

Influenza Vaccine: An Engineering Vision from Virological Importance to Production

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Abstract According to data from the World Health Organization (WHO) every year, millions of people are affected by flu. Flu is a disease caused by influenza viruses. For preventing this, seasonal influenza vaccinations are widely considered the most efficient way to protect against the negative effects of the flu. To date, there is no “one-size-fits-all” vaccine that can be effective all over the world to protect against all seasonal or pandemic influenza virus types. Because influenza virus transforms its genetic structure and it can emerge as immunogenically new (antigenic drift) which causes epidemics or new virus subtype (antigenic shift) which causes pandemics. As a result, annual revaccination or new subtype viral vaccine development is required. Currently, three types of vaccines (inactivated, live attenuated, and recombinant) are approved in different countries. These can be named “conventional influenza vaccines” and their production are based on eggs or cell culture. Although, there is good effort to develop new influenza vaccines for broader and longer period of time protection. In this sense these candidate vaccines are called “universal influenza vaccines”. In this article, after we mentioned the short history of flu then virus morphology and infection, we explained the diseases caused by the influenza virus in humans. Afterward, we explained in detail the production methods of available influenza vaccines, types of bioreactors used in cell culture based production, conventional and new vaccine types, and development strategies for better vaccines.

Keywords: influenza virus, influenza vaccine, vaccine manufacturing, cell culture-based vaccine production, vaccine production in bioreactors, influenza vaccine development strategies

1. Introduction

Influenza, or colloquially referred to as flu, is a very contagious respiratory disease. Influenza has caused many pandemics and epidemics by affecting animals as well as humans since ancient times. According to the oldest records available to date, a flu-like illness was first described by Hippocrates in 412 BC [1,2]. When looking at more recent history, it can be seen that the oldest flu pandemic that can be determined with certainty occurred for the first time in Russia between 1890-1891. When the time comes to 1918, a flu pandemic had occurred, and this pandemic was the worst flu pandemic to have happened worldwide. This pandemic was named "Spanish Flu" because it first appeared in Spain and spread to the whole world from there. It is estimated that this pandemic caused the death of nearly 100 million people in 1918-1919 and affected 500 million people in total. Due to the Spanish flu pandemic in 1918, awareness of influenza has increased in the scientific community since that date, and studies on the production of vaccines to protect against flu have accelerated from year to year [3-6] (Fig. 1).

Flu is divided into pandemic and epidemic (or seasonal). Similar to the Covid-19, pandemic flu outbreaks also emerge suddenly and have a huge impact on the World. On the other hand, seasonal flu outbreaks regularly affect many people every year. Pandemic flu outbreaks occur due to emerging new subtypes of influenza viruses. Since people have not been encountered these new viruses before, there is no strong immune response against them in community.

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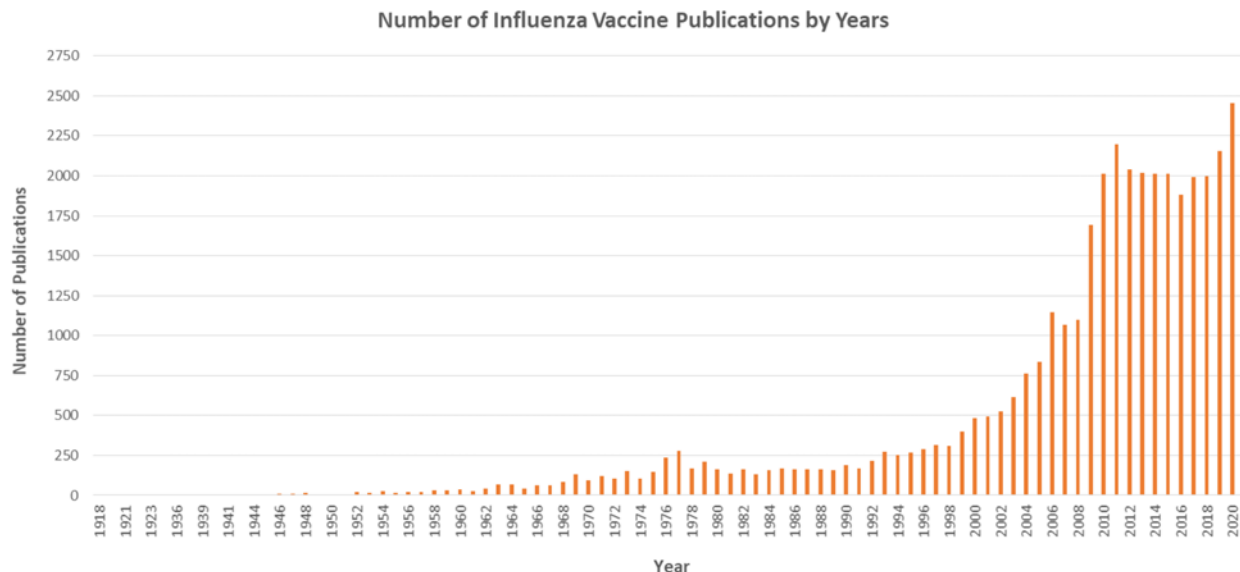


Fig. 1. Number of influenza vaccine publications that found between 1918–2021 searched by “influenza vaccine” keyword, according to PubMed.

On the other hand, seasonal flu shows itself every year with little immunogenic change. So thanks to pre-existing immunity in people against the seasonal influenza viruses, the effect of seasonal flu is not as devastating as pandemic. The viruses that cause seasonal flu are called “circulating influenza agents”. The term “circulation” here indicates that the related viruses that cause seasonal flu continue to spread among humans [4,6–9].

According to the data from the World Health Organization (WHO), 3–5 million people are hospitalized worldwide every year due to seasonal flu epidemics, and 290,000–650,000 people die from flu. In addition, when seasonal flu cases are considered, it is stated that 5–10% of adults and approximately 20–30% of children all over the world are infected every year [8,10–14]. In addition to all of these, the costs of medical care during the hospitalization and the inability to work during the illness, can cause large economic losses. Statistical research results estimated that, annual economic damage of seasonal flu in the United States (USA) varies between 6.3 billion and 25.3 million dollars [15,16]. At the same time, due to flu cases in livestock (especially in poultry) infected animals can be culled. So the culling of these animals in large quantities also creates problems in terms of economical and food availability [2,4,6,9,13].

2. Virus Structure and Morphology

All viruses discovered to date have been classified based on their viral structure and genetics. Influenza viruses are

included in the virus class Orthomyxoviridae. There are 4 types of influenza virus identified so far. These are; alpha (A), beta (B), gamma (C), and delta (D) influenza viruses. This distinction in influenza viruses was made on the basis of the antigenic differences of the two main structural proteins. These are nucleoprotein (NP) and matrix protein 1 (M1) whose are glycoproteins [8,9,17–20]. Influenza virus type A and B responsible for all seasonal flu cases in worldwide. However, clinical virus identification studies have shown that the influenza A virus is responsible for most of the seasonal flu cases worldwide alone. Also, identification tests made for investigating the influenza pandemics that have occurred so far have shown that influenza virus type A is responsible for all these pandemics. Along with that, influenza B viruses are responsible for approximately 15–30% of the total seasonal flu cases [9,21].

The influenza A virus is subdivided by the antigenic characterization of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 18 HA and 11 NA subtypes have been described. However, only three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes have been observed in circulating human influenza viruses that cause seasonal flu and human influenza viruses that historically cause pandemics. Based on historical progress, the WHO determined the circulating influenza A virus subtypes among humans as H1N1, H2N2, and H3N2. These also called human influenza viruses. In these, H2N2 is no longer circulating and has not been seen since 1968. According to current data, only H1N1 (similar to the virus that caused the 2009 pandemic) and H3N2 viruses are in the circulation today. For this reason, antiviral drugs and

vaccine development studies are carried out only for these virus types [3,20,22].

Influenza B viruses not divided to subtypes like influenza A viruses. They are just divided into two lineages. These are "Yamagata" and "Victoria" lineages. Besides, influenza B viruses do not cause pandemics like influenza A viruses. Because it has been show that their mutation rate is quite lower than influenza A viruses, and even if they mutate, their antigenic characteristics remains similar to its original lineage. In this way, even if the influenza B virus was newly formed by mutation, the possibility of a pre-exist immune response against this influenza B virus is quite high in society (especially in adult and elderly individuals). Thus, this pre-existing immune response to influenza B viruses in the population provides a great deal of protection. This makes the probability of a pandemic caused by influenza B viruses very low. According to the general assumptions to date, influenza B viruses cannot cause a pandemic, since there is no a new influenza B virus that emerges "suddenly" as a result of mutations. For this reason, there are also only two influenza B virus strains that have been causing infection in humans for many years around the world. These strains are Yamagata and Victoria like strains. In addition to all these, it has been shown that influenza C virus can only infect humans, dogs, cattle, and pigs. However, this infection is very mild in humans and does not require hospitalization or special treatment. Influenza D virus has not been found to infect humans so far. Infections due to this virus type are also seen only in pigs and cattle. For this reason, the whole scientific world is

working only on the prevention and treatment of flu caused by influenza A and B viruses in humans [5,7,9,15,20,23].

Influenza viruses are single-stranded RNA viruses with a viral envelope. The general structure of influenza virus can be divided into capsid and viral envelope. The capsid is basically composed of M1. Within this capsid there are Non-Structural Protein 2 (NS2 and also called nuclear export protein, NEP) and Non-Structural Protein 1 (NS1), along with segmented single-stranded RNA molecules (Fig. 2), which is the genetic material of the influenza viruses. On the surface of the virus, there are viral protein structures on the double-layered lipid layer that forms the envelope, which is specific by influenza virus types [9,24-27].

Influenza A and B viruses both contain 8 part, single-stranded RNA molecules wrapped in NP and packaged as a complex with single-stranded RNA-dependent RNA polymerase enzymes (RdRp). All of these are collectively referred to as the viral Ribonucleoprotein structure. On the double-layered lipid membrane layer (viral envelope) of these virions, there are glycoproteins HA, NA, and Matrix Protein 2 (M2) proteins (a transmembrane protein that acts as an ion channel, for influenza B viruses; this protein is BM2). Unlike these proteins, there is also the recently discovered NB protein, which is thought to act as an ion channel specific for influenza B viruses. However, for both types of these influenza viruses, in the double-layered membrane layer that forms the viral envelope, HA is the most abundant viral protein and then NA (Fig. 2). These protein structures are seen on the virus as 10-14 nm long

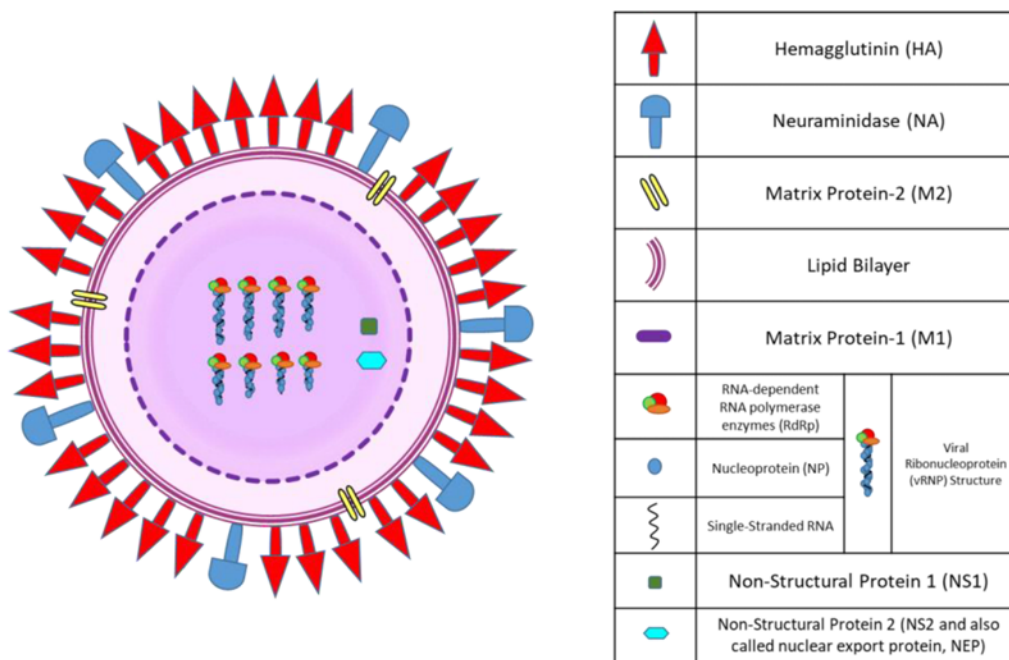


Fig. 2. Influenza virus structure.

protruding spikes. In particular, HA's shape is more like the thorn and beside HA, NA is depicted as a capped mushroom. Also it was shown that an influenza virion with an average diameter of 120 nanometers (nm) contains approximately 300–400 HAs and 40–50 NAs in virus surface. In addition to all of these, it has been shown that influenza virus can be found in 3 different morphologies. These are (i) filamentous, (ii) elliptical (also mentioned as bacilliform), and (iii) spherical. Although there are different theories about why the influenza virus has different morphologies, the exact reason for this is not fully understood [6,7,9,20,24,25,27,28].

