Performance of a Dual-Target Monkeypox Virus Real-Time PCR Assay

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Introduction

- In May 2022, an increasing number of monkeypox (Mpox) virus cases in non-endemic countries, including the United States, was noted.
- At the onset of the current outbreak, the US Centers for Disease Control and Prevention (CDC) diagnostic algorithm included a tiered testing approach that utilized non-variola *Orthopoxvirus* (OPXV) polymerase chain reaction (PCR) testing followed by confirmatory testing for monkeypox virus (MPXV) at the CDC (https://www.cdc.gov/poxvirus/monkeypox/pdf/Monkeypox-lab-process.pdf).
- However, simultaneous testing of OPXV and MPXV may have advantages over tiered testing.
- This study describes the performance of a dual-target, real-time PCR assay that can detect OPXV and MPXV clade II in a single-well format.

Methods

- DNA was extracted from deidentified lesion specimens submitted for clinical testing collected in viral transport media and tested by real-time PCR using an OPXV target (E9L-NVAR)[1] and an MPXV clade II (West African) target (MPXV-WA).[2]
- Analytical sensitivity, specificity, precision, accuracy, and specimen stability during storage were assessed.
- Real-time PCR cycle threshold (Ct) distributions were determined for specimens submitted for clinical testing, deidentified, and stratified by patient age and sex.
- To investigate OPXV-positive and MPXV-negative cases, 9 samples were provided to the Los Angeles County Department of Public Health for Mpox whole genome sequencing.[3]

Results

- The initial limits of detection (LOD) for the MPXV and OPXV targets were established using gBlocks Gene Fragments (Integrated DNA Technologies) and found to be 100 copies/mL and 250 copies/mL (not shown), respectively.
- Confirmatory testing of an individual Mpox-positive specimen spiked into viral transport media collected from negative lesion swabs demonstrated 100% detection for both the MPXV and OPXV targets at 100 copies/mL, confirming the initial LODs (**Table 1**).
- The maximum coefficient of variation (CV) of the replicate Ct values for the MPXV gene target for 5 replicates assessed for intra-assay reproducibility was <3% for all viral load levels (high, medium, and low). Likewise, that for the OPXV gene target was <5% for all viral load levels (**Table 2**).

Results (continued)

Table 1. Limit of Detection Confirmation for Mpox Clinical Specimens[a]

Viral load, copies/mL	Mean Ct			Specimens in Which Target Detected/Specimens Tested		Detection Rate
	MPXV	OPXV	IPC	MPXV	OPXV	
500	32.6	34.1	25.4	5/5	5/5	100%
300	33.8	35.7	25.5	5/5	5/5	100%
200	34.0	34.9	25.3	5/5	5/5	100%
100	34.8	35.8	25.5	20/20	20/20	100%
Positive control	30.0	31.7	25.5	NA	NA	NA
Negative control	-	-	25.9	NA	NA	NA

Ct, cycle threshold; IPC, internal positive control; LOD, limit of detection; MPXV, monkeypox virus; OPXV, *orthopoxvirus*; NA, not applicable.

[a] Testing of 1 specimen spiked at concentrations of 2x, 3x, and 5x the assay LOD (100 copies/mL) and 5 individual Mpox-positive specimens at the assay LOD.

Table 2. MPXV and OPXV Intra- and Inter-assay Precision[a]

Study	Sample	Replicates	Viral load, copies/mL	Mean Ct (CV, %)		
				MPXV	OPXV	IPC
Intra-assay	MPXV-High	5	5,000	30.5 (0.4)	Undet	26.7 (2.1)
	MPXV-Med	5	1,000	32.8 (0.9)	Undet	26.5 (1.6)
	MPXV-Low	5	250	34.6 (1.5)	Undet	26.8 (1.4)
	Negative	5	0	Undet	Undet	25.9 (1.1)
Inter-assay	MPXV-High	5 x 5 runs	5,000	30.6 (1.7)	Undet	27.4 (7.7)
	MPXV-Med	5 x 5 runs	1,000	32.7 (1.4)	Undet	27.0 (6.3)
	MPXV-Low	5 x 5 runs	250	34.6 (1.7)	Undet	27.7 (8.7)
	Negative	5 x 5 runs	0	Undet	Undet	26.3 (3.3)
Intra-assay	OPXV-High	5	10,000	Undet	28.8 (1.9)	27.0 (2.6)
	OPXV-Med	5	2,500	Undet	29.4 (1.3)	27.8 (2.7)
	OPXV-Low	5	500	Undet	33.8 (4.9)	26.3 (1.7)
	Negative	5	0	Undet	Undet	25.9 (1.1)
Inter-assay	OPXV-High	5 x 5 runs	10,000	Undet	29.6 (3.0)	27.1 (5.0)
	OPXV-Med	5 x 5 runs	2,500	Undet	29.9 (4.7)	28.2 (6.8)
	OPXV-Low	5 x 5 runs	500	Undet	34.5 (3.5)	26.1 (1.1)
	Negative	5 x 5 runs	0	Undet	Undet	26.3 (3.3)

Ct, cycle threshold; IPC, internal positive control; MPXV, monkeypox virus; OPXV, *orthopoxvirus*; NA, not applicable; Undet, Undetermined.

