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Development of a recombinase polymerase amplification isothermal assay for rapid visual and lateral flow detection of porcine parvovirus-7

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Abstract

Porcine parvoviruses (PPVs) are globally recognized as significant contributors to reproductive failure in swine, primarily due to their association with fetal death. Infection in pregnant sows can lead to severe reproductive disorders including stillbirth, mummification, embryonic death and infertility. A recombinase polymerase amplification assay targeting the variable region of the outer capsid protein gene of the PPV-7 genome was developed and systematically optimized under a range of reaction conditions. The assay showed optimal amplification at a constant temperature of 35.00 ˚C for 25 min, using 0.72 µM of each forward and reverse primer and 14.00 mM magnesium acetate. It demonstrated high sensitivity, reliably detecting as few as 2,050 copies of viral nucleic acids in both the conventional and fluorescent dye-based formats. The assay also showed high specificity, exhibiting no cross-reactivity with other common porcine pathogens such as porcine sapelovirus, porcine circovirus and classical swine fever virus. Of the 167 field samples tested, 23 were positive for PPV-7, corresponding to a positivity rate of 13.77%. Operating at a low and constant temperature, the assay eliminates the need for advanced laboratory equipment, making it highly suitable for pen-side application in field settings. In conclusion, this novel assay demonstrated strong potential for field-based detection of PPV-7 circulating within the swine population of Haryana, India, marking the first report of its kind from this region. Further validation using samples from clinically affected herds will strengthen its diagnostic applicability.

Introduction

Among livestock species, pigs play a crucial role, particularly for socio-economically disadvantaged communities. Piggery not only serves as a potent source of meat production but also offers significant potential for rapid economic returns to farmers. The success of swine production hinges largely on preventing infectious diseases that can negatively impact reproductive performance. Over the past two decades, several notable swine viruses have emerged in Asia, causing significant economic losses. These include the porcine reproductive and respiratory syndrome virus, porcine epidemic diarrhoea virus, foot-and-mouth disease virus, African swine fever virus, porcine parvovirus (PPV), porcine circovirus (PCV) and classical swine fever virus (CSFV).[1] The PPV-7, a member of the family *Parvoviridae, subfamily Hamaparvovirinae* and genus *Chapparvovirus*, is an autonomous virus characterized by its negative, single-stranded DNA genome of approximately 5.00 kb.[2,3] This genome contains two open reading frames and features complex palindromic hairpin structures at both terminal sequences, typically ranging from 120 to 200 bases.[4] The PPV-7 encodes two non-structural proteins, NS1 and NS2, transcribed from promoter P4 and two structural proteins, VP1 and VP2, from promoter P40. The major structural protein, VP2, serves as the primary target for neutralizing antibodies.[1] The genetic determinants of virulence are confined to the structural proteins with low pathogenic strains (e.g., NADL-2 and MSV) being less capable of crossing the placental barrier compared to more virulent strains.[5]

Laboratory diagnosis of PPV typically involves cell culture isolation and genome characterization using established cell lines such as Embryonic Swine Kidney (ESK), Porcine Kidney 15 (PK-15), Swine Kidney 6 (SK6), Swine Testicle Epithelial (STE) and Suspension Porcine Embryonic Kidney (SPEV).[6] Viral isolation is often

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complemented by immunofluorescence microscopy to distinguish PPV from other viral infections.[7] The hemagglutination inhibition test is a standard technique for quantifying PPV-specific antibodies. Notably, antibody titers induced by commercial vaccines tend to differ significantly from those resulting from natural infections. In the hemagglutination inhibition test, vaccine-induced antibody titers are usually below 1:512,[8] while antibody titers from field infections often exceed 1:2,000.[9] Other serological techniques, including serum neutralization, modified direct complement fixation and enzyme-linked immunosorbent assay (ELISA) are also commonly employed to detect PPV-specific antibodies. Sensitive nucleic acid detection methods offer precise information on both the presence and quantity of viral pathogens. Nested PCR systems, for instance have been developed for diagnosing various swine diseases by amplifying specific genomic regions such as the *VP2 capsid (Cap)* gene of PPV and the hexon of porcine adenoviruses. However, conventional PCR and qPCR assays are not well-suited for field applications because they require sophisticated laboratory infrastructure, including thermal cyclers, calibrated power sources and temperature-controlled environments. In addition, these assays demand trained personnel and stringent sample handling conditions to avoid contamination making them impractical for on-site or resource-limited diagnostic settings.

