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Genetic Analysis of the Nonstructural (NS) Genes of H9N2 Chicken Influenza Viruses Isolated in China During 1998–2002

SHUAI WANG,[1] WEI-MIN SHI,[1] AARON MWEENE,[2] HUI-LING WEI,[1] GUI-RONG BAI[2] & JIN-HUA LIU[1,*]

[1]*Department of Infectious Disease, College of Veterinary Medicine, China Agricultural University, 100094, Beijing, People's Republic of China*

[2]*Laboratory of Microbiology, Department of Disease Control, Hokkaido University Graduate School of Veterinary Medicine, 060-0818, Sapporo, Japan*

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Abstract. H9N2 subtype avian influenza viruses are widespread in domestic poultry. Genetic analysis indicated that three lineages of H9N2 viruses have been established in Eurasia and only one lineage is present on chicken farms in mainland China. Here, NS1 genes of eight H9N2 chicken influenza viruses, isolated in mainland China during 1998–2002, were completely sequenced and phylogenetically analyzed. By comparison, the homology of the NS1 of the A/chicken/Neimenggu/ZH/02 (Ck/NM/ZH/02) strain had a high identity (93.8%) with that of A/chicken/Korea/323/96 (Ck/Kor/323/96), which is an A/duck/Hong Kong/Y439/97 (Dk/HK/Y439/97)-like virus. NS1 peptides of seven strains possessed 217 amino acids, while that of the strain Ck/NM/ZH/02 coded 230 amino acids. Except for the amino acid at position 225, the additional amino acid sequence (13 AAs) of NS1 of Ck/NM/Zh/02 at the carboxy-terminus is identical with that of Ck/Kor/323/96. Phylogenetic analysis showed that seven of the tested strains belong to the A/duck/Hong Kong/Y280/97 (DK/HK/Y280/97)-like lineage, while the NS1 gene of Ck/NM/Zh/02 belongs to the Dk/HK/Y439/97-like lineage and has a close relationship with that of the Ck/Kor/323/96-like viruses. Therefore, although most of the H9N2 influenza viruses circulating on chicken farms in mainland China belong to the DK/HK/Y280/97-like lineage, the present results indicate that the other two of the three H9N2 lineage viruses also circulate in the chicken population in mainland China.

Key words: avian influenza virus, genetic analysis, H9N2, NS1 gene

Introduction

The genome of avian influenza virus comprises eight separate segments of single-stranded negative-sense RNA. The smallest of these gene segments encodes two nonstructural proteins, non-structural protein 1 (NS1), which is not present in mature virions [1,2] and non-structural protein 2 (NS2) of which small amounts are packaged into the viral particle [3,4]. Encoded by a co-linear mRNA, NS1 consists of 124–237 amino acids, depending on the virus strain. Splicing of the NS1 mRNA leads to the formation of the NS2 mRNA which encodes the NS2 that comprises 121 amino acids [5]. The NS genes of influenza viruses are different from the other gene segments and are divided into two groups called alleles A and B [6]. Allele A accounts for viruses from human, equine, swine and avian species, whereas allele B comprises one equine and many avian influenza isolates [7,8]. Nucleotide sequence similarities within alleles A and B have been found to be 86.5–99.4% and 89.4–99.6%, respectively, and at most only 72.3% between the alleles [9]. There

*Author for all correspondence:
E-mail: ljh@cau.edu.cn

exists a strict correlation between the year of isolation of the strain and evolution of the human H3N2 influenza virus under selection pressure [10]. Three distinct lineages of NS1 genes of H9N2 influenza viruses have been observed in Hong Kong [11]. The H9N2 influenza viruses have been the most prevalent in the chicken population and have caused great economic losses in mainland China since 1992. Domestic researchers mostly focus on the study of the haemagglutinin (HA) gene and the immunity of H9N2 influenza viruses [12–14]. In contrast, there have been only a few studies of the NS1 genes of these viruses. Therefore, in this study, the NS1 genes of eight strains of H9N2 influenza viruses isolated from diseased chickens during 1998–2002, in mainland China, were sequenced and analyzed. This was to address issues concerning the gene constellation that the NS1 genes of H9N2 influenza viruses prevailing in mainland China belong to, the characteristics of the NS1 genes of the isolates and the relationships of these NS1 genes with those of H9N2 influenza viruses prevalent elsewhere.

