

Recombinant infectious bronchitis virus (IBV) H120 vaccine strain expressing the hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) protects chickens against IBV and NDV challenge

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Abstract Infectious bronchitis (IB) and Newcastle disease (ND) are common viral diseases of chickens, which are caused by infectious bronchitis virus (IBV) and Newcastle disease virus (NDV), respectively. Vaccination with live attenuated strains of IBV-H120 and NDV-LaSota are important for the control of IB and ND. However, conventional live attenuated vaccines are expensive and result in the inability to differentiate between infected and vaccinated chickens. Therefore, there is an urgent need to develop new efficacious vaccines. In this study, using a previously established reverse genetics system, we generated a recombinant IBV virus based on the IBV H120 vaccine strain expressing the haemagglutinin-neuraminidase (HN) protein of NDV. The recombinant virus, R-H120-HN/5a, exhibited growth dynamics, pathogenicity and viral titers that were similar to those of the parental IBV H120, but it had acquired hemagglutination activity from NDV. Vaccination of SPF chickens with the R-H120-HN/5a virus induced a humoral response at a level comparable to that of the LaSota/H120 commercial bivalent vaccine and provided significant protection against challenge with virulent IBV and NDV. In summary, the results of this study indicate that the IBV H120 strain could serve as an effective tool for designing vaccines against IB and other infectious diseases, and the generation of IBV R-H120-HN/5a provides a solid foundation for the

development of an effective bivalent vaccine against IBV and NDV.

Introduction

Infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) are important disease agents causing respiratory diseases in chickens, resulting in severe economic losses in the poultry industry [1]. Live attenuated vaccines and inactivated vaccines are important for the control of infectious bronchitis (IB) and Newcastle disease (ND) [2, 3]. Several strains have been developed as vaccines strains, including IBV vaccine strains H120, M41, and Beaudette [4, 5] and NDV vaccine strains LaSota, Clone30, and B1 [6, 7]. The IBV H120 strain, an attenuated live vaccine strain of the Massachusetts (Mass) type, is widely available and reliable vaccine strain that is used as a primary vaccine in broilers, breeders and future layers [4, 8], and the NDV LaSota strain, a naturally occurring low-virulence NDV strain, has been used routinely as a live vaccine throughout the world [9]. The current live attenuated vaccines, although effective, are expensive and result in the inability to differentiate infected from vaccinated chickens. Therefore, we are now at a stage where it is crucial to develop innovative live bivalent vaccines.

IBV, a coronavirus of chickens, is an enveloped virus that contains a single-stranded, positive-sense RNA genome of approximately 27.6 kb in size, with a 5' cap and a 3' polyA tail [10, 11]. As in other coronaviruses, the 5' approximately two thirds of the IBV genome encode two polyproteins, 1a and 1b, which are further processed to form the proteins necessary for RNA replication [12]. The remaining 3' approximately one-third of the genome

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encodes four major structural proteins: the spike (S) glycoprotein, the small envelope (E) protein, the membrane (M) glycoprotein, and the nucleocapsid (N) protein [13]. In addition, there are also two accessory genes interspersed among the structural protein genes: gene 3, which encodes the accessory proteins 3a and 3b, and gene 5, which encodes the accessory proteins 5a and 5b [2, 14].

The large size of the IBV genome and its potential for expressing heterologous genes has made this virus attractive for use in the development of viral-vector vaccines [15]. Previous research by us and others using reverse genetics systems has allowed the production of full-length cDNAs from various IBV vaccine strains, including H120 and Beaudette [5, 16]. Furthermore, it has been shown that heterologous genes can be incorporated into and expressed from a range of IBV (Beaudette strain) genome locations [15]. All of these advances in the field now mean we can explore the uses of IBV strains in vaccine vector development.

NDV is a member of the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae* [17]. The genome of NDV encodes at least six proteins, one of which, the haemagglutinin-neuraminidase (HN) protein, is the viral surface glycoprotein and neutralising antigen, and it is responsible for attachment of the virus to host cell [17, 18]. Therefore, NDV HN was selected as a putative protective antigen in this study. We engineered the genome of the IBV H120 strain to encode the HN protein of the NDV LaSota strain, using our previously established reverse genetics system [16]. The recombinant virus was then evaluated for growth dynamics, pathogenicity, virus titers, levels of induced humoral responses, and protection against challenge with IBV and NDV in SPF chickens.

