マイクロプレートハイブリダイゼーション（MPH）法

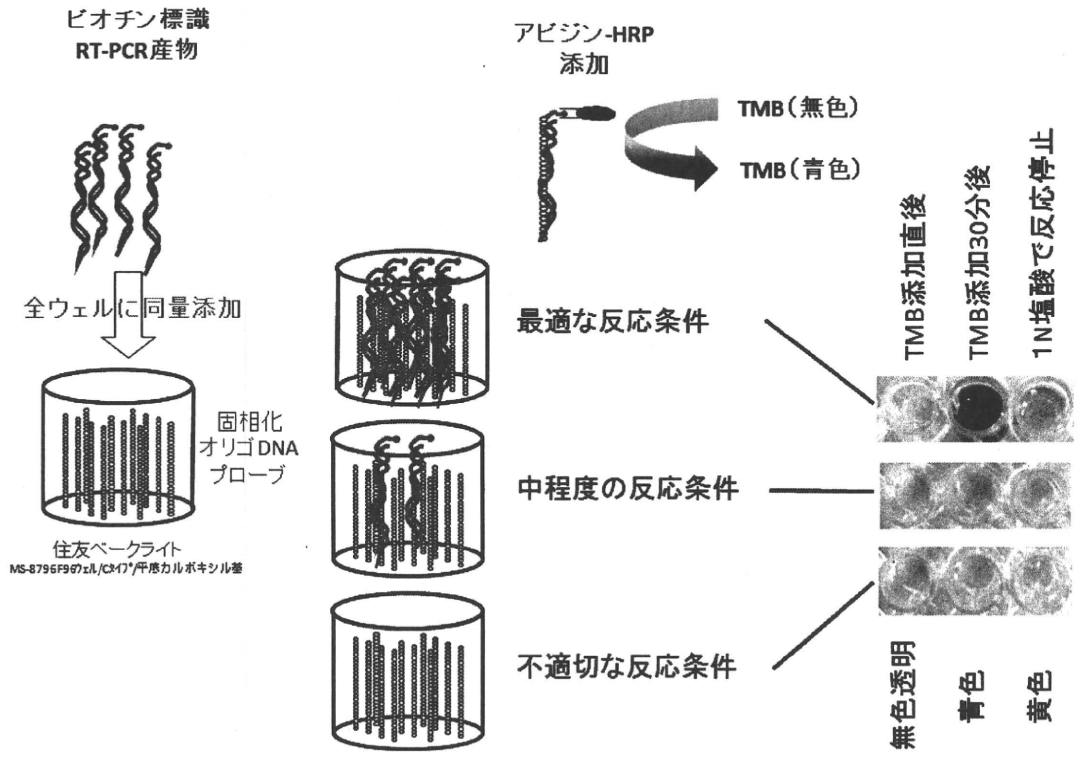


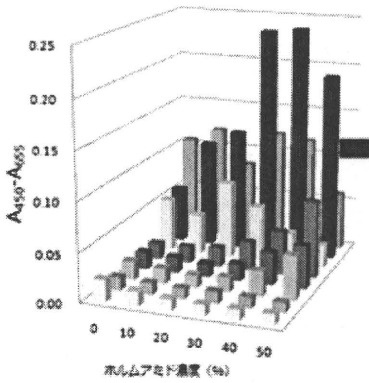
図 1.本研究で検証したマイクロプレートハイブリダイゼーション法の概要。ビオチン修飾プライマーを用いて増幅したインフルエンザウイルス RNA の RT-PCR 産物はホルムアミドを含む 5x SSC 緩衝液と混合し、固相化オリゴ DNA プローブと反応させる。相同しなかったまたは、余剰な RT-PCR 産物を洗浄し、アビジン HRP と反応させた後 TMB で発色させ、1N 塩酸で反応を停止させた。サンプルである RT-PCR 産物とオリゴ DNA プローブの配列が完全に相補し、反応条件が適切であれば発色基質（TMB）を添加後青く発色し、停止液で黄色く変色する。RT-PCR 産物とプローブが相補しなければ、TMB を添加しても発色しない。

A

		1	2	3	4	5	6	7	8	9	10	11	12
5xSSC + ホルムアミド濃度		0%		10%		20%		30%		40%		50%	
nH1 60mer probe mix	A												
nH1 50mer probe mix	B												
nH1 40mer probe mix	C												
nH1 25mer probe mix	D												
H1 25mer probe mix	E												
H5 25mer probe mix	F												
BSA	G												
H2O	H												

