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Development and characterization of a recombinant infectious bronchitis virus expressing the ectodomain region of *S1* gene of H120 strain

Yan-Quan Wei · Hui-Chen Guo · Hu Dong · Hai-Ming Wang · Jin Xu · De-Hui Sun · Shou-Guo Fang · Xue-Peng Cai · Ding-Xiang Liu · Shi-Qi Sun

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Abstract Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is a highly contagious chicken disease, and can lead to serious economic losses in poultry enterprises. The continual introduction of new IBV serotypes requires alternative strategies for the production of timely and safe vaccines against the emergence of variants. Modification of the IBV genome using reverse genetics is one way to generate recombinant IBVs as the candidates of new IBV vaccines. In this study, the recombinant IBV is developed by replacing the ectodomain region of the *S1* gene of the IBV Beaudette strain with the corresponding fragment from H120 strain, designated as rBeau-H120(S1e). In Vero cells, the virus proliferates as its parental virus and can cause syncytium formation. The peak titer would reach $10^{5.9}$ 50 % (median) tissue culture infective dose/mL at 24 h post-infection. After inoculation of chickens with the recombinant virus, it demonstrated that rBeau-H120(S1e) remained nonpathogenic and was restricted in its replication in vivo. Protection studies showed that vaccination with rBeau-H120 (S1e) at 7-day after hatch provided 80 % rate of immune protection against challenge with 10^3 50 % embryos infection dose of the virulent IBV M41 strain. These results indicate that rBeau-H120 (S1e) has the potential to be an alternative vaccine against IBV based on excellent propagation property and immunogenicity. This finding might help in providing further information that replacement of the ectodomain fragment of the IBV Beaudette *S1* gene with that from a present field strain is promising for IBV vaccine development.

Keywords Infectious bronchitis virus · Recombinant virus · H120 · Immunogenicity

Yan-Quan Wei and Hui-Chen Guo contributed equally to this work.

Y.-Q. Wei · H.-C. Guo · H. Dong · H.-M. Wang · J. Xu · D.-H. Sun · X.-P. Cai · D.-X. Liu · S.-Q. Sun (✉)
State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Xujiaping 1, Lanzhou, Gansu 730046, People's Republic of China
e-mail: sunshiqi@caas.cn

D.-X. Liu (✉)
School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore
e-mail: dxliu@ntu.edu.sg

S.-G. Fang
Agricultural School, Yangtze University, 88 Jingmilu, Jingzhou City, Hubei Province 434025, China

Introduction

Infection with infectious bronchitis virus (IBV) reduces the performance of broilers and egg production, making a severe economic impact on domestic commercial poultry industry (De Wit et al. 2010). Traditional prevention against IBV infection usually involves live attenuated or inactivated vaccines (Bijlenga et al. 2004; Tang et al. 2008). However, some disadvantages have been found in both of the two types of vaccines: the live attenuated vaccines, which generally induce long-lasting immunity, can induce both humoral and cellular immune responses, but have a risk of insufficient attenuation and/or genetic instability (Cook et al. 1986; Wareing and Tannock 2001); the inactivated vaccines can elicit high titers of antibody but usually with lower cytotoxic T-lymphocyte

responses (CTL) (Yang et al. 2004). The single dose of inactivated vaccines usually results in less than 50 % protection in chickens (Cavanagh 2003). Therefore, a new generation of vaccines is necessary.

IBV contains an unsegmented, single-stranded, positive-sense RNA genome of approximately 28 kb in length (Sjaak de Wit et al. 2011), which specifies the production of the four structural proteins—spike glycoprotein (S), small membrane protein (E), integral membrane protein (M), and phosphorylated nucleocapsid protein (N) which interacts with the genomic RNA (Bayry et al. 2005). The S glycoprotein (1,162 amino acids in length), that is responsible for binding to the target cell receptor and fusion of the viral and cellular membranes, consists of the amino terminal S1 (535 amino acids, 90 kDa) and carboxyl terminal S2 (627 amino acids, 84 kDa), which are generated by post-translational cleavage (Shil et al. 2011). S1 and S2 associate to form the viral envelope, in which S1 is exposed on the virion surface, anchored by S2 (de Groot et al. 1987). The S1 glycoprotein induces virus neutralization and hemagglutination inhibition antibody and it has also been identified as a major inducer of protective immune responses (Cavanagh et al. 1984, 1986; Koch and Kant 1990; Mockett et al. 1984). Thus, the best candidate gene for inclusion in a recombinant vector to protect against IBV would seem to be the one encoding S1 (Shil et al. 2011). In recent years, *S1* gene has become established as the major gene for recombinant vaccine research against IBV infection (Shi et al. 2011).

Swapping the ectodomain region of the IBV S protein through the use of a reverse genetics system is a precise and effective way of generating genetically defined candidate IBV vaccines (Britton et al. 2012). It has been reported that replacement of the ectodomain of the IBV Beaudette *S* gene with the corresponding region from the pathogenic IBV did not confer virulence to Beau-R but did result in recombinant IBVs, which were able to act as vaccines for the protection of chickens against challenge with the parental virulent viruses from which the substituted ectodomain region of the *S* gene was derived (Armesto et al. 2011; Hodgson et al. 2004).

In this study, the IBV Beaudette strain genome have been modified by exchanging the ectodomain region of the *S1* gene with the corresponding fragment from the IBV H120 strain, the recombinant IBV was referred to as rBeau-H120(S1e) (this recombinant IBV strain had been deposited in China General Microbiological Culture Collection Center (CGMCC); the accession number, 7103). The potential of this recombinant IBV for vaccine development have been evaluated in the following aspects. First, the growth properties would be tested in Vero cells. Second, the virulence would be analyzed in chickens infected with the recombinant IBV. Finally, the immunized chickens would be challenged with the virulent M41strain to see if this recombinant IBV, the first to be made, had the capacity to induce protective immunity.

Materials and methods

Virus, cells, and chickens

The IBV H120 vaccines were purchased from Qingdao Yebio Biotechnology Co., Ltd., China. The pathogenic M41 strain, which was purchased from China Veterinary Culture Collection Center, was used as challenge virus in this study. The 50 % embryos infection dose (EID_{50}) was determined by inoculating serial tenfold dilutions of virus into 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The SPF chicken embryos were purchased from Beijing Laboratory Animal Research Center, China. Vero cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, USA) supplemented with 5 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at pH 7.2 and were kept at 37 °C with 5 % carbon dioxide. All the chickens were derived from an SPF flock of White Leghorn parents and were housed in positive-pressure isolators at the Laboratory Animal and Resources Facility, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS).

Amplification and cloning of the ectodomain of the IBV H120 *S1* gene

Total RNA was extracted from IBV H120 vaccines. Primers, which were used to amplify the ectodomain region of the *S1* gene of IBV H120 strain, were H20421-forward: 5′-GAGGTCTCTGCTGTTTTGTATGACAGTAG-3′; H21711-reverse: 5′-ACGGTCTCCACATTAGTAATAAAACC-3′. *Bsa* I restriction enzyme sites (shown by underline) were added to the 5′-ends of the primers and the fragment cloned into pGEM -T Easy vector (Promega, Madison, USA).

Construction of recombinant virus

The procedure for constructing a full-length cDNA clone of a recombinant IBV was similar to construct a full-length IBV clone of a Vero cell-adapted IBV Beaudette strain (p65) with the slight modification (Fang et al. 2007). The assembly of full-length cDNA clone of the recombinant IBV derived from IBV Beaudette strain and H120 strain is shown in Fig. 1. Briefly, five fragments of IBV Beaudette strain (p65) genome were obtained by reverse transcriptase PCR (RT-PCR) of total RNA extracted from Vero cells infected with a Vero cell-adapted IBV Beaudette strain (p65) as described previously (Fang et al. 2005). The PCR products were purified from agarose gel and cloned into pCR-XL-TOPO vectors (Life Technologies, California, USA).

The six fragments were prepared by digestion of the corresponding constructs with either *Bsm* BI or *Bsa* I and purified. The full-length clone was made by ligation of the

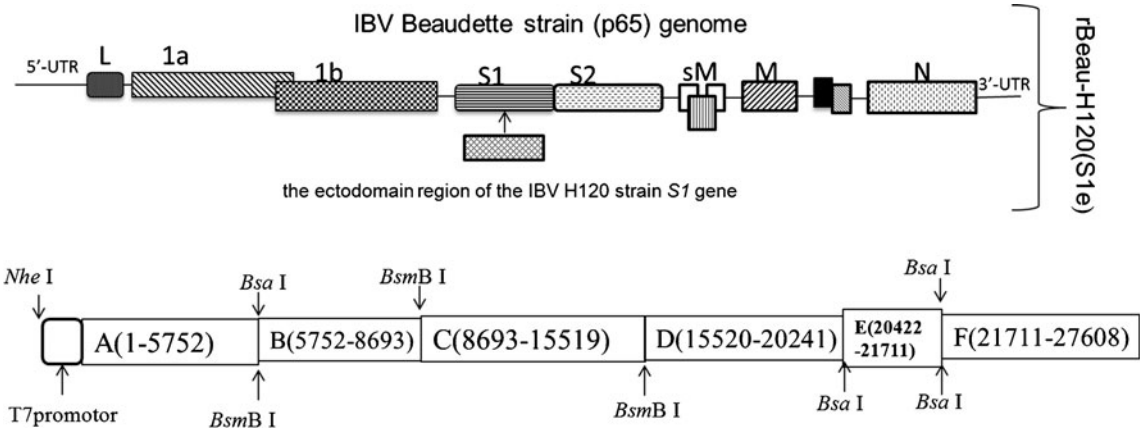

Fig. 1 Diagram of the genome organization of IBV and the swapped gene fragment. The recombinant IBV rBeau-H120(S1e) is developed by replacing the ectodomain region of the *S1* gene of the IBV Beaudette strain with the corresponding fragment from H120 strain. The regions of the six RT-PCR fragments and the T7 promoter at the 5′-end of fragment A are shown

purified fragments and used as the template for in vitro transcription. The full-length transcripts were generated using the mMessage mMachine T7 kit (Ambion, Austin, USA), and were introduced into Vero cells by electroporation. The rescued virus was designated as rBeau-H120(S1e) (the CGMCC accession number, 7103).

