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The convergent evolution of influenza A virus: Implications, therapeutic strategies and what we need to know

Zheng Yao Low [a], Ka Heng Wong [a], Ashley Jia Wen Yip [a], Wee Sim Choo [a,*]

[a] School of Science, Monash University Malaysia, 47500 Subang Jaya, Selangor, Malaysia

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ABSTRACT

Influenza virus infection, more commonly known as the 'cold flu', is an etiological agent that gives rise to recurrent annual flu and many pandemics. Dated back to the 1918- Spanish Flu, the influenza infection has caused the loss of many human lives and significantly impacted the economy and daily lives. Influenza virus can be classified into four different genera: influenza A-D, with the former two, influenza A and B, relevant to humans. The capacity of antigenic drift and shift in Influenza A has given rise to many novel variants, rendering vaccines and antiviral therapies useless. In light of the emergence of a novel betacoronavirus, the SARS-CoV-2, unravelling the underpinning mechanisms that support the recurrent influenza epidemics and pandemics is essential. Given the symptom similarities between influenza and covid infection, it is crucial to reiterate what we know about the influenza infection. This review aims to describe the origin and evolution of influenza infection. Apart from that, the risk factors entail the implication of co-infections, especially regarding the COVID-19 pandemic is further discussed. In addition, antiviral strategies, including the potential of drug repositioning, are discussed in this context. The diagnostic approach is also critically discussed in an effort to understand better and prepare for upcoming variants and potential influenza pandemics in the future. Lastly, this review encapsulates the challenges in curbing the influenza spread and provides insights for future directions in influenza management.

1. Introduction

The influenza virus is an etiological agent known as the cause of acute respiratory disease in mammals and numerous other domestic poultry. Despite its first discovery in 1918, the influenza virus progression shows no signs of slowing down, with multiple pandemics and seasonal endemics pestering the everyday life of many. Accounting for 290,000–650,000 deaths annually, the conquest and demand for effective influenza antivirals and vaccines must be addressed (WHO, 2023). Depending on the severity of the infection, the symptoms of influenza infection range from fever, cough, headaches, fatigue, sore throat, runny nose, and body aches to name a few (CDC US, 2022). Influenza viruses are RNA viruses belonging to the family of Orthomyxoviridae, subdivided into four different types: A, B, C and D (Javanian et al., 2021). The type A and B influenza viruses are more related to human infections, with the former contributing to multiple pandemics over a few decades, thus making influenza A virus (IAV) the focus of this review. Generally, the rapid evolution of influenza A virus infection lies within its capacity of HA and NA reassortments, wrong nucleotide integrations accompanied with extension mutations that adapt towards human host receptors, giving various combinations from the 18 different HA subtypes and 11 different NA subtypes, e.g., H1N1, H3N2, to name a few (Kosik and Yewdell, 2019). Due to these catastrophic events, new variants have contributed to immune evasion and multiple antiviral resistance.

There are four recorded instances of influenza pandemics in the 20th and 21st centuries, namely the 1918 Spanish Flu (A/H1N1), 1957 Asian Influenza (A/H2N2), 1968 Hong Kong Influenza (A/1968), and the 2009 pandemic (A/H1N1pdm09) (Kilbourne, 2006). The first known influenza pandemic, the Spanish flu, had a notoriously high mortality rate with a case fatality rate (CFR) greater than 2.5%, accompanied by 50–100 million deaths, recognised as an exceptionally deadly virus (Taubenberger and Morens, 2006). The prevalence of secondary bacterial pneumonia caused by *Haemophilus influenzae, Staphylococcus aureus*, and *Streptococcus sp*. (*S. pneumoniae, S. pyogenes*) in influenza-infected individuals was later discovered to attribute to the greater severity of the disease (Piret and Boivin, 2021; Morens et al., 2008). Moving on to the Asian flu, the virus affects individuals of school age and adults aged

* Corresponding author.
E-mail address: choo.wee.sim@monash.edu (W.S. Choo).

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between 30 and 45 years old more severely, with surprisingly lower infection rates on other age spectrums (Piret and Boivin, 2021). It has a comparatively lower CFR (~0.13%) and deaths recorded (~1 million) (Piret and Boivin, 2021). The impact of the 1968 Hong Kong influenza, on the other hand, was the mildest amongst the aforementioned pandemics due to the pre-existing immunity within the community, affecting mainly older and immunocompromised individuals with recorded deaths of approximately 0.5–2 million (Piret and Boivin, 2021). The 2009 influenza pandemic, termed the Swine Flu, originated from swine and has a CFR of ~0.5%. Notably, older individuals have a lower risk of infection due to pre-existing immunity from prior exposure to the influenza A/H1N1 virus, which has then been replaced entirely by the A/H1N1pdm09 strain (Piret and Boivin, 2021). The aetiology for such events is believed to be zoonotic spillover events, widely referred to as reassortment events in influenza virus between human/mammalian adapted, avian adapted species (1918 Spanish, 1957 Asian and 1968 Hong Kong influenza) and swine adapted (2009 Swine flu) influenza strains (Kessler et al., 2021). In accord with those mentioned above, the rapid progression of the influenza A virus poses a significant concern for epidemiologists and public health officials, especially the increasing possessions of livestock that may lead to an appalling global outbreak over time (Kessler et al., 2021).

The emergence of influenza pandemics mentioned above lies within its capacity for antigenic changes. There are a few known factors for antigenic changes. As such, the high rate of wrong nucleotide integration during viral replication resulting an antigenic drift and RNA reassortment of two or more influenza strains via co-infections that confers an antigenic shift, to name a few. In accordance with those mentioned above, the review aims to investigate and study the evolution, risk factors, diagnostic, antiviral and vaccination strategies to further contain the spread of influenza infection, especially amid the global COVID-19 outbreak.

2. Variants and evolution of influenza virus

Influenza virus infection is a notable scenario that poses an imminent hazard to global health security. Commonly well known as the 'flu', it has disrupted our daily lives, leaving significant impressions until today. The etiological agents that contributed to this disastrous episode are the influenza A and influenza B viruses, where the former gave rise to multiple pandemics accompanied by fatal outcomes. The influenza virus is an enveloped, negative-sense single-stranded RNA virus. There are generally four different types of influenza virus, namely the influenza A (genus *Alphainfluenzavirus*), B (genus *Betainfluenzavirus*), C (genus *Gammainfluenzavirus*), and D (genus *Deltainfluenzavirus*), with typically the former two causing human Influenza (Wong and Lal, 2023). There are eight different RNA segments in the influenza virus which capable of encoding up to 12 different proteins, namely the PB2, PB1, PA, HA, NP, NA, M1 & M2, and NS1 & NS2 proteins (Tripathi et al., 2015). Amongst those mentioned above, the HA and NA partake in virus infection, release, and the emergence of new variants that cause recurrent infections and immune evasions (Eisfeld et al., 2015). The rapid evolution of the influenza virus is attributed to the lack of proofreading mechanisms in the RNA-dependent RNA polymerase (RdRp), a critical viral RNA replicative enzyme, followed by the gradual accumulation of mutations and reassortment in progenies. Antigenic drift and shift explain the abovementioned phenomenon, with the latter asserting on novel strain associated with random reassortment of co-infecting influenza strains, which may also contribute to altered host preference. These concomitantly give rise to numerous variants that hamper the acquired immunity by means of previous infections or vaccinations.

To date, there is a library of reported variants that cause infections in humans, with more to come. There are constituted from 18 different hemagglutinin subtypes and 11 different neuraminidase subtypes (CDC, 2022). For instance, the various combinations of HA and NA proteins can give rise to the 'A(H5N1)' virus that carries an HA5 protein and an NA1 protein. Generally, the naming of influenza A virus (IAV) is based on the HA and NA protein as described above, and sometimes in accordance to host specificity and pathogenicity [(low pathogenic avian influenza (LPAI); highly pathogenic avian influenza (HPAI)) as IAV is known to infect numerous hosts such as human, bird, equine, swine, canine, and bat. Influenza B virus, on the other hand, follows the designated lineage, namely the Yamagata and Victoria (e.g., B/Yamagata) (CDC, 2022). Of which, the known subtypes that have been confirmed to be responsible for the disastrous human pandemic are the A/H1N1 Spanish flu 1918, A/H1N1 Russian flu 1977, A/H1N1pdm09 Swine flu 2009, A/H2N2 Asian flu 1957, A/H3N2 Hong Kong flu 1968, A/H5N1 global, A/H7N9 China, and the A/H1N1/H3N2 & B/Yamagata/Victoria typical seasonal flu that occurs every year.

As mentioned above, the recombination and mutation of HA and NA have contributed to more than 130 known variants (CDC, 2022). In addition to that, altered host preference was reported in numerous incidents. To recap, the host specificity of the HA for avian IAV is the α-2,3 SA receptors, which differ from the α-2,6 SA receptors predominantly found in humans, making it non-infectious to humans. However, the HPAI A(H5N1) avian influenza has been reported to transmit between mammals, making human cross-infection plausible (Shao et al., 2017). Recently, there have been 240 reported cases of human infection with HPAI A(H5N1) across four countries, Cambodia, China, Lao PDR, and Vietnam. Of these, 135 people died, scoring an astonishing 56% case fatality rate (CFR) (WHO, 2023). To date, there have been 457 deaths since the discovery of human infection with avian HPAI A(H5N1) in January 2003. Numerous genetic studies have been conducted to unravel the evolutionary dynamics within the HPAI A(H5N1) variants to characterise the abovementioned further. The N220K substitution in HA and E627K substitution in PB2 are among the notable cause for the change in receptor preference from avian α2,3-linked to human α2, 6-linked SA in HPAI A(H5N1) (Pawestri et al., 2020; Imai et al., 2018). Interestingly, another study has also introduced other PB2 substitutions via site-directed mutagenesis. As such, the Q73E, Q74R, L183S, and Q507R mutations can achieve >10-fold increases in polymerase activity than the wild type, coming close to E627K, suggesting that host adaptation is highly dynamic, especially during human H5N1 infection (Welkers et al., 2019).

Other avian strains, such as the H7N9, have also been reported to adapt to human host receptors. There have been 1568 laboratory-confirmed cases of human infection with the A(H7N9) virus accompanied by 616 fatal cases, scoring a 39% CFR since 2013 (WHO, 2023). Of the reported cases, 33 patients from China have been associated with haemagglutinin gene mutations in poultry (HPAI A(H7N9)) (WHO, 2023). Studies have pointed towards G186V, Q226L, K581, and G219S mutations, to name a few, have conferred enhanced adaptation towards human host α-2,6 SA receptors, atop of the usual α-2,3 SA receptors (Schrauwen et al., 2016; Xiong et al., 2013). In particular, the G228S mutation in H7N9 HA has been reported to bind to human tracheal tissue extensively (Bisset and Hoyne, 2020). Similar to H5N1, the E627K and D701N mutations in PB2 have also been evidently contributing to an enhanced mammalian adaptation accompanied by increased viral polymerase activity (Mok et al., 2014; Yamayoshi et al., 2015; Chan et al., 2016). In particular, a study has reported on the alteration of amino acid sequences upon viral-cell serial passages in human airway epithelial cells. There were six changes in the A(H7N9) sequences (R54G, T160A and Q226L in HA; K289/292R in NA; V363V/I for NP, and L/R332R for PB2) that occurred during the 35 serial passages. Of which, the HA mentioned above mutations would increase affinity for α2,6-linked receptors in humans (53.4% binding affinity compared to 39.2% in the first passage), suggesting the capacity of the virus to gradually adapt to the environment and host changes, thereby conferring enhanced viral replication (Huang et al., 2017).

