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DEVELOPMENT OF HIGHLY PATHOGENIC AVIAN INFLUENZA VACCINE FOR PANDEMIC INFLUENZA PREPAREDNESS: A REVIEW

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ABSTRACT

The Highly Pathogenic Avian Influenza (HPAI) H5N1 virus caused extraordinary incidents in several countries, especially Asia, Africa, and Europe. The disease's fatality rate caused by the H5N1 virus reaches 60%. This incident raised concerns about a wider HPAI H5N1 virus pandemic if effective human-to-human transmission occurred. Therefore, the World Health Organization (WHO) calls on all countries to prepare themselves for a pandemic that may occur. One of the strategies in preparation for this pandemic is to develop a vaccine. So far, vaccines are effective in disease prevention efforts because they can work to trigger a natural immune response and induce a memory response against the pathogen in question. Several strategies are needed to develop an effective influenza vaccine in carrying out vaccine development research and in-depth understanding of (i) virology, (ii) virus life cycle, (iii) host immune response to infection, (iv) pathology, and (v) HPAI H5N1 vaccine development strategy. Until now, many HPAI H5N1 vaccines have been developed, from vaccines developed using conventional methods to alternative vaccines using more modern methods. The results of the development of the vaccine are expected to provide provisions for a possible pandemic caused by the HPAI H5N1 virus.

Keywords : *Influenza, virus, vaccine*

INTRODUCTION

Influenza viruses are known as one of the pathogens that can cause epidemics and pandemics (Kamps et al ., 2006; Peiris et al ., 2007). Three pandemics that caused significant morbidity and mortality have occurred, and all three were caused by influenza viruses, namely the pandemic that occurred in 1918 (Spanish flu) by influenza A virus subtype H1N1, 1957 (Asian flu) by subtype H2N2 and 1968 (Hong Kong). Kong flu) by the H3N2 subtype (Peiris et al ., 2007; Gillim-Ross et al ., 2006). Casualties from viral pandemics are estimated at 50-100 million people worldwide (Kamps et al ., 2006; Johnson et al ., 2002). Even in 2009, WHO announced that there had been another pandemic caused by the H1N1 subtype with a different strain from pdmH1N1 in 1918, with 30,000 cases in 74 countries (WHO, 2009).

The HPAI H5N1 case originated from an influenza virus isolated from geese in Guangdong, China, 1996. Then in 1997, there was an outbreak for the first time in a poultry farm and animal market in Hong Kong, and 18 cases were also found in humans; 6 of them died. This case was increasingly

widespread in late 2003 and early 2004. This case did not only occur in Hong Kong but also found similar cases in the southern and southeastern Asian continents, namely South Korea, Japan, Indonesia, Vietnam, Thailand, Laos, Cambodia, and China. (Kamps et al ., 2006; Gillim-Ross et al ., 2006). Until now, HPAI H5N1 cases have occurred in many countries. Not only in Asia but also in Africa and Europe (WHO, 2012).

The HPAI H5N1 virus has caused substantial economic losses because apart from causing disease in poultry and humans, millions of poultry were burned on a large scale during outbreaks. It harms poultry farms (Kamps et al ., 2006; Peiris et al ., 2007). In addition, the possibility of human-to-human virus infection transmission has been reported (Ungchusak et al ., 2005). However, the transmission process from human to human is less effective than from avian to human. However, considering that H5N1 is a new antigen that can mutate, is highly pathogenic to humans, and may acquire the ability to efficiently transmit from human to human, WHO urges all countries to prepare for the next pandemic that may occur soon (WHO, 2011). WHO also launched the Pandemic Influenza Preparedness Framework program to improve preparation for and response to pandemic influenza and strengthen protection against pandemic influenza by strengthening global surveillance and response systems (WHO, 2011). Antiviral therapy and vaccines are essential to the strategy against the HPAI H5N1 virus.

Several drugs that have the ability as an antiviral have been known. Adamantan (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) are two classes of drugs that are available and active against influenza viruses (Peiris et al ., 2007; Stephenson et al ., 2006). Adamantan is an inhibitor of the influenza virus's M2 membrane protein ion channel. However, several research results stated that several strains of the H5N1 virus were resistant to amantadine due to mutations in the genome segment that encodes the M2 protein, which had mutations at a ratio of >60 and >90% (Stephenson et al., 2006; Dharmayanti et al . 2010). The virus's resistance made amantadine and rimantadine no longer effective as therapy for the H5N1 virus. Several reports of resistance to oseltamivir also appeared due to mutations in the neuraminidase active site (Stephenson et al ., 2006).

Due to the weakness of some of these antivirals, the best protection against the influenza virus is vaccination. Vaccination is a procedure to increase immunity, providing protective immunity by inducing a memory response to specific pathogens/toxins using nonvirulent/nontoxic antigen substances (Bratawidjaja & Rengganis, 2009). Apatogenic/nonvirulent antigen entities or substances that can

stimulate the immune system itself are called vaccines. Influenza vaccines are considered effective in preventing illness and death, especially in high-risk groups, and in their report, WHO states that influenza vaccines are the most effective prevention strategy (Kamps et al ., 2006). In the Pandemic Influenza Preparedness Framework program, which describes "Pandemic influenza preparedness vaccine virus" or PIP vaccine virus, namely viruses developed from recombinant viruses with high growth rates or several reference influenza viruses, influenza viruses recommended by WHO for the use of vaccines or materials from influenza virus developed with the latest technology, which is provided to develop influenza vaccine prototypes (WHO, 2011). The encouragement from WHO spurred research on developing various types of H5N1 virus vaccines to meet demand and prepare for a possible H5N1 influenza virus pandemic. Several strategies are needed in carrying out vaccine development research. It requires in-depth knowledge and understanding of (i) virology, (ii) virus life cycle, (iii) host immune response to infection, (iv) pathology, and (v) HPAI H5N1 vaccine development strategy. Discussion of all these things will be discussed in this chapter.