Detection of virus titer by $TCID_{50}$

Titrations of the rBeau-H120 (S1e) infectivity were performed in Vero cells after successive 10 passages, and the titers were expressed as the 50 % (median) tissue culture infective dose ($TCID_{50}$). The procedure for titration of viral infectivity is similar to the previous description with the slight modification (Wang et al. 2012). Briefly, confluent Vero cell monolayers were infected with serial 10-fold dilutions of viruses and incubated in medium with DMEM without FBS. At 1–3 days post-infection, cytopathic effect (CPE) was monitored and $TCID_{50}$ of virus was calculated using the Reed–Muench method (Reed and Muench 1938).

Growth curves of recombinant virus in cells

Vero cells were seeded into 12-well dishes, and confluent monolayers were infected with 10-fold diluted virus stock in triplicate for 1 h. The inoculum was removed, and the cell monolayers were washed three times with PBS (pH 7.4). Supernatant and cells were collected at 6 h intervals after infection, the titers of viruses were determined by $TCID_{50}$ assay in Vero cells.

Detection of gene replication of recombinant virus by RT-PCR

Vero cells were seeded into seven dishes (35×10 mm). The cells in each dish were infected with $1\times10^{4.9}$ $TCID_{50}$ rBeau-H120(S1e) after confluent monolayer was formed, then the infected cells were harvested at selected time points (0, 6, 12, 18, 24, 28, 32 h post-infection (hpi)), total RNA was extracted and cDNA was synthesized using PrimeScript™ Reverse Transcriptase (Takara, China). The target gene was amplified by PCR using synthesized cDNA and specific primers pairs. The *S1* gene and the housekeeping β-*actin* gene had been detected. The primers used to amplify the *S1* and β-*actin* genes are S1-forward: 5′-TGAGATTGAAAGCAACGCCAGTTG-3′, S1-reverse: 5′- CTTACCATAACTAACATAAGGGC-3′; β-actin-forward: 5′-CGG-CATCCACGAAACTAC-3′, β-actin-reverse: 5′-ATCTTCATCGTGCTGGGCG-3′.

Immunoblot

Western blots were performed with infected Vero cell lysates. Briefly, ~2×10^5 Vero cells/dish (35×10 mm) were infected with $1\times10^{4.9}$ $TCID_{50}$ rBeau-H120(S1e), the cells were harvested in 150 μL of NET buffer at the time points indicated. Of the cell lysate from each sample, 10 μL was run on a denaturing polyacrylamide gel electrophoresis gel. Proteins were transferred from the gel onto polyvinilidene fluoride (PVDF) membranes and were blocked overnight in TBS-T buffer with 5 % skim milk. PVDF membranes were incubated with the corresponding primary antibodies and second antibodies, respectively, and then incubated with ECL reagent (Pierce, Rockford, USA).

Biosafety of recombinant virus for chicken

In order to test the pathogenicity of rBeau-H120(S1e) and to determine whether the viruses are able to propagate in SPF chickens, a single experiment was carried out to test these two attributes of the recombinant IBV. Two groups of 7-day-old SPF chickens were housed in positive pressure, HEPA-filtered isolation rooms in which each group of chickens was housed

in a separate room. One group ($n=10$) of chickens were inoculated with 2×10^5 $TCID_{50}$ of rBeau-H120(S1e) in infected Vero cells lysates via conjunctival (eye drop) and intranasal routes; the other group ($n=5$) of chickens were inoculated with 0.1 mL lysates of mock-infected Vero cells in the same manner as controls. Chickens were monitored daily for clinical signs to assess of pathogenicity. From days 2–10 after inoculation, oropharyngeal swabs from all chickens of the two groups were collected for virus isolation. The swabs were soaked in PBS with 1,000 U each of penicillin and streptomycin per milliliter, vortexing briefly, then the suspensions were centrifuged, and the supernatants were used for inoculating into 10-day-old SPF embryonated chicken eggs. At 72 h post-inoculation, the allantoic fluid was collected for detecting of rBeau-H120(S1e) by RT-PCR. All animal experiments were carried out in strict accordance with animal ethics guidelines and approved protocols and were approved by the Lanzhou Veterinary Research Institute, CAAS.

Vaccination of chicken with recombinant virus

The 7-day-old SPF chickens were randomly divided into four groups ($n=20$ each). The chickens in one group were inoculated with 150 μL supernatant of cell lysates infected with rBeau-H120(S1e) (1×10^5 $TCID_{50}$) by the nasal–ocular route (group 1). The second group was administered with 150 μL commercial IBV H120 vaccine according to the manufacturer's instructions (group 2). The third group was given 150 μL supernatant of cell lysates mock infected (group 3). The fourth group was given 150 μL PBS only (group 4).

