Possibility of cross-species/subtype reassortments in influenza A viruses
An analysis of nonstructural protein variations

Shaomin Yan and Guang Wu*

State Key Laboratory of Non-food Biomass Enzyme Technology; National Engineering Research Center for Non-food Biorefinery; Guangxi Key Laboratory of Biorefinery; Guangxi Academy of Sciences; Guangxi, PR China

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The reassortment of genetic segments from different host species and from different subtypes of influenza A viruses occurs frequently, which may generate new strains causing flu epidemic or pandemic. However, the underlined mechanisms of reassortment were less addressed from the viewpoint of protein variations. Recently, we used the amino-acid pair predictability as an indicator to convert eight types of influenza A virus proteins into predictable portion of amino-acid pairs, and then applied the models I and II ANOVA to estimate their differences in terms of subtypes and host species. In order to get a full picture, 2729 and 1063 non-structural 1 and 2 proteins of influenza A viruses were analyzed in this study. The results are consistent with those obtained from hemagglutinin, neuraminidase, nucleoprotein, polymerase acidic protein, polymerase basic proteins 1 and 2, and matrix proteins 1 and 2, indicating that inter-species/subtypes variations are smaller than intra-species/subtype ones. Our findings provide statistical evidence that can partially explain why cross-subtype mutation and cross-species infection easily occur during co-infecting of different strains.

Introduction

It is well known that unpredictable mutations of influenza viruses can trigger seasonal influenza epidemics and occasional pandemics, which have been threatening humans and other species worldwide. Among various mutation patterns, the reassortment of genetic segments from different host species and from different subtypes is more serious because such a mutation can form a new strain that may become a pathogen no longer sensitive to anti-infection drugs and induces the outbreak of influenza, such as the last outbreak of influenza A/H1N1 pandemic that was due to a new strain resulting from the reassortant of swine, avian, and human influenza viruses.

In general, influenza viruses are classified as different subtypes according to their two surface proteins and as different species according to their affected host, thus viral differences between subtypes and between species are remarkable, because they prevent the formation of cross-subtype mutation, which may come from multiple mutations leading to a virus to change from one subtype to another subtype, and cross-host infection. For example, H5N1 influenza A virus was considered to have higher virulence than other subtypes of influenza A viruses. Equine influenza viruses hardly affect humans because of substantial incompatibility among their gene products. However, frequent emergence of the reassortment of genetic segments from different subtypes and from different species challenges the barriers between subtypes and between species of influenza viruses, and many studies have been addressed this issue. If genetic segments of influenza viruses would be quite different between species/subtypes, a formation of reassortment from different species and subtypes would be easily prevented. Indeed, this reassortment implies smaller difference in genetic segments between species/subtypes. An intriguing question raised here is how to estimate the difference of viral proteins from different species and subtypes.

In statistics, the one-way ANOVA is widely used to determine difference of certain characteristic among different groups. However, this method works on numbers rather than alphabets, which represent amino acids in proteins. In order to overcome this obstacle, we have developed dynamic protein features, which can numerically present proteins and then can be used in various models. Recently, we applied our method to compare inter- and intra-species/subtype variations in eight proteins from influenza A viruses. The results showed that the inter-species/subtype variations were generally smaller than intra-species/subtype ones, indicating that reassortment is a tendency in influenza A viruses, which would form new strains leading cross-species infection and cross-subtype mutation.
The segmented genome of influenza A virus encodes 10 or 11 proteins depending on the strain. So far, we have yet to analyze the non-structural (NS) proteins that have multiple functions during viral infection. They can affect the host immune response through many virus and host-cell processes. Amino acid sequence analysis of the NS1 proteins revealed that some nonsynonymous substitutions play a critical role in shaping the genetic diversity. Animal experiment showed that a single residue change in the highly conserved SH2bm within NS1 protein (Y89F) can strongly reduce virulence. The combination of all these functions makes the NS1 protein a very potent inhibitor of immunity and allows influenza virus to efficiently escape the immune surveillance and to establish infection in the host. As a nuclear export protein, the NS2 protein can change RNA levels by specific alteration of the viral transcription and replication machinery. Thus, non-structural proteins have the potential application benefits for drug target and vaccination against influenza viruses.