[a] Performed using synthetic gene blocks or vaccinia virus.

- In addition, the maximum CV assessed for inter-assay reproducibility in 5 runs was <2% for the MPXV target and <5% for the OPXV target at all levels.
- Analytical specificity was 100% when tested among unrelated pathogens that can also cause lesions (eg, herpes simplex virus, varicella zoster virus). An Mpox-positive clinical specimen diluted in negative swab media was stable for 24 hours at room temperature, 7 days refrigerated, and 30 days frozen.
- A Bland–Altman difference plot from positive clinical samples submitted for testing showed that the mean MPXV Ct values were lower than the mean of the MPXV and OPXV Ct values (**Figure 1**).
- Less than 0.75% of clinical specimens tested had only a single positive target. Mpox whole genome sequencing of 9 OPXV-positive/MPXV-negative specimens by Los Angeles County Department of Public Health identified a 600 bp deletion in both copies of the *crmB* gene that removed the MPXV probe target region.[3]
- Multiple swabs were collected from 26.3% (6,577/24,981) of patients. Ct values from patients with multiple swabs with both Mpox-positive and -negative results were 4.2 Cts (95% CI: 3.4-5.5) higher than those from patients with exclusively Mpox-positive swabs (**Table 3**).
- MPXV Ct value distribution differences by age group were small, with mean values of 21.5-22.0 (**Figure 2**), though still statistically significant (Kruskal-Wallis p=0.018).

Results (continued)

Table 3. Demographics of Patients With Positive, Mixed and Negative Mpox Specimens

Category[a]	Sex	N (%)	Median Age (IQR)	Age Range	Median Ct (IQR)[b]
Positive	Male	6179 (96.8)	34.0 (26.0-44.0)	1-99	20.3 (17.9-24.7)
	Female	153 (2.4)	31.0 (22.0-46.8)	1-96	
	Unknown	51 (0.8)	33.0 (25.0-44.3)	1-71	
Mixed	Male	266 (91.7)	35.0 (29.9-42)	1-70	25.5 (20.0-33.7)
	Female	22 (7.6)	30.5 (24.8-46.1)	16-92	
	Unknown	2 (0.7)	32.5 (32.0-33.0)	32-33	
Negative	Male	10712 (58.5)	35.0 (30.0-43.0)	3-81	NA
	Female	7492 (40.9)	32.0 (25.0-38.3)	1-77	
	Unknown	104 (0.6)	33.0 (29.2-42.0)	21-63	

Ct, cycle threshold; IQR, interquartile range; Mpox, monkeypox.

[a] Positive: all specimens submitted for Mpox testing produced a positive result. Mixed: specimens from patients submitting multiple swabs yielded positive and negative Mpox test results. Negative: all specimens submitted for Mpox testing produced a negative result.

[b] For the MPXV target. Hodge-Lehman shift for mixed vs positive median Cts: 4.2 [95% CI: 3.4-5.1]; p<0.0001.

