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Genetic analysis of two influenza A (H1) swine viruses isolated from humans in Thailand and the Philippines

N. Komadina · V. Roque · P. Thawatsupha · J. Rimando-Magalong · S. Waicharoen · E. Bomasang · P. Sawanpanyalert · M. Rivera · P. Iannello · A. C. Hurt · I. G. Barr

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Abstract Influenza viruses A/Philippines/341/2004 (H1N2) and A/Thailand/271/2005 (H1N1) were isolated from two males, with mild influenza providing evidence of sporadic human infection by contemporary swine influenza. Both viruses were antigenically and genetically distinct from influenza A (H1N1 and H1N2) viruses that have circulated in the human population. Genetic analysis of the haemagglutinin genes found these viruses to have the highest degree of similarity to the classical swine H1 viruses circulating in Asia and North America. The neuraminidase gene and the internal genes were found to be more closely related to viruses circulating in European swine, which appear to have undergone multiple reassorting events. Although transmission of swine influenza to humans appears to be a relatively rare event, swine have been proposed as the intermediate host in the generation of potential pandemic influenza virus that may have the capacity to cause human epidemics resulting in high morbidity and mortality.

Keywords Influenza A · Swine influenza · Haemagglutinin · Reassortants · Neuraminidase · Phylogenetic

N. Komadina (✉) · P. Iannello · A. C. Hurt · I. G. Barr
WHO Collaborating Centre for Reference and Research on Influenza, 45 Poplar Rd, Parkville, VIC 3052, Australia
e-mail: Naomi.Komadina@influenzacentre.org

V. Roque · J. Rimando-Magalong
National Epidemiology Centre, Bldg. 9, San Lazaro Compound, Sta Cruz, Manila, Philippines

E. Bomasang · M. Rivera
Virology Department, Research Institute for Tropical Medicine, Filinvest Corporate City Compound, Alabang, Muntinlupa City, Philippines

P. Thawatsupha · S. Waicharoen · P. Sawanpanyalert
WHO National Influenza Centre, National Institute of Health, 88/7 Tivanonda Rd., Nonthaburi, Thailand

A. C. Hurt · I. G. Barr
Monash University Gippsland, Churchill, VIC 3842, Australia

Introduction

Influenza A viruses are known to infect a wide variety of species, including humans, swine and birds. Swine are thought to play an important role in interspecies transmission, having receptors for both human and avian viruses and the possibility of acting as an intermediate host thereby creating novel reassortant viruses of interspecies origin [1]. Influenza A subtypes, H1N1, H3N2 and to a lesser extent H1N2 are known to circulate in the human population [2] and have also been reported in circulating in swine [3, 4]. Infection of humans with swine influenza is a relatively uncommon occurrence but humans infected with H1N1 and H3N2 of swine origin have been reported previously in North America, Europe and Asia [5–7] The 'classic' H1N1 swine influenza which appears to have infected pigs in North America in 1918 at about the same time as the H1N1 virus emerged to cause 1918–1919 pandemic, was originally isolated in 1930 [8]. A common origin has been suggested for the human 1918 virus and the classic swine viruses which appear to have a genetic 'sister group' relationship [9]. The H3N2 influenza virus that was responsible for the 1968 pandemic was found to have both avian and human genes and similar viruses have since become established in pig populations in various parts of the world [10]. In North America during 1998, a new

influenza A H3N2 triple reassortant emerged in swine, which contained haemagglutinin (HA), neuraminidase (NA) and PB1 gene of the polymerase unit of human origin, matrix (M), nucleoprotein (NP) and the non structural protein (NS) genes from the classical swine influenza and PA and PB2 genes from the polymerase unit of avian origin [11]. A further new subtype emerged in North American pigs in 1999 which had inherited the HA and NA genes of the classical swine virus and the remaining six genes from the 1998 triple reassortant [12].

