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Genetic and Antigenic Analyses of Bovine Respiratory Syncytial Virus Detected in Japan

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ABSTRACT. Genetic and antigenic analyses of bovine respiratory syncytial virus were conducted on 12 field strains from Tohoku and Hokuriku districts in Japan during from 2002 to 2004. On the phylogenetic tree of the nucleotide sequences of the glycoprotein region, the examined strains fell in the same cluster as the strain isolated in Nebraska and were classified as the subgroup III. The examined strains were subdivided into 2 lineages (A, B). Isoleucine 200 of the epitope domain was replaced by threonine as a feature of the lineage B strains. The examined strains showed the nucleotide sequence homologies of 88.3–93.3% with the known Japanese strains classified as the subgroup II and of 86.1–96.6% with those in the subgroup III. No significant difference was found on the neutralization index between the examined strain and the 52–163–13 phylogenetically similar to the Japanese vaccine one. The results suggest that the subgroup III strains have existed in Japan and that epidemics of the strains could be protected due to the present vaccination.

KEY WORDS: antigenicity, bovine respiratory syncytial virus, field strain, glycoprotein, nucleotide sequence.

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Bovine respiratory syncytial virus (BRSV) causes respiratory diseases ranging from asymptomatic to severe in cattle. The virus infections generally affect calves, although the reinfections occasionally occur in adult cattle [1, 3, 5, 11, 18, 20–23]. The glycoprotein (G protein) is responsible for the virus binding to the cell surface receptor which plays an important role in the virulence and immunogenicity of the virus [4, 7, 9, 10, 12, 15, 16].

The antigenic diversity among the viruses has been revealed by investigations with monoclonal antibodies to the G protein [2, 4, 6, 7, 10, 13, 14, 16], and confirmed by phylogenetic analyses [19]. The virus strains are divided into 6 subgroups from I to VI based on the phylogenetic tree of the G protein, nucleoprotein (N protein) or fusion protein (F protein) regions, although the main differences among these subgroups are located in the G protein [19]. The subgroups are suggested to correlate with the geographical and temporal distributions of the strains [6, 19]. A continuous evolution of the sequences of the G, N and F protein regions has been occurring in the strains from European countries where vaccination was widely practiced, and the significant residues substitution in the G protein resulting in a notable antigenic variant was demonstrated in the recent strains from France [19].

There are no reports on the genetic analysis of the virus strains isolated in Japan except for the NMK7 strain [6, 8] detected in Kanto district in 1969. The present report describes the genetic and antigenic characteristics of 12 field strains of BRSV detected in Japan during from 2002 to 2004.

MATERIALS AND METHODS

Twelve field strains of BRSV (IW01–05, AK01, MY01–02, YM01–03 and FKI11) had been recovered from 9 calves and 3 cows developing severe respiratory symptoms, on 12 farms located in Tohoku and Hokuriku districts in Japan during from October 2002 to January 2004. Three farms with detection of 3 strains (IW01, 02 and 05) were located within the neighboring geographical areas in Iwate Prefecture, but other 9 farms were scattered throughout Tohoku and Hokuriku districts. There were no direct contacts among the cattle or men in all farms.

Viruses and field samples: IW03 strain was isolated with Vero cell culture passages from nasal swabs of a nonvaccinated calf and 11 others obtained directly due to the RNA extraction method from the same materials. All samples were stored at −80°C until analysis. These strains were never passaged in cell cultures before RNA extraction. For the reference in this study, the RNA of rs-52 strain was obtained from a Japanese vaccine bottle (Kyoto Biken, Japan), and the 52–163–13 strain phylogenetically similar to the rs-52 provided with cell culture passages. The nucleotide sequences of the NMK7 isolated previously in Japan and the strains from other countries were available from the GenBank detabase.

Extraction of RNA and Nested RT-PCR: The viral RNA was extracted from the samples using TRIzol (GibcoBRL, U.S.A.) according to the manufacturer's instructions. Briefly, RNA was extracted with a TRIzol regent, purified with chloroform, and precipitated with isopropanol 15,000 rpm at 4°C. After washed with 70% ethanol, the RNA pellet was dried and resuspended in 30 μl of Tris-EDTA buffer.

