博士論文

論文題目　Controlling monkeypox virus infection in human: Establishment of a rapid diagnostic system and evaluation of highly attenuated smallpox vaccine for prevention of monkeypox using non-human primate model

(ヒトサル痘の迅速診断法の開発および高度弱毒痘そうワクチンの霊長類をモデルとした評価)

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Controlling monkeypox virus infection in human: Establishment of a rapid diagnostic system and evaluation of highly attenuated smallpox vaccine for prevention of monkeypox using non-human primate model

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ABSTRACT

Monkeypox (MPX) virus (MPXV) causes a smallpox-like disease in non-human primates and humans, and is endemic to central and western Africa. In this study, we approached for the control of MPX in two aspects as a public health concern. One is the development of diagnostic system, and the other is evaluation of highly attenuated smallpox vaccine against MPX.

In chapter I, a rapid diagnostics of MPX was developed using LAMP technology. Since MPXV is divided into two genetically different groups, more virulent Congo Basin and West African MPXV, the assay developed was designed to differentiate each MPXV. The sensitivity and specificity of the LAMP were evaluated with nested polymerase chain reaction (PCR) using specimens from MPXV-infected monkeys. The sensitivity of LAMP was about 70% and the specificity was 100%. The viremia level determined by LAMP assays was positively correlated with the severity of the MPX-associated symptoms. The newly developed LAMP assay was confirmed to be a quantifiable, highly sensitive and specific system.

In chapter II, the long-term efficacy of highly attenuated smallpox vaccine LC16m8 in single vaccination was evaluated in a nonhuman primate model. Monkeys were inoculated with LC16m8, parental strain Lister, or mock and were challenged with MPXV subcutaneously at 6 or 12 months after the vaccination. The efficacy of LC16m8 was evaluated in comparison with Lister and mock vaccination. LC16m8-monkeys showed no MPX associated symptoms as Lister-monkeys did, while most of the naïve-monkey died. The present study indicates that vaccination of humans with LC16m8 has a potential to induce long-term protection against MPX.

ABBREVIATIONS

%	: Percent
μg	: Microgram
μl	: Microliter
A	: Adenine
BIP	: backward inner primer
bp	: Base pair
C	: Cytosine
CDC	: Centers for Disease Control and Prevention
CPE	: a cytopathic effect
DRC	: Democratic Republic of Congo
EC_{50}	: 50 % effective concentration
ELISA	: Enzyme-linked immunosorbent assay
FBS	: Fetal bovine serum
FIP	: forward inner primer
hr	: Hour(s)
ID	: Infectious dose
IDSR	: Integrated Disease Surveillance and Response technical guidelines
LAMP	: Loop-mediated amplification
LC16m8	: Highly attenuated smallpox vaccine LC16m8
LD_{50}	: The 50 % lethal dose
LP	: Loop primers
MEM	: Eagle's Minimal essential medium

MEM-5FBS	: Minimal essential medium supplemented with 5% fetal bovine serum
MPXV	: Monkeypox virus
MPX	: Monkeypox
MVA	: Modified Vaccinia Ankara
ml	: Milliliter
m.o.i.	: Multiplicity of infection per cell
NC	: Negative control
NIID	: National Institute of Infectious Diseases, Tokyo, Japan
ND	: Not detected
NT	: Not tested
PBS	: a phosphate-buffered saline solution
PCR	: Polymerase chain reaction
PFU	: Plaque forming unit(s)
qPCR	: Quantitative real-time PCR
ROC	: Republic of Congo
T	: Thymidine
US	: The United States of America
UDL	: Under determination level
UV	: Ultra violet
WHO	: World Health Organization
VACV	: Vaccinia virus

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GENERAL INTRODUCTION

Monkeypox (MPX)

Monkeypox (MPX) is a smallpox-like disease in nonhuman primates (NHP) caused by infection with a zoonotic orthopoxvirus, monkeypox virus (MPXV) (1, 2). MPXV was first isolated from vesiculopustular lesions in cynomolgus monkeys**,** in Copenhagen in 1958 (3). MPXV belongs to the genus *Orthopoxvirus* in family *Poxviridae*, together with variola virus, which is a causative agent of variola (smallpox). MPXV is a double stranded DNA virus, and contains genome of about 200 kbp, encoding approximately 200 genes (4, 5). It has a large and complex virion with dimensions of approximately 250×350 nm (6) (Fig. 1, 2).

Generally orthopoxviruses including MPXV produce two distinct virions: the intracellular mature virion (IMV) and extracellular enveloped virion (EEV) (7). IMV and EEV differ in that EEV has an additional lipid envelope surrounding an IMV particle, which is also surrounded by a single lipid membrane (8-10). Inside this membrane, a virus core is included, and the virus structural proteins, DNA genome and transcriptional associated enzymes are located within the virus core (7, 10). EEV is released from infected cells before cell lysis, whereas IMV is released only after cell lysis (11, 12) (Fig. 2A, B).

Vaccinia virus (VACV) is closely related to both variola virus and MPXV, and was used for eradication of smallpox (13). Recent studies revealed that attenuated VACV strains induced the protective efficacy in NHP against a lethal challenge of MPXV (14-16).

Epidemiology of human MPX

MPXV infections in humans, called as human MPX (Table 1), were first described in central Africa through the smallpox surveillance continued from 1970 to 1976 in Africa (1, 17-19) (Table 1B). Afterwards, the sporadic outbreaks were reported in tropical rain forest in Cote d'Ivoire, Liberia, Nigeria, and Zaire, where smallpox had already been eliminated at that time (18-26) (Table 1B). All the cases reported have been from small villages in the rain forest of western and central Africa because the contact with small forest animals was reported to be the most common mode of transmission in cases identified up to 1996. The true geographic range of human endemic disease has yet to be determined (26, 27). MPX is a zoonotic infectious disease, the reservoirs of MPXV being some rodent species in Africa and humans acquire the virus through direct contact with infected animals or patients (23, 28-31). When the eradication of smallpox was issued at the global committee in 1979, it was thought that MPX could not become a threat like smallpox. Therefore the smallpox vaccination program was terminated (13, 22). Since 1979, when surveillance was started by the World Health Organization (WHO) in Zaire, 4 cases of possible human-to-human transmission were reported among the 45 cases of human MPX, suggesting the necessity for more understanding of MPXV infection in nature (32, 33) (Table 1A). An active surveillance program was conducted by the WHO during 1981-1986 in the Democratic Republic of Congo (DRC, former Zaire), resulting in the identification of 338 human MPX cases, including 33 fatal cases (34) (Table 1A). From these data, a stochastic model for interhuman spread of MPXV was constructed by Jezek and colleagues, and it was concluded that MPXV was not as serious as a public health problem (35, 36).

After the active surveillance program that ended in 1986, there was a long period until 1995 without epidemiologic data of human MPX in the endemic regions. An active surveillance was conducted again during 1995-1997. Approximately 500 cases of suspected human MPX were reported in the DRC, including 88 cases laboratory confirmed (37, 38) (Table 1A). The surveillance was continued in the DRC during 2001-2004, reporting 51 PCR based laboratory-confirmed human MPX cases (37, 39) (Table 1A). Out of these confirmed patients, most people (94%) were aged less than 25, and all the patients lived mainly in tropical rainforests (37). Cases of human MPX reported had also increased in the neighboring Republic of Congo (ROC), and a cluster of cases were reported for the first time in Sudan in 2006 (40, 41) (Table 1A). Additionally, an active surveillance program was continued in the DRC, where MPXV was known to circulate during 2005-2007 (42, 43) (Table 1A). An increase in the number of the reported human MPX (760 laboratory-confirmed cases (44)) was identified among younger persons without smallpox vaccination in comparison with the previous data in 1980's (34, 42, 43, 45) (Table 1A). In 2010, the WHO and the Centers for Disease Control and Prevention (CDC) issued Integrated Disease Surveillance and Response technical guidelines (IDSR) for Africa in order to early identification and report of infectious diseases including MPX, and initiated an intensive community education for improving health standards in DRC and ROC (46). Remarkably, in 2003, the first outbreak of human MPX was reported in the United States of America (US) (47, 48), occurring as a form of multi-states outbreaks. The total number of the patients was 71, and 35 of them were laboratory-confirmed (49) (Table 1A). Fortunately, the outbreak was sporadic, and no fatal cases were reported (49). Thirty-seven patients were virologically confirmed to be infected with MPXV (47, 48). The patients in this outbreak in the US were infected with MPXV through a

direct contact with pet prairie dogs (*Cynomys* species) that got disease from the rodents such as African dormice and Gambian giant rodents imported from West Africa through Accra, Ghana (47, 49). The outbreaks of human MPX in the US highlight the capacity of this virus to cause epidemic in the regions where MPXV is not prevalent yet. From these findings, preparedness against human MPXV infections is considered as one of the most important orthopoxvirus infections in humans in the society, where smallpox has already been eradicated since late 1970's.

Pathogenicity of MPXV infections in human

Clinical manifestations in humans MPX are mostly similar to those in patients with smallpox (50) (Fig. 3). The initial symptoms of human MPX are fever, headache, general malaise and prostration. The incubation period is typically 8-14 days. This period of prodrome is followed by the development of a characteristic pustular rash (50-52). The number of skin lesions in individual cases varies from a few to several thousands. The skin lesion appears on the entire skin, with preference to the face and the extremities, including the palms and soles (51). This latter feature helps clinicians to distinguish human MPX from chickenpox, which is the infection often misdiagnosed (53). The skin lesions of patients with smallpox and human MPX are shown in Fig. 3.

The lesions are also seen on the mucous membranes in patients, who suffer from sore throats, and painful ulcers in the oral cavity (54). Coughing with or without sputum production is a relatively common symptom (33, 39, 55). The major difference in the clinical features between smallpox and human MPX is temporary enlargement of lymph nodes in human MPX (54). Lymphadenopathy usually occurs at an early stage of illness, occasionally coinciding with the onset of fever (33, 39, 55).

The disease lasts 2-4 weeks, depending on its severity (33, 55). In most cases of human MPX, the initial site of infection is the upper respiratory tract infected by viruses released in the oropharyngeal secretions and/or shed from rash of patients (29).

Two clades of MPXV

MPXV is divided into two genetically distinct groups, Congo Basin and West African MPXV clades (56). According to the epidemiological analyses, >90% of human MPX cases occurred in the Congo Basin (42, 57). Congo Basin MPXV is reported to be more virulent than West African MPXV; the case fatality rate of Congo Basin MPXV is 1-17% (27, 31, 58, 59), whereas there have been no reported cases of fatal human MPX due to West African MPXV (56). The strain that caused the human MPX epidemic in the US was West African MPXV (56, 57, 60). Virulence of these two MPXV clades also differs in non-human primates, similarly to that in human. Therefore, there is a need to develop a rapid and accurate diagnostic system not only to detect MPXV, but also to discriminate the clades of Congo Basin and West African MPXV (57, 60).

