

Genetic Analysis of the S1 Gene of 4/91 Type Infectious Bronchitis Virus Isolated in Japan

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ABSTRACT. S1 gene sequences for infectious bronchitis virus (IBV) strains of the 4/91 genotype (commonly called 793B) isolated from field outbreaks in Japan were analyzed to ascertain the relationship to 4/91 vaccine strain. Three field isolates (JP/Wakayama/2003, JP/Iwate/2005 and JP/Saitama/2006) from flocks not immunized with a 4/91 type live IBV vaccine and one isolate (JP/Wakayama-2/2004) from a flock immunized with a 4/91 type live vaccine were examined. The amino acid identities among JP/Wakayama/2003, JP/Iwate/2005 and JP/Saitama/2006 were about 98%, whereas the identities to the 4/91 vaccine strain and JP/Wakayama-2/2004 were about 90%. Three of the field isolates, JP/Wakayama/2003, JP/Iwate/2005 and JP/Saitama/2006, were classified into a cluster closely related to French and Spanish isolates, but different from the cluster including the vaccine and JP/Wakayama-2/2004. These results indicate that JP/Wakayama/2003, JP/Iwate/2005 and JP/Saitama/2006 were derived from foreign field isolates, but not from the vaccine strain. On the other hand, the S1 gene of JP/Wakayama-2/2004 revealed high sequence similarity with that of the 4/91 vaccine strain and appeared to be a vaccine-like virus derived from a vaccine. The field isolates of 4/91 genotype IBV could be distinguished from other genotypes by using the *Bal*I and *Pst*I enzymes in addition to the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) methods of Mase *et al.* [16] using *Hae*II and *Eco*RI enzymes. Furthermore, the 4/91 vaccine strain and vaccine-like isolate (JP/Wakayama-2/2004) could be differentiated from the other field isolates by *Bgl*II digestion. This method, therefore, would assist in identification of field isolates of the 4/91 genotype as outbreaks of IBV in vaccinated flocks.

KEY WORDS: 4/91, genotype, infectious bronchitis virus, 793B, vaccine.

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Infectious bronchitis (IB) is an economically important disease in chickens that causes respiratory disease and egg production losses and has a high rate of mortality. The causal agent, IB virus (IBV), readily undergoes mutation in chickens, resulting in the emergence of new variant serotypes and genotypes [6, 15, 22].

Results of molecular studies have shown that the spike (S) protein of IBV is responsible for determining its serotype [11, 12, 13]. Therefore, genotypes based on the sequence of the S gene, especially for the hyper variable region (HVR) in S1, have been used for genotyping of IBV. In a study of Japanese IBV strains isolated between 1951 and 2000, isolates were classified into five genotypes, JP-I, JP-II, JP-III, Mass and Gray, based on the sequence in the N-terminus of the S1 protein including HVR [16]. Recently, some field isolates recovered from commercial chickens have been classified into the 4/91 genotype, and some of the isolates were pathogenic in chicks compared with vaccine strains [20]. These facts indicate that a new variant of the 4/91 genotype has emerged among flocks in Japan.

IBV strains of the 4/91 type, which are also known as

793B, were first reported and characterized in Britain in 1991 [1, 9] and have been the dominant genotype in Europe [21]. Serological survey has revealed high incidences of IBV infection of the 4/91 type in layer and broiler chickens worldwide [5]. In Japan, the 4/91 genotype was first isolated in 2003, and isolates of the 4/91 genotype have been obtained sporadically in distant areas of Japan [20].

Various serological types of live or inactivated vaccines have been available for control of IBV infections in Japan. Vaccines have generally been effective in controlling clinical disease associated with IBV infections, but new variants may continue to emerge and cause clinical disease and production problems in vaccinated flocks [8, 18]. Some studies [7, 10] have reported frequent isolation of isolates related to vaccine strains from diseased flocks in the field, and reversion of the virulence of vaccine strains and recombination has been suspected.

A vaccine containing the 4/91 strain was approved in Japan in 2001 and has been used since 2002. In regard to Japanese isolates of the 4/91 genotype, the possibility that the isolates might be derived from the 4/91 vaccine remains uncertain. Therefore, to ascertain the relationship between 4/91 genotype field isolates and the 4/91 vaccine strain, we investigated the genetic characterization of the S1 gene and compared it with that of the vaccine and foreign isolates.

To detect and determine the serotype and genotype of

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IBV, reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) analysis have been used as a simple and fast typing method [2, 14, 17]. In regard to Japanese isolates, Mase *et al.* have developed a genotyping method using RT-PCR RFLP analysis [16]. However, isolates cannot divide into the new 4/91 genotype by this method using the *Hae* II and *Eco*R I restriction enzymes, and isolates of the 4/91 genotype have been classified into the Massachusetts (Mass) genotype. Furthermore, this method has limited capacity to discriminate between vaccine strains and virulent IBV. Therefore, on the basis of our data, we attempted to improve the original method to distinguished between the field isolates and vaccine strain of the 4/91 genotype.

MATERIALS AND METHODS

IBV field isolates of the 4/91 genotype and vaccine: Four IBV field isolates, JP/Wakayama/2003, JP/Wakayama-2/2004, JP/Iwate/2005 and JP/Saitama/2006, that were classified into the 4/91 genotype based on their S1 gene sequences, were investigated as 4/91 field isolates (Table 1). All field isolates were derived from sick birds, and JP/Wakayama-2/2004 was isolated from a flock immunized with 4/91 live vaccine strain. Each virus was isolated from the tracheas of chicks during outbreaks of disease using embryonated eggs in regional laboratories. These isolates were propagated once in 10-day-old embryonated specific pathogen free (SPF) eggs, and the virus stock was used for sequence analysis and RT-PCR RFLP.

For RFLP analysis, 4/91 genotype and Mass type vaccines (Ma5, H120, Kita-1, Nerima and KU strains) were used.

Viral RNA extraction, RT-PCR: Whole S1 gene sequences were determined based on the direct sequence. Viral RNA was extracted from infected allantoic fluids using ISOGEN-LS (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The RNA was air-dried for 10 min, redissolved in 0.05 ml RNase-free water and stored at -70°C until use. Reverse transcription was carried out with a kit (Takara AMV RT-PCR kit, Tokyo, Japan) using random 9 mers. PCR was performed to amplify the whole S1 gene using the primer pair comprised of S1oligo 5' and S1oligo 3' described in the report of Kwon *et al.* [14]. For RT-PCR of RFLP, the primer pair comprised of IBV S1

(forward primer, 5'-AGG-AAT-GGT-AAG-TTR-CTR-GTW-AGA-G-3') and IBV S2M (reverse primer, 5'-GCG-CRG-TAC-CRT-TRA-YAA-ART-ARG-C-3'), modified according to the previous reports of Mase *et al.* [16], was used.

Sequence analysis of the S1 protein gene: PCR products were cut from 1.2% agarose gels and purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified RT-PCR products were sequenced in the forward and reverse directions using PCR primers and others to various regions within the S1 gene of each strain. Purified PCR products were used as templates for sequencing on an Applied Biosystems 310 automated DNA sequencer using dye terminator cycle sequencing chemistry (Perkin-Elmer/Applied Biosystems, Foster City, CA, U.S.A.). Analysis of the S1 sequences obtained from GenBank and our laboratory was performed using GENETYX ver. 9.0 (Software Development, Tokyo, Japan). Phylogenetic analysis was conducted by the neighbor-joining (NJ) method [19] in Clustal W.

The accession numbers of the S1 gene sequences obtained from GenBank are AB363949 (JP/Wakayama/2003), AB363959 (JP/Iwate/2005), AB465727 (JP/Saitama/2006), AB363951 (JP/Wakayama-2/2004), AF093793 (4/91 attenuated, vaccine), AF093794 (4/91 pathogenic), AJ618987 (FR-94047-94), DQ386098 (Spain/00/336), AJ618984 (UK/1233/95), AJ618986 (FR-88061-88), AJ618985 (FR-85131-85), Z83977 (UK/3/91), Z83978 (UK/5/91), Z83976 (UK/2/91), DQ064802 (Spain/95/194), AF093795 (Israel variant 1), AY837465 (TA03), DQ386093 (Spain/95/193), DQ064805 (Spain/97/307), EU350550 (IS/1366) and Z83979 (UK/7/93).

RFLP analysis for typing: The RFLP method was performed according to a previous report [16]. The PCR products were excised from agarose gel and purified by the same method as for sequence analysis. The restriction endonucleases *Pst*I, *Bal* I and *Bgl* II (Takara, Tokyo, Japan) were selected on the basis of the S1 sequence analysis. RFLPs were determined after agarose gel electrophoresis.

