Emerging Microbes & Infections
2024, VOL. 13, 2337666 (7 pages)
https://doi.org/10.1080/22221751.2024.2337666

RESEARCH ARTICLE

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Evaluation of analytical performance of the STANDARD™ M10 MPX/OPX assay for the simultaneous DNA detection and clade attribution of *Monkeypox virus*

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ABSTRACT

Monkeypox virus (MPXV) infection confirmation needs reliable polymerase chain reaction (PCR) assays; in addition, viral clade attribution is a key factor in containment measures, considering a more severe syndrome in clade I and the possibility of simultaneous circulation. This study evaluates the performance of all-in-one STANDARD M10 MPX/OPX (SD BIOSENSOR, South Korea – M10). Frozen samples from 205 subjects were selected and stratified according to routine test results (RealStar® Orthopoxvirus PCR Kit 1.0, Altona DIAGNOTICS, Germany – RS; RS-1): in detail, 100 negative skin lesions (SL) and 200 positive samples at the variable stage of infection were analysed. Positive samples were retested with RS (RS-2). Positive and Negative Percent Agreements (PPA, NPA) were calculated. The median (IQR) C_t values of RS and M10 (OPXV target) assays were highly similar. The PPA of M10 compared to RS-1 was 89.5% considering system interpretation, and 96.0% when the operator classified results as positive if any target was detected; NPA was 100%. Comparing the RS-2 run and M10, an overall concordance of 95.3% between assays was found; however, considering operator interpretation, M10 returned more positive results than RS-2. The occurrence of False-Negative results was likely associated with the influence of thawing on low viral concentration; no False-Positive tests were observed. All samples collected at the time of Mpox diagnosis were positive and M10 correctly attributed the clade (West-Africa/II). The M10 MPX/OPX assay demonstrated high reliability in confirming MPXV infection and clade attribution.

ARTICLE HISTORY Received 12 November 2023; Revised 17 March 2024; Accepted 27 March 2024

KEYWORDS Monkeypox virus; clade determination; methods comparison; all-in-one RealTime-PCR; Orthopoxvirus

Introduction

Monkeypox (Mpox) is a viral disease endemic in several African countries, that is caused by the *Monkeypox virus* (MPXV), a double-stranded DNA virus belonging to the *Orthopoxvirus* (OPXV) genus, which includes also the *Variola virus*, the aetiological agent of human Smallpox [1]. Two distinct viral clades were identified, the Congo Basin (Central African) and the West African, recently renamed as clade I and clade II, respectively: such genetic differentiation seems to influence host immune response, with clade I expressing several proteins linked to the suppression of cellular and humoral functions [2–5].

MPXV animal reservoir remains still uncertain but is thought to be one or more African rodents or other small mammals (e.g. *Funisciurus* spp., *Heliosciurus* spp.) [6]. Apart from incidental zoonotic transmission [7], MPXV can be transmitted between humans through respiratory droplets, direct contact with skin lesions and body fluids, and indirect contact with infected materials [3,8].

Since the virus discovery in Denmark in 1958 from a primate (*Macaca fascicularis*) skin lesion, periodic outbreaks of Mpox have been reported in West African countries and the Congo basin [9].

In May 2022, the World Health Organization was notified of a Mpox case in the United Kingdom, in a subject recently returned from Nigeria; however, in a few days the number of infected individuals increased and a multi-country outbreak was identified [10]. Such epidemiological expansion claimed a huge effort in terms of preventive measures and diagnostic tools. The STANDARD M10 MPX/OPX test is a rapid, multiplex real-time PCR intended for use with the STANDARD M10 system. The assay can detect OPXV-DNA and MPXV-DNA, also differentiating viral clades. This assay works on a single device with results obtained after one-hour run. It is based on an all-in-one working cartridge: nucleic acid extraction

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and amplification are consecutively executed in a fully automated and closed system, requiring minimal training with a consequent less cross-contamination risk [11,12]. Such characteristics would improve rapid Mpox case confirmation and definition of preventive measures.

A comparative retrospective study was conducted to evaluate the STANDARD M10 assay performance as the primary aim. Clinical samples collected from patients with confirmed or excluded MPXV infection, already tested with available reference tests, were selected.

