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Original Article

The global epidemic trend analysis of influenza type B drug resistance sites from 2006 to 2018

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Abstract

Introduction: Influenza is a severe respiratory viral infection that causes significant morbidity and mortality, due to annual epidemics and unpredictable pandemics. With the extensive use of neuraminidase inhibitor (NAI) drugs, the influenza B virus has carried different drug-resistant mutations. Thus, this study aimed to analyze the prevalence of drug-resistant mutations of the influenza B virus.

Methodology: Near full-length sequences of the neuraminidase (NA) region of all influenza B viruses from January 1, 2006, to December 31, 2018, were downloaded from public databases GISAID and NCBI. Multiple sequence alignments were performed using Clustal Omega 1.2.4 software. Subsequently, phylogenetic trees were constructed by FastTree 2.1.11 and clustered by ClusterPickergui_1.2.3.JAR. Then, the major drug resistance sites and surrounding auxiliary sites were analyzed by Mega-X and Weblogo tools.

Results: Among the amino acid sequences of NA from 2006 to 2018, only Clust04 in 2018 carried a D197N mutation of the NA active site, while other drug resistance sites were conserved without mutation. According to the Weblogo analysis, a large number of N198, S295, K373, and K375 mutations were found in the amino acid residues at the auxiliary sites surrounding D197, N294, and R374.

Conclusions: We found the D197N mutation in Clust04 of the 2018 influenza B virus, with a large number of N198, S295, K373, and K375 mutations in the helper sites around N197, N294, and R374 from 2006 to 2018. NA inhibitors are currently the only kind of specific antiviral agent for the influenza B virus, although these mutations cause mild NAIs resistance.

Key words: drug resistance mutation; influenza B virus; phylogenetic tree.

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Introduction

Influenza is an acute viral respiratory tract infection that can spread globally between humans, infect any age group, and cause a serious public health problem. The typical symptoms observed are high fever, runny nose, sore throat, muscle aches, headaches, cough, and feeling of tiredness [1]. According to the latest estimates by the US Centers for Disease Control and Prevention, World Health Organization (WHO), and global health partners, every year, 650,000 people die due to respiratory illness caused by seasonal influenza [2]. The human influenza virus belongs to the orthomyxoviride virus family in the virological classification [3]. It is divided into A, B, and C types according to the antigenicity of their nuclear proteins. While influenza B viruses have been the primary pathogens causing human influenza in recent years, with minor outbreaks in some areas [4].

Since 1983, circulating influenza B viruses have split into two distinct lineages, B/Victoria (B/Vic) and B/Yamagata (B/Yam), represented by B/Victoria/2/87 and B/Yamagata/16/88 strains, respectively [5]. The HA proteins of B/Vic and B/Yam viruses were significantly different, with about a 5% amino acid difference. Most of the differences located at the antigenic sites of the influenza B virus HA protein would lead to antigenicity differences between strains. In addition, the NA amino acid difference between

B/Vic and B/Yam viruses is about 2%. Studies found NA amino acid differences in various flu B strains mainly located in the open reading frame of the NB gene [6], coding the head and stem of NA proteins. The recombination from those differential fragments forms new strains, which is more likely to lead to the popularity of influenza B [7].

Nowadays, neuraminidase inhibitors, such as Oseltamivir, Zanamivir, Peramivir, etc., are the main clinical drugs for type B influenza treatment [8]. With the worldwide prevalence of influenza caused by the type B influenza virus and the wide use of NAI drugs, the drug resistance mutations of influenza type B viruses have been accumulating. It has been reported that influenza B viruses have evolved varying degrees of resistance to clinically used NAI drugs as mentioned above [9]. However, there is no systematic study on drug resistance of NAI all over the world. In this study, we analyzed the near full-length neuraminidase (NA) gene sequences of influenza type B virus that had been submitted to the public databases around the world to characterize the crucial drug resistance mutations in NA proteins to provide a theoretical basis for better scientific prevention and control of influenza B [10].

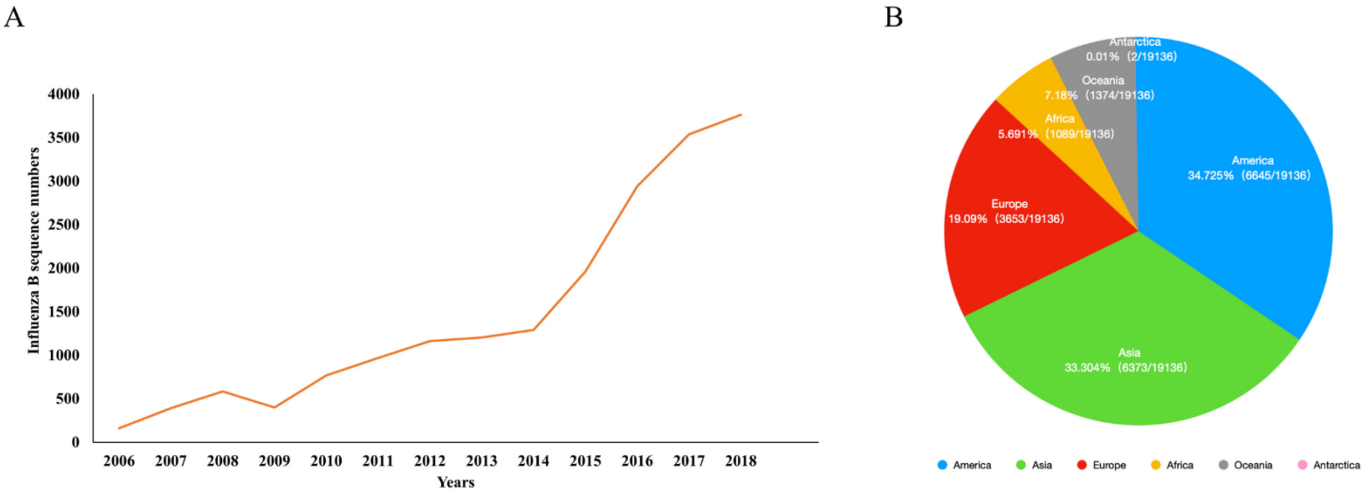
Methodology

As the internationally approved anti-influenza B virus drugs have mainly been developed for neuraminidase, the NA region sequences of all influenza type B viruses in the public databases between January 1, 2006 and December 31, 2018 were downloaded for this study. The databases used to retrieve the sequences were from Global Initiative on Sharing All Influenza Data (GISAID, http://platform.gisaid.org/epi3/frontend) and National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?Go=database). The Python script was used to get rid of the repeated sequences and edit the unified sequence name with the format{accession}{continent}{country}{year}. The Clustal Omega 1.2.4 software was used to carry out multi-sequence alignment, retain the qualified NA sequences, and delete the sequences missing more bases at both ends to obtain the near full-length NA sequences.

In order to understand the molecular evolution of the influenza B virus, we used FastTree 2.1.11 software to perform a phylogenetic analysis of the near full-length NA sequences, and constructed approximately-Maximum-likelihood (ML) phylogenetic trees. The command used was fasttree-gtr-nt < alignment. File > tree_file. Then, clustering analysis was performed using ClusterPickerGUI_1.2.3.jar. Parameters were set as initial Threshold = 0.9, Main Support Threshold = 0.9, Genetic Distance Threshold = 4.5, and Large Cluster Threshold = 20 to extract in-cluster sequences and non-cluster sequences.

Using the online Consensus Maker tool (https://www.hiv.lanl.gov/content/sequence/CONSENSUS/SimpCon.html), consensus sequences of in-clusters and non-cluster were computed. The consensus sequences of in-clusters and non-cluster were translated into amino acid sequences using MEGA-X [11], the differences in amino acids were compared, and the changes in in-clusters' drug-resistant mutations of amino acid sequences in each year were counted. The ML tree was constructed using the consensus sequences

Figure 1. Number and location of influenza B virus NA sequences per year from 2006 to 2018. **A:** From 2006 to 2018, the related research on the NA sequences of the influenza B virus showed an upward trend year by year; **B:** A distribution map of 19136 sequences in all continents showed that the influenza B virus spread worldwide.



of all the clusters to show the agglomerates of sequences. Ten amino acids surrounding the resistance loci were selected and analyzed by WebLogo (http://weblogo.threeplusone.com/) to reflect the mutation of auxiliary amino acid residues around the drug-resistant sites.

