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Development of Chimeric Hepatitis B (HBV) – Norovirus (NoV) P particle as candidate vaccine against Hepatitis B and norovirus infection

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ABSTRACT

Introduction: Hepatitis B remains a global problem with no effective treatment. Here, a mucosal vaccine candidate was developed with HBsAg and HBcAg, to provide both prophylactic and therapeutic protection against hepatitis B. The antigens were presented using the P particle of human norovirus (HuNov). As a result, the chimeric HBV – HuNoV P particle can act as a dual vaccine for hepatitis B and HuNoV.

Methods: The vaccine candidate was expressed and purified from *Escherichia coli* BL21 (DE3) cells. HBV-HuNoV chimeric P particles were successfully expressed and isolated, with sizes of approximately 25.64 nm. Then, the HBV-HuNoV chimeric P particles were evaluated for safety and immunogenicity in mice and gnotobiotic (Gn) pigs. After three doses (5 μg/dose in mice and 200 μg/dose in Gn pigs) of intranasal immunization, humoral and cellular immune responses, as well as toxicity, were evaluated.

Results: The vaccine candidate induced strong HBV-HuNoV specific IFN-γ producing T-cell responses in the ileum, spleen, and blood of Gn pigs. Serum IgG and IgA antibodies against HBV-HuNoV chimeric P particles also increased significantly in Gn pigs. Increased HBsAg- and HuNoV-specific serum IgG responses were observed in mice and Gn pigs, although not statistically significant. The vaccine candidate did not show any toxicity in mice.

Conclusions: In summary, the chimeric HBV-HuNoV P particle vaccine given intranasally was safe and induced strong cellular and humoral immune responses in Gn pig. Modifications to the vaccine structure and dosage need to be evaluated in future studies to further enhance immunogenicity and induce more balanced humoral and cellular responses.

Introduction

The Hepatitis B virus (HBV) is an enveloped DNA virus that belongs to the Hepadnaviridae family. The genome of HBV is partially double-stranded circular DNA which will be circularized into a covalently closed circular DNA (cccDNA) upon entry of the genome into the nucleus of hepatocytes. The cccDNA of HBV is the key to the persistence of HBV inside the infected cells as it is a stable template that is relatively resistant to antiviral action and immune clearance [1].

Despite routine prophylactic efforts through vaccination programs for infants that have been endorsed by WHO since 1991 [2], HBV remains one of the major health problems globally [3]. One of the concerns regarding HBV is the development of chronic infection. There are more than 250 million people in the world suffering from chronic HBV infection, which puts them at high risk for developing terminal liver diseases and hepatocellular carcinoma [4]. Among untreated patients, 40% of chronic HBV patients progress into cirrhosis [5]. In other cases, chronic infection can also manifest in the form of asymptomatic infection. However, this does not mean decreased risk, as chronically infected patients are still able to transmit HBV to nonimmune partners through

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various routes, including sexual contact [6].

This clearly necessitates an effective therapy for HBV patients. However, effective curative intervention is still absent as current approaches in HBV therapies still possess various drawbacks [7]. Direct antiviral agents (DAAs) such as entecavir and tenofovir were able to reduce viral replication by hampering reverse transcription of the viral genome – however, they have little impact on the cccDNA of HBV [8]. Moreover, this approach also does not affect the integrated viral genome, which accounts for the constitutive expressions of Hepatitis B surface antigen (HBsAg) as well as the HBV-associated carcinogenicity. Therefore, this approach does not promote "functional cure" – loss of HBV surface antigen, whether accompanied with or without the production of anti-HBs [7]. Another approach involves the administration of pegylated interferon. However, response towards interferon therapy largely depends on the genotype of infecting HBV [8], with patients infected by HBV genotype A responding more favorably towards interferon administration [9]. Furthermore, usage of interferon therapy is limited by lack of tolerability and adverse consequences such as suppression of bone marrow and exacerbation of existing neuropsychiatric symptoms such as depression [5].

Effective clearance of HBV infection, as demonstrated in adult patients after acute HBV infection, is associated with the induction of long-lasting helper and cytotoxic T cells targeting several HBV proteins and the expression of antibodies against the HBV envelope. This immune response is comprised of T cells able to secrete Th1 cytokines, proliferate, and lyse infected hepatocytes. In cases of chronic patients, they fail to develop such a response [10]. Considering this immune profile, a therapeutic approach that restores the adaptive immune response in chronic HBV patients becomes an attractive prospect. One such approach is the HBV therapeutic vaccine that specifically aims to overcome immunosuppression caused by high viral load, tolerogenic liver environment, and dysfunction of T cells [2]. However, several of them have been tested in clinical trials with disheartening outcomes. They often did not induce HBV-specific T-cell response, or when such a response was boosted, it did not demonstrate therapeutic efficacy [10]. Therefore, the development of an effective therapeutic vaccine for chronic HBV infections remains an area for active research.

One of the approaches employed in the development of a therapeutic vaccine against HBV infection to potentiate necessary immune response is the utilization of VLP-based vaccines, which by definition are particles that contain viral antigens and show similar size and shape as viruses [11]. Because of their versatility and proclivity to self-assemble, these particles can be used in vaccine development to construct antigen presentation systems. Among particles that are studied as a prospective antigen presentation system is the P particle of human norovirus (HuNov) [12].

Norovirus, an important cause of epidemic gastroenteritis worldwide [13], is a positive single-stranded RNA virus from the family Calciviridae. The genome of HuNoV is contained inside a protein capsid composed of a single major structural protein (VP1) which consists of a shell (S) and protruding (P) domains connected by a short hinge [12]. The P particle of HuNoV is a 24-mer octahedral nanoparticle formed upon expression of the P domain in *Escherichia coli* [14]. What makes this particle an appealing platform for antigen presentation is the existence of three surface loops on the outermost surface of the P domain. These three loops have been shown to be good sites for foreign antigen insertion for improved immune responses against inserted antigens [12]. Foreign antigens that can be inserted varies greatly in size, ranging from small peptides such as the Histidine tag (7 amino acids [aa]) to larger antigen such as VP8 of rotaviruses (159 aa) [14]. Additionally, P particle chimeric also has high immunogenicity to induce immune responses against Norovirus [12,15].

In terms of inducing immunological response, utilizing HuNoV P particle as a platform in the therapeutic HBV vaccine has several advantages. Limited evidence has suggested that HuNoV infection induces a primarily Th1-type immune response [16] – a type of response lacking in the case of chronic HBV infection. Furthermore, P particles can be administered intranasally. Intranasal vaccination elicited T cell immune response in gnotobiotic (Gn) pigs [16], a well-established animal model for Norovirus vaccine research [17,18]. Intranasal vaccination of HuNoV P particle in mice also induces humoral and cellular immune responses [19]. In addition to being a less invasive approach, intranasal delivery of vaccine is able to increase immunity in human vaginal mucosa via the common mucosal immune system [20]. Thus, the intranasal HBV vaccine might also be able to prevent sexual transmission of HBV.

