A highly pathogenic avian-derived influenza virus H5N1 with 2009 pandemic H1N1 internal genes demonstrates increased replication and transmission in pigs

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Abstract

This study investigated the pathogenicity and transmissibility of a reverse-genetics-derived highly pathogenic avian influenza (HPAI) H5N1 lineage influenza A virus that was isolated from a human, A/Iraq/755/06. We also examined surface gene reassortant viruses composed of the haemagglutinin and neuraminidase from A/Iraq/755/06 and the internal genes of a 2009 pandemic H1N1 virus, A/New York/18/2009 (2Iraq/06 : 6NY/09 H5N1), and haemagglutinin and neuraminidase from A/New York/18/2009 with the internal genes of A/Iraq/755/06 (2NY/09 : 6Iraq/06 H1N1). The parental A/Iraq/755/06 caused little to no lesions in swine, limited virus replication was observed in the upper respiratory and lower respiratory tracts and transmission was detected in 3/5 direct-contact pigs based on seroconversion, detection of viral RNA or virus isolation. In contrast, the 2Iraq/06 : 6NY/09 H5N1 reassortant caused mild lung lesions, demonstrated sustained virus replication in the upper and lower respiratory tracts and transmitted to all contacts (5/5). The 2NY/09 : 6Iraq/06 H1N1 reassortant also caused mild lung lesions, there was evidence of virus replication in the upper respiratory and lower respiratory tracts and transmission was detected in all contacts (5/5). These studies indicate that an HPAI-derived H5N1 reassortant with pandemic internal genes may be more successful in sustaining infection in swine and that HPAI-derived internal genes were marginally compatible with pandemic 2009 H1N1 surface genes. Comprehensive surveillance in swine is critical to identify a possible emerging HPAI reassortant in all regions with HPAI in wild birds and poultry and H1N1pdm09 in pigs or other susceptible hosts.

INTRODUCTION

Since its identification in China and subsequent spread through Europe, Asia and Africa, the Eurasian lineage of highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype has remained largely restricted to avian species, while causing severe outbreaks in poultry [1, 2]. Occasional transmission of H5N1 viruses to humans has been reported, and despite a lack of efficient human-to-human transmission [3, 4], H5N1 viruses are an important global health concern due to the high case fatality rate observed in humans (~60%) [5]. A lineage of HPAI H5N1 emerged outside of Asia around 2005, called the Euro-African lineage, and was subsequently associated with a number of human cases, including those in Iraq and Egypt [6]. Due to the high number of human cases associated with the Middle Eastern HPAI H5N1 viruses, this lineage was selected to conduct the risk assessment for mammalian adaptation described here. More recently, another genetic clade of HPAI H5 was detected in North America that included H5N2, H5N8 and H5N1 subtypes [7–9]. The viruses were detected in wild bird, backyard poultry and commercial chicken and turkey flocks, with the H5N2 subtype responsible for many of the commercial poultry outbreaks in the Midwest.

A key step in infection is viral entry into the host cell but also requires an adequate constellation of internal genes that allow for robust replication in the host and the ability to sustain host-to-host transmission. Terminal sialic acids on cell surface glycoproteins and glycolipids are an important factor in host cell binding, and influenza A virus (IAV) differentially bind to α(2,3)-linked and α(2,6)-linked sialic acids.
acids, although a recent study suggests that the glycan backbone, modifications and valency of sialic acids, in addition to linkage, are important [10]. The haemagglutinin (HA) of human viruses preferentially binds to α(2,6)-linked sialic acids, and avian viruses preferentially bind to α(2,3)-linked sialic acids [11–13]. Pigs express both types of sialic acids, and there is abundant and dominant expression of α(2,6)-linked sialic acids in trachea and bronchi and the presence of both α(2,3)-linked and α(2,6)-linked sialic acids in bronchioles and alveoli [12, 14–23]. Substitutions at multiple positions within the HA have been reported to either alter receptor-binding specificity or increase HA stability of HPAI H5 HA and confer increased transmission by respiratory droplets in ferrets [24–27] and guinea pigs [28], but it is unknown if these changes have the same effect in pigs.