In European swine herds, the classical H1N1 swine viruses were replaced by 'avian' like H1N1 viruses in 1981 and these have since co-circulated with H3N2 swine viruses [13, 14]. Genetic reassortment between these two subtypes has produced reassortant H3N2 viruses containing six internal genes from the 'avian-like' H1N1 virus along with swine derived HA and NA genes. A H1N2 swine virus derived by reassortment between H1N1 and H3N2 viruses, has also been isolated [15]. In Asia all three subtypes H1N1, H1N2 and H3N2 have been reported circulating in swine and studies of the prevalent viruses circulating in pigs in southern China show that H3N2 viruses were closely related to the human H3N2 that circulated during 1976–1978 and in 1982 [16, 17]. In contrast during 1977–1980, the classical H1N1 swine strain accounted for a most infections in pigs. During the intervening period there appeared to be variation in the prevalence of each subtype and while there was co-circulation of the H1N1 and H3N2 subtypes the incidence of reassortants was low and there was no evidence of the acquisition of avian influenza virus segments during this period [18]. However in 1993, there was antigenic and genetic evidence that two different groups of H1N1 were circulating in China, one group, similar to the original classical lineage and a second group more closely related to the 'avian-like' H1N1 viruses circulating in pigs in Europe since 1979 [19]. Japan, Korea and Thailand all have variously reported H1N2 and or H1N1 swine viruses circulating at different times since the 1970s which were related to swine viruses circulating in the USA [20, 21, 22, 23].

During February 2004, influenza A/Philippines/344/2004 (H1N2), was isolated at the Research Institute for Tropical Medicine, Manila from a 25-year-old male who had mild influenza. In July 2005, influenza A/Thailand/271/2005 (H1N1), was isolated by the WHO National Influenza Centre, Bangkok, from a 4-year-old male. Both samples were forwarded to the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne for testing where they were found to be antigenically and genetically distinct to the viruses circulating in the human population at the time. Genetic analysis determined that these viruses were of swine origin and were genetically similar to H1 viruses circulating in swine in Asia in recent years.

Materials and methods

Viruses

The viruses received from the Research Institute Tropical Medicine, Manila, Philippines and WHO National Influenza Centre, Bangkok, Thailand were passaged in MDCK cells. Growth was monitored by CPE and the presence of haemagglutination activity using Turkey red blood cells (RBC's) as previously described [24]. The isolates were tested using a standard haemagglutination inhibition assay (HAI) against a panel of reference viruses and their homologous ferret antisera [24]. Virus isolates were further analysed using the BD Directigen^{TM} EZ Flu A + B (New Jersey, USA) and Remel Xpect Flu A&B rapid test kits (Kansas, USA). Resistance to neuraminidase inhibitors was determined using the neuraminidase inhibition assay [25].

Sequencing and phylogenetic analysis

RNA extraction, RT-PCR and sequencing were performed as previously described [24]. Sequences were assembled using the Lasergene Seqman package IV (DNAStar 6) and phylogenetic relationships determined using PHYLIP V 3.5.7 [26] neighbour-joining method on ANGIS (Australian National Genomic Information Service) and dendograms were drawn using Treeview [27]. Bootstrap confidence values were calculated using 100 replicates before determining phylogenetic distances. Amantadine resistance was determined by analysis of the sequence of the M2 gene [28]. Gene sequences were submitted to Genbank and the accession numbers are given in Table 1.

Results

Influenza A/Philippines/344/2004 was isolated from a 25 year old male who had presented at the local health

Table 1 Genbank accession numbers of the eight gene segments of A/Philippines/344/2004 and A/Thailand/271/2005

Gene	A/Philippines/344/2004	A/Thailand/271/2005
PB2	EF101747	EF101754
PB1	EF101748	EF101753
PA	EF101746	EF101755
HA	EF101741	EF101749
NP	EF101745	EF101752
NA	EF101743	EF101756
MP	EF101742	EF101750
NS	EF101744	EF101751

