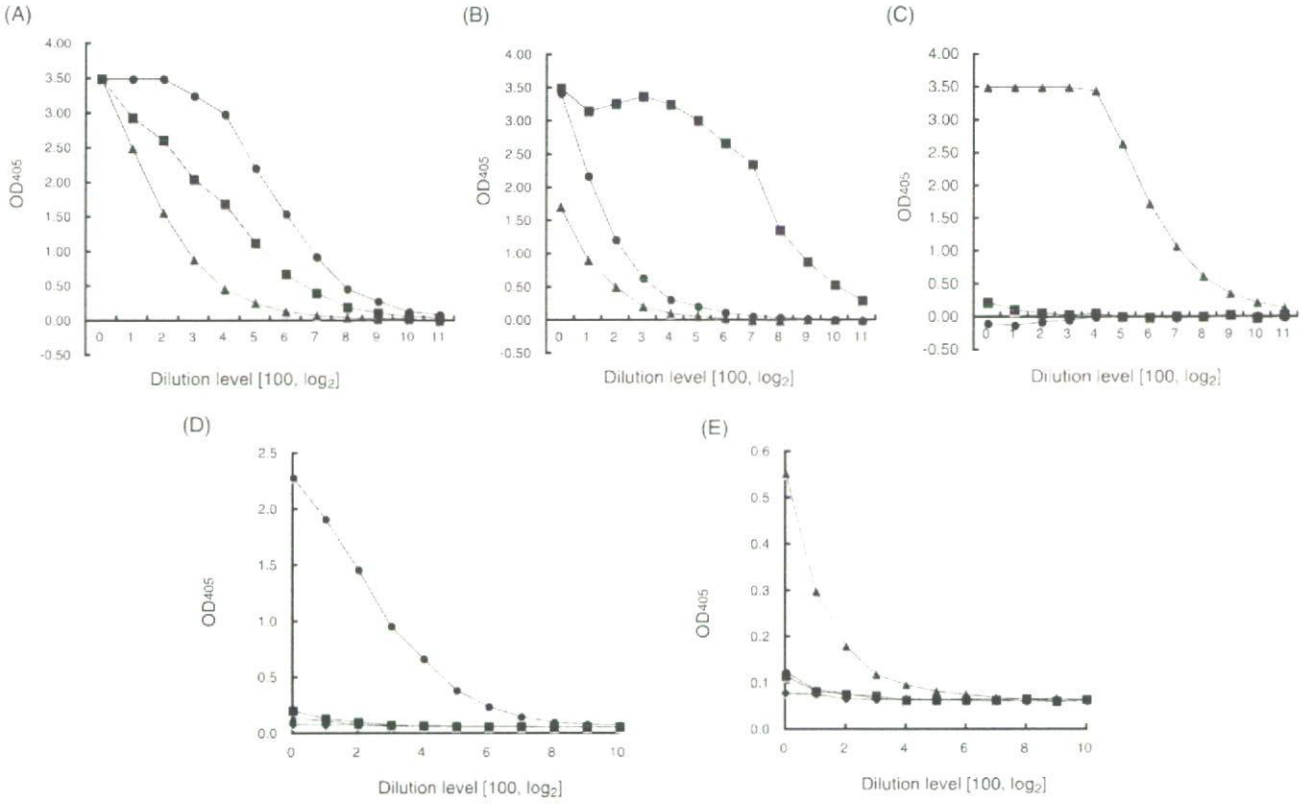


FIG. 4. Reactivity of antibodies to arenaviruses (LASV, LCMV, and JUNV) to the rNPs of these viruses. The reactivities of rabbit sera raised to LASV-rNP (●), LCMV-rNP (■), or JUNV-rNP (▲) with the antigens His-LASV-rNP (A), LCMV-rNP (B), and JUNV-rNP (C) in an IgG-ELISA are shown. The reactivities of the sera collected from patients with LF (D) and AHF (E) with the antigens LASV-rNP (●), LCMV-rNP (■), and JUNV-rNP (▲) and negative control antigen (◆) in an IgG-ELISA are also shown.

treatment, whereas the rNPs of these viruses remained insoluble. After centrifugation at 15,000 × *g* for 10 min, pellet fractions were collected. The rNPs, which were still present in the pellet fractions, were completely solubilized in PBS–8 M urea. The samples were then centrifuged at 15,000 × *g* for 10 min, and the supernatant fractions of the PBS–8 M urea were confirmed to contain highly purified recombinant rNPs of arenaviruses (Fig. 2).

Development of indirect immunofluorescence. The LASV-rNP was expressed in HeLa cells by transfection with the expression vector, pKS336-LASV-NP. The transfected cells were stained by anti-His-LASV-rNP rabbit serum and human serum samples from LF patients (Fig. 3). All 4 serum samples collected from two LF patients showed a positive staining, but 96 control serum samples did not. The LASV-rNP-based IIFA was also evaluated using serum samples collected from monkeys experimentally infected with LASV. All of the sera collected from five LASV-infected monkeys showed a positive staining, but those from four mock-infected monkeys did not.

Development of His-LASV-rNP-based IgG-ELISA. Four serum samples collected from LF patients were determined to be positive by His-LASV-rNP-based IgG-ELISA, whereas 94 of the 96 control serum samples were determined to be negative. Thus, the sensitivity and specificity of the ELISA were 100 and 96%, respectively. All serum samples collected from five LASV-infected monkeys were determined to be positive, whereas those from four mock-infected monkeys were negative.

In order to examine cross-reactivity among arenaviruses in the LASV-rNP-based IgG-ELISA, antisera against LASV-rNP, LCMV-rNP, or JUNV-rNP were examined (Fig. 4). The anti-LASV-rNP serum showed a strongly positive reaction, and anti-LCMV rNP and anti-JUNV-rNP sera showed strongly positive reactions in the IgG ELISA using the respective antigens (Fig. 4A, B, and C). Anti-LCMV-rNP and anti-JUNV-rNP sera showed a less strongly positive reaction in the His-LASV-rNP-based IgG-ELISA than anti-LASV-rNP serum (Fig. 4A). Anti-LASV-rNP and anti-JUNV-rNP also showed a less strongly positive reaction in the His-LCMV-rNP-based IgG-ELISA than anti-LCMV-rNP serum (Fig. 4B). However, anti-LASV-rNP and anti-LCMV-rNP sera showed a negative reaction in the JUNV-rNP-based IgG-ELISA (Fig. 4C). Human sera from LF patients showed a highly positive reaction in the LASV-rNP-based IgG-ELISA, but sera from patients with Argentine hemorrhagic fever (AHF), which is caused by JUNV, did not (Fig. 4D). Serum from an AHF patient showed a highly positive reaction in the JUNV-rNP-based IgG-ELISA (Fig. 4E). These results suggest that cross-reactive antibody among arenaviruses may be detected by the newly developed LASV-rNP-based IgG-ELISA.

Development of LASV Ag-capture ELISA. Three clones of a hybridoma that excreted an MAb to His-LASV-rNP were es-

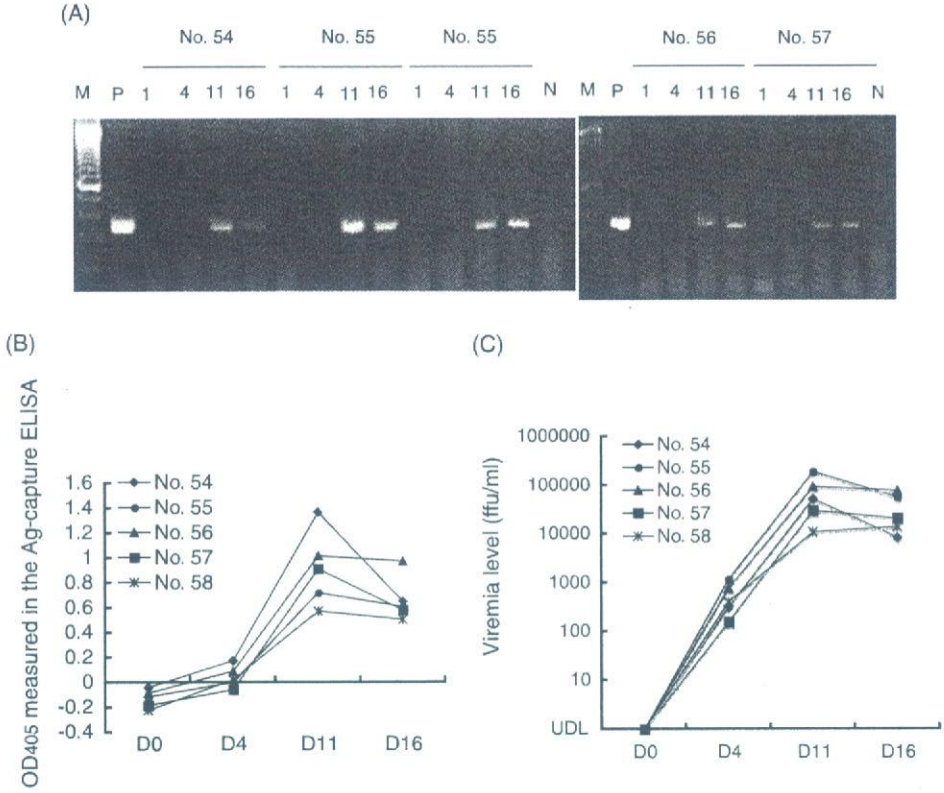


FIG. 5. Detection of the LASV genome by the RT-PCR (A), LASV-NP by the LASV-NP-Ag-capture ELISA (B), and the infectious dose of LASV (C) in serially collected sera of hamsters experimentally infected with LASV. The OD_{405} values in panel B were obtained at a dilution of 1:40.

tablished. The isotype of the three MAbs were identified as IgG1. These MAbs were designated MAb 4A5, MAb 6C11, and MAb 2-11. Of these MAbs, MAb 4A5 was the most efficient in capturing His-LASV-rNP in the Ag-capture ELISA format. The Ag-capture ELISA with MAb 4A5 detected His-LASV-rNP concentrations as low as 800 pg/ml (data not shown). Furthermore, the Ag-capture ELISA detected the MOPV-NP but not the rNPs of LCMV and JUNV (data not shown).

