RESEARCH REPORT
SWINE HEALTH

**Title:** Efficacy of Multiplex SIV TaqMan® RT-PCR and PRDC Microarray Hybridization in Predicting Swine Influenza Virus Genotype – **NPB#03-096**

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**Abstract:** Multiplex SIV TaqMan® RT-PCR end-point analyses to rapidly diagnose both the hemagglutinin (HA) and neuraminidase (NA) genes of swine influenza virus (SIV) were developed and analyzed for sensitivity and specificity using viral field isolates. The TaqMan® results were compared to those obtained by traditional diagnostic assays as well as HA nucleotide sequence analysis, and found to be accurate and rapid. Microarray hybridization data showed that this relatively new technique was not very sensitive and produced uninformative data. As a result, the hybridization experiments were not included in our diagnostic test comparison. Nucleotide sequence analysis of the SIV HA gene revealed that there are now three major H1 and five H3 genotypes circulating in U.S. swine herds.

**Introduction:** Swine influenza virus (SIV) is one of the most commonly diagnosed agents of porcine respiratory disease complex (PRDC). PRDC is a serious concern for the swine industry as respiratory disease is the major cause of swine mortality (http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/swine/swine.htm). In addition, SIV has become more troubling for swine producers and veterinarians. The virus has changed dramatically over the last decade, leading to the evolution of several distinct SIV genotypes circulating in U.S. swine herds. These changes have complicated the management and prevention of the disease to the point where herds are experiencing severe SIV-induced respiratory disease despite vaccination. In some herds, the virus is endemic, continually resulting in respiratory disease problems and poor performance in growing swine. Yet the diagnostic assays for SIV are time-consuming and often unproductive, and as a result, the selection of appropriate vaccine type is sometimes obscure.

The aim of this research proposal was to evaluate the outcomes of both a new TaqMan® RT-PCR multiplex and a porcine respiratory disease complex (PRDC) microarray in genotyping the SIV hemagglutinin (HA) and neuraminidase (NA) swine respiratory disease samples. The genotypes were then precisely elucidated by nucleotide sequence analysis and compared to other established serotyping analyses. The new diagnostic format developed with the aid of this research, Multiplex TaqMan® RT-PCR assays for rapid SIV typing, will be faster and more informative than current tests for influenza, allowing for improved surveillance of SIV strains and leading to better prevention strategies.

**Objectives:**

1. To evaluate the use of multiplex SIV TaqMan® RT-PCR on defined SIV isolates.
2. To evaluate the use of PRDC microarray hybridization on defined SIV isolates.
3. To perform diagnostic multiplex SIV TaqMan® RT-PCR and microarray hybridization on diagnostic submissions from new SIV outbreaks.

**Materials & Methods:** We have designed our multiplex assay (SIV TaqMan® RT-PCR Multiplex) as two separate tests run in parallel on 96-well plates, one for H1N1 isolate typing and one for typing H2N3 isolates. We found that although there are several available fluorescent indicator tags for product identity (product probes), four separate sets of primers for RT-PCR amplification and four different probes for identification were too technically difficult to include in one reaction with absolute diagnostic assurance. In brief, the viral RNA is extracted from nasal or bronchial swabs, lung tissue or viral isolates using RNA extraction kits (Qiagen, Valencia, CA). The RNA is separately subjected to both H1N1 and H3N2 TaqMan® RT-PCR using specific primers and probes for each type of hemagglutinin (HA) and neuraminidase (NA) gene. The plates are read by an Applied Biosystems 7000 Sequence Detector at the appropriate wavelengths before and after nucleic acid amplification, which means it is an endpoint assay, not in “real time” and thus the assay is not quantitative. The results define whether a SIV isolate is H1N1, H3N2, H1N2 or a genotype combination.

