

Molecular Epidemiology of Avian Influenza H5N1 in Thailand

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ABSTRACT: Avian influenza H5N1 epidemics have been reported in Asia since 2003. Early in 2004, this virus emerged in Thailand, which necessitated the culling of nearly one hundred million domestic poultry. The distinctive feature of this virus is that it can cross from avian species to humans. Molecular characterization of Avian influenza virus H5N1 from this outbreak showed evidence of HPAI on the cleavage site of the H gene (poly basic amino acids in HA), a 20 codon deletion in the neuraminidase gene (stalk region), a 5 codon deletion in the NS gene and mutations or polymorphisms in the M2 gene leading to Amantadine resistance. Our group analyzed the entire genome sequences from multiple virus isolates in Thailand between 2004 and 2007 and concluded that H5N1 can be divided into two clades. The avian influenza outbreaks appeared to emerge in five major episodes between 2004 and 2007 with the first three waves caused by genotype Z, clade I. During the last two waves in Thailand in 2006-2007, two genotypes, V and Z, clades 1 and 2, subclade 3 predominated with surveillance still in progress. The resulting data might be crucial for developing preventive measures, such as an efficient vaccine in order to prepare the population for the potential occurrence of a pandemic of avian influenza.

KEYWORDS: Epidemiology, Avian influenza, H5N1, Thailand.

Whenever an epidemic strikes, it is essential to coordinate organizations most qualified to study the etiology of the underlying infection and thus ensure prompt treatment, effective management and instigation of preventive measures. The spread of avian influenza A subtype H5N1 in Thailand served as a prime example that drew attention to research collaboration. Since 2004, our group's aim has been to explore the characteristics of H5N1 influenza virus all across Thailand¹⁻³. The data gathered will help develop diagnostic techniques for humans and animals, as well as advance research into interspecies transmission, virus resistance to antiviral drugs and annual genetic characterization of the virus, all of which will eventually lead to vaccine development suitable for humans and animals.

Avian influenza A subtype H5N1 has been reported to have emerged in Hong Kong in 1997. From late 2003 to early 2004, the virus reached endemic levels among poultry in several south-east Asian countries and spread to Europe and Africa during 2005, with H5N1 virus infected birds discovered in more than 50 countries. The 2004-2007 outbreaks in various countries have highlighted the highly pathogenic avian influenza (HPAI) subtype H5N1 virus as the cause of a major epidemic, with potentially vast repercussions on economics, public health and society at large. Not only has this AI virus infected poultry but it has also proven highly pathogenic and fatal to mammalian species including humans and other mammals⁴⁻⁷. By July 2007, many countries had been affected by the spread of

influenza H5N1 virus infections in poultry, and transmission to humans engenders a high mortality rate with nearly 60% of cases diagnosed as H5N1 infection having proved fatal.

Avian influenza virus subtype H5N1 are type A influenza viruses and thus, members of the *Orthomyxoviridae* family. The genome of this virus consists of eight unique segments of single-stranded RNA of negative polarity. The eight influenza A viral RNA segments encode 10 recognized gene products. These are PB1, PB2, and PA polymerases, HA, NP, NA, M1 and M2 proteins, and NS1 and NS2 proteins. HA and NA are important antigenic determinants, with HA facilitating virus entry into the host cell via its attachment to cell surface sialic acid receptors. NA catalyzes cleavage of glycoside linkages to sialic acid on the host cell and virion surfaces, thus preventing aggregation of virions and facilitating the release of progeny virus from infected cells. Further subtyping of influenza A viruses is based on antigenic differences between the two surface glycoproteins HA and NA. Up to now, 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) of influenza A viruses have been identified.

