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GENETIC RELATIONSHIP BETWEEN MASSACHUSETTS INFECTIOUS BRONCHITIS FIELD VIRUSES AND MASSACHUSETTS VACCINE STRAINS

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ABSTRACT

BACKGROUND: Infectious Bronchitis Virus (IBV) is causative agent of a highly contagious and acute viral disease. IBV cause significant economic losses to the poultry industry in worldwide. Only a few differences in amino acids of the S1 protein of vaccine and virulent strains of IBV can cause impaired immunity of vaccinated birds.
Objective: To determine the genetic relationship between circulating field strains of infectious bronchitis virus, Massachusetts serotype with vaccine strain.
Methods: Viral RNA was extracted from infected allantoic fluid and the S1 sequence was amplified by RT-PCR. The PCR products were sequenced and their homology with the standard strain of IBV (M41, Baudette, H-120, H-52 and Ma5) derived from the gene bank were compared with BioEdite and MegAlign.
Results: Sequence analysis of isolates showed that they are closely related to Massachusetts serotype. Alignment of the S1 sequences of the isolated viruses with standard IBV strains, revealed high homology of 99% and 97% in nucleotide and amino acid sequences respectively with M41 virus. The nucleotide and amino acid sequences of 14 isolates were very close (99% - 100%) with vaccines H-120, H52, Ma5.
Conclusion: The results of this investigation suggest that comprehensive knowledge on infectious bronchitis virus circulating in different areas is essential for design and probably update the right strategy vaccination against the disease.

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Introduction

Infectious bronchitis virus (IBV) is a member of group 3 coronavirus, family Coronaviridea, orders Nidovirales. The virus possesses a positive-sense, single-stranded RNA genome and contains 27.6 kb nucleotide [1] encoding four structural proteins: the nucleocapsid protein (N), membrane glycoprotein (M), spike glycoprotein (S), a small membrane envelops protein (E)

The surface glycoprotein (S) is post-translationally cleaved into S1 and S2 subunits. The cleavage site of S1 protein in Massachusetts serotype has 5 sequential alkaline amino acids including Arg-Arg-Phe-Arg-Arg [2, 3, 4 , 5]. Highly variable regions of virus genome (HVR 1, 2 , 3) are located in S1 protein [2, 6, 1 , 7].

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HVR one and two possesses sequences which are serotype-specific and virus neutralizing indicators [1, 8]. New serotypes and variants are related to S1 glycoprotein variations that used for distinguish IBV serotypes and variants [8, 9, 10, 11, 7]. The difference between some IBV serotypes is only due to a few, 7 amino acid residues, which 5 of them located in HVR one and two regions of S1 glycoprotein [4]. Between coronaviruses, emergence of new variants has the highest frequency in IBV. Since IBV were identified in 1930 by Schalk and Hawn [12], serotypes and variants of the virus have increased and more than 50 serotypes are mentioned (2000), and new variant still are emerging [13]. However Massachusetts serotype is the main serotypes of the virus in the world and it is believed that other serotypes are derived from it therefore most poultry flocks are vaccinated with this serotype at the beginning of life.

Infectious bronchitis (IB) is an acute and highly contagious viral disease of the poultry industry with worldwide distribution [11]. IB is a disease of respiratory tract, nephritis and reproductive system and causes severe economic losses due to respiratory complications (rales in the trachea, sneezing and coughing), mortality (10% - 30%) in young chickens, reduced feed efficiency, weight loss, decreased egg production (50%) and its poor quality in laying hens, and renal failure along with losses (30%) by nephropathogenic strains [14 , 15].

Chickens are the most natural host of IBV and are infected at all ages. The virus exists in the respiratory secretions and feces of infected birds and spreads through aerosol particles. The contaminated cloths and equipment in poultry farms play a critical role in the mechanical spread and transmission of virus. IBV enters the body of birds through breathing or contaminated food and drinking water and spreads in the upper respiratory tract. This disease has long been prevalent in poultry fields in Iran [1, 10 , 14].

Prevention and control of infectious bronchitis is based on biosecurity and vaccination. Using live attenuated and killed vaccines prepared with Massachusetts serotype are common in the poultry industry in most countries.

Although high-quality live attenuated and killed vaccines have long been used to induce the initial immunity for controlling infections in industrial poultry, the disease has been reported even in vaccinated poultry due to the unpredictable cross-protection between serotypes [6 , 17]. However, a high level of cross-protection has been observed in poultry vaccinated with the available vaccines during the occurrence of the disease [17].

