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EVALUATION OF STABILITY OF INACTIVATED SPLIT TRIPVALENT SEASONAL INFLUENZA VACCINE (IVACFLU-S) PRODUCED BY IVAC

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SUMMARY OF PhD THESIS ON MEDICINE

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LIST OF PUBLISHED SCIENTIFIC ARTICLES RELATED TO THE THESIS

1. Nguyen Hoang Tung, Le Van Be, Nguyen Le Khanh Hang, Nguyen Van Hung (2020). Results of preclinical assessment of IVACFLU-S seasonal influenza vaccine produced by IVAC in Vietnam in 2014-2017. *Journal of Vietnam Preventive Medicine*, Vol.5 (30): 134-142.
2. Nguyen Hoang Tung, Nguyen Thi Ly, Hoang Minh Hung (2018). Stability of seasonal flu vaccine IVACFLU-S 2014-2015 and 2016-2017, *Journal of Vietnam Preventive Medicine*, Vol.11 (28): 29-34.

INTRODUCTION

Influenza viruses belong to Orthomyxoviridae family. The influenza viruses caused influenza in humans are mainly of influenza type A and B. Influenza is one of the dangerous infectious diseases not only because of its adverse health effects due to the annual influenza pandemic, it is also capable in causing global influenza pandemics with a large number of deaths.

According to the World Health Organization (WHO), vaccination against influenza contributes to reduction about 60% of flu-related illness, reducing the risk of death from influenza up to 70-80% as well as reducing up to 70-90% the risk of getting flu among healthy people.

In Vietnam, the Institute of Vaccines and Medical Biologicals (IVAC) is the only unit that has an influenza vaccine production line on chicken eggs with a capacity of 1.5 million doses / year meeting GMP-WHO standards, served a solid foundation for research, development and production of seasonal trivalent influenza vaccines in Vietnam. Along with the research to produce seasonal trivalent influenza vaccine, the assessment of vaccine's quality stability as well as study on its ability to create the immune response is a necessary issue for introducing the fragment seasonal trivalent influenza vaccine in human clinical trials. This study, therefore was conducted with the following objectives:

1. Assess pre-clinically the inactivated seasonal trivalent split influenza vaccine for use in clinical trial.

2. Evaluate the efficacy and stability of inactivated seasonal trivalent split influenza vaccine, 2014 - 2017.
3. Evaluate the stability of some chemical and physical properties of inactivated seasonal trivalent split influenza vaccine, 2014 – 2017.

New contributions in science and practical value of the thesis

The results of preclinical assessment given in this thesis have contributed in confirmation of the quality of IVACFLU-S seasonal trivalent influenza vaccine produced by IVAC meeting the standards of preclinical testing. It is an important premise to ensure the research and development, proceed to commercial production and use the vaccines for disease prevention.

The study has evaluated the stability of IVACFLU-S seasonal trivalent influenza vaccine and obtained results showed that a temperature of 5 ± 3^0C is suitable for storage of the vaccine. After 15 months of storage at a 5 ± 3^0C, the virus HA antigen concentration of the IVACFLU-S seasonal trivalent influenza vaccine tended to decrease but still ensured to meet the manufacturer's registration standards and as the result, the vaccine shelf life of 12 months from the date of manufacture is completely suitable.

Influenza vaccine production technology based on chicken eggs with embryos has been applied in reputable vaccine manufacturers in developed countries around the world. Research results contribute to confirm that this technique can be completely applied in Vietnam.

The structure of thesis

Thesis consisted of 109 pages (excluding the administrative sections, lists of published articles, references and appendices),

35 tables and 22 figures. Of which, 2 pages of the Introduction; 34 pages of Literature overview; 21 pages of Study subjects and methods; 28 pages of Research Results; 20 pages of Discussion; 2 pages of Conclusion and 1 page of Recommendation.

Thesis has used 121 scientific articles and documents for references.

CHAPTER 1. LITERATURE REVIEW

1.1. Influenza virus

1.1.1 Morphology, structure

Influenza viruses are very diverse in shape: spherical, ovoid or sometimes filamentous of up to 2000 nm, with an average diameter of 80-120 nm. Influenza A and B virus particles have an envelope. The viral envelope is essentially a protein derived from the cytoplasmic membrane of the host, including some glycoproteins and non-glycosylated bare proteins.

