

Monkeypox virus detection in rodents using real-time 3'-minor groove binder TaqMan[®] assays on the Roche LightCycler

David A Kulesh¹, Bonnie M Loveless¹, David Norwood¹, Jeffrey Garrison², Chris A Whitehouse³, Chris Hartmann³, Eric Mucker³, David Miller³, Leonard P Wasieloski Jr¹, John Huggins³, Gregory Huhn⁴, Lori L Miser⁵, Carroll Imig⁵, Mark Martinez⁶, Tom Larsen⁶, Cynthia A Rossi¹ and George V Ludwig¹

¹Diagnostic Systems Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA; ²Battelle, Columbus, OH, USA; ³Virology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA; ⁴Illinois Department of Public Health; ⁵Illinois Department of Agriculture, Springfield, IL, USA and ⁶Pathology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA

During the summer of 2003, an outbreak of human monkeypox occurred in the Midwest region of the United States. In all, 52 rodents suspected of being infected with monkeypox virus were collected from an exotic pet dealer and from private homes. The rodents were euthanized and submitted for testing to the United States Army Medical Research Institute of Infectious Diseases by the Galesburg Animal Disease Laboratory, Illinois Department of Agriculture. The rodent tissue samples were appropriately processed and then tested by using an integrated approach involving real-time polymerase chain reaction (PCR) assays, an antigen-detection immunoassay, and virus culture. We designed and extensively tested two specific real-time PCR assays for rapidly detecting monkeypox virus DNA using the *Vaccinia* virus *F3L* and *N3R* genes as targets. The assays were validated against panels of orthopox viral and miscellaneous bacterial DNAs. A *pan*-orthopox electrochemiluminescence (ECL) assay was used to further confirm the presence of *Orthopoxvirus* infection of the rodents. Seven of 12 (58%) animals (seven of 52 (15%) of all animals) tested positive in both monkeypox-specific PCR assays and two additional *pan*-orthopox PCR assays (in at least one tissue). The ECL results showed varying degrees of agreement with PCR. One hamster and three gerbils were positive by both PCR and ECL for all tissues tested. In addition, we attempted to verify the presence of monkeypox virus by culture on multiple cell lines, by immunohistology, and by electron microscopy, with negative results. Sequencing the PCR products from the samples indicated 100% identity with monkeypox virus strain Zaire-96-I-16 (a human isolate from the Congo). These real-time PCR and ECL assays represent a significant addition to the battery of tests for the detection of various orthopoxviruses. In light of the recent monkeypox virus transmissions, early detection of the virus is crucial for both natural outbreaks and potential acts of bioterrorism.

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Monkeypox virus (MPXV) is a zoonotic virus in the family *Poxviridae*, genus *Orthopoxvirus*. It was first isolated from lesions seen among captive monkeys

in Copenhagen, Denmark.¹ Human monkeypox was subsequently identified in 1970 in the Democratic Republic of the Congo (DRC).² The virus causes a disease in humans that is similar to smallpox, but results in a lower case-fatality rate. The disease is endemic in the rainforests of central and western Africa, where squirrels and monkeys have been suggested to play a role in the life cycle of the virus.^{3–5}

With the eradication of smallpox and widespread discontinuation of smallpox vaccination, human monkeypox has re-emerged as a human health threat with major outbreaks occurring in 1996–1997 and 2001 in the DRC.^{6,7} More recently, MPXV was found

Correspondence: Dr DA Kulesh, PhD, LtCol, USAF, BSc, Diagnostic Systems Division, USAMRIID, 1425 Porter St., Fort Detrick, MD 21702-5011, USA.

E-mail: David.Kulesh@amedd.army.mil

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14. ABSTRACT During the summer of 2003, an outbreak of human monkeypox occurred in the Midwest region of the United States. In all, 52 rodents suspected of being infected with monkeypox virus were collected from an exotic pet dealer and from private homes. The rodents were euthanized and submitted for testing to the United States Army Medical Research Institute of Infectious Diseases by the Galesburg Animal Disease Laboratory, Illinois Department of Agriculture. The rodent tissue samples were appropriately processed and then tested by using an integrated approach involving real-time polymerase chain reaction (PCR) assays, an antigen-detection immunoassay, and virus culture. We designed and extensively tested two specific real-time PCR assays for rapidly detecting monkeypox virus DNA using the Vaccinia virus F3L and N3R genes as targets. The assays were validated against panels of orthopox viral and miscellaneous bacterial DNAs. A pan-orthopox electrochemiluminescence (ECL) assay was used to further confirm the presence of Orthopoxvirus infection of the rodents. Seven of 12 (58%) animals (seven of 52 (15%) of all animals) tested positive in both monkeypox-specific PCR assays and two additional pan-orthopox PCR assays (in at least one tissue). The ECL results showed varying degrees of agreement with PCR. One hamster and three gerbils were positive by both PCR and ECL for all tissues tested. In addition, we attempted to verify the presence of monkeypox virus by culture on multiple cell lines, by immunohistology, and by electron microscopy, with negative results. Sequencing the PCR products from the samples indicated 100% identity with monkeypox virus strain Zaire-96-I-16 (a human isolate from the Congo). These real-time PCR and ECL assays represent a significant addition to the battery of tests for the detection of various orthopoxviruses. In light of the recent monkeypox virus transmissions, early detection of the virus is crucial for both natural outbreaks and potential acts of bioterrorism.		

