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Developments of Subunit and VLP Vaccines Against Influenza A Virus*

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Abstract: Influenza virus is a continuous and severe global threat to mankind. The continuously re-emerging disease gives rise to thousands of deaths and enormous economic losses each year, which emphasizes the urgency and necessity to develop high-quality influenza vaccines in a safer, more efficient and economic way. The influenza subunit and VLP vaccines, taking the advantage of recombinant DNA technologies and expression system platforms, can be produced in such an ideal way. This review summarized the recent advancements in the research and development of influenza subunit and VLP vaccines based on the recombinant expression of hemagglutinin antigen (HA), neuraminidase antigen (NA), Matrix 2 protein (M2) and nucleocapsid protein (NP). It would help to get insight into the current stage of influenza vaccines, and suggest the future design and development of novel influenza vaccines.

Key words: Influenza; Subunit vaccine; VLP; Recombinant expression

Influenza virus is a lipid-enveloped orthomyxovirus, possessing a segmented, single stranded RNA genome with negative orientation. Based on their genetic and antigenic differences they are divided into three types: A, B and C, among which influenza A virus causes the most significant morbidity and mortality in the human population and domestic animals on a global scale. There have been several global pandemics for the last 100 years. The 1918 'Spanish influenza' killed as many as 50 million people worldwide[38]; the 1957'Asian influenza' H2N2, 1968'Hong Kong influenza' H3N2[15,21] and 2009 swine-origin H1N1[38] all gave rise to the world-wide panic. Besides, influenza A virus causes yearly epidemics of respiratory illness of varying severity worldwide in people of all ages, and results in thousands of deaths and enormous economic losses[41].

Vaccination is the primary method to prevent or lower the burden of influenza disease. As the polymerase complex of influenza virus does not possess a proof reading activity, it is easy to bring in mutations to the progeny virus (called "antigenic drift"), especially to the HA and NA parts. Those mutations lead to the conformational alterations of epitopes recognized by neutralizing antibodies, thus

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making existing vaccines less- or non-effective. On the other hand, due to the nature of the segmented genome, influenza virus can independently recombine segments upon the infection to a cell (called "antigenic shift"). For influenza A virus, 16 HA-subtypes (H1-H16) and 9 NA-subtypes (N1-N9) have been discovered, and recombinations among the subtypes may lead to new antigenic properties[32,51]. Consequently, the vaccines against influenza virus need to be adjusted annually. Each year, the World Health Organization (WHO) identifies new strains of influenza viruses through the international surveillance system, summaries the epidemiological data twice a year in two meetings (one in February, the other in September), and decides which strains will be included in the trivalent influenza vaccine production[18].

Most of present available influenza vaccines are generated in embryonated hen's eggs. Seed strain is inoculated in the allantoic cavity of embryo. Three days after inoculation, allantoic fluid is collected for the purification of progeny virions, which is then inactivated by formalin or β-propriolactone and detergent treatment. Then we can either use the harvested virus as wholevirus vaccine, or further get the purified hemagglutinin and neuraminidase proteins as subvirion or subunit vaccines[10,18]. Although this traditional vaccine production system has served well for decades, there are several insurmountable defects for the egg-based vaccine: 1) In order to produce enough influenza vaccines, a large amount of SPF level embryonated chicken eggs are needed[49]; 2) The manufacture of influenza vaccines is limited to influenza virus strains that replicate well in eggs, but some vaccine strains such as high pathogenic avian influenza H5N1 which can kill the embryos could not be produced in this way[48]; 3) The vaccine strain that grows in embryonated chicken eggs sometimes undergoes antigenic variations, resulting in a less effective vaccine; 4) last but not least, the vaccine has potential safety concerns in individuals with egg allergies[5,26]. Considering all this defects of traditional egg-based vaccine, efforts have been made to develop alternative vaccine production methods, among which the present influenza subunit and VLP vaccines are produced in a much more superior way.

