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HNB-RT-LAMP Coupled with CRISPR/Cas12a for the Simultaneous Detection of Enteroviruses and Enterovirus A71 in a Single Tube

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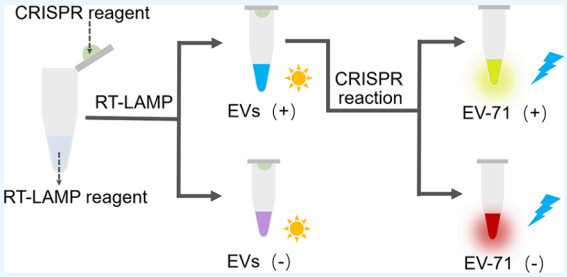
ABSTRACT: **Background:** Enterovirus (EV) infections encompass a spectrum of diseases, with severe cases often linked to enterovirus A71 (EV-A71), highlighting its significance in EV screening. Although the CRISPR/Cas12a diagnostic system shows promise in nucleic acid detection, enhancing specificity and sensitivity when combined with reverse transcription-loop-mediated isothermal amplification (RT-LAMP), its limitation lies in targeting a single sequence, thus preventing the simultaneous detection of both EVs and EV-A71. **Results:** This study presents a novel one-tube strategy by integrating HNB (hydroxynaphthol blue)-RT-LAMP with CRISPR/Cas12a for the simultaneous detection of EVs and EV-A71 in a single tube. The assay initially detects EVs using an HNB colorimetric approach under natural light, followed by the specific detection of EV-A71 through CRISPR/Cas12a under blue light. The limit of detection for EVs was 10 copies/μL, and for EV-A71, it was 1 copy/μL. Clinical sample assays demonstrated that, compared to qPCR, the accuracy of HNB-LAMP-CRISPR detection for EVs and EV-A71 was 95.7 and 100%, respectively. **Significance:** In summary, this strategy offers a reliable and user-friendly approach for EV screening. Also worth mentioning is that the provided method has beneficial effects on rapid visualized detection.



1. INTRODUCTION

Enteroviruses (EVs), members of the Enterovirus family of small RNA viruses,[1] have been associated with a range of human illnesses, including Hand Foot and Mouth Disease (HFMD),[2] acute flaccid paralysis,[3] acute hemorrhagic conjunctivitis,[4] aseptic meningitis, and myocarditis.[5] These viruses spread via both respiratory and digestive routes, posing a potential risk of large-scale epidemics.[6] The general population can be affected by EV infections, while children have an infection rate that is seven-fold higher, which consequently makes EVs a leading cause of infection in the pediatric group.[7−9] Furthermore, HFMD caused by EVs is highly contagious, and in some children with severe comorbidities, it progresses rapidly and can lead to death. As a result, there is an imperative to conduct timely and effective detection of EVs so as to carry out treatments and prevention at an early stage to control their spread.[10,11]

The International Committee on Taxonomy of Viruses categorizes EVs into groups A, B, C, and D based on biological and genetic characteristics.[12] Among these, enterovirus A71 (EV-A71), which once caused large outbreaks of HFMD,[13] stands out as a cause of severe cases and fatalities in EV infections.[14] EV-A71, highly neurotropic, can lead a considerable number of infected patients to develop fatal neurological and cardiopulmonary complications such as meningitis, cerebellar ataxia, acute flaccid paralysis, encephalitis, and pulmonary edema.[15,16] Moreover, follow-up studies have shown that patients with severe infections may still suffer from serious neurological sequelae after recovery.[2] In view of this, the early detection and diagnosis of EV-A71 are essential for reducing severe cases and mortality, warranting significant attention and prompt implementation.[17,18]

Nowadays, while EV-A71 still remains a major cause of fatal cases of HFMD, its proportion has been declining year by year, with the implementation of vaccination work.[19−21] The proportion of fatal cases caused by other EVs such as Coxsackievirus A16 (CV-A16), Echovirus, Enterovirus C95 (EV-C95), and Enterovirus D68 (EV-D68) has shown a significant upward trend by contrast.[22] Moreover, the fact that the EV-A71 vaccine has not yet achieved cross-protection against infections caused by other EVs makes the control of EVs' prevalence even more difficult.[23] Therefore, only by simultaneously detecting both EVs and EV-A71 can we clarify

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Table 1. Sequences Used in the Study

name	sequences (5′-3′)	length (bp)	category
5′-UTR-F3	ACGGGACGCTAGTTGTGA	18	DNA
5′-UTR-B3	ATTGTCACCATAAGCAGCCA	20	DNA
5′-UTR-FIP	ATTAGCCGCATTCAGGGGCC-ACAGGGTGTGAAGAGCCTAT	40	DNA
5′-UTR-BIP	TGTCGTAACGCGCAAGTCCG-GAAACACGGACACCCAAAGT	40	DNA
5′-UTR-LF	GGATTCTTATGTAGCCTC	18	DNA
5′-UTR-LB	TGGCGGAACCGACT	14	DNA
VP1-F3	GCGGAGTTCACTTTTGTTGC	20	DNA
VP1-B3	CGCAGGTGACATGAATGGTA	20	DNA
VP1-FIP	GGCTCCAGGTGGCACAAACAT-CACACCCACAGGGGAAGT	39	DNA
VP1-BIP	AGCCAGATTCCAGGGAATCCCT-AGGGTCTGACAGCTTGACAA	42	DNA
VP1-LB	GTATTGGAGCAATTGTGGGACA	22	DNA
VP1-LF	CATGGCAAACCGCCACCAA	19	DNA
VP1-crRNA	AAUUUCUACUCUUGUAGAU-TGCCACCTGGAGCCCCTAAGCCA	42	RNA
DNA reporter	FAM-TTATT-BHQ1	5	DNA

