academicJournals

Vol. 8(9), pp. 908-914, 26 February, 2014
DOI: 10.5897/AJMR12.2206
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Comparison of DNA extraction methods for polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis of the infant fecal microbial communities

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Accepted 6 August, 2013

The study of the intestinal microflora has been developed mainly using conventional microbiological approaches. These studies have made great progress, but it is imperative that new methods will be applied to facilitate scientific progress. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been used to study microbial communities in many environmental samples. So, we must do our best to enhance the quality and accuracy of upstream analyses, such as DNA extraction. In this study, the relative efficacy of four DNA extraction methods (QIAamp DNA stool mini kit method, Q; QIAamp DNA stool mini kit+ Bead beating method, QB; QIAamp DNA stool mini kit+ Frozen thawed method, QF; E.Z.N.A. Stool DNA Kit method, E) were evaluated. Further, PCR-DGGE technique was also assessed in detecting diversity in infant intestinal bacterial fingerprint profiles. The total DNA was extracted from the infant fecal specimens using four different methods, followed by PCR amplification of bacterial 16S rRNA-V3 region, and DGGE separation of the amplifications. The number of extracted DNA of the infant feces using the Q method was larger than the QB, QF and E methods, and the produced bands of its DGGE profiles were more than the other three methods, which was due to its cracking temperature (95°C). We concluded that DGGE of 16S rRNA gene PCR products was suitable for capturing the profiles of the infant intestinal microbial community and the QIAamp DNA stool mini kit method was appropriate for infant fecal DNA extraction. PCR-DGGE could be an important tool for DNA studies.

Key words: DNA extraction method, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), infant intestinal microflora.

INTRODUCTION

The microbial community colonizing the human gastrointestinal (GI) tract is diverse (Eckburg et al., 2005) and plays an important role in digestion, production of essential vitamins, as well as protecting the GI tract from pathogen colonization. The influence of diet on intestinal microflora has been largely studied using conventional microbiological techniques. Many limitations are associated with these techniques, and a significant drawback is their

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Abbreviations: DGGE, Denaturing gradient gel electrophoresis; **DNA,** deoxyribonucleic acid; **PCR,** polymerase chain reaction.

reliance on the identification of appropriate con-ditions. Now, new analytical tools that can be applied in the studies are needed to overcome these limitations.

In the past, molecular techniques based on16S rRNA gene and other genetic markers have been developed to analyze bacterial communities in environmental samples (Nakatsu, 2007). The DNA extraction is crucial for the molecular technique. The ability to obtain the represent-tative total DNA will determine the credibility of subse-quent intestinal microbial diversity analysis. DNA extrac-tion methods can be divided into two categories (Patrick et al., 2003). The extraction efficiency of the direct extrac-ted method is high and the total number of extracted DNA is large, but the content of impurities is also higher, it may affect the subsequent PCR amplification; the purity of the indirect extracted DNA is high, but the total number is smaller; it is not suitable for microbial diversity research, so some suitable methods are demanded.

Compared with other environmental samples, the fecal samples contained large quantities of food residue, gas-trointestinal ingredients and PCR inhibitors, so the extrac-tion and purification of fecal microbial DNA was difficult (Cordova et al., 2010). Bead beating was also a common method which was used in the fecal and intestinal microbial DNA extraction (Zhang et al., 2007). In addition, some commercial fecal microbial DNA extraction kits were also widely used in molecular studies of the gut microflora, such as QIAamp DNA Stool Mini Kit (Johnson et al., 2005) , Fast DNA kit and EZNA Stool DNA Kit. These kits are fast, efficient, high-throughput, suitable for large-scale extracted DNA, and can effectively wipe off PCR inhibitors; they may be the effective methods for the intestinal microbial genomic DNA extraction.

The goal of this study was to compare the relative effi-cacy of four DNA extraction methods, which were used to extract bacterial genomic DNA from the infant fecal specimens. Ultimately, we would aim to illustrate the opti-mized method which was used to extract DNA obtained from the infant fecal specimens.

METHODS

Fecal specimens collection

Healthy infants aged 1-6 months were recruited from a rural area in Handan, He bei province. The collected fecal specimens were divided into two groups: the feces were from the breast-fed infants, denoted by number 1; the others were from formula-fed infants, denoted by number 2, then stored at -80°C prior to being analyzed. The infants did not have any food allergies, antibiotics and any GI diseases (stomach ulcers, colon cancer, and recent bouts of diarrhea, acid reflux disease, and heartburn).

