Minor adverse events were reviewed from clinical records of participants in the third and fourth rounds. In general, frequencies of minor adverse events were smaller than those obtained with conventional first-generation vaccines. Rather than total sample size, representativeness was better reflected by correctly interpreting minor adverse events; ie, the mean age of the sampled vaccinees appeared to be significantly higher than that of nonsampled vaccinees. We did not observe a significant difference in the outcome (ie, take) between sampled and nonsampled vaccinees, and we stratified analyses of minor adverse events by previous vaccination history (which mirrors ages; see Table 1). We therefore believe that our findings of minor adverse events are valid.

One of the major concerns with adult smallpox vaccination has been the myopericarditis observed in the United States program.[8] Inflammatory cardiac disease was recognized in adult recipients of Dryvax and the ACAM2000 vaccine in the United States,[8] but we observed no abnormal ECG tracings or symptomatic heart diseases in the present study. As discussed previously, the sample size was limited to sufficiently verify the absence of myopericarditis. In addition, asymptomatic myopericarditis could not be excluded, because the sensitivity of ECG and measurement of troponin T levels 30 days postvaccination is limited.[5,7,8]

A more appropriate time to perform ECG and quantify troponin T levels to detect myopericarditis is 7 to 14 days after vaccination.[5,7,8] We recognized this issue after starting the vaccination program but could not revise the protocol. Future studies performing ECG and measuring troponin T levels 7 to 14 days postvaccination could confirm our findings and may strengthen the evidence of the safety of LC16m8. Other explanations for the difference between our study and investigations with other vaccines are the younger age of vaccinees compared with those in a previous study[8] as well as the possible influence of the ethnic and genetic backgrounds.

Vaccination of individuals diagnosed with atopic dermatitis is another concern for implementation of widespread vaccination. In our program, individuals with atopic dermatitis were vaccinated if their lesions were stable, and we observed no features suggestive of eczema vaccinatum. A preclinical study using a murine atopic dermatitis model supports the safety of LC16m8 vaccine for immunocompromised individuals as well as for those with atopic dermatitis.[32] The low proliferation rate of LC16m8 in the peripheral skin may make it safer than first- and second-generation vaccines. Given the absence of severe adverse events, further evaluation of the performance of LC16m8 in individuals with atopic dermatitis is warranted.

CONCLUSION

We demonstrated the immunogenicity of LC16m8 vaccine in vaccinia-naive adults by a single vaccination. LC16m8 vaccine also induces a good booster response in previously vaccinated individuals. Our study also offers supportive evidence for the safety of LC16m8 vaccine in adults; LC16m8 vaccine appears to be a viable alternative to first-, second-, and other third-generation vaccines in a smallpox preparedness program.

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There are no new truths, but only truths that have not been recognized by those who have perceived them without noticing. A truth is something that everyone can be shown to know and to have known, as people say, all along.
—Mary McCarthy (1912-1989)

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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

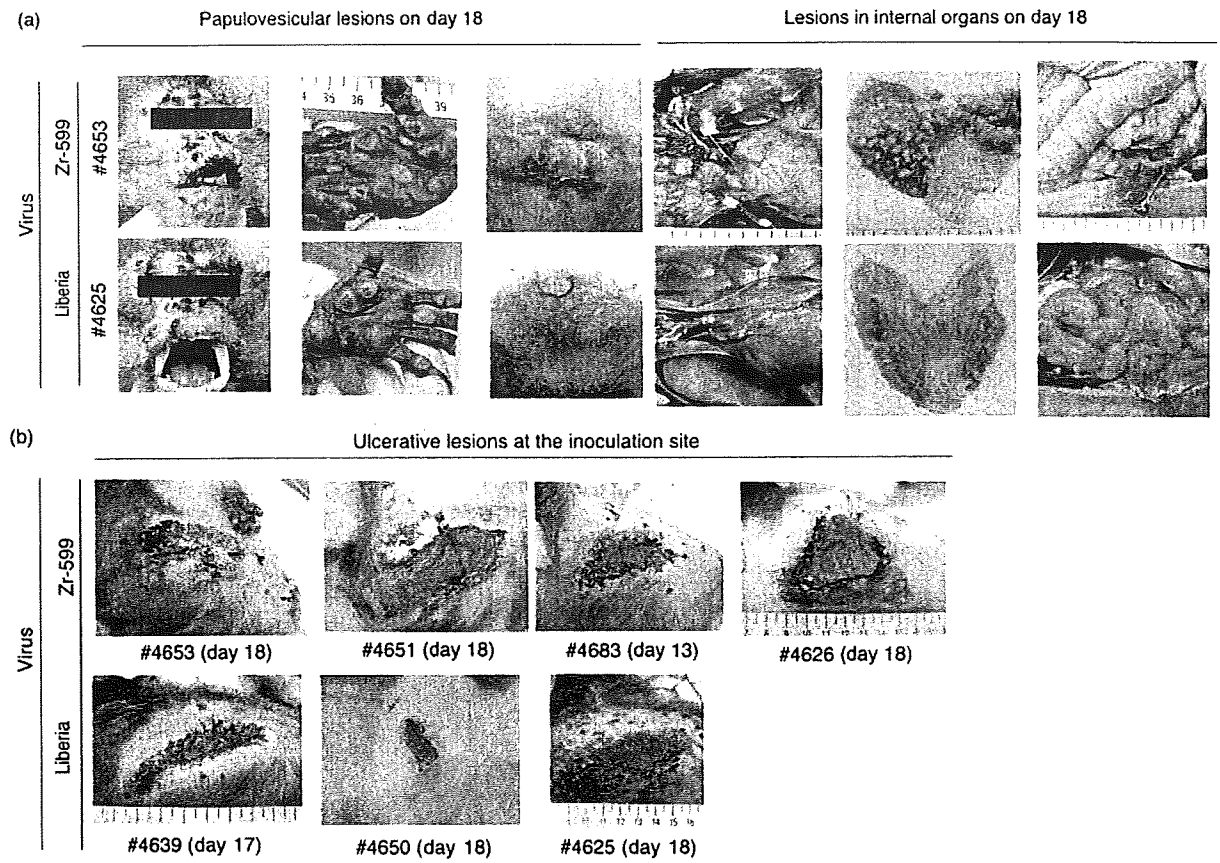

Fig. 1. (a) Monkeypox-associated lesions in skin and internal organs of fatal cases of Zr-599 (#4653) and Liberia (#4625) infection. (b) Ulcerative lesions at the monkeypox virus inoculation site in each subject.

(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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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.

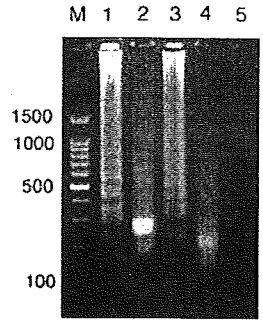

Fig. 1. DNA products from MPXV and camelpox virus amplified with COM-LAMP and separated in a 3% agarose gel by electrophoresis (**lanes 1** and **3**, respectively). To discriminate MPXV from camelpox, MPXV-LAMP and camelpox virus-LAMP products were treated with a restriction enzyme (*Taq*I) (**lanes 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

