NOTE

Avian Pathology

Molecular survey of infectious bronchitis virus on poultry farms in Gifu Prefecture, Japan from 2021 to 2022 by RT-PCR with an enhanced level of detection sensitivity for the S1 gene

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ABSTRACT. Infectious bronchitis virus (IBV) is the causative agent of infectious bronchitis (IB) in chickens. There is a correlation between cross-protection and percentage of similarity between nucleotide sequences encoding the S1 subunit, which is responsible for generating neutralizing and serotype-specific antibodies. Therefore, RT-PCR is commonly used to amplify the IBV-S1 gene following DNA sequencing in order to predict the efficacy of vaccines against IBV strains. We successfully enhanced the sensitivity for detection of the IBV-S1 gene by second PCR after purification of the 1st RT-PCR product. Using that method, we obtained detailed information on the prevalence of IBV on poultry farms in Gifu Prefecture, Japan. The IBV-S1 gene detection method used in the current study will enable accurate information on the prevalence of IBV in Japan to be obtained.

KEYWORDS: genotyping, infectious bronchitis virus, spike gene, survey

Infectious bronchitis (IB) is an acute, highly contagious upper respiratory tract disease in chickens, but it can also occur in the kidneys and reproductive tract, leading to nephritis and reproductive disorders [8]. Infectious bronchitis virus (IBV), which is the causative agent of IB, is distributed worldwide. While inactivated vaccines and live attenuated vaccines have been developed, IBV has not been completely eliminated in any country, and IB has caused a significant economic loss to commercial chicken industries worldwide [6]. It is estimated that an IBV infection in a commercial flock with the best possible management practice reduces income by approximately 3%, in comparison with a hypothetical flock free from IBV [8]. Reduction of the economic losses associated with IB has been a challenge for the poultry industry for a long time.

IBV belongs to the *Gammacoronaviru*s genera in the subfamily *Coronavirinae* and family *Coronaviridae*. The viral genome enclosed by an envelope is a single-stranded positive-sense strand of RNA that is approximately 27.5–28 kb in length. The virions are made up of four canonical structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins [1]. Among them, the S protein is the most important for antigenic and immunogenic reasons. The S protein is a trimer made up of two subunits, S1 and S2 (approximately 520 and 625 amino acids, respectively). The S1 subunit is associated with virus attachment to host cells and is a major inducer of neutralizing antibodies in chickens, meaning that mutations in the antigenically important S1 subunit lead to the emergence of variant serotypes associated with disease outbreaks [8]. Since there are many serotypes of IBV worldwide, it is important to know the serotypes of IBV and to use vaccines with the corresponding serotypes for control of IB in chickens.