Taking these potentials into account, we hereby attempt to develop a novel intranasal of dual HBV and Norovirus vaccine by inserting the Hepatitis B core antigen (HBcAg) and the HBsAg into the first and second loop of the HuNoV P domain, respectively. Hepatitis B surface antigen has been proven for over 20 years to be an effective antigen for prophylactic HBV vaccine [21,22], while the core antigen is an excellent antigen for the therapeutic vaccine as it is a potent immunogen even without adjuvants [22] and can induce HBsAg- and HBcAg-specific immunity as well as endogenous dendritic cell (DC) without liver damage in mice [23]. By intranasally administering the chimeric HBV - HuNoV P particle to mice and Gn pig animal models, we aim to elicit not only the immune response required for inhibiting HBV but also the immune response against Norovirus infection.

Materials and Methods

Expression construct for recombinant HBV - HuNoV P particle

All of the construct design process described in this research was done in silico. The P domain sequence was derived from the VP1 sequence of the norovirus variant Hu/GII.4/2006b/092895/2008/USA (GenBank no. KC990829). To determine the surface loops of the P particle which will be used as the antigen presentation platform, we previously compared the structure of this norovirus variant with a reference norovirus variant GII.4/1997/VA387 (GenBank no. JQ47807), which was used to generate P particle vaccine candidates in previous studies [14,16,24].

Subsequently, two different HBV antigens were inserted into separate surface loops. Sequence encoding epitope 18–27 (F18 to I27) of HBcAg (GenBank no. AKA94096.1) was inserted into loop 1 of the HuNoV P Particle, between I293 and R297. The 18–27 epitope of HBcAg has been previously demonstrated to trigger T cell response *in vitro* [25] and is one of the epitopes that is recognized frequently by cytotoxic T lymphocytes [26]. Meanwhile, sequence encoding "a determinant" of the HBsAg (a.a 123–147) (GenBank no. AB466417.1) was inserted into loop 2 of the HuNoV P Particle, between T371 – D374. To stabilize the interaction between P domains in the P particle, we supported the construct with the sequence encoding cysteine-containing peptide (CNGRC) in the C terminal. To ensure an optimal level of expression in *Escherichia coli* BL21 (DE3) cells, the gene fragment was optimized by using codon usage of the *E. coli* B strain. The model of the constructed protein is then generated using I-TASSER.

The designed construct was then synthesized as a gene fragment in the pET-15b backbone (Fig. 3A). Synthesis of the construct was carried out by GenScript, USA.

Expression and purification

Recombinant P particle was expressed in and purified from the inclusion body in *E. coli* BL21 (DE3) cells, as described in a protocol adapted from Shaohua Li (unpublished). In this protocol, cells containing recombinant plasmid were grown to an optical density (OD) of 0.5–0.7. The culture was then induced by adding 1 mM of Isopropyl ß-D-1-thiogalactopyranoside (IPTG) (1st Base) and incubated at 37 °C for 28–29 h. Cells were then harvested and washed with PBS buffer. Afterward, they were mixed with 1 mg/mL lysozyme (Sigma-Aldrich) and binding buffer containing 20 mM sodium phosphate (Merck Milipore),

300 mM sodium chloride (Merck Milipore), and 20 mM imidazole (pH 7.4) (Bio Basic). The cell and buffer mixture was incubated on ice for 30 min before undergoing the sonication process. Post-sonication, the mixture was centrifuged, and the pellets were collected. The pellets were then resuspended in a binding buffer containing 3% of EBB detergent (Sigma-Aldrich) and incubated overnight at 4 °C. After overnight incubation, the mixture was centrifuged, and solubilized protein was collected in the supernatant.

Purification of the solubilized protein was performed in prepacked cOmplete™ His-Tag Purification Column (Roche) using NGC Medium-Pressure Liquid Chromatography Systems (Bio-Rad). Fractions were washed using wash buffer (pH 7.4) containing 20 mM sodium phosphate (Merck Milipore), 300 mM sodium chloride (Merck Milipore), 40 mM imidazole (Bio Basic), 10% Glycerol (Merck Milipore), and 1% EBB (Merck Milipore). Elution was done using elution buffer (pH 7.4) containing 20 mM sodium phosphate (Merck Millipore), 300 mM sodium chloride (Merck Millipore), 250 mM imidazole (Bio Basic), 10% Glycerol Merck Millipore, and 1% EBB (Sigma-Aldrich).

The fractions containing purified protein were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gel. Fractions that were confirmed to contain the chimeric protein were then pooled. To eliminate EBB detergent in the samples, the pooled protein was dialyzed at 4 °C four times in PBS buffer, with buffer exchanged every 8 to 12 h. Finally, the protein was concentrated using the VivaSpin column (Sartorius). This concentrated pure chimeric protein was analyzed by SDS-PAGE and quantified using the Lowry assay protocol described by the Chemical Department of the Faculty of Mathematics and Natural Sciences ITB (2017) [27].

Transmission electron microscopy (TEM) imaging of the recombinant protein

Before usage, the grid was treated on a drop of 1% aqueous Alcian blue for 5 min and then washed with 3 to 5 drops of water until the rinsed droplets were clear. A drop of the diluted P particle sample (5 ug/mL) was added to the grid and left to absorb for a minute. The excess droplet was removed using filter paper. Afterward, the grid was stained using 3% Ethanolic Phosphotungstic acid (PTA) in H_2O (pH 7.0) for 1 min. The excess stain was removed, and the grid was dried in a grid container. Dried grids were then checked immediately in TEM (200,000–250,000 magnitudes).

Mouse immunization and sample preparation

Male 7–8 week old BALB/c mice (3 mice/experimental group) were obtained from PT. Indo Anilab in Bogor. Mice were maintained in the animal enclosures of the School of Life Sciences and Technology, Institut Teknologi Bandung, Indonesia. All animal procedures were approved by the Animal Research Ethics Committee of Institut Teknologi Bandung (Ethical approval No. 11/KEPHP-ITB/4–2018).

The immunization scheme and groups for mice are summarized in Fig. 1(A). Mice (n = 3) were immunized intranasally (IN) three times on days 1, 14, and 28 with a 5 μg dose of the HBV-HuNoV chimeric P particle into the mouse nostril. Control mice (n = 3) were administered IN with only the diluent (sterile PBS). Body temperature was measured 6 and 24 h after IN administration. Blood and urine were collected on days 0, 38, and 42. Body weights were monitored weekly. Mice were euthanized at the end of the experiments, then nasal, liver, and kidney were obtained for histological analysis. The liver and kidney were weighed for organ index (organ weight/body weight × 100), as described previously [28].

Gnotobiotic (Gn) pig immunization and sample preparation

A total of six Gn pigs were divided into two groups: (i) HBV-HuNoV chimeric P particle (n = 3) and (ii) Adjuvant only (n = 3). All animal procedures were approved by Institutional Animal Care and Use Committee-Virginia Tech with the protocol numbers 16–214-CVM and 17–110-CVM.

These pigs were either administered intranasally with 200 μg/dose HBV-NoV chimeric P particle vaccine plus monophosphoryl Lipid A (MPL) adjuvant (50 μg/dose) or mock vaccinated in the control group (PBS + MPL adjuvant only) at 5 days of age (post-inoculation day [PID] 0). Booster doses were administered at PID 10 and PID 21. An intranasal mucosal atomization device (MAD NasalTM, Teleflex) was used for the intranasal vaccination. Serum was collected from each pig at PID 0, 10, 21, and, upon euthanasia, at PID 28. Meanwhile, blood, spleen, and ileum were collected on PID 28 at necropsy for the isolation of mononuclear cells. The immunization scheme and groups for Gn pigs are summarized in Fig. 1(B).

