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LETTER

An on-site visual assay detecting Langya henipavirus based on recombinase polymerase amplification technology

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Highlights

- A visual assay of Langya henipavirus (LayV) nucleic acids was developed based on recombinase polymerase amplification technology (RPA), combined with an immunochromatographic test device.
- The detection limit of our assay reaches 1.22 copies μL^{-1}, with no observed cross-reactivity with other henipaviruses.
- This assay is well-suited for the requirements of point-of-care and field detection and has the potential for broader application.

The viruses of the genus *Henipavirus* within the family *Paramyxoviridae* are highly pathogenic and often associated with severe diseases in animals and humans (Basler 2012). For example, Nipah virus (NiV) and Hendra virus (HeV), two members of the genus *Henipavirus*, are known to infect humans and cause fatal disease (Field 2016; Singh *et al.* 2019). Recently, a newly identified henipavirus, Langya henipavirus (LayV), was reported to be associated with respiratory symptoms in humans (Zhang *et al.* 2022). The natural hosts of LayV are found to be the shrew species *Crocidura lasiura* and *C. shantungensis* (Zhang *et al.* 2022). Multiple systems are the targets of LayV; apart from clinical symptoms and signs of viral infection (such as fever, cough, headache), liver and kidney function are also affected (Uwishema *et al.* 2023). Considering the extreme danger of henipaviruses and the close genetic relatedness between LayV and other henipaviruses, LayV has the potential to cause a global outbreak.

As an emerging zoonotic pathogen, few diagnostic methods have been reported. He *et al.* (2024) developed a reverse transcription real-time PCR (RT-qPCR) method for LayV detection, but it requires sophisticated instruments and equipment, as well as skilled operators to ensure the accuracy and precision of results (He *et al.* 2024). However, Wu *et al.* (2024) established a HRP-integrated CRISPR-Cas12a biosensor assay for the point-of-care detection of LayV, but the result needs to be colorimetrically read out by naked eyes, which lacks an objective indicator (Wu *et al.* 2024). With the biosafety level 4 restriction, there was no specificity assessment involving LayV in the field detection methods targeting other henipaviruses. Jin *et al.* (2024) established a field-deployable one-pot RT-RPA CRISPR/Cas12a assay for NiV diagnosis, which could reach 5.5 copies μL^{-1} in sensitivity evaluation, but LayV was not involved in specificity evaluation (Jin *et al.* 2024). To facilitate field detection and meet the point-of-care requirement, we developed an on-site assay targeting the *N* gene of LayV, based on recombinase polymerase amplification (RPA) technology, which achieves rapid and specific detection of LayV nucleic acids using lateral flow-based visualization.

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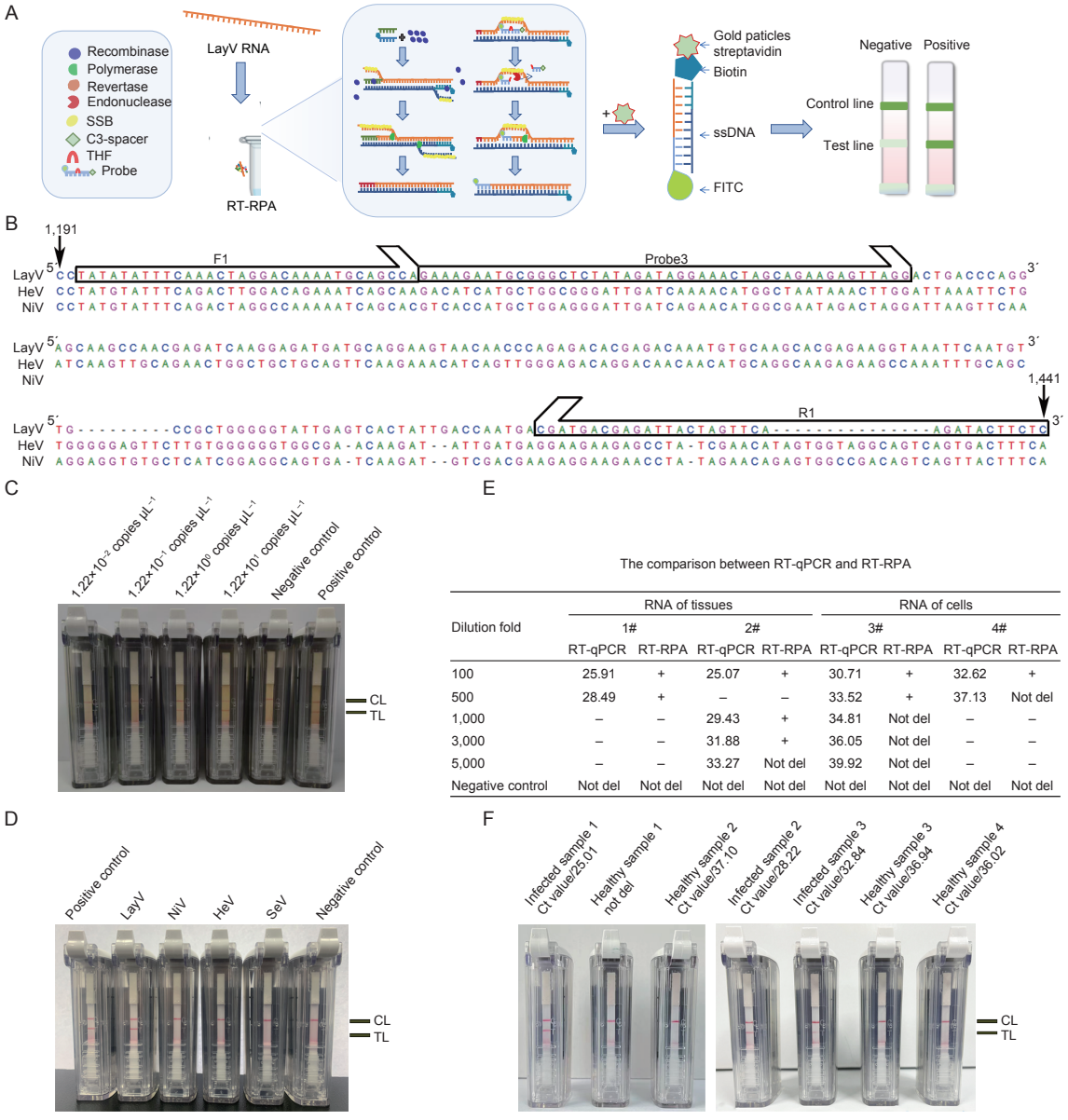
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First, we designed specific primers and probes for the *N* gene of the LayV isolate SDQD_H1801 (GenBank no. OM101125.1). To analyze the conservation of the henipaviruses *N* gene, we performed a sequence alignment. The analysis revealed that the *N* gene is highly conserved within LayV but exhibits significant divergence in its nucleic acid sequence from other henipaviruses, such as NiV and HeV (Appendix A).

To combine with immunochromatographic technology and realize visual detection, we labeled one of the primers and the probe at the 5′ end with fluorescein isothiocyanate (FAM) and biotin, respectively. In addition, we used tetrahydrofuran (THF) to replace a thymine near the 3′ end of the probe and introduced a blocking group, a three-carbon spacer (C3 spacer), at the 3′ end of the probe, which was cleaved by endonuclease IV (Nfo), thereby removing the blocking group and forming a new 3′ end that can be extended (Lai *et al.* 2018). Different combinations of primers and probes were designed, and all the details and sequences of primers and probes are shown in Appendix B. The schematic of our assay is illustrated in Fig. 1-A. When viral RNA or an amplification template is detected, followed by multiple cycles



The comparison between RT-qPCR and RT-RPA

Dilution fold	RNA of tissues				RNA of cells			
	1#		2#		3#		4#	
	RT-qPCR	RT-RPA	RT-qPCR	RT-RPA	RT-qPCR	RT-RPA	RT-qPCR	RT-RPA
100	25.91	+	25.07	+	30.71	+	32.62	+
500	28.49	+	–	–	33.52	+	37.13	Not del
1,000	–	–	29.43	+	34.81	Not del	–	–
3,000	–	–	31.88	+	36.05	Not del	–	–
5,000	–	–	33.27	Not del	39.92	Not del	–	–
Negative control	Not del	Not del	Not del	Not del	Not del	Not del	Not del	Not del

