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RESEARCH ARTICLE

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Detection of *Eperythrozoon wenyoni* by PCR assay

Abstract The objective of this research was to develop a detection method for *Eperythrozoon wenyoni* infection using polymerase chain reaction (PCR) assay technique. A pair of primers was designed and synthesized according to the conservative sequence 16S rRNA. The PCR assay was performed with the primers. A 985-bp fragment was amplified by using PCR. The amplified fragments with the expected size were identified by *Eco*R I restriction digestion. The crossing-reaction, specific-reaction and duplicate-reaction indicated that the PCR method is a specific, sensitive, fast and effective method for diagnosing *E. wenyoni* infection at group level.

Keywords cattle, *Eperythrozoon wenyoni*, PCR

1 Introduction

Eperythrozoonosis is a parasitic disease caused by *Eperythrozoon* parasitizing on the erythrocyte surface, plasma and marrow of cattle. The cattle parasitized by *Eperythrozoon* pathogens have the symptoms of anemia, icterus, fever and reproductive disorders (Zinn et al., 1952; Shang, 1994; Lin, 2000). In 1934, Adler et al. (Adler and Ellenbogen, 1934; Smith and Rahn, 1975) found a kind of microorganism named *E. wenyoni* which was similar to blood parasites in morphology. At present, direct microscopy is almost the most common method used in clinical checkups. Very few domestic reports associated with serology diagnosis have been published, whereas there are many methods which have been reported abroad, such as CFT (Daddow, 1977; Splitter, 1985), IHA (Smith and Rahn, 1975; Baljer et al., 1989), ELISA (Nicholls and Veal, 1986; Lang et al., 1987; Schuller et al., 1990; Hsu et al., 1992; Cha and Liu, 2002; Zhang and Zhang, 2006), IFA (Hua et al., 1970) etc. However, a lot of factors could cause morphological changes in erythrocytes, and antibodies often present transient features so the methods above usually result in misdiagnosis (Mason and Statham, 1991; Huang et al., 2003). A PCR assay is a specific, sensitive and fast method which has been applied generally in the detection of antigens. In this study, two primers were synthesized based on the conservative sequence of *E. wenyoni* 16S rRNA and amplified by using PCR. Then the result of sequencing of the amplified fragments was compared with the homology in bases of the 16S rRNA gene of *E. wenyoni* that is published in the NCBI. Their homologies determined their class attribute.

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2 Materials and methods

2.1 Animal

Cows from eight big cattle farms in Hebei Province, China, were used in our research. Ten 12-month-old cows were housed in separate rooms during the period of the study. Body temperature and blood smears were taken daily between 8 and 9 o'clock and the blood smears were stained by the Giemsa method. The percentage of erythrocytes infected with *E. wenyoni* was determined. The highly infected blood was used for producing the antigen or for serological testing. The 80 cows used in the experiment were chosen randomly from the eight cattle farms.

2.2 Design and synthesis of primers

According to the conservative sequence (AF016546) of *E. wenyoni* 16S rRNA, a pair of primers was designed using the software Primer 5.0. It was synthesized by the

manufacturer Shanghai Bioasis Biological Technology Co., Ltd. The sequences were as follows:

P1: 5'-AGTGGCAAACGGGCGAGTAATA-3',

P2: 5'-TAACCAAACATCTCAAGACACG-3'.

2.3 Isolation of *E. wenyoni* from blood sample

Each blood sample collected by venipuncture from the bovine ear was added to a volume-equal physiological saline solution. When greater than 90% of erythrocytes were infected with *E. wenyoni* as determined by microscopic examination of blood smears, the whole blood sample was collected from the jugular vein. The blood sample was then washed with double physiological saline solution and centrifuged at 1500 $r \cdot min^{-1}$ for 10 min. The supernatant was discarded and the sediment washed twice. Then the precipitate was added to a volume-equal 0.15 $mol \cdot L^{-1}$ PBS-T solution (containing 0.15% tween-20, 1% EDTA, pH 7.2), shaken lightly for 1 h, followed by water-curing treatment at 56°C for 1 min and centrifuged at 1500 $r \cdot min^{-1}$ for 10 min again to get the supernatant. This supernatant was placed into another centrifuge tube and centrifuged at 12000 $r \cdot min^{-1}$ for 1.5 h at 4°C. The precipitates obtained through centrifugation were preserved at −70°C.

2.4 Extraction of whole organism DNA

Whole organism DNA was recovered from purified *E. wenyoni* with phenol-chloroform (Sambrook et al., 2001).

