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RESEARCH ARTICLE

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A field diagnostic method for rapid and sensitive detection of mpox virus

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Abstract

The mpox outbreak has subdued with fewer reported cases at the present in high-income countries. It is known that mpox virus (MPXV) infection has been epidemic for more than 50 years in African countries. The ancestral MPXV strain has changed into multiple clades, indicating the ongoing evolution of MPXV, which reflects the historical neglect of mpox in Africa, especially after smallpox eradication, and bestows the danger of more severe mpox epidemics in the future. It is thus imperative to continue the development of mpox diagnostics and treatments so we can be prepared in the event of a new mpox epidemic. In this study, we have developed an MPXV detection tool that leverages the recombinase-aid amplification assay by integrating lateral flow strips (RAA-LF) and one-step sample DNA preparation, with visible readout, no need of laboratory instrument, and ready for field deployment. The detection limit reaches 10 copies per reaction. The performance of our RAA-FL assay in diagnosing mpox clinical samples is on par with that of the quantitative polymerase chain reaction (PCR) assay. Taken together, we have developed a point-of-care RAA-LF method of high accuracy and sensitivity, readily deployable for field detection of MPXV. This diagnostic tool is expected to improve and accelerate field- and self-diagnosis, allow timely isolation and

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treatment, reduce the spread of MPXV, thus effectively mitigate MPXV outbreak in the future.

KEYWORDS
field-deployable, instrument free, lateral flow strips, MPXV detection, RAA

1 | INTRODUCTION

Mpox is caused by a zoonotic virus belonging to the *Orthopoxvirus* genus and the *Poxviridae* family.[1] Mpox viruses (MPXVs) are divided into two genetic clades: Clade I (the Central African or Congo Basin clade) and Clade II (the West African clade).[2] Clade II is less virulent but more widely spread, and is the dominant subtype in the 2022 outbreak (classified as Clade IIb),[3] which caused the first widespread human-to-human transmission.[4] To date, MPXV has spread to 115 countries and regions worldwide, led to over 90 000 mpox cases and 167 deaths.[5] At present, the frequency of mpox cases reported to World Health Organization (WHO) has decreased substantially, as a result WHO has declared that mpox is no longer a global health emergency on May 11, 2022. However, recent weeks have seen an increase in mpox cases in 10 countries including China which now has 2024 cases of mpox since the first imported mpox case in September 2022.[5,6] Therefore, diagnosis and prevention of mpox transmission represent an ongoing task to prepare for future mpox outbreaks.

Mpox is a self-limiting disease characterized by skin rashes, lymphadenopathy, fever, chills, headache, and other symptoms similar to those observed in smallpox, but milder.[7] Most infected people recover within a few weeks, while children, pregnant women, and those with a weakened immune system are at a higher risk of developing severe disease.[8] The incubation period of mpox has historically been reported to range from 5 to 21 days and may vary depending on the type of exposure and transmission routes.[9] Currently, there are no approved treatments for mpox, and mpox vaccines are unavailable to individuals younger than 18 years.[10] Therefore, early detection is central to identify the infected person, alert them to take timely isolation and treatments, thereby reducing the spread of MPXV and mitigating the impact of the outbreak.[11]

The main method currently used to detect the MPXV is real-time quantitative polymerase chain reaction (qPCR). Together with the patient's symptoms, a preliminary diagnosis of the disease can then be made.[12] qPCR has the advantages of high-quantity throughput and great sensitivity. However, the PCR-based detection assays were often longer than 90 min and the results relied on sophisticated temperature control equipment or fluorescence signal capture equipment, which is only suitable for laboratory-based testing.[13] The coronavirus disease 2019 (COVID-19) pandemic has led to the improved capacity of many low-income and middle-income countries in scaling up molecular diagnostics, however, the lack of essential reagents and well-trained personnel hampers the establishment of PCR laboratories and therein the PCR-based diagnosis platform in these countries.[14] More efforts have been made to improve MPXV detection. These include clustered regularly interspaced Short palindromic repeats (CRISPR), combined with surface plasmon resonance or plasmonic fiber tip which provides more convenient and reliable techniques for the rapid and specific detection of MPXV.[15–19] Isothermal amplification methods have been explored as an alternative to real-time PCR. Recombinase polymerase amplification (RPA) coupled CRISPR/Cas12 facilitates the rapid and sensitive diagnosis of mpox within 20 min.[20,21] However, the need of laboratory instruments and the complexity of sample extraction and manipulation remain a great challenge for field use.

Recombinase-aid amplification (RAA) are a promising alternative due to its simplicity, high sensitivity, compatibility with multiplexing, rapid amplification, as well as its operation at room temperature.[22] The RAA process consists of a biotin-labeled reverse primer and a fluorescein isothiocyanate (FITC)-labeled probe which together determine the sensitivity and specificity, using the recombinase enzyme to insert primers and probes into the double-stranded template at a single temperature, enabling low-resource capture of amplicons using simple lateral flow detection of the biotin-FITC dual-labeled amplicon.[23] RAA-coupled lateral flow strips allow the simplest detection procedure with the same sensitivity. Moreover, the biggest impediment for field adaptation is sample preparation, which is often a multistep procedure requiring columns or magnetic beads and depends on laboratory instruments.[24,25] It is believed that one-step sample preparation and fast detection technology together enable rapid, point-of-care detection of MPXV, allow early identification and self-isolation of mpox.

