法である ELISA を組み合わせる事で、迅速、安全かつ詳細なハンタウイルス血清診断システムが構築出来ると考えられる。これは公衆衛生上重要な情報を迅速に収集できることを示すものである。

疫学的調査からは東南アジア、南アジア、東アジアにおいてそれぞれ固有 SEOV および THAIV 関連ウイルスがあり、これら宿主である、ドブネズミ、クマネズミ、bandicoot 類が重要であることが示唆された。また、これらのラットがハンタウイルスのみならず他の病原体を同時に媒介していることも明らかとなった。しかしながら未だに限定された情報しか得られていないことから、さらなる情報の蓄積が必要であると考えられた。

E. 結論

近年、我が国でのハンタウイルス感染例の報告はないが、近隣諸国や世界的にもハンタウイルスは流行しており、また新しいウイルスも発見されている。今の所これらの新しいウイルスは病原性が不明なものも多いが、輸入症例の出現する可能性には常に配慮する必要がある。とくに、南北アメリカ大陸で流行している HPS は経過が早くかつ重症になりやすいため、早期診断が求められている。このような状況のなかで、本研究で示した各種診断抗原やスクリーニングおよび鑑別診断用抗原の開発は、本疾患に対する公衆衛生対策上必要であると考えられる。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

1) Luan VD, Yoshimatsu K, Endo R, Taruishi M, Huong VT, Dat DT, Tien PC, Shimizu K, Koma T, Yasuda SP, Nhi L, Arikawa J. Studies on hantavirus infection in small mammals captured in southern and central highland area of Vietnam. J Vet Med Sci 74(9): 1155-1162, 2012

2) Koma T, Yoshimatsu K, Yasuda SP, Li T, Amada T, Shimizu K, Isozumi R, Mai LT, Hoa NT, Nguyen V, Yamashiro T, Hasebe F, Arikawa J. A survey of rodent-borne pathogens carried by wild Rattus spp. in Northern Vietnam. Epidemiol Infect: 1-9, Epub ahead of print

3) Koma T, Yoshimatsu K, Taruishi M, Miyashita D, Endo R, Shimizu K, Yasuda PS, Amada T, Seto T, Murata R, Yoshida H, Kariwa H, Takashima I, Arikawa J. Development of a serotyping enzyme-linked immunosorbent assay system based on recombinant truncated hantavirus nucleocapsid proteins for New World Hantavirus infection. J Virol Mehods 185(1): 74–81, 2012

4) Schlegel M, Tegshduuren E, Yoshimatsu K, Petraityte R, Sasnauskas K, Hammerschmidt B, Friedrich R, Mertens M, Groschup MH, Arai S, Endo R, Shimizu K, Koma T, Yasuda S, Ishihara C, Ulrich RG, Arikawa J, Kollner B. Novel serological tools for detection of Thottapalayam virus, a Soricomorpha-borne hantavirus. Arch Virol 157(11): 2179-2187, 2012

2. 学会発表

1) Amada T, Yoshimatsu K, Yasuda SP, Koma T, Shimizu K, Isozumi R, Hayashimoto N, Takakura A, Arikawa J. Development of immunochromatographic test strips for the detection of HFRS and HPS hantavirus antibody in the human and rodent serum. The 9th Japan-China International Conference of Virology, Sapporo, Japan. June 12-13, 2012.
2) Amada T, Hayashimoto N, Yoshimatsu K, Yasuda S, Shimizu K, Koma T, Isozumi R, Takakura A, Arikawa J. Development of immunochromatographic test for the detection of HFRS and HPS Hantavirus antibody in the human and rodent serum. XVIII International Congress for Tropical Medicine and Malaria, Rio de Janeiro, Brazil. September 23-27, 2012.
3) Amada T, Yoshimatsu K, Yasuda SP, Koma T, Shimizu K, Nishio S, Hayashimoto N, Takakura A, Arikawa J. Rapid and whole blood diagnostic test for detecting anti-hantavirus antibody in human and rodent. The 5th Asian Federation of Laboratory Animal Science Associations Congress, Bangkok, Thailand. October 10 -12, 2012.
4) 有川二郎, 天田貴子, 林元展人, 吉松組子, 安田俊平, 清水健太, 駒貴明, 五十棲理恵, 高倉彰. イムノクロマト法によるラットとヒト血清のハンタウイルス抗体迅速検出法の開発. 日本実験動物科学・技術九州 2012, 別府市. 2012 年 5 月 24 日－26 日
5) 天田 貴子, 吉松 組子, 安田 俊平, 清水 健太, 駒 貴明, 林元 展人, 高倉 彰, 有川 二郎. イムノクロマト法による抗ハンタウイルス抗体の迅速抗体検出法の開発. 第 60 回日本ウイルス学会学術集会, 大阪市. 2012 年 11 月 13 日－15 日(一般演題・ポスター)

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

厚生労働科学研究費補助金(地球規模保健課題推進研究事業(国際医学協力研究事業))
分担研究報告書

ウイルス性出血熱や新興ウイルス感染症の診断法の開発に関する研究

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研究要旨

2006 年に中国広西自治区のサル繁殖施設(31,260 頭)でアカゲザル 10,000 頭が発症し4,250 頭死亡(2006 年)する事例があり、イヌディステンパーウイルス(CDV)が分離同定された。その後、中国で複数回の CDV によるサルの致死的感染症流行が報告されている。国内でもカニクイザルに致死的 CDV 感染症が報告されている。本研究では、流行時のサルの血清学的解析、病理学的解析等により、流行の全容を明らかにした。

研究協力者: 酒井宏治、永田典代、竹田誠、西條政幸(国立感染症研究所)

A. 研究目的

CDV はモルビリウイルス属に分類されヒトの麻疹ウイルスに近縁なウイルスである。麻疹ウイルスは、ヒト及び霊長類に感染発症させるが、CDV はイヌ及びその他の肉食獣を宿主とする。これは、それぞれのウイルスが宿主動物やヒトのレセプターに適応していることが原因の一つであると考えられる。本研究ではCDVによる霊長類の致死的感染症に関して全容を明らかにすることを目的とする。

B. 研究方法

カニクイザルコロニーで発生した CDV 感染症の剖検例の病理学的解析を行った。臓器、組織は 10%ホルマリン緩衝液固定後、パラフィン包埋切片を用いた組織検索と免疫組織化学法によるウイルス抗原の検索を実施した。免疫組織化学法には CDV NP に対するモノクローナル抗体(CDV-NP, VMRD Inc)を用いた。

また、サル血清中の抗体応答を ELISA により行った。重症例のケモカイン・サイトカイン反応を human cytokine 25-plex antibody bead kit (Invitrogen)を用いて Luminex100 により解析した。

(倫理面への配慮)

人の検体は用いていない。サルは導入時検疫中であり、動物検疫官の指示により適切に処置されたものを分与された。国立感染症研究所での動物実験には該当しない。

C. 研究結果

1)CDV 感染により重症化したサルの病理学的解析:

重症サル 2 頭(#1, #2 とする)の病理組織学的解析から以下の所見が得られた。#1 は、全身性CDV 感染症を呈し感染は広範囲で重症であった。巨細胞性肺炎、リンパ球脱落による免疫能低下、全身の皮膚における巨細胞形成による痂皮化、ウイルス血症、中枢神経系における巨細胞形成のグリオーシスおよび脱髄病変がみられた(図 1)。唾液腺、腎、表皮、腸管、

脈絡叢、血管内皮、リンパ球にウイルス抗原がみられた。一方#2も同様に全身性CDV感染症を呈していたが、リンパ球の脱落が#1と比較して軽度であった。しかしウイルスは#1と同様殆どの体液、排泄物に含まれているため、感染源となり得ると考えられた。いずれの症例でも、リンパ球系、上皮系細胞へのCDV感染が認められたことから、流行時のCDVは、少なくともサルのリンパ球レセプターであるSLAM及び上皮系レセプターであるnectin4を介した感染を効率よく起こしていると考えられた。#1では、ニューロンへの感染も確認されている。