Detection of antibody titers and specific antibodies for S/M/N proteins in sera

The prevaccination sera were collected from the wing vein of chickens in each group. Then, sera were collected every week after vaccination until challenge. Total serum immunoglobulin G (IgG) specific for IBV was measured by indirect enzyme-linked immunosorbent assay (ELISA). The test sera were diluted to 1:500 and then processed following the instruction of a commercial antibody capture ELISA kit for IBV (IDEXX, Westbrook, USA). Negative and positive control sera were included in each assay. The optical density at 650 nm was measured in a microplate reader (Bio-Rad model 680, California, USA). The total serum IBV-specific IgG was represented by the value of optical density. Endpoint titers were calculated with the equation that was recommended by the manufacturer's protocol.

To confirm whether the sera from chickens immunized with rBeau-H120(S1e) contained specific antibodies for S, M, or N proteins, Western blots were performed with the sera collected 21 days after immunization. The loading samples were 10 μl of rBeau-R-infected Vero cell lysates, 10 μl of rBeau-H120(S1e)-infected Vero cell lysates, and 10 μl of mock-infected Vero cell lysates, respectively. The primary antibodies used were the sera from the chickens immunized with rBeau-H120(S1e)-infected Vero cell lysates, mock-infected Vero cell lysates, and PBS.β-actin proteins were also probed as a loading control.

Virus challenge experiment, necropsy, and histopathology

At 21 days after immunization, all chickens were challenged with 10^3 EID_{50} of the IBV M41 strain in 0.1 mL by the nasal–ocular route. The challenged chickens were examined daily for clinical symptoms such as sneezing, tracheal rales, wheezing, nasal discharge, or death for 2 weeks. Dead chickens were necropsied to confirm death by IBV infection. The surviving chickens in each group were euthanized at 14 days post-challenge. Necropsies were performed immediately postmortem, then tracheal sections and kidney tissues were collected for further detection of viruses by RT-PCR. At necropsy, the tracheas were removed for histopathological analysis. Pieces of trachea were fixed in 10 % neutral buffered formalin, and transverse sections were prepared and stained with hematoxylin and eosin.

Statistical analyses

Data was analyzed using the GraphPad software package. Comparisons between groups were done using the one-tailed Student's t test. Differences were considered statistically significant with $P<0.05$.

Results

CPE monitoring, titers, and one-step growth curves of rBeau-H120(S1e)

The first generation of rBeau-H120(S1e) stock was passaged 10 times successively in Vero cells, and then CPE of rBeau-H120(S1e) and its parental virus, rBeau-R, was monitored at the time points indicated (Fig. 2a). The recombinant IBVs infection could cause syncytium formation as its parental virus in Vero cells, and the areas of viral CPE had been expanded along with prolonged time. One-step growth curves were analyzed to determine the replication kinetics of rBeau-H120(S1e) and rBeau-R (Fig. 2b). The peak titer of the rBeau-H120(S1e) suspension reached $10^{5.9}$ $TCID_{50}$/mL at 24 hpi and began to decline at 30 hpi. In contrast, the peak titer of the rBeau-R suspension reached $10^{5.1}$ $TCID_{50}$/mL at 18 hpi and then began to decline.

