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Establishment of a duplex SYBR green I-based real-time polymerase chain reaction assay for the rapid detection of canine circovirus and canine astrovirus

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ABSTRACT

The similar clinical characteristics of canine circovirus (CaCV) and canine astrovirus (CaAstV) infections and high frequency of co-infection make diagnosis difficult. In this study, a duplex SYBR Green I-based real-time polymerase chain reaction (PCR) assay was established for the rapid, simultaneous detection of CaCV and CaAstV. Two pairs of specific primers were designed based on the *Rep* gene of CaCV and the *Cap* gene of CaAstV. By using the real-time PCR assay method, the two viruses can be distinguished by the difference in melting temperatures, 79 °C and 86 °C for CaCV and CaAstV, respectively. This assay had high specificity, showing no cross-reaction with other common canine viruses, as well as high sensitivity, with minimum detection limits of 9.25×10^1 copies/μL and 6.15×10^1 copies/μL for CaCV and CaAstV, respectively. Based on the mean coefficient of variation, the method had good reproducibility and reliability. In a clinical test of 57 fecal samples, the rates of positive detection by real-time PCR were 14.04% (8/57) and 12.28% (7/57) for CaCV and CaAstV, respectively, and the rate of co-infection was 8.77% (5/57). In conclusion, the newly established duplex SYBR Green I-based real-time PCR assay is sensitive, specific, reliable, and rapid and is an effective tool for the detection of co-infections with CaCV and CaAstV.

1. Introduction

Canine circovirus (CaCV), belonging to the genus *Circovirus* and family *Cricoviridae*, is a non-enveloped, single-stranded virus. The virus contains two transcription units with two open reading frames (ORFs), ORF1 and ORF2, with genes encoding the replicase protein (*Rep*) and the capsid protein (*Cap*), respectively [1]. The *Rep* gene is more highly conserved than the *Cap* gene [2] and is, therefore, suitable for the design of specific primers. CaCV was first reported in the USA in 2012 [3]. Since then, the virus has been reported in Italy, Germany, China, Thailand, and other countries [1,2,4–6]. CaCV-infected dogs usually exhibit diarrhea; however, others show a lack of clinical symptoms.

The canine astrovirus (CaAstV) belongs to the genus *Mamastrovirus*. The virus contains three ORFs: ORF1a, ORF1b, and ORF2 [7]. Each ORF encodes a different protein, with different degrees of sequence conservation. Compared with other genes, the *Cap* gene is most highly conserved [8], indicating that the design of specific primers in this gene is appropriate. CaAstV was first reported in 1980 in the USA [9]. The virus has since been reported in Hungary, Australia, China, and other regions [8,10,11]. According to previous reports, susceptibility to CaAstV is higher in puppies (<12 months of age) than adult dogs. Co-infection with other enteric viruses, such as the canine parvovirus (CPV), is common [12]. Moreover, CaAstV can be found in healthy or diarrheic dogs [13].

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With both CaCV and CaAstV being enteric viruses, the clinical symptoms of infections are extremely similar [4,5,14,15]. Therefore, it is necessary to establish a rapid method with high specificity and sensitivity for the simultaneous detection of CaCV and CaAstV. An effective method would also contribute to the epidemiological investigations of these viruses.

A duplex SYBR Green I-based quantitative real-time polymerase chain reaction (PCR) assay for CaCV and CaAstV has not been reported to date. Thus, two pairs of specific primers were designed to establish a duplex SYBR Green I-based real-time PCR assay that can detect both viruses simultaneously. This study provides a rapid and sensitive detection method for CaCV and CaAstV.

2. Materials and methods

2.1. Viruses and nucleic acid extraction

Viral DNA and RNA were extracted from positive samples after centrifugation using the DNA/RNA Mini Kit (Tiangen, Beijing, China) following the instructions of the manufacturer. Extracted RNAs were reverse transcribed into cDNA using the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Kusatsu, Japan) following the instructions of the manufacture [16]. The strains of CaCV, CaAstV, CPV, canine distemper virus (CDV), canine coronavirus (CCV), and canine kobuvirus (CaKoV) used in this study were stored in our laboratory.

