Pharmaceutical Sciences Asia

Pharm Sci Asia 2022; 49(5), 454-463

DOI:10.29090/psa.2022.05.22.098

Research Article

Characterization and *in vivo* assay of chitosan alginate microencapsulation to deliver the combination of HBcAg and HBsAg as a hepatitis B oral vaccine candidate

Nurlaili Ekawati[1,2], Mohamad Taufik[2], Apon Zaenal Mustopa[1*], Ari Estuningtyas[3], Imelda Rosalyn Sianipar[4], Ai Hertati[1], Maritsa Nurfatwa[1], Djadjat Tisnadjaja[6], Tri Isyani Tungga Dewi[5]

[1] Research Center for Genetic Engineering, National Research and Innovation Agency (BRIN), Bogor, Indonesia
[2] Master Programme in Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[3] Department of Pharmacology and Therapeutics Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[4] Department of Physiology Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[5] Faculty of Veterinary Medicine, IPB University, Bogor, West Java, Indonesia
[6] Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Bogor, Indonesia

ABSTRACT

Chronic hepatitis B infection is one of main factors of cirrhosis primary cause, which can develop into hepatocellular carcinoma. Medically, there are various disadvantages associated with administering hepatitis B vaccine through injection, include pain, reduced patient compliance, higher production costs, and inadequate mass vaccination. Therefore, it is necessary to develop an oral vaccine. This study aims to develop and characterize oral vaccine through combination of Hepatitis B Surface Antigen (HBsAg) and Hepatitis B Core Antigen (HBcAg) encapsulated within chitosan alginate. The vaccine formula was prepared by ionic gelation method that consists of HBcAg (marked as MPS) and a combination of HBcAg and HBsAg (which is marked as MPC) microparticles. Examined parameters include loading efficacy, particle characteristics, anti-HBcAg immune response, ALT & AST, and liver histology. It was found that loading efficacy of MPS and MPC were 82.5±9.57 and 75.0±11.78%. The mean particle size (Z-average), polydispersity index (PdI), and zeta potential of MPS and MPC were 4,869±739 nm and 8,712±2,110 nm, 0.32±0.032 and 0.37±0.088, -7.50±1.82 mV and -2.10±1.59 mV, respectively. The Hepatitis B core antibody (HBcAb) started forming on the 35th day after first vaccination. The results show that both AST and ALT serum were in normal range. Therefore, antigen dose given in this study had no pathological effects on liver histology. Furthermore, based on its parameters such as loading efficacy, PdI, zeta potential, particle size, FTIR, and formation of HBcAb from the 35th day after vaccination, it concluded that combination of HBcAg and HBsAg is safely encapsulated within microparticles (MP) chitosan alginate.

Keywords:
Combination of HBcAg and HBsAg, Chitosan alginate encapsulation, Oral vaccine, Hepatitis B

1. INTRODUCTION

Global cirrhosis cases have recently manifested a significant increase, including Indonesia. This disease can be caused by chronic hepatitis B infection, which is able to develop further into terminal hepatocellular carcinoma. According to the World Health Organization (WHO), various limitations are associated with the treatment of this virus and existing intramuscular vaccines which contain HBsAg are less effective against patients[1]. The intramuscular vaccine is generally less effective in developing countries with a large population, such as Indonesia because it is more expensive and requires services from experts[2]. Therefore, designing and developing an oral vaccine, instead of intramuscular vaccine, using a combination of hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) will hopefully improve hepatitis vaccine's effectiveness in developing

***Corresponding author:**
*Apon Zaenal Mustopa Email: azae001@brin.go.id

countries. This can also be a starting point to develop hepatitis therapeutic vaccine in the near future.

Furthermore, as both HBsAg and HBcAg antigens/ proteins are degradable in digestive system[3], optimized methods should be designed to ensure effective immune responses towards the mucosal barrier. Czerkinsky and Holmgren stated alternatives to improve vaccination delivery methods through oral route[4], by modification on formulation and vaccine delivery system, which is adopted in this study by combining both HBcAg and HBsAg encapsulated in chitosan alginate.

Polysaccharide polymers are widely used in vaccine development as antigen carriers due to their intrinsic, biocompatible, biodegradable, non-toxic, and safe immunomodulating characteristics. Chitosan is a natural polysaccharide that has potential as carrier of HBcAg and HBsAg for oral vaccination. The cationic load and the mucoadhesive properties of chitosan make it an ideal candidate as an agent to deliver the vaccine into mucosal tissue and acting as a paracellular transport[5-6]. Additionally, despite the feasibility of vaccine encapsulation design and well functioned-chitosan as vaccine carrier as stated by Islam et al.[7], chitosan also increases the immune response in mucosal vaccination[8].

On the other hand, chitosan microparticles have limited ability in controlling the release of encapsulated antigens due to the presence of an ammonium group, which makes it dissolve readily under acidic conditions. This characteristic limitation can be solved by coating with acid-resistant anionic polysaccharide polymers, such as sodium alginate, which has a carboxylic group[9]. Coating process of chitosan microparticles (MP) using alginate is easily processed using light stirring[10]. Furthermore, alginate-coated chitosan acts as an antigen carrier in delivering mucosal vaccines to prevent degradation. According to Borges study, intranasal/oral delivery of recombinant HBsAg using nanoparticles (NP) and chitosan alginate with or without cytosine-guanine oligodeoxynucleotide (CpG ODN) as an adjuvant demonstrates an efficient humoral immune response in mice[6]. Single chitosan combined with alginate induces the body's immunity and increases humoral, cellular, and mucosal immune effects[11-13]. *In vivo* studies showed that the antibody response towards administration with NP chitosan is higher than those without NP chitosan[12]. Furthermore, chitosan alginate nanoparticles also act as effective adjuvants for hepatitis B when it was administered subcutaneously in mouse models[11]. In this study, some characterization of chitosan alginate encapsulation was carried out to prove the intact formation between combination of HBcAg and HBsAg and the encapsulants during oral vaccine delivery.

2. MATERIALS AND METHODS

2.1. Experimental Materials

2.1.1. Materials

This research was carried out using chitosan with medium molecular weight (75-85% deacetylated) (Sigma), sodium tripolyphosphate (Sigma), $CaCl_2$ (Sigma), commercial recombinant HBcAg (Fitzgerald), HBsAg (Indofarma), mice feed (Indofeed), Elisa Kit (EMIGA-Thermo Scientific), BCA kit assay (Thermo Fisher Scientific), ketamine (Agrovet market), xylazine (Interchemie), anti-mouse IgG (whole molecule) (Sigma), Na_2CO_3 (Sigma), $NaHCO_3$ (Sigma), NaCl (Sigma), Tween 20 (Sigma), aquabidest (Ikapharmindo), Skim milk (Sunlac), Mouse Hepatitis B Virus Core Antibody ELISA Kit (Colorimetric) from Novus Biologicals, ASAT (GOT) & ALAT (GPT) FS* (IFCC mod.) from DiaSys, and ultrapure water (Milli-Q, Merck).

