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Vaccination with Lipid Nanoparticle-Delivered VP2-DNA Elicits Immune Protection in Chickens Against Novel Variant Infectious Bursal Disease Virus (nVarIBDV)

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Abstract

Background/Objective: Infectious bursal disease (IBD) is an acute and highly contagious immunosuppressive disease in chickens caused by infectious bursal disease virus (IBDV). In recent years, a novel variant IBDV (nVarIBDV) has emerged and spread widely, inducing severe immunosuppression and posing a substantial threat to the poultry industry. More importantly, owing to antigenic variations, nVarIBDV can escape the immune protection of the existing vaccines. Therefore, it is imperative to develop a new vaccine that is antigenically matched to nVarIBDV. Methods: The major protective antigen gene VP2 of the representative nVarIBDV strain SHG19 was inserted into the eukaryotic expression plasmid pCAGGS to construct the recombinant plasmid pCASHGVP2. Subsequently, pCASHGVP2 was encapsulated in lipid nanoparticles (LNPs) to form pCASHGVP2-LNP nanoparticles. Finally, using the SPF chicken model, the immune efficacy of pCASHGVP2-LNP was preliminarily assessed by administering two vaccine doses (10 and 20 μg) and two immunization regimens (single or double immunization). Results: Efficient VP2 protein expression from pCASHGVP2 was confirmed by in vitro transfection experiments. The prepared pCASHGVP2-LNP nanoparticles exhibited an optimal particle size distribution and acceptable polydispersity index, indicating a homogeneous formulation. Furthermore, animal experiments showed that the candidate DNA vaccine elicited specific neutralizing antibodies after double immunization and protected immunized chickens from disease induced by nVarIBDV challenge. Conclusions: This study reports the first development of an LNP-encapsulated VP2 DNA vaccine (pCASHGVP2-LNP) against nVarIBDV, highlighting its potential application for the prevention of nVarIBDV.

Keywords: infectious bursal disease (IBD); novel variant infectious bursal disease virus (nVarIBDV); VP2; DNA vaccine; lipid nanoparticle (LNP)

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1. Introduction

Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV), is a highly immunosuppressive disease affecting chickens and poses a serious threat to the poultry industry [1]. Since the 1980s, a very virulent IBDV (vvIBDV) associated with high mortality has swept the world and severely impacted the poultry industry, making IBD an essential immunization disease [2]. A series of inactivated and attenuated live vaccines have been developed to prevent and control IBD, including inactivated vaccines and attenuated live vaccines [3]. These vaccines have gradually contributed to controlling the spread of IBD. However, in recent years, a novel variant IBDV (nVarIBDV) has emerged in immunized chicken flocks [4] and rapidly spread in many countries, including Japan, South Korea, Malaysia, Egypt, and Argentina [5–9]. nVarIBDV is the primary pathogen responsible for atypical IBD, which can induce severe immunosuppression and reduced production performance, and has emerged as a significant threat to the poultry industry [10,11]. Notably, compared with vvIBDV, nVarIBDV has undergone antigenic mutations, resulting in existing vvIBDV vaccines being unable to provide complete protection against it [12,13]. Consequently, there is a critical need to develop vaccines with improved safety and efficacy to prevent nVarIBDV infection [14,15].

DNA vaccines represent a modern approach to immunization, which has proven effective in conferring protection against various pathogens and offers showcasing advantages such as straightforward preparation, low production cost, high stability, and ease of storage [16]. Additionally, they are inherently safer than conventional live-attenuated or inactivated vaccines, because they eliminate the risk of viral spread [17]. As a novel gene delivery vector, lipid nanoparticles (LNPs) can effectively protect plasmid DNA from nuclease degradation, thereby enhancing stability and improving transfection [18,19]. The LNP-DNA complex can be internalized by cells via endocytosis, subsequently releasing the plasmid DNA into the cytoplasm to enable transgene expression.

As a member of the genus *Avian birnavirus* and family *Birnaviridae*, infectious bursal disease virus (IBDV) has a genome composed of two segments of double-stranded RNA (segments A and B). The VP2 protein encoded by segment A is the major capsid protein and the primary protective antigen of IBDV, making it a key target for the development of effective vaccines against emerging variants [20–22]. In this study, for the first time, an LNP-encapsulated VP2 DNA vaccine (pCASHGVP2-LNP) against nVarIBDV was developed and preliminarily evaluated, representing a potentially valuable approach for the comprehensive prevention and control of IBD.