In this study, we report a rapid, sensitive, field-deployable assay that combines one-step sample preparation, RAA, and lateral flow strip (RAA-LF) for the point-of-care detection of MPXV. This system is equipped with primers and probes that are optimized for the detection of the conserved viral genes in orthopoxvirus and the specific viral genes in MPXV. The entire reaction, including DNA extraction, can be completed within 20 min at body temperature with a detection sensitivity of viral DNA under 10 copies. The assay can be performed without the need of pipettors or laboratory instruments, and results are presented as a visual readout. Our system can be developed into rapid diagnostic test kits that can be used for fast, accurate, and point-of-care detection of MPXV especially in resource-limited settings.

2 | RESULTS

2.1 | Design and test primers and probes targeting MPXV

To ensure the high specificity of the RAA primers, we designed two pools of primer pairs. One pool targets viral genes D6R and E9L that are conserved among orthopoxviruses including MPXV, the other pool targets regions in the N3R and N4R genes that are nearly 100% conserved among multiple species of MPXV but exhibit low homology with other orthopoxviruses, which is consistent with previous studies.[20,26] The D6R and E9L genes were amplified from vaccinia virus (VACV) and cloned into plasmid vectors. The N3R and N4R genes were synthesized. Twenty pairs of primers were designed to target each viral gene. The specificity and sensitivity of primer pairs were tested with isothermal amplification method using each viral gene plasmid or empty vector as a template (Figure 1A–D). Considering the identities and structures of primer pairs, for each gene we chose one of the best primer pairs (P19 in D6R, P15 in E9L, P11 in N3R, and P6 in N4R) and designed matched probes.

We next evaluated the performance of each set of primers and probes with lateral-flow readout using viral gene plasmid as a template. When the gold-labeled anti-FITC antibody and streptavidin fixed on the strip capture the amplicon which is labeled with FITC and biotin, gold particles will accumulate to form a dark band at the test line (Figure 1A). All sets of primers and probes successfully detected each target gene, the empty vector control did not generate a band at the test position (Supporting Information S2: Figure S2A–D). All sets of primers and probes were blasted and aligned. Primer and probe sets targeting N3R and N4R are highly conserved among MPXV genomes, while those targeting D6R and E9L show a few mismatches among orthopoxvirus genomes (Supporting Information S2: Figure S2E–H), among which D6R is more conserved than E9L, N4R is more conserved than N3R.

2.2 | Specificity and sensitivity of the RAA-LF system

To demonstrate the specificity of the RAA-LF system, we first tested all sets of primers and probes using MPXV DNA as the template. MPXV DNA was detected by all primer sets as visualized as positive test bands (Figure 1B). When VACV DNA was tested as the target, the D6R and E9L primer pairs produced the positive results, while the N3R and N4R primer pairs produced negative results because VACV does not have the target sequence for the N3R and N4R primer pairs (Figure 1C). To determine the sensitivity of this assay, different copy numbers of MPXV DNA were used as the template. MPXV DNA was diluted to 10^3 copies, 10^2 copies, 10 copies, and 1 copy as absolute quantified by digital PCR [27](Supporting Information S3: Figure S3A). Results showed that all four sets of primers and probes were able to detect MPXV DNA as low as 1 copy (Figure 1D).

The recommended amplification condition of RAA reaction is 39°C for 30 min. Considering the application of our assay in resource-limited settings, particularly the lowest temperature that allows the reactions to take place without compromising the sensitivity and specificity, we performed our RAA-LF reaction at temperatures 10°C, 20°C, and 45°C for a duration from 15 to 120 min. We observed that in low temperatures, it needs more time to detect MPXV DNA, while from 20°C to 45°C, MPXV DNA was detected within 15 min (Figure 1E and Supporting Information S3: Figure S3B).

We next assessed the capacity of the RAA-LF system in the detection of MPXV in clinical samples. Primers were initially designed to amplify both N3R and N4R genes that are specific in MPXV. Since the N4R primers and probe are more conserved than the N3R ones of which the N3R probe has a mismatch in the aligned MPXV sequences (Figure 2G,H), the blinded concordance testing was conducted with primers targeting N4R. One hundred and twelve clinical samples were tested, containing swabs from six different sites of the body. Results were compared with an in-house qPCR assay targeting the E9L gene. RAA-LF results of two cases are shown and all swab samples from six different sites presented positive results (Figure 2A). Of the swab samples from each site and total samples, the C_t values were calculated and divided into four groups:C_t values less than 30, C_t values between 30 and 35, C_t values between 35 and 40, and C_t value beyond the qPCR detection limit (N/A). The graph shows the number of samples in each C_t value interval (Figure 2B). For all swab samples, positivity varies based on the C_t value cutoff. At a C_t cutoff of 30, 53 samples were MPXV-positive and 59 samples were MPXV-negative by qPCR. At a C_t cutoff of 35, 75 samples were MPXV-positive and 37 samples were MPXV-negative by qPCR. At a C_t cutoff of 40, 102 samples were MPXV-positive and 10 samples were beyond the detection limit by qPCR. The RAA-LF visual results of each site and total samples were also calculated, and the results showed that the RAA-LF system correctly identified all positive samples (102 of 112) even at a C_t cutoff of 40 (Figure 2B). To rule out the false positive results, we tested the standard control DNA including Herpes simplex virus type 1/2, varicella-zoster virus, *Candida albicans*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* that are listed in the National Reference Panel for Mpox Nucleic Acids Detection requirements provided by National Institutes for Food and Drug Control (NIFDC). We also tested the clinical samples for common respiratory virus which may exist in oro-pharyngeal swab or nasopharyngeal swab. None of these pathogens generated positive bands, confirming that these primer and probe sets are highly specific and suitable for the diagnosis of clinical samples (Figure 2C,D).