RESULTS

Sequence similarity of the S1 gene for the 4/91 type vaccine and field isolates: The nucleotide and amino acid identities between the 4/91 vaccine strain and field isolates are

Table 1. IBV strains of the 4/91 genotype isolated in Japan

Strain	Age of flock	IBV vaccine strain	Form of disease
JP/Wakayama/2003 ^{a)}	36-day broiler	Kita-1	Depression, diarrhea
JP/Iwate/2005	47-day broiler	— ^{b)}	Increased mortality
JP/Saitama/2006	2-wk layer	Ma5, C78	Respiratory signs, increased mortality
JP/Wakayama-2/2004	37-day broiler	4/91	Depression, diarrhea

a) Japan/location/year of isolation.

b) Not vaccinated.

Table 2. Sequence similarity of the whole S1 gene for the 4/91 type vaccine and Japanese field isolates

Amino acid similarity (%)		4/91 vaccine	JP/Wakayama-2/2004	JP/Wakayama/2003	JP/Iwate/2005	JP/Saitama/2006
Nucleotide similarity(%)						
4/91 vaccine		100	99.4	89.6	89.8	89.6
JP/Wakayama-2/2004		99.8	100	90.0	90.2	90.0
JP/Wakayama/2003		92.3	93.1	100	98.3	98.0
JP/Iwate/2005		93.1	93.2	99.3	100	98.3
JP/Saitama/2006		92.8	93.0	98.9	98.9	100

JP/Wakayama/2003	1	MLGKPLLLVTLWYALCSALLYDNGTYVYYYQSAFRPGPGWHLYGGAYAVDRVFNATNSAGSASDCTAGTFYESYNISAVSVAMTVPLNGM	90
JP/Iwate/2005	1N...V.A.....	90
JP/Saitama/2006	1N...V.....	90
JP/Wakayama-2/2004	1KN.....Q.....H.....K...E...N.V.V.....A.....PA..	90
4/91vaccine	1KN.....Q.....H.....K...G...N.V.V.....A.....PA..	90
4/91pathogenic	1KN.....Q.....H.....K...G...N.V.V.....A.....PA..	90
JP/Wakayama/2003	91	SWSAQFCTAHCFNSDFTVFVTHCFKNQPGSCPLTGMIPQNHIRISAMRDRTLFYNSTVSVSKYPRFKSLQCVSNFTAVYLNGLDVLFTSN	180
JP/Iwate/2005	91V.....I...Q.....	180
JP/Saitama/2006	91V.....Q.....Y.....	180
JP/Wakayama-2/2004	91VA.....S.Q.....SGF...L.....K.....G.S.S.....	180
4/91vaccine	91VA.....S.Q.....SGF...L.....K.....G.S.S.....	180
4/91pathogenic	91V.....S.Q.....SGF...L.....K.....G.S.S.....	180
JP/Wakayama/2003	181	ETSYVTGAGVYFKSGGPVYTKVMKEVRALAYFINGTAQEVILCDNSPRGLLACQYNTGNFSDGFYFPFTNSSLVKDRFVVYRESSINTTLE	270
JP/Iwate/2005	181K...F.....	270
JP/Saitama/2006	181K...F.....	270
JP/Wakayama-2/2004	181	..TH.....K.....I.....T.....	270
4/91vaccine	181	..TH.....K.....I.....T.....	270
4/91pathogenic	181	..TH.....K.....I.....T.....	270
JP/Wakayama/2003	271	LTNFTFTNVSNAAPNNSGDIGTFQLYQQTQAQDGYYNFNLISFLSSFFVYIPSNMYGSGYHPNCNFRPEGINNGLWFNSLSVSLTYGPIQGGC	360
JP/Iwate/2005	271	360
JP/Saitama/2006	271I.....	360
JP/Wakayama-2/2004	271S.....GVD.....H.....K...DF.....N.....	360
4/91vaccine	271S.....GVD.....H.....K...DF.....N.....	360
4/91pathogenic	271S.....GVD.....H.....K...DF.....N.....	360
JP/Wakayama/2003	361	KQSVFHNKATCCYAYSQGPISCKGVYRGELTQNFECGLLVYVTKSDGSRIQTRSEPLVLTQHNNYNNITLNKCVDYNIYGRVGGQFITNV	450
JP/Iwate/2005	361K.....	450
JP/Saitama/2006	361QK.....	450
JP/Wakayama-2/2004	361S.....R...TR.....Y.....Y.....E.....	450
4/91vaccine	361S.....R...TR.....Y.....Y.....E.....	450
4/91pathogenic	361S.....R...TR.....Y.....Y.....E.....	450
JP/Wakayama/2003	451	TEATANYSYLADGGLAILDTSGAIDIFVVKGEYGLNYYKVNPCEDVNQQFVVSQGLVGLTISRNETDSQPLENFYIKKLINGSRRFR	539
JP/Iwate/2005	451Q.....	539
JP/Saitama/2006	451Q.....	539
JP/Wakayama-2/2004	451R.A.....N.....H.....EFI.....T...S...	539
4/91vaccine	451R.A.....N.....H.....EFI.....P...T...S...	539
4/91pathogenic	451R.A.....N.....H.....EFI.....T...S...	539

