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Letter

Development of rapid nucleic acid assays based on the recombinant polymerase amplification for monkeypox virus

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Dear Editor,

Monkeypox virus (MPXV), an enveloped double-stranded DNA virus with 190 open-reading frames and a genome length of about 200 kb, belongs to the *genus Orthomyxovirus* (OPXV; subfamily *Chordopoxvirinae*, family *Poxviridae*), which causes a disease with symptoms similar to, but less severe than, smallpox. MPXV is subdivided into two clades: clade I for the former Congo Basin clade and clade II for the former West African, and the clade I is more pathogenic. The clade II consists two subclades, clade IIa and clade IIb, with the latter referring primarily to the group of variants largely circulating in the 2022 global outbreak (ICTV, 2022; Bunge et al., 2022; Mauldin et al., 2022).

Monkeypox can be transmitted from animals to humans. Cases often occur close to tropical rainforests. Though human-to-human transmission of MPXV is limited, it can occur through direct contact with infectious skin or mucocutaneous lesions. The incubation period of monkeypox has historically ranged from 5 to 21 days. Typically, the prodromal phase of clinical illness lasts 1–5 days, during which time patients may experience fever, headache, back pain, muscle aches, and lymphadenopathy. The second phase which occurs typically after the fever subsides, and is characterized by a rash that may involve the oral mucous membranes, conjunctiva, cornea and/or genitalia (Adler et al., 2022; Miura et al., 2022).

Since 1 January and of Oct 7, 2022, 71,237 confirmed Monkeypox-infected cases in 107 countries have been reported to World Health Organization (WHO), and total deaths reached 26. Globally, the overall risk is assessed as moderate, considering this is the first time that cases and clusters are reported concurrently in five WHO Regions. The risk in the European Region is considered to be high due to its report of a geographically widespread outbreak involving several newly affected countries, as well as a somewhat atypical clinical presentation of the cases. In other WHO Regions, the risk is considered moderate with consideration for epidemiological patterns, possible risk of importation of cases and capacities to detect cases and respond to the outbreak. In newly affected countries, this is the first time that cases have mainly, but not exclusively, been confirmed among men who have had recent sexual contact with a new or multiple male partners (WHO, 2022a, 2022b).

For MPXV test confirmation, the best diagnostic specimens are directly from the rash-skin, fluid or crusts, or biopsy where feasible (Erez et al., 2019; Thornhill et al., 2022). Methods that detect antigens and antibody may not be useful as they do not distinguish between orthomyxoviruses (Stern et al., 2016). At present, detecting viral DNA by quantitative polymerase chain reaction (qPCR) is the preferred laboratory test for monkeypox; indeed, a positive PCR result is part of case definition, regardless of associated symptoms or their absence (WHO, 2022a, 2022b; Erez et al., 2019). However, qPCR method is time consuming (> 1 hours) and requires complex and expensive equipment. Therefore, there is a need for a faster and simpler method for MPXV testing.

Recombinant polymerase isothermal amplification (RPA) technology utilizes a recombinant enzyme obtained from bacteria or fungi. At room temperature, the recombinant enzyme binds tightly to the primer DNA to form a polymer. First, the primer recognizes a complementary sequence in the template DNA. Next, with the help of a single-stranded DNA binding protein, the double-stranded structure of the template DNA is unwounded and opened by DNA polymerase. A new DNA strand is formed, and the amplification product grows exponentially. The RPA technology can amplify a target gene within 15–20 min at room temperatures (25–42 °C), meaning that the amplification reaction is faster than qPCR; also, and the equipment is both compact and cheap, making it more suitable than qPCR for application in local unit (Jia et al., 2019; Wang et al., 2022; Davi et al., 2019; Tang et al., 2022).

In this study, we developed two RPA methods to detect MPXV, fluorescence-RPA (F-RPA) and vertical flow strip RPA (VF-RPA). F-RPA is performed in a small portable fluorescence-RPA device, and the

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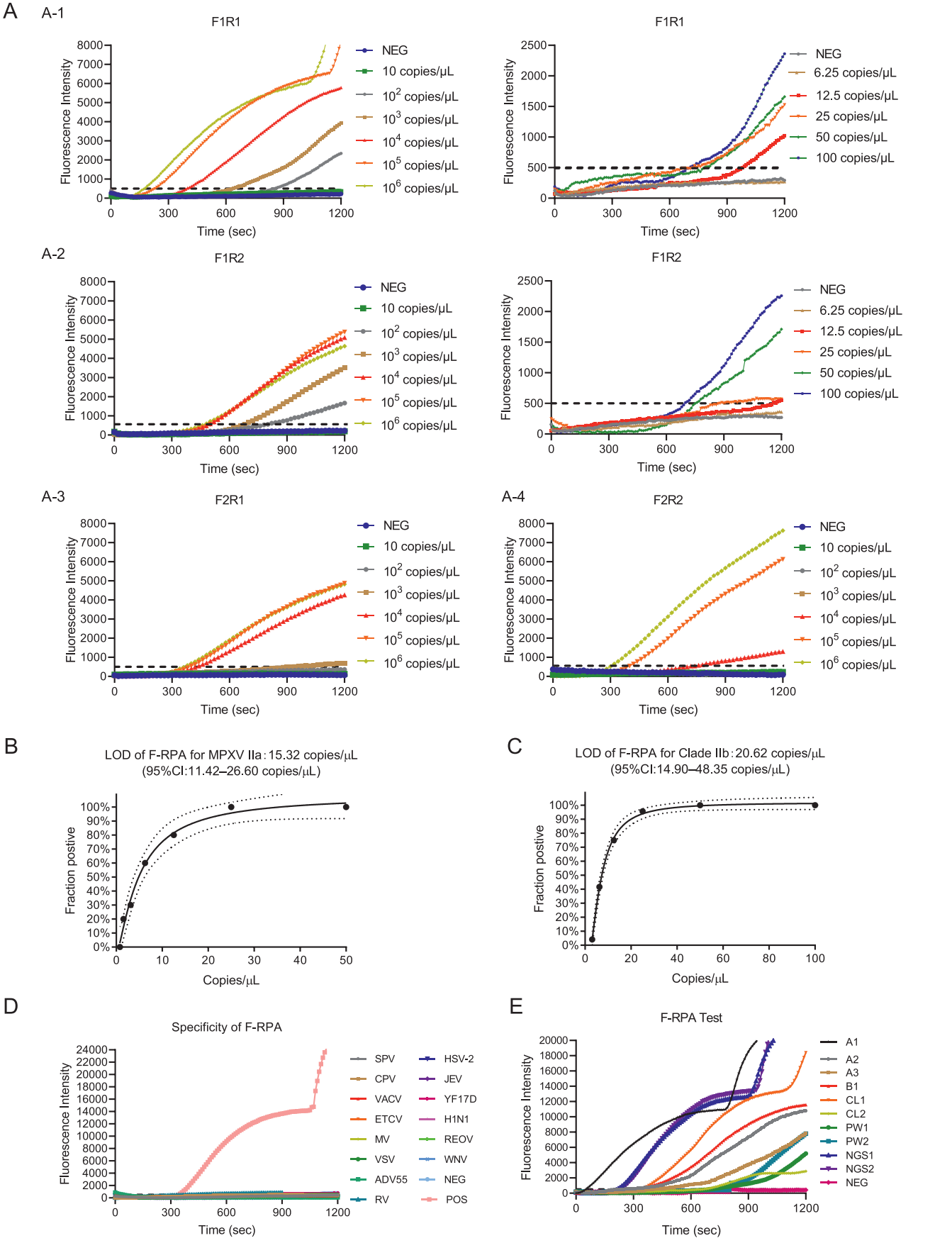

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fluorescence curve can be observed in real time, which is suitable for local medical unit; VF-RPA requires only a small thermostatic bath; the VF-RPA results can be visualized on a strip, making the assay sensitive, easy to operate and low-cost. Thus, the VF-RPA is of potentially valuable for MPXV detection in local units. One concern, however, is avoiding DNA contamination during tube opening and sampling. In this study, the VF-RPA reaction takes place within a simple disposable vertical-flow strip cassette, which is designed for instrument-free, cross-contamination-proof detection of amplicons, enabling rapid identification of MPXV in field. Therefore, both of F-RPA and VF-RPA would be potentially useful in local medical unit for rapid MPXV infection identification.