On the other hand, the occurrence of avian H3N8, H7N4, H9N2, and H10N3 infections in humans is relatively low, with no circulating cases (WHO, 2023). Moving into the recurrent influenza infections, the

influenza A/H1N1(pdm09)/H3N2 & B/Yamagata/Victoria, commonly known as the seasonal cold 'flu', occurs every year. The current H1N1 virus is related to the pandemic H1N1 2009 A/H1N1(pdm09). H3N2, on the other hand, originated from the 1968 Hong Kong pandemic and has since evolved and been circulating until today (Neumann and Kawaoka, 2019). Notably, H3N2 is less prevalent in South-East-Asia despite being subjected to more frequent mutation and has surpassed H1N1 in seasonal flu occurrence (Rahman et al., 2022). Similar to the significant pandemics mentioned above, the circulating variants result from amino acid mutations in HA and NA, also known as 'antigenic drift ', contributing to immune evasion. HA mutations are more profound around the receptor-binding site, whereas NA mutations more commonly reside around the active enzymatic center (Yamayoshi and Kawaoka, 2019). For instance, the H3N2 has evolved over 35 years via single amino acid substitutions adjacent to the RBS at seven positions (145, 155, 156, 158, 159, 189, and 193) (Yamayoshi and Kawaoka, 2019).

3. Risk factors and outcomes

Influenza is generally self-limiting within healthy individuals and is typically presented as fever, headache, dry cough, sore throat, myalgia and coryza (Tanner et al., 2021; Boktor and Hafner, 2022). Despite so, influenza infection may lead to severe complications and death in high-risk groups (Boktor and Hafner, 2022). Influenza infection aggravates underlying lung diseases, prompting pneumonia development and extrapulmonary symptoms involving gastrointestinal and neurological systems. Reports have shown that young children (less than 5 years old), older adults (aged > 65 years), pregnant women, and individuals with existing comorbidities, to name a few are at higher risk of morbidity and mortality (Tanner et al., 2021). Notably, the associated risk has increased drastically due to the expanding ageing society and rising disease of affluence (Becker et al., 2021). It is no stranger that vaccines have been the most effective mode to curb transmissions, preventing severe influenza. To no avail, the individuals above are more likely to experience vaccination failure, posing a greater risk of disease severity (Becker et al., 2021; Tanner et al., 2021). Here, we aim to discuss and elucidate Influenza infection's plausible risks and outcomes.

3.1. Age

Ageing has been a well-characterised risk for many diseases; influenza is no exception. Young children and individuals of older age are most likely to experience severe complications due to influenza infection, raising the fatality risk (Martínez et al., 2019; Yang et al., 2020). Accounting for one-fifth of the influenza-associated deaths worldwide (> 120,000), immunosenescence explains the relationship between increased age and morbidity, especially in individuals aged > 75 years old (Tanner et al., 2021). As the term immunosenescence suggests, the weakened immune response and function in older adults contribute to the development of infections and poor vaccine response (Allen et al., 2020; Lian et al., 2020). Although the associated mechanisms are not well elucidated, the compromised immune system in aged individuals may be related to both humoral and adaptive responses atop of any pre-existing comorbidities (Hernandez-Vargas et al., 2014). Of which, "Inflammageing", also known as low-level chronic inflammation that elevates basal levels of various inflammatory cytokines, has been commonly seen in older people, predisposing affected individuals to chronic disease, frailness, disabilities and premature death (Hernandez-Vargas et al., 2014; Ferrucci and Fabbri, 2018). Additionally, the impaired antibody production, accompanied by altered maturity and proportions of memory and naïve B cells and T cells, changes the quality of immune response in older individuals (Howard et al., 2006; Alam et al., 2013). These put the elderly at greater risk of disease complications and death from a seemingly harmless influenza infection (Quandelacy et al., 2014; Van Kerkhove et al., 2015; Hauge et al., 2020).

Young children (< 5 years) are more likely to be hospitalised from seasonal influenza, especially given the low vaccine prevalence in newborns. Notably, children aged 6 months to 2 years are at a higher risk of developing severe symptomatic influenza (Lynfield et al., 2014; Hardelid et al., 2018; Hauge et al., 2020). A study recorded an astounding 26% of pediatric intensive care unit (PICU) admissions for children under 6 months from 2003 to 2015 in the UK (Hardelid et al., 2018). It was estimated that 0.41 deaths occur annually for every 100,000 children infected with influenza (Quandelacy et al., 2014). Another study in Shanghai, China, found that 58.5% of influenza patients were male children aged 1 month – 8 years, with relatively low vaccination rates at 13.6% (Shi et al., 2021). Of these, 12.7% of the children died despite receiving supporting treatment such as mechanical ventilation, continuous renal replacement therapy, immunoglobulin, corticosteroid therapy, and antiviral treatment, to name a few (Shi et al., 2021). The common causes of influenza-associated hospitalisation and death in children include reduced haemoglobin levels, elevated alanine aminotransferase, high urea nitrogen, high lactate levels and influenza-associated neurological encephalitis/encephalopathy (IAE) (Liu et al., 2014; Seki et al., 2019; Shi et al., 2021). Comorbidities in children have also been reported to contribute to poorer disease outcomes (Mastrolia et al., 2019; Robinson and Saux, 2020).

For instance, IAE has significantly contributed to influenza morbidity and mortality, especially in East Asian ethnicities, where 47.5% of hospitalised patients were Asian patients aged 16 and below in the UK (Mastrolia et al., 2019; Seki et al., 2019; Shi et al., 2021; Hardelid et al., 2018). A study in Italy over two influenza seasons (2017 – 2019) also found that 13.1% of the 114 cases of influenza in patients aged 1 month – 14 years exhibited neurological manifestations (Hardelid et al., 2018). Akin to the abovementioned, a study conducted in Bangkok documented multiple neurological complications (seizures and acute encephalopathy) in 16.9% of the 73.1% of influenza-hospitalised children (Jantarabenjakul et al., 2022). Similar occasions were reported in the US and Australia, affecting approximately 4 per 100,000 children and 28 per 1000,000 children annually, respectively (Mastrolia et al., 2019).

Vaccination is undoubtedly the best way to prevent infection and poor influenza outcomes. Concerning the adverse effects, children, especially those aged below 6 months, are not entitled to vaccination and have been cited as a significant casualty of influenza infection (Hardelid et al., 2018; Mastrolia et al., 2019). Transplacental transfer of maternal antibodies acquired via vaccines has been shown to confer immune protection against severe influenza for infants with reduced hospitalisations rates by 91.5% (Benowitz et al., 2010; Steinhoff et al., 2010; Lindley, 2019). Thus, vaccinations for the elderly, young children and pregnant women are highly encouraged.

3.2. Underlying health

Comorbidities put the affected individual at a greater risk for severe clinical outcomes and death. Adults are more likely to suffer from influenza-associated complications when they have existing comorbidities, such as chronic renal disease, underlying respiratory system disease, chronic lower respiratory disease, myocardial infarction, renal disease, cancer, cardiovascular disease, to name a few (Quandelacy et al., 2014; Van Kerkhove et al., 2015; Hauge et al., 2020). Among these, neurological issues (such as epilepsy), respiratory diseases (asthma) and immunocompromised states also affect children (Hardelid et al., 2018; Hauge et al., 2020). It seems that certain comorbidities are more significant risk factors than others. Cardiovascular disease (CVD) and chronic renal disease seem prevalent in people who perished from influenza-associated complications (Quandelacy et al., 2014; Van Kerkhove et al., 2015). A study conducted between 2000 and 2012 on 11,086 US military personnel with renal, CVD (hypertension, coronary heart disease) and liver disease was revealed to be significantly associated with developing severe influenza that requires hospitalisations (Van Kerkhove et al., 2015). In addition, CVD and cerebrovascular

patients were among the most commonly found comorbidities in patients with nosocomial influenza (Yang et al., 2020). While the exact mechanisms are yet to be unravelled, several studies support kidney disease as a risk factor for influenza infection (Van Kerkhove et al., 2015; Álvarez-Lerma et al., 2017; Martínez et al., 2019; Yang et al., 2020). It was postulated that the compromised immune functions, such as reduced chemotaxis and phagocytosis of monocyte or macrophages, B-cell lymphopenia, and decreased $CD4^+$ and $CD8^+$ T-cell responses contributed to the increased morbidity and mortality in infected renal disease patients (Yang et al., 2020).

Obesity (BMI 30 or higher) may also lead to non-favourable outcomes, with studies revealing increased morbidity in influenza A subtype H1N1pdm09 infection (Fezeu et al., 2011; Kwong et al., 2011; Gómez-Gómez et al., 2014). This may be explained by impaired immune functions (reduced cytokine production, antigen/mitogen stimulation, macrophage and dendritic function, natural killer cell impairment) and metabolic homeostasis in obese individuals (Karlsson and Beck, 2010; Gutiérrez-Pizarraya et al., 2012; Zhao et al., 2020). However, the relationship between obesity and influenza mortality rates may be confounded by early antiviral treatment and therefore, discretion on the interpretation is advised (Lynfield et al., 2014; Braun et al., 2015; Sun et al., 2016; Álvarez-Lerma et al., 2017).

3.3. Compromised immunity

Immunocompromised individuals, such as individuals with human immunodeficiency virus (HIV) infection, cancers (chronic lymphoproliferative disorders, multiple myeloma), and those receiving antineoplastic therapy are vulnerable to severe influenza outcomes and death (Safdar and Cox, 2007; Lynfield et al., 2014; Shehata and Karim, 2014; Martínez et al., 2019). In particular, HIV patients were found to be more susceptible to acute lower respiratory tract infection from influenza, up to 4 to 8 times higher than normal individuals (Cohen et al., 2013). Vaccine response is not always ideal; cancer patients undergoing chemotherapy must be administered at a specific time window to ensure proper vaccine response, especially those with a disrupted immune system (Meerveld-Eggink et al., 2011; Shehata and Karim, 2014). Despite that, vaccination has been proven safe and immunogenic in HIV patients (Madhi et al., 2011, 2013; Nunes et al., 2020). Thus, to further protect the compromised community from severe influenza outcomes, family members and caretakers are also advised to be vaccinated (Shehata and Karim, 2014).

3.4. Pregnancy

Pregnancy has been associated with a higher risk of influenza-related pneumonia and death, especially during the 2nd and 3rd trimesters (Koul et al., 2016; Gunnes et al., 2020). It was reported that four of the five hospitalised pregnant women who developed respiratory failure were in the 3rd trimester, and three out of five of them deceased in a study conducted in India (Koul et al., 2016). The exact mechanisms for such occurrences are not well understood. It was postulated that a combination of physiological changes that occurs to accommodate the foetus, including the respiratory, hormone, cardiovascular and immune systems, disrupts the proper response to infection. Influenza has also been reported to increase the risk of perinatal death (Gunnes et al., 2020). As mentioned above, the transplacental transfer of maternal antibodies provides passive immunity for the child and reduces the risk of infection for the mother (Benowitz et al., 2010; Steinhoff et al., 2010; Lindley, 2019). Thus, the importance of vaccination cannot be further stressed to protect the mother and the young infant.

3.5. Medication

The use of acetylsalicylic acid, commonly referred to as aspirin, in individuals younger than 19 years or under long-term aspirin treatment increases the risk of developing rare but fatal Reye's syndrome, which inadvertently increases the risk of influenza-associated death (McGovern, 2001; Noor and Gradidge, 2018). Reye's syndrome is a non-inflammatory encephalopathy with presentations of convulsions, vomiting, disturbed consciousness, fever, disturbed respiratory rhythm, altered muscle tone and altered reflexes (Noor and Gradidge, 2018). Several case reports cited that aspirin intake in children during the viral prodrome phase cause increased death in influenza infection (McGovern, 2001; Noor and Gradidge, 2018). The use of corticosteroids may enhance the viral replication of influenza (Tsai et al., 2020; Yang et al., 2020). Corticosteroid is commonly used in treating severe influenza and acute respiratory distress syndrome (ARDS) (Yang et al., 2020; Tsai et al., 2020). Higher dosage and early corticosteroid treatment were believed to be correlated to higher mortality rates in hospitalised influenza patients (Ni et al., 2019; Tsai et al., 2020). Similarly, several studies have reported that prolonged hospitalisation, increased duration of virus shedding, mechanical ventilation, increased rate of secondary infections and mortality in patients receiving corticosteroid treatment (Cao et al., 2016; Ni et al., 2019; Tsai et al., 2020).

