Pakistan J. Zool., vol. 43(5), pp. 927-931, 2011.

Entire Genome Sequence Analysis of Avian Influenza Virus Isolate A/Mallard/ZhaLong/88/04(H4N6)

Chai Hongliang[1,3], Yang Siyuan[1,2], Hou Zhijun[1], Xing Mingwei[1], Sun Ying[1] and Hua YuPing[1,3]*

[1] *College of Wildlife Resources, Northeast Forestry University, Harbin 150040, China*

[2] *College of ethical profession of HeiLongjiang, Harbin 150081, China*

[3]*Key Laboratory of Wildlife Conservation, China State Forestry Administration, Harbin 150040, China*

Abstract.- In 2004, an H4N6 subtype avian influenza virus (AIV) was isolated from mallard ducks (*Anas platyrhynchos*) in Zhalong Nature Reserve of Heilongjiang Province in China. The virus was named A/Mallard/Zhalong/88/04(H4N6) (MZL88/04). In this study, eight gene segments of MZL88/04 were amplified by reverse transcription polymerase chain reaction (RT-PCR). Nucleotide and deduced amino acid sequence homology and phylogenetic relationships were determined between MZL88/04 and other H4N6 subtype isolates. Isolate MZL88/04 was classified into the Eurasian lineage, and was closely related to isolates from Nanchang, indicating close evolutionary relationships between the genes studied. To our knowledge, this is the first report of an H4N6 subtype AIV strain isolated from wild birds in the mainland of China.

Keywords: H4N6, avian influenza virus, wild birds, ZhaLong.

INTRODUCTION

The H4N6 subtype of avian influenza virus (AIV) has not consistently been reported to cause highly pathogenic avian influenza (HPAI). However, in 1999, pigs that potentially had contact with wild birds became infected naturally with H4N6 AIV, resulting in animal mortality on a Canadian commercial swine farm (Karasin *et al.,* 2000b). Since pigs can serve as intermediate hosts for the mammalian adaptation of AIVs, subsequent infection of H4N6 AIV in other mammals, including humans, can potentially occur. Hence, studying the H4N6 subtype of AIV is beneficial to both the social economy and public health.

Wild birds are proven natural hosts of AIV, with all 16 hemagglutinin (HA) subtypes and nine neuraminidase (NA) subtypes of AIV having been isolated from various bird species (Webster *et al.,* 1992; Fouchier *et al.,* 2005). Long-term epidemiological investigation has indicated that, in many cases, H4N6 subtype is the predominant subtype of AIV that parasitizes wild waterfowl in many areas of North America and Asia (Shortridge, 1992; Hanson, 2003).

In August 2004, an H4N6 subtype of AIV was isolated from mallard ducks (*Anas platyrhynchos*) collected in the ZhaLong Nature Reserve in China, and subsequently named A/Mallard/ZhaLong/88/04(H4N6) (MZL88/04) (Hua *et al*., 2005). Previous investigation indicated that H4N6 subtype was the most abundant subtype of AIV in wild birds in Taiwan and poultry in Hong Kong and southern China (Shortridge, 1992; Cheng *et al*., 2004). However, the H4N6 subtype of AIV has not been isolated from wild birds in the mainland of China. In our study, entire genes of the isolate MZL88/04 were cloned and comparative analysis was conducted based on nucleotide sequences and their deduced amino acid sequences to assess if this isolate was highly pathogenic and infectious to mammals or birds. This study further provides valuable experimental data for future research into the molecular epidemiology of avian influenza.

