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Molecular Comparison of Three Different Regions of the Genome of Infectious Bronchitis Virus Field Isolates and Vaccine Strains

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Abstract

Rapid detection and differentiation of infectious bronchitis virus (IBV) involved in the disease outbreak is very important for controlling disease and developing new vaccines. In the present study, 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 restriction fragment length polymorphism (RFLP) using three different endonucleases. Amplicons from S1 gene and N-3'UTR generated four RFLP patterns, grouping IBV strains into four similar groups, while amplicons of gene 3 generated three RFLP patterns classifying examined IBVs in different groups from those of S1 and N-3' UTR. 4/91 strain and MNS-7862-1field isolate both belong to 793/B serotype were differentiated from each other based on gene 3, N-3'UTR and S1gene. IBVs belonged to different serotypes showed different RFLP patterns based on RFLP patterns of all three regions. S1 gene and N-3'UTR RFLP analysis differentiated IB88, MNS-7862-1 and 4/91 from each other. This is the first report on the molecular analysis of the gene 3 for IBV strain differentiation. Our results revealed that RFLP analysis of N-3'UTR and S1 gene had the higher discriminatory power than gene 3. None of the RFLP patterns of different regions differentiated 4/91 vaccine strain from its field isolate.

Keywords: Infectious bronchitis virus, RT-PCR, RFLP, Gene 3, N gene, 3'UTR

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Introduction

Infectious bronchitis is an acute, highly contagious, viral disease of poultry with worldwide distribution.[1-3] The causative agent is infectious bronchitis virus (IBV), a corona virus which primarily infects respiratory tract, though some isolates replicate in the kidney and oviduct, resulting in nephritis and reduced egg production.[4]

The genome of IBV, a member of the family *Coronaviridae*, contains a single-stranded positive sense RNA of 27.6 kb.[5,6] Four major structural proteins, the glycosylated spike (S) protein, the envelope or small membrane (E) protein, the membrane (M) protein, and the phosphorylated nucleocapsid (N) protein make up the IBV virions. Four non-structural proteins (3a, 3b, 5a, and 5b) are also encoded.[2,7] Typically, the disease has been controlled with serotype-specific vaccines.[8]

Prevention of the disease heavily depends on vaccination using attenuated viruses prepared according to circulating strains in the region. Although commercial poultry flocks are routinely vaccinated for IBV, outbreaks of infectious bronchitis still happen due to naturally occurring variant viruses that continue to arise[8] and although many countries share some common antigenic types, IBV strains within a geographic region are unique and distinct.[9] Generally, different serotypes do not cross-protect. Therefore, the serotype of the virus causing the disease must first be determined so that the birds can be properly vaccinated. Reverse transcription-polymerase chain reaction (RT-PCR) along with restriction fragment length polymorphism (RFLP) is a rapid technique for detection IBV strains and has led to the identification of a tremendous number of virus isolates, which was not possible with the traditional virus-neutralization test in embryonating eggs.[8]

The purpose of the present study was to genetically characterize vaccine strains and field isolates of IBV and investigate the similarity between these IBVs based on three different region of their genome including S1 gene, gene 3 and nucleocapsid (N) gene with part of 3'UTR (N-3'UTR) using PCR-RFLP technique.

Materials and Methods

Viruses. Two field isolates and five vaccine strains were used in this study. Field isolate MNS-7862-1, isolated from a commercial broiler farm with respiratory signs in Tehran was obtained from Faculty of Veterinary Medicine (Tehran University, Iran) and field isolate 4/91 isolated from a commercial broiler flock of 2-18 weeks of age was provided by Razi Vaccine and Serum Research Institute (Karaj, Iran). The IBV vaccine strains H120, MA5 and 4/91 were obtained from the Intervet Ltd (The Netherlands), vaccine H52 was obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran) and IB88 was obtained from Merial Ltd (France). All vaccine strains examined in this study are in use in poultry industry in Iran.

Viral RNA extraction. Viral RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. About 50 µl of each virus suspension was used for each extraction, and purified RNA was resuspended in 30 µl elution buffer and used immediately for cDNA synthesis or stored at -70 ˚C.