3.6. Environmental conditions

Healthcare workers are at a greater risk of influenza infection and developing severe complications (Safdar and Cox, 2007; Godoy et al., 2020). Nosocomial influenza infection poses a huge threat to public health and is associated with longer hospitalisation accompanied by a greater significance of morbidity and mortality (Yang et al., 2020: Godoy et al., 2020). Generally, individuals aged 65 and above, with chronic cardiovascular, renal and liver diseases, and immunodeficient individuals are more susceptible to hospital-acquired infections (Yang et al., 2020; Godoy et al., 2020). A study conducted in Spain showed that about 5.6% of subjects ($n = 412$) admitted into the hospital for reasons other than acute respiratory diseases had developed influenza-like illness within 48 h upon admission (Godoy et al., 2020). Another study in Spain reported 9.3% of hospitalised patients ($n = 2421$) acquired nosocomial infections within the same admission timeframe (Álvarez-Lerma et al., 2017). Studies from the UK and Australia have also documented nosocomial cases at ~2.0% ($n = 152$) and 4.3% ($n = 598$) in hospitalised patients, respectively (Enstone et al., 2011; Macesic et al., 2013). Notably, a study in China found that 22.6% of hospitalised patients ($n = 412$) during the 2018–2019 influenza season were hospital-acquired, much higher compared to other countries. The low vaccination rates in the overall Chinese population (~0.8% – 2.2%) between 2004 and 2014 may explain the above (Yang et al., 2020). On the other hand, it was found that H1N1pdm09 strains acquired from hospital environments are more likely to be fatal in critically ill patients since the manifestations are more severe, requiring a greater need for mechanical ventilation and the administration of vasoactive drugs (Álvarez-Lerma et al., 2017). Poor hospital infection control, entailing cross-contamination between healthcare workers and infected healthcare workers, could contribute to nosocomial infection in patients (Safdar and Cox, 2007; Godoy et al., 2020; Yang et al., 2020). Additionally, patient-to-patient and visitor-to-patient transmission cannot be ruled out, with infected individuals in constant proximity to non-infected individuals (Godoy et al., 2020; Yang et al., 2020).

3.7. Pandemic co-infections

COVID-19, also referred to as SARS-CoV-2 infection; its etiological agent contributed to the current pandemic, is a betacoronavirus, akin to the SARS-CoV infection that occurred in the past decade (Yip et al., 2022). The manifestation of COVID-19 is similar to the common cold, or influenza, in the upper respiratory tract. With the death toll surpassing the 6900,000 mark and the 690,000,000 infected individuals, the presence of influenza-SARS-CoV-2 co-infection pose a critical company, especially in elderly, children, pregnant women, and

immunocompromised individual, given the similarity in symptoms (fever, cough, shortness of breath, fatigue, sore throat, runny nose, muscle aches, headache, vomiting, diarrhoea, to name a few) and conflicting findings (Khorramdelazad et al., 2021). In addition to the pandemic, there has been an increase in respiratory syncytial virus (RSV) spread recently, the common viral infection in the elderly, newborns, and immunocompromised individuals during the winter (Ambrosch et al., 2023). Akin to the SARS-CoV-2 and influenza, RSV is also characterised by cough, wheezing, and an increased respiratory effort, to name a few. Apart from the similar symptoms, the current absence of approved antiviral and vaccines for RSV poses a great challenge and concern to many as the potential 'tripledemic' (SARS-CoV-2, IAV and RSV) might further aggravate the already critical situation (MacMillan, 2023; Xing and Proesmans, 2019).

Acknowledging the above, several co-infection cases have been reported in several countries. These were China, the USA, Brazil, Switzerland, Italy, and Iran, to name a few (Dadashi et al., 2021). In the collective study above, 76 SARS-CoV-2 influenza co-infections in the 3070 participants scored a 0.8% prevalence (Dadashi et al., 2021). Despite its low prevalence, the impact of co-infection on high-risk groups (elderly, pregnant women, immunocompromised individuals, and children) cannot be understated. An investigation by the CDC stated that 6% of paediatric hospitalisation (32 of 575) had SARS-CoV-2 influenza co-infection, and 16% of deaths were paediatric influenza-SARS-CoV-2 during the 2021–2022 seasonal influenza (Adams et al., 2022). In which, most of them have received invasive mechanical ventilation compared to non-co-infected individuals (13% vs 4%) (Adams et al., 2022). In another study 20.7% (24/116) of patients were found to be infected with SARS-CoV-2 and other respiratory viruses (Kim et al., 2020). Of these, they are the rhinovirus/enterovirus (6.9%), respiratory syncytial virus (5.2%), influenza virus (0.9%), parainfluenza (2.7%) etc. (Kim et al., 2020). Apart from that, a study reported 0.31% SARS-CoV-2 RSV co-infections in New York City during March-April 2020 (Nowak et al., 2020).

While co-infection prevalence might not be significant in the overall picture, the increasing trend for plausible co-infections between the SARS-CoV-2, Influenza viruses and RSV should not be overlooked. Thus, a call for further monitoring and a long-standing investigation to better prepare for the unexpected and avoid underplaying the severity of disease progression. As a summary, individuals above 65 and below 5 years old, especially those with existing comorbidities, are the most vulnerable to severe complications of influenza infection and thus are highly recommended for vaccination. Individuals with altered physiological states such as pregnancy and immunodeficiency, healthcare settings, certain medications, and co-infection of one or more other viruses can also place the influenza-infected individual with a worse disease outcome. Hence, the identification of these risk factors can assist clinical decisions and the management of disease control.

4. Antiviral strategies for influenza A virus

4.1. Current antiviral approach for influenza A virus

The approach for managing influenza infection comes down to antiviral drugs and vaccination. However, the constant mutations in influenza that confer host immune evasions and drug resistance pose a significant challenge in developing a new antiviral approach. There are currently three approved drug types that inhibit the life cycle of the influenza virus, namely the M2 proton channel antagonists (amantadine, rimantadine), neuraminidase inhibitors - NAI (Peramivir, Zanamivir, Oseltamivir and Laninamivir), and polymerase acidic- PA endonuclease inhibitor (Baloxavir Marboxil), with the latter, emerged as a new class of antiviral over the years (Świerczyńska et al., 2022). In addition, other alternatives like the RNA-based therapeutics and drug repositioning to mitigate the challenges of IAV are discussed.

4.1.1. Amantadine- M2 ion inhibitor

Amantadine (1-amino adamantane hydrochloride) is the first antiviral drug to treat influenza infection. It was approved in 1976 for prophylaxis use upon discovering its antiviral properties in 1963 by Grunert et al. (1965). The study covers 735 college students pre-treated with 100 mg of amantadine 18 h before the introduction of attenuated live influenza virus was found to reduce serological infection by up to 50% (Hubsher et al., 2012). Upon gaining full approval from the FDA in 1973, the use of Amantadine has since been extended to levodopa-induced Dyskinesia, multiple sclerosis and traumatic brain injury (Hauser et al., 2019; Loggini et al., 2020; Rejdak and Grieb, 2020). The mode of the mechanism of amantadine lies within its ability to target and binds to the N-terminal pore of the transmembrane domain, specifically on the His-37 residue proton sensor of the influenza A M2 ion channel protein via its hydrophilic amine, disrupting the protonation equilibrium, causing the proton gate Trp41 to remain close as it would at high pH (Mtambo et al., 2021). To recap, the M2 ion channel is a vital transmembrane protein that forms small protein channels that allow the pass-through of protons to acidify viral content for viral RNA release in infected cells (Pielak and Chou, 2011). Thus, blocking the M2 channel inhibits viral replication upon viral attachment. However, the rapid mutation of IAV strains has rendered resistance towards amantadine and its similar derivative, rimantadine (1-methyl-1-adamantane methylamine hydrochloride). Studies have shown amino acid changes in the M2 protein to be the culprit for this event. For instance, the L26F, V27A, A30V/T, S31N, G34E, and L38F amino acids substitutions, found in H1, H3, H5, H9 and H9 subtypes of influenza A virus have conferred enhanced viral fitness, accounting for an astonishing 100% amantadine resistance for certain countries (South Korea, Taiwan, Japan, Hong Kong, China, and Canada) during the 2005–2006 influenza season (Deyde et al., 2007; Hayden et al., 1989). Of which, the S31N mutation is the most prevalent mutant in today's circulating variants entailing both H1N1 and H3N2 (up to 95%), thereby gaining the interest of many (Li et al., 2016). With the increasing cases of resistance and severe side effects, the emergence of NAIs and PAs aims to curb the aforementioned.

4.1.2. Oseltamivir- Neuraminidase inhibitors (NAIs)

Neuraminidase inhibitors (NAIs) are the currently most prescribed antivirals for influenza infection. The discovery of NAI was made by Edmond and colleagues in 1966 in an attempt to understand the structure and activity of what was known as the N-acetylneuraminic acid (Edmond et al., 1966). The synthetic compound 2-deoxy-2,3-dihydro--N-acetylneuraminic acid (Neu5Ac2en), also known as DANA, was found to inhibit the release of virus progeny (Laborda et al., 2016; Edmond et al., 1966). The IAV NA is a 60 kDa homotetrameric glycoprotein with a slender stalk region and an enzymatic active boxed-shaped head that is comprised of eight highly conserved catalytic residues that are responsible for the interaction with sialic acids (Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371, and Tyr406) (Mtambo et al., 2021; Colman et al., 1983). Upon infection, the IAV NA cleaves the α-(2–3 or 2–6)-ketosidic linkage between the terminal sialic acid and residue of Neu5Ac2en containing receptor on the host cell surface via four intermediatory steps, the binding incidence, the formation of endocyclic sialosyl cation intermediate, formation, and release of sialic acids (Palese et al., 1974; Mtambo et al., 2021). NA plays a profound role in immobilising new viral progeny from the host cells, aiding the subsequent host cell invasion and infection. As mentioned above, there are currently 4 NAIs marketed for IAV, namely the Peramivir, Zanamivir, Oseltamivir and Laninamivir. The route of administration of the NAIs differs where Oseltamivir is delivered orally, Zanamivir and Laninamivir are inhaled as a dry powder, and Peramivir is given intravenously (Zaraket and Saito, 2016). Today, Oseltamivir (Trademark name: Tamiflu) is the most commonly used NAIs for IAV (Farrukee and Hurt, 2017).

Oseltamivir was first discovered and patented by Bischofberger and

colleagues in 1995 with excellent influenza inhibitory activity and greater bioavailability than Zanamivir, the first-ever approved NAI for IAV infection (McKimm-Breschkin, 2013). The subsequent release of Laninamivir and Peramivir were derivatives from the Zanamivir with modifications on the C4-guanidino group and an additional 7-methoxy group, respectively. The mode of action of NAIs revolved in the binding of NA protein, preventing cleavage of the sialic acid of host cell surface receptors, thereby preventing the new viral progeny from budding off and infecting new cells (Mtambo et al., 2021). Until recently, there has been a rise in the number of NAIs resistance cases due to the rapid changes of NA proteins of the influenza virus; in particular, the H275Y mutation in NA was known to confer resistance to the Influenza A (H1N1)pdm09 (CDC USA, 2022). The incidence of Oseltamivir-resistant H1N1 viruses was reported at 95% worldwide during the 2008/2009 influenza season, but thankfully remained low incidence at current circulating H1N1 strains (O'Hanlon and Shaw, 2019). The past concerning incidence calls for an urgent demand for a new class of antivirals to overcome the aforementioned resistance. This brought us to baloxavir marboxil, a novel mechanism of action against the IAV after the two-decades-old NAIs (O'Hanlon and Shaw, 2019).

