Original article

Emergence of truncated PB1-F2 protein of H3N2 influenza virus during its epidemic period in Jiangsu Province, China

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Keywords: *H3N2 subtype influenza A virus; PB1-F2 protein; truncation*

Background PB1-F2 protein has been proven to increase the pathogenicity of influenza A virus (IAV) strains in primary infection and in secondary bacterial infection. It can also regulate the activity of viral polymerase. However, it was shown in another retrospective study that a portion of IAVs do not express full-length PB1-F2 protein during virus development; different kinds of stop codons cause exits in the open reading frames and form PB1-F2 gene products with the corresponding genotypes. Truncated PB1-F2 in human H3N2 IAVs has long been detected in North America but its evolution in China is still unclear.

Methods Influenza-like illnesses (ILIs) from the whole of Jiangsu Province were collected and inspected to determine the type and subtype of the viruses. A portion of isolates collected in the epidemic period were selected as samples for later whole-genome sequencing, and the exact sequences were determined and analyzed.

Results H3N2 influenza virus was one of the epidemical strains which had been prevalent during 2009–2010, in Jiangsu. Five H3N2 isolates with truncated PB1-F2 protein (25aa) were detected in influenza samples from Nanjing and Xuzhou, while seven similar H3N2 isolates were also reported in Niigata, Japan.

Conclusion This emergence indicates the possibility that there has been transmission of the H3N2 virus between the two countries.

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Influenza A viruses (IAVs) can infect different species; have caused three pandemics during the twentieth century with large-scale morbidity and mortality in 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2);[1] and can also spread worldwide annually from global circulation of the virus; for instance, seasonal influenza viruses (H3N2/H1N1/B) can result in 250 000–500 000 deaths every year.[2]

Factors such as gene mutation of the virus, climatic condition changes, and population activities can all affect the epidemics of influenza virus worldwide.[3] IAVs include a single negative-sense RNA chain which consists of eight segments (1–8), and at least 10 kinds of protein are encoded in all subtypes of IAVs.[4] At present, 16 hemagglutinin (HA) subtypes and nine neuraminidase subtypes have been classified by the characteristics of the genes which express these two kinds of viral surface protein.[5] H3N2 subtype viruses have the highest variation rate among the three main influenza subtypes (H3N2/H1N1/B) currently circulating.[6] Gene variation monitoring is regarded as an important tool for the analysis of antigenic shift status and antigenic drift as well as for "gene re-assortment" between different segments of the influenza virus.[7]

Another kind of polypeptide expressed by segment 2 mRNA which is encoded by an alternative reading frame in the PB1 gene was discovered in 2000. The full-length PB1-F2 protein consists of 90 amino acids, and the ORF of the PB1-F2 gene (273bp) starts at position 119 and ends at position 391 with the whole PB1 gene fragment containing 2341bp.[8] This short life cycle protein was first discovered in the human influenza strain A/Puerto Rico/8/34 (H1N1) as one of the non-structure protein types expressed when the host cells were infected. Its ORF was identified in all types of IAVs but is absent in the influenza B virus. Former studies have proven that the PB1 segment was reassorted from viruses of the avian reservoir in all three pandemics of the twentieth century;[9] so the PB1-F2 gene may be a clue to explain the exact function of the PB1 segment, and thus a series of experiments were carried out in the past few years and the effects of this protein have been determined in the animal model.[10] The PB1-F2 protein has a pleiotropic effect as recent studies have shown that it[11,12] promotes apoptosis, promotes inflammation, and enhances virus polymerase complex activity.

