus isolation, or RT-

PCR(136) for influenza-specific RNA(137). Immunofluorescence antibody (IFA) staining of original clinical samples or isolates can be completed with commercially available monoclonal antibodies. Viral isolation is preferred for a larger quantity of antigen to be present yet suitable clinical specimens can be used. The sensitivity of IFA depends on sample quality, specificity of reagents used, and the level of experience for test performance, reading, and interpretation.

Commercially available rapid influenza antigen detection tests (RIDTs) are used within the first 4 days of ILI symptom onset. RIDTs are used for: detection of influenza A only, detection of both influenza A and B, or detection and differentiation between influenza A and B(137). For detection of nucleoprotein (NP) for pandemic H1N1 2009, RIDTs, compared to RT-PCR, has a sensitivity range of 10-69%(138-142); detection of NP from seasonal influenza with RIDTs, compared to RT-PCR or viral culture, has a sensitivity range of 10-96%(143-146). The use of RIDTs is not encouraged if RT-PCR of immunofluorescence assays are available and the WHO recommends IFA, viral culture, or RT-PCR should be used in conjunction to confirm and extend results(147).

The proximity ligation assay utilizes quantitative real time PCR (qRT-PCR) for the detection of antigen-antibody interaction(148). For this assay: (1) oligonucleotide-linked monoclonal antibodies are incubated with the analyte in question; (2) if in close proximity, the oligonucleotides can be ligated together; and (3) presence of analyte will be shown by amplification of ligated products with qRT-PCR. The assay reporter signal is dependent on a proximal and dual recognition of each target analyte providing high specificity(149).

Genomic sequencing and data analyses

Sequencing of IAV is useful to identify the origin of novel reassortant viruses as well as identify molecular characteristics of a virus, such as host range, replication efficiency, transmissibility, or virulence(150-153). The traditional method of Sanger sequencing is widely used in influenza surveillance and research(154). Next-generation sequencing (NGS) can sequence the entire genome(155, 156) and is helpful in the identification of genetic variation within a viral quasispecies and to monitor vaccine composition(157). The massively parallel sequencing of influenza virus by the NGS platforms allow for increased sensitivity of the study of the populations. This is in contrast to the traditional method and NGS is a highly sensitive sequencing method that does not require molecular cloning or plaque generation for phenotypic selection(158-160) for an output of multiple gigabases of DNA(161). Because the whole IAV genome is only 13,000 nucleotide bases of RNA, coverage is high, yet the segmented nature of the genome is challenging to achieve full genome coverage(155, 162-166). In the analysis of NGS data for IAV, it is important to accurately assemble the short reads into the 8 genomic sequences while retaining the polymorphisms within the quasispecies or true mixed infection of multiple viruses.

Phylogenetic analyses

Genetic differences can be visualized by relatedness with other influenza viruses by phylogenic trees, typically HA and NA gene segments. The length of the horizontal branches indicates the number of nucleotide differences and the vertical distance of the branches indicate the genetic difference. Phylogenic analysis can determine a common ancestor and evolution of IAV(167).

Antigenic cartography

Genetic differences, alone, are unreliable in the determination of antigenic differences in novel viruses. Additionally, antigenic properties through immunological datasets are not always straightforward. The relative antigenic relationship and evolution can be visualized with the production of two- or three- dimensional maps, known as antigenic cartography(168). The antigenic map produced can quantify antigenic differences between viruses; antigenically similar viruses cluster closely while antigenically different viruses are farther away.

Objectives, hypotheses, and specific aims of this study

The goal of this study is to understand antigenic evolution of influenza A virus directly using clinical samples. The hypotheses of this study are that antigenic variants can be detected directly from clinical samples and that antigenic characterization from clinical samples, especially uncultivable samples, would provide new knowledge of the antigenic evolution of influenza A viruses. To test the hypotheses of this study, a novel polyclonal antibody based proximity ligation assays (polyPLA) was developed and validated to characterize influenza antigenic variants directly using clinical samples and further to apply this method in clinical samples from influenza surveillance to understand antigenic evolution of influenza A virus in human and swine populations.

CHAPTER II

US FERAL SWINE WERE EXPOSED TO BOTH AVIAN AND SWINE IFNLUENZA A VIRUSES

Influenza A viruses (IAVs) in swine can cause sporadic infections and pandemic outbreaks among humans, but how avian IAV emerges in swine is still unclear. Unlike domestic swine, feral swine are free ranging and have many opportunities for IAV exposure through contacts with various habitats and animals, including migratory waterfowl, a natural reservoir for IAVs. During 2010–2013, 8,239 serum samples were collected from feral swine across 35 US states and tested against 45 contemporary antigenic variants of avian, swine, and human IAVs; of these, 406 (4.9%) samples were IAV-antibody positive. Among 294 samples selected for antigenic subtyping, 271 cross-reacted with ≥1 testing virus whereas the other 23 did not cross-react with any testing virus. Of the 271 IAV-positive samples, 236 cross-reacted with swine IAVs, 1 with avian IAVs, and 16 with avian and swine IAVs, indicating that feral swine were exposed to both swine and avian IAVs but predominantly to swine IAVs. Our findings suggest that feral swine could potentially be infected with both avian and swine IAVs, generating novel IAVs by hosting and reassorting IAVs from wild birds and domestic swine and facilitating adaptation of avian IAVs to other hosts, including humans, before their spillover. Continued surveillance to monitor the distribution and antigenic diversities of

IAVs in feral swine is necessary to increase our understanding of the natural history of IAVs.

Introduction

Influenza A virus (IAV), a negative-stranded RNA virus with 8 genomic segments, can infect a wide range of hosts, including humans, wild birds and domestic poultry, swine, canines, felines, equines, mink, ferrets, sea mammals, and bats. IAVs have been recovered from at least 105 wild bird species of 26 different families (91). Migratory waterfowl, such as *Anseriformes* spp. birds (e.g., ducks, geese, and swans) and *Charadriiformes* spp. birds (e.g., gulls, terns, and waders), are considered the major natural reservoirs of IAVs (7). Sixteen IAV HA (H1–H16) and NA (N1-N9) subtypes have been recovered from migratory waterfowl. The prevalence of IAV infection is up to 30% among wild birds (7), and virus transmission typically occurs via exposure to virus shed in the feces of infected animals (92, 93). It has been conceptually proposed that antigenic evolution in migratory waterfowl could be static (94); for example, this theory is supported by recent studies indicating a lack of antigenic diversity among H3 and H7 IAVs in migratory waterfowl in North America (169, 170).

IAVs in domestic swine are genetically and antigenically diverse. In the past decade, the predominantly circulating domestic swine strains in the United States were IAV subtypes H1N1, H1N2, and H3N2 (57, 84). Subtypes such as H4N6, H2N3, and H3N1 were also identified in North American domestic swine (82, 171, 172), but these viruses did not become endemic. The H1 subtypes circulating in domestic swine form four genetic clades: swH1α (classic H1N1), swH1ß (reassortant H1N1-like), swH1γ (H1N2-like), and swH1δ (human-like H1). Clade swH1γ is further divided into subclades

swH1γ1 and swH1γ2, and clade swH1δ is subdivided into swH1δ1 (human-like H1N2) and swH1δ2 (human-like H1N1) (173). In addition, the 2009 H1N1 pandemic virus, A(H1N1)pdm09 emerged from a classic H1N1 virus and evolved into a distinct genetic and antigenic lineage (173). There are 4 genetic clusters (I-IV) of the H3N2 subtype of IAVs present in the US swine population (174-176). Cluster IV, currently the predominant IAV cluster circulating among domestic swine, can be further divided into 2 antigenic clusters H3N2-α and H3N2-ß (85). Antigenic characterization suggests that these genetically diverse H1 and H3 viruses are antigenically distinct, showing different extents of cross-reactivity in serologic assays (85, 177). Influenza surveillance studies in domestic swine from 2009 through 2012 identified the co-circulation of H1N1, H1N2, and H3N2 IAVs, including 6 H1 genetic clades (H1α, H1ß, H1γ, H1δ1, H1δ2, A[H1N1]pdm09) and 2 H3 cluster IV antigenic clusters (H3SIVα and H3SIVß) (29, 178).

Avian- and human-origin IAVs typically preferentially bind to receptor saccharides containing terminal α2,3-linked sialic acid-galactose (SA2,3Gal) or α2,6-linked sialic acid-galactose (SA2,6Gal), respectively (8, 9). Swine tracheal epithelium expresses both SA2,3Gal and SA2,6Gal receptors (10), and swine are therefore proposed as the intermediate host for avian IAV adaptation and as a "mixing vessel" for generating novel viruses by reassortment between avian-origin and human-origin IAVs (11, 12, 179). In addition to avian-origin H2N3 and H4N6 IAVs, which were identified in domestic swine in North America, avian-origin H1N1(77, 78), H1N2 (180), H3N3 (83), H5N1 (81), H6N6 (181), and H9N2 (79, 80). IAVs were also identified in domestic swine in Eurasia. Among these avian-origin IAVs, only subtypes H1N1 and H3N2 have

become endemic in domestic swine; the other avian-origin IAVs caused only low seroconversion rates and has been transient in domestic swine. Nevertheless, under laboratory conditions, avian-origin IAVs of subtypes H1-H13 can infect and replicate in swine (12).

Feral swine in the US are domestic swine that escaped from commercial operations or were intentionally released, descendants of Eurasian wild boar introduced for hunting purposes, or hybrids of the two (182). In 2013, an estimated 5 million feral swine were found in at least 35 US states, with both numbers and geographic range increasing. H1N1 and H3N2 IAVs have been recovered from feral swine, and serologic surveillance conducted during 2011–2012 showed that 9.2% of 1,983 serum samples from feral swine in 31 states were IAV-seropositive (85). Similar to domestic swine, feral swine can be infected with IAVs under laboratory conditions (183). Feral swine have opportunities to encounter wild waterfowl by frequenting the same bodies of water, feeding in the same areas, and preying or scavenging on wild waterfowl, which can provide potential for IAV transmission from wild birds to feral swine. Because feral swine are highly mobile, they can also have opportunities to come into contact with IAVs from infected domestic swine, poultry, and even humans via contaminated fomites or aerosol dispersal (89).

Our objective was to conduct a serological survey of feral swine for IAV exposure and to assess the role of feral swine at the interface between wild birds and domestic swine. Utilizing 8,239 serum samples collected from feral swine in 35 US states from 2010-2013, we explored patterns of IAV seropositivity and further characterized

seropositive samples' cross-reaction to 45 antigenically diverse prototype IAVs from avian, domestic swine and human hosts.

Materials and Methods

Sample collection and serology testing.

From October 1, 2009–September 30, 2013, the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service's Wildlife Services collected 8,239 serum samples from 8,239 individual feral swine, postmortem, across 35 US states. This collection period included fiscal years (FY) 2010 (October 1, 2009–September 30, 2010; 1,818 samples), 2011 (October 1, 2010–September 30, 2011; 2,467 samples), 2012 (October 1, 2011–September 30, 2012; 1,846 samples), and 2013 (October 1, 2012–September 30, 2013; 2,108 samples) (Table 1). Serum samples from October 1, 2011–September 30, 2012, were previously reported as 1,989 totally, yet 143 serum samples had duplicate information and were ignored for this study. The date of collection, geographic location, age (juvenile, subadult, adult, and unknown), and sex were recorded. Antibody status was determined with the IDEXX AI MultiS-Screen Ab Test (IDEXX, Westbrook, Maine, USA). Serum samples with a sample-to-negative control ratio of ≤0.681 were determined to be IAV-positive (183). The ELISA results for a subset of feral swine samples (76 of 111) from FY2012 are reported elsewhere (85) in an assessment of the seropositivity rate of subtype H3 IAV in feral swine. To ensure complete results, we included all feral swine serum samples collected for the study. Of the samples tested, 406 were identified as IAV-positive; to make this study more cost-effective, 294 of these were randomly selected for subtyping by HI assay.

Table 1 Summary of feral swine serum samples used to determine the predominant source of feral swine exposure to endemic influenza A virus (IAV) in the United States.

Fiscal year samples collected[a]	No. samples collected	No. IAV-positive samples (% positive)[b]	No. positive samples selected for testing by HI assay[c]
2010	1,818	112 (6.2%)	0 [d]
2011	2,467	95 (3.9%)	95
2012	1,846	111 (6.0%)	111
2013	2,108	88 (4.2%)	88
Total	8,239	406 (4.9%)	294

[a]Fiscal years run from October 1st of one year through September 30th of the next year; the FY is named according to the second year.
[b]Serum samples were considered IAV-positive if the sample-to-negative control ratio was ≤0.681by ELISA (IDEXX AI MultiS-Screen Ab Test; IDEXX, Westbrook, Maine, USA) (183).
[c]HI, hemagglutinin inhibition.
[d] Not analyzed to make more cost effective.

Viruses and Ferret Reference Sera.

A total of 45 IAVs were selected to represent the following antigenic groups of contemporary IAVs: endemic swine IAVs H1 (α, ß, γ1, γ2, δ1, δ2) and H3 cluster IV (α and ß); human influenza viruses (A[H1N1]pdm09, swine-origin influenza viruses A[H3N2]v, and seasonal H3N2); and avian influenza viruses H1–H14 (Table 2). These viruses were used in the serologic characterization. The ferret reference sera used to assess cross-reactivity among testing viruses were generated as described elsewhere(178).

Table 2 Cross-reactivities of feral swine serum samples against testing influenza A viruses in hemagglutinin inhibition assays

Virus	Antigenic group	Source of virus*	No. seropositive samples (%)†	GMT (LB-HB)‡
A/swine/Minnesota/02093/2008	H1N1-α	domestic swine	44 (14.67)	138.85 (40-1280)
A/swine/Minnesota/A01394082/2013	H1N2-α	domestic swine	20 (6.67)	80 (40-320)
A/swine/Nebraska/A01399642/2013	H1N1-ß	domestic swine	58 (19.33)	124.49 (40-1280)
A/swine/Nebraska/A01240348/2011	H1N1-ß	domestic swine	57 (19.00)	146.94 (40-1280)
A/swine/Indiana/13TOSU0832/2013	H1N1-γ	domestic swine	54 (18.00)	204.19 (40-1280)
A/swine/Indiana/13TOSU1154/2013	H1N1- γ	domestic swine	53 (17.67)	187.19 (40-1280)
A/swine/Illinois/A01076767/2010	H1N1- γ2	domestic swine	5 (1.67)	45.95 (40-80)
A/swine/South Dakota/A01349306/2013	H1N1- γ2	domestic swine	63 (21.00)	271.32 (40-1280)
A/swine/Iowa/15/2013	H1 δ1	domestic swine	12 (4.00)	75.51 (40-160)
A/swine/Iowa/18/2013	H1 δ2	domestic swine	22 (7.33)	600.92 (40-1280)
A/swine/Iowa/19/2013	H1 δ2	domestic swine	12 (4.00)	59.93 (40-320)
A/swine/Iowa/7/2013	H1 2009p	domestic swine	91 (30.33)	264.51 (40-1280)
A/swine/Iowa/8/2013	H1 2009p	domestic swine	81 (27.00)	301.39 (40-1280)
A/California/04/2009	H1 2009p	human	61 (20.33)	212.57 (40-1280)
A/mallard/Wisconsin/A00751454/2009	H1N1	avian	13 (4.33)	75.85 (40-640)
A/mallard/Oregan/A0030758/2007	H2N3	avian	0	0
A/swine/Ohio/09SW96/2009	H3N2 α	domestic swine	84 (28.00)	105.04 (40-1280)
A/swine/Ohio/10SW215/2010	H3N2 ß	domestic swine	117 (39.00)	140.45 (40-1280)
A/swine/Ohio/11SW347/2011	H3N2 ß	domestic swine	93 (31.00)	146.31 (40-1280)
A/swine/Texas/A01104013/2012	H3N2 ß	feral swine	123 (41.00)	64.58 (40-1280)
A/Perth/16/2009	H3N2	human	177 (59.00)	109.86 (40-1280)
A/Wisconsin/112/2010	H3N2v	domestic swine	119 (39.67)	153.61 (40-1280)
A/Pennsylvania/14/2010	H3N2v	domestic swine	89 (29.67)	89.91 (40-640)
A/Minnesota/10/2011	H3N2v	domestic swine	105 (35.00)	106.96 (40-640)
A/Iowa/07/2011	H3N2v	domestic swine	118 (39.33)	148.24 (40-1280)
A/Victoria/361/2011	H3N2	human	195 (65.00)	154.18 (40-1280)
A/mallard/Wisconsin/A00661715/2009	H3N2	avian	1 (0.33)	80 (±0.00)
A/blue-winged teal/Colorado/A00170379/2006	H3N8	avian	0	0
A/mallard/Washington/A00714770/2009	H4N6	avian	0	0
A/mallard/Wisconsin/10os3845/2010	H5N2	avian	0	0
A/mallard/Oregon/A00571208/2007	H6N1	avian	0	0
A/mallard/Ohio/648/2002	H6N2	avian	1 (0.33)	40 (±0.00)
A/bufflehead/Virginia/A00120022/2008	H7N2	avian	1 (0.33)	40 (±0.00)
A/American black duck/Delaware/A00870108/2010	H7N3	avian	0	0
A/northern shoveler/Illinois/10os3632/2010	H8N4	avian	0	0
A/mallard/Minnesota/10os4670/2010	H9N2	avian	0	0
A/northern shoveler/Arkansas/11os386/2011	H9N2	avian	0	0
A/mallard/South Dakota/A00536114/2007	H10N7	avian	0	0
A/mallard/Illinois/10OS3249/2010	H11N2	avian	0	0
A/mallard/Wisconsin/10OS2889/2010	H11N9	avian	0	0
A/American green-winged teal/Missouri/10OS4622/2010	H12N4	avian	0	0
A/bufflehead/Wisconsin/10OS3204/2010	H12N5	avian	0	0
A/hooded merganser/New Brunswick/03750/2009	H13N6	avian	0	0
A/white-winged scooter/Wisconsin/10OS3922/2010	H14	avian	0	0
A/long-tailed duck/Wisoconsin/10OS3912/2010	H14N6	avian	0	0

*The host from which the virus was isolated;
†Serum samples were determined to be positive against a testing virus if the associated HI titer was ≥1:40;
‡The geometric mean titer (GMT) was calculated for each group of positive samples. HB, high boundary of HI titer; LB, low boundary of HI titer.

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Assays.

HI assays were performed according to the World Health Organization Global Influenza Surveillance Network Manual for the laboratory diagnosis and virologic surveillance of influenza (184). In brief, we treated 1 volume of feral swine serum with 3 volumes of receptor-destroying enzyme (RDE) overnight at 37°C and then heat-inactivated the serum at 56°C for 30 minutes. After returning to room temperature, treated antisera were diluted with 6 volumes of 1× PBS (pH 7.4). To minimize nonspecific agglutination, we treated RDE-treated serum samples with 0.5% turkey red blood cells (RBCs) and then incubated them at 4°C for 1 hour, followed by centrifugation at 1,200 rpm for 10 minutes; we then collected the serum samples without disturbing the packed RBCs. RBC absorption was repeated until no nonspecific agglutination was associated with any serum sample. In the HI assay, 0.5% turkey RBCs were used for absorption; serum samples were determined to be positive against a specific virus if the HI titer was ≥1:40, as described previously (85).

Virus neutralization inhibition assays.

RDE-treated feral swine serum was serially diluted 1:2 in a microtiter plate, and 100 µL of 50% Tissue Culture Infectious Dose ($TCID_{50}$) virus was added to each well, and then the plate was incubated at 37°C for 1 hour. The sera–virus mixture was then incubated with Madin-Darby canine kidney (MDCK) cells for one hour in a 96-well tissue culture plate (USA Scientific; Ocala, FL, USA), washed twice with 1× PBS (pH =7.4), washed with 200 µL of Opti-MEM (Thermo Fisher; Waltham, MA, USA), and then incubated for 96 hours at 37°C in 5% CO_2. Virus titration was conducted using HA

assays with 0.5% turkey RBCs. Serum samples were determined to be positive against a specific virus if the neutralization inhibition titer was ≥1:40(185).

Data analyses.

Chi-square tests were used to assess the differences of IAV seropositivity rate between sex, age group, and month, year, and state of sample collection. We developed a multilevel multivariable logistic regression model to test potential risk factors associated with IAV seropositivity rate in feral swine, focusing on the population sizes of domestic swine and poultry. The logistic regression model was developed using generalized estimation equations with binomial distribution and logit link function and accounted for clustering of swine samples on the same date and location. Variables were manually selected if they contributed significance to the likelihood ratio statistic for Type 3 analysis at an alpha level of <0.05. IAV seropositivity rate for sex and age group of feral swine, month, fiscal year, and state of sample collection were analyzed as individuals and feral swine groups and best fit was assigned based on QIC (Quasilikelihood under the Independence model Criterion). Feral swine groups were defined to eliminate confounding variables; we grouped samples if collected on the same date at the same location; in theory, swine from the same group would be exposed to the same virus. In addition, an odds ratio (OR) was estimated using the GLIMMIX procedure for generalized linear mixed models with binomial distribution and logit link function. Observations from states with less than 100 samples were excluded from the analysis. We obtained population data for domestic swine and poultry per state from the 2012 USDA Census of Agriculture (https://www.agcensus.usda.gov/Publications/2012/). Small domestic swine farms were considered as those which had less than or equal to 1000 total

swine. All statistical analyses were conducted in SAS9.5 (SAS Institute Inc., Cary, NC, USA).

Results

IAVs exposure in feral swine.

To evaluate the overall seropositivity rate of IAVs among feral swine, we used 8,239 serum samples collected across 35 US states during October 1, 2009–September 30, 2013; this collection period included FY2010–FY2013 (Figure 1; Table 1). Serologic testing by the IDEXX AI MultiS-Screen Ab test suggested that 4.9% (406) of the samples were IAV-positive.

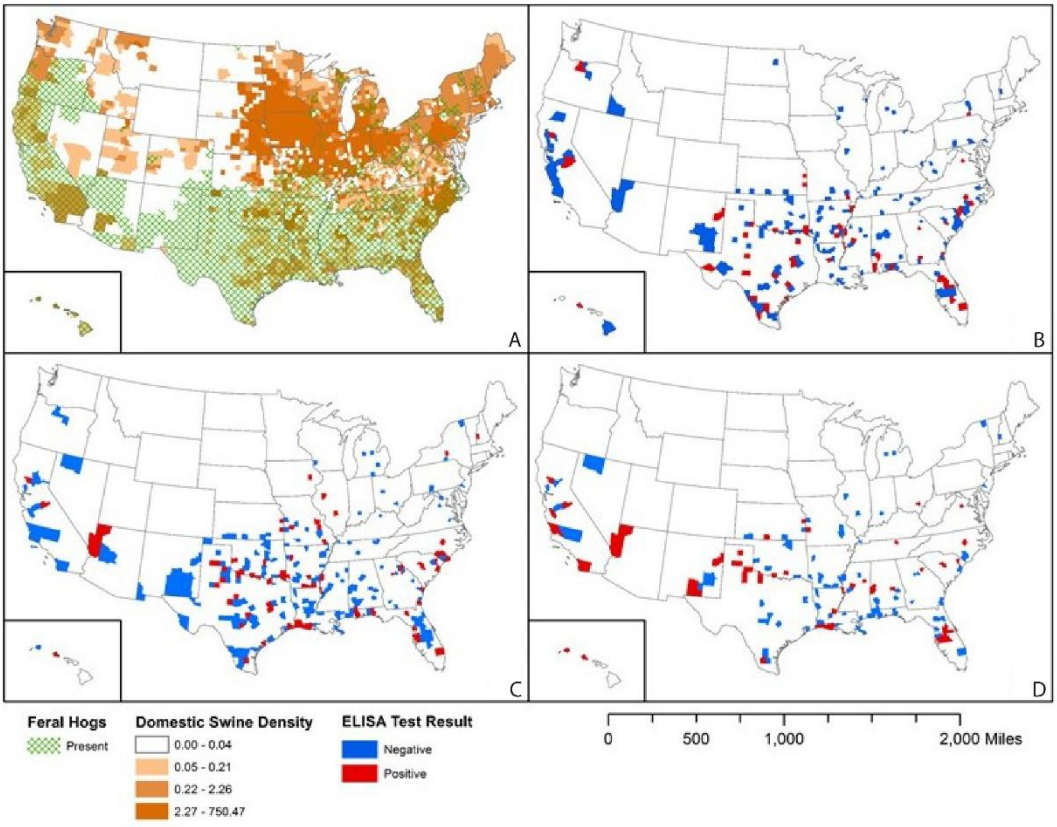

Figure 1 Geographic distribution of swine and of influenza A virus (IAV)–positive and IAV-negative serum samples per county collected from feral swine across the United States during fiscal years (FY) 2010–2013.

A) Distribution of feral and domestic swine, and the density unit was 1,000,000. B–D) Distributions of IAV ELISA–negative and –positive feral swine serum samples collected in FY2011 (October 1, 2010–September 30, 2011) (B), FY2012 (October 1, 2011–September 30, 2012) (C), and FY2013 (October 1, 2012–September 30, 2013) (D).

An association was identified between fiscal years and IAV seropositivity rate ($p = 0.0002$) and months and IAV seropositivity rate ($p < 0.0001$) for individual feral swine but not for groups ($p = 0.1717$ and $p = 0.1184$ respectively). An analysis of seropositivity rate by month determined that during FY2010 (October 1, 2009–September 30, 2010), the highest and lowest seropositivity rates were detected among samples collected in

April (12.3%, 20 of 162 swine) and September (1.8%, 1 of 57 swine), respectively. This temporal pattern, with a relatively higher IAV seropositive rate in spring and winter, was similar to the patterns seen in FY2011 and FY2012 and to those seen in FY2013 in our previous report (85) (Figure 2A).

To understand variables that are associated with IAV seropositivity among feral swine, we analyzed our results by swine age group and sex. IAV seropositivity rate was highest among the adult swine (5.8%, 350/5,984) and lowest among juvenile swine (1.6%, 3/190); this pattern was consistent for all years; 2010 had the largest percent positives only due to having the smallest sampling of juveniles (14.29%; 1/7 samples), (Table 3). We identified an association between swine age group and IAV seropositivity rate ($p < 0.0001$) for both individual swine and groups. In addition, IAV seropositivity rate was higher among female (5.4%, 233/4,333) than male (4.5%, 173/3,871) swine; this pattern was consistent for all fiscal years except FY2011 (Table 3), in which seropositivity rate was higher among male (4.1%; 48/1,170) than female (3.7%; 47/1,287) swine. However, no association was identified between sex and IAV-positive samples for individual swine ($p = 0.0583$) and groups ($p = 0.0665$). Overall, most IAV-percent positive samples were from adult (86.2%) and female (57.4%) feral swine (Figure 2B).

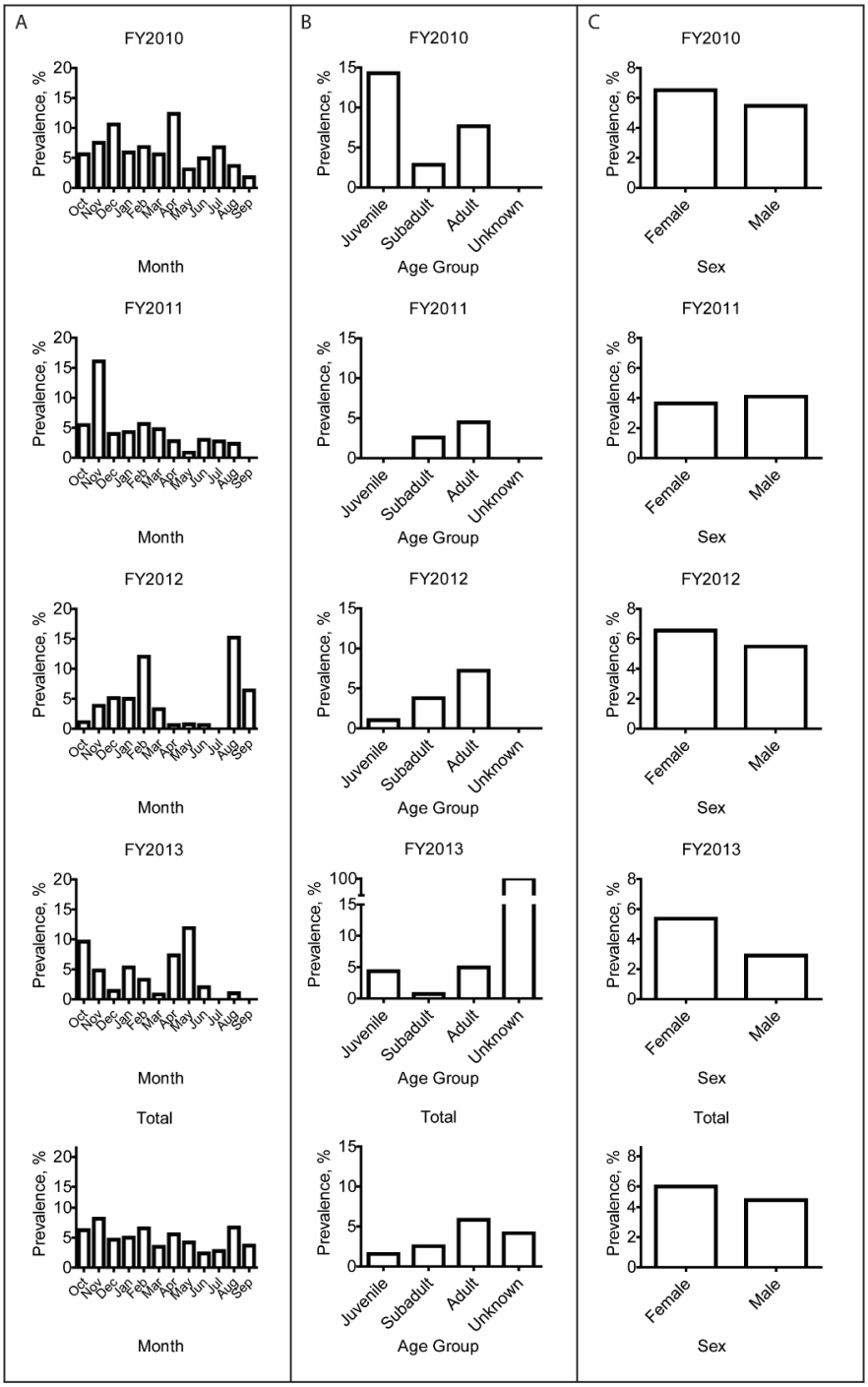

Figure 2 Epidemiologic analyses of the percentage of influenza A virus–positive feral swine serum samples collected across the United States during fiscal years (FY) 2010–2013.

Samples were determined to be positive by ELISA. A) Temporal distribution of positive serum samples. B) Age distribution of feral swine with positive serum samples. C) Sex distribution of feral swine with positive serum samples. FYs run from October 1st of one year through September 30th of the next year; the FY is named according to the second year.

Table 3 Total number of influenza A virus-positive (N=406) and total number of feral swine serum samples collected during fiscal years 2010-2013* for month, age group, and sex.

	No. positive samples/total no. samples† (%)				
Month	2010	2011	2012	2013	Total
January	13/220 (5.91)	16/373 (4.29)	4/80 (5.00)	10/188 (5.32)	43/861 (4.99)
February	16/235 (6.81)	19/337 (5.64)	25/208 (12.02)	9/274 (3.28)	69/1054 (6.55)
March	14/250 (5.60)	18/378 (4.76)	8/245 (3.27)	3/364 (0.82)	43/1237 (3.48)
April	20/162 (12.35)	7/253 (2.77)	1/159 (0.63)	16/218 (7.34)	44/792 (5.56)
May	5/162 (3.09)	2/234 (0.85)	1/131 (0.76)	22/185 (11.89)	30/712 (4.21)
June	4/81 (4.94)	4/134 (2.99)	1/153 (0.65)	2/99 (2.02)	11/467 (2.36)
July	9/133 (6.77)	5/184 (2.72)	0/86 (0.00)	0/101 (0.00)	14/504 (2.78)
August	9/246 (3.66)	4/170 (2.35)	47/309 (15.21)	2/201 (1.00)	62/926 (6.70)
September	1/57 (1.75)	0/112 (0.00)	18/280 (6.43)	0/67 (0.00)	19/516 (3.68)
October	6/107 (5.61)	6/110 (5.45)	1/91 (1.10)	18/187 (9.63)	31/495 (6.26)
November	6/80 (7.50)	9/56 (16.07)	1/26 (3.85)	4/83 (4.82)	20/245 (8.16)
December	9/85 (10.59)	5/126 (3.97)	4/78 (5.13)	2/141 (1.42)	20/430 (4.65)
Age group	2010	2011	2012	2013	Total
Juvenile	1/7 (14.29)	0/63 (0.00)	1/97 (1.03)	1/23 (4.35)	3/190 (1.58)
Subadult	15/526 (2.85)	17/658 (2.58)	17/451 (3.77)	3/406 (0.74)	52/2041 (2.55)
Adult	98/1281 (7.49)	78/1737 (4.49)	93/1288 (7.22)	83/1678 (4.95)	350/5984 (5.85)
Unknown	0/0 (0.00)	0/9 (0.00)	0/10 (0.00)	1/1 (100.00)	1/24 (4.17)
Sex					
Female	63/968 (6.51)	47/1287 (3.65)	64/977 (6.55)	59/1101 (5.36)	233/4333 (5.38)
Male	46/843 (5.81)	48/1170 (4.10)	47/858 (5.48)	29/1000 (2.90)	173/3871 (4.47)
Unknown	0/7 (0.00)	0/10 (0.00)	0/11 (0.00)	0/7 (0.00)	0/35 (0.00)
Total	112/1818 (6.16)	95/2467 (3.85)	111/1846 (6.01)	88/2108 (4.17)	406/8239 (4.93)

* Fiscal years run from October 1st of one year through September 30th of the next year; the FY is named according to the second year.
†A total of 8,239 samples were tested using the IDEXX AI MultiS-Screen Ab Test (IDEXX, Westbrook, Maine, USA).

Although our data set is comprised of samples collected from 35 states, sample sizes were not evenly distributed since the feral swine populations vary widely between states. Consequently only 23 states had samples which tested IAV-positive. The

seropositivity rate of IAV was highest in North Carolina (16.1%, 34/211 samples) and Texas (10.5%, 164/1,561 samples) (Table 4).

