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Original Article

Novel RFLP Method for Detection of Four Avian Infectious Bronchitis Virus Genotypes (Massachusetts, 793/B, Variant2, and QX)

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Abstract

Background and Aims: Infectious bronchitis (IB) is one of the significant economically critical poultry diseases distributed worldwide, including Iran. Different IB virus (IBV) genotypes are circulating in different geographical regions. Typing of IBV strains is useful for implementation of control measures and for understanding the epidemiology and evolution of IBV. The S1 gene sequencing is used for IBV genotyping. Massachusetts, 793/B, QX, and IS-14194 IBV genotypes currently exist in Iranian poultry farms. This study aims to design an RFLP-based method to do genotyping and confirm of mentioned IBV genotypes.

Materials and Methods: After amplification of parts of the S1 gene (RT-PCR), the PCR products treated with four enzymes (SfoI: Massachusetts, BtsCI: 793/B, MspI: IS-1494, and NruI: QX) and finally visualized on agarose gel electrophoresis.

Results: Results showed 100 percent of the specificity of the newly designed method (in comparison with sequencing).

Conclusions: This method can be used for primary confirmation and fast screening of Massachusetts, 793/B, QX, and IS-14194 IBV genotypes and even in local laboratories without the need for sequencing.

Keywords: Avian Infectious Bronchitis, Iran, RFLP, Genotyping.

Introduction

Infectious bronchitis virus (IBV) belongs to the genus Gamma-coronavirus within the order Nidovirales. It is highly contagious and causes infectious bronchitis (IB) in chickens, resulting in economic losses in the poultry industry [1]. IBV genome consists of positive-stranded RNA, with 27.6 kb in length. The 3' end of IBV genome is approximately 7.0 kb in length and encodes four structural proteins, of which the spike protein (S) localized on the IBV particle surface, is composed of two subunits, namely, S1and S2 [2]. The S1 subunit is responsible for the fusion between the virus envelope and the cell membrane of the host [3]. It contains virus neutralization and serotype-specific epitopes that are formed by amino acid within the defined hypervariable region (HVR); therefore, the molecular characterization of IBV is based on an analysis of the S1 gene [4].IBV genomes are continuously evolving as a result of

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frequent point mutations and genomic recombination events [5]. Classification systems of IBV strains are divided into two major groups: functional tests, which regard the biological function of a virus; and non-functional tests, which look at the viral genome. The classification of IBV strains based on the characterization of the genome results in genotypes. The main genotyping methods include nucleotide sequencing, determining the position of enzyme cleavage sites on a relevant gene by the restriction fragment length polymorphism technique (RFLP), or detection of genotype-specific parts of the genome by RT-PCR [6].

More than 20 different IBV serotypes are differentiated worldwide that evolved from genomic insertions, deletions, substitutions, and RNA recombination of the S1 gene. Some of the variants are endemic only in particular regions, while others circulate worldwide [6]. In Iran, several serotypes and genotypes of IBV have been reported from different regions; the first isolation of IBV in Iranian chicken flocks was reported in 1994. Later, several Iranian researchers identified the 793/B serotype, which turned out to be one of the most predominant types of IBV circulating in the country [7]. Genotyping of IBV strains isolated in Iran were classified into seven distinct phylogenetic groups (Mass, 793/B like IS/1494 like, IS/720-like, QX-like, IR-1, and IR-2) based on analysis of mainly HVRs of the S1 gene [8, 9]. Since rapid detection of IBV genotypes and the sequencing is time-consuming, we developed RFLP for accurate and specific detection of Mass, 793/B, IS-1494, and QX IBV genotypes. This paper presents the new method for the rapid detection of four IBV genotypes through RFLP analysis.

