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Virus neutralization study using H120, H52, 793/B antisera against Iranian infectious bronchitis virus genotypes

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Summary. – Infectious bronchitis virus (IBV), a major pathogen of the domestic fowl, exhibits extensive antigenic variation. IBV is a member of the *Coronaviridae* family and the genus *Gammacoronavirus*. A new infectious bronchitis virus serotype can emerge from only very few amino acid changes within the major peplomer glycoprotein, namely in its S1 part forming the virion spike. Principally, the serotypes are identified by virus neutralization (VN) tests. This study is aimed to investigate the neutralizing efficiency of H52, H120, and 4/91 antiserum against IBV genotypes (IS-1494, IS-720, 793/B, IR-1) recently circulating in Iran. For the first time, we have used cross-neutralization tests for the serological classification of these isolates. In this study, all antisera failed to neutralize all IBV strains. According to the results of our research, cross-protection studies are necessary for the design of a proper vaccination program for IBV circulating genotypes in Iran. The data are useful for the development of new vaccine strategies.

Keywords: avian infectious bronchitis; Iran; virus neutralization

Introduction

Infectious bronchitis (IB) is an acute, highly contagious viral respiratory disease of chickens characterized by coughing, sneezing, and tracheal rales. Infectious bronchitis virus (IBV) causes poor weight gain and feed efficiency (Cavanagh, 2007). IBV is a member of the *Coronaviridae* family and the genus *Gammacoronavirus*. Coronaviruses are enveloped viruses that replicate in the cell cytoplasm and contain a non-segmented, single-stranded, positive-sense RNA genome of 28–32 kb (Armesto *et al*., 2011). The IBV genome encodes of four major structural proteins: the phosphorylated nucleocapsid (N) protein, the membrane (M) glycoprotein, the envelope (E) protein, and the spike (S) glycoprotein (Liu *et al*., 2006). The appearance of antigenic variants of IBV causes a major problem in the poultry industry. Natural outbreaks of IBV are often the results of infections with strains that serologically differ from the vaccine strains (Xu *et al*., 2007). IBV is not a single homogeneous type but occurs in different serotypes. Variant serotypes continue to be associated with outbreaks of disease in many countries (Kumar *et al*., 2007). Spike (S) antigen, which is known as the main immunogenic antigen of IBV, has two parts called S1 and S2. The S1, which is the most ever-changing part, is responsible for viral variations and recombinations. There are hundreds of IBV serotypes, and the majority of them differ from each other by 20%–25% of S1 amino acids (Cavanagh *et al*., 1997; Jackwood *et al*., 2001). However, some serotypes differ by approximately 50% of S1 amino acids (Bande *et al*., 2015). The S1 subunit of the S protein is the main inducer of protective immunity, and it carries the majority of the virus neutralizing epitopes, including serotype-specific epitopes, which are usually conformation-dependent (Mockett *et al*., 1984). New variant strains may differ as much as 55% in their S1 amino acid sequences compared with vaccine

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Abbreviations: IB = infectious bronchitis; IBV = IB virus; NI = neutralization index; VN = virus neutralization

strains (Gelb *et al.*, 2005; Kusters *et al.*, 1989). The concept of classifying strains according to their "prototype" has been proposed. However, given the logistical limitations (facilities and costs) of performing cross-challenge tests in chickens, scientists must often rely on using *in vitro* tests to establish the relationship of IBV field strains and vaccines (Ladman *et al.*, 2006). The first isolation of IBV from Iranian chicken flocks was reported in 1994. Later, several Iranian researchers identified the 793/B serotype. This serotype turned out to be one of the predominant types of IBV in Iran (Bijanzad *et al.*, 2013). Until now, several genotypes of IBV (Massachusetts, 793/B, QX, IS-1494 like, IS-720 like, IR-1, and IR-2) have been reported in Iran (Hosseini *et al.*, 2015; Najafi *et al.*, 2015). At present, different vaccines (Massachusetts and 793/B type) are used in Iranian poultry farms, but IB outbreaks are still present. Despite extensive studies on genotyping of Iranian IBV isolates, no serotyping study has been done.