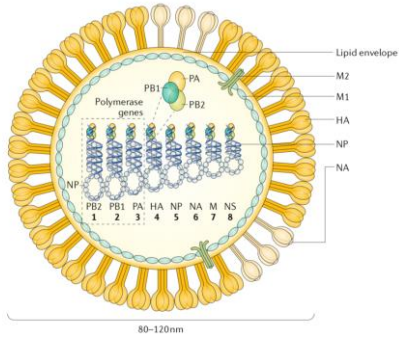

Figure 1.1. Structural model of influenza virus

**Source:* https://www.nature.com/articles/s41572-018-0002-y[48]

1.1.2 HA and NA antigens in the immune response to influenza virus infection

Influenza virus antigens are mainly surface antigens consisting of HA and NA proteins. HA antigen consists of 1,742-1,778

nucleotides encoding 562-566 amino acids, capable of causing red blood cell agglutination, attaching to receptors containing sialic acid on the cell surface, helping the invasion of virus to host cells.

NA antigen is a protein of 1413 nucleotides encoding 453 amino acids, acts as an important chain in the release of virus from infected cells and spread them in the respiratory tract. NA can produce immunity more durable and therefore can be use for developing the universal vaccine against influenza.

1.2. Influenza surveillance system

1.2.1 Global influenza surveillance system

The Global Influenza Surveillance and Response System (GISRS) has carried out surveillance for influenza since 1952.

This GISRS System is coordinated by the World Health Organization (WHO) to provide following information:

- Rates of influenza infection, rate of circulating influenza virus strains, results of monitoring virus antigenic change.
- The important properties of the outbreak.
- Support the selection of influenza virus strains suitable for influenza vaccine production.

1.2.2 Influenza surveillance system in Vietnam

According to surveillance data from the National Influenza Center – National Institute of Hygiene and Epidemiology in 2006 – 2011, 17.8% of samples tested were positive for influenza virus. Monthly statistic analysis showed that influenza is circulating throughout the year with the peaks between July and August. Influenza A/H1N1 virus strains and B virus strains were reported with the highest frequency in 2006 (53.7% and 40.8%, respectively) and in 2008 (43.5% and 41.3%,

respectively), while strains of influenza A/H3N2 (73.9%) and of B virus (25.1%) were reported the most in 2007.

In 2 years, 2009 – 2010, along with the circulation of influenza A/H3N2 virus strain, the emerging epidemic of influenza A/H1N1*pdm09* was accounted for 46.6% in 2009 and 28.0% in 2010. In 2011, A/H1N1*pdm09* was the most reported influenza virus (74.1%), followed by influenza B virus (22.1%).

1.3. IVACFLU-S seasonal influenza vaccine

1.3.1 Virus strain used for vaccine production

A/H1N1 strain

- Strain name: NYMC X-179A A/H1N1 Code 09/124
- Source: Standard virus strain NYMC X-179A, derived from strain A/California/7/2009), dated July 17, 2009.

A/H3N2 strain

- Strain name: NYMCX-223A
- Source: Standard virus strain NYMCX-223ª, derived from strain A/Texas/50/2012, dated July 28, 2014.

Influenza B virus strain

- Strain name: NYMC BX-51B
- Source: Standard virus strain NYMCBX-51B, derived from strain B/Massachusetts/02/2012, dated December 06, 2013.

1.3.2 Production stages for IVACFLU-S vaccine

Egg incubation: The automatic egg incubation is processed in a specific incubator under strictly controlled temperature conditions of about 36^0C - 38^0C, humidity of 70% - 80% with a capacity of 20,000 eggs / batch.

Infection: The strains used for egg infection are influenza strains NYMC X-179A (H1N1), NYMC X-223A (H3N2) and influenza B NYMC BX-51B.

Culture: Infected eggs are transferred to incubators. After enough incubation time, check to remove the incubation failed eggs and transfer all achieved requirement eggs to cold storage of 2^0C – 8^0C for 20-24 hours.

Harvesting the fluid containing virus from the infected eggs: After being cooled, eggs are transferred to the specific room for collection of the egg fluid for harvesting fluid containing virus.

Centrifuge: The harvested egg fluid containing virus is stored in a tank and are centrifuged to obtain the clear supernatant fluid.

Concentration: The clear supernatant containing virus obtained after centrifugation or filtration, is transferred to the condensing system before going through the ultracentrifugation stage.

Ultra-centrifugation: The concentrated egg fluid containing virus is centrifuged by ultracentrifugation.

Split: After ultracentrifugation stage, treat the obtained influenza virus particles with 0.75% Triton X-100 to cell break down for 1 hour at room temperature.

Inactivation: Use 0.01% formalin to inactivate virus solution.

Pre-filtration: Use 5 µm filter to remove sediment before lysis.

Lysis, sterilization by filtration: After inactivation, egg solution is lysed through hollow fiber column system or TFF 50 kD column to remove sucrose, triton X-100 from the product.

Preparation of flu vaccine: Preparing semi-finished vaccines in laminar flow cabinet (grade 100K class) in the grade cleanroom.

