

Prediction of mutations engineered by randomness in H5N1 neuraminidases from influenza A virus

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Summary. In this proof-of-concept study, we attempt to determine whether the cause-mutation relationship defined by randomness is protein dependent by predicting mutations in H5N1 neuraminidases from influenza A virus, because we have recently conducted several concept-initiated studies on the prediction of mutations in hemagglutinins from influenza A virus. In our concept-initiated studies, we defined the randomness as a cause for mutation, upon which we built a cause-mutation relationship, which is then switched into the classification problem because the occurrence and non-occurrence of mutations can be classified as unity and zero. Thereafter, we used the logistic regression and neural network to solve this classification problem to predict the mutation positions in hemagglutinins, and then used the amino acid mutating probability to predict the would-be-mutated amino acids. As the previous results were promising, we extend this approach to other proteins, such as H5N1 neuraminidase in this study, and further address various issues raised during the development of this approach. The result of this study confirms that we can use this cause-mutation relationship to predict the mutations in H5N1 neuraminidases.

Keywords: Influenza – Logistic regression – Mutation – Neuraminidase – Prediction

Introduction

In preparation for the possible epidemics and pandemics of influenza, an important issue is the prediction of mutated proteins of influenza A virus, because the unpredictable mutations lead humans to have little immunity against this deadly disease. Among various subtypes of influenza viruses, the H5N1 viruses are highly pathogenic (Lee et al., 2005; Chen et al., 2006), of which the mutations mainly occur in the RNA genes coding for ten virus proteins (Hilleman, 2002).

Neuraminidase is a sialidase (Gottschalk, 1957) that prevents virion aggregation by removing cell and virion surface sialic acid (Paulson, 1985), is the major antigen for neutralizing antibodies and is involved in the binding

of virus particles to receptors on host cells (Zambon, 1999). Still, neuraminidase is the target of several anti-influenza drugs (Hochgürtel et al., 2002; Garman and Laver, 2004; Oxford et al., 2004). Of subtypes, H5N1 neuraminidase is important as H5N1 virus is currently threatening humans and the mutations in neuraminidase may lead to the dysfunction of anti-influenza drugs.

The preparedness is currently conducted along various approaches, of which the modeling is playing its role in this battle against influenza A virus. A prominent approach in developing inhibitors is conducted at several levels. At receptor protein level, the modeling helps to determine the “binding pocket” of the receptor protein with its ligands (Chou, 2004a–e, 2005; Chou et al., 1997, 1999, 2000, 2003, 2006; Li et al., 2007; Wang et al., 2007a, c). At “cleavage-site” level, the modeling is trying to find the target residue for mutagenesis (Poorman et al., 1991; Elhammer et al., 1993; Chou, 1993a, b, 1996; Thompson et al., 1995). Upon two levels above, it is generally possible to find the target residues, the next level study is directed to the mutagenesis and the designing of effective inhibitors (Althaus et al., 1993a–c; Chou et al., 1994; Du et al., 2005, 2007; Gan et al., 2006; Gao et al., 2007; Wei et al., 2007). The fourth level of modeling is the determination of 3D structure of binding interaction in proteins of interests (Wei et al., 2006; Wang et al., 2007b).

Recently we have tried to use the modeling approach to predict mutations in proteins from influenza A virus, which is related to several levels too, say, the prediction of mutation positions, the prediction of would-be-mutated amino acids at predicted positions, the timing of mutations, and the prediction of new functions resulting from

the mutations. The first three types of predictions are relevant to the primary structure of proteins, while the last one is relevant to 3D structure of proteins.

It is no doubt that the best way for the prediction of mutation is to find the cause for mutations, and then we can build a cause-mutation relationship, and predict the occurrence of mutation when its cause appears. This approach is quite straightforward, however it is challenged by three facts. First, many causes, which led mutations in the past, might have left any trace due to the huge changes in environments, so we would have a relatively detailed record of mutations, but a poor record of their causes. Thus, we could not establish the one-to-one relationship between causes and mutations. Consequently, we even could not define the scale of causes for monitoring. Second, the current version of proteins might not be subject to the causes, which led the historic mutations, because of evolution, the multi-drug resistance could be an example for the evolution of bacteria. Third, it is difficult to find the historically macro- and micro-environmental surroundings, under which historic causes triggered the mutations.