Recombinase polymerase amplification (RPA) is a novel isothermal amplification technique that offers a user-friendly, rapid and highly sensitive and specific alternative for pathogen detection, making it suitable for field diagnostics. The RPA can simultaneously process multiple samples and requires only a single pair of primers, eliminating the need for an initial denaturation step typically required in other amplification methods.[10-12] Yang *et al.* successfully developed an RPA assay targeting the *PPV NS1* gene, employing both real-time fluorescence detection (PPV real-time RPA) and lateral flow (LF) dipstick visualization (PPV RPA-Lateral Flow Device). Their assay demonstrated high sensitivity, detecting as few as 400 copies per reaction within 20 min at a constant temperature.[13] Given these advantages, this study aimed to develop a robust isothermal RPA assay for accurate detection of PPV-7, facilitating timely and effective disease management in swine populations.

Materials and Methods

Collection of samples and viral DNA extraction. Between March 2021 and October 2021, a total number of 167 field samples, comprising fecal samples and nasal swabs, were collected from both sick and healthy pigs of various age groups across organized and unorganized farms in Hisar (29.1492° N, 75.7217° E), Bhiwani (28.7975° N, 76.1322° E), Sirsa (29.5321° N, 75.0318° E) and Jhajjar (28.6055° N, 76.6538° E; Table 1 Supplementary data). Pigs were categorized as 'sick' based on observable clinical signs such as diarrhoea, anorexia, fever, lethargy, nasal discharge, vaginal discharge and poor body condition. These animals were identified during routine health monitoring and sampling visits. Faecal samples were collected in sterile containers and nasal swabs were obtained using sterile swabs. All specimens were transported under cold conditions and stored at – 20.00 ˚C until further processing. As the molecular analyses were conducted within a short period following sample collection, storage at – 20.00 ˚C was sufficient to preserve DNA integrity. However, for long-term storage or biobanking purposes, – 80.00 ˚C is recommended to ensure maximal stability of viral nucleic acids. The ethical committee approval was taken prior to sample collection. The animal experiment was conducted as per guidelines approved by the Institutional Animal Ethics Committee, registered as 1669/GO/ReBiBt/S/12/CPCSEA dated 6.12.2012 in 25th meeting held on 28th October, 2022. To prepare the fecal samples for analysis, large debris was removed by diluting the samples in 10.00% w/v phosphate-buffered saline, followed by vortexing and centrifugation at 12,000 ×*g* for 10 min. The processed samples were then frozen at – 20 .00 ˚C for future use. The DNA isolation was conducted using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, Irvine, USA), and a conventional method involving phenol-chloroform extraction was also performed for comparison. The Quick-DNA™ Miniprep Plus Kit was used to obtain high-purity DNA suitable for sensitive downstream molecular assays, while the phenol–chloroform method was included to validate extraction efficiency and serve as a cost-effective alternative for resource-limited settings. The isolated DNA was quantified and assessed for purity using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Waltham, USA).

Design of primers and probes. For the development of RPA assay, the primers and probe were designed targeting the outer variable Cap protein gene of the PPV-7 genome resulting in an amplicon of 110 bp. The sequence of primers and probes are given in Table 1. The probes were modified with a 5-Carboxyfluorescein (5'-FAM) at the 5'end, a polymerase extension blocking group the C3 phosphoramidite spacer at the 3' end and an internal abasic nucleotide analogue (a tetrahydrofuran residue (THF) – sometimes referred to as a 'dSpacer') which replaces a nucleotide. The reverse primer is biotin labelled at its 5' end. The primers and probes were got synthesized from Eurofins Scientific (Luxembourg, Luxembourg) and Integrated DNA Technology (Coralville, USA) and were high-performance liquid chromatography purified.

Gene construct. A synthetic gene construct having variable outer Cap protein gene region of PPV-7 genome in pUC57 vector was obtained from Bio Basic Inc. (Markham,

Canada). This recombinant plasmid was used as positive control for the development of the assay. The plasmid was transformed in *Escherichia coli* DH5 alpha cells for further multiplication. The plasmid extraction was done using Monarch Plasmid Miniprep kit (New England Biolabs, Ipswich, USA) as per manufacturer's protocol. The concentration and quality of plasmid DNA was determined using Nanodrop 2000 spectrophotometer.

