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Diagnosis of Monkeypox infection: Validation of two diagnostic kits for viral detection using RT-PCR

Meital Elbaz [a,*], Ora Halutz [b], Yaniv Ali [b], Amos Adler [b,c]

[a] Infectious disease unit, Tel Aviv Sourasky Medical Center, Israel
[b] Clinical Microbiology Laboratory, Tel Aviv Sourasky Medical Center, Israel
[c] Sackler Faculty of Medicine, Tel Aviv University, Tel-Aviv, Israel

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ABSTRACT

Monkeypox virus, a zoonotic Orthopox DNA virus was rarely reported outside of African regions until April 2022. Since then, thousands of cases have been reported worldwide. In order to cope with the increasing need for laboratory diagnosis, the availability of reliable commercial PCR assays is of paramount importance. In this study we compared the diagnostic performance of two commercial real-time (RT)-PCR assays, the Novaplex™ MPXV Assay and the Bio-Speedy® Monkeypox Virus qPCR Kit, for the detection of Monkeypox virus (MPXV) DNA from 154 human samples. These assays were compared to a recently published in-house assay that included a general MPXV target (G2T) and a West African specific target (genericWA). All assays demonstrated 100% specificity. While sensitivity of the Novpalex assay was 100% the sensitivity of the other assays was lower; 94% for the Bio-speedy assay and G2R assay and 88% for the genericWA assay. The sensitivity differences between the methods manifested almost entirely in those pharyngeal samples in which the Ct values were high (≥35). The Novaplex™ MPXV Assay showed higher Ct values compared with the other methods with a median of 27.1 compared with the Bio-Speedy assay (median 15.8, $p < 0.001$), the G2R assay (median 23.5, $p < 0.001$) and the genericWA assay (median 23.6, $p < 0.001$). For all 4 methods, the Ct values were higher in samples taken from oropharynx compared with samples from rectal and pustule swabs.

Monkeypox virus, a zoonotic Orthopox DNA virus related to the virus that causes smallpox, was first described in humans in 1970 in the Democratic Republic of Congo (formerly Zaire) (Ladnyj et al., 1972). Since then, Sporadic outbreaks of infection have been reported in Africa (CDC, 1997). Until the 2003 US outbreak (Reed et al., 2004), no human cases had been reported outside of Africa, and therefore research of the disease and its diagnosis has been limited. Since May 2022, more than 47000 Monkeypox virus infections have been reported by the CDC in countries where the disease is not endemic (https://www.cdc.gov/-poxvirus/monkeypox/response/2022/index.html, 2022), prompting the World Health Organization to declare the ongoing Monkeypox outbreak a Public Health Emergency of International Concern on June, 2022 (Multi-country monkeypox outbreak: situation updateWorld Health Organization, 2022). In Israel, 215 patients were diagnosed between May 2022 and August 2022 (https://www.gov.il/he/departments/guides/disease-monkeypox, 2022). The laboratory diagnosis is based on PCR testing, as the disease may resemble other types of infections (e.g., HSV1) and is new in many parts of the world. So far, methods for testing were based on in-house protocols published by the WHO (WHO, 2022), NIH (Laboratory Guidelines for the Detection of Monkeypox Virus, 2022) and by Li et al (Li et al., 2010). In order to cope with the increasing need for laboratory diagnosis, the availability of reliable commercial PCR assays is of paramount importance. In this study we aim to compare the diagnostic performance of two commercial real-time (RT)-PCR assays, the Novaplex™ MPXV Assay and the Bio--Speedy® Monkeypox Virus qPCR Kit, for the detection of Monkeypox virus (MPXV) DNA from human samples. A previously published assay (Li et al., 2010) served as a reference method.