In comparison with other eight analyzed proteins from influenza A viruses, the aim of this study is to estimate the inter-species/subtype and intra-species/subtype variations of non-structural proteins to see their role on reassortment and get whole insights into this issue from different proteins of influenza A viruses.

**Results**

Figures 1 and 2 show the comparisons of NS1 and NS2 proteins, where the upper, middle and lower panels represent the results obtained from HA and NA subtypes, and species, respectively. For example, of various species there are 467 NS1 proteins in H1 subtype with the predictable portion of 39.1 ± 3.2% (the first bar in the upper panel of Fig. 1). Statistical differences are found among subtypes and species in both NS1 and NS2 proteins tested by ANOVA (P < 0.001). Figure 3 elucidates the results obtained from all paired comparisons. In this figure, the upper right and lower left triangles show the data from NS1 and NS2 proteins, respectively. Among 13 HA subtypes in the upper panel, difference and no difference are found in 43 and 35 pairs in NS1 proteins, and in 16 and 62 pairs in NS2 proteins; Among 9 NA subtypes in the middle panel, difference and no difference are found in 16 and 20 pairs in NS1 proteins, and in 9 and 27 pairs in NS2 proteins; In the lower panel, difference and no difference are found in 21 and 7 pairs among 8 species in NS1 proteins, while difference and no difference are found in 3 and 7 pairs among 5 species in NS2 proteins. Comparing both proteins, the number of pairs with statistical difference is larger in NS1 proteins than that of NS2 ones, for instance, NS1 proteins reveal P < 0.001 and P = 0.021 (Chi-square test) in HA subtypes and in species.

Figure 4 displays difference between 3 main host species (avian, human, and swine) among 9 subtypes in NS1 proteins and 6 subtypes in NS2 ones, which further reveal that NS2 proteins have a smaller species difference than NS1 ones. As can be seen, among 19 pairs for comparison there are 12 pairs with difference and 7 ones without difference in NS1 proteins (the upper panel), whereas among 14 pairs in NS2 proteins (the lower panel) there are only 4 pairs with difference but 10 ones without difference.

The above results suggest larger intra-subtype/species variations in comparison with inter-subtypes/species ones. This issue can be analyzed using the standard ANOVA table and the result is listed in Table 1, which demonstrates a tendency that inter-subtypes/species variation is general smaller than intra-subtype/species one, which appears in nine out of 13 objectives in NS1 proteins and in all nine objectives in NS2 proteins. Furthermore, inter- and intra-subtype/species variations can be
presented by percentage instead of the sum of squares as our previous studies.17-24 Figure 5 displays such results calculated from NS1 and NS2 proteins in different subtypes and species, in comparing with other eight influenza A virus proteins. This figure highlights the fact that inter-subtype/species variation is generally smaller than intra-subtype/species one in all of influenza A virus proteins. Figure 6 shows variation percentages of inter- and intra-species calculated from different subtypes, where dominant intra-species variations can be further found in H3N2, H5N1, and H9N2 subtypes of 10 viral proteins.

**Discussion**

Because influenza A viruses are negative-sense and have eight single-stranded RNA segments,10 which is the foundation for genetic reassortment that provides genomic and phenotypic changes through genetic materials directly exchanging from different subtypes and species;11,12,38 however, the effects of reassortment are still difficult to analyze. Our efforts pave the way to estimate the issue that why the genetic reassortment is so easily occur in influenza A viruses by means of statistical analysis. Hereby we analyzed the inter- and intra-subtype/species variations in NS1 and NS2 proteins using both models I and II ANOVA, and the results are consistence with our previous studies and show that small inter-subtype/species variations can be found in all of 10 proteins of influenza A viruses, which can partially explains why cross-subtype mutation and cross-species infection easily occur during co-infecting of different strains.