B

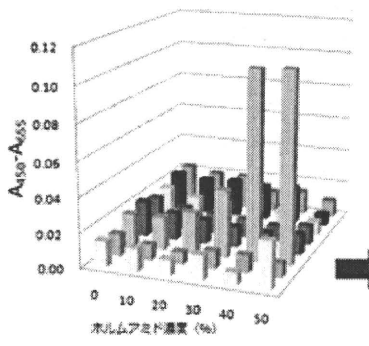
H1 Narita株
RT-PCR産物のハイブリダイゼーション



	A450-A655					
Formamide (%) + 5xSSC	0%	10%	20%	30%	40%	50%
nH1 60mer probe mix	0.11	0.12	0.08	0.12	0.12	0.06
nH1 50mer probe mix	0.06	0.11	0.13	0.24	0.24	0.20
nH1 40mer probe mix	0.05	0.04	0.08	0.06	0.03	0.02
nH1 25mer probe mix	0.02	0.02	0.02	0.03	0.05	0.08
H1 25mer probe mix	0.02	0.02	0.01	0.02	0.03	0.05
H5 25mer probe mix	0.02	0.02	0.01	0.02	0.03	0.05
BSA	0.01	0.01	0.01	0.01	0.01	0.01
H2O	0.02	0.02	0.01	0.01	0.01	0.01

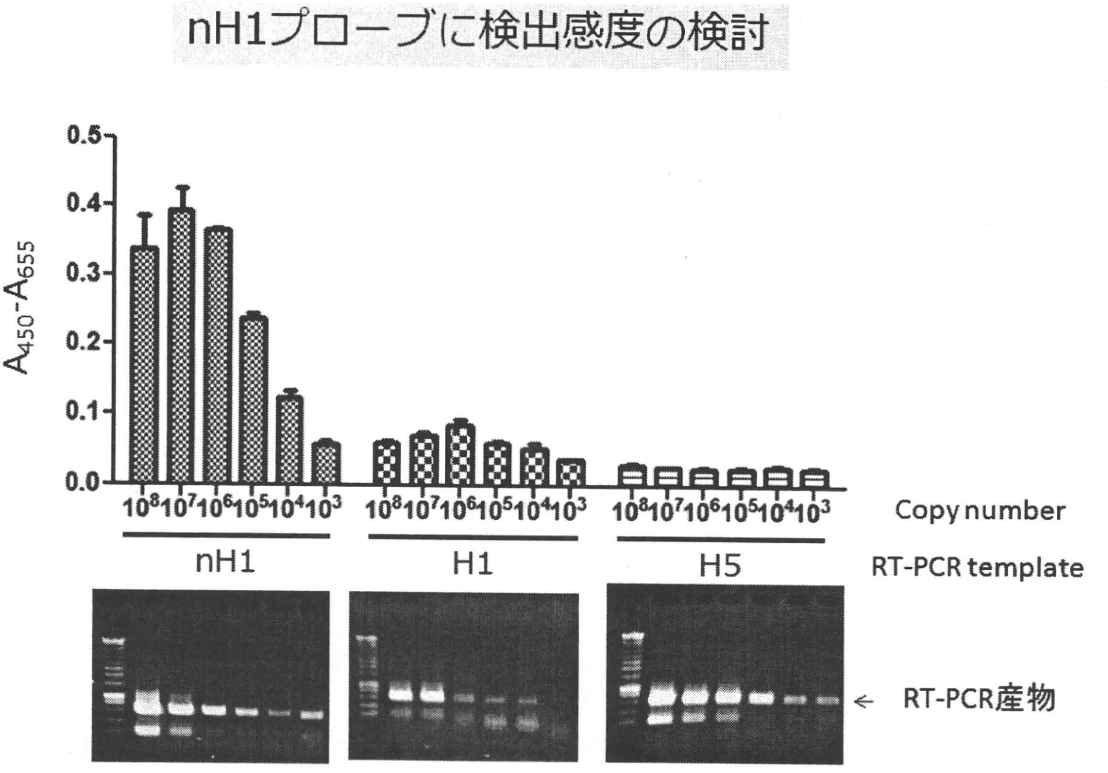
C

H5 Hyogo株
RT-PCR産物のハイブリダイゼーション



	A450-A655					
5xSSC + x% formamide	0%	10%	20%	30%	40%	50%
nH1 60mer probe mix	0.02	0.02	0.02	0.01	0.01	0.01
nH1 50mer probe mix	0.02	0.02	0.02	0.02	0.02	0.01
nH1 40mer probe mix	0.02	0.02	0.01	0.01	0.01	0.01
nH1 25mer probe mix	0.02	0.01	0.01	0.01	0.01	0.01
H1 25mer probe mix	0.02	0.02	0.01	0.01	0.01	0.01
H5 25mer probe mix	0.02	0.02	0.02	0.04	0.11	0.11
BSA	0.01	0.01	0.01	0.01	0.01	0.01
H2O	0.02	0.01	0.01	0.01	0.01	0.03