MATERIALS AND METHODS

Virus

From September 2003 to May 2005, staff from the Research Section of Wild Animal Diseases in Northeast Forestry University collected 429 wild

* Corresponding author yuping_hua@126.com Chai Hongliang and Yang Siyuan contributed equally to this work as co-first authors

0030-9923/2011/0005-0927 $ 8.00/0

Table I.- Primer sequences used for RT-PCR

RT primer		AGCAAAAGCAGG
Genes	Forward primer	Reverse primer
HA1-3	ATGCTATCAATCGCGATTTTGTT	CTTGCCAGCCATTCTCTATGAAC
HA2-4	CCCAAATATGTCAAACAGGGATC	CTTTAAATGCAAATCTGGCACCG
PA1-3	AGCAGGTACTGATCCAAAATGGAAG	AATGTGTTCAATTGGTGCAACGTC
PA2-4	AATTCAACAAAGCATGCGAATTGAC	GCXAATAGTAGCATTGCCACAAYT
PB11-3	CATTTGAATGGATGTCAATCCGA	AAXTCATCAGAGGATTGGAGTCCG
PB12-4	AGGACAAAAGAGGTACACCAAAAC	TTCATGAAGGACAAGCTAAATTCAC
PB21-3	GCAGGTCAAATATATTCAATATGGA	GCTCTATTGACAAAXTTCAGATCXC
PB22-4	CATGGTXTTTTCACAAGAGGATTGC	ACAATTCGACACTAATTGATGGC
M	AGCAAAAXCAGGTAGATXTTGAA	ACAAGGTAGTTTTTTAYTCCAXC
NS	AAGCAGGGTGACAAAAACA	TAGAAACAAGGGTGTTTTTTATCA
NP	AAAAGCAGGGTAGATAATCACTC	TTCTYYAATTGTCATAYTCCTCTGC
NA	ATGAATCCAAATCAGAAGATAATATGC	CTACTTAAAGTAGATGATTTCAGCC

* X=A/G Y=C/T

bird specimens of 72 species in Heilongjiang Province, among which a H4N6 subtype avian A influenza virus named A/Mallard/ZhaLong/88/04 (H4N6) (MZL88/04) was isolated. Virus stocks were propagated in specific pathogen-free chicken eggs, and the allantoic fluids were harvested and stored at -70°C.

RT-PCR method

Twelve primers pairs were designed and synthesized according to the relevant nucleotide sequences in GenBank and used for amplifying Hemagglutinin (HA), Neuraminidase (NA), polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), Nucleoprotein (NP), Matrix protein (M), Nonstructural protein (NS) genes of isolate MZL88/04. HA, PA, PB1 and PB2 genes were all divided into two fragments to be amplified. The detailed sequences of primers were listed in Table I.

Viral RNA was obtained from samples of allantoic fluid using TRIzol LS reagent (Invitrogen, USA). The eight MZL88/04 genes were amplified by RT-PCR. HA, PA, PB1 and PB2 were each amplified as two fragments.

Cloning, identification and sequencing of PCR products

Purified cDNA was inserted into the PMD18-T vector (TaKaRa, Dalian, China), and the competent DH5-α *Escherichia coli* was transformed with recombinant plasmid. Three positive recombinant plasmids for each gene segment were sequenced by Shanghai Sangon Company (Shanghai, China).

Sequences analysis

Multiple sequences was aligned using Clustal X (version 1.81) and phylogenetic trees were generated with MEGA package, version 4.0.2 using the neighbor-joining algorithm. Bootstrap values of 1,000 were used.

RESULTS

Amplified genes were successfully sequenced and sequences were submitted to the GenBank database (accession numbers FJ349247–FJ349254). The eight sequences were analyzed and compared with related H4N6 isolates from GenBank using MEGA package, version 4.0.2. Examples indicative of the HA, M, NP, NS, PA and PB1 gene phylogenetic analyses are shown in Figure 1 and that of the NA and PB2 gene phylogenetic analyses are shown in Figure 2.

DISCUSSION

Influenza A virus can generally be divided into two branches, the Eurasian lineage and North American lineage. Alaska Lake is the main region of propagation of the North American lineage, whereas southern China in Asia is the reservoir of the Eurasian lineage. Many unique environmental

characteristics have created a biological niche for this virus. These include a vast water area where migratory birds breed and live in winter, a warm and humid climate for virus preservation, and open contact among human, pigs, poultry and waterfowl. Furthermore these reservoirs of infection are located on international bird migratory routes. As a result, novel influenza virus strains can potentially be preserved and transmitted among poultry, waterfowl, other wild birds, pigs and human. ZhaLong Nature Reserve, where the virus MZL88/04 was isolated, is also on the same bird migratory route as these reservoirs.

