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Short communication

Virulence characteristics of *Yersinia pseudotuberculosis* isolated from breeding monkeys in Japan

Taketoshi Iwata [a], Yumi Une [b], Alexandre Tomomitsu Okatani [b],
Yukio Kato [b], Aya Nakadai [a], Ken-Ichi Lee [a], Maiko Watanabe [a],
Takahide Taniguchi [a], AbdelAzim Elsayed Elhelaly [a],
Yoshikazu Hirota [a], Hideki Hayashidani [a],*

[a] *Division of Animal Science, Institute of Symbiotic Science and Technology,
Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*
[b] *School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan*

Abstract

Between April 2001 and 2007, 18 *Yersinia pseudotuberculosis* outbreaks occurred in breeding monkeys at 12 zoological gardens in Japan, and 28 monkeys of 8 species died. A total of 18 *Y. pseudotuberculosis* strains from the dead monkeys, comprising one strain per outbreak, were examined for serotype and the presence of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB* and *ypmC*) and *irp2*. Of the 18 *Y. pseudotuberculosis* strains, 7 (38.9%) were serotype 4b, 7 (38.9%) were serotype 1b, and there was one each of serotypes 2b, 3, 6 and 7. All the 18 strains examined harbored *virF* and *inv*. Sixteen (88.9%) strains, including the strain of serotype 7, harbored *ypmA*. However, no strain harbored *ypmB*, *ypmC* and *irp2*.

This study demonstrated that among other pathogenic factors, almost all the *Y. pseudotuberculosis* isolated from the outbreaks had the *ypm* gene encoding the superantigenic toxin, YPM. As most of the monkeys who died in those outbreaks originated from South America and other regions, where the presence of the *ypm* gene have not been reported, YPM might be the cause, or at least the most important factor for, the high mortality of the breeding monkeys infected by *Y. pseudotuberculosis* in Japan. This is also the first report of a fatal case due to *Y. pseudotuberculosis* serotype 7 infection in the world.
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Keywords: Yersinia pseudotuberculosis; Breeding monkey; Virulence genes; YPM

1. Introduction

Yersinia pseudotuberculosis is known to be an important causal agent of zoonosis. Monkey species are especially sensitive to *Y. pseudotuberculosis*, and many fatal cases of *Y. pseudotuberculosis* infection in

* Corresponding author. Tel.: +81 42 367 5775;
fax: +81 42 367 5775.
E-mail address: eisei@cc.tuat.ac.jp (H. Hayashidani).

breeding monkeys have been reported throughout the world, including in Japan (Buhles et al., 1981; Hirai et al., 1974; Kageyama et al., 2002; MacArthur and Wood, 1983; Maruyama et al., 1983; Murata and Hama, 1992; Rosenberg et al., 1980; Sasaki et al., 1996; Taffs and Dunn, 1983; Une et al., 2003). Affected monkeys may die unexpectedly or after a very short illness, and at the present time there is no effective preventive method against *Y. pseudotuberculosis* infection. Therefore, monkey *Y. pseudotuberculosis* infection poses a serious problem for zoological gardens engaged in monkey breeding.

The pathogenicity of *Y. pseudotuberculosis* is associated with several virulence factors. Pathogenic strains of *Y. pseudotuberculosis* harbor 70-kb virulence plasmid (pYV), which encodes a number of important virulence and virulence-associated proteins. Additionally, a high-pathogenicity island (HPI), encoding an iron uptake system represented by its siderophore yersiniabactin (Carniel, 1999), and *Y. pseudotuberculosis*-derived mitogen (YPM), which is a superantigenic toxin, are known to play important roles in causing severe systemic infection (Abe et al., 1997). However, it remains unclear which virulence factor is connected with the high mortality of monkeys in *Y. pseudotu-*

berculosis infection. In the present study, we investigated the characteristics of *Y. pseudotuberculosis* isolated from dead breeding monkeys in Japan.

2. Materials and methods

2.1. Bacterial strains

Eighteen *Y. pseudotuberculosis* strains isolated from monkeys that died in 18 outbreaks (one strain per outbreak) were analyzed. These outbreaks occurred between April 2001 and 2007 at 12 zoological gardens (A–L) in Japan, and a total of 28 monkeys of 8 species, comprising 19 squirrel monkeys (*Saimiri sciureus*), 2 hamadryas baboons (*Papio hamadryas*), 2 white-faced sakis (*Pithecia pithecia*), 1 agile gibbon (*Hylobates agilis*), 1 dusky leaf monkey (*Presbytis obscurus*), 1 orangutan (*Pongo pygmaeus*), 1 ring-tailed lemur (*Lemur catta*) and 1 ruffed lemur (*Varecia variegata*), died (Table 1). Pathological findings such as swelling of the Peyer's patch and abscesses in the spleen and liver were typical of yersiniosis. Outbreaks occurred two, three and four times in the zoological gardens C, H and G, respectively.

Table 1
Sources of *Y. pseudotuberculosis* isolated from breeding monkeys in Japan

No.	Strain	Institution	Region	Isolation month year	Source (number and species of other monkeys dead in the same outbreak)
1	NP011001	A	Kanto	April 2002	Squirrel monkey
2	NP031103	B	Kanto	November 2003	Orangutan
3	NP031101	C	Kanto	November 2003	Squirrel monkey (1 squirrel monkey)
4	NP050101	C	Kanto	January 2005	Squirrel monkey
5	NP070401	D	Kanto	April 2007	Dusky leaf monkey
6	NP031201	E	Kinki	December 2003	Squirrel monkey (2 squirrel monkeys)
7	NP040301	F	Chugoku	March 2004	Squirrel monkey
8	NP010401	G	Sikoku	April 2001	Squirrel monkey
9	NP030401	G	Sikoku	April 2003	Squirrel monkey
10	NP050102	G	Sikoku	January 2005	Squirrel monkey
11	NP051201	G	Sikoku	December 2005	Squirrel monkey
12	NP020501	H	Kyusyu	May 2002	Squirrel monkey
13	NP030601	H	Kyusyu	June 2003	Squirrel monkey
14	NP070201	H	Kyusyu	February 2007	Squirrel monkey
15	NP030701	I	Kyusyu	July 2003	Squirrel monkey (1 squirrel monkey)
16	NP050201	J	Kyusyu	February 2005	Hamadryas baboon (1 hamadryas baboon and 1 agile gibbon)
17	NP050301	K	Kyusyu	March 2005	Squirrel monkey (1 squirrel monkey)
18	NP050303	L	Kyusyu	March 2005	White-faced saki (1 white-faced saki, 1 ruffed lemur and 1 ring-tailed lemur)

2.2. Isolation and identification of Y. pseudotuberculosis

The samples (liver and spleen) collected from the dead monkeys were homogenized or suspended in phosphate-buffered saline (PBS: 7.2), and 10-fold serial dilutions of the suspension were plated on irgasan-novobiocin (IN) agar plates (Fukushima et al., 1990). These PBS suspensions were incubated at 4 °C for 3 weeks and then subcultured on IN agar plate after alkali (KOH) treatment (Aulisio et al., 1980). The plates were incubated at 25 °C for 48 h. Colonies morphologically similar to those of Yersinia spp. were subcultured on trypticase soy agar (TSA) (BBL, Sparks, MD, USA) and submitted for biochemical examination for identification, as described elsewhere (Wauters et al., 1988).

2.3. Serotyping

Serotyping of Y. pseudotuberculosis isolated from the monkeys was performed by slide agglutination with a commercial rabbit anti-Y. pseudotuberculosis sera set (Denka-Seiken Co., Tokyo, Japan), and with the rabbit immune sera made in our laboratory. Additional serotyping was performed by PCR as described by Bogdanovich et al. (2003).

2.4. PCR detection of virulence genes

Six sets of primers, designed in Table 2, were used for detection of the virulence genes virF, inv, ypm (ypmA, ypmB and ypmC) and irp2. The virF and irp2 genes were used as the markers for the presence of pYV and HPI, respectively. Chromosomal DNA for PCR was isolated with a wizard genomic DNA purification kit (Promega Co., Madison, WI, USA) following the manufacturer's instructions. PCRs were performed in 50 μl volumes containing 5 μl of template DNA, 0.1 mM each of the four deoxynucleoside triphosphates, 5 μl of 10× PCR buffer, 3 mM MgCl$_2$, 0.1 μM of each primer, and 0.5 U of Taq DNA polymerase (Promega Co., Madison, WI, USA). The PCR amplifications were carried out at 94 °C for 5 min as an initial denaturation step and then subjected to 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C for detection of virF, inv, ypmA and irp2, or at 52 °C for detection of ypmB, or at 49 °C for ypmC (Table 2), 1 min at 72 °C, followed by a final 5 min extension step at 72 °C. Amplifications were performed with a Program Temperature Control System PC-701 (ASTEC, Fukuoka, Japan). Ten microliters of the PCR amplification products were subjected to electrophoresis in a 1.5% agarose gel. A 1-kb PLUS DNA ladder (Invitrogen Co., Carlsbad, CA, USA) was used as a DNA size marker. The gels were stained with ethidium bromide for 10 min, and photographed under UV light.

3. Results

3.1. Serotyping of Y. pseudotuberculosis strains

By slide agglutination, 7 (38.9%) strains of the 18 were serotype 4b, 7 (38.9%) were serotype 1b, and

Table 2
Primers for PCR detection of virulence genes

Virulence factor	Taget gene	Sequence (5'–3')	Annealing temperature (°C)	Size of product (bp)	Reference
pYV	virF	TCATGGCAGAACAGCAGTCAG, ACTCATCTTACCATTAAGAAG	55	590	Wren and Tabaqchali (1990)
Inv	inv	TAAGGGTACTATCGCGGCGGA, CGTGAAATTAACCGTCACACT	55	295	Nakajima et al. (1992)
YPMa	ypmA	CACTTTTCTCTGGAGTAGCG, GATGTTTCAGAGCTATTGTT	55	350	Ito et al. (1995)
YPMb	ypmB	TTTCTGTCATTACTGACATTA, TTTCTGTCATTACTGACATTA	52	453	Ramamurthy et al. (1997)
YPMc	ypmA and ypmC	ACACTTTTCTCTGGAGTAGCG, ACAGGACATTTCGTCA	49	418	Carnoy and Simonet (1999)
HPI	irp2	AAGGATTCGCTGTTACCGGAC, TCGTCGGGCAGCGTTTCTTCT	55	280	Schubert et al. (1998)

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139 there was one each of serotypes 2b, 3, 6 and 7. *Y.*
140 *pseudotuberculosis* serotype 7 has not been isolated
141 from clinical samples in humans or in animals, and
142 thus PCR-based serotyping was used to eliminate any
143 doubt about the serotype of strain NP030601, which
144 was identified as serotype 7 by the slide agglutination.
145 The PCR result of strain NP030601 matched with the
146 above condition for serotype 7 (data not shown),
147 eliminating any doubt about the serotype of this strain.
148 The results of the PCR-based serotyping of the other
149 17 strains also matched with those of the slide
150 agglutination (data not shown). All the *Y. pseudotu-*
151 *berculosis* strains isolated from the monkeys who died
152 in the same outbreak were of the same serotype of
153 strains chosen for analysis in this study.

