

The Establishment of the PCR Detection Method of Babesia Canis with Internal Amplification Control

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Abstract

The aim of this study is to establish a PCR method for detection of *Babesia canis*, which contains amplified internal standard, to improve the accuracy of detection. According to *Babesia canis*, the 18S rRNA gene of *Babesia canis* design primers for PCR detection of *Babesia canis*, using the gene fragment of chicken plasmid constructed and amplified internal standard method using a composite primer containing the recombinant. The amplified internal standard was added into the PCR detection system of *Babesia canis* to establish a PCR method for detection of *Babesia canis*. The results showed that the detection sensitivity of PCR was 10^5 copies/25 L in 25 L reaction system by adding 10^6 copies internal standard. In this study, a PCR assay with amplified internal standard was developed for rapid, accurate and simple detection of *Babesia canis* infection, and it could effectively avoid false negative phenomena.

Keywords —*Babesia canis* PCR; canine; internal amplification control; false negative

I. INTRODUCTION

Babesia canis disease is a canine serious blood protozoa disease caused by a Piroplasmida, *Babesia* parasites infecting body within the red blood cells in the canine's erythrocytes, spreading by the tick blood [1]. *Babesia canis* disease routine pathogen diagnosis is blood smear microscopy, the main basis of diagnosis. But after acute infection or initial infection with *Babesia*, and the canine after the natural rehabilitation or rehabilitation through the drug, at this point the protozoa disease in the blood is too low to detect worm PCR under optical

microscope technique for the early accurate diagnosis of infection disease and insect canine provides high sensitivity and good specificity of diagnosis methods. Moreover, PCR technology has the advantages of simple operation, easy access to reagents and rapid detection, and can be used to identify insect species according to sequence analysis, restriction fragment length polymorphism analysis or nested PCR [2-4]. In the process of PCR detection, to prevent the production of false-positive results, negative control and blank control were set in the test samples, and the false-positive results were detected. The positive control is usually set in the prevention of false-negative results. However, many false-negative results are caused by the presence of PCR inhibitors, PCR instrument failures, reaction system errors, polymerase inactivation, and so on. These problems are not found in the pure positive control. Many scholars believe that it is necessary to put in the internal amplification control (IAC) in the PCR reaction system [5]. Therefore, in the process of PCR, this experiment introduced the amplification internal standard to avoid the occurrence of false negative phenomenon.

II. MATERIALS AND METHODS

A. Clinical Disease Collection and DNA Extraction

Collecting animal hospital diagnosis for *Babesia canis* trypanosomiasis disease of the blood and ACD anticoagulation, save them 20 ° C for later use. The total DNA in the blood was extracted by means of protease K digestion and phenol extraction [6]. The DNA template of chicken genome was preserved.

B. Main Reagents

PMD18 -T Simple Vector purchased from Bio-Engineering (Dalian) Co., Ltd.; 2 X Taq PCR Mix, DH5 alpha-sensing cell, ampicilline, x-gal, IPTG, plasmid - small test kit, common agarose gel DNA recycling kit, etc., and other products are purchased from Tiangen Biochemical Technology (Beijing) Co., Ltd.;

C. Primer Design and Synthesis

Using the chicken genome microsatellite ABR0389 as the internal standard gene, the primers gallus-F and gallus-R were designed and the genomic DNA was used as template to amplify the target fragment 103 bp. B.com 339-F and B.com 339-R were designed according to the genetic sequence of the 18S rRNA gene sequence of the *Babesia canis*, and the genome of the *Babesia canis* genome was used as the template, and 339 bp was amplified from the genomic DNA of *P. bassiana*. According to the genetic sequence of the 18S rRNA gene sequence of the *Babesia canis*, B.com 1700-f and B.com 1700-r were used as the template for the genome of the *Babesia canis* genome, and the expanded target fragment was about 1700 bp. The primers were designed as BEA 339-F-gallus-F and B.com 339-F-gallus-R. The primers were used to amplify the target fragment with 103 bp PCR product amplified by gallus-F and gallus-R Length of 150 bp, the target fragment containing the chicken genomic DNA fragment also contains *Babesia canis* primers B.com 339-F and B.com 339-R. The primer sequences are shown in Table 1 and are synthesized by Invitrogen Corporation Shanghai Representative Office.