4.1.3. *Baloxavir Marboxil- polymerase acidic inhibitor (PAIs)*

Baloxavir Marboxil (BM), proprietary name Xofluza, is a single-dose oral prodrug cap-dependent endonuclease protein inhibitor made for both influenza A and B (Baker, 2019). The discovery of Baloxavir Marboxil was designed based on the Dolutegravir DTG molecule by Shionogi and Co., Japan, in 2016 (Dufrasne, 2021). It was the first new-in-class drug to be FDA-approved for influenza treatment after two decades of the approval of Peramivir in 2014. The mode of the mechanism of BM lies within the hydrolysis to its active form, baloxavir acid, in the intestinal epithelial cells, blood and liver by Acrylacetamine deacetylase (AADAC) (Shirley, 2020). The active baloxavir acid then targets and binds to the C-terminal end (Ala20, Tyr24, Lys34, Ala37, and IIe38) at one of the three polypeptides (PA, PB1, PB2) of RNA dependent RNA polymerase (RdRp) complex, namely the Polymerase Acidic (PA) protein (Dufrasne, 2021; Świerczyńska et al., 2022; O'Hanlon and Shaw, 2019). To recap, upon infection, the replication of the influenza viral RNA was done by the RdRp, which functions as an RNA polymerase that synthesises three different RNAs, the complementary RNA (cRNA) and messenger RNA (mRNA) from the viral RNA (vRNA) and back to cRNA, cap binding mechanism and endonuclease activities. In which, the PB1 subunit assembles the RdRp and catalyses the cRNA synthesis, the PB2 and PA subunits capture the host cell mRNA cap (cap-snatching) to initiate vRNA transcription (Dou et al., 2018; Miyake et al., 2017). Today, BM has been a preferred alternative medication for IAV with fruitful outcomes. As such, a study has concluded that BM has a much lower risk of complications and adverse events than Oseltamivir, accompanied by a significant decline in virus titres (Kuo et al., 2021).

4.2. *Genome-targeted antiviral approach for IAV*

Despite the discovery of new class drugs such as Baloxavir Marboxil, the rapid emergence of resistant IAV poses a serious concern to the already limited therapeutic options. This calls for an alternative approach to mitigate the rapid evolution of IAV, especially on the recurrent annual influenza infection (cold flu). The use of RNA-targeting technologies such as RNA interference (RNAi), antisense oligonucleotides (ASO), and clustered regularly interspaced short palindromic repeat (CRISPR) systems may provide insight into exterminating current influenza endemic.

4.2.1. *RNA interference (RNAi)*

RNA interference, or RNA silencing pathways, is a small RNA employed for sequence-specific mRNA degradation triggered by long double-stranded RNA (dsRNA) (Svoboda, 2020). RNAi was first discovered in the nematode *Caenorhabditis elegans* (Lee et al., 1993). It is first converted to a 21–23 nucleotide-long non-coding RNA fragment that exists in almost every eukaryotic organism that controls the expression of genetic information. RNAi can be triggered via various sources such as RNA viruses, transposons, exogenously introduced dsRNAs (siRNA), endogenous small non-coding miRNAs, etc. (Rana, 2007). The mode of action of RNAi depends on the trigger source. For instance, the siRNA long dsRNA molecules first work in tandem with the RNase type III enzyme, Dicer, which then cleaves it to the appropriate 21–23 nucleotide length with 2 nucleotides at their 3′ ends overhangs and 5′ ends phosphate group. The end product is then assembled and incorporated into the RNA-induced silencing (RISC) complex, which contains the ribonuclease Argonaute 2 (AGO2), Dicer and other vital cellular factors (Rana, 2007). The activated Argonaute2-RISC complex then forms a sequence-specific gene silencing sequence that binds and cleaves the target-mRNA sequence in accordance with the Watson–Crick base pairing with the help of an antisense guide strand. miRNAs, on the other hand, work in a similar fashion with additional transcription steps. The miRNA is transcribed by RNA polymerase II, giving a Pri-miRNA containing a 5′- cap structure and a polyA-tail. The Pri-miRNA then works in tandem with Drosha–DGCR8 (DiGeorge syndrome critical region gene-8) complex, an RNase type III enzyme, to produce an appropriate 70 nucleotide length pre-miRNA structure with 2 nucleotides at their 3′ end overhangs and 5′ ends phosphate group. The Pre-miRNAs then bind to exportin-5–RanGTP in the cytoplasm and cleave by the Dicer to give into 22 nucleotides long miRNA capable of binding to the AGO-RISC complex, akin to the siRNA. Differing from the siRNA, the miRNA RISC complex (miRISC) binds to the target mRNA, forms a bulge sequence, and accumulates in P-bodies, repressing translational machinery (Fig. 1).

It is evident that the mammalian host cell produces abundant virus-derived siRNAs, PIWI-interacting RNAs (piRNAs), and transfer RNAs (tRNAs) upon RNA virus infection in response to antiviral immunity; influenza infection is no exception (Takahashi et al., 2021; Ding et al., 2018). As mentioned above, the vital genes for IAV entail the PB2, PB1, PA, HA, NP, NA, M1 & M2, and NS1 & NS2 proteins. Of which, the HA and NA that conferred rapid antigen shift, drifts and immune evasions should be avoided as target studies. For instance, NS1 deletion in IAV has produced virus-derived siRNAs (vsiRNAs) in humans, whereas intact viruses do not (Li et al., 2016). Besides that, several studies have shown significant viral inhibitory effects upon silencing IAV NP at up to a staggering 56-fold reduction in virus titre (Tompkins et al., 2004; Ge et al., 2003). Other vital genes, such as the M2 mRNA of H1N1, have also been covered in other studies; in which the use of anti-M2 siRNA has yielded up to 54.7% reduction in matrix RNA accompanied by a statistically significant decrease in viral load (McMillen et al., 2016).

In accordance with the abovementioned, siRNA-directed RNAi therapy is a great exemplary idea for a promising alternative therapeutic approach for influenza infection. To date, the FDA has approved various types of RNAi-directed short-length RNAs, including nine antisense oligonucleotides (ASOs), one aptamer, four siRNAs, and one CpG oligonucleotide (Takahashi et al., 2021). In parallel with the recent success of mRNA vaccines for COVID-19, no doubt the future of small RNAs antiviral and vaccines for IAV infection remains a bright spot. With the high potency, short production hours and the advancement of polymer-carriers and liposomal formulations, the siRNA can be readily administered via non-invasive hand-held inhalers, conferring great advantage over the conventional means for antiviral therapy (Barik, 2010; Tompkins et al., 2004)

4.2.2. *Antisense oligonucleotides (ASO)*

Antisense oligonucleotides (ASO), as the name suggests, is a short single-stranded DNA oligonucleotides (12–25 nucleotides) designed to complement the mRNA targets in accordance with the Watson-Crick base pairing. The use of ASO for gene silencing or suppression is no stranger since the FDA approval of the Fomivirsen drug for cytomegalovirus retinitis in August 1998 (Henahan, 1998). Since then, several

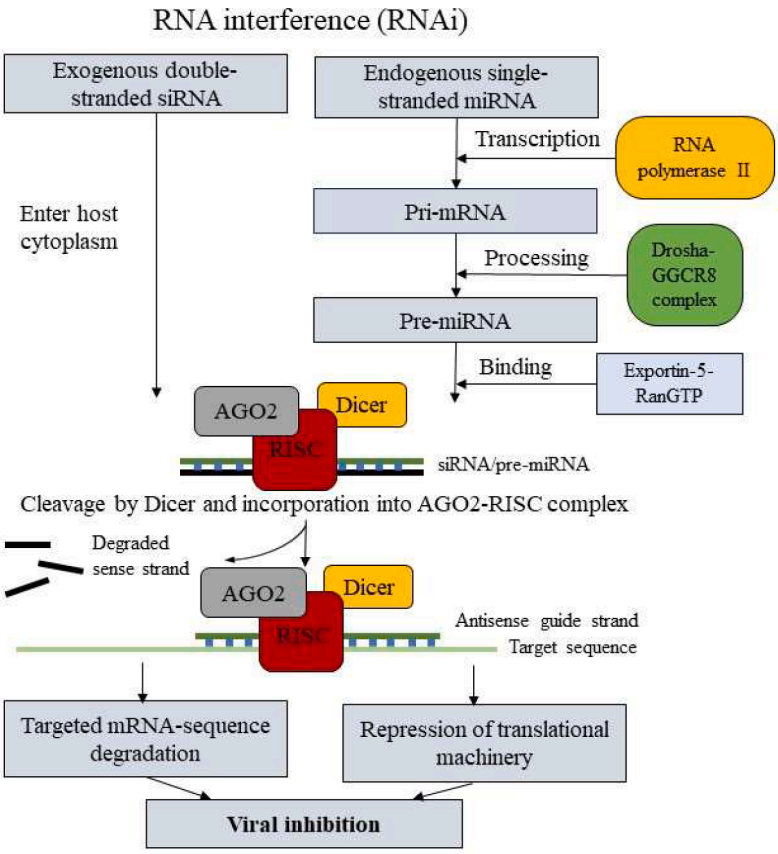

Fig. 1. The mode of the mechanism of RNA interference (RNAi) against IAV.

FDA approved ASO have been used to treat Batten disease, Duchenne Muscular dystrophy (Eteplirsen, Golodirsen), familial hypercholesterolemia (Mipomersen), hereditary transthyretin-mediated amyloidosis (Patisiran, Inotersen), and spinal muscular atrophy (Nusinersen) (Roberts et al., 2020). The use of ASO continues to gain interest from many due to its capability to be designed to target various mRNA, leading to the reduction of gene-mRNA translation. The downregulation of genes can be done via several modes, to name a few. As such, the binding of Gapmer ASOs to the flanking region of target mRNA forms a DNA-RNA heteroduplex which in turn recruits RNASEH1 for mRNA degradation. Steric block ASOs, on the other hand, can bind to the AUG region and disrupt the translational activity of the primary open reading frame of mRNA by introducing upstream open reading frames (uORFs) or blocking the attachment of ribosomes for translation (Roberts et al., 2020). Besides that, steric ASOs also bind to the pre-mRNA and modulate alternative splicing to promote exon inclusion/splicing, producing

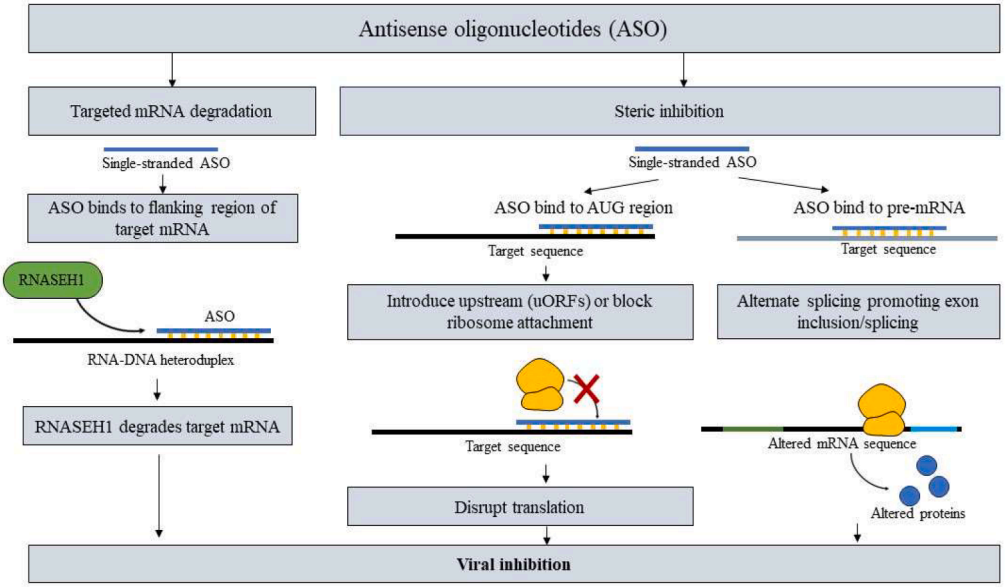

Fig. 2. An overview of antisense oligonucleotides (ASO) as an antiviral approach for IAV.

mRNA that produces altered protein production (Roberts et al., 2020) (shown in Fig. 2). In layman, the ASO is a small molecule used to prevent or alter the production of proteins, effectively bringing down harmful protein production in many of the diseases mentioned above. The knowledge of ASO may give us an insight into targeting viral RNAs, producing an efficacious and potent viral inhibitor.