154 *3.2. Detection of virulence genes in Y.*
pseudotuberculosis strains

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156 All strains were *inv* and *virF* positive, and 16
157 (88.9%) of the 18 strains were *ypmA* positive by PCR.
158 Of the 2 *ypmA* negative strains, one was serotype 4b,
159 and another was serotype 3. On the other hand, all
160 strains were *ypmB*, *ypmC* and *irp2* negative (Table 3).

Table 3
Characteristics of *Y. pseudotuberculosis* isolated from breeding monkeys

No.	Virulence genes						Serotype
	virF	inv	ypm			irp2	
			ypmA	ypmB	ypmC		
1	+	+	+	–	–	–	4b
2	+	+	+	–	–	–	4b
3	+	+	+	–	–	–	4b
4	+	+	+	–	–	–	4b
5	+	+	+	–	–	–	1b
6	+	+	+	–	–	–	4b
7	+	+	+	–	–	–	4b
8	+	+	+	–	–	–	1b
9	+	+	+	–	–	–	6
10	+	+	+	–	–	–	1b
11	+	+	+	–	–	–	2b
12	+	+	–	–	–	–	4b
13	+	+	+	–	–	–	7
14	+	+	+	–	–	–	1b
15	+	+	+	–	–	–	1b
16	+	+	–	–	–	–	3
17	+	+	+	–	–	–	1b
18	+	+	+	–	–	–	1b

+: PCR positive; –: PCR negative.

4. Discussion

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161 In the present study, the predominant serotypes of
162 *Y. pseudotuberculosis* isolated from dead monkeys
163 were serotypes 1b and 4b. In Japan, these serotypes
164 have also been the predominant serotypes isolated
165 from clinical samples, for example, of human patients,
166 and the majority of the strains of these serotypes are
167 highly pathogenic, with the *ypmA* (Fukushima et al.,
168 2001). In the present study, almost all of the strains
169 isolated from dead monkeys also had *ypmA* genes. It is
170 known that the presence of the *ypmA* is pretty much
171 limited to the Far East (Japan, Korea and Far-Eastern
172 Russia), and also that it exacerbates the toxicity of *Y.*
173 *pseudotuberculosis* in systemic infection in mice
174 (Fukushima et al., 2001). Moreover, it has been
175 reported that the clinical signs of *Y. pseudotubercu-*
176 *losis* infection found in the Far East include not only
177 fever, gastroenteric symptoms, and mesenteric lym-
178 phadenitis, which are the main symptoms in Europe,
179 but also a variety of systemic manifestations such as
180 rash, desquamation, erythema nodosum and arthritis
181 (Sato et al., 1983). In zoological gardens in Japan, a
182 variety of primates are bred, including monkey species
183 from South America, Southeast Asia or Africa, listed
184 in Table 1, as well as the Japanese macaque (*Macaca*
185 *fuscata*). It has been noted that monkeys from those
186 regions, where the presence of *Y. pseudotuberculosis*
187 with the *ypm* gene has not been identified, frequently
188 die when infections with this pathogen occur, while
189 there has been little mortality of Japanese macaques
190 due to *Y. pseudotuberculosis* infection (Kageyama
191 et al., 2002). Because of the persistent exposure of the
192 Japanese macaque to *Y. pseudotuberculosis* with the
193 *ypm* gene from ancient times they may have acquired
194 resistance to that pathogen, unlike the imported
195 monkeys. Thus, YPM seems to be the main cause
196 of the high mortality of the monkeys imported from
197 abroad.
198 This is the first report of isolation of *Y.*
199 *pseudotuberculosis* serotype 7 from a clinical sample
200 anywhere in the world. This serotype has been isolated
201 from dogs, raccoon dogs, moles, wild mice and water.
202 However, there have been no reports about *Y.*
203 *pseudotuberculosis* serotype 7 isolated from samples
204 of primate origin. Pathological analysis of the squirrel
205 monkey, from which the serotype 7 were isolated,
206 showed swelling of the spleen and liver and multiple

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white abscesses in the spleen, and the PCR analysis demonstrated that the strain of serotype 7 also harbored pYV and *ypmA* genes. These results possibly suggest that the strain serotype 7 isolated in the present study has the same degree of pathogenicity as the other pathogenic serotypes. Therefore, we should pay attention to the possibility of humans and other animal species infected by serotype 7.

Many monkey species kept at zoological gardens are formally recognized as "threatened" by The World Conservation Union (IUCN), and their deaths pose a serious loss to the zoological gardens involved. Thus, preventive measures against *Y. pseudotuberculosis* infection in breeding monkeys should be established as soon as possible. However, most breeding monkeys kept at zoological gardens are maintained in outdoor cages or enclosures for exhibition. These conditions lead to the exposure of the monkeys to animals living in the wild, such as birds and rodents, and as *Y. pseudotuberculosis* is widely distributed in wild animals, the probability of transmission of this pathogen from those animals is very high. Moreover, it is very difficult to completely prevent wild animals from invading the cages of the monkeys, and thus the foods and water provided for the monkeys can easily become contaminated. Therefore, development of effective vaccines is important for preventing pathogenic *Y. pseudotuberculosis* infection in breeding monkeys.

