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Rapid and sensitive real-time recombinase polymerase amplification for detection of Marek's disease virus

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ABSTRACT

Marek's disease (MD) is one of the most devastating diseases of poultry. It's caused by the highly infectious alphaherpesvirus MD virus serotype 1 (MDV-1). In this study, a rapid and easy-to-use assay based on recombinase polymerase amplification (RPA) was developed for MDV detection. Primer-probe sets targeting the highly conserved region of Meq gene were designed and applied to the RPA assay. The assay was carried out on a real-time thermostatic fluorescence detector at 39 °C for 20 min. As revealed by the results, no cross-reactions were found with the Newcastle disease virus (NDV), chicken infectious anemia virus (CAV), infectious bursal disease virus (IBDV), avian infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avain influenza virus (AIV), avian leucosis virus (ALV), avian reovirus (ARV), Marek's disease virus serotype 2 (MDV-2) and turkey herpes virus (HVT), indicating appropriate specificity of the assay. Plasmid DNA standards were used to determine the sensitivity of the assay and the detection limit was 10^2copies/μL. To further evaluate the clinical performance, 94 clinical samples were subjected to the RPA assay and 28 samples were tested MDV positive, suggesting that the real-time RPA assay was sufficient enough for clinical sample detection. Thus, a highly specific and sensitive real-time RPA assay was established and validated as a candidate for MDV diagnosis. Additionally, the portability of real-time RPA assay makes it suitable to be potentially applied in clinical diagnosis in the field, especially in resource-limited settings.

1. Introduction

Marek's Disease Virus (MDV) is the causative agent of Marek's disease (MD) in chickens [1]. It is a highly contagious oncogenic herpesvirus of poultry that can infect chickens, turkeys and pheasant. The virus is shed from feather follicles of infected chicken or birds and contaminates the living environment. Chickens will be infected by inhalation of contaminated air [2,3]. The prevalence of MD in all poultry-rearing countries causes huge economic losses every year [4,5]. Isolates of MDV can be classified into three serotypes, only serotype 1 (MDV1) or Gallid herpesvirus 2 (GaHV-2) is pathogenic and oncogenic. The severity of MD varies depending on virus stains, host genotypes and the vaccination status of the infected hosts [6]. Despite the wide use of vaccines, the economic burden of the disease will substantially increase. Therefore, timely and accurate detection of MDV in poultry farms is extremely important.

Conventional methods such as virus isolation and serum neutralization tests are labor-intensive and time-consuming [7]. To date, real-time PCR, GeXP-multiplex PCR assays and xTAG assay have been developed for MDV detection [8–10]. These methods are specific, sensitive and even enable high-throughput detection, which make them perfect choices for laboratory diagnosis. However, the need of expensive equipment such as Luminex-200 reader or GeXP analyser and well-trained technicians limits their applications in poultry farms [11,12].

Recombinase polymerase amplification (RPA), represents a hugely

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versatile alternative to PCR for the development of fast, portable nucleic acid detection assay [13–16]. The RPA reaction exploits enzymes known as recombinases, which will form complexes with oligonucleotide primers and pair the primers with their homologous sequences in duplex DNA [17]. A single-stranded DNA binding (SSB) protein binds to the displaced DNA strand and stabilizes the resulting D loop [18]. DNA amplification by polymerase is then initiated from the primer and progresses rapidly, reaching detectable levels within minutes. RPA is very forgiving of operating temperature. The reaction works optimally at a temperature of around 37–42 °C, but will also work over a wide range of ambient temperatures. The amplification products can be analyzed by gel electrophoresis. Besides, a probe-based system with fluorescent or lateral flow detection that increased specificity and sensitivity was developed [19].

Until now, there is no report on detection of MDV with RPA. In this study we described the development and validation of real-time RPA assay for rapid detection of MDV. It is highly specific and user friendly, making it a useful tool for MDV diagnosis in poultry farms.

2. Material and methods

2.1. Viruses and clinical samples

The Marek's disease virus (MDV) serotype 1 (MDV-1) GA and Marek's disease virus serotype 2 (MDV-2), turkey herpes virus (HVT) Fc-126, infectious bronchitis virus (IBV) Massachusetts 41, infectious laryngotracheitis virus (ILTV) N-71851 and Newcastle disease virus (NDV) F48E9 were preserved in our laboratory. Avian leucosis virus (ALV) subgroup J was purchased from the China Veterinary Culture Collection. The avian influenza virus (AIV) (H7N2), avian reovirus (ARV) GD-2, infectious bursal disease virus (IBDV) B87 and chicken infectious anemia virus (CAV) GD-2014 were purchased from Guangdong epidemiology prevention and control center. 94 clinical samples including feather follicles, spleen, kidney, thymus, bursa of Fabricius and skin were collected from poultry farms in different provinces of China.