By means of specific deletions, truncations, and elongations, influenza viruses can synthesize their proteins that are variable in length, which relates to virulence and host adaptation.39 Also, the genetic reassortment provides the way for fast changing viral host range and virulence through adaptive evolution, where influenza viruses reveal an extraordinary ability to diversify.40 Phylogenetic analysis shows that the degree of variation is low within the alleles of non-structural protein 1. Bogoyavlenskiy et al. studied different combinations from 7 HA subtypes and 5 NA subtypes, and they found that the maximum of amino acid divergence in clade was 5% and 4% in allele A and B viruses.41 Our studies provide statistical evidence that ten types of influenza A virus proteins have small inter-subtypes variations, indicating that the barrier between subtypes is general weaker to prevent cross-subtype mutation.

For transmission and maintenance in certain species, there may be a competitive advantage in some genetic segments over other combinations, although within a host multiple reassortments can take place.33 Our results demonstrate that the intra-species variations are quite larger than the inter-species ones in H3N2, H5N1, and H9N2 subtypes, which suggests that the barrier between species is very weak in these subtypes. No differences are found between many paired comparisons, for example, human vs. swine in H1N2 subtype; human vs. avian in H1N2, H3N2, and H5N1 subtypes; and swine vs. avian in H1N2, H3N2, and H9N2 subtypes in NS1 proteins, proving theoretical reason for easy cross-species infection. Thus, reassortments potentially transmit the genetic diversity from the donor host to the recipient one, which may make influenza A viruses adapt to new host species, although it is originally perceived that transmission bottlenecks is narrow.43

Host range variants or antigenic escape may be induced during the course of single infections, where potentially advantageous mutations can orrue.44 Our findings can shed some lights on the strategy for designing anti-viral vaccines and drugs. The vaccines and drugs would easily suffer inefficacy...
if they are designed against single subtype/species because variations are generally small between subtypes and species. A large-scale analysis revealed that universally conserved residues can serve as candidates for protein-protein interactions, basing on the amino acid sequences of 2620 NS1 proteins. Therefore the heterogeneity of anti-viral vaccines and drugs can benefit for pandemic preparedness.

Many studies from various research laboratories around the world have indicated that mathematical analysis, computational modeling, and introducing novel physical concept to solve important problems in biology and medicine, such as protein structural class prediction, modeling 3D structures of targeted proteins for drug design, diffusion-controlled reaction simulation, cellular responding kinetics, biomacromolecular internal collective motion simulation, identification of proteases and their types, membrane protein type prediction, viral protein misfolding, and protein–protein interaction, can timely provide very useful information and insights for both basic and clinical research, and hence are widely welcome by science community. The present study is related to the fundamental problems in system biology, network biology, and structural biology of proteins. The relationship between these systems will be of use for the global research.

**Materials and Methods**

**Data**

In order to let this study be comparable with previous studies, 7826 full-length NS1 proteins of influenza A viruses sampled from 1918 to 2008 and 6635 NS2 sampled from 1931 to 2009 were obtained from the Influenza Virus Resources. After excluded identical sequences, 2729 NS1 and 1063 NS2 proteins were actually used in this study.

**Quantification of NS proteins**

As mentioned in the Introduction, the one-way ANOVA deals with decimal data rather than alphabets that represent amino acids in proteins, thus all NS proteins must be converted into numbers, and the amino-acid pair predictability has been used to do so.

Taking the ABB86874 NS2 protein (strain A/swine/Ontario/57561/03[H1N1]) as an example, it has 121 amino acids, which construct 120 adjacent amino-acid pairs. This NS2 protein has 15 leucines, “L”, and the appearance of amino-acid pair LL should be twice according to the permutation (15/121 × 14/120 × 120 = 1.74). There are 2 pairs of LL found in the NS2 protein, so the amino-acid pair LL is predictable. By contrast, there are 7 phenylalanines “F” and 14 glutamic acids “E” in this protein, and the appearance of amino-acid pair FE should be once (7/121 × 14/120 × 120 = 0.81). However, it appears 3 times in reality, so the amino-acid pair FE is unpredictable. In this way, all amino-acid pairs in this NS2 protein can be classified as predictable and unpredictable, and their portions are 31.11% and 68.89%, respectively. Thus, ABB86874 NS2 protein can be represented by either 31.11% or 68.89%. Another swine NS2 protein (accession number ABB86944) has only one amino acid different from ABB86874 NS2 protein at position 6, but its predictable and unpredictable portions are 30.77% and 69.23% so the ABB86944 NS2 protein can be represented by either 30.77% or 69.23%, which is different from ABB86874 NS2 protein.

