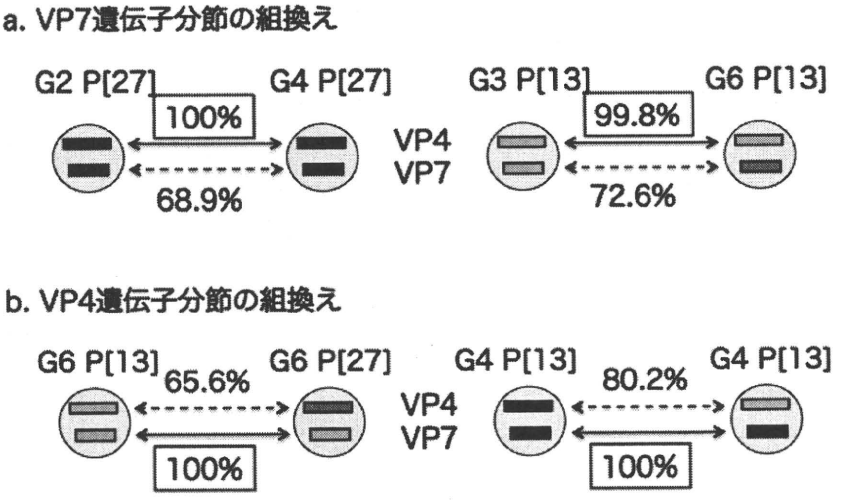

図 3. 健常豚においてみられた遺伝子分節の組換え（リアソートメント）

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表雑誌名	巻	ページ	出版年
Shivakoti S, Ito H, Otsuki K, Ito T.	Characterization of H5N1 highly pathogenic avian influenza virus isolated from a mountain hawk eagle in Japan.	J. Vet. Med. Sci.	72	459-463	2010
Tsunekuni R, Ito H, Otsuki K, Kida H, Ito T.	Genetic comparisons between lentogenic Newcastle disease virus isolated from waterfowl and velogenic variants.	Virus Genes	40	252-255	2010
Shivakoti S, Ito H, Murase T, Ono E, Takakuwa H, Yamashiro T, Otsuki K, Ito T.	Development of reverse transcription-loop-mediate d isothermal amplification (RT-LAMP) assay for detection of avian influenza viruses in field specimens.	J. Vet. Med. Sci.	72	519-523	2010
Takakuwa H, Yamashiro T, Le MQ, Phuong LS, Ozaki H, Tsunekuni R, Usui T, Ito H, Yamaguchi T, Ito T, Murase T, Ono E, Otsuki K.	Possible circulation of H5N1 avian influenza viruses in healthy ducks on farms in northern Vietnam.	Microbiol. Immunol.	54	58-62	2010
Fujimoto Y, Ito H, Shivakoti S, Nakamori J, Tsunekuni R, Otsuki K, Ito T.	Avian Influenza Virus and Paramyxovirus Isolation from Migratory Waterfowl and Shorebirds in San-in District of Western Japan from 2001 to 2008.	J. Vet. Med. Sci.	72	963-967	2010
Fujimoto Y, Ito H, Shinya K, Yamaguchi T, Usui T, Murase T, Ozaki H, Ono E, Takakuwa H, Otsuki K, Ito T.	Susceptibility of two species of wild terrestrial birds to infection with a highly pathogenic avian influenza virus of H5N1 subtype.	Avian Pathol.	39	95-98	2010

Sugiyama M, Ito H, Hata Y, Ono E, Ito T.	Complete nucleotide sequences of avian metapneumovirus Virus Genes subtype B genome.	Virus Genes	41	389-395	2010
Tsunekuni R, Ito H, Kida H, Otsuki K, Ito T.	Increase in the neuraminidase activity of a nonpathogenic Newcastle disease virus isolate during passaging in chickens.	J. Vet. Med. Sci.	72	453-457	2010
Inoue, E., Wang, X., Osawa, Y., Okazaki, K.	Full genomic amplification and subtyping of influenza A virus using a single set of universal primers.	Microbiol. Immunol.	54	129-134	2010
Matsumura, K., Inoue, E., Osawa, Y., Okazaki, K.	Molecular epidemiology of bovine leukemia virus associated with enzootic bovine leukosis in Japan.	Virus Res.	155	343-348	2011
Inoue, E., Matsumura, K., Maekawa1, K., Nagatsuka,K., Nobuta, M., Hirata, M., Minagawa, A., Osawa, Y., Okazaki, K.	Genetic heterogeneity among bovine leukemia viruses in Japan and its relationship to leukemogenicity.	Arch. Virol.			In press
Abe, M., Ito, N.a, Masatani, T., Nakagawa, K., Yamaoka, S., Kanamaru, Y., Suzuki, H., Shibano, K., Arashi, Y. and Sugiyama, M.	Whole genome characterization of new bovine rotavirus G21P[29] and G24P[33] strains provides evidence for interspecies transmission.	J. Gen. Virol.			In press
三井寛子、赤崎　創、久保田智江、池田秀利	処理場で採材した豚扁桃に対する各種ウイルス遺伝子調査	家畜衛生学雑誌	36	10-11	2010

Virus Research 155 (2011) 343–348

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Molecular epidemiology of bovine leukemia virus associated with enzootic bovine leukosis in Japan☆

Keiko Matsumura[a], Emi Inoue[a], Yoshiaki Osawa[a], Katsunori Okazaki[a,b,*]

[a] Laboratory of Microbiology and Immunology, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan
[b] Division of Medicinal Science, The Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan

ARTICLE INFO

Article history:
Received 22 September 2010
Received in revised form
16 November 2010
Accepted 16 November 2010
Available online 21 November 2010

Keywords:
BLV
env gene
Phylogenetic analysis
Japan

ABSTRACT

Bovine leukemia virus (BLV) infection of cattle has been increasing yearly in Japan although several European countries have successfully eradicated the infection. In the present study, phylogenetic analysis on the *env* gene obtained from 64 tumor samples found in different regions in Japan was carried out in order to define the genetic background of BLV strains prevailing in the country. Most of the Japanese isolates were found to reside in the consensus cluster or genotype 1 of BLV strains (Rodriguez et al., 2009). Out of them, 21 isolates and 10 isolates exhibited the identical sequences, respectively. Only one isolate was classified into the different genotype related to the US isolates. Analysis on the deduced amino acids of gp51 demonstrated the sequence diversity in the neutralizing domain. These data may indicate that two major populations of BLV prevailed throughout Japan, whereas antigenic variants also exist. It was further proved that multiple invasion of the genetically different BLV strains have occurred in Japan.

1. Introduction

Bovine leukemia virus (BLV) is a member of the family *Retroviridae*, genus *Deltaretrovirus* and the causative agent of enzootic bovine leukosis (Kettmann et al., 1976). Cattle infected with BLV usually shows no clinical sign; only 30–70% of infected animals develop persistent lymphocytosis, and 0.1–10% develop tumors (Burny et al., 1998; Kettmann et al., 1980). The virus is present in blood lymphocytes and in tumor cells as provirus integrated into the genome and found in the cellular fraction of various body fluids. Enzootic bovine leukosis are prevalent all over the world and in Japan, where a number of cases shows a yearly increase recently. Since transmission of BLV may occur via contact with the affected animals, parturition, and mechanical transmission by insects, blood transfusion, and the use of common needles, it is emphasized to carry out surveillance study for the control of the disease.

The envelope glycoproteins of viruses play a crucial role in their life cycle. The BLV envelope contains two glycoproteins; gp30 and gp51, which are disulfide-linked and derived by posttranslational proteolytic cleavage of precursor (gpr72) encoded by the *env* gene (Johnston and Radke, 2000; Mamoun et al., 1983). Since gp51 is responsible for virus attachment and entry into the host cells, the glycoprotein serves as a target for neutralizing antibodies (Bruck et al., 1982; Callebaut et al., 1993; Portetelle et al., 1989). The glycoprotein also induces a cell-mediated immune response, which may play a role in protective immunity against BLV infection, especially tumorigenesis (Gatei et al., 1993).

