

Molecular characterization of infectious bronchitis virus based on RNA-dependent RNA polymerase gene

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Abstract

Extensive rate of variations in the *SI* gene (spike glycoprotein subunit gene) of infectious bronchitis virus (IBV) causes challenges for clinicians in counting variants for differentiation of infected from vaccinated birds and addressing the variants of unknown significance. This study investigated the possibility of using an *RNA-dependent RNA polymerase* gene (*RdRp*) as a target for molecular characterization of IBV strains in Iran. Trachea samples were collected from commercial broiler flocks ($n = 52$) showing respiratory syndrome. Specific PCR primers were designed for a variable region located in the *RdRp* gene flanked by highly conserved regions. Reverse transcriptase PCR followed by sequence analysis identified eight IBV variants, with an overall prevalence of 44.2%. Deduced nucleotide and amino acid sequences were compared with published sequences for IBV strains. Because of the long-distance similarities, the field samples could be discriminated from vaccine strains. Phylogenetic analysis of *RdRp* gene sequences resulted in clustering of the IBV strains related to each area. Using *RdRp* as a genetic marker eliminates the challenges arising from the enormous variations that make it difficult to discriminate between field and vaccine strains as well as affiliate certain variants to various geographical areas.

KEYWORDS

broiler, chicken, infectious bronchitis virus, *RdRp*, replicase

1 | INTRODUCTION

Coronaviruses can cause respiratory disease in a wide variety of animals including chicken (*Gallus gallus*). Infectious bronchitis virus (IBV), a member of the type 3 coronavirus (*Gammacoronavirus*), seriously affects the performance of both meat-type and egg-laying birds by

tendency for replicating not only in the respiratory tract, but also in alimentary tract, kidney, oviduct, and testes. IBVs, as well as other members of *Coronaviruses*, have a unique mechanism of viral replication which permits a high frequency of recombination in their structural proteins.¹ Their propensity for variations based on the high mutation rates and recombination results in viral

Abbreviations: E, envelope; IBV, infectious bronchitis virus; M, integral membrane; N, nucleocapsid; PL(pro), papain-like proteinase; *RdRp*, RNA-dependent RNA polymerase; RT-PCR, reverse transcriptase PCR; S, spike.

tissue tropism, pathogenicity, and adaptation to immunologically and/or genetically resistant hosts.^{1–3}

The IBV genome codes four kinds of structural proteins, including spike (S), envelope (E), integral membrane (M), and nucleocapsid (N). There is also a set of accessory protein genes of unknown function. Besides the structural and accessory genes, two-thirds of the coronavirus genome comprises the replicase complex that codes for functional domains such as a papain-like proteinase (PL[pro]), main protease (M[pro]), RNA helicase, and RNA-dependent RNA polymerase (RdRp).⁴ The replicase is a multifunctional polyprotein which, after cleavage, produces a number of functional products that are indispensable for viral transcription and replication.⁵

Mutation and recombination in the IBV genome are the main causes of phenotypic variations. Consequently, emergence of newly variant strains is mainly considered as a potential for new pathotypes, serotypes, and proctotypes. Nucleotide sequencing and subsequent genetic analysis indicated a continuous spatiotemporal dynamics of IBV variations. Most investigations on the IBV strains are conducted based on the S and N protein gene sequences.^{6–8} It is believed that structural proteins are imperative target parts of the virus particles for immunization and achieving protective immunity. Therefore, variations in IBV S and N sequences are mostly considered for selecting the best vaccine strains for vaccination programs. However, extensive rate of variations in the S1 gene of IBV poses multiple challenges for clinicians in counting variants for differentiation of infected from vaccinated birds and addressing the variants of unknown significance.⁹ Furthermore, in molecular epidemiology approaches, high polymorphism means that there is a low probability that a new variant could be classified in an identified profile or group. Because of high degree of polymorphisms on the S1 sequence, diverse genetic group designations for IBV strains are usually inconsistent with phylogenetic history.¹⁰ Therefore, another powerful target for monitoring the virus variations seems to be necessary. As a genetic marker, it must also be capable of being used for phylogenetic and epidemiological evolution of IBV variants. Here we suggested the molecular characterization of IBV based on RNA-dependent RNA polymerase gene.