2.2. Preparation of standard plasmids

The *Rep* gene of CaCV and the *Cap* gene of CaAstV were used as template DNA. Target fragments were PCR-amplified, and the primers are shown in Table 1 (CaCV-F/CaCV-R and CaAstV-F/CaAstV-R). Products were cloned into the pMD-19T vector in accordance with the protocol of the manufacturer and transformed into *Escherichia coli* DH5α cells. The recombinant plasmids were extracted using the TIANprep Mini Plasmid Kit II (Tiangen, China), and sequencing was conducted by Sangon Company (Shanghai, China). The concentration of the positive plasmid was quantitated using the NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Dreieich, Germany), and copy numbers were calculated using the following formula: (plasmid concentration [ng] $\times$ 6.02 $\times$ 10^{23})/(genome length $\times$ 10^{9} $\times$ 660 Da/bp). The plasmids were serially diluted 10-fold and stored at −20 °C until usage.

2.3. Primer design

In the CaCV accessions, MN863535, MN863536, and MN863537 sequences were compared using MegAlign (DNASTAR, Madison, WI, USA), and the *Rep* region exhibited the highest conservation. Additionally, the CaAstV accessions MF973500, MK026166, and KX599351 were compared, and the *Cap* region exhibited the lowest variation. Two specific primers were designed using Primer Premier 5 for the *Rep* and *Cap* genes of CaCV (CaCV-SYBR-F and CaCV-SYBR-R) and CaAstV (CaAstV-SYBR-F and CaAstV-SYBR-R), respectively. Primers for conventional PCR were also designed using the same conserved region. All primer sequences used in this study are shown in Table 1. All primers were synthesized by Sangon Company.

Table 1
Primer sequences designed in this study.

Primer name	Sequence (5′-3′)	Product size (bp)
CaCV-F	ATAGTCTACACACAAATGGACCAGC	912
CaCV-R	TCAGTAGTTATACATGTGTGGAAAC	
CaAstV-F	ACTTGTAACAGGTGTGTTCCAACAA	1000
CaAstV-R	ATTCCCTCGATCCTACTCGGCGTGG	
CaCV-SYBR-F	GCACCAGGATGTCATTCT	76
CaCV-SYBR-R	GTACCGATCCAACAGTCTAA	
CaAstV-SYBR-F	TTCCCTGCTTCTGATCAG	126
CaAstV-SYBR-R	CTCACTTAGTGTAGGGAGAG	

2.4. Optimization of the duplex SYBR green I real-time PCR assay

Standard plasmids of CaCV and CaAstV were used as templates to optimize the duplex real-time PCR assay. Prior to performing duplex real-time PCR assay, the concentrations of both forward and reverse primers, annealing temperature, and cycle number were preliminarily optimized by traditional PCR, and the PCR products were separated by 2% agarose gel electrophoresis using Gold View (Solarbio, Beijing, China). Duplex real-time PCR assay amplification was performed using the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). While holding other conditions constant, different concentrations of the two pairs of forward and reverse primers were evaluated (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 μM). Moreover, different annealing temperatures were evaluated.

2.5. Standard curves for CaCV and CaAstV

The 10-fold serially diluted recombinant plasmid standards of CaCV and CaAstV were used as templates with each concentration repeated thrice. According to the optimized reaction system, duplex SYBR Green I-based real-time PCR assay was performed to obtain standard curves for CaCV and CaAstV.

2.6. Sensitivity analysis

Standard plasmid concentrations for CaCV and CaAstV ranging from 10^{1} to 10^{8} copies/μL were applied to the optimized duplex real-time PCR assay to determine the sensitivity of the method. Traditional PCR was also used to allow the comparison of the sensitivity of both methods.

2.7. Specificity of the duplex SYBR green I real-time PCR assay

For the evaluation of specificity, duplex real-time PCR was performed using the DNA of CaCV and CPV and the cDNA of CaAstV, CCV, CDV, and CaKoV as templates, and their concentrations were all 1 $\times$ 10^{7} copies/μL. A negative control (ddH_2O) was also included. After each run, 2% agarose gel electrophoresis was used to verify whether the expected amplification product was obtained.

