Characterization of an influenza A virus isolated from pigs during an outbreak of respiratory disease in swine and people during a county fair in the United States

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1. Introduction

Influenza in swine is an acute respiratory disease caused by influenza A viruses within the Orthomyxoviridae family, first isolated and identified in North America in 1930 (Shope, 1931). Orthomyxoviruses have negative stranded RNA genomes that are segmented, allowing for reassortment and production of novel viruses. There are two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), that define subtypes and are important for host range, antigenicity, pathogenesis, and diagnostic detection. Following the introduction of the triple reassortant H3N2 in 1998, three predominant swine influenza virus (SIV) subtypes have circulated in US swine, H1N1, H1N2, and H3N2. The H1N1 viruses contain the HA and NA from the classical swine virus and the internal genes from the triple reassortant H3N2 viruses (rH1N1); the H1N2 viruses contain the HA from the classical swine virus and the NA and internal genes from the triple reassortant H3N2 viruses (Karasin et al., 2002; Webby...
et al., 2004). Contemporary triple reassortant viruses were demonstrated to have acquired a PB1 gene of human virus origin; PA and PB2 genes of avian virus origin; and the remaining internal genes, M, NS, and NP, of swine virus origin, thus giving rise to the triple reassortant designation (Zhou et al., 1999). Since the introduction of the triple reassortant internal gene (TRIG) cassette, an increase in the rate of genetic change in North American swine influenza isolates appears to have occurred in H1 virus subtypes, and distinct genetic and antigenic clusters have evolved (Vincent et al., 2006). H1N1 and H1N2 viruses with human-like HA and NA were isolated from pigs in Canada in 2003 and 2004 (Karasin et al., 2006). These viruses were wholly human or reassortants with internal genes of classical swine virus lineage. Since that time, reassortant viruses with human-like HA and NA but with the TRIG cassette have been isolated from pigs across the U.S. (M. R. Gramer, personal communication). These human-like H1 viruses have become endemic in the U.S. pig population in concurrent circulation with the contemporary SIV described above.

The tracheal epithelium in pigs expresses the receptors for avian and human influenza viruses, leading to the suggestion that the pig is a mixing vessel for the emergence of new isolates with human pandemic potential (Ito et al., 1998; Scholtissek et al., 1993). There are documented cases as well as serologic evidence for pig to human transmission of SIV (reviewed in (Myers et al., 2007)); however, the frequency of human infections with SIV and potential risk to the human population at large is less clear (Van Reeth, 2007). Moreover, it is unknown what conditions and virulence properties are required for cross-species transmission of SIV. In August 2007, pigs and people became clinically affected by an influenza-like illness during attendance at an Ohio county fair. Influenza A virus was identified from pigs and people, and the virus isolates were characterized as swine H1N1 similar to the swine H1N1 viruses currently circulating in the U.S. pig population. The swine isolate, A/SW/OH/511445/2007, was evaluated in an experimental challenge and transmission study reported here. Sequences from each gene segment of A/SW/OH/511445/2007 were analyzed and compared with phylogenetically related H1 SIV viruses. Additionally, the antigenic properties of the virus were evaluated by cross-reactivity with a panel of reference H1 SIV anti-sera.

