

LETTER TO THE EDITOR

Evolutionary changes in avian influenza H5N1 viruses in Thailand

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H5N1 avian influenza outbreak in Thailand is now well under control and no human infection has been reported since July 2006. Only a small number of cases and outbreaks, mostly in domestic poultry, are reported in a limited area of the country. This is in contrast to the explosive widespread epidemic in Thailand as well as other countries in Southeast Asia in 2004–2005 (1–4). These H5N1 avian influenza viruses were classified as genotype Z by their genome constellation (1, 4, 5).

The change in epidemic pattern may influence selective pressure and the evolution of the virus. H5N1 viruses from the early outbreaks in 2004–2005 are believed to be under positive selective pressure for the adaptation to new transmission environments and new hosts. Evidence of positive selection, using the method of dN/dS analysis, can be observed in M2 and PB1-F2 genes of H5N1 viruses from Southern China and Southeast Asia in 2003–2005 (6). Selective pressure can change over time. Positive selective pressure appears when organisms encounter a new environmental constraint, and disappears when the organisms have adapted to this new environment. The nature of epidemic of H5N1 avian influenza in Thailand has changed from the early rapid expansion phase to an endemic phase. Therefore, it would be

interesting to investigate whether the positive selection has disappeared, keeping in mind that the control measures and the limitation of transmission may also impose a different type of positive selection on viral genes.

In order to compare the selective pressure on the viruses from the early extensive outbreaks and the current viruses, the rates of dN/dS in these two groups of viruses were analyzed. All of the full-length viral gene sequences for the genotype Z clade 1 H5N1 from Thailand available in the GenBank database were collected. The sequences were separated according to the early phase of epidemic from 2003–2005 and more recent sequences from 2006–2011. The numbers of sequences for each gene of the two groups are shown in Table 1. The early virus group contains sequences of isolates from human, mammalian and avian species, whereas the more recent virus group contains mostly viruses from domestic avian species. Information on specific location is available only for some isolates, but, in general, the recent virus group was mostly confined to the repeated outbreak area in the upper central region of the country, while the early viruses were more widely spread. The selective pressure acting on each gene was estimated using the CODEML program in the PAML version 4.4 package (7, 8). Likelihood ratio test accepted the M7 model (does not allow dN/dS value to be >1) for most of the genes except for the group of PB1-F2 gene of current strains, for which the test rejected the M7 model in favor of the M8 model (allows dN/dS value to be >1). The dN/dS value of the M2 genes of the early virus group was estimated to be 1.4 (95% confidence

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Abbreviations: dN/dS = non-synonymous/synonymous changes

Table 1. dN/dS of individual genes of genotype Z clade 1 H5N1 viruses from Thailand (The analyses were performed separately for 2003–2005 and 2006–2011 viruses. The dN/dS value indicating positive selection for M2 in the 2003–2005 viruses is marked in bold.)

Gene	2003–2005 viruses		2006–2011 viruses	
	No. of sequences	dN/dS	No. of sequences	dN/dS
PB2	44	0.1627	13	0.1373
PB1	40	0.2057	17	0.0948
PA	31	0.2844	19	0.1027
HA	111	0.2172	32	0.2574
NP	35	0.0954	19	0.1264
NA	72	0.3678	25	0.2343
M1	36	0.0562	31	0.1214
M2	14	1.4031	16	0.6639
NS1	88	0.5392	32	0.3290
NS2	44	0.7778	19	0.5406
PB1-F2	31	0.9970	24	?

interval; 0.0000–3.6977). Due to the limited number of PB1-F2 sequences, positive selection of this gene could not be statistically concluded, but a trend of positive selection (higher dN/dS value) was still observed. These results are in agreement with previously published data for genotype Z viruses from southern China and Southeast Asia, showing the dN/dS value of >1 for M2 and PB1-F2 (6). In contrast, dN/dS values of current viruses are < 1 for all genes, except for that of PB1-F2. The number of available PB1-F2 sequences of current viruses is also very small and the data set contains no synonymous changes, therefore the dN/dS value was estimated by the program to be ∞ , denoted as '?' in the Table. Although there are more PB1 sequences in the database, the overlapping PB1-F2 sequences from the recent viruses showed truncation in 8 out of 32 sequences. This truncation was not observed in the early virus group.

The results indicate that the nature of evolution of H5N1 avian influenza viruses in Thailand has changed and some of the positive selective pressure found in the early epidemic has disappeared. This could mean either that the genotype Z virus has already adapted to poultry as the new host or that the outbreak conditions causing the positive selection no longer exist, although these two reasons are not mutually exclusive. Although inconclusive, PB1-F2 gene seemed to be continuously evolving under positive selective pressure. The high dN/dS value for PB1-F2 is difficult to interpret. It can be an artifact related to the location of PB1-F2 in the +1 ORF of the PB1 gene (9). This is based on the assumption that PB1-F2 is more flexible to changes than PB1 and the negative selective pressure on PB1 reduces dS in PB1-F2, while the dS at the third codon position of PB1 results in an increase of dN at the second codon position of PB1-F2. However, our analysis compared the two groups of PB1-F2,

which probably had the similar bias by the overlapping PB1. Although it cannot be made certain whether dN/dS value of PB1-F2 from the recent virus group really means a positive selection, it can be inferred that the recent virus group either had an increased positive selection or became less constrained compared to the early virus group. The latter alternative is probably more likely because the truncation of PB1-F2 sequences was observed more frequently in recent viruses from Thailand. As PB1-F2 is considered a virulence factor of H5N1 viruses, the observed change in selective pressure of this gene may reflect an adaptation to optimize the viral virulence, probably toward lower virulence. Alternatively, the increase in the dN/dS value may reflect a reduced constraint and may indicate that the gene is no longer needed in the recent viruses, while it was essential in the early viruses probably for establishment of the rapidly spreading outbreaks.

In order to further understand the nature of the change in selective pressure, we analyzed viral sequences from Indonesia, where outbreaks are more active than in Thailand. In this analysis, 70 and 96 M2 sequences; and 33 and 95 PB1-F2 sequences from 2003–2005 and 2006–2011 viruses, respectively, were analyzed. The dN/dS value of M2 sequences from the early and late viruses were 0.8172 (0.3725–1.2619) and 0.5080 (0.1563–0.8145), respectively, whereas the value of PB1-F2 sequences from the early and late viruses were 1.8128 (0.0000–4.0758) and 7.3665 (0.0000–18.5912), respectively. The likelihood ratio test favored the M8 model only for the PB1-F2 of the recent virus group.

It was previously shown that the recent viruses in Thailand stemmed from only a few lineages of indigenous viruses, while the viruses of the initial outbreaks were more diverse (10). This should limit the number of common changes between the early and recent virus groups in the dN/dS analyses. These common changes would reflect the earlier changes in the recent virus group, which would reduce the difference between the two virus groups. This bias may reduce the sensitivity of the tests to detect differences of dN/dS between the two groups, but should not affect the conclusion for the differences, which have been observed.

Our analyses show that the nature of H5N1 viral evolution is a dynamic process and positive selective pressure found in the early phase of epidemic was transient. While the PB1-F2 gene is still evolving, probably to optimize the viral virulence, the disappearance of positive selection on M2 in recent strains suggests that the virus may have reached equilibrium with its new hosts and transmission environment in a low level endemicity. It was previously shown that the H5N1 viral population size in central Thailand has markedly declined, which further reduces the risk of new strain emergence (2, 10). However, this should not be a reason for complacency, and surveillance and vigilance should be continued, since influenza virus is usually hard to predict.

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