2)CDV感染により重症化したサルのケモカイン・サイトカイン反応:

上記2頭に加え安楽殺された1頭のサルの血清中のケモカイン・サイトカインを解析した結果、proinflammatory cytokines と chemokines (IL-1 β, IL-6, macrophage MIP-1 α, MIP-1 β, MCP-1, eotaxin)の有意な上昇が認められた。また、proinflammatory cytokines associated with T cell activation (IFN-γ, IL-15) 及び anti-inflammatory responses of IL-1 receptor antagonist (IL-1ra)が上昇していた(表1)。

3)流行時のサルの血清抗体:

重症例の3頭の血清中のCDV抗体は陰性であった。一方、臨床的に発症していないサルの血清抗体を調べた結果、多くのサルがCDV抗体陽性であり麻疹ウイルス抗体陽性は殆どいなかった。このことから、発症していないサルも殆どがCDVに感染していたと考えられる。このため、最終的に流行は終息したと考えられる。

D. 考察

モルビリウイルス属に分類されるCDVや麻疹ウイルスは互いに近縁なウイルスである。しかし、麻疹ウイルスは、ヒト及び霊長類に感染発症させるが、CDVはイヌ及びその他の肉食獣を宿主とする。これは、それぞれのウイルスが宿主動物やヒトのレセプターに適応していることが原因の一つであると考えられる。

モルビリウイルスは、まずリンパ球上のSLAMをレセプターとして感染し、感染リンパ球によるウイルス血症を起こす。感染リンパ球から毛細血管内皮を通して上皮細胞に感染し全身感染を起こす。上皮細胞上のレセプターは昨年nectin4であることが明らかにされた。サルの感染症の原因となったCDV株がサルのSLAM, nectin4を有効に使って感染するかを解析した結果、本来の宿主であるイヌのSLAM, nectin4と同程度の効率でレセプターとして機能していることが分かっている。イヌから分離されたCDVと比較してサルから分離されたCDVは、N, P, F, H, L遺伝子に変異が認められる。特にFとH遺伝子にアミノ酸置換を伴う変異が多い。このため、本来のイヌを宿主とするCDVから何らかの変異導入がサルへの馴化に寄与していると考えられる。どの様な変異がサルへの馴化に寄与しているかは今後の解析が必要である。いずれにしても、このCDVのサルのレセプターの効率良い利用によりサルからサルへの効率良いウイルス伝播がおきたと考えられる。病原性獲得に関しては、レセプターの利用とは別の変異が関与しているかは不明である。

一方、このウイルスはヒトのSLAMは有効に利用できなかった。ヒトとサルのSLAMはアミノ酸配列が非常に良く一致することから、麻疹ウイルスに共通に感受性があるが、このCDV株が何故ヒトのSLAMを有効に利用できないかは、今後の解析が必要である。しかし、このCDVによるヒトへの感染リスクは、このままでは高くないと考えられる。今後、ヒトのSLAMを効率よく利用できる変異が容易に生じるのかを明らかにしたい。

E. 結論

本来、イヌなどの肉食動物を宿主とするCDV が霊長類に大規模な致死的感染症を起こした。この CDV は、サルのレセプターを有効に利用できることが、サルからサルへの伝播が容易におきた原因であると考えられた。霊長類への病原性獲得に関しては、レセプターの利用とは別のウイルス遺伝子内の変異が関与しているかは不明である。

G. 研究発表

1. 論文発表

1. 論文発表

1) Sakai K, Nagata N, Ami Y, Seki F, Suzaki Y, Iwata-Yoshikawa N, Suzuki T, Fukushi S, Mizutani T, Yoshikawa T, Otsuki N, Kurane I, Komase K, Yamaguchi R, Hasegawa H, Saijo M, Takeda M, Morikawa S. Lethal Canine Distemper Virus Outbreak in Cynomolgus Monkeys in Japan in 2008. J Virol 87(2): 1105-1114, 2013

2) Sharma N, Hotta A, Yamamoto Y, Fujita O, Uda A, Morikawa S, Yamada A, Tanabayashia K. Detection of *Francisella tularensis*-specific antibodies in patients with tularemia using a novel competitive enzyme-linked immunosorbent assay. Clin Vaccine Immunol, *in press*

3) Taniguchi S, Sayama Y, Nagata N, Ikegami T, Miranda ME, Watanabe S, Iizuka I, Fukushi S, Mizutani T, Ishii Y, Saijo M, Akashi H, Yoshikawa Y, Kyuwa S, Morikawa S. Analysis of the humoral immune responses among cynomolgus macaque naturally infected with Reston virus during the 1996 outbreak in the Philippines. BMC Vet Res 8(1):189, 2012

4) Fukushi S, Tani H, Yoshikawa T, Saijo M, Morikawa S. Serological assays based on recombinant viral proteins for the diagnosis of arenavirus hemorrhagic fevers. Viruses 4(10):2097-2114, 2012 (special issue: Arenaviruses)

5) Katano H, Sato S, Sekizuka T, Kinumaki A, Fukumoto H, Sato Y, Hasegawa H, Morikawa S, Saijo M, Mizutani T, Kuroda M. Pathogenic characterization of a cervical lymph node derived from a patient with Kawasaki disease. Int J Clin Exp Pathol 5(8):814-823, 2012

6) Sayama Y, Demetria C, Saito M, Azul RR, Taniguchi S, Fukushi S, Yoshikawa T, Iizuka I, Mizutani T, Kurane I, Malbas FF Jr, Lupisan S, Catbagan DP, Animas SB, Morales RG, Lopez EL, Dazo KR, Cruz MS, Olveda R, Saijo M, Oshitani H, Morikawa S. A seroepidemiologic study of Reston ebolavirus in swine in the Philippines. BMC Vet Res 18(8):82, 2012

7) Lihoradova O, Kalveram B, Indran SV, Lokugamage N, Juelich TL, Hill TE, Tseng CT, Gong B, Fukushi S, Morikawa S, Freiberg AN, Ikegami T. The dominant-negative inhibition of double-stranded RNA-dependent protein kinase PKR increases the efficacy of Rift Valley fever virus MP-12 vaccine. J Virol 86(14):7650-7661, 2012

8) Fukushi S, Nakauchi M, Mizutani T, Saijo M, Kurane I, Morikawa S. Antigen-capture ELISA for the detection of Rift Valley fever virus nucleoprotein using new monoclonal antibodies. J Virol Methods 180(1-2):68-74, 2012

9) Arai S, Gu SH, Baek LJ, Tabara K, Bennett SN, Oh HS, Takada N, Kang HJ, Tanaka-Taya K, Morikawa S, Okabe N, Yanagihara R, Song JW. Divergent ancestral lineages of newfound hantaviruses harbored by phylogenetically related crocidurine shrew species in Korea. Virology. 424(2):99-105, 2012

10) Kennedy JS, Gurwith M, Dekker CL, Frey SE, Edwards KM, Kenner J, Lock M, Empig C, Morikawa S, Saijo M, Yokote H, Karem K, Damon I, Perlroth M, and Greenberg RN. Safety and Immunogenicity of LC16m8, an Attenuated Smallpox Vaccine in Vaccinia-Naive Adults. J Inf Dis 204(9):1395-1402, 2012

11) Shirato K, Maeda K, Tsuda S, Suzuki K, Watanabe S, Shimoda H, Ueda N, Iha K, Taniguchi S, Kyuwa S, Endoh D, Matsuyama S, Kurane I, Saijo M, Morikawa S, Yoshikawa Y, Akashi H, Mizutani T. Detection of bat coronaviruses from Miniopterus fuliginosus in Japan. Virus Genes 44(1):40-44, 2012

