Rapid and Visual Detection of the Emerging Novel Duck Reovirus by Using a Specific and Sensitive Reverse Transcription Recombinase Polymerase Amplification Method

Weiwei Wang
Institute for Poultry Science and Health, Guangxi University
Yan Zhang
Institute of Poultry Sciences and Health, Guangxi University
Yu Huang
Institute for Poultry Science and Health, Guangxi University
Guo Chen
Institute for Poultry Science and Health, Guangxi University
Mengya Shi
Institute for Poultry Science and Health, Guangxi University
Teng Huang
Institute for Poultry Science and Health, Guangxi University
Tianchao Wei
Institute for Poultry Science and Health, Guangxi University
Merlan Mo
Institute for Poultry Science and Healtry, Guangxi University
Xiumiao He
Institute for Poultry Science and Healtry, Guangxi University
Ping Wei (Pingwei8@126.com)
institute for poultry science and health, Guangxi University https://orcid.org/0000-0002-2306-1688

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Abstract

Background

Duck spleen necrosis disease (DSND) caused by Novel Duck Reovirus (NDRV), is an emerging infectious disease that causes severely threaten to duck industry. Currently, the popular conventional PCR technique for detecting NDRV is time consuming. So, it is essential to develop a rapid and accurate molecular diagnosis techniques of viral pathogens for the purpose to prevent further disease transmission or outbreaks. Recombinase polymerase Amplification (RPA) is a new generation of simple, rapid and cost-effective molecular diagnosis technology, which has been applied to the molecular detection of various pathogens.

Methods

In our study, a simple, rapid and reliable detection method was developed target NDRV by an isothermal reverse transcription-recombinase polymerase amplification (RT-RPA). The RT-RPA primers were designed based on the S3 gene of NDRV, and a series of other waterfowl-origin pathogens were detected by RT-RPA. A total of 20 field and experimental infected samples were tested by RT-RPA and compared with the results of conventional RT-PCR and quantitative RT-PCR simultaneously.

Results

The RT-RPA method proved to be repeatable and could detect as little as 3.48×10^{-6} ng/μl of the standard plasmid DNA inserted with the viral S3 gene. This was a 10 × higher sensitivity rate than that of conventional RT-PCR. The major advantage of this RT-RPA method is that it could be performed as an isothermal reaction at 37 ℃ and completed within 20 min. In addition, no cross-reactivity was detected with other waterfowl-origin viruses. Also, the amplified products could be visualized faster, without the gel electrophoresis, by adding the SYBR Green I and observing them under an ultraviolet light.

Conclusions

This newly developed RT-RPA method offers a simple, rapid and accurate for rapid detection of NDRV, which especially useful in on-site facilities and resource-limited areas.

Background

Novel duck reovirus (NDRV), an avian *Orthoreovirus*, is an emerging important viral pathogen causing Duck Spleen Necrosis Disease (DSND) and infecting a variety of duck species recently [1–4]. The emerging NDRV can cause severe damage to the immune organs, and co-infected with *Salmonella spp.* can greatly enhance its pathogenesis and further raise the risk of missed and/or mistaken diagnoses [4]. Compared with all previously reported duck reovirus (DRV) infections, NDRV infection exhibits higher virulence and widely host species [5] and the viral S3 gene have been used to distinguish NDRV from the Muscovy duck reovirus (MDRV) or broiler/layer-origin reovirus (ARV) [6, 7]. Even though vaccination against ARVs or MDRVs could reduce the risk

of the reoviruses infections, an outbreak of the emerging, highly contagious, and fatal disease caused by NDRV still brings severely threaten to duck industry [1, 8]. This especially has a profound effect on the food supply in China because about 4.43 billion meat-type ducks were produced in 2019, up an increase of 33.2% over 2018, and because ducks are one of the alternatives for the shorter supply of pork due to the outbreaks of African swine fever over the country [9]. So, it is pivotal to develop a simple, rapid and accurate on-site detection method to assist with the diagnosis and effective control of this disease for the duck industry.

Traditional NDRV diagnostic techniques, such as electron microscopy, virus isolation and immunofluorescence assay, could identify the pathogen in tissues and/or cell culture. Compared with traditional methods, reverse transcription polymerase chain reaction (RT-PCR) and quantitative PCR (qRT-PCR) techniques to detect NDRV with higher sensitivity and specificity [10–12]. RT-PCR and qRT-PCR for detecting NDRV in tissue samples requires expensive thermal cycler equipment and is time-consuming. In addition, the enzyme-linked immunosorbent assay (ELISA) have been reported to be effective in detecting NDRV [13], but it relies on high antibodies quality and specificity and easy to produce false positives. The reverse transcription loop-mediated isothermal amplification (RT-LAMP) was established to detect NDRV [14, 15], which needs six primers, high temperature reaction (60–65 ℃) and long reaction time (50–65 min) makes it difficult as an on-site facilities rapid detection method [16].