VIROLOGI OF HPAI H5N1

Highly Pathogenic Avian Influenza (HPAI) H5N1 is part of the Orthomyxoviridae family group, which consists of four genera, namely influenza A virus, influenza B virus, influenza C virus, and Thogovirus (Kamps et al ., 2006; Peiris et al ., 2007). Influenza B and C viruses infect humans, while influenza A viruses infect humans and a variety of poultry, pigs, and several other mammalian species (Gillim-Ross et al ., 2006; Stephenson et al ., 2006). Of all the influenza genera, only the influenza A virus can cause a pandemic. HPAI H5N1 belongs to the influenza A virus genus. Influenza A is an enveloped RNA virus with a genome with eight segments, a single strand, and negative polarity.

The morphology of the HPAI H5N1 virus appears to have an envelope, usually round, and a diameter of around 80-120 nm (Figure 1). Eight gene segments of the influenza A virus encode 11 viral genes, namely hemagglutinin (HA), neuraminidase (NA), matrix proteins M2 and M1, nonstructural proteins (NS) NS1 and NS2 or also known as NEP (nuclear export protein), nucleocapsids, and three polymerases namely PB 1 (polymerase basic 1), PB2 and PA (polymerase acidic) and PB1-F2 (Samji, 2009) (Figure 2).

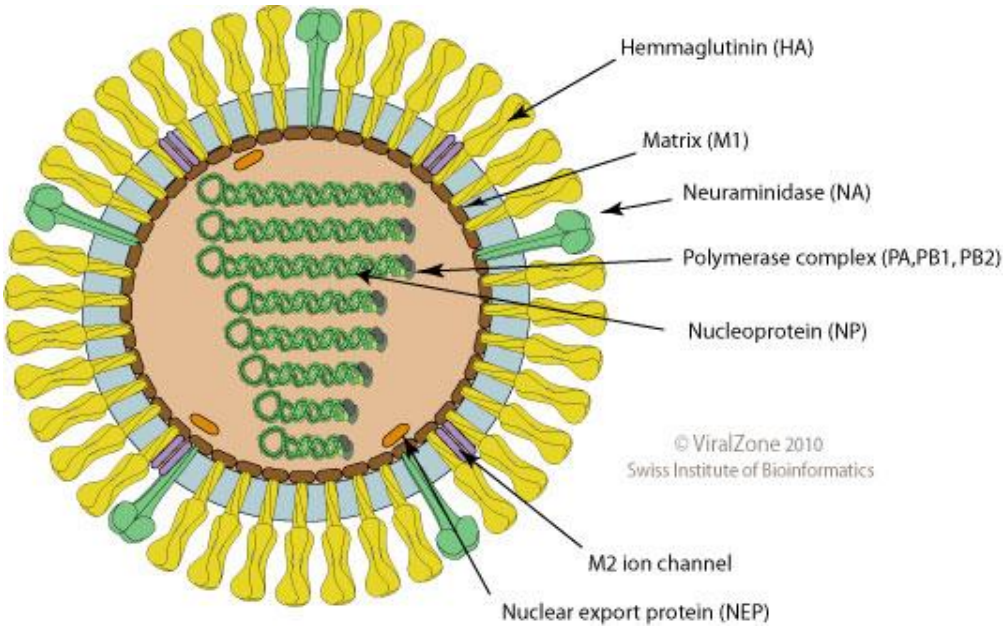

Figure 1. Morphology and structure of the HPAI H5N1 virus. (Source: http://viralzone.expasy.org)

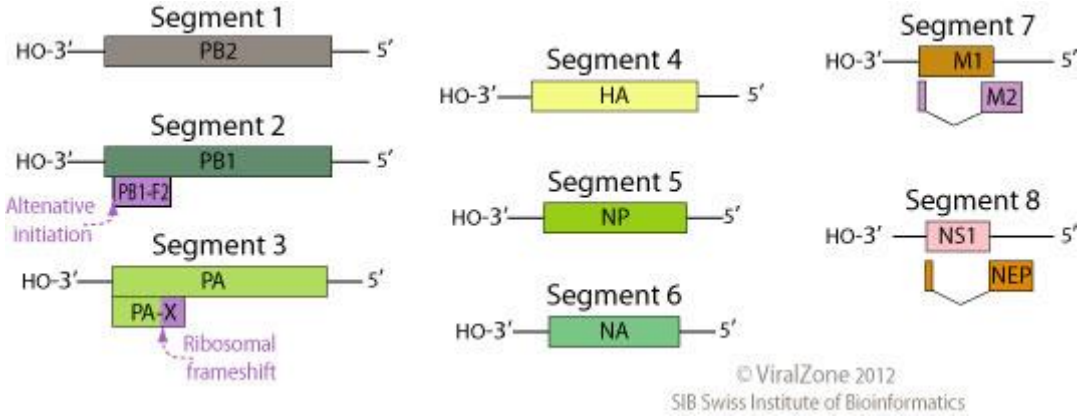

Figure 2. The genome structure of the HPAI H5N1 virus. (Source: http://viralzone.expasy.org)

Influenza A viruses are classified into several subtypes based on their surface protein components: HA and NA. To date, 16 HA subtypes (H1-H16) and nine N subtypes (N1-N9) have been found. All of these subtypes are found in several species of aquatic birds, but only a few subtypes infect humans (HA [H1, H2, and H3] and NA [N1 and N2]) (Kamps et al., 2006; Peiris et al ., 2007; Stephenson et al ., 2006). Hemagglutinin, an acylated and glycosylated protein consisting of 562-566 amino acids, is embedded in the viral envelope. The globular portion of the protein head is closely related to receptor recognition on the host cell because it will cross-link with the sialic acid group on the host cell surface receptor. Meanwhile, neuraminidase is a protein with sialolytic enzyme activity that removes new viruses from infected cells (Kamps et al ., 2006).

