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Development of two multiplex real-time PCR assays for simultaneous detection and differentiation of monkeypox virus IIa, IIb, and I clades and the B.1 lineage

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ABSTRACT

An ongoing multicountry outbreak of monkeypox was reported in May 2022 with several deaths, affecting 107 countries of all six World Health Organization (WHO) regions. The WHO has declared the current monkeypox outbreak a Public Health Emergency of International Concern. It is, thus, necessary to rapidly and accurately detect and distinguish different monkeypox virus (MPXV) clades. We designed primers and probes based on the alignment of 138 complete genomes of poxviruses. In Panel 1, we mixed one pair of primers and three probes to detect and differentiate the MPXV Western Africa (IIa, IIb clade) and Congo Basin (I clade) and other orthopoxviruses. In Panel 2, we mixed one pair of primers and two probes to detect the 2022 MPXV (B.1 lineage and its descendant lineages). In addition, we tested the specificity and sensitivity of the assay using real-time PCR. In Panel 1, the assay reproducibly identified various concentrations of two plasmids of the monkeypox virus, whereas other orthopoxviruses did not cross-react. In Panel 2, the probe annealed well to MPXV B.1 and showed the expected linearity. These two multiple real-time assays are inclusive and highly specific for identifying different clades of MPXV.

1. Introduction

Monkeypox virus (MPXV), a contagious zoonotic pathogen, belongs to the *Orthopoxvirus* genus within the family *Poxviridae* and subfamily *Chordopoxvirinae*. Monkeypox was first identified in captive monkeys in 1958 and a child from the Democratic Republic of Congo in 1970 [1]. Since eradication of smallpox in 1980 [2], Monkeypox has gradually become the most severe *Orthopoxvirus* infection in humans [3]. After a 10- to the 14-day incubation period, patients develop a prodromal fever, malaise, headache, myalgia, and a generalized skin rash [4]. Approximately 90% of individuals infected with MPXV develop lymphadenopathy, a key clinical feature differentiating it from smallpox [2,5]. MPXV transmission is thought to occur through direct contact [6], or indirect transmission [5], including contact with contaminated bedding, clothing, or surface spread [7,8]. In addition, it can be transmissible via respiratory droplets over short distances. Sexual transmission is also possible, especially in homosexual communities [7,9,10].

Monkeypox is endemic to the rainforests of central and west Africa [10–12]. Monkeypox first emerged in the Western Hemisphere in 2003, causing an outbreak in the Midwestern United States, affecting 81 people exposed to ill prairie dogs infected by exotic rodents from Ghana in West Africa [13,14]. Subsequent studies demonstrated the existence of two genetic variants of the virus: Western Africa (MPXV-WA, now named as IIa, IIb clade) and the Congo Basin (MPXV-CB, now named as I clade) clades [5,13]. MPXV-WA circulates primarily from western Cameroon to Sierra Leone [14], whereas MPXV-CB circulates mainly from central and southern Cameroon to the Republic of the Congo [15], sharing 99% sequence identity with MPXV-WA [14]. MPXV-WA produces milder infections than MPXV-CB [16], with a less severe rash and fewer lesions [2]. In addition,

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intraperitoneal injection of MPXV-USA into SCID-BALB/c mice resulted in a longer mean time to death than Congo Basin strains [13].

Between January 1 and October 7, 2022, 71,237 laboratory-confirmed monkeypox cases with 26 deaths were reported, affecting 107 countries of all six WHO regions [17]. On July 23, 2022, the WHO Director-General declared that the multicountry outbreak of monkeypox constituted a Public Health Emergency of International Concern. Thus, there is a pressing need to develop new diagnostic assays to rapidly and accurately detect and distinguish between MPXV clades during the current outbreak.