Quantification of IFN-γ- producing CD4 + and CD8 + T-cells

Upon euthanasia on Day 28, freshly isolated mononuclear cells were stimulated *in vitro* for 17 h with either (i) HBV-HuNoV P particle antigen used in the vaccine or (ii) Homologous P particle developed from the same norovirus strain GII.4/2006b. At 12 h, CD49d and Brefeldin A were added to each of the samples. Samples were then stained using respective fluorochrome-conjugated secondary antibodies and detected for GII.4 NoV-specific IFN- γproducing effector T cell responses using flow cytometry as described previously [16].

Specific IgG determination using indirect ELISA

Pre each dose of vaccination, blood serum samples were collected from animals (Figs. 1 and 2). The serum samples were used to examine the immunogenicity of the chimeric HBV-HuNoV P particles by relatively quantifying the IgG antibody produced by using indirect ELISA. Two types of IgG antibodies were detected: IgG specific for HBV and IgG for HuNoV P Particles.

To perform indirect ELISA, a PVC microtiter plate was first coated with 100 μL of antigens, of which concentration was diluted up to 1 μg/mL using a coating buffer. The HBsAg (Arista Biologicals, USA; #AGHBS-0120) and HuNoV P particles were used as antigens to detect IgG against HBV and HuNoV P particles, respectively. The plate was then wrapped tightly using plastic wraps and then incubated overnight at 4 °C. Following incubation, the coating solution was removed and then washed three times using 200 μL/well of PBS buffer. The solution was then removed by flicking the plate over a sink, and the remaining droplets were then patted onto paper towels. The plate was then blocked by adding 200 μL/well of blocking buffer, wrapped tightly using plastic wraps, and incubated overnight at 4 °C again. The plate was then rinsed using 200 μL/well PBS buffer twice. Afterward, 100 μL of diluted serum samples were added to the plate. For pig and mouse serum, the samples were subsequently diluted at 1:10 and 1:100 using a blocking buffer. The plate was then wrapped and incubated overnight at 4 °C again before being rinsed four times with 200 μL/well PBS. The plate was then further incubated with 100 μL/well of HRP-conjugated secondary antibody for 2 h at room temperature before being washed four times with 200 μL PBS. To detect the antibodies, 100 μL TMB was added to each well. After waiting for 15–30 min or after the sufficient color had developed, 100 μL acidic stop solution is added. Lastly, The optical density values were read at 450 nm using an ELISA reader. The detailed explanation is referred to in supplement Table S1.

IgA and IgG determination of Gn pig sera against HBV-HuNoV using ELISA

ELISA was done using “U” bottom well polyvinyl microtiter plates coated with 50 uL of Mucin from porcine stomach Type III (PGM) – SIGMA (50 μg/mL in PBS pH 7). The plate was then washed six times with 0.05% Tween 20-PBS. An overnight blocking was done using 200 μL of 5% dry milk/0.05%Tween 20-PBS at 4 °C. After incubation, the

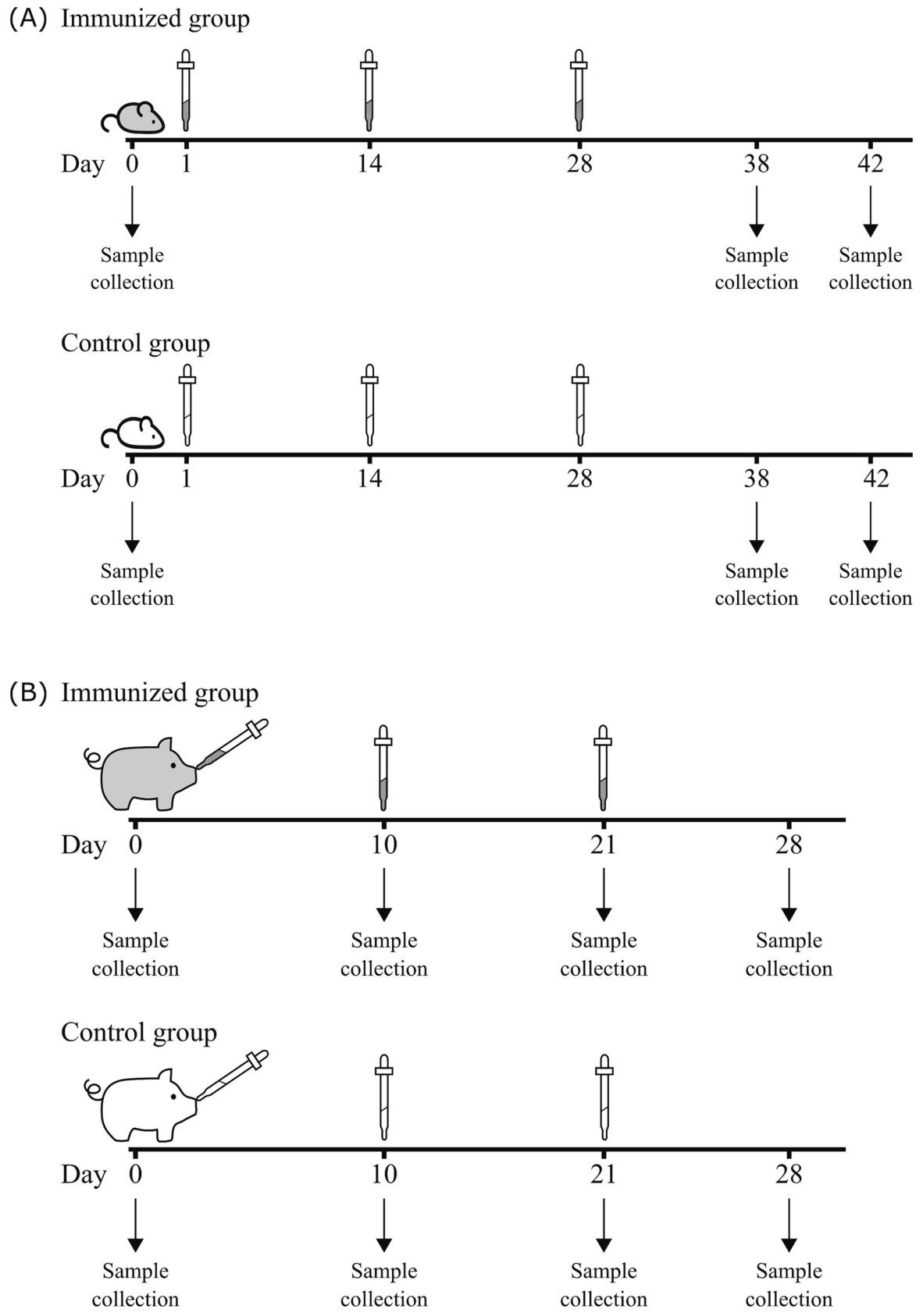


Fig. 1. Mice and Gnotobiotic (Gn) pig immunization group and schemes. (A) Intranasal immunizations of mice (days 1, 14, and 28) were done using a 5 μg dose of HBV-HuNoV chimeric P particles (grey) or sterile PBS (white) for immunized (n = 3) and control groups (n = 3), respectively. Sample collection (blood, urine, and vaginal fluid) was done on days 0, 38, and 42. Mice were euthanized on day 42, and nasal, liver, and kidney were collected. **(B)** Intranasal immunizations of Gn pig (days 0, 10, and 21) were done using a 200 μg dose of chimeric P particle with MPL adjuvant (grey) or MPL adjuvant only (white) for immunized and control groups, respectively. Serum collection was done on days 0, 10, 21, and 28. Pigs were euthanized on day 28, and blood, spleen, and ileum were collected.