2.3 | One-step sample DNA preparation and RAA-LF detection

The utility of RAA-LF system is, however, dependent on the quality of nucleic acid templates to be detected, and the amplification-inhibiting factors may affect assay's efficacy as well as accuracy.[28]

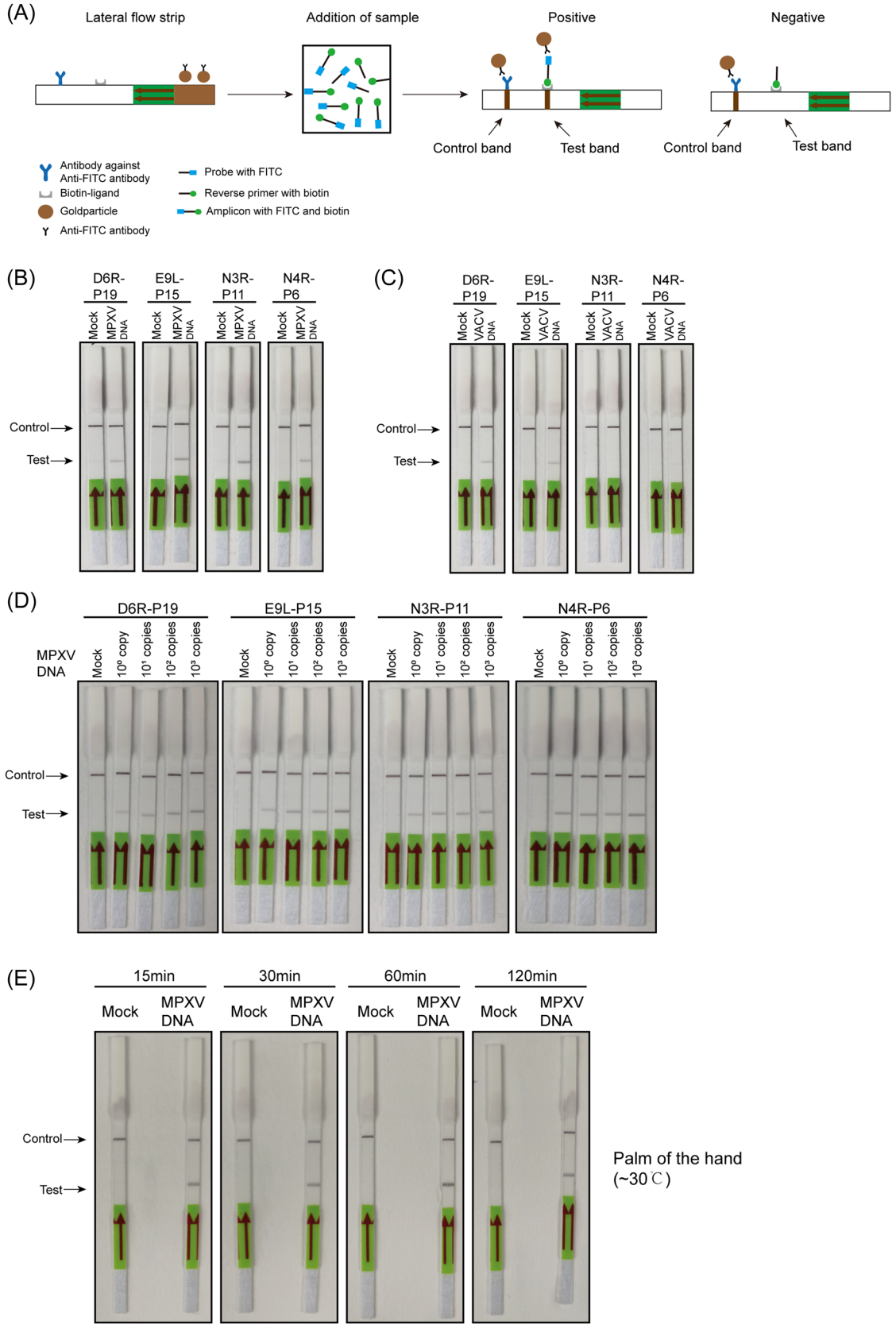


FIGURE 1 (See caption on next page).

Sample preparation is often a multistep procedure requiring columns or magnetic beads and other laboratory instruments. To develop an one-step sample preparation method suitable for the RAA-LF assay, we infected mice with VACV via the nasal route for 7 days, used swabs to collect samples from different sites, and directly soaked the swabs in 1× sample lysis buffer. Visual readouts were performed using laboratory instruments (Figure 3A). Viral DNA was detected using both RAA-LF and qPCR, while samples of uninfected control mice showed negative results (Figure 3B).

We further determined whether the RAA-LF assay can be completed without the need of any laboratory instruments. We first used a tiny swab to collect DNA extracted from mouse swab samples and added into the RAA reaction buffer. After incubation at hand palm temperature for 15 min, the RAA reaction buffer mix was added into dilution buffer and then applied onto strips for detection (Figure 3C). The RAA-LF assay generated results consistent with those of the above laboratory tests, that is, swabs from infected mice gave rise to positive results, while swabs from uninfected mice showed negative results (Figure 3D).

We next tested whether the composition of clinical samples, such as feces and urine, has an effect on one-step DNA extraction. Feces- or urine-containing clinical sample swabs were soaked in 1× sample lysis buffer and examined with RAA-LF (Figure 4A). Results of two cases are shown, and swab samples from six different sites of the body presented positive results (Figure 4B). To validate the RAA-LF data, we examined all clinical samples by qPCR and calculated the C_t values the same as Figure 2B. The RAA-LF visual results of each site and total samples were also calculated, and the results showed that the RAA-LF system correctly identified all positive samples extracted with one-step lysis buffer. If sample positivity is defined as C_t<40 cycles, the accuracy of RAA-LF is fully consistent with the PCR data (Figure 4C). Altogether, this RAA-LF detection system is operational in the absence of any laboratory instruments and suitable for field testing.