Laboratory diagnosis is essential because the clinical appearance of monkeypox is indistinguishable from other pox-like illnesses, such as smallpox and chickenpox [2,5,18]. Virus isolation is reliable and specific [11,19]; however, it carries a high risk of exposure. Also, serological tests can produce false-positive results due to cross-reactivity with other orthopoxviruses [20]. Polymerase chain reaction (PCR) assays have a high sensitivity and require a lower detection limit [21]. However, traditional PCR assays that use combined restriction fragment-length polymorphism analysis are labor-intensive and time-consuming [22–24]. Therefore, rapid and scalable TaqMan probes based on real-time PCR tests are needed [25].

Therefore, we developed two multiplex real-time PCR assays for simultaneous detection and differentiation of MPXV-IIa, IIb clade, MPXV-I clade, and the 2022 outbreak MPXV B.1 lineage. In addition, we designed probes specific to different MPXV clades and other orthopoxviruses. Finally, using real-time PCR, we determined the specificity and limit of detection of the two assays.

2. Materials and methods

2.1. Nucleic acid extraction

We used the Tiantan vaccinia virus strain preserved in our laboratory. According to the manufacturer's instructions, the virus culture (200 μL) was extracted using a TIANamp Genomic DNA kit (Tiangen Biotech Beijing Co., ltd.). The extracted DNA was resolved in 50 μL of TE buffer and stored at −20 °C.

2.2. Plasmid synthesis and extraction

The F3L gene and other target sequences of MPXV-IIa, IIb, and MPXV-I clade were synthesized and cloned to the vector PUC57 (Sangon Biotech). These plasmids were transformed into *Escherichia coli* cells using *E. coli* DH5α Electro-Cells (TaKaRa). Extraction was performed using the Endofree Maxi Plasmid Kit V2 (Tiangen Biotech Beijing Co., ltd.), according to the manufacturer's instructions. These synthetic plasmids were diluted tenfold, or twofold using RNase-free and DNase-free water, respectively, and used as standards.

2.3. Primers and probes

Primers and probes specific to MPXV-IIa, IIb, MPXV-I clade, and other orthopoxviruses were designed using Primer-BLAST (US National Library of Medicine). All primers and probes were synthesized by Beijing Tianyi Huiyuan Life Science & Technology, Inc.

In Panel 1, the probe specific to MPXV-IIa, IIb was labeled [26,27] with FAM at the 5′-end, and with the quencher Blackhole Quencher 1 at the 3′-end. The probe specific to MPXV-I clade was labeled with Cyanine 5 at the 5′-end and with the quencher Blackhole Quencher 2 at the 3′-end. The probe specific to other orthopoxviruses contained HEX at the 5′-end and the quencher Blackhole Quencher 1 at the 3′-end. In Panel 2, the probe specific to MPXV B.1 was labeled with HEX at the 5′-end and Blackhole Quencher 1 at the 3′-end. The probe specific to other clades was labeled with FAM at the 5′-end and minor groove binding (MGB) was conjugated to the 3′-end. The working concentration of the primers and probes was 10 μM.

2.4. Real-time PCR assays

In Panel 1, a real-time PCR assay was carried out in 20-μL reaction volumes, including 10 μL of 2 × Taq Pro U^+ Probe qPCR Mix (Vazyme-innovation in enzyme technology), 0.4 μL of forwarding primer (10 μM), 0.4 μL of reverse primer (10 μM), 0.4 μL of MPXV-I clade probe (10 μM), 0.1 μL of MPXV-IIa, IIb probe (10 μM), 0.4 μL of other *Orthopoxvirus* probe (10 μM), 2 μL of template DNA, and 6.3 μL of RNase-free and DNase-free water. In Panel 2, a 20-μL mix contained 10 μL of 2 × ChamQ Geno-SNP Probe Master Mix (Vazyme-innovation in enzyme technology), 0.4 μL of forwarding primer (10 μM), 0.4 μL of reverse primer (10 μM), 0.4 μL of each probe (10 μM), 2 μL of template, and 6.4 μL of RNase-free and DNase-free water. The PCR assay conditions were optimized by adjusting the concentrations of the primers and probes. Three replicates were prepared for serial dilution. RNase-free and DNase-free water were used as the negative controls.

