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First whole genome characterization of swine influenza virus subtype H3N2 in Thailand

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ABSTRACT

H3N2 swine influenza viruses (SIV) were first detected in Asia shortly after the 1968 pandemic emerged in humans. Subsequently, human H3N2 viruses have sporadically reappeared in swine. In Thailand, a human-like H3N2 SIV was reported in 1978 although the genetic sequence of this virus is unknown. In this study, we undertook cross sectional syndromic surveillance in pigs in four provinces in Thailand. Seven genetically similar H3N2 viruses were isolated. A representative, A/SW/Thailand/KU5.1/04, was fully sequenced and shown to contain genes from human-like influenza viruses and North American and European SIV. The results restate that transmission of influenza A virus among human and swine populations is common and that genes from both American and Eurasian SIV lineages cocirculate in Thailand.

1. Introduction

Influenza A viruses have been isolated from a number of animals including humans, birds, dogs, seals, horses, and swine (Webster et al., 1992). Taxonomically, along with Influenza B and C viruses, Influenza A viruses are genera within the family *Orthomyxoviridae*. Influenza B and C virus infections are primarily restricted to humans, although influenza C virus has been isolated from swine (Guo and Ulrich, 1984) and influenza B virus from seals (Osterhaus et al., 2000). Influenza A viruses are divided into subtypes based on the antigenic nature of their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA); 16 different subtypes of HA (H1 to 16) and 9 different subtypes of NA (N1–9) have thus far been identified. The influenza A virus genome is comprised of eight individual pieces of single-stranded negative sense RNA that, in addition to HA and NA, encode 8 or 9 other proteins; non structural 1 (NS1), non structural 2 (NS2), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP) and three polymerase proteins, PB1, PB2, and PA (some viruses also produce a small protein PB1-F2) (Ghedin et al., 2005).

Swine influenza viruses (SIV) are able to cause a transient, acute respiratory disease in swine. To date, and although other subtypes have been sporadically identified, three major subtypes of SIV (H1N1, H1N2, and H3N2) are routinely found in global swine populations (Jung and Chae, 2004).

In North America, influenza-like symptoms were first recognized in swine during the 1918 Spanish Flu pandemic in humans (Webster et al., 1992) and the virus was first isolated in 1930 (Shope, 1931). These so-called "classical" swine H1N1 viruses circulated almost exclusively in US swine populations until 1998 when H3N2 viruses

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established (Karasin et al., 2000). These H3N2 viruses were reassortants containing human virus (HA, NA and PB1), swine virus (NP, M, and NS), and avian virus (PB2, and PA) gene segments (Zhou et al., 1999). Subsequent reassortment events between H1N1 and H3N2 viruses led to the generation of H1N2 viruses within the US swine industry (Karasin et al., 2000; Vincent et al., 2008).

In Europe, the first reports of classical H1N1 SIV were made around 1950 (Brown, 2000). These viruses seemingly disappeared from European swine, but reemerged in 1976 at a time that coincided with the emergence of human-like H3N2 SIV (Pensaert et al., 1981). In 1979 the classical H1N1 SIV was replaced by an avian-like H1N1 virus, which later reassorted with the human-like H3N2 viruses (Pensaert et al., 1981). Similarly as has been seen in the US, subsequent H1N2 viruses were generated but this time through reassortment between contemporary human H1N1 viruses and the reassortant H3N2 viruses (Marozin et al., 2002).

All three subtypes, H1N1, H1N2 and H3N2 of SIV have also been found in Asian swine populations. H1N1 SIV has been reported in several countries (Guan et al., 1996) such as Hong Kong (Yip, 1976), Japan (Yamane et al., 1978), China, Taiwan (Shortridge and Webster, 1979), and Thailand (Kupradinun et al., 1991). In 1968, H3N2 viruses antigenically similar to the contemporary human H3N2 virus were isolated from Asian swine (Shortridge and Webster, 1979). In Thailand, H3N2 SIV was first isolated in 1978 (Nerome et al., 1981), followed by H1N1 in 1988 (Kupradinun et al., 1991). The H1N1 isolates were antigenically related to the classical H1N1 SIV as determined by hemagglutination-inhibition tests using monoclonal antibodies. Recently, H1N1 and H3N2 virus activity was detected by serum neutralization test (SNT) in swine sera collected from 5 provinces in Thailand (Parchariyanon et al., 2006).