Materials and Methods

Viruses

Eight H9N2 influenza virus strains used in this study were isolated from diseased or dead chickens in Beijing, Hebei, Jiangsu, Neimenggu, Yunan and Henan provinces during 1998–2002. Fig. 1 illustrates the locations where the viruses were isolated in China. Initial isolations of the viruses were performed in 10-day-old specific pathogen free (SPF) embryonated chicken eggs (ECE). Subtype identification of the viruses were determined by standard hemagglutination inhibition and neuraminidase inhibition assays using specific antisera to the reference strains of influenza viruses [15]. Allantoic fluid were harvested from ECE-passaged viruses and used as a stock for sequence analysis.

RNA Extraction and RT-PCR

Viral RNA was extracted from allantoic fluid by Trizol reagents (Gibco-BRL) and reverse transcription was performed with oligonucleotide

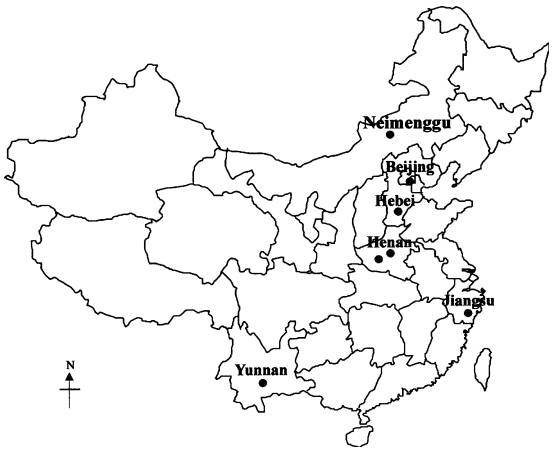

Fig. 1. Location of the 6 different places where the samples were collected in mainland China.

influenza universal primer Uni 12: 5′-AGC AAA AGC AGG-3′. After reverse transcription, cDNA was amplified by PCR as described by Shu [16]. The primers used for amplification were NSF (5′-ACA GAA TTC TAA TGG ATT CCA AC-3′) and NSR (5′-TTA CTC GAG ACT TCA AGC AAT AG-3′), corresponding to positions −2–12 and 785–798, respectively.

Gene Sequence

PCR products were purified with the QICquick PCR purification kit (Qiagen). The purified PCR products were then sequenced by BGI LifeTech Co., Ltd. Assembly of sequences, translation of nucleotide sequence into protein sequence, and initial multiple sequence alignments were performed with the DNASIS software version 3.5 (Hitachi Software Engineering Co., Ltd) and Genetyx software version 10.1 (Software Development Co., Ltd).

Phylogenetic Analysis

Phylogenetic analysis was carried out by analyzing the data obtained here with those of other sequences of influenza viruses from the GenBank database by using PHYLIP software package (http://www.-ddbj.nig.ac.jp/E-mail/clustalw-e.html) The tree was drawn using TREEVIEW software (version 1.40, Roderic D.M. Page, 1997).

Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in this study are available from GenBank under accession numbers AY912495-AY912502.

Results

Homology of Nucleotide and Deduced Amino Acid Sequences of NS1 of the Isolates

The NS1 genes of the eight H9N2 chicken influenza strains were completely sequenced. The results showed that the NS1 genes of seven of the eight isolates had the same nucleotide number, 654 bp, while that of the Ck/NM/Zh/02 strain possessed 690 bp nucleotides. The homologies of the nucleotides of the isolates were 84.4–99.9% with each other (Table 1). The amino acid sequences of the NS1 of the eight isolates were deduced from the nucleotide sequence and the identity among them was 81.7–99.5%. It is noteworthy that the homologies for the NS1 nucleotide and amino acid sequences of the Ck/NM/Zh/02 isolate with the other isolates ranged from 84.4–87.6% and 81.7–85.7%, respectively, while they had a high homology (93.8% and 91.3%, respectively) with that of the Korea isolate, Ck/Kor/323/96, suggesting that the NS1 genes of Ck/NM/Zh/02 and the Korea isolate might have a common ancestral origin.

