A Research-Use Multiplex qPCR Assay* that Detects Monkeypox Virus Clades I and IIa/IIb

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Abstract

The current outbreak of mpox (monkeypox) disease has spread outside its prior endemic region in Africa and the virus has emerged as a more infectious pathogen associated with newer sequences found in Clade II. Due to its expansion to previously non-endemic countries, the World Health Organization (WHO) has designated it a public health emergency. PCR-based methods have shown utility in tracking the spread of viral pathogens in human samples. To accelerate the surveillance of Monkeypox virus (MPXV) globally, we show that primers and probes originally designed for Droplet Digital™ PCR (ddPCR™) can be used in a multiplex quantitative PCR (qPCR) format to detect both Clade I and Clade IIa/IIb. Samples containing synthetic MPXV DNA were evaluated with an MPXV-specific qPCR assay performed on a common commercial real-time PCR system. The dual-target assay performed well using contrived samples with or without PCR inhibitors, suggesting its utility to expand surveillance detection.

Introduction

Mpox is a zoonotic disease caused by monkeypox virus (MPXV), a DNA-based virus in the genus *Orthopoxvirus*. It is historically endemic in African countries (WHO 2022a, European Centre for Disease Prevention and Control [ECDC] 2022). Mpox became a disease of global public health importance affecting over 50 non-endemic countries, with 77,650 cases reported globally as of this writing. The Centers for Disease Control and Prevention (CDC) Mpox Outbreak Global Map has up-to-date case information (CDC 2022a). MPXV is separated into two distinct clades that differ considerably — Congo (Clade I) and West African (Clade II), with the latter leading to less severe symptoms. Clade II has mutated significantly since the 1970s and the WHO designated the group of variants largely circulating in the 2022 global outbreak with its own subclade, IIb (WHO 2022b). Clades IIa and IIb are closely related through a common ancestor and contain key differences of 50–100 single nucleotide polymorphisms.

For clinical diagnosis, due to serologic cross-reactivity with other *Orthopoxvirus*-associated infections, antigen and antibody detection methods cannot provide MPXV-specific confirmation needed for clinical diagnosis. As of August 2022, the CDC's Non-variola *Orthopoxvirus* (NVO) Generic Real-Time PCR Test is the sole FDA 510(k)–cleared assay to detect NVO in the USA. While the NVO qPCR assay is designed to detect non-variola *Orthopoxvirus*–associated infections, it does not specifically detect MPXV sequences (Aden TA et al. 2022), requiring secondary confirmation for positive samples. The preferred confirmatory test for mpox disease is qPCR using MPXV-specific primer/probe designs and samples from skin lesions of infected patients, given the accuracy and sensitivity of qPCR assays (WHO 2022a). The first real-time PCR assays for MPXV used primer/probe designs for singleplex detection of either Clade I or Clade II (Li Y et al. 2010).

To determine the geographic spread of MPXV, wastewater-based epidemiology using the Droplet Digital PCR assays for MPXV is the preferred technique. Researchers in public health may also benefit from rapid, cost-effective multiplex qPCR assays to understand the epidemiology, sources of infection, and transmission patterns for MPXV. In this study, we evaluated whether ddPCR primer/probe sequences** currently available for wastewater surveillance of the two main Clades, I and IIa/IIb, could also be used in a qPCR

* For research use only, not for use in diagnostic procedures.

** As of September 2, 2022, the CDC's Laboratory Outreach Communication System (LOCS) provided an alert that, in cases where samples contain a significant deletion in the *TNF* receptor gene, assays using the CDC published primers and probes that specifically target MPXV may not detect MPXV Clade IIa/IIb (CDC, 2022b).

