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NOTE

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Genotyping of infectious bronchitis viruses isolated in Japan during 2008–2019

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ABSTRACT. Seventeen isolates of infectious bronchitis virus (IBV) were obtained from various prefectures of Japan during 2008–2019 and genetically analyzed. The IBV isolates were classified into six genetic groups, based on phylogenetic analysis of the S1 gene. The S1 genotypes were distinguishable by a newly developed restriction fragment length polymorphism (RFLP) method using three endonucleases, *Hae* II, *Hpa* I, and *Fok* I. Moreover, the isolates were classified into four genetic groups, based on phylogenetic analysis of the S2 gene. However, novel genetic groups based on a combination of S1 and S2 genotypes, which were undetected previously, were confirmed in this study, indicating that various recombinant IBV strains were prevalent in poultry in Japan.

KEY WORDS: diversity, genotype, infectious bronchitis virus

Avian infectious bronchitis virus (IBV) causes a highly contagious respiratory and sometimes urogenital disease in chickens that leads to problems with egg production and shell quality in layer chickens. The causative coronaviruses are an enveloped and positive-stranded RNA virus, containing an unsegmented genome of approximately 27.6 kb [8]. To protect poultry from IBV infection, live or inactivated vaccines are used [8]. However, the protection afforded by vaccination is incomplete, because the high mutation frequency of IBV leads to the emergence of new strains [8].

IBV has three major virus-encoded structural proteins: spike (S) glycoprotein, membrane (M) protein, and nucleocapsid (N) protein. The IBV spike is formed by the post-translational cleavage of S1 and S2 polypeptides [3]. The S1 glycoprotein is associated with virus attachment and is a major target of neutralizing antibodies in chickens [4, 11]. The genetic grouping of IBV has been performed based on the nucleotide sequence of the S1 gene [10, 13, 14, 20, 21]. Although a major antigenic site does not exist in S2, this region is associated with antigenicity that is affected by the conformation [2], and the S2 subunits of IBV were identified as determinants of cellular tropism [1]. Therefore, analysis of the S2 gene is important for understanding the antigenicity and cellular tropism of the isolates. Most importantly, the antigenic diversity in IBV may arise from a recombination between strains classified into different genetic groups [9, 12].

We have reported the Japanese epidemic genotype based on S1 gene analysis [16, 18, 19] and that genetic recombination had occurred in poultry and recombinant viruses might be epidemic strains in Japan, based on S2 gene analysis [17]. To obtain the finding about the genotypes of recent IBV isolates in Japan, we analyzed 17 IBV isolates, based on S1 and S2 genes, obtained from various prefectures in Japan during 2008–2019.

The IBV isolates were obtained from prefecture-based regional animal hygiene service centers in Japan (Table 1). The allantoic cavities of embryonated eggs were used for virus propagation. In the inoculated embryos, IBV was detected through IBV-specific reverse transcriptase (RT)-PCR, as described [19] and by observing characteristic embryonic changes such as dwarfing, stunting, or curling.

First, we amplified the S1 gene, the major determinant IBV antigenicity, using PCR, as reported [18]. Viral RNAs from infected culture fluids were extracted using the QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, USA). PCR amplification, sequencing, and phylogenetic analysis were performed as described [18]. The following primer set was used for the S1 gene: forward, 5′-AGGAATGGTAAGTTRCTRGTWAGAG -3′ and reverse, 5′- GCGCAGTACCRTTRAYAAAATAAGC-3′ [18]. The predicted size of the PCR products was approximately 700 bp and included the hypervariable region and the region used to

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Table 1. Infectious bronchitis virus (IBV) isolates from field cases in chickens during 2008–2019 in Japan