Synthesis of cDNA. cDNAs were synthesized in 25 µl reaction mixture, according to previous studies[10] with minor modifications. For each cDNA synthesis reaction, 5 µl of extracted RNA was mixed with 1 µl oligo (dt) (25 µM) (Fermentas, Cinnagen, Iran).The premix was incubated at 100 ˚C for 1 min, subsequently the tubes were placed on ice for 5 min. After incubation on ice, an amount of 19 µl premix containing 24 U RNA guard (Fermentas, Cinnagen, Iran) 50 µM each of dATP, dTTP, dGTP and dCTP, 5 µl of

5 X reaction buffer (Fermentas) and 200 U moloney murine leukaemia virus reverse transcriptase (MMLV) (Fermentas, Cinnagen, Iran), was added to each tube. The reaction mixtures were incubated at 42 ˚C for 1 hour followed by inactivation of the reverse transcriptase enzyme at 100 ˚C for 5 min. The resultant cDNAs was immediately used in a PCR or stored at -70 ˚C for later use.

Polymerase Chain Reaction

Amplification of S1 gene, gene 3 and N-3'UTR. For the amplification of S1 gene, gene 3 and N-3' UTR of the IBV genome, three pairs of primers were used (Table 1). The three primers which were designed in this study were based on the genome sequence of Beaudette strain from GenBank (accession number: NC_001451.1). The PCR reactions was carried out in 50µl mixture containing 50 µM each of dNTP, 0.5 µM each of primers, 5µl of 10X PCR buffer (CinnaGen, Iran), 2 mM magnesium chloride, 5 U SmarTaq DNA polymerase (CinnaGen, Iran) and 6 µl cDNA as template. Amplification of the S1 gene was performed using 35 cycles of incubation at 94 ˚C for 45 s, 55 ˚C for 40 s and 72 ˚C for 2min with a final extension at 72 ˚C for 5 min (this study). Amplification of gene 3 was performed using 35 cycles of incubation at 94 ˚C for 45 s, 55 ˚C for 40 s and 72 ˚C for 90 s, with a final extension at 72 ˚C for 5 min (this study). For the N-3' UTR, the PCR profiles involved an initial denaturation for 2 min at 94 ˚C followed by 35 cycles of incubation at 94 ˚C for 45 s, 55 ˚C for 40 s and 72 ˚C for 2 min with a final extension at 72 ˚C for 5 min (this study). The obtained PCR products were separated on 1.5 % agarose gel and results were observed using ultraviolet transillumination. For amplifying S1 gene and N-3'UTR a few modifications in $MgCl_2$ concentration and annealing temperature was performed and finally PCR reactions were optimized for amplifying two fragments of 1.8 kb of S1 gene and N-3'UTR. The first attempt to amplifying gene 3 (1.2 kb) of all IBV field and vaccine strains was successful. All strains examined in this study gave only one band about 1.2 kb as it was expected.

PCR product purification. Before digestion, all PCR products were purified using DNA extraction Kit (Fermentas, Cinnagen, Iran), according to kit's manufacture instructions.

Restriction Fragment Length Polymorphism. Purified PCR products for S1 gene, N-3'UTR and gene 3 were digested using two of three restriction endonuclease enzymes *Alu*I (Fermentas, Cinnagen, Iran), *Rsa*I (Fermentas, Cinnagen, Iran) and *Mnl*I (Fermentas, Cinnagen, Iran) for each part of IBV genome. For digestion with each endonuclease four µl of purified PCR product and 10 U of each *Alu*I, *Rsa*I and *Mnl*I in separate tubes were mixed and incubated at 37 ˚C for three h. Digested products were separated on 2 % agarose gel and visualized using ultraviolet transillumination.

Table 1.Primer sequences, their target gene and amplicon sizes used in this study

Primer name	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
PolyF1	GATTGTGCATGGTGGACAATG	S1 gene	1806	This study
S1-R1	CCACCAGAAACTACAAACTG			Mardani *et al*., 2006 [10]
S2-F1	GGTGGAATGATACTAAGCATG	Gene 3	1231	This study
M-R1	ACACCTACTGCAATGTTAAGGG			This study
5b-F2	CCTTTTCGCGGAGCAATAG	N-3'UTR	1829	This study
UTR-R1	CTGTACCCTCGATCGTACTC			Mardani *et al*., 2006 [10]