Materials and Methods

Sample selection. IBV was isolated from clinical samples (trachea and kidney from IB-suspected broiler flocks) during 2014–2015 in SPF chicken embryonic eggs. After confirmation, we identified the genotypes of the isolates based on S1 gene according to the methods that were described previously (data not shown) (Najafi *et al.*, 2015). The isolates were selected based on their virus genotype. Information about the isolates may be found in Table 1. We ensured that the samples were free from contamination by other avian pathogens using PCR and culture assays detecting Newcastle disease virus, avian influenza virus, reovirus, infectious bursal disease virus, adenovirus, infectious laryngotracheitis virus, bacteria, and mycoplasma *spp*.

Antisera preparation. The antisera to the Iranian IBV isolates, 793/B, H120, and H52 types, were made in SPF chickens hatched and reared in isolators kept in a Horsfall isolation unit. Each three-week-old SPF chicken was inoculated intratracheally with $10^{\wedge 5}$ EID_{50} of IBV. The chickens were inoculated by intravenous injection of $10^{\wedge 5}$ EID_{50} of the IBV strains at the age of five weeks. Blood was obtained two weeks after the last inoculation, and the collected serum was heat inactivated (at 56°C for 30 min) and stored at -20°C (Wang and Huang, 2000). (According to University of Tehran Animal Protocol, UT#AN53)

Virus neutralization. A one-direction virus neutralization, constant-virus, and constant-serum procedure developed by Cowen and Hitchner were used for serotyping in SPF chicken embryos (Cowen and Hitchner, 1975). One hundred EID_{50} of the virus isolates reacted with 20 units of anti-H120 and anti-Conn antisera. Viruses were considered to be of the same serotype if the serum protected 50% or more of the embryos. A cross-virus neutralization (VN) test was then performed. The beta VN method with the constant virus and diluted serum was employed in SPF chicken embryos for serotyping. Serial four-fold dilutions of serum were incubated with 100 EID_{50} of IBV at room temperature for 1 h. Virus-serum mixtures were inoculated into the allantoic cavity of SPF chicken embryos. The embryos were observed for seven days. Eight days after inoculation, the eggs were opened and examined for lesions regarded as typical for infection with IBV. By definition, neutralization index (NI) values equal to or less than 2.0 represent no neutralization.

Bioinformatics analysis. The multiple alignments of partial S1 amino acid sequences were constructed by using the MEGA 5.1 software with the Clustal W method, and a homology matrix was produced using a consensus of 1000 bootstrap replicates (Tamura *et al.*, 2011). The nucleotide sequences of a partial segment of the S1 gene were compared with S1 sequences from a gene bank: H120 (KR605489), H52 (KR605488), and 793/B (AF093793). The correlation between S1 partial amino acid homology and the NI was calculated by Microsoft Excel software.

Results

After genotyping results based on sequence of S1 gene, we detected IR-1, IS-1494, IS-720, and 793/B in the samples. As the mentioned genotypes were circulating in Iran, we decided to do VN on these isolates.

By definition, NI values equal to or are less than 2.0, represent no neutralization. In this study, all used antisera failed to neutralize all genotypes, and there was no difference between any antiserum. NI values are presented in Table 2. Most cross-reactions were more or less heterologous. The amino acid homology and alignment are available in Table 3 and Fig. 1. The homology is low between Iranian IBV field isolates and vaccine IBV strains. The correlation (R-value) between amino acid homology and the NI index is high and around 0.9 (Fig. 2).

Table 1. Description of avian infectious bronchitis virus strains investigated in this study

Name	Type of flock	Tissue	Genotype	Passage No.	Acc. No.
UTIVO-96	broiler	trachea	IS-1494	5	KT583587
UTIVO-120	broiler	trachea	IR-1 like	5	KT583582
UTIVO-15	broiler	kidney	IS720	6	KT583583
UTIVO-21	broiler	trachea	793/B	5	KT583574

Table 2. Cross-neutralization (neutralization index, NI) of new Iranian infectious bronchitis virus isolates by antisera to 793/B, H120, and H52 types

Antigen	H52	H120	793/B vaccine type	UTIVO-96	UTIVO-120	UTIVO-15	UTIVO-21
UTIVO-96 (IS-1494)	1.2	1	1.5	5	ND	ND	ND
UTIVO-120 (IR-1)	1.4	1	2	ND	5	ND	ND
UTIVO-15 (IS-720)	0.9	0.8	1	ND	ND	5.5	ND
UTIVO-21 (793/B)	ND	1	3.5	ND	ND	ND	5.5

The NI is obtained by dividing the virus titration titre by the virus and antibody mixture titre. ND = not detected.