the overall epidemic situation of the viruses, ensure that no other types of EVs are overlooked, identify children who may develop severe symptoms, and conduct risk assessments for potential HFMD outbreaks. Many researchers, such as Zhou et al.,[24] Puenpa et al.,[25] have recognized this issue and are committed to developing detection strategies that can simultaneously detect EV-A71 and other EVs to reduce outbreaks of HFMD related to EVs. The ideal diagnostic testing method should be able to simultaneously detect EV and differentiate EV-A71,[26] which would facilitate the assessment of the potential severity of the EV outbreak and thus trigger appropriate public health intervention measures.[27]

Traditional methods for detecting EVs, such as serological assays,[28] have low sensitivity. Molecular biology detection techniques, like real-time quantitative polymerase chain reaction (RT-qPCR),[29] require complex thermal cycling equipment, expensive probes, nucleic acid extraction methods, and considerable expertise, making them less suitable for rapid diagnosis.[30] Loop-mediated isothermal amplification (LAMP) has emerged as a promising alternative to RT-qPCR, offering benefits such as tolerance to inhibitors without the need for extraction and isothermal amplification without complex thermal cycling.[31] Introducing LAMP colorimetric indicators like hydroxynaphthol blue (HNB) enables visual evaluation of results, enhancing its usability.[32]

Recent advancements in CRISPR/Cas-based diagnostics, particularly the fusion of CRISPR/Cas12a with LAMP (LAMP-CRISPR/Cas12a),[33] have further improved sensitivity and specificity, making it suitable for accurate point-of-care (POC) diagnostics. CRISPR/Cas12a, guided by CRISPR RNA (crRNA), can recognize LAMP amplification products and trigger its cleavage of a fluorescent DNA reporter, yielding a visible fluorescent signal.[34] However, one tube can detect only one target at a time, whether for EVs or EV-A71.[35] Furthermore, when performing LAMP-CRISPR detection in two steps, opening the tube can allow the target virus that might be present in the air to enter the sample or reaction system, leading to false positive results. The limitation underscores the need for advancements in multiplex capabilities and specificity to enhance diagnostic potential.

To address this, we proposed a strategy coupling HNB-RT-LAMP with CRISPR/Cas12a (HNB-LAMP-CRISPR) for the simultaneous detection of EVs and EV-A71, which allowed for single-tube reactions and visual readout. Clinical validation using nasopharyngeal (NP) swabs from children suspected of HFMD further confirmed the performance of HNB-LAMP-CRISPR for rapid and accurate detection.

2. METHODS

2.1. Materials and Reagents. WarmStart LAMP Kit and rCutSmart buffer were obtained from New England Biolabs (NEB). HNB, TAE, and agarose were purchased from Sigma-Aldrich (Shanghai). AsCas12a was procured from Shanghai Tolo Biotech Co., Ltd. RNase inhibitor, DEPC water, and DNA marker were obtained from Nanjing Vazyme Medical Technology Co., Ltd. All sequences, including RNA templates, primers, crRNA, and ssDNA reporter, were synthesized by Beijing Tsingke Biotech Co., Ltd.

2.2. Design of Primers and crRNA. Primers were designed based on the highly conserved 5′-UTR sequence of EVs, referencing previous studies.[36] Utilizing the VP1 gene of EV-A71 (GenBank accession No: GQ855293.1), LAMP-specific primers for EV-A71 were designed using Primer Explorer V5, and crRNAs were designed near the PAM site of the LAMP product. The specific sequences of primers, crRNAs, and other sequences used in this study are given in Table 1.

2.3. Virus Genomes and RNA Templates. Virus genome RNA, including Enterovirus A71, Coxsackievirus A16 (CV-A16), Coxsackievirus A6 (CV-A6), Coxsackievirus A10 (CV-A10), Coxsackievirus B3 (CV-B3), Coxsackievirus B5 (CV-B5), and Echovirus 30 (EV-30), were provided by the Qinhuai District Center for Diseases Control and Prevention (Nanjing, China). A mixture of EV-A71 and EVs was used for feasibility testing. Amplification products of the 5′-UTR and VP1 gene were used for sensitivity testing.

