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Letter

A visual assay panel for the identification of monkeypox virus DNA belonging to the clades I and II

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Dear Editor,

Monkeypox virus (MPXV) is an enveloped double-stranded DNA virus belonging to the family *Poxviridae*, subfamily *Chordopoxvirinae*, and genus *Orthopoxvirus* (Hraib et al., 2022; Gong et al., 2022). MPXV forms Congo Basin clade (clade I) and West African clade (clade II) (Durski et al., 2018). Additionally, clade II consists of two subclades, clade IIa and clade IIb. The 2022 MPXV isolates in global outbreak were found to belong phylogenetically to the clade IIb, which caused the first widespread human-to-human transmission (WHO, 2022). To date, MPXV has spread to 103 countries and regions worldwide. On July 23, 2022, the World Health Organization declared the monkeypox (Mpox) outbreak a Public Health Emergency of International Concern (WHO, 2022; Peng et al., 2023). With the increasing number of infection cases worldwide, developing a rapid detection tool for MPXV to improving surveillance and detection capacities in endemic countries and regions is of great significance.

Detecting is essential for preventing the spread of the virus. Previous studies have found that *Orthopoxvirus* members cannot be fully distinguished by antibody testing, due to there was a cross-reactivity between MPXV and smallpox virus (Hughes et al., 2014). And the production of antibodies has a certain delay, which is not conducive to the rapid diagnosis of early disease. Based on this, we established a nucleic acid viusal assay panel for rapid identification and detection of the MPXV clade I. Several PCR assays, recommended by WHO, had detection limits ranging from 3.5 to 40.4 copies, which can distinguish orthopoxviruses by multiple real-time PCR (Maksyutov et al., 2016; Durski et al., 2018). Compared with other diagnostic methods, real-time PCR has the advantages of high-quantity throughput and increased sensitivity. However, the detection period of these assays was longer than 90 min and the results relied on sophisticated temperature control equipment or fluorescence signal capture equipment.

Accordingly, to enhance MPXV surveillance with rapid detection capacities and to prevent the spread of the virus into nonendemic countries, in this study, recombinase-aid amplification and closed vertical flow visualization strip (RAA-VF) were combined to assemble a nucleic acid visual assay panel for rapid identification of the clades I and II of MPXV.

First, we analyzed the gene sequences of the MPXV and selected the *F3 L* and *D18 L* genes as targets to distinguish the two clades of MPXV. Among them, primers and probe designed against the *F3 L* gene can be used as universal targets for clade I and clade II, and primers and probe for the *D18 L* gene can identify clade II. It should be noted that the genome of the clade I (Genbank No. NC_003310.1) has an extra 1953 bp of gene fragments compared to clade II (Genbank No. ON563414.3), located at positions 18,953 to 20,905 nt, including *D14 L*, *D15 L*, *D16 L* and *D17 L* genes. Previous studies have confirmed that the RAA fails to produce amplicons of 1500 base or more (Daher et al., 2016). Considering this shortcoming, we designed a forward primer targeting the overlapping sequence between *D13 L* and *D18 L* genes. With clade II as a template in RAA reaction system, forward primer D18 L-F (Biotin-CCAATTCATTTCTAATAGTATCAAACTCTCCA), reverse primer D18 L-R (TATAAGGATGTTTAACTTTTCTAAAAGATA) and probe D18 L-P [FAM-AAACTATATAATACGTCGATGTCAGAAATAATCAACA(THF)TAT TACTGCGGTGAC-C3 spacer] eventually extend to form a large number of double-stranded nucleotide fragments labelled with both FAM and biotin at 195 bp. With clade I as a template, RAA failed to amplify the 2188 base between the primers. Hence, the D18 L assay based on the above scheme ensures that there is no cross-reactivity with clade I. This provides a new strategy to enhance surveillance and epidemiological investigation of MPXV epidemics.