Serological and genetic typing methods have been used to classify IBV strains. Since it has been shown that there is a correlation between percentage of similarity between S1 gene sequences and cross-protection [22], IBV strains that fall into the same genetic type based on the S1 gene sequence are generally related serologically. Accordingly, RT-PCR is currently used to amplify the S1 gene following nucleic acid sequencing in most laboratories [8, 11, 14]. Mase *et al*. established RT-PCR for genotyping Japanese IBV
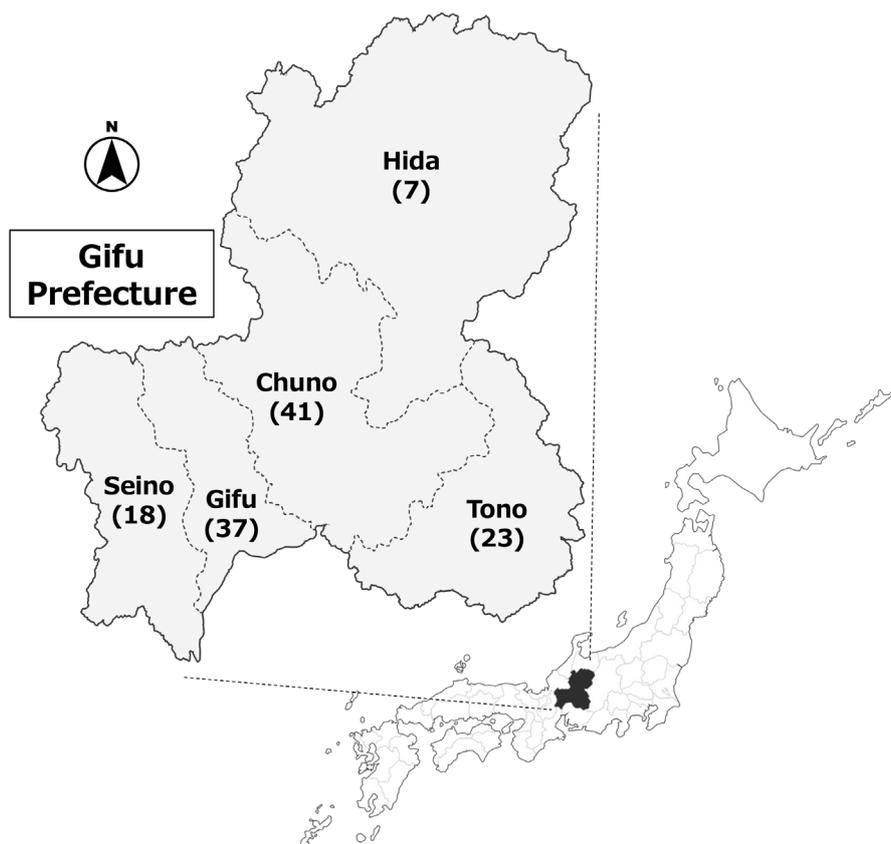
Fig. 1. Map of sampling regions in Gifu Prefecture. Gifu Prefecture includes Seino, Gifu, Chuno, Tono and Hida regions. The numbers in parenthesis indicate the number of samples collected from each region.

strains based on S1 gene analysis, which enabled identification of seven genotypes (JP-I, JP-II, JP-III, JP-IV, Mass, Gray, and 4/91) [15, 17, 18]. The method developed by Mase *et al.* has been used for molecular surveys of IBV in Japan by several groups [7, 9, 10], and the utility of the method for selection of appropriate vaccines against IB in Japan has been demonstrated. Kaneda *et al.* recently reported the results of a nationwide survey for IBV in Japan by using Mase's method [9]; however, there was a lack of information on the prevalence of IBV in Gifu Prefecture, which is part of the Chubu area of Japan. So far, there has been little information of the prevalence of IBV in Chubu and Kinki areas in Japan.

In the current study, we investigated the prevalence of IBV on poultry farms in Gifu Prefecture (including Seibu, Gifu, Chuno, Tono, and Hida regions) by using RT-PCR for the N gene, which is relatively conserved in the IBV genome, and the S1 gene according to Mase's method. Furthermore, we simply conducted the 2nd PCR by using the same primer pairs for the S1 gene after purification of the 1st RT-PCR reactions, which successfully led to a significant enhancement of detection sensitivity for the S1 gene. Our data obtained in this study provided information on the genotypes of prevailing IBV strains in Gifu Prefecture, which could not be detected by the 1st RT-PCR for the IBV-S1 gene. RT-PCR with an enhanced level of sensitivity for detection of the IBV-S1 gene will provide information on the exact prevalence of IBV and will contribute to future molecular surveys of IBV in order to use appropriate IB vaccines in Japan.