3. Influenza Virus Infection

Influenza viruses can infect cells in the human respiratory tract. However, the human respiratory tract has a highly viscous mucosal layer with a continuous flow to trap airborne microbes and prevent potential infection. Therefore, influenza viruses must first overcome this mucosal barrier in order to cause infection [12,25,29,30]. In the evolutionary process, influenza viruses have developed an HA and NA-based mechanism to penetrate this layer and infect the epithelial cells under this mucus layer. According to this mechanism, initially inhaled influenza viruses cling to mucin chains containing sialic acid (SA) on the mucus surface with HA. Next, the SA at the attachment site is cut by the NA and the virus attaches to the next SA extension. In this way, the virus moves from the mucin chain to the epithelial tissue in a rolling-like motion. When the virus reaches the cell, it finds the appropriate receptor that will enable the virus to be taken into the cell with a similar motion on the cell and binds to it. After this binding, the virus is taken into the cell [24,29,30]. The balance of HA and NA on the virus is also very important in this respect for a successful infection of the virus [8,24,31].

Receptors with SA molecule bound to the galactose chains from the α -2,3 region (α -2,3Gal or also abbreviated as SA α 2,3Gal) are dominantly found on the epithelial cell surfaces in the upper region of the human respiratory system. After passing through the mucus layer, influenza viruses specifically bind to cells by recognizing these receptor motifs or patterns to enter the cell. Human influenza viruses generally recognize α -2,3Gal pattern and attach to these receptors with their HAs. For this reason, the influenza viruses mostly infects the cells in the upper respiratory tract. Therefore, the infection is usually confined just in the upper respiratory tract [5,7,8,24,25,29–32]. However, some type of cells in the human respiratory tract, also express large amounts of the α -2,6Gal motif along with α -2,3Gal on their surface. Different from α -2,3Gal

pattern, in this motif SA binds to galactose chains at the α -2,6 region. Especially, lung alveolar epithelial cells in the lower respiratory tract are one of these cell types. Therefore, other influenza virus subtypes like avian influenza viruses, which be included in influenza A viruses and mostly circulate among birds, can also infect the humans by recognizing this α -2,6Gal motif. For this reason, the fact that cells in the lower part of the human respiratory system have α -2,6Gal motif is the main reason why avian influenza viruses cause infection in humans [6,7,25,29,30]. In cases recorded to date shows that, avian influenza viruses (particularly H5N1 and H7N9) usually cause more severe disease in humans, and it is also shown that, their lethality rate is much higher than human influenza viruses [32].

3.1. Antigenic shift and antigenic drift

Influenza viruses are constantly changing viruses, and there are two basic mechanisms that enable the antigenic structures of viruses to change. These are antigenic drift and antigenic shift mechanisms. Antigenic drift (Fig. 3A) mostly occurs due to the rapid evolution of the virus and the absence of proof-reading (correction of incorrect base pairings that occur during replication) mechanism in RdRp [33,34]. Therefore, point mutations occur by errors from RdRp and assistance of the continuous evolutionary process for better adaptation of the virus to the host organism that the virus infects. These point mutations lead to changes in amino acid sequences, especially in HA and NA surface glycoproteins. When these point mutations occur in a particular gene region cumulatively, the corresponding antigenic traits of viral protein can change dramatically. At the same time, the rate of change via antigenic drift continues to increase with each infection-replication cycle and during this process it occurs very frequently. In this context, if newly emerged mutant virus's surface glycoproteins, which formed after small point mutations, is antigenically similar to its first form before mutation, some of the previously formed antibodies can also provide protection against to this new virus. This is called "cross-protection". However, when multiple point mutations occur in the same region of the surface antigen-encoding viral genes (such as HA and NA encoding viral gene segments) because of the antigenic drift mechanism, the rate of change of the antigenic properties of surface glycoproteins can be quite high. In this way, mutant influenza viruses remain the same in terms of their viral subtype, but their surface antigens are immunologically distinct from its original antigenic form. Therefore, antigenically distinct new mutant viruses can escape from the immune system and cause infection, because the specificity of existing antibodies in the organism decreases to this new virus strain. As a result, continuously every year seasonal flu

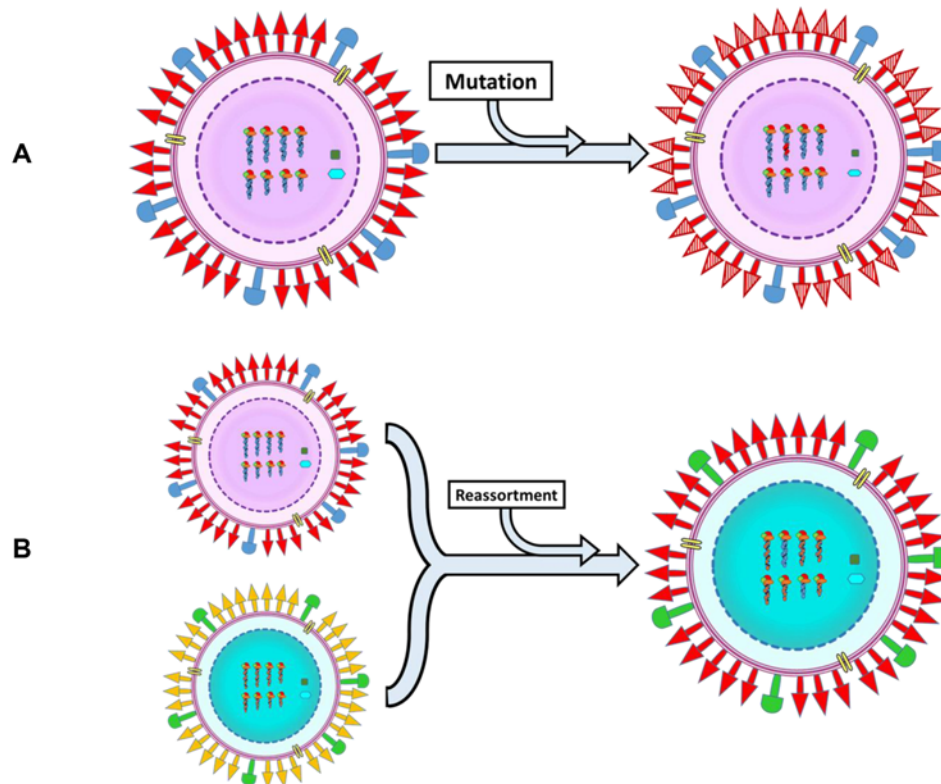


Fig. 3. (A) Antigenic drift: Alteration of the antigenic structures of the virus in terms of immunogenicity. (B) Antigenic shift: The forming of a single new virus subtype with the characteristics of both viruses as a result of the change of gene segments of two different virus.

cases occur [3,4,6,7,12,22,35].

The influenza genome's segmented structure makes it easier to genetic material exchange between different subtypes of influenza viruses. This process is called antigenic shift (Fig. 3B) and the change of the virus takes place here in one step. So, this mechanism allows the virus to take unpredictable evolutionary leaps. Rather, this situation occurs as a result of different subtypes of viruses infecting a single organism at the same time, and the viral genomes replicated here during infection reassort randomly among themselves. This exchange also called "reassortment" and the viruses that form in this way are called "reassortment viruses". For this mechanism, pigs in particular act as a "viral mixing tank", since both α -2,3Gal, which human influenza viruses recognize and bind to, and α -2,6Gal, which avian influenza viruses recognize and bind to, patterns are found in almost equal amounts on their cell surfaces. This definition also can be partially applies to humans. However, since α -2,6Gal cell surface motifs are found only on certain types of cells in humans, the probability of this happening in the human body is much lower than in pigs. Antigenic shift mechanism make it possible for some influenza viruses that do not normally infect humans or that do not transmit from person to person, to become infective for humans. As a result of this,

new virus subtypes can be formed with this mechanism. Also, since they are new viruses that have never circulated among humans before, the possibility of there is pre-existing immunity in people against them is quite low. Therefore, these new viruses can create a huge impact in society in the mean of public health as in the definition of pandemic, and because of them global influenza pandemics can occur [5-7,13,22,23,31,32].

3.2. Diseases caused by influenza virus infection in humans

Flu typically occurs after an incubation period of 1 to 2 days following the introduction of the influenza virus to the body. Symptoms are usually systemic and these are; fever, chills, headache, severe muscle pain, weakness and loss of appetite. These symptoms are accompanied by symptoms such as dry cough, runny nose, and sore throat. Especially during the examination, if patient has dry cough with fever and also considering the current season, the diagnosis of the disease becomes easier. Additionally, at the onset of the disease, the patient may experience eye inflammation, watering or redness of the eyes, and photophobia. In the later stages, the most prominent complaints are dry cough and fatigue. One or two weeks after the beginning of the disease, the patient almost completely returns to their former state [2,6,7,13,19,27,32].

However, influenza can cause more serious complications in humans. These can be divided into pulmonary complications which is mostly affecting the lungs and non-pulmonary complications affecting other organ systems of the body. Pulmonary ones can be classified as viral origin (or primary pneumonia) and bacterial origin (or secondary pneumonia). Viral pneumonia occurs as a result of the influenza virus lodged in the lower respiratory tract, especially in avian influenza cases. On the other hand bacterial pneumonia occurs mostly caused by the growth of pathogens in the lungs as a result of weakened immune system while fighting with the influenza virus. Therefore, individuals with weak immune systems are at great risk for secondary pneumonia while they suffering from flu [2,6,7,32].

Non-pulmonary complications can mainly affect the muscles, heart, and neurological systems. These complications are more common in children compared to adults. For the muscular system, flu can rarely cause myositis and rhabdomyolysis. Although complications of flu are rare in the muscle system, it can affect the heart more intensely. Cardiac complications of the flu include myocarditis, pericarditis, and it can worsen existing heart problems that patients have. Influenza has also been associated with neurological complications, including Reye's syndrome, encephalomyelitis, transverse myelitis, Guillain-Barré syndrome, aseptic meningitis, and encephalitis [6,13].

4. Human Immune Response to Influenza Infection

As soon as any pathogen enters the cell, the human immune system begins to fight it. The human body also creates this immune response against influenza viruses. The immune response emerges as intrinsic (or innate immunity) and subsequently adaptive immunity. These two cannot be fully separable because they stimulate the immune system in highly linked co-operation with the cellular and molecular level [25,36].

4.1. Innate immunity

Innate immunity is the immune response that humans have developed in the evolutionary process and shown against a wide variety of pathogens (bacteria, viruses, fungi, *etc.*). For this reason, the human immune system also creates a natural response against the influenza virus. Also, another natural defense mechanism is the mucus layer in the respiratory system that prevents the virus from reaching the cell for infection. So, as a respiratory virus influenza virus have to penetrate the mucus layer first and then reaches and infects the cells there. This infection, which starts especially in the lung epithelium, over time also occurs in resident macrophages and dendritic cells located in the

lungs. However, due to the specific nature of these cell types, viral infection occurring in these cells is limited thanks to their enhanced internal defence mechanisms (such as intracellular nitric oxide release) [25,29,37]. Interferon stimulation is performed by activation of pattern recognition receptors and toll like receptors (TLR), which are especially abundant in these cells. Although these receptors are mostly specific to immune cells such as macrophages and dendritic cells, all these intracellular receptor structures are also found in human lung epithelial cells. Thus, the immune system is stimulated via a variety of cytokines without waiting for the virus to reach the immune cells [18,25,29,35,37].