Recent phylogenetic study on the BLV *env* gene of the strains isolated in worldwide demonstrated that the virus can be classified into 7 genotypes (Moratorio et al., 2010; Rodriguez et al., 2009). In the present study, to define the genetic background of BLV prevailing in Japan, we determined the nucleotide sequences of the *env* gene obtained from 64 tumor samples found in different regions in Japan and analyzed them phylogenetically. We also compared the deduced amino acid sequences of the regions of gp51 that elicit the immune responses against BLV in order to reveal antigenic property of the viruses. The results of these studies revealed that most of the Japanese isolates belonged to genotype 1 and that two major populations of the virus cocirculated throughout the country. It is also surmised that multiple invasion of genetically distinct strains occurred by importation.

2. Materials and methods

2.1. Tumor samples

Tumors were collected from 64 cattle diagnosed as lymphosarcoma at various slaughterhouses in Japan in 2008–2010. Fifty-three cattle were Holstein, 8 were Japanese Black and the others were

☆ DDBJ Accession Nos.: Nucleotide sequence data from this article have been deposited with DNA Data Bank of Japan under Accession No. AB598781–AB598805.

* Corresponding author at: Department of Microbiology and Immunology, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan. Tel.: +81 133 23 1333; fax: +81 133 23 1333.
E-mail address: kokazaki@hoku-iryo-u.ac.jp (K. Okazaki).

0168-1702/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2010.11.005

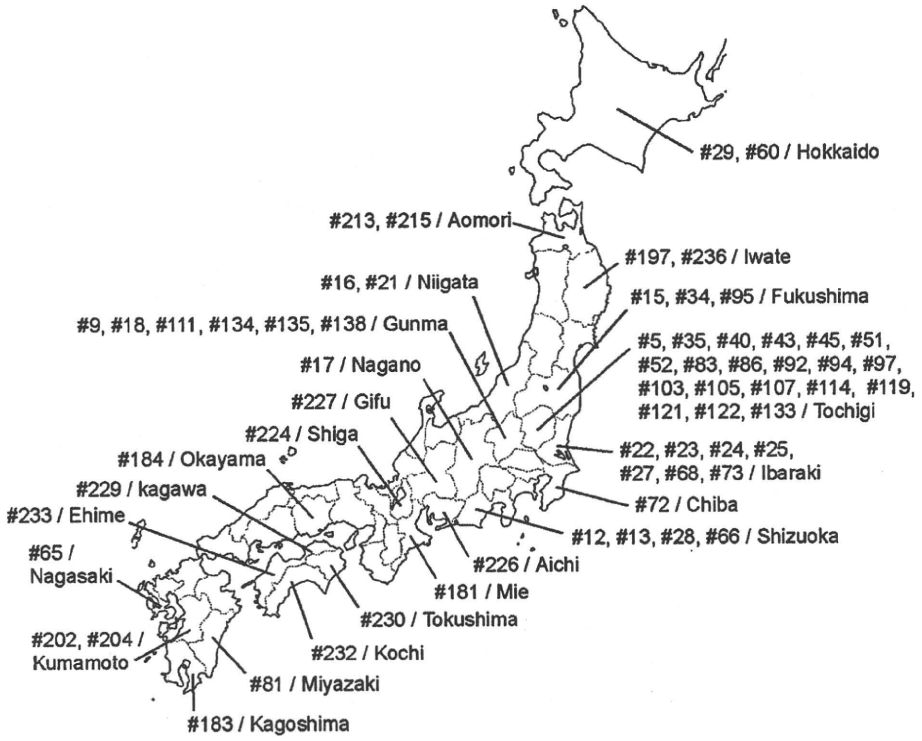

Fig. 1. Raising places of cattle examined in this study. The animals harboring #60 and #81 were raised for 3 years in Hokkaido and 1 year in Miyazaki, followed by 2.5- and 5-year raising in Tochigi, respectively. The remaining animals were raised in a single prefecture.

Jersey and hybrid. Raising places of the animals, which were traced on the basis of the Information for Individual Identification of Cattle provided by National Livestock Breeding Center, Japan, are shown in Fig. 1. The tissues were put into cryotubes, the lids sealed, and transported in frozen conditions to Health Sciences University of Hokkaido and stored at −80 °C until used.

2.2. DNA isolation and PCR

Provirus DNA was obtained from the tumor using QuickGene DNA tissue kit S (FUJIFILM, Tokyo, Japan) according to manufacture's instruction. The PCR was done as described in the diagnostic manual of OIE (The World Organisation for Animal Health, 2004). In brief, a 440-bp part of the *env* gene, corresponding to the nucleotide positions from 5029 to 5468 of the BLV genome (Sagata et al., 1985), was amplified by using GoTaq Flexi DNA polymerase (Promega, Madison, WI) and a set of primers, OBLV1A (5′-CTTTGTGTGCCAAGTCTCCCAGATACA-3′) and OBLV6A (5′-CCAACATATAGCACAGTCTGGGAAGGC-3′) (Ballagi-Pordany et al., 1992). The reaction mixtures contained 26.75 μl of distilled water, 10 μl of 5× GoTaq Flexi buffer (Promega, Madison, WI), 2 μl of 10 μM each primer, 5 μl of template DNA, 4 μl of 25 mM $MgCl_2$ and 0.25 μl of GoTaq Flexi DNA polymerase. The amplification was carried out by 5 cycles consisting of 94 °C for 45 s, 60 °C for 60 s and 72 °C for 90 s and followed by 30 cycles of 94 °C for 45 s, 55 °C for 60 s and 72 °C for 90 s. The program ended with one cycle at 72 °C for 7 min. The amplification products were analyzed in 1.5% agarose gels stained with ethidium bromide.

2.3. Nucleotide sequencing and analysis

The amplification products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The amplicons were directly sequenced with the primers OBLV1A, OBLV6A, OBLV3 (5′-CTGTAAATGGCTATCCTAAGATCTACTGGC-3′) and OBLV5 (5′-GACAGAGGGAACCCAGTCACTGTTCAACTG-3′) (Ballagi-Pordany et al., 1992) using BigDye® Terminator ver1.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA), according to manufacture's instruction. The sequences were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster, CA).

Phylogenetic analysis was based on 346-bp sequences within the *env* gene, which excluded the primer sequences and covered the first and second neutralizing domains (Callebaut et al., 1993) and $CD8^+$-T cell epitope of gp51 (Gatei et al., 1993). Sequence data of each sample, together with those from GenBank, were analyzed by UPGMA method using GENETYX-MAC version 12.2.0 program (Genetyx, Tokyo, Japan).