The real-time PCR assay was performed using a LightCycler 96. The following thermal cycling program was used: 37 °C for 2 min, 95 °C for 30 s, followed by 45 cycles at 95 °C for 10 s and 60 °C for 30 s. The performance of multiple real-time PCR was evaluated using a standard curve. Amplification efficiency, slope, and R^2 were determined using LightCycler 96 analysis software.

The assay's limit of detection (LOD) on the LightCycler 96 instrument was determined from serial dilutions of plasmids and was defined as a > 95% detection rate at a given DNA concentration.

3. Results

3.1. Sequence alignment and design of primers and probes

We aligned 138 complete genome sequences of MPXV-WA (now IIa, IIb clade), MPXV-CB (clade I), and other orthopoxviruses, including variola virus, vaccinia virus, ectromelia virus, horsepox virus, and camelpox virus (data not shown). We discovered four mutations in the MPXV-I clade compared with MPXV-IIa, IIb at locations 46422–46446 of MPXV-UK_P2 (MT903344.1) (Fig. 1A). Orthopoxviruses other than MPXV, possessed two mutations across the complete genome compared to the MPXV-IIa, IIb sequences (Fig. 1A). Flanking the probe at the locations of 46313–46339 and 46467–46491, these two regions were conserved in all the full-length genomes that we chose. The forward and reverse primers were designed to amplify these regions (Fig. 1A, Table 1).

Sequences of the 2022 MPXV outbreak strain were clustered into a new clade of MPXV clade IIb [28], the MPXV B.1 lineage [29]. Therefore, it is necessary to design real-time PCR assays to detect MPXV B.1 and to differentiate it from other clades. Compared to the complete genome of MPXV-UK_P2, there were 46 single-nucleotide mutations across the complete genome. So we designed two single-nucleotide polymorphism (SNP)-typing probes targeting MPXV B.1 and other clades of MPXV-IIa and IIb at locations 183519–183542 and 183524–183539 of MPXV_USA_2022_MA001 (ON563414.2), respectively (Fig. 1B, Table 1), and mixed a pair of primers with these two probes.

3.2. Specificity of the real-time PCR assays

To further evaluate the specificity of the probe, we mixed a pair of primers and three probes in Panel 1 to test the three types of samples. The plasmid that targeted the IIa, IIb sequence only combined the probe labeled FAM on the 5′-end (Fig. 2A). The plasmid containing the MPXV-I clade sequence was only efficiently annealed to the probe

