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LETTER

Rapid and visual on-site detection system for Epizootic Hemorrhagic Disease Virus based on a combination of CRISPR-Cas12a and RT-ERA

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Highlights

- First development of a RT-ERA/CRISPR-Cas12a platform for rapid and visual detection of Epizootic Hemorrhagic Disease Virus (EHDV), enabling on-site diagnosis within 1 h without specialized equipment.
- High sensitivity and broad serotype coverage: The assay detects as low as 1.7×10^{1} copies/reaction and recognizes 8 EHDV serotypes (1, 2, 4–8, and 10), overcoming the limitation of serotype-specific antisera required in traditional methods.
- Integration with HUDSON sample processing allows direct detection from crude samples without RNA extraction, making it suitable for field use in resource-limited settings and supporting point-of-care testing in ruminant populations.

Epizootic Hemorrhagic Disease (EHD), a vector-borne disease affecting both wild and domestic ruminants, is transmitted by biting midges of the genus *Culicoides*. Since 2008, it has been classified as a notifiable disease by the World Organization for Animal Health (WOAH). The causative agent, Epizootic Hemorrhagic Disease Virus (EHDV), belongs to the genus *Orbivirus* within the family *Reoviridae* and possesses a viral genome comprising ten double-stranded RNA (dsRNA) segments (Jiménez-Cabello *et al.* 2023). To date, ten distinct serotypes of EHDV, designated as EHDV-1, 2, and 4 through 11, have been identified globally (Anthony *et al.* 2009; Maan *et al.* 2017; Shirafuji *et al.* 2017; Yang *et al.* 2020). For instance, EHDV-7 has caused widespread outbreaks in cattle in Israel, leading to substantial economic losses with morbidity rates ranging from 5 to 80%, a mortality rate of 1%, and a 10–20% decline in milk production (Yadin *et al.* 2008; Kedmi *et al.* 2010; Wilson *et al.* 2015). Similarly, Ibaraki virus (IBAV), classified under EHDV-2, was responsible for 43,793 reported infections and 4,298 fatalities in cattle in Japan during 1959–1960 (Omori *et al.* 1969). Additionally, over 1,000 cases of abortions and stillbirths were documented among pregnant cows infected with IBAV during an epidemic in Japan from 1997 to 1998 (Ohashi *et al.* 2002; Potgieter *et al.* 2009). Although no large-scale EHD outbreaks have been officially reported in China, recent surveillance studies have identified multiple EHDV serotypes (1, 5, 6, 7, 8, 10, and 11) circulating in sentinel cattle and *Culicoides* populations across southern provinces (Qi *et al.* 2019; Xin *et al.* 2023; He *et al.* 2024). Alarmingly, epidemiological surveys revealed a high seroprevalence of EHDV (75.1–100%) in cattle populations across southern and northern China (Yang *et al.* 2024), indicating widespread subclinical infections and an imminent outbreak risk. Given China's rapidly expanding livestock industry, the lack of field-deployable diagnostic tools for EHDV poses a critical bottleneck for early outbreak containment. This gap underscores the urgent need for a portable, specialized instrument-free detection platform that integrates high sensitivity with visual readouts, enabling rapid on-farm decision-making.

Conventional methods for EHDV detection and typing involve virus isolation in embryonated chicken eggs or cell cultures, along with ELISA or virus neutralization tests (the "gold standard" recommended by WOAH). However, these

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methods are time-consuming, costly, and require biosafety laboratories and specific reference antisera, which are unavailable in most rural veterinary clinics. Although RT-PCR and real-time RT-PCR-based nucleic acid assays for EHDV offer enhanced speed and reliability, they necessitate expensive specialized equipment, limiting their suitability for on-site testing (Maan *et al.* 2010, 2017; Xin *et al.* 2023). Therefore, there is an imperative need for a rapid, sensitive, and specific detection method that yields visual results without relying on precision laboratory instruments. The CRISPR-Cas system has emerged as a prominent diagnostic tool for nucleic acids (Chen *et al.* 2024; Zhou *et al.* 2024). CRISPR sequences are transcribed and processed into small crRNA, which activate Cas proteins that stimulate RNase or DNase activity, leading to a "collateral effect" that non-specifically cleaves adjacent ssRNA or ssDNA (Abudayyeh *et al.* 2016; Li *et al.* 2018). Leveraging CRISPR-Cas systems in conjunction with isothermal amplification technologies, several nucleic acid testing platforms have been developed, including DETECTR (Chen *et al.* 2018), SHELOCK (Gootenberg *et al.* 2017), and HOLMES (Li *et al.* 2023). The CRISPR-Cas system is characterized by its programmability, high specificity, sensitivity, and cost-effectiveness, and has been successfully used to detect SARS-CoV-2 (Wang *et al.* 2020b), MPXV (Wang *et al.* 2024) and ASFV (Cao *et al.* 2024). Furthermore, when combined with a lateral flow strip, it leverages the rapid and portable characteristics of point-of-care testing (POCT) to facilitate nucleic acids detection without the need for professional personnel (Wang *et al.* 2020a).