The microarrays were completed by preparing microscope slides with ordered arrays of tiny DNA spots containing segments of relevant genes (HA, NA, NP) of various swine, avian and human influenza viruses and several other pathogens. The slides were then incubated with a preparation of purified viral RNA that had been processed into fluorescently tagged complementary DNA. After incubation (termed hybridization) and washing, then plates were dried and then read into computer software programs (Scanarray and Quantarray), available in the Advanced Genetic

Analysis Center at the University of Minnesota. The results were expressed as the amount of fluorescence over background for each spot and then analyzed for test accuracy in predicting viral genotype.

SIV genotypes were confirmed by several methods. They included MVDL serotype analysis of HA by hemagglutinin inhibition (HI) and NA by neuraminidase inhibition (NI), nucleotide sequence analysis using established methods and primer pairs used routinely in our Molecular Diagnostic Laboratory to prepare phylogenetic analyses for MVDL clients, and RT-PCR followed by gel analysis (Choi et al., 2002).

**Results**

**Objective 1:** The two multiplex TaqMan RT-PCR diagnostic tests appear to perform well, although we have not yet finalized the assays and performed complete validation. The development was more difficult than we had envisioned and involved several versions of primers and of probes due to novel SIV isolates unmasked by sequence analysis. At present, the H1N1 multiplex is fairly sensitive and able to detect H1 and N1 vRNA equivalent to 3,981 $TCID_{50}$/ml of reference viral H1N1 strain A/Sw/Minnesota/2002. The H3N2 multiplex was sensitive and able to detect H3 and N2 vRNA equivalent to 630 $TCID_{50}$/ml of reference viral H3N2 strain A/Sw/Texas/1998. The H3N2 multiplex was specific and did not produce reactions with common viruses seen in the MVDL such as BVDV and PRRSV. H1N1 multiplex specificity tests have not been completed, but are expected to be similar to the results of the H2N2 multiplex specificity tests. Table 1 is a summary of the number field isolates analyzed for this proposal and their genetic makeup.

**Table 1.** Number of Field Isolates Examined in this Study

| Multiplex Results | No. of Samples |
|---|---|
| H3N2 | 75 |
| H1N1 | 36 |
| H1N2 | 12 |
| H1N1, H3N2 | 2 |
| H1N1, H1N2 | 6 |
| HA and NA tested; neither conclusive | 6 |
| Total | 137 |

Table 2 presents the breakdown of the isolates and the confirmatory assays that were completed to support the multiplex TaqMan® RT-PCR results. In no case were the results derived by Multiplex TaqMan® RT-PCR contradictory to other confirmatory tests. However, there were some HA or NA genes as well as some entire cases that were not resolved because the confirmatory tests did not yield diagnostic results. There were 28 H3, 69 N2, 12 H1, and 30 N2 unconfirmed genes where one strain was present, and 6 HA and 8 NA unconfirmed genes when two genotypes were present. Sequence analysis was not completed on many of the isolates, although most of the isolates were confirmed as SIV by screening them with the SIV NP TaqMan® RT-PCR.

These results highlight the power of rapid genetic typing by multiplex RT-PCR. Not only are the HA and NA results more quickly generated, but they are capable of discerning both genes at once and uncovering the presence of reassortment SIV viruses as well as mixed infections.

**Objective 2.** To evaluate the use of PRDC microarray hybridization on defined SIV isolates.

Microarray hybridization results were disappointing, in that all trials revealed a lack of diagnostic method sensitivity. Although we performed several experiments with RT-PCR amplified known genes and also tested concentrated viral isolates, we could not reliably obtain positive signals (data not shown). We discontinued research on the microarray because of insufficient funds and focused our efforts on the multiplex assays described above. From analysis of present literature, it appears that sensitivity of this test format is a concern to many laboratories.