All of the sera collected from five LASV-infected hamsters on days 11 and 16 postinfection were antigen positive in the Ag-capture ELISA using MAb 4A5 as a capture antibody, whereas the sera collected on days 0 and 4 were antigen negative. The OD_{405} values in the ELISA were highest on day 11. The reactivity patterns in each hamster in the ELISA were similar to the viremia levels (Fig. 5). The sera collected on days 11 and 16 were found to be LASV genome positive by RT-PCR (10). Thus, the sensitivity of the Ag-capture ELISA was similar to that of the RT-PCR.

Determination of the epitope recognized by the monoclonal antibodies. The epitope recognized by MAbs was determined. MAb-4A5 reacted in Western blots with GST-LASV-rNP1-6 (full-length LASV-rNP), GST-LASV-rNP1-5, and GST-LASV-rNP1-4 but not with the other truncated LASV-rNPs shown in Table 1, suggesting that MAb 4A5 reacted with a conformational epitope located on the amino-terminal portion of LASV-rNP. The epitope was maintained when the extreme amino-terminal portion, LASV-rNP1, was present but was lost when LASV-rNP1 was removed. These results suggest that the extreme amino-terminal portion, LASV-rNP1, is essential for the maintenance of the conformational epitope. MAbs 6C11 and 2-11 reacted in Western blots with GST-LASV-rNP1 and GST-LASV-rNP5, respectively (Table 1).

The Pepscan analyses indicated that MAbs 6C11 and 2-11

TABLE 1. Reactivities of the MAbs developed in the present study with the GST-tagged truncated LASV-rNP in Western blot analyses

Truncated LASV-rNP	Reactivity with MAb[a]:		
	6C11	4A5	2-11
LASV-rNP1	+	–	–
LASV-rNP2	–	–	–
LASV-rNP3	–	–	–
LASV-rNP4	–	–	–
LASV-rNP5	–	–	+
LASV-rNP6	–	–	–
LASV-rNP1-2	ND	–	ND
LASV-rNP1-3	ND	–	ND
LASV-rNP1-4	ND	+	ND
LASV-rNP1-5	ND	+	ND
LASV-rNP1-6[b]	ND	+	ND
LASV-rNP2-7	ND	–	ND
LASV-rNP3-6	ND	–	ND
LASV-rNP4-6	ND	–	ND
LASV-rNP5-6	ND	–	ND

[a] "+" and "–" indicate positive and negative reactions, respectively. ND, not determined.

[b] LASV-rNP1-6 indicates LASV-rNP.

TABLE 2. Reactivities of the MAbs developed in the present study with the NPs of LASV, MOPV, LCMV, and JUNV in Western blot analyses

MAb	Reactivity of MAb[a] with NP of:			
	LASV	MOPV	LCMV	JUNV
4A5	+	+	–	–
6C11	+	ND	+	–
2-11	+	ND	–	–

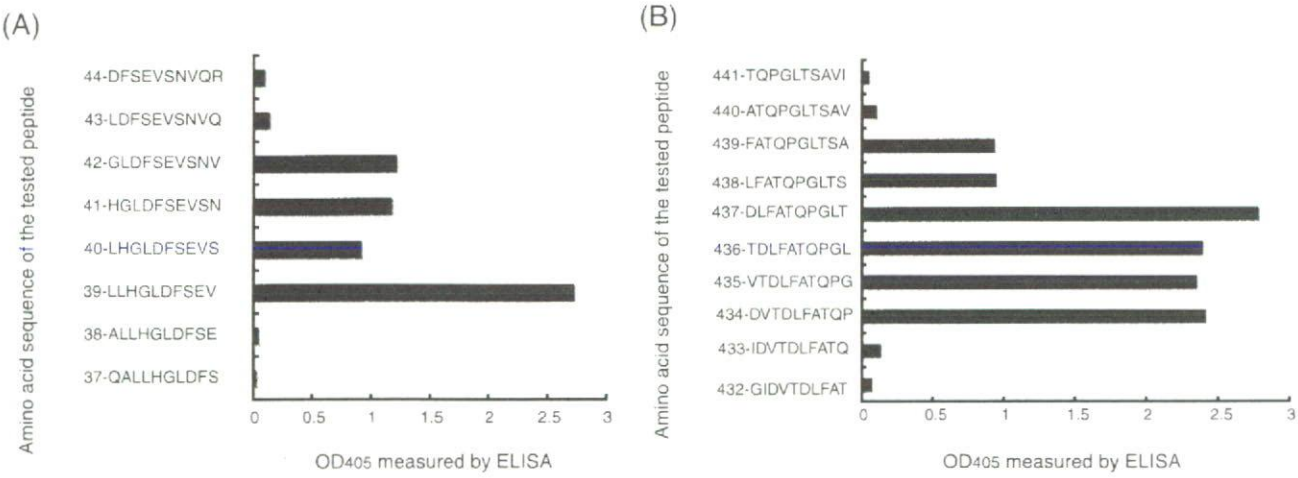
[a] "+" and "–" indicate positive and negative reactions, respectively. ND, not determined. The reactivities of MAb 6C11 and MAb 2-11 were not evaluated with MOPV-NP. However, theoretically, MAb 6C11 should be reactive with MOPV-NP due to the presence of the amino acid residues that can react with MAb 6C11, but MAb 2-11 should not react with MOPV-NP due to the absence of the amino acid residues that can react with MAb 2-11.

recognized linear epitopes. MAbs 6C-11 and 2-11 recognized GLDFSEV (aa 41 to 47) within LASV-rNP1 and FATQP (aa 439 to 443) within LASV-rNP5, respectively (Fig. 6). The reactivity patterns of these MAbs with NPs of LASV, MOPV, LCMV, and JUNV are summarized in Table 2.

DISCUSSION

We report here the development of diagnostic systems (antibody and antigen detection systems) for LF using LASV-rNP. The LASV-rNP-based IgG-ELISA was sensitive and specific in detecting anti-LASV-IgG. Although the data were not shown, an IgM-capture ELISA using purified LASV-rNP as an antigen was developed in the same way as that shown in previous reports and detected LASV-IgM antibody (42, 43). All sera collected from LF patients and monkeys infected with LASV showed positive reactions in the LASV-rNP-based IIFA. The staining patterns of the rNP with these sera were granular in the IIFA (Fig. 3), making it easy to distinguish positives from negatives. IIFA using LASV-rNP-expressing HeLa cells was also highly sensitive and specific in detecting LASV-IgG. In the preliminary study, ca. 15% of the sera collected from 334 Ghanaians and only less than 1% of 280 Zambians showed positive reactions in the LASV-rNP-based IgG ELISA (our data). The results are considered to be compatible with the fact that LF is endemic to the western African region, including Ghana, but not to the eastern African region. The LASV-rNP-based antibody detection systems such as ELISA and IIFA were suggested to be useful not only in the diagnosis of but also in the seroepidemiological study of LF.

The LASV-rNPs were expressed by a transformation system in *E. coli* or by recombinant baculovirus systems and have already been applied as antigens in ELISA, Western blotting, and IIFA for the detection of antibodies to LASV (4, 14, 16, 22, 23, 44). In the present study, an Ag-capture ELISA using



(C)

Arenavirus-NP	Amino acid sequence of the corresponding region: MoAb-6C11	MoAb-2-11
LASV-NP	31-VVKDAQALLHGLDFSEVNVQRLMRKERRD	431-HGIDVTDLFATQPGLTSAVI
MOPV-NP	31-VIKDAQALLHGLDFSEVANVQRLMRKEKRD	432-HGIDIQDLFSVQPGLTSAVI
LCMV-NP	31-VIKDATNLLNGLDFSEVSNVQRIMRKEKRD	424-HGMDLADLFNAQPGLTSSVI
JUNV-NP	31-VLKDAKLIADSIDFNQVAQVQRALRKTKRG	422-HGILMKDIEDAMPGVLSYVI

FIG. 6. Pepscan analyses to determine the epitopes of MAb 6C11 (A) and MAb 2-11 (B). The vertical bar indicates the amino acid residues with an amino acid position within the LASV-NP. MAb 6C11 was confirmed to react with 7 aa residues positioned from aa 42 to 48 (GLDFSEV) within LASV-NP1. MoAb-2-11 was confirmed to react with 5 aa residues positioned from aa 439 to 443 (FATQP) within LASV-NP5. (C) The corresponding amino acid residues to the epitope of the MAb 6C11 and MAb 2-11 among MOPV, LCMV, and JUNV are shown. The GenBank accession numbers for the S genes of LASV, MOPV, LCMV, and JUNV are NC_004296, AY772170, AY847350, and DQ272266, respectively. The epitope of the MAb 6C11 is present not only in the nucleoprotein of LASV but also in those of MOPV and LCMV—but not in that of JUNV.

MAbs to LASV-rNP was also developed. Furthermore, detection of the cross-reactive antibody by LASV-rNP-based IgG-ELISA was examined. The results for cross-reactivity indicate that the LASV-rNP-based IgG-ELISA detects not only antibodies to LASV but also those to LCMV.

The Ag-capture ELISA using MAb 4A5 was confirmed to be useful in the detection of authentic LASV antigen in sera serially collected from hamsters infected with LASV. The sensitivity of the MAb 4A5-based Ag-ELISA was similar to that of conventional RT-PCR, the efficiency of which in the diagnosis of LF was previously reported (10). Therefore, the MAb 4A5-based Ag-capture ELISA is regarded as useful in the diagnosis of LF. Unfortunately, the efficacy of the MAb 4A5-based Ag-capture ELISA in the diagnosis of LF was not evaluated using serum samples from patients. Thus, further study is still required. The three MAbs, including MAb 4A5, were characterized, and the corresponding amino acid residues within the nucleoproteins of LASV, MOPV, LCMV, and JUNV to the epitopes of MAb 6C11 and MAb 2-11 are summarized in Fig. 6C. It was of interest that LASV, MOPV, LCMV, and JUNV might be identified by analyses of the reactivity patterns of MAbs 4A5, 6C11, and 2-11 to the nucleoproteins of each virus. The nucleoproteins of all LASV strains circulating in the western and central parts of Africa would be detected by the MAb 4A5-based Ag-capture ELISA, since this ELISA was able to detect MOPV-NP that was different from LASV in terms of genetic and evolutional characteristics.

