RESEARCH REPORT
SWINE HEALTH

Title: Genetic assessment of VDL SIV isolate pool for evidence of the swine flu strain reported to be infecting people and development of a high-throughput differential test for the novel strain
NPB #09-206

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Date Submitted: August 6, 2011

Industry Summary

A novel H1N1 influenza virus (initially named "Swine Flu" and now referred to as "2009 pandemic H1N1") emerged and caused widespread clinical disease in US and other countries. The novel virus is a reassortant between North American lineage and Eurasian lineage of swine influenza viruses (SIVs) which has not been reported in human and swine populations throughout the world previously. Although human-to-human transmission was the main mode after initial outbreak, the potential and perception that pigs might have been the initial source of the novel strain had detrimental impact on both domestic and export markets of U.S. pigs and pork products. Although preliminary tests indicated otherwise, uncertainty existed as to whether or not the novel strain had been circulating undetected in the US swine population. The following study was conducted to address this concern through retrospective and proactive surveys.

First, archived H1N1 SIV isolates (n=118) and H1N1-positive clinical specimens from cases submitted from Iowa and major swine producing states to the Iowa State University Veterinary Diagnostic Laboratory during 2008 and the first quarter of 2009 were sequenced and analyzed for HA, NA and M genes in comparison to those of the novel H1N1 strain. Sequence analyses did not reveal the presence of novel H1N1 virus among the 118 banked H1N1 SIV isolates and H1N1-positive clinical specimens, strongly suggesting that 2009 pandemic H1N1 was unlikely circulating undetected in the US swine population prior to its emergence in humans. Second, the effort was made to develop a multiplex PCR assay for rapid detection and differentiation of the novel H1N1 strain from endemic SIVs which have been circulated in the US swine population. As a differential PCR was successfully developed, specimens collected from 165 animals after the emergence of the novel H1N1 virus in humans were examined by the test. In contrast to the observation on the archived isolates/samples, the novel H1N1 strain was detected in various animal species (pigs, cats and dog) although its incidence was low (3%).

In conclusion, evidence that the novel H1N1 virus was circulating undetected in US swine population prior to its emergence in human is lacking. Cross-species transmission of the novel H1N1 strain from affected

humans to animals including pigs were apparent, suggesting that good biosecurity measure and farm personnel management should be practiced when an influenza epidemic occurs in humans. Development of PCR-based assay for rapid detection and differentiation of the novel H1N1 strain would be of help for a proactive surveillance program as its incidence in pigs is expected to continue to increase. Please contact Dr. Yoon for details and further information.

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Keywords: Influenza A virus; Swine; 2009 pandemic H1N1; sequence analysis; differential PCR

Scientific Abstract

A novel H1N1 influenza virus (now referred to as "2009 pandemic H1N1") emerged and infected human in Mexico and caused widespread clinical disease in US and other countries. The novel virus is a reassortant between North American lineage and Eurasian lineage of swine influenza viruses (SIVs) which has not been reported in human and swine populations throughout the world previously. Although human-to-human transmission was the main mode, the potential and perception that pigs might have been the initial source of the novel strain (hereafter, 'pH1N1') had significantly negative impact on both domestic and export markets of U.S. pigs and pork products. Although preliminary tests indicated otherwise, uncertainty existed as to whether or not the novel strain had been circulating undetected in the US swine population. Therefore, retrospective and proactive surveillance for the new virus was necessary to address this concern.

First, archived H1N1 SIV isolates (n=118) and clinical specimens from 55 H1N1-positive cases submitted from Iowa and surrounding major swine producing states to the Iowa State University Veterinary Diagnostic Laboratory during 2008 and the first quarter of 2009 were selected, sequenced and analyzed for HA, NA and M genes in comparison to those of the novel H1N1. Second, the effort was made to develop an M gene-based PCR assay in a multiplex format for rapid differentiation of pH1N1 from endemic SIVs which have been circulated in US swine population since the novel virus contains the unique M gene (i.e., Eurasian lineage) as compared to endemic SIVs (North American lineage).