2. Materials and methods

2.1. In vivo study

Thirty-nine 4-week-old cross-bred pigs from a herd free of SIV and porcine reproductive and respiratory syndrome virus (PRRSV) were divided into three groups. All pigs were treated with ceftiofur crystalline free acid (Pfizer, New York, NY) to reduce bacterial contaminates prior to the start of the study. Groups were housed in individual isolation rooms and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. Pigs were humanely euthanized with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) at the appropriate times during the course of the study. Twenty pigs were inoculated intratracheally with $2 \times 10^5$ TCID$_{50}$/pig of A/SW/OH/511445/2007 (OH07) rH1N1 isolated and prepared in MDCK cells. Nine control pigs were inoculated with noninfectious cell culture supernatant. The OH07 virus and sham inocula were given intratracheally while the pigs were anesthetized with an intramuscular injection of a cocktail of ketamine (8 mg/kg), xylazine (4 mg/kg), and Telazol (6 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA). Ten contact pigs were co-mingled with inoculated pigs on 2 days post inoculation (dpi) to study transmission efficiency. Pigs were observed daily for clinical signs of disease. Nasal swabs were taken and placed in 2 ml minimal essential medium (MEM) on 0, 3, 5, and 7 dpi or days post contact (dpc) to evaluate nasal shedding and stored at −80 °C until study completion. Five inoculated pigs and three control pigs were euthanized on 3, 5, and 7 dpi to evaluate lung lesions and viral load in the lung.

After euthanasia, each lung was lavaged with 50 ml MEM to obtain bronchioalveolar lavage fluid (BALF). Each nasal swab sample was subsequently thawed and vortexed for 15 sec, centrifuged for 10 min at 640 $\times g$ and the supernatant passed through 0.45 μm filters to reduce bacterial contaminants. An aliquot of 200 μl of the filtrate was plated onto confluent phosphate buffered saline– (PBS) washed MDCK cells in 24-well plates. After 1 h incubation at 37 °C, 200 μl serum-free MEM supplemented with 1 μg/ml TPCK trypsin and antibiotics was added. All wells were evaluated for cytopathic effect (CPE) between 24 and 48 h and subsequently frozen. Ten-fold serial dilutions in serum-free MEM supplemented with TPCK trypsin and antibiotics were made with each BALF sample and virus isolation positive nasal swab filtrate sample. Each dilution was plated in triplicate in 100 μl volumes onto PBS-washed confluent MDCK cells in 96-well plates. Plates were evaluated for CPE between 48 and 72 h post infection. At 72 h, plates were fixed with 4% phosphate-buffered formalin and stained using immunocytochemistry with an anti-influenza A nucleoprotein monoclonal antibody as previously described (Kitikoon et al., 2006). A TCID$_{50}$ was calculated for each sample using the method of Reed and Muench (Reed and Muench, 1938).

2.2. Pathologic examination of lungs

At necropsy, lungs were removed and evaluated for the percentage of the lung affected with purple-red consolidation typical of SIV infection. The percentage of the surface affected with pneumonia was visually estimated for each lung lobe, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume (Halbur et al., 1995). Tissue samples from the trachea and right cardiac lung lobe and other affected lobes were taken and fixed in 10% buffered formalin for histopathologic examination. Tissues were routinely processed and stained with hematoxylin and eosin. Lung sections were given a score from 0–3 to reflect the severity of bronchial epithelial injury based on previously described methods (Richt et al., 2003).
lung sections were scored according to the following criteria: (0.0) no significant lesions; (1.0) a few airways affected with bronchiolar epithelial damage and light peribronchiolar lymphocytic cuffing often accompanied by mild focal interstitial pneumonia; (1.5) more than a few airways affected (up to 25%) often with mild focal interstitial pneumonia; (2.0) 50% airways affected often with interstitial pneumonia; (2.5) approximately 75% airways affected, usually with significant interstitial pneumonia; (3.0) greater than 75% airways affected, usually with interstitial pneumonia. A single pathologist scored all slides and was blinded to the treatment groups.

2.3. Serologic assays

Serum samples were collected by jugular venipuncture prior to challenge and at approximately 14, 21 and 35 dpi. For use in the HI assay, sera were heat inactivated at 56 °C for 30 min, then treated to remove non-specific hemagglutinin inhibitors and natural serum agglutinins with a 20% suspension of Kaolin (Sigma–Aldrich, St. Louis, MO) and adsorption with 0.5% turkey red blood cells (RBC), respectively. The HI assays were done with the OH07 virus as antigen and turkey RBC using standard techniques (Palmer et al., 1975). In addition to homologous HI assays, the OH07 virus was evaluated against a panel of previously described H1 antisera (Vincent et al., 2006). Reciprocal HI titers were log2 transformed for analysis and reported as geometric means. The HI cross-reactions with the H1 reference strain (Caton et al., 1975). Putative antigenic sites in the OH07 HA were identified by alignment with the PR8 H1 reference strain (Caton et al., 1982), and putative receptor binding and host determinant sites were identified as previously described (Matrosovich et al., 1997).