The G protein gene was amplified using outer primers (G2.5, F2.7) for RT-PCR and inner primers (VG1, VG4) for nested PCR as described previously [19]. The RT-PCR was conducted using the SuperScript One-Step RT-PCR Kit (Invitrogen, U.S.A.) and the PCR Thermal Cycler (TAKARA, Japan) for the reaction. Following cDNA synthesis at 50°C for 30 min and pre-denaturation at 94°C for 2

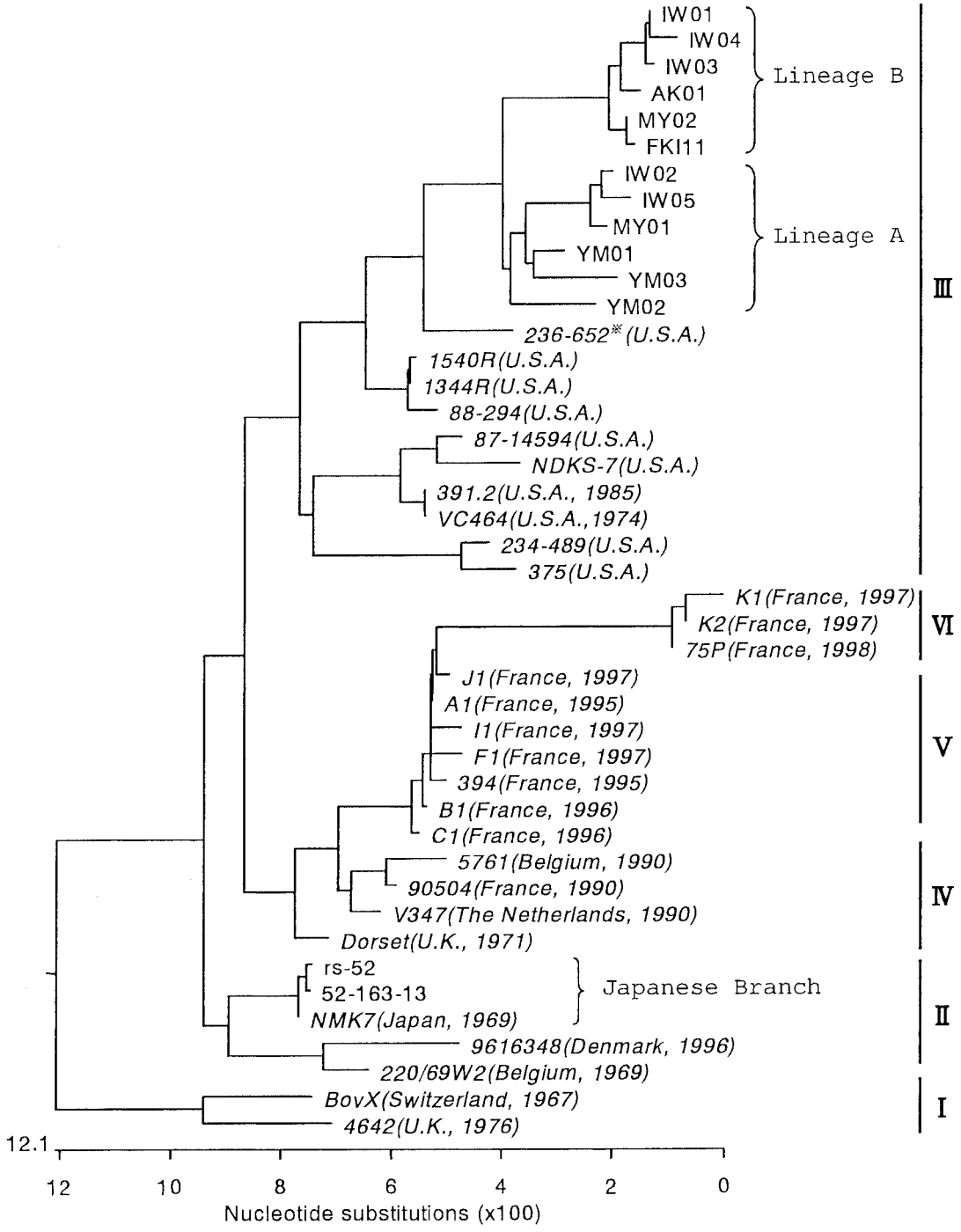

Fig.1. Phylogenetic tree of G protein nucleotide sequences of BRSV. The scale below measures the evolutionary distances between the sequences. Units indicate the number of substitution events. ※: The italics indicate the sequence data obtained from the GenBank database, with their detected countries and years in parenthesis. DDBJ accession numbers are 4642 (Y08718), BovX (U57823), 220/69W2 (AF188577), 9616348 (U92114), NMK7 (U24713), Dorset (U24715), V347 (AF188584), 90504 (AF188580), 5761 (AF188583), C1 (AF188590), B1 (AF188589), 394 (AF188596), F1 (188591), I1 (AF188594), A1 (AF188588), J1 (AF188592), 75P (AF188587), K2 (AF188586), K1 (AF188585), 375 (L10925), 234–489 (L08413), VC464 (AF188582), 391.2 (M58307), NDKS-7 (L08417), 87–14594 (L08412), 88–294 (L08411), 1344R (L08415), 1540R (L08416) and 236–652 (L08414).

