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REVIEW

The emerging influenza virus threat: status and new prospects for its therapy and control

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Abstract

Influenza A viruses (IAVs) are zoonotic pathogens that cause yearly outbreaks with high rates of morbidity and fatality. The virus continuously acquires point mutations while circulating in several hosts, ranging from aquatic birds to mammals, including humans. The wide range of hosts provides influenza A viruses greater chances of genetic re-assortment, leading to the emergence of zoonotic strains and occasional pandemics that have a severe impact on human life. Four major influenza pandemics have been reported to date, and health authorities worldwide have shown tremendous progress in efforts to control epidemics and pandemics. Here, we primarily discuss the pathogenesis of influenza virus type A, its epidemiology, pandemic potential, current status of antiviral drugs and vaccines, and ways to effectively manage the disease during a crisis.

Introduction

Influenza viruses belong to the family *Orthomyxoviridae* and are the leading cause of severe respiratory illness across the world. They are enveloped viruses containing a single-stranded, negative-sense RNA genome, and they account for a large number of deaths each year. In an electron microscope, influenza A and B viruses look similar and are virtually indistinguishable. They are either spherical (100 nm in diameter) or filamentous (often in excess of 300 nm in length) in form [1]. Of the four influenza virus types (A, B, C and D), influenza A virus (IAV) causes the most severe disease and infects a variety of animals, including humans, pigs, horses, sea mammals, and various bird species (reviewed in reference [2]). Type A mutates more rapidly and exhibits a higher degree of variability in its antigenicity and virulence than the other influenza types [3, 4]. It can cause zoonotic infections and adapts easily to humans, leading to a sustained human-to-human transmission, which favors the emergence of novel strains. In this review, we have focused primarily on the contemporary aspects of influenza A virology and new prospects for its treatment and prevention.

Influenza virus genetics, epidemiology and pandemic history

The genome of influenza A and B viruses consists of eight single-stranded viral RNA (vRNA) segments, while influenza C virus has a seven-segment genome. Each segment codes for one of the viral proteins, which include the major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), the nucleocapsid protein (NP), three subunits of the viral RNA-dependent RNA polymerase (RdRP) (PA, PA-X, PB1, PB2, PB1-F2), the matrix proteins (M1, M2) and the nonstructural proteins NS1 and NS2 [5]. All influenza A viruses are classified based on their surface glycoproteins, HA and NA. HA is responsible for binding to sialic acid (SA) (N-acetyl neuraminic acid) at the termini of glycans, which act as receptors on the host cell plasma

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membrane, while the NA, a type II integral membrane glycoprotein with sialidase enzymatic activity, is involved in the final step of the replication cycle and helps in release of mature virions. The two surface glycoproteins, HA and NA, are present in a ratio of 4:1 [3]. Co-evolutionary adaptation between HA and NA allows them to perform the complimentary functions of SA binding (by HA) and SA removal (by NA). The segmented nature of the genome and the high frequency of mutations during replication in multiple hosts is responsible for regular epidemics and occasional pandemics.

The two major factors in influenza epidemics and pandemics are genetic drift and genetic shift [6]. Genetic drift occurs due to point mutations in the influenza virus genome, as the viral RNA polymerase, unlike DNA polymerase, lacks a proofreading function making coding errors and multiple mutations more likely. A genetic shift occurs when two or more different influenza virus strains infect the same cell in a host, leading to recombination of genetic materials, an event that occasionally generates a new strain with a novel combination of hemagglutinin and neuraminidase. These genetic shifts lead to pandemics when the novel strain acquires the capacity for sustained efficient human-to-human transmission. To date, 18 novel hemagglutinins (H1-H18) and 11 neuraminidases (N1-N11) have been identified [7]. Most of the combinations of H and N types (144) are found in wild birds, which serve as reservoirs for influenza viruses and pose a severe risk, because they can be infected with multiple strains and serve as potential mixing vessels. H17-18 and N10-11 have not been detected in birds but have been found in bats [7, 8]. IAVs that infects birds have an HA receptor-binding specificity for α2-3 SA, while HAs from IAVs that infect humans have a higher specificity for α2-6 SA, with the major exception of the highly pathogenic avian influenza (HPAI) strain H5N1, which has a preference for α2-3 SA. The differences in preferred cellular binding sites allow different strains of influenza virus to infect either birds or humans, thereby creating lineages that are host specific, and so far, only H1N1, H2N2, H3N2, H5N1, H7N7 and H9N2 viruses are known to infect humans. However, the respiratory epithelial cells of pigs (swine) express both α2-3- and α2-6-linked sialic acids and can therefore support infections with both avian and human influenza virus strains. This makes pigs a mixing vessel for producing novel strains with the ability to infect humans, and some of these strains can cause fatal infections (Fig. 1) [9]. These novel reassortant viruses, due to a lack of existing immunity in human population, can lead to pandemic situations, as witnessed in the year 2009.