To develop the F-RPA and VF-RPA, we first designed the primer and probe sequences for MPXV-RPA assay. MPXV genome sequences used for sequence alignment and primer/probe design (including both the West African clade and central African clade, as well as the emerging MPXV strains) were obtained from the NCBI websites. The downloaded MPXV strains and the gene accession numbers are shown in Supplementary Figure S1.

The sequences were aligned using Meg Align and a 399 nucleotide (nt) fragment of B7R (168,791–169,189, sequence ID: KP849470.1) was selected as target gene. This fragment was synthesized and cloned into plasmid pUNC57 via the *Kpn*I and *Hin*dIII restriction sites (Sango Bio Inc., Shanghai, China). The recombinant plasmid pUNC57-B7R was used as the template for the MPXV-RPA. The RPA primers and probe were also designed and synthesized by Sango Bio Inc. (Shanghai, China). The primer and probe sequences are listed in Supplementary Table S1 and partial alignments of oligonucleotide binding regions are in Supplementary Figure S1. The sequence of the candidate primer and probe are identical to that of MPXV Clade I and IIa, and have only a single base mutation when compared with the strain circulating in 2022 (MPXV Clade IIb), this mutation occurs immediately after the THF cleavage site in the probe. In theory, the single base mutation will not affect target gene detection.

To screen the primer sets, two forward primers, two reverse primers and one probe were designed; thus, four candidate primer sets could be used to amplify the serially diluted plasmid containing target genes (10^2–10^6 copies/μL) to screen the most sensitive primer sets for MPXV-RPA assay. The F-RPA reaction was performed to screen the primer sets screening. The protocol is as follows: 45.5 μL of a master mixture comprising 2.1 μL of forward prime B7R–F1 (10 μmol/L), 2.1 μL of reverse primer B7R-R1, 0.6 μL of B7R-Probe (10 μmol/L), and 40.7 μL of rehydration buffer A (Zhongce Ltd., Hangzhou, China) was placed in a 0.2 mL reaction tube containing a dried enzyme pellet (Zhongce Ltd., Hangzhou, China). The tube was vortexed to dissolve the dried enzyme pellet. Next, 2 μL of DNA template was added, and 2.5 μL of magnesium acetate (MgAc; 280 mmol/L) was pipetted onto the inside of tube lids, the total reaction volume was 50 μL. Next, the lids were closed, and the RPA tubes were centrifuged using a mini-spin centrifuge to deposit the MgAc; the tubes were immediately placed into an isothermal fluorescence scanner (Qitian, China) at 42 °C for 20 min since the reaction will proceed immediately after the addition of magnesium acetate. The real-time fluorescence signals were measured over time. The fluorescence signal threshold was set as three standard deviations above the background signal determined within 1 min; the slope (mV/time) was calculated by F1620 software. Signals were interpreted using combined threshold and signal slope analysis. A positive reading was set as a slope greater than 15 mV/min, with a fluorescence signal >500.

The results showed that among the four primer sets (F1R1, F1R2, F2R1 and F2R2), the limit of detection for F2R1 and F2R2 was about 10^4 copies/μL, which was significantly higher than that of F1R1 and F1R2 (10^2 copies/μL), therefore, the F2R1/F2R2 pairs were discarded first. The LODs of both F1R1 and F1R2 reached 100 copies/μL (Fig. 1A). To select the most sensitive primer set, further dilutions (100, 50, 25, 12.5, 6.25 copies/μL) of the template were tested using F1R1 and F1R2, the F-RPA results showed that F1R1 detected a minimum concentration of 12.5 copies/μL, while F1R2 detected only 50 copies/μL, indicating that F1R1 has higher sensitivity (Fig. 1A). Therefore, we selected F1R1 as the primer pair for monkeypox detection.