**Table 2.** Typing Methods Utilized to Confirm the SIV Multiplex TaqMan® Assays

| Multiplex RT-PCR Result | Number of Samples | Alternate Successful Confirmatory Method(s)* | | | | |
|---|---|---|---|---|---|---|
| | | HA HI | NA NI | HA seq | NA seq | NA RT-PCR |
| H3N2 | 1 | X | X | X | X | |
| H3N2 | 4 | X | | X | | |
| H3N2 | 5 | | X | X | | |
| H3N2 | 17 | X | | | | |
| H3N2 | 20 | | | X | | |
| H3N2 | 28 | | | | | |
| H1N1 | 1 | X | X | | | |
| H1N1 | 12 | X | | X | | |
| H1N1 | 5 | | | X | X | |
| H1N1 | 6 | | | X | | |
| H1N1 | 12 | | | | | |
| H1N2 | 1 | X | | X | | |
| H1N2 | 3 | | X | X | | |
| H1N2 | 1 | | | X | X | |
| H1N2 | 3 | | | X | | X |
| H1N2 | 1 | X | | | | |
| H1N2 | 2 | | | X | | |
| H1N2 | 1 | | | | | |
| H1N1/H3N2 | 2 | | | | | |
| H1N1/H1N2 | 1 | X | | X | | |
| H1N1/H1N2 | 1 | | | X | | |
| H1N1/H1N2 | 4 | | | | | |

*Methods: HA serotyping by hemagglutination inhibition (HA HI),
NA serotyping by neuraminidase inhibition (NA NI)
HA genetic sequencing (HA seq)
NA genetic sequencing (NA seq)
NA gel-electrophoresis RT-PCR (NA RT-PCR)

**Objective 3.** To perform diagnostic multiplex SIV TaqMan™ RT-PCR and microarray hybridization on diagnostic submissions from new SIV outbreaks.

Antigenic characterizations conducted via hemagglutination inhibition (HI) tests performed using a panel of antisera for several H1 and H3 viruses had revealed antigenic diversity between both H1 and H3 viruses. The reassortant H1N1 viruses recently isolated from swine were inhibited by classical H1N1 antisera to a much lesser extent than was found in homologous HI reactions. Furthermore, detection of convalescent antibodies in sera from swine infected naturally with reassortant H1N1 viruses was inconsistent or absent, especially when compared to seroconversion to the homologous infecting field strain of reassortant H1N1 virus. H3 viruses were increasingly non-reactive to reference A/Sw/TX/98 antisera. Overall, the percent of SIV isolates that were untypeable by HI serotyping with reference classical H1N1 or A/Sw/TX/98 antisera increased from 2001 (11%) to 2003 (13%). The TaqMan results, described above, illustrate the advancement we have initiated in rapid genotypic analysis of SIV HA and NA genes. The MVDL also began to genetically characterize SIV isolates of interest to swine practitioners and producers. 335 HA gene sequences have now been completed and have been deposited in the Influenza Sequence Database (Macken et al., 2001). HA gene phylogenies of both H1 and H3 influenza viruses have revealed several groups of divergent virus variants (Figures 1 and 2). The figures have been reduced in font (best read at 300% magnification) and their names simplified in order to more easily obtain SIV temporal and geographical evolutionary trends.

Genetic characterizations of both H1 and H3 influenza viruses completed through comparisone of the HA gene nucleotide sequence has revealed several groups of divergent virus variants. In an analysis of 206 H1 field viruses (Figure 1), three phylogenetic groups were apparent – a reassortant H1N1-like group that included approximately 28% of the viruses sequenced an H1N2-like group (45%), and a classical H1N1-like group (27%). The H1 sequence information did not predict NA type, nor did it reveal the presence of avian internal genes (data not shown). Within groups, there was a maximum of 5% nucleotide difference and between groups the nucleotide differences were as high as 12%. H3 virus phylogenetic analysis (Figure 2) was consistent with previous analyses by Webby et al., 2000. The results demonstrated the three clusters of H3 viruses, but also a few isolates that were quite divergent from the three clusters. An analysis of 129 field viruses revealed that the majority of H3N2 viruses (117 isolates) were grouped with A/Sw/IL/99 and A/Sw/OK/99 in Cluster III (red typeset in Figure 2). Within a group, the H3 nucleotide differences ranged from 0 to 8%.

