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Detection of *monkeypox virus* with real-time PCR assays

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Abstract

**Background:** Human monkeypox, a zoonotic disease, was first reported outside of Africa during the 2003 US outbreak.

**Objectives:** We present two real-time PCR assays critical for laboratory diagnosis of monkeypox during the 2003 US outbreak.

**Study design:** A TaqMan-based assay (E9L-NVAR) targets the *orthopoxvirus* DNA polymerase gene and detects Eurasian *orthopoxviruses* other than *Variola*. A hybridization assay, utilizing a MGB EclipseTM (Epoch Biosciences) probe, targets an envelope protein gene (B6R) and specifically detects *monkeypox virus* (MPXV). Assays were validated using coded orthopoxvirus DNA samples and used to evaluate lesion samples from five confirmed US monkeypox cases.

**Results:** E9L-NVAR did not detect variola (48 strains), North American orthopoxviruses (2), or DNA derived from non-poxviral rash illnesses. The assay reproducibly identified various concentrations of 13 Eurasian orthopoxvirus strains and was sensitive to 12.5 vaccinia genomes. The B6R assay recognized 15 different MPXV strains, while other orthopoxvirus (9) and bacteria (15) strains did not cross-react. Of the 13 human samples tested from confirmed cases, both assays identified 100% as containing MPXV DNA.

**Conclusions:** E9L-NVAR and B6R assays demonstrate 100% specificity for non-variola Eurasian orthopoxvirus and MPXV, respectively. Using two discrete viral gene targets, these assays together provide a reliable and sensitive method for quickly confirming monkeypox infections.

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**Keywords:** Orthopoxvirus; Monkeypox virus (MPXV); Real-time PCR; Diagnostic

1. Introduction

Orthopoxvirus *monkeypox*, first isolated in 1958 from captive primate rash specimens (Magnus et al., 1959), was recognized to cause human illness in 1970 during smallpox eradication campaign intensification (Jezek and Fenner, 1988; Ladvany et al., 1972). Between 1970 and 1986, >400 monkeypox cases were reported in Africa, 95% within Zaire (now the Democratic Republic of Congo, DRC) (Jezek and Fenner, 1988). Until the 2003 US outbreak (Reed et al., 2004), no human cases had been reported outside of Africa.

Human monkeypox, as described in the Congo Basin, typically presents with symptoms similar to discrete, ordinary smallpox. After about a 2-week, asymptomatic incubation period, infected individuals develop fever followed by disseminated rash. Both illnesses are transmissible between humans and can result in death. The case fatality rate for monkeypox (~0–10%) (Jezek and Fenner, 1988) is much lower than for variola major (10–30%) (Fenner et al., 1998), as is the rate of human-to-human transmissibility. Control measures for these two diseases would differ, and recent events emphasize the need for monkeypox diagnostics. After discontinuation of smallpox vaccination, susceptibility to zoonotic monkeypox increased, likely contributing to increased disease reports in DRC (Hutin et al., 2001; Meyer et al., 2002; Mwanbal et al., 1997). The US monkeypox outbreak demonstrated the ability of the virus to exploit new hosts and move globally (Reed et al., 2004).
Several nucleic acid test methods have been developed for monkeypox virus (MPXV) detection and characterization (Kulesh et al., 2004; Meyer et al., 2004). Compared with other diagnostic methods, real-time PCR has the advantages of fast, high-quantity throughput and increased sensitivity. This is the first report of real-time PCR assays used to diagnose human monkeypox from clinical rash samples. The assays target different orthopoxvirus genes: DNA polymerase (E9L) and envelope protein (B6R). We report the analytic sensitivity and specificity of both assays and demonstrate their utility for US monkeypox outbreak human rash specimens. These assays represent two sensitive, rapid diagnostic tools for identification of orthopoxviral infection within clinical samples.