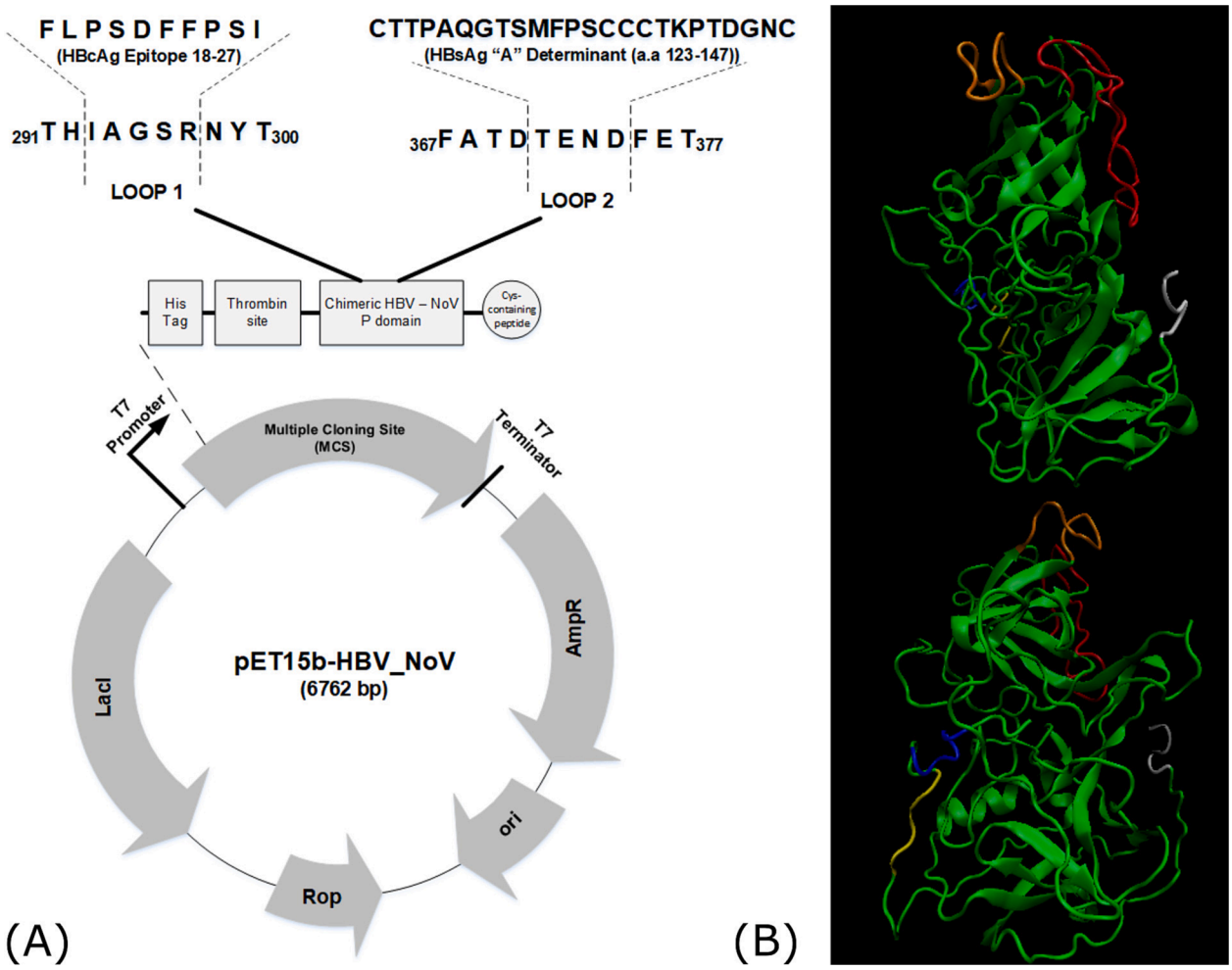


Fig. 2. The expression construct and in silico modeling of chimeric HBV - HuNoV P domain. (A) Construct used in this paper. The first and second loops of HuNoV genotype GII.4 were used as a platform to present HBV antigens. HBcAg was inserted into the first loop of the HuNoV P domain between I293 and R297. Meanwhile, a determinant of the HBsAg was inserted into the second loop of the P domain between T371 – D374. **(B)** In silico model protein generated by I-TASSER has shown that both loops are exposed on the surface of the P domain. The HBcAg in loop 1 is shown in orange, the HBsAg in loop 2 is shown in red, the cys-containing peptide is shown in white, His-tag is shown in blue, and the thrombin cleavage site is shown in yellow.

plate was washed six times with 0.05% Tween 20-PBS. A 50 μL solution of HBV-HuNoV chimeric P particles in concentration 1.5 μg/mL in 5% dry milk/0.05% tween 20-PBS was added to each well. Then the plate was incubated for one hour at 37 °C. The plate was then washed six times with 0.05% Tween 20-PBS. Gn pig serum was serially four fold diluted from 1:4 to 1:65536 in 5% dry milk/0.05% tween 20-PBS and added to each well. The plate was incubated for one hour at 37 °C and washed six times with 0.05% Tween 20-PBS. A horseradish peroxidase (HRP) conjugated goat anti-pig IgA or goat anti-pig IgG (Bethyl Laboratories, Inc. USA) at 1:2000 dilution in 5% dry milk/0.05% tween 20-PBS was added at volume 50 μL per well. After an hour of incubation at 37 °C, the plate was washed six times with 0.05%Tween 20-PBS. Then, 100 μLof ABTS peroxidase substrate (1:1 ratio of KPL ABTS® peroxidase substrate solution A and KPL peroxidase substrate solution B from Seracare Life Sciences, Inc. USA)) was added. An optical density reading was taken at 405 nm and another 30 min later. End point titers were defined as the reciprocal of the final serum dilution giving a mean OD405 nm of $\geq$ 0.200 after background subtraction. Antibody values were log transformed for subsequent statistical analysis.

Mouse blood biochemistry and urinalysis

Mouse blood samples were collected, and plasma was processed by centrifugation at 2000 g for 10 min and used for clinical biochemistry parameters (blood bilirubin, alanine amino transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) by diagnostics kit (ReiGed Diagnostics, Italy) using a spectrophotometer. Urine samples were collected for urinalysis parameters (specific gravity, pH, protein, glucose, ketones, blood, bilirubin, nitrites, WBC, RBC, and sediment) using urine strips.

Mouse histological analysis

After mice were sacrificed, organs were collected and placed in bouin fixative solution, embedded in paraffin, and sectioned at 8–10 μm thickness with a microtome. Liver and kidney staining was done with Hematoxylin-Eosin (HE), while nasal tissue was stained with Alcian Blue at pH 2.5 and counterstained with Eosin.

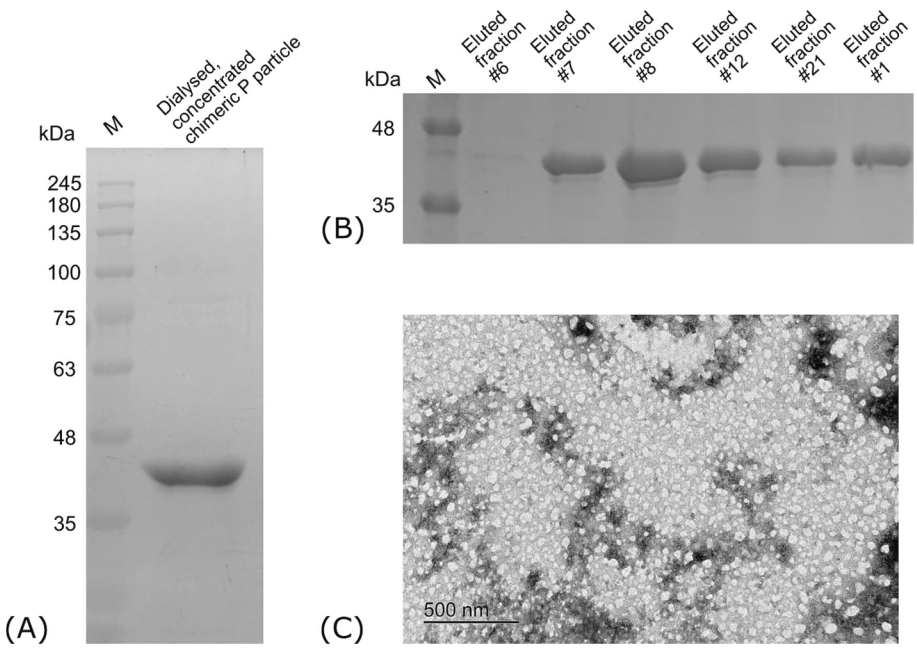


Fig. 3. Production, purification, and characterization of recombinant chimeric HBV – HuNoV P domain. (A) SDS-PAGE analysis of eluted fractions in purification using His-tag affinity chromatography has shown a single band with molecular weight ~ 35–48 kDa, which is expected to be the chimeric P domain. (**B**) Further processing of pooled eluted fractions through dialysis and spin concentrator also yield consistent result as (B). (**C**) Observation of the purified chimeric protein using TEM microscopy revealed that all of the P particles appear to be globular (scale bar 500 nm).

Statistical analysis

Statistical analysis was done using Graph Pad Prism 8 (GraphPad Software) software and R (R Foundation). Data was analyzed using Student's T-test and Tukey's test.

Results

Expression and characterization of chimeric HBV – HuNoV P particle

Both the HBsAg and HBcAg encoding genes were inserted in the frame into the HuNoV P domain to ensure proper translation of the P domain (Fig. 2A). The protein model from I-TASSER predicted that both HBcAg (in loop 1) and HBsAg (in loop 2) were exposed on the surface of the recombinant P domain (Fig. 2B). The exposure of antigens on the surface would allow them to interact with the immune system molecules [23].

The chimeric HBV – HuNoV P particle was predicted to have a molecular weight of 40.9 kDa, according to the SnapGene software. The chimeric protein was then expressed in *E. coli* BL21 (DE3) cells. SDS-PAGE analysis confirmed the expression of the chimeric P domain, consistently yielding a band of 35–48 kDa (Fig. 3A and 3B).

In order to confirm the formation of 24-mer P particles in the purified and dialyzed protein, the sample was studied under a TEM microscope (Fig. 3C). The chimeric particles had a size of approximately 25.64 nm, according to ImageJ. This size is larger than the parental 24-mer P particle (~20 nm) [14]. Several recombinant P particles described in previous studies have also shown similar sizes [29] and patterns [16], suggesting that the recombinant P domain was successfully assembled into 24-mer P particles *in vitro*.

HBV – HuNoV immunization in gnotobiotic pigs elicits CD4-Th1-Type and CD8 immune response

All three tissues of vaccinated Gn pigs contained higher frequencies of HBV-HuNoV P particle-specific CD4 + IFN-γ + and CD8 + IFN-γ + T cells compared to control pigs (Fig. 4). Significantly higher frequencies of antigen-specific IFNγ + CD4 in the ileum, PBL, and spleen and IFNγ + CD8 + T cells in the spleen were observed, suggesting strong Th1 type and CD8 + T cell type immunogenicity of the vaccine. As a control, the T cell response was compared to the T cell response stimulated with homologous HuNoV-P particle (Figure S1). The result in Fig. 4 and Figure S1 showed that CD4 and CD8 T cells in all three tissues were activated to secrete IFNγ by the HBV-HuNoV chimeric P articles and not by the HuNoV-P particles.

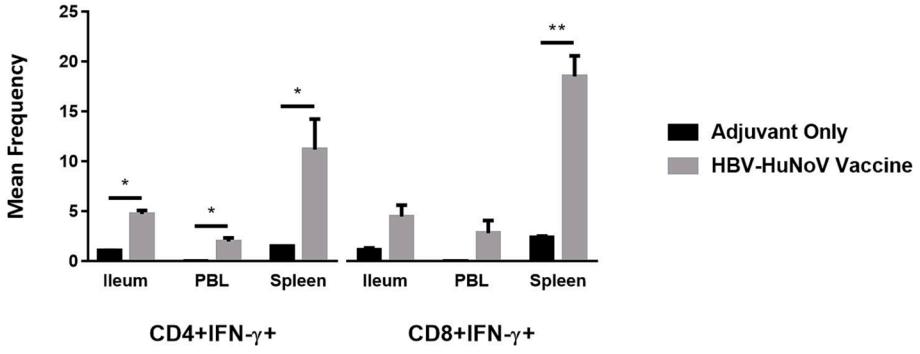


Fig. 4. Frequencies of HBV-HuNoV P particle-specific IFN-γ + CD4 + and IFN-γ + CD8 + T cells in the ileum, peripheral blood (PBL), and spleen at PID 28. Two-way ANOVA followed by Multiple t-tests were carried out for comparisons. Significant differences are identified by * ($n = 3$; $p < 0.05$); and ** ($n = 3$; $p < 0.01$).

Intranasal administration of HBV-HuNoV P particle increased immune response in mice and Gn pigs

There was a significant increase in IgG against HBV-HuNoV P Particle at PID 28 in Gn pigs (Fig. 5). Additionally the vaccination also induced serum IgG antibody response against HBsAg in both mice and Gn pigs (Fig. 6A and 6C). However, the responses were not statistically significant compared to the control (Fig. 6A and 7). Similarly, both animals exhibited a slight increase, yet non-significant of antibodies against HuNoV after 21 days post vaccination (Fig. 6B and 6C). However, in general, both anti-HBsAg and anti-HuNoV IgG titer increase following booster immunization with the HBV – HuNoV P particle.