Table 4 Total number and number of influenza A virus–positive feral swine serum samples collected from each of 35 US states during fiscal years 2010–2013.*

State	no. positive samples/total no. (%) positive†
Alabama	11/268 (4.10)
Arkansas	7/437 (1.60)
Arizona	1/100 (1.00)
California	39/499 (7.82)
Colorado	0/3 (0.00)
Florida	17/916 (1.86)
Georgia	12/553 (2.17)
Hawaii	36/559 (6.44)
Iowa	1/1 (100.00)
Idaho	0/1 (0.00)
Illinois	4/41 (9.76)
Indiana	0/22 (0.00)
Kansas	10/274 (3.65)
Kentucky	0/41 (0.00)
Louisiana	5/390 (1.28)
Michigan	0/47 (0.00)
Missouri	4/188 (2.13)
Mississippi	7/440 (1.59)
North Carolina	34/211 (16.11)
North Dakota	0/5 (0.00)
New Hampshire	0/20 (0.00)
New Jersey	0/8 (0.00)
New Mexico	2/224 (0.89)
Nevada	0/16 (0.00)
New York	2/63 (3.17)
Ohio	0/45 (0.00)
Oklahoma	27/735 (3.67)
Oregon	1/94 (1.06)
Pennsylvania	0/5 (0.00)

Table 4 (Continued)

South Carolina	14/274 (5.11)
Tennessee	4/127 (3.15)
Texas	164/1,561 (10.51)
Virginia	3/52 (5.77)
Wisconsin	0/4 (0.00)
West Virginia	1/15 (6.67)
Total	406/8,239 (4.93)

* Fiscal years run from October 1st of one year through September 30th of the next year; the FY is named according to the second year.
†A total of 8,239 samples were tested using the IDEXX AI MultiS-Screen Ab Test (IDEXX, Westbrook, Maine, USA).

Of 438 counties, 112 (25.56%) were IAV-positive (Figure 1). Texas had the most IAV-positive samples (40.4%; 164/406 total). Of 31 counties sampled in Texas, the highest seropositivity rates were in Dickens County (36.2%; 42/116 total), Hall County (42.3%; 22/52 total), and Freestone County (35.7%; 5/14 total). The number of IAV-positive samples varied by year, Hall County for example: in 2010, 0 of 1 samples were positive; in 2011, 0 of 12 were positive; in 2012, 6 (30.0%) of 20 were positive; and in 2013, 16 (84.2%) of 19 were positive.

Distinction between swine and avian IAVs.

HI assays were performed on 294 ELISA positive samples and tested against 45 IAVs, including HA subtypes H1–H14 and diverse antigenic clusters of contemporary avian-, swine-, and human-origin IAVs (Table 2). Of note, among the testing strains, there were different extents of cross-reactivity against the reference sera against these

viruses although most of these viruses are antigenically different with ≥4-fold loss in HI activity relative to homologous titer(Table 5).

Table 5 Hemagglutination inhibition cross-reactivity among reference influenza A viruses and homologous antisera.

Virus[ab]	Antigenic group	Serum										
		A/California/04/2009[c]	A/swine/Iowa/18/2013[c]	A/swine/Ohio/09SW96/2009[c]	A/swine/Ohio/10SW215/2010[c]	A/swine/Ohio/11SW347/2011[c]	A/swine/Texas/A01104013/2012[d]	A/Perth/16/2009[c]	A/Victoria/361/2011[c]	A/mallard/Ohio/648/2002[e]	A/American black duck/Delaware/A00870108/2010[e]	A/bufflehead/Virginia/A00120022/2008[e]
A/swine/Minnesota/02093/2008	H1N1-α	20	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Minnesota/A01394082/2013	H1N2-α	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Nebraska/A01399642/2013	H1N1-ß	640	20	<10	20	<10	<10	20	<10	20	<10	<10
A/swine/Nebraska/A01240348/2011	H1N1-ß	80	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Indiana/13TOSU0832/2013	H1N1-γ	80	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Indiana/13TOSU1154/2013	H1N1- γ	640	<10	<10	<10	<10	<10	<10	<10	<10	20	<10

Table 5 (Continued)

A/swine/Illinois/A01076767/2010	H1N1- γ2	20	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/South Dakota/A01349306/2013	H1N1- γ2	640	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Iowa/15/2013	H1 δ1	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Iowa/18/2013	H1 δ2	<10	**2560**	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Iowa/19/2013	H1 δ2	<10	1280	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Iowa/7/2013	H1 2009p	1280	<10	<10	<10	<10	<10	10	<10	<10	<10	<10
A/swine/Iowa/8/2013	H1 2009p	1280	<10	10	<10	<10	<10	<10	<10	<10	<10	<10
A/California/04/2009	H1 2009p	**160**	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Wisconsin/A00751454/2009	H1N1	80	<10	<10	<10	<10	<10	<10	<10	<10	<10	20
A/mallard/Oregan/A0030758/2007	H2N3	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Ohio/09SW96/2009	H3N2 α	<10	<10	**1280**	320	320	80	<10	<10	<10	<10	<10
A/swine/Ohio/10SW215/2010	H3N2 ß	<10	<10	40	**1280**	640	320	<10	<10	<10	<10	<10
A/swine/Ohio/11SW347/2011	H3N2 ß	<10	<10	80	1280	**1280**	640	10	<10	<10	<10	<10
A/swine/Texas/A01104013/2012	H3N2 ß	<10	<10	40	1280	640	**320**	<10	<10	<10	<10	<10
A/Wisconsin/112/2010	H3N2v	<10	<10	80	2560	1280	640	20	<10	<10	<10	<10
A/Pennsylvania/14/2010	H3N2v	<10	<10	160	640	640	640	<10	<10	<10	<10	<10
A/Minnesota/10/2011	H3N2v	<10	<10	40	640	320	320	<10	<10	<10	<10	<10
A/Iowa/07/2011	H3N2v	<10	<10	80	1280	1280	640	20	<10	<10	<10	<10
A/Perth/16/2009	H3N2	<10	<10	<10	<10	<10	20	**320**	80	<10	<10	<10
A/Victoria/361/2011	H3N2	<10	<10	<10	40	<10	40	160	**640**	20	<10	<10
A/mallard/Wisconsin/A00661715/2009	H3N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/blue-winged teal/Colorado/A00170379/2006	H3N8	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Washington/A00714770/2009	H4N6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Wisconsin/10os3845/2010	H5N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Oregon/A00571208/2007	H6N1	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Ohio/648/2002	H6N2	<10	<10	<10	<10	<10	<10	<10	<10	**80**	<10	<10

Table 5 (Continued)

A/American black duck/Delaware/A00870108/2010	H7N3	<10	<10	<10	<10	<10	<10	<10	<10	<10	**80**	160
A/bufflehead/Virginia/A00120022/2008	H7N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	160	**160**
A/northern shoveler/Illinois/10os3632/2010	H8N4	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Minnesota/10os4670/2010	H9N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/northern shoveler/Arkansas/11os386/2011	H9N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/South Dakota/A00536114/2007	H10N7	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Illinois/10OS3249/2010	H11N2	<10	<10	<10	20	<10	<10	<10	<10	40	<10	<10
A/mallard/Wisconsin/10OS2889/2010	H11N9	<10	<10	<10	20	<10	<10	<10	<10	<10	<10	<10
A/American green-winged teal/Missouri/10OS4622/2010	H12N4	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/bufflehead/Wisconsin/10OS3204/2010	H12N5	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/hooded merganser/New Brunswick/03750/2009	H13N6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/white-winged scooter/Wisconsin/10OS3922/2010	H14	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/long-tailed duck/Wisoconsin/10OS3912/2010	H14N6	<10	<10	<10	20	<10	<10	<10	<10	<10	<10	<10

[a]These viruses were also used in a panel against feral swine antisera (n = 45).
[b]Bold indicates homologous titers.
[c]Sera generated in ferrets
[d]Sera generated in feral swine
[e]Sera generated in chickens

Of the 294 feral swine serum samples tested, 271 from 71 counties within 21 states tested positive by HI assay for at least one virus in the reference panel, and 23 samples from 17 counties within 13 states tested negative to all viruses in the reference panel. Of the feral swine samples tested 38.4% were positive against H1 swine IAVs (113 out of 294), and 53.7% were positive against H3 swine IAVs (158 out of 294). Totally, 52 (17.7%) were positive to both H1 and H3 swine IAVs; among these H1- and H3-positive samples, 106 (36.1%) and 233 (79.3%) were also positive against H1 and H3 human IAVs, respectively (Figure 3). The serologic characterization suggests that the swine-origin IAVs in H3α and H3ß clusters cross-reacted with the ferret reference sera against H3 seasonal and A(H3N2)v human IAVs, and vice versa (Table 6).

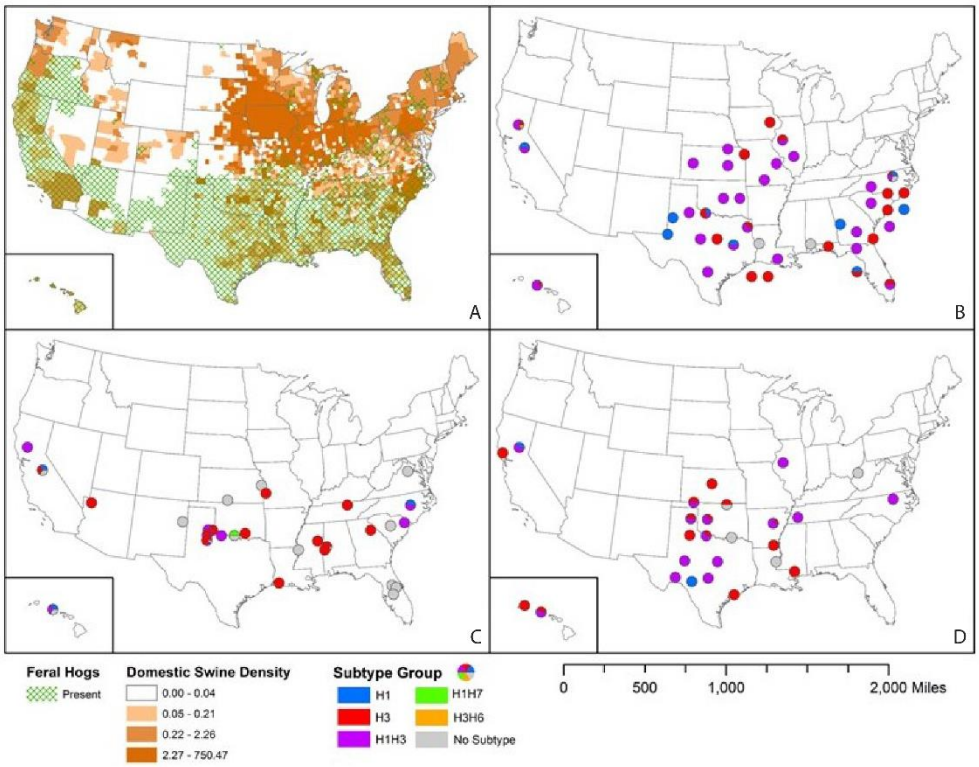

Figure 3 Geographic distribution of domestic and feral swine across the United States and distribution of influenza A virus (IAV)–positive serum samples (by hemagglutination [HA] subtype) collected from feral swine during fiscal years (FYs) 2011–2013.

A) Distribution of feral and domestic swine, and the density unit was 1,000,000. HA subtypes were determined by hemagglutination inhibition assay for FY2011 (B), FY2012 (C), and FY2013 (D). FYs run from October 1st of one year through September 30th of the next year; the FY is named according to the second year. Dots (pie charts) indicate US counties where samples positive for different IAV subtypes were collected.

Table 6 Virus neutralization assay of feral swine sera HI seropositive against avian influenza viruses.

Virus[a]	Antigenic group	Serum ID0003350[b]	ID0005300	ID0006148	ID0007830	ID0008267	ID0008278	ID0008850	ID0016217	ID0016798	ID0016850	ID0017995	ID0018023	ID0018061	ID0018062	R10003296	R10008481
A/swine/Minnesota/02093/2008	H1N1-α	<10	<10	40	<10	320	1280	1280	160	160	160	<10	320	16	40	320	<10
A/swine/Minnesota/A01394082/2013	H1N2-α	<10	<10	20	<10	40	80	80	20	40	<10	<10	<10	<10	20	320	<10
A/swine/Nebraska/A01399642/2013	H1N1-ß	<10	<10	160	<10	1280	1280	320	80	320	320	80	1280	80	160	1280	<10
A/swine/Nebraska/A01240348/2011	H1N1-ß	<10	<10	320	<10	1280	1280	1280	80	320	160	80	320	80	80	1280	<10
A/swine/Indiana/13TOSU0832/2013	H1N1-γ	<10	<10	<10	<10	1280	1280	1280	160	320	320	160	1280	160	80	1280	<10
A/swine/Indiana/13TOSU1154/2013	H1N1- γ	<10	<10	<10	<10	1280	1280	320	160	160	320	80	1280	160	20	1280	<10
A/swine/Illinois/A01076767/2010	H1N1- γ2	<10	<10	<10	<10	<10	40	<10	<10	<10	<10	<10	<10	<10	<10	40	<10
A/swine/South Dakota/A01349306/2013	H1N1- γ2	<10	<10	320	<10	320	1280	1280	160	1280	160	320	1280	1280	160	1280	<10
A/swine/Iowa/15/2013	H1 δ1	80	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Iowa/18/2013	H1 δ2	<10	<10	<10	<10	<10	<10	<10	<10	<10	40	<10	<10	<10	<10	10	<10
A/swine/Iowa/19/2013	H1 δ2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	10
A/swine/Iowa/7/2013	H1 2009p	<10	<10	160	<10	1280	1280	1280	160	1280	320	160	1280	320	160	640	<10
A/swine/Iowa/8/2013	H1 2009p	<10	<10	1280	<10	1280	1280	1280	1280	1280	1280	320	1280	1280	80	80	<10
A/California/04/2009	H1 2009p	10	<10	160	10	1280	1280	640	160	1280	320	160	1280	320	320	640	<10
A/mallard/Wisconsin/A00751454/2009	H1N1	<10	<10	40	40	160	320	640	40	160	80	40	40	40	40	40	<10
A/mallard/Oregan/A0030758/2007	H2N3	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/swine/Ohio/09SW96/2009	H3N2 α	<10	1280	320	<10	<10	<10	<10	40	<10	<10	<10	<10	<10	<10	160	320
A/swine/Ohio/10SW215/2010	H3N2 ß	<10	1280	320	<10	<10	<10	<10	20	<10	<10	40	<10	<10	40	80	<10
A/swine/Ohio/11SW347/2011	H3N2 ß	<10	1280	1280	<10	<10	<10	<10	10	<10	<10	<10	<10	<10	20	20	<10
A/swine/Texas/A01104013/2012	H3N2 ß	10	1280	320	20	<10	<10	<10	20	<10	<10	40	<10	<10	40	40	1280
A/Wisconsin/112/2010	H3N2v	10	1280	640	20	<10	<10	<10	40	<10	<10	40	<10	<10	40	160	640
A/Pennsylvania/14/2010	H3N2v	10	320	320	20	<10	<10	<10	10	<10	<10	<10	<10	<10	<10	80	160
A/Minnesota/10/2011	H3N2v	10	160	160	20	<10	<10	<10	<10	<10	<10	80	<10	<10	20	40	320
A/Iowa/07/2011	H3N2v	10	640	320	20	<10	<10	<10	20	<10	<10	40	<10	<10	40	80	320
A/Perth/16/2009	H3N2	10	40	160	10	<10	80	80	40	40	40	80	160	80	80	80	40
A/Victoria/361/2011	H3N2	<10	80	160	10	40	160	80	40	40	80	20	160	320	320	160	80
A/mallard/Wisconsin/A00661715/2009	H3N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	80

Table 6 (Continued)

A/blue-winged teal/Colorado/A00170379/2006	H3N8	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Washington/A00714770/2009	H4N6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Wisconsin/10os3845/2010	H5N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Oregon/A00571208/2007	H6N1	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Ohio/648/2002	H6N2	<10	40	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/American black duck/Delaware/A00870108/2010	H7N3	20	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/bufflehead/Virginia/A00120022/2008	H7N2	40	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/northern shoveler/Illinois/10os3632/2010	H8N4	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Minnesota/10os4670/2010	H9N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/northern shoveler/Arkansas/11os386/2011	H9N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/South Dakota/A00536114/2007	H10N7	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Illinois/10OS3249/2010	H11N2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/mallard/Wisconsin/10OS2889/2010	H11N9	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/American green-winged teal/Missouri/10OS4622/2010	H12N4	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/bufflehead/Wisconsin/10OS3204/2010	H12N5	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/hooded merganser/New Brunswick/03750/2009	H13N6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/white-winged scooter/Wisconsin/10OS3922/2010	H14	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/long-tailed duck/Wisoconsin/10OS3912/2010	H14N6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

[a]These viruses were also used in a panel against feral swine antisera (n = 45).

[b]Identification number

Although 271 of the 294 feral swine serum samples tested HI positive to swine and human IAV, only 16 (5.4%) cross-reacted to one of the four avian IAVs included in the reference panel: 13 (4.4%) samples cross-reacted to subtype H1 virus, one (0.3%) cross-reacted to H3 virus, and one (0.3%) each cross-reacted to H6 or H7 virus (Table 2). HI with reference sera showed H1 avian IAVs cross-reacted with ferret reference sera against H1N1 human IAVs and that avian subtypes H3N2, H6N2, and H7N3 did not cross-react with the reference sera against the testing human and swine IAVs. To confirm the HI results, we performed neutralization inhibition assays, which also showed that the 16 feral swine serum samples were indeed cross-reactive against avian IAVs (Table 6).

Distribution of avian subtypes detected in feral swine.

Sixteen feral swine serum samples from 10 counties in six US states were positive for avian IAV (Figure 3). The states with the highest number of positive samples were Texas (5), California (4), and Hawaii (4); the remaining samples were from Iowa, Kansas, and Ohio. The surveillance year with the highest number of avian IAV–positive samples was 2013 (7 samples), followed by 2011 (6 samples) and 2012 (3 samples). Thirteen samples were positive for avian subtype H1. Linn County, Iowa, was the only location with an avian H3 subtype. Colusa County, California, was the only location with an avian H6 subtype, and Jefferson County, Oklahoma, was the only place where an avian H7 subtype was identified.

Factors associated with IAV seroprevalance in feral swine.

Using manual forward variable selection to determine factors that affect the likelihood for IAV exposure for IAV exposure in feral swine, we first tested four

variables using a multivariable model: the number of domestic swine farms, small domestic swine farms, poultry farms, and human population per state. We considered farms with 1,000 or fewer domestic swine to be small farms. Small farms were of interest due to less biosecurity and increased chance for contact with feral swine. Individually, the number of domestic swine farms, small domestic swine farms, and poultry farms were significant ($p < 0.05$) but the number of small domestic swine farms had a better fit based on QIC (2.29). The number of domestic swine farms was highly correlated with the other variables and were confounded, making each not significant. There were more IAV seropositive samples from states with more small domestic swine farms.

Discussion

Feral swine are a potential reservoir for infectious pathogens of domestic swine, including IAVs, because bidirectional transmission of pathogens occurs through direct and indirect contact between feral and domestic swine, primarily through backyard farming operations with poor biosecurity (186). A previous study suggested that IAV circulating among feral swine are antigenically and genetically similar to those circulating among domestic swine (85). Laboratory experiments have demonstrated that swine IAV can infect feral swine and transmit efficiently among them (183). In addition, feral swine may be exposed to avian IAVs through direct and indirect contact with wild birds via scavenging or preying and by using common sources of water and forage. Our study findings confirm that although feral swine may be exposed to swine or avian IAVs, exposure to swine IAVs is much more common, especially with subtypes H1 and H3. Exposure to avian IAV was rare from our finding reported here; yet, there is concern that

feral swine could have a mixed IAV infection, and generate reassortants between swine and avian IAVs that could ultimately be transmitted to domestic swine or humans.

Based on serological evidence, our findings suggest that IAV-positive feral swine in the United States were predominantly exposed to subtypes H1 and H3. H3N2 and H1N1 IAVs have been isolated from feral swine and are genetically close to endemic domestic swine IAVs. It is unclear if there is an epidemiological link; however, this finding is consistent with a scenario where domestic swine IAV's occasionally spill over into feral swine populations. Feral swine may be more likely to have contact with domestic swine in backyards or small farming operations that have less biosecurity than large swine operations and it is possible that direct or indirect (i.e. through fomites) transmission occur between feral swine and domestic swine. Another possible source of domestic-like IAVs in feral swine could be from escaped infected domestic swine, however, the recruitment rate of domestic swine into the feral swine population is not clear. Additionally, some feral swine serum samples cross-reacted with both H1 and H3 subtypes of IAVs. These findings indicate that these swine could have been exposed to more than one IAV and is consistent with previously reported findings (85). These possibilities need to be investigated by isolating IAVs currently circulating in feral swine and comparing their genetics to those of nearby IAVs circulating in the domestic swine populations.

A significant portion of the tested feral swine serum samples (78.57%; 231/294) cross-reacted with human-origin IAVs, including H1N1 and H3N2 viruses. The source of feral swine exposure to human H1N1 and H3N2 viruses is unknown. In 1934, Elkeles demonstrated the susceptibility of swine to human influenza virus strains (26). In the past

five decades, genomic analyses suggested at least 20 introductions of IAVs from humans to swine, the majority being human seasonal subtype H3N2 viruses (27). Human-to-swine transmission of influenza A(H1N1)pdm09 virus was determined in domestic swine approximately one month after the virus was detected in humans (28). After this "reverse zoonosis" event, A(H1N1)pdm09 virus co-circulated with endemic swine influenza virus, including triple-reassortant H3N2, human-origin H1N2 (H1 δ1), and classical H1N1 (H1 γ) swine influenza viruses (29), resulting in reassortment events (30-33). Additionally, in our reference sera panel, H3N2 seasonal viruses did not cross-react with any reference sera against any contemporary H3 swine IAVs, this is consistent with the H3 human-like viruses found in domestic swine(187). As early as 2010, within the domestic swine population, novel HAs of H3 viruses emerged and were most genetically similar to human H3N2 strains from the 2010-2011 season; this spillover event of human H3N2 into swine is currently being sustained within the domestic swine population.

In this study, only 4.9% of sera samples were IAV-positive, which is less than previously reported(85), yet a more stringent ELISA cutoff (S/N ≤0.681) was used among 4 years of sample collection; we believe this more truly represents the true seropositivity rate of IAV in the feral swine population. The nucleoprotein for influenza of different species can have up to 18.5% nucleotide differences(188). Therefore, the IDEXX AI MultiS-Screen Ab Test may not be appropriate for use in feral swine due to the low seropositivity rate of avian influenza in feral swine and the IDEXX Swine Influenza Virus Ab Test may be used for further studies.

In summary, feral swine were predominantly exposed to H1 and H3 swine IAV, with only 5.4% of IAV seropositive samples cross-reacted with avian IAV. Thus, there is

still potential for feral swine to generate novel IAVs by hosting and reassorting IAVs from wild birds with those from domestic swine and facilitating adaptation of avian IAVs before their spillover to other hosts, including humans. Continued surveillance is warranted to monitor the distribution and genomic/antigenic diversity of IAVs in feral swine to assess their risk to human health and commercial livestock producers.

CHAPTER III

DETECTION OF INFLUENZA ANTIGENIC VARIANTS DIRECTLY FROM CLINICAL SAMPLES USING POLYCLONAL ANTIBODY BASED PROXIMITY LIGATION ASSAYS

Identification of antigenic variants is the key to a successful influenza vaccination program. The empirical serological methods to determine influenza antigenic properties require viral propagation. Here a novel quantitative PCR-based antigenic characterization method using polyclonal antibody and proximity ligation assays, or so-called polyPLA, was developed and validated. This method can detect a viral titer that is less than 1,000 $TCID_{50}$/mL. Not only can this method differentiate between different HA subtypes of influenza viruses but also effectively identify antigenic drift events within the same HA subtype of influenza viruses. Applications in H3N2 seasonal influenza data showed that the results from this novel method are consistent with those from the conventional serological assays. This method is not limited to the detection of antigenic variants in influenza but also other pathogens. It has the potential to be applied through a large-scale platform in disease surveillance requiring minimal biosafety and directly using clinical samples.

Introduction

The influenza virus is a perpetual threat to public health. Seasonal influenza infections are associated with thousands of deaths every year in the United States (1). A

worldwide pandemic could increase the death toll to millions in a short period of time. The hallmark of the influenza virus is antigenic variation, which comes in two forms: antigenic drift and antigenic shift; leading to the recurrence of influenza virus infections (98). Mutations in the hemagglutinin and neuraminidase glycoproteins cause antigenic drift. Meanwhile, antigenic shift is caused by the replacement of a new subtype of hemagglutinin and sometimes neuraminidase through genetic reassortment.

The influenza vaccine is the most viable option in counteracting and reducing the impact of influenza outbreaks (189). Since influenza viruses are continuously changing their antigenicity in order to escape the host immunity (3, 4), the vaccine strains need to be updated almost annually to obtain antigenic matches between the vaccine strain and the strain potentially causing future outbreaks (6, 190). Identification of influenza antigenic variants is the key to a successful influenza vaccination program for both pandemic preparedness as well as seasonal influenza prevention and control (98).

Routinely, immunological tests, such as hemagglutination inhibition (HI) assays and microneutralization (MN) assays, have been relied upon to identify antigenic variants among the circulating strains (122). The HI assay is an experiment to measure the level to which a test influenza antigen and a reference antigen (e.g. a current vaccine strain) match through the immunological reaction between the test antigen and the reference antiserum. This reference antiserum is usually generated in ferrets using the reference antigen. HI assays are limited due to their use of red blood cells (RBCs), e.g. turkey red blood cells, as indicators for the binding affinity of antigen and antiserum (191). A higher interaction between antigen and antisera will lead to less hemagglutination of RBCs (192). Compared to HI assays, MN assays seem to be more sensitive and specific but are

much more time-consuming. Moreover, for influenza viruses requiring biosafety-level 3 (BSL-3) or higher, MN assays are difficult to perform (193). For this reason, HI assays have been one of the routine procedures used to identify influenza antigenic variants for vaccine strain selection while MN assays are generally used to validate the results from HI assays.

However, the data from HI assays are notoriously noisy, and HI experiments are affected by many factors. For example, RBCs used from different species and even variation in RBC sialic acid receptors can produce varied results (122). The data are subjective interpretations and the HI assays have difficulty in automating and standardizing operations. Minor antigenic variants within a heterogeneous population cannot be assessed by the serological method of HI (132). More importantly, mutations of the receptor binding site in HA (4) (antigenic drift) are causing human seasonal H1N1 (100, 123) and H3N2 influenza A viruses (124) to lose the ability to bind to RBCs. For example, the mutations at residue 193, 196, 197, and 225 in the human epidemic H1N1 influenza A viruses in 1988 or later caused the loss of their abilities to agglutinate chicken RBCs due to four amino acid changes (125). For H3N2 viruses, the Gly190Asp substitution has been correlated to the loss of the ability to agglutinate chicken erythrocytes (122, 124, 126, 128-130, 194). Since 2000, human seasonal H1N1 and H3N2 influenza A viruses have been losing their binding abilities to turkey red blood cells (122, 131). This may be attributed to a reduced affinity for sialic acid-linked receptors (particularly α2-6-linked receptors), which are at lower levels on chicken and turkey RBC compared to levels on guinea pig RBC (122, 131). Consequently, a critical

demand exists for the development of a red blood cell independent assay for influenza antigenic variation.

Proximity ligation assay utilizes quantitative real time PCR (qRT-PCR) for the detection of antigen-antibody interaction (148). For this assay: (1) oligonucleotide-linked monoclonal antibodies are incubated with the analyte in question; (2) if in close proximity, the oligonucleotides can be ligated together; and (3) presence of analyte will be shown by amplification of ligated products with qRT-PCR. The assay reporter signal is dependent on a proximal and dual recognition of each target analyte providing high specificity (149).

In this study, we developed a novel antigenic characterization method using polyclonal antibody-based proximity ligation assays (polyPLA). This method was found to be useful in detecting influenza antigenic variants in clinical samples.

Materials and Methods

Viruses and antibodies

The H3N2 viruses used in this study were obtained from the Centers of Disease Control and Prevention, Department of Health & Human Services and BEI Research Resources Repository (http://www.beiresources.org/) (Table 7), and the monoclonal antibodies against nucleoprotein (NP) from Millipore, the United States. The viruses were propagated at Madin-Darby Canine Kidney (MDCK) cells and stored at -80 °C before usage. The polyclonal antisera were generated using 4- to 6-month-old ferrets (Triple F Farm, PA). The ferrets were anesthetized with isoflurane and inoculated intranasally with 10^6 50% egg infectious doses (EID_{50}) of a challenging virus. The ferret

sera were collected three weeks post-infection. The viral isolation was performed using MDCK cells.

Table 7 The H3N2 influenza A viruses used in this study.

Virus	Abbreviation	Antigenic Cluster[a]
A/Sichuan/2/87(H3N2)	SI/2	ND
A/Johannesburg/33/94(H3N2)	JO/33	BE92
A/Nanchang/933/95(H3N2)	NA/933	WU95
A/Sydney/05/97(H3N2)	SY/05	SY97
A/Brisbane/10/07(H3N2)	BR/10	BR07
A/Perth/16/09(H3N2)	PE/16	PE09
A/Victoria/361/11(H3N2)	VI/361	VI11

[a]the antigenic cluster was described in Sun et al. (195), and ND, not determined.

Labeling of antibodies.

The IgG was purified from ferret polyclonal antisera and mice monoclonal antibodies using Pierce Chromatography Cartridges Protein A/G according to the manufacturer's instruction (Pierce, Rockford, IL) and then biotinylated with Biotin-XX Microscale Protein Labeling Kit according to manufacturer directions (Life Technologies, Carlsbad, CA). To remove the free biotin, Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific Pierce, Rockford, IL) were used; dialysis was performed at 4 ºC in cold 1X PBS (pH 7.4), and the buffer was changed at least 5 times each 2 hours.

Forced proximity probe test.

An aliquot of biotinylated antibody stock solution was diluted to 200 nM (30 µg/mL); 2 µL of diluted biotinylated antibody was added to 2 µL of equal mixture of 200 nM 3' and 5' TaqMan Prox-Oligo, designated probe A and probe B (Life Technologies, Carlsbad, CA), and incubated at room temperature for 60 minutes. A negative control was made replacing diluted biotinylated antibody with 2 µL antibody dilution buffer. After incubation, 396 µL of assay probe dilution buffer was added and incubated for another 30 minutes at room temperature. Then 96 µL of ligation solution was added to 4 µL of the probe and virus mixture, incubated again at 37 ºC for 10 minutes, and cooled at 4 ºC for 10 minutes. After incubation, 9 µL were transferred to a new 0.2 mL microcentrifuge tube with 11 µL qPCR mix (10 µL Fast Master Mix, 2x plus 1 µL Univeral PCR Assay, 20x) and was briefly centrifuged. The qPCR cycling was as follows: 95 ºC for 2 minutes, 40 cycles of 95 ºC for 15 seconds, and 60 ºC for 1 minute.

The change in threshold cycle (ΔCt) values was calculated for each biotinylated antibody: Average Ct (negative control) – Average Ct (forced proximity probe). If the $\Delta Ct \geq 8.5$, the test biotinylated antibody was considered suitable for use in the PLA.

Probe preparation.

Two assay probes for each antibody were prepared by combining the biotinylated antibodies with either 3' or 5' TaqMan Prox-Oligo (probe A and probe B). For example, for each probe, 2.5 µL 200 nM biotinylated antibody was combined with 2.5 µL of either 200 nM 3' prox-Oligo or 200 nM 5' prox-Oligo, and the mixture was incubated at room temperature for 60 minutes. Then, 45 µL Assay Probe Storage Buffer was added, briefly centrifuged, and incubated at room temperature for 20 minutes.

PLA and quantification of polyΔCt and monoΔCt.

The Taqman PLA was performed by first diluting equal parts of probes A and B mixture 1:10 with phosphate-buffered saline (PBS, pH 7.4). For the non-protein control (NPC), 2 µL were combined with 2 µL diluted virus (virus lyzed for NP detection) or 2 µL 1x PBS, pH 7.4. The mixture was centrifuged briefly and incubated at 37ºC for one hour. Then, 96 µL of ligation solution was added to 4 µL of the probe and virus mixture, incubated again at 37 ºC for 10 minutes, and cooled at 4 ºC up to 10 minutes. After incubation, 2 µL of 1x protease was added to each ligation reaction and incubated at 37ºC for 10 minutes, 95 ºC for 5 minutes, and 4ºC for holding. Lastly, 9 µL of the product was transferred to a new 0.2 mL microcentrifuge tube with 11 µL qPCR mix (10 µL Fast Master Mix, 2x plus 1 µL Universal PCR Assay, 20x) and was briefly centrifuged. The qPCR cycling was as follows: 95 ºC for 2 minutes, 45 cycles of 95 ºC for 15 seconds, and 60 ºC for 1 minute.

The NPC was used as a reference background and the threshold cycle (Ct) value given dictated the non-target ligation signal noise of the assay. A total of three replicates for each sample and control were performed. To calculate the ΔCt values: Average Ct (NPC) – Average Ct (sample), which represented the true target-mediated signal above background. The cutoff of $\Delta Ct \geq 3.00$ was used for qualitative analysis of viral and antibody binding, as according to the Taqman Protein Assays Sample Prep and Protocol(196).

HI and virus neutralization assays.

In the HI assay, the receptor destroying enzyme (RDE, Denka Seiken Co., Japan) was used to treat the ferret sera in the ratio of 1:3 (RDE : sera, volume : volume) for 18 hours at 37 ºC, then heat inactivated at 56 ºC for 30 minutes. The treated ferret sera were diluted to 1:10 with phosphate-buffered saline (PBS) then 2-fold serial diluted and reacted with 4-hemmagglutination-units viruses. The HI titers were expressed as the reciprocal of the highest dilution at which virus binding to the 0.5% turkey red blood cells (RBC) was blocked.

For the virus neutralization assay, serially diluted ferret sera were first incubated with 100 $TCID_{50}$ viruses at 37 ºC for 1 hour. The virus-sera mixtures were then adsorbed to MDCK cells for 1 hour. The infected cells were washed twice with PBS buffer and replenished with Opti-Mem Reduced Serum Media (Life Technologies, US). The supernatants from the infected cells were harvested 4 days post-infection and were analyzed using hemagglutination assay.

