Short Communication

Diagnosis and Assessment of Monkeypox Virus (MPXV) Infection by Quantitative PCR Assay: Differentiation of Congo Basin and West African MPXV Strains

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SUMMARY: Human monkeypox, an infectious disease caused by monkeypox virus (MPXV), is endemic to western and central Africa. A LightCycler quantitative PCR (LC-qPCR) system was developed for the diagnosis of this disease, targeting the A-type inclusion body gene (ATI gene) of MPXV. One naïve monkey was infected with MPXV Zr-599 (Congo Basin strain) and one with MPXV Liberia (West African strain). Another three monkeys were immunized with smallpox vaccine on 0, 3, or 7 days, respectively, before infection with MPXV Zr-599. Peripheral blood cell (PBC) and throat swab (TS) specimens were serially collected. The LC-qPCR was validated for the diagnosis of monkeypox using virus isolation. Sequencing of the partial ATI gene revealed the insertion of a unique 453-nucleotide residue in the West African strains but not in the Congo Basin strains. Specific reverse primers for Congo Basin and West African strains were designed based on the unique sequence insertion. The LC-qPCR detected the MPXV genome, but not those of the other orthopoxviruses tested nor the varicella-zoster virus. Both the sensitivity and specificity of the LC-qPCR were over 90% in comparison to virus isolation when TS specimens were tested. Fourteen of the 15 virus isolation-positive PBC specimens showed positive reactions in the assay. Further, most PBC specimens collected from symptomatic monkeys in the later stage of illness showed positive reactions in the assay but negative reaction in virus isolation. It was possible to differentiate between these two groups with the LC-qPCR. Thus, the newly developed LC-qPCR is a useful and reliable diagnostic tool for MPXV infection.

Monkeypox virus (MPXV) infection in humans, known as human monkeypox and having symptoms similar to those of smallpox, is endemic to the western and central regions of Africa (1,2). MPXV belongs to the Family *Poxviridae*, Genus *Orthopoxvirus*. A sporadic human monkeypox outbreak was reported in the United States in 2003, suggesting that the disease must be regarded as an important re-emerging infectious disease (3-7). MPXVs form two genetically distinct clades, Congo Basin and West African strains, which are prevalent in the central and western regions of Africa, respectively (7). It has been reported that human monkeypox in western Africa is less severe than that in central Africa (3). Therefore, a rapid diagnostic tool for MPXV infection with the ability to differentiate the two clades is desirable. In the present study, quantitative real-time polymerase chain reaction (PCR) for amplification of the MPXV genome and differentiation of the two clades was developed, and its efficacy in the diagnosis of monkeypox was examined.

MPXV strains, Zr-599, Congo-8, Sierra Leone, Orangutan, Copenhagen, Liberia, SEN-79, and clinical isolates (1996-97 isolates in DRC) recovered from patients with human monkeypox in the Democratic Republic of Congo in 1996 and 1997 were used (8). Ectromelia virus (strain Hampstead),

camelpox virus (strain J1E3), cowpox virus (strain Brighton Red) and vaccinia virus (strain Lister) were also used. Cynomolgus monkeys were immunized with smallpox vaccine, LC16m8 (8,9). DNA of varicella-zoster virus (VZV) (strain Webster), whose infections (chickenpox and zoster) are important virus infections that need to be differentiated from monkeypox, was used.

Five male cynomolgus monkeys (*Macaca fascicularis*) weighing 3,080 - 4,500 g were used in the experiments. Three monkeys were immunized with a smallpox vaccine, LC16m8, and another 2 monkeys were mock-immunized. The 3 LC16m8-monkeys, Zr-LC-0, Zr-LC-3, and Zr-LC-7, were inoculated subcutaneously with the challenge virus (10^6 pfu, MPXV Zr-599) at either 0, 3, or 7 days post immunization, respectively. One of the mock-monkeys, Zr-Mock, was inoculated with MPXV Zr-599 and the other, Liberia-Mock, with MPXV Liberia at the same dose.

Before and after the MPXV challenge, 5 ml of total peripheral blood cells (PBC) was drawn and the surface of the throat of each monkey was swabbed with a cotton-tipped swab every 3 - 4 days. The swab was inoculated onto and mixed in 2 ml of MEM supplemented with 2%FBS (MEM-2FBS). The virus was isolated using Vero E6 cell-monolayers from buffy-coat fractions obtained from monkeys challenged with MPXV as reported previously (8). The throat swab (TS) specimens were centrifuged at 3,500 rpm for 5 min, and 1 ml of the supernatant fraction was inoculated onto Vero E6 cell mono-layers seeded in a 25-cm²-culture bottle for virus isolation.

