

SWINE INFLUENZA H3N2 VIRUSES CIRCULATING IN PIG POPULATION IN CHINA IN 2001 CLOSELY RELATED TO AVIAN INFLUENZA VIRUSES

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Introduction

Influenza viruses are negative sense RNA viruses with single-stranded genomes composed of eight or seven segments. The viruses can be divided into different subtypes based on differences in the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). In general, influenza viruses are species specific; however, whole viruses may occasionally be transmitted from one species to another and genetic reassortment between viruses from two different hosts can create a virus that is infected to a third host. Pigs are peculiar in that they replication of avian, human, and porcine influenza A viruses, and therefore they are regarded as “mixing vessels” for the creation of new pandemic strains^[1]. Previous studies have showed that the human influenza pandemic strains in twenty century were the result of reassortment: HA gene of the 1918 “Spanish Flu” originated by reassortment of swine-lineage and human-lineage influenza viruses, which probably changed the virulence of the virus^[2]; three genes (HA, NA, and PB1) of the Asian/57 strain were of avian influenza virus (AIV) origin, and remaining genes were of human influenza virus origin^[3]; the Hong Kong/68 strain obtained its HA and PB1 genes from an avian virus and the other genes were characteristic of human influenza virus^[4]. However, the available evidence indicates that influenza viruses of avian origin don’t undergo productive replication in humans, other than human infection and mortality in Hong Kong in 1997 associated with AIV H5N1/97 and human disease associated with H9N2 virus^[5,6]. Therefore, a more likely explanation for the reassortment events responsible for the three human influenza pandemic viruses is that mixing between human and avian influenza viruses occurred in another animal that served as a “mixing vessels”, such as pig.

Large-scale virological and serologic surveillance in China in 1998–2002 has provided ample evidence for the extensively prevalence of SIV H3N2 subtype in the swine population in China. To Provide further evidence in regard to interspecies infection among avian, swine and human hosts, some H3N2 influenza viruses from pig flocks in China in 2001 were analysed by homology and genetic evolutionary means.

Material and methods

Virus strains

All SIV (SWFJ668, SWFJ670, SWFJ674, SWNM526, SWNM547, SWHN703) examined in this study were isolated from samples collected in China during an epidemiological survey from 2001^[7]. These isolates were passaged in the amniotic cavities of 10-day old embryonated chicken eggs and purified by finity dilution cloning.

Antigenic analysis

Hyperimmune guinea pig antisera of SWFJ668, SWFJ670, SWFJ674, SWHN703 and Hyperimmune rabbit antisera of SWNM526, SWNM547, DKUK63 and SWCO77 were prepared as previously described (Kendal et al, 1982). All sera were treated with “Trypsin-Heat-Periodate”^[8]. The hemagglutination -inhibition (HI) tests and neuraminidase inhibition (NI) tests were performed as previously described^[8].

Gene sequencing and phylogenetic analyses

Virus RNA was extracted from infective allantoic and amniotic fluid using TRIzol LS Reagent. RT-PCR was performed using specific primers for HA1 and NA gene segment (primer sequences are available on request). PCR product were purified by agarose gel electrophoresis and sequenced by TaKaRa Biotechnology (Dalian) Co., Ltd. Using an ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase and an ABI PRISM™ model 377XL DNA Sequencer (Perkin-Elmer/Applied Biosystems).

Sequence data were edited and analyzed using the LASERGENE System of Wisconsin Package. es were carried out using PAUP (Phylogenetic Analysis Using Parsimony). The nucleotide sequences obtained from this study are available from GenBank.

Result

Antigenic characteristics

HI tests with absorbed antisera defined at least two antigenic variants of H3N2 influenza virus are present in the pig population in China in 2001 (Table 1). NI tests using hyperimmune goat or rabbit antisera against various N2 virus showed that the NA of these SIV isolates was related to the early human H2N2 viruses such as A/Singapore/1/57, A/Japan/305/57 and the recent avian virus such as A/Duck/Gremany/1215/73 (H2N2) and A/Chicken/Shandong/6/96 (H9N2).

Table 1 Antigenic analysis of HA of swine H3N2 virus in China

Virus ^a	HI titers with rabbit antisera ^b	with	HI titers with guinea pig antisera
	SWCO77	to To	To
SWFJ668	160	640	320
SWFJ670	1280	1280	1280
SWFJ674	160	2560	640
SWNM524	160	2560	640
SWNM547	160	1280	40
SWHN703	160	< ^c	2560

^a Virus abbreviations denote type of animal (swine, SW) and place isolated (Fujian, FJ; Neimeng, NM; Henan, HN).

^b Diluted 1:40. ^c < below the level of detection.