2.5 PCR amplification of genomic DNA of *E. wenyoni*

With the extracted DNA as a template, the DNA fragment was amplified by Taq DNA polymerase, adopting a 20 μL reaction system. The conditions of the system were as follows: 0.5 μL Taq DNA polymerase, 2 μL 10×Buffer, 2 μL dNTP, 2 μL DNA, 1 μL primer P1, 1 μL primer P2, and adding ddH_2O up to 20 μL. The amplifications were performed as follows: pre-denaturation at 94°C for 10 min, denaturation at 94°C for 1 min, annealing at 52°C for 1 min, elongation at 72°C for 2 min for 30 cycles, followed by prolonged elongation at 72°C for 10 min. The amplified products were saved at 4°C. The PCR products were visualized under UV light on ethidium bromide-stained 0.8% agarose gel after electrophoresis.

2.6 Identification of amplified fragment by restriction digestion

The PCR products were identified by *Eco*R I enzyme digestion. Based on the analysis of previous 16S rRNA gene sequences, amplified DNA fragments should have an *Eco*R I enzyme-cutting site. Two fragments were obtained by *Eco*R I restriction digestion.

2.7 Sensitivity of the constructed PCR method

The purity and concentration of the purified DNA were determined with the constructed PCR method. The purified DNA concentration was then diluted at the proportion of 1:10; the PCR was performed respectively according to the serial diluted concentrations. The lowest detectable concentration of antigen was determined by PCR.

2.8 Specificity of PCR

In order to test the specificity of the PCR detection method, crossing-tests were performed among *Toxoplasma gondii*, *E. coli*, *Staphylococcus*, and *E. wenyoni* using the developed PCR method.

2.9 Duplicate test

Using the developed method of PCR, *Toxoplasma gondii*, *E. coli*, *Salmonella* and *E. wenyoni* were detected three times in order to validate their dependence.

2.10 Application test

Eighty blood samples obtained from the above cow farms in Hebei Province, China, were detected by PCR, sandwich ELISA and direct microscopy, respectively, for the comparison of detection rates.

3 Results

3.1 Extraction of whole organism DNA

The *E. wenyoni* DNA fragment was characterized on 0.8% agarose gel. There was only one specific band with a high molecular weight (Fig. 1). No other obvious bands were detected. The result showed that the purity of the extracted total DNA was applicable for DNA amplification.

3.2 Result of PCR of genomic DNA of *E. wenyoni*

A band of specific *E. wenyoni* gene was visualized by 0.8% agarose gel electrophoresis. The obtained specific band was approximately 985 bp (Fig. 2). This result was consistent with the theoretical value. PCR could only amplify a specific *E. wenyoni* fragment by the primers.

3.3 Result of cloning and identification of amplified target fragment

Two fragments of 410 bp and 757 bp were retrieved by *Eco*R I restriction digestion (Fig. 3). This result was also in accordance with that anticipated.

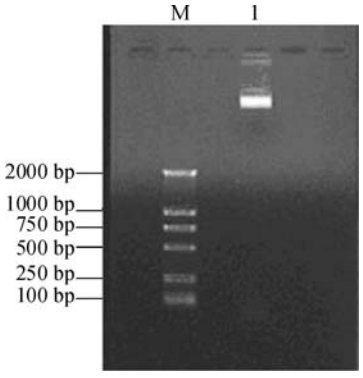

Fig. 1 Purified genomic DNA of *E. wenyoni*
Note: M stands for DL2000 DNA marker; 1 stands for purified DNA of *E. wenyoni*.

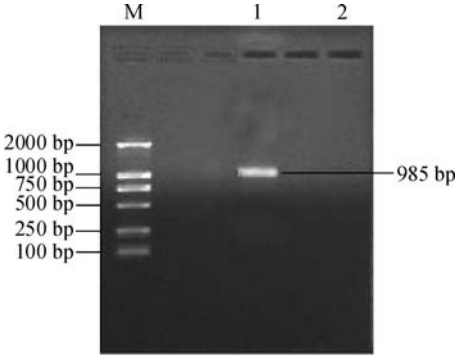

Fig. 2 DNA fragment amplified by PCR
Note: M stands for DL2000 DNA marker, 1 stands for PCR products, and 2 stands for the negative control.

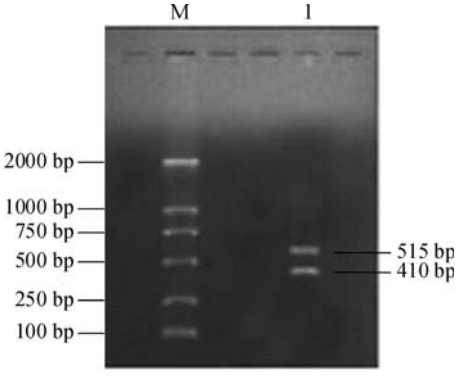

Fig. 3 Enzyme-cutting identification of amplified products
Note: M stands for DL2000 DNA marker, and 1 stands for *Eco*R I digestion results.