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After incubation for 1 h, the cells were cultured in MEM-2FBS for 5 days. When a cytopathic effect (CPE) was observed in the cell culture, the CPE agent was confirmed as MPXV by indirect immunofluorescence assay. Plaque numbers were then counted after fixation of cells with a 10% formalin solution and staining with crystal violet solution.

The nucleotide sequence of the A-type inclusion body (ATI) gene of MPXV strains used in the present study was determined by the direct sequencing method. The partial ATI gene was amplified from the DNA of MPXV using primer set ATI-up-1 (5′-AATACAAGGAG GATCT-3′) and ATI-low-1 (5′-CTTAACTTTTTCTTTCTC-3′) (10). The PCR product was used as a template for direct sequencing.

DNA was isolated from 200 μl of PBC and TS specimens using a Viral Nucleic Acid purification kit™ (Roche Diagnostics, Mannheim, Germany). Purified DNAs were used as templates. The LightCycler real-time quantitative PCR (LC-qPCR) was developed using primers and probes that were designed based on the sequence of ATI gene in the MPXV genome (8). The sequences of the primers and probes were as follows: LC-forward primer, 5′-GAGATTAGCAGACT CCAA-3′; fluorescein (FC)-probe, 5′-GCAGTCGTTCAACT GTATTTCAAGATCTGAGAT-3′-Fluorescein; LCRed640 probe, 5′-LCRed640-CTAGATTGTAATCTCTGTAGCATT TCCACGGC-3′-phosphorylation; and reverse primers (Reverse primer 1: 5′-GATTCAATTTCCAGTTTGTAC-3′ and Reverse primer 2: 5′-TCTCTTTTCCATATCAGC-3′). The reverse primers, Reverse primer 1 and 2, were designed according to the specific nucleotide sequences in MPXV Congo Basin and West African strains, respectively. The LC-qPCR using the Reverse primer 1 and Reverse primer 2 were designated "LC-qPCR-C" and "LC-qPCR-W", respectively. Internal controls for the measurement of viral genome copy numbers of the MPXV Congo Basin and West African strains were pGEM-T-easy vectors (Promega, Madison, Wis., USA) carrying the ATI gene of MPXV Zr-599 and Liberia strains, respectively, and included in each assay. Amplification conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 57°C for 10 s, and 72°C for 6 s, and melting reaction.

PCR products, which were amplified from each of the MPXV strains by PCR using the primer set ATI-up-1 and ATI-low-1, were classified into 2 groups (1,520 bp and 1,070 bp, respectively, including the primer lesions) based on the size of the product (data not shown). An additional 453 nucleotide residues were present in the ATI gene of the Liberia, Copenhagen, SEN-79, Sierra Leone, Anteaten, and Orangutan MPXV strains at a point corresponding to position 2147/2148 of the nucleotide sequence of MPXV strain Gabon (one of the MPXV Congo Basin strains), counted from the initiation codon (GenBank accession no. MVU84504), resulting in a larger-size PCR product. On the other hand, Zr-599, Congo-8, and the 1996-97 isolates in DRC showed a smaller-size PCR product. These results indicate that the West African strains of MPXV have a longer ATI gene than the MPXV Congo Basin strains.

The LC-qPCR detected at least several copies of the MPXV ATI gene. Twenty-four PBC specimens and 24 TS specimens were serially collected from 4 monkeys on days 0-21 after infection with MPXV-Zr-599. Seven PBC specimens and 7 TS specimens were also serially collected from one monkey on days 0-21 after infection with MPXV-Liberia (Table 1). All the 18 virus-isolation-positive TS specimens showed a positive reaction in the LC-qPCR, while 12 of the 13 virus isolation-negative specimens showed a negative reaction.