The human population remains at risk of an influenza pandemic each year due to the high mutation rate of the virus. Influenza pandemics have occurred several times, with inter-pandemic intervals averaging approximately 40 years [10]. Type A has been responsible for several widespread

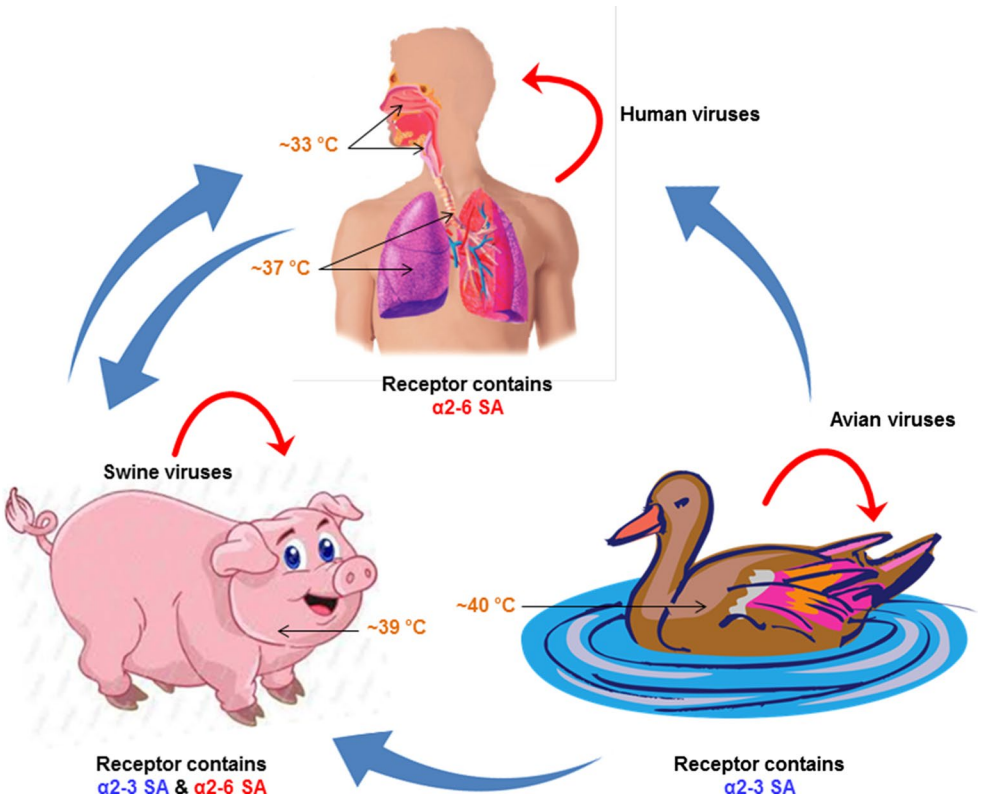

Fig. 1 Mechanisms for the emergence of pandemic influenza virus strains. The virus keeps circulating among own species and sometimes jump the species barrier to generate a novel strain of pandemic potential

pandemics since the 16th century. The three major pandemics were the Spanish flu (1918-19), the Asian flu (1957), and the Hong Kong flu (1968–69), which resulted in a large number of deaths [11] (Fig. 2).

The 1918 (H1N1) pandemic has been recorded as the worst pandemic in history. It infected 500 million people globally, caused approximately 675,000 deaths in the United States [12], and killed up to 50-100 million people worldwide [13]. The viral genome reconstructed from the lung tissues of several victims demonstrated that it was an avian-descended H1N1 virus [14]. Waterfowl, of the order Anseriformes, such as ducks, swans and geese, serve as reservoirs of all IAVs. Charadriiformes, including shore birds, gulls, and terns, also harbor influenza virus, but of a different gene pool from those of the Anseriformes, and the two remain the most important orders for the transmission and spread of HPAI [15]. Influenza viruses from these birds are able to infect other bird species, such as chickens, as well as mammals, and they adapt to a new host by accumulating mutations through genetic drift or genetic shifts [12]. Due to the unavailability of any IAV sequences from prior to 1918, the possibility of involvement of an intermediate host in the emergence of the virus in humans during the 1918 pandemic remains an unresolved mystery [16]. However, the virus was readily transmitted to pigs, as was also observed during the 2009 pandemic of H1N1 [17]. Most of the deaths resulted from respiratory complications, such as bronchopneumonia with bacterial invasion and progressive cyanosis and collapse. Scientists believe that the pathogenicity of the 1918 H1N1 virus was amplified by concomitant infection of influenza virus with bacteria such as *S. pneumoniae* and *S. pyogenes* [18]. The 1918 pandemic spread in three rapid waves within an approximately 9-month period. The large number of deaths could also be attributed to several other factors, such as unpreparedness for an influenza virus strain of pandemic potential and the lack of effective vaccines to prevent influenza and antibiotics to treat secondary bacterial pneumonia. After the pandemic period, the virus kept accumulating mutations for several years and disappeared in 1957, only to reappear in circulation in 1977 [2].

Following the Spanish flu in 1918, another influenza pandemic occurred in 1957 and was called the Asian flu. The 1957 pandemic was caused by the H2N2 strain of IAV and resulted in ~115,700 excess deaths. The overall impact on mortality was one-tenth of that observed during the 1918 Spanish flu (H1N1) pandemic [19]. This new influenza strain was detected in February 1957 in Yunnan Province of China, and by April, the virus had spread to Hong Kong, followed by Singapore, Taiwan, Japan and the rest of the world by the summer of 1957 [20]. In the USA alone, this strain caused