As the searching of each historically instant cause appears difficult, we might need to direct our effort forward the searching of constant causes, because the protein constantly evolves although its evolutionary speed is not constant. Randomness should be one of such constant causes, which engineer mutations through generation, not only because the pure chance is now considered to lie at the very heart of nature (Everitt, 1999) and the occurrence of mutation is generally considered a random event (Fitch et al., 1997), but also because randomness suggests that an event does not occur deliberately, but naturally. This further suggests that the event with a bigger probability would occur more easily than the event with a smaller probability. Although nature should deliberately construct the absolutely necessary structure for a protein with more time and energy, there must be some structures that can be explained by random mechanism, because not only nature follows parsimony, but also nature cannot predict the future by constructing the structure for the future, which are currently useless.

Once we could measure and quantify this randomness, we could compare the quantified randomness before and after mutations to determine whether randomness plays a role. If so, we could build a quantitative cause-mutation relationship to predict the mutations engineered by randomness.

Although it is difficult to measure and quantify the randomness in nature, we could measure and quantify the

randomness in a protein, which mirrors the randomness in nature. Since 1999, we have developed three methods to quantify the randomness in protein, and find the quantified randomness sensitive to mutations. This means that the randomness does play an important role in engineering mutations or we can use the random mechanism to explain some mutations.

Furthermore, this also means that we can build a cause-mutation relationship accounting for the mutations engineered by randomness. This is possible, because we can classify the occurrence and non-occurrence of mutation as unity and zero. This way, the cause-mutation relationship is switched into the classification problem, which can be solved either using logistic regression or neural network. However, the occurrence and non-occurrence of mutation is a binary event, which means that we can only use this cause-mutation relationship to predict the mutation positions rather than the would-be-mutated amino acids at predicted positions.

For prediction of would-be-mutated amino acids, we have more difficulties to build a deterministic relationship or classification model. However, there are several common ways (Dayhoff et al., 1978; Feng et al., 1985; Karlin and Ghandour, 1985; Müller et al., 2002) as well as the amino acid mutating probability developed by us (Wu and Yan, 2005g, 2006a, 2007b) to solve this issue.

All these indicate that the prediction of mutations includes at least two steps, say, the prediction of mutation position and the prediction of would-be-mutated amino acids at predicted positions. Along this two-step frame, we very recently conducted several concept-initiated studies to test whether we can apply this cause-mutation relationship with logistic regression as well as neural network to predicting the mutation positions, and then apply the amino acid mutating probability to predicting the would-be-mutated amino acids at predicted mutation positions in hemagglutinins from influenza A virus (Wu and Yan, 2006e, f, 2007a, c, d).

As the results of these concept-initiated studies appear promising, we need to conduct many more proof-of-concept studies to determine whether this approach is dependent on different proteins, subtypes, etc., and to refine the approach and to clarify the related issues. Hence, we attempt to apply this approach to predicting the mutations in H5N1 neuraminidase from influenza A virus in this study.

Materials and methods

429 H5N1 neuraminidases from influenza A virus from 1996 to 2006 are obtained from influenza virus resources (Influenza virus resources, 2006).

As our approaches are not familiar to most researchers, we will explain them in great details.

Prediction model

In our two-step frame, the prediction model is only related to the cause-mutation relationship, which is switched to the classification problem. Thus, we use the logistic regression, whose output ranges between zero and unity, $P(y) = \frac{1}{1 + e^{b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_6x_6 + b_7x_7}}$, where x_i is the independent, y is the dependent, and b_i is the model parameters. As our previous studies shows that seven independents work better than six independents (Wu and Yan, 2006f, 2007a, d), we will use seven independents in modeling of neuraminidase in this study.

Independent I – amino-acid pair predictability

This quantification is calculated according to permutation, and we have used it to study various proteins (Wu, 1999, 2000a–g; Wu and Yan, 2000a–c, 2001a–c, 2002a–d, 2003a–h, 2004a–e, 2005a–d, f, 2006b, d–f, 2007a, c). Its rationale includes: (i) this is the simplest way to quantify the randomness in a protein, (ii) the counting of amino-acid pairs was inspired from modern encryption technology by counting the frequency of basic unit in an unknown language, and (iii) a good signature pattern of a protein must be as short as possible, but the conserved sequence is not longer than four or five residues (PROSITE, 2002), while our previous studies show the amino-acid pair the best for our aim. The practical meanings are that this amino-acid pair predictability is very sensitive to the change in neighboring amino acids, and answers why a type of amino acid is adjacent to a certain type of amino acid but not to the others.