centre with high grade fever, dizziness and occasional vomiting. The virus was initially identified in Manila as influenza A by immunofluorescence and further subtyped as H1 utilising influenza A (H1) and influenza A (H3) specific monoclonal antibodies supplied by the WHO Collaborating Centre for the Surveillance, Epidemiology and Control of Influenza, Centre for Disease Control (CDC), Atlanta. Influenza A/Thailand/271/2005 was isolated from a 4 year old male suffering from rhinorrhea, fever and myalgia. Viruses were passaged in MDCK cells at the Melbourne Centre and the cells exhibited typical CPE for influenza and agglutinated turkey RBCs. Both the isolates gave positive influenza A results when tested using the two rapid immunochromatic based influenza detection kits. Both the isolates were negative in HI tests using specific ferret antisera raised against contemporary human influenza A, H1 and H3 viruses. When the isolates were tested against broadly reactive hyper immune rabbit and sheep antisera, both isolates were identified as Influenza A virus of the H1 subtype. These results were further confirmed by RT-PCR using gene specific primers.

The HA gene sequences of A/Philippines/344/2004 and A/Thailand/271/2005 were subjected to a BLAST search (www.flu.lanl.gov) that revealed the sequence with the greatest degree of similarity was A/Wisconsin /4755/94, a virus isolated from a human but of swine origin (U53136, Table 2). The NA gene of A/Philippines/344/2004 was found to be of the N2 subtype and of swine origin while the NA gene of A/Thailand/271/2005 was found to be of N1 subtype and also of swine origin (Table 2). The internal genes for both viruses were sequenced and the viruses with the closest homology to the individual genes are shown in Table 2. The internal genes of A/Philippines/344/2004 were found to have the highest degree of homology with swine viruses for the PB2, PB1 and PA genes whereas for the MP and NP genes the highest degree of similarity was with viruses of avian origin. For A/Thailand/271/2005 the internal genes had the highest degree of homology with swine derived viruses, with the exception of the NP gene which had the highest match with an avian virus NP gene. Both viruses were examined for their antiviral drug susceptibility. Adamantane susceptibility was determined by sequencing the matrix gene and both viruses were found to possess an asparagine at residue 31 of the M2 protein, which would make them resistant to amantadine and rimantadine [29]. Both virus exhibited normal sensitivity to the neuraminidase inhibitor drugs zanamivir and oseltamivir. A/Philippines/344/2004 had an IC_{50} of 0.51 nM with zanamivir and 0.20 nM with osteltamivir versus historical ranges of 0.35 ± 0.18 nM and 1.30 ± 0.42 nM respectively for human influenza viruses with an N2 type neuraminidase. For A/Thailand/271/2005 the IC_{50} was 0.34 nM and 0.54 nM for zanamivir and osteltamivir respectively compared to historical ranges of 0.35 ± 0.18 nM and 0.76 ± 0.76 nM for human influenza viruses with an N1 type neuraminidase.

Phylogenetic analysis of the nucleotide sequences of the HA genes of the two viruses indicated that they were related to swine viruses from the classical H1 lineage, which include swine viruses isolated in North America and Asia (Fig. 1). When the sequences were compared to that of a contemporary human H1N1 virus, influenza A/New Caledonia/20/99, the HA gene of each virus was found to be clearly distinct with only 74% similarity to the HA gene of A/New Caledonia/20/99. The HA of the two viruses were also only distantly related to each other with 86% nucleotide similarity.

Genetic relationship analysis of the NA nucleotide sequences for the viruses showed that the N1 gene of influenza A/Thailand/271/2005 was more closely related to the 'avian-like' European lineage of H1N1 swine viruses (Fig. 2). The N2 gene of influenza A/Philippines/344/2004 also appeared to be more closely related to H3N2 and H1N2 swine viruses circulating in Asia. The nucleotide sequences for the six internal genes of both the viruses were compared with sequences available on public databases. The six internal genes of influenza A/Philippines/344/2004 were found to have the greatest degree of

Table 2 Percentage of similarity between A/Philippines/344/2004 and A/Thailand/271/2005 and viruses with the highest degree of homology based on nucleotide comparisons

