Development of Novel Multiplex Panels for Detection and Differentiation of Monkeypox / Mpox Virus from Other Orthopox Viruses

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ABSTRACT: The 2022 outbreak of the Mpox virus (MPXV) spread to at least 75 countries, with about 85,115 cases worldwide. The 2022 outbreak was declared a public health emergency on July 23, 2022, by the World Health Organization (WHO). That prompted a renewed interest in the scientific community to develop rapid detection and diagnostic assays for identifying this enveloped DNA virus belonging to the family *Poxviridae*, genus orthopoxvirus (OPXV). Other closely related members are the variola virus (VARV) and zoonotic vaccinia (VACV), cowpox (CPXV), Alaskapox, and Akhmeta viruses. These viruses cause human febrile disease with a rash, ranging from benign lesions to a severe fatal systemic infection such as smallpox. We have developed and evaluated two multiplex assays; 1) multiplex antigen test to detect and differentiate MPXV from other OPXV, 2) multiplex field-portable real-time polymerase chain reaction (PCR) test to detect and differentiate MPXV DNA from OPXV. The multiplex antigen detection assay uses Luminex xMAP® technology with a set of three capture antibodies immobilized on three different microspheres and a biotinylated detection antibody. Using this assay, we tested two Mpox strains/isolates, hMPXV/USA/MA001/2022 and USA-2003. This assay detected both isolates in the 200 - 1000 pfu/mL range. The reactivity pattern on three beads differentiated the MPXV from VACV and CPXV. Multiplex real-time PCR assay reagents for the detection of MPXV include three forward and three reverse primers and two probes; FAM-labeled for MPXV and CFR-labeled for OPXV. The *in silico* characterization of all primers and probes by screening them against all publicly available MPXV sequences downloaded from GISAID and NCBI on October 10, 2022, shows high specificity for the MPXV targets. We observed that the depositors label many MPXV sequences present in the dataset as partial or unverified. The analytical sensitivity of the assay was determined to be 20 copies per reaction using synthetic DNA from BEI Resources (Catalog Number NR-58627). We performed the assay specificity test using VACV and CPXV. Further characterization of the two multiplex assays is in progress. These two assays will be helpful for orthogonal detection and confirmation of this disease of low prevalence.

MATERIALS AND METHODS: We have developed and evaluated two different methods for orthogonal detection and differentiation of MPXV from OPXV. We have evaluated a field potable thermocycler T-COR 8™ for detection of molecular signature and xMAP® technology based immunoassay for antigenic signature of MPXV and OPXV.

Multiplex PCR Reagents and Method:

- We tested a multiplex assay that includes primers and probes for detection and differentiation of MPXV from OPXV. In addition we have two controls, one inhibition control and another RNase P control added in this assay.
- The MPXV detection reagents include three forward and three reverse primers and two FAM-labeled probes. This assay has one primer-probe set designed by CDC. The other two primer sets and probe were designed at Tetracore.
- The OPXV assay reagents are same as CDC Non-Variola Orthopox virus assay.
- The assay can be completed in 30 minutes.

Multiplex Immunoassay Reagents and Method:

- Three different antibodies, two mouse monoclonal (Mab) and one rabbit polyclonal were covalently coupled to carboxylated magnetic microspheres.
- A biotinylated Mab was used as detection antibody and streptavidin-phycoerythrin (SAPE) was used as the reporter molecule.
- In addition to three analyte microspheres we added 4 internal controls to each well to monitor the assay performance at every step for proper addition of assay reagents, matrix effects, and correct performance of the reader in each well.
- Briefly, in a 96 well plate the capture antibody coupled microspheres were mixed with sample, buffer or standard and incubated at room temperature while shaking for 30 minutes. The plate was washed using automated plate washer and three times and incubated with biotinylated antibody for 30 minutes while shaking. Plates were washed again as before and incubated with SAPE for 15 minutes. Plates were washed at the end of the incubation and microspheres were then re-suspended in wash buffer and analyzed on MAGPIX® reader.

Evaluation Reagents and Method:

(Table1). Reagents used for evaluation of the two methods. MPXV was from BEI resources. Crude virus preps were inactivated by 4.4 x 10^6 Rads of γ-irradiation

#	Test materials (PFU/mL)
1	Cowpox virus, Norway94 (3 X 10^6)
2	Cowpox virus, Germany98 (3.5 X 10^7)
3	Cowpox virus, Finland (3 X 10^7)
4	Lesion swab eluates from 45 clinical samples

The lesion swab eluates were tested in BSL-2 lab for PCR. We heat inactivated the lesion swab eluates and stock MPXV at 60°C for three hours for testing in immunoassay in a BSL-1 lab.

Figure 1. Portable Thermocycler T-COR 8™, with a portable mixer, test cartridge, T-COR 8 tubes, and simple sample collection device.

RESULTS OF PCR TEST EVALUATION

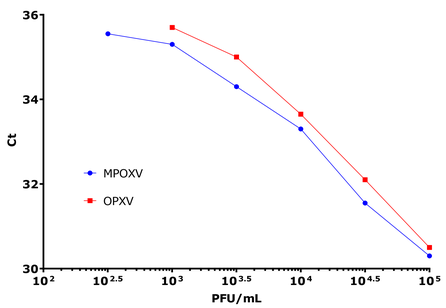

Figure 2. Analytical sensitivity determination by direct testing of heat inactivated Mpox virus in the range of 100 to 10^5 PFU /mL.