2. Materials and methods

2.1. Viruses, bacteria, and clinical samples homogenization

Origins, propagation, and harvesting procedures for viral and cellular isolates are documented (Esposito and Knight, 1985; Esposito et al., 1987; Frenkel et al., 1976; Gispen et al., 1967; Hanrahan et al., 2003; Likos et al., 2005; Loparev et al., 2001; Olson et al., 2004; Pulford et al., 2004; Regnery, 1971; Ropp et al., 1995; Sarmiento et al., 1979; Seki et al., 1990) or briefly described (Table 1). Viral samples were processed as described for clinical samples. Bacteria and Rickettsia (Table 2) were gifts (Holmes H and Massung R, CDC). Those bacteria with potential to contaminate clinical samples (Table 3) were propagated on blood agar plates, suspended in 0.85% sterile saline (0.5 McFarland turbidity), spotted (10^6/H9262L) onto slides, and processed to replicate conditions within a clinical sample.

Clinical samples were obtained from vesicular lesions as skin biopsies (scab or vesicle roof), vesicular fluid slide (“touch prep”), or vesicular fluid swab. Recommendations for lesion sampling can be found at http://www.bt.cdc.gov/agent/smallpox/response-plan/files/guide-d.pdf. In brief, samples were processed under biosafety containment conditions to form homogenates suitable for DNA extraction:

1. Skin biopsies were homogenized in sterile water or PBS (500/L) by freezing, disruption with a disposable pestle, and vortexing. If physical disruption was insufficient, the sample underwent further freeze–thaw/grinding cycles. Finally, samples, in closed tubes, were sonicated (cup-horn sonicator, 40% maximum output).

2. Nuclease-free water (100/L) was added to each spot on the slide, scraped, and recovered into a sterile tube. Water addition/scraping was repeated twice and pooled into the same tube.

3. Shafts of vesicular fluid swabs were broken near the top of the swab material. The swab was hydrated (300/L PBS) for 5–10 min in a sterile tube and then transferred to a Swab Extraction Tube System tube (Roche Applied Science, Indianapolis, IN). The swab tube was spun for 1 min to rinse the swab and collect the eluent.

2.2. DNA extraction

Crude virus (viral-infected cell lysates harvested 48 hpi), semi-purified virions (Esposito et al., 1981), purified virions, scabs, bacteria, and clinical samples were homogenized (as described above), and DNA extracted using the AquaPure Genomic DNA Isolation Kit (Bio-Rad, Hercules, CA), suspended in 50 L AquaPure DNA hydration buffer, and stored at −20 °C.

Table 1

| Organism        | Sample ID | Location     | Year | Patient or supplier | Material sent to CDC          |
|-----------------|-----------|--------------|------|---------------------|--------------------------------|
| Variola V74-227| Congo 9    | Somalia      | 1974 | R. Gipsen           | Chicken chorioallantoic membrane |
| Variola V77-1605| Somalia    | 1977         | Female, 9 years old | Creutz                       |
| Variola V78-4-903| Somalia    | 1978         | Female camel        | Creutz                       |
| Camelopox V78-4-2370| Somalia | 1978         | Female camel        | Creutz                       |
| Monkeypox V77-4-813| Zaire   | 1977         | Female, 7 years old | Creutz                       |
| Monkeypox V77-4-823| Zaire   | 1977         | Male, 15 years old | Creutz                       |
| Monkeypox V70-266| Sierra Leone | 1970      | Male, 24 years old | Creutz                       |
| Monkeypox V81-187| Zaire   | 1981         | Male, 2 years old  | Creutz                       |
| Monkeypox V81-12-179| Ivory Coast | Côte d’Ivoire | 1981 | Female, 3 years old | Creutz                       |
| Monkeypox V82-167| Zaire   | 1982         | Female, 29 years old | Swab                        |
| Monkeypox V83-036| Zaire   | 1983         | Female, 3 years old | Swab                        |
| Monkeypox I2003ki-DRC| DRC, formally Zaire | 1998 |                   |                                |
| Vaccinia       | Lister    | Great Britain |      |                     |                                |
| Vaccinia       | Temple of Heaven | China |      |                     |                                |
| Vaccinia       | Wyeth/Doyva | US          |      |                     |                                |
| Human-E lymphoblast | SUP-FVRI |                |      | ATCC CRCL-1942     |                                |
| African Green Monkey | BS-C-40 |                |      | ATCC #CCL-38       |                                |
| Herpesvirus Varicella Zoster-OKa | ATCC # VR-795 |      | |                                |                                |
| Herpesvirus Varicella-Webster | ATCC # VR-916 |      | |                                |                                |
Table 2