2. Materials and Methods

2.1. Animals and Ethics Statement

Specific-pathogen-free (SPF) chickens were obtained from the National Poultry Laboratory Animal Resource Library and housed in negative-pressure isolators under standard conditions at the Experimental Animal Center, Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences (CAAS). The chickens were provided food and water ad libitum and maintained under a standard light–dark cycle. All animal procedures were approved by the HVRI Experimental Animal Welfare Ethics Committee (Approval No. 250116-01-GR) and conducted in accordance with welfare guidelines. At the end of the experiment, all surviving animals were euthanized. by high-concentration carbon dioxide inhalation, and tissue samples were subsequently collected for analysis.

2.2. Viral Strain and Cell Lines

The Avian Immunosuppressive Disease Division of HVRI isolated and characterized the nVarIBDV reference strain SHG19 [4], which served as the VP2 gene donor and chal-

lenge virus. The recombinant virus rGtVarVP2 [1], constructed by inserting the VP2 gene from SHG19 into the genome of a cell-adapted strain, was used to measure neutralizing antibodies against nVarIBDV. DF-1 and 293T cells were cultured in Dulbecco's Modified Essential Medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) at 38.5 °C (DF-1) or 37 °C (293T) with 5% CO_2.

2.3. Construction and Characterization of the Recombinant Plasmid pCASHGVP2

To construct the recombinant plasmid pCASHGVP2, VP2 from the nVarIBDV SHG19 strain (GenBank accession number: MN393076.1; Figure S1) was amplified by PCR, digested with *Eco*RI and *Bgl*II, and ligated into the pCAGGS vector under the control of the chicken β-actin promoter (primers are listed in Table S1). After confirmation of the plasmid sequence, the construct was prepared using a midi-prep kit and transfected into DF-1 cells. Expression of VP2 protein was then verified by indirect immunofluorescence assay (IFA) and Western blot analysis using an anti-VP2 monoclonal antibody (MAb). Briefly, 2 μg of the pCASHGVP2 was transfected into DF-1 cells cultured in six-well plates. At 24 h post-transfection, IFA and Western blot were performed. For IFA, cells were incubated with an anti-VP2 MAb (1:200), followed by an FITC-conjugated goat anti-mouse secondary antibody (Sigma, Livonia, MI, USA; 1:400). Images were acquired using an EVOS M5000 imaging system (Life, Waltham, MA, USA). For Western blot, an anti-VP2 MAb was used at a dilution of 1:2000, followed by an IRDye® 800CW goat anti-mouse secondary antibody (Li-COR, Lincoln, NE, USA; 1:20,000). Blots were visualized using an Odyssey CLX imaging system (LI-COR, Lincoln, NE, USA) with a 680 nm solid-state laser.

2.4. Preparation and Characterization of pCASHGVP2-LNP

The pCASHGVP2 was mixed with LNPs (ALC-0315, SCINDY, Suzhou, China) using a nanomedicine preparation system (Model: INano L, Shanghai, China) to form LNP-encapsulated pCASHGVP2 particles and ensure controlled and reproducible nanoparticle formation. First, the aqueous phase was prepared by mixing the pCASHGVP2 nucleic acid stock solution with acetate buffer (50 mM, pH 4.0) and ultrapure water to achieve a final nucleic acid concentration of 0.13 μg/μL. The LNPs were formulated by combining at the four-component ethanol phase with the aqueous phase at a volumetric flow ratio of 1:3 in a microfluidic device. The resulting product was collected and purified using a Millipore ultrafiltration centrifuge tube. The prepared pCASHGVP2-LNPs were characterized for particle size, polydispersity index (PDI), and zeta potential using a NanoZS90 nanoparticle analyzer (Malvern, Malvern, UK). Encapsulation efficiency of the plasmid DNA was determined directly using the Quant-iT™ PicoGreen™ dsDNA assay kit (Thermo Scientific, Waltham, MA, USA). Typically, acceptable criteria for the DNA-LNP formulation were defined as follows: an encapsulation efficiency (EE) of $\geq$85%, a polydispersity index (PDI) of $\leq$0.30, and a zeta potential within $\pm$15 mV [23].

2.5. Immunization of pCASHGVP2-LNP

2.5.1. Single Immunization Experiment

Fourteen-day-old chickens (n = 40) were randomly divided into eight groups with five chickens per group. Groups 1 and 2 were designated as the negative controls (NC); Groups 3 and 4 were designated as the non-immune challenge controls (CC); Groups 5 and 6 were immunized intramuscularly with 10 μg of pCASHGVP2, and Groups 7 and 8 were immunized intramuscularly with 20 μg of pCASHGVP2. Group 1 (NC), Group 3 (CC), Group 5 (pCASHGVP2, 10 μg), and Group 7 (pCASHGVP2, 20 μg) were used to evaluate the immune efficacy of the single immunization experiment (Figure 1a).