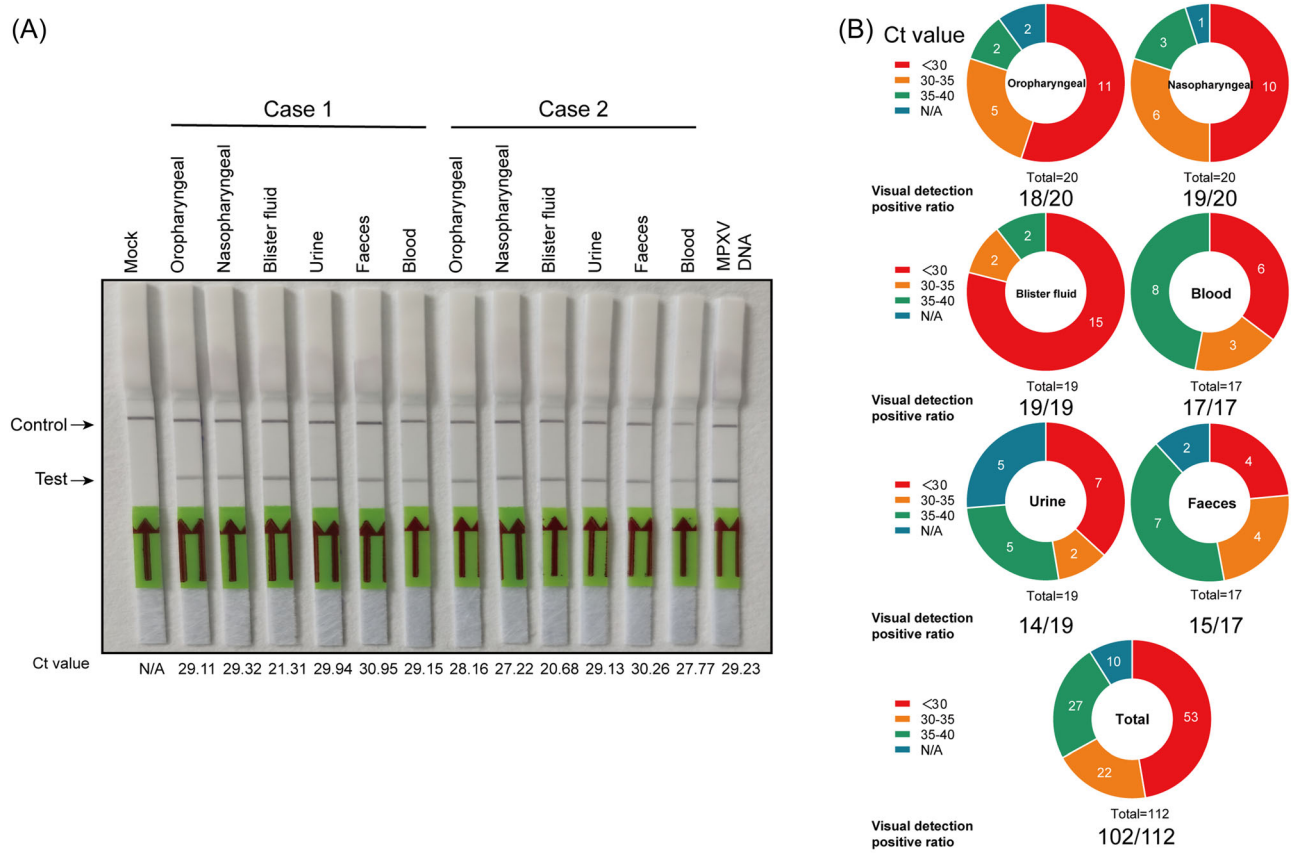
3 | DISCUSSION

Mpox has long been considered an endemic disease. Although historically it has been mostly present in Central and West Africa,[29] the currently circulating MPXV strain is likely a descendant of the strain that caused the 2018–2019 mpox outbreak. MPXV has evolved from Clade I to Clade IIb, lineage C.1.[30] With global travel and trade, caution has to be taken for a potential Clade I MPXV outbreak which is more virulent than Clade II MPXV. Rapid and accurate diagnostic methods are the foundation of effective prevention and control of mpox outbreaks.[25] In addition to antigen/antibody-based serology tests, PCR-based diagnosis has also becoming widely adopted as a result of its specificity and high sensitivity.[31,32] These assays often need laboratory equipment and involve complex procedures, which discourage their utility as point-of-care tools particularly in resource-limited settings. In this context, isothermal amplification technologies present advantage in their independence on special laboratory instruments and their feasibility of being developed into molecular diagnostic tools for field testing.

Compared with other isothermal amplification techniques including loop-mediated isothermal amplification (LAMP) and helicase-dependent amplification (HDA) nucleic acid sequences-based amplification (NASBA), the RAA/RPA method has a shorter reaction time, a simpler primer design and much less dependence on special equipment. As a result, RPA has been used in the detection of MPXV coupled with the CRISPR/Cas12a system.[20,21,33] Yet, this assay still requires a multistep procedure involving column or magnetic beads to prepare DNA samples before starting the RPA and CRISPR/Cas12a assays. To simplify the detection system and aim for field testing, we have developed an one-step sample preparation method and coupled RAA with lateral flow for visual readout, without the need of any laboratory instruments for MPXV detection.

