

# Chitosan Nanoparticle as a Novel Delivery System for A/H1n1 Influenza Vaccine: Safe Property and Immunogenicity in Mice

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**Abstract**—The aims of this paper are to study the efficacy of chitosan nanoparticles in stimulating specific antibody against A/H1N1 influenza antigen in mice. Chitosan nanoparticles (CSN) were characterized by TEM. The results showed that the average size of CSN was from 80nm to 106nm. The efficacy of A/H1N1 influenza vaccine loaded on the surface of CSN showed that loading efficiency of A/H1N1 influenza antigen on CSN was from 93.75 to 100%. Safe property of the vaccine were tested. In 10 days post vaccination, group of CSN 30 kDa and 300 kDa loaded A/H1N1 influenza antigen were the rate of immune response on mice to be 100% (9/9) higher than Al(OH)<sub>3</sub> and other adjuvant. 100% mice in the experiment of all groups had immune response in 20 days post vaccination. The results also showed that HI titer of the group using CSN 300 kDa as an adjuvant increased significantly up to 3971 HIU, over three-fold higher than the Al(OH)<sub>3</sub> adjuvant, chitosan (CS), and one hundred-fold than the A/H1N1 antigen only. Stability of the vaccine formulation was investigated.

**Keywords**—Chitosan nanoparticles, A/H1N1 influenza antigen, vaccine, immunogenicity, adjuvant, antibody titer

## I. INTRODUCTION

INFLUENZA is a major cause of mortality, morbidity, and economic loss. There are about 5-10% of the world's population affected by influenza each year. Influenza is a respiratory disease caused by influenza viruses [1]. Pandemic influenza viruses have caused substantial morbidity, mortality worldwide and threatened public health in which A/H5N1 and A/H1N1 influenza viruses are highly toxic strains and widespread public. Recently, A/H1N1 influenza pandemic (Swin Flu) occurred in Mexico on March, 2009 and expanded quickly over 74 countries and 29,696 H1N1 confirmed cases and hundreds deaths until 12<sup>th</sup>, June, 2009. The World Health Organization (WHO) had announced it raised influenza pandemic alert to phase 6 [2].

Vaccination is the best choice of influenza prevention and the main method of prophylaxis to control pandemic influenza. Using a whole inactivated virus (WIV) vaccine

based on hemmagglutinin (HA) is low immunogenic when administered without adjuvant. To enhance immune response and HI titer, influenza antigen is formulated commonly with adjuvant such as interleukin 12, cationic cholesterol, interferon  $\alpha$ , lectin C and  $\alpha$ -galactosylceramide [3]-[8]. Chitosan and its derivatives have been used as a novel adjuvant for influenza vaccines [9]-[12].

Chitosan is a biopolymer of glucosamine residues, processed from seafood wastes such as shrimp, crab shell or cell wall of fungi. Chitosan and its derivatives are natural polymers, non-toxic, biocompatible, biodegradable and adsorption properties [13]-[14]. Therefore, chitosan nanoparticles and their derivatives have been applied for biomedical field such as antibacterial agents [15]-[19], gene delivery vectors [20]-[23], carriers for release of protein and drugs [24]-[29]. As non-toxic, biodegradable, biocompatible and immune-enhancing effects, chitosan and its derivatives have been used as potential adjuvant for vaccine. Chitosan emulsion and Zn-chitosan particles using as adjuvant may be formulated with beta-human chorionic gonadotropin and vaccinated in mice and guinea pig and the new adjuvant are very effective in sensitizing mice for antigen specific DTH response and stimulated both B and T lymphocytes [30]. Chitosan is able to up regulate expression of CD 69 on B cells and CD4+T lymphocytes [31] and enhances immune properties of GM-CFS [32]. Chitosan has been used as a novel nasal delivery system for vaccines [9]. Particularly, chitosan nanoparticles are suitable for mucosal drugs and vaccine delivery. Chitosan nanoparticles enhance antigen uptake by mucosal lymphoid tissues, induce strongly immune responses against the antigens. Efficacy of chitosan microparticles and nanoparticles adjuvant seem to be dependent on molecular weight and degree of deacetylation [33], [34]. As potential characteristics in vaccine application, chitosan nanoparticle and its derivatives have been used as novel adjuvant for some vaccines such as hepatitis B [35]-[37], piglet paratyphoid vaccine [38], H3N2 influenza vaccine [11]. A influenza (A/PR/8/34) is loaded on N, N, N- trimethyl chitosan [39], H3/N2 and H6/N2 influenza vaccine are loaded on nanoparticles which make from different materials such as poly hydroxyethyl methacrylate (Phema) and amylopectin starch [40], [41].