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家庭で飼育されている爬虫類における
サルモネラの保有状況

中臺　文*¹　　塩谷　亮*²　　加藤行男*³　　黒木俊郎*⁴　　岩田剛敏*¹　　廣田好和*¹　　林谷秀樹*¹

要　約

2005〜2006年に我が国の一般家庭で飼育されていた爬虫類30種115検体からサルモネラの分離を行った。その結果，これら爬虫類の32.2%（37/115）がサルモネラを保有していた。分離されたサルモネラは11種類の血清型に型別され，爬虫類が感染源となった人のサルモネラ症の原因として報告のあるものや，我が国の胃腸炎患者からの分離頻度の高いものが含まれていた。これらのことから，家庭で飼育されている爬虫類は人のサルモネラ症の感染源として重要な存在であり，ペットとして飼育する場合には，サルモネラを高率に保有していることを良く認識し，その取扱いには十分注意する必要があると思われる。

はじめに

サルモネラは代表的な細菌性人獣共通感染症の原因菌の1つとして知られている。欧米諸国では爬虫類はペットとして人気が高く，多くの種類の爬虫類がペットとして飼育されているが，これらの爬虫類が感染源となった人のサルモネラ症が報告されている[1,2]。近年，我が国でも爬虫類のペットとしての人気が高まっており，さまざまな種類の爬虫類が飼育されるようになってきているが，それに伴いこれら爬虫類に起因する人のサルモネラ症が発生しており，最近ではミドリガメに起因し，敗血症や髄膜炎などの重症に至った事例や，イグアナやリクガメに起因する事例が報告されている[3]。したがって，ペットの爬虫類から人へのサルモネラの感染予防対策を図ることは，公衆衛生上緊急の課題となっている。しかし，我が国の家庭で実際に飼育されている爬虫類におけるサルモネラの保有状況は依然明らかになっていない。そこで今回，日本小動物獣医師会の協力を得て，我が国の家庭で飼育されている爬虫類におけるサルモネラ保有状況について調査した。

調査方法

供試材料として，2005〜2006年に日本の13都道府県の一般家庭76軒で飼育されていた爬虫類30種115検体の糞便を用いた。サルモネラの分離・同定は定法に従い，サルモネラと同定された株については，各種生化学性状試験により生物群を，市販免疫血清を用いた凝集試験により血清型を型別した。

調査成績

我が国の家庭で飼育されている爬虫類の32.2%（37/115）からサルモネラが分離された。爬虫類の種別に

*¹ Aya NAKADAI, Taketoshi IWATA, Yosikazu HIROTA & Hideki HAYASHIDANI（写真・コメント）：東京農工大学大学院共生科学技術研究院（〒183-8509 府中市幸町3-5-8）
*² Ryo SHIOTANI：ジェントルマン・ルーサー（〒229-0002　相模原市淵野辺本町5-29-7）
*³ Yukio KATO：麻布大学獣医学部（〒229-8501 相模原市淵野辺1-17-71）
*⁴ Toshiro KUROKI：神奈川県衛生研究所（〒253-0087 茅ヶ崎市下町屋1-3-1）

爬虫類におけるサルモネラの保有状況を調べて，早4年が過ぎました。これまでにペットショップで販売されている爬虫類や外国から輸入されてくる爬虫類が高率にサルモネラを保有していることを報告し，これら爬虫類が人へのサルモネラの感染源として危険であることを指摘してまいりましたが，最近，ペットの爬虫類から人へのサルモネラ感染事例が次々に報告され，危惧していたことが現実となってしまいました。

表1. 家庭で飼育されている爬虫類におけるサルモネラの保有状況		生物群（%）				
爬虫類の種類	陽性検体数／調査検体数（%）	I	II	IIIb	IV	UTᵃ
カメ類	16/89（18.0）	54.2	33.3	4.2	4.2	4.2
トカゲ類	12/16（75.0）	53.8	7.7	23.1	15.4	0.0
ヘビ類	9/10（90.0）	8.3	16.7	75.0	0.0	0.0
計	37/115（32.2）	42.9	22.4	26.5	6.1	2.0

ᵃUT：型別不能

みた本菌の保有率は，カメ類が 18.0%（16/89），トカゲ類が 75.0%（12/16）およびヘビ類が 90.0%（9/10）で，ヘビ類が最も高率であった（表1）。また，分離株の生物群の分布をみると，人や家畜のサルモネラ症と関連の深い I 群が 42.9% で最も多く，次いで IIIb 群が 26.5%，II 群が 22.4% および IV 群が 6.1% であった。菌株の血清型は 11 種類に型別され，*Salmonella* Hvittingfoss および *S.* Poona など，欧米諸国や我が国で実際に爬虫類に起因する人のサルモネラ症の原因として報告されている血清型や，*S.* Newport ならびに *S.* Typhimurium など，我が国の人の胃腸炎患者から高頻度に分離される血清型も含まれていた。

今回の調査成績から，我が国の家庭で飼育されている爬虫類はサルモネラを高率に保有しており，人のサルモネラ症の感染源として重要な存在であることが示唆された。これまでの我々の調査で，我が国のペットショップで販売されている爬虫類はサルモネラを高率に保有していること[4]，また，爬虫類は日本に輸入された時点ですでにサルモネラを高率に保有しており，野生の状態でもすでに本菌を高率に保有していることが明らかとなっている[5,6]。今回供試した家庭飼育の爬虫類のほとんどはペットショップで購入された個体であったことから，家庭で飼育されている爬虫類の多くは原産国からサルモネラを保有した状態で我が国に輸入され，小売店を通じて家庭に流通している可能性が高いと考えられる。最近，我が国では，ペットとして飼育される爬虫類に起因する小児のサルモネラ感染事例の報告が相次いでいることから，爬虫類をペットとして飼育する際には爬虫類がサルモネラを保有している可能性が高いことを十分に認識して取り扱う必要があろう。