Recombinase polymerase amplification assay optimization for PPV-7. The RPA assay was developed using the TwistAmp™ Basic Kit (TwistDx Inc., Maidenhead, UK), which included a rehydration buffer, an enzyme pellet and magnesium acetate (MgOAc). To initiate the assay, the enzyme pellet was dissolved in the rehydration buffer and MgOAc was added as the final step to start the reaction. Self-designed primers targeting the PPV-7 genome were employed, utilizing a recombinant plasmid as a positive control template. The reaction was conducted in a total volume of 50.00 µL, comprising 29.50 µL of rehydration buffer, 2.40 µL each of RPA forward and reverse primers (10.00 µM), 13.20 µL of nuclease-free water, and DNA template. An initial addition of 2.50 µL of MgOAc (14.00 mM) was necessary to initiate the reaction. To minimize the risk of cross-contamination, all RPA reactions were prepared and amplified using a unidirectional workflow with physically separated areas for reagent preparation, template addition and post-amplification analysis. Aerosol-resistant filter tips were used throughout, and all work surfaces and pipettes were routinely decontaminated with 70.00% ethanol and ultraviolet exposure. Each batch of reactions included a no-template control and an extraction negative control processed in parallel to monitor contamination. Following assay development, key reaction parameters including temperature, incubation time, primer concentration, and MgOAc concentration were systematically optimized. The optimization of the RPA assay was guided by the relative band intensities observed after agarose gel electrophoresis, which served as the primary indicator of amplification efficiency. The optimal reaction temperature was determined by evaluating temperatures from 35.00 to 45.00 at 2.00 ˚C increments over a 20-min incubation period using a known concentration of plasmid DNA as the template. The optimal reaction time was evaluated by conducting the assay for 5, 10, 15, 20, 25, and 30 min at the previously determined optimal temperature. Primer concentrations were optimized using a checkerboard titration approach, in which forward and reverse primers were tested across 16 combinations ranging from 0.24 to 0.96 µM. To further enhance amplification performance, MgOAc concentrations of 10.00, 12.00, 14.00, 16.00, 18.00, and 20.00 mM were systematically assessed. A detailed summary of reagent compositions, concentrations and total reaction volumes for the RPA, LF-RPA, and PCR assays is presented in Table 2 to ensure clarity and reproducibility.

Analytical sensitivity evaluation. The analytical sensitivity of the assay was assessed using tenfold serial dilutions of the positive control, ranging from 1.00×10^{-1} to 1.00×10^{-10}. The limit of detection (LOD) was established by calculating the plasmid copy number corresponding to the lowest dilution detectable by the assay. Sensitivity experiments were performed in triplicate to assess the assay repeatability and reproducibility. For each dilution, the mean and standard

Table 1. Details of primers and probes used for recombinase polymerase amplification (RPA) assay of porcine parvovirus (PPV)-7.

Primers	Modification	Primer orientation	Sequence (5'-3')	Product size (bp)
PPV7/Cap/3563-3584F	None	Forward primer	ATTAGGATGGCCAGGYACMGAA	
PPV7/Cap/3589-3636P	FAM	Probe	CAACACCAAAATATCCACCCATGAATAACT TCAHCAAGGGAATACCAC	110
PPV7/Cap/3673-3653R	Biotin	Reverse Primer	GTGACTGTTTTACYAKGGTT	

Cap: Capsid.

Table 2. Reagent compositions and concentrations for different assay types.

Assay type	Total volume (µL)	Rehydration buffer (µL)	Primer concentration (µM)	Probe concentration (µM)	MgOAc (mM)	Template (µL)	Remarks
Basic RPA	50.00	29.50	0.72 (each)	-	14.00	1.00	Optimized for 35.00 ˚C, 25 min
LF-RPA	50.00	29.50	0.72 (each)	0.12	14.00	1.00	Detection via Milenia HybriDetect strip
PCR	25.00	-	0.40 (each)	-	-	2.00	Thermal cycling with optimized parameters (54.00 ˚C annealing)

LF-RPA: Lateral flow-recombinase polymerase amplification.

deviation (SD) from three independent runs were calculated and reported as mean ± SD (Graphpad prism software version 8.0; Boston, USA). Statistical comparisons between replicates were conducted using a two-tailed Student's *t*-test, with $p < 0.05$ considered statistically significant. The plasmid copy number was derived from the measured DNA concentration using the following formula.

$$Copies\ per\ \mu L = \frac{DNA\ concentration\ (ng\ \mu L^{-1}) \times 10^{-9}}{amplicon\ length \times 660} \times 6.022 \times 10^{23}$$

Standardization of RPA assay in dye format. A DNA binding dye (PicoGreen®; Invitrogen, Waltham, USA) was used for the calorimetric detection of RPA amplicons. One microliter of the dye (1:10 dilution in nuclease-free water) was added to 10.00µL of the RPA reaction product and fluorescence was observed under an ultraviolet transilluminator.