First, we used primer set F1R1 to develop the MPXV F-RPA assay. The limit of detection (LOD) determination experiment was conducted as follows: seven different concentrations (1–10^6 copies/μL) of synthesis plasmid pUNC57-B7R (containing the target gene) were used as templates. All DNAs were tested in duplicate and in three independent runs. The results revealed that F-RPA generated positive results in test samples containing 100 copies/μL or more; not all samples containing 10 copies/μL produced positive results. Next, the template DNAs were serially diluted 2-fold (100–6.25 copies/μL) to yield five different concentrations. These were applied to the F-RPA in three individual replicate reactions (eight replicates for each sample at each experiment). The positive percentage was calculated for each dilution, and the LOD with 95% confidence interval was calculated with nonlinear probit regression analysis (SPSS 2.0 software). Based on the probit regression average, the LOD for MPXV F-RPA was calculated as 15.32 copies/μL, with a 95% CI of 11.42–26.60 copies/μL (Fig. 1B).

To test the utility of the RPA for MPXV Clade IIb, we synthesized the corresponding *B7R* gene fragment in the MPXV/Germany/2022/ON/RKI169 strain (sequence ID: OP0188588.1; nt166236-166,634), the LOD reached 20.62 copies/μL, which was comparable with that for the previous MPXV virus gene, indicating that this RPA system could not only detect the previously prevalent monkeypox virus, but can also be used to detect the current monkeypox virus strain (Fig. 1C).

The specificity of the F-RPA assay was determined using a representative panel of inactivated viruses, including vaccinia virus (VACV), cowpox virus (CPV), Ectromelia virus (ECTV), measles virus (MV), rubella virus (RV), vesicular stomatitis virus (VSV), human herpes virus 2 (HSV-2), adenovirus 55 subtype (ADV55), yellow fever virus (YF17D), Japanese encephalitis virus (JEV), West Nile virus (WNV), H1N1 influenza virus (H1N1), reovirus (REOV), and smallpox virus (SPV) synthetic plasmid, all of the concentration of virus were higher than 10^6 pfu/mL. No positive signals were observed in any of these samples (Fig. 1D).

Next, we combined the RPA reaction with a disposable sealed nucleic acid detection cassette to generate the VF-RPA. The schematic diagram of VF-RPA is shown in Fig. 2A. To carry out the VF-RPA protocol, the VF-RPA reaction was performed first; the protocol is similar to the F-RPA, but the method of labeling the reverse primer and probe is different. The concentration of the primers and the reaction time were optimized to achieve higher sensitivity and specificity. The optimized VF-RPA was conducted as following: The final volume (50 μL) comprised 1.4 μL of forward primer B7R–F1 (10 μmol/L), 1.4 μL of biotin labeled reverse

Fig. 1. Establishment and evaluation of the fluorescence-RPA (F-RPA) for monkeypox virus (MPXV). **A** Sensitivity of the four candidate primer sets. **A-1,** Sensitivity of the F1R1 primer set; **A-2**, Sensitivity of the F1R2 primer set; left: the concentration of the template ranging from 10–10^6 copies/μL, right: the concentration of the template ranging from 100 to 6.25 copies/μL; **A-3**, Sensitivity of the F2R1 primer set; **A-4**, Sensitivity of the F2R2 primer set. Primer set F1R1 showed the strongest signals and highest sensitivity. A positive reading was set as a slope (mV/time) greater than 15 mV/min, with the fluorescence signal >500. The dotted/dashed lines represent the cutoff value the fluorescence signal. **B** Limit of detection (LOD) of the F-RPA assay for MPXV Clade IIa. **C** Limit of detection (LOD) of the F-RPA assay for MPXV Clade IIb. The inner line is the probit curve (dose-response rule). The outer dotted/dashed lines are the 95% confidence intervals. **D** Specificity of the F-RPA. DNA/RNA samples from fever- or rash-associated inactivated virus cultures were tested. Samples included vaccinia virus (VACV), cowpox virus (CPV), Ectromelia virus (ECTV), measles virus (MV), rubella virus (RV), vesicular stomatitis virus (VSV), human herpes virus 2 (HSV-2), adenovirus 55 subtype (ADV55), yellow fever virus (YF17D), Japanese encephalitis virus (JEV), west Nile virus (WNV), H1N1 influenza virus (H1N1), reovirus (REOV), and smallpox virus (SPV). No positive signal appeared in any of these samples. POS, positive sample; NEG, negative sample. **E** F-RPA test conducted using ten MPXV-positive samples.

