Phylogenetic analysis of avian infectious bronchitis virus strains isolated in Japan

Brief Report

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Summary. To define the origin and evolution of recent avian infectious bronchitis virus (IBV) in Japan, a genetic analysis was performed. By phylogenetic analysis based on the S1 gene including the sequence of the hypervariable regions, IBV isolates in Japan were classified into five genetic groups, which included two already-known groups (Mass and Gray). Among them, three major genetic groups were associated with the recent outbreaks of IB in Japan. One group is indigenous to Japan and could not be placed within the known existing groups in other countries. The remaining two groups, which have emerged recently, are related to isolates in China and Taiwan.

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Infectious bronchitis (IB), which is caused by the IB virus (IBV), is one of the most highly infectious and contagious pathogens of chickens worldwide [4]. The primary tissue of IBV infection is the respiratory tract, though some isolates also replicate in the kidney and oviduct, resulting in nephritis and reduced egg production, respectively. Generally, IB has been controlled with serotype-specific vaccines, but outbreaks of IB still occur because vaccines offer little or no cross-protection between serologically distinct viruses [10]. A high mutation frequency and RNA recombination leads to the emergence of new viruses capable of causing disease in vaccinated chickens [24]. It is extremely important to identify field isolates for the selection of vaccines.

Members of the *Infectious bronchitis virus* (IBV) species, which is included in the family *Coronaviridae*, are enveloped and positive-stranded RNA viruses containing a genome of approximately 27-kb [1]. IBV has three major virus-encoded structural proteins, which include the spike (S) glycoprotein, the membrane (M) protein, and the nucleocapsid (N) protein. The spike of IBV is formed by

post-translational cleavage of two separate polypeptide components, designated S1 and S2 [2]. Of these, the S1 glycoprotein is associated with virus attachment and is a major target of the neutralizing antibodies in chickens, so serotypic evolution in IBV is associated primarily with the sequences of the S1 glycoprotein [3, 12]. Hence, the molecular characterization of IBV is based mainly on analysis of the S1 gene [16, 25].

Classically, IBV strains have been placed into serologically related groups on the basis of virus neutralization tests (VNT). However, VNTs are very laborious and time-consuming. In addition, there is considerable evidence that serologic relationships among IBV isolates, determined by in vitro VNTs, have not been reflected by the results of in vivo cross-immunity studies [8]. Moreover, the antigenic variants emerging from wild-type or vaccine viruses by point mutation or RNA recombination cannot be detected by VNTs [11]. Recently, genetic grouping of IBV on the basis of nucleotide sequences have been applied for virus classification [15, 16]. Since anyone can apply the genetic information of viruses deposited into the GenBank, genetic comparison with other virus strains for epidemiological analysis can be performed quickly.

Since the first isolation of IBV was recorded in Japan in 1954 [20], the outbreaks have been ongoing. However, the epidemiological analysis of IBV isolates in Japan has not been thorough except for a few strains [17, 18]. The relationships between Japanese IBV isolates and foreign IBV isolates have also remained unknown. We were particularly interested to know whether the current IBV isolates in Japan were newly introduced from other countries or whether they arose by mutations of circulating Japanese IBV strains.

In this study, to define the origin and evolution of recent IBV in Japan, we determined the nucleotide sequences of IBV isolated in Japan using the reverse transcriptase-polymerase chain reaction (RT-PCR) method coupled with direct sequencing, and analyzed the sequences phylogenetically with viruses isolated in other countries. This information is important for determining strategies to control IB.

The IBV isolates employed in this study are listed in Table 1. Most of the IBV isolates were obtained from regional laboratories in Japan. Most of the specimens of IBV were isolated by two or three passages using embryonated specific pathogen-free (SPF) eggs.

The materials were submitted to our laboratory, and were propagated once in SPF eggs. The presence of IBV in the inoculated embryos was initially determined by immunofluorescence assay of allantoic cells using anti-IBV chicken serum and observation of characteristic embryo changes such as dwarfing, stunting or curling, according to the procedures of a previous report [7]. The following commercial attenuated live vaccine strains in Japan were also used in this study: C-78, Miyazaki, ON/74, Kita-1, KU and *TM86*. They were derived from field cases of IB in Japan.