2.4. Sequence analysis

Sequencing of the viruses isolated from the Ohio outbreak will be reported elsewhere (S. Swenson, accession numbers: EU604689-EU604696). The gene sequences from the OH07 isolate used in this in vivo study were analyzed using SeqMan (DNASTar, Inc., Madison, WI). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007).

3. Results

3.1. OH07 virus inoculated pigs

All pigs were seronegative for specific antibodies to SIV by the hemagglutination inhibition (HI) assay and PRRSV by ELISA prior to the start of the study. Pigs inoculated with OH07 virus became clinically ill, demonstrating lethargy, anorexia and dyspnea. Necropsy revealed severe macroscopic lung lesions typical of SIV (purple-red colored, consolidated areas) in inoculated pigs but none in control pigs. Macroscopic lung lesions averaged 23.5% on 3 days post infection (dpi), 25.1% on 5 dpi, and 21.0% on 7 dpi. Microscopic lung lesion scores (0–3) averaged 2.4 on 3 dpi, 2.4 on 5 dpi, and 2.3 on 7 dpi, reflecting approximately 75% of the airways in each section being affected by necrotizing bronchiolitis in combination with significant interstitial pneumonia. Control pigs showed no macroscopic or microscopic lung changes.

Virus titers in BALF averaged $10^{6.4}$ TCID<sub>50</sub>/ml on 3 dpi, $10^{5.0}$ TCID<sub>50</sub>/ml on 5 dpi, and were negative by 7 dpi in the OH07 inoculated group. In the OH07 inoculated group, 85% of the pigs were shedding virus in nasal swab samples on 3 dpi, with an average titer of $10^{2.9}$ TCID<sub>50</sub>/ml. On 5 dpi, 100% of nasal swabs were positive with an average titer of $10^{2.4}$ TCID<sub>50</sub>/ml. All of the nasal swab samples from OH07 inoculated pigs were negative on 7 dpi by virus isolation in tissue culture. The 5 remaining OH07 inoculated pigs were seropositive at 14 dpi, with reciprocal HI titers ranging from 40 to 80 and a geometric mean reciprocal titer of 78. Nasal shedding and seroconversion are summarized in Table 1.

3.2. Transmission to contact pigs

The OH07 virus was efficiently transmitted from the primary challenge pigs to all contact pigs and is summarized in Table 1. Contact pigs also demonstrated clinical illness similar to the primary challenge group, although somewhat more sporadic. In the contact group, 100% of the pigs were positive by nasal swab samples on 3 dpc, with an average of $10^{3.8}$ TCID<sub>50</sub>/ml. On 5 dpc, 100% of the pigs were shedding virus with an average of $10^{3.8}$ TCID<sub>50</sub>/ml. In contrast to the OH07 inoculated pigs, 90% of the contact pigs continued to shed virus on 7 dpc, with an average titer of $10^{2.0}$ TCID<sub>50</sub>/ml. All of the contact pigs were seropositive by 12 dpc, with reciprocal HI titers ranging from 40 to 160 and a geometric mean reciprocal titer of 102.

### Table 1

<table>
<thead>
<tr>
<th>Nasal shedding&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Seroconversion</th>
<th>Day 12/14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>Day 5</td>
<td>Day 7</td>
</tr>
<tr>
<td>17/20 (2.9 ± 0.4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15/15 (2.4 ± 0.3)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>10/10 (2.9 ± 0.3)</td>
<td>10/10 (3.8 ± 0.3)</td>
<td>9/10 (2.0 ± 0.4)</td>
</tr>
<tr>
<td>Contact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10/10 (2.9 ± 0.3)</td>
<td>10/10 (3.8 ± 0.3)</td>
<td>9/10 (2.0 ± 0.4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number positive out of total number on Days 3, 5, 7, 12 or 14 post infection or post contact.

