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Fine level epitope mapping and conservation analysis of two novel linear B-cell epitopes of the avian infectious bronchitis coronavirus nucleocapsid protein

Zongxi Han, Fei Zhao, Yuhao Shao, Xiaoli Liu, Xiangang Kong, Yang Song, Shengwang Liu*

Division of Avian Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Harbin 150001, People's Republic of China

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ABSTRACT

The nucleocapsid (N) protein of the infectious bronchitis virus (IBV) may play an essential role in the replication and translation of viral RNA. The N protein can also induce high titers of cross-reactive antibodies and cell-mediated immunity, which protects chickens from acute infection. In this study, we generated two monoclonal antibodies (mAbs), designated as 6D10 and 4F10, which were directed against the N protein of IBV using the whole viral particles as immunogens. Both of the mAbs do not cross react with Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILTV) and subtype H9 avian influenza virus (AIV). After screening a phage display peptide library and peptide scanning, we identified two linear B-cell epitopes that were recognized by the mAbs 6D10 and 4F10, which corresponded to the amino acid sequences ^{242}FGPRTK^{247} and ^{195}DLIARAAKI^{203}, respectively, in the IBV N protein. Alignments of amino acid sequences from a large number of IBV isolates indicated that the two epitopes, especially ^{242}FGPRTK^{247}, were well conserved among IBV strains. This conclusion was further confirmed by the relationships of 18 heterologous sequences to the 2 mAbs. The novel mAbs and the epitopes identified will be useful for developing diagnostic assays for IBV infections.

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1. Introduction

Coronaviruses (CoVs) are found in a wide variety of animals where they cause respiratory, enteric, and neurological diseases with variable severity. Based on genotypic and serological analyses, CoVs are divides into 3 genera; alpha-, beta- and gamma-coronaviruses (Carstens, 2009). Alpha- and beta-coronaviruses have been isolated from mammals, while gamma-coronaviruses cause avian infectious bronchitis (IBV), as well as the genetically closely related Turkey coronavirus (Cao et al., 2008; Cavanagh et al., 2001; Gomaa et al., 2008; Guy, 2000) and pheasant coronavirus (Cavanagh et al., 2002). Numerous variants and serotypes of IBV continue to be discovered in poultry flocks worldwide (Cavanagh et al., 1988, 1992; Dolz et al., 2006; Farsang et al., 2002; Gelb et al., 1991; Han et al., 2011; Wang and Huang, 2000) that cause infectious bronchitis (IB), which is responsible for mortality in young chickens, economic losses due to poor weight gain, and a reduction in the egg quality and quantity (Cavanagh and Gelb, 2008).

Like the typical genomic organization found in other gamma-coronaviruses, the 3′ end of the IBV genome contains the main structural genes; the spike glycoprotein (S), the small membrane protein (E), the integral membrane protein (M), and the nucleocapsid protein (N), as well as several accessory genes, usually in the order S-Gene 3-E-M-Gene 5-N (Boursnell et al., 1987). The S1 subunit of the S protein carries virus-neutralizing and serotype-specific determinants, but it exhibits high sequence diversity among different IBV serotypes. By contrast, the N protein is highly conserved with 91.0–96.5% similarity in different IBV strains (William et al., 1992). Its primary function is the formation of the viral ribonucleoprotein complex, but it is also considered that the IBV N protein is multifunctional. Its intracellular localization suggests that it is a likely component of the coronavirus replication and transcription complex. Furthermore, the N protein can induce high titers of cross-reactive antibodies and cell-mediated immunity, which protects chickens from acute infections (Ignjatovic and Galli, 1994; Seo et al., 1997; Tang et al., 2008). Most of the IBV N protein is composed of 409 amino acids with a predicted molecular weight of 45 kDa. The N protein is a phosphoprotein that can bind viral RNA with high affinity (Chen et al., 2005) and it is expressed abundantly during infections (Cavanagh, 2005). Thus, it is a target protein when designing infectious bronchitis (IB) vaccines (Tian et al., 2008) and a frequent target of diagnostic applications (Chen et al., 2003; Gibertoni et al., 2005; Ndifuna et al., 1998). However, most of the diagnostic assays had been focused on the antibody

* Corresponding author at: Division of Avian Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Harbin 150001, People's Republic of China. Tel.: +86 451 85935065; fax: +86 451 82734181.
E-mail address: swliu@hvri.ac.cn (S. Liu).

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detection using recombinant N proteins. In the sampling practices in poultry farms in China, it is very common to take tracheal swabs to look for respiratory virus infections, though it is also very common to take blood samples to detect antibody. Hence, new assays focusing on virus detection, such as using mAb(s) against N protein, would be an improvement on current available diagnostic assays.