D. PCR Detection Method

All the PCR reaction systems in this experiment were treated with 25 L PCR reaction system: 2.5 µL 2 × Taq PCR Mix, 2.5 µL Primer F (10 µM), 2.5 µL Primer R (2 µM), 2.5 µL template DNA, and water was added to 25 µL. All the PCR reaction program adopts touchdown PCR reaction: pre-denaturation is at 94 ° C for 3 min. Denaturation is at 94 ° C, 15 s; Annealing 65° C, 15 s, each loop drop 1 ° C; extension 72 ° C, 30-90s (adjusted for extension of time according to product size); 10 cycles. Finally, it should stretch up to 72 ° C, 5 min. The PCR product was tested by 2% agarose gel electrophoresis (embedded with Goldview 30 mu L/L), constant

pressure 120V, electrophoresis about 30 min, and was photographed under the bio-rad gel imaging system.

E. Construction of Internal Standard Plasmids

According to the method described in the bibliography [7], the plasmid containing the *Babesia canis* primer B.com 339-F and B.com 339-R and the chicken genomic DNA of B.com 339-F and B.com was constructed. First, gallon-F and gallus-R were used as template to amplify the 103 bp fragment of chicken microsatellite ABR0389, and then using 10³ bp PCR product as template, B.com 339-F-gallus-F and B.com 339-F-gallus-R as a primer, amplified PCR product length 150 bp. After the PCR products of 150 bp obtained by agarose gel electrophoresis, the DNA fragments were recovered by using normal agarose gel DNA recovery kit, and the DNA fragment was connected to pMD18-T Simple Vector (2692 bp), which was constructed as pMD18-T-B.com-gallus plasmid. And then transformed into DH5α competent cells, coated with LB / Amp / X-Gal / IPTG medium plate (Ampline 0.1mg·mL⁻¹, X-Gal 0.04 mg·mL⁻¹, IPTG 0.024 mg·mL⁻¹) White colonies were identified by PCR using pUC series primers Primer RV-M and Primer M13-47, and the target fragment was 305 bp. The positive clones were inoculated with LB liquid medium (Ampline 0.1 mg / mL) and cultured at 37 ° C overnight. The plasmid extracted with plasmid kit. TE was dissolved and the DNA concentration was measured with Nanodrop, and stored at -70 ° C. The plasmid copy number was calculated according to the formula (1) according to pMD18-T-B.com-gallus plasmid size of 2842 bp (2692 bp + 150 bp).

Genome copy number = genomic DNA amount (µg) × Avogadro coefficient (mol⁻¹) / DNA length (bp) / 10⁶(µ g g⁻¹) / 650(g mol⁻¹ bp⁻¹), Avogadro coefficient: 6.022×10²³ (mol⁻¹); 650: Average molecular weight per base pair

Table 1 Information of primers

Primer sequences	Fragment length
TTGCCTTAGGactgtaag	103
CCTCTATCAGcatccatc	
gtcttgaattggaatgatggtgac	339
atgcccccaacegttcttatta	
gtcttgaattggaatgatggtgac-TTGCC TTAGG	150
atgcccccaacegttcttatta- CCTCTATCAG	
aacctggtgatcctgccagtagtcat	1700
gaatgatcctccgcaggttcacctac	

F. The construction of the standard heliotype granules with the total length of the 18S rRNA of Babesia canis

Using B.com 1700-F and B.com 1700-R primers for *Babesia canis* genome DNA as a template to amplify the PCR product length of 1700 bp and 1700 bp by agarose gel electrophoresis of PCR products, the DNA fragment was recovered by ordinary agarose gel DNA recovery kit, and the DNA fragment was ligated into pMD18-T Simple Vector (2692 bp) to construct pMD18-T-B.com1700 plasmid method with 1.5 amplification internal standard plasmid.

G. Amplification of Internal Standard pMD18-T-B.com-gallus PCR Detection Sensitivity

The amplification of the internal standard plasmid pMD18-T-B.com-gallus was diluted 10 times with sterile water and PCR amplification was carried out using B.com 339-F and B.com 339-R primers to determine the sensitivity of PCR detection of the internal standard.

H. Standard Positive Particle pMD18-T-B.com1700 PCR Detection Sensitivity

The plasmid pMD18-T-B.com1700 with full length of 18S rRNA gene of *Babesia canis* PCR was diluted 10 times with sterile water, and amplified by B.com 339-F and B.com 339-R primers. The sensitivity of PCR detection of canine BABEL was determined.

I. Determination of the optimum amount of added in the PCR detection system of Babesia canis

A standard positive plasmid pMD18-TB.com1700 (10^6 - 10^4 copies / 25 μ L reaction system) with different copy numbers was added to the same PCR reaction system and the internal standard plasmid pMD18-TB.com-gallus (10^6 - 10^4 copies / 25 μ L reaction system) as a template, and PCR amplification was carried out using B.com 339-F and B.com 339-R primers to perform a comprehensive trial optimization to determine the best internal standard plasmid that did not affect the PCR Add the amount.

J. Actual Sample Detection Application

The blood of 0.5 dogs suspected of being infected with BABEL's disease was collected, and the total DNA in blood was extracted by 0.1mL, and finally dissolved by 50 μ L water DNA mL. The

sample PCR reaction system: 2 \times Taq PCR Mix 12.5 μ L, upstream and downstream primer B.com 339-F and B.com 339-R 0.5 μ L, amplification of internal standard plasmid pMD18-TB.com-gallus 10^6 copies, sample DNA 10 μ L, replenishing water to 25 μ L; Positive control was added to standard positive control plasmid pMD18-T-B.com 1700; Negative control of healthy canine genomic DNA; Blank control does not add any templates. The reaction conditions and electrophoresis methods are the same as the 1.4 PCR. The results showed that: ① the standard positive control lane contains 2 bands, and one band size is 339bp, the other stripe size 150bp; Standard negative control lane contains 1 stripe, and the size is 150bp; there is no stripe in the blank control lane. Positive control, negative control, blank control did not meet the above results to determine that the test results were invalid and re-examined.

III. RESULTS AND ANALYSIS

A. Construction of the internal standard plasmid pMD18-T-B.com-gallus and PCR detection sensitivity

PCR was carried out using the pUC series universal Primer RV-M and Primer M13-47 to amplify the target band of 305 bp (lane 3 in Figure 1) using the plasmid pMD-18-TB.com-gallus as template. (Fig. 1, lane 1) can be amplified by gallus-F and gallus-R. The target bands of 150 bp were amplified by B.com 339-F and com 339-R (Fig. 1 Lane 2). The results indicated that the gene fragment of chicken was inserted into pMD18-T Simple Vector, and the fragment contained the primers for identification of *Pseudomonas aeruginosa* and the plasmid pMD-18-T-B.com-gallus was constructed successfully. Sensitivity tests were performed by using B.com 339-F and com 339-R and the results showed that the amplified internal standard plasmid pMD-18-T-B.com-gallus had a sensitivity of 10^5 copies / 25 μ L PCR reaction system (Fig. 2).

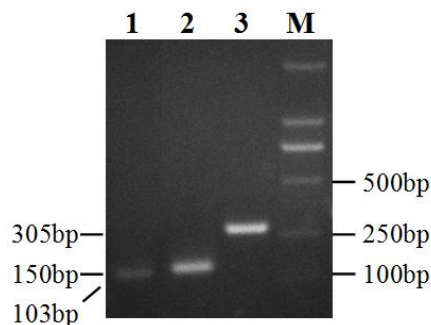


Figure 1 Amplification of the Internal Standard Plasmid pMD-18-T-B.com-Gallus PCR Identification Results

1: gallus-F/gallus-R primers; 2: B.com 339-F/com 339-R primers; 3: Primer RV-M/Primer M13-47; M: Marker (2000, 1000, 750, 500, 250, 100 bp)

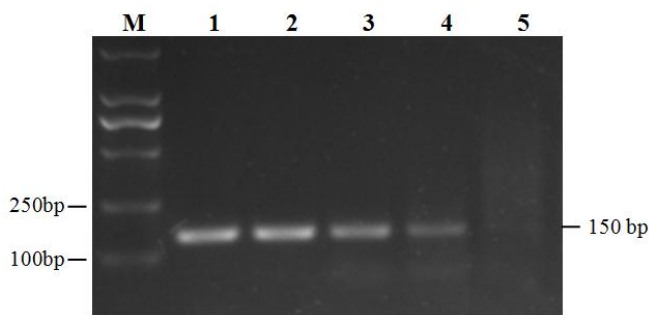


Figure 2 Amplification of the Internal Standard Plasmid pMD-18-T-B.com-Gallus PCR Detection Sensitivity

M: Marker (2000, 1000, 750, 500, 250, 100 bp) ; 1-8: 10^8 copies- 10^4 copies/25 μ L PCRreaction system