Pigs have susceptibility to some avian and human influenza virus lineages, leading to concern that pigs could potentially serve as an intermediary for the emergence of a reassorted HPAI-derived virus adapted to a mammalian host, posing an important global health concern that must be carefully monitored. Additionally, the emergence of a novel virus strain in pigs can have a detrimental effect on the swine industry due to production losses in infected animals and/ or loss of consumer confidence and potential trade restrictions. Serological assays suggest sporadic natural infections of avian H5N1 viruses in pigs, with limited pig-to-pig transmission [29–32], and experimental infection studies determined limited susceptibility to HPAI H5N1 [33, 34] and low-pathogenic avian H5 viruses in pigs [35, 36]. In contrast, the H1N1pdm09 transmits efficiently and causes disease in pigs. H1N1pdm09 produced similar clinical symptoms as other swine influenza viruses in both natural and experimental infections [32, 37–45], and additional human-to-swine transmission events continue to be reported [46]. By 2014, H1N1pdm09 had been detected in swine from 37 countries due to multiple spillover events [47, 48], and anecdotal evidence suggests widespread distribution [49]. Furthermore, the 2009 H1N1 pandemic-origin internal genes in combination with enzootic IAV in swine have been detected more frequently [50, 51], suggesting that the pdm09 internal genes are well adapted to swine and are widely incorporated into reassorted endemic swine viruses in many geographic locations.

Since swine may be susceptible to some lineages of H5 HPAI, the potential for a novel reassortant to emerge from swine that could be a public health concern warrants further investigation. We observed a relative increase in infectivity and transmission in swine of an HPAI-derived H5N1 following incorporation of H1N1pdm09 internal genes. Although we evaluated a different neuraminidase (NA) subtype and a different genetic H5 lineage compared to the current North American H5N2 lineage (Fig. 1), reassortment may be a potential scenario in the USA as well as many countries where HPAI H5 viruses circulate in wild birds or poultry and viruses with H1N1pdm09 internal genes circulate in swine. Infection and transmission studies in pigs were performed with parental virus strains HPAI-derived A/Iraq/755/06 H5N1 and A/New York/18/2009 H1N1pdm09 and 2:6 reassortants, all with wild-type gene segments, to determine the effects of swapped HA and NA genes paired with the internal gene constellations of the two selected parental virus strains.

**RESULTS**

**The HA of A/Iraq/755/06 belongs to the 2.2 HPAI lineage**

The HA sequence of A/Iraq/755/06, along with representative H5 HA sequences, was aligned, and a phylogenetic analysis was performed (Fig. 1). The HPAI-derived HA sequences isolated from humans in 2006 were highly similar and were within the monophyletic clade 2.2. Within the HA1 domain, Iraq/06 shares relatively low amino acid homology with recently detected North American 2.3.4.4 HPAI, ranging from 87.6 to 87.9 % (Table 1).

Molecular markers in the HA1 domain known to be associated with adaptation to mammals for increased transmissibility [24–28, 52, 53] were compared among wild-type Iraq/06, low-pathogenic H5N2 isolated from pigs in Korea and recent HPAI H5 isolated in North America (Table 1). The low-pathogenic H5N2 strains were selected for sequence comparison because both strains were isolated from pigs and one strain was capable of transmitting in swine [54], and the North American HPAI strains were selected as representatives of clade 2.3.4.4. Iraq/06 does not encode amino acids previously associated with conferring increased transmissibility with the exception of the absence of a glycosylation site at position 158.