Nowadays influenza subunit and VLP vaccines have been produced in bacterial[3], mammalian[4,39,54], and recombinant baculovirus (rBV) expression systems[11,52]. Owing to the development of protein purification technology, as well as the commercially available purification systems[24,42], it is now much easier to purify proteins expressed in prokaryote or eukaryote systems. So under these favorable backgrounds, influenza subunit and VLP vaccine technology is developed from strength to strength. This paper is focused on the recent development of subunit (for viral vector vaccine such as intranasal influenza vaccine designed by Vaxin, the active ingredient of which was the protein subunit, is also classified as subunit vaccine) and VLP vaccines against influenza A virus. According to the difference in the expression of influenza antigen, subunit vaccines can be further divided into HA subunit vaccines, NA subunit vaccines, NP subunit vaccines and M2 subunit vaccines[8]. As to VLP vaccine, which is produced by co-expressing of influenza M1 in combination with HA, NA, and/or M2 protein, is also discussed in the following text.

HA subunit vaccine

Hemagglutinin (HA) is the dominant surface

glycoprotein on the influenza virus, which is recognized as the key antigen in the host response to influenza virus in natural infection and vaccination. Besides, the host resistance to infection correlates with serum anti-HA antibody levels[7], and the antigenic structure of the HA is of primary importance in strain selection for inclusion in the influenza vaccine each year. Thus HA is a logical candidate for recombinant vaccine production.

Progress in recombinant DNA technology has allowed for the rapid cloning of influenza virus HA genes, expression of correctly folded and biologically active recombinant HA (rHA). The most familiar influenza vaccine produced in this way is FluBlØk, which is produced in baculovirus-insect cell expression system[10,11,23,48,49]. FluBlØk is similar to the licensed egg-grown vaccines in that it contains antigens (HA proteins) derived from three strains of the influenza virus that are selected for inclusion in the annual influenza vaccine by WHO. FluBlØk presents a potential solution to the insurmountable limitations associated with the licensed vaccines that are grown in eggs. Antigens are developed using recombinant DNA technology and manufactured in cell culture but not eggs. Besides, unlike the licensed vaccines and many cell culture vaccines in development, the manufacturing of FluBlØk does not generate live influenza viruses, thus biocontainment facilities and harmful chemicals such as formalin are dispensable[49]. The vaccine produced in this way consists only of three strains of influenza HA antigens: two strains of influenza A virus HA antigen (eg: H1N1,H3N2) and one strain of influenza B virus HA antigen. They can be stored in sterile buffered salt water, avoiding preservatives such as thimerosal (a mercury derivative currently used in egg production) or other adjuvants which may do harm to the inoculators. Compared with the traditional egg-based vaccine as long as six months production cycle, new vaccines can be developed quickly in just 8 weeks[8]. In clinical trials, the vaccine was shown to be well tolerated and immunogenic in adults older than 18 years. More importantly, this vaccine has demonstrated protective efficacy in a field efficacy trial against drifted influenza viruses[9,10,48]. Besides, the rHA can be also expressed in mammalian expression system[4,39,54], and even in prokaryote such as *E.coli*[3]. In order to mimic the natural form of HA at the virus surface, now more and more scientists are trying to express the trimerization HA, and it turns out that this kind of rHA vaccine exerts a much better protection efficiency than the monomer rHA in animal model[3].

NA subunit vaccine

Influenza neuraminidase (NA) is also an integral membrane glycoprotein and a second major surface antigen of the virion. NA cleaves the α-(2,3) or α-(2,6) glycosidic linkage between a terminal sialic acid residue and its adjacent carbohydrate moiety (glycoproteins or glycolipids) on the host receptor, allowing progeny virus particles escaping from infected cell surfaces, and thus facilitating virus spread[46,51]. NA has been proven as a valid therapeutic target for antiviral drugs due to its essential role in the viral replication cycle[35]. Zanamivir and Oseltamivir are such drugs directed to influenza neuraminidase, and now approved for general use in some developed countries[19].

In addition to being a well-recognized target for antiviral drugs, NA can also be a candidate in influenza vaccine design. The immunogenicity of NA

have been intensively studied in experimental animal models[5,25,27,31]. Given the role that NA plays in the virus replication cycle, antibody to NA is infection permissive, but has been correlated with resistance to challenge or natural infection[30]. In one research the author found that volunteers' clinical response to the wild-type virus was related to the level of serum anti-neuraminidase antibody[37], proving that anti-NA antibodies have been linked with reducing the severity of clinical disease and contributing to the prevention of epidemic spread in humans. The proposed mechanism of action is that antibodies against NA may prevent the influenza virus from spreading through infected body[8]. Therefore, for NA subunit vaccine, rather than being a standing alone vaccine, maybe it is better to be used as a supplement to the licensed vaccine[28]. At present, a phase Ⅱ challenge by addition of a NA subunit vaccine "FluNhance" to a licensed seasonal vaccine was conducted by the National Institute of Allergy and Infectious Diseases, results from the study showed that the subjects who injected this mixed vaccine experienced milder illness that was of shorter duration when compared to subjects only injected the licensed seasonal vaccine (Protein Sciences Co., USA).