Data analysis.

To compare the antigenic properties across the testing antigens (viruses), we computed the polyPLA unit between antigen (virus) and antibody using the following equation:

$$\text{polyPLA} = a * (\text{poly}\Delta\text{Ct} - \text{mono}\Delta\text{Ct}) + b \quad (1)$$

To improve our computation, we simply used a =1.00 and b=10.00. The b=10.00 enabled us to avoid negative numbers. If monoΔCt < 3.00, polyPLA will be assigned as "<0", meaning that the viral loads were too low for analyses. As mentioned above, in general, polyPLA is sensitive in detecting viral loads of approximately 10^3 $TCID_{50}$/mL.

Genomic sequencing and GenBank accession number.

The HA genes of 21 H3N2 isolates recovered from human clinical specimens were sequenced using Sanger Sequencing, and they were deposited in Genbank with the accession numbers KM244531-KM244551.

Molecular characterization and phylogenetic analyses.

The multiple sequence alignments were conducted using the MUSCLE software package (197). The phylogenetic analyses were performed using maximum likelihood by GARLI version (198), and bootstrap resampling analyses were conducted with 1,000 runs using PAUP* 4.0 Beta (199) with a neighborhood joining method, as previously described (200).

Results

polyPLA for influenza antigenic variant detection.

polyPLA quantifies the antibody antigen binding avidity using the amplification signals in quantitative PCR (qPCR) from the pairs of primers attached to a reference polyclonal antiserum. The first step of this experiment is to biotinylate a reference polyclonal antiserum (Figure 4A), which will be then labeled with sodium azide-linked 5' and 3' oligonucleotides (Figure 4B). The ligation efficiency will be assessed with qPCR. A labeled polyclonal antiserum with $\Delta Ct \geq 8.5$ in the ligation efficiency test is then incubated with a reference antigen (virus) or a testing antigen (Figure 4C), followed by the proximity ligation of the two oligos (Figure 4D). The antibody antigen binding avidity is quantified using the amplification signals ΔCt in qPCR (Figure 4E). The ΔCt values among the polyclonal antisera and antigens can be compared to assess antigenic

differences among these tested antigens. These ΔCt values can be viewed as similar to the serological titers, such as HI and neutralization titers, from conventional serological assays (Figure 4F).

To make the ΔCt values comparable across reference sera, we have to ensure the testing antigens have the same quantities across quantification assays. In HI assays, we usually standardize the antigens to be 4 or 8 units of hemaglutinnation titer before HI; in neutralization assays, we usually standardize antigen quantities using $TCID_{50}$(196). In this assay, we use the quantities of nucleoproteins (NPs) to normalize the amount of viruses in the analyses. For data consistency, we used a monoclonal antibody targeting conserved regions of NPs in the proximity ligation assay (148). Thus, for a test antigen, the polyPLA units were normalized by its ΔCt values for polyclonal antiserum (polyΔCt) with its ΔCt values for monoclonal antibody against NP (monoΔCt) (Figure 4G).

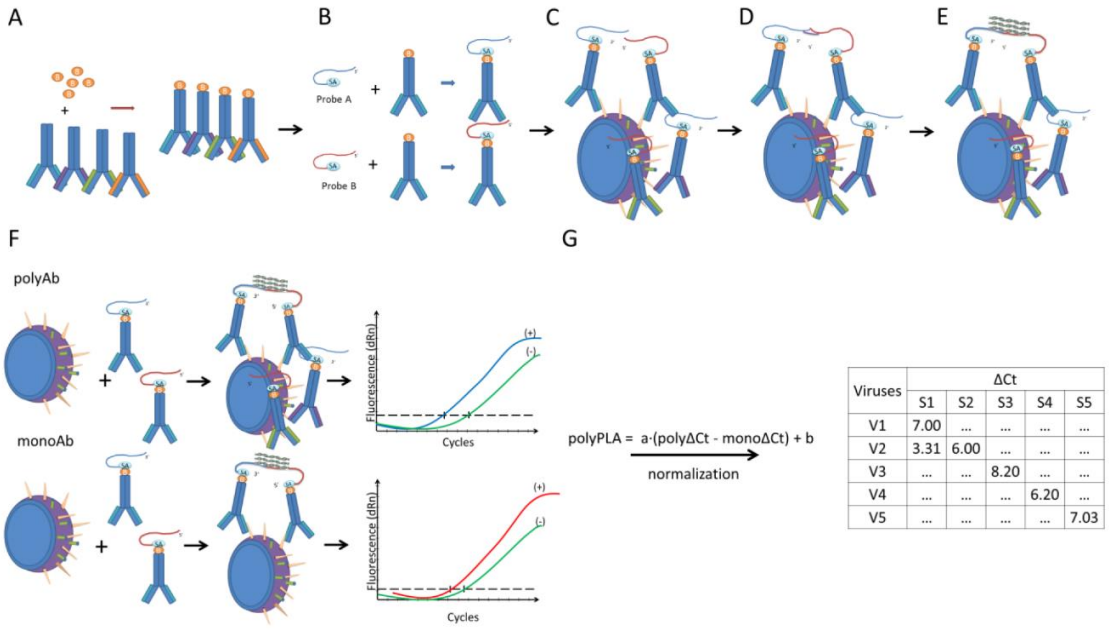

Figure 4 The simplified diagram of polyPLA.

polyPLA quantifies the antibody antigen binding avidity using the amplification signals in quantitative PCR (qPCR) from the pairs of primers attached to a reference polyclonal antiserum. First, the IgG purified from a reference polyclonal antiserum is biotinylated (*A*), which will be then labeled with sodium azide-linked 5' and 3' oligonucleotides (*B*). A labeled polyclonal antiserum with $\Delta Ct \geq 8.5$ in the ligation efficiency test is then incubated with a reference antigen (virus) or a testing antigen (*C*), followed by the approximate ligation of the two oligos (*D*). The antibody antigen binding avidity is quantified using the amplification signals ΔCt in qPCR (*E*). The ΔCt values among the polyclonal antisera and antigens can be compared to assess antigenic differences among these tested antigens, and these ΔCt values can be viewed as similar as the serological titers, such as HI and neutralization titers, from conventional serological assays (*F*). The polyPLA units were normalized by its ΔCt values for polyclonal antiserum (polyΔCt) with its ΔCt values for monoclonal antibody against NP (monoΔCt) (*G*).

Viral particles must be completely lysed to release NPs and allow for an accurate measure of these protein quantities. We compared two commonly used methods for viral lysis: freeze/thaw and treatment with lysis buffer. The results showed that lysis buffer treated virus has a significantly higher ΔCt value of 7.46 (± 0.45) for A/Sydney/05/1997 (SY/05), $p<0.05$ (Figure 5) compared to the freeze/thaw method of viral lysis. However,

for A/Sichuan/2/1987 (SI/2), lysis buffer treated virus did not have a significantly different ΔCt value compared to the freeze/thaw method of viral lysis. In the following assays, all the samples used in normalization were treated with lysis buffer.

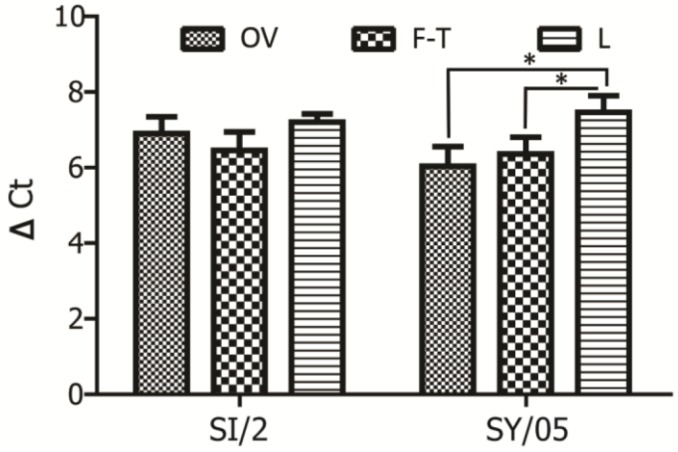

Figure 5 Optimization of the methods in detecting NP proteins using proximity ligation assays.

OV denotes the control the control viruses, which were harvested directly after viral propagation in MDCK cells; F-T denotes the viruses, which were frozen and thawed 5 times; L denotes the viruses, which were treated with lysis buffer.

HA specific IgG predominates polyclonal antisera.

PolyPLA quantifies the interactions between influenza viral proteins and all IgG present in the polyclonal antisera. To assess the impacts of NA and other internal proteins on PolyPLA, we constructed three reassortants between SY/05 and PR8, including SY/05xPR8(H3N2), SY/05xPR8(H3N1), and SY/05xPR8(H1N2). The signals from NPC were used as the control to calculate ΔCt value from proximity ligation assays. Our results showed that SY/05, SY/05xPR8(H3N2), and SY/05xPR8(H3N1) had ΔCt values of 5.40(± 0.74), 5.67(± 0.17), and 5.26(± 0.34), respectively (Figure 6). The ΔCt values from PR8 and the reassortant SY/05xPR8(H1N2) were negligible.

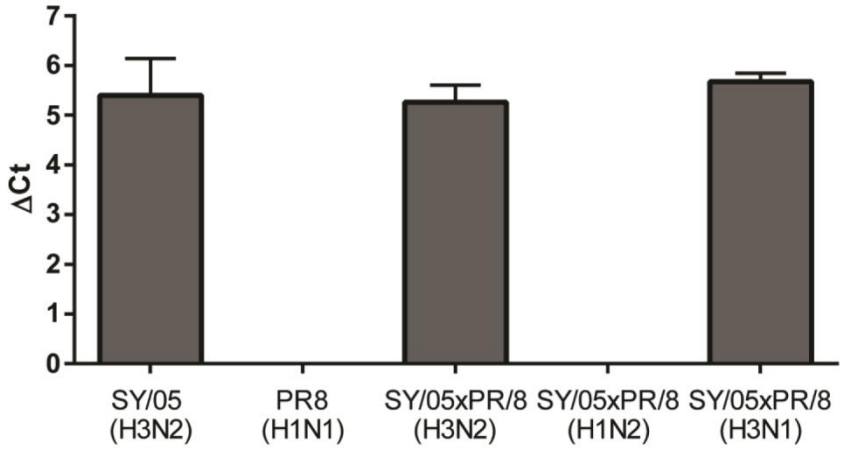

Figure 6 PolyPLA detects predominant IgG against HA gene.

The ΔCt of proximity ligation assays for SY/05(H3N2), PR8(H1N1), and three reassortants SY05xPR8(H3N2), SY05xPR8(H1N2), and SY05xPR8(H3N1) were measured using reference polyclonal sera against SY/05(H3N2).

Viral quantities are linearly correlated with ΔCt values.

To assess the sensitivity of polyPLA, we performed PLA on influenza A viruses with serial dilutions. Regression analyses demonstrated that the polyΔCt values are linearly correlated with the influenza viral quantities, with Pearson's coefficient R = 0.98 for the testing strain SY/05 ($p<0.001$) (Figure 7). The cutoff ΔCt value 3.00 was equivalent to 4.90×10^4 $TCID_{50}$/mL against its homologous polyclonal antibodies. Similarly, the mono-ΔCt values were also linearly correlated with HA titers, and the R was 0.92 for SY/05 ($p<0.001$). The cutoff ΔCt value was equivalent to 9.80×10^4 $TCID_{50}$/mL against the NP-specific monoclonal antibody. Similar linear correlations were also observations in A/Johannesburg/33/1994(H3N2) (JO/33) and A/Nanchang/933/1995(H3N2) (NA/933) (data not shown). Linear correlation between viral quantities and ΔCt allows us to normalize the viral titers by using a simple equation such as a*(polyΔCt-monoΔCt)+b, where a and b are constant parameters. This

normalization method enables us to compare the antigenic properties between the testing antigens (viruses) without justifying the viral quantities before measuring polyΔCt, having been used in HI and neutralization assays to ensure the equivalency of the viral quantities before assays.

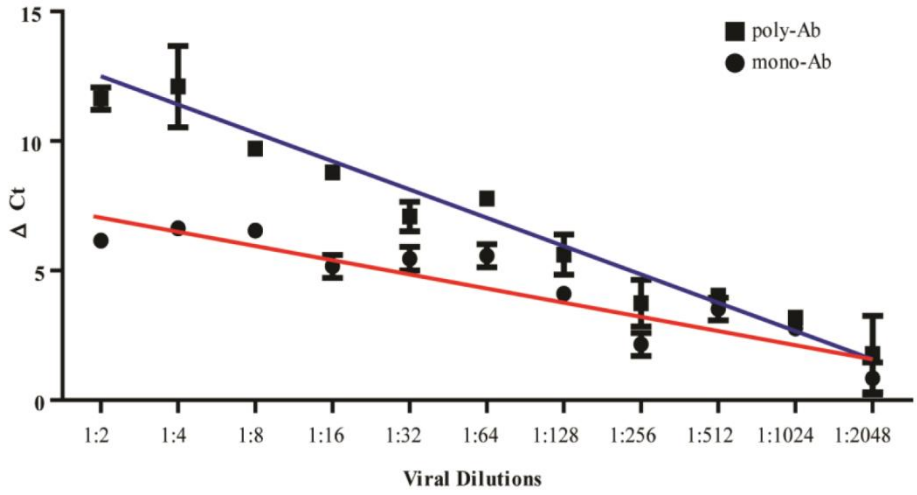

Figure 7 The linear correlation of polyΔCt (R = 0.98) and monoΔCt (R = 0.92) with viral quantities.

The cutoff ΔCt value 3.00 was equivalent to 4.90 x 10^4 $TCID_{50}$/mL against its homologous polyclonal antibodies and 9.80 x 10^4 $TCID_{50}$/mL against NP specific monoclonal antibody.

Sensitivities of polyPLA.

To test the sensitivity of polyPLA, we evaluated the viral loads from nasal swabs collected from ferrets infected with A/swine/K6/2011(H6N6). After only the first day of infection, a polyΔCt titer of 3.20 (± 0.06, standard deviation) was obtained, corresponding to a $TCID_{50}$ titer of 1.00 x 10^3 for the infected ferret (Figure 8). After two days of infection, a polyΔCt value of 5.19 (± 0.06) was obtained, corresponding to a $TCID_{50}$ titer of 1.00 x 10^4. A higher polyΔCt titer corresponded to a higher $TCID_{50}$ titer

among the nasal wash samples post-infection. All the samples collected from the control ferrets had polyΔCt titers of less than 3.00, and no viruses were recovered from these control ferrets (Figure 8). Thus, this method is sensitive sufficiently to detect not only the viruses propagated from the laboratory, but also those in animal specimens. The detection limit is approximately 10^3 $TCID_{50}$/mL, which is much less than the viral loads from most patients at the peak time of virus shedding. For example, man can shed 2.6, 5.0, 5.1, 4.9, 3.8, and 1.9 $TCID_{50}$/mL from one through six days post inoculation of H1N1 seasonal influenza virus, respectively (201).

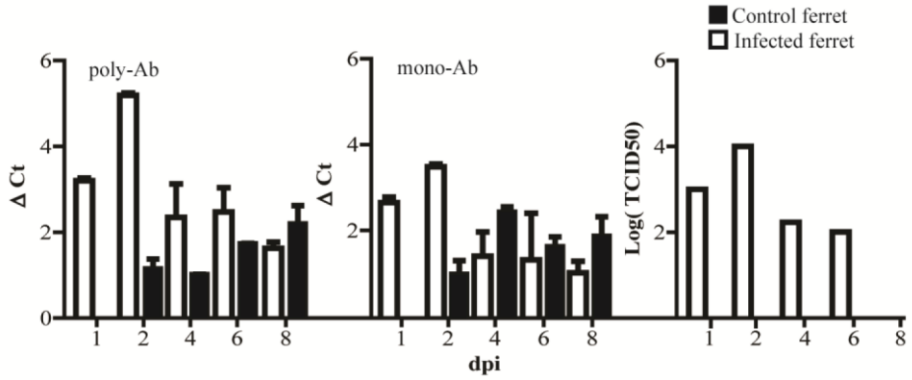

Figure 8 Sensitivity of polyPLA.

Comparison of polyΔCt and monoΔCt titers with viral culture titers using the nasal swabs collected from the ferrets infected with A/swine/Guangdong/K6/2010(H6N6). After only the first day of infection, a polyΔCt value of 3.20 (± 0.06, standard deviation) was obtained, corresponding to a $TCID_{50}$ titer of 1.00 x 10^3 for the infected ferret. After two days of infection, a polyΔCt value of 5.19 (± 0.06) was obtained, corresponding to a $TCID_{50}$ titer of 1.00 x 10^4.

Detecting antigenic variants of H3N2 historical seasonal influenza viruses.

The H3N2 viruses have been causing seasonal epidemic outbreaks since its first introduction into the human population, resulting in the pandemic of 1968. During the past four decades, at least 12 antigenic drift events have been detected; six of which

occurred from 1997 to 2010 (195, 202). In this study, six historical H3N2 isolates, JO/33, NA/933, SY/05, A/Brisbane/10/2007 (BR/10), A/Perth/16/2009 (PE/16), A/Victoria/361/2011(VI/361) representing antigenic cluster BE92, WU95, SY97, BR07, PE09, and VI11 (195), and their corresponding homologous ferret antisera were used to validate polyPLA. Our method was expected to identify significant differences in homologous and heterologous polyPLA titers.

Our results showed that the homologous titers were approximately 10.00 polyPLA units. The polyPLA titers for NA/933 and SY/05 against JO/33 antisera were 9.37 (±0.22) and 6.725 (±0.32) polyPLA units, which were significantly less than the homologous JO/33 titer 11.43 (±0.01) units, $p<0.0001$ (Figure 9A). The homologous titer for NA/933 was 11.71 (±0.28) whereas the titers for JO/33 and SY/05 against NA/933 antisera were 8.44 (±0.32) and 8.16 (±0.43) units, respectively; the titers for JO/33 against NA/933 antisera were significantly less than the homologous titers for JO/33, $p<0.001$. The homologous titer for SY/05 was 13.19 (±0.06) units whereas the titers for JO/33 and NA/933 against SY/05 antisera were 10.59 (±0.15) and 8.21 (±0.07) units, respectively; the titers for both JO/33 and NA/933 against SY/05 antisera were significantly less than the homologous titers for SY/05, $p<0.0001$.

Similarly, the homologous titers for BR/10 were 9.91 (±0.16) whereas the titers for PE/16 and VI/361 against BR/10 sera were 7.97 (±0.12) and 7.72 (±0.21), respectively (Figure 9B); the titers for PE/16 and VI/361 against BR/10 sera were significantly less than the homologous titers for BR/10, $p<0.001$. PE/16 had the highest polyΔCt value of 12.00 (±0.20) against PE/16 sera whereas the titers for BR/10 and VI/361 against PE/16 sera were 7.92 (±0.10) and 10.01 (±0.15), respectively; the titers

for BR/10 and VI/361 against PE/16 sera were significantly less than the homologous titers for PE/16, $p<0.001$. The homologous titer for VI/361 was 10.87 (±0.22) whereas the titers for BR/10 and PE/09 against VI/361 sera were 5.90 (±0.10) and 7.93 (±0.15), respectively; the titers for BR/10 and PE/09 against VI/361 sera were significantly less than the homologous titers for VI/361, $p<0.001$.

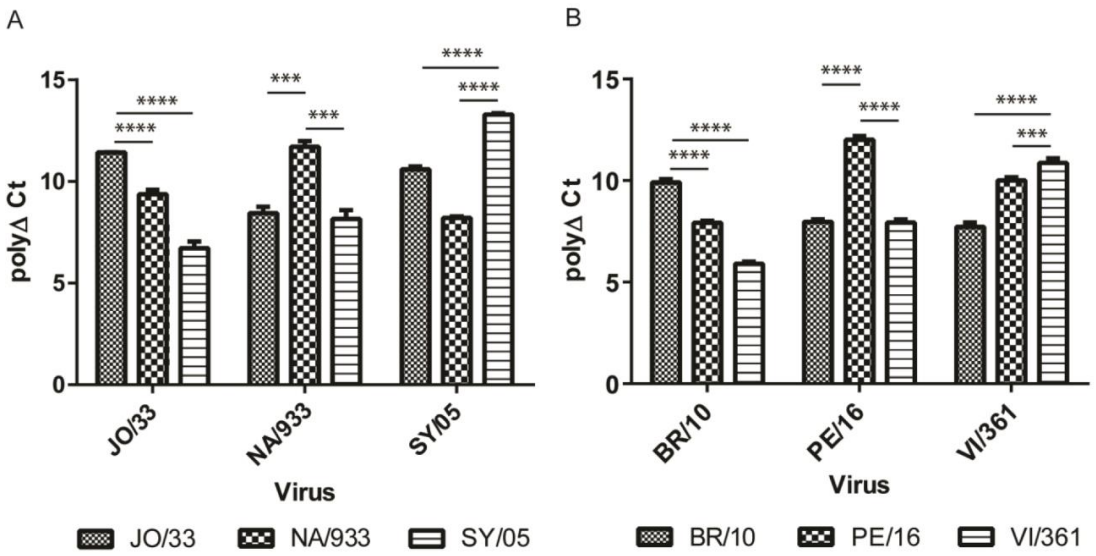

Figure 9 Detecting antigenic variants of H3N2 historical seasonal influenza viruses.

JO/33, NA/933, SY/05, BR/10, PE/16, VI/361 representing antigenic cluster BE92, WU95, SY97, BR07, PE09, and VI11. The homologous polyPLA titers were significantly higher than the heterologous titers for both the antigenic drift event BE92->WU95->WY97 (*A*) and BR07->PE09->VI11(*B*). These results were consistent with those measured from conventional HI and neutralization assays (Tables 8 and 9).

Table 8 The correlation among the titers from polyPLA and those from HI and MN assays for JO/33, NA/33, and SY/05.

	Ferret Antisera[a]								
	JO/33			NA/933			SY/05		
Virus	HI	MN	polyΔCt (Standard Deviation)	HI	MN	polyΔCt (Standard Deviation)	HI	MN	polyΔCt (Standard Deviation)
JO/33	**640**	**ND**	**11.43(0.01)**	40	ND	8.44(0.32)	<10	40	10.59(0.15)
NA/933	160	ND	9.37(0.22)	**1280**	**ND**	**11.71(0.28)**	160	30	8.21(0.07)
SY/05	<10	ND	6.725(0.32)	<10	ND	8.16(0.43)	**1280**	**1280**	**13.29(0.06)**

[a] the number in bold is the homologous titer.

Table 9 The correlation among the titers from polyPLA and those from HI and MN assays for BR/10, PE/16, and VI/361.

	Ferret Antisera[a]								
	BR/10			PE/16			VI/361		
Virus	HI	MN	polyΔCt (Standard Deviation)	HI	MN	polyΔCt (Standard Deviation)	HI	MN	polyΔCt (Standard Deviation)
WI/67	ND	ND		40	160		320	320	
BR/10	**1280**	**2560**	**9.91(0.16)**	40	160	7.92(0.10)	40	40	5.90(0.10)
PE/16	80	80	7.97(0.12)	**640**	**640**	**12.00(0.20)**	160	160	7.93(0.15)
VI/361	20	20	7.72(0.21)	320	640	10.01(0.15)	**640**	**640**	**10.87(0.22)**

[a] the number in bold is the homologous titer.

Detecting antigenic variants in human clinical specimens.

To test the applicability of polyPLA in clinical samples, we applied this same method to characterize antigenic profiles of H3N2 influenza A viruses, which came directly from clinical samples collected in the 2012-2013 influenza season. A total of 100 nasal swabs were collected from September of 2012 to April of 2013, and confirmed as H3N2 positive using quantitative RT-PCR.

About 50% of these samples had at least a 3-polyPLA-unit decrease compared to BR/10 homologous titers and that about 18% and 1% of these samples had at least 3-polyPLA-unit decrease when compared to PE/16 and VI/361 homologous titers, respectively (Figure 10A). The polyPLA titers of the majority clinical samples were highest when using VI/361 sera, followed by PE/16 and BR/10. We compared the polyΔCt titers of the clinical samples with the HI titers for these 21 isolates, and the HI titers were positively correlated with polyΔCt titers (Figure 10B).

To better understand the antigenic and genetic background of the H3N2 viruses in these samples, 21 samples were randomly selected and subjected to viral isolation using MDCK cells. A total of 21 viruses were recovered, and the HA sequences of these viruses were sequenced. The sequence analyses showed that no consistent mutations at the reported antibody binding sites (203) were observed in the isolates we recovered from this study (Table 10).

Table 10 Molecular characterization of amino acid changes in the HA1 proteins of H3N2 isolates recovered from 2012-2013 season.

	Amino acid position[a]																	
	2	8	33	45	48	53	94	128	142	144	145	158	198	199	223	278	280	312
Isolate				(C)	(C)	(C)	(E)	(B)	(A)	(A)	(A)	(B)	(B)			(C)	(C)	(C)
WI/67	K	N	Q	S	T	D	Y	T	R	N	N	K	A	S	I	N	E	N
BR/10	K	N	Q	S	T	D	Y	T	R	N	N	K	A	S	V	N	E	N
PE/16	K	N	Q	S	T	D	Y	T	R	K	N	N	A	S	V	N	E	N
VI/361	K	N	Q	N	I	D	Y	T	R	N	N	N	S	S	I	N	E	S
TX/50	K	N	R	N	I	D	Y	N	R	N	N	N	P	S	I	K	E	S
MS/3	K	N	R	N	I	D	Y	T	R	N	S	N	S	S	I	K	E	S
MS/4	K	N	R	N	I	D	Y	A	G	N	S	N	S	S	I	K	E	S
MS/17	K	N	R	N	I	D	Y	A	G	N	S	N	S	S	I	K	E	S
MS/20	K	N	R	N	I	D	Y	T	R	N	S	N	S	S	I	K	E	S
MS/27	N	N	R	N	I	D	Y	T	R	N	S	N	S	S	I	K	E	S
MS/29	K	N	R	N	I	D	Y	T	R	N	S	N	S	S	I	K	E	S
MS/32	K	N	R	N	I	D	Y	T	R	N	S	N	S	S	I	K	E	S
MS/33	K	N	R	N	I	D	Y	T	R	N	S	N	S	S	I	K	E	S
MS/35	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/36	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/40	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/41	K	N	R	N	I	D	Y	T	R	N	S	N	S	S	I	K	E	S

Table 10 (Continued)

MS/44	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/45	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/47	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/48	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/49	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/51	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/53	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N
MS/65	K	N	R	N	I	D	Y	T	R	N	S	N	S	S	I	K	E	S
MS/6	E	D	Q	S	T	N	H	T	R	N	N	N	A	S	V	N	A	N

[a]The antibody binding sites were annotated based on previous report (203).

Since 2007, the H3N2 seasonal influenza viruses have six genetic clusters (196) The genetic clusters 1 and 2 were PE/16 like-viruses. The viruses from 2010 were scattered into genetic clusters 3, 4, 5, and 6. The phylogenetic analyses showed that these 21 viruses belonged to genetic cluster 3C and 5 (Figure 10C).

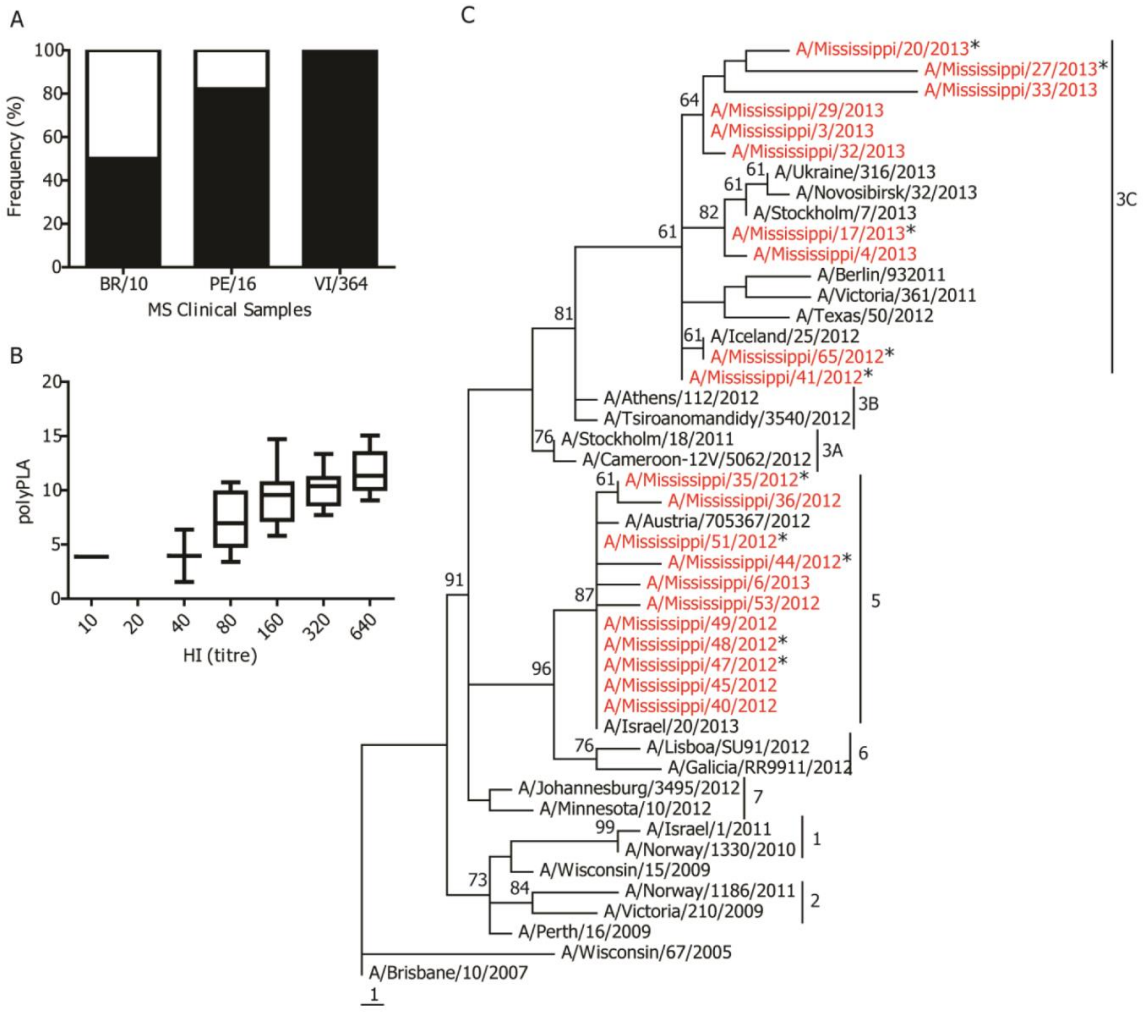

Figure 10 Detecting antigenic variants in human clinical specimens.

(*A*) The percentile of antigenic variants (white portion) compared to their corresponding homologous titers, and a 3-polyPLA-unit threshold was used; (*B*) the correlation between polyPLA units and HI titers, which were measured using PE/16 polyclonal antisera; (*C*) phylogenetic tree of HA protein sequences of H3N2 seasonal influenza viruses. The 21 isolates recovered from the 2012-2013 influenza season from Mississippi were marked in red, and the antigenic variants proposed by polyPLA assays using clinical samples against PE/16 were marked with stars. The phylogenetic tree was constructed by maximum parsimony based on HA protein sequences, and genetic clusters were defined based on the reports from Community Network of Reference Laboratories (CNRL) for Human Influenza in Europe (http://www.ecdc.europa.eu/en/publications/Publications/Influenza-visus-characterisation-June-2012.pdf).

Discussion

Identification of antigenic variants in disease surveillance is essential, and an ideal antigenic characterization platform should meet the following four criteria: (1) robust, the results should be repeatable; (2) simple and economic, the methods can be carried out in a common diagnosis laboratory; (3) high throughput, the method should be able to perform on a large-scale; and (4) sensitive, the method will have optimal performance in identifying antigenic variants directly from clinical samples, avoiding viral isolation. For many diseases, the pathogen isolation process is not only time-consuming but also can change virus antigenic properties, resulting in data that does not accurately represent those antigenic properties in circulating viruses. Furthermore, some pathogens cannot even be recovered from the specimen. The polyPLA developed in this study was designed to meet these four criteria, as confirmed in influenza antigenic variant identification in this study.

The polyPLA quantifies antibody-antigen interactions for influenza viral proteins, including both HA and NA, against their corresponding antibodies in the antisera. The principle of this method is more similar to neutralization assays rather than HI assays. More importantly, it can avoid the red blood cell binding problems usually seen in HI assay. For example, egg-adaptation substitutions affect the architecture of the HA receptor-binding site and alter the interactions of the HA with the terminal sialic acid moiety(133).

The high concentration of influenza A viruses could lead to the saturation of viruses over antibody, which is 200 nM used in this study. For example, when the undiluted, MDCK cell derived NA/933 virus was used, there were no significant

differences between the ΔCt values against NA/933 antibody and those against JO/33 antibody (data not shown). After the NA/933 viruses were diluted to 1:40, there was a significant difference between the ΔCt values against NA/933 and those against JO/33 antibodies (Table 8 and Figure 9). However, we do not believe this potential limitation will affect the application of this method in clinical samples, from which the virus loads are much smaller than a single isolate.

Compared to seasonal influenza virus surveillance, the antigenic characterization of emerging pathogens for pandemic preparedness, especially those pathogens in areas without sufficient biosafety facilities, has been challenging. Propagation of these emerging viruses usually requires a higher biosafety containment such as BSL-3 and even BSL-4. In most cases, the specimens need to be shipped to a laboratory with appropriate biosafety containment, and sometimes even the paperwork for such collaborative agreements, especially among countries, can cause delays when an outbreak occurs. Because the polyPLA can use the clinical samples directly and also use a common qRT-PCR platform, it can perform large-scale analyses with minimal biosafety requirements in laboratory conditions. For example, Biosafety Level 2 level will be sufficient for the use of the polyPLA in influenza surveillance(196) Thus, this method will be very useful in detecting antigenic variants for the H5N1 highly pathogenic avian influenza viruses and emerging H7N9 low pathogenic avian influenza viruses. In resource limited areas, although the RBCs in conventional serological assays such as HI can be more accessible, it would be much easier and more economical to set up a qRT-PCR platform than a high containment environment for viral propagation.

In summary, polyPLA is a simple method quantifying the binding avidity of antibody-antigen interactions. Beyond influenza applications, this method can also be used for other pathogens including those cannot be propagated in laboratory.