Recombinase polymerase amplification (RPA) is an emerging next-generation molecular diagnostic technique, which considered as a simple, rapid and cost-effective isothermal amplification method [17]. RPA is a considerably simpler technique and does not require a thermal cycler and could be completed at a low temperature (37 ℃ to 42 ℃) in less than 20 min in time [18, 19]. RPA has been widely applied in the clinical diagnosis of animal and plant viral including porcine circovirus-2 [20], porcine parvovirus [21], foot-and-mouth disease virus [22, 23], avian influenza [24], potato virus Y [25], maize chlorotic mottle virus [26] and apple stem grooving virus [27]. To our knowledge, the RT-RPA method for the rapid detection of NDRV in ducks has not yet been reported.

This study aims to establish and apply the RT-RPA as a reliable and alternative method for the rapid and specific detection of NDRV. Initially, the RT-RPA primers were designed and the reaction conditions were optimized for the detection of NDRV. Secondary, the sensitivity and specificity of the RT-RPA were conducted with NDRV and other common waterfowl-origin viruses. Lastly, the performance of this method was conducted with field and experimental challenged samples for the detection of NDRV. This method could provide a simple, rapid and reliable on-site detection method for NDRV in both field and experimental sample tests.

Results

RT-RPA primers evaluation

Analysis of the RT-RPA primer sequences using BLASTn indicated 100% query coverage with that of NDRV strains. No matches were detected with other waterfowl-origin viruses and bacteria. Only two reactions with the standard plasmid DNA generated amplicons of the expected size, while no amplification was detected by RPA analysis in blank controls (Fig. 1.). So, the RPA3 primer-set could be utilized in the later assays.

Temperature range, incubation time and primer concentration of the RT-RPA

Initially, we assessed a series of temperature (33–43 ℃) during incubation for 30 minutes. Amplification signals can be detected at 33, 35, 37, 39, 41 and 43 ℃, among which the amplification signal was in the highest at 37 ℃ (Fig. 2A). Then the performance of RT-RPA assay was tested at 37 ℃ with the incubations for 5, 10, 20, 30 and 40 min, respectively. Amplification signals could be detected at 37 ℃ with the incubation for 5 min, and the signal intensity increased with time increasing (Fig. 2B). However, there was no significant difference in the products yielded among the 20-, 30- and 40-min reactions, so the optimal incubation time was considered to be 20 min in the following experiments. As shown in Fig. 2C, the most suitable primer concentrations for RT-RPA assays were performed with 0.48 μM each from here onwards.

Clear DNA band was visualized by using the purified RPA products

Although DNA products directly from RPA could be visualized in agarose gels through electrophoresis, better results could be obtained by using the purified products before electrophoresis, due mainly to the presence of proteins in the RPA reaction mixtures. It has been reported that the presence of enzymes in the mixture can inhibit DNA migration in the gel [28]. Although better results could be achieved by usage of Universal DNA Purification Kit, it increased the cost of RPA-based assays. However, Jiao et al. reported that the phenol/chloroform/isoamyl alcohol- or heat-treated RPA DNA products showed stronger DNA bands in the gels when compared to those purified by using the Universal DNA Purification Kit [26].

Analytical specificity, sensitivity and repeatability

To test the specificity of the RT-RPA, the reactions were conducted with NDRV and 11 other waterfowl-origin viruses DNAs as the templates and ddH_2O as the blank control. As clearly shown in Fig. 3A, only NDRV was used as template for the positive reaction, and no other virus were amplified. The RT-RPA assay showed high sensitivity, with the detection limit is 3.48×10^{-6} ng/μl, which had 10 × higher sensitivity than that found with the conventional RT-PCR (3.48×10^{-5} ng/μl) (Fig. 3B and C). Three positive NDRV samples were randomly selected for repeatability test, indicating that the RT-RPA method was stable and repeatable (Fig. 3D).