Sequence analyses for HA, NA and M genes revealed no pH1N1 among the 118 archived H1N1 SIV isolates and H1N1-positive clinical specimens. The vast majority of the virus isolates were classified into one of β, γ, and δ clusters based on their HA sequences. To our surprise, M gene showed a high degree of variability and the degree of homology was related to year of submission. As a differential PCR assay was successfully developed for detecting and differentiating pH1N1 from endemic SIVs, specimens collected from 165 animals after emergence of pH1N1 in humans were tested by the assay. In contrast to the observation on the archived isolates/samples, 5 animals (2 porcine, 2 feline and 1 canine) were determined to be positive for pH1N1 (i.e., EA matrix) while 6 animals were positive for endemic (NA matrix) influenza A virus (5 porcine and 1 feline).

In conclusion, evidence that the novel H1N1 virus was circulating undetected in US swine population prior to its emergence in human is lacking. Furthermore transmission of novel H1N1 strain from affected humans to pigs was apparent, suggesting that good biosecurity measure and farm personnel management should

be practiced when an influenza epidemic due to a new strain occurs in humans. Continuous monitoring of swine herds for emergence of a novel strain may be necessary as reassortment between pH1N1 and endemic SIVs is expected.

Introduction

Iowa is by far the #1 producer of pork in the U.S. and pork sales represent nearly half ($5 billion) of the animal agriculture receipts for Iowa. A profitable pork industry is critical to the economic success of Iowa and other swine-producing states and countries.

Evidence indicated that a 'novel' swine influenza virus (SIV), which is now referred to as "2009 pandemic H1N1 virus (pH1N1)", from pigs had infected people in Mexico causing widespread clinical disease and over 150 deaths in certain regions. The novel virus is a reassortant between North American lineage and Eurasian lineage of SIVs which has not been reported in human and swine populations throughout the world previously. As of April 29, 2009, approximately 91 cases and 1 death were reported in 10 states in the U.S. and several of these had some link to traveling or being in contact with people who had traveled to Mexico. The incidence rate was expected to continue to increase as suspect cases continue to be reported from other countries.

Although human-to-human transmission had been the main mode so far, the potential and perception that pigs might have been the initial source of the novel strain has significant impact on both domestic and export markets of U.S. pigs and pork products. Although preliminary tests indicated otherwise, uncertainty existed as to whether or not the novel strain had been circulating undetected in the US swine population. Therefore, retrospective and proactive surveillance for the new virus was necessary. The proactive surveillance would require a reliable rapid differential test which can fit high throughput testing.

Objectives

The working hypothesis was that the newly identified "swine flu" isolate (i.e., pH1N1) causing serious disease in Mexico is not yet present in the Iowa swine population. Specific objectives were to:

1. Analyze achieved viruses and clinical specimens by molecular tools;
2. Develop a high throughput differential assay(s) for detecting and differentiating the novel strain from endemic SIV;
3. Evaluate the utility of M-Chip microarray technology as differential testing; and
4. Assess the utility of oral fluid sampling for better population-based surveillance for influenza virus in comparison to other clinical specimens.

However, due to constraint in funding amount ($25,000 of $75,000 total request), the focus of the NPB-funded study was objective 1 and 2.

Materials & Methods

Banked isolates and specimens from cases submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) in 2008 and the first quarter of 2009 were selected for the study. The isolates (n=118) and clinical specimens represented commercial hog operations in major swine producing areas of Iowa (27 counties) and some surrounding states (IL, MI, MO, NC, NE, OH, PA, WI, VA). Clinical specimens were selected from 55 cases (each represent different herd/farm) with a history of respiratory diseases, SIV vaccine

failure and/or influenza-like illness in human. Sequencing for hemagglutinin (HA), neuraminidase (NA) and matrix (M) genes was done using standard protocol established in the ISUVDL. Sequence analysis was done by using Lasergene (DNAstar) and included HA, NA and M sequences of 11 known pH1N1 isolates which were obtained from GenBank.