CHAPTER IV

GENETIC AND ANTIGENIC VARIATIONS AMONG SUBTYPE H3N2 INFLUENZA A VIRUSES IN HUMAN NASOPHARYNGEAL AND VIRAL ISOLATES

Influenza A virus (IAV) is a zoonotic pathogen which consists of a large genetically and antigenically diverse viral population. Swine IAVs not only cause disease outbreaks among swine, but can also be transmitted bi-directionally between swine and humans, causing sporadic infections and even pandemic outbreaks apart from human seasonal IAVs. Antigenic variant identification is fundamental for an effective vaccination program. Conventional red blood cell based immunological tests have been used to identify antigenic variants among circulating IAV strains. Because these assays require viral isolation, they are time consuming and labor intensive. Thus only limited numbers of virus isolates are subjected to antigenic characterization in influenza surveillance studies and much of this important information is lost. In this project, a novel polyclonal antibody based proximity ligation assays (polyPLA) was developed and validated to characterize IAV antigenic variants directly using clinical samples. The application of this method with clinical samples from influenza surveillance had aided in the understanding of the antigenic evolution of IAV in human and swine populations. In the comparison of clinical sample and their isolates, both can antigenically and genetically differ. Further analyses of those samples with inconsistent antigenic

properties between swab and isolates showed that polymorphisms were identified in the isolates, including those in the antibody binding sites. This study demonstrated polyPLA can be effective in characterizing antigenic profiles in clinical samples and culture adapted mutations can lead to antigenic variations in human clinical samples and skew the antigenic data. polyPLA would be useful influenza vaccine strain selection by the antigenic characterization of viruses directly with the use of clinical samples.

Introduction

Antigenic variants

The surface glycoprotein of influenza A virus (IAV), hemagglutinin (HA) and neuraminidase (NA), are under positive selection in order to evade the host's immunological responses (204). The most common antibodies against influenza target the highly variable head of HA; such antibodies prevent reinfection with similar antigenic properties. Variability of IAV is a product of adaptation to host cell factors as well as change of antigenicity to escape from host immune response (3, 4). Evasion tactics of the host acquired immune system are 1) antigenic drift (204), which is mainly caused by accumulative mutations in HA and/or NA of IAVs, and 2) antigenic shift, which is caused by acquiring a new HA and/or NA by reassortment (205).

Surveillance of human clinical samples

Worldwide epidemiological surveillance is necessary in order to identify and monitor the emergence and spread of novel antigenic variants, to project their impacts on population health, and to provide scientific evidence for making decision vaccine composition each year; this is accomplished by the isolation and characterization of

currently circulating viruses. Human IAV continually circulate in yearly epidemics, with most frequency in the months from late fall through the spring. Many speculate that the infectious virus is transmitted mainly by small-particle aerosols, which are maintained at low temperature (7ºC to 8ºC) and low relative humidity (< 50%) due to lower osmotic pressure on the influenza virus particles (Reviewed in (206, 207)).

Early detection of IAV infection by reliable laboratory results can prevent spread of the virus by providing information to public health authorities about early intervention as well as assist with optimal treatment decisions. The use of molecular diagnostic methods, RT-PCR, rapidly decreases after one week from onset of symptoms (208). Diagnostics through viral cultures are more likely to obtain a positive result if the clinical sample is collected within three days of onset of symptoms (208). Specimens from nasal and nasopharyngeal specimens (e.g. nasal swab, nasopharyngeal swab, nasopharyngeal aspirate, nasal wash, combined nasal and throat swab, and throat swab) have a higher yield of virus detection than oropharyngeal specimens (208).

The global influenza surveillance network coordinated by the World Health Organization (WHO) characterizes antigenic properties of influenza viruses using the golden standard antigenic characterization method of hemagglutinin inhibition assay and sequencing of HA1 domain (54, 55). Antigenic, genetic, and epidemiological data are examined to make recommendation of candidate vaccine strains.

Pandemic preparedness

Typically, 30,000 deaths, a year in the United States, are caused by seasonal IAV. Influenza epidemics, every a few years, increase annual mortality level to 40,000 to 45,000 deaths. Global pandemics of influenza have occurred unpredictably, resulting in

the infection of 20% to 40% of the population within a single year, dramatically raising death rates above normal levels. There was incomplete historical surveillance information before the 2009 H1N1 pandemic which lead to a limited ability to evaluate the severity of the novel virus within the human population (208).

With the standardization of influenza data collection by the WHO, the policy makers can better understand risk factors for the potential severity of a pathogen, variation of influenza severity from season to season and its relationship to virus types or subtypes, the burden of disease related to influenza, and other factors critical to public health decision-making (208). Rapid comparative assessment of each influenza season is necessary for preparation of future pandemics locally and globally (208).

Human H3 3c.3a and 3c.2a

In the 2014-2015 influenza season, two H3 genetic subclades mutated independently and the rapid spread of novel antigenic mutations occurred due to a lack of herd immunity to the novel viruses. Previously, in the H3 genetic clade 3C.1, phenylalanine (F) was at residue 159 and asparagine (N) was at residue 225. Both clades mutated at residue 225 first to aspartic acid (D), followed by the mutation of residue 159 to serine (S) or tyrosine (Y) for clades 3C.3a or 3C.2a, respectively. A/Switzerland/9715293/2013-like virus (3C.3a) was chosen as the 2015-2016 vaccine virus because the majority of viruses analyzed were antigenically related to those viruses in 3C.3a (118, 119). The following season, 2015-2016, the majority of viruses analyzed were of the genetic subclade 3C.2a with 3C.3a co-circulating but at a lower proportion and A/Hong Kong/4801-like virus (3C.2a) was chosen as the 2016-2017 vaccine virus (118).

Adaptive mutations

Viral isolation is not only time-consuming, but also can change the antigenic properties of the original viruses due to the laboratory adaptation in cells or embryonated eggs(121, 133-135, 209). Egg-adaptation substitutions have been shown to affect the HA receptor-binding site and to change the interactions between influenza HA and the terminal sialic acid of the host cell(133). Isolated H3N2 viruses can potentially have mutations across the whole genome level when cultured in Madin-Darby Canine Kidney (MDCK) cells(196). Within the HA itself, most residue changes were within to near receptor binding sites (A138S, G218R, R221L, V223I), altering receptor binding, and one residue change (N165K) was within an antigenic site.

Uncultivable (low viral titer) clinical samples

Only a small quantity of influenza infections can be laboratory confirmed per season. Recommendations of influenza vaccine strains are based on the prediction of circulating viruses, before full epidemiological significance is known (97). A real-time surveillance system for influenza outbreaks is necessary to produce earlier interventions for a decrease of morbidity and mortality (210-214). Influenza surveillance is not fully complete without the inclusion of clinical samples, which are uncultivable (low viral titer) in both cells and eggs. In a previous study, influenza A and B, as well as subtypes H1, H3, and H5, were detected with RT-PCR in uncultivable clinical samples with a sensitivity of 90.4%(215). Although subtype can be distinguished by PCR and sequencing, information about antigenicity is still missing.

Viral quasispecies Selection and fitness

Infection within a host is not limited to one identical viral genome; the proliferating population of viruses is closely related, yet is not identical (42-45). The viral quasispecies of IAV within a host are subject to continuous competition because of the error-prone genome replication (44, 46). Some mutations can be positively selected in order for a virus to escape from host-antibody neutralization or to replicate more efficiently, leading to virus variants, which could become predominant in the population (47). Population-level fitness has also been shown to be increased by cooperative interactions between variants within a quasispecies(48-52).

Genomic sequencing and data analyses

Sequencing of IAV is useful to identify origin of novel reassortant viruses as well as identify molecular characteristics of a virus, such as host range, replication efficiency, transmissibility, or virulence (150-153). The traditional method of Sanger sequencing is widely used in influenza surveillance and research (154). Next-generation sequencing (NGS) can sequence the entire genome (155, 156) and is helpful in the identification of genetic variation within a viral quasispecies and to monitor vaccine composition (157). The massive parallel sequencing of influenza virus by the NGS platforms allow for increased sensitivity of the study of the original populations. This is in contrast to the traditional method that requires molecular cloning or plaque generation (158-160) for an output of multiple gigabases of DNA (161). Because the whole IAV genome is only 13,000 nucleotide bases of RNA, coverage is high, yet the segmented nature of the genome can be challenging to achieve full genome coverage (155, 162-166). In the analysis of NGS data for IAV, it is important to accurately assemble the short reads into

the 8 genomic sequences while retaining the polymorphisms within the quasispecies or true mixed infection of multiple viruses.

Antigenic Cartography

Most retrospective studies only use a partial sequence of the HA gene to understand and predict, the evolution of H3N2 strains (126, 168, 216-218). Genetic differences, alone, are unreliable in the determination of antigenic differences in novel viruses and antigenic properties through immunological datasets are not always straightforward. The relative antigenic relationship and evolution can be visualized with the production of two- or three- dimensional maps, known as antigenic cartography (168). The antigenic map produced can quantify antigenic differences between viruses; antigenically similar viruses cluster closely while antigenically different viruses are farther away.

the polyclonal sera–based proximity ligation assay (polyPLA)

Proximity ligation assay utilizes quantitative real time PCR (qRT-PCR) for the detection of antigen-antibody interaction (148). For this assay: (1) oligonucleotide-linked monoclonal antibodies are incubated with the analyte in question; (2) if in close proximity, the oligonucleotides can be ligated together; and (3) presence of analyte will be shown by amplification of ligated products with qRT-PCR. The assay reporter signal is dependent on a proximal and dual recognition of each target analyte providing high specificity(149).

MATERIALS AND METHODS

Viruses

The 11 reference H3N2 viruses were received from the Food & Drug Administration, Health and Human Services, to represent the past 9 influenza vaccine reformulations of the Northern Hemisphere winter seasons of November 2008-April 2009 until November 2016-April 2017. The twelve viruses represent 6 genetic clades, including 1-Brisbane/10, 1-Perth/16, 2-3c.1, 1-3c.2, 3-3c.2a, 3-3c.3a. Additionally, 30 H3N2 clinical samples were received from the Mississippi Department of Health (MSDH) from the 2014-2015 influenza season and 60 H3N2 clinical and isolated samples were received from the Centers of Diseases Control and Prevention, Department of Health & Human Services (CDC) from 2014-2015 and 2015-2016 seasons. All viruses were isolated and propagated in Madin-Darby Canine Kidney (MDCK) cells and stored at -80ºC before usage (Table 11).

Table 11 Paired sample information.

Reference Viruses	Genetic Clade	Clinical	Isolate
A/Brisbane/10/2007 (BR/10)	BR/10	no	yes
A/Perth/16/2009 (PE/16)	PE/16	no	yes
A/Victoria/361/2011 (VI/361)	3c.1	no	yes
A/Texas/50/2012 (TX/50)	3c.1	no	yes
A/Costa Rica/4700/2013 (CR/4700)	3c.2	no	yes
A/Utah/07/2013 (UT/07)	3c.3a	no	yes
A/Switzerland/9715293/2013 (SW/9715293)	3c.3a	no	yes
A/Palau/6759/2014 (PA/6759)	3c.3a	no	yes
A/Hong Kong/4801/2014 (HK/4801)	3c.2a	no	yes
A/Victoria/503/2015 (VI/503)	3c.2a	no	yes
A/Fiji/2/2015 (FI/2)	3c.2a	no	yes
A/Brisbane/82/2015 (BR/82)	3c.2a	no	yes
MSDH Viruses	Genetic Clade	Clinical	Isolate
A/Mississippi/161/2014	3C.2a	yes	yes
A/Mississippi/162/2014	3C.3a	yes	yes
A/Mississippi/163/2014	3C.3a	yes	yes
A/Mississippi/164/2014	3C.3a	yes	yes
A/Mississippi/165/2014	3C.3a	yes	yes
A/Mississippi/166/2014	3C.3a	yes	yes
A/Mississippi/167/2014	3C.3a	yes	no
A/Mississippi/168/2014	3C.3a	yes	yes
A/Mississippi/169/2014	3C.3a	yes	yes
A/Mississippi/180/2014	3C.3a	yes	yes
A/Mississippi/181/2014	3C.3a	yes	yes
A/Mississippi/182/2014	3C.3a	yes	yes
A/Mississippi/183/2014	3C.3a	yes	yes
A/Mississippi/198/2014	3C.3a	yes	yes
A/Mississippi/199/2014	3C.3a	yes	yes
A/Mississippi/200/2014	3C.3a	yes	yes
A/Mississippi/201/2014	3C.3a	yes	yes
A/Mississippi/202/2014	3C.3a	yes	yes
A/Mississippi/203/2014	3C.3a	yes	yes
A/Mississippi/204/2014	3C.2a	yes	yes
A/Mississippi/205/2014	3C.3a	yes	yes
A/Mississippi/206/2014	3C.2a	yes	yes
A/Mississippi/207/2014	3C.3a	yes	yes

Table 11 (Continued)

A/Mississippi/223/2014	3C.3a	yes	yes
A/Mississippi/224/2014	3C.3a	yes	yes
A/Mississippi/225/2014	3C.2a	yes	yes
A/Mississippi/226/2014	3C.3a	yes	yes
A/Mississippi/227/2014	3C.2a	yes	yes
A/Mississippi/228/2014	3C.3a	yes	yes
A/Mississippi/229/2014	3C.3a	yes	no
CDC Viruses	Genetic Clade	Clinical	Isolate
A/Missouri/04/14	3C.3	yes	yes
A/Alaska/05/2014	3C.3	yes	yes
A/Louisiana/9/13	3C.3	yes	yes
A/Hawaii/2/14	3C.3	yes	yes
A/Washington/14/14	3C.3	yes	yes
A/Maryland/10/14	3C.3	yes	yes
A/New Mexico/20/14	3C.3	yes	yes
A/New York/1/15	3C.3	yes	yes
A/Florida/3/15	3C.3	yes	yes
A/Utah/1/15	3C.3	yes	yes
A/Colorado/1/15	3C.3	yes	yes
A/Ohio/11/15	3C.3	yes	yes
A/Iowa/6/15	3C.3	yes	yes
A/Massachusetts/1/15	3C.3	yes	yes
A/Arkansas/10/14	3C.3	yes	yes
A/Wisconsin/37/13	3C.3	yes	yes
A/Indiana/29/14	3C.3	yes	yes
A/Wyoming/19/14	3C.3	yes	yes
A/California/4/14	3C.3a	yes	yes
A/North Carolina/12/14	3C.3a	yes	yes
A/North Carolina/14/14	3C.3a	yes	yes
A/California/18/14	3C.3a	yes	yes
A/Mississippi/3/14	3C.3a	yes	yes
A/Hawaii/49/14	3C.3a	yes	yes
A/Texas/11/16	3C.3a	yes	yes
A/California/2/16	3C.3a	yes	yes
A/Montana/13/16	3C.3a	yes	no
A/Oklahoma/2/16	3C.3a	yes	yes
A/South Carolina/17/16	3C.3a	yes	yes

Table 11 (Continued)

A/Colorado/22/16	3C.3a	yes	yes
A/Idaho/10/15	3C.3a	yes	yes
A/Nebraska/3/15	3C.3a	yes	yes
A/Pennsylvania/6/15	3C.3b	yes	yes
A/Washington/53/14	3C.3b	yes	yes
A/Montana/3/15	3C.3b	yes	no
A/Hawaii/34/15	3C.3b	yes	yes
A/Pennsylvania/1/15	3C.3b	yes	yes
A/Pennsylvania/27/14	3C.2	yes	yes
A/New Mexico/23/13	3C.2	yes	yes
A/California/58/13	3C.2	yes	yes
A/Vermont/3/14	3C.2a	yes	yes
A/Minnesota/7/14	3C.2a	yes	yes
A/Rhode Island/11/14	3C.2a	yes	yes
A/Connecticut/4/14	3C.2a	yes	yes
A/New Jersey/13/14	3C.2a	yes	yes
A/New Mexico/11/14	3C.2a	yes	yes
A/Florida/1/16	3C.2a	yes	yes
A/Minnesota/54/15	3C.2a	yes	yes
A/North Carolina/4/16	3C.2a1	yes	yes
A/Texas/73/14	3C.2a	yes	yes
A/Maine/4/15	3C.2a	yes	yes
A/Florida/5/15	3C.2a	yes	yes
A/West Virginia/1/15	3C.2a	yes	yes
A/Washington/19/16	3C.2a1	yes	yes
A/Wyoming/19/15	3C.2a	yes	no
A/Colorado/27/15	3C.2a	yes	no
A/Oregon/1/15	3C.2a	yes	yes
A/Utah/2/15	3C.2a	yes	yes
A/Delaware/44/14	3C.2a	yes	yes
A/Hawaii/49/15	3C.2a	yes	yes

Serum generation with ferrets

Twenty-two 4-month old female ferrets were confirmed to be seronegative for circulating H3N2 influenza viruses prior to purchase from Triple F Farms (Sayre, PA,

USA). All animal experiments described here were approved by Mississippi State University Institutional Animal Care and Use Committee. Animals were anesthetized by intramuscular administration of Ketamine 20mg/kg and Xylazine 1mg/kg (by body weight) prior to all procedures, including infection, bleeding, and nasal swabbing. The ferret sera were collected three weeks post-infection. Briefly, there were 11 groups consisting of 2 ferrets each, each group was inoculated with one reference virus. Monoclonal antibodies anti-nucleoprotein (NP) were obtained from BEI Resources (Bethesda, MD, USA).

Nasal swabs

Nasal swabs were taken for two groups of ferrets infected with viruses BR/10 and HK/4801, representing two antigenically distinct viruses, on 0, 3, 5, 7, and 10 days post infection, stored in 1.5 mL tubes with 1 mL Opti-MEM Reduced Serum Media (Thermo Fisher; Waltham, MA, USA), and titrated by $TCID_{50}$ in MDCK cells. $TCID_{50}$ was calculated by the Reed-Muench method(219).

HI

Hemagglutinin inhibition assay (HI) was performed for all 11 viruses and ferret sera as well as the 28 isolated Mississippi clinical samples. Briefly, ferret sera, were treated at a 1:3 ratio with receptor destroying enzyme (RDE, DenkaSeiken Co.; Japan) overnight at 37ºC, then heat inactivated at 56ºC for 30 minutes, and diluted 1:10 with 1x phosphate-buffered saline (PBS; pH 7.4). The treated and diluted ferret sera were 2-fold serially diluted in 1x PBS and reacted with 4-hemagglutination-units of viruses. HI titres

were expressed as the reciprocal of the highest dilution in which virus binding to 0.5% turkey red blood cells (RBCs) were blocked. Each experiment was repeated in triplicate.

polyPLA

polyPLA, was completed as previously described (220). Briefly, Pierce protein A/G chromatography 1 mL cartridge (Thermo Scientific Pierce, Rockford, IL, USA) were used to purify IgG from ferret polyclonal antisera. Monoclonal antibodies anti-NP and purified IgG were biotinylated with Biotin-XX miscroscale protein labeling kit, as according to manufacturer's directions (Life Technologies, Carlsbad, CA, USA). Excess biotin was removed with Slide-A-Lyzer dialysis cassettes (Thermo Scientific Pierce, Rockford, IL, USA) in cold 1X PBS (pH 7.4) at 4ºC overnight.

Two assay probes for each antibody were prepared by incubating the biotinylated antibodies with either 3' or 5' TaqMan Prox-Oligos (Thermo Scientific Pierce, Rockford, IL, USA). For example, for each probe, 2.5 µL 200 nM biotinylated antibody was combined with 2.5 µL of either 200 nM 3' prox-Oligo or 200 nM 5' prox-Oligo, incubated at room temperature for 60 minutes, and 45 µL Assay Probe Storage Buffer was added, briefly centrifuged, and incubated at room temperature for 20 minutes.

Antigenic cartography

The antigenic maps of H3N2 swine IAVs were constructed using AntigenMap (http://sysbio.cvm.msstate.edu/AntigenMap) and data derived from the HI assay or the polyPLA (25, 26). The data entry with an HI titer of <1:10 or a polyPLA unit of <5.0 were determined as a low reactor for the data from HI or the polyPLA, respectively.

Sequencing

Viral RNA was extracted from both clinical samples (n=30) and viral isolates (n=28) with the GeneJet Viral DNA/RNA Purification Kit (Thermo Scientific Pierce, Rockford, IL, USA) according to manufacturer's instructions. Using previously described primers (221, 222), full-length cDNA for the eight influenza A gene segments was amplified with SuperScript One-Step RT-PCR (Invitrogen). Amplified viral DNA products were quantified with use of High Sensitivity DNA kit (Agilent Technologies; Santa Clara, CA, USA) on Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA). Sequencing libraries were prepared with equal amounts of each sample for the Illumina Nextera DNA Sample Prepartion kit (Illumina, San Diego, CA, USA), quantified, normalized, and pooled together. Sequencing was completed with MiSeq Reagent kit v2 (Illumina, San Diego, CA, USA) with the MiSeq sequencing system (Illumina, San Diego, CA, USA), according to the manufacturer's suggested protocol.

Next Generation Sequence Data Analyses

All the pair-end reads were trimmed according to their sequence quality with Trimmomatic (version 0.36)(223). For assembly, a hybrid assemble pipeline was implemented to assemble pair-end reads using either reference or de novo method. Using Bowtie2 (version 2.3.1)(224), the pipeline maps all assembled reads against template sequences and then generates consensus sequences as the results. In order to detect co-infection in our sample, samples were assembled using the de novo method by Velvet (version 1.2.10)(225). Different from reference method, Velvet will generate contigs from all reads, which is able to identify different HA/NA genes from the same sample. Polymorphisms were identified by mapping all trimmed reads against their consensus

sequences using Bowtie2. After mapping steps, we generate all polymorphisms under a 10% cutoff using SAMtools (version 1.4)(226) and igvtools (version 2.3)(227).

Molecular characterization and phylogenetic analyses.

Full length consensus sequences for 12 HA and 16 NA clinical and 58 HA and 48 NA isolate were downloaded for CDC samples from GISAID EpiFlu database (http://platform.gisaid.org/). The multiple sequence alignments of the consensus HA and NA gene segments, from GISAID and MSDH, were conducted using the MUSCLE software package (197). The phylogenetic analyses were performed using maximum likelihood by GARLI version (198), and bootstrap resampling analyses were conducted with 1,000 runs using PAUP* 4.0 Beta (199) with a neighborhood joining method, as previously described (200).

Statistical Analysis

Linear regression analyses were performed using the HI titers of the isolated viruses (n=84) versus the 11 reference antisera and polyPLA units of these 84 virus isolated verses polyclonal antibodies of TX/50, SW/9715293, PA/6759, FI/2 and HK/4801. The 90 clinical samples were assessed for monoΔC_T and ΔpolyPLA cutoffs by the frequency procedure using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) to determine sensitivity and specificity for detection of IAVs and antigenic variants with confidence intervals at 95%. The mathematical product of sensitivity × specificity, given the term efficiency, was calculated and graphed for each polyPLA value to provide the probability of correct classification for unknown sample status(228). Comparison of proportions calculator

(MedCalc; Ostend, Belgium) was used to test for significant difference in residue changes of the HA protein of clinical and isolate samples.

Results

Sensitivity of nasal swabs

To determine whether polyPLA is sensitive enough to identify H3N2 human IAVs in clinical samples, we used 20 nasal swabs from 2 ferrets infected with BR/10 and 2 ferrets infected with HK/4801. Of the 20 samples, 6 had virus titers up to 2.8×10^8 $TCID_{50}$/mL and both swabs and virus isolates for each sample were IAV-positive ($\Delta C_T \geq 3.00$) using NP monoclonal antibodies. Further analyses using polyclonal antibodies from BR/10, PE/16, and TX/50 were used to detect BR/10 infected ferrets and TX/50, SW/9715293, and HK/4801 were used to detect HK/4801 infected ferrets. polyPLA detected viral shedding from 3 and 5 days after virus challenge and titers ranged from 6.0 to 10.9 polyPLA units for swabs and 6.2 to 11.8 polyPLA units for virus isolates (Table 12).

Table 12 Virus titration and polyPLA of nasal swab from ferrets challenged with A/Brisbane/10/2007 and A/Hong Kong/4801/2014.

Ferret[a]	DPI[b]	$TCID_{50}$/mL[c]	polyPLA (SD[d])									
			BR/10[e]		PE/16		TX/50		SW/9715293		HK/4801	
			Swab[f]	Isolate[g]	Swab	Isolate	Swab	Isolate	Swab	Isolate	Swab	Isolate
50	5	2.81E+06	**9.776 (0.406)[h]**	**7.878 (0.062)**	6.273 (0.132)	6.196 (0.132)	6.590 (0.592)	8.006 (0.592)				
51	3	1.58E+07	**10.818 (0.317)**	**7.923 (0.001)**	8.144 (0.977)	5.873 (0.977)	8.228 (0.512)	7.907 (0.512)				
54	3	1.58E+08					10.915 (0.850)	9.608 (0.850)	8.568 (0.406)	10.023 (0.086)	**10.675 (0.475)**	**10.554 (0.035)**
54	5	2.81E+08					10.618 (0.450)	10.617 (0.450)	8.232 (0.135)	9.216 (0.097)	**10.761 (0.797)**	**11.776 (0.249)**
55	3	8.89E+06					8.760 (0.484)	9.398 (0.484)	5.962 (1.134)	9.478 (0.377)	**8.468 (0.176)**	**11.311 (0.199)**
55	5	8.89E+07					8.048 (0.856)	9.163 (0.856)	5.747 (0.567)	8.935 (0.579)	**7.867 (0.170)**	**9.658 (0.061)**

[a] Ferret identification number.
[b] Days post infection.
[c] 50% tissue culture infectious dose per mL.
[d] Standard deviation.
[e] Polyclonal antibodies used against original samples and isolates. BR/10 – A/Brisbane/10/2007; PE/16 – A/Perth/16/2009; TX/50 – A/Texas/50/2012; SW/9715293 – A/Switzerland/9715293/2013; HK/4801 – A/Hong Kong/4801/2014.
[f] Nasal swabs directly from ferrets.
[g] Virus isolated from nasal swabs.
[h] Homologous titers in bold.

Detecting antigenic variants of H3N2 historical seasonal influenza viruses.

The 11 reference viruses were tested against the polyclonal antibodies for TX/50, SW/9715293, PA/6759, HK/4801, and FI/2 as well as the monoclonal NP antibody. The results from polyPLA indicated that the titers for these five testing isolates ranged from 2.80 to 12.38 polyPLA units. In support of the HI cartography–derived data, polyPLA-based cartography also showed that the two genetic subclades 3C.2a and 3C.3a were grouped in two antigenic clusters. The average distance between genetic subclusters was 1.50 units (± 1.11 standard deviation; Fig. 11a) and 2.40 units (±1.46 standard deviation;

Fig. 11b) in HI and polyPLA cartography, respectively. Correlation association analyses through linear regression showed the fold increment titers from homologous virus isolates and fold increment in polyPLA values had a coefficient of $R^2 = 0.82$ ($p<0.0001$; Fig. 11c). An 8-fold increment in HI titer was correlated with a 1.57-fold increment in polyPLA units.

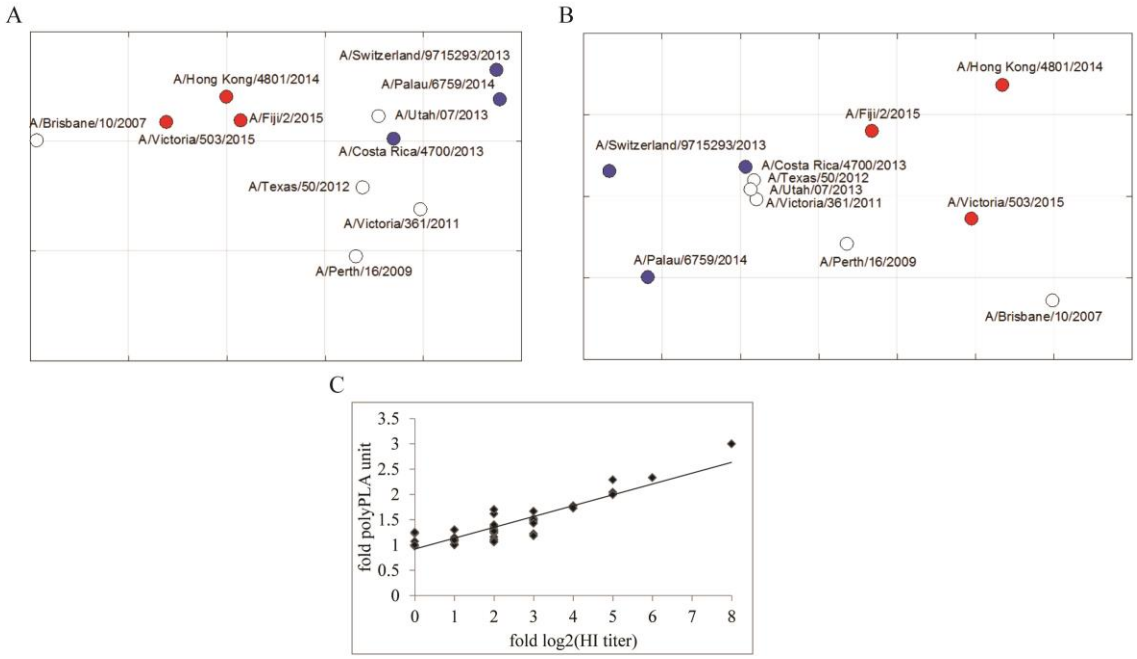

Figure 11 Comparison of antigenic characterization of H3N2 reference IAVs using hemagglutination inhibition (HI) assays and polyclonal sera–based proximity ligation assay (polyPLA).

(A) Antigenic map derived from HI data; 3C.2a are marked in green and 3C.3a are marked in blue. (B) Antigenic map derived from polyPLA data; 3C.2a are marked in green and 3C.3a are marked in blue. (C) Correlation of the fold increments in HI titers and those in polyPLA values; fold increment in polyPLA values can be predicted from fold increment in HI titers by the following formula: ΔpolyPLA values = $0.21 \times \Delta\log_2$(HI titers) – 0.92, $R^2 = 0.82$.

Correlation with golden standard assay

To assess the effectiveness of the polyPLA, we compared the antigenic data for the same set of viral isolates derived from the HI assay and the polyPLA. The total of 84 H3N2 IAVs isolates used for testing in our study had cross-reaction titers ranging from <1:10 to 1:1,280 against all reference sera (Table 13). HI-based antigenic cartography showed that these 84 isolates can be grouped in two antigenic clusters, each is corresponding to genetic cluster 3C.2a and 3C.3a, respectively (Fig. 12a).

The results from polyPLA using five reference sera against TX/50, SW/9715293, PA/6759, FI/2, and HK/4801 indicated that the titers for these 84 testing isolates ranged from 2.58 to 15.43 polyPLA units. polyPLA-based cartography also showed that these 84 isolates were could be grouped according to genetic subclade (Fig. 12b). The average distance between genetic subclusters was 1.14 units (± 0.62 standard deviation) and 2.78 units (± 1.06 standard deviation) in HI and polyPLA cartography, respectively. Correlation association analyses through linear regression showed the fold increments in HI titers and polyPLA values had a coefficient of $R^2 = 0.82$ ($p<0.0001$) (Fig. 12c). An 8-fold increment in HI titer was correlated with a 3.60-difference in polyPLA units.

a. Clinical samples

To measure the sensitivity of polyPLA on the clinical samples, we used the assay on the 95 samples with three reference polyclonal antibodies against TX/50, SW/9715293, PA/6759, FI/2, and HK/4801. The frequency distribution of ΔC_T values for NP monoclonal antibodies for the 90 IAV positive– and 5 IAV negative–clinical samples showed that the greatest efficiency (98.9%) was observed at a ΔC_T cutoff of 3.0 (Fig. 12d). The optimum combination for detecting IAVs in clinical samples is an assay

sensitivity of 98.9% (95% CI = 93.1%, 99.9%) and specificity of 100.0% (95% CI = 46.3%, 100.0%).

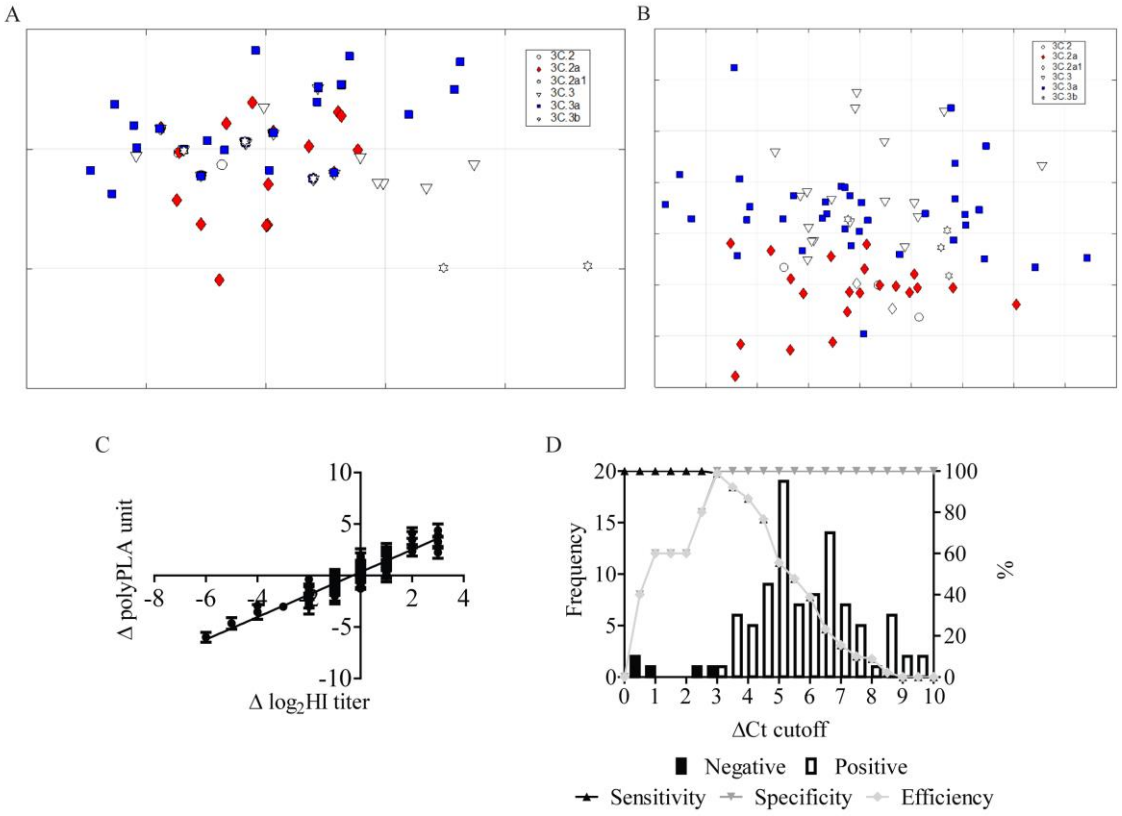

Figure 12 Comparison of antigenic characterization of H3N2 IAVs using hemagglutination inhibition (HI) assays and polyclonal sera–based proximity ligation assay (polyPLA).