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assay format, which offers high-throughput and a well-established technique available to many research laboratories. The primer/probe sequences used here were the same sequences used in earlier published assays specific to Clades I and IIa (Li et al. 2010), with the Clade I assay targeting the *C3L* gene and the Clade IIa/IIb assay (G2R_WA primers and probe) targeting a region of the *OPG002* gene that is specific to Clade II. Using contrived samples containing synthetic MPXV DNA, the results showed that the qPCR detection was sensitive for low-input samples and was completed in less than an hour with the Bio-Rad™ CFX Opus 96 Real-Time PCR System.

Materials and Methods

MPXV qPCR Primers and Probes

Available individual ddPCR PCR primer/probe sets targeting sequences specific to MPXV Clade I and Clade IIa/IIb based on prior published sequences (Li et al. 2010) were used in a qPCR format. The MPXV Clade I assay includes a FAM labeled probe (Bio-Rad Laboratories, Inc., Assay ID #dEXD77548788) and the MPXV Clade IIa/IIb assay uses a HEX labeled probe (Bio-Rad, Assay ID #dEXD51818561). The oligonucleotides in the Clade IIa/IIb MPXV assay primer/probe sets show 100% sequence homology to all 121 MPXV genomic sequences deposited in the Global Initiative on Sharing Avian Influenza Data (GISAID) database*** for the 2022 international MPXV outbreak, as of July 15, 2022 (data not shown), and are thus expected to recognize samples containing the currently circulating mpox pathogen. The MPXV Clade I assay will not detect the currently circulating MPXV pathogen in human specimens; it serves as a contingency assay for surveillance of new outbreaks of MPXV Clade I.

Quantification of Synthetic MPVX DNA

As noted by Huggett et al. (2022), Droplet Digital PCR is a suitable technique to determine the quantity of MPXV control materials (in genome copies or equivalent). Synthetic double-stranded DNA, approximately 400 base pairs in length, containing regions specific to MPXV Clade I or Clade IIa/IIb genomes were obtained (Integrated DNA Technologies). The synthetic MPXV DNA was quantified by Droplet Digital PCR using either Clade I or Clade IIa/IIb MPXV ddPCR Assays (Bio-Rad, Assay ID #dEXD77548788 and Assay ID #dEXD51818561), ddPCR Supermix for Probes (No dUTP) (Bio-Rad, catalog #1863023) and the QX200 AutoDG Droplet Digital PCR System (Bio-Rad, #1864100) according to the manufacturer's instructions.

qPCR Reaction Conditions

Real-time PCR was performed with MPXV ddPCR Assays (Bio-Rad, Assay ID #dEXD77548788 and Assay ID #dEXD51818561) and Reliance One-Step Multiplex Supermix (Bio-Rad, #12010220), run on the CFX Opus 96 Real-Time PCR Instrument (Bio-Rad, #12011319) with CFX Maestro 2.3 Software for analysis (Bio-Rad, #12013758) according to the manufacturer's instructions, with the modifications to the qPCR reaction conditions that follow. While Reliance One-Step Multiplex Supermix is designed to quantify RNA targets by conversion to cDNA templates using reverse transcriptase (RT), it can also quantify DNA targets with outstanding sensitivity. Therefore, the RT step of the published Reliance One-Step Multiplex Supermix protocol was eliminated, and a shorter PrimePCR qPCR protocol was used in which the PCR process involved an initial 2 min denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 5 sec and amplification/extension (with plate read) at 60°C for 30 sec. The entire qPCR protocol was accomplished in less than 1 hour.

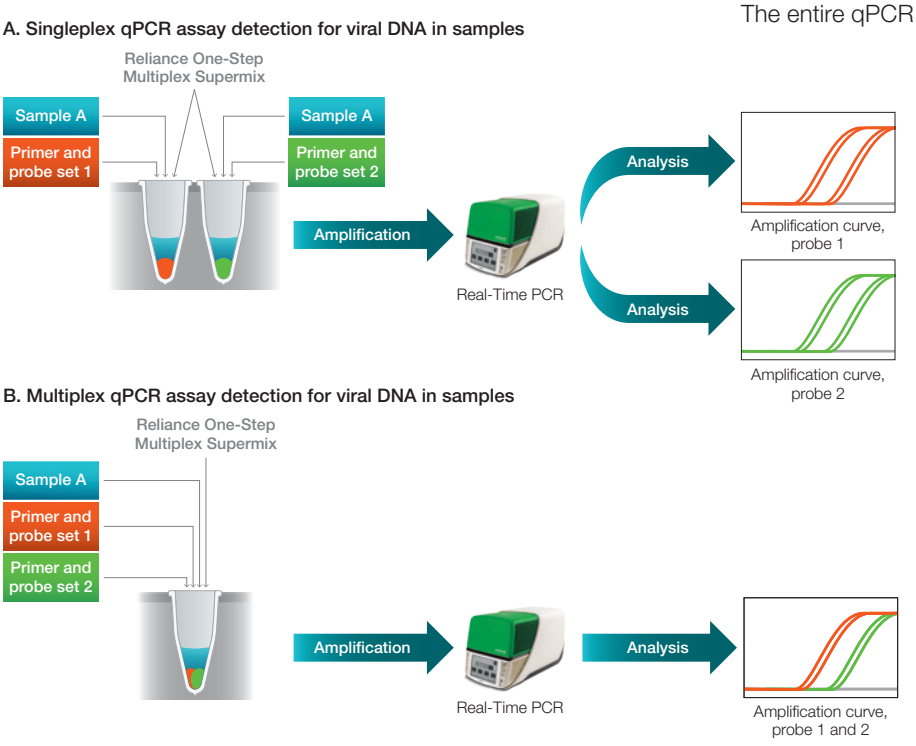

Fig. 1. Schematic of qPCR assay experimental designs. **A**, singleplex qPCR assay, each primer/probe set is tested individually; **B**, multiplex qPCR assay, primer/probe sets are combined into a single test. Reliance One-Step Multiplex Supermix contains reverse transcriptase, DNA polymerase, buffer reagents, and dNTPs.

*** As of the 2022 MPXV outbreak, GISAID has added a database specifically for MPXV genomes.

Evaluation of MPXV qPCR Assays in Singleplex and Multiplex Formats

A dilution series, from 100,000 to 10 copies of synthetic MPXV Clade I or Clade IIa/IIb DNA per reaction, was analyzed by qPCR using Clade I and/or Clade IIa/IIb MPXV assays, described above, in either a singleplex or multiplex format (Figure 1). A no template control (NTC) reaction containing water instead of MPXV synthetic DNA was also analyzed. Each diluted standard in the series was analyzed in triplicate.

Single-Copy Detection of MPXV Clade IIa/IIb DNA

Synthetic Clade IIa/IIb MPXV DNA was diluted to a concentration so that one MPXV DNA molecule was analyzed per qPCR reaction well, on average. A set of 40 reactions containing an average of one MPXV DNA molecule and 20 control reactions containing no MPXV DNA were analyzed by qPCR using multiplex Clade I and Clade IIa/IIb assays. A reaction was scored as positive if the Clade IIa/IIb MPXV PCR trace showed a quantification cycle (Cq) <40 and was chosen as the lower limit of detection for the assay.

Detection of Clade IIa/IIb MPXV DNA in Contrived Human Samples

Contrived human samples with a range of MPXV DNA concentrations as input material were prepared in viral transport media (VTM) (Neuromics, #VTM-4) containing 10% v/v human serum (BioIVT, #HUMANSRMMNN) and spiked-in Clade IIa/IIb MPXV DNA and diluted to a standard concentration of 800–50 copies per ml sample. Nucleic acid extraction was performed on the contrived samples with the QIAamp Viral RNA Mini Kit (QIAGEN, #52906) according to the manufacturer's instructions. Multiplex qPCR assays for Clade I and Clade IIa/IIb using 10 µl of purified sample were tested in 20 replicate reactions for each MPXV Clade IIa/IIb DNA concentration. A reaction was scored as positive if the MPXV Clade IIa/IIb PCR trace showed a Cq <40.