終わりに

家庭で飼育される爬虫類から人へのサルモネラの感染を予防するため，爬虫類に触れた後の手洗い・うがいを励行するとともに，爬虫類は家庭内で人の生活圏とはできる限り区別した場所で飼育し，また免疫機能の低い乳幼児や高齢者の居る家庭では飼育を控えるなどの注意が必要である。また，現在法的な規制がない爬虫類の輸入，流通および販売に関して，今後は何らかの規制や対策を講じる必要があると思われる。

最後に，本調査にご協力いただいた日本小動物獣医師会の会員の先生方に，この場をお借りして深謝いたします。

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

Fumio Ike,[1,*] Franck Bourgade,[2] Kazutaka Ohsawa,[3] Hiroshi Sato,[3] Shigeru Morikawa,[4] Masayuki Saijo,[4] Ichiro Kurane,[4] Kazuhiro Takimoto,[5] Yasuko K Yamada,[5] Jean Jaubert,[2] Marion Berard,[6] Hatsumi Nakata,[1] Noriko Hiraiwa,[1] Kazuyuki Mekada,[1] Akira Takakura,[7] Toshio Itoh,[7] Yuichi Obata,[1] Atsushi Yoshiki,[1,*] and Xavier Montagutelli[2]

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 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

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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-

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

| | Frequency of evaluation at | |
Pathogen	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 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 micro-

scope (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-

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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, Carlsbad, 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 transmitted 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
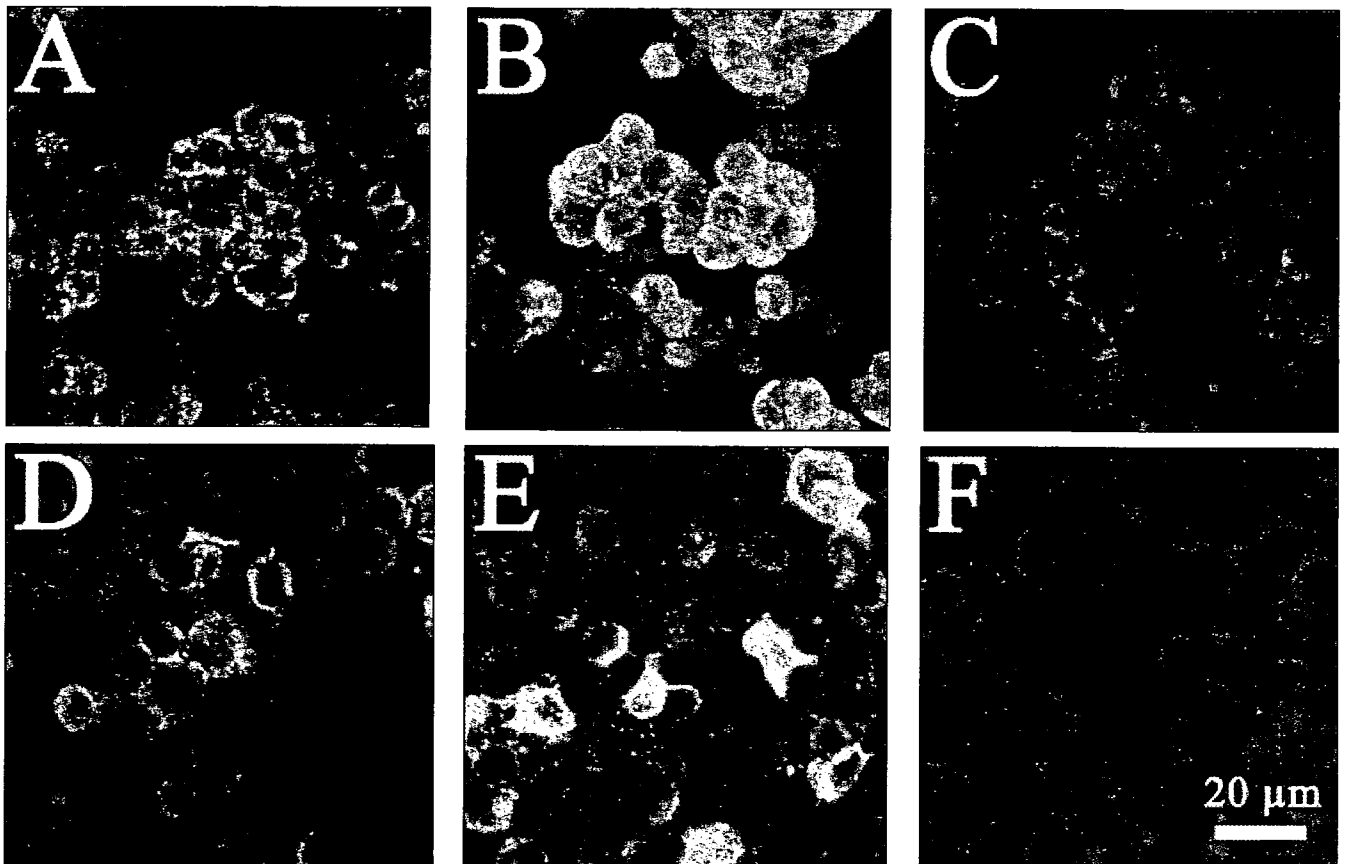
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Figure 1. IFA test for detection of antiLCMV antibody. IFA slides were prepared using (A through C) Vero E6 cells infected with LCMV Armstrong and (D through F) L cells infected with LCMV WE. (A and D) Serum from a BALB/cA-*nu*/+ mouse caged with MAI/Pas 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% ho-

mologous, 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 d 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-