Samples for the current study were collected during the period from March 2021 to January 2022. Cloacal swabs were collected from 10 subclinical chickens in each poultry farm and pooled into a single sample. We collected 118 cloacal samples from subclinical chickens in poultry farms in Seino, Gifu, Chuno, Tono, and Hida regions of Gifu Prefecture, Japan. The samples from subclinical chickens were collected for monitoring highly pathogenic influenza virus (HPAI) and low pathogenic influenza virus (LPAI), and the samples were negative in RT-PCR for both viruses, meaning that these monitoring samples were derived from chickens with no clinical signs. Similar to the sampling method described above for subclinical chickens, we collected 4 cloacal samples from poultry farms with egg drop. For tracheal samples, we collected a tracheal swab from a specific chicken showing a clinical sign (e.g., depression) in a farm with a rise in mortality rate and counted it as a single sample. Consequently, we obtained 4 tracheal samples from 4 farms. A total of 126 samples from 99 poultry firms were used in the current study. A map of Gifu Prefecture and the number of samples obtained from each region are shown in Fig. 1.

RNAs from the samples collected were extracted by using TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) following the instructions in the manufacturers' product manuals. RT-PCR was performed using the One-Step RT-PCR Kit (TaKaRa Bio Inc., Kusatsu, Japan) and IBV-N gene specific primers (IBV-N forward,

5′-AGCACCCTTAGCAGCAACCC-3′; IBV-N reverse, 5′-ATCTTCAGTTTVGGAGGTAA-3′) [25]. RT reaction was performed at 50°C for 30 min, and PCR amplification for the N gene was successively carried out with the initial step at 95°C for 2 min followed by 35 cycles of amplification: 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec. Subsequently, from each N gene-positive sample by RT-PCR, a partial DNA fragment of the S1 gene that includes the hypervariable region was amplified by the same method as the N gene amplification. Primers used for the IBV-S1 gene were 5′-AGGAATGGTAAGTTRCTRGTWAGAG-3′ (forward) and 5′-GCGCAGTACCRTTRAYAAAATAAGC-3′ (reverse) [15]. After purification of the 1st RT-PCR products for the IBV-S1 gene with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany), they served as templates for the 2nd PCR for the IBV-S1 gene by using KOD FX Neo (TOYOBO Co., Ltd., Osaka, Japan) and the same primer sets as those shown above. Second PCR amplification for the S1 gene was carried out with the initial step at 95°C for 2 min followed by 20 cycles of amplification: 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec. PCR products of the IBV-S1 gene were purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Tokyo, Japan) on an ABI Prism 3100 DNA analyzer (Thermo Fisher Scientific). The sequencing was performed with the forward and reverse primers used for PCR of the IBV-S1 gene. Sequence alignments and construction of a phylogenetic tree were performed using MEGA version X software [12]. A phylogenetic tree was constructed by the maximum likelihood method [24] with 1,000 bootstrap replicates.

A total of 126 samples from 99 farms in Gifu Prefecture were used for analysis in our laboratory. The samples came from broiler and layer farms in Seino (18 samples), Gifu (37 samples), Chuno (41 samples), Tono (23 samples), and Hida regions (7 samples) (Fig. 1). Forty-two of 118 monitoring samples were positive for the IBV-N gene by RT-PCR (Table 1 and Fig. 2), whereas 4 of 8 clinical samples were positive for the IBV-N gene, indicating that about one third of the flocks in Gifu Prefecture retain IBV. The numbers of samples positive for IBV-N gene by RT-PCR in Seino, Gifu, Chuno, Tono, and Hida regions were 6, 12, 14, 10, and 4, respectively. The positive rates in the regions ranging from 32.4% to 57.1% showed no significant differences. Interestingly, we could not find IBV genotype JP-I in Tono and Hida regions, whereas that was the majority in Seino, Gifu and Chuno regions. These data imply that IBV genotype JP-I may not prevail in these regions. To clarify this point, we need to increase the number of samples for an IBV survey in the future.