Development of LF RPA assay. TwistAmp® nfo probe kit (TwistDX Ltd., Maidenhead, UK) or AmplifyRP® isothermal amplification kit (Agdia Inc., Elkhart, USA) were used initially for RPA reactions and detection using LF methods. The designed probe contained the target sequence and the reverse primer, in the opposite orientation to the probe, was biotin-labeled at the 5′ end. The probe contained FAM at 5' and THF site with C3-spacer at 3' end. For LF detection, the RPA reaction was prepared according to optimized parameters. The reaction mixture (total volume 50.00 µL) contained 29.50 µL of rehydration buffer, 2.40 µL each of forward and reverse primers (10.00 µM), 13.20 µL of nuclease-free water, 2.50 µL of MgOAc (14.00 mM), and 1.00 µL of DNA template. For LF visualization, 10.00 µL of the amplified product was applied to the HybriDetect test strip following manufacturer's instructions. Milenia strips from Milenia Gen Line Hybri Detect (MileniaBiotec GmbH, GienBen, Germany) were used for LF assays. For each sample to be analyzed, 40.00 µL Hybri Detect Assay Buffer was mixed with 10.00 µL of the amplified product in the reaction tube. The sample solution was poured on the sample application area and incubated for 5-15 min. At the end of incubation period, dipsticks were checked for development of control and test line and results were interpreted immediately.

Analytical specificity evaluation. To assess the specificity, the assay was tested using purified nucleic acids from other viruses related to the target pathogen, such as porcine sapelovirus (PSV), PCV and CSFV. All specificity assays included appropriate negative and extraction controls to verify the absence of contamination during nucleic acid handling and reaction setup.

Evaluation of developed RPA assay. The developed assay was evaluated using 167 field samples, comprising fecal samples and nasal swabs collected from both healthy and clinically affected pigs in organized and unorganized farms across Haryana (29.0588° N, 76.0856° E). Each sample was tested using the optimized RPA assay and conventional PCR, which served as the reference method. For PCR, the same primer set was used at a concentration of 0.40 µM (forward and reverse), and 2.00 µL of DNA extracted from each field sample served as the template. A previously confirmed PPV-7-positive plasmid was included as the positive control, while a no-template control and an extraction negative control were incorporated into each run to monitor potential contamination. The PCR conditions were optimized as follows: Initial denaturation at 95.00 ˚C for 2 min; 40 cycles of denaturation at 95.00 ˚C for 1 min, annealing at 54.00 ˚C for 30 sec, and extension at 72.00 ˚C for 30 sec, followed by a final extension at 72.00 ˚C for 10 min. Amplified products were resolved on a 1.50% agarose gel stained with ethidium bromide and visualized under ultra-violet illumination.

Results

Extraction of DNA. The DNA was extracted using the Quick-DNA™ Miniprep Plus Kit and by a conventional phenol–chloroform extraction method. The concentration and purity of the extracted DNA were quantified spectrophotometrically. DNA obtained using the kit-based method yielded a concentration of 25.00 ngµL^{-1} with high purity (A_{260}/A_{280} = 1.78), whereas, the phenol–chloroform method produced a higher DNA concentration of 102 ngµL^{-1} but with lower purity (A_{260}/A_{280} = 1.55). The reaction conditions for the RPA assay were optimized at 35.00 ˚C for 20 min. Although the RPA reaction was effective across a temperature range of 35.00 ˚C to 45.00 ˚C, there was an increase in non-specific amplification with rising temperatures. Consequently, the lowest temperature that still yielded amplification at 35.00 ˚C was determined to be the optimal temperature for the PPV-7 RPA assay, based on band intensity (Fig. 1).

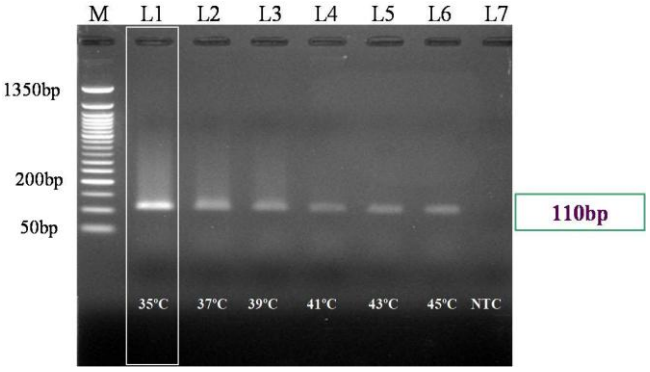

Fig. 1. Agarose gel electrophoresis of porcine parvovirus-7 recombinase polymerase amplification (RPA) amplified product at variable temperatures. Lane M: 50bp DNA marker; Lane 7: Non template control; L1, L2, L3, L4, L5, L6: RPA amplified product at temperature 35.00, 37.00, 39.00, 41.00, 43.00 and 45.00 ˚C. Optimized temperature is 35.00 ˚C in L1.

