Establishment of reverse genetics system for infectious bronchitis virus attenuated vaccine strain H120

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ABSTRACT

Infectious bronchitis virus (IBV) strain H120 was successfully rescued as infectious clone by reverse genetics. Thirteen 1.5–2.8 kb fragments contiguously spanning the virus genome were amplified and cloned into pMD19-T. Transcription grade complete length cDNA was acquired by a modified "No See'm" ligation strategy, which employed restriction enzyme Bsa I and BsmB I and ligated more than two fragments in one T4 ligase reaction. The full-length genomic cDNA was transcribed and its transcript was transfected by electroporation into BHK-21 together with the transcript of nucleocapsid gene. At 48 h post transfection, the medium to culture the transfected BHK-21 cells was harvested and inoculated into 10-days old SPF embryonated chicken eggs (ECE) to replicate the rescued virus. After passage of the virus in ECE five times, the rescued H120 virus (R-H120) was successfully recovered. R-H120 was subsequently identified to possess the introduced silent mutation site in its genome. Some biological characteristics of R-H120 such as growth curve, EID50 and HA titers, were tested and all of them were very similar to its parent strain H120. In addition, both R-H120 and H120 induced a comparable titer of HA inhibition (HI) antibody in immunized chickens and also provided up to 85% of immune protection to the chickens that were challenged with Mass41 IBV strain. The present study demonstrated that construction of infectious clone from IBV vaccine strain H120 is possible and IBV-H120 can be use as a vaccine vector for the development of novel vaccines through molecular recombination and the modified reverse genetics approach.

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1. Introduction

avian infectious bronchitis virus (IBV), the causative agent of infectious bronchitis (Cavanagh, 1997; Cavanagh et al., 1999), is a gammacoronavirus in the family Coronaviridae (Casais et al., 2005; Saif et al., 2008; Tidona and Darai, 2011). IBV is worldwide distributed and difficult to control because of the existence of multiple serotypes and variants of the virus (Cavanagh, 2007).

IBV is an enveloped, unsegmented, positive sense ssRNA virus and has a genome of approximately 27.6 kb in length (Casais et al., 2001, 2003; Hodgson et al., 2006). Similar to other coronaviruses, the 5′ two thirds of the IBV genome encodes two polyproteins, pp1a and pp1ab, and the latter is an extension product of pp1a as a result of a −1 frameshifting (Almazan et al., 2004; Brierley et al., 1989). The remaining one third of the genome encodes the structural proteins and group-specific ORFs, including spike glycoprotein (S), envelope protein (E), membrane

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protein (M) and nucleocapsid protein (N), which are essential for replication of the virus (Armesto et al., 2009; Fang et al., 2007; Youn et al., 2005). In addition, IBV also encodes a set of accessory proteins of unknown function that may be absent in some strains and not essential for virus replication in vitro (Armesto et al., 2009; Casais et al., 2005; Hodgson et al., 2006; Youn et al., 2005).

The reverse genetic system for IBV was firstly established using vaccinia vector (Casais et al., 2001). Subsequently, a more elegant protocol to obtain viral infectious RNA was developed. In this system, full-length viral cDNA was assembled in vitro by orderly ligating viral genomic cDNA fragments and directly used as DNA template for reverse transcription of viral infectious RNA. The new technique was successfully applied to the studies on the role of accessory genes in viral replication (Youn et al., 2005), virulence determinant of Beaudette strain (Fang et al., 2007) and the relationship between S gene and tissue tropism of the virus (Britton et al., 2006; Casais et al., 2001, 2003, 2005; Youn et al., 2005). However, to the best of our knowledge, all existing reverse genetics systems for IBV were based on Vero cell-adapted Beaudette strain, which was considered to be poorly immunogenic and never used as a vaccine strain (Geilhausen et al., 1973). Therefore, application of the reverse genetics techniques to other strains including vaccine strains could improve the technique on modification of viral genome and provide a powerful tool for novel vaccine development.

H120, an attenuated live vaccine strain of Massachusetts (Mass) serotype, was originally obtained by serial passage of strain H that was isolated in the Netherlands in 1956 in embryonated chicken eggs up to the 120th passage (Bijlenga et al., 2004). In recent 50 years, H120 was considered to be one of the safest vaccine strains and used worldwide as a primary vaccine in broilers, breeders, and future layers. The complete genome of H120 was sequenced in our previous study (Zhang et al., 2010). In this study, we describe the in vitro assembly and recovery of an infectious clone of IBV-H120, the biological and immune characteristics of the rescued H120 virus (R-H120), and the potential to use R120 as a candidate of vaccine vector in the future vaccine development.

2. Materials and methods

2.1. Virus and cell

The IBV strains, H120 and Mass41, obtained from China Institute of Veterinary Drug Control (IVDC), were propagated in the allantoic cavities of the 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECE), and the allantoic fluid was harvested 36 h post inoculation. BHK-21 cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2. RT-PCR, fragment cloning and ligation strategy

Viral RNA was extracted from H120-infected allantoic fluid with TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Reverse transcription was performed with Superscript III (Invitrogen).