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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 used 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
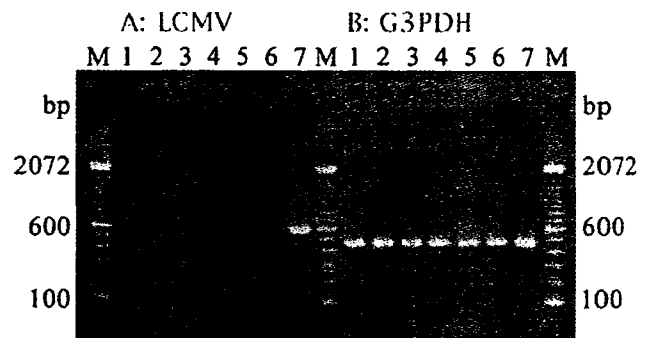

Figure 2. Detection of LCMV by RT-PCR. RNA was extracted from kidneys of mice of the wild-derived strains WMP/Pas (*M. m. domesticus*), SEG/Pas (*M. spretus*), STF/Pas (*M. spretus*), and MAI/Pas (*M. m. musculus*). These strains were transferred from the Institut Pasteur to RIKEN BRC during overlapping 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-

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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 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 d 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 d 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, sug-

gesting 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.

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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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Development of Recombinant Nucleoprotein-Based Diagnostic Systems for Lassa Fever[▽]

Masayuki Saijo,[1]*† Marie-Claude Georges-Courbot,[2] Philippe Marianneau,[2] Victor Romanowski,[3]
Shuetsu Fukushi,[1] Tetsuya Mizutani,[1] Alain-Jean Georges,[4] Takeshi Kurata,[5]
Ichiro Kurane,[1] and Shigeru Morikawa[1]†

*Department of Virology 1, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan[1];
Unit of Biology of Viral Emerging Infections, Institute Pasteur, IFR 128 Biosciences Lyon-Gerland, 21 Avenue Tony Garnier,
69365 Lyon Cedex 07, France[2]; Instituto de Bioquímica y Biológica Molecular, Facultad de Ciencias Exactas, Universidad
Nacional de a Plata, 1900 La Plata, Argentina[3]; Laboratory P4 Jean-Merieux-INSERM, 21 Avenue Tony Garnier 69365
Lyon Cedex 07, France[4]; and Department of Pathology, National Institute of Infectious Diseases,
1-23-1 Toyama, Shinjuku-ku, Tokyo 162-864, Japan[5]

Diagnostic systems for Lassa fever (LF), a viral hemorrhagic fever caused by Lassa virus (LASV), such as enzyme immunoassays for the detection of LASV antibodies and LASV antigens, were developed using the recombinant nucleoprotein (rNP) of LASV (LASV-rNP). The LASV-rNP was expressed in a recombinant baculovirus system. LASV-rNP was used as an antigen in the detection of LASV-antibodies and as an immunogen for the production of monoclonal antibodies. The LASV-rNP was also expressed in HeLa cells by transfection with the expression vector encoding cDNA of the LASV-NP gene. An immunoglobulin G enzyme-linked immunosorbent assay (ELISA) using LASV-rNP and an indirect immunofluorescence assay using LASV-rNP-expressing HeLa cells were confirmed to have high sensitivity and specificity in the detection of LASV-antibodies. A novel monoclonal antibody to LASV-rNP, monoclonal antibody 4A5, was established. A sandwich antigen capture (Ag-capture) ELISA using the monoclonal antibody and an anti-LASV-rNP rabbit serum as capture and detection antibodies, respectively, was then developed. Authentic LASV nucleoprotein in serum samples collected from hamsters experimentally infected with LASV was detected by the Ag-capture ELISA. The Ag-capture ELISA specifically detected LASV-rNP but not the rNPs of lymphocytic choriomeningitis virus or Junin virus. The sensitivity of the Ag-capture ELISA in detecting LASV antigens was comparable to that of reverse transcription-PCR in detecting LASV RNA. These LASV rNP-based diagnostics were confirmed to be useful in the diagnosis of LF even in institutes without a high containment laboratory, since the antigens can be prepared without manipulation of the infectious viruses.

Lassa fever (LF) is a viral hemorrhagic fever caused by Lassa virus (LASV), an Old World arenavirus. Many cases of LF occur in western Africa in countries such as Guinea, Sierra Leone, and Nigeria (7, 23, 27, 29–31). It is thought that LASV infects tens of thousands of humans annually and causes hundreds to thousands of deaths (34). Humans become infected through contact with infected excreta, tissue, or blood from the peridomestic rodent, *Mastomys natalensis*, the reservoir host of LASV (34). LASV can be transmitted to other humans via mucosal or cutaneous contact or through nosocomial contamination (27). More than 20 imported cases of LF have been reported outside the endemic region in areas such as the United States, Canada, Europe, and Japan (1, 2, 13, 15, 18, 24, 25). Recently, the potential for the use of hemorrhagic fever viruses, including LASV, as a biological weapon has been emphasized (5, 6). Therefore, the development of diagnostic systems for LF is important even in countries free from LF outbreaks to date.