Viral RNA was extracted from infected allantoic fluids using a kit (ISOGEN-LS, Nippon Gene, Tokyo, Japan). A reverse transcriptase (RT) reaction was carried out with Superscript II (Life Technologies, Gaithersburg, MD, U.S.A.) using random 9 mers. The N-terminus of the S1 glycoprotein, which includes

Table 1. IBV isolates from field cases in chickens in Japan examined in this study

Viruses[a]	Isolation year	Major clinical sign	Genetic groups
JP/Ishida/51	1951	respiratory	Mass
JP/Nerima/53	1953	respiratory	Mass
JP/KH/64	1964	respiratory	JP-I
JP/Shizuoka/71	1971	respiratory	JP-I
JP/Chiba/74	1974	respiratory	JP-I
JP/Chiba/77	1977	egg drop	JP-I
JP/Chiba/80	1980	respiratory	JP-I
JP/Miyazaki/89	1989	nephritis	JP-II
JP/Mie/92	1992	nephritis	JP-I
JP/Akita/92	1992	respiratory	JP-I
JP/Yamanashi/93	1993	nephritis	JP-II
JP/Nagano/95	1995	nephritis	JP-I
JP/Yamanashi/95	1995	respiratory	JP-I
JP/Shizuoka/98	1998	nephritis	JP-I
JP/Shimane/98	1998	respiratory	JP-III
JP/Chiba/98	1998	nephritis	JP-I
JP/Toyama/2000	2000	nephritis	JP-I
JP/Aichi/2000	2000	nephritis	JP-III
JP/Fukui/2000	2000	respiratory	JP-III
JP/Okayama-1/2000	2000	nephritis	JP-I
JP/Okayama-2/2000	2000	nephritis	JP-I
JP/Osaka/2000	2000	nephritis	JP-II
JP/Kanagawa/2001	2001	nephritis	JP-II
JP/Shimane/2002	2002	nephritis	JP-III
JP/Ibaraki/2003	2003	nephritis	JP-I

[a]Named as suggested by Cavanagh [5]: country/region of origin/year of isolation except JP/KH/64 strain. JP, Japan

important structures such as hypervariable regions (HVR) associated with antigenic properties [3, 12, 13], was selected for this analysis. The following primers were used: IBV-S1 (forward), 5′ AGG-AAT-GGT-AAG-TTR-CTR-GTW-AGA-G 3′, and IBV-S2 (reverse), 5′ GCG-CAG-TAC-CRT-TRA-YAA-AAT-AAG-C 3′. (The expected product was about 670 base pairs). These were designed based on a comparison and alignment of the GenBank sequences of several known IBV strains.

PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified PCR products were used as a template for sequencing on an Applied Biosystems 373S automated DNA sequencer using dye terminator cycle sequencing chemistry (Perkin-Elmer/Applied Biosystems, Foster City, CA, U.S.A.). Purified PCR products were sequenced from both directions. The derived nucleotide sequences were analyzed using GENETYX-Mac ver. 10.0 (Software Development Corp., Tokyo, Japan), and through GenBank searches. The phylogenetic analysis with available sequences from GenBank was conducted using the Clustal

Table 2. Summary of PCR and restriction endonuclease analysis of IBV isolates in Japan

Genetic groups	Length of PCR products[a]	The sizes (base pairs) of fragments generated by	
		Hae II	*Eco*R I
JP-I	692[b]	461, 231[c]	Not digested[d]
JP-II	674	287, 175, 162, 50[e]	Not digested
JP-III	674–680	Not digested	502, 172–178
Mass	665–671	Not digested	Not digested[f]
Gray	689	566, 123	Not digested

[a]Base pairs including the length of primers

[b]Except JP/Shizuoka/98 (689 base pairs)

[c]Except JP/Shizuoka/98, which was digested into 305, 228, and 156 bp for addition of one cutting site

[d]Except JP/Yamanashi/95, which was digested into 381 and 311 bp for addition of one cutting site

[e]Except JP/Osaka/2000, which was digested into 287, 212, and 175 bp for loss of one cutting site

[f]Except JP/Nerima/53, which was digested into 409 and 262 bp for addition of one cutting site

X program [23], and the tree was constructed by the neighbor-joining (NJ) method [21].