2.2.2. Animals

This study was conducted using SPF male BALB/c mice strains that were 7 to 8 weeks old, weighing 18-25 g, and purchased from PT Bogor Life Science and Technology (BLST)-IPB. The animals used were adapted for 14 days at room temperature of 25-28°C and 12 hours light/dark cycle. Furthermore, the mice were feed with INDO FEED® at amount of 15 g/100 g of body weight and provided free access to water. Ethical approval was obtained from The Animal Ethics Committee, Faculty of Veterinary Medicine, The IPB University (authorization No.149/KEH/SKE/VIII/2019).

2.2. The Preparation of Chitosan Microparticles

Chitosan microparticles were manufactured using modified Borges method[14]. This process was carried out by dissolving 0.25% (w/v) chitosan (Sigma) in 1% (v/v) acetic acid (Merck) at a constant stirring rate of 700 rpm. After the dissolution process, the substance was mixed with sodium tripolyphosphate (STPP) solution (Sigma) using a peristaltic pump and sonicated with amplitude 20%. Then followed by centrifuging the solution at 13,000 rpm for 20 minutes, pellet re-suspended in ultrapure water, and repeated centrifugation at 30,000 rpm for 10 minutes, and re-washed with ultrapure water. The solution was stored at -80°C and freeze dried. The chitosan microparticles were measured to determine the Z average using Malvern Zetasizer.

2.3. Antigen Encapsulation

Encapsulation was performed on MPS (HBcAg) and MPC (combination of HBcAg and HBsAg) in a ratio of 1:0.2[15]. Chitosan microparticles were dissolved in PBS pH 7.4 with a concentration of 4 mg/mL. Furthermore, 10 µg/mL of HBcAg or a combination of HBcAg and HBsAg consisting of 10 µg/mL and 2 µg/mL was added.

The solution was further incubated in a rotator at 4°C for two hours and centrifuged for 20 minutes at 13,000 rpm. The resulted supernatant was collected and used to determine the loading efficacy. Meanwhile the pellets were resuspended in PBS pH 7.4, sonicated with amplitude 40% for 10 seconds then mixed gently with 10 mg/mL sodium alginate solution. Furthermore, the resuspended pellets were centrifuge for 20 minutes at 13,000 rpm then the supertanat was discarded. The pellets were resuspended with 0.524 mM $CaCl_2$, then treated by sonication of 40% amplitude for 10 seconds. It was further re-centrifuged to obtain the antigen encapsulated within chitosan alginate.

2.4. Particle Characterization

Particle characterization was carried out by determining the loading efficacy, particle size (Z average), polydispersity index (PdI), and zeta potential using particle size analyzer, SEM and FTIR analyses (Fourier transform infrared spectroscopy)[2,6,13-14,16].

2.4.1. Loading Efficacy (%)

Loading efficacy of vaccine formulation of MPS (HBcAg microparticles) and MPC (HBcAg and HBsAg microparticles) was determined by quantifying free antigen remaining in the supernatant after antigen encapsulation in chitosan particles (Method 2.3). The mixture was centrifuged at 15,000 rpm for 20 minutes then protein concentration determination using the BCA kit assay (Thermo Fisher Scientific). The loading efficacy values were calculated using the following formula[6,13].

Loading Efficacy (%) =

$$\frac{\text{Total amount of HBcAg or combination-Free HBcAg or combination}}{\text{Total amount of HBcAg or Combination}} \times 100\%$$

2.4.2. Particle Size Analyzer

The microparticles size (Z average), PdI, and zeta potential were determined using PT DKSH's Malvern Zeta sizer, with each sample dissolved in PBS, poured into a cuvette, and measured on a Malvern zeta sizer, UK.

2.4.3. Scanning Electron Microscopy (SEM) Analysis

SEM analysis was performed to determine the morphological shape of the samples according to manual of the equipment. Encapsulated samples (chitosan alginate microparticles, MPS, and MPC) that have been freeze dried glued to the specimen stub using a double-sided silver tape. Specimen was coated with Au using an ion coater, then placed at the sample holder of SEM (JEOL JSM-IT200, Japan). The equipment was set in a vacuum (0 Pa) and voltage 15 kV. Samples morphology was observed in 5,000 magnification times.

2.4.4. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier transform infrared spectroscopy (FTIR) analysis was performed according to instrument manual. Chitosan alginate microparticles, MPS, and MPC were dissolved in 1 mL PBS pH 7.4. Samples in 200 µL of volume was poured into potassium bromide platform, had it covered, then analyzed with FTIR (Bruker, Germany) to determine sample's function groups.

2.5. *In vivo* Assay

BALB/c mice (*Mus musculus*) were randomly divided into 5 groups containing 5 mice in each group. Group A is normal control, group B was given HBcAg without encapsulation, group C was given HBcAg microparticles (MPS), group D was given combination of HBcAg and HBsAg without encapsulation, and group E was given combination of HBcAg and HBsAg microparticles (MPC).

2.5.1. Immunization

Immunizations were given 9 times (days 0, 1, 2, 14, 15, 16, 28, 29, and 30) by oral administration at a dose of 1 µg/0.1 mL per 20 g body weight (BW) of mice for HBcAg. Furthermore combination of HBcAg and HBsAg at amount of 1.0 µg and 0.2 µg/0.1 mL, respectively, were administered to 20 g BW of mice. The volume of each oral administration was adjusted to the mice body weights at a maximum limit of 0.2 mL. Blood collections were carried out 4 times (days 0, 21, 35 and 51), using hematocrit tubes (Marienfeld) from orbital veins after it was anesthetized, using ketamine-xylazine at a dose of 1% mice BW. Furthermore, blood collection was carried out in the morning before feeding. Sera were separated by centrifugation at 6,000 rpm for 10 minutes and stored at -80°C until upcoming bioassay.