Comparison of Amino Acid Sequences of NS1 Between the Isolates and the Reference H9N2 Viruses

Sequence analyses of the HA genes of avian H9N2 influenza viruses indicated that all the HA genes of the strains isolated in mainland China belonged to the A/duck/Hong Kong/Y280/97 (Dk/HK/Y280/97)-like lineage and shared high homology with each other [12,14]. Therefore, we chose Dk/HK/Y280/97 as a reference strain to compare its amino acid sequence with those of deduced amino acid sequences of the isolates, together with those of the A/quail/Hong Kong/G1/97 (Qa/HK/G1/97)-like viruses and the Korea H9N2 avian strain, as shown in Fig. 2. Except for that of Ck/NM/Zh/02 sequenced here, the NS1 peptides of all the other seven H9N2 avian influenza viruses sequenced in this study and those sequenced previously [17] had 217 amino acids. The NS1 of Ck/NM/Zh/02 has 230 amino acids, the same number as most of the H9N2 strains isolated elsewhere. The additional amino acid sequence (13 AAs) of the NS1 of Ck/NM/Zh/02 at the carboxy-terminus which is identical to that of Ck/Kor/323/96 except for position 225, a further indication of their close relationship.

Phylogenetic Analysis

The NS1 genes of eight H9N2 avian influenza viruses sequenced in this study and 21 NS1 genes of H9N2 reference strains obtained from the GenBank were used for phylogenetic analysis (Fig. 3). The results revealed that the NS1 genes of all the H9N2 influenza viruses isolated in mainland China were located in allele A. The NS1 genes of the Eurasian H9 viruses are divided into three different lineages, whose representative strains are A/duck/Hong Kong/Y439/97 (DK/HK/Y439/97), Qa/HK/G1/97 and DK/HK/Y280/97 [11]. Six of the virus isolates belonged to the Dk/HK/Y280/97-like lineage. The NS1 gene of Ck/BJ/Zh/02 had a sister-

Table 1. Percentage homology comparison of nucleotide sequence and deduced amino acid sequence of NS1 genes

	Ck/HN/1/98	Ck/BJ/8/99	Ck/JS/W1/00	Ck/HN/1/01	Ck/YN/3/01	Ck/BJ/Zh/02	Ck/HB/11/02	Ck/NM/Zh/02
Ck/HN/1/98		96.8	96.8	99.5	99.1	91.2	95.4	82.6
Ck/BJ/8/99	96.6		99.5	97.2	96.8	92.6	97.7	83.5
Ck/JS/W1/00	96.3	99.7		97.2	96.8	92.2	97.2	83.9
Ck/HN/1/01	99.9	96.8	96.5		99.5	90.8	95.9	82.2
Ck/YN/3/01	99.7	96.6	96.3	99.9		90.3	95.4	81.7
Ck/BJ/Zh/02	93.1	94.5	94.2	93.0	92.8		93.1	85.7
Ck/HB/11/02	96.2	98.3	98.0	96.3	96.2	95.3		83.9
Ck/NM/Zh/02	84.7	85.2	85.2	84.5	84.4	87.6	86.3	

The lower left data stands for nucleotide percentage homology while the upper right group represents amino acid percentage homology. Ck, chicken; BJ, Beijing; HN, Henan; HB, Hebei; JS, Jiangsu; YN, Yunan; NM, Neimenggu.

