VALIDATION OF POTENCY TEST OF THE INFLUENZA VACCINE BY SRD METHOD

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Abstract: There are currently 3 types of influenza vaccines: single-strain influenza vaccines (H1N1, or H5N1, or H7N9...), trivalent vaccines (containing 3 strains at the same time: H1N1, H3N2 and B/Victoria or B/Yamagata) and quadrivalent vaccines (containing 4 strains at the same time: H1N1, H3N2, B/Victoria and B/Yamagata). The potency of inactivated influenza vaccines is determined through the HA antigen component by the single immunodiffusion method (SRD). This is a standard procedure already in the pharmacopoeia, so we only evaluated a partical validaiton which included accuracy, precision, linearity and specificity. The results showed that the procedure met the requirements for specificity, linearity and accuracy (the recovery rate of the standard was 102.01%, in the range of 80-125%); Repeatability (intra-assay) CV = 3.73%; intermediate precision given with CV % was 6.46%.

Keywords: *Validation, influenza vaccine, influenza potency, SRD method, H1N1.*

1. Introduction

Seasonal influenza viruses are among the pathogens that undergo continuous antigenic variation, with two major changes a year, around April in the Northern Hemisphere and October in the Southern Hemisphere. Consequently, Vietnam imports updated seasonal influenza vaccines twice a year. Therefore, quality control, particularly potency test of influenza vaccines is essential to ensure protective efficacy against the continuously evolving seasonal influenza strains [1,2].

Currently, there are two types of influenza vaccines: live attenuated vaccines and inactivated vaccines. The efficiency of live attenuated vaccines is tested by CCID50 or PFU method, while the potency of inactivated flu vaccines is determined by HA antigen component through Single Radial Diffusion (SRD) method [1-3]. In this article, research team focus on potency test of inactivated influenza vaccine.

SRD technique is based on the diffusion of antigens in a gel containing type-specific antibodies, and the size of the precipitation zone formed is directly proportional to the antigen concentration. This method has high specificity, short duration (2 days), and can detect antigen levels as low as approximately 3µg. Therefore, this method is widely used for all currently available inactivated influenza vaccines, with a

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standardized procedure applicable to all strains of the flu virus [1-3]. Therefore, we only performed the procedure validation on a representative strain (type) of a particular inactivated influenza vaccine.

In this research, we reported the result of procedure validation on potency test of H1N1 component in Vaxigrip trivalent vaccine by SRD at laboratory of Quality control of Viral vaccine department, National Institute for Control of Vaccines and Biologicals (NICVB). The indicators include accuracy, precision, linearity and specificity [4- 6].

2. Research method

2.1 Research objects, duration and location

Research object is potency procedure of influenza vaccine by SRD

Research duration: From 6/2014 to 12/2014.

Research location: Quality control of Viral vaccine department, National Institute for Control of Vaccines and Biologicals (NICVB).

2.2 Research design: Laboratory method description

2.3 Material and chemicals

2.3.1 Material and chemical

Standard antigen HA strain H1N1 (Influenza Antigen A/Canifornia/7/2009 (H1N1)); Lot: 09/146 (NIBSC); Quantity: 2 vials/ sample

Standard antibody H1N1 (Influenza Anti- A/Canifornia/7/2009 HA serum H1N1***);*** Lot: 10/118 (NIBSC); Quantity: 2 vials/ sample.

Standard antigen HA strain H3N2 (InfluenzaAntigenB/Massachsetts/2/2012***);*** Lot: 13/106 (NIBSC); Quantity: 1 vial/ sample

Sample: Vaxigrip vaccine, lot: L7008, quantity: 24 dose, expired date: 2/2015;

Sterile water, PBS (-), Agarose, Zwittergent, Comasie brilliant blue, destrain solution: methanol, sterile water, acetic acid (ratio 5:5:1).

2.3.2 Equipment and comsumables

Vortex (IKA), incubator 56^{o}C (Panasonic), fridge (2-8^{o}C) (Panasonic), freezer (-20^{o}C) (Panasonic), incubator 37^{o}C (Memmer), microwave (Vietnam), Pippettte (Eppendorf); syringe 3ml (Vinahankook); Eppendorf tube 2ml (Eppendorf, code: 30120094); pippette tip, gel mold, hole punch, specilized microscope.

2.4. Procedure summary

The procedure is following to SOP VR07-01 [8]

a/ Gel containing specific antibody preparation

- Prepare 100 ml agarose 1% then keep in the water bath at 60 ^{o}C for 15 minutes.

- Pour agarose to gel mold: Each mold contained 13 ml agarose uniformed mixed with 195 µL specific antibody.

- After 15 minutes, the gel is set then use the hole punch to circular wells, storage at room temperature, use in a day.

b/ Sample preparation

- Mix 450 µl vaccine sample with 50

µl Zwittergent 10%; 450 µl HA standard antigen (H1N1) with 50 µl Zwittergent 10% then incubate at room temperature for 30 minutes.

- Sample dilution:

+ Sample: Dilute Vaxigrip vaccine to 4 dilutions: 1; 3/4; 1/2; 1/4;

+ Dilute HA standard antigen (H1N1) concentrated 50 µg/ml to 5 dilutions: 40 µg/ml, 30µg/ml, 22,5 µg/ml, 15 µg/ml, 7,5 µg/ml.

+ Negative control: Diluent.

+ Positive control: HA standard antigen (H1N1) concentrated 50 µg/ml.

c. Procedure

- Add 18-20 µl each dilution of mỗi HA standard antigen (H1N1) and Vaxigrip vaccine to respective wells.

- After 15 minutes, incubate gel in the containers at 20°C- 25°C for 18 -24 hours.

d. Gel processing

- Clean the glass by sterile water then dry by tissue paper to remove all bubbles on the surface of the gel, then cover the gel by filter paper.

- Put a thick glass 600g on the gels for 30 minutes.

- Remove the glass then dry the gel in incubator 37°C.

- Note: Always cover the gel surface with filter paper.

- Stain the gel by dying solution.

- Destain the gel by destain solution then let it dry.

- Measure the diameter of precipation ring

e. Calculation of antigen concentration HA (H1N1)

- Use Excel solfware:

+ Create the standard curve y = ax + b.

+ Calculate the recovery factor of the standard curve R^2.

+ Calculate the concentration of HA (H1N1) of vaccine based on standard curve.

f. The test is valid if:

- The recovery factor of standard curve $R^2 \geq 0.98$;

- Negative control: No formed precipitation ring;

- Chứng dương: Formed precipitation ring.

* *Specification:* The concentration of HA antigen of each strain ≥ 30µg/ml) [1-3].

2.5. Accuracy

a/Test design

- Dilute HA standard antigen (H1N1) from concentration 50 µg/ml to 40µg/ml with the ratio 4:1.

- Add HA standard antigen (H1N1) with concenrtation 40µg/ml to undiluted vaccine sample with the ratio 1:1 as spike sample

- Spike sample and vaccine sample then were diluted to 4 dilutions: 1; 3/4; 1/2; 1/4.

- Determine the HA content (H1N1) of standard, vaccine samples and spike samples in 6 different days.

b/Calculation method

- Excel software is used to calculate HA content (H1N1) of vaccine samples and spike samples in each day.

- Calculate the average of HA content (H1N1), SD

- Calculate % recovery (Spike sample).

$$R\% = \frac{2Cm + c - Cm}{Cc} x100$$

In which:

R%: Recovery factor

C_{m+c} : Analyte concentration in spike sample

C_m: Analyte concentration in vaccine sample

C_c: Concentration of added standard

c/Specification

- According to EU Pharmacopeia: 80% ≤ R% ≤ 125% [4-6].

2.6. Precision

** Repeatability (intra-assay)*

a/ Test design

- Perform with vaccines samples and replicate 6 times within a day and use 1 standard curve.

b/Calculation method

Excel software is used to calculate the antigen concentration in vaccine sample, average, SD và %CV

+ Average factor: $\overline{X} = \sum C_i/n$

In which: C_i: Antigen concentration in sample

n: Number of replication

+ SD: Standard derviation (by Excel)

+ %CV = SD/$\overline{X}$ x100%

c/Specification

- % CV ≤ 10 % [4-6]

** Intermediate precision (inter-assay)*

a/ Test design

- Determine HA antigen concentration (H1N1) in vaccine sample 6 times in 6 different days.

b/ Calculation method

- Excel software is used to calculate the antigen concentration in vaccine sample, average, SD và %CV

c/ Specification

- %CV ≤ 10% [4-6]

2.7. Linearity

a/ Test design

- Antigen standard HA (H1N1) concentrated 50 µg/ml was diluted to 4 dilutions: 30µg/ml, 22,5 µg/ml, 15 µg/ml, 7,5 µg/ml.

b/ Calculation method

- Excel is used to:

+ Determine linear curve y = ax + b and linear recovery factor (R^2)

+ Calculate SD, % CV of each concentration on standard curve in 6 days.

+ Calculate concentration of HA antigen in samples of each concentration based on standard curve in 6 days

- Calculate Δi by the following formula:

$$\Delta_i = \left|\frac{C_t - C_c}{C_c}\right| \times 100$$

In which: Δ_i: Deviation of each standard point

C_t : Calculated concentration of each standrd point

C_c : Theoretical concentration of each standard point

c/Specification [4-6]

- The method has linearity when 0,99 ≤ R^2 ≤ 1

- The method is accepted when:

+ 0,99 ≤ R^2 ≤ 1.