2.5.2. Antibody Anti-HBcAg Detection

Anti-HBc screening was performed to ensure the expression of anti-HBc antibody (HBcAb) in the serum of experimental animals after vaccination. Anti-HBcAg detection was done in accordance with the kit protocol from Mouse Hepatitis B Virus Core Antibody ELISA Kit (Colorimetric) NBP2-6004 (Novus Biologicals) with a slight modification. The measurement used ELISA (Enzyme-Linked Immunosorbent Assay) technique with competitive ELISA method. Samples were collected using a serum separator tube (SST) and allowed to clot for two

hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 × g. The microtiter plate provided in this kit was pre-coated with HBcAg (antigen), while the serum samples were pipetted into the wells with HBcAb conjugated Horseradish Peroxidase(HRP). A substrate solution was added into the wells, and we can observe the color development. Then continued to washing process to remove any unbound reagent. The color development was stopped, and the intensity was measured. Calculation was made through the valence of mouse HBcAb samples compared to control. Meanwhile, $OD_{sample}/OD_{negative} \leq 0.3$ is interpreted as Positive, and $OD_{sample}/OD_{negative} > 0.3$ is interpreted as Negative.

2.5.3. Measurement of Aspartate Transaminase (AST) and Alanine Transaminase (ALT)

Both ALT and AST activities were measured by spectrophotometric techniques using the ASAT (GOT) & ALAT (GPT) FS* (IFCC mod.) kit from DiaSys with modifications. Measurements of ALT and AST were performed on serum samples of post-vaccination. The first step was done by preparing serum samples by centrifugation of serum samples at 6,000 rpm at 4°C for 5 minutes. Then followed by preparation of mono-reagent ALT and AST separately. Mono-reagent is prepared by mixing reagent 1 and reagent 2 by ratio of 4:1. The mono-reagent was put into a microplate followed by mixing the sample (serum or calibration solution) in a ratio 10:1. The mixed solution (mono-reagent+sample) in the microplate then transferred to the Thermo Multiskan EX Microplate Photometer. Samples were measured at 340 nm, 334 nm and 365 nm waves. Measurements were carried out 3 times with an interval of 1 minute for each OD reading. Measurements of enzyme activities (ALT and AST) were completed by plotting sample's the absorbance values to a calibrator.

The value of ALT/AST activity was obtained from ΔA/min×factor = enzyme activity [U/L] or using the following formula:

$$\text{Enzyme activity U/L} = \frac{\Delta A/\min \text{ sample}}{\Delta A/\min \text{ calibtaror}} \times \text{Conc.Calibrator [U/L]}$$

2.5.4. Preparation of Liver Histology

The liver specimens were fixed in 4% formalin solution for ≥24 hours and stored in paraffin. Furthermore, 4 µm of tissue slices were routinely stained with haematoxylin & eosin (HE), while the overviews of liver tissue were analyzed descriptively.

2.6. Statistical Analysis

Data were presented as mean±SD. Meanwhile, the significance tests were performed using the t-test to compare the Z average, PdI, zeta potential, and loading efficacy of MPS and MPC. One-way ANOVA test was used to determine the AST and ALT data followed by Post Hoc LSD. The difference is considered significant, assuming the value of $p<0.05$[17]. Anti-HBc antibody screening data and histological features were analyzed statistically and descriptively using the SPSS 23 software.

3. RESULTS AND DISCUSSION

Selection of chitosan as a carrier agent is performed to deliver protein to the target, while alginate is added to control the release of antigen in the gut. Before using this substance for *in vivo* tests, the microparticles need to be characterized to measure loading efficacy, particle size, distribution, zeta potential, morphological shape, and FTIR.

3.1. Particle Characterization

3.1.1. Loading Efficacy

The loading efficacy is measured using a BCA kit in 4 samples with 3 repetitions. Results show that the vaccine formula with a single MPS antigen (HBcAg microparticles) have loading efficacy of 82.50±9.57% and the HBcAg and HBsAg combination microparticles (MPC) is 75.00±11.78%. Furthermore, the t-test statistical test results show no significant difference between the means of two formulas with a value of $p=0.36$. This value is greater than the results obtained by Soares in 2018 which stated that encapsulated HBsAg in chitosan coated with alginate, namely 77.1±3.0% is lower than the loading efficacy of HBsAg[18]. Another comparable experiment done by AbdelAllah and his colleagues in 2016 about formulation HBsAg in Aluminum suspension coated with chitosan and sodium alginate were having loading efficacy at 89.73±3.67%. On the other hand, another study reveals loading efficacy of HBsAg encapsulated into NP chitosan alginates microparticles is 85.90±4.70%[13].

3.1.2. Particle Size Analyzer

In this study, the average of particle size (Z average), polydispersity index (PdI), and zeta potential of particles values were obtained by Zeta sizer (Malvern Instruments, UK), as shown in Table 1. Based on the statistical analysis results of their variability in MPS and MPC, it is known that p value for each parameter is 0.271, 0.226, and 0.823 for Z average, PdI and zeta potential, respectively, which means that there is no significant difference.

In preliminary studies carried out by Borges and Sarmento, the particle sizes of ovalbumin and insulin loaded in chitosan alginate were 700-4,000 nm[14,19]. The average particle size has a coefficient of variance (COV)

Table 1. Measurements results of Z average, PdI and zeta potential of samples (n=3).

Groups	Z Average (nm)	PdI	Zeta potential (mV)
HBcAg	1,046.00 ± 93.00	0.6640 ± 0.0060	-0.025 ± 0.02
HBcAg and HBsAg	3,455.00 ± 980.23	0.4630 ± 0.1658	-8.840 ± 2.70
Chitosan Alginate	1,731.00 ± 5.56	0.9150 ± 0.0760	+3.040 ± 0.47
MPS	4,869.00 ± 739.40	0.3246 ± 0.0320	-7.500 ± 1.82
MPC	8,712.00 ± 2110.00	0.3770 ± 0.0882	-2.100 ± 1.59

Note: MPS=HBcAg microparticles and MPC=combination of HBcAg and HBsAg microparticles

value <30%, which is homogeneous. However, for the combination of HBcAg and HBsAg without encapsulation and MPC, a high COV value range of 20<COV<30 is obtained in the Gomes classification[20]. In addition, previous studies carried out by encapsulating lysozyme and BSA was in the range of 700-800 nm.

Original size of chitosan particles was known to have the Z average at 123.83±2.70 nm with a PdI of 0.44± 0.01. However, encapsulation causes an increase of its size from nano to micro range, where the largest size was belonged to MPC (combination of HBcAg and HBsAg) counted at 8,712±2,110 nm. The result shows that encapsulation with chitosan alginate leads to the increase in particle size because chitosan alginate without antigens has a micro-size of 1,731 nm. Besides, the molecular weight of HBcAg is 21 kDa, while HBsAg is 24 kDa, the combination between the two leading to doubling size of HBcAg microparticles. Polydispersity index (PdI) of MPS and MPC showed a value <0.5, which indicates that the value of distribution size is within recommended range in the tool manual and in line with the PdI values[14,16].