DNA extraction methods

Q- QIAamp DNA stool mini kit method

First, directly weigh 200 mg stool sample in a centrifuge tube (2 ml), then the subsequent steps was performed according to the QIAamp DNA stool mini kit's instructions. In order to improve the wall-breaking efficiency of the G+ bacteria in the feces , we incubated them at 95°C for 5 min after joining the ASL. The final extracted DNA was dissolved in 50 µL AE.

QB-QIAamp DNA stool mini kit+Bead beating method

First, 200 mg stool sample was directly weighed into a centrifuge tube (2 ml), 1.4 ml ASL buffer and 300 mg glass beads (≤ 0.1mm) were added, vortex oscillation for 5 min, then the subsequent steps were performed according to the QIAamp DNA stool mini kit's instructions. The final extracted DNA was dissolved in 50 µL AE.

QF- QIAamp DNA stool mini kit+ Frozen thawed method.

First, 200 mg stool sample was directly weighed into a centrifuge tube (2 ml), 1.4 ml ASL buffer was added, water bath cracking for 5 min at 95° C was done, and then melted immediately at 70°C, repeated freezing and thawing two times was, then the subsequent steps were performed according to the QIAamp DNA stool mini kit's instructions. The final extracted DNA was dissolved in 50 µL AE.

E- E.Z.N.A. Stool DNA Kit method

200 mg stool sample was directly weighed into a centrifuge tube (2 ml), and then the subsequent steps were performed according to the QIAamp DNA stool mini kit's instructions. The final extracted DNA was dissolved in 50 µL AE.

DNA electrophoresis

The quality of the extracted DNA was detected by agarose gel electrophoresis. We used a 0.8% agarose gel electrophore because of the large DNA molecular weight.

PCR-DGGE Analysis

The total extracted DNA in this experiment was mixture containing the DNA of the microorganisms and the intestinal cell. In order to improve the specificity of the PCR product, we used nested PCR amplification. First, we used the total DNA as a template to amplify the bacterial 16SrRNA full-length genes, and then amplified the V3 variable region using the whole 16S rRNA gene as a template. The PCR primers were bacterial universal primers (Wen et al., 2009). The PCR product was separated using DGGE, which was conducted using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA).

Analysis of Bacterial DGGE Banding Profiles and Sequencing

Similarities between banding patterns in the DGGE profile were calculated based on the presence and absence of bands and expressed as a similarity coefficient. Quantity One 4.6 software was applied to count the bands of the DGGE profiles, Unweighted Pair Groupmean Average (UPGMA) was used to cluster analysis, and the Shannon-Weaver index of the samples was calculated. The Shannon-Weaver index's formula was $Shannon\text{-}Weaver = -\Sigma PilnPi = -\Sigma (Ni/N)\ ln\ (Ni/N)$. *Pi* is a ratio of one band's strength which accounted for all bands' strength in the sample; *Ni* is the optical density of the *i*-th band; *N* is a sum of the optical density value of all bands in lanes. Higher index indicates that the species of the sample is more abundant. From the above analysis, we evaluated the efficiency of the different DNA extraction methods.

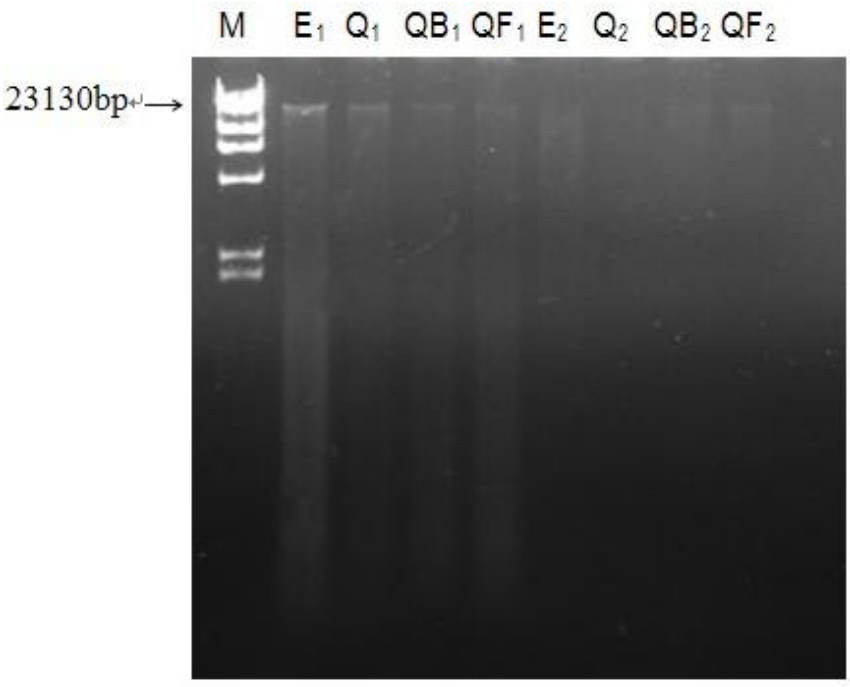

Figure 1. Electrophoresis of infant fecal DNA with different extraction methods. 1, Samples of breast-fed infant; 2, Samples of formula-fed infant.