Table 1. Homology percentages of pairwise distances within and between each group on G protein nucleotide and amino acid sequences of the strains examined and available from GenBank

	I	II		III			IV	V	VI
			(Japan)*		(LineageA)	(LineageB)			
I	95.9 *92.5*	89.1–89.8	90.8–92.9	85.8–90.2	85.7–87.4	85.8–88.3	87.8–89.8	88.9–90.3	89.0–90.1
II	*81.9–84.3*	95.1 *93.7*	94.7–96.4	80.2–94.3	80.2–92.4	83.1–91.1	90.2–96.1	87.3–93.4	84.9–90.3
(Japan)	*83.0–84.4*	*89.7–93.7*	99.8–100 *100*	92.8–95.3	91.5–93.3	88.3–92.2	95.1–97.1	93.1–94.2	89.8–90.6
III	*77.0–83.0*	*84.1–90.6*	*89.1–91.8*	92.4–100 *86.4–100*	98.6–96.6	86.1–95.0	91.4–95.8	90.8–93.9	87.4–90.3
(LineageA)	*74.1–78.2*	*80.7–85.8*	*83.0–87.8*	*80.3–93.7*	96.3–99.3 *91.8–99.3*	87.2–97.1	90.4–93.2	89.4–91.4	86.6–87.8
(LineageB)	*75.2–78.2*	*78.7–84.3*	*83.7–85.0*	*81.0–92.1*	*91.2–94.6*	94.4–99.8 *98.0–100*	88.8–91.9	86.7–91.4	84.7–89.5
IV	*79.5–83.5*	*83.5–92.1*	*89.3–91.3*	*85.0–92.1*	*80.3–86.6*	*78.0–85.0*	97.1–98.7 *94.5–98.4*	95.3–97.8	90.9–93.9
V	*81.7–85.8*	*81.7–87.5*	*86.7–88.3*	*84.4–89.2*	*79.2–83.3*	*80.0–82.5*	*91.3–96.7*	98.9–99.7 *97.5–100*	95.0–95.8
VI	*79.6–83.3*	*77.9–83.3*	*82.3–83.3*	*78.2–84.2*	*74.1–77.5*	*76.2–79.2*	*84.3–90.0*	*90.5–93.3*	99.7–100 *99.2–100*

*: Japan strains comprise the NMK7, rs-52 and 52–163–13. Values for nucleotide and amino acid sequences are given by normal and italic letters, and subgroup designations by block letter.

min, 40 amplification cycles were performed consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min.

The nested PCR was carried out in 20 μl of a reaction mixture containing 2 μl of 10 × reaction buffer (Applied Biosystems, U.S.A.) , 2 μl of 25 mM $MgCl_2$, 2 μl of dNTP (2 mM each dNTP), 1 μM of the inner primers, 0.5 U of Taq DNA polymerase (Applied Biosystems, U.S.A.) and 2 μl of the RT-PCR products under the same reaction conditions, with the exception that the annealing temperature was set at 58°C. RT- and nested PCR amplifications resulted in a 1,030 and 541 base pair products, with an ultimate elongation step at 72°C prolonged to 10 min. The products were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide, and detected under UV light.

Sequence analysis: The nested PCR-amplified fragments were purified by the Microcon-PCR (Millipore, U.S.A.) and sequenced using the BigDye Terminator Cycle Sequencing FS Kits (Applied Biosystems, U.S.A.). Sequences were resolved with the ABI 377 DNA sequencer (Applied Biosystems, U.S.A.). The nucleotide and amino acid sequences were analyzed using the GENETYX-MAC sequence analysis program (Software Development, Japan).

The nucleotide and amino acid sequences of G protein region were aligned by the multiple program DNASTAR (DNASTAR, U.S.A.) with the CLUSTALW method. The phylogenetic tree was constructed due to the MegAlign PROGRAM DNASTAR PACKAGE based on the distance matrix method.