The IBV-H120 sequence (GenBank, accession number FJ888351) was used for primer design and nucleotide sequencing. Each DNA fragment was amplified from cDNA templates by PCR using KOD plus polymerase (Toyobo, Japan). PCR primer pairs used to amplify genomic regions are listed in Table 1. PCR amplification of cDNA fragments was performed in the following conditions: denaturation at 94 °C for 5 min, 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2–6 min depending on the size of the products and final extension at 72 °C for 10 min. The PCR products were isolated from agarose gels and TA-cloned into pMD19-T vector (TaKaRa, Japan), according to the manufacturer's directions. The entire nucleocapsid (N) ORF, including the 3′-UTR, was amplified by RT-PCR from total RNA extracted from H120-infected allantoic fluid, and the RT-PCR product was also cloned into pMD19-T vector. In order to transcribe RNA by using T7 RNA polymerase, T7 promoter sequence was incorporated to 5′ end primers of F1 and N-3′ fragments. In addition, a silent nucleotide change of A to T at position 20,132 nt was incorporated in to F10F primer as a molecular marker. To determine the consensus clone, two to four independent clones of each amplicon were sequenced by Sangon Biological Engineering Technology & Services Co., Ltd. Each amplicon that represents the consensus sequence of IBV-H120 was then released from cloning vector by restriction enzyme digestion and recovered for construction of the full-length IBV genome in vitro. Briefly, the plasmid carrying F1 amplicon was digested with Sal I and Nci I, and treated with calf intestine alkaline phosphatase (CIAP: TaKaRa), before digesting with the BsmB I; the plasmid with F13 amplicon was digested with Xho I, CIAP treated and digested with BsmBI; the plasmids carrying amplicons F2 and F10 were digested with Nci I and BsmB I; and the other plasmids inserted amplicon F3, F4, F5, F6, F7, F8, F9, F11 and F12, were digested with BsaI. All the digested plasmids were separated on 0.8% agarose gels and the bands corresponding to each amplicon were cut from the gels and purified with QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA). The cDNA amplicons were ligated orderly with T4 DNA ligase as following: (1) ligation of the amplicons into four fragments (F1 + F2 + F3, F4 + F5 + F6, F7 + F8 + F9, and F10 + F11 + F12 + F13) in equal mole ratio and recovering the appropriately sized four fragments from 1% agarose gels and (2) ligation of the four fragments into full-length IBV genome (Fig. 1). The final ligation product was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol, and detected by electrophoresis on 0.4% agarose gel.

2.3. In vitro transcription, transfection and generation of the rescued virus R-H120

Full-length transcript was generated in vitro using mMESSAGE mMACHINE® T7 kit (Ambion, Austin, TX) according to the manufacturer's instructions with certain modifications. Briefly, 30 μl of transcription reaction with a 1:1 ratio of GTP to cap analog was sequentially incubated at 37 °C for 120 min. A similar reaction was performed for the N transcripts, which were generated by using a linearized pMD19-T-N-3′ containing the IBV N gene and the 3′-UTR region as templates. A 1:2 ratio of GTP to cap

Table 1
Primers for amplifying the fragments of genome cDNA of IBV H120 strain.

	Length	Primers	sequences (5´-3´)
F1	1621bp	Forward	<u>GTCGAC</u>*TAATACGACTCACTATAGGG*ACTTAAGATAGATATTAATATATATCTATTGCA
		Reverse	<u>CGTCTC</u>AACAATGCACATCATGCAATGC
F2	2779bp	Forward	<u>CGTCTC</u>ATTGTTGTGAGCGTGTTGTCTGTCT
		Reverse	TCAGTTG<u>CGTCTC</u>CACCCAAAAGTT
F3	2841bp	Forward	ACAACTTTTGGGTG<u>GAGACC</u>CAACT
		Reverse	CCAGATCTTGTTATAAAGAAAC<u>GAGACC</u>
F4	2310bp	Forward	<u>GGTCTC</u>GTTTCTTTATAACAAGATCTG
		Reverse	<u>GGTCTC</u>TATCCAACCATTTAGGCAG
F5	2329bp	Forward	<u>GGTCTC</u>TGGATAGTACTACTGTCAGTGTTG
		Reverse	<u>GGTCTC</u>CCAAGTACCATACCCCTAA
F6	2379bp	Forward	<u>GGTCTC</u>ACTTGGTGCTATATCTAATGTTG
		Reverse	<u>GGTCTC</u>TGCCCTATCACACTTTGG
F7	2043bp	Forward	<u>GGTCTC</u>AGGGCAATGCCTAATTTGT
		Reverse	<u>GGTCTC</u>ACAACTAACTTCTGGCAAGG
F8	1965bp	Forward	<u>GGTCTC</u>AGTTGTGATATTCTTTTGGTTG
		Reverse	<u>GGTCTC</u>GTTCACATACAGACTACCACC
F9	2020bp	Forward	<u>GGTCTC</u>GTGAACAAACATGCATTTC
		Reverse	<u>GGTCTC</u>TTTTTCCACTAACTTTAACCTTTG
F10	2107bp	Forward	<u>CGTCTC</u>GAAAAACT̲CTGCACGCAAATTA (A→T)
		Reverse	<u>CGTCTC</u>ATATGTTGTCGCAAACAGGACC
F11	1985bp	Forward	<u>GGTCTC</u>ACATATTGTCTGTAGTAAATAGTG
		Reverse	<u>GGTCTC</u>CATTCTCCTCTAGCGACT
F12	1921bp	Forward	<u>GGTCTC</u>AGAATGGAAGTTTTCTAACAG
		Reverse	CTTGA<u>GGTCTC</u>CAGTACCCATGTTG
F13	1559bp	Forward	ACATGGGTACTG<u>GAGACG</u>TCAAGCC
		Reverse	<u>CTCGAG</u>(T)$_{30}$TGCTCTAACTCTATACTAGCCTATAA
N-3´	1758bp	Forward	<u>GAATT</u>*CTAATACGACTCACTATAGGG*ATGGCGAGCGGTAAGACAACTG
		Reverse	<u>CTCGAG</u>(T)$_{30}$TGCTCTAACTCTATACTAGCCTATAA