Statistical analysis

All extractions were performed in triplicate to account for analytical variability. Means of DNA yield were analyzed using SAS (version 9.1; SAS Institute, Cary, NC); Differences were considered as significant when P was < 0.05.

RESULTS

The results of extracted DNA electrophoresis

We extracted DNA from 1 and 2 samples respectively, and used agarose gel electrophoresis; the results are shown in Figure 1. The size of the DNA fragment was about 21kb; it indicated that the large fragment of genomic DNA had been acquired, but the effect of all DNA bands obtained by the four methods was not very good, the brightness was low and fuzzy, which was related to the reason that the sample itself contained fewer microorganisms. In addition, some bands also had obvious smearing. From the above, DNA bands spectrum did not accurately evaluate the extracted efficiency of the four DNA methods. DNA bands spectrum were evaluated only in the quantity of the DNA extraction, but could not reflect the quality. In order to evaluate the effect of DNA extraction in quality, we needed to further detect the biodiversity of microorganisms.

The results of 16SrRNA-V3 region amplification

In order to improve the specificity of the PCR amplification, we used nested PCR amplification. First the whole 16S rRNA gene was amplified and the electrophoresis pattern are shown in Figure 2, and then the V3 region was amplified using the whole 16S rRNA gene as a template; the results are shown in Figure 3. From the electrophoresis pattern (Figure 2), the full-length sequence of 16S rRNA gene was well amplified, its objective band was complete and consistent, had no impurity band, and the size was about 1.5 kb. The quality of extracted DNA by the four methods was suitable for the normal PCR amplification; the consistency of the PCR amplification product could provide a good template for the second round's amplification of the V3 variable region. Seen by the electrophoresis pattern (Figure 3), the size of the target gene fragment was about 250 bp.The objective band was complete, consistent and had not specific band dispersion; it indicated that the specificity of the targeted product was high and less mismatched, and it could be used for the following DGGE analysis.

Analysis of DGGE Fingerprint Profiles

The difference of DGGE profiles was mainly reflected in the number and brightness of the bands (Figure 4). The bands had some differences in the brightness and location. In the spectrum of No. 2 sample, the brightness of zone a, b from the bands obtained by the method E was stronger than the other three methods, but the brightness of zone c, d, e from the bands obtained respectively by the methods Q, QB, QF was significantly higher than the

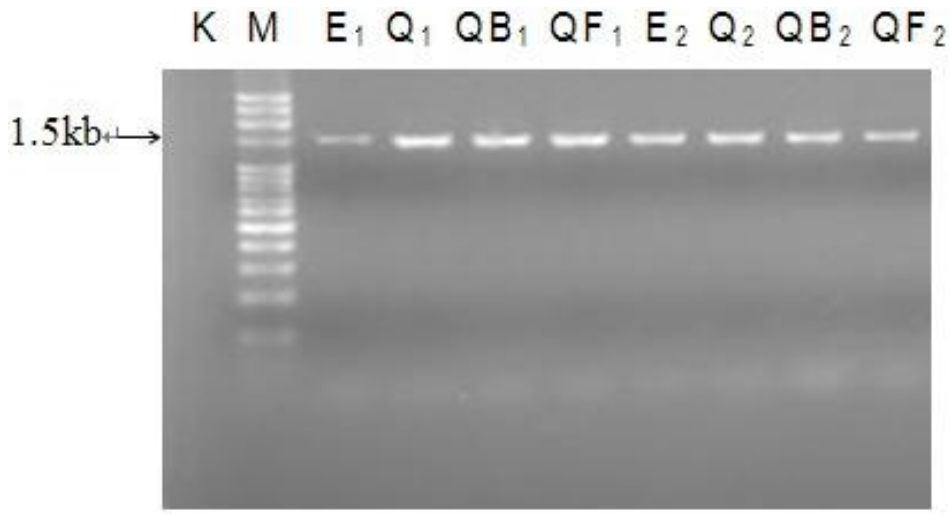

Figure 2. Electrophoresis of amplified product of 16S rRNA of infant fecal DNA with different extraction methