る　という条件を満たす場合，医薬品やワクチン製剤の使用を緊急許可することのできる制度（Emergency Use Authorization；EUA）を制定した。EUAは，非常事態宣言の終結までの期限付きの許可ではあるが，1年単位の更新もありうる。ただし，使用条件として，医療従事者や受療者が緊急時の使用であることや既知の便益とリスクの可能性，代替治療の有無を理解し，受療にあたり個人の選択自由があることが明記されている。また，適切に有害事象のモニタリングと記録，報告を行うことも義務づけられている。

さらに，生命に重篤な危機のある致死的な化学物質，生物物質，放射線物質，核物質の治療方法が必要な際に，既存治療に比して意味のある便益の提供ができるとの判断のもと，万が一臨床試験が不可能あるいは非倫理的な場合には，動物実験のデータによって緊急に薬剤の使用許可を与えるという制度（animal rule）が制定されている。この場合には，動物実験の有効性のデータが科学的に適切であり，FDAの規制による有効性の評価が困難であることが条件とされ，人のデータとして，薬物動態（PK）や免疫原性，使用する人口における安全性（子供，妊婦などの考慮）は必要とされている。

また，2005年11月には「インフルエンザ汎流行国家戦略」とよばれる計画を発表し，H5N1新型インフルエンザのパンデミックについては国家安全保障の枠でとらえて様々な対策を講じている。その中で，保健福祉省は製薬企業とH5N1新型インフルエンザワクチンの製造委託契約を締結し，製造設備建設への補助金を拠出した。さらに特筆すべきことに，2007年1月には製造物責任免責法を発出し，H5N1新型インフルエンザに対するワクチン製造企業に製造物責任訴訟が提起された場合，責任を免除する措置も講じた。なお，パンデック時のワクチンの国民への安定供給に対しては，米国は欧州諸国と同様，ワクチン製造企業との間に事前購入契約を締結し，一定の枠を確保している。

以上のように，米国の感染症対策，ワクチン行政は，パンデミック感染症や病原体に対する健康保障にも施策がなされているのである。

1.6　おわりに

感染症ワクチンの研究開発と適正使用は，国民の健康を維持し社会を健全に機能させるために重要なことはいうまでもない。ところが，海外諸国での出願のみならず，日本国内でのワクチンの特許も，外資系企業や米国DHHSからの出願で占められているのが現状である，すなわち，わが国におけるワクチンの研究開発の低調さが問題となっている。近年世界100カ国以上で承認されている子宮頸癌予防を目的としたヒトパピローマウイルスも，日本では未だ承認に至っていない。これは日本の臨床試験環境もさることながら，疫学調査，データベースが十分とはいえない健康行政のあり方も関連しているといえよう。今後は，米国での例も参考にして，わが国に

おいて，国家の健康保障としてしっかりとワクチンを研究開発し，また同時に疫学的根拠データの収集も怠りなく実施していくべきである。国民への十分な啓発や理解促進などのよって適正使用を推進し，さらに，新型インフルエンザなどのパンデミック感染症への脅威にも対応できるような行政施策が強く望まれるところである。

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Loop-Mediated Isothermal Amplification-Based Diagnostic Assay for Monkeypox Virus Infections

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Monkeypox virus (MPXV) causes a smallpox-like disease in non-human primates and humans. This infection is endemic to central and western Africa. MPXV is divided into two genetically different groups, Congo Basin and West African MPXV, with the former being the more virulent. A real-time quantitative MPXV genome amplification system was developed for the diagnosis of MPXV infections using loop-mediated isothermal amplification (LAMP) technology. Primers used for genome amplification of Congo Basin (C-LAMP), West African (W-LAMP), and both Congo Basin and West African (COM-LAMP) MPXV by LAMP were designed according to the nucleotide sequences of the Congo Basin-specific D14L gene, the West African-specific partial ATI gene, and the partial ATI gene that is shared by both groups, respectively. The sensitivity and specificity of the LAMP were evaluated with nested PCR using peripheral blood and throat swab specimens collected from Congo Basin MPXV or West African MPXV-infected monkeys. The sensitivity and specificity of COM-LAMP, C-LAMP, and W-LAMP were 80% (45/56) and 100% (64/64); 79% (19/24) and 100% (24/24); and 72% (23/32) and 100% (40/40), respectively. The viremia level determined by LAMP assays increased with increases in the severity of the monkeypox-associated symptoms. The newly developed LAMP assay was confirmed to be a rapid, quantifiable, and highly sensitive and specific system effective in the diagnosis of MPXV infections. The LAMP assays made it possible to discriminate between Congo Basin and West African MPXV. The LAMP developed in this study is useful not only for diagnosis of but also for the assessment of MPXV infections. ***J. Med. Virol. 81:1102–1108, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: loop-mediated isothermal amplification; LAMP; monkeypox; monkeypox virus; diagnosis

INTRODUCTION

Monkeypox virus (MPXV) belongs to the genus *orthopoxvirus* in family *Poxviridae*, and, together with variola virus, is a causative agent for smallpox. MPXV was first isolated in 1958 from cynomolgus monkeys with symptoms similar to those of smallpox in humans [von Magnus et al., 1959]. It was identified that MPXV causes a smallpox-like disease in humans (human monkeypox) in 1970 [Ladnyj et al., 1972]. The disease is endemic to the rainforests of central and western Africa, where some species of ground squirrels, which are suggested to be the reservoir, are prevalent [Meyer et al., 2002]. People living in the endemic regions still suffer from this infection. A sporadic outbreak of human monkeypox occurred in the USA in 2003 [Likos et al., 2005]. This was the first outbreak of human monkeypox outside Africa. The source of the outbreak was MPXV-infected ground squirrels, such as African dormice and Gambian giant rats, imported from western Africa through Accra, Ghana. This event indicates that there is the possibility that MPXV could spread outside Africa and cause human monkeypox even in countries currently free from this infection. Furthermore, MPXV is categorized as an important bio-weapons. MPXV is divided into two genetic distinct groups, Congo Basin and West African MPXV. Congo Basin MPXV was

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reported to be more virulent than West African MPXV [Chen et al., 2005]. Therefore, there is a need to develop rapid and accurate diagnostic systems with which to discriminate between Congo Basin and West African MPXV.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that works by auto-cycling strand displacement DNA synthesis by *Bst* DNA polymerase, and has been confirmed to be simple to use [Notomi et al., 2000]. The amplification is conducted under isothermal conditions ranging from 60 to 65°C with DNA polymerase and, usually, four primers recognizing six distinct target regions (4-primer-based LAMP), making this assay highly specific. If two additional "loop primers" are included in the LAMP assay, the reaction time can be reduced (6-primer-based LAMP) [Nagamine et al., 2002]. Recently, the LAMP-based diagnosis for several virus infections have been developed [Hong et al., 2004; Kaneko et al., 2005; Imai et al., 2006; Parida et al., 2006, 2007; Suzuki et al., 2006; Shirato et al., 2007].

In the present study, 6-primer-based LAMP was applied for the development of diagnostic systems for MPXV infections. Three LAMP assays, which detected specifically Congo Basin and West African MPXV, Congo Basin but not West African MPXV, and West African but not Congo Basin MPXV, respectively, were developed. The usefulness of the LAMP assays for the diagnosis and assessment of MPXV infections was evaluated using peripheral blood and throat swab specimens collected from MPXV-infected monkeys.

MATERIALS AND METHODS

Viruses and Cells

Congo Basin MPXV (Zr-599, Congo-8, and V97-I-008 strains), West African MPXV (Sierra Leone, Liberia, Copenhagen, and Anteatan strains), cowpox virus (Brighton Red strain), camelpox virus (J1 strain), ectromelia virus (Hamstead strain), and vaccinia virus (Lister strain) stored at the National Institute of Infectious Diseases, Tokyo, Japan (NIID), were used. The MPXV strains and other orthopoxviruses were grown on Vero and HeLa cells, respectively.

Extraction and Purification of Virus Genome

Viral DNA was extracted from MPXV (Zr-599)-, MPXV (Liberia)-, cowpox virus-, camelpox virus-, ectromelia virus-, or vaccinia virus-infected cells using the Hirt extraction method [Hirt, 1967]. Viral DNA in the peripheral blood and throat swab specimens was purified using a Viral Nucleic Acid Purification Kit™ (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and stored at −30°C until use.

Real-Time Quantitative PCR for Orthopoxvirus

A SYBR Green-based real-time quantitative PCR (pox-qPCR) was developed. Primers, forward primer [H2Rf (5′-CGGTTAACGATTGGAAATCATTAACGG-3′)] and reverse primer [H2Rr (5′-CCTCGCCTAA-TAGCTTGCG-3′)], used in the pox-qPCR were designed according to the nucleotide sequences of the *H2R* gene shared by viruses in the genus *Orthopoxvirus*. Standard DNA, a pGEM-T easy vector (GH Health Care Japan, Tokyo, Japan) inserted with the partial *H2R* gene amplified in PCR with the above primer set (H2f and H2r), was used for the determination of the copy number of the virus genome of MPXV, cowpox virus, camelpox virus, ectromelia virus, and vaccinia virus. The reaction conditions were as follow: one cycle of 95°C for 10 min for denaturation, 45 cycles of 95°C for 15 sec, 63°C for 5 sec, 72°C for 10 sec, followed by one cycle of 73°C for 15 sec. PCR amplification was performed using a LightCycler FastStart DNA Master SYBR Green I™ kit (Roche Diagnostics Ltd.) in a 20 μl-volume format containing 5 μl of template DNA according to the manufacturer's instructions.

LAMP

The 6-primer-based LAMP consists of six primers; two outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and two loop primers (LF and LB) [Nagamine et al., 2002]. Three LAMP assays were developed for the amplification of the genomes of both Congo Basin and West African MPXV (COM-LAMP), of the genomes of Congo Basin but not West African MPXV (C-LAMP), and of the genomes of West African but not Congo Basin MPXV (W-LAMP). The primers for COM-LAMP, C-LAMP, and W-LAMP were designed according to the nucleotide sequences of the A-type inclusion body (ATI) shared by both Congo Basin and West African MPXV, those of the Congo Basin MPXV-specific *D14L* gene [Likos et al., 2005], and those of the West African MPXV-specific partial ATI gene [Saijo et al., 2008]. The primer Loop-B-COM in the COM-LAMP was designed to anneal the region containing a deletion of eight nucleotide residues observed only in MPXV but not in other orthpoxviruses [Neubauer et al., 1998]. The nucleotide sequences of the primers used in each LAMP assay are shown in Table I. The LAMP reaction was performed with a Loopamp DNA Amplification kit™ (Eiken Chemical Co., Ltd., Tochigi, Japan). The reaction mixture (25 μl) containing 40 pmol of each inner primer, FIP and BIP, 5 pmol of each outer primer, F3 and B3, and 20 pmol of each loop primer, LF and LB, 2 times concentrated reaction mix (12.5 μl), *Bst* polymerase (1.0 μl), and 2.0 μl of sample was incubated at 63°C with a Loopamp real-time turbidmeter (LA-200; Teramecs, Tokyo, Japan) for 1 hr, followed by incubation at 80°C for 2 min to terminate the reaction. To confirm whether the LAMP amplification products were authentic, they were digested with a designated restriction enzyme and electrophoresed in a 3% agarose gel containing ethidium bromide for separation. The DNA fragments were then visualized. The amplified COM-LAMP and C-LAMP products were digested with *Taq*I, and the W-LAMP product was digested with *Bgl*II.

TABLE I. Names and Sequences of Primers for COM-LAMP, C-LAMP, W-LAMP

Assay	Target gene	Primers	
		Name	Nucleotide sequence
COM-LAMP	ATI	FIP-COM	5′-TGGAGTCTGCTAATCTCTGTAAGATTAGAGAACTAGAGAATAAGTTGACC-3′
		F3-COM	5′-CACAAGAAGTTGATGCACTG -3′
		BIP-COM	5′-TGAGTGAATGCCGTGGAAATGCGCAGTCGTTCAACTGTA-3′
		B3-COM	5′-CAGCATTGATTTCATTATTACGT-3′
		Loop-F-COM	5′-CGCTCTCGATGCAGTC-3′
		Loop-B-COM	5′-CAGAGATTACAATCTAGAATCTCAG-3′
C-LAMP	D14L	FIP-C	5′-TGGGAGCATTGTAACTTATAGTTGCCCTCCTGAACACATGACA-3′
		F3-C	5′-TGGGTGGATTGGACCATT-3′
		BIP-C	5′-ATCCTCGTATCCGTTATGTCTTCCCACCTATTTGCGAATCTGTT-3′
		B3-C	5′-ATGGTATGGAATCCTGAGG-3′
		Loop-F-C	5′-GATATTCGTTGATTGGTAACTCTGG-3′
		Loop-B-C	5′-GTTGGATATAGATGGAGGTGATTGG-3′
W-LAMP	ATI	FIP-W	5′-CCGTTACCGTTTTTACAATCGTTAATCAATGCTGATATGGAAAAGAGA-3′
		F3-W	5′-TACAGTTGAACGACTGCG-3′
		BIP-W	5′-ATAGGCTAAAGACTAGAATCAGGGATTCTGATTCATCCTTTGAGAAG-3′
		B3-W	5′-AGTTCAGTTTTATATGCCGAAT-3′
		Loop-F-W	5′-GATGTCTATCAAGATCCATGATTCT-3′
		Loop-B-W	5′-TCTTGAACGATCGCTAGAGA-3′

Standard DNA for Quantification in Each LAMP Assay

Standard DNA for the determination of the copy number of MPXV DNA was the pGEM-T easy vector inserted with the partial ATI gene amplified using the primer set, ATI-up-1 and ATI-low-1, for COM-LAMP and W-LAMP [Meyer et al., 1994]. The standard DNA for C-LAMP was the pGEM-T easy vector inserted with the partial *D14L* gene amplified using the primer set D14L-F (5′-GTTGTATGAGAGTATGATC-3′) and D14L-R (5′-TATGAAGGTGGAGAGCGTGAC-3′).

Nested PCR

A nested PCR, with which very low copies of the MPXV genomes could be amplified, was developed. The first round PCR was carried out using the primer set Gabon-1 (5′-GAGAGAATCTCTTGATAT-3′) and Gabon-2 (5′-ATTCTAGATTGTAATC-3′) [Neubauer et al., 1998]. The primers for the second round PCR were designed as follows: forward primer nest-Fa1 (5′-GCACACGCAATCAAGAAGAC-3′) and reverse primer nest-Ra1 (5′-ATTGTAATCTCTGTAGCATTTC-3′) to amplify the inner region of the product in the first round PCR. The reaction conditions were as follow: one cycle of 94°C for 5 min for denaturation, followed by 25 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, followed by one cycle of 72°C for 5 min. The PCR were performed using the GeneAmp PCR system 9700 (Applied Biosystems, Carlsbad, CA) with the Expand High Fidelity System™ (Roche Diagnostics Ltd.) according to the manufacturer's instructions.

Monkeys and MPXV-Challenge Experiments

Thirteen cynomolgus monkeys (*Macaca fascicularis*) born and raised at the Tsukuba Primate Center for Medical Science, National Institute of Biomedical Innovation, Tsukuba, Japan, were used. The monkeys were infected with MPXV in a highly contained laboratory, in which glove-box type safety cabinet was installed, at the NIID, Tokyo, Japan. All monkeys were infected with MPXV (Zr-599 or Liberia strain) at a dose of 10^6 plaque forming units. Smallpox vaccine, LC16 m8 and Lister, was confirmed to be effective in protecting the monkeys from generalized monkeypox [Saijo et al., 2006]. To demonstrate the various levels of the clinical manifestations of monkeypox, monkeys were immunized with LC16m8 or Lister for a designated period before MPXV challenge as shown in Table II. One monkey was infected with Zr-599 followed by immunization with LC16 m8. Blood samples and throat swab specimens were collected every 3 or 4 days after challenge. Clinical manifestations, such as changes in body weight, volume of food and water consumed, skin lesions, and the appearance of feces, were observed daily. The skin surface was observed carefully, and body temperature and weight were measured every 3–4 days while the monkeys were anesthetized.

Quantitative Real-Time PCR for MPXV

MPXV genome copies in the peripheral blood and throat swab specimens were determined by real-time quantitative PCR (qPCR) as reported previously [Saijo et al., 2006].

Statistical Analysis

Sensitivity and specificity were defined as the probability that the LAMP assay result was positive when the nested PCR showed a positive result, and as the probability that the LAMP assay result was negative when the nested PCR showed a negative result, respectively. The correlation coefficient(r) between the viremia levels detected by COM-LAMP and by qPCR was obtained using Pearson's correlation coefficient.

TABLE II. Experimental Data and Clinical Manifestations of Monkeypox for Monkey Subjects Used in This Study

ID	Virus inoculated	Route	Vaccination	Days from vaccination to challenge	No. of clinical samples tested		No. of papulovesicular lesions	Severity	Outcome
					PBC	TS			
Z-01-SC	Zr-599	SC	—	—	5	5	388	Severe	Sacrificed
Z-02-SC		SC	LC16m8	0	7	7	691	Severe	Survived
Z-03-SC		SC	LC16m8	3	6	6	286	Mild	Survived
Z-04-SC		SC	LC16m8	7	6	6	0	Asymptomatic	Survived
L-01-SC	Liberia	SC	—	—	7	7	196	Moderately severe	Survived
L-02-SC		SC	—	—	4	0	—	Severe	Sacrificed
L-03-SC		SC	—	—	7	0	29	Moderately severe	Survived
LC-04-IN		IN	—	—	8	0	10	Mild	Survived
LC-05-IN		IN	—	—	8	0	16	Mild	Survived
LC-06-IN		IN	Lister	42	8	0	0	Asymptomatic	Survived
LC-07-IN		IN	Lister	42	9	0	0	Asymptomatic	Survived
LC-08-IN		IN	Lister	42	7	0	0	Asymptomatic	Survived
LC-09-IN		IN	LC16m8	42	7	0	0	Asymptomatic	Survived

TS, throat swab specimens; PBC, peripheral blood cells; SC, subcutaneous inoculations; IN, intranasal inoculations.

RESULTS

Detection of Orthopoxviruses by COM-LAMP

At least 10^2 copies of MPXV Zr-599 and Liberia genomes were detected by the COM-LAMP assay. 10^6 copies of camelpox virus genomes, but only 10^5 copies of the virus genome, were positive by the COM-LAMP assay (Table III). However, the amplified DNA from the MPXV genomes could be differentiated from that from camelpox virus by restriction enzyme treatment (Fig. 1).

Detection of the Genomes of Congo Basin and West African MPXV by C-LAMP and W-LAMP

MPXV Zr-599 was detected by C-LAMP, but not by W-LAMP. On the other hand, MPXV Liberia was detected by W-LAMP but not by C-LAMP (Table III). All the Congo Basin MPXV genomes but not the West African MPXV genomes were detected by C-LAMP. On the other hand, all the West African MPXV genomes but not the Congo Basin MPXV genomes were detected by W-LAMP (data not shown).

Detection Limit of COM-LAMP, C-LAMP, and W-LAMP

The detection limits of COM-LAMP, C-LAMP, and W-LAMP were approximately $10^{2.0}$, $10^{2.4}$, 10^3 copies/reaction of standard DNA, respectively. These values were calculated from the results obtained from 9 independently repeated experiments.

Sensitivity and Specificity of the LAMP Assays

Detection of MPXV genomes in clinical samples, peripheral blood cells and throat swab specimens, was tested by each of the LAMP assays and nested PCR. The sensitivity and specificity of COM-LAMP, C-LAMP, and W-LAMP were 80% (45/56) and 100% (64/64); 79% (19/24) and 100% (24/24); and 70% (23/32) and 100% (40/40), respectively (Table IV).

Relationship Between Severity of Symptoms and Viremia Level as Determined by COM-LAMP

The severity of monkeypox symptoms observed during the challenge experiments is shown in Table II. The

TABLE III. The Reactivity of the Tested Orthopoxvirus DNAs in COM-LAMP and of MPXV DNAs in C-LAMP and W-LAMP

LAMP	Virus	Virus genome (copies/reaction)					
		10^1	10^2	10^3	10^4	10^5	10^6
COM-LAMP	MPXV Zr-599	−	+	+	+	+	+
	MPXV Liberia	−	+	+	+	+	+
	Ectromelia	NT	NT	NT	−	−	−
	Cowpox	NT	NT	NT	−	−	−
	Camelpox	NT	NT	NT	−	−	+
	Vaccinia	NT	NT	NT	−	−	−
C-LAMP	MPXV Zr-599	−	−	+	+	+	+
	MPXV Liberia	−	−	−	−	−	−
W-LAMP	MPXV Zr-599	−	−	−	−	−	−
	MPXV Liberia	−	+	+	+	+	+

NT, not tested.

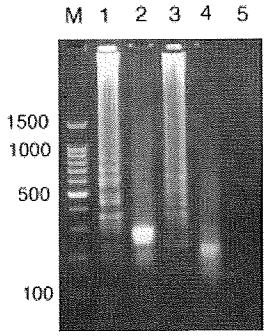

Fig. 1. DNA products from MPXV and camelpox virus amplified with COM-LAMP and separated in a 3% agarose gel by electrophoresis (**lanes 1** and **3**, respectively). To discriminate MPXV from camelpox, MPXV-LAMP and camelpox virus-LAMP products were treated with a restriction enzyme (*TaqI*) (**lanes 2** and **4**, respectively). A 100 bp-DNA ladder marker and negative control are also shown (**lanes M** and **5**, respectively).

infection was lethal in one naive monkey (Z-01-SC). The viremia level determined by the COM-LAMP assay continued to increase until sacrifice. The symptoms in the monkey pre-immunized 3 days before challenge (Z-03-SC) were less severe than those of the post-exposure vaccinated monkey (Z-02-SC). The viremia level in monkey (Z-02-SC) was significantly higher than that in monkey (Z-03-SC) throughout the observation period. The monkey pre-immunized 7 days before challenge (Z-04-SC) showed an asymptomatic infection. No viremia was demonstrated in this subject by the COM-LAMP assay (Fig. 2A). The severer the level of monkeypox-associated symptoms observed, the higher the viremia level determined by COM-LAMP. A similar phenomenon was observed in experiments in which monkeys were infected with MPXV Liberia through intranasal inoculation or the subcutaneous route at a dose of 10^6 PFU (Fig. 2B). The monkeypox symptoms in one monkey (L-03-SC) were so severe that the subject was sacrificed due to ethical considerations. The two monkeys (L-01-SC and L-020SC) infected with MPXV Liberia through the subcutaneous route showed moderately severe symptoms and survived. The two monkeys (L-04-IN and L-05-IN) showed mild symptoms with less than 20 papulovesicular skin lesions. Furthermore, viremia was demonstrated on Day 3 in the subcutaneously infected monkeys but not in the intranasally infected subjects.

Relationship Between Virus Loads as Determined by COM-LAMP and qPCR

The relationship between the virus load in the clinical samples determined by COM-LAMP and those determined by qPCR is shown in Figure 3. The correlation coefficient was 0.60, which represents a strong positive correlation.

DISCUSSION

Nucleic acid amplification-based diagnostic assays have become a gold standard for the rapid diagnosis of viral infections. Several PCR assays, such as conventional PCR and real-time quantitative PCR, have been reported for MPXV [Ibrahim et al., 1997; Neubauer et al., 1998; Kulesh et al., 2004; Aitichou et al., 2005, 2008; Saijo et al., 2006, 2008; Scaramozzino et al., 2007]. The real-time quantitative PCR assays have the advantages of rapidity, quantification-capacity, detection in a real-time manner, and high sensitivity. However, these nucleic acid amplification methods require high-precision instruments such as LightCycler instruments (Roche Diagnostics Ltd.). On the other hand, LAMP can be carried out without using such instruments. Furthermore, virus genomes can be detected within a shorter time and in a real-time manner. If turbidity detection is performed using a Loopamp real-time turbidmeter (LA-200), the virus genomes can be detected in a real-time manner along with genome quantification.

Three LAMP assays, COM-LAMP, C-LAMP, W-LAMP, were developed in this study. Using these assays, it was possible to detect the genomes of the Congo Basin and West African MPXVs and to differentiate between the genomes of the Congo Basin and of West African MPXVs by a combination of the three LAMP assays.

10^6 copies/reaction of the camelpox virus genome showed a positive reaction in the COM-LAMP (Table III), indicating that a positive reaction in the COM-LAMP assay does not always indicate an MPXV infection. The homology of the Loop-B-COM with

TABLE IV. Relationship Between the Results Obtained From the Nested PCR and LAMP Assays

	Samples from Zr-599-challenged monkeys Nested PCR		Samples from Liberia-challenged monkeys Nested PCR	
LAMP method	Positive	Negative	Positive	Negative
COM-LAMP				
Positive	21	0	24	0
Negative	3	24	8	40
C-LAMP				
Positive	19	0	0	0
Negative	5	24	33	39
W-LAMP				
Positive	0	0	23	0
Negative	24	24	9	40

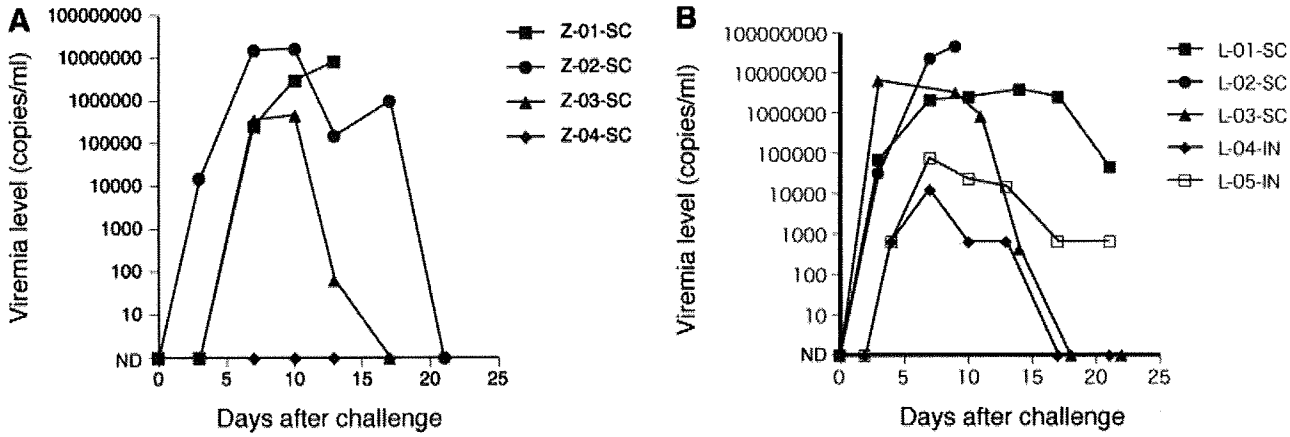

Fig. 2. Viremia level in peripheral blood collected from MPXV Zr-599-infected monkeys (**A**) and those in MPXV Liberia-infected monkeys (**B**) as determined by COM-LAMP. "ND" indicates it was below the detection level.

camelpox virus ATI-gene (80%) was higher than those with vaccinia virus and cowpox virus (76%), while there were no significant differences in homology of the other primers among these orthopoxviruses. Although further study is needed, the difference might be responsible for positive reaction of camelpox virus in COM-LAMP assay. Although a positive reaction in COM-LAMP does not indicate MPXV genome-positive, the amplified products of MPXV DNA and the other orthopoxvirus DNAs could be differentiated by restriction enzyme treatment or a combination of the COM-LAMP, C-LAMP and W-LAMP assays. Furthermore, when the viremia level determined by COM-LAMP was much less than that determined by C-LAMP, the samples can be understood to contain orthopoxviruses other than MPXV as orthopoxviruses such as camelpox and vaccinia viruses possesses a similar nucleotide sequence to the *D14L* gene of Congo Basin MPXV (data not shown). Although the data is not shown here, the genomes of herpes simplex virus and varicella zoster virus, which cause vesicular skin infections in humans and must be differentiated from human monkeypox, showed negative reactions in the newly developed LAMP assay. The corresponding genomes in variola virus, a causative agent for smallpox, to the partial ATI gene amplified by the COM-LAMP assay do not possess the *Taq*I restriction site, suggesting that the differentiation of MPXV from variola virus is possible by the COM-LAMP assay.

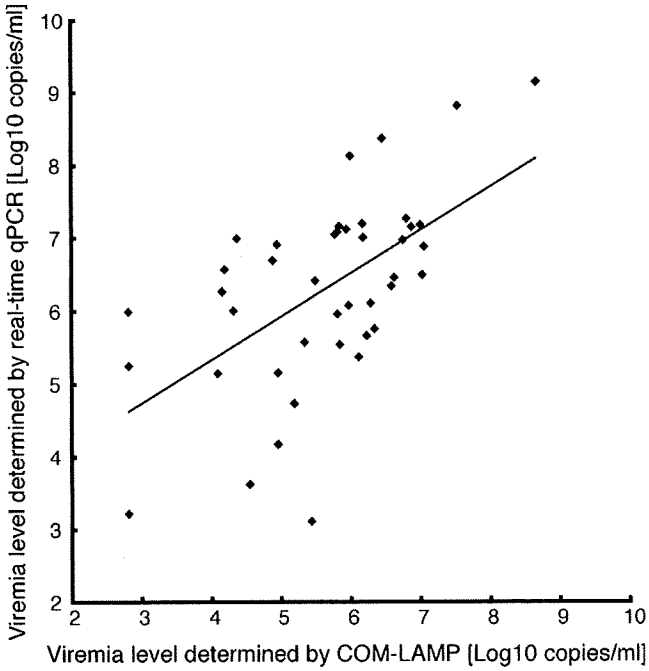

Fig. 3. Correlation between viral loads as determined by LAMP assays and real-time qPCR.

Three LAMP assays were evaluated in comparison with nested PCR. The sensitivity and specificity of the three LAMP assays when compared with the nested PCR were approximately 70–80% and 100%, respectively (Table IV). Because the nested PCR for the amplification of genome sequences is quite sensitive, the sensitivity of the LAMP assay was calculated to be 70–80% in this study. However, the sensitivity of the COM-LAMP assay was much higher when compared with the conventional PCR reported previously [Neubauer et al., 1998]. The viremia level determined by COM-LAMP was associated with the severity of clinical symptoms of monkeypox. The most significant advantages of LAMP assay over conventional PCR and real-time quantitative PCR is that the assay is simpler to perform. No highly specialized instruments are necessary for the LAMP assay, and even the detection of the turbidity derived from the accumulation of byproduct can be done visually [Mori et al., 2001]. It is concluded that the newly developed LAMP assays afford a valuable tool not only for the diagnosis of but also for the assessment of MPXV infections.

In summary, a sensitive, specific and rapid LAMP system for the detection of the MPXV genome was developed. Using this technology, MPXV can be differentiated into Congo Basin strains or West African

strains. This technology offers great benefits in the control of outbreaks of MPXV infections and in the assessment of the course of MPXV infections. Furthermore, the newly developed LAMP system may offer advantages in the diagnosis of human monkeypox, which would need to be differentiated from smallpox in the event of a variola virus-associated bioterrorism attack.

ACKNOWLEDGMENTS

The challenge experiments with MPXV were conducted in the high-containment laboratory at the NIID, Japan. All animal procedures were approved by the Committees on Biosafety and Animal Handling and Ethical Regulations of the National Institute of Infectious Diseases, Japan. Animal research was undertaken in compliance with the guidelines issued from the Ministry of Health, Labor and Welfare, "The Fundamental guidelines for proper conduct of animal experiment and related activities in institutions under jurisdiction (June 2006)." Our animal work also adhered to the principles stated in the guidelines.

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Short Communication

Virulence and pathophysiology of the Congo Basin and West African strains of monkeypox virus in non-human primates

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Monkeypox virus is divided into Congo Basin and West African strains. The virulence and pathophysiology of two strains, Zr-599 (a Congo Basin monkeypox virus) and Liberia (a West African monkeypox virus), were evaluated in non-human primates. Four monkeys were infected by the subcutaneous (SC) and two by the intranasal (IN) inoculation routes for Zr-599 and Liberia at a dose of 10^6 p.f.u. One monkey in the Liberia/SC group was demonstrated to be co-infected with Gram-positive cocci and was excluded from analyses. Infections in three of the four Zr-599/SC monkeys and in one of the three Liberia/SC monkeys were fatal. Virus genome levels in blood in the Zr-599/SC monkeys were approximately 10 times higher than those in the Liberia/SC monkeys. Zr-599 affected respiratory, genito-urinary and gastrointestinal tract organs more severely than Liberia. Zr-599 was more virulent than Liberia and one of the factors might be the difference in organ tropism.

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The species *Monkeypox virus* belongs to the genus *Orthopoxvirus*, family *Poxviridae*. Monkeypox viruses cause human monkeypox in humans inhabiting the rainforests of central and western Africa (Arita *et al.*, 1985; Damon, 2007; Heymann *et al.*, 1998). Wild rodents (*Funiscius anerythrus* and *Helioscirus rufobrachium*) were implicated as the most probable reservoir (Khodakevich *et al.*, 1987). Human monkeypox is endemic to central and western Africa (Khodakevich *et al.*, 1988). An outbreak of human monkeypox was reported in the Democratic Republic of Congo (DRC) (Khodakevich *et al.*, 1988; Learned *et al.*, 2005; Mukinda *et al.*, 1997). Human monkeypox outbreaks outside Africa were first reported in the USA in 2003 (Di Giulio & Eckburg, 2004; Guarner *et al.*, 2004; Reed *et al.*, 2004). In that outbreak, patients acquired the virus from prairie dogs (*Cynomys* spp.) that became ill after contact with various exotic rodents shipped from Ghana, Africa (Reed *et al.*, 2004).

Two clades of monkeypox virus exist: West African and Congo Basin monkeypox virus (Likos *et al.*, 2005). Human and monkey disease virulence differs between Congo Basin and West African strains, the former being more virulent in non-human primates (Chen *et al.*, 2005). The clinical manifestations of human monkeypox are reported to be similar to those of smallpox (Arita *et al.*, 1985; Breman *et al.*, 1977, 1980; Foster *et al.*, 1972; Janseghers *et al.*, 1984; Jezek & Khodakevich, 1987; Stagles *et al.*, 1985). This study describes the clinical manifestations and laboratory and pathological findings in cynomolgus monkeys infected with Congo Basin or West African monkeypox virus. The virulence of Congo Basin and West African monkeypox virus was compared. Furthermore, the pathophysiological mechanisms behind the difference in virulence between these two monkeypox viruses were elucidated.

Monkeypox virus strains Zr-599 (a representative Congo Basin strain) and Liberia (a representative West African strain) were used. Zr-599, isolated from a patient in the DRC, and Liberia, isolated from a patient with human monkeypox in Liberia, were assigned to the Congo Basin and the West African clades, respectively, according to A-type inclusion body gene sequence (Likos *et al.*, 2005). Virus solution for challenge experiments was prepared by disruption of Vero E6 cells infected with each monkeypox

Supplementary figures and tables are available with the online version of this paper.

virus strain in a sonicator (TITEC Ultra S Homogenizer UP-5) for 30 s at full power, followed by high-speed centrifugation (3500 r.p.m. for 5 min at 4 °C). The infectious dose of the virus was determined by plaque assay.

Twelve cynomolgus monkeys (*Macaca fascicularis*) were used (see Supplementary Table S1, available in JGV Online). They were classified into four groups: Zr-599/SC, Liberia/SC, Zr-599/IN and Liberia/IN. Monkeys #4651 and #4653 (Zr-599/SC) and #4595 and #4596 (Liberia/IN) were used in a previous study in which the efficacy of a smallpox vaccine, LC16m8, was evaluated (Saijo *et al.*, 2006). Other monkeys were also used as control subjects in the study for the evaluation of LC16m8 efficacy. A monkey in the Liberia/SC group (#4567) died on day 10 post-inoculation. This subject was demonstrated to be co-infected with Gram-positive cocci and was excluded from further analyses.

Complete blood-cell counts in peripheral blood collected in sodium heparinized tubes were measured. C-reactive protein (CRP) was measured as an indicator of inflammation level.

Vaccinia virus-specific antibody levels were measured by ELISA using the entire suite of vaccinia virus proteins as antigens, as reported previously (Morikawa *et al.*, 2005; Saijo *et al.*, 2006). Although the IgG response in monkeys #4651 and #4653 from the Zr-599/SC group and #4595 and #4596 from the Liberia/IN group had already been determined as reported previously (Saijo *et al.*, 2006), the IgG response in all of the monkeys, including the four previously tested monkeys, was determined simultaneously. Virus genome levels were determined by a quantitative real-time PCR (qPCR) method as reported previously (Saijo *et al.*, 2006, 2008). Although the virus genome level in monkeys #4651 and #4653 from the Zr-599/SC group and #4595 and #4596 from the Liberia/IN group had already been determined in a previous study (Saijo *et al.*, 2006), the virus genome level in the peripheral total blood of all of the monkeys, including the four previously tested monkeys, was determined simultaneously. All challenge experiments were conducted in a highly contained laboratory in which a glovebox class III safety cabinet was installed. The monkeys were anaesthetized and inoculated intranasally (IN) with 0.5 ml virus solution containing 1×10^6 p.f.u. Zr-599 or Liberia by using an atomizer (Keytron Co.) to atomize the virus solution, or inoculated subcutaneously (SC) with 0.5 ml virus solution containing 1×10^6 p.f.u. Zr-599 or Liberia. After the challenge, blood was drawn every 2–4 days. Clinical manifestations, such as volume of food and water consumed, appearance of faeces, etc., were observed every day. The skin surface was observed carefully, and body (anal) temperature and mass were measured.

After sacrifice under anaesthesia, skin, lymphoreticular system structures (lymph nodes, spleen, thymus, pharynx and tonsils), gastrointestinal tract organs (including the liver and pancreas), genito-urinary tract organs (kidneys, bladder, testes, ovaries and uterus), endocrine organs (adrenal glands and thyroid), respiratory tract organs (trachea and lungs), the heart as the cardiovascular organ, and central nervous system (CNS) organs (brain and spinal cord) were excised, fixed in 10 % formalin in PBS and embedded in paraffin. They were then examined for micropathology and the presence of monkeypox virus antigens by immunohistochemical analyses as reported previously (Nagata *et al.*, 2001, 2002; Saijo *et al.*, 2006).

Infections in three of the four Zr-599/SC monkeys and one of the three Liberia/SC monkeys were fatal, whereas all Zr-599/IN and Liberia/IN monkeys survived (see Supplementary Table S1, available in JGV Online). In Zr-599/SC monkeys, body mass decreased sharply by 10–20 % after challenge without any sign of recovery except for one subject that survived, whereas body mass in Liberia/SC monkeys decreased less sharply. There was a tendency for body temperature to rise for the first week after virus inoculation in all groups (see Supplementary Fig. S1, available in JGV Online).

Papulovesicular rashes appeared on days 7–9 after monkeypox virus inoculation. The general condition of the monkeypox virus-infected monkeys deteriorated and their activity decreased from day 6 to day 11 post-inoculation. The mean number of papulovesicular lesions in the Zr-599-infected monkeys (n=369) was higher than that in the Liberia-infected monkeys (n=226) (Supplementary Table S1). Skin and gross lesions in the internal organs in a Zr-599-infected subject (#4653) and a Liberia-infected subject (#4625) on day 18 post-inoculation are shown in Fig. 1. Both subjects were sacrificed because of severe symptoms on day 18 post-inoculation. The papulovesicular lesions demonstrated in both monkeys were morphologically similar. Lymph nodes and thymus in both subjects were affected. The most significant differences were that gross lesions with a granulomatous appearance were demonstrated in the gastrointestinal tract organs, such as stomach, small intestine and colon, in the Zr-599-infected monkey, but not in the Liberia-infected monkey (Fig. 1). The peritoneal membrane of the Zr-599-infected subject (#4653) became thickened and had granulomatous lesions, whereas that of the Liberia-infected subject (#4625) was intact. In Zr-599/IN monkeys, one (#4654) showed severe monkeypox-associated symptoms and the other (#4655) showed very mild symptoms. In the Liberia/IN monkeys, the symptoms were relatively mild and of short duration, with only a small number of papulovesicular lesions. The Zr-599-induced ulcerative lesions were still exudative on day 18, whereas the Liberia-induced lesions were dried and covered with scar tissues (Fig. 1). The Zr-599-induced ulcerative lesions seemed to be more severe than the Liberia-induced lesions.

There was a statistically significant difference in virus genome levels as determined by qPCR between Zr-599-infected and Liberia-infected monkeys (see Supplementary Table S2, available in JGV Online), the highest level assessed in combinations of SC and IN group monkeys

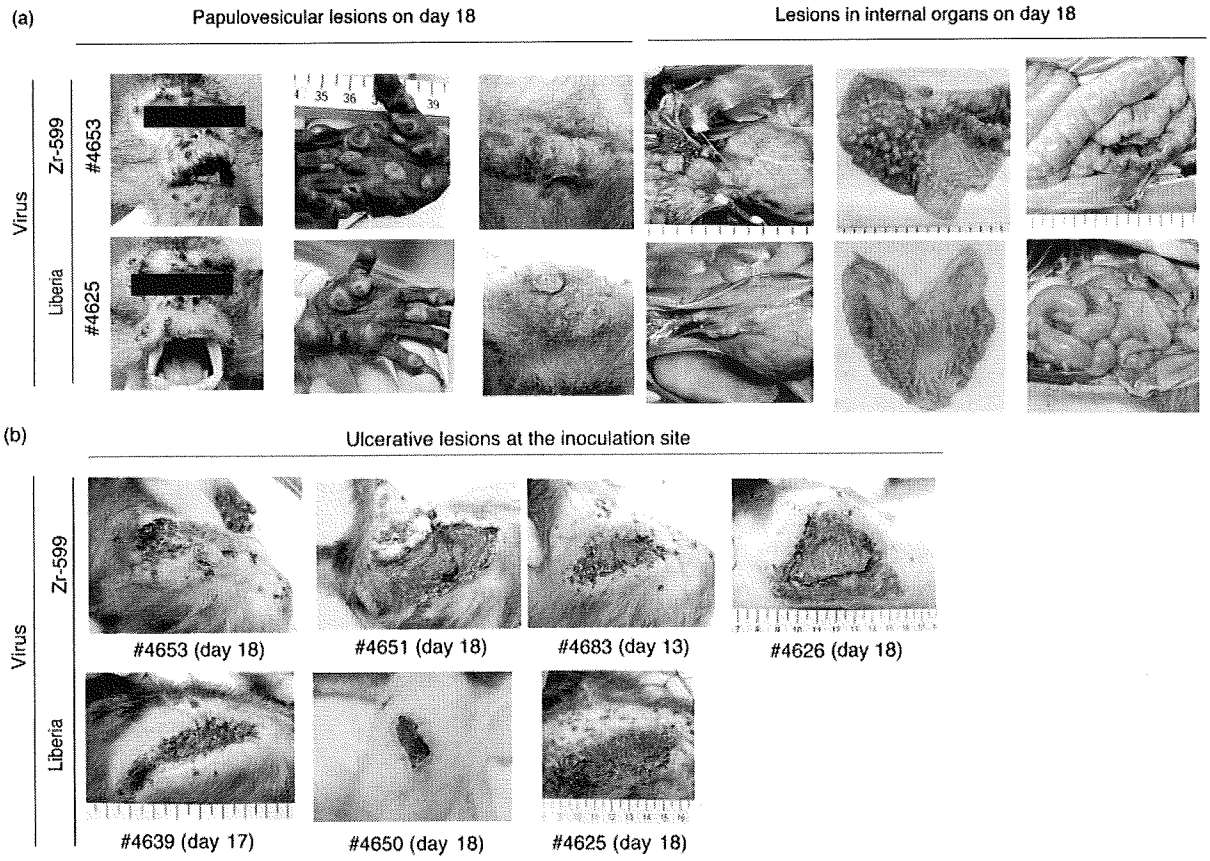

Fig. 1. (a) Monkeypox-associated lesions in skin and internal organs of fatal cases of Zr-599 (#4653) and Liberia (#4625) infection. (b) Ulcerative lesions at the monkeypox virus inoculation site in each subject.

(Student's *t*-test, $P=0.03$) and the mean virus genome level per day in the first 14 days in IN group monkeys (Student's *t*-test, $P=0.02$). All indices in Zr-599-infected monkeys were approximately 10 times higher than those in Liberia-infected monkeys.

There was a significant difference in the IgG response between the Zr-599-infected and Liberia-infected monkeys (Supplementary Fig. S1). In five of the six Zr-599-infected monkeys, an obvious IgG response was demonstrated even on day 10 post-inoculation, whereas the IgG ELISA was negative in all Liberia-infected monkeys except one on day 10. The IgG response was detected 2–3 days earlier in the Zr-599-infected monkeys than in the Liberia-infected monkeys.

The data on monkeypox virus antigen distribution indicated that Zr-599 infected the organs of the respiratory tract, gastrointestinal tract, lymphoid and reticuloendothelial systems, genito-urinary tract and skin, but not the CNS, and that Liberia mainly infected the lymphoid and reticuloendothelial systems and skin (Table 1). Micrographs of immunoperoxidase-stained tissue sections of Zr-599/SC monkeys are available in Supplementary Fig. S2 (in JGV Online).

To compare the severity of monkeypox-associated symptoms, a scoring system was developed and used in this study. The score is composed of two categories: items associated with clinical symptoms and those associated with laboratory findings. The following variables were recorded: decrease in body mass, duration of decreased activity with ill appearance, duration of decreased food consumption (<75 %), body temperature (fever and lower temperature), diarrhoea with/without blood contamination, number of papulovesicular lesions except for the lesions associated with the challenge virus inoculation, outcome, virus genome level (maximum value during the course of observation and the final value when the observation finished), total peripheral blood-cell counts (increased and/or decreased numbers of white blood cells, decreased number of platelets, haemoglobin level) and CRP level. The points for each item are shown in Table 2. It is indicated that the higher the score, the more severe the monkeypox. The mean severity point of monkeypox caused by Zr-599, 25.2, was higher than that caused by Liberia, 17.0. When the same analysis was conducted based on points of the subjects that were infected with virus through the SC route, the mean point of the Zr-599/SC group, 30.0, was higher than that of the Liberia/SC group,

Table 1. Number of subjects with gross monkeypox-associated lesions confirmed by positive monkeypox virus antigen in each organ, as determined by immunohistochemical analyses

Organ	No. subjects with monkeypox virus antigen in each organ			
	Zr-599/SC	Zr-599/IN	Liberia/SC	Liberia/IN
Total *n*	4	2	3	2
Respiratory				
Trachea	3	0	0	1
Lung	3	0	0	2
Cardiovascular				
Heart	0	0	0	0
Gastrointestinal tract				
Liver	4	1	1	0
Pancreas	3	1	0	1
Oesophagus	0	0	0	0
Stomach	3	0	0	0
Ileum	2	1	0	0
Colon	1	0	0	0
Rectum	4	1	0	0
Endocrine system				
Thyroid	2	0	0	0
Adrenal gland	1	0	0	0
Lymphoreticular system				
Radial lymph node	4	1	1	1
Submandibular lymph node	4	2	1	2
Inguinal lymph node	4	1	2	1
Axillar lymph node	4	1	2	2
Tonsil	4	1	2	2
Thymus	4	1	2	2
Spleen	3	1	0	2
Pharyngeal	4	1	1	1
Genito-urinary tract				
Kidney	0	0	0	0
Bladder	1	0	0	0
Prostate/uterus	2	0	1	1
Testis/ovary	3	1	0	0
Skin				
Skin lesions	4	2	3	2
CNS				
Basal ganglia, lateral lobe, frontal lobe, thalamus or spine	0	0	0	0

20.7 (Table 2). The mean point of the Zr-599/IN group, 15.5, was also higher than that of the Liberia/IN group, 11.5.

The pathology of experimental monkeypox virus infections in non-human primates infected with isolate V79-I-005, which was originally obtained from a fatally infected human from Zaire in 1979, through the respiratory route was reported previously (Zaucha *et al.*, 2001). However, the pathology in monkeys infected with monkeypox virus West African strain has not been studied. Monkeypox-associated gross lesions were demonstrated in the following organs: respiratory system, skin, oral cavity, gastrointestinal tract and lymphoid systems. The pathological findings observed in monkeys infected with the Zr-599 strain were similar to those observed in the previous study (Zaucha *et al.*, 2001). The value of the present study is that the pathology of both Congo Basin and West African monkeypox viruses has been investigated. Differences were observed in fatality rate, severity of monkeypox-associated symptoms, virus genome level and the organs affected. Zr-599 replicated in skin, lymphoid and reticuloendothelial systems, genito-urinary tract organs, respiratory tract organs and gastrointestinal tract organs, whereas Liberia replicated only in the skin, lymphoid and reticuloendothelial systems of the monkeys (Table 1). Whilst the lung of a Zr-599/SC-infected monkey was entirely and diffusely affected by the infection (Supplementary Fig. S2), the lung of a Liberia/SC-infected monkey was histopathologically intact (data not shown). Haemorrhagic diarrhoea was observed only in the Zr-599-infected monkeys. These results suggest that the respiratory and gastrointestinal functions were more severely impaired

Table 2. Difference in virulence between the Zr-599 and Liberia strains of monkeypox virus in non-human primates, as determined by the proposed scoring system for analysis of monkeypox severity

Abbreviations: UDL, under detection level; WBC, white blood cells.

Item	No. animals with score	Mean score in each group			
		Zr-599/SC	Zr-599/IN	Liberia/SC	Liberia/IN
Decrease in body mass (%)	<3, 0; 3–<8, 1; 8–<13, 2; >13, 3	2.3±0.5	1.5±0.7	2.3±1.2	2.0±0.0
Recovery signs in body mass	Positive, 0; negative, 3	2.3±1.5	0	1.0±1.7	0
Duration of decreased activity (days)	None, 0; 1–5, 1; 6–10, 2; >10, 3	2.3±1.0	1.5±0.7	2.0±0.0	1.5±0.7
Duration of decreased meal consumption (days)	None, 0; 1–5, 1; 6–10, 2; >10, 3	2.5±0.6	2.0±1.4	2.3±0.6	1.0±0.0
Fever >1 °C	Negative, 0; positive, 1	0.3±0.5	0	0.3±0.6	0
Drop in body temperature >1.5 °C	Negative, 0; positive, 3	2.3±0.5	0	1.0±1.7	0
Faecal appearance	Normal, 0; watery diarrhoea, 1; haemorrhagic diarrhoea, 3	1.8±1.5	1.5±2.1	0.7±0.6	1.0±0.0
Papulovesicular lesions (no.)	None, 0; 1–50, 1; 51–499, 2; >500, 3	2.3±0.5	1.5±0.7	2.0±1.0	1.0±0.0
Outcome	Non-fatal, 0; fatal, 6	4.5±3.0	0	2.0±3.5	0
Maximum virus genome level [$\log_{10}$(copies ml^{-1})]	UDL, 0; <5, 1; 5–7, 2; >7, 3	2.8±0.5	2.5±0.7	2.3±0.6	1.5±0.7
Virus genome level when sacrificed [$\log_{10}$(copies ml^{-1})]	UDL, 0; <5, 1; 5–7, 2; >7, 3	2.0±1.4	1.0±1.4	1.0±1.0	0.5±0.7
Peripheral WBC count <5000 μl^{-1}	Positive, 0; negative, 1	0.5±0.6	1.0±0.0	1.0±1.0	0.5±0.7
Thrombocytopenia <20 000 μl^{-1}	Positive, 0; negative, 1	0.8±0.5	0.5±0.7	0.3±0.5	1.0±0.0
Anaemia with decrease in haemoglobin level >1.5 g dl^{-1}]	Positive, 0; negative, 1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Maximum CRP level [mg dl^{-1}]	<1, 0; 1–<5, 1; 5–<10, 2; >10, 3	2.8±0.5	1.5±0.7	2.3±0.6	1.0±1.4
Mean		30.0±10.4	15.5±9.2	20.7±9.1	11.5±3.5

in the Zr-599-infected monkeys than in the Liberia-infected monkeys.

The difference in the level and course of virus genome detection was consistent with the difference in the pathological findings. Zr-599 replicated more efficiently in the internal organs than did Liberia (Fig. 1; Table 1). The higher level of virus genome detected in the later phase of infection in the Zr-599/SC monkeys might be due to the more efficient replication of Zr-599, particularly in the generalized lymphoid and reticuloendothelial systems, skin, genito-urinary tract organs, respiratory organs and gastrointestinal organs, than that of Liberia in these organs. This feature of Zr-599 might lead to multi-organ failure with malfunctions of respiratory, gastrointestinal and genito-urinary tract organs, resulting in stronger virulence of Zr-599 than of Liberia in non-human primates.

It is believed that Congo Basin strains are more virulent than West African strains in humans, based on clinical studies (Breman *et al.*, 1980; Foster *et al.*, 1972; Ladnyj *et al.*, 1972). Recently, it was reported that a Congo Basin strain, Zr79, which was isolated from a fatal case of monkeypox in Zaire in 1979, was more virulent than the West African strain US03, which was isolated from a non-fatal case of monkeypox in the USA in 2003, using a ground squirrel model for monkeypox virus infection (Sbrana *et al.*, 2007). The clinical course and virological and pathophysiological features of monkeypox virus infections in non-human primates obtained in this study were different from those reported in the ground squirrel model (Sbrana *et al.*, 2007; Tesh *et al.*, 2004). Monkeypox virus infections in non-human primates resemble human monkeypox in terms of pathophysiological profile, making the present study of particular value.

The genetic and molecular mechanism(s) underlying the differences in pathogenesis between Congo Basin and West African strains should be clarified through further studies. It was reported that *D10L*, *D14L*, *B10R*, *B14R* and *B19R* were possibly responsible genes, with *D14L*, an orthologue of vaccina complement protein, as a leading candidate and with *D10L* and *B19R* as less likely candidates (Chen *et al.*, 2005). Although the data are not shown here, the *D14L* gene was confirmed to be absent in the Liberia strain, as in the case of an West African strain, SL-70 (Chen *et al.*, 2005).

In conclusion, it was demonstrated that Zr-599, a Congo Basin strain, was more virulent than Liberia, a West African strain. The difference in virulence might be due to the difference in the sites of virus replication resulting in organ dysfunction: Zr-599 replicated in skin, lymphoid and reticuloendothelial systems, genito-urinary tract organs, respiratory organs and gastrointestinal organs, whereas Liberia replicated only in skin, lymphoid and reticulo-endothelial systems.

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Characterization of Monoclonal Antibodies to Junin Virus Nucleocapsid Protein and Application to the Diagnosis of Hemorrhagic Fever Caused by South American Arenaviruses▽

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Junin virus (JUNV), Machupo virus, Guanarito virus, Sabia virus, and Chapare virus are members of New World arenavirus clade B and are the etiological agents of viral hemorrhagic fevers that occur in South America. In this study, we produced three monoclonal antibodies (MAbs) to the recombinant nucleocapsid protein of JUNV, designated C6-9, C11-12, and E4-2. The specificity of these MAbs was examined by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay, and an epitope-mapping method. Using these MAbs, we developed antigen (Ag) capture ELISA systems. We showed that by using MAb C6-9, JUNV Ag was specifically detected. On the other hand, by using MAb C11-12 or E-4-2, the Ags of all human pathogenic South American arenaviruses were detected. The combined use of these Ag capture ELISA systems in the present study may be useful for the diagnosis of acute-phase viral hemorrhagic fever due to infection by a South American arenavirus.

The South American arenaviruses Junin virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Sabia virus (SABV), and Chapare virus (CHPV) are members of New World arenavirus clade B. JUNV, MACV, GTOV, and SABV are the etiological agents of Argentine hemorrhagic fever (AHF), Bolivian hemorrhagic fever (BHF), Venezuelan hemorrhagic fever (VHF), and Brazilian hemorrhagic fever, respectively (4). CHPV was also recently shown to be associated with cases of hemorrhagic fever in Bolivia (5). AHF emerged in the 1950s, and since then, outbreaks have occurred annually without interruption (4). The mortality rate for AHF is estimated to be 15 to 30%, but early treatment with immune plasma reduces the rate to less than 1% (6). The region at risk has been progressively expanding into northern central Argentina, and almost 5 million people are currently considered to be at risk for AHF (6, 13). Phylogenetic analysis indicates that JUNV is more closely related to MACV than to SABV or CHPV, whereas SABV and CHPV are more closely related to each other than to other New World arenaviruses (5).

Arenaviruses are enveloped and contain a bisegmented RNA genome. The genome consists of two ambisense single-stranded RNA molecules, one designated L, which encodes the RNA-dependent RNA polymerase and a zinc-binding matrix protein, Z, and the other designated S, which encodes the major structural components of the virion, i.e., the nucleocapsid protein (NP) and the envelope glycoprotein precursor (15). The arenavirus NP is the most abundant protein among the viral structural proteins both in infected cells and in virions (2) and is commonly used as a target for detecting viral antigens (Ags) (20). Moreover, arenavirus NPs have been known to be the most conserved among the same virus species and, to some extent, among different arenavirus species (3, 8). Therefore, it seems likely that monoclonal antibodies (MAbs) raised against the NP of an arenavirus would also be useful for detecting other arenaviruses (20). Recently, an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was developed by using a recombinant NP (rNP) of JUNV, obtained from a recombinant baculovirus system, and was proposed to be useful for etiologic confirmation of AHF in seroepidemiological studies (20, 26). It is considered that an Ag capture ELISA using MAbs specific for viral Ags allows rapid diagnosis of the acute phase of viral hemorrhagic fever by detecting viral Ags in blood or tissue homogenates (20). In this study, we produced MAbs to the rNP of JUNV. These MAbs were characterized by ELISA, indirect immunofluorescence assay (IFA), and an epitope-mapping method. Ag capture ELISAs were developed by using these MAbs that are specific for JUNV and that are broadly applicable for the detection of human pathogenic New World arenaviruses.

MATERIALS AND METHODS

Cell culture. Hybridomas and their parental cell line, P3/Ag568, were maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (Invitrogen), and antibiotics (streptomycin and penicillin G; Invitrogen). Hypoxanthine-aminopterin-thymidine supplement (Invitrogen) was added to the me-

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dium for selection of hybridomas, as recommended by the supplier. BTI-TN-5B1-4 (High Five; Invitrogen) insect cells were maintained in TC100 (Invitrogen) supplemented with 10% FBS, 2% tryptose phosphate broth (Difco, Detroit, MI), and kanamycin (Invitrogen). HeLa cells were maintained in minimal essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FBS and antibiotics (streptomycin and penicillin G; Invitrogen).

Recombinant baculoviruses. The baculoviruses Ac-JUNV-NP and Ac-His-Lassa virus (LASV)-NP, expressing the JUNV and His-LASV rNPs, respectively, were generated as described previously (20).

The cDNAs of the MACV, GTOV, SABV, and CHPV NPs were obtained by chemical synthesis (Codon Devices, Cambridge, MA). The GenBank accession numbers of the nucleotide sequences of the MACV, GTOV, SABV, and CHPV NP genes are NC_005078, AF485258, NC_006317, and NC_010562, respectively. The cDNAs of the MACV, GTOV, SABV, and CHPV NPs were digested with BamHI and subcloned into the BamHI restriction site of pAcYM1 (14), and the resulting plasmids were designated pAcYM1-MACV-NP, pAcYM1-GTOV-NP, pAcYM1-SABV-NP, and pAcYM1-CHPV-NP, respectively. High Five cells were transfected with mixtures of linearized BacPAK6 DNA (Clontech, Mountain View, CA) and the recombinant transfer vector according to the manufacturer's instructions and the procedures described by Kitts and Possee (10), and recombinant baculoviruses were obtained from them. The baculoviruses expressing the MACV, GTOV, SABV, and CHPV rNPs were designated Ac-MACV-NP, Ac-GTOV-NP, Ac-SABV-NP, and Ac-CHPV-NP, respectively.

Expression and purification of rNPs. High Five cells infected with Ac-JUNV-NP, Ac-MACV-NP, Ac-GTOV-NP, Ac-SABV-NP, Ac-CHPV-NP, or 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. The High Five cells were lysed in PBS containing 1% NP-40 and 2 M urea. After the cell lysates were centrifuged at 15,000 × *g* for 10 min, the pellet fractions were collected and then solubilized in PBS containing 8 M urea. After the samples were centrifuged, the supernatant fractions were used as the purified Ags. The control Ag was produced from High Five cells infected with Ac-ΔP, which lacks the polyhedrin gene, in the same manner as for the negative control Ags. All Ags were aliquoted and kept at −80°C until use.

Establishment of MAbs. BALB/c mice were immunized three times with the purified JUNV rNP. Spleen cells were obtained 3 days after the last immunization and fused with P3/Ag568 cells by using polyethylene glycol (Invitrogen). The culture supernatants of the hybridoma cells were screened by ELISA with purified JUNV rNP as an Ag in the presence of 2 M urea. MAbs were purified from the culture supernatant by using a MAbTrap GII antibody purification kit (GE Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. The concentration of each purified MAb was also determined by use of a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Polyclonal antibodies. Polyclonal antibodies were induced in rabbits by immunization with the purified rNPs of JUNV, MACV, GTOV, SABV, and CHPV, respectively. Rabbit sera collected before immunization were used as controls.

IgG ELISA. The IgG ELISA was performed as previously described, except for Ag preparation (20–22). Briefly, ELISA plates (96 wells, Pro-Bind; Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with the predetermined optimal quantity of purified JUNV, MACV, GTOV, SABV, CHPV, or His-LASV rNP (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was covered with 200 μl of PBS containing 5% skim milk and 0.05% Tween 20 (PBST-M), followed by incubation for 1 h for blocking at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and then inoculated with MAbs (100 μl/well), which were diluted 1:1,000 with PBST-M. After a 1-h incubation period, the plates were washed three times with PBST and then the plates were inoculated with goat anti-mouse IgG antibody labeled with horseradish peroxidase (HRP; 1:1,000 dilution; Zymed Laboratories, Inc., South San Francisco, CA). After a further 1-h incubation period, the plates were washed and 100 μl of ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at room temperature, and the optical density at 405 nm (OD_{405}) was measured against a reference of 490 nm. The adjusted OD_{405} value was calculated by subtracting the OD_{405} value of the negative Ag-coated wells from that of the corresponding wells.

IFA. The full-length cDNA of the JUNV NP obtained from Ac-JUNV-NP, which possessed a BamHI restriction site at both extremities, was cloned into the BamHI site of the pKS336 vector (23), and the resulting plasmid was designated pKS-JUNV-NP. Also, the chemically synthesized full-length cDNAs of the MACV, GTOV, SABV, and CHPV NPs were cloned into the BamHI site of the pKS336 vector and the resulting plasmids were designated pKS-MACV-NP, pKS-GTOV-NP, pKS-SABV-NP, and pKS-CHPV-NP, respectively. HeLa cells were then transfected with each of these expression plasmids by using a transfection reagent (FuGENE6; Roche Diagnostics) according to the manufacturer's instructions. The transfected cells were selected with 4 μg of blasticidin *S*-hydrochloride/ml in culture medium. The HeLa cell clones were obtained by analyzing the expression of each rNP by IFA with rabbit serum raised against the JUNV, MACV, GTOV, SABV, or CHPV rNP, as previously described (20).

Ag capture ELISA. Purified MAb C6-9, C11-12, or E4-2 was used to coat microwell immunoplates (Falcon; Becton Dickinson Labware) at 100 ng/well in 100 μl of PBS at 4°C overnight, followed by blocking with PBST-M for 1 h at room temperature. After the plates were washed with PBST, 100 μl of samples containing serially diluted rNP of JUNV, MACV, GTOV, SABV, CHPV, or LASV was added and the plates were incubated for 1 h at 37°C. The plates were then washed with PBST, and 100 μl of rabbit polyclonal antibody raised against the rNP of JUNV diluted 1:500 with PBST-M was added to each well. After 1 h of incubation at 37°C, the plates were washed with PBST, and HRP-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) was added. The plates were incubated for 1 h at room temperature. After another extensive washing with PBST, 100 μl of ABTS substrate solution (Roche Diagnostics) was added and the OD_{405} was measured with a reference wavelength of 490 nm after 30 min of incubation at room temperature. As a negative control, the OD of control Ag-inoculated wells was measured. The adjusted OD_{405} values were calculated by subtracting the OD_{405} value of the negative control well from the corresponding OD_{405} values. Means and standard deviations were calculated from the ODs of 12 negative control wells, and the cutoff value for the assay was defined as the mean plus 3 standard deviations.

Expression of truncated rNPs of JUNV. In order to determine the epitope on the JUNV rNP for the MAbs, a series of truncated JUNV rNPs were expressed as fusion proteins with glutathione *S*-transferase (GST). The DNA corresponding to each of the truncated NP fragments was amplified by PCR with specifically designed primer sets. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). The GST-tagged full-length rNP (GST-JUNV frNP) or truncated forms of the rNP (GST-JUNV trNPs) were expressed in *Escherichia coli* BL21 and then partially purified.

Western blotting. The MAbs were tested for reactivity to GST-JUNV frNP and a series of GST-JUNV trNPs by Western blotting as reported previously (9, 18, 24).

MAb epitope mapping. The epitopes for MAbs C6-9 and C11-12 were determined by epitope-blocking ELISA using synthetic peptides. The decapeptides were chemically synthesized by shifting one amino acid, with a consecutive overlap of nine amino acids to cover the JUNV NP (amino acids [aa] 5 to 26 for C6-9 and aa 543 to 564 for C11-12). ELISA plates were coated with purified JUNV rNP prepared by using a baculovirus expression system (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was inoculated with 200 μl of PBS-M, followed by incubation for 1 h for blocking. MAb C6-9 or C-11-12 was mixed with each peptide (1 μg/well) and incubated for 1 h at 37°C, and then the mixture was added to each well of the plates. After a 1-h incubation period, the plates were washed three times with PBST, and then the plates were inoculated with goat anti-mouse IgG antibody labeled with HRP (1:1,000 dilution; Zymed). The following procedure was performed as described in the IgG ELISA section above.