2.4. HNB-RT-LAMP Assay. The 20 μL singleplex HNB-RT-LAMP system for LAMP primer testing comprises 1× WarmStart LAMP mixture, 0.2 μM primers F3 and B3, 1.6 μM primers FIP and BIP, 0.16 μM loop primers LF and LB, 120 μM HNB, and 2 μL of virus genome RNA. For the 20 μL HNB-RT-LAMP detection system designed for dual-target detection, its components consisted of 1 × WarmStart LAMP mixture, 0.1 μM of each primer F3 and B3, 0.8 μM of each primer FIP and BIP, 0.08 μM of each loop primer LF and LB, 120 μM HNB, and 2 μL of virus genome RNA. The reaction mixture was incubated at 65 °C for 40 min, followed by termination at 80 °C for 10 min. Colorimetric analysis was performed under natural light, where blue indicated a positive

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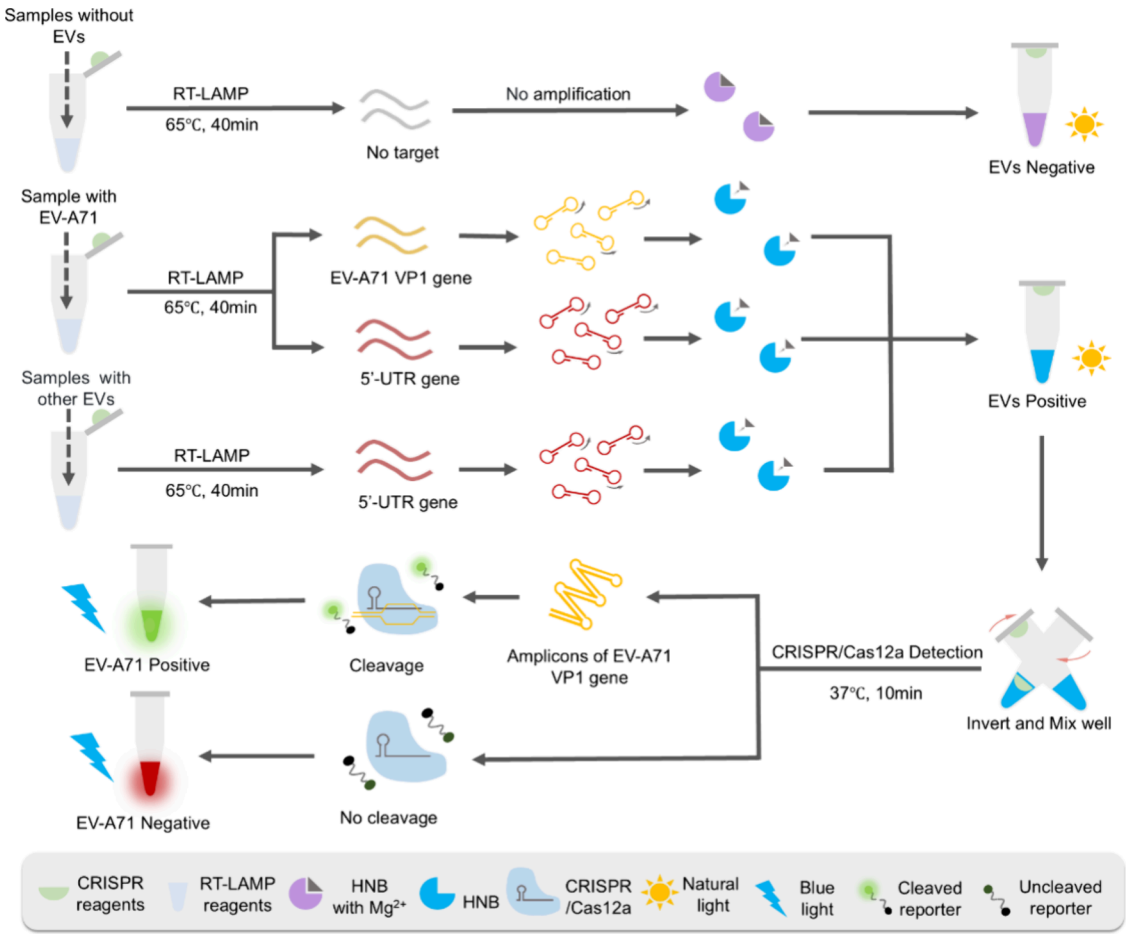

Figure 1. HNB-LAMP-CRISPR principle.

result. Gel electrophoresis with 2.5% agarose was used for validation.

2.5. CRISPR/Cas12a Trans-Cleavage Assay. The 20 μL CRISPR/Cas12a trans-cleavage system included 10 μL of RT-LAMP amplification products and a total volume of 10 μL of the CRISPR/Cas 12a system, which consisted of 1× rCutSmart buffer, 50 nM AsCas12a, 100 nM crRNA, and 500 nM DNA reporter. Incubation was carried out at 37 °C for 10 min, followed by visual analysis under a blue light transilluminator (BlueVision200A/BV200, Clinx Science Instruments Co., Ltd.). Then, the sample with EV-A71 that can activate the trans-cleavage ability of Cas12a, thus generating fluorescent signals, was placed in a microplate reader (Infinite 200 PRO, TECAN) to quantitatively detect the fluorescence intensity. The RT-LAMP amplification products were obtained separately using EV-A71 and the EV genome.

2.6. HNB-LAMP-CRISPR Assay. The one-tube HNB-LAMP-CRISPR detection system comprised equal volumes of the HNB-RT-LAMP system and the CRISPR/Cas12a system, both of which had a volume of 10 μL. The concentration of the HNB-RT-LAMP system was as described in Section 2.4, while the CRISPR/Cas12a system was added at twice the initial concentration on the tube lid, compensating for the dilution that occurred after mixing with the HNB-RT-LAMP system, to ensure that the concentration in the reaction remained constant. After adding 2 μL of plasmid or sample, incubation was first carried out at 65 °C for 40 min, followed by observation of the colorimetric result under natural light.