(A) Antigenic map derived from isolate HI data; 3C.2a are marked in red and 3C.3a are marked in blue. (B) Antigenic map derived from isolate polyPLA data; 3C.2a are marked in red and 3C.3a are marked in yellow. (C) Correlation of the fold increments in HI titers and the difference in polyPLA values; difference in polyPLA values can be predicted from fold increment in HI titers by the following formula: difference in polyPLA values = $1.09 \times \Delta\log_2$(HI titers) – 0.38, R^2 = 0.82. (D) Distribution of IAV-positive samples (white bars, N = 90) vs. IAV-negative samples (grey bars, N = 5) obtained using NP monoclonal antibody and various ΔC_T values. A total of 12 representative H3N2 swine influenza A viruses (IAVs) were selected to represent the past 9 influenza vaccine reformulations of the Northern Hemisphere winter seasons of November 2008-April 2009 until November 2016-April 2017 (Table 11). The homologous ferret antisera for these viruses were used to perform the HI assay and polyPLA. The HI assays were performed using 0.5% red blood cells. Antigenic maps were constructed using AntigenMap (http://sysbio.cvm.msstate.edu/AntigenMap) (229, 230).

polyPLA units for the paired 84 clinical samples ranged from 2.80 to 16.73 polyPLA units. The clinical samples had larger polyPLA values against the polyclonal antibodies from the same genetic subclade. With the data from five reference polyclonal antibodies, the antigenic map of clinical samples with polyPLA showed variation in the samples but with little distinction of genetic subclusters grouping together (Fig. 13). The average distance between subclusters was 2.45 units (± 0.78 standard deviation) in polyPLA cartography, respectively.

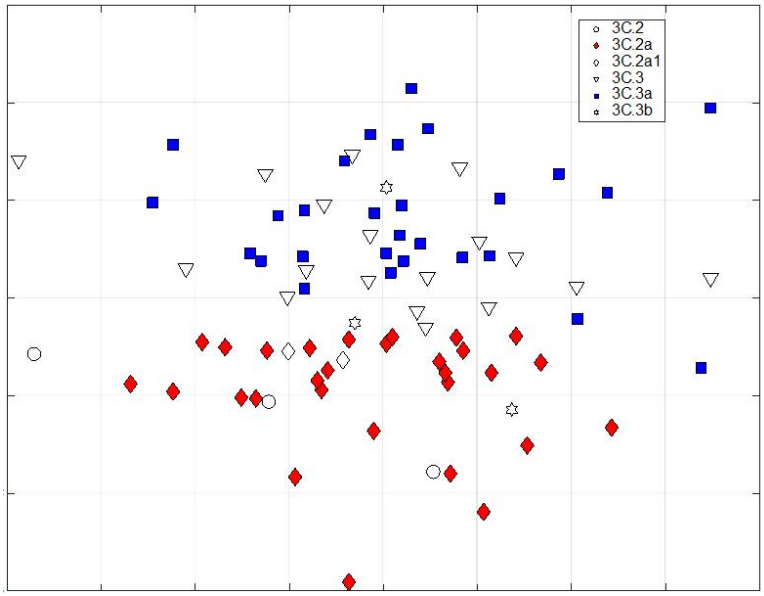

Figure 13 Antigenic map derived from 90 clinical samples polyPLA data. 3C.2a are marked in red and 3C.3a are marked in blue.

Table 13 Antigenic characterization of clinical and isolate viruses.

		TX/50		SW/9715293		HK/4801		PA/6759		FI/2	
Genetic Clade	Clinical Virus	polyPLA	std dev	polyPLA	std dev	polyPLA	std dev	polyPLA	std dev	polyPLA	std dev
3C.2a	A/Mississippi/161/2014	6.888	0.288	7.852	0.100	10.555	0.565	11.567	0.261	13.809	0.256
3C.2a	A/Mississippi/162/2014	1.319	0.282	5.802	0.167	8.227	0.596	6.316	0.284	8.018	0.269
3C.3a	A/Mississippi/163/2014	4.023	0.636	9.943	0.492	7.017	0.256	9.799	0.277	7.222	0.307
3C.3a	A/Mississippi/164/2014	9.995	0.961	12.547	0.225	8.304	0.013	10.936	0.285	6.788	0.331
3C.2a	A/Mississippi/165/2014	7.203	0.164	11.895	0.085	13.275	0.449	10.391	0.306	10.891	0.282
3C.3a	A/Mississippi/166/2014	3.960	0.146	8.330	0.423	5.367	0.330	9.340	0.271	5.378	0.315
3C.3a	A/Mississippi/1667/2014	3.472	0.393	7.060	0.267	5.334	0.029	6.478	0.286	5.326	0.301
3C.3a	A/Mississippi/168/2014	4.381	0.234	8.953	0.359	5.817	0.129	8.053	0.274	5.610	0.281
3C.3a	A/Mississippi/169/2014	4.898	0.807	9.341	0.173	7.457	0.295	9.630	0.275	8.381	0.291
3C.2a	A/Mississippi/180/2014	4.773	0.612	9.111	0.004	10.637	0.424	7.695	0.270	11.998	0.244
3C.3a	A/Mississippi/181/2014	6.435	0.266	12.260	0.391	8.118	0.503	13.031	0.267	7.455	0.328
3C.3a	A/Mississippi/182/2014	6.696	0.253	9.676	1.321	12.630	0.463	12.369	0.266	6.943	0.326
3C.3a	A/Mississippi/183/2014	7.277	0.699	11.674	0.417	10.145	0.520	11.822	0.281	9.817	0.305
3C.3a	A/Mississippi/198/2014	8.522	0.709	12.550	0.111	7.179	0.199	13.959	0.254	11.530	0.261
3C.3a	A/Mississippi/199/2014	2.795	0.719	11.784	0.424	8.180	0.310	12.023	0.238	6.459	0.294
3C.3a	A/Mississippi/200/2014	9.395	0.100	14.544	0.560	10.999	0.365	11.320	0.274	10.175	0.267
3C.3a	A/Mississippi/201/2014	7.516	0.199	11.852	0.320	8.790	0.351	11.940	0.276	10.547	0.272
3C.2a	A/Mississippi/202/2014	4.963	0.775	7.366	0.331	8.792	0.531	5.717	0.321	8.235	0.297
3C.3a	A/Mississippi/203/2014	8.158	0.529	9.601	0.158	8.232	0.770	10.725	0.283	9.025	0.298
3C.2a	A/Mississippi/204/2014	9.424	0.410	7.218	1.167	11.143	0.265	7.086	0.335	8.982	0.318
3C.2a	A/Mississippi/205/2014	5.432	0.507	11.600	0.443	13.029	1.603	12.437	0.247	14.144	0.248
3C.2a	A/Mississippi/206/2014	4.028	0.111	7.519	0.232	10.340	0.307	7.042	0.098	9.238	0.322

Table 13 (Continued)

3C.2a	A/Mississippi/207/2014	5.938	0.296	9.585	0.308	11.939	0.246	8.706	0.051	13.555	0.546
3C.2a	A/Mississippi/223/2014	5.022	0.181	7.916	0.321	9.657	0.123	8.386	0.276	10.522	0.272
3C.3a	A/Mississippi/224/2014	7.482	0.770	13.751	0.424	8.744	0.020	11.225	0.277	10.121	0.270
3C.2a	A/Mississippi/225/2014	7.631	0.456	9.901	0.339	9.190	0.597	8.861	0.271	10.615	0.271
3C.2a	A/Mississippi/226/2014	6.076	0.297	10.695	0.039	12.518	0.461	10.111	0.274	11.091	0.283
3C.2a	A/Mississippi/227/2014	4.975	0.327	7.258	1.326	10.317	0.381	6.975	0.265	8.780	0.264
3C.3a	A/Mississippi/228/2014	9.859	0.186	10.324	0.452	14.067	0.243	16.730	0.236	9.317	0.296
3C.3a	A/Mississippi/229/2014	4.494	0.704	10.584	0.517	9.568	1.117	11.373	0.277	10.027	0.272
3C.3	A/Missouri/04/14	6.268	0.564	9.894	0.318	9.509	0.196	10.259	0.284	8.777	0.290
3C.3	A/Alaska/05/2014	3.090	0.899	10.526	0.228	9.038	0.231	6.294	0.440	6.291	0.417
3C.3	A/Louisiana/9/13	4.405	0.532	11.599	0.048	10.526	0.194	8.125	0.359	7.638	0.388
3C.3	A/Hawaii/2/14	4.952	0.327	11.018	0.109	9.607	0.080	10.329	0.407	6.321	0.397
3C.3	A/Washington/14/14	N/A	N/A	9.162	0.298	9.442	0.168	11.801	0.419	9.802	0.421
3C.3	A/Maryland/10/14	7.516	0.683	9.569	0.609	9.116	0.371	13.578	0.390	7.861	0.376
3C.3	A/New Mexico/20/14	5.912	0.706	11.394	0.469	12.734	0.269	10.268	0.428	9.162	0.420
3C.3	A/New York/1/15	8.071	0.500	9.762	0.415	8.853	0.677	11.685	0.428	11.389	0.409
3C.3	A/Florida/3/15	4.218	1.441	9.061	0.605	8.246	0.206	8.762	0.413	8.369	0.441
3C.3	A/Utah/1/15	8.383	0.020	13.296	0.040	13.182	0.095	11.180	0.416	10.696	0.444
3C.3	A/Colorado/1/15	5.970	0.213	11.358	0.316	11.052	0.266	11.375	0.388	5.822	0.372
3C.3	A/Ohio/11/15	5.033	0.216	11.024	0.177	8.776	0.124	5.044	0.409	2.141	0.433
3C.3	A/Iowa/6/15	8.589	0.202	14.383	0.370	12.772	0.161	13.953	0.400	13.336	0.421
3C.3	A/Massachusetts/1/15	8.594	0.690	9.774	0.299	8.035	0.428	12.472	0.300	10.906	0.127
3C.3	A/Arkansas/10/14	7.800	0.485	10.089	0.050	8.921	0.946	12.735	0.077	9.671	0.318
3C.3	A/Wisconsin/37/13	5.631	0.513	11.073	0.319	10.502	0.343	10.793	0.440	7.662	0.466
3C.3	A/Indiana/29/14	7.326	0.177	11.757	0.779	9.141	1.046	8.401	0.411	5.473	0.449

Table 13 (Continued)

3C.3	A/Wyoming/19/14	6.473	0.123	10.212	0.410	10.128	0.509	10.615	0.425	10.282	0.406
3C.3a	A/California/4/14	5.164	0.720	9.197	0.594	8.803	0.554	9.489	0.348	7.916	0.355
3C.3a	A/North Carolina/12/14	5.126	0.836	10.510	0.471	10.037	0.357	11.805	0.700	8.450	0.105
3C.3a	A/North Carolina/14/14	6.190	0.605	10.626	0.302	10.285	0.631	9.638	0.444	5.884	0.478
3C.3a	A/California/18/14	5.957	0.460	11.551	0.414	10.882	0.387	11.067	0.355	5.864	0.407
3C.3a	A/Mississippi/3/14	5.656	0.704	10.653	0.032	9.326	0.725	16.103	0.380	14.224	0.390
3C.3a	A/Hawaii/49/14	N/A	N/A	11.340	0.100	9.558	0.688	10.547	0.436	8.566	0.461
3C.3a	A/Texas/11/16	4.949	1.452	9.219	0.351	8.653	0.082	12.189	0.151	8.684	0.486
3C.3a	A/California/2/16	7.517	0.545	12.469	0.434	11.190	0.261	10.269	0.347	7.684	0.364
3C.3a	A/Montana/13/16	6.523	0.692	10.781	0.166	7.953	0.421	10.629	0.162	7.790	0.411
3C.3a	A/Oklahoma/2/16	5.792	0.583	9.184	0.375	9.131	0.377	8.420	0.349	6.082	0.364
3C.3a	A/South Carolina/17/16	4.545	1.106	11.537	0.194	11.359	0.426	10.952	0.311	7.150	0.342
3C.3a	A/Colorado/22/16	5.928	0.291	11.491	0.375	11.249	0.133	10.267	0.328	8.712	0.354
3C.3a	A/Idaho/10/15	5.195	0.439	11.047	0.394	10.520	0.365	7.883	0.343	6.653	0.366
3C.3a	A/Nebraska/3/15	6.986	1.117	10.246	0.081	9.462	0.923	13.764	0.322	12.614	0.325
3C.3b	A/Pennsylvania/6/15	5.502	0.451	9.780	0.163	9.603	0.359	9.172	0.274	9.853	0.021
3C.3b	A/Washington/53/14	6.636	0.558	8.633	0.055	8.571	0.454	11.672	0.224	13.108	0.008
3C.3b	A/Montana/3/15	5.829	0.707	11.498	0.214	11.387	0.145	11.181	0.209	9.959	0.141
3C.3b	A/Hawaii/34/15	5.771	0.271	13.494	0.264	9.451	0.076	9.870	0.484	8.625	0.052
3C.3b	A/Pennsylvania/1/15	6.265	0.363	10.461	0.316	9.990	0.313	12.046	0.304	10.420	0.330
3C.2	A/Pennsylvania/27/14	7.370	0.330	12.224	0.313	13.131	0.311	7.796	0.711	11.421	0.079
3C.2	A/New Mexico/23/13	2.665	0.351	6.678	0.323	7.041	0.326	4.039	0.008	6.212	0.020
3C.2	A/California/58/13	5.120	0.358	6.923	0.344	8.701	0.320	8.412	0.335	9.877	0.329
3C.2a	A/Vermont/3/14	7.040	0.338	10.708	0.340	10.848	0.332	10.294	0.657	11.912	0.122
3C.2a	A/Minnesota/7/14	5.980	0.312	9.143	0.335	15.217	0.160	10.298	0.264	11.661	0.021

Table 13 (Continued)

3C.2a	A/Rhode Island/11/14	6.111	0.155	9.045	0.144	16.556	0.368	7.970	0.418	8.167	0.438
3C.2a	A/Connecticut/4/14	6.549	0.671	11.029	0.206	11.900	0.438	9.612	0.327	11.521	0.316
3C.2a	A/New Jersey/13/14	6.976	0.348	10.047	0.073	10.765	0.864	9.316	0.357	10.188	0.369
3C.2a	A/New Mexico/11/14	5.120	0.387	11.639	0.185	11.177	0.111	7.165	0.352	9.163	0.341
3C.2a	A/Florida/1/16	5.873	0.404	9.986	0.026	10.114	0.187	8.340	0.052	9.863	0.030
3C.2a	A/Minnesota/54/15	6.642	0.432	11.085	0.243	11.687	0.563	10.279	0.402	11.166	0.413
3C.2a1	A/North Carolina/4/16	7.704	0.430	9.285	0.911	10.380	0.569	7.164	0.396	8.324	0.404
3C.2a	A/Texas/73/14	6.971	0.277	9.052	0.111	9.637	0.180	11.302	0.052	12.522	0.118
3C.2a	A/Maine/4/15	5.117	0.557	7.289	0.023	7.575	0.695	7.377	0.057	8.412	0.143
3C.2a	A/Florida/5/15	4.059	0.343	8.498	0.333	9.350	0.317	8.283	0.341	10.200	0.330
3C.2a	A/West Virginia/1/15	3.739	0.347	11.545	0.295	11.712	0.287	6.071	0.357	8.057	0.345
3C.2a1	A/Washington/19/16	5.980	0.300	7.972	0.438	9.147	0.279	9.062	0.333	10.053	0.332
3C.2a	A/Wyoming/19/15	2.352	0.495	7.972	0.445	6.490	0.243	5.842	0.435	6.962	0.237
3C.2a	A/Colorado/27/15	4.040	0.481	5.946	0.076	10.314	0.174	9.807	0.074	10.785	0.170
3C.2a	A/Oregon/1/15	6.853	0.522	9.194	0.122	10.308	0.111	7.475	0.211	8.998	0.119
3C.2a	A/Utah/2/15	4.772	0.395	9.128	0.048	9.815	0.497	6.901	0.462	8.935	0.423
3C.2a	A/Delaware/44/14	9.470	0.685	10.516	0.419	10.329	0.004	10.849	0.447	12.520	0.439
3C.2a	A/Hawaii/49/15	6.639	0.372	8.876	0.620	9.389	0.359	10.906	0.445	11.733	0.421

Table 13 (Continued)

Genetic Clade	Isolate Virus	TX/50		SW/9715293		PA/6759		HK/4801		FI/2	
		polyPLA	std dev	polyPLA	std dev	polyPLA	std dev	polyPLA	std dev	polyPLA	std dev
BR/10	A/Brisbane/10/2007	4.668	0.561	2.165	0.256	2.786	0.667	8.915	0.599	7.270	0.983
PE/16	A/Perth/16/2009	10.112	0.576	6.137	0.656	5.333	0.341	8.419	0.723	10.708	0.855
3c.1	A/Victoria/361/2011	10.653	0.423	6.762	0.158	8.197	0.270	8.438	0.382	10.556	0.543
3c.1	A/Texas/50/2012	10.205	0.324	8.353	0.416	6.678	0.162	9.225	0.294	9.174	0.823
3c.3a	A/Costa Rica/4700/2013	9.496	0.208	9.529	0.712	6.364	0.307	8.538	0.502	7.204	0.118
3c.2	A/Utah/07/2013	9.718	0.644	8.513	0.391	7.939	0.285	8.400	0.624	10.767	0.080
3c.3a	A/Switzerland/9715293/2013	9.533	0.117	12.378	0.473	9.882	0.271	7.656	0.229	10.779	0.514
3c.3a	A/Palau/6759/2014	8.386	0.159	9.199	0.785	11.331	0.236	6.799	0.184	9.370	0.518
3c.2a	A/Hong Kong/4801/2014	8.776	0.225	5.406	0.560	0.300	0.272	11.474	0.656	11.746	0.764
3c.2a	A/Victoria/503/2015	6.778	0.576	4.494	0.477	2.268	0.173	8.327	0.314	10.079	0.557
3c.2a	A/Fiji/2/2015	8.557	0.578	6.726	0.787	4.989	0.332	10.853	0.439	11.633	0.146
3C.1	A/Texas/50/12	10.487	0.016	6.842	0.349	7.561	0.246	7.009	0.151	7.086	0.380
3C.3a	A/Switzerland/9715293/13	7.563	0.667	9.438	0.092	9.202	0.204	7.313	0.286	7.313	0.315
3C.2a	A/Hong Kong/4801/14	9.000	0.737	8.099	0.204	8.218	0.315	10.175	0.003	10.169	0.486
3c.2a	A/Mississippi/161/2014	6.074	0.535	6.622	0.495	11.906	0.110	14.412	0.161	15.084	0.034
3c.3a	A/Mississippi/162/2014	8.829	0.332	13.090	0.168	13.393	0.699	11.074	0.342	10.674	0.631
3c.3a	A/Mississippi/163/2014	10.647	0.173	11.457	0.293	13.298	0.328	9.591	0.284	10.745	0.464
3c.3a	A/Mississippi/164/2014	N/A	N/A	14.913	0.304	14.241	0.175	12.926	1.575	12.835	0.439
3c.3a	A/Mississippi/165/2014	3.421	0.392	9.176	0.155	9.247	0.219	6.922	0.713	6.102	0.166
3c.3a	A/Mississippi/166/2014	5.914	0.240	11.043	0.750	11.987	0.841	8.043	0.404	9.733	0.131
N/A	A/Mississippi/167/2014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3c.3a	A/Mississippi/168/2014	3.093	0.372	9.584	0.057	9.897	0.501	8.904	0.500	7.317	0.095
3c.3a	A/Mississippi/169/2014	4.681	0.100	7.778	0.271	9.432	0.033	6.109	0.083	7.720	0.128

Table 13 (Continued)

3c.3a	A/Mississippi/180/2014	3.214	0.170	4.232	0.259	6.805	0.653	3.704	0.100	3.667	0.255
3c.3a	A/Mississippi/181/2014	6.818	0.127	11.439	0.195	11.789	0.548	9.972	0.068	9.163	0.168
3c.3a	A/Mississippi/182/2014	7.572	0.518	9.459	0.430	14.355	0.556	13.268	0.037	10.272	0.569
3c.3a	A/Mississippi/183/2014	10.443	0.378	13.552	0.414	13.842	0.015	12.487	0.557	12.336	0.027
3c.3a	A/Mississippi/198/2014	6.232	0.321	13.784	0.570	13.756	0.323	12.725	0.531	11.421	0.421
3c.3a	A/Mississippi/199/2014	6.863	0.298	11.456	0.166	11.729	0.004	9.985	0.530	9.571	0.867
3c.3a	A/Mississippi/200/2014	12.076	0.349	14.299	0.075	14.733	0.684	11.857	0.127	10.368	0.553
3c.3a	A/Mississippi/201/2014	6.866	0.381	11.079	0.738	13.182	0.693	11.497	0.752	10.693	0.151
3c.3a	A/Mississippi/202/2014	5.649	0.365	10.754	0.248	10.794	0.360	11.075	0.414	8.636	0.730
3c.3a	A/Mississippi/203/2014	9.799	0.429	8.101	0.286	9.588	0.445	9.622	1.957	7.195	0.088
3c.2a	A/Mississippi/204/2014	9.695	0.116	7.611	0.310	11.109	0.581	13.470	0.354	13.940	0.199
3c.3a	A/Mississippi/205/2014	4.627	0.680	5.860	0.448	6.918	0.812	5.226	0.149	5.221	0.007
3c.2a	A/Mississippi/206/2014	9.666	0.651	7.982	0.171	8.798	0.438	13.102	0.497	12.375	0.691
3c.3a	A/Mississippi/207/2014	5.518	0.232	9.197	0.270	10.826	0.371	10.518	0.420	9.943	0.723
3c.3a	A/Mississippi/223/2014	3.235	0.400	9.034	0.334	10.061	0.101	6.350	0.526	6.850	0.025
3c.3a	A/Mississippi/224/2014	2.991	0.263	6.074	0.391	9.043	0.238	7.539	0.498	6.718	0.212
3c.2a	A/Mississippi/225/2014	6.317	0.458	7.607	0.498	8.228	0.078	9.541	0.450	9.019	0.356
3c.3a	A/Mississippi/226/2014	7.771	0.343	14.029	0.585	15.429	0.185	13.194	0.407	13.520	0.460
3c.2a	A/Mississippi/227/2014	8.262	0.404	6.687	0.677	8.597	0.284	11.848	0.844	11.323	0.008
3c.3a	A/Mississippi/228/2014	8.302	0.492	12.955	0.406	12.062	0.137	9.361	0.790	9.964	0.285
N/A	A/Mississippi/229/2014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3C.3	A/Missouri/04/14	5.915	0.919	10.602	0.317	11.085	0.284	9.277	1.191	9.603	0.290
3C.3	A/Alaska/05/2014	7.520	0.208	10.718	0.246	10.279	0.440	11.992	0.670	10.276	0.417
3C.3	A/Louisiana/9/13	7.567	0.443	11.481	0.045	10.854	0.359	N/A	N/A	10.367	0.388
3C.3	A/Hawaii/2/14	5.245	0.062	10.026	0.021	11.889	0.407	8.011	0.525	7.881	0.397

Table 13 (Continued)

3C.3	A/Washington/14/14	N/A	N/A	12.722	0.294	11.119	0.419	11.680	0.117	9.120	0.421
3C.3	A/Maryland/10/14	6.557	0.670	13.470	0.505	14.922	0.390	9.968	0.861	9.205	0.376
3C.3	A/New Mexico/20/14	N/A	N/A	12.884	0.424	11.485	0.428	9.494	0.014	10.379	0.420
3C.3	A/New York/1/15	7.127	0.344	11.633	0.363	11.239	0.428	10.337	0.441	10.943	0.409
3C.3	A/Florida/3/15	7.911	0.194	10.487	0.562	11.334	0.413	9.783	0.508	10.941	0.441
3C.3	A/Utah/1/15	7.203	0.931	13.305	0.359	11.212	0.416	9.671	0.319	10.729	0.444
3C.3	A/Colorado/1/15	N/A	N/A	10.807	0.830	12.207	0.388	7.208	0.424	6.654	0.372
3C.3	A/Ohio/11/15	6.577	0.986	9.752	0.376	8.170	0.409	N/A	N/A	5.267	0.433
3C.3	A/Iowa/6/15	7.571	0.388	10.812	0.674	11.132	0.400	10.726	0.446	10.515	0.421
3C.3	A/Massachusetts/1/15	3.409	0.788	9.805	0.278	9.635	0.300	8.114	0.511	8.070	0.127
3C.3	A/Arkansas/10/14	7.519	0.881	12.566	0.761	12.648	0.077	9.267	0.816	9.584	0.318
3C.3	A/Wisconsin/37/13	6.616	0.674	9.609	0.514	10.519	0.440	6.455	0.320	7.388	0.466
3C.3	A/Indiana/29/14	6.847	0.471	9.689	0.168	9.062	0.411	9.459	0.136	6.134	0.449
3C.3	A/Wyoming/19/14	6.057	0.048	12.429	0.608	10.843	0.425	8.776	0.555	10.510	0.406
3C.3a	A/California/4/14	N/A	N/A	8.942	0.408	9.665	0.348	7.682	0.141	8.092	0.355
3C.3a	A/North Carolina/12/14	5.497	0.699	12.277	0.493	12.103	0.700	9.033	0.765	8.749	0.105
3C.3a	A/North Carolina/14/14	4.016	0.804	8.604	0.572	9.163	0.444	8.297	0.218	5.409	0.478
3C.3a	A/California/18/14	7.239	0.329	10.579	0.645	11.970	0.355	N/A	N/A	6.767	0.407
3C.3a	A/Mississippi/3/14	5.076	0.139	10.925	0.160	11.497	0.380	8.425	0.259	9.618	0.390
3C.3a	A/Hawaii/49/14	6.965	0.010	9.553	0.077	10.429	0.436	N/A	N/A	8.448	0.461
3C.3a	A/Texas/11/16	5.126	0.629	11.846	0.197	12.215	0.151	9.316	0.158	8.710	0.486
3C.3a	A/California/2/16	7.320	0.285	12.380	0.465	11.199	0.347	9.609	0.530	8.614	0.364
N/A	A/Montana/13/16	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3C.3a	A/Oklahoma/2/16	6.367	0.443	9.106	0.620	9.198	0.349	9.421	0.406	6.860	0.364
3C.3a	A/South Carolina/17/16	4.145	0.339	10.365	0.035	10.470	0.311	N/A	N/A	6.668	0.342

Table 13 (Continued)

3C.3a	A/Colorado/22/16	4.971	0.484	10.653	0.092	10.432	0.328	9.736	0.022	8.877	0.354
3C.3a	A/Idaho/10/15	9.364	0.209	10.758	0.095	11.191	0.343	9.687	0.045	9.961	0.366
3C.3a	A/Nebraska/3/15	6.142	0.125	10.773	0.040	9.913	0.322	9.806	0.119	8.763	0.325
3C.3b	A/Pennsylvania/6/15	3.880	0.142	8.601	0.311	6.865	0.274	9.426	0.189	7.545	0.021
3C.3b	A/Washington/53/14	1.674	0.693	9.441	0.338	8.063	0.224	10.153	0.027	9.499	0.008
N/A	A/Montana/3/15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3C.3b	A/Hawaii/34/15	2.953	0.500	9.757	0.405	8.144	0.484	9.639	0.112	6.899	0.052
3C.3b	A/Pennsylvania/1/15	5.690	0.037	11.442	0.076	10.480	0.304	10.297	0.116	8.854	0.330
3C.2	A/Pennsylvania/27/14	6.116	0.476	7.241	0.782	6.520	0.711	8.390	0.866	10.145	0.079
3C.2	A/New Mexico/23/13	7.054	0.004	8.613	0.678	8.061	0.008	9.057	0.571	10.235	0.020
3C.2	A/California/58/13	5.897	0.128	11.415	0.058	10.328	0.335	12.847	0.167	11.793	0.329
3C.2a	A/Vermont/3/14	5.239	0.684	7.787	0.696	7.271	0.657	9.369	0.826	8.889	0.122
3C.2a	A/Minnesota/7/14	4.400	0.302	7.542	0.368	8.638	0.264	8.139	0.501	10.001	0.021
3C.2a	A/Rhode Island/11/14	7.295	0.254	8.734	0.112	9.463	0.418	9.088	0.187	9.660	0.438
3C.2a	A/Connecticut/4/14	9.848	0.272	11.094	0.045	8.911	0.327	9.850	0.029	10.820	0.316
3C.2a	A/New Jersey/13/14	6.538	0.450	9.858	0.111	10.432	0.357	10.104	0.236	11.304	0.369
3C.2a	A/New Mexico/11/14	7.751	0.033	8.477	0.125	8.306	0.352	9.949	0.229	10.304	0.341
3C.2a	A/Florida/1/16	2.586	0.295	6.151	0.368	5.344	0.052	7.660	0.101	6.866	0.030
3C.2a	A/Minnesota/54/15	4.122	0.478	9.989	0.106	9.473	0.402	10.240	0.687	10.359	0.413
3C.2a1	A/North Carolina/4/16	7.159	0.431	7.478	0.309	7.561	0.396	10.262	0.332	8.721	0.404
3C.2a	A/Texas/73/14	6.195	0.394	12.177	0.201	11.921	0.052	13.687	0.130	13.141	0.118
3C.2a	A/Maine/4/15	6.619	0.413	7.580	0.477	8.957	0.057	9.277	0.485	9.991	0.143
3C.2a	A/Florida/5/15	6.997	0.110	8.015	0.341	9.190	0.341	10.361	0.241	11.107	0.330
3C.2a	A/West Virginia/1/15	5.309	0.187	7.546	0.277	7.497	0.357	9.472	0.239	9.483	0.345
3C.2a1	A/Washington/19/16	7.542	0.513	8.419	0.097	9.242	0.333	9.893	0.035	10.233	0.332

Table 13 (Continued)

N/A	A/Wyoming/19/15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
N/A	A/Colorado/27/15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3C.2a	A/Oregon/1/15	4.071	0.555	6.989	0.847	7.010	0.211	7.938	0.729	8.533	0.119
3C.2a	A/Utah/2/15	8.940	0.247	8.940	0.209	7.774	0.462	10.622	0.122	9.808	0.423
3C.2a	A/Delaware/44/14	7.682	0.503	9.649	0.104	10.703	0.447	11.596	0.993	12.374	0.439
3C.2a	A/Hawaii/49/15	6.821	0.498	10.826	0.046	11.790	0.445	12.040	0.044	12.618	0.421

Genetic Clade	Isolate Virus	BR/10	PE/16	VI/361	TX/50	CR/4700	UT/07	SW/9715293	PA/6759	HK/4801	VI/503	FI/2
BR/10	A/Brisbane/10/2007	**1280**	40	<10	20	40	<10	<10	<10	320	80	160
PE/16	A/Perth/16/2009	40	**1280**	320	640	640	320	80	40	320	<10	640
3c.1	A/Victoria/361/2011	80	640	**640**	640	320	640	320	320	320	<10	640
3c.1	A/Texas/50/2012	160	640	640	**1280**	640	320	160	160	320	160	640
3c.3a	A/Costa Rica/4700/2013	<10	320	320	320	**1280**	160	320	160	160	80	320
3c.2	A/Utah/07/2013	160	640	320	320	640	**1280**	320	320	320	320	640
3c.3a	A/Switzerland/9715293/2013	<10	160	320	640	40	640	**1280**	1280	320	80	640
3c.3a	A/Palau/6759/2014	<10	160	320	320	160	640	1280	**1280**	160	80	640
3c.2a	A/Hong Kong/4801/2014	80	320	160	320	320	40	40	<10	**1280**	640	1280
3c.2a	A/Victoria/503/2015	40	320	80	80	160	20	10	10	640	**640**	1280
3c.2a	A/Fiji/2/2015	80	320	160	320	320	80	80	20	1280	640	**1280**
3C.1	A/Texas/50/12	40	640	1280	640	160	320	160	160	320	<10	320
3C.3a	A/Switzerland/9715293/13	<10	<10	<10	40	<10	<10	160	160	80	<10	320
3C.2a	A/Hong Kong/4801/14	40	160	40	160	160	<10	<10	<10	640	640	640
3c.2a	A/Mississippi/161/2014	40	80	80	160	80	80	80	80	160	80	160
3c.3a	A/Mississippi/162/2014	<10	320	160	160	160	160	160	160	160	<10	320
3c.3a	A/Mississippi/163/2014	160	640	640	640	640	640	320	640	320	320	640

Table 13 (Continued)

3c.3a	A/Mississippi/164/2014	80	320	640	320	320	320	160	320	320	160	640
3c.3a	A/Mississippi/165/2014	40	320	160	160	160	320	160	160	160	160	320
3c.3a	A/Mississippi/166/2014	80	640	640	640	640	640	160	320	640	320	1280
N/A	A/Mississippi/167/2014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3c.3a	A/Mississippi/168/2014	80	640	640	640	640	640	320	320	640	320	1280
3c.3a	A/Mississippi/169/2014	80	320	320	320	320	640	320	320	320	160	640
3c.3a	A/Mississippi/180/2014	160	640	640	320	320	640	320	320	320	160	640
3c.3a	A/Mississippi/181/2014	80	640	320	640	640	640	320	320	320	<10	640
3c.3a	A/Mississippi/182/2014	40	320	160	320	320	320	160	160	320	160	640
3c.3a	A/Mississippi/183/2014	80	640	320	320	320	640	320	320	320	160	1280
3c.3a	A/Mississippi/198/2014	80	320	160	320	320	320	160	320	320	160	320
3c.3a	A/Mississippi/199/2014	80	320	320	320	320	320	160	160	320	160	640
3c.3a	A/Mississippi/200/2014	80	320	320	320	320	640	160	160	320	160	640
3c.3a	A/Mississippi/201/2014	80	320	320	320	320	320	160	160	160	160	320
3c.3a	A/Mississippi/202/2014	<10	320	160	320	160	320	80	160	160	80	320
3c.3a	A/Mississippi/203/2014	80	320	320	320	320	640	160	160	160	160	640
3c.2a	A/Mississippi/204/2014	80	320	320	320	320	320	160	160	640	320	640
3c.3a	A/Mississippi/205/2014	80	320	320	320	320	320	160	160	160	160	320
3c.2a	A/Mississippi/206/2014	40	80	80	80	80	80	160	80	80	80	320
3c.3a	A/Mississippi/207/2014	80	640	640	320	320	640	160	320	320	160	640
3c.3a	A/Mississippi/223/2014	80	320	320	320	160	320	160	160	160	160	320
3c.3a	A/Mississippi/224/2014	80	320	320	320	320	320	160	160	320	160	640
3c.2a	A/Mississippi/225/2014	<10	80	80	80	80	80	80	160	80	40	320
3c.3a	A/Mississippi/226/2014	80	320	320	320	160	320	160	160	320	160	640
3c.2a	A/Mississippi/227/2014	160	320	320	320	320	320	320	320	320	160	640