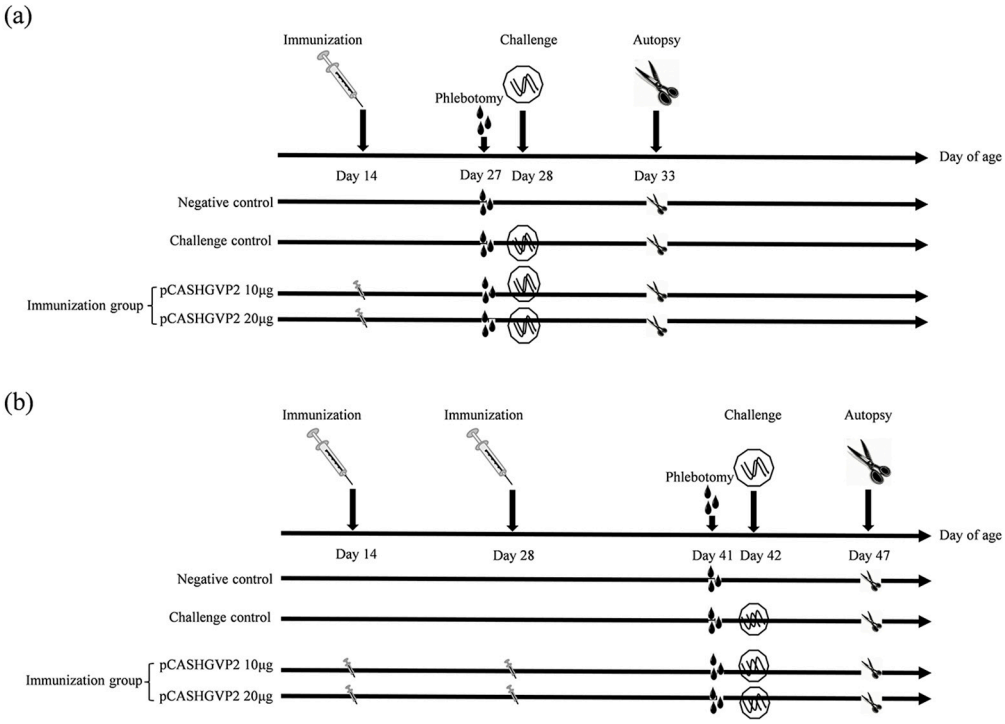


Figure 1. The flow chart of the animal experiments. (**a**) Single immunization experiment. (**b**) Double immunization experiment.

2.5.2. Double Immunization Experiment

To systematically evaluate the immune efficacy of the DNA vaccine, a double immunization experiment was performed. Fourteen days after the prime immunization, a booster immunization with 10 μg and 20 μg of pCASHGVP2 was administered to Groups 6 and 8, respectively, as described above. Group 2 (NC), Group 4 (CC), Group 6 (pCASHGVP2, 10 μg), and Group 8 (pCASHGVP2, 20 μg) were used to evaluate the immune efficacy of the double immunization experiment (Figure 1b).

2.6. Determination of Neutralizing Antibody Titers

On day 13 post-immunization (single immunization trial), serum samples were collected from all chickens to determine neutralizing antibody titers against the SHG19 strain antigen. A neutralization assay was performed using rGtVarVP2 in DF-1 cells. Serum samples were heat-inactivated at 56 °C for 30 min and subsequently filtered through a 0.22 μm membrane. Twofold serial dilutions beginning at $1:2^3$ were prepared in DMEM containing 2% fetal bovine serum. Next, 100 μL of each diluted serum was mixed with an equal volume of rGtVarVP2 virus (200 $TCID_{50}$) and incubated at 37 °C for 1 h. After removal of the culture supernatant from DF-1 monolayers in 96-well plates, 100 μL of the virus–serum mixture was added and incubated at 37 °C for 72 h. Negative and positive controls were included. Neutralizing antibody titers were determined based on cytopathic effects (CPE) observed under a microscope. In the double immunization experiment, neutralizing antibody titers were measured in serum samples collected on day 13 after booster immunization.

2.7. Challenge with nVarIBDV

2.7.1. The Challenge in the Single Immunization Experiment

In the single immunization experiment, a challenge test was performed at 14 days post-immunization (dpi). Chickens in Groups 3, 5, and 7 were challenged with the nVarIBDV

strain SHG19 via the intranasal and ocular routes at a dose of 10 BID50 (50% chicken bursa infectious dose) per chicken. At 5 days post-challenge (dpc), all surviving chickens in Groups 1, 3, 5, and 7 were humanely euthanized, and a gross pathological examination was performed; bursa and anal-swab specimens were collected immediately after death. Tissue samples from three bursae per group were fixed in 10% neutral buffered formalin, processed for routine hematoxylin and eosin (H&E) staining, and examined under a light microscope for histopathological analysis. Infection with nVarIBDV (SHG19 strain) does not directly cause mortality in chickens, but induces characteristic pathological changes, primarily manifested as grayish-yellow discoloration of the bursa accompanied by obvious inflammatory exudate, as well as damage to the lymphoid follicular structure, lymphocyte disintegration and necrosis [4]. Chickens in the immunized groups without specific gross lesions in the bursa were considered protected by the vaccine.