Next, we tried to detect the IBV-S1 gene from the 46 samples that were positive for the IBV-N gene. While nested RT-PCR and semi-nested RT-PCR are promising methods for increasing the sensitivity for detection of a target gene, a primer(s) for the 2nd PCR should be designed to be annealed to a sequence internal to the sequence amplified by the 1st PCR. Therefore, nested RT-PCR and semi-nested RT-PCR might miss detection of broad genotypes of IBVs due to the presence of a hypervariable region in the S1 gene region. To avoid that potential problem, we designed RT-PCR with two PCRs by using the same primer pairs after purification of the 1st RT-PCR products. Importantly, we demonstrated that detection efficacy for the IBV-S1 gene with the 2nd PCR was significantly higher than that with the 1st RT-PCR (Fig. 2), indicating that we successfully established a method with an enhanced level of detection sensitivity for the IBV-S1 gene. Methods for detection of the IBV-S1 gene with a high level of sensitivity will provide detailed information on the prevalence of IBV, which is important for control of IB on poultry farms. Furthermore, since RT-PCR with the 2nd PCR used in the current study is technically simple, it may be useful as a method for enhancing the sensitivity for detections of other genes coding a hypervariable region.

At the 1st RT-PCR, the number of IBV-N gene-detected samples (46 samples) were larger than the number of IBV-S1 gene-detected samples (18 samples) (Fig. 2). Recently, Shirato et al. showed that detection efficiency for the SARS-CoV-2-N gene in clinical samples from patients is higher than that for the SARS-CoV-2-S gene with qRT-PCR [21]. During viral replication, a coronavirus generates genomic RNA and several subgenomic mRNAs for expression of the viral protein, and subgenomic mRNAs coding the coronavirus N gene are the most abundant in coronavirus-infected cells [4, 13]. The high efficiency for detection of the IBV-N gene suggests that clinical samples in the current study may contain a significant amount of subgenomic RNAs. In addition, there remained the possibility of the primers' mismatch to the IBV-S1 gene due to the presence of a hypervariable region in the S1 gene region.

Usually, we could not get many samples for diagnosis from layers with suspected IB because it is difficult to identify the exact layers with egg drop from their appearance. Hence, ten (or more) cloacal swabs were randomly collected from layers, and the swabs were pooled into a single sample and subjected to diagnosis of IBV with RT-PCR. This means that detection sensitivity of RT-PCR for the viral gene is critical for the diagnosis of IB in chickens with egg drop. In the current study, we successfully detected the IBV-S1 gene in two pooled samples derived from layers with egg drop (Sample names: Gifu_2021-8 and Gifu_2021-10) by the 2nd PCR, but not by the 1st RT-PCR (Table 1). We hope that the RT-PCR with the 2nd PCR used in this study will contribute to the exact diagnosis of IBV-derived egg drop.

From 30 samples in which the IBV-S1 gene was detected, we successfully determined 27 partial S1 gene nucleic acid sequences (Accession number, LC685308-34). Based on homology rates of the S1 gene region between IBV strains identified in this study and IB vaccine strains in GenBank, the detected IBV strains were classified into two main groups: potential vaccine strains and field strains. Namely, strains with <99% homology with commercial vaccine strains were considered to be field strains, whereas those with 99–100% homology were considered to be vaccine strains [3, 23]. According to these criteria, 13 and 14 samples out of a total 27 samples in which the IBV-S1 gene was detected were classified as field strains and vaccine strains, respectively. These results suggest that field strains, not vaccine strains, invade poultry farms via unidentified routes. While several groups in other countries have found avian coronaviruses similar to IBV in wild birds [1, 2, 5, 19, 20], there is no information on this in Japan. Wild birds might mechanically transport and actively multiply IBV, playing the role of a source for IBV transmission.

In the clinical samples, we detected potential vaccine strains (Gifu_2021-9 and Gifu_2021-10) (Table 1). Interviews with the poultry owners of S1 and O2 farms, where we detected the potential vaccine strains, revealed that they did not use IB vaccines with the same genotype as we detected (data not shown), suggesting a low possibility of vaccine-related IB cases. Although further investigation of

Table 1. Overview of the cases and samples in which the gene(s) was detected in the present study

