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Review Article
Changes in Germs: A Potential Preemptive Strike Against the Next Pandemic

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Abstract

With recent multiple outbreaks around the world in the past ten years especially with current massive Asian flu outbreak, HPAI H5N1 is considered to be next pandemic threat. Studies and experiences from three previous 20th-century pandemics influenza A in 1918, 1957 and 1968, teach us lessons in the dynamic nature of influenza virus classification, its mutation and epitope structure. The combined effort in studying epidemiology, molecular structure and analysis of the bioinformatics data, could hopefully prepare us facing the next flu pandemic. The discussion also touches a controversial subject on "weaponized virus," engineering the wild-type strain to become more pathogenic and highly contagious.

Key words: Influenza A, H5N1, pandemic, mutation, bioinformatics

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

During the 20th century, history recorded at least four major pandemics caused by influenza virus. From the onset of first world war until the end of 1960, the total cause of human life was estimated to reach 7 million patients. Each pandemic was unique in their way. It includes the country of origin, the viral subtype and the geopolitical situation at the time of outbreak[1]. All the pandemics had similarities such as in method and speed of transmission, vulnerabilities of the urban population and the cycle of the outbreak [2]. Understanding the pattern of the outbreak and the study of virus epitope with their neutralizing protein lead us to one step closer to win our constant warfare against the global pandemic[3]. Advanced in bioinformatics studies and technologies provide additional data on classification and genome comparison among different clades of viruses[4]. Protein folding prediction and visualization have improved our effort in designing agents for viral epitopes. It will difficult for the virus to attach to the host cells as well as to increase selection for certain type of haemagglutinin (HA) and neuraminidase (NA)[5]. The insight from "continuous struggle for survival" by the Darwinian view of evolution and a new finding on the mutability of virus epitope against recent drugs, should be a dire warning for alternative solutions to preventing a new pandemic problem. It should be acknowledged that we are a long way from winning the battle against a pandemic. At this moment, researchers would like to show their effort in fighting mutability of viral genome of Highly Pathogenic Avian Influenza (HPAI) H5N1 against a variety of drugs. It will increase our awareness in preparing for the next pandemics H5N1[6]. The review focused on three different areas: (a) Evolution of the virus, (b) The molecular analysis on virus epitopes and (c) A recent unusual approach to make avian influenza more virulence. Thus, this review gave an additional insight for the researchers on how to prevent the next flu pandemic to occur globally shortly.

EPIDEMIOLOGY OF INFLUENZA OUTBREAK

The first recorded outbreak caused by influenza virus could be traced back about 300 years into the 19th century as it is shown in Fig. 1[1]. The well-known influenza pandemic was the "1918 Spanish flu". A large number of people gathered in Spain after the end of the great war and it fueled the spread of virus transmission around the world. There was a high rate of mortalities in both infant and elderly individuals. The two waves of the outbreak, were in 1918 and 1919, leaving a devastating effect on while world population. The total death during this pandemic is around 100 million worldwide[2]. By the end of 1919, the pandemic mysteriously stopped. Many studies proposed that the reasons for this phenomena were due to (a) Better patient care, (b) Increasing pandemic preparedness and (c) The natural cycle of virus pathogenicity[3].