Materials and methods

Study population

The study population included subjects already tested for MPXV infection, for whom frozen samples and data on viral clade were available. Patients were selected among those attending two Mpox Lombardia clinical referral centres, *L. Sacco* University Hospital (ASST Fatebenefratelli Sacco, Milan) and *San Raffaele* Hospital (Gruppo San Donato, Milan): during the 2022 outbreak, the two centres accounted for the majority of Mpox cases in Italy and for both the laboratory diagnosis was performed at the Laboratory of Clinical Microbiology, Virology and Bioemergencies of Sacco Hospital identified as Regional Monkeypox Laboratory Reference Centre. A total of 100 negative and 200 positive specimens were included, and data on biological matrixes, time of collection, and Cycle threshold (C_t) values were recorded.

The following matrixes were considered: swabs of skin lesion (SL), plasma (PL), anal swabs (AS), oropharyngeal swabs (OPS), and urethral swabs (US).

All 100 negative samples were selected from the SL subgroup (SL-), while all 100 positive skin samples were retrospectively selected based on the C_t value obtained from the reference test performed: sample selection was conducted to have a rather similar distribution among high, medium and low positive, to investigate analytical sensitivity. The SL + samples were consequently stratified according to C_t at diagnosis in three groups: C_t 14–24 = 34 (SL + 1), C_t 25–32 = 35 (SL + 2), and C_t >35 = 31 (SL + 3). All samples were thawed at room temperature and then assayed.

To analyse other samples than skin lesions, AS (20), OPS (16), and US (16) were collected from 9 patients with a median time of collection of 7 days each (±2 days) and then classified as Follow-Up (FUP) controls for a total of 52 positive samples FUP.

Moreover, a total of 48 positive plasma samples were included in the evaluation (Table 1).

Positive samples were simultaneously tested with index and reference assays to cope with freeze-thawing influence on results: an equal amount of viral DNA was tested with both methods.

Index test

The STANDARD™ M10 (SD Biosensor, Inc., Republic of Korea) is a user-friendly, rapid molecular platform that integrates nucleic acid extraction and amplification in an all-in-one cartridge, returning a result in approximately 60 min. The STANDARD™ M10 MPX/OPX (henceforth referred to as M10 MPX/OPX) allows the detection of four genetic viral targets: E9L of OPXV genus; G2R of MPXV; F3L~F4L (intergenic region) of MPXV clade I (formerly Congo Basin), and OPG181~OPG185 (the intergenic region) of MPXV clade II (formerly West Africa). The bacteriophage MS2 gene is included as Internal Control. The tests were performed according to the manufacturer's instructions: after thawing, samples were briefly centrifuged, the cartridge safe lap was removed, and the sealing layer punched; a 300 µL sample volume was transferred into the cartridge, which was then loaded on the analyser. The algorithm resulted in one of the following: Invalid = IC C_t > 30 or E9L negative plus G2R and/or clades positive; Negative: C_t > 38 for all targets; Positive: C_t < 38 at least for E9L. Positive results were further classified as OPXV = only E9L positive; MPXV = E9L plus G2R positive; WA or CB, according to amplification of clade-specific probe. Besides automatic results,

Table 1. Assay results stratified according to sample group. The table shows a loss of positive rate for both RealStar® OPX-2 and M10 MPX/OPX as compared to RealStar® OPX-1, probably due to freezing-thawing process; beside that, M10 appeared to be more sensitive than RealStar® OPX-2. Moreover, after the operator's interpretation the concordance with RealStar® OPX-1 sensitively increases, evidencing better performance of the index test.

Sample group	RealStar OPX-1			RealStar OPX-2			M10 MPX/OPX automatic			M10 MPX/OPX operator		
	POS	NEG	INV	POS	NEG	INV	POS	NEG	INV	POS	NEG	INV
SL-1	34	0	0	34	0	0	34	0	0	34	0	0
SL-2	35	0	0	33	2	0	33	2	0	33	2	0
SL-3	31	0	0	20	11	0	23	7	1	28	3	0
FUP	52	0	0	41	11	0	47	3	2	51	1	0
PL	48	0	0	46	2	0	42	4	2	46	2	0
NEG	0	100	0	0	100	0	0	100	0	0	100	0
Total	200	100	0	174	126	0	179	116	5	192	108	0

POS: positive; NEG: negative; INV: invalid.

operator interpretation was performed, considering all positive samples with at least one target amplified; samples with a $C_t > 40$ were classified negative.