Collection year/month	Region	Monitoring or clinical sample (Symptom)	Farm	Breed	Tissue	N gene detection	S1 gene detection (1st or 2nd PCR)	Genotype	Vaccine strain with the highest homology rate (%)	Potential vaccine or field strain [a]	Accession # of partial S1 gene (Sample name)
2021/Mar	Gifu	Monitoring	A1	Layer	Cloacal swab	+[b]	−[c]				
2021/Mar	Tono	Monitoring	B1	Layer	Cloacal swab	+	−				
2021/Mar	Hida	Monitoring	C1	Layer	Cloacal swab	+	+ (1st PCR)	JP-II	Miyazaki (98.9%)	Field strain	LC685320 (Gifu_2021-1)
2021/Mar	Chuno	Monitoring	D2	Layer	Cloacal swab	+	−				
2021/Mar	Chuno	Monitoring	E1	Layer	Cloacal swab	+	+ (1st PCR)	JP-I	GN (93.3%)	Field strain	LC685321 (Gifu_2021-2)
2021/Mar	Tono	Monitoring	F1	Layer	Cloacal swab	+	−				
2021/Mar	Gifu	Monitoring	G1	Layer	Cloacal swab	+	+ (2nd PCR)	JP-I	C78 (99.3%)	Vaccine strain	LC685316 (Gifu_2021-3)
2021/Mar	Tono	Monitoring	H1	Layer	Cloacal swab	+	−				
2021/Apr	Seino	Monitoring	I1	Layer	Cloacal swab	+	−				
2021/Apr	Gifu	Monitoring	J1	Layer	Cloacal swab	+	+ (2nd PCR)	JP-II	Miyazaki (98.4%)	Field strain	LC685317 (Gifu_2021-4)
2021/Apr	Tono	Monitoring	K1	Layer	Cloacal swab	+	−				
2021/Apr	Tono	Monitoring	L1	Layer	Cloacal swab	+	−				
2021/Apr	Hida	Monitoring	M1	Layer	Cloacal swab	+	−				
2021/Apr	Hida	Monitoring	N1	Broiler	Cloacal swab	+	+ (2nd PCR)	Gray	ON (99.6%)	Vaccine strain	LC685318 (Gifu_2021-6)
2021/Apr	Chuno	Monitoring	O1	Layer	Cloacal swab	+	+ (1st PCR)	JP-II	TM86 (97.7%)	Field strain	LC685322 (Gifu_2021-7)
2021/May	Gifu	Monitoring	P1	Layer	Cloacal swab	+	−				
2021/May	Tono	Monitoring	Q1	Layer	Cloacal swab	+	+ (2nd PCR)	ND [d]			
2021/Jul	Gifu	Monitoring	R1	Layer	Cloacal swab	+	−				
2021/Jul	Chuno	Monitoring	S1	Broiler	Cloacal swab	+	+ (1st PCR)	JP-I	C78 (99.5%)	Vaccine strain	LC685311 (Gifu_2021-11)
2021/Aug	Gifu	Monitoring	T1	Layer	Cloacal swab	+	+ (1st PCR)	JP-III	AK01 (92.5%)	Field strain	LC685308 (Gifu_2021-15)
2021/Aug	Chuno	Monitoring	U1	Layer	Cloacal swab	+	+ (1st PCR)	JP-I	C78 (99.7%)	Vaccine strain	LC685309 (Gifu_2021-16)
2021/Sep	Gifu	Monitoring	V1	Layer	Cloacal swab	+	+ (1st PCR)	JP-I	C78 (96.7%)	Field strain	LC685315 (Gifu_2021-12)
2021/Sep	Seino	Monitoring	W1	Broiler	Cloacal swab	+	+ (1st PCR)	JP-I	C78 (99.1%)	Vaccine strain	LC685312 (Gifu_2021-13)
2021/Sep	Gifu	Monitoring	X1	Broiler	Cloacal swab	+	+ (1st PCR)	JP-I	C78 (99.7%)	Vaccine strain	LC685313 (Gifu_2021-14)
2021/Oct	Seino	Monitoring	Y1	Layer	Cloacal swab	+	+ (2nd PCR)	JP-I	C78 (98.6%)	Vaccine strain	LC685324 (Gifu_2021-22)
2021/Oct	Gifu	Monitoring	Z1	Layer	Cloacal swab	+	−				
2021/Nov	Tono	Monitoring	A2	Broiler	Cloacal swab	+	+ (1st PCR)	JP-II	Miyazaki (99.3%)	Vaccine strain	LC685323 (Gifu_2021-17)
2021/Nov	Seino	Monitoring	B2	Broiler	Cloacal swab	+	+ (2nd PCR)	JP-I	C78 (99.7%)	Vaccine strain	LC685325 (Gifu_2021-23)
2021/Nov	Chuno	Monitoring	C2	Broiler	Cloacal swab	+	+ (2nd PCR)	4/91	4/91 (92.7%)	Field strain	LC685327 (Gifu_2021-26)
2021/Nov	Chuno	Monitoring	S1	Broiler	Cloacal swab	+	+ (2nd PCR)	JP-I	C79 (99.3%)	Vaccine strain	LC685326 (Gifu_2021-24)
2021/Nov	Tono	Monitoring	D2	Layer	Cloacal swab	+	+ (1st PCR)	JP-II	Miyazaki (99.8%)	Vaccine strain	LC685334 (Gifu_2021-18)
2021/Nov	Tono	Monitoring	E2	Layer	Cloacal swab	+	+ (2nd PCR)	ND			
2021/Dec	Seino	Monitoring	F2	Layer	Cloacal swab	+	−				
2021/Dec	Chuno	Monitoring	G2	Layer	Cloacal swab	+	−				
2021/Dec	Gifu	Monitoring	H2	Layer	Cloacal swab	+	+ (1st PCR)	JP-III	AK01 (93.1%)	Field strain	LC685328 (Gifu_2021-27)
2021/Dec	Gifu	Monitoring	I2	Layer	Cloacal swab	+	+ (1st PCR)	JP-II	Miyazaki (98.2%)	Field strain	LC685329 (Gifu_2021-28)
2021/Dec	Chuno	Monitoring	J2	Layer	Cloacal swab	+	−				
2021/Dec	Chuno	Monitoring	K2	Broiler	Cloacal swab	+	+ (1st PCR)	JP-III	AK01 (96.2%)	Field strain	LC685330 (Gifu_2021-29)
2021/Dec	Hida	Monitoring	L2	Layer	Cloacal swab	+	−				
2022/Jan	Chuno	Monitoring	E1	Layer	Cloacal swab	+	+ (1st PCR)	JP-I	C78 (99.7%)	Vaccine strain	LC685331 (Gifu_2021-30)
2022/Jan	Chuno	Monitoring	M2	Layer	Cloacal swab	+	+ (2nd PCR)	ND			
2022/Jan	Chuno	Monitoring	N2	Layer	Cloacal swab	+	+ (1st PCR)	JP-I	GN (90.9%)	Field strain	LC685332 (Gifu_2021-32)
2021/Jun	Gifu	Clinical (Egg drop)	T1	Layer	Cloacal swab	+	+ (2nd PCR)	JP-III	AK01 (93.3%)	Field strain	LC685319 (Gifu_2021-8)
2021/Jul	Chuno	Clinical (Rise in mortality)	S1	Broiler	Tracheal swab	+	+ (1st PCR)	4/91	4/91 (99.8%)	Vaccine strain	LC685310 (Gifu_2021-9)
2021/Sep	Seino	Clinical (Egg drop)	O2	Layer	Cloacal swab	+	+ (2nd PCR)	JP-I	C78 (99.5%)	Vaccine strain	LC685314 (Gifu_2021-10)
2022/Jan	Tono	Clinical (Rise in mortality)	F1	Broiler	Tracheal swab	+	+ (1st PCR)	JP-II	Miyazaki (97.6%)	Field strain	LC685333 (Gifu_2021-33)