In addition to sequence analysis of archived SIV isolates, the effort was also made to develop a PCR-based assay in a multiplex format for rapid differentiation of the 2009 pandemic H1N1 strain from endemic SIVs which have been circulated in US swine population. The target gene was the M gene since the novel H1N1 contained the unique M gene (i.e., Eurasian genotype) as compared to endemic SIVs. Matrix gene sequences from U.S. human pH1N1 cases and U.S. SIVs were aligned to determine a suitable region for an assay target. Primers were selected to amplify all influenza A. Two probes were designed to differentiate pH1N1 viruses (i.e., Eurasian matrix) from endemic SIVs (i.e., North American matrix). The assay was initially validated using the first U.S. pH1N1 strain, 10 human pH1N1-positive specimens and 9 U.S. SIV isolates, then evaluated on 165 specimens (32 cases) of swine and other animal origin submitted to ISUVDL. Specimens included a variety of sample matrix (i.e., lungs, nasal swab, treacheal swab, phargyneal swab, lung lavages). Results were compared to other influenza A PCR assays (i.e., ISUVDL in-house SIV PCR, NAHLN SIV surveillance PCR and CDC pH1N1 and seasonable flu PCRs) and/or sequencing for confirmation of virus classification. In addition sequences from additional pH1N1 strains and contemporary H1N1 SIVs were used to bioinformatically assess robustness of the selected primers and probes for the intended purpose.

Results

Objective 1 Sequence analysis for HA, NA and M genes did not show the presence of pH1N1 among the 118 archived H1N1 SIV isolates and clinical specimens from 55 respiratory cases in which H1N1 SIV was identified by PCR. Based on HA sequence, vast majority of the virus isolates were classified into one of β, γ, and δ clusters (Figure 1).

To our surprise, the virus isolates showed a high degree of variability in M sequences, ranging from 90% to 99.5% homology, even though none of them contained the M gene of Eurasian lineage. The virus isolates could be classified into 5 distinct clades. Although M gene homology was independent of H1 subtype, degree of homology was related to year of submission.