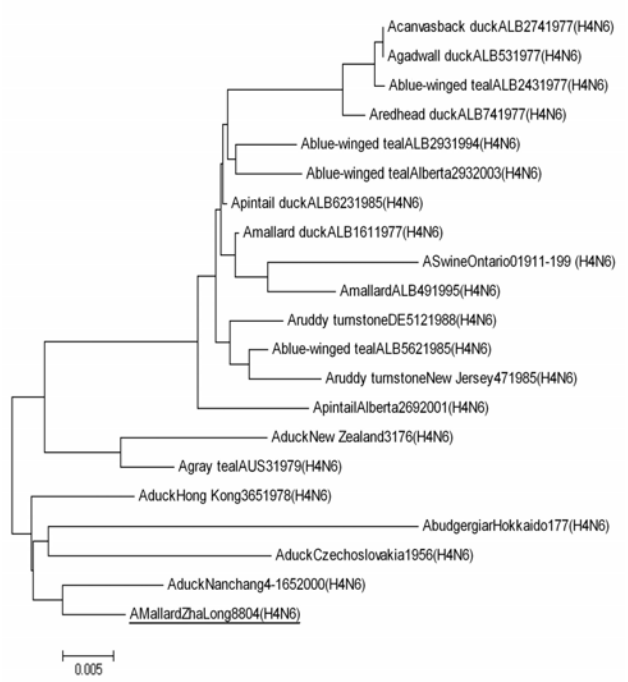

Fig. 1. Phylogenetic tree of the HA gene based on the neighbor-joining algorithm. Scale bar indicates nucleotide substitutions per position.

ZhaLong Nature Reserve, located in the Songnen Plain in the western part of Heilongjiang Province, is one of the largest national nature reserves in China. It has widespread reed swamps, acts as a conservation area for waterfowl such as cranes, and also facilitates the preservation of swamp ecosystems. Therefore, this reserve has become an important breeding and migratory resting place for birds that live in the south during winter and breed in the north in spring.

AIV has become increasingly important in recent years because of its potential as a human pathogen. Of particular interest is whether bird specific subtypes of influenza virus, especially those not producing HPAI, have the capacity to develop into pandemic human influenza.

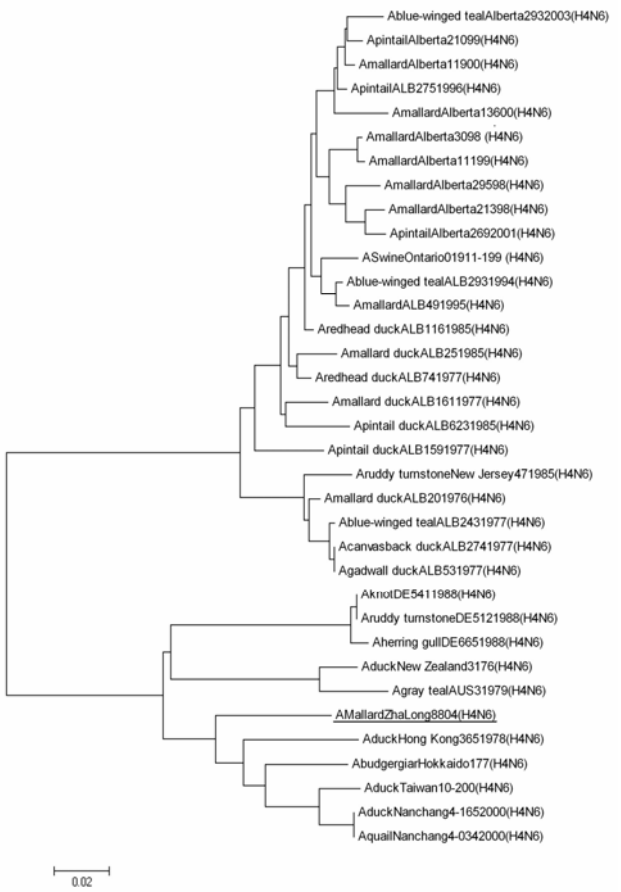

Fig. 2. Phylogenetic tree of the NA gene based on the neighbor-joining algorithm. Scale bar indicates nucleotide substitutions per position.