The influenza virus genome is very susceptible to mutation, especially mutations in the surface envelope proteins (HA and NA), which cause the antigen diversity of the influenza A virus to be very high. Mutations that occur can cause antigenic drift and antigenic shift. Antigenic drift occurs when a mutation occurs in an influenza virus subtype so that it evolves and causes a change in the immune response in the same host. Thus, even if a host is infected with the same virus subtype, it cannot be recognized by the memory cells formed from the previous infection. Antigenic drift can cause annual or repeated epidemics and outbreaks caused by the same virus subtype. Antigenic shift occurs when gene reassortment occurs, namely the recombination of genome segments of two or more influenza A viruses that infect one host cell simultaneously. These mutations lead to new virus subtypes that increase the genetic diversity of influenza viruses and increase the possibility that these new generations can carry out interspecies transmission and avoid the host's immune response. A pandemic can occur when a new HA subtype (sometimes also NA) acquires the ability to transmit effectively and efficiently from human to human in a population that has never been infected with the virus so that the immune system does not yet recognize the new antigen type (Stephenson et al . , 2006; Carter & Saunders, 2007).

Until now, it has been known that the HPAI H5N1 virus is generally transmitted through livestock to humans, and there is no substantial evidence that this virus can be transmitted from human to human (Peiris et al ., 2007). The HA protein of the avian influenza virus has a different binding type in humans and birds. Avian influenza viruses form a specific bond to sialic acid in galactose with an α-2,6 (SA α-2,6) bond in human cells, whereas in avian cells, the bond is α-2,3 (SA α-2,3). Pig cells have both receptors, so viral genome re-sorts could likely occur in this host species (Samji, 2009). The specificity of these receptors is believed to be a barrier to interspecies infection. However, mutations in HA were found in the H5N1 virus isolated from humans, which caused changes in affinity for SA receptors α-2,3 and α-2,3 (Yamada et al ., 2006). This affinity change allows the H5N1 virus to infect human cells.

Various cases of H5N1 infection reported that the source of infection generally came from poultry farms and markets such as chickens, ducks, geese, and other poultry species that humans widely consume (Peiris et al ., 2007). Because the HPAI H5N1 virus in livestock is associated with viral infections in various organs of poultry and many viruses are also found in poultry faeces, diseased birds are the leading cause of human infection. Humans can be infected through several mechanisms, such as direct handling of sick birds, slaughtering or preparing sick birds for consumption, consuming poultry products that are not cooked thoroughly, or direct contact with poultry on farms. Direct contact with

contaminated environments, such as water and poultry faeces, also allows infection to occur. Transmission from birds to humans, the entry of the virus into the human body can be through the respiratory tract, digestive tract, or conjunctiva. Transmission of the H5N1 virus from human to human is still very little or almost non-existent, so until now, it is still believed that human-to-human transmission is inefficient (Peiris et al ., 2007). Nevertheless, it also need to be aware of the possibility that a mutation of the virus could occur at any time and cause the virus to adapt and be able to transmit from human to human. If that happens, it is not impossible that the H5N1 pandemic that WHO is worried about will happen.

LIFE CYCLE OF HPAI H5N1

HPAI H5N1 is a member of the influenza virus, so the mechanism of its life cycle is generally the same as other influenza viruses. Understanding this virus's life cycle can be used to develop vaccines and drugs to fight infection with the H5N1 virus. The influenza virus life cycle can be divided into the following stages: entry of the virus into the host cell, entry of vRNPs into the nucleus, transcription and replication of the viral genome, export of vRNPs from the nucleus, and assembly and budding on the host cell plasma membrane (Peiris et al ., 2010).

Viral attachment to host cells is mediated by the interaction of HA proteins with sialic acid-containing receptors. The HA precursor, HA0, consists of two subunits, HA1, which has a binding domain for the receptor, and HA2, which contains a peptide for fusion. Then the virus enters through the mechanism of receptor-mediated endocytosis, and the virus enters the cell by being in the endosome. Endosomes are in moderately acidic conditions with a pH range of 5-6. It causes a conformational change in HA, exposing the HA2 subunit initially hidden by the HA1 subunit. The fusion peptide domain on HA1 triggers fusion activity between the viral membrane and the endosome.

The acidic state also triggers the opening of the ion channels of the M2 protein. The opening of the M2 channel makes the state of the virus core also become acidic. This acidic state encourages the release of vRNP (viral ribonucleoprotein) to enter the cell cytoplasm freely. After that, vRNP enters the nucleus. The viral proteins that form vRNP are NP, PA, PB1, and PB2.

The viral genome is a negative sense ssRNA, so a positive sense RNA (cRNA) sequence must be made as a template for viral RNA production. Viral genome replication does not require a primer, but RNA-dependent RNA polymerase (RdRp) initiates RNA synthesis. RdRp consists of three viral proteins, namely PB1, PB2, and PA. PB2 has endonuclease activity. In the nucleus, the transcription process of

the genes that express viral proteins occurs. Six segments of viral RNA each code for one type of protein. However, segments 7 and 8 encode two proteins through a splicing process that utilizes the splicing 'machinery' of the host cell. Segment 7 encodes M1 and M2 proteins, while segment 8 encodes NS1 and NEP. Splicing influenza virus mRNA can inhibit the splicing of host cell mRNA. NS1 can inhibit the release of host cell mRNA from the nucleus by preventing cleavage at the polyadenylated cleavage site.

