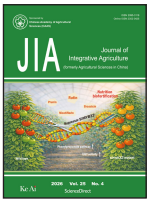
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LETTER

A rapid and visual detection method for porcine deltacoronavirus with single-copy sensitivity based on the CRISPR/Cas12a assay

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Highlights

- By integrating RT-RAA with CRISPR/Cas12a, a portable, one-pot visual detection system was engineered, significantly minimizing the potential for false positives caused by aerosol contamination.
- RT-RAA-CRISPR/Cas12a assay can complete the detection within 40 min, with a sensitivity as high as 3.9 copies μL^{-1} for single copy.

Porcine deltacoronavirus (PDCoV) disease is an acute and highly contagious illness caused by the PDCoV. It was first detected in Hong Kong, China in 2012 and has since caused sporadic outbreaks globally. It primarily targets the digestive system of piglets, inducing symptoms such as vomiting, diarrhea, dehydration, and potentially leading to death, with a case fatality rate that may reach 100% (Hu *et al.* 2015). Such outbreaks pose a significant threat to the swine farming industry, resulting in substantial economic losses (Dong *et al.* 2015). In addition to swine, PDCoV can also infect other species, such as chickens, mice, and calves (Jung *et al.* 2017; Liang *et al.* 2019; Zhang *et al.* 2022). It is noteworthy that PDCoV nucleic acid was detected in three children in Haiti in 2020, indicating a significant risk of cross-species transmission and posing potential threats to human public health (Lednicky *et al.* 2021). As an emerging infectious disease, PDCoV presents considerable challenges for effective management and control. Currently, there is a notable lack of commercially available vaccines and specific antiviral treatments targeted against this pathogen. Furthermore, research into novel detection methodologies remains limited, with existing preventive and control measures proving inadequate for containing its spread (Zhai *et al.* 2022). In recent years, PDCoV has caused escalating economic losses in the livestock industry, highlighting the critical need for reliable, early-detection diagnostic tools. The development of such tools is crucial for implementing effective prevention strategies and controlling the epidemic before it causes further devastation to the swine industry and potentially crosses over to affect human populations (Wang *et al.* 2019).

Currently, the primary detection methods for PDCoV include PCR, quantitative real-time PCR (qPCR), enzyme-linked immunosorbent assay (ELISA), among others (Zhao *et al.* 2023). However, these methods all depend on laboratory equipment and impose high technical demands on operators, making it challenging to effectively serve clinical applications (Wang *et al.* 2021). Therefore, the development of a rapid, convenient, and sensitive diagnostic method that does not rely on complex instruments for early PDCoV detection could significantly mitigate the economic losses caused by the disease. In recent years, the CRISPR-associated protein 12a (CRISPR-Cas12a) system has gained popularity in molecular diagnostics owing to its efficient DNA trans-cleavage activity, which enhances signal amplification (Manghwar *et al.* 2019; Liu *et al.* 2024). However, its sensitivity is limited without a target amplification step. Recombinase Aided Amplification (RAA), an isothermal amplification method, is often combined with Cas12a system to establish specific and sensitive nucleic acid detection

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assays (Huang *et al*. 2023). This combination is favored due to RAA's efficient amplification and optimal reaction temperature range of 37°C to 42°C, which aligns well with the Cas12a system's requirements (Lobato and O'sullivan 2018).

Here, we represent a single-copy sensitive, field-deployable, one-pot visual detection method that combines reverse transcription (RT)-RAA with CRISPR/Cas12a for the detection of PDCoV nucleic acids (Fig. 1-A). By