2.2. DNA/RNA extraction

The nucleic acid of viruses and clinical samples were extracted using the TIANamp Virus genomic DNA/RNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instruction and stored at −80 °C until used.

2.3. RPA primers and probes

4 specific primer-probe sets were designed targeting the conserved region of MDV Meq gene in accordance with TwistDx RPA kits guidelines (TwistDx Inc. Cambridge, UK). All primers and probes were carefully analyzed for elimination of primer dimers. Besides, Primer-blast and Blast of NCBI were run to check and ensure the specificity of the primers and probes before synthesis by Sangon (Sangon Biotech, Shanghai, China). All the probes were fluorescently labeled to collect signals during the amplification. The sequences of designed primers and probes were summarized in Table 1.

2.4. Generation of DNA standard

The open reading frame (ORF) of Meq gene (GenBank accession No. KU744561.1) was amplified using the forward primer 5′- TGGAAACC ACCAGACCGT-3′ and reverse primer 5′- GTCTCAGGAGCCAGAGCC-3’. The PCR product was purified by using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) before cloning into the pMD-18T vector (Takara, Dalian, China). The positive clone was selected and sequenced before further steps. Plasmid extraction was carried out with the SanPrep Plasmid MiniPrep Kit (Sangon Biotech, Shanghai, China) following the manufacturer's instruction. DNA concentration was determined by spectrophotometry and the copy number was calculated before using as DNA standard for sensitivity analysis.

2.5. Real-time RPA assay

The RPA reaction was performed with a TwistAmpTM exo lyophilized kit (TwistDX, Cambridge, United Kingdom). A total of 50 μL reaction volume contained 420 nM forward primer, 420 nM reverse primer, 120 nM probe, 1 μg of viral DNA and 280 mM magnesium acetate and incubated at 39°Cfor 20 min in the real-time thermostatic fluorescence detector (DEAOU Biotechnology, China). All the samples were run in duplicate with nucleic acid of MDV as positive control and the genome of normal chicken tissue as negative controls.

2.6. Sensitivity and specificity analysis

To determine the sensitivity of the RPA assay, 10-fold serial dilutions of DNA standard ranging from 10^6 to 10^0copies/μL were tested. All the samples and positive/negative controls were performed in duplicate and the whole assay was carried out for three times.

The nucleic acid of NDV, CAV, IBDV, MDV-2, HVT, IBV, ILTV, AIV, ALV and ARV were tested to evaluate the cross-reactivity of the assay, also the positive and negative controls were simultaneously run.

2.7. Clinical sample detection

The performance of RPA assay was evaluated with 94 clinical samples. For comparison, all the clinical samples were tested with a reference real-time PCR method targeting the Meq gene. Briefly, 20 μL reaction volume containing 10 μL 2 × Probe qPCR Mix (Takara, Dalian, China), 2 μM of each forward primer: 5′-GGAGCCGGAGAGGCTTT ATG-3′, reverse primer: 5′-ATCTGGCCCGAATACAAGGAA-3′ and probe 5′(FAM)-CGTCTTACCGAGGATCCCGAACAGG(BHQ-1)-3′ and 25 ng DNA template. The amplification conditions were as previously described [20].

3. Results

3.1. Optimal primer-probe set selection

In this study, four primer-probe sets were designed and subjected to the real-time RPA assay respectively. As revealed by the results, all 4 primer-probe sets could recognize and amplify MDV. However, striking difference was observed during the amplification. Compared with the other three sets, primer-probe set 1 exhibited higher fluorescence signal during the whole process, which made it the optimal primer-probe set and was chosen for the subsequent experiments (Fig. 1).

3.2. Sensitivity and specificity analysis

The detection limit of RPA was tested by a dilution series of DNA standard. As shown in Fig. 2, the assay was sufficiently sensitive for detecting 10^2copies/μL, but no typical amplification curves were observed from 10^2 copies/μL to 10^0 copies/μL, suggesting that 10^2 copies/μL was the detection limit of the assay.