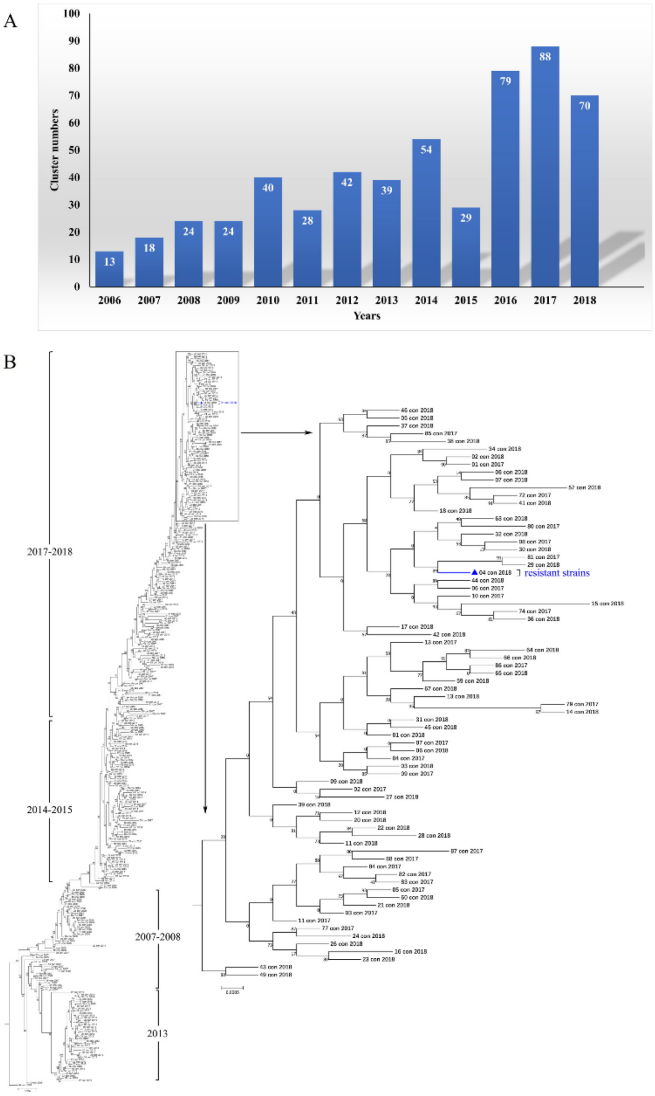
Results

A total of 23,357 NA sequences of influenza B virus from 2006 to 2018 were retrieved from NCBI and GISAID on November 01, 2019. The repeated sequences and sequences with dozens of missing nucleotide base pairs at both ends were removed. 19,136 nucleic acid sequences in the near full-length NA region were obtained after preprocessing. The above NA nucleic acid sequences were classified by year to analyze the epidemic distribution of NA sequences each year. From 2006 to 2018, the related research on the NA sequences of the influenza B virus showed an upward trend year by year (Figure 1A). These data provided the basis for epidemiological and drug resistance analysis of the influenza B virus. We counted the number of influenza B sequences. A total of 19,136 sequences are found in the Americas, Asia, Europe, Africa, Oceania, and Antarctica. A distribution map of 19,136 sequences in all continents was made. As shown in Figure 1B, the proportion of sequences in the Americas is the most, accounting for 34.73% (6,645/19,136), followed by Asia, accounting for 33.30% (6,373/19,136). Europe ranks third, accounting for 19.09% (3,653/19,136), while Oceania and Africa account for 7.18% (1,374/19,136) and 5.69% (1,089/19,136), respectively. Antarctica is the least, accounting for only 0.01% (2/19,136).

The nearly full-length NA sequences of the influenza B virus were aligned, and the ML phylogenetic tree was generated by FastTree 2.1.11 each year. We clustered the NA sequences through ClusterPicker, and the sequence number of in-Cluster from 2006 to 2018 was obtained and statistically plotted each year. The number of epidemic clusters of influenza B virus' NA sequences also increased year by year (Figure 2A), indicating that the NA sequence of the influenza B virus never stopped evolving, and it was constantly mutated with a diversified variation. With the increased number of Clusters, the variation of influenza B is becoming more and more complex, which is likely to contribute to NAIs resistant mutants.

The resistance of influenza viruses to NAIs drugs is mainly due to the mutation of NA protease catalytic activity center sites. At present, the reported mutation sites of influenza B virus resistance to NAIs drugs are mainly G407S, R374K, N294S, H273Y, I221 (I221V, I221T), D197 (D197E, D197Y, D197N), R150K and E117A (N1 numbering) [12]. We counted mutations in NA resistance sites each year between 2006 and 2018. It was found that only Clust04 in 2018 had the mutation of neuraminidase active site D197N, and other drug resistance sites were conserved without mutation. Further analysis found that there were 73 Ns, 6 Es, and 1 G, at position 197, out of 19,136 sequences in total. D197N mutation is 0.35% of the whole infected population. Subsequently, we built an ML tree using all the consensus sequences of clusters, folded branches,

