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Characteristics of Influenza Virus Genome Mutations

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Although the viral factors of host adaptation from domestic poultry to humans have been studied several times since the first cases of direct transmission of highly pathogenic avian influenza viruses from domestic poultry to humans were confirmed in 1997, the host-specific adaptation mechanisms from waterfowl to domestic poultry remain unknown. To study the mechanisms involved, a waterfowl-derived virus was passaged in a chicken fibroblast cell line. This passaged virus was found to have much higher growth titer than that of the original virus and several mutations were discovered in its genome. One of the most characteristics was an increase of the polymorphism of the internal genes. In addition, the general applicability of this property to the field isolates of influenza A viruses by database sequences analysis was confirmed, with the smallest amount of amino acid polymorphism in viral internal proteins observed in waterfowl-derived viruses, more in domestic poultry and the most in human-derived viruses. Although specific amino acid changes conserved in human-derived viruses were found, such amino acid changes were not observed in poultry-derived viruses.

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The influenza A virus is classified into the Orthomyxoviridae family and is known to be zoonotic, having the ability to infect a range of species including birds and mammals [1]. Waterfowl, such as the mallard, are the natural hosts of the influenza virus and show no clinical signs when infected [1-4]. Historically observed outbreaks of influenza in other species, such as domestic birds, humans, swine, horses and canines [4], have recorded both the severity of the disease's symptoms and its high mortality rate. These viruses are potentially capable of interspecies transmission [4-9], therefore it is important to elucidate the mechanism by which these viruses adapt from species to species.

Since the outbreaks of the highly pathogenic avian influenza (HPAI) H5N1 virus in domestic poultry in 1997, many cases of direct transmission of HPAI from domestic poultry to humans have been reported [5, 6]. Although the viral factors involved in host adaptation from avian species to mammals in cases of interspecies transmission have been studied [10-12], the adaptation from waterfowl to domestic poultry has not yet been investigated in depth. The viral susceptibility, pathogenicity and replication efficiency of waterfowl (the natural host of the virus) and domestic poultry (a non-natural host) are quite different. Several studies have shown that, while some were still sustainable, waterfowl-derived viruses replicated with little success in chickens [13].

Studies of the amino acid substitutions important in the adaptation of the virus to domestic poultry have, to date, focused mainly on the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [14, 15]. However, little work has been done on the internal viral proteins: polymerase basic protein 1 and 2 (PB1, PB2), polymerase acidic protein (PA) and nucleoprotein (NP). In order to understand the mechanisms used by waterfowl-derived viruses in their adaptation to domestic poultry, such virus was serially passaged in a chicken fibroblast cell line and then characterized. In addition, the database sequences of waterfowl, domestic poultry and human-derived viruses were compared in order to find out the mutation propensities in each species.

MATERIALS AND METHODS

1. Viruses

A waterfowl-derived virus strain, A/duck/Shimane/188/99 (H1N1) (DK188), was isolated during surveillance activity at Tottori University [16]. This virus was grown in 10-day-old embryonated chicken eggs and stored at -80 ^{o}C until use. Virus was titrated on Madin-Darby canine kidney (MDCK) cells by plaque assay.

2. Cell lines

MDCK cells were maintained in Eagle's Minimum Essential Medium supplemented with 5% fatal bovine serum, $NaHCO_3$ (Cat#S8761, Sigma-Aldrich, Steinheim, Germany), MEM vitamins solution (Cat#M6895, SIGMA), MEM amino acids solution (Cat#0683, Gibco, Auckland, New Zealand), 4mM L-glutamine (Cat#G7513, Sigma) and penicillin-streptomycin solution (Cat# P0781, Sigma-Aldrich).

The chicken fibroblast cell line, DF-1, was maintained in high glucose Dulbecco's modified Eagle's medium (Cat#12430, Gibco), supplemented with 10% fatal bovine serum and penicillin-streptomycin solution (Cat#171012, Cell culture bioscience).

All cells were cultured at ^{o}C in 5% CO_2.

3. Viral passages in DF-1 cells

DF-1 cells were infected with the virus at 0.1 multiplicity of infection (MOI) with 1 μg/ml of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Cat#3740, Worthington, Lakewood, New Jersey, USA). After 80% of the cells showed cytopathic effect (CPE), supernatant was harvested using a QIAshredder (Cat#79656, QIAGEN, Germantown, Maryland, USA) centrifuge at 15,000 rpm for 2 min, and blind passage was continued for seven generations at 1:10 dilution. After these seven passages, DK188 was propagated in 10-day-old embryonated chicken eggs and then passaged in DF-1 for additional three times. It resulted in total ten passages (DK188-p10).