To optimize the incubation time, the RPA reaction was evaluated at several intervals: 5, 10, 15, 20, 25, and 30 min, all conducted at 35.00 ˚C. The assay demonstrated amplification across all six-time frames, however, the combination of the lowest temperature and the highest amplification intensity was observed at 25 min. Thus, 25 min was selected as the optimal reaction time (Fig. 2).

In terms of primer concentration, a combination of 0.72 µM each of the forward and reverse primers produced amplicons with significant intensity, making this pair the optimal choice for the assay (Fig. 3). The concentration of MgOAc was also optimized, tested between 10.00 and 20.00 mM. Amplification was observed across all concentrations; however, the best intensity was achieved at 14.00 mM, which aligned with the recommendations provided in the kit literature (Fig. 4). Therefore, MgOAc concentration of 14.00 mM was established as the optimal level for the RPA assay.

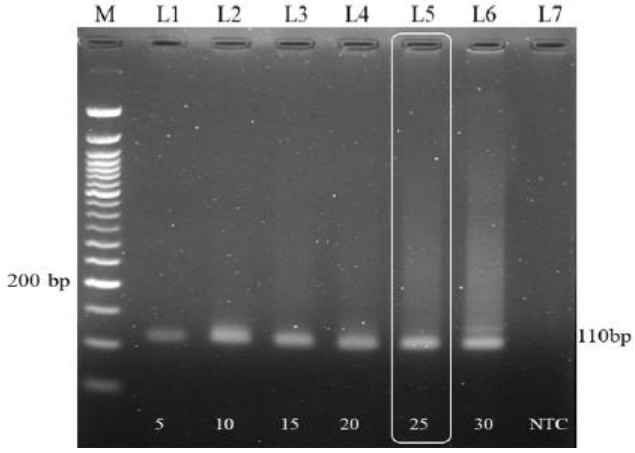

Fig. 2. Agarose gel electrophoresis of PPV-7 RPA amplified plasmid for variable time. Lane M: 50 bp DNA marker; Lane 7: Non template control; Lanes L1-L6: RPA amplified product at variable time 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min. Optimized time was 25 min in L5.

Analytical sensitivity of RPA assay. The analytical sensitivity of the assay was evaluated using 10 fold serial dilutions of the positive control (plasmid DNA), ranging from 1.00×10^{-1} to 1.00×10^{-10}. The results showed detectable amplicons up to the ninth dilution, detecting approximately 2,050 ± 125 copies per reaction (Fig. 5). Each dilution was tested in triplicate and mean ± SD values were calculated to determine the assay LOD. Statistical comparison across replicates showed no significant variation ($p > 0.05$), confirming the reproducibility of the assay. In contrast, the non-template control exhibited no false positive result. The RPA assay showed high repeatability and reproducibility, with consistent amplification across replicates. Intra- and inter-assay evaluations, performed using three dilutions near the detection limit, exhibited a coefficient of variation below 5.00%, confirming the assay precision and robustness.

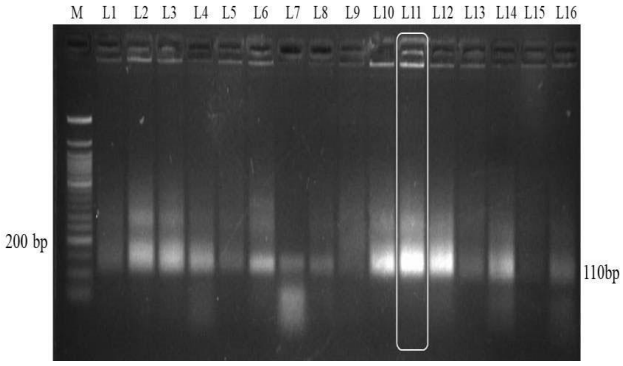

Fig. 3. Agarose gel electrophoresis of porcine parvovirus PPV-7 recombinase polymerase amplification (RPA) amplified product for forward and reverse primer concentration (0.24 µM to 0.96 µM). Final optimized primer concentration for RPA reaction is 15F15R *i.e.* 0.72 µM of F primer and 0.72 µM of R primer. Lane M: 50 bp DNA marker; L1: 5F5R, L2: 10F5R, L3: 15F5R, L4: 20F5R, L5: 5F10R, L6: 10F10R, L7: 15F10R, L8: 20F10R, L9: 5F15R, L10: 10F15R, L11: 15F15R, L12: 20F15R, L13: 5F20R, L14: 10F20R, L15: 15F20R, L16: 20F20R.