The result of replication of the viral genome that comes out of the nucleus is only a vRNP that contains negative sense RNA. vRNPs are excreted from the nucleus via a CRM1-dependent nucleopore. The NP protein shows direct interaction with CRM1 even without GTP hydrolysis. After the vRNP leaves the nucleus, it forms new virus particles. Because influenza is an envelope virus, the virus uses the host cell membrane to form new virus particles that will leave the cell. Virus particle buds form from the apical side of the host cell. Therefore HA, NA, and M2 are transported to the apical portion of the plasma membrane. M1, under the lipid bilayer layer, is essential in the final step of forming new shoots. One of the most critical steps in the formation and release of new viral particles is the cleavage of sialic acid residues from glycoproteins and glycolipids by NA proteins so that the virus can be released from the plasma membrane (Peiris et al., 2010).

PATHOLOGY OF HPAI H5N1

Even though the body's immune system is active against infection, the H5N1 virus can still escape the resistance of the body's immune system, replicate and cause acute illness in humans that can even cause death. Disease caused by HPAI H5N1 virus is clinically and pathologically different from seasonal influenza virus caused by H3N2 or H1N1 (Peiris et al ., 2007). Acute disease caused by H5N1 infection in humans may occur through several mechanisms. These mechanisms include: (i) spreading of the virus beyond the respiratory tract (different from seasonal flu); (ii) higher and prolonged viral replication, which causes direct damage to cytolysis by the virus; (iii) differences in tissue tropism of H5N1 viruses (different from seasonal influenza viruses); and (iv) differences in host responses induced by H5N1 viruses (Peiris et al ., 2009).

Although the H5N1 virus can spread beyond the respiratory tract, it can be isolated from faeces, serum, and occasionally from the nervous system. 2,9 The cell targets for H5N1 replication are not entirely known, but alveolar pneumocytes and macrophages have been identified by immunohistochemistry at autopsy, virus-binding studies, and lung fragment cultures (Peiris et al ., 2007).

The H5N1 virus can replicate well in the alveolar and nasopharyngeal epithelium. Pathology of the lungs (ARDS- Acute Respiratory Disease Syndrome) is the leading cause of death in H5N1 disease (Peiris et al ., 2010). Clinical manifestations of the H5N1 influenza virus include diarrhoea, liver and kidney dysfunction, acute lymphopenia, and reactive hemophagocytosis that occurs in several organs (Peiris et al ., 2007).

Clinical observations and animal experiments indicate that viral disease is associated with cytokine dysregulation. However, because the effects of high viral replication and immune response are closely related, it is also unclear whether cytokine dysregulation is a consequence of acute disease or contributes to viral infection pathology (Peiris et al., 2010). Patients generally have higher serum levels of macrophage and neutrophil chemoattractant chemokines (CXCL-2, IL-8) and pro-inflammatory and anti-inflammatory cytokines (IL-6, IL-10, IFN-γ). The NS1 protein plays a vital role in modulating the immune response. The H5N1 viral NS gene segment partially mediates cytokine hyper induction in macrophages. The NS H5N1 gene causes an imbalance in the concentration of cytokines in the lungs, where pro-inflammatory cytokines increase while anti-inflammatory cytokines decrease. In addition, the NS1 protein plays a role in avoiding the innate immune response in the host, namely by inhibiting the activation of the interferon response in the host (Peiris et al ., 2007).

HPAI H5N1 VACCINE DEVELOPMENT STRATEGY

The previous discussion has explained the pathogenesis of the H5N1 virus. The H5N1 virus can cause acute clinical symptoms, especially in the respiratory system (ARDS) (Peiris et al ., 2007), even if the infection fatality reaches 60%. Therefore, it is unsurprising that WHO launched a program to prepare for the possibility of an H5N1 virus pandemic (WHO, 2011). One strategy is to develop an effective vaccine to prevent casualties from diseases caused by the H5N1 virus during an actual pandemic. However, developing an effective pandemic vaccine faces practical and immunological challenges (Subbarao et al ., 2006).

Several important considerations must be considered in developing a vaccine. First, the influenza virus replicates very quickly within the host. Peak titers (which correlate with disease) are reached before a cellular immune response is generated de novo or from memory cells. Thus, the main goal of vaccine development is to induce antibodies that can weaken the ability of virus replication. Second, influenza is a respiratory tract infection; vaccine-induced antibodies are expected to build up along the respiratory tract from top to bottom. Live attenuated viral vaccines administered intranasally efficiently induce

mucosal immune responses as well as systemic immune responses. Mucosal antibodies are more effective at stopping viral replication than systemic antibodies in the upper respiratory tract.

In contrast, parenteral inactivated vaccines effectively induce systemic antibodies (serum) that stop the influenza virus from replicating in the lower respiratory tract. Thus, inactivated vaccines prevent acute illness and complications of influenza but are less effective than attenuated vaccines in protecting the upper respiratory tract. Third, the ability of the virus to mutate and avoid detection of the immune response and the less conservative HA protein epitope, which can induce cross-reaction of antibodies for neutralization and protection, are challenges that must be faced in vaccine development. The strategy that can be done is to develop a vaccine that can produce an immune response against conservative antigens. The fourth relates to vaccine doses. Clinical studies have determined that two doses of inactivated vaccine are needed to elicit adequate antibody titers in the immune response in naïve individuals. Given the enormous need for vaccines worldwide, the required dose of vaccine should be minimized but still immunogenic, perhaps by adding adjuvants, as is done in current vaccine development (Subbarao et al., 2006).