4. RNA extraction, reverse transcription polymerase chain reaction (RT-PCR) and sequencing

Viral RNA was extracted from the viral stock by employing a QIAamp Viral RNA Mini Kit (Cat#52906, QIAGEN). This was followed by amplifying the complementary DNA (cDNA) using SuperScript one-step RT-PCR Platinum (Cat#12574-035, Invitrogen, Carlsbad, California, USA). In order to detect the viral RNA, a set of universal primers for matrix (M) segment was used; forward: atgagycttytaaccgaggtcgaaacg, reverse: tggacaaancgtctacgctgcag (referring to the guideline of the Infectious Disease Surveillance Center, at the National Institute of Infectious Diseases in Japan). For full segment cloning, universal primers set specific to each segment were used [17]. Each cDNA fragment was inserted into pCR 2.1-TOPO vector (Cat#45-0641, Invitrogen) and transformed in COMPETENT high DH5α (Code#DNA-903, TOYOBO, Osaka, Japan). The plasmids were extracted from the DH5α with Wizard Plus SV Minipreps (Cat#A1460, Promega, Madison, Wisconsin, USA) and used as templates for sequencing reactions. Sequencing reactions were carried out using a set of M13 forward primers: gtaaaacgacggccag and reverse primers: caggaaacagctatgac, with the sequence data being collected with a ABI Model 3730*x*/ DNA Analyzer (Applied Biosystems, Forster City, California, USA). Accession number of DK188 were; PB2: CY091589, PB1: CY091590, PA: CY091591, HA: CY091592, NP: CY091593, NA: CY091594, M: CY091595, NS: CY091596.

5. Analysis of viral growth in DF-1 cells

Growth efficiency of both DK188 and DK188-p10 were compared in DF-1 cells. Cells were infected with the virus at 0.01 MOI with 1μg/μl TPCK-Trypsin, and supernatants were collected at periods of 24, 48 and 72 hours post-infection (hpi). Each virus was titered in MDCK cells by plaque assay.

6. Analysis of mutational variations using the Influenza Research Database (IRD)

Sequence data were obtained from the influenza virus recourses of the National Center for Biotechnology Information (NCBI). In order to count the expected mutations of DK188-p10 in the field isolates database, the number of sequences listed in table 1 was used. The number of sequences listed in table 1 was compared with the variation in mutations among waterfowls, domestic poultry and human. These were analyzed with web-based sequence polymorphism calculation software provided by the Influenza Research Database (IRD; http://www.fludb.org) [18] and a polymorphism score on each amino acid was calculated for each segment.

Table 1 The number of the sequence used in analysis

Sequences used for comparison with DK188-p10

Gene	waterfowl	domestic poultry
PB2*	980	736
PB1*	852	613
PA*	1048	731
NP*	815	709
M1*	1583	1156
NS2*	867	778
HA1**	63	12
HA2**	63	12
NA***	371	531

Sequences used for caliculation of polymorphism score

Gene	waterfowl	domestic poultry	Human
PB2*	688	383	1000
PB1*	673	383	1000
PA*	763	430	995
NP*	627	328	747

* All subtype except for H5N1 and H7N7
** H1N1
** All N1 of waterfowls except for H5N1 and H7N1, all N1 of domestic poultry

RESULTS

1. Biological characterization of the passaged virus in a chicken cell line

After seven blind passages in DF-1, the viral M gene of DK188 was still undetectable by RT-PCR. The DK188 was then propagated in 10-day-old embryonated chicken eggs before being passaged three additional times. DF-1 infected with this re-passaged DK188 (DK188-p10) rapidly showed CPE, and the viral M gene was detected by RT-PCR. To examine the properties of the passaged virus, the growth of DK188 and DK188-p10 in DF-1 was compared. The viral titer of DK188-p10 was found to be approximately 10^5-fold higher than in the original DK188 (Figure 1). These data indicate that DK188-p10 had successfully adapted to DF-1, acquiring the ability to grow significantly in chicken fibroblasts.

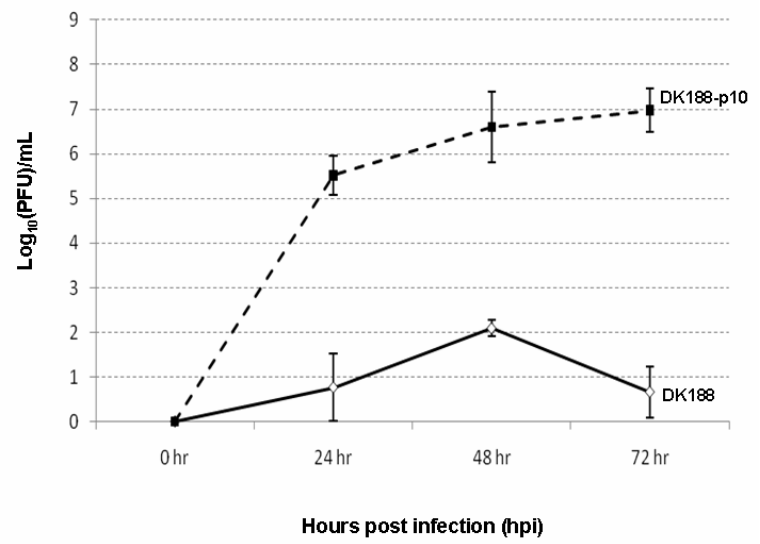


Figure 1. Replication of original DK188 and DK188-p10 viruses in DF-1
The viral titer of DK188-p10 (dotted line) was found to be much higher than in the original DK188 (solid line), indicating the successful adaptation of the DK188-p10 to DF-1. Each virus infected DF-1 cells at M.O.I 0.01 with 1μg/μl TPCK-Trypsin. The supernatants were collected at 24 hr, 48 hr and 72 hpi.

2. Molecular changes in the genome of the passaged virus in a chicken cell line

The genome sequences of DK188-p10 were analyzed and compared to the original DK188 (Table 2). When compared to the original DK188 virus, DK188-p10 was discovered to have acquired two HA mutations A509T (K172M; 3 of 3 clones) and T1039C (F348L; 3 of 3 clones): the first located at the receptor binding site of the HA1 protein and the second at the N-terminal of the HA2 protein. In addition, the DK188-p10 also showed variations in NA stalk region. Two of the four clones showed NA stalk deletion, while the other two showed T180A, T180C (W61R; 2 of 4 clones) mutations.

In PB2 of the DK188-p10 virus, variants appeared at T728A (M243L; 2 of 8 clones). In PB1, the virus showed variants at A578G (D193G; 3 of 9 clones) and G1082T (N382D; 2 of 5 clones) and, in PA, it showed additional variations in mutation in the PA N-ter region, G79A (D27N; 3 of 10 clones) and A160G (I54V; 2 of 10 clones). In NP, DK188-p10 showed one missense variation at G251A (S84N; 3 of 8 clones). In M1, DK188-p10 had variants at T539C (V180A; 3 of 7 clones) and G616T (A206S; 4 of 7 clones), but no mutations were found in the M2 gene. The NS gene encodes two non-structured proteins: NS1 and NS2/NEP [1]. In NS2/NEP, DK188-p10 showed variations at G722A (M100I; 3 of 7 clones), but did not mutate at all in NS1. Most of these mutations in the internal genes located at the amino acid positions where several amino acid variations are found in field's isolates (Table 2).

This analysis shows that the waterfowl-derived virus has an apparent propensity to completely replace amino acids in the HA molecule during passage, while mutations in the virus' internal genes appeared with only a slight increase in the viral heterogeneity in the population.