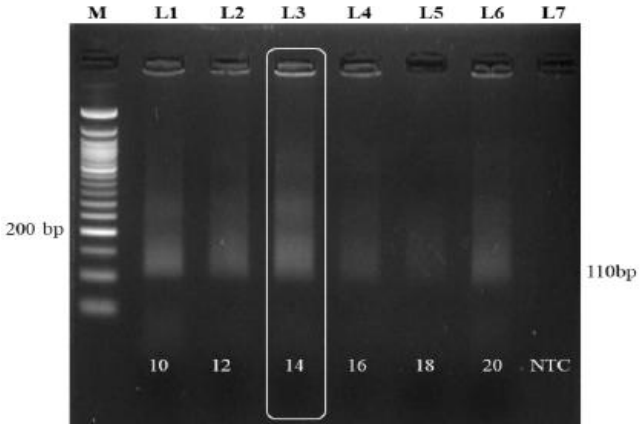

Fig. 4. Agarose gel electrophoresis of PPV-7 RPA amplified product with variable MgOAc concentration. Lane M: 50 bp DNA marker; Lane 7: Non template control; Lanes 1-6: RPA amplified gene product with different concentration of MgOAc (10 mM, 12 mM, 14 mM, 16 mM, 18 mM, and 20 mM). Optimum MgOAc concentration 14mM.

Visual RPA assay. The assay was visually detected using different dilutions of plasmid DNA (ranging from 1.00×10^{-1} to 1.00×10^{-10}) with PicoGreen dye. A negative result appeared colourless, while a positive result exhibited green fluorescence. In terms of sensitivity with the visual assay, the green fluorescence was detectable up to ninth dilution and thus the LOD was up to 2,050 copies similar to the conventional format. The specificity was also assessed in visual RPA by testing the related porcine viruses *viz*. CSFV, PSV, PCV using developed assay. There was no change in colour with other related viruses confirming that the RPA assay is PPV-7 specific (Fig. 6).

Analytical specificity of RPA assay. To evaluate the specificity of PPV-7 specific RPA assays testing was conducted with various related porcine viruses, including

CSFV, PSV and PCV. Amplification was detected solely with the PPV-7 positive control, while no amplification was observed with the nucleic acids derived from the other viruses (Fig. 7).

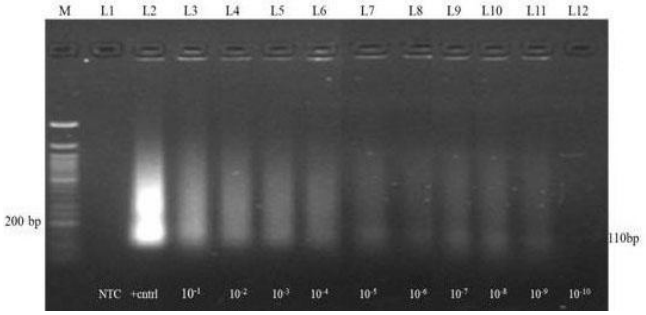

Fig. 5. Agarose gel electrophoresis of recombinase polymerase amplification (RPA) amplified product with different dilutions of recombinant plasmid DNA. Lane M: 50 bp DNA marker; Lane 1: Non template control; Lane 2: positive control; Lanes 3-12: RPA amplified gene product with different dilutions (1.00×10^{-1} - 1.00×10^{-9}).

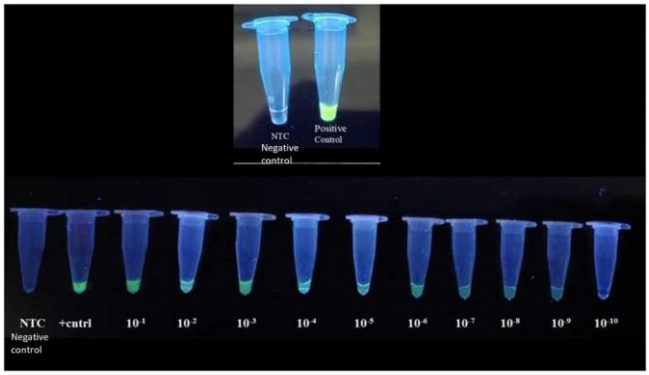

Fig. 6. Visual detection of recombinase polymerase amplification product with different dilutions of plasmid DNA (1.00×10^{-1} - 1.00×10^{-10}) with PicoGreen® dye. Negative is colorless, positive is green and with the increase in dilution green fluorescence is decreasing. The green fluorescence was detectable up to ninth dilution and therefore the LOD was up to 2,050 copies. NTC: Non template control.