Actually, such quantification is based on random principle, i.e., the issue that an amino acid joins into an amino-acid pair is probabilistically independent event. As predictable amino-acid
pair requires the least time and energy for construction, and thus nature would intuitively build a protein with a maximal predictable portion, while unpredictable portion implies probabilistically unstable, which is more likely subject to mutations.\textsuperscript{14-16}

Comparing variation between and within species/subtype

After converting all NS proteins into numerical data, their predictable portions were grouped according to their subtypes and species because influenza A viruses are characterized by their surface proteins and by the species affected host.\textsuperscript{3,7} The one-way ANOVA (model I ANOVA) followed by the Holm–Sidak comparison test was used to compare the difference among subtypes/species and the Holm–Sidak comparison test was used to compare the difference between subtypes/species. The single classification model II ANOVA with unequal sample sizes\textsuperscript{75,76} was used to determine the inter- and intra-subtype/species variations. Analysis of variance requires samples to be normally distributed, sample size in our cases is large enough to guarantee the normal distribution because data distribution is generally considered to be normal when data set is large than 50 as indicated by Statistica software. It turns out that the difference detected by ANOVA in terms of inter- and intra-subtype/species variations is how difference in protein sequences among subtypes/species, while the bigger the difference is, the more different in protein sequence is, the more difficult for mutations to lead cross-subtype mutation and cross-species infection is. Thus, the difference detected by ANOVA can be viewed as barriers between species and subtypes. The SigmaStat software was used to conduct all comparisons. P < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 4. Comparison of species difference among different subtypes. The data were presented as mean ± SD. The one-way ANOVA and Holm–Sidak comparison test were used to compare the difference among and between species. # and * indicated the statistical significance from corresponding avian and human at P < 0.05 level.
| Objective                  | Source of variation | Degree of freedom | Sum of squares | Mean squares | F value |
|---------------------------|---------------------|-------------------|----------------|--------------|---------|
| **NS1 proteins**          |                     |                   |                |              |         |
| HA subtypes               | Inter-subtype       | 12                | 6979.32        | 581.61       | 89.20   |
|                           | Intra-subtype       | 2709              | 17664.31       | 6.52         |         |
|                           | Total               | 2721              | 24643.63       |              |         |
| NA subtypes               | Inter-subtype       | 8                 | 649.32         | 81.17        | 9.15    |
|                           | Intra-subtype       | 2698              | 23940.66       | 8.87         |         |
|                           | Total               | 2706              | 24589.98       |              |         |
| All species               | Inter-species       | 7                 | 4946.57        | 706.65       | 97.26   |
|                           | Intra-species       | 2716              | 19734.14       | 7.27         |         |
|                           | Total               | 2723              | 24680.71       |              |         |
| Species hosting H1N1     | Inter-species       | 2                 | 997.76         | 498.88       | 75.21   |
|                           | Intra-species       | 391               | 2593.73        | 6.63         |         |
|                           | Total               | 393               | 3591.49        |              |         |
| Species hosting H1N2     | Inter-species       | 2                 | 52.68          | 26.34        | 2.33    |
|                           | Intra-species       | 66                | 746.29         | 11.31        |         |
|                           | Total               | 68                | 798.98         |              |         |
| Species hosting H2N2     | Inter-species       | 1                 | 39.03          | 39.03        | 9.20    |
|                           | Intra-species       | 54                | 229.14         | 4.24         |         |
|                           | Total               | 55                | 268.16         |              |         |
| Species hosting H2N3     | Inter-species       | 1                 | 44.29          | 44.29        | 27.58   |
|                           | Intra-species       | 12                | 19.28          | 1.61         |         |
|                           | Total               | 13                | 63.57          |              |         |
| Species hosting H3N1     | Inter-species       | 1                 | 33.63          | 33.63        | 18.40   |
|                           | Intra-species       | 4                 | 7.31           | 1.83         |         |
|                           | Total               | 5                 | 40.94          |              |         |
| Species hosting H3N2     | Inter-species       | 2                 | 148.30         | 74.15        | 12.66   |
|                           | Intra-species       | 518               | 3035.17        | 5.86         |         |
|                           | Total               | 520               | 3183.47        |              |         |
| Species hosting H3N8     | Inter-species       | 1                 | 91.77          | 91.77        | 40.53   |
|                           | Intra-species       | 44                | 99.63          | 2.26         |         |
|                           | Total               | 45                | 191.40         |              |         |
| Species hosting H5N1     | Inter-species       | 2                 | 54.43          | 27.21        | 6.59    |
|                           | Intra-species       | 554               | 2286.96        | 4.13         |         |
|                           | Total               | 556               | 2341.38        |              |         |
| Species hosting H9N2     | Inter-species       | 2                 | 38.94          | 19.47        | 4.91    |
|                           | Intra-species       | 310               | 1228.35        | 3.96         |         |
|                           | Total               | 312               | 1267.29        |              |         |
| **NS2 proteins**          |                     |                   |                |              |         |
| HA subtypes               | Inter-subtype       | 12                | 857.05         | 71.42        | 8.47    |
|                           | Intra-subtype       | 1045              | 8814.60        | 8.44         |         |
|                           | Total               | 1057              | 9671.66        |              |         |
| NA subtypes               | Inter-subtype       | 8                 | 733.47         | 91.68        | 10.77   |
|                           | Intra-subtype       | 1045              | 8893.88        | 8.51         |         |
Table 1. Standard ANOVA table regarding subtype and species of N1 and N2 from influenza A viruses (continued)