With the above considerations in mind, there are two approaches to developing vaccines for pandemic preparations that can be exploited. The first and most likely to be developed is to use existing technology to improve the ability of vaccines to induce antibodies with high effectiveness and protection. The second approach is to build basic research to explore vaccines that can induce immunity or antibodies that produce cross-protection against conservative epitopes such as HA and M2 proteins. However, this approach requires a longer time (Subbarao et al ., 2006).

Several types of vaccines have been developed to prevent the danger of infection with the HPAI H5N1 virus. Vaccines can be divided into dead virus vaccines and live virus vaccines. Apart from these two types, other types of vaccines have been developed by utilizing current advances in biotechnology, especially those related to genetic engineering (Kamps et al ., 2006). Inactive virus vaccines can be subdivided into whole, split, and subunit (Gillim-Ross et al ., 2006). The live virus vaccine that has been developed is a cold-adapted (ca) live, attenuated influenza virus vaccine (Suguitan et al., 2006). Furthermore, other alternative vaccines that utilize genetic engineering technology include reverse genetics, DNA vaccines, universal vaccines (conservative proteins), virus-like particle (VLP) vaccines, viral vector vaccines and vaccines with various adjuvants (Kamps et al ., 2006; Gillim -Ross et al ., 2006).

Inactivated Vaccine

The whole virus vaccine was the first vaccine to be developed. This vaccine is produced by culturing the virus in chick embryos; after harvesting, the virus is inactivated with formaldehyde. The virus was concentrated and purified in a graded gradient sucrose solution using continuous centrifugation. Next, purification was carried out with a membrane filter (Kamps et al ., 2006). In general, influenza virus cultures for vaccine production do use chicken embryos. In addition, currently, many viral cultures use cell culture media such as Madin-Darby Canine Kidney (MDCK) cells or Vero cells (derived from the kidneys of African green monkeys) (Kamps et al., 2006; Peiris et al . , 2007). The production capacity is hoped to be more significant using this cell culture.

The results of phase 1 and 2 clinical studies for the safety of whole virus vaccines produced in Vero cell cultures demonstrated their ability to induce antibodies that neutralize various strains of the H5N1 virus. The vaccine elicited a neutralizing immune response against clade 1 virus strains (A/Vietnam/1203/2004) and against clade 2 and 3 strains. The use of adjuvants did not increase antibody response. Maximum response to vaccine strains was obtained with formulations containing 7.5 µg of hemagglutinin antigen and 15 µg without adjuvants. Mild pain at the injection site (in 9-27% of subjects) and headache (in 6 to 31% of subjects) were the most common side effects for all vaccine formulations (Ehrlich et al., 2008). Among other inactivated vaccines, whole virus vaccines are significantly more immunogenic than split or subunit vaccines but are more reactogenic, causing more side effects, especially in children.

To date, there is only one H5N1 vaccine that has received a license from the Food and Drug Administration (FDA), namely "Influenza Virus Vaccine, H5N1", which is an inactivated vaccine of type A monovalent HA protein subunit used intramuscularly, which was developed using the split virus method. The split virus method is almost the same as the whole virus. However, in a split virus, the virus is disturbed by a chemical solution using a non-ionic surfactant, polyethene glycol p-isooctinynil ether (Triton X-100). The split virus was then purified by chemical means and suspended in an isotonic sodium chloride solution with sodium phosphate buffer. Each 1mL dose contains 90 µg HA protein from influenza virus strain A/Vietnam/1203/2004 (H5N1, clade1). This vaccine is indicated for active immunization for those aged 18 to 64 years with a risk of exposure to influenza virus subtype H5N1.

Belshe et al. (2011), in their research results, reported on the effect of schedule on antibody response to 2 doses of H5 vaccine (one HA vaccine from strain clade 1 A/Vietnam/04 and one vaccine

from the clade two strain A/Indonesia/05) containing 90 µg antigen in adults aged 18-49 years. Two vaccine doses generally induce an antibody titer of >1:10. The immunization schedule on days 0 and 14 and 0 and 28 produced a better response than those on days 0 and 7 for the same type of vaccine immunization. In heterologous vaccination, the best response appears on the vaccination schedule with a difference of 6 months compared to the difference in the schedule of 1 month. Cross-reactions occurred in several study subjects who were induced by the H5N1 A/Vietnam/04 virus vaccine against the HA antigen of the H5N1 A/Indonesia/05 virus, but on the contrary, there was almost no cross-reaction in the H5N1 A/Indonesia/05 virus vaccine against the HA antigen of the H5N1 A virus. /Vietnam/04. Even so, administering heterologous vaccines for the H5N1 A/Vietnam/04 virus vaccine and the H5N1 A/Indonesia/05 virus vaccine produced antibodies against the two virus antigens.

Live, Attenuated Virus Vaccine

A cold-adapted (ca) influenza vaccine for intranasal use has been available in the United States since July 2003, and in the Soviet Union, this live vaccine has been used for several years. This vaccine consists of a mother virus that has taken the HA gene (already attenuated) and the NA gene to be inserted into the master virus. The master viruses used were A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66. The master virus used as a vaccine can adapt to cold conditions. In other words, it has adapted to grow ideally at 25 0 C, which means that at average body temperature, humans are weakened. The adaptation process has been shown to cause stable mutations of three viral polymerase genes, namely PA, PB1, and PB2, making their replication ability weak (Kamps et al ., 2006).