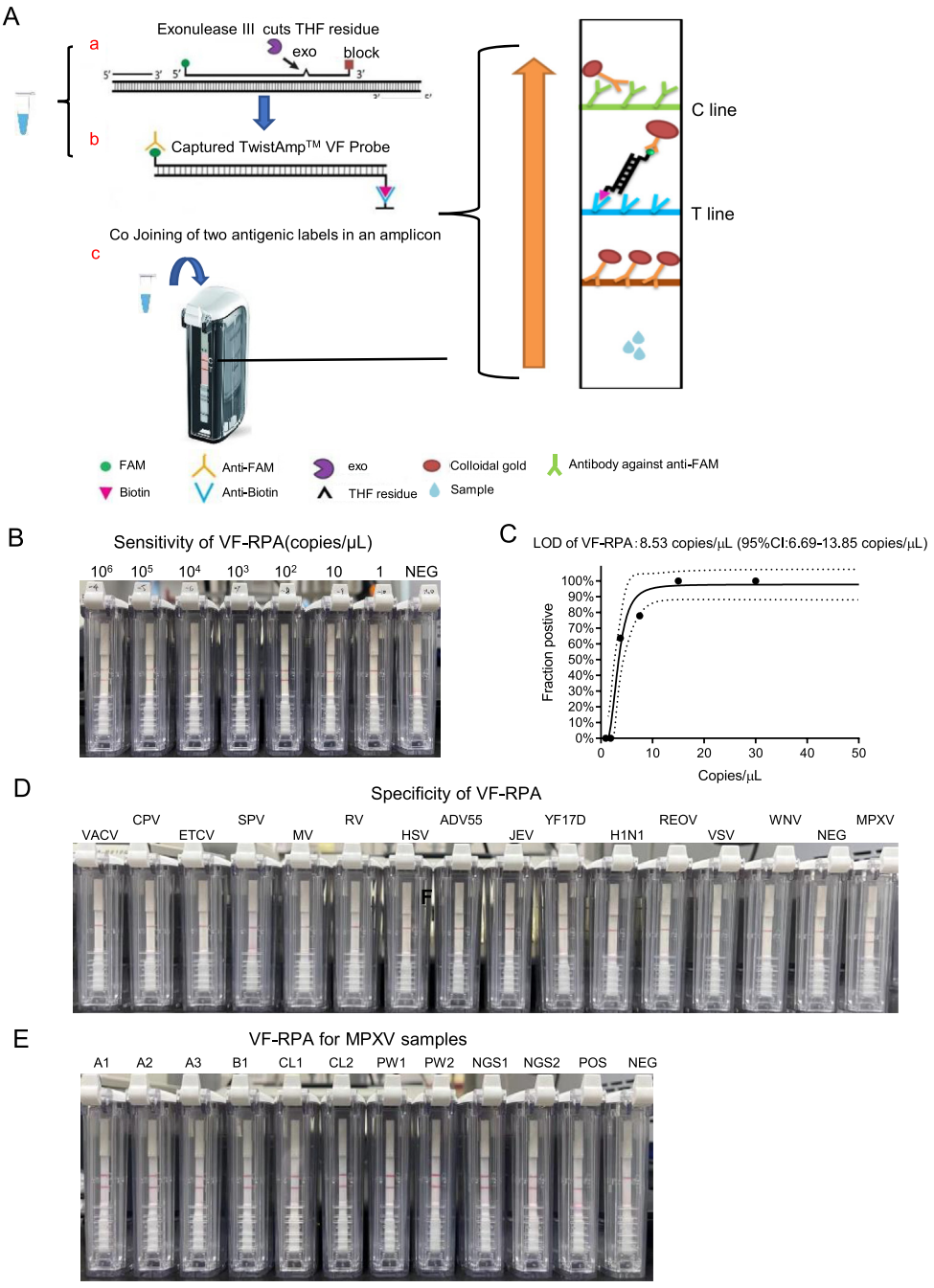

Fig. 2. Evaluation of the vertical flow strip RPA (VF-RPA) for monkeypox virus (MPXV). **A** Schematic illustration of the VF-RPA. **A-a**, RPA reaction; **A-b**, Hybridization of the VF-probe with the biotin-labeled amplicons; **A-c**, Color development in the type 3 disposable nucleic acid detection cassette. **B** VF-RPA results for DNA samples (diluted 10-fold; 10^0–10^6 copies/μL). **C** Limit of detection (LOD) of the VF-RPA assay for MPXV. The inner line is the probit curve (dose-response rule). The outer dotted/dashed lines are the 95% confidence intervals. **D** Specificity of the VF-RPA. DNA/RNA samples from fever- or rash-associated inactivated virus cultures were tested. Viruses included vaccinia virus (VACV), cowpox virus (CPV), Ectromelia virus (ECTV), measles virus (MV), rubella virus (RV), vesicular stomatitis virus (VSV), human herpes virus 2 (HSV-2), adenovirus 55 subtype (ADV55), yellow fever virus (YF17D), Japanese encephalitis virus (JEV), West Nile virus (WNV), H1N1 influenza virus (H1N1), reovirus (REOV), and smallpox virus (SPV). No positive result appeared in any of these samples. **E** VF-RPA test conducted using ten MPXV-positive samples.

primer B7R-R1-VF, 0.4 μL of FAM labeled B7R-Probe-VF (10 μmol/L) (see Supplementary Table S1 for sequences and labeling description), 2 μL of template DNA, 2.5 μL of magnesium acetate and 42.3 μL of rehydration buffer. After incubating at 42 °C for 15 min, the positive VF-RPA reaction generated a dual-labeled hybrid fragment: a FAM-labeled B7R -probe -VF and a biotin-labeled amplicon. Next, the tubes were placed in a type 3 disposable nucleic acid detection cassette (USTAR BioTech Corporation, Hangzhou, China), at room temperature for 5–10 min for vertical flow strip detection. Pushing the handle cuts the tube, allowing the RPA product to flow into the strip. In the cassette, a strip with a test (T) line (which captures the dual-labeled probe-amplicon) and a control (C) line (which captures gold-labeled rabbit anti-FAM antibody). A positive result occurs when both the T line and the C line were visible through the detection window. A negative read occurs when only the C

line is displayed. The assay is regarded as invalid if neither the T line nor the C line is displayed.

The LOD of the VF-RPA was determined using serial dilutions of the synthetic plasmid as templates, the method is similar with the F-RPA, based on the nonlinear probit regression analysis by SPSS 2.0 software, the LOD of the MPXV VF-RPA was calculated as 8.53 copies/μL (95% CI: 6.69–13.85 copies/μL) (Fig. 2B and C), which is a little lower than that of the F-RPA (15.32 copies/μL), meaning that VF-RPA is more sensitive. Actually, the sequences of the two sets of primers and probes are the same, which means that the amplification efficiency of the target gene is similar for the F-RPA and VF-RPA. The main differences between F-RPA and VF-RPA are the way in which the primers and probes are labeled, and the way in which the results are presented. For the F-RPA, the probe was labeled with FAM, and the results were based mainly on fluorescence intensity. In the VF-RPA, the probe was labeled with FAM, and the reverse primer was labeled with biotin. Therefore, the amplicon was captured by the specific binding to avidin on the test strip, and colloidal gold aggregation and color development were performed simultaneously by the specific binding of FAM to anti-FAM (conjugated with colloidal gold); thus, the signalling cascade in VF-RPA is stronger than in the F-RPA, resulting in higher sensitivity. Since the signal in the VF-RPA is amplified, the risk of a false positive via nonspecific amplification is increased. Therefore, to reduce the nonspecific signal, we shortened the amplification reaction to 15 min. The specificity of the VF-RPA assay was also determined as for the F-RPA; no positive signals were observed, (Fig. 2D), meaning that both the F-RPA and VF-RPA are specific for MPXV.