A zeta potential above +30 mV or below -30 mV is considered as stable and it shows the information on the surface charge of the particles[21]. The zeta potential value of microparticles chitosan alginate was +3.04, which indicates less stable condition and in line to previous report[21]. The results also illustrate that HBcAg without encapsulation has a zeta potential of -0.025, supported by a PdI value >0.5. Therefore, the particles are less homogeneous and easy to form sedimentation[16,22]. Combination of non-encapsulated HBcAg and HBsAg, MPS and MPC produce a zeta potential -8.84, -7.50, and -2.10 mV, respectively. The results show that a single HBcAg antigen or combination of HBcAg and HBsAg has negative surface charge. Both formulas have a negative charge on the surface and the isoelectric point of the two antigens when dissolved in PBS at a pH of 7.4. Although the zeta potential value is not above +30 mV or below -30 mV[21], the PdI is <0.5, which indicate a narrow particle size distribution within the recommended range[16].

3.1.3. Scanning Electron Microscopy

Chitosan alginate microparticles have spherical shape with smooth surface[14]. The results of morphological analysis was carried out using SEM with 5,000 magnification times showed that the shape of chitosan alginate (A) is spherical and similar to previous research[14]. Meanwhile, for HBcAg microparticles/MPS (B), the shape is irregular with a bumpy surface and so does the combination of HBcAg and HBsAg microparticles/ MPC's morphology (C), as shown in Figure 1.

3.1.4. FTIR Analysis

FTIR analysis was carried out to provide information on chemical bonds, molecular structure, and solubility of polymers in a multicomponent system[23]. FTIR spectrum of alginate was at 3,415 cm^{-1} (OH), 2,931 cm^{-1} (CH), 1,618-1,605 cm^{-1} (COO- asymmetric stretching), 1,417-1,412 cm^{-1} (COO- symmetrical stretching), and 900-1,400 cm^{-1} (CO or OCO)[14,24]. Meanwhile, chitosan has strong peaks at 3,400-3,200 cm^{-1} (OH, NH, and intermolecular hydrogen bonds), 1,655-1,633 cm^{-1} (Amide), and 1,089-1,065 cm^{-1} (CO)[25]. Chitosan alginate has strong peaks at 3,565-3,400, 1,651-1,643, and 1,072 cm^{-1}, respectively[23,26]. The FTIR spectrum in the range of 2,170-2,000 cm^{-1} shows linear CO bonds[27]. The shifting peak from 1,610 to 1,643 cm^{-1} was due to the overlap between chitosan amide group and carboxyl alginate[23].

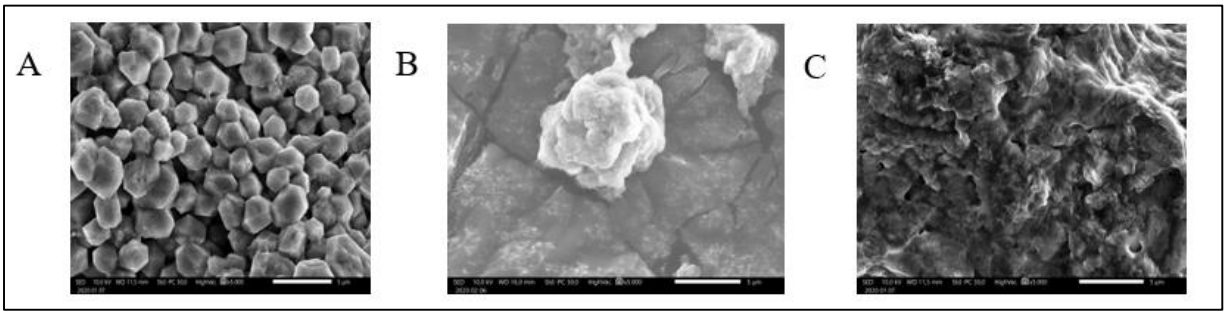

Figure 1. The results of morphological analysis of samples under SEM magnified 5000 times (A) Morphological form of chitosan alginate (B) Morphological form of HBcAg/MPS microparticles (C) Morphological forms of combination between HBcAg and HBsAg/MPC microparticles.

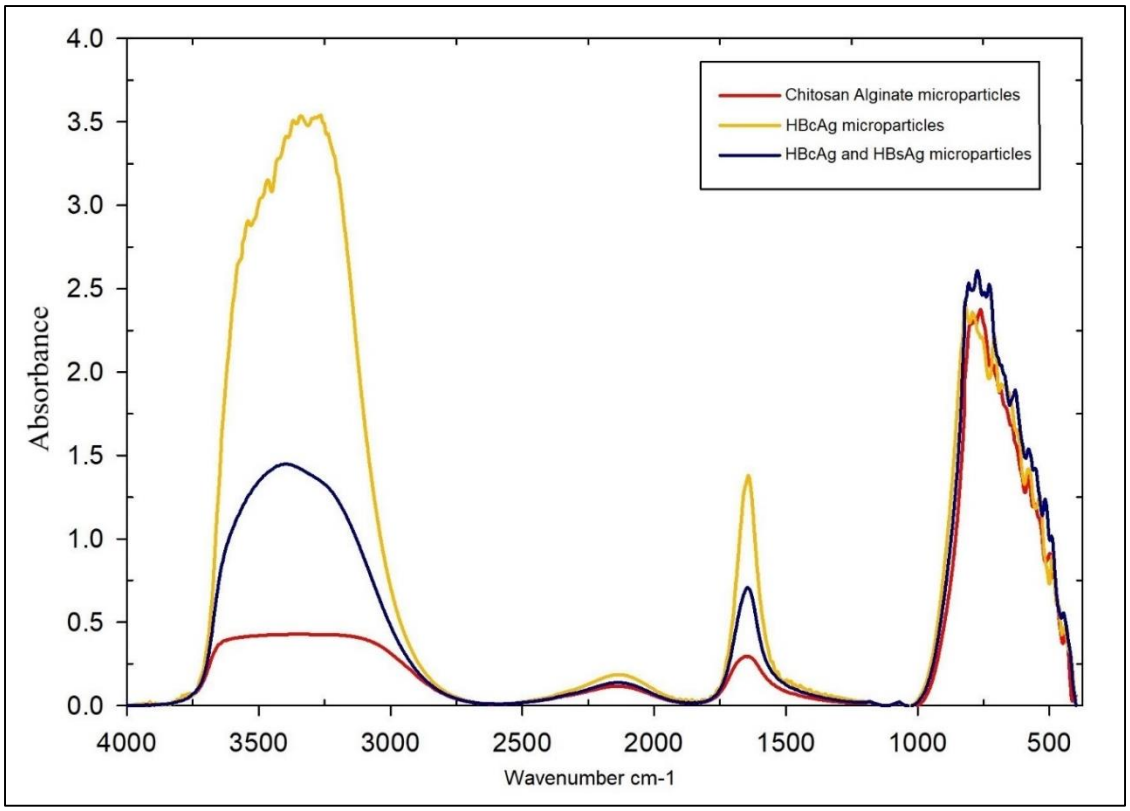

Figure 2. The FTIR Analysis Results.