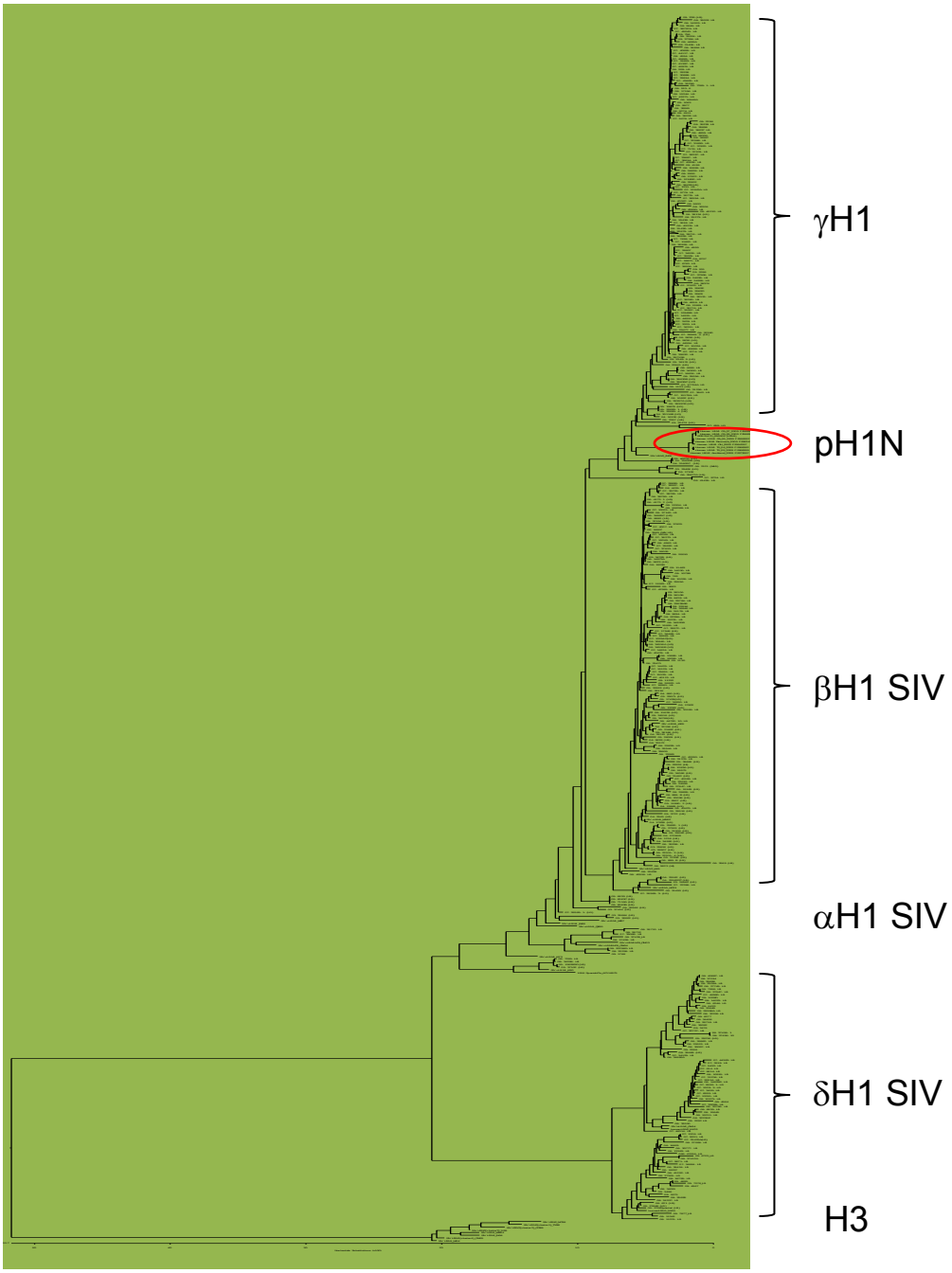

Figure 1. Dendogram showing HA-gene based phylogenetic relationship and clusters among H1N1 swine influenza viruses (SIVs) isolated from or identified in cases submitted to the Iowa State University Veterinary Diagnostic Laboratory during 2008 and the first quarter of 2009. Sequences of 2009 pandemic H1N1 viruses were obtained from GenBank and included in the analysis for comparison. Sequences of H3N2 SIVs were included as outlier to construct a phylogenetic tree.

Objective 2 M-gene based multiplex PCR (hereafter, 'differential M-gene RT-PCR') was successfully developed for detecting and differentiating pH1N1 from endemic SIVs. The 10 pH1N1-positive human respiratory specimens provided by Iowa State Hygienic Laboratory were detected by only the VIC dye, as expected for the Eurasian (EA) M gene of the pH1N1 virus. The A/Swine/Iowa/73 (H1N1, α clade) and A/Swine/TX/98 (H3N2, cluster I) viruses were detected only by the FAM dye, confirming specificity of the assay for the North American (NA) matrix gene.

Classification of the 8 SIV isolates sent to the State Hygienic Laboratory is summarized in Table 1. Although all 8 isolates were endemic SIVs, 5 of the 8 viruses were identified as positive for pH1N1 by CDC PCRs and the remaining 3 isolates as reassortant between pH1N1 and human seasonal influenza viruses. In contrast, the NAHLN SIV surveillance PCR did not identify any of the 8 isolates as pH1N1 as shown in Table 1. These isolates were all tested NA matrix gene positive (i.e., endemic SIV) by the assay described herein.

Specimens collected from 165 animals after emergence of pH1N1 in humans were tested by the differential M-gene RT-PCR. All results (e.g., positive for influenza A, identification of virus genotype) were confirmed by ISU-VDL in-house RT-PCR assay (NP,H, N genes) or by the NAHLN surveillance PCR (M and N genes) and/or by sequencing for H and M genes. In summary, 6 samples were positive for endemic (NA matrix) influenza A virus (5 porcine and one feline), 5 samples were positive for pandemic (EA matrix) H1N1 influenza A virus (2 porcine, 2 feline and one canine) and the remaining 154 samples were negative for influenza A virus.

Sequencing was performed on 122 clinical porcine samples or virus isolates from samples which had previously tested as H1N1 by the ISU-VDL assay and on 2 pH1N1 samples. Analysis revealed total homology between the EA matrix primer/probe sequences and that from the pH1N1 matrix sequences. However, minor mismatches in the probe target region were found in 6 of the endemic H1N1 matrix sequences from clinical specimens. A probe reflecting this mismatch (M_NAPR2) was synthesized and incorporated into the PCR reaction mixture. All 6 samples were tested as NA Matrix positive with the updated reagent mix.