Materials and methods

Cells, viruses and nucleic acid isolation

BHK-21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with a 10 % fetal bovine serum (FBS) in the presence of penicillin (100 units/ml) and streptomycin (100 g/ml) at 37 °C in a 5 % CO₂ environment. The attenuated vaccine strains IBV H120 and NDV LaSota and the standard virulent strains IBV M41 and NDV F48E9 were obtained from the Chinese Institute of Veterinary Drug Control. All of the strains were propagated in the allantoic cavities of 10-day-old SPF fertilized chicken eggs (Merial-Vital Experimental Animal Technology Co. Ltd. Beijing), and the allantoic fluid was harvested 36 hours after inoculation. Viral RNA was isolated from the allantoic fluid of

infected chicken embryos using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The rescued virus, the IBV R-H120 strain, was generated as described previously [16]. Plasmid DNA containing 13 fragments (F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, F12, and F13) constituting the IBV R-H120 full-length cDNA [16] was extracted from transfected *E. coli* strain DH5 α , using a GenEluteTM Plasmid Mini-prep Kit (Sigma-Aldrich, USA).

Construction of the Δ F12 fragment for replacing the 5a gene of IBV-H120 with the HN gene of NDV-LaSota

The HN gene of NDV was amplified by PCR from the cDNA of LaSota using the primers HN F and HN R (Table 1). Because the F12 fragment from our previously established reverse genetics system of IBV H120 contained the 5a gene, the F12 fragment was used to carry the HN gene for replacing the 5a gene. The upstream region of F12, named F12-1, was amplified by PCR using the F12 amplicon as a template together with primers F12 F and F12-1 R, while the downstream region of F12, named F12-2, was amplified from the F12 amplicon using primers F12-2 F and F12 R (Table 1). Next, the three amplicons (HN, F12-1, and F12-2) were cloned into the vector pMD-19 (TAKARA, Japan) to generate pT-HN, pT-F12-1, and pT-F12-2, respectively. Two to four independent clones of each amplicon were sequenced by the Sangon Biological Engineering Technology & Services Co., Ltd. Each amplicon that contained the consensus sequence was released from the cloning vector by restriction enzyme digestion (HN with BsmBI, F12-1 and F12-2 with BsaI) and then purified using a QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Using T4 DNA ligase, the cDNA amplicons were then ligated in the correct order (F12-1+HN+F12-2) in an equimolar ratio to the Δ F12 fragment to replace the 5a gene of IBV-H120 with the HN gene of NDV-LaSota.

Construction of a recombinant H120 cDNA containing an HN gene

To generate a recombinant IBV H120 cDNA clone with the NDV HN gene replacing the 5a gene of IBV, 13 cDNA amplicons (F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, Δ F12, and F13) were ligated in the correct order using T4 DNA ligase as described previously [16]. The final ligation product, named IBV R-H120-HN/5a, was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol, and detected by electrophoresis on a 0.4 % agarose gel.

Table 1 Primers for amplifying fragments of F12-1, HN, and F12-2

Product	Length (bp)	Primer name	Sequence (5'-3') ^a
F12-1	1325	F12 F	<u>GGTCTC</u> CAGAATGGAAGTTTCTAACAG
		F12-1 R	<u>GGTCTC</u> TTCTAACGTCTGTATTGTTAAGC
HN	1838	HN F	<u>CGTCTCA</u> ATACGGGTAGAACACTAGTCCGCC ACCATGGACCGCGCCGTAGCCA
		HN R	<u>CGTCTC</u> CTAGCCAGACC TGGCTTCTCTA
F12-2	422	F12-2 F	<u>GGTCTC</u> GGCTAG ATAATAGTAAAGATAATCCT
		F12 R	CTTG <u>AGGTCTC</u> CAGTACCCATGTTG

^a Underlined nucleotides indicate inserted restriction sites for BsaI (GGTCTC) or BsmBI (CGTCTC)

Generating the rescued virus R-H120-HN/5a

Recombinant viruses were rescued by transfection of BHK-21 cells with the full-length cDNA, using an mMACHINE mMACHINE[®] T7 kit (Ambion, Austin, TX) according to the manufacturer's instructions as described previously [16]. The transfected cells were collected, and the virus was subjected to five passages in 10-day-old SPF embryonated chicken eggs, after which the allantoic fluid was harvested in preparation for the next round of experiments. The complete genomic sequence of the rescued virus was determined by direct sequencing of the RT-PCR products, which were amplified from the viral genomic RNA as described previously [16].

The biological characteristics of the rescued virus R-H120-HN/5a

HA assay

To test the hemagglutination activity of R-H120-HN/5a, the virus was serially diluted twofold in the range of 1:2-1:2048 and titrated with 0.75 % chicken erythrocytes. Parental H120, R-H120, parental LaSota, SPF allantoic fluid, and phosphate-buffered saline (PBS) were used as controls.

Pathogenicity in embryonated chicken eggs (ECEs) and in 1-day-old SPF chickens

To determine the pathogenicity in ECEs and the 50 % egg infection dose (EID₅₀) of R-H120-HN/5a, serial tenfold dilutions (10⁻¹ to 10⁻⁹) of the recombinant virus were inoculated into 10-day-old SPF ECEs. For each dilution, 0.2 ml of virus suspension was injected into each egg. Four eggs were used for each dilution. The parental strain H120 and PBS were used as positive and negative controls, respectively. The EID₅₀ was calculated by the method of Reed and Muench [19]. Forty 1-day-old SPF chickens were housed in biosafety isolator cages. The birds in this experimental group were then inoculated via the eye drop

method with IBV strain R-H120-HN/5a, H120, or R-H120 at equivalent doses of 10³ EID₅₀/0.2 ml, while the control group was inoculated with PBS only. To determine the pathologic characteristics of the strains, we observed the birds twice daily for any clinical signs for 14 days.

Growth of recombinant viruses in chicken embryos

To analyze the replication kinetics of recombinant viruses in chicken embryos, 0.2 mL of virus suspension containing 10³ EID₅₀ of R-H120-HN/5a, H120, or R-H120 was inoculated into the allantoic cavities of 10-day-old SPF chicken embryos. The allantoic fluids of six eggs were harvested at 12-hour intervals, and the virus titers were determined by titration in ECEs.

Immunization and challenge experiments

Immunization of chickens

One hundred 7-day-old SPF chickens were randomly divided into five groups of 20 birds. Chickens inoculated with PBS (group 1) were used as a control group. Each bird was inoculated via the oculo-nasal route with H120 (10³ EID₅₀) (group 2), LaSota (10³ EID₅₀) (group 3), R-H120-HN/5a (10³ EID₅₀) (group 4) or LaSota/H120 commercial bivalent vaccine (10³ EID₅₀) (group 5).

Determination of the specific anti-IBV IgG and anti-NDV hemagglutination inhibition (HI) titer

Serum samples were taken every week from the five groups of chickens on days 0, 7, 14, 21, and 28 postvaccination for antibody titer assay. The total serum immunoglobulin G (IgG) specific for IBV was measured by indirect enzyme-linked immunosorbent assay (IDEXX, Westbrook, MA, USA). Anti-NDV antibodies were detected using an HI assay, as described by the World Organization for Animal Health (OIE) [20]. The data on antibody titers were analyzed using the Statistics Package for Social Science (SPSS).

Protective efficacy of R-H120-HN/5a against IBV and NDV challenge

To evaluate the protective efficacy of R-H120-HN/5a, the IBV M41 strain, a pathogenic strain of the Mass type, as well as the highly virulent NDV strain F48E9 (IX genotype), were used for challenge tests. Chickens were inoculated with 10^3 EID₅₀ of the M41 strain in 0.1 ml or 10^3 EID₅₀ of the F48E9 strain in 0.1 mL via the oculo-nasal route on the 14th day post-vaccination. The chickens were then observed daily for 14 days for any signs of disease and mortality.

Results

Generation of a recombinant virus R-H120-HN/5a

The 13 cDNA fragments (F1-F11, ΔF12, and F13) were prepared by digestion of the corresponding plasmids with restriction enzymes and recovered from an 0.8 % agarose gel. The full-length cDNA was assembled by ligation of the fragments in the correct order as described previously [16] (Fig. 1). The cDNA was then used as a template for *in vitro* transcription. After transfection of BHK-21 cells and subsequent propagation of virus in SPF chicken embryonated eggs, the H120-strain-based recombinant virus containing the HN gene was successfully generated. Nucleotide sequence analysis of the RT-PCR products of the viral genome confirmed the correct sequence of the rescued virus, which was designated a R-H120-HN/5a.

The biological characteristics of the rescued virus R-H120-HN/5a

The HA activity, pathogenicity, and growth characteristics of the rescued virus were tested to determine the biological characteristics of R-H120-HN/5a. The hemagglutination titers of both R-H120-HN/5a virus and LaSota were $10 \log_2/0.2$ mL, whereas R-H120 and parental H120 exhibited no characteristics of hemagglutination. The successfully rescued R-H120-HN/5a produced typical embryo lesions of curling, stunting and dwarfing and its EID₅₀ titer (EID₅₀ = $10^{-6.70}/0.2$ mL) was comparable to that of R-H120 (EID₅₀ = $10^{-6.84}/0.2$ mL) and H120 (EID₅₀ = $10^{-6.73}/0.2$ mL). The growth pattern of R-H120-HN/5a in chicken embryos was similar to that of R-H120 and H120 (Fig. 2). All of the one-day-old SPF chickens that received R-H120-HN/5a inoculations remained healthy throughout the 14 days of observation, and they did not display any clinical symptoms. These data indicated that the rescued virus R-H120-HN/5a retained the same characteristics of pathogenicity, growth, and virus titer as the parental H120, but it possessed hemagglutination activity obtained from the NDV HN gene.

Anti-IBV-specific IgG antibody and anti-NDV HI titers in chickens after immunization with rescued virus

To evaluate the immunogenicity of the rescued virus, R-H120-HN/5a, H120, LaSota, the LaSota/H120 commercial bivalent vaccine, and PBS, were each injected into

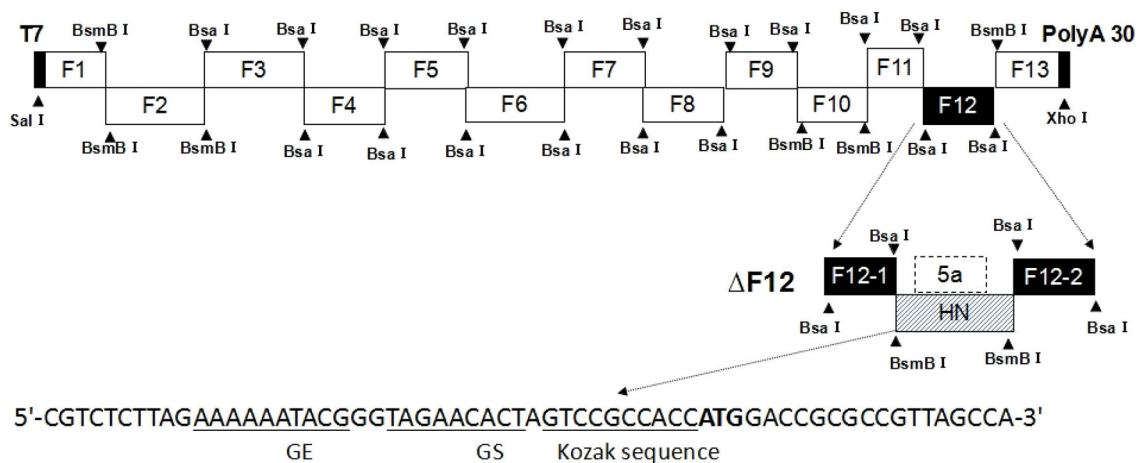


Fig. 1 Scheme of full-length cDNA of IBV R-H120-HN/5a construction. The underlined sequences introduced before ATG of the HN gene were gene end (GE), gene start (GS), and **Kozak** sequences. The HN gene of NDV was amplified by PCR from cDNA from the LaSota strain. The F12 fragment was used to carry the HN gene for replacing the 5a gene. The upstream region of F12, named F12-1, was amplified by PCR using the F12 amplicon, and the downstream region