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to be the cause of a cluster of cases of disease in the Midwest region of the US.^{8–10} Epidemiologic investigations confirm that the human cases of monkeypox resulted from contact with infected prairie dogs that had been housed or transported with African rodents imported from Ghana.¹¹ This represents the first time that a monkeypox outbreak has occurred in the Western Hemisphere and highlights the need for rapid and accurate diagnostics to detect these emerging viral pathogens.

Several molecular-based techniques have been developed to detect and discriminate orthopoxviruses;^{12–15} however, they require conventional polymerase chain reaction (PCR) followed by restriction endonuclease digestion and subsequent gel electrophoresis. Recently, rapid real-time PCR methods were developed for detecting *Orthopoxvirus* DNA.^{16–18} The goal of this study was to develop real-time LightCycler PCR assays based on the conserved *F3L* and *N3R* genes for specifically detecting MPXV and validating these assays by using a panel of DNAs from orthopox viral isolates that vary both temporally and geographically and tissues from various MPXV-infected rodents from the recent US outbreak.⁸ Two previously developed *pan*-orthopox real-time PCR assays based on the hemagglutinin (*HA*) and *E9L* genes¹⁹ and a *pan*-orthopox electrochemiluminescence (ECL) immunoassay were also used to evaluate potentially monkeypox-infected tissues.

Materials and methods

PCR Primers, Target Sequences, and Fluorogenic Probe

The real-time PCR assay primers and TaqMan[®]-MGB probe sequences are listed in Table 1. The *Vaccinia F3L* gene (also called the *D14L* gene) (GenBank Accession #'s: AF380138 for MPXV-ZRE (bp 48 048–48 509); M35027 for VAC-COP (bp 50 911–51 483); Y16780 for VMN-GAR (bp 39 619–40 197); L22579 for VAR-BSH (bp 39 258–39 836); AF482758 for CPXV-BR (bp 64 465–65 037); NC_001611 for VAR-IND (bp 38 632–39 204); AF095689 for VAC-TAN (bp 47 348–47 921); AF438165 for CMPV-M96 (bp 50 654–51 226)) and

the *N3R* gene (GenBank Accession #'s: AF380138 for MPXV-ZRE (bp 190 003–190 733); X94355 for CPXV-BR (bp 19 910–20 625 reverse)) sequences were selected as potential monkeypox-specific targets. All sequence alignments were carried out using the EMBL-EBI ClustalW (1.82) Multiple Sequence Alignment Tool (<http://www.ebi.ac.uk/clustalw/index.html>). Regions of nonhomology were used as target sequences for each potential MPXV-specific assay. The specific primer and TaqMan[®]-MGB probe sequences were designed using *Primer Express* Version 2.0 for Windows (Applied Biosystems). All primers were synthesized using standard phosphoramidite chemistry with an ABI 394 DNA/RNA synthesizer. The TaqMan[®]-MGB probes were also synthesized by ABI and contained 6-carboxyfluorescein (6FAM) at the 5' end. An NFQ and the MGB were added to the 3' end.

5' Nuclease PCR (TaqMan[®]-MGB) Assays

Using the *Primer Express* 2.0. software to design potential MPXV-specific TaqMan[®]-MGB assays (*F3L*-MGB and *N3R*-MGB), we optimized each assay according to a standard protocol instituted by the Diagnostic Systems Division at US Army Medical Research Institute of Infectious Diseases (USAMRIID). Briefly, potential primer pairs were initially tested in the LightCycler by the fluorescent dye SYBR Green I (Roche Biochemicals). The optimum primer pair was selected based on specificity (a single, appropriately sized amplicon) and efficiency of amplification (lowest *C_t* value which is defined as the real-time PCR cycle at which the LightCycler software determines the reaction to be positive). The selected primer pair was then optimized to the final concentration (0.1–1.0 μ M) with the lowest *C_t* value and the highest fluorescent signal. Next, several potential TaqMan[®]-MGB probes were tested with the optimized primer pair by varying the probe and MgCl₂ concentrations. The final assay consisted of the primer/probe pair concentrations and reaction conditions that combined the lowest level of detection (LOD—the gene copy number that was detected by the assay at least 58/60 times), lowest *C_t* value, and highest fluorescent signal-to-noise ratio. The

Table 1 Primer/probe sequence of the monkeypox-specific assays

Organism	Gene target	Amplicon size (bp)	Primers/probe	Sequence	Final conc (μ M)	MgCl ₂ (mM)
Monkeypox	<i>F3L</i>	107	F3L-F290	5'-CTC ATT GAT TTT TCG CGG GAT A-3'	0.5	5
			F3L-R396	5'-GAC GAT ACT CCT CCT CGT TGG T-3'	0.5	
			F3Lp333S-MGB	5'-6FAM-CAT CAG AAT CTG TAG GCC GT-MGBNFQ-3'	0.05	
Monkeypox	<i>N3R</i>	139	N3R-F319	5'-AAC AAC CGT CCT ACA ATT AAA CAA CA-3'	0.5	5
			N3R-R457	5'-CGC TAT CGA ACC ATT TTT GTA GTC T-3'	0.5	
			N3Rp352S-MGB	5'-6FAM-TAT AAC GGC GAA GAA TAT ACT-MGBNFQ-3'	0.05	

LODs of the assays were determined from serial dilutions of genomic DNA purified from monkeypox virions (Zaire 79-I-05).

All assays were carried out in 20 µl volumes for the LightCycler with each reaction made up in PCR buffer (50 mM Tris, pH 8.3); 25 µg/ml of bovine serum albumin (BSA) and 0.2 mM dinucleotide triphosphate (dNTP) mix (Idaho Technology, Salt Lake City, UT, USA). Eight-tenths (0.8) unit of Platinum *Taq* DNA polymerase (Invitrogen) was added to each reaction. The final MgCl₂, primer and probe concentrations for each assay are listed in Table 1. Thermal cycling for the LightCycler was performed as follows: one cycle at 95°C for 2 min, followed by 45 cycles of 95°C for 1 s and 60°C for 20 s. A fluorescence reading was taken at the end of each 60°C step. Each reaction capillary tube was read in Channel 1 (F1) at a gain setting of 16 with data being analyzed by the LightCycler Data Analysis software (version 3.5.3). Sample curves were analyzed using the 'second derivative maximum' with the baseline adjustment set to *Arithmetic*.

Extended Assay Evaluation

Both assays were extensively evaluated, first against various genomic orthopox and nonorthopox virus nearest-neighbor DNAs (Table 2). The panel consisted of 17 monkeypox viral isolate DNAs (10–100 pg/µl), 25 *Variola* isolate DNAs (100 pg/µl), and DNAs from *Vaccinia*, camelpox, cowpox, *Herpes simplex*, *Varicella zoster* viruses, and human genomic DNA. Utrecht is a nonhuman primate monkeypox isolate. The other MPXV DNAs are human monkeypox isolates from Zaire/DRC; MPX-Sierra Leone is an isolate from 1970, 12003KI is an isolate from 1999 (DRC), and V96-I-16 is a strain of MPX-monkeypox Zaire. Within the MPXV panel are 11 monkeypox-human isolates obtained from clinical samples during the 1996 outbreak in the Congo (unpublished data). All testing with *Variola* virus genomic DNA was conducted at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA. Both assays were also tested against various bacterial isolates and strains from the USAMRIID bacterial DNA cross-reactivity panel (100 pg of each DNA) (data not shown). In all, 5 µl of each sample was added to the appropriate MPXV assay LightCycler Master Mix (15 µl) and cycled as described above. All test runs included at least one positive control that contained 2.3×10^4 copies (5 pg total) of purified monkeypox genomic DNA (Zaire 79-I-05) and two no-template controls (NTC): reagent NTC and sample NTC.

Study Animals

All animals used in this study were collected from either an exotic pet dealer or private homes in the Chicago, Illinois metropolitan area. The animals

Table 2 USAMRIID/CDC DNA panel

DNA species (Genomic)	Concentration (pg/µl)	F3L-MGB	N3R-MGB
Human genomic DNA	1000	—	—
MPV Zaire 79-I-05	10	+	+
MPXR Zaire 79-I-05 (Cidofovir-resistant)	10	+	+
MPV Utrecht	10	+	+
MPV Sierra Leone	10	+	+
MPV 12003k	10	+	+
MPV DCR (Zaire) 96-I-16	10	+	+
MPX V97-I-04	100	+	+
MPX V97-I-16	100	+	+
MPX V97-I-03	100	+	+
MPX V98-I-0035	100	+	+
MPX V97-I-07	100	+	+
MPX V97-I-05	100	+	+
MPX V97-I-12	100	+	+
MPX V97-I-17	100	+	+
MPX V97-I-11	100	+	+
MPX V97-I-06	100	+	+
MPX V97-I-08	100	+	+
<i>Variola</i>			
Horn	100	—	—
V77-I-1605	100	—	—
V70-I-46	100	—	—
Afgan Variolator 4	100	—	—
Nepal V73-I-175	100	—	—
India 7124	100	—	—
Bangladesh 1975 (V75-I-550)	100	—	—
Kali Muthu 50	100	—	—
Iran 2602	100	—	—
K1629	100	—	—
Higgins	100	—	—
Hinden	100	—	—
Juba	100	—	—
Lee	100	—	—
Rafiq-Lahore	100	—	—
Harper	100	—	—
ETH-17R14-X-72	100	—	—
102	100	—	—
Yamada	100	—	—
V70-I-222	100	—	—
Butler	100	—	—
V72-I-119	100	—	—
V72-I-143	100	—	—
V73-I-225	100	—	—
V77-I-1252	100	—	—
IHD— <i>Vaccinia</i> strain IHD	100	—	—
MVA—modified <i>Vaccinia</i> Ankara	100	—	—
MDV—Maerek's Disease Vaccine virus	100	—	—
VAC WR— <i>Vaccinia</i> Western Reserve	100	—	—
CML—Camelpox Somalia	100	—	—
CMLR—Cidofovir-resistant Camelpox Somalia	100	—	—
CPX—cowpox Brighton Red (BR)	100	—	—
CPXR—Cidofovir-resistant cowpox Brighton Red	100	—	—
VAC— <i>Vaccinia</i> Copenhagen	100	—	—
VACR—Cidofovir-resistant <i>Vaccinia</i> Copenhagen	100	—	—
HSV-1—Herpes Simplex virus type 1	100	—	—
HSV-2—Herpes Simplex virus type 2	100	—	—
<i>Varicella</i> OKA (purified virus)	4000	—	—
<i>Varicella</i> WEB (purified virus)	18 000	—	—
<i>Varicella</i> V2V 123j (purified virus)	48 000	—	—

Table 3 Animal real-time PCR

<i>Animal</i>	<i>Total # of animals</i>	<i>panHA-MGB</i>	<i>panE9L-MGB</i>	<i>MPXV F3L-MGB</i>	<i>MPXV N3R-MGB</i>	<i>Total^a</i>
Prairie dog	8 ^b	3/8	2/8	3/8	3/8	3/8
Rat	2	0/2	0/2	0/2	0/2	0/2
Mole rat	1	0/1	0/1	0/1	0/1	0/1
Jerboas	2	0/2	0/2	0/2	0/2	0/2
Hamster	12	2/12	0/12	1/12	1/12	2/12
Gerbil	13	4/13	5/13	6/13	5/13	6/13
Dwarf Hamster	12	0/12	0/12	0/12	0/12	0/12
Chinchilla	2	1/2	1/2	1/2	1/2	1/2
	52					12/52

^aTotal positive animals for at least one *pan*-orthopox/MPXV PCR assay in one tissue = 12/52 (23% positive animals for orthopox).