Reference test

The RealStar® Orthopoxvirus PCR Kit 1.0 (altona Diagnostics GmbH, Germany), henceforth referred to as RealStar® OPX, was used as a reference test, being the one used in the diagnostic routine. It is a standard RealTime-PCR assay, allowing detection and differentiation between non-variola Orthopoxvirus species (*Cowpox virus*, *Monkeypox virus*, *Racoonpox virus*, *Camelpox virus*, and *Vaccinia virus*) and *Variola virus*-specific DNA. Nucleic acid extraction and plate set-up were performed on the AltoStar® Automation System AM16 (altona Diagnostics GmbH, Germany), according to the manufacturer's instructions; the RT–PCR was performed with a CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad, Inc., USA). Results with a $C_t > 40$ were classified as negative. The original test at the diagnosis was identified as RealStar® OPX-1, while the repetition was RealStar® OPX-2.

Performance evaluation

Positive Percent Agreement (PPA), Negative Percent Agreement (NPA), and overall concordance were calculated for RealStar® OPX-2 vs M10 MPX/OPX, RealStar® OPX-1 vs M10 MPX/OPX and RealStar® OPX-1 vs RealStar® OPX-2. PPA, NPA, and overall concordance were defined as follows: PPA was the percentage of positive samples classified as positive by both methods; NPA was the percentage of negative samples classified as negative by both methods; overall concordance was the percentage of samples with the same result by both methods. Concordance for clade attribution was evaluated for M10 MPX/OPX against sequence analysis. The minimum sample size was determined in 93 for both PPA and NPA, assuming individual discordant pairs $k = 1$, discordance rate $\alpha = 0.05$, and tolerance probability $\beta = 95\%$ [13].

Results

A total of 300 samples were assayed, representing 205 subjects: the 100 negative samples were from 100 individuals, while the 200 positive ones were distributed among the remaining 105 patients. The positive patients were all infected with MPXV clade II as per previous characterization with sequencing analysis. RealStar® OPX-2 did not return any invalid result, while a rerun was performed with M10 MPX/OPX on two samples (0.7%), with negative IC: both retests were successful and the samples were included. Five tests were classified as invalid according to M10 interpretation rules: these results were excluded from the initial comparison since it was not possible to compare them with the corresponding result by OPX-2 (negative or positive).

Table 1 shows the comparison of data obtained with RealStar® OPX-2 and M10 MPX/OPX against the RealStar® OPX-1. For both assays, a loss in positive rate was observed, while all negative samples were accordingly identified: the overall concordance with original results was estimated at 92.9% (274/295) and 94.6% (279/295) for RealStar® OPX-2 and M10 MPX/OPX, respectively. All False-Negative (FN) mismatches were found in the presence of a RealStar® OPX-1 $C_t \geq 32$; a viral DNA amplification was detected in 174/200 (87.0%) RealStar® OPX-2 and 179/200 (89.5%) M10 tests, suggesting a slightly higher sensitivity of M10.

The agreement between parallel assays was higher: excluding the M10 MPX/OPX 5/300 invalid specimens, the same result was obtained in 95.3% (281/295) cases, while in the remaining ones, M10 MPX/OPX was more frequently positive than RealStar® OPX-2 (11/14 versus 3/14).