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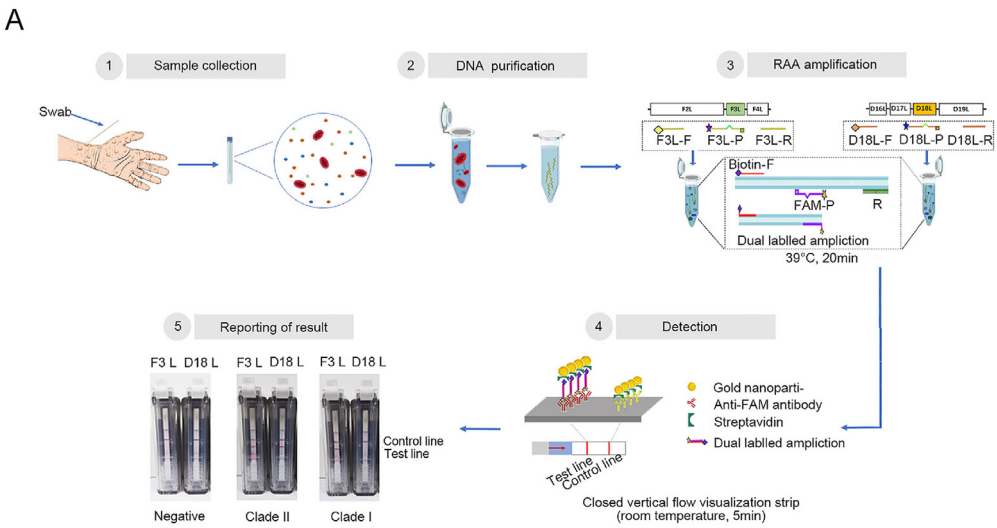