Table 2 Comparison of Mutations in DK188-p10 with in field isolates

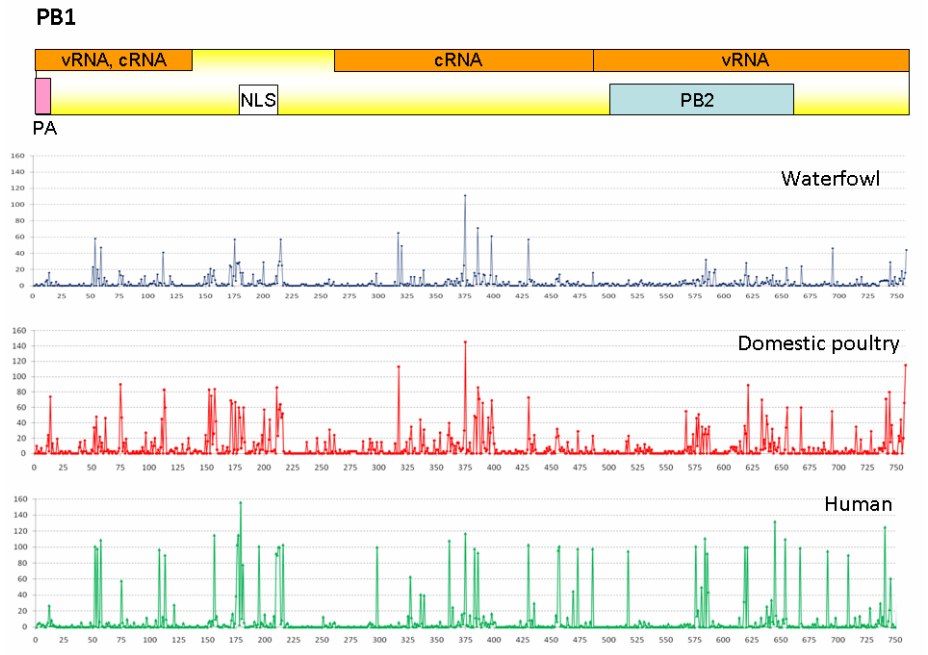
Protein	Substitution	Mutation	Waterfowl		Domestic poultry		Function
PB2	T728A	M243L	M	(978/980)	M	(701/736)	Cap binding site
			L	(2/980)	L	(28/736)	
					S	(5/736)	
					T	(1/736)	
					F	(1/736)	
PB1	G399A	-*	-		-		
	A578G	D193G	D	(851/852)	D	(611/613)	Nuclear localization signal
			A	(1/852)	E	(1/613)	
					G	(1/613)	
	G1082T	N382D	N	(852/852)	N	(613/613)	cRNA binding site
	T1532C	-*	-		-		
PA	G79A	D27N	D	(1035/1048)	D	(683/731)	Endonuclease activity
			N	(63/1048)	N	(46/731)	
			V	(2/1048)	V	(2/731)	
			T	(1/1048)			
			Y	(1/1048)			
			E	(2/1048)			
			K	(1/1048)			
	A160G	I54V	I	(1044/1048)	I	(725/731)	Endonuclease activity
			V	(4/1048)	V	(5/731)	
					A	(1/731)	
NP	G251A	S84N	S	(807/815)	S	(692/709)	
			N	(7/815)	N	(16/709)	
			H	(1/815)	I	(1/709)	
M1	T539C	V180A	V	(1583/1583)	V	(1156/1156)	vRNA binding site
	G616T	A206S	A	(1583/1583)	A	(1156/1156)	vRNA binding site
NS1	T219C	-*	-		-		
NS2	G772A	M100I	M	(691/867)	M	(654/778)	M1 binding site
			L	(155/867)	L	(91/778)	
			I	(21/867)	I	(21/778)	
					V	(11/778)	
					K	(1/778)	
HA1	A509T	K172A**	K	(62/63)	K	(12/12)	Receptor binding site
			E	(1/63)			
HA2	T1039C	F348L**	F	(62/63)	F	(12/12)	
			I	(1/63)			
NA	del***	del	del	(4/371)	del	(473/531)	NA stalk region
			full	(367/371)	full	(58/531)	
	T180A/C	W61R	W	(371/371)	W	(531/531)	NA stalk region

All sequence data are available in influenza sequence database at NCBI
* silent mutation
** HA residue is numbered according to H3 number
*** del: deletion

3. Analysis of mutational variations in PB1, PB2, PA and NP

To understand the general applicability of the increasing variation of mutations to field isolates from domestic poultry's population, the variations found in the virus' internal genes, especially in PB2, PB1, PA and NP, of the database sequences were analyzed using sequence polymorphisms analysis software (Figure 2). Hotspots for mutations as well as areas of considerable conservation were discovered in the proteins. Although these mutation hotspots were largely not related to the specific function of the protein, the patterns of mutation in each gene were quite similar in both the waterfowl (Figure 2, blue line) and domestic poultry (Figure 2, red line). The most interesting finding was a small increase in the polymorphism score of the domestic poultry-derived virus when compared to that of the waterfowl-derived virus. Further, when compared to that of the human-derived viruses, the human-derived viruses showed the highest mutation score (Figure 2, green line) and had mutation patterns in PA and NP slightly different from those of the avian-derived viruses. Sequence alignment analysis also showed some amino acids in the human-derived viruses were highly-conserved when compared to those of the waterfowl-derived viruses (Figure 3).