Table 2. The FTIR Analysis Result of Chitosan alginate, MPS and MPC.

Chitosan alginate vibration (cm^{-1})	MPS vibration (cm^{-1})	MPC vibration (cm^{-1})	Prediction of Assignment
3368	3340	3397	O-H, N-H stretch
2138	2135	2137	C=O linear
1652	1643	1646	C=O Amide
1070	1071	1070	C-O

Note: MPS=HBcAg microparticles and MPC=combination of HBcAg and HBsAg microparticles

Furthermore, results of FTIR analysis are shown in Figure 2. The chitosan alginate (red) spectrum depicts several bands at 3,368, 2,138, 1,652, and 1,070 cm^{-1}. The MPS (yellow) and MPC (blue) spectrum show similar absorption patterns with a slight uptake shift, at 3,340, 2,135, 1,643, and 1,071 cm^{-1} for MPS and 3,397, 2,137, 1,646, and 1,070 cm^{-1} for MPC. Functional group assumptions from the results of the FTIR analysis shown in Table 2. The band at 3,390-3,340 cm^{-1} indicates a vibrational strain of hydroxyl group with an increase of band's shape, which overlaps the hydroxyl group of amines in the presence of an intermolecular hydrogen bond in line with previous study[23,26]. Another spectrum at 2,138-2,135 cm^{-1} acts as a vibrational strain of linear CO[27]. The presence of absorption bands at 1,652-1,643 cm^{-1} originates from C=O vibration of amide group, whereas the C-O bonds in chemical structures are evidenced by the presence of peaks in fingerprint region at 1,070 cm^{-1} [23,26].

3.2. *In vivo* Assay

3.2.1. Antibody Anti-HBcAg Screening

Antibody screening was done using semi-quantitative ELISA kit to ensure the antibodies were properly formed. This assay is well known for its high sensitivity and excellent specificity for detecting mouse antibody with no significant cross-reaction or interference between HBcAb and its analogues. The bioassay used serum samples without any dilution. Therefore, the results of anti-HBc measurements out of subject experiments after administration of various antigens groups are, shown on Table 3. All subjects in the group B (HBcAg) showed HBcAb (positive) in their blood starting on the 35th day after first vaccination. Further observation of the antibody on days 51st confirmed a slight decrease, in 80% of subject group. Meanwhile in group C (MPS/MP HBcAg) shows a different response. 80% of all tested animals were able to produce anti-HBc antibodies detected on the 51st day after vaccination. This result is quite similar to group E (MPC/MP HBcAg and HBsAg). The antibody was detected on days 51st after vaccination, but only in 60% of group population. Group D (HBcAg and HBsAg without encapsulation) was found to show a good adaptive response by producing HBcAb detected on the

Table 3. The results of HBc antibodies detection in serum by ELISA.

Groups	Anti-HBc days to		
	21	**35**	**51**
A	(-)	(-)	(-)
B	(-)	(+) 100%	(+) 80%
C	(-)	(-)	(+) 80%
D	(-)	(+) 100%	(-)
E	(-)	(-)	(+) 60%

Note: A=normal, B=HBcAg, C=MPS/MP HBcAg, D=HBcAg and HBsAg, E=MPC/MP HBcAg and HBsAg. (Percentage positive = ∑ positive/n)

35th day after vaccination, but then extremely decreased on days 51st. However, all of these results might differ when more animals were added to the experiment or increase the antigen doses.

Antibody expression in the form of anti-HBc confirms the formation of antibodies (HBcAb) against the presence of HBcAg in the serum of tested animals as response to hepatitis B virus for successful encapsulation. The administration of single and combined antigens, with and without encapsulation showed that these antigens were able to provide an adaptive immune response marked by positive HBcAb (+) and distinguishable from the normal group, which was proven to be HBcAb negative (-) on the 21st, 35th and 51st days after antigen administration. A positive anti-HBcAg value of single or combined administration with HBsAg on day 51 indicates that the encapsulation could elicit immune response over a prolonged period. Therefore, research and development are still needed to determine the doses as well as the time and amount of antigen administered using optimized encapsulation techniques.

The immune response-ability of tested animals was carried out for 51 days to determine stability and type of immune response, especially the adaptive type. In this study, animal groups were injected with a single form of HBcAg (B) and combination form of HBcAg and HBsAg (D). Research outcomes showed that all subjects (100%) in both groups presented antibodies (HBcAb) on the 35th day, but then decreased on the 51st day, as shown in Table 3. Anti-HBc or HBcAb is antibody in the form of immunoglobulin IgM or IgG and specific to the presence of HBcAg. Our study in agreement with previous report which demonstrated that BALB/c mice immunized with single HBcAg or in combination with IFNα-2b showed humoral immune response on the 35th day[28]. The oral administration of the vaccine candidate in this study was similar to HBcAg-based vaccine study administered via nasal route. In phase I of the clinical trial process conducted by NASVAC HBcAg+HBsAg-based vaccine via the nasal route, healthy adult males showed a significant immune response in the form of anti-HBc formation 30 days after vaccination[29-30].

Different results were obtained for antigen encapsulated with chitosan alginate. Group C that were administered with encapsulated HBcAg (MPS) and combination of HBcAg and HBsAg with encapsulation (MPC/ group E) showed 80% and 60% of population developed anti-HBc on days 51st, respectively. This result relates to the size of the microparticles which affected the absorption power and the time needed to deliver antigens to site of action (intestines) and target cells (hepatocytes). Hence, this causes target cells need longer while responding to a series of specific antibody formations. According to Eldridge et al. and Singh et al., particle size affects absorption by Peyer's intestinal patches[31-32]. Therefore, the delivery power and the resulting immune response become smaller[32].

This study confirms that the HBcAg (exogenous) antigen has the potential to elicit immune response, whether it is administered alone or in combination with HBsAg. In addition, the immune response formed using specific antibodies (HBcAb) in the group of animals given the encapsulated antigen proves the ability of chitosan and alginate to be administered as oral vaccine.