Specific fluorescence signal was observed only from the corresponding MDV nucleic acid. Viruses such as MDV2, HVT, NDV, CAV, IBDV, IBV, ILTV, AIV, ALV and ARV showed no cross-reactions, demonstrating the appropriate specificity of the RPA assay (Fig. 3).

3.3. Comparison of real-time RPA assay and real-time PCR assay in clinical sample detection

The practicality of real-time RPA assay was evaluated with 94 clinical samples. The results were compared with real-time PCR assay

Table 1
The sequence of the primers and probes.

name	Sequence	Product size (bp)	Length(bp)	Location (KU744561.1)
F1	TTGTCTACATAGTMCGTCTGCTYCCTGCGTC	154	31	133667–133697
R1	AAAGGAAAAGTCACGACATCCCCAACAGCC		30	133544–133573
P1	CACGATTCCTTTTTCTCCTCCTTTCCAGCT(dT-FAM)(THF)(dT-BHQ1)GTTTCTCCTCCTCAG-C3spacer		48	133604–133651
F2	TCGTGCGGGGTGGTAAGCAGTCCAAGGGTC	128	30	133842–133871
R2	GCCGCTCGGAGAAGACGCAGGRAGCAGACG		30	133654–133683
P2	ACGTACTATGTAGACAAACTCCATGAAGCA(dT-FAM)(THF)(dT-BHQ1)GAAGAGCTGCAGAGG-C3spacer		48	133681–133728
F3	CGTCTGCTYCCTGCGTCTTCTCCGAGCGGCGTCA	121	34	133650–133683
R3	CCCCAACAGCCCCTCCAAACACCCCTTCCC			133563–133592
P3	CACGATTCCTTTTTCTCCTCCTTTCCAGCT(dT-FAM)(THF)(dT-BHQ1)GTTTCTCCTCCTCAG-C3spacer		48	133604–133651
F4	TCTCCCGTCACCTGGAAACCACCAGACCGT	293	30	134431–134460
R4	CCCTCTTCTGCCCTCCCCAGCCTCCATCTC		30	134168–134197
P4	GCCGGGGACGGTTTACGCTCAGCTTTGTCC(dT-FAM)(THF)(dT-BHQ1)TGGCCAGGCTCCCCT-C3spacer		48	134253–134300

F: forward primer.
R: reverse primer.
P: probe.
THF: tetrahydrofuran, it was an abasic nucleotide analogue residue.

(Table 2). Among these clinical samples, 28 samples were detected MDV positive by both real-time RPA and real-time PCR and sequenced for confirmation. As revealed by the results, these two methods were highly consistent on clinical sample detection (Fig. 4), suggesting the potential of RPA for clinical diagnosis.

4. Discussion

MDV has become more virulent and poses a serious threat to poultry industry [21]. Rapid and sensitive diagnosis of MDV is extremely important for control of the disease. Isothermal amplification of nucleic acid offers a better choice to poultry farms especially those with limited settings [22].

In fact, there are reports on the loop mediated isothermal amplification (LAMP) of MDV for clinical diagnosis [23,24]. Compared with LAMP, RPA assay can be operated at lower temperature and the results are typically generated within 5–20 min. In the lyophilized format, RPA reagents exhibit excellent stability at ambient temperatures and are easily transportable, making the RPA assay available for field application as a mobile suitcase laboratory [25–27].

Establishing a rapid and sensitive RPA assay depends on selecting suitable amplification primers. Until now, no automated software is available for designing primer-probe sets for RPA. All the primers and probes used in this study were manually designed following the manufacturer's instruction. RPA primer design will be simplified if specialized software is developed in the future.

Previous studies showed that the Meq gene is unique to MDV1 and is considered to be associated with viral oncogenicity and pathogenicity [28,29]. In this study, the highly conserved region of Meq gene was targeted for screening of candidate primers and probes. 4 primer-probe sets were identified and primer-probe set 1 could effectively amplify MDV. Despite the fact that primer-probe set 1 is highly conserved within the compared Meq gene, mutations may exist in different strains. Though previous studies have proven that up to nine point mutations were tolerated in RPA assay, more works should be done for avoiding false negative detection results of MDV in the future [30,31].

