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Letter to the Editor

Rapid detection of monkeypox virus and monkey B virus by a multiplex loop-mediated isothermal amplification assay

Dear Editor,

Monkeypox virus (MPXV) and Monkey B virus (BV) are two emerging zoonotic viruses that naturally infect monkeys. MPXV was first reported in monkeys in 1959.[1] Since the first human monkeypox case was recognized in 1970, numerous cases of monkeypox have been reported in humans and wildlife in Central and West Africa. In particular, a global monkeypox outbreak first emerged in England, and then rapidly spread to other non-African countries since May 2022. As of Jan 27, 2023, the outbreak had resulted in 85,155 confirmed cases, the vast majority (98.5%) of which were in countries with no history of monkeypox cases.[2] Faster and sensitive Point-of-Care testing (POCT) assays are urgently needed for rapid detection of MPXV infection.

BV was firstly identified in 1932.[3] It belongs to α-herpesvirus and is usually transmitted by direct contact and exchange of body secretions. BV infections usually do not cause significant clinical symptoms in its natural host, but result in severe central nervous system diseases in human. Although zoonotic infections of BV in humans were rare and sporadic, the mortality was 70–80%. The high fatality rate makes BV one of the most important zoonotic agents of concern for persons having frequent or close contact with macaques. On June 30, 2021, the first human case of BV infection was reported in China,[4] highlighting the importance of detecting BV infection.

Recently, Li et al. reported a monoclonal antibody-based antigen detection assay for monkeypox virus (MPXV) in this journal.[5] The MPXV antigen detection assay is rapid, and cost-effective, and its result can be identified with the naked eye. However, the sensitivity of antigen detection assay is inferior to nucleic acid amplification tests (NAATs).[6] Among various NAATs methods, the real-time polymerase chain reaction (qPCR) method is the most widely used strategy for the diagnosis of various viruses because of high sensitivity, high specificity and single-tube multiplex detection. However, the need for specialized equipment, highly trained personnel, and time-consuming (often 1–1.5 h) limits the application of qPCR in resource-limited areas. The loop-mediated isothermal amplification (LAMP) method, which uses strand displacement DNA synthesis by Bst DNA polymerase, and amplifies the targets without thermal cycler, was considered to be a promising POCT method.[7] However, the prominent shortage of LAMP is the frequent non-specific amplification, which inevitably results in false-positive results when non-specific dyes (e.g., DNA-binding fluorescent dye and pH-sensitive indicator dyes) are used.

Three LAMP assays with non-specific dyes were recently developed to detect MPXV even though there is an uncertain risk of false-positive.[8–10] Thanks to the advancement in the probe-based multiplex real-time LAMP method,[11] here we reported a rapid, sensitive and specific LAMP assay for simultaneous detection of both MPXV and BV using HFman probes (Fig. 1A).

Based on MPXV (GenBank accession: OP150923.1) and BV (GenBank accession: AF533768.1) genomic sequences, we designed seventeen sets of LAMP primers (5, 7, and 5 in OPG002 and ATI of MPXV, and UL30 genes of BV, respectively) using the open access Primer Explorer V.5 software tool (http://primerexplorer.jp/lampv5e/index.html). The primer set with the highest amplification efficiency for each gene was selected as previously described[12] (Fig. S1). A 25 μL multiplex LAMP system contains 1 × isothermal amplification buffer, 8 mmol/L MgSO4, 1.8 mmol/L dNTPs, 8 U Bst 4.0 DNA/RNA polymerase (Haigene, China), 0.15 U High-fidelity DNA polymerase (NEB, Beijing, China), the primer mix for each of MPXV and BV, and 3 μL of DNA template. The primer mix for each virus includes 0.1 μM each of F3 and B3, 1.0 μM each of FIP and BIP, 0.6 μM LF, 0.3 μM LB and 0.3 μM HFman probe (Table S1). The LAMP reaction was performed at 64 ℃ for 50 min in the CFX 1000 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA).