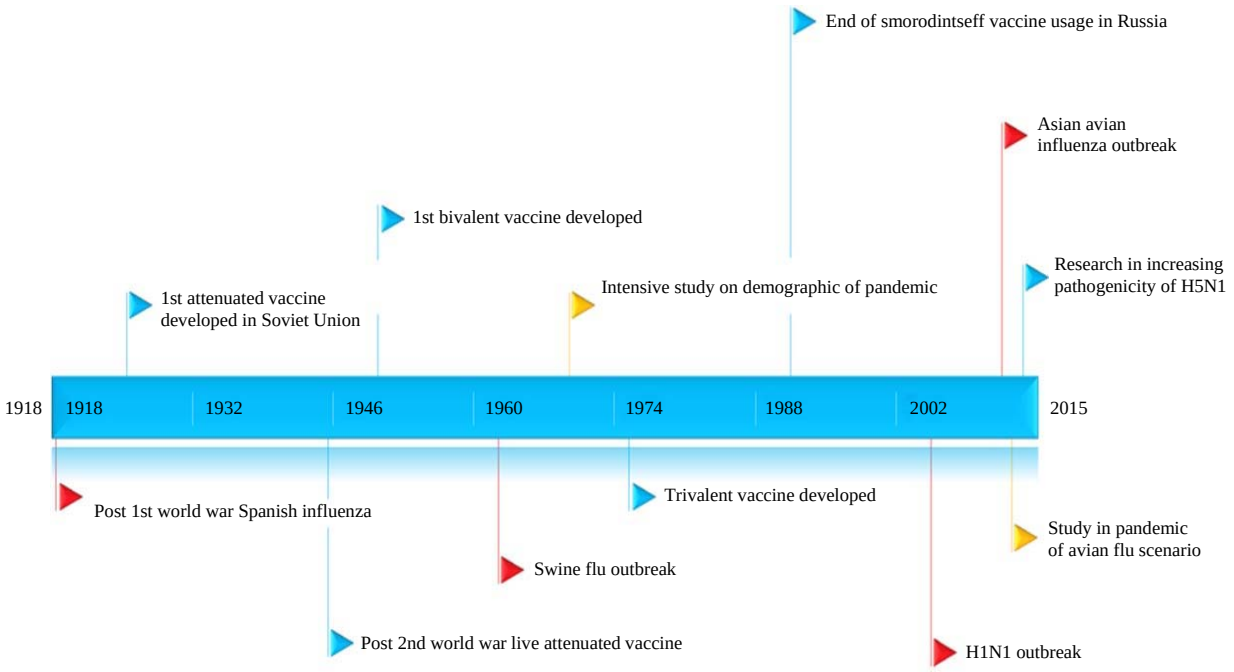

Fig. 1: Timeline of outbreak cases, vaccine development and epidemiology studies in the last 100 years [1,5]

Recent cases of influenza outbreak were from 2002-2007 in multiple waves, causing the massive death of waterfowl and some mammals species in Asia. The casualties found in Indo-China countries such as Myanmar, Cambodia, Laos and Vietnam, Thailand, mainland China and Indonesia[4]. Although it considered as an outbreak, H5N1 avian influenza did not have pandemic status[6,7]. Human casualties caused by direct contact with avian via living or dead domesticated bird, waterfowl and chicken infected with H5N1[8]. Human to human infection had not reported yet. Therefore, there is a possibility that human to human infection may develop in future[9,10].

Country preparedness is the key issue in regarding health policy in the aftermath of the first avian influenza outbreak[11]. Among many factors evaluated, the important factors are health system surveillances which monitor the onset of the outbreak and the preparedness of clinical management in the local hospital. These two important factors could increase our prediction on next outbreak. A case from the 2012 H5N1 outbreak in Indonesia gave us a valuable lesson in the country preparedness. While the global mortality rates for avian influenza was 59%, a relatively high percentage, the mortality rate in Indonesia mortality reached a new high of 83% from 185 cases[12]. The delay in health care service plays important roles in the high mortality rate. Patients who contracted mild symptoms of influenza did not hesitate to go to local health service. The delay was mostly on the limited ability of health care providers to manage their patients, identify the symptoms and prepare certain antiviral drugs. Moreover, the level of a highly contagious suspicion HPAI H5N1 was low in certain rural areas[12].

Among countries affected by 2012 HPAI H5N1 outbreak, Thailand has made significant progress by utilizing and combining spatial analysis of the sequence data from the viruses. A genome comparison, especially on HA genes, showed different clades for different outbreak areas. Further analysis of the gene comparison identified the areas where virus reassortments were taking place. Therefore, that approach will assist effort in localizing research on mutation of avian influenza virus in small areas[4]. While analysis of viral genome in Taiwan also shed light on how H5N1 came into Formosa from the mainland China through a smuggling operation via Kinmen Islands[13]. In both cases, they showed how the finding from basic research on virus molecular and phylogenetic integrate into epidemiological domain would give invaluable information to the policymaker.

The role of phylogenetic in mapping virus mutations also important in predicting the spread of the disease. The spatial map combines with data from the phylogenetic tree from various variants could provide new insight into the evolutionary pathway of a recent virus outbreak. Pybus and Rambaut[14] introduced the term phylodynamic to answer questions such as how swift the disease spread in the case of the outbreak from a general scale (i.e., country-to-country or people-to-people) or even in the small spatial scale (i.e., from tissues to tissues inside a host). The case from Indonesian HPAI H5N1 infections in November, 2002 is a good example of how phylodynamic gave insight into viral spreading to the Westward and Eastward from its center origin in the East Java. The phylogeny of viral strains and spatial data coming from infection as well as suspected reports were combined to give new information in predicting disease behavior across the Java Island. The phylodynamic analysis also shows how selection pressure act on HA protein that arose from a rapid burst of virus genetic diversity and stabilization of genetic material couple months after the initial invasion[15].

