Development of a real-time TaqMAN probe PCR assay for the detection of bacteroide☆

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Abstract

Objective. To identify and quantify bacteria of bacteroide group by developing a real-time fluorescent quantitative PCR (FQ-PCR) assay. *Methods*. A pair of universal primers and an oligonucleotide probe were designed based on 16S rRNA gene sequences of bacterium, and the oligonucleotide probe was labeled with FAM and TAMRA. Standard sample, bacteria of bacteroide group as the positive control, and 40 strains of other bacteria, HBV-DNA and genome DNA of human monocyte as the negative control, were tested by FQ-PCR. At the same time, the reproducibility, sensitivity and specificity of the FQ-PCR assay were evaluated. *Results.* (1) The developed FQ-PCR could specially detect bacteria of bacteroide group, while the negative control could not be amplified. (2) The FQ-PCR assay showed excellent sensitivity and detected minimum concentration was 10 genomes/μl. (3) The standard curve showed excellent linear correlation between cycle threshold and template concentration, with the coefficient 0.99. (4) The obtained CV values were 0.74% and 0.704%, respectively, which demonstrated that the assay had good reproducibility. *Conclusion.* The developed FQ-PCR assay could successfully detect the bacteroide group with high sensitivity, specificity and reproducibility. [Life Science Journal. 2007; 4(2): 42 – 45] (ISSN: 1097 – 8135).

Keywords: bacteroides; FQ-PCR; TaqMAN-probe; 16S rRNA

1 Introduction

Bacteria of bacteroide group are G- anaerobic bacteria, the natural bacterium of human intestines tract, which also can cause kinds of diseases in clinic. Bacteroide group are frequently isolated from human infections such as peritonitis, abscesses and bacteremia[1]. In G- anaerobic bacteria without spore isolated from patients, the isolated rate of bacteroide group was approximate 90%[2]. So it is important to detect these anaerobic bacterium for diagnosis and treatment. However, conventional methods need 2 – 7 days for detecting these anaerobic bacterium, and the sensitivity and specificity are not satisfactory[3]. Among bacteroide group, *B. fragilis, B. thetaiotaomicron, B. vulgatus, B. ovatus, B. distasonis* and *B. uniformis* were major bacterium that could cause diseases under certain condition. Especially *B. fragilis,* and *B. thetaiotamicron* were very important in clinic[4,5]. The report[6] showed that 54%, 16%, 11%, 10%, 6% and 3% of the infection were caused by *B. fragilis, B. thetaiotamicron, B. vulgatus, B. distasonis, B. ovatus,* and *B. uniformis* respectively. So it is vital to develop a rapid identification method. Detection of *B. fragilis* and *B. thetaiotamicron* by PCR had been reported[5,7,8], but the detection of bacteroide group by PCR had not been found. In this study, a real-time fluorescent quantitative PCR (FQ-PCR) assay was developed to identify and quantify bacteria of bacteroide group, and a pair of universal primers and a universal oligonucleotide probe were designed based on standard 16S rRNA gene sequences from GenBank for the FQ-PCR. The specificity of the primers and the probe were determined by detecting posi-

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tive control bacterium strains and negative control bacterium strains and comparing sequences of primers and probe with sequences in GenBank using Blast program.

2 Materials and Methods

2.1 Design of primers and probe

The universal primers and universal probe were designed based on 16S rRNA gene sequences available in GenBank with ClustalW1.83 software. Respectively, 5′ end and 3′ end of probe were labeled with FAM and TAMRA. The following is sequences of primers and probe. Forward primer: 5′-CGATGAATACTCGCTGTTTGCGAT-3′; reverse: 5′-TGCAATTTAAGCCCGGGTAA-3′. Probe: 5′-FAM-AAGCGGCCAAGCGAAAGC-TRAMA-3′. The length of product is 182bp. Primers and probe were synthesized by TaKaRa Biothechnology Company (Dalian, China).

2.2 Materials

Reagents: FQ-PCR kit, TaqMAN probe, bacteria genome extraction kit and primers were offered by TaKaRa Biothechnology Company (Dalian, China). Bacterial strains were listed in Table 1. *B. fragilis, B. thetaiotamicron, B. vulgatus* and *B. ovatus* were bacteria group, others were negative control group.

Table 1. Bacterial strains in test

Name of bacteria	Number	Origin
B. fragilis	ATCC25285	Stored in our laboratory
B. thetaiotamicron	12002	
B. vulgatus	14002	Offered by Fudan university microorganism laboratory
B. ovatus	2005.12	
The rest (aerobe intestine bacillus)		Offered by clinical laboratory of the First Affiliated Hospital and the Third Affiliated Hospital of Zhengzhou university
Escherichia coli	ATCC25922	
B. longum	DQ259034	
Fusobacterium		Stored in our laboratory
G+ cocci		
G+ bacillus		
Genome of human monocyte		Offered by incretion department of the First Affiliated Hospital of Zhengzhou university
HBV-DNA genome		Offered by molecule bio-laboratory of Xinxiang infection hospital of Henan, china

2.3 Methods

2.3.1 Standard samples preparation and genome DNA of bacteria extraction: The operation was performed by following: Respectively, one colony-forming unit (CFU) of *B. fragilis, B. thetaiotamicron, B. vulgatus,* and *B. ovatus* was picked, and inoculated into liquid culture medium at 37ºC under anaerobic condition for 48 hours; the number of bacterium were measured under microscope[9] and diluted to 6×10^8 cell/ml. 1 ml liquid culture medium was taken out for bacterium genome DNA extraction according to manufacture instructions. 60 μl extraction was obtained, with genome DNA concentration 1×10^7 bacterium genome DNA/μl. The primary extracted liquid was diluted by 10-fold dilution from 1×10^7 to 1×10^0 bacteria genome DNA/μl, and obtained series of dilutions (1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, 1×10^0 bacterium genome DNA/μl). The dilutions of concentration were used as standard samples when the FQ-PCR assay was carried out.

2.3.2 FQ-PCR reaction system: Taq DNA polymerase, 10 μl; primer, 0.8μl; probe, 0.8 μl; ROX Reference DyeII, 0.4 μl; DNA template, 2.0 μl; H_2O, 6.0 μl. Denaturalization 10 seconds at 94ºC, one cycle, followed by 40 cycles of 5 seconds at 94ºC, 60 seconds at 34ºC.

2.3.3 Sensitivity and specificity: The sensitivity was determined by detecting the above 8 dilutions (from 1×10^7 to 1×10^0 bacterium genome DNA/μl). The specificity was evaluated with following two methods: one was that

non-bacteroides (40 strains intestine bacillus, *E. coli, B. longum*, Fusobacterium, G+ cocci, G+ bacillus, genome of human monocyte and HBV-DNA genome) were used as negative control. The other was to repeatedly detect the same mixed sample which was composed of 10% bacteroides genome (1×10^4 genome DNA/μl) and 90% non-bacteroides genome (9.6×10^9 genome DNA/μl) to investigate whether the detection result was influenced by non-bacteroides genome DNA.

2.3.4 Reproducibility: The reproducibility was evaluated with CV values obtained by detecting dilutions with two different concentrations (1×10^5, 1×10^4 bacteria genome DNA/μl) which were detected five times respectively.

3 Results

3.1 Standard amplification curve (Figure 1) and standard curve (Figure 2)

Amplification curves of standard sample (1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2 and 1×10^1) from the left to the right were demonstrated that the detection limit was 10 bacteria genomes DNA/μl by the FQ-PCR assay. There was no amplification curve around 1×10^0 genome DNA/μl.

The standard curve showed excellent correlation between Ct (cycle threshold) and template concentration, and the correlation coefficient was 0.997.

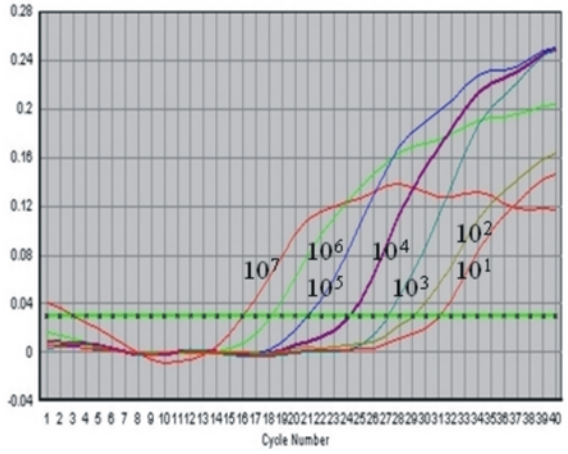

Figure 1. Standard amplification curve.

3.2 Sensitivity and specificity

Standard amplification curve and standard amplification curve showed a very high sensitivity: as small as 1×10^1 bacterium genome DNA /μl could be detected. As for the specificity, fluorescent signals could be detected from positive control, but not from non-bacteroides. Therefore, the universal primers and the probe did not cross-react with genome DNA from non-bacteroides, and were highly specific for bacteriode group. The following was the amplification curve of mixed sample (Figure 3). The amplification curve showed that the detection of bacteroides genome DNA(1×10^4 genome DNA/μl) was not influenced by non-bacteroides genome DNA (9.6×10^9 genome DNA/μl) with the assay.