To date, the use of ASOs for influenza infection studies involved targeting vRNA segments 5, 7, and 8. For instance, a study conducted by Michalak and colleagues targeting vRNA segment 5 (encoding for NP) has revealed a staggering 88% viral titre reduction and 55% vRNA reduction with ASO 883–11L (targeting region 878–888 nucleotide) (Michalak et al., 2019). A clinical study conducted by Beigel and colleagues has also illustrated effective and safe viral inhibition with Radavirsen (a peptide-conjugated phosphorodiamidate morpholino oligomer) that binds to the M1 and M2 viral mRNA sequence, physically blocks the mRNA translation as well as the processing of pre-mRNA via splicing prevention of M1 and M2 transcripts (Beigel et al., 2018). The study has shown an acceptable dose accumulation of 57.9 μg*h ml^{-1} AUC_{24} (area under the concentration-time curve at time 0–24) (a metric to determine the concentration of a drug in blood plasma as a function of time) in multiple dosing studies with 66 subjects receiving 8 mg kg^{-1} single dose or multi-dose daily for 5 days or placebo intravenously (Beigel et al., 2018). In parallel with the increasing antiviral drug resistance for IAV and the positive findings for ASOs, it opens a new window for influenza eradication.

4.2.3. *Clustered regularly interspaced short palindromic repeat (CRISPR)*

The term clustered regularly interspaced short palindromic repeat, or CRISPR, has emerged in view of the recent SARS-CoV-2 pandemic. The usage of the CRISPR-Cas system has been extensive and practical, ranging from a promising diagnostic tool to a potential antiviral therapeutic approach. For instance, the EUA-approved CRISPR-Cas 12a (DETECTR) and CRISPR-Cas 13a (SHERLOCK) employ a guide RNA (gRNA) to recognise a specific sequence of the N gene or E gene in SARS-CoV-2, providing a simple, efficient, and fast detection of SARS-CoV-2 under an hour without the need for expensive reagents and equipment (Sharma et al., 2021, 2022). Akin to the SARS-CoV-2, the CRISPR-Cas 12a/Cas13a was also developed to detect the matrix (M) gene, H1 and H2 genes in influenza A/B (Mayuramart et al., 2021). By nature, the bacteria utilise the CRISPR/Cas system to protect against invading viruses and foreign nucleic acids (Baddeley and Isalan, 2021). Therefore, we can employ the same principle against the virus, giving a new window for antiviral therapeutics. To date, Cas9, Cas12, and Cas13 have been widely investigated for different dsRNA (e.g. HIV, HBV) and ssRNA viruses (e.g. influenza, SARS-CoV-2) (Baddeley and Isalan, 2021). The principle of the CRISPR-Cas system lies within its ability to target and cleave the positive RNA strand of the influenza virus, effectively halting the virus's replication cycle.

The CRISPR-Cas 13d contains 22-nucleotide spacer CRISPR-associated RNAs (crRNAs) or known as the guide RNA (gRNA), that direct the Cas13d to the specific targeted RNA for cleavage and subsequent degradation via a protospacer flanking sequence (PFS) (Abbott et al., 2020). In a study conducted by Abbott and colleagues, a prophylactic antiviral CRISPR in human cells (PACMAN) was employed as an intervention strategy for the influenza virus. To note, it is vital to target highly conserved viral packaging segments for effective inhibitory effect across all the different influenza strains (Muramoto et al., 2006). Of these, 6 of the 8 IAV viral genome segments were highly conserved, and the respective crRNAs were transfected into A549 lung epithelial cells for this study. Following that, the crRNAs transfected cells were challenged with fluorescent reporter strain of influenza (A/Puerto Rico/8/1934)-mNeonGreen gene at an MOI of 2.5 or 5.0. The results suggest a robust 72% and 52% reduction in viral titre at MOI 2.5 and MOI 5 for the crRNA pool targeting segment 6 (encodes for NA), respectively (Abbott et al., 2020). Besides that, crRNA targeting segment 4 (encodes for HA) also showed a moderately consistent inhibition at 26% and 55% reduction in viral titre at MOI 2.5 and 5, respectively (Abbott et al., 2020). Given the above, using the CRISPR-Cas system would allow an alternative precise antiviral approach to influenza infection.

4.3. *Alternative approaches against the increasing resistance of influenza A antivirals*

4.3.1. *Drug repositioning*

As known to many, existing anti-viral drugs have saved millions of lives over the years and remain essential in treating existing, re-emerging, and future infections (Andrei, 2021). However, there is still a need for a more robust, non-toxic, and accessible anti-viral to overcome the challenges faced, as previously described, in particular, antiviral drug resistance. Drug development is a lengthy and utterly costly process that requires approximately 17 years of development, accompanied by many hurdles before FDA approvals (Low et al., 2020). The term drug repositioning is no stranger to many, especially in this urgent time of need to address the immediate demand for efficacious antiviral therapy against COVID-19. Drug repositioning, also known as drug repurposing, drug recycling or drug reprofiling, is an alternate approach to finding new uses to treat other diseases aside from the intended ones (Low et al., 2020). The classic examples of drug repositioning are Minoxidil for hair loss atop the intended use for hypertension treatment, the Sildenafil for erectile dysfunction atop the intended use for angina, to name a few (Low et al., 2020). The trend of drug repositioning for influenza is no exception and has gained much-needed traction and attention. For instance, nitazoxanide (NTZ) for parasitic infections showed anti-influenza properties by selectively blocking the maturation of the viral hemagglutinin, impairing hemagglutinin intracellular trafficking and insertion into the host plasma membrane, effectively inhibiting the assembly and viral exit from the host cell (Rossignol et al., 2009). Other notable drugs repurposing potential for IAV include triamterene, a diuretic medication used to treat oedema by inhibiting epithelial sodium channels was recently found to significantly reduce influenza A/WSN/33 and A/CA/04/09 replications with concentrations of 100–125 uM in Calu-3 cells. The purported mode of action was the specific inhibitory of δ subunit of the sodium channel gene (SCNN1D), contributing to the collapse in ion gradients, affecting the Ca^{+2}-dependent influenza virus entry into the host cells (Orr-Burks et al., 2021). Concerning the advancements in computational approach, data mining, *in silico* bioinformatics, cell-based screening, multiplex assays and cheminformatics databases, the antiviral potential of a drug repositioning candidate can be assessed quickly in several ways before the *in-vitro* and *in-vivo*-setup, such as signature matching, molecular docking, genome-wide association studies (GWAS), pathway mapping, to name a few, putting drug repositioning a much more feasible approach to exterminate the IAV infection (Low et al., 2020).

4.3.2. *Natural occurring compounds*

Apart from drug repositioning, naturally occurring compounds can contain a hidden trove of anti-viral agents. Identifying and purifying active ingredients from natural sources such as plants presents an attractive and logical strategy for novel drug discovery. Various plant compounds have long been investigated for their anti-inflammatory, antioxidant, anti-tumour (Owen et al., 2022), and even antimicrobial activity (Urgaonkar et al., 2005; Ginoyvan et al., 2017; Valle et al., 2015). Today, many investigations pursue natural compounds as lead candidates for anti-viral agents. A great example is silymarin, which has been found to inhibit virus replication of EV-A71, chikungunya virus (CHIKV), Hepatitis C virus (HCV), and dengue virus (DENV) (Lani et al., 2015; Lalani et al., 2020; Low et al., 2021). However, the development of silymarin resistance has been observed during in vitro analysis (Lalani et al., 2020; Esser-Nobis et al., 2013). Another flavonoid, baicalein, a compound isolated from the medicinal plant *Scutellaria baicalensis*, showed inhibitory effects of IAV *in-vitro* and *in-vivo* experiments,

where orally administered baicalein has significantly reduced the mortality of H1N1-infected mice (Ding et al., 2014; Xu et al., 2010). Atop of that, Ding et al. (2014) have illustrated that the anti-viral activity was achieved by inhibiting IAV neuraminidases, which in turn impedes the budding of virus progeny. Further investigations have also identified the antiviral potential of baicalein against herpes simplex viruses (HSV) (especially in acyclovir-resistant strains), Japanese encephalitis virus (JEV), and DENV (Luo et al., 2020; Johari et al., 2012; Zandi et al., 2012; Low et al., 2021; Moghaddam et al., 2014).

4.3.3. Antiviral peptides

The potential of anti-viral peptides (AVP) against IAV infection should also be further investigated. Several AVPs targeting IAV have been identified, which have been isolated from human (APD3, LL-37), mammalian (mBD1, mBD3, P9, P9R) and non-mammalian (M1, VY1, alloferin 1 and 2, urumin) sources (Lee et al., 2022). In particular, chemically synthesised AVPs based on structural and ligand-based design can ensure highly specific interactions with the virus protein compared to those obtained naturally (Lee et al., 2022). These agents operate via various principles, such as promoting neutrophil clearance by neutralizing and aggregating IAV; diminishing IAV replication and associated inflammation based on mouse model studies; directly inhibiting IAV attachment and subsequent infection; preventing IAV endosomal escape by targeting virus-host endosomal acidification; and via immunomodulation (Saito et al., 2021; Lee et al., 2022). AVP is cited as less susceptible to developing drug resistance and has broad-spectrum activity, which may help us overcome the challenges presented by current antivirals (Agamennone et al., 2022; Jones et al., 2006; Zhao et al., 2020). For example, P96, a modified peptide derived from mouse β-defensin-4, showed broad-spectrum and potent inhibitory activity of the enveloped A(H1N1)pdm09 virus, avian A(H7N9) virus, SARS-CoV, SARS-CoV-2, MERS-CoV and non-enveloped rhinoviruses by preventing endosomal acidification responsible for successful virus infection (Zhao et al., 2020). Furthermore, passaging the A(H1N1)pdm09 virus in the presence of P9R did not cause the emergence of drug resistance, even at higher passages (~40) (Zhao et al., 2020). However, AVPs have yet to be developed into feasible therapeutics as it faces issues such as poor stability and bioavailability, high cytotoxicity, and potential for inducing immunogenicity (Lee et al., 2022; Skalickova et al., 2015; Vilas Boas et al., 2019).

4.3.4. Chemical modifications and drug combinations

Rapid progress in structural biology and computational chemistry expedites the design and modification of existing anti-viral therapeutics to overcome the issue of resistance, which remains a major challenge in developing antivirals (Lee et al., 2022). Since existing drugs are relevant molecular scaffolds for drug discovery, rational drug design involves making structural modifications to a chemical to change the potency of the associated drug (Andrei, 2021). Adamantane is a class of anti-influenza drugs that are rendered clinically obsolete due to widespread drug resistance; however, creating derivatives by the addition of chemical groups to the primary amine of adamantanes showed promising outcomes (Scott et al., 2020; Shibnev et al., 2012). Guanidation of oseltamivir carboxylate and Zanamivir has also yielded derivatives with promising antiviral activity (Chayrov et al., 2020). Favipiravir, first identified in an in vitro study against the IAV H1N1 PR8 strain in Japan, was a promising broad-spectrum anti-viral drug that targets the viral RNA polymerase (Konstantinova et al., 2022; Goldhill et al., 2018). However, the use of this potent antiviral is hampered by its potential teratogenic and embryogenic effects, in addition to drug resistance (Scott et al., 2020; Goldhill et al., 2018; Ertem et al., 2022). Notably, despite the downsides of Favipiravir, it has been noted that Favipiravir targets the SARS-CoV-2 viral RNA polymerase, a potential drug for repositioning use against SARS-CoV-2 (Sada et al., 2020). Thus, chemical modification to Favipiravir may improve the safety of the drug, overcome drug resistance, and give great potential for a broad-spectrum antiviral drug against the IAV and SARS-CoV-2 infection, posing a rational approach to breathing new life into the old drug that once developed resistance. In addition, molecular docking programs such as Autodock Vina, SwissDock, and CB-Dock can assist in screening potential chemicals that interact with virus proteins. Employing an *in silico*-assisted approach, isolating promising compounds for further in vitro and pre-clinical evaluation can be done more quickly and economically (Andrei, 2021).

