DEVELOPMENT AND APPLICATION OF PCR ASSAY FOR DETECTION OF *BORDETELLA BRONCHISEPTICA* IN RABBITS

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ABSTRACT

With a pair of specific primers designed to amplify fim2 gene of *Bordetella bronchiseptica* BJL06 in rabbits, a fragment of 425 bp in length of the target gene was amplified by PCR. The sequencing result showed that the homology was 100% compared with the reference sequence published in GeneBank. Specificity assays revealed that the assay did not cross react with *Escherichia coli*, *Pasteurella multocida* and *Clostridium welchii*. The least amount of the bacteria that could be detected was 3.6 CPU, and a PCR assay was developed for quick detection of *B. bronchiseptica* infection. One hundred and forty six samples of nasal mucus from rabbits from Jiangsu and Shandong province were examined by the PCR assay. Ninety two samples were positive and positive rates were 63.01%.

Key words: Rabbits, *Bordetella bronchiseptica*, PCR, Detection.

INTRODUCTION

B. bronchiseptica has complete typeⅣ pili synthesis system which is encoded by fimN. TypeⅣ pili is recognized as virulence factors, contributing to the strong adherence of *B. bronchiseptica* to mucosa epithelial cells of animal alimentary respiratory and genital tract. In the study, we designed a pair of specific primers on the basis of fim2 gene sequence. A simple, rapid, sensitive and specific PCR detection method was developed to provide technical support on clinical examination and investigation on epidemiology of *B. bronchiseptica* from rabbits.

MATERIALS AND METHODS

Bacterial samples and strains

Bordetella bronchiseptica BJL06, *Escherichia coli* Lu1, *Pasteurella multocida* C51-17 and *Clostridium welchii* 84-A were isolated and conserved by Jiangsu Academy of Agriculture Sciences. We collected 146 samples of nasal mucus from rabbits from Jiangsu and Shandong provinces.

Main reagent

Common Taq enzyme, $MgCl_2$, 10×buffer, dNTP, EB, Marker and agarose gels were from Takara Biotechnology (Dalian) Co,Ltd; Mac Conkey agar medium and triple sugar-iron-agar medium were from Nanjing ZhuoYue Biotechnology Co.Ltd.

Primers

The primers were chosen to amplify the fim2 gene of the *Bordetella bronchiseptica* :upstream primer: 5'-TGAACAATGGCGTGAAAGC-3';downstream primer: 5'-TCGATAGTAGGACGGGAGGAT-3' A fragment of the expected size (425 bp) was amplified.

Establishment of PCR diagnostic method 1.4.1 Standard strains and template DNA preparation

Picked colony of standard strains were inoculated into 5 ml of nutrient broth. Bacterial suspension was prepared after growth for 18-24 h at 37°C by shake cultivation and then agitated to uniformity. One ml of suspension was centrifuged at 10,000 rpm/min at 4°C for 5 min in sterile. The supernatant was discarded. The pellet was suspended in sterile water and boiled for 10 min. Then the suspension was kept frozen for about 10 min and centrifuged at 10,000 rpm/min at 4°C for 5 min. The supernatant was used as template and stored at -20°C.

Optimization of PCR reaction system. The concentration of Mg^{2++} and primers, the temperature of denaturation, annealing and elongation were sieved repeatedly. When the target band was clear and specific, the optimal reaction condition was determined. We used ddH_2O instead of template DNA as the negative control.

PCR assays. DNA amplifications were performed in a final volume of 25 μl. The reaction mixture consisted of 0.2 μl of Taq polymerase, 2.5 μl of 10×buffer, 1.5 μl of $MgCl_2$, 2 μl of dNTP, 0.5 μl of each primer, 4 μl of template and 13.8 μl of ddH_2O. Reactions were performed for 30 cycles with the following parameters: 5 min of preheating at 95°C, 1 min of denaturation at 95°C, 30 s of annealing at 57.4°C, 1 min of renaturation at 72°C and 10min of elongation at 72°C.

Detection of PCR products. 10 μl of the PCR reaction mixture were analyzed by gel electrophoresis in 1% agarose gel containing 0.25 μl of ethidium bromide per ml. The observation and analysis of the image of the PCR reaction mixture was performed by use of the gel imaging system.

Target fragments cloning and nucleotide sequence analysis. For nucleotide sequence analysis, the PCR products were purified with Gel Extraction Kit. According to the illustrations of pMD-19T-Vector Kit, the target fragments were inserted into the pMD-19T-Vector. Plasmid constructions were performed with *E. coli* DH5α.These inserts were digested with Hind Ⅲ and Kpn Ⅰ at 37°C for 2-4 h. Positive colony was sieved and sequenced by Takara Biotechnology (Dalian) Co. Ltd.

