

# Molecular characterization of recent Iranian infectious bronchitis virus isolates based on S2 protein gene

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characterization, infectious bronchitis virus, genotyping, phylogenetic study, spike

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## Abstract:

**BACKGROUND:** Avian infectious bronchitis (IB), with avian infectious bronchitis virus (IBV) as the causing agent, is a ubiquitous endemic disease of the chicken with devastating effects on its industry. A viral membrane surface protein called S not only induces neutralizing antibodies but also plays an important role in virus binding and entry to host cells. Technically, S1 protein gene sequencing also helps greatly in IBV genotyping. **OBJECTIVES:** The aim of this study was to characterize Iranian IBV based on S2 gene. **METHODS:** After RT-PCR amplification, the S2 gene of nine Iranian IBV isolates were sequenced and then compared with reference strains. **RESULTS:** The isolates were classified into genotype I as Massachusetts like IBVs, genotype VII which clustered into two branches, VIIa (IS-1494 like IB viruses), and VIIb, and was related to QX-like viruses and Genotype VIII as 793/B like IBVs. **CONCLUSIONS:** As far as we know, this is the first S2-based classification study on Iranian IBV isolates providing a firm experimental basis to correlate with genotypic characterization.

## Introduction

Infectious bronchitis (IB) is a ubiquitous and highly contagious disease of chickens caused by Infectious Bronchitis Virus (IBV). IBV is a member of genus Gammacoronavirus, subfamily Coronavirinae, family Coronaviridae, order Nidovirales with a single-stranded positive sense RNA genome, 27.6 kb (Jackwood, 2012). By infecting the respiratory tract, kidneys and oviduct, the virus results in reduced

performance and egg quality and quantity while predisposing the chickens to other pathogens (Mo, et al., 2013). The viral genome encodes proteins including the viral RNA-dependent RNA-polymerase (RdRp), envelope (E), membrane (M), nucleocapsid (N) and structural proteins spike (S) (Jackwood, 2012). The latter, also called surface glycoprotein (S), is located on the surface of the viral membrane and is a major inducer of neutralizing antibodies while playing a vital role in virus binding and entry to host

cells. It is cleaved into two subunits, amino-terminal S1 (92 kDa, ~535 amino acids) and the carboxyl-terminal S2 (84 kDa, ~627 amino acids) post-translationally. The S1 subunit forms the distal and bulbous part of the spike whereas the S2 subunit anchors S1 to the viral membrane. The antigenic region of the S2 subunit has been proposed to play a role in protection (Mo, et al., 2013). This subunit also has a fusion peptide-like region and two heptade regions, approximately 100 to 130 Å in length (771-879 amino acid in IBV), involved in oligomerization of the protein and entry into susceptible host cells (Abro, 2012). More studies should be performed on this subunit though the S1 subunit has been examined extensively. Apparently based on the highly conservative nature of the S2 subunit among different members of the Coronavirus genus and different strains of IBV, it plays a little or no role in inducing a host immune response. Mostly, the molecular studies aimed to detect IBV genotypes were based on S1 glycoprotein. On the other hand, the S2 subunit possibly induces serotype-specific neutralizing antibodies while it is conserved within a serotype but not between serotypes (Ammayappan & Vakharia, 2009; Scott Andrew Callison, Mark W Jackwood, & Deborah Ann Hilt, 1999).

The first report of IBV isolation from Iranian chicken flocks goes back to 1994 (Seyfi Abad Shapouri, Mayahi, Assasi, & Charkhkar, 2004). Based on analyzing mainly hypervariable regions of the S1 glycoprotein gene, Genotyping of IBV strains isolated in Iran were classified into seven distinct phylogenetic groups as Mass, 793/B like, IS/1494 like, IS/720-like, QX-like, IR-1, and IR-2 (Hosseini, Bozorgmehri Fard, Charkhkar, & Morshed, 2015; Najafi, et al., 2015). At present, different vaccines

for Massachusetts and 793/B types are administered in Iranian poultry industry. Nevertheless, IB outbreaks are still present. However, genotyping of Iranian IBV isolates based on sequence analysis of the S2 protein gene have not been performed, hence, the aim of this study was to understand genotype identification of the S2 gene of IBVs isolated in Iran by sequencing and phylogenetic analysis.