3. Results

3.1. Phylogenetic analysis of BLV

The 440-bp fragments were amplified from every tumor samples obtained from the cattle diagnosed as lymphosarcoma. The nucleotide sequence of the *env* gene was detected in each of the fragments, indicating that all of the animals tested were affected with enzootic bovine leukosis caused by BLV. The 346-bp sequences of the *env* gene from the 64 samples were analyzed by UPGMA method with those from viruses isolated at different places in the world. The phylogenetic tree indicates that BLV strains found all over the world were divided into 7 genotypes reported by Rodriguez et al. and an extra cluster, which included 3 Iranian isolates (Hemmatzadeh, 2007) (Fig. 2). Out of the 64 strains tested here, 63 (98.4%) were found to belong to genotype 1 together with other isolates in Japan, Brazil, Australia, Iran, Korea, USA, and Uruguay. Only #60 as well as JPFU, which was isolated in Fukuoka, Japan, was allied to genotype 3 consisting of isolates in North America. The tree also indicates that #12-, #35-, and #5-like strains were

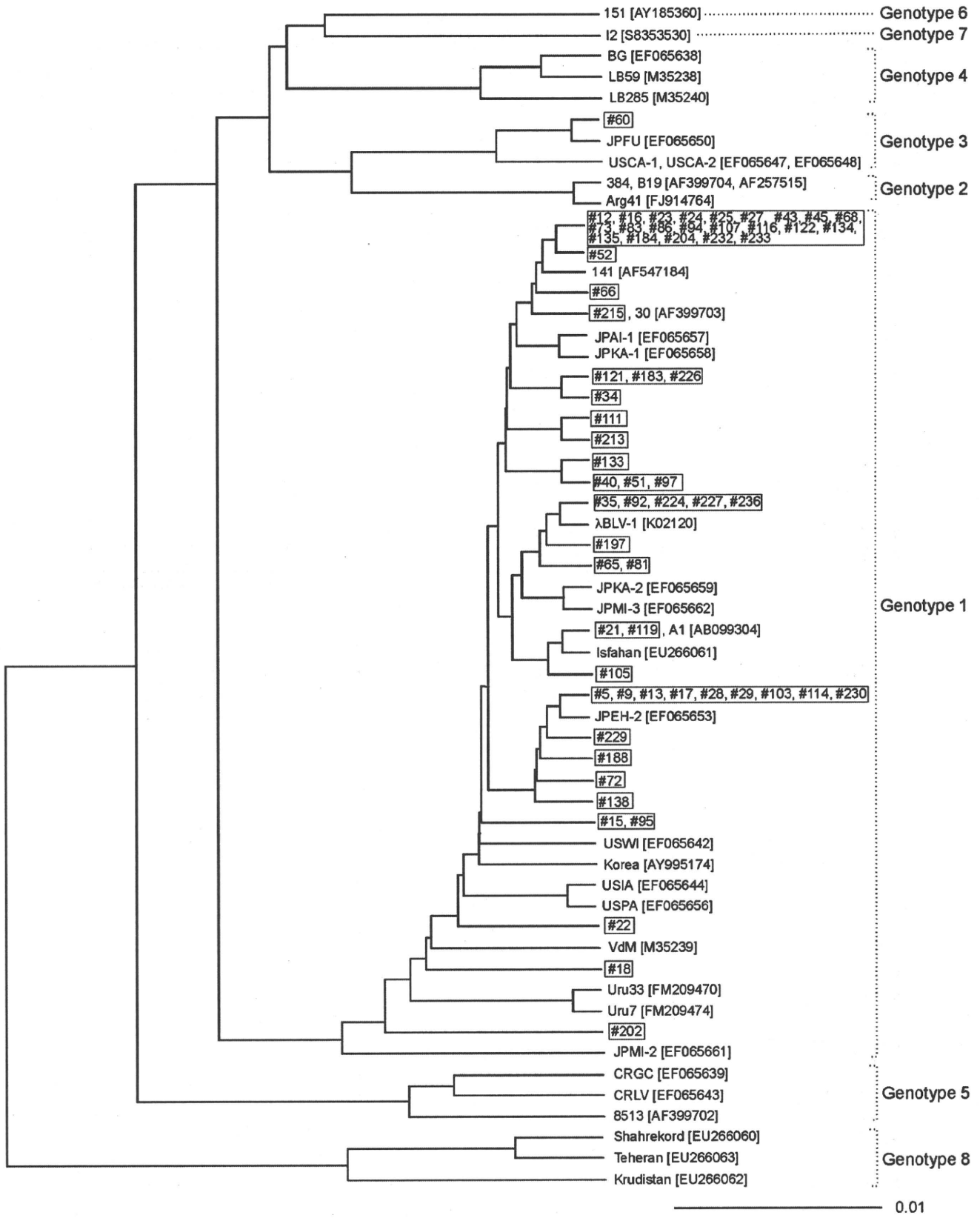

Fig. 2. Phylogenetic tree based on partial sequences of BLV *env* gene. The 346-bp sequences of the *env* gene from 64 tumor samples collected in different places in Japan were analyzed by UPGMA method with those from viruses isolated throughout the world. Sequences obtained in this study are boxed. The GenBank accession numbers are shown in brackets. Genotypes 1–7 were described by Rodriguez et al. (2009).

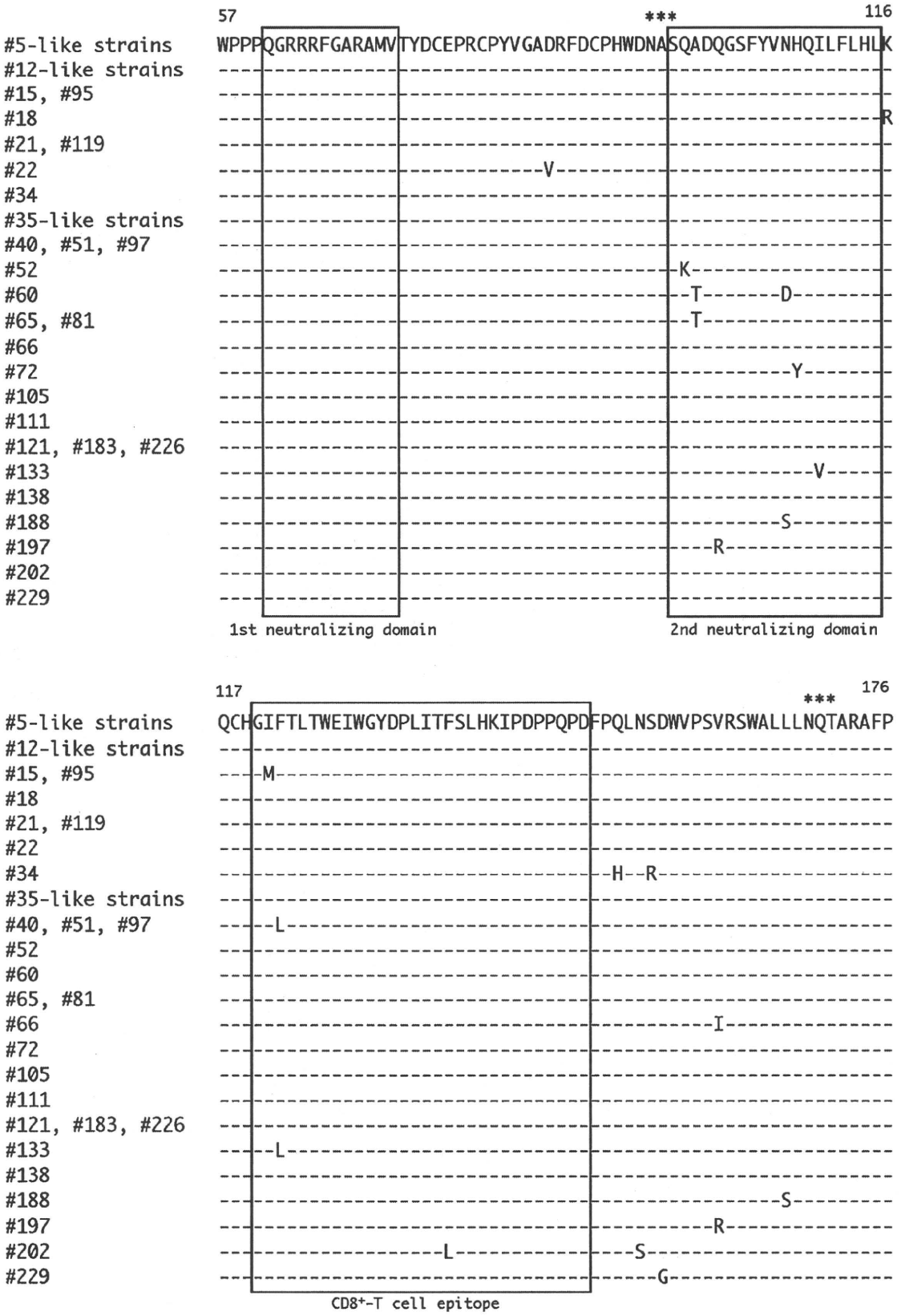

Fig. 3. Amino acid sequence alignment of partial sequences of BLV gp51 glycoprotein. The amino acid sequences were deduced from the *env* gene fragment of 64 isolates in this study. The first and second neutralizing domains (Callebaut et al., 1993) and $CD8^+$-T cell epitope (Gatei et al., 1993) of the glycoprotein are enclosed by boxes. Potential glycosylation sites and conserved amino acid positions are indicated by asterisks and bars, respectively.

prevailing in Japan. Interestingly, 3 of 8 Japanese Black cattle tested in this study harbored #35-like strains and #197 and #65, which were genetically related to these strains, were also isolated from Japanese Black ones.