Detection using LF dipsticks. The end point detection of RPA was also performed using LF dipsticks. In the negative reaction only control line was visible, while in a positive reaction a test line along with a control line was developed.

Evaluation of the developed assay. The developed RPA assay was validated using 167 field samples collected from various piggery farms of Haryana. Out of 167 samples tested, 23 samples were found positive for PPV-7 depicting a positivity rate of 13.77% (23/167). The samples were also tested using the RPA primers in conventional PCR. The result of PCR testing showed a positivity rate of 11.97% (20/167). A total number of three samples yielded positive results in the RPA assay but were negative by conventional PCR, indicating potential false negatives in PCR and underscoring the superior sensitivity of the RPA assay. There were no samples which were positive by PCR but not detected by RPA assay. To assess the degree of agreement between RPA and conventional PCR results, Cohen's kappa statistic was calculated. The kappa value ($\kappa = 0.92$) indicated an almost perfect agreement between the two assays, demonstrating the reliability and diagnostic consistency of the developed RPA method.

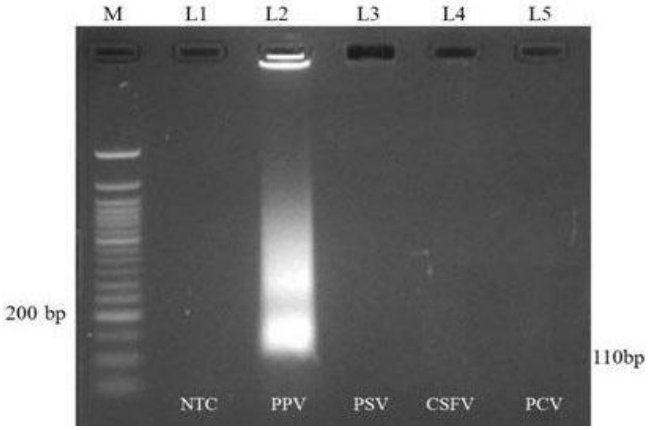

Fig. 7. Agarose gel electrophoresis of recombinase polymerase amplification (RPA) amplified product with different porcine viruses. Lane M: 50 bp DNA marker; Lane 1: Non template control; Lanes 2-5: RPA amplified gene product with nucleic acid from viruses including porcine parvovirus (PPV)-7, porcine sapelovirus (PSV), classical swine fever virus (CSFV) and porcine circovirus (PCV). Amplification was only observed with PPV-7 DNA (Lane 2).

Discussion

Porcine parvovirus 7 is a newly discovered virus belonging to the *Parvoviridae* family, first identified in China in 2017. This virus is associated with reproductive failures in pigs, leading to significant economic losses in the swine industry.[14] Timely and effective diagnosis is critical to mitigating the economic impact of this disease. Various diagnostic methods for PPV have been developed, including serological tests such as ELISA,[15] and indirect immunofluorescence assays for detecting PPV-7 antibodies in serum samples. However, these methods have certain limitations.

Nucleic acid-based molecular techniques, such as conventional PCR, nested PCR and real-time PCR using SYBR Green dye have been developed for the confirmatory diagnosis of PPV.[16-18] Unfortunately, these techniques are often impractical for field conditions due to their reliance on complex equipment and laboratory setups. To address these challenges and facilitate on-site testing for the rapid identification of PPV, an isothermal amplification method known as RPA was developed. Since its introduction in 2006, RPA has gained popularity for detecting both RNA and DNA, offering high specificity and sensitivity, and is compatible with multiplexing.[19,20]

The primers designed for this assay specifically targeted the variable Cap protein gene region of the PPV-7 genome, a region that has been utilized in various studies for detecting PPV-7.[21-23] The variable region of the *Cap* gene was selected as the assay target because it encodes the major antigenic protein responsible for eliciting neutralizing antibodies and displays sufficient sequence variability to enable specific detection of PPV-7 field strains.[23] Previous studies have also highlighted the immunological and diagnostic relevance of this gene. The RPA assay was optimized for several parameters, including temperature, incubation time, primer concentration and MgOAc concentration. The reaction temperature is a crucial factor affecting RPA efficiency and sensitivity with the optimal range typically between 37.00 and 42.00 ˚C. Some studies have indicated that RPA can function efficiently even at temperatures as low as 25.00 ˚C.[13,24,25] In our study, effective amplification was achieved in a temperature range of 35.00 to 45.00 ˚C, with 35.00 ˚C being selected as the optimal temperature to minimize non-specific amplification while ensuring ease of operation.