## **Materials and Methods**

**Samples:** Nine Iranian IBV isolates with different genotypes were studied in this paper. The isolates information including the type of flock, specimens, and S1 gene-based genotypes were indicated in Table 1; the broiler chickens have shown respiratory manifestations, nephritis and mortalities. The surveillance was done from 2014 to 2015.

**RNA extraction and cDNA synthesis:** The total RNA of viruses was extracted using Cinna Pure RNA extraction kit (Sinaclon, Iran) and then stored at -70°C until further use. The extracted RNA was used in reverse transcription (RT) reaction to generate cDNA using Revert Aid Reverse Transcriptase (Thermo Scientific, Canada), Ribolock Rnase inhibitor (Thermo Scientific, Canada), dNTP mix (Sinaclon, Iran), and DEPC-treated water (Sinaclon, Iran). For cDNA synthesis, 5 µL of the extracted RNA was mixed with 1 µL of random hexamer primer and incubated at 65°C for 5 min, followed by addition of 14 µL of master mix (7.25 µL distilled water, 4 µL buffer 5X, 2 µL dNTP mix, and 0.5 µL RT enzyme) to make the final volume 20 µL. Then, the mixture was incubated at 25°C for 5 min, 42°C for 60 min, 95°C for 5 min, and 4°C

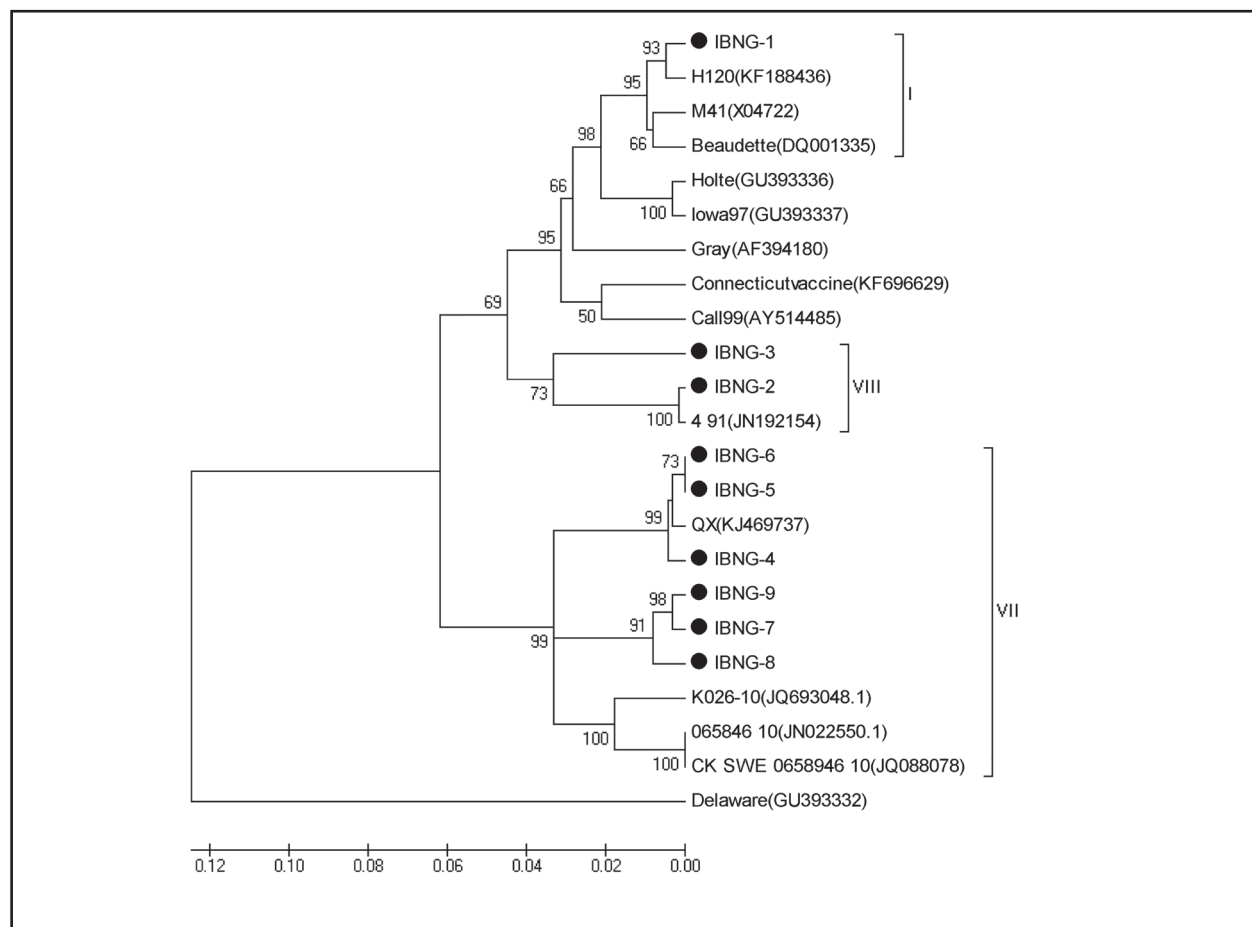


Figure 1. Phylogenetic tree based on amino acid of S2 gene of IBVs, black circles: nine Iranian strains among other reference strains; The phylogenetic tree was constructed with the MEGA version 5.0 using the neighbor-joining method with bootstrap replicates (bootstrap 1,000 values as shown on the tree). The branch number represents the percentage of the branch showing up in the tree. Bootstrap values greater than 50% are shown.

Table 1. IBV strains used in this study.

Name	Chicken species	Organ	Genotypes based on S1 gene
IBNG-1	Broiler	Trachea	Massachusetts
IBNG-2	Broiler	Trachea	793/B
IBNG-3	Broiler	Kidney	793/B
IBNG-4	Broiler	Trachea	QX
IBNG-5	Broiler	Kidney	QX
