JOURNAL OF VIROLOGY, Dec. 2009, p. 13015–13018 Vol. 83, No. 24
0022-538X/09/$12.00 doi:10.1128/JVI.01714-09

Ostrich Involvement in the Selection of H5N1 Influenza Virus Possessing Mammalian-Type Amino Acids in the PB2 Protein▽

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Received 15 August 2009/Accepted 14 September 2009

Amino acids at positions 627 and 701 in the PB2 protein (PB2-627 and PB2-701, respectively) of avian influenza A viruses affect virus replication in some mammalian cells. Highly pathogenic H5N1 influenza viruses possessing mammalian-type PB2-627 were detected during the Qinghai Lake outbreak in 2005 and spread to Europe and Africa. Via a database search, we found a high rate of viral isolates from *Ratitae*, including ostrich, possessing mammalian-type PB2-627 or -701. Here, we report that H5N1 avian influenza viruses possessing mammalian-type amino acids in PB2-627 or -701 are selected during replication in ostrich cells in vitro and in vivo.

Influenza A virus is a zoonotic pathogen whose natural reservoir is aquatic birds.

Outbreaks of highly pathogenic H5N1 avian influenza viruses in poultry and occasional transmission to humans have continued since the virus was first reported in 1997 in Hong Kong (8, 28), with a fatality rate of more than 60% in humans (http://www.who.int/csr/disease/avian_influenza/en/). Moreover, a poultry outbreak of highly pathogenic H7N7 avian influenza virus occurred in 2003, causing one fatality among 83 people infected (10). Efficient human-to-human transmission of these avian-origin viruses, however, has not been observed thus far, suggesting that an amino acid substitution(s) in the avian viral proteins may be required for human adaptation of avian influenza viruses.

In fact, several amino acids associated with host range restriction of influenza A viruses have been identified in viral proteins (1, 3, 4, 7, 9, 22, 24, 27), some of which were detected in avian viruses isolated from humans (2, 13, 23, 25, 29). Two amino acids in the PB2 protein, which comprises the heterotrimeric viral polymerase with the subunit proteins PA and PB1, are known to affect viral host range (11, 13, 27); most avian viruses, including the H5N1 subtype (with the exception of the descendants of the virus strain responsible for the Qinghai Lake outbreak; see below), possess glutamic acid at position 627 (PB2-627Glu), whereas most mammalian isolates have lysine at this position (PB2-627Lys). During the poultry outbreak of H7N7 avian viruses, PB2-627Lys was detected only in an isolate from a human with a fatal case of pneumonia; the other isolates, from humans with conjunctivitis and from chickens, possessed PB2-627Glu (10). Li et al. demonstrated that asparagine at position 701 of PB2 (PB2-701Asn) is responsible for the high virulence of A/duck/Guangxi/35/2001 (H5N1) virus in mice (19). Some H5N1 human isolates possess PB2-701Asn (15, 16), whereas most other viruses, regardless of their origin, possess aspartic acid at this position (PB2-701Asp). Previously, we demonstrated that H5N1 viruses possessing PB2-627Lys or PB2-701Asn are selected during replication in humans (18). Thus, amino acid substitutions at positions 627 and 701 in PB2 are important indicators for human adaptation of H5N1 viruses (9, 11, 26).

The amino acid at position 627 in PB2 is also known to affect the temperature sensitivity of viruses. That is, viruses with PB2-627Lys replicate better at the low temperature of the human upper respiratory tract (33°C) than those with PB2-627Glu, which supports efficient viral replication at higher temperatures (e.g., 37°C in the human lower respiratory tract and 41°C in the avian body) (14, 21). In fact, H5N1 viruses possessing PB2-627Lys efficiently replicate in the upper respiratory organs in mice, unlike viruses with PB2-627Glu (14). Taken together, these observations indicate that PB2-627Lys thus has an advantage over PB2-627Glu in virus replication and transmission in mammalian hosts.

In 2005, however, highly pathogenic H5N1 influenza virus possessing mammalian-type PB2-627Lys caused an outbreak among wild aquatic birds at Qinghai Lake in China, killing more than 6,000 birds (5, 6). Their descendant strains (the so-called Qinghai Lake lineage) have spread to Europe and Africa. As of the end of 2008, about 20% of H5N1 avian virus PB2 genes in the Influenza Sequence Database encode PB2-

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▽ Published ahead of print on 30 September 2009.

TABLE 1. Number of avian virus isolates encoding mammalian-type amino acids at positions 627 and 701 in PB2 in the Influenza Virus Resource database[a]

Avian species	No. of viruses possessing:		Total no. of viruses
	PB2-627Lys	PB2-701Asn	
Duck	1	1	375
Chicken	2	0	426
Ratitae			
Ostrich	2	0	6
Emu	2	1	4
Rhea	1	0	2

[a] Numbers of viruses isolated prior to 2005 from duck, chicken, and three *Ratitae* species (ostrich, emu, and rhea) are shown.

