

Drug resistance in influenza A virus: the epidemiology and management

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Abstract: Influenza A virus (IAV) is the sole cause of the unpredictable influenza pandemics and deadly zoonotic outbreaks and constitutes at least half of the cause of regular annual influenza epidemics in humans. Two classes of anti-IAV drugs, adamantanes and neuraminidase (NA) inhibitors (NAIs) targeting the viral components M2 ion channel and NA, respectively, have been approved to treat IAV infections. However, IAV rapidly acquired resistance against both classes of drugs by mutating these viral components. The adamantane-resistant IAV has established itself in nature, and a majority of the IAV subtypes, especially the most common H1N1 and H3N2, circulating globally are resistant to adamantanes. Consequently, adamantanes have become practically obsolete as anti-IAV drugs. Similarly, up to 100% of the globally circulating IAV H1N1 subtypes were resistant to oseltamivir, the most commonly used NAI, until 2009. However, the 2009 pandemic IAV H1N1 subtype, which was sensitive to NAIs and has now become one of the dominant seasonal influenza virus strains, has replaced the pre-2009 oseltamivir-resistant H1N1 variants. This review traces the epidemiology of both adamantane- and NAI-resistant IAV subtypes since the approval of these drugs and highlights the susceptibility status of currently circulating IAV subtypes to NAIs. Further, it provides an overview of currently and soon to be available control measures to manage current and emerging drug-resistant IAV. Finally, this review outlines the research directions that should be undertaken to manage the circulation of IAV in intermediate hosts and develop effective and alternative anti-IAV therapies.

Keywords: influenza A virus, drug resistance, M2 ion channel inhibitors, neuraminidase inhibitors, oseltamivir, zanamivir

Introduction

Influenza virus is one of the most successful, persistent, and unpredictable human pathogens. Influenza continues to cause regular seasonal epidemics, unpredictable pandemics, and frequent and deadly zoonotic outbreaks worldwide. Influenza virus transmits through aerosol and causes an acute febrile respiratory disease in humans, commonly known as “flu”, which is particularly severe in young children, elderly, and immunocompromised individuals. The burden of influenza virus on global human population and economy is significant.^{1–23} According to the World Health Organization (WHO) estimate, influenza virus annually causes 1 billion cases of flu, 3–5 million cases of severe illness, and 300,000 to 500,000 deaths worldwide. The annual influenza vaccination program alternating in the Northern and Southern Hemispheres is the major tool to prevent or control seasonal influenza epidemics. In addition, two classes of antiviral drugs, 1) M2 ion channel inhibitors

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(amantadine and rimantadine) and 2) neuraminidase (NA) inhibitors (NAIs; oseltamivir, zanamivir, peramivir, and laninamivir), have been approved to treat influenza virus infections. However, like antibiotic resistance, emergence of antiviral drug resistance in influenza virus is a major concern. Consequently, NAIs are the only class of anti-influenza drugs currently in use as most of the circulating influenza viruses have acquired resistance to M2 ion channel inhibitors.²⁴ Nevertheless, many circulating influenza viruses have also acquired the resistance to NAIs. This review highlights the emergence and epidemiology of drug-resistance mutations in M2 and NA with focus on influenza A virus (IAV), the prototypic and most significant member of the *Orthomyxoviridae* family.

Natural history and epidemiology of IAV

IAV virion particles exhibit both spherical and filamentous morphology and possess the negative-sense, segmented and single-stranded RNA genome. Each of the eight IAV gene segments encodes at least one major viral protein. However, some IAV segments encode more than one viral protein through mechanisms including leaky ribosomal scanning, alternative splicing, ribosomal frameshifting, and use of alternative start codon.^{25,26} So far, IAV has been reported to encode at least 17 viral proteins, although not all IAV subtypes encode every protein. IAV is an enveloped virus, and each virion contains ~300 hemagglutinin (HA) and ~40 NA glycoprotein spikes on the surface.^{27,28} The HA is the receptor-binding protein and facilitates IAV entry to host cell, whereas NA facilitates the release of newly produced virions from the host cell.²⁸ A third protein, M2 that forms an ion channel and is critically involved in virus entry, is also embedded in the viral envelope, which is derived from the host cell plasma membrane.²⁸ Underneath the envelope is a rigid layer comprised of matrix protein 1 (M1), which maintains the shape and integrity of IAV virion.²⁹ M1 also interacts with the cytoplasmic domains of IAV envelope proteins and viral ribonucleoprotein (vRNP) core. The vRNP core is primarily composed of viral genome, nucleoprotein (NP), and polymerase complex, which consists of PA, PB1, and PB2 proteins.²⁸

IAV has global presence and a broad host range that includes humans, seals, horses, pigs, dogs, cats, and birds (Figure 1). The aquatic birds, such as waterfowl and shorebirds, are the reservoir host of IAV.³⁰ IAV is subtyped based on the type and antigenicity of its surface glycoproteins, HA and NA. So far, 18 HA and 11 NA subtypes have been described,