Table 3. Amino acid homology between Iranian IBV isolates H120, H52, and 793/B vaccine strain

	H120(KR605489)	H52(KR605488)	793/B(AF093793)
793 B/UTIVO/21 (KT583574)	73.03	73.03	100
IR-1/UTIVO/120 (KT583582)	75.28	75.28	79.77
IS-720/UTIVO/15 (KT583583)	74.15	74.15	80.89
IS-1494UTIVO-96 (KT583587)	77.52	77.52	82.02

Discussion

Avian infectious bronchitis virus (IBV) causes a highly contagious disease in chickens. It mainly affects the respiratory tract and frequently causes damage to the kidneys and the reproductive system (Jackwood, 2012). New variants of IBV have emerged due to spontaneous mutations and recombinations during virus replication, followed by replication of those phenotypes favored by selection (Dhama *et al.*, 2014). Currently, in Iran, different vaccine serotypes, Massachusetts and 793/B, are used. According to different vaccination programs that take place throughout the country, the high-performance program is very important. However, there have been frequent reports of IB-suspected cases from all over the country because of immune failure. The reason for this immune failure is the poor cross-protection between the field virus and the vaccine strain, as well as the continual emergence of new variants (Najafi *et al.*, 2015). The most antigenic of the virus neutralization antibody-inducing epitopes is formed by a few amino acids that occur in the first and third quarters of the S1 subunit, especially in the hypervariable regions (HVR) (Wang and Huang, 2000). Vasfi-Marandi and Bozorgmehrifard (2001) did the first VN study on Iranian IBV isolates between 1997–2000. Thirty-seven IBVs were isolated in embryonated chicken eggs. The group-specific polyclonal antiserum was produced and used in dot-immunoblotting and immunofluorescent assays. There was 80% similarity between these tests in the identification of IBV field isolates. A field IBV strain designated as 2100/I was selected for comparison of the NI with H120, a pooled and negative control sera. The NI of the 2100/1 strain through the use of pooled antiserum was 2.4 times greater than that of the H120 Massachusetts antiserum, indicating that this strain may antigenically differ from the H120 Massachusetts strain (Vasfi-Marandi and Bozorgmehrifard, 2001). Carrying out the VN test on IBV isolates has a long history in the world. In 1975, Johnson and Marquardt worked on the neutralizing characteristics of strains of IBV (Massachusetts 41, Connecticut, Iowa 97, Iowa 609, Holte, JMK, Clark 333, SE 17, Florida, and Arkansas 99) as measured by the constant-virus variable-serum method in chicken tracheal cultures. These viruses were all proved to be serologically distinct by this method

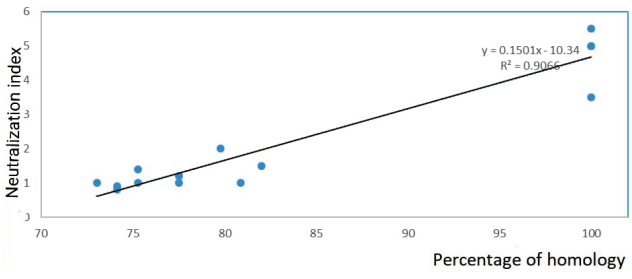

Fig. 1

Comparison of the amino acid sequence of an 89-residue fragment of the S1 polypeptide of Iranian IBV isolates and H120 (KR605489), H52 (KR605488), and 793/B (AF093793) isolates