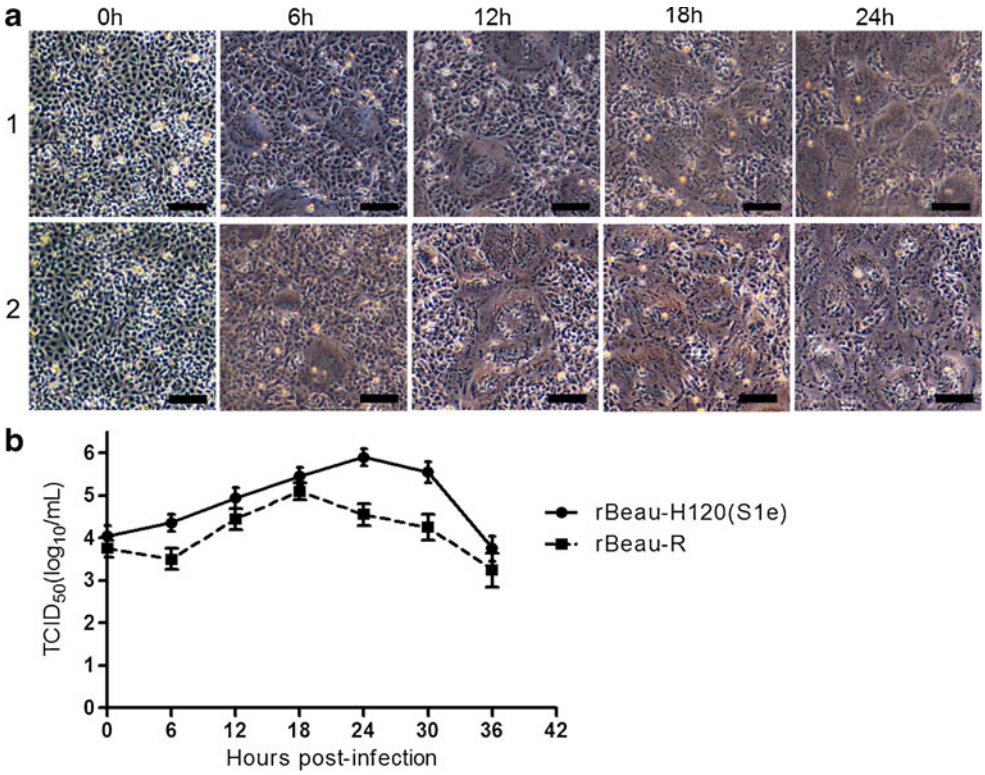

Fig. 2 Analysis of the growth properties of rBeau-H120(S1e) and its parental virus, rBeau-R, by cytopathic effect (CPE) monitoring and viral one-step growth curves. **a** CPE of rBeau-H120(S1e) and rBeau-R was monitored at the time points indicated (0, 6, 12, 18, and 24 hpi). The rBeau-H120(S1e) infection could cause syncytium formation as its parental virus, rBeau-R, in Vero cells. *1* and *2* rBeau-H120(S1e) and rBeau-R infected Vero cells, respectively. The bottom scale indicated 100 μm. **b** One-step growth curves for rBeau-H120(S1e) and rBeau-R. Vero cells were infected with rBeau-H120(S1e) or rBeau-R and harvested at 0, 6, 12, 18, 24, 30, and 36 hpi. The titers were determined by $TCID_{50}$ assay, and were expressed as the $TCID_{50}$/mL. Data represent the mean±SD from three independent experiments

Gene replication and expression of rBeau-H120(S1e)

The replication profile of viral genomic RNA was determined by RT-PCR. The results showed that the copies of viral RNA were increasing when infection period had been extended, whereas the copies of β-*actin* gene decreased as the cells were dying. The peak of viral RNA copies reached at 28 hpi (Fig. 3a). Western blots were performed on infected Vero cell lysates taken at selected time points to determine the expression profile of S, M, and N proteins of the recombinant virus. The number and staining intensity of these proteins increased from 12 to 32 hpi (Fig. 3b). This result was consistent with the accumulation of the copies of viral RNA during the same time period.

Pathogenicity and propagation property of rBeau-H120(S1e) in chickens

In terms of clinical observations, chickens inoculated with rBeau-H120(S1e) or mock-infected cell lysates did not display any clinical symptoms such as sneezing, tracheal rales, wheezing, or nasal discharge. From 2 to 10 days after inoculation, oropharyngeal swabs were collected for virus isolation, and then the supernatants of the collected swabs were inoculated into 10-day-old SPF embryonated chicken eggs. The gene fragment from rBeau-H120(S1e) had been detected by RT-PCR from 2 to 6 days after inoculation with rBeau-H120(S1e) while there was no gene fragment of rBeau-

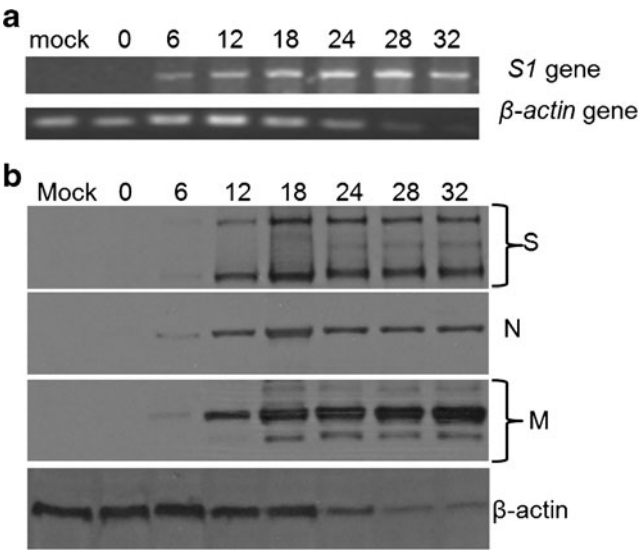

Fig. 3 The characteristics of gene replication and expression of rBeau-H120(S1e) in Vero cells. **a** The replication profile of Viral genomic RNA of rBeau-H120(S1e) in Vero cells. Vero cells infected with rBeau-H120(S1e) ($1\times10^{4.9}$ $TCID_{50}$) were harvested at the selected time points (0, 6, 12, 18, 24, 28, and 32 hpi), total RNA was extracted and analyzed by RT-PCR. The *S1* gene and β-*actin* gene fragments were taken as the indicator. **b** Western blot analysis of viral protein expression in cells infected with rBeau-H120(S1e). Vero cells infected with rBeau-H120(S1e) ($1\times10^{4.9}$ $TCID_{50}$) were harvested at the selected time points (0, 6, 12, 18, 24, 28, and 32 hpi), lysates prepared and separated on 10 % SDS-polyacrylamide gel. The expression of S, N, and M proteins was analyzed by Western blot with polyclonal anti-S, anti-N, and anti-M antibodies, respectively. The same membrane was also probed with anti-actin antibody

H120(S1e) that had been detected in the mock-infected cell lysates group (Fig. 4a). These results showed that replacement of the ectodomain region of the Beaudette *S1* gene with the sequence of the IBV H120 strain did not confer pathogenicity to the resulting virus rBeau-H120(S1e) and this recombinant IBV was restricted in its replication in vivo.

Detection of antibody titers post vaccination

The ability of the recombinant IBV to stimulate antibodies production was detected by indirect ELISA. The mean antibody titers of chickens in each group were shown in Fig. 4b. The specific antibodies against IBV were not detectable in prevaccination chickens. After vaccination, the antibody titers in chickens vaccinated with rBeau-H120(S1e) began to rise, then there was a statistical difference in antibody titers between the chickens inoculated with rBeau-H120(S1e) and those of the two control groups (mock-infected cell lysates and PBS controls) from 2 to 3 weeks after immunization, however, there was no significant difference in antibody titers between the chickens inoculated with rBeau-H120(S1e) and those inoculated with commercial available IBV H120 vaccine during the whole detection period. Before challenge, the mean value of antibody titers in rBeau-H120(S1e)-immunized group and commercial H120 vaccine-immunized group up to 341 and 399, respectively.

Detection of specific antibodies for S/M/N proteins in sera

Western blots were used to confirm whether the sera from chickens immunized with rBeau-H120(S1e) contained specific antibodies for S, M, or N proteins. The results revealed that the sera from chickens immunized with rBeau-H120(S1e) included specific antibodies for S, M, and N proteins while there were no antibodies for S, M, or N proteins in the sera from chickens immunized with mock-infected cell lysates or PBS controls (Fig. 5).

Protection against IBV M41 challenge

After chickens were challenged with the IBV M41 strain, morbidity, trachea infection, and percent protection of chickens were summarized in Table 1. No protection was obtained in the two control groups (mock-infected cell lysates and PBS groups), where all chickens showed the typical symptoms of IBV infection, sneezing, tracheal rales, wheezing, and nasal discharge. Necropsies of chickens in the two control groups exhibited typical pathological lesions of acute IB, which were restricted to the upper respiratory tract, such as the presence of mucus in the upper respiratory tract, swelling of the throat, bleeding in the trachea. In contrast, 85 % (17/20) rate of the chickens in both the rBeau-H120(S1e) group and the

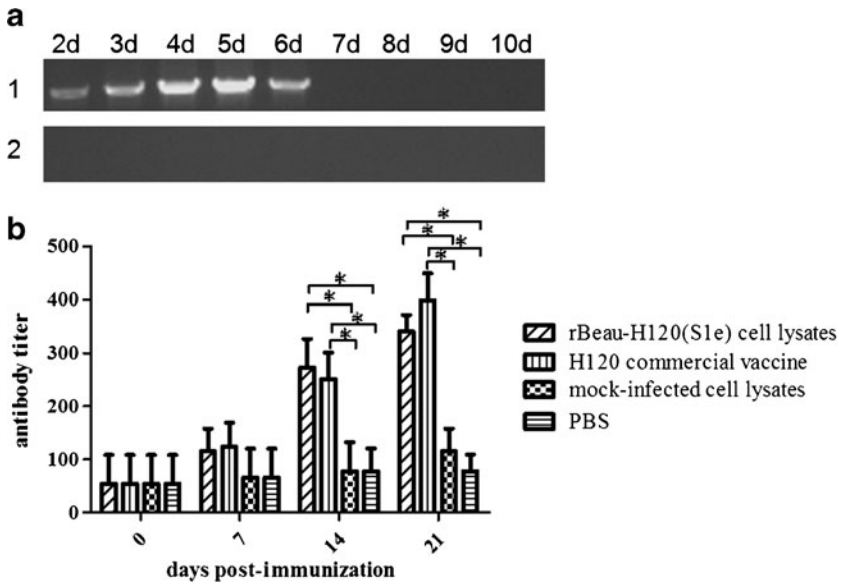

Fig. 4 Dynamics of viral replication and anti-IBV antibodies in SPF chickens. **a** Two groups of 7-day-old SPF chickens were inoculated with rBeau-H120(S1e)-infected Vero cells lysates (2×10^5 $TCID_{50}$, *row 1*) and 0.1 mL of mock-infected Vero cells lysates (*row 2*) via the nasal–ocular route, respectively. From 2 to 10 days after inoculation, oropharyngeal swabs from all chickens were collected for virus isolation. The supernatants of the swabs were inoculated into 10-day-old SPF embryonated chicken eggs. At 72 h post-inoculation, the allantoic fluid was collected for detecting of rBeau-H120(S1e) by RT-PCR. **b** Anti-IBV serum antibodies were detected by indirect ELISA. The 7-day-old SPF chickens were randomly divided into four groups ($n=20$ each). The chickens were inoculated with 150 μL supernatant of the rBeau-H120(S1e)-infected cell lysates (1×10^5 $TCID_{50}$), commercial IBV H120 vaccine, mock-infected cell lysates, or PBS by the nasal–ocular route, respectively. The prevaccination sera were collected from the wing vein of chickens in each group. Then sera were collected every week after vaccination until challenge. The ELISA assays were performed according to the manufacturer's protocol. Variation (mean ± SD based on triplicate assays) were represented as histograms

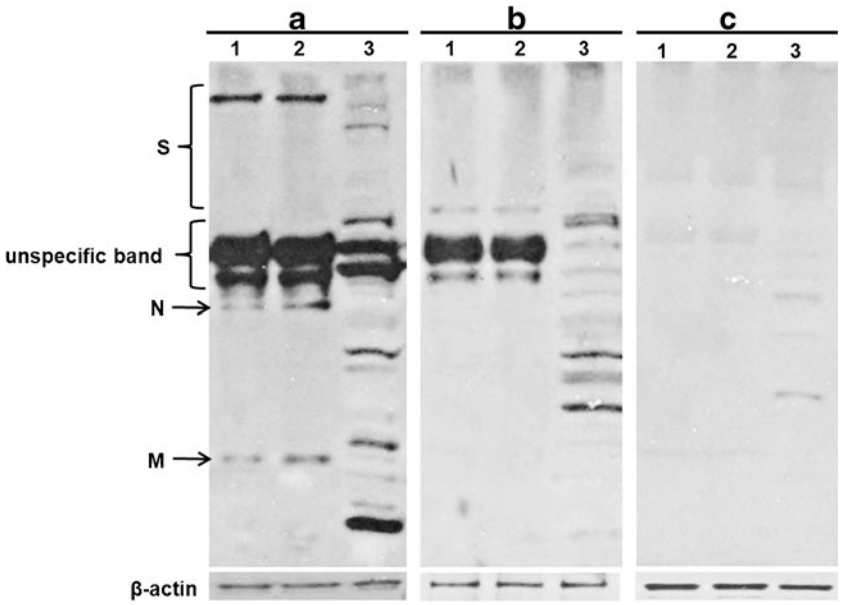

Fig. 5 Detection of specific antibodies for S/M/N protein in the sera collected at 21 days after immunization with western-blotting. The loading samples in *1*, *2*, and *3* were 10 μl of rBeau-R-infected Vero cell lysates, 10 μl of rBeau-H120(S1e)-infected Vero cell lysates, 10 μl of mock-infected Vero cell lysates, respectively. The primary antibodies used were the sera from the chickens immunized with rBeau-H120(S1e)-infected Vero cell lysates (*a*), mock-infected Vero cell lysates (*b*), and PBS (*c*). β-actin proteins were also probed as a loading control

commercial H120 vaccine group did not display clinical symptoms. Histopathological analysis revealed that there were mild deciliation, desquamation, monocytic infiltration, occasional epithelial hyperplasia, and vascular congestion in the two control groups. These phenomena were not observed with the 80 % (16/20) rate of the chickens in the rBeau-H120(S1e) group or 85 % (17/20) rate of the chickens in the commercial H120 vaccine group. Thus, lesions in the immunized with rBeau-H120(S1e) or commercial H120 vaccine group were significantly less severe than those in the two control groups. RT-PCR of the collected samples demonstrated that all chickens in the two control groups were positive for IBV M41 strain, while the positive percent of chickens in rBeau-H120(S1e)-immunized group and commercial H120 vaccine-immunized group were 20 and 15 %, respectively. These results revealed that rBeau-H120(S1e) provided a reliable resistance against the virulent IBV challenge, as well as the commercial H120 vaccine.

Table 1 The morbidity and protection rate of chickens challenged by the IBV virulent strain M41

Groups	No. IBV positive[a]	Morbidity[b]	Protection rate (%)[c]
rBeau-H120(S1e)	4/20	3/20	80
H120 vaccine	3/20	3/20	85
Mock	20/20	20/20	0
PBS	20/20	20/20	0

[a] The number of chickens positive for IBV was determined by RT-PCR from the trachea

[b] Morbidity was recorded for each day after challenge and is presented as total number of chickens that displayed the typical symptoms in each group

[c] Percent of protection was determined by the number of chickens negative for IBV divided by the total number of chickens