Results

Restriction endonuclease digestion of S1 gene and N-3'UTR . The RFLP patterns of amplified fragments of S1 gene of seven IBV strains by using *Alu*I and *Rsa*I are shown in figures 1 and 2 respectively and N-3'UTR RFLP patterns were generated using *Alu*I and *Mnl*I are illustrated in figures 3 and 4 respectively. Each enzyme produced distinguishable fragments in the range of 100-1000 base pairs. Both pair of enzymes used for digesting S1 gene and N-3'UTR PCR products were generated four distinct RFLP patterns, grouping IBV strains used in this study in exactly similar groups. Strains H120, H52 and MA5 were generated identical RFLP patterns named pattern I, 4/91 field isolate and 4/91 vaccine strain had the same RFLP pattern named pattern II and strains IB88 and MNS-7862-1 had two distinct RFLP patterns which named pattern III and IV respectively (Table 2).

Table 2. RT-PCR RFLP patterns of IBV genes S1, N-3'UTR and 3 generated using *Alu*I, *Rsa*I and *Mnl*I enzymes

Genes	Enzyme	Pattern	
S1	*Alu*I and *Rsa*I	I.	H120, H52, MA5
		II.	4/91 (F), 4/91 (V)
		III.	IB88
		IV.	MNS-7862-1
N-3'UTR	*Alu*I and *Mnl*I	I.	H120, H52, MA5
		II.	4/91 (F), 4/91 (V)
		III.	IB88
		IV.	MNS-7862-1
Gene 3	*Alu*I	I.	H120, H52, MA5
		II.	4/91 (F), 4/91 (V)
		III.	IB88, MNS-7862-1
Gene 3	*Rsa*I	I.	H120, H52, MA5
		II.	4/91 (F), 4/91 (V), MNS-7862-1
		III.	IB88

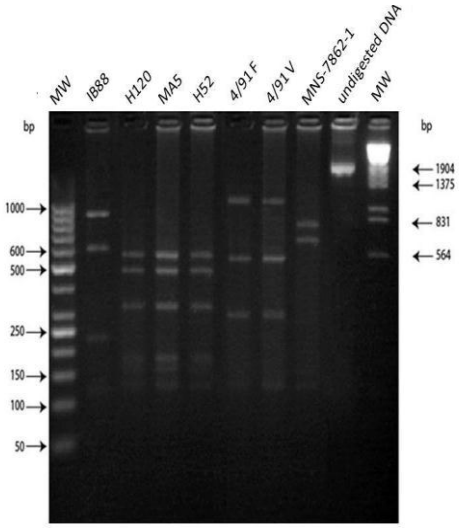

Fig 1. RFLP patterns of the S1 gene of seven IBV vaccine strains and field isolates generated using *Alu*I. First lane MW: molecular weight GeneRuler™ 50 bp DNA Ladder, 50-1000 bp, last lane MW: molecular weight Lambda *Eco*RI/*Hind*III ladder

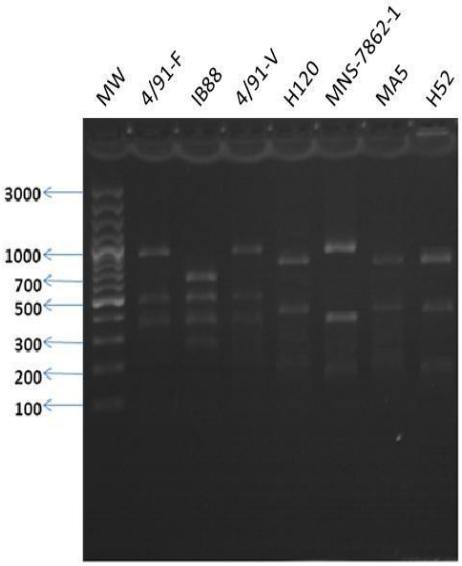

Fig 2. RFLP patterns of the gene S1 of IBV vaccine strains and field isolates generated using *Rsa*I. MW: molecular weight GeneRuler™ 100 bp DNA ladder, 100-3000 bp

Restriction endonuclease digestion of gene 3. RFLP analysis of the gene 3 of seven IBV strains using both *Alu*I and *Rsa*I enzymes generated three RFLP patterns (figures 5 and 6). However the grouping of the IBVs according to generated RFLP patterns were different based on the enzymes used. Both enzymes grouped H120, H52 and MA5 together named