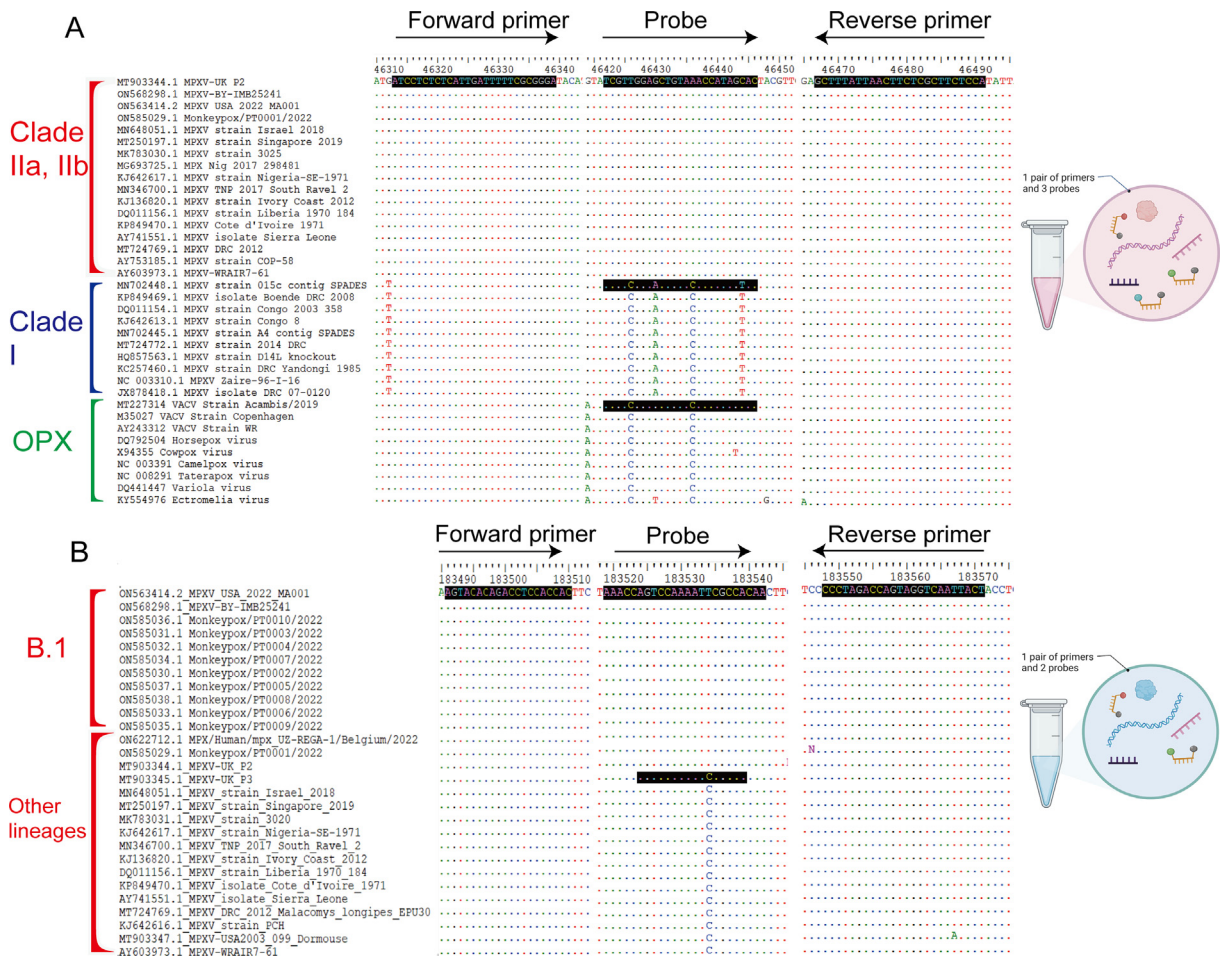

Fig. 1. Sequence alignments of target regions for the monkeypox viruses and other orthopoxviruses. The alignments were made using MAFFT (Multiple Alignment using Fast Fourier Transform). The nucleotide positions of target regions were shown above the sequences based on the reference genome. Dotes represent identical nucleotides compared to the reference genome. Mutations are denoted as "A", "T", "C or "G". A) Sequence alignment of monkeypox virus clade IIa, IIb, clade I, and other orthopoxviruses. The target region of MPXV_UK_P2 was used as the reference sequence. The arrows indicate the forward primer, the reverse primer, and three TaqMan probes. The probes were designed specifically for IIa, IIb, I, and other orthopoxviruses. In Panel 1, we mixed a pair of primers and these three probes to distinguish different clads of monkeypox viruses. B) Sequence alignment of MPXV B.1 lineage and other sequences of MPXV IIa, IIb clade. The sequence of MPXV_USA_2022_M001 was used as the reference genome. The arrows indicate the forward primer, reverse primer, and two probes. The probes were designed specifically for the MPXV B.1 lineage and other sequences of IIa and IIb, respectively. In Panel 2, we mixed a pair of primers and these two probes to differentiate the MPXV B.1 lineage.

Table 1
Primers and probes designed in this study.