Furthermore, it was determined that similar viral isolates were detected in several states, either in the same year or in successive years. Thus, there are many SIV HA genotypes (3 H1 and 3-5 H3) circulating, many times in close proximity to each other. The study data must now be assembled and rigorously reviewed in order to establish any link between the isolates that were untypeable by traditional HI tests and their respective nucleic acid sequence.

**Discussion:** In an attempt to determine the implications that reassortant or variant SIV have on swine health and production, antigenic and genetic characterizations have been performed by the Minnesota Veterinary Diagnostic Laboratory (MVDL) on numerous virus isolates from pigs with swine influenza-induced respiratory disease. A microarray hybridization assay was designed that would hopefully characterize SIV isolates in a timely manner as to their hemagglutinin, neuraminidase, polymerase A, and polymerase B2 genes. However, during development, it was determined that this

method was presently unreliable, laborious, expensive, and would not provide more rapid, accurate or useful information when compared to more conventional diagnostic methods such as hemagglutination inhibition serotyping of SIV isolates, genetic sequence analysis of the hemagglutinin gene, and genotypic analysis of both HA and NA genes multiplex SIV TaqMan® RT-PCR. As stated in the results section, we believe the new multiplex TaqMan® methodology will enable fast and accurate typing of both SIV HA and NA genes. However, determination of the HA nucleic acid sequence will sometimes be necessary in order to determine which available vaccine to utilize.

We discovered in the course of nucleic acid sequence analysis that the virus is changing quickly and dramatically. Instead of one stable SIV strain circulating in herds throughout the US as it was prior to the last decade, now we have several isolates of divergent HA genotype circulating in several swine producing states. As a result, available vaccines and their protection against the novel HA types may have to be reevaluated in light of these findings.

**Lay Interpretation:** New Multiplex TaqMan® RT-PCR genotyping assays for HA and NA genes of SIV were developed. These new diagnostic tests will advance available SIV diagnostics, both in speed and accuracy. We found that the TaqMan® assays, at present, can detect as little as 3,981 $TCID_{50}$/ml of reference viral H1N1 strain A/Sw/Minnesota/2002 and 630 $TCID_{50}$/ml of reference H3N2 viral strain A/Sw/Texas/1998. A new microarray format, having potential to advance the field of swine diagnostics, was not sensitive or reliable at present. Lastly, we provided HA sequence analysis for 335 SIV isolates, which showed that there are now several genotypes circulating in many US swine producing states. Please contact MVDL (mvdl@umn.edu) or K. S. Faaberg (kay@mail.ahc.umn.edu) for further information on these assays.