Diagnostics for MPXV

The standard diagnostic assays for MPXV are virus isolation, detection of MPXV DNA using nucleic acid amplification system, such as PCR (61-65), detection of a MPXV virions using electron microscopy, and demonstration of significant rise in antibody titers against MPXV between acute and convalescence phases with an enzyme-linked-immunosorbent assay (ELISA) (16, 66) for confirmation of MPXV infection in laboratories. However, orthopoxviruses closely resemble each other

morphologically and genetically; MPXV is indistinguishable from the other orthopoxviruses on the basis of electron microscopy and immunological assay.

Loop-mediated isothermal amplification (LAMP) method

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method, which depends on autocycling strand displacement DNA synthesis performed by *Bst* DNA polymerase large fragment (New England Biolabs) and confirmed to be simple for use (67). The amplification is conducted under isothermal conditions ranging from 60 °C to 65 °C with DNA polymerase. Usually, 4 primers recognize 6 distinct target regions (4-primer-based LAMP), making this assay highly specific. If the two additional primers, "loop primers", are included in the LAMP assay, the reaction time could be shortened (6-primer-based LAMP) (67, 68). The most significant advantage of the LAMP over conventional PCR and real-time quantitative PCR is that there is no need of denaturation step of DNA strand. LAMP does not require the highly advanced instrument for the amplification, leading to simplicity of the assay (67-69). The visual detection of the turbidity, derived from the accumulation of byproduct, is possible (68). During DNA polymerization by DNA polymerase, pyrophosphate ion is released from dNTP as a byproduct (70). When a large amount of this pyrophosphate ion is produced, it reacts with magnesium ion in the LAMP reaction buffer, and is observed as precipitates (70). When potassium pyrophosphate was added to the LAMP reaction buffer containing magnesium sulfate and reacted for 60 min at 65°C, turbidity is increased when the concentration of pyrophosphate ion exceeds 0.5 mM (68). When the LAMP reaction synthesizes 10 mg/25 ml or more DNA, the produced pyrophosphate ion reacts with magnesium ion to induce the precipitate (68). However, DNA yield by PCR is about 0.2 mg/25 mg

and the resulting pyrophosphate ion approximates 0.02 mM (68). LAMP has the advantages of its safety, easiness, and low-cost suitable for use as just described in the epidemic countries. Recently, the LAMP-based diagnostics for several viral infections have been developed (71-82).

Vaccines for MPXV

For the countermeasure against MPXV, the first choice is the use of next generation smallpox vaccines (83).

Smallpox vaccines are classified into three generations according to their manufacturing method. The traditional smallpox vaccines (1st generation vaccines; e.g., Dryvax, NYCBH, and Lister) were live vaccinia viruses, and were used for the smallpox eradication campaign (84). Although the 1st generation vaccines were highly efficacious, they induced severe adverse effects, such as encephalitis, encephalopathy, progressive and generalized vaccinia, ocular vaccinia virus infections, and cardiac dysfunction (84, 85). Subsequently, the 2nd and the 3rd generation vaccines [e.g. LC16m8, modified vaccinia Ankara (MVA)] were developed to reduce the risk of severe adverse events (84). These vaccines were not tested for the efficacy in protection of humans from smallpox and not actually used in the endemic regions for the smallpox eradication campaign (84).

A highly attenuated smallpox vaccine, LC16m8

The highly attenuated smallpox vaccine, LC16m8, was developed in Japan (66). LC16m8 smallpox vaccine was developed by multiple passages of smallpox vaccine, the first generation Lister strain (Elsree), in the primary rabbit kidney cells under lower temperature conditions (86, 87). Due to a single nucleoside deletion

mutation in the *B5R* viral gene, LC16m8 does not express the whole 45-kDa B5, a very immunogenic membrane protein, and is defective in producing EEV particles responsible for cell-to-cell spread. The function of B5 in detail remains unclear (16, 66) (Fig. 2B, C). Although the 10-kDa, truncated B5 protein of LC16m8 (Fig. 2B,C) affects its efficacy as a vaccine for smallpox in animal model, this virus induces neutralizing antibodies against both IMV and EEV, and protects animals from a lethal orthopoxvirus challenge (88). In the previous studies, a single vaccination of NHPs with LC16m8 protects NHPs from lethal MPXV infections after 5 weeks from vaccination (16, 66). Approximately 100,000 Japanese children were immunized in the clinical trial in 1970's (89-91). No severe adverse events were reported in the clinical study. The safety of LC16m8 in immunocompromised subjects has also been studied (88, 89, 91). Neither severe combined immune deficient (SCID) mice nor T or B cell depleted macaques showed a fatal outcome after immunization with LC16m8 (89, 91).

Safeguard against bioterrorism

Variola virus is still stocked in the US and Russian Federation. Variola virus and MPXV are categorized to be the pathogens with a risk of being used as a bioweapon, and pose a possible threat to the public (92). Additionally, clinical manifestations and immunological responses observed in monkeys infected by lethal MPXV resemble those of humans with smallpox (Fig. 4). This fact prompted investigators to evaluate the new generation smallpox vaccines (15, 16). Therefore, smallpox vaccine is still important even though smallpox has already been eradicated from the general society.

OBJECTIVES

The main objectives of the present study are the followings:

1. Development and evaluation of a novel LAMP method for detection and classification of MPXV infections in human

2. Evaluation of smallpox vaccine LC16m8 in long-term protection efficacy against lethal MPXV infection with cynomolgus monkey

I. Development and evaluation of LAMP-based assay for MPXV infections

INTRODUCTION

MPXV infections in humans are endemic to central and western Africa (17, 21, 54, 93). MPXV is divided into two genetically different groups, Congo Basin and West African MPXV, with the former being more virulent than the latter and sometimes causes lethal infection (56, 57, 60). Since it is difficult to distinguish human MPX from chickenpox and the other orthopoxvirus infections, it is necessary to develop a rapid, accurate, and easy-to-use diagnostic system suitable in endemic countries for detection of MPXV genome in specimens collected from patients infected with MPXV (94). LAMP technology is a real-time quantitative MPXV genome amplification system (67). Moreover, the most remarkable feature of this technology is that DNA can be amplified isothermally, which doesn't require the special instruments such as the thermal cycler (67, 68). In this study, we developed an LAMP assay for the first time for detection of MPXV with the ability of discrimination of the two clades. They might be useful in endemic countries.

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 the 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 (95). Viral DNA in the peripheral blood and throat swab specimens was purified using a Viral Nucleic Acid Purification kit^{TM} (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and stored at -30 °C until use.

Real-time quantitative PCR for Orthopoxvirus

A Cyber Green-based real-time quantitative PCR (pox-qPCR) was developed. Primers, forward primer [H2Rf (5'-CGGTTAACGATTGGAAATCATTAACGG-3')] and reverse primer [H2Rr (5'-CCTCGCCTAATAGCTTGCG-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 (Pharmacia, Sweden) inserted with the partial H2R gene amplified with PCR using the primer set (H2f and H2r), was used for the determination of the copy number of

the virus genome of viruses used. The reaction conditions were as follows: 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. The pox-qPCR amplification was performed using a LightCycler FastStart DNA Master SYBR Green I™ kit (Roche Diagnostics Ltd., Rotkreuz, Switzerland) in a 20 µl-volume format containing 5 µl of template DNA according to the manufacturer's instructions.

LAMP

The 6-primer-based LAMP consisted 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) (96). 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 (56), and those of the West African MPXV-specific partial ATI gene (65) (Fig. 5). The primer Loop-B-COM in the COM-LAMP was designed to anneal the region containing a deletion of 7 nucleotide residues observed only in MPXV but not in other orthpoxviruses (64) (Fig. 5). The nucleotide sequences of the primers used in each LAMP assay are shown in Table 1. The LAMP reaction was performed with a Loopamp DNA Amplification kit™ (Eiken Chemical Co., Ltd., Tochigi, Japan). The reaction mixture (25µl) 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* DNA 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 that 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 (Fig. 5).

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 (97). The standard DNA for C-LAMP was the pGEM-T easy vector inserted with the partial D14L gene amplified with PCR 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') (64). 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 in the product of 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, and one cycle of 72°C for 5 min. The PCR were performed using the GeneAmp PCR system 9700 (Applied Biosystems) with the Expand High Fidelity System™ (Roche Diagnostics) according to the manufacturer's instructions.

Monkeys and MPXV-challenge experiments

Seventeen 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 experiment was conducted under the approval of the Animal Experiment Ethical Committee of NIID. The monkeys were experimentally infected with MPXV in a highly contained laboratory 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 (PFU). Smallpox vaccine, LC16m8 and Lister, was confirmed to be effective in protecting the monkeys from generalized MPX (16). To demonstrate the various levels of the clinical manifestations of MPX, monkeys were immunized with LC16m8 or Lister for a designated period before MPXV challenge as shown in Table 2. One monkey was infected with Zr-599 followed by immunization with LC16m8. Blood samples and throat swab specimens were collected every 3-4 days after challenge. Clinical manifestations, such as changes in bodyweight, 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 (qPCR)

MPXV genome copies in the peripheral blood and throat swab specimens were determined by real-time quantitative PCR as reported previously (16).

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 real-time quantitative PCR was obtained using Pearson's correlation coefficient.

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, showed a positive reaction in the COM-LAMP assay (Table 4). However, the amplified DNA from the MPXV genomes could be differentiated from that from camelpox virus by restriction enzyme treatment (Fig. 6).

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 4). All the Congo Basin MPXV genomes but not the West African MPXV genomes were detected by C-LAMP (data not shown). 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, 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 more than 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 73% (57/78) and 100% (67/67), 70% (32/46) and 100% (27/27), and 70% (23/32) and 100% (40/40), respectively (Table 5).

Relationship between severity of symptoms and viremia level as determined by COM-LAMP

The severity of MPX symptoms observed during the challenge experiments is shown in Table 3. The infection was lethal in two naïve monkeys, one was infected subcutaneously (Z-01-SC) and the other was intranasally (Z-06-IN). The viremia level determined by the COM-LAMP assay continued to increase until sacrifice. In the subcutaneously inoculated group, 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. 7A). We observed that the severer the level of monkeypox-associated symptoms, the higher the viremia level determined by COM-LAMP. In the intranasally inoculated group, since all monkeys were naïve, severe MPX-associated symptoms were observed. One monkey was scarified before the end of the experiment, because of general prostration, represented by decreased in body temperature, decreased weight, rhinorrhea, diarrhea, decreased activity, and loss of appetite. Although the other 3 monkeys also showed severe symptoms, all became recovered

18 days post infection. 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. 7B). The MPX symptoms in one monkey (L-02-SC) were so severe that the subject was sacrificed due to ethical considerations. The two monkeys (L-01-SC and L-03-SC) 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 (Table 3). 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 real-time qPCR

The relationship between the virus load in the clinical samples determined by COM-LAMP and those determined by real-time qPCR is shown (Fig. 8). The correlation coefficient was 0.60, which represents a strong positive correlation.