3.2. Estimation of antigenic property of gp51

Amino acid sequences were deduced from the nucleotide sequences of the *env* gene derived from the tumors and compared with each other in order to estimate antigenic property of gp51. As shown in Fig. 3, the second neutralizing domain showed diversity of the sequences. Although 54 of the 64 strains possessed the same sequences, amino acid substitutions were observed in #52, #60, #65, #72, #81, #133, #188, #197, #202, and #229. Substitutions from neutral Gln to basic Lys and Arg were observed at amino acid residues 98 and 101 of gp51 of #52 and #197, respectively. Substitutions from hydrophobic Ala to hydrophilic Thr were observed at residue 99 of those of #60, #65, and #81. Additional substitution from neutral Asn to acidic Asp at residue 107 was observed in #60, which was genetically distinct from the others. Substitution from basic His to polar Tyr at residue 108 was found in #72 and those from Ile to Val at residue 110 and from Asn to Ser at residue 107 were observed in #133 and #188, respectively. These findings possibly indicate antigenic diversity of BLV strains circulating in Japan. The potentially N-glycosylation site just prior to the second neutralizing domain was conserved in the glycoproteins tested here. The $CD8^+$-T cell epitope of gp51 also showed diversity although 57 strains exhibited the identical sequence. #15 and #95 showed substitution from Ile to Met at residue 122. Substitution from Phe to Leu at residue 123 were observed in #40, #51, #97, and #133 and the same substitution at 138 was in strain #202. These substitutions were accompanied by changes in hydrophobicity. No substitution was found within the first neutralizing domain among the 64 BLV strains.

4. Discussion

Phylogenetic analysis based on the partial sequence of the *env* gene indicates that most of the Japanese isolates belonged to genotype 1 (Rodriguez et al., 2009), which was referred to as the consensus cluster (Zhao and Buehring, 2007). Although many forkings in the tree indicate the genetic diversity of the virus in this lineage, the sequences identical to those of #12 and #5 were obtained from the animals raised at different places in Japan. #12-like strains were found in Niigata (#16), Tochigi (#43, #45, #83, #86, #94, #107, #122), Gunma (#134, #135), Ibaraki (#23, #24, #25, #27, #68, #73), Shizuoka (#12), Ehime (#233), Kochi (#232), and Kumamoto (#204). #5-like strains were in Hokkaido (#29), Tochigi (#5, #103, #114), Gunma (#9), Nagano (#17), Shizuoka (#13, #28), and Tokushima (#230). These findings suggest that these two series of strains cocirculated throughout Japan. #35-like viruses were also found in Iwate (#236), Tochigi (#35, 92), Gifu (#227), and Shiga (#224). On the other hand, #40-like strains were found only in Tochigi and #15-like strains were also found only in Fukushima. The animals harboring strains #45 and #86, which possessed the identical sequences, were raised in the same farm at the same time although they were born from different mothers.

Out of the 64 samples tested here, only #60, of which host animal was raised for 3 years in Hokkaido, followed by 2.5-year raising in Tochigi, were allied to genotype 3 together with JPFU, which was isolated from cattle with persistent lymphocytosis in Fukuoka in 2002 or 2003 (Asfaw et al., 2005). They were genetically closely related to the US isolates. Although it was ambiguous where the animals harboring #60 and JPFU were infected with the virus, their common ancestor might be introduced into Japan by importation of infected cattle from the US. Since no other Japanese strain genetically related to them was found, the ancestor was supposed to invade Japan not so ago. It was certain that multiple invasion of BLV strains occurred in Japan.

Our phylogenetic analysis also demonstrated an extra cluster other than 7 genotypes indicated by neighbour-joining method (Rodriguez et al., 2009). Three BLV strains isolated in Iran, which were sorted into genotype 1 previously, was mapped to the outgroup by UPGMA method. Since BLV infection is almost confined to cattle, the evolutionary rate of the virus is assumed to be constant. Hence, it was accepted that UPGMA method is adequate to analyze the evolution of BLV. The present findings may indicate that BLV can be classified into at least 8 genotypes.

Antigenic diversity was estimated in the second neutralizing domain and the $CD8^+$-T cell epitope of gp51 among the viruses tested. Thr99 within the second neutralizing domain of #60, #65, and #81 was found in other Japanese, German, and US isolates (Fechner et al., 1997; Zhao and Buehring, 2007). Asp107 of #60 was also found in many isolates in South America (Camargos et al., 2007; Monti1 et al., 2005; Moratorio et al., 2010). Both Thr99 and Asp107 of #60 were found in US isolates, which constituting genotype 3 together with #60 (Zhao and Buehring, 2007). Arg101 of #197 was found in a Brazilian isolate (Felmer et al., 2005). Tyr108 of #72 was found in an Argentina isolate [AC037626] and a Japanese isolate [BAH23661]. Lys98 of #52, Ser107 of #188, and Val110 of #133 were unique to these strains. Met121 found within the $CD8^+$-T cell epitope of #15 and #95 as well as Leu122 of #40, #51, #97, and #133 was also unique to these strains. Leu137 of #202 was found in an Uruguayan isolate [CAW30943]. Much more divergent sequences were observed in the second neutralizing domain than the first neutralizing domain or the $CD8^+$-T cell epitope of the glycoprotein. A previous report demonstrated that a synthetic peptide corresponding to the second neutraling domain stimulated T-cell proliferation from 3 of 5 cattle infected with BLV (Callebaut et al., 1993). It is, therefore, considered that this reagion was dominant in the bovine immune system and that possibly escape mutants arose in many parts of the world. Amino acid sequence between residues 104 and 123 was also reported to be involved in receptor binding of gp51 (Gatot et al., 2002). Substitutions at residues 107, 108, and 110 within the second neutralizing domain as well as those at residues 116, 121, and 122 may affect viral fusion and infectivity. Johnston et al. (2002) reported that amino acid substitutions at residue 107, 112, and 120 influenced syncytium formation by cells expressing the BLV envelope glycoproteins.

In conclusion, our phylogenetic analysis on BLV strains isolated in Japan showed that most of them belonged to genotype 1 or the consensus cluster and that two major populations of the virus prevailed throughout the country although genetic and probably antigenic variants also exist. It was further proved that the genetically distinct BLV strain(s) have continued to invade Japan. Hence, extreme care in introducing cattle is necessary for control of BLV infection.

Acknowledgements

This work was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan. The authors thank Hokkaido Obihiro, Hayakita, and Yakumo, Aomori Prefecture Towada, Tochigi Prefecture Kenkita, Ibaraki Prefecture Kenkita and Kennishi, and Shizuoka Prefecture Eastern District Meat Inspection Offices, Iwate, Gunma, Gifu, Okayama, Kagawa, and Tokushima Prefectural Meat Inspection Offices, Yokohama and Kumamoto City Meat Inspection Offices and Nara Prefectural Food Inspection Office for their extensive help in collecting the samples.