+%CV ≤ 20%.

+ $\Delta_i \leq 15\%$ *(according to SOP KĐQG-34)*.

2.8. Specificity [4-6]

a/ Test design

- Negative control: Diluent and standard antigen H3N2 without HA antigen (H1N1).

- Positive control: Standard antigen H3N2 was spiked HA antigen (H1N1) concentrated 40 µg/ml with the ratio 1:1 and performed 6 times in 6 different days.

b/ Calculation method

- Observation the diffusion/ precipitation ring in negative and positive control

c/ Specification

- Negative control: No formed precipitation ring.

- Positive control: Form precipitation ring.

3. Result

a. Accuracy

Table 1. Recovery factor of HA antigen (H1N1) concentration in spike samples

Duration	Concentration of HA antigen (H1N1) (µg/ml)		Recovery (%)
	Sample	Spike sample	
Day 1	40,36	41,20	105,10
Day 2	37,71	39,47	103,07
Day 3	34,17	36,89	99,03
Day 4	39,67	41,25	107,07
Day 5	36,62	38,06	98,78
Day 6	35,36	37,48	99,01
Mean	**37,32**	**39,06**	**102,01**
SD	2,41	1,88	

- According to table 1: The recovery of HA antigen (H1N1) concentration in spike samples (which was prepared by adding standard antigen concentrated 40µg/ml to undiluted vaccine sample with the ratio 1:1) was 98,78 – 107,07%, that meet the specification of EU Pharmacopeia (80-125%). Therefore, the procedure of determination of antigen concentration HA (H1N1) met the requirement of accuracy.

b. Precision

- *Repeatability:*

Table 2. Repeatability of Vaxigrip vaccine

Duration	Concentration of HA antigen (H1N1) in Vaxigrip vaccine (µg/ml)
1	37,99
2	40,32
3	36,33
4	39,66
5	37,64
6	38,35
Average	**38,39**
SD	**1,43**
%CV	3,73

According to table 2: %CV=3,73 < 10%. Therefore, the procedure of determination of antigen concentration HA (H1N1) met the requirement of repeatability.

- ***Intermediate precision:***

Table 3. Intermediate precision of Vaxigrip vaccine

Duration	Concentration of HA antigen (H1N1) in Vaxigrip vaccine (µg/ml)
Day 1	40,36
Day 2	37,71
Day 3	34,17
Day 4	39,67
Day 5	36,62
Day 6	35,36
Average	**37,32**
SD	**2,41**
%CV	**6,46**

According to table 3: %CV= 6,46 < 15%. Therefore, the procedure met the criteria of intermediate precision.

c. Linearity

Table 4. Linear correlation coefficient R^2 và %CV at each standard point

HLKN (*) (µg/ml)	Diameter of diffusion ring (mm)						Mean	SD	%CV
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6			
30	6,35	6,55	6,75	6,45	6,55	6,55	6,53	0,13	2,03
22,5	6	6,2	6,1	5,95	6,05	6,15	6,07	0,09	1,53
15	5,75	5,8	5,65	5,65	5,55	5,55	5,65	0,10	1,80
7,5	5,45	5,45	5,25	5,35	5,15	5,15	5,30	0,13	2,60
R^2	0,996	0,999	0,980	0,981	0,997	0,993	0,991		

*HLKN: Antigen concentration.

Table 5. Δ_i at each standard point

C_c (µg)	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
	$C_{t(\mu g)}$	$\Delta_{i\ \%}$	$C_{t(\mu g)}$	$\Delta_{i\ \%}$	$C_{t(\mu g)}$	$\Delta_{i\ \%}$	$C_{t(\mu g)}$	$\Delta_{i\ \%}$	$C_{t(\mu g)}$	$\Delta_{i\ \%}$	$C_{t(\mu g)}$	$\Delta_{i\ \%}$
30	30,76	2,53	30,1	0,33	31,25	4,17	31,25	2	30,64	2,13	29,68	1,07
22,5	21,79	3,15	22,95	2	21,09	6,27	20,83	7,42	22,58	0,36	23,44	4,18
15	15,38	2,53	14,79	1,4	14,06	6,25	14,58	2,8	14,51	3,27	14,06	6,27
7,5	7,69	2,53	7,65	2	7,81	4,13	8,33	11,1	8,06	7,47	7,81	4,13

+ According to table 4: R^2=0,991; %CV=1,539-2,601 < 20%

+ According to table 5: Δ_i = 0,33-11 (%) ≤ 15%

Therefore, linearity met the requirement.