Since the M10 MPX/OPX analyser algorithm classifies as negative or invalid also samples for which at least one target was amplified, results were manually reinterpreted considering invalid only tests with negative IC and negative those with no viral target detection. The total of positive specimens increased to 192/200, of which 158/192 (82.3%) for all targets, 9/192 (4.7%) for two targets, and 25/192 (13.0%) for one. The MPX G2R gene was the most frequently positive target (184/200, 92.0%), followed by OPX E9L (177/200, 88.5%) and MPX WA (169/200, 84.5%). Table 1 shows how the operator interpretation changed the M10 results' categorization, inducing a reduction of RealStar® OPX-2/M10 concordance (92.7% versus 95.3%), as well as the increase of PPA (96.0% versus 89.5%) and overall concordance (97.3% versus 93.0%) between RealStar® OPX-1 and M10. The original C_t of the 8 still negative samples ranged 32–38 by RealStar® OPX-1. The MPXV clade II was always correctly attributed by M10 OPX/MPX, with both algorithm and operator interpretations. Considering C_t values of samples positive with both M10 and RealStar® OPX-2, the assays demonstrated high correlation, with differences among single M10 target genes: even with an $R^2 \geq 0.93$ for all targets (Figure 1), M10 showed lower median C_t in all targets (ΔCt range 0.82–3.06), being MPX G2R the one with the highest difference in median C_t (28.34 versus 31.40).

Regarding the different materials, no biological matrix-related interference was detected: the IC signal was always present, while FN results were attributable to genome concentration variation, as shown above. An additional clinically relevant evaluation was made considering the time of collection, calculating

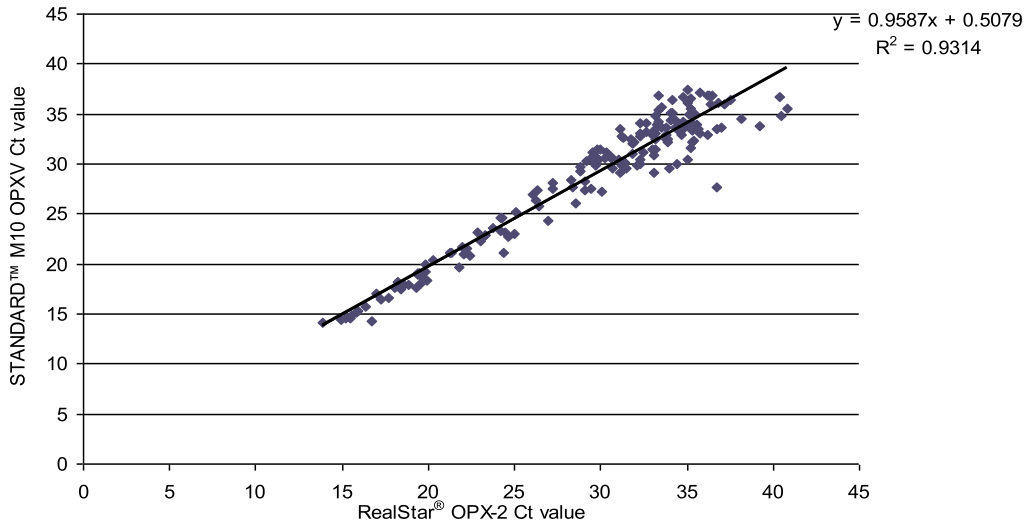

Figure 1. Assay correlation of cycle threshold (C_t) values. Correlation between the RT-PCR cycle threshold values obtained by RealStar® OPX-2 and STANDARD™ M10 MPX/OPX gene E9L targeting for Orthopoxvirus genus. C_t = cycle threshold.

the positive rate of samples collected at patients' first admission ($N = 88$), to identify any missed diagnosis: 97.7% were positive and skin lesions, the WHO recommended matrix for laboratory diagnosis, represented the 48.9% of specimens; notably, the 2/88 (2.3%) negative tests, also confirmed by RealStar® OPX-2 and supported by the C_t was 32 in RealStar® OPX-1, belonged to this group.

Discussion

The present study evaluated the performance of the new M10 MPX/OPX assay, a single-test cartridge that combines nucleic acids extraction, amplification, and detection of genomic targets: results were compared to the laboratory RT–PCR for *Orthopoxvirus* infection diagnosis, showing high concordance, PPA, and NPA.