Sensitivity testing of HNB-LAMP-CRISPR was conducted using serially diluted 5′-UTR and VP1 gene sequences. For the 5′-UTR, colorimetric analysis was performed under natural light after the HNB-RT-LAMP reaction. For the VP1 gene, subsequent to the CRISPR/Cas12a trans-cleavage process, a visual analysis was carried out under a blue light trans-illuminator. After that, the absorbance spectra were examined and analyzed by the utilization of a microplate reader at an excitation wavelength of 490 nm.

2.7. Clinical Sample Testing. 94 NP swab samples from suspected HFMD patients under 12 years of age were provided by the Qinhuai District Center for Disease Control and Prevention. These samples were lysed for RNA prior to testing, according to the instructions provided with the nucleic acid lysis solution kit (Suzhou Jiennuo Biomedical Technology Co., Ltd.). HNB-LAMP-CRISPR was then used to detect EVs and EV-A71. Standard RT-qPCR testing was conducted using the Enterovirus (EV) RT-PCR Kit and Enterovirus type 71 (EV71) RT-PCR Kit (Shanghai Bolsen Biotechnology Co., Ltd.) with a real-time fluorescence quantitative PCR system (QuantStudio 5, Thermo Fisher).

3. RESULTS AND DISCUSSION

3.1. Overview of the HNB-LAMP-CRISPR System. In the HNB-LAMP-CRISPR system, we placed the HNB-RT-LAMP reagents for EV detection at the bottom of the tube and the CRISPR/Cas12a reagents for EV-A71-specific detection at the top. Initially, samples were directly added to the HNB-RT-

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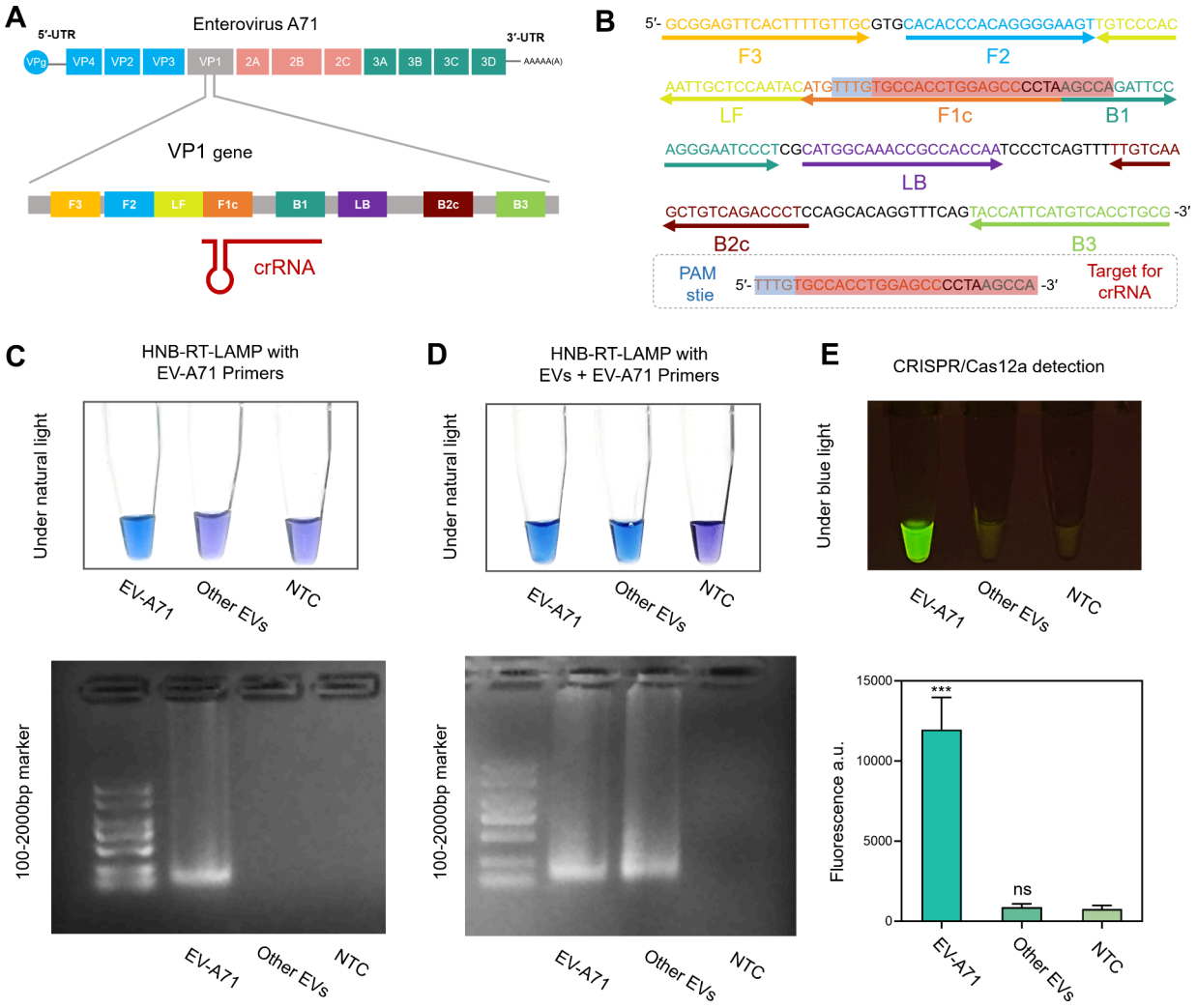