Visualization of amplification products of RT-RPA reactions

Aliquots (5 μl) of the amplification products were analyzed respectively by 2% agarose gel electrophoresis and directly SYBR Green I staining. After the amplification reactions of RT-RPA, the tubes were spun briefly and then the SYBR Green I was added and mixed with the amplified product. At 37 ℃, 3 min incubation for RPA reaction containing positive templates turned green under the ultraviolet light, and the signal intensity increased with time increasing. And the negative sample retained its original orange color. Thus, our results indicate that the sensitivity of the SYBR Green I staining detection is the same as that of the agarose gel electrophoresis (Fig. 4.) but with much shorter time.

Performance of the RT-RPA assay on the field and experimentally infected samples

The performance of the NDRV RT-RPA for rapid detection was tested and validated with spleen samples collected from the clinical ducklings with suspected NDRV infection (n = 8) and the experimentally infected

ducklings (n = 8) and the healthy uninfected control ducklings (n = 4). As shown in Fig. 5., the NDRV RT-RPA method can 100% accurately identify all the samples. The performance of RT-RPA was consistent with the conventional RT-PCR assays (16 positive and 4 negative cases), and the results were also identical to that of the qRT-PCR. In addition, the samples collected from the field and experimentally infected ducklings were all positive while the samples from the healthy ducklings were all negative by NDRV RT-RPA assay.

Discussion

An emerging novel duck reovirus (NDRV) disease, called Duck Spleen Necrosis Disease, was recently found in China and the pathogen, distinct from the MDRV isolates previous, was identified to be a Orthoreovirus [1, 3]. Importantly, Wang et al. recently report that the emerging NDRV XT18 has extensive tissue tropism and could cause severe damage to the immune organs [3, 8]. Furthermore, the NDRV co-infection with other pathogens can result in serious duck diseases [4]. At present, with the frequent trade of agricultural products between countries or regions, the possibility of virus transmission is greatly increased. So, it is essential to develop molecular diagnosis techniques for the purpose of correct and rapid detection of viral pathogens in order to prevent further disease transmission or outbreaks.

Multiple diagnostic techniques, including RT-PCR, RT-LAMP and several serological assays, have been used to detect viruses in various samples [11, 13, 14]. Although test results obtained through RT-PCR are reliable, this technique is too costly and time-consuming for companies or persons who have limited laboratory equipment, including a thermal cycler, and can only conduct mostly field surveys [26]. RPA is a rapid and simple isothermal gene amplification method and does not require thermal cycler and high temperatures that can overcome the shortcomings of conventional PCR-based methods. At 37 ℃, the reaction can be completed within 20 minutes with only a pair of primers and a simple device such as water bath and heating block. Rapid and specific detection of NDRV using the RT-LAMP procedure has also been reported for ducklings [14, 15]. But RT-LAMP method requires high temperature (60–65 ℃), six primers and more difficult downstream analysis, such as cloning and direct sequencing, limitations that are overcome by the RT-RPA procedure. In our study, we first developed a new method for rapid detection of NDRV based on an isothermal gene amplification, with high sensitivity and specificity.

Currently, all kinds of modified RPA assay have been applied to the diseases diagnosis in the field of human and veterinary medicine, as well as the detection of pathogenic bacteria in the food safety and agriculture respectively [29]. The RT-RPA assays has some advantages over the ELISA, conventional RT-PCR and RT-LAMP currently used for detection NDRV due to its short reaction time and only require a single constant temperature (33 to 43 ℃). The RT-RPA assays was enough to produce sufficient amount of NDRV amplicon at 37 ℃ incubated for 3 min for rapid detection of the virus. On the contrary, conventional RT-PCR requires more than 95 min to complete before gel electrophoresis [11] and 60–65 min was required for RT-LAMP [14]; and two days were require for ELISA detection [13]. In addition, the high specificity and amplification of the RT-RPA method also allows for the easy and rapid visualization of the amplified products without the need for gel electrophoresis, thus making it a very simple and rapid diagnostic tool.

Compared with the conventional RT-PCR, RT-RPA assays has higher sensitivity and specificity in detecting NDRV. This method has high specificity and no cross-reactivity was detected with other waterfowl-origin

viruses. Furthermore, the detection limit of RT-RPA was 3.48×10^{-6} ng/μl with the standard plasmid DNA, which is a 10-fold higher sensitivity rate than that of the conventional RT-PCR previously reported. RT-LAMP and RT-PCR are both reliable methods for detecting NDRV with high sensitivity and specificity. However, aerosol pollution and false positives are prone to occur in RT-LAMP reactions [30]. The RPA method developed in our study did not produce this pollution and/or false positives, possibly since its reagents used are provided in a lyophilized pellet [31]. Finally, we evaluated the newly established RT-RPA method in this study by using field and experimental infected samples, and found that the test results were highly consistent those with the conventional RT-PCR and qRT-PCR assays.