of the F12, named F12-2, was amplified from the F12 amplicon as well. Then, the three amplicons (HN, F12-1, and F12-2) generated ΔF12 (F12-1+HN+F12-2), in which the 5a gene of IBV-H120 was replaced with the HN gene of NDV-Lasota. The thirteen DNA amplicons (F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, ΔF12, and F13) were ligated in order, and the final ligation DNA product was named R-H120-HN/5a

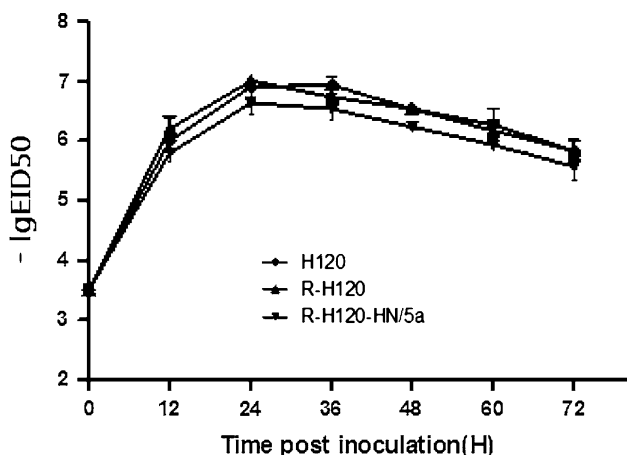


Fig. 2 Growth curves of recombinant viruses in chicken embryos. The growth pattern of R-H120-HN/5a in chicken embryos was similar to that of R-H120 and H120

a group of chickens, and the induced serum antibody was titrated. Dynamic changes in the anti-IBV-specific IgG antibody titer and the anti-NDV HI titer were observed after inoculation with R-H120-HN/5a, H120, and LaSota/H120. The dynamic changes in the mean anti-IBV-specific ELISA OD₆₅₀ titer of 10 birds showed no specific differences among the R-H120-HN/5a, H120 and LaSota/H120 groups on the same day after vaccination, but a significant difference was detected between the three vaccinated groups and the PBS group ($p < 0.05$) (Fig. 3). No statistical difference in anti-NDV-specific HI titer was found in the R-H120-HN/5a, LaSota, and LaSota/H120 groups, but a significant difference was observed between the R-H120-HN/5a group and the H120 and PBS groups ($p < 0.05$) (Fig. 4).

Protection against challenge with IBV and NDV

To evaluate the protective efficacy of recombinant virus R-H120-HN/5a against IBV and NDV, groups of SPF chickens were inoculated by the **intranasal** and **intraocular** routes with PBS, H120, LaSota, R-H120-HN/5a or the LaSota/H120 bivalent live vaccine and challenged with a lethal dose of the IBV M41 virus or NDV F48E9. As shown in Table 2, in subgroup A, 80 % of the chickens that had been immunized with R-H120-HN/5a, IBV H120 or LaSota/H120 combined live vaccine were protected against IBV challenge and showed no signs of disease. In contrast to this, every bird that was inoculated with PBS or LaSota displayed signs of disease, with a 50 % and a 40 % mortality rate, respectively. In subgroup B, 80 % of the chickens vaccinated with R-H120-HN/5a were protected, but the protection rate was lower than those induced by LaSota (90 %) and LaSota/H120 (90 %). These results suggested that the protection provided by R-H120-HN/5a