We have thus far reported the development of antibody and antigen detection systems using the recombinant nucleoproteins of the viruses for Ebola hemorrhagic fever, Marburg hemorrhagic fever, and Crimean-Congo hemorrhagic fever (32, 33, 36–42). Recently, a number of highly pathogenic emerging virus infections in humans appeared, such as Nipah virus encephalitis (8), SARS-coronavirus infections (21, 35), and highly pathogenic avian influenza virus infections (9, 45, 46). The strategy shown here might be applicable to the development of diagnostic systems for severe viral infections whose etiologic agents are highly pathogenic to humans as an alternative method to methods using infectious viruses.

ACKNOWLEDGMENTS

We thank M. Ogata, Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan, for great clerical assistance.

This study was partly supported by a grant-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of Japan; by the Japan Health Sciences Foundation (grant 16171301); and by the Japan Society for Promotion of Science (grant 18591210).

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Comparative Medicine
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17-5

Vol 57, No 3
June 2007

Lymphocytic Choriomeningitis Infection Undetected by Dirty-bedding Sentinel Monitoring and Revealed after Embryo Transfer of an Inbred Strain Derived from Wild Mice

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Persistent LCMV infection in wild-derived MAI/Pas mice housed under conventional conditions remained undetected for a decade, despite periodic health monitoring using dirty-bedding sentinels. When MAI/Pas mice were rederived by embryo transfer, recipient mothers produced antiLCMV antibodies, which first revealed the presence of the virus in the colony. Before this information was obtained, MAI/Pas mice had been shipped to another facility, undergone cesarean rederivation there, and been introduced into the recipient barrier. The foster mothers of rederived pups were LCMV-negative according to enzyme-linked immunosorbent assay, but sera of both cesarean-rederived MAI/Pas mice and their foster mothers were positive for LCMV infection by immunofluorescent assay (IFA). LCMV was isolated from the MAI/Pas mice, and its genomic RNA was sequenced. Examination of animal technicians in contact with LCMV-infected mice and of other mouse samples by IFA or a reverse transcriptase-polymerase chain reaction test (or both) revealed that neither the workers nor other animals had been infected with LCMV. Experimental data showed that LCMV transmission from persistently infected mice to naïve ones occurred only after direct contact of animals housed in the same cage. This experience demonstrates the importance of careful viral monitoring in the transfer of laboratory rodents between institutions, the limitation of dirty-bedding sentinels for detection of LCMV infection, and the inadequacy of cesarean rederivation for elimination of enzootic LCMV infection.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescent assay; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; RIKEN BRC, RIKEN BioResource Center; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase–polymerase chain reaction

Most laboratory inbred strains of mice have been established from a small pool of ancestors of 3 major subspecies: *Mus musculus domesticus*, *M. m. musculus*, and *M. m. castaneus*.[3,14,39] The pedigrees of laboratory inbred strains are long known based on historical data and recently have been confirmed by the genotyping of thousands of single-nucleotide polymorphisms. This effort has made allowed a global assessment of genetic relationships among the most commonly used inbred strains.[1,25,39] Compared with human populations, inbred mice do not exhibit a great variety of natural genetic polymorphisms. Furthermore, most strains of inbred mice share the same maternal DNA,[10,38] which, together with this lack of polymorphism, may limit the number of potentially informative phenotypes that might be useful models of human conditions or reveal alternative biologic pathways.[39]

This limitation can be overcome by studying stocks or inbred strains derived from recently trapped wild mice that belong to different subspecies or species of genus *Mus*. Such strains have been valuable in establishing high-density genetic maps of the mouse,[5,13] which then served as a foundation for the construction of physical maps.[3,39] François Bonhomme and colleagues in Montpellier, France, have established numerous colonies from various taxa (in particular *M. m. domesticus*, *M. m. musculus*, and *M. spretus*).[3,14] In the 1980s, Jean-Louis Guénet imported several of these colonies to the Institut Pasteur (Paris, France) and maintained them there as closed colonies. These colonies have been used extensively for gene mapping and positional cloning of mouse mutations. These colonies also have proven valuable in the study of various biologic systems, thus leading to dissemination of these mice from the Institut Pasteur to other research institutions worldwide. One of these strains, MAI/Pas, was established from *M. m. musculus* wild mice trapped near Illmitz, Austria, in 1985.[3] The strain name MAI/Pas stands for *M. m. musculus*, Austria, Illmitz, Pasteur.

Of particular concern with wild-derived colonies is the risk of introducing microorganisms that are common in the wild but that should be excluded from research animal facilities.[4,8,12,16,19,20,31,33] Great care must be taken to check the health status of the progenitors, by using sentinel mice or by directly analyzing a sample of

Received: 9 Jun 2006. Revision requested: 25 Sep 2006. Accepted: 20 Dec 2006.
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the trapped specimens. In our experience, decontamination by embryo transfer or cesarean section is often more difficult in these wild-derived colonies than in laboratory strains of mice because of both the decreased viability of embryos after in vitro manipulation and the increased difficulty of obtaining vigorous, live-born neonates.

One of the pathogens currently found in wild rodents is lymphocytic choriomeningitis virus (LCMV), a prototypic member of the arenaviridae family.[19,20,31-33,35] LCMV is maintained in nature by lifelong persistence in wild mice but is transmissible to a broad range of mammalian hosts, including humans, by direct contact with chronically infected rodents.[9,33] Human infections are usually subclinical or mild, but a minority of cases result in lethal meningoencephalitis.[6,11,12,15,17,24,30] Adult mice infected with LCMV develop an immune response and clear the infection after a period of viremia. However, when mice are infected by intrauterine transmission, or when neonates are experimentally infected, the animals fail to respond to the virus, which then persists throughout their life and is chronically excreted at a high titer in the urine.[33] Because of these peculiar properties, LCMV has been used to study acute and persistent infections and the role of the immune system in viral pathogenesis. This work has contributed to several basic concepts in modern virology and immunology.[20,40]

Before MAI/Pas mice were introduced into the Institut Pasteur barrier breeding facility, they were rederived by embryo transfer. This process revealed the presence of LCMV in a colony of MAI/Pas mice. Within this colony, the infection had remained undetected for more than 10 y despite health monitoring using sentinel mice exposed to dirty bedding. Before LCMV was detected, infected MAI/Pas mice had been transferred from the Institut Pasteur to RIKEN BioResource Center (BRC) in Tsukuba, Japan, where the mice were kept in conventional and barrier facilities. Cesarean rederivation was attempted at RIKEN BRC, but LCMV was detected in the rederived pups. The LCMV was isolated, its genomic RNA was sequenced, and a reverse transcriptase–polymerase chain reaction (RT-PCR) assay was established for detection of LCMV. This experience reveals the substantial difficulties in diagnosing and monitoring enzootic LCMV infection in a mouse colony and indicates the need for advanced technology to aggressively re-examine the effectiveness of sentinel programs.

Materials and Methods

MAI/Pas mice. The colony of MAI/Pas mice was established in 1990 from *M. m. musculus* progenitors from François Bonhomme (Montpellier, France) and has been maintained at the Institut Pasteur since then as a closed colony of 18 pairs or trios. Although breeding did not strictly follow typical guidelines for inbreeding, mice sharing the same grandparents were mated for more than 45 generations, so that this colony can be considered highly inbred. MAI/Pas mice were housed in a room that also contained 6 other wild-derived inbred strains, as part of the Institut Pasteur Genetic Repository. Breeding pairs have been sent to several research institutions, including RIKEN BRC, during the last decade.

Humane animal care and use. All animal experiments were approved by the animal care and use committee of each organization and performed according to institutional and university regulations. Virus infection experiments using mice were performed at National Institute of Infectious Diseases (Tokyo, Japan) and Nagasaki University (Japan) under Biosafety Level 3 regulations. Mice were euthanized by exposure to increasing concentrations of carbon dioxide, ether overdose, or cervical dislocation.

Health monitoring at the Institut Pasteur. For the past decade, MAI/Pas mice have been maintained in filter-top cages with pine shavings as bedding, autoclaved water, and irradiated food. Cages were changed once each week on a tabletop. Forceps were used to handle mice; 2 forceps were alternated and disinfected between cages. Animal technicians wore disposable clothing and gloves. Dirty cages were replaced with autoclaved cages prefilled with bedding.

Two health-monitoring cycles were completed every year. A single sentinel cage with 2 NMRI mice (Charles River Laboratories France, Les Oncins, France) was assigned to a group of 108 cages (18 rows of 6 cages). Every week, sentinel mice were placed in a dirty cage from one of the 18 rows, taken serially according to a pre-established scheme. The location of the sampled cage in the row changed to eventually allow sampling of all cages on the row. After 6 mo of exposure, the 2 sentinels were analyzed for viruses, parasites, and bacteria according to recommendations from the Federation of European Laboratory Animal Science Associations[22] and replaced with new sentinels for the next health-monitoring campaign. However, testing for LCMV and other seldom-identified viruses was done only once a year (Table 1). Because the cages containing the MAI/Pas colony occupy 3 rows, sentinel mice were exposed to soiled bedding from MAI/Pas for at least 3 consecutive weeks during the 6-mo period.

