Virus Research 250 (2018) 104–113

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Effects of hypervariable regions in spike protein on pathogenicity, tropism, and serotypes of infectious bronchitis virus

Dan Shan[a], Shouguo Fang[b], Zongxi Han[a], Hui Ai[a], Wenjun Zhao[a], Yuqiu Chen[a], Lei Jiang[a], Shengwang Liu[a,*]

[a] Division of Avian Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, The Chinese Academy of Agricultural Sciences, Harbin 150026, China
[b] Agricultural School, Yangtze University, 266 Jingmi Road, Jingzhou City, Hubei Province 434025, China

ARTICLE INFO

Keywords:
Hypervariable region
Infectious bronchitis virus
Infectious clone
Recombinant IBV
Reverse genetic vaccine

ABSTRACT

To study the roles of hypervariable regions (HVRs) in receptor-binding subunit S1 of the spike protein, we manipulated the genome of the IBV Beaudette strain using a reverse genetics system to construct seven recombinant strains by separately or simultaneously replacing the three HVRs of the Beaudette strain with the corresponding fragments from a QX-like nephropathogenic isolate ck/CH/LDL/091022 from China. We characterized the growth properties of these recombinant IBVs in Vero cells and embryonated eggs, and their pathogenicity, tropism, and serotypes in specific pathogen-free (SPF) chickens. All seven recombinant IBVs proliferated in Vero cells, but the heterogenous HVRs could reduce their capacity for adsorption during *in vitro* infection. The recombinant IBVs did not significantly increase the pathogenicity compared with the Beaudette strain in SPF chickens, and they still shared the same serotype as the Beaudette strain, but the antigenic relatedness values between the recombinant strain and Beaudette strain generally decreased with the increase in the number of the HVRs exchanged. The results of this study demonstrate the functions of HVRs and they may help to develop a vaccine candidate, as well as providing insights into the prevention and control of IBV.

1. Introduction

IBV primarily replicates in the epithelial surface of the respiratory tract, although some strains are nephropathogenic (Cavanagh, 2003). IBV infections reduce egg production, quality, and hatchability, as well as increasing the feed conversion ratio and carcass condemnation in slaughterhouses. Thus, infectious bronchitis causes severe economic losses in the poultry industry worldwide.

The enveloped IBV belongs to the genus *Coronavirus,* family Coronaviridae, order Nidovirales (Cavanagh, 2003). The main structural protein is the spike (S) glycoprotein, which comprises a divergent S1 subunit and conserved S2 subunit (de Groot et al., 1987; Shil et al., 2011). The S1 subunit contains the receptor-binding domain (Wickramasinghe et al., 2011), and it carries virus-neutralizing and serotype-specific determinants. The S1 domain exhibits high sequence diversity, where 20%–25% (even up to 50%) of the amino acids differ within the S1 subunit among IBV serotypes. After comparing the S1 subunit from a number of distinct IBV isolates, a previous study defined particularly variable segments at the amino terminus of the S1 subunit as hypervariable regions (HVRs) (Kusters et al., 1989).

The HVRs possibly account for the antigenicity and serotypic variation, and evidence indicates that five neutralizing peptides mainly mapped onto the S1 subunit are co-located within the HVRs (Cavanagh et al., 1992, 1988; Moore et al., 1997; Niesters et al., 1987). In addition, the HVRs are possibly associated with receptor binding. It has been reported that the critical amino acids for attachment of the M41 spike overlap with a HVR in the S1 subunit (Promkuntod et al., 2014). Thus, the HVRs may affect the tropism, serotype, and pathogenicity of IBV, and it is important to elucidate their biological functions.

Previous studies applied forward genetics methods to determine the roles of the HVRs by characterizing their phylogenetically closely related isolates with mutations in other parts of the IBV genome, but it is difficult to establish a precise model for further analysis based on these studies. Thus, in this study, we manipulated the IBV Beaudette genome by reverse genetics to exchange the HVRs in the Beaudette strain with those in ck/CH/LDL/091022 in order to precisely determine their biological functions. We also explored the possibility of developing a reverse genetic vaccine candidate cultured in Vero cells to provide protection from the prevalent field strains. The results of this study contribute to our understanding of the prevention and control of IBV.

* Corresponding author.
E-mail addresses: swliu@hvri.ac.cn, liushengwang@caas.cn (S. Liu).

https://doi.org/10.1016/j.virusres.2018.04.013
Received 13 December 2017; Received in revised form 13 April 2018; Accepted 17 April 2018
Available online 21 April 2018
0168-1702/

2. Materials and methods

2.1. Cells and viruses

Vero cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Saint Louis, MO, USA), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C with 5% carbon dioxide. The ck/CH/LDL/091022 strain was isolated from H120-vaccinated chickens with renal lesions in 2009 (Liu et al., 2013). The Beaudette strain was routinely propagated in Vero cells (Liu et al., 1995, 1998; Tay et al., 2012) and the 50% tissue culture infection dose ($TCID_{50}$) of the viral stock was calculated using 96-well plates with 10-fold serial dilutions. The 50% egg infection dose (EID_{50}) of the viral stock was determined by inoculating 10-fold dilutions into groups of 9-day-old embryonated chicken eggs.