analog was used for the transcription of IBV N gene (Fang et al., 2007; Youn et al., 2005). In the same time, BHK-21 cells were grown on monolayers to 80% confluence, treated with trypsin, washed with cold DEPC-treated PBS twice, and resuspended in DEPC-treated PBS at a concentration of 10^7 cells/ml. RNA transcripts were added to 600 µl of BHK-21 cell suspension in microfuge tubes on ice, gently pipetted and transferred to electroporation cuvettes. Three consecutive electrical pulses of 850 V at 25 AF were given, using the Gene Pulser Xcell^{TM} Electroporation System (Bio-Rad). The transfected BHK-21 cells were incubated at 37 °C for 48 h in DMEM supplemented with 10% FBS (Invitrogen). After 48 h, the virus containing medium was collected and inoculated into the 10-day-old SPF embryoned chicken eggs. After 5 passages in ECE, allantoic fluid was harvested according to the previous description (Cavanagh, 1983).
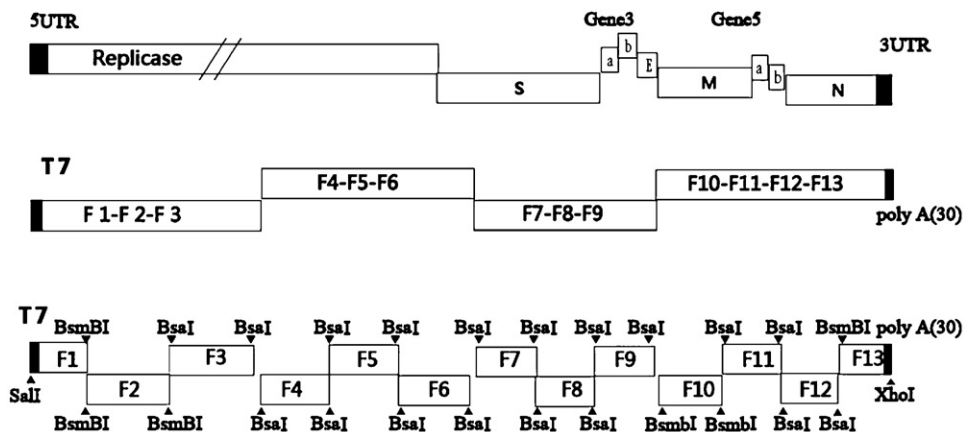
Fig. 1. In vitro assembly of full-length cDNA clone derived from a vaccine IBV H120 strain. (A) Diagram of the genome organization of IBV. The regions coding for the replicase polyproteins, the structural proteins S, E, M, and N, the accessory proteins gene 3 (a, b, E), gene 5 (a, b), and the 5′ and 3′ UTR are shown. Also shown are the regions of the 13 RT-PCR fragments, the T7 promoter at the 5′ end of fragment 1 and the 30 polyAs at the 3′ end of fragment 13. There are 1621 bp, 2779 bp, 2841 bp, 2310 bp, 2329 bp, 2379 bp, 2043 bp, 1965 bp, 2020 bp, 2107 bp, 1985 bp, 1921 bp, 1559 bp, respectively assembly of the 13 fragments into four 1/4 full-length cDNA fragments (FA: F1 + F2 + F3 (7.2 kb), FB: F4 + F5 + F6 (7 kb), FC: F7 + F8 + F9 (6 kb), and FD: F10 + F11 + F12 + F13 (7.6 kb)), then assembly of the four 1/4 full-length cDNA fragments into a full-length cDNA clone, and in vitro transcription of the full-length transcripts.

2.4. Detection of the rescued viruses R-H120

2.4.1. RT-PCR detection of the silent mutation point

To differentiate the rescued viruses from the parental viruses, viral RNAs were extracted from allantoic fluid (R-H120 and parental H120) as described previously. RT-PCR was performed with selected primer pairs (5′-aatataaga-cagagcacaag-3′, 5′-ctgtcatacaaagcagcactaca-3′) to amplify the fragment consisting of the silent nucleotide change of A to T at position 20,132 nt. The PCR fragment was cloned and sequenced to check the silent mutation site.

2.4.2. EID50 assay

To determine 50% egg infection dose (EID50) of the R-H120, serial 10-fold dilutions (10^{-1}–10^{-9}) of the amplified virus were inoculated into the 10-day-old SPF ECE. For each dilution, 0.2 ml of virus solution was injected into each egg and four eggs were used for each dilution. The parental strain H120 was used as a positive control. The EID50 calculation was based on Reed and Muench (1938) method.

2.4.3. HA assay

R-H120 virus hemagglutination antigen was prepared by using the filtrate from a clostridium welchii to treat the 100-fold concentration. HA antigens were doubly diluted in a range of 1:2–1:2048 and titrated with 0.75% chicken erythrocytes. Parental H120, SPF allantoic fluid (free IBV) and PBS were used as controls.