In a previous study, we studied using chitosan oligomer (dp = 8-16) as a potential adjuvant for A/H5N1 influenza vaccine. We found that chitosan oligomer stimulated and enhanced the rate of immune response and HI titer in mice [42]. Therefore, chitosan nanoparticles have been regarded as a promising

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adjuvant for A/H1N1 influenza vaccine to protect public health from A/H1N1 influenza pandemic.

The aims of this paper are to investigate the efficacy of chitosan nanoparticles in stimulating specific antibody against A/H1N1 influenza antigen and effects of molecular weight of chitosan nanoparticles and storage time of chitosan nanoparticles loaded A/H1N1 antigen on the rate of immune response and HI titer in mice.

## II. MATERIALS AND METHODS

### A. Materials

Chitosan was purchased from Keumho Chemical Products Co. Ltd, South Korea with molecular weight 20, 30 and 300 kDa and degree of deacetylation (DD) from 80-85%.

Tripolyphosphate (TPP), acetic acid and other chemicals were purchased from Merck (Germany).

Influenza Antigen A/California/7/2009 (H1N1) v (NYMC-X179A). NIBSC code: 09/146 was supplied by National Institute for Biological Standard and Control (NIBSC), UK.

A/H1N1 influenza antigen (30 µg. ml<sup>-1</sup>) was whole inactivated virus (WIV) prepared by Nha Trang Vaccine and Medical Biological Substance Institute (Vietnam) following WHO protocol in briefly following steps [43]. Process of H1N1 influenza vaccine production has main three steps:

First step: egg incubation, virus inoculation, virus incubation and cooling (from 1<sup>st</sup> day to 16<sup>th</sup> day). Standard eggs for vaccine production were disinfected by Ethanol 70% and formalin gas, the eggs were incubated at 34.5°C, 65 to 85% humidity for 11 days. During the incubation, the eggs were disinfected three more time on 3<sup>rd</sup>, 7<sup>th</sup> and 10<sup>th</sup> day. After egg incubation, incubated eggs were candled to reject un-embryonated eggs. The embryonated eggs were inoculated by H1N1 strain at 34.5°C and 65-85% humidity for 60-72hrs, the eggs were cooled at 5°C for 20-24hrs.

Second step: Decapping, harvesting and ultra centrifugation (from 17<sup>th</sup> day to 18<sup>th</sup> day). The eggs were decapped and checked to remove infected eggs. The eggs were harvested and added PBS 7.2 and centrifuged at 12,000 rpm. The suspension was filtrated with 2 µm filtration and tested HA, OD, pH and protein and kept at 2-8°C overnight. The suspension was gradient centrifuged with 60% sucrose and tested HA, SRID, pH, protein, bioburden and endotoxin.

Third step: Inactivation, sterilized filtration (from 18<sup>th</sup> to 24<sup>th</sup> day). The suspension was inactivated by 0.05% formalin at 22-24°C, 50-70 rpm, for 15-30min. The vaccine was sterilized by 0.22µm filtration and kept at 2-8°C.

The H1/N1 influenza antigen contained 30 µg protein.ml<sup>-1</sup> with 1280 HAU.ml<sup>-1</sup> (Lot. 01/09).

Mice (*Mus musculus* Swiss) were used for immunogenicity tests in animal models with weight of 14 to 16g.mouse<sup>-1</sup> and 4 to 6 weeks age.

### B. Preparation of Chitosan-TPP Nanoparticles

#### + Chitosan purification

Further purification of purchased chitosan was to ensure that starting chitosan materials having the highest purity

before preparation of chitosan-TPP nanoparticles and immunogenicity tests in animals. Purification process was involved as follow: 1g the chitosan powder was mixed with 25ml 1M NaOH and then heated for 2hrs at 60°C. Chitosan was filtered using a Buchner funnel then washed thoroughly and dried for 12h at 40°C. After NaOH treating, chitosan was dissolved in 0.1 M acetic acid and filtered to remove insoluble particles. The chitosan solution was precipitated by 1M NaOH solution and washed thoroughly with deionized water and the chitosan was vacuum dried for 24h at room temperature (25°C).

#### + Chitosan nanoparticles preparation

Chitosan nanoparticles were prepared according to ionic gelation method [23]. In brief, chitosan solution (0.2% w/v) with different molecular weight (20, 30 and 300 kDa) was prepared by dissolving 0.2g the purified chitosan powder in 100ml 0.35% acetic acid solution and kept it overnight. The chitosan solution was adjusted to pH 5.5 by 1M NaOH solution.