H. 知的財産権の出願・登録状況

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

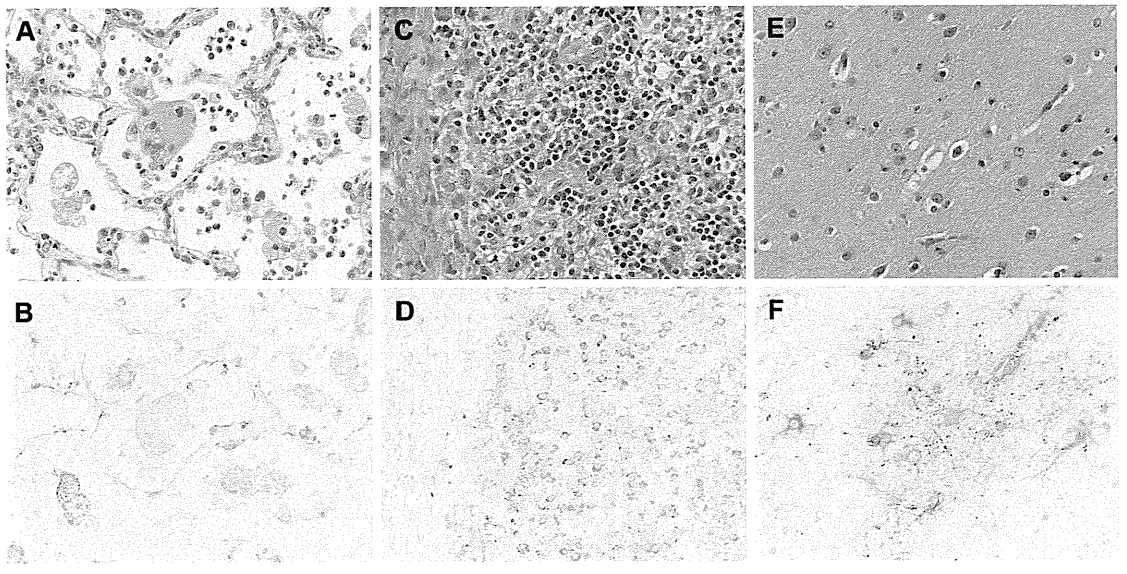
該当なし

表１．重症サルの血中サイトカイン・ケモカイン

	monkeys naturally infected with CDV			normal monkeys				
Cytokine	Median (pg/ml)	Standard deviation	No. positive/ no. tested	Median (pg/ml)	Standard deviation	No. positive/ no. tested	*P* value	
IL-1β	120	66	2/3	<17		0/8	0.023	*
IL-6	94	72	3/3	<9		0/8	0.011	*
MIP-1α	228	78	3/3	84	48	8/8	0.000	**
MIP-1β	157	61	3/3	40	36	7/8	0.001	**
MCP-1	3,917	2,286	3/3	461	245	8/8	0.043	*
Eotaxin	2,121	1,096	3/3	413	203	8/8	0.023	*
IFN-γ	509	150	3/3	261	183	3/8	0.002	*
IL-15	89	65	2/3	64	23	1/8	0.115	

Asterisks indicate statistically significant differences between monkeys naturally infected with CDV and normal monkeys (* $p < 0.05$, ** $p < 0.005$)

図 1．#1 サルの病理組織学的解析



上段の HE 染色では、肺胞上皮のシンシチウム形成(A)、リンパ節のリンパ球脱落(C)、大脳へのミクログリアの軽度浸潤(E)が認められる。下段の免疫組織染色では、肺胞上皮(B)、リンパ球(D)、ニューロンとグリア(F)に CDV 抗原が認められる。

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	発表誌名	巻号	ページ等	出版年
Okamoto K, Kinoshita H, Parquet Mdel C, Raekiansyah M, Kimura D, Yui K, Islam MK, Hasebe F, Morita K.	Dengue virus strain DEN2 16681 utilizes a specific glycochain of syndecan-2 proteoglycan as a receptor.	J Gen Virol	93(4)	761-770	2012
Furuta T, Murao LA, Lan NTP, Huy NT, Huong VTQ, Thuy TT, Tham VD, Nga CTP, Ha TTN, Ohmoto Y, Kikuchi M, Morita K, Yasunami M, Hirayama K, Watanabe N.	Association of Mast Cell-Derived VEGF and Proteases in Dengue Shock Syndrome.	PLoSNTD	6(2)	e1505	2012
Hasebe F, Thuy NTT, Inoue S, Yu F, Kaku Y, Watanabe S, Akashi H, Dat DT, Mai LTQ, Morita K.	Serologic Evidence of Nipah Virus Infection in Bats, Vietnam.	Emerg Infect Dis	18(3)	536-537	2012
Lauber C, Ziebuhr J, Junglen S, Drosten C, Zirkel F, Nga PT, Morita K, Snijder EJ, Gorbalenya AE.	Mesoniviridae: a proposed new family in the order Nidovirales formed by a single species of mosquito-borne viruses.	Arch Virol.	157(8)	1623-1628	2012
Shimasaki T, Ishigaki Y, Nakamura Y, Takata T, Nakaya N, Nakajima H, Sato I, Zhao X, Kitano A, Kawakami K, Tanaka T, Takegami T, Tomosugi N, Minamoto T, Motoo Y.	Glycogen synthase kinase 3β inhibition sensitizes pancreatic cancer cells to gemcitabine.	J Gastroenterol	47	321-333	2012
Nukuzuma S, Kameoka M, Sugiura S, Nakamichi K, Nukuzuma C, Miyoshi I, Takegami T.	Exogenous Human Immunodeficiency Virus-1 Protein, Tat enhances replication of JC virus efficiently in neuroblastoma cell lines.	J Med Virol	84	555-561	2012
Ishigaki Y, Nakamura Y, Oikawa Y, Yano Y, Kuwabata S, Nakagawa H, Tomosugi N, Takegami T.	Observation of live ticks (Haemaphysalis flava) by scanning electron microscope under high vacuum pressure.	PLoS One	7	e32676	2012
Zhang S, Kim T-S, Dong Y, Kanazawa S, Kawaguchi M, Gao N, Minato H, Takegami T, Nojima T, Miura Y.	AT motif binding factor 1 (ATBF1) is highly phosphorylated in embryonic brain and protected from cleavage by calpain-1.	Biochem Biophys Res Commun	427	537-541	2012
Mulyatno KC, Yamanaka A, Yotopranoto S, Konishi E.	Vertical transmission of dengue virus in Aedes aegypti collected in Surabaya, Indonesia, during 2008–2011.	Jpn J Infect Dis	65 (3)	274-276	2012
Yamanaka A, Tabuchi Y, Mulyatno KC, Susilowati H, Hendrianto E, Soegijanto S, Konishi E.	Dengue virus infection-enhancing and neutralizing antibody balance in children of the Philippines and Indonesia.	Microbes Infect	14(13)	1152-1159	2012
Yamaji H, Segawa M, Nakamura M, Katsuda T, Kuwahara M, Konishi E.	Production of Japanese encephalitis virus-like particles using the baculovirus-insect cell system.	J Biosci Bioeng	114(6)	657-662	2012
Konishi E, Kitai Y, Nishimura K, Harada S.	Follow-up survey of Japanese encephalitis virus infection among humans in Kumamoto Prefecture, south-west Japan: status during 2009–2011.	Jpn J Infect Dis	65(5)	448-450	2012