In this study, we aimed to develop a rapid and visual test strip for EHDV RNA detection based on RT-ERA and CRISPR-Cas12a. This approach is designed to achieve high sensitivity and specificity in detecting EHDV within ruminant populations, making it suitable for POCT in field settings. To develop a CRISPR-Cas12-based detection system for EHDV, we first aligned and analyzed the reference sequences of ten genomic segments of EHDV obtained from GenBank (Appendix A) using MEGA 11. Through this analysis, we identified a conserved region within segment 1 (S1), confirming its potential as a target for crRNA design. Subsequently, two crRNA candidates (crRNA1 and crRNA2, detailed in Table 1) were designed based on the PAM sites. To identify the optimal crRNA for sensitive detection of EHDV, we individually incubated crRNA1, crRNA2, and a mixture of both (crRNA1/2) with plasmid p57-ES1 containing the complete S1 sequence of EHDV. The effectiveness of these candidates was then evaluated using the CRISPR-Cas12a (GeneScript, China) reaction volume according to the manufacturer's instructions. The reaction was conducted at 37°C for 20 min using the Applied Biosystems QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, United States), with fluorescence measurements recorded every 30 s. Fluorescence detection indicated that crRNA1 exhibited the highest endpoint fluorescence value and optimal fluorescence efficiency, making it the most effective (Fig. 1-A). Consequently, crRNA1 was identified as the preferred choice for the EHDV CRISPR-Cas12a detection system and was utilized in subsequent experiments. To optimize the CRISPR-Cas12a system, we conducted additional experiments to evaluate the concentrations of Cas12a protein (50–500 ng) and crRNA1 (200–1,000 nmol L^{-1}). A combination of 75 ng Cas12a and 400 nmol L^{-1} crRNA1 in a 10 μL reaction volume was selected as the optimal condition, balancing fluorescence intensity and cost-effectiveness (Fig. 1-B and C). Therefore, the CRISPR-Cas12a detection system was composed of the following components: 75 ng of Cas12a, 400 nmol L^{-1} of crRNA1, 1 μmol L^{-1} of either ssDNA FQ reporter gene or ssDNA biotin-labeled nucleic acid probe, 10 U of recombinant RNase inhibitor (RRI), 1 μL of 10× Cas12a buffer, 1–2 μL of template, and with RNase/DNase-free H_2O added to bring the total volume to 10 μL.

Table 1 Sequences of designed crRNAs and RT-ERA prime specific to Epizootic Hemorrhagic Disease Virus (EHDV)

Name	Sequence (5′→3′)
crRNA1	UAAUUUCUACUCUUGUAGAUAACCACUCGUUUAAUGAGYU
crRNA2	UAAUUUCUACUCUUGUAGAUACCACUCGUUUAAUGAGYUS
F1	GTTAAAATGCAATGGTCGCAATYACCGTGC
F2	GCAATGGTCGCAATYACCGTGCAAGGTGC
F3	ATGCAATGGTCGCAATYACCGTGCAAGGTGC
F4	GTTAAAATGCAATGGTCGCAATYACCGTG
F5	GTTAAAATGCAATGGTCGCAATYACCGT
F6	GTTAAAATGCAATGGTCGCAATYACCG
R1	TTYYCTCTDAYRCGYCTKATATGDTCTG
R2	TGYTTYYCTCTDAYRCGYCTKATATGDTCTG
R3	TCCCARGTYGARTCAYGYARAACRGG'
R4	CYTCCCARGTYGARTCAYGYARAACRG
R5	TCYTCCCARGTYGARTCAYGYARAACRGG
R6	TTCYTCCCARGTYGARTCAYGYARAACRGG
R7	CGYCTKATATGDTCTGAAAAYTTATARAAYTC
R8	CTKATATGDTCTGAAAAYTTATARAAYTC