LCMV serology by enzyme-linked immunosorbent assay (ELISA). At the Institut Pasteur, antibodies against LCMV were tested by ELISA using precoated plates and reagents purchased from Charles River Laboratories. This ELISA used recombinant LCMV nucleoprotein (NP) antigen obtained from Dr. Michael B. Oldstone at the Scripps Research Institute.[34] ELISA reactions were performed according to the manufacturer's instructions. Briefly, a skilled animal technician collected blood samples from the retro-orbital sinus of unanesthetized mice, and sera were obtained from these samples. Precoated plates were thawed and saturated for 15 min at room temperature in blocking buffer containing nonfat dry milk (Blotto, Pierce Biotechnology, Rockford, IL). After blocking buffer was removed, mouse sera were diluted 1:60 in phosphate-buffered saline (PBS) containing the blocking agent, added to wells, and incubated for 45 min at 37 °C. Plates were washed at room temperature and goat antimouse antibody conjugated to peroxidase (Charles River Laboratories France, Les Oncins, France) was diluted 1:7000 and added for 40 min. Plates were washed again, and substrate (0.4 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), 2 mM H_2O_2) was added for 40 min. The reaction was stopped by the addition of 0.12% fluorhydric acid. Plates were read in an ELISA spectrometer at 405 nm. Each sample was tested in 2 wells, with and without viral antigen. Positive and negative reference sera and PBS were used as controls and for establishing the threshold for positivity, according to the manufacturer's recommendations.

Between April 2002 and November 2005, the ICLAS Monitoring Center performed LCMV serology by an ELISA method using LCMV NP of WE strain[18] as an antigen. Although the ELISAs used at the ICLAS Monitoring Center and the Institut Pasteur both used recombinant LCMV NP antigens, the 2 gene constructs and antigens were prepared independently. The IFA method using LCMV WE-infected L cells (prepared by Nagasaki University) had been used before April 2002 and was chosen again after November 2005. All clinical samples (84,527 samples) tested by ELISA during April 2002 to November 2005 had been stored fro-

Table 1. Pathogens assessed during routine health monitoring of MAI/Pas mice

Pathogen	Frequency of evaluation at	
	Institut Pasteur[a]	RIKEN BRC[b]
Mouse hepatitis virus	6 mo	3 mo
Mouse rotavirus (enzootic diarrhea of infant mice)	6 mo	On request
Minute virus of mice	6 mo	On request
Mouse parvovirus	6 mo	On request
Pneumonia virus of mice	6 mo	On request
Sendai virus	6 mo	3 mo
Theiler murine encephalomyelitis virus	6 mo	On request
Ectromelia virus	Annually	3 mo
Lymphocytic choriomeningitis virus	Annually	3 mo
Hantaan virus	Not done	3 mo
Mouse adenovirus type 1 (FL)	Annually	On request
Mouse adenovirus type 2 (K87)	Annually	On request
Mouse cytomegalovirus	Annually	On request
Reovirus type 3	Annually	On request
Cilia-associated repiratory bacillus	Not done	3 mo
*Citrobacter rodentium**	3 mo	3 mo
Clostridium piliforme (Tyzzer disease)*	3 mo	3 mo
*Corynebacterium kutscheri**	3 mo	3 mo
Mycoplasma spp.*	3 mo	3 mo
*Mycoplasma pulmonis**	3 mo	3 mo
Pasteurellaceae*	3 mo	Not done
*Pasteurella pneumotropica**	3 mo	3 mo
Salmonella spp.*	3 mo	3 mo
Streptococci β-hemolytic (not group D)*	3 mo	Not done
*Streptococcus pneumoniae**	3 mo	Not done
Helicobacter spp.	Not done	3 mo
Helicobacter hepaticus	Not done	3 mo
Helicobacter bilis	Not done	3 mo
Streptobacillus moniliformis	Annually	Not done
Ectoparasites[c]	6 mo	3 mo
Endoparasites[c]	6 mo	3 mo

[a]Health monitoring of MAI/Pas mice at the Institut Pasteur nearly matched that recommended by the Federation of European Laboratory Animal Associations.[22] Before rederivation, the MAI/Pas colony was maintained under conventional housing conditions. In that regard, listed pathogens were monitored as indicated, except that bacteriology (*) was done only annually. In the barrier breeding facility, all mice were monitored for and remained free of the listed pathogens.

[b]RIKEN BRC has listed pathogens to be monitored in laboratory mice according to a guide for the transfer and procurement of mice and rats in Japan by the Japanese Association of Laboratory Animal Facilities of National Universities and the ICLAS Monitoring Center.[37] In the barrier facility, listed pathogens were monitored regularly as indicated, and all the mice remained free of them. Pathogens indicated as 'on request' are rare in Japan;[37] therefore these pathogens are examined only when necessary. The cesarean-rederived MAI/Pas colony was tested by the ICLAS Monitoring Center for pathogens all pathogens listed, and all results were negative.

[c]Identified species would be designated.

zen, and all were re-examined by the IFA method (described in detail later).

Embryo transfer of MAI/Pas. To superovulate 4- to 5-wk-old MAI/Pas mice, 5 IU pregnant mare serum gonadotropin (Folligon, Intervet, Beaucouze, France) was injected intraperitoneally and followed 45 to 48 h later by intraperitoneal injection of 5 IU human chorionic gonadotropin (Pregnyl, Organon, Peutaux, France); 1-cell embryos were recovered 12 h after injection of human chorionic gonadotropin. The embryos were washed 3 times in KSOM culture media (Specialty Media, Phillipsburg, NJ) and implanted in the oviducts of 8-wk-old pseudopregnant NMRI mice, which were maintained in positive-pressure isolation caging. Adult mice were anesthetized by intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (15 mg/kg). Pups were weaned at 4 wk of age, and the female recipient mice were underwent complete health-monitoring analysis (Table 1).

Cesarean rederivation of MAI/Pas. In June and December 2004, MAI/Pas mice were shipped from the Institut Pasteur to RIKEN BRC. The mice were pair-housed in filter-top cages (F-Cage, Japan Clea, Tokyo, Japan) within a negative-pressure system (bioBubble, Cololado Clean Room, Fort Collins, CO). The mice were given irradiated food (CE2, Japan Clea) and autoclaved water ad libitum. Fir shavings (Soft Chip, Oriental Yeast, Tokyo, Japan) were used for bedding material. The stage of pregnancy of MAI/Pas mice was determined by abdominal palpation, and full-term fetuses were obtained aseptically after euthanization of the dams by cer-

Table 2. PCR primers, conditions, and product length

	Primer orientation and sequence		PCR conditions	Product length (basepairs)
PCR1-F	Forward	ATT GAA TTC ACA GTG GAT CCT AGG CAT TTG ATT	94 °C for 40 s, 60 °C for 40 s, and 72°C for 75 s; 40 cycles	584
PCR1-R	Reverse	GGC ATT GTG CCR AAY TGA TTG TTC		
PCR2-F	Forward	GTG CAA GTG GTG TRG TAA GRG TTT	94 °C for 40 s, 64 °C for 40 s, and 72 °C for 60 s; 40 cycles	830
PCR2-R	Reverse	AAG ATC CAT GCC GTG TGA RTA CTT		
PCR3-F	Forward	GGC ART TCA TAC AYT TTT ACA GRG AA	94°C for 30 s, 62 °C for 40 s, and 72 °C for 60 s; 40 cycles	476
PCR3-R	Reverse	TTA GAG TGT CAC AAC ATT GGG TCC T		
NP5-001	Forward	TCC ATR AGW GCA CAG TGY GGG GTG AT	95 °C for 15 min; then 30 cycles of 94 °C for 30 s, 60 °C for 90 s, and 72 °C for 90 s; 72 °C for 10 min	590
NP3-001	Reverse	GCA TGG GAR AAY ACR ACA ATT GAY C		

R, A or G; W, A or T; Y, C or T.
Sets of PCR primers used for the identification of LCMV by RT-PCR were PCR1 (PCR1-F and PCR1-R), PCR2 (PCR2-F and PCR2-R), and PCR3 (PCR3-F and PCR3-R).[26] NP5-001 and NP3-001 were newly designed during the course of this study and recognize LCMV Armstrong and WE and the 2 isolates from MAI/Pas tissues.

vical dislocation. The rederived MAI/Pas pups were examined for spontaneous respiration and then were moved to quarantine in a barrier facility, where they were fostered by BALB/cA-*nu*/+ mothers obtained at 6 wk of age from Japan Clea. After 6 wk in quarantine, the foster mothers were examined for health monitoring at the ICLAS Monitoring Center (Table 1). Mouse rooms in the barrier facility at RIKEN BRC were maintained with constant temperature (24 ± 2 °C), relative humidity (55% ± 10%), and ventilation rate (7 air changes hourly) and were equipped with individually ventilated microisolation caging. The mice were supplied with irradiated food, autoclaved water, and paper bedding material. Animal technicians showered prior to the entry into the animal area and wore sterilized clothing.

AntiLCMV antibodies. To generate polyclonal antiLCMV antibodies, 6-wk-old female C57BL/6 mice (Japan SLC, Hamamatsu, Japan) were inoculated intraperitoneally with 1×10^5 focus-forming units LCMV (Armstrong strain) according to the peroxidase–antiperoxidase method using antiLCMV NP rabbit serum.[36] The immunized mice were euthanized by ether anesthesia and bled by cardiocentesis 4 wk after inoculation. Serum was prepared by centrifugation and inactivated at 65 °C for 30 min. Mouse monoclonal antiLCMV antibody (clone M104) was purchased from American Research Products (Belmont, MA).

Immunofluorescent assay (IFA) for LCMV. IFA was carried out according to the protocol described on the RIKEN website.[27] Briefly, Vero E6 and L cells were infected with LCMV Armstrong and WE strains, respectively, the cells were trypsinized, washed with PBS, spotted on 14-well Teflon-coated glass slides (AR Brown, Tokyo, Japan), air-dried, and fixed with acetone at room temperature for 5 min. Prepared slides were stored at –80 °C until use. IFA slides using LCMV Armstrong-infected Vero E6 cells were prepared at National Institute of Infectious Diseases, and those using LCMV WE-infected L cells were made at Nagasaki University. Mouse sera were diluted 1:20 with PBS and applied to IFA slides for 30 min at room temperature. After slides were washed with PBS, fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin (heavy and light chain; Southern Biotech, Birmingham, AL) was used to detect primary antibodies under fluorescence microscopy. In some experiments, cells were counterstained with To-Pro3 (Molecular Probes, Invitrogen, Carlsbad, CA) at 1:1000 dilution, and images were recorded by a confocal laser microscope (LSM510, Carl Zeiss Co.Ltd., Tokyo, Japan).

