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Detection and characterization of an H4N6 avian-lineage influenza A virus in pigs in the Midwestern United States

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ABSTRACT

H4Nx viruses were reported in swine in Canada and China, but had not been recognized in swine in the USA. In late 2015, an avian-origin H4N6 influenza A virus was isolated from pigs in the United States during a routine diagnostic investigation of clinical respiratory disease in the herd. Serological analysis from additional pigs at the farm and other pigs within the swine production system indicated that the virus did not efficiently transmit from pig-to-pig and the mode of transmission to swine could not be determined. The isolate was characterized at the molecular level and the pathogenesis and transmission was experimentally evaluated in pigs. Although the virus replicated in the lungs of pigs and caused mild pulmonary lesions, there was no evidence of replication in the upper respiratory tract or transmission to indirect contacts, supporting the findings on the farm.

1. Introduction

Influenza A virus (IAV) is an enveloped virus with a negative-sense RNA genome comprised of 8 segments. Wild aquatic bird populations are the natural reservoir for most IAV strains, including those of the subtypes H1-16/N1-9 (Joseph et al., 2016; Yoon et al., 2014), while bats have recently been described as a reservoir for H17-18/N10-11 (Tong et al., 2012, 2013). Despite the large amount of diversity of HA and NA subtypes circulating in birds, only H1N1, H1N2 and H3N2 subtypes are endemic in swine worldwide (Nelson et al., 2015b). Even though there are only few HA and NA subtypes co-circulating in swine, the ecology of IAV in swine is complex and is characterized by large genetic and antigenic diversity due primarily to human-to-swine spillover events. The diversity of contemporary strains co-circulating in the United States has been relatively well characterized in large part due to the US Department of Agriculture (USDA) surveillance efforts operated through the National Animal Health Laboratory Network and the National Veterinary Services Laboratories. The increased reporting and accessibility to publically available sequence data resulted in a more comprehensive genetic and antigenic understanding of circulating strains (Abente et al., 2016; Anderson et al., 2015, 2013; Lewis et al., 2014). Furthermore, it facilitated the early detection of novel HA introductions into the swine population, such as a 2010-11 human H3 that has sustained onward transmission in the pig population (Rajao et al., 2015). Early detection of novel incursions such as these, particularly of antigenically distinct HA's for which there is little to no population immunity through vaccination or exposure to circulating strains, are critical to prevent and intervene in such events and to identify potential vaccine strains for preventing spread of new introductions.

Avian lineage IAV have also played an important role in the ecology of IAV in swine through incursion of surface and internal genes. Historically, the first report of IAV in swine was concomitant with the spread of the 1918 “Spanish flu” (Koen, 1919), and an H1N1 was later isolated (Shope, 1931) and identified as belonging to what is now described as the classical swine H1N1 lineage. While the details of how the pandemic 1918 strain emerged have not been fully determined (Smith et al., 2009; Taubenberger, 2006; Taubenberger et al., 2005; Worobey et al., 2014a, 2014b), genes of avian-origin were associated with the genesis of the classical swine H1N1 and have been associated with the emergence of other pandemic strains (Smith et al., 2009). Another major IAV swine lineage endemic in pigs on multiple continents is a direct incursion of an avian lineage IAV, the Eurasian avian-like H1N1 (Castrucci et al., 1993; Guan et al., 1996; Vincent

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et al., 2014), but spillover and onward transmission of non-reassortant avian viruses in swine is not common. Additionally, avian-origin PB2 and PA genes comprise the well-established triple reassortant internal gene cassette that continues to circulate in North America (Zhou et al., 1999), migrated to parts of Asia, and contributed to the 2009 H1N1 pandemic genome constellation.

Avian H4 IAV are known to have been circulating at least since 1956 when an H4N6 virus was isolated from a duck, with two well supported lineages, North American and Eurasian (Donis et al., 1989). Among the H4 HA sequences detected worldwide, publicly available in the Influenza Research Database, the majority were identified in avian species (2140/2235), a small number were found in mammals (5 in swine, 3 in sea mammals, and 2 in muskrats), 84 were detected in environmental samples and one was of unknown origin (Zhang et al., 2017). Pigs were experimentally challenged with 5 avian H4 strains from ducks in a comprehensive study that assessed the susceptibility of swine to 38 avian IAV strains (Kida et al., 1994). Virus was isolated from nasal swabs in 4 out of 5 H4-challenged pigs and duration of shedding varied from 4 to 7 days post infection. A recent study characterized 32 contemporary H4 viruses that were isolated from live poultry markets in China, and consistent with previous findings, all viruses could replicate in mice without adaptation (Liang et al., 2016). Additionally, they determined from a subset of 10 H4 viruses that 6 efficiently transmitted in guinea pigs, an animal model used to assess the potential risk of IAV to infect and circulate in mammals.

To date, three reports have documented the isolation of avian H4 viruses from naturally infected pigs exhibiting influenza-like clinical signs (Hu et al., 2012; Karasin et al., 2000; Su et al., 2012). All three cases were self-limiting without sustained onward transmission or spread from the index herds. In two cases, H4 viruses were isolated from commercial farms during an outbreak that displayed respiratory disease (Karasin et al., 2000; Su et al., 2012). Pre- and post-outbreak serologic data were reported only from the outbreak that occurred in Ontario, Canada (Karasin et al., 2000). In that report, a small subset of sera (10) tested post-outbreak were all HI-positive against the H4N6 virus associated with the outbreak, suggesting potential pig-to-pig transmission, although this was not experimentally confirmed.