Yamaji H, Nakamura M, Kuwahara M, Takahashi Y, Katsuda T, Konishi E.	Efficient production of Japanese encephalitis virus-like particles by recombinant lepidopteran insect cells.	Appl Microbiol Biotechnol	Epub ahead of print	5-Sep	2012
Sjatha F, Takizawa Y, Yamanaka A, Konishi E.	Phylogenetic analysis of dengue virus types 1 and 3 isolated in Jakarta, Indonesia in 1988.	Infect Genet Evol	12(8)	1938-1943	2012
Kuwahara M, Kitai Y, Kondo T, Konishi E.	Survey of antibodies specific for West Nile virus in horses from 2006 to 2010 in Japan.	Jpn J Infect Dis	65(6)	553-555	2012
Doan YH, Nakagomi T, Nakagomi O.	Repeated circulation over 6 years of intergenogroup mono-reassortant G2P[4] rotavirus strains with genotype N1 of the NSP2 gene.	Infect Genet Evol	12(6)	1202-1212	2012
Nakagomi T, Nakagomi O, Dove W, Doan YH, Witte D, Ngwira B, Todd S, Duncan Steele A, Neuzil KM, Cunliffe NA.	Molecular characterization of rotavirus strains detected during a clinical trial of a human rotavirus vaccine in Blantyre, Malawi.	Vaccine	30 Suppl 1	A140-151	2012
Noguchi A, Nakagomi T, Kimura S, Takahashi Y, Matsuno K, Koizumi H, Watanabe A, Noguchi H, Ito T, Ohtsuka M, Uemura N, Takeda O, Komatsu A, Kikuchi W, Komatsu M, Fukaya H, Miura S, Toda H, Nakagomi O, Takahashi T.	Incidence of intussusception as studied from a hospital-based retrospective survey over a 10-year period (2001-2010) in Akita Prefecture, Japan.	Jpn J Infect Dis	65(4)	301-305	2012
Matthijinssens J, Nakagomi O, Kirkwood CD, Ciarlet M, Desselberger U, Van Ranst M.	Group A rotavirus universal mass vaccination: how and to what extent will selective pressure influence prevalence of rotavirus genotypes?	Expert Rev Vaccines	11(11)	1347-54	2012
Doan YH, Nakagomi T, Aboudy Y, Silberstein I, Behar-Novat E, Nakagomi O, Shulman LM.	Identification by Full Genome Analysis of a Bovine Rotavirus Transmitted Directly to, and Causing Diarrhea in a Human Child.	J Clin Microbiol	51(1)	182-189	2013
Hosssain M, Ahmed K, Bulbul T, Hossain S, Rahman A, Biswas M N U, Nishizono A.	Human rabies in rural Bangladesh.	Epidemiol Infect	140(11)	1964-1971	2012
Ahmed K, Wimalaratne O, Dahal N, Khawplod P, Nanayakkara S, Rinzen K, Perera D, Karunanayake D, Matsumoto T, Nishizono A.	Evaluation of a Monoclonal Antibody–Based Rapid Immunochromatographic Test for Direct Detection of Rabies Virus in the Brain of Humans and Animals.	Am J Trop Med Hyg	86(4)	736-740	2012
Nishizono A, Yamada K, Khawplod P, Shiota S, Perera D, Matsumoto T, Wimalaratne O, Mitui M T, Ahmed K.	Evaluation of an improved rapid reutralizing antibody detection test (RAPINA) for qualitative and semiquantitative detection of rabies neutralizing antibody in humans and dogs.	Vaccine	30(26)	3891-3896	2012
Jamil K M, Ahmed K, Hossain M, Matsumoto T, Ali M A, Hosssain S, Hossain S, Islam A, Nasiruddin M, Nishizono A.	Arctic-like Rabies Virus, Bangladesh.	Emerg Infect Dis	18(12)	2012-2024	2012

Yamada K, Park CH, Noguchi K, Kojima d, Kubo T, Komiya N, Matsumoto T, Mitui M T, Ahmed K, Morimoto K, Inoue S, Nishizono A.	Serial passage of a street rabies virus in mouse neuroblastoma cells resulted in attenuation : Potential role of the additional N-glycosylation of a viral glycoprotein in the reduced pathogenicity of street rabies virus.	Virus Res	165(1)	34-45	2012
Virojanapirom P, Khawplod P, Sawangvaree A, Wacharapluesadee S, Hemachudha T, Yamada K, Morimoto K, Nishizono A.	Molecular analysis of the mutational effects of Thai street rabies virus with increased virulence in mice after passages in the BHK cell line.	Arch Virol.	157(11)	2215-2205	2012
Yamada K, Noguchi K, Nonaka D, Morita M, Yasuda A, Kawazato H, Nishizono A.	Addition of a single N-glycan to street rabies virus glycoprotein enhances virus production.	J Gen Virol	94(2)	270-275	2013
Watanabe I, Yamada K, Aso A, Suda O, Matsumoto T, Yahiro T, Ahmed K, Nishizono A.	Relationship between Virus-Neutralizing Antibody Levels and the Number of Rabies Vaccinations: a Prospective Study of Dogs in Japan.	Jpn J Infect Dis	66(1)	17-21	2013
Luan VD, Yoshimatsu K, Endo R, Taruishi M, Huong VT, Dat DT, Tien PC, Shimizu K, Koma T, Yasuda SP, Nhi L, Huong VT, Arikawa J.	Studies on Hantavirus Infection in Small Mammals Captured in Southern and Central Highland Area of Vietnam.	J Vet Med Sci	74(9)	1155-1162	2012
Koma T, Yoshimatsu K, Taruishi M, Miyashita D, Endo R, Shimizu K, Yasuda SP, Amada T, Seto T, Murata R, Yoshida H, Kariwa H, Takashima I, Arikawa J.	Development of a serotyping enzyme-linked immunosorbent assay system based on recombinant truncated hantavirus nucleocapsid proteins for New World hantavirus infection.	J Virol Methods	185(1)	74-81	2012
Schlegel M, Tegshduuren E, Yoshimatsu K, Petraityte R, Sasnauskas K, Hammerschmidt B, Friedrich R, Mertens M, Groschup MH, Arai S, Endo R, Shimizu K, Koma T, Yasuda S, Ishihara C, Ulrich RG, Arikawa J, Köllner B.	Novel serological tools for detection of Thottapalayam virus, a Soricomorpha-borne hantavirus.	Arch Virol	157(11)	2179-2187	2012
Koma T, Yoshimatsu K, Yasuda SP, Li T, Amada T, Shimizu K, Isozumi R, Mai LT, Hoa NT, Nguyen V, Yamashiro T, Hasebe F, Arikawa J.	A survey of rodent-borne pathogens carried by wild Rattus spp. in Northern Vietnam.	Epidemiol Infect	[Epub ahead of print]		
Sakai K, Nagata N, Ami Y, Seki F, Suzaki Y, Iwata-Yoshikawa N, Suzuki T, Fukushi S, Mizutani T, Yoshikawa T, Otsuki N, Kurane I, Komase K, Yamaguchi R, Hasegawa H, Saijo M, Takeda M, Morikawa S.	Lethal Canine Distemper Virus Outbreak in Cynomolgus Monkeys in Japan in 2008.	J Virol.	87(2)	1105-1114	2013
Sharma N, Hotta A, Yamamoto Y, Fujita O, Uda A, Morikawa S, Yamada A, Tanabayashia K.	Detection of *Francisella tularensis*-specific antibodies in patients with tularemia using a novel competitive enzyme-linked immunosorbent assay.	Clinical and Vaccine Immunology		in press	2013
Taniguchi S, Sayama Y, Nagata N, Ikegami T, Miranda ME, Watanabe S, Iizuka I, Fukushi S, Mizutani T, Ishii Y, Saijo M, Akashi H, Yoshikawa Y, Kyuwa S, Morikawa S.	Analysis of the humoral immune responses among cynomolgus macaque naturally infected with Reston virus during the 1996 outbreak in the Philippines.	BMC Veterinary Research	8(1)	189	2012