The specificity of RAA heavily depends on the primers and probes used in the assay. Given the increasing diversity of MPXV, we have designed 20 pairs of primer on each viral target gene and, based on the experimental data, chose the top-ranking one to avoid false positive results. Sequences of primer and probes have also been blasted against available genome databases to choose the sequences with the highest specificity (Supporting Information S2: Fig S2E–H). The recommended amplification condition of RAA reaction is 39°C for 30 min. It has been reported that RPA can take place with body heat, especially held in the axilla.[34] Our data showed that, when the temperature is lower than 20°C, RAA needs more than 30 min to produce positive results, while in hand palm, positive results can be observed as short as 15 min. Thus, in field testing, palm of hand is sufficient for RAA to proceed without compromising the sensitivity and specificity. Compared with the qPCR-based golden standard diagnosis, our RAA-LF system is cost effective, with less than one

FIGURE 1 Specificity and sensitivity of the recombinase-aid amplification assay by integrating lateral flow strips (RAA-LF) system. (A) Illustration of lateral flow strips. FITC-biotin-labeled RAA amplicons were first conjugated with colloidal gold-labeled anti-FITC antibodies. As the conjugates flew, the complex was captured by streptavidin on the test line. The remaining complex continues to diffuse forward and was captured by antibodies against anti-FITC antibodies at Control line. Visual bands at both test and control lines indicate a positive readout, while only a single band at control lines indicates a negative readout. (B,C) RAA-LF detection of MPXV and VACV DNA with primer and probe sets targeting the D6R, E9L, N3R, and N4R genes. Controls include no template control (Mock). (D) Detection limit of the D6R, E9L, N3R, and N4R primer and probe sets using MPXV DNA as a template with lateral flow readout. MPXV DNA concentrations are presented as genome copies/μL. Controls include no template control (Mock). (E) Lateral flow results of the N4R primer and probe set amplified at hand palm temperature. A volume of 10 μL reaction products were removed and tested with lateral flow readout at the indicated times. All experiments were repeated three times, one representative result is shown.



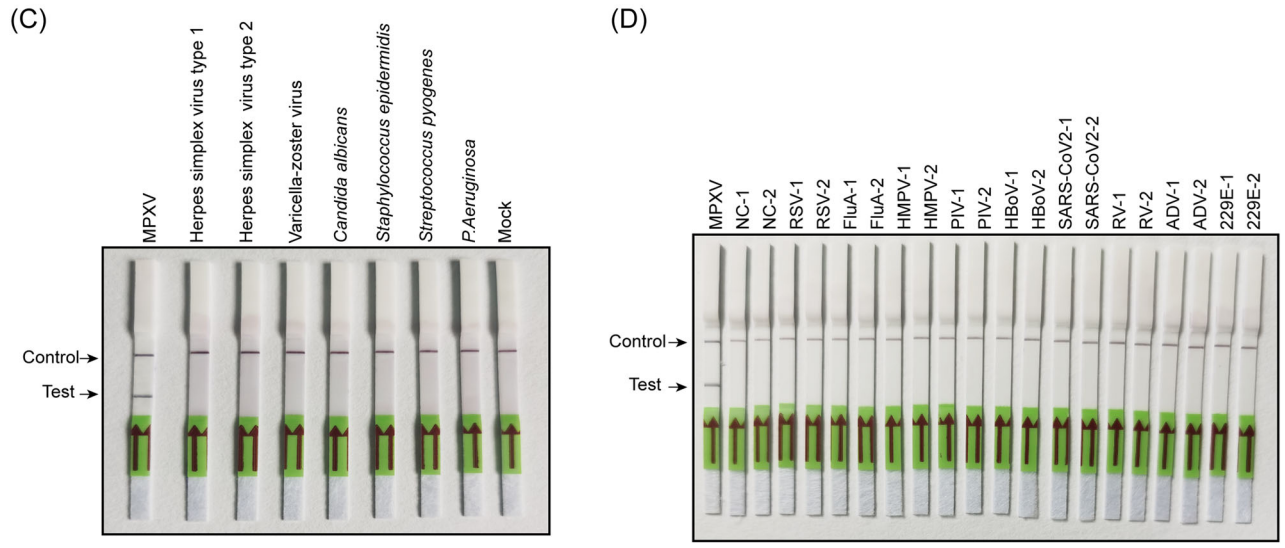


FIGURE 2 Specificity and sensitivity of the recombinase-aid amplification assay by integrating lateral flow strips (RAA-LF) system with clinical samples. (A) RAA-LF system readout using the N4R primer and probe set testing swab samples of six different body sites from two of 10 clinical cases. The C_t values were shown at the bottom of lateral flow readout. Controls include no template control (Mock) and positive control (MPXV DNA). (B) Statistical analysis of all swab samples of each body sites and total samples. C_t values were assessed at three cutoffs: $C_t = 30$, $C_t = 35$, and $C_t = 40$. Each number in the graph corresponds to the number of samples in the C_t value interval. Visual interpretation of the strips from three times experimental repeats was performed by three independent observers, and a sample was scored as positive by each individual when at least two of the repeats were interpreted as positive. (C) Lateral flow results of the N4R primer and probe set analyzing the standard DNA. Controls include no template control (Mock), positive controls (MPXV). (D) Lateral flow results of the N4R primer and probe set analyzing the clinical samples infected with multiple viruses. Controls include positive controls (MPXV). RSV, respiratory syncytial virus; FluA, influenza A virus; HMPV, human metapneumovirus; PIV, parainfluenza virus; HBoV, human bocavirus; RV, rotavirus; ADV, adenovirus; 229E, human CoV-229E. All experiments were repeated three times and one representative result is shown.