Molecular determinants that allow adaptation of avian IAV to a new host species, particularly humans, have been described. Most studies examined the HA and its interaction with the host receptor, but the requirements for host-switch are complex. Interactions between the HA and sialic acids are important for initiating infection and can define species tropisms, and while this interaction has been studied extensively (de Graaf and Fouchier, 2014), other viral genes are also involved in host-switch events. The polymerase and non-structural genes are associated with the ability of viruses to replicate efficiently in the host cell, and specific interactions with host cellular proteins and modifications of the virus genome have been described (Long et al., 2016; Xu et al., 2016). Reassortment of gene segments is a hallmark of genetic evolution of IAV and can play a role in cross-species spread of IAV (Steel and Lowen, 2014).

Here, we report an H4N6 that was detected and isolated in December 2015 from pigs on a farm in Missouri that exhibited influenza-like symptoms and contributed to the USDA IAV surveillance system in swine. We describe the epidemiology associated with this avian H4N6 detection, the genetic characterization of the virus genome, and the results of a pathogenesis and transmission challenge study in weaned pigs.

2. Materials and methods

2.1. Collection and processing of clinical samples

On December 16, 2015, five nasal swab and five serum samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) from a group of mature female pigs that had not yet given birth to a litter of piglets (gilts). The five gilts (approximately 7–8 months of age), pregnant at the time, demonstrated respiratory disease at a breeding and gestation farm located in Northeast Missouri. Clinical signs were observed only in the replacement gilt population and included coughing, lethargy, and anorexia. RNA was extracted from the five nasal swab samples and evaluated for the presence of porcine reproductive and respiratory syndrome virus (PRRSV) by one-step, reverse transcription RT-PCR (VetMAX™ NA and EU PRRSV, ThermoFisher Scientific, Waltham, MA) and serum was used to test for PRRSV-specific antibodies by ELISA (IDEXX PRRS ×3 Ab Test, IDEXX Laboratories, Inc., Westbrook, ME). Samples tested negative for PRRS virus and antibody. The samples were also tested by one-step, reverse transcription RT-PCR for the presence of influenza A virus (VetMAX™-Gold SIV detection Kit, ThermoFisher Scientific, Waltham, MA), then tested for HA and NA subtyping specific for H1/H3 and N1/N2 (VetMAX™-Gold SIV Subtyping Kit, ThermoFisher Scientific, Waltham, MA). Samples were then tested for IAV isolation on Madin Darby Canine Kidney (MDCK) cells, and whole-genome sequencing and sequence analysis were performed on the successfully recovered virus isolate (described below).

As a follow-up investigation, additional nasal swab and sera diagnostic samples were collected from the commercial breeding and gestation farm (index farm) to investigate the origin and presence of the H4N6. Diagnostic samples were also collected from additional production sites linked to the flow of pigs associated with the index farm, including an upstream breeding and gestation farm (gilt multiplier) that supplied new breeding-age females to the index herd and its associated nursery (~3–10 weeks of age) and grow-finish sites (~11–30 weeks of age), designated 1, 2 and 3 and known as contract facilities (Fig. 1). On January 22, 2016, thirty-seven days after the initial H4N6 detection, the submitting veterinarian collected sixty-five nasal swab samples and sixty sera from the H4-positive index farm. Samples were collected from twenty-four gilts approximately eight-months-old that included four of the five original gilts tested in December 2015 (one died prior to re-sampling), from sixteen sows (approximately 1–2 years of age and older) that had farrowed at least one litter, from five three-week-old piglets at weaning, and from twenty four-week-old piglets that were already weaned and moved to a downstream commercial nursery. On March 15, 2016, thirty nasal swabs and sera were collected from randomly selected gilts and sows approximately 1–4 years of age from the upstream gilt multiplier breeding and gestation farm. On March 25, March 28, and April 20, 2016, ten nasal swabs and sera were randomly collected from nursery pigs and grow-finish pigs located in the contract facilities 1, 2 and 3, respectively. Samples were processed and nasal swabs tested individually or pooled in groups of 5 for one-step, reverse transcription RT-PCR analysis for the presence of IAV RNA and sera tested individually for antibody to investigate if the H4N6 virus continued to circulate.

2.2. Viruses and cell lines

A virus was isolated on Madin-Darby canine kidney (MDCK) cells from the single one-step, reverse transcription RT-PCR positive nasal swab specimen collected on December 16, 2015 and submitted to the Swine IAV Surveillance System repository held at the USDA National Veterinary Service Laboratories (NVSL) in conjunction with the USDA National Animal Health Laboratory Network. Whole genome sequencing was performed at NVSL in accordance with guidelines stated in the National Swine Influenza Virus Surveillance Plan (Anderson et al., 2013; APHIS, 2010) and sequences submitted to GenBank (Benson et al., 2017; Clark et al., 2016). All eight segments of the virus were amplified using universal primers for each segment. cDNA was purified and cDNA libraries were prepared using the Nextera XT DNA Library Preparation Kit with Nextera XT Index Kit barcode adapters (Illumina, San Diego, CA). Prepared libraries were pooled and loaded onto a MiSeq Reagent Cartridge and a 500–cycle (2 × 250) PE kit v2 was used