The effectiveness of inactivated vaccines in general influenza virus prevention studies in humans produced less than optimal results because they required high doses with twice the dose. So it is hoped that the development of influenza ca virus vaccine will produce a more robust immune response with a smaller dose than inactivated vaccines. However, the main concern regarding using live virus vaccines is the risk of reassortment with circulating influenza viruses. This reassortment can produce a new influenza subtype that can spread to the human population and is feared to have a higher virulence. This concern has not been proven until now, so a more in-depth study in experimental and clinical research still needs to be done (Luke & Subbarao, 2006).

Karron et al. (2009) report the results of an evaluation of a phase I clinical trial for applying two types of H5N1 influenza ca virus vaccine in healthy adults. The ca vaccine used in this study was developed from ca H5N1 VN 2004 AA and ca H5N1 HK 2003 AA. Each of these vaccines contains

modified hemagglutinin H5 and unmodified neuraminidase N1 from the wild-type parent virus and six internal protein gene segments from the cold-adapted A/Ann/Arbor/6/60 virus master donor (ca). Two vaccine doses are administered intranasally to healthy adults at 4-8 week intervals. The results obtained, the spread of the virus was minimal, as well as the antibody response to HI and neutralization.

Only 52% of ca H5N1 VN 2004 AA vaccine recipients produced antibodies in their serum. These results indicate that the two live attenuated vaccines have minimal replication capabilities and are weaker than similar vaccines using H1, H3, or HA antigens of the influenza B virus.

Apart from being developed using conventional methods, live, attenuated virus vaccines are also developed using reverse genetic technology (described below). Live, attenuated virus candidate vaccines possessing a modified gene encoding the hemagglutinin (HA) H5 protein and wild type (wt) N1 neuraminidase from influenza A H5N1 virus isolated in Hong Kong and Vietnam in 1997, 2003 and 2004. The gene segments that form the backbone are derived from cold-adapted (ca) influenza A vaccine donor strain, influenza A/Ann Arbor/6/60 ca (H2N2). Four weeks after receiving the two doses of the vaccine, the rats and ferrets were fully protected against heavy viral replication in the lungs. The promising results in this pre-clinical study are a good provision for research in the next stage, namely clinical research in humans (Suguitan et al., 2006).

Reverse Genetic Vaccines

The previous discussion explained that the main virulence factor in influenza viruses is the HA protein because this protein plays a role in the initial viral infection by recognizing host cell receptors and then entering the host cell. Therefore, another strategy in vaccine development is to weaken the virulence ability by cutting the gene encoding the polybasic amino acid that causes virulence in the HA protein. A fast and reproducible system is needed for modification, one of which is the reverse genetic method. The beneficial potential of reverse genetics to produce a new generation of vaccine candidates against highly and low pathogenic influenza viruses is enormous (Nicolson et al., 2005; Webby et al ., 2004). 41,42

The use of reverse genetics in vaccine manufacturing has several advantages compared to traditional methods: (i) the reverse genetic approach is a more direct rational approach compared to the traditional approach; (ii) reverse genetics can reduce contamination of wild-type viruses that may be carried due to invalid systems or cultures that may contain other pathogens; (iii) at the plasmid stage, this method can engineer HA so that it eliminates its pathogenicity (Nicolson et al., 2005). In general,

genetic reverse viruses for influenza vaccine production utilize internal genes from the influenza A/Puerto Rico/8/34 (PR8) virus strain which has adapted well to egg culture to produce high-growth reassortant (HGR) viruses (Lekcharoensuk et al., 2012). However, several studies carried out optimization on Vero cell culture. 41,42 Viruses produced in laboratory facilities that comply with Good Manufacturing Practice (GMP) standards require less than four weeks from virus isolation. This virus was also non-pathogenic in chickens and ferrets and showed stable growth when cultured into embryonated chick eggs (Nicolson et al., 2005).

The results of Govorkova et al. (2006) reported that an inactivated virus vaccine produced from the reverse genetic PR8-H5N1 virus triggered an immune response that protected ferrets from H5N1 viruses A/HK/213/03, A/HK/156/97, and A/Vietnam/1203/04. Previously Subbarao et al. (2003) stated that a similar virus vaccine triggered an immune response and protected mice against the H5N1 influenza virus strains A/HK/486/97 and A/HK/491/97.

In addition, Lekcharoensuk et al. (2012) developed another master donor virus, namely influenza virus strain A/swine/Iowa/15/30 (H1N1) (rg1930), which showed good growth in Madin-Darby canine kidney (MDCK) cell culture. The result is that the rg1930H5N1 virus has low pathogenic ability in vivo studies. Inactivated vaccine rg1930H5N1 can protect chickens from morbidity and mortality against HPAI H5N1. Compared to reverse genetic viruses using PR8, rg1930H5N1 produced higher virus titers in MDCK and Vero cell cultures. Reverse genetic technology is used to produce inactivated virus vaccines and live attenuated vaccine viruses described previously (Suguitan et al., 2006).

DNA vaccine

Previously, it has been explained about the development of several conventional vaccines against H5N1, namely inactivated and live attenuated vaccines. Even though it is practical, this vaccine strategy requires a relatively long time and requires the supply of raw materials. Therefore, another approach is needed to overcome this problem. Using current biotechnology advances, DNA vaccines are an appropriate alternative (Chen et al . 2008).

Since its discovery more than one and a half decades ago, the results of genetic engineering DNA can be used as a vaccine and cause an immune response. Vast amounts of data have been generated in pre-clinical research. More continuous and consistent cellular and antibody responses are observed in the clinic with several vaccines. Four DNA vaccine products have just been approved, all in veterinary medicine. These results indicate the future of DNA vaccines as the optimal technology for

vaccine production (Kutzler & Weiner, 2008). DNA vaccines' working principle is inoculating DNA that codes for antigens into the host's body. Then the DNA is expressed into viral proteins, presented by antigen-presenting cells (APC). Antigens are detected by the immune system, causing humoral and cellular responses (Kutzler & Weiner, 2008).