**2Iraq/06 : 6NY/09 H5N1 transmitted more efficiently than Iraq/06 H5N1 to direct-contact pigs**

NY/09 H1N1 and other H1N1pdm09 challenge studies are well-described in pigs [32, 37–45]; thus, NY/09 H1N1 was used as a control to compare with the parental HPAI-derived H5N1 and two reassortant viruses. Because virus levels were often near the limit of detection for Iraq/06 and the two reassortant viruses, two passages of virus isolation were performed with parental virus strains HPAI-derived A/Iraq/755/06 H5N1 and A/New York/18/2009 H1N1pdm09 and 2:6 reassortants, all with wild-type gene segments, to determine the effects of swapped HA and NA genes paired with the internal gene constellations of the two selected parental virus strains.
In pigs challenged with H5N1 Iraq/06, infectious virus was successfully isolated from the nasal secretions in 15/15 of the principal pigs through 3 days p.i. (Fig. 2a). The viral RNA copies in nasal swab samples in the principal pigs peaked at 1 day p.i. and subsequently declined. Intermediate replication was observed with the 2:6 reassortant virus that encoded Iraq/06 H5N1 surface genes and NY/09 internal genes (2Iraq/06:6NY/09 H5N1). Infectious virus was successfully isolated from the nasal secretions in all H5N1 2Iraq/06:6NY/09 principal pigs at 3 and 5 days p.i. and in 3/5 pigs at 7 days p.i. The viral RNA in nasal swab samples in the principal pigs displayed delayed kinetics compared to other inoculated pigs and peaked at 3 days p.i., followed by declining levels at 5 and 7 days p.i.

Direct-contact pigs were introduced at 2 days p.i. to evaluate transmission, and nasal swabs were collected to test for virus isolation and viral RNA in nasal secretions (Fig. 2b). Virus was isolated from all contact pigs exposed to NY/09-
Table 1. Molecular analysis and comparison of amino acids associated with adaptation to mammals and the cleavage site of representative H5 HA sequences

<table>
<thead>
<tr>
<th>Iraq/06*</th>
<th>C12/HA-Korea/08* (91.1%)*</th>
<th>C13/HA-Korea/08* (90.5%)*</th>
<th>North American HPAI* (87.6–87.9%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA†</td>
<td>H110Y</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>N158D</td>
<td>D</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>T160A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>K193R‡</td>
<td>R</td>
<td>K</td>
<td>K</td>
</tr>
<tr>
<td>Q196R‡</td>
<td>Q</td>
<td>Q</td>
<td>Q</td>
</tr>
<tr>
<td>N224K§</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Q226L§</td>
<td>Q</td>
<td>Q</td>
<td>Q</td>
</tr>
<tr>
<td>G228S§</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>T318I</td>
<td>T</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Cleavage</td>
<td>GERRRKR</td>
<td>RE-RE-</td>
<td>RERRR-KR</td>
</tr>
</tbody>
</table>


† HA1 amino acid homology compared to Iraq/06.
‡ H3 numbering [101]; H110Y denotes that a histidine at position 110 was substituted with a tyrosine to acquire a new characteristic that benefits the HA in mammals, and so forth for all the examples.
§ Substitutions associated with receptor-binding specificity.

challenged pigs through 7 days post-contact (dpc), and viral RNA copies in the nasal epithelium reached similar levels compared to the primary challenged pigs. In contact pigs from the 2NY/09:6Iraq/06 H1N1-challenge group, virus was isolated from only 2/5 pigs, and viral RNA was below the limit of quantification with the RT-PCR assay.

Virus was isolated from 3/5 pigs after two passages, but viral RNA was below the limit of quantification in contact pigs in the Iraq/06 H5N1 group (Fig. 2b). In the 2Iraq/06:6NY/09 H5N1-contact group, virus was isolated after two passages from nasal swabs in 5/5 contact pigs at 1 dpc, although only low levels of viral RNA were quantified by RT-PCR in the nasal swab samples of contact pigs at 5 and 7 dpc.

**Lung pathology was minimal in all virus challenge groups**

Overt clinical illness was not observed in pigs in any groups. Necropsy was performed on principal pigs at 3 and 5 days p.i. to assess signs of disease in the lung and collect bronchoalveolar lavage fluid (BALF) to perform virus isolation and test for viral RNA. The pathology of NY/09 H1N1-infected pigs presented with modest macroscopic and microscopic scores (Table 2) and similar to 2009 pandemic H1N1 experimental intranasal challenge results previously reported. Higher macroscopic pneumonia percentages were observed in 2NY/09:6Iraq/06 H1N1-challenged pigs at 5 days p.i. compared to HPAI H5N1 Iraq/06-challenged pigs, but less than NY/09-challenged pigs. Pneumonia percentages in the H5N1 Iraq/06-infected pigs were low, and macropathic pneumonia at 5 days p.i. was not significantly higher than the negative control pigs. The percentage of pneumonia in the 2Iraq/06:6NY/09 H5N1-challenged pigs was also minimal.

Microscopic lung lesions in all challenged pigs were characterized by minimal to moderate amounts of inflammation adjacent to and within affected bronchi and/or bronchioles and arranged in a lobular pattern typical of IAV infection in swine (Table 2). At 3 days p.i., NY/09-challenged pigs demonstrated more severe but moderate necrotizing bronchiolitis compared to the other three groups. In contrast, Iraq/06 H5N1-, 2Iraq/06:6NY/09 H5N1- and 2NY/09:6Iraq/06 H1N1-challenged pigs demonstrated similar but minimal microscopic lesions with few affected lobules, mild bronchi and bronchial epithelial attenuation and low numbers of neutrophils in upper airways and adjoining alveoli. Airway epithelium ranged from mild attenuation to necrosis with moderate numbers of neutrophils observed in multifocal bronchi, bronchioles and adjacent alveoli. Alveolar septa were mildly infiltrated with low numbers of macrophages and lymphocytes.

At 5 days p.i., microscopic lesions were most severe in NY/09-challenged pigs and consistent with experimental infection with pandemic H1N1. Multifocal bronchi and bronchioles demonstrated affected epithelium that ranged from necrotic to proliferative. Low numbers of sloughed epithelial cells were admixed with neutrophils in bronchiolar lumens, and mild to moderate lymphocytic cuffs were apparent around affected bronchi and bronchioles. Alveolar septa contained mild to moderate numbers of macrophages and lymphocytes in affected lobules. There was a mild increase in the number of affected lung lobules in the 2NY/09:6Iraq/06 H1N1-challenged group. Multifocal bronchi and bronchioles demonstrated necrotizing bronchiolitis, and low numbers of neutrophils and sloughed epithelial cells were apparent in the lumen of affected airways. Multifocal peribronchial infiltrates of lymphocytes were also mild. Microscopic lung lesions in Iraq/06 and 2Iraq/06:6NY/09 H5N1 groups remained minimal at 5 days p.i. and comparable to lesions observed at 3 days p.i. Few affected bronchioles demonstrated mild epithelial attenuation, low numbers of neutrophils and sloughed epithelial cells.

2Iraq/06:6NY/09 H5N1 replicated more efficiently than Iraq/06 H5N1 in the lungs

Consistent with replication in the upper respiratory tract, virus was isolated at 3 and 5 days p.i. from all NY/09-challenged pigs, and high levels of viral RNA were detected in BALF samples (Fig. 3). Virus was also isolated from BALF after two passages at 3 and 5 days p.i. (5/5) in all 2NY/09:6Iraq/06 H1N1-challenged pigs, but the viral RNA.
Fig. 2. Quantitative RT-PCR of viral RNA in nasal secretions. Average viral RNA copy numbers (matrix gene) detected in nasal secretions collected from (a) principal and (b) contact pigs intranasally inoculated with NY/09, Iraq/06, 2NY/09:6Iraq/06 H1N1 and 2Iraq/06:6NY/09 H5N1. Contact pigs were placed in the same pen with each group at 2 days p.i. The numbers in parentheses indicate the number of pigs positive by two passages of virus isolation in Madin–Darby canine kidney London cells and immunocytochemistry staining with monoclonal influenza A nucleoprotein (NP)-specific antibody. Letters a–d indicate significant difference (P<0.05) between groups within the same days p.i.
Table 2. Percent pneumonia and microscopic scores at 3 and 5 days p.i.

<table>
<thead>
<tr>
<th>Group</th>
<th>3 days p.i.</th>
<th>5 days p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung lesion (%)</td>
<td>Microscopic scores (0–22)</td>
</tr>
<tr>
<td>Negative control</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>NY/09 (H1N1)</td>
<td>3.1±0.9†</td>
<td>4.1±0.6*</td>
</tr>
<tr>
<td>2NY/06:6NY/09 (H1N1)</td>
<td>1.9±0.9*</td>
<td>0.2±0.2*</td>
</tr>
<tr>
<td>Iraq/06 (H5N1)</td>
<td>0.8±0.4*</td>
<td>0.1±0.1*</td>
</tr>
<tr>
<td>2Iraq/06:6NY/09 (H5N1)</td>
<td>0.3±0.1*</td>
<td>0.2±0.2*</td>
</tr>
</tbody>
</table>