2.4.4. Growth kinetics of the rescued viruses R-H120 in ECE

To determine the growth kinetics of the rescued virus R-H120, the viral titre (EID50) were tested. In this procedure, the parental strain H120 was a postive control. Briefly, 0.1 ml of virus $10^{3.0}$ EID50 titre of R-H120 and H120 were inoculated into the allantoic cavities of 10-days old embryonated eggs, and the allantoic fluid of six eggs from each group were harvested at the time points of 12, 24, 36,48, 60 and 72 h post inoculation and pooled for the determination EID50, were carried out as described by the

Office of International des Epizooties. All the assays were run in triplicates and the EID50 of each virus were calculated according to standard curve.

2.5. Immunization characteristics of R-H120 as a vaccine vector candidate

2.5.1. Immunization of chickens with R-H120

To determine the immunization characteristics of R-H120 virus, 60 chickens of 7-day-old were randomly divided into three groups and raised separately. R-H120 virus at $10^{3.0}$ EID50/ml (group 1) was injected intramus-cularly to both quadriceps; H120 virus at $10^{3.0}$ EID50/ml (group 2) was injected in the same way; and 0.5 ml PBS (group 3) was used as control injection. All groups were boosted with an equivalent dose on 14 days post the initial inoculation.

2.5.2. Detection of specific anti-IBV antibodies (HI)

Serum samples were taken weekly from the three groups of immunized chickens on 7, 14, 21, 28, and 35-day post-hatch for Haemagglutination inhibition (HI) assay. The HI tests were carried out as described by the Office of International des Epizooties (OIE, 2008). The maximum dilution of each serum sample that caused HA inhibition (HI) was recorded as the endpoint of dilution and the HI titers were determined as the geometric mean titer (GMT) of $\log_2$.

2.5.3. Protection efficacy against IBV challenge

The immunized chickens were challenged with 10^3 EID50 of IBV strain Mass41 in 0.1 ml by the nasal ocular route on the 36th day after the hatching. Chickens were examined daily for 2 weeks for the clinical symptoms such as coughing, sneezing, dyspnea or death. Dead chickens were necropsied to confirm that the death was due to IBV infection. The chickens in each group were euthanized on 14 days post challenge. Kidney and lung tissues were collected individually from either the dead or euthanized chickens and virus in these tissues was detected by RT-PCR.

3. Results

3.1. Generation of the fragments and clones spanning the entire IBV genome

Thirteen 1.5–2.8 kb fragments spanning the entire IBV genome, designating F1–F13, were obtained by RT-PCR and all the fragments do have the same size as the predicated from H120 sequence. To facilitate the assembly of the full-length cDNA in vitro, each fragment has been ranged between two restriction enzymes in H120-IBV genome, e.g., BsmBI or BsaI, either at the 5′ or 3′ ends of each fragment (Fig. 1). The amplified PCR fragments were recovered from agarose gel and all of them were successfully cloned into pMD19-T cloning vector. Determined by sequencing, the consensus clones including those that contains the correctly introduced sequences such as T7 promoter at 5′-end of F1 and poly(A)$_{30}$ at 3′-end of F13 were chosen to construct the full-length cDNA clone.

3.2. Generation of the rescued H120 virus (R-H120)

The 13 cDNA fragments were prepared by digestion of the corresponding plasmids with assigned restriction enzymes such as BsmBI and BsaI and recovery from agarose gel. The full-length cDNA was assembled by orderly ligation of the purified fragments in vitro, which in turn was used as the template for in vitro transcription. Coronavirus N gene transcripts were shown to enhance the recovery of the rescued virus from the in vitro-synthesized full-length transcripts (Casais et al., 2001; Youn et al., 2005; Yount et al., 2000, 2002, 2003), therefore, N-gene ORF was also prepared for transcription in vitro (Fig. 2A3). The full-length transcript together with the N transcript was electroporated into BHK-21 cells and cytopathic effects (CPE) including cell clustering and falling-off were observed on two days post transfection. To amplify the rescued virus, the supernatant from transfected cell culture was harvested and inoculated into allantoic cavities of embryonated chicken eggs for replication of the rescued virus. After 5 passages in ECE, typical embryo lesions such as curling, stunting and dwarfing were observed, which indicated the successful rescue of H120 virus from infectious RNA. Finally, the rescued virus was designated as IBV R-H120.

