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Protocols

Development of a multiplex real-time PCR assay for the simultaneous detection of mpox virus and orthopoxvirus infections

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ABSTRACT

Since May 2022, the multi-country outbreak of monkeypox (mpox) has raised a great concern worldwide. Early detection of mpox virus infection is recognized as an efficient way to prevent mpox transmission. Mpox specific detection methods reported up to now are based on the SNPs among mpox virus and other orthopoxviruses. We have therefore developed a real-time PCR based mpox detection method targeting mpox virus specific sequences (N3R and B18Rplus). We have also optimized an orthopoxvirus detection system which targets the highly conserved E9L and D6R genes. The mpox and orthopoxvirus real-time PCR assays have a high sensitivity (1 copy/reaction) and specificity. Mpox viral DNA and clinical samples from mpox patients are detected with the mpox detection system. Furthermore, we have established a multiplex real-time PCR detection system allowing simultaneous and efficient detection of mpox and orthopoxvirus infections.

1. Introduction

Orthopoxviruses are a group of large DNA viruses with a genome of about 200 kbp, including vaccinia virus (VACV), Variola virus (VARV), Cowpox virus (CPXV), mpox virus (also known as monkeypox virus, MPXV), Camelpox virus (CMLV), Ectromelia virus (ECTV), Raccoonpox virus, Skunkpox virus, and Taterapox virus (Li et al., 2007; Babkin et al., 2022). Some of these orthopoxviruses have been reported to infect human, including CPXV (Diaz-Cánova et al., 2022), MPXV (Lahariya et al., 2022), VACV, CMLV (Bera et al., 2011) and the eradicated VARV (the cause of smallpox) (Jacobs et al., 2009). In the early 20th century, smallpox was prevalent worldwide with a fatality rate of approximate 30% (Meyer et al., 2020). As a result of the world-wide inoculation of VACV as smallpox vaccine, the World Health Organization (WHO) announced that smallpox was eradicated in 1980 (Sánchez-Sampedro et al., 2015). The genomes of orthopoxviruses are conserved up to 80–90% (Li et al., 2007; MacNeill, 2022; Babkin and Babkina, 2012), and the vaccination against VARV can also protect against infection by other orthopoxviruses (Dubois et al., 2012; Gilchuk et al., 2016). After 1980, with the termination of smallpox vaccination, infection cases of other orthopoxviruses, especially mpox, have been rising (Peiró-Mestres et al., 2022; Koenig et al., 2022; Happi et al., 2022).

The first case of mpox was reported in the Congo region of Africa in 1970. Since then mpox outbreaks were mostly reported in Central and West Africa, and sporadically spread to other regions and countries by infected travelers (Alakunle et al., 2020; Nalca et al., 2005). Sequences of MPXV differ based on ancestral origin of the virus. There are two major Clades of MPXV genetic sequence known as Clade I and Clade II, which are prevalent in Central and West Africa respectively (Chen et al., 2005). Clade II consists of two subclades, IIa and IIb. Recently in May

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2022, mpox cases were reported in European countries, and have been spread worldwide quickly afterwards (Lahariya et al., 2022). All of these newly-reported MPXV belonged to Clade IIb (Koenig et al., 2022; Happi et al., 2022; Huo et al., 2022). On July 23, 2022, WHO declared mpox a public health emergency of international concern (Vaughan et al., 2022).

Developing an economic, effective and fast diagnostic method for mpox is central to timely identify mpox virus infection. Conventional polymerase chain reaction (PCR) (Neubauer et al., 1998) or combination of restriction fragment length polymorphism (Ropp et al., 1995; Loparev et al., 2001) were originally used to detect orthopoxvirus infection, followed by real-time PCR which is quantitative and sensitive (J, 2013; Bustin et al., 2009). Li *et al (*Li et al., 2006*)*. first reported an mpox specific real-time PCR assay targeting the B6R gene in 2006. In this assay, the primer and probe sequences were designed according to Clade I MPXV genome sequence, and MPXV was differentiated from other orthopoxviruses by 2 single nucleotide polymorphisms (SNPs) in the probe sequence. Another mpox generic real-time PCR assay was reported in 2010 (Li et al., 2010), which targets the G2R gene and is cited as standard detection method in USA now (Centers for Disease Control and Prevention, 2022). This mpox-specific detection method differentiates mpox virus from other orthopoxviruses by SNPs in the probe sequence. The SNP-based scheme was also used in few more mpox specific detection assays based on SNPs in the F3L gene (Maksyutov et al., 2016; Mondolfi et al., 2022). However, the probes used in mpox-specific detection methods based on SNPs are relatively expensive and hard to design, and may resulted false-negative detection in the mutants with large deletions and genomic rearrangements (Gigante et al., 2022; Kozlov, 2022a). In this study, we improved the real-time PCR detection of mpox and orthopoxviruses by developing assays which amplifies E9L and D6R that are conserved in all orthopoxviruses, or N3R and B18R that are specific for mpox virus. The detection assays are capable of detecting 1 copy viral DNA/reaction. We further developed a multiplex real-time PCR assay which simultaneously amplifies E9L and B18R, and accurately distinguishes mpox virus from other orthopoxviruses in clinical samples of mpox patients.

2. Methods and materials

2.1. Cells, plasmids, virus strain and clinical samples

Cells used in this study were all cultured in Dulbecco's modified Eagle's medium supplemental with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 conditions. E9L, D6R, N3R and B18Rplus cDNAs were synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China). The E9L and D6R gene sequences are derived from the VACV genome (JX489139), and N3R and B18Rplus sequences of different branches are derived from the Mpox Clade IIb (ON674051.1), Clade IIa (KJ642617.1) and Clade I (KJ642613.1) separately. VACV strain used in this study is VACV Tiantan strain as reported previously (Mei et al., 2022). The mpox virus isolate used was MPVX-B.1-China-C-Tan-CQ01 strain (GISAID ID：EPI_ISL_15293815hMpxV/China/CQ-IVDC-0001/2022), which was isolated from the first mpox case in mainland China (Huang et al., 2022). DNA of herpes simplex virus type 1, herpes simplex virus type 2, varicella-zoster virus, *Candida albicans*, *Staphylococcus epidermidis*, *P. aeruginosa* and *streptococcus pyogenes* were purchased from GeneWell Company (Shenzhen). Clinical samples including blister fluid, nasopharyngeal and oropharyngeal swabs from mpox cases reported in Chinese mainland (Huang et al., 2022) were collected. Viral DNAs were extracted using QIAamp®DNA Mini Kit and detected by our newly constructed assays.