*a: not determined.
†The letters a–c indicate significant differences (*P<0.05) compared between groups.

quantities were significantly lower when compared to NY/09-challenged pigs. Virus was isolated at 3 and 5 days p.i. in 3/5 pigs challenged with Iraq/06, although viral RNA was below quantification limits by RT-PCR. In 2Iraq/06:6NY/09 H5N1-challenged pigs, virus was isolated in 5/5 pigs at 3 and 5 days p.i., and while viral RNA levels were low at 3 days p.i., there were significantly higher copy numbers at 5 days p.i. when compared to the Iraq/06-challenged group.

**2Iraq/06:6NY/09 H5N1 had increased infectivity and transmission compared to Iraq/06 H5N1 as measured by seroconversion**

Seroconversion as measured by antibody detection against the nucleoprotein (NP) and the HA [measured by haemagglutination inhibition (HI) titres] was evaluated as another parameter to indicate exposure in primary pigs and transmission in contact pigs. NP ELISA and homologous HI results at both time points were not identical within individual pigs, so positivity on either was used as seropositive criteria. All five principal pigs challenged with NY/09 were NP and HI positive at 12 and 21 days p.i., as were the contact pigs at the same time points (Fig. 4). 2NY/09:6Iraq/06 H1N1-challenged pigs were also NP positive and HI positive at 12 and 21 days p.i., and many contact pigs were positive at 21 dpc, although antibodies detected by HI appeared to be waning.

At 12 days p.i., 4/5 pigs challenged with Iraq/06 were NP and HI antibody positive, and at 21 days p.i., 3/5 were NP and HI antibody positive (Fig. 4). In contrast, contact pigs within the Iraq/06-challenge group were variably seropositive (3/5) at 12 dpc, and only 1/5 pigs were positive at 21 dpc. At 12 days p.i., 4/5 2Iraq/06:6NY/09-challenged pigs were seropositive, and at 21 days p.i., all pigs were positive by NP or HI (5/5 and 3/5, respectively). Contact pigs for the 2Iraq/06:6NY/09-challenged pigs were also all positive at 12 dpc for NP or HI antibodies (5/5 and 4/5, respectively), as well as at 21 dpc (3/5 and 5/5, respectively).

**DISCUSSION**

HPAI H5N1 continues to be a public health concern due to high case mortality rates, although to date, an H5N1 with human-to-human transmission and greater pandemic potential has yet to emerge. However, the continued detection of HPAI in poultry and humans in Asia, the Middle East and North Africa, as well as the recent spread of an Asian HPAI H5 lineage into wild birds and subsequently into domestic poultry in Europe and North America, underscores the need for ever-present vigilance in monitoring IAV in wild bird and domestic animal populations. An important mechanism of influenza evolution is the reassortment of gene segments between different viruses to generate a new virus that may replicate more robustly and/or escape the host immune response more efficiently.

Reverse-genetics-derived Iraq/06 and NY/09 2:6 reassortants with wild-type gene segments were examined to test if infection and transmissibility properties were conferred by the acquisition of internal genes by either pair of surface genes. Both reassortant viruses displayed intermediate properties of infection and transmission in the spectrum between the wild-type parental H1N1 and H5N1 viruses, with 2Iraq/06:6NY/09 H5N1 acquiring increased infectivity and transmissibility relative to Iraq/06 H5N1. This would suggest that low levels of onward transmission of an HPAI H5 virus that incorporated mammalian adapted internal genes could occur, potentially allowing the virus to evolve in the new host to acquire adaptive mutations. Despite some of the infection and transmissibility properties of the H1N1pdm09 virus being partially conferred to the reassortant viruses, both reassortants were significantly less fit than the NY/09 wild-type virus, indicating that other gene segments and/or adaptive mutations are required to be comparable to the parental NY/09 in the pig. Consistent with our results, a low-pathogenic H5N2 reassortant isolated from a pig in Korea (C13/Korea/08) that was evaluated in a pig challenge study transmitted to naive pigs, in contrast to a highly similar H5N2 with only avian-lineage genes [54].
reassortant H5N2 contained gene segments of swine lineage (PB2, PA, NP, M) and avian lineage (HA, NA, PB1, NS), and no amino acid differences were observed in virus recovered from challenged pigs or from contact pigs. Similarly, in this study, the reassortant virus 2Iraq/06 : 6NY/09 H5N1 demonstrated modest replication and transmissibility advantages over the parental H5N1.