Results

To assess the performance of the MPXV Clade I and Clade IIa/IIb assays, we first tested the primers/probes individually in singleplex qPCR assays and then together in a multiplex qPCR assay format (Figure 1). A serial dilution series covering 5 orders of magnitude for synthetic MPXV DNA ranging from 100,000 copies per well to 10 copies per well was performed in both the singleplex and the multiplex assay formats. PCR amplification traces (Figures 2A and 2B) and associated Cq values from the dilution series (Table 1) showed very comparable results for both Clade I and Clade IIa/IIb when the qPCR assays were performed individually or multiplexed together.

The performance metrics of these assays for both MPXV Clades I and IIa/IIb demonstrated key qPCR attributes of broad dynamic range, sensitivity down to 10 copies of the MPXV gene per reaction, and R^2 values >0.99, thus approaching the estimated limit according to the Poisson distribution (Yates and Goodman 2014). PCR efficiency was >92% for the Clade I assay and >98% for the Clade IIa/IIb assay (Table 1).

Therefore, based on the qPCR amplification traces and Cq values showing no loss of sensitivity when combined into a single reaction (Table 1), both assays can be successfully multiplexed and allow detection of all 121 MPXV clades banked in GISAID as of August 2022 (data not shown).

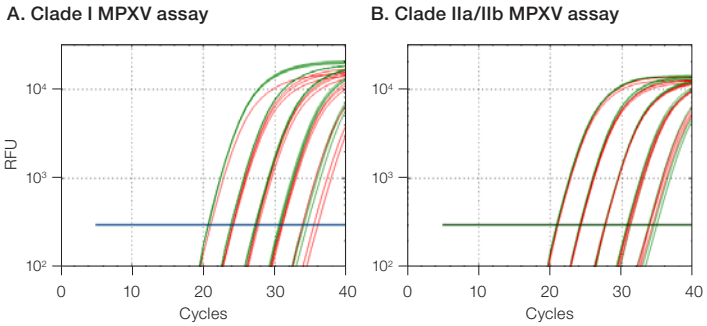

Fig. 2. Comparison of singleplex and multiplex assay performance. A dilution series of synthetic MPXV DNA corresponding to appropriate regions of the MPXV Clade I and Clade IIa/IIb genomes was analyzed by qPCR in either singleplex assay format (■) or multiplex assay format (■). The amount of synthetic DNA analyzed varied from 100,000 copies to 10 copies per reaction. **A**, detection of MPXV Clade I by qPCR; **B**, detection of MPXV Clade IIa/IIb by qPCR. RFU, relative fluorescence units.

Table 1. MPXV qPCR Cq values and assay performance. Average Cq values shown for single and multiplex assays in Figure 2. All assays achieved a dynamic range of 100,000 to 10 copies of MPXV per reaction. NTC, no template control.

	Clade I MPXV Assay		Clade IIa/IIb MPXV Assay	
	Singleplex	Multiplex	Singleplex	Multiplex
MPXV Copies per Reaction				
100,000	20.61	20.95	20.85	21.08
10,000	23.80	24.10	24.12	24.20
1,000	27.24	27.48	27.58	27.61
100	30.58	30.97	30.84	30.99
10	34.08	35.05	34.30	33.96
NTC	N/A	N/A	N/A	N/A
Parameter				
Dynamic range linearity (R^2)	0.999	0.991	0.997	0.999
PCR efficiency	98.0%	92.8%	98.4%	102.8%

Huggett et al. (2022) note that a well-designed, optimized assay can demonstrate near single-copy performance. Therefore, we evaluated MPXV multiplex assay format sensitivity using a dilution series starting with an average of one MXPV Clade IIa/IIb DNA molecule per reaction. As shown in Table 2, we performed 40 reactions with synthetic MPXV DNA and 20 control reactions without MPXV DNA. We observed that 60% of the reactions containing MPXV DNA detected MPXV Clade IIa/IIb, in contrast to the control reactions, which did not. The ability to detect near single-copy levels of MPXV IIa/IIb synthetic DNA in a reaction suggests that the multiplex MPXV assay can be validated by researchers to provide sensitive detection.