The clinical performance of real-time RPA assay was determined by detecting 94 samples corrected from unvaccinated flocks. As revealed by the results, 28 samples including feather follicles and different tissues were tested MDV positive by RPA. The same results were obtained from the real-time PCR assay, indicating that the real-time RPA assay is sufficient for clinical diagnosis of MDV in different sample types. Though, the newly developed RPA assay could not distinguish pathogenic MDV from the CVI988 or mMDV814, most of the broiler flocks are not vaccinated in China and the RPA assay is useful to detect MDV in non-vaccinated broiler flocks, but would have limited application in

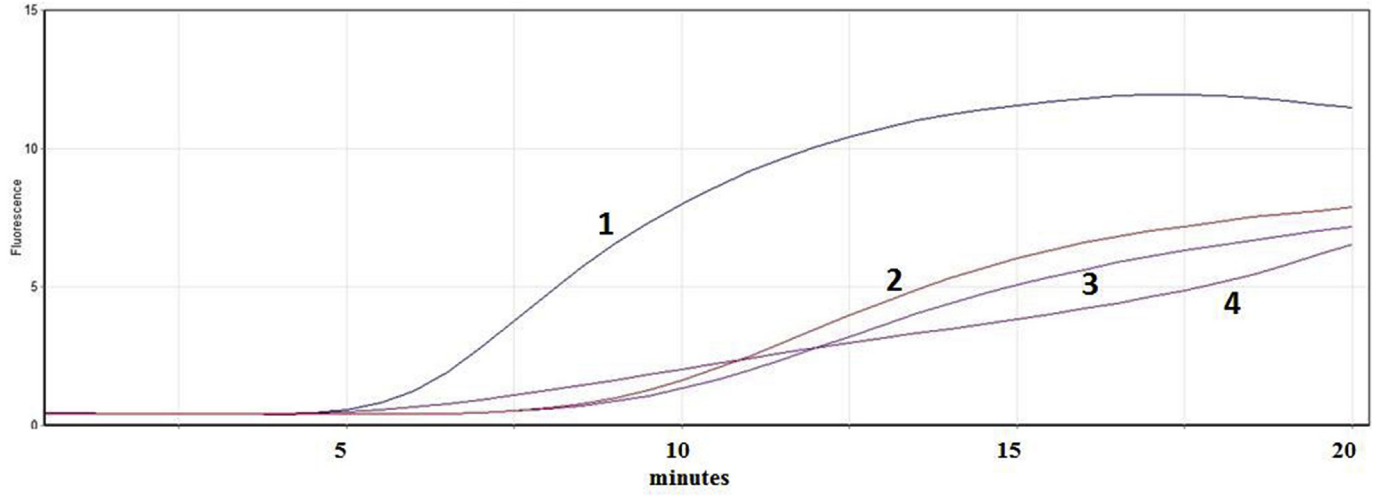

Fig. 1. Selection of optimal primer-probe set.
4 primer-probe sets targeted the Meq gene of MDV were designed and applied to the RPA assay. X-axis is time and the Y-axis is fluorescence intensity. 1: primer-probe set 1; 2: primer-probe set 2; 3: primer-probe set 3; 4: primer-probe set 4. The primer-probe set 1 produced the optimal amplification curve and was selected for the following trials.