In addition, dendritic cells have a very important role in the stimulation of both intrinsic and adaptive immunity and in the development of immune response in viral infections. These cells begin to present influenza virus antigens on their surface as a result of phagocytosis (with MHC-II) or their direct infection (with MHC-I) at the site of infection. In this way, antigen-presenting dendritic cells go to the lymph nodes with the circulation and stimulate B and T lymphocytes, which will enable the realization of adaptive immunity [29,37,38].

4.2. Adaptive immunity

Firstly, innate immunity fights with influenza virus, which enters the body. Then, this system stimulates many cascades via special receptors. Following innate immunity, an adaptive immune response is induced for a more specific response. One of the biggest reasons for this is that natural immunity cannot fully prevent infection. Adaptive immunity is divided into two pathways. These are; cellular immunity through T lymphocytes, and humoral immunity through antibodies secreted from B lymphocytes [12,27,29,37].

4.2.1. Cellular immunity

As a result of the stimulation of antigen presenting cells, the production of T lymphocytes is stimulated by the immune system against the related infection. T lymphocytes (CD4+ and CD8+) form the basis of immunity at the cellular level. These T cells can only recognise viral antigens when presented to them as short peptide fragments on the major histocompatibility complexes (MHC). Therefore, an antigen-presenting cell must present the relevant influenza antigen to T cells and activate them [25,29,36]. Studies have shown that, when influenza virus infection occurs, T cells (especially cytotoxic T cells, T_c) elicit a specific response to intrinsic viral proteins (such as NP and M1) which they are more conserved antigenic structures in the influenza virus. Therefore, the immune response of T_c cells against intrinsic viral proteins provides much broader spectrum protection than the response against the more variable HA and NA proteins of the virus. Because, intrinsic viral

proteins is more conserved in terms of antigenic structure in most subtype of influenza viruses than HA and NA. When influenza virus-infected cells display these proteins on their MHC-I, it has been observed that Tc rapidly recognizes them. After this recognition Tc cell, which bound to infected cell, secrete perforin and granzyme. These two component are lead the infected cell to apoptosis. On the other hand, T CD4⁺ cells (or generally named T helper cells) provide a versatile immune response. They involved in stimulation of phagocytic macrophages, activation of Tc cells, and cytokine expression to limit infection while at the same time promoting B-cell activation for humoral immunity. However, T CD4⁺ cells only recognize the infection via antigen presenting cells. When these cells display the viral antigens on their MHC-II, T CD4⁺ cells bind to them and stimulation of different immune responses occurs, which is mentioned above [25,27,29,35].

4.2.2. Humoral immunity

Humoral immunity is based on B cells. These B cells can produce antibodies which is highly specific to the different antigenic parts of influenza viruses [12,13,25,35]. The mechanism of immunodominance determines which antigenic region of the virus the antibodies produced by B cells are mostly specific for. In short, the immunodominance mechanism can be explained as which of the antigenic regions on the influenza virus are more preferred by the human immune system to form antibodies. Accordingly, B cells mostly prefer the head region of HA to produce HA specific antibodies. Therefore, high amounts of antibodies, which are specific to the HA head region are produced after influenza infections. Then, head region of NA, the outer region of M2 in the viral envelope (ectodomain, M2e), and stalk region of HA respectively [12,23,25]. Although antibodies against other antigenic regions are also produced in the human body, it has been shown that most of the total antibodies produced by the human body against the influenza virus are specific to the HA head region. Besides these, it was observed that an antibody response was also formed against the internal proteins of the virus, such as NP and M1. However, the level of these antibody responses is quite low. Because these are only available when the infected cell is in the viral replication cycle. In this cycle, when cellular mechanisms synthesizing a new virus, during the viral assembly for a short period of time these proteins structures presented on the cell surface via MHC-I. So, the antibodies which are specific to them can bind these proteins [12,25,32].

In addition to all of these, vaccines aim to induce an immune response in which both adaptive and intrinsic immune responses are involved. They can show long-term protective activity against the related disease, especially

thanks to well-induced adaptive immune responses in terms of both cellular and humoral. In this respect, conventional and new candidate vaccines, try to evoke a good immune response by imitating these whole infection processes occurring in the body [12].

5. Influenza Vaccines

Vaccines have been most effective achievement of human-kind against pathogens for many years. Therefore, vaccines have been developed against many pathogens by scientists that threaten human and animal life. Developing a vaccine against influenza started right after the discovery of the virus in 1933, and an influenza vaccine was first developed for military purposes in the USA in 1940. This vaccine was produced in embryonated chicken eggs and served to human use in partially purified. However, in the trials this vaccine was found highly pyrogenic and lack of protective efficacy. For this reason, studies have been carried out to develop a more successful vaccine. As a result of this, a licensed monovalent (for a single virus type) flu vaccine was produced for the first time in 1945. This vaccine was used successfully until 1947. Because, in 1947 studies showed that this vaccine's efficacy was significantly reduced. When the reason for this was investigated, it was observed that the influenza virus was antigenically changed. Thus, for the first time, antigenic shift and drift mechanisms began to be discovered in those years [1,7,12,39]. Consequently, it has been understood that long term immunity provided by a one-time vaccine (such as a smallpox vaccine) will not be applicable for flu caused by influenza viruses. For this reason, in 1950, guidelines and studies were published regarding the need for regular renewal of influenza vaccines every year [1].

For the first time, a multinational organization for the surveillance of circulating influenza virus strains in various countries around the world was established by the WHO in 1952. The purpose of this organization was to examine only virus changes at first. Then, it merged with WHO for monitoring virus changes and circulating virus incompatibilities reported in seasonal flu cases. In this context, WHO organize two large meetings annually. As a result of these meetings, the types of viruses circulating in the different countries and their effects are evaluated. After these evaluations, virus strains, and types that may be involved in the next year's circulation are determined while considering possible mutations. Then, the viruses that are most suitable to be used in influenza vaccines are selected. These selected viruses are also mentioned as "candidate vaccine virus". Then, selected virus strains are shared with flu vaccine manufacturers and scientists all over the world.

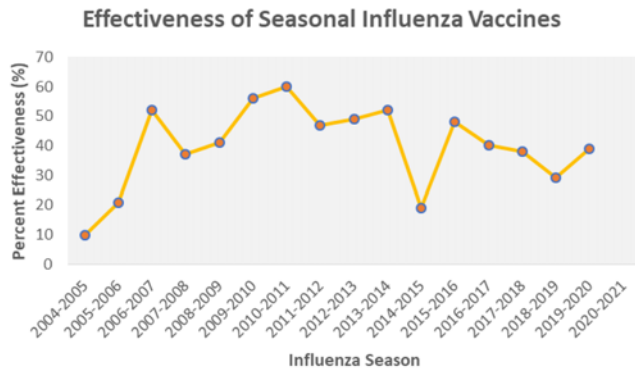


Fig. 4. Past seasons vaccine effectiveness estimates from 2004-2021 in USA, according to CDC.

This process is repeated every year by WHO [40].

After WHO shares the candidate vaccine viruses with vaccine manufacturers, WHO and their collaborating organizations (e.g., Centers for Disease Control and Prevention) also monitors the protective effectiveness of these vaccines. As a result of this surveillance, the accuracy of the virus predictions evaluated based on the vaccine effectiveness of the relevant year (Fig. 4). In this context, if the effectiveness of the vaccine is high, it can be said that selected candidate vaccine viruses for vaccine production are very similar to relevant year's circulation viruses in terms of antigenicity. However, if vaccine effectiveness is low, it can be said that predictions are insufficient or that the immunogenic parts of the circulating viruses in the relevant year have changed a lot as a result of the continuously changing nature of influenza viruses. So, vaccines that are produced for the relevant year are not antigenically matches to the circulating ones [40,41].

5.1. Currently produced influenza vaccine types

Currently produced vaccine types against influenza, which are already in commercial use are basically divided into 3 groups by their differences in production process. These are (i) inactivated vaccines, (ii) live attenuated vaccines, and (iii) recombinant vaccines. All these vaccine types are produced using one of the egg-based, cell culture-based and recombinant expression platform production methods [6,23,42] (Table 1 [43-48]).

5.1.1. Inactivated influenza vaccines

Inactivated vaccines are the most common type of vaccine used in the world for the prevention of influenza and all other diseases. Inactivated influenza vaccines were also divided into three groups. These are (i) whole virus vaccine, (ii) split virus vaccine, and (iii) inactivated subunit vaccine [49,50] (Fig. 5). The first licensed influenza vaccine, which is mentioned above, is an inactivated whole virus vaccine. This type of vaccine was used for many years and its main production process can be outlined as; production of virus (propagated in eggs or cell culture), inactivation, purification and then formulation, respectively. The inactivation step is quite important here and for this process, usually certain concentrations of formalin or β -propiolactone are used in vaccine production. However, the use of inactivated whole virus vaccines was restricted in 1970. Because, it has been shown that this type of vaccine caused severe side effects in many individuals. The main reason of this is inactivated whole virus vaccines contain non-cleaved viral RNA, which is highly reactogenic. Currently, there is no inactivated whole virus vaccine commercially available in Europe and America. Nonetheless, in several Asian countries are still produce and administered the inactivated whole virus

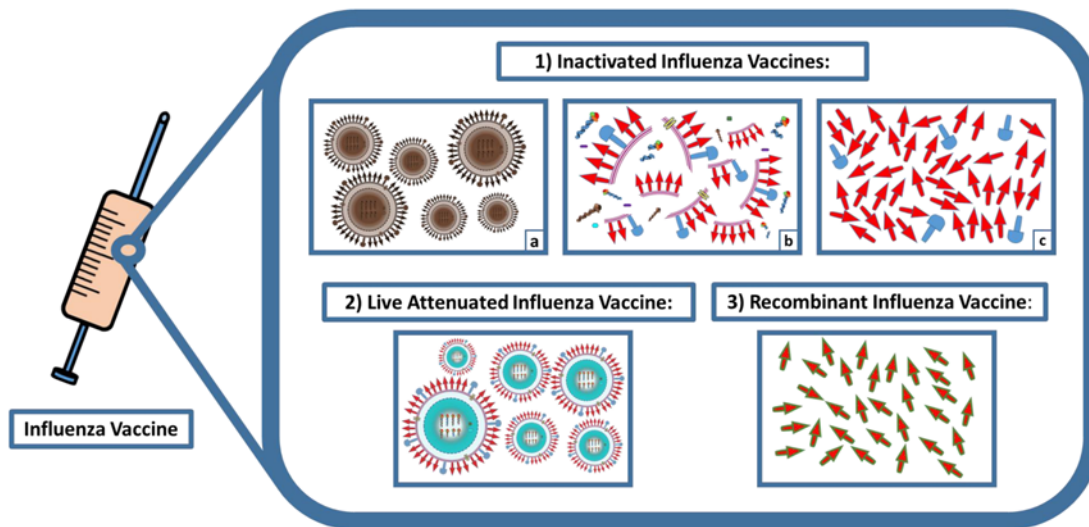


Fig. 5. Currently produced influenza vaccine types. 1) Inactivated influenza vaccines; (a) Whole virus vaccine, (b) Split virus vaccine, (c) Subunit vaccine. 2) Live attenuated influenza vaccine. 3) Recombinant influenza vaccine (also referred as recombinant subunit vaccine).

Table 1. Commercially available influenza vaccines [43-48]

Production platform of vaccine	Vaccine type	Dominant influenza antigen type that stimulates the immune system	The amount of antigen that provides immunization in the vaccine	Adjuvant in the vaccine	Trade name of the vaccine	The company that manufactured the vaccine
Egg based	Inactive/Split virion	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	-	Afluria®	Seqirus
	Inactive/Whole virus	HA	6 µg/0.5 mL of HA of each virus used in the vaccine	Alum	3Fluart®	Fluart Innovative Vaccines Kft
	Inactive/Split virion	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	-	Fluarix®	GlaxoSmithKline
	Inactive/Subunit	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	-	Agrippal®	Seqirus
	Inactive/Subunit	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	MF59	Fluad®	Seqirus
	Inactive/Subunit	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	-	Imuvac®	Abbot Biologicals/ Mylan Products (Marketing Authorisation Holder)
	Inactive/Subunit	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	-	Influvac®/ Xanaflu®	Mylan Products
	Inactive/Split virion	HA	15 µg/0.5 mL of HA of each virus used in the vaccine (60 µg/0.7 mL of HA in high-dose form of the vaccine)	-	Vaxigrip® (EU)/ Fluzone® (USA)	Sanofi Pasteur
	Inactive/Split virion	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	-	FluLaval®	ID Biomedical Corporation of Quebec
	Attenuated infective virus	Contain 10 ^{6.5-7.5} FFU/0.2 mL of each virus type	-	-	FluMist® (EU)/ Fluenz® (USA)	MedImmune/ AstraZeneca
Cell culture based (MDCK cell line)	Inactive/Subunit	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	-	Flucelvax®	Seqirus
	Inactive / Subunit	HA	15 µg/0.5 mL of HA of each virus used in the vaccine	-	*SKYCellflu®	SK Bioscience
Based on recombinant expression system (Sf9 cell line)	Recombinant HA antigen	HA	45 µg/0.5 mL of HA of each virus used in the vaccine	-	Supemtek® (EU)/ FluBlok® (USA)	Sanofi Pasteur

HA: hemagglutinin, Alum: aluminum phosphate gel, MF59: oil-in-water emulsion of squalene oil, EU: European Union, USA: United States, MDCK: Madin Darby Canine Kidney cells, Sf9: *Spodoptera frugiperda* derived insect cells, FFU: fluorescence-forming unit.

vaccines [1,2,7,50].

Different from the whole inactivated virus vaccine production, in the manufacture of split (or fragmented) virus vaccines, diethyl ether or sodium dodecyl sulfate, which cleaves the viral envelope, is added to the bulk production medium after the inactivation step. Thus, the inactivated whole virus is broken apart and virus fragments are revealed. So, split virus vaccines only contain viral fragments (Fig. 5). Although there is still viral RNA in the inactivated split virus vaccine composition, these are no longer as reactogenic as inactivated whole virus vaccines. Because, they are mostly broken into pieces or structurally degraded in the fragmentation process [49-52].

In the production of inactivated subunit vaccines, the

only difference is that the disintegration and purification processes are carried out thoroughly. In this process, like split virus vaccine production, firstly, the virus is produced and inactivated. After that, the viral envelope is broken down with detergents. Here, different detergents (such as ammonium deoxycholate and Triton X-100) are added to the bulk production medium for further separation. Then, with further purification steps mostly the HA, and little amount of NA parts of the virus are obtained for final vaccine formulation [23,49,51] (Fig. 5).

The adaptive immune response created by inactivated vaccines is mostly carried out by the antigen presenting cells taking in viral parts that exist in the vaccine and processing them and then presenting them to T and B cells.

Specific cellular immunity against endogenous viral proteins is usually very low, especially since inactivated vaccines contain a large amount of HA (and a small amount of NA). Thus, production of high amounts of HA specific antibodies is induced by inactivated vaccines. However, specific T cell mediated cellular immunity (which is stimulated by the presence of intrinsic viral proteins) is not sufficiently developed, because of the very little amount of endogenous viral proteins in inactive vaccine contents. Therefore, inactivated vaccines can not provide broad protection against other viral antigens successfully. In addition, analyses show that all the three types of inactive influenza vaccines induce similar levels of immunogenic responses in the human body. However, some studies point out that whole inactivated influenza virus vaccines can induce slightly more broad antibody responses than split and subunit vaccines. Also, since split virus vaccines are less purified compared to subunit vaccines, it has been observed that they can mimic the natural infection better and develop a higher immune response, since other viral proteins can also be found in the vaccine [7,12,23,49-51].

5.1.2. *Live attenuated influenza vaccine*

Live attenuated vaccines are vaccines developed to provide more effective immunity on the grounds that they will better mimic real virus infection. Thus, the immune response via attenuated vaccines is very similar to natural infection. Also, the route of administration of attenuated vaccines, especially for influenza, was developed accordingly. While all inactivated vaccines are usually administered intramuscularly (there is only one inactivated influenza vaccine administered intradermally, Fluzone Intradermal®), attenuated influenza vaccines are administered as intranasal. This route of administration helps to imitate a natural infection from the moment viruses enter the body. Thus, both mucosal and systemic immunity are stimulated [5-7,23,49,50,53].

The first step in the production of attenuated vaccines is to co-infect the selected candidate vaccine viruses with the virus called "master donor". Master donor viruses are influenza viruses that are passaged for long periods in the cold (at 25°C) and lose their ability to infect humans. These viruses are also called "cold-adapted" viruses. There are two master donor viral strains that have long been used in the production of live attenuated vaccines for influenza A and B viruses in Europe and America. These are A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66 [7,54,55]. As a result of this co-infection, a new 2+6 reassortant virus is created from a candidate vaccine virus and master donor viruses whose infect the same cell. This virus also has the HA and NA gene segments belonging to the selected candidate vaccine virus, and the remaining 6 gene segments from the main donor virus (Fig. 5). This selected new virus

is also called "inoculation strain" or "inoculation virus". This new reassortment virus can induce similar immune responses as WHO selected candidate vaccine virus, while it also has the ability to replicate at low temperatures of the master donor strain from which it receives other gene segments. After the inoculation strains are obtained, these viruses are propagated and purified in eggs or cell cultures and prepared in the appropriate formulation [6,7,23,27,55].

Since the temperature of the human nose and trachea parts is below the normal human body temperature (30-33°C) due to air flow, this inoculation virus can only reproduce here. In this way, although the inoculation virus is an infective virus, it will only reproduce in the upper respiratory tract and it will not be able to reproduce in the lower respiratory tract or any other part of the body. The live attenuated influenza vaccines safety based on this [7,54,55].

Live attenuated vaccines are the best vaccine type that mimics the actual viral infection. They are especially preferred in infants because to form the initial basis for their first immune response to the influenza virus. However, there are three concerns regarding the safety of attenuated influenza vaccines. These are;

- Since the application site of the vaccine is very close to the central nervous system, the brain and nervous system are affected by the vaccine side effects or possible high immune response,
- A mutation occurs that will make the live virus given as a vaccine again highly pathogenic,
- The formation of new and highly pathogenic viruses as a result of reassortment with another possible influenza virus in the human body during or after vaccine production [6,7,12,55].

5.1.3. *Recombinant influenza vaccine*

Currently, the first and only recombinant vaccine, Flu Blok®, was approved by the Food and Drug Administration (FDA) for commercial use in 2013. Firstly, in the production process of this vaccine, viral gene sequencing has to be made. After sequencing of the viral genes, HA genes from the candidate vaccine viruses (which were selected by the WHO) were identified. Then, HA gene carrier baculoviruses are produced via plasmids and these HA gene carrier baculoviruses are used to infect the Sf9 cells in culture medium. As a result of this infection candidate vaccine viruses HAs are expressed by Sf9 cells. Finally, when production is done purification and formulation are carried out. In addition, when we look at the production process, it can be said that this type of vaccine is a subunit vaccine. So, they can also be mentioned as "recombinant subunit vaccines" (Fig. 5). Although the mechanism of action of this vaccine is similar to that of inactivated subunit vaccines,

unlike them, the HAs produced with these systems have higher purity. However, these higher purity levels cause much lower immunogenicity than inactivated ones. For this reason, recombinant influenza vaccines have recently been presented to the market with only partial purification instead of higher purification [12,23,42,49,50,53,56-58].

5.2. Current strategies for better influenza vaccines

Different strategies are being tried to be implemented by focusing on increasing the protective effectiveness and longevity of protection of influenza vaccines. In order to increase the efficacy of commercially available vaccines, adjuvants were tried in the first place among these strategies. Because, adjuvants are well-known systems and they were primarily used as excipients in vaccines for many years. Besides the use of adjuvants, different approaches and strategies are being developed to create more strong and specific immune responses. Therefore, these are mostly targeting the viral proteins of the influenza virus, which are known they can efficiently stimulate the immune system. As a result of these strategies, new vaccine candidates are showing up [14-16].

5.2.1. Adjuvants (or immunomodulators)

Adjuvants can enhance the immunostimulating effects of viral antigens and their safety has been proved in many studies. They can create strong immune responses because they are ligands of TLR. For instance, an influenza vaccine (sub-unit Fludax®) containing the adjuvant MF59 found in seasonal flu vaccines is now licensed and released to the market (Table 1). This vaccine is used, especially in elderly individuals to increase the immunogenicity of HA in the vaccine. In addition to the MF59 adjuvant, Alum, AS03 and AF03 adjuvants were also used in the H1N1 vaccines, which were produced with emergency approval in the flu pandemic in 2009 [39,42,50,53].

5.2.2. NA-targeted approach

After the administration of influenza virus vaccines, NA-specific antigens are also produced. However, their titers are quite low when they compared with HA-specific antibody titers. The main reason for this is the immunodominance mechanism which is mentioned before. Importance of NA-specific antibodies has been neglected for a long time. Because, even if they have high potential for stimulation of the immune system, the main mechanism behind virus-cell entry is directly related to HA. Thus, NA-specific antibodies usually slow infection and do not fully prevent cell entrance of the virus. However, it has been shown that they are very important in the release of new viruses formed in the host cell. Thus, if viral NAs are inhibited by NA-specific antibodies, the newly synthesized viruses cannot be released

from the host cell. This leads to the ease of fighting viral infections for the immune system. In addition, NA antigenic regions are more stable than HA. Thus, with NA-specific cross-protective antibodies, broader protection can be provided in the long-term. For this reason, studies have been carried out in recent years to develop more effective influenza vaccines that can stimulate the production of NA-specific antibodies by the immune system. In this sense, two strategies are being considered. One of these is vaccines which only contain recombinant NA, and the other one is adding the high amount of recombinant NA to conventional vaccine formulations [14,16,42,53,56].

5.2.3. M2-targeted approach

The M2 protein is a very important transmembrane protein for influenza virus infection. Studies have shown that, this protein's antigenic regions are highly conserved, especially in influenza A viruses. Also, this protein exhibits good antigenic properties. For this reason, many studies have been carried out in recent years to develop a vaccine which can stimulate a specific immune response against M2. In these studies, especially, the M2e protein draws all the attention. Because, antibodies can easily access to the this region, when compared with the intrinsic viral proteins. In particular, positive results were obtained in studies made by over-M2-synthesizing virus-like particles (VLPs) on their surface. There are also vaccine trials by creating a fusion protein of the M2e peptide with flagellin, which has a high immunogenic effect, and production trials of M2-encoding RNA vaccines. Both high antibody and cellular (via Tc cells) responses were obtained in these trials [15,16,23,59,60].

5.2.4. Intrinsic viral proteins of influenza virus

Intrinsic proteins of the influenza virus were generally much more highly conserved than its surface proteins. This makes them potential antigenic targets for the universal influenza vaccine approach. However, since these proteins are not usually present on the surface of the virus, they are not easily accessible by the immune system. On the other hand, as mentioned earlier, they can be presented directly on MHC-I on the surfaces of the influenza-infected cells. In particular, it has been shown that NP and M1 can induce high T cells response. Therefore, especially NP and M1 proteins or their combinations are great for stimulation of strong cellular immunity. In addition, this also makes them excellent antigenic targets for creating universal influenza vaccines [15,23,42,52,59].

5.2.5. HA-targeted approaches

Although HA is the most ideal antigenic target for the influenza vaccine development studies according to the immunodominance mechanism, this protein's ever-changing

structure makes it difficult. Especially the head region of HA changes very quickly in antigenically. Thus, mismatching occurs very often for existing antibodies. For this reason, HA's continuously changing head region is excluded from the HA-based new vaccine development studies and different approaches are tried for the other antigenic regions of HA. These approaches have also been included in universal flu vaccine development studies [15,53,59].

5.2.5.1. Chimeric hemagglutinins (cHA)

This approach aims to increase antibodies against the HA stem (or stalk) region, which is much more conserved than the HA head region, with serial vaccinations. In this approach, H1 or H3 subtypes head region is replaced with the head region of other HA subtypes such as H5, H6 or H7 (they are called exotic head regions) which they are not normally seen in human influenza circulation. These are called cHA. In this process, different cHAs obtained by changing the head region constantly. However, each cHA's stalk region remains the same here. After production, each different cHA is made into a vaccine separately. Then these vaccines are applied to the person in series. This approach aims to harness the immune system's tendency to continuously expand pre-existing memory responses. Thus, while the body tries to develop new antibodies against the renewed head region each time, stalk region-specific antibodies are secreted for a rapid immune response. As a result of this, stalk region-specific antibodies titer is elevated after each vaccination [12,14,15,23]. In addition, the immune system also tries to develop an immune response to these exotic head regions. Therefore, this approach can also create an immune response against exotic HA subtypes. It has also been shown, the use of H5 and H7 in these vaccines provide protection against avian influenza viruses [15,16,42,53].

5.2.5.2. Mosaic hemagglutinins (mHA)

This approach is very similar to the cHA process. The main difference here is that only the antigenic parts are changed, not the whole HA head region, in each cycle. Here, the antigenic parts in the head regions of circulating human influenza viruses are replaced with the antigenic parts of exotic HAs. These are called mHAs. Again, the main goal in this approach is increasing the antibody response against the HA stalk region with the serial vaccinations [15,16,23,61].

5.2.5.3. The using Computationally Optimized Broadly Reactive Antigens (COBRA) approach for HA

COBRA is another HA-based vaccine strategy. This approach aims to design a new and universal HA by scanning the consensus genetic sequences of all HAs of the influenza

viruses. According to this approach, if individuals are vaccinated with COBRA-based influenza vaccine, which contains universal HAs, their immune system should also develop universal antibodies. Therefore, it is thought that these universal antibodies can provide protection against all influenza virus HA subtypes. Additionally, successful results have also been obtained in studies using this approach *in silico* [15,61].

5.2.5.4. HAs without head region

Another alternative approach is to completely remove the head region of the HA. In this context, the stalk region of HA (which is highly conserved) becomes visible or accessible by the immune system. However, these structures are difficult to construct because HAs without head regions are very unstable and they are prone to misfolding. This misfolding causes the formation of variable regions which are not actually exist in the HA stalk region. This cause unnecessary development of misled antibodies. In a few studies, this unstable headless HA structure was stabilized with different methods. In these studies, it has been reported that stalk region-specific antibodies can be developed when these headless HAs are used as vaccine [15,53,59,61].

5.3. New candidate influenza vaccines

Although current influenza vaccines provide good immunity, vaccine development studies are still continuing due to some problems. These problems are; the need for annual renewal of influenza vaccines, the low protection of some of them, disadvantages in current production systems, safety problems, inability to create a sufficiently high cellular immune response, and provide a very short immunity period, respectively [15,56,57]. Vaccine development efforts are generally focused on reducing the cost and production time of vaccines, stimulating both humoral and cellular immunity for a much more effective and long-lasting response, and identifying conserved antigenic regions of the influenza virus. For this reason, different strategies have been used to develop better influenza vaccines [15,39,42,62], which is mentioned above. In this sense, various studies have been carried out, and as a result of these strategies, many different new candidate vaccine types have been developed. Some of these have achieved high success experimentally and some of them have begun clinical trials (Table 2 [63]). Apart from these new vaccines, the ideal point wanted to be reached in influenza vaccines is "universal" influenza vaccines. Universal influenza vaccines aim to end the production of influenza vaccines with different virus strains obligatory for every year, and to provide immunity against all influenza virus types or subtypes with a single shot [12,16,49,53,59].

Table 2. New candidate influenza vaccines in clinical trials [63]

New candidate influenza vaccines	Candidate vaccine name	Candidate vaccine description	Vaccine target	Clinical development stage	Sponsor	Clinical trial identifier
Virus-like particles (VLP)	FLU-A	M2e fusion with hepatitis B virus core protein (HBc) expressed in <i>Escherichia coli</i>	M2e	Phase 1	Sanofi	NCT00819013
	Uniflu	Four copies of conserved regions of M2e selected from human influenza A viruses fused with HBc (all proteins expressed in <i>E. coli</i>)	M2e	Phase 1	VA Pharma Limited Liability Company	NCT03789539
	Monovalent Avian Influenza VLP (H7N9)	HA and NA from A/Anhui/1/13 (H7N9) strain with M1 from A/Indonesia/5/05 (H5N1) VLP produced in Sf9 cell line via baculoviruses	HA, NA, and M1	Phase 1	Novavax	NCT01897701
	Quadrivalent VLP (QVLP) Influenza Vaccine	Four different HA from A/California/07/2009 H1N1, A/Hong Kong/4801/2014 H3N2, B/Brisbane/60/08 and B/Phuket/3073/2013 strain containing VLPs produced in <i>Nicotiana benthamiana</i>	HA	Phase 3	Medicago	NCT03321968
Nucleic acid-based vaccines	mRNA-1010	HA encoding mRNA (seasonal type viruses)	HA	Phase 2	ModernaTX, Inc.	NCT04956575
	VAL-339851	HA encoding mRNA (H7N9 avian influenza virus)	HA	Phase 1		NCT03345043
	VAL-506440	HA encoding mRNA (H10N8 avian influenza virus)	HA	Phase 1		NCT03076385
	VGX-3400	DNA plasmids encoding the HA, NA, and M2e-NP antigen of the H5N1 avian influenza virus	HA, NA, M2e, and NP	Phase 1	GeneOne Life Science, Inc.	NCT01184976
	INO-3401	DNA plasmids encoding the HA, NA, and NP antigen of the H5N1 avian influenza virus	HA, NA, and NP	Phase 1	Inovio Pharmaceuticals	NCT01403155
Viral vector vaccines	AVX502	Replication deficient alphavirus replicon vector system expressing HA from influenza A/Wyoming/03/2003 (H3N2)	HA	Phase 1-2	AlphaVax, Inc.	NCT00440362 NCT00706732
	NasoVAX	Replication deficient Adenovirus type 5 (Ad5) displaying the full-length HA from an A/California/04/2009(H1N1)-like influenza strain produced in PER.C6 cell line	HA	Phase 2	Altimmune, Inc.	NCT03232567
	MVA/ChAdOx1-NP+M1	Chimpanzee origin adenoviral vector ChAdOx1 and Modified Vaccinia Virus Ankara (MVA) displaying NP and M1 proteins	NP and M1	Phase 1	Jenner Institute and University of Oxford	NCT01818362 NCT01623518
	MVA-NP+M1	MVA viral vector (produced in the novel immortalised duck retinal cell line AGE1.CR.pIX) expressing the influenza antigens NP and M1 as a fusion protein	NP and M1	Phase 1-2	Vaccitech and University of Oxford	NCT03277456 NCT00993083 NCT03883113
	VXA-A1.1	Adenoviral vector sytem expressing HA from A/California/04/2009 (H1N1) oral influenza vaccine	HA	Phase 1-2	Vaxart	NCT03121339 NCT02918006

Table 2. Continued

New candidate influenza vaccines	Candidate vaccine name	Candidate vaccine description	Vaccine target	Clinical development stage	Sponsor	Clinical trial identifier
Protein or peptide-based vaccines	Multimeric-001 (M-001)	Nine peptides which are highly conserved regions of linear epitopes of HA, NP and M1 from seasonal and pandemic influenza viruses	HA, NP, and M1	Phase 3	BiondVax Pharmaceuticals and National Institute of Allergy and Infectious Diseases (NIAID)	NCT03450915
	FLU-v	Equimolar mixture of four synthetic polypeptides from conserved regions of NPA, NPB, M1 and M2 viral proteins	NPA, NPB, M1, and M2	Phase 2	PepTcell Limited	NCT02962908
	FP-01.1	Six synthetic polypeptides selected from highly conserved regions of NP, M1, PB1 and PB2 viral proteins across H1-H9 subtypes	NP, M1, PB1, PB2	Phase 1	Immune Targeting Systems Ltd	NCT01701752
	VAX102	Recombinant fusion protein that links four copies of the influenza virus M2e antigen to <i>Salmonella typhimurium</i> flagellin	M2e	Phase 1-2	VaxInnate Corporation	NCT00921947 NCT00921973 NCT00921206
	VAX125	Recombinant fusion protein that consists of <i>S. typhimurium</i> flagellin and globular head of the HA1 domain of the A/Solomon Islands/3/2006 (H1N1) influenza virus	HA	Phase 1-2		NCT00730457 NCT00966238
	D-SUIV (GSK38163 02A)	Chimeric HAs (cH8/1N1, cH5/1N1 and cH11/1N1) with conserved stalk region and headless HAs (adjuvanted or not adjuvanted)	HA	Phase 1	GlaxoSmithKline	NCT03275389
Nanoparticle-based vaccines	FluMos-v1	Lumazine synthase based nanoparticles with different HAs from circulating seasonal viruses on its surface	HA	Phase 1	National Institute of Allergy and Infectious Diseases (NIAID)	NCT04896086
	H1ssF_3928	Ferritin based nanoparticles with stabilized H1 subtype HAs stalk region on its surface	HA	Phase 1		NCT03814720 NCT03186781
	HA-F A/Sing	Ferritin based nanoparticles with A/Singapore/1/57 (H2N2) strain influenza virus HA on its surface	HA	Phase 1		NCT03186781
	OVX836	oligoDOM® self-assembling nanoparticle technology with NP influenza virus protein from A/WSN/1933 (H1N1) strain	NP	Phase 2	Osivax Ltd with Centre for vaccinology (CEVAC), University of Ghent	NCT05060887
	Nano-Flu	Polysorbate 80 (PS80) based nanoparticles with the four influenza strains HAs recommended by WHO (for the 2019-20 Northern hemisphere influenza season) produced in Sf9 cell line via baculovirus and Matrix-M1 adjuvanted	HA	Phase 3	Nanovax	NCT04120194

HA: hemagglutinin, NA: neuraminidase, NP: nucleoprotein, NPA: influenza virus A nucleoprotein, NPB: influenza virus B nucleoprotein, M1: matrix protein 1, M2: matrix protein 2, M2e: matrix protein 2 ectodomain, PB1 and PB2: viral RNA polymerase components (polymerase basic 1 and polymerase basic 2), Sf9: *Spodoptera frugiperda* derived insect cells, WHO: World Health Organization.

5.3.1. VLP

VLPs have morphological and structural features similar to viruses but lack the viral genome. Therefore, they cannot cause problems such as infection and reassortment. In addition, since they are in the form of a complete virus, the immune system recognizes them as viruses. In a study, it was shown that high immunity is provided with VLPs containing only viral envelope components (HA, NA, and M2). Similar to recombinant productions, VLPs are obtained by transfecting the structural proteins that make up the VLP into a cell on expression vectors and expressing them by the cell [15,23,42,49,50,57,58,60].

5.3.2. Nucleic acid-based vaccines

Nucleic acid-based vaccines include DNA and RNA vaccines. On this platform, the genes encoding the desired antigenic region of the influenza virus are sequenced and then converted into DNA or RNA and become a vaccine. Especially, for RNA vaccines only mRNA used for this purpose. Because, mRNAs are the fundamental molecules in the cellular protein expression mechanism. Therefore, mRNAs are the most suitable formats for triggering expressional mechanisms for producing the antigens which have the capability of inducing the immune system. In this context, mRNA which has the code of the desired antigenic protein is given in the vaccine to the body. Then, these mRNAs are taken into human cells and the desired viral antigenic proteins are expressed by these cells. Afterward, the immune system is stimulated by these synthesized antigenic viral proteins. In DNA containing (in plasmid form) nucleic acid vaccines have a similar mechanism. The only difference here is that mRNAs carrying the antigenic viral protein codes are synthesized over this DNA given into the cell this time. In addition, generated DNA or mRNA molecules which are produced for use as vaccines can be produced very quickly, independently of cell culture or egg production, by means of special nucleic acid sequencing and amplification devices/techniques. They are the most advantageous systems in the mean of rapid vaccine development, especially in major pandemics [23,49,50,52, 57,60].