Virus	Isolation year	Type of chicken	Major clinical sign	Tissue	Genetic group based on S1	Genetic group based on S2*
JP/Kagoshima/2008	2008	Broiler	Depression, respiratory	Trachea	JP-I	V
JP/Kagoshima-1/2009	2009	Broiler	Depression, respiratory	Trachea	JP-III	VII
JP/Kagoshima-2/2009	2009	Broiler	Respiratory, nephritis	Kidney	JP-III	VII
JP/Chiba/2010	2010	Layer	Nephritis	Kidney	JP-IV	IX
JP/Yamagata/2011	2011	Layer	Nephritis	Kidney	JP-II	VI
JP/Kochi/2013	2013	Homebred chicken	Rise in mortality	Kidney	JP-III	VII
JP/Nagasaki/2013	2013	Layer	Depression, diarrhea	Kidney	JP-III	VII
JP/Kagoshima-1/2014	2014	Broiler	Respiratory, nephritis	Kidney	JP-I (*Hpa*I+)	VII
JP/Kagoshima-2/2014	2014	Broiler	Respiratory, nephritis	Kidney	Mass	VII
JP/Kagoshima-3/2014	2014	Broiler	Respiratory, nephritis	Trachea	JP-IV	VII
JP/Kagoshima-4/2014	2014	Broiler	Depression, rise in mortality	Trachea	Gray	VII
JP/Nagasaki/2015	2015	Broiler	Respiratory	Trachea	JP-II	VI
JP/Gifu/2015	2015	Layer	Egg drop	Oviduct	JP-III	VII
JP/Nagasaki/2016	2016	Broiler	Respiratory, diarrhea	Kidney	JP-III	VII
JP/Yamagata/2017	2017	Broiler	Rise in mortality	Kidney	JP-I	VI
JP/Chiba/2018	2018	Broiler	Respiratory	Lung	JP-III	VII
JP/Kumamoto/2019	2019	Layer	Nephritis	Kidney	JP-I (*Hpa*I+)	VII

*According to previous report [17].

construct a phylogenetic tree, as described [18].

DNA fragments of the expected size were successfully amplified from all IBV samples. Determination of the nucleotide sequences of the obtained PCR products revealed the diversity in their lengths (671–692 bp). The S1 sequences of IBV obtained in this study were deposited in GenBank (Acc No. LC588320-36).

Using phylogenetic analysis, the isolates were classified into the JP-I, J-II, JP-III, JP-IV, Mass, and Gray genotypes (Table 1, Fig. 1A). JP-III was the most prevalent genotype; the previously identified 4/91 genotype [16] was not detected.

We have previously reported the RFLP method for simple genotyping using *Hae* II and *Eco* RI [18]; however, some strains identified in this study could not be distinguished by the RFLP method. For example, JP/Nagasaki/2013, JP/Kochi/2013 and JP/Nagasaki/2016 strains are classified into the genotype JP-III by phylogenetic analysis. However, these strains had lost the *Eco* RI digestion site, leading to misidentification as Mass type by a previous RFLP method. Therefore, we have modified the RFLP method for consistency of classification by phylogenetic analysis. After comparing all sequences obtained (GENETYX-Mac, ver. 18, Software Development Corp., Tokyo, Japan), we used *Hae* II, *Hpa* I, and *Fok* I (Takara, Tokyo, Japan) to differentiate between the prevalent S1 genotypes in Japan (Table 2, Fig. 2). Each prevalent genotype had a specific restriction profile and they were easily distinguishable. In addition, we examined 50 or more sequences of the domestic IBV strains deposited with GenBank to further verify this method. Most strains consistent with any of the profiles of genotype based on S1 gene are shown in Table 2 and are consistent with S1 genotype based on phylogenetic analysis. The only exception was JP/Shizuoka-1/2015 (genotype JP-I, Acc. No.LC428324) strain [10], which had lost the *Hae* II digestion site, but the profile of digestion using *Hpa* I was consistent with that of JP-I.

To further examine the genetic diversity of these isolates, we analyzed the S2 gene, based on a previous report. For the S2 gene, the following primer set was used: forward, 5′-TGGATAAGGTCCAAATTAATTG-3′ and reverse, 5′-GCTGCTGTAATACCACCAAAAGCCAT-3′ [17]. The predicted size of the PCR product was approximately 490 bp. DNA fragments of the expected size were successfully amplified by RT-PCR from all IBV samples, confirming that these primers could be used to detect field isolates. Using phylogenetic analysis, the isolates were classified into four genetic groups based on the S2 gene, namely V, VI, VII, and IX (Table 1, Fig. 1B). The S2 sequences of IBV obtained in this study were deposited in GenBank (Acc No. LC588337-53).