The simplest calculations are as follows: according to the permutation, for example, there are 44 glycines (G) and 34 isoleucines (I) in 2005 AB239126 neuraminidase, the randomly predicted frequency of amino-acid pair “GI” is $3 (44/449 \times 34/448 \times 448 = 3.3318)$, that is, “GI” would appear three times in this neuraminidase, which is the predicted frequency and is the reference for comparison. Actually we do find 3 “GI”, so “GI” is predictable and the difference between its actual and predicted frequency is 0. Again, there are 28 threonines (T) in AB239126 neuraminidase, and the randomly predicted frequency of “TI” is $2 (28/449 \times 34/448 \times 448 = 2.1203)$, i.e. there would be two “TI” in the neuraminidase. But the “TI” appears five times in reality, so the difference between its actual and predicted frequency is 3. After such calculations, each amino-acid pair has its difference between actual and predicted frequency. As a point mutation is related to a single amino acid, it connects with two neighboring amino acids except for the terminal one and constructs two amino-acid pairs, so each amino acid can have the sum of difference between actual and predicted frequency in two neighboring amino-acid pairs (SDAPF).

Independent II – percentage of SDAPF

This is a derivative quantification, because our previous studies show that the mutation minimizes the difference between actual and predicted frequency, and the bigger the difference is, the more vulnerable the amino-acid pair to mutation is (Wu and Yan, 2002a–c, f, 2003a–h, 2004a–c). As each amino acid in neuraminidase has its SDAPF, which generally ranges from –5 to 9, we count how many amino acids have SDAPF of 1, SDAPF of 2, and so on, then calculate their percentage with respect to all amino acids, and each amino acid has this percentage.

Independent III – interaction between SDAPF and its percentage

This quantification is based on the common consideration in regression, that is, the first-order interaction between independents is frequently included in regression analysis (Draper and Smith, 1981; Hosmer and Lemeshow, 2000). In our case, SDAPF and its percentage are closely related one another, and our previous studies suggest that

the interaction significantly enhances the predictability (Wu and Yan, 2006e, f, 2007a, d). We therefore assign the first-order interaction to each amino acid.

Independent IV – amino-acid distribution probability

This quantification is calculated according to the occupancy of subpopulations and partitions (Feller, 1968), and we have used this quantification to study various proteins (Gao et al., 2006; Wu and Yan, 2000d, 2001d, e, 2002c–f, 2004f, 2005d, e, 2006c–f, 2007a, c, d).

The quantification is developed along such line of thought, for example, there are two methionines (M) among 141 amino acids in human hemoglobin α -chain (Wu and Yan, 2000d). With regard to their random distribution, our intuition may suggest that there would be one “M” in the first half of the chain and another “M” in the second half, which is true in real-life case. In fact, there are only three possible distributions of “M”s in human hemoglobin α -chain, i.e. (i) both “M”s are in the first half, (ii) one “M” is in each half and (iii) both “M”s are in the second half. If we do not distinguish either first half or second half but are simply interested in whether both “M”s are in both halves or in any half, we will have the probability of 1/2 for each distribution.

If we are interested in the distribution probability of three amino acids in a protein sequence, we naturally imagine to group the protein into three parts, and our intuition may suggest that each part contains an amino acid. If we do not distinguish the first, second and third part, actually there are three types of distributions, i.e. (i) each part contains an amino acid, (ii) two amino acids are in a part and an amino acid in another part, and (iii) three amino acids are in a part. However, the distribution probabilities are different for them, say, 0.2222 for (i), 0.6667 for (ii) and 0.1111 for (iii). Clearly the protein can only adopt one type of distribution for these three amino acids, which is the actual distribution probability, and we may guess that the distribution (ii) is more likely to happen because of its biggest probability, which is the predicted distribution probability and is the reference for comparison.

For four amino acids, we will have five distribution probabilities, i.e. (i) each part contains an amino acid, (ii) a part contains two amino acids and two parts contain an amino acid each, (iii) two parts contain two amino acids each, (iv) a part contains an amino acid and a part contains three amino acids, and (v) a part contains four amino acids. Their distribution probabilities are 0.0938, 0.5625, 0.1406, 0.1875, 0.0156, respectively. Further, we have seven distributions for five amino acids, we have 11 distributions for six amino acids, we have 15 distributions for seven amino acids, and so on.