Fig. 1. Sequence alignment of the amino acid residues of the whole S1 protein among the 4/91 type Japanese IBV isolates and vaccine strains. Identical amino acids are shown by dots(.). Black arrows indicate different positions between the vaccine strain and JP/Wakayama-2/2004.

shown in Table 2. JP/Wakayama-2/2004 is similar to the 4/91 vaccine strain, and its nucleotide and amino acid similarities to the 4/91 vaccine strain were 99.8% and 99.4%, respectively. Of the field isolates, JP/Wakayama/2003, JP/Iwate/2005 and JP/Saitama/2006, the similarities to the vaccine strain were somewhat low, and the identities of the nucleotide and amino acid sequences of the S1 gene were 92.3 to 93.1% and 89.6 to 89.8%, respectively. The S1 sequences of these three isolates were similar, and the nucleotide and amino acid similarities were 98.9 to 99.3% and 98.0 to 98.3%, respectively.

Sequences alignment of the amino acid residues of the S1 protein: The amino acid sequence of the whole S1 gene was compared between the Japanese field isolates, 4/91 vaccine

strain and field isolates of UK/4/91(4/91 pathogenic) as the origin of vaccine strain. As shown in Fig. 1, almost all of the amino acid residues of JP/Wakayama-2/2004 were identical to the vaccine strain, and only three amino acid (positions at 55, 508 and 530 indicated by black arrows in Fig. 1) were different. Various parts of the S1 gene contained amino acids that were identical among JP/Wakayama/2003, JP/Iwate/2005 and JP/Saitama/2006, but different in the 4/91 vaccine strain and JP/Wakayama-2/2004.

Phylogenetic analysis of the nucleotide sequences of the S1 genes of Japanese and foreign 4/91 type isolates: The S1 gene sequence of the field isolates were compared with available sequences from GenBank for foreign IBV strains of the 4/91 genotype. The phylogenetic tree based on the

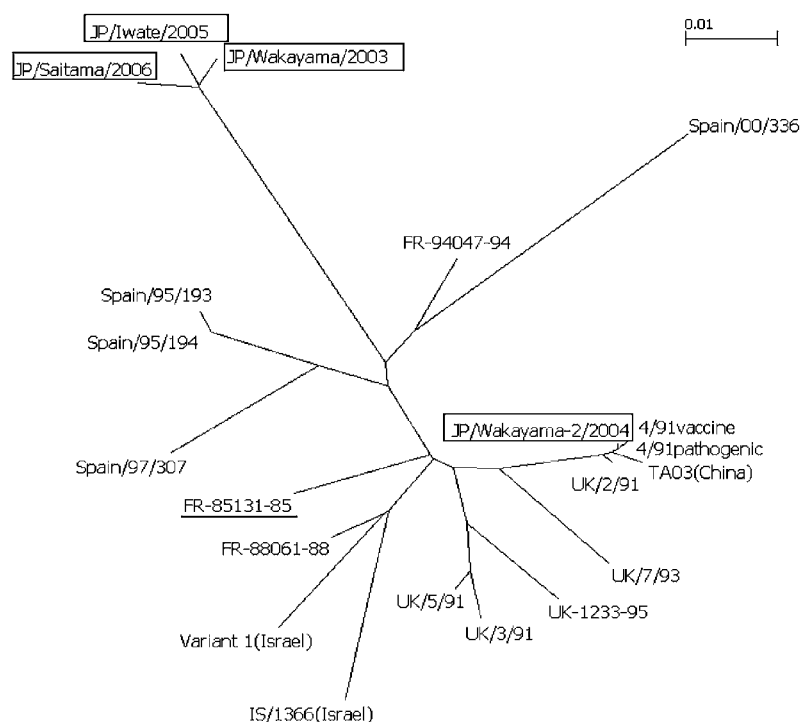


Fig. 2. Phylogenetic analysis of the nucleotide sequences of the whole S1 gene. Relationships between Japanese isolates and foreign isolates of the 4/91 type illustrated by a maximum likelihood phylogeny unrooted tree. The Japanese isolates have been boxed. The oldest strain, FR-85131-85, is underlined.