Retrospective research has found stop codons can exist in

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the PB1 gene. The full-length PB1-F2 protein could not be expressed and the truncated PB1-F2 loses the functions previously described.[11] PB1-F2 protein expressed by IAVs in different hosts or different subtypes showed significant differences in both length and evolutionary features.[10] Seasonal influenza subtype H3N2, H1N1, and B-type influenza were the main circulating strains from 2006 to 2009; H3N2 subtype virus mostly spread in the northwest and northern as well as southern parts of China. East and Southeast Asia were the center of virus circulation in temporally overlapping epidemics and epidemics in the temperate regions each year as revealed by antigenic and genetic analysis during 2002–2007.[2] Additionally, analysis on migration dynamics of seasonal H3N2 influenza virus during 2008 and 2010 had supported a similar conclusion;[13] influenza surveillance of these areas is an important part of global control and prevention.

Recently, IAV isolates with a kind of truncated PB1-F2 protein were detected in surveillance samples of clinical illnesses. Five H3N2 isolates showed high similarity in PB1 gene sequence with Niigata isolates detected in the same period. And more isolates that encode other kinds of truncated PB1-F2 proteins were reported in 2010–2011 around the world.

METHODS

Influenza-like illness surveillance

Influenza surveillance network

The influenza surveillance network in Jiangsu Province includes 14 network laboratories and 29 sentinel hospitals. Throat swab samples of influenza-like illnesses (ILIs) were collected and inspected by 14 influenza surveillance network laboratories. Each week, 5–15 samples were required for every sentinel hospital following the National Influenza Surveillance Program (2010) protocols.

ILI sample virus isolation and nucleic acid identification

Throat swab samples were inoculated and cultured in Madin–Darby canine kidney cells to isolate influenza viruses. Real-time RT-PCR detections were performed to identify the type and IAV subtype of influenza virus in the culture products.

Data entry and statistical analysis

ILI information and sample test results were reported to the "National Disease Surveillance System" by influenza surveillance laboratory staff and data sets were generated automatically. Data in "National Disease Surveillance System" were exported to Excel files, we calculated percentages of RNA-positive cases in all ILIs as RNA-positive rates, and counted the case numbers of different virus types, charts about RNA-positive rates and different case numbers of each month in three years were generated by Excel.

Gene detection and PB1 sequencing

Sample selection and virus proliferation

The principle of convenience sampling was adopted. By determining the peak positive-detection period and the period of seasonal H3N2 influenza virus dissemination in ILI epidemiological surveillance, isolates were selected in the two periods with at least one case in each year. Viruses were cultured minimally in MDCK cells to generate products with a titer greater than 1:8, and the RNA was extracted from the clarified supernatant.

Gene segment amplification and DNA sequencing

Viral RNA was extracted from 140 μl nasopharyngeal aspiration samples with an auto nucleotide extraction set (Apply Bio Company, USA). Reverse transcriptions were carried out by using the SuperScript II Reverse Transcriptase Kit (Invitrogen); cDNA was generated and sequence-specific primers for segment 2 were designed to keep the amplified segment lengths less than 1000 bp. Polymerase chain reactions were performed by using the LA-Taq DNA polymerase (TaKaRa) kit to amplify gene segments. PCR products were gel recovered, purified, dye labeled, and examined with a 3730 sequencer (Applied Biosystem).

Sequence collation and analysis

Sequence results were exported as sequence files from the sequencing computer; reference sequences were downloaded from the NCBI database. DNAStar software was used for sequence comparison. Phylogenetic trees were deduced from data sets described above using the neighbor-joining algorithm available in the MEGA4 software package. And to assess the reliability of key nodes on the phylogenetic tree, a bootstrap re-sampling analysis was also undertaken in each case. This involved the inference of 1 000 replications of neighbor-joining trees using the maximum likelihood substitution model inferred for data sets.

RESULTS

RNA detection of ILI specimens and monthly RNA-positive rates

A total of 69774 ILI cases were accepted during 2009–2011 of which 56054 cases were RNA negative and 13720 cases were RNA positive following fluorescent quantitative PCR detection; the overall positive rate of nucleic acid detection was approximately 19.7% (Figure 1). Among positive cases, 210 cases were seasonal H1 (1.5%), 6178 cases were 2009 pandemic influenza strain (45%), 3479 cases were seasonal H3 (25.5%), 3531 cases were B-type influenza (25.7%), and 322 cases were other influenza A subtypes (2.3%). The amount of samples in a single month after January 2010 was between 1300 and 2700.