Manipulation of infectious LASV is necessary for the detection of specific antibodies. However, a high-containment laboratory (biosafety level 4 [BSL-4]) is required for handling infectious LASV and, therefore, the preparation of LASV antigens cannot be implemented in institutes without a BSL-4 facility. Within this framework, it is important to develop sensitive and specific diagnostic systems for LF that eliminate the need for the manipulation of infectious LASV. In the present study, the recombinant nucleoprotein (rNP) of LASV (LASV-rNP) was expressed and evaluated for its ability to detect LASV antibodies. LASV-rNP-based enzyme-linked immunosorbent and indirect immunofluorescence assays (ELISA and IIFA) were developed. Furthermore, novel monoclonal antibodies to LASV-rNP were generated and used in combination with the recombinant antigen to develop an LASV antigen (nucleoprotein) capture ELISA. The present study presents an alternative strategy to develop diagnostic systems without handling infectious LASV.

MATERIALS AND METHODS

Cells. A HeLa cell line was cultured in the Eagle minimum essential medium supplemented with 10% fetal bovine serum and the antibiotics penicillin G and streptomycin (MEM-10FBS). Tn5 insect cells were used for the expression of the rNPs of arenaviruses (LASV, lymphocytic choriomeningitis virus [LCMV], and Junin virus [JUNV]) in a baculovirus system. The Tn5 insect cells were cultured as reported previously (38).

* Corresponding author. Mailing address: Special Pathogens Laboratory, Department of Virology 1, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 320. Fax: 81-42-561-2039. E-mail: msaijo@nih.go.jp.
† M.S. and S.M. contributed equally to this study.
▽ Published ahead of print on 18 July 2007.

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Viruses. LASV (strain AV), which was isolated from an imported case of LF to Germany from West Africa, was used (13). The experimental process that required manipulation of infectious LASV was carried out in the BSL-4 laboratory in the P4 laboratory, INSERM, Lyon, France. Mopeia virus (MOPV), which belongs to the family *Arenaviridae*, genus *Arenavirus*, was also used. Recombinant NPs of LCMV (26) and JUNV (11), designated LCMV-rNP and JUNV-rNP, respectively, were also expressed in a baculovirus system and used in the study. A baculovirus (Ac-ΔP), which lacks polyhedrin expression, was used as a control virus (26). The virus titer of LASV in serum samples was determined by using a focus-forming unit (FFU) assay as described previously (3).

Sera. Four human serum samples—three samples serially collected from one patient with LF and one additional sample from another patient with LF—and ninety-six human sera collected from Japanese subjects with no history of travel to areas where LF is endemic were used as positive and negative controls, respectively. The patient with LF, from whom three serial serum samples were collected, was the first case of LF to be imported in Japan in 1987 (15). The other human serum sample was provided from the Special Pathogens Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA.

Serum samples collected from five monkeys (*Macaca fascicularis*) subcutaneously infected with LASV strain AV at 10^3 FFU (two monkeys) or 10^7 FFU (three monkeys) and those collected from four monkeys with mock infection were also used. The serum samples used in the study were collected at 4 to 5 weeks postchallenge.

Five hamsters were subcutaneously infected with 10^3 FFU of LASV, strain AV, and blood was drawn on days 0, 4, 11, and 16 postinfection, taking the day on which the virus was inoculated as day 0. Serum fractions of the collected blood specimens were separated and tested for LASV antigen by antigen capture (Ag-capture) ELISA and reverse transcription-PCR (RT-PCR).

Rabbit sera (polyclonal antibodies) were raised against LASV-rNP, LCMV-rNP, and JUNV-rNP by immunization of rabbits with the purified LASV-rNP, LCMV-rNP, and JUNV-rNP, respectively, in the form of a mixture with the adjuvant, Inject Alum (Pierce). Rabbits were immunized with sufficient amount of the purified nucleoproteins of each virus three times with an interval of 2 weeks. After confirmation of the increased titer, >10,000 times as determined by indirect immunofluorescence assay, which was developed in the present study, blood was drawn from the rabbits, and the serum fraction was used in the present study.

Recombinant baculovirus. In order to construct the transfer vector, a cDNA clone of NP from LASV strain Josiah was used. The cDNA was kindly provided by J. B. McCormick, former Director of the Special Pathogens Branch, National Centers for Infectious Diseases, Centers for Disease Control and Prevention. The complete nucleotide sequence of the NP gene is registered in GenBank under the accession number NC_004296. The DNA of the LASV-NP was amplified by PCR from the source using the primers LAS-NfB (5'-GTGGATCCA ACACAACAATCTGG-3'; the BamHI restriction site is underlined) and LAS-NrB (5'-CCGGATCCATTTACAGAACGACTC-3'). The PCR conditions were the same as previously reported (38). The 1,743-bp amplification product was digested with BamHI and subcloned into the BamHI site of pQE32 vector DNA (QIAGEN GmbH, Hilden, Germany) to construct pQE32-LASV-NP. The inserted LASV-NP DNA was sequenced by using appropriate primers with an ABI Prism 310 genetic analyzer (PE Applied Biosystems, Foster City, CA) and confirmed to be in proper orientation downstream the promoter and identical to the original sequence. The DNA fragment of LASV-NP with a histidine (His) tag was isolated from the plasmid, pQE32-LASV-NP, by digestion with EcoRI and HindIII. It was then blunt repaired with Klenow enzyme and ligated into the blunt-ended BamHI site of pAcYM1 (26). The resulting recombinant transfer vector with the correct orientation with respect to the polyhedrin promoter was constructed (pACYM1-His-LASV-NP). Tn5 insect cells were transfected with mixtures of purified *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA and pAcYM1-His-LASV-NP according to the procedures described by Kitts et al. (20), with the modification of Matsuura et al. (26). Recombinant baculovirus was then isolated. The baculovirus, which expressed His-tagged LASV-rNP (His-LASV-rNP), was designated Ac-His-LASV-NP.