Figure 2. Number of clusters of influenza B virus per year from 2006 to 2018 and the ML tree from all the consensus sequences of clusters. **A:** The number of epidemic clusters of influenza B virus' NA sequences also increased year by year, indicating that the NA sequence of influenza B virus never stopped evolving, and it was constantly mutated with a diversified variation; **B:** Clust04 in 2018 harboring D197N mutation might be derived from sequences from years 2015, 2014, 2008, and 2007.



and strains without resistant mutations and found that Clust04 in 2018 harboring D197N mutation might be derived from sequences from the years 2015, 2014, 2008, and 2007 (Figure 2B). Moreover, there are only three sequences of Clust04 in 2018 harboring D197N drug-resistant mutation, located in Australia in Oceania, Russia in Europe, and the United States in the Americas, respectively.

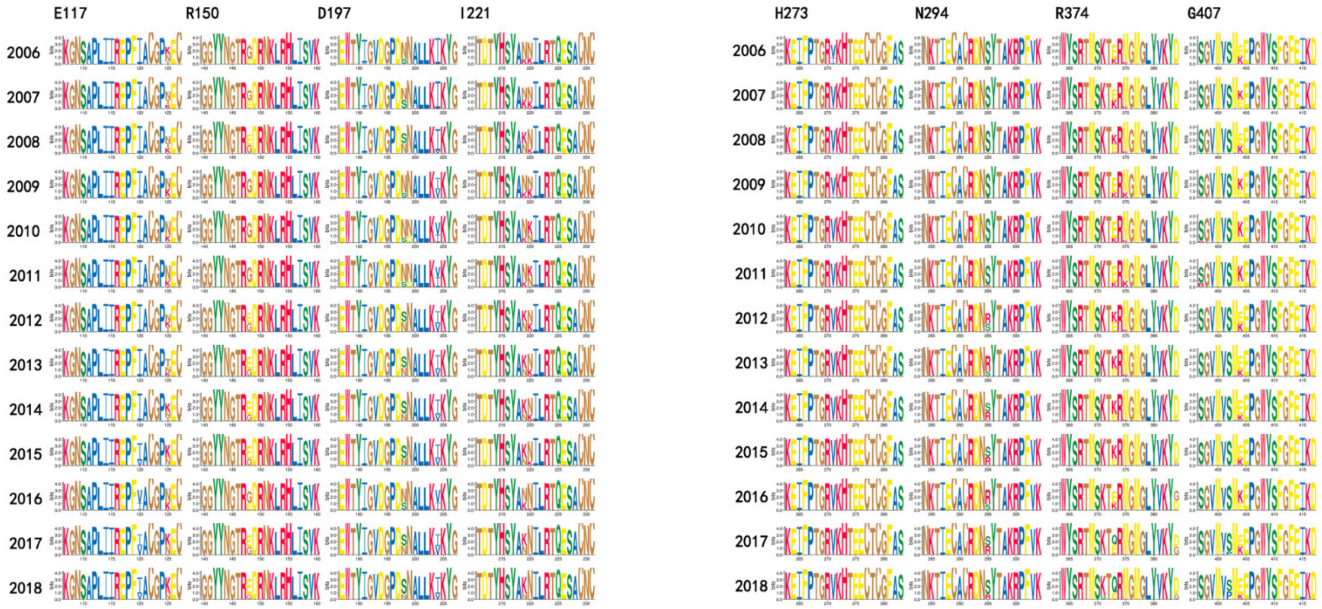
Weblogo (http://weblogo.threeplusone.com) was used to describe the frequency of changes in 10 amino acids surrounding the drug resistance sites of the influenza B virus from 2006 to 2018 (Figure 3). According to the statistical results, many mutations of N198, S295, K373, and K375 (N1 numbering) occurred in the amino acid residues at the auxiliary sites around D197, N294, and R374.

Discussion

NAIs can mimic the natural substrate, sialic acid, to bind to NA and block its active sites so that it cannot catalyse the hydrolysis of sialic acid and prevent the release of virus particles. The RNA polymerase of the influenza virus is prone to cause mismatches. With the increased clinical use of NAIs, the corresponding NAIs drug-resistant strains gradually appeared. At present, it is believed that the molecular mechanism of influenza virus resistance to NAIs is mainly the mutation of the viral RNA sequence encoding NA, which changes one or more amino acid residues constituting NA. The most common changes include amino acid residue substitution [13] and deletion [14]. Drug-resistant strains with the substitution or deletion of NA protease active sites or nearby amino acid residues can directly or indirectly cause the spatial conformation change of NA protease active sites and the damage of enzyme function, the failure of NA binding to NAIs with high affinity.