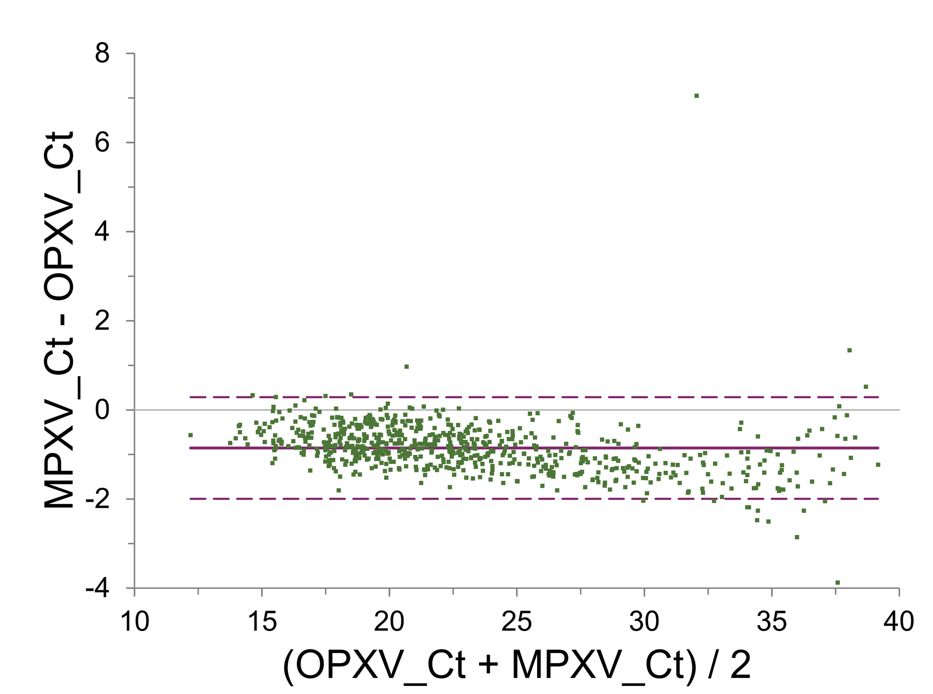

Figure 1. Bland–Altman analysis of MPXV and OPXV Ct values. The mean difference for the Cts between the MPXV and OPXV targets (solid line) was -0.85 (95% CI [dashed lines]: -2.0-0.28).

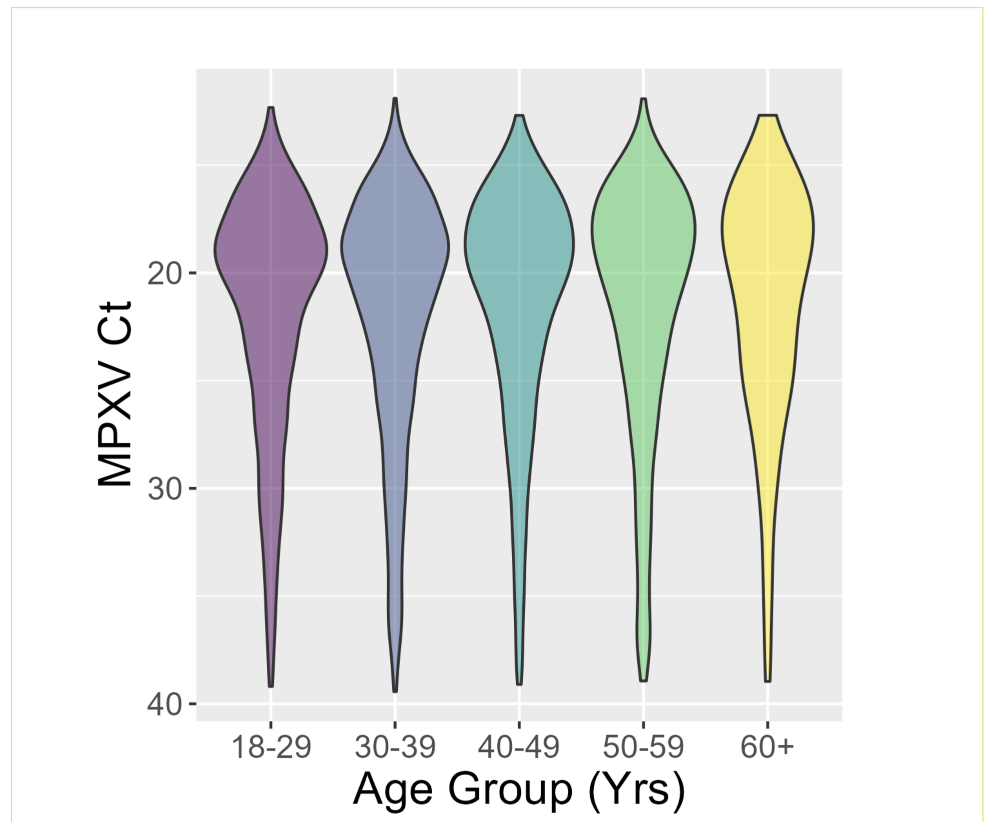

Figure 2. Violin plot of MPXV Ct values by age. For patients with multiple positive results, the lowest Ct value for the MPXV target was used.

Conclusions

- A sensitive, dual-target, real-time PCR assay to enable Mpox diagnosis without the use of tiered testing demonstrated excellent performance in clinical specimens.
- The inclusion of 2 targets in a single well provided redundancy in detection and reduced the risk of false negatives resulting from genomic deletions.
- Overall, these data suggest that both targets, which were originally published as single-target assays, retain high levels of sensitivity and precision when combined into a single-well, dual-target assay.
- Patients with discordant positive and negative swabs had higher Ct values than those with concordant swabs, which may be associated with variability in lesion stage and swabbing efficiencies.
- Ct values varied slightly by patient age group but did not display a bimodal distribution in younger age groups as in a previous study.[4]
- Access to an accurate, high-throughput assay for MPXV is critical to support national testing capacity and will allow for improved patient care and outbreak management.

References

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