2 | MATERIALS AND METHODS

2.1 | Sampling

This study was conducted in commercial broiler flocks showing respiratory syndrome. Trachea samples were collected between February 1, 2018, and June 30, 2019,

from 52 flocks in farms located at north of Iran, namely, from Mazandaran (35 flocks) and Golastan (17 flocks) provinces. From each flock, comprising 10,000 chickens, three or five tissue samples were collected and pooled. The samples were frozen and stored in 1.5 mL RNase- and DNase-free microtubes at -70°C until use.

2.2 | RNA isolation and complementary DNA synthesis

Total RNA extraction was performed using the Viral Gene-spin Viral DNA/RNA Extraction Kit (Intron Biotechnology, Seoul, South Korea). Extracted RNA was then reverse transcribed to complementary DNA (cDNA) in a 20 μL volume containing 1 μg of RNA, 200 ng random hexamer, and 0.5 mM deoxynucleoside triphosphate mix. The mixture was heated to 65°C for 5 min, which was followed by the addition of 40 U RNase inhibitor, reverse transcriptase (RT) buffer (50 mM Tris-HCl, 75 mM KCl, and 3 mM MgCl_2), 10 mM dithiothreitol, and 200 U Moloney murine leukemia virus RT (Thermo Fisher Scientific, Schwerte, Germany). This mixture was incubated for 10 min at 25°C and then for 50 min at 37°C . The prepared cDNA was heated at 75°C for 15 min to denature the Moloney murine leukemia virus RT and then stored at -20°C .

2.3 | Reverse transcriptase-polymerase chain reaction of IBV genes

All available IBV RdRp gene sequences were retrieved from the GenBank and aligned using ClustalW implemented in the BioEdit version 7.0.5.3 software package. Two PCR primer sets were designed spanning the variable region of RdRp gene sequences. Based on the reference sequence for avian IBV (AY692454), the forward PCR primer (F: 5'-ACCCGATTCTYATGGGTTGGG-3') is located in polyprotein 1b, nucleotides 14,167 to 14,188, and the reverse PCR primer (R: 5'-AGACGCGCAACATTAGCAGA-3') is located in polyprotein 1b, nucleotides 14,436 to 14,455. Amplification was performed in a final volume of 25 μL mixture containing 20 ng template cDNA, 1.5 mM MgCl_2 , 250 μM of each deoxynucleoside triphosphate, PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1 μL (2 μM), 1 U/ μL of *Taq* DNA polymerase (Cinaclon, Tehran, Iran), and 20 pmol of specific primers. The thermal cycling profile was 1 cycle of 95°C for 2 min, 30 cycles of 95°C for 30 s, 60.5°C for 30 s, and 72°C for 40 s, with a final extension step of 5 min at 72°C .

The S1 gene amplification was carried out according to Homayounimehr *et al.*¹¹ The N gene was amplified as previously described by Masoudian *et al.*¹²

2.4 | Sequencing and data analysis

The direct sequencing method was performed for the analysis of the amplified RdRp, S1, and N genes. The PCR products were purified using the purification kit (Bioneer, Seoul, Korea) and each amplicon was separately sequenced in both directions using specific forward and reverse primers. All procedures were carried out by Sanger sequencing on an ABI 3730 XL automatic DNA sequencer (Macrogen Inc., Seoul, Korea). Sequences were analyzed by BLAST through the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Alignments and the amino acids prediction were made using the BioEdit version 7.0.5.3 software package.

2.5 | Phylogenetic analysis

For phylogenetic analysis, partial sequence of the RdRp gene, belonged to Iranian IBV field strains, were aligned with nonredundant sequences from all available strains that was retrieved from the GenBank database. The partial sequence of S and N genes, which is conventionally used for IBV genotyping, was also used for phylogenetic analysis and making comparisons. Phylogenetic analysis was conducted using MEGA6 Beta (<http://www.megasoftware.net/mega41.html>) and the tree was constructed by the neighbor-joining method. Bootstrapping over 1000 replicates was performed to assess the confidence level of the branch pattern.