2.7.2. The Challenge in the Double Immunization Experiment

In the double immunization experiment, at 14 days post-booster immunization, a challenge test with the SHG19 strain at a dose of 10 BID_{50} was performed in Groups 4, 6, and 8. At five days post-challenge (dpc), all surviving chickens in Groups 2, 4, 6, and 8 were humanely euthanized, and a gross pathological examination was performed. All data collection and analyses were conducted using the same procedures as those described for the first challenge experiment above.

2.8. Virus Load Detection

At 5 dpc, bursa and cloacal swab samples from all surviving chickens in the immunized groups were analyzed for IBDV viral load using RT-qPCR with specific primers and probes (Table S1) [24]. Samples from all five chickens in the challenge control (CC) group were served as controls. Bursa tissues and cloacal swabs were subjected to three freeze–thaw cycles and subsequently homogenized using a high-throughput tissue grinder (Tissuelyser II, QIAGEN, Hilden, Germany). Total RNA was extracted from the supernatant using TRIzol reagent. cDNA was synthesized using the HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China). For bursa samples, viral genome copy numbers were normalized to 28S rRNA cDNA levels. For cloacal swabs, viral loads were expressed as IBDV RNA copies per 100 μL of sample.

2.9. Statistical Analysis

All data are expressed as mean ± standard deviation (mean ± SD). Statistical analyses were performed using GraphPad Prism software (version 8.0). Groups comparisons were conducted using an independent samples *t*-test, with a p value ≤ 0.05 considered statistically significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

3. Results

3.1. Construction and Protein Expression Validation of the Recombinant Plasmid

The nVarIBDV VP2 gene was obtained by RT-qPCR amplification, and a 1362 bp product was obtained, corresponding to the expected size of the target fragment. The VP2 gene fragment was subsequently inserted into the pCAGGS vector, resulting in the successful construction of the recombinant plasmid pCASHGVP2. IFA and Western blot analyses using an anti-VP2 MAb demonstrated efficient VP2 protein expression in the pCASHGVP2 plasmid. A specific fluorescent signal was observed in DF-1 cells transfected with pCASHGVP2, confirming successful expression of the viral VP2 protein (Figure 2a). No such signal was detected in cells transfected with the empty pCAGGS vector control.

Western blot analysis using an anti-VP2 MAb revealed a specific band of approximately 45 kDa, corresponding to the expected size of the recombinant VP2 protein (Figure 2b).