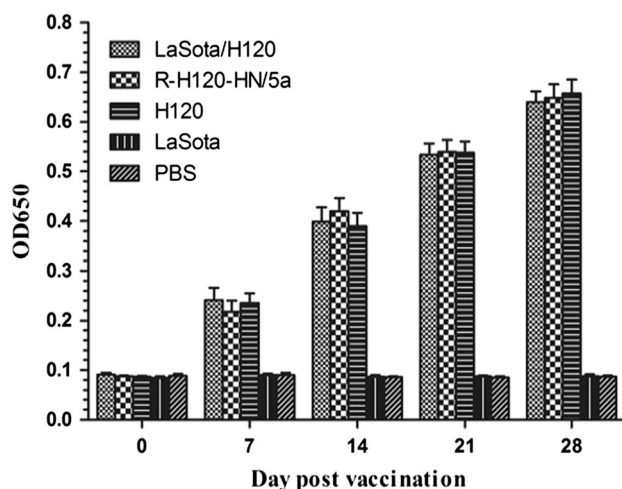


Fig. 3 Specific anti-IBV IgG antibody titers of different vaccination groups measured by ELISA. Serum samples were collected weekly from all chickens. Antibody titer data were analyzed using the Statistics Package for Social Science (SPSS). The mean antibody titers of each immunized group on days 0, 7, 14, 21, 28 after vaccination are shown

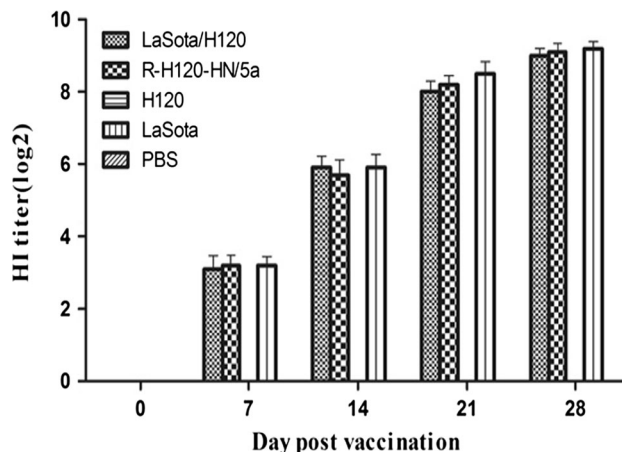


Fig. 4 Specific anti-NDV HI antibody titers of different vaccination groups measured by HI. Serum samples were collected weekly from all chickens. Antibody titer data were analyzed using Statistics Package for Social Science (SPSS). Mean antibody titers of each immunized group on days 0, 7, 14, 21, 28 after vaccination are shown

against challenge with virulent IBV and NDV was similar to that of the LaSota/H120 commercial vaccine.

Discussion

The Mass type of IBV was first isolated in the USA and other regions of the world in the 1950s [21]. Since then, many other serotypes and genotypes of IBV have emerged, such as SAIBK-like, QX-like, and TW/97-4-like [22]. However, the Mass type has been continually isolated from

Table 2 Protection rate in each group after virulent IBV or NDV challenge

Group number	Group	Subgroup number	Virus for challenge	No. of chickens with clinical signs/total	No. of survivors/total	Protection rate (%)
1	PBS	A1	IBV M41	10/10	5/10	0
2	H120	A2	IBV M41	2/10	10/10	80
3	LaSota	A3	IBV M41	10/10	6/10	0
4	R-H120-HN/5a	A4	IBV M41	2/10	10/10	80
5	LaSota/H120	A5	IBV M41	2/10	10/10	80
1	PBS	B1	NDV F48E9	10/10	2/10	0
2	H120	B2	NDV F48E9	10/10	1/10	0
3	LaSota	B3	NDV F48E9	1/10	10/10	90
4	R-H120-HN/5a	B4	NDV F48E9	2/10	9/10	80
5	LaSota/H120	B5	NDV F48E9	1/10	9/10	90

chicken flocks with respiratory clinical signs up to the present day [23–25]. In this study, R-H120-HN/5a induced complete protection against challenges with IBV M41 in SPF chickens, as did H20 and LaSota/H120 in this study and in a previous report [1], indicating that the recombinant vaccine is protective against the Mass type of IBV and has practical application value in chicken flocks. Although H120 attenuated vaccine has been shown to be able to protect against a wide range of virulent IBV strains [25], it is not yet known whether the recombinant vaccine is protective against other IBV strains. Cross-protection experiments are therefore still needed.