3.3. The biological characteristics of R-H120 virus

The recovery of R-H120 from the ligated cDNA was confirmed by RT-PCR amplifying and sequencing the genomic region that contained molecular marker to distinguish R-H120 from H120. As expected, a nucleotide
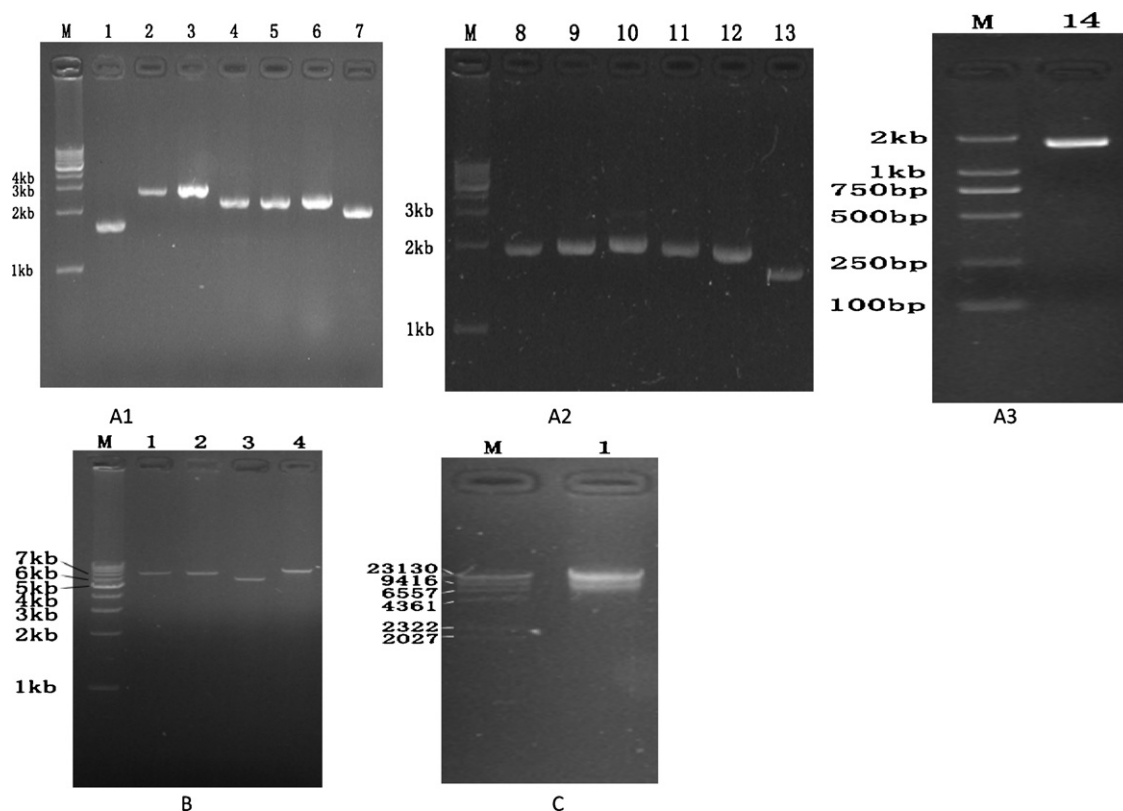

Fig. 2. (A1 and A2) Purified 13 fragments used for in vitro ligation. Each fragment was digested with the appropriate enzyme which were analyzed on a 0.8% agarose ge. Lane M represents the 1 kb DNA ladder, the lane 1 to lane 13, fragment F1–F13. (A3) lane 14 is purified N-3′ fragment. (B) Purified ligation products of 1/4 length cDNA fragments, lane M represents the 1 kb DNA ladder, the lane 1 to lane 4, represents the 4 1/4 fragment respectively. (C) Concentrated ligation product of complete length cDNA of IBV H120 strain. Lane Ml represent the λ-Hind III digest marker, lane 1 is concentrated ligation product of complete length cDNA of H120 genome.

change at 20,132 nt was identified in R-H120 genome. The virus titer and growth curve of R-H120 were also estimated and compared with the parental strain H120. The results showed that (1) R-H120 was pathogenic to chicken embryos and the EID50 titer was comparable to its parental virus H120 (Fig. 3a); (2) the tested HA titers of R-H120 was almost equal to that of H120 (Fig. 3b); and (3) like the parental strain H120, R-H120 could replicate in ECE cells and both viruses had very similar growth pattern (Fig. 3c). The above experimental data indicated that the rescued virus shared several essential properties with its parental virus H120.