図 2.マイクロプレートハイブリダイゼーション法の基礎的条件検討の結果。(A) 固相化オリゴ DNA プローブと反応溶液中のホルムアミド濃度の 96 穴プレートレイアウト。様々な長さの H1 Narita 株に対するオリゴ DNA プローブ (nH1) を 12 穴分固相化した。また、H1 Narita 株以外を認識する汎 H1 および汎 H5 用プローブも別途固相化した。反応溶液の条件検討の際に用いたホルムアミド濃度は、2 連で反応させた。(B) H1 Narita 株の RT-PCR 産物を各種固相化プローブと反応させたときの吸光度。図に示した値は 2 穴分の平均値を示した。(C) H5 Hyogo 株の RT-PCR 産物を各種固相化プローブと反応させたときの吸光度。図に示した値は 2 穴分の平均値を示した。



10^4コピーまでMPH法にて検出できる可能性が示唆された。

図 3.マイクロプレートハイブリダイゼーション法の検出感度と特異性の検討。各濃度の H1 Narita 株（nH1）、H1 Brisbane 株（H1）、H5 Hyogo 株（H5）RNA を鋳型とし RT-PCR で増幅後、50mer の H1 Narita 株プローブと反応させた時の吸光度を棒グラフで示した。H1 Narita 株（nH1）の RT-PCR 産物をハイブリさせた場合、10^4 コピーのサンプルでも検出可能であった。一方、H1 Brisbane 株（H1）、H5 Hyogo 株（H5）の RT-PCR 産物をハイブリさせてもバックグランドレベルの吸光度しか得られなかった、このことから本研究で開発したオリゴ DNA プローブは、高感度・高特異性を有している可能性が示唆された。

厚生労働科学研究費補助金（食品の安心・安全確保推進研究事業）
分担研究報告書

と畜場に搬入された豚におけるA群ロタウイルスの感染状況及び簡便なウイルス全遺伝子解析法の確立とその応用

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研究要旨：食肉に供される家畜におけるA群ロタウイルスのリスクを把握するために、と畜場に搬入された豚における本ウイルスの排泄状況を解析した。また、前年度に引き続き簡便なロタウイルス遺伝子解析法の開発を進めた。食肉に供される健康な豚177例の9.6%から感染量として十分なA群ロタウイルス遺伝子の排泄を確認した。これらの遺伝子には人や豚に病原性を示すタイプも含まれており、感染源として直接的なリスクを示唆する結果となった。さらに、混合感染や分節遺伝子交換（リアソートメント）も観察され、直接的なリスクだけでなく、遺伝子の供給源としての間接的なリスクも示唆された。野外サンプルに直接適応できるロタウイルスの全分節遺伝子を解析する簡便法に改良を加え、前年度の検出率54.5%から81.8%と改善に成功した。今後、本法の疫学的調査への応用が期待できる。

A研究目的

A群ロタウイルスは、人を含め哺乳類および鳥類に感染し急性胃腸炎を起こし、種を越えての感染が報告されている。食用に供される家畜におけるロタウイルスの感染は人の健康障害のリスクと考えることが出来る。一方、多くの家畜から本ウイルスは分離・検出されているが、その自然界での生態は明らかにされていなかった。そこで、平成21年度の本研究において、食肉に供される家畜におけるA群ロタウイルスのリスクを把握するために、牛と豚における本ウイルスの感染環の解析を行った。その結果、健常な豚においてA群ロタウイルスの常在性が示唆されたことから、2県のと畜場に搬入された豚を材料に同ウイルスの検出を行い、排泄状況を把握し、食肉を介しての人へのリスクの評価を試みた。また、A群ロタウイルス流行の原因究明を図るため、前年度に引き続き糞便材料から直接的に多種の分節遺伝子解析を可能とする簡便な方法の改良を進めた。

B.研究方法

1. 2009年5月に群馬県及び2010年2月に名古屋市食肉衛生検査所に搬入された健康な豚それぞれ87例及び90例の計177例より採材された豚糞便サンプルについて、RT-PCR法によりA群ロタウイルスVP4遺伝子の検出を行った。これまで自然界での感染状況調査の際には、感度を上げるためSemi-nested RT-PCR法（検出限界0.5～1 ffu）を採用してきたが、今回は食肉からのリスクを評価するために感染源

として問題となる量の観点から、感度が低いRT-PCR法（検出限界 10^2～10^3 ffu）を採用した。陽性検体についてダイレクトシークエンスあるいはTAクローニングにより同ウイルスVP4及びVP7遺伝子の部分配列を決定し、P及びG遺伝子型を決定した。

2. 本プロジェクトで開発した簡便な分節遺伝子解析法であるRT-PCR法に改良を加え、RT-semi-nested PCR法の確立を行った。-80℃で保存されていたロタウイルス遺伝子陽性の牛糞便20%乳剤11例を用いて、VP4、VP7、NSP2、NSP3、NSP4及びNSP5の6つの分節を対象に簡便法を試みた。従来の方法でRT-PCRを行った後、得られたPCR産物を鋳型にしてsemi-nested PCRを行った。