nucleotide sequence of the whole S1 gene is shown in Fig. 2. A diagram based on the amino acid sequence was similar (data not shown). From the oldest strain FR-85131-85, the 4/91 genotype isolates differentiated in two directions, one consisting of French and Spanish strains and another consisting of British strains. Almost all foreign isolates of the 4/91 genotype tended to segregate along geographical lines, and three Japanese field isolates, JP/Wakayama/2003, JP/Iwate/2005 and JP/Saitama/2006, formed a cluster that was apparently separated from the 4/91 vaccine cluster in which JP/Wakayama-2/2004 were classified.

Genotyping and identification of field isolates by PCR-RFLP: The 4/91 isolates were classified into the Mass group by PCR-RFLP using *Hae* II and *Eco*R I. To distinguish the 4/91 and Mass genotypes, *Pst* I and *Bal* I were used. Using these endonuclease, 5 vaccine strains (KU, H120, Ma5, Kita-1 and Nerima) of the Mass genotype were digested; isolates of the 4/91 genotype were not digested (Fig. 3 A and B). For all IBVs of the 4/91 genotype in the present study, it was confirmed that the *Pst* I and *Bal* I sites did not exist in the objective sequence. According to the sequence differences among the 4/91 vaccine and field isolates, the *Bgl* II restriction enzyme was expected to distinguish the 4/91 field isolates from the 4/91 vaccine and vaccine-like isolate (JP/Wakayama-2/2004). All field isolates of 4/91 type strains and the vaccine strain were tested (Fig. 3C). The PCR products of the three field strains, JP/Wakayama/2003, JP/Iwate/

2005 and JP/Saitama/2006, were not digested, but the PCR products of the vaccine strain and vaccine-like strain were digested by *Bgl* II into 440 bp and 237 bp fragments. The *Bgl* II site of the PCR products was confirmed in the sequences of the 4/91 pathogenic, UK/2/91, UK/7/91 and TA03 strains, but not in the other 4/91 genotype IBVs in our study, by analysis of the sequence data.

DISCUSSION

Since it was first described in the early nineties in United Kingdom, 4/91 type IBVs was identified in many other countries and became one of the most predominant genotype in Europe [4, 6]. Almost all IBVs recently isolated in Japan have been classified into three major genotypes, JP-I, JP-II and JP-III, but isolates of the 4/91 genotype have been sporadically detected since 2003. The 4/91 genotype vaccine strain has already been used in Japan, and it has been assumed that this new genotype emerged due to the vaccine strain. We previously confirmed that some of these isolates are more pathogenic in chickens compared with a vaccine strain [20]. However, the origin of the Japanese isolates of the 4/91 genotype, especially in relation to the vaccine strain, has been uncertain. Recombination with field isolates and reversion to virulence has been reported for the IBV vaccine [7, 10]. Therefore, it is important to verify the relationship between field isolates and the vaccine strain of