3.2.2. AST and ALT Enzyme Activity in Blood

A substance or antigen must meet several requirements to be used as a vaccine candidate. One of such requirements is its ability to remain stable and safely tolerated by the body. A toxicity test on the liver (hepatotoxicity) is one method commonly used to determine antigen's safety. Hepatotoxicity is a state of dysfunction or the impact of liver damage by antigens or toxic exogenous compounds that can be seen through liver damage biomarkers analysis (ALT and AST).

The activity of ALT and AST in the serum of the tested animals showed some fluctuations. Most of the enzyme activity increase in immunized group is proportional to the duration of experimental animals were kept, which is shown in Figure 3. This means that the greater and stronger the antigen is given to the subject, the higher the immune response generated. Interaction between antigens and immune cells causes inflammation, thus, inflammation will increase the activity of inflammatory enzymes marker in the liver.

The measurement of control group activity (A) after 51 days of treatment illustrates that the average ALT and AST values are 89.21 U/L and 157.05 U/L, respectively. The data presents an increase in ALT and AST values of B, C, D, and E treatment groups, which are recorded at 115.61 U/L: 229.36 U/L, 95.10 U/L: 260.01

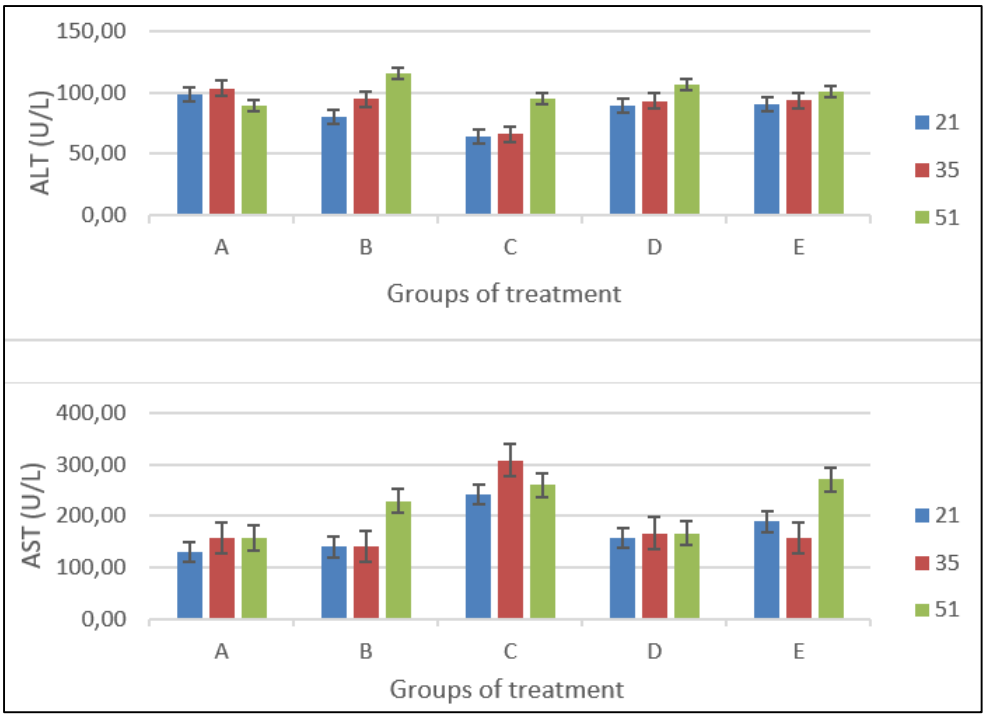

Figure 3. The ALT and AST activities of animal subject during treatment on day 21, 35 and 51 after first vaccination. A=normal, B=HBcAg, C=MPS/MP HBcAg, D=HBcAg and HBsAg, E=MPC/MP HBcAg and HBsAg.

U/L, 106.23 U/L: 166.65 U/L, and 101.21 U/L: 270.91 U/L. Based on statistical analysis results, comparison between control (A) and treatment groups (B, C, D, and E) showed that there was no significant difference in the mean of ALT (p>0.05) between control (non-vaccinated) and all immunized groups. Meanwhile, the AST value showed a significant difference (p<0.05) between the control group (A) and the treatment groups (HBcAg, MP HBcAg, and MP HBcAg and HBsAg). Furthermore, change in AST level does not fully referring the condition of liver but an increase of AST enzyme activity in blood damages the liver cells, and the cardiovascular system. Hepatotoxicity biomarkers are usually a manifestation of the increase of hepatic enzymes in serum, which consists of AST and ALT of which secreted in parallel[33-34].

The unchanged value or deviation of ALT and AST (as shown in Figure 3) in each group that was administered with antigens is because they were within the normal range limit. According to experiment conducted in 2009 by Murray and his colleagues, ALT and AST levels in hepatic pathological conditions increases up to 10-100 times of the normal value because the liver transaminase enzymes are released into circulatory system. Normal ALT range in mice blood is 17-77 U/L, however, according to preliminary studies, within 25-200 U/L is also categorized as normal range. On the other hand, the range of normal AST levels in mice's blood is 54-298 U/L, while in other literature, a range of 70-400 U/L is categorized as normal value[36-37]. Sharon (2015) stated that the level of liver infection is grouped based on the increase in hepatic enzyme activity. In small animal groups, an increase of enzyme activity above normal reference range is considered mild if it is less than 3 times of upper reference range, considered as moderate when it is 3-9 times, and severe when it increases above 10 times[38].

3.2.3. Liver Histology after Vaccination

In addition to biological markers, assessment of toxicity effect from an exogenous compound on liver function is determined by analyzing liver tissue under microscope (histopathology overview). This helps the diagnosis of a pathological condition obtained from the response of antigen-target cell interactions. In this study, all liver tissue specimens were prepared from cross-sectional slices of mice liver on the 51st day after vaccination. One specimen represented the treatment group, as shown in Figure 4.

The liver tissue in control subject (A) shows hepatocyte cells and a few immune cells in physiological conditions. Meanwhile, normal infiltration indicated a small number of inflammatory neutrophil cells, with lymphocytes and plasma cells illustrated in the multifocal parenchyma area. In the treatment groups (B, C, D, and E), the overview of liver tissue shares similar morphology between groups given a single and combined antigen. The four test groups showed the presence small numbers of macrophages in the multifocal parenchyma area. The description of histological picture confirms that liver function conditions is in accordance with previous normality seen on ALT and AST values.