2.2. Orthopoxvirus sequence alignment

We retrieved the genome information of orthopoxviruses from NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/), and the genome pairwise identity of the E9L, D6R, N3R and B18Rplus from orthopoxviruses were aligned by DNAMAN. Sequence alignments of primers and probes were performed with BLAST in the NCBI database (website: https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Viral genomic DNA extraction

Viral genomic DNA was extracted using the QIAamp®DNA Mini Kit following the manufacturer's instructions. Briefly, 20 μL of proteinase K were added to 200 μL virus-containing suspensions, and another 200 μL of buffer AL were added to the mix. After vortexing, virus in the mix were further incubated in 56 °C for 10 min. After adding 200 μL ethyl alcohol, the mixture was added to centrifugal column, and washed with buffer AW1 and AW2. The DNA was eluted using buffer AE.

2.4. Quantification of virus genome by droplet-digital PCR

Viral genomic DNA was quantified with droplet-digital PCR (ddPCR) using QuantStudio™ 3D Digital PCR System (Applied Biosystems) following QuantStudio™ 3D Digital PCR System USER GUIDE.

2.5. Construction of recombinant VACV-NB virus

N3R and B18Rplus sequence as well as eGFP coding sequence were inserted into the thymidine kinase (TK) locus of the VACV Tiantan strain by homologous recombination. The inserted N3R and B18Rplus can't be transcribed or translated as there were no promoter or ATG initiation codons, and eGFP with an initiation codon under H5 promoter was inserted to facilitate the screening of recombinant virus. Recombinant VACV-NB virus was selected by eGFP fluorescence and purified with plaque assay. Five rounds of plaque purification were performed to obtain purified recombinant VACV-NB virus.

2.6. Detection of VACV or VACV-NB infected samples

VACV or VACV-NB infected samples were prepared as follows: 2×10ˆ5 Vero cells were seeded in 12-well plate, and infected with VACV or VACV-NB virus at MOI of 0.0001, 0.001, or 0.01 for 24 hours. Then cells were suspended in 200 μl of PBS and treated with 20 μl of proteinase K (QIAamp®, Cat. 19157) at 56 ℃ for 10 minutes. After centrifugation at 12,000 rpm for 10 minutes, the supernatant was used as template for real-time PCR.

2.7. Real-time PCR

The real-time PCR assay contains target-specific forward and reverse primers, FAM-labeled probes, master mix, template and ddH_2O. Primers and probes were synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China), their sequences are shown in Fig. 1A and Table 1. Each real-time PCR was performed in a 20 μl reaction mix, containing 10 μl TSINGKE TSE301 2×T5 Fast qPCR Mix, 0.75 μl probe (0.325 μM), 2 μl forward primer (1 μM) and 2 μl reverse primer (1 μM) for E9L, B18Rplus and N3R method, and 1.5 μl forward primer (0.75 μM) and 1.5 μl reverse primer (0.75 μM) for D6R method, 2 μl template, and 3.75 μl/4.75 μl RNase and DNase-free water. The real-time PCR program starts with 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 20 s. Fluorescence was recorded for each cycle using Bio-Rad CFX96.

All the newly established real-time PCR (E9L, D6R, N3R and B18Rplus) assays and reported real-time PCR (D6R-reported, E9L-reported and G2R) assays used the same mastermix, and the published assays used the same protocol and cycling parameters provided in the literature.