Conclusions

To our knowledge, this is the first report of the newly RT-RPA method for use-friendly and reliable detection and diagnosis of NDRV infection. It is believed that the RT-RPA assay, as a next-generation gene amplification technology, is an ideal for rapid and efficient detection of NDRV especially suitable for laboratories or field facilities with limited resources.

Methods

Viruses and RNA/DNA extraction

The NDRV, MDRV, ARV, Duck Hepatitis Virus (DHAV, -1 and − 3 types), Newcastle Disease Virus (NDV), Duck Tanzuru virus (DTMUV), Duck parvovirus (DPV), Infectious Bursal Disease Viruses (IBDV), Infectious Bronchitis Virus (IBV) and Avian Metapneumovirus (aMPV) used in this study were stocked at − 80 °C in our laboratory. A total of 8 ducks suspected to be infected with NDRV were collected from the clinic (Guiliu Animal Husbandry Co., Ltd., China). Another 12 samples collected from the NDRV experimentally infected ducklings (n = 8) and non-infected ducklings (n = 4) were also used in the study (the local commercial duck hatchery). All the ducks used in the study were euthanized using the carbon dioxide method. The extraction of total RNA/DNA as noted in our previous description [4].

Primer’s design and evaluation

The primers were designed according to the manufacturer’s protocol suggested in the TwistAmp™ Basic Kit. Based on the alignment of 6 different NDRV virus sequences representing the known NDRV strains, a total of 3 primer pairs were designed to target the S3 gene. In addition, the primers NDRV-S3F/R were used to amplify the S3 gene of NDRV to construct the plasmid as the standard positive DNA sample. The specificity of the primer was assessed using the Primer-Blast program with the NCBI. The RPA primers and PCR primers were synthesized by BGI Technology Co., Ltd., Beijing, China. The sequences of all primers used in this study are shown in Table 1.

Table 1
Primers used for amplification of the conventional RT-PCR, real time RT-PCR and RT-RPA assays.

Primer pairs	Primer [a]	Sequence 5'-3'	Position [b]	Product length (bp)	Reference
NDRV-PCR1	NDRV-S3F	GCTTTTTGAGTCCTTAGCGTGCAA	1–24	1202	[4]
	NDRV-S3R	GATGAATAGGTGAGTCCCGCTAACC	1202 – 1178		
NDRV-PCR2	NDRV-S3-P1	ACCTCAGGATATCGCTGAAACT	390–411	586	[11]
	NDRV-S3-P2	CTCCATCCCTGCAGCACATGAAAAG	951–975		
NDRV-qPCR	NDRV-S3-S	GTTATCAGGGTCGGCAACGCTTA	154–176	270	[12]
	NDRV-S3-A	TGCGATTGACTCAGTTTCAGCGATA	399–423		
NDRV-RPA1	NDRV-RPA-1F	CACACACCTGACGTTATCAGGGTCGGCAACG	142–172	123	Our research
	NDRV-RPA-1R	CTGTTGATGACACTTGTGATGTGGAAAACAG	234–264		
NDRV-RPA2	NDRV-RPA-2F	CTGTTTTCCACATCACAAGTGTCATCAACAG	234–264	178	
	NDRV-RPA-2R	CACTGCGAATAGATTCAGTCCTGGTTACGATATC	430–463		
NDRV-RPA3	NDRV-RPA-3F	CTTGGATCATGATGTCGAGGACTGTTGGATGGATG	756–790	293	
	NDRV-RPA-3R	CACAGATATCCGGAAGAGAACGTCCGAACATAGTTC	1013–1048		

[a] F: forward primer; R: reverse primer.

[b] Positions in the genome segments of NDRV/GX-Y7/China/2018.

Recombinant plasmid construction

A recombinant plasmid used in the study was constructed by using the S3 gene of a Guangxi NDRV reference strain DRV/GX-Y7/2018/China (GenBank ID: MN747004-MN747013). The target gene was amplified, purified and cloned as the descriptions by our group have described previously [4]. Plasmid DNA was purified using the Endo-free Plasmid Midi Kit (Cwbio, Beijing, China) and quantified by BioDrop spectrophotometry (BioDrop,

Cambridge, England). The positive clone was verified by sequencing (BGI, Guangzhou, China) and the sequence obtained was analyzed using the BLAST nucleotide option in the GenBank database.