For MAb E4-2, the epitope was determined by ELISA using GST-JUNV frNP and trNPs. ELISA plates were coated with purified GST-JUNV frNP or trNPs (approximately 100 ng/well) according to the method described in the IgG ELISA section above. MAb E4-2 or an anti-GST MAb was used for detection at a 1:2,000 or a 1:500 dilution, respectively. The adjusted OD_{405} was calculated by dividing the OD_{405} of MAb E4-2 by that of the anti-GST MAb from the corresponding wells.

RESULTS

Generation of MAbs. In order to obtain MAbs against the JUNV NP, BALB/c mice were immunized with the purified rNP of JUNV. The MAbs were purified and tested for reactivity to the rNP of JUNV by IgG ELISA. Three MAbs, designated MAb C6-9, MAb C11-12, and MAb E4-2, reacted with the rNP of JUNV by IgG ELISA even in the presence of 2 M urea.

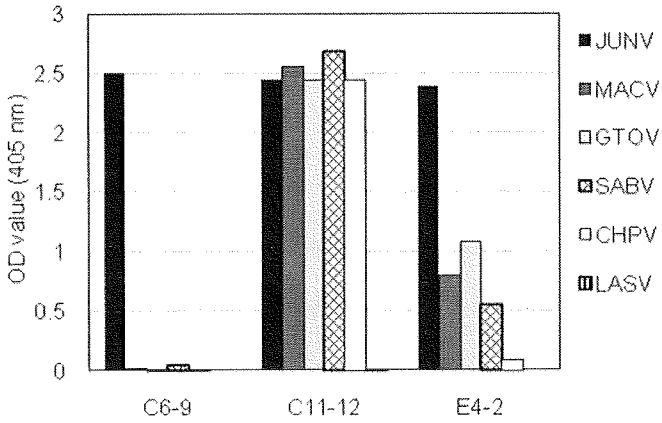

FIG. 1. Reactivity of each MAb with arenavirus rNP. Each purified rNP (100 ng/well) was used to coat microplates as described in the text, and the reactivities of each MAb to the rNPs of JUNV, MACV, GTOV, SABV, CHPV, and LASV were measured. The MAbs are shown on the *x* axis. Results are expressed as the OD_{405}.

Reactivities of MAbs to rNPs of arenaviruses. The reactivities of MAbs to the rNPs of human pathogenic arenaviruses were examined by ELISA. MAb C6-9 reacted specifically with the rNP of JUNV but did not react with those of the other pathogenic South American arenaviruses (Fig. 1). On the other hand, MAb C11-12 reacted at the same level with the rNPs of all of the pathogenic South American arenaviruses, including JUNV, GTOV, MACV, SABV, and CHPV. MAb E4-2 reacted strongly with the rNP of JUNV, slightly more weakly with those of GTOV, MACV, and SABV, and very weakly with that of CHPV. However, MAb E4-2 reacted clearly with the rNP of CHPV when ELISA plate wells were coated with more-concentrated CHPV Ag (data not shown). None of the three MAbs reacted with the rNP of the human pathogenic Old World arenavirus LASV.

Reactivity was also examined by IFA. Consistent with the ELISA result, MAb C6-9 reacted only with HeLa cells expressing the rNP of JUNV and MAb C11-12 reacted with HeLa cells expressing the rNPs of all of the pathogenic South American arenaviruses (Table 1). On the other hand, MAb E4-2, which showed cross-reactivity to other arenaviruses by ELISA, reacted only with HeLa cells expressing the rNP of JUNV (Table 1). None of the three MAbs reacted with LASV NP-expressing HeLa cells (Table 1).

Development of Ag capture ELISAs. Ag capture ELISAs were developed by using three MAbs as capture antibodies, and sensitivity and specificity were determined. The Ag capture ELISA with MAb C6-9 specifically detected the rNP of JUNV, whereas it could not detect the rNPs of the other South American arenaviruses. No less than 62.5 ng/well of the rNP of JUNV was detected by the Ag capture ELISA using MAb C6-9 (Fig. 2A). On the other hand, the Ag capture ELISAs using

TABLE 1. MAb reactivity with NPs of arenaviruses in IFA

MAb	Reactivity[a] with NP of:					
	JUNV	MACV	GTOV	SABV	CHPV	LASV
C6-9	+	−	−	−	−	−
C11-12	+	+	+	+	+	−
E4-2	+	−	−	−	−	−

[a] The symbols + and − indicate positive and negative reactions, respectively. The expression of each NP in HeLa cells was confirmed by IFA with a rabbit polyclonal antibody produced against each NP.

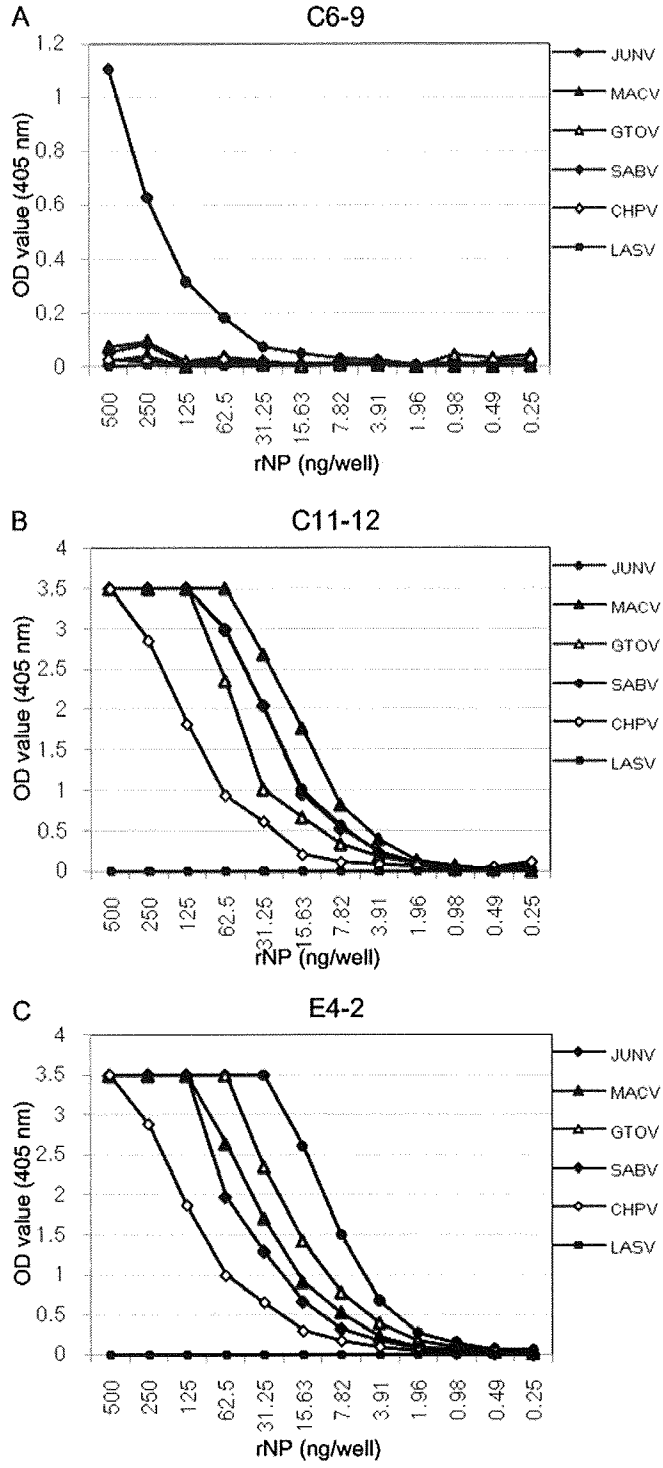

FIG. 2. Reactivity of each MAb in an Ag capture ELISA. Purified MAbs C6-9 (A), C11-12 (B), and E4-2 (C) were used to coat microplates as described in the text, and their abilities to capture the rNPs of JUNV, MACV, GTOV, SABV, CHPV, and LASV were examined at various concentrations in the Ag capture format. Results are expressed as the OD_{405}.

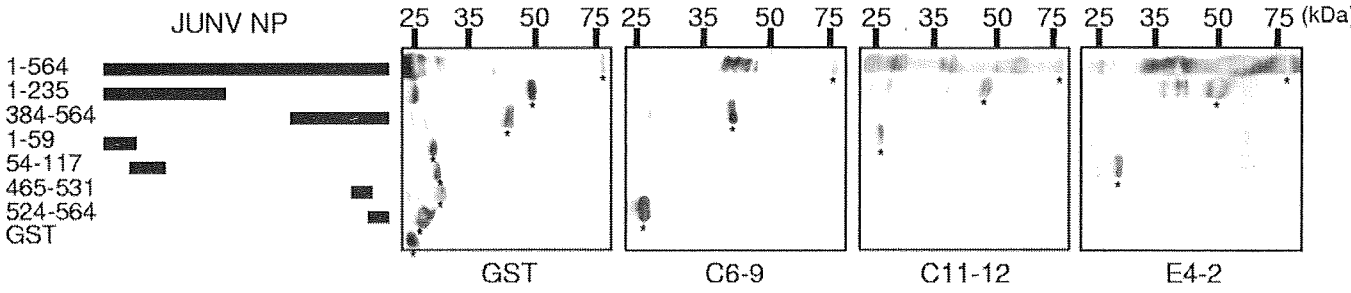

FIG. 3. Reactivities of MAbs C6-9, C11-12, and E4-2 with the GST-tagged JUNV NP by Western blotting. Schematic drawings of polypeptides of the JUNV NP and the amino acid positions of the polypeptides are shown on the left. The reactivities of MAbs and a control MAb against GST to these polypeptides by Western blotting are shown on the right. The asterisks indicate the polypeptides to which the MAbs reacted.

MAbs C11-12 and E4-2 were more sensitive at detecting the rNP of JUNV, with detection limits of 7.82 and 3.91 ng/well, respectively, and these Ag capture ELISAs also detected the rNPs of the other South American arenaviruses GTOV, MACV, SABV, and CHPV (Fig. 2B and C). In contrast, the LASV NP was not detected by any of the Ag capture ELISAs.

Determination of the epitope on the JUNV rNP recognized by the MAbs. In order to determine regions including epitopes on the JUNV rNP recognized by the MAbs, the reactivity of these MAbs was tested by Western blotting using the GST-JUNV frNP and a series of GST-JUNV trNPs as Ags. MAb C6-9 reacted with GST-JUNV frNP and trNPs at aa 384 to 564 and 524 to 564 (Fig. 3). MAb C11-12 reacted with GST-JUNV frNP and trNPs at aa 1 to 235 and 1 to 59 (Fig. 3). MAb E4-2 reacted with GST-JUNV frNP and trNPs at aa 1 to 235 and 54 to 117 (Fig. 3).