| Objective | Source of variation | Degree of freedom | Sum of squares | Mean squares | F value |
|-----------|---------------------|-------------------|----------------|--------------|--------|
| All species | Inter-species | 4 | 271.90 | 67.98 | 7.55 |
| | Intra-species | 1054 | 9487.38 | 9.00 | | |
| | Total | 1058 | 9759.28 | | | |
| Species hosting H1N1 | Inter-species | 2 | 366.72 | 183.36 | 26.73 |
| | Intra-species | 170 | 1165.99 | 6.86 | | |
| | Total | 172 | 1532.71 | | | |
| Species hosting H1N2 | Inter-species | 2 | 5.41 | 2.70 | 0.29 |
| | Intra-species | 38 | 355.88 | 9.37 | | |
| | Total | 40 | 361.28 | | | |
| Species hosting H2N2 | Inter-species | 1 | 0.22 | 0.22 | 0.06 |
| | Intra-species | 21 | 74.05 | 3.53 | | |
| | Total | 22 | 74.27 | | | |
| Species hosting H3N2 | Inter-species | 2 | 2.96 | 1.48 | 0.27 |
| | Intra-species | 141 | 784.78 | 5.57 | | |
| | Total | 143 | 787.74 | | | |
| Species hosting H5N1 | Inter-species | 1 | 46.67 | 46.67 | 6.43 |
| | Intra-species | 228 | 1654.66 | 7.26 | | |
| | Total | 229 | 1701.33 | | | |
| Species hosting H9N2 | Inter-species | 2 | 53.47 | 26.74 | 3.22 |
| | Intra-species | 130 | 1077.92 | 8.29 | | |
| | Total | 132 | 1131.39 | | | |

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Figure 5. Between species/subtype variations (black) and within species/subtype variations (gray) in ten proteins from influenza A viruses. HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein; PA, polymerase acidic protein; PB1 and PB2, polymerase basic protein 1 and 2; M1 and M2, matrix protein 1 and 2; NS1 and NS2, nonstructural protein 1 and 2.

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