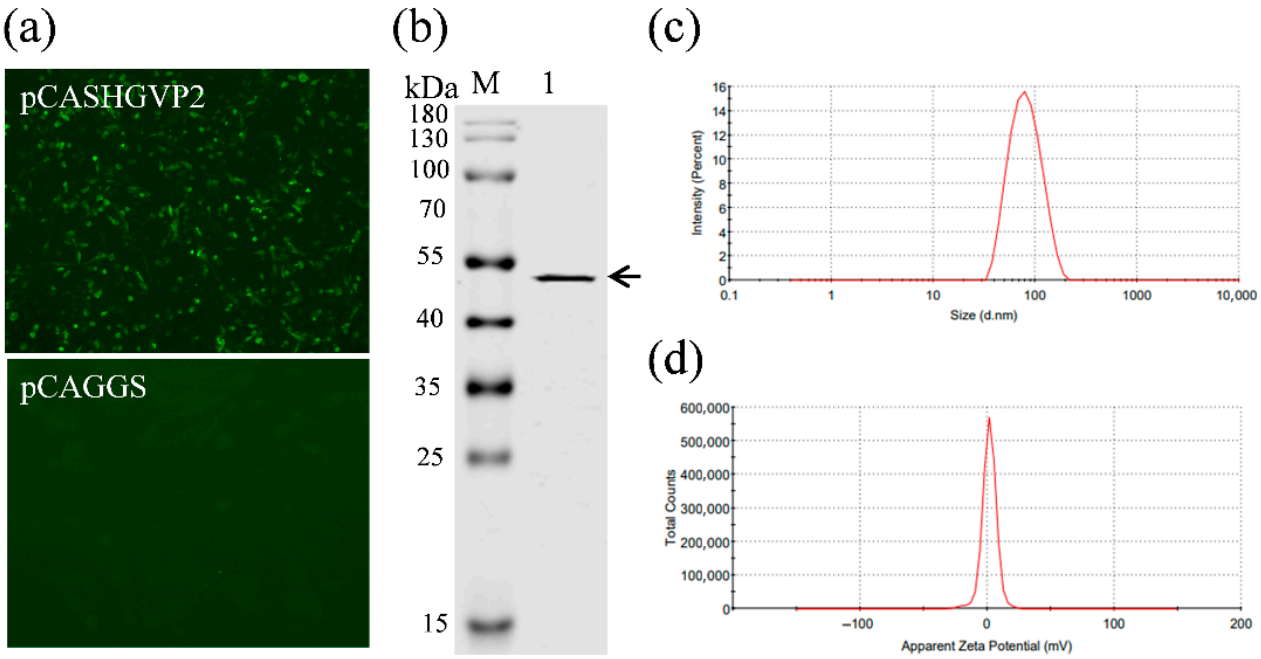


Figure 2. Identification in vitro of pCASHGVP2-LNP. (**a**) VP2 expression detection of the recombinant plasmid pCASHGVP2 with indirect immunofluorescence assay. (**b**) VP2 expression detection of the recombinant plasmid pCASHGVP2 with Western blot. M, protein molecular weight standard; Lane 1, pCASHGVP2(Indicated by the black arrow). (**c**) Particle size of pCASHGVP2-LNP. (**d**) Zeta potential of pCASHGVP2-LNP.

3.2. Preparation and Characterization Results of pCASHGVP2-LNP

The pCASHGVP2-LNPs formed an opalescent suspension. Dynamic light scattering analysis revealed a uniform, monodisperse size distribution with an average hydrodynamic diameter of about 74.7 nm and a polydispersity index (PDI) of 0.1 (Figure 2c), indicating high sample homogeneity. Characterization revealed that the pCASHGVP2-LNPs had a zeta potential of +1.63 mV (Figure 2d) and a high encapsulation efficiency of 87.7%.

3.3. pCASHGVP2-LNP-Induced Immune Responses in Chickens

In the single immunization experiment, both the 10 μg and 20 μg doses of pCASHGVP2-LNP elicited IBDV neutralizing antibodies in chickens. At 13 dpi, the positive rates of neutralizing antibodies (>3 log2) in chickens immunized with 10 μg pCASHGVP2 were 60% (3/5), with a mean titer of 6 ± 0.58 log2; whereas pCASHGVP2 (20 μg) induced 80% (4/5) positive of neutralizing antibodies (>3 log2), with a titer of 6.4 ± 1.73 log2 (Figure 3a).

In the double immunization experiment, at 13 days post-booster-immunization, the 10 μg group induced 80% (4/5) positive neutralizing antibodies (>3 log2), with a titer of 5.25 ± 0.5 log2; whereas the positive rates of neutralizing antibodies (>3 log2) in the 20 μg group were 100% (5/5) with a mean titer of 6.8 ± 0.4 log2 (Figure 3b).

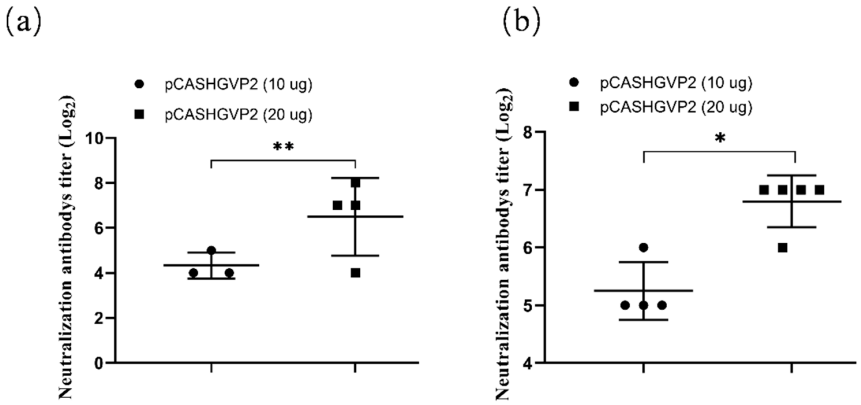


Figure 3. Detection of neutralizing antibody against IBDV induced by immunization with pCASHGVP2-LNP. (**a**) Single immunization with two immune dose (10 μg or 20 μg). (**b**) Double immunization with two immune dose (10 μg or 20 μg). Only serum samples with neutralizing antibody titers above 3 log2 in each group were counted. Experimental data are presented as mean ± standard deviation. * $p < 0.05$; ** $p < 0.01$.