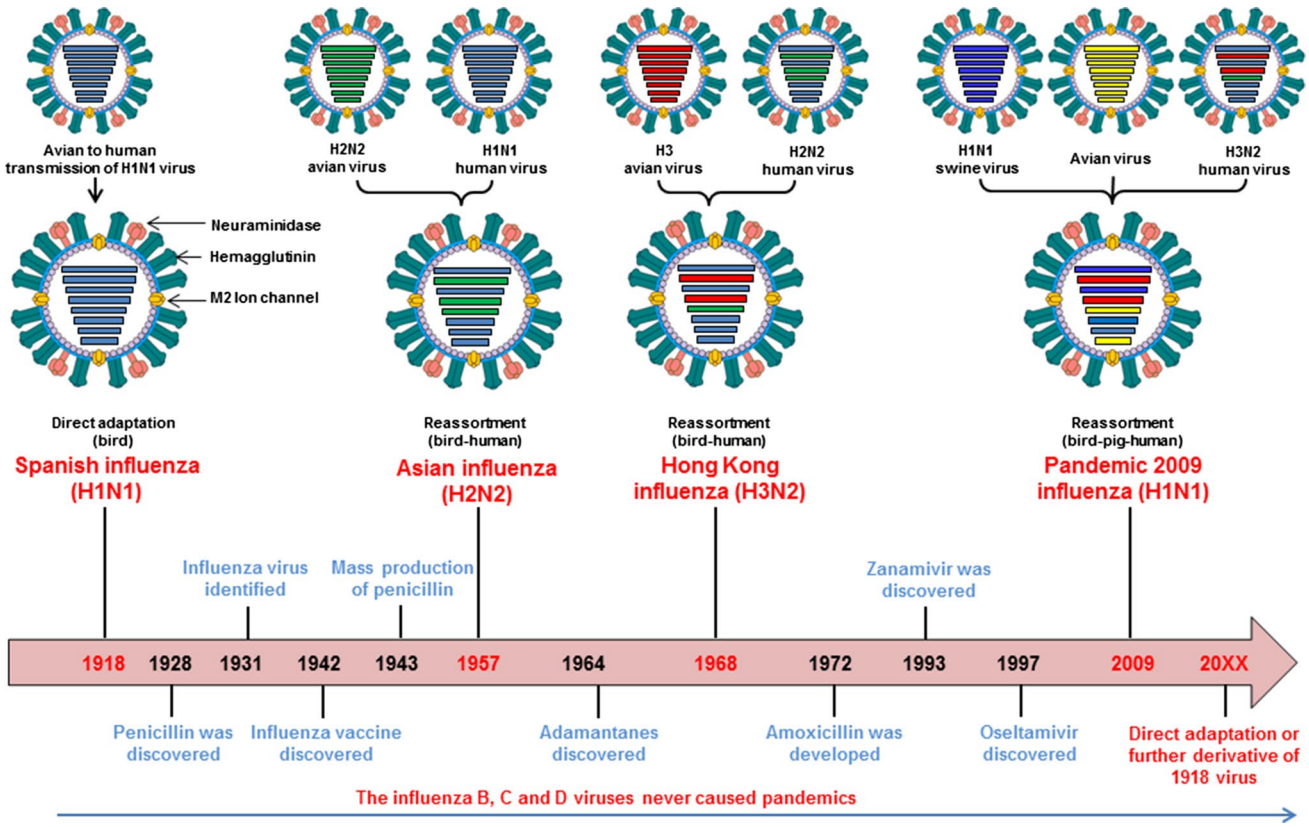

Fig. 2 A time line of major influenza pandemics and the responsible influenza strains

almost 60,000 excess deaths from September 1957 to March 1958 [21, 22].

"Original antigenic sin" is a phenomenon where a prior exposure to an antigen leads to an optimal immune response to the related antigen. Thus, during the Asian flu pandemic, individuals, except those who were 60 years and older, had no prior exposure to the H2N2 strain and therefore had no previous immunity, leading to a large susceptible population in the United States becoming infected [21]. The effectiveness of an influenza vaccine may decrease if the antigenic distance between the vaccine and circulating strains increases. Also there is a possibility that the original antigenic sin could make people who are vaccinated, more susceptible to the virus than those who are not vaccinated [23].

In the year 1968, a new influenza virus strain (H3N2) that differed from the Asian pandemic strain (H2N2) by its HA glycoprotein but had the same NA glycoprotein, replaced the H2N2 strain that had been circulating in all countries since 1957, and this led to the third pandemic causing a large number of deaths [24]. The H3N2 strain, which was first isolated in Hong Kong in July 1968 [25], was highly transmissible but caused disease milder than the Asian flu. The virus mainly spread due to international air travel and resulted in an increase in the mortality rate in United States during the pandemic season (1968/1969), especially in persons <65 years old [25]. The H3N2 strain caused an estimated 98,100 excess deaths over the 4-year period 1968–1971 [19]. The 1957 (H2N2) and 1968 (H3N2) influenza pandemic viruses were avian-human reassortants in which avian gene segments were introduced into a human-adapted virus that was already in circulation [26]. The spring of 2009 again marked the emergence of a novel subtype of influenza A virus (pandemic H1N1-2009), which caused the first pandemic of the 21st century. The newly emerged virus subtype spread worldwide with unprecedented speed and proved its ability to be transmitted from human to human [27]. The health authorities gained momentum, and strict surveillance programs started globally to combat the threat by this novel virus [28], which was a fourth-generation descendant of the 1918 H1N1 virus [29]. The World Health Organization (WHO) declared a pandemic in June 2009, and the phase ended by August 2010. According to WHO, 50.7% of subtyped influenza A viruses collected globally from July 11-17, 2010 and reported on July 28 were the pandemic H1N1-2009 strain [30]. By October 2009, around 191 countries had reported more than 375,000 laboratory-confirmed cases of pandemic (H1N1) 2009 and more than 4500 deaths [31]. The rates of hospitalization and death varied among countries. According to a study done from 15 April 2009 through 23 January 2010 in the USA, 272 pediatric deaths were found to be associated with laboratory-confirmed pandemic H1N1-2009 [32]. While the rate of hospitalization was higher in children, the adult population aged 65 years of age or older showed the lowest rate [33]. According to one study, the death toll due to this novel pandemic H1N1-2009 strain was 18,631, as declared in the WHO reports. However, they reported that the actual mortality burden due to the pandemic was substantially higher and that the number of cases was underreported in Africa and Asia [34]. The 2009 influenza pandemic came to an end, and just like any other pandemic strain, the pandemic H1N1-2009 strain has been considered a seasonal strain since July 2010.

