Establishment of Real-time PCR for detecting Bibersteinia trehalosi for rpoβ gene

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Abstract. Bibersteinia trehalosi is a zoonosis bacterial species, There are few reports of the disease in our country, and there is no fast, sensitive and efficient detection method have been developed. Therefore, the Quantitative Real-time PCR (q-PCR) for detecting Bibersteinia trehalosi has been established, and the specific primers designed based on the conserved sequence of Bibersteinia trehalosi rpoβ gene, the method was constructed by optimizing reaction conditions and then subjected the sensitivity, specificity, reproducibility, and clinical samples test. The results indicated a good linear relations hip when the concentration of standard substance ranged between 1.20×10¹~1.20×10⁵ copies/μL. Inaddition, the minimum detectable concentration was 1.20×10⁻² copies/μL. There are no cross reaction with 20 kinds of bacteria and viruses. All of the CV values of intra-groups and intra-groups were less than 3%. The results showed that the method has the advantages of high sensitivity, strong specificity, good stability, high accuracy and rapid detection. It can be used for early diagnosis of infections in Bibersteinia trehalosi, as well as rapid detection and quantitative analysis of samples.

1. Introduction
Bibersteinia trehalosi is a member of the genus Bibersteinia pasteurellla. According to its ability to ferment arabinose or trehalose in biochemical experiments, it can be subdivided into two biological types: A and T [1]. In 1999, according to the results of nucleic acid hybridization and 16s RNA gene sequence analysis, the four serotypes (T3, T4, T10, T15) of the T biological type of Pasteurella haemolyticus were renamed as Pasteurella trehalosi. In 2007, the bacterium was reclassified and renamed Bibersteinia trehalosi [2].

Bibersteinia trehalosi belongs to a gram-negative coccidiella. A majority of the disease reports in China were mainly ruminants, especially cause respiratory diseases of cattle and sheep, but occasionally infect humans. In animals, the bacteria can cause systemic and septic bacteraemia in sheep and calves, and the main clinical symptoms are dyspnea, skin ulceration and joint enlargement and the body was dissected and showed the typical symptoms of sepsis. Blood spots and anemic infarcts were found in liver [3]. In humans, the bacteria were isolated from the blood of a patient with liver cirrhosis [3], there ar
e also cases of pulmonary infection consistent with animals' clinical symptoms [5]. Cases of intracranial infection [6], brain swelling [6], facial carbuncles [8], and urinary tract infection [9] have also been reported.

To date, the detection methods of trehalose biberstam are mainly based on some biochemical experiments or bacterium growth characteristics, and there are also PCR detection methods for specific genes, however, it takes multiple PCR to distinguish the genus manzanella from the genus trehalose biberstam, and if the two strains were mixed in one sample, they become indistinguishable [10]. rpoβ gene is much conserved in Bibersteinia trehalosi [10], therefore, it was selected as a target gene for q-PCR detecting.

2. Materials and methods

2.1. Bacteria and viruses used for detection
A total of 20 species of bacteria and viruses were isolated, identified and preserved by Sichuan key laboratory of animal diseases and human health, college of veterinary medicine, sichuan agricultural university. They are Bibersteinia trehalosi, Escherichia coli, Proteus, Lactic acid bacteria, Acinetobacter baumannii, Kill pap bacillus, Salmonella, Hay bacillus coli, Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium tuberculosis, Tetanus bacillus, Streptococcus pneumoniae, Clostridium wheelchair, Klebsiella bacteria, Chlamydia, Mycoplasma pneumoniae, Brinell bacillus, Herpes virus, Pseudo rabies virus.

2.2. Experimental materials
TIANamp Bacteria DNA Kit, TIANprep Mini Plasmid Kit, Universal DNA Purification Kit All purchased from tiangen biochemical technology (Beijing) co., LTD. 2×SG Fast q-PCR Master Mix Purchased from Sangon Biotech (Shanghai) Co., Ltd. pMD19-T vector Purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. Lysozyme, Proteinase K and absolute ethanol were purchased from Vanke Enterprise (Chengdu) Co., Ltd.

2.3. The design of primers and synthesis
DNAMAN software was used to compare the sequences of rpoβ of more than 20 strains of biberstam and mansiella from National Center for Biotechnology Information (NCBI), a conserved sequence was founded and a pair of primers were designed according to it (forward primer: CGCACAGGCGAACTCAA; reverse primer: CATCGTCGTGCTCAAGGAAT), the expected amplified fragment size was 187 bp. The primer was synthesized by Sangon Biotech (Shanghai) Co., Ltd.