Alteration of advantages for epidemic strains

The 2009 pandemic strain and seasonal H3N2 influenza as well as B-type accounted for 96.2% of all influenza cases (Figure 2). Briefly, seasonal H3N2 was highly detected from July to November of 2009 and 2010; the 2009 pandemic strain was highly detected from July 2009 to

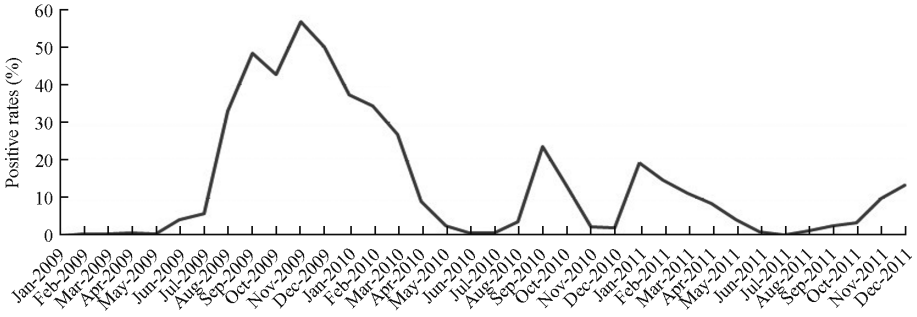

Figure 1. Overall RNA-positive detection rates for each month from 2009 to 2011. Among the positive rates for all 36 months, three peaks appeared in 3 years: August 2009–March 2010, October 2010–March 2011, and December 2011.

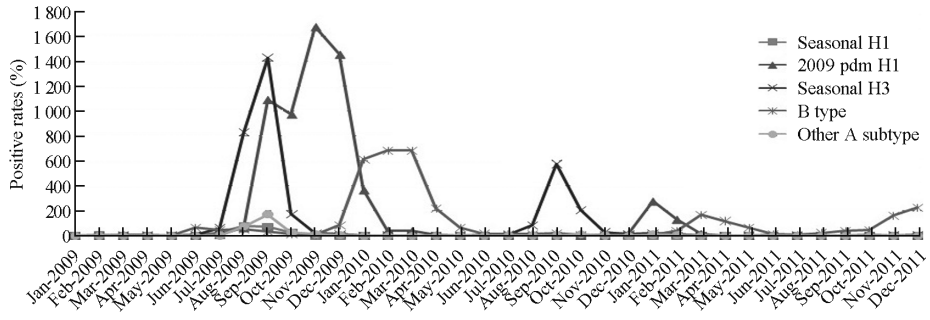

Figure 2. Alteration of advantages for epidemic strains over 3 years.

March 2010 and December 2010 to February 2011; B-type influenza was highly detected from July 2009 to June 2010 and January 2011 to June 2011 as well as October 2011 to December 2011.

Gene detection and PB1 sequencing

Nineteen isolates were examined in accordance with the convenience sampling method, and they are representative of 3 years and two incidence periods: six viruses were isolated before April 2009 (outbreak of swine-derived H1N1) and 13 after April 2009; the northern region of Huaihe River (9) and the southern region (11), the peak incidence period (12), and the dissemination period (7). The virus titers of the 19 isolates were all greater than 1:8. PCR of segment 2 was successfully amplified in all 19 isolates (Table 1).

Genetic variations were mastered by phylogenetic tree construction and amino acid sequences analysis

Phylogenetic trees

A/New York/572/1996 (2003) was the earliest isolate with truncated PB1-F2 (63aa) in H3N2 IAVs, and isolates with the other kind of truncated PB1-F2 (11aa) also emerged in 2004 in this area. So, eight former New York isolates (1996–1997) and four swine strains as well as isolates in 2010–2011 were introduced in the construction of the phylogenetic tree, and sequence data for PB1 segments of seasonal IAVs (H3N2) sampled during the period 1968 and 1999–2009 were downloaded from GenBank, using GenBank accession numbers available from *http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html* (Figure 3).

Twelve isolates from Nanjing and Xuzhou as well as Niigata encoded PB1-F2 (25aa) while 24 other isolates encoded PB1-F2 (34aa). The amino acid sequences of the 19 isolates in Jiangsu and other representative isolates are shown in Figure 4. Mitochondrial targeting sequences (MTSs) in these truncated PB1-F2 proteins are missing. The proportion of PB1-F2 (<79aa) was approximately 5% in all known human H3N2 isolates as shown by an earlier study;[10] comparatively, 37 isolates in our investigation were

Table 1. Illness information for the 19 isolates detected

Name of isolates	Gender	Age (years)	Sampling date	Inoculating date	HA titer	PCR result	City
A/JSNC/122/2009(H3N2)	Male	7	2009/2/4	2009/3/3	1:8	Seasonal H3	Wuxi
A/JSBT/123/2009(H3N2)	Male	23	2009/1/15	2009/2/4	1:8	Seasonal H3	Wuxi
A/JSBT/130/2009(H3N2)	Female	39	2009/2/18	2009/3/3	1:16	Seasonal H3	Wuxi
A/JSBT/133/2009(H3N2)	Male	35	2009/2/21	2009/3/3	1:8	Seasonal H3	Wuxi
A/JSNC/136/2009(H3N2)	Male	4	2009/2/20	2009/3/3	1:8	Seasonal H3	Wuxi
A/JSBT/151/2009(H3N2)	Female	55	2009/2/13	2009/3/3	1:32	Seasonal H3	Wuxi
A/JSQS/1418/2009(H3N2)	Female	21	2009/8/31	2009/9/1	1:16	Seasonal H3	Xuzhou
A/JSXY/396/2009(H3N2)	Male	10	2009/9/8	2009/9/11	1:16	Seasonal H3	Xuzhou
A/JSXY/397/2009(H3N2)	Female	8	2009/9/8	2009/9/11	1:32	Seasonal H3	Xuzhou
A/JSXZ/1595/2009(H3N2)	Female	8	2009/9/23	2009/10/16	1:16	Seasonal H3	Xuzhou
A/JSSN/325/2009(H3N2)	Female	11	2009/9/7	2009/9/11	1:16	Seasonal H3	Xuzhou
A/JSXZ/1909/2009(H3N2)	Female	50	2009/10/19	2009/11/2	1:32	Seasonal H3	Xuzhou
A/JSQS/11484/2009(H3N2)	Male	1	2010/9/16	2010/9/28	1:32	Seasonal H3	Xuzhou
A/JSXZ/2257/2010(H3N2)	Female	28	2010/9/20	2010/10/10	1:32	Seasonal H3	Xuzhou
A/JSXZ/1766/2010(H3N2)	Male	48	2010/9/27	2010/10/25	1:32	Seasonal H3	Xuzhou
A/Nanjing/1663/2010(H3N2)	Male	61	2010/8/28	2010/9/10	1:16	Seasonal H3	Nanjing
A/Nanjing/1655/2010(H3N2)	Female	48	2010/8/26	2010/9/10	1:16	Seasonal H3	Nanjing
A/Nanjing/1654/2010(H3N2)	Male	46	2010/8/26	2010/9/10	1:16	Seasonal H3	Nanjing
A/JSJR/1646/2011(H3N2)	Female	25	2011/11/9	2011/11/10	1:16	Seasonal H3	Zhenjiang