Determination of the concentration of bacterial suspension. One ml of bacterial suspension was diluted serially from 10^{-1} to 10^{-10}. A 100 μl volume of each dilution from 10^{-5},10^{-6},10^{-7} and 10^{-8} was spread on blood agar culture-medium. The concentration of bacterial suspension was determined by counting colonies after growth for 24-48 h at 37°C.

Sensitivity of PCR assay. After the template was extracted from standard *B. bronchiseptica* strains, PCR amplifications were performed according to the reaction system and conditions as described above. The bacterial suspension with the highest dilution ratio was the limit of detection if the specific fragment could be amplified.

Specificity of PCR assay. The template DNA was extracted from *B. bronchiseptica* BJL06, *Escherichia coli* Lu1, *Pasteurella multocida* C51-17 and *Clostridium welchii* 84-A. Then the PCR amplifications were performed synchronously under the same conditions.

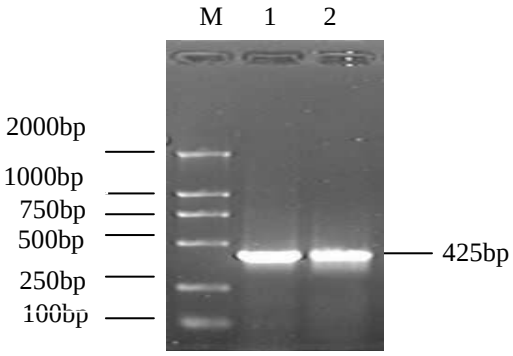
Use of Bb PCR in clinical samples. The performance of the PCR was investigated with 146 selected clinical samples of nasal mucus to evaluate the applicability of the PCR diagnostic method.

Contrast test

Bacterioscopy was performed as conventional laboratory method.

RESULTS

Establishment and use of the PCR assays As shown in Figure 1, after optimizing the reaction system, a fragment of the expected size (425 bp) which was distinctive was amplified with standard *B.bronchiseptica* DNA.The target fragment was cloned and digested with enzyme after reclaiming. The clones were verified by restriction enzyme analysis that they had been cloned into T vector successfully (Figure 2).The nucleotide sequences of the *B. bronchiseptica* fim2 gene was submitted to EMBI Data Library under accession number X74119.The identity between the nucleotide sequences of the fim2 genes is 100% (Figure 3).



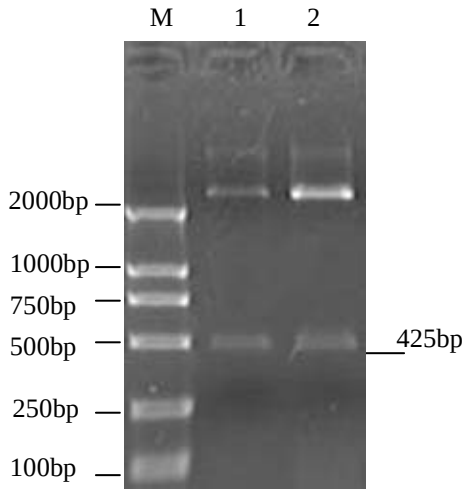


Figure 1: The PCR testing for Bb
M: DL2000 DNA Marker.
1-2: *Bordetella bronchisepti a* (425 bp)

Figure 2: Enzyme digestion of recombinant plasmid fim2 gene/T-vector. M: DL2000 DNA Marker.
1-2: fim2 gene/T-vector(Kpn Ⅰ/HindⅢ)