The 2004-2007 outbreaks of highly pathogenic avian influenza (HPAI) subtype H5N1 virus in Thailand occurred in 5 episodes with 17 out of 25 infected humans succumbing to the disease. In the first episode between January and May 2004, the genome sequence analysis of H5N1 avian Influenza A was promptly reported¹⁻⁶ from viruses isolated from poultry, wild and domestic bird populations, a domestic cat infected by

eating a pigeon carcass, a tiger and leopard from Suphanburi province in Thailand. Upon molecular characterization, the HA gene revealed a characteristic common to all highly pathogenic AI (HPAI) viruses, a 20-codon deletion in the neuraminidase gene, a 5-codon deletion in the NS gene and polymorphisms of the M2 gene, amantadine resistance, and a single amino acid substitution at position 627 of the polymerase basic protein 2 (PB2). Moreover, the HA and NA genes of the Thai avian influenza virus displayed high similarity to those of the AI viruses isolated from human cases during the same epidemic. Subsequently, our group developed a rapid single-step multiplex RT-PCR based on conventional PCR and real-time PCR for influenza A virus subtype H5N1 detection, in order to screen the copious amount of samples^{8,9}. Based on virulence, the H5 influenza virus subtype can be further differentiated into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). The HPAI virus causes systemic lethal infection, which can kill numerous birds rapidly, whereas it is uncommon for the LPAI virus to generate outbreaks of severe disease. Hence, morbidity and mortality rates of LPAI virus infections are lower than those of HPAI viruses. Due to different post-translational proteolytic cleavage of the HA precursor molecule (HA0) into HA1 and HA2 subunits, LPAI do not contain a series of basic amino acids at the protease cleavage site but are cleaved by proteases localized in respiratory and intestinal organs, resulting in mild localized infections. In contrast, the HA of HPAI virus harbors multiple basic amino acids at the cleavage site, for example RERRRKKR, which are cleaved by ubiquitous proteases in a wide range of organs, resulting in lethal systemic infection. The method of choice to discriminate between HPAI and LPAI is sequencing, however, use of one-step real-time RT-PCR with melting curve analysis was attractive for large-scale screening of samples suspected of subtype H5N1 influenza A virus during outbreaks, to identify candidate LPAI that could be used as vaccine strains¹⁰. The details of the cleavage site of 2004-2007 HPAI isolated were shown in Figure 1.

The second wave of the outbreaks occurred between July 2004 and April 2005 during which time avian influenza H5N1 virus was most widespread among poultry in Thailand. In October 2004, H5N1 virus

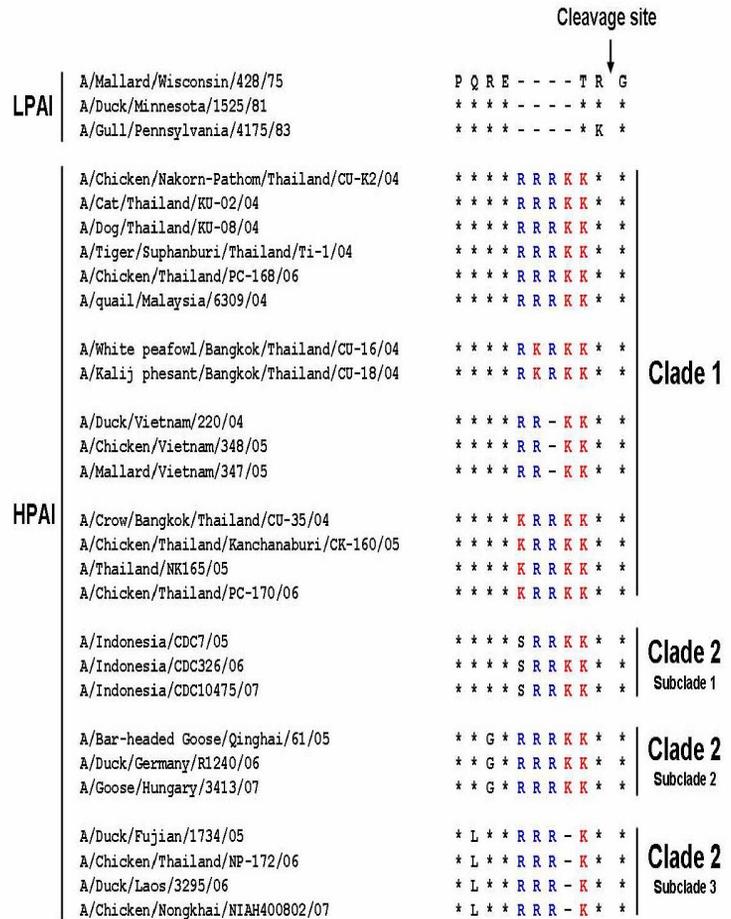


Fig 1. Alignment comparison of amino acid sequences of the H5N1 HA cleavage sites. Available sequences of LPAI and HPAI viruses from GenBank since 1975 to 2007 (LPAI, Low pathogenic avian influenza; HPAI, High pathogenic avian influenza).