So we view the positions of each kind of amino acids in a protein as a certain distribution, whose probability can be calculated according to the equation of $r!/(q_0! \times q_1! \times \dots \times q_n!) \times r!/(r_1! \times r_2! \times \dots \times r_m!) \times n^{-r}$ (Feller, 1968), where ! is the factorial function, r is the number of a kind of amino acid, q is the number of parts with the same number of amino acids and n is the number of grouped parts in the protein for a kind of amino acid. In fact, this distribution probability can be referred to the statistical mechanics, which classifies the distribution of elementary particles in energy states according to three assumptions of whether or not distinguishing of each particle and energy state, i.e. Maxwell-Boltzmann, Fermi-Dirac and Bose-Einstein assumptions (Feller, 1968). In plain words, this distribution probability is the probability if we would receive seven letters in a week but the letters distribute randomly.

The practical meanings are that this quantification is mainly subject to any change in the position of amino acid, and answers why the majority of amino acids cluster in some regions rather than homogeneously distribute along the primary structure of a protein.

With respect to neuraminidases in this study, for instance, there are 18 cysteines (C) in AB239126 neuraminidase. Its predicted and actual distribution probabilities are 0.1246 and 0.0138, so the ratio of predicted versus actual distribution probabilities is 9, whose natural logarithm is 2.1972 (LRPADP). In this way, each amino acid has its LRPADP.

Table 1. Amino acid mutating probability based on the translation probability between RNA codons and translated amino acids

Amino acid	Mutated amino acid with its translation probability
A	12/36A + 2/36D + 2/36E + 4/36G + 4/36P + 4/36S + 4/36T + 4/36V
R	2/54C + 6/54G + 2/54H + 1/54I + 2/54K + 4/54L + 1/54M + 4/54P + 2/54Q + 18/54R + 6/54S + 2/54T + 2/54W + 2/54STOP
N	2/18D + 2/18H + 2/18I + 4/18K + 2/18N + 2/18S + 2/18T + 2/18Y
D	2/18A + 2/18D + 4/18E + 2/18G + 2/18H + 2/18N + 2/18V + 2/18Y
C	2/18C + 2/18F + 2/18G + 2/18R + 4/18S + 2/18W + 2/18Y + 2/18STOP
E	2/18A + 4/18D + 2/18E + 2/18G + 2/18K + 2/18Q + 2/18V + 2/18STOP
Q	2/18E + 4/18H + 2/18K + 2/18L + 2/18P + 2/18Q + 2/18R + 2/18STOP
G	4/36A + 2/36C + 2/36D + 2/36E + 12/36G + 6/36R + 2/36S + 4/36V + 1/36W + 1/36STOP
H	2/18D + 2/18H + 2/18L + 2/18N + 2/18P + 4/18Q + 2/18R + 2/18Y
I	2/27F + 6/27I + 1/27K + 4/27L + 3/27M + 2/27N + 1/27R + 2/27S + 3/27T + 3/27V
L	6/54F + 2/54H + 4/54I + 18/54L + 2/54M + 4/54P + 2/54Q + 4/54R + 2/54S + 1/54W + 6/54V + 3/54STOP
K	2/18E + 1/18I + 2/18K + 1/18M + 4/18N + 2/18Q + 2/18R + 2/18T + 2/18STOP
M	3/9I + 1/9K + 2/9L + 1/9R + 1/9T + 1/9V
F	2/18C + 2/18F + 2/18I + 6/18L + 2/18S + 2/18V + 2/18Y
P	4/36A + 2/36H + 4/36L + 12/36P + 2/36Q + 4/36R + 4/36S + 4/36T
S	4/54A + 4/54C + 2/54F + 2/54G + 2/54I + 2/54L + 2/54N + 4/54P + 6/54R + 14/54S + 6/54T + 1/54W + 2/54Y + 3/54STOP
T	4/36A + 3/36I + 2/36K + 1/36M + 2/36N + 4/36P + 2/36R + 6/36S + 12/36T
W	2/9C + 1/9G + 1/9L + 2/9R + 1/9S + 2/9STOP
Y	2/18C + 2/18D + 2/18F + 2/18H + 2/18N + 2/18S + 2/18Y + 4/18STOP
V	4/36A + 2/36D + 2/36E + 2/36F + 4/36G + 3/36I + 6/36L + 1/36M + 12/36V
STOP	1/27C + 2/27E + 1/27G + 2/27K + 3/27L + 2/27Q + 2/27R + 3/27S + 2/27W + 4/27Y + 4/27STOP