The ancestor of the currently circulating HPAI lineage, A/goose/Guandong/96, caused an outbreak in China in 1996 and has not only been sustained in avian hosts but has also continued to genetically diversify and evolve [2, 55–61]. Additionally, distinct sublineages have spread throughout Asia, Europe, Africa, the Middle East and most recently North America [2, 62–67]. The maintenance of the HPAI H5 in North America remains to be determined [68, 69], although HPAI continues to circulate worldwide. Recent studies have described multiple incursions of human IAV strains into the swine population [48, 70–72], and genes from the H1N1pdm09 are frequently detected in various combinations in swine populations worldwide [48, 50, 73–75]. If an avian virus spilled over into swine, even transiently, there would be a diverse gene pool with which to reassort and evolve, increasing the chances that a successful virus may emerge.

Avian H5 virions (low-pathogenic H5N9 and highly pathogenic H5N1) were capable of moderately attaching to alveolar cells, but not to trachea, bronchus or bronchiole tissue in virus histochemistry assays with swine explants [76]. In another study, H5N1 infection of swine explants with histochemical analysis found consistent evidence of replication in pig lung explant but not the trachea [20]. This suggests that limitations are likely for an H5 virus to initiate infection in the upper respiratory tract when exposed by the intranasal route. Species tropism is likely due to the combination of internal and surface genes, with PB2 having been demonstrated to be particularly important to species adaption among the replicase genes [52, 77]. The Iraq/06 HA does not encode any of the amino acids associated with α(2,6)-linked sialic acid binding that have been previously identified in ferret and guinea pig transmission studies (Table 1). The Iraq/06 HA lacks a glycosylation site at position 158 and additionally encodes an arginine at position 193, and these two factors have been shown to confer binding to α(2, 6)-linked sialic acids by in vitro glycan assays [53], although

![Fig. 3. Quantitative RT-PCR of viral RNA in lungs. Average viral RNA copy numbers (matrix gene) detected in BALF at 3 and 5 days p.i. in principal pigs. The numbers in parentheses indicate the number of pigs positive by two passages of virus isolation in Madin–Darby kidney L cells and immunocytochemistry staining with monoclonal influenza A NP-specific antibody. Letters a–c indicate significant difference (P<0.05) compared between groups within the same days p.i.](image)
its significance has not been shown in the swine host. Further studies are required to better understand the molecular mechanism of the establishment of infection with delayed kinetics in the upper respiratory tract of Iraq/06:6NY/09 H5N1-challenged pigs and transmission to naive pigs.

One of the limitations of this study is that the viruses were generated using reverse genetics, and therefore, bias was introduced in determining the genome of the reassortant. A previously reported study performed coinfections with two viruses and subsequently characterized the reassortants naturally generated in vivo [78], while another study generated all possible reassortants in vitro and tested for transmissibility and virulence using guinea pigs and mice, respectively [28]. In a similar study that also looked at reassortants with an H1N1pdm09 backbone, studying a different potential pandemic HA (H9), it was found that H1N1pdm09 internal genes were compatible with the H9 gene and that the resultant virus could transmit via aerosolization in the ferret model [79]. It remains to be determined if such a virus with a non-human seasonal HA and an H1N1pdm09 backbone emerged in nature would be able to outcompete other circulating viruses or subsist long enough to adapt. Experiments analysing HPAI H5N1 reassorted with contemporary genotypes (H3N2 and H1N1pdm09) have shown that avian H5N1 readily reassorts [78, 80–85], and H5N1 viruses in wild birds have been shown to frequently reassort in nature.
to acquire new lineages of internal gene segments [63, 66, 86–88]. Due to the complex dynamics of IAV ecology, global surveillance efforts across multiple species are critical for monitoring the emergence of novel viruses with pandemic potential.