By combining the S1 and S2 genotypes, the IBV strains isolated in Japan during 2008–2019 were classified into nine groups, which include undetected five groups in previous report (Table 3) [17]. Notably, several viruses classified into group VII based on S2 gene analysis were divided into various genotypes based on S1 gene analysis. For example, the three strains isolated from the Kagoshima prefecture in 2014 were classified into three genotypes (JP-IV, Mass, and Gray) based on the S1 gene, but were classified into type VII based on the S2 gene. The sequence similarity between the S2 genes of the three isolates was found to be 96–98%, suggesting that genetic recombination between virus strains classified into different genetic groups had occurred in poultry. These three isolates were derived from the same farm, which strongly reveals that such a recombination had occurred on the farm. The mechanism of how virus strains with closely related S2 genes were generated needs to be elucidated.

In the present study, the identified novel genetic groups based on a combination of S1 and S2 genotypes, which were undetected previously, showed that various recombinant IBV strains were prevalent in poultry in Japan (Table 3). Our results strongly suggest that in addition to the analysis of the S1 gene, regions such as the S2 gene are important for characterizing IBV isolates in detail.

Using the first analysis of the S1 gene, IBV isolates collected between 2008–2019 were classified into six genetic groups, which

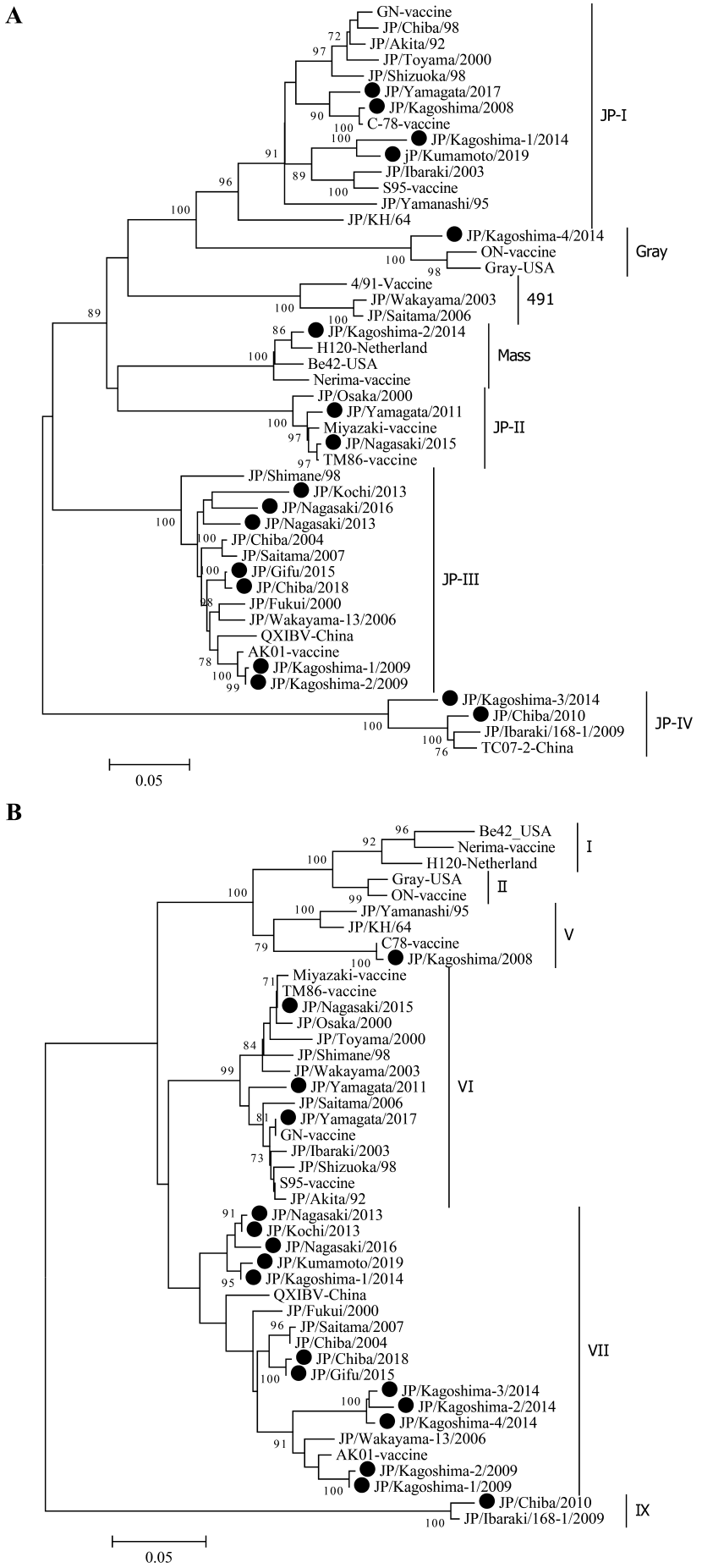

Fig. 1. (A) Phylogenetic tree based on the S1 glycoprotein gene of infectious bronchitis virus (IBV). Nucleotides 20368-20988 (621 bases) of the S1 gene of IBV Beaudette (GenBank Accession No. NC001451) were subjected to phylogenetic analysis. (B) Phylogenetic tree based on the S2 glycoprotein gene of infectious bronchitis virus (IBV). Nucleotides 22197-22552 (356 bases) of the S2 gene of IBV Beaudette (GenBank Accession No. NC001451) were subjected to phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. The viruses studied here are shown by a black circle.

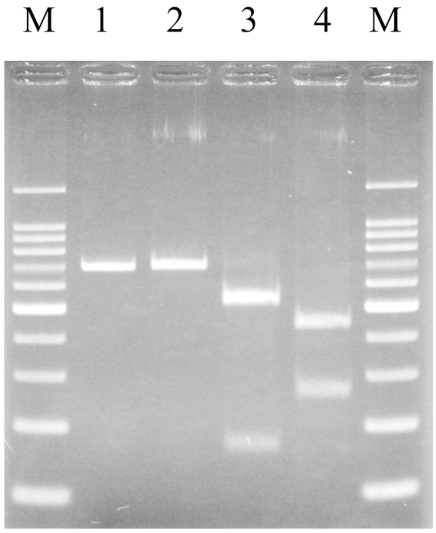

Fig. 2. An example of restriction endonuclease analysis of the PCR-amplified S1 gene. The strain JP/Chiba/2018, which was classified into genotype JP-III, was used. Lane M: 100 bp ladder marker. Lane 1: undigested. Lane 2: *Hae* II digest. Lane 3: *Hpa* I digest. Lane 4: *Fok* I digest.

have been reported previously, namely Mass, Gray, JP-I, II, III, and VI, using phylogenetic analysis based on the S1 gene. The S1 glycoprotein is a major target of neutralizing antibodies in chickens; therefore, serotype evolution of IBV is associated primarily with the sequences of the S1 glycoprotein [4, 11], and the genetic relationship between viruses based on S1 amino acid sequences can be used to predict the level of cross-protection between different IBV types, with some exceptions [5, 15]. We have reported that the IBV strains isolated in Japan are classified phylogenetically into seven genotypes [19]. Consistently, a recent nationwide survey of IBV in layer farms in Japan revealed the prevalence of the JP-I, JP-II, JP-III, and Mass genotypes based on the S1 gene [10]; however, the 4/91 genotype, prevalent in Europe, was not detected in this study. Thus, no new genotype based on the S1 gene of IBV has been confirmed in Japan in the last 10 years.