C. 研究結果

1. と畜上に搬入された豚におけるロタウイルスの排泄状況

と畜場に搬入された177例中17例（9.6%）の豚糞便からVP4遺伝子が検出でき、地域に関係なく健常な豚で感染量として十分なA群ロタウイルスを排泄していることが確認された（表1）。VP4及びVP7遺伝子の解析から、G及びP遺伝子型の組み合わせが9通りあった（表2）。また、牛で検出されるG6遺伝子型に極めて近縁な同遺伝子型（AIP67株、図1、相同率99.8%）、同様に人ロタウイルスに近縁なG1遺伝子型（AIP21株、図2、相同率94.8%）のA群ロタウイルスも健常豚より検出された。17例中2例において、ダイレクトシークエンスで二重の波形が、TAクローニングにより異なるクローンが見出され、異なった遺伝子型ウイルスの混合感染が確認できた。さらに、混合感染の結果起こる遺伝子分節の組換え（リアソートメント）を示すサンプルも複数株見出された（図3）。

2.全分節遺伝子の簡便解析法の糞便サンプルへの応用と改良

従来のRT-PCR法で、11例の糞便サンプルを用いてVP4、VP7、NSP2、NSP3、NSP4及びNSP5の6つの分節遺伝子の増幅を試みたところ、延べ66遺伝子中36（54.5%）が増幅でき、6つの全ての遺伝子が増幅できたのは11例中4例であった（表3）。一方、新たにプライマーを設計し、これまでの方法で得られたPCR産物を鋳型にしてsemi-nested PCRを行った結果、66遺伝子中56（84.5%）の増幅が可能となった（表4）。結果として、11例中5例で6つの遺伝子を増幅することができ、これらの塩基配列を解読することにより、遺伝子型を決定することができた。VP7遺伝子分節は、全てのサンプルで増幅可能となり、少なくとも主要な遺伝子型であるP及びG遺伝子型の決定は可能となった。

D.考察

前年度、Nested RT-PCR法により健康な豚169例からA群ロタウイルスのVP4遺伝子を検出したところ、84例（49.7%）が陽性となり、豚では、本ウイルスが常在している可能性が考えられた。そこで、実際の食肉を介しての感染リスクを解析するために、と畜場に搬入された豚における本ウイルスの調査を実施した。これまで、本ウイルスの感染環を解明するために、感度の高いsemi-nested RT-PCR法を採用し、解析を行ってきた。本法は、計算上0.5～1粒子のウイルス遺伝子の検出が可能であることが予備的実験から明らかとなっている。一方、実際の本ウイルスの感染には100粒子程度が必要と考えられている。食肉でのリスクを過剰に評価しないためには、感度の低い検出法が必要である。そこで、今回は$10^{2\sim3}$のウイルス粒子の遺伝子が検出可能な通常のRT-PCR法によりウイルス遺伝子の検出を行った。その結果、前年度の検出率40.2%には及ばな

いものの、約1割の豚より本ウイルス遺伝子が検出され、食肉でのリスクが示唆された。さらに、遺伝子解析により人や牛で下痢を起こすタイプの遺伝子型が検出され、混合感染やリアソートメントが活発に起きている様相も明らかとなった。以上より、ロタウイルス感染症の感染源あるいは遺伝子の供給源として豚がヒトへのリスクとなると考えられた。

近年、A群ロタウイルスの疫学的研究には、全11分節のゲノムを解析する必要があると考えられている。全ゲノムを解析することは煩雑であり、簡便な方法が求められている。昨年度は、開発した簡便法を直接糞便サンプルに応用したが、54.5%と低い検出率であった。今年度はsemi-nested RT-PCR法を採用することにより80%以上の検出率となり、大幅に改善することができた。完全ではないものの高率で各分節遺伝子型の同定が可能になったことから、本法の疫学的調査への応用が期待できる。

E.結論

食肉に供される健康な豚の約 1 割から感染量として十分な A 群ロタウイルス遺伝子の排泄を確認した。これらの遺伝子には人や豚に病原性を示すタイプも含まれており、感染源として直接的なリスクを示唆する結果となった。さらに、混合感染や分節遺伝子交換（リアソートメント）も観察され、直接的なリスクだけでなく、遺伝子の供給源としての間接的なリスクも示唆された。野外サンプルに直接適応できるロタウイルスの全分節遺伝子を解析する簡便法に改良を加え、検出率の改善に成功した。今後、本法の疫学的調査への応用が期待できる。

F. 健康危険情報

なし

G. 研究発表

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2) 岡寺康太、安部昌子、伊藤直人、杉山　誠：と畜場に搬入された豚における A 群ロタウイルスの検出およびその遺伝学的解析. 第 150 回日本獣医学会学術集会（2010 年 9 月、帯広）