	Panel 1	Panel 2
Forward Primer	5′-ATCCTCTCTCATTGATTTTTCGCGGGA-3′	5′-AGTACACAGACCTCCACCAC-3′
Reverse Primer	5′-TGGAGAAGCGAGAAGTTAATAAAGC-3′	5′-AGTAATTGACCTACTGGTCTAGGG-3′
Probe 1	5′CY5-TCGTCGGAACTGTACACCATAGTAC-3′BHQ2	5′HEX-AAACCAGTCCAAAATTCGCCACAA-3′BHQ1
Probe 2	5′FAM-TCGTTGGAGCTGTAAACCATAGCAC-3′BHQ1	5′ FAM -AGTCCAAAATCCGCCA-3′MGB
Probe 3	5′HEX-TCGTCGGAGCTGTACACCATAGCAC-3′BHQ1	

labeled CY5 at the 5′-end (Fig. 2B). However, the vaccinia virus strain Tiantan could be detected only by the probe labeled HEX at the 5′-end (Fig. 2C), and no cross-reactivity was detected among the MPXV-IIa, IIb, MPXV-I, and other orthopoxviruses, demonstrating the high specificity of Panel 1. The specificity of the assays we developed was further confirmed by one serum sample collected from a patient infected with the MPXV-II in Sierra Leone. Similarly, this real sample could be detected by the probe labeled with FAM at the 5′-end (Fig. 2D). In the other two fluorescent light channels, no amplification curves were observed (Fig. 2D). The cycle threshold value was 31.95 (Table 2). We also detected the virus load in different sample types of one monkeypox case found in Chongqing, China in 2022 [30]. The cycle threshold value of blister fluid, oropharyngeal swabs, nasopharyngeal swab, and whole blood was 22.39, 29.46, 34.09, 35.45, respectively (Table 2).

In Panel 2, we compared the amplification curves obtained by mixing the two probes. There were amplification curves in two fluorescence channels when we put into the plasmid of MPXV B.1 lineage (Fig. 2E). MPXV B.1 could be identified by the TaqMan probe labeled HEX at the 5′-end, whereas other lineages did not cross-react with this probe (Fig. 2E & F). There were apparent amplification curves in the FAM fluorescence channel on testing other lineages of MPXV clade II (Fig. 2F). The virus load in two flurescence channels of the monkeypox