IBNG-6	Broiler	Trachea	QX
IBNG-7	Broiler	Kidney	IS-1494 like (Var-2)
IBNG-8	Broiler	Trachea	IS-1494 like (Var-2)
IBNG-9	Broiler	Kidney	IS-1494 like (Var-2)

for 1 min.

**PCR for amplification of partial segment of S2 gene:** PCR was performed to amplify the S2 gene using the primer pairs as forward primer S2F2, 5'' GCTG-CGTCRTTTAATAAGGCCAT 3'' (22756-

22778 according to Beaudette CK strain) and reverse primer S2R2, 5'' CTAGGCTGCCACAACAATAAC 3'' (23752 - 23732) (PCR length : 997 pb). The reaction mix was prepared in a volume of 20µL with 3µL of cDNA, 13µL of master mix (Sinaclon, Iran),

1 µl of each primer. The PCR reaction was carried out under the following conditions in a thermal cycler (Eppendorf, Germany): 35 cycles of 95°C for 3 min, denaturation 95 °C for 30 Sec, Annealing at 58°C for 30 Sec. Extension 72 °C for 60 Sec. The expected amplified fragment of PCR product is one Kb. The RT-PCR products were visualized by electrophoresis of 5 µl of each product in a 1% agarose gel, containing red safe stain, followed by UV trans illumination.

**Phylogenetic analysis:** The AccuPrep® PCR Purification Kit (Bioneer Co., Korea) was used for purification of the PCR products. Sequencing was performed with the same primers used in the PCR (Bioneer Co., Korea). Then, chromatograms were evaluated with ChromasPro (ChromasPro Version 1.5) and the multiple alignments of S2 nucleotide was achieved using Clustal W using the MEGA 5.1 software. The phylogenetic tree was constructed by using the same software with the neighbor-joining method and each tree was produced using a consensus of 1000 bootstrap replicates (Tamura, et al., 2011). The amino acid sequences of the S2 gene were compared with several S2 sequences from Gene bank including H120(KF188436), 4/91(JN19215.4), M41(X04722), Beaudette (DQ001335), Iowa97 (GU 393337), Holte (Gu39336), Gray (AF394180), Call99 (AY514485), QX (KJ469737) and Delaware (GU393332).