3) 安部昌子、伊藤直人、正谷達謄、中川敬介、山岡理子、杉山　誠：A 群ロタウイルスの感染環における野生イノシシの関与. 第 58 回日本ウイルス学会学術集会（2010 年 11 月、徳島）

H. 知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

表 1. と畜場に搬入された豚における RT-PCR 法によるロタウイルス遺伝子の検出

糞便採取地	由来地域	例数（農家数）	検出例数（農家数）	検出率(%)
名古屋市食肉衛生検査所	愛知県	55 (11)	8 (5)	14.5
	岐阜県	15 (3)	2 (1)	13.3
	三重県	15 (3)	2 (1)	13.3
	静岡県	5 (1)	1 (1)	20.0
	計	90 (18)	13 (8)	14.4
群馬県食肉衛生検査所	地域 A	20	0	0
	地域 B	12	0	0
	地域 C	9	1	11.1
	地域 D	2	0	0
	地域 E	44	3	6.8
	計	87	4	4.6
計		177	17	9.6

表 2. 健常豚より検出されたロタウイルスの G/P 遺伝子型

	G1	G2	G3	G4	G5	G6	計
P[7]	2						2
P[13]			3	3	3	1	10
P[27]		3		1		1	5
P[34]		2					2
計	2	5	3	4	3	2	19

表 3. 糞便サンプルを用いた簡便法による各分節遺伝子型別（RT-PCR 法）

サンプル	VP4	VP7	NSP2	NSP3	NSP4	NSP5
GB1-76	P11	G10	N2	T9	E2	H3
GB2-6	P29	—	—	—	—	—
GB9-3	P29	G21	N2	T9	E2	H3
GB11-3	P29	G21	—	—	—	—
GB12-4	P11	G10	—	—	—	—
GB12-22	P11	G8	N2	T6	E2	H3
GB14-45	P11	G6	N2	T6	E2	H3
GB15-35	P11	—	N2	—	E2	H3
GB16-44	P29	—	—	—	—	—
GB18-10	P29	—	—	—	—	—
GB20-40	P29	—	—	—	—	—

表 4. 糞便サンプルを用いた簡便法による各分節遺伝子型別（semi-nested RT-PCR 法）

サンプル	VP4	VP7	NSP2	NSP3	NSP4	NSP5
GB1-76	P11	G10	N2	T9	E2	H3
GB2-6	P29	G21	—	—	E2	H3
GB9-3	P29	G21	N2	T9	E2	H3
GB11-3	P29	G21	—	T9	E2	—
GB12-4	P11	G10	N2	T6	E2	—
GB12-22	P11	G8	N2	T6	E2	H3
GB14-45	P11	G6	N2	T6	E2	H3
GB15-35	P11	G8	N2	T6	E2	H3
GB16-44	P29	G21	—	T9	E2	H3
GB18-10	P29	G21	N2	—	—	—
GB20-40	P29	G8	N2	T6	E2	—