For diagnosis of infection in humans, blood was collected from animal technicians prior to working and used as pairwise and negative controls. Human sera were diluted at 1:20 with PBS and applied to IFA slides as described, fluorescein isothiocyanate-conjugated goat antihuman immunoglobulin G (Zymed Laboratories, San Francisco, CA) was used, and slides were prepared and evaluated at National Institute of Infectious Diseases.

Isolation of LCMV and evaluation of its pathogenicity. Kidneys and spleens from MAI/Pas mice were emulsified in Dulbecco modified minimum essential media (Gibco, Carlsbad, CA) containing 1% fetal calf serum and 60 mg/ml kanamycin to prepare 10% homogenates of the specimens, and 0.1-ml aliquots of the homogenates were inoculated into Vero E6 cell cultures. LCMV infection was identified by IFA using the cultured cells as targets and antiLCMV NP rabbit serum for detection.

In addition, 3-wk-old Crlj:CD1 male mice purchased from Charles River Laboratories Japan were anesthetized with ether and inoculated intracranially with 0.05-ml aliquots of the homogenates. At 6 to 10 d after inoculation, mice showing ruffled fur or hunched posture were diagnosed with meningitis after observation of clonic convulsive seizures that occurred after the sick mice were handled.[2,29] The mice were euthanized by ether overdose after the clinical diagnosis was determined.

RT-PCR and sequencing of LCMV genomic RNA. Virus RNA was extracted from 5-mg samples of spleen and kidney by use of QuickGene RNA tissue kit S (Fujifilm, Tokyo, Japan) by a bead cell disrupter (ZB-50 and MicroSmash MS-100R, Tomy Seiko, Tokyo, Japan) and automated extraction system (QuickGene-800, Fujifilm). cDNA was synthesized by using SuperScript II (Invitrogen, Carlsbad, CA) and random primers (Promega, Madison, WI) according to the manufacturer's instructions. Three overlapping fragments covering the entire small (S) RNA coding region were generated by RT-PCR using reported primers[26] (Table 2). Three PCR products of expected sizes in agarose gel were extracted and directly sequenced with the same primer sets used for their amplification. Additional primer sets were designed from newly generated as well as published sequence data, and PCR amplification was extended to integrate and determine the entire sequence of LCMV S RNA from 2 isolates, M1 and M2, isolated from the spleens of MAI/Pas mice. GENETYX-MAC (version 12.2.0; GE-

NETYX, Tokyo, Japan) was used for the sequence analysis. Two nearly complete sequences of S RNA encoding the viral NP and glycoprotein precursor protein have been identified from M1 and M2 isolates, submitted to the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) and registered under the accession numbers AB261990 and AB261991, respectively.

Establishment of RT-PCR test for detection of LCMV. Using the sequence data from the isolated LCMV, we designed a degenerate primer set (NP5-001 and NP3-001, Table 2) capable of detecting the NP region of LCMV Armstrong, WE, and the 2 isolates derived from the MAI/Pas colony; glyceraldehyde-3-phosphate dehydrogenase also was amplified from the same templates as a control. Amplified products were separated by 1% agarose gel electrophoresis and visualized by ethydium bromide staining under UV irradiation. 100-basepair ladder marker (Invitrogen, Calsbad,CA) was used to estimate product size. This RT-PCR test was utilized for repeat evaluation of LCMV infection at RIKEN BRC. The detailed protocol for the RT-PCR assay for LCMV is available on the RIKEN BRC website.[28]

Results

LCMV infection undetected by routine health monitoring. Although sporadic microbiologic controls had been performed earlier, systematic periodical health monitoring was implemented at the Institut Pasteur since 1996. The monitoring program used exposure of sentinel mice to dirty bedding. Every week, each cage of sentinel mice received dirty bedding from 1 of 108 cages. Because the MAI/Pas colony was maintained in 18 cages (that is, 3 rows) in this 108-cage rack, sentinel mice received dirty bedding from a cage located in 1 of these 3 rows (of 18 rows) sequentially for 3 consecutive weeks at least once during the 26-wk duration of each health-monitoring cycle. LCMV was never detected—neither in the animal room where the MAI/Pas colony was maintained nor in any other animal room—from 1996 through 2005. Notably, none of the research institutions that received MAI/Pas mice reported detection of LCMV infection in this strain or in their animal facility after the introduction of MAI/Pas mice.

LCMV infection detected after embryo transfer. Transfer of the MAI/Pas colony from the conventional facility into the barrier breeding facility at the Institut Pasteur required embryo transfer to decontaminate the mouse strain. The first recipient female gave birth to 6 (4 male and 2 female) pups. Assessment of the recipient female after weaning revealed a high titer of antibodies against LCMV by ELISA (Charles River Laboratories); these results were confirmed by IFA. AntiLCMV antibodies also were found in all progeny at 5 wk of age and were assumed to be of maternal origin. These data validate the ELISA test used at the Institut Pasteur for the detection of LCMV infection in the MAI/Pas colony and support its use for further investigations.

Detection of persistent infection in the MAI/Pas colony at the Institut Pasteur. Blood samples then were collected from 1 adult mouse in each of the 18 cages of the MAI/Pas breeding colony and were submitted for serologic examination by ELISA. All 18 sera were negative for LCMV. Similar examinations of all cages of the other strains maintained in the same animal room did not reveal LCMV-positive sera. We formulated the hypotheses that MAI/Pas mice were persistently infected, mothers transmited the infection to their progeny either during gestation or just after birth, and the virus was excreted from infected mice in low titer or was labile in the cage environment.

To test these hypotheses, we introduced an NMRI female mouse into each of the 18 breeding cages. After 4 wk of direct contact, all 18 of these female mice were strongly positive for antiLCMV antibodies by ELISA. These results supported the hypothesis that MAI/Pas mice were persistently infected and were in fact excreting LCMV in their secretions, such as urine and saliva. Efficient transmission and infection occurred when naïve individuals were placed in the same cage with infected mice for 4 wk. As soon as this result was obtained, all MAI/Pas mice were placed in negative-pressure isolation caging. Simultaneously, all laboratories that had received MAI/Pas mice (including RIKEN BRC) were alerted.

Using the procedure of direct contact with sentinels, the Institut Pasteur group confirmed that LCMV infection had not spread to the other strains maintained in the same animal room. In addition, serologic examination of the animal technician who had cared for the MAI/Pas colony for more than 9 y and of the animal facility personnel who had been in contact with dirty bedding from this animal room revealed that all 6 persons tested were negative for LCMV, indicating that the use of protective gear such as disposable clothing and gloves had been sufficient to prevent human contamination over a long period of time.

Possible elimination of LCMV infection from the MAI/Pas colony by embryo transfer. To determine whether the progeny born from embryo transfer carried the virus, 1 sentinel mouse was introduced into each of 4 cages containing individually housed, 2-mo-old, rederived MAI/Pas progeny, in negative-pressure isolation caging at the Institut Pasteur. After 4 wk, all contact sentinel mice were negative by ELISA for antiLCMV antibodies, strongly suggesting that the progeny were not persistently infected. Our results suggested that embryo transfer could be used to eliminate the persistent LCMV infection in the mouse colony.

Rederivation of imported MAI/Pas strain at RIKEN BRC. Breeding pairs of the MAI/Pas mice were received from the Institut Pasteur and maintained in the conventional facility at RIKEN BRC to prepare pregnant dams for cesarean rederivation. Neonates were collected aseptically by cesarean section from 5 pregnant mice and were fostered by BALB/cA-*nu*/+ mothers for 6 wk in the quarantine area of the barrier facility. After neonates were weaned, foster mothers were transported to the ICLAS Monitoring Center and examined with a complete set of microbiologic tests (Table 1). Because none of the 5 foster mothers were positive for any tested organism, 3 litters of rederived MAI/Pas mice were introduced into the barrier facility in individually ventilated microisolation caging. The remaining 2 litters of rederived MAI/Pas mice were retained in the quarantine room because RIKEN BRC had received the LCMV contamination alert from the Institut Pasteur before they were moved into the barrier. The health status of the mice in the barrier facility was monitored 4 times per year at the ICLAS Monitoring Center by using sentinel mice that received dirty bedding from all cages in each rack every 2 wk at cage change. The health reports of the sentinel mice indicated that the MAI/Pas colony in the barrier facility was LCMV-negative.

Detection of LCMV in the MAI/Pas colony at RIKEN BRC. After notification of the LCMV infection at the Institut Pasteur, RIKEN BRC re-examined the LCMV status of its MAI/Pas colony by using the frozen sera from the 5 BALB/cA-*nu*/+ foster mothers. The ICLAS Monitoring Center previously had performed an ELISA test using recombinant NP antigen of LCMV WE strain.[18] Although the ELISA used at the Institut Pasteur successfully detected LCMV infection, the ELISA used by the ICLAS Monitoring Center did not detect antiLCMV antibodies in the BALB/cA-*nu*/+ foster mothers. Meanwhile, the Nagasaki University group