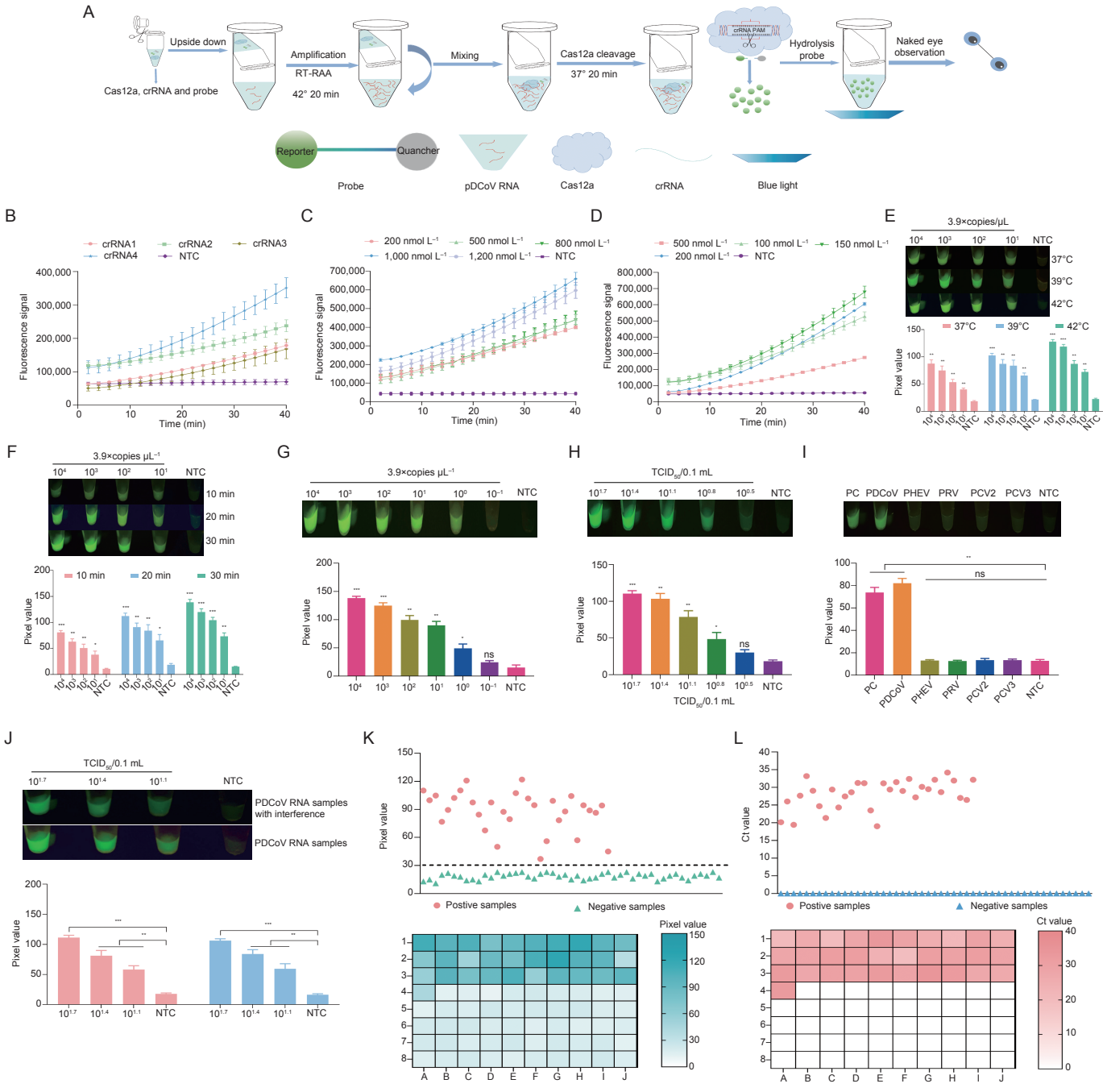


Fig. 1 Development and evaluation of the RT-RAA-CRISPR/Cas12a assay for porcine deltacoronavirus (PDCoV) detection. A, schematic illustration of the one-pot visual RT-RAA-CRISPR/Cas12a assay for detecting of PDCoV infection. B, screening of four crRNAs targeting the PDCoV *N* gene plasmid, with fluorescence intensity monitored in real-time PCR. C, optimization of cas12a protein concentrations in the one-pot assay. D, optimization of crRNA concentrations in the one-pot assay. E, determination of optimal temperature for the one-pot assay using tenfold serially diluted PDCoV *N* gene plasmid. F, evaluation of optimal reaction time for the one-pot assay using tenfold serially diluted PDCoV N gene plasmid. G, sensitivity assessment of the one-pot assay using tenfold serially diluted PDCoV *N* gene plasmid (3.9×10^{4}–10^{-1} copies μL^{-1}). H, sensitivity evaluation of PDCoV RNA detection using twofold serially diluted stock PDCoV CC23 strain solution (initial concentration of $10^{3.8}$ $TCID_{50}$/0.1 mL). I, specificity assessment of the one-pot assay using nucleic acids from PDCoV, PHEV, PRV, PCV2, and PCV3. J, evaluation of the one-pot assay's capability to detect PDCoV in mixed viral infections by combining PDCoV RNA with other viral nucleic acids. K, clinical validation of the one-pot assay using fecal or intestinal content samples from 80 diarrheic pigs (Pixel value≥30 considered positive). L, clinical validation *via* real-time PCR using fecal or intestinal content samples from 80 diarrheic pigs (Ct value≤35 considered positive; negative samples consistently categorized as Ct=0). PC, positive control; NTC, nontarget control. Error bars represent standard errors of the mean. *, $P\leq0.05$; **, $P\leq0.01$; ***, $P\leq0.001$.