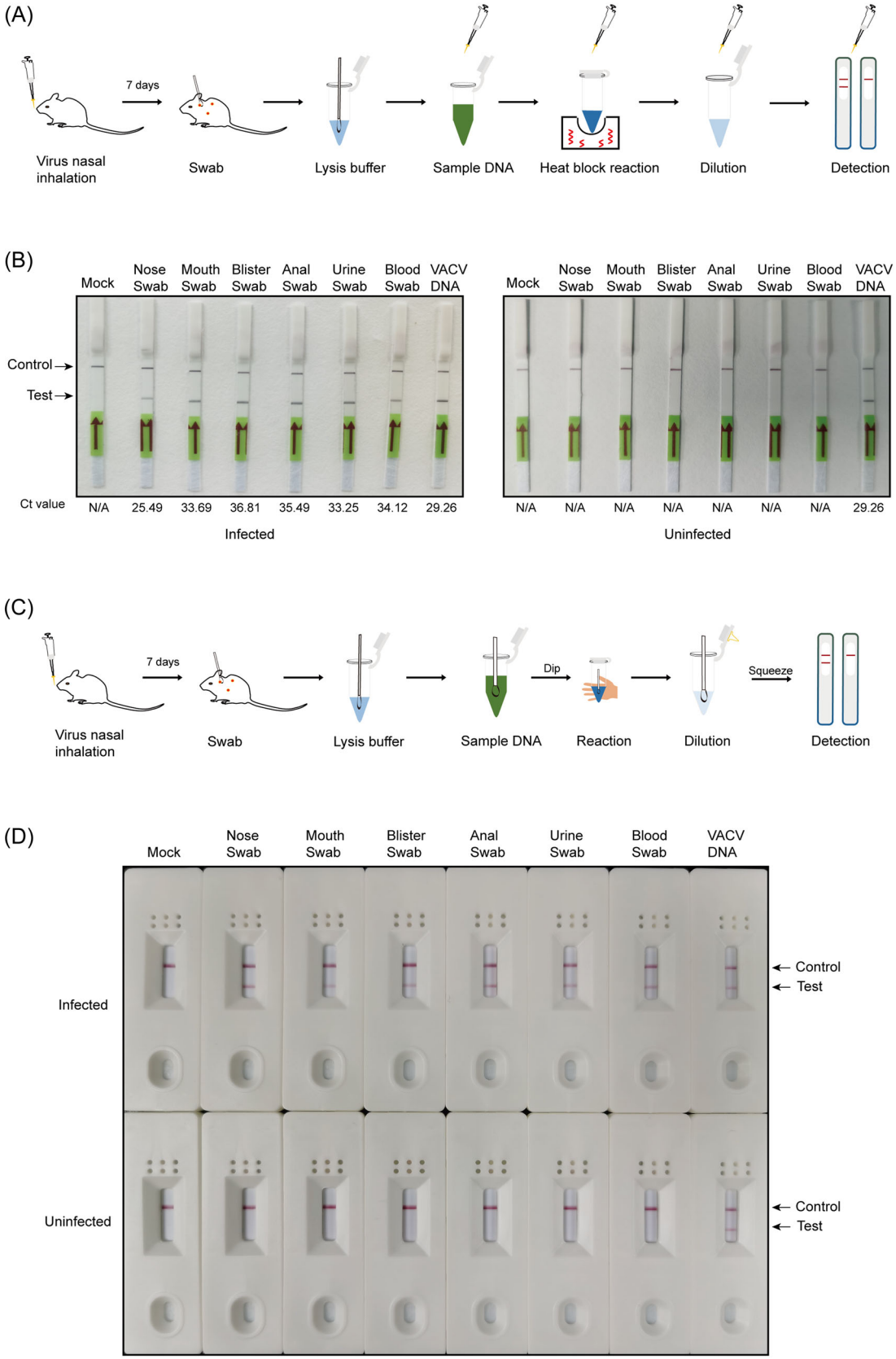


FIGURE 3 (See caption on next page).

dollar per test, is simple to use and easy to follow, takes only 10–15 min to deliver the results, and does not need any laboratory instruments.

Skin lesions in mpox cases are the most accessible for sample collection with swabs and also the most reliable for producing high-level sensitivity and specificity in diagnostic testing. However, when skin lesions are not present, sample collections with oropharyngeal, nasopharyngeal, urine or feces swabs become necessary for mpox diagnosis, and these swab samples have a high rate of negative results (Figure 2B). Our RAA-LF assay is able to present positivity of these latter swab samples as being validated by the results of real-time PCR. Thus, the RAA-LF system is expected to be more sensitive and effective in early diagnosis of asymptomatic mpox cases who do not present skin lesions but already begin shedding MPXV.

With the success of our RAA-LF system in testing clinical samples come certain limitations. First, the number of mpox cases tested is relatively small, and a much larger study is warranted to further validate the accuracy of the RAA-LF system. Second, different stages of the mpox disease may exhibit varying viral loads, the diagnostic accuracy with RAA-LF can be further verified with samples of different stages of mpox including post infections. Furthermore, more types of clinical samples can be tested to improve the clinical utility of the RAA-LF system. Third, current RAA-LF system is a semi-open testing system and may be contaminated by environment DNA. An intact and closed RAA-LF system including sample DNA extraction would be more beneficial for field testing. Addressing these limitations projects future directions to improve and optimize the RAA-LF method so that it can be adopted as a new and effective mpox diagnostic tool. In this study, we have demonstrated the accuracy, specificity, and sensitivity of the RAA-LF system in detecting MPXV. This method can be readily adapted to simultaneously detect two or more pathogens to achieve multiplex testing, which can be achieved by designing and testing multiple pathogen-specific probes.

In summary, we have developed a specific, sensitive, fast, and practical RAA-LF assay for field testing of MPXV infection. This system shows robust performance with a variety of different clinical samples, does not need laboratory instruments, operates well at hand palm temperature, thus is suitable for point-of-care mpox testing, especially in resource-limited settings. Several features of the RAA-LF system make it a potentially valuable tool in safeguarding public health. For example, the low cost and easiness to use of RAA-LF is expected to accelerate self-testing including in the resources-limited settings, which is known to play a large role in mitigating the transmission of infectious agents including MPXV and initiating early treatment. The RAA-LF diagnostic method can also be incorporated into the arsenal of global surveillance of MPXV when properly integrated with digital reporting and the national and international database.