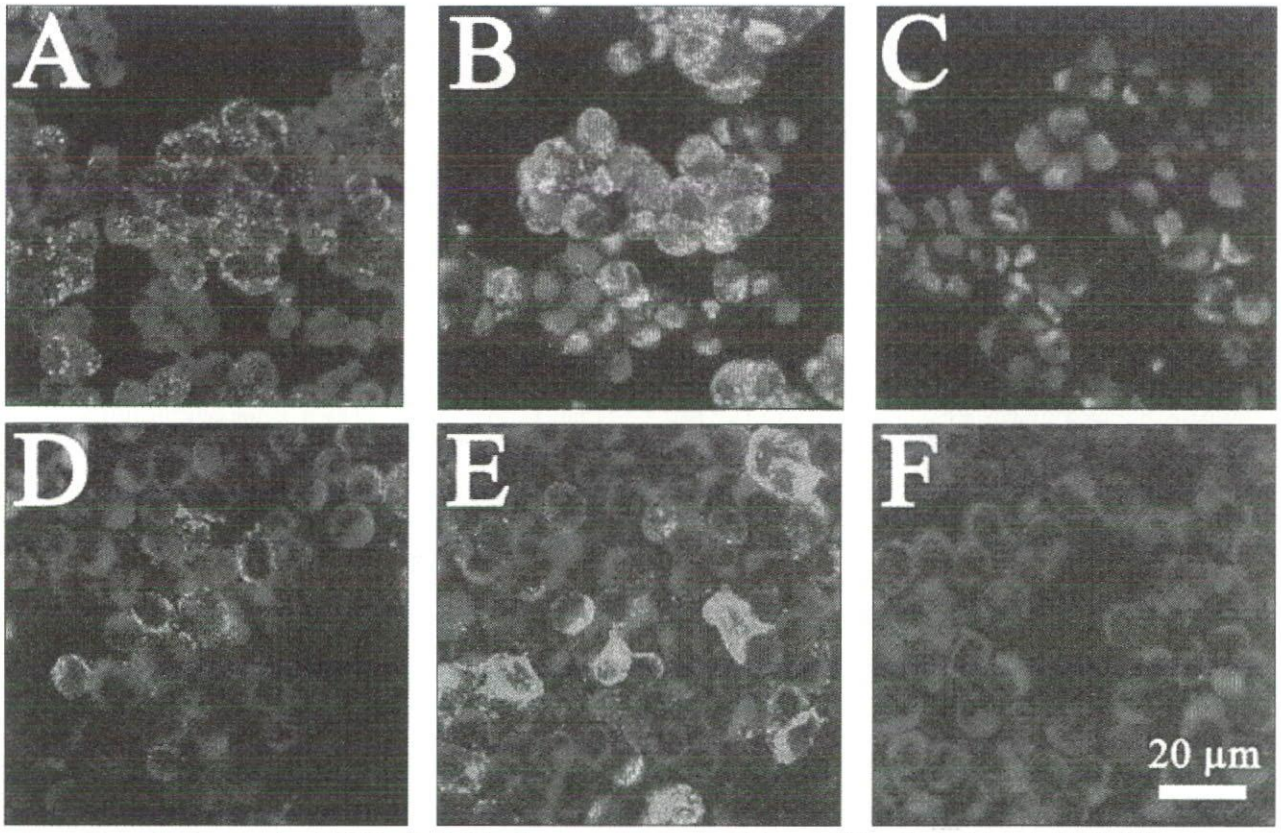

Figure 1. IFA test for detection of antiLCMV antibody. IFA slides were prepared using (A through C) Vero E6 cells infected with LCMV Armstrong and (D through F) L cells infected with LCMV WE. (A and D) Serum from a BALB/cA-*nu*/+ mouse caged with MAI/Pas mouse showed positive staining for LCMV. (B and E) Strong signals were detected with antiserum raised against LCMV Armstrong. (C and F) Preimmune normal mouse serum was used as a negative control.

detected high-titer antiLCMV antibodies in 4 of the 5 BALB/cA-*nu*/+ foster mothers by using IFA slides made from L cells infected with LCMV WE strain. These results indicated the necessity of further re-evaluation regarding the recombinant LCMV antigen in the ELISA used at the ICLAS Monitoring Center. Consequently IFA was used to detect LCMV for the remainder of the diagnostic effort at RIKEN BRC.

Serodiagnosis of human LCMV infection was done by IFA at National Institute of Infectious Diseases. None of the 22 animal technicians from RIKEN BRC who were tested were positive for LCMV infection.

Isolation of virus and determination of its genomic RNA sequence. At Nagasaki University a single LCMV isolate was obtained from the kidney tissue of a MAI/Pas mouse. Intracranial inoculation revealed that this isolate caused typical meningitis and mortality. In addition, LCMV isolates M1 and M2 were obtained at National Institute of Infectious Diseases from the spleen tissues of MAI/Pas mice. Nearly complete sequences (3381 basepairs) derived from genomic RNA encoding NP and glycoprotein precursor protein regions (S RNA) were determined for the LCMV M1 and M2 isolates and were found to be identical. Nucleotide and amino-acid sequences of WE, Armstrong 53b, and LCMV M1 were aligned. At the nucleotide level, Armstrong 53b and M1 were 78.9% homologous, WE and M1 were 78.8% homologous, and Armstrong 53b and WE were 84.7% homologous. At the amino-acid level, Armstrong 53b and M1 were 93.2% homologous, WE and M1 were 93.2% homologous, and Armstrong 53b and WE were 95.7% homologous. Taken together the clinical observation of experimental infection, specific antibody production and amino-acid level homology, these data showed that the virus isolated from the MAI/Pas colony belonged to the LCMV group but, from nucleotide level homology data, was genetically distinct from the Armstrong and WE strains.

Re-examination of MAI/Pas colony at RIKEN BRC. Five cesarean sections were performed for the rederivation of MAI/Pas mice. Preserved sera of the foster mothers showed 4 highly positive results and 1 negative result by IFA against LCMV (Figure 1), confirming the results obtained at Nagasaki University. In addition, 22 MAI/Pas progeny derived from those 5 cesarean sections and 3 BALB/cA-*nu*/+ mice that had been caged with MAI/Pas mice for 12 days were further analyzed by RT-PCR and IFA (Table 3). The results showed that all 22 MAI/Pas progeny tested were positive for LCMV and the 3 BALB/cA-*nu*/+ all were negative by RT-PCR. However, antiLCMV antibodies were detected in 27.3% of MAI/Pas mice and 33.3% of BALB/cA-*nu*/+ mice. These data suggested that cesarean section had not eliminated LCMV and that the MAI/Pas progeny were vertically infected with the virus. Among the 22 MAI/Pas progeny that produced antiLCMV anti-

Table 3. Results of re-examination of MAI/Pas colony by RT-PCR and IFA

Antibody (IFA)	Virus (RT-PCR)	MAI/Pas	BALB/c
positive	positive	6 (27.3%)	0 (0%)
positive	negative	0 (0%)	1 (33.3%)
negative	positive	16 (72.7%)	0 (0%)
negative	negative	0 (0%)	2 (66.7%)

IFA and RT-PCR were used to evaluate LCMV infection among 22 MAI/Pas mice progeny rederived by 5 cesarean sections as well as 3 BALB/cA-*nu*/+ mice caged with MAI/Pas mice for 12 d.

bodies by the age of 5 mo, the antibodies seemed unable to clear the LCMV infection. In addition, 1 BALB/cA-*nu*/+ produced antiLCMV antibodies, but the virus was not detected by RT-PCR. To contain the infection, infected mice were euthanized immediately after detection of the virus.

Overall re-examination of mouse colonies. Serologic procedures revealed that the LCMV infection at the Institut Pasteur had remained strictly confined to the MAI/Pas strain and had not spread to any of the other strains maintained in the same animal room or to related mice strains. At RIKEN BRC 116 serum samples of the foster mothers of 20 wild-derived strains were re-examined by IFA and diagnosed as LCMV-negative. Progeny of those wild-derived strains after cesarean section also were analyzed by RT-PCR using RNA extracted from kidneys. The only LCMV-positive strains detected were the MAI/Pas mice (Figure 2). Meanwhile 84,527 serum samples initially evaluated at the ICLAS Monitoring Center during 2002 through 2005 were re-examined by IFA, which found that all the samples were negative except for the sera of the foster mothers of MAI/Pas mice derived from RIKEN BRC. We conclude that LCMV infection also was contained within the MAI/Pas colony at RIKEN BRC.

Discussion

Mouse strains created from trapped wild mice belonging to different taxa of the *Mus* genus have been for various fields of biologic research including genetics, immunology, oncology, and infectious diseases. However, unlike typical inbred strains of laboratory mice, which have been maintained under highly regulated conditions in animal facilities for decades, wild-derived strains have often been bred in conventional facilities and may harbor pathogens that are circulating among natural populations of wild rodents. Among the likely pathogens in trapped animals is LCMV, which is of particular concern because of the zoonotic risk.

This study has shown that LCMV infection can escape detection from routine health monitoring of sentinel mice exposed to dirty bedding, even when a substantial number of mice are persistently infected. The observation that naïve sentinels placed in the same cage with persistently infected MAI/Pas mice were consistently highly positive for antiLCMV antibodies shows that MAI/Pas mice were able to shed enough of the virus to constitute an infective dose during the 4 wk of cohabitation. In our experience, efficient contamination required close and substantial contact between mice, because sentinel mice that had been exposed to dirty bedding from infected MAI/Pas mice for at least 3 consecutive weeks over a 6-mo period did not develop antibodies. This observation should be kept in mind when designing health-monitoring programs for animals with some risk of LCMV infection. Our data also explain why the infection had not spread to other

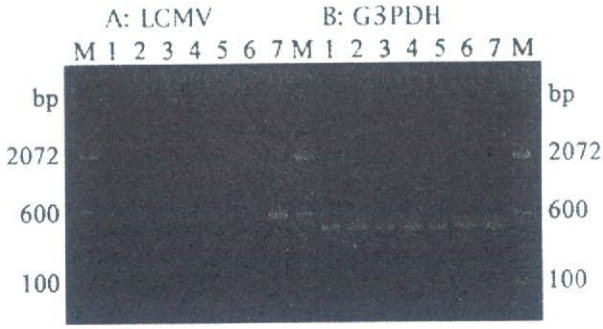

Figure 2. Detection of LCMV by RT-PCR. RNA was extracted from kidneys of mice of the wild-derived strains WMP/Pas (*M. m. domesticus*), SEG/Pas (*M. spretus*), STF/Pas (*M. spretus*), and MAI/Pas (*M. m. musculus*). These strains were transferred from the Institut Pasteur to RIKEN BRC during overlapping periods. (A) The degenerate primers NP5-001 and NP3-001 amplified a 590-basepair fragment of LCMV NP only from the MAI/Pas mouse. Lanes 1 and 2, WMP/Pas; 3 and 4, SEG/Pas; 5 and 6, STF/Pas; 7, MAI/Pas. (B) A 450-basepair fragment of glyceraldehyde-3-phosphate dehydrogenase was amplified uniformly from all samples. M, 100-basepair ladder marker.

breeding colonies in the same animal room over a 10-y period.