The baculovirus, Ac-LCMV-NP, which expressed LCMV-rNP, was used in the study (26).

The recombinant baculovirus that expressed JUNV-rNP, Ac-JUNV-NP, was generated as follows. The gene encoding the NP of JUNV (strain MC2) was reconstructed from cloned cDNA. The nucleotide sequence of the interest gene was deposited in GenBank under accession number D10072 (12). A complete NP gene with the initiation and stop codons amplified by PCR using appropriate primers, which possessed BamHI restriction sites. The entire DNA product of JUNV-NP was digested with BamHI and ligated into the transfer vector
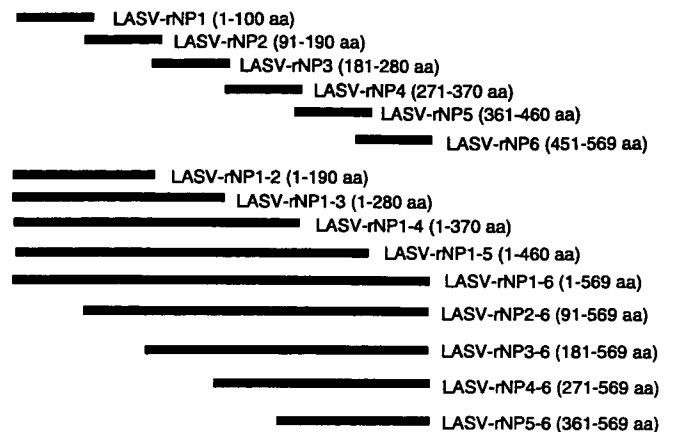

FIG. 1. Schematic representation of truncated LASV-rNP expressed as a form of GST fusion protein in *E. coli* transformed with the corresponding expression vector. The description "LASV-NP1-6" in the middle portion of the figure indicates full-length LASV-rNP.

pAcUW2B (28). Clones containing the insert in the correct orientation were selected, and the plasmid DNA was used for cotransfection in Sf21 cells with a polyhedrin-positive AcMNPV DNA, and the supernatant culture was screened for a polyhedrin-negative phenotype by plaque assay (19). Finally, recombinant baculovirus clones overexpressing JUNV-rNP were obtained after three successive plaque purifications. One of them, designated AcNMPV-Jun-N122, was used in the present study and is referred to hereafter as Ac-JUNV-NP.

Expression and purification of His-LASV-rNP, LCMV-rNP, and JUNV-rNP. Tn5 cells infected with Ac-His-LASV-NP were incubated at 26°C for 72 h. The cells were then washed twice with cold phosphate-buffered saline (PBS) solution. A preliminary study demonstrated that most of the Tn5 cellular proteins were solubilized in PBS containing 2 M urea (PBS-2 M urea) but that the His-LASV-rNP was insoluble and that the LASV-rNP could be solubilized in PBS containing 8 M urea (PBS-8 M urea). Therefore, the Tn5 cells infected with Ac-His-LASV-NP were first suspended in PBS-2 M urea. After the centrifugation of the cell suspensions at 15,000 × *g* for 10 min, the pellet fractions were collected and then were solubilized in PBS-8 M urea. After the centrifugation of the samples, the supernatant fractions were used as the purified antigens. LCMV-rNP and JUNV-rNP showed dissolution characteristics in urea similar to those of His-LASV-rNP; therefore, LCMV-rNP and JUNV-rNP were also fractioned in the same way as the His-LASV-rNP. The control antigen was produced from Tn5 cells infected with Ac-ΔP in the same manner as that for the positive antigens. The His-LASV-rNP was also purified by using the Ni^{2+} column purification method as reported previously (38). The source for His-LASV-rNP-purification was the supernatant fraction of the PBS-8 M urea-treated Tn5 cells infected with Ac-His-LASV-NP after sufficient dilution with PBS in order to reduce the urea concentration.

SDS-PAGE. The expression and purification efficiency of His-LASV-rNP, LCMV-rNP, and JUNV-rNP were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12% polyacrylamide) after staining with Coomassie blue.

Establishment of MAbs. Monoclonal antibodies (MAbs) were generated as previously described (32, 41). BALB/c mice were immunized with purified His-LASV-rNP in the present study. Isotypes of the MAbs were determined by using a mouse MAb isotyping kit (Life Technologies).

Expression of truncated NPs of LASV. In order to determine the epitope of the MAbs to the His-LASV-rNP, truncated LASV-rNPs were expressed as a form of fusion protein with glutathione S-transferase (GST) as shown in Fig. 1. The DNA corresponding to each of the truncated NP fragments was amplified with the designed primers. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). Each insert was sequenced and confirmed to be in the correct frame and identical to the original sequence. The GST-tagged nucleoprotein fragments were expressed in an *Escherichia coli* BL21 system.

Western blotting. The MAbs were tested for reactivity to His-LASV-rNP and its fragments by Western blotting as reported previously (17, 32, 41).

Pepscan analyses. ELISA was performed as reported previously with the purified rNP or partial nucleoprotein peptides as the antigen (33). The peptides