References

Asfaw, Y., Tsuduku, S., Konishi, M., Murakami, K., Tsuboi, T., Wu, D., Sentsui, H., 2005. Distribution and superinfection of bovine leukemia virus genotypes in Japan. Arch. Virol. 150, 493–505.

Ballagi-Pordany, A., Klintevall, K., Merza, M., Klingeborn, B., Belak, S., 1992. Direct detection of bovine leukaemia virus infection: practical applicability of a double polymerase chain reaction. J. Vet. Med. [B] 39, 69–77.

Burny, A., Cleuter, Y., Kettman, R., Mammerickx, M., Marbaix, G., Portelle, D., Vanden Broeke, A., Willems, L., Thomas, R., 1998. Bovine leukaemia: facts and hypotheses derived from the study of an infectious cancer. Vet. Microbiol. 17, 197–218.

Bruck, C., Portetelle, D., Burny, A., Zavada, J., 1982. Topographical analysis by monoclonal antibodies of BLV gp51 epitopes involved in viral functions. Virology 122, 353–362.

Callebaut, I., Voneche, V., Mager, A., Fumiere, O., Krchnak, V., Merza, M., Zavada, J., Mammerickx, M., Burny, A., Portetelle, D., 1993. Mapping of B-neutralizing and T-helper epitopes on the bovine leukemia virus external glycoprotein gp51. J. Virol. 67, 5321–5327.

Camargos, M.F., Pereda, A., Stancek, D., Rocha, M.A., dos Reis, J.K., Greiser-Wilke, I., Leite, R.C., 2007. Molecular characterization of the *env* gene from Brazilian field isolates of bovine leukemia virus. Virus Genes 34, 343–350.

Fechner, H., Blankenstein, P., Looman, A.C., Elwert, J., Geue, L., Albrecht, C., Kurg, A., Beier, D., Marquardt, O., Ebner, D., 1997. Provirus variants of the bovine leukemia virus and their relation to the serological status of naturally infected cattle. Virology 237, 261–269.

Felmer, R., Muñoz, G., Zuñiga, J., Recabal, M., 2005. Molecular analysis of a 444 bp fragment of the bovine leukaemia virus gp51 env gene reveals a high frequency of non-silent point mutations and suggests the presence of two subgroups of BLV in Chile. Vet. Microbiol. 108, 39–47.

Gatei, M.H., Good, M.F., Daniel, R.C., Lavin, M.F., 1993. T-cell responses to highly conserved CD4 and CD8 epitopes on the outer membrane protein of bovine leukemia virus: relevance to vaccine development. J. Virol. 67, 1796–1802.

Gatot, J.S., Callebaut, I., Van Lint, C., Demonté, D., Kerkhofs, P., Portetelle, D., Burny, A., Willems, L., Kettmann, R., 2002. Bovine leukemia virus SU protein interacts with zinc, and mutations within two interacting regions differently affect viral fusion and infectivity in vivo. J. Virol. 76, 7956–7967.

Hemmatzadeh, F., 2007. Sequencing and phylogenetic analysis of gp51 gene of bovine leukaemia virus in Iranian isolates. Vet. Res. Commun. 31, 783–789.

Johnston, E.R., Albritton, L.M., Radke, K., 2002. Envelope proteins containing single amino acid substitutions support a structural model of the receptor-binding domain of bovine leukemia virus surface protein. J. Virol. 76, 10861–10872.

Johnston, E.R., Radke, K., 2000. The SU and TM envelope protein subunits of bovine leukemia virus are linked by disulfide bonds, both in cells and in virions. J. Virol. 74, 2930–2935.

Kettmann, R., Cleuter, Y., Mammerickx, M., Meunier-Rotival, M., Bernardi, G., Burny, A., Chantrenne, H., 1980. Genomic integration of bovine leukemia provirus: comparison of persistent lymphocytosis with lymph node tumor form of enzootic. Proc. Natl. Acad. Sci. U.S.A. 77, 2577–2581.

Kettmann, R., Portetelle, D., Mammerickx, M., Cleuter, Y., Dekegel, D., Galoux, M., Ghysdael, J., Burny, A., Chantrenne, H., 1976. Bovine leukemia virus: an exogenous RNA oncogenic virus. Proc. Natl. Acad. Sci. U.S.A. 73, 1014–1018.

Mamoun, R.Z., Astier, T., Guillemain, B., Duplan, J.F., 1983. Bovine lymphosarcoma: processing of bovine leukemia virus-coded proteins. J. Gen. Virol. 64, 2791–2795.

Monti1, G., Schrijver, R., Beier, D., 2005. Genetic diversity and spread of bovine leukaemia virus isolates in Argentine dairy cattle. Arch. Virol. 150, 443–458.

Moratorio, G., Obal, G., Dubra, A., Correa, A., Bianchi, S., Buschiazzo, A., Cristina, J., Pritsch, O., 2010. Phylogenetic analysis of bovine leukemia viruses isolated in South America reveals diversification in seven distinct genotypes. Arch. Virol. 155, 481–489.

Portetelle, D., Dandoy, C., Burny, A., Zavada, J., Siakkou, H., Gras-Masse, H., Drobecq, H., Tartar, A., 1989. Synthetic peptides approach to identification of epitopes on bovine leukemia virus envelope glycoprotein gp51. Virology 169, 34–41.

Rodriguez, S.M., Marcelo, D., Golemba, Campos, R.H., Trono, K., Jones, L.R., 2009. Bovine leukemia virus can be classified into seven genotypes: evidence for the existence of two novel clades. J. Gen. Virol. 90, 2788–2797.

Sagata, N., Yasunaga, T., Tsuzuku-Kawamura, J., Ohishi, K., Ogawa, Y., Ikawa, Y., 1985. Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. Proc. Natl. Acad. Sci. U.S.A. 82, 677–681.

The World Organisation for Animal Health, 2004. Enzootic bovine leukosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. http://www.oie.int/eng/normes/mmanual/A_00055.htm.

Zhao, X., Buehring, G.C., 2007. Natural genetic variations in bovine leukemia virus *envelope* gene: possible effects of selection and escape. Virology 366, 150–165.

NOTE *Virology*

Development of Reverse Transcription-Loop-Mediated Isothermal Amplification (RT-LAMP) Assay for Detection of Avian Influenza Viruses in Field Specimens

Sakar SHIVAKOTI[1], Hiroshi ITO[1,2], Toshiyuki MURASE[2,3], Etsuro ONO[2,4], Hiroki TAKAKUWA[5], Tetsu YAMASHIRO[6], Koichi OTSUKI[2,5] and Toshihiro ITO[1,2]*

[1]Laboratory of Veterinary Public Health, Faculty of Agriculture, Tottori University, Tottori 680–8553, [2]Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, Tottori 680–8553, [3]Laboratory of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori 680–8553, [4]Laboratory of Biomedicine, Center of Biomedical Research, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812–8581, [5]Avian Influenza Research Center, Kyoto Sangyo University, Kyoto 603–8555 and [6]Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852–8523, Japan

(Received 22 October 2009/Accepted 3 December 2009/Published online in J-STAGE 22 December 2009)

ABSTRACT. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is an established gene amplification method for rapid diagnosis of various infectious diseases. In order to detect avian influenza viruses, particularly in field specimens, specific primers targeting the matrix gene were designed. Thirty-four virus samples, including isolates from wild and domestic avian hosts belonging to various geographical areas, were used to confirm the validity of the primers. All samples were confirmed to be positive in less than 1 hr. The RT-LAMP assay was also able to detect avian influenza virus in the various field samples, such as swabs, tissues, and feces. These results indicate that the developed RT-LAMP assay with uniquely designed primers is potentially useful in comprehensive avian influenza surveillance.