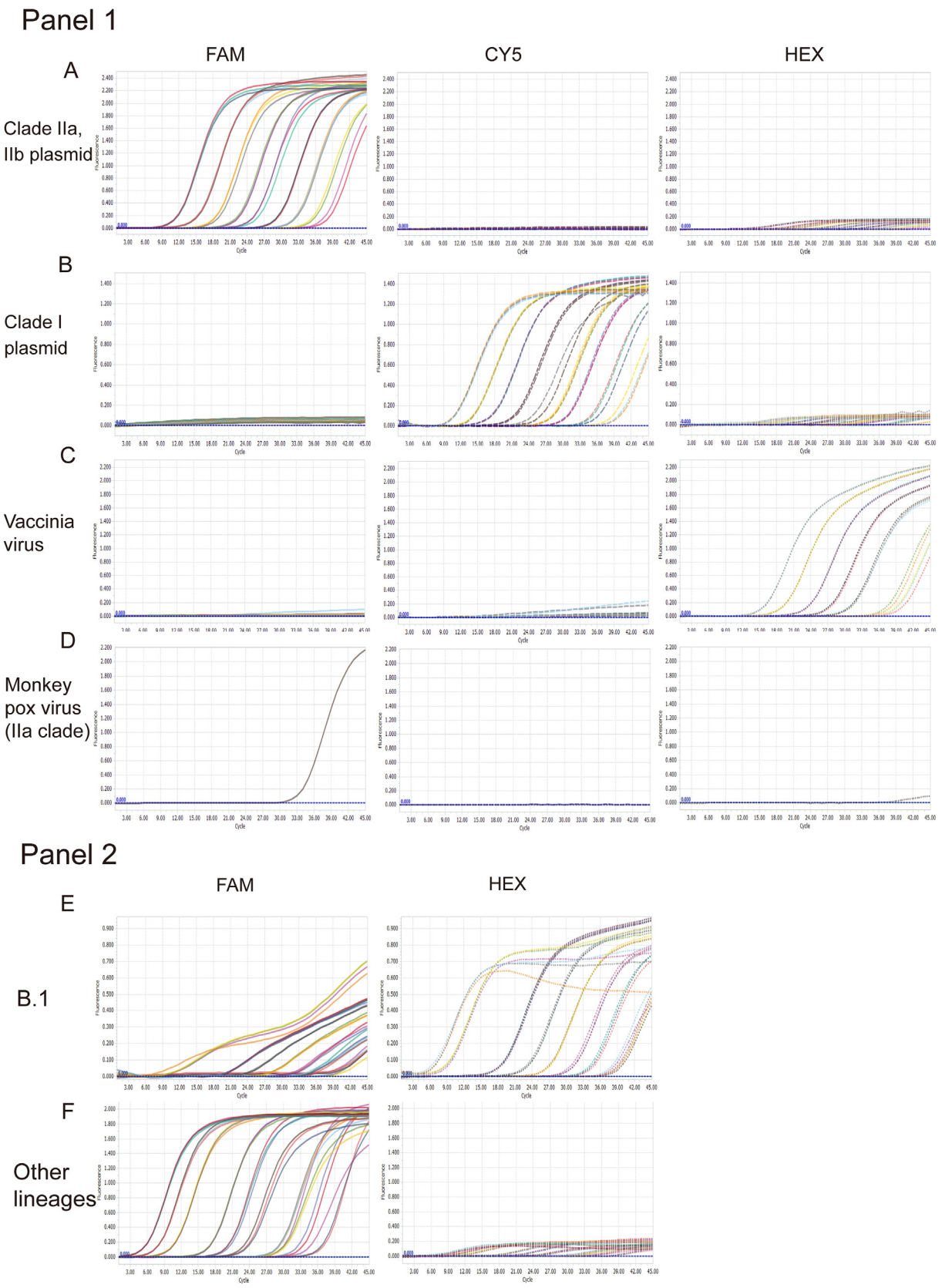

Fig. 2. Amplification curves of different types of templates. In Panel 1, three probes were labeled with FAM, CY5, and HEX at the 3′-end. A) The amplification curve of the different dilutions of plasmids of monkeypox virus (MPXV) IIa, IIb clade. B) The amplification curve of the different dilutions of plasmids of MPXV I clade. C) The amplification curve of the different dilutions of vaccinia virus strain Tiantan. D) The amplification curve of one clinical serum sample of MPXV-IIa. The X-axis shows cycle threshold (Ct) and the Y-axis indicates relative fluorescence. In Panel 2, two probes were labeled with HEX and FAM at the 3′-end. E) The amplification curve of the different dilutions of plasmids of MPXV B.1 lineage. F) The amplification curve of the different dilutions of plasmids of other sequences of MPXV non-B.1 lineages.

Table 2
Cycle threshold of different types of clinical samples of monkeypox cases found in Chongqing and Sierra Leone.

Sample type	Panel 1			Panel 2	
	FAM	CY5	HEX	FAM	CY5
Blister fluid[a]	22.39	Negative	Negative	25.79	23.91
Oropharyngeal swabs[a]	29.46	Negative	Negative	32.58	31.08
Nasopharyngeal swab[a]	34.09	Negative	Negative	36.03	34.29
Whole blood[a]	35.45	Negative	Negative	40.27	38.66
Serum[b]	31.95	Negative	Negative	31.37	Negative

[a] Sample from the case found in Chongqing in 2022.
[b] Sample from the case found in Sierra Leone.

case found in Chongqing were shown in Table 2. However, the serum sample of the case in Sierra Leone was only detected by the MGB probe in FAM florescence channel (Table 2).