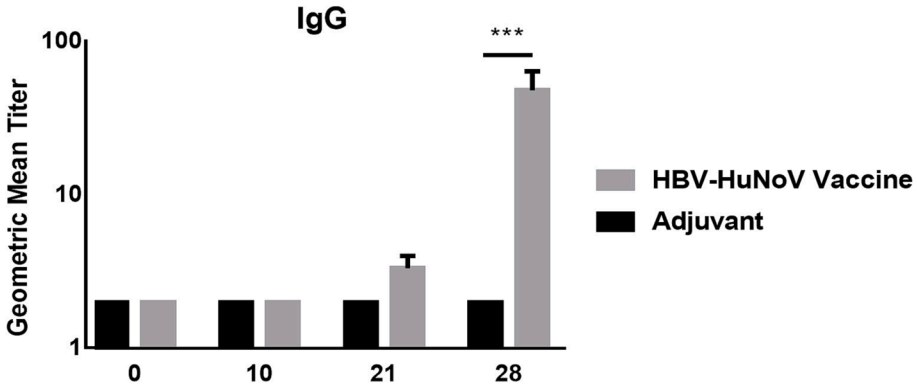
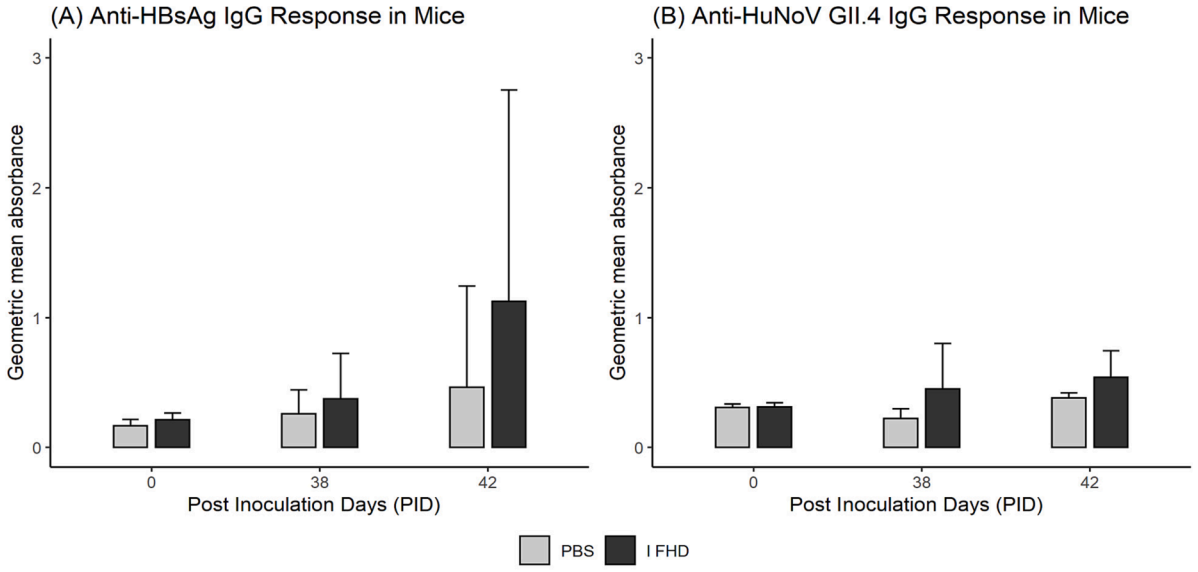


Fig. 5. Geometric mean end-point titers of HBV-HuNoV P particle-specific IgG antibody titers in Gn pig serum. Each serum sample was tested at an initial dilution of 1:4. Negative samples were assigned an arbitrary value of 2 for calculation and graphical illustration purposes. Bars represent the geometric mean end-point titers with 95% confidence intervals. Comparisons between groups at the same time points were carried out using Student's *t*-test (n = 3 for each group). Significant differences are identified by **P < 0.01 and ***P < 0.0001.



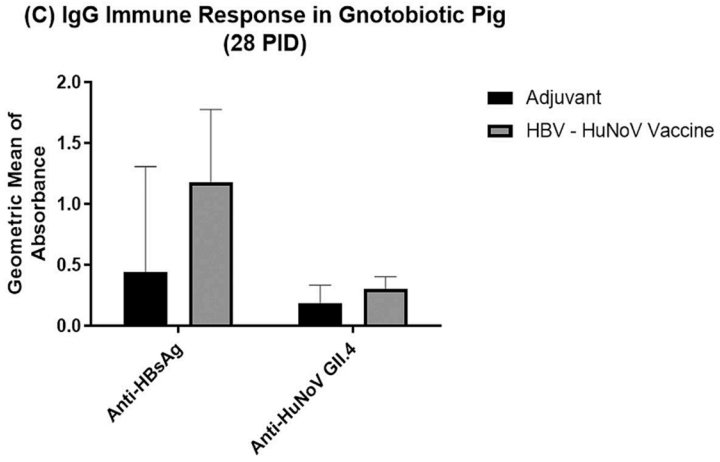


Fig. 6. Serum IgG antibody response to HBV – HuNoV P particle in BALB/c mice BALB/c mice (A and B) and Gn pig (C). (A) In mice, there was an increase in anti-HBsAg IgG over time, although not significant (P for days 0, 38, and 42 are P = 0.3477, 0.5418, and 0.4501). **(B)** A similar trend was also observed in anti-HuNoV GII.4 IgG following three times of intranasal administration of HBV-HuNoV chimeric P particle in mice (P for days 0, 38, and 42 are P = 0.8332, 0.2303, and 0.1994). **(C)** In Gn pig, there was an increase in anti-HBsAg IgG and slightly increase of anti-HuNoV 28 days after vaccination. Data are shown as geometric mean ± geometric s.d. Statistical significance was assessed using Student's T-test (n = 6 for mice and n = 3 for Gn pig for each group).

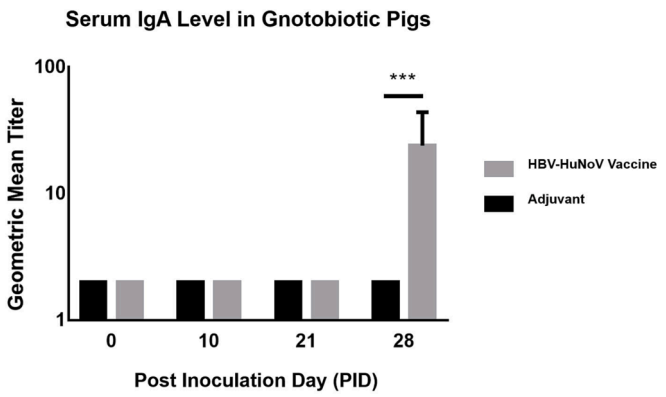


Fig. 7. Geometric mean titers of HBV-HuNoV P particle-specific IgA antibody titers in Gn pig serum. Each serum sample was tested at an initial dilution of 1:4. Negative samples were assigned an arbitrary value of 2 for calculation and graphical illustration purposes. Bars represent log10 geometric mean end-point titers with 95% confidence intervals. Comparisons between groups at the same time points were carried out using Student's *t*-test ($n = 3$ for each group). Significant differences are identified by $^{**}P < 0.01$ and $^{***}P < 0.0001$.

In Gn pigs, while the first two immunizations did not elicit a significant rise in serum IgA compared to mock groups, the third immunization at PID 21 induced a strong IgA response in serum (Fig. 7). The high titer of HBV–HuNoV IgA antibodies in pig serum indicated that the vaccine plausible induced mucosal antibody immune responses.