2.8. Multiplex real-time PCR

Each multiplex real-time PCR was performed in a 20 μl reaction mix,

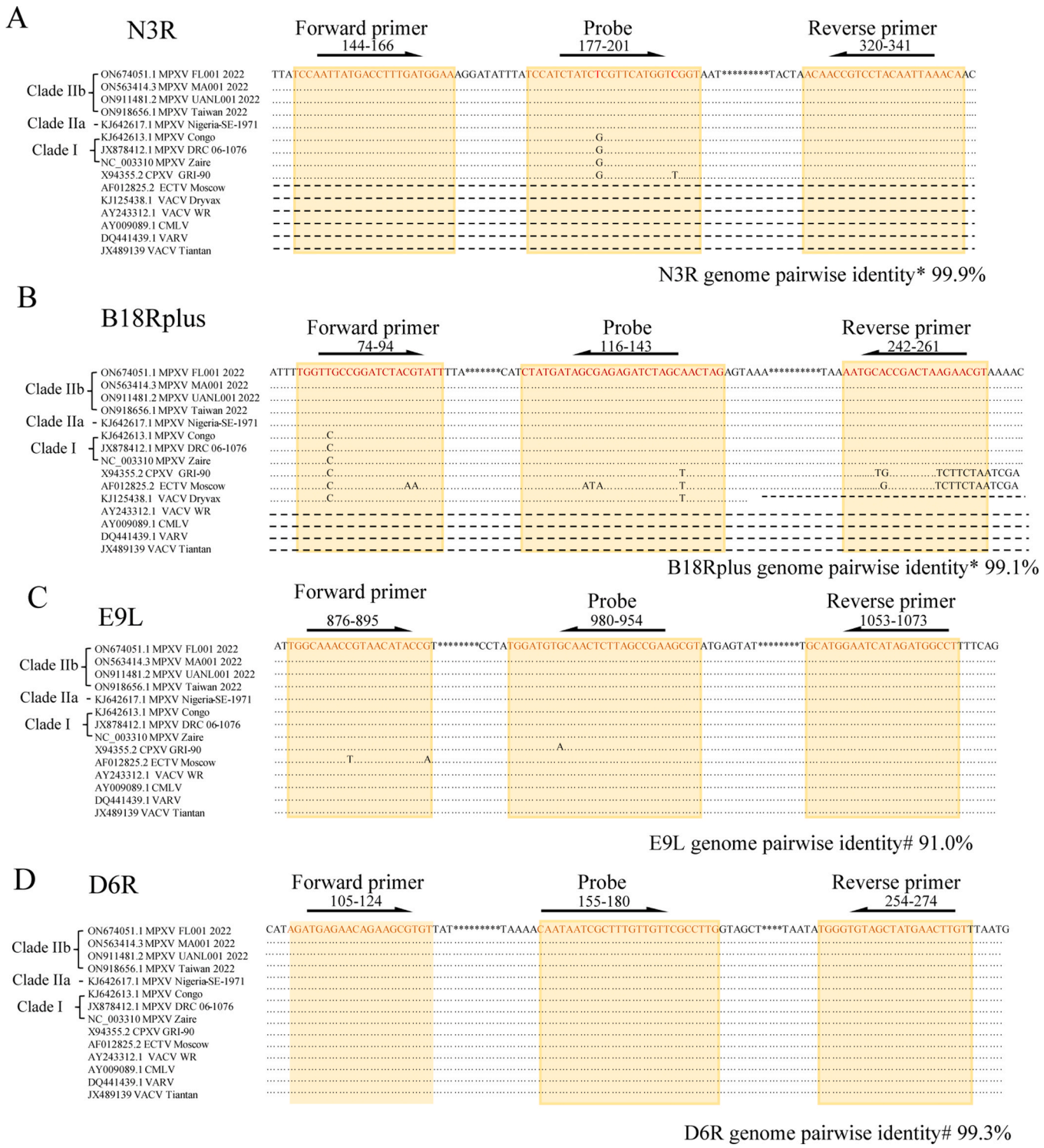


Fig. 1. Conservation of real-time PCR target sequences among mpox, VACV, VARV, CPXV, CMLV and ECTV. A. N3R sequences were aligned among MPXV Clade I and Clade II linages as well as other orthopoxviruses. The starting site of primers and probes were labeled according to N3R gene sequence of MPXV ON674051.1 strain. B. B18Rplus sequences were aligned among MPXV Clade I and Clade II linages as well as other orthopoxviruses. The starting site of primers and probes were labeled according to B18Rplus gene sequence of ON674051.1 strain. C. E9L sequences were aligned among Clade I and Clade II MPXV strains, CPXV, ECTV, VACV and VARV. The starting site of the primers and probes were labeled according to the E9L gene sequence of VACV Tiantan strain JX489139 genome. D. D6R sequences were aligned among Clade I and Clade II MPXV strains, CPXV, ECTV, VACV and VARV. The starting site of the primers and probes were labeled according to the D6R gene sequence of JX489139 genome. *: The identity shown was aligned among MPXV genomes. #: The identity shown was aligned among orthopoxviruses.

containing 10 μl TSINGKE TSE301 2×T5 Fast qPCR Mix, 1 μl forward primer (0.5 μM) and 1 μl reverse primer (0.5 μM) for both E9L and B18Rplus, 0.75 μl FAM -labeled E9L probe (0.325 μM), 0.75 μl Cy5-labeled B18Rplus probe (0.325 μM), 2 μl template, and 2.5 μl RNase and DNase-free water. The real-time PCR program starts with 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. Fluorescence was recorded for each cycle using Bio-Rad CFX96.

Table 1
primer and probe sequences in orthopoxvirus and mpox real-time PCR assays.

Target	Oligonucleotide Name	Sequence
orthopoxvirus generic (E9L gene)	E9L-F	TGGCAAACCGTAACATACCG
	E9L-R	AGGCCATCTATGATTCCATGC
	E9L-P (E9L-P*)	FAM-ACGCTTCGGCTAAGAGTTGCACATCCA-TAMRA
orthopoxvirus generic (D6R gene)	D6R-F	AGATGAGAACAGAAGCGTGT
	D6R-R	ACAAGTTCATAGCTACACCCA
	D6R-P	FAM-CAATAATCGCTTTGTTGTTCGCCTTG-TAMRA
Mpox generic (N3R gene)	N3R-F	TCCAATTATGACCTTTGATGGAA
	N3R-R	TGTTTAATTGTAGGACGGTTGT
	N3R-P	FAM-TCCATCTATCTCGTTCATGGTCGGT-TAMRA
Mpox generic (B18Rplus gene)	B18R-F	TGGTTGCCGGATCTACGTATT
	B18R-R	ACGTTCTTAGTCGGTGCATT
	B18R-P	FAM-CTAGTTGCTAGATCTCTCGCTATCATAG-TAMRA
	B18R-P*	Cy5-CTAGTTGCTAGATCTCTCGCTATCATAG-BHQ

*: Probe used in multiplex real-time PCR assay

3. Results

3.1. Construction of mpox virus and orthopoxvirus real-time PCR

As previously reported mpox detection methods were mostly based on SNPs among mpox virus and other orthopoxviruses, we aimed to construct a new method based on genes that are conserved and unique among mpox virus strains but heterogenous in other orthopoxviruses. We compared the mpox virus genomes and found that N3R and B18R genes are conserved among mpox virus and absent or exhibited low homology in other orthopoxviruses (Figs. 1A and 1B). As for B18R, to better distinguish mpox virus from other orthopoxviruses, we extended 54 bp after the ORF of B18R as the target, which was named as B18Rplus in this study. The pairwise identity among the analyzed mpox virus genomes was 99.9% for N3R and 99.1% for B18Rplus, and both N3R and B18Rplus targeting sequences were absent in ECTV, VACV, VARV and CMLV (Fig. 1A and B). Next, we selected highly conserved sequence as primers and probes for real-time PCR, and all the primers and probes selected for N3R and B18Rplus were 100% conserved among mpox virus Clade II genomes (Fig. 1A and B). Thus, N3R and B18Rplus primers and probes were highly conserved among mpox virus genomes and had strong specificity against other orthopoxviruses.

To detect orthopoxvirus infections, we developed a pan-orthopoxvirus detection system, in which the highly conserved E9L (DNA polymerase encoding gene) (Kulesh et al., 2004) and D6R (early transcription factor subunit encoding gene) (Luciani et al., 2021) genes were targeted. All orthopoxvirus genomes share E9L and D6R genes, and the pairwise identity among analyzed orthopoxvirus genome was 91.0% for E9L and 99.3% for D6R (Fig. 1C and D). The primers and probe sequences for E9L were 100% conserved among VACV, VARV, CMLV and mpox Clade I and Clade II strains, except two SNPs in E9L-F of ECTV and one SNP in the E9L-P of CPXV (Fig. 1C). As for D6R, the primers and probe sequences were 100% conserved among VACV, VARV, CPXV, CMLV, ECTV and mpox Clade I and Clade II strains (Fig. 1D).