Influenza A virus has a wide range of hosts

IAVs are also widely distributed in avian species (ducks, geese, swans, gulls, terns, etc.) around the world and are predominantly maintained in asymptomatic infections termed "low-pathogenic avian influenza" (LPAI). Around 105 different species of birds have been documented to harbor IAVs [35]. The virus predominantly infects the epithelial cells of the intestinal tract [36] and is subsequently excreted in the faeces. IAVs are known to cross species barriers and be transmitted to other species. A recent example could be seen in the harbour seals (*Phoca vitulina*) of the North-European coastal waters, where H10N7 (LPAI) infection caused high mortality [37]. There are also instances where LPAI has jumped from birds to long-finned pilot whales (*Globicephala melas*) and balaenopterid whales [38]. Influenza viruses circulating in mammalian species including dogs (*Canis lupus familiaris*) and horses (*Equus ferus caballus*) are also thought to be derived from avian influenza viruses [39]. Strains of subtypes H3N8 and H3N2 are currently circulating amongst dogs [39] while H3N8 virus has long been circulating amongst horses [39], and Bactrian camels (*Camelus bactrianus*) [40]. The LPAI subtypes H5 and H7 can subsequently evolve into highly pathogenic avian influenza (HPAI) viruses by insertion of a multi-basic cleavage site in the viral HA [41]. Recent years have seen the occurrence of two such HPAI strains of subtypes H5N1 and H7N9 in Asian countries that resulted in a high fatality rate and hospitalization [42]. An H7N9 strain is currently in circulation and is the cause of significant public health concern in China [42, 43]. Another HPAI strain of subtype H5N6 was first reported to infect humans in April 2014 in Sichuan Province and again in December 2014 in Guangdong Province, followed by four more cases in December 2015 in China [44].

The recent presence of H5N8 and H5N5 infections in various duck species also poses a threat of evolution of this lineage of HPAI H5 viruses in the future [45]. HPAI viruses have also been documented in some non-primate mammals living in captivity, such as tigers (*Panthera tigris*), cats (*Felis catus*), leopards (*Panthera pardus*), and Owston's palm civets (*Chrotogale owstoni*) [46].

Influenza virus infections and clinical course of disease

Influenza virus primarily spreads from one person to another through respiratory droplets when the infected person comes in close contact with a healthy person (generally within a distance of a meter). The virus can survive for 24 to 48 hours on hard, non-porous surfaces and thus may also spread when a person comes in contact with any such surface or item contaminated with the respiratory droplets from an infected person [47]. A typical influenza infection is often characterized by sudden onset of fever, chills, headache, malaise and myalgia, followed by prominent upper respiratory tract symptoms, such as rhinorrhea, cough, sore throat and inflammation of the upper respiratory tract. Apart from these, gastrointestinal symptoms such as nausea, vomiting and diarrhea are very common [48]. However, the duration of illness in cases of pandemic H1N1-2009 infections were found to be slightly longer than that of seasonal influenza infections [49], and gastrointestinal symptoms, especially diarrhea, appeared to be more prominent than in seasonal influenza [50–52]. The incubation period of influenza virus from the time of infection to appearance of symptoms typically varies from 1 to 4 days [53], but it may extend up to 7 days in some cases [54, 55], and weakness and fatigue can sometimes last for weeks. An infected person typically sheds virus one day prior to the appearance of symptoms, which spreads infection before the sick can be isolated, and the virus continues to be shed until the symptoms resolve. The peak viral load is generally observed on the day of the onset of symptoms and gradually decreases with time. Children and younger adults often shed the virus for 10 days or more [56], while an immunocompromised person may shed the virus for weeks [57]. The virus can be detected in easily clinical specimens such as nasal/throat swabs and nasopharyngeal aspirates. There are also reports of viral load detection in urine and stool of infected patients [58, 59].