Fukushi S, Tani H, Yoshikawa T, Saijo M, Morikawa S.	Serological assays based on recombinant viral proteins for the diagnosis of arenavirus hemorrhagic fevers.	Viruses	4(10)	2097-2114	2012
Katano H, Sato S, Sekizuka T, Kinumaki A, Fukumoto H, Sato Y, Hasegawa H, Morikawa S, Saijo M, Mizutani T, Kuroda M.	Pathogenic characterization of a cervical lymph node derived from a patient with Kawasaki disease.	Int J Clin Exp Pathol	5(8)	814-823	2012
Sayama Y, Demetria C, Saito M, Azul RR, Taniguchi S, Fukushi S, Yoshikawa T, Iizuka I, Mizutani T, Kurane I, Malbas FF Jr, Lupisan S, Catbagan DP, Animas SB, Morales RG, Lopez EL, Dazo KR, Cruz MS, Olveda R, Saijo M, Oshitani H, Morikawa S.	A seroepidemiologic study of Reston ebolavirus in swine in the Philippines.	BMC Vet Res	8	82	2012
Lihoradova O, Kalveram B, Indran SV, Lokugamage N, Juelich TL, Hill TE, Tseng CT, Gong B, Fukushi S, Morikawa S, Freiberg AN, Ikegami T.	The dominant-negative inhibition of double-stranded RNA-dependent protein kinase PKR increases the efficacy of Rift Valley fever virus MP-12 vaccine.	J Virol	86(14)	7650-7661	2012
Fukushi S, Nakauchi M, Mizutani T, Saijo M, Kurane I, Morikawa S.	Development and Applications of VSV Vectors Based on Cell Tropism.	J Virol Methods	180	68-74	2012
Arai S, Gu SH, Baek LJ, Tabara K, Bennett SN, Oh HS, Takada N, Kang HJ, Tanaka-Taya K, Morikawa S, Okabe N, Yanagihara R, Song JW.	Divergent ancestral lineages of newfound hantaviruses harbored by phylogenetically related crocidurine shrew species in Korea.	Virology	424(2)	99-105	2012
Shirato K, Maeda K, Tsuda S, Suzuki K, Watanabe S, Shimoda H, Ueda N, Iha K, Taniguchi S, Kyuwa S, Endoh D, Matsuyama S, Kurane I, Saijo M, Morikawa S, Yoshikawa Y, Akashi H, Mizutani T.	Detection of bat coronaviruses from Miniopterus fuliginosus in Japan.	Virus Genes	44(1)	40-44	2012
中込とよ子, 中込 治	ロタウイルス感染症とロタウイルスワクチン	メディカル・テクノロジー	40(9)	1005-1008	2012
山田健太郎, 西園 晃	イムノクロマト法による狂犬病とデング熱の迅速診断法	臨床と微生物	39(6)	713-719	2012
西園 晃, 山田健太郎	ラブドウイルス	ウイルス	62(2)	183-196	2012

研究成果の刊行に関する一覧表（書籍）

著者氏名	論文タイトル名	書籍全体の編集者氏名	書籍名	出版社名	出版地	出版年	ページ
森田公一	アルボウイルス	柳雄介、堤裕幸	新編ウイルスの今日的意味	医薬ジャーナル社	東京	2012	82-90

Ⅳ. 研究成果の刊行物・別刷

Arctic-like Rabies Virus, Bangladesh

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Arctic/Arctic-like rabies virus group 2 spread into Bangladesh ≈32 years ago. Because rabies is endemic to and a major public health problem in this country, we characterized this virus group. Its glycoprotein has 3 potential *N*-glycosylation sites that affect viral pathogenesis. Diversity of rabies virus might have public health implications in Bangladesh.

Rabies virus causes severe encephalitis in a wide range of mammals, including humans. Conservative estimates suggest that 55,000 persons worldwide die of rabies each year (*1*). Although the case-fatality rate in humans is 100%, rabies is preventable by vaccination. Bangladesh has the world's third highest death rate for human rabies, an estimated 2,100 deaths per year (*2*). Dogs are the main reservoir of the virus and are responsible for spillover infections in humans (*2*). Therefore, dogs should be the principal target for successful rabies elimination.

With political will and solid global epidemiologic information, rabies elimination is possible. Molecular typing of circulating rabies viruses is necessary to identify and develop effective control measures, and to understand the spread of certain rabies virus variants and their incursion into new regions (*3*). For rabies elimination, this knowledge is needed for establishing cooperative approaches between neighboring countries to which the disease is endemic.

Bangladesh is one of several countries in which no molecular study has been conducted to identify types of rabies virus circulating within its boundaries. A lack of knowledge of phylogenetic relationships of Bangladesh rabies virus with viruses in other countries continues to hinder coordinated rabies control efforts in the region. This study was conducted to characterize rabies virus circulating in Bangladesh and to determine its relationship with viruses in neighboring countries to clarify its epidemiologic relationships, origin, and transmission dynamics.

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The Study

Seven brain samples were collected from animals with suspected rabies in 3 districts of Bangladesh (Dhaka, Narayanganj, and Narshingdi) in 2010 (Table 1). A portion of brainstem was removed from each sample and preserved in TRizol (Invitrogen, Carlsbad, CA, USA) at –20°C. Total RNA was extracted from brain homogenate, cDNA was synthesized by using random hexamer primers, reverse transcription PCR was conducted to amplify gene fragments, and nucleotide sequencing of genes was performed (*4*).

Full-length nucleoprotein (N) and glycoprotein (G) gene sequences from samples were determined. Nucleotide identities of N and G genes were 98%–100%. Amino acid identities of N and G genes were 100% and 98%–100%, respectively. Complete genomic sequencing (11,928 nt) of strain BDR5 was also conducted.

Evolutionary analysis was performed by using full-length N gene. We created a maximum clade credibility phylogenetic tree using the Bayesian Markov chain Monte Carlo method available in BEAST version 1.6.1 (*5*). Analysis was conducted by using a relaxed (uncorrelated lognormal) molecular clock and a generalized time reversible + Γ + proportion invariant model (*6*). All chains were run for 90 million generations and sampled every 3,000 steps and an effective sample size >1,383 was obtained for all estimated parameters. Posterior densities were calculated with 10% burn-in and checked for convergence by using Tracer version 1.5 in BEAST.

The mean rate of nucleotide substitution estimated for the N gene was 2.3×10^4 substitutions/site/year (95% highest posterior density [HPD] $1.4–3.1 \times 10^4$ substitutions/site/year). This rate is consistent with that of a previous study (*7*). The phylogenetic tree showed that rabies viruses in Bangladesh belong to Arctic/Arctic-like group 2 (AAL2) (*3*) also known as Arctic-like-1 (*8*), in close association with the strain from Bhutan.

Approximately 397.0 years ago (95% HPD 273.5–589.5 years), AAL and cosmopolitan rabies virus segregated from their most recent common ancestor (Figure 1). Approximately 225.6 years ago (95% HPD 157.4–324.2 years), AAL3 segregated. Approximately 187.4 years ago (95% HPD 129.0–271.9 years), AAL1 and AAL2 segregated. The AAL2 clade had a common progenitor that circulated ≈133.1 years ago (95% HPD 91.3–193.4 years), which has evolved into several different lineages. One lineage evolved 91.5 years ago (95% HPD 63.1–132.2 years) and currently circulates in Bangladesh, India, and Bhutan. Separate linages circulate in others

Table 1. Characteristics of 7 animal samples tested for rabies virus, Bangladesh

Sample no.	Animal	Age, y	District	History	Signs and symptoms	GenBank accession no.*
BDR1	Dog	Unknown	Dhaka	Unknown	Angry, biting tendency, excessive salivation, gradually became drowsy	Not determined
BDR2	Cow	8	Narsingdi	Calf died of suspected rabies 1 wk earlier	Angry, salivation, drooping of tongue, inability to drink or eat	AB699208
BDR3	Cow	10	Dhaka	Unknown	Angry, salivation, frequent micturition, inability to drink or eat	AB699209
BDR4	Goat	3	Narayanganj	Dog bite 2.5 mo earlier	Angry, inability to eat and drink, biting tendency	AB699210
BDR5	Goat	2	Narayanganj	Dog bite to head 2 mo earlier	Angry, salivation, inability to eat and drink	AB699220 (whole genome)
BDR6	Cow	6	Dhaka	Unknown	Angry, salivation, trying to attack	AB699212
BDR7	Cow	5	Narayanganj	Dog bite 2 mo earlier	Angry, salivation, trying to attack	AB699213

*For glycoprotein gene.

countries in this region, including Iran, Nepal, Pakistan, and Afghanistan. AAL2 spread into central Bangladesh 32.3 years ago (95% HPD 18.4–50.6 years) in ≈1978 (95% HPD range 1958–1991).

Compared with the AAL2 strain from India (AY956319), BDR5 had several amino acid substitutions (Table 2). Sizes of their 2 genomes, leader RNA, trailer RNA, and intergenic regions were similar. The *N*-glycosylation site was predicted by using the NetNGlyc 1.0 server (www.cbs.dtu.dk/server/netnglyc). With the exception of BDR6, the G gene of all strains had potential glycosylation sites at position 37, 146, and 319.