Another interesting observation is that the high-titer antibodies against LCMV were detected in recipient mothers after embryo transfer, clearly indicating the difficulty of eliminating LCMV from the embryos and their surrounding media. Although the recipients of the embryos produced antiLCMV antibodies, the progeny rederived by embryo transfer were free from LCMV as tested with direct sentinels. Fazakerley and colleagues[9] reported that LCMV nucleic acid in persistently infected mice was detected in various tissues, including epithelial cell layers of the bladder and the ductus epididymis. Those investigators also reported the presence of virus-associated signals in spermatogonia but not in differentiated germ cells, such as spermatocytes and spermatids, and suggested that transmission of infection by infected sperm was unlikely.[9] We speculate that washing embryos before transfer efficiently reduced or inactivated the virus particles so that the recipient mothers were immunized but not infected. Further improvement of washing methods and use of in vitro fertilization or intracytoplasmic sperm injection[21] will be considered for future use to completely eliminate LCMV contamination. For the time being, careful monitoring of the progeny rederived from embryo transfer must be continued by IFA and RT-PCR to confirm complete elimination of the LCMV.

The ELISA used at the ICLAS Monitoring Center incorporated recombinant antigen from LCMV NP of WE strain[18] and did not detect antiLCMV antibodies in the foster mothers of persistently infected MAI/Pas mice. These foster mothers were strongly LCMV-positive according to IFA. In contrast to that at the ICLAS Monitoring Center, the ELISA performed at the Institut Pasteur, which used different plates and recombinant LCMV NP antigen, revealed high antibody titers in the recipient mothers of MAI/Pas embryos. This result indicated that the ELISA at the Institut Pasteur was highly sensitive. The exact reasons for the low sensitivity of ELISA at the ICLAS Monitoring Center must be clarified by comparing the gene constructs of the expression vectors and preparation procedures for the LCMV NP antigens in the 2 different ELISAs.

Sequence analysis demonstrated that LCMV WE strain was dis-

tinct and genetically divergent from the MAI/Pas-derived LCMV isolates. This difference might substantially diminish the sensitivity of the ELISA test used at the ICLAS Monitoring Center and its ability to detect antibodies raised against the LCMV isolated from the MAI/Pas colony. Thus, the LCMV-infected MAI/Pas mice imported from France accidentally revealed that the sensitivity and utility of the ELISA test performed at the ICLAS Monitoring Center was suboptimal to detect natural infection with LCMV.

The observation that 100% of MAI/Pas progeny of cesarean section rederivation were LCMV-positive by RT-PCR (Table 3) confirms the efficiency of this assay when used with kidney tissue. However, to apply RT-PCR to monitoring persistent infection of LCMV, samples must be collected without euthanisia. Therefore; we must test its efficacy when using blood or urine as samples. In addition, RNA viruses including LCMV use low-fidelity RNA polymerase, such that mutations in its genome occur with high frequency, enabling the virus to adapt to the host environment.[32] Therefore, knowing the applicability of the present primer set to newly emerging and genetically divergent LCMV that have not yet appeared in the public database is difficult. Information regarding the sequences of primers used to detect of LCMV must be updated frequently so that users can evaluate the coverage of the primer set.

Table 3 shows that all 3 BALB/cA-*nu*/+ direct sentinels housed with persistently infected MAI/Pas mice for 12 days were RT-PCR-negative for LCMV. However 1 of the 3 direct sentinels produced high-titer antiLCMV antibodies, indicating that this mouse had been acutely infected and had cleared the virus. Because the RT-PCR results indicated that all MAI/Pas progeny were producing virus, these data suggest either that insufficient shedding of the virus to infect all sentinels within 12 days or lability of the virus in the environment. As the data of the Institut Pasteur showed, direct exposure of sentinel mice to test mice for more than 4 wk should be sufficient to ensure infection of sentinels from mice persistently infected with LCMV. In light of the IFA and RT-PCR test results of MAI/Pas progeny and BALB/cA-*nu*/+ direct sentinels, both a serologic test for acute infection and an RT-PCR test for persistent infection are necessary to efficiently detect LCMV in an animal facility.

All adult MAI/Pas mice tested at the Institut Pasteur were seronegative by ELISA. In contrast, 27% of MAI/Pas progeny from cesarean section produced weak antiLCMV antibodies detectable by IFA (Table 3) at RIKEN BRC. In one report, antiLCMV antibodies that developed in mice chronically infected with LCMV were difficult to detect in the circulation because of an excess of viruses (antigens).[23] These authors suggested that imbalance between the amounts of circulating antibodies and viruses influenced the amount of serologically detectable free antiLCMV antibodies, leading to inconsistent serologic results among animals. The MAI/Pas strain and the LCMV isolated from it would provide a good experimental model to study this possibility.

Previously, a study using IFA to survey LCMV infection in laboratory mice in Japan revealed that approximately 5% of mouse colonies were infected with LCMV.[31] Other reports showed 7% of wild mice trapped in the Yokohama Bay area were LCMV-positive by serology in 1991,[19] and LCMV was isolated successfully from wild mice in the Osaka Bay area.[20] These studies suggest the likelihood of natural LCMV infection in Japanese mouse colonies. However, re-examination by IFA of 84,527 preserved serum samples collected between 2002 and 2005 revealed that all were negative except those from foster mothers of MAI/Pas mice, suggesting that LCMV infection in laboratory mice is rare in Japan, at least during these 4 y. Systematic epidemiologic surveillance of human serology for LCMV has not yet been performed in Japan.

In our case, no animal technicians were infected with LCMV, even at the Institut Pasteur where the MAI/Pas colony was maintained for more than 10 y. This study clearly demonstrates the low infectivity of LCMV in the MAI/Pas colony. Protective measures taken by animal technicians, including wearing masks and disposable gloves and using disinfected forceps when handling the mice, are believed to have contributed greatly to preventing this zoonotic hazard for long periods in our animal facilities. We principally have described the LCMV infection in the MAI/Pas mouse colony. However, hamsters (*Mesocricetus auratus*) are a natural LCMV reservoir, and human LCMV infection from hamster to pet owners and laboratory workers has been reported.[6,33] Furthermore cell lines or tissues contaminated by LCMV cannot be cleared of the virus because of its broad host range.[33] Several LCMV outbreaks have originated from contaminated cell lines or tissues.[8,11,12,15,24]

The number of mouse resources has been growing exponentially for a decade worldwide,[7] and transportation of mouse strains is expected to increase between institutions and countries. Because the distribution of pathogens varies around the globe, excluded pathogens and methods and frequency of testing may differ among institutions, resource centers, and countries. To protect workers and mouse resources from unacceptable infectious agents and zoonotic hazards, we must assess the health monitoring protocol of exporting institutions and understand the limitation of the methods used. The recipient institution is responsible to recipient investigators regarding the health status of transferred mice. International cooperation is necessary to share both health information and the positive controls to establish valid detection systems.

Seventeen resource centers worldwide founded the Federation of International Mouse Resources (http://www.fimre.org) to promote global access to valuable mouse resources. One of the specific goals of this organization is to establish consistent and rigorous animal health standards. Individual mouse resource centers are important in establishing a favorable international environment for the safe and smooth transfer of mouse resources.

Here we report the experiences of the Institut Pasteur, which used embryo transfer, and RIKEN BRC, which used cesarean section, for rederivation of MAI/Pas mice. Both institutions used exposure to dirty bedding as the sentinel system. The details of protocols for embryo transfer, cesarean section, and the sentinel health monitoring program are critical to successful protection against LCMV infection. In addition, the established monitoring system should be tested periodically using samples from naturally infected animals to confirm the effectiveness of the system. Worldwide sharing of information and reagents (including pathogens, antigens, and antisera) would be invaluable.

Acknowledgments

We especially are grateful to Yves Rivière (Institut Pasteur) for sharing his expertise in LCMV epidemiology and biology. We thank the reviewers for their helpful comments and suggestions to improve the manuscript. We thank Kazuo Moriwaki (special consultant to RIKEN BRC) and Jean-Louis Guenet (Institut Pasteur) for their support and Yukiko Kitasaka, Reiko Kojima, Naoki Otaka, Maimi Yuri, and other staff in the Experimental Animal Division (RIKEN BRC) for their devoted cooperation through their efforts in containing the LCMV outbreak. We also thank Kazuko Orimoto (Occupational Health Center, RIKEN Tsukuba Institute, Japan) for her technical assistance. We are grateful to Isabelle

Lanctin for her expertise in breeding wild mice and Yannick Boulet for the embryo transfers. We acknowledge Kazuya Yamanouchi (professor emeritus, University of Tokyo, Japan) and Clarence J Peters (University of Texas, Galveston, TX) for their helpful comments at the beginning of the search in Japan. FI is supported in part by the Strategic Programs for Research and Development (President's Discretionary Fund) of RIKEN. RIKEN BRC collects and preserves mouse resources as a core facility of the National Bio-Resource Project in Japan and is supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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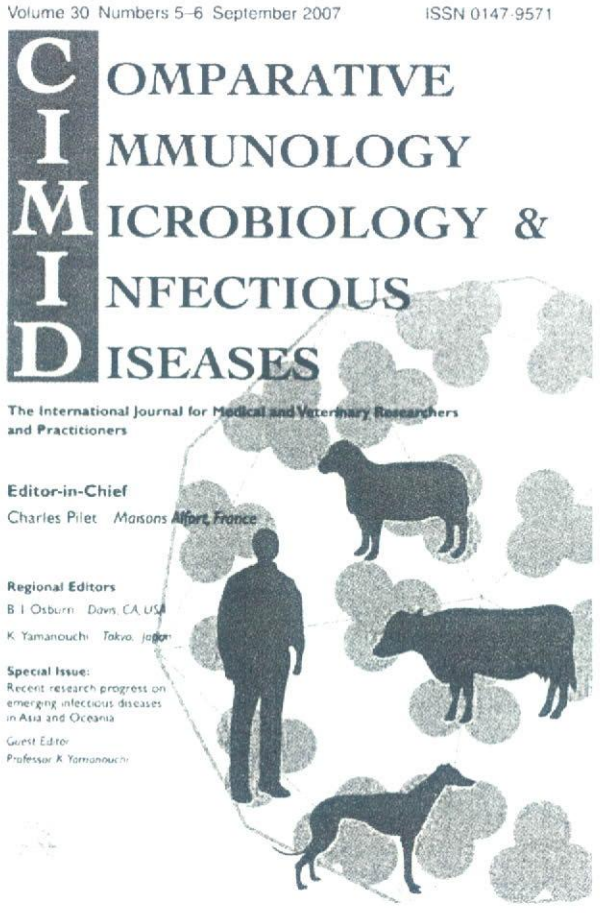