KEY WORDS: avian influenza virus, LAMP primers, Matrix gene, rapid detection, RT-LAMP.

J. Vet. Med. Sci. 72(4): 519–523, 2010

Influenza A viruses belong to the family Orthomyxoviridae, having an eight-segmented, single-stranded, negative-sense RNA genome [23], and are further classified into 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes [6]. Influenza A viruses have been isolated from a number of different animal hosts, including humans, pigs, horses, seals and whales, as well as a variety of domestic and wild birds [21, 22]. To date, most HA/NA combinations have been identified in the domestic and wild bird reservoir [23]. Surveillance of influenza A virus infection and outbreaks in non-primate mammals and birds is essential in understanding the ecology of influenza viruses, and to determine the molecular basis of host range transmission and spread in new hosts.

Various molecular diagnostic tests have been used for detection of influenza A viruses, such as reverse transcription-PCR (RT-PCR) [5], microarray [4], nucleic acid sequence-based amplification (NASBA) [3], real-time RT-PCR [16], mismatch amplification mutation assay (MAMA) PCR [8] and multiplex RT-PCR assay [2, 24]. However, most of these methods require expensive and specialized instruments and reagents, and therefore may not be readily applicable, particularly in diagnostic laboratories in developing and underdeveloped countries or in frontline laboratories. In contrast to other molecular techniques, loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method in which reagents react under isothermal conditions with high specificity, efficiency and rapidity [14].

The method is applicable to amplification of RNA templates by combination with a reverse transcription reaction (RT-LAMP), and this approach has been applied successfully to the detection of numerous viruses, such as West Nile virus [15], viral hemorrhagic septicemia virus (VHS) [19], Spring viremia of carp virus [18], respiratory syncytial virus (RSV) [20], H3 swine influenza virus [7], infectious bursal diseases virus [25], hepatitis E virus [11] and Newcastle disease virus [12]. Moreover, RT-LAMP has been used for diagnosis of infection by influenza viruses, including H5 and H9 subtypes [1, 10]. However, this method has not been reported for the detection of all subtypes of influenza A virus. In this study, we have selected the most conserved region of the matrix gene sequence of the influenza A virus in order to design common primers and to sensitively and specifically detect a wide range of avian influenza viruses using the RT-LAMP assay.

Thirty-four avian influenza virus isolates and three non-avian influenza viruses were used in order to determine the sensitivity and specificity of the present RT-LAMP assay. These strains represent viruses from wild and domestic avian hosts from various geographical distributions in both Eurasian and North American regions over the 60-year period prior to 2009.

In addition to infected allantoic fluid, tracheal swabs from a whooper swan infected with HPAI virus A/whooper swan/Aomori/1/2008 (H5N1), fecal samples from a whistling swan and duck infected with A/whooper swan/Shimane/148/09 and A/duck/Saga/62/08, respectively, and tissue and swab samples from the carcass of a mountain hawk-eagle, collected from Kumamoto Prefecture, infected with HPAI

* CORRESPONDENCE TO: ITO, T., Department of Veterinary Public Health, Faculty of Agriculture, Tottori University, 4–101 Minami, Koyama-cho, Tottori 680–8553, Japan.
e-mail: toshiito@muses.tottori-u.ac.jp

virus A/mountain hawk-eagle/Kumamoto/01/07 (H5N1) were used to determine the sensitivity of the present RT-LAMP assay for field specimens. RNA isolation of the H5N1 HPAI virus samples was performed in a Biosafety level 3 laboratory at Tottori University.

Genomic viral RNA was extracted from 140 μl of allantoic fluid or 10% suspensions of swab, tissues and fecal samples using the QIA amp viral RNA mini kit (Qiagen, Valencia, CA, U.S.A.) in accordance with the manufacturer's protocols. RNA was eluted from QIAamp Mini spin columns in a final volume of 60 μl of elution buffer. The eluted RNA concentration was determined with a ND-1000 spectrophotometer (Nano Drop, ND-1000, NanoDrop Technologies, Wilmington, DE, U.S.A.) and the RNA concentration of each sample was adjusted to 60–80 $ng/\mu l$. The RNA samples were stored at –80°C until use.

The matrix gene sequences of Influenza A viruses from wild and domestic avian hosts belonging to different geographical distributions were obtained from the influenza A database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). As identifying conserved regions for primer construction was nearly impossible with a single alignment from all the available viral sequences, total virus sequences were divided into the following bird groups: Eurasian wild; Eurasian domestic; North American wild; and North American domestic. All sequences were aligned (BioEdit version 7.0.8.0) and conserved regions for each group were traced out. One set of primers from each group, with each set having four specific primers, including two inner primers (one forward inner primer, one backward inner primer) and two outer primers (forward outer primer and backward outer primer), recognizing six distinct genomic sequences of the matrix gene of influenza A virus, and all falling in conserved regions, were designed manually following the standard parameters of the prime explorer LAMP primer designing software (http://primerexplorer.jp/e/).

RT-LAMP was carried out using a Loopamp RNA Amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). Briefly, 5 μl of extracted RNA was added to 20 μl of master mix containing 12.5 μl of 2× reaction mixture, 1 μl of enzyme (mixture of Bst polymerase and AMV reverse transcriptase), 40 pmol of each inner primer and 5 pmol of each outer primer, and distilled water to make a final volume of 20 μl. The mixture was incubated using a Loopamp real-time turbidimeter (RT-160C; Eiken Chemical). Turbidity of the reaction was measured by real time and the result was indicated with the graph in the monitor of real time turbidimeter verifying the startup of the amplification. RNA was extracted from the allantoic fluid samples of 37 strains and RT-LAMP was carried out separately with all the four sets of primers for 120 min at 63°C constant temperature. After determining the best set of primers in terms of sensitivity, specificity and speed, RT-LAMP was further carried out at 60 to 65°C in order to determine the optimum temperature. The optimum temperature was recorded as 63°C and time was set for 60 min, followed by 5 min at 80°C in order to terminate the reaction. The experiment was replicated twice and average detection time for each sample was calculated.

For the visual detection of RT-LAMP results, fluorescent detection reagent (Eiken Chemical) was added to the master mix (1 μl/test), followed by incubation for 60 min in a conventional water bath maintained at 63°C, followed by 5 min at 80°C. The same RNA extracts from six different avian influenza A viruses (A/duck/Memphis/546/74, A/Turkey/Ontario/6118/68, A/mountain hawk-eagle/Kumamoto/01/07, A/chicken/Germany/N/49, A/whooper swan/Aomori/ 2/2008 and A/chicken/VN/G14-TC5&6/08) and one influenza B virus, B/Lee, used for the RT-LAMP assay, were used for this purpose and the products were observed under a UV lamp (λ= 365 nm) after termination of the reaction.

In order to design specific primers for detecting a wide range of avian influenza viruses, a total of 823 sequences were classified into Eurasian wild, Eurasian domestic, North American wild and North American domestic bird groups, and were then aligned. One set of primers from each group was designed, based as far as possible on conserved regions. Of the four sets of primers, the set with the best results (prepared from the North American wild group) was selected. The primers used in this study are listed in Table 1. All avian influenza viruses were detected in less than 60 min and as rapidly as 27 min (Table 2). The three non-avian influenza viruses (infectious bronchitis virus, influenza B virus and Newcastle disease virus) were not detected. These results indicate that there is no cross-reactivity among non-avian influenza viruses, and that the primers are specific to the avian influenza virus (Table 2).