Besides that, using a combination of antiviral drugs for IAV treatment may produce a synergistic antiviral effect and prevent drug resistance. This is not new as a combination of drug cocktails in HIV treatment to prevent the development of drug resistance has been in place to avoid devastating resistance outcomes (Matthew et al., 2021). Resistance develops easily and rapidly against baloxavir, a recently approved anti-influenza drug, exacerbating the race to look for alternative antiviral drugs against influenza (Matthew et al., 2021). Drug combinations, in particular, may overcome the complications associated with individuals with lower genetic barriers to resistance. For instance, M2WJ332 (modified adamantane with activity against full-length M2-N31) and L1.1 induced an anti-viral synergistic impact, possibly due to its binding to distinct sites. Synergy was also observed on M2WJ332 with DP9 and increased DP9 concentration (Scott et al., 2020). The L1.1 also showed synergistic effects in combination with Zanamivir. A combination of oseltamivir and baloxavir led to reduced selection for drug-resistant A/H3N2 viruses as compared to monotherapy of baloxavir in ferret models (Koszalka et al., 2022; Scott et al., 2020). However, there was no synergistic effect between the oseltamivir and baloxavir combination therapy (Koszalka et al., 2022). Additionally, favipiravir has also exhibited a similar synergistic antiviral effect with peramivir, another NA inhibitor, in mice models infected with A/California/04/2009 (H1N1) virus (Tarbet et al., 2012).

4.3.5. Targeting host factors

To achieve effective virus inhibition, host-targeting inhibitors shall be the priority to be exploited due to the rapid mutation of viral protein and the dependency on the virus-host life cycle. For instance, a study has shown promising results using Diltiazem (a calcium channel blocker currently used for hypertension) in combination with Oseltamivir, a neuraminidase inhibitor against IAV, after shortlisting 35 potential candidates upon computational screening and transcriptomic signature analysis of 1309 FDA-approved bioactive molecules (Pizzorno et al., 2019). The study has shown the combination mentioned above has induced $a > 3$-log reduction in viral replication in Human Airway Epithelia (HAE) at MOI of 0.1, 48 h post-infection compared to untreated control (Pizzorno et al., 2019). Interestingly, the Diltiazem alone successfully rescued 40% (4/10) of the mice in an in vivo setting, a more significant achievement than the 20% rescue from Oseltamivir (Pizzorno et al., 2019). To date, more than 15 drug molecules have been selected for the repositioning approach. Of which, Nitazoxanide (anti-parasitic), Celecoxib and LASAG (anti-inflammatory), Statins (cholesterol modulators), Naproxen (NSAID anticancer), Midodrine (anti-hypotensive), Diltiazem (anti-hypertensive) have successfully reached clinical phase II and III trials (Pizzorno et al., 2019). As a summary, the rapidly evolving nature of the virus has conferred rapid resistance to different antivirals and vaccines, and influenza is no exception. Developing novel strategies such as drug repositioning, exploring naturally occurring compounds, antiviral peptides, and drug combinations are critical in producing cost-effective yet efficacious antiviral drugs against IAV.

5. Vaccinations

Vaccination against influenza has been proven to be the most effective approach to prevention and management (Martínez et al., 2019; Shasha et al., 2020; Tanner et al., 2021). Given the perpetual antigenic shifts and drifts in the influenza virus, the rise of antigenic

variation requires the vaccines to be kept up to date to prevent vaccine inefficiency. The composition of the vaccine strains is updated biannually according to the World Health Organization (WHO) recommendations, which tracks clinical data on the circulating and emerging influenza strains worldwide under a collaborative effort (Baxter, 2016; Tanner et al., 2021). Akin to many vaccines, influenza vaccines work by stimulating antibody production against the surface antigens of influenza, but the effectiveness of the conferred immunity may vary due to age, lifestyle factors, and initial exposure to prior strains (Becker et al., 2021). Thus, we aim to discuss the types and mechanism of actions of currently available influenza vaccines, plus the factors contributing to vaccine inefficacy.

5.1. Current vaccines

Influenza vaccines exist in trivalent or quadrivalent forms. Trivalent vaccines target the influenza A subtypes H1N1 and H3N2, in addition to one of the two influenza B subtypes recommended by WHO. Trivalent vaccines were the "mainstay" flu vaccine until 2015, when quadrivalent vaccines were introduced (Shasha et al., 2020; Tanner et al., 2021). No doubt, the risk of vaccine mismatch between the circulating strain and vaccine strain in the trivalent vaccine contributes to the increasing preference for the quadrivalent vaccine (Baxter, 2016). The quadrivalent vaccine targets the IAV strains H1N1 and H3N2 plus both influenza B lineages (Tanner et al., 2021). Studies have shown that quadrivalent vaccines are more effective in reducing patient morbidity and mortality, leading to a lower load of healthcare services, effectively giving a higher "cost-effective ratio of quality-adjusted life years versus non-vaccinated" (Shasha et al., 2020; Tanner et al., 2021). Depending on the formulations, vaccines can also be further classified into inactivated influenza vaccine (IIV), live attenuated influenza vaccine (LAIV), and the emerging recombinant baculovirus vaccine (Table 1).

5.1.1. Inactivated influenza vaccine

IIVs have an excellent safety record and are commercially available as a whole, split, and HA and NA subunit-only vaccine (Becker et al., 2021). In the egg-based system, the vaccine influenza strains are grown in the allantoic fluids of embryonated, pathogen-free chicken eggs and subsequently purified in sterile conditions before extraction (Baxter, 2016; Becker et al., 2021). The cell-based system is also used to produce inactivated subunit vaccines (Becker et al., 2021). Initially, the whole inactivated vaccines were widely used, but slowly obscure in the modern days due to the presence of bacterial endotoxins and high reactogenicity produced by the purification methods (Baxter, 2016; Trombetta et al., 2022). Split virion IIVs, on the other hand, subject the whole influenza viruses produced in the egg-based system to ether treatment to disrupt the bilipid membrane and subsequently produce a preparation that includes all the viral proteins and lipid bilayer constituents. Meanwhile, subunit vaccines are manufactured by adding a detergent such as sodium dodecyl sulfate (SDS) to the whole virus, producing a preparation mainly made of HA and NA before undergoing an enrichment process to obtain the final vaccine product (Baxter, 2016). Both the split virion and subunit influenza vaccines are now markedly preferred to the whole virus vaccine (Trombetta et al., 2022).

The associated mechanism of action of the IIVs involves the induction of a systemic Immunoglobulin G (IgG) response. Interestingly, given that influenza viruses rarely enter the bloodstream, the IgG confers protection by entering the respiratory tract's mucosal membrane, providing a neutralising antibody effect without inducing an IgA response (Baxter, 2016). The vaccine's key target is the HA surface antigen of influenza; with a standard dose of 15 μg of HA of each vaccine strain, it is sufficient to generate an immune response in healthy adults (Trombetta et al., 2022). For elderly and immunodeficient people, a high-dose IIV with 60 μg of HA can be used to prevent insufficient antibody induction for protective response (Safdar and Cox, 2007; Trombetta et al., 2022).

5.1.2. Live attenuated influenza vaccine (LAIV)

LAIVs are produced by inserting genes of the desired seasonal HA and NA surface antigens into a HA/NA-deleted influenza virus vessel containing all internal genes and grown in an egg-based culture system (Baxter, 2016; Becker et al., 2021). LAIV is administered intranasally to individuals between 2 and 49 years old (Becker et al., 2021; Trombetta et al., 2022). This route of administration imitates the natural pathway of influenza infection, leading to a broader humoral and cellular response compared to the inactivated vaccines (Trombetta et al., 2022). Additionally, the vaccine induces a strong local mucosal IgA response and activates both CD4+ and CD8+ T-cell responses (Becker et al., 2021). Studies showed LAIV confers a level of protection comparable to IIV in healthy adults. Notably, the protection levels varied between age groups, offering less protection to older people than IIVs. Thus, unless contraindicated, LAIVs are preferred for children aged between 2 and 17 years (Trombetta et al., 2022). Unfortunately, children with severe asthma and wheezing are not encouraged for LAIV and are given IIVs instead (Becker et al., 2021; Trombetta et al., 2022).

Egg-adapted systems are well established for LAIVs and IIVs, producing approximately 85 – 90% of influenza vaccines. Besides being cost-effective, it attains high viral titers, as evidenced by the global production capacity of 413 million trivalent influenza vaccines annually, effectively facilitating global access to the vaccine (Trombetta et al., 2022). However, this system is labour-intensive since it requires pathogen-free flocks that potentially an allergen and entail a relentless effort to maintain production sterility (Trombetta et al., 2022). Apart from that, it has been suggested that passage adaptations during the culturing process could affect the vaccine's effectiveness. Tentatively, the cell-based system may be a more feasible alternative production method than the egg-based approach. Flucelvax, a quadrivalent IIV produced via cell-based methods, was approved for use during the 2019 – 2020 flu season, demonstrating a possible future for cell-based production (Trombetta et al., 2022). Despite this, DNA-based production offers a more significant advantage over egg- or cell-based production systems, making it a game-changer in modern vaccine production. Ultimately, this brings us to discuss the baculovirus expression system.

The baculovirus recombinant HA (rHA) vaccine produced in *Lepidopteran* insect cells presents a modern alternative to overcome the issues associated with embryonated chicken egg system (Becker et al., 2021; Trombetta et al., 2022). The rHA antigens are produced in insect cells by recombinant baculovirus vectors and grown in animal-free suspension culture sera to produce high amounts of desired antigens strains (Safdar and Cox, 2007; Manini et al., 2017). This relieves the

Table 1
An overview of current influenza vaccines.

Influenza vaccines	Method of production	Vaccine formulation	Route of administration	Recommended age groups
Inactivated	Embryonated chicken egg system; cell-based system	Trivalent or quadrivalent	Intramuscular or subcutaneous administration	18 years and older High dose for ≥ 60 years
Live attenuated	Embryonated chicken egg system	Quadrivalent	Intranasal administration	2 – 49 years
Recombinant	Baculovirus insect expression system	Quadrivalent	Intramuscular	18 years and above

dependence on chicken eggs for production, a huge advantage considering the current trend of global egg shortage that may put vaccine production in a precarious situation (Treanor et al., 2007; Manini et al., 2017; White, 2023). Additionally, this method reduces mutations in the baculovirus-expressed rHA, as the double-stranded DNA vector containing the HA gene is susceptible to DNA repair mechanisms (Safdar and Cox, 2007). Clinical trials showed the vaccines were well tolerated and effective in young and older adults (65 years and above), with doses of 15 – 135 μg per component administered to elderly individuals (Treanor et al., 2007). It induces an appropriate immunogenic response in older people at a rate commensurate to the currently available options while complying with the safety requirements, proposing a promising alternative platform for vaccine production.

Manufactured by Protein Sciences, a baculovirus recombinant vaccine (FLUBLOK) obtained FDA approval for commercial use among individuals aged 18 – 49 in the US in 2013 (Trombetta et al., 2022). Meanwhile, Supemtek, a quadrivalent influenza vaccine produced with the baculovirus expression system, has also been approved for use in the European Union by the European Medicine Agency (EMA) in 2020 (Manini et al., 2017). The vaccine contains 45 μg of HA, three times higher than the standard of other vaccines of a 15 μg dose (Manini et al., 2017). Of course, production cost remains a limiting factor in the commercial use of the recombinant influenza vaccine. With the recent developments in vaccine production after the COVID-19 pandemic, it is possible to overcome the associated impediments to recombinant influenza vaccine production.

5.2. Issue of influenza vaccine inefficacy

Vaccine inefficacy significantly hinders public health management of influenza, particularly the influenza A/H3N2 subtype (Chen et al., 2019). As mentioned above, vaccinations are the most effective method of control. The protective effects of influenza vaccines involve generating virus-specific and broadly reactive antibodies with virus-specific T-cells to combat the invading pathogen, thereby reducing disease severity (Becker et al., 2021). Nevertheless, the efficacy of vaccines can be influenced by the state of the immune system and how vaccines are manufactured (Hardelid et al., 2018; Martínez et al., 2019; Hauge et al., 2020; Yang et al., 2020). Additionally, individual factors such as the prior encounter with previously circulating strains or closely related viruses were also found to diminish vaccine efficiency, which gives in to the concept of "original antigenic sin (OAS)" (Becker et al., 2021). However, the "original antigenic sin" theory and its role in limiting the effectiveness of influenza vaccines have faced much scepticism (Henry et al., 2018). The concept refers to the lifelong impact of an individual's first influenza variant encounters through natural infection or vaccination means. In which, the anti-influenza antibody response from childhood remains dominant despite encounters with other strains over the lifetime (Henry et al., 2018; Tanner et al., 2021).