Strong efforts were made to contain Mpox diffusion and to better characterize the disease, also improving diagnostic capacities: the usually restricted virus circulation limited research opportunities, while the 2022 outbreak allowed interdisciplinary cooperation to grow scientific and clinical communities' knowledge [14,15]. Before the epidemic started, few commercial assays for *Orthopxvirus* were available, requiring advanced expertise for reliable diagnosis: in-house PCR protocols, sequence analysis, virus isolation, and serological tests were indeed performed in referral centres, complicating and delaying the diagnostic process [16–21]. However, the rapid changes in the epidemiology scenario claimed for larger diagnostic networks and tools, to save time and to maximize management and containment [14]. More RT–PCR assays were then marketed, with different characteristics and performances. Papadakis et al. compared results obtained using 5 homemade and 11 commercial assays, targeting the *Orthopoxvirus* genus or *Monkeypox* virus, finding a good correlation among them; however, clinical samples represented a limited proportion of the total amount [22]. Similarly, two other groups validated MPXV-specific RT–PCR. The first assayed 154 clinical samples, of which 130 were from patients with a suspected MPXV infection and 24 were positive for HSV-1, HSV-2 or VZV, with the Novaplex™ MPXV Assay (Seegene, Seoul, Republic of Korea) and the Bio-Speedy® Monkeypox Virus assays (Bioeksen, Istanbul, Turkey): both assays showed high sensitivity and specificity, also in comparison with the reference protocol [17], even for Novaplex™ also samples with a C_t value up to 44 were considered positive; no cross-reactivity with Herpesviruses was found [23]. The second work, by Paniz-Mondolfi et al. [24], validated the PKamp™ Monkeypox Virus RealTime-PCR RUO Kit (Perkin Elmer Inc., USA), using 20 Non-Variola OPXV (NVO) negative and 20 NVO-positive samples: data were weighed against a Centers for Disease Control and Prevention (CDC) protocol and virus isolation by cell culture. Molecular assays demonstrated 100% concordance, while no replication was observed in 1/19 cellular substrate, for which the RT–PCR C_t was >31 [24]. As expected, all the kits demonstrated high performances, representing reliable diagnostic tools; nonetheless, they guarantee high-throughput results, working in batches with large amounts of specimens and requiring medium-high expertise. A different approach was used by Cui et al., who developed a Recombinase-aided amplification (RAA)-based isothermal assay, which would benefit from a significant reduction of analysis time (10 min), costs (5$/sample), and laboratory requirements (i.e.: expertise, cold-chain, RT–PCR instruments); however, a minor analytical sensitivity, compared to RT–PCR, could impair the potential of this technology, especially in a low prevalence context [25]. The epidemiological

scenario extremely changed starting in late 2022, with a highly significant drop in new cases [26]; nevertheless, the virus continues its circulation under the radar, requiring a new approach: smaller numbers and the need to perform tests in limited expertise settings represent two important issues for surveillance. The M10 assay is rapid, user-friendly, and in the single-test format, which allows to return of a result in less than two hours from sample reception to validation. The STANDARD M10 platform was previously evaluated in the context of SARS-CoV-2 detection, also for differential diagnosis with Influenza A, Influenza B, and Respiratory Syncytial Virus (RSV), proving its reliability in terms of PPA, NPA, short time-to-result, and easy usage and an appropriate diagnostic tool for those scenarios requiring a rapid response [22, 27–30]. The present study is the first on the use of STANDARD M10 MPX/OPX: as for respiratory pathogens, the assay showed high-quality performance, with a higher concordance with first results as per RealStar® OPX-1, especially when the operator interpretation was used. The discrepancies were mainly observed in samples with low C_t values in RealStar® OPX-1: such observation suggests a possible influence of freeze-thawing on nucleic acids, as well as of the different distribution of low target DNA in the independent samples aliquots used for assays.