3.4. Protective Efficacy of Single Immunization Against nVarIBDV

3.4.1. Histopathological Lesions of the Bursa

Necropsy revealed severe pathological changes in the bursa of the CC group compared with the negative controls group. These changes were characterized by grayish-yellow discoloration and serous inflammatory exudate (Figure 4a), lymphoid follicle atrophy with lymphocyte depletion, extensive necrosis presenting as vacuole-like structures and cellular debris, interstitial edema, and heterogeneous granulocyte infiltration (Figure 4b). Necropsy data showed that 20% (1/5) of the bursa in the 10 μg immunization group is normal, and 60% (3/5) of the bursa in the 20 μg immunization group were normal, whereas severe lesions were observed in 100% (5/5) of the bursae in the CC group (Figure 4a,b, Table 1). The incidence of normal bursae in immunized chickens corresponded to the protection rate observed in each group.

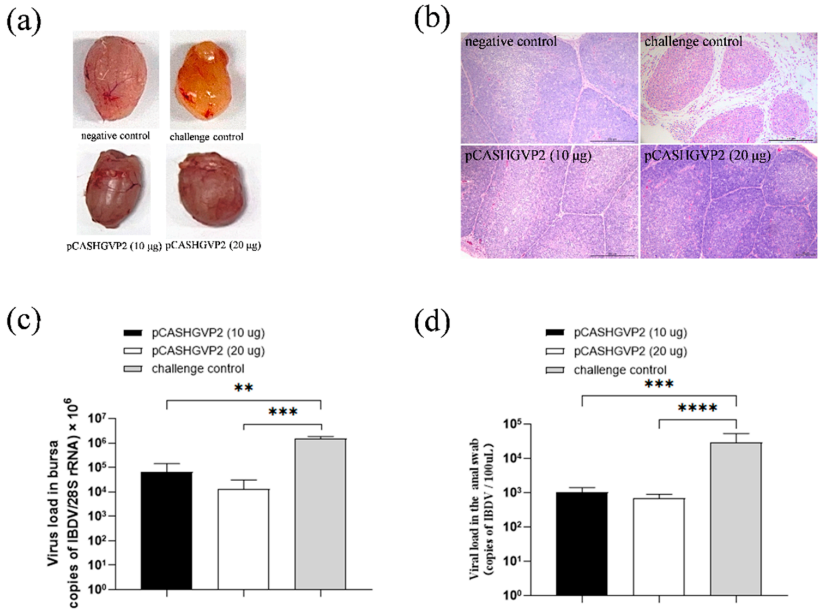


Figure 4. Protective efficacy of single immunization with pCASHGVP2 (10 μg or 20 μg) against SHG19 challenge in chickens. (**a**) Observation of bursa lesions. (**b**) Histopathological changes of bursa. (**c**) Viral loads of IBDV in bursa of immunization groups on 5 days post challenge (dpc). (**d**) Viral loads of IBDV in cloacal swab of immunization groups on 5 dpc. Experimental data are presented as mean ± standard deviation. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 1. Protection effect against nVarIBDV challenge in single immunization and double immunization experiments.

Groups	Protection Rate *	
	Single Immunization	Double Immunization
pCASHGVP2 (10 μg)	20% (1/5)	80% (4/5)
pCASHGVP2 (20 μg)	60% (3/5)	80% (4/5)
Challenge group (CC)	0% (0/5)	20% (1/5)
Negative group (NC)	100% (5/5)	100% (5/5)

* The percentage of chickens with normal appearance of bursa in each group.

3.4.2. Viral Load Results

RT-qPCR analysis revealed significantly lower viral loads in the bursae of both immunized groups (10 and 20 μg) compared with the CC group ($p < 0.01$); the 20 μg group was more effective than the 10 μg group in reducing the viral load (Figure 4c). Viral loads were also quantified in cloacal swabs collected from IBDV-challenged chickens. Viral shedding was significantly lower in both vaccinated groups (10 and 20 μg) than in the CC group ($p < 0.01$) (Figure 4d).

3.5. Protective Efficacy of Double Immunization Against nVarIBDV

3.5.1. Histopathological Lesions of the Bursa

After the challenge, 80% (4/5) of bursae remained normal in the 10 μg pCASHGVP2 group and 20 μg pCASHGVP2 group, whereas the CC group showed severe lesions in 80% (4/5) of bursae (Figure 5a,b; Table 1).