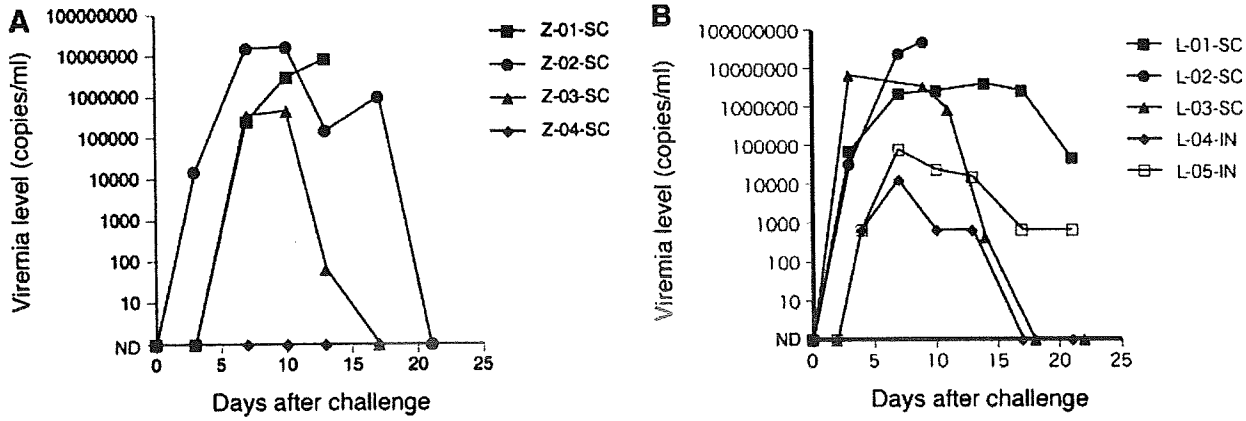

Fig. 2. Viremia level in peripheral blood collected from MPXV Zr-599-infected monkeys (**A**) and those in MPXV Liberia-infected monkeys (**B**) as determined by COM-LAMP. "ND" indicates it was below the detection level.

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.

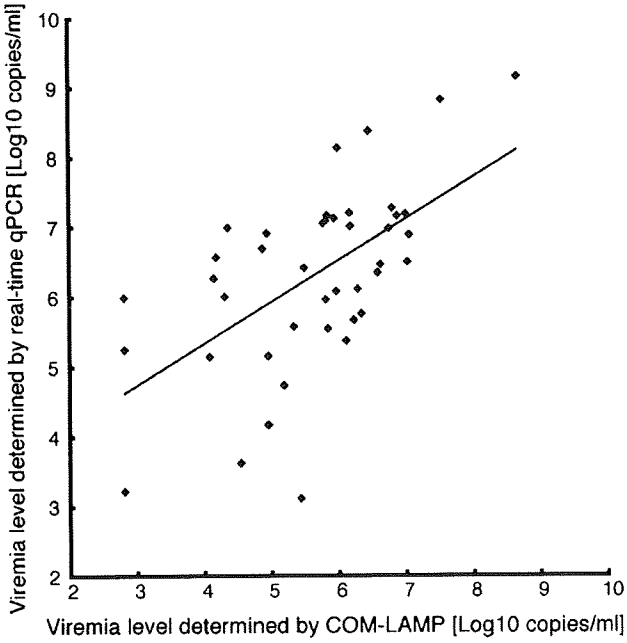

Fig. 3. Correlation between viral loads as determined by LAMP assays and real-time qPCR.

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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BRIEF REPORT

Novel virus discovery in field-collected mosquito larvae using an improved system for rapid determination of viral RNA sequences (RDV ver4.0)

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Abstract In this study, we improved a method for rapid determination of viral RNA sequences (RDV) to overcome the limitations of previous versions. The RDV ver4.0 method can detect RNA sequences with at least 1,000 copies as starting material. A novel virus, which was isolated from field-collected *Aedes aegypti* larvae in the Phasi Charoen district of Thailand using C6/36 cells, was identified using the RDV ver4.0 protocol. The virus was named Phasi Charoen virus (PhaV). We used a high-throughput pyrosequencing approach to obtain more information about the genome sequence of PhaV. Analysis of a phylogenic tree based on amino acid sequences strongly suggested that PhaV belongs to the family *Bunyaviridae*.

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Mosquitoes transmit various pathogenic microorganisms, including viruses and parasites. The epidemic areas of mosquito-borne disease are predicted to spread due to the difficulty of controlling mosquito populations and widening mosquito distribution as a result of global warming. For example, the geographic distribution of *Aedes aegypti*, which transmits several flaviviruses such as yellow fever and dengue virus, has spread northward in North America [14]. The surveillance of mosquito-borne disease is dependent upon determining the viral infection transmitted by mosquitoes. Research has focused on the development of viral detection tools using reverse transcription-polymerase chain reaction (RT-PCR). Recently, we developed a rapid determination system for viral RNA sequences (called RDV) that is useful for determining a viral genome sequence without cloning in a plasmid vector [11]. In addition, the RDV method allows exhaustive identification of viruses in comparison with previous viral detection systems such as RT-PCR because a primer specific for a target viral nucleotide sequence is not used in RDV. In our previous research, RDV version 1 (RDV ver1.0) was used to detect some mosquito-borne RNA viruses, such as West Nile virus, Japanese encephalitis virus, and dengue virus type 2, from cell culture supernatant [11]. We applied RDV

ver1.0 to homogenates of *Aedes aegypti* adult females collected from a dengue epidemic area in Thailand, using the mosquito cell line C6/36. Co-infection of dengue virus type 4 and cell fusing agent virus was detected [7]. To increase sensitivity over RDV ver1.0, the sequence-independent amplification step was improved (RDV ver2.0), and avian paramyxovirus was detected in the allantoic fluid of embryonated chicken eggs [13]. In the RDV ver2.0 method, a multiplex PCR system (Takara Bio Inc., Japan) was used, and many amplicons were obtained at the final step. The AmpliTaq Gold PCR system (Applied Biosystems, USA) was used in RDV ver2.1 instead of the multiplex PCR system. A new adenovirus, Ryukyu virus (RV) 1, belonging to the family *Adenoviridae,* which was isolated from *Pteropaus dasymallus yayeyamae*, was successfully detected in the culture supernatant of primary kidney cells using the RDV method (RDV-D) [10]. We further developed the RDV method to produce RDV ver3.0, with the number of primer sets reduced to 256 [16]. The sensitivity of these RDV methods was approximately 10,000 copies per reaction. In addition, short-length RNA (<1 kb) was difficult to amplify. Therefore, in this study, we further improved the RDV method for detecting a wide range of viral genomic RNA and to increase the sensitivity of amplification.

Short-length RNA (<1 kb) is difficult to amplify by using the whole transcriptome amplification kit (Sigma-Aldrich), REPLI-g kit (Qiagen), and Genomiphi V2 kit (GE Healthcare) due to the use of quasi-random or random primers and/or nuclease activity, whereas the QuantiTect whole transcriptome kit (Qiagen) improves the problem by using ligation of cDNA. This kit is optimized for whole transcriptome amplification and delivers high cDNA yields by following a simple three-step protocol. RNA is first transcribed to cDNA using T-Script reverse transcriptase. The cDNA is ligated using a high-efficiency ligation mix and then amplified using REPLI-g. In this study, SuperScript III (Invitrogen) as a reverse transcriptase and GenomiPhi V2 for sequence-independent amplification of cDNA were applied to the QuantiTect whole transcriptome kit. The results showed that although a simple three-step process was not achieved due to a different buffer system, the sensitivity was increased compared with the original protocol by Qiagen as described below. To further increase sensitivity, a 5′phosphorylated 20 mer oligonucleotide was added at the ligation step. The improved RDV method developed in this study, with increased sensitivity, is called RDV ver4.0 (Fig. 1).

The RDV ver4.0 method includes the following four procedures (Fig. 1).

(1) RNA extraction was described in the original RDV ver1.0 method [11]. (2) cDNA synthesis was also described in the RDV ver1.0 method. cDNA was synthesized using

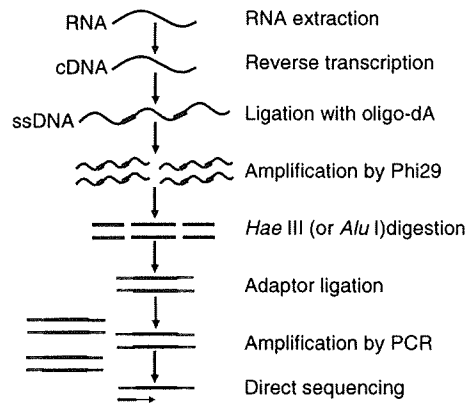

Fig. 1 Overall scheme for RDV ver4.0

random hexamer [11]. In order to amplify a low amount of short-length cDNA using RDV ver4.0, oligonucleotide was added at the ligation step for constructing bridges amongst cDNAs. Ligation buffer and enzyme from the QuantiTect Whole Transcriptome kit (Qiagen) was added to 10 μl of cDNA in the presence of 5 μl of 100 μM oligo-dA (5′-P-AAAAAAAAAAAAAAAAAA-3′) or oligo-1 (5′-P-GTNNNANNCGNNNGTNNNAN-3′). After the reaction mixture was incubated at 22°C for 2 h, 1 μl of ligation solution was amplified using Phi29 DNA polymerase (GenomiPhi V2 DNA amplification kit) at 30°C for 2 h (first cDNA library). (3) Construction of the second cDNA library was described in RDV ver1.0 method [11]. In this study, DNA was digested with *Hae*III or *Alu*I (Takara Bio Inc.). Ligation-convenience kit (Nippon Gene, Tokyo, Japan) was used for adaptor ligation. The second cDNA library was amplified by PCR using specially designed primer sets [11]. (4) Direct sequencing [11].

To investigate the sensitivity of RDV ver4.0 to short-length RNA, in vitro-synthesized albumin mRNA was used as the template. RDV ver4.0 has the potential to detect at least 1,000 copies of short-length RNA (data not shown). The RDV ver4.0 method was successfully used to detect dengue fever virus type 4, cell fusing agent virus and Yokose virus (data not shown).

Aedes aegypti larvae were collected at the homes of dengue fever patients at Phasi Charoen, Bang Khun Thian, Bang Khae, Bang Bon and Chom Thong Districts in Bangkok Province, Thailand, in May 2007. The larvae were homogenized in 200 μl of MEM with 2% FBS. Each homogenate was centrifuged at 550 *g* for 10 min at 4°C, and supernatant was filtered through 0.22 μm Millex-GX filters (Millipore, Billerica, MA, USA). The supernatant (25 μl) of 33 groups of field-collected mosquito larvae was exposed to the C6/36 cells in a 24-well plate. After 8 days, a cytopathic effect (CPE) was observed in 14 groups. In particular, group number 12 exhibited a strong CPE. The supernatant of group number 12 was collected and was

used for RDV ver4.0 after centrifugation at 550 *g* for 10 min at 4°C. A total of 149 PCR products at the final step of RDV ver4.0 were extracted from agarose gels, and direct sequencing was performed using forward primers. Each nucleotide sequence was used to determine homologous sequences using blastx on the National Center for Biotechnology Information (NCBI) website. Four read sequences, which consisted of 37 amino acids, were found to have low homology with a sequence of RNA-dependent RNA polymerase of rice stripe virus (RSV; GenBank accession number EF141327) (Fig. 2a, b). The amino acid sequence also had low homology to Uukuniemi virus (UUKV) (accession number D10759) and Rift Valley fever virus (RVFV) (accession number Z30318) of the genus *Phlebovirus* (Fig. 2b).

To eliminate the possibility that the RSV-like sequence originated from C6/36 cellular DNA and RNA, mock-infected cells were used as negative controls. RNA and DNA were prepared from mock-infected C6/36 cells at Oita University and Nagasaki University. Primers (P1-3: 5′-GAACATATGAAGTTCTGCAA-3′ and P2-2: 5′-GCAACCTAATAATCCAGGGC-3′) were designed for amplification of the RSV-like sequence. The expected size of PCR product was 92 bp. No amplification was observed for these C6/36 cells (data not shown). To investigate how many mosquito larva groups have RSV-like RNA in homogenate-inoculated cells, PCR was performed for amplification of RSV-like sequences. Eighteen of 33 groups comprised the RSV-like sequence (data not shown). In addition, there was no relationship between CPE and the appearance of an RSV-like sequence.

To investigate the infectivity of the RSV-like agent, the supernatant of C6/36 cells inoculated with homogenate from group number 12 was inoculated onto fresh Vero (African green monkey kidney) cells and C6/36 cells (second passage). After 5 days, RNA was extracted from the supernatant and PCR was performed. However, an RSV-like fragment was not detected in either of the cell lines (data not shown). After 10 days, an RSV-like fragment was amplified in the supernatant RNA of C6/36 cells, but not of Vero cells. The supernatant of C6/36 cells inoculated with PCR-positive group number 12 was inoculated onto fresh C6/36 cells (third passage), and RNA was extracted from cells and supernatant after 10 days. RNAs extracted from C6/36 cells and supernatant were positive for the RSV-like fragment. The supernatant of C6/36 cells inoculated with group number 12 homogenate was further inoculated onto fresh C6/36 cells (fourth passage), and RNA was extracted after 10 days. The RSV-like fragment was detected in both cells and supernatant (data not shown). These results indicated that the RSV-like agent was infectious to C6/36 cells. We named this RNA virus Phasi Charoen virus (PhaV). PhaV does not induce CPE after the second passage. Therefore, there may be another virus that causes CPE in C6/36 cells in group number 12. This virus may replicate slowly compared to PhaV, or PhaV may have mutation(s) in genes that are responsible CPE after the first passage.

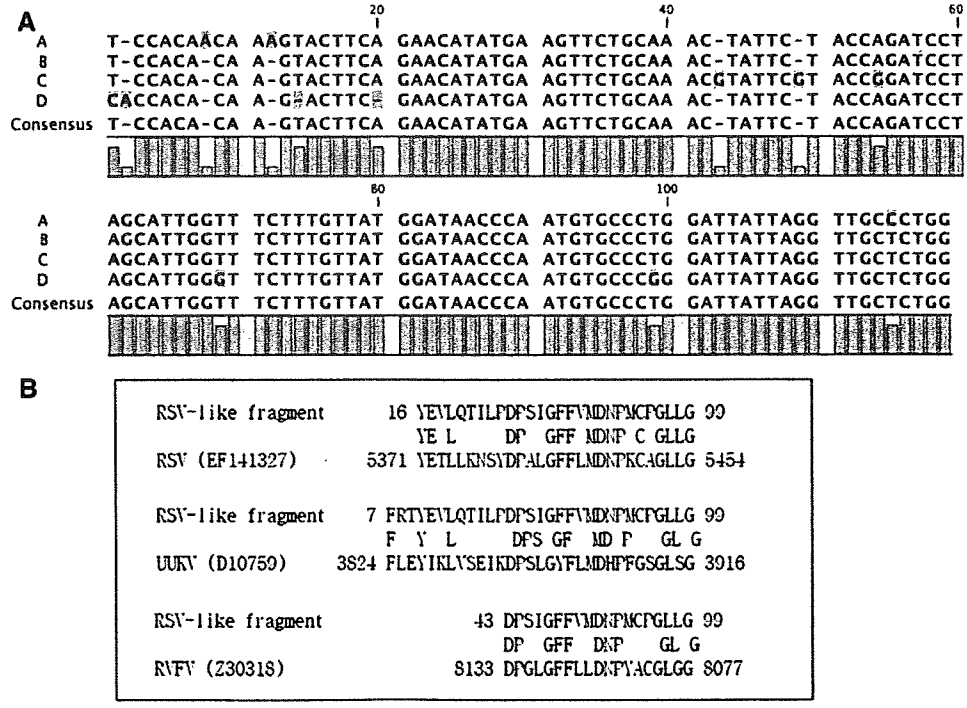

Fig. 2 Rice stripe virus-like sequence obtained using RDV ver4.0. **a** Alignment of four nucleic acid sequences (**a–d**) obtained from number 12 homogenate using RDV ver4.0. **b** Homology of RSV-like sequence to RSV, Uukuniemi virus (UUKV), and Rift Valley fever virus (RVFV)

To obtain information regarding the genome sequence of PhaV, we used the Genome Sequencer FLX System of Roche and 454 Life Sciences. The cDNA synthesized from RNA in the second passage supernatant, as described above, was amplified by using GenomiPhi V2. DNA sequencing libraries for Genome Sequencer FLX were constructed and sequenced by Takara Bio Inc. The obtained 5,000 read sequences were analyzed by Phred/