DISCUSSION

Nucleic acid amplification-based diagnostics have become a 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 (16, 64, 65, 98). The real-time quantitative PCR assays have the advantages over the rapidity, quantification-capacity, detection in a real-time manner, and high sensitivity. However, these nucleic acid amplification methods are complicated and require high-precision instruments. On the other hand, LAMP can be carried out without using such instruments. Furthermore, virus genomes can be detected within a relatively short time and in a real-time manner. If turbidity detection is performed in a real-time manner using a Loopamp DNA Amplification kit^{TM} (Eiken Chemical Co., Ltd., Tochigi, Japan), the virus genomes can be detected along with genome quantification. In this study, We successfully developed LAMP assay, which enabled us to detect MPXV genome DNA with both high sensitivity and specificity, for the first time.

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 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 4), indicating that a positive reaction in the COM-LAMP assay does not always indicate an MPXV infection. However, 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 judged 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). The genomes of herpes simplex virus and varicella zoster virus, which cause vesicular skin infections in humans and must be differentiated from human MPX, showed negative reactions in the newly developed LAMP assay (data not shown). 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 might be possible by the treatment of COM-LAMP products with *Taq* I enzyme.

Three LAMP assays were evaluated in comparison with nested PCR. This study is the first to develop nested PCR to specifically amplify MPXV gene. This method was modified from the PCR previously reported (64), whose detection limit is 10^6 copies/reaction (data not shown). The detection limit of the novel nested PCR was 10 copies/reaction (data not shown). The sensitivity and specificity of the three LAMP assays when compared with the nested PCR were approximately 70% and 100%, respectively (Table 5). Although the sensitivity of the LAMP assays was not higher than the nested PCR, COM-LAMP successfully detected the MPXV genome DNAs in the monkey's peripheral blood before the clinical symptoms appear (Table 5, Fig. 7). The viremia level determined by COM-LAMP was clearly associated with the severity of clinical symptoms of MPX. We conclude that the newly developed LAMP assays afford a valuable tool not only for the diagnosis of but also for the assessment of human MPX severity.

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. The LAMP technique is highly efficient for DNA amplification (up to 10^{10} times amplification within 60 minutes), is faster than PCR, and is easily performed in basic laboratories without the need for specialized infrastructure. This diagnostic assay offers great benefits in the control of outbreaks of MPXV infections and in the assessment of the morbidity and mortality in patients with human MPX. Furthermore, the newly developed LAMP system offers advantages over the diagnosis of human MPX, which needs to be differentiated from smallpox in the event of a variola virus-associated bioterrorism attack.

II. Evaluation of smallpox vaccine LC16m8 in long-term protection efficacy against lethal MPXV infection with cynomolgus monkey

INTRODUCTION

As a countermeasure against human MPX, the effective method is vaccination (45, 51). Fortunately, smallpox vaccine is proved to be effective in protection of NHP against MPXV infection (22, 99). However, smallpox vaccine used for smallpox eradication campaign was reported to cause severe adverse effects mostly in allergic people (83, 90, 100). LC16m8, a novel highly attenuated smallpox vaccine, was developed in Japan (16, 66, 90). In previous studies, a single vaccination with LC16m8 protected monkeys from lethal MPXV infection, as an alternative model for smallpox in humans, when vaccinated one month before infection (16). However, the persistence of protection efficacy still remains unclear. In this study, we evaluated the protection efficacy of LC16m8 using monkeys infected with lethal MPXV a half or one year after vaccination.

MATERIALS AND METHODS

Virus, smallpox vaccine, and cells

MPXV strains Zr-599, a highly virulent strain, was used in the challenge experiments (16). The virus was confirmed to be MPXV by determining the specific nucleotide sequence of the A-type inclusion body (ATI) gene, of MPXV (16, 64). The infectious doses of MPXV were determined by plaque assays on Vero cells (16). Vero cells were grown in Eagle's minimum essential medium (MEM) supplemented with the penicillin G and streptomycin, and with 5% fetal bovine serum (MEM-5FBS). Smallpox vaccines, LC16m8 and Lister, were used in this study (14). These vaccines do not contain adjuvants described in the package insert of LC16m8 (Kaketsuken, Kumamoto, Japan). The titers of the two vaccines were higher than 1×10^8 PFU/ml (16).

Non-human primates (NHP)

One female and 13 male cynomolgus monkeys (*Macaca fascicularis*), aged 2 years old and weighing 3000-4000g, were used in the experiments (Table 6). The monkeys were subjected to 5 groups: naïve-group and groups LC16m8-6M, LC16m8-12M, Lister-6M, and Lister-12M. The naïve group consisted of 4 monkeys without vaccination but challenged subcutaneously with MPXV as described below. Notably, only 1 out of 4 naïve-group monkeys was inoculated with MPXV simultaneously with the other group subjects. The other 3 naïve-group subjects were inoculated with 10^6 PFU of MPXV Zr-599 in the same way as in the present study separately (16, 60). The virus dose inoculated was always confirmed to be the dose expected by back titration confirmation. LC16m8-6M (3 monkeys) and Lister-6M (2 monkeys) group

monkeys were challenged with MPXV at 6 months after vaccination with LC16m8 and Lister, respectively. LC16m8-12M (3 monkeys) and Lister-12M (2 monkeys) group monkeys were challenged with MPXV at 12 months after vaccination in the same way with 6M groups. These monkeys were born and raised in Tsukuba Primate Research Center, National Institute of Biomedical Innovation Tsukuba, Japan.

Vaccination with smallpox vaccines and challenge with MPXV

All animal experiments were conducted by myself under the directorship of Dr. Masayuki Saijo with collaboration of Dr. Yasushi Ami, Dr. Noriyo Nagata in National Institute of Infectious Diseases, Japan. Monkeys were vaccinated with each of the vaccines by the multiple-puncture method with standard bifurcated needles holding a drop of vaccine, pressured more than 15 times onto the skin at the vaccination site (16). In the present study, Day 0 was defined as the day when monkeys were challenged subcutaneously with MPXV at the 10^6 PFU of MPXV Zr-599. Monkeys were challenged with MPXV at the dose on Day 0 and were observed until the humane endpoints designed for maximum 3 weeks.

Challenge with MPXV

All the challenge experiments with MPXV were conducted in a highly contained laboratory at National Institute of Infectious Diseases, Tokyo, Japan (16, 60) under the approval of the Animal Experiment Ethical Committee of NIID. The monkeys, which were mock-immunized or immunized with either vaccine of LC16m8 or Lister, were anesthetized and inoculated subcutaneously with 0.5ml of a virus solution containing 1×10^6 PFU of MPXV strain Zr-599 (16). After the challenge, body temperature and weight were measured. Peripheral blood was drawn, and throat

swab was collected every 3-4 days as described previously (16). Clinical manifestations including skin lesions were also observed every day (16).

Assays of IgG antibody to orthopoxvirus

Levels of antibody to vaccinia virus were measured by the ELISA using the entire vaccinia virus proteins as antigens, as reported previously (16, 66), except for the secondary antibody conjugated with horse-radish peroxidase purchased from ZyMed laboratories (South San Francisco, CA) (16).

Virus isolation from PBMCs

Virus was isolated using Vero cells from the buffy coat fractioned cells from peripheral blood and throat swab specimens obtained from the monkeys as reported previously (16, 101). The virus isolation methods from the peripheral blood were as follows. Four ml of heparinated peripheral blood was collected and centrifuged at 1,500 rpm. The entire buffy coat fraction was collected carefully and was washed twice with a phosphate-buffered saline solution (PBS) and then the cells were cocultivated with Vero cells as described above in MEM-2FBS. When a cytopathic effect (CPE) was observed in cell culture, the cytopathic causative agent was confirmed to be MPXV by indirect immunofluorescence with an anti-vaccinia virus antibody prepared in our laboratory (16, 66). Furthermore, the plaque number was also counted. When CPE did not appear, blind passage was further carried out.

Determination of MPXV loads in total peripheral blood by quantitative PCR

The viremia level was determined as reported previously (16, 60). Briefly, DNA was isolated from total peripheral blood using a Viral Nucleic Acid purification

kit™ (Roche Diagnostics) according to the supplier's instructions. The quantitative real-time PCR (qPCR) was performed by Light Cycler PCR-based methods (65). The primers and probes were designed based on the specific ATI gene on the MPXV genome as previously reported (16, 65).

Scoring system in determination of severity of MPX

To compare the level of MPX severity, a scoring system developed in a previous study was used in the present study (60). Briefly, the score is composed of two categories: items associated with clinical symptoms, and those associated with laboratory test results (60). The higher the score is, the more severe the symptoms are.

Histopathological examination

Histopathological examination was conducted by myself under the directorship of Dr. Masayuki Saijo with collaboration of Dr. Noriyo Nagata.

After sacrifice under deep anesthesia using ketaral, skin, lymph nodes, brain, lungs, heart, liver, spleen, pancreas, kidneys, bladder, gastrointestinal organs, and genitourinary tract structures were excised, fixed in 10% formalin in PBS, and embedded in paraffin. Macroscopic and histological examinations were performed on the excised tissues and organs. Paraffin sections, 4 μm thick, were stained with hematoxylin and eosin, and with Luxol-Fast Blue for the brain. Immunohistochemistry for the detection of MPXV antigens was performed using paraffin sections according to the method described previously (16, 66). For detection of MPXV antigens, a rabbit anti-vaccinia virus serum was used (16).

Laboratory data and cytokine responses

Total peripheral blood cell counts, serum biochemical tests such as AST, ALT, and LDH, CRP levels, were done with the standard methods (60). The concentrations of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6, and IL-10 were determined in serum samples using Human Cytokine 24-plex Antibody Bead Kit (BioSource Invitrogen, Camarillo, CA) according to the manufacturer's instructions. Samples were analyzed using a Luminex 100TM (Luminex Corporation, Austin, US).

Statistical analysis

Student *t*-test was performed for comparison of body temperature change and maximum viremia level between LC16m8-group and Lister-group. Analysis was two-tailed and conducted using JMP 11 (SAS institute, Cary, NC). Results were considered significant at $P<0.05$.

RESULTS

Clinical manifestations and skin lesions after immunization with LC16m8 and Lister

Local effects at the site of vaccination with LC16m8 and Lister were observed. Immunization with LC16m8 and Lister induced "vaccine take": pustules, scabs, and scarring. The lesions were similar to those reported in a previous study (16). The lesions induced by Lister were severer than those induced by LC16m8 (data not shown) as described previously (16, 60).