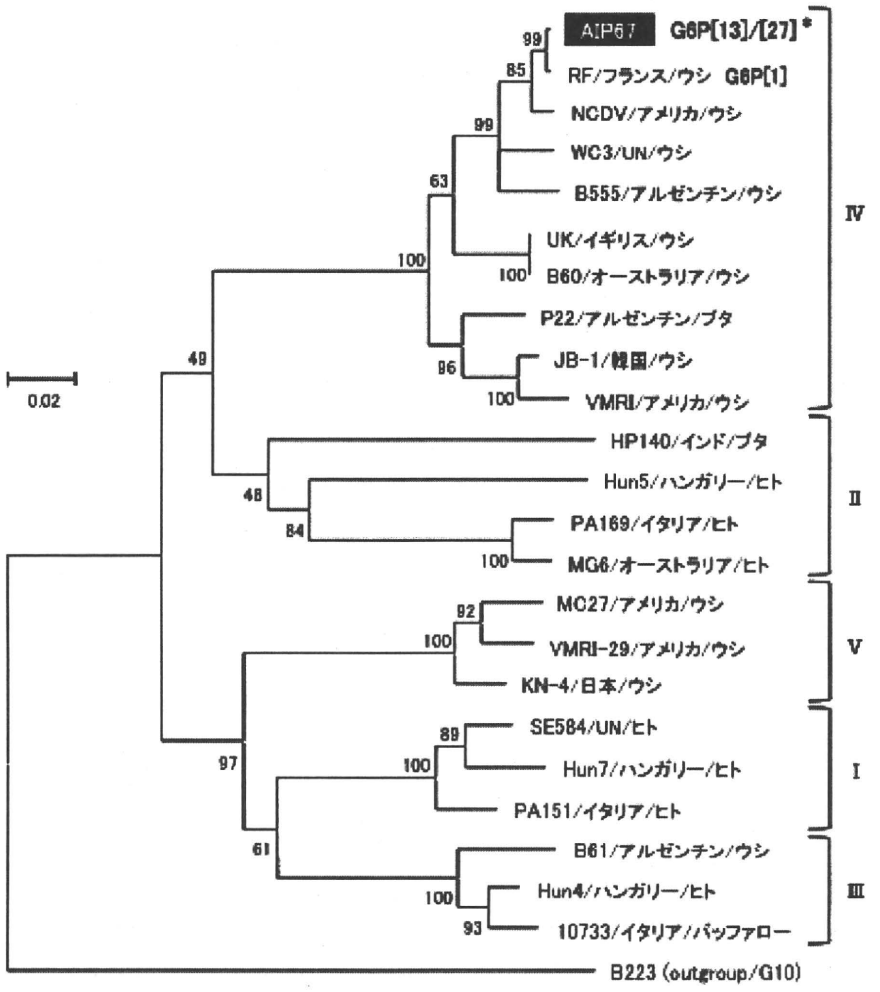

図１. G6 遺伝子型ロタウイルスの VP7 遺伝子部分塩基配列に基づいた系統解析

解析領域：NCDV 株 nt 81-894 (814 bp)

UN ： 検出国不明

*TA クローニングにより２株（VP4 遺伝子）の混在を確認

解析に用いた株の accession number を下に示す。

RF (X65940), NCDV (M12394), WC3 (AY050272), B555 (DQ631820), UK (X00896), B60 (M64680), P22 (EF474079), JB-1 (AY596798), VMRI (U53924), HP140 (DQ003293), Hun5 (EF554109), PA169 (EF554131), MG6 (EF554098), MC27 (AF162435), VMRI-29 (U50332), KN-4 (D12710), SE584 (EF672609), Hun7 (AJ488134), PA151 (L20881), B61 (DQ637812), Hun4 (AJ487833), 10733 (AY281360), B223 (X57852)

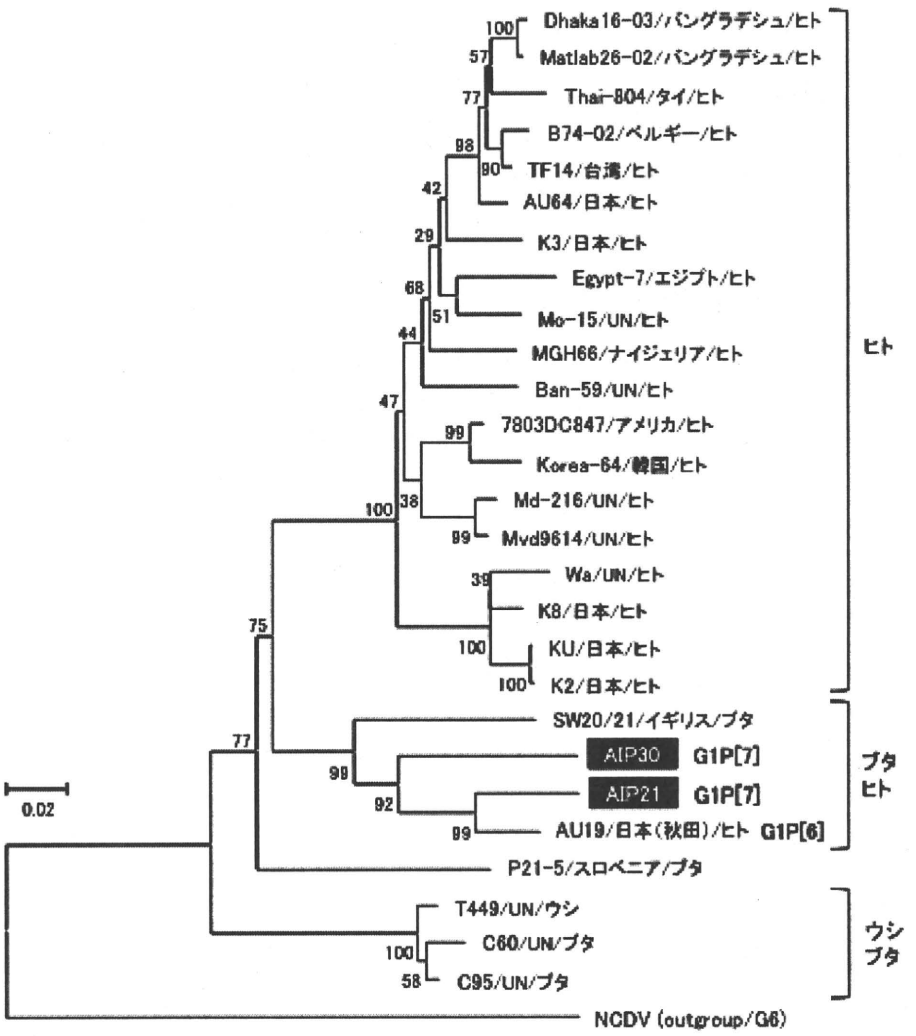

図 2. G1 遺伝子型ロタウイルスの VP7 遺伝子部分塩基配列に基づいた系統解析

解析領域：NCDV 株 nt 141-888 (748 bp)