Laboratory diagnosis of influenza

Accurate diagnosis and prompt treatment with antiviral drugs can have positive effects on human health and reduce the economic burden of influenza illness each year. However, because several other respiratory viruses, including adenoviruses, rhinoviruses, respiratory syncytial virus (RSV), coronaviruses, metapneumoviruses and parainfluenza viruses, can cause common symptoms of influenza-like-illness (ILI), many cases are misdiagnosed as influenza [60]. Proper specimen collection is of paramount importance, regardless of the diagnostic method used. Nasopharyngeal specimens are always preferred over throat swabs or other specimens [61]. The best time to collect the clinical specimen is on the second or third day of symptoms (when viral shedding is at its peak), as the results obtained will be more reliable than when samples are obtained earlier or later in the course of disease [28, 51, 55, 61, 62]. There are a number of methods available for influenza diagnosis including rapid antigen tests, viral culture, serology, conventional reverse transcription polymerase chain reaction (PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP), real-time reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence assay. For rapid antigen (influenza) tests, the preferred specimens are nasopharyngeal or nasal swabs or throat swabs collected within 3-4 days of infection for more accurate testing. These tests provide results in less than 15 minutes with 40-70% sensitivity [63] when compared with viral culture (3-10 days) or RT-PCR, which has greater than 90% specificity and is moderately fast. Therefore, false negative results are more common than false positive results during influenza seasons when bedside rapid antigen tests are used [63, 64]. These rapid antigen tests can differentiate between seasonal influenza A and B types, but they are unable to detect pandemic H1N1-2009 viruses exclusively [30]. Health care professionals, during the time of year when outbreaks of ILI are common, can perform tentative diagnosis of influenza using various commercially available rapid immunoassay kits, but due to the limitations of rapid viral tests, confirmatory laboratory testing should be done to determine the treatment of choice [64]. While virus culture is believed to be one of the most accurate methods for identifying viral strains and subtypes, it can sometimes be an impractical choice for physicians who usually need to initiate antiviral drug therapy within 48 hours of the onset of symptoms [59]. The virus culture method also becomes a secondary choice during pandemic situations when a large number of infected people rush to hospitals for diagnosis and treatment [51, 62]. The most sensitive diagnostic tool available to date is the real-time reverse transcription polymerase chain reaction (RRT-PCR) test [62, 65]. RRT-PCR detects the viral RNA with high sensitivity in a few hours and requires relatively little effort. It targets the matrix gene to detect influenza viruses and the HA gene, not only to broadly distinguish between influenza A from B types but also to detect different strains of influenza A viruses (H3N2, H1N1, H1N1pdm09, etc. with high sensitivity and specificity [62]. The TaqMan chemistry is the most commonly used, as it gives high accuracy and specificity; however, it also comes with the burden of slightly higher costs when compared to the SYBR Green chemistry. The SYBR Green chemistry is

cost effective, as it does not require dual-labelled probes like the TaqMan chemistry and is highly sensitive. The SYBR Green chemistry however, has not been widely used for clinical diagnostics, as it uses an intercalating dye that can produce fluorescence with any mis-amplified DNA, thus compromising the specificity of the test [66]. A new diagnostic method named RT-SmartAmp assay was developed in Japan during the 2009 H1N1 pandemic to reduce the time required for detection. The RT-SmartAmp assay includes reverse transcription and isothermal DNA amplification in one step, and RNA extraction and PCR are not required. An exciton-controlled hybridization-sensitive fluorescent primer specifically detected the HA segment of the pandemic H1N1-2009 influenza A virus within 40 minutes without cross-reacting with seasonal A (H1N1), A (H3N2), or B-type virus. It was found to be an efficient method for detection of IAV in patient's swab samples in early stages of infection [67]. A recent study demonstrated the diagnostic potential of recombinant scFv antibodies generated against the hemagglutinin protein of influenza A virus for diagnosis and treatment of human influenza A virus infections. In that study, an ELISA was developed that demonstrated 83.9% sensitivity and 100% specificity for H1N1 influenza A viruses and promised to be a cheaper alternative to the costly RRT-PCR test [68]. At research institutes and in reference or hospital laboratories, where sophisticated equipment is available, electron microscopy, cytology and histology may also be used to diagnose influenza virus infections.

Treatment of influenza

Effective management of influenza lies in following good health practices and preventive measures laid down by health authorities. Appropriate treatment of the patients can be done after accurate and timely diagnosis, and this can further reduce the inappropriate use of antibiotics and antiviral therapy. Usually, **??**antiviral**??** therapy is preferred, as bacterial co-infection usually occurs only after viral infection. The first line of antiviral therapeutics that are chosen are inhibitors of viral proteins. The antiviral drugs currently available against influenza viruses are adamantane derivatives (amantadine and rimantadine) and neuraminidase (NA) inhibitors (zanamivir, oseltamivir and peramivir). A viral infection can be inhibited at several crucial steps, such as entry, signaling, assembly, and egress (Fig. 3).

Adamantane derivatives inhibit virus multiplication by interfering with the transmembrane domain of the matrix protein (M2) of influenza type A viruses and also interferes in viral assembly during viral replication [69, 70] (Fig. 3). Amantadine was approved for clinical use in 1966, and rimantadine was approved in 1993 [71, 72]. In the United States, three FDA-approved neuraminidase inhibitor antiviral drugs are currently recommended by the US Centers for Disease Control and Prevention (CDC): oseltamivir (available under the trade name Tamiflu), zanamivir (trade name, Relenza), and peramivir (trade name, Rapivab). The recently circulating influenza A and B viruses in the USA are susceptible to neuraminidase inhibitors, but amantadine and rimantadine are not recommended because of resistance to these drugs