Chitosan nanoparticles were prepared by dropping 0.5% TPP (Sodium tripolyphosphate) solution to the chitosan solution with the rate 6:1 (w/w) of chitosan: TPP under stirring condition with 1000 rpm (magnetic stirrer) for 30 min at room temperature. The chitosan nanoparticles were collected by spray dryer (ADL 31, Yamato, USA) and kept at 2 to 5°C.

Chitosan nanoparticles were investigated morphology by Transmission electro microscope (Jeol, JEM 1400, Japan) at an acceleration voltage of 100 kV. The 10 µL suspension chitosan nanoparticles were placed on copper grid and kept at room temperature to be dry. The samples were taken with suitable magnification.

The average particle size and Zeta potential (mV) value of chitosan nanoparticles were investigated by using zetasizer nano ZS (Malvern, UK).

### C. Loading H1N1 Influenza Antigen on Chitosan nanoparticles

0.9 mL chitosan nanoparticles (0.5 mg/ml) with different molecular weight (20 kDa, 30 kDa and 300 kDa) were mixed 0.1 ml (3µg) H1N1 antigen containing 128 HA for 30 min at room temperature. Then, the mixture was centrifuged at 12,000 rpm, 4°C for 10 min. The amount of free antigen (HA) in the supernatant was determined by hemagglutinin assay.

Loading efficiency and loading capacity of chitosan nanoparticles were calculated according to following formulas:

LE (Loading efficiency) = (Total HA – Free HA)/ Total HA x 100%

LC (loading capacity) = (Total HA – Free HA)/1 mg chitosan nanoparticles dry weight.

### D. Hemagglutinin (HA) Assay

The hemagglutinine assay is a method for titering influenza viruses based on their ability attach to molecular present on the surface of chicken red blood cells. One hemagglutinine unit (HAU) is equal to approximately 5 to 6 logs of the virus [44].

#### + Chicken red blood preparation

Rooster blood was collected from specific pathogen free chickens were preferred for use in HA assay. Preparation of chicken red blood was carried out with briefly following steps: Preparation of 15 ml sterile conical tube with a lid containing 4ml Alsever's solution for anticoagulation, collected 4ml of blood from wing vein of the chicken and topped off with PBS. The tube was centrifuged at 800 rpm for 10 min. Aspirate the supernatant without disturbing the erythrocytes.- Add 12 ml PBS and mix gently by inverting. Centrifuge at 800 rpm for 5 min and wash repeatedly the erythrocytes two more times in PBS. Make a final working solution of 0.5% chicken red blood cells in PBS.

#### + Viral dilution and Assay

Using a round bottomed 96 well dish is preferred for this assay. Add 50  $\mu$ L PBS to each well.

Add 50  $\mu$ L of virus sample in the first column. Mix each well and transfer 50  $\mu$ L to next well on its right. Repeat mixing and transferring 50  $\mu$ L down the length of the plate. Discard 50  $\mu$ L from the last well into a bleach solution. Add 50  $\mu$ L of 0.5% red blood cell to each well and mix gently, keep at room temperature for 30 to 60 min. Negative results will appear as dots in the center of round bottomed plates and positive results will form a uniform reddish color across the well. The virus's HA titer is a simple number of the highest dilution factor that produced a positive reading.

#### *E. Specific Safe Test of A/H1N1 Antigen Loaded Chitosan Nanoparticles in Mice*

The specific safe and non-specific tests were conducted seven groups designed as follow:

- + 1<sup>st</sup> group: H1N1 antigen (Ag).
- + 2<sup>nd</sup> group: H1N1 antigen and Al(OH)<sub>3</sub> adjuvant.
- + 3<sup>rd</sup> group: H1N1 antigen and chitosan solution (CS).
- + 4<sup>th</sup> group: H1N1 antigen and 20kDa chitosan nanoparticles (CSN 20)
- + 5<sup>th</sup> group: H1N1 antigen and 30kDa chitosan nanoparticles (CSN 30).
- + 6<sup>th</sup> group: H1N1 antigen and 300kDa chitosan nanoparticles (CSN 300).
- + Control group: Saline solution.

Ten mice for one group (14 to 16 grs and 4 to 6 weeks old) were injected 0.5 ml H1N1 influenza vaccin mixed with chitosan nanoparticles or adjuvants into abdomen of the mice and saline solution was used for the control. The weight of the mice was observed for 3 days and 7 days after injection. Weight gain of the mice was compared to the control injected with saline solution.