Conclusions

Genetic analysis and phylogenetic studies can contribute to understanding the epidemiology of rabies virus in disease-endemic countries. Molecular analysis of animal rabies viruses showed that AAL2 appeared in central Bangladesh only 32 years ago. A close association between N genes sequences from rabies viruses in Bangladesh and

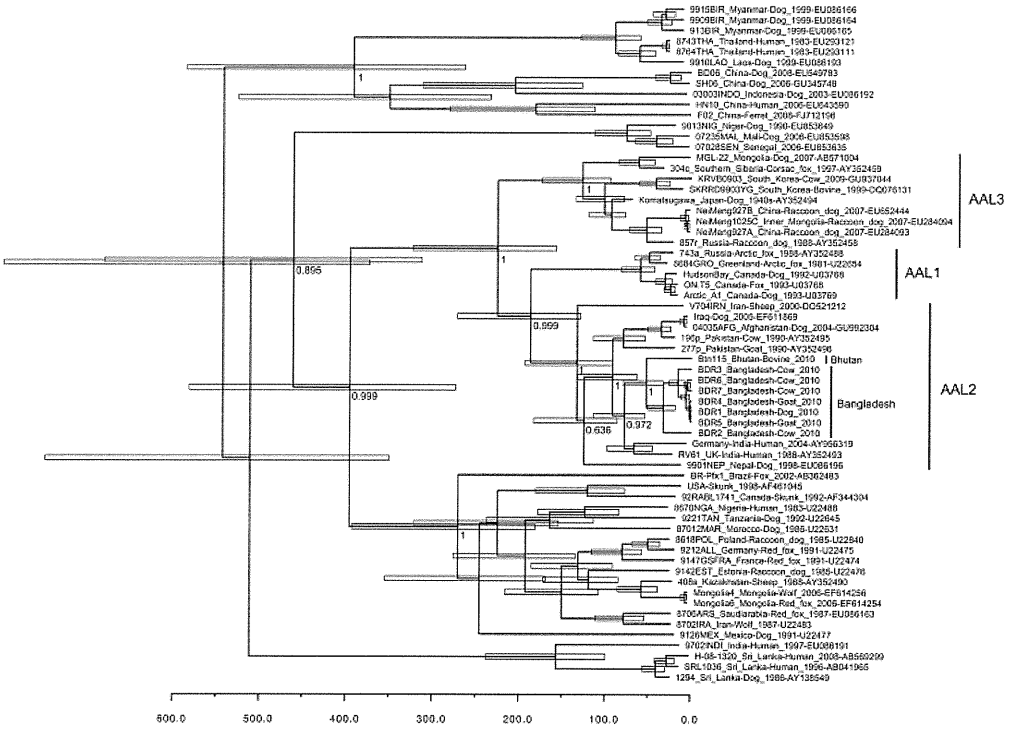

Figure 1. Bayesian maximum credibility tree showing genealogy of rabies virus obtained by analyzing nucleotide sequences of full nucleoprotein (N) gene sequences (1,350 nt), Bangladesh. Nodes indicate the mean age at which they are separated from the most recent common ancestor, and white horizontal bars at nodes indicate 95% highest posterior density values of the most recent common ancestor. Numbers at the main nodes indicate posterior values. Scale bar indicates time scale in years starting from 2010. Each strain name is followed by country of origin, host, year of detection, and GenBank accession number.

Nucleotide sequence data of the N gene of rabies viruses from Bangladesh appear in the DDBJ/EMBL/GenBank nucleotide sequence databases: accession nos.: AB699214 (rabies virus strain BDR1), AB699215 (strain BDR2), AB699216 (strain BDR3), AB699217 (strain BDR4), AB699218 (strain BDR6), AB699219 (strain BDR7), and AB699220 (whole genome of strain BDR5). AAL, Arctic/Arctic-like.

Table 2. Substitutions in genome sequence of rabies virus BDR5 from Bangladesh compared with genome sequence of strain from India (AY956319), 2010*

Protein, amino acid substitution	Site/domain/region of protein†
N	
$Asp_{378} \rightarrow Glu_{378}$	Antigenic site IV
$Gln_{422} \rightarrow Arg_{422}$	–
P	
$Ser_{64} \rightarrow Pro_{64}$	Variable domain I
$Gln_{71} \rightarrow Thr_{71}$	Variable domain I
$Asn_{90} \rightarrow Ser_{90}$	N protein binding site in variable domain II
$Pro_{159} \rightarrow Ser_{159}$	N protein binding site in variable domain II
$His_{162} \rightarrow Ser_{162}$	N protein binding site in variable domain II
$Asn_{166} \rightarrow Ser_{166}$	N protein binding site in variable domain II
$Ala_{174} \rightarrow Val_{174}$	N protein binding site in variable domain II
M	
$Leu_{21} \rightarrow Pro_{21}$	–
$Ser_{46} \rightarrow Gly_{46}$	–
$Ile_{168} \rightarrow Val_{168}$	–
G	
$Ala_{-(minus)15} \rightarrow Val_{-15}$	Signal peptide
$Val_{-(minus)6} \rightarrow Phe_{-6}$	Signal peptide
$Val_{7} \rightarrow Ile_{7}$	–
$Asp_{146} \rightarrow Asn_{146}$	Putative additional *N*-glycosylation: NKS
$Val_{427} \rightarrow Ile_{427}$	–
$Arg_{462} \rightarrow Gly_{462}$	Transmembrane domain
$His_{465} \rightarrow Arg_{465}$	Transmembrane domain
$Gly_{473} \rightarrow Ser_{473}$	Transmembrane domain
L	
$Asp_{18} \rightarrow Glu_{18}$	–
$Ala_{19} \rightarrow Thr_{19}$	–
$Arg_{315} \rightarrow Lys_{315}$	Conserved domain I
$Val_{361} \rightarrow Leu_{361}$	Conserved domain I
$His_{640} \rightarrow Gln_{640}$	Conserved domain III
$Lys_{657} \rightarrow Arg_{657}$	Conserved domain III
$Ala_{966} \rightarrow Thr_{966}$	Conserved domain IV
$Pro_{1133} \rightarrow Ser_{1133}$	Conserved domain V
Arg_{1307} Lys_{1307}	Conserved domain IV
$Asp_{1373} \rightarrow Gly_{1373}$	–
$Leu_{1626} \rightarrow Val_{1626}$	–
$Leu_{1654} \rightarrow Ser_{1654}$	–
$Val_{1755} \rightarrow Ile_{1755}$	–
$Cys_{1825} \rightarrow Tyr_{1825}$	–
$Asn_{1841} \rightarrow Lys_{1841}$	–
$Gln_{1845} \rightarrow His_{1845}$	–
$Cys_{1872} \rightarrow Phe_{1872}$	–
$Asn_{2091} \rightarrow Ser_{2091}$	–

*N, nucleoprotein; P, phosphoprotein; M, matrix protein; G, glycoprotein; L, polymerase.
†– indicates that the amino acid substitution was in a location that has no site/domain/region name. NKS, asparagine-lysine-serine.

Bhutan indicates that they originated from a common ancestor. If one considers the ease of human movement between countries, AAL2 most likely entered Bangladesh from India rather than from Bhutan.

Circumstantial evidence suggests that rabies virus spread from India to Bhutan (*9*). AAL2 circulates in many states of India. It has spread into southern India and has replaced older strains (*10*,*11*). It is likely that AAL2 is also circulating in states of India that are between Bhutan and Bangladesh. Estimated time of AAL2 spread is based on 7 samples that are representative of central Bangladesh (Figure 2). Therefore, further surveillance might identify the extent to which AAL2 has spread and the diversity of rabies viruses in other parts of Bangladesh that might alter the estimated date of spread. It has been reported that arctic rabies virus and other variants can co-circulate in the same region (*12*).