d. Specificity

Table 6. Specificity indicator

Performance	Positive control (H3N2 + H1N1 antigens)	Negative control (Diluent)	Negative control (H3N2 Antigen)
1	Formed precipitation ring	No precipitation ring	No precipitation ring
2	Formed precipitation ring	No precipitation ring	No precipitation ring
3	Formed precipitation ring	No precipitation ring	No precipitation ring
4	Formed precipitation ring	No precipitation ring	No precipitation ring
5	Formed precipitation ring	No precipitation ring	No precipitation ring
6	Formed precipitation ring	No precipitation ring	No precipitation ring

During 6 performance in 6 different days gave the results:

- Negative control (Diluent and standard antigen H3N2 without HA antigen (H1N1)): No precipitation rings were formed

- Positive control (Standard antigen H3N2 was spiked HA antigen (H1N1) concentrated 40 µg/ml with the ratio 1:1): Precipitation rings were formed.

Therefore, the procedure of determination of antigen concentration HA (H1N1) met the requirement of specificity.

4. Discussion

There are 4 inactivated influenza vaccines circulated in Vietnam: Vaxigrip from Sanofi Pasteur (50-70% in the market); Influvac vaccine from Abbott (15-30%); GC-Flu vaccine from Korea (7-10%) and a domestic vaccine IvacfluS (3-5%) that all produced in egg and determined potency by SRD in NICVB. Therefore, we validated the procedure in Vaxigrip, the most common vaccine to ensure the accuracy and reliability of the test result. During the validation, we chose H1N1 strain because it appeared in both monovalent and multivalent vaccine.

SRD method has been used for many years, we also have been trained at many manufacturers since 2000 and WHO, NIBSC in 2010. However, to ensure the result, procedure validation is necessary to prove the suitability, accuracy, reliability of the test.

This is a standard procedure that written in Pharmacopeia so we just performed a partical validation with 4 indicators: accuracy, precision, linearity and specificity under conditions of laboratory of Quality control of viral vaccine department, NICVB, such as equipment, devices, specific chemicals for SRD; the international standard was provided by NIBSC; the indicators for validation were designed according to the guidelines of WHO, EMA [5,6]. All the staffs performed the validation were trained and experienced for SRD therfore, the result was reliable.

To evaluate the accuracy indicator, we used standard concentrated 40µg/ml to spike into samples with the ratio 1:1 then calculate % recovery according to WHO recommendation, performed 6 times in 6 different days and the result showed that the recovery was 98,78 – 107,07%, in the range of 80-125% based on EU pharmacopeia; the average value of 6 performance was 102,01%, higher 0,8 µg than the initial value, %CV = 4,8% (<10%). Therefore, the accuracy met the requirement.

Intermediate precision (Perform 6 times in 6 days) and repeatability (Perform 6 times in 1 day) results in table 2 and 3 showed that % CV was repectively 6,46% and 3,73%, both were lower than 10% as specification. Therefore, the precision met the requirement.

SRD used the standard curve to calculate

the concentration for samples, therefore, linearity is an essential indicator. The result in table 4 and 5 determined R^2 >99%; %CV ≤ 20% and Δ_i ≤ 15% *(according to the criteria of SOP KĐQG-34).* Hence, the validation is passed for linearity.

In multivalent vaccine, there are 4 components include antigens H1N1, H3N2, B/Victoria and B/Yamagata, so that it is necessary to ensure the specificity. Consequently, we designed test samples using H3N2 standard antigen (matching the H3N2 strain in Vaxigrip vaccine), along with a negative control and a sample spiked with H1N1 standard antigen as a positive control. These samples were applied onto gel plates containing strain-specific antibodies against H1N1. We selected H3N2 strain because it belongs to the same influenza A group, which implies a higher degree of similarity. If sample containing H3N2 antigen forms a diffusion ring, it indicates that the method lacks specificity. The result showed that if there was no appearance of H1N1 antigen, there would not have the antigen-antibody reaction and precipitation ring. It proved that the procedure has high specificity.

As a reference, we also revised that validation dossier of Vaxigrip potency test for registration. The result for H1N1 of the manufacturer met the requirement of specificity, accuracy (with % recovery equals to 98,83%); precision (CV<10%), linearity (R^2 >99%) as similar as our results [8].

5. Conclusion

The procedure of potency test for inactivated inffluenza vaccine by SRD in NICVB is a standard operation procedure that met all the criteria of validation including accuracy, precision, specificity and linearity according to international specification. The result of this research confirmed the reliability of the procedure in quality control of inactivated influenza vaccine in Vietnam.

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