627Lys, suggesting that PB2-627Lys is maintained even in avian populations.

Via a database search (Influenza Virus Resource, http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) of virus gene sequences uploaded prior to 2005, we detected a high prevalence of PB2-627Lys in isolates from ostrich, emu, and rhea, all of which belong to the *Ratitae* (ratites or running birds, with a flat sternum) (Table 1). To clarify the contributions of these avian species to the emergence of viruses possessing mammalian-type amino acids in PB2, we infected ostrich-derived cells and ostrich chicks with H5N1 viruses and analyzed the nucleotide sequences of recovered viral genes.

For our in vitro analysis, we prepared ostrich embryo fibroblasts (OEF) and chicken embryo fibroblasts (CEF) from 20-day-old ostrich embryos and 10-day-old chicken embryos, respectively. Both cells were maintained in Dulbecco's minimum essential medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and antibiotics at 37°C in 5% CO_2. We infected OEF and CEF with A/duck/Vietnam/5001/2004 (H5N1; VN5001) or with A/duck/Vietnam/NCVD18/2004 (H5N1; NCVD18) virus at a multiplicity of infection of 0.01. By sequencing 50 (each) of the molecularly cloned PB2 for both of the original viruses, we found that all of the gene clones encoded avian-type amino acids at positions 627 (PB2-627Glu) and 701 (PB2-701Asp) in PB2, suggesting that the proportion of viruses possessing mammalian-type amino acids in PB2 in each of the original virus populations was less than 2% (i.e., <1 in 50). All procedures using H5N1 viruses were done in biosafety level 3 facilities at the University of Tokyo. After several passages in OEF or CEF, viral RNA was extracted from the culture supernatants by using a viral RNA extraction kit (Qiagen, Hilden, Germany). After a one-step reverse transcriptase PCR reaction (Superscript III High Fidelity Platinum *Taq* system; Invitrogen), the amplified PCR products were cloned into a TA-cloning vector (TOPO-TA cloning system; Invitrogen) and processed for sequencing.

We analyzed the nucleotide sequences of the PB2 genes that cover the regions that include PB2-627 and -701 from at least six clones of VN5001 and NCVD18 viruses that were passaged in OEF or CEF one to six times (Table 2). VN5001 virus possessing the PB2-701Asn mutation and NCVD18 virus possessing the PB2-627Lys mutation were detected after three and one passage(s) in OEF, respectively. After six passages in OEF, the proportion of PB2-701Asn-possessing VN5001 virus and PB2-627Lys-possessing NCVD18 virus reached 80% and 100%, respectively. In contrast, we did not detect these mammalian-type amino acids in viruses passaged the same number of times in CEF. These results indicate that viruses possessing mammalian-type amino acids in PB2 were selected during replication in ostrich-derived cells.

TABLE 2. Alteration of the PB2 gene following virus propagation in CEF and OEF[a]

Fibroblast type and passage no.	% Viral clones encoding a mammalian-type amino acid in the PB2 protein of the indicated H5N1 virus (no. of clones analyzed)			
	A/duck/Vietnam/5001/2004		A/duck/Vietnam/NCVD18/2004	
	PB2-627Lys	PB2-701Asn	PB2-627Lys	PB2-701Asn
CEF				
1	0 (6)	0 (6)	0 (8)	0 (8)
2	0 (6)	0 (6)	0 (8)	0 (8)
3	0 (14)	0 (14)	0 (8)	0 (8)
4	0 (6)	0 (6)	0 (6)	0 (6)
5	0 (6)	0 (6)	0 (6)	0 (6)
6	0 (11)	0 (11)	0 (10)	0 (10)
OEF				
1	0 (8)	0 (8)	11.1 (9)	0 (6)
2	0 (8)	0 (8)	37.5 (8)	0 (6)
3	0 (8)	12.5 (8)	70.0 (10)	0 (6)
4	0 (6)	16.6 (6)	100 (6)	0 (6)
5	0 (6)	16.6 (6)	100 (6)	0 (6)
6	0 (10)	80.0 (10)	100 (10)	0 (10)

[a] We sequenced the PB2 gene of viruses grown in OEF or CEF at the indicated passage number and calculated the proportion of viruses possessing mammalian-type amino acids at positions 627 and 701 in PB2.