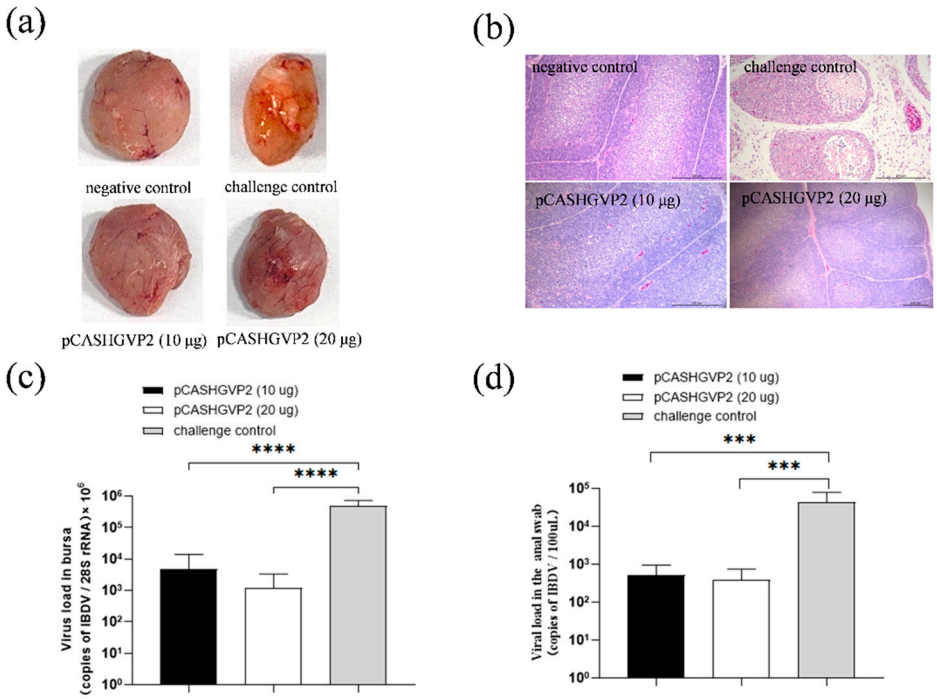


Figure 5. Protective efficacy of double immunizations with pCASHGVP2 (10 μg or 20 μg) against SHG19 challenge in chickens. (**a**) Observation of bursa lesions. (**b**) Histopathological changes of bursa. (**c**) Viral loads of IBDV in bursa of immunization groups on 5 days post challenge (dpc). (**d**) Viral loads of IBDV in cloacal swab of immunization groups on 5 dpc. Experimental data are presented as mean ± standard deviation. *** $p < 0.001$; **** $p < 0.0001$.

3.5.2. Viral Load Results

RT-qPCR results showed that viral loads in bursae of both immunized groups (10 μg and 20 μg) were significantly lower than those in the CC group ($p < 0.01$) (Figure 5c); the 20 μg group demonstrated greater efficacy in reducing viral load compared with the 10 μg group. Additionally, RT-qPCR analysis of cloacal swabs showed that viral shedding levels in both immunized groups (10 μg and 20 μg) were significantly lower than those in the CC group ($p < 0.01$) (Figure 5d).

4. Discussion

nVarIBDV infection in chickens is primarily characterized by severe bursal atrophy and irreversible immunosuppression [25]. The primary reason for the widespread occurrence of nVarIBDV in immunized chicken flocks is its ability to evade immunity conferred by existing vvIBDV vaccines and corresponding maternal antibodies [26,27]. The molecular mechanism underlying nVarIBDV immune escape is primarily attributed to its significant antigenic differences from vvIBDV vaccine strains [15]. Therefore, it is imperative to develop new vaccines with matched antigenicity to effectively control nVarIBDV.

DNA vaccines encode only specific pathogen antigens, thereby eliciting protective immunity against IBDV while avoiding the risks of virulence reversion, mutation-driven immune escape, and environmental contamination associated with traditional live vaccines [28]. However, a key limitation is that IBDV DNA plasmids typically require high-dose, multiple-injection regimens, which hinders their translation into commercially feasible vaccines [29]. In this study, we aimed to overcome the limitations of conventional DNA vaccination by employing an LNP delivery system for the pCASHGVP2 vaccine, and assessing its potential to induce robust protection against nVarIBDV challenge in chickens.