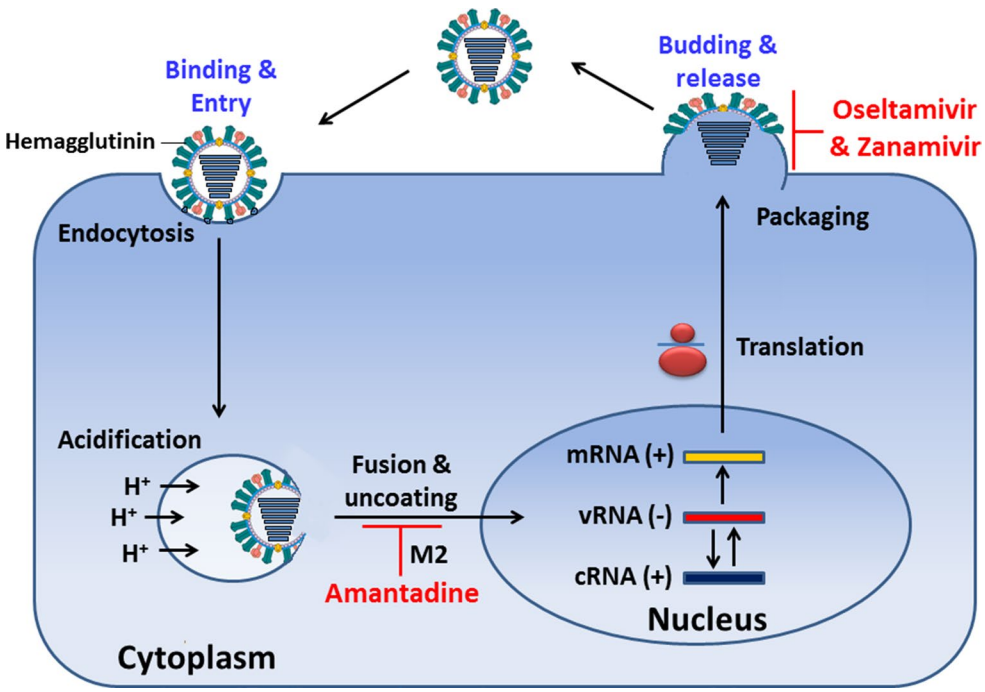

Fig. 3 A schematic diagram depicting the crucial steps of influenza virus infection

and also because they are not effective against influenza B viruses. All of these drugs are partially licensed against influenza in various countries. Controlled clinical trials have shown sufficient effectiveness of these classes of drugs, which also prevent influenza-related illness. Oseltamivir and zanamivir are recommended for all individuals with suspected or confirmed influenza requiring hospitalization and patients in high-risk groups, such as children under the age of two years, adults 65 years or older, pregnant women, immunosuppressed individuals, and **??**women who have given birth within the previous two weeks**??**.

In addition to the antiviral drugs that are available for treating influenza infections, there are new alternatives with better therapeutic potential, which studies suggest may prove to be beneficial in the near future. The long-acting inhaled neuraminidase inhibitor (NAI) CS-8958 (also known as R-118958) has shown promising results in murine models of influenza treatment [73]. A polymerase inhibitor, T-705 (Toyama Chemical), whose mechanism of action is the inhibition of the viral RNA polymerase, has not only been found effective against all three influenza virus types (A, B and C) but is also effective to some extent against other RNA viruses, including hemorrhagic fever viruses [74]. Another drug, DAS181, which is a fusion construct that includes the sialidase from *Actinomyces viscosus*, targets the viral attachment process during the early stages of replication of influenza virus [75]. Another recent study showed that chlorogenic acid (CHA) has antiviral properties and shows an inhibitory effect on A/PuertoRico/8/1934(H1N1) (EC_{50} = 44.87 μM), A/Beijing/32/92(H3N2) (EC_{50} = 62.33 μM), and oseltamivir-resistant strains in the late stage of the infectious cycle by blocking neuraminidase activity [76]. CHA (100 mg/kg/d) administered as an intravenous injection, showed 60% and 50% protection from death against the H1N1 and H3N2 strains, respectively, by reducing the viral titers and alleviating virus-associated inflammation in lungs of infected mice [76]. There are several other novel influenza antiviral drugs under clinical development in the United States, such as AVI-7100, which is a 20-mer phosphorodiamidate morpholino oligomer (PMO) IV formulation that hampers the translation and splicing of mRNA derived from the matrix gene [77]. CR6261 and CR8020 are monoclonal antibodies that bind to the conserved stalk region of HA and inhibit the entry and fusion stages [78]. EV-077 is a dual thromboxane receptor antagonist and thromboxane synthase inhibitor that inhibits virus replication by inhibiting the increase of prostanoids that is associated with influenza virus infections. The drug prevents inhibition of the host immune response by the virus, thus increasing virus replication [79]. A recent study also showed the anti-influenza activity of a natural product, aureonitol, a compound obtained from fungi that has shown inhibitory effects against both influenza A and B virus replication. Aureonitol inhibits influenza virus hemagglutination and thus impairs virus adsorption [80].

Influenza drug resistance

The use of antiviral drugs is preferred during pandemic situations until an effective and sufficient vaccine is available. However, these drugs have a number of side effects, and viruses tend to develop resistance against these drugs over the course of time. The emergence of antiviral-drug-resistant seasonal influenza A viruses is a major concern. Initially both amantadine and rimantadine were successful in inhibiting IAV infection, and the efficacy was around 90% [81]. The first adamantine-resistant viruses were reported during the 1980 epidemic, and since then, the number has continued to increase [82]. Surveillance for adamantane resistance among A (H3N2) viruses from 1991 to 1995 revealed that the global frequency of resistance was as low as 0.8% [83]. Another study conducted in 2004 showed that this global resistance frequency increased to 12.3%, and a year later it reached 96%, 72%, and 14.5% in China, South Korea, and the United States, respectively [84]. The alarming increase in drug-resistant H3N2 strains in the USA in 2005 led the US-CDC to issue a public health warning recommending clinicians not to prescribe adamantanes for the remainder of the 2005 and 2006 season [85]. Most of the adamantine-resistant H3N2 isolates (98.2%) were found to contain an S31N mutation in the M2 transmembrane domain, while L26F, V27A, and A30T mutations accounted for the rest (1.8%) [86]. Starting in 2005, the number of cases of resistance increased exponentially, and from 2005 to 2006, almost 90.6% of H3N2 strains and 15.6% of H1N1 were adamantane resistant [87]. In the USA alone, 96.4% of H3N2 isolates and 25% of H1N1 isolates were adamantane resistant [84, 87], and the resistance-conferring mutation was S31N in the M2 gene in both H1N1 and H3N2 strains. The pandemic H1N1-2009 strain [88] as well as the H5N1 and H7N9 strains that caused fatal zoonotic infections in humans in 2003 and 2013, respectively, were also observed to have the same S31N mutation in the M2 gene [89, 90]. By 2013, almost 45% of all the IAV isolates were resistant to adamantanes [91]. The neuraminidase inhibitors (NAIs) are the second class of anti-influenza drugs, and the only one currently being used worldwide. These drugs target the surface protein NA to produce an antiviral effect [92]. The NAIs oseltamivir (Tamiflu) and zanamivir (Relenza) are most effective if administered within 36-48 hours of the onset of symptoms [93]. Zanamivir was approved for prophylaxis and treatment of IAV infection in humans in July 1999, followed by oseltamivir in October 1999 [94]. The global Neuraminidase Inhibitor Susceptibility Network (NISN), established in 1999, reported that during the period 1996-1999, all human