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COMPARATIVE IMMUNOLOGY MICROBIOLOGY & INFECTIOUS DISEASES
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Review

Current knowledge on lower virulence of Reston Ebola virus (in French: Connaissances actuelles sur la moindre virulence du virus Ebola Reston)

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Received 10 February 2007

Abstract

Ebola viruses (EBOV) and Marburg virus belong to the family *Filoviridae*, order *Mononegavirales*. The genus *Ebolavirus* consists of four species: Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SEBOV), Ivory Coast ebolavirus (ICEBOV) and Reston ebolavirus (REBOV). Three species of ebolaviruses, ZEBOV, SEBOV, ICEBOV, and Marburg virus are known to be extremely pathogenic in primates and humans and cause severe hemorrhagic fever leading up to case fatality rate of some 90%, while REBOV is thought to be pathogenic in Asian monkeys but not in African monkeys and humans. Recent studies indicated several factors involved in different virulence between African EBOV and REBOV. This article reviews the history, epidemiology, and virulence of REBOV.

Keywords: Reston ebolavirus; Virulence; Epidemiology

Résumé

Les virus Ebola (EBOV) et le virus Marburg appartiennent à la famille des *Filoviridae*, ordre des *Mononegavirales*. Le *virus Ebola* comporte quatre espèces: le virus Ebola-Zaïre (ZEBOV), le virus Ebola-Soudan (SEBOV), le virus Ebola-Côte d'Ivoire (ICEBOV) et le virus Ebola

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doi:10.1016/j.cimid.2007.05.005

Reston (REBOV). Trois espèces de virus Ebola, ZEBOV, SEBOV, ICEBOV et le virus de Marbourg, sont extrêmement pathogènes chez les primates et les hommes, et se caractérisent par une fièvre hémorragique foudroyante entraînant la mort dans 90% des cas. Le virus REBOV, quant à lui, est pathogène chez les singes asiatiques, mais pas chez les singes africains et l'homme. De récentes études ont montré que la différence de virulence entre le virus africain EBOV et REBOV était liée à plusieurs facteurs. Le présent article analyse l'histoire, l'épidémiologie et la virulence du virus REBOV.

Mots clés: Le virus Ebola Reston; La virulence; Épidémiologie

Contents

1. Introduction

Epizootic caused by Reston Ebola virus (REBOV) was first recognized in 1989 at a quarantine facility in Reston, Virginia where a shipment of cynomolgus monkeys (*Macaca fascicularis*) from the Philippines was quarantined. Introduction of REBOV through infected cynomolgus monkeys from the Philippines was also identified in Philadelphia, Pennsylvania in 1989, Reston and Alice, Texas in early 1990, Siena, Italy in 1992, and Alice in 1996. Epidemiological study in the Philippines showed that REBOV-infected monkeys were originated from one monkey facility but not from other facilities in the Philippines. After the facility was closed in 1997, any epizootic or sporadic case of Ebola hemorrhagic fever-like disease caused by REBOV has not been documented. It is still unclear how the monkeys in the facility were infected with REBOV. Epidemiological study indicated that REBOV was avirulent in humans and experimental infection of nonhuman primates showed that REBOV was less virulent compared to ZEBOV and SEBOV.

Recently, many studies revealed the pathogenesis of ZEBOV especially the impairment of innate and adapted immune response, vascular disfunction and mechanism of virus infection to a variety of cell types. The recent review excellently summarized the current view on pathogenesis [1]. A majority of these studies did not answer the question why REBOV was less virulent compared to African EBOVs, however, several studies implicated the reason why REBOV was less virulent compared to African EBOV. These studies indicated that lower virulence of REBOV was, at least, due to several viral factors.

2. Epizootics and epidemiology of Reston Ebola

In late 1989, an Ebola virus antigenically related to ZEBOV was identified in cynomolgus monkeys (*M. fascicularis*) at a quarantine facility in Reston, Virginia [2–5]. These wild-caught cynomolgus monkeys were imported from the Philippines, and a higher mortality rate was noted in the monkeys during quarantine. The clinical features were characterized by abrupt onset of complete anorexia. Splenomegaly, puffy eyelids, lacrimation, nasal exudates, and coughing were observed in some monkeys. Epistaxis, subcutaneous hemorrhage, dehydration, bloody diarrhea and fever were observed infrequently [6]. Marked increase in lactate dehydrogenase, asparatate aminotransferase and alanine aminotransferase, increase in alkaline phosphatase, blood urea nitrogen, creatine, and triglycerides, and decreased platelet counts were observed [6]. These clinical features were similar to simian hemorrhagic fever, which is caused by infection of simian hemorrhagic fever virus, a member of family *Arteriviridae*. Actually, simian hemorrhagic fever virus was isolated from some monkeys but many of them were also shown to be infected with an Ebola virus.

The virus, named Reston Ebola virus (REBOV), was shown to be closely related but clearly distinct from previously known ZEBOV and SEBOV. Transmission of REBOV among monkeys were mainly caused by direct contact with blood or secretion, or by injection with virus-contaminated syringes, however, droplet or aerosol transmission was also suspected since the disease among monkeys spread within rooms despite discontinuation of all direct contact with animals [6]. Similar outbreak was also reported in Philadelphia, Pennsylvania in 1989, and REBOV was also isolated from monkey specimens. Antigen capture ELISA showed that 52.8% of 161 monkeys that died over a 2.5-month period at export facilities in the Philippines [7]. A case fatality rate of 82.4% was documented for the monkeys infected with REBOV and simian hemorrhagic fever virus at an export facility. Any illness associated with the epizootic in monkeys was not observed in any human, however, four animal handlers at the quarantine facility in Reston had serologic evidence of recent infection with the virus [8,9]. Seroepidemiological survey in the Philippines showed that three out of five workers in the animal hospital at the export facility at Laguna, with which the epizootics in the USA were associated, were positive for REBOV antibodies. In the facility, there was no illness associated with REBOV [10]. These indicated the REBOV can be transmitted to humans when heavily exposed to the infected monkey or its specimens but may not induce illness in humans. In 1990, REBOV was again introduced to primate facilities in Reston, Virginia and Alice, Texas by infected monkeys imported from the same export facility in the Philippines.

In 1992, REBOV was introduced in Siena, Italy and the virus was isolated from monkeys imported from the same export facility the Philippines [11]. It has been reported that all monkeys in the export facility in the Philippines has been destroyed following the epizootics in 1989–1990 [7]. It has been suggested that depopulation of the monkeys was not completely carried out [12].

In 1996, 4 cynomolgus monkeys among 100 monkeys imported from the Philippines were shown to be infected with REBOV in a quarantine facility in Alice, TX, USA [13]. Epidemiological study in the Philippines showed that 113 out of 353 dead or

moribund monkeys collected during May–September in 1996 were REBOV antigen positive, and 18 out of 465 healthy monkey were shown to be antigen positive [12,14]. All the monkeys from the other four facilities were negative for virus antigen. After the complete depopulation of the facility, in which the REBOV-infected monkeys found, was executed by the Philippine government and eventually the facility was closed in 1997. Since then, any epizootic or sporadic case of Ebola hemorrhagic fever-like disease caused by REBOV has not been documented. It is still unclear how the monkeys in the facility were infected. However, a recent study in Gabon and the Republic of the Congo during human and ape Ebola outbreaks between 2001 and 2005 [15,16] has demonstrated ZEBOV RNAs in liver and spleen specimens of three different species of fruit bats, *Hypsignathus monstrosus*, *Epomops franqueti* and *Myonycteris torquata* [17,18]. These bats were asymptomatically infected and their distribution in Africa included regions where human Ebola outbreaks were documented. These findings support earlier studies that indicated bats as potential reservoirs of filoviruses [19,20]. An in-depth survey especially focused on bat species in south Asia is necessary to elucidate reservoir wildlife of REBOV as to whether the virus originated in south Asia or introduced from Africa.

3. Molecular structure of REBOV

Filoviruses contain a single stranded, negative sense RNA genome with approximately 19 kb. The genome of REBOV is composed of seven genes as like other filoviruses; major nucleoprotein (NP), P protein (VP35), matrix protein (VP40), glycoprotein (GP), minor nucleoprotein (VP30), minor matrix protein (VP24) and RNA-dependent RNA polymerase (L) [21,22]. Phylogenetically REBOV is clearly distinct from other EBOVs, even though REBOV is relatively related to SEBOV. Nucleotide and amino acid sequences among REBOV isolates were highly conserved. However, significant differences in GP sequence were observed among REBOVs isolated in different year, while no amino acid substitutions were observed within the different isolates in the same year. This strongly indicated independent introduction of REBOV into the monkey facility from yet-identified reservoir.

4. Virulence of REBOV *in vivo*

As described above, epidemiological study indicated that REBOV is pathogenic in cynomolgus monkeys but not pathogenic in humans. Similar differences in pathogenicity between cynomolgus monkeys and humans were also observed in experimentally infected nonhuman primates. Intraperitoneal infection of 1000 $TCID_{50}$ of ZEBOV, SEBOV, or REBOV in cynomolgus monkeys and African green monkeys (*Cerocopithecus aethiops*) demonstrated that ZEBOV caused lethal infection in both monkeys while SEBOV and REBOV caused lethal infection only in cynomolgus monkeys. Clinical disease in monkeys caused by REBOV infection was similar to that caused by ZEBOV and SEBOV, but the former was characterized by slower onset of disease and viremia [23]. Higher doses of subcutaneous infection