DNA vaccines are the primary alternative method of producing several vaccines. Several DNA vaccines that have been developed include DNA vaccines for both homologous and heterologous HA proteins as well as consensus results (Chen et al ., 2008), DNA vaccines for conservative NP and M proteins (Jimenez et al ., 2007), and also DNA vaccines a combination of several viral proteins (Lalor et al ., 2008). All of these types of vaccines have been proven to cause an immune response in preclinical trials using experimental animals.

Formulated DNA vaccines have been developed from DNA vaccine formulations (Jimenez et al ., 2007), pre-clinical trials on mice and ferrets (Lalor et al ., 2008), to phase I clinical trials (Smith et al., 2010). From the results of these studies, it is evident that DNA vaccines can generate a better immune response than conventional vaccines and have great potential to eradicate the H5N1 pandemic.

Universal Vaccine

Influenza A virus is a virus that has a wide antigenic variety and causes many epidemics. The currently available influenza vaccines are primarily specific, only protective against certain antigens, although there is also a trivalent influenza vaccine against seasonal influenza (Pearce et al ., 2012), but it is only effective for specific subtypes. If a new subtype causes an outbreak, it will be challenging to prevent morbidity and mortality because it has antigens that are alien to the human population. Therefore, in current vaccine development research, researchers are thinking of making a universal influenza vaccine that can cause an immune response against all types of influenza viruses (Du et al., 2010; Heiny et al . , 2007; Thompkins et al . , 2007). The strategy was to utilize the conservative genome regions of all influenza A viruses, including the HA1, HA2, NP and M2e genes (Thompkins et al ., 2007).

Several studies reporting on the development of universal vaccines focus on utilizing conservative regions on the M2 protein (Fiers et al., 2009; De Filette et al., 2005) and HA (Gerhard et al., 2006). The conservative region of the gene encoding the M2 protein is in its external domain (M2e). This area is low immunogenic in natural infection and conventional vaccination. DeFilette et al. (2005) explained that previous research showed that when the M2e protein can bind to carrier particles such as hepatitis B virus core particles (HBc), this protein becomes highly immunogenic and provides complete protection

in mice against lethal viral infections. Then after further investigation, even though HBc is very immunogenic, the optimum M2e-HBc vaccine induces higher anti-M2e titers than anti-HBc titers. Fiers et al. (2009) also explained that in several experimental animals, the M2e vaccine administered parenterally or intranasally could protect these animals against disease and death caused by various influenza A virus strains, and the addition of adjuvants could increase the antibody titer higher. The results of a phase I clinical trial also indicated the safety and immunogenicity of this vaccine.

The study of the conservative region in the HA gene region is also developed to develop a universal vaccine. Vaccine studies have proven that the HA protein is most effective in triggering an immune response, so studies of conservative sites in this region are a plausible strategy for universal vaccine development. Although the degree of HA diversity in the various vaccine subtypes is high, especially in the HA1 polypeptide subunit (homology between subtypes 34%-59%), some conservative areas are present in the HA2 polypeptide subunit (homology between subtypes 51%-80%). The most abundant conservative area is the sequence around the cleavage site, particularly at the N-terminal 11 aa HA2, called the fusion peptide, which is conserved in all influenza A subtypes. Some areas are seen as surface curvature on the HA molecular precursor (HA0) (Gerhard et al., 2006).

In addition, Heyni et al. (2007) reported 55 conservative sequences, most of which were predicted to be relevant to T cell-recognized epitopes. These conservative sequences were found in the PB1, PB2, PA, NP, M1 and HA genes.

Virus-like Particle (VLP) Vaccine

The VLP vaccine is another alternative vaccine developed to produce a vaccine that does not depend on egg embryo culture to increase the production capacity of the H5N1 vaccine. Several current studies have focused on research on non-replicating VLPs (Kang et al ., 2009a). Virus-like particle (VLP) is a multiprotein structure similar to the organization and conformation of the original virus. However, it lacks a viral genome, can generally be produced in insect or yeast cells, and can express important viral membrane components (Khurana et al., 2011). Several VLP vaccine products have been licensed, VLP vaccines for hepatitis B virus and human papillomavirus (HPV), and all of them have proven that VLP vaccines can trigger an immune response and are safe to use (Kang et al., 2009a; Khurana et al ., 2011).

Virus-like particle (VLP) is one of the results of recombinant DNA technology, which can potentially be used to develop various types of vaccines (Rizqoh, 2021). As previously explained,

subunit vaccines have weaknesses, such as being less immunogenic and requiring large doses. It is probably because subunit vaccines experience inaccurate folding of subunit proteins, so the immune system is less able to recognize the structure of the antigen (WHO, 2011). VLP includes a subunit vaccine that contains one or more specific types of antigen, but VLP has the same structure and mimicry as the original virus without containing infectious, genetic material. VLP vaccines are more immunogenic than ordinary subunit vaccines and even match the ability of the original virus to induce humoral and cellular immune responses (Peiris et al., 2010; WHO, 2011).

The VLP vaccine for the influenza virus is currently being developed. Influenza VLP vaccines are generally produced with recombinant baculoviruses that express the HA, NA, and M1 genes (Kang et al., 2009b; Bright et al., 2008). The non-infectious nature of VLP and the absence of a viral genome have the potential to be a safe vaccine candidate and can be administered to all human populations, including high-risk groups. In addition, VLPs possessing a portion of antigens can activate APCs, such as dendritic cells, which present antigens to T and B lymphocyte cells (Kang et al ., 2009a).