3.3. Amplification efficiencies of the real-time PCR assays

The standard curves of the real-time PCR assays were constructed by plotting the cycle threshold (Ct) versus the viral load (copies/μL) of plasmids (Fig. 3A, B, D, E) or plaque-forming units (PFU)/mL of the vaccinia virus (Fig. 3C) and displayed good linearity and repeatability. In Panel 1, the concentrations of plasmid of MPXV-WA (IIa, IIb) and MPXV-CB (clade I) ranged from 1.19×10^9 to 1.19×10^1 copies/μL and 1.83×10^9 to 9.15 copies/μL, respectively (Fig. 3A & B). The limits of detection were 119 and 183 copies/μL, respectively. For vaccinia virus, the dynamic range was seven orders of magnitude and represented 8.6×10^8 to 2.15×10^2 PFU/mL. Vaccinia virus DNA was reproducibly detected in a linear fashion to 8.6×10^3 PFU/mL (Fig. 3C). In Panel 2, we established the expected linearity of the MPXV B.1 assay between 1.27×10^{10} to 1.27 copies/μL, and the LOD was 127 copies/μL (Fig. 3D). The LOD for the other strains was 190 copies/μL (Fig. 3E).

4. Discussion

Since May 13, 2022, an increasing number of cases of monkeypox have been reported in Western European and North American countries [25]. It is the first time such an unusually high number of cases and sustained chains of human-to-human transmission have been reported in countries outside the West or Central Africa [29,31]. Furthermore, compared to past outbreaks, the current cases seem to have occurred mainly in homosexuals [31], even without excluding pregnant women and babies [28,32]. Therefore, early detection of the virus is crucial to counteract the spread of the disease. In this study, we developed multiple real-time PCR assays for the specific detection of MPXV-IIa, IIb, MPXV-I clade, and MPXV B.1 to track the ongoing monkeypox outbreak early.

Some serological tests, such as neutralization and hemagglutination inhibition assays, have been designed for MPXV antibody detection [33,34]. However, cross-reactivity of surface antigens among