It must be underlined that in this study all clinical information, including those on patients' follow-up, were available: hence, laboratory managers must check results, also considering epidemiological and anamnestic data. The operator interpretation was possible because of the already confirmed infection as per laboratory results and clinical evidence, but it requires more supporting data and careful interpretation. Besides that, the M10 MPX/OPX successfully classified as positive all but two samples collected from lesions at the time of patients' first admission, detecting all targets: the result is a prompt detection of any *Orthopoxvirus* genus DNA, plus the identification of *Monkeypox virus* species and clades. Such a feature could represent a highly useful tool for patients' management. On the one hand, a rapid result would minimize the isolation of negative individuals, with a diminution of psychological impact and healthcare system burden. On the other hand, OPXV is widely diffused, with different species localizations, and human cases are described, claiming for reliable and flexible diagnostic tests to fulfil the evolving clinical needs, as demonstrated by the 2022 MPXV outbreak: a positive result for OPX E9L, coupled with epidemiological and anamnestic data, would indicate a highly probable case, obviously requiring confirmation tests [31–34]. To the same extent, clade identification is a relevant aspect. In March 2023, a case of Mpox caused by clade I virus was identified in the Democratic Republic of the Congo (DRC), classified as the first documented infection by this clade through sexual contact: the patient reported two intercourse in Belgium with a symptomatic individual, with frequent visits in DRC, and further nine partners (six men and three women) after home return [35]. According to a recent WHO report, Mpox by Clade I demonstrated a Case Fatality Rate (CFR) of up to 4.5%, especially in children under 15 years of age, and the cases are significantly rising [36]. The main gap in spread containment is the limited diagnostic capacity in DRC and in Africa in general [36]. The STANDARD M10 MPX/OPX could, therefore, represent a valid solution, especially considering that it is user-friendly, rapid, and stable at room temperature: the operator only needs to transfer the appropriate sample volume in the cartridge, to load it in the analyser and to start the run; in contrast, as for other molecular systems, the need of specialized assistance and reliable power supply could limit the deployment, requiring an intervention by international authorities to overcome such a major constrain. A main limitation in this study was the absence of samples positive for other pathogens, such as VZV, inducing rash syndromes: given the similar clinical picture, differential diagnosis is mandatory, requiring highly specific assays without cross-reactivity. Molecular primers and probes are designed to minimize unspecific annealing, and assay development always includes product challenge against possible interfering substrates; nevertheless, samples from real-life settings are the best option to address the issue. In addition, no other OPXV were tested, because of the unavailability of positive specimens. To overcome these issues, further study should 1. include samples positive for other targets, which are of interest for differential diagnosis; 2. include at least mock samples containing DNA of other OPXV; 3. design assays to include OPXV and other targets of interest in the context of rash syndromes.

The M10 OPX/MPX assay demonstrated its reliability in detecting MPXV infection, with high analytical sensitivity and in different biological matrixes, also allowing a rapid clade identification. Moreover, the rapid processing, the Point-of-Care approach, and being highly user-friendly make the platform an interesting means of testing for expanded diagnostic networks, in which resources and expertise might greatly differ across different laboratories: trustworthy and quick results are mandatory to put in place successful containment measures.

Acknowledgements

The "PerBiocid" project of the Italian National Research Council (progetto@CNR2020: SAC.AD002.173.032) is gratefully acknowledged. We thank Doctor Francesca Garbarino from SD Biosensor Inc. (Republic of Korea), Roberta

Gullotta and Davide Antoniani from Relab S.r.l. (Italy) for their technical support and test provision.

Disclosure statement

DM had sponsorship for attending the 50th AMCLI Congress from Relab S.r.l. (Italy) and the 33rd European Congress of Clinical Microbiology and Infectious Diseases from SD Biosensor Inc. (Republic of Korea). The other 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.

Funding

This research was funded by SD Biosensor Inc. (Republic of Korea). The sponsor had no role in study design, data analysis, or manuscript preparation.

Ethics

The study was conducted in accordance with the Declaration of Helsinki, using appropriately frozen samples in the framework of a larger protocol approved by the institutional Ethics Committee "Comitato Etico Milano Area 1" (protocol number n. 2022/ST/124).

Authors' contribution

AR, DMo, AG, SA, GA, GR, AC, and SN collected samples and clinical data, in the context of patient management. GG, CC, and FB performed assays. AR and FS managed the database. MRG managed the study funding. AM and DMi planned the study, interpreted the results, and wrote the paper draft and final version. All the authors reviewed and agreed on the final manuscript version.

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