Table 1. Comparative performance of various PCR-based assays for influenza A viruses on known reference H1 and H3 swine influenza viruses and 2009 pandemic H1N1 (A/California/04/2009)*

Virus	ISU SIV PCR			ISU M-gene PCR		NAHLN SIV PCR		CDC pH1N1 PCR			CDC seasonal flu PCR	
	NP	H1	H3	NA-M	EA-M	M	N1 diff	M	swHA	swNP	huH1	huH3
α H1 SIV	+	+	-	+	-	+	-	+	+	+	-	-
β H1 SIV	+	+	-	+	-	+	-	+	+	+	-	-
γ H1 SIV	+	+	-	+	-	+	-	+	+	+	-	-
δ H1 SIV	+	+	-	+	-	+	-	+	-	+	+	-
H3N2 SIV	+	-	+	+	-	+	-	+	-	+	-	+
pH1N1	+	+	-	-	+	+	+	+	+	+	-	-

*NOTE: Adopted from a publication by Harmon K, Bower L, Kim W-I, Pentella M and Yoon K-J (*Influenza and Other Respiratory Viruses* 4:405-410, 2010) with modifications.

Discussion

Sequence analysis of H1N1 SIV isolates obtained before the emergence of pH1N1 in human clearly demonstrated the absence of pH1N1 in swine specimens tested. Although a relatively small number of isolates were examined, they represented respiratory cases in all major swine producing regions in the US (Midwest, East coast). USDA ARS scientists reported that experimental inoculation of pH1N1 demonstrated that the virus is pathogenic to pigs (USDA report). Therefore it would be logical to conclude that pH1N1 was not circulating undetected in the US swine population prior to its emergence in humans in Mexico and US.

It was unexpected that the M gene had a high degree of sequence variation among SIV since matrix protein (i.e., M gene product) has been used as one of type-specific antigens for influenza viruses. Sequence variation and evolution of the M gene was in relation to time of isolation although its mutation rate was a lot less than that of HA and NA genes. While it remains to be further studies what implication M gene variation plays in virulence, immunity and cross protection, the study observation should be taken into consideration when one plans to develop a molecular diagnostic assay targeting the M gene. Otherwise, misdiagnosis (i.e., false negative) can occur. The study observation also emphasizes that primers and/or probes should be designed with good bioinformatics support. Like other RT-PCR assays, monitoring the variation of sequences in field strains and updating the primers and/or probes is necessary to ensure reliable performance of the assay.

The newly developed real-time PCR assay targeting the M gene was able to detect and differentiate pH1N1 and US influenza A viruses in various sample matrices and species. It was used to detect the first reported human-to-feline reverse zoonotic transmission of pH1N1 virus and has also been used to detect this virus in canine and porcine specimens. (For the USDA's list of pH1N1 influenza presumptive and confirmed results in non-human species, see: http://www.usda.gov/documents/FINAL_RESULTS_2009_PANDEMIC_H1N1_INFLUENZA_CHT.pdf). Since pH1N1 virus has spread from humans to pigs and the virus appears to co-circulate with endemic SIVs (data not shown), care must been taken in interpreting the test result in the future as reassortment between pH1N1 and endemic influenza A viruses is expected to occur. Use of the assay in combination with NAHLN SIV PCR may overcome this concern since NAHLN PCR differentiates pH1N1 from endemic SIVs based on the NA gene. Otherwise, sequencing for HA, NA and M genes of each H1N1 virus will be necessary.

The CDC protocol was reported to differentiate pH1N1 from human influenza virus and may be useful in monitoring the emergence of reassortant influenza viruses, but cannot differentiate pH1N1 from endemic H1 SIVs as shown in our study, raising a concern on its differential diagnostic utility at public health laboratories since incidence of SIV infection in humans has been reported. The differential matrix assay described herein may be a good supplement to the CDC testing protocol and to the NAHLN PCRs.