Despite the development and production of high quality vaccines that are used to control clinical disease, monitoring of virus variants emerged in flocks must be followed[6, 18]. Different antigenic serotype and new variants which have emerged in the field sometimes cause vaccine failure In spite of prevention programs based on using H-120 and 4/91 vaccines in Iran, several cases of disease have been observed. Therefore, to identify and characterize field isolates and better understanding of pathogenicity of IBV constant effort to control the disease should be considered. This research was performed to identify and characterize new field isolate of the IBV Massachusetts serotypes and establish relationship between field isolate and vaccine strains.

Material and Method

Samples were collected from broiler flocks with respiratory and autopsy symptoms suspected to IB disease of different organs (trachea, lungs, kidneys and cecal tonsils) in Alborz, Yazd, Tehran, Kerman, Chahar Mahal and Fars provinces in Iran. At least five sample tissues and a total of 68 samples were taken.

Virus isolation in SPF embryonated eggs:

Specimens were homogenized with TPB medium containing antibiotics, clarified by centrifugation at 1000 g at 4°C for 10 minutes and the supernatants of each sample was inoculated to the allantoic cavity of 9-11-day-old SPF eggs [14]. On day 2 after inoculation, three eggs were chilled at 4°C, and the allantoic fluids were harvested and stored at −40°C until RNA extraction and RT-PCR test. The remaining eggs were further incubated till seven days post inoculation and observed for typical embryo lesions of IBV such as dwarfing, stunting, and curling. The stored allantoic fluids were further passage 3 to 5 times into specific-pathogen-free eggs before samples, free of any specific lesions, were declared negatives.

RNA Extraction

Viral nucleic acid (RNA) was extracted from the allantoic fluid of inoculated eggs using RNA purification kit (High Pure Viral RNA Kit, Roche, Inc.) and was stored at -80˚C in diethyl pyrocarbonate–treated water until used.

RT-PCR (Reverse Transcription-polymerase Chain Reaction)

RT-PCR was carried out on the viral RNAs using the Roche Titan One tube RT-PCR kit. Amplification of the S1 gene was performed using two sets of primer flanking a variable region between strains in virus genome [4]. These primers are SX1+, SX2-, SX3+ and SX4- which proliferate 497 and 393 nucleotide parts of S1 gene (Table 1).

Table 1. Primers used for RT-PCR reaction of IBV

Polymer	Sequence	Position in Gene*
SX1+	5'-CACCTAGAGGTTTG T/C T A/T GCAT-3'	677-698
SX2-	5'-TCCACCTCTATAAACACC C/T TT-3'	1148-1168
SX3+	5'-TAATACTGG C/T AATTTTTCAGA-3'	705-725
SX4-	5'-AATAC AGATTGCTTACAACCACC-3'	1075-1097

*Nucleotide positions matches with the strain sequence of UK/7/93 with accession number of Z83979 in gene bank.

Sequence Analysis of Isolates

Analysis of sequence data were conducted using the BioEdit program. Nucleotide and amino acid deduced sequences were aligned using ClustalW. Phylogenetic analyses and tree construction, performed by the neighbor-joining method with 1000 bootstrap replicates, were conducted with MEGA software (fig 1). The phylogenetic tree of the aforesaid strains was plotted in comparison to the reference Massachusetts virus and their similarities and differences in nucleotide and amino acid of the strains were identified using sequence distance (Table 2).

GenBank accession numbers of the reference strains:

M41 (X04722), H120 (FJ807652); H-52 (AF352315.), Ma5 (45549460), Baudette 42 (DQ830981)

Table 2. Similarity (%) of nucleotide and amino acid between standard strains and isolates

Strains	Nucleotide distance range (%) in the group	Amino acid distance range (%) in the group
Isolates	0.0 – 0.02	0.0 – 0.03
Vaccine Strains	0.0 -0.04	0.0 – 0.06
M41 Virus	0.01 – 0.03	0.01 – 0.05

Results and Discussion

Infectious bronchitis disease is controlled mainly by vaccination with live attenuated or inactivated vaccines mostly based on Massachusetts serotypes. IB vaccination programs must be based on the characterization and identification of IBV type in the field [19] Since the nucleotide differences between S1 gene of virus isolates are sufficient to change the virus serotypes and the cross-protection between the serotypes is poor [10 , 3], it is important to identify the field viruses for the selection of the best vaccine to control the disease.