A alanine; R arginine; N asparagine; D aspartic acid; C cysteine; E glutamic acid; Q glutamine; G glycine; H histidine; I isoleucine; L leucine; K lysine; M methionine; F phenylalanine; P proline; S serine; T threonine; W tryptophan; Y tyrosine; V valine

Independents V and VI – percentage of LRPADP and interaction between LRPADP and its percentage

With the similar consideration of independents II and III, we give the percentage of LRPADP and the first order interaction between LRPADP and its percentage to each amino acid.

Independent VII – future composition of amino acids

This quantification is calculated according to the translation probability between RNA codons and translated amino acids (Wu and Yan, 2005g, 2006a, 2007b), and we have used this quantification to study various proteins (Wu and Yan, 2005g, 2006a, f, 2007a–d).

This quantification is developed along such line of thought, for example, we are interested in the amino acid threonine and its mutated amino acids with their mutating probability. As the RNA codons have the unambiguous relationship with their translated amino acids, we can extend this question to RNA level, this is, a point mutation in RNA codon leads to the mutation at amino acid level.

Threonine is related to RNA codons ACU, ACC, ACA, and ACG, the mutation at the first position of ACU can lead ACU to mutate to CCU, GCU, and UCU, which correspond to threonine to mutate to proline, alanine, and serine at amino acid level. Similarly, the mutation at second position of ACU can result in isoleucine, asparagine, and serine, the mutation at the third position of ACU can result in threonine, threonine and threonine. Taken four RNA codons together, threonine would mutate in such a way, say, 4 alanines + 2 arginines + 2 asparagines + 3 isoleucines + 2 lysines + methionine + 4 prolines + 6 serines + 12 threonines. Thus we have the threonine mutating probability to these amino acids, say, $4/36 + 2/36 + 2/36 + 3/36 + 2/36 + 1/36 + 4/36 + 6/36 + 12/36$. For all 20 kinds of amino acids, we have the amino acid mutating probability in Table 1.

For the calculation of future composition of amino acids, we have the following steps: (i) We would expect that “A” has the 12/36 chance of mutating to “A” (line 2 in Table 1), “R” and “N” have no chance of mutating to “A” (lines 3 and 4 in Table 1), “D” has 2/18 chance (line 5 in

Table 1), “C” has no chance (line 6 in Table 1), “E” has 2/18 chance, and so on. (ii) Meanwhile, AB239126 neuraminidase has 18 “A”, 16 “R”, 29 “N”, 21 “D”, 18 “C”, 20 “E”, and so on. (iii) So we can estimate how many “A” can be mutated using $18 \times 12/36 + 16 \times 0 + 29 \times 0 + 21 \times 2/18 + 18 \times 0 + 20 \times 2/18 +$, and so on. In total, this is the future composition of amino acid “A”. (iv) After calculated all 20 kinds of amino acids, “A” contributes 6.3374% to the future composition of neuraminidase, which is the predicted composition and is the reference for comparison. (v) On the other hand, “A” contributes 4% (18/450) to the current composition of AB239126 neuraminidase. (vi) Thus, we have the ratio of future versus current compositions, for example, the ratio of “A” is 1.5844 (6.3374%/4%), which can be assigned to each “A” in AB239126 neuraminidase.

The practical meanings are that this quantification is mainly subject to the future mutation trend, and answers with what probability an amino acid mutates to another type of amino acid.

Dependent – occurrence or non-occurrence of mutation

The phylogenetics analyses the evolutionary process of neuraminidases in question. Along same branch of the evolutionary tree, we can compare the parent and daughter neuraminidases, the difference between them indicates the occurrence of mutation, which is marked as unity, whereas no difference between them indicates the non-occurrence of mutation, which is marked as zero.

Would-be-mutated amino acid

To predict the would-be-mutated amino acids at predicted positions, we can also use Table 1 to make the estimation, for example, we would like to know which type of amino acid “T” would mutate to, according to Table 1, we find that “T” has the highest mutating probability (12/36), however this is only the case that “T” mutates to “T”, then the next to the highest probability is the one that would be likely to be mutated, that is, “S” has 6/36 probability of occurrence. This way, we can approximately predict the would-be-mutated amino acids at the predicted positions.