^bPrairie dog #5 was *pan*-OP (PCR) and MPXV positive for 8/8 tissues (although liver was only positive for 2/4 assays: *panE9L*-MGB and MPXV-*F3L*-MGB; all other tissues were positive for 4/4 assays).

were voluntarily released to public health and veterinary health officials as a result of either cohabitation of these animals with known positive animals or those that exhibited signs of orthopox infection. All animals were euthanized and frozen for transport to USAMRIID. Animal species tested during the course of this study included: prairie dog (*Cynomys* sp.), rat (*Rattus* sp.), hamster (*Cricetus* spp.), dwarf hamster (*Allocricetulus* sp.), gerbil (*Gerbillus* sp.), Jerboa (*Jaculus* sp.), mole rat (*Heterocephalus* sp.), and chinchilla (*Chinchilla* sp.).

Postmortem Examination and Tissue Collection

The 52 frozen rodent carcasses (see Table 3) collected in Illinois that were linked to possible MPXV transmission were thawed overnight in a Class II laminar hood. Complete necropsies were performed on each animal by or under the direct supervision of a board-certified veterinary pathologist. Tissue samples including brain, lung, liver, spleen, kidney, and ovary/testis of each rodent, and dermal lesions with the draining lymph nodes of affected rodents were harvested for PCR, immunoassay, and viral isolation. However, not all tissues were taken from each animal.

Tissue Preparation and DNA Extraction

A 10% homogenate of each tissue specimen was made in Dulbecco's high-glucose medium using a PCR tissue homogenizer (Omni International, Inc., Warrenton, VA, USA). The homogenate was centrifuged for 10 min at 2000 rpm at 4°C. DNA was extracted from 100 µl of the supernatant using the QIAamp DNA Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions with the following minor modification: during the initial lysis step, the incubation time was increased to overnight at 56°C to ensure complete lysis of any virus present in the sample. The remaining super-

natant was stored at -70°C for immunoassay and virus isolation.

Inhibition Testing of Animal DNA

Inhibition testing of DNA isolated from animals was performed incorporating an internal positive control (IPC) testing system developed at USAMRIID (manuscript submitted). Briefly, both forward and reverse primer sites and a TaqMan[®] probe site of the *Bacillus anthracis* protective antigen gene was mutated to create a novel sequence with no known homology to published sequences with a resulting amplicon of 153 bp. The IPC was shown to be sensitive to a variety of inhibitors including hemoglobin, heparin, ethylenediamine tetraacetic acid (EDTA), humic acids, and fulvic acid. All extracted animal samples were tested for inhibition by adding either 5 µl of the sample to 15 µl of the same IPC LightCycler Master Mix and compared to an IPC control (5 µl of H₂O instead of sample). Cycling conditions on the LightCycler were as described above. All sample curves were analyzed using the LightCycler Data Analysis software (LCDA version 3.5.3) with 'second derivative maximum' and baseline adjustment set to arithmetic.

Testing for inhibition with the IPC assay resulted in three possible outcomes: (a) complete inhibition; (b) partial inhibition (the cycle threshold (*C_t*) was delayed and/or end point fluorescence (EPF) was reduced); or (c) no inhibition (*C_t* and EPF were comparable to the H₂O blank). A *C_t* shift of three cycles was quantitatively equivalent to an approximately one log decrease in copy number (detection limit). Therefore, a *C_t* shift of greater than three cycles beyond the water blank was considered inhibitory. Inhibition was indicated when a sample had a *C_t* shift of greater than three cycles OR a reduction in EPF of greater than or equal to 50% when compared to water controls. If inhibition was observed in a sample, two-fold serial dilutions were performed until inhibition was completely elimi-

nated. C_t shifts less than three cycles or less than 50% loss of EPF were not considered significant, and dilution of these samples would unnecessarily dilute out the target DNA.

Assaying of animal tissues

Real-Time PCR (*panHA*-MGB, *panE9L*-MGB, *F3L*-MGB, and *N3R*-MGB)

Each animal tissue-extracted DNA (appropriately diluted based on the IPC assay results) was tested with four real-time PCR assays. The first two assays, *panHA*-MGB and *panE9L*-MGB, were described previously¹⁹ and detect the presence of orthopoxvirus DNAs. Each tissue DNA was then tested in singlet with the MPXV-specific assays, *F3L*-MGB and *N3R*-MGB. All resulting positive samples were then retested in triplicate. A 3/4 or 4/4 positive result on any given assay indicated that the sample was positive for either/both *Orthopoxvirus* DNA and/or MPXV DNA. If a sample was only 1/4 positive, the sample was called negative. If the sample was 2/4 positive, the sample was re-extracted and retested by both the *F3L*-MGB and *N3R*-MGB PCR assays. If any sample remained only '50% positive', the sample result would have been labeled 'indeterminate.'

pan-Orthopox ECL

A mixture of four monoclonal antibodies (Mab) was used as capture antibodies. An additional two Mabs were added as detector antibodies (total of six Mabs). All antibodies were originally developed against *Vaccinia* virus and were selected for this assay based on their ability to recognize four different *Vaccinia* proteins and their cross-reactivity with multiple members of the *Orthopoxvirus* genus including *Vaccinia*, monkeypox, cowpox, camel pox, *Variola major*, and *Variola minor*. These antibodies were produced in bioreactors, purified using protein G chromatography medium, and labeled with biotin or ruthenium (II) tris-bipyridal chelate (Ru) using standard coupling methods.²⁰ Biotinylated antibodies were prebound to streptavidin-coated 2.8 μ m diameter paramagnetic beads. Concentrations of labeled antibodies were then optimized as previously described.²⁰ Finally, the optimized capture antibody-bound beads and Ru-labeled detector antibodies were mixed and lyophilized into single-use assay tubes.