Naïve B-cells, which are the principal agents of humoral immune responses, are stimulated by the specific recognition and binding of B-cell receptors to a region of the antigen known as the epitope. Together with co-stimulation by T-lymphocytes, naïve B-cells become fully activated then proliferate and differentiate into memory and plasma cells, while the latter act as key engines for producing specific antibodies. The identification and mapping of B-cell epitopes on antigens has been a subject of intense research because knowledge of these markers has profound implications for the development of peptide-based diagnostics, therapeutics, and vaccines. B-cell epitopes may consist of linear, contiguous stretches of amino acids in a protein, or they can be discontinuous stretches of amino acids that are brought together spatially via protein folding. The majority of B-cell epitopes are discontinuous in nature, but difficulties in the design of such epitopes have led to an emphasis on the identification of linear B-cell epitopes. Monoclonal antibodies (mAbs) are used widely as powerful tools for identifying linear epitopes, or for mimicking the epitopes of a variety of infectious agents (Deng et al., 2007; Kaverin et al., 2007; Zhang et al., 2011). In this study, we prepared mAbs against the N protein of IBV strain tl/CH/LDT3/03I and used them to screen for linear B-cell epitopes. The results provided important insights that could facilitate the development of possible specific diagnostics for IBV infection and that further our understanding of the antigenic structure of N protein.

2. Materials and methods

2.1. Viruses and their propagation in specific pathogen-free embryonated eggs

IBV strain tl/CH/LDT3/03I was isolated in 2003 from a teal in Guangdong Province, China (Liu et al., 2005), and it was used for the preparation and identification of mAbs, as well as for N gene cloning and expression. To investigate the reactivity of the 2 mAbs, 25 heterogeneous IBV strains, i.e., 20 field strains and 5 vaccine strains, were used as representatives of different IBV types (Liu et al., 2006; Ma et al., 2012). The backgrounds of the 25 heterogeneous IBV strains are shown in Table 1. All IBV strains were propagated once in 9–11-day-old specific pathogen-free (SPF) embryonated chicken eggs and the presence of viral particles in the allantoic fluids of inoculated eggs was confirmed using a negative contrast electron microscope (JEM-1200, EX) and by RT-PCR, as previously described (Liu and Kong, 2004).

Newcastle disease virus (NDV) La Sota vaccine strain, infectious laryngotracheitis virus (ILTV) (Tong et al., 2001) and subtype H9 avian influenza virus (AIV) (Yu et al., 2008) were used for evaluating the cross-reactivity with the 2 mAbs. All these virus strains were propagated once in 9–11-day-old SPF embryonated chicken eggs and the presence of NDV and subtype H9 AIV viral particles in the allantoic fluids of inoculated eggs was confirmed by HI using specific antibodies, respectively (Majiyagbe and Hitchner, 1977). The ILTV was confirmed by RT-PCR as previously described (Tong et al., 2001).

Fertile white Leghorn embryonated SPF chicken eggs were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China.

2.2. Generation and identification of mAbs

Six 8-week-old BALB/c female mice were immunized subcutaneously with condensed IBV tl/CH/LDT3/03I virus-infected allantoic fluids (Yu et al., 2010) mixed with Freund's complete adjuvant, followed by two booster immunizations. The protocols used for the preparation of mAbs and ascetic fluids were as previously described (Ruf et al., 1983; Vilella et al., 1983; Yu et al., 2010). All hybridomas were cloned via at least 3 rounds of limiting dilution. Primary screening of hybridomas was by enzyme-linked immunosorbent assay (ELISA) using a commercial total antibody ELISA kit (IDEXX Corporation, Westbrook, ME, USA), according to the manufacturer's instructions. The mAbs were reacted with both IBV tl/CH/LDT3/03I virus particles and recombinant N protein as coating antigens for ELISA and Western blotting, respectively. The mAb classes and subclasses were determined using an SBA Clonotyping System/HRP kit (Southern Biotechnology Associates, Birmingham, AL, USA). Two mAbs, designated as 6D10 and 4F10, were identified and used for further fine-level epitope mapping.

Table 1
Background information of IBV strains used in Western blotting in the present study.

IBV strain	Country[a]	Year[b]	Type	GenBank accession number
H120	Vaccine	–	Mass	AY856349
H94	Vaccine	–	Mass	EF602438
IBN	Vaccine	–	Mass	AY856349
M41	US	1965	Mass	FJ904720
CK/CH/LHN/00I	China (Henan)	2000	N1/62 associated strain	EF602456
JAAS	Vaccine	–	N1/62 associated strain	AY839138
J9	Vaccine	–	N1/62 associated strain	EF602440
CK/CH/LDL/97I	China (Dalian)	1997	CK/CH/LDL/97I	EF602445
tl/CH/LDT3/03	China (Guangdong)	2003	tl/CH/LDT3/03	AY702975
CK/CH/LHLJ/04V	China (Heilongjiang)	2004	LX4	FJ821744/FJ821725
CK/CH/LSD/03I	China (Shandong)	2003	LX4	EF602457
CK/CH/LTJ/95I	China (Tianjin)	1995	LX4	DQ287917
CK/CH/LGD/04II	China (Guangdong)	2004	LX4	EF602444
CK/CH/LXJ/02I	China (Xinjiang)	2002	LX4	EF602458
CK/CH/LLN/98I	China (Liaoning)	1998	LX4	EF602451
LX4	China (Xinjiang)	1999	LX4	AY338732
TW2575/98	China (Taiwan)	1998	TW-II	AY606327
CK/CH/LGD/04III	China (Guangdong)	2004	Variant	EF602447
CK/CH/LSD/05I	China (Shandong)	2005	Variant	EU637854/EU637824

[a] Country (province) where the viruses were isolated.
[b] Year when viruses were isolated.

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