## Results

Obtained Amino acid sequences of S2 gene of nine IBV isolates were aligned and compared with the reference strains from GenBank and available from the national center for biotechnology information (NCBI). Phylogenetic analysis based on the

S2 gene showed that the Iranian IBV isolates were grouped into three distinct clusters I, VII & VIII. Group I (IBNG-1) closely related to Massachusetts viruses, Group VII, related to QX IBV isolate, clustered into two sub-clusters including VIIa containing IBNG-4, IBNG-5 and IBNG-6 (Variant 2 like virus) and second sub cluster VII b containing IBNG-7, IBNG- 8 and IBNG-5, and finally group VIII (IBNG-2 & IBNG-3) which were in the same cluster with 793/B viruses (Fig. 1). The amino acid identity within groups VIII and VII was 90.21% and 93.81%-100%, respectively. On the other hand, the amino acid identity between group I and group VIII was 88.19% while it was 85% between group VII and group VIII. In the case of vaccine strains, the identity level of group I with H120, M41, and Beaudette was 99.35%, 98.58%, and 98.36% respectively. Meanwhile, sequence identity between group VIII and 4/91(JN192154) and between group VII and QX (KJ1469737) was also 99.39%-99.89 % and approximately 99%, respectively.

## Discussion

The infectious bronchitis virus (IBV) causes huge losses in the poultry industry. In the S2 subunit, regions of sequence variation were found interspaced with regions of high conservation causing the overall diversity of the S gene. Genotyping is often the preferred serotyping method due to its convenience and time-saving aspects compared with virus neutralization (VN) or other traditional serotyping tests (Lee, Hilt, & Jackwood, 2003). Playing a role in many biofunctions such as cell receptor's attachment and tissue tropism together with inducing IBV neutralizing antibodies makes

S1 gene the principal method to study the genetic diversity of infectious bronchitis virus. Meanwhile, S2 gene does not have any major antigenic sites. However, it was considered to be associated with antigenicity, which is affected by its conformation. This makes the analysis of the S2 gene critical to further determine the antigenicity of isolates, which might be diverse among IBV strains because of some recombination events between the virus strains classified into different genetic groups (Callison, et al., 1999). IBV in Iranian chicken flocks was first isolated by Aghakhan et al (1994) (Aghakhan, Abshar, Fereidouni, Marunesi, & Khodashenas, 1994) and found to belong to Massachusetts serotype. Hosseini et al. isolated Iran/QX/H179/11 strain in 2011 followed by Iran/QX/H255/12, Iran/QX/H281/12, and Iran/QX/H284/12 strains in 2012 in Iran. QX-type isolates of the current study showed more than 96% homology with these Iranian strains (Bozorgmehri-Fard, Charkhkar, & Hosseini, 2014). Also, the presence of two variant viruses (IS/1494/06 like) in Iranian commercial flocks has been demonstrated in another study (Hosseini, et al., 2015). Based on phylogenetic analysis of the S1 glycoprotein gene, six distinct phylogenetic groups as IS/1494/06 [Var2] like, 4/91-like, IS /720-like, QX-like and IR-1 and Mass-like were described recently (Najafi, Madadgar, Jamshidi, Langeroudi, & Lemraski, 2014). In case of Phylogenetic analysis based on S2 gene, the current study's IBV strains could be classified into three genetic groups: genotypes I (Massachusetts like), VII (QX & IS 1494 like) & VIII (793/B like). The present study was partially consistent with Mase et al (2008) who developed a grouping method of IBV based on the S2 glycoprotein gene and di-