The expected sizes of DNA fragments were successfully amplified by RT-PCR from all the employed IBV samples with our designed primers. The determination of nucleotide sequences of obtained PCR products revealed the diversity of those lengths (665–692 bp) (Table 2). By phylogenetic analysis, the IBV isolates in Japan used in this study were divided into five genetic groups (Fig. 1). In particular, recent isolates in Japan were assigned to three major genetic groups. Among them, one group (JP-I) is indigenous to Japan and is not present in other countries. The two other groups that have emerged recently (JP-II and JP-III) are related to isolates in China and Taiwan. Some isolates that were mainly isolated in the 1950s, such as JP/Ishida/51 or JP/Nerima/53, and two local attenuated vaccine strains, Kita-1 and KU, were classified into the Mass group. Another local attenuated vaccine strain, C-78, was classified into the JP-I group, and Miyazaki and *TM86* strains were classified into the JP-II group. Only one strain (ON/74), which was also a local attenuated vaccine strain, was classified into the Gray group.

The deduced amino acids from obtained nucleotide sequences of PCR products were aligned with various IBV strains isolated in different geographic regions. The results are shown in Fig. 2. Many insertions or deletions of amino acids among IBV strains including the Japanese isolates were found in the amplified region.

The generated PCR products were employed in restriction endonuclease analysis for the development of a simple and rapid classification of genetic groups. After comparing the obtained sequences in this study, we selected two endonucleases, *Hae* II and *Eco*R I (Takara, Tokyo, Japan), for this analysis. The restriction profiles
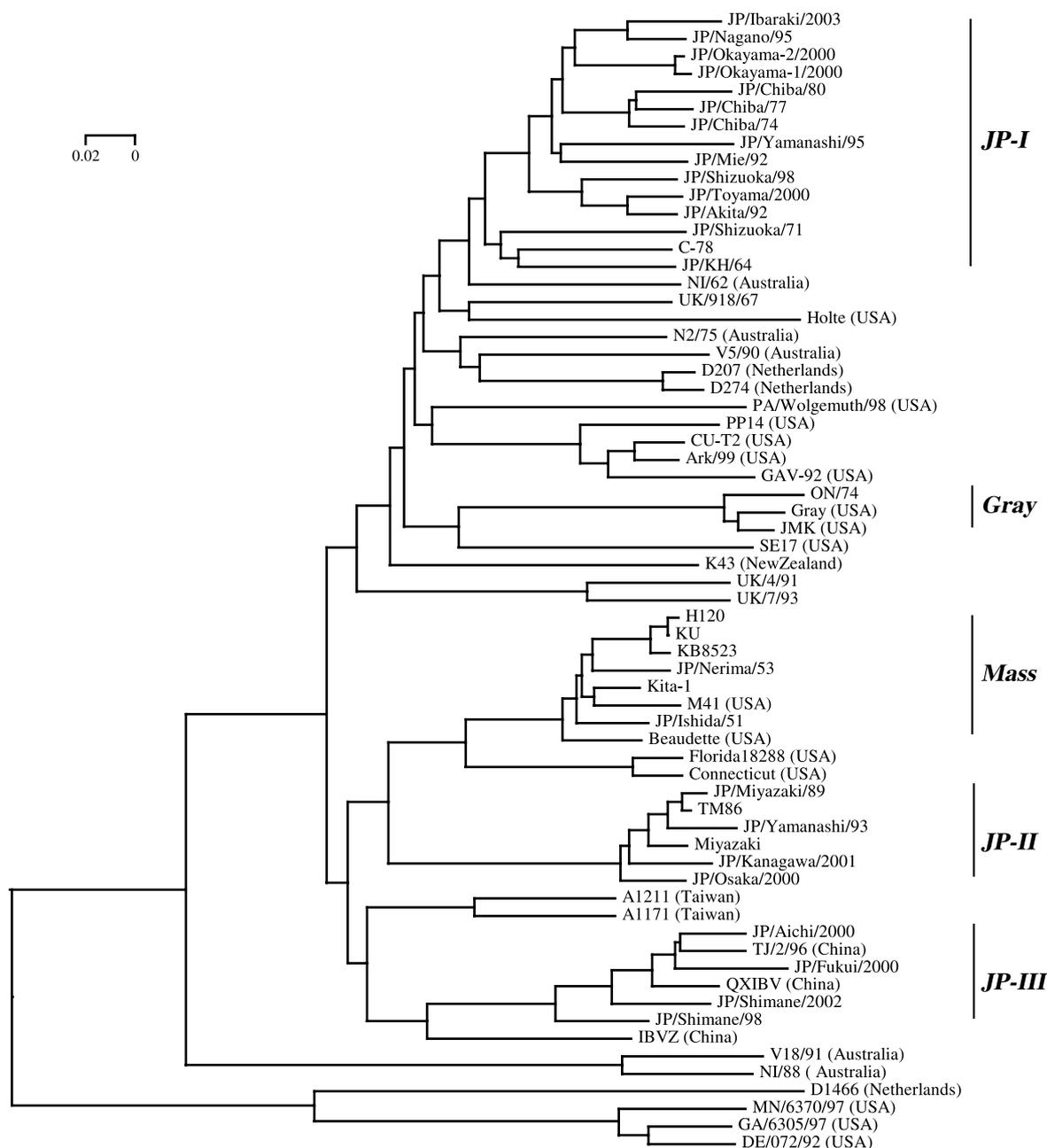