The optimizations of the RT-RPA primer and the reaction conditions

RT-RPA primer optimization was carry out in a 39 ℃ water bath for 30 min using the TwistAmp™ Basic Kit (TwistDx, Ltd.). Then the different reaction temperatures (33 ℃ to 43 ℃), different reaction times (5 min to 40 min) and different primer concentrations (0.32 μM to 0.48 μM) were evaluated respectively. For RT-RPA amplification, 2.0 μl of the standard plasmid DNA was mix with 2.4 μl of each NDRV-RPA primer (10 μM), 29.5 μl of rehydration buffer, 11.2 μl of ddH_2O and 2.5 μl of magnesium acetate (280 mM). RPA amplicons were purified using a Universal DNA Purification Kit (TIANGEN, Beijing, China) and aliquots (5 μl) of the purified RPA products analyzed by 2% agarose gel electrophoresis or added SYBR Green I to RT-RPA amplification products for visual detection.

Analysis of the RT-RPA products

Aliquots (5 μl) of the RT-RPA products were analyzed by 2% agarose gel electrophoresis as the routine or/and SYBR Green I staining for visual inspection by using 1.0 μl of 100-fold diluted SYBR Green I nucleic acid gel stain (Solarbio, Beijing, China) added to the reaction tube and observed for the color change. The reaction solution changed to green if RT-RPA reaction products were present, otherwise it remained orange, the color of the unreacted SYBR Green I dye.

Evaluation on the Specificity, sensitivity and repeatability of the RT-RPA

To evaluate the specificity of the established RT-RPA method, NDRV and MDRV, ARV, DHAV, NDV, DTMUV, DPV, IBDV, IBV, and aMPV were used, ddH_2O were used as a negative control. For sensitivity analysis, ten-fold serial dilutions of 10^{-1}-10^{-10} of the standard plasmid DNA (174 ng/μl) were prepared. In order to compare the sensitivity of RT-RPA method, the diluted standard plasmid DNA were also analyzed by conventional RT-PCR and qRT-PCR according to the developed detection methods, respectively [11, 12]. Three repeated tests were carried out to evaluate the reliability of the newly established RT-RPA method.

Testing of the field and experimental challenged samples

To determine the practical application of RT-RPA on-site field diagnostics, a total of 8 natural field samples suspected having NDRV infection were collected from Cherry Valley ducklings in Guangxi, China. Also, another 12 samples collected respectively from NDRV experimentally infected 8 ducklings and non-infected 4 ducklings were used. For the sake of comparison, the same samples were also subjected to NDRV detection by conventional RT-PCR and qRT-PCR assays respectively, as noted in previously described [11, 12].

Abbreviations

DSND: Duck spleen necrosis disease; NDRV:Novel Duck Reovirus; RPA:Recombinase polymerase Amplification; RT-RPA:reverse transcription-recombinase polymerase amplification; MDRV:Muscovy duck reovirus; ARV:broiler/layer-origin reovirus; RT-PCR:reverse transcription polymerase chain reaction; qRT-PCR:quantitative

PCR; ELISA:enzyme-linked immunosorbent assay; RT-LAMP:reverse transcription loop-mediated isothermal amplification; DHAV:Duck Hepatitis Virus; NDV:Newcastle Disease Virus; DTMUV:Duck Tanzuru virus; DPV:Duck parvovirus; IBDV:Infectious Bursal Disease Viruses; IBV:Infectious Bronchitis Virus; aMPV:Avian Metapneumovirus.

Declarations

Ethics approval and consent to participate

All samples collection from the field and experimental challenged in this study were approved by the Animal Welfare and the Animal Experimental Ethical Committee of Guangxi University (No. GXU2019-080).

Availability of data and materials

The datasets during in our study are available from the corresponding author upon request.

Competing interests

All the authors in this study declare that there is no potential competing interest.

Consent for publication

Not applicable.

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Authors' contributions

Conceived and designed the study: PW, XMH and WWW. Performed the study: WWW, YZ and YH.The data analysis and manuscript revision: WWW, GC, MYS, TH, TCW, MLM, XMH and PW.Wrote the manuscript: WWW.All authors read and approved the final manuscript.