Vaccine bottle filling: After checking the sterility and safety, vaccine solution is filled into the bottles. The flu vaccine is packaged in a 2ml glass bottle containing a single dose of 0.5ml.
Packing: Labeling, packaging the vaccine bottles**.**
Storage: Vaccines are stored at a temperature of 2^0C - 8^0C.

1.3.3 Producing and control the inactivated seasonal flu vaccine in Vietnam

Table 1.2 Quality standards of IVACFLU-S vaccine

No	Criteria	Applied standards
1	Sensory	TCCS
2	Volume	TCCS
3	Sterility	Vietnamese pharmacopoeia IV, 2009
4	pH	TCCS
5	HA identification	WHO- TRS 927, 2005
6	HA concentration	TCCS
7	Endotoxin	Europe pharmacopoeia 2008
8	Total protein	Europe pharmacopoeia 2008
9	Formaldehyde residue	Vietnamese pharmacopoeia IV, 2009
10	Safety	Vietnamese pharmacopoeia IV, 2009

CHAPTER 2. STUDY MATERIALS AND METHODS

2.1. Study materials

2.1.1. Study samples

❖ ***Formula of IVACFLU-S flu vaccine for the 2014-2015 epidemic season:***

In 1 dose of 0.5 ml of IVACFLU-S seasonal flu vaccine contains the following antigen components:

- NYMC X-179A (A/California/7/2009) (H1N1)
- NYMCX-223A (A/Texas/50/2012) (H3N2)
- NYMC BX-51B (B/Massachusetts/02/2012) (B)
- Phosphat buffer solution pH = 7.2

❖ ***Formula of IVACFLU- S flu vaccine for the 2016-2017 epidemic season:***

In 1 dose of 0.5 ml of IVACFLU-S seasonal flu vaccine contains the following antigen components:

- NYMC X-179A (A/California/7/2009) (H1N1)
- NYMCX-263B (A/Hong Kong/4081/2014) (H3N2)
- NYMC BX-35B (B/Brisbane/60/2008) (B)
- Phosphat buffer solution pH = 7.2

❖ ***Formula of Placebo:***

In 1 placebo dose of 0.5 ml contains:

- NaCl 4.5 mg
- $Na_2HPO_4.2H_2O$ 0.695 mg
- $NaH_2PO_4.2H_2O$ 0.181 mg
- Distilled water

2.1.2. Biokits

No	Biokits
1	Anti-standard influenza (H1N1) HA antibody

2	Anti standard influenza (H3N2) HA antibody
3	Anti standard influenza (B) HA antibody
4	Standard influenza (H1N1) HA
5	Standard influenza (H3N2) HA
6	Standard influenza (B) HA

2.1.3. Equipment and consumable materials

No	Equipment
1	The thermocouple with an accuracy of 0.1^0C
2	pH meter
3	Microscope

2.1.4. Experimental animals

Table 2.1 Experimmental animals for pre-clinical asessment

Type	Breed	Gender	Source
Mice	*Swiss*	Both sex	IVAC
Guinea pigs		Male	IVAC
Rabbits	*New Zealand*	Both sex	IVAC