Protection of monkeys from MPXV infections by immunization with either of LC16m8 or Lister

Naïve-group monkeys subcutaneously inoculated with MPXV Zr-599 developed severe symptoms (Table 6, Fig. 9). Body weight decreased after 7 days post challenge by approximately 10 % in the group (Fig. 9A). On the other hand, the monkeys in the vaccinated groups (LC16m8-6M, Lister-6M, LC16m8-12M, Lister-12M) maintained their body weight during the observation period (Fig. 9B). No significant difference was demonstrated between the LC16m8 vaccinated and Lister vaccinated monkeys. Papulovesicular skin lesions appeared on day 7 after challenge in the naïve-group (Table 6 and Fig. 10). To the contrary, the LC16-m8- or the Lister-vaccinated monkeys in all the groups did not develop any MPX-associated skin lesions (Table 6). Whereas the ulcerative lesions appeared locally at the site of MPXV inoculation in all the LC16m8-6M and LC16m8-12M group monkeys, the subjects of Lister-6M and Lister-12M groups showed no MPX associated symptoms, even at the site of MPXV inoculation (Fig. 10).

Viremia determined by the virus isolation and qPCR

Virus isolation results are summarized in Table 6. MPXV was isolated from the buffy coat fractions obtained from 5 ml of peripheral blood collected between days 3 and 21 from monkeys in each group. MPXV was isolated from the buffy coat fractions of naïve-group monkeys between day 3 and 14. The plaque number was so many that the number was not counted particularly in the acute phase of infections in naïve monkeys (Table 6). The isolation-positive period in naïve-group monkeys was also longer than those of the vaccinated groups. In the vaccinated groups, MPXV-isolation was positive in the buffy coat fractions of 2 (#4636, 4638) out of the 3 LC16m8-6M monkeys from day 7, whereas in none of the Lister-6M-group subjects were positive (Table 6). A significant feature of virus isolation in the LC16m8-6M and LC16m8-12M-groups was that plaque number was small and the isolation period was short. On the other hand, MPXV was not isolated from the buffy coat fractions collected from any of and Lister vaccinated group subjects (Table 6).

In the naïve-group subjects, viremia was demonstrated with more than 10^5 copies/mL at Days 3 or 4. The level peaked at more than 10^7 copies/mL around on Day 10, and then decreased (Fig. 9A). In the LC16m8-6M-group, all monkeys showed viremia with being more than 10^5 copies/mL, but the viremia period was shorter than that of the naïve-group (Fig. 9). All 2 Lister-6M-group monkeys showed viremia (the maximum level was more than 10^4 copies/mL) around at Day 3-7. The levels of Lister-6M-group monkeys were lower and the period was shorter than those of the LC16m8-6M group (Fig. 9B). In the LC16m8-12M group, all monkeys showed viremia (two monkeys; more than 10^4 copies/mL, one monkey; more than 10^5

copies/mL) from Day 3 to 10, whereas one of the 2 Lister-12M-group monkeys showed viremia at Day 3 (more than 10^4 copies/mL) (Fig. 9C).

Statistical analysis confirmed maximum viremia level between LC16m8-6M-group and LC16m8-12M-group subjects have no significant differences (data not shown).

Comparison of MPX-associated symptoms of LC16m8-6M and LC16m8-12M monkeys with those of Naïve-group monkeys

The clinical severity of the LC16m8-6M and LC16m8-12M subjects was compared with those of the Naïve-group by implementing the scoring system. The mean and SD value of each group were 4.3±0.5 (LC16m8-6M) and 2.2±1.0 (LC16m8-12M), respectively. Both were much lower than that of the naïve monkeys (30.0±10.6) (Table 7).

Histopathological examination

All the monkeys were sacrificed for virological and histopathological examination at the end of observation. MPX-associated lesions with positive MPXV antigens were observed in the lymphoid systems (lymph node, thymus, and tonsils), respiratory tract (lung and trachea), digestive tract (stomach, small intestine, colon, rectum, and liver), urogenital tract (bladder, uterus, and ovary), or skin in naïve-group as previously described (16, 60). On the other hand, no MPX-associated lesions were detected in any organs of vaccinated monkeys with either of Lister or LC16m8, except for the skin lesions at the site of MPXV challenge in the LC16m8-6M and LC16m8-12M groups (Fig. 10).

Assays of IgG antibody to orthopoxvirus

The levels of circulating IgG to vaccinia virus antigens were measured by ELISA in pre- and post-infection in all the vaccination groups (Fig. 11), while the pre-challenge sera of the naïve-group subjects were negative for IgG to vaccinia virus antigens (Fig. 11A). OD values representing the level of IgG antibody to vaccinia virus antigen in the ELISA among the LC16m8-6M and LC16m8-12M groups were lower than those of the Lister-6M and Lister-12M group monkeys (Fig. 11B, C) at the time of MPXV challenge.

In detail, the kinetics of the antibody response in the LC16m8-vaccinated monkeys were different from those in the Lister-vaccinated monkeys; the antibody titer showed a large increase from the baseline levels, reached the maximum level around 28 days post immunization in all monkeys, and then decreased slowly thereafter (Fig. 11B, C). The OD values representing the antibody titer in LC16m8-6M group were significantly lower than those detected in the Lister-6M group after 185 days (approximately 6 months) after vaccination.

After infection, OD values measured by IgG-specific ELISA in plasma from the Naïve monkeys increased and reached the maximum level around 10 days after MPXV challenge (Fig. 11A). On the other hand, the antibody responses were induced immediately in both the LC16m8- and Lister-vaccinated groups (Fig. 11B, C). As described above, the OD values reached the maximum level at 4 weeks from vaccination, and then decreased gradually until the challenge only in the LC16m8-vaccinated monkeys (both the LC16m8-6M and LC16m8-12M groups). Nevertheless, an immune response was rapidly induced within 7 days of infection (Fig. 11B, C).

Laboratory findings and cytokine responses

CRP levels were significantly increased in naïve-group but not in the animals immunized with either vaccine (Table 7). The Luminex assay showed no increase in IFN-γ in the serum of subjects in both naïve (#4626) and the vaccinated monkeys (data not shown). However, inflammatory cytokines, such as IL-6 and TNF-α, were increased in the naïve monkey but not in the vaccination groups (Fig. 12), with the exception of a positive TNF-α response in 1 (#4637) of 3 LC16m8-6M subjects (Fig. 12C).

DISCUSSION

Current knowledge on the immunological mechanisms of protection against smallpox and MPX suggests that both humoral and cellular immunities play an important role (102-105), although the underlying mechanisms are not fully understood because of difficulties in the use of animal models for experimental infection.

LC16m8 is reportedly effective in the protection of NHPs from MPXV infection, when challenged with MPXV after 4-5 weeks from vaccination (16). In this study, We confirmed for the first time that a single vaccination of NHP with LC16m8 induced a long-lasting protective immunity against MPXV infection. The protective efficacy persisted for 6 and 12 months. The protective efficacy demonstrated at 6 and 12 months after LC16m8 vaccination was similar to that demonstrated at 4-5 weeks after LC16m8 vaccination (16) in terms of level of the clinical manifestations, viremia, cytokine responses, and pathological findings. These results suggest that a single vaccination with LC16m8 provides a long-term protective immunity against smallpox.

In the present study, we also demonstrated that Lister conferred stronger protective efficacy against MPX in NHPs than LC16m8 did, as reported previously (16). Viremia levels determined by qPCR were consistent with those of virus isolation experiments in naïve, LC16m8-, and Lister-vaccinated groups (Table 6, Fig. 9). Interestingly, MPXV was isolated from the throat swab specimens collected from 1 of the 2 Lister-12M-group monkeys, in which no MPX-associated symptoms were demonstrated and no viremia was detected by sensitive qPCR (data not shown). This result indicates that complete protection of MPXV infections was impossible even by non-attenuated Lister vaccine. Lister induced stronger protective efficacy than

LC16m8 against MPXV infection. The evidence was obtained by demonstrating skin lesions at the site of MPXV inoculation: mild ulcerative lesions in LC16m8-immunized groups versus no lesions in Lister-immunized groups (Fig. 10). The difference in the MPXV replication at the inoculation site may account for the difference in MPXV viremia level between LC16m8- and Lister-immunized groups (Figs. 9, 10).

To evaluate the efficacy of LC16m8 from the aspects of immune response, the levels of circulating IgG to vaccinia virus antigens were measured by ELISA. For analyzing the IgG value of naïve monkeys, the data of prior studies were included. As a methodological limitation, the kinetics of IgG response in naïve monkeys differ substantially between individuals (16, 106, 107). IgG-specific ELISA in plasma from #4626 monkey showed a strong antibody response that reached a plateau by 10 days post infection, while the other monkeys prior studied attained the maximum level around 10 days post infection (Fig. 11A). These results suggest that the clinical symptoms and viremia level observed in #4626 were milder than in the prior monkeys (Table 6, Fig. 9). The kinetic of OD values representing the antibody titer was followed until 12 months from LC16m8 or Lister vaccination in NHPs. It was demonstrated that OD values reached the maximum level 1-month later, and decreased gradually thereafter (Fig. 11). However, the protective efficacy maintained. It suggests that in LC16m8 vaccinated subjects, in which circulating IgG antibody became decreased, memory B cells, however, still existed and were activated immediately after infection (102). Not only the humoral responses but also cellular responses play a role in protection.

In LC16m8 vaccinated groups, OD values in IgG antibody response measurement of LC16m8-6M groups were lower than those of LC16m8-12M group

(Fig. 11B, C), which was consistent with the results that the lesions at MPXV inoculated site seemed to be slightly severer in LC16m8-6M group than in LC16m8-12M group (Fig. 10). This phenomenon suggests that the efficacy induced by vaccination seems to be dependent on the individual difference in response to the vaccination in each monkey. To compare the severity in LC16m8-6M group and LC16m8-12M group, the scoring system was introduced (Table 7). In summary, the severity of LC16m8-6M monkeys was similar to those of LC16m8-12M group monkeys. The results of Luminex assay suggested a reduction in cytokine production the vaccinated groups, except for #4637 monkey (Fig. 12).

The naïve-group included 4 subjects, but only 1 subject was simultaneously infected with MPXV with those of the other groups' subjects. The 3 in the naïve-group subjects were those infected with MPXV in the same way as this present study in the previous studies. The 3 monkeys were included. Ideally, all the subjects should be simultaneously infected with the virus with the other group subjects. However, we had a limitation in using NHPs based on the ethical consideration. Because we had already confirmed that each of the naïve monkeys infected with MPXV Zr-599 strain at a dose of 10^6 PFU showed almost similar level of severity from lethal to severe infections through the previous and ongoing studies (16, 60). In each course of experiment, the infectious dose administered was confirmed to be the expected amount by back titration (16). We consider that this modification by reducing the number of NHPs used can be acceptable scientifically and ethically.

It was demonstrated as a new finding in this study that single vaccination with LC16m8 induced a long-term protection of NHPs from MPX, although the IgG antibodies to vaccinia virus antigens decreased. The protective efficacy at 12 months after vaccination was almost the same level as those induced at 4-5 weeks from

vaccination (16). The results in this study suggest that LC16m8-vaccination protects humans not only against smallpox but also against MPXV infection, and that the efficacy remains for a long period of time.