Next, we cloned N3R, B18Rplus of mpox Clade IIb (ON674051.1) into plasmid DNAs to evaluate the efficiency of newly established real-time PCR. The standard curve of the N3R real-time PCR was shown in Supplemental Figure 1 A, with a R^2 of 0.9995 and slope of -3.484, and B18Rplus real-time PCR had a R^2 of 0.9995 and slope of -3.432 in its standard curve (Supplemental Figure 1B). Then we evaluated the efficiency of our method for mpox Clade I, Clade IIa and Clade IIb detection using N3R or B18R containing plasmids. B18Rplus real-time PCR had a similar efficiency for Clade I, Clade IIa and IIb detection, and N3R had a similar efficiency for Clade IIa and IIb detection, with a little higher Ct value for Clade I (Supplemental Figure 1 C and D).

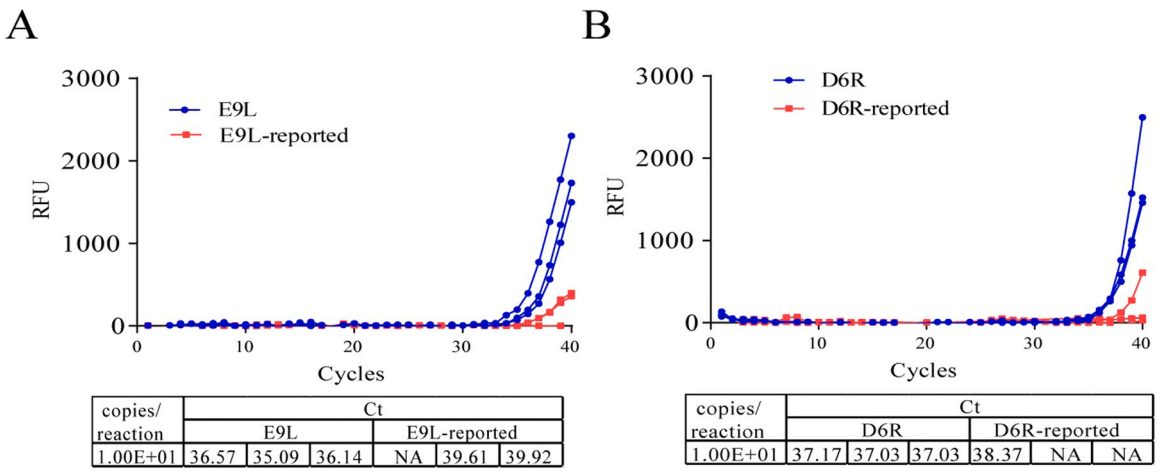
We also evaluated the efficiency of newly established E9L and D6R method. The standard curve of the E9L real-time PCR is shown in Supplemental Figure 1E, with a R^2 of 0.9968 and the slope is -3.455. The standard curve of D6R is shown in Supplemental Figure 1 F, with a R^2 of 0.9985 and slope of -3.434. Kulesh *et al* (Kulesh et al., 2004). and Luciani *et al* (Luciani et al., 2021). separately reported a pan-orthopoxvirus diagnosis targeting E9L and D6R. We compared the detection efficiency of the newly constructed E9L and D6R real-time PCR with the reported ones. 10 copies of DNA can be detected in 100% of replicates by newly constructed E9L and D6R real-time PCR (e.g., 3 of 3), while cannot by the reported assay (Fig. 2 A and B). The Ct value and limit of detection (LOD) were both lower in the new E9L and D6R real-time PCR. As for unusual orthopoxviruses, such as Skunkpox virus, Raccoonpox virus, Volepoxvirus, Alaskapox virus and Ahkmeta virus, it is expected that the discussed methods would fail to detect, since there were several SNPs in primer and probe sequences in both our and reported (Kulesh et al., 2004; Luciani et al., 2021) E9L and D6R methods (Supplemental Figure 2). We have also tested E9L method, which failed to detect Skunkpox virus (data was not shown). Thus, another specific method may be needed for these unusual orthopoxvirus detections.

3.2. Orthopoxvirus and mpox real-time PCR can detect viral genomic DNA at 1 copy per reaction

To further evaluate the efficiency of the designed mpox and orthopoxvirus detection real-time PCR assays, we constructed a recombinant VACV-NB virus, in which mpox virus specific N3R and B18Rplus sequence of Clade IIb strain (ON674051.1), without promoter and initiation codon ATG, were inserted into the TK locus of VACV Tiantan strain (Fig. 3A). VACV and VACV-NB genomic DNAs were used as template for real-time PCR amplification. As shown in Figs. 3B to 3E, VACV-NB DNA could be detected in N3R, B18Rplus, E9L and D6R real-time PCR, while VACV DNA can only be detected in E9L and D6R real-time PCR, indicating N3R and B18Rplus systems detect MPXV genome and have no cross reaction with VACV.

To evaluate the LOD of our systems for viral genomic DNA, the VACV-NB genomic DNA was extracted and quantified by digital-droplet PCR (ddPCR). As shown in Fig. 3F, all four real-time PCR assays can detect viral genomic DNA with a LOD of 1 copy/reaction. We further validated the LOD of these newly established real-time PCR assays with mpox viral DNA. Mpox viral DNA was also diluted, quantified by ddPCR, and tested by E9L, D6R, N3R and B18Rplus real-time PCR. As shown in Figs. 4, 1 copy of mpox viral DNA could be detected with all four real-time PCR assays (Fig. 4A-D). As G2R-targeting real-time PCR assay had been cited as standard detection method in USA (Centers for Disease Control and Prevention, 2022), we further compared the efficiency of N3R and B18Rplus method with G2R method. As shown in Fig. 5, our newly established mpox detection method had a similar LOD to reported method, with a slightly lower Ct value than G2R method.

To test the specificity of the detection method, we used the DNAs of herpes simplex virus type 1, herpes simplex virus type 2, varicella-zoster virus, *Candida albicans*, *Staphylococcus epidermidis*, *P. aeruginosa* and *streptococcus pyogenes* as template in our detection real-time PCR assays. As shown in Supplemental Figure 3, only VACV produced positive amplification signals in the E9L and D6R test, and all other tests were negative in E9L, D6R, N3R and B18Rplus real-time PCR assays. Thus, our newly constructed mpox and orthopoxvirus real-time PCR assays exhibited specificity in mpox and orthopoxvirus detections.



copies/ reaction	Ct					
	E9L			E9L-reported		
1.00E+01	36.57	35.09	36.14	NA	39.61	39.92

copies/ reaction	Ct					
	D6R			D6R-reported		
1.00E+01	37.17	37.03	37.03	38.37	NA	NA

Fig. 2. Comparison of newly constructed real-time PCR assay to reported E9L (Kulesh et al., 2004) (A) or D6R (Luciani et al., 2021) (B) real-time PCR assay.

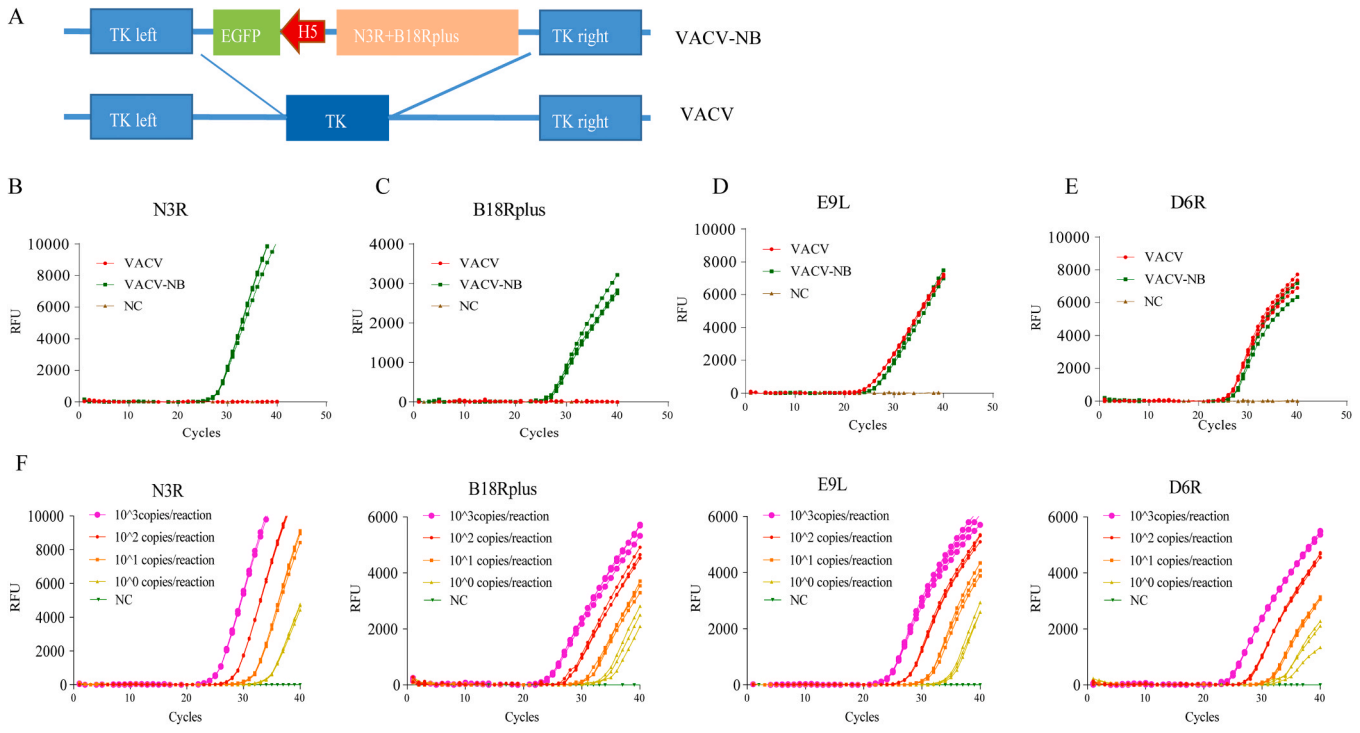


Fig. 3. Orthopoxvirus and mpox real-time PCR assays can detect viral genomic DNA at 1 copy/reaction. A. Construction of the recombinant VACV-NB virus by homologous recombination. B-E. Viral DNAs were extracted from VACV and VACV-NB virus and detected by N3R(B) B18Rplus(C), E9L(D) and D6R(E) real-time PCR amplification assays. F. LOD of the constructed real-time PCR assays based on N3R, B18Rplus, E9L and D6R amplification systems. VACV-NB genomic DNA was extracted, quantified by ddPCR, and series dilutions were used as template. NC: negative control.

3.3. Real-time PCR assays are able to detect VACV and VACV-NB in infected cells and mpox in clinical samples

We used virus infected cells and clinical specimens to further evaluate the real-time PCR methods. First, we validated the real-time PCR with virus infected cells directly. Vero cells infected with VACV-NB at different MOIs were treated with proteinase K, and the supernatant of the lysis was directly used as template in real-time PCR. The results showed that VACV-NB was detected in all infected specimens with the newly constructed N3R, B18Rplus, E9L and D6R methods (Supplemental Figure 4).

The efficiency of the real-time PCR assays were further evaluated using samples from the first mpox patient in Chinese mainland, who was tested positive for mpox infection by China CDC (Huang et al., 2022). Blister fluid swab, oropharyngeal swab and nasopharyngeal swab were collected, and virus DNAs were extracted and tested in our real-time PCR assays. All of these samples had positive amplifications in our tests (Fig. 6).

3.4. Multiplex real-time PCR amplification

To further optimize the orthopoxvirus and mpox specific detection method, we constructed a multiplex real-time PCR assay to detect mpox and orthopoxviruses simultaneously. E9L and B18Rplus real-time PCR were selected to construct the multiplex real-time PCR assay. E9L-F, E9L-R, E9L-P* with FAM fluorophore, and B18R-F, B18R-R, B18R-P* with Cy5 fluorophore were combined in this multiplex real-time PCR assay (Table 1). First, we verified the efficiency of the multiplex real-