M2 subunit vaccine

The M2 protein is a transmembrane pH-activated proton-selective ion channel, playing crucial roles in viral replication and protection of HA maturation and structural integrity[40]. In mature virus particles, M2 exists as a homotetramer, and each monomer consists of 97 amino acid residues[22]. The extracellular domain of the M2 protein, known as M2e, consists of N-terminal 24 residues, and is remarkably conserved especially at the residues 2-9 (SLLTEVET) that is shared among all subtypes of influenza A viruses[16].

M2e is located at the outside of membrane and most importantly of its high conservation, makes it a promising target for development of universal influenza vaccines. A recombinant vaccine using recombinant virus-like particles derived from Hepatitis B virus core (HBc) as carrier for efficacious presentation of the M2e antigen (M2e-HBc) elicited high level anti-M2e antibodies, which protected mice against mouse adapted (m.a.) X47 virus (H3N2) challenge[13]. Another research group demonstrated the potential of using a papaya mosaic virus (PapMV) carrying the universal M2e influenza epitope (PapMV-CP-M2e) as a vaccine can induce anti-M2e antibodies that recognize influenza-infected cells[14]. Furthermore, human anti-M2e monoclonal antibodies have been proved to protect mice from lethal challenges with either H5N1 or H1N1 influenza viruses. These results suggest the potential of the anti-M2e human monoclonal antibody (MAb) has a broad binding spectrum, and could be regarded as a universal passive immunotherapeutic agent to influenza A virus infection[20,50]. Recently, Phase I clinical studies have been conducted with M2e vaccine candidates, demonstrating their safety and immunogenicity in humans[45], further implying that M2e-based vaccines have great potential to be developed as effective and safe universal influenza vaccines. So if the M2e as the target antigen for producing universal vaccine is to be approved, it can not only save much time in picking of vaccine strain in each epidemic season, but also make it possible in reservation of vaccines against pandemic human flu.

NP subunit vaccine

The nucleoprotein (NP), which has multiple

functions during the virus life cycle, possesses regions that are highly conserved among influenza A, B, and C viruses[36]. It stands to reason that NP is another promising candidate for the design of universal influenza vaccines. Currently, a number of recombinant expressions of NP protein have been shown to stimulate protective immune responses and provide cross-protection against homologous or heterosubtypic influenza viruses, resulting in the potential to protect against a broader range of viruses.

Altstein *et al*. reported that a recombinant vaccinia virus expressing NP protein of influenza A/PR8/34 (H1N1) virus induced specific antibodies and protected mice against low-dose challenge of mouse-adapted heterosubtypic variants of human A/Aichi2/68 (H3N2) and avian A/Mallard/ Pennsylvania/ 10218/84 (H5N2) influenza virus strains[1]. Similar studies also demonstrated that recombinant vesicular stomatitis viruses (rVSVs) expressing influenza nucleoprotein (VSV NP) induces robust anti-NP CD8 T cells, thus enhances antibody-mediated protection after lethal challenge of mice with influenza virus A/PR/8/34[2]. Since influenza NP is a highly conserved antigen, and can induce protective anti-NP responses, it may be a productive strategy for generating heterologous protection against divergent influenza strains.

VLP vaccine

Co-expression of the influenza virus proteins in the same cell may result in the production of Virus-like particles (VLPs), which mimics the organization and conformation of authentic native viruses but lack of viral genomic material[44]. Influenza A virus VLPs have been studied extensively nowadays, and a wide variety of them have shown favorable results in mouse and ferret models. Those VLPs could enhance the production of neutralizing antibodies, target dendritic cells (DCs) to activate the innate and adaptive immune responses, thus enable the subject animals to survive from a lethal challenge[43].