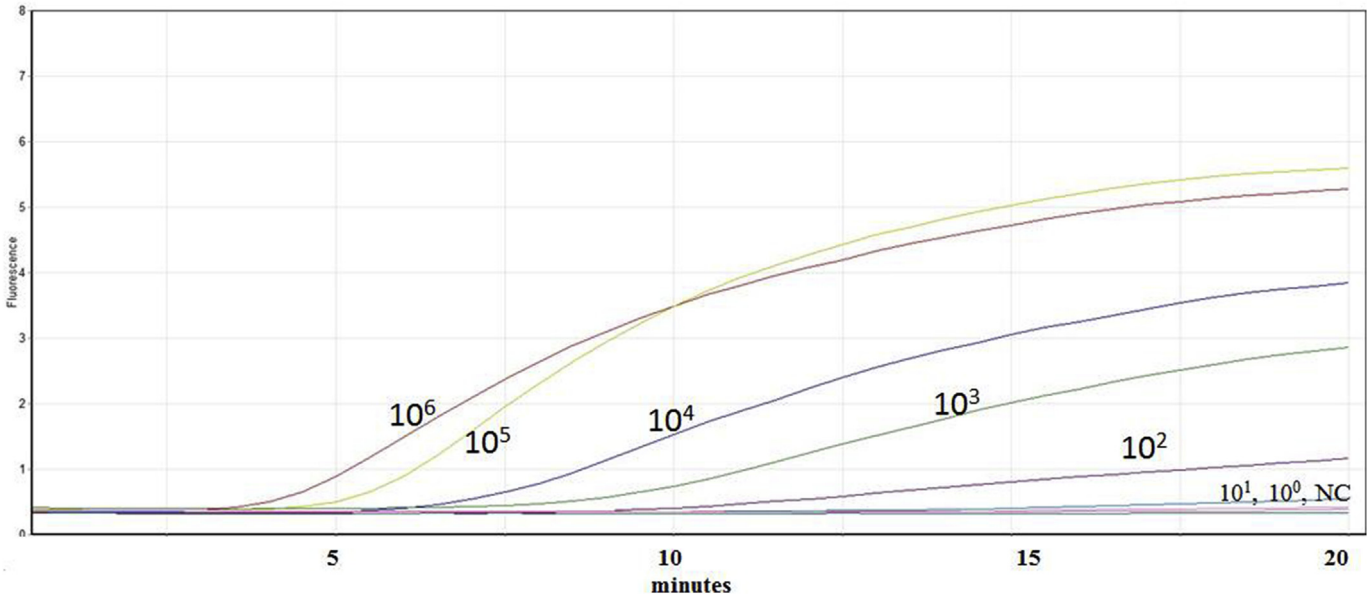

Fig. 2. Sensitivity analysis of real-time PRA assay.
10-fold serial dilutions of DNA strandard ranging from 10^6 to 10^0 copies/uL were used as template for the determination of the limit of detection. X-axis is time and the Y-axis is fluorescence intensity. As revealed by the results, no fluorescence signal was observed at the concentration of 10^1 and 10^0 copies/uL, which made the detection limit of the RPA assay was 10^2 copies/uL.

any flock that is vaccinated with CVI988 or mMDV814. Hopefully it may be useful for the discrimination of Meq-deleted vaccine strain and pathogenic MDV in the future when the new vaccine is available. For now, it is more suitable for the rapid diagnosis of pathogenic MDV in poultry industry.

5. Conclusions

In summary, this study demonstrates a rapid and highly specific real-time RPA assay for MDV detection. The detection limit of RPA assay was 10^2copies/μL, which was sufficient enough for clinical sample detection. It can be a useful tool for routine monitoring of the disease especially in poultry farms with limited resources.

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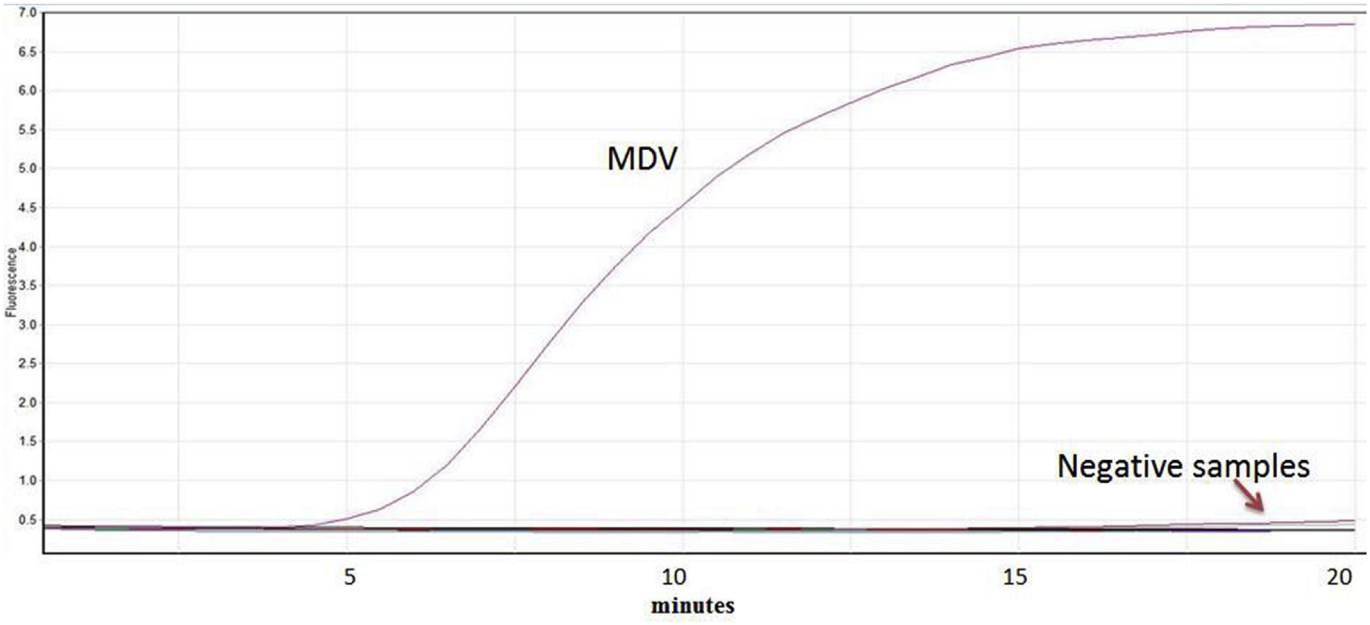