Statistics

The SigmaStat (SPSS 1992–2003) and Systat (Systat Software 2004) are used to conduct all the logistic regressions. The outlier (3SD) is calculated according to Healy (1979). The prediction sensitivity, specificity and total correct rate are calculated according to the method mentioned in Systat software (Systat Software 2004). The Chi-square test is performed for comparison.

Results and discussion

After all the calculations, each parent neuraminidase has seven independents and one dependent for each amino acid of its sequence, for example, Table 2 shows a fraction of a neuraminidase after the calculation, where each amino acid is associated with seven independents and one dependent, which is determined by comparing 1996 AAD51926 and 1997 AAK38299 neuraminidases. Thus, we can input this format of data into the logistic regression to obtain the model parameters.

In modeling, we use the so-called population estimates to make predictions, and we have technically two ways to obtain the population estimates, either by calculating mean $\pm$ SD of all obtained model parameters or by pooling the data into a representative. We use the second method in this study because the logistic regression does not appear powerful enough to capture the mutation in each sequence, which is particularly related to the ratio of number of mutations to the length of sequence, although neuraminidase is generally longer than hemagglutinin and the logistic regression functions better.

In our previous studies (Wu and Yan, 2006e, f), we used the linear regression to evaluate the prediction performance, which is a very traditional method for evaluation of prediction performance. However, we soon realized the limitation of linear regression in context with the prediction of mutation. This is because we generally have the

paired datasets for linear regression, for example, we might have the measured and predicted blood drug concentrations at certain time points, and then we can use the linear regression to regress them and get the correlation coefficient. However, this is not suitable for the prediction of mutation, for example, we might have five actual mutation positions, but four predicted mutation positions. In such a case, it would be difficult to use the linear regression because of unpaired datasets. Still, we also cannot use the linear regression for evaluation of would-be-mutated amino acids, because an amino acid can mutate to several different types of amino acids, which cannot be considered as paired cases.

To overcome this difficulty, we used the percentage of captured positions for evaluation (Wu and Yan, 2007a, d), and more recently we use the prediction sensitivity, specificity and total correct rate (Wu and Yan, 2007c) according to the method mentioned in Systat software (Systat Software 2004) because we can classify the predicted mutation positions as the positives, false positives, negatives and false negatives when comparing the predicted with the actual mutation positions. Thus, the percentage of captured positions in our previous studies (Wu and Yan, 2007a, d) is in fact equal to the total correct rate.

As can be seen in Fig. 1, the prediction pattern of H5N1 neuraminidase is similar to the prediction patterns of other hemagglutinins although we can find the statistical difference. However, the statistical difference is mainly found between the prediction in hemagglutinins with distinguishing arginine, leucine and serine and others. This is very suggestive because it implies our research direction in near future, say, to conduct the prediction at RNA codon level as a single mutation in RNA codon level may not lead to the mutation at amino acid level such as “A” has 12/36 chance of mutating “A” in Table 1.

Table 2. Independents and dependent of AAD51926 neuraminidase

Position	Amino acid	Independents							Dependent
		I	II	III	IV	V	VI	VI	
1	M	2	22.3404	44.6809	0.4055	1.4894	0.6039	1.1468	0
...	...								
16	V	1	21.4894	21.4894	1.4962	6.8085	10.1868	0.9826	0
17	V	0	12.3404	0.0000	1.4962	6.8085	10.1868	0.9826	1
18	G	0	12.3404	0.0000	2.1752	9.5745	20.8265	0.7309	0
19	I	2	22.3404	44.6809	2.4812	8.0851	20.0611	0.7020	0
20	I	5	3.6170	18.0851	2.4812	8.0851	20.0611	0.7020	1
21	S	3	16.5957	49.7872	1.0498	11.4894	12.0618	0.8347	0
...	...								
469	K	2	22.3404	44.6809	2.9957	4.0426	12.1104	0.9620	0

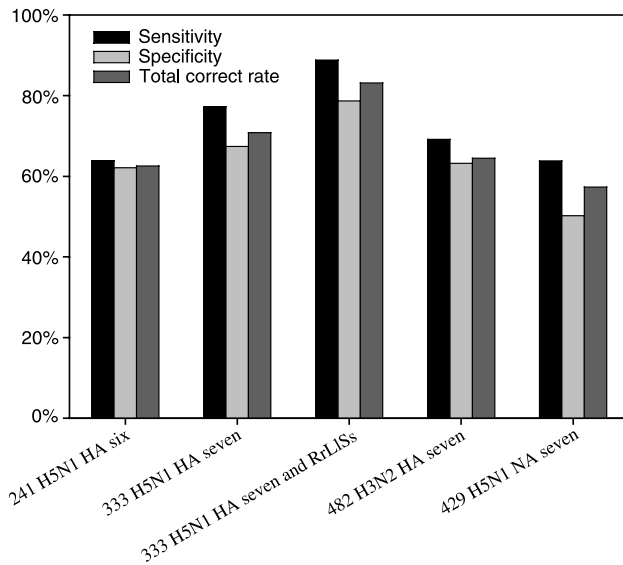


Fig. 1. Prediction performance in studied proteins. The sensitivity is equal to the predicted positives/the actual mutations (%), the specificity is equal to the predicted negatives/the actual non-mutations (%), and the total correct rate is equal to (predicted positives + predicted negatives)/length of hemagglutinin (%). 241 H5N1 HA six is the predictions using 241 H5N1 hemagglutinins with six independents; 333 H5N1 HA seven is the predictions using 333 H5N1 hemagglutinins with seven independents; 333 H5N1 HA seven with RrLISs is the predictions using 333 H5N1 hemagglutinins with seven independents with distinguishing arginine, leucine and serine; 482 H3N2 HA seven is the predictions using 482 H3N2 hemagglutinins with seven independents; 429 H5N1 NA seven is the predictions using 429 H5N1 neuraminidases with seven independents. The Chi-square test indicates the statistically significant difference in sensitivity between 241 H5N1 HA six and 333 H5N1 HA seven with RrLISs, between 333 H5N1 HA seven and 333 H5N1 HA seven with RrLISs, between 333 H5N1 HA seven with RrLISs and 482 H3N2 HA seven, between 333 H5N1 HA seven with RrLISs and 429 H5N1 NA seven; the statistically significant difference in specificity between 241 H5N1 HA six and 333 H5N1 HA seven with RrLISs, between 333 H5N1 HA seven and 429 H5N1 NA seven, between 333 H5N1 HA seven with RrLISs and 482 H3N2 HA seven, between 333 H5N1 HA seven with RrLISs and 429 H5N1 NA seven; the statistically significant difference in total correct rate between 241 H5N1 HA six and 333 H5N1 HA seven with RrLISs, between 333 H5N1 HA seven with RrLISs and 482 H3N2 HA seven, between 333 H5N1 HA seven with RrLISs and 429 H5N1 NA seven

Still, Fig. 1 suggests that the cause-mutation relationship defined is independent of subtypes of protein as well as proteins, at least for hemagglutinins and neuraminidases. In this case, it means that the randomness does play a mutating role not only in hemagglutinins but also in neuraminidases although we need to conduct more proof-of-concept studies to further determine this issue.

This way, we can obtain the population estimates from regressing historical data. Thereafter, we can input seven independents of recent neuraminidases, whose mutations are yet to know, into the logistic regression with population estimates and get the output, which is ranged from 0 to 1 in each position. For example, the human H5N1 neuraminidase (AB239126) is a relatively new sequence, which can serve for our prediction. For this neuraminidase, we have seven independents (Table 3), and then we put them into $P(y) = \frac{1}{1 + e^{0.42 - 0.214x_1 - 0.061x_2 - 0.156x_3 - 0.021x_4 + 0.11x_5 + 0.016x_6 + 0.035x_7}}$ which is based on the population of 90 neuraminidases from 2000 to 2004.

Figure 2 displays the prediction of mutation in AB239126 H5N1 neuraminidase according to our two-step frame. The solid line in the lower panel is the predicted mutation probability with respect to each position, and the dash-dotted line is the cut-off mutation probability of 0.5, that is, the amino acid whose mutation probability is larger than 0.5 risks mutation. The pie picture in the upper panel shows how to predict the would-be-mutated amino acid from serine at position 319 according to the amino acid mutating probability in Table 1.