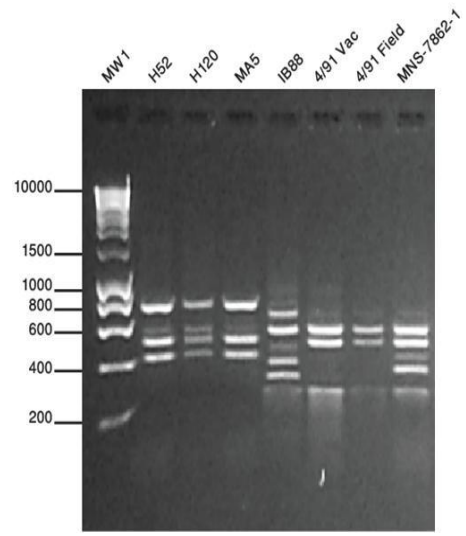

Fig 3. RFLP patterns of the N gene and 3' UTR of seven IBV vaccine and field isolates, digested with *Alu*I. MW1: molecular weight marker Bioline hyperladder I

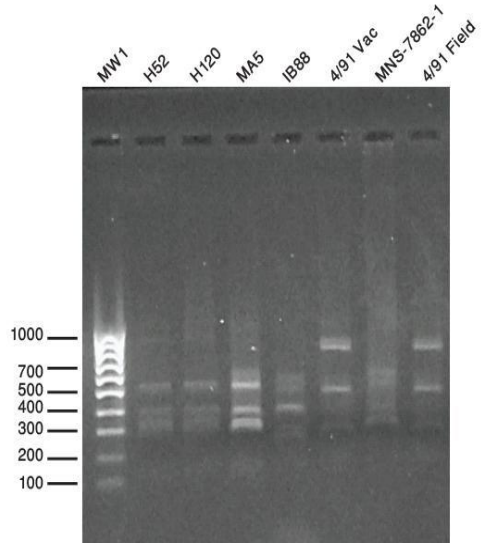

Fig 4. RFLP patterns of the N gene and 3' UTR of seven IBV vaccine and field isolates, digested with *Mnl*I. MW1: molecular weight marker Bioline hyperladder VI

pattern I, exactly same as the results obtained by analysing gene S1 and N-3' UTR. 4/91 vaccine strain and its field isolate showed identical pattern (RFLP pattern II) using both enzymes, however *Alu*I was not able to differentiate IB88 from MNS-7862-1 (RFLP pattern III) while *Rsa*I was able to discriminate these two strains from each other (Table 2).

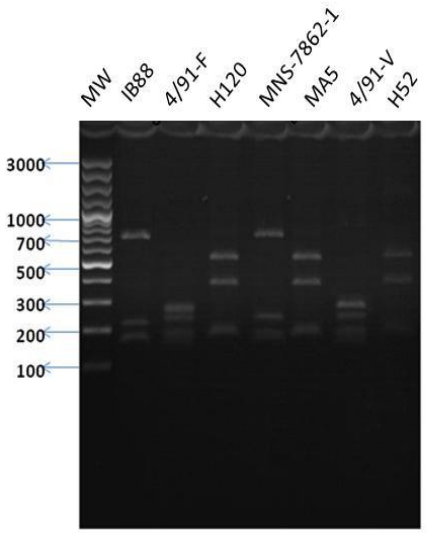

Fig 5. RFLP patterns of the gene 3 of IBV vaccine strains and field isolates generated using *Alu*I. MW: molecular weight GeneRuler™ 100 bp DNA ladder, 100-3000 bp

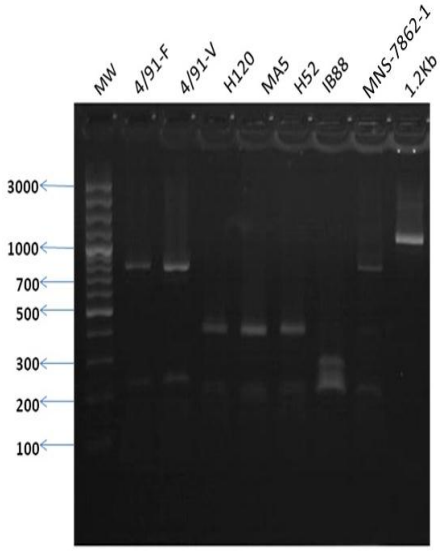

Fig 6. RFLP patterns of the gene 3 of IBV vaccine strains and field isolates generated using *Rs*I. MW: molecular weight GeneRuler™ 100 bp DNA ladder, 100-3000 bp