Table 2. Single-copy detection of Clade IIa/IIb MPXV DNA in contrived human samples. An average of one copy per well of synthetic MPXV Clade IIa/IIb DNA was analyzed by qPCR with the Clade IIa/IIb MPXV assay in 40 replicate wells. Twenty no template controls were also analyzed.

Average MPVX Copies per Reaction	Total Reactions Analyzed	Number Positive Reactions	Number Negative Reactions	Percent Positive Reactions
1	40	24	16	60
0	20	0	20	0

Sensitive Detection of Clade IIa/IIb MPXV in Contrived Human Samples Containing Contaminants

Next, we explored the sensitivity of the multiplex MPXV assay against a background of human contaminants using contrived human samples with synthetic MPXV Clades IIa/IIb DNA in a background of viral transport media containing 10% v/v human serum; the amount of MPXV DNA ranged from 800 copies per ml down to 50 copies per ml sample. Nucleic acid was isolated from the samples and analyzed by qPCR with 20 replicate reactions per spiked MPXV DNA concentration. Our results, shown in Table 3, demonstrate that the assay was able to detect Clade IIa/IIb MPXV DNA even when present at exceptionally low titer in contrived samples, further supporting assay sensitivity. While the experimental data using contrived samples showed sensitive detection with a broad dynamic range, synthetic material may perform differently than that of intact viral DNA from human specimens.

Table 3. Sensitivity of detecting Clade IIa/IIb MPXV DNA in contrived human samples. Contrived human samples spiked with Clade IIa/IIb MPXV synthetic DNA at a concentration range from 800 to 50 copies per ml were prepared. Nucleic acid was isolated from the samples and analyzed by qPCR with 20 replicate reactions per spiked MPXV DNA concentration.

MPXV Copies/ml in Contrived Human Sample	Total Reactions Analyzed	Number Positive Reactions	Number Negative Reactions	Percent Positive Reactions
800	20	20	0	100
400	20	20	0	100
200	20	18	2	90
100	20	20	0	100
50	20	9	11	45

Conclusions

A highly sensitive qPCR assay can enable the detection of MPXV DNA in human specimens with low viral titer. Based on these results, we present a new multiplex qPCR assay approach that shows sensitive detection of MPXV Clade I and Clade IIa/IIb DNA and can effectively differentiate MPXV specifically from other closely related *Orthopoxvirus* pathogens by distinguishing between Clade I and Clade IIa/IIb. These assays are for research use only (RUO) and could be helpful in public health surveillance of the current mpox outbreak by screening for both MXPV Clades I and IIa/IIb in a single reaction and distinguishing the presence of MPXV separately from other *Orthopoxvirus* species.

This study determined that the multiplex qPCR assays can detect all banked genomic sequences of MPXV (as of August 2022). As shown, the multiplex MPXV assay can detect samples containing currently circulating MPXV Clades IIa/IIb with the G2R_WA primers. Moreover, it can detect MPXV Clade I, which targets the *C3L* gene (Huggett et al. 2022) and will be useful if this viral strain re-emerges. From a public health perspective, tracking Clade I is important, as it is the most severe strain in terms of symptoms.

The ready access to qPCR instruments and reagents in molecular laboratories globally may greatly expand the possibility of routine surveillance for both Clade I and Clade IIa/IIb. While this study has shown that these research-use assays are compatible when used in a multiplex format, further validation with human samples is needed to determine their utility in evaluating the presence of either Clade I or Clade II in human specimens.

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Ordering Information

Catalog #	Description
12010176	**Reliance One-Step Multiplex RT-qPCR Supermix**, 200 x 20 µl reactions, 1 ml
12011319	**CFX Opus 96 Real-Time PCR System**

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