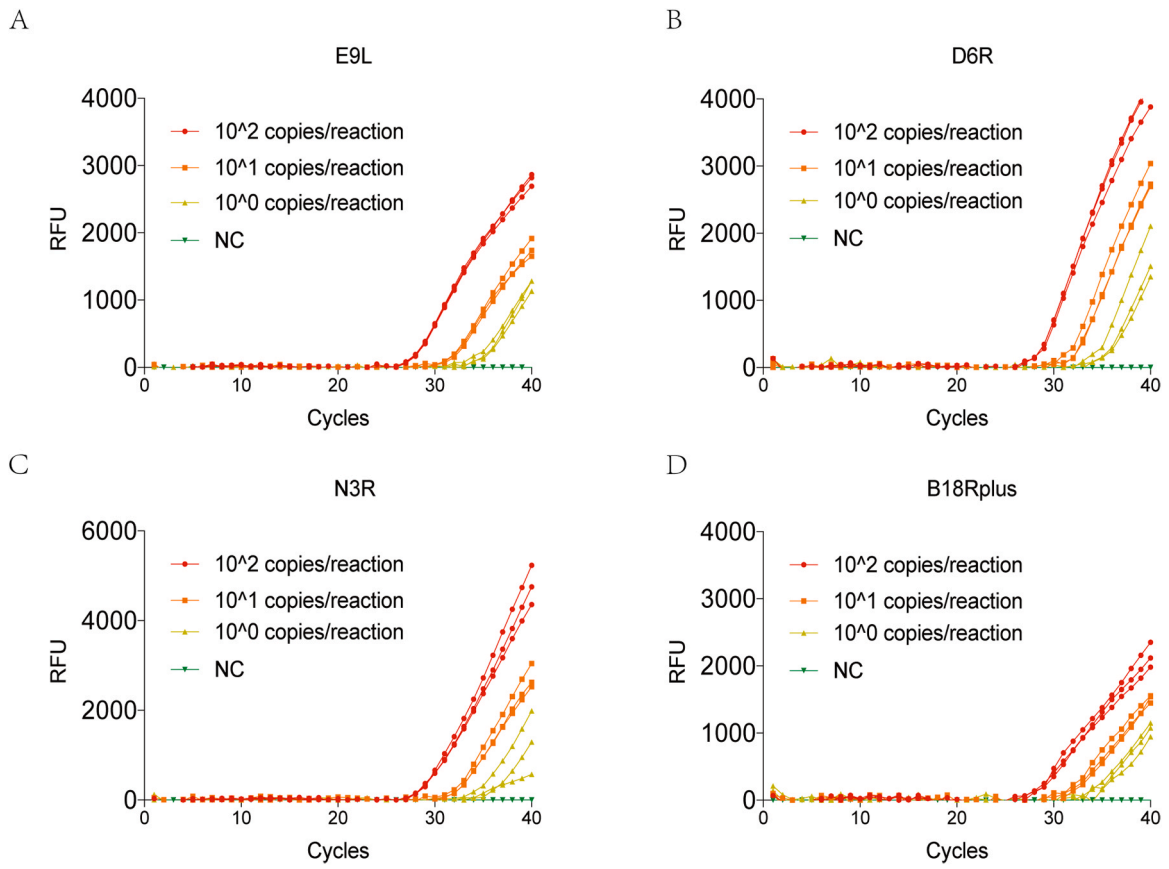
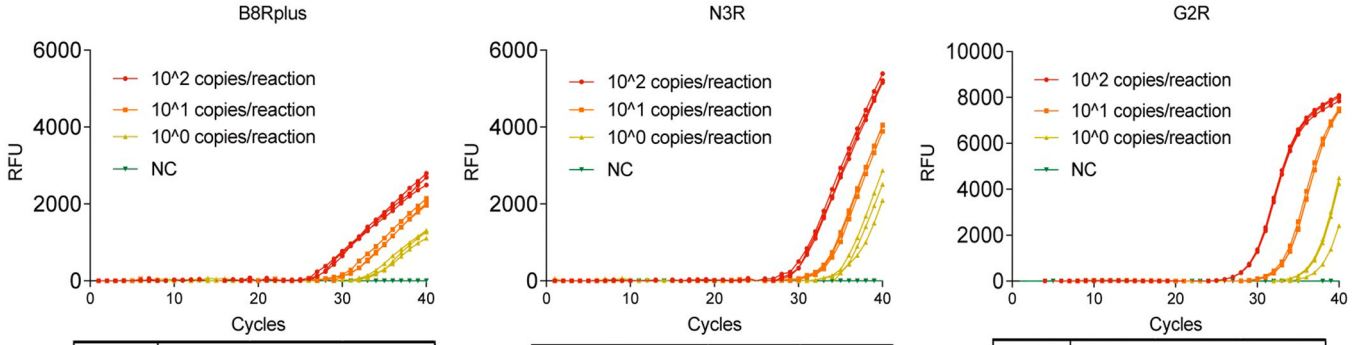


Fig. 4. Newly established real-time PCR assays can detect MPXV DNA at 1 copy/reaction. MPXV genomic DNA was extracted, quantified by ddPCR, and series dilutions were detected by E9L (A), D6R (B), N3R (C), B18Rplus (D) real-time PCR assays.



Copies/reaction	Ct		
10^0	33.35	34.14	33.86
10^1	30.80	30.90	30.18
10^2	26.73	27.56	27.53

Copies/reaction	Ct		
10^0	33.64	33.52	34.03
10^1	30.55	30.10	30.61
10^2	27.42	27.47	27.72

Copies/reaction	Ct		
10^0	38.6	37.28	37.15
10^1	33.68	33.4	33.73
10^2	29.68	29.74	29.61

Fig. 5. Comparison of the LOD of B18Rplus and N3R methods to reported G2R (Centers for Disease Control and Prevention, 2022) method.

time PCR with VACV, VACV-NB and MPXV DNA. As shown in Figs. 7A to 7C, only FAM fluorophore signal was positive with VACV DNA (Fig. 7A), while both FAM and Cy5 fluorophore signals were positive with VACV-NB (Fig. 7B) and MPXV (Fig. 7C) DNA. We also examined the LOD of multiplex real-time PCR assay for mpox detection. As shown in Fig. 7D, the multiplex real-time PCR assay can detect MPXV DNA at 1 copy/reaction.

Next, the efficiency of multiplex real-time PCR assay was further evaluated with virus infected cells and clinical samples. First, we applied this multiplex real-time PCR assay to detect VACV or VACV-NB infected Vero cells. As shown in Supplemental Figure 5, both VACV and VACV-NB infected samples had positive FAM amplification signals, and only VACV-NB infected samples had positive Cy5 amplification signals. We also evaluated our multiplex real-time PCR assay with samples from mpox patients reported in Chinese mainland. Blister fluid swabs, oropharyngeal swabs and nasopharyngeal swabs from patients all