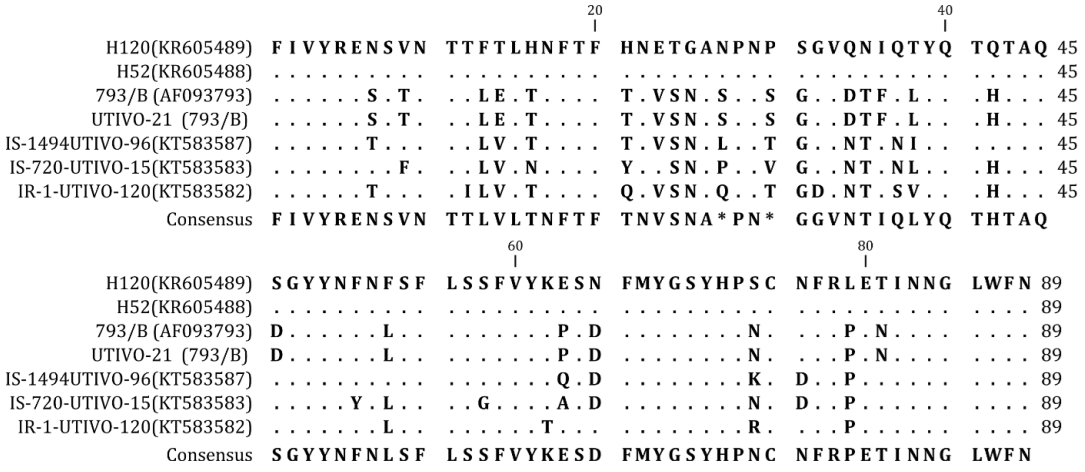

Fig. 2

Correlation between S1 partial amino acid homology and neutralization index

(Johnson and Marquardt, 1975). Between 1976 and 1980, 24 isolates of IBV were obtained from Quebec flocks. The serological classification of these isolates was demonstrated through cross-neutralization tests using antisera to compare 13 different reference virus strains. Of the 24 isolates, ten were identified as Connecticut, six as Holland, and one as SE-17 type. Seven strains did not react with any of the specific antisera (Marsolais and Marois, 1982). Otsuki did a study on the serological relationship among ten Japanese strains of avian IBV. Most cross-reactions were partially heterologously showing a one-way-relationship; heterologous relationships were observed less frequently than with chicken sera (Otsuki *et al.*, 1987). Through cross-neutralization tests, the Hungarian isolates differed from IBV reference strains M41 and H52 and can be considered distinct variants (Al Tarcha *et al.*, 1989). Fifteen isolates of IBV were made from a total of 126 Brazilian poultry flocks of all ages that were examined. One of the isolates was of the Massachusetts serotype. The remainder were examined using cross-neutralization tests in tracheal organ cultures and were shown to belong to at least four antigenic groups, all different from ones described previously in other countries (Di Fabio *et al.*, 2000). Ducatez characterized a new genotype and serotype of IBV in Western Africa (Ducatez *et al.*, 2009). Phylogenetic analysis of full-length sequences of the S1 gene revealed a new genotype of IBV that we refer to as "IBADAN" (NGA/A116E7/2006). NGA/A116E7/2006 did not cross-react with antisera against IT02, M41, D274, Connecticut, or 793/B strains in VN assays. NGA/A116E7/2006 cross-reacted with the QX-like strain ITA/90254/2005 but only to a low level (antigenic relatedness of 33%), suggesting that IBADAN also represents a new serotype (Ducatez *et al.*, 2009). Ladman's findings indicated that partial S1 sequence identity values were more strongly correlated with protective relatedness values than with antigenic relatedness values (Ladman *et al.*, 2006). To group IBV isolates, a genetic grouping method based on hypervariable region 1 (HVR 1, nucleotides 168 to 197) was compared with that based on the whole S1 gene. Therefore, the grouping method based on HVR 1 could represent the grouping method based on the whole S1 gene. Taiwan isolates could not be placed within the existing groups. To test the correlation between genotype and serotype, a one-way VN test was used to compare nine Taiwan isolates selected from different genotypes with Massachusetts (H120) and Connecticut (Conn) standard strains. Also, a two-way cross-neutralization test was performed on chicken embryonated eggs with the beta method (constant virus, diluted serum), and the reciprocal neutralization titers were calculated to give the relatedness (r) values. The results of two kinds of neutralizing tests showed that the serotypes of nine isolates were different from H120 or Conn. Based on the R-values, nine isolates were divided into two serotypes which correlated with their genotypes (Wang and Huang, 2000). According to the results of our study, to evaluate the cross-protective immunity, antisera are used in combination. In addition, cross-protection studies are necessary for the design of a proper vaccination program for IBV circulating genotypes in Iran.

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