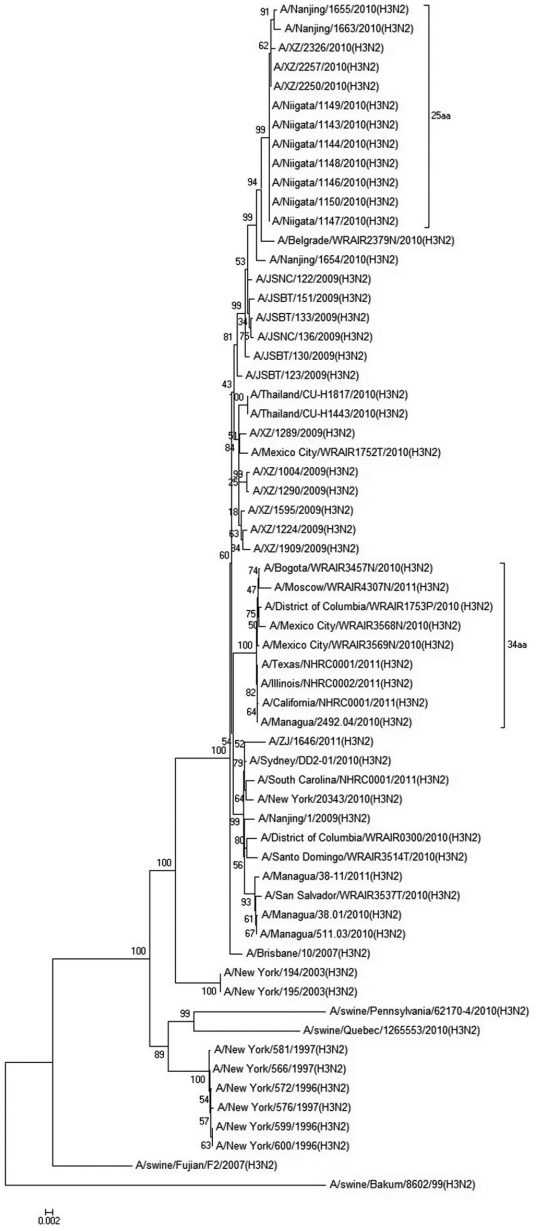

Figure 3. Phylogenetic tree based on sequences of the PB1 ORF. The phylogenetic tree was generated by the neighbor-joining algorithm, based on 1000 replications in MEGA4. All isolates which were collected after 2010 can be classified in one main branch. Two isolate groups that contain different kinds of PB1-F2 genes were labeled group (25aa) and (34aa), respectively.

truncated.

The similarity between any 2 of the total 13 isolates (25aa PB1-F2 protein) was ≥99.0%. Of the five H3N2 viruses surveyed, the PB1-F2 protein was truncated to 25 amino acids theoretically, while isolates such as Nanjing/1654 can encode a complete PB1-F2 (90aa). Accordingly, H3N2 influenza viruses with two different PB1-F2 genotypes were examined in this area and compared; still no evidence was found to prove that these kinds of truncated PB1-F2 genes in recent isolates are not inherited from earlier H3N2 viruses or other species, so it is possibly the result of gene mutation from recent seasonal H3N2 viruses.

DISCUSSION

Seasonal H1N1 virus is expected to maintain a low level of prevalence in this area in the future. Swine-origin H1N1 virus has replaced seasonal H1N1 as the main epidemic strain of this region and is expected to co-circulate with H3N2 and influenza B virus in the next few years. The epidemic period of seasonal H3N2 was 2009–2010; it was highly detectable from July to November. The PB1 ORF sequence similarities of the 12 total isolates from 2010 indicated that these viruses were highly homologous, and the study on whole-genome sequences of the three Nanjing isolates and the 8 Niigata isolates proved that a high similarity also existed between the other seven RNA segments. In conclusion, the high degree of similarity suggests that this kind of H3N2 virus was probably spread between the populations of two different countries.[14]

As studies have shown, the coding sequence of the PB1-F2 protein developed with higher variation rates than that of the entire PB1 segment, indicating that host selection pressure on PB1-F2 proteins was rather strong. A notable feature of the PB1-F2 gene mutations in human H1N1 subtype IAVs is the termination codons which have existed in its ORF and can cause protein truncation. Besides human H1N1 strains, the PB1-F2 of strains which infect different hosts have length polymorphisms and evolutionary characteristics that have also shown heterogeneity.[9]