2.8. Reproducibility analysis

Different dilutions of several standard plasmids (10^{7}, 10^{5}, 10^{3}, and 10^{1} copies/μL) were used as templates to verify the reproducibility of the optimized duplex real-time PCR assay. Each concentration was tested thrice at equal intervals. The coefficient of variation (CV) and standard deviation were calculated to determine the intra-assay and inter-assay variation based on cycle threshold (Ct) values.

2.9. Detection of clinical samples

A total of 57 clinical samples obtained from diarrheic dogs were collected from different veterinary hospitals. The nucleic acid of the virus was extracted using DNA/RNA Mini Kit, enabling simultaneous nucleic acid extraction. After that, all products were reverse transcribed, allowing the system to contain both DNA and cDNA. With the methods described above, the optimized duplex real-time PCR method was used for amplification, and the same samples were subjected to conventional PCR detection. Differences between the two methods at the end of the reaction were compared.

3. Results

3.1. Optimization of the duplex SYBR green I real-time PCR assay

The optimal total reaction volume for duplex real-time PCR was 20 μL, including 10 μL of SuperReal PreMix Plus (Tiangen), 0.3 μL (10 μmol/L) of the forward and reverse primers for CaCV, 0.2 μL (10 μmol/L) of the forward and reverse primers for CaAstV, 1 μL of each template, and ddH_2O. The reaction conditions were 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. As shown in Fig. 1a–b, the melting temperatures (T_m) were 79 °C for CaCV and 86 °C for CaAstV. The duplex melting curve showed that the T_m value of CaCV was 79 °C, whereas it displayed a T_m value of CaAstV at 86 °C (Fig. 2), which is consistent with the results of the single curve analysis.

3.2. Establishment of CaCV and CaAstV standard curves

Based on the concentration of the standard plasmid, the estimated copy numbers were 9.25×10^{10} copies/μL and 6.15×10^{10} copies/μL for CaCV and CaAstV, respectively. We established standard curves for CaCV and CaAstV, which showed good linear relationships with their respective Ct values. The standard curves were fit with the equations $y = -3.331x + 36.493$ and $y = -3.366x + 36.648$, and the amplification efficiencies were 99.6% and 98.2%, for CaCV and CaAstV, respectively. The correlation coefficients (R^2) were all 0.999 for CaCV and CaAstV, respectively (Fig. 3a–b).

3.3. Sensitivity analysis

After mixing the CaCV and CaAstV templates in equal proportions, amplification was performed by the optimized duplex real-time PCR assay. The lowest sensitivity that can be achieved was 10^1 copies/μL (Fig. 4a and b). The duplex real-time PCR was approximately 100 times more sensitive than traditional PCR. These results show that the sensitivity of the duplex real-time PCR assay was higher than that of traditional PCR.

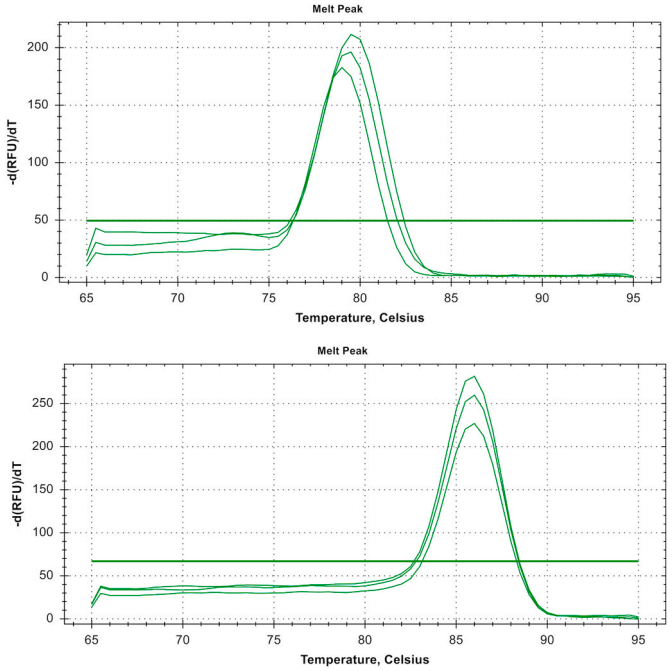

Fig. 1. Melting curve analysis. (a) Melting curve analysis of CaCV; the single peak has good specificity (T_m value = 79 °C). (b) Melting curve analysis of CaAstV; the single peak has good specificity (T_m value = 86 °C).