The presence of macrophages in hepatocytes is used to induce a cellular immune response, which starts with naive T lymphocytes activated through the CD4^{+}Th-1

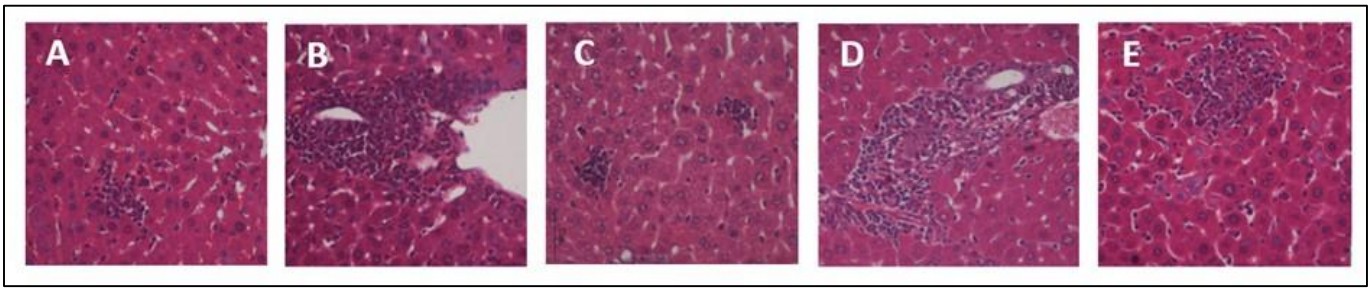

Figure 4. Histology of liver specimens of mice after the 51st day of vaccination with magnification 40x. A=normal, B=HBcAg, C=MPS/MP HBcAg, D=HBcAg and HBsAg, E=MPC/MP HBcAg and HBsAg.

cell activation pathway. This process leads to cytotoxic T cells and macrophage activation, which tend to eliminate intracellular pathogens or cells affected by the given antigen. Furthermore, the presence of antigen-induced from the vaccine candidate can be controlled, both in terms of dose and amount of administration, to generate cellular and humoral immune responses without causing significant hepatocyte damage.

The result of this study is similar to administration of a non-adjuvant recombinant vaccine consisting of HBcAg and HBsAg. It could form cellular and humoral immunity, as well as a strong antibody (Ab) against HBcAg, such as HBcAb. The intranasal and subcutaneous routes of vaccine administrations provide stability and safety to subjects by administering multiple doses[39].

4. CONCLUSION

Based on the parameters of loading efficacy, PdI, zeta potential, particle size, FTIR, and antibody presented (anti-HBc), it was concluded that the combination of HBcAg and HBsAg can be encapsulated in microparticle chitosan alginate. Furthermore, the formation of HBcAb appeared on the 35th day after the first vaccination. The ALT and AST enzyme activity is still within normal range. In addition to histological mice's liver observation, we found that administration of HBsAg and HBcAg antigens encapsulated by chitosan alginate to mice via oral route aided the adaptive immune response without causing liver damage.

5. ACKNOWLEDGEMENT

We would like to thank dr. R. Fera Ibrahim, M.Sc., PhD, SpMK (K) and Dr. Rudi Heriyanto, M.Si for the discussions. We also thank to PT DKSH Indonesia for their permission to use the zeta sizer facility, and thanks to the Saintek-Kemenristekdikti Scholarship 2017.

Conflict of interest
None to declare.

Funding
This study was fully funded and supported by Research Center for Biotechnology, Indonesian Institute of Science by Prioritas Nasional (PN) Obat 2019 and Prioritas Nasional (PN) LIPI 2021 scheme.

Ethics approval
Ethical approval was obtained from The Animal Ethics Committee, Faculty of Veterinary Medicine, The IPB University (authorization No.149/KEH/SKE/VIII/2019).

Article info:
Received March 15, 2022
Received in revised form June 14, 2022
Accepted July 26, 2022

Author contribution
NE: Performed experiments, analyzed the data, drafted the manuscript; **MT**: performed experiments, analyzed the data; **AE & IRS**: Supervision; **TID**: Performed experiments; **AZM**: Conseptualization, supervision, acquired funding; **MN**: Performed experiments; **DT**: Analyzed the data, provided technical support in manuscript preparation; **AH** Analyzed the data, drafted the manuscript, supervision; **All authors**: edited and reviewed the manuscript.

REFERENCES

1. WHO. Global Hepatitis Report 2017 [document on the Internet]. Geneva: World Health Organization; 2018. Available from: https://www.who.int/news-room/fact-sheets/ detail/hepatitis-b.
2. Lebre F, Bento D, Jesus S, Borges O. Chitosan-Based Nanoparticles as a Hepatitis B Antigen Delivery System. Methods Enzymol. 2012;509:127-42.
3. Biswas S, Chattopadhyay M, Sen KK, Saha MK. Development and characterization of alginate coated low molecular weight chitosan nanoparticles as new carriers for oral vaccine delivery in mice. Carbohydr Polym. 2015;121:403-10.
4. Czerkinsky C, Holmgren J. Enteric vaccines for the developing world: A challenge for mucosal immunology. Mucosal Immunol. 2009;2(4):284-7.
5. Mody N, Sharma R, Dubey S, Vyas SP. Microparticles for Vaccine Delivery. In: Skwarczynski M, Toth I, editors. Micro and Nanotechnology in Vaccine Development. William Andrew Publishing; 2017. p. 259-78.
6. Borges O, Tavares J, Sousa A De, Borchard G, Junginger HE, Cordeiro da silva A. Evaluation of the immune response following a short oral vaccination schedule with hepatitis B antigen encapsulated into alginate-coated chitosan nanoparticles. Eur J Pharm Sci. 2007;32:278-90.
7. Islam MA, Firdous J, Choi YJ, Yun CH, Cho CS. Design and application of chitosan microspheres as oral and nasal vaccine