UN ： 検出国不明

解析に用いた株の accession number を下に示す。

Dhaka16-03 (DQ492674), Matlab26-02 (AY631054), Thai-804 (DQ512979), B74-02 (AY635048), TF14 (AF183860), AU64 (AB081801), K3 (D16317), Egypt-7 (U26373), Mo-15 (U26382), MGH66 (Y08033), Ban-59 (U26366), 7803DC847 (GU723354), Korea-64 (U26378), Md-216 (U26380), Mvd9614 (AF480296), Wa (K02033), K8 (D16344), KU (AB222787), K2 (D16323), SW20/21 (AF426162), AU19 (AB018697), P21-5 (DQ629928), T449 (M92651), C60 (L24164), C95 (L24165), NCDV (M12394)

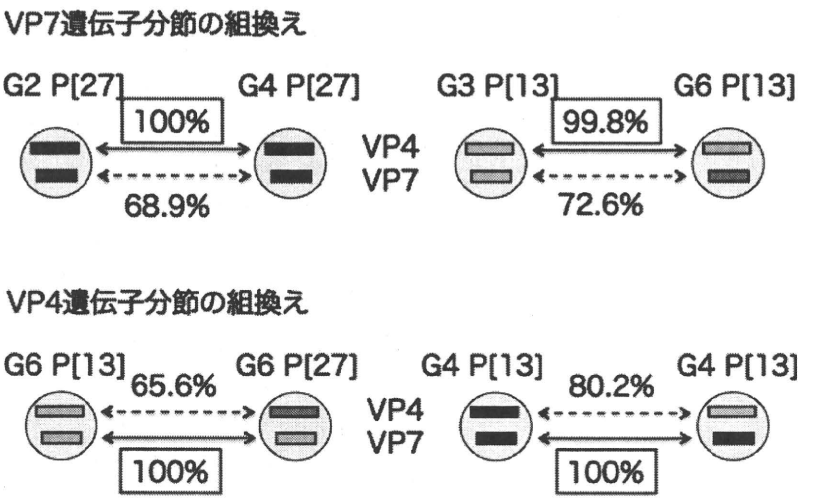

図 3. 健常豚においてみられた遺伝子分節の組換え（リアソートメント）

Ⅲ. 研究成果の刊行に関する一覧表

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-mediated 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

Ⅳ. 研究成果の刊行物・別刷

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

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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

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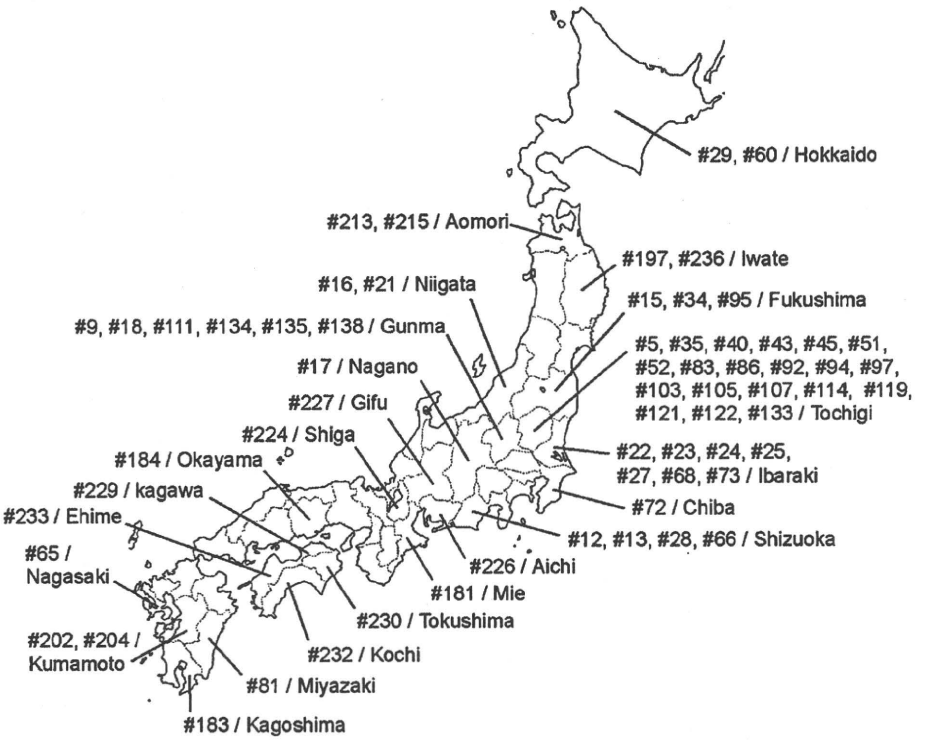

Fig. 1. Raising places of cattle examined in this study. The animals harboring #60 and #81 were raised for 3 years in Hokkaido and 1 year in Miyazaki, followed by 2.5- and 5-year raising in Tochigi, respectively. The remaining animals were raised in a single prefecture.