The G protein is the major factor responsible for the pathogenesis of rabies virus and contains 2 glycosylation sites (*13*). The G protein of strains from Bangladesh uniquely evolved to contain 3 potential glycosylation sites, which has been reported in only fixed (laboratory adapted) strains and proposed to be responsible for their reduced pathogenicity (*13*). However, the site for additional

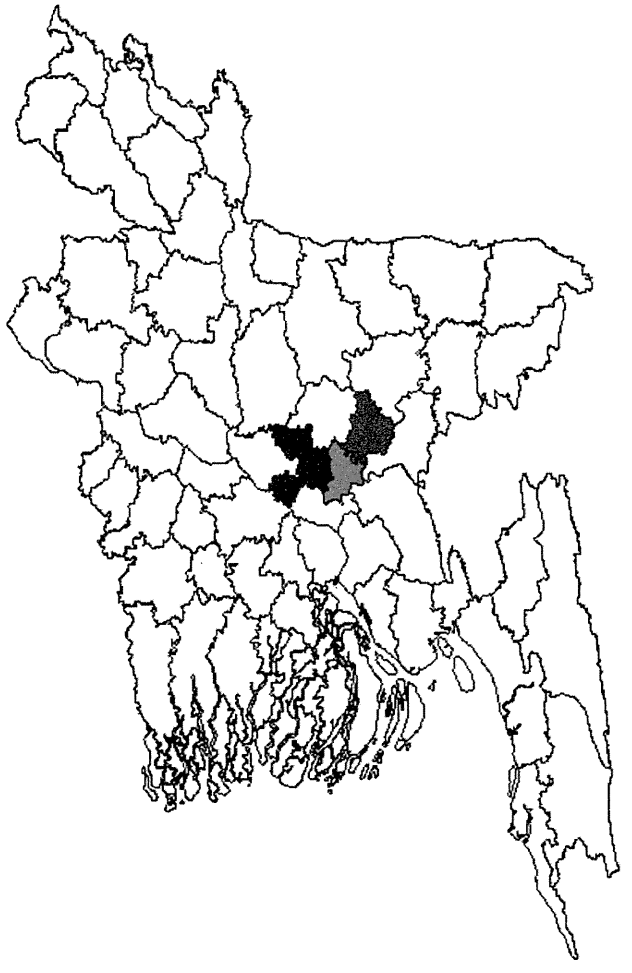

Figure 2. Three districts of Bangladesh from which samples were tested for Arctic/Arctic-like rabies virus and strains were found. Black, Dhaka District, strains BDR1, BDR3, and BDR6; light gray, Narayanganj District, strains BDR4, BDR5, and BDR7; dark gray, Narshingdi District, strain BDR2.

glycosylation differs between Bangladeshi and fixed strains. Detection of an additional glycosylation site and amino acid substitutions deserve further investigations.

AAL viruses could have moved southward from Siberia or other northern regions of the former Soviet Union into Nepal, India, and other countries in Asia by a species jump from fox to dog at some point (*3*). Another possibility is that AAL viruses first emerged in dogs in southern Asia and subsequently spread to northern climes, where they are now maintained in fox populations (*3*,*8*). Extensive surveillance of viruses from Iran, Iraq, Afghanistan, and countries north of them is necessary to determine the origin and spread pattern of AAL rabies virus.

The timeline of divergence of different lineages determined in this study was similar to that previously reported (*8*). That study and our study used the full-length N gene to determine the time of divergence. Another study reported the timeline of divergence as a more recent event (*14*). This study used partial sequences of N genes, which might be responsible for different results. Rabies virus from Nepal also belongs to AAL2, and as reported in a previous study (*15*), seemed to be forming a different lineage. However, the speculation was not supported by a significant a posterior density value (0.6355). Thus, a network of countries is urgently needed to exchange information on molecular typing of circulating strains of rabies virus that might be useful in controlling rabies in this region.

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References

1. Knobel DL, Cleaveland S, Coleman PG, Fevre EM, Meltzer MI, Miranda ME, et al. Re-evaluating the burden of rabies in Africa and Asia. Bull World Health Organ. 2005;83:360–8.
2. Hossain M, Ahmed K, Bulbul T, Hossain S, Rahman A, Biswas MN, et al. Human rabies in rural Bangladesh. . Epidemiol Infect. 2011;140:1–8. http://dx.doi.org/10.1017/S095026881100272X
3. Nadin-Davis SA, Turner G, Paul JP, Madhusudana SN, Wandeler AI. Emergence of Arctic-like rabies lineage in India. Emerg Infect Dis. 2007;13:111–6. http://dx.doi.org/10.3201/eid1301.060702
4. Matsumoto T, Ahmed K, Wimalaratne O, Yamada K, Nanayakkara S, Perera D, et al. Whole-genome analysis of a human rabies virus from Sri Lanka. Arch Virol. 2011;156:659–69. http://dx.doi.org/10.1007/s00705-010-0905-8
5. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007;7:214. http://dx.doi.org/10.1186/1471-2148-7-214
6. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics. 1998;14:817–8. http://dx.doi.org/10.1093/bioinformatics/14.9.817
7. Bourhy H, Reynes JM, Dunham EJ, Dacheux L, Larrous F, Huong VT, et al. The origin and phylogeography of dog rabies virus. J Gen Virol. 2008;89:2673–81. http://dx.doi.org/10.1099/vir.0.2008/003913-0
8. Kuzmin IV, Hughes GJ, Botvinkin AD, Gribencha SG, Rupprecht CE. Arctic and Arctic-like rabies viruses: distribution, phylogeny and evolutionary history. Epidemiol Infect. 2008;136:509–19. http://dx.doi.org/10.1017/S095026880700903X
9. Tenzin, Dhand NK, Dorjee J, Ward MP. Re-emergence of rabies in dogs and other domestic animals in eastern Bhutan, 2005–2007. Epidemiol Infect. 2011;139:220–5. http://dx.doi.org/10.1017/S0950268810001135
10. Nagarajan T, Nagendrakumar SB, Mohanasubramanian B, Rajalakshmi S, Hanumantha NR, Ramya R, et al. Phylogenetic analysis of nucleoprotein gene of dog rabies virus isolates from southern India. Infect Genet Evol. 2009;9:976–82. http://dx.doi.org/10.1016/j.meegid.2009.04.004
11. Reddy GB, Singh R, Singh RP, Singh KP, Gupta PK, Desai A, et al. Molecular characterization of Indian rabies virus isolates by partial sequencing of nucleoprotein (N) and phosphoprotein (P) genes. Virus Genes. 2011;43:13–7. http://dx.doi.org/10.1007/s11262-011-0601-0
12. Mansfield KL, Racloz V, McElhinney LM, Marston DA, Johnson N, Ronsholt L, et al. Molecular epidemiological study of Arctic rabies virus isolates from Greenland and comparison with isolates from throughout the Arctic and Baltic regions. Virus Res. 2006;116:1–10. http://dx.doi.org/10.1016/j.virusres.2005.08.007
13. Yamada K, Park CH, Noguchi K, Kojima D, Kubo T, Komiya N, et al. Serial passage of a street rabies virus in mouse neuroblastoma cells resulted in attenuation: Potential role of the additional N-glycosylation of a viral glycoprotein in the reduced pathogenicity of street rabies virus. Virus Res. 2012;165:34–45. http://dx.doi.org/10.1016/j.virusres.2012.01.002
14. Nadin-Davis SA, Sheen M, Wandeler AI. Recent emergence of the Arctic rabies virus lineage. Virus Res. 2012;163:352–62. http://dx.doi.org/10.1016/j.virusres.2011.10.026
15. Pant GR, Horton DL, Dahal M, Rai JN, Ide S, Leech S, et al. Characterization of rabies virus from a human case in Nepal. Arch Virol. 2011;156:681–4. http://dx.doi.org/10.1007/s00705-010-0868-9

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Original Article

Relationship between Virus-Neutralizing Antibody Levels and the Number of Rabies Vaccinations: a Prospective Study of Dogs in Japan

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SUMMARY: A mass rabies vaccination of dogs has been conducted annually in Japan over the last 60 years. To assess both current levels of rabies virus-neutralizing antibody (VNA) in dogs and the rationale for current vaccination procedures, we used a rapid fluorescent focus inhibition test to determine VNA levels in 756 dogs that had visited animal hospitals in Japan. We found that 51.1% of the dogs that had received 1 rabies vaccination had protective VNA levels ($\geq$0.5 IU/ml) with a geometric mean of 0.61 IU/ml. In contrast, 97.8% of the dogs that had been vaccinated at least twice had protective VNA levels with a geometric mean of 7.86 IU/ml. Furthermore, 97.9–100% of the dogs vaccinated at least twice retained protective VNA levels into the second year after the last vaccination. Although VNA levels in the dogs vaccinated at least twice tended to decline 2 years after the last vaccination, 78.9% retained protective VNA levels. Thus, the current rabies vaccination schedule provides adequate protection, but the registration system and vaccination schedule needs to be improved to ensure that increased numbers of dogs are vaccinated against rabies.