Even though recombinant influenza A virus VLPs can be produced by only expression of HA and M1[43] or HA and NA[6] , most of them have been generated by co-expressing the M1 in combination of HA, NA, and/or M2 protein[44]. Nowadays the Influenza VLPs can be produced in plant cell[12], mammalian expression system 293T cell[47] or Vero cell[53], but the best way to manufacture influenza A virus VLP vaccine is to use the baculovirus-insect cell expression system[17,34]. Novavax (MD, USA) is such an example, which conducted three clinical trials in parallel to evaluate safety and immunogenicity of 2009 pandemic H1N1(Phase II), seasonal influenza(Phase IIa) and H5N1 (Phase I/IIa) VLPs in humans. In 2011, the company published the Phase II clinical results of their 2009 pandemic H1N1 VLP vaccine, demonstrated the VLP vaccine was safe and well-tolerated, and robust HAI immune responses were detected after vaccination with high rates of seroprotection（≥40 HAI titer) in 82–92% of all subjects[33]. Also in 2010, they released the final results of its trivalent (H1N1 A/Brisbane/59/2007, H3N2 A/Brisbane/10/2007, B/Florida/04/2006) seasonal influenza VLP vaccine that was conducted both in older adults (60 years or higher in age) and health adults (18 to 49 years in age), and the results showed the vaccine was well-tolerated and immunogenic in both groups (Novavax, USA). As for H5N1 VLPs, the Phase I/IIa clinical research demonstrated strong neutralizing antibody titers and robust hemagglutination

inhibition (HAI) responses[29]. All those inspiring clinical results outline the promising future for the influenza VLP vaccines.

CONCLUSION

The current licensed influenza vaccine provides an economical and effective means to reduce the impact of an influenza infection. However, as discussed above, conventional egg-based influenza vaccines have a number of limitations. All the limitations call for a new vaccine production method, through which the influenza vaccine can be produced in a much shorter time period and in a much easier scalable way. The recombinant influenza vaccine, taking the advantage of recombinant DNA technologies, can be produced in such a novel way.

The influenza subunit and VLP vaccines (Table 1) offer the following potential advantages compared with the conventional egg based vaccines. Firstly, selection or adaptation of influenza virus strains for production at high levels in eggs is not required; meanwhile the cloning, expression and manufacture of recombinant influenza virus can be accomplished within a short period of time (probably less than 8 weeks) much less than the traditional egg based vaccine production (about 6 months)[8]; Secondly, the influenza subunit and VLP vaccines are produced in a scalable, reproducible, and quality-stable, as well as low bio-burden way, which results in lower cost and quicker response in the epidemic season with a new epidemic or pandemic strain of influenza virus[22], thus providing sufficient vaccine for inoculators[16]; Thirdly,

Table 1. Summary of the potential subunit and VLP vaccines against influenza

Type	Name	Company/Researcher	Stage	Description	Reference
HA Subunit Vaccine	FluBlØk	Protein Sciences Corporation	Phase Ⅲ (completed)	HA protein produced by BEVS*	[13,14,52]
	VAX125	VaxInnate	Phase Ⅱ	HA fused to flagellin	VaxInnate Corporation. USA
	Intranasal Influenza Vaccine	Vaxin	Phase Ⅱ	HA produced in a non-replicating adnovirus	Vaxin Inc. UK
NA Subunit Vaccine	FluNhance	Protein Sciences Corporation	Phase Ⅱ	NA produced by BEVS	Protein Sciences Corporation, USA
M2 Subunit Vaccine	ACAM-FLU-A	Acambis	Phase Ⅰ	M2e linked to the Hepatitis B core protein	[17]
	PapMV-CP-M2e	J. Denis, *et al.*	Preclinical study	M2e fused to the PapMV-CP	[18]
NP Subunit Vaccine	Vaccinia NP	A. D. Altstein, *et al.*	Preclinical study	NP expressed by vaccinia virus	[5]
	VSV NP	B.E. Barefoot, *et al.*	Preclinical study	NP expressed by vesicular stomatitis virus	[6]
VLP accine	A(H1N1) 2009 Influenza VLP	Novavax	Phase Ⅱ	coexpression of HA, NA and M1 of H1N1 with BEVS	[37]
	H5N1 VLP	Novavax	Phase Ⅰ	Coexpression of HA, NA and M1 of H5N1 with BEVS	[33]