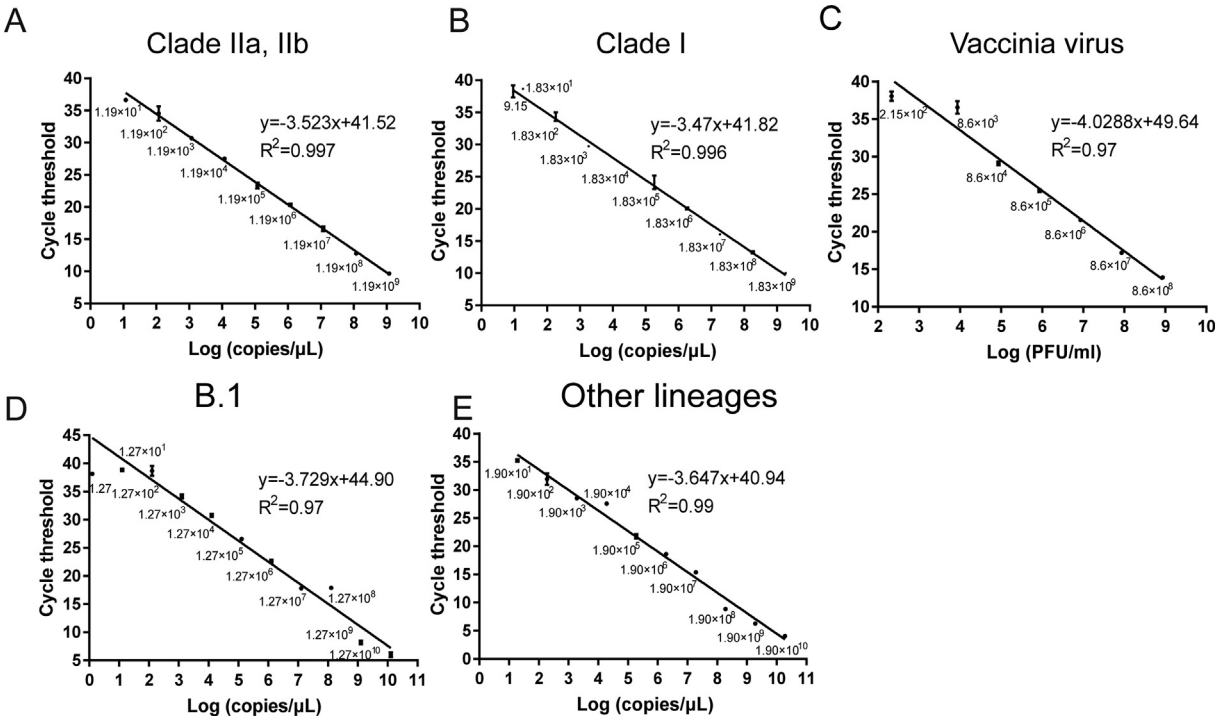

Fig. 3. Standard curves of the different plasmids of monkeypox viruses or vaccinia virus. The standard curves were plotted against the log (copies/μL) or log (PFU/mL) against the cycle threshold from tenfold or twofold serial dilutions of plasmid DNA or vaccinia viruses. The regression equation and R^2 are shown on each curve. A) Tenfold serial dilutions of purified plasmid DNA of the monkeypox virus (MPXV) IIa, IIb clade. B) DNA plasmids of the MPXV I clade. C) 7 orders of magnitude of vaccinia virus. D) Plasmid DNA of B.1 lineage. E) DNA plasmids of other strains of the MPXV IIa, IIb.

orthopoxviruses is difficult to avoid. PCR or real-time PCR can be used alone or in combination with sequencing.

Several designed primers and probes have been developed, targeting B6R [35], B7R [18], F3L [12,36], N3R [12], J7R [37], and O2L [38] of monkeypox viruses, specifically from orthopoxviruses such as variola virus, cowpox virus, vaccinia virus, and varicella-zoster virus. However, these real-time assays cannot differentiate between the two clades. Li *et al.* [16] designed specific probes to draw this distinction. There were three deletions in the MPXV-CB of the *tumor necrosis factor receptor* gene (G2R); therefore, they designed a probe specific to MPXV-WA. For MPXV-CB, the complement binding protein (C3L) gene was used because of the C3L deletion in MPXV-WA. Other studies have designed conventional PCR [22] and real-time PCR [39] due to the insertion a unique 453-nucleotide residue of the A-type inclusion body gene partial sequences of MPXV-WA. Therefore, specific reverse primers for MPXV-WA and MPXV-CB were designed based on this insertion. In this study, we designed specific probes based on four nucleotides between MPXV-WA (IIa, IIb) and MPXV-CB (clade I). We then mixed them to detect and differentiate the two clades rapidly. Our assay would be a universal test that can find wide applications. In light of current monkeypox outbreaks, we designed two probes specific to MPXV B.1 and other strains of MPXV-WA (IIa, IIb) based on single-base differences. The TaqMan probe, which targets MPXV B.1, did not anneal to other strains, showing good linearity and specificity. However, the MGB probe, designed for other strains combined with MPXV B.1, weakly with low amplification efficiency. One of the reasons could be that MGB can stabilize combinations of probes and templates, and there was only one mutation between MPXV B.1 and the other MPXV-WA strains.

Based on sensitive real-time PCR assays, we designed primers and probes specific to sequences of different clades of monkeypox viruses or other orthopoxviruses. As a result, our assay provides a reliable and simple method to rapidly identify MPXV and differentiate the strain in ongoing monkeypox outbreaks.

Author contributions

Shuting Huo: Investigation, Validation, Data Curation, Formal Analysis, Visualization, Writing – Original Draft. **Yuda Chen:** Investigation, Validation, Data Curation. **Roujian Lu:** Investigation, Validation, Data Curation. **Zhongxian Zhang:** Investigation, Validation. **Gaoqian Zhang:** Investigation, Validation. **Li Zhao:** Investigation, Validation. **Yao Deng:** Investigation, Validation. **Changcheng Wu:** Data Curation, Conceptualization, Writing – Review & Editing. **Wenjie Tan:** Conceptualization, Writing – Review & Editing, Project Administration, Funding Acquisition, Resources.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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