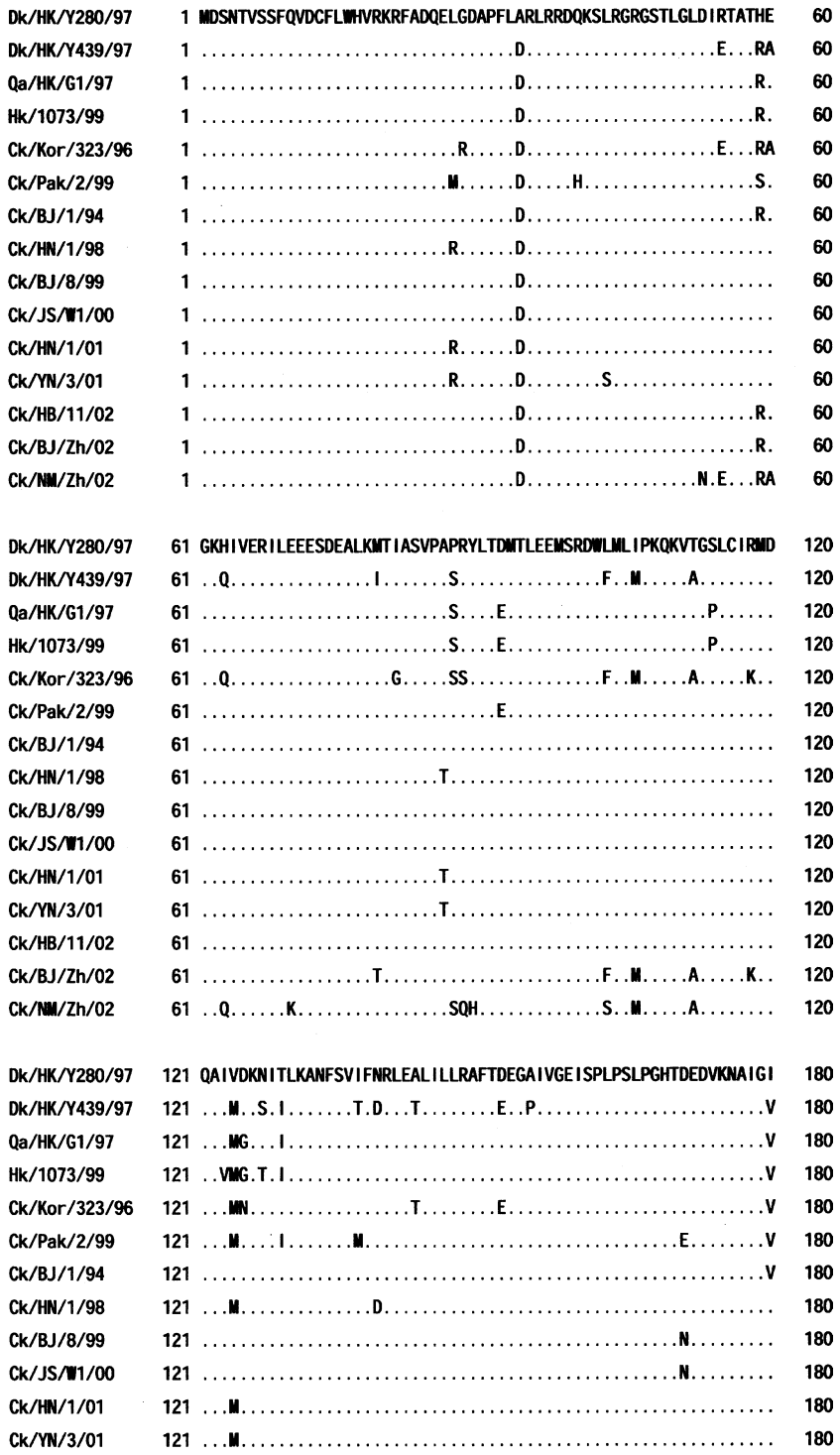

Fig. 2. Continued.