Discussion

The majority of IB vaccines manufacturing relies on embryonated chicken eggs to propagate the vaccine virus. If the circulating virus strain is highly virulent for embryos, the corresponding attenuated virus has to be obtained by serial passages. As a consequence, attenuated viruses have only a few mutations responsible for the loss of virulence, and can revert to virulence. Besides, this process is time-consuming and costly. Hodgson et al. had constructed a recombinant IBV with the genome of Beau-R, except that the ectodomain of S glycoprotein had been replaced with corresponding region from the pathogenic M41 strain to produce BeauR-M41(S), the recombinant IBV replicated very poorly or not at all in Vero, BHK, and primary chicken embryo fibroblasts (Casais et al. 2003). In this study, the recombinant IBV, rBeau-H120(S1e) is developed by replacing the ectodomain region of the *S1* gene of the IBV Beaudette strain with the corresponding fragment from H120 strain, and it could replicate equally well in Vero cells, and the peak titers would reach at 24 hpi, which had been exhibited in the one-step growth curves. Furthermore, the replication kinetics of rBeau-H120(S1e) had been investigated from gene replication to protein expression. The results indicated that the accumulations of S, M, and N proteins and the viral RNA copies were increasing when infection period had been extended. They reached peak value at 28 hpi. In comparison with Hodgson's studies, the length of swapping gene segment in this study is shorter. The location of replacing gene section in our study is only the ectodomain region of the *S1* gene, instead, the location in Hodgson's studies is the ectodomain of *S* gene (*S* gene contain two fragments, *S1* gene and *S2* gene). The results implied that a recombinant IBV which can replicate in Vero cells might be related to the length and location of swapping

gene segment. In our previous study, furin-mediated cleavage of the IBV S protein at two furin sites ($RRFRR_{537}$/S and $RRRR_{690}$/S) was observed in IBV-infected cells. The $RRRR_{690}$/S motif at the second IBV furin site was not necessary for, but could promote, syncytium formation and the infectivity of IBV in Vero cells (Yamada and Liu 2009). In this study, the $RRRR_{690}$/S site had not been changed, however, the site had been changed in Hodgson's studies because the replaced gene segment contains the site, so it is rational that the $RRRR_{690}$/S site in *S* gene of rBeau-H120(S1e) supports IBV infection in Vero cells. Our finding that rBeau-H120(S1e) could propagate in Vero cells with high titer is promising with respect to vaccine development.

The H120 vaccine is possibly the most widely used live attenuated IB vaccine globally to this day. It has proved to be one of the most enduring live attenuated IB vaccines because of its ability to provide heterologous cross-protection against a number of IB viruses of different serotypes (Bijlenga et al. 2004). The efficacy, safety, and long-term immunity associated with the H120 vaccine serves as a prototype for the feasibility of a live-attenuated recombinant IBV vaccine. Moreover, the S1 protein subunit is necessary and sufficient to induce protective immunity and has been successfully constructed as a DNA vaccine against IBV (Kapczynski et al. 2003; Yang et al. 2009). Therefore, the substituted ectodomain region of the Beaudette strain *S1* gene was derived from the corresponding segment of the H120 strain. The results from inoculation of chickens with rBeau-H120(S1e) demonstrated that the recombinant IBV remained nonpathogenic and was restricted in its replication in vivo. Thus, rBeau-H120(S1e) might be an ideal strain to produce live-attenuated vaccines.

In this study, serum antibody titers of the chickens inoculated with rBeau-H120(S1e) were statistically higher than those of the two control groups, but the titers (around 340) were considered as negative according to the criterion in the ELISA kit (the antibody titer, which is greater than 396, is considered as positive). This observation is in line with a previous study that inoculation with attenuated vaccines generated by successive passages of local strains in Taiwan did not elicit higher antibody titers than the controls (Huang and Wang 2006). The precise role of antibodies for the control of IBV infection remains controversial (Tang et al. 2008). Some reports have demonstrated that protection against IBV did not positively correlate with serum antibody titers (Gelb et al. 1998; Gough and Alexander 1979). Other studies displayed that the humoral antibody response plays a significant role in virus clearance and disease recovery (Thompson et al. 1997; Toro and Fernandez 1994). Thus, the capability of rBeau-H120(S1e) for protection against IBV had to be evaluated through virus challenge experiment.

Armesto et al. had constructed a recombinant IBV, BeauR-4/91(S), with the genetic backbone of Beau-R but expressing the spike protein of the pathogenic IBV strain 4/91(UK), which belongs to a different serotype as Beaudette. Protection studies showed BeauR-4/91(S) conferred protection against challenge with wild-type 4/91(UK) virus (Armesto et al. 2011). In the present study, the results of protection assay demonstrated that vaccination with rBeau-H120(S1e) at 7 days after hatch provided 80 % rate of immune protection against challenge with 10^3 EID_{50} of the IBV M41 strain. This finding confirmed again that low serum antibody titers do not always correlate with lack of protection against IBV, and provided an effective approach to protect chickens from IBV.

Although both live attenuated and inactivated IBV vaccines have played a major role in controlling of IBV infection, the existence and continual introduction of new IBV serotypes requires alternative strategies for the production of timely and safe vaccines against the emergence of variants (Britton et al. 2012). Given the important role of the S1 protein in immunity (Cavanagh and Davis 1986; Ignjatovic and Galli 1994; Johnson et al. 2003; Song et al. 1998), it is possible that the rBeau-H120(S1e) could be updated if the ectodomain region of *S1* gene was replaced by that from a present field strain of a different serotype. In summary, the recombinant IBV rBeau-H120(S1e) was avirulent but was able to act as vaccine for the protection of chickens against challenge with the virulent M41 strain.

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