To further determine exact epitope positions on the rNP of JUNV, we performed epitope-blocking ELISAs with a series of overlapping synthetic peptides. As shown in Fig. 4A, peptides containing PPSLLFLP (aa 551 to 558) blocked the reaction of MAb C6-9 with the purified rNP of JUNV. Similarly, peptides containing WTQSLR (aa 12 to 17) blocked the reaction of MAb C11-12 with the purified rNP of JUNV (Fig. 4B).

Because the epitope recognized by MAb E4-2 could not be determined by epitope-blocking ELISA, it was analyzed more in detail by using a series of GST-JUNV trNPs by ELISA (Fig. 4C). The reactivity of MAb E4-2 was normalized by dividing the OD_{405} value of MAb E4-2 by that of an anti-GST MAb. MAb E4-2 reacted with the GST-JUNV trNP containing the polypeptide KEVDRLMS (aa 72 to 79). The ELISA result was consistent with that of Western blotting (data not shown). The epitopes recognized by the MAbs are summarized in Fig. 5.

DISCUSSION

Detection of a viral Ag and/or the viral genome is crucial for rapid diagnosis of patients with hemorrhagic fever caused by South American arenaviruses, especially for patients in the acute phase. The application of reverse transcriptase PCR (RT-PCR) and TaqMan PCR for detection of the JUNV, MACV, and GTOV genomes has been reported (1, 11, 12, 27). Serological diagnosis is also useful for the diagnosis of AHF, especially in patients in the convalescent phase (7, 17, 19, 20, 26).

An Ag capture ELISA using a cocktail of MAbs against JUNV (25) was applied in an epidemiological study of rodents in Argentina (16). MAbs reactive with the NP of JUNV have been shown to cross-react with those of MACV and other nonpathogenic arenaviruses (25). In the present study, by using MAbs raised against the rNP of JUNV, we developed Ag capture ELISAs specific for JUNV and broadly reactive to human pathogenic New World arenaviruses.

The three MAbs to JUNV NP (designated C6-9, C11-12, and E4-2) reacted with the rNP of JUNV prepared using a baculovirus expression system by IgG ELISA and with rNP expressed in mammalian cells by IFA (Fig. 1 and Table 1). All Ag capture ELISAs using MAbs E4-2, C11-12, and C6-9 detected the rNP of JUNV (Fig. 2), suggesting that these ELISAs are useful tools for the diagnosis of AHF.

Interestingly, an Ag capture ELISA using MAb E4-2 detected the Ags of all of the pathogenic South American arenaviruses tested, in addition to that of JUNV (Fig. 2). IgG ELISA showed that the reactivity of MAb E4-2 with the rNP of JUNV was stronger than that with the rNPs of other South American arenaviruses (Fig. 1). The minimal length of the epitope required to be recognized by MAb E4-2 was 8 aa with the sequence KEVDRLMS (Fig. 4 and 5). However, the GST-JUNV trNP at aa 1 to 80 was more reactive than that at aa 1 to 79, which includes minimal epitope sequences, but was still less reactive than those at aa 72 to 564, 67 to 564, and 1 to 564 (Fig. 4). Even though we could not express GST-JUNV trNPs at aa 1 to 81 or more in *E. coli* because of their toxicity, it is possible that some additional amino acids at the C terminus of the minimal epitope are required for complete reaction with MAb E4-2. Actually, comparison of the amino acid sequences of NPs at positions 72 to 83 among South American arenaviruses showed that the amino acid differences with respect to JUNV were 1 aa for GTOV, 2 aa for MACV, 3 aa for SABV, and 5 aa for CHPV (Fig. 5), and these differences correlated well with the levels of reactivity of MAb E4-2 to the rNPs of the viruses (Fig. 1).

The Ag capture ELISA using MAb C11-12 also detected the Ags of all of the other pathogenic South American arenaviruses (Fig. 2). MAb C11-12 reacted with the rNPs of all of the pathogenic South American arenaviruses by IgG ELISA and IFA (Fig. 1 and Table 1). These results suggest that MAb C11-12 would be useful for detecting the Ags of all South American arenaviruses by Ag capture ELISA and IFA. Furthermore, the amino acid sequence (WTQSLR) of the epitope recognized by MAb C11-12 was located at the N terminus of the JUNV NP and was conserved among all of the pathogenic

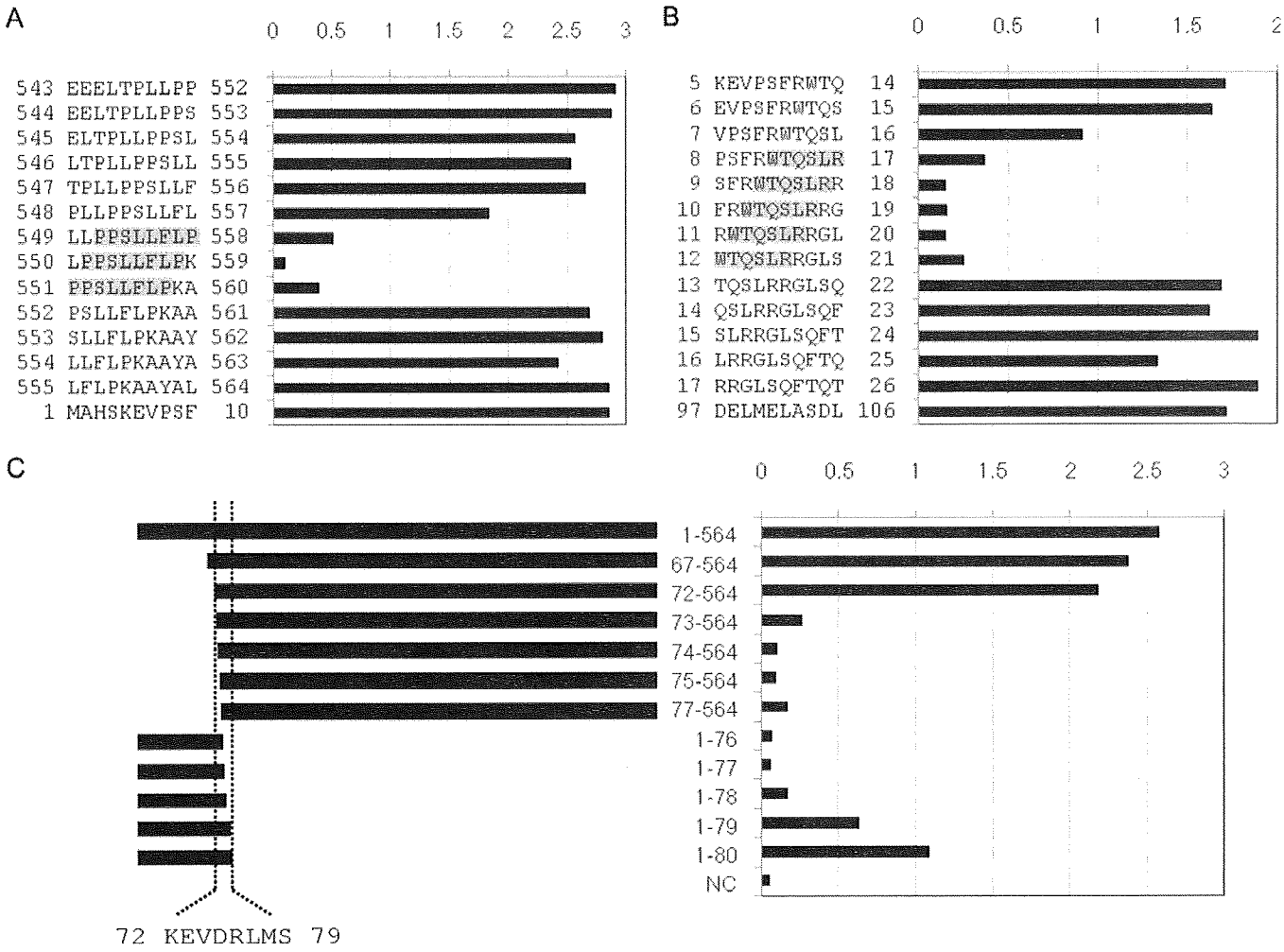

FIG. 4. Determination of the epitope on JUNV NP recognized by MAbs C6-9 (A), C11-12 (B), and E4-2 (C). (A, B) The ability of synthetic decapeptides to block the reactivity of MAbs to the JUNV rNP was examined by ELISA. The amino acid sequences and positions of synthetic peptides used in the assay are shown on the *y* axis. The synthetic peptides at aa 1 to 10 and 97 to 106 were used as negative control peptides for MAbs C6-9 and C11-12, respectively. Results indicate the OD_{405}. MAb C6-9 was confirmed to react with the 8 aa residues (PPSLLFLP) at positions 551 to 558, as represented by the shaded box (A). Similarly, MAb C11-12 was confirmed to react with the 6 aa residues (WTQSLR) at positions 12 to 17 (B). (C) The reactivity of MAb E4-2 with GST-tagged partial polypeptides of the JUNV NP was examined by ELISA. Schematic drawings of polypeptides of the JUNV NP are shown on the left, and the amino acid positions of the polypeptides are indicated on the *y* axis. NC represents the GST protein without any JUNV NP sequences. The reactivity of MAb E4-2 to each partial JUNV NP is indicated by the adjusted OD_{405}, which was calculated by dividing the OD_{405} of MAb E4-2 by that of the anti-GST MAb to the corresponding Ag. MAb E4-2 was confirmed to react with the 8 aa residues (KEVDRLMS) at positions 72 to 79, as indicated at the bottom.

South American arenavirus isolates so far deposited in GenBank (Fig. 5). However, slight differences in the sensitivity of detection of the NPs of the South American arenaviruses by Ag capture ELISA were observed. This may be due to the reactivity of the detector antibody, anti-JUNV NP rabbit serum, which was raised against the purified rNP of JUNV. Since the N-terminal region of the NPs recognized by MAbs C11-12 and E4-2 (aa 1 to 80) was relatively conserved among the NPs of South American arenaviruses, Ag capture ELISAs using MAbs C11-12 and E4-2 are considered to be useful for detecting most South American arenavirus isolates. Therefore, these Ag capture ELISAs may be applicable not only for the diagnosis of AHF but also for the diagnosis of BHF, VHF, and Brazilian hemorrhagic fever and may also be applicable for newly emerging viral hemorrhagic fevers caused by CHPV, although further study is needed.

On the other hand, the Ag capture ELISA using MAb C6-9 only detected JUNV Ag (Fig. 2). Furthermore, MAb C6-9 only reacted with the rNP of JUNV by IgG ELISA and IFA (Fig. 1 and Table 1). The amino acid sequence (PPSLLFLP) of the epitope recognized by MAb C6-9 was conserved among JUNV isolates so far deposited in GenBank (data not shown) but differed from those of other South American arenavirus isolates (Fig. 5). Since only the proline at position 552 in the epitope sequence is different in MACV, this proline is likely to be critical in the reaction of MAb C6-9. Therefore, the Ag capture ELISA using MAb C6-9 may detect most, if not all, JUNV isolates. Considering that the symptoms due to JUNV infection in humans are indistinguishable from those due to other South American arenaviruses, the Ag capture ELISA using MAb C6-9 may be a useful diagnostic tool, especially for AHF.