NDVs are separated into two classes. Class I includes two genotypes, and class II includes 18 genotypes [26]. The LaSota strain, belonging to genotype II of class II, has been commonly used as a live-virus NDV vaccine strain since the 1950s [27], but several studies have reported that using LaSota vaccine to immunize chickens could provide cross-protection against other genotypes of NDV, such as VIb, VIg, VIId, XVII and IX [28–30]. In the present study, the recombinant vaccine expressing LaSota HN protein displayed 80 % protective efficacy against challenge with NDV F48E9 (IX genotype), in contrast to 90 % protective efficacy in this study and 100 % protective efficacy reported by Liu et al. induced by LaSota live vaccine [28]. Recently, Kumar et al. reported that a recombinant avian paramyxovirus type 3 vector expressing both the HN and F proteins could be used to protect against virulent NDV strain, but birds immunized with the vector expressing HN alone were only partially protected [31]. Furthermore, it has been reported that not only the HN protein but also the fusion glycoprotein (F), which mediates the fusion of the viral envelope with the cell membrane, are the neutralizing and protective antigens of NDV [18, 32]. Therefore, the development of an R-H120 recombinant vaccine that expresses both the HN and F genes needs to be further

investigated in order to investigate its protective efficacy against virulent NDV.

Immunization of SPF chickens is commonly used to evaluate the protective efficacy and immune response to IB and ND vaccines [29, 30, 33, 34]. It seems probable that the ideal protective effect will be achieved when using SPF chickens. However, poor protection rates will be possibly be obtained when vaccines are applied to commercial chickens in chicken flocks due to interference from maternal antibodies, asymptomatic infections with these viruses, or immune tolerance of the chickens [35, 36]. Thus, the protective efficacy of the recombinant vaccine reported here needs to be evaluated in commercial chickens. Although the efficacy of the recombinant vaccine in this study should be further improved and evaluated, it has two obvious advantages when compared with conventional IBV and NDV live vaccines. First, as the recombinant virus can express both the IBV and NDV antigens, it will reduce the number of embryonated chicken eggs by a half for virus cultures during vaccine production and thus cut down on production costs. Second, in veterinary clinical practice, it is difficult to differentiate IBV-infected from IBV-vaccinated chickens by RT-PCR due to the usage of conventional live vaccines. However, the 5a IBV gene, which is absent in the recombinant vaccine in this study, can be used as a target gene for RT-PCR detection if this recombinant vaccine is widely used.

Viral vectors are promising tools for making vaccines, and genetically altered vaccine vectors have been developed to improve efficacy and safety, reduce administration dose, and enable large-scale manufacturing [37]. IBV H120 strain has several features that make it attractive as an avian vaccine vector. First, IBV possesses the largest known avian viral RNA genome, at 27.6 kb [12], which makes it possible to incorporate larger heterologous gene sequences. Second, the deletion of nonessential genes such

as 5a, 3a and 3b is not a disadvantage for viral replication [38, 39], and this provides suitable targets for the insertion of heterologous genes. Third, it has been shown that nonessential genes of IBV can be replaced with heterologous genes and that recombinant viruses show the characteristics of stable replication and the expression of heterologous genes. Fourth, IBV H120, an attenuated live vaccine strain used worldwide in chicken flocks, is considered to be a safe and widely available vaccine strain for protecting against IB [4, 8]. Therefore, we chose IBV H120 to carry the NDV HN gene, replacing the 5a gene, which was generally found to have little effect on viral growth and protein expression in this study. These results also indicate that IBV H120 can be engineered to stably express a foreign protein and can be manipulated in the future for use as a vaccine vector. In summary, for the first time, to our knowledge, we have generated and evaluated the potential of the IBV H120 strain as a vaccine vector for the expression of the NDV HN protein.

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Compliance with ethical standards

Conflict of interest We declare that there is no conflict of interest.

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