BIOINFORMATICS APPROACH

The study on virus classification gives insight on several things, including the ability to trace back the origin of the outbreak with the help of spatial analysis and to shed light on the relationship between the recent outbreak and the past data[4]. Moreover, the classification of the viral genomes from the recent outbreak can divide into several clades and this classification can help us focus researchers efforts on designing drugs for a specific clade only. The case from Banten in Indonesia showed the changes in HA and NA epitopes that were detected using the *in silico* approach. Moreover, the change detected as a result of antigenic drift and it provided information about the negative finding of human-to-human transmission[10]. The *in silico* approach will not only give additional data on certain type mutation but will also reduce the social cost due to rumors and false news on the recent outbreak. This quick fix attempt proved effective in the case of a major outbreak. It should be known that a comprehensive study will need the threat of outbreak dissipates[16].

Bioinformatics can provide several tools, either as data provider or analytical approach. Query on viral genomes and aligning important genes responsible attachment of certain protein on virus epitope cannot be done without a computational power. The data accumulated from the past outbreak stored neatly with their annotation in NCBI virus database, thereby in most cases, give emerging properties apart from the data itself[17]. The effectiveness of two major antiviral drugs against HPAI H5N1 is also making a problem in the face rapid evolution and selection of viral genome. Both oseltamivir and zanamivir have frequently been used to target NA enzyme on avian influenza virus. But, the emergence of

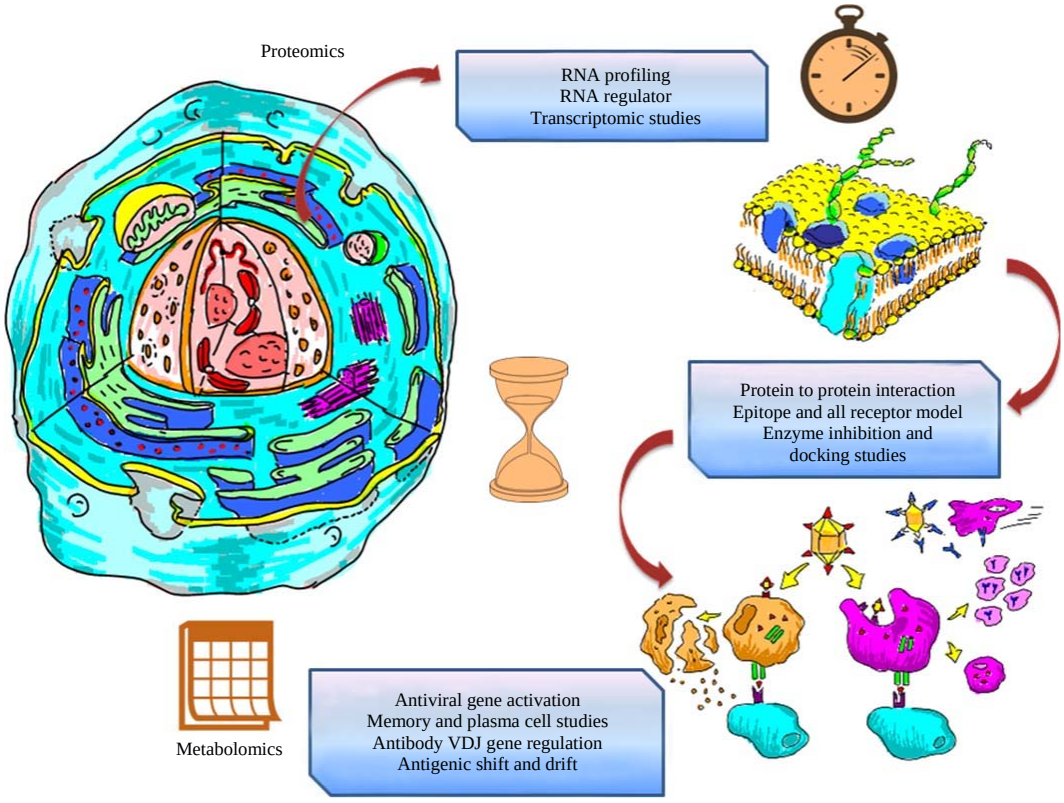

Fig. 2: Multiple and integrated approaches to understanding viral infection from gene expression to complexity and emerging properties in the immune system