The detection limit of the RT-LAMP assay in the 60-min amplification period was assessed by titrating and serially diluting (10-fold) A/chicken/Ibaraki/01/05 (Ibaraki/05). For viral titration, 100 μl of 10-fold diluted Ibaraki/05 virus was inoculated into five SPF embryonated hen's eggs and the median embryo infectious dose (EID_{50}) was calculated according to the Reed and Muench formula [17]. From each dilution, RNA was extracted and RT-LAMP was carried out. The results showed that the detection limit of RT-LAMP within 60 min was a 10^5 fold dilution of the initial virus titer (i.e., approximately 10^3 EID_{50}/reaction tube), as determined by electrophoresis with LAMP-specific ladder-shaped bands (Fig. 1).

The ability of the developed RT-LAMP analysis to detect avian influenza viruses in swab, tissue and fecal samples was assessed by testing three tissue samples and two swab samples of a H5N1 HPAI virus A/mountain hawk-eagle/Kumamoto/01/2007-infected mountain hawk-eagle and a tracheal sample from a whooper swan infected with the H5N1 HPAI virus A/whooper swan/Aomori/1/2008. RT-LAMP confirmed all the swab and tissue samples to be positive when the reaction was carried out for 60 min (Table 3). Viral RNA extracted from frozen fecal samples of wild waterfowl infected with A/whistling swan/Shimane/148/2009 and A/duck/Saga/62/2008 were also confirmed to be positive by RT-LAMP assay. Unlike A/duck/Saga/62/2008, the A/whistling swan/Shimane/148/2009-infected fecal sample was confirmed to be positive in 67 min when the

Table 1. Oligonucleotide primers used for RT-LAMP amplification of matrix gene of avian influenza viruses

Primer name	Type	Length(s)	Genome position	Sequence (5'–3')
F3	Forward outer primer	19-mer	26–44	ATGAGTCTTCTAACCGAGG
B3	Reverse outer primer	19-mer	238–256	TCTACGCTGCAGTCCTCGC
FIP	Forward inner primer	37-mer; F1C,18-mer F2, 19-mer	F1c;94–111 F2;45–63	TCAAGTCTCTGCGCGATC-TCGAAACGTACGTTCTCTC
BIP	Reverse inner primer	39-mer; B1C,20-mer B2,37-mer	B1C;167–186 B2;219–237	ACAAGACCAATCCTGTCACC-TCACTGGGCACGGTGAGCG

Table 2. Detection of avian influenza viruses of different subtypes by RT-LAMP assay

Virus	Detection time in min
Eurasian isolate	
A/duck/Hong Kong/319/78 (H2N2)	30
A/duck//Hokkaido/8/80 (H3N8)	35
A/duck/Vietnam/G114C20/2006 (H3N8)	35
A/duck//Ukraine/1/63 (H3N8)	34
A/chicken/Vietnam/G14-TC5&6/2008 (H3N8)	35
A/whistling swan/Shimane/227/2001 (H3N9)	28
A/budgregriar/Hokkaido/1/77 (H4N6)	35
A/duck/Czechoslovachia/56(H4N6)	34
A/mountain hawk- eagle/Kumamoto/01/07 (H5N1)	29
A/whooper swan/Aomori/1/2008 (H5N1)	37
A/whooper swan/Aomori/2/2008 (H5N1)	36
A/duck/Vietnam/G12/2008 (H5N1)	38
A/chicken/Ibaraki/01/2005 (H5N2)	35
A/E.woodcock/Vietnam /WB8–9/01 (H5N2)	28
A/crow/Kyoto/T2/2004 (H5N1)	41
A/whistling swan/Shimane/499/83(H5N3)	35
A/whistling swan/Shimane/150/2002 (H5N3)	27
A/muscovy duck/Vietnam /G33-TC15&16/2007 (H6N2)	38
A/whistling swan/Shimane/35/80 (H6N3)	33
A/duck/Saga/62/2008 (H7N7)	33
A/quail/Aichi/1/2009 (H7N6)	40
A/whistling swan/Shimane/42/80 (H7N7)	34
A/teal/Tottori/130/2002 (H8N4)	35
A/duck/Hokkaido/26/99 (H9N2)	35
A/whistling swan/Shimane/148/2009 (H9N2)	33
A/chicken/Germany/N/49 (H10N7)	29
A/duck/England/1/56 (H11N6)	33
North American isolate	
A/mallard/NY/6750/78 (H2N2)	35
A/turkey/Massachusetts/65 (H6N2)	36
A/turkey/Ontario/6118/68 (H8N4)	35
A/turkey/Wisconsin/1/66 (H9N2)	35
A/duck/Memphis/546/74 (H11N9)	34
A/mallard duck/Alberta/60/76 (H12N5)	33
A/gull/Maryland/704/77 (H13N6)	31
Non avian influenza virus	
Infectious bronchitis virus strain Beaudette	negative
Influenza B virus strain B/Lee	negative
Newcastle disease virus strain HB-1	negative

Detection time is the average turbidity increment time of the twice replicated experiment.

reaction was carried out for 120 min. Some inhibitors or impurities may have delayed the detection time.

Detection of positive samples by RT-LAMP assay, without the use of a turbidimeter, can be achieved by visual observation of the white precipitate formed by magnesium pyrophosphate in the positive RT-LAMP reaction mixture [12, 13, 25]. Alternatively, RT-LAMP products in a reaction tube can be visualized under ultraviolet light by adding a fluorescence detection reagent to the reaction mixture [10]. In order to confirm whether the primers designed for RT-LAMP are feasible under conditions other than in a turbidimeter, 63°C isothermal conditions were maintained in a conventional water bath and the reaction was carried out for the same reaction time. When observed under a UV lamp at 365 nm, all avian influenza viruses were found to be fluorescent, in contrast to the negative control and influenza B virus strain B/Lee (data not shown). Thus, visual florescence detection assay is compatible with the real-time turbidity detection assay, suggesting that the LAMP system can be performed in a laboratory possessing a simple water bath and a UV light.

In the present study, we developed a rapid and highly sensitive diagnostic system based on RT-LAMP technology to detect the wide range of avian influenza viruses by targeting the matrix gene. Among the 4 sets of the primer, other than that prepared from the North American wild bird group could not detect all the influenza A viruses, or were very slow with regard to turbidity increment time (data not shown). All of the primer sets other than the North American wild bird group had two or more mixed nucleotides in the sequence, as it was not possible to make all primers fall on a conserved region, while in the case of the North American wild bird group primer set, all primers were in the conserved region of the virus sequences within that group.

A spacer comprising several thymidines has been inserted between F1c or B1c and F2 or B2 in other primers (FIP or BIP) [14, 15]. However, the spacer was not used in this study because that LAMP reaction progresses with inner primers lacking spacers, as reported elsewhere [9, 13].

We assessed the sensitivity of the designed primers in the RT-LAMP when detecting avian influenza virus in the tracheal and cloacal swabs, tissues extracts and fecal samples. RT-LAMP was able to detect the avian influenza virus in all specimens within 60 min, except for frozen the fecal sample of the A/whistling swan/Shimane/148/2009. The assay was

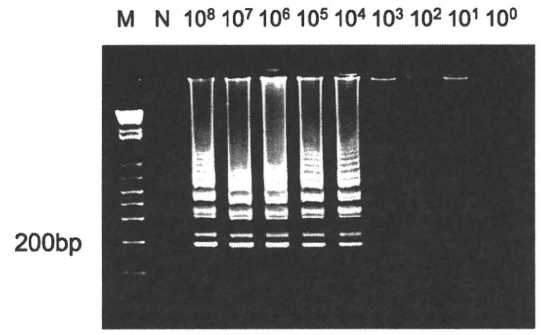

Fig. 1. Detection limit for RT-LAMP assay within a 60-min reaction period using A/chicken/Ibaraki/01/05 viral RNA at a concentration ranging from 10^8 to 10^0 EID_{50}/0.1 m*l*. RT-LAMP products (1 μl) were observed on a 2% agarose gel stained with ethidium bromide. M; DNA marker, NC; Negative control.