influenza isolates were found to be susceptible to NAIs; however, in the later years 2005 and 2007, the frequency of oseltamivir resistance in global H1N1 isolates increased slightly by 0.4% and 0.6%, respectively [95]. In 2007-2008, there was a significant 7% global rise in oseltamivir-resistant H1N1, but not H3N2 strains, and all oseltamivir-resistant H1N1 isolates from that season were sensitive to zanamivir [96]. In the 2008-2009 season, more than 90% of the globally circulating H1N1 subtypes were found to be oseltamivir resistant [97]. In the year 2009, the circulating NAI-resistant H1N1 strains were replaced by the novel pandemic H1N1-2009 strain, which, fortunately, was sensitive to NAIs [98]. These influenza A (H1N1 and H5N1) viruses have shown resistance due to mutation of a histidine to a tyrosine at residue 274 of the NA (H274Y), which confers a high level of resistance to oseltamivir but has no effect on susceptibility to zanamivir or to the adamantanes [99].

Vaccines for influenza

Although antiviral drugs against influenza are readily available worldwide, the administration of vaccines remains at the forefront for managing influenza virus infections because prevention is still better and more cost-effective than cure. The administration of influenza and pneumonia vaccines is one of the highest priorities in primary-care medicine [100]. Since their first introduction in the 1940s, influenza vaccines have come a long way [101]. These early vaccines were inactivated whole-virus vaccines that were generated in embryonated chicken eggs and inactivated by treatment with formalin. The genetic drift in viral genome has made it necessary to formulate new vaccines each year. Although separate vaccines are now available for individual influenza viruses, a universal influenza vaccine has not yet been developed due to the highly variable nature of the surface glycoproteins HA and NA. WHO maintains surveillance of circulating strains of influenza viruses in both the Northern and Southern Hemisphere, and influenza vaccines are formulated annually to be administered to healthy individuals and those at higher risk of complications prior to the start of the flu season. There are currently several types of influenza vaccines available, of which the major types are conventional inactivated influenza vaccines and live attenuated influenza virus (LAIV) vaccines. The conventional inactivated influenza vaccines consist of purified virus particles that have been inactivated by treatment with formalin or β-propiolactone. Live attenuated influenza vaccines are made using virus strains that are cold adapted, temperature sensitive, and attenuated to prevent them from causing illness. LAIV vaccines have been successfully made that can be administered via nasal spray (FluMist). They have shown high efficacy in children when compared to inactivated vaccines [102], as the LAIV activates mucosal, systemic humoral, and cellular immunity, just like natural influenza viruses. In the USA and Canada, an LAIV vaccine is licensed under the trade name FluMist, while in Europe it is licensed under the trade name Fluenz. Since LAIV vaccines have been observed to be less effective in adults, inactivated split vaccines are recommended for adults. Traditional trivalent vaccines containing two influenza A strains (H1N1 and H3N2) and one influenza B strain sometimes show limited protection due to a lineage mismatch between the vaccine B strain and the circulating B strain. To minimize the limitation in protection by trivalent vaccines, the FDA, for the first time in 2009, considered the inclusion of an additional influenza B strain, thus making a quadrivalent vaccine. Both live-attenuated quadrivalent influenza vaccines and inactivated quadrivalent influenza vaccines are known to confer significant protection against the drifted circulating influenza B viruses [103]. Apart from the traditional vaccine approaches, other approaches includes the development of DNA vaccines against different influenza virus antigens [104], the development of a possible universal influenza vaccine targeting the HA stalk domain [105, 106], and the use of influenza-virus-like particles as vaccines [107]. The long delivery time frame for egg-based vaccines can be a critical factor during a pandemic, and therefore, cell-culture-based vaccines (e.g., Optaflu, Flucelvax, Preflucel, and Celvapan) are also being used to overcome this issue [108].

Alternative approaches to combating antiviral resistance and developing vaccine formulations

Due to the increasing burden of vaccine formulations and cases of antiviral-drug-resistant influenza virus isolates turning up every year, it has become necessary to search for alternatives to the current treatment and prevention strategies. The last few decades have seen a tremendous effort being made to develop inhibitors and blockers of vital genes of influenza viruses. Novel drugs have been formulated against the viral nucleoprotein [109] and non-structural proteins [110]. Several studies have also been performed using siRNA and antisense oligonucleotides as gene silencing tools to inhibit influenza virus replication in cell lines, chicken embryonated eggs, and mice [111–113]. The potential of siRNAs as antivirals was first recognized in 2011, when this approach was used against the viral transcription factor, P (phosphoprotein), and viral F (fusion) protein of RSV [114]. In 2003, Chen's laboratory published the first report of the use of siRNAs against NP, PA, PB1, PB2, M, and NS genes that showed various degrees of inhibition of multiple subtypes of influenza viruses [113]. A study conducted using antisense oligonucleotides against the 3'