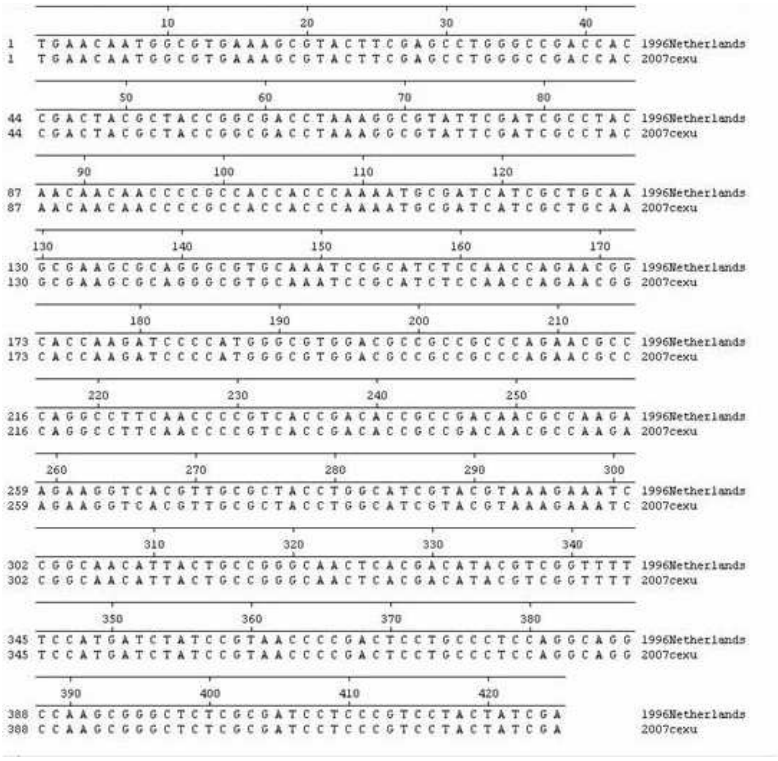


Figure 3: Sequence of fim2 gene and comparison analysis

Sensitivity of PCR assay

The concentration of the bacterial suspension was 9.0×10^{6}CPU/μl. The bacterial suspension was diluted from 10^{-1} to 10^{-10} and then the templates were extracted. The PCR assay was performed with 4 μl template DNA of each dilution. It indicated that the target DNA fragment (425bp) was amplified as long as the content was more than 3.6CPU (Figure 4).

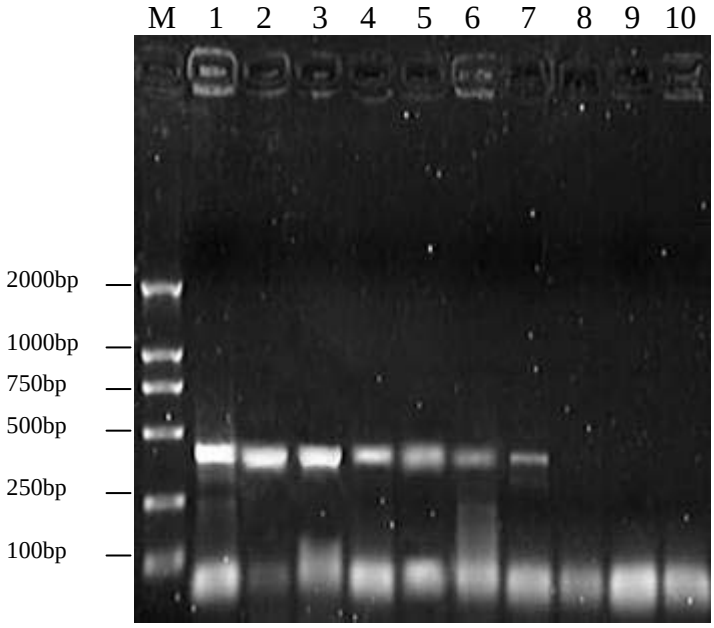


Figure 4: The result of sensitivity assay of the PCR. M: DL2000 DNA Marker; 1: 3.6×10^6 CPU; 2: 3.6×10^5 CPU; 3: 3.6×10^4 CPU; 4: 3.6×10^3 CPU, 5: 3.6×10^2 CPU; 6: 3.6×10CPU; 7: 3.6 CPU

Specificity of PCR assay

The PCR assay was used to detect standard *Bordetella bronchiseptica* strains BJL06,*Escherichia coli* Lu1, *Pasteurella multocida* C51-17and *Clostridium welchii* 84-A. Using the Bb primers, a 425-bp PCR product was detected only from *B. bronchiseptica* (Figure 5).