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Figures

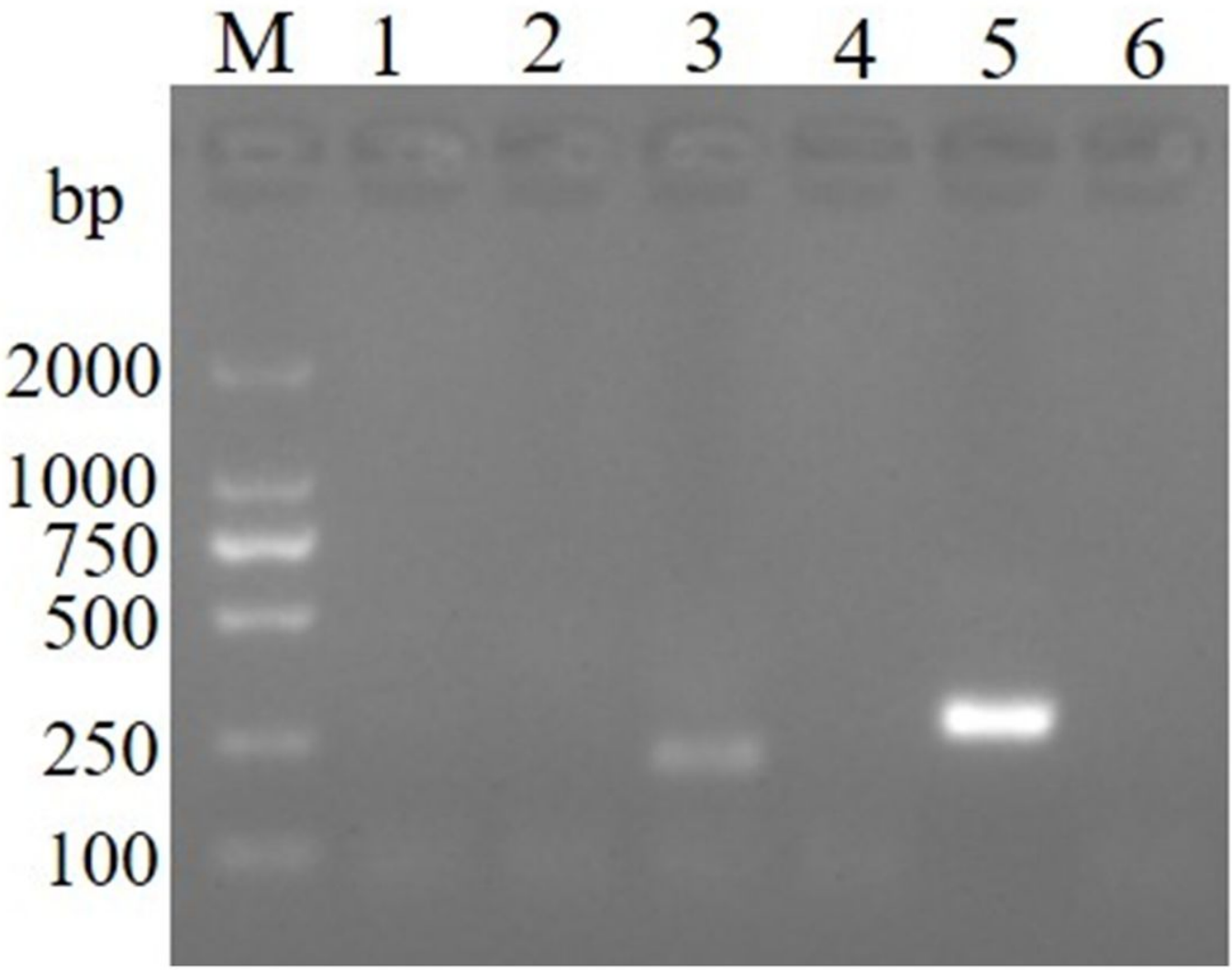

Figure 1

RPA primer optimization. Every two lines of primer sets are the same, the front line is the NDRV standard plasmid DNA and the latter line as a blank control. M: DL2000 marker. Lanes 1–2: NDRV-RPA-1F/R primer; lanes 3–4, NDRV-RPA-2F/R primer; 5–6, NDRV-RPA-3F/R primer.