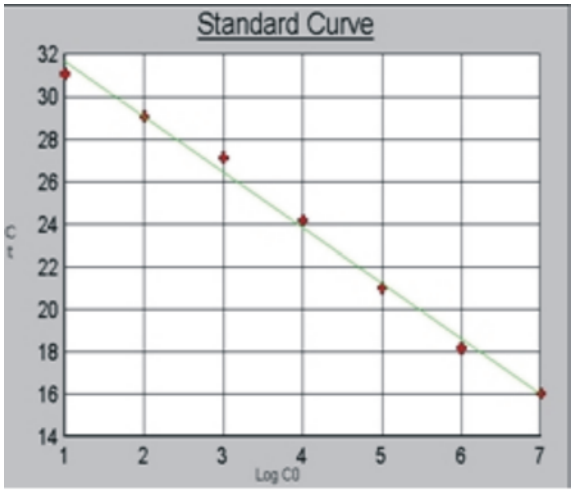

Figure 2. Standard curve.

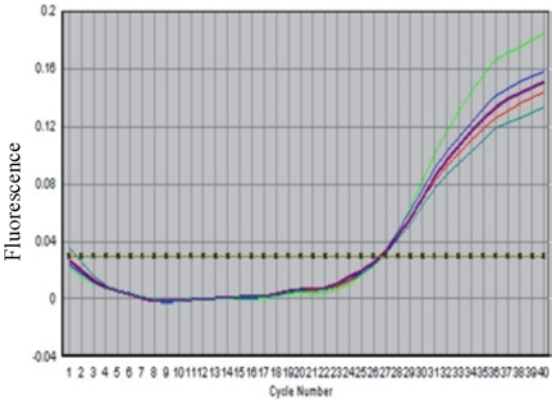

Figure 3. Amplification curve of mixed sample.

3.3 Reproducibility

The following was the graph of amplification of two different concentration dilutions (Figure 4). Detected Ct values of one concentration samples were 24.5, 24.6, 24.6, 24.37 and 24.2, respectively. The other series of Ct values were 26.5, 26.7, 26.4, 26.8 and 26.4, respectively.

CV values were 0.74% and 0.704%, which demonstrated that this FQ-PCR assay had excellent reproducibility.

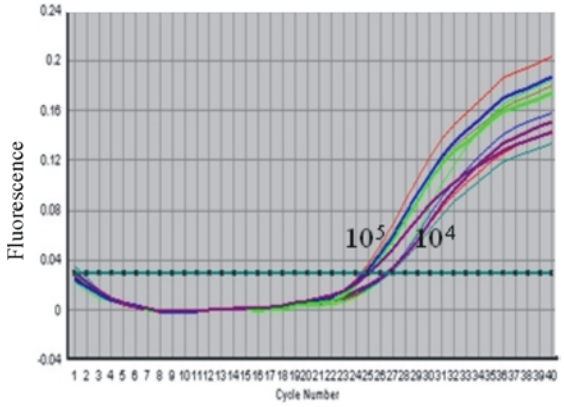

Figure 4. The amplification curves of two different concentration sample (1×10^4, 1×10^5 μl/L).

4 Discussion

Bacteroide group put a significant threat to human health. Consequently, considerable efforts have been devoted to the development of rapid, sensitive, and specific assays for detecting these organisms. At present, the report which detected bacteroide group by real-time FQ-PCR method was not found. In this FQ-PCR assay, fluorescent probe with high specificity was adopted.

This developed FQ-PCR assay used the method of direct microscopic counting bacteria as standard sample, which will obtain more accurate result. Standard sample usually were PCR products or the plasmids with PCR product. The former can not be stored for a long time, while the latter's results were unstable in despite of its long storage. The present standard sample had advantages of long storage and easy preparation. The lost of bacterium genome was not considered when extracted bacterium genome with using purified product of PCR or plasmid as standard sample. The reports showed that the detection result of PCR, product of PCR amplification as standard sample, was approximately 1/10 of the actual quantity[11].

FQ-PCR has high request on sites of primers and probe in genome. One side, gene sequences of primers and probe must be high homologous with bacteroides genome, not with non-bacteroides genome, human monocyte genome, virus genome etc, otherwise, false positive result will be caused; the other side, the sequences must be universal in bacteroides genome, otherwise, false negative result will be caused. The study showed that sensitivity, specificity and reproducibility of the developed FQ-PCR assay were satisfied. As small as 10 bacteria genomes DNA/μl could be detected by this assay. When detecting mixed sample, composed of 10% bacteroides genome (1×10^4 genome DNA/μl) and 90% non-bacteroides genome (9.6×10^9 genome DNA/μl), the assay was not influenced by high concentration of non-targeted genome DNA. Two different concentration samples were deceted repeatedly, and the obtained two CV values were 0.74% and 0.704%, respectively, which demonstrated the good reproducibility.

In total, the FQ-PCR assay could detect bacteroide group successfully and had high sensitivity, specificity, reproducibility, and shortened detecting time. It might be applicable for clinic practices.

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