Table 3. Detection of avian influenza viruses in swab, tissue and fecal samples by RT-LAMP

Sample	Time of detection
A/mountain hawk-eagle/Kumamoto/1/2007	
Tracheal swab	39[a)]
Cloacal swab	36
Brain tissue	29
Tracheal tissue	39
Muscles	45
A/whooper swan/Aomori/1/2008	
Tracheal swab	50
A/whistling swan/ Shimane/148/09	
Fecal sample	67[b)]
A/duck/Saga/62/08	
Fecal sample	56

a) Time for positive detection (in min).
b) The reaction was carried out for 120 min.

able to detect the $10^{3.5}$ EID_{50} in tracheal swabs and $10^{0.5}$ EID_{50} in the case of muscle tissue extracts among A/mountain hawk-eagle/Kumamoto/01/07-infected tissue samples. As the duck fecal sample infected by A/duck/Saga/62/08 was successfully detected, even with 10-fold diluted RNA samples, the previous results may have been due to the presence of inhibitors, or as a result of some unknown factor that delays detection time. The A/whistling swan/Shimane/148/2009 fecal sample was confirmed to be positive when a separate reaction was carried out for 2 hr. Thus, for fecal samples, the reaction time could be increased in order to avoid the false negative cases.

In conclusion, we report a newly developed RT-LAMP assay with uniquely designed primers that is sensitive and specific to avian influenza viruses, extremely rapid, cost-effective and has potential usefulness in comprehensive avian influenza surveillance and diagnosis.

ACKNOWLEDGMENT(S). The first author was supported by the Monbukagakusho Scholarship of Japan for Ph.D. study, and all authors are grateful to the Ministry of Education, Culture, Sports, Science and Technology of Japan for financial support.

REFERENCES

1. Chen, H. T., Zhang, J., Sun, D. H., Ma, L. N., Liu, X. T., Cai, X. P. and Liu, Y. S. 2008. Development of reverse transcription loop-mediated isothermal amplification for rapid detection of H9 avian influenza virus. *J. Virol. Methods* **151**: 200–203.
2. Choi, Y. K., Goyal, S. M., Kang, S. W., Farnham, M. W. and Joo, H. S. 2002. Detection and subtyping of swine influenza H1N1, H1N2 and H3N2 viruses in clinical samples using two multiplex RT-PCR assays. *J. Virol. Methods* **102**: 53–59.
3. Collins, R. A., Ko, L. S., Fung, K. Y., Chan, K. Y., Xing, J., Lau, L. T. and Yu, A. C. 2003. Rapid and sensitive detection of avian influenza virus subtype H7 using NASBA. *Biochem. Biophys. Res. Commun.* **300**: 507–515.
4. Dawson, E. D., Moore, C. L., Dankbar, D. M., Mehlmann, M., Townsend, M. B., Smagala, J. A., Smith, C. B., Cox, N. J., Kuchta, R. D. and Rowlen, K. L. 2007. Identification of A/H5N1 influenza viruses using a single gene diagnostic microarray. *Anal. Chem.* **79**: 378–384.
5. Ellis, J. S. and Zambon, M. C. 2002. Molecular diagnosis of influenza. *Rev. Med. Virol.* **12**: 375–389.
6. Fouchier, R. A., Munster, V., Wallensten, A., Bestebroer, T. M., Herfst, S., Smith, D., Rimmelzwaan, G. F., Olsen, B. and Osterhaus, A. D. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79**: 2814–2822.
7. Gu, H., Qi, X., Li, X., Jiang, H., Wang, Y., Liu, F., Lu, S., Yang, Y. and Liu, F. 2009. Rapid and specific detection of H3 swine influenza virus using reverse transcription loop-mediated isothermal amplification method. *J. Appl. Microbiol.* (Epub ahead of print).
8. Hata, M., Tsuzuki, M., Goto, Y., Kumagai, N., Harada, M., Hashimoto, M., Tanaka, S., Sakae, K., Kimura, T., Minagawa, H. and Miyazaki, Y. 2007. High frequency of amantadine-resistant influenza A (H3N2) viruses in the 2005–2006 season and rapid detection of amantadine-resistant influenza A (H3N2) viruses by MAMA-PCR. *Jpn. J. Infect. Dis.* **60**: 202–204.
9. Hong, T. C., Mai, Q. L., Cuong, D. V., Parida, M., Minekawa, H., Notomi, T., Hasebe, F. and Morita, K. 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. *J. Clin. Microbiol.* **42**: 1956–1961.
10. Imai, M., Ninomiya, A., Minekawa, H., Notomi, T., Ishizaki, T., Tu, P. V., Tien, N. T., Tashiro, M. and Odagiri, T. 2007. Rapid diagnosis of H5N1 avian influenza virus infection by newly developed influenza H5 hemagglutinin gene-specific loop-mediated isothermal amplification method. *J. Virol. Methods* **141**: 173–180.
11. Lan, X., Yang, B., Li, B. Y., Yin, X. P., Li, X. R. and Liu, J. X. 2009. Reverse transcription-loop-mediated isothermal amplification assay for rapid detection of hepatitis E virus. *J. Clin. Microbiol.* **47**: 2304–2306.
12. Li, Q., Xue, C., Qin, J., Zhou, Q., Chen, F., Bi, Y. and Cao, Y. 2009. An improved reverse transcription loop-mediated isothermal amplification assay for sensitive and specific detection of Newcastle disease virus. *Arch. Virol.* **154**: 1433–1440.

13. Mori, Y., Hirano, T. and Notomi, T. 2006. Sequence specific visual detection of LAMP reactions by addition of cationic polymers. *BMC Biotechnol.* **6**: 3.
14. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* **28**: e63.
15. Parida, M., Posadas, G., Inoue, S., Hasebe, F. and Morita, K. 2004. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. *J. Clin. Microbiol.* **42**: 257–263.
16. Playford, E. G. and Dwyer, D. E. 2002. Laboratory diagnosis of influenza virus infection. *Pathology* **34**: 115–125.
17. Reed, L. J. and Muench, H. 1938. A simple method of estimating fifty percent end point. *Am. J. Hyg.* **27**: 493–497.
18. Shivappa, R. B., Savan, R., Kono, T., Sakai, M., Emmenegger, E., Kurath, G. and Levine, J. F. 2008. Detection of spring viraemia of carp virus (SVCV) by loop-mediated isothermal amplification (LAMP) in koi carp, Cyprinus carpio L. *J. Fish Dis.* **31**: 249–258.
19. Soliman, H. and El-Matbouli, M. 2006. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) for rapid detection of viral hemorrhagic septicaemia virus (VHS). *Vet. Microbiol.* **114**: 205–213.
20. Valette, M. and Aymard, M. 2002. Quality control assessment of influenza and RSV testing in Europe: 2000–01 season. *Euro. Surveill.* **7**: 161–165.
21. Webby, R. J., Webster, R. G. and Richt, J. A. 2007. Influenza viruses in animal wildlife populations. *Curr. Top. Microbiol. Immunol.* **315**: 67–83.
22. Webster, R. G. 1998. Influenza: an emerging disease. *Emerg. Infect. Dis.* **4**: 436–441.
23. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. and Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**: 152–179.
24. Wu, C., Cheng, X., He, J., Lv, X., Wang, J., Deng, R., Long, Q. and Wang, X. 2008. A multiplex real-time RT-PCR for detection and identification of influenza virus types A and B and subtypes H5 and N1. *J. Virol. Methods* **148**: 81–88.
25. Xu, J., Zhang, Z., Yin, Y., Cui, S., Xu, S., Guo, Y., Li, J., Wang, J., Liu, X. and Han, L. 2009. Development of reverse-transcription loop-mediated isothermal amplification for the detection of infectious bursal disease virus. *J. Virol. Methods* **162**: 267–271.