Fig. 1. Dendrogram of the S1 glycoprotein of infectious bronchitis viruses. Amino acids 1–207 (in GenBank accession number P11223) of the Beaudette strain S1 glycoprotein were used for phylogenetic analysis at the amino acid level. The dendrogram is rooted to murine hepatitis virus ML-10 strain. Horizontal distances are proportional to the minimum number of amino acid differences required to join nodes and sequences

of the PCR products of the five genetic groups in this study, as revealed by two restriction endonucleases are shown in Table 2 and Fig. 3. Each Japanese genetic group has a specific profile pattern. The other genetic groups represented, such as

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JP/KH/64            1 MLAKSLFIVTLLFALCSATLYDNGSYVYYYQSAFRPPFGWHLHGGAYAVV    50
JP/Toyama/2000        ..V...S...........I....DT...........GV............
JP/Yamanashi/93       ..VR...LA..S......I.................D....Q.......
JP/Fukui/2000         ..G....L..I.C.....N.V.FDNNYV..YQSAFR.PNGWHLQ.GAYA.
Beaudette             ..VTP.LL....C.....V...SS.............S....Q.......
Connecticut           ..VTP.LL.........A....S.............N............
D207                  ..ER..LLA...S.....N.FG.N............D.........E..
UK/4/91               ..G.P.LL...WY.....L...KNT...........GQ...........D
Ark99                 ..V....L..I......N....E.F.........GH............

JP/KH/64           51 NVTTENNNA-GTSAECTAGAIYWSKNFSASSVAMTAPLGGMTWSTRQFCT    99
JP/Toyama/2000        ..SQ.T...-.SASK......H.......A.....T.QS..A...SE...
JP/Yamanashi/93       ..SSVT...-..APV.IG.SLQG.RVVN...I..I..GD......S....
JP/Fukui/2000         VNS.NYT.NA.LAN...V.V.KDVY.Q.VAAI.....FQ..A..KS...S
Beaudette             .ISS.F...-..S.SG..V.I.HGGRVVN...I.....SS..A..SS....
Connecticut           .TFI.S-----NLR..IV.I.GGDRVVN...I.....QP..G..S.....
D207                  ..S..SS..-..TGCTAGAIYWSKNFSA..VAMTAPQN.MSWSTEQFCTA
UK/4/91               K.FNGT...-VSVSD....TF.E.Y.I..A.....V.PA..S..VS....
Ark99                 ..SS.....-..APS......GY......A.....S..S..ASS...