In general, OAS refers to the first encountered strain with the highest levels of anti-influenza antibodies compared to the other strains (Henry et al., 2018). Forasmuch, as some epitopes remain conserved in antigenically drifted strains, cross-reaction of such epitopes with pre-existing antibodies further boosts the existing antibody responses (Henry et al., 2018). Furthermore, a mismatch between the vaccine strain and circulating strains due to genetic alterations causes poor vaccine effectiveness when the vaccines are available (Tanner et al., 2021). The 2018/19 flu season in Europe showed poor vaccine efficiency with rates of 25% to 52% against the co-circulating influenza A (H1N1)pdm09, A(H3N2) and B strains (Rondy et al., 2018). In particular, the B/Yamagata lineage was the dominant influenza B strain circulating during the season, but it was not included in the trivalent vaccine (Rondy et al., 2018). Vaccine mismatch could also have occurred with passage adaptations, an additional adaptation that happens in the vaccine production process due to the accumulation of substitution mutations during the propagation of viral strains in culturing media (Chen et al., 2016, 2019). The deviations of the passaged viruses from the original ultimately led to poorer vaccine effectiveness.

5.3. Current approach to improving vaccine efficacy and future directions

Current methods to overcome the issues of vaccine inefficiency include increasing the dose of immunogens, adding adjuvants to the vaccine formulation, and using different modes of administration (Haq and McElhaney, 2014). The former is an established method of inducing a protective immune response, especially in older people with minimal safety issues in multiple studies (Safdar and Cox, 2007; Izurieta et al., 2015; Chang et al., 2019; Li et al., 2021; CDC, 2022; Trombetta et al., 2022). A double-blind, randomised trial also showed improved immunogenicity in patients with long-term immunosuppression when administered with a high-dose influenza vaccine (60 μg of HA) than the standard dose vaccines (Natori et al., 2018). Overall, the high-dose vaccine is well-tolerated and effective (Trombetta et al., 2022). Currently, the Fluzone High-Dose Quadrivalent vaccine manufactured by Sanofi Pasteur contains 4 times the amount of influenza antigen (60 μg) than the standard dose (15 μg). It has been approved for use on individuals aged over 65 years in the US since 2009 and is recommended for the 2022–2023 flu season (Chang et al., 2019; CDC, 2022; Trombetta et al., 2022). It was later approved in 25 European countries for older adults aged $\geq$ 60 years in 2020 (Trombetta et al., 2022). Meanwhile, adjuvants, such as insoluble complexes (alum), virosomes or oil-in-water emulsions (MF59, AS03 and AF03), adsorb and increase the visibility of antigens to the immune systems. These greatly improve the antigen presentation to antigen-presenting cells (APCs), thus conferring a greater antigen-specific antibody response (Haq and McElhaney, 2014; Tregoning et al., 2018). High-dose and adjuvanted vaccines also boosted titres of neutralising antibodies and T-cell responses against influenza viruses (Li et al., 2021). Vaccines containing the MF59 adjuvant, an oil-in-water squalene emulsion, were shown to improve CD4+ helper T cell and IFN-γ+-activity (Li et al., 2021). Vaccines adjuvanted with AS03 offer better reactogenicity but induce local and general adverse events such as redness, pain and swelling at the injection site more often than non-adjuvanted vaccines (Beran et al., 2013).

In addition, different routes of administration also boost vaccine responses against influenza. As previously mentioned, mucosal immunisation is beneficial as it mimics natural infection (Trombetta et al., 2022). Intranasal administration of the influenza vaccine has been well established, but intradermal vaccination is also a promising delivery route (Becker et al., 2021). Intradermal vaccination of older adults showed improved immunogenicity compared to the intramuscular route while maintaining the same safety profile (Holland et al., 2008; Chi et al., 2010). The dermal dendritic cells, an APC, are directly presented with the influenza antigens that will naturally trigger the immune response by migrating to the lymph nodes and presenting the antigens to T cells (Holland et al., 2008; Chi et al., 2010; Quach and Kennedy, 2022). Since the intradermal technique for vaccination is challenging to perform, microinjection and microneedle systems are used to maintain proper needle placement to administer the vaccine, which has proven reliable and successful (Holland et al., 2008; Kang et al., 2012).

Broad-spectrum vaccines have been proposed for next-generation influenza vaccines to target highly conserved epitopes of the HA stem, surface membrane proteins, and internal proteins, inducing a broadly protective response against influenza infection and disease severity (Tregoning et al., 2018; Becker et al., 2021). Moreover, the mRNA vaccine technology used against SARS-CoV-2 in the COVID-19 pandemic brings great promise to develop a novel and improved influenza vaccine. Investigations on novel adjuvants for influenza vaccines are underway, such as toll-like receptor (TLR) ligands, variants of oil-in-water emulsions or liposomes, cytokines, and immunostimulators, which have been reviewed in detail in (Tregoning et al., 2018). Combining technology from other vaccines against infectious diseases and using rational

approaches in vaccine design may address vaccine inefficiency in vulnerable individuals and improve influenza management globally.

6. Diagnostics

Influenza shares several symptoms with many respiratory viruses, leading to complications in distinguishing influenza infections based on clinical features. Despite the advancements in influenza prevention, the rapid evolution of influenza viruses has resulted in many deaths today. Therefore, pursuing fast and effective diagnostic methods is an urgent need, as early administration (within 48 h) of antiviral treatment provides the most significant effect against influenza viruses (Koonin and Patel, 2018). While viral isolation of the nasopharyngeal or throat secretions is the gold standard for influenza diagnosis, the time taken (days to weeks) for results exceeds the therapeutic window. Thus, several approaches were developed for inexpensive assays with short turn-around times. Current diagnostic assays can be classified into molecular-based and antigen-detection assays. Molecular-based assays include reverse transcription polymerase chain reaction (RT-PCR), loop-mediated isothermal amplification (LAMP), and CRISPR-Cas12a DETECTR. In contrast, antigen detection assays consist of the rapid influenza diagnostic test (RIDT), immunofluorescence assay, hemagglutination inhibition assay and others (summarised in Fig. 3).

6.1. Molecular-based assays

6.1.1. Reverse transcription polymerase chain reaction (RT-PCR)

Other than viral culture, RT-PCR also serves as a gold standard to diagnose influenza due to its ability to detect influenza subtypes. The basis of influenza diagnosis via RT-PCR lies in the amplification of target viral genes from cDNA or extracted RNA using specific primers, followed by fluorescent detection via RT-PCR thermal cyclers (Lee et al., 2001). As such, apart from allowing influenza A, B, and C identification, the RT-PCR also further identify specific seasonal influenza subtypes, such as H1N1. This is especially important for effectively preventing and controlling highly pathogenic influenza subtypes (Lee et al., 2001; Dziąbowska et al., 2018). Amongst the conventional techniques, RT-PCR confers greater sensitivity with a detection rate of 93% compared to ELISA (62%) and viral isolation (80%). The RT-PCR diagnosis is age-independent atop the greater sensitivity at 103- and 106 times greater than viral isolation and ELISA, respectively (Steininger et al., 2002). Since then, RT-PCR has been the gold standard for many assays. For instance, multiplex RT-PCR allows the detection of several respiratory viruses in a single reaction using respective primers, allowing the diagnosis of potential co-infection (Stockton et al., 1998). Additionally, real-time RT-PCR can detect influenza virus up to seven days after the onset of symptoms accompanied by a lower contamination rate than viral culture (van Elden et al., 2001). However, the RT-PCR is laborious, expertise taxing and requires expensive laboratory equipment; hence, less reach for public use. As RT-PCR uses specific primers, influenza strains with novel NA or HA can also be missed (Gavin and Thomson, 2004).

6.1.2. Loop-mediated isothermal amplification (LAMP)

Isothermal amplification assays have been commonly used to diagnose numerous viruses, such as SARS-CoV, rhinovirus, and adenovirus. Given the advantage of the single-tube reaction with constant temperature in isothermal amplification assay, they are relatively cheap, rapid, and energy-efficient than the traditional thermal cycling technique that requires a series of alternating temperatures, steps and cycles (Dziąbowska et al., 2018). Loop-mediated isothermal amplification (LAMP) is a DNA loop-mediated isothermal nucleic acid amplification that utilizes DNA or RNA polymerases and inner and outer loop primer pairs. The primer pairs can recognize and amplify the six distinct regions in the viral cDNA, leading to high specificity (McMullen et al., 2016). Poon et al. (2005) reported a 100% sensitivity using LAMP for detecting seasonal influenza A virus subtypes, H1N1 and H3N2. The results are photometrically determined by detecting the magnesium pyrophosphate by-product or observing the colour change upon adding SYBR green (Poon et al., 2005). LAMP has since been incorporated into many diagnostic assays, such as reverse transcription-LAMP (RT-LAMP), in which its sensitivity and specificity are equivalent to RT-PCR (Chen et al., 2021). RT-LAMP demonstrated a 97.8% sensitivity and 100% specificity against the H1N1 virus compared to RT-PCR (Kubo et al., 2010). Notably, the sensitivity of RT-LAMP was shown to be 10-fold higher than the WHO-approved RT-PCR against patients exhibiting influenza-like illness (Parida et al., 2011). The requirement for simple equipment and short-time nature have brought RT-LAMP uses in mobile

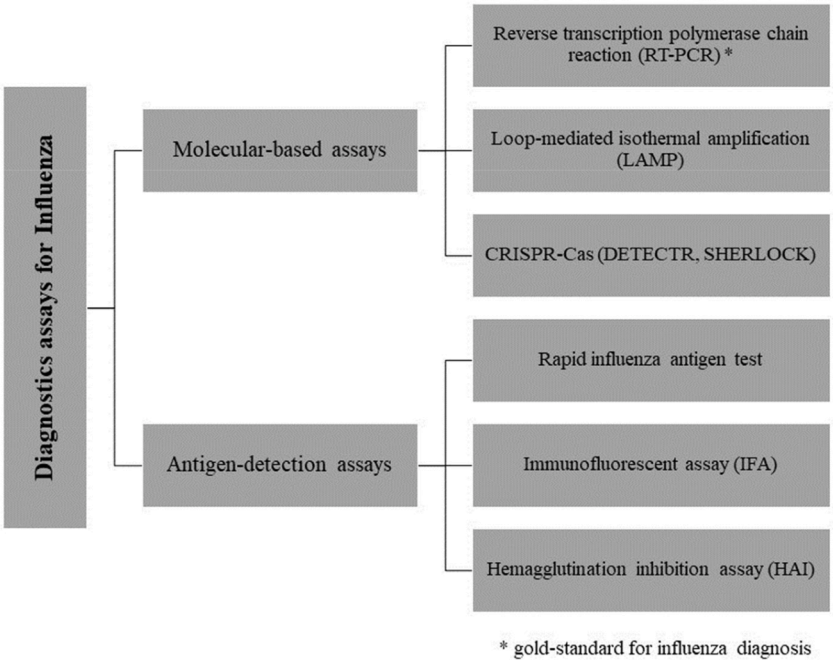

Fig. 3. An overview of current diagnostics assays for influenza.

laboratories (Kubo et al., 2010). Despite so, the major downside for LAMP is the interpretation of colour change, and turbidimetric signals, as it can be subjective to individuals (Gadkar et al., 2018). Besides that, determining the DNA size poses another challenge for adopting LAMP as the band smears as opposed to a single defined band seen in PCR (Wong et al., 2018).