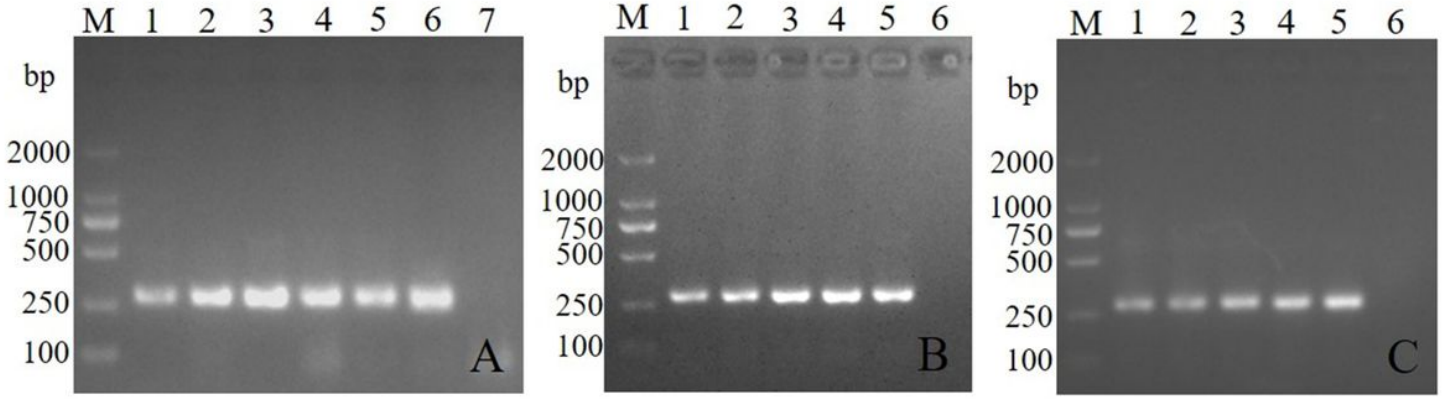

Figure 2

Optimization of RPA assay components. (A) RPA reaction temperature; M: DL2000 marker. Lanes 1–7: 33 ℃, 35 ℃, 37 ℃, 39 ℃, 41℃ and 43℃, and the negative control; (B) Optimization of the RPA reaction time; M: DL2000 marker. Lanes 1–6: the RPA reaction incubated for 5,10, 20, 30, 40 min and negative control, respectively; (C) Optimal primer concentration screening. M: DL2000 marker. Lanes 1-6: 0.32 μM, 0.36 μM, 0.4 μM, 0.44 μM, 0.48 μM and the negative control.

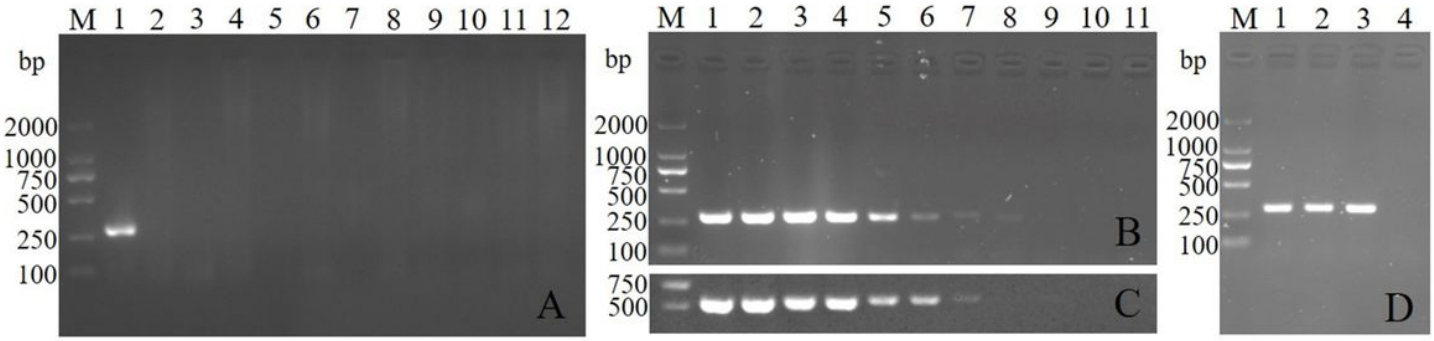

Figure 3

The specificity, sensitivity and repeatability of the RPA. (A) RPA specificity test; M: DL2000 marker. Lanes 1–12: NDRV, MDRV, ARV, DHAV-□, DHAV-□, NDV, DTMUV, DPV, MPV, IBDV and IBV, and ddH2O. (B) Sensitivity of the RT-RPA (B) and RT-PCR (C); M: DL2000 marker. 10-fold gradient dilution of the DNA samples (10-1–10-10, lanes 1–10). Lane 11: negative control. (D) Repeatability of the RPA assay. M: DL2000 marker. Lanes 1-3: NDRV standard plasmid DNA, Lanes 4: the negative control.