JP/KH/64          100 AHCNFTSFTVFVTHCFKSGHGYCPLTGLIQSGYIRISAMKNGGSSAADLF   149
JP/Toyama/2000        .....S.............V.S...................Q...GPS...
JP/Yamanashi/93       .....SQV.......YN.SQ.A------CPT.FVPRNHIRISAMKNGS..
JP/Fukui/2000         .....SEI.......YS..S.S..I..Q.-----PQNHIRISAMKNGS..
Beaudette             .....SDT.......Y-------KHG.CPLT.MLQQNLIRVSAMKNGQ..
Connecticut           .....SDI.......Y-------KHN.CPIT.S.PQHSIRVSAMKKGR..
D207                  HCN-..D.V......Y.K.P.S.......-----PQYHIRISAMKNSS..
UK/4/91               .....SD...........QQ.S.....M.PQNH......RS-----GF..
Ark99                 .......YI..........SNS.......P......A...H.SRTPGH..

JP/KH/64          150 YNLTVSVAKYPRFMSLQCVNNQTSVYLNGDLVFTSNETKDVSAAGVYFKA   199
JP/Toyama/2000        .....P.T..SK.R.........................T..VG...S...
JP/Yamanashi/93       ....I..S.H.K.K.F.....Y..........Y.....T..TS...H...
JP/Fukui/2000         .......SN..N.K.FE....F.............K.T..TS.......
Beaudette             ..........T.R.F.....L..........Y.....I..TS......
Connecticut           .......N...T.K.F.....F..........Y.....T..TS.....N.
D207                  .....A.T.....K......M......................H...
UK/4/91               .......S...K.K.....G.S...............TH.TG......S
Ark99                 .......T...K.R......H.............GY.E..V....H..S

JP/KH/64          200 GGPITYKVMREVKAL
JP/Toyama/2000        ..............
JP/Yamanashi/93       .......I.......
JP/Fukui/2000         ...VN.NI.KDFNV.
Beaudette             ..............
Connecticut           ..............
D207                  ..............
UK/4/91               ...V.....K.....
Ark99                 ..............
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Fig. 2. Multiple alignment of deduced amino acids of representative isolates of three major genetic groups in Japan with various IBV strains isolated in different geographic regions. Identical amino acids are shown by dots (.) and the positions of deletions by dashes

Connecticut, D207, UK/4/91, and Ark99, do not have sites for these two enzymes in their reported sequences. Hence, the three major genetic groups in Japan were easily differentiated from other genetic groups.
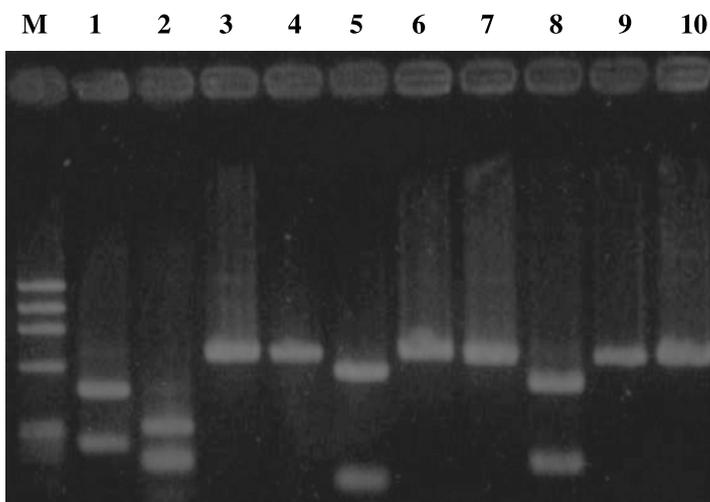
Fig. 3. Restriction endonuclease analysis of the PCR-amplified S1 genes from the five representative genetic groups of IBV in Japan, digested with *Hae* II (*1–5*) and *Eco*RI (*6–10*). M, molecular weight marker ΦX174-*Hae* III digest; *1* and *6*, JP/Toyama/2000 (JP-I); *2* and *7*, JP/Yamanashi/93(JP-II); *3* and *8*, JP/Fukui/2000(JP-III); *4* and *9*, JP/Ishida/51(Mass); *5* and *10*, ON/74(Gray)