Our results showed the prevalence of influenza B in America, Asia and Europe; most are countries and regions with better economic development. It suggests that continuous monitoring of the influenza B should be strengthened in developing and low-income countries. Meanwhile, previous research has shown that the NAIs resistant strains of the influenza B virus are not as common as the influenza A virus, and the detection rate is very meagre at 0-1% [15]. Similarly, our research shows 0.35% (73/19,136) D197N mutation in the infected population between 2006 and 2018. As most of the strains harboring D197N mutation came from developed countries, we speculate the D197N mutations will keep growing in the future with the widely used antiviral drugs. Furthermore, our current analysis revealed that the three sequences, harboring the D197N, of Clust04 in 2018, did not originate from the same continent or country or neighboring continents, but were distributed in completely different continents and countries. The reason might be that only a few influenza B transmission clusters contain drug-resistant strains. Meanwhile, our ongoing research on influenza A drug-resistant mutations got more transmission

Figure 3. Weblogo analysis of auxiliary amino acid mutations nearby the drug resistance sites of influenza B virus from 2006 to 2018. Weblogo (http://weblogo.threeplusone.com) was used to describe the frequency of changes in 10 amino acids surrounding the drug resistance sites of influenza B virus from 2006 to 2018.



clusters with a wider distribution worldwide (unpublished data).

Drug resistance mutations of the influenza B virus mainly occurred at D197 (D197E, D197Y, D198N) and R150K (N1 numbering) [16]. The mutation of neuraminidase active site D197 is most common among the mutations associated with resistance [17]. It is very consistent with our analysis. As D197N mutation located in the highly conserved NA enzyme active site, works as one of the framework residues, and causes reduced NA activity significantly in vivo, the emergence of NAI resistance could be a major clinical concern [16]. However, some studies have pointed out that the three-dimensional structure of the D197N site is not directly related to the substrate or inhibitor [18,19], although D197N can destroy the interaction between the D197 site and the R150 site, reduce the stability of critical inhibitory binding sites, which will lead to potential drug resistance. Meanwhile, it is investigated by Hurt AC that Influenza B with a D197N shows little reduction in reproduction or transmission in the contact ferret model [16]. Those results suggest that the influenza B virus might still be highly sensitive to NAIs drugs.

In addition, mutations in I221T, G407S, R374K, and E117 (E119A, E119D, E119G) can also lead to resistance to oseltamivir (I221T, G407S, R374K, and E119A/D/G), paramivir (E119A/D/G), and zanamivir (G407S, R374K, and E119A/D/G) [19]. However, we did not find any transmission cluster with those mutations, only a few drug-resistant strains were found. Nevertheless, the mutation rate of the auxiliary sites surrounding NAI drug-resistant mutations is very high, indicating that NA's active sites are highly conserved. However, the functional research of the auxiliary site gene mutations found in this study is not investigated. Hopefully, it may affect the binding of NA to NAI drugs or increase the transmission of the influenza B strain harboring those mutations.

Although the mechanism of NAI resistance caused by some sites of the influenza virus has been studied, the specific mechanism has not been generally accepted and fully explained. HA mainly recognizes and binds receptors, which recognize and act on the same receptor. NA is mainly involved in the release of virus particles from cells. Therefore, the balance of HA and NA plays a vital role in viral replication. Mutations in some HA sites cause the decrease of affinity between HA and receptors and reduce the virus's dependence on NA enzyme activity. Therefore, the resulting NAIs resistance is a kind of multiple cross-resistance. In addition, HA mutation may also have a particular compensation effect on the decline of virus viability caused by NA resistance mutation [20].

Conclusions

According to this study, we know that neuraminidase inhibitors are still the only specific antiviral drugs for the treatment of the influenza B virus, and their early use will significantly improve the prognosis of patients. Although some viruses have drug resistance mutations, the currently prevalent influenza B virus is generally sensitive to NAIs.

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Authors' Contributions

Yihong Hu conceived and designed the analysis. He Li, Dong Wei, Jing Wang and Die Yu collected the data and performed the analysis. Dan Qian, Die Yu, Lihuan Yue, Jing Wang, Yaming Jiu and Yihong Hu contributed reagents, materials and analysis tools. He Li and Yihong Hu wrote the paper. All authors contributed to the article and approved the submitted version.

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