Table 2. Analytical sensitivity determination by testing of synthetic DNA from BEI Resources NR-58627 in the copy number range of 0.1 to 3 copies/µL.

Testing on T-COR 8	Mpox		Orthopox	
Copies of synthetic DNA /µL	FAM-Average Ct	# pos/ total	CFR-Average Ct	# pos/ total
NR-58627 3E0	34.1	20/20	33.9	20/20
NR-58627 1E0	35.1	24/24	35.0	24/24
NR-58627 3E-1	35.7	19/20	35.6	17/20
NR-58627 1E-1	35.6	10/24	35.5	4/24

- During initial development, we evaluated assay repeatability using quantitative synthetic DNA standard at low levels of DNA copies using 20 replicates. The assay was 95% sensitive at 0.3 copies/µL (Table 2). The assay was further modified for direct detection using cultivated Mpox virus.
- We obtained Mpox virus hMPXV/USA MA002/2022 that was isolated from a human in Massachusetts, in May 2022 (NR-58622) from BEI resource and used this as a standard. The virus was cultivated as per BEI instructions. The concentration of the virus stock was determined to be 1.95 X 10^6 PFU/mL. This stock was heat inactivated and tested by plaque assay. No plaques were seen in heat inactivated virus.
- This stock was tested directly by diluting in water. The analytical sensitivity of the assay was determined to be 300 PFU/mL (Figure 2).
- The diagnostic sensitivity was assessed using 45 clinical samples obtained as lesion swab eluates in PBS or UTM. There was 100 % positive concordance between CDC and Tetracore assays.
- We also tested three different Cowpox strains for which PCR results were unknown. They were all negative for Mpox and 2 out of three were positive for non-variola orthopox assay.
- A ZeptoMetrix positive control (NATMPXV-ERC) was also used as an external control. It tested positive in both Mpox and Orthopox assays.
- We also performed *in-silico* characterization of all primers and probes for MPXV detection by screening them against all publicly available MPXV sequences downloaded from GISAID and NCBI on October 10, 2022. The MPXV target sequences for the CDC and Tetracore assays are ~18,000 bases apart.

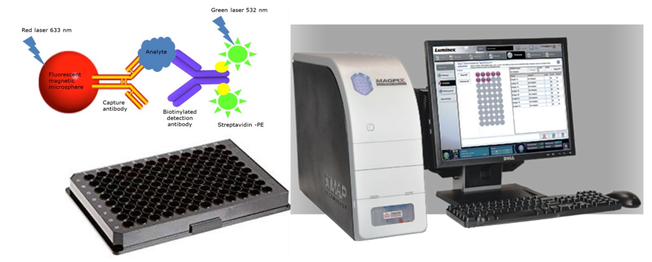

Figure 3. Schematic representation of a multiplex antigen detection assay using xMAP® Technology.

RESULTS OF IMMUNOASSAY EVALUATION

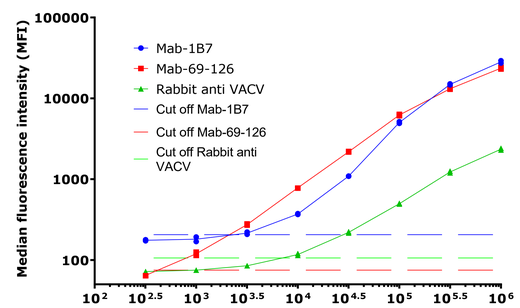

Figure 4. Analytical sensitivity determination by titration of heat inactivated Mpox virus in the range of 100 to 10^6 PFU /mL.

- The heat inactivated MPXV stock was serially diluted to determine the analytical sensitivity of the multiplex immunoassay. We determined that the analytical sensitivity of the assay was 1000 PFU/mL (Figure 3).
- We observed a differential immunoreactivity pattern for CPXV and MPXV viruses in this multiplex assay.
- The Mab-69-126 has higher immune reactivity in this assay compared to Mab-1B7 and rabbit polyclonal for detection of MPXV.
- Three CPXV strains tested in this study display similar immunoreactivity pattern. The reactivity of these viruses is higher with Mab –1B7 and rabbit polyclonal.
- Mab-69-126 displays higher specificity of detection for MPXV.
- We tested the 45 clinical lesion swab eluates and found only 6 out of 45 clinical samples to be positive using this assay. Two out of six clinical samples showed a reactivity pattern similar to CPXV. The clinical testing needs to be re-evaluated further.

Concluding remarks:

- The Tetracore multiplex PCR assay reagents, simple easy-to-use assay protocol in combination with T-COR 8 portable thermocycler can be effectively used for detection of MPXV DNA in lesion swabs in the field.
- The analytical sensitivity of multiplex immune assay is determined to be about 3– 10 fold less sensitive than PCR.
- The multiplex immunoassay can be performed in less than 2 hours and up to 96 samples may be tested in one run.
- We observed that 27 out of 45 clinical samples had Ct values below 30. 6 out of these 27 samples were positive for antigen detection. There was no concordance or relationship between Ct values in PCR assay for 45 clinical samples and antigen detection.
- We plan to test negative lesion swab samples in future studies. We are looking for collaborators and partners for evaluation of these assays in future.

Acknowledgments:

The following reagent was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: Monkeypox virus, hMPXV/USA/MA001/2022 (Lineage B.1, Clade IIb), NR-58622; Monkeypox Virus, USA-2003, NR-2500; Quantitative Synthetic DNA from Monkeypox Virus, NR- 58627