Neutralization test: The neutralization test was applied for the comparison of antigenicity between the following 2 strains. One is the IW03 and another the 52–163–13. The tests were performed by the viral dilution method using the antiserum to the NMK7 and Vero cells grown in 96-well culture microplates, and the obtained neutralization indices were compared. The antiserum was kindly provided from National Institute of Animal Health, Japan.

RESULTS

Genetic analysis: The phylogenetic tree obtained with the G protein regions of the examined and the known strains is shown in Fig. 1. On the tree, the sequences of 12 examined strains fell in the same cluster as the 236–652 strain from Nebraska [17] in U.S.A., and were assigned as the subgroup III on the classification due to Valarcher *et al.* [19]. The examined strains were subdivided into 2 lineages, A and B. Six strains of IW01, 03, 04, AK01, MY02 and FKI11 belonged to the lineage B, and the remaining to the A.

As shown in Table 1, the nucleotide sequence homologies of the G protein regions ranged from 87.2 to 97.1% between both lineages, 96.3–99.3% within the lineage A and 94.4–99.8% within the lineage B. While, the homologies were 88.3–93.3% between the examined and the known Japanese strains consisting of the NMK7, rs-52 and 52–163–13 in the subgroup II [6, 19]. The homologies between the lineages A or B strains and those of the subgroups from I to VI were as highest as 86.1–96.6% for III, 85.7–88.3%, 80.2–92.4%, 88.8–93.2%, 89.4–91.4% and 84.7–89.5% for I, II, IV, V and VI.

Deduced amino acid sequence analysis: The deduced amino acid sequence homologies within the examined strains were as high as 91.2–100%. The examined strains showed the homologies of 80.3–93.7% with those in the subgroup III but of 83.0–87.8% with the NMK7. While, the amino acid alignment of linear epitope regions in the exam-

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          69                                                                                                  148
I         4642      AKPTSKPTTQQTQQLQNHTPPPLTEHNYKSTHTSIQSTTLSQPPNIDTTSGTTYGHPTNRTQNRKIKSQSTPLATRKPPI
          BovX      ..............P.....LLP....H......T.....................I.................L..

          220/69W2  ........I....RP......LF....H.............L......R....S...DE...........LPT..Q...
          9616348     ..F...I.....P....S.LF....H.............L.....R....S...DE...........LP...Q...
II        NMK7      ........I.....P..L.S.LF..................LL.....R........DG...........LP..G....
          52-163-13 ........I.....P..L.S.LF..................LL.....R........DG...........LP..G....
          rs-52     ........I.....P..L.S.LF..................LL.....R........DG...........LP..G....

          375       ........I.....P....S.FF..N..............LI.....R......S.DE..S.......ALPT......
          234-489   ........I.....P....S.FF..N..............LI.....R......S.DE..S...R...SLPT......
          VC464     ........I.....P....S.FF.................LL.....R.I....S..E.......G...LP.......
          391.2     ........I.....P....S.FF.................LL.....R.I....S..E.......G...LP.......
          NDKS-7    ........I.....P....S.FF.................LL...A.R.I....S..E...I...G...LS.......
          87-14594  ........I.....P....S.FF.................LL...A.R.I....S..E...I...GL..LP.......
III       88-294    ........V.....P....S.FF.........I.........LL.....R....S.S..E....R......LP.......
          1344R     ........V.....P....S.FF.........I.........LL.....R....S.S..E...........LP.......
          1540R     ........V.....P....S.FF.........I.........LL.....R....S.S..E...........LP.......
          236-652   ........I.....P....S.FF.K...............LL.....R.A..S.S..E...........LP.......
               (Lineage A)
          YM02      ........I.....P....S.FS.KQ..............LL.....R.A..S.S.HG...........LP.......
          YM03      ........I.....P....S.FS.KQ...................S....R.A..S.S..E...........LPV......
          YM01      ........I.....P....S.FS.KQ..............LLS....R.A..S.S..E...........LP.....S.
          MY01      ........I.....P....S.LS.KQ..............LLS....R.V..S.S.IE..........LP.......
          IW05      ........IH....P....S.LS.KQ..............LLS....R.A..S.S.IE..........LP...N...
          IW02      ........I.....P....S.LS.KQ..............LLS....R.A..S.S.IE..........LP...N...
               (Lineage B)
          FKI11     ........F.....P....S.FS.KQ..............LLS....R.A..S.S..E...........LP.......
          MY02      ........F.....P....S.FS.KQ..............LLS....R.A..S.S..E...........LP.......
          AK01      ........F.....P....S.FS.KQ..............LLS....R.A..S.S..E...........LP.......
          IW03      ........F.....P....S.FS.KQ.......L.....LLS....R.A..S.S..E...........LP.......
          IW04      ........F.....P....S.FS.KQ..............LLS....R.A..S.S..E...........LP.......
          IW01      ........F.....P....S.FS.KQ..............LLS....R.A..S.S..E...........LP.......