To examine whether selection of mammalian-type PB2 amino acids occurs in vivo, one 3-week-old ostrich (Japan Ostrich Council, Chiba, Japan) per virus strain was intratracheally inoculated with either VN5001 or NCVD18 virus at a titer of 10^6 PFU (total volume, 500 μl). The infected ostrich chicks were housed at a biosafety level 3 facility in the University of Tokyo. Animal care and experimental procedures were approved by the Animal Care and Use Committee of the Institute of Medical Science, University of Tokyo. Three days after infection, we euthanized the animals and collected trachea, lung, and brain tissue samples for virus isolation. The collected samples were homogenized in phosphate-buffered saline (10 times the volume per sample weight), and 100 μl of each supernatant was inoculated into 10-day-old embryonated chicken eggs. After incubation for 36 h at 35°C, viral RNA was extracted from the allantoic fluid and amplified by reverse transcriptase PCR. The amplified DNA fragments were cloned into a TA-cloning vector, and at least 50 clones per sample were sequenced.

Under these conditions, the substitution to mammalian-type amino acid in PB2 was detected at position 627, but not 701 (Fig. 1). The prevalence of PB2-627Lys-possessing viruses in the trachea, lung, and brain tissues of VN5001 virus-infected ostriches was 84.6%, 40.7%, and 100%, respectively, and that for NCVD18 virus-infected ostriches was 100%, 80.3%, and 100%, respectively. These results indicate that viruses possessing PB2-627Lys were selected during replication in several organs.

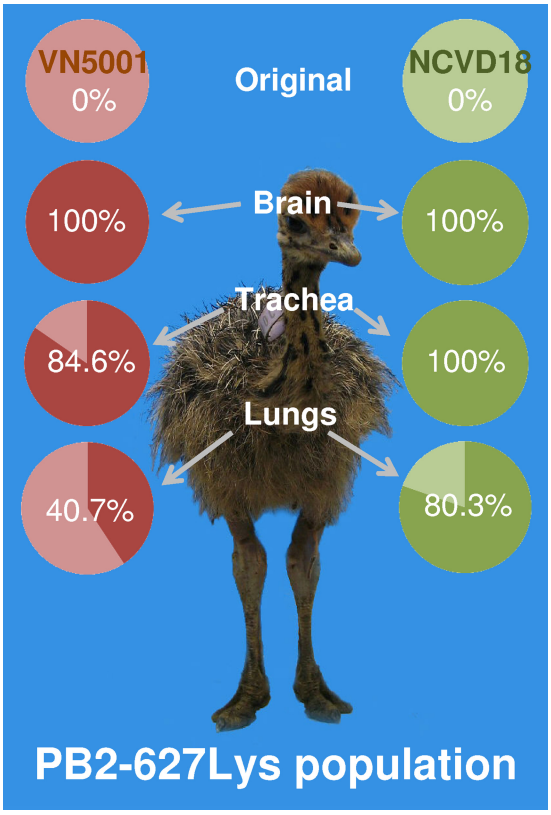

FIG. 1. Virus populations from trachea, lung, and brain samples from infected ostriches, characterized by the amino acid at position 627 in PB2. The percentage of viruses possessing the PB2-627Lys mutation is indicated for each organ.

Here, we demonstrated that H5N1 avian influenza viruses possessing a mammalian-type amino acid in PB2 were selected during replication in ostrich (Table 2 and Fig. 1). Our results are consistent with the influenza virus database finding of a high prevalence of PB2 sequences isolated from ostrich that encode PB2-627Lys or PB2-701Asn (Table 1) and suggest that both the ostrich and other *Ratitae*, such as the emu and the rhea, may contribute to the emergence of viruses possessing mammalian-type PB2-627Lys and/or PB2-701Asn.

We observed a difference in the proportion of PB2-627Lys-possessing viruses among the organs of infected ostriches (Fig. 1): PB2-627Lys-possessing viruses were more prevalent in the trachea and brain than in the lungs of ostriches infected with VN5001 or NCVD18 virus. Consistent with these findings, viruses isolated from the upper and lower respiratory tracts of an H5N1 virus-infected patient possessed PB2-627Lys and PB2-627Glu, respectively (20). These findings suggest that the selective pressure for viruses to possess PB2-627Lys in ostriches is similar to that seen in humans.

Since the amino acid at position 627 in PB2 is known to affect the RNA binding property of PB2 (17), Ratitae, unlike most other birds, may share with mammals functionally and/or structurally similar molecules involved in PB2 RNA binding. In fact, the *Ratitae* are evolutionarily categorized between birds and dinosaurs (12). Although the ostrich may not have contributed to the emergence of H5N1 viruses possessing a mammalian-type amino acid in PB2 during the Qinghai Lake outbreak, there may be other birds with properties similar to those of the ostrich that have played an intermediate role in the emergence of such mutant viruses. A thorough assessment of influenza virus infection in such birds will be important for influenza control.

We thank Susan Watson and Krisna Wells for editing the manuscript.

This work was supported by Precursory Research for Embryonic Science and Technology (PRESTO) and ERATO (Japan Science and Technology Agency); by a grant-in-aid for Specially Promoted Research; by a contract research fund for the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases from the Ministries of Education, Culture, Sports, Science, and Technology; by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan; and by National Institute of Allergy and Infectious Diseases Public Health Service research grants.

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