2.2. Study methods

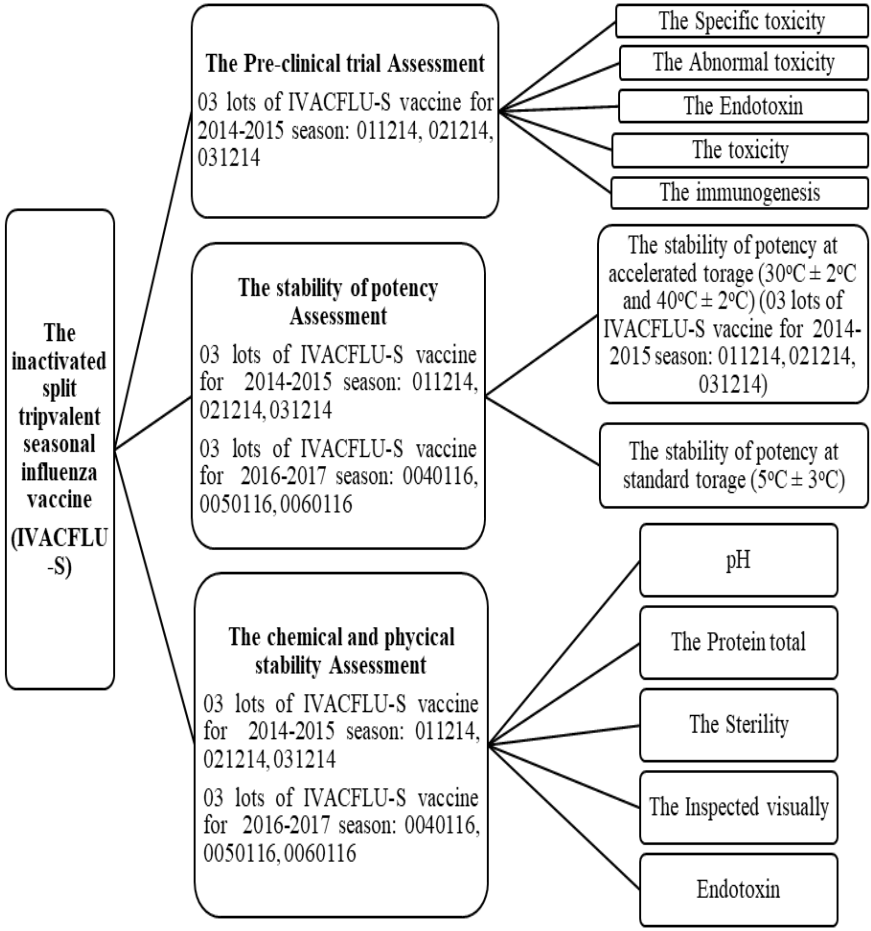

Figure 2.1 Research diagram

The study was conducted with 3 contents corresponding to 3 research objectives, including: Pre-clinical assessment, evaluation of stability and effectiveness, evaluation of stability of some physical and chemical properties (Figure 2.1).

CHAPTER 3. STUDY RESULTS

3.1. Results of pre-clinical assessment of IVACFLU- S influenza seasonal vaccine

3.1.1. Specific safety test

Research results showed that, 3 batches of seasonal flu vaccine bulks (CT-S/H1N1/01, CT-S/H3N2/04, CT-S/B/07) used to produce IVACFLU-S seasonal flu vaccine achieved the specific safety test requirements according to WHO/TRS 927, 2005 guidance. The percentage of tested embryos alive after 1st and 2nd culture passage both reached 100% at all batches of influenza monovalent vaccine bulks.

3.1.2. General safety test

The testing results in mice showed that the general safety of 3 series of IVACFLU-S seasonal influenza vaccines, lots 011214, 021214, and 031214, all met the requirements. The average increase in mice weight after 7 days of testing ranged from 9.46 to 10.03g/mouse/7 days.

3.1.3. Fever test

The increase in body temperature of rabbits after vaccination ranged from 0.1 to 0.3^0C, while this of rabbits injected with placebo ranged from 0 to 0.1^0C. The total increase in body temperature of 3 tested rabbits immunized with the same vaccine batch ranged from 0.4 to 0.7^0C. The total increase in body temperature of 3 rabbits injected with placebo after injection was only 0.1^0C. Test results showed that the batches of IVACFLU-S seasonal influenza trivalent vaccine, in particular batches 011214, 021214, and 031214, all met the requirements according to the guidelines of Vietnam Pharmacopoeia IV,

2009. The increase in body temperature of each rabbit after vaccination was not more than 0.6^0C/rabbit, the total increase in body temperature of 3 tested rabbits was not more than 1.3^0C/3 rabbits.

3.1.4. Toxicity test

3.1.4.1. Blood biochemical index

Average values of blood biochemical indices including gluocse, Creatinine, GOT, GPT of vaccinated rabbits were found within normal physiological limits. There was no difference in the blood biochemical indices tested in the vaccinated and placebo rabbits, except for the glucose index on day 16 of follow-up. However, result of glucose testing showed that this index of experimental rabbits is within normal physiological limits.

3.1.4.2 Pathology and cytology

For the rabbits vaccinated with 3 batches of IVACFLU-S seasonal trivalent influenza vaccine, testing results showed the difference of the muscle in the injection site observed by the 40X lens before and after vaccination. However, post-injection pathology images of the muscles at the injection site of all experimental rabbits showed the parallel striated muscle bundles, normal morphology, some flatern veins, no congestion. There was no microscopic injury to the muscle at the injection site.

After 16 days and 28 days of vaccination with IVACFLU-S seasonal trivalent influenza vaccine, the images of bone marrow anatomy, lung tissue, liver tissue, spleen tissue, kidney tissue of vaccinated rabbits on a 40X-image lens showed a significant differences before and after injections. However, anatomic analysis result expressed normal pathological morphology.

Microscopic observations of all experimental rabbits showed no abnormal pathophysiology.

3.1.5. Test for immunogenicity

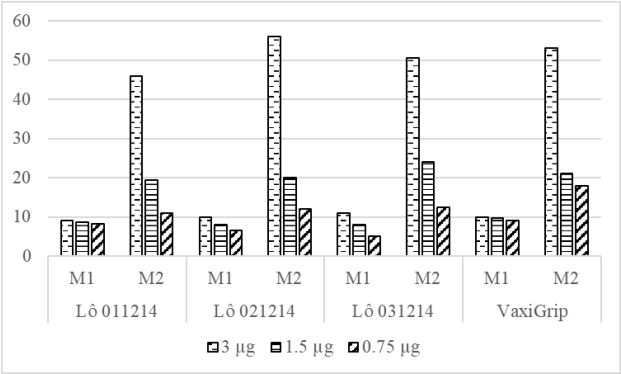

Figure 3.7 Immune response to X-179A (H1N1) strain of IVACFLU-S seasonal influenza vaccinated mice

After 21 days of the first injection (M1), the immune response to X-179A (H1N1) strain in mice injected with all 3 batches of tested vaccines and placebo was low at all 3 doses of the tested antigen (3µg/dose, 1.5µg/dose, 0.75µg/dose). The immune response level to X-179A (H1N1) strain in all experimental mice tested with 3 vaccine batches was about less than 10 IU/ml. Mice immune response increased after 14 days vaccinated with 2nd injection (M2). In which, the highest immune response was found in mice injected with lot vaccine IVACFLU-S 021214 with the average amount of antibodies obtained among the experimental mouse group was 57.6 IU/ml, the lowest response was obtained in mice injected with vaccine lot IVACFLU-S 011214 (47.2 IU/ml). Meanwhile, antibody level obtained from mouse group injected with the control commercial vaccine was found of 52.3 IU/ml.

Similar to experimental results on X-179A (H1N1) strain, immune response to X-223A (H3N2) strain of vaccinated mice after 21 days of the first injection (M1) was found fluctuated in the range below 20 IU/ml. Their immune response has increased after 14 days of 2nd injection (M2).

Experimental results on the immune response to BX-51B (B) strain showed that, after 21 days getting the first shot (M1), the antibody response level in all vaccinated mice with 3 batches of vaccine and control vaccines were low at all 3 experimental antigen doses (3 µg/dose, 1.5µg/dose, 0.75µg/dose), the maximum amount of the antibody obtained was averaged in the tested mouse group was 6.2 IU/ml.

3.2 The efficacy and stability of inactivated seasonal trivalent split influenza vaccine

3.2.1 The efficacy of IVACFLU- S seasonal flu vaccine

Table 3.8 The efficacy of IVACFLU-S seasonal trivalent flu vaccine 2014-2015

Virus strain	Lots of IVACFLU- S vaccines			p*
	011214	021214	031214	
B strain	24.0	23.9	24.6	>0.05
H1N1 strain	25.6	24.4	25.3	>0.05
H3N2 strain	27.9	28.7	27.5	>0.05

* *T-test*

Effectiveness of IVACFLU-S seasonal trivalent flu vaccine batches of 2014 – 2015 was tested immediately after production, the obtained average concentration of HA antigens of each strain in the vaccine batches was found ranged in between 23.9 - 28.7µg/dose.

Table 3.9 The efficacy of IVACFLU-S seasonal trivalent flu vaccine 2016-2017

Virus strain	Batch of IVACFLU- S vaccine			p*
	0040116	0050116	0060116	
B strain	17.9	17.8	19.6	>0.05
H1N1 strain	19.7	19.7	20.4	>0.05
H3N2 strain	20.0	19.8	18.9	>0.05

* *T-test*

The effectiveness of batches of IVACFLU-S seasonal flu vaccines in 2016 - 2017 season was also tested immediately after production, the result showed an average concentration of HA antigens of each strain in the vaccine batches ranged of 17.8-20.4 µg/dose.

3.2.2 The effective stability of IVACFLU-S seasonal influenza vaccine under accelerated conditions

At the challenge temperature of $30^0C \pm 2^0C$, the HA antigen concentration in all 3 batches of IVACFLU-S seasonal flu vaccine in the 2014-2015 season has decreased. After a trial period of 14 days at a temperature of $30^0C \pm 2^0C$, the concentration of this HA antigen of IVACFLU-S seasonal flu vaccine batches in the 2014-2015 season was found meeting reference standards, with the monovalent antigenic concentration achieved the minimum of ≥15µg/dose.

At the challenge temperature of $40^0C \pm 2^0C$, the concentration of HA antigens in the IVACFLU-S seasonal flu vaccine after 3 days of storage was decreased with an average rate ranging from 5.04 - 7.99% compared with the initial HA antigen content in the vaccine.