This validation study was performed on a collection of 154 clinical samples collected between January 1, 2022 and August 10, 2022. Samples were submitted to the Clinical Virology Laboratory at the Tel Aviv Sourasky Medical Center (TASMC), a tertiary care center at the heart of the MPXV epidemic in Israel. The validation included 130 samples that were sent for MPXV testing (including pustule fluid swabs, oropharyngeal swab and rectal swabs) and 24 skin samples that were previously tested positive for HSV1, HSV2 or VZV. The study was

* Correspondence to: Infectious Disease Unit, Tel Aviv Sourasky Medical Center, 6 Weizman St, Tel Aviv, Israel.
E-mail address: meitale@tlvmc.gov.il (M. Elbaz).

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Table 1
Diagnostic performance of the four assays used in the study for the detection of MPXV.

Assay	Novaplex™ MPXV (%)	Bio-Speedy (%)	G2R (%)	Generic WA (%)
Sensetivity	100	94	94	88
Specificity	100	100	100	100

MPXV: Monkeypox virus; G2R: MPXV generic (G2R G) assay; Generic WA: MPXV West African specific assay.

approved by the TASMC ethics committee.

Samples were collected by swabs that were inoculated into UTM tubes and transported to the laboratory. In general, the sampling collection policy based on the Israeli Ministry of Health instructions (Israeli ministry of health guidelines for sampling and diagnosis of Monkeypox, 2022) was to collect additional pharyngeal samples in patients with skin lesions, irrespective of pharyngeal symptoms. Nucleic acid extraction was done using the easyMAG® system (BioMérieux, Marcy-l'Étoile, France). Two commercial multiplex RT-PCR assays were evaluated in this study: 1) the Novaplex™ MPXV Assay (Seegene, Seoul, Republic of Korea); 2) the Bio-Speedy® Monkeypox Virus assays (Bioeksen, Istanbul, Turkey). Both assays included one MPXV target and one internal control target and were prepared and performed according to the manufacturers' instructions (https://www.seegene.com/assays/novaplex_mpxv_assay and https://www.bioeksen.com.tr/Media/Documents/biospeedy–monkeypox-virus-qpcr-kit-brochure8dcea.pdf, respectively). These assays were compared to a recently published in-house assay (Li et al., 2010) that included a general MPXV target (G2T) and a West African specific target (genericWA), both targeting the TNF receptor gene. The MPXV target gene in the Bio-Speedy® Monkeypox Virus assay was the F3L gene and was designed to cover both the West Africa and the Congo clades (personal communication). The target gene was not disclosed by the manufacturer of the Novaplex™ MPXV Assay, but according to the company's statement it was designed to cover both the West Africa and the Congo clades. All four assays were run on a Bio-Rad CFX96 RT-PCR instrument. The results of the G2T and the genericWA assays were analyzed using the instrument's software, while the Novaplex™ MPXV and the Bio-Speedy® Monkeypox assays were analyzed by a designated software of the manufacturers. The Ct maximal positivity threshold were determined according to the manufacturer instructions as follows: 1) Novaplex™ MPXV- 45 cycles; 2) the Bio-Speedy® Monkeypox-35 cycles; 3) genericWA and G2T- 45 cycles.

Since none of the tests had long lasting experience to allow it's use as a gold standard, the definition of 'true positive' was based on the following criteria: 1) positive result obtained by ≥one assay; 2) positive result obtained by one assay from a patient with additional positive sample from another site (e.g., pharyngeal and pustular samples).

Of the 154 samples, 104 (67.5%) were true positive for MPXV by at least one assay and 50 (32.5%) were tested negative, including all of the 24 HSV1/HSV2/VZV-positive samples.

Samples positive for MPXV were mostly from pustule fluid (n = 63, 60.5%), followed by oropharyngeal swab (n = 32, 30.8%,) and rectal swab (n = 6, 5.8%), collected from the same patients with pustular lesions. Sample source was not documented in three samples.

In the MPXV -negative group, 36 samples (72%) were collected from pustule fluid, followed by oropharyngeal and rectal swab (n = 10,20%, and n = 3, 6%, respectively). In one sample, the source was not documented.

The sensitivity and specificity values of all four assays are presented in Table 1.