Enzymatic recombinase amplification (ERA) is an isothermal amplification technique that has been progressively integrated with the CRISPR-Cas system for the detection of microbial pathogens (Xia and Chen 2020). To improve the sensitivity of EHDV detection, we integrated RT-ERA with the CRISPR-Cas12a Detection System. Following the principles of isothermal amplification primer design (Euler *et al.* 2013), six upstream primers (F1–F6) and eight downstream primers (R1–R8) were designed based on crRNA1 (Table 1; Fig. 1-D). To identify the optimal RT-ERA primer pairs, standard RT-ERA reactions were conducted utilizing EHDV S1 RNA as the template, adhering to the manufacturer's instructions provided in the RT-based Nucleic Acid Amplification Kit (GenDx Biotech, China). Following RT-ERA amplification at 40°C for 20 min, the resulting products from the RT-ERA amplification were utilized as substrates for detection using the CRISPR-Cas12a system. Initially, R3 was employed to screen primers F1-F6, revealing that F6 exhibited the highest amplification efficiency (Fig. 1-D). Subsequently, using F6 as an anchor, primers R1–R8 were evaluated. The results indicated that the combination of primers F6 and R3 demonstrated the greatest amplification efficiency (Fig. 1-D). Consequently, this pair was selected as the optimal primers for RT-ERA.

To assess the sensitivity of the RT-ERA/CRISPR-Cas12a detection system, EDHV RNA at varying concentrations was