B. Construction of standard positive plasmid pMD18-T-B.com1700 and detection sensitivity of PCR

Using the standard positive plasmid pMD-18-TB.com1700 as a template, the pUC series of universal Primer RV-M and Primer M13-47 were used to amplify the target band of 1855 bp (lane 3) com 1700-F and B.com 1700-R can amplify the target band of 1700 bp (Figure 3, Lane 2); B.com 339-F and com 339-R can be used to amplify 339 bp (Figure 3 lane 1). The results showed that the 18S rRNA gene fragment was inserted into the pMD18-T Simple Vector, and B.com 339-F and com 339-R were correctly amplified. The standard positive plasmid pMD-18-TB.com1700 was successfully constructed The Sensitivity tests were performed using B.com 339-F and com 339-R and the results showed that the standard positive plasmid pMD-18-T-B.com1700 showed a sensitivity of 10^4 copies / 25 μ L of the PCR reaction system (Figure 4).

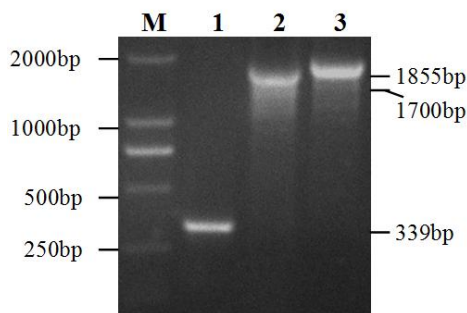


Figure 3 Standard Positive Plasmid pMD-18-T-B.com1700PCR Identification Results

M: Marker (2000, 1000, 750, 500, 250, 100 bp) ; 1 : B.com 339-F/com 339-R primergallus-F/gallus-R primer; 2: B.com 1700-F/B.com 1700-R primer; 3: Primer RV-M/Primer M13-47 primer

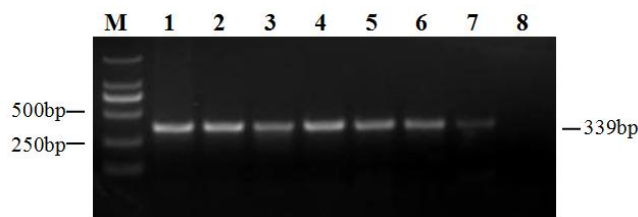


Figure 4: Standard Positive Plasmid pMD-18-T-B.com1700 PCR Detection Sensitivity

M: Marker (2000, 1000, 750, 500, 250, 100 bp) ; 1-8: 10^{10} copies- 10^3 copies/25 μ L PCRreaction system

C. Determination of the optimum amount of amplified internal standard in the PCR detection system of Babesia canis

The standard positive plasmids of different copy numbers and the amplified internal standard plasmids were added to the same PCR reaction system at the same time. PCR amplification was carried out with B.com 339-F and B.com 339-R primers. The results are shown in Fig.5. When 10^8 μ L of the amplified internal standard plasmid was added to the 25 μ L reaction system, the standard positive plasmids were amplified by no PCR bands (lanes 5, 10, 15 in Figure 5); when 10^7 μ L was added to the 25 μ L reaction system, the sensitivity of the standard positive plasmid was 10^6 copies / 25 μ L PCR reaction system (Fig. 5 lanes 4, 9, 14). When 10^6 μ L of the amplified internal standard plasmid was added to the 25 μ L reaction system, the standard positive plasmid assay sensitivity was 10^5 copies / 25 μ L PCR reaction system (lanes 3, 8, 13 in Figure 5); When 10^5 copies of the amplified internal standard plasmid were added to the 25 μ L reaction system, the standard positive plasmid assay sensitivity was 10^4 copies / 25 μ L PCR reaction system (lanes 2, 7, 12 in Figure 5), but the standard positive plasmid exceeded 10^6 copies / 25 μ L PCR reaction system, and the internal standard did not amplify the corresponding fragments. Therefore, it is appropriate to add 10^6 copies of the amplified internal standard plasmid to the 25 μ L reaction system in this experiment. The detection sensitivity is 10^5 copies / 25 μ L PCR reaction system at this time.