3.4 Result of sensitivity of PCR

The purified PCR products were diluted at the proportion of 1:10. One pg DNA could be detected by the PCR assay method (Fig. 4).

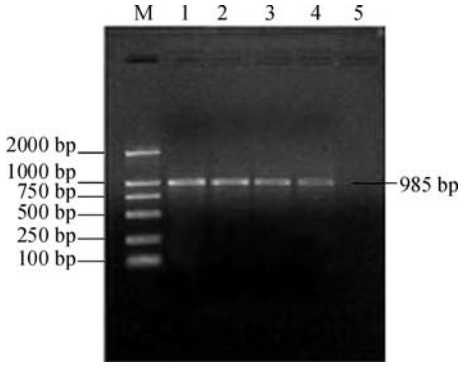

Fig. 4 Sensitivity of PCR products
Note: M stands for DL2000 DNA marker; 1–5 for the PCR results with the serial concentration of template DNA of 1 ng, 100 pg, 10 pg, 1 pg, and 0.1 pg, respectively.

3.5 Result of specificity of PCR

Proven by specificity tests, this diagnostic sequence could not amplify *Toxoplasma gondii*, *E. coli* and *Staphylococcus*, but could do so for *E. wenyoni* (Fig. 5). Results remained the same for three repeated performances under the same conditions.

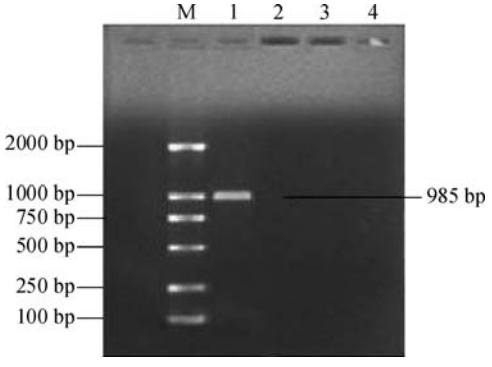

Fig. 5 The result of specificity of PCR
Note: M stands for DL2000 DNA marker; 1, 2, 3, and 4 stand for the amplified fragment of *E. wenyoni*, the amplified fragment of *Staphylococcus*, the amplified fragment of *E. coli*, and the amplified fragment of *Toxoplasma gondi*, respectively.

3.6 Result of application test

Eighty blood samples were identified, and the positive rate through direct microscopy was 72.5% (58/80), through sandwich ELISA 87.5% (70/80), and through PCR 92.5% (74/80). PCR diagnosis was found to be the most sensitive assay method due to its high detection rate.

4 Discussion

The quantity and purity of template DNA were the key points in the completion of the PCR. Because there were

lymphocyte DNAs in the blood and many enzymes could digest DNA, the template DNA must have high purity without other pathogenic proteins and non-pathogenic materials. The condition of pre-denaturation at 94°C for 10 min was applicable for further tests.

The whole DNA could not be extracted when the infection rate in erythrocytes was lower than 90% because *Eperythrozoon* was difficult to purify. A DNA isolating reagent kit was used in the extraction of *Eperythrozoon* DNA, which was amplified as the template by PCR to detect Eperythrozoonosis (Vandervoort et al., 2001; Zhang et al., 2005). Therefore, Cathepin-K was used in this research to digest the proteins, especially the histones attached to the DNA. The *E. wenyoni* DNA was then extracted with phenol-chloroform and precipitated with ethanol.

According to the conservative sequence (AF016546) of *E. wenyoni* 16 S rRNA published in NCBI, a pair of primers with high specificities was designed. A 985-bp fragment was amplified with the extractive DNA by PCR. It was indicated by *Eco*R I restriction digestion which had two fragments (410 bp and 575 bp) with the expected size. The diagnostic sequence could not amplify *Toxoplasma gondii*, *E. coli* and *Staphylococcus*, but could do so for *E. wenyoni*. Joanne et al. (1999) once designed four pairs of primers, three of them able to amplify the DNA of *Eperythrozoon*. One pg DNA was detected by the PCR method according to the serial concentrations. Our test proved that this method was an accurate detection method for *E. wenyoni* infection.

By the result of the application test, we found that there was a serious infection of *E. wenyoni* in Hebei Province. The positive rate by PCR was 92.5% (74/80), which was higher than the positive rate of 30%–80% in Guangxi (Chen, 2006). PCR was the most sensitive method compared with the methods of direct microscopy and sandwich ELISA, according to their positive rates. The positive rate of PCR was comparatively better than the method of direct microscopy, and direct microscopy was not as accurate because of other pathogens and impurities which could bring forth misdiagnosis and interfere with the results. The establishment of this PCR assay method may offer great support in diagnosing *E. wenyoni* infections.

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