In an effort to monitor avian influenza in migrating birds, 10,945 fecal samples were collected for virus isolation between 1998 and 2002, and 232 AIV isolates were obtained. Up to 51% were identified to be H4N6 virus, which was deemed the most common subtype (Cheng MC *et al*., 2004). At least 10 subtypes of influenza A virus were isolated

from ducks, geese and chickens in Hong Kong and southern China between 1975 and 1980 for epidemiologic investigation. It was discovered that the frequency (in decreasing order) of different HA subtype viruses isolated from ducks was H4 (29%), H3 (25%), H6 (22%), H10, H5, H9, H11, H2, H1 and H7, respectively (Shortridge, 1992). These results further indicate that H4N6 or H4 AIV exist extensively in wild waterfowl and poultry in Taiwan and southern China, although isolate MZL88/04 is reportedly the first strain of H4N6 subtype AIV isolated from wild birds in the mainland of China.

Sequence homology and phylogenetic analysis

Comparative sequence homology analysis and the assessment of phylogenetic relationships indicated that the HA, M, NP, NS, PA and PB1 genes of isolate MZL88/04 were all closely related to those observed in isolates from Nanchang (China). Although each gene formed a separated branch, they may share a common ancestor. Phylogenetic trees based on the NA and PB2 genes of isolate MZL88/04 were very similar. Isolate MZL88/04 clustered within a clade that also contained isolates from Nanchang, Hong Kong, Taiwan and Hokkaido (Japan). Thus, isolate MZL88/04 can potentially be classified within the Eurasian lineage.

Speculation on viral pathogenicity

The discovery of H4N6 AIV in pigs in Canada in 1999 (Karasin *et al*., 2000a; Hatta *et al*., 2001) suggests that this virus can induce illness in a porcine host and has the ability to spread from pig to pig (Karasin *et al*., 2000b; Christopher, 2002). Outbreaks of this AIV in pigs are of both veterinary and human health concern, especially considering the potential for these H4N6 viruses, or H4 reassortment viruses, to enter the human population.

The HA of MZL88/04 does not contain any additional basic amino acids at the putative HA1/HA2 cleavage site (K-A-S-R/G), compared with other H4 HAs. As a result, it can be concluded that MZL88/04 does not cause HPAI, considering the virus infection did not result in SPF embryo mortality.

Speculation on viral host range

Reverse genetics studies have shown that residue 627 in the PB2 protein influences the outcome of infection in mice. Moreover, high cleavability of the HA glycoprotein is considered an essential requirement for lethal infection (Subbarao *et al*., 1993; Chen *et al*., 2004). The PB2 protein of MZL88/04 was classified as 627E, which binds preferentially to the receptors of birds.

Although the exact effect of the receptor component-binding site on the range of hosts is not clear and the appetency of the cell is still uncertain, most human isolates were classified as HA-196H and most isolates from birds were HA-196N (Weis *et al*., 1988). Previous studies suggest that mammals can become infected with H4 AIV (Hinshaw *et al*., 1984; DONIS *et al*., 1989; Webster *et al*., 1992), and all 33 isolates in Genbank, including isolate MZL88/04, were classified as HA-196H. Consisting of isolates from human, and as a result, H4 AIV has the possibility of infecting humans.

However, it should also be noted that the detection of AIV of H4N6 subtype in certain mammal species does not necessarily mean that this subtype can also infect humans or other mammals. Rather, the mechanism of infection might relate to the characteristics of a species or individual, which require time for adaption to the host. Additional supporting data are therefore required before it can be concluded whether the H4N6 subtype of AIV can infect humans.