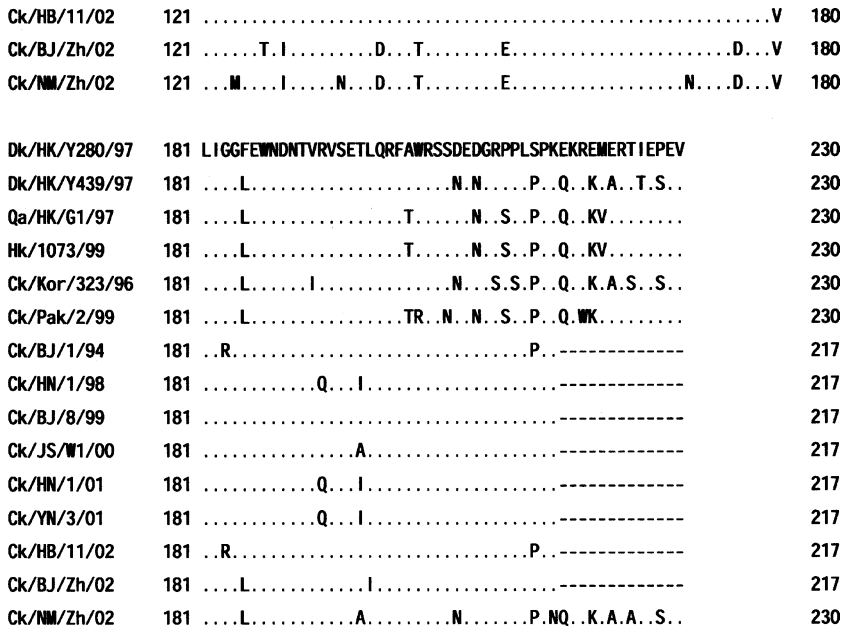

Fig. 2. Comparison of amino acid sequences of NS1 of H9N2 influenza viruses. Dots indicate residues identical to those of the Dk/HK/Y280/97 virus. Dashes indicate the deletion positions. Dk, duck; Qa, quail; HK, Hong Kong; Kor, Korea; Pak, Pakistan.

group relationship with those of most of the mainland China H9N2 viruses. None of the NS1 of the Qa/HK/G1/97-like viruses was detected among the H9 subtype viruses tested here. Remarkably, the NS1 gene of Ck/NM/Zh/02 was located in the Dk/HK/Y439/97-like lineage, to which most of the Korea H9N2 avian strains belonged. Thus, phylogenetic analysis also showed that the NS1 gene of Ck/NM/Zh/02 had a close relationship with those of the Korea H9N2 avian strains and that two of the three distinct lineages of NS1 genes of the H9N2 influenza viruses were circulating in the chicken population in mainland China.

Discussion

Since the first outbreak of the H9N2 chicken influenza occurred in Guangdong province in 1992, sporadic and endemic outbreaks of the H9N2 influenza have often occurred and caused great economic losses in many provinces from Northern to Southern China [17]. Compared with the HA genes of the H9N2 viruses isolated in mainland China, only a few phylogenetic analyses of the NS1 genes have, hitherto, been reported. In this study, the NS1 genes of eight H9N2 avian influenza viruses isolated during 1998–2002 from different farms were completely sequenced and phylogenetically analyzed. The results showed that the homology of the nucleotides and amino acids of the NS1 genes were 84.4–99.9% and 81.7–99.5%, respectively. The NS genes of avian influenza viruses are divided into alleles A and B on the basis of their nucleotide sequence homology [6]. Nucleotide sequence similarities within alleles A and B have been found to be 86.5–99.4% and 89.4–99.6%, respectively; however, the two alleles are, at most, only 72.3% similar [9]. In this study, homology as well as phylogenetic analyses indicated that all the isolates of mainland China belonged to allele A.

Except for that of Ck/NM/Zh/02, the NS1 peptides of the seven mainland China isolates had a deletion of 13 amino acids at the carboxyl terminal compared with those of the other reference strains. Shortening of the NA stalk by deletion of the amino acid is characteristic of the highly pathogenic H5 and H7 chicken influenza viruses [18]. But it is not yet clear as to whether the deletion in NS1 at the carboxy-terminus has any effect on the function of the NS1. The NS1 gene of Ck/NM/Zh/