caused major devastation in a tiger zoo in Sriracha, Chonburi where 147 out of 441 tigers either died or had to be culled. The animals had been fed raw chicken carcasses that were possibly contaminated with the HPAI H5N1 virus. Microscopic findings showed moderate congestion of the brain with mild nonsuppurative meningoencephalitis, severe diffuse lung hemorrhage and edema, and moderate multifocal necrotizing hepatitis. Approximately 12 days after stopping the feeding of the tigers with raw chicken carcasses, these symptoms were still present. Moreover, except for the tigers no other avian or mammalian species kept in the zoo had been infected during this outbreak. Hence, after cessation of feeding raw chicken carcasses, the tigers were probably infected by horizontal transmission¹¹. Administration of oseltamivir therapy might suppress H5N1 virus and prolong its incubation period but this is unlikely. Not only is avian influenza H5N1 virus known to cross the species barrier and infect humans and felines; in this period, fatal

H5N1 infection in a dog following ingestion of an H5N1-infected duck was reported from Suphanburi province. H5N1 influenza virus can be isolated from lung, liver, kidney, and urine of dog's specimens and this study is the first report of H5N1-related systemic disease in a domestic dog⁶. The studies performed on felines and canines demonstrate that H5N1 virus infection causes systemic disease and can spread within and between mammalian hosts. Although no direct transmission of H5N1 from cats to humans has been reported, the possibility of humans acquiring H5N1 infection from direct contact with infected cats and dogs warrants concern and highlights the necessity for monitoring domestic animals during H5N1 outbreaks

The third wave of the outbreaks occurred between July and November 2005. The H5N1 viruses in this period were investigated following isolation from one human and three poultry cases. A plasma sample from an H5N1 infected patient, initially stored at -20°C for 12 days and then stored at -70°C, was processed for virus isolation by embryonated egg inoculation for 48 hours, and the allantoic fluid was shown to contain 2,048 hemagglutinin (HA) units. The entire genome was sequenced and compared with chickens and quail from this particular outbreak. Sequence analysis of eight gene segments revealed that the 2005 H5N1 viruses isolated in October 2005 were closely related to those recovered from chicken, tiger(s) and human(s) between January and July 2004². Furthermore, we discovered genetic alterations at the HA cleavage site of the AI isolates. Ubiquitous administration of Tamiflu (Oseltamivir), which has been stockpiled in many countries potentially affected by the influenza A virus subtype H5N1 epidemic, has led to the emergence of oseltamivir resistant H5N1 viruses. In order to identify this mutation in Oseltamivir-treated patients, a method based on real-time PCR using two labeled TaqMan probes was developed to detect the substitution of amino acid H274Y in the Neuraminidase gene of H5N1 influenza A virus in many species and various specimens with high sensitivity and specificity^{12,13}.

The fourth wave began on July 23, 2006 and had spread by July 29, 2006. These outbreaks affected