Another critical factor in RPA efficiency is primer concentration. Higher concentrations can lead to non-specific amplification, while lower concentrations may compromise assay sensitivity.[26] For our developed RPA assay, we optimized the primer concentrations to 0.72 μM for both the forward and reverse primers. Adding MgOAc is essential for initiating the RPA reaction, so optimizing its concentration and the timing of its addition to the reaction mixture is crucial.

The method of mixing the reaction components such as vortexing or gentle pipetting is also important for achieving efficient and rapid amplification of target DNA due to the viscous nature of the reaction mixture. The incubation time required for the RPA reaction is often very short, with results detectable within 30 min. Some studies have reported successful amplification in less than 5 min.[27] In our optimization, a 25-min incubation time yielded the most intense amplicon bands.

The specificity of the RPA assay was thoroughly analyzed, confirming that it is specific for PPV-7 and does not cross-react with other related porcine viruses, including PCV, porcine sapelovirus, and CSFV, all of which can exhibit clinical signs similar to PPV infections. *In silico* analysis, examining genome sequences of other swine-infecting viruses such as CSFV, foot-and-mouth disease virus, PCV type 1, porcine reproductive and respiratory syndrome virus, porcine teschovirus, encephalomyocarditis virus, porcine enterovirus B, transmissible gastroenteritis virus, and porcine epidemic diarrhea virus showed no cross-reactivity affirming the specificity of the primers and probes.

For validation of the developed RPA assay, a total number of 167 nasal and faecal samples were collected from pigs of various age groups from organized farms of Haryana. None of the samples were collected from pigs showing stillbirth, mummification, embryonic death and infertility symptoms. Although PPV-7 has been detected in pigs showing various clinical conditions, including diarrhoea and reproductive disorders, conclusive evidence linking its presence to overt disease remains lacking.[28] The current study was limited to molecular detection and did not involve pathological or serological correlation. Therefore, the diagnostic findings should be interpreted cautiously, and future investigations involving clinical, histopathological and serological data are warranted to elucidate the pathogenic significance of PPV-7. Out of these, 23 samples (13.77%) tested positive for PPV-7 using the developed RPA assay targeting the cap region of the PPV-7 genome. The high concordance between the two assays (Cohen's $\kappa = 0.92$) further supported the diagnostic robustness of the developed RPA system and validated its suitability for field-level detection of PPV-7. The occurrence rate of PPV-7 in Haryana (13.77%) was notably higher compared to previous studies in other Indian states, which reported seropositivity rates of 7.14,[29] and 41.10% of porcine parvo virus.[30] However, both of studies did not specify the strain of circulating virus. This finding represented the first detection of PPV-7 in the state of Haryana, based on RPA/PCR results indicating its circulation within the local pig population. As all samples in this study were collected exclusively from pig farms located within the state of Haryana, the results primarily reflected the local epidemiological scenario of PPV-7. Therefore, these findings should be interpreted with caution when extrapolating to other geographical regions. Broader surveillance involving different states and management systems would help establish a more comprehensive picture of PPV-7 circulation in India.

To better contextualize the diagnostic utility of the developed RPA assay, it is essential to compare it with other isothermal amplification platforms commonly applied for swine pathogen detection, particularly loop-mediated isothermal amplification (LAMP). Compared to LAMP, which typically requires 30 - 60 min and relies on multiple primers the PPV-7 RPA assay produces results in less than half an hour using only a single primer pair, greatly simplifying assay design and optimization. While LAMP is generally more economical due to the use of low-cost enzymes like Bst polymerase, the higher cost of RPA reagents is balanced by its superior speed, minimal equipment requirements and operational simplicity at the point of care. Collectively, these advantages make the developed RPA assay a more practical and efficient option for rapid field-level detection of PPV-7.

The present study demonstrated that the developed RPA assay for PPV-7 was rapid, sensitive, specific, and operationally simple. Its minimal equipment requirements and ease of use made it highly suitable for field deployment and point-of-care diagnostics. The assay

performance showed strong agreement with conventional PCR, although validation using larger sample sets would further strengthen these findings. The inclusion of both dye-based visualization and lateral-flow probe detection enhanced its applicability under varied field conditions. Importantly, this assay enabled the first detection of PPV-7 within the swine population of Haryana, India. As the sampling was geographically limited, the results should be interpreted as region-specific and broader surveillance across diverse regions is recommended to better understand the epidemiological distribution of PPV-7.

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Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

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