To date, there are no clinical samples of monkeypox infection or MPXV strains in China up to now, which led to great difficulty with respect to clinical validation of the F-RPA/VF-RPA. Fortunately, in 2018 and 2019, we participated in the international viral External Quality Assurance Exercise (EQAEs) for *Orthomyxovirus* samples identification test, which was sponsored by the Robert Koch Institute, Germany; therefore, we used EQAE samples for F-RPA/VF-RPA validation. These included ten inactivated MPXV samples (four MPXV Clade IIa and six Clade I), two vaccinia virus samples, four cowpox virus samples, and five negative matrix samples (a total of 21 samples). The CT value of the ten MPXV samples in the qPCR assay varied from 28.59 to 37.02 (The qPCR protocol was in supplemental material and the CT value for the ten MPXV samples was demonstrated in Supplementary Table S2), indicating that the concentration of MPXV gene varied from high to low. Although the species of the tested samples were revealed later by the sponsor, the actual concentration of the ten MPXV was unknown. About 22 measles samples from Hebei Children's Hospital and 23 common fever samples from Chinese PLA General Hospital, were used as negative samples; in total, we evaluated 10 positive MPXV samples and 56 negative samples. Both of the F-RPA and VF-RPA provided accurate result; the *Kappa* value reached 1.00 by consistency test with qPCR using SPSS 2.0 software, (Figs. 1E and 2E and Table 1), demonstrating that both the F-RPA and VF-RPA are applicable for detection of MPXV nucleic acid.

Table 1
Result of the F-RPA/VF-RPA validation.

Sample (no.)	F-RPA		P-RPA		qPCR	
	Negative	Positive	Negative	Positive	Negative	Positive
MPXV (10)	0	10	0	10	0	10
CPV (4)	4	0	4	0	4	0
VCV (2)	2	0	2	0	2	0
Matrix (5)	5	0	5	0	5	0
MV (22)	22	0	22	0	22	0
Fever (23)	23	0	23	0	23	0
Total	56	10	56	10	56	10

MPXV: monkeypox virus samples; CPV: cowpox virus samples; VCV: vaccinia virus samples; MV: swab samples from measles virus infected patients; Fever: throad swab samples from common fever patients. The consistency of F-RPA/VF-RPA with qPCR results was analyzed by SPSS 2.0 software, the Kappa value was 1.00.

In nucleic acid detection methods, target gene selection greatly affects the sensitivity and specificity. The abundance of different target genes varies between samples, which affects sensitivity, whereas the similarity of the target gene sequence to that of other species affects the specificity. With respect to primers and probe, formation of dimers and hairpins can have a marked effect on amplification efficiency, again affecting the sensitivity and specificity of an assay. In 2019, Davi et al. used *G12R* gene as the detection target and established a fluorescence-RPA assay for monkeypox virus; the LOD of the assay was 12 copies/μL (Davi et al., 2019). In this study, we used *B7R* gene as target and the sensitivity of F-RPA/VF-RPA established herein is comparable with that, suggesting that both *G12R* and *B7R* are suitable targets for MPXV RPA assays. The genome of monkeypox virus is as long as 200 kb, therefore, there will be other suitable target genes and primers (in addition to *G12R* and *B7R*) for RPA, resulting in more options for rapid detection of MPXV.

The VF-RPA/F-RPA described herein requires prior extraction of DNA, which may limit its use for in-field MPXV detection. In a previous study, we developed an integrated RPA assay for SARS-CoV-2, which can amplify the target gene directly from a sample without nucleic acid extraction; this method should be applicable to other viruses such as MPXV. However, because no MPXV strain is available in China, we cannot optimize and validate the integrated RPA for MPXV detection (Tang et al., 2022). Once we obtain MPXV, we will develop an integrated RPA assay, thereby providing a more convenient and powerful detection method for prevention and control of monkeypox infection.

Footnotes

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All the data generated during the current study are included in the manuscript. Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.12.001.

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