*BEVS: Baculovirus Expression Vector System

purification procedures for influenza subunit and VLP vaccines is relatively easier which does not include influenza virus inactivation or organic extraction procedures, thus avoiding additional safety concerns because of residual harmful chemicals in the vaccine. Perhaps what is the most important, from a clinical perspective, vaccine produced in this way is highly purified and does not contain ovalbumin or other antigenic proteins present in eggs[11,23]. Besides, if the universal influenza vaccine would be produced, it will leave out the trouble of adjusting the vaccine strain each year, and will have the possible reservation of large amount of vaccine against the emerging pandemic human flu. Though the universal vaccine is still in the early stage of research, this concept and further manufacturing of product deserve the expectation. All those advantages fully demonstrated the great potential for the production of recombinant influenza vaccines to prevent influenza infection in epidemic even pandemic seasons.

Above all, our comprehensive review on the recent developments of subunit and VLP vaccines against human influenza virus would help to get insight into the current situation of influenza vaccines, and suggest the future design and development of novel influenza vaccines.

References

1. **Altstein A D, Gitelman A K, Smirnov Y A, *et al.* 2006.** Immunization with influenza a np-expressing vaccinia virus recombinant protects mice against experimental infection with human and avian influenza viruses. **Arch Virol**, 151(5): 921-931.
2. **Barefoot B E, Sample C J, Ramsburg E A. 2009.** Recombinant vesicular stomatitis virus expressing influenza nucleoprotein induces cd8 t-cell responses that enhance antibody-mediated protection after lethal challenge with influenza virus. **Clin Vaccine Immunol**, 16(4): 488-498.
3. **Biesova Z, Miller M A, Schneerson R, *et al.* 2009.** Preparation, characterization, and immunogenicity in mice of a recombinant influenza h5 hemagglutinin vaccine against the avian h5n1 a/vietnam/1203/2004 influenza virus. **Vaccine**, 27(44): 6234-6238.
4. **Brands R, Visser J, Medema J, *et al.* 1999.** Influvac: A safe madin darby canine kidney (mdck) cell culture-based influenza vaccine. **Dev Biol Stand**, 98: 93-100; discussion 111.
5. **Brett I C, Johansson B E. 2005.** Immunization against influenza a virus: Comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. **Virology**, 339(2): 273-280.
6. **Chen B J, Leser G P, Morita E, *et al.* 2007.** Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. **J Virol**, 81(13): 7111-7123.
7. **Couch R B, Cate T R. 1983.** Managing influenza in older patients. **Geriatrics**, 38(9): 61-74.
8. **Cox M M. 2005.** Cell-based protein vaccines for influenza. **Curr Opin Mol Ther**, 7(1): 24-29.
9. **Cox M M, Hollister J R. 2009.** Flublok, a next generation influenza vaccine manufactured in insect cells. **Biologicals**, 37(3): 182-189.
10. **Cox M M, Patriarca P A,Treanor J. 2008.** Flublok, a recombinant hemagglutinin influenza vaccine. **Influenza Other Respi Viruses**, 2(6): 211-219.
11. **Cox M M J,Karl Anderson D. 2007.** Production of a novel influenza vaccine using insect cells: Protection against drifted strains. **Influenza Other Respi Viruses**, 1(1): 35-40.
12. **D'Aoust M A, Lavoie P O, Couture M M, *et al.* 2008.** Influenza virus-like particles produced by transient expression in nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. **Plant Biotechnol J**, 6(9): 930-940.
13. **De Filette M, Martens W, Smet A, *et al.* 2008.** Universal influenza a m2e-hbc vaccine protects against disease even in the presence of pre-existing anti-hbc antibodies. **Vaccine**, 26(51): 6503-6507.