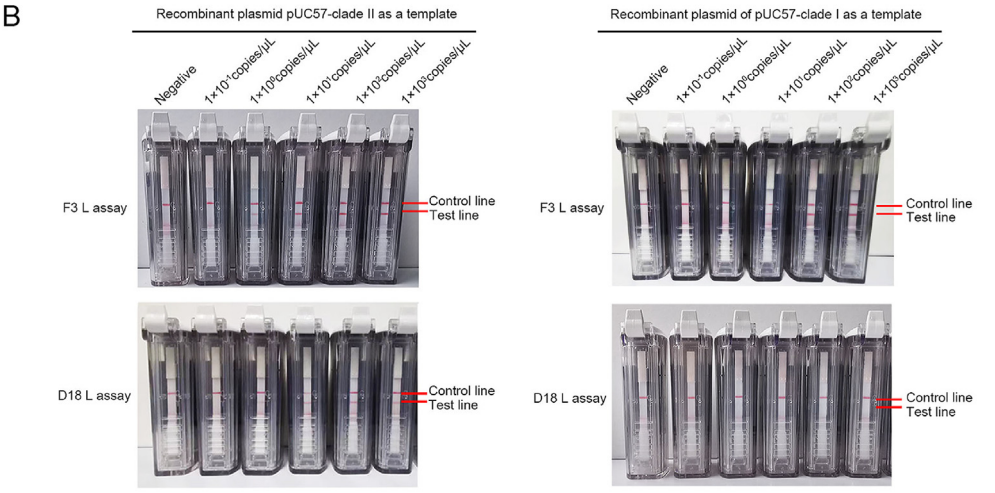

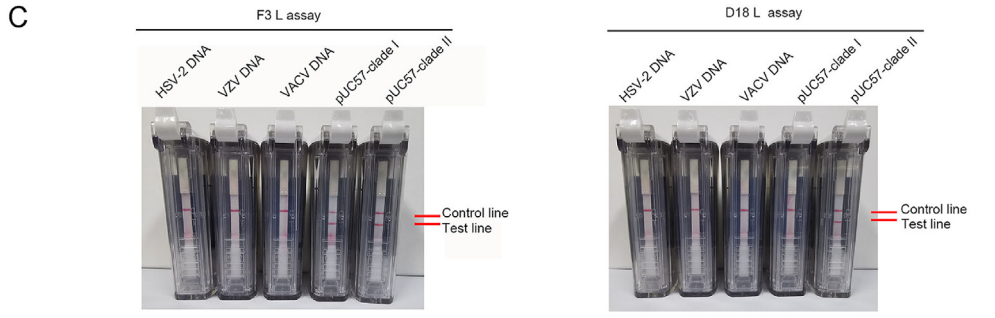

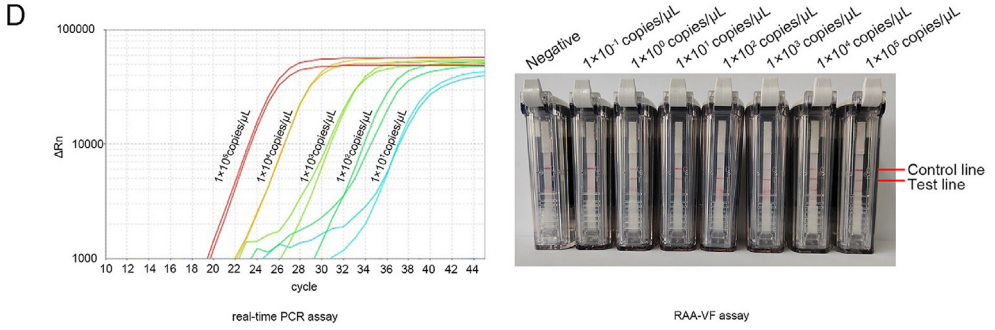

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Fig. 1. A nucleic acid visual assay panel for MPXV two clades. **A** Schematic illustrating the visual assay panel targeting the MPXV two clades. **B** Sensitivity of the assay panel was evaluated by the tenfold series dilution (concentrations in the range of 10^{-1} to 10^{3} copies/μL) of recombinant plasmids pUC57-clade I and pUC57-clade II as template. And RAA reaction system contained 0.4 μmol of each primer, 0.12 μmol of probe, 25 μL of rehydration buffer, 2 μL of template, 14 mmol/L magnesium acetate solutionand and 15.9 μL of ddH_2O was added to the reaction tube with lyophilized powder. Then, the reaction tube was incubated at 39 °C for 20 min. Empty vectors pUC57 was used as negative controls. **C** Specificity of the assay panel was evaluated using several viral DNA, including VACV (TianTan), HSV-2, VZV, and recombinant plasmids of MPXV clades I and II. The RAA reaction was performed at 39 °C for 20 min. **D** Comparison of real-time PCR and nucleic acid visual assay panel for MPXV *F3 L* gene. Empty vectors pUC57 was used as negative controls.

The executed process of the visual assay panel for distinguishing two clades was shown in Fig. 1. First, suspicious samples from human or susceptible animals were processed by heat inactivation or a DNA purification kit with magnetic beads without centrifugation to isolate viral DNA (TIANGEN Company, Beijing, China) after collection. Next, the extracted DNA was amplified by RAA, result in the production of abundant amplicon dual-labelled with FAM and biotin. Finally, the tube containing the amplicon was placed in a closed vertical flow visualization strip device to detect the RAA products. As shown in Step 4 of Fig. 1, the gold-labelled anti-FAM antibody and streptavidin fixed on the strip will capture the amplicon labelled with FAM and biotin, so gold nanoparticle accumulate to form a red band in test line. If there is no red band in test line of both assays, the patient had been not infected with MPXV. If the test line shows red band for both the F3 L and D18 L assays, the patient had been infected with clade II. If only the test line of the F3 L assay appeared red bands but with no band for D18 L assay, the patient had been infected with the clade I (Step 5 of Fig. 1).