vided IBV genotypes into eight clusters. The strains belonged to the Massachusetts, Gray and Connecticut types were classified into group I, II and III, respectively while those belonging to the foreign Iowa-609 type were classified as group IV and Group V consisted of Japanese strains, including the C-78 vaccine strain, only. It is necessary to mention that Group VI also mainly consisted of Japanese strains, including the comparatively newly established vaccine strains, the Miyazaki, TM86 and GN strains. Since viruses belonging to groups VII or VIII were of strains isolated after 2000, they were not used in the previous study (Mase, Inoue, Yamaguchi, & Imada, 2009). Zulperi et al studied the phylogenetic analysis and sequencing of both S1 and S2 genes of Malaysian IBV strains concluding that even with only 77% identity, MH5365/95 and V9/04 belong to non-Massachusetts strain. The S2 gene-based phylogenetic tree analysis of the study also indicated that both MH5365/95 and V9/04 isolates grouped together in a separate sub-cluster (Zulperi, Omar, & Arshad, 2009). Though showing sequence variability among different strains of IBV, which generally differ by less than 10%, Callison et al concluded S2 gene sequence is more conserved than that of the S1 gene and in fact, the serotypes were grouped based on S2 gene sequence (Callison, Jackwood, & Hilt, 1999). The diversity of the N-terminal region of the S2 gene of IBV in Iran was revealed in this study confirming the applicability of this region for IBV strain grouping. While some studies reported Iranian IBV genotyping based on S1 gene, this study highlighted the first time use of S2 gene-based IBV genotyping in Iran as far as we know. There are different reports around Iranian IBV genotyping

based S1 gene. Our findings have highlighted for the first time IBV genotyping based on the S2 gene in Iran. More studies should be performed with a full-length sequence of S2 gene while genotyping the S2 gene on more IBV isolates.

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## ژنوتایپینگ و تقسیم بندی ویروس های برونشیت عفونی پرندگان بر اساس ژن کد کننده پروتئین S<sup>2</sup>

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### چکیده

**زمینه مطالعه:** ویروس برونشیت عفونی، عامل بیماری برونشیت عفونی از عوامل مهم در ایجاد خسارات اقتصادی در صنعت طیور دنیا می باشد. ویروس برونشیت عفونی به سروتیپ ها و ژنوتیپ های مختلفی طبقه بندی می شود. پروتئین اسپایک (S) (~۳۴۶۲)، واقع در سطح غشاء ویروسی، القاء عمده ای از آنتی بادی های خنثی است و مسئول ویروس اتصال و ورود به سلول های میزبان است. جدایه های ویروس برونشیت عفونی بطور گسترده بوسیله سکانس ژن پروتئین S<sup>1</sup> ژنوتایپینگ می شوند. هدف: هدف از این مطالعه، ژنوتایپینگ جدید و طبقه بندی جدایه های ویروس برونشیت عفونی در ایران بر اساس پروتئین S<sup>2</sup> می باشد. روش کار: ژن S<sup>2</sup> نه جدایه ویروس برونشیت عفونی ابتدا توسط روش RT-PCR تکثیر داده شدند و مورد سکانس قرار گرفتند. سکانس آمینواسیدی این جدایه ها با سکانس جدایه های غیر ایرانی مورد مقایسه قرار گرفتند. نتایج: جدایه های ویروس برونشیت عفونی ایران به سه ژنوتیپ (I, VII, VIII) تقسیم بندی گردیدند. ژنوتیپ I شامل ویروس های مشابه سروتیپ ماساچوست بود. ژنوتیپ VII به دو شاخه تقسیم بندی شدند، اولین شاخه VIIa (مشابه IS-1۴۹۴) و دومین شاخه VIIb نیز مربوط به ویروس های مشابه QX بودند. ژنوتیپ VIII شامل ویروس های مشابه B/۷۹۳ بودند. نتیجه گیری نهایی: مطالعه حاضر نخستین اطلاعات در مورد ژنوتایپینگ و طبقه بندی جدایه های ویروس برونشیت عفونی ایران می باشد.

**واژه های کلیدی:** تعیین هویت، برونشیت عفونی، شجره شناسی، پروتئین S<sup>2</sup>، اسپایک

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