Methods

Restriction enzymes selection. S1 gene sequences four genotypes Massachusetts ، 793/B ،QX and IS-1494 (var2) (Reference, global, regional, and Iranian IBVs) were extracted from NCBI® gene bank. Also, unpublished S1 sequences in our laboratory were used. After multiples alignment (MEGA 7) [10], the target region that was amplifies by Worthington's primers was selected (390 bp) [11]. For each mentioned genotypes, specific restriction enzymes were selected using NEBcutter V2.0 (http://nc2.neb.com/ NEBcutter2/). The enzymes have been shown in table.1.

IBV isolates and RNA extraction. Four to Seven IBV isolates from each mentioned IBV genotypes (table 2) were selected from the virus bank (Ghalyanchi lab, University of Tehran). Viral RNA was extracted from the allantoic fluid using a CinnaPure RNA extraction kit (Sinaclone, Iran) as recommended by the supplier[12].

cDNA synthesis and PCR for amplification of partial S1 gene. For cDNA synthesis, 1 µL (0.2 µg) of random hexamer primer (SinaClon, Iran) was added to 5 µL of extracted RNA, and the mixture was heated at 65 C for 5 minutes. 14 µL of cDNA master mix containing 7.25 µL of DEPC-treated water (SinaClon, Iran), 2 µL of dNTP mix (SinaClon, Iran), 0.25 µL of RiboLock RNase Inhibitor (Thermo Fisher Scientific, USA), 0.5 µL of Revert Aid Reverse Transcriptase (Thermo Fisher Scientific, USA), and 4 µL of 5X RT reaction buffer was added to each tube, resulting in a final volume of 20 µL [13]. Then, the mixture was incubated at 25 C for 5 min, 42 C for 60 min, 95 C for 5 min, and 4 C for 1 min. The cDNA was stored at -20 C until use.

Nested PCR was performed using spike gene primers that were designed to amplify a 390-bp fragment of the gene [11].

First-round amplification (494 bp) was performed in a final volume of 20 µL containing 2 µl of distilled water, 13 µl of Sina ClonPCR master mix (SinaClon, Iran), 2 µl of SX1 (5' CACCTAGAGGTTTGYTWG-CATG 3') and SX2 (5' TCCACCTCTATAAACAC-CYTTAC 3') primers (10 µM), and 3 µl of cDNA . The amplification was performed with a 35-cycle thermal profile (94 C for 2 min, 94 C for 30 s, 58 C for 30 s, 72 C for 30 s, and 72 C for 10 min). In the second round of the nested PCR, SX3 (5'TAATACTGGYAA-TTTTTCAGATGG 3') and SX4 (5' AATAC-AGATTGCTTACAACCACC3') primers were used.

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The second round of amplification was performed in a volume of 20 µL (4.5 µL of distilled water, 13 µL of SinaClon PCR master mix (SinaClon, Iran), 2 µL of SX3 and SX4 primers (10 µM), and 0.5 µL of the first-round PCR product). The reaction was carried out under the same cycling conditions.

PCR product digestions. PCR products were treated with four (SfoI, BtsCI, MspI, and NruI)enzymes according to the instructions. The supplier of all enzymes was Thermo Scientific™ (Thermo Fisher Scientific, USA). For digestion with each endonuclease 4 µl of purified PCR product and 1 unit of each SfoI, BtsCI, MspI, and NruI in separate tubes were mixed and incubated at 37 ˚C for three hours. PCR products was run on 2% agarose gel, and results were recorded according to restriction sites (Table 1). Restriction band sizes were calculated by visual comparison to the molecular weight marker GeneRuler 100 bp (Thermo Fisher Scientific, USA).

Results

We analyzed 24 IBV samples from four IBV genotypes (Massachusetts ،793/B ،QX and IS-1494 (var2)), including Iranian and Iraqi IBV isolates and vicinal stains. You can find the IBVs in Table 2. After successful amplification of all IBV genotypes, the PCR product was digested by four (SfoI, BtsCI, MspI, and NruI) restriction enzymes. All enzyme digested the products (as designed and selected) with a specificity of 100%. Results were re-confirmed by restriction enzyme mapping of the S1 gene sequence (Figure 1).