integrating these technologies in a closed-one-tube system, we significantly avoided false positives from aerosol contamination. First, given the high conservation of the PDCoV *N* gene (Lee and Lee 2015), we conducted a comparative analysis across different strains and designed four specific crRNAs targeting *N* gene (Table 1). After the double-stranded DNA was obtained *via* PCR amplification, it was purified and subsequently transcribed into RNA. Further purification steps resulted in high-purity crRNA (Jin *et al*. 2024). Next, the four crRNAs were screened using qPCR with a system including Cas12a protein, fluorescent probe, a recombinant plasmid containing the PDCoV *N* gene, and buffer solution. The results demonstrated that the fourth crRNA exhibited the highest fluorescence intensity (Fig. 1-B). Subsequently, the concentrations of Cas12a protein and crRNA were further optimized. It was determined that the fluorescence intensity reached its peak when the concentration of Cas12a protein was 1,000 nmol L^{-1} (Fig. 1-C) and the concentration of crRNA was 150 nmol L^{-1} (Fig. 1-D). In the next step, we systematically optimized RT-RAA amplification conditions, evaluating temperatures of 37°C, 39°C, and 42°C, and reaction times of 10, 20, and 30 min. The highest amplification efficiency was achieved at 42°C (Fig. 1-E), with significantly higher efficiency at 20 and 30 min compared to 10 min (Fig. 1-F). A 20-min amplification time was selected for subsequent experiments to meet rapid detection requirements.

Next, the sensitivity of the one-pot assay was evaluated using 10-fold serially diluted PDCoV *N* gene plasmids (ranging from 3.9×10^{4} to 10^{-1} copies μL^{-1}). The results revealed that the detection limit of the one-pot assay was as low as 3.9 copies μL^{-1} of the positive plasmid (Fig. 1-G). Subsequently, the sensitivity of this assay was further assessed using 2-fold serial dilutions of the PDCoV CC23 strain (initial concentration of $10^{3.8}$ $TCID_{50}$/0.1 mL) as detection templates. The assay successfully detected the virus at a concentration of $10^{0.8}$ $TCID_{50}$/0.1 mL (Fig. 1-H), demonstrating a sensitivity approximately twice that of RT-qPCR (Appendix A). These findings collectively indicate that the one-pot assay possesses high sensitivity for PDCoV detection.

To assess the specificity of our one-pot assay, we conducted tests using viral RNA from porcine hemagglutinating encephalomyelitis virus (PHEV), nucleic acids from pseudorabies virus (PRV), porcine circovirus Type 2 (PCV2), and porcine circovirus Type 3 (PCV3), as well as positive plasmid and PDCoV RNA. The results demonstrated that only the positive plasmid and PDCoV RNA groups generated significant fluorescence signals, whereas no fluorescence was detected in the other groups or the negative control group (Fig. 1-I). Furthermore, we simulated co-infection scenarios by combining PDCoV RNA with nucleic acids from other viruses to evaluate the anti-interference performance of our assay. The data revealed that no significant difference between the detection outcomes of the mixed samples and those of PDCoV RNA alone (Fig. 1-J). These findings confirm that RT-RAA/CRISPR/Cas12a one-pot assay is highly specific and can effectively respond to the detection of PDCoV in the case of multiple virus co-infection.

To evaluate the clinical applicability of our assay, we collected 80 fecal or intestinal content samples from diarrheic pigs and tested them using both RT-RAA CRISPR/Cas12a (Fig. 1-K) and RT-qPCR (Fig. 1-L) methods. The results indicated that 31 clinical samples tested positive by both techniques, yielding a 100% concordance rate between the two methods. These findings demonstrate that the RT-RAA/CRISPR/Cas12a one-pot assay is suitable for rapid clinical detection. In veterinary clinical practice, multiple viral co-infections are a common cause of pig diarrhea.Therefore, developing a CRISPR/Cas12a-based system capable of identifying these mixed infections will be a crucial direction for future research.

In summary, we have developed a one-pot visual assay that combines RT-RAA amplification with CRISPR/Cas12a system for highly sensitive and specific detection of PDCoV infection. The entire detection process can be completed within 40 min. This assay is performed in a closed system, thereby effectively minimizing the risk of false positives caused by aerosol contamination during lid opening. Given its exceptional clinical performance, this method holds promise as a valuable tool for the early diagnosis of PDCoV infections, facilitating effective prevention and control of PDCoV outbreaks.

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Table 1 Oligonucleotide sequences used in this study

Name	Sequence (5´ to 3´)
crRNA-F	GAAATTAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGAT
crRNA-1R	ACCAGTACGAGACCGGTTGCATCTACACTTAGTAGAAATTA
crRNA-2R	GACATGTCCCTGGTGTTCAGATCTACACTTAGTAGAAATTA
crRNA-3R	AAGGGTAATTGCCCCTGCCTATCTACACTTAGTAGAAATTA
crRNA-4R	GACGCGTCTTGGTTTCAGGTGCTATCTACACTTAGTAGAAATTA
ssDNA reporter	FAM-TTATT-BHQ1
RAA-F	GGCTGCACCAGTGGTCCCTACTACTGAC
RAA-R	GTCTGGTGTAACGCAGCCAGTAGCCATG
RT-qPCR-F	ACAAGAAGCCTGACAAACAAGACC
RT-qPCR-R	AGCATCATCCCACTCCCAATCC

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 Youdao Dictionary 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

This research did not involve animal ethics experiments.

Data availability

Data will be made available on request.

Appendix associated with this paper is available at https://doi.org/10.1016/j.jia.2025.07.030

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