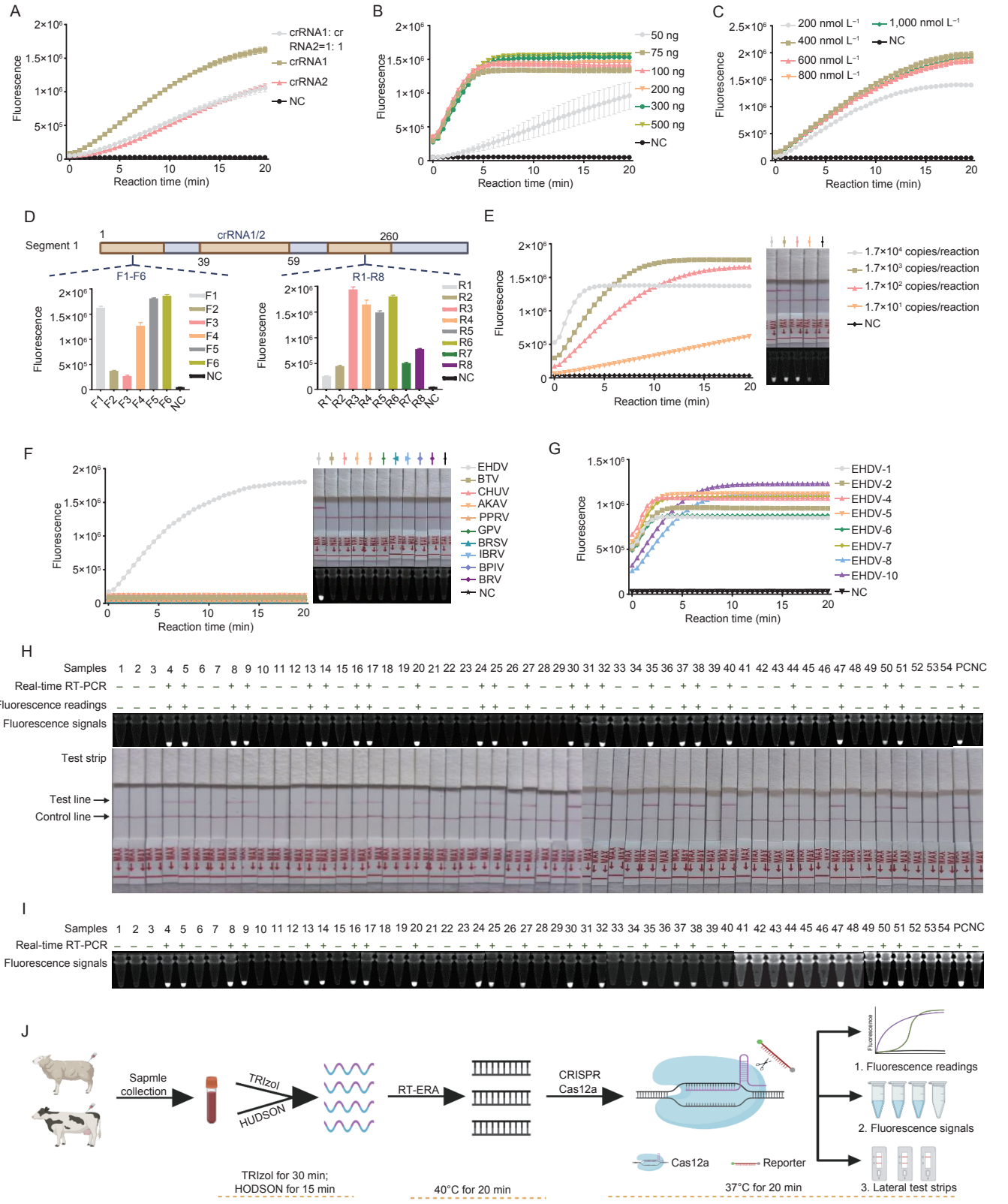


Fig. 1 Establishment and validation of a rapid and visual on-site detection system for Epizootic Hemorrhagic Disease Virus (EHDV) based on CRISPR-Cas12a and RT-ERA. A, detection results for three crRNA combinations targeting EHDV. B, detection results for different concentrations of Cas12a protein. C, detection results for different concentrations of crRNA. D, schematic diagram of RT-ERA primer locations and screening results for RT-ERA primers. E, sensitivity analysis of EHDV detection using the RT-ERA/CRISPR-Cas12a system. F, specificity analysis of EHDV detection using the RT-ERA/CRISPR-Cas12a system. G, universality analysis of EHDV detection using the RT-ERA/CRISPR-Cas12a system. H, detection results from 54 clinical samples using RT-qPCR and RT-ERA/CRISPR-Cas12a following RNA extraction with TRIzol. I, visual detection results from 54 clinical samples using RT-ERA/CRISPR-Cas12a following HUDSON sample processing. J, schematic workflow for visual detection based on RT-ERA/CRISPR-Cas12a. PC, positive control; NC, negative control. Error bars in panels represent the mean±SD, where *n*=3 replicates.

utilized as a template for the RT-ERA reaction. Subsequently, 1 μL of the RT-ERA product served as a substrate for the CRISPR-Cas12a detection system. As shown in Fig. 1-E, the RT-ERA/CRISPR-Cas12a system achieved detection limits of 1.7×10^{1} copies reaction^{-1} and 1.7×10^{2} copies reaction^{-1} for fluorescence-based and lateral flow strip readouts, respectively. Notably, this sensitivity approaches the performance of the established real-time RT-PCR assay for EHDV S9 (Maan *et al.* 2017), while enabling rapid visual interpretation without specialized instrumentation.

Additionally, considering that other vector-borne viruses, aside from EHDV, can produce similar clinical symptoms and taking into account the prevalence of co-infections with various pathogens in ruminant populations, it is imperative to validate the specificity of the RT-ERA/CRISPR-Cas12a detection system for EHDV. To achieve this objective, the genomic material from inactivated BTV, CHUV, AKAV, PPRV, GPV, BRSV, BRV, BPIV3, and IBRV was used at equivalent concentrations as non-target controls, while inactivated EHDV (EHDV-5 and 7) and synthetic EHDV S1 RNA (EHDV-1, 2, 4, 6, 8, and 10) were used as target controls. All inactivated viruses mentioned above were provided by the Livestock Infectious Diseases Research Team at the Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences (CAAS). No fluorescence readings, fluorescence signals, or test strip results were observed when the non-target pathogens were evaluated using the RT-ERA/CRISPR-Cas12a assay (Fig. 1-F), whereas significant fluorescence was observed when the EHDV-1, 2, 4, 5, 6, 7, 8, and 10 were tested (Fig. 1-G). These findings demonstrate that the RT-ERA/CRISPR-Cas12a detection system developed in this study exhibits a high level of specificity for EHDV detection, encompassing at least 8 distinct EHDV serotypes.