Fig. 1 The development and evaluation of on-site visual assay of Langya henipavirus (LayV). A, schematic diagram of the visual assay of LayV. B, details (location and sequence) for primers and probe used in our visual assay. The GenBank accession numbers of indicated viruses are OM101125.1 (LayV), AF212302.2 (Nipah virus (NiV)), and AF017149.3 (Hendra virus (HeV)), respectively. C, the sensitivity of the visual assay was evaluated by tenfold serial dilution of recombinant plasmids containing the LayV *N* gene from 10^{1} to 10^{-2} copies μL^{-1}. Diethyl pyrocarbonate (DEPC)-treated water and recombinant plasmid (without dilution) were used as negative and positive controls, respectively. CL, control line; TL, test line. D, the specificity of the visual assay was evaluated by using RNA from indicated viruses; negative control (DEPC-treated water) and positive control (LayV *N* gene-containing recombinant plasmid without dilution) were set and analyzed. SeV, Sendai virus. E, the comparison of detection limits between RT-qPCR and visual assay. RT-qPCR and visual assay were performed to detect viral RNA of infected cells and tissues. +, positive result; not del, negative result; –, the sample under indicated dilution was not used for detection. F, the visual assay test results of clinical samples of field detection. The Ct values of the samples detected by RT-qPCR are shown.

of amplification reactions, numerous amplification products with FAM and biotin are synthesized (Liu *et al*. 2024). The products are then placed in a disposable nucleic acid visualization test device to finish result readout (Cao *et al.* 2016).

The *N* gene of LayV was cloned into the pCAGGS vector by *Not*I and *Nhe*I restriction sites to generate the pCAGGS-N recombinant plasmid. Tenfold serial dilutions of this plasmid were used as positive control templates for subsequent assay evaluation and optimization. We screened different primer and probe sets using the RPA reaction, with results interpreted *via* a visualization device. The set of primers and probe that produced a successful amplification reaction with a strong signal was selected for the development of our assay, and the details are shown in Fig. 1-B. To determine the optimal reaction conditions for maximum sensitivity, we performed RPA at various temperatures and for different durations. The results showed that the visual assay we established here reached the highest sensitivity (1.22 copies μL^{-1}) at 42°C with 30 min of amplification (Fig. 1-C).

Given that the henipaviral *N* genes are relatively conserved, assay specificity is critical for distinguishing LayV from related viruses. We therefore evaluated specificity using viral RNA from several sources, including the henipaviruses NiV and HeV, as well as Sendai virus (SeV), a member of the related *Respirovirus* genus. The data suggested that positive results were detected only in the presence of positive control or LayV RNA, with no observed cross-reactivity with other viruses, indicating the high specificity of this assay (Fig. 1-D).

Due to its high sensitivity, RT-qPCR is widely used in the field of virus detection. As a newly identified virus, there is no commonly accepted RT-qPCR method for LayV. Therefore, we developed an RT-qPCR method targeting the LayV *N* gene to benchmark the sensitivity of Appendix C. Both assays were used to detect serial dilutions of nucleic acid extracted from LayV-positive cells and shrew tissues. The results demonstrated that the two methods had comparable detection limits (Fig. 1-E). Notably, our visual assay offers significant advantages in being easy-to-use, faster, and requiring only visual readout. To assess the feasibility of our visual assay for field deployment, we conducted a double-blind trial using seven tissue samples from healthy or LayV-infected shrews from a previous study (Zhang *et al.* 2022). The detection results from our visual assay were fully consistent with both the prior study data and our RT-qPCR results (Fig. 1-F).

In summary, we have developed a rapid, specific, and user-friendly visual assay for the on-site detection of LayV nucleic acid. This method is well-suited for point-of-care and field detection and can be readily scaled for broader application.

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Declaration of competing interest

Sandra Chiu, Zengguo Cao, Wei Liu, Xiaoai Zhang, Lin Cheng and Fangxu Li are co-inventors on a pending patent application (China Patent Bureau, 2024119250623.X). The other authors declare that they have no competing interests.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

This study did not involve any experiments on humans or live animals.

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2025.12.008

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