14. **Denis J, Acosta-Ramirez E, Zhao Y, *et al.*** 2008**.** Development of a universal influenza a vaccine based on the m2e peptide fused to the papaya mosaic virus (papmv) vaccine platform. **Vaccine**, 26(27): 3395-3403.
15. **Doherty P C, Turner S J, Webby R G, *et al.*** 2006**.** Influenza and the challenge for immunology. **Nat Immunol**, 7(5): 449-455.
16. **Fiers W, De Filette M, Birkett A, *et al.*** 2004**.** A "universal" human influenza a vaccine. **Virus Res**, 103(1-2): 173-176.
17. **Gavrilov V, Orekov T, Alabanza C, *et al.*** 2011**.** Influenza virus-like particles as a new tool for vaccine immunogenicity testing: Validation of a neuraminidase neutralizing antibody assay. **J Virol Methods**, 173(2): 364-373.
18. **Gerdil C.** 2003**.** The annual production cycle for influenza vaccine. **Vaccine**, 21(16): 1776-1779.
19. **Govorkova E A, Leneva I A, Goloubeva O G, *et al.*** 2001**.** Comparison of efficacies of rwj-270201, zanamivir, and oseltamivir against h5n1, h9n2, and other avian influenza viruses. **Antimicrob agents ch**, 45(10): 2723-2732.
20. **Grandea A G, Olsen O A, Cox T C, *et al.*** 2010**.** Human antibodies reveal a protective epitope that is highly conserved among human and nonhuman influenza a viruses. **Proc Natl Acad Sci USA**, 107(28): 12658-12663.
21. **Holmes E C, Ghedin E, Miller N, *et al.*** 2005**.** Whole-genome analysis of human influenza a virus reveals multiple persistent lineages and reassortment among recent h3n2 viruses. **Plos Biology**, 3(9): e300.
22. **Holsinger L J, Alams R.** 1991**.** Influenza virus M_2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. **Virology**, 183(1): 32-43.
23. **Holtz K M, Anderson D K, Cox M M J.** 2003**.** Production of a recombinant influenza vaccine using the baculovirus expression vector system. **Bioprocess J**, 2: 25-32.
24. **Bac-to-bac® baculovirus expression system.** 2010. An efficient site-specific transposition system to generate baculovirus for high-level expression of recombinant proteins. **Invitrogen.** p1-69.
25. **Johansson B, Kilbourne E.** 1994**.** Immunization with purified n1 and n2 influenza virus neuraminidases demonstrates cross-reactivity without antigenic competition. **Proc Natl Acad Sci USA**, 91(6): 2358.
26. **Johansson B E, Brett I C.** 2007**.** Changing perspective on immunization against influenza. **Vaccine**, 25(16): 3062-3065.
27. **Johansson B E, Price P M, Kilbourne E D.** 1995**.** Immunogenicity of influenza-a virus n2 neuraminidase produced in insect larvae by baculovirus recombinants. **Vaccine**, 13(9): 841-845.
28. **Johansson B E, Pokorny B A, Tiso V A.** 2002**.** Supplementation of conventional trivalent influenza vaccine with purified viral n1 and n2 neuraminidases induces a balanced immune response without antigenic competition. **Vaccine**, 20(11-12): 1670-1674.
29. **Khurana S, Wu J, Verma N, *et al.*** 2011**.** H5n1 virus-like particle vaccine elicits cross-reactive neutralizing antibodies that preferentially bind to the oligomeric form of influenza virus hemagglutinin in humans. **J Virol**, 85(21): 10945-10954.
30. **Kilbourne E D, Cerini C, Khan M, *et al.*** 1987**.** Immunologic response to the influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin. I. Studies in human vaccinees. **J Immunol**, 138(9): 3010.
31. **Kilbourne E D, Pokorny B A, Johansson B, *et al.*** 2004**.** Protection of mice with recombinant influenza virus neuraminidase. **J Infect Dis**, 189(3): 459-461.
32. **Lamb R, Krug R.** 2001. Orthomyxoviridae: The viruses and their replication. In: **Fields Virology.** Knipe D N, Howley P M, Griffin D E, *et al*, ed. Philadelphia: Lippincott Williams and Wilkins Publishers, pp1487-1532.
33. **Lopez-Macias C, Ferat-Osorio E, Tenorio-Calvo A, *et al.*** 2011**.** Safety and immunogenicity of a virus-like particle pandemic influenza a (h1n1) 2009 vaccine in a blinded, randomized, placebo-controlled trial of adults in mexico. **Vaccine**, doi:10.1016/j.vaccine.2011.07.099.
34. **Mahmood K, Bright R A, Mytle N, *et al.*** 2008**.** H5n1 vlp vaccine induced protection in ferrets against lethal challenge with highly pathogenic h5n1 influenza viruses. **Vaccine**, 26(42): 5393-5399.
35. **McKimm-Breschkin J, Trivedi T, Hampson A, *et al.*** 2003**.** Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. **Antimicrob Agents Ch**, 47(7): 2264-2272.
36. **Mena I, Jambrina E, Albo C, *et al.*** 1999**.** Mutational analysis of influenza a virus nucleoprotein: Identification of mutations that affect rna replication. **J Virol**, 73(2): 1186-1194.