#### *F. Non-specific Safe Test of H1N1 Influenza Antigen and Chitosan Nanoparticles in Mice*

Five mice for one group (17 to 22 grs) were injected 0.5 ml H1N1 influenza vaccin mixed with chitosan nanoparticles or adjuvants into abdomen of the mice. The rate of survival or fatal of the mice of each group after 3 days and 7 days injection was calculated. The standard of non specific safe test of vaccin is 100% survival and no unusual symptom.

#### *G. In vivo Immunological Study*

TABLE I  
IMMUNIZATION PROTOCOL IN MICE

| Groups          | Vaccine Formulation                               | Immunization schedule (days) | Route of administration | Serum sampling days |
|-----------------|---------------------------------------------------|------------------------------|-------------------------|---------------------|
| Control         | Saline solution                                   | 1, 20                        | Subcutaneous injection  | 10, 20, 30          |
| 1 <sup>st</sup> | 0.375 $\mu$ g H1N1 Antigen                        | 1, 20                        | Subcutaneous injection  | 10, 20, 30          |
| 2 <sup>nd</sup> | Al(OH) <sub>3</sub> + 0.375 $\mu$ g H1N1 antigen  | 1, 20                        | Subcutaneous injection  | 10, 20, 30          |
| 3 <sup>rd</sup> | 0.2mg CS + 0.375 $\mu$ g H1N1 antigen             | 1, 20                        | Subcutaneous injection  | 10, 20, 30          |
| 4 <sup>th</sup> | 0.375 $\mu$ g H1N1 antigen + 0.2 mg of NCS 20kDa  | 1, 20                        | Subcutaneous injection  | 10, 20, 30          |
| 5 <sup>th</sup> | 0.375 $\mu$ g H1N1 antigen + 0.2 mg of CSN 30kDa  | 1, 20                        | Subcutaneous injection  | 10, 20, 30          |
| 6 <sup>th</sup> | 0.375 $\mu$ g H1N1 antigen + 0.2 mg of CSN 300kDa | 1, 20                        | Subcutaneous injection  | 10, 20, 30          |

The mice of 4 to 6 weeks age (14-16 grs) were injected into their subcutaneous legs with 0.2 ml vaccine containing 0.2 mg chitosan nanoparticles or other adjuvant loaded amount of H1N1 antigen of 0.375  $\mu$ g as Table I (ten mice for each group with triplicate). The second vaccination was carried out after 20 days with the same dose as the first. In 10, 20 and 30 days post vaccination, the blood of the mice was collected to prepare sera for HI assay.

\* 1<sup>st</sup> group to 4<sup>th</sup> group was to compare the rate of immune response and HI titer on the mice of chitosan nanoparticles loaded H1N1 antigen to common adjuvants.

\* 4<sup>th</sup> group to 6<sup>th</sup> group was to study effect of molecular weight of chitosan nanoparticles on the rate of immune response and HI titer in mice.

#### *H. Hemagglutination Inhibition Assay (HI assay)*

HI assay was determined by WHO protocol. Blood samples were collected after 10, 20 and 30 days of each immunization. The serum samples were separated from blood cell and protein coagulated protein by centrifugation at 14,000 rpm and 4<sup>o</sup>C for 5 min. The serum samples were inactivated at 56<sup>o</sup>C for 30 min. 100  $\mu$ L the serum was mixed with 300  $\mu$ L kaolin solution (25%w/v) and 0.01 M PBS (pH=7.2) incubated for 20 min at room temperature. Then, kaolin was removed from the mixture by centrifugation at 1,200 rpm, the supernatant was added 50% chicken red blood cell and incubated at 4<sup>o</sup>C for 60 min.

HI assay carried out with steps as follow: Add 25  $\mu$ L PBS/BSA buffer to each well in microtiter plate. Add another 25  $\mu$ L PBS/BSA buffer to all well of column 10, 11, 12. Discard 25  $\mu$ L dilution serum from column 9 and 12. Add 25  $\mu$ L sample dilution (4 HA units) to all wells of column 1 to 9. Mixing for 1 min and incubated for 1 hour at room temperature. Finally, 25  $\mu$ L of 1% erythrocyte solution were

added to the wells. Seal the plates and incubate for 0.5-4 hours at room temperature. The HI titer was expressed as the reciprocal value of the highest serum dilution capable of inhibiting the agglutination of the turkey erythrocytes by the influenza virus.