| Organism Sample ID | DNA | 2 ng | 200 pg | 20 pg | 2 pg | 200 fg | 20 fg | 2 fg |
|--------------------|-----|------|--------|-------|------|--------|-------|-----|
| Eurasian orthopoxvirus* |     |      |        |       |      |        |       |     |
| Camelpox E2379 | Partially pure | 21.26 | 24.60 | 29.01 | 34.18 | ND | ND | ND |
| Camelpox v78-1-903 | Crude | 21.12 | 20.67 | 38.48 | 36.84 | ND | ND | ND |
| Coopst Brighton | Partially pure | 16.05 | 24.85 | 33.11 | ND | ND | ND | ND |
| Ectromelia Moscow | Partially pure | 17.10 | 20.55 | 25.54 | 28.57 | 31.48 | 37.43 | ND |
| Taterapox Gerbitopsis | Partially pure | 23.56 | 24.66 | 28.60 | 31.00 | ND | 39.25 | ND |
| Monkeypox MPXV-ZAI-1986-016 | Partially pure | 15.29 | 19.94 | 22.49 | 37.10 | ND | ND | ND |
| Monkeypox V70-286 Sierra Leone | Partially pure | 16.71 | 28.23 | 33.73 | ND | ND | ND | ND |
| Vaccinia Lister | Partially pure | 17.48 | 26.34 | 34.55 | ND | ND | ND | ND |
| Vaccinia Temple of Heaven | Partially pure | 17.13 | 28.32 | 35.86 | ND | ND | ND | ND |
| Vaccinia BE9 | Crude | 26.19 | 30.43 | 34.21 | 36.04 | ND | ND | ND |
| Vaccinia Wyeth/Dryvax | Crude | 26.69 | 38.19 | ND | ND | ND | ND | ND |
| Vaccinia WYH pG68-5-v1-1-1 | Partially pure | 19.33 | 23.60 | 36.71 | ND | ND | ND | ND |
| Variola SA6F5-102 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola SA6F5-103 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola 7124 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola 7125 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Virolog 4 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Guccio | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola BSH | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Butler | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola ETH22-17 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Harper | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Harvey | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Heindberg | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Higgins | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Hinden | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Horo | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Jean 2602 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Juba | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola K125 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Kali Mathu | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Hembula | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Minnesota 124 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Lee | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola New Delhi | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Nur Islam | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Lahore | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Rambree | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Stulzmann | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Soliz | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Stubbell | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V66-39 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V68-258 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V68-59 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V70-222 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V70-228 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Congo | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V72-119 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V72-143 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Nepal 73 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V73-225 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V74-227 Congo 9 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V77-125 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola V77-1665 | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Yamala | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Bombay | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Hembula | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Maimun | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Kudano | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Parvin | Crude | ND | ND | ND | ND | ND | ND | ND |
| Variola Soliz | Crude | ND | ND | ND | ND | ND | ND | ND |
2.3. E9L-non-virotela (NVAR) assay

The primer/probe sequences were selected from the DNA polymerase gene (E9L; GenBank L22579) with Primer Express (version 1.5; Applied Biosystems). These included E9L forward primer (5′-TCA.ACT.GGA.AAG.GC.CC.ATC.TAT.GA-3′), E9L reverse primer (5′-GAG.TAT.AGA.GCA.CTA.TTT.CTA.AAT.CCC.A-3′), and E9L-NVAR probe (5′-TET.CCA.TGC.AAT.ATA.CGT.ACA.AGA.TAG.TAG.CCA.AC-3′). Primers and probe were synthesized in the Biotechnology Core Facility (CDC, Atlanta GA), utilizing standard phosphoramidite chemistry. The detection probe contained 5′ amino group after synthesis.

2.4. B6R MPXV-specific assay

The primer sequences were selected from the extracellular enveloped virus protein gene (B6R; GenBank L22579) using Primer Express (version 1.5; Applied Biosystems, Foster City, CA). These included B6R forward primer (5′-ATT.GCT.CAT.TAT.TTT.TGT.CAC.AGG.AAC.A-3′), and B6R reverse primer (5′-AAT.GGC.GTG.TAT.GAC.AAT.TAT.GGG.TG-3′). The MPXV-specific probe (5′-MGB/DarkQuencher-MGA.GAT.TAG.AAA.TA.FAM-3′) was selected from the B6R sequence with the aid of MGB Eclipse™ By Design software (Epoch Biosciences, Bothell, WA) and has a conjugated minor groove binding (MGB) ligand and a dark quencher at the 5′-end, with the fluorophore at the 3′-end. Fluorescence of the single-stranded probe is efficiently quenched by the interaction of the terminal dye and quencher groups when not hybridized (Afonina et al., 2002a,b). Each reaction (20 μL) contained 1 x Eclipse Gene Expression Buffer (20 mmol/L Tris–HCl pH 8.7, 50 mmol/L NaCl, 5 mmol/L MgCl2), 200 nmol/L MGB Eclipse™ probe, 0.25 μL 100 nmol/L deoxynucleoside triphosphate mixture, 0.4 μmol/L each primer, 0.75 μL JumpStart TaqDNA polymerase (Sigma, St. Louis, MO), and 2 μL template DNA. Thermal cycling conditions for the iCycler (Bio-Rad, Hercules, CA): one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 40 s. PCR amplification is based on fluorescent emission after annealing/elongation (60 °C).
| Eurasian orthopoxvirus        | Sample ID   | DNA    | Average Ct for samples with different amounts of viral DNA |
|-------------------------------|-------------|--------|----------------------------------------------------------|
|                               |             |        | 2 ng | 200 pg | 20 pg | 2 pg | 200 fg | 20 fg | 2 fg |
| Camelpox v78.1279             | Purified    | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |
| Cowpox CP58                   | Purified    | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |
| Cowpox Brighton               | Purified    | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |
| Monkeypox MPXV-ZAI-1979-005   | Purified    | 17.30b | 21.01b| 26.00b | 29.79b| 33.69b| 36.74b | 39.70b | ND   |
| Vaccinia WythDryvax           | Purified    | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |
| Vaccinia BHDI                 | Purified    | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |
| Vaccinia Wyth                 | Purified    | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |
| Variola V73-143               | Crude       | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |
| Variola BSH                   | Purified    | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |
| Variola Horn                  | Purified    | ND     | ND   | ND     | ND    | ND   | ND     | ND     | ND   |

| Bacteria                      | Average Ct |        |
|-------------------------------|------------|--------|
| Streptococcus Pyogenes ATCC 19615 | ND         |        |
| Diphtheria CDC8143-02          | ND         |        |
| Peptostreptococcus anaerobius ATCC 15689 | ND         |        |
| Propionibacterium acnes ATCC 6910 | ND         |        |
| Staphylococcus aureus strain 1 ATCC 12600 | ND         |        |
| E. coli ATCC 13867             | ND         |        |
| S. epidermidis (strain 3) ATCC 12228 | ND         |        |
| S. epidermidis (strain 1) ATCC 49134 | ND         |        |
| Pseudomonas aeruginosa ATCC27853 | ND         |        |
| Enterococcus faecalis ATCC 29212 | ND         |        |
| Streptococcus hens (alpha-Strep) ATCC 49147 | ND         |        |
| S. aureus (strain 3) CD003-06 (TSST1 positive) | ND         |        |
| S. aureus (strain 2) ATCC 2925 | ND         |        |
| Escherichia coli 29222         | ND         |        |

* Each sample was tested in triplicate; all results were negative except as indicated. ND, not detected.
* All assays were positive and the average Ct value is shown.
* Each sample was spotted onto a slide to mimic a clinical sample (see Section 2). The slides were processed, DNA extracted, and each DNA tested in triplicate. ND, not detected.

Based on the fluorescent emission with annealed probe (57°C).

2.5. Statistical probit analysis

Analytical sensitivity was determined using purified, photometrically quantified vaccinia DNA diluted in water (24 replicates of 5 concentrations). Probit analysis as a model of non-linear regression was accomplished with commercial software (SPSS 11.0, for Mac® OS X; SPSS, Inc., Chicago, IL.). The software determines a continuous 95% confidence interval of the probability of achieving a positive result at any given input DNA concentration within the concentration range of the experiment.

3. Results and discussion

3.1. E9L-NVAR orthopoxvirus assay

Several TaqMan-based real-time PCR assays have been developed as rapid orthopoxvirus diagnostics. One diagnostic assay for orthopoxviral infections other than variola targets the viral DNA polymerase gene (E9L), amplifying a conserved gene segment within all Eurasian orthopoxviruses. The probe, however, targets 32 bases within the E9L gene containing a three nucleotide difference between variola and other orthopoxviruses (Fig. 1), and thus efficiently anneals to Eurasian orthopoxviruses other than variola. This orthopoxviral diagnostic can be used without raising concern that variola has been detected.

The specificity and sensitivity of the assay, designated E9L-NVAR, was determined utilizing a coded panel of multiple orthopoxviruses. Each sample was tested singly, and positive samples are denoted by the cycle where fluorescence crossed the threshold (Ct) (Table 2). E9L-NVAR identified all non-variola Eurasian orthopoxviruses (13 species) at concentrations between 2 pg and 20 fg of viral DNA, depending upon DNA quality. The assay detected 20 fg of partially purified MPXV DNA (~100 genomes). Partially purified cowpox, ectromelia, and vaccinia were identified at similar efficiencies (Table 2). Partially purified camelpox and taterapox demonstrated a diminished interaction with the E9L-NVAR probe (Table 2) due to a single base difference...
Fig. 1. Alignment of primers and probes with orthopoxviral DNA. The primers and probe for each of the real-time PCR assays are aligned with the targeted sequence of DNA within several orthopoxviral species. The E9L-NV AR primers and probe are completely homologous with the vaccinia Copenhagen DNA sequence. The B6R primers and probe are completely homologous with the monkeypox (CB) DNA sequence. Virus strains: monkeypox (CB) MPXV-ZAI-1996-016 (Genbank AF380138); monkeypox (US) MPXV-USA-2003-039 (Genbank DQ011154); vaccinia Copenhagen (Genbank M35027); cowpox Brighton (Genbank AF482758); ectromelia Moscow (Genbank AF012825); camelpox Kazakhstan M-96 (Genbank AF438165); taterapox (Smith GL, personal communication); variola major Bangladesh (Genbank L22579); variola minor Garcia (Genbank Y16780). CB, Congo Basin; US, United States.

between these viruses and variola in the probe target region (Fig. 1).

Vaccinia and MPXV sequences are identical in this region (Fig. 1), and serially diluted purified vaccinia DNA established linearity of the E9L-NV AR assay from 2 ng to 20 fg (83% reaction efficiency) (Table 2). Probit regression analysis determined assay sensitivity using the same preparation of purified DNA in 24 replicate amplification reactions. Amplification was positive in all 24 replicate reactions containing 20, 10, and 5 fg of vaccinia input DNA. Only 22 of 24 replicates containing 2.5 fg were detected, while no reactions containing 1.25 fg vaccinia DNA were positive (data not shown). Therefore, 2.5 fg viral DNA (∼12.5 genomes) is the calculated detection limit for 95% confidence.

The E9L-NV AR assay is specific for six non-variola Eurasian orthopoxviruses, not cross-reacting with variola (48 strains) or North American orthopoxviruses (2 strains). Furthermore, E9L-NV AR assay did not cross-react with any DNA derived from rash illnesses potentially confused with orthopoxviral infection, such as herpesvirus and rickettsial infections, or with human cellular DNA (Table 2), even at high concentrations (2 ng). Overall, the E9L-NV AR assay can reproducibly detect as few as 12.5 genomes of purified vaccinia or MPXV DNA without giving false positive results.