Influenza viruses with the potential to cause pandemics in the human population continue to circulate in nature, yet influenza experts have not been able to predict the next emerging pandemic strain. In this study, we found that while wild-type Iraq/06 H5N1 did not replicate or transmit efficiently in pigs, a reassortant H5N1 with H1N1pdm09 internal genes demonstrated a relative increase in replication and transmissibility properties. An important feature of many IAVs in swine, as with the 2Iraq/06:6NY/09 H5N1 in this study, is the possibility that a virus causing minimal clinical disease in pigs and going undetected could have pandemic potential in humans. It is also noteworthy that weak and/or intermittent serologic responses were detected in the H5N1-infected pigs. These findings highlight the importance of continued passive and active virologic surveillance of circulating strains in swine, perhaps combined with paired serology for specific high-risk subtypes or strains, such as HPAI H5, in areas of high concentration of these viruses of concern. Further research on the genetic requirements for transmission of non-human seasonal IAV to human hosts, complemented with comprehensive surveillance of circulating strains in other natural mammalian hosts, will help prioritize and improve the ability to assess the risk of emergence of potential pandemic viruses in non-human animal populations such as swine.

**METHODS**

**Viruses**

Reverse-genetics-derived viruses of A/Iraq/755/06 (Iraq/06) H5N1 and A/New York/18/2009 (NY/09) pandemic H1N1 were generated as described previously [89, 90]. Reverse genetics was used to recover 2:6 reassortants that expressed the HA and NA of Iraq/06 with the internal genes of NY/09 (2Iraq/06:6NY/09 H5N1) or the HA and NA of NY/09 with the internal genes of Iraq/06 (2NY/09:6Iraq/06 H1N1), all with wild-type gene segments, and kindly provided by the Influenza Division at the Centers for Disease Control and Prevention. Rescued viruses replicated to high titres in Madin–Darby kidney L cells [91]. Laboratory experiments were conducted under biosafety level 3 containment (www.cdc.gov/OD/ohs/biosfty/bmb15/bmb15toc.htm) including enhancements required by the U.S. Department of Agriculture (USDA) and the Federal Select Agent Program [92], available at www.selectagents.gov.

**Pig challenge and transmission study**

Animal experiments were conducted under biosafety level 3 agriculture containment (www.cdc.gov/OD/ohs/biosfty/bmb15/bmb15toc.htm) including enhancements required by the USDA and the Federal Select Agent Program [92]. A total of 85 pigs were obtained from a healthy herd free of IAV, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and *Mycoplasma hyopneumoniae*. Upon arrival, pigs were confirmed seronegative to IAV with a NP-specific ELISA (Idexx Laboratories). All pigs were treated with ceftriaxone crystalline-free acid (Pfizer) and enrofloxacin (Bayer HealthCare AG) prior to the start of the study to prevent bacterial infection. Pigs were divided into four groups of 20 and one group of 5 (sham), housed in individual isolation rooms and cared for following the guidelines of the Institutional Animal Care and Use Committee of the National Animal Disease Center. Fifteen pigs from groups 1–4 were inoculated intranasally (1 ml) with 10^6 50% tissue culture infective dose (TCID_{50}) per ml. PBS was given as a sham inoculum in the negative control group (group 5). Inoculation of pigs was performed under anesthesia, using an anesthetic cocktail of ketamine (8 mg kg^-1), xylazine (4 mg kg^-1) and Telazol (6 mg kg^-1; Fort Dodge Animal Health), administered intramuscularly. Five contact pigs were comingle’d with each of the virus-infected groups at 2 days p.i. to evaluate the transmissibility of wild-type parental and reassortant viruses. Pigs were observed daily for clinical signs of respiratory disease. Sera were obtained from blood samples collected from principal (12 and 21 days p.i.) and contact (12 and 21 dpc) pigs to test for IAV NP seroconversion with the Flock Check AI MultiScreen diagnostic kit (Idexx Laboratories) and homologous HI responses. Five principal pigs from each group were necropsied at 3, 5 and 21 days p.i. following humane euthanasia with a lethal dose of pentobarbital sodium (Fatal Plus; Vortech Pharmaceuticals). All five contact pigs from groups 1–4 were necropsied at 19 dpc.