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).

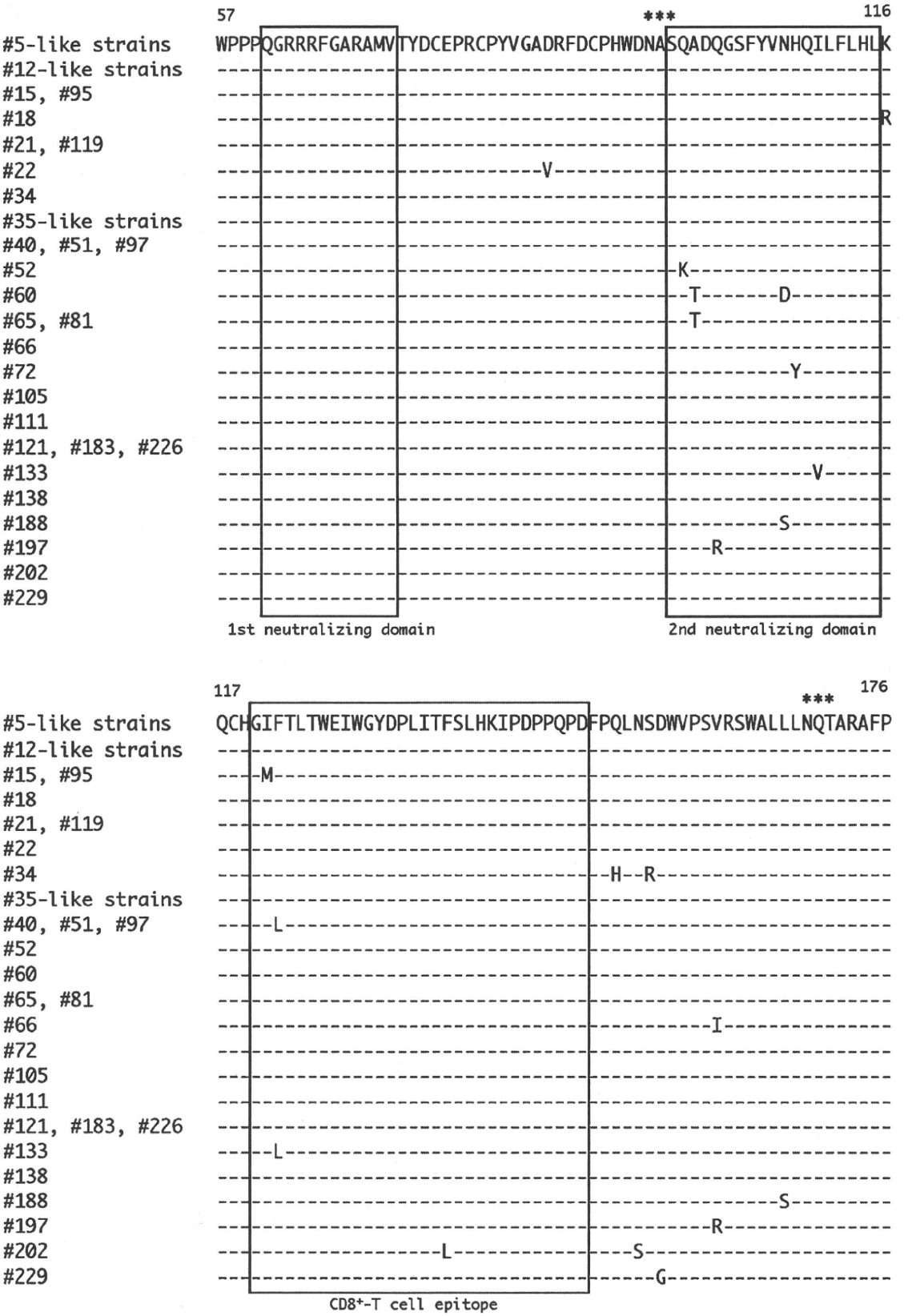

Fig. 3. Amino acid sequence alignment of partial sequences of BLV gp51 glycoprotein. The amino acid sequences were deduced from the *env* gene fragment of 64 isolates in this study. The first and second neutralizing domains (Callebaut et al., 1993) and $CD8^+$-T cell epitope (Gatei et al., 1993) of the 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

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