Recent investigations demonstrated a major increase in human MPX incidence 30 years after the discontinuation of smallpox vaccination in Congo Basin, most endemic region (45, 46, 108). Improved surveillance and epidemiological analysis are necessary for assessment of the public health threat and develop management strategies for reducing the risk of spread of human MPX over wide area. The novel findings in this study will contribute to consider the adequacy of the targeted vaccination in endemic countries, and assist people living where at risk for MPXV to participate in preventing the spread of disease.

CONCLUSIONS

The conclusions of this study were the followings:

1. A novel diagnostic assay for MPXV infection in human was developed with application of LAMP technology. This method for the detection of MPXV genome is sensitive, specific, rapid and in a real-time quantitative manner. In addition, using this novel assay, MPXV can be differentiated into Congo Basin strains and West African strains.

2. A single dose vaccination of cynomolgus monkey with highly attenuated smallpox vaccine, LC16m8, provides protection against MPXV for a long term that persists at least one-year post immunization.

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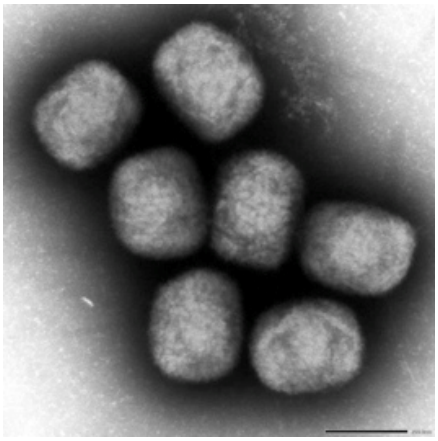
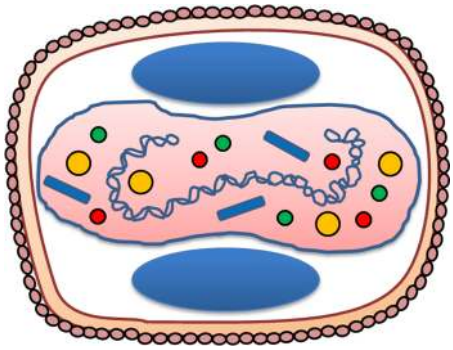


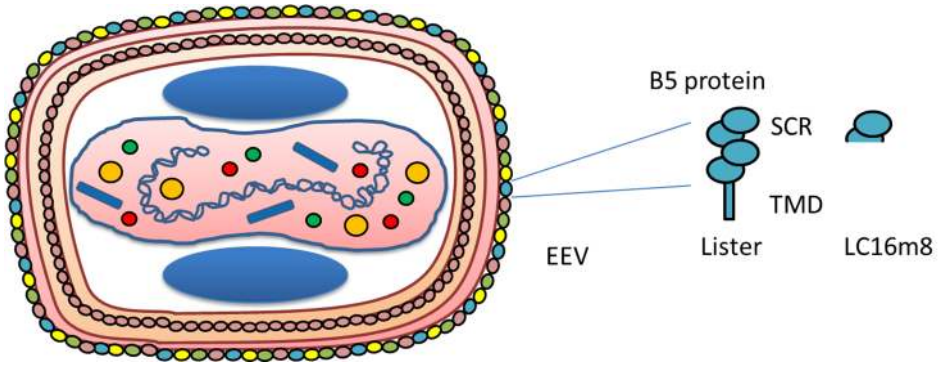
Fig. 1.

Negative stain electron micrograph of orthopoxviruses. Scale bar indicates 200 nm. (It is provided under the courtesy of Dr. Noriyo Nagata in Department of Pathology, National Institute of Infectious Diseases, Japan).

(A)



(B)



(C)

Fig. 2. Two distinct infectious forms of VACV, the intracellular mature virus (IMV) (A) and the extracellular enveloped virus (EEV), which have different numbers of membranes and distinct surface antigens. B5 protein produced by LC16m8 is truncated (C). SCR indicates “short consensus region” , TMD indicates “trans membrane domain” and CT indicates “cytoplasmic region”.

Table 1. The cases of human MPX reported in central and western Africa, and US.

(A) The case of human MPXV reported in Africa and US from 1970 to 2010.

Year	No. of cases		country	refrence
1970	1	the first case	DRC (Zaire)	(26)
1970-1979	47	17 (fatal cases)	DRC (Zaire)	(27)
1980-1984	214		DRC (Zaire)	(30)
1980-1986	338	33 (fatal cases)	DRC	(39)
1995-1997	500 (approximately)	88 (laboratory confirmed)	DRC	(38)
2001-2004	51	5 deaths	DRC	(37, 39)
2005-2007	760		DRC	(44, 45)
2005	19	10 (laboratory confirmed)	Sudan	(41, 42)
2010	10	2 (laboratory confirmed)	ROC	(109)
Imported case				
2003	71	35 (laboratory confirmed)	US	(49)

(B) The cases of human MPXV reported from 1970 to 1986 in 7 African countries.

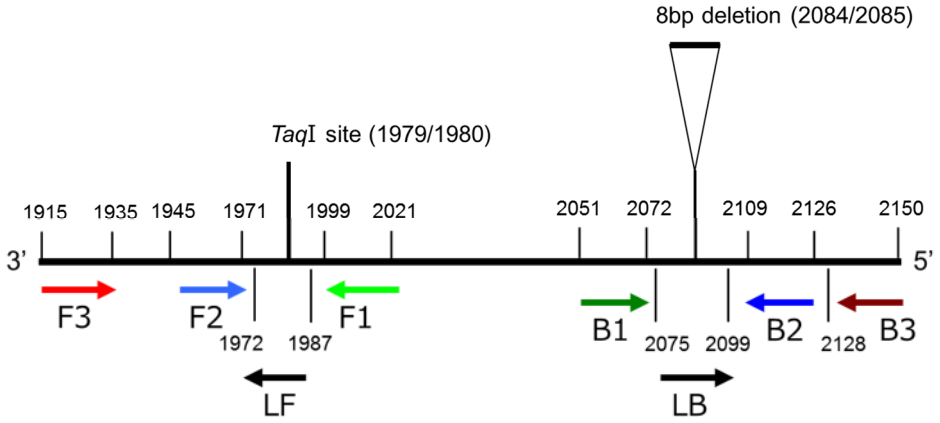
Year	Zaire (DRC)	Liberia	Nigeria	Sierra Leone	Cameroon	Central African Republic	Cote d'Ivoire
1970	1	4	-	1	-	-	-
1971	-	-	2	-	-	-	1
1972	5	-	-	-	-	-	-
1973	3	-	-	-	-	-	-
1974	1	-	-	-	-	-	-
1975	3	-	-	-	-	-	-
1976	5	-	-	-	-	-	-
1977	6	-	-	-	-	-	-
1978	13	-	1	-	-	-	-
1979	10	-	-	-	2	-	-
1980	4	-	-	-	-	-	-
1981	8	-	-	-	-	-	1
1982	40	-	-	-	-	-	-
1983	84	-	-	-	-	-	-
1984	92	-	-	-	-	6	-
1985	62	-	-	-	-	-	-
1986	59	-	-	-	-	-	-

Source : (26)

Fig. 3. The skin lesions of patients with smallpox (left panel) and human MPX (right panel). (It is provided from Dr. Masayuki Saijo in Department of Virology 1, National Institute of Infectious Diseases, Japan).

Fig. 4. Papulovesicular skin lesions appeared on the skin surface of NHP infected with MPXV [face (left panel) and inguinal part (right panel)].

(A)



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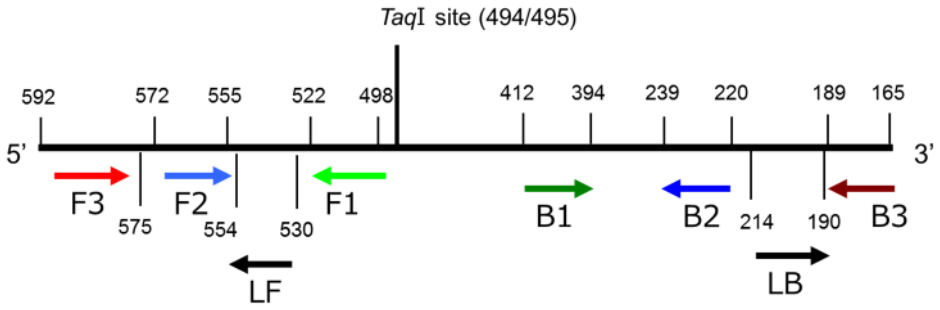

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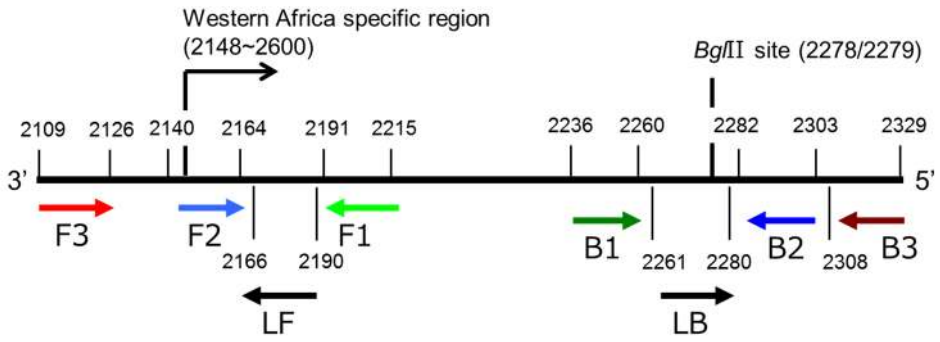

Fig. 5. Target region of primer design for COM-LAMP, C-LAMP, W-LAMP

Primers for COM-LAMP (A), W-LAMP (C) were designed to amplify the specific region in ATI gene respectively. Primers for C-LAMP (B) were designed to amplify the specific region for Congo Basin MPXV in D14L gene.

Table 2. 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’-TGGGAGCATTGTAACTTATAGTTGC-CCTCCTGAACACATGACA-3’
		F3-C	5’-TGGGTGGATTGGACCATT-3’
		BIP-C	5’-ATCCTCGTATCCGTTATGTCTTCC-CACCTATTTGCGAATCTGTT-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’-ATAGGCTAAAGACTAGAATCAGGGA-TTCTGATTCATCCTTTGAGAAG-3’
		B3-W	5’-AGTTCAGTTTTATATGCCGAAT-3’
		Loop-F-W	5’-GATGTCTATCAAGATCCATGATTCT-3’
		Loop-B-W	5’-TCTTGAACGATCGCTAGAGA-3’

Table 3. Experimental conditions and clinical manifestations of MPX for monkey subjects.