Figure 2. Feasibility Testing. (A) Design of the LAMP primer set and crRNA using the VP1 gene sequence of the EV-A71. (B) Specific sequences of the primers and crRNA are shown, with arrows indicating the 5′ to 3′ direction. The crRNA is designed downstream of the PAM site (5′-TTTG-3′). (C) HNB-RT-LAMP colorimetric and gel electrophoresis analyses were conducted using the standalone EV-A71 primers set. (D) Multiplex HNB-RT-LAMP colorimetric and gel electrophoresis analyses were conducted using the mixed primer set for EV-A71 and universal EVs. (E) CRISPR/Cas12a detection was performed on the amplification products of the multiplex RT-LAMP using the mixed primer set for EV-A71 and EVs. Observation was made under a blue light transilluminator, and fluorescence intensity was analyzed using a microplate reader. The difference in fluorescence intensity between the EV-A71 group and the NTC group was statistically significant ($P < 0.001$, ****), whereas other EVs showed no significant difference from the NTC ($P > 0.05$, ns). The experiment was carried out three times.

LAMP reagents, and LAMP reactions were conducted at 65 °C for 40 min. The LAMP reagents contained specific primer sets for EVs and EV-A71, which facilitated the amplification of the EVs 5′-UTR and EV-A71 VP1 gene. In the case of positive amplification (indicating the presence of EVs), a blue color would be presented through the HNB colorimetric detection, whereas negative amplification would appear violet under natural light. Subsequently, the CRISPR reaction tube was inverted to thoroughly mix the LAMP reaction products with the CRISPR system and then placed in a constant temperature incubation device for incubation at 37 °C for 10 min. If CRISPR/Cas12a can specifically recognize the amplification product of the EV-A71 VP1 gene, it will trigger the trans-cleavage activity and cleave surrounding fluorescent reporter molecules. Through this method, EV-A71 can be detected under blue light, differentiating other EVs (Figure 1).

At the bottom of the tube is the HNB-RT-LAMP reagent, with the CRISPR/Cas12a reagent added to the top. The sample is added to the HNB-RT-LAMP reagent for RT-LAMP to detect EVs. Samples without EVs do not show RT-LAMP amplification, so there is no change in the HNB, appearing violet under natural light. Samples with EV-A71 undergo amplification of the VP1 gene and 5′-UTR gene, causing the precipitation of free Mg^{2+} in HNB and appearing blue under natural light. For samples with other EVs, only the amplification of the 5′-UTR gene occurs, appearing blue under natural light. After the detection of EVs, the tube containing the EV-positive sample is inverted to mix the two reagents for CRISPR/Cas12a detection. Samples with the VP1 gene activate the trans-cleavage activity of Cas12a, generating fluorescent signals and appearing green under blue light. While Samples without the VP1 gene cannot activate the trans-cleavage process, no fluorescent signals are generated, appearing red under blue light.

3.2. Testing of Primers and crRNA. RT-LAMP introduces a set of primers targeting EVs and a specific set of primers designed for EV-A71. The 5′-UTR region is conserved among EVs.[37] We referred to previous studies and

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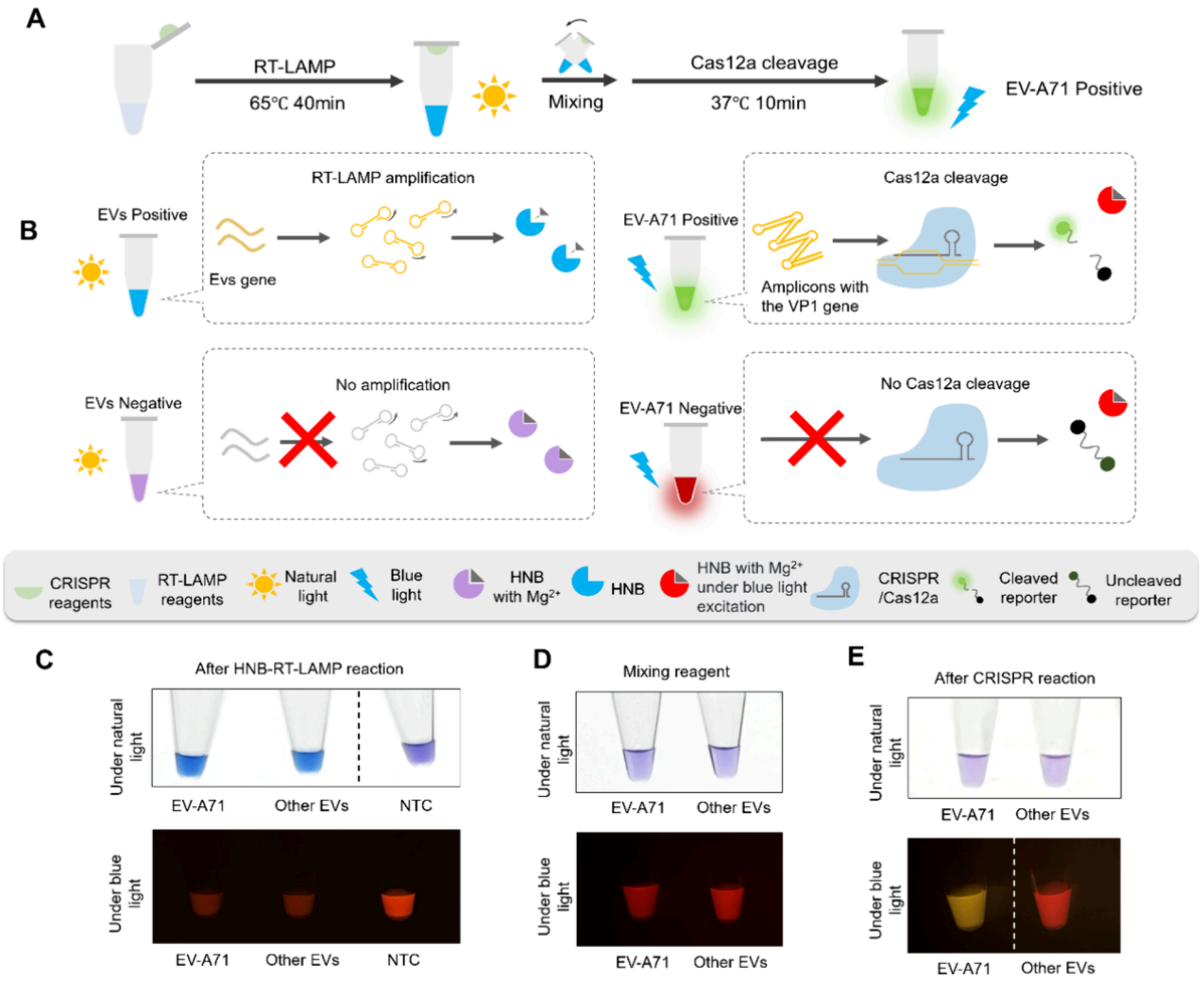

Figure 3. One-tube strategy for screening EVs and genotyping EV-A71. (A) Protocol of "One-Tube" strategy. At the bottom of the tube is the HNB-RT-LAMP reagent, with the CRISPR/Cas12a reagent added on top. The sample is added to the HNB-RT-LAMP reagent for RT-LAMP, enabling the screening of EVs. By mixing HNB-RT-LAMP and CRISPR/Cas12a reagents, the CRISPR/Cas12a assay is carried out to screen for EV-A71 positive samples. The entire procedure is performed in a single tube. (B) Principle of visual colorimetric detection. For EV samples, after RT-LAMP amplification, the precipitation of $Mg_2P_2O_7$ results in a blue color change under natural light, while samples without EVs appear violet as the RT-LAMP amplification does not occur in them. The EV-A71 positive samples emit green fluorescence under blue light, resulting from the trans-cleavage activity of CRISPR/Cas12a, which generates fluorescent signals. Samples with other EVs appear red due to the fluorescence characteristics of HNB under blue light. (C) Colorimetric results of EV-A71, other EVs, and NTC samples after the HNB-RT-LAMP reaction under natural light and blue light excitation. (D) Colorimetric results of EV-A71 and other EV samples under natural light and blue light excitation after mixing both systems and before the CRISPR/Cas12a reaction. (E) Colorimetric results of EV-A71 and other EV samples under natural light and blue light excitation after the CRISPR/Cas12a reaction. The experiment was carried out three times.

used the 5′-UTR as a target for universal EV LAMP detection.[36] The specific set of primers designed for EV-A71 was used to amplify DNA for CRISPR/Cas12a detection (Figure 2A). Moreover, based on the principles of multiplex primer design,[38] we equally distributed the concentration of these two sets of LAMP primers. Six primers of LAMP and crRNA were designed targeting sequences in the VP1 gene of EV-A71[39] (Figure 2B).

To test the feasibility of the LAMP primers, we used the EV-A71 primers set alone in HNB-RT-LAMP reactions. The amplification product containing the VP1 gene sequence of EV-A71 exhibited blue under natural light, thus distinguishing it from other EVs and negative controls (NTC). Agarose gel electrophoresis confirmed the amplification bands of EV-A71 LAMP (Figure 2C), indicating successful amplification of the VP1 gene. We performed multiplex HNB-RT-LAMP reactions by mixing the EV-A71 primer set with the EV primer, which resulted in positive signals for both EV-A71 and other EVs (Figure 2D). Therefore, these mixed primer sets will be used in the subsequent HNB-LAMP-CRISPR system.

Next, we tested the feasibility of crRNA for EV-A71 detection using CRISPR/Cas12a. Based on the success of the RT-LAMP multiplex primers, the genomic sequences of EV-A71 and other EVs were first subjected to RT-LAMP amplification, followed by the addition of CRISPR/Cas12a reagents for trans-cleavage detection. Finally, visual analysis was conducted under a blue light transilluminator. The results indicated that the detection of RT-LAMP products by the CRISPR/Cas12a system was rapid, with fluorescence signals of the EV-A71 groups being observed within 10 min, clearly distinguishing them from other EVs (Figure 2E). These results confirmed the feasibility of both primer sets and the crRNA.