<sup>b</sup> Numbers in parentheses are the log<sub>10</sub> geometric mean nasal swab TCID<sub>50</sub>/ml titer or the geometric mean reciprocal HI titer.
3.3. Antigenic cross-reactivity with H1 SIV anti-sera

The fold-decrease between the geometric mean titer of homologous virus/anti-serum and the geometric mean titer of OH07 virus/heterologous anti-serum was calculated with $\leq 4$-fold decrease being cross-reactive; between 4 and 8-fold reduction being moderately cross-reactive; and $>8$-fold reduction being a considerable loss in cross-reactivity. The OH07 virus was weakly-to-moderately cross-reactive with H1 SIV anti-sera from the same genetic cluster (defined in Section 3.4 below), and exhibited significant loss in cross-reactivity with most of the other H1 SIV anti-sera in our panel (Table 2). The highest antigenic cross-reactivity was with anti-serum from A/Swine/Kansas/00246/2004 H1N2 (KS04).

### Table 2
Serologic cross-reactivity of OH07 with H1 reference anti-sera.

<table>
<thead>
<tr>
<th>Anti-sera</th>
<th>H1α cluster</th>
<th>H1β cluster</th>
<th>H1γ cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA30</td>
<td>90$^a$</td>
<td>NC02</td>
<td>MN03</td>
</tr>
<tr>
<td>WI68</td>
<td>6</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>IA73</td>
<td>23</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>MN99</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>5120/57$^b$</td>
<td>1280/57</td>
<td>1810/320</td>
<td>640/57</td>
</tr>
</tbody>
</table>

$^a$ Fold-decrease as compared to homologous virus/anti-sera geometric mean titer.
$^b$ Geometric mean titers for homologous antigen and antisera/OH07 antigen with heterologous antisera.

3.4. Genetic analysis of the OH07 isolate

Hemagglutinin genes of H1 SIV isolates from the US cluster into three distinct phylogenetic groups, labeled here as H1α, H1β, and H1γ (Fig. 1A). The HA gene of the OH07 isolate was shown to be phylogenetically related to the H1γ cluster (H1N2-like, IN00 reference strain) of contemporary H1 SIV (Fig. 1A). The HA genes ranged from 96.4 to 97.2% identity at the nucleotide level and 95.9 to 97.9% identity at the amino acid level to the H1γ reference viruses shown to have moderate cross-reactivity with the OH07 virus in the H1 serologic panel. The HA gene was also evaluated for amino acid changes at putative antigenic and receptor binding sites (Caton et al., 1982; Matrosovich et al., 1997). No amino acid changes were identified at the putative receptor binding site D225 (H3 numbering) compared to contemporary swine H1 reference viruses.
however changes were identified at putative antigenic determinant sites (Table 3). This is consistent with the reduction in serologic cross-reactivity shown in Table 2. Unique changes at antigenic determinant sites were identified in the OH07 HA at positions 71 and 162 and may play a role in the loss of cross-reactivity. The NA gene was shown to be related to the swine N1 phylogenetic cluster (Fig. 1B). The internal genes (Fig. 2A–F) were shown to be of the triple reassortant SIV lineage and group with those of the cluster IV H3N2 viruses reported by Olsen, et al. (Olsen et al., 2006). The PB2 gene was determined to contain the conserved avian amino acid residue glutamic acid at position 627, reported to be important in avian and human host specificity (Subbarao et al., 1993).

4. Discussion

The OH07 isolate was evaluated in an in vivo challenge and transmission model. Our results indicate that the OH07 virus was pathogenic in pigs, was transmissible among pigs, and exhibited significant loss in cross-reactivity with many swine H1 reference anti-sera. The OH07 virus induced macroscopic and microscopic lesions that are indistinguishable from that induced by contemporary SIV; however, the clinical signs and lesions were increased in severity as compared to many of the previously evaluated U.S. H1 SIV (Vincent et al., 2006). Virus was transmitted from inoculated pigs to contact pigs as demonstrated by nasal swab virus isolation and seroconversion. Contact pigs that were infected via natural transmission routes shed virus longer than the intratracheally inoculated pigs. All contact pigs began to shed substantial amounts of virus as early as 3 dpc, and 9 out of 10 continued to shed for as long as 7 dpc. Nasal swabs were not taken after Day 7 post contact, so an endpoint was not reached for nasal shedding in the contact pigs.

The limited cross-reactivity between OH07 and reference H1 anti-sera from viruses in the same genetic cluster suggests antigenic drift that may lead to a lack of herd immunity in the swine population. Amino acid changes were demonstrated in putative antigenic determinant sites consistent with the loss in cross-reactivity (Caton et al., 1982), especially those at positions 71 and 162. Viruses with HA genes similar to the OH07 virus have continued to be identified in the pig population (M. R. Gramer, R. A. Hesse, personal communications), and these viruses tend to be associated with severe respiratory disease outbreaks in young to mature pigs. To our knowledge, no further reports of human illness associated with these H1 SIVs have been made.

In the 2007 outbreak at the Ohio county fair, two-thirds of the approximately 235 market size pigs being housed in a single barn at the county fairgrounds became clinically affected. The genetic analysis of the OH07 isolate

### Table 3

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino Acid Position</th>
<th>71</th>
<th>73</th>
<th>74</th>
<th>142</th>
<th>156</th>
<th>162</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/SW/OH/511445/07 (OH07)</td>
<td>S</td>
<td>A</td>
<td>S</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>A/SW/MN/1192/01 (MN01)</td>
<td>F</td>
<td>T</td>
<td>S</td>
<td>S</td>
<td>N</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>A/SW/MN/00194/03 (MN03)</td>
<td>F</td>
<td>A</td>
<td>S</td>
<td>N</td>
<td>N</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>A/SW/KS/00246/04 (KS04)</td>
<td>F</td>
<td>A</td>
<td>R</td>
<td>K</td>
<td>D</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

a Swine H1 numbering of mature polypeptide.
demonstrated that the 8 gene segments were similar to that of contemporary circulating SIV. However, numerous nucleotide changes leading to amino acid changes were demonstrated in the HA gene and throughout the genome (data not shown) when compared to viruses in the same HA genetic cluster. It remains unknown if any of the amino acid changes were related to the ability of this virus to infect people. The amount and duration of nasal shedding demonstrated in our study would suggest that the exposure of the people caring for the pigs at the fair could have been quite high. Approximately 26 people in close association with the fair pigs were affected by an influenza-like illness. Viruses from at least two individuals were isolated, sequenced and analyzed at the Centers for Disease Control and determined to be nearly identical to the swine virus studied here (A. Klimov, personal communication). It is unknown if the people that became ill and/or were positive for SIV had pre-existing immunity to contemporary SIV.

A number of determinants have been proposed for barriers against interspecies transmission of influenza A viruses (reviewed in (Landolt and Olsen, 2007)). The binding of the HA protein to the host receptor has been demonstrated to be a major barrier. A number of putative sites important for receptor recognition have been described, with two amino acid residues having a fundamental role in avian and human species specificity of H1 viruses (E190 and G225 in avian viruses and D190 and D225 in human viruses, H3 numbering) (Matrosovich et al., 2000), however D190 and D225 are conserved between human and swine H1 isolates. As predicted, these positions were demonstrated to be D190 and D225 in the HA of OH07 rH1N1 isolate. Other amino acid sites in the H1 HA thought to play a role in differential recognition of avian or human receptor types (positions 77, 138, 194, 226, and 228) (Matrosovich et al., 1997) were conserved between the OH07 isolate and the recent classical and reassortant H1 SIV evaluated. Since the HA determinants of receptor recognition are common between swine and human H1 viruses and the amino acids are conserved among recent swine H1 viruses, HA sequence analysis alone fails to explain why this particular swine isolate crossed into humans whereas other H1 SIV have been apparently less successful in human transmission. The PB2 gene has also been associated with interspecies transmission barriers for avian viruses to infect people, especially the amino acid mutation E627K (Subbarao et al., 1993). Since the PB2 in the contemporary swine influenza viruses is avian in origin, it was of interest to evaluate the PB2 at this amino acid position; however the avian type (E627) was present in the swine OH07 PB2 gene. It appears that
E627 is not restrictive for avian PB2 genes to be maintained in the swine triple reassortant virus lineage. The NP and M genes in their entirety have been demonstrated to confer species specificity (Scholtissek et al., 1985; Scholtissek et al., 2002); however, the NP, M, and NS genes in the OH07 virus are of swine virus lineage. The internal genes demonstrated genetic variability among contemporary swine isolates, but it is unknown if a specific mutation or series of mutations contributed to the pig to human transmission of OH07. The OH07 rH1N1 virus was divergent from the swine rH1N1 isolated from a swine farmer in Iowa, A/Iowa/CEID23/2005 (Gray et al., 2007). The HA gene had 91.5% identity and NA had 91.8% identity. The internal genes were less variable, but ranged from 95.7% to 98% identity. Taken together, there is no evidence for molecular changes that would enhance transmission of the OH07 SIV to humans. Based on observations in our experimental setting, the OH07 virus was efficiently transmitted to contact pigs and subsequently shed at high titers. In addition, the antigenic drift suggests that previous exposure to H1 SIV or vaccine may not provide adequate protection against the OH07-like viruses in the pig population. This would indicate that a large group of naïve swine such as in the Ohio county fair setting could have produced a considerable exposure dose to the human exhibitors in close contact with the pigs.

There have been numerous but sporadic accounts of pig to human transmission confirmed by isolation of SIV including those of triple reassortant swine H3N2 lineage (Olsen et al., 2006); however, all have seemingly failed to become adapted to the human host (reviewed in (Myers et al., 2007; Van Reeth, 2007)). Additionally, the presence of antibodies against SIV in human populations is highly correlated with occupational exposure to swine, such as pig farmers and veterinarians (Myers et al., 2006; Olsen...
et al., 2000). The pig has been suggested to be a "mixing vessel" for influenza viruses with pandemic potential due to the presence of both avian and mammalian receptors located on the epithelial cells of the respiratory tract (Ito et al., 1998). It is apparent that pigs may be infected at least transiently with wholly avian and/or human viruses, but long enough for reassortment to occur, allowing swine viruses to acquire avian and/or human virus gene segments (Karasin et al., 2006; Karasin et al., 2004; Olsen et al., 2003; Olsen et al., 2006; Yu et al., 2007; Zhou et al., 1999). Further supporting the occurrence of such events is our recent identification of a swine and avian reassortant H2N3 virus (Ma et al., 2007), an HA subtype not seen in the human population since the 1957 H2N2 pandemic virus disappeared in 1968. The TRIG cassette from the swine triple reassortant viruses found in North America seems to have lent contemporary SIV the ability to acquire new HA and NA genes as well as an increased rate of antigenic drift. This was not previously observed with classical swine H1N1 viruses. The potential for a successful swine to human jump of contemporary SIV is unknown, but the rapid rate at which SIVs in North America are evolving and emerging potentially increases the risk. The characteristics of the OH07 virus as well as the documented pig-to-human transmission warrant close monitoring of the spread of this virus in pig and human populations. This report underscores the need for vigilance in examining influenza A viruses from swine (and other species) for human potential in addition to the major focus currently placed on avian influenza viruses.

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