[a] Strains with <99% homology with commercial vaccine strains were considered to be field strains, and those with 99–100% homology were considered to be vaccine strains. [b][c] + and − indicate that the IBV gene was detected and was not detected, respectively. [d] ND means "not determined".

the clinical samples is needed, the clinical signs of the chickens in the farms might be caused by pathogens other than IBV.

The clinical sample of Gifu_2021-8 and the monitoring sample of Gifu_2021-15 were collected from the same farm and belong to the JP-III genotype (Table 1 and Fig. 3). According to the owners of the farm, IB vaccines belonging to the JP-III genotype were not used in their poultry flock, suggesting invasion of genotype JP-III IBV to the poultry flock. In Japan, cloacal samples are collected
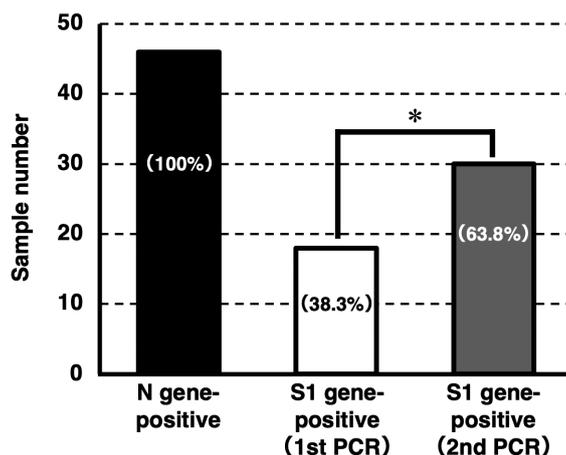
Fig. 2. Number of samples in which each viral gene was detected. Bars indicate the numbers of sample positive for the gene by each PCR condition. The number in parenthesis shows relative detection efficiency by each PCR condition taking that of infectious bronchitis virus (IBV)-N gene-positive as a base of 100%. An asterisk represents statistically significant differences in detection efficiency with Fisher's exact test ($P<0.05$).

from poultry farms throughout the year for monitoring HPAI and LPAI. Application of the monitoring samples to genotyping of prevailing IBV strains with the method for the S1 gene detection used in this study would provide the exact information for selecting appropriate IB vaccines with less time and effort.

The phylogenetic tree based on the IBV-S1 gene showed that the field strains detected in this study belong to the JP-I, JP-II, JP-III, Gray, or 4/91 genotype (Fig. 3). Interestingly, we found field strain (Gifu_2021-26) belonging to the 4/91 genotype by RT-PCR with the 2nd PCR, although previous investigations in Japan by Mase's method with 1st RT-PCR could not detect IBV strains belonging to the 4/91 genotype [9, 16]. Even when we used RT-PCR with 2nd PCR, an IBV strain belonging to the JP-IV genotype, for which no vaccine has been developed, was not detected, suggesting a low prevalence of IBV strains belonging to the JP-IV genotype in Gifu Prefecture. Taken together, the results indicate that RT-PCR with 2nd PCR and sequence analysis of amplified IBV-S1 genes provide the exact information on prevailing IBV strains.