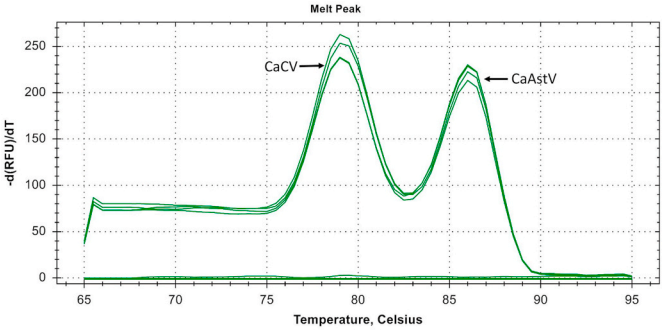

Fig. 2. Duplex melting curve analysis of CaCV (T_m value = 79 °C) and CaAstV (T_m value = 86 °C). This result is highly consistent with the result of single curve analysis.

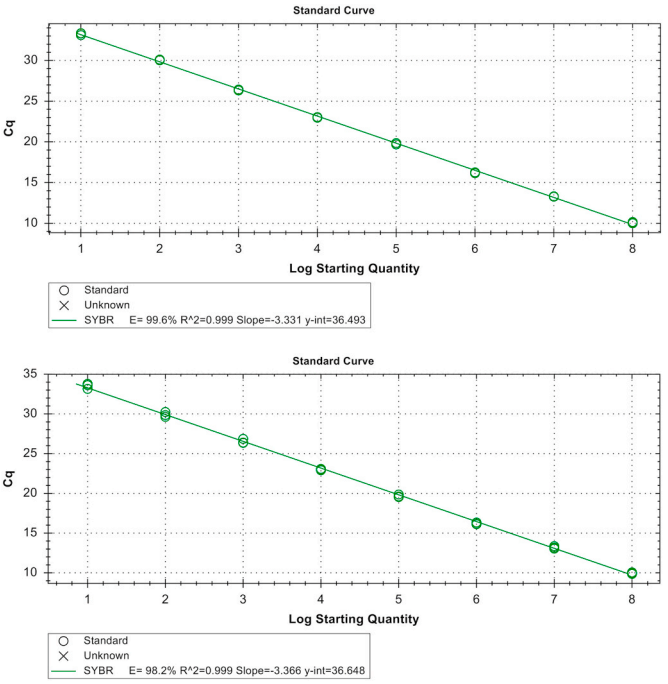

Fig. 3. Standard curve analysis of the standard plasmids. (a) Standard curve of CaCV (concentrations ranging from 9.25×10^8 to 9.25×10^1 copies/μL; y = −3.331x + 36.493; R^2 = 0.999; Eff = 99.6%). (b) Standard curve of CaAstV (concentrations ranging from 6.15×10^8 to 6.15×10^1 copies/μL; y = −3.366x + 36.648; R^2 = 0.999; Eff = 98.2%).

3.4. Specificity analysis

CaCV, CaAstV, CPV, CCV, CDV, and CaKoV templates were amplified using the newly developed duplex real-time PCR method, and ddH_2O was used as a negative control. There were no differences in T_m values among the viruses and negative control groups. Importantly, the results of all control groups were below the threshold values (Fig. 5). These results showed that the duplex real-time PCR assay had no cross-reactivity with other viruses and showed good specificity.

3.5. Reproducibility analysis

In the reproducibility analysis, intra- and inter-assay CVs were 0.11–0.23% and 0.18–0.32% for CaCV, while they were 0.13–0.27% and 0.17–0.33% for CaAstV. These results indicate that the method has good reproducibility and reliability.

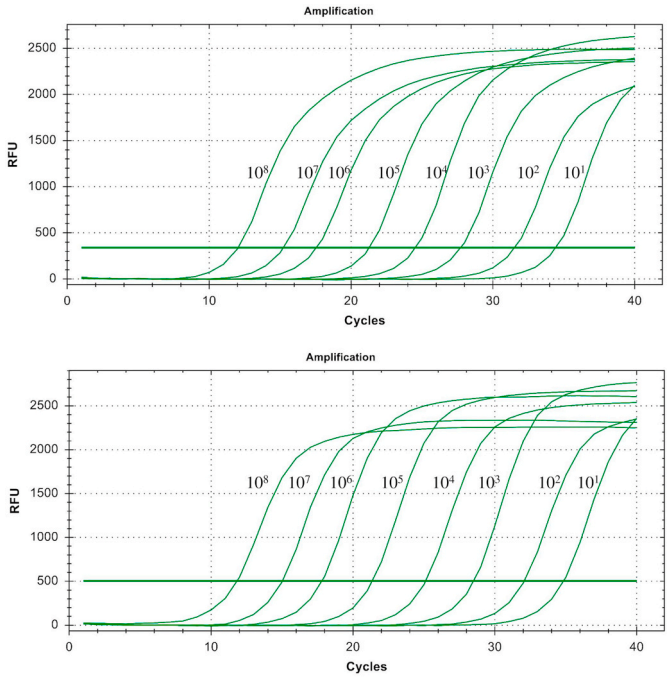

Fig. 4. Sensitivity analysis. Amplification curve of SYBR Green I real-time PCR using the standard plasmids of CaCV and CaAstV. (a) The recombinant plasmid standard of CaCV was used as a template after 10-fold dilution in the concentrations ranging from 9.25×10^8 copies/μL to 9.25×10^1 copies/μL. (b) The recombinant plasmid standard of CaAstV was used as a template after 10-fold dilution in the concentrations ranging from 6.15×10^8 copies/μL to 6.15×10^1 copies/μL.

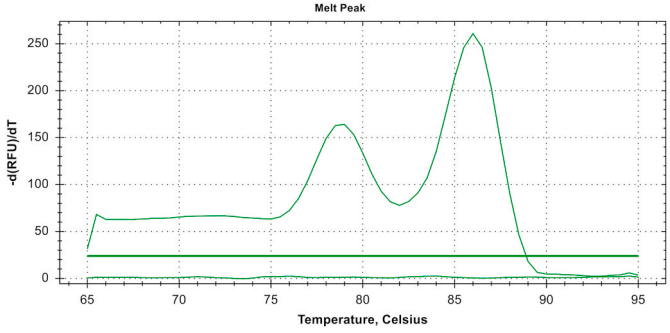

Fig. 5. Specificity analysis. There is no cross-reactivity with CPV, CCV, CDV, and CaKoV in the duplex melting curve except for CaCV and CaAstV.

3.6. Detection of clinical samples

A total of 57 unknown clinical samples from diarrheic animals were evaluated by the duplex SYBR Green I-based real-time PCR assay and traditional PCR assay. As shown in Table 2, the positive detection rates for CaCV and CaAstV by the duplex real-time PCR method were 14.04% (8/57) and 12.28% (7/57), respectively, and the co-infection rate was 8.77% (5/57) (Fig. 6). However, using the traditional PCR, the positive detection rates for CaCV and CaAstV were 10.53% (6/57) and 5.26% (3/57), respectively. These results show that the positive detection rate is higher for the real-time PCR assay than for traditional PCR assay, supporting the use of the newly developed method for clinical detection.

Table 2
Detection of CaCV and CaAstV in clinical samples by conventional PCR and real-time PCR.

Virus	Total clinical samples	Positive rata (%)	
		Real-time PCR	Conventional PCR
CaCV	57	14.04% (8/57)	10.53% (6/57)
CaAstV	57	12.28%(7/57)	5.26% (3/57)
co-infection	57	8.77% (5/57)	5.26% (3/57)

4. Discussion

Infectious diseases in animals are a serious issue that can lead to mortality. Common causes of diarrhea include CPV, CDV, and CCV [17–19] as well as some novel viruses, such as CaCV and CaAstV [1,3,8]. CaCV and CaAstV infections in dogs have been reported in various countries and may cause severe diseases and economic losses [8]. It is particularly important to study co-infections with CaCV and CaAstV, both of which are enteric viruses that can cause serious diarrhea and are widespread in dogs. Dogs infected with these two viruses can exhibit similar clinical symptoms, ranging from asymptomatic to subclinical and acute-chronic forms. The symptoms of infections caused by these viruses are extremely similar, making differential diagnosis difficult. Therefore, identification of novel diagnostic methods for the differentiation of these viruses has been a general focus of recent research [20–26].