Table 1. The restriction enzymes data that used for RFPLF base IBV genotyping.

Genotype	Enzyme	Size of Products after digestion
Massachusetts	SfoI	297,35
793/B	BtsCI	211,137
IS-1494 (Var -2)	MspI	298,34
QX	NruI	311,77

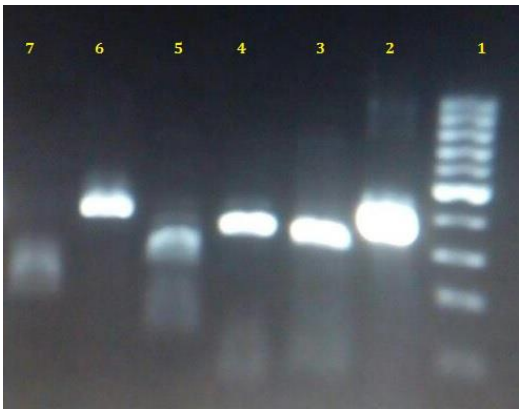


Fig. 1. RFLP pattern of different IBV genotypes .1: ladder 100 bp Fermentase, 2: undigested PCR product; 3: Var2+MSPI, 4: QX+NruI, 5: 793/B FoKI, 6: undigested PCR product .

Discussion

In recent years, the Iranian poultry industry has experienced an increased incidence of respiratory and nephritis problems with severe economic losses related to infection with IBV in flocks. Massachusetts, 793/B, QX, and IS-14194 genotypes are the most common IBV genotypes circulating in Iranian poultry farms [14]. A high frequency of antigenic variation is common in IBV, probably due to high rates of genetic mutations. The major antigenic determinant of IBV is the viral surface glycoprotein called spike (S) protein. Anti- S protein antibody protein has been known to play an important role in virus neutralization [15].

The IBV genotyping is not necessary only for understanding virus evolution but also for effective modification of the vaccination programs. In the present study, the efficacy of PCR-RFLP procedure using four different restriction enzymes were examined and compared with sequencing. It is the first time that this kind of method has been used for IBV genotyping. This study thus provides a simple and rapid method for typing of IBV. Lin et al. (1991) worked on the RFLP method for IBV genotyping. A 400 bp apart on the S1 protein gene of IBV were prepared. Using RT-PCR and RFLP, the amplified segment showed

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Table 2. The results of RFLP based genotyping of IBVs.

Name	Accession Number	Genotype	*SfoI*	*BtsCI*	*MspI*	*NruI*
H120 (Vaccine, Merial)	KR605489.1	Massachusetts	+*	-	-	-
Ma5(Vaccine, Intervet)	AY561713	Massachusetts	+	-	-	-
UTIVO-22	KT583564.1	Massachusetts	+	-	-	-
UTIVO-46	KT583565.1	Massachusetts	+	-	-	-
UTIVO-111	KT583566.1	Massachusetts	+	-	-	-
4/91 (Vaccine, Intervet)	AF093793	793/B	-	+	-	-
IB 88 (Vaccine, Merial)	KM067900.1	793/B	-	+	-	-
SGK-29	KU143912.1	793/B	-	+	-	-
UTIVO-86	KT583577.1	793/B	-	+	-	-
UTIVO-80	KT583578.1	793/B	-	+	-	-
UTIVO-108	KT583579.1	793/B	-	+	-	-
UTIVO-90	KT583599.1	793/B	-	+	-	-
UTIVO-99	KT583598.1	IS-1494 (Var -2)	-	-	+	-
UTIVO-27	KT583593.1	IS-1494 (Var -2)	-	-	+	-
UTIVO-65	KT583586.1	IS-1494 (Var -2)	-	-	+	-
UTIVO-58	KT583596.1	IS-1494 (Var -2)	-	-	+	-
UTIVO-56	KT583595.1	IS-1494 (Var -2)	-	-	+	-
UTIVO-39	KT583594.1	IS-1494 (Var -2)	-	-	+	-
SGK-5	KU143897.1	IS-1494 (Var -2)	-	-	+	-
QX Antigen	----------	QX	-	-	-	+
UTIVO-2	KT583567.1	QX	-	-	-	+
UTIVO-9	KT583569.1	QX	-	-	-	+
UTIVO-103	KT583570.1	QX	-	-	-	+
UTIVO-105	KT583571.1	QX	-	-	-	+