We conducted an IBV survey in Gifu Prefecture by RT-PCR with an enhanced level of detection sensitivity for the IBV-S1 gene region using samples from clinical/subclinical chickens. Our study revealed the detailed status of IBV prevalence in Gifu Prefecture. The IBV-S1 gene detection method used in this study will enable obtain information on the exact prevalence of IBV to be obtained and will contribute to future molecular surveys of IBV in order to use appropriate IB vaccines in Japan.
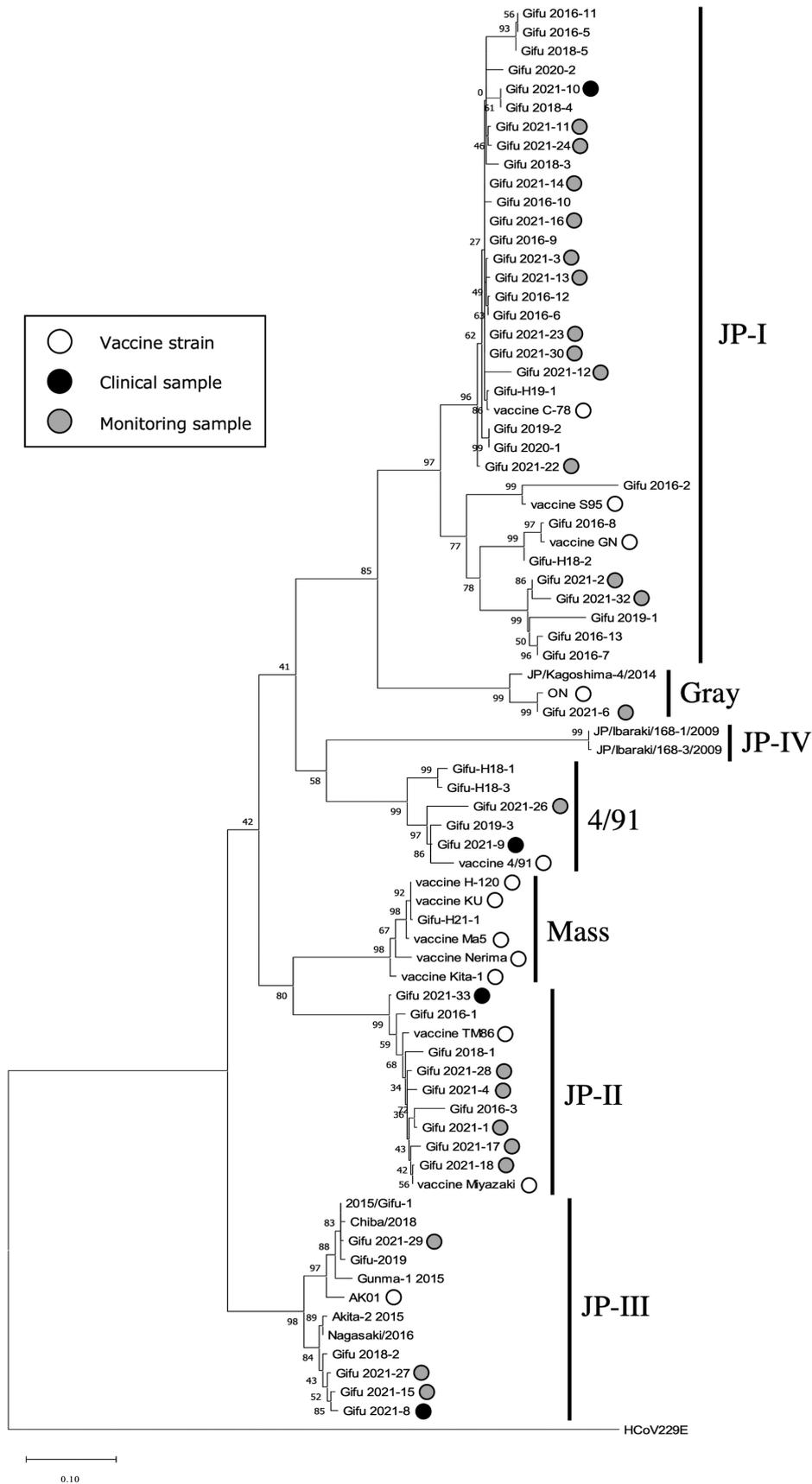
Fig. 3. Phylogenetic tree based on the infectious bronchitis virus (IBV)-S1 gene detected in this study. The tree was generated by the maximum likelihood method (bootstrap value: 1,000 replicates) using MEGA version X. Blank, shaded, and black circles indicate vaccine strains, monitoring samples, and clinical samples, respectively. The bar indicates nucleotide substitution per site. The S1 gene sequences were classified into 7 genotypes (JP-I, JP-II, JP-III, JP-IV, Gray, Mass, and 4/91) as previously reported [16, 18].

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