3 | RESULTS

The RT-PCR with specific primers for the RdRp gene of avian IBV resulted in the amplification of a 288 bp

sequence as expected. By targeting the RdRp gene, from 52 flocks with respiratory disease, 23 flocks were identified to be positive for IBV. Based on the sequencing analysis and by comparisons to reference strain, eight sequences were assigned to new variants including M (Mazandaran) and G (Golastan). Nucleotide alignments indicated that Iranian strains were 92%–94% identical to the reference sequence (AY692454) (Figure 1). Compared with the reference sequence, almost all strains showed variations in 16 nucleotide sites.

M6 was the most prevalent strain detected in seven flocks in Mazandaran, in comparison with other strains which were detected in one to four flocks. Each of the M16 and M28 strains was detected in three different flocks (collectively six flocks). Strain M14 that is identical to G53 was found in two flocks of Mazandaran and one flock in Golastan. Strain M18 was also identical to G102 and was found in four flocks of Mazandaran and one flock of Golastan. There were three distinct strains in Golastan province (G31, G55, and G82; Table 1).

Among the 79 amino acid sites in the translated sequence of the IBV RdRp replicase gene, one site was different (S/T) in all Iranian field strains. In comparison with the reference strain, 22 nucleotide variations (9.24%) were identified in Iranian sequences. Only one of the substitutions was nonsynonymous (Figure 2).

A tree was constructed based on the IBV RdRp nucleotide sequences using the neighbor-joining method (Figure 3). The evolutionary distances were computed using the maximum composite likelihood method and were reported in the units of the number of base substitutions per site. Phylogenetic analysis of 57 nucleotide sequences over a total of 236 positions (primer sequences not included) indicated that all eight Iranian IBV strains clustered into a distinct group. This group only contained a *Gammacoronavirus* sequence reported from Poland

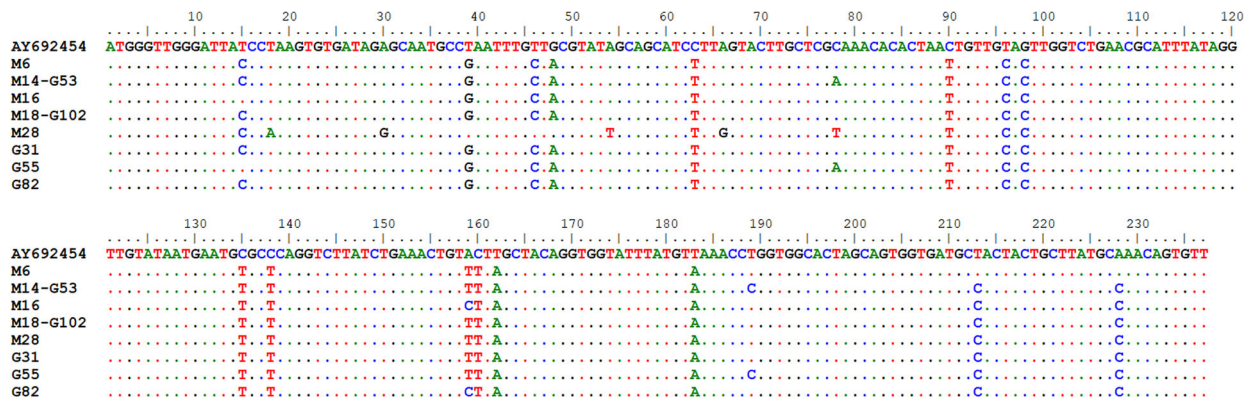


FIGURE 1 Partial RNA dependent RNA polymerase (RdRp) gene sequence homology between Infectious Bronchitis virus (IBV) strains detected in the provinces of Mazandaran (M) and Golastan (G). A comparison with the IBV reference sequence (AY692454) shows the specific detection of Iranian IBV strains

TABLE 1 Prevalence of lock-level IBV strains among affected commercial broiler chicken flocks sampled between February 2018 and June 30, 2019, in Mazandaran ($n = 18$ flocks) and Golastan ($n = 5$ flocks)

IBV strains	Samples origin (Iranian provinces)	Number of affected flocks	% of samples positive for each strain
M6	Mazandaran	7	39
M14/G53	Mazandaran	2	11
	Golastan	1	20
M16	Mazandaran	3	17
M18/G102	Mazandaran	3	17
	Golastan	1	20
M28	Mazandaran	3	17
G31	Golastan	1	20
G55	Golastan	1	20
G82	Golastan	1	20