ACKNOWLEDGMENTS

This research was funded by Northeast Forestry University (07042) and supported by the 'Fundamental Research Funds for the Central Universities' (DL10BA03). We greatly appreciate the support of the ZhaLong Nature Reserve, SanJiang Nature Reserve and Harbin Veterinary Research Institute.

REFERENCES

CHEN, H., DENG, G., LI, Z., TIAN, G., LI, Y., JIAO, P., ZHANG, L., LIU, Z., WEBSTER, R.G. AND YU, K., 2004. The evolution of H5N1 influenza viruses in ducks in southern China. *Proc. natl. Acad. Sci. USA.*, **101**: 10452-10457.

CHENG, M.C., WANG, C.H. AND KIDA, H., 2004. Influenza A virological surveillance in feral waterfowl in Taiwan

from 1998 to 2002. *Int. Congr. Ser*., **1263**: 745-748.

CHRISTOPHER, W. AND OLSEN, C.W., 2002. The emergence of novel swine influenza viruses in North America. *Virus Res.*, **85**: 199-210.

FOUCHIER, R.A., MUNSTER, V., WALLENSTEN, A., BESTEBROER, T.M., HERFST, S., SMITH, D., RIMMELZWAAN, G.F., OLSEN, B. AND OSTERHAUS, A.D., 2005. Characterization of a novel influenza A virus hemaglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.,* **79:**2814-2822.

HANSON, B.A., STALLKNECHT, D.E., SWAYNE, D.E., LEWIS, L.A. AND SENNE, D.A., 2003. Avian influenza viruses in Minnesota ducks during 1998–2000. *Avian Dis*., **47**: 867-871.

HATTA, M., GAO, P., HALFMANN, P. AND KAWAOKA, Y., 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science,* **293:**1840–1842.

HINSHAW, V.S., BEAN, W.J., WEBSTER, R.G., REHG, J.E., FIORELLI, P., EARLY, G., GERACI, J.R. AND ST AUBIN, D.J., 1984. Are seals frequently infected with avian influenza viruses? *J. Virol*., **51**: 863-865.

KARASIN, A.I., BROWN, I.H., CARMAN, S. AND OLSEN, C.W., 2000a. Isolation and Characterization of H4N6 Avian Influenza Viruses from Pigs with Pneumonia in Canada. *J. Virol*., **74**: 9322–9327.

KARASIN, A.I., OLSEN, C.W., BROWN, I.H., CARMAN, S., STALKER, M. AND JOSEPHSON, G., 2000b. H4N6 influenza virus isolated from pigs in Ontario. *J. Can. Vet. J.*, **41**: 938–939.

DONIS, R.O., BEAN, W.J., KAWAOKA, Y. AND WEBSTER, R.G., 1989. Distinct lineages of influenza virus H4 hemagglutinin genes in different regions of the world. *Virology,* **169:**408-417.

SHORTRIDGE, K.F., 1992. Pandemic influenza: a zoonosis? *Sem. Resp. Infect.,* **7:** 11-25.

SUBBARAO, E.K., LONDON, W. AND MURPHY, B.R., 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J. Virol.,* **67:** 1761-1764.

WEBSTER, R.G., BEAN, W.J., GORMAN, O.T., CHAMBERS, T.M. AND KAWAOKA, Y., 1992. Evolution and ecology of influenza A viruses. *Microbiol. mol. Biol.,* **56:**152-179.

WEIS, W., BROWN, J.H., CUSACK, S., PAULSON, J.C., SKEHEL, J.J. AND WILEY, D.C., 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature,* **3:**426-431.

HUA, Y.P., CHAI, H.L., YANG, S.Y., ZENG, X.W. AND SUN, Y., 2005. Primary survey of AIV and NDV infection in wild birds in some areas of Heilongjiang Province. *J. Vet. Sci.,* **6:**311-315.

(Received 13 January 2011, revised 12 April 2011)