The sensitivity of the novel singleplex assay was tested for each gene using 10-fold serially diluted DNA standards from 10^3 to 1 copies/μL. The results showed that assay can detect 3 copies of each gene of OPG002, ATI, and UL30 (Fig. S2). Then, the multiplex LAMP assay was established and optimized for MPXV (ATI gene) and BV. A ten-fold serial diluted MPXV and BV DNA standards from 10^3 to 1 copies/μL were used to determine the sensitivity of the multiplex LAMP assay. The results showed that the multiplex LAMP assay can detect as low as 3 copies of each of MPXV and BV within 30 min (Fig. 1B). The limit of detection (LOD) of the multiple LAMP assay was estimated to be 28.7 and 27.8 copies per reaction for MPXV and BV, respectively (Table 1). The LOD of the novel assay for MPXY was more sensitive than previous ones (111.8 copies per reaction).[8] To determine the specificity of the multiplex LAMP assay for MPXV and BV, six common human viruses, including BK polyomavirus, JC polyomavirus, Cytomegalovirus, human adenovirus type 5, human immunodeficiency virus type 1, and Hepatitis B virus, were tested. None amplification signals were observed, indicating a high specificity (Fig. 1C). According to our previous study,[11] the novel multiplex LAMP assay is believed to be comparable to qPCR in sensitivity and specificity. Furthermore, a multiplex LAMP assay was also developed for simultaneous detection of both OPG002 and ATI genes of MPXV, and showed high sensitivity (Fig. S3 and Table S2).

Because of lack of positive clinical samples in China, we prepared simulated clinical samples using 6 serum samples that were collected from monkeys and tested as negative for both MPXV and BV (Fig. S4). The simulated samples with mono-infection of MPXV or BV, and coinfection with both MPXV and BV were prepared by mixing MPXV and/or BV plasmid standards into monkey sera, and diluted 10-fold into eight concentrations from 2×10^7 to 2×10^0 copies/μL. Original monkey sera were used as negative controls. The

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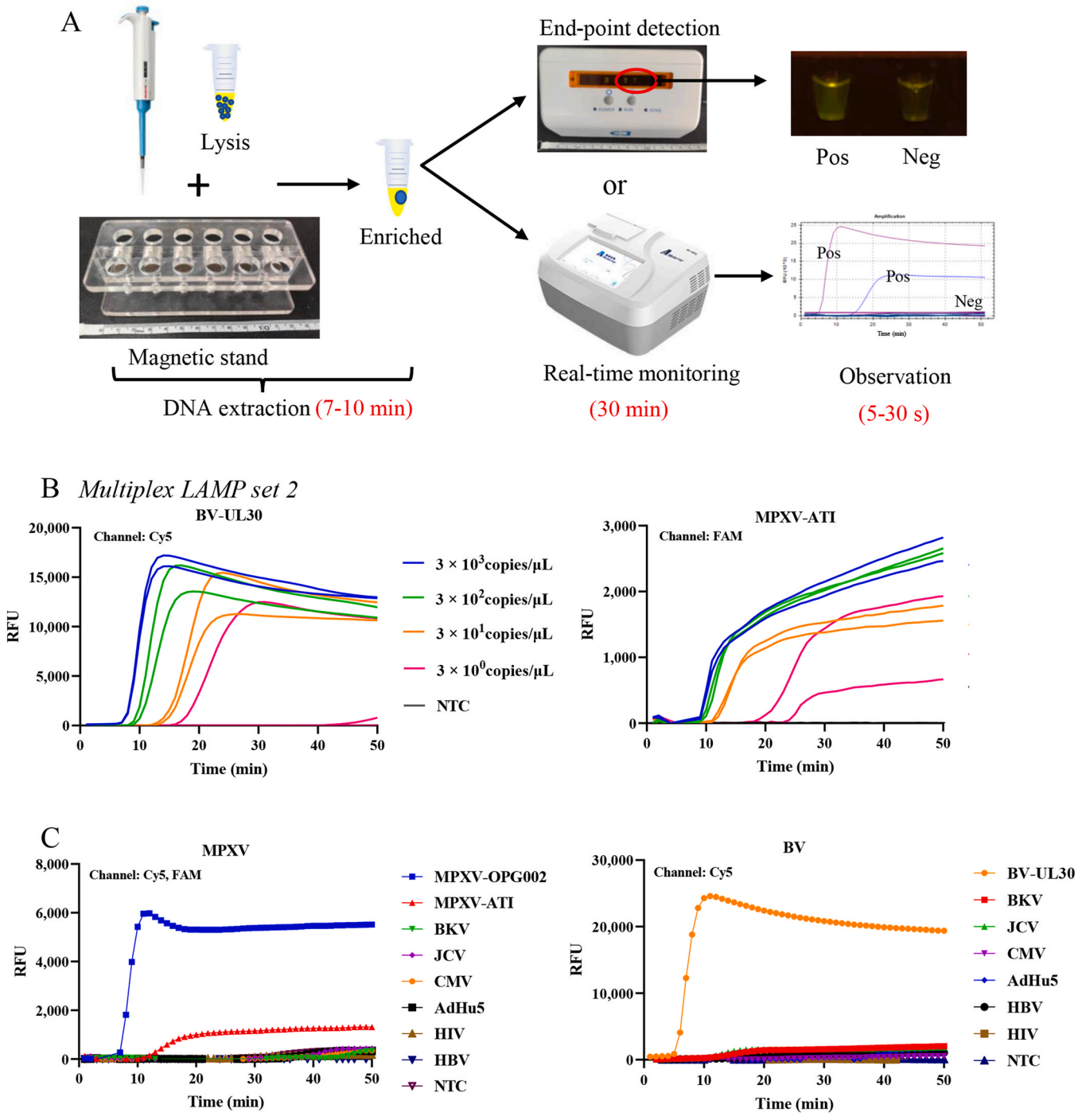