Discussion

One of the major problems with IBV is the frequent emergence of new variants. New variant strains of IBV have appeared all over the world in the last few years.[11-17] Because of low rate of cross-immunity between IBV strains, fast and easy identification and differentiation of new strains in different geographical areas is very important for controlling the disease with appropriate attenuated vaccines against homologous strains.

In the present study the RFLP patterns of three different regions of the IBV genome (genes S1, N-3'UTR and gene 3) were compared. All previous reports were focused on S1 gene RFLP analysis for strain differentiation and to date the RFLP analysis of the gene 3 has not been reported elsewhere. Some molecular analysis based on N gene and also 3'UTR has accomplished in a few studies.[18,19] But there is not enough information about N gene and 3'UTR of IBV stains circulating in Iran. In a report by Callison *et al*.,[8] 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. In Korea, 15 isolates was analyzed using RT-PCR RFLP analysis of the S1 gene and the results revealed four different RFLP patterns.[20] Bouqdaoui *et al*.,[21] reported five different RFLP patterns based on S1 gene analysis of 30 IBV field isolates in Morocco.

In this study, two field isolates and five vaccine strains of IBV were analyzed based on RT-PCR RFLP analysis of genes S1, N-3'UTR and 3. According to the both of S1 and N-3'UTR RFLP analysis using both restriction enzymes, strains H120, H52 and MA5 all from same serotype generated identical patterns while IB88 and MNS-7862-1 again belong to the same serotype of 793/B, generated different RFLP patterns which indicates the variation in the spike protein within the same serotype as reported by Cavanagh *et al*.[22] There are different reports about differentiating IBV strains based on N gene. In another study, Brazilian isolates clustered together in a distinct group based on N gene RFLP analysis [23] but some other surveys reported different cluster assignments for some Italian and Korean strains based on N gene and S1 molecular analysis.[24,25] The region of 3'UTR that has

been used in recent studies for differentiate the IBV strains,[26] is used in this study. According to the RT-PCR RFLP analysis of gene 3, IBV strains were classified in three different groups using both *Alu*I and *Rsa*I.

The PCR-RFLP procedures based on only one part of IBV genome may have a limited sensitivity for discrimination of IBV vaccine strains and field isolates. Furthermore, recombination is an important mechanism in the evolution of coronaviruses [27] and can occur outside the S1 gene,[28] and viruses that differ as a result of recombination are less likely to be distinguished by examination of a single gene. In the present study, the efficacy of PCR-RFLP procedure using three different regions of IBV genome simultaneously were examined and compared.

Our results revealed that RFLP analysis of S1 and N-3'UTR had the same discriminatory power. Otherwise, RFLP analysis of gene 3 couldn't differentiate IBV strains as well as two other regions used in this study. This difference is may be due to the smaller size of the gene 3 that has been used in the study (1.8 for S1 and N-3' UTR and 1.2 for gene 3). In spite of some previous studies about more conservative sequence of N gene, its RFLP analysis could differentiate strains as S1 gene. All of three genomic fragments had differentiated IBV strains and they can be used for IBV differentiation. Analysis of S1gene and N-3'UTR are better than the gene 3 for IBV discrimination. In the cases that S1 gene amplification was not successful because of the more sequence variation, N-3'UTR and gene 3 could be used for strain identification. For gene 3, using both enzymes will increase the discrimination power to the same level as S1 gene and N-3'UTR.

In conclusion, our results suggest that IBV strains could be differentiated by PCR-RFLP procedure based on different regions of IBV genome. It was also revealed that PCR-RFLP analysis of different regions of IBV genome had different discriminatory power for differentiating IBV strains. Compared with the conventional immunological methods for differentiation of IBV strains, the PCR-RFLP technique described here is quick and relatively inexpensive. It does not require isolation and propagation of the viral isolates and could be performed directly on clinical samples, as well as previously isolated viruses. Further studies will require developing a procedure for discriminating IBV vaccine strains from field isolates.

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