INTRODUCTION

Rabies, an acute and fatal encephalitis, is caused by the rabies virus. An estimated 55,000 people die from rabies each year worldwide; 99% of transmission is caused by dogs (the urban form) (1). Although rabies remains endemic in many countries, the virus has been eradicated in several others, including Japan. In 1950, the government of Japan enacted the Rabies Prevention Law to regulate and enforce a dog registration system, mandatory vaccination of dogs, management of unleashed dogs, stray dog regulations, a quarantine system for overseas dogs, and other practices (2–4). As a result, no cases of indigenous human rabies have been reported in Japan since 1954; further, it has not been reported in indigenous dog and cat rabies since 1956 and 1957, respectively (5).

Globalization has increased the travels of humans and animals from developing countries endemic with rabies, and thus, the risk of rabies entering Japan is growing. Natural disasters increase rabies risks as well. For example, the number of rabies cases in Japan grew during the years following the Great Kanto Earthquake of 1923 because of the increase in stray dogs (5). The number of stray dogs also increased following the huge earthquake and tsunami on March 11, 2011 (i.e., the Great East Japan Earthquake). The accidental introduction of a rabid animal to a large population of unvaccinated stray dogs promotes the rapid spread of rabies, thereby threatening Japan's current rabies-free status.

Mass vaccination of dogs against rabies is a highly rational strategy for interrupting the natural transmission cycle of urban rabies. According to the World Health Organization (WHO), immunization of at least 70% of a dog population minimizes the risk of endemic rabies (1). However, the percentage of dogs that meet recommended virus-neutralizing antibody (VNA) levels in Japan is unknown. Furthermore, few studies have attempted to determine the adequacy of the current annual rabies vaccination program for dogs (6).

Therefore, we conducted a prospective, hospital-based study to evaluate the effects of the frequency of vaccination and intervals on serum VNA levels in pet dogs in Japan.

MATERIALS AND METHODS

Collection and storage of serum samples: A total of 756 serum samples were collected from dogs in 30 animal hospitals (28 from Oita Prefecture and 2 from Tokyo) between May 2010 and November 2011, along with information such as date of birth, number of rabies vaccinations, number of days since previous vaccination, and the sample collection date. Of the 756 serum samples, 649 were obtained from vaccinated dogs and 107 from unvaccinated dogs. Mean dog age was 6.2 years (range, 5 days to 19 years). Of the vaccinated

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dogs, 249 were male, 280 were female, and the sex of 120 dogs was unknown. The sex of the unvaccinated dogs (age range, 0–12 months) was unknown. We were unable to confirm maternal protective VNA levels against rabies; however, since all subjects' mothers had official records of multiple vaccinations against rabies, we assume that most also mounted protective VNA levels. The dog immunization formulations included 6 different commercially available rabies vaccines, all derived from the RC-HL strain.

The blood samples were obtained from dogs that had visited animal hospitals for vaccinations or other reasons. These dogs were not afflicted with any diseases related to the immune system. Verbal consent was obtained from each dog owner after a detailed explanation of the testing procedure was provided. We collected 10 ml of whole blood in plain glass tubes. After the sample was stored at room temperature for 15 min, the serum was separated from the blood by centrifugation at 2,000 $\times g$ for 10 min at 4°C. Serum samples were stored at −20°C and sent to our laboratory at Oita University. Frozen samples were thawed and serum complement was inactivated by incubation for 30 min at 56°C prior to VNA titration. This study was approved by the ethics committee of Oita University (M010003).

Determination of VNA levels: We used a rapid fluorescent focus inhibition test (RFFIT) (7,8) to determine the rabies VNA levels. First, serial serum sample dilutions were incubated in a 96-well plate with the challenge virus standard (CVS-11) rabies strain for 90 min at 37°C. BHK-21 cells were added to each well before incubating for 18 h at 37°C. Finally, the culture media was discarded and the cells were fixed in 90% acetone, stained with fluorescein isothiocyanate-conjugated anti-rabies N monoclonal antibody (Fujirebio Diagnostics, Inc., Malvern, Pa., USA) for 45 min at 37°C, and observed with a fluorescence microscope. The VNA levels were calculated based on comparison to the WHO standard serum. According to the WHO, VNA levels of ≥0.5 IU/ml provide adequate protection against rabies. This value is also recommended for dogs by the World Organisation for Animal Health (9,10).

Statistical analysis: Statistical analysis was performed using the Mann-Whitney *U* test to compare VNA levels. Correlations between variables were tested using Spearman's rank correlation coefficient test. A graph was prepared with the DeltaGraph version 5 program.

RESULTS

Relationship between VNA levels and number of rabies vaccinations: We determined the rabies VNA levels in serum samples collected from dogs in animal hospitals in Japan. A VNA level of ≥0.5 IU/ml, the accepted protective level against rabies (1,9,10), was considered protective in this study. Table 1 shows the relationship between VNA levels and the number of rabies vaccinations. Of the 107 unvaccinated dogs (age range, 0–12 months), only 1 dog exhibited protective VNA levels. The geometric mean of VNA in 92 dogs that had received a single vaccination was 0.6 IU/ml; 51.1% of these dogs exhibited protective VNA levels. In contrast, the geometric mean of VNA in 557 dogs that had received multiple vaccinations was 7.86 IU/ml; 97.8% of these dogs exhibited protective VNA levels.

Duration of protective VNA after rabies vaccination: Next, we analyzed the duration of VNA in dogs that had received either a single or multiple rabies vaccinations and prepared a box and whisker plot to enhance our understanding of VNA distribution under each condition. As shown in Figs. 1A and 1C, of the dogs that had received a single vaccination, 33.3–57.7% exhibited protective VNA levels within 12 months of vaccination. The geometric mean of VNA in these dogs was 0.43–0.83 IU/ml; however, 13 months after the vaccination, 76.9% of the single vaccination dogs exhibited protective VNA levels.

In contrast, 97.3–100% of dogs that had received multiple vaccinations exhibited protective VNA levels for up to 24 months after the last vaccination (Figs. 1B and 1C). The geometric mean of VNA was elevated substantially within 18 months after the last vaccination

Table 1. Relationship between the VNA levels and number of rabies vaccination

No. of vaccinations	No. of samples	Average age (yr)	Average days after vaccination	Geometric mean of the VNA level (IU/ml)	No. of samples with VNA ≥ 0.5 IU/ml (%)
0	107	0.22	—	0.12	1/107 (0.9)
1	92	2.25	156	0.61	47/92 (51.1)
2–	557	8.08	233	7.86	545/557 (97.8)
2	78	4.09	239	7.49	75/78 (96.2)
3	71	5.76	187	10.59	69/71 (97.2)
4	59	6.36	268	9.53	58/59 (98.3)
5	67	7.06	246	10.17	65/67 (97.0)
6	56	8.30	238	8.82	56/56 (100)
7	42	8.14	274	8.35	40/42 (95.2)
8	30	9.57	267	9.71	30/30 (100)
9	42	10.38	238	5.44	42/42 (100)
10	42	11.22	209	8.55	42/42 (100)
11	25	12.11	241	4.34	24/25 (96.0)
12	19	12.96	236	4.71	19/19 (100)
13–	26	14.43	177	3.04	25/26 (96.2)