However, additional analyses of the S2 gene revealed diversity among virus strains and suggested that recombination events were likely to be major determinants of viral evolution, as reported [9]. The combination of JP-III (S1) and VII (S2) was most commonly detected in this study (Table 3), with a detection rate of approximately 40%. The rate of this combination was nearly 20% [17], and this VII (S2) type was limited to only JP-III (S1) in a previous study [17]. Moreover, the combination VII (S2) with various S1 genotype (JP-I, IV, Mass, and Gray) were detected in this study, suggesting that various genetic recombination between different S1 genotype virus strains have occurred in domestic poultry.

Table 2. Summary of modified restriction endonuclease analysis of PCR products from infectious bronchitis virus (IBV) isolates in Japan

Genetic groups based on S1 gene	Lenngth of PCR products	Example strain in this study	The size (base pairs) of fragments generated by		
			Hae II	*Hpa* I	*Fok* I
JP-I	689–692	JP/Yamagata/2017	458–461, 231[a]	Not digested	Not digested[b]
JP-I (*Hpa*I+)		JP/Kagoshima-1/2014		393–396, 296	
JP-II	674	JP/Nagasaki/2015	287, 175, 162, 50[c]	511, 163	Not digested
JP-III	674–680	JP/Chiba/2018	Not digested	511–517, 163	426, 248–254[d]
JP-IV	683	JP/Chiba/2010	Not digested	520, 163	Not digested
Mass	665–671	JP/Kagoshima-2/2014	Not digested	Not digested	498–504, 167
Gray	683–689	JP/Kagoshima-4/2014	560–566, 123	Not digested	Not digested
4/91	677	JP/Saitama/2006[e]	Not digested	Not digested	Not digested

a) There is one exception strain JP/Shizuoka/98, which was digested into 305, 228, and 156 bp for addition of one cutting site reported previously [18]. And except JP/Kumamoto/2019, which was digested into 311, 231, and 150 bp for an additional cutting site. b) There are two exception strains, JP/Yamanashi/95 and JP/Chiba/98, which were digested into 459–464 and 228–233 bp for addition of one cutting site. c) There is one exception strain JP/Osaka/2000, which was digested into 287, 212, and 175 bp for loss of one cutting site reported previously [18]. d) There are two exception strains JP/Nagasaki/2013 and JP/Nagasaki/2016, which were digested into 426, 153–156, and 98 bp for an additional cutting site. e) This strain cited in a previous report [16].

Table 3. Summary of genetic groups based on S1 and S2 genes of IBV isolated in Japan during 2008–2019

Groups based on		Example strain in this study	Number of strains in this study	Number of strains in previous report [17]	Index strain in previous report [17]
S1	S2				
JP-I	V	JP/Kagoshima/2008	1	6	C-78-vaccine
JP-I	VI	JP/Yamagata/2017	1	10	GN-vaccine
JP-I	VII	JP/Kagoshima-1/2014	2	0	Not found
JP-II	VI	JP/Yamagata/2011	2	4	Miyazaki-vaccine
JP-III	VII	JP/Kagoshima-1/2009	7	7	JP/Fukui/2000
JP-IV	IX	JP/Chiba/2010	1	0	Not found
JP-IV	VII	JP/Kagoshima-3/2014	1	0	Not found
Mass	VII	JP/Kagoshima-2/2014	1	0	Not found
Gray	VII	JP/Kagoshima-4/2014	1	0	Not found

The effect of recombination on the antigenicity, virulence, replication, shedding, and tissue tropism of the virus warrants further study. Two field IBVs used to successively infect chickens in a short-period on a farm were suggested to generate recombinant strains in infected poultry [12]. Therefore, clarifying the mechanism of emergence of recombinant viruses due to infection with multiple IBV strains is important.

Although this study analyzed the partial nucleotide sequences of the S1 and S2 genes, complete genome analysis of many strains of the virus and various genetic recombinants have been reported [6, 7, 9, 22]. However, IBV strains that have undergone complete genome analysis have not been reported in Japan. Examining the genetic diversity of IBV strains for each genotype by complete genome analysis in Japan is more warranted. At present, we are working on determining the complete genome sequence of some IBV strains, including the strains used in this study.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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