3.4. Serum HI antibody assay in chicken after immunizing the H120 and R-H120

To estimate the immunogenicity of the rescued virus, an important factor for using it to develop vaccine, R-H120 and H120 viruses were immunized to two groups of chickens and the induced serum antibody was titrated. The dynamic changes of HI antibody titer following the inoculation of R-H120 and H120 were observed (Table 2) and no statistic difference of HI antibody titer was found between R-H120 and H120 groups. However, a significant difference was
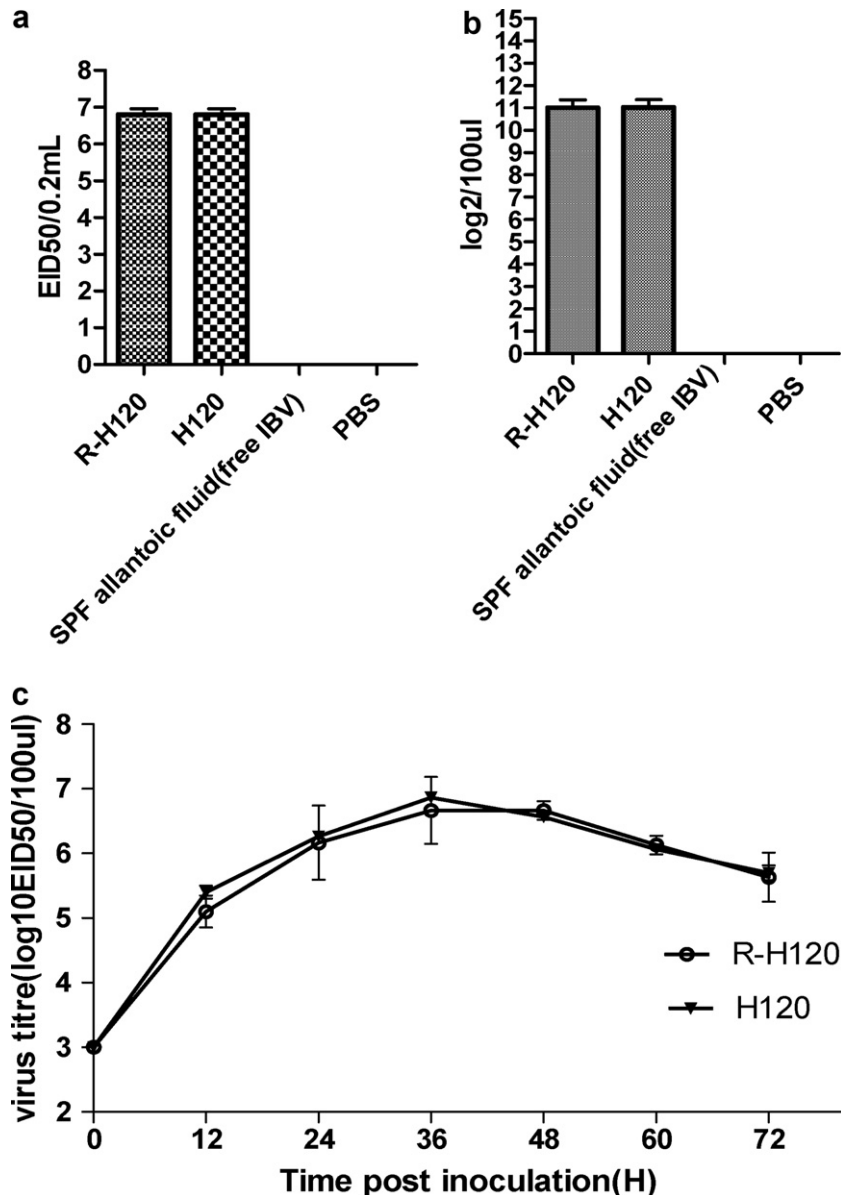
Fig. 3. Biological characteristic of the R-H120. (a) The EID50 of the R-H120, the parental strain H120 was the positive control, SPF allantoic fluid (free IBV) and PBS were the negative controls. (b) HA titer of the R-H120, parental strain H120 was the positive control, SPF allantoic fluid (free IBV) and PBS were the negative controls. (c) Comparison of the replication kinetics of R-H120 and H120 in ECE cell. The rescued virus H120 (R-H120 and Parent H120 (0.1 ml of 100 EID50) were inoculated into the allantoic cavities of 10-day old embryonated eggs, and the allantoic fluid of six eggs from each group was harvested at the time points of 12, 24, 36, 48,60 and 72 h post inoculation and pooled for the determination of EID50 in ECE.

Table 2
The dynamic variation of antibody (HI) titers in chicken ($\log_2$).

Group	(days post immunization) dpi				
	7 day	14 day	21 day	28 day	35 days
R-H120	2.8 ± 0.3^a	4.0 ± 0.3^a	5.1 ± 0.2^a	6.0 ± 0.3^a	7.5 ± 0.4^a
H120	2.8 ± 0.4^a	4.0 ± 0.4^a	5.1 ± 0.3^a	6.0 ± 0.3^a	7.5 ± 0.4^a
PBS	2.8 ± 0.4^a	2.83 ± 0.3^b	2.8 ± 0.3^b	2.8 ± 0.3^b	2.8 ± 0.3^b

Data within a column with different letters differ significantly ($p < 0.05$); the same letters means no significance.

detected between PBS and virus inoculated groups ($p < 0.05$).

3.5. Protection efficacy against challenge with IBV

Three groups of chickens were challenged with the virulent strain Mass41 to test if the rescued virus could protect the immunized animals from IBV infection. On the 5th day post challenge, chickens started to show clinical signs of infection or death. The data shown affected chickens in each group after challenge, such as mortality rate and protection rate, were listed in Table 3. The chickens injected with PBS alone were not protected from IBV infection and developed cough, nasal discharge and dyspnea. The death rate in the group injected with PBS reached to 85%, but it was only 5% for both groups 1 and 2. To evaluate the level of protection, shielding virus in the collected lung and kidney samples were estimated by RT-PCR. The results showed that 15% of the chickens in groups 1 and 2 had virus shielding in their lungs and/or kidney in compared with 100% in control group (PBS). The results suggested that R-H120 and H120 both provide protection to immunized chickens from virulent IBV challenge.

4. Discussion

Through different methodologies, vaccinia virus vectors or BAC as cloning systems and the in vitro assembly strategy, reverse genetics systems of IBV and other coronaviruses (TGEV, HCoV 229E, MHV-A59, and SARS-CoV) have been constructed (Almazán et al., 2000; Casais et al., 2001; Yount et al., 2002, 2003). For the purposes of studying the mechanisms of pathogenesis, or building transfer vectors, so far, Casais et al. (2001), Youn et al. (2005) and Fang et al. (2007) established the reverse genetic system for Vero cell-adapted Beaudette strain. After that, this reverse genetic system for Vero cell-adapted Beaudette strain are used to research the pathogenicity, tissue tropism and functionality of accessory gene of the

IBV (Armesto et al., 2009, 2011; Britton et al., 2006; Casais et al., 2003; Hodgson et al., 2006).