37. **Murphy B R, Kasel J A, Chanock R M.** 1972**.** Association of serum anti-neuraminidase antibody with resistance to influenza in man. **New Engl J Med**, 286(25): 1329-1332.

38. **Neumann G, Noda T, Kawaoka Y.** 2009**.** Emergence and pandemic potential of swine-origin h1n1 influenza virus. **Nature**, 459(7249): 931-939.

39. **Pau M G, Ophorst C, Koldijk M H, *et al.*** 2001**.** The human cell line per.C6 provides a new manufacturing system for the production of influenza vaccines. **Vaccine**, 19(17-19): 2716-2721.

40. **Pinto L H, Lamb R A.** 2006**.** The m2 proton channels of influenza a and b viruses. **J Biol Chem**, 281(14): 8997-9000.

41. **Pleschka S, Ludwig S, Wolff T, *et al.*** 2006**.** Anti-viral approaches against influenza viruses. **New Concepts of Antiviral Therapy**, 115-167.

42. **QIAexpressionist A.** 2002. **A handbook for high-level expression and purification of 6xhis-tagged proteins.** Qiagen. p1-125.

43. **Quan F S, Huang C, Compans R W, *et al.*** 2007**.** Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. **J Virol**, 81(7): 3514-3524.

44. **Roldao A, Mellado M C, Castilho L R, *et al.*** 2010**.** Virus-like particles in vaccine development. **Expert Rev Vaccines**, 9(10): 1149-1176.

45. **Schotsaert M, De Filette M, Fiers W, *et al.*** 2009**.** Universal m2 ectodomain-based influenza a vaccines: Preclinical and clinical developments. **Expert Rev Vaccines**, 8(4): 499-508.

46. **Sylte M J, Suarez D L.** 2009**.** Influenza neuraminidase as a vaccine antigen. In:**Vaccines for Pandemic Influenza**. Compans R W, Orenstein W A, eds. Heidelberg: Springer, p227-241.

47. **Szecsi J, Boson B, Johnsson P, *et al.*** 2006**.** Induction of neutralising antibodies by virus-like particles harbouring surface proteins from highly pathogenic h5n1 and h7n1 influenza viruses. **Virol J**, 3: 70.

48. **Treanor J J, Schiff G M, Hayden F G, *et al.*** 2007**.** Safety and immunogenicity of a baculovirus-expressed hemagglutinin influenza vaccine: A randomized controlled trial. **JAMA**, 297(14): 1577-1582.

49. **Wang K, Holtz K M, Anderson K, *et al.*** 2006**.** Expression and purification of an influenza hemagglutinin—one step closer to a recombinant protein-based influenza vaccine. **Vaccine**, 24(12): 2176-2185.

50. **Wang R, Song A, Levin J, *et al.*** 2008**.** Therapeutic potential of a fully human monoclonal antibody against influenza a virus m2 protein. **Antiviral Res**, 80(2): 168-177.

51. **Webster R G, Bean W J, Gorman O T, *et al.*** 1992**.** Evolution and ecology of influenza-a viruses. **Microbiol Rev**, 56(1): 152-179.

52. **Weldon W C, Wang B Z, Martin M P, *et al.*** 2010**.** Enhanced immunogenicity of stabilized trimeric soluble influenza hemagglutinin. **PLoS One**, 5(9).

53. **Wu C Y, Yeh Y C, Yang Y C, *et al.*** 2010**.** Mammalian expression of virus-like particles for advanced mimicry of authentic influenza virus. **PLoS One**, 5(3): e9784.

54. **Youil R, Su Q, Toner T J, *et al.*** 2004**.** Comparative study of influenza virus replication in vero and mdck cell lines. **J Virol Methods**, 120(1): 23-31.