Finally, to assess the feasibility of the RT-ERA/CRISPR-Cas12a method for detecting clinical samples, we extracted RNA from 54 whole-blood or tissue samples using TRIzol reagent under controlled laboratory conditions. Subsequently, these samples were subjected to the real-time RT-PCR (Maan *et al.* 2017) and the RT-ERA/CRISPR-Cas12a assay. As shown in Fig. 1-H, the fluorescence readings, fluorescence signals, and test strip outcomes derived from the RT-ERA/CRISPR-Cas12a were in agreement with those obtained from the real-time RT-PCR method. Among the 54 tested samples, 23 were positive and 31 were negative, yielding a sensitivity of 100% (95%CI: 85–100%) and a specificity of 100% (95%CI: 89–100%). This indicates that the efficiency of the RT-ERA/CRISPR-Cas12a detection system developed in this study is comparable to that of the real-time RT-PCR method. Additionally, in light of the limited availability of experimental equipment and the economic constraints associated with detection under field conditions, we employed the HUDSON (heating unextracted diagnostic samples to obliterate nucleases) rapid sample processing method to extract nucleic acids from 54 whole-blood and tissue samples. HUDSON is a method designed to lyse viral particles and inactivate the elevated levels of ribonucleases present in bodily fluids through the application of heat and chemical reduction (Myhrvold *et al.* 2018). Samples treated with HUDSON can be directly incorporated into RT-ERA reaction mixtures without requiring any dilution or purification steps. The rapid detection method combining HUDSON with the RT-ERA/CRISPR-Cas12a assay demonstrated a sensitivity of 96% (95%CI: 80–100%) and a specificity of 100% (95%CI: 89–100%) (Fig. 1-I). This indicates that the EHDV detection method based on HUDSON and RT-ERA/CRISPR-Cas12a can provide visible results without requiring sophisticated instruments, thereby rendering it suitable for on-site detection work of ruminant populations.

The whole process of the RT-ERA/CRISPR-Cas12a detection system for EHDV identification is illustrated in Fig. 1-J. Firstly, viral genomic RNA is extracted from samples using TRIzol reagent or HUDSON and subsequently amplified through RT-ERA, after which the product is transferred to the CRISPR-Cas12a system. Finally, the results were rapidly analyzed *via* fluorescence readings, fluorescence signals, or lateral test strips. Our RT-ERA/CRISPR-Cas12a system achieves two critical advancements for EHDV surveillance: First, its broad serotype coverage (EHDV-1, 2, 4-8, and 10) addresses a key limitation of traditional serotyping methods, which require serotype-specific antisera that are often unavailable for emerging strains. Second, the integration of HUDSON sample processing eliminates RNA extraction steps, reducing both cost and turnaround time. While these features render the system highly applicable to resource-limited settings, two limitations warrant consideration: First, it does not differentiate between EHDV serotypes, which is critical for accurately tracing outbreak origins. Second, field validation was limited to 54 clinical samples, and larger-scale trials are necessary to evaluate its robustness across diverse environmental conditions (e.g., varying temperatures or sample hemolysis). Future work will focus on multiplexing crRNA designs to enable simultaneous serotyping and integration with smartphone-based fluorescence readers for quantitative analysis.

In conclusion, we present the first CRISPR-Cas12a/RT-ERA platform for rapid and specialized instrument-free detection of EHDV. This system demonstrates three key advantages over existing methods: (1) the detection limit (1.7×10^{1} copies/reaction) comparable to real-time RT-PCR but without requiring expensive thermocyclers; (2) serotype-agnostic recognition of 8 major EHDV strains, overcoming antisera dependency; (3) compatibility with crude samples *via* HUDSON pretreatment, enabling on-farm use. With a turnaround time of <1 h and visual readouts, this platform enables on-farm decision-making for rapid containment of suspected EHDV outbreaks, significantly reducing reliance on centralized laboratories. With further optimization for multiplexed serotyping and field ruggedness, this technology could serve as a model for decentralized diagnostics of other vector-borne livestock pathogens, ultimately strengthening global One Health initiatives.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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

During the preparation of this manuscript, the authors used DeepSeek to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

Ethical statements

The Animal Ethics Committee of Harbin Veterinary Research Institute, CAAS, determined this study did not require ethical review or approval, as it complied with local regulations and institutional policies regarding clinical samples.

Appendix associated with this paper is available at https://doi.org/10.1016/j.jia.2025.09.023

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