different cleavage patterns by the restriction enzymes. The 12 IBV strains were classified into five groups. The strain typing based on the comparison of the cleavage patterns was consistent with the previous serological typing [15].To differentiate IBV isolates in Taiwan, Wang did PCR-PFLP technique.

Two conserved sequences that flank the HVR I in the N-terminus of the S1 protein gene were chosen as primers. PCR products were digested with five restriction endonucleases, BsoFI, DdeI, MboII, AluI, RsaI . The data from PCR-RFLP and sequencing of IBV genomes showed that the 24 Taiwan isolates could be divided into two distinct groups, I and II. Seven RFLP patterns are identified in group I and only 1 in group II [16]. The entire S1 glycoprotein gene of twelve Brazilian isolates and one reference vaccine strain of IBV was analyzed by (RT-PCR-RFLP), using the restriction enzymes HaeIII, XcmI, and BstyI. The RFLP patterns led to the classification of these isolates into five distinct genotypes: A, B, C, D, and Massachusetts [17]. Kwon et al. used RT-PCR-RFLP to differentiate between serotypes of several IBV strains A sequence of 1720 base pairs (bp) that contains the S1 gene of IBV was amplified by PCR, purified, and digested with restriction enzymes. Eleven reference IBV strains were grouped according to the RFLP patterns. The IBV Holte, Arkansas DPI, SE 17, Md 27, and Iowa 97 strains could be differentiated from the other IBVs Strains using the restriction enzyme HaeIII. The

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Beaudette, Massachusetts, Connecticut, and Florida 88 strains had the same HaeIII RFLP pattern but could be differentiated using XcmI and BstYI restriction enzymes. The Gray and JMK strains could not be differentiated by their RFLP patterns following digestion with 23 different restriction enzymes [18]. RT-PCR-RFLP classified forty Korean IBV isolates and four reference strains. RFLP patterns of an amplified DNA fragment (1722 bp) containing the S1 gene of IBV digested by restriction enzyme HaeIII showed that the Korean isolates were classified into five genotypes, I to V [19]. Lee et al. (2003) after working on 7un-typed field IBV isolates obtained in 1999, and 2000 with RT-PCR-RFLP found that typing this method will serve as a fast typing method for IBV diagnosis [20]. Bouqdaoui et al. reported five different RFLP patterns based on the S1 gene analysis of 30 IBV field isolates in Morocco [21]. In a report by Callison et al., in the United States, 11 IBV strains from US foreign places analyzed based on their S1 gene RFLP patterns, and they showed eight different RFLP patterns [22]. In Iran, three regions of the genome of IBV vaccine and field isolates including S1 gene, gene 3 and nucleocapsid (N) gene along with 3' untranslated region (3' UTR) were amplified and subjected to RFLP using three different endonucleases (AluI, RsaI, and MnlI). The results revealed that RFLP analysis of N-3'UTR and S1 gene had higher discriminatory power than gene 3 [23].

The PCR-RFLP procedures based on only one part of the IBV genome may have limited sensitivity for discrimination of IBV vaccine strains and field isolates. Also, our developed RFLP method shows promising results and provide quick and cheap genotyping without sequencing. According to the IBV effects on poultry farms (layer and breeders), this method can diagnose these genotypes quickly and without the need for sequencing. If we can detect or suspected some genotype, especially QX in one-two weeks of layer and breeder chicken, we could prevent false layer. The developed approach can be used in the local laboratories, even without professional equip-ment.

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