Gene		A/Philippines/344/2004		A/Thailand/271/2005
PB2	94%	a/sw/germany/2/81 (H1N1)	94%	a/swi/italy/2064/99 (H1N2)
PB1	93%	a/sw/cotes d'armor/790/97 (H1N2)	94%	a/sw/italy/1521/98 (H1N2)
PA	94%	a/sw/cotes d'armor/2433/98 (H1N2)	94%	a/sw/cotes d'armor/790/97 (H1N2)
HA	94%	a/wisconsin/4755/94 (H1N1)	90%	a/wisconsin/4755/94 (H1N1)
NP	95%	a/turkey/germany/3/91 (H1N1)	96%	a/turkey/germany/3/91 (H1N1)
NA	96%	a/sw/Belgium/220/92 (H3N2)	94%	a/sw/England/195852/92 (H1N1)
MP	98%	a/turkey/germany/3/91 (H1N1)	95%	a/sw/hong kong/5200/99 (H3N2)
NS	96%	a/sw/germany/8533/91 (H1N1)	91%	a/sw/tennessee/26/77 (H1N1)

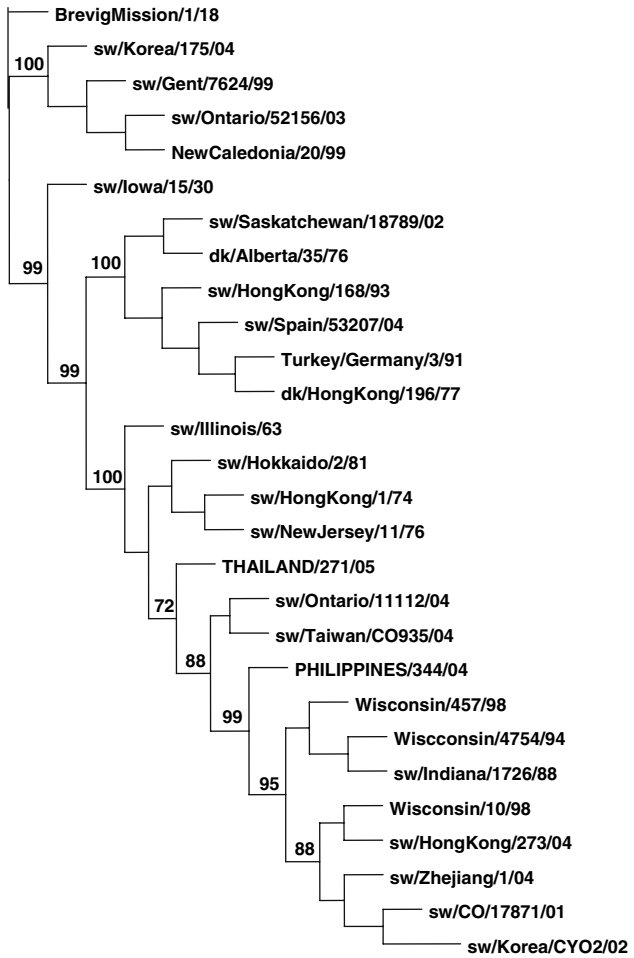

Fig. 1 Phylogenetic comparisons of the nucleotide sequences encoding the influenza A(H1) haemagglutinins. Bootstrap values are shown for selected nodes (only for those with a frequency greater than 70%). All other sequences were obtained from Genbank