HBV – HuNoV P particle administration exhibited no toxicity in organs

The HBV – HuNoV P particle did not exhibit toxicity in the mouse model, as measured through body temperature (Figure S2), weight (Figure S3), blood (Figure S4), and urine biochemistry (Figure S5), as well as histology analysis. Intranasal immunization did not adversely affect the liver (Fig. 8) or nasal cavity (Fig. 9) of mice. The liver organ index was similar between immunized and control groups, further supporting the safety of this immunization in the mouse model (Fig. 10). Furthermore, administration of HBV – HuNoV P particles did not significantly alter body weight (Figure S3), temperature (Figure S2), or blood and urine biochemistry profiles (Figures S4 and S5).

Discussion

In this study, we developed an intranasal vaccine candidate for hepatitis B using the P particle of HuNoV. We inserted two HBV epitopes (HBcAg and HBsAg) into the P particle to develop a vaccine candidate that can act as a protective vaccine and potentially a therapeutic vaccine for hepatitis B. Effective clearance of HBV remains a challenge, especially for chronic patients [10]. So, therapeutic vaccines for HBV are an attractive prospect to help restore the adaptive immune response for chronic HBV infection. Additionally, the chimeric P particle could potentially protect against HuNoV as well.

The P particle has been demonstrated to be an excellent vaccine carrier because it presents epitopes on its outermost surface to induce specific antibodies against the epitopes [14]. For instance, chimeric P particles inserted with influenza virus antigen M2e protected mice against subsequent lethal challenges against human influenza virus PR8 (H1N1) compared to mice supplemented with free M2e antigens and PBS saline [14]. Chimeric P particles inserted with murine rotavirus (EDIM) antigen VP8 also reduced viral shedding [14]. Moreover, P particles can self-assemble into 24 polymer forms, which are conducive to presenting more epitopes and inducing high levels of specific antibodies [14].

Thus, we developed recombinant P particle proteins by inserting two different epitopes (HBcAg and HBsAg) into the particle's loop one and loop 2, respectively (Fig. 2A). We successfully expressed the HBV–HuNoV chimeric P particles using *E. coli* BL21 (DE3). In silico analysis predicted the chimeric protein's molecular weight of 40.9 kDa, later confirmed in SDS-PAGE analysis (Fig. 3A-B). Further TEM microscope analysis revealed that the HBV-HuNoV chimeric P particles were globular and around 25.64 nm. The observations suggest that the recombinant P domain was successfully assembled into 24-mer P particle *in vitro*.

Immunogenicity evaluation in animal models demonstrated that the chimeric P particle could induce strong T-cell responses specific to HBV–HuNoV (Fig. 4). We observed statistically significant increased activity of effector CD8 + and CD4 + T cells in pigs (Fig. 4). The increased CD4 + T cell activities were significant in different tissues: the systemic lymphoid tissues (PBL and spleen) and ileum, suggesting activation of Th1 type response. Since CD4 + T cells are necessary for CD8 + T-cell-mediated cell lysis [30], the observations suggest that the chimeric P particle has a strong T cytotoxic response. Additionally, the detection of increased IFN-γ + CD4 + and IFN-γ + CD8 + T cells in different tissues indicates that intranasal vaccination might deliver HBV-HuNoV chimeric P particles to mucosal and peripheral immune organs.

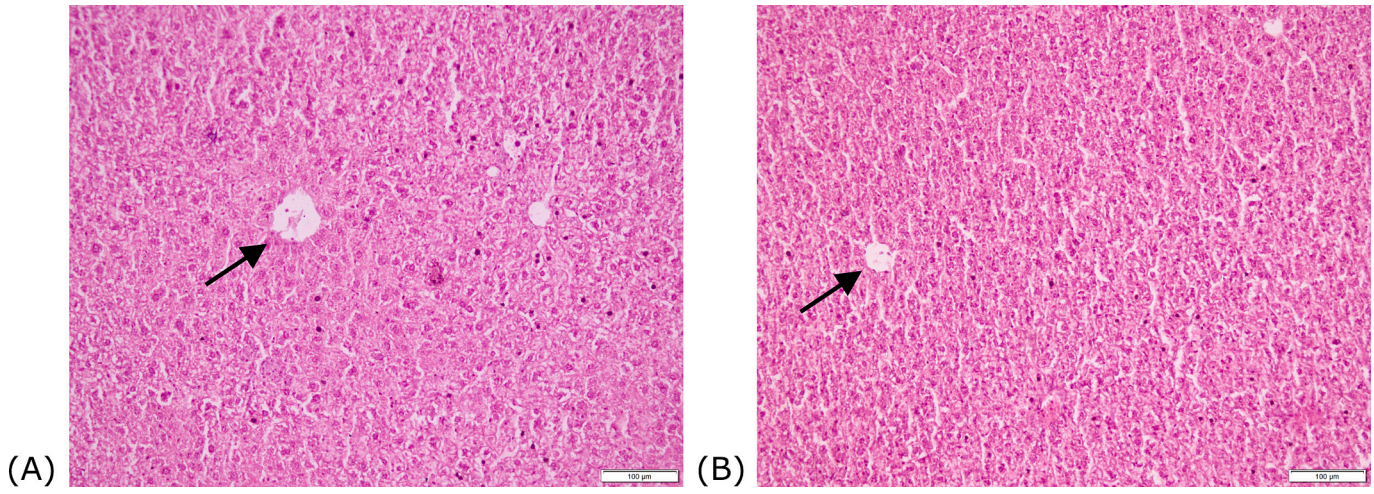


Fig. 8. Hematoxylin and eosin staining of mouse liver sections of mice immunized with HBV – HuNoV chimeric P particle (20x magnification). (A) PBS, **(B)** 1 FHD. Black arrows indicate the central vein. PBS = Phosphate Buffer Saline group served as control; 1 FHD group = 1 Full Human Dose or 5 μg of HBV – HuNoV chimeric P particle.