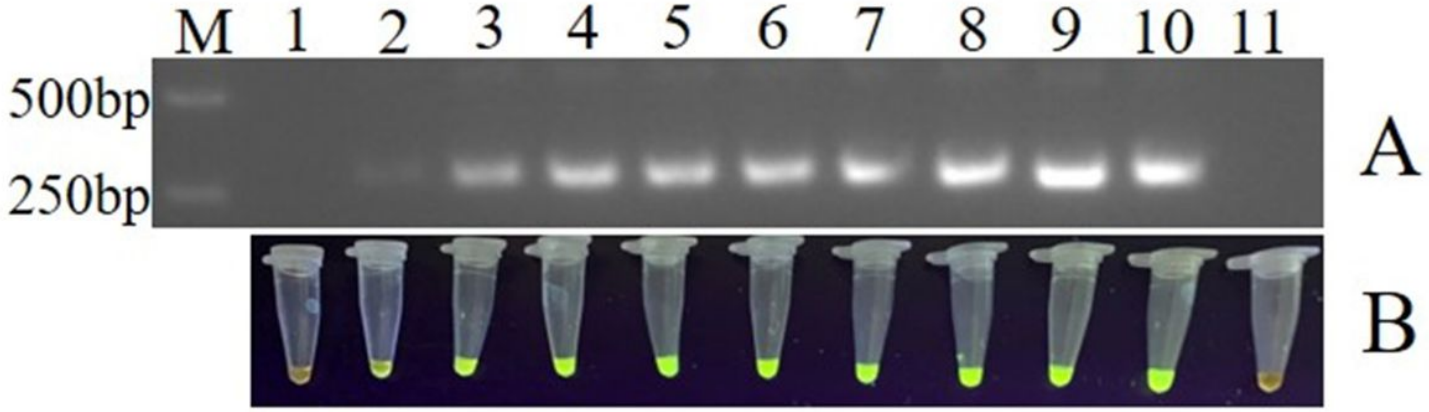

Figure 4

Visualization of RPA products under ultraviolet light. Aliquots (5 μl) of the RT-RPA products were analyzed by 2% agarose gel electrophoresis (A) and SYBR Green I staining (B). M: DL2000 marker. Lanes 1–11: the RT-RPA reactions incubated for 1, 3, 5, 7, 10, 15, 20, 25, 30, 40 min and negative control, respectively.

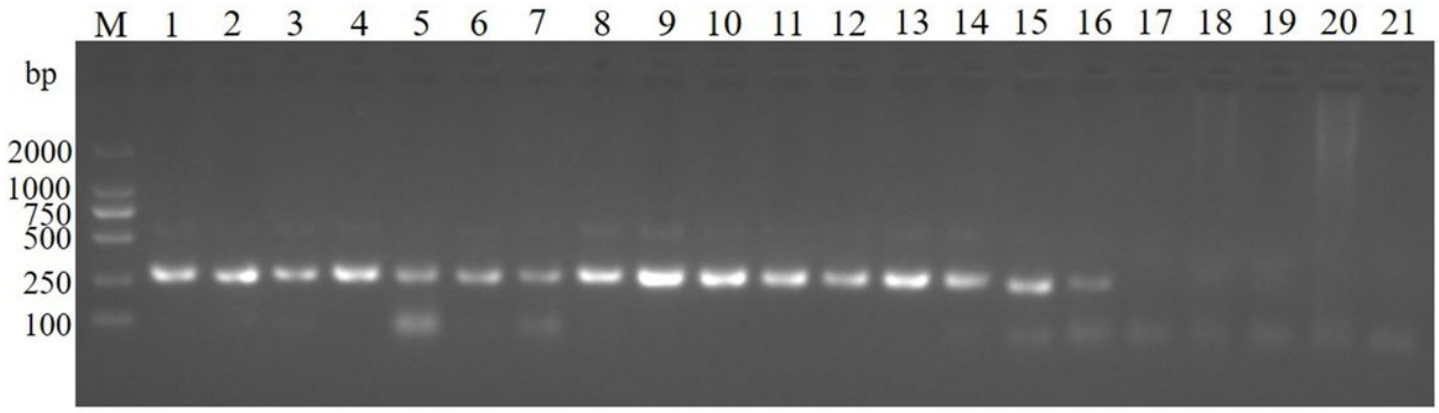

Figure 5

Detection of NDRV in field-collected and artificially inoculated samples by RT-RPA. M: DL2000 marker. Lanes 1–8: field-collected samples; Lanes 9–16: artificially inoculated samples. Lanes 17–20: the samples obtained from healthy ducklings. Lanes 21: the negative control.

Supplementary Files

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- additionalfiles.docx