#### I. Stability of H1N1 Antigen Loaded Chitosan Nanoparticles Vaccine

0.375 µg A/H1N1 antigen was loaded on 0.2 ml containing 0.20 mg chitosan nanoparticles for 30 min at room temperature and then, kept it at 2-5°C for 15, 45, 90 and 135 days. After each storage time, the 10 mice for each group were injected with 0.2 ml (0.2mg) chitosan nanoparticles loaded 0.375 µg A/H1N1 antigen. In 10, 20 and 30 days post vaccination, the rate of immune response and HI titer of the mice were examined.

#### J. Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) and followed by Duncan's multiple range tests with triplicate by MSTATC software. P-value ≤0.05 and 0.01 considered as significant.

### III. RESULTS AND DISCUSSION

#### A. Physical Characterization of Chitosan Nanoparticles

Chitosan nanoparticles were prepared according to ionic gelation method of Gan [23]. Chitosan solution (0.2% w/v) with different molecular weight of 20 kDa, 30 kDa and 300 kDa was mixed with 0.5% TPP solution (CS:TPP mass ratio was 6:1).

Physical characterization of the chitosan nanoparticles was shown in Table II indicated that average size of the chitosan nanoparticles were from 80nm with chitosan 20 kDa to 106 nm with chitosan 300 kDa (Fig 2) and the size were bigger if using higher Mw of chitosan. Zeta potential of the chitosan nanoparticles distributed from +41mV to +48mV and smaller Mw and higher DD% of chitosan, zeta potential of chitosan nanoparticles were higher. These results were better than the results which found by previous works [20], [22], [44].

TABLE II

EFFECT OF MOLECULAR WEIGHT OF CHITOSAN ON SIZE AND ZETA POTENTIAL OF CHITOSAN NANOPARTICLES

| Mw chitosan (DD %)     | 20 kDa 85%   | 30 kDa 83%   | 300 kDa 80%  |
|------------------------|--------------|--------------|--------------|
| Size distribution (nm) | 33.33-142.86 | 54.42-112.24 | 73.82-138.15 |
| Average size (nm)      | 80.90        | 82.83        | 106.77       |
| Zeta potential (+ mV)  | 48.0         | 46.0         | 42.0         |

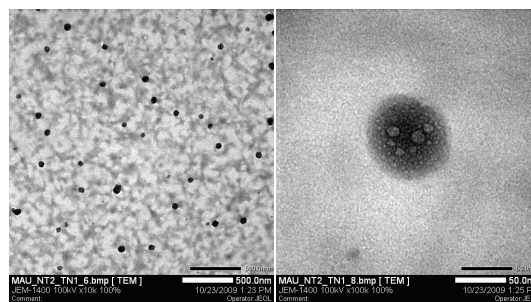


Fig. 1 TEM photograph of chitosan nanoparticles

Chitosan solution (0.2% w/v) with different molecular weight of 30kDa was mixed with 0.5% TPP solution (CS:TPP mass ratio was 6:1) at pH=5.5, room temperature and magnetic stirring at 1,000 rpm.

Gan, Wang, Cochrane & McCarron (2005) also prepared chitosan-TPP nanoparticles with high, medium and low molecular weight of chitosan. The results also showed that the size and zeta of the nanoparticles were 200 nm, +46 mV (Low molecular weight) to 250 nm, +42 mV (high molecular weight) [23]. Chitosan nanoparticles prepared from chitosan of 220 kDa and by same method were 48 nm and +51 mV [17].

#### B. Effect of Molecular Weight of Chitosan Nanoparticles on Loading Efficiency (LE) and Loading Capacity (LC) of A/H1N1 Influenza Antigen

0.9 mL chitosan nanoparticles (0.5 mg/mL) with different molecular weight (20 kDa, 30 kDa and 300 kDa) were mixed with 0.1 mL (3µg) H1N1 antigen containing 128 HA for 30 min at room temperature. Chitosan with molecular weight of 20 kDa, 30 kDa and 300 kDa was used for this experiment.

Loading efficiency and loading capacity of H1N1 influenza antigen on chitosan nanoparticles was shown in Table III indicated that chitosan nanoparticles were able to load highly A/H1N1 influenza antigen from 93.75% to 100% and loading capacity was up to 256 HA.mg<sup>-1</sup>.

Chitosan nanoparticles with low molecular weight lead to smaller size and higher zeta potential, they had higher LE (%) and higher LC. These results were higher than the Vila's work when loading Tetanus toxoid on chitosan nanoparticles achieved loading efficiency to be only 50-60% [34] and H3N2 influenza subunit antigen loaded on N-trimethyl chitosan nanoparticles with average size 800 nm achieved loading efficiency to be 78% [11].