Recently, novel IBV strains have been proliferating abroad, and genetic analysis of these strains have been mainly based on the S1 gene [25]. IBV strains in Japan have been considered variants different from foreign IBVs such as Massachusetts or Connecticut types by VNT or PCR-restriction enzyme fragment length polymorphism based on the S2 gene [9, 17, 18]. Still, it remains unknown whether the current IBV isolates in Japan were newly introduced from other countries or whether they arose by mutations of circulating Japanese IBV strains.

In this study, it was revealed that five genetic groups, which could be differentiated by simple restriction endonuclease analysis, were present in Japan. Although sequencing is recommended to obtain precise genetic information, restriction endonuclease analysis is simple, easy and convenient for primary characterization in routine diagnosis. Among these genetic groups, three major genetic groups were associated with recent outbreaks of IB in Japan. A previously published study divided IBV strains into several distinct genetic groupings by analyzing the S1 gene [25]. However, most IBV isolates in Japan formed genetic groups distinct from these. Among three major genetic groups of IBV in Japan, one group (JP-I) has not been found in other countries; this group may be indigenous and has been prevalent in Japan for a long time, from at least the 1960s. The other two groups that have emerged recently (JP-II and JP-III) are related to isolates in China and Taiwan. Recent IBV isolates in China and Taiwan also form distinct genetic groups [26, 27]. It is unknown whether these genetic groups originated in Japan or in these neighboring countries. Interestingly, recently isolated Newcastle disease viruses in Japan were also closely related, genetically, to those isolated in

Taiwan and China [19]. Furthermore, it was recently revealed that the coronavirus isolated from pheasants was genetically closely to IBV [6]. One possibility for the migration of IBV strains into this region is dissemination by some kind of wild birds close to chickens, such as pheasants.

The virus neutralization antibodies that form the basis for comparison of IBV isolates are induced largely by the N-terminus in the S1 glycoprotein [3, 13]. In this study, prevalent IBV genetic groups in Japan were shown to be completely distinct from other known serotypes from Europe and North America by comparing amino acid sequences of the N-terminus in the S1 glycoprotein. On the basis of the relationships between genotypes and serotypes of IBV [25], it was suggested that the serotypes of recent IBV in Japan are novel. Further analysis would be required to examine the antigenic property of isolates for establishment of a control strategy for IB outbreaks in Japan.

On the other hand, the variation of IBV may also be attributed to recombination following co-infection of a few distinct strains [11, 24]. Previously, the KB8523 strain isolated in Japan [22] was revealed to be genetic recombinant [14]. In Japan, in addition to major Massachusetts (H120 strain) and Connecticut (L2 strain) type live vaccines, local attenuated vaccines isolated from outbreaks of IB in Japan have been developed and used. They proved to derive from the genetic groups, Mass, Gray, JP-I or JP-II, although the genetic backgrounds of these attenuated vaccines have not been clarified. So far, it has been suggested that the recombination events occur between vaccine strains and field strains [27]. It is unknown whether or not vaccine strains in Japan are associated with the emergence of recombinant viruses. To detect recombinant viruses, it would be required to analyze two or more different genetic regions of isolates. Further analysis of viruses would clarify whether recombinant variants have become prevalent in Japan.

Nucleotide sequence accession numbers

All sequences used in this study were sent to DDBJ, and their accession numbers are AB120628 to AB120658.

Acknowledgements

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