Table 13 (Continued)

3c.3a	A/Mississippi/228/2014	80	640	640	320	320	640	160	320	320	160	640
N/A	A/Mississippi/229/2014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3C.3	A/Missouri/04/14	160	320	320	320	320	320	160	320	320	160	640
3C.3	A/Alaska/05/2014	80	320	160	320	320	320	160	160	160	80	320
3C.3	A/Louisiana/9/13	80	320	160	320	160	320	160	160	80	80	320
3C.3	A/Hawaii/2/14	320	640	320	320	320	640	320	320	320	160	640
3C.3	A/Washington/14/14	80	320	160	320	160	320	160	160	160	80	320
3C.3	A/Maryland/10/14	40	80	80	80	80	160	40	40	80	20	160
3C.3	A/New Mexico/20/14	80	160	80	160	80	160	80	80	160	80	320
3C.3	A/New York/1/15	<10	160	160	160	80	160	80	80	80	40	160
3C.3	A/Florida/3/15	<10	80	80	160	80	160	40	80	80	40	160
3C.3	A/Utah/1/15	<10	160	80	160	80	160	80	80	160	80	160
3C.3	A/Colorado/1/15	80	320	320	320	320	640	320	320	640	160	640
3C.3	A/Ohio/11/15	<10	40	40	80	80	80	160	160	80	40	320
3C.3	A/Iowa/6/15	40	320	160	320	320	320	160	160	320	80	640
3C.3	A/Massachusetts/1/15	40	320	160	160	160	320	160	160	160	80	320
3C.3	A/Arkansas/10/14	80	320	160	320	160	320	160	160	160	80	320
3C.3	A/Wisconsin/37/13	40	80	80	160	80	160	80	40	80	40	160
3C.3	A/Indiana/29/14	<10	80	40	80	40	80	40	40	40	20	80
3C.3	A/Wyoming/19/14	40	320	160	320	320	160	160	160	160	160	320
3C.3a	A/California/4/14	40	160	80	160	80	160	80	80	160	80	160
3C.3a	A/North Carolina/12/14	<10	40	40	80	80	80	160	160	80	40	160
3C.3a	A/North Carolina/14/14	<10	10	20	40	40	40	80	80	40	20	80
3C.3a	A/California/18/14	40	160	80	160	160	160	80	80	160	80	320
3C.3a	A/Mississippi/3/14	<10	10	40	80	80	80	160	160	80	20	320

Table 13 (Continued)

3C.3a	A/Hawaii/49/14	<10	40	40	80	80	80	160	160	80	40	320
3C.3a	A/Texas/11/16	<10	20	40	40	80	80	160	160	80	40	320
3C.3a	A/California/2/16	<10	20	20	40	40	40	80	80	80	20	160
N/A	A/Montana/13/16	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3C.3a	A/Oklahoma/2/16	<10	<10	20	20	40	20	80	80	40	20	160
3C.3a	A/South Carolina/17/16	<10	<10	40	80	80	80	160	160	80	40	160
3C.3a	A/Colorado/22/16	<10	<10	40	80	80	160	160	160	160	10	160
3C.3a	A/Idaho/10/15	<10	40	40	80	80	80	160	160	80	40	160
3C.3a	A/Nebraska/3/15	<10	40	80	80	80	160	320	320	160	40	320
3C.3b	A/Pennsylvania/6/15	80	320	160	320	160	320	160	160	160	80	320
3C.3b	A/Washington/53/14	80	320	160	320	160	320	160	320	320	160	640
N/A	A/Montana/3/15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3C.3b	A/Hawaii/34/15	40	160	80	160	80	160	80	80	160	40	320
3C.3b	A/Pennsylvania/1/15	40	320	160	320	160	320	160	160	160	80	320
3C.2	A/Pennsylvania/27/14	80	320	160	320	160	320	160	160	320	80	320
3C.2	A/New Mexico/23/13	<10	640	320	320	160	320	320	160	320	160	640
3C.2	A/California/58/13	80	320	160	320	160	320	160	160	320	160	640
3C.2a	A/Vermont/3/14	<10	160	160	160	80	160	80	80	320	160	640
3C.2a	A/Minnesota/7/14	80	160	80	160	160	160	160	160	160	80	320
3C.2a	A/Rhode Island/11/14	40	160	80	160	160	320	160	160	160	160	320
3C.2a	A/Connecticut/4/14	20	80	80	160	80	160	160	80	160	20	160
3C.2a	A/New Jersey/13/14	80	320	160	160	160	160	160	320	160	160	320
3C.2a	A/New Mexico/11/14	40	80	40	160	80	160	80	80	160	40	160
3C.2a	A/Florida/1/16	<10	80	160	320	80	160	80	160	640	160	640
3C.2a	A/Minnesota/54/15	80	160	80	160	160	320	160	80	320	160	320

Table 13 (Continued)

3C.2a1	A/North Carolina/4/16	<10	40	<10	80	40	80	40	<10	160	80	160
3C.2a	A/Texas/73/14	40	40	40	80	80	160	80	80	160	80	160
3C.2a	A/Maine/4/15	80	160	160	320	160	320	320	160	320	<10	640
3C.2a	A/Florida/5/15	5	80	80	160	80	80	80	80	320	160	640
3C.2a	A/West Virginia/1/15	160	320	160	320	320	320	320	320	320	160	640
3C.2a1	A/Washington/19/16	<10	10	<10	20	10	20	<10	<10	80	40	80
N/A	A/Wyoming/19/15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
N/A	A/Colorado/27/15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3C.2a	A/Oregon/1/15	<10	160	160	160	80	160	80	80	640	160	1280
3C.2a	A/Utah/2/15	40	160	80	160	80	160	80	80	160	80	320
3C.2a	A/Delaware/44/14	80	160	160	160	160	320	160	320	320	80	320
3C.2a	A/Hawaii/49/15	80	160	160	160	160	80	160	160	160	160	320

Uncultivable samples

Totally, six clinical samples were unable to be isolated with MDCK cells. Sequence analyses of isolated viral RNA directly from the clinical samples concluded that two samples were of the 3C.2a subclade (A/Colorado/27/2015, A/Wyoming/19/2015), three samples were of the 3C.3a subclade (A/Mississippi/167/2014, A/Mississippi/229/2014, A/Montana/13/2016), and one sample was of the 3C.3b subclade (A/Montana/3/2015). With the use of polyPLA, the samples were able to be antigenically characterized. The clinical samples had larger polyPLA values against the polyclonal antibodies from the same genetic subclade.

Phylogenetic analysis

Twenty-eight out of 30 MSDH viruses were isolated from MDCK cells. Of the 58 total clinical and isolate samples, 24 clinical samples and 28 isolate samples were sequenced; totally, 23 pairs were sequenced. Additionally, 58 out of the 60 CDC viruses were isolated from MDCK cells; from GISAID EpiFlu database (http://platform.gisaid.org/), full length consensus sequences for 12 HA and 16 NA clinical and 58 HA and 48 NA isolate were downloaded.

Since 2007, the H3N2 seasonal influenza viruses have six genetic clusters (231). The viruses from 2013 belong to genetic clades 3c.2a and 3c.3a.The phylogenetic analyses of the consensus sequences for HA gene showed that all 90 viruses belong to genetic clusters 3c.2a and 3c.3a (Fig. 14a). For H3, 14 out of 33 (42.4%) pairs differed in sequence from clinical to isolate and for N2, 9 out of 27 (33.3%) pairs differed in sequence from clinical to isolate (Fig. 14b).

Figure 14 Phylogenic analysis for HA and NA genes of all viruses.

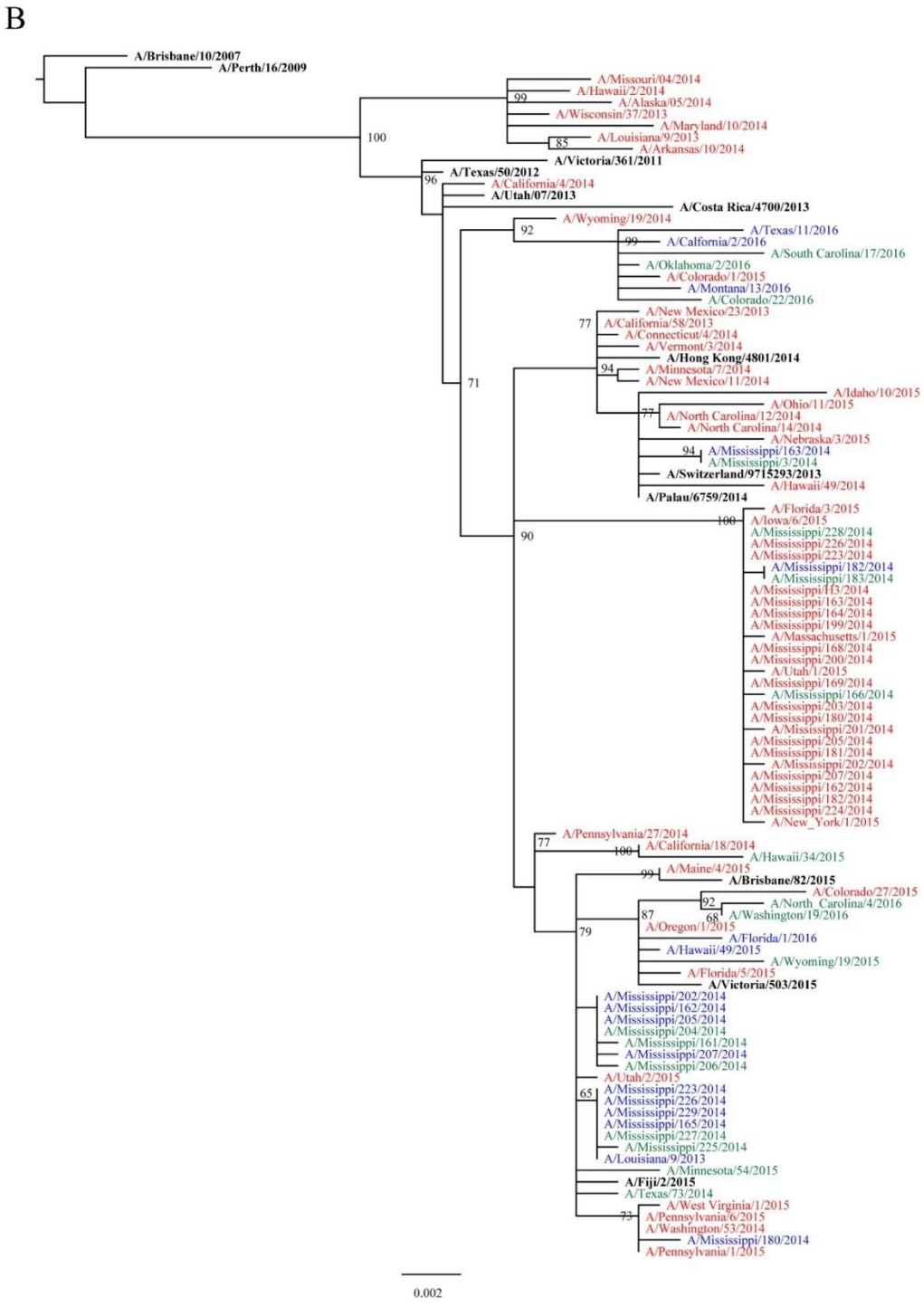

Figure 14 (Continued)

(A) Phylogenetic tree of HA nucleotide sequences of H3N2 seasonal influenza viruses. (B) Phylogenetic tree of NA nucleotide sequences of H3N2 seasonal influenza viruses. The 90 pairs of viruses are from 2013-2016 H3N2 IAV from MSDH and CDC. Unique clinical sequences were marked in blue, unique isolate sequences were marked in red, similar clinical and isolate sequences were marked in green, and reference sequences are in bold. The phylogenetic tree was constructed by maximum likelihood based on HA nucleotide sequences.

HA polymorphism distribution

Full protein consensus sequences for 23 pairs of clinical and isolate HA proteins of the MSDH viruses were analyzed for polymorphism distribution. The average depth of coverage for the polymorphic sites identified in HA is 8,275 with a range from 1,186 to 18,544. Overall, 21 residues had significantly different proportions of amino acids between 3C.2 and 3C.3 of both clinical and isolated samples. For 3C.3, 12 residues were significantly different between clinical and isolate samples but there were no residues significantly different between clinical and isolate samples for 3C.2 (Fig. 15).

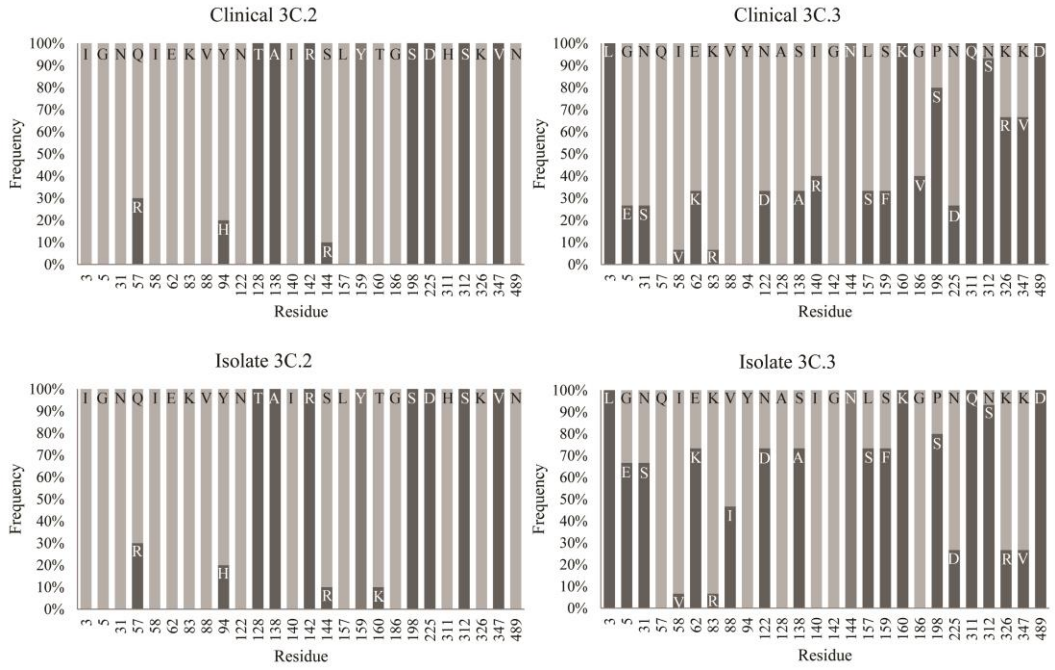

Figure 15 The polymorphism distribution in 84 clinical and isolate pairs.

Using the comparison of proportions calculator (MedCalc) significant difference between proportions of residue changes from H3 genetic clades 3C.2 and 3C.3 are shown.

polymorphisms in the sequences for the HA protein in the pubic databases

Totally, protein consensus sequences of HA for 827 original and isolate H3N2 pairs from 2012 until 2017 were downloaded from GISAID. Of these, 600 pairs were genetically related to 3C.2 and 227 were genetically related to 3C.3. Totally, 29 residues had significantly different proportions of amino acids between 3C.2 and 3C.3 of both original and isolated samples. There were no residues that were significantly different between original and isolate samples (Fig. 16).

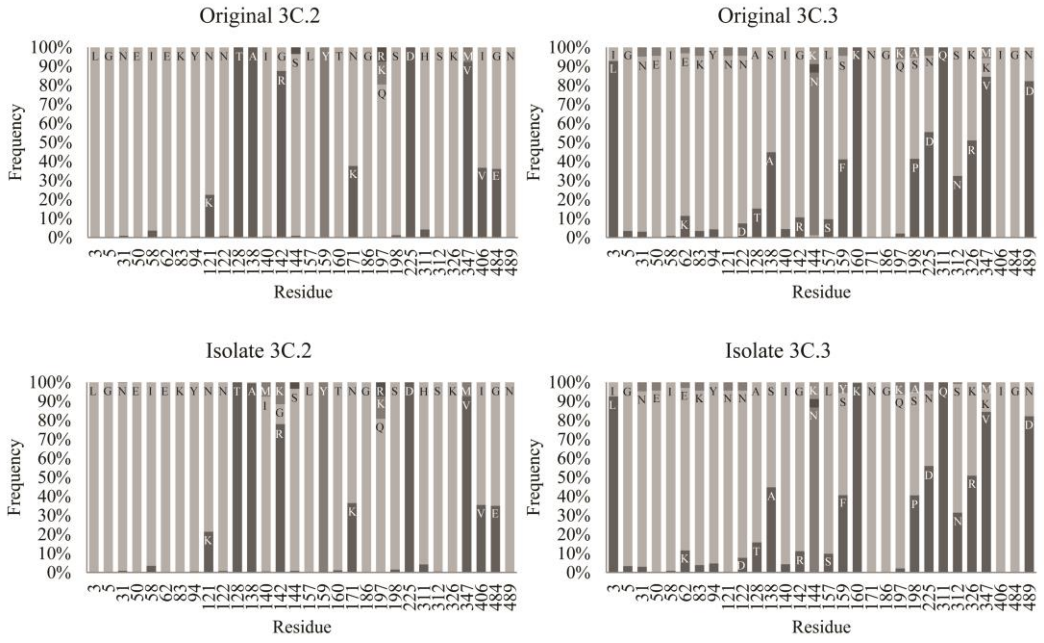

Figure 16 The polymorphism distribution in human H3N2 from 2012 to 2017, 3C.2 original and isolate pairs (n=600) and 3C.3 original and isolate pairs (n=227) samples downloaded from GISAID.

Using the comparison of proportions calculator (MedCalc) significant difference between proportions of residue changes from H3 genetic clades 3C.2 and 3C.3 are shown.

All viruses in the genetic subgroup 3C.2a have amino acids changes at residues L3I, F159Y, K160T, N335D, Q311H, and D489N. K160T is shown to be an addition of a glycosylation motif at 158-160. 90% of viruses have the loss of glycosylation motif at N144S. 20% of samples have the same amino acid changes at residues N171K I406V, and G484E. Genetic subgroup 3C.3 contains amino acid changes at residues T128A, a loss of glycosylation motif, and R142G. Subgroup 3C.3a has additional residue changes at A138S, F159S, N225D, and K326R.

Using paired data for both clinical and isolate, a genetic and antigenic switch occurred in eight MSDH samples; all of these eight clinical samples belonging to 3C.2a and all the eight corresponding isolates belonging to 3C.3a. Through NGS analysis, two clinical samples (A/Mississippi/207/2014, A/Mississippi/226/2014) have polymorphisms within the HA protein yet no polymorphisms within the isolate sample, which is what is expected. While the other six clinical samples have no polymorphisms, more interestingly enough, four of those samples have polymorphisms within the isolate HA protein. Two samples have no polymorphisms present in either clinical or isolate.

Discussion

In this study, a polyPLA assay was used to detect antigenic variants of contemporary human H3 IAVs in the United States. The previously defined detection limit for polyPLA is approximately 10^3 TCID50/ mL(220, 232), which is much less than the viral loads from most patients at the peak time of virus shedding (201). This assay was used to differentiate viruses in genetic clusters 3C.2a from those in 3C.3a directly in clinical samples, such as nasal swab samples.

With the Δ Ct PLA unit of 3.00, this assay can detect antigenic variants with a specificity of 100% and a sensitivity of 98.9% in influenza positive samples collected during influenza surveillance. Thus, the polyPLA is specific and sensitive enough to be used for antigenic variant detection in human H3 IAVs. Because it is developed based on a qRT-PCR platform, polyPLA can be used for high-throughput antigenic screening in most laboratories; this assay reduces the necessary time of 3-5 days for conventional methods to only a few hours after specimen collection for antigenic characterization, because there is no need for virus isolation. Detection of possible emerging antigenic variants can increase effectiveness of vaccination programs.

Low or undetectable hemagglutination activity was common, particularly for 3C.2a, which caused antigenic characterization to be more difficult of the novel viruses (118). Ferret sera from cell-propagated 3C.3a and 3C.2a inhibited most circulating viruses, so WHO has concluded that 3C.3a and 3C.2a were antigenically related. With the use of polyPLA, antigenic clades could be better distinguished. The antigenic map of polyPLA units is in agreement with the antigenic map of HI titers, both show slight separation of 3C.2a and 3C.3a.

In this study, as well as others, have seen a variety of viruses with different HA1 mutations for both 3c.3a and 3c.2a(119). Additionally, mutations occurred in each antigenic site (A-E); typically the accumulation of amino acid changes in HA are clustered in the antigenic sites located close to the receptor binding sites (233). In a previous study, 42% of H3N2 viruses isolated from MDCK cells contained substitutions in most viral segments as compared to their original clinical sample (196). In our study,

we saw that in HA and NA gene segments specifically, 42.4% and 33.3%, respectively contained substitutions in viral isolates as compared to clinical samples.

It has been well established genetic evolution is a continuous process, hence relying on consensus sequences alone for influenza genetic analysis can miss information about the true population of viruses within a sample. Additionally, it is possible that with the use of reverse transcriptase or Taq, template switching can occur during the first amplification cycles, leading to artificial chimaeras(234). Even in conventional Sanger sequencing, *in silico* recombination could occur by assembling fragments that have been sequenced separately(235). With NGS, the entire genome of influenza can be sequenced at once and the true genetic variation within a viral quasispecies can be identified (155, 156). Further analysis on NGS of all 84 clinical and isolate pairs is still needed in order to analyze polymorphisms within each sample population.

Additionally, the original viral population is not limited one identical viral genome and the cell-propagated population of viruses should be more homogeneous (42-45). Of the 23 pairs of clinical and isolate HA sequences, eight pairs had polymorphisms in clinical samples, yet not in isolates, as expected; nine pairs did not contain polymorphisms in either clinical or isolate; six pairs contained polymorphisms in isolates but not in clinical samples. The average coverage for NGS was approximately 8,000; the reason for the eight MSDH samples that have a switch in genetic subclade is still unclear.

Conclusions

Detection of antigenic variants of H3 human influenza viruses from clinical samples can give additional information that can be missed in traditional surveillance techniques. Both genetic subclusters 3C.2a and 3C.3a of H3N2 are currently circulating

in the human population. The direct use of influenza positive clinical samples can help to give better insight into the true circulating population of viruses. In this chapter, we see both genetic and antigenic differences in original clinical samples as compared to the isolated viruses with the use of polyPLA and NGS. Further analyses will be completed, including sparse machine learning, to establish a connection between genetic and antigenic data for clinical and isolate pairs.

CHAPTER V

DETECTION OF ANTIGENIC VARIANTS OF SWINE SUBTYPE H3 INFLUENZA A VIRUSES FROM CLINICAL SAMPLES

A large population of genetically and antigenically diverse influenza A viruses (IAVs) are circulating among the swine population, playing an important role in influenza ecology. Swine IAVs not only cause outbreaks among swine, but they can also be transmitted to humans, causing sporadic infections and even pandemic outbreaks. Antigenic characterization of swine IAVs are key to understanding the natural history of these viruses in swine and to select strains for effective vaccines. However, influenza outbreaks generally spread rapidly among swine, and the conventional methods for antigenic characterization require virus propagation, a time-consuming process that can significantly reduce the effectiveness of vaccination programs. We developed and validated a rapid, sensitive, and robust method, the polyclonal sera–based proximity ligation assay (polyPLA), to identify antigenic variants of subtype H3N2 swine IAVs. This method utilizes oligonucleotide–conjugated polyclonal antibodies and quantifies antibody–antigen binding affinities by quantitative RT-PCR. The results showed that the assay can rapidly detect H3N2 IAVs directly from nasal wash or nasal swab samples collected from laboratory-challenged animals or during influenza surveillance at county fairs. In addition, the polyPLA can accurately separate the viruses at two contemporary swine IAV antigenic clusters (H3N2 swine IAV-α and H3N2 swine IAV-ß) with a

sensitivity of 84.9% and a specificity of 100.0%. The polyPLA can be routinely used in surveillance programs to detect antigenic variants of influenza viruses and to select vaccine strains for use in controlling and preventing disease in swine.

Introduction

Influenza A virus (IAV) can infect swine, causing typical signs of respiratory disease (e.g., fever, depression, coughing, nasal discharge, sneezing, and breathing difficulty) that generally last 5–7 days. IAVs can be transmitted between humans and swine, both of which have *N*-acetylneuraminic acid–α2, 6–linked galactose (NeuAcα-2,6Gal) serving as receptors for IAVs in their upper respiratory tracks (9). In addition to NeuAcα-2,6Gal, swine also have *N*-acetylneuraminic acid–α2, 3–linked galactose (NeuAcα-2,3Gal) serving as receptors for IAVs in their upper respiratory tracks. Thus, swine have been proposed to serve as "mixing vessels," generating novel IAVs by reassorting avian IAVs and swine IAVs and facilitating adaptation of avian IAVs before their spillover to the human population (236, 237). Swine IAVs cause sporadic cases of influenza among humans as well as a pandemic in human, e.g. by a swine-origin IAV, influenza A(H1N1)pdm09 (21). On the other hand, it is not uncommon for IAVs to be introduced from humans to swine (238). For subtype H3N2 specifically, at least 13 infections caused by the introduction of virus from humans to swine were identified from 1965 to 2013 (239). Thus, understanding the genetic and antigenic diversity of IAVs in swine is key to developing an effective strategy for influenza prevention and control among domestic swine and to protect public health.

In the United States, surveillance has shown that a genetically diverse population of IAVs are circulating among domestic swine, and these viruses are subtype H1 or H3

(57, 84). At least seven genetic clusters of swine subtype H1 IAVs (H1α, H1ß, H1γ1, H1γ2, H1δ1, H1δ2) and A(H1N1)pdm09 are circulating in the swine population, and the viruses in these H1 genetic clusters are antigenically distinct (173). A recent study suggested the proportion for the number of swine subtype H3N2 IAVs over the total number of swine IAVs recovered from domestic swine have increased, and these viruses are widely spread across the United States (29). Molecular characterization suggested that the hemagglutinin (HA) genes of contemporary swine subtype H3 IAVs belong genetically to cluster IV, and these viruses can be antigenically separated into two groups, H3N2 swine IAV-α and H3N2 swine IAV-ß (abbreviated as H3α and H3ß) (240).

Vaccination has been routinely utilized in the United States to prevent virus transmission at swine farm operations (241) and swine exhibits at agricultural fairs (239). Swine vaccines, which are available as licensed commercial products, are typically produced from culture-derived virions incubated in crude allantoic fluid from special pathogen–free chicken eggs. After incubation, the virus is chemically inactivated and formulated into a mineral oil emulsion vaccine (242). Unlike the process for producing vaccines for humans, this process for producing swine vaccines avoids costly purification steps for enrichment of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (58); however, potential booster vaccinations are necessary to achieve and maintain protective levels of systemic HA-inhibiting antibodies (59).

Production of an effective vaccine requires an antigenic match between circulating strains and the vaccine strain, but that is not a trivial task. IAV transmission occurs rapidly among swine populations, especially on farms with middle- or large-scale operations; thus, vaccine types must be determined quickly, and vaccines must be rapidly

produced and administered (within days of an outbreak) to effectively mitigate the spread of disease. However, the conventional methods for antigenic characterization (e.g., hemagglutination inhibition [HI]) require virus isolation, which usually takes at least 3 days to accomplish. Thus, because of the antigenic diversity of swine IAVs, conventional methods cannot be used to determine immunologic reactions between circulating viruses and available vaccines. Therefore, many vaccines for swine IAVs in the United States have been produced locally and autogenously without efficacy testing (243).

It is not uncommon for vaccines that are antigenically inaccurate for circulating viruses to lead to vaccine-induced immunity, causing antigenic drift and the silent spread of virus (244). Moreover, the generation of novel escape mutants could gradually lead to the replacement of circulating virus strains (61, 62), further complicating the antigenic and genetic evolution of swine IAVs. The occurrence of such antigenic drift events in swine is well documented (57, 63, 64). To develop an effective and robust swine IAV vaccination program, a rapid and robust method for antigenic characterization in swine is needed. Ideally, the assay would not require virus isolation but would be sensitive enough to directly perform antigenic characterization using clinical samples.

A polyclonal serum–based proximity ligation assay (polyPLA) was developed recently to measure antibody–antigen binding affinities without using red blood cells (RBCs) (220). The polyPLA attaches probes to antibody, and then quantifies the binding affinities between antibody and antigen by quantitative RT-PCR. This method is highly sensitive and was shown to effectively detect antigenic variants for human seasonal influenza subtype H3N2 virus in human nasopharyngeal swab samples, thus avoiding the need for virus isolation (220). Our goal was to develop a specific polyPLA method to

quantify the antibody–antigen interaction for swine H3 IAVs. This method would be useful for vaccine strain selection for swine IAVs.

Materials and Methods

Viruses and serum samples.

Seven contemporary (2009–2011) swine H3N2 IAV isolates and their homologous ferret serum samples were chosen to represent the swine IAV antigenic groups H3α and H3ß (Table 14); antigenic characterization of these isolates is described elsewhere (240). The strain of A/California/04/2009(H1N1) (abbreviated as CA/04) [A(H1N1pdm09] and homologous ferret serum was used as a negative control. The anti-NP monoclonal antibody was obtained from BEI Resources (Manassas, VA, USA).

Table 14 Antigenic characterization of H3N2 swine influenza viruses using hemagglutination inhibition assay and polyPLA

		Ferret Antiserum[a]							
		09SW96[b]		10SW215[b]		11SW347[b]		CA/04[b]	
	Antigenic		polyPLA		polyPLA		polyPLA		polyPLA
Virus	Cluster	HI[c]	(SD)[d]	HI[c]	(SD)[d]	HI[c]	(SD)[d]	HI[c]	(SD)[d]
A/swine/Ohio/09SW64/2009(H3N2)	H3α	1,600	10.37 (0.10)	40	6.14 (0.69)	<10	5.20 (0.36)	<10	N/A (N/A)
A/swine/Ohio/09SW96/2009(H3N2)	H3α	**1,280**	**11.06 (0.72)**	40	4.53 (0.71)	<10	1.71 (0.52)	<10	N/A (N/A)
A/swine/Ohio/10SW130/2010(H3N2)	H3ß	40	6.62 (0.86)	640	11.87 (0.33)	640	10.62 (0.28)	<10	N/A (N/A)
A/swine/Ohio/10SW156/2010(H3N2)	H3ß	20	4.12 (0.37)	1,280	14.85 (0.36)	640	9.52 (0.56)	<10	N/A (N/A)
A/swine/Ohio/10SW215/2010(H3N2)	H3ß	40	4.01 (1.32)	**1,280**	**16.47 (0.11)**	640	11.64 (0.10)	<10	N/A (N/A)
A/swine/Ohio/11SW208/2011(H3N2)	H3ß	40	7.24 (0.65)	320	11.59 (0.46)	1,024	12.26 (0.28)	<10	N/A (N/A)
A/swine/Ohio/11SW347/2011(H3N2)	H3ß	20	4.99 (0.14)	320	8.15 (0.44)	**1,280**	**12.63 (0.39)**	<10	N/A (N/A)
A/California/04/2009(H1N1)[f]	H1pdm09	10	6.66 (0.95)	<10	4.83 (0.51)	<10	N/A (N/A)	**1,280**	**9.70 (0.61)**

[a] The viruses were collected from pigs at agricultural fairs in Ohio, USA, 2009–2011 (240)
[b] Homologous titers are in bold.
[c] 09SW96, A/swine/Ohio/09SW96/2009(H3N2); 10SW215, A/swine/Ohio/10SW215/2010(H3N2); 11SW347, A/swine/Ohio/11SW347/2011(H3N2); CA/04, A/California/04/2009(H1N1).
[d] HI, hemagglutination inhibition assay. Each experiment was repeated two times, and each HI value reported in this table is an average number from three independent experiments.
[e] polyPLA, polyclonal sera based proximity ligation assay; SD, standard deviation. Values represent the average of three replicate experiments.
[f] A/California/04/2009 (H1N1) was used as a negative control.

Clinical samples.

A total of 120 nasal wash and nasal swab samples were collected for 10 consecutive days post infection (dpi) from 8 feral swine infected with A/swine/Texas/A01104013/2012 (H3N2) and 4 sentinel feral swine. The details for the experimental design and sample collection are available from a prior publication (183). Among these samples, 42 were tested with a detectable $TCID_{50}$, and these samples were used in this study to test the sensitivity and specificity of the proposed polyPLA method.

We also tested 81 nasal swab samples that had been collected from swine at the pig exibits at agricultural fairs in Ohio during 2009–2013; 61 of the samples were

positive for IAV, using matrix gene–based quantiative RT-PCR (qRT-PCR) (240, 245); and 20 of the samples were negative for IAV. A power analysis (OpenEpi, Version 3) suggested that a sample size of 20 gave 95% probability to detect ±10% with expected specificity (246). Of those 61 IAV-positive samples, 50 were subtype H3 and 11 were subtype H1.

HA and HI assays.

HI was performed as previously described (247). In brief, receptor-destroying enzyme (RDE; Denka Seiken Co., Ltd., Tokyo, Japan) was incubated overnight at a 1:3 ratio (vol:vol) with ferret antisera. After incubation, the mixture was heat-inactivated at 56ºC for 30 min and then diluted 1:10 with 1× phosphate-buffered saline (PBS, pH 7.4). The treated ferret antiserum was then 1:2 serially diluted in 96-well v-bottom plates with 1× PBS, reacted with 4 HA units of virus, and then incubated for 30 min at 37ºC, after which 0.5% turkey RBCs were added to each well and incubated for 30 min at 37ºC. Each experiment was repeated two times and each HI value reported is an average number from three independent experiments; the highest dilution in which virus binding to the RBCs was blocked was expressed as the reciprocal HI titer.

polyPLA.