Homology and evolutionary analysis of HA genes

The nucleotide sequence analysis indicated that HA1 gene formed 2 markedly different lineages. SIV isolates from China in 2001 all belonged to Euroasian pig lineage and had formed a stable sub-lineage. All the strains in this sub-lineage evolved from water-fowl AIV strain. The nucleotide and amino acid homology of 6 Chinese SIV isolates with A/Aichi/2/68 and A/Duck/Ukraine/1/63 (DKUK63) were given in Table 2. There are 5 potential glycosylation sites between the 12th to 322nd amino acid sequence of HA1 domain of HA gene, which completely the same with the virus of DKUK63 in the number and position of the 22nd, 38th, 81st, 165th and 285th amino acid residue respectively.

Table 2 Homology of HA1 and NA gene segments of swine H3N2 virus in China with reference strain

SIV Isolates	% Homology ^a of HA1 gene with:		% Homology of NA gene with:
	A/Aichi/2/68	DKUK63	DKHKY280-97
SWFJ6 68	90.4 (95.1)	99.9 (100)	97.8 (98.3)
SWFJ6 70	89.8 (92.8)	99.0 (99.7)	97.6 (96.7)
SWFJ6 74	90.4 (95.1)	99.9 (100)	98.0 (98.4)
SWNM 526	90.5 (95.1)	100 (100)	97.5 (98.1)
SWNM 547	90.5 (95.1)	100 (100)	92.6 (93.8)
SWHN7 03	90.4 (95.1)	99.9 (100)	92.2 (93.2)

^a Calculated based on the nucleotide sequences of HA1 (nucleotides 33 to 954) and NA (31 to 1380). Values in front are nucleotide homology and in parentheses are corresponding amino acid homology.

Homology and evolutionary analysis of NA genes

The majority of SIV isolates in China shared high nucleotide and amino acid homology with strain A/Duck/Hong Kong/Y280/97 (DKHKY280-97) in the range of 92.2% to 98.0% and 93.2% to 98.4% respectively (Table 2). SIV isolates except SWHN703 all show deletions of the 187th to 195th nucleotides, and have led to lack of amino acid residues at the site of 63rd to 65th and of the potential glycosylation sites at the 61 amino acid residue. The nucleotide and amino acid of NA gene of Influenza viruses evolve along 2 distinctly separate lineages, in both of which occurred gene recombination. SIV isolates from China belonged to a large lineage, and further evolved into 2 stable sub-lineages. SIV isolates from Inner Mongolia belonging to 2 different sub-lineages evolved at rather high speeds. SWNM547 strain provided NA gene to SIVs in Henan Provinces, whereas the NA gene in Fujian isolates and SWNM547 descended from DKHKY280-97. These results indicated the closely relationship in genetic evolution between SIVs isolated from China to H9N2 AIV. The routes and speeds of genetic evolution of amino acids of NA gene were similar to those of the nucleotide.

Prediction of secondary structure of glycoproteins HA1 and NA

Significant secondary structural difference existed among SIVs from China and reference strain viruses. These differences were mainly reflected by the numbers and positions of α -helix, β -sheet, β -turn and irregular coil. In some strains, a single substitution of amino acid caused changes in neighboring secondary structures. Thus point mutations, deletions and insertions of nucleotides led to changes in secondary structure and function of the protein.

Discussion

Close monitoring of the evolution of the HA and NA genes of SIV is important in the selection of appropriate vaccine strains. Antigenic and genetic analyses revealed that SIVs isolates from China in 2001 at least could be divided into two antigenic groups. Molecular analysis of influenza virus genes is useful for studying virus evolution and emphasized the need to sequence both the HA and the NA genes of field strains in order to achieve as close a match as possible for both surface antigens between the vaccine and circulating strains.

Study in the evolution of HA genes of these SIV isolates indicated that all SIV H3N2 isolates in 2001 in China were genetically close to H3N2 type duck influenza viruses, they are in the same phylogenetic sub-lineages and evolve almost in the same speeds, we can make a conclusion that these 6 SIV isolates from China are DKUK63-like viruses, it has been suggested avian-originating influenza virus have crossed the species barrier and transmitted to the pig population in China and formed a stable lineage. The question arises as to there is no further evolution and lineage among them while they have spanned 38 years in time! It was surprised that where these DKUK63-like viruses from 1963 had survived over the previous 38 years in pig flocks in China without significant evolution, this means that there is mildly selection pressure with time. This observation seem to indicate that the evolutionary rate under national conditions might depend on a number of factors, ecological environment, density of animal group as well as the condition in breeding management, and such strains can survive in certain places in the world for quite a while before they showed up again as a pandemic strain in pig population that is not sufficiently protected by the corresponding antibodies. From the nucleotide and amino acid sequence analyses of N2 neuraminidase of these SIV, it was shown that two sub-lineages have co-circulated in the swine populations in China in 2001.

These above molecular epidemiological and ecological findings indicated that cross infections and gene recombination occurred between human, swine and avian influenza viruses. This phenomenon confirmed "avian to pig to human" and "human to pig to human" infection route by influenza viruses. The cross-species transmission of SIVs isolated in China and their roles in genetic evolution were established, which facilitated unraveling of molecular mechanisms of cross-species transmission of SIVs and molecular evolution. These would also significantly assist prediction, forecast and measures formulation in preventing Swine Influenza.

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