          Dorset    ........I.....P....S.FF..................L..T...R......SIDE...........LP.......
          V347      ........I.....P....S.FFK........I.........L..T...R......SI.E...........PV......
IV        90504     ........I.....P....S.LF.........I.........L..T...R......SIDE...........PV......
          5761      ........I.....P....S.LF.........I.........L..T...R...D..SIDE...........PV......

          C1        ........I.....P....S.FF.........I.........L..T...R......LI.E...........PV......
          B1        ........I.....P....S.FF.........I.........L..T...R......LI.E...........PV......
          394       ........I.....P....S.FF.........I.........L..T...R......LI.E...........PV......
V         F1        ........I.....P....S.FF..Y......I.........L..T...R.N....LI.E...........PV......
          I1        ........I.....P....S.FF.........I.........L..T...R......LI.E...........PV......
          A1        ........I.....P....S.FF.........I.........L..T...R......LI.E...........PV......
          J1        ........I.....P....S.FF.........I.........L..T...R......LI.E...........PV......

          75P       ........I.....P....S.FF.........I.........L..T...R......LI.E...........P.......
VI        K2        ........I.....P....S.FF.........I.........L..T...R......LI.E...........P.......
          K1        ........I.....P....S.FF.....E...I.........L..T...R......LI.E...........P.......
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ined strains was similar in the same degree to the those in NMK7 as compared with those in the subgroup III strains. Alanine (Ala) 205 of an epitope domain was replaced by threonine (Thr) in all examined strains, the residue substitution which is found in those in the subgroups III, IV, V and VI [10, 19] as well as NMK7 [12]. All 6 strains in the lineage B showed a uniform pattern of the residue substitution that isoleucine (Ile) 200 of the epitope domain was replaced by Thr. All 4 cysteine (Cys) residues involved in the two disulfide bridges, Cys 173-Cys 186 and Cys 176-Cys 182, shown to be immunodominant in the G protein [10], were conserved in all examined strains without any substitutions (Fig. 2).

Neutralization indices: The neutralization indices of the strains IW03 and 52–163–13 were 1.4 and 1.1 log10 without the significant difference between both strains.

DISCUSSION

It is pointed out that the NMK7 strain isolated in Japan is not related to any subgroups on the phylogenetic tree obtained with the G protein regions [19], although no other protein regions of the same strain such as N and F proteins has been analyzed. Twelve field strains detected in Japan during from 2002 to 2004 fell in the same cluster as the 236–652 from Nebraska and in the subgroup III. The strains