ID	Virus inoculated	Route	Vaccination	Days from vaccination to challenge	No. of clinical samples tested		No. of papulovesicular lesions	Severity	Outcome
					PBC[*1]	TS[*2]			
Z-01-SC	Zr-599	SC[*3]	-	-	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
Z-05-IN		IN[*4]	-	-	5	0	106	Severe	Survived
Z-06-IN		IN	-	-	7	0	131	Severe	Sacrificed
Z-07-IN		IN	-	-	7	0	136	Severe	Survived
Z-08-IN		IN	-	-	7	0	53	Severe	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

*1 PBC: Peripheral blood cells
*2 TS: Throat swab specimens

*3 SC: Subcutaneous inoculations
*4 IN: Intranasal inoculations

Table 4. 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-	MPXV Zr-599	-	+	+	+	+	+
LAMP	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	-	+	+	+	+	+

* Not tested

Table 5. Relationship between the results obtained from the nested PCR and LAMP assays.

LAMP method		Samples from Zr-599-challenged monkeys		Samples from Liberia-challenged monkeys		Sensitivity	Specificity
		Nested PCR		Nested PCR			
		Positive	Negative	Positive	Negative		
COM-LAMP	Positive	33	0	24	0	73% (57/78)	100% (67/67)
	Negative	14	27	8	40		
C-LAMP	Positive	32	0	0	0	70% (32/46)	100% (27/27)
	Negative	15	27	33	39		
W-LAMP	Positive	0	0	23	0	70% (23/32)	100% (40/40)
	Negative	24	24	9	40		

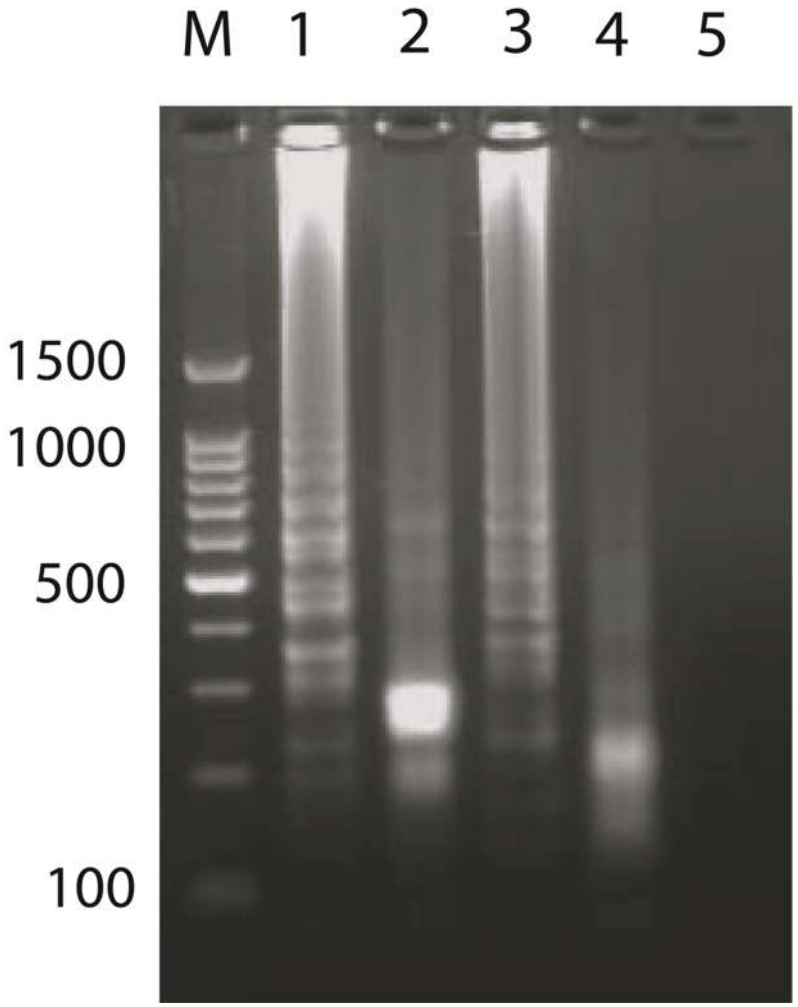

Fig. 6. DNA products from MPXV and camelpox virus amplified with COM-LAMP and separated by electrophoresis (lanes 1 and 3, respectively). To discriminate MPXV from camelpox, MPXV-LAMP and camelpox virus-LAMP products were treated with a restriction enzyme (*Taq* I) (lanes 2 and 4, respectively). A 100bp-DNA ladder marker and negative control are also shown (lanes M and 5, respectively).

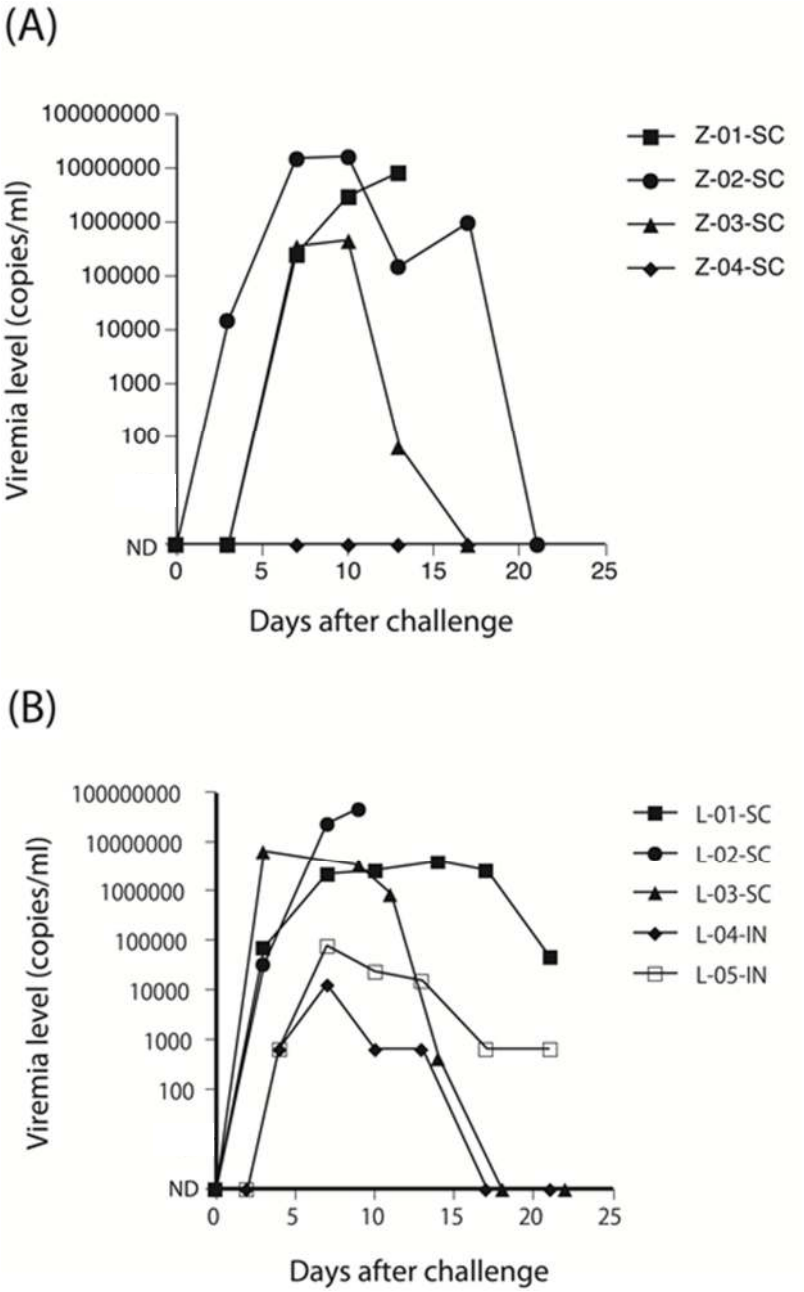

Fig. 7. Viremia level in peripheral blood collected from MPXV Zr-599-infected monkeys (A) and those in MPXV Liberia-infected monkeys (B) as determined by COM-LAMP. "ND" indicates that the level was below the detection level.

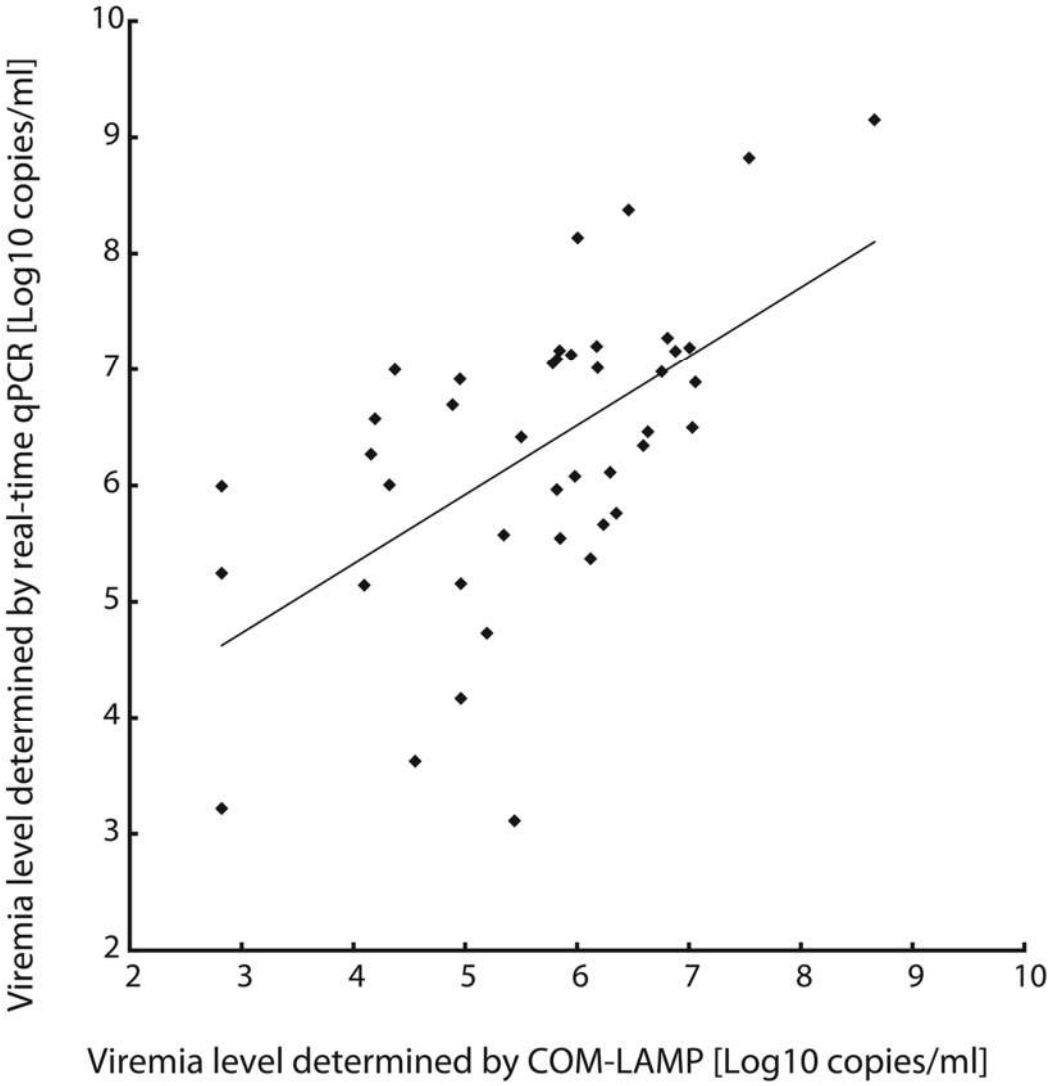

Fig. 8. Correlation between viral loads determined by real-time qPCR and those by the COM-LAMP developed in this study.