To evaluate the sensitivity of the visual assay panel, the different concentrations of plasmids containing the *F3 L* and *D18 L* genes of the clade I or clade II were used as the template for the RAA-VF assay (Supplementary Fig. S1). Following the instructions, the RAA reaction mixture was prepared using the RAA nfo kit (See Supplementary Data for a detailed description). The recommended amplification condition of RAA reaction was 39°C for 20 min (Supplementary Figs. S2 and S3). After amplification, the reaction solution was loaded onto a fully closed vertical flow visualization strip for detection within 5 min. As shown Fig. 1B, the detection limit of F3 L assay was 1 copy/μL plasmid of the clades I and II. The D18 L assay could detect as low as 1 copy/μL plasmid of clade II, and showed no reaction for clade I. Compared with the previous isothermal amplification detection, Iizuka et al. developed an assay based on loop-mediated isothermal amplification, which requires only 60 min for detection of two clades of MPXV, nevertheless it is prone to cause aerosol contamination due to the need to open the cap for agarose gel electrophoresis of the amplified product. And the method is capable of detecting more than 100 copies of viral DNA (Iizuka et al., 2009). Therefore, the visual assay panel in this study has higher sensitivity. Recently, a rapid recombinase polymerase amplification (RPA) assay targeting MPXV was established, which can complete detection within 10 min with a limit of detection of 16 molecules/μL DNA (Davi et al., 2019). Further, Mao et al. established three recombinase-based isothermal amplification assays (real time RPA, RPA-Cas12a, RAA-LFS) for the rapid detection of MPXV *G2R* gene, which can detect as low as 1 copy/reaction plasmid within 20–30 min (Mao et al., 2022). The above three assays and the visual assay panel in this study showed almost consistent sensitivity. However, it is worth mentioning that our assay panel can achieve the identification of two clades of MPXV within 25 min.

Several orthopoxviruses and herpesviruses, such as vaccinia virus vaccine (VACV, TianTan strain), human herpesvirus 2 (HSV-2), and varicella-zoster virus (VZV), can cause skin blistering lesions with clinical symptoms similar to those of Mpox, and it is thus difficult for medical workers confirm to the pathogen causing infection. Thus, VACV (TianTan), HSV-2, VZV, recombinant plasmids pUC57-clade I and pUC57-clade II (the MPXV genes contained in the recombinant plasmid were described in the Supplementary Data) were used as templates to evaluate the specificity of the visual assay panel. The results showed that the recombinant plasmids pUC57-clade I and pUC57-clade II reacted positively with the F3 L assay, and only plasmids pUC57-clade II indicated a positive result with the D18 L assay (Fig. 1B); VACV (TianTan), HSV-2 and VZV all showed negative results, indicating that this visual assay panel has high specificity.

To verify the utility of the assay panel, mixtures containing serial tenfold dilutions of the plasmid (ranging from 10^{6} to 10^{0} copies/μL) of the *F3 L* and *D18 L* genes spiked separately with genomes derived from 293T cells were detected by this panel and real-time PCR. Among them, real-time PCR was performed with reference to the multiplex real-time PCR established by Maksyutov et al. (2016). The result shown that the sensitivity of the RAA-VF assay for the *F3 L* gene was 1 copy/μL plasmid included in spiked genomes, which was higher than that of real-time PCR for the *F3 L* gene with limit of detection as 10 copies/μL (Fig. 1D).

In conclusion, the visual assay panel is a rapid, portable, highly specific and sensitive tool for MPXV DNA detection that can differentiate the clade I and clade II. In addition, the detection limit of the panel was 1 copy/μL recombinant plasmids within 25 min; the sensitivity is higher than that of previous methods (Iizuka et al., 2009; Li et al., 2010; Maksyutov et al., 2016; Davi et al., 2019). It is expected that this panel will allow for point-of-care MPXV detection. Its high sensitivity and specificity indicate that this panel is a promising alternative that will improve current diagnostic capabilities.

Footnotes

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