resistance among patients treated with those two drugs has been detected and brought grim news to us. The 3D structural studies using x-ray crystallography on a particular type of NAs showed the emergence structural correlation. It opens up an opportunity to exploit this feature for next generation avian influenza drug[18]. Several studies have been conducted to find a novel drug candidate that can be used to replace both oseltamivir and zanamivir as NA inhibitors[19-27]. In the next outbreak, vaccines will play an important role in preventing escalation of the disease into a pandemic level. The study on the HA and NA proteins will lead to insight on how to design vaccine based on B-cell and T-cell responses to viral epitopes. *In silico* vaccine design with the help of software binding prediction, BCPREDS[28] and TAPPRED[29] server, along with ProPred[30] and NetMHCpan[31,32] servers for identification bond region for a class I HLA antigens can increase the efficiency of drug discovery as well[33]. Not only research on drug design but bioinformatic also contributes to how to prevent or reduce the danger of pandemic problem of human-to-human transmission in future as is shown in Fig. 2. Vaccine design by using samples taken from various regions gives us opportunities to develop broad-spectrum vaccines. The importance of broad spectrum of vaccines in necessary due to various ethnic type susceptibilities to different types of viruses in South East Asia. Next, the problems in various regions are due to a different policy, health care access and geographical barrier that proved to cause a delay in treating the suspected influenza patient[34].

One attempt to develop a broad spectrum vaccine was using Computationally Optimized Broad Reactive Antigens (COBRA). A sample from HA clade 2 of H5N1 has been used as a template for development non-infectious VLP vaccines. The vaccines designed and produced were proved to have protective properties against the clade of two viruses[35]. Although the goal of creating a broad spectrum vaccine had not achieved, the efficiency and speed of creating a protective blanket for the wide geographic population on the certain area were improved.

Vaccine development since Pasteur time resembles the Red Queen phenomena on the famous Lewis Carrol's story. Smorodintsef experiment with a live attenuated vaccine could last for decades. But in the same cases on the other side of Atlantic, a bivalent vaccine developed by United States Army provides protection only for infants with primo-infection advantages. The two big problems with vaccine development are (a) The strain resistance caused by selection pressure on

strong antibody triggered by vaccine and (b) The problem regarding different vaccine's efficacy with the different subject group. The attenuated vaccine has its payoff with two most vulnerable age group, including infants and elder people. The former suffers from low responses on their immunity to their humoral response[5].

MOLECULAR ANALYSIS OF VIRAL STRUCTURE

While the virus is considering as a simple organism by the size of their genomes, our knowledge of their metabolism is still in its infancy related to the surrounded proteins as the protective layer, regulating their mechanism while infecting a host cell. Their genes have a wide range ability to penetrate cell membranes and their ability to reverse the central dogma of genetic using reverse transcriptase enzyme. The former is the notorious feature of a retrovirus. Its ability to create DNA from their RNA genome to put their gene on host genome is the key to understanding how our fight against them proved futile until now.

Influenza A virus has eight segments of genes and segments 1-3 play a role in their replication by expressing RNA polymerase in a host cell. While the first three segments are important in reversing the transcription process and further incorporating viral genome into a host cell, discussing segments 4-6 were much more interesting. The HA and NA are two important coat glycoproteins for Influenza A virus. The HA and NA are coded on segments 4 and 6 of the viral genome. HA acts as an entry point to host cells by binding its binding to cell receptors and initiating endocytosis while NA cleaves the sialic acids on the virus. The rest of segments are important in constructing viral matrix structure (segment 7) and to encode for nonstructural protein (segment 8)[36].

While there is 16 major variety of HA and 9 NA, only a few considered highly pathogenic (H5 and H7). Though considered low pathogenic H1, H2 and H3 type of HA were causing three global pandemics in 1918, 1957 and 1968, consequently. Further analysis of HA structure reveals some conserved region in all type of HA. There are four residues of amino acid on HA that plays an important role in receptor binding site with host cells, targeting sialic acid on cell's membrane. The study also revealed mutation on one amino acid could change virus preference host from avian into a human[37].

MODIFYING PATHOGENICITY AS PREEMPTIVE STRIKE

Phylogenetic tree and protein analysis of HPAI H5N1 shows the striking difference between HA and NA. Both proteins are responsible for virus ability to bind to host cell by forming attachment into glycoprotein or glycolipid on the cell surface membranes. Molecular analysis shows, HA responsible for attachment into a human host cell, has sialic acid 2,6 α-galactose binding site (SA α-2,6Gal), comparing with sialic acid 2,3 α-galactose preference that often found on HPAI H5N1. The difference in binding site differences, causing a human to human transmission does not occur efficiently so far[38,39].