6.1.3. CRISPR-Cas (DETECTR and SHERLOCK)

Clustered regularly interspaced short palindromic repeats, or CRISPR for short, have been recently used to develop diagnostic assays with increased specificity. The well-investigated systems include the DNA endonuclease-targeted CRISPR trans reporter (DETECTR) and specific high-sensitivity enzymatic reporter unlocking (SHERLOCK). The differences between the two lie in the type of CRISPR-associated protein (Cas) involved, where DETECTR utilizes Cas12a and SHERLOCK exploits Cas13 (Chen et al., 2018; Gootenberg et al., 2017). Both DETECTR and SHERLOCK use fluorescently labelled ssDNA/RNA probes, in which Cas proteins will emit a fluorescent signal upon cleavage (Mustafa and Makhawi, 2021). In DETECTR, the viral genetic material is amplified via recombinase polymerase amplification (RPA) and reverse transcription, followed by the cleavage of target DNA by Cas12a. Akin to DETECTR, SHERLOCK utilises Cas13, which cleaves RNA instead of DNA. Compared with conventional PCR, DETECTR and SHERLOCK demonstrate comparable sensitivity and specificity at a lower cost and shorter duration in less established laboratory equipment. In particular, SHERLOCK allows sensitive multiplex analysis of clinical samples (Chen et al., 2018; Kellner et al., 2019). As such, Park et al. (2021) successfully detected influenza A and B virus titers as low as 1 pfu/reaction via DETECTR, RT-RPA, and RT-LAMP in 75 – 85 min without cross-reactivity observed (Park et al., 2021). Moreover, a diagnostic platform that uses SHERLOCK, Cas13-assisted restriction of viral expression readout (CARVER), was reported to detect the influenza A virus and other ssRNA viruses in less than two hours (Freije et al., 2019).

6.2. Antigen detection assays

6.2.1. Rapid influenza antigen test

Rapid antigen test kits for influenza are a qualitative method with moderate accuracy used in influenza detection, offering users point-of-care testing. It provides convenience and accessibility for the public due to its rapid (results within 15–30 min), simplicity, cost-effectiveness, and mass production capacity, making it a popular choice within clinical settings (Green and StGeorge, 2018). These test kits use antibodies specific to the target protein to produce a qualitative, easily interpreted result (Green and StGeorge, 2018; Chartrand et al., 2010). Generally, a nitrocellulose membrane containing anti-nucleoprotein antibodies is immobilized on a thin test line (Chartrand et al., 2010). Dye-labelled free antibodies targeting the viral protein are present on the lower end of the membrane. In contrast, antibodies specific to these dye-labelled antibodies will be bound to another line known as the control line. Freshly collected specimens are mixed with a buffer and added to the membrane, which is taken up across the two test strips on the membrane (Chartrand et al., 2010). In the presence of the antigen, dye-labelled antibodies are trapped on the test and control lines, showing two lines indicating a positive result. Meanwhile, the dye-labelled antibodies will only bind to the control line without the antigen to produce results with a single line, showing a negative result (Chartrand et al., 2010).

Various rapid antigen detection kits are commercially available for detecting the presence of the influenza antigen, usually the nucleoprotein protein, in fresh nasopharyngeal samples or throat swabs, with a broad range of specificity and sensitivity ranging from as low as 58% and 52%, respectively, depending on the brands, site of collection and pathogenesis (Takeuchi et al., 2022; Chartrand et al., 2010; Dinc et al., 2023). In addition, the emergence of SARS-CoV-2 and influenza A/B co-detection rapid test kit allows users to identify and distinguish the causative agent for the infection, which is often challenging due to the similar clinical symptoms (Takeuchi et al., 2022). The rapid antigen test kits facilitate the diagnosis and clinical decisions, thereby preventing further transmission and aggravation of these contagious agents throughout the community (Dinc et al., 2023; Takeuchi et al., 2022). Despite so, the broad range of specificity and sensitivity are prone to false-negative results (Drexler et al., 2009; Faix et al., 2009; Dinç et al., 2023). A study reported 97.9% and 33.3% sensitivities at the 20-cycle threshold (Ct) but only 36.5% and 11.1% below 20 Ct for IAV and IBV, respectively. This suggests that the sample collection timing impacts the test's performance, as a higher amount of infectious viral particles is required to produce more accurate results (Dinc et al., 2023; Green and StGeorge, 2018). Despite the limitations, the potential of rapid test kits for diagnosing and subtyping influenza infections cannot be overlooked. For instance, Iwatsuki-Hiromoto and colleagues have designed a rapid test kit that is capable of detecting specific anti-H7 avian influenza HA antigens with high specificity, allowing for rapid influenza subtypes detection (Iwatsuki-Hiromoto et al., 2018).

6.2.2. Immunofluorescence assay

The direct immunofluorescent antibody (DFA) test, also termed the immunofluorescent antibody test (IFA), is an influenza diagnostic tool that detects influenza antigen via a modified antibody labelled fluorescent (Vemula et al., 2016). Despite the ability to differentiate influenza A and B infections, it cannot be used for subtyping (Vemula et al., 2016). Clinical specimens such as nasopharyngeal swabs or aspirates containing respiratory epithelial cells are affixed onto glass slides and directly stained with fluorescently labelled influenza-specific antibodies (Vemula et al., 2016; Peaper and Landry, 2014). These slides are then examined under a fluorescent microscope in a dark room to visualize the presence of viral proteins within infected cells (Vemula et al., 2016; Peaper and Landry, 2014).

Numerous studies have evaluated the accuracy of DFA and other tests against the RT-PCR. As such, in a study conducted by Vizcaya and colleagues, the DFA is more sensitive overall (35 to 87%) than rapid antigen tests (45 – 66%) for A/H1N1 diagnosis; however, rapid antigen tests show better specificity (83 – 96%) than DFA (74 – 94%) (Vizcaya et al., 2010). Similarly, another study has also reported moderate sensitivity (51.8%) with high specificity (99.6%) when used for A/H1N1 diagnosis (Bakerman et al., 2011). In addition, the DFA assay is age sensitive. The assay was shown to be moderately sensitive (60 - 62.2%) with high specificity (96 - 97.1%) for detecting A/H1N1 in children but poor among individuals $>$ 55 years of age, which is also consistent with an earlier study by Sandora and colleagues, presenting another challenge for diagnostics (Nitsch-Osuch et al., 2012; Sandora et al., 2010). To overcome this, Cytospin-modification has been used to enhance the sensitivity of DFA for influenza detection, but it still falls short compared to molecular-based assays (Vemula et al., 2016; Peaper and Landry 2014). Despite the short turn-around time (1–4 h), it requires laboratory expertise and specialized equipment to perform the assay (Peaper and Landry 2014; Chartrand et al., 2010). Hence, the usage of IFA restricts point-of-care testing, unlike RIDTs. To top off, IFA provides valuable information on preliminary diagnosis and the pathogenesis of the influenza virus but with limited sensitivity and general use.

6.2.3. Hemagglutination inhibition assay

The hemagglutination inhibition (HAI) assay is referred to 'gold standard' recommended by the European Union (EU) and WHO that is widely used in vaccine licensure and assessment of vaccine protection and infection in seroepidemiologic studies (Pedersen, 2014; Zacour et al., 2016; Ravina et al., 2021). The assay lies within the ability of subtype-specific antibodies to inhibit the agglutination of erythrocytes caused by the influenza HA protein (Ravina et al., 2021). Thus, this assay is used for subtyping influenza hemagglutinin (HA) in unidentified isolates or accessing existing antibody specificity toward influenza HA subtypes in extracted samples (Pedersen, 2014; Zacour et al., 2016). It is

important to note that although the HAI assay and the hemagglutination assay both use the basis of erythrocyte agglutination, the latter only ascertains agglutinating agents in general and does not specifically identify influenza viruses (Pedersen, 2014).

The influenza HA protein is a glycoprotein found on the surface of influenza viruses, which interacts with the sialic acid (SA) receptors on the surface of erythrocytes (Pedersen, 2014; Ravina et al., 2021). When there are sufficient quantities of the virus HA, the virus and host proteins interact to form a matrix of erythrocytes, thus preventing the erythrocytes from precipitating into a "button" or "pellet" at the bottom of microtiter plates or tubes (Spackman and Sitaras, 2020). This process is termed agglutination. Specific HA-subtype antibodies are added to different microtiter plate/tube rows to identify the influenza subtype. This prevents agglutination if the subtype-specific antibodies successfully bind to the target viruses in the HAI assay. A series of dilutions are usually prepared to quantify the antibody concentration and a panel of sera containing subtype-specific antibodies against the 16 different HA subtypes of influenza for subtyping (Pedersen, 2014). The HAI titer is obtained from the reciprocal of the highest dilution that inhibits precipitation (Spackman and Sitaras, 2020).

The HAI assay is cost-effective and relatively simple, with a shorter turnaround time than other molecular tests (Pedersen, 2014). However, this assay is not without caveats. The HAI assay has a history of poor result reproducibility due to the inconsistencies between erythrocyte batches and its dependence on the concentration of viruses and antibodies that are highly susceptible to the personnel, reagents, and protocols used in the assay across different laboratories (Spackman and Sitaras, 2020; Ravina et al., 2021). Notably, the lacking of standardization of the HAI assay for the A/H3N2 strain and influenza B viruses poses a considerable challenge to consistent results (Zacour et al., 2016). Apart from requiring a library of reference reagents, the production and optimization of the reagents are crucial to ensure the success and reliability of the assay (Pedersen, 2014). Notable, this assay cannot detect influenza viruses with novel HA subtypes, making it prone to produce false-negative results (Pedersen, 2014). Thus, the HAI assay has to be supplemented with real-time reverse-transcriptase polymerase chain reaction, gene sequencing or antigen immunoassays to rule out false negatives (Pedersen, 2014). Apart from the aforementioned, human sera and many other mammal sera also contain non-specific influenza inhibitors that reduce HAI assay specificity (Ananthanarayan and Paniker, 1960). The inhibitors may compete with influenza HA to bind to the host SA, thereby disrupting the agglutination reaction, causing false positives or elevated antibody titers (Boliar et al., 2006). Hence, sera pre-treatments are common with the use of potassium periodate or receptor-destroying enzymes to diminish inhibitory activity (Pedersen, 2014).

7. Conclusion

The rapid progression of influenza virus infection has put enormous economic pressure, especially given the need for annual changes in vaccine formula. Given the rapid emergence of new variants and amid the SARS-CoV-2 (COVID-19) pandemic, it is crucial to study the importance of viral evolution and associated risk factors. Considering the similarities of symptoms between influenza and SARS-CoV-2 infection, unravelling the underlying mechanism and preparing for any possible coinfections is imperative. Current antiviral strategies for the influenza virus entail Amantadine, Oseltamivir, and Baloxavir Marboxil. Alternatives such as RNAi, ASO, CRISPR and other drug repositioning candidates such as Nitazoxanide (anti-parasitic), Celecoxib and LASAG (anti-inflammatory), Statins (cholesterol modulators), Naproxen (NSAID anticancer), Midodrine (anti-hypotensive), to name a few are in great potential to overcome the significant concerns such as the antiviral resistance over time. Besides antiviral, vaccinations play an essential role in eradicating the influenza virus. Current vaccines include the inactivated, live attenuated, and recombinant influenza vaccine. In which, all of the vaccines could be in a trivalent or quadrivalent formulation to confer better protection to individuals from the different variants of influenza A. In such urgent needs, multiple diagnostic approaches exist where RT-PCR, LAMP, and CRISPR-Cas play a vital role in accurate and early detection. Other antigenic-based diagnostic assays, such as the rapid influenza antigen test, immunofluorescence assay, and hemagglutination inhibition assay, are also much needed to improve the detection time to respond to the effective therapeutic window. To further identify potential vaccine candidates, continuous monitoring efforts must be in place to understand the evolution of the influenza virus and, of course, to design better preventive and therapeutic strategies. It is hoped that with the information garnered from the past influenza pandemic and the vaccine advancement for SARS-CoV-2, the urgency for a broad-spectrum vaccine and antiviral that covers the SARS-CoV-2 and influenza virus for managing the influenza endemic could be brought forth.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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