Fig. 3. Specificity analysis of real-time RPA assay.
The nucleic acid of MDV-2, HVT, NDV, CAV, IBDV, IBV, ILTV, AIV, ALV and ARV were subjected to the real-time RPA assay for specificity analysis. X-axis is time and the Y-axis is fluorescence intensity and the fluorescence signal was observed only in the MDV positive control, no cross-reaction was found during the amplification.

Table 2
The results of clinical sample detection.

sample	method		sample	method		sample	method		sample	method	
	RPA	PCR Ct value		RPA	PCR Ct value		RPA	PCR Ct value		RPA	PCR Ct value
F1	–	–	F25	+	22	SK8	+	15	T4	–	–
F2	–	–	F26	+	13	SK9	–	–	T5	–	–
F3	+	16	F27	–	–	SK10	+	19	T6	+	21
F4	–	–	F28	–	–	B1	+	22	T7	–	–
F5	–	–	SP1	–	–	B2	–	–	T8	–	–
F6	–	–	SP2	–	–	B3	+	28	T9	–	–
F7	–	–	SP3	–	–	B4	+	21	T10	–	–
F8	+	18	SP4	–	–	B5	+	27	F-101	–	–
F9	+	21	SP5	–	–	B6	–	–	F102	+	14
F10	–	–	SP6	–	–	B7	–	–	F103	–	–
F11	–	–	SP7	+	28	B8	–	–	F-201	–	–
F12	–	–	SP8	–	–	B9	–	–	F-202	–	–
F13	–	–	SP9	+	26	B10	+	26	F-203	–	–
F14	+	25	K1	–	–	B11	–	–	F-204	+	17
F15	–	–	K2	+	23	B12	–	–	F-205	–	–
F16	–	–	K3	+	19	B13	–	–	F-206	–	–
F17	+	24	K4	–	–	B14	–	–	F-207	–	–
F18	–	–	SK1	+	13	B15	+	24	F-208	–	–
F19	–	–	SK2	–	–	B16	–	–	F-209	–	–
F20	–	–	SK3	+	18	B17	–	–	F-210	–	–
F21	–	–	SK4	+	17	B18	–	–	F-211	–	–
F22	–	–	SK5	–	–	T1	+	29	F-212	–	–
F23	–	–	SK6	+	22	T2	–	–			
F24	+	10	SK7	–	–	T3	–	–			

1. Results: +: Positive; -: Negative.
2. Sample types: F: feather follicles; SP: spleen; K: kidney; T: thymus; SK: skin; B: bursa of Fabricius.

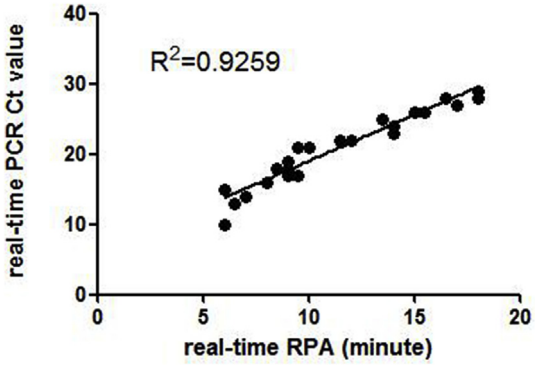

Fig. 4. Performance of real-time RPA in comparison with real-time PCR. Comparison of clinical performance between the threshold time of real-time RPA assay (X-axis) and Ct value of real-time PCR (Y-axis) on positive clinical samples (n = 28).

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