(KT886454). There were another two main clusters divided from the ancestor node. One cluster contained vaccine strains including H120, Ma5, and 4/91 together with some Chinese, Pakistani, Indian, and Jordanian strains. The strain 4/91, segregated with a Chinese strain and separated by a long branch from Georgia and Massachusetts strains. Besides these two clusters, there was a cluster that contained a well-defined branch which only contained Conn 46 strains reported in the United States and one branch which only contained strains reported in South Korea. The trees were also constructed based on the IBV S and N nucleotide sequences. The S1 tree consisted of three main clusters divided from the ancestor node. Serotypes Gray and JMK were separated by a long branch from other groups. *Gammacoronavirus*, one Chinese strain and two QIA strains, segregated with one vaccine strain (4/91). The third cluster contained H120, Ma5, Pakistani, Massachusetts, and Chinese strains. The N tree also consisted of three main clusters

divided from the ancestor node. Iranian strains separated in one cluster containing Chinese, Italian, Canadian, and 4/91 vaccine strains. Pakistani, Jordanian, and H120 strains segregated in the second cluster and Polish, Chinese, and SNU strains in the third cluster.

4 | DISCUSSION

The replicase gene of IBV encodes PL (pro), M (pro), RNA helicase, and RdRp domains that are important for virus replication. The genotypic characterization assay developed in this study focused on the variable regions of the RdRp gene. This idea came from the observation that IBV S1 is a high variability-prone region, especially due to the lack of RNA polymerase proofreading.⁶ It is also a shifting viewpoint from the assays targeting the incessant S1 gene variation to the characterization/detection based on the cause or origin of variation, which is the RdRp gene. However, this idea must be elaborated by studying the numerous variations as well as the association with pathogenicity and virulence.

Based on the RT-PCR detection and sequence analysis of the IBV RdRp gene, the overall prevalence of field strains was estimated as 44.2%. A similar prevalence has been reported for farms with clinical respiratory signs, which varies from 45% to 54.4% between districts.^{11–14} Eight variants of the virus were identified in 23 farms, with the noticeable dominance of M6. The emergence of new IBV variants, which is an ongoing process, presents a great challenge for identification, discrimination, and characterization of the novel variants. In our experiment identifying the viruses that did not overlap with the vaccine strains suggests the capability of the test for detecting IBV and differentiating infected from vaccinated animals. In addition, differentiations made by sequence analysis could be used for further characterization, such as affiliation of certain variants to certain geographical area.

Among the replicase domains of *Coronavirus*, RdRp is a highly conserved domain with 48%



FIGURE 2 Multiple amino acid sequence alignment of the partial RNA dependent RNA polymerase (RdRp) gene. Translated amino acid sequences from Infectious Bronchitis Virus (IBV strains) detected from the provinces of Mazandaran (M) and Golastan (G) in Iran were compared with variant reference strains (AY692454)

conserved residues. IBV strains at this domain have shown 92.7% of conserved residues.¹⁵ Our finding also indicated 98.7% conserved residues among Iranian strains. According to the nucleotide sequence variability analysis, the IBV RdRp gene comprised two conserved regions (42 single-nucleotide polymorphisms), whereas in the Iranian strains four conserved regions were detected (22 single-nucleotide polymorphisms). By contrast, RdRp nucleotide substitution frequencies (per 100 nucleotides) varied from 17.64 in the world strains to 9.28 when compared in the Iranian strains. A higher nucleotide substitution frequency has been reported for the genus *Coronavirus* (50.13).¹⁶ Viruses like the members of *Coronavirus*, despite the alterations in their structural proteins, apparently have similarities in their replication and expression strategies. Conserved regions in the RdRp gene of IBV form the basis for developing a common RT-PCR that could be used for the detection of virus in clinical samples. Moreover, the central variable region flanked by highly conserved regions is a suitable target for making phylogenetic comparisons.

Phylogenetic tree constructed based on the RdRp gene sequences resulted in clustering which is closely related to the geographical areas. This analysis further revealed that in published IBV sequences for each area, the percentage of nucleotide sequence identities ranged from 89.8% to 98.9%. Our results are not in agreement with the findings based on the S1 gene that have been previously reported for Iranian strains.^{12,13} In addition, Iranian strains are found on a common branch distantly related to vaccinal strains. The trees which are constructed based on the S1 and N genes data indicated different patterns. Interestingly, in comparison with RdRp tree, the same strains in one cluster arranged in different clusters when compared with the RdRp S1 and N tree were constructed (Figures 3,4). Although data obtained from S1 and N analysis provide information about the variability of strains, different strains do not appear to be closely related to the geographical areas as indicated by RdRp analysis.

Our findings could be considered as a novel standpoint for different reasons. First, the genetic characterization developed in this study, which is based on the RdRp gene, displayed an analytical procedure clearer than that based on the S1 gene. Second, using RdRp as a genetic marker eliminates the challenges that arise from the enormous variations that make it difficult to affiliate certain variants to various geographical areas. Third, by using the sequence base analysis, the field samples could be easily discriminated from vaccine strains. Finally,

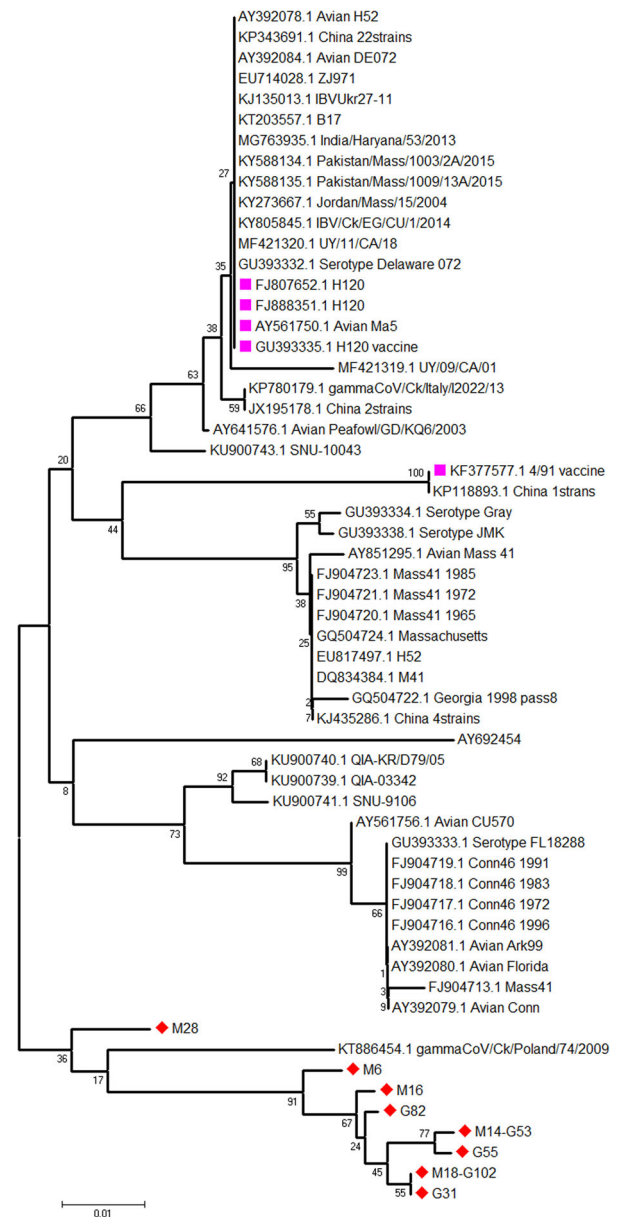


FIGURE 3 Phylogenetic tree constructed based on RNA dependent RNA polymerase (RdRp) gene sequence of Infectious Bronchitis Virus (IBV). RdRp sequences of Iranian IBV strains together with previously reported sequences from other countries (GenBank accession numbers are shown) were used for phylogenetic tree construction. Numbers at the nodes indicate bootstrap (1000 replicates). The evolutionary distances were computed using the maximum composite likelihood method and are reported in the units of the number of base substitutions per site. The analysis involved 54 nucleotide sequences. There were a total of 236 positions (primer sequences not included) in the final data set. Evolutionary analyses were conducted in MEGA6

because major changes occur at the S1 gene, targeting the RdRp gene might be considered an alternative strategy for selecting the appropriate homologous strains for vaccines in various regions.