Table 6. Groups, MPX-associated symptoms, and virus isolation in naïve monkeys, and monkeys immunized with LC16m8 or Lister

Group	ID[a]	Vaccination	months before infection	Sex[b]	MPXV-associated symptoms				
					Virus isolation[c]	No. of plaques at the indicated day of collection[d]	Symptom(s) at the site of MPXV inoculation	No. of papulovesicular	Outcome
Naïve	4626	Mock	0	M	Positive	3+ at day3, 3+ at day7, 2+ at day10	Oral cavity, papulovesicles, decreased activity, loss of appetite	95	Survival
	4683			M	Positive	2+ at day4, 3+ at day6, 3+ at day10, 3+ at day13	Diarrhea, papulovesicles, decreased activity, loss of appetite	388	Sacrificed
	4651			F	Positive	1+ at day3, 1+ at day7, 3+ at day9, 3+ at day14	Diarrhea, rhinorrhea, depressed, ulcer, loss of appetite	390	Survival
	4653			M	Positive	3+ at day3, 2+ at day7, 1+ at day9, 3+ at day11, 2+ at day14, 1+ at	Diarrhea,severely decreased activity, papulovesicles, ulcer, loss of appetite	1150	Survival
LC16m8-6M	4636	LC16m8	6M	M	Positive	2+ at day7	None	0	Survival
	4637			M	Negative		None	0	Survival
	4638			M	Positive	1+ at day3	None	0	Survival
Lister-6M	4642	Lister		M	Negative		None	0	Survival
	4646			M	Negative		None	0	Survival
LC16m8-12M	4631	LC16m8	12M	M	Negative		None	0	Survival
	4632			M	Negative		None	0	Survival
	4633			M	Negative		None	0	Survival
Lister-12M	4634	Lister		M	Negative		None	0	Survival
	4635			M	Negative		None	0	Survival

[a]ID, monkeys identification number.
[b]F, female; M, male.
[c]Positive or negative, MPXV was or was not isolated, respectively, during the observation periods from challenge to sacrifice. Virus isolation was attempted from the buffy coat fractions collected from 4-ml aliquots of total peripheral blood collected from monkeys every 3 or 4 days during the observation period after virus was challenged.
[d]No. of plaques at the indicated day of collection, 1+ indicates 1 to 5 plaques, 2+ indicates 6 to 20 plaques, 3+ indicates more than 21 plaques observed through virus isolation.

Table 7. Comparison of severity among the Naïve-group LC16m8-6M, and LC16m8-12M groups by using the scoring system developed for assessment of monkeypox severity.

Item	No. animals with score	Mean score in each group		
		Naïve	LC16m8-6M	LC16m8-12M
Number of monkeys		4	3	3
Decrease in body mass (%)	<3, 0; 3-<8, 1;8-<13, 2; >13,3	2.3 ± 0.5	0	0.3 ± 0.6
Recovery signs in body mass	Positive, 0; negative, 3	2.3 ± 1.5	0	0
Duration of decreased activity (days) with ill appearance	None, 0; 1-5, 1;6-10, 2; >10, 3	2.3 ± 1.0	0	0
Duration of decreased meal consumption (days)	None, 0; 1-5, 1;6-10, 2; >10, 3	2.5 ± 0.6	2.3 ± 0.6	0.3 ± 0.6
Fever > 1℃	Negative, 0; positive, 1	0.3 ± 0.5	0	0
Drop in bodt temperature > 1.5℃	Negative ,0; positive, 3	2.3 ± 1.5	0	0
Faecal appearance	Normal, 0; watery diarrhoea, 1; haemorrhagic diarrhoea, 3	1.8 ± 1.5	0	0
Papulovesicular lesions (no.)	None, 0; 1-50, 1; 51-499, 2; >500,3	2.3 ± 0.5	0	0
Outcome	Non-fatal, 0; fatal,6	4.5 ± 3.0	0	0
Maximum virus genome level [log_{10}(copies/mL)]	UDL[a], 0; <5, 1; 5-7, 2; >7, 3	2.8 ± 0.5	2	1.6 ± 0.7
Virus genome level when sacrificed [log_{10}(copies/mL)]	UDL, 0; <5, 1; 5-7, 2; >7, 3	2.0 ± 1.4	0	0
			0	0
Peripheral WBC count <5000μL-1	Positive, 0; negative, 1	0.5 ± 0.6	0	0
Thrombocytopenia < 20000μL-1	Positive, 0; negative, 1	0.8 ± 0.5	0	0
Anaemia with decrease in haemoglobin level >1.5g/dL	Positive, 0; negative, 1	1.0 ± 0.0	0	0
Maximum CRP level [mg/dL]	<1, 0; 1-<5, 1;5-<10, 2; >10, 3	2.8 ± 0.5	0	0
Mean		30.0 ± 10.6	4.3 ± 0.5	2.2 ± 1.0

[a]UDL, UDL indicates “under detection level”.

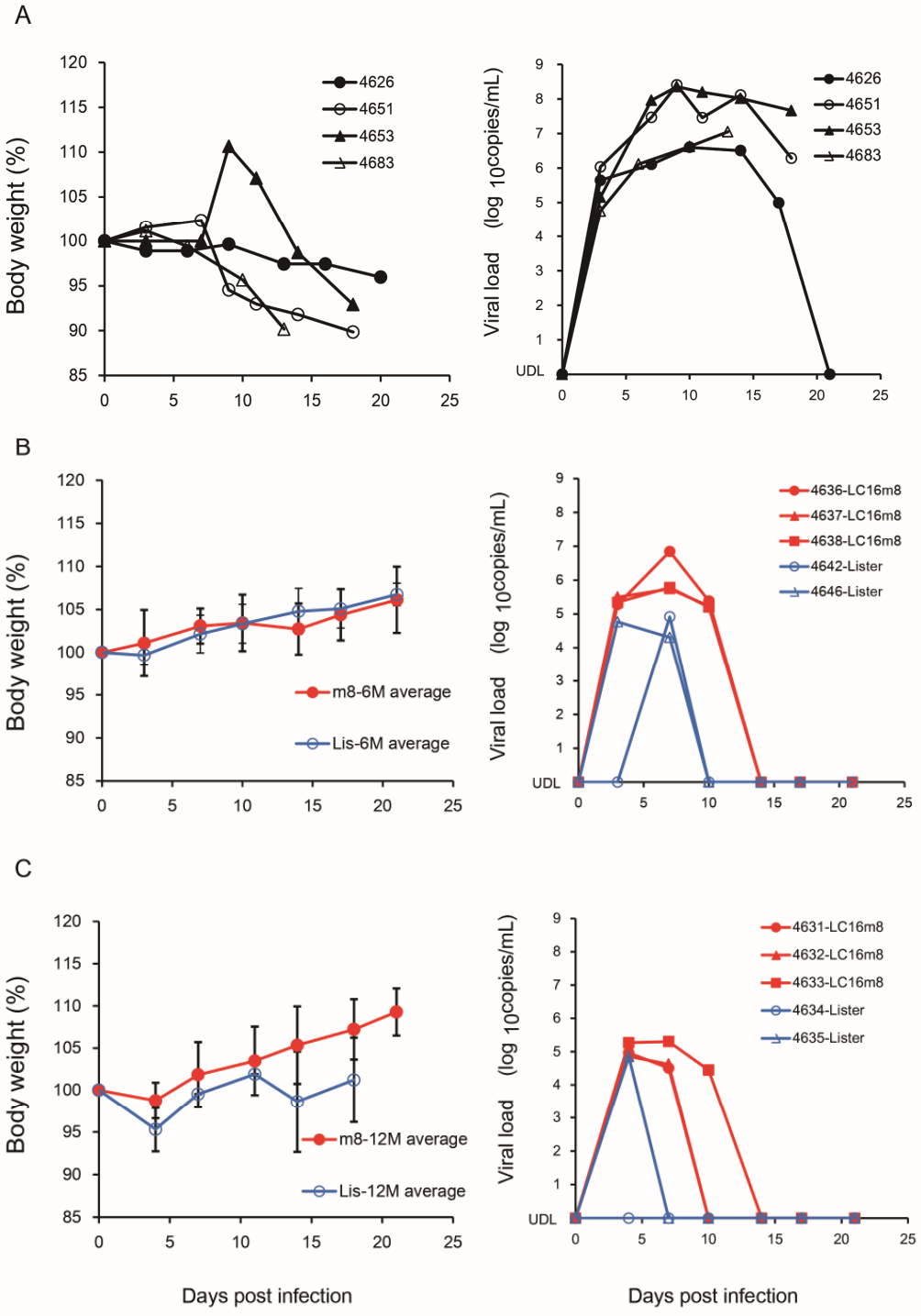

Fig. 9. Changes in body weight of Naïve-group monkeys (A, left), LC16m8-6M and Lister-6M group monkeys (B, left), and LC16m8-12M and Lister-12M group monkeys (C, left). MPXV viremia levels in the total peripheral blood determined by qPCR in Naïve-group monkeys (A, right), LC16m8-6M and Lister-6M group monkeys (B, right), and LC16m8-12M and Lister-12M group monkeys (C, right). UDL indicates "under the detection level". The error bars were shown in the line graph above represents standard deviations of the mean values.