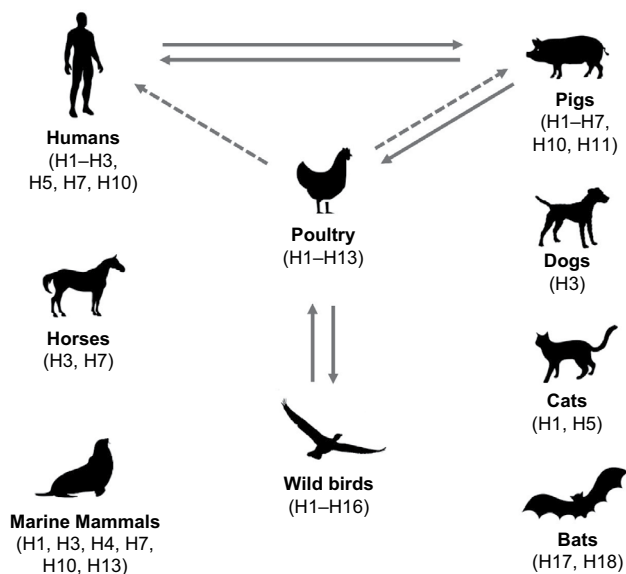


Figure 1 The host range of influenza A virus.

Notes: The IAV HA subtypes isolated from each host are mentioned in parenthesis. The significant interspecies IAV transmission is shown by solid (common) and dashed (sporadic) arrows.

Abbreviations: IAV, influenza A virus; HA, hemagglutinin.

of which 16 HA and 9 NA have been found to circulate in avian species, whereas 2 HA and 2 NA subtypes have been detected in bats (Figure 1).^{31,32} However, the bat IAV subtypes, H17N10 and H18N11, are remarkably different from other IAV subtypes prompting suggestion that these bat viruses should be labeled as influenza-like viruses.³³

The interspecies transmission of IAV occurs and is common as well as significant between humans and pigs and poultry and pigs, while it is sporadic in others (Figure 1). The ability of IAV to transmit between species is determined by its capability to change specificity to target species. IAV is also well adapted to promote antigenic diversity by using two particular mechanisms known as antigenic drift and antigenic shift.³⁴ Antigenic drift causes mutations in HA and NA resulting in antigenic variants, which can reinfect a host and avoid the pre-existing immunity.³⁴ The error-prone nature of viral RNA polymerase is the major contributor to antigenic drift, which along with frequent reassortment and natural selection is the main cause of recurring seasonal influenza epidemics.³⁵ These epidemics are capable of lasting at least 6 to 12 weeks, with observed infection rates of 10–30% in adults and 20–50% in children.³⁶ On the other hand, antigenic shift is the reassortment of gene segments between two different parental viruses within the same host, giving rise to a novel pandemic IAV. The H1N1 subtype, which caused the first recorded IAV pandemic in 1918, was originated from the reassortment between a human H1

subtype and an avian N1 subtype.³⁷ The next IAV pandemic of 1957 was caused by an H2N2 subtype, which originated when circulating 1918 H1N1 subtype reassorted with an avian H2N2 subtype.³⁸ Subsequent IAV pandemic in 1968 was caused by the H3N2 subtype. This subtype arose when circulating 1957 H2N2 subtype reassorted with an avian H3 subtype.³⁸ The most recent IAV pandemic in 2009 was caused by a swine-origin H1N1 subtype, which originated from the sequential reassortment events between human H3N2, swine H1N1 and avian H1N2 subtypes of North American and Eurasian lineages.³⁸ A pandemic IAV has the potential to spread quickly and infect ~50% of the global human population within a short period of time. Therefore, all four pandemics combined resulted in the deaths of millions of people worldwide. Lately, several pure avian-origin IAV subtypes (e.g., H5N1, H7N9, and H10N8) have been found to cause deadly outbreaks in humans. Many of these IAV subtypes cause a disease that is clinically distinct and more severe than the disease caused by a human IAV subtype and results in a significantly high mortality rate (~35–50%).³⁹ Fortunately, none of these avian IAV subtypes have acquired the capability of direct human-to-human transmission by aerosol; they mainly spread through a direct contact. The spread of avian IAV to humans is limited by differing receptor specificities.⁴⁰ IAV utilizes carbohydrate moiety, sialic acids present on host cell surface as its receptor. The HA of human IAV subtypes specifically binds to sialic acids with α -2,6-linkages, whereas the HA of avian IAV subtypes binds to sialic acids with α -2,3-linkages.⁴⁰ The upper respiratory tract in humans predominantly contains sialic acid with α -2,6-linkages. However, sialic acids with α -2,3-linkages are found in the lower respiratory tract of humans, meaning that avian IAV subtypes are capable of infecting humans upon exposure. Interestingly, both α -2,6- and α -2,3-linkages are found in swine upper respiratory tract; therefore, pigs are regarded as the “mixing vessels” for the generation of human and avian IAV reassortant subtypes.⁴¹