2.4. Preparation of standard substance
The genome of Bibersteinia trehalosi was extracted by bacterial genomic DNA extraction kit, rpoβ gene was amplified by PCR with a pair of primers designed above, purification the PCR production, and connected with pMD19-T vector, transferred the plasmid into DH5α (escherichia coli).The correct recombinant plasmid was used to prepare the standard substance, and its concentration was measured with Nano drop 2000 (Thermo Fisher).

2.5. DNA extraction
The genomic DNA of 18 kinds of bacterias were extracted according to the manufacture’s instructions (TIANamp Bacteria DNA Kit tiangen biochemical technology (Beijing) co., LTD), genomic DNA of sheep herpesvirus and pseudorabies virus obtained by phenol extraction method and stored at -20°C

2.6. Q-PCR protocol optimize and establish standard curve
The standard substance was used as a template for the optimization of reaction conditions and the establish standard curves. For primer concentration, a total of 6 gradients were set between 0.1 μM to 0.6 μM; for the annealing temperature, a total of 8 gradients are set between 52°C to 60°C. The fluorescence
signal was detected during annealing to determine the optimal primer concentration and annealing temperature. DdH2O was used to dilute the standard into 10 gradients (1.20×10^10~1.20×10^1 copies/μL), and 7 of the standard gradients (1.20×10^7~1.20×10^1 copies/μL) were taken as templates respectively. Negative control was set at the same time for amplification under the optimal reaction conditions. After the reaction, ABI7500 Software v2.0.1 was used to analyze the software to automatically generate the amplification kinetics curve and the standard curve.

2.7. Sensitivity test
DdH2O was used to dilute the standard substance into 15 gradients (1.20×10^1~1.20×10^3 copies/μL) as templates, and the optimal reaction conditions were used for detection to determine the minimum detection amount of the reaction and to evaluate the sensitivity of the reaction.

2.8. Specificity test
DNA from different bacteria and viruses were used as templates, meanwhile, negative control was set up, and q-PCR was performed under optimized reaction conditions and systems. The specificity of different templates was evaluated according to the amplification curve.

2.9. Repeatability and reproducibility test
Using standard plasmids with a 10-fold gradient dilution, three dilution gradient standard plasmids of 1.20×10^8, 1.20×10^6, 1.20×10^4 copies/μL were used as templates, and the repeatability test was conducted; each template was subjected to three different batches of analysis tests for inter-group reproducibility, and the inter-assay variance and inter-assay variance were calculated respectively.

2.10. Testing of clinical samples
Taking 11 samples of sheep lung tissue with dyspnea, skin ulceration, and systemic sepsis as samples, the bacterial genomic extraction kit was used to extract the sample genomic DNA as a template, q-PCR was performed on 11 clinical samples under optimal reaction conditions, and negative and positive controls were set.

3. Result

3.1. Identification of recombinant plasmid
The concentration of recombinant plasmid was 38.05 ng/μL, converted to 1.20×10^11 copies/μL. PCR amplification of the standard plasmid was performed with rpoβ primers, and the products were detected by 1% agarose gel electrophoresis. The results showed that there was a specific band at about 187 bp (Fig.1), and the sequencing results were correct.

![Figure 1](image)

M. Marker; 1. rpoβ gene.

**Figure 1.** Amplification of rpoβ gene by PCR
3.2. Q-PCR protocol optimize and establish standard curve

The best reaction system: 2×SG Fast q-PCR Master Mix 10 μL, RNase-Free H2O 5.8 μL, 1.4μL primer (10μM) each, template 2 μL. The best reaction conditions: 95 ℃ for 3 min; 95 ℃ 10 s, 58 ℃ 10 s, 72 ℃ 30 s; 95 ℃ 10 s; a total of 39 cycles. With the best system and conditions, take 7 dilutions of the standard (1.20×10^7~1.20×10^1 copies/μL) for amplification, and the ABI 7500 Software v2.0.1 analysis software automatically generates the amplification kinetic curve (Fig.2) and standard curve (Fig.3), melting curve (Fig.4). It can be seen that the standard concentration has a good linear correlation in the range of 1.20×10^5 to 1.20×10^1 copies/μL. The slope is -3.680 and the intercept is 37.507. The correlation coefficient R^2 = 0.999, that is, the standard curve Y = -3.680x + 37.507, where X represents the log of the initial copy concentration (copies/μL) of the template, and Y represents the CT value of the sample amplification.

1-5: The recombinant plasmid dilution from 1.20×10^5 to 1.20×10^1 copies/μL, respectively

**Figure 2.** Amplification curves of different dilutions of standards of q-PCR

**Figure 3.** Standards curve of q-PCR
3.3. **Sensitivity test**

The recombinant plasmids with 15 dilution gradients (1.20×10^{11}~1.20×10^{-3} copies/μL) were tested under the optimal reaction conditions, and the minimum concentration of the tested templates was determined to be 1.20×10^{-2} copies/L (Fig.5), indicating that the method was highly sensitive.