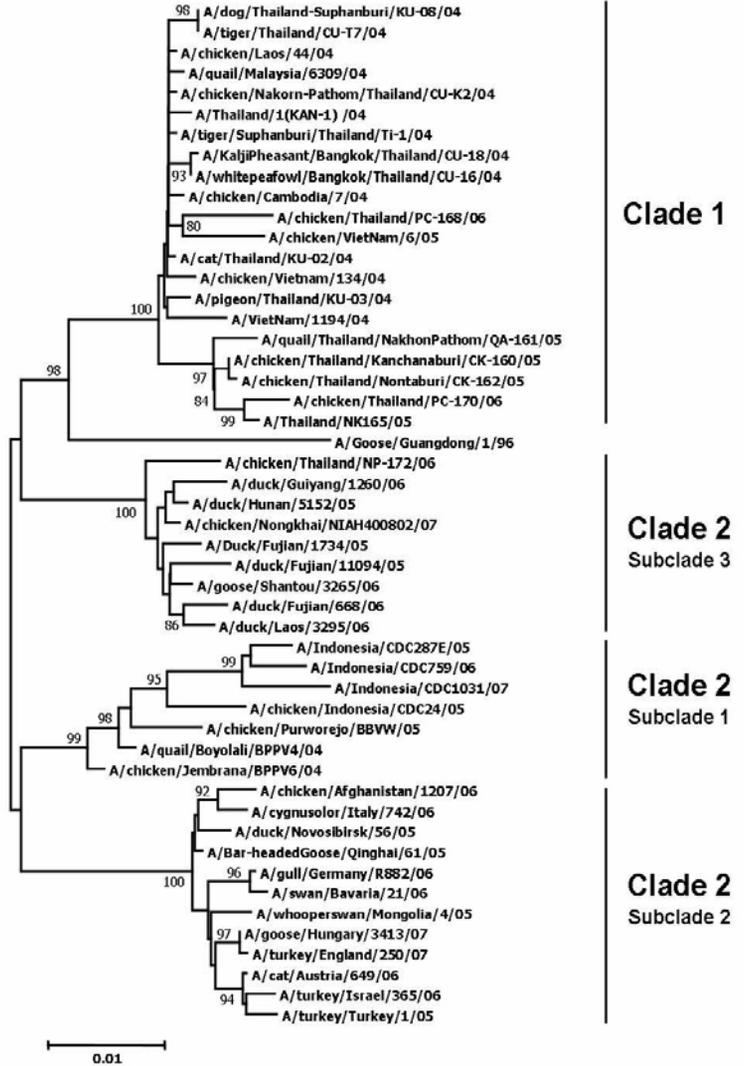


Fig 2. Phylogenetic analysis among hemagglutinin (HA) genes from H5N1 avian influenza viruses which were inferred from nucleotide sequences by the neighbor-joining method using MEGA 3.1 program. The clusters of H5N1 virus in the year of 2004 to 2007 are able to distinguish to clade 1 and clade 2 including 3 subclades.

chickens and encompassed 2 distinct areas: Pichit and Nakhon Phanom Province. We sequenced all 8 gene segments of three virus isolates obtained from both provinces. Whole genome analysis showed that all three samples had undergone minor mutations typical of circulating influenza A viruses. Suddenly, this outbreak was associated with two strains of the virus. The samples from Pichit closely resembled H5N1 strains that had circulated in Thailand during 2004 and 2005, but the samples from Nakhon Phanom were newly emerged in Thailand and more closely related to H5N1 strains, that had been circulating since 2005 in south-east China and Laos. Phylogenetic analysis also showed that the viruses isolated from Pichit belonged

to genotype Z, whereas virus isolated from Nakhon Phanom belonged to genotype V³. According to previous World Health Organization reports, the HA sequences of most influenza (H5N1) viruses, that circulated in avian species during the past three years, are separated into two distinct phylogenetic clades. In Thailand, H5N1 influenza virus isolated during the four episodes of the outbreak can be separated into Clade 1 and Clade 2 subclade 3 (Figure 2).

The most recent outbreaks occurred between January and March 2007 affecting four provinces: Ang Thong, Phitsanulok, Nong Khai and Mukdahan. Isolates from this episode can be divided into two genotypes, genotype V and Z, Clade 1 and Clade 2 subclade 3, as during the fourth wave.

The H5N1 influenza virus epidemics have provided us with crucial insight. Some evidence has pointed to probable virus transmission among humans in Thailand, Vietnam and Indonesia, as well as transmission among tigers. Symptomatic H5N1 avian influenza infection in humans and mammals incurred a high mortality rate. The virus is highly invasive, infecting not only the pulmonary tract, but also the extra-respiratory system such as CNS (encephalitis) and kidneys (renal failure). H5N1 avian influenza virus might acquire mutations facilitating transmission among humans or it might recombine with a common human influenza strain. Either scenario would lead to a pandemic once the virus has sufficiently adapted to humans, the majority of whom would lack the required immune response. We are convinced that additional H5N1 outbreaks will occur in the near future. To be prepared for a potential pandemic, extensive influenza surveillance and influenza related research aimed at rapid and accurate diagnosis, vaccine development and production, and antiviral therapy are imperative.

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