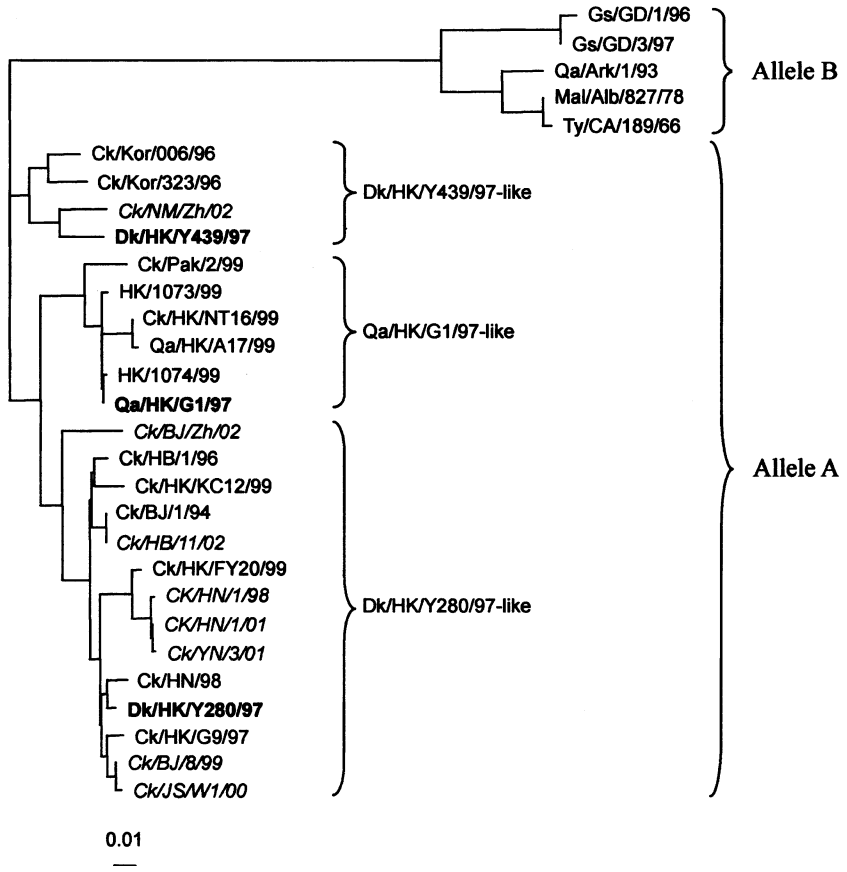

Fig. 3. Phylogenetic tree for the NS1 gene of influenza viruses. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Virus strains sequenced in the present study are in italics. The prototype viruses are in bold. Gs, Goose; Ty, Turkey; Mal, Mallard; Ark, Arkansas; Alb, Albert; CA, California; others are presented in Table 1 and Fig. 2.

02 has a high homology of nucleotides and amino acids (93.8% and 91.3%) with that of Ck/Kor/323/96 and has the same amino acid sequence (13 AAs) at the carboxy-terminus except for the position 225. These results, taken together, indicate a common ancestral origin for the two viruses.

Although three stable lineages of H9N2 viruses have been established in Asia [11], the existence in mainland China of the viruses of all the three lineages has not been reported. All of the NS1 genes of the H9N2 mainland China isolates have, hitherto, been reported to belong to the Dk/HK/Y280/97-like lineage with none of them belonging to the Qa/HK/G1/97 or Dk/HK/Y439/97 lineages. In this study, genetic analysis showed that the NS1 gene of Ck/NM/Zh/02 belonged to the Dk/HK/Y439/97-like lineage. All the gene fragments of the Ck/NM/Zh/02 were sequenced and phylogenetic analysis showed that except for the NS gene, the other seven gene fragments belonged to the Dk/HK/Y280/97-like lineage (unpublished data). The present results indicated that the Ck/NM/Zh/02 was a reassortant virus. So, what is the source of the NS1 gene? Do the Dk/HK/Y439/97-like viruses exist in the environment of those chicken farms? Previous studies have shown that the Korea H9N2 avian viruses might have been derived from the viruses isolated from migratory ducks [19]. Ck/NM/Zh/02 was isolated from a chicken in Neimenggu province, Northern China, which is located on the flyway of migration for the ducks from Siberia to the South. Aquatic birds, especially migratory ducks, appear to serve as a reservoir of all of the known subtypes of influenza A