Previous reverse genetic system set for coronavirus was assembly of a genomic length cDNA from 6 to 8 contiguous cDNA subclones in size 3–7 kb (Fang et al., 2007; Youn et al., 2005). Although this segmentation strategy can decrease the number of cDNA fragments and is easier to link to full-lengthen cDNA, it is difficult to get the longer PCR product with correct sequence and clone these cDNA fragments. In addition, the recombined plasmid containing larger fragments may be harmful to host bacteria, resulting some of them could not replicate in Escherichia coli host cells. In order to solve these problems, certain modifications for assembling of IBV genomic RNA in vitro were made to improve the efficiency of generating full-length cDNA. In this study, two improvements for assembly of full-length viral genome were applied. At first, the IBV genome was cloned as thirteen 1.5–2.8 kb fragments. The shortened length of cDNA fragments made them easier to clone, with less sequence mutation in each fragment and more stable in bacteria. Secondly, three or four fragments were ligated in one T4 DNA ligase reaction to reduce the number of recovery times which was negative correlated to DNA recovery rate from agarose gels.

The genome of the Avain coronavirus was established to provide a tool not only for the mechanism studies but also for the development of new vaccines (Matthijs et al., 2005; Ziebuhr, 2005). In this study, we were established a reverse genetics system based on H120 strain of IBV, a worldwide used attenuated live vaccine strain of Massachusetts (Mass) serotype. In China, H120 is always considered as the first vaccine selection. Compared with the Vero cell-adapted Beaudette strain, H120 can be developed as the vaccine vector to express other antigens since it has a stronger immunogenicity than that of Beaudette strain, although the latter was a widely used model of IBV on reverse genetics study and never considered as a vaccine strain (Geilhausen et al., 1973). According to the present data, the biological and immune

Table 3
Protective effects in 4-week-old SPF chickens immunized with R-H120 and H120 against challenge of IBV Mass41 strain.

Virus immunized	No. of death chickens[a]	No. of affected chickens[b]	Mortality (%)	Protection (%)[c]
R-H120	1/20	3/20	5	85
H120	1/20	3/20	5	85
PBS	17/20	20/20	85	0

[a] The death chicken was recorded each day after challenge and is presented as total number of dead chickens in each group.
[b] The dead and euthanized chicken was determined as affected chicken by RT-PCR amplification of IBV N protein cDNA band out of the lung and/or kidney tissues.
[c] Percent protection was determined by the number of unaffected chickens/total number of chickens in each group.

characteristics of R-H120 including the EID50 and HA titer, growth curve and protection of immunized animals from challenge were all very similar to its parental strain H120. The same situation was found in other CoVs such as IBV Beaudette strain (Fang et al., 2007), MHV (Yount et al., 2002), TGEV (Yount et al., 2000), NDV (Hu et al., 2009) and avian influenza virus (Hoffmann et al., 2002; Jackson et al., 2008). It might imply that H120 was a good strain for the development of engineering avian vaccines. In addition, the 27.6 kb genome of H120 as a vaccine vector can be recombined with larger pathogenic antigen gene compared with 15 kb Newcastle disease virus vaccine vector, which was a vector used presently to express different pathogenic antigen genes as vaccine to protect the immunized animals from correspondence pathogen's challenge (DiNapoli et al., 2007; Nakaya et al., 2001; Veits et al., 2006).

It would make the procedure of constructing reverse genetics system labor intensive if a continuous cell line could not be used in infectious RNA transfection and rescued virus replication. In our study, the continuous cell line, BHK-21, was applied to generating modified virus after electroporation, indicating that BHK-21 can be utilized to rescue virus from infectious RNA although BHK-21 was never used to replicate H120 virus before. At present, most IBV strains replicate in primary cells like CK and CEK and do not adapt to continuous cell lines (Casais et al., 2003). Therefore, in vitro assembly strategy is applicable to IBV strains that were not adapted to the commonly used continuous cell lines through the established approach in the present study. It is interesting to find that the rescued virus R-H120 and H120 viruses cannot efficiently replicate in BHK21 and make the cells emerge obvious CPE, but R-H120 do rescue from BHK21 cell. In next step, the S gene of H120 may be modified by reverse genetics to find out BHK21 adapted H120 that may reveal the information on cell tropism of the virus.

In conclusion, the present study demonstrated that the rescued virus R-H120 has similar biological and immunogenic characteristics with its parental strain H120 and it would be possible to develop novel avian vaccine through reverse genetics approach by modification or recombination of the present vaccine virus H120.

Acknowledgments

References

Almazán, F., González, J.M., Pénzes, Z., Izeta, A., Calvo, E., Plana-Durán, J., Enjuanes, L., 2000. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc. Natl. Acad. Sci. U.S.A. 97, 5516.

Almazan, F., Galan, C., Enjuanes, L., 2004. The nucleoprotein is required for efficient coronavirus genome replication. J. Virol. 78, 12683.

Armesto, M., Cavanagh, D., Britton, P., 2009. The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. PLoS One 4, e7384.

Armesto, M., Evans, S., Cavanagh, D., Abu-Median, A.B., Keep, S., Britton, P., 2011. A recombinant avian infectious bronchitis virus expressing a heterologous spike gene belonging to the 4/91 serotype. PLoS One 6, e24352.

Bijlenga, G., Cook, J.K.A., Gelb Jr., De Wit, J., 2004. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol. 33, 550–557.

Brierley, I., Digard, P., Inglis, S.C., 1989. Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot. Cell 57, 537–547.

Britton, P., Casais, R., Hodgson, T., Davis, M., Cavanagh, D., 2006. Genes 3 and 5 of infectious bronchitis virus are accessory protein genes. Nidoviruses 363–368.

Casais, R., Davies, M., Cavanagh, D., Britton, P., 2005. Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. J. Virol. 79, 8065.

Casais, R., Dove, B., Cavanagh, D., Britton, P., 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J. Virol. 77, 9084.

Casais, R., Thiel, V., Siddell, S.G., Cavanagh, D., Britton, P., 2001. Reverse genetics system for the avian coronavirus infectious bronchitis virus. J. Virol. 75, 12359–12369.

Cavanagh, D., 1983. Coronavirus IBV: further evidence that the surface projections are associated with two glycopolypeptides. J. Gen. Virol. 64, 1787–1791.

Cavanagh, D., 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. 142, 629.

Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. Vet. Res. 38, 281–297.

Cavanagh, D., Mawditt, K., Britton, P., Naylor, C., 1999. Longitudinal field studies of infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions. Avian Pathol. 28, 593–605.

DiNapoli, J.M., Kotelkin, A., Yang, L., Elankumaran, S., Murphy, B.R., Samal, S.K., Collins, P.L., Bukreyev, A., 2007. Newcastle disease virus, a host range-restricted virus, as a vaccine vector for intranasal immunization against emerging pathogens. Proc. Natl. Acad. Sci. U.S.A. 104, 9788.

Fang, S., Chen, B., Tay, F.P.L., Ng, B.S., Liu, D.X., 2007. An arginine-to-proline mutation in a domain with undefined functions within the helicase protein (Nsp13) is lethal to the coronavirus infectious bronchitis virus in cultured cells. Virology 358, 136–147.

Geilhausen, H., Ligon, F., Lukert, P., 1973. The pathogenesis of virulent and avirulent avian infectious bronchitis virus. Arch. Virol. 40, 285–290.

Hodgson, T., Britton, P., Cavanagh, D., 2006. Neither the RNA nor the proteins of open reading frames 3a and 3b of the coronavirus infectious bronchitis virus are essential for replication. J. Virol. 80, 296–305.

Hoffmann, E., Krauss, S., Perez, D., Webby, R., Webster, R.G., 2002. Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine 20, 3165–3170.

Hu, S., Ma, H., Wu, Y., Liu, W., Wang, X., Liu, Y., Liu, X., 2009. A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. Vaccine 27, 904–910.

Jackson, D., Hossain, M.J., Hickman, D., Perez, D.R., Lamb, R.A., 2008. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proc. Natl. Acad. Sci. U.S.A. 105, 4381.

Matthijs, M.G.R., van Eck, J.H.H., De Wit, J., Bouma, A., Stegeman, J., 2005. Effect of IBV-H120 vaccination in broilers on colibacillosis susceptibility after infection with a virulent Massachusetts-type IBV strain. Avian Dis. 49, 540–545.

Nakaya, T., Cros, J., Park, M.S., Nakaya, Y., Zheng, H., Sagrera, A., Villar, E., García-Sastre, A., Palese, P., 2001. Recombinant Newcastle disease virus as a vaccine vector. J. Virol. 75, 11868–11873.

OIE, A.H.S., 2008. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Office International des Epizooties, Paris, France, pp. 1092–1106.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27, 493–497.

Saif, L.J., Perlman, S., Gallagher, T., Snijder, E., 2008. Coronaviruses of domestic livestock and poultry: interspecies transmission, pathogenesis, and immunity. Nidoviruses 279.

Tidona, C., Darai, G., 2011. The Springer Index of Viruses, second ed. Springer Verlag, pp. P403–P413.

Veits, J., Wiesner, D., Fuchs, W., Hoffmann, B., Granzow, H., Starick, E., Mundt, E., Schirrmeier, H., Mebatsion, T., Mettenleiter, T.C., 2006. Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. Proc. Natl. Acad. Sci. U.S.A. 103, 8197–8202.

Youn, S., Leibowitz, J.L., Collisson, E.W., 2005. In vitro assembled, recombinant infectious bronchitis viruses demonstrate that the 5a open reading frame is not essential for replication. Virology 332, 206–215.

Yount, B., Curtis, K.M., Baric, R.S., 2000. Strategy for systematic assembly of large RNA and DNA genomes: transmissible gastroenteritis virus model. J. Virol. 74, 10600.

Yount, B., Curtis, K.M., Fritz, E.A., Hensley, L.E., Jahrling, P.B., Prentice, E., Denison, M.R., Geisbert, T.W., Baric, R.S., 2003. Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. U.S.A. 100, 12995.

Yount, B., Denison, M.R., Weiss, S.R., Baric, R.S., 2002. Systematic assembly of a full-length infectious cDNA of mouse hepatitis virus strain A59. J. Virol. 76, 11065–11078.

Zhang, Y., Wang, H.N., Wang, T., Fan, W.Q., Zhang, A.Y., Wei, K., Tian, G.B., Yang, X., 2010. Complete genome sequence and recombination analysis of infectious bronchitis virus attenuated vaccine strain H120. Virus Genes 41, 377–388.

Ziebuhr, J., 2005. The coronavirus replicase. Coronavirus Repl. Reve. Genet. 57–94.