The ECL *Orthopoxvirus*-genus detection assay was conducted as follows: 50 μ l of an unknown sample (liquid) or controls were added in duplicate to tubes containing the lyophilized capture and detector antibodies. The tubes were then incubated for 15 min on the vortexing Origen[®] Analyzer 1.5 carousel (Igen International Inc., Gaithersburg, MD, USA) after which 300 μ l of 10 mM phosphate-

buffered saline with 0.3% Tween-20 (PBS-T) was added to each tube. The analyzer then drew the processed sample from the carousel to a flow cell where it captured and washed the magnetic beads and measured the ECL signal. Tissue preparations from uninfected mice and Dulbecco's high glucose medium containing 1% fetal calf serum served as matrix controls and were used to determine assay cutoffs. The data were analyzed using custom prepared Microsoft[®] Excel 2000 spreadsheets. Samples were considered positive if the ECL signal was greater than the average plus three times the standard deviation or 1.2 times the average ECL signal of the negative matrix controls, whichever was higher. Differences in ECL signals of the various machines was negated by determining the *S/N* ratio of each sample, calculated by dividing the sample ECL value by the average ECL value of the negative matrix controls. Positive controls near the sensitivity level of the assay were included on every run. The assay could detect as little as 5×10^5 plaque-forming units (PFU)/ml or 2.5×10^4 PFU per assay of *Vaccinia* virus and was quantitative within a dynamic range of at least 2.5 logs of virus. The size of the dynamic range and sensitivity level varied slightly depending on the *Orthopoxvirus* strain tested. The assay was not used in a quantitative manner for this work. The total assay time is 15 min plus up to 1 min per sample reading time.

Results

Development of Assays

The final primer/TaqMan[®]-MGB probe assay sequences and reaction conditions for each MPXV-specific assay are shown in Table 1. The *pan*-orthopox assays (*panHA*-MGB and *panE9L*-MGB) were previously published.¹⁹ The MPXV assays reproducibly detected 11–55 fg of monkeypox genomic DNA, which represented approximately 50–250 copies of each gene. Results from the genomic DNA LOD experiments also correlated linearly with a dynamic range of six orders of magnitude representing approximately 25 to 2 500 000 copies (data not shown).

USAMRIID/CDC DNA Panel Evaluation

Both MPXV-specific assays were tested against two DNA panels: (1) a USAMRIID/CDC orthopox DNA panel (Table 2) and the USAMRIID DNA cross-reactivity panel (99 DNAs—data not shown). The results in Table 2 indicate that both the *F3L*-MGB and *N3R*-MGB assays were capable of detecting all of the MPXV genomic DNA species available in the orthopox DNA panel. Non-MPXV DNAs (to include human DNA, 25 strains of *Variola* virus DNA, five strains of *Vaccinia*, camelpox, cowpox, fowlpox viruses, two strains of *Herpes simplex* virus, and

three strains of *Varicella* virus) were not detected by either assay. There were no false positives among the non-MPXV DNA samples for either of the assays on the LightCycler. Data from the USAMRIID bacterial cross-reactivity panel demonstrated that both assays were also negative (not detected) for 99 different isolates and strains of bacterial DNA.

Animal Results

A total of 52 animals were screened for the presence of orthopox DNA using the two previously tested orthopox spp-MGB assays: *panHA*-MGB and *panE9L*-MGB.¹⁹ The sensitivities of the *panHA*-MGB and *panE9L*-MGB were retested with monkeypox DNA. The LODs for both assays was 2.5 fg (12 copies). The animals were also tested for the presence of monkeypox DNA using the *F3L*-MGB and *N3R*-MGB assays. Results (Table 3) indicate that a total of 12 out of the 52 animals had at least one tissue PCR positive for MPXV DNA.

Table 4 shows the results of testing 318 total tissue samples from the 52 animals. Note that some tissues (particularly from the prairie dogs) exhibited some level of PCR inhibition. The inhibition was overcome by diluting the sample 1:2, 1:4, or 1:8 with the dilution selected, which restored the C_t values when compared to the noninhibited control. The total positives for one or more PCR assays were 25 tissues from 12 different animals (Table 5). The ECL data are also presented in Table 5. In all, 12.6% of all tissues exhibited some level of PCR inhibition. However, the vast majority (75%) of PCR-inhibited tissue samples were from prairie dogs. We attribute this fact to the possible way the prairie dogs were handled both before and during shipment to USAMRIID. Also noted during virus culture was the fact that the prairie dog tissues were highly

contaminated with bacteria that may have contributed to the PCR inhibition. Table 5 shows the breakdown of each positive animal, which tissue was positive, and the results of the individual PCR and ECL tests. All other tissue samples (a total of 292 with the exception of the spleen of gerbil #32, which was PCR-negative but ECL-positive) were negative for all four PCR tests. An additional 17 tissues from randomly chosen PCR-negative animals were also negative for ECL (data not shown). Animals testing positive for all four PCR assays (in at least one tissue) were 7/12 (58%) of positive animals or 7/52 (15% of all animals tested). Animals testing positive for at least three PCR assays (in at least one tissue) were 10/12 (83%) of positive animals or 10/52 (19% of all animals tested). Animals testing positive for at least two PCR assays (in at least one tissue) were 10/12 (83%) of positive animals or 10/52 (19% of all animals tested). Animals testing positive for only one PCR assay (in at least one tissue) were 2/12 (17%) of positive animals or 2/52 (3.8% of all animals tested). Interestingly, there was only one case where a tissue (liver of hamster #21) was positive for *panHA*-MGB but not positive in either MPXV assay. This tissue was also ECL positive. We, therefore conclude that all virus detected by PCR was MPXV and that the liver tissue from hamster #21 contained MPXV virus DNA very near the detection limit of our four PCR assays. It is also possible that the tissue may have been infected by some other orthopox virus (ie, ectromelia).

All eight tested tissues from prairie dog #8 tested positive by PCR. Three of these tissues were also ECL positive. This animal was therefore chosen for culture attempts of the virus (specifically the skin and lung tissue) because they had the lowest PCR C_t values indicating that they potentially contained the highest concentration of viable virus.

Table 4 Animal tissue real-time PCR assays

Tissue	Total # of tissues ^a	# Tissues w/inhibition ^b	rtPCR				Total
			<i>panHA</i> -MGB	<i>panE9L</i> -MGB	MPXV <i>F3L</i> -MGB	MPXV <i>N3R</i> -MGB	
Brain	52	0	4/52	3/52	3/52	4/52	4/52
Kidney	52	11	3/52	2/52	3/52	3/52	3/52
Liver	51	7	2/51	3/51	3/51	2/51	4/52 ^c
Lung	51	4	2/51	2/51	4/51	2/51	4/51
Lymph node	10	2	2/10	2/10	2/10	2/10	2/10
Skin	3	0	2/3	1/3	2/3	2/3	2/3
Spleen	49	9	3/49	3/49	3/49	3/49	3/49
Testis/ovary	49	6	3/49	3/49	3/49	2/49	3/49
Scab ^d	1	1	0/1	0/1	0/1	0/1	0/1
Subtotal	318	40	21/318	19/318	23/318	20/318	25

^aNot all tissues were taken from all animals.

^bIn all, 12.6% of all tissue exhibited some level of inhibition; 75% (20/30) of tissues showing a level of inhibition were from prairie dogs.

^cOne liver sample was only positive for *panHA*-MGB.

^dScab was from prairie dog #6 (Department of Agriculture #5587).

Table 5 Positive animal tissue assays

Animal #	Animal type	Tissue type	Inhibition assay	Dilution	panHA-MGB	panE9L-MGB	MPXV F3L-MGB	MPXV N3R-MGB	pan-OP ECL
5	Prairie dog	Brain	No	0	+	+	+	+	+
5	Prairie dog	Kidney	Yes	1:2	+	+	+	+	+
5	Prairie dog	Liver	Yes	1:2	—	+	+	—	+
5	Prairie dog	Lung	No	0	+	+	+	+	—
5	Prairie dog	Lymph node	No	0	+	+	+	+	—
5	Prairie dog	Skin	No	0	+	+	+	+	—
5	Prairie dog	Spleen	Yes	1:8	+	+	+	+	—
5	Prairie dog	Testis/ovary	No	0	+	+	+	+	—
7	Prairie dog	Lung	No	0	+	+	+	+	—
7	Prairie dog	Lymph node	No	0	+	+	+	+	—
2	Prairie dog	Skin	No	0	+	—	+	+	—
19	Hamster	Brain	No	0	+	—	—	+	+
19	Hamster	Kidney	No	0	+	—	+	+	+
21	Hamster	Liver	No	0	+	—	—	—	+
24	Gerbil	Brain	No	0	+	+	+	+	+
24	Gerbil	Liver	No	0	+	+	+	+	+
25	Gerbil	Brain	No	0	+	+	+	+	—
27	Gerbil	Lung	No	0	—	—	+	—	—
28	Gerbil	Liver	No	0	—	+	+	+	+
30	Gerbil	Spleen	No	0	+	+	+	+	+
49	Gerbil	Kidney	No	0	+	+	+	+	+
49	Gerbil	Spleen	No	0	+	+	+	+	+
49	Gerbil	Testis/ovary	No	0	+	+	+	—	—
45	Chinchilla	Lung	No	0	—	—	+	—	—
45	Chinchilla	Testis/ovary	Yes	1:2	+	+	+	+	—
32	Gerbil	Spleen	No	0	—	—	—	—	+

DNA Sequencing

The 139 and 107-bp amplicons produced from the MPXV-specific *N3R* and *F3L* assays, respectively, were sequenced and compared to related orthopoxviruses in GenBank. When aligned to other MPXV sequences, using BLASTN, both amplicons exhibited 100% identity to the Zaire-96-I-16 strain of MPXV (data not shown).