3.2.3 The effective stability by the time of IVACFLU-S vaccine at standard storage conditions

The effectiveness of batchs 011214, 021214, 031214 of IVACFLU-S seasonal influenza vaccine was found decreased over time when stored at a temperature of $5^0C \pm 3^0C$. After 15 months from the manufacturing date, the content of HA antigens of strains BX-51B (B), X-179A (H1N1), X-223A (H3N2) in batch 011214 was found of 36.4 µgHA/ml, 41.2 µgHA/ml and 34.4 µgHA/ml, respectively. Lot 021214 showed HA antigens concentration of 33.8µg HA/ml, 38.6µg HA/ml and 34.8µg HA/ml, respectively. Lot 031214 has this antigen concentration of 34.4µg HA/ml, 37.4µg HA/ml and 34.2µg HA/ml respectively.

The similar results were obtained with the 2016-2017 seasonal trivalent flu vaccine. The effectiveness of IVACFLU-S seasonal trivalent flu vaccine in batches 0040116, 0050116, 0060116 decreased over time when stored at $5^0C \pm 3^0C$. After 15 months from the date of manufacture, the content of HA antigens of strains BX-35B (B), X-179A (H1N1), X-263B (H3N2) was 31.8µg HA/ml, 31.6µg HA/ml, 31.4µg HA/ml, respectively, in batch 0040116; 31.2µg HA/ml, 32.0µg HA/ml, 31.6µg HA/ml, repectively, in batch 0050116, and 31.4µg HA/ml, 31.4µg HA/ml, 31.8µg HA/ml, respectively, in batch 0060116.

After 15 months from the date of manufacture, under the storage temperature condition of $5^0C \pm 3^0C$, the HA antigen content of each strain in the trivalent vaccine was still found meeting the manufacturer's reference standards with the HA concentration of monovalent strain of ≥ 15.0µg HA/dose.

3.3 The stability of some chemical and physical properties of inactivated seasonal trivalent split influenza vaccine, 2014 – 2017

Research results showed that pH of IVACFLU-S seasonal trivalent influenza vaccine batches in the 2014-2015 and 2016-2017 seasons was stable after 15 months of storage at a temperature of $5^0C \pm 3^0C$ from the date of manufacture, fluctuating by between 6.97 - 7.04 and 7.03 - 7.22, respectively.

The total protein content of the tested vaccine vials decreased over time. After 15 months of storage at $5^0C \pm 3^0C$, the average total protein content of vaccines 011214, 021214, 031214 batches was 205.5µg/45µgHA, 215.7µg/45µgHA, 196.2µg/45µg/HA, respectively and of the vaccine batches 0040116, 0050116, 0060116 was 281.3µg/45µgHA, 275.1µg/45µgHA, 279.1µg/45µgHA, respectively.

Results of assessing the stability of some other physicochemical properties of the vaccine (sterility, sensory and endotoxins) showed that after 15 months of storage at a temperature of $5^0C \pm 3^0C$, the IVACFLU-S seasonal trivalent flu vaccines produces in the 2014-2015 season still met the sterility, sensory and endotoxin requirements. The same results were obtained from the assessment of the sterility, sensory and endotoxin of the vaccine produced in 2016-2017 season: after 15 months of storage at a temperature of $5^0C \pm 3^0C$, the IVACFLU-S seasonal trivalent flu vaccines still met the sterility, sensory and endotoxin requirements.

CHAPTER 4. DISCUSSION

4.1 Assessment method for pre-clinical and its application in research the production of IVACFLU-S trivalent seasonal influenza vaccine in Vietnam

4.1.1. Method and value of preclinical assessment in research and production of vaccine

The ultimate goal of preclinical studies is to accurately modelized the desired biological effect of vaccines on laboratory animals to predict the efficacy of vaccines in humans. In vitro studies were performed including determination of pH, sensory, sterility, HA antigen content. The in vivo studies included determination of toxicity, specific safety, general safety, and immunogenicity. These tests were established in accordance with WHO recommendations and MOH regulations for preclinical evaluation applicable to vaccine research and vacine production. Many preclinical studies have shown a correlation between preclinical data and clinical trial results.

4.1.2 The assessment of safety and immunogenicity in research and produc the IVACFLU-S inactivated seasonal influenza vaccine

A specific safety test was performed with bulk of IVACFLU-S seasonally priced seasonal influenza vaccine to determine the specific toxicity of vaccine HA antigen for usage. Result of specific safety testing should show that the HA antigen of the influenza virus in the product is still capable to multiply or produce the toxicity to chicken embryos. Several studies have shown 20 different biomarkers for the same HA antigen that may characterize influenza vaccine-specific safety.