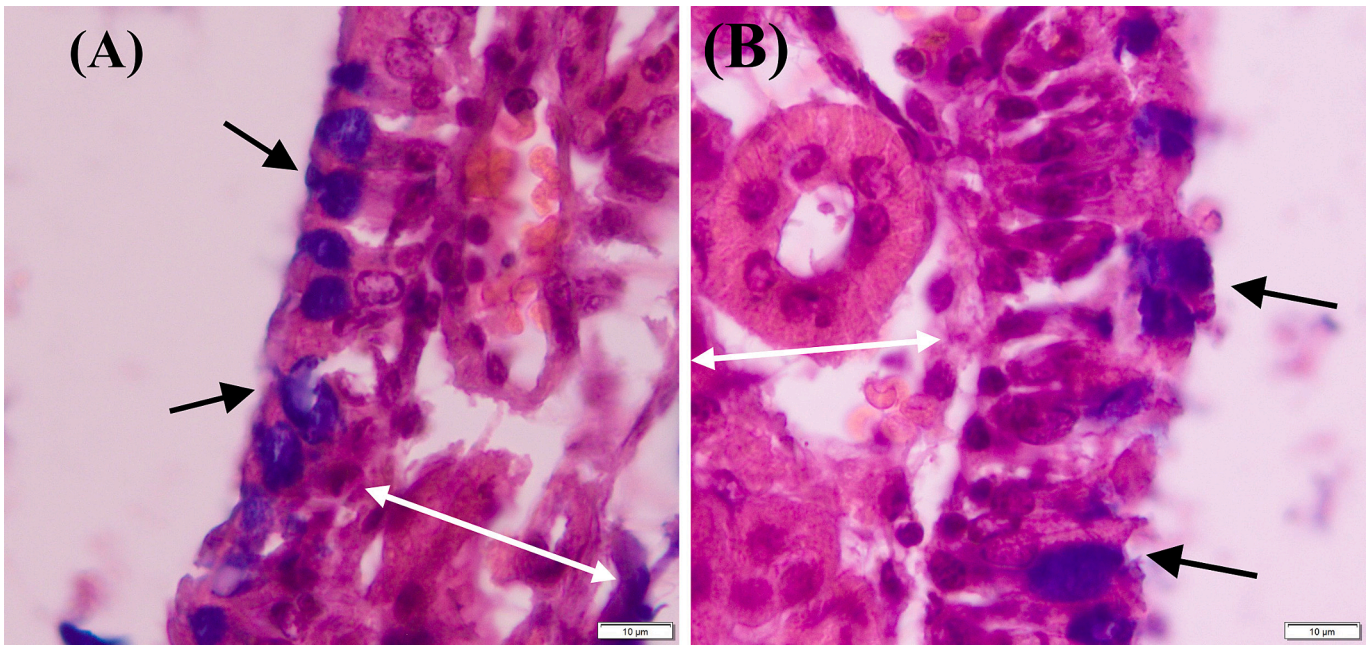


Fig. 9. Alcian Blue stained nasal cavity of mice (100x magnification). (A) PBS, **(B)** 1 FHD. Black arrows indicate goblet cells, and lamina propria are indicated by white lines. PBS = Phosphate Buffer Saline group served as control; 1 FHD group = 1 Full Human Dose or 5 µg of P particle.

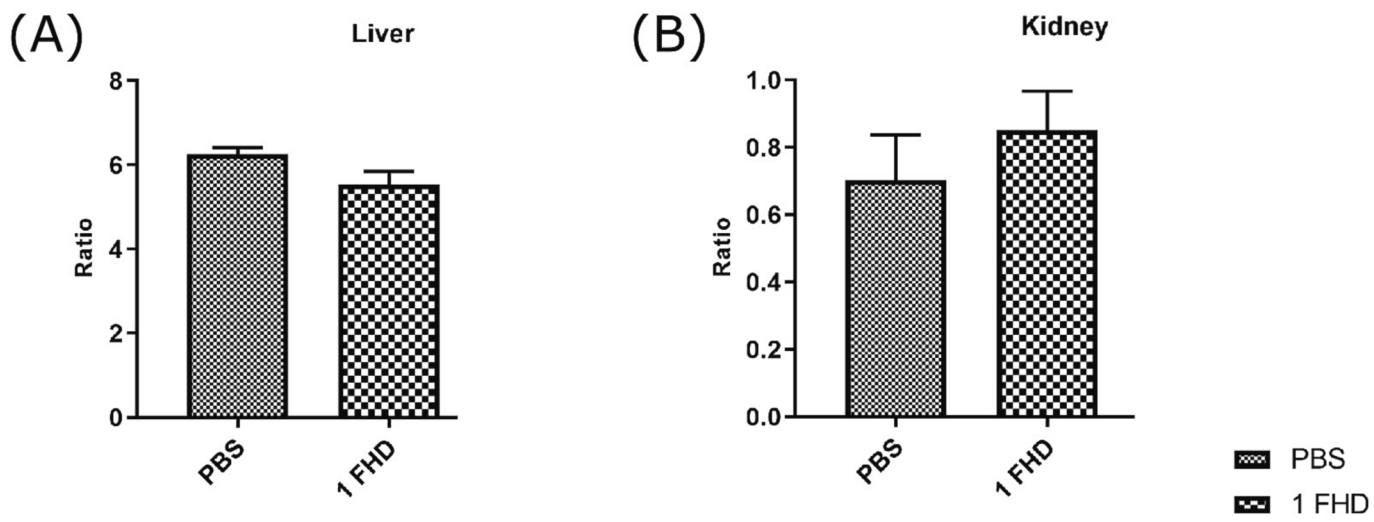


Fig. 10. Liver (A) and kidney (B) index after p-particle administration compared to the control in mice immunized with PBS and 1 FHD. PBS = Phosphate Buffer Saline group served as control; 1 FHD group = 1 Full Human Dose or 5 µg of HBV-HuNoV chimeric P particle.

Further investigation should be performed to verify the mucosal immune response.

Regarding antibody responses in mice and pigs, although the number of animals in each group is low (n = 3, due to the budget limit), there was a tendency for increased IgG activity (Figs. 6 and 7). Result in Fig. 5 demonstrated a significant increase of IgG in Gn pig against chimeric HBV- HuNoV P particles. Both animals exhibited an enhanced immune response against HBsAg, as indicated in Fig. 6A and 6C. Nevertheless, the increase in immune response against HBsAg was not statistically significant. Additionally, in mice, there was a slight increase in Anti-HuNoV IgG levels, as depicted in Fig. 6B. Conversely, the response level in pigs closely resembled the control, as shown in Fig. 6C. This result implies that the IgG response is likely to be primarily driven by the complex chimeric P particles, which probably contributed from both HBcAg and HBsAg. However, further investigations are required to validate this finding.

Regarding the lack of significant increase in the specific IgG response to HBsAg and HuNoV when compared to IgG response against the entire HBV-HuNov chimeric P particle, this maybe attributed to several factors. It is known that IFN-γ inhibits certain IgG isotype switching [31], so the observed increased IFN-γ after P particle vaccination might be related to the less-pronounced IgG activation. We also identified a possible salt bridge between the chimeric P particle's epitopes, which might influence antigen exposure for IgG induction. This salt bridge could inhibit the exposure of antigens and hinder IgG activation. Additionally, we only evaluated one dose of vaccination (5 µg/dose in mice and 200 µg/dose in Gn pigs). An increased dosage might elicit higher antibody responses, but safety evaluations would also be needed.

The chimeric P particle was also able to elicit an IgA response in Gn pig. Serum IgA antibodies against the chimeric P particle were increased significantly after the third vaccination in pigs. Previous studies on live-attenuated Salmonella vaccine and rotavirus vaccine in pigs

demonstrated that mucosal IgA and IFN-γ + CD8 T cell immunity are important for vaccine efficacy and protection against infection [32,33]. IgA antibodies play an important role in mucosal immunity, so the chimeric P particle might also induce mucosal immunity [34]. It is a pitall of this study that we did not evaluate IgA activity in mucosal tissues. Future studies of IgA responses in mucosal tissues of mice and Gn pigs are needed to confirm the ability of HBV-HuNoV chimeric P particles to elicit a mucosal immune response.

Conclusions

In summary, the study described here further demonstrates the versatility of norovirus P particle as a platform in vaccine development. Through the non-invasive intranasal approach, the chimeric HBV - HuNoV P particles elicited humoral and cellular immune responses in pigs and induced humoral response against HBsAg in both animals. Future studies on the chimeric HBV-HuNoV P particles should address the potential salt bridge between the epitopes, evaluate different vaccination doses, evaluate the specific immune response against HBcAg, and further evaluation of other cytokines for T cell responses (such as IL-4, IL-2, IL-5), and evaluate IgA activity in mucosal tissues.

Data statement

The data that support the findings of this study are presented in this manuscript and raw data is available from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2023.100354.

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