Pandemic H1N1 strains before 1947 such as laboratory strains A/Brevig Mission/1/18 (H1N1) and A/Puerto Rico/8/34(H1N1) encoded full-length PB1-F2 proteins, which were 90aa and 87aa, respectively; while PB1-F2 proteins of H1N1 strains isolated after 1947 were usually truncated. And the 2009 pandemic strains such as A/California/07/2009 (H1N1) possessed a kind of PB1-F2 protein which fractured at the 11th and 57th amino acid sites.[10-12] Recently, PB1-F2 truncation has become an important genetic characteristic of the current seasonal H1N1 influenza strains; it can be speculated that such changes are more conducive to propagation of the virus in crowds. This phenomenon can also be found in the PB1 segments of swine H1N1 strains; classical swine H1N1 viruses from before 1947 also expressed full-length PB1-F2 proteins but since 1947 this protein has developed fractures at the amino acid sites 12, 26, and 35. Accordingly, intact PB1-F2 proteins of human H1N1 viruses gradually disappeared in approximately 30 years of evolution since 1918.

The prevalence of truncated PB1-F2 can also be demonstrated in recent H3N2 strains. Intact PB1-F2 proteins of H3N2 subtype IAVs have gradually fractured since 1968 and considerable evidence has been discovered to prove this trend. Retrospective research has demonstrated that theoretical PB1-F2 of H3N2 influenza viruses named "North America pig tripe reassortants" were full length