In the pre-clinical study, for testing the general safety, 3 batches of IVACFLU-S seasonal influenza vaccine in the 2014-2015 season, batch 011214, 021214 and 031214, were used. After 7 days of vaccination, the tested mice gained weight, healthy and showed no difference in weight gain among the vaccinated and placebo injected group. This result is consistent with general safety standards applied to seasonal flu vaccines when assess vaccine finished products. This result is similar to the trial of T. Mizukami et al. (2014) in a study on preclinical evaluation of 4 types of seasonal flu vaccines.

According to WHO guidelines, evaluation of vaccine toxicity in preclinical research also includes physical harmfull assessment of target tissues and organs related to vaccines. In particular, histopathology of any lesions detected during vaccine preclinical assessment should also be reported. In out study, in the preclinical assessment of IVACFLU-S seasonal influenza vaccine, the histopathology of experimental rabbits was performed with targested organs such as liver, kidney, marrow, spleen, and lungs.

4.1.2. Immunogenicity control in preclinical assessment of IVACFLU-S seasonal trivalent influenza vaccine

Immunogenicity has always been the first rangking criterion of manufacturers evaluating the success of a vaccine against disease. For vaccines to be able to produce good immunity, it’s not only neccessary to have a strong antigen, but also the active support of excipients. Although that could bring some of the side effects associated with the overall safety of the vaccine. Hemagglutinin (HA) is one of two main glycoproteins important for influenza, that is expressed on the surface of influenza virus

particles. It is also the reason that HA is used as a target antigen for influenza vaccine production and the immunogenicity of the seasonal influenza vaccine depends on the properties of the HA antigen. In which, its concentration and used regimen will determine the immunological response of host to HA antigens. HA antigens in different species also induce different immune responses.

Results of preclinical evaluation of IVACFLU-S seasonal trivalent influenza vaccine have contributed to prove this theory. With 3 different strains of influenza virus X-179A (H1N1), X-223A (H3N2) and BX- 51B (B), the immune response to strain X-223A (H3N2) was found the strongest and to strain BX-51B (B) was the weakest. However, this results were only obtained after mice received a second vaccine shot and evaluated at 14 days of post-injection. At the same time, the immune response also showed significant differences at different antigen doses. At the antigen dose of 0.75µg HA/dose, the immune response was very low and there was almost no difference between the first and the second injections. Large difference in immuno response was determined after injection of second dose of vaccine using HA antigen concentration of 1.5µg HA/dose and 3.0µg HA/dose. With a dose of 3.0µg HA/dose, the immune response to all 3 antigen types after the second injection were significantly higher than that obtain after the first injection.

This result suggested the development of a single-dose or 2-dose injection regimen when the IVACFLU-S seasonal flu vaccine is licened for manufactory and put into use in disease prevention. Injection administration route is also a determinant of the antigen's immunological response. For different pathogens, the

detection of entry path and subsequent replication sites of the pathogen is essential for identifying appropriate strategy for selection of vaccination route. In this study, the results of preclinical assessment have determined that the intramuscular route is appropriate for IVACFLU-S seasonal trivalent influenza vaccine.

4.2 The stability of IVACFLU-S seasonal trivalent flu vaccine produced in Vietnam

4.2.1 Effect and stability of IVACFLU-S vaccines stored at different temperatures

Vaccine effectiveness

The effectiveness of IVACFLU-S seasonal trivalent influenza vaccine is determined by the total HA antigen concentration in the vaccine. With 3 monovalent season influenza strains selected suitable for season 2014 – 2015, in particular X-179A (H1N1), X-223A (H3N2), BX-51B (B) and for season 2016 - 2017 [BX-35B (B), X-179A (H1N1), X-263B (H3N2)], the effectiveness of the vaccine was determined in order to provide the appropriate dose of the vaccine. In the case of the IVACFLU-S trivalent influenza vaccine in particular and the seasonal influenza vaccine in general, the production process and vaccine efficacy trials are often difficult due to the continuous mutation of the flu virus strain.

In principle, the efficacy and immune response of seasonal influenza vaccines can be evaluated based on animal models. Although, the vaccine basic standard determined the antigen concentration in IVACFLU-S seasonal trivalent influenza vaccine of ≥15µg HA/dose for each strain of virus, but for mouse immune response testing, the lower doses were used. The

immunological response level of experimental animals showed that the immunostimulated dose of IVACFLU-S seasonal influenza vaccine was 1.5µg HA/dose with the first 2-dose regimen 21 days apart. And there is a difference in immune response of animals between 03 strains of virus used to produce IVACFLU-S seasonal influenza vaccine. This result is also consistent with studies on different strains of influenza virus H5N1.