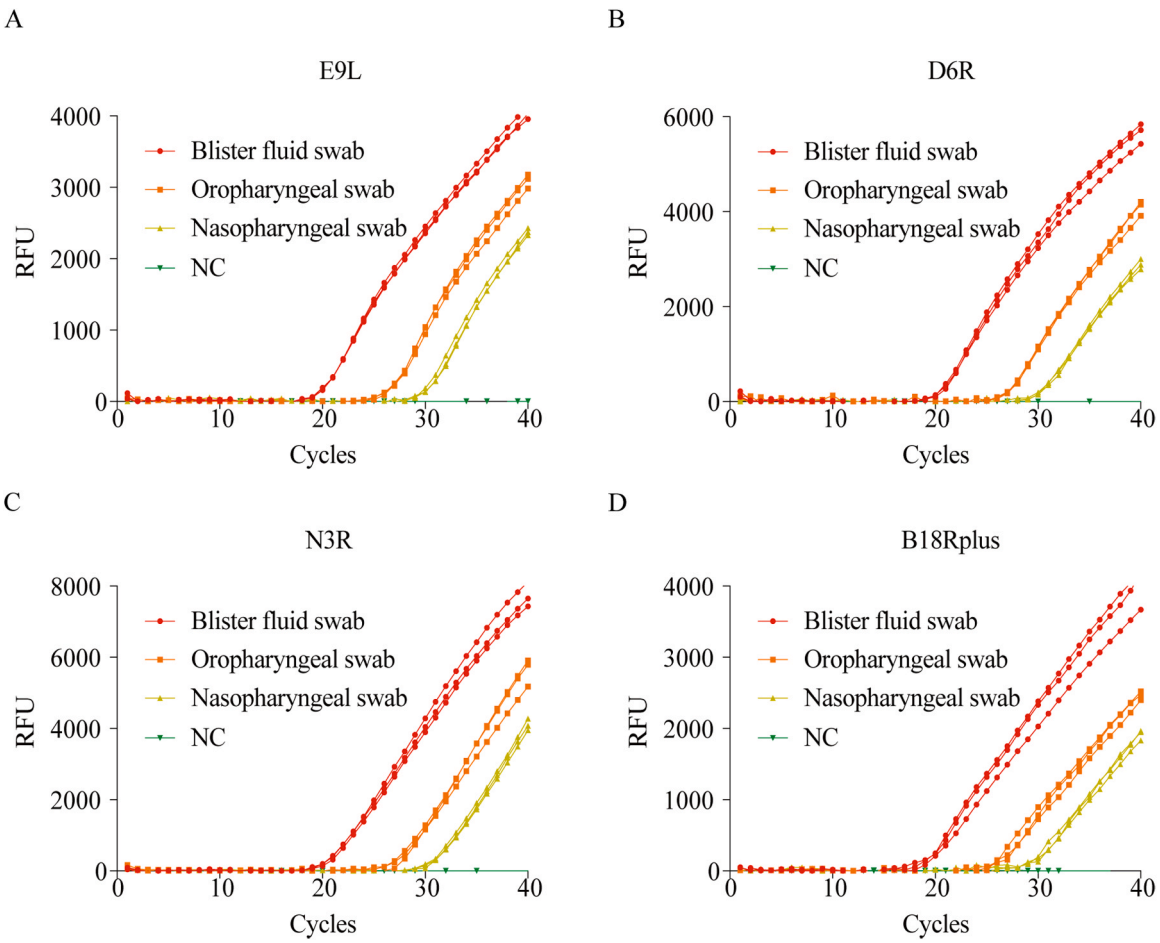


Fig. 6. Orthopoxvirus and mpox real-time PCR assays can detect mpox virus in samples from mpox patient. Clinical specimens were examined with E9L (A), D6R (B), N3R (C), and B18Rplus (D) real-time PCR assays.

produced positive amplification signals in both FAM and Cy5 channels, while positive signals could not be observed in samples from healthy donors (Supplemental Figure 6). All these data indicated that the newly constructed multiplex real-time PCR assay can simultaneously detect mpox and orthopoxviruses, and can distinguish mpox virus from other orthopoxviruses.

4. Discussion

Since the termination of smallpox vaccination, orthopoxvirus infection, especially the mpox outbreak since May 2022, had brought concerns to clinicians and scientists ($author1$ et al., 2021; Kozlov, 2022b). Early detection and diagnosis of mpox virus infection would be the most efficient way to mitigate the transmission. The reported mpox detection methods are based on SNPs of the consensus genes, such as B6R, G2R and F3L, to differentiate mpox virus from other orthopoxviruses (Li et al., 2006, 2010; Maksyutov et al., 2016). Here we developed a new mpox detection method based on mpox specific gene sequences, N3R and B18Rplus. These newly selected real-time PCR targets are conserved among epidemic mpox genomes and absent or exhibited low homology in other orthopoxviruses. The newly designed mpox-specific real-time PCR assays can differentiate mpox virus from VACV and exhibit an estimated limit of detection of 1 copy/reaction based on the quantification methodology utilised.

There are several mutations among MPXV strains during the 2022 mpox outbreak, and the mutation rate appears to be increasing (Isidro et al., 2022). APOBEC3 has been reported to generate those mutations and promote MPXV adaptation in humans (Chen et al., 2022). Recently, Gigante et al. reported seven MPXV strains with large deletions (Gigante et al., 2022), in which the G2R gene, the target site of USA CDC diagnosis assay for mpox (Li et al., 2010), was lost. Ongoing mutations, such as large deletions and genomic rearrangements in the future, may result in false-negative detections (Gigante et al., 2022; Kozlov, 2022a). Consistent with these reports, Garrigues et al. reported interference of mpox diagnosis caused by G2R-targeting sequence deletions in MPXV genomes from mpox patients (Garrigues et al., 2022). Although the newly reported DC0009 and MD0005 strains have a deletion of the B18R gene and the FL0052 strain has a deletion of the N3R gene (Gigante et al., 2022), the combination of our four targets can detect all MPXV strains reported.

Since mutations will continue to emerge, combining the highly conserved orthopoxvirus detection targets and mpox specific detection targets are deemed more practical to identify the infection of mpox virus and orthopoxviruses. In this context, pan-orthopoxvirus detection will be needed in the long-run. Several real-time PCR assays have been reported for orthopoxviruses (Luciani et al., 2021; Yang et al., 2022; Schroeder and Nitsche, 2010), among which the conserved genes E9L and D6R were targeted. In our study, we optimized the pan-orthopoxvirus detection method by targeting E9L and D6R, and the optimized pan-orthopoxvirus detection method has a LOD of 1 copy/reaction.