5.3.3. Viral vector vaccines

Viral vector vaccines are vaccines produced by expressing the antigenic parts of the virus to be used as a vaccine on another carrier virus that does not show pathogenicity in humans. There are some successful studies done in this area. One of them was used Modified Vaccinia Virus Ankara (MVA) and in this study they synthesized the HA, M1, and NP viral proteins from the influenza virus on MVA. In an other study, they developed adenoviruses that expressed the HA antigen on the surface of the virus.

When the potential of using these viral vectors created in both studies as a vector vaccine was investigated, it was observed that they provided a very high immunity against influenza virus [15,57,64]. This is because these vector vaccines stimulate both humoral and cellular immune responses, mimicking natural infection. Also, most viral vectors lack replication in mammalian cells and are therefore safe to use. However, previous immunity against the carrier vector virus may cause problems. Because when these vectors enter the body, the immune system quickly recognizes the antigenic parts of the carrier virus and ignores the antigenic influenza virus parts that are expressed on the carrier virus for the rapid protective reaction. As a result of that, the immune response will mostly be against only to the carrier virus antigenic parts instead of for the specifically expressed influenza virus part (or parts) by immune system [39,50,57,60,64].

5.3.4. Protein or peptide-based vaccines

In protein or peptide-based vaccines, the peptides or the proteins structures which have antigenically important is used. Especially, the conserved regions, chimeric proteins such as cHA and the unfolded whole proteins are used in these vaccines. These protein or peptide structures are synthesized by synthetically or via a host organism. When a host organism is used for this production recombinant techniques are highly involved in the process. Therefore, these vaccines can also be mentioned as “recombinant protein-based” vaccines. In particular, it has been observed that a good immune response is obtained as a result of combining the synthesized peptides of NP and M1 proteins, which induce the high involvement of CD8 cytotoxic T cells in the immune response, with adjuvants or by transporting them in/on the surface of nanoparticles or VLP's [14,15,50, 59,65].

5.3.5. Nanoparticle-based vaccines

Nanoparticle vaccine technology is similar to the VLP platform. However, instead of a structure consisting of viral proteins, different components (such as gold, silver, and chitosan) at the nanometer level are used here. Also, unlike VLP, most nanoparticles alone can not trigger an immune response. Nanoparticles are actually carriers that are used to increase the immunogenicity of antigenic viral proteins. For the production of nanoparticle-based vaccines, targeted viral proteins (HA, NA, NP, M2, *etc.*) are needed primarily to be used as vaccines. These proteins are then either attached to the surface of the nanoparticles or trapped inside them. While virus-like structures are created by the surface binding method, viral infection is imitated by incorporating the nanoparticle with viral proteins into the cell by the confinement method [14-16,49,66].

5.4. Production methods of commercially available influenza vaccines

The most effective method against the viruses, including influenza virus, is vaccines in terms of preventing the disease directly before it occurs. Also, vaccines are the least expensive way to fight with the flu, while considering the cost of antiviral drug treatments, hospitalization, and healthcare worker service fees. For this reason, vaccine production is an area that has been and continues to be emphasized for years [6,19,39,51,67,68].

Conventional influenza vaccines are designed to only contain the current circulating virus types. Thus, basically two different vaccine formulations are produced in terms of viral content. These are trivalent and quadrivalent vaccines. Trivalent vaccines are designed for protection against H1N1, H3N2 influenza A subtypes and influenza B Victoria lineage. Additional to trivalent vaccines, only influenza B Yamagata lineage is added to the quadrivalent vaccine composition [7,49,59]. Many influenza vaccines were produced as trivalents in the past. However, since quadrivalent vaccines provide a broader spectrum of protection, in recent years, most of the influenza vaccine is produced as quadrivalent [13,49,50].

The targeted antigenic part of the influenza virus is HA in all influenza vaccines produced today. Because, according to the immunodominance mechanism, the immune system gives the strongest response against the head region of HA than other antigenic parts of the virus. Nonetheless, while the head region of HA is a very good target for the immune system, this region antigenically changes very quickly. So, this situation makes it necessary to prepare vaccines annually. At the same time, most of the conventional vaccines do not contain only HA. Many commercially available vaccines also contain large amounts of NA and different antigenic regions of the influenza virus. However, calculations are still being made based on the amount of HA in the vaccine, while determining the appropriate dose in vaccine formulations [7,42,52,53,58].

Most dominant antigenic parts of the virus are HA and then NA. These are glycosylated proteins. Post-translational mechanisms in host cells are very important for these proteins that gain functionality thanks to the glycosylation process. Therefore, this situation makes influenza viruses species-specific. Thus, they can only infect developed eukaryotic vertebrates [11]. On the other hand, due to the parasitic nature of viruses, they cannot reproduce on their own. So, they need living organisms or cells that they can infect. For this reason, in all productions, a suitable host organism or cell should be selected appropriately. Thus, the virus can infect or properly express its antigenic structures in terms of immunogenicity. In this context, basically 3 production methods are used for all conventional vaccines

produced today. These are: (1) production in specific pathogen-free (SPF) embryonated eggs (in short, egg-based), (2) cell culture-based production, and (3) production on a recombinant expression platform (Fig. 6). All these production platforms are selected from cells that can perform the post-translational modifications required for HA and NA and allow the influenza virus infection [1,6,11,51,53-55,67,68].

Egg-based production is used for the influenza vaccines for nearly 70 years. In this method, since the viruses selected for vaccine production are human influenza viruses, they generally need to be adapted to produce in eggs. Because, human viruses are accustomed to human mammalian cells. In particular, the H3N2 virus reproduces very little in the egg. Since the SPF eggs used in egg-based production contain cells belonging to the bird species, a new inoculation strain has to be created for an effective viral infection. For this reason, the egg is infected simultaneously at the same time with selected candidate vaccine virus by WHO and an influenza strain (this is called a high-producing strain) that shows very good replication in the eggs. The aim of this co-infection process is to create new viruses that are antigenically similar to the candidate vaccine viruses, while they are able to reproduce in high quantities in the egg. A high-producing virus strain A/Puerto Rico/8/1934 (H1N1) (abbreviated PR8), which replicates in eggs very efficiently, is used for this co-infection for a long time [6,51,68].

As a result of this co-infection process, many new viruses emerge. However, among these, a special 2+6 reassortment virus must be selected. The expression of “2+6 reassortment virus” means this special virus must have HA and NA gene segments from the candidate vaccine virus and its remaining 6 gene segments from the high-producing strain. This special reassortment virus is selected by elaborate screening. This selected virus is called the “inoculation strain”. After the inoculation strain is determined, actual production takes place. For this production, many SPF eggs are infected with the inoculation strain. This process begins with the inoculation of the inoculation strains into the allantoic fluid of the SPF eggs. The virus replicates in large numbers here. Then, in order to obtain the viruses produced here, allantoic fluids of all eggs whose infected with the inoculation strain are collected. After that, other separation/purification processes and formulation processes are performed based on which type of vaccine (inactive or attenuated) to be produced (Fig. 6). All of these processes are performed separately for all virus types and subtypes that will be included in the vaccine. Finally, all viruses produced are combined according to the determined formulation of the vaccine. Additionally, almost all influenza vaccines contain equal amounts of all virus types and subtypes [6,7,13,50, 51,55].

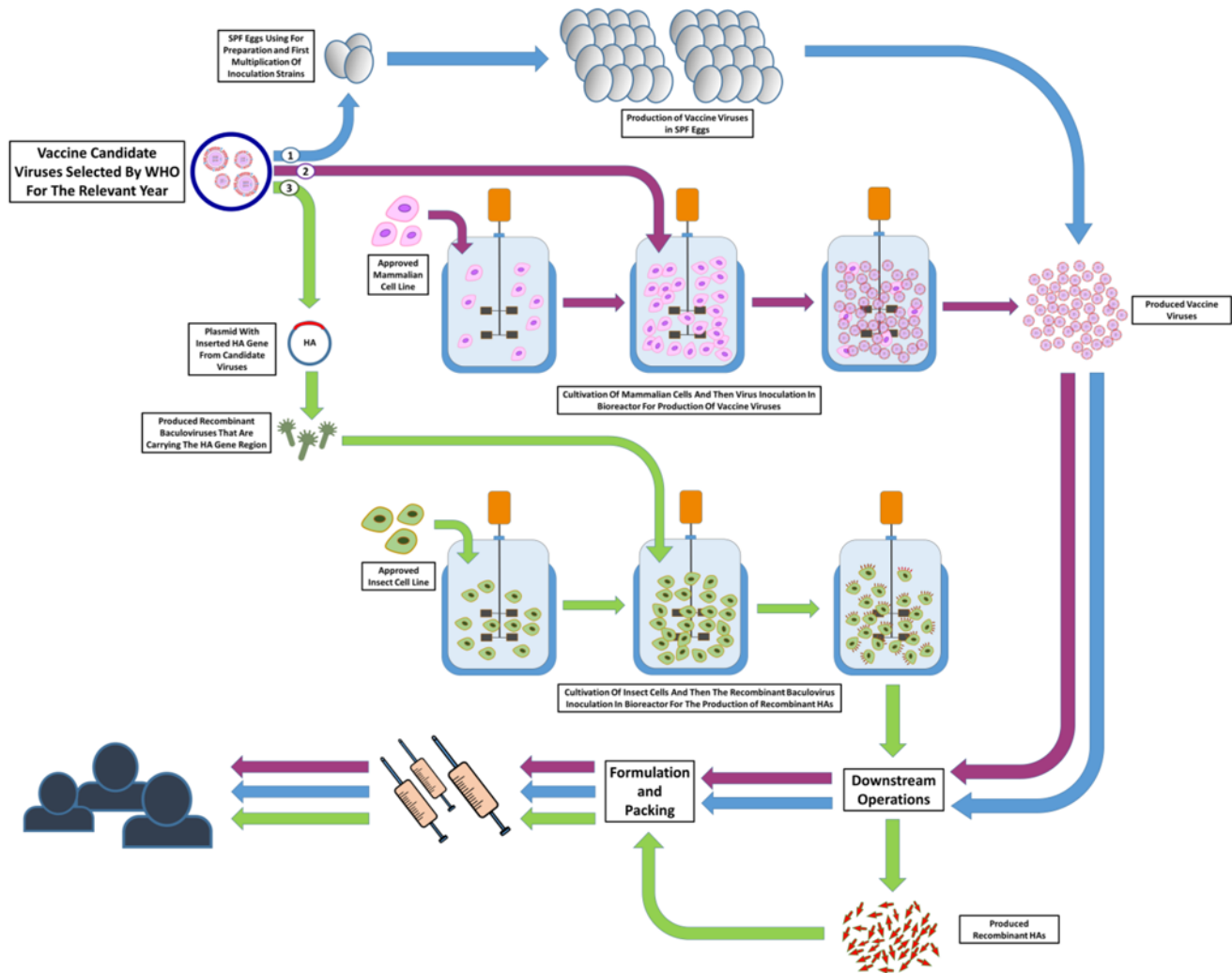


Fig. 6. Influenza vaccine production methods flow chart. 1) Egg-based vaccine production (blue line); 2) Cell culture-based vaccine production (purple line); 3) Recombinant vaccine production (green line). WHO: World Health Organization, SPF: specific pathogen-free, HA: hemagglutinin.