3.2. Monkeypox-specific B6R assay

Although the E9L-NVAR assay reliably detects Eurasian orthopoxviruses, other than variola, it is unable to make a species-specific identification. Due to the low G + C content (∼30%) and 90% sequence similarity to other Eurasian orthopoxviruses, it is difficult to design a monkeypox-specific TaqMan assay. To improve the reliability of a MPXV-specific assay, we utilized the MGB-based real-time PCR technology. Linking a DNA double helix MGB protein to the probe permits use of shorter probe sequences, which can detect single nucleotide polymorphisms (SNPs) (Afonina et al., 2002a,b; Belousov et al., 2004) such as within the MPXV envelope protein gene (B6R) (Fig. 1). The probe 5′-MGB molecule stabilizes probe-template interaction (Afonina et al., 2002a,b) and enhances assay specificity and sensitivity. A coded test panel containing orthopoxviral and bacterial DNAs was assayed in triplicate using the iCycler platform (Bio-Rad, Hercules, CA) to monitor reproducibility and specificity of the B6R assay (Table 3). MPXV DNA was reproducibly detected in a linear fashion to ∼10 viral copies (2 fg). The B6R assay did not cross-react with any other orthopoxviral DNA (variola, cowpox, camelpox, and vaccinia) or with 15 bacterial species. Although certain Gram-positive bacterial DNAs were less efficiently extracted, all
Table 4

| Monkeypox strain | DNA | Geographic area | 10 ng |
|------------------|-----|-----------------|-------|
| Ivory Coast V81-4-179 | Crude | Côte d’Ivoire (West Africa) | 16.43 |
| MPXV-LIB-1970-184 | Purified | Liberia (West Africa) | 14.30 |
| Umohé | Crude | The Netherlands (original origin unknown) | 22.87 |
| MPXV-NIG-1978 | Crude | Nigeria (West Africa) | 17.17 |
| V70-266 Sierra Leone | Crude | Sierra Leone (West Africa) | 15.10 |
| MPXV-CAM-1990 | Crude | Cameroon (Congo Basin) | 20.23 |
| MPXV-GAB-1988-001 | Crude | Gabon (Congo Basin) | 22.53 |
| 1200/S-li-BEC-1998 | Crude | Zaire (Congo Basin) | 13.90 |
| MPXV-ZAI-1979-005 | Crude | Zaire (Congo Basin) | 15.00 |
| MPXV-ZAI-1996-016 | Crude | Zaire (Congo Basin) | 13.97 |
| V77-823 | Crude | Zaire (Congo Basin) | 14.20 |
| V77-813 | Crude | Zaire (Congo Basin) | 14.57 |
| V81-187 | Crude | Zaire (Congo Basin) | 14.33 |
| V82-187 | Crude | Zaire (Congo Basin) | 14.00 |
| V83-036 | Crude | Zaire (Congo Basin) | 14.03 |

| Sensitivity to purified DNAb | 2 ng | 200 pg | 20 pg | 2 pg | 200 fg | 20 fg | 2 fg |
|-----------------------------|------|--------|-------|------|--------|-------|------|
| MPXV-ZAI-1996-016 (old)    | 16.43 | 20.37  | 24.23 | 28.97 | 34.07  | 38.63 | ND   |
| MPXV-ZAI-1996-016 (fresh)  | 16.96 | 20.74  | 24.76 | 27.50 | 31.14  | 33.85 | 38.21 |

b | Ability of the assay to detect multiple strains of monkeypox virus. Each sample was tested in triplicate; all three assays were positive and the average Ct value is shown.

Assay limit of detection for various quantities of purified monkeypox DNA. Each sample was tested in triplicate using either freshly diluted DNA (fresh) or diluted DNA that had undergone multiple freeze-thaw cycles (old). Where all three assays were positive, the average Ct value is shown. ND, not detected.

b | Ability of the assay to detect multiple strains of monkeypox virus. Each sample was tested in triplicate; all three assays were positive and the average Ct value is shown.

Assay limit of detection for various quantities of purified monkeypox DNA. Each sample was tested in triplicate using either freshly diluted DNA (fresh) or diluted DNA that had undergone multiple freeze-thaw cycles (old). Where all three assays were positive, the average Ct value is shown. ND, not detected.