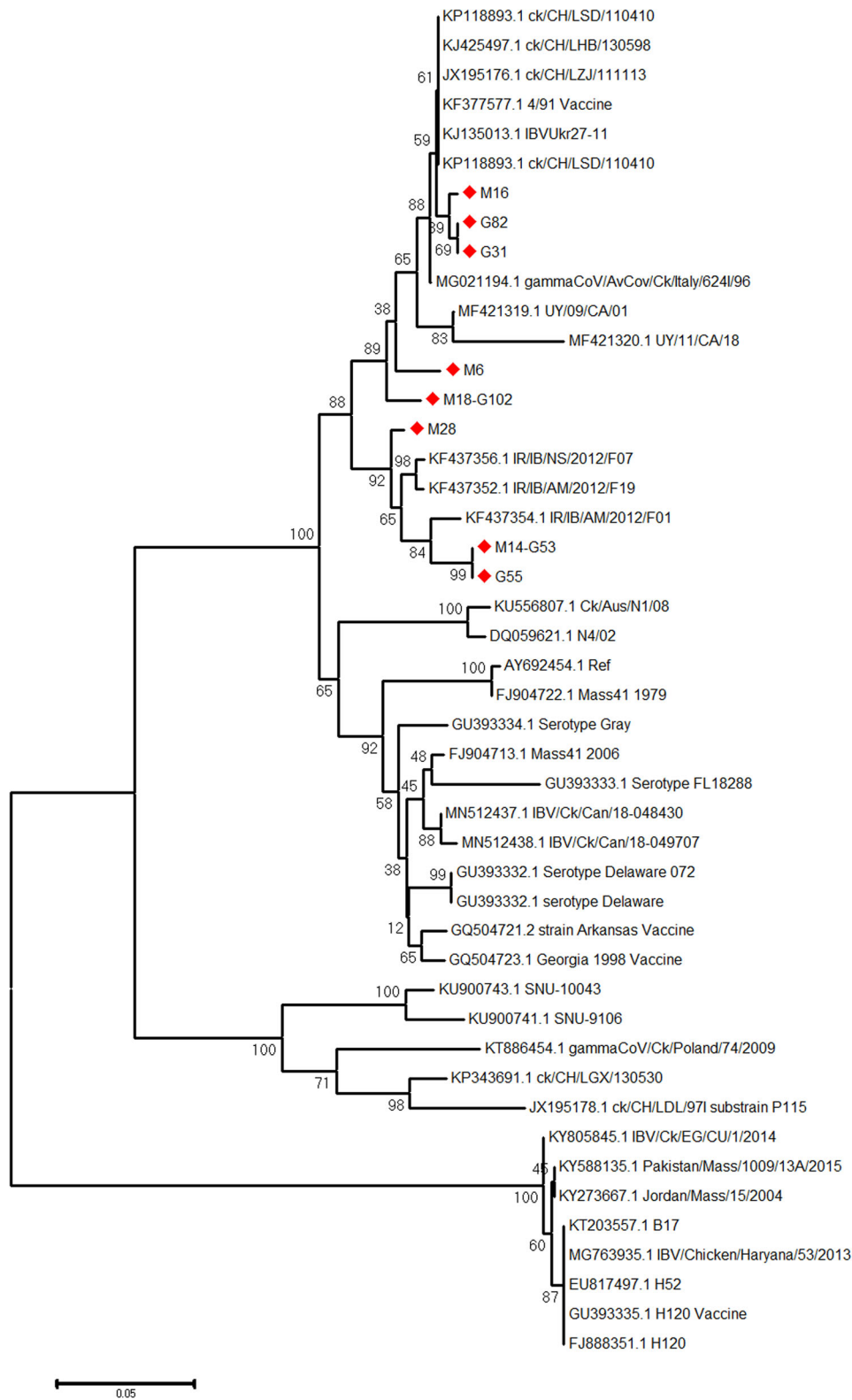



FIGURE 4 Phylogenetic tree constructed based on IBV N gene sequences. GenBank accession numbers are shown. Numbers at the nodes indicate bootstrap (1000 replicates). The evolutionary distances were computed using the maximum composite likelihood method and are reported in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6

DISCLOSURE

The authors declare that they have no conflict of interests.

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