Although egg-based vaccine production has been used very efficiently for a long time, it has some disadvantages. These are (i) the need for continuous SPF eggs (using at least 1 or 2 eggs for a dose of vaccine), (ii) individuals with egg allergies (allergic reaction to the vaccine), (iii) doubting the cleanliness of eggs in an avian flu-based pandemic, and (iv) difficulty in supplying clean eggs. However, egg-based influenza vaccine production is still the most preferred production method in worldwide. The main reason for this is that large companies do not want to change their production systems that are established and already meet Good Manufacturing Practice (GMP) standards. Also, the agreements they have made with the companies that supply the SPF eggs used in these productions are another reason for this. On the other hand, there has been a search for overcome the disadvantages of the egg-based flu vaccine production among manufacturers and researchers around

the world search for different production platforms. The most suitable among these is cell culture-based productions. The first cell culture-based flu vaccine in the world was released by Novartis in Europe under the name Optaflu®, approved by European Medicines Agency in 2007, and in the USA under the name Flucelvax® (today, the production rights of this vaccine have been acquired by Seqirus Company) with FDA approval in 2012 [7,51,55,60,67-70].

In cell culture-based productions also, an inoculation strain can be created for a higher yield production, or production can be started directly with a candidate vaccine virus selected by the WHO. Generally, in the cell culture-based production process, viruses are directly inoculated into the culture medium. Afterwards, the cells inoculated with the virus are cultured for a certain period of time, and the virus is expected to reach high amounts and production is ended. Then, suitable downstream processes are applied

according to the formulation and type of vaccine to be produced, as in egg-based production (Fig. 5). Canine kidney epithelial cell line (Madin-Darby Canine Kidney, MDCK) is the most used cell line in cell culture-based influenza vaccine production. However, it has been shown that the human cell lines such as human embryonic retinal cell line (PER.C6) and the human embryonic kidney cell line (HEK-293), can also be used in influenza virus production in recent studies. In addition, cell culture systems provide an advantage in terms of quality standardization due to the fact that production is carried out in bioreactors that require less labor and provide a more controlled and sterile environment compared to egg-based production. In particular, they are preferred because they minimize the processing area required for production [15,23,49,51,54,55,60,67,70,71]. In a study, for the production of a certain amount of vaccine; operating a 1,000 L stirred tank bioreactor (STR) or 12,000 roller bottles was found sufficient for cell culture-based systems, while 30,800 eggs are needed for the egg-based production of the same amount of vaccine was calculated [60].

Vaccine production on a recombinant expression platform is a new approach compared to others. Flu Blok®, the first and only recombinant influenza vaccine in the world, was licensed in 2013 and approved by the FDA. In this recombinant vaccine produced, an insect cell line Sf9 (a cell line derived from *Spodoptera frugiperda* cells), is used as an expression system. In this production, the Sf9 cell

line is first transfected with baculoviruses carrying the HA gene. Then, these cells are grown in bioreactors, and then purification processes are applied to obtain this HA at the end of production (Fig. 6). Although the production of recombinant products is usually carried out on easier and cheaper expression platforms such as *Escherichia coli*, eukaryotic expression platforms that can synthesize viral proteins in the appropriate antigenic structure are needed for influenza virus production [23,39,52,58,60,67].

5.5. Types of bioreactors used in cell culture-based influenza vaccines production and case studies

Vaccines produced in embryonated eggs have serious problems such as the difficulty of finding SPF eggs, and the burdensome procedures, as well as allergic reactions that may develop in susceptible individuals [72]. On the other hand, cell culture systems provide an advantage in quality standardization because production is carried out in bioreactors that require less labor and provide a more controlled, and sterile environment than egg-based production. In addition, production time and production costs, which are critical targets in vaccine production, are reduced [73]. In conventional cell culture-based influenza vaccine production and other cell culture-based vaccine production studies, different types of bioreactors are used. These are; STR, wave bioreactor, orbitally shaken bioreactor, single-use bioreactor, and hollow fiber bioreactor (HFBR) (Table 3). In addition, disposable bioreactors have been an innovative

Table 3. Overview of small-scale bioreactor studies for production of different influenza viruses reported in the literature

Produced Influenza Virus	Type of Bioreactor	Production Mode	Cell Line	Cell Proliferation Character	Maximum Influenza Virus Production Efficiency That Was Reached	Reference
A/PR/8/34 (H1N1)	STR/Plug Flow Bioreactor	Continuous	MDCK.SUS2	Suspension	7.7×10^{10} virions/hour	[75]
	STR (Stainless steel and Single-use)	Batch	MDCK.SUS2	Suspension	For Stainless Steel: 3.8×10^{10} virions/hour For Single-use: 4.7×10^{10} virions/hour	
A/Wisconsin/67/2005 HGR (H3N2)	STR	Batch	MDCK Vero	Adherent	10,000 virions/cell 4,976 virions/cell	[76]
A/PR/8/34 (H1N1)	Wave (Wave Biotech® AG)	Batch	MDCK Vero	Adherent	33,255 virions/cell 3,990 virions/cell	[77]
A/PR/8/34 (H1N1)	Wave (Wave Biotech® AG)	Batch	MDCK.SUS2	Suspension	7,000 virions/cell 5,000 virions/cell	
B/Malaysia/2506/2004	STR	Batch Fed-Batch Perfusion	PBG.PK2.1	Suspension	5,375 virions/cell	[78]
A/PR/8/34 (H1N1)	STR				5,006 virions/cell	
A/PR/8/34 (CAP adapted)	STR				3,929 virions/cell	
A/PR/8/34 (H1N1)	STR	Batch	CAP	Suspension	6,400 virions/cell	[79]
A/PR/8/34 (H1N1)	STR	Batch	MDCK	Adherent	22,891 virions/cell	[80]
			MDCK.SUS2	Suspension	11,411 virions/cell	

Table 3. Continued

Produced Influenza Virus	Type of Bioreactor	Production Mode	Cell Line	Cell Proliferation Character	Maximum Influenza Virus Production Efficiency That Was Reached	Reference
A/California/07/2009 (H1N1)	STR	Batch	DuckCelt-T17	Suspension	5.7×10^9 virions/mL	[81]
A/PR/8/34 (H1N1)	STR	Batch	HEK-293	Suspension	5.3×10^{10} TCID ₅₀ unit/mL	[82]
		Perfusion			3.3×10^{11} TCID ₅₀ unit/mL	
A/PR/8/34 (H1N1)	STR	Batch	MDCK	Suspension	10,476 virions/cell	[83]
	ATF/STR	Perfusion			11,690 virions/cell	
A/PR/8/34 (H1N1)	STR with inclined settler	Perfusion	AGE1.CR.pIX	Suspension	3,474 virions/cell	[84]
A/PR/8/34 (H1N1)	STR	Batch	HEK-293	Suspension	643 virions/cell	[85]
A/Sydney/5/97 (H3N2)	STR	Perfusion	PER.C6	Suspension	10^{10} TCID ₅₀ unit/mL	[86]
H1N1 A/Singapore/1/57 ca × A/Singapore/ 2339/2000 (Cold adapted live attenuated virus)	STR	Batch	AGE1.CR.pIX	Suspension	1×10^8 virions/cell	[87]
NIBRG-14 (derived from A/Vietnam/1194/ 2004 (H5N1))	STR	Batch	MDCK	Adherent	$7.1 - 7.6 \times 10^8$ PFU/mL	[88]
A/Chicken/Guangdong/SS/ 94 (H9N2)	STR	Batch	MDCK	Suspension	13,151 virions/cell	[48]
A/PR/8/34 (H1N1)	Orbital Shaken Bioreactor (Adolf Kühner® AG)	Batch	AGE1.CR.pIX	Suspension	1,286 virions/cell	[91]
	ATF / Orbital Shaken Bioreactor (Adolf Kühner® AG)	Perfusion			3,059 virions/cell	
	TFF / Orbital Shaken Bioreactor (Adolf Kühner® AG)				3,487 virions/cell	
RG6 (derived from A/Anhui/ 01/2005 (H5N1))	BelloCellA® fixed-bed bioreactor	Periodically Medium Immersion	MDCK	Adherent	1519 TCID ₅₀ unit virions/cell	[97]
RG30 (derived from A/ Hubei/1/2010 (H5N1))					2 TCID ₅₀ unit virions/cell	
RG268 (derived from A/Anhui/01/2013 (H7N9))					4 TCID ₅₀ unit virions/cell	
H5N1 clade II (RG6M13C4 derived from A/Anhui/01/ 2005 (H5N1))					12 TCID ₅₀ unit virions/cell	
H5N1 clade II (RG6M13C4 derived from A/Anhui/01/ 2005 (H5N1))	BelloCellP® fixed-bed bioreactor				33 TCID ₅₀ unit virions/cell	
RG268 (derived from A/Anhui/01/2013 (H7N9))	TideCell002® fixed-bed bioreactor				51 TCID ₅₀ unit virions/cell	
RG6 (derived from A/Anhui/ 01/2005 (H5N1))					16 TCID ₅₀ unit virions/cell	
A/PR/8/34 (H1N1)	Hollow Fiber Bioreactor (PRIMER HF®, Biovest International Inc.)	Recirculation	MDCK	Adherent	8,110 virions/cell	[98]
			MDCK.SUS2	Suspension	19,138 virions/cell	
A/Mexico/4108/2009			MDCK	Adherent	84 virions/cell	
			MDCK.SUS2	Suspension	3,219 virions/cell	

STR: stirred tank bioreactor, ATF: alternating tangential flow, TFF: tangential flow filtration, MDCK: Madin Darby Canine Kidney Cells, Vero: African Green Monkey Kidney Epithelial Cells, AGE1.CR.pIX: Modified Muscovy Duck Retina Cells, MDCK.SUS2: Modified MDCK Cells For Suspension Grow, PBG.PK2.1: Derived From Porcine Kidney Cells, CAP: Human Amniocyte Cells, DuckCelt-T17: Derived From *Cairina moschata* Duck Embryonic Cells, HEK-293: Human Embryonic Kidney Cells, PER.C6: Human Embryonic Retinal Cells, TCID₅₀: median tissue culture infectious dose, PFU: plaque forming unit.

alternative to stationary equipment in recent years. It is highly preferred in terms of economy, quality, and ease of use, leaving traditional stainless steel behind for many types of equipment in the production process. Pressure on capital and operating costs, risk of cross-contamination, increase in sterilization and validation costs in vaccine and pharmaceutical productions with conventional equipment systems lead manufacturers away from traditional equipment to disposable systems for production facilities under GMP conditions [74,75].

5.5.1. STR

Cell culture operations are carried out in a variety of STR with well-defined hydrodynamic parameters; these bioreactors may also be employed on an industrial scale. In this system, various studies and modifications are applied that can keep shear forces low and increase oxygen transfer rates to maintain optimal cultivation conditions at large scales [73]. If we look at the studies carried out for the production of influenza vaccine in the STR; In a study by Genzel *et al.* [76], a direct comparison of influenza virus production with adherent MDCK and Vero cells in T-flasks, cylinder bottles, and different laboratory-scale bioreactors (stirred tank and wave bioreactor) was made. Production was carried out in batch mode and 5 L STR, by infecting the A/Wisconsin/67/2005 HGR (H3N2) virus strain with MDCK and Vero cells. As a result of this production, MDCK cells showed 10,000 virions/cell and Vero cells 4,976 virions/cell virus production efficiency. It was shown that MDCK cells were more productive for influenza virus production than Vero cells [76]. MDCK.SUS2 suspended cells cultivated using a microcarrier system in a working volume of 1 L were infected with influenza strain B/Malaysia/2506/2004, and a high viral concentration of 2.75 log HA units/100 μ L titer was established at the conclusion of production. The MDCK cell line was found to be an excellent model cell line for influenza virus generation in this investigation [77].

Gränicher *et al.* [78] conducted research to see if the PBG.PK2.1 cell line, which is derived from immortal porcine kidney cells, could be a new candidate cell line for influenza vaccine production in a 700 mL working volume, using various production modes (batch, fed-batch, perfusion) and the A/PR/8/34 virus strain. The results obtained achieved a maximum HA titer of 3.93 log HA units/100 μ L with cell viability, which is 95%. They have produced viruses at concentrations of 5,375 virions/cell, 5,006 virions/cell, and 3,929 virions/cell in batch, fed-batch, and perfusion systems, respectively. A comparison of the glycosylation pattern of the expressed viral HA antigen with that generated in MDCK and Vero cell lines was

done, and they found significant discrepancies. Ultimately, they demonstrated that PBG.PK2.1 cells might be a good option for the development of next-generation influenza virus vaccines [78]. In another promising investigation, a newly developed CAP cell line originating from human amniocytes was cultivated with A/PR/8/34 (CAP adapted) virus strain in a STR in a 1 L working volume and batch production mode for its potential in influenza virus production. Results obtained resulted in HA titers of up to 3.2 log HA units/100 μ L and maximum cell-specific virus productivity of 6,400 virions/cell. They showed that CAP cells can produce high viral yields for several influenza strains and that they can provide a well-characterized human cell line that meets industrial standards as an alternative to influenza virus vaccine manufacturing [79].

Another study was made by comparing the production efficiency of adherent and suspension adapted MDCK cells in influenza vaccine production. In this context, adherent MDCK cells were adapted to the suspension culture, and their production efficiency was compared with the MDCK.SUS2 cell line. The production studies was made with a 1 L working volume and batch mode STR. As a result, MDCK.SUS2 suspended and MDCK adherent cells, showed similar HA titers of 2.94 and 3.15 log HA units/100 μ L and a TCID₅₀ of 1×10^9 and 2.37×10^9 virions/mL respectively [80].

Suspended DuckCelt-T17 cell line (a new avian cell line developed by Transgene company, produced from primary embryo duck cells with constitutive expression of duck telomerase reverse transcriptase) obtained a virus titer of 5.7×10^9 virions/mL by culturing with A/California/07/2009 (H1N1) virus strain in 400 mL working volume and batch production. The DuckCelt-T17 cell line showed that a suitable alternative model for the industrial production of influenza viruses, particularly avian viral strains, according to the findings [81]. HEK293SF cells have recently been utilized to effectively create influenza viruses, giving the highest HA and infectious viral particle titers ever recorded. Perfusion culture mode was evaluated with batch culture mode in one investigation to boost virus titers at high cell densities. In this sense, HEK-293 cells cultured with A/PR/8/34 (H1N1) virus strain in a 3 L working volume reached titers of 5.3×10^{10} TCID₅₀ units/mL in batch mode and 3.3×10^{11} TCID₅₀ units/mL in perfusion mode. The HEK293SF cell is a good platform for high-throughput influenza virus generation, according to these findings [82].

In another study by Wu *et al.* [83], the production efficiency of A/PR/8/34 (H1N1) infected MDCK cells in STR systems in batch mode and ATF/STR systems in perfusion mode were compared, a virus titer of 11,690 virions/cell was obtained in the ATF/STR system. Also, in

a study conducted by Coronel *et al.* AGE1.CR.pIX cells used for influenza A virus production. For this purpose STR system with inclined settler used and this system operated in perfusion mode. As a result of this study 3,474 virions/cell was obtained [84]. Additionally, there are other promising studies using STR for the efficient production of influenza viruses. In these studies, different cell lines and different influenza viruses (including avian influenza viruses) are used for the virus production [48,85-88].

5.5.2. Wave bioreactor

Over the last few years, disposable bioprocess systems for culture and medium preparation that can be quickly scaled up have seen fast progress in animal cell bioprocess engineering. The wave bioreactor, with a maximum working volume of 500 L, has been in high demand for a variety of applications, ranging from plant cells to insect cells infected with baculoviruses. When the cultivation is done in disposable cell bags, which allows for speedier reactor preparation while eliminating bioreactor system investment expenditures. To mix the culture, cell bags with suspension cells or adherent cells on microcarriers can be shaken at various angles and speeds. Headspace ventilation provides oxygen delivery. For diverse manufacturing processes, different cell bags are now available in varied sizes and materials (*e.g.*, batch, fed-batch, perfusion, ...). Sensors in these flexible bags need the use of specific cell bags, which are now available and allow for simpler pH and pO₂ control as well as perfusion culture [89]. If we look at the studies performed with the wave bioreactor, in a study by Genzel *et al.* [76], MDCK and Vero cells were infected with A/PR/8/34 (H1N1) virus strain in a working volume of 2 L, in batch mode and serum-free medium. MDCK cells provided a virus titer of 33,255 virions/cell, and Vero cells 3,990 virions/cell. These results were compared with the production in the STR and a 3-fold titer difference was found, especially in MDCK cell [76]. A/PR/8/34 infection in the wave bioreactor induced a quick decline in viability and an early increase in HA, with a maximal HA value of 2.90 for this strain 48 h after cell infection. The wave bioreactor was compared to the STR, and the findings showed that the wave bioreactor generated 7,000 virus particles per cell whereas the STR produced 5,000 virus particles per cell [77]. Considering these results, it is seen that the wave bioreactor is an important model bioreactor in influenza vaccine production.

5.5.3. Orbital shaking bioreactors

Perfusion culture is more efficient and adaptable than other growth systems that allow for high cell concentrations (10⁷-10⁸ cells/mL) and volumetric output. Furthermore, in perfusion cultures, product quality can be enhanced by

lowering product heterogeneity or limiting the buildup of growth inhibitors and metabolic waste products. Spin filters [90], tangential flow filtration (TFF) [91,92], or alternative tangential flow filtration (ATF) [78,93,94] were utilized in most perfusion investigations in laboratory-scale bioreactors for viral vaccine manufacturing. In a study conducted for this purpose, AGE1.CR.pIX cells infected with A/PR/8/34 (H1N1) were cultivated in 3 different bioreactor systems and the effects of the systems on efficiency were investigated. In the results obtained; While 1,286 virions/cell was obtained in batch mode in orbital shaking bioreactor, virus titers of 3,059 virions/cell and 3,487 virions/cell were obtained in perfusion mode in orbital shaking bioreactor systems with ATF and TFF, respectively [91].

5.5.4. Disposable fixed bed bioreactors

Disposable bioreactors have cheaper initial costs, are easier to operate, have shorter turnaround times, and have less cleaning verification needs. Cescio Bioengineering, Taiwan, recently created two novel bioreactors: BelloCell (lab scale) and TideCell002 (industrial scale). BelloCell bioreactors have been successfully utilized to culture mammalian cells for HDV (hepatitis delta virus)-like particles, Japanese encephalitis virus, and insect cells for baculovirus synthesis. When we look at the studies, bioreactors have shown efficient properties in studies by providing the ability to collect cells and components with features such as high biomass due to their large surface area, moving the ambient liquid without creating gas bubbles due to low shear stress, and being ready for use and sterile before production [95,96].

One study used H5N1 clade II and H7N9 candidate vaccine viruses cultured in both MDCK and Vero cells. MDCK cells in BelloCellA® fixed-bed bioreactor; RG6 (derived from A/Anhui/01/2005 (H5N1)), RG30 (derived from A/ Hubei/1/2010 (H5N1)), RG268 (derived from A/ Anhui/01/2013 (H7N9)) and H5N1 clade II (RG6M13C4 derived from A/Anhui/01/2005 (H5N1)) virus strains and titers of 1,519 TCID₅₀ units virions/cell, 2 TCID₅₀ units virions/cell, 4 TCID₅₀ units virions/cell and 12 TCID₅₀ units virions/cell, respectively, were obtained. In BelloCellP® fixed-bed bioreactor, MDCK cells were infected with H5N1 clade II (RG6M13C4 derived from A/Anhui/01/2005 (H5N1)) virus strain, and 33 TCID₅₀ units virions/cell virus titer was obtained. In the TideCell002® fixed-bed bioreactor, MDCK cells were infected with RG268 (derived from A/Anhui/01/2013 (H7N9)) and RG6 (derived from A/Anhui/01/2005 (H5N1)) with 51 and 16 TCID₅₀ units virions/cell titers respectively, were obtained. BelloCell is a novel form of bioreactor with a high cell density and the ability to produce viruses. BelloCell may readily be expanded

up to the size of a TideCell system while maintaining identical operating conditions. This innovative bioreactor worked well for adherent cells in the generation of influenza virus, and this study shows how disposable bioreactors may generate vaccines fast and efficiently [97].

5.5.5. HFBRs

HFBRs have long been considered to be capable of producing highly concentrated monoclonal antibodies and recombinant proteins. HFBRs were just recently presented as a novel disposable platform for producing high-titer influenza A virus. A number of hollow fiber capillaries separate the bioreactor in an intracapillary and extracapillary space in these bioreactors. Cells are frequently cultivated in the extracapillary space, where they can reach extremely high cell concentrations. In a study, the virus production activities of MDCK and MDCK.SUS2 cells were compared in a HFBR in recirculation mode, and according to the results obtained, MDCK cells provided 8,110 virions/cell virus titers, while MDCK.SUS2 cells produced 19,138 virions/cell titers, allowing suspended cultures to be grown in this system showed that they are the most efficient [98].

6. Conclusion

Influenza is a highly contagious respiratory disease and flu cases continue throughout the year. Especially, seasonal flu cases usually reach their top point from October through late March or early April. Many people around the world have had the flu at least once in their lives. Also, many people recover from the flu completely within a few weeks. However, especially in the elderly, young children, pregnant women, and those with chronic diseases are at great risk. In these people, the influenza infection can lead to hospitalization and even death. Vaccination has been demonstrated to be the most efficient strategy to protect against the flu in scientific studies all around the world. As a result, it is suggested that who's in the high-risk groups should get the seasonal flu vaccine.

The targeted antigenic viral part of all currently produced influenza vaccines is HA. This antigen has been targeted in conventional vaccine production for many years. Therefore, in conventional vaccines, productions are still carried out based on HA. Vaccine productions are carried out by using egg-based, cell culture-based, and recombinant expression methods. Vaccines produced from the egg-based method have major drawbacks, including the difficulty in finding SPF eggs, as well as the possibility of adverse allergic reactions in susceptible individuals. In comparison to egg-based production, cell culture-based methods can provide

more standard production since production is carried out in bioreactors which have a more controlled environment. Also, in the cell culture-based method, vaccine production requires less labor and process time. Thus, vaccine production time and overall costs can be reduced with this method. In cell culture-based production bioreactors are used and the most commonly studied bioreactor types used in influenza vaccine production are STR, wave, orbital shaking, disposable fixed-bed, and HFBRs.

Vaccine types that are licensed and commercially available against flu include inactivated, live attenuated, and recombinant vaccines. These are produced with appropriate production processes, which are mentioned above, by their type. Even though current influenza vaccines provide good immunity, they have drawbacks. These are the necessity for annual viral strain renewal, low protection due to antigenic mismatching, protection against only certain viral subtypes, drawbacks of conventional production systems, difficulty to develop a sufficient adaptive cellular immune response, and short-term protection. Thus, great efforts to develop better influenza vaccines are being made. For this purpose, different strategies are being implemented and developed by focusing on increasing the efficacy and duration of protection. In this context, very different approaches were tried in various studies. While these studies include more traditional approaches like adding adjuvants to currently produced vaccines, new strategies are also being tried. These new strategies are mostly viral surface antigens (NA, M2, and HA) and viral intrinsic antigens (NP and M1) targeted approaches. Although HA is the most ideal target for inducing the immune response, the constantly changing nature of this antigenic structure complicates this situation. Therefore, in the HA-based studies, the immune system is tried to induce by focusing on conserved regions of HA. As a result of these different strategies, new candidate vaccines have come out. These new candidate vaccines are VLP, nucleic acid-based vaccines, viral vector vaccines, peptide or protein-based vaccines, and nanoparticle-based vaccines. While, most of them are still in the experimental stage, their pre-clinical results are very promising. However, some of these new vaccine candidates have already started clinical trials, and few ones are really close to the commercial stage.

Basically, all current approaches have one common point, and this is developing a vaccine that can provide broad and long-lasting protection against influenza virus infection. For this reason, creating a universal influenza virus vaccine, which protects against seasonal, zoonotic (like avian influenza viruses), and pandemic influenza virus infections for a long period of time, is at the center of all of these efforts.

Ethical Statements

The authors declare no conflict of interest.

Neither ethical approval nor informed consent was required for this study.

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