PB2
PB1
NP
Cap
NLS
Cap
PB1
Impa5
NP
NLS
Waterfowl
Domestic poultry
Human


PB1
vRNA, cRNA
cRNA
vRNA
NLS
PB2
PA
Waterfowl
Domestic poultry
Human

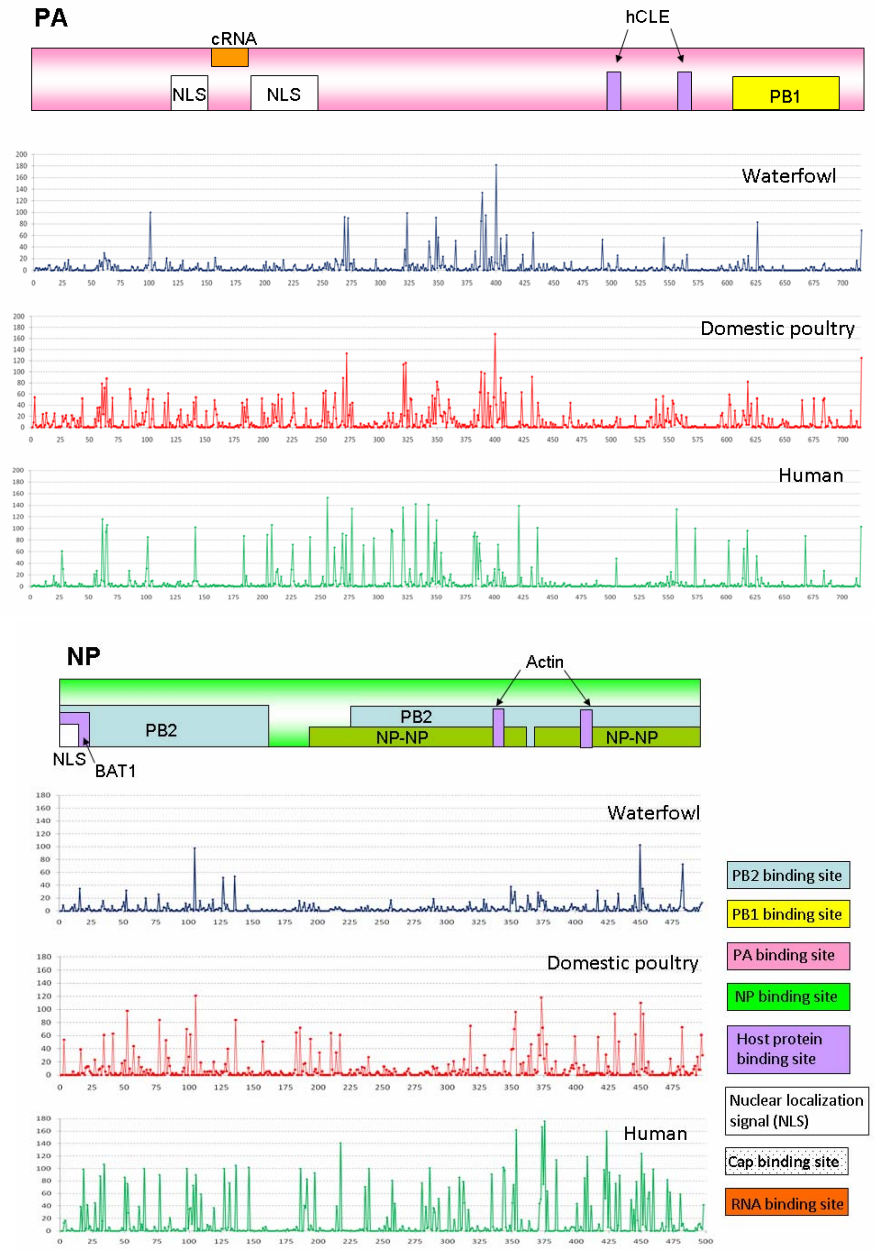


Figure 2. A comparison of mutation variation in waterfowls and domestic poultry.
The mutation hot spot patterns of waterfowl (blue) and domestic poultry (red) were quite similar, but that the polymorphism score of the domestic poultry-derived virus in each segment was slightly higher than that of the waterfowl-derived virus. The human-derived viruses (green) had much higher polymorphism scores than the avian species-derived viruses (blue and red).
The mutation scores were calculated using tools provided by Influenza Research Database (IRD). All the sequence data used in this analysis were from the NCBI Influenza Virus Recourses Database. The scheme of the functional domain for each protein was referred to Naffakh et al [19].