Antiviral drugs and drug resistance in IAV

Adamantanes: M2 ion channel inhibitors

The adamantanes, amantadine (Symmetrel), and rimantadine (Flumadine) were the first-approved class of anti-IAV drugs.⁴² Adamantanes target the M2 protein of IAV to exert their antiviral function.^{43,44} The M2 is a tetrameric, type III integral membrane protein, and the single transmembrane domain of M2 forms a 4-helix bundle that acts as a pH-sensitive gated ion channel in the IAV envelope.^{45–49} IAV

enters the host cell via receptor-mediated endocytosis and uncoats through HA-mediated fusion of viral envelope with endosomal membrane.²⁸ Prior to the fusion, the low pH in endosome activates and opens the M2 ion channel allowing the entry of protons and causing the internal acidification of virion. This event leads to the dissociation of M1 protein from vRNP core and subsequent release of the latter into host cell cytoplasm, initiating the IAV replication.⁵⁰ The adamantanes bind to M2 channel pore and block proton conductance either directly or allosterically, consequently inhibiting the vRNP release and IAV replication.^{51–54} To acquire resistance to adamantanes, IAV mutated several amino acids (L26F, V27A, A30T/V, S31N, G34E, and L38F) in M2 transmembrane domain that line the channel pore (V27, A30, and G34) or are involved in the tetramer helix–helix packing (L26, S31, and L38), leading to increase in pore size and hydrophilicity of the channel or destabilization of helix–helix assembly and narrowing of the pore size, respectively.^{51–53,55–58}

Epidemiology of adamantane-resistant IAV

Amantadine was first of the two adamantanes to be approved for clinical use in 1966 followed by rimantadine in 1993.^{43,56,59} Initially, both drugs were highly successful in inhibiting and preventing the IAV infection with an efficacy rate of up to 90%.^{60–62} The resistance of IAV to adamantanes was first detected during 1980 epidemic.⁶³ However, the resistance to both drugs in seasonal IAV subtypes was rare with only 1–2% frequency until 2000^{59,64–66} but has risen dramatically since then.⁶⁷ From 2000 to 2004, the resistance to adamantane among IAV H3N2 subtype isolates across Asia increased from 1.1% to 27%.⁶⁷ The numbers from China (73.8%), Hong Kong (69.6%), and Taiwan (22.7%) were the main contributors to this 27% increase in Asia.⁶⁷ However, during the same period, the increase in H3N2 subtypes resistant to adamantanes in Europe, North America, and South America was only 4.7%, 3.9%, and 4.3%, respectively, whereas no resistant virus was detected in the Oceania region. Nevertheless, during 2000–2004, the overall resistance to adamantanes among H3N2 subtypes rose to 12.3% globally.⁶⁷ In majority of the H3N2 isolates (98.2%), the adamantane resistance was due to the S31N mutation while the L26F, V27A, and A30T mutations accounted for the rest (1.8%).⁶⁷ On the other hand, only 0.3% H1N1 subtypes were resistant to adamantanes during this period. Similarly, during 2004–2005 season, only 15% of the H3N2 and 4.1% of the H1N1 global isolates were resistant to adamantanes.⁶⁸ However, from 2005 onward, the resistance to adamantanes started to increase almost

exponentially. Remarkably, during 2005–2006 season, 90.6% of the H3N2 and 15.6% of the H1N1 global isolates were adamantane resistant.⁶⁸ The Asia, Europe, North and South America, and Oceania regions contributed almost equally to these statistics, although no significant data were available from Africa. Astonishingly, 100% of the H3N2 isolates from South Korea, Taiwan, Japan, Hong Kong, and China were resistant to adamantanes.⁶⁸ Similarly, in the USA, up to 96.4% of the H3N2 isolates and up to 25% of the H1N1 isolates were adamantane resistant.^{68,69} These data prompted the Centers for Disease Control and Prevention (CDC), USA, to issue an advisory against the use of adamantanes to treat IAV infections.⁷⁰ Again, in 90–98% of the isolates, the resistance-conferring mutation was S31N in both H1N1 and H3N2 subtypes. Similarly, the swine-origin IAV H1N1 subtype that caused the pandemic in 2009 also contained the S31N mutation in M2, hence, was resistant to adamantanes.^{71,72} Furthermore, the avian IAV subtypes H5N1 and H7N9 that emerged and caused severe zoonotic infections in humans in 2003 and 2013, respectively, also possess the S31N mutation in M2, hence, were resistant to adamantanes.^{39,73}

As of 2013, ~45% of all IAV subtypes circulating in the world were resistant to adamantanes.⁷⁴ Particularly, over 69% of the H1, 43% of the H3, 28% of the H5, 12% of the H7, and 23% of the H9 subtypes carried adamantane resistance-conferring mutations in M2. However, the adamantane-resistant mutations were rare in H2, H4, H6, H10, and H11 subtypes, and no such mutations were identified in H8 and H12–16 subtypes. Interestingly, all three bat-origin H17N10 isolates were also adamantane resistant. The adamantane-resistant H1 and H3 subtypes were mainly found in humans and swine and were widely distributed in the world. However, most of the adamantane-resistant H1 and H3 subtypes were detected in the Americas (52% and 56%), followed by Asia (26% and 34%), Europe (19% and 4.6%), Oceania (1.8% and 1.8%), and Africa (0.7% and 2.5%). In the Americas, USA had the largest distribution of adamantane-resistant H1 and H3 subtypes, whereas China, Singapore, and Hong Kong led the numbers in Asia and UK followed by Spain in Europe. In contrast, majority of the adamantane-resistant H4–H11 subtypes were detected in avian species and were mainly distributed in Asia. Particularly, the distribution of adamantane-resistant H5, H6, H7, and H9 subtypes in Asia was at 91%, 100%, 67%, and 100%, respectively. Vietnam had the highest number of adamantane-resistant H5 subtypes followed by Thailand, China, and Indonesia. On the other hand, China had the largest distribution of adamantane-resistant H7 and H9 subtypes followed by Hong Kong. The next

largest distribution of the adamantane-resistant H7 (31.3%) and H5 (6.4%) subtypes was found in Americas (exclusively USA) and Africa (primarily Egypt), respectively.⁷⁴