4 | MATERIALS AND METHODS

4.1 | Ethical consideration

This clinical samples were collected at Beijing Ditan Hospital Capital Medical University, which is the referral hospital for mpox cases in China. Written informed consent was obtained from each individual. The study was approved by the Institutional Review Boards of Beijing Ditan Hospital Capital Medical University (2023-025).

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Beijing Protein Innovation Co., Ltd.

4.2 | Animals

Female mice used in this study (aged between 6 and 8 weeks) were of BalB/C, purchased from Vital River.

4.3 | Plasmids construction

Full-length D6R gene (nucleotide positions 100 176–102 089) and E9L gene (nucleotide positions 53 110–56 130) were amplified by PCR from vaccinia virus Tiantan strain DNA (GenBank accession number JX489139.1) and cloned into pUC57 vector using EcoRI and BamHI restriction sites. Full-length N3R gene (positions 190 389–190 919) and N4R gene (positions 191 033–192 346) from MPXV (GenBank accession number ON563414.3) were synthesized by Beijing Ruibio BioTech Co., LTD and cloned into pUC57 vector using the same restriction sites.

FIGURE 3 One-step sample DNA preparation and recombinase-aid amplification assay by integrating lateral flow strips (RAA-LF) detection. (A) Illustration of one-step sample DNA preparation with samples from mice. VACV was given to mice via nasal inhalation. Seven days postinfection, samples from different body sites were collected with swabs which were soaked in 1× lysis buffer. DNA samples were prepared and tested with laboratory instruments. (B) Lateral flow method with the D6R primer and probe set to detect VACV from infected mice. Controls include no template control (Mock), positive controls (VACV DNA). (C) Illustration of RAA-LF assay without any laboratory instruments. One-step prepared DNA was added into the amplification buffer, reaction was carried out in hand palm for 15 min. Reaction products were directly subject to lateral flow detection. (D) Hand palm temperature is sufficient to power the lateral flow assay to detect VACV from infected mice. Swab samples from different body sites of mice were examined. Controls include no template control (Mock), positive controls (VACV DNA). All experiments were repeated three times, and one representative result is shown.