IgG was purified from polyclonal serum and monoclonal antibody and labeled separately with 5′ and 3′ TaqMan Prox-Oligos (Thermo Fisher Scientific, Waltham, MA, USA) for use in a proximity ligation assay, as described elsewhere (220). In brief, 5′- and 3′- labeled IgG was diluted (1:10) in assay probe dilution buffer, and 2 µL was added to 2 µL of viruses or 1× PBS (non-protein control [NPC]) and incubated at 37ºC for 1 h. The

96 µL of ligation mixture (0.1 µL of diluted [1:500] ligase, 5 µL of 20X ligation reaction buffer, and 90.9 µL of dH_2O) was added to each incubation product, incubated at 37ºC for 10 min, and then put on ice. Diluted protease was then added to the ligation products and incubated at 37ºC for 10 min and at 95ºC for 5 min and then put on ice. Last, 4.5 µL of protease products was added to 5 µL of TaqMan Protein Assays Fast Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA) and 0.5 µL 20X Universal PCR Assay (Thermo Fisher Scientific, Waltham, MA, USA), and quantitative RT-PCR was performed as follows: 95ºC for 20 sec, 40 cycles at 95ºC for 1 sec, and 60ºC for 20 sec. The threshold was set at 0.2, and change in the cycle threshold (ΔC_T) were calculated by [average C_T (NPC) – average C_T (sample)]; quantitative RT-PCR was performed on each sample in triplicate.

Antigenic cartography.

The antigenic maps of H3N2 swine IAVs were constructed using AntigenMap (http://sysbio.cvm.msstate.edu/AntigenMap) for the data derived from the HI assay or the polyPLA (229, 230). The data entry with an HI titer of <1:10 or a ΔC_T of <3.00 were determined as a low reactor for the data from HI or the polyPLA, respectively.

Data analyses.

To make the antigenic properties across the testing antigens (viruses) comparable, we calculated the polyPLA units between virus and antibody as previously described (220): polyPLA = $a \times (\text{poly}\Delta C_T - \text{mono}\Delta C_T) + b$, in which $a = 1.00$ and $b = 10.00$, to eliminate negative numbers. A monoΔC_T cutoff of <3.00 has been traditionally used to distinguish if virus loads are too low for analyses.

Linear regression analyses were performed using the HI titers of the 7 swine IAV isolates (2 to antigenic clade H3α and 5 to H3ß) verses their homologous antisera and polyPLA units of these 7 swine IAV isolates verses 3 polyclonal antibodies (1 to H3α and 2 to H3ß). The 81 nasal swab samples were assessed for monoΔC_T and ΔpolyPLA cutoffs by the frequency procedure using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) to determine sensitivity and specificity for detection of IAVs and antigenic variants with confidence intervals at 95%, receiver operator characteristic area under the curve, and linear regression. The mathematical product of sensitivity × specificity, given the term efficiency, was calculated and graphed for each polyPLA value to provide the probability of correct classification for unknown sample status (228). Kappa analyses and all descriptive graphs were created using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Comparison of HI assay and polyPLA in antigenic characterization of subtype H3 swine IAVs.

To assess the effectiveness of the polyPLA, we compared the antigenic data derived from the HI assay and the polyPLA. The seven H3N2 swine IAVs used for testing in our study had cross-reaction titers ranging from <1:10 to 1:1,600 (Table 14). HI-based antigenic cartography showed that the seven isolates were separated into two antigenic clusters: two isolates from 2009 were in cluster H3α, and the five other isolates were in cluster H3ß (Figure 17a). The results from polyPLA indicated that the titers for these seven testing isolates ranged from 1.71 to 16.47 polyPLA units. In support of the HI cartography–derived data, polyPLA-based cartography also showed that these seven

isolates were grouped in two antigenic clusters (Figure 17b). The average distances between clusters were 5.14 units (± 0.15 standard deviation) and 6.27 units (± 1.22 standard deviation) in HI and polyPLA cartography, respectively. Correlation association analyses through linear regression showed that the titers between these two types of data had a coefficient of $R^2 = 0.82$ ($p<0.0001$) (Figure 17c) and that the fold increments in HI titers and polyPLA values had a coefficient of $R^2= 0.85$ ($p<0.0001$) (Figure 17d). Similar to HI assay results, polyPLA results suggested that subtype H3N2 swine IAVs did not react with the negative-control H1N1 virus and polyclonal antibodies.

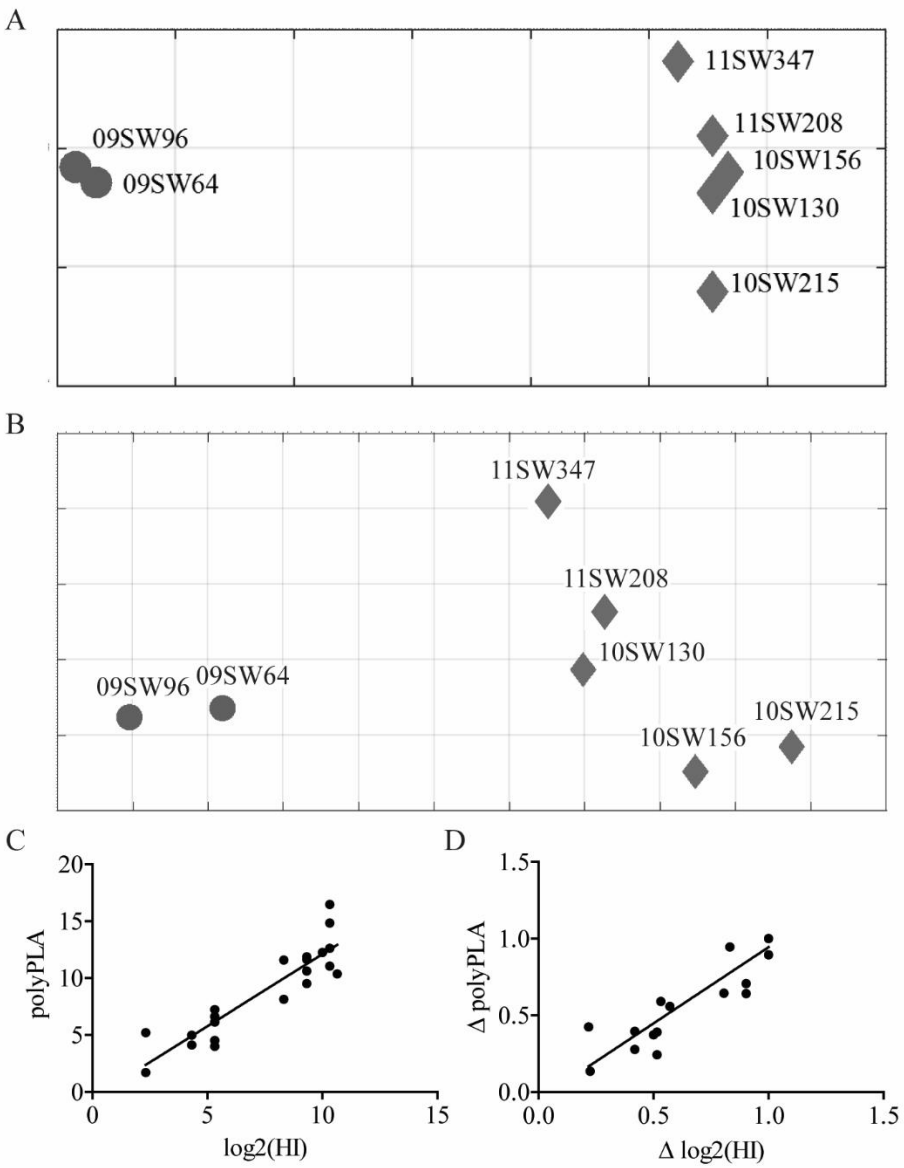

Figure 17 Comparison of antigenic characterization of H3N2 swine IAVs using hemagglutination inhibition (HI) assays and polyclonal sera–based proximity ligation assay (polyPLA).

(A) Antigenic map derived from HI data. (B) Antigenic map derived from polyPLA data. (C) Correlation of HI titers and polyPLA values; polyPLA values can be predicted from HI titers by the following formula: polyPLA values = 1.27 × $\log_2$(HI titers) – 0.56, R^2 = 0.82. (D) Correlation of the fold increments in HI titers and those in polyPLA values; fold increment in polyPLA values can be predicted from fold increment in HI titers by the following formula: ΔpolyPLA values = 0.10 × Δ$\log_2$(HI titers) – 0.05, R^2 = 0.85. A total of 7 representative H3N2 swine influenza A viruses (IAVs) were selected to represent antigenic clusters H3 swine IAV-α and H3 swine IAV-ß (abbreviated as H3α and H3ß) (Table 14). The homologous ferret antisera for these viruses were used to perform the HI assay and polyPLA. The HI assays were performed using 0.5% red blood cells. Antigenic maps were constructed using AntigenMap (http://sysbio.cvm.msstate.edu/AntigenMap) (229, 230). Viral isolates are 09SW64, A/swine/Ohio/09SW64/2009 (H3α); 09SW96, A/swine/Ohio/09SW96/2009 (H3α); 10SW130, A/swine/Ohio/10SW130/2010 (H3ß); 10SW156, A/swine/Ohio/10SW156/2010 (H3ß); 10SW215, A/swine/Ohio/10SW215/2010 (H3ß); 11SW208, A/swine/Ohio/11SW208/2011 (H3ß); 11SW347, A/swine/Ohio/11SW347/2011 (H3ß).

Detection of H3N2 swine IAVs in clinical samples from feral swine.

To determine whether polyPLA is sensitive enough to identify H3N2 swine IAVs in clinical samples, we used 42 nasal swab and nasal wash samples from 8 feral swine infected with A/swine/Texas/A01104013/2013(H3N2) (belonging to antigenic clade H3ß) and 4 sentinel feral swine with virus titers up to 5.00×10^5 $TCID_{50}$/mL. Of the 42 samples, 34 were IAV-positive ($\Delta C_T \geq 3.00$) using NP monoclonal antibodies. Further analyses using polyclonal H3ß antibodies showed that polyPLA can detect H3N2 swine IAV virus titers of <1,000 $TCID_{50}$/mL (Figure 18a); this finding is similar to that for human IAVs which can detect virus titers of <1,000 $TCID_{50}$/mL, as previously published (220). Furthermore, in the animal experiments, polyPLA could detect viral shedding from 1 to 10 days after virus challenge, and titers ranged from 4.35 to 14.83 polyPLA units (Figure 18b; Table 15).

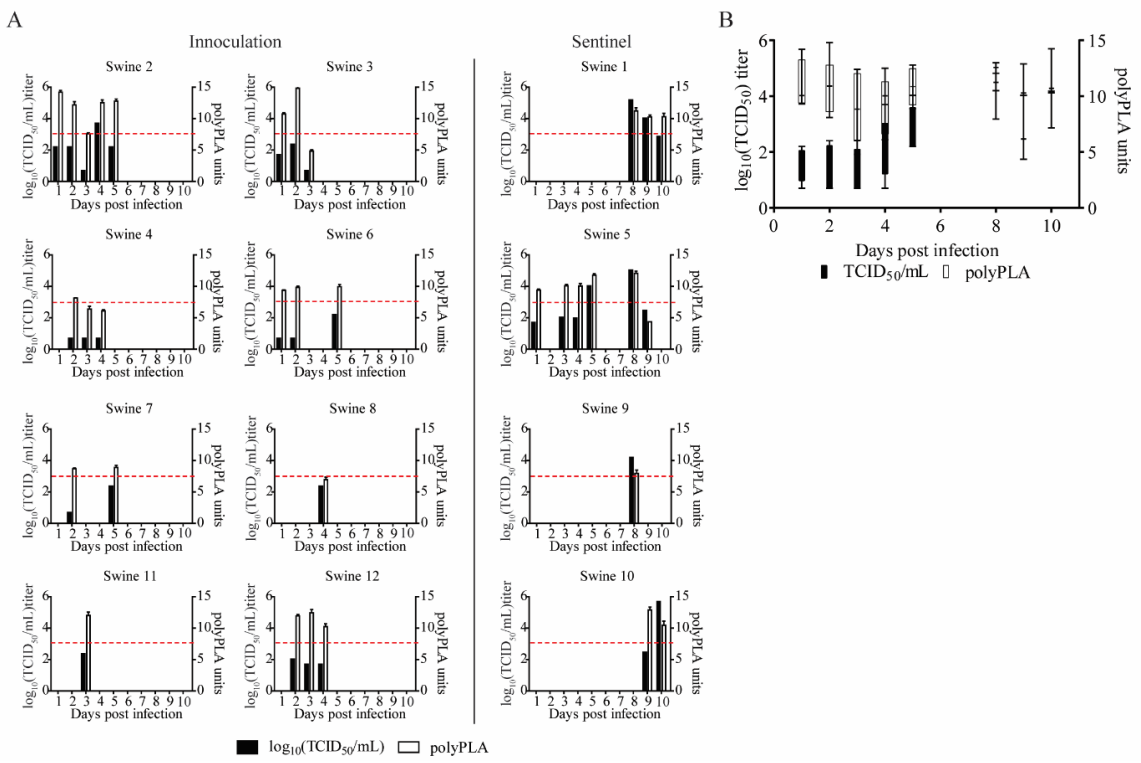

Figure 18 Comparison of sensitivity of cell culture based viral titration and polyclonal sera–based proximity ligation assay (polyPLA) in detecting influenza A viruses (IAVs) in nasal wash and nasal swab samples collected from feral swine infected with A/swine/Texas/A01104013/2012(H3N2).

(A) Variations of $TCID_{50}$ and polyPLA titers in 12 swine. Horizontal dashed line indicates 1000 $TCID_{50}$/mL. (B) Average number of days after virus challenge that virus could be detected by $TCID_{50}$ and polyPLA. The whiskers of the box-and-whisker plots denote the smallest value to the larger value, while the box extends to the 25th and 75th percentiles, with the median in the middle. The infecting virus belongs to swine influenza A virus (IAV) antigenic cluster H3N2 swine IAV-ß. Swine 1–8 were inoculated nasally with virus; swine 9–12 were sentinel swine housed in the same room. Among a total of 120 samples collected from these 12 swine, 42 samples with detectable TCID50 were used in this study (Table 15) (183).

Table 15 Virus titration in nasal swab and nasal wash from feral swine challenged with A/swine/Texas/A01104013/2012 for 10 dpi[a]

Group, swine no.[b]	1 dpi			2 dpi			3 dpi			4 dpi			5 dpi		
	$TCID_{50}$[c]	polyPLA[d]	SD	$TCID_{50}$	polyPLA	SD	$TCID_{50}$	polyPLA	SD	$TCID_{50}$	polyPLA	SD	$TCID_{50}$	polyPLA	SD
Inoculation															
2	2.199	14.211	0.257	2.199	12.182	0.505	0.699	7.594	0.161	3.699	12.532	0.449	2.199	12.792	0.328
3	1.699	10.725	0.188	2.366	14.83	0.352	0.699	4.826	0.217	N/A	8.940	0.420	3.032	9.546	0.207
4	N/A	N/A	N/A	0.699	8.132	0.058	0.699	6.380	0.435	0.699	6.084	0.182	3.199	11.729	0.433
6	0.699	9.338	0.078	0.699	9.828	0.185	N/A	N/A	N/A	N/A	N/A	N/A	2.199	9.944	0.354
7	N/A	N/A	N/A	0.699	8.682	0.129	N/A	N/A	N/A	N/A	N/A	N/A	2.366	8.895	0.341
8	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.366	6.965	0.399	N/A	N/A	N/A
11	N/A	N/A	N/A	N/A	N/A	N/A	2.366	12.024	0.540	N/A	N/A	N/A	N/A	N/A	N/A
12	N/A	N/A	N/A	2.032	11.960	0.202	1.699	12.434	0.523	1.699	10.244	0.451	2.032	9.218	0.592
Sentinel	1 dpi			2 dpi			3 dpi			4 dpi			5 dpi		
1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	1.699	9.394	0.172	N/A	N/A	N/A	2.032	10.078	0.236	1.980	10.032	0.406	4.032	11.77	0.211
9	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Inoculation	6 dpi			7 dpi			8 dpi			9 dpi			10 dpi		
2	2.199	6.745	0.813	N/A	15.955	1.151	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3	N/A	11.703	0.429	N/A	11.712	0.730	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	0.699	5.89	0.267	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 15 (Continued)

Sentinel	6 dpi			7 dpi			8 dpi			9 dpi			10 dpi		
1	N/A	N/A	N/A	2.930	11.407	0.610	5.199	11.232	0.508	4.032	10.283	0.281	2.866	10.307	0.534
5	4.032	10.403	0.155	3.199	7.913	0.722	5.032	12.059	0.345	2.468	4.352	0.000	N/A	N/A	N/A
9	N/A	N/A	N/A	N/A	N/A	N/A	4.199	7.942	2.158	N/A	N/A	N/A	N/A	N/A	N/A
10	N/A	N/A	N/A	N/A	N/A	N/A	4.262	8.965	1.193	2.468	12.893	0.470	5.699	10.457	0.658

[a] dpi, day after infection; $TCID_{50}$, 50% tissue culture infectious dose; polyPLA, polyclonal sera–based proximity ligation assay; SD, standard deviation; N/A, not available.

[b] Feral swine randomly assigned to treatment group or sentinel group.

[c] Virus titers [$\log_{10}(TCID_{50}/mL)$] were previously measured in Madin–Darby canine kidney cells (183).

[d] polyPLA values of feral swine clinical samples; assays were performed in triplicate using NP monoclonal antibodies and 10SW215, A/swine/Ohio/10SW215/2010(H3N2) polyclonal antibodies.

Application of polyPLA in detecting H3N2 swine IAV antigenic variants from clinical samples collected from swine at agricultural fairs.

To measure specificity of polyPLA in the clinical setting, we used the assay on 81 samples taken from swine at agricultural fairs. The frequency distribution of ΔC_T values for NP monoclonal antibodies for the 61 IAV positive– and 20 IAV negative–clinical samples from domestic swine showed that the greatest efficiency (77.0%) was observed at a ΔC_T cutoff of 7.0 (Figure 19a). Thus, the optimum combination for detecting IAVs in clinical samples is an assay sensitivity of 77.0% (95% CI = 64.5%, 86.8%) and specificity of 100.0% (95% CI = 83.2%, 100.0%). Accuracy of the polyPLA was measured by the receiver operating characteristic area under the curve, which was 0.90, an excellent test for separating IAV-positive from IAV-negative clinical samples (Figure 20). There was 82.7% overall agreement between qRT-PCR and polyPLA, with a kappa of 62.4% (95% CI = 45.8%, 79.0%), which suggests a good strength of agreement. The typical ΔC_T cutoff of 3.0 showed that the polyPLA had high sensitivity (96.7%; 95% CI = 88.7%, 99.6%) but low specificity (15.0%; 95% CI = 3.2%, 37.9%), and 59 true-positive and 17 false-positive samples were detected. At the higher ΔC_T cutoff of 7.0, false positives were eliminated, and 47 true positive samples were detected.

To distinguish between antigenic group H3α and antigenic group H3ß viruses, we calculated the frequency distribution of ΔpolyPLA values for H3α and H3ß polyclonal antibodies for the 47 IAV-positive clinical samples from domestic swine. At the ΔpolyPLA threshold of 3.5, the greatest efficiency was observed at 85.0%, with a sensitivity of 85.0% (95% CI = 77.0%, 91.0%) and specificity of 100.0% (95% CI = 87.7%, 100.0%) (Figure 19b; Table 16). Correlation association analyses through linear

regression showed the fold increment titers from homologous virus isolates and fold increment in polyPLA values had a coefficient of $R^2 = 0.88$ ($p<0.0001$; Figure 21). An 8-fold increment in HI titer was correlated with a 3.26-fold increment in polyPLA units. polyPLA was able to distinguish between the two swine IAV H3 antigenic groups with complete agreement: 10 samples were H3α-positive (sensitivity 95% CI = 69.2%; 100%), and 33 were H3ß-positive (sensitivity 95% CI = 89.4%; 100%); 4 were negative to both polyclonal antibodies because they were previously identified as H1 qRT-PCR-positive (Tables 17 and 18).

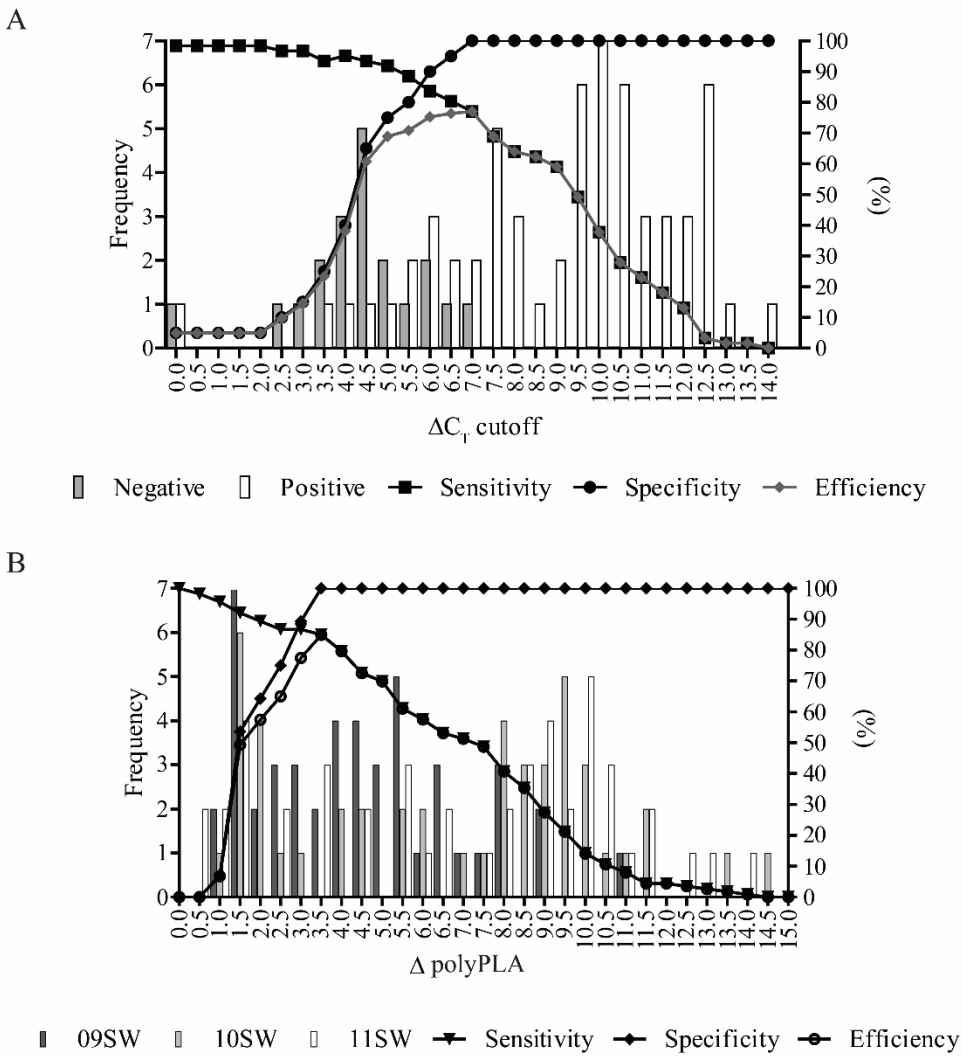

Figure 19 Optimization of the polyclonal sera–based proximity ligation assay (polyPLA) in detecting antigenic variants in clinical samples from swine infected with IAV.

(A) Distribution of IAV-positive samples (white bars, N = 61) vs. IAV-negative samples (grey bars, N = 20) obtained using NP monoclonal antibody and various ΔC_T values. (B) Distribution of H3 swine IAV-α vs. H3 swine IAV-ß samples at various ΔpolyPLA values. 09SW, A/swine/Ohio/09SW96/2009(H3N2); 10SW, A/swine/Ohio/10SW215/2010(H3N2); 11SW, A/swine/Ohio/11SW347/2011(H3N2); shown in dark grey, light grey, and white bars, respectively. Data analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with 95% CIs.

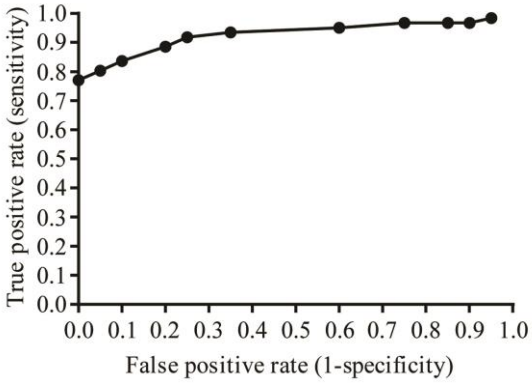

Figure 20 Receiver operating characteristic was performed to assess the accuracy of polyPLA results for determining the presence of IAV in clinical samples from swine.

The area under the curve was 0.8973, which is a very good test for the separation of IAV-positive from IAV-negative clinical samples with NP monoclonal antibody.

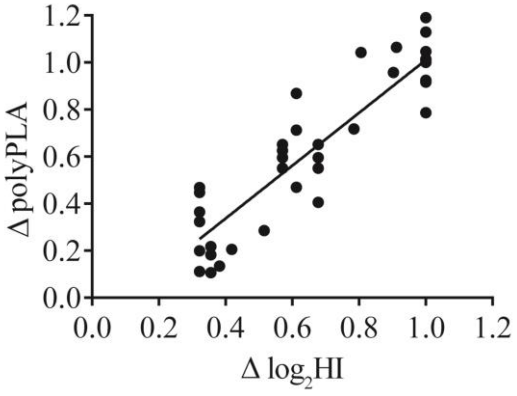

Figure 21 Correlation of fold change of log_2(HI) titer and fold change of polyPLA values.

Fold change in polyPLA values can be predicted from the fold change in HI titers by the following formula: fold change polyPLA values = 1.1228 × fold change log_2HI – 0.11124, R^2=0.8836 ($p<0.0001$). An 8-fold change of HI titer correlates with a 1.604 (± 0.125)–fold change of polyPLA value: these fold changes were used to distinguish 10 H3α-positive and 33 H3ß-positive clinical samples. HI, hemagglutination inhibition.

Table 16 Antigenic differences in subtype H3 influenza A virus–positive samples from swine, using the HI assay and the polyPLA[a]

Virus[b]	Subtype[c]	GenBank Accession No	Antibody[d]											
			09SW96			10SW215			11SW208			CA/04		
			HI[e]	polyPLA[f]	SD	HI	polyPLA	SD	HI	polyPLA	SD	HI	polyPLA	SD
A/swine/Indiana/13TOSU482/2013	H3	KF428880	N/A	4.64	0.29	N/A	9.87	0.84	N/A	9.37	0.16	N/A	3.06	0.94
A/swine/Indiana/13TOSU490/2013	H3	KF428888	N/A	3.30	0.37	N/A	7.53	0.12	N/A	8.29	0.60	N/A	2.09	0.45
A/swine/Indiana/13TOSU1122/2013	H3	KF704130	N/A	0.75	0.16	N/A	4.13	0.83	N/A	4.06	0.67	N/A	N/A	N/A
A/swine/Indiana/13TOSU1128/2013	H3	KF704138	N/A	1.52	0.63	N/A	5.01	0.55	N/A	5.52	0.44	N/A	0.23	0.41
A/swine/Ohio/09SW63/2009	H3	CY130709	1280	6.02	0.37	80	4.29	1.51	80	5.22	0.93	N/A	0.31	0.13
A/swine/Ohio/09SW64/2009	H3	CY130717	640	5.80	0.65	40	3.62	1.20	<10	0.61	0.10	N/A	N/A	N/A
A/swine/Ohio/09SW74/2009	H3	N/A	N/A	7.70	0.21	N/A	3.58	0.30	N/A	4.15	0.10	N/A	N/A	N/A
A/swine/Ohio/09SW77/2009	H3	N/A	N/A	8.51	0.24	N/A	1.87	0.02	N/A	3.41	0.19	N/A	N/A	N/A
A/swine/Ohio/09SW84/2009	H3	CY130829	640	4.61	0.24	80	0.00	0.72	40	0.41	0.20	N/A	0.49	0.18
A/swine/Ohio/09SW85/2009	H3	CY130837	640	5.05	0.25	80	0.42	0.34	40	0.72	0.13	N/A	N/A	N/A
A/swine/Ohio/09SW89/2009	H3	CY130861	640	4.19	0.11	80	1.70	0.37	160	0.35	0.24	N/A	0.28	3.37
A/swine/Ohio/09SW90/2009	H3	CY130869	640	5.46	0.57	80	0.18	0.23	40	0.65	0.25	N/A	1.33	0.15
A/swine/Ohio/09SW98/2009	H3	CY130925	1280	6.39	0.05	80	1.83	0.09	40	2.01	0.05	N/A	2.96	0.39
A/swine/Ohio/09SW99/2009	H3	CY130933	N/A	6.38	0.49	N/A	0.89	1.63	N/A	2.46	0.61	N/A	1.35	0.39
A/swine/Ohio/10SW122/2010	H3	CY130949	10	1.17	0.30	1280	5.89	0.12	1280	5.40	0.07	N/A	N/A	N/A
A/swine/Ohio/10SW125/2010	H3	CY130957	20	1.16	0.30	1280	5.63	0.41	1280	5.21	0.06	N/A	0.69	0.36
A/swine/Ohio/10SW137/2010	H3	CY131037	10	3.53	0.24	1280	5.06	0.09	1280	6.02	0.19	N/A	0.45	0.12
A/swine/Ohio/10SW139/2010	H3	CY131045	10	0.87	0.05	1280	7.85	0.37	1280	6.18	0.14	N/A	1.74	0.06
A/swine/Ohio/10SW156/2010	H3	CY131053	<10	1.04	0.06	640	4.82	0.30	640	7.67	0.02	N/A	2.40	0.15
A/swine/Ohio/10SW202/2010	H3	CY131061	10	1.50	1.32	640	8.26	0.24	640	8.64	0.27	N/A	0.42	0.09
A/swine/Ohio/10SW204/2010	H3	CY131077	10	5.05	0.23	1280	11.31	0.62	1280	11.47	0.14	N/A	2.62	0.45
A/swine/Ohio/10SW207/2010	H3	CY131101	20	1.33	0.63	2560	9.91	1.32	1280	10.54	0.21	N/A	3.30	0.18
A/swine/Ohio/10SW210/2010	H3	CY131125	10	2.96	0.06	1280	9.16	0.25	1280	10.35	0.33	N/A	1.95	0.01
A/swine/Ohio/10SW214/2010	H3	CY131157	10	3.58	0.37	1280	7.64	1.44	640	9.94	0.27	N/A	0.36	0.52
A/swine/Ohio/10SW218/2010	H3	CY131173	10	3.00	0.10	1280	9.32	1.10	640	8.93	1.11	N/A	1.40	0.74
A/swine/Ohio/10SW220/2010	H3	CY131189	10	2.91	0.12	1280	8.01	0.67	1280	8.10	1.16	N/A	1.73	0.95
A/swine/Ohio/11SW87/2011	H3	CY131269	N/A	1.73	1.00	N/A	1.73	0.10	N/A	6.02	0.16	N/A	0.10	0.43
A/swine/Ohio/11SW96/2011	H3	CY131301	N/A	2.27	0.42	N/A	6.83	0.41	N/A	8.75	0.13	N/A	N/A	N/A
A/swine/Ohio/11SW98/2011	H3	CY131317	N/A	4.28	0.02	N/A	9.23	0.31	N/A	9.79	0.21	N/A	0.42	0.70
A/swine/Ohio/11SW103/2011	H3	CY131197	N/A	7.15	0.31	N/A	10.45	0.22	N/A	12.21	0.13	N/A	2.75	0.88
A/swine/Ohio/11SW109/2011	H3	CY131213	N/A	2.04	0.25	N/A	8.35	0.48	N/A	7.71	1.01	N/A	2.75	0.17
A/swine/Ohio/11SW172/2011	H3	CY131529	N/A	7.75	1.29	N/A	10.53	0.26	N/A	11.23	0.57	N/A	3.69	0.20
A/swine/Ohio/11SW180/2011	H3	CY131579	N/A	6.91	0.54	N/A	9.35	0.14	N/A	10.35	1.46	N/A	4.01	0.43
A/swine/Ohio/11SW191/2011	H3	CY131639	N/A	7.96	0.68	N/A	11.11	0.05	N/A	10.04	0.38	N/A	4.30	0.17
A/swine/Ohio/11SW187/2011	H3	CY131621	N/A	3.62	0.20	N/A	7.40	0.39	N/A	8.33	0.25	N/A	1.57	0.92

Table 16 (Continued)

A/swine/Ohio/11SW211/2011	H3	CY131767	N/A	3.86	0.89	N/A	8.70	0.17	N/A	8.86	0.26	N/A	0.74	0.32
A/swine/Ohio/11SW347/2011	H3	CY131957	<10	5.42	0.09	320	9.96	0.43	1280	9.55	0.57	N/A	4.06	0.84
A/swine/Ohio/12TOSU347/2012	H3	N/A	N/A	10.57	0.39	N/A	14.14	0.30	N/A	13.90	0.08	N/A	4.70	0.99
A/swine/Ohio/12TOSU293/2012	H3	JX534969	N/A	8.95	0.14	N/A	13.45	0.16	N/A	12.63	0.02	N/A	4.92	0.16
A/swine/Ohio/12TOSU268/2012	H3	JX534961	N/A	4.22	0.56	N/A	8.87	0.54	N/A	9.82	0.31	N/A	0.76	0.50
A/swine/Ohio/12TOSU307/2012	H3	JX565505	N/A	5.08	0.48	N/A	9.48	0.47	N/A	7.04	2.91	N/A	3.83	0.28
A/swine/Ohio/12TOSU467/2012	H3	JX565473	N/A	4.24	0.66	N/A	8.88	0.29	N/A	9.97	0.29	N/A	N/A	N/A
A/swine/Ohio/12TOSU527/2012	H3	KC020452	N/A	3.16	0.07	N/A	7.81	0.13	N/A	9.28	0.31	N/A	N/A	N/A
A/swine/Indiana/13TOSU486/2013	H1	KF428868	N/A	1.14	0.59	N/A	1.90	0.32	N/A	3.35	0.67	N/A	7.02	0.97
A/swine/Ohio/09SW1484/2009	H1	N/A	N/A	1.32	0.52	N/A	1.63	0.01	N/A	1.27	0.38	N/A	4.55	0.51
A/swine/Ohio/09SW1487/2009	H1	N/A	N/A	2.83	0.70	N/A	2.49	0.71	N/A	2.02	0.03	N/A	5.10	0.58
A/swine/Ohio/11SW174/2011	H1	CY131552	N/A	2.14	0.48	N/A	2.65	0.10	N/A	1.44	0.02	N/A	6.21	0.25

[a] HI, hemagglutination inhibition; polyPLA, polyclonal sera–based proximity ligation assay; SD, standard deviation.