A vast majority of adamantane-resistant IAV subtypes (95%) contained the S31N mutation.^{74,75} Particularly, over 96% of the H1, 93% of the H3, 83% of the H5, 86% of the H7, and 87% of the H9 subtypes harbored the S31N mutation. Other mutations (L26F, V27A, A30T/V, G34E, and L38F) were sporadic, but over 11.8% of the H5 and 9.8% of the H9 subtypes contained V27A mutation. In addition, 2.8% of the H1, 5.4% of the H3, 2.6% of the H5, and 10.4% of the H7 viruses possessed the double mutation V27A+S31N and few of the H1, H3, and H5 viruses had the double mutation L26F+S31N. Furthermore, S31N was the most common mutation in human (98%), avian (88%), and swine (77%) IAV subtypes distantly followed by V27A in the same species. Interestingly, over 20% of the swine IAV subtypes had the double mutation V27A+S31N, frequency of which is believed to be rising in swine IAV.⁷⁶ Predictably, S31N mutation was distributed worldwide with majority of the distribution in Americas followed by Asia, Europe, Africa, and Oceania. However, V27A, A30T and L26F mutations were more prevalent in Asia, with majority V27A distribution in Indonesia followed by China. The most common double mutation was V27A+S31N with major distribution in the Americas (mainly USA) followed by Europe and Asia. The next most common double mutation was L26F+S31N with similar geographical distribution.⁷⁴

Temporally, the resistance to adamantanes in IAV subtypes H1 and H3 increased consistently in many countries from 2001 and spiked in 2009. Particularly, in Hong Kong, UK, and Germany, the frequency of adamantane-resistant H1N1 subtypes has increased consistently since 2001, and in China and USA since 2005 and 2006, respectively. Similarly, China, Hong Kong, Taiwan, and USA had a consistent increase in adamantane-resistant H3N2 subtypes since 2000, and Vietnam, Taiwan, and Malaysia since 2004.⁷⁴ The most likely reason for this dramatic increase in IAV resistance to adamantanes is the widespread usage of these drugs to treat IAV infections. However, increase in resistance has also been noted in countries where adamantanes use was low, indicating the stable circulation of the fit mutant viruses, which may have emerged without drug pressure and have replication potential and virulence similar to the wild type.^{59,77–80} It has been proposed that S31N mutation independently emerged multiple times and introduced at least 11 times in H3N2 subtypes during 1997–2007. It was the seventh introduction in H3N2 during 2003 in Hong Kong that subsequently

reassorted with another H3N2 subtype in 2005 to acquire a novel HA and gave rise to the “N-lineage”.^{75,79,81} The N-lineage then acquired other fitness-enhancing mutations elsewhere in the genome and spread globally; hence, all currently circulating adamantane-resistant H3N2 subtypes belong to a single lineage.⁷⁹ The emergence of adamantane resistance in H1N1 subtypes was delayed and temporally different to H3N2 subtypes.^{68,69,74} It is believed that adamantane resistance in H1N1 subtypes did not arise through reassortment with a resistant H3N2 subtype, but through a separate introduction and spread process.^{68,82}

NAIs

NAIs were the second approved and are the only currently used class of anti-influenza drugs. As the name suggests, NAIs target the IAV surface protein NA to exert their antiviral function.⁸³ NA possesses the sialidase enzyme activity that cleaves the cell surface sialic acid to which the newly formed IAV progeny is attached.^{28,84,85} This action releases the IAV progeny from infected cells that go on to infect naive cells and spread the infection. In addition, NA sialidase activity also facilitates the movement of IAV particles through sialic acid-rich human respiratory tract. The concept of an inhibitor of the NA sialidase activity as an antiviral agent was envisaged as early as 1948, not long after the discovery of a receptor-destroying activity on influenza virus surface.⁸³ The first such inhibitors were tested during 1966–1976 but exhibited low specificity and potency.^{83,86–88} However, the determination of the three-dimensional crystal structure and catalytic sites of NA led to the rational design of first two potent NAIs, now known as zanamivir (Relenza) and oseltamivir (Tamiflu).^{89–92} The NA is a mushroom-shaped, tetrameric glycoprotein, which is anchored in IAV envelope with exposed globular head.⁸³ The enzyme active site is situated in the head and consists of 8 functional amino acids (R118, D151, R152, R224, E276, R292, R371, and Y406 – N2 numbering throughout) and 11 structural amino acids (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, D293, and E425) that are conserved in almost all IAV subtypes. The functional amino acids form the catalytic core and directly contact the sialic acid, whereas the structural amino acids form the active site framework.^{93,94} The NAIs are sialic acid or transition state structural analogs that compete with cell surface sialic acid–viral NA interactions and inhibit the enzymatic reaction and release of the newly formed IAV progeny, consequently inhibiting the spread of the IAV and further infection.^{83,91} However, similar to M2, IAV has mutated several amino acids, notably E119V, I222V, H274Y, R292K, and N294S, in or around NA active site to

acquire the resistance to NAIs.^{95,96} Many of these mutations alter the architecture of NA active site and reduce the binding of NAIs by many fold.^{95,97–103}