TABLE III

EFFECT OF MOLECULAR WEIGHT OF CHITOSAN NANOPARTICLES ON LOADING EFFICIENCY AND LOADING CAPACITY OF A/H1N1 ANTIGEN

| Mw of chitosan nanoparticle | Chitosan nanoparticle (mg.ml <sup>-1</sup> ) | HA total (HAU) | HA in the supernatant (HAU) | Loading efficiency (%) | Loading capacity (HA.mg <sup>-1</sup> ) |
|-----------------------------|----------------------------------------------|----------------|-----------------------------|------------------------|-----------------------------------------|
| CSN 20                      | 0.5                                          | 128            | 0                           | 100.00                 | 256                                     |
| CSN 30                      | 0.5                                          | 128            | 4                           | 96.87                  | 248                                     |

|         |     |     |     |       |     |
|---------|-----|-----|-----|-------|-----|
| CSN 300 | 0.5 | 128 | 8   | 93.75 | 240 |
| Control | 0.0 | 128 | 128 | 0.00  | 0   |

### C. Safety of A/H1N1 Antigen and Vaccine Formulation in Mice

Safety is the one of the most importance in studying and applying of vaccine. Before testing in vivo study, A/H1N1 influenza antigen and all vaccine formulations were tested safety in mice. In the safe test, ten mice of each group were injected 0.5 ml H1N1 influenza vaccine mixed with chitosan nanoparticles or adjuvants into abdomen. Control group was injected with saline solution. According to WHO's vaccine safe requirements, weight gain of vaccinated mice are minimum 60% comparison to the control [46].

TABLE IV  
SAFETY OF A/H1N1 ANTIGEN AND VACCINE FORMULATIONS IN THE MICE

| Groups          | Weight increase (g) |           | Weight gain rate (%) | Survival (%) |
|-----------------|---------------------|-----------|----------------------|--------------|
|                 | 3 days              | 7 days    |                      |              |
| Control         | 3.56±0.29           | 8.76±0.46 | 100.0                | 100          |
| 1 <sup>st</sup> | 3.20±0.20           | 8.36±0.78 | 109.9                | 100          |
| 2 <sup>nd</sup> | 3.26±0.47           | 8.12±0.58 | 102.0                | 100          |
| 3 <sup>rd</sup> | 3.14±0.27           | 7.86±0.52 | 90.1                 | 100          |
| 4 <sup>th</sup> | 3.26±0.56           | 7.9±0.43  | 84.21                | 100          |
| 5 <sup>th</sup> | 3.23±0.24           | 8.26±0.24 | 96.00                | 100          |
| 6 <sup>th</sup> | 3.61 ±0.63          | 8.50±0.48 | 90.81                | 100          |

Result shown in Table IV indicated that the mice in all groups gained weight from 84.21% to 109.9% comparison to the control after vaccination and 100% survival and no significant difference at  $P<0.01$  between experimental groups and control group. Mice in all groups were no allergic or unusual symptom. Therefore, it concluded that all vaccine formulations were safe for mice. These results showed that the dose of A/H1N1 antigen and chitosan nanoparticles was safe for mice.

### D. Effect of Vaccine Formulation on Immune Response and Antibody HI Titer in Mice

The mice of 4 to 6 weeks age (14-16 grs) were injected into their subcutaneous legs with 0.2 mL vaccine containing 0.2 mg chitosan nanoparticles (CSN 20, CSN 30 and CSN 300) or other adjuvant loaded amount of H1N1 antigen 0.375 µg. The vaccine formulations were shown in Table I. There were two times of vaccination, the second vaccination was boosted in 20 days post vaccination with the same dose as the first. In 10, 20 and 30 days post vaccination, the blood of the mice was collected to prepare sera for HA and HI assay.

Results shown in Table V indicated that the rate of immune response in mice was from 60 to 100% after 10 days post vaccination. Particularly, 5<sup>th</sup> group (CSN 30 + A/H1N1 antigen) and 6<sup>th</sup> group (CSN 300 + A/H1N1 antigen) were able to be early immune response. The immune response of these groups in 10 days post vaccination was 100% whereas the

rate of immune response with Al(OH)<sub>3</sub> and A/H1N1 antigen groups were only 64-78%. In 20 days post vaccination, immune response rate was 100% in all groups except A/H1N1 antigen only. It is clear that chitosan nanoparticles are able to enhance immune response better than Al(OH)<sub>3</sub> adjuvant and chitosan (CS). Early and stable immune response of 5<sup>th</sup> group (CSN 30 + A/H1N1 antigen) and 6<sup>th</sup> group (CSN 300 + A/H1N1 antigen) is very necessary when threat of H1N1 influenza pandemic [42].