2.2. Recovery of infectious recombinant IBVs

Five fragments spanning the entire IBV genome were obtained by RT-PCR from Vero cells infected with the IBV Beaudette strain, as described previously (Fang et al., 2007). The three HVRs in the isolate ck/CH/LDL/091022 were identified based on alignments of the amino acid sequences, as described previously (Zhang et al., 2015). The sequences of the IBV cDNA covering the three HVRs in the S gene were replaced separately or simultaneously with those from ck/CH/LDL/091022, and subsequently ligated into the full-length IBV cDNA (Fig. 1a and Table 1). Full-length transcripts generated *in vitro* were introduced into Vero cells by electroporation. IBV N gene transcripts were also generated to enhance the efficiency of viral recovery. Total RNA was prepared from the electroporated Vero cells or infected allantoic liquid. Viral RNA replication was investigated based on RT-PCR of negative-strand genomic RNA (Tan et al., 2006). The S gene in the recovered IBV clones (third passage in Vero cells and embryonated eggs) was amplified by RT-PCR and subsequently confirmed by DNA sequencing analysis. The gene was characterized during the third passage of viruses in specific pathogen-free (SPF) embryonated eggs or Vero cells. The seven rescued recombinant IBVs were designated as rHVR I, rHVR II, rHVR III, rHVR I/II, rHVR I/III, rHVR II/III, and rHVR I/II/III.

2.3. Growth kinetics of the recombinant IBVs

To determine the growth kinetics of the rescued recombinant IBVs, a dose of 100 × EID_{50} was inoculated into the allantoic cavities of 9-day-old embryonated eggs, and the allantoic fluid was harvested from three eggs in each group at 12, 24, 36, 48, and 60 h post-inoculation, where the fluids from three eggs were pooled for EID_{50} determination with three replicates. Vero cells were infected with Beaudette and recombinant IBVs, and three wells were harvested at 4, 8, 12, 16, 24, and 36 h post-infection. Viral stocks were prepared by freezing/thawing the cells three times to determine the $TCID_{50}$ with three replicates for each time.

2.4. Immunofluorescent (IF) staining

IBV infected cells cultured in six-well plates were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.2% Triton X-100 for 10 min. IF staining was performed with a monoclonal antibody 6D10 against IBV N protein (Han et al., 2013) and subsequently with FITC-conjugated anti-mouse IgG (Sigma-Aldrich). Cells were examined by fluorescence microscopy.

2.5. Pathogenicity test

All of the animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China (License no. SQ20160408).

We randomly assigned 150 7-day-old SPF layer chickens to 10 groups, *i.e.*, the seven recombinant strains, Beaudette, ck/CH/LDL/091022, and PBS groups. There were 15 SPF chickens in each group. The challenge strain (10^5 × EID_{50} per bird) was applied *via* the intranasal and ocular routes. The clinical signs were monitored in 10 randomly selected challenged birds from each group, and the morbidity and mortality rates were recorded daily. We evaluated the challenged chickens blindly for respiratory rales at 5 days after challenge. Signs were scored as 0 = absent, 1 = mild, 2 = moderate, or 3 = severe. At 5 days after IBV challenge, the other five birds in each group were killed humanely using carbon dioxide over inhalation, followed by exsanguination. The cranial third of the trachea, lungs, kidneys, and cecum tonsils were collected, partly fixed in formalin, and embedded in paraffin. Longitudinal 5-μm sections were stained with hematoxylin-eosin (H&E stain). The mucosal thickness, deciliation, goblet cells, and lymphocytes scores for the tracheal mucosa were evaluated blindly and scored from 1 to 5 based on their severity (*i.e.*, normal, mild, moderate, marked, and severe) (van Ginkel et al., 2015). Moreover, viral shedding was quantified in the oropharyngeal secretions from 10 infected chickens at 4, 8, 12, 16, and 20 days after challenge by real-time RT-PCR (Jones et al., 2011).

2.6. In vivo Tissue tropism

The tissues collected at 5 days post-IBV challenge, *i.e.*, tracheas, lungs, kidneys, and cecum tonsils, were also subjected to immunohistochemistry (IHC) using monoclonal antibody 6D10, as described previously (de Wit et al., 2011; Xu et al., 2016). Viral loads in selected tissues were determined based on IBV RNA detection by real-time RT-PCR, as described previously (Zhao et al., 2017).

2.7. Cross-virus neutralization assay

The seven IBV recombinant strains, Beaudette, and ck/CH/LDL/091022 were analyzed in cross-virus neutralization tests. Sera against the IBV strains were prepared as previously described (Zhang et al., 2015). In the virus neutralization tests, sera were serially diluted two times with sterile PBS and mixed with 200 × EID_{50} or $TCID_{50}$ for the IBV strains. The two-way cross-neutralization test between the Beaudette and recombinant IBVs was performed in Vero cells. After incubation for 1 h at 37 °C, the virus–serum mixtures were cultured in 96-well microplates for 5 days. We could not detect the replication with ck/CH/LDL/091022 in Vero cells, so the neutralization tests were performed in 9-day-old SPF embryonated eggs to confirm whether the serotypes of the recombinant IBVs belonged to ck/CH/LDL/091022. The end-point titer for each serum sample was calculated using the Reed–Muench method. Antigenic relatedness values were calculated (Archetti and Horsfall et al., 1950; Wadey and Faragher, 1981).

2.8. Adsorption and internalization assessments using a cellular enzyme-linked immunosorbent assay (cELISA)

The cELISA protocol used in this study is similar to the conventional ELISA. The viral solution for each strain was serially diluted two times with sterile PBS and coated on the plate. Standard curves were drawn according to the standard ELISA method. Vero cells were cultured overnight in 96-well microplates (Corning, USA) (10^5 cells/well). For the adsorption assay, each strain was incubated in cells at a multiplicity of infection (MOI) of 1 for 1 h at 4 °C with three replicates (Fang et al., 2010; Sun et al., 2017). After two washes with PBS, the microplates were fixed with methanol and 1% hydrogen peroxide for 30 min, and then permeabilized with 0.2% Triton X-100 for 10 min at room temperature. Next, the microplates were washed three times and blocked with 8% skim milk for 30 min at room temperature. After washing three