Kang et al . (2009b) presented the results of a study on VLP vaccine immunization containing HA, NA, and M virus H5N1 A/VietNam 1203/04 in mice intranasally. This VLP vaccine produces high H5N1-specific antibodies and is 100% protective against the H5N1 virus. Then Bright et al. (2008) and Khurana et al. (2011) research on VLP vaccines with different H5N1 virus strains proved that VLP vaccines could produce an immune response and cross-protection between clades 1 and 2. Apart from being made in insects, VLP can also be made using the *Nicotiana bethamiana* plant. The results of a pre-clinical and clinical phase I study conducted by Landry et al. (2010) showed cross-protection in ferrets and good safety in humans.

Virus Vector Vaccine

Many delivery systems are available to enhance immunogenicity or stimulate specific immune responses against target antigens and immune responses generated by the innate immune system to protect the body from pathogens. One of the delivery systems currently widely used as vaccine antigen carriers is DNA and RNA viral vectors. Interestingly, the type of vaccine that was first developed became one of the most widely used viral vectors, namely the poxvirus vector. Since then, many DNA viruses have been used in vaccine development, including adenoviruses, herpesviruses, and baculoviruses. In addition, several types of RNA virus vectors were developed from paramyxoviruses, rhabdoviruses, alphaviruses, coronaviruses, retroviruses, and flaviviruses (Brun et al ., 2008).

Several H5N1 vaccine developments also utilize viral vectors as carriers of vaccine viral antigens, such as adenovirus (Toro et al., 2007), fowlpox virus (Bublot et al., 2007), modified vaccinia virus Ankara (MVA) (Kreijtz et al., 2009), baculovirus (Wu et al ., 2009), alphavirus (Yang et al., 2009), alphaherpesvirus (duck enteritis virus) (Fragane et al., 2010), and others. These virus vector vaccines have generally been studied in animals, such as mice, chickens and pigs, and have produced an immune response in these animals. Research on viral vector vaccines in humans has reached phase I clinical trials, namely research on replicating adenovirus serotype four vector vaccine (Gurwith et al., 2013). The results of this study indicate that this vaccine is immunogenic and safe because it does not cause serious side effects.

Vaccine Adjuvants

Adjuvants are molecules, components or macromolecular complexes that have the potential to strengthen and prolong the immune response to antigens but only cause minimal toxicity (Reed et al., 2008). Adjuvants have been used in several vaccines against pathogens and are being investigated for a role in influenza vaccines. The purpose of the adjuvant is to increase the immune response to the vaccine, thereby allowing a decrease in the dose of antigen in the vaccine and increasing the success of vaccination. Alum is the only adjuvant registered in the United States, and MF59, an oil/liquid emulsion, has been used in European influenza vaccines since 1997. A vaccine using the outer membrane protein of *Neisseria meningitidis* as an adjuvant has also shown success in early clinical trials (Kamps et al., 2006).

Adjuvants can be classified according to their source of components, physicochemical properties or mechanism of action. Two classes of adjuvants commonly used in modern vaccines are immunostimulants and vehicles (drug delivery systems). Adjuvants as immunostimulants are adjuvants that directly trigger an immune response to increase the immune response to antigens. Examples include TLR ligands, cytokines, saponins, and bacterial exotoxins that stimulate an immune response. Then, the adjuvant as a vehicle is tasked with presenting the vaccine antigen to the immune system, including controlling the release and placement (delivery system) to enhance the desired specific immune response against the antigen. These vehicles can also play a dual role as immunostimulants. Examples of adjuvants of this type include mineral salts, emulsions, liposomes, virosomes (nanoparticles made from viral proteins such as influenza hemagglutinin and phospholipids),

biodegradable microspherical polymers and so-called immunostimulant complexes (ISCOM, ISCOMATRIX TM) (Reed et al . , 2008).

Adjuvant application in H5N1 virus development has been widely used. Vaccines containing MF59 adjuvants have increased the immune response (Fragapane et al., 2010; Stephenson et al., 2005). This adjuvant is also relatively safe because it only causes side effects that are not severe and generally only occur locally (Bernstein et al., 2008). This MF59 adjuvant can induce an earlier T-CD4 cell response and is predicted to make the antibody last a long time at a protective level (Galli et al., 2009). Vaccines using oil-in-water emulsions as phase I clinical trials also show an immunogenic reaction and are safe to use (Levie et al., 2008).

The delivery system has also been used as an adjuvant and can cause a cross-protective immunogenic reaction in mice against the H5N1 virus (Dong et al., 2012). A cationic lipid-based adjuvant, Vaxvectin®, can increase IgG2 titer and Th1 cell response and is also used in DNA vaccines (Jimenez et al., 2007). There is also an H5N1 vaccine using ISCOM which can also induce protection against the H5N1 virus in chickens (Rimmelzwaan et al., 1999).

CONCLUSION

HPAI H5N1 virus is an influenza virus that attacks the respiratory tract and causes a significant impact on morbidity and mortality. Even though it can only be transmitted through animal-to-human intermediaries, the possibility of human-to-human transmission can occur because this virus can mutate. Mutations that occur can make the virus adapt to increase the effectiveness of the virus in transmission between humans. If that happens, the feared pandemic could occur, especially considering the history of pandemics, most of which were caused by the influenza virus. Therefore, a vaccine development strategy must be carried out as a preventive measure to deal with the HPAI H5N1 virus pandemic.

Currently, many types of vaccines have been developed, both by conventional methods and by utilizing alternative technologies that are more modern and are currently being developed. Most of the vaccines developed have completed pre-clinical studies with good results, some of which have reached phase I clinical studies and some have even been licensed. It is hoped that these vaccines will meet the target and meet the demand for vaccines worldwide in preparation for the HPAI H5N1 virus pandemic.

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