First, the major protective antigen gene VP2 of a representative nVarIBDV representative strain SHG19 was inserted under the CMV enhancer and chicken β-actin promoter in the eukaryotic expression plasmid pCAGGS, which has been reported to confer more efficient protein expression than other conventional expression vectors. To confirm VP2 expression, the constructed DNA vaccine pCASHGVP2 was transfected into DF-1 cells and analyzed by IFA and Western blotting using a VP2-specific MAb. Strong fluorescence signals were observed in IFA and a band of approximately 45 kDa was detected by Western blotting, confirming the successful expression of VP2 from the DNA vaccine pCASHGVP2. Furthermore, pCASHGVP2 was encapsulated in LNPs to generate pCASHGVP2-LNP. LNPs have proven to be highly efficient delivery vehicles for nucleic-acid vaccines, as underscored by the worldwide impact of COVID-19 mRNA-LNP vaccines [30,31]. In addition to mRNA, this platform readily accommodates plasmid DNA, offering a versatile tool for gene therapy and genetic immunization. LNP-formulated DNA vaccines that encode antigens from a broad spectrum of viral and bacterial pathogens have been shown to elicit protective immunity across diverse pre-clinical species, including mice, rabbits, hamsters, pigs, transgenic cattle, and non-human primates [32–34]. Owing to their rapid self-assembly, LNPs stably encapsulate plasmid DNA within biocompatible and storage-stable nanoparticles, thereby shielding the payload from nuclease degradation, markedly enhancing DNA integrity, and ensuring robust in vivo gene delivery [35]. LNPs are internalized by cells via endocytosis, facilitating the plasmid DNA delivery and subsequent transgene expression. Collectively, accumulating evidence demonstrates the versatility of the LNP-DNA platform in eliciting protective immunity across diverse species [36].

Next, we preliminarily evaluated the DNA vaccine using an nVarIBDV challenge model based on SPF chickens and compared the immune effects of two immunization doses (10 or 20 μg) and two immunization regimens (single or double immunization). Humoral immunity is critical for protection against IBDV and serum neutralizing antibodies

are key indicators for assessing the level of this immune response. For single immunization, pCASHGVP2 (10 μg) induced 60% (3/5) positive neutralizing antibodies, while the neutralizing antibody positive rate of pCASHGVP2 (20 μg) was 80% (4/5). Furthermore, facing the challenge with lethal doses of nVarIBDV, pCASHGVP2 (10 μg) provided only 20% (1/5) pathogenic protection. Comparatively, pCASHGVP2 (20 μg) demonstrated greater immune protection, providing 60% (3/5) pathogenic protection. In the double immunization, pCASHGVP2 (10 μg) induced an 80% (4/5) neutralizing antibody positive rate and provided 80% (4/5) protection against nVarIBDV challenge, while pCASHGVP2 (20 μg) induced 100% (5/5) neutralizing antibody positivity and provided 80% (4/5) protection against nVarIBDV. Protected chickens showed no clinical symptoms or bursa damage, and viral loads in the bursa and cloacal swabs were significantly reduced. The challenge dose of 10 BID_{50} for the nVarIBDV SHG19 strain was determined based on extensive experimental data generated in our laboratory. This dose induces 100% morbidity in infected chickens at approximately 28 days of age, as demonstrated by the results from the present study. Therefore, this dose was appropriate for evaluating candidate vaccines.

The immune efficacy of vaccines is determined not only by the antigen itself, but also by the delivery system, which plays a key role in shaping vaccine-induced immune responses [37,38]. In a previous study on IBDV DNA vaccines, even with doses as high as 100 μg per chicken, the immunogenic potential of the antigen was not fully realized when naked plasmid DNA was administered directly to chickens [29]. In the present study, the DNA vaccine utilized LNP encapsulation and delivery, which significantly reduced the required dosage and improved immune efficiency. A regimen of two immunizations with 10 μg per chicken provided significant protection. Additionally, adjuvants are crucial for enhancing the immune responses to vaccines. Adjuvants enhance the immunogenicity of antigens and effectively stimulate stronger and more durable antibodies and cellular immune responses. Second, adjuvants help reduce the amount of antigen required, lower vaccine production costs, and broaden the protective efficacy of vaccines [39–41]. Based on these considerations, future studies will focus on adjuvant screening to further reduce the immunization dose, minimize the number of immunizations required, and enhance immune efficacy.

5. Conclusions

This study reports the development of an LNP-encapsulating VP2 DNA vaccine (pCASHGVP2-LNP) against nVarIBDV. With double immunization, this DNA vaccine can induce specific neutralizing antibodies, protecting immunized chickens from diseases induced by nVarIBDV, highlighting its potential for clinical application in the prevention of nVarIBDV.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vaccines14020113/s1, Table S1. Primers and probes used in this study. Figure S1. Gene sequence of IBDV SHG19 VP2. (a) Nucleotide sequence. (b) Amino acid sequence.

Author Contributions: X.Q. and Y.G. supervised the project; X.Q., Y.Z. (Yulong Zhang) and Y.G. designed the experiments; Y.Z. (Yulong Zhang), H.Y., Z.W., G.W., R.L., D.L., E.K., X.L. and T.X. performed the experiments; Y.G., S.W., Y.C., Y.L., H.C., Y.Z. (Yanping Zhang) and Y.D. provided resources; X.Q. and Y.Z. (Yulong Zhang) analyzed the data and wrote the manuscript; X.Q. and Y.G. provided funding. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (Approval No. 250116-01-GR, 16 January 2025).

Data Availability Statement: Data can be requested by writing to the author.

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