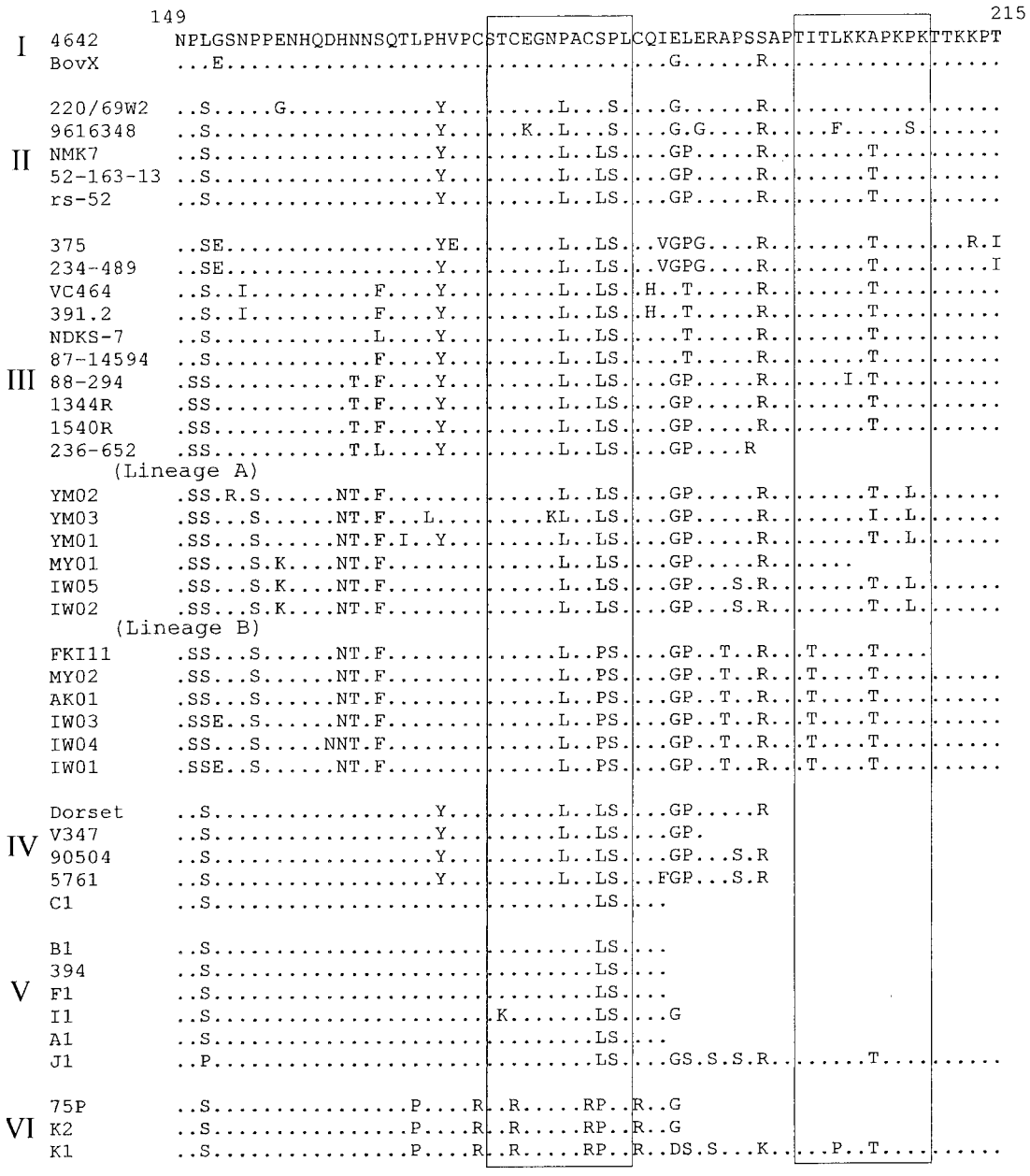

Fig. 2. Amino acid sequences from 69 to 215 of BRSV G protein region. Subgroup designations are shown by block letter. Dots indicate the identity with 4642, and Boxes the linear epitope [10].

were subdivided in 2 lineages (A, B). The sequence homologies were 94.4–99.8% within the examined strains, and 88.3–93.3% between the examined and the known Japanese strains. Although small numbers of strains were treated in this study, it seems likely that the known Japanese strains containing the NMK7 are no representative strains recently circulating in Tohoku and Hokuriku districts and that the subgroup III strains have existed in Japan.

On the antigenic analysis with specific monoclonal antibodies to the G protein, the NMK7 revealed different reaction patterns from European strains [6] but the identical patterns with American ones containing the 236–652 [12]. The examined strains showed the sequence homologies of 86.1–96.6% with those in the subgroup III originating exclusively from U.S.A. [19] and of 88.3–93.3% with the known Japanese strains. While, the amino acid alignment of the linear epitope regions in the examined strains was similar in the same degree to those in the NMK7 as compared with those in the subgroup III strains. And there was no significant difference on the neutralization indices between an

examined strain (IW03) and the 52–163–13 phylogenetically similar to the Japanese vaccine strain. The results suggest that epidemics of the present strains could be protected due to the vaccination and that the present strains might possess closer relationship to American strains than to the known Japanese ones. Although the exact reasons for 2 lineages in the examined strains remain unknown, the both lineages might express an evolving process from the original strain under the Japanese vaccine pressure.

As the characteristic residue substitution in the G protein of the examined lineage B strains, Ile 200 was replaced by Thr which was a potential O-glycosylation site. A carbohydrate at the position could result in a very different antigenic variant [10]. The field strains of BRSV should continue to be phylogenetically and antigenetically investigated to reevaluate the level of protection provided by the present vaccine strain.

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