NCR of vital segments of the IAV genome showed significant inhibition of viral replication. The designed antisense molecules were tested against the A/PR/8/34 (H1N1), A/Udorn/307/72 (H3N2), and A/New Caledonia/20/99 (H1N1) strains of IAV and were found to reduce the cytopathic effect caused by these viruses for almost 48 hours postinfection in cell lines and to increase the survival of experimental mice [112]. Ribozymes (Rz) and DNAzymes (Dz) are yet another class of gene-silencing tools that have been demonstrated to control IAV replication [115, 116]. A study conducted on the A/PR/8/34 (H1N1) strain showed that Rz and Dz along with antisense molecules accomplish a synergistic cleavage of the matrix (M1) gene of influenza virus, thus inhibiting virus replication in host cells [117]. Another recent study conducted on influenza B virus also confirmed the role of Dz in inhibiting IAV replication [70]. The designed Dz showed a significant 52% inhibition of the B/Yamagata/1/73 strain of influenza B virus [70]. In another recent study, the authors revealed that self-assembling influenza nanoparticle vaccines could elicit broadly neutralizing H1N1 antibodies. They genetically fused the viral hemagglutinin to ferritin, a protein that naturally forms nanoparticles, and showed that this influenza nanoparticle vaccine generated more than tenfold higher HA inhibition antibody titres than those induced by the licensed inactivated vaccine [118]. Another prospective approach to achieving high virus-neutralizing activity is the use of monoclonal antibodies and recombinant antibody fragments [119, 120]. Several host-cell molecules have been known to play a crucial role during influenza virus infection, thereby representing targets for designing inhibitors of virus-cell interactions. One such target is the vacuolar proton-ATPase, which when inhibited, renders viral M2 ion channels inactive [121]. A few other studies have also focused on inhibitors of cellular proteases [122] that block the proteolytic activation of HA and blockers of the cellular ubiquitin-proteasome system [123]. In the past few years, there has been a remarkable increase in the number of studies describing the use of a new class of influenza-virus-neutralizing antibodies targeting conserved sites in the HA stem. These molecules have shown varying levels of cross-reactivity toward group 1 [124, 125], group 2 [126, 127] and group 1 & 2 viruses [128, 129]. Despite these efforts, antibodies that can react with the stem region of both group 1 and 2 subtypes are rare and do not cover all subtypes. In view of this, in a recent study, a broad spectrum human monoclonal antibody (mAb- MEDI8852) was developed, which unlike other stem-reactive antibodies, used a rare heavy chain VH (VH6-1) gene and carried a low level of somatic mutations [130]. MEDII8852 was effective in mice and ferrets and was better than oseltamivir. Its broad neutralizing capability makes this molecule a good candidate for development as an immunotherapy for influenza-virus-infected humans [130]. These alternative approaches, which, when backed up with clinical trials, will provide promising tools for managing influenza virus infections effectively.

Summary

Influenza viruses have successfully evolved with striking survival strategies. They circulate worldwide with established lineages in avian and mammalian species. Of the four influenza virus types (A, B, C and D), influenza A viruses are the most virulent and have the potential to cause both epidemics and pandemics due to genetic drift and genetic shift, respectively. Types B, C and D are not known to cause pandemics. Influenza A virus has a wide range of hosts, which provides opportunities to cross species barriers, thus increasing the chances of an influenza pandemic. The virus circulates around the world and causes annual outbreaks resulting in about 3-5 million cases of illness and up to 500,000 deaths [131]. Two classes of anti-influenza drugs (adamantanes and NAIs) are available, of which only the NAIs are currently effective against circulating strains of IAV. Vaccination is one of the best approaches to prevent influenza infections annually. However, due to frequent mutations in the surface glycoprotein HA, IAV acquire enough mutations each year to escape the protectivity of the annually formulated vaccines, and some of them show a high level of resistance against antiviral drugs [132]. Alternative approaches such as the use of siRNA, antisense nucleotides, Dz and Rz have gained importance in the past few decades and have shown promising results in cell lines and mouse models [69, 70, 112, 113, 115, 117]. Several other studies are still being performed to develop a universal influenza vaccine that can neutralize all types of IAV. Influenza viruses have evolved in parallel to humans to establish successful infections and continue to pose a significant threat to both life and economy. The health authorities invest large amounts of money into annual vaccine formulations, and the virus acquires several mutations to render those vaccines ineffective within a year. Thus, new alternative approaches to combating antiviral resistance and the development of universal vaccine formulations are currently needed in order to manage future influenza threats.

Future perspective

Influenza viruses have been the cause of annual epidemics throughout the world. The A subtypes occasionally cause pandemics that lead to the death of millions of people. The history of influenza suggests that the virus is highly unpredictable in its ability to jump species barriers and cause threatening situations for mankind. Health authorities across the world have influenza preparedness plans that are

based on combined surveillance data received from both the Southern and Northern Hemisphere. Advancements in science have brought together several antiviral therapeutic strategies combined with novel drugs that can be used to manage influenza during annual epidemics. Recent attempts to produce a universal influenza vaccine have also shown promise for combating future flu pandemics.

Compliance with ethical standards

Disclosure of potential conflicts of interest All of the authors declare that they have no conflict of interest (financial or non-financial).

Research involving human participants and/or animals The authors further declare that this is a review article and it did not require research involving human participants and/or animals.

Informed consent All of the authors declare that this is a review article and did not require research involving human participants; thus, no informed consent was needed.

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