The stability of vaccine

Assessment of vaccine stability at real-time/real condition, including studies of the physical, chemical, biological, pharmaceutical, and microbiological properties of vaccines, during and before the end of its shelf life and expected storage time of samples under expected handling and storage conditions. Stability indicators are direct or indirect indicators of the efficacy or safety of vaccines demonstrated in clinical trials. They are used to assess the suitability of a product over its shelf life.

Based on these recommendations, the study assesses the stability of IVACFLU-S seasonal trivalent influenza vaccine under the accelerated storage conditions and basic storage conditions. The evaluated indexes included efficacy, total protein content, endotoxin content, pH, sterility, and the sensory. In which, sterility and sensory are two qualitative indicators used in this study. Effectiveness was assessed under 3 storage conditions: $30^0C \pm 2^0C$, $40^0C \pm 2^0C$ and $5^0C \pm 3^0C$.

At the standard storage conditions of $5^0C \pm 3^0C$, the stability of the IVACFLU-S seasonal trivalent influenza vaccine was assessed more comprehensively using quality indicators directed

by WHO. At the same time, the evaluation period was also extended to 15 months from the factory time. This period was longer than the shelf life of the vaccine (12 months), thus can bring certain benefits to the users when the vaccine is commercially produced. The characteristics of vaccine concerning the total protein concentration, pH, endotoxin, sterility, and sensory were found did not change compared with that of the factory date and all met basic standards of the IVAC.

4.3 The limitations of the study

The pre-clinical and stability assessment of IVACFLU-S seasonal trivalent influenza vaccine conducted in this study strictly complied with WHO recommendations. At the same time, at each stage of the study, a full set of experiments was carried out to provide evidence for the process of building quality standards of vaccines. However, research still has certain limitations.

In this study, the assessment of vaccine stability was performed for completetion the production profile in accordance with WHO regulations and Vietnamese standards. According to the regulations on completing documents, assessing stability at this stage only required to be performed at least on 3 consecutive series of vaccines. However, the overall evaluation found that the stability assessment performed on 6 vaccine consecutive series is still a modest number and it is still difficult to apply in-depth statistical analysis for evaluating vaccine stability.

Although each vaccine batch is consisted a large number of samples, responsed to the instructions of WHO and of the national testing agency, the National Institute for Control Vaccines and Medical Biologicals, but the number samples of 6

batches of vaccine is still less than the standard number of 30 - 50 vaccine lots to be able to apply the mathematical model and Shewhart graph. We expect that once the vaccine is licensed for production and use, the number of batches of vaccine will be sufficient to conduct a stability assessment using this method.

Similar to other vaccine production processes, the production of IVACFLU-S seasonal influenza vaccine also has passed through many stages with intermediate and semi-finished products. Except for specific safety assessment performed on the bulk of monovalent seasonal influenza vaccine, intermediate and semi-finished products have not been selected for the study. This is also a limitation of research results when describing the stability of the vaccine.

CONCLUSION

The bulk batches of monovalent seasonal influenza vaccine used in the production of IVACFLU-S seasonal trivalent influenza vaccine meet the requirements of specific safety, general safety when tested on mice and guinea pigs, as well as of toxicity and immunegenicity test.

All tested batches of IVACFLU-S seasonal trivalent fluenza vaccine of 2014-2015 season and 2016-2017 season meet the requirements for fluenza vaccine effectiveness according to IVAC's basic standards. These batches achieved an effective stability test when tested under accelerated conditions at $30^0C \pm 2^0C$ for 14 days, at $40^0C \pm 2^0C$ for 3 days and at standard storage conditions of $5^0C \pm 3^0C$ for 15 months.

The batches of IVACFLU-S seasonal trivalent fluenza vaccine of 2014 - 2015 and of 2016 - 2017 season achieved the

requirement of stability test of physical and chemical properties including sterility, sensory, pH, endotoxins, total protein content at storage condition of $5^0C \pm 3^0C$ for 15 months.

RECOMMENDATION

From research obtained results, we have some recommendations:

1. Continued implementation of vaccine stability assessment with higher number of vaccine batches in order to increase the value of control data.

2. Research results at 15 months after the production and preservation of vaccine at standard conditions indicated the ensurance of quality standards in general and in effective standards in particular. Therefore, it is advisable to continue to conduct research on vaccine stability for up to 18 months after production and store under standard conditions for futher accurate assessment the stability of vaccines.

IVACFLU-S seasonal trivalent influenza vaccine has been licensed, manufacture and put into commercial use. The futher in-depth research on target vaccine user audience need to be developed. In particular, studies may prioritize the effects of vaccines on certain target groups, for example in those at high risk of influenza such as the age of 6 months - 18 years old and the group over 60 years old.