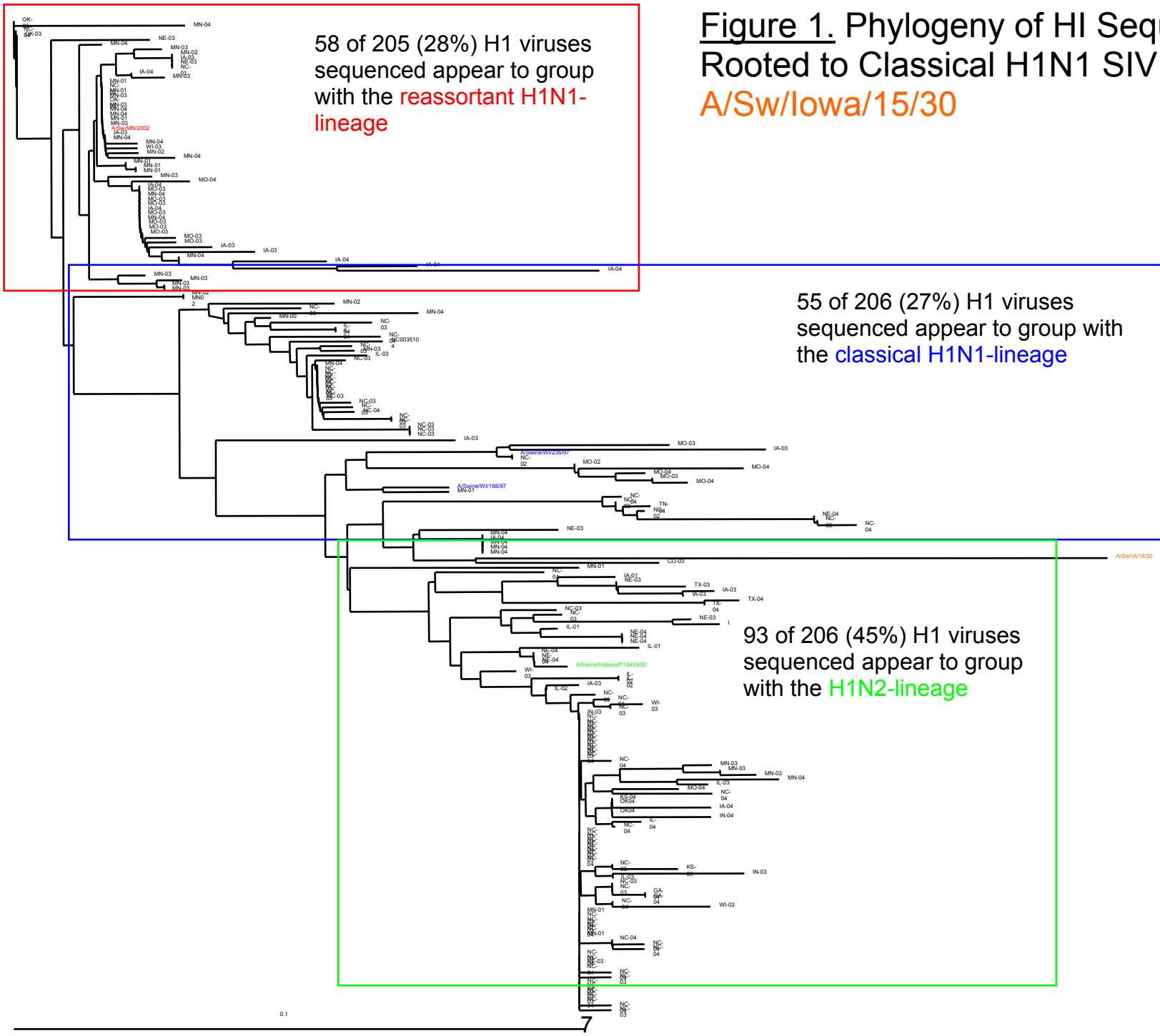
**References:**

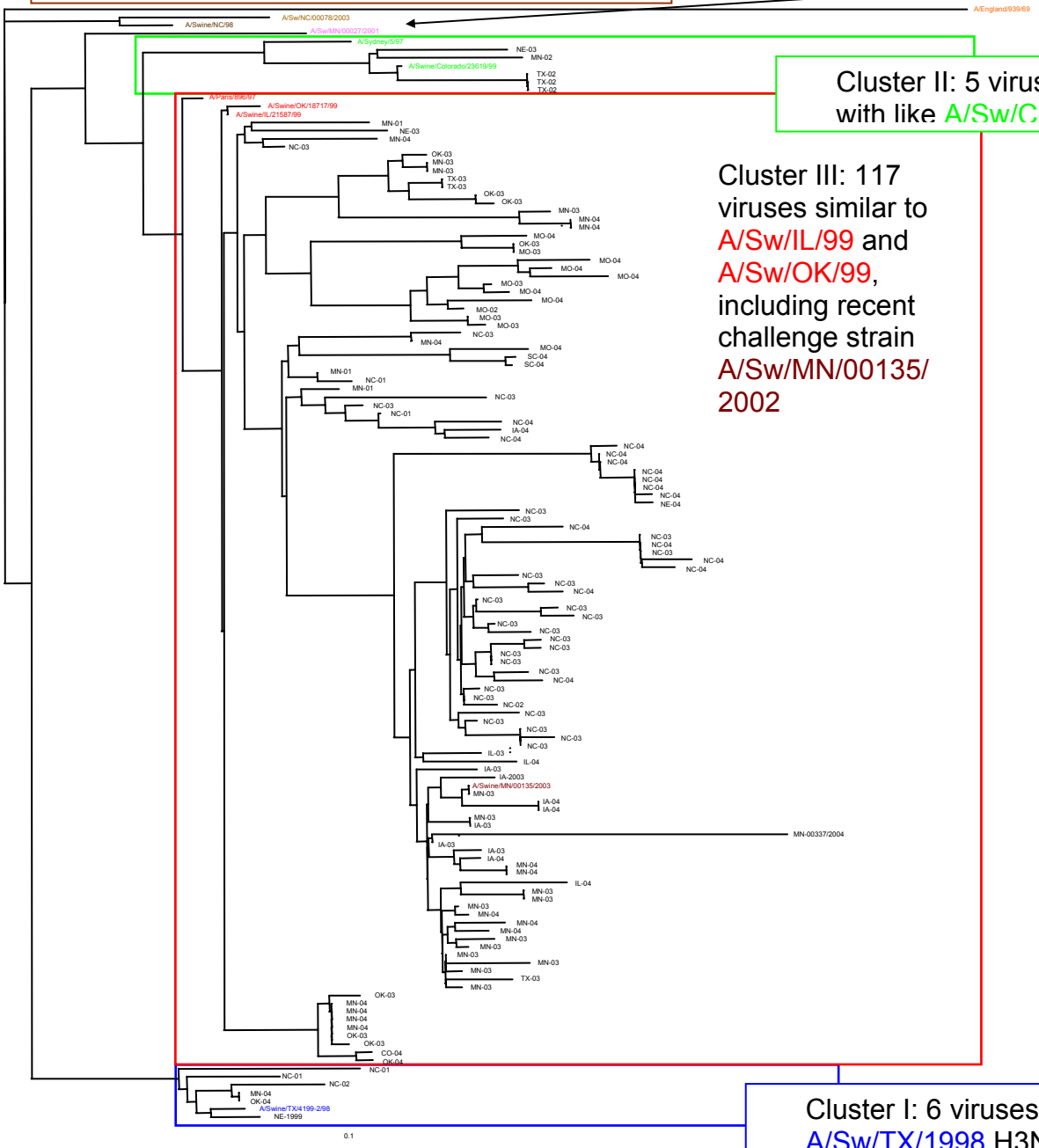

Choi Y. K., Goyal, S. M., Kang, S. W., Farnham, M. W., and Joo, H. S. 2002. Detection and subtyping of swine influenza H1N1, H1N2 and H3N2 viruses in clinical samples using two multiplex RT-PCR assays. J. Virol. Methods. 102:53-59.

Macken, C., Lu, H., Goodman, J., and Boykin, L. 2001. The value of a database in surveillance and vaccine selection, p. 103-106. In *Options for the Control of Influenza IV.* A.D.M.E. Osterhaus, N. Cox & A.W. Hampson , eds. Elsevier Science, Amsterdam.

Webby, RJ, SL Swenson, SL Krauss, PJ Gerrish, SM Goyal, RG Webster. 2000. Evolution of swine H3N2 influenza viruses in the United States. J. Virol. 74:8243-8251.

# Figure 1. Phylogeny of HI Sequences Rooted to Classical H1N1 SIV A/Sw/Iowa/15/30






Only one virus – A/Sw/NC/00078/2003 groups with A/Sw/NC/98 double reassortant H3N2.

1 virus (A/Sw/MN/00027/2001) that does not group with the others very tightly.

Cluster II: 5 viruses (from 3 cases) of 129 cluster with like A/Sw/Colorado/1999 Human A/Sydney/5/97

Cluster III: 117 viruses similar to A/Sw/IL/99 and A/Sw/OK/99, including recent challenge strain A/Sw/MN/00135/2002

Cluster I: 6 viruses of 129 are similar to A/Sw/TX/1998 H3N2 viruses

Figure 2: H3 Sequence Phylogeny Rooted to A/England/1969 human H3N2