carriers: An updated review. Int J Nanomedicine. 2012;7:6077-93.
8. Ahire VJ, Sawant KK, Doshi JB, Ravetkar SD. Chitosan microparticles as oral delivery system for tetanus toxoid. Drug Dev Ind Pharm. 2007;33(10):1112-24.
9. Kim B, Bowersock T, Griebel P, Kidane A, Babiuk LA, Sanchez M, et al. Mucosal immune responses following oral immunization with rotavirus antigens encapsulated in alginate microspheres. J Control Release. 2002;85:191-202.
10. Borges O, Cordeiro da silva A, Romeijn SG, Amidi M, Sousa A De, Borchard G, et al. Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination. J Control Release. 2006;114 (3):348-58.
11. Borges O, Silva M, de Sousa A, Borchard G, Junginger HE, Cordeiro da silva A. Alginate coated chitosan nanoparticles are an effective subcutaneous adjuvant for hepatitis B surface antigen. Int Immunopharmacol. 2008;8:1773-80.
12. Heffernan MJ, Zaharoff DA, Fallon JK, Schlom J, Greiner W. *In vivo* efficacy of a chitosan/IL-12 adjuvant system for protein-based vaccines. Biomaterials. 2011;32(3):926-32.
13. AbdelAllah NH, Abdeltawab NF, Boseila AA, Amin MA. Chitosan and Sodium Alginate Combinations Are Alternative, Efficient, and Safe Natural Adjuvant Systems for Hepatitis B Vaccine in Mouse Model. Evid Based Complement Altern Med. 2016;2016:1-8.
14. Borges O, Borchard G, Verhoef JC, Sousa A De, Junginger HE. Preparation of coated nanoparticles for a new mucosal vaccine delivery system. Int J Pharm. 2005;299:155-66.
15. Li J, Ge J, Ren S, Zhou T, Sun Y, Sun H, et al. Hepatitis B surface antigen (HBsAg) and core antigen (HBcAg) combine CpG oligodeoxynucletides as a novel therapeutic vaccine for chronic hepatitis B infection. Vaccine. 2015;33(35):4247-54.
16. Farhadian A, Dounighi NM, Avadi M. Enteric Trimethyl Chitosan nanoparticles containing Hepatitis B surface antigen for oral delivery. Hum Vaccin Immunother. 2015;11(12):2811-8.
17. Witte RS, Witte JS. Statistics, 11th ed. In: Hoffman G, Visentin V, editors. Hoboken, New Jersey: John Wiley & Sons, Inc; 2017.
18. Soares E, Jesus S, Borges O. Chitosan : β -glucan particles as a new adjuvant for the hepatitis B antigen. Eur J Pharm Biopharm. 2018;131:33-43.
19. Sarmento B, Ferreira D, Veiga F, Ribeiro A. Characterization of insulin-loaded alginate nanoparticles produced by ionotropic pre-gelation through DSC and FTIR studies. Carbohydr Polym. 2006;66:1-7.
20. Couto MF, Peternelli LA. Classification of the coefficients of variation for sugarcane crops. Ciência Rural St Maria. 2013;43 (6):957-61.
21. Silva M dos S, Cocenza DS, Grillo R, Melo NFS de, Tonello PS, Oliveira LC de, et al. Paraquat-loaded alginate/chitosan nanoparticles: Preparation, characterization and soil sorption studies. J Hazard Mater. 2011;190:366-74.
22. Serruys B, Van Houtte F, Farhoudi-moghadam A, Leroux-roels G, Vanlandschoot P. Production, characterization and *in vitro* testing of HBcAg-specific VHH intrabodies. J Gen Virol. 2010;91:643-52.
23. Azhar FF, Olad A. A study on sustained release formulations for oral delivery of 5-fluorouracil based on alginate-chitosan/montmorillonite nanocomposite systems. Appl Clay Sci. 2014;101: 288-96.
24. Vijaya Y, Popuri SR, Boddu VM, Krishnaiah A. Modified chitosan and calcium alginate biopolymer sorbents for removal of nickel (II) through adsorption. Carbohydr Polym. 2008;72(2): 261-71.
25. El-Sherbiny IM, Smyth HDC. Poly(ethylene glycol)-carboxymethyl chitosan-based pH-responsive hydrogels: Photo-induced synthesis, characterization, swelling, and *in vitro* evaluation as potential drug carriers. Carbohydr Res. 2010;345(14):2004-12.
26. Katuwavila NP, Perera ADLC, Samarakoon SR, Soysa P, Karunaratne V, Amaratunga GAJ, et al. Chitosan-Alginate Nanoparticle System Efficiently Delivers Doxorubicin to MCF-7 Cells. J Nanomater. 2016;2016:1-12.
27. Manzoli M. Boosting the characterization of heterogeneous catalysts for H2O2 direct synthesis by infrared spectroscopy. Catalysts. 2019;9(1):30.
28. Meilina L. Imunogenesitas Kandidat Vaksin Oral Lactococcus lactis Rekombinan HBcAg dan IFNα-2B untuk Pencegahan Hepatitis B [dissertation]. Bogor Regency: Institut Pertanian Bogor; 2019.
29. Betancourt AA, Delgado CAG, Estévez ZC, Martínez JC, Ríos GV, Aureoles-Roselló SRM, et al. Phase I clinical trial in healthy adults of a nasal vaccine candidate containing recombinant hepatitis B surface and core antigens. Int J Infect Dis. 2007;11(5): 394-401.
30. Al-Mahtab M, Akbar F, Uddin H, Rahman S, Rubido JCA, Nieto GEG, et al. 760 A Phase III Clinical Trial with a Nasal Vaccine Containing Both HBsAg and HBcAg in Patients with Chronic Hepatitis B. J Hepatol. 2013;58:S309.
31. Eldridge JH, Staas JK, Meulbroek JA, McGhee JR, Tice TR, Gilley RM. Biodegradable microspheres as a vaccine delivery system. Mol Immunol. 1991;28(3):287-94.
32. Singh B, Maharjan S, Cho K, Cui L, Park I, Choi Y, et al. Chitosan-based particulate systems for the delivery of mucosal vaccines against infectious diseases. Int J Biol Macromol. 2018;110: 54-64.
33. Guo H, Zhu B, Li S, Li J, Shen Z, Zheng Y, et al. Development and validation of a novel model to predict liver histopathology in patients with chronic hepatitis B. Biomed Res Int. 2019;2019: 1-8.
34. Giannini EG, Testa R, Savarino V. Liver enzyme alteration: A guide for clinicians. CMAJ. 2005;172(3):367-79.
35. Murray R, Granner K, Rodwell V. Biokimia Harper, 27th ed. Jakarta: Buku Kedokteran EGC; 2009.
36. Hall RL. Clinical pathology of laboratory animals in animal model in toxicology, 2nd ed. USA: CRC Press; 2007.
37. Sass P. Reference values for laboratory animal normal hematology values [document on the Internet]. Missoula: Research of Animal Resources; 2009 [cited 2021 Feb 6]. Available from: http://www.ahc.umn.edu/rar/refvalues.html.
38. Center SA. Enzyme activity in hepatic disease in small animals [document on the Internet]. New York; 2015. Available from: https://www.msdvetmanual.com/digestive-system/hepatic-disease-in-small-animals/enzyme-activity-in-hepatic-disease-in-small-animals#:~:text=Liver disease is often first,the prevalence of liver disease.
39. Bourgine M, Crabe S, Lobaina Y, Guillen G, Cesar J, Michel M. Nasal route favors the induction of CD4 + T cell responses in the liver of HBV-carrier mice immunized with a recombinant hepatitis B surface- and core-based therapeutic vaccine. Antiviral Res. 2018;153:23-32.