viruses [20,21]. Although Dk/HK/Y439/97-like viruses were not found on chicken farms of the mainland China, the present results, at least, indicated their existence on those premises. Therefore, the molecular epidemiological surveillance of H9N2 viruses not only from domestic chickens but also from migratory ducks should be continued and intensified. On the other hand, the Qa/HK/G1/97-like viruses have been considered to have potential public health risks as exemplified by the donation of the internal genes to the H5N1 viruses that resulted in the Hong Kong incident in 1997 in which avian influenza A viruses transmitted directly from chickens to humans. Even though the Qa/HK/G1/97-like viruses were not actually found on chicken farms, in this study, the implications of the zoonotic risks further stress the importance of the continued and sustained surveillance for the ultimate goal of the control of influenza.

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References

1. Sarkar G., Pelletier J., Duby R.B., Jayasuriya A., Fields B.N., and Sonenberg N., J Virol *54*, 720–725, 1985.
2. Zweerink H.J., McDowell M.J., and Joklik W.K., Virology *45*, 716–723, 1971.
3. Richardson J.C. and Akkina R.K., Arch Virol *116*, 69–80, 1991.
4. Yasuda J., Nakada S., Kato A., Toyoda T., and Ishihama A., Virology *196*, 249–255, 1993.
5. Lamb R.A. and Lai C.J., Virology *135*, 139–147, 1984.
6. Treanor J.J., Snyder M.H., London W.T., and Murphy B.R., Virology *171*, 1–9, 1989.
7. Ludwig S., Schultz U., Mandler J., Fitch W.M., and Scholtissek C., Virology *183*, 566–577, 1991.
8. Guo Y., Wang M., Kawaoka Y., Gorman O.T., Ito T., Saito T., and Webster R.G., Virology *188*, 245–255, 1992.
9. Kawaoka Y., Gorman O.T., Ito T., Wells K., Donis R.O., Castrucci M.R., Donatelli I., and Webster R.G., Virus Res *55*, 143–156, 1998.
10. Ludwig S., Schultz U., Mandler J., Fitch W.M., and Scholtissek C., Virology *183*, 566–577, 1991.
11. Guan Y, Shortridge K.F., Krauss S., and Webster R.G., Proc Natl Acad Sci USA *96*, 9363–9367, 1999.
12. Liu H.Q., Cheng J., Peng D.X., Jia L.J., Zhang R.K., and Liu X.F., Acta Microbiologica Sinica *42*, 288–299, 2002.
13. Cheng J., Liu X.F., Peng D.X., and Liu H.Q., Acta Microbiologica Sinica *42*, 442–447, 2002.
14. Li X., Yu K.Z., Tian G.B., Tang X.Y., Deng G.H., Wang X.R., and Meng Q.W., Chin J Prev Vet Med *24*, 249–251, 2002.
15. Kendal A.P., Pereira M.S., and Skehel J.J., *Concepts and Procedures for Laboratory-Based Influenza Surveillance*. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, GA, 1982.
16. Shu L.L., Lin Y.P., Wright S.M., Shortridge K.F., and Webster R.G., Virology *202*, 825–833, 1994.
17. Liu J.H., Okazaki K., Ozaki H., Sakoda Y., Wu Q.M., Chen F.Y., and Kida H., Avian pathol *32*, 551–560, 2003.
18. Matrosocich M., Zhou N.N., Kawaoka Y., and Webster R.G., J Virol *73*, 1146–1155, 1999.
19. Liu J.H., Okazaki K., Shi W.M., and Kida H., Virus Genes *27*, 291–296, 2003.
20. Rohm C., Zhou N., Suss J., Mackenzie J., and Webster R.G., Virology *217*, 508–516, 1996.
21. Webster R.G., Bean W.J., Gorman O.T., Chambers T.M., and Kawaoka Y., Microbiol Rev *56*, 152–179, 1992.