To further optimize the mpox and orthopoxvirus detection, we combined the mpox virus specific B18Rplus and pan-orthopoxvirus E9L real-time PCR assays and developed a multiplex real-time PCR assay. We tested the efficiency of the multiplex real-time PCR assay with virus infected cells and clinical samples, and the modified detection method

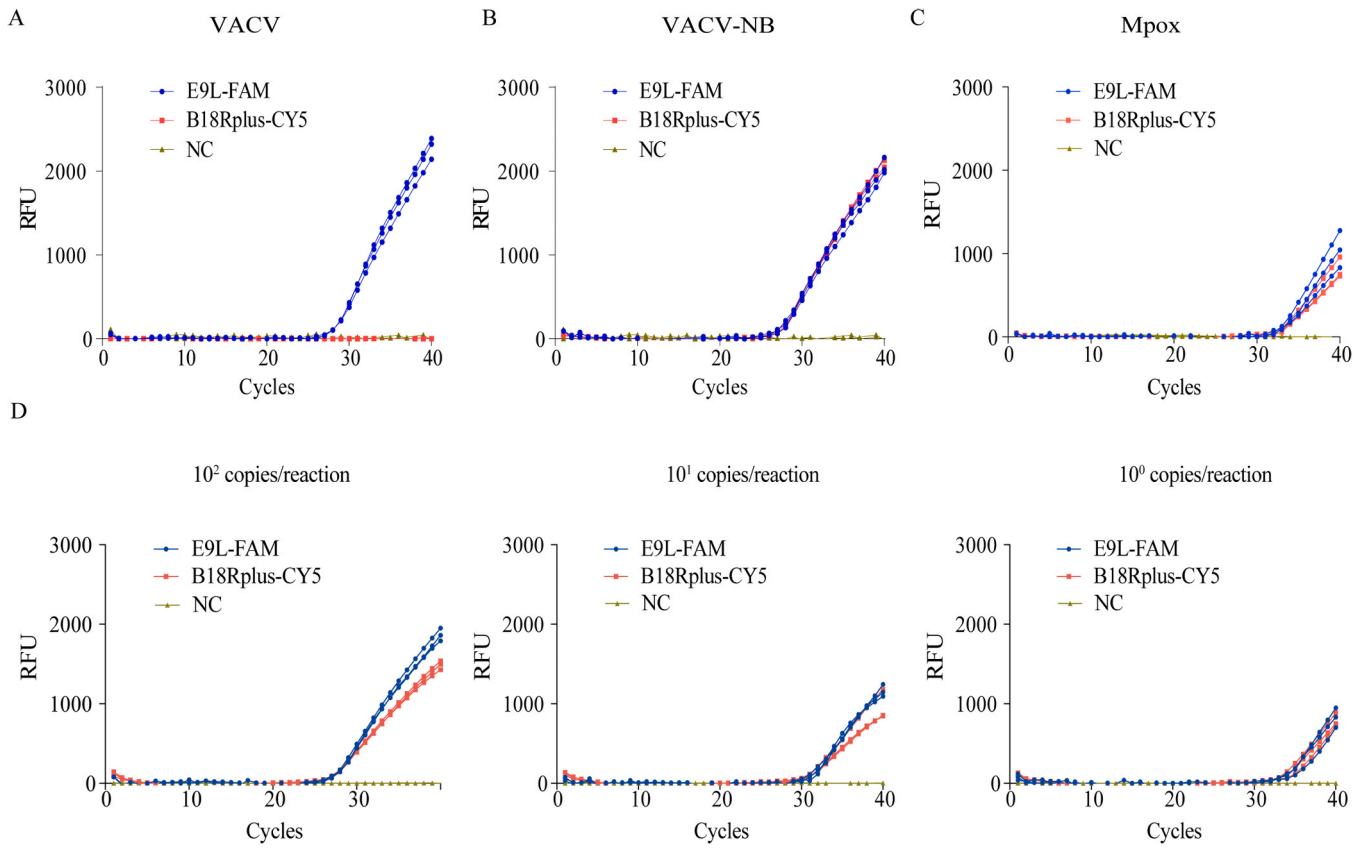


Fig. 7. Multiplex real-time PCR detection system. VACV genomic DNA (A), VACV-NB DNA (B), and mpox viral DNA (C) was detected by multiplex real-time PCR, and amplification curves from FAM and Cy5 channel were shown. D. 10-fold dilutions of mpox virus DNA were detected by multiplex real-time PCR assay.

could detect mpox virus and orthopoxvirus simultaneously. More clinical samples may be needed in the future to further evaluate the sensitivity and specificity of our method for clinical applications.

In conclusion, we have developed a new mpox detection method based on mpox virus specific sequences with a high sensitivity and specificity and can detect mpox virus from clinical samples efficiently. The optimized pan-orthopoxvirus real-time PCR assay can diagnose mpox and other orthopoxvirus infections efficiently and specifically. We have further combined mpox and pan-orthopoxvirus detection into one system, and developed a multiplex real-time PCR assay, which can detect mpox virus and orthopoxvirus infections simultaneously with rapidity and simplicity.

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Ethical statement

Informed consent may be exempted as a public health response to an emerging infectious disease outbreak. The collection of data and samples from human cases is determined by the National Bureau of Disease Control and Prevention of the People's Republic of China as part of an ongoing public health outbreak investigation and does not require approval by institutional review board (Huo et al., 2022).

CRediT authorship contribution statement

Lingwa Wang: Formal analysis, Data curation. **Jugao Fang:** Visualization. **Yu Xie:** Writing – original draft, Validation, Methodology. **Chen Liang:** Writing – review & editing, Visualization. **Baoying Huang:** Writing – original draft, Validation. **Zhao Gao:** Formal analysis, Data curation. **Bin Ai:** Visualization. **Zhangling Fan:** Writing – original draft, Validation, Methodology. **Fei Guo:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Yu Huang:** Formal analysis, Data curation. **Shan Mei:** Formal analysis, Data curation. **Fengwen Xu:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Fei Zhao:** Formal analysis, Data curation. **Wenjie Tan:** Writing – review & editing, Supervision, Conceptualization. **Yamei Hu:** Formal analysis, Data curation. **Liang Wei:** Formal analysis, Data curation. **Liming Wang:** Formal analysis, Data curation.

Declaration of Competing Interest

F.G., F.X., F.Z. and S.M. are inventors on pending and issued patents on mpox detection system. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationship that could be constructed as a potential conflict of interest.

Data Availability

All the original data were included in the article and supplementary materials. Further inquiries can be directed to the corresponding authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.114957.

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