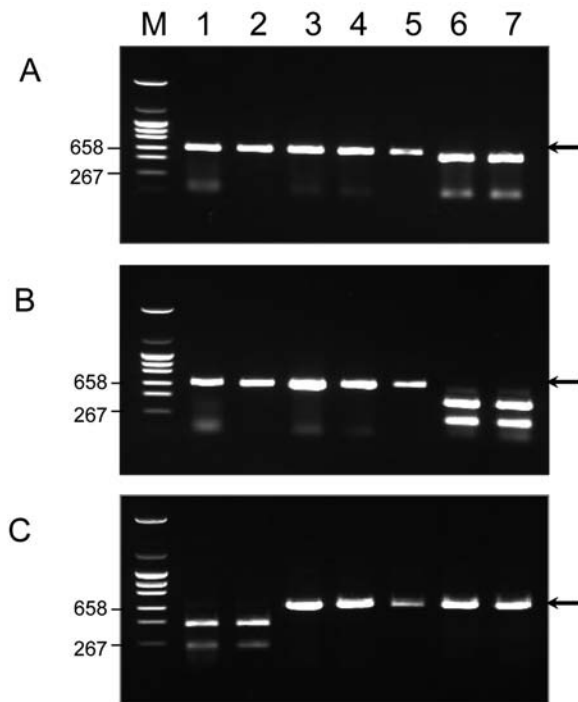


Fig. 3. RFLP patterns of the PCR-amplified S1 genes from IBV strains of the 4/91 and Mass genotypes digested with *Pst* I (A), *Bal* I (B) and *Bgl* II (C). M=pHY Marker; Lane 1=4/91 vaccine strain; Lane 2=JP/Wakayama-2/2004; Lane 3=JP/Wakayama/2003; Lane 4=JP/Iwate/2005; Lane 5=JP/Saitama/2006; Lane 6=Ma5 vaccine strain; Lane 7=H120 vaccine strain. Arrows indicate non-digested PCR products (about 670 bp).

the 4/91 genotype.

The results of our sequence analysis of JP/Wakayama-2/2004 showed that the S1 sequence similarity to the 4/91 vaccine strain was high. During collection of this isolate, bacterial agents, such as *Escherichia coli*, were also recovered from lesions, and mixed infection was suspected. The present results confirm the results of our previous study, which showed that the pathogenicity of JP/Wakayama-2/2004 in chicks was as low as that of vaccine strain [20]. These facts suggest that JP/Wakayama-2/2004 is derived from a vaccine.

In the S1 sequence of this vaccine-derived isolate, three amino acids (positions 55, 508 and 530, where position 1 is the start of the open reading frame of the S1 gene) were different compared with the vaccine strain. Callison *et al.* [3] reported that three amino acids, amino acid positions 95, 508 and 530, were observed to be different between a UK/4/91 field isolate (called 4/91 pathogenic in this paper) and its embryo-passaged, attenuated derivative (called 4/91 vaccine in this paper). Of these three differences, the two amino acids at positions 508 and 530 of JP/Wakayama-2/2004 were the same as the 4/91 pathogenic strain. However, these changes would be not associated with pathogenicity because JP/Wakayama-2/2004 had low pathogenicity as

seen in the vaccine strain [20].

Cavanagh *et al.* [4] examined passage of the 4/91 vaccine strain in chickens, compared the S1 sequences of vaccine strains and examined the pathogenicity of egg-passaged and chick-passaged strains. In their report, they recognized one amino acid substitution at position 95, alanine in the vaccine strain and serine (S) in the chick-passaged strain, in the strain after 10 passages in the chicken, but the pathogenicity of the chick-passaged strain did not change. They suggested that replication in chickens and embryonated eggs is associated with this amino acid difference. For the Japanese field isolates, except for JP/Wakayama-2/2004, the amino acid residue at position 95 was serine (S), which is the same as the chick-passaged isolate described above. These results might suggest that the field isolates have adapted to propagate in chickens. This position was alanine (A) in JP/Wakayama-2/2004, which is the same as the vaccine strain described above. Therefore, this also supports the conclusion that this strain is derived from vaccine.

Unlike JP/Wakayama-2/2004, our results for the S1 sequences of the other 4/91 genotype field isolates (JP/Wakayama/2003, JP/Iwate/2005 and JP/Saitama/2006) raise the possibility that these isolates are derived from a 4/91 genotype strain similar to the French or Spanish isolates, but not from the vaccine. Because the identities of the S1 gene among these isolates were high, these isolates appear to be derived from the same origin. In regard to the origin, imported live chick or livestock products and wild birds from overseas might be of concern; however the precise origin is still unknown. One possible scenario is that a 4/91 genotype strain might have entered Japan in the 1990s and spread without being noticed in conjunction with small mutations in various areas.

In the field, vaccine-like strains have been isolated by inoculation of embryonated eggs, and this makes it difficult to find the actual cause. To uncover the true cause, it is important to confirm whether the isolate is a vaccine strain or not, but there is a little information available concerning methods to distinguish vaccine strains.

Our RT-PCR RFLP method using *Bgl* II and *Bal* I was able to distinguish the vaccine and vaccine-derived strains such as JP/Wakayama-2/2004, from field isolates.

This method would be useful for confirmation of isolates of the 4/91 genotype in the field.

Our results indicate that the pathogenic IBV strains of the 4/91 genotype isolated in Japan were not derived from the vaccine strain but were instead derived from another strain that is similar to French and Spanish isolates. Some subgroups of this genotypes have been recognized in other countries. Therefore, it is necessary to pay attention to the prevalence of the 4/91 genotype in Japan.

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