TABLE V  
EFFECT OF VACCINE FORMULATION ON RATE OF IMMUNE RESPONSE

| Groups          | Rate of immune response, % |                          |                          |
|-----------------|----------------------------|--------------------------|--------------------------|
|                 | 10 days post vaccination   | 20 days post vaccination | 30 days post vaccination |
| Control         | 0                          | 0                        | 0                        |
| 1 <sup>st</sup> | 60                         | 80                       | 100                      |
| 2 <sup>nd</sup> | 80                         | 100                      | 100                      |
| 3 <sup>rd</sup> | 90                         | 100                      | 100                      |
| 4 <sup>th</sup> | 80                         | 100                      | 100                      |
| 5 <sup>th</sup> | 100                        | 100                      | 100                      |
| 6 <sup>th</sup> | 100                        | 100                      | 100                      |

In 10, 20 and 30 days post vaccination, the sera of vaccinated mice were collected to determine HI titer. Results shown in Fig.2 indicated that HI titer depended clearly on Mw of chitosan nanoparticles. Antibody HI titer achieved up 1270 HIU in 6<sup>th</sup> group (CSN 300+H1N1 antigen) in 20 days post vaccination, whereas 4<sup>th</sup> group (CSN 20+H1N1 antigen) and 5<sup>th</sup> group (CSN 30+H1N1 antigen) was only 325 to 355 HIU, although this results were higher than the 2<sup>nd</sup> group (Al(OH)<sub>3</sub>) and 3<sup>rd</sup> group (CS). The difference of antibody HI titer of 6 groups was significance at  $P<0.01$ .

In 30 days post vaccination, HI titer of all groups increased significantly, 1210 HIU (Al(OH)<sub>3</sub>); 1350 HIU (CS), 1520 HIU (CSN 20 + A/H1N1 antigen), 2000 HIU (CSN 30 + A/H1N1 antigen) and up to 3971 HIU (CSN 300 + A/H1N1 antigen). HI titer of 6<sup>th</sup> group was higher two-fold than the 5<sup>th</sup> group and approximately four-fold than the 2<sup>nd</sup> group. It concluded that chitosan nanoparticles with high molecular weight (300 kDa) stimulated strongly the rate of immune response and specific HI titer against A/H1N1 influenza antigen in mice and proposed that it is should be study continuously with higher molecular weight of chitosan nanoparticles.

These results were same as some previous works. Zaharoff (2007) found that chitosan solution enhanced both humoral and cell mediated immune responses. Chitosan enhanced antigen specific antibody titers over five fold and antigen specific splenic CD<sup>+</sup> proliferation over six fold and chitosan adjuvant was better than Freund and Al(OH)<sub>3</sub> [32]. A/H3N2 influenza vaccine was also loaded on trimethyl chitosan nanoparticles, the results showed that HI and IgG titer was much higher than the antigen in PBS. Specific HI titer against the antigen was under 10<sup>2</sup> in TMC+ antigen group and under 10<sup>3</sup> in TMC nanoparticles + antigen [11].

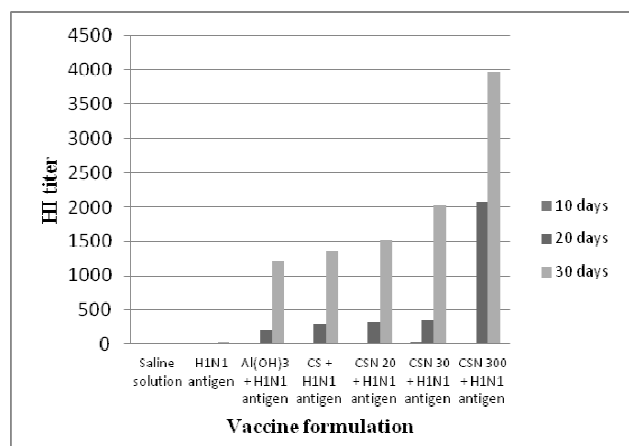


Fig. 2 Effect of vaccine formulation on HI titer

Hagennars (2009) loaded A influenza (WIV) on trimethyl chitosan nanoparticles (TMC) and found that TMC nanoparticles strongly enhanced immunogenicity and protection of the vaccine with WIV [39]. Borges (2007) also reported that hepatitis B antigen encapsulated into alginate-coated chitosan nanoparticles showed significant higher values of CD69 expression in CD4<sup>+</sup>, CD8<sup>+</sup> and T lymphocytes in comparison to the control [31].