3.4. **Specificity test**

The q-PCR for 20 common pathogens, only Bibersteinia trehalosi was detected as positive; other bacteria and viruses and negative controls did not show any obvious amplification curve (Fig.6).
1. Bibersteinia trehalosi; 2. Escherichia coli; 3. Proteus mirabilis; 4. Lactobacillus;
5. Acinetobacter baumannii; 6. Pasteurella multocida; 7. Salmonella; 8. Bacillus subtilis;
9. Pseudomonas aeruginosa; 10. Staphylococcus aureus; 11. M. tuberculosis;
12. Clostridium tetani; 13. Streptococcus pneumonia; 14. Clostridium welchii;
15. Klebsiella pneumonia; 16. Chlamydia; 17. M. Pneumonia;
18. Brucella; 19. Herpes Simplex; 20. Pseudorabies virus; 21. Negative control

Figure 6. Specificity test of q-PCR.

3.5. Repeatability and reproducibility test
The recombinant plasmid samples with 3 dilution degrees were used as templates to carry out the intra-assay repeatability and inter-assay reproducibility experiments by q-PCR under the optimal reaction conditions. The results showed that the CV was both less than 3%, indicating that the method had good repeatability and reproducibility.

Table 1. intra-assay and inter-assay variation of recombinant plasmid.

| Concentration (copies/μL) | Intra-assay variation | Inter-assay variation |
|--------------------------|-----------------------|-----------------------|
|                          | Mean(CT value)        | SD                    | CV (%)     | Mean(CT value) | SD | CV (%) |
| 1.20×10⁸                 | 11.910                | 0.187                 | 1.57%      | 11.038         | 0.196 | 1.78% |
| 1.20×10⁶                 | 18.295                | 0.201                 | 1.10%      | 18.458         | 0.239 | 1.29% |
| 1.20×10⁴                 | 24.645                | 0.349                 | 1.42%      | 24.912         | 0.268 | 1.08% |

3.6. Testing of clinical samples
Among the 11 samples to be tested, two samples showed positive results after q-PCR, and the results of bacterial isolation and identification were consistent with q-PCR.

4. Conclusion
At present, there have been reports of methods for diagnosing Bibersteinia trehalosi mainly including the isolation and identification of bacteria and common PCR methods, but these diagnostic methods still have some deficiencies. For example, the isolation and identification of bacteria takes too long, and common PCR methods it is not suitable for the detection of a large number of clinical samples, and it is not easy to distinguish it from the genus Mannella. The q-PCR is currently the standard method for qualitative and quantitative detection with high accuracy and good reproducibility.

The rpoβ, the DNA-dependent RNA polymerase, located on the bacterial chromosome is a highly conserved housekeeping gene. Each bacteria has at least one copy of the rpoβ gene to meet the needs of
bacterial growth. And it can be used for the identification and genetic relationship of bacteria [11]. In molecular diagnosis, this gene has been used as a new molecular marker for the identification of different brucella species. This gene can identify and distinguish all known brucella species at the biological type level [12].

According to the species specificity of rpoβ, this study compared the rpoβ gene sequences of more than 20 different strains of Bieberstein and Pasteurella in GenBank, and designed a pair of specific primers. The program performed a simulated PCR amplification reaction, and found that there is no non-specific amplification in all databases, which theoretically proves the feasibility and specificity of primers. The primers were used to detect the Bibersteinia trehalosi stored in the laboratory, and the result was positive; at the same time, 20 other bacteria and viruses were tested, and the results did not have any obvious amplification, indicating that this method has good specificity.

When the sample concentration is within a certain range, the CT value and the sample concentration are linearly correlated, and the correlation coefficient is close to 1, indicating that the method is very accurate. A coefficient of variation of less than 3% indicates its repeatability and reproducibility, and its test results are reliable. The testing of 11 clinical samples also proved the reliability of the method. The above results indicate that this method can be used for the rapid detection of Bibersteinia trehalosi in clinical samples of sheep, and it can also achieve the differential diagnosis of Mannella.

Although lambs infected with Bibersteinia trehalosi showed more subclinical symptoms and were not the main pathogenic bacteria in China's breeding industry, but still brought huge hidden dangers to the breeding industry. The bacterium is also a zoonotic bacterium, which should be highly valued in many aspects such as animal breeding industry, human disease prevention and public health safety. In addition, there are few reports and studies on the pathogen in China. Therefore, this study provides a scientific reference for the clinical detection of Bibersteinia trehalosi infection, and also provides a basis for other related research of Bibersteinia trehalosi materials.

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