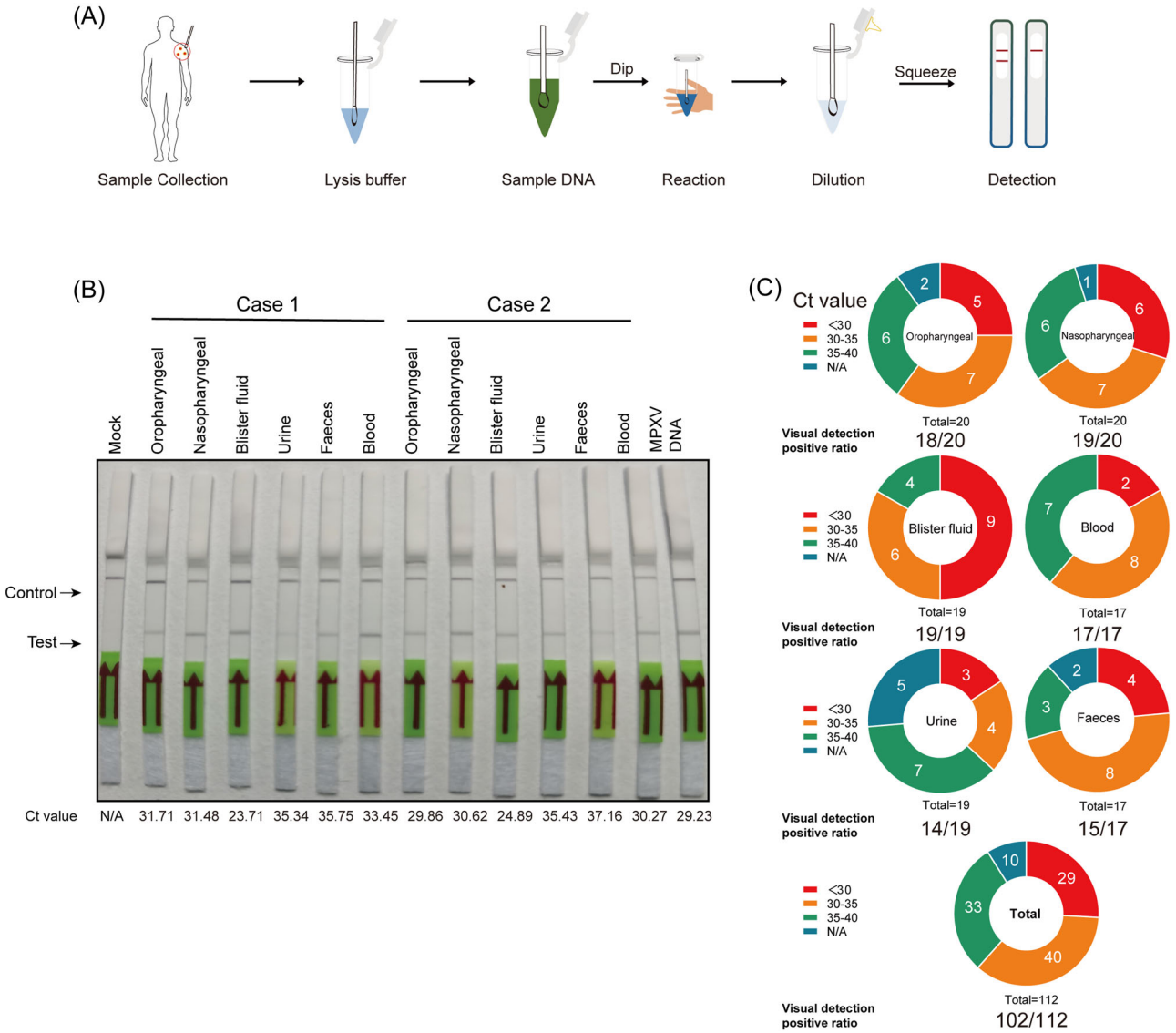


FIGURE 4 One-step clinical sample DNA preparation and RAA-LF detection. (A) Illustration of one-step sample DNA preparation with clinical samples. Clinical sample swabs from different sites of the body were soaked in 1× lysis buffer for 1 min, the prepared DNA was added into the amplification buffer. The reaction was carried out in hand palm for 15 min. Reaction products were subject to lateral flow detection. (B) RAA-LF readout using the N4R primer and probe set to test swab samples at six different sites of the body from two of 10 clinical cases. Sample DNA was extracted with one-step lysis as shown in Figure 3A. C_t values were presented at the right side of lateral flow readout. Controls include no template control (Mock) and positive control (MPXV DNA). (C) Statistical analysis of all swab samples of each body sites and total samples. DNA samples were prepared using the one-step lysis protocol. C_t values of the swab samples were assessed at three cutoffs: C_t=30, C_t=35 and C_t=40. Each number in the graph corresponds to the number of samples falling within the C_t value interval of the same color code. Visual interpretation of the RAA-LF data from three experimental repeats was performed by three independent observers, and a sample was scored as positive by each individual when at least two of the repeats were interpreted as positive.

4.4 | DNA preparation

VACV was amplified by infecting Vero cells. DNA of virus and clinical samples were extracted using QIAamp DNA minikit (Qiagen), in accordance with the manufacturer's instructions. For one-step DNA extraction, we used the nucleic acid extraction-free regents (Warbio) for the purpose of nucleic acids release.

4.5 | RAA primers and probes design

For RAA amplification, the amplicons were determined based on the target sequences of D6R, E9L, N3R, and N4R. RAA primers were designed based on RAA-nfo Design Manual. We also confirmed the specificity of selected RAA primers and probes using NCBI BLAST (Figure 2). See Supporting Information S4: Table S1 for sequences of primers and probes.

4.6 | RAA and lateral flow detection

RAA reaction was conducted using RAA-nfo kit (Hangzhou ZC Bio-Sci& Tech Co. Ltd). In brief, 2 μL of primer RAA-F (2 μM), 2 μL of primer RAA-R-Biotin (2 μM), 0.5 μL of probe (2 μM), 25 μL of RAA reaction buffer, 13 μL of ultrapure water were mixed, then pipetted 2.5 μL of magnesium acetate (280 mM, MgOAc), and 5 μL of the template DNA into the tube lids, upside down and incubated at a constant temperature of 37°C for 15–30 min.

For lateral flow detection, 10 μL of the reaction was removed and mixed with 90 μL of HybriDetect assay buffer. Then, 10 μL of the diluted sample was pipetted into 100 μL HybriDetect Assay Buffer, the strips were placed with the sample application area facing downwards into, and incubated for 5–15 min at room temperature in an upright position. If both the test and control bands are displayed, it is a valid positive result. If only the control band is displayed, it is considered to be a valid negative result. Moreover, if the control band is not visible after the incubation period, the result is invalid, and the test must be repeated with a new strip.

4.7 | Droplet digital PCR

The viral DNA was quantified by droplet-digital PCR (ddPCR) using QuantStudio 3D Digital PCR System (Applied Biosystems) following QuantStudio 3D Digital PCR System USER GUIDE. The ddPCR reaction system contained target-specific forward and reverse primers, carboxy-fluorescein (FAM)-labeled probe, master mix, template, and ddH2O. The final concentration of primers used for E9L were 1 μM. Probe (E9L: FAM-ACGCTTCGGCTAAGAGTTGCACATCCA-TAMRA) was synthesized by Tsingke Biotechnology Co., Ltd. and the final concentration was 0.325 μM. Procedure of the ddPCR were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s. The ddPCR primers are E9L-F (5′-GAACATTTTTGGCAGAGAGAGCC-3′) and E9L-R (5′-CAACTCTTAGCCGAAGCGTATGAG-3′).

4.8 | Real-time PCR

Copy number of viral and sample DNA were determined by quantitative real-time PCR (qPCR) using the Luna Universal qPCR Master Mix (M3003L, NEB). The qPCR primers are E9L-F (5′-GAACATTTTTGGCAGAGAGAGCC-3′) and E9L-R (5′-CAACTCTTAGCCGAAGCGTATGAG-3′). Reactions were conducted in 20 μL following the manufacturer's instructions. Reaction cycle parameters were set as follows: 50°C for 2 min, denaturation at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and at 60°C for 1 min.

AUTHOR CONTRIBUTIONS

Lili Ren, Jianwei Wang, and Fei Guo conceived the project. Fei Zhao, Fengwen Xu, Xinming Wang, Rui Song, and Yamei Hu performed the experiments and prepared the manuscript. L.W., Yu Xie, Yu Huang, Shan Mei, Liming Wang, Lingwa Wang, and Zhao Gao performed the data curation and formal analysis. All authors contributed to the experimental design. Li Guo, Jugao Fang, Lili Ren, Ronghua Jin, Jianwei Wang, and Fei Guo composed the manuscript. All authors reviewed the manuscript and discussed the work.

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CONFLICT OF INTEREST STATEMENT

Fei Guo, Fei Zhao, Fengwen Xu, and Shan Mei are inventors on pending and issued patents on Field detection of mpox. The remaining authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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