Despite their repeated identification, information regarding the genetic composition of SIV in Thailand is limited. Increasing the importance of such information is the circulation of HPAI H5N1 viruses in the region; data on endemic swine viruses is needed to identify any potential H5N1/SIV reassortment events. The emergence of the pandemic A (H1N1) 2009 virus from undetermined swine virus ancestry reservoirs also raises the need for further genetic characterization of SIV. In this study, we describe the results of SIV surveillance in Thailand and describe for the first time the characterization of all eight genes of H3N2 SIV from Thai swine.

2. Materials and methods

2.1. Clinical samples and virus isolation

To isolate influenza viruses for characterization, nasal and/or lung tissue was taken from animals from a number of swine herds across Thailand (Fig. 1). Samples were collected from four provinces where most of swine farms exist on two occasions: once in 2004 and another in 2006. To increase the chances of isolating influenza viruses, where possible, samples were taken from diseased animals. Virus was isolated from the clinical material by inoculation onto Madin Darby Canine Kidney (MDCK) cell monolayers maintained in Dulbecco's modified eagle medium (Invitrogen) supplemented with 0.3% bovine serum albumin (Sigma) and 2 μg/ml trypsin. The inoculated cells were incubated in a CO_2 incubator at 37 °C and were examined for the presence of cytopathic effect (CPE) each day for 3 days. Supernatants were harvested when the CPE involved more than 80% of the monolayer or at 72 h post inoculation, whichever came first.

2.2. Immunoperoxidase monolayer assay

To confirm the presence of influenza A virus in cultures, we used an Immunoperoxidase Monolayer Assay (IPMA). Trypsinized infected cells were transferred to 15 ml tubes containing 5 ml of phosphate buffered saline, pH 7.2, and spun at 1500 rpm for 5 min. The cell pellets were spread on a glass slide and air-dried prior to fixation with 4% formalin buffer for 10 min at room temperature. Slides were then incubated in 0.5% saponin for 10 min at room temperature and washed 3 times with PBS containing 0.05% Tween 20 (PBST). Viral antigen was detected by using a murine monoclonal antibody specific for the influenza A virus nucleoprotein (NP) (ATCC). This antibody was diluted at 1:2,000 in PBST, applied to the cells, and then incubated on the slides at 37 °C for 1 h in a humidified incubator. After incubation, the cells were washed 3 times with PBST. Antibody-bound cells were identified through the use of a peroxidase labeled goat anti-mouse IgG (KPL) at dilution of 1:250 at 37 °C for 1 h in a humidified incubator. Excess secondary antibody was removed by washing slides 3 times with PBST and cells were stained with the Diamino Benzidine (DAB) substrate solution (sigma) for 10–15 min at room temperature. Positive, i.e., brown, cells were observed by using an inverted microscope.

2.3. Virus subtype determination

The subtype of any isolated virus was determined by performing RT-PCR with HA and NA specific primers on positive cell supernatants. RNA was isolated from the supernatants by using Trizol® (Invitrogen) according to the manufacturer's instruction and the subtype was determined by using 2 different multiplex reverse transcription-polymerase chain reactions (RT-PCR) as previously described (Choi et al., 2002).

2.4. Genomic sequencing and analysis

Partial gene sequencing for purposes of genotyping was done on each of the isolated viruses while the full genomic sequence of A/SW/Thailand/KU5.1/04 was determined for additional phylogenic studies. Sequencing templates were generated by amplification of each gene segment using one-step RT-PCR (SuperScrip™III RT/Platinum® *Taq* Mix (Invitrogen)) and segment-specific primers as described by Hoffmann et al. (2001). PCR products were purified using the Nucleospin® kit (Macherey-Nagel) and sequenced by using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) at Bioservice Unit, Bangkok. The primers used for sequencing were as previously described (Hoffmann et al., 2001; Zou, 1997)