Only 92 samples were positive in all four methods. The Novaplex™ MPXV Assay identified 104 positive samples, of which 98 samples were tested positive by additional one method and five samples were from patients that were tested positive from additional site. Hence, the sensitivity value was 100%. The Bio-Speedy® Monkeypox kit identified 98 positive samples out of 104 true positive samples, thus found to have 94% sensitivity. The G2T and the genericWA assays found 98/104 and 92/104 positive samples, respectively with sensitivity values of 94% for the G2T assay and 88% for the genericWA assay.

Table 2
Ct values for different sample sites.

Sample site	Novaplex™ MPXV, median Ct value (IQR)	Bio- speedy, median Ct value (IQR)	G2R, median Ct value (IQR)	genericWA, median Ct value (IQR)
Pustule fluid (n = 63)	24.4 (22.9–27.6)	14.2 (11.7–16.6)	22.3 (21.3–24.7)	22.2 (21.2–24.7)
Oropharyngeal swab (n = 32)	37.8 (32.2–40.5)	25.7 (21.1–27.8)	32.3 (28.8–34.8)	33.4 (29.5–35.5)
Rectal swab (n = 6)	24.7 (23.8–28.1)	14.3 (12.6–16.1)	22.2 (20.9–25.6)	23.4 (21.5–26.4)
Total[a] (n = 101)	27.1 (23.5–36.4)	15.8 (12.8–22.2)	23.5 (21.4–29.5)	23.6 (21.5–29.7)

Ct: cycle threshold
[a] excluding the three samples from unknown site.

All false negative results in the Bio-Speedy assay (n = 6) and the G2R assay (n = 6) were in samples taken from oropharyngeal swab. For the genericWA assay, most false negative results were in sample from oropharyngeal swab (n = 9), followed by pustule fluid swab (n = 2) and 1 sample for whom the source was not documented.

Positive samples were detected with a Ct values of 17–44 and 8–32 in the Novaplex™ MPXV Assay and Bio-Speedy® Monkeypox Virus qPCR Kit, respectively (Table 2). For the G2R assay and the genericWA assay the Ct values were 17–39 and14–35, respectively. For all 4 methods, the Ct values were higher in samples taken from oropharynx compared with samples from rectal and pustule swabs (Table 2).

In this study we validated two new RT-PCR assays, the Novaplex™ MPXV and the Bio-Speedy® Monkeypox Virus qPCR Kit assays in comparison with a previously described in-house assays (9) for the detection of Monkeypox infection. The new assays demonstrated 100% specificity, while sensitivity of the Novpalex assay was 100%, higher compared with the other methods. In theory, mutation at the TNF receptor gene could have explained the lower sensitivity of the in-house assays, as was recently reported by the CDC (https://www.cdc.gov/locs/2022/09–02–2022-lab-alert-MPXV_TNF_Receptor_Gene_Deletion_May_-Lead_False_Negative_Results_Some_MPXV_Specific_LDTs.html#print). However, as the decreased sensitivity was observed also by the Bio-Speedy® Monkeypox Virus assay, this is less likely.

The sensitivity differences between the methods manifested almost entirely in those pharyngeal samples in which the Ct values were high (≥35). Although we do not possess clinical data on these cases, we can assume that in many of these cases, samples were collected even without pharyngeal symptoms (Israeli ministry of health guidelines for sampling and diagnosis of Monkeypox, 2022). A recent report (Paran et al., 2022) found that samples with a Ct values of ≥ 35 are unlikely to contain a viable virus. However, that study used only the reference method that was used in our study (Li et al., 2010), whereas our results indicate that Ct values cannot be considered equivalent when different methods are being used. Hence, the clinical-epidemiological significance of these positive pharyngeal samples and the exact Ct threshold determining infectivity (by using the other PCR methods) remains uncertain.

In conclusion, our study provides valuable methodological data about two novel commercial assays, that could serve as an important tool for the diagnosis of MPXV in clinical laboratories.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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