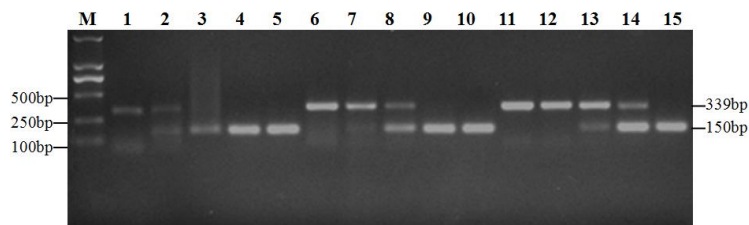


Figure 5 Determination of the Addition of the Internal Standard Plasmid pMD-18-T-B.com-Gallus

M: Marker (2000, 1000, 750, 500, 250, 100 bp) ; 1-5 : pMD-18-T-B.com1700p additive amount is 10^4 copies/25 μ L PCR reaction system , pMD-18-T-B.com-gallus additive amount is 0, 10^5 copies, 10^6 copies 10^7 , copies 10^8 /25 μ L PCR reaction system; 6-10: pMD-18-T-B.com1700p additive amount is 10^5 copies/25

μL PCR reaction system, pMD-18-T-B.com-gallus additive amount is 0, 10⁵ copies, 10⁶ copies, 10⁷ copies, 10⁸ copies/25 μL PCR reaction system; 11-15: pMD-18-T-B.com1700p additive amount is 10⁶ copies/25 μL PCR reaction system, pMD-18-T-B.com-gallus additive amount is 0, 10⁵ copies, 10⁶ copies, 10⁷ copies, 10⁸ copies/25 μL PCR reaction system.

D. Actual Sample Detection

The actual sample test results shown in Figure 6. The standard positive control lane contains two bands, and one strip size is 339bp and the other stripe size is 150bp; the standard negative control lane contains one band with a size of 150bp; there is no band in the blank control lane; only one band of 150bp in the lane of the sample was negative judgments. There were two bands in the lane 2, and one band size was 339bp and the other one is positive for 150bp.

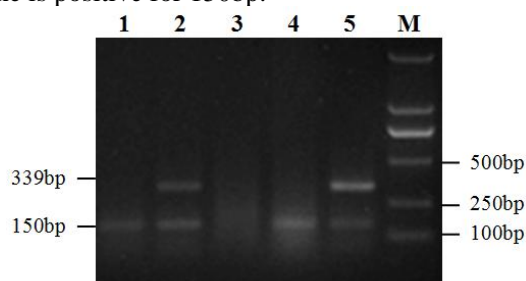


Figure 5 Actual Sample Test Results

1: sample to be tested 1; 2: sample to be tested 2; 3: blank control; 4: standard negative control; 5: standard positive control; M: Marker (2000, 1000, 750, 500, 250, 100 bp)

IV. DISCUSSION

Internal Amplification Control (IAC) is a DNA sequence that is added to the PCR reaction system and is not homologous to the target gene but can be simultaneously amplified [8]. It is also a family of gene sequences that can be used to indicate false negative phenomenon. If there is no IAC in the reaction system, detected negative results may be due to the absence of the target gene in the reaction system, and the presence of the inhibitor in the reaction system, PCR equipment failure, system

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errors, false negative results caused by the inactivation of the polymerase [9]. If IAC is added to the reaction system, the signal of the internal standard will always appear whether the target gene is present in the reaction system. If the internal amplification control without signal PCR failure, so IAC can solve the false negative phenomenon effectively.

At present, there are many reports on the detection methods of *Babesia canis* PCR. However, there are few reports on the inclusion of internal standard in the detection system to avoid false negative phenomena. The amplified internal standard was designed according to the gene fragment of chicken. The gene is special for chicken. It has no homology with canine genome and *Babesia canis* genome, and is very suitable as an extension of the internal standard gene. Using the complex primers constructs competitive internal standards and internal standard genes and target genes compete in the same reaction system using the same primer, so you need to optimize the concentration of IAC. If IAC concentration is too high, IAC amplification will increase, thereby reducing the detection sensitivity of the target gene. The results of this study also show that high concentrations of IAC will reduce the detection sensitivity of the target gene, while the high concentration of target genes will also affect the amplification of IAC.

According to comprehensive consideration, 10⁶ copies of IAC were added to the 25 μL reaction system at the time of detection of *Babesia canis*, and the detection sensitivity of the target gene was 10⁵ copies. In the actual test, the number of copies of 18S rRNA genes in the blood is about 10⁶ copies, so the sensitivity of this experiment can reach the test requirement. (calculation basis: infection with canine erythrocytes 2.5 × 10⁶ / μL [10], red blood cell dyeing rate of 0.1%, this experiment taking 100 μL of blood to extract DNA, the final solution with 50 μL of water, taking 10 μL DNA as a template, usually 18S rRNA in the genome of the number of copies 100 [11], 2.5 × 10⁶ / μL × 0.1% × 100 μL × 10 μL ÷ 50 μL × 100 = 5 × 10⁶ copies). Actual sample test results also showed that the IAC test with 106 copies was normal.

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