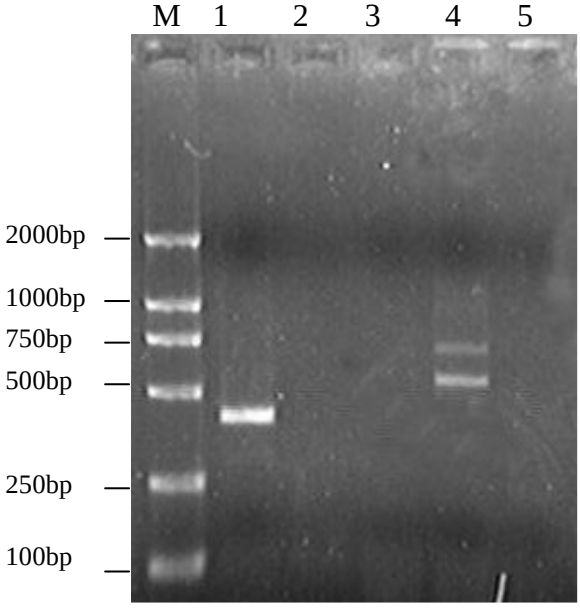


Figure 5: The result of specificity assay of the PCR. M: DL2000 DNA Marker; 1: *B.bronchiseptica* BJL06; 2: *C. welchii* 84-A; 3: *P. multocida* C51-17; 4: *E. coli* Lu1; 5: Negative control

PCR assay for clinical samples

We evaluated the PCR assay by analyzing 146 samples of nasal mucus from rabbits. Ninety two samples were positive and the positive rates were 63.01%. Part results of the detection were shown in Figure 6.

The comparison of the PCR assay with the bacterioscopy

On the 146 samples of nasal mucus from rabbits analyzed by PCR assay and bacterioscopy. 92 were positive by PCR assay (positive rate 63.01%) while 49 samples were positive by bacterioscopy (positive rate 33.56%).

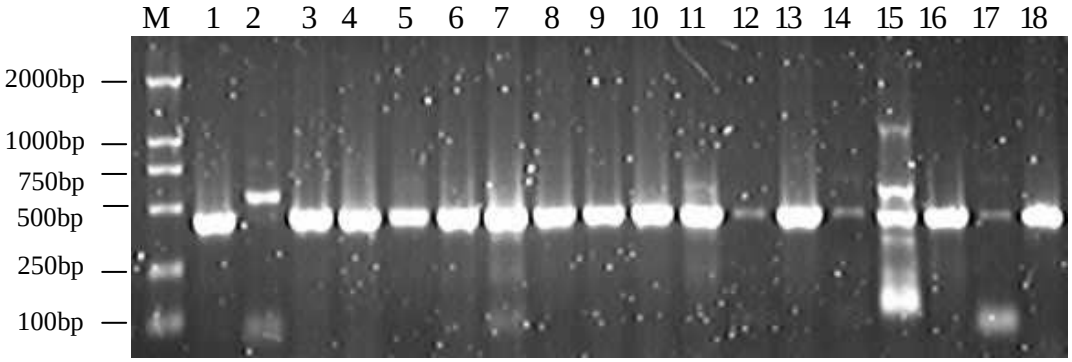


Figure 6: The part results of the detection of nasal mucus from rabbits

DISCUSSION

B. bronchiseptica was primarily isolated from respiratory tract of dogs which suffered from canine distemper by Ferry in 1910. The three species of the genus *Bordetella* are *B. bronchiseptica, pertussis* and *parapertussis*.The respiratory tract infections of rabbits caused by *B. bronchiseptica* usually take rhinitis as mainly clinical symptom. When the season changes, the infection rate of rabbits is very high. If Bb admixing with *Pasteurella multocida* will aggravate the symptom. Spanoghe and Okerman (1978) investigated 32 warrens, the rate of infection of *B. bronchiseptica* was 20-47%. The researchers in china examined rabbits infected with rhinitis and peri-pneumonia. The positive rate of detection for *B. bronchiseptica* was 35.8% (Dong, 1987) and 46.0% (Ma, 1985) respectively. Type IV pili of *B. bronchiseptica* has been shown to serve as essential adhesion factor for colonization, which is encoded by fimN gene. In this study, we designed a PCR method based on fim2 gene. It will take only 18 hours to give result. PCR products are identified when the concentration of bacteria are up to 0.9 CPU/μl, neither need isolation nor enrichment. After reclamation, recombination and cleavage, the DNA sequences of amplified fragment of *B.bronchiseptica* was identical to X74119 submited on GeneBank. This method sufficiently presented high specificity and reliability. We analyzed 146 samples of nasal mucus of rabbits from Jiangsu and Shandong provinces. It has been showed that the PCR assay used were adapted to severe infections as well as carrier status in clinical samples. It will get twice the result with half the effort to control the disease that the infected rabbits and bacteria carriers are isolated and cleared from warrens combined with drug treatments and vaccine therapy, using the PCR assay to detect clinical samples and investigate the state of infections. Compared with conventional bacterioscopy, the developed PCR assay displays many advantages like simple, rapid, efficient, sensitive and specific. It has a widely application foreground on clinical diagnosis and investigation on epidemiology of Bb from rabbits.

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