Discussion

In the United States, smallpox vaccinations were discontinued after the eradication of the disease in 1979. However, recent world events have prompted the US military to begin vaccinating troops against smallpox. The anthrax attacks during the fall of 2001 demonstrate that there is a high potential for additional bioterrorism attacks. It is conceivable that an individual or possibly a rogue government could use their technical expertise to mass-produce an infectious *Orthopoxvirus* such as smallpox or MPXVs (or a genetically engineered variant of either). It is, therefore, crucial that fast, reliable yet simple molecular diagnostic tests be developed with the latest detection and identification technology available. We present the development and extended evaluation of two real-time PCR assays to confirm the presence of monkeypox DNA (MPXV-specific assays) on the LightCycler. Each assay is an entirely new assay (*F3L*-MGB or *N3R*-MGB) incorporating MPXV-specific target sequences. The pri-

mers were specifically designed to generate amplicons less than 150 bp because these assays were to be used in a rapid cycling machine (one cycle/15–20 s). We chose primer/TaqMan[®]-MGB probe pairs that exhibited the maximum efficiency of amplicon synthesis, the lowest *C_i* value, and the maximum LOD. We opted to use TaqMan[®]-MGB probe technology because it possesses significantly improved hybridization properties.²¹ TaqMan[®]-MGB probes are more stable, display increased mismatch discrimination, and have an improved *S/N* ratio due to the use of an NFQ instead of the fluorescent quencher dye TAMRA.²² In addition, the MGB stabilizes A/T rich duplexes, resulting in increased probe *T_m* (that temperature at which 50% of an oligonucleotide is annealed to its complement strand). Therefore, the MGB probes simplified assay design for the MPXVs, which have a high A/T ratio (~66%).²³

In this study, the LOD for each TaqMan[®]-MGB assay was evaluated on the LightCycler using purified monkeypox (Zaire 79-I-05) virus genomic DNA. The LOD with genomic DNA was 11–55 fg (50–250 copies). Both assays were also highly quantitative over a range of 1 ng (5×10^6 copies) to 11 fg (50 copies) when tested in the LightCycler. Initial testing against genomic DNAs available at USAMRIID and from the CDC increased our confidence in the overall specificity of each assay. While each assay detected only the appropriate DNA samples in the USAMRIID/CDC orthopox DNA panel, none of the assays detected any DNAs in the

cross-reactivity panel. These data showed that both MPXV-specific TaqMan[®]-MGB assays were highly specific and exceptionally sensitive.

The MPXV-infected animal results presented in this study confirm that both previously reported *pan*-orthopox assays¹⁹ along with the two new MPXV-specific assays (*F3L*-MGB and *N3R*-MGB) achieve the same level of sensitivity and specificity with clinical (animal) samples as they do with pure cultures. Of the 52 animals ultimately tested, 12 were shown to have at least one tissue positive for MPXV DNA with one animal positive for all eight of its tissues tested. ECL testing of this same animal's tissues showed that only three of the PCR-positive tissues were ECL-positive. This result is not surprising for two reasons: ECL for orthopox viruses has been shown to be less sensitive than PCR (ECL limit of detection for the orthopox viruses is approximately 5×10^5 PFU/ml or 2.5×10^4 PFU/assay); secondly, we were not able to detect monkeypox virions by electron microscopy and no viable virus was isolated from the PCR-positive skin and lung tissue of prairie dog #5. These observations suggest that viral loads were either present at concentrations below the LOD of these techniques or samples were degraded such that antigen was not available in a form that could be recognized by the antibodies used in the ECL assay. Spleen from one animal was ECL-positive but PCR-negative. This result is at present not understood but could represent a false-positive ECL result or a false-negative PCR result. Alternatively, it is possible that viral antigen could have been present in the absence of agent-specific nucleic acid.

Sequencing of the PCR products obtained from the MPXV-infected tissues revealed a 100% identity with the Zaire 96-I-16 strain of MPXV. This strain was originally isolated from an infected human during the 1996 outbreak in the Democratic Republic of Congo (formerly Zaire).^{24,25} It is known that two clades of MPXV exist in Africa, those isolated from Zaire and those from West Africa (H Meyer, personal communication). As the source of this outbreak was from rodents collected from Ghana in West Africa, we presumed that they carried viruses of the West African clade. It is possible, although highly unlikely, that our samples and/or PCRs were contaminated with MPXV strain Zaire 96-I-16. We do not believe this to be the case for several reasons. First, testing of additional samples from this outbreak, which were processed independently by the Poxvirus Section at the CDC, yielded PCR products that also exhibited 100% identity to the Zaire 96-I-16 strain. Second, although our laboratory possesses the Zaire 96-I-16 strain of MPXV, it had not been worked with or grown in the laboratory in over 2 years, and it was not used as positive control DNA for any of the PCR assays used in this study. Another, more likely possibility, is that either the size of the amplicons sequenced, or the specific gene targets themselves (ie, *F3L* and *N3R*) do not allow for

differentiation of the two clades. In support of this idea, Reed *et al*¹⁰ recently showed that the sequence of a PCR amplicon, using the *HA* gene as a target, from a patient and a prairie dog from the 2003 outbreak grouped within the West African clade. Complete genome sequencing of isolated virus will be needed to resolve these issues.

In addition to using these assays for diagnostics during an outbreak, they have potential uses in monitoring viral-load in real-time in animal models of *Orthopoxvirus* disease. For example, Jahrling and colleagues recently developed a monkey model for studying the progression of smallpox infections.²⁶ In these studies, our quantitative *pan*-orthopox *HA*-MGB assay was used to monitor viral load in monkey blood and tissues after infection with smallpox virus. Smallpox infections in monkeys are an important surrogate model for human smallpox infections.²⁷ Likewise, the two MPXV-specific assays reported here could be used to monitor, in real time, the progression of monkeypox in nonhuman primates as has been done in smallpox animal models (unpublished data).