**Viral RNA detection and shedding**

Dacron nasal swabs were taken at 0, 1, 3, 5 and 7 days p.i. from principal pigs and 1, 3 and 5 dpc from contact pigs and placed in 2 ml minimal essential medium (MEM) to evaluate viral shedding. Whole lungs were removed from the principal necropsied pigs at 3 and 5 days p.i., and BALF was collected for virus titre analysis by performing a lavage with 50 ml MEM. All nasal swabs and BALF samples were stored at −80 °C.

Prior to virus isolation, nasal swab samples were thawed, vortexed for 15 s and passed through a 0.45-µm-pore-sized filters to reduce potential bacterial contaminants. Virus isolation was performed by passaging twice in Madin–Darby canine kidney (London cells in serum-free MEM supplemented with toly sulphomethyl phenylalanyl chloromethyl ketone trypsin and antibiotics. Virus detection was determined on the second passage by fixing cells after 48 h with 4% phosphate-buffered formalin and performing immunocytocytochemistry with an anti-IAV NP monoclonal antibody as described previously [93]. Viral RNA was extracted from nasal swab, and BALF samples were collected at 3 and 5 days p.i. using the MagMax Viral RNA isolation kit (Life Technologies). A quantitative TaqMan RT-PCR assay that targets a...
conserved region of the IAV matrix gene was performed as described previously [94].

Pathological examination of lungs
Lungs were removed from necropsied pigs and examined to determine the percentage of surface area with purplered consolidation typical of the pathology caused by IAV. For each lung lobe, an estimate of the percentage of surface area affected with pneumonia was performed visually, and total percentage for the entire lung was estimated based on weighted proportions of each lobe to total lung volume [95, 96]. Tissue samples from the trachea and right middle or affected lung lobe were fixed in 10% buffered formalin for histopathologic examination. Microscopic lesions were evaluated by a veterinary pathologist blinded to treatment groups. Scores were adapted from a previously described scoring system [97], where individual scores were assigned to six parameters: bronchial and bronchiolar epithelial necrosis or proliferation, suppurative bronchitis or bronchiolitis, peribronchiolar lymphocytic cuffing and alveolar septal thickening with inflammatory cells (interstitial pneumonia). The first two scores focused on the intrapulmonary airways typical of IAV: (i) percentage of bronchi and bronchioles that contained purulent exudate (suppurative bronchitis or bronchiolitis) (0–4) and (ii) percentage of bronchi and bronchioles affected with epithelial lesions (necrotizing or proliferative bronchitis and bronchiolitis) (0–4). Four additional lung lesion scores associated with vaccine-associated enhancement of respiratory disease were based on the following: (iii) magnitude of peribronchiolar lymphocytic cuffing (0–4), (iv) presence and severity of alveolar septal inflammation (interstitial pneumonia) (0–4), (v) presence and severity of alveolar and interlobular oedema (0–3) and (vi) presence and magnitude of epithelial exocytosis (intraepithelial microabscesses) (0–3). A composite score was computed with the sum of the six individual scores (0–22).

Phylogenetic analysis
Reference sequences provided by the World Health Organization (www.who.int/influenza/gisrs_laboratory/) were compiled with representative H5 HA sequences downloaded from the National Center for Biotechnology Information GenBank. Sequences were aligned using default settings in MUSCLE (v3.8.31) [98] with subsequent manual correction. For the alignment of 165 HA sequences, we inferred the best-known maximum-likelihood tree with RAxML (v8.2.0) [99], implementing the rapid bootstrap algorithm with a general time-reversible model of nucleotide substitution with Γ-distributed rate variation among sites. Statistical support for individual branches was estimated by bootstrap analysis with the number of bootstrap replicates determined automatically using an extended majority-rule consensus tree criterion [100].

Statistical analysis
All statistical analyses were performed by using GraphPad Prism 7 software. ANOVA was used to test differences between different groups at different time points, followed by Tukey’s multiple comparison test. P values <0.05 were considered statistically significant.

Dual-use research
All experiments described here were conducted before the moratorium and federal funding pause on H5N1 influenza virus research. After completion of the studies, an internal USDA-Agricultural Research Service (ARS) Dual-Use Research of Concern review process evaluated this work and concluded that the agents and results described herein are not Dual-Use Research of Concern or Gain-of-Function Research of Concern as defined by the National Institutes of Health at the time of submission and thus approved the manuscript for publication.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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