Since the difference between those two binding site preferences only in few amino acids, a mutation on HA gene could give the H5N1 new ability for not just from avian-to-human infection but also for human-to-human transmissions that lead to a new global pandemic. The famous H1N1 Spanish flu virus has both SA α-2,6Gal and SA α-2,3Gal, respectively, on its genome. It's duality on binding site preferences given its ability to infect on two areas. Since upper human respiratory tract filled with SA α-2,6Gal, it becomes the attachment site and also an effective transmission method via airborne droplet. On the lower side, SA α-2,3Gal receptors lie on bronchioli and terminal alveolus to limit the transmission of the virus but not its pathogenicity. It's preference infection site on the soft lung tissue and lower airways cause massive cytokine response, accumulation of fluid, alveolar damage and pulmonary congestion with hemorrhage[40].

Comparison between viral, another glycoprotein, NA of 1918, 1957 and 1968 pandemics showed stability at low pHs. The analysis from HPAI H5N1 from 1997 Hongkong outbreak until 2005 showed low stability at low pHs. Contrary to HA, NA plays important roles in virus mechanism to detach itself from the surface of host cells. It removes sialic acid from the cell and separating glycoconjugates and its glycoproteins component[39]. The high stability at low pH is an indication of the virus survival ability. Since most infections must pass through the stomach acid digestive system, its ability to withstand low pHs is crucial for the longevity of the viral genome. The low stability NA at a low pH environment was the important feature of virus found in human to make it easier to infect the human population[37,39].

Efforts to create artificial reassortment of viral components to make it more virulence and highly pathogenic were investigated for a preemptive strike against virus ability to mutate and causing the next global pandemic. Since H5N1 subtype and 2009 H1N1 are closely related, one attempt was tried to generate virus from both subtypes. The reassortant has HA from avian H5N1 and H1N1 to provide its contributions to the remaining seven genes. Mutated HA from experimental studies has airborne transmission from droplet as shown in the ferret model, which is a mammal model for flu research. While the wild-type HA did not prefer binding to SA α-2,6Gal in the cell surface binding test, mutated HA showed a high

affinity to SA α-2,6Gal. These laboratory controlled experiments demonstrated the possibilities of avian influenza reassortment in nature that have two properties of a pandemic such as highly pathogenic and human to human transmission[41].

Another approach to predicting mutability of avian influenza is by mimicking the natural process of reassortment in nature. Comparisons between the wildtype and mutated version of H5N1 (A/H5N1HA Q222L, G224S PB2 E627K) were made by running multiple series of infections in ferrets. Viruses adapted to mammalian host cells showed preferences into SA α-2,6Gal binding site, which is key to airborne transmission. The wild-type used in these experiments was acquired from influenza virus A/Indonesia/5/2005. Consideration was made from high-level infections and fatalities in Indonesia during the 2005 outbreak. Another reason is that there was no known reassortant between A/H5N1 with the seasonal flu, therefore, it limited the possibilities of impurity from another subtype of influenza virus. Ferret infected with mutated version showed symptom and spread the virus within two days period of viral airborne. Short incubation period combined with high pathogenicity and airborne transmission, one more recipe of a global pandemic[36].

CONCLUSION

Understanding complex phenomena such as global pandemic require a combined effort of research from multi-disciplinary areas. Epidemiology itself is a multi-subject approach science. The essential needs are ranging from the health system, medical science, human behavior, mathematical modeling and spatial analysis using recent technology from Geographical Information System. Additional help from other subjects such as molecular protein structure, bioinformatics and microevolution is essential in our arsenal against virus infection. The unconventional method that seems contrary to our effort battling the pandemic, by increasing virus pathogenicity, should also consider as a genuine effort in understanding the complexity to give great insight on influenza virus. Along with strict protocols and tight regulations with high safety procedures to eliminate the threat of bioterrorism, this weaponizing virus research could better prepare us for the next Pandemic in the world.

SIGNIFICANCE STATEMENT

The review focused on three different areas: (a) Evolution of the virus, (b) The molecular analysis on virus epitopes and (c) A recent unusual approach, to make avian influenza more virulence. Thus, this review gave an additional insight for the researchers on how to prevent the next flu pandemic to be occurred globally in the near future.

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