Table 1. Relationship between the results of virus isolation and LC-qPCR among PBC- and TS-specimens

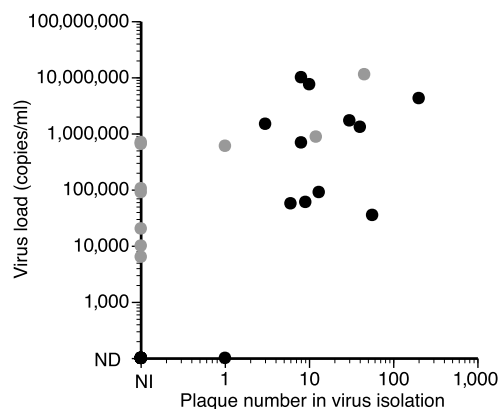|		LC-qPCR with					
		TS-specimens			PBC-specimens		
		+	−	Total	+	−	Total
Virus isolation	+	18	0	18	14	1	15
	−	1	12	13	7	9	16
Total		19	12	31	21	10	31

Fig. 1. Relationship between the virus load determined by the LC-qPCR and the plaque number in the virus isolation among PBC specimens. The specimens, which showed positive reactions in either virus-isolation or LC-qPCR, collected between day 0-10 and day 11-21 are shown in black circle and gray circle, respectively. The specimens, which showed positive reactions both in virus isolation and qLC-PCR are also shown in black circle. "ND" and "NI" indicate "undetectable" and "not isolated", respectively.

The sensitivity and specificity of the LC-qPCR were 100% (12/12) and 93% (12/13), respectively, in comparison with the virus isolation method. In contrast, 14 of the 15 virus isolation-positive PBC specimens showed a positive reaction in the LC-qPCR, while only 9 of the 16 virus isolation-negative specimens showed a negative reaction (Table 1). Most of the PBC specimens collected from symptomatic monkeys (Zr-Mock, Liberia-Mock, and Zr-LC-0) in the later stage of illness showed a negative reaction in virus-isolation negative but a positive reaction in the LC-qPCR (Fig. 1). IgG-response is usually demonstrated in monkeys subcutaneously infected with MPXV after approximately 10 days post inoculation (8). It is speculated that the discrepancy between the virus isolation and the LC-qPCR in PBC specimens in the later stage of illness is due to IgG-response. The antibody reactive to MPXV may make the virus isolation test negative, even though PMXV is represented in the PBC specimens.

The LC-qPCR-C detected only MPXV Congo Basin strains, whereas LC-qPCR-W detected only MPXV West African strains. The LC-qPCR detected MPXV DNA, but not the DNAs of camelpox virus, cowpox virus, ectromelia virus, vaccinia virus, or VZV, whereas the DNAs of these viruses were detected by conventional PCR methods using the primer set, ATI-up-1 and ATI-low-1, or the in-house primer set designed for the thymidine kinase gene of VZV (data not shown). There is a unique specific nucleotide sequence with a unique 8-nucleotide residue deletion only in the ATI gene of MPXV (11). An LCRed probe was designed to anneal this MPXV-specific nucleotide sequence. The specific reaction to MPXV in the LC-qPCR is thought to be due to the specific nucleotide sequences (11). The DNA of variola virus, the

causative agent for smallpox, expected to show a negative reaction in the LC-qPCR, as variola virus does not contain the specific 8-nucleotide residue deletion. The fact that the homology of the target region in MPXV with the corresponding region in the variola virus is lower than those with the corresponding regions in the orthopoxviruses tested supports this assumption.

We succeeded in producing various levels of clinical symptoms in 4 MPXV-infected monkeys. The naïve-monkey (Zr-Mock) died, while the Zr-LC-0 and Zr-LC3 monkeys survived. The ZR-LC-7 monkey did not show any symptoms. The viremia level determined by LC-qPCR in the Zr-Mock PBC continued to increase during the course of illness, while viremia was not demonstrated in the asymptomatic monkey, Zr-LC-7, by the assay. LC-qPCR is considered to be efficacious not only in diagnosis of MPXV infections but also in the assessment of the severity of MPXV infection-associated symptoms and outcome.

Recently, there have been several reports that describe real-time qPCR assays for the detection of MPXV genomes (12-16). These real-time qPCR assays, including that described herein, are sensitive, rapid, and useful in the diagnosis of MPXV infections. The significant advantage of this newly developed assay, LC-qPCR, over previously reported methods is that it enables the differentiation of MPXV into West African and Congo Basin strains.

In summary, we developed a sensitive, specific, and rapid LC-qPCR system for detection of the MPXV genome targeting specific nucleotide sequences in the ATI gene. This technology should offer great benefits in the control of outbreaks of MPXV infections and in the assessment of the course of MPXV infections.

ACKNOWLEDGMENTS

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