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Assay limit of detection for various quantities of purified monkeypox DNA. Each sample was tested in triplicate using either freshly diluted DNA (fresh) or diluted DNA that had undergone multiple freeze-thaw cycles (old). Where all three assays were positive, the average Ct value is shown. ND, not detected.

b | Ability of the assay to detect multiple strains of monkeypox virus. Each sample was tested in triplicate; all three assays were positive and the average Ct value is shown.

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Table 5

Analysis of clinical samples

| Case number | Sample type | Lab diagnosis | Tissue culture | Standard PCR |
|-------------|-------------|---------------|----------------|--------------|
|             |             | HA            | HA RFLP        | ATI ATI RFLP |
| Monkeypox   |             |               |                |              |
| 2003-038 (Patient 7*) | Slide of vesicle fluid | MPX + (1 day) | + | MPX + | MPX |
|              | Skin biopsy  | MPX + (6 days) | - | NA - | NA |
|              | Swab of vesicle fluid | MPX + (2 days) | + | Not done | Not done |
|              | Vesicle roof | MPX + (3 days) | + | MPX + | MPX |
|              | Swab of vesicle fluid | MPX + (6 days) | + | - | + |
|              |               | NA            | NA             | Not done     |
| 2003-039 (Patient 4*) | Slide of vesicle fluid | MPX + (3 days) | + | Inconclusive | MPX |
|              | Skin biopsy  | MPX + (3 days) | + | Inconclusive | MPX |
|              | Swab of vesicle fluid | MPX + (3 days) | + | Inconclusive | MPX |
|              | Swab of vesicle fluid | MPX + (6 days) | + | - | + |
|              |               | NA            | NA             | Not done     |
| 2003-040 (Patient 8*) | Slide of vesicle skin | MPX + (6 days) | + | Inconclusive | MPX |
|              | Skin biopsy  | MPX + (6 days) | + | Inconclusive | MPX |
|              | Swab of vesicle fluid | MPX + (6 days) | + | Inconclusive | MPX |
|              | Swab of vesicle fluid | MPX + (6 days) | + | - | + |
|              |               | NA            | NA             | Not done     |
| 2003-045 (Patient 11*) | Swab of vesicle skin | MPX + (2 days) | + | Inconclusive | MPX |
| 2003-073 (Patient 6*) | Skin biopsy  | MPX + (1 day) | + | MPX + | MPX |
| Varicella zoster virus | Vesicle skin | Negative | - (7 days) | - | NA |
| 2003-072 | Vesicle skin | Negative | - (7 days) | - | NA |

Results from tissue culture, standard PCR, and real-time PCR assays are shown for each sample. MPX, monkeypox; OPX, orthopox; NA, not applicable; ND, not detected.

* Each sample was tested in triplicate and the average Ct for each positive sample is shown. Negative samples were not detected (ND).

Results from tissue culture, standard PCR, and real-time PCR assays are shown for each sample. MPX, monkeypox; OPX, orthopox; NA, not applicable; ND, not detected.

4. Summary

Two rapid real-time PCR assays for the detection of orthopoxvirus and MPXV DNA have been developed. The E9L-NVAR and B6R assays target orthopoxvirus DNA polymerase and extracellular enveloped protein genes, respectively. These assays are highly sensitive (2 fg or ~10 viral genomes) and specific. The E9L-NVAR assay detects 13 Eurasian orthopoxviruses but not variola or North American orthopoxviruses, and the B6R assay detects MPXV isolates but no other orthopoxviruses. Neither assay gave false positives with other rash illness-causing viruses or bacteria. The E9L-NVAR assay, initially developed upon the ABI7700, has provided similar results with other real-time PCR platforms such as the Lightcycler (Roche) and iCycler (data not shown). Similarly, the B6R assay, validated on the iCycler, is compatible with the ABI7700 real-time PCR technology.
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digned to detect Congo Basin MPXV; West African/US MPXV has one SNP within
the B6R probe (Fig. 1). The lack of complete homology to the
US monkeypox isolates did not adversely affect the detection of
MPXV DNA within human samples (Table 5), confirming
the B6R assay diagnostic utility for both known MPXV
clades (Likos et al., 2005). Furthermore, monkeypox spread
outside of Africa suggests these diagnostic assays may be rel-
evant worldwide for identification of smallpox-like orthopox

diseases.

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