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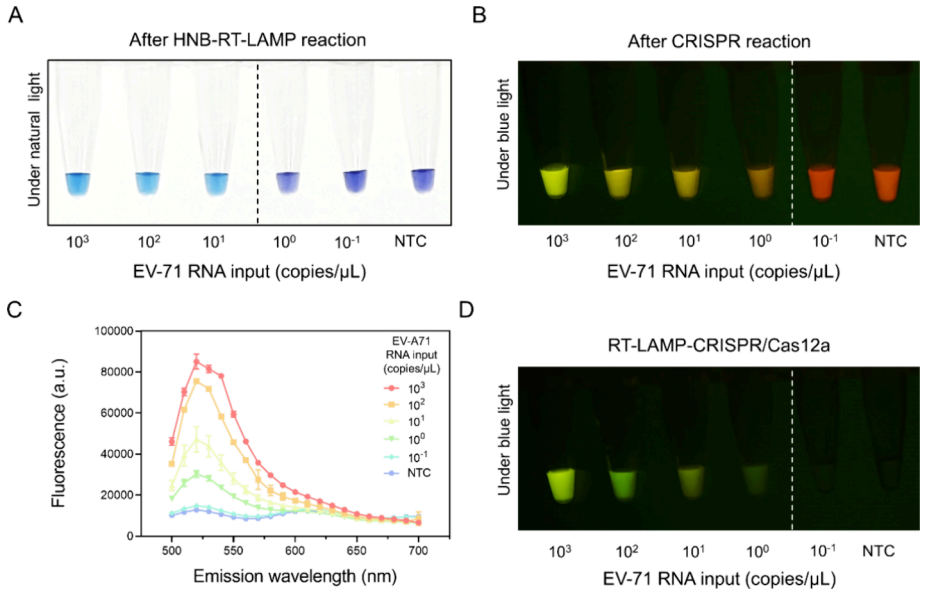

Figure 4. Sensitivity testing. (A) Sensitivity testing of consecutive dilutions of the EV 5′-UTR region using HNB-RT-LAMP. (B) Sensitivity testing of consecutive dilutions of the EV-A71 VP1 gene using HNB-LAMP-CRISPR. (C) Emission spectrum of consecutive dilutions of the EV-A71 VP1 gene using HNB-LAMP-CRISPR under 590 nm excitation. (D) Sensitivity testing of the EV-A71 VP1 gene using conventional RT-LAMP-CRISPR. The experiment was carried out three times.

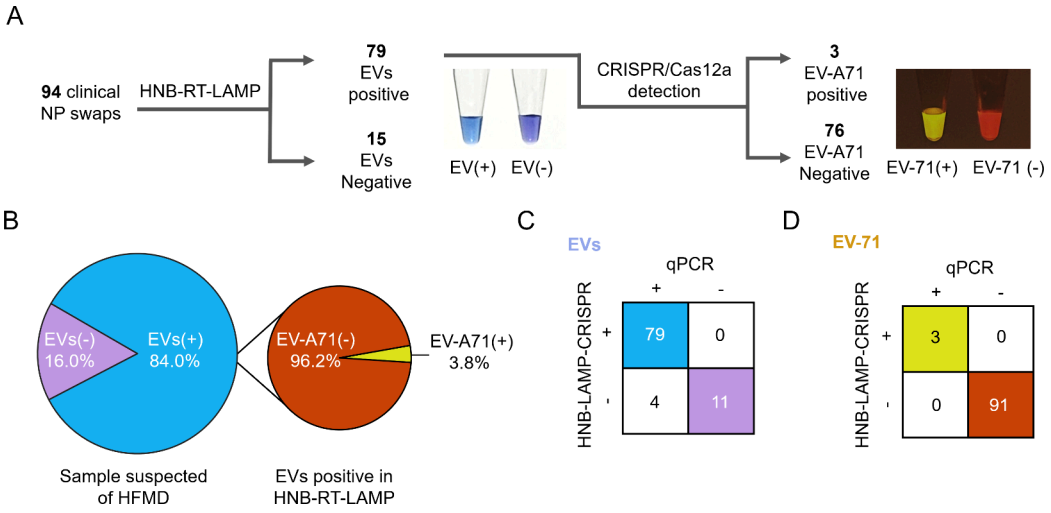

Figure 5. Clinical sample testing of HNB-LAMP-CRISPR. (A) HNB-LAMP-CRISPR results of NP swab samples from 94 suspected HFMD patients under 12 years old. (B) Positivity rates of EVs and EV-A71 Based on HNB-LAMP-CRISPR. (C) Sensitivity and specificity of HNB-LAMP-CRISPR in detecting EVs are compared to RT-qPCR. (D) Sensitivity and specificity of HNB-LAMP-CRISPR in detecting EV-A71 compared to RT-qPCR.

3.3. Feasibility Testing of HNB-LAMP-CRISPR. We developed a "One-tube" strategy to simultaneously detect EVs and EV-A71, which could effectively avoid aerosol contamination caused by opening the tube (Figure 3A). In principle, after LAMP amplification, the precipitation of magnesium pyrophosphate ($Mg_2P_2O_7$) occurs with the reduction of free Mg^{2+},[40] subsequently leading to an observable color alteration of HNB. Colorimetric detection under natural light allows the distinction of EV samples from NTC. Following the mixing of the HNB-RT-LAMP system and the CRISPR/Cas12a system, the VP1 gene amplification products can be detected by CRISPR/Cas12a, and fluorescence signals were generated through the trans-cleavage process, which could be subsequently observed under blue light excitation. This result indicated the presence of EV-A71 (Figure 3B).

We tested the feasibility of HNB-LAMP-CRISPR with EV-A71 and other EVs. After RT-LAMP amplification, EV-A71 and other EVs exhibited a blue color, distinguishable from the violet color of the NTC. Simultaneously, under blue light excitation, we observed that HNB could be excited to red fluorescence, but the fluorescence of positive amplification was weaker than that of the NTC (Figure 3C). The fluorescence characteristics of HNB have been reported in previous studies.[41] Mixing the two reagents in the tube supplemented the CRISPR buffer with Mg^{2+}, which replenished the Mg^{2+} consumed during amplification. Therefore, both EV-A71 and other EVs appeared violet under natural light and red under blue light excitation before the CRISPR/Cas12a trans-cleavage reaction (Figure 3D). After 10 min of CRISPR/Cas12a detection, EV-A71 was excited to green fluorescence under

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blue light, while other EVs remained red, enabling the discrimination of EV-A71 (Figure 3E). This indicated the feasibility of HNB-LAMP-CRISPR for one-tube detection of EVs and EV-A71.

3.4. Sensitivity Testing. We conducted sensitivity testing of the HNB-LAMP-CRISPR system. For EV detection, the 5′-UTR gene sequence of EVs was serially diluted, and the HNB-RT-LAMP assay was performed. The limit of detection (LoD) under natural light was determined to be 10^1 copies/μL (Figure 4A), consistent with the sensitivity of conventional LAMP assays.[42] For EV-A71 detection, the VP1 gene of serially diluted EV-A71 was subjected to the HNB-LAMP-CRISPR assay. As the RNA concentration decreased, the reaction product transitioned from green to yellow under blue light, with an LoD of 10^0 copy/μL (Figure 4B). Under 490 nm blue light excitation, the HNB-LAMP-CRISPR positive reaction reached the highest absorption peak at 520 nm, while the negative sample showed a relatively high absorption peak at 610 nm (Figure 4C). We also compared the results with and without the addition of HNB in the LAMP-CRISPR/Cas12a assays (Figure 4D). It was observed that, for untrained testers, without the addition of HNB, it would be difficult to differentiate between low-copy and negative samples due to background signals when testing unknown samples. In contrast, in HNB-LAMP-CRISPR, since the negative reaction appeared red, it was easier to observe the difference between the red color of the negative samples and the yellow color of the low-copy samples, making HNB-LAMP-CRISPR more user-friendly.

3.5. Clinical Performance Evaluation. A total of 94 clinical samples were utilized to assess the performance of HNB-LAMP-CRISPR. These samples consisted of NP swabs from children under 12 years old suspected of HFMD. Overall, 79 samples were positive for EVs, and three samples were positive for EV-A71 (Figure 5A). HNB-LAMP-CRISPR determined an EV positivity rate of 84.0% and an EV-A71 positivity rate of 3.8% among suspected HFMD patients (Figure 5B). RT-qPCR detected four additional EV-positive cases (Figure 5C), with HNB-LAMP-CRISPR exhibiting complete consistency in EV-A71 detection compared to RT-qPCR (Figure 5D). In conclusion, the results demonstrate that the false positive rate and false negative rate of HNB-LAMP-CRISPR in detecting EVs are 0% and 4.8%, respectively, while those in the detection of EV-A71 are both 0%, demonstrating the commendable detection performance of HNB-LAMP-CRISPR.

4. CONCLUSIONS

We developed HNB-LAMP-CRISPR for the simultaneous detection of EVs and EV-A71. This streamlined process, conducted in a single tube, eliminates the risk of aerosol contamination. The sensitivity of HNB-LAMP-CRISPR for detecting EVs and EV-A71 is impressive, reaching 10^1 and 10^0 copies/μL, and the results are more interpretable. The detection results of clinical samples demonstrated the high sensitivity and specificity of HNB-LAMP-CRISPR. However, it should be noted that more comprehensive and extensive studies are needed to further evaluate its performance and reliability. At present, it may serve as a supplementary tool rather than a fully reliable substitute for RT-qPCR in clinical settings. It is hoped that this research can contribute to the exploration of new diagnostic methods and provide new ideas for improving diagnostics in the future.

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Author Contributions

T.Z.: experiments, formal analysis, data curation, methodology, visualization, and writing-review and editing. X.C.: writing-review and editing. W.Z.: writing-review and editing. Z.Z.: writing-review and editing. C.G.: writing-review and editing. Y.C.: formal analysis. Y.L.: project administration and supervision. M.J.: methodology and writing-review and editing. T.Z. and X.C. contributed equally.

Notes

The authors declare no competing financial interest.

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