Figure 3. Conserved amino acids specific to species.
Comparing the amino acids of avian and human isolates, several highly conserved amino acids in internal proteins specific to humans were found; however, such differences were not observed between waterfowl and domestic poultry throughout the segments. The ratios of the residues at the each amino acid position were colored by each amino acid.

DISCUSSION

Here we characterized genome mutations of influenza A viruses for host adaptation. When the mutation variations between waterfowl and domestic poultry were compared using sequence polymorphisms analysis, with special focus on PB2, PB1, PA and NP genes (Figure 2), it was found that the mutation hotspots were not necessarily located in the functional area of the proteins [20]. It was also found that although the mutation hotspot patterns of waterfowl and domestic poultry were quite similar the polymorphism score of the domestic poultry-derived virus in each segment was slightly higher than in that of the waterfowl-derived virus. This indicates that variants of the virus in domestic poultry appeared at a much higher rate than in waterfowl. This finding is consistent with the *in vitro* passage-model data above, which showed that serial passages of waterfowl-derived viruses in chicken cells resulted in the appearance of many variants mainly at the mutation hotspots. The study also showed that human-derived viruses in general had much higher polymorphism scores than those of the avian-derived viruses. The concept of *mutator mutants*, a hypothesis in which a virus having mutations in its polymerase unit shows a high genomic mutation rate, has previously been proposed [20, 21]. Based on this theory,

increasing variation in influenza A viruses during infection to new hosts could be the cause and/or result of mutator mutants. This feature may contribute to the acceleration of the adaptation process of the influenza virus from its natural waterfowl host to other species.

When comparing the amino acids of avian and human isolates, it is typical to find several amino acids conserved in internal proteins specific to humans (Figure 3) [22]. However, such a pattern of amino acid mutation was not observed in the internal proteins of domestic poultry-derived viruses, and the major amino acid residues were similarly conserved in both waterfowl and domestic poultry-derived viruses (Figure 3). This finding indicates that there are no selective pressures in the internal molecules of waterfowl viruses during passages in domestic poultry. In contrast, in HA molecule in our *in vitro* passage model, the passaged virus showed a complete replacement of amino acids in the molecules K172M and F348L, located at the receptor binding site of the HA1 and N-terminal of the HA2 protein (Table 2). Three dimensional (3D) protein structure of HA1 showed that Lys172 located proximal to amino acid residues Trp169 and Tyr211 which are known to be important for receptor binding [23]. Therefore mutation K172M has possibility to affect receptor binding activity of HA1. Although these mutations were not found in the field H1N1 avian isolates, it is well demonstrated that mutations at the HA receptor biding regions and cleavage site as well as mutations in NA stalk region are the most frequently observed phenomenon in the adaptation to chickens [24-26]. In addition, a close relationship between viral pathogenicity in chickens and the mutations observed in these surface molecules has been reported [14, 27, 28]. These findings indicate that, distinct from internal molecules, there are strong selective pressures on the surface molecules during passage of waterfowl viruses in chicken.

From this experiment, several important findings should be noted: (i) the waterfowl-derived virus, DK188, originally replicated poorly in the chicken fibroblasts; (ii) another egg-passage allowed the poorly replicated strain to continue additional viral passages in the cell; and (iii) the passaged virus acquired the ability to replicate efficiently in the chicken fibroblast (Figure 1).

These data support the conclusion, as has been reported in other studies [13], that most waterfowl-derived viruses show low growth efficiency in chickens. It also shows that, in spite of this extremely low growth efficiency, small virus populations retain the potential to infect chicken fibroblasts. Once the population of the virus is large enough, it is able to efficiently infect chicken cells. This biological characteristic of the influenza A virus may play an important part in the origins of outbreaks in domestic poultry under certain selective pressures.

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