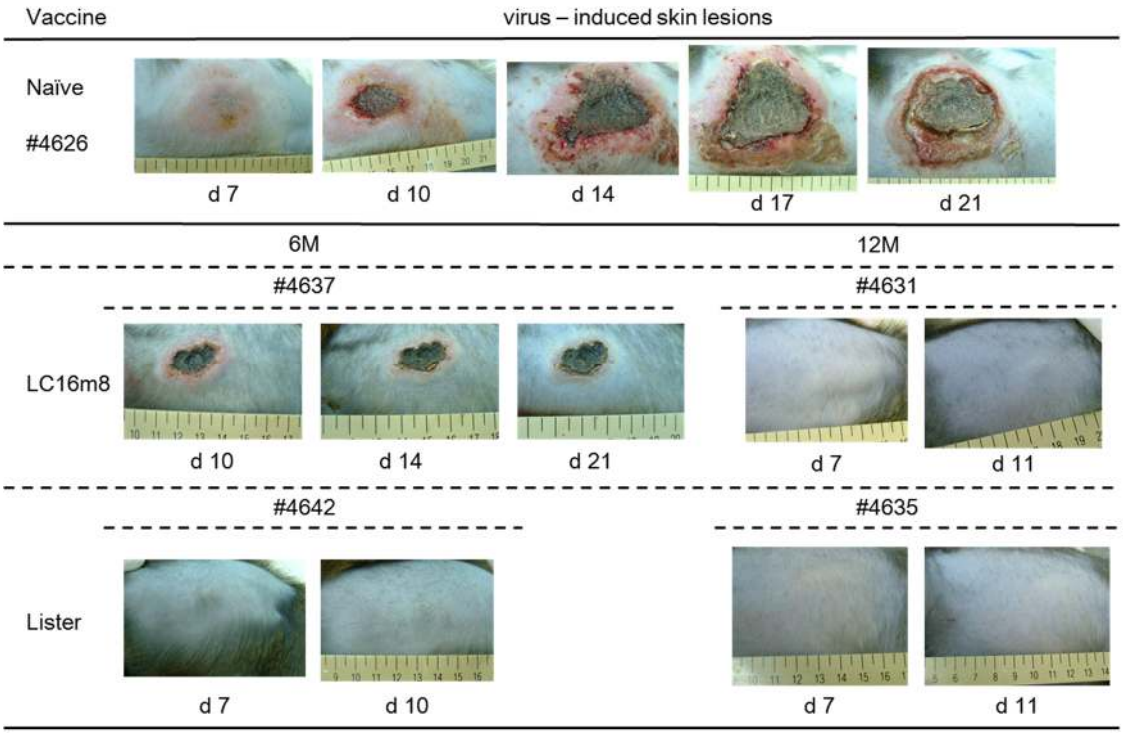

Fig. 10. Development of local cutaneous lesions at the site (upper right leg) of MPXV Zr-599 inoculation in Naïve-group monkeys (#4626), LC16m8-6M and Lister-6M group monkeys (#4637 : LC16m8, #4642 : Lister), and LC16m8-12M and Lister-12M group monkeys (#4631 : LC16m8, #4635 : Lister). d7, d10, d14, d17, d21 indicates day7, day10, day14, day 17 and day 21 post infection.

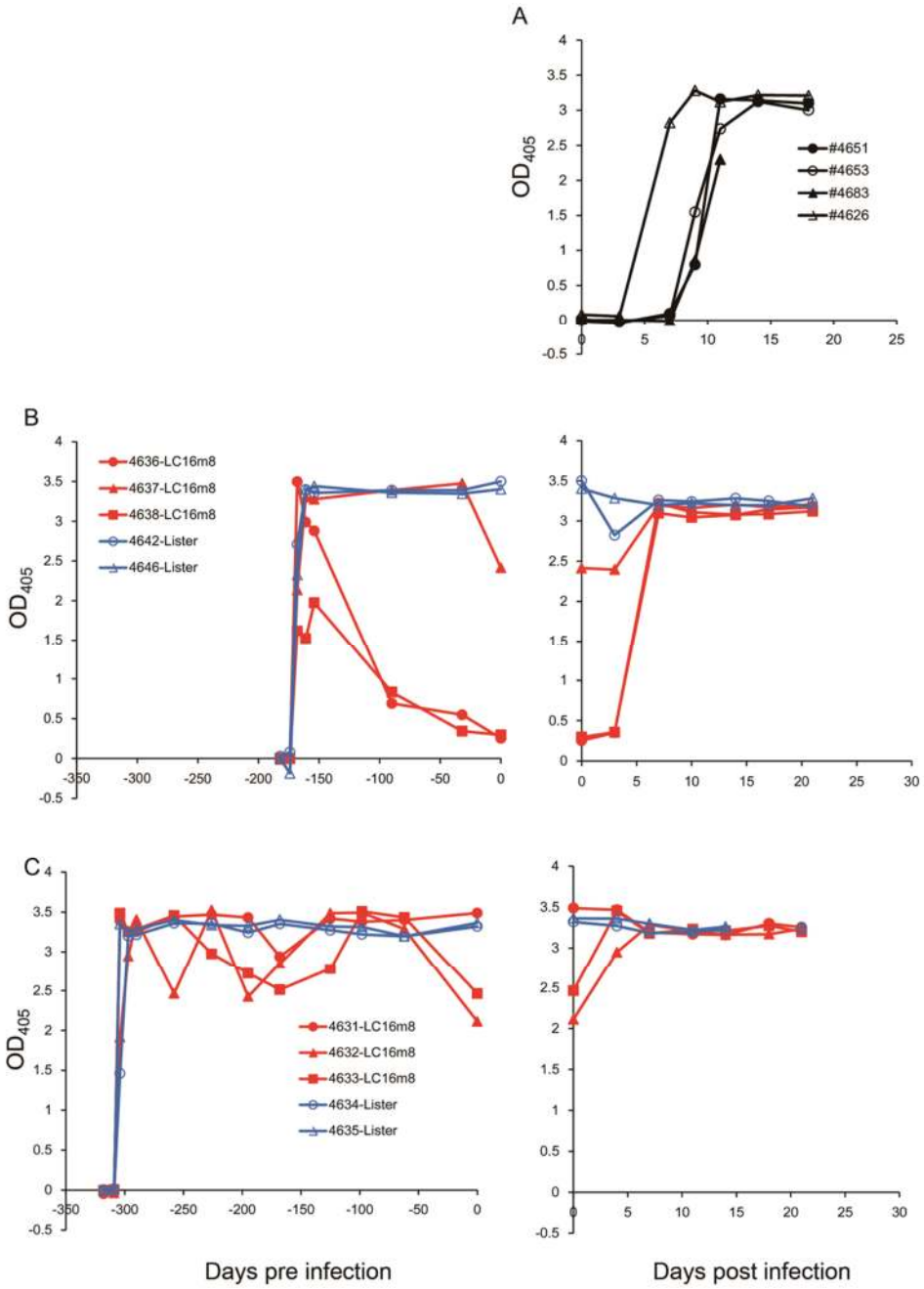

Fig. 11. Vaccinia virus-specific IgG responses determined by IgG-ELISA. The optical densities measured at 405 nm (OD405) at the serum sample dilutions level of 1:100 are shown in Naïve-group monkeys after MPXV challenge (A, right), LC16m8-6M and Lister-6M group monkeys (B, left panel: after vaccination until MPXV challenge; right panel: after MPXV challenge), and LC16m8-12M and Lister-12M group monkeys (C, left panel: after vaccination until MPXV challenge; right panel: after MPXV challenge). The day of MPXV challenge was taken as day 0.

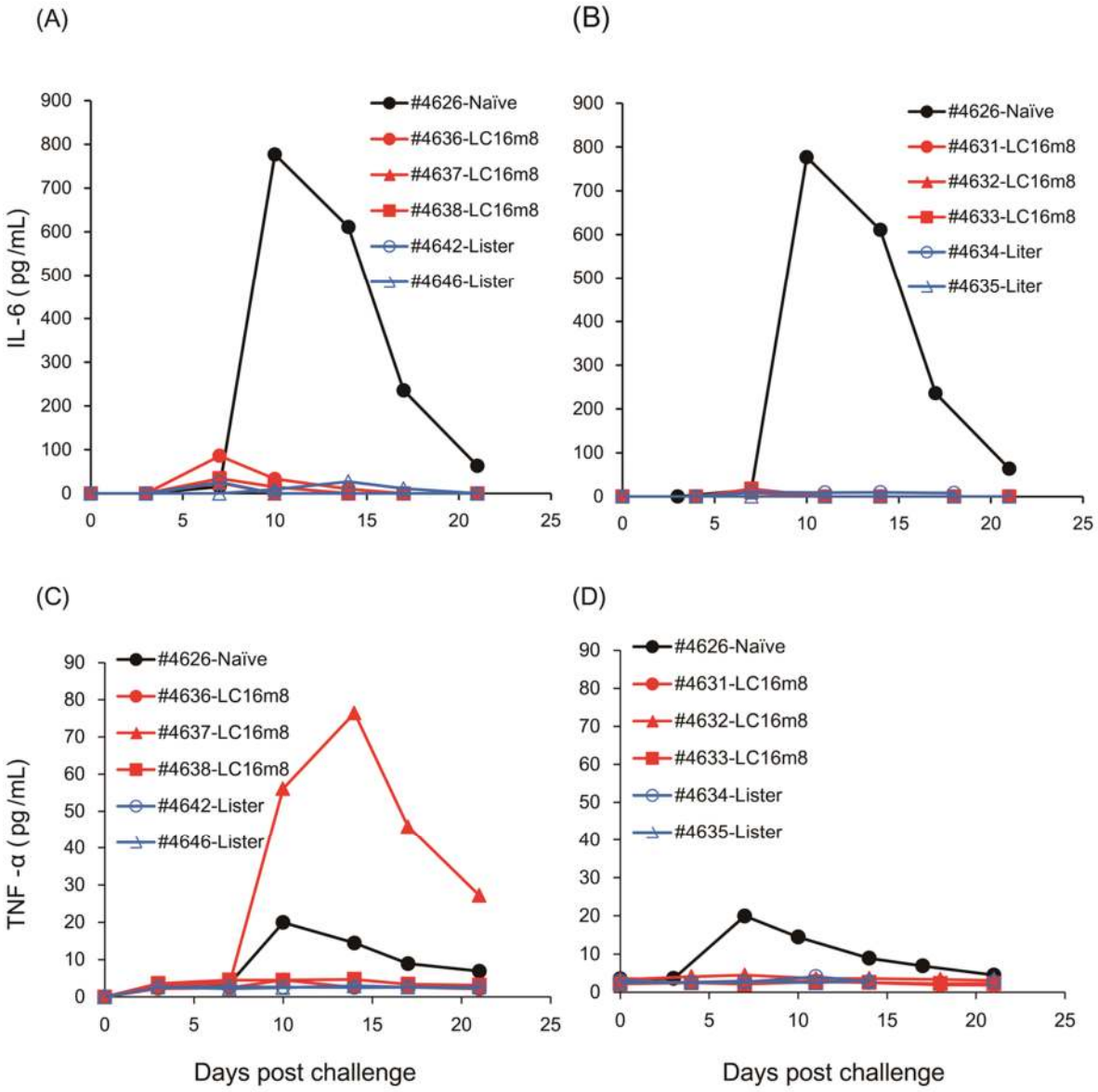

Fig. 12. Changes in serum IL-6 levels in each group subjects inoculated with MPXV after 6 months from vaccination, LC16m8-6M and Lister-6M groups (A), and those after 12 months from vaccination, LC16m8-12M and Lister-12M groups (B). Changes in TNF-α serum levels in LC16m8-6M and Lister-6M groups (C), and those after 12 months from vaccination, LC16m8-12M and Lister-12M groups (D). The response in the naïve-group subject (#4626) is included as the control.