In conclusion, this study demonstrates the reliable and specific identification of MPXV virus DNA from clinical (animal) samples by TaqMan[®]-MGB real-time PCR on the LightCycler. By using these assays as part of a battery of PCR and ECL tests, one can first establish the presence of orthopox viral DNA and/proteins in a sample. Then MPVX can be quickly and accurately distinguished from smallpox virus by the use of the MPXV-specific PCR confirmatory assays in conjunction with three previously published smallpox-specific assays.¹⁹

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References

- 1 Magnus P, Anderson E, Petersen K, *et al*. A pox-like disease in cynomolgus monkeys. *Acta Pathol Microbiol Scand* 1959;46:156–176.
- 2 Ladnyj ID, Ziegler P, Kima E. A human infection caused by monkeypox virus in Basankusu Territory, Democratic Republic of the Congo. *Bull World Health Organ* 1972;46:593–597.

- 3 Jezek Z, Feener F. Human monkeypox. *Monogr Virol* 1988;17:80–110.
- 4 Khodakevich L, Jezek Z, Kinzanzka K. Isolation of monkeypox virus from wild squirrel infected in nature. *Lancet* 1986;1:98–99.
- 5 Khodakevich L, Szczeniowski M, Manbu mD, *et al.* The role of squirrels in sustaining monkeypox virus transmission. *Trop Geogr Med* 1987;39:115–122.
- 6 Hutin YJ, Williams RJ, Malfait P, *et al.* Outbreak of human monkeypox, Democratic Republic of Congo, 1996 to 1997. *Emerg Infect Dis* 2001;7:434–438.
- 7 Meyer H, Neubauer H, Pfeffer M. Amplification of variola virus-specific sequences in German cowpox virus isolates. *J Vet Med B Infect Dis Vet Publ Health* 2002;49:17–19.
- 8 Multistate outbreak of monkeypox—Illinois, Indiana, and Wisconsin, 2003. *MMWR*. 2003;13:537–540.
- 9 Enserink M. Infectious diseases. U.S. monkeypox outbreak traced to Wisconsin pet dealer. *Science* 2003;300:1639.
- 10 Reed KD, Melski JW, Graham MB, *et al.* The detection of monkeypox in humans in the Western Hemisphere. *N Engl J Med* 2004;350:342–350.
- 11 Update: multistate outbreak of monkeypox—Illinois, Indiana, Kansas, Missouri, Ohio, and Wisconsin, 2003. *MMWR*. 2003;52:642–646.
- 12 Meyer H, Pfeffer M, Rziha HJ. Sequence alterations within and downstream of the A-type inclusion protein genes allow differentiation of Orthopoxvirus species by polymerase chain reaction. *J Gen Virol* 1994;75(Pt 8):1975–1981.
- 13 Ropp SL, Jin Q, Knight JC, *et al.* PCR strategy for identification and differentiation of small pox and other orthopoxviruses. *J Clin Microbiol* 1995;33:2069–2076.
- 14 Neubauer H, Pfeffer M, Meyer H. Specific detection of mousepox virus by polymerase chain reaction. *Lab Anim* 1997;31:201–205.
- 15 Loparev VN, Massung RF, Esposito JJ, *et al.* Detection and differentiation of old world orthopoxviruses: restriction fragment length polymorphism of the crmB gene region. *J Clin Microbiol* 2001;39:94–100.
- 16 Ibrahim MS, Esposito JJ, Jahrling PB, *et al.* The potential of 5' nuclease PCR for detecting a single-base polymorphism in Orthopoxvirus. *Mol Cell Probes* 1997;11:143–147.
- 17 Ibrahim MS, Kulesh DA, Saleh SS, *et al.* Real-time PCR assay to detect smallpox virus. *J Clin Microbiol* 2003;41:3835–3839.
- 18 Espy MJ, Cockerill III FR, Meyer RF, *et al.* Detection of smallpox virus DNA by LightCycler PCR. *J Clin Microbiol* 2002;40:1985–1988.
- 19 Kulesh DA, Baker RO, Loveless BM, *et al.* Smallpox and *pan*-Orthopox virus detection by real-time 3'-minor groove binder TaqMan Assays on the Roche LightCycler and the Cepheid Smart Cycler platforms. *J Clin Microbiol* 2004;42:601–609.
- 20 Kijek TM, Rossi CA, Moss D, *et al.* Rapid and sensitive immunomagnetic-electrochemiluminescent detection of *Staphylococcal enterotoxin B*. *J Immunol Methods* 2000;236:9–17.
- 21 Kutyavin IV, Afonina IA, Mills A, *et al.* 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res* 2000;15:655–661.
- 22 Afonina IA, Reed MW, Lusby E, *et al.* Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. *Bio-techniques* 2002;32:940–949.
- 23 Afonina I, Zivarts M, Kutyavin I, *et al.* Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res* 1997; 25:2657–2660.
- 24 Mukinda VB, Mwema G, Kilundu M, *et al.* Re-emergence of human monkeypox in Zaire in 1996. Monkeypox Epidemiologic Working Group. *Lancet* 1997;349:1449–1450.
- 25 Mwanbal P, Tshioko K, Moudi A, *et al.* Human monkeypox in Kasai Oriental, Zaire (1996–1997). *Euro Surveill* 2004;2:33–35.
- 26 LeDuc JW, Damon I, Relman DA, *et al.* Smallpox research activities: U.S. interagency collaboration, 2001. *Emerg Infect Dis* 2002;8:743–745.
- 27 Zaucha GM, Jahrling PB, Geisbert TW, *et al.* The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (*Macaca fascicularis*). *Lab Invest* 2001;81:1581–1600.