The present study was intended to show the status of Massachusetts IBV serotypes in Iran by identification of Massachusetts virus isolates and investigate their relationship with reference strains. During 2011-2012, about 14 IBV isolates were isolated from the trachea, lungs, kidney and cecal tonsils of broiler chickens with suspected clinical symptoms in different provinces.

As shown in Table 1, the primers used in RT-PCR were common for most detected IBVs and cover a S1-gene region that has already been reported [20]. These primers were standardized by reference genotypes. The region of S1gene that is surrounded by universal primers varies for each individual genotypes of IBV. These primers are SX1+, SX2-, SX3+ and SX4- which primed 497 and 393 nucleotide segments of S1 gene and confirm the identity of 14 virus isolates.

The nucleotide sequence of S1 HVR region of 14 isolates was determined and compared with each other (497 and 393 nucleotide parts) and their nucleotide sequence showed 98% to 100% homology and they all belonged to one genotype (Figure 1).

The nucleotide sequence of 128, 289, 301 and 302 isolates have 100% homology. The homology of isolates 322 and 253 was 100%. The isolates 90, 253, 306 and 71 had the least homology (98%). The comparison of nucleotide sequence of S1-gene isolates with standard viruses (M41, H-120, H-52, Ma5 and Baudette) showed that they are 97% to 100% homologous. The isolates 128 were similar to the vaccine strains by 100%. The isolates 104 and 128 have 99% homology with M41 virus while other isolates had the least homology with M41 (98%) and the most homology with Ma5, H-120 and H52 viruses (99%).

The amino acids sequences of all isolates have 97% to 100% homology. The isolates 104, 128, 301, 302 and 289 as well as the isolates of 253, 322 and 71 were 100% homologous with each other (Figure 2). The isolates 104 and 301 (Yazd), 322 and 166 (Tehran), 253 (Kerman), 289 (Chahar Mahal), 128 (Fars), and 70, 17 and 43 (Alborz) were 99% homologous with M41, H-120, H-51 and Ma5 viruses.

There was 100% homology between the amino acid sequence of genotypes 104 (Yazd), 128 (Fars), and 253 (Kerman) and vaccine viruses of H-120, H-52 and Ma5 belonging to Massachusetts serotype. The results of phylogeny (Figure 3) showed that the isolates of 104, 128 and 253 belonged to the same branch where H-120 was located; therefore, it is concluded that these isolates are the vaccine strains of H-120. On the other hand, the isolates of 43, 70 and 17 were homologous with M41 viruses and H-120 and H-52 vaccine viruses respectively by 96% and 99%. The isolates of 301, 306, 322 and 71 had the least homology with standard viruses (98%) and the isolates of 90 and 302 had the least homology with M41 (97%).

Amino acid sequence comparison of proliferated segment of S1 glycopeptide of 14 isolates with standard viruses, showed that similar to M41 virus, the amino acid 266 was transformed from isoleucine to threonine in 14 isolates. (Figure 2)

In three isolates of 70, 43 and 17 in which whole of the S1 gene was amplified and its sequence was determined, the transformation of amino acid was completely similar to vaccine viruses with some exceptions. In isolate 17, similar to M41 virus the amino acid 39, was transformed from alanine to valine, but in the other two isolates this amino acid similar to vaccine viruses was Alanine. Furthermore, the amino acid 117 was transformed from phenyl alanine to valine in the isolate 43 while it is similar with M41 in other isolates. In the isolates 17 and 70, the amino acids 137 and 138 in comparison with M41 strain, similar to H-120 and H-52 strains, were transformed from tyrosine and asparagine to histidine and valine respectively.

The sequence of cleavage site of S1 glycoprotein in three isolates of 43, 70 and 17, including amino acids 553 and 557 (Arg-Arg-Phe-Arg-Arg (PRFRR)), is similar to standard viruses of Massachusetts serotype.

The amino acids of HVR 2 region contain 76-173 amino acids and this region is associated with the serotype-specific neutralizing epitopes. Moreover, the region between amino acids 123 and 152 plays an important role in the differentiation and virulence of some IBV strains [6].

The changes in amino acids have a direct effect on the polarity of protein and its secondary structure and subsequently changes three-dimensional form of protein and it antigenicity [4]. On the other hand, it was realized that amino acids 99 to 127 may have been involved in organ tropism and virulence [21]. Point mutation or any deletion or change in amino acids of HVR region of S1 gene modify glycosylation and antigenic site of D, S1 and E antigens respectively in amino acids 49-61 and 131-149. These changes may lead to significant changes in antigenicity and virulence of IBV [6, 22, 7].