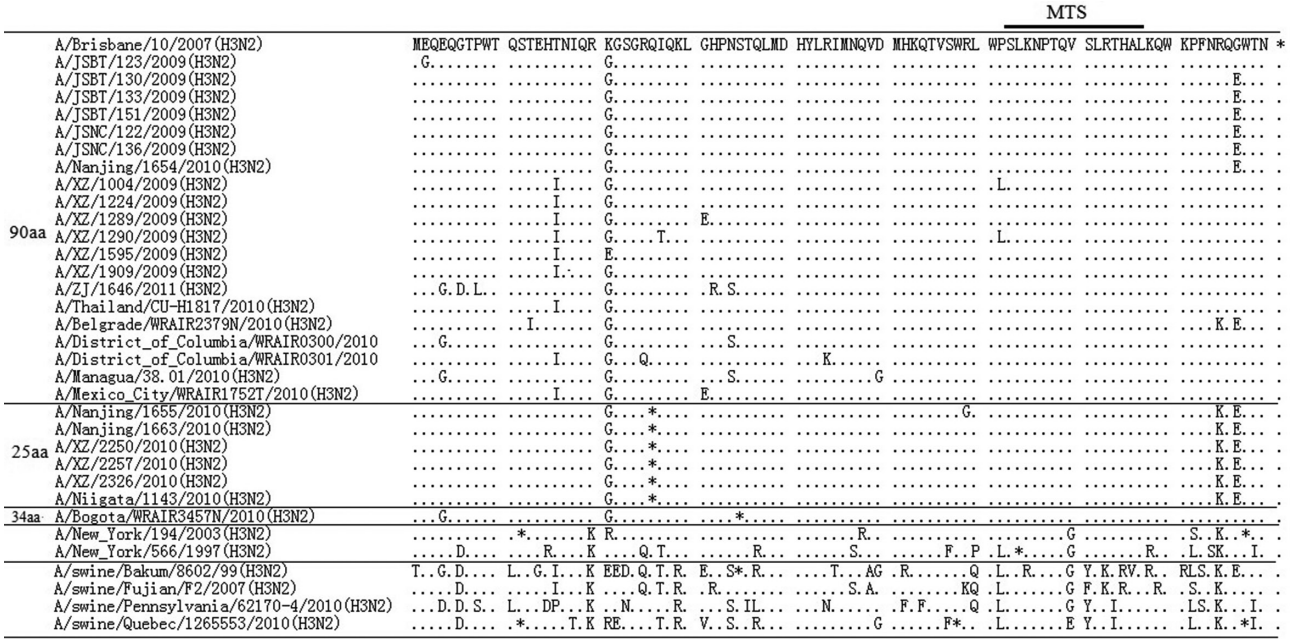

Figure 4. Theoretical PB1-F2 amino acid sequences of different isolates. *Each amino acid sequence was inferred in MEGA4. Each line represents one single isolate's PB1-F2 amino acid sequence from a vaccine strain. A/Brisbane/10/2007 (H3N2) is shown in the top line. Stop codons are labeled with *. MTS is referred to by the black line. Eight New York isolates with truncated PB1-F2 were included as a reference, and part of the PB1 sequences submitted to NCBI after January 2010 were compared.

while European swine strains fractured in the amino acid sites 12, 26, and 35.[10,11] The current H3N2 subtypes of influenza virus circulating in the human population have all developed from the pandemic strain that formed in 1968. The seasonal H3N2 virus before 1996 encoded a full-length PB1-F2. However, strains which encoded a truncated PB1-F2 began to become prevalent after approximately 40 years from when the H3N2 strain was formed, and its influence on the pathogenic capacity and transmission as well as ecology of the H3N2 subtype should be researched in future.

The full-length PB1-F2 protein was previously shown to enhance apoptosis in response to cytotoxic stimuli as is described by the localization of the 87 amino acid proteins in PR8 to the mitochondria, a property consistent with its pro-apoptotic functions. (1) An MTS exists in the C-terminal portion of the PB1-F2 protein[15] and mainly bonds with the adenine nucleotide translocator 3 in the inner mitochondrial membrane and the voltage-dependent anion channel 1 in the outer mitochondrial membrane. This combination results in the formation of the mitochondrial surface holes which change the mitochondrial membrane permeability resulting in the release of mitochondrial cytochrome C and the initiation of the intrinsic apoptosis pathway.[16,17] (2) Experiments in mice have shown that PB1-F2 protein significantly induces apoptosis in alveolar macrophages and causes changes in the level of host immune factors;[18] it can also lead to lung injury associated with secondary bacterial infections of the host; in addition, the PB1-F2 protein upregulates inflammatory cells in the host blood which elevates serum cytokines and increases the amount of bacteria in lung tissue.[19,20] (3) The PB1-F2 protein was found to locate to the host cell nucleus where it is phosphorylated by protein kinase C and binds with the PB1 protein to increase the activity of the virus polymerase complex by increasing nuclear retention time.[21,22]

The effects mentioned above were not found in every kind of PB1-F2 protein; not all roles of the PB1-F2 protein in the PR8 virus were demonstrated in other full-length PB1-F2 proteins. For instance, the PB1-F2 protein of pandemic strain A/Brevig Mission/1/1918 (H1N1) and A/Hong Kong/1/1968 (H3N2) could not promote the apoptosis of experimental cells. By comparison, truncated PB1-F2 protein (57aa) encoded by A/Beijing/11/1956 (H1N1) could still enhance the activity of the virus polymerase complex.[23-25] The differences in functionality are possibly related to specific regions of the PB1-F2 protein. For example, the N-terminal region of the PB1-F2 protein was responsible for increased expression of viral PB1 protein in a recent study.[26]

The exact functions of the PB1-F2 protein in seasonal H3N2 viruses are still not as clear as in H1N1 viruses. However, these five isolates are H3N2 strains with truncated PB1-F2 protein first found in the Chinese mainland. The viruses were detected in the Nanjing District, which is regarded as an important communication hub with a large floating population. Its unique geographic position and population mobility would provide a suitable condition for the rapid spread of IAVs.

Mortality caused by influenza is concentrated at the extremes of age, but is mainly above 64 years of age; in contrast, morbidity mainly occurs in younger people. Population immunity status is recognized to play an important role in the formation of this clinical feature.[27]