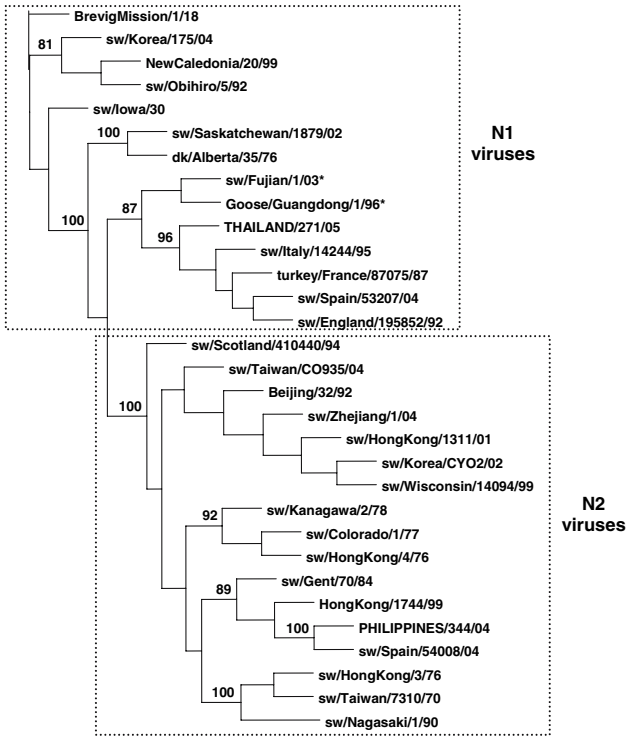

Fig. 2 Phylogenetic comparisons of the nucleotide sequences encoding the N1 and N2 neuraminidase genes. Bootstrap values are shown for selected nodes (only for those with a frequency greater than 70%), * denotes H5N1 viruses. All other sequences were obtained from Genbank

similarity to the internal genes of H1N1, H1N2 and H3N2 viruses circulating in European swine. With the exception of the NS gene of A/Thailand/271/2005 the remaining internal genes also appear to have originated from the European lineage. The NS gene differed in that it appears to be more closely related to North American swine viruses.

Discussion

Influenza viruses, A/Philippines/304/2004 (H1N2) and A/Thailand/271/2005 (H1N1) were isolated from patients with influenza-like illness and were found, following sequence analysis of the HA gene, to be swine-like viruses. These viruses most closely resembled viruses that have circulated in swine of North America in the 1990s and more recently in Asia. Little is known of the epidemiology of influenza in swine in the Philippines as outbreaks have not been reported to date, therefore it is unknown if this strain is representative of strains that are currently circulating in swine in the Philippines. The isolation of a swine-like influenza virus from man appears to be the first such case reported from the Philippines. Thailand first reported the isolation of influenza A H1N1 viruses in swine in 1988 and these viruses were found to be related to those prevalent in the USA, Japan and Hong Kong [23]. The NA and internal genes of the two viruses isolated in this study were found to have a closer genetic relationship to viruses circulating in European swine, which appear to have undergone multiple reassorting events [30]. The isolation of influenza A/Philippines/304/2004 and A/Thailand/271/2005 provides further evidence of swine to human transmission of viruses which are genetically distinct from those circulating in the human population and for which there would be expected to be little protective immunity in the population. The emergence of H3N2 and H1N2 swine viruses in the US with genomes composed of influenza A genes of human, swine and avian origin coupled with the relative ease with which this reassortment occurs in swine, reinforces the potential for the emergence of multiple origin reassortants with the capacity to become established in the human population. The amantadine resistant phenotype

which has been evident in swine viruses for some time and in human influenza A(H1) and A(H3) viruses recently, [28, 31] was also present in these two viruses. These viruses were however sensitive to the newer neuraminidase inhibitor antiviral drugs, oseltamivir and zanamivir.

This report highlights two recent infections of humans with swine influenza viruses, who both appeared to recover without further complications following their illness. No other cases of swine influenza were identified in humans at a similar time in these locations, suggesting that there was no extensive human to human transmission, however further transmission cannot be excluded as no comprehensive analysis or serological survey was conducted at either site. It is not known how the swine influenza viruses were contracted, as neither patient appeared to have direct contact with swine, however, as pigs are commonly raised in backyard farms in these regions incidental contact with pigs cannot be excluded. While such infections with swine influenza viruses appear to be relatively rare, they highlight the potential for these viruses to cross the species barrier and possibly reassort with other influenza viruses to enable the efficient transmission of novel viruses from person to person. Such an event has the potential to initiate a new pandemic as was feared in the USA in 1976 [32]. While a pandemic did not eventuate from this outbreak, the fact that man has little or no immunity against infections with many animal influenza A viruses, still raises the possibility that novel influenza A viruses new to the human population that are able to efficiently transmit from person to person and cause illness, may represent a pandemic threat. As such human infections with animal influenza viruses should be further investigated where possible to determine the genetic composition and origin of the virus, the source of infection, the extent of the spread and any evidence of human to human transmission.

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