The variations of amino acids in the present study did not have any effect on the epitopes of neutralization, pathogenicity and tissue tropism of these isolates and they are completely homologous with vaccine viruses. Therefore, sequences and phylogenic data of fourteen isolates confirm that there were not any changes in Massachusetts serotype in the field.

Despite the use of prophylactic program, based on the H-120 and 4/91 vaccine in Iran, the results are disappointing and outbreak of IBV is still possible. The difference in the nucleotide and amino acid between isolated viruses and standard viruses is stable (Table 3).

Variation in S1-gene sequence is used to differentiate IBV serotypes [23, 15, 24, 19 , 25]. This variation in S1 is probably caused by mutation, recombination and positive selection under in-vivo conditions [3]. The presence of genetic variants is supposed to be the result of the variation of small number of amino acids in IBV S1 protein [26]. Identifying genetic characteristics of circulating IB viruses in the field is essential to investigate the genetic relationship between circulating viruses and vaccine strains and this helps us select the suitable vaccine and increases our ability to control the disease.

Conclusion

Since vaccination against IB disease with Massachusetts vaccine strain is widely done with live attenuated vaccines, it is essential to study their pathogenicity, antigenicity and cross-protection in order to characterize and differentiate between field and vaccine strains.

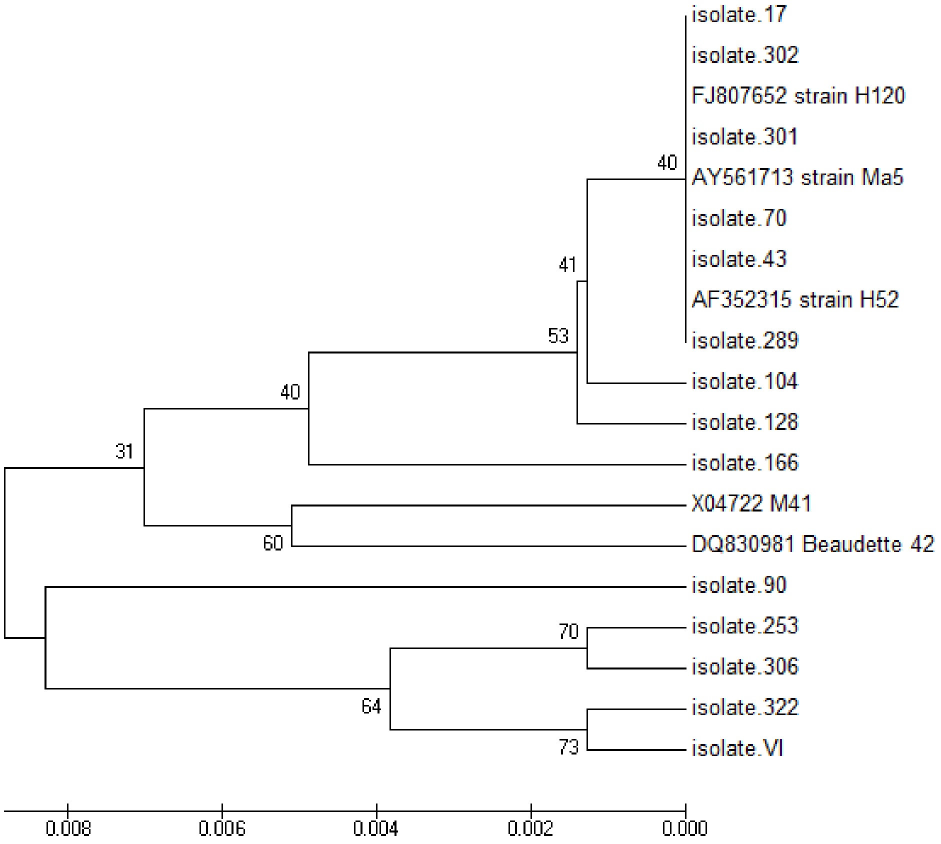

Figure 1. Phylogenic relationship between 14 isolates of IBV filed and standard IBV viruses of Massachusetts serotype

M41 (X04722), H120 (FJ807652); H-52 (AF352315.), Ma5 (45549460), Baudette 42 (DQ830981)

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