Chitosan nanoparticles stimulate immunity system in mice and increase specific HI titer against H1N1 antigen by some ways. The first, this is to base on bioactivity of chitosan. Chitosan is more effective in inducing antigen specific DTH response and stimulates both B and T lymphocytes in experimental mice [30]. Chitosan is able to up regulate expression of CD 69 on B cells and CD4<sup>+</sup>T lymphocytes and enhances immune properties of GM-CFS [31-32]. The second, Chitosan nanoparticles loaded H1N1 antigen with nanometer size are easy to go through cell membrane and release H1N1 antigen to stimulate B cells and T cells of immune system. Chitosan nanoparticles enhance antigen uptake by mucosal lymphoid tissues, induce strongly immune responses against the antigen. In addition, H1N1 influenza antigen loaded chitosan nanoparticles can be maintainable in tissue longer than the other carriers and the duration of the response is longer. Zaharoff (2007) also found that chitosan can be maintained in tissue for 7 days after injection whereas control (PBS) was only 3hrs [32].

In comparison to other adjuvants, chitosan nanoparticles stimulate and enhance HI titer in mice more strongly than previous works. Kunzi (2009) used Inflexal<sup>®</sup>V adjuvant for A/Solomon islands/ 3/2006/H1N1 vaccine, the result showed that HI titer of experimental groups was about 1000 after 3 weeks vaccination [47]. Nishino (2009) studied using surfactant (protein: lipid) as an adjuvant for PR8/34/H1N1 and a New York 55/2004/H3N2 antigens in minipig. Specific HI titer against A/H1N1 antigen and H3N2 antigen were under 40 after 2weeks first vaccination and 4 weeks second vaccination [48].

#### E. Stability of Chitosan Nanoparticles Loaded A/H1N1 Influenza Antigen

Stability of vaccine is one of the most important in formulation and storage of vaccine. 0.1 mL containing 0.375 $\mu$ g A/H1N1 influenza antigen was loaded on 0.2 mg chitosan nanoparticles (Mw 300kDa) in 0.9mL PBS, sealed in 2mL glass vials and kept at 2-5<sup>o</sup>C. After storage time 0 (without storage), 15, 45, 90 and 135 storage days, the vaccine was used to immunize in mice with the same above protocol. The rate of immune response that the rate of immune response in 10 days post vaccination had a slight reduction, but this value also achieved 100% in 20 and 30 days post vaccination.

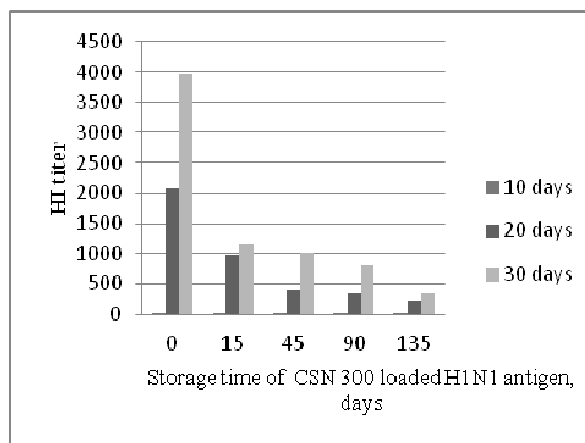


Fig. 3 Effect of storage of the vaccine on HI titer

Specific antibody HI titer against H1N1 influenza antigen reduced strongly in the first period of 15 days and reduced slightly from 15 to 135 storage days (Fig.3). After 135 storage days, HI titer was of 365 HIU and nine fold higher than the WHO's requirement (> 40 HI). It is should be study continuously storage condition to inhibit reduction in vaccine activity and check stability vaccine for longer.

#### IV. CONCLUSION

Chitosan nanoparticles prepared by gel ionic method with TPP are able to load highly A/H1N1 influenza antigen. The loading efficiency and loading capacity depend on molecular weight and DD% of chitosan nanoparticles. A/H1N1 influenza antigen and chitosan nanoparticles are safe for experimental mice. Chitosan nanoparticles can stimulate early and enhance strongly the immune response and specific HI titer in mice against H1N1 influenza antigen. Chitosan nanoparticles prepared from high molecular weigh (300kDa) show the better efficacy on enhancing specific HI titer than the lower molecular weight. It is clear that the stability of the vaccine reduces significantly in the first period of 15 days storage time and then it can be stable immune activity.

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