Fig. 1. Sensitivity and specificity of the novel multiplex LAMP assay. (A) Workflow of POCT detection of MPXV and BV using the multiplex LAMP assay. Pos: positive results; Neg: negative results; min: minutes; s: seconds. (B) Sensitivity. The LAMP reaction was carried out with serial dilutions of $3 \times 10^3 - 3 \times 10^0$ copies/μL of MPXV and BV DNA standards. (C) Specificity. Tested viruses included BK polyomavirus (BKV), JC polyomavirus (JCV), Cytomegalovirus (CMV), human adenovirus type 5 (AdHu5), human immunodeficiency virus type 1(HIV-1), and Hepatitis B virus (HBV). NTC, non-template control.

Table 1
Detection limit of LAMP for MPXV and BV.

Template input (copies/ 25 μL reaction)	MPXV ATI gene (positive/total)	BV UL30 gene (positive/total)
3000	10/10	10/10
600	10/10	10/10
120	10/10	10/10
24	8/10	9/10
5	0/10	1/10
LOD (copies/25 μL reaction)	28.7	27.8

minimal detectable concentrations in simulated serum samples were 20 copies/reaction for mono-infection with MPXV or BV and co-infection with both viruses (Table S2).

In summary, we developed a simple, rapid, sensitive and specific multiplex LAMP assay for simultaneous detection of MPXV and BV. The novel assay LOD of 28.7 and 27.8 copies per reaction for MPXV and BV, respectively. As there is no outbreak of MPXV and/or BV, we are unable to obtain real clinical samples positive for both viruses. Therefore, we hope the researchers in epidemic area to test and evaluate the novel multiplex assay, which will be especially helpful

for providing a new POCT tool to facilitate the surveillance and diagnosis of both MPXV and BV.

Ethical approval

All animal studies were approved by Laboratory Animal Welfare & Ethics Committee of Shanghai Public Health Center (2022-A053–01). The study is compliant with all relevant ethical regulations regarding animal research.

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Authors' contributions

Chiyu Zhang: designed the study, analyzed data, and reviewed draft. Yi Zeng: carried out the experiments, analyzed data, and wrote the original draft. Yongjuan Zhao: investigation and interpreted the results. Zhenzhou Wan, Yi-Qun Kuang, and Xiaohui Zhou interpreted the results. Xiaonan Ren and Xiaohui Zhou: collected samples.

Conflict of interest

No conflicts of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2023.02.003.

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