[b] Clinical samples from swine at Ohio, USA, agricultural fairs and virus isolates from Madin–Darby canine kidney cells.

[c] Subtypes positive for IAV using matrix gene-based quantitative RT-PCR.

[d] 09SW96, A/swine/Ohio/09SW96/2009 (H3N2); 10SW215, A/swine/Ohio/215/2010 (H3N2); 11SW208, A/swine/Ohio/11SW208 (H3N2); CA/04, A/California/04/2009 (H1N1pdm09).

[e] Hemagglutination inhibition virus titers in swine H3N2 viral isolates. Each assay was performed in triplicate, repeated twice, and published previously (240).

[f] polyPLA values of swine clinical samples performed in triplicate.

Table 17 Antigenic differences in subtype H1 isolates and clinical samples from swine, using the HI assay and the polyPLA[a] with polyclonal antibody against A/California/04/2009(H1N1).

Clinical Sample ID [b]	Isolate	GenBank ID	Genetic cluster [c]	HI [e]	ΔCt[f]	ΔCt		polyPLA [g]	
				CA/04[d]	MAB	CA/04	SD	CA/04	SD
N/A	A/California/04/2009(H1N1)	GQ117044	A(H1N1)pdm09	**160**	**3.27**	**7.10**	**0.17**	**13.83**	**0.17**
N/A	A/swine/Iowa/8/2013(H1N1)	N/A	A(H1N1)pdm09	320	3.15	9.02	1.09	15.87	1.09
N/A	A/swine/Minnesota/A01394082/2013(H1N1)	KF824480	H1α	<10	3.32	2.67	0.10	ND	ND
N/A	A/swine/Nebraska/A01240348/2011(H1N1)	JX657786	H1ß	640	7.83	9.84	0.85	12.02	0.85
N/A	A/swine/Indiana/13TOSU1154/2013(H1N1)	KJ640612	H1γ	320	3.47	4.48	0.17	11.01	0.17
N/A	A/swine/Indiana/13TOSU0832/2013(H1N1)	KJ640531	H1γ1	40	3.06	3.28	0.21	10.22	0.21
N/A	A/swine/Illinois/A01076767/2010(H1N1)	CY114575	H1γ2	<10	6.24	3.43	0.28	7.19	0.28
N/A	A/swine/Iowa/15/2013(H1N1)	N/A	H1δ1	<10	4.20	3.69	0.25	9.49	0.25
N/A	A/swine/Iowa/18/2013(H1N1)	N/A	H1δ2	<10	3.07	2.54	0.24	ND	ND
TOSU56	A/swine/Ohio/11SW174/2011(H1N2)	CY131545	H1δ1	ND	9.38	5.58	0.25	6.2	0.25
TOSU58	A/swine/Kentucky/12TOSU1053/2012(H1N2)	KC866470	H1δ1	ND	6.22	3.28	0.48	7.06	0.48
TOSU55	A/swine/Ohio/11SW129/2011(H1N2)	CY131373	H1δ1	ND	5.84	5.47	1.12	9.63	1.12
TOSU59	A/swine/Kentucky/12TOSU1054/2012(H1N2)	KC866542	H1δ1	ND	5.7	3.09	0.83	7.39	0.83
TOSU54	A/swine/Ohio/11SW128/2011(H1N2)	CY131365	H1δ1	ND	5.48	5.37	1.07	9.89	1.07
TOSU57	A/swine/Ohio/11SW192/2011(H1N2)	CY131647	H1δ1	ND	<3.0	ND	ND	ND	ND
TOSU51	A/swine/Indiana/13TOSU486/2013(H1N1)	KF428864	H1γ	ND	7.02	4.04	0.97	7.02	0.97
TOSU61	A/swine/Ohio/12TOSU45/2012(H1N1)	KC866566	H1γ	ND	5.08	2.22	1.68	ND	ND
TOSU52	N/A	N/A	N/A	ND	9.34	3.9	0.51	4.56	0.51
TOSU53	N/A	N/A	N/A	ND	10.04	5.14	0.58	5.1	0.58
TOSU60	N/A	N/A	N/A	ND	4.55	0.53	0.12	5.98	0.12

[a] HI, hemagglutination inhibition; polyPLA, polyclonal sera–based proximity ligation assay; SD, standard deviation. [b] Clinical samples from swine at Ohio, USA, agricultural fairs; [c]Genetic cluster defined by the Influenza Research Database Swine H1 Classification Tool. [d] CA/04, A/California/04/2009 (H1N1).
[e] Hemagglutination inhibition virus titers in H1 viral isolates. Each assay was performed in three independent experiments, each of which with two replicates. [f] The numbers in bold denote homologous titers, and the numbers underlined denotes those samples as IAV positive using polyPLA with a ΔC_T cutoff of 7.0.
[g] polyPLA values of swine clinical samples and viral isolates performed in triplicate.

Table 18 Antigenic differences in subtype H3 influenza A virus–positive samples from swine.

Clinical Sample[a]	Subtype[b]	Antigenic Group	
		H3α[c]	H3ß[d]
TOSU 1	H3	-	+
TOSU 2	H3	-	+
TOSU 3	H3	-	+
TOSU 4	H3	-	+
TOSU 5	H3	+	-
TOSU 6	H3	+	-
TOSU 7	H3	+	-
TOSU 8	H3	+	-
TOSU 9	H3	+	-
TOSU 10	H3	+	-
TOSU 11	H3	+	-
TOSU 12	H3	+	-
TOSU 13	H3	+	-
TOSU 14	H3	+	-
TOSU 15	H3	-	+
TOSU 16	H3	-	+
TOSU 17	H3	-	+
TOSU 18	H3	-	+
TOSU 19	H3	-	+
TOSU 20	H3	-	+
TOSU 21	H3	-	+
TOSU 22	H3	-	+
TOSU 23	H3	-	+
TOSU 24	H3	-	+
TOSU 25	H3	-	+
TOSU 26	H3	-	+
TOSU 27	H3	-	+
TOSU 28	H3	-	+
TOSU 29	H3	-	+
TOSU 30	H3	-	+
TOSU 31	H3	-	+
TOSU 32	H3	-	+
TOSU 33	H3	-	+

Table 18 (Continued)

TOSU 34	H3	-	+
TOSU 35	H3	-	+
TOSU 36	H3	-	+
TOSU 37	H3	-	+
TOSU 38	H3		
TOSU 39	H3		
TOSU 40	H3	-	+
TOSU 41	H3	-	+
TOSU 42	H3	-	+
TOSU 43	H3		
TOSU 44	H3	-	+
TOSU 45	H3		
TOSU 46	H3		
TOSU 47	H3	-	+
TOSU 48	H3	-	+
TOSU 49	H3		
TOSU 50	H3		
TOSU 51	H1	-	-
TOSU 52	H1	-	-
TOSU 53	H1	-	-
TOSU 54	H1		
TOSU 55	H1		
TOSU 56	H1	-	-
TOSU 57	H1		
TOSU 58	H1		
TOSU 59	H1		
TOSU 60	H1		
TOSU 61	H1		
TOSU 62	Negative		
TOSU 63	Negative		
TOSU 64	Negative		
TOSU 65	Negative		
TOSU 66	Negative		
TOSU 67	Negative		
TOSU 68	Negative		
TOSU 69	Negative		
TOSU 70	Negative		

Table 18 (Continued)

TOSU 71	Negative
TOSU 72	Negative
TOSU 73	Negative
TOSU 74	Negative
TOSU 75	Negative
TOSU 76	Negative
TOSU 77	Negative
TOSU 78	Negative
TOSU 79	Negative
TOSU 80	Negative
TOSU 81	Negative

[a] Swine clinical samples from Ohio, USA, agricultural fairs; samples were given arbitrary names of The Ohio State University (TOSU) 1 to 81.
[b] Of the 81 samples, 61 were positive for IAV using matrix gene-based quantitative RT-PCR; this includes 50 that were subtyped as H3 and 11 that were subtyped as H1(2).
[c]Results of polyPLA (polyclonal sera–based proximity ligation assay) using 09SW96 polyclonal antibodies to determine samples positive (+) or negative (-) for antigenic group H3-α swine influenza virus (SIV).
[d] polyPLA results distinguishing SIV H3-ß positive (+) and negative (-) antigenic group with 10SW215 and 11SW208 polyclonal antibodies.

Effectiveness of polyPLA in detecting H1 swine IAV antigenic variants.

To evaluate whether polyPLA was effective in identifying antigenic variants for subtype H1 IAVs, cross-activities were measured using both the polyPLA and HI assays between the CA/04 polyclonal serum and a panel of H1N1 isolates, which belong to 8 antigenically distinct clades, including clade H1α , H1ß, H1γ, H1γ1, H1γ2, H1δ1, H1δ2, and A(H1N1)pdm09. Results from polyPLA showed that CA/04 polyclonal serum cross-reacted with the homologous virus CA/04 and A/swine/Iowa/8/2013(H1N1) (both to clade A(H1N1)pdm09) with two highest polyPLA units, 13.83 and 15.87, respectively; this serum cross reacted to A/swine/Nebraska/A01240348/2011(H1N1) (H1ß) with 12.02 polyPLA unit, to A/swine/Indiana/13TOSU1154/2013(H1N1) (H1γ) with 11.01 polyPLA unit, to A/swine/Indiana/13TOSU0832/2013(H1N1) (H1γ1) with 10.22 polyPLA unit. The polyPLA values for the other four viruses (H1α, H1γ2, H1δ1, and H1δ2) were less

than 10.00 (Table 17). Such results were consistent with the corresponding HI titers, validating that this method is effective in antigenic characterization of H1 viruses. Furthermore, we performed polyPLA assays using CA/04 serum against the 47 IAV-positive clinical samples. Results showed that CA/04 serum did not cross-react with 43 H3 IAV positive samples but did to 4 H1 IAV positive samples to different extents. Among these 4 H1 viruses, 2 were sequenced, 1 genetically belong to H1γ and the other one to H1δ1 (Table 17).

Discussion

In this study, a polyPLA assay was used to detect antigenic variants of contemporary subtype H3 IAVs in swine in the United States. This assay was validated to differentiate viruses in antigenic cluster H3α from those in H3ß directly in clinical samples, such as swine nasal swab or nasal wash samples. With the optimized **Δ**polyPLA unit of 3.500, this assay can detect antigenic variants with a specificity of 100% and a sensitivity of 84.9% in samples collected during influenza surveillance. Thus, the polyPLA is specific and sensitive enough to be used for vaccine strain selection for swine IAVs. Because it does not require virus isolation, this assay shortens the time needed for antigenic characterization using conventional methods (3–5 days) to only a few hours after specimen collection and, therefore, can increase effectiveness of vaccination programs on swine farm operations and at agricultural fairs. In addition, because it is developed based on a qRT-PCR platform, polyPLA can be used for high-throughput screening and for clinical diagnosis in most laboratories.

The HI assay is used routinely in influenza antigenic characterization because of its ease of access and its capacity for medium–throughput screening. The principle of HI

is based on the competition of the glycan receptors on animal RBCs and antibody against surface glycoproteins, especially hemagglutinins of IAVs. Thus, antigenic characterization results can be affected not only by changes at antibody binding sites, but also by the source of the RBCs and the variation of receptor binding sites. In addition, HI requires a large amount of virus particles, so, in general, it is necessary to recover and propagate viruses using cells or chicken eggs, which can lead to unwanted adaptive mutations, especially those at the receptor binding sites of viral hemagglutinin proteins. These mutations can skew HI data and even cause loss of binding affinity to some RBCs (122, 124). In addition, because no standard RBCs are used in HI assays, the variation in the binding affinities of IAVs to different sources of RBCs make it difficult to interpret those results across HI assays using different RBCs. Unlike the HI assay, polyPLA does not use RBCs and is not affected by variations in receptor binding sites. Instead, polyPLA utilizes oligonucleotide-labeled antibodies and quantifies the binding affinities between antibody and antigen through qRT-PCR. More strikingly, this method can be applied directly in clinical samples and can minimize biases due to virus adaptation in virus isolation. Results showed that polyPLA values are similar to those in HI assays, although the scale of fold increments are different (Figure 21). In this study, both HI and polyPLA clearly separated swine IAVs in antigenic cluster H3α from those in antigenic cluster H3ß. Furthermore, an eight-fold increment in HI titer was approximately equal to a 3.256-fold increment in polyPLA (Figure 21).

Compared with clinical samples from laboratory animals, samples derived from the field could be complicated with high background in qRT-PCR due to low quantities of virus analyte, inappropriately collected/handled/transported specimens, presence of

viral inhibitor, and/or presence of proteins from other viruses or bacterial pathogens (248). Nevertheless, for vaccine strain selection in the clinical setting, it is critical to use assays with 100% specificity and relatively high sensitivity. Relatively low assay sensitivity can be overcome by using a larger number of samples in the assays; in general, the availability of multiple samples from swine herds will not be an issue, especially during an outbreak. Based on the 81 samples we tested, polyPLA has a sensitivity of 77.0% (95% CI of 55.7%, 80.1%) and specificity of 100.0% (95% CI of 83.2%, 100.0%) when the ΔCt cutoff is set at 7.0 (Figure 19a).

The number of polyPLAs conducted in experiments can be reduced if the IAV subtype in samples is known prior to testing. Thus, the performance of polyPLA can be maximized and the cost can be reduced if the assay is coupled with subtype-specific IAV antigen assays, such as qRT-PCR. The recommended procedure for polyPLA application includes three steps: 1) determine whether the testing sample is IAV-positive by using a matrix gene–based qRT-PCR (245) rapid influenza antigen detection test (249) or an influenza test strip; 2) use qRT-PCR to determine the subtype of IAV in the sample; and 3) perform antigenic characterization by using subtype-specific polyPLA. As with the conventional methods for antigenic characterization, polyPLA can be used with a panel of reference sera to quantify antigenic diversity among the viruses; thus, polyPLA would be useful for antigenic characterization of IAVs and, potentially, other pathogens.

CHAPTER VI

MULTIPLEX POLYPLA FOR ANTIGENIC CHARACTERIZATION OF INFLUENZA VIRUSES USING CLINICAL SAMPLES

Introduction

For the past six influenza seasons in the United states, 2010-2011 until 2015-2016, the estimated deaths associated with influenza have ranged from low of 12,000 (2011-2012 season) to high of 56,000 (2012-2013 season)(250). Influenza vaccination is the basis for the control and treatment of the influenza virus(2). To create a successful influenza vaccination program for pandemic preparedness and seasonal influenza prevention and control, antigenic variant identification is crucial (98). One major limitation for implementing changes to the influenza vaccine is the time restriction on current virus strain assessment. Currently, recommendations are based on the future impact of circulating viruses, most notably, emerging antigenic variants, before their full epidemiological significance is known (97).

Clinical signs and symptoms of influenza can include: fever, muscle aches, headache, lethargy, dry cough, sore throat, nasal congestion, and possible runny nose (120). Influenza diagnosis based on clinical signs and symptoms alone is difficult because they are similar to those caused by many other pathogens. There are several diagnostic tests available for the detection of influenza virus, including: viral culture, serology, immunofluorescence, enzyme immunoassay, immunochromatorgraphy,

fluoroimmunoassay, rapid influenza antigen detection tests (RIDTs), and reverse transcription polymerase chain reaction (RT-PCR). Sensitivity and specificity of these tests depend on type of test used, time from onset of illness until sample collection, type of sample collected, sample handling, and test performance.

Detection of influenza virus alone is not useful for vaccine preparation and identification of novel, circulating antigenic variants is crucial. Antigenic variant detection is conventionally dependent upon the serological assays such as hemagglutination inhibition (HI) assay and microneutralization (MN) assays (122). The use of red blood cells (RBCs) as an indicator for binding affinity of antigen and antiserum is a limitation of the HI assays (251). Compared to HI assays, MN assays seem to be more sensitive and specific but are much more time-consuming and have predominantly been used to validate the results from HI assays. Another drawback of the HI assay is that it is notoriously noisy due to variation in types of RBCs used in the assays (122) and subjective interpretations due to difficulty in automation and standardization of the operation.

The conventional serological assays require a large quantity of viruses, and thus viral isolation from clinical sample is a must. The pathogen isolation process is not only time-consuming but also can change the antigenic properties of the original infecting virus due to culture adaptation. This will result in data that does not accurately represent those antigenic properties in the circulating viruses in the hosts. Additionally, heterogeneous populations which contain minor antigenic variants can be skewed by cell culture and will not be able to be characterized by conventional serological assays (132).

Therefore, there is a critical need for a viral isolation-free and RBC independent assay to assess antigenic changes in circulating viruses.

As shown in earlier chapters, the novel antigenic variant detection method of polyPLA was developed to address this need (220). Proximity ligation assay uses quantitative real time PCR (qRT-PCR) to detect antigen-antibody interactions (148). The polyPLA assay quantifies antibody-antigen interactions for influenza viral proteins, including both HA and NA, against their corresponding antibodies in the polyclonal antisera. This novel method avoids the use of viral isolation with the direct use of clinical samples. Therefore no adaptation substitutions are created which can affect the architecture of the HA receptor-binding site, thus avoiding altering interaction of the HA protein with terminal sialic acid moiety (133).

With the scalability of qRT-PCR, an ideal polyPLA assay shall be able to characterize viruses using a panel of reference sera, which can be either for a single HA subtype, multiple HA subtypes or both. A multiplex polyPLA will be able to use a small volume of samples to achieve the testing results for multiple sera. In addition, mixed infections of influenza viruses are not uncommon, presenting a great challenge in antigenic characterization because viral propagation could skew the viral population. A multiplex polyPLA assay can potentially detect antigenic variants in clinical samples with mixed infections, providing antigenic profiles for multiple variants in a single assay. The objective of this chapter is to develop and validate a multiplex assay to detect H1 and H3 antigenic variants in human clinical samples at the same time.

Materials and Methods

Viruses and Sera.

Isolates and their homologous ferret serum for human influenza a virus (IAV) A/California/04/2007 (H1N1; CA/04) and A/Switzerland/9715293/2013 (H3N2; SW/9715293) were chosen to represent IAV vaccine virus strains. The anti-NP monoclonal antibody was obtained from BEI Resources (Manassas, VA, USA).

Biotinylation of monoclonal and polyclonal antibodies.

IgG, purified from polyclonal serum, and monoclonal antibody were separately biotinylated with Biotin-XX Microscale Protein Labeling Kit (Invitrogen; Carlsbad, CA, USA), according to manufacturer's specifications, at a protein molar ratio (MR) of 18. To remove excess biotin, dialysis was performed for 12 hours with 1X phosphate buffered saline (1X PBS, pH 7.2) at 4°C with Slide-A-Lyzer MINI dialysis device (7 kDa molecular weight cut off, Thermo Fisher Scientific; Waltham, MA, USA).

Random oligonucleotide generation.

Using the PLA oligonucleotide design program (252), which uses RNAfold and RNAplex from the ViennaRNA package(253), oligonucleotides of 40 nucleotides (nt) were designed (Table 19). Each of the 40 nt DNA oligos (Eurofins; Louisville, KY, USA) has 20 nt sequence for primer targeting in PCR pre-amplification and qRT-PCR (CAATCGAGAGGTGAGTGCAT and TACAGCGGCGTCTATATCAG; Eurofins; Louisville, KY, USA) and 20 bp unique connector sequence. Two 40 nt oligonucleotides, Probe B and Probe D, contain 5'-thiol labeled free 3' -OH and one 40 bp oligonucleotide and two, Probe A and Probe C, contain 3' –thiol labeled free 5'-phosphate end for

ligation. Two RNA connector oligos (CC1, CC2; IDT; Coralville, IA, USA) of 20 nt were included to make ligation of the two 40 bp oligos more specific. Additionally, two TaqMan probes of the homologous DNA sequence include different reporter dyes FAM or HEX for TaqMan probe A or TaqMan probe B, respectively (Eurofins; Louisville, KY, USA).

Table 19 Multiplex polyPLA oligonucleotides, RNA connectors, primers, and TaqMan probes.

Sequence Name	Sequence 5' to 3'	
Probe A	CTCAAAGAAAGTTGGCCGGACTGATATAGACGCCGCTGTA	5' free
Probe B	CAATCGAGAGGTGAGTGCATGATCCCTGTCCAAACTTACG	3' free
Probe C	GACACGTCCCTGAATTCGATCTGATATAGACGCCGCTGTA	5' free
Probe D	CAATCGAGAGGTGAGTGCATAGCGACACAGTTCATTGAGG	3' free
CC1:	TTTCTTTGAGCGTAAGTTTG	
CC2:	GGGACGTGTCCCTCAATGAA	
Forward Primer	CAATCGAGAGGTGAGTGCAT	
Reverse Primer	TACAGCGGCGTCTATATCAG	
TaqMan probe A	CAAACTTACGCTCAAAGAAA	FAM, BHQ1
TaqMan probe B	TTCATTGAGGGACACGTCCC	HEX, BHQ1

Oligonucleotides of 40 nucleotides (nt) were designed using the PLA oligonucleotide design program (252), which uses RNAfold and RNAplex from the ViennaRNA package (253), Each of the 40 nt DNA oligo has 20 nt sequence for primer targeting in PCR amplification and qRT-PCR (underlined) and 20 bp unique connector sequence. Two 40 nt oligonucleotides, Probe B and Probe D, contain 5'-thiol labeled free 3' -OH and one 40 bp oligonucleotide and two, Probe A and Probe C, contain 3' –thiol labeled free 5'-phosphate end for ligation. Two RNA connector oligos (CC1, CC2) of 20 nt were included to make ligation of the two 40 bp oligos more specific. Taqman probes of the homologous DNA sequence include different reporter dyes, FAM or HEX for TaqMan probe A or TaqMan probe B, respectively.

Proximity probe preparation.

Thiol labeled oligos (100 µg) were DTT-reduced (50 mM DTT; Thermo Fisher Scientific; Waltham, MA, USA) and excess DTT was removed with Quick Spin Columns, Sephadex G-25, fine (Sigma-Aldrich; St. Louis, MO, USA). The eluate was directly combined with 0.5 nmol Maleimide-derived streptavidin (STV; Thermo Fisher Scientific; Waltham, MA, USA), incubated for 2 hours at 37°C, and reaction quenched with 0.5 µL of 0.5 M 2-mercaptoethanol. STV labeled oligos were then purified from free DNA with protein precipitation, followed by DNA precipitation to remove free protein. For quality control, protein concentration was quantified with use of the Quant-iT Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA) and oligo concentration was measured at A260 on NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). Biotinylated antibodies (as prepared previously) were combined with STV-oligos in equal molar ratios (100 nM each), and incubated for 1 hour at room temperature. Lastly, proximity probes were diluted to 10 nM in 1x PBS with EDTA with 1% BSA and 0.02% sodium azide and stored separately (5' and 3') at 4°C.

Multiplex polyPLA.

Equal molar concentrations of each proximity probe were combined and 2 µL proximity probe mixture was incubated with 2 µL antigen (lysed or unlysed virus) or 2 µL non-protein control (1X PBS) at 37°C for 1 hour to allow the probes to bind to analytes. Then 96 µL of ligation mixture will be added to each sample, incubated at 16°C for 60 minutes, followed by heat inactivation 65°C for 10 minutes. The ligation mixture is as follows: 1X T4 ligation buffer (New England Biolabs), 10 µM connector oligonucleotide, 80 units T4 DNA ligase. For preamplification, to increase sensitivity, in

a total volume of 25 µL, 20 µL ligation product was added to 5 µL pooled PCR mix (1X PCR buffer, 1.5 mM $MgCl_2$, 1 mM dNTP, 0.2 µM each forward and reverse preamplification primer, 2 units Platinum Taq polymerase (Thermo Fisher Scientific; Waltham, MA, USA)) and PCR was completed as follows: 95°C for 10 minutes, 2 cycles of 95°C for 15 seconds, 46°C for 10 minutes, and 60°C for 2 minutes, followed by 15 cycles of 95°C for 15 seconds, 54°C for 2 minutes, and 60°C for 2 minutes. Finally, 10 µL of each sample was added to 1X Fast Universal Master Mix (Thermo Fisher Scientific; Waltham, MA, USA), 1 µL of 18 µM qRT-PCR primers, 5 µM each of TaqMan probes, and H_2O to each a total 20 µL. The qRT-PCR cycling was 95°C for 5 minutes and 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The threshold was set at 0.2, and each change in the cycle threshold (ΔC_T) was calculated by [average C_T (NPC) – average C_T (sample)]; qRT-PCR was performed on each sample in triplicate.

Data Analysis.

polyPLA units were calculated to make antigenic properties across the testing antigens comparable, as previously described polyPLA = $a \times (\text{poly}\Delta C_T - \text{mono}\Delta C_T) + b$, in which $\text{poly}\Delta C_T$ = polyclonal antiserum ΔCt values, $\text{mono}\Delta C_T$ = monoclonal antibody against NP ΔCt values, a = 1.00, and b = 10.00, to eliminate negative numbers. A $\text{mono}\Delta C_T$ cutoff of <3.00 has been traditionally used to distinguish if virus loads are too low for analyses.

Results

Polyclonal antibody panel evaluated.

In order to determine if the two polyclonal proximity probe pairs, in multiplex, could distinguish the homologous antigen from the heterologous antigen, polyclonal antibodies for CA/04 and SW/9715293 were used as proximity probes in multiplex against CA/04 or SW/9715293 viruses alone. Each of the proximity probes had polyPLA units significantly higher for their homologous virus ($p < 0.001$ [Figure 22; Table 20]). CA/04 polyclonal antibodies against CA/04 virus had 12.8 (± 1.3) polyPLA units, whereas CA/04 polyclonal antibodies against SW/971293 virus had 2.6 (± 0.5) polyPLA units ($p < 0.001$). SW/971293 polyclonal antibodies against CA/04 virus had 0.4 (± 0.1) polyPLA units, while CA/04 polyclonal antibodies against SW/971293 virus had 15.2 (± 0.1) polyPLA units.

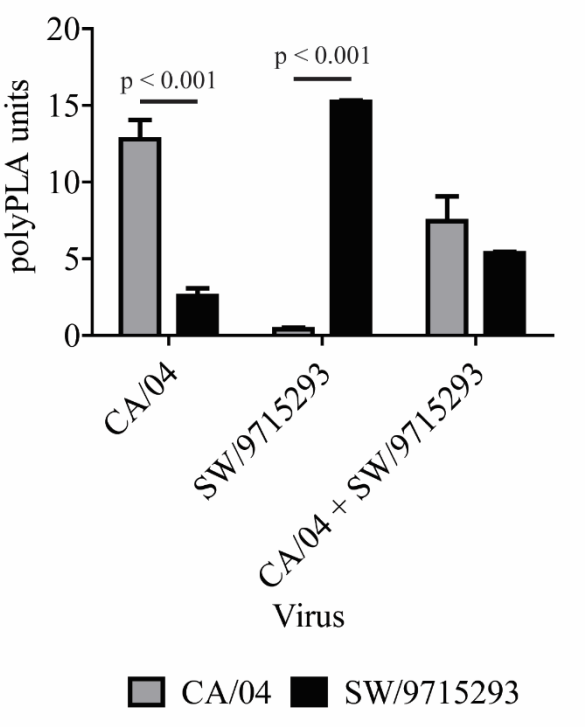

Figure 22 Oligonucleotide-labeled antibodies for A/California/04/2009 (CA/04; H1N1) and A/Switzerland/9715293/2013 (SW/9715293; H3N2) tested against CA/04, SW/9715293, and equal ratios of CA/04:SW/9715293 viral isolates.

Table 20 Oligonucleotide-labeled antibodies for A/California/04/2009 (CA/04; H1N1) and A/Switzerland/9715293/2013 (SW/9715293; H3N2) tested against CA/04, SW/9715293, and equal ratios of CA/04:SW/9715293 viral isolates.

	Virus					
Antibody	CA/04		SW/9715293		CA/04 + SW/9715293	
	polyPLA	SD	polyPLA	SD	polyPLA	SD
CA/04	12.785	1.265	0.404	0.1	7.46	1.62
SW/9715293	2.572	0.496	15.229	0.104	5.356	0.098

Next, we wanted to determine if each of the polyclonal proximity probe pairs, in multiplex, could identify a mixed sample with both CA/04 and SW/9715293 viruses that were diluted to 1,000 $TCID_{50}$/mL and mixed together. CA/04 polyclonal antibodies had 7.5 (± 1.6) polyPLA units and SW/9715293 had 5.356 (± 0.1) polyPLA units against their respective homologous viruses. While in the multiplex, each of the polyclonal proximity probe pairs could identify a mixed sample with no significant difference in polyPLA value.

Discussion

Influenza viruses are continuously changing their antigenicity in order to escape host immunity (3, 4) causing a need to update the vaccine strains, almost annually, to obtain antigenic matches between the vaccine strain and the strain potentially causing future outbreaks(5, 6). One major challenge for selecting an influenza vaccine strain is the time restriction of at least six months are required for vaccine manufacture before the next influenza season begins. For the Northern Hemisphere, the vaccine strain for the fall season has to be determined early spring (usually on February 15th each year). The rapid identification of antigenic variants is essential for a successful influenza vaccine (98, 99).

Although this method has proved its high-throughput capabilities, polyPLA still has to prove its robustness (220, 232). To further improve the high-throughput capabilities of polyPLA, the multiplex assay was generated in order to detect antigenic variants from a panel of polyclonal antibodies. In this chapter, both H1N1 and H3N2 polyclonal antibodies were used for subtype verification and proved the feasibility for a multiplex polyPLA assay. The ability for multiple probes sets to amplify with limited

reagents in a single reaction can lead to the detection of a mixed infection or assessing of antigenic profiles using multiple sera.

Historically, both H1 and H3 have lost the ability to bind certain species of RBCs, which can impede typical serological assays(100, 123, 124). Rapid subtype verification in human clinical samples can be accomplished with the use of the polyPLA multiplex assay in a panel consisting of H1 and H3 polyclonal antibodies as well as NP monoclonal antibodies. Because subtype alone does not fully give knowledge about novel viruses, additionally, a panel of contemporary H1 and/or H3 variants can be constructed in order to further antigenically characterize circulating virus.

In the 2014-2015 influenza season, novel antigenic variants from two antigenically distinct H3N2 clades (3C.3a and 3C.2a) emerged and rapidly caused epidemic outbreaks. Low or undetectable hemagglutination activity was common, particularly for 3C.2a, which caused antigenic characterization to be more difficult for the novel viruses (118). The majority of the viruses analyzed were antigenically related to 3C.3a and A/Switzerland/9715293/2013-like virus (3C.3a) that was chosen as the 2015-2016 vaccine virus (118, 119). However, the following season, 2015-2016, the majority of endemic viruses were 3C.2a but with 3C.3a co-circulating at a lower proportion (118). Like the previous year, antigenic characterization was technically challenging due to low or undetectable hemagglutination activity. As a result, A/Hong Kong/4801-like virus (3C.2a) remained as the 2016-2017 vaccine virus by the WHO (118). With the multiplex polyPLA, a panel of polyclonal antibodies consisting of H3N2 variants 3C.2a and 3C.3a as well as A(H1N1)pdm09 could rapidly screen for circulating variants.

Mixed infections of influenza viruses are not uncommon, presenting a great challenge in antigenic characterization because viral propagation could skew the viral population. Infection within a host is not limited to one viral genome and the proliferating population of viruses is closely related, but not identical (42-45). Additionally, cooperative interactions among two distinct variants of human viruses has been shown to increase population fitness(254). A panel of currently circulating variants should reveal clinical samples that consist of more than just one variant from the same subtype of influenza virus. Additionally, as seen in this application, mixed infections with different subtypes of influenza can be screened. This novel multiplex assay can detect antigenic variants in clinical samples with mixed infections, providing a robust detection method for antigenic variant identification.

In a natural mixed infection, viral population diversity is in a transient state (254), unlike in equal ratios as shown here. Further studies need to be completed to measure the sensitivity and specificity at different viral titrations within a mixed infection. A cutoff will need to be established for the lowest concentration of a single variant within a mixed infection that can be detected with this method.

In summary, a multiplex assay was developed and validated for the detection of H1 and H3 antigenic variants in clinical samples in this chapter. The novel multiplex polyPLA has the potential to give the complete antigenic profile of clinical samples within a single assay.

CHAPTER VII

CONCLUSIONS

The studies in this dissertation have generated the following conclusions:

First, a novel polyPLA was developed and optimized to detect antigenic variants for influenza viruses in clinical samples, including those uncultivable samples. PolyPLA is a viral isolate- and red blood cell-independent method and has been shown to be a high throughput, sensitive assay for the identification of antigenic variants in clinical samples from human and animals (i.e. swine). A multiplex polyPLA was further developed to assess profiles for multiple antigenic variants simultaneously. This method can be used in diagnosis and surveillance (i.e. vaccine strain selection) not only for influenza viruses but also other pathogens with potential antigenic variations. The novel polyPLA also provides a useful tool for us to understand antigenic evolution of influenza A viruses directly by using clinical samples. Further studies are needed to develop and optimize a more comprehensive multiplex polyPLA for IAVs and other pathogens.

Second, this study further demonstrated the complexity of the ecology for influenza A virus. This study suggested antigenic diversity of IAVs in feral swine, an emerging and expanding natural host for IAVs. This study suggested that feral swine of the United States were exposed to H1, H3, H6, and H7 subtypes of IAVs, majority of which were swine and human influenza A viruses but a few avian influenza A viruses.

Third, this study demonstrated the discrepancy of antigenic profiles for the H3N2 IAVs in human clinical samples and the corresponding viral isolates, which could be generated by culture adaptation in the process of virus isolation. This study indicated the potential biases in antigenic data derived from viral isolates, which might not represent those for the viruses circulating in hosts.

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