With the population estimates as model parameters for prediction, an important issue is the sampling strategy (Wu et al., 1995, 1996; Wu, 1997), that is, from which population we get the population estimates, not only because there are many subtypes in neuraminidases (Air et al.,

Table 3. Independents of AB239126 neuraminidase

Position	Amino acid	Independents						
		I	II	III	IV	V	VI	VI
1	M	1	22.2222	22.2222	2.1972	5.7778	12.6951	0.9433
2	N	1	22.2222	22.2222	0.3365	6.4444	2.1684	0.6475
3	P	4	8.8889	35.5556	3.5766	4.8889	17.4854	0.9764
4	N	5	4.6667	23.3333	0.3365	6.4444	2.1684	0.6475
5	Q	2	21.3333	42.6667	2.8134	2.4444	6.8772	0.9832
6	K	0	12.8889	0.0000	0.8755	4.6667	4.0855	0.7919
7	I	-1	5.1111	-5.1111	1.0527	7.5556	7.9535	0.7410
8	I	1	22.2222	22.2222	1.0527	7.5556	7.9535	0.7410
...	...							
449	K	2	21.3333	42.6667	0.8755	4.6667	4.0855	0.7919

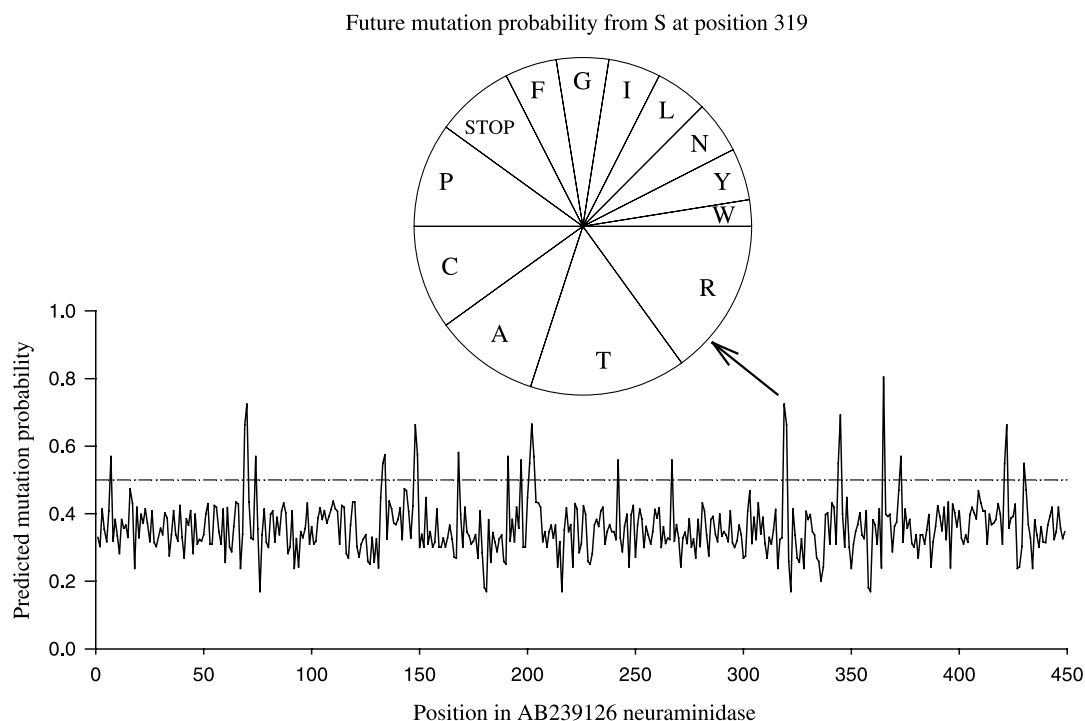


Fig. 2. Prediction of mutations in AB239126 neuraminidase based on logistic regression (lower panel) and amino acid mutating probability (upper panel)

1985; Schreier et al., 1988; Harley et al., 1989; Liu et al., 2003; Campitelli et al., 2004; Suzuki et al., 2004; Bragstad et al., 2005) but also the migration of wild birds is different one from another (Donis et al., 1989; Rohm et al., 1995; Hoffmann et al., 2000; Guan et al., 2004; Krauss et al., 2004; Wu and Yan, 2005e). This implies that the population estimates obtained from Asian wild bird may not be suited for the prediction of mutation in wild bird in North America, which nevertheless needs more studies. Suggestive is that we may have many different population estimates, based on which we make the predictions, which of course needs more studies.

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