Quantitative real-time PCR is now widely used in clinical settings. Compared with loop-mediated isothermal amplification, enzyme-linked immunosorbent assay, and indirect immunofluorescence assay, quantitative real-time PCR has advantages with respect to time and sensitivity [27–30]. Quantitative real-time PCR also has higher sensitivity, specificity, and positive detection rates than traditional PCR. The SYBR Green I-based real-time PCR assay had numerous advantages, including high sensitivity, strong specificity, rapidity, and easy operation, making this method the best option; compared with TaqMan-based real-time PCR, the former is cheaper and easily applied [31]. TaqMan-based real-time PCR and SYBR Green-based real-time PCR are two methods employed in real-time PCR (quantitative PCR). Both the methods help quantify PCR products efficiently and rely on the emission of fluorescence. The TaqMan method uses dual-labeled probes for the detection of accumulated DNA, whereas the SYBR Green method uses a fluorescent dye. In addition, both the methods have different applications in molecular biology. However, owing to its ability to bind to any double-stranded DNA chain, nonspecific binding can lead to over quantification of the PCR product in the SYBR Green method. Therefore, it is crucial to verify the amplified PCR products. Typically, in cases of mixed viral infections in clinical settings, only one of the infections is diagnosed, and the other is ignored. Therefore, there is an urgent need to establish a method that can detect both CaCV and CaAstV simultaneously.

In this study, two pairs of specific primers were designed based on conserved regions of the CaCV and CaAstV genomes, and reaction conditions were optimized. We successfully established a duplex real-time SYBR Green I-based real-time PCR assay for the simultaneous detection of CaCV and CaAstV. This method was specific and showed no cross-reactivity with common canine enteric viruses, such as CPV, CCV,

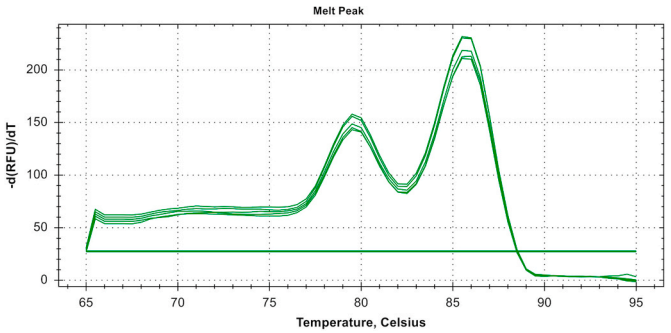

Fig. 6. Dissolution curve of co-infected samples.

CDV, and CaKoV. The sensitivity of the duplex real-time SYBR Green I-based real-time PCR method showed that the minimum detection limit was 101 copies/μL, which is higher than that of traditional PCR. The intra- and inter-assay CVs indicated that the assay shows good reproducibility. The positive co-infection rate shown by the duplex real-time SYBR Green I-based real-time PCR method was 8.77%, while that of the traditional PCR was only 3.51%, indicating the suitability of the duplex real-time SYBR Green I-based PCR method in clinical diagnosis.

We found that the primer concentration was a significant factor for optimizing the duplex real-time SYBR Green I PCR assay. Notably, the competitive inhibition between pairs of primers can result in the preferential amplification of a particular target and can affect annealing. In this study, the concentration of SuperReal PreMix Plus was also important. The ratio of reagents and PreMix should be carefully considered to ensure good specificity and the efficient use of PreMix.

In summary, we successfully established a rapid, sensitive, and specific duplex real-time SYBR Green I-based PCR method. For clinical samples with multiple target viruses, this method may become a rapid and convenient diagnostic tool because it allows the simultaneous detection of viruses. In addition, rapid and accurate diagnostic methods can be an effective tool for the epidemiological investigations during outbreaks of novel viral infections.

Author contributions

Yong Wang and Yeqiu Li conceived of the study, carried out the experiment and drafted the manuscript, contributed equally to this work. Yongqiu Cui participated in the data collection and analysis. Shudong Jiang involved in drafting of the manuscript. Guangqing Liu and Jing Wang participated in statistical analysis. Yongdong Li conceived of the study, revising the manuscript critically. All authors have read and approved the final manuscript.

Compliance with ethical standards

All experiments were compliant with the ethical standards of Anhui Agricultural University.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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