Epidemiology of NAI-resistant IAV

Initial clinical studies showed that confirmed cases of IAV infection can be treated with both zanamivir and oseltamivir, and if administered within 36 to 48 h of the onset of clinical symptoms, both drugs reduced the duration of illness by up to 3 days in all ages.^{104–106} The prompt initiation of treatment after the onset of clinical symptoms was key in reducing the duration of illness proportionally.¹⁰⁷ Accordingly, both zanamivir and oseltamivir also prevented the IAV infection by 70–90% when used as a prophylaxis before or after the exposure to close contacts infected with IAV.^{104,106} In July 1999, zanamivir was the first NAI approved for the prophylaxis and treatment of IAV infection in humans followed by oseltamivir in October 1999.¹⁰⁸ Two related NAIs, peramivir^{109,110} and laninamivir,^{111–113} have also been recently approved in multiple countries and Japan, respectively.

Due to the experience with rapid emergence of adamantane-resistant IAV, several *in vitro* and preclinical studies were performed to select and isolate NAI-resistant IAV. Only few drug-resistant IAV mutants were isolated from *in vitro* passage cultures as well as patients treated with both zanamivir and oseltamivir.¹¹⁴ These mutants predominantly had two mutations, E119G/A/D/V and R292K in NA.¹¹⁴ In addition, two resistant mutants containing the H274Y mutation in NA were also isolated from healthy volunteers experimentally infected with IAV H1N1 subtype and subsequently treated with oseltamivir.¹¹⁵ Therefore, to monitor IAV resistance to NAIs, a global Neuraminidase Inhibitor Susceptibility Network (NISN) was established in 1999.¹¹⁶ The main objectives of NISN were to set the guidelines to test sensitivity of NAIs and monitor the susceptibility and resistance to NAIs in clinical influenza virus isolates collected from various parts of the world. In their first study published in 2003, NISN concluded that all clinical IAV isolates collected during 1996–1999 were susceptible to NAIs.^{117,118} Furthermore, during first 3 years (1999–2002) of NAI usage, basically no resistant IAV was detected, except few isolates from untreated individuals that exhibited reduced susceptibility to oseltamivir,^{118,119} and a disproportionately high rate (18–27%) of oseltamivir resistance observed in both H1N1 and H3N2 isolates from children.^{120,121} Similarly, no resistance was detected during 2004–2005 season.¹²² In the following 2005–2006 and 2006–2007 seasons, the frequency of oseltamivir resistance in global H1N1 isolates was only 0.4% and

0.6%, respectively.¹²³ Particularly in USA, no resistance was detected during 2005–2006 season, but during 2006–2007 season, it was detected to be ~0.9%. However, 2007–2008 season had a significant 7% increase in oseltamivir resistance in global H1N1 isolates, but no resistant H3N2 isolates were detected, and all 2007–2008 oseltamivir-resistant H1N1 isolates were sensitive to zanamivir.¹²² Nevertheless, 2.3% of the H1N1 isolates circulating between 2006 and early 2008 in Australasia and Southeast Asia exhibited resistance to zanamivir.¹²⁴ During 2007–2008, several European countries also had ~20% increase in oseltamivir-resistant H1N1 subtypes.^{125–127} Particularly, Norway witnessed an unprecedented 65% increase in the circulation of oseltamivir-resistant H1N1 subtypes followed by France (46%), Belgium (37%), and rest of the Europe. According to the WHO, by mid-2008, 15% of the H1N1 isolates circulating worldwide were oseltamivir resistant. Remarkably, in 2008–2009 season, more than 90% of the circulating H1N1 subtypes globally were oseltamivir resistant.^{128,129} According to CDC (USA), until April 2009 (just before the emergence of 2009 pandemic H1N1 subtype), over 99% of the H1N1 isolates were resistant to oseltamivir; however, all were sensitive to zanamivir and none of the H3N2 isolates were resistant to oseltamivir. Similarly, during 2008–2009 season, 100% of the H1N1 isolates from Japan were oseltamivir resistant.^{130,131} Further, 86%, 32%, and 100% of the H1N1 subtypes isolated in Oceania, South East Asia, and South Africa, respectively, were oseltamivir resistant.¹³²

In April 2009, a novel swine-origin IAV H1N1 subtype emerged and caused the first IAV pandemic of 21st century. Fortunately, the 2009 pandemic H1N1 subtype was sensitive to NAIs.⁷¹ Therefore, the only positive outcome of 2009 pandemic was that in the following seasons, the circulating pre-pandemic oseltamivir-resistant H1N1 subtype was replaced by the oseltamivir-sensitive pandemic H1N1 subtype.¹³³ Almost all of the pandemic H1N1 global isolates collected between April 2009 and January 2010 were sensitive to NAIs, except an odd 0.7% and other few H1N1 subtypes isolated from local cases that were resistant to oseltamivir.^{129,133–137} In April 2011, only 1.6% of the global pandemic H1N1 isolates were oseltamivir resistant.¹³⁸ Furthermore, only 3.2% of the pandemic H1N1 isolates collected between 2009 and 2012 from Asia, Africa, and Oceania were resistant to peramivir, but none to laninamivir.¹³⁹ Therefore, more than 98% of the pandemic H1N1 and seasonal H3N2 isolates collected globally till May 2015 remain largely sensitive to all NAIs.^{140–142} Nevertheless, a high transmission (15–29%) of oseltamivir-resistant pandemic H1N1 virus in some local communities^{141,143–146} and the rapid emergence and transmission of

resistant viruses in immunocompromised patients have been detected.^{147–151}

Unlike adamantanes resistance, which initially emerged and was more widespread in IAV H3N2 subtypes, the NAI resistance first emerged and was predominant in H1N1 subtypes.^{75,152} Majority of avian IAV H5N1 subtypes (except some isolates from Indonesia and Vietnam that exhibit reduced sensitivity to oseltamivir) and H7N9 subtypes circulating in nature are largely susceptible to NAIs.^{39,153} Further, the resistance in circulating IAV was mainly against oseltamivir and peramivir, and not zanamivir, and it is primarily limited to human IAV of N1 subtype.⁷⁵ The most common oseltamivir (and peramivir) resistance-conferring mutation in NA of the H1N1 and H5N1 subtypes was H274Y, whereas in H3N2 and H7N9 subtypes, the E119V and R292K mutations were more common (Table 1).^{122,125,127,130–133,138,141,142} Further, few of the H1N1 and H3N2 isolates carrying single mutations I222K/T/R, N142S, D198E, S246G/N, N294S, and G320E and double mutations T156I+D213G, I222T+S331R, I222R/V+H274Y, and S246N+H2724Y in their NA also exhibited reduced sensitivity to oseltamivir and peramivir and seldom to laninamivir (Table 1).^{140–142} In addition, the H1N1 subtypes with H274Y mutation also acquired secondary permissive mutations R193G, R221Q, V233M, V240I, D343N, D353G, and N368K in NA and T82K, K141E, R189K, and A193T in HA to improve their fitness.^{130,154–159} On the other hand, the resistance or reduced sensitivity to zanamivir in H1N1 and H3N2 isolates was conferred by the Q136K mutation in NA (Table 1).^{124,140,142,160}

Although use of NAIs is high in some countries such as Japan and USA, the emergence of oseltamivir-resistant IAV is largely attributed to the spread of a fit H274Y mutant, both pre- and post-pandemic period of 2009. Except in Japan (where the use of oseltamivir dramatically increased in 2001–2002 and was at its peak during 2008–2009) and USA, little or no oseltamivir was in use in Europe, Australia, New Zealand, and Southeast Asian countries where H274Y mutant was also pre-dominantly circulating during 2008–2009.^{119,127,130,132} Furthermore, rapid transmission of 2009 pandemic H1N1 (H274Y) mutant has been detected in communities with little or no previous exposure to oseltamivir.^{144,145} The pre-pandemic oseltamivir-resistant IAV H1N1 lineage (A/Brisbane/59/2007), first detected in Europe, emerged without drug pressure and subsequently acquired permissive mutations in NA and spread globally.^{127,130–132,152,157,161} The NAI-sensitive 2009 pandemic H1N1 subtype displaced the pre-pandemic oseltamivir-resistant H1N1 lineage and remains largely NAI sensitive and is predominantly circulating at present. However,

Table 1 Susceptibility of IAV subtypes with naturally acquired NA mutations to NAIs

Mutation	Subtype	Inhibition level (IC ₅₀ fold-change) [#]				Reference
		Oseltamivir	Zanamivir	Peramivir	Laninamivir	
V116A	H5N1	NI/RI (7.2–18)	RI (32.8)	NT	NT	180,181
E119A	H5N1	RI (35.3)	HRI (1,254)	NT	NT	181
E119V	H3N2	RI/HRI (25–535)	NI (1.2–5.7)	NI (0.9–3.7)	NI (1.1–3.0)	140–142,160,182
Q136K	H3N2	NI (0.2–1.1)	RI (11)	NI (2.5–9.0)	NI (0.9–5.2)	141,142,160
Q136K/R	pH1N1	NI (0.6)	HRI (185–200)	HRI (143–234)	RI (33–42)	140
N142S	H3N2	HRI (595)	HRI (244)	RI (40)	RI (53)	142
D198E	pH1N1	RI (16)	NI (7.1)	NT	NT	141
I222K	pH1N1	RI (23–39)	NI (5–6)	NI (4)	NI (3.6)	141,183
I222R	pH1N1	RI (13–58)	NI/RI (7–10)	NI/RI (5.3–10.0)	NI (2.2–2.3)	141,142,184
I222T	pH1N1	NI/RI (8.9–15.0)	NI (3.1–3.2)	NI (1.8–1.7)	NI (1.7–2.0)	141
S246N	pH1N1	NI (4–8)	NI (2–5)	NI (1)	NT	185
	H5N1	RI (24)	NI (2)	NT	NT	180
H274Y	pH1N1	HRI (151–4,010)	NI (0.1–6.5)	RI/HRI (87–2,045)	NI (0.3–5.1)	140–142,186–190
	H5N1	HRI (941–1,813)	NI (2.5–3.4)	NT	NT	181,191
R292K	H3N2	HRI (27,146–110,000)	RI (12–53.0)	HRI (271–474)	NI (2.7–5.3)	120,140,142,186,192
N294S	pH1N1	HRI (124–208)	NI (3–9)	NI/RI (4–12)	NT	140,193,194
	H5N1	RI (57–93)	NI (3–3.4)	NI (4)	NT	181,194
G320E	H3N2	RI (17)	RI (8.4)	NI (3.3)	NI (1.9)	142
T156I+D213G	pH1N1	RI (14)	NI (7.0)	NI (1.6)	NI (1.3)	142
D198N+H274Y	pH1N1	HRI (318)	NI (3.0)	HRI (108)	NT	187
I222R+H274Y	pH1N1	HRI (1,674–16,307)	NI/RI (2–22)	HRI (7,530–17,347)	RI (17)	183,184,189
I222V+H274Y	pH1N1	HRI (1,733)	NI (2)	HRI (1,331–2,707)	NT	184,193
I222T+S331R	H3N2	RI (12–31)	NI (2.9–7.2)	NI (1.6–2.4)	NI (2.5–3.6)	142
S246N+H274Y	pH1N1	HRI (5,880)	NI (5)	HRI (334)	NT	185

Notes: Bold indicates resistance; pH1N1, 2009 pandemic H1N1. [#]As per the WHO GISRS guidelines.^{140–142}

Abbreviations: IAV, influenza A virus; NA, neuramidase; NAI, neuramidase inhibitor; NI, normal inhibition; RI, reduced inhibition; NT, not tested; HRI, highly reduced inhibition; GISRS, Global Influenza Surveillance and Response System.

some oseltamivir- and peramivir-resistant 2009 H1N1 subtypes found circulating in local community clusters acquired two permissive NA mutations V240I and N368K, which improved their fitness.^{158,162} Concernedly, almost all currently circulating oseltamivir-sensitive 2009 H1N1 subtypes possess the V240I and N368K substitutions in NA.¹⁴¹ Therefore, similar to pre-pandemic H1N1 variants, a potential exists for the fit oseltamivir-resistant 2009 H1N1 variants to emerge and spread globally in the future.

It is somewhat intriguing that only some IAV subtypes (predominantly H1, H3, and H5) primarily circulating in birds, humans, and pigs – the three most important IAV hosts, have been reported to acquire the adamantane- or NAI-resistant mutations. This could be explained partly by the circulation and transmission frequency as well as epidemiological fitness of these subtypes in above hosts. One theory is that the acquisition of drug resistant, but potentially fitness-compromising mutations by these IAV subtypes under drug pressure in one host (humans) helps them acquire the fitness-enhancing permissive or epistatic mutations when transmitted to the other host (birds or pigs) under no drug pressure and a favorable immune environment. Consequently,

such drug-resistant variants then become epidemiologically fit and prevail in multiple host populations.

Management of drug-resistant IAV

The trio – vaccines, antiviral drugs, and surveillance – are key to control and eradicate viral pathogens, and the same is true for IAV.

Vaccines

The vaccines are at the forefront to prevent and manage the spread of drug-resistant IAV. Although a universal influenza vaccine is yet to be developed due to highly variable nature of its surface antigens HA and NA, vaccines specific to individual influenza viruses are available and have come a long way in terms of their composition and administration since first introduction in 1940s.¹⁶³ Now, intramuscularly, intradermally, and intranasally administered subunit and live-attenuated vaccines are available against seasonal IAV as well as zoonotic IAV such as H5N1 and H7N9. Based on a worldwide surveillance program in both Northern and Southern hemispheres spearheaded by the WHO, seasonal IAV vaccines are annually reformulated, manufactured, and

delivered for administration to global human population just before the start of flu season. Numerous individual and meta-analysis studies have found the seasonal IAV vaccines to be effective, albeit modestly in some instances, for both healthy and at-risk population.^{164–167} Nevertheless, influenza vaccine effectiveness is an important issue, and efforts are being made continuously to improve the efficacy of current vaccines and develop next-generation influenza vaccines. These include new vaccine formulations, development of universal influenza vaccine targeting the HA stalk domain, M2 ectodomain or inducing T-cell response, DNA vaccine against different influenza antigens, recombinant HA vaccine using baculovirus expression system (e.g., FluBlock) and other viral vectors (e.g., adenovirus and poxvirus), and influenza virus-like particles as vaccine.^{168–170} Furthermore, cell culture-based platforms are being used to prepare vaccines (e.g., Optaflu, Flucelvax, Preflucel, and Celvapan) to circumvent the issues faced with egg-based virus culture and vaccine delivery timeframe, a critical factor in the event of a pandemic or zoonotic outbreak.¹⁶⁸

Antiviral drugs

In the absence of a universal influenza vaccine, antiviral drugs become the first line of defense against a pandemic and zoonotic IAV. The adamantane-resistant IAV with S31N mutation appears to have established itself in nature and it is unlikely that this mutation will ever be lost; hence, adamantanes have become practically obsolete as anti-IAV drugs. Luckily, majority of currently circulating IAV H1N1 and H3N2 subtypes are sensitive to NAIs, and primarily human IAV H1N1 subtypes have acquired the resistance to oseltamivir. Therefore, NAIs are still effective in treating the infections with adamantane-resistant IAV and newly emerging IAV, provided they are administered at least within 48 hours of the appearance of clinical symptoms. Furthermore, majority of the oseltamivir- and peramivir-resistant IAV are still largely sensitive to zanamivir and laninamivir (Table 1). Nevertheless, the emergence and global spread of the fit IAV variants resistant to all NAIs is a concern. Therefore, a variety of new antiviral agents targeting either existing targets M2 and NA or other IAV components HA, NP, NS1, and polymerase complex are being developed, and several of them have shown promising results in clinical trials. Particularly, a small-molecule inhibitor of HA, nitazoxanide, and polymerase inhibitors, VX-787 and S-033188 are undergoing Phase 3 and Phase 2 clinical trials, respectively. One of the influenza polymerase inhibitors, Favipiravir (T-705) has already been approved in

Japan and is undergoing Phase 3 clinical trial in USA and Europe. Several other polymerase inhibitors (L-742001, Compounds “1”, “7”, and “367”, ASN2, ANA1) and NP inhibitors (Curcumin, Naproxen, Nucleozin, RK424) are at the experimental stages. In addition, therapeutic monoclonal antibodies are also being developed against IAV. Some of them (e.g., CR6261, CR8020, MHAA4549A, and VIS410) targeting the HA are in Phase 2 clinical trials.^{24,171–173} Furthermore, host factors that are involved in IAV replication and pathogenesis are also being explored as targets to develop anti-IAV strategies.^{24,172} Finally, viral RNA is also being targeted by antisense and short-interfering RNAs to develop the alternative anti-IAV therapeutics; however, this approach would have its own challenges and it will be interesting to know whether and how IAV develops resistance to it. Once more than one class of anti-IAV drugs is available, a combination therapy involving different classes of drugs could be more effective and beneficial in reducing the emergence of antiviral resistance in IAV.

Surveillance

IAV transmission in humans via aerosol and intercontinental spread by migratory birds makes surveillance a crucial player in the global management of IAV.^{174,175} The WHO Global Influenza Surveillance and Response System (GISRS) through its collaborating centers and reference laboratories in 113 member states conducts influenza virus surveillance and provides recommendations regarding the laboratory diagnosis, vaccines, antiviral susceptibility, and risk assessment. In addition, GISRS also provides global alerts on the emergence of novel influenza viruses. Therefore, GISRS serves as a single and timely source on worldwide status and management of the influenza virus, including drug-resistant IAV. In addition, CDC (USA) also puts out timely updates and advisories on influenza virus.

Future research directions

The broad host range of IAV and interspecies transmission are critical factors for its continuous circulation and evolution in nature (Figure 1).¹⁷⁵ The intermediate hosts such as pigs, birds, and horses play a crucial role in maintaining the IAV in nature and its transfer to humans (Figure 1). Therefore, in addition to continuous surveillance and developing a universal vaccine and effective antivirals, an effective management of such hosts to restrict the circulation and generation of new and more virulent IAV variants is needed. Eradication of IAV from its zoonotic hosts using existing knowledge and approaches is practically impossible. There

is a need to gain molecular and genetic understanding of why some animals (e.g., sheep and rabbits) are resistant to IAV infection and some (e.g., ducks) are resistant to IAV disease and identify the genes that confer such resistance. Then, by inserting those genes or using gene-editing methods (e.g., RNA interference and CRISPR-Cas), the intermediate hosts could potentially be made resistant to IAV infection. This will reduce and potentially eliminate IAV from the intermediate hosts and consequently its maintenance in nature and transfer to humans. Initial proof-of-concept studies in this direction have already begun to restrict IAV transmission or replication in transgenic animals.^{176–179}

The current understanding of IAV biology in a host is mainly acquired using cell culture or animal models, because it is not possible to experimentally infect humans. Due to lack of the insight into IAV infection dynamics in human respiratory tract, many antiviral drugs fail to advance beyond experimental and clinical trial stage. Therefore, large animals such as pigs may be engineered to exhibit respiratory tract physiology akin to humans to make advances in this area. Finally, the relationship between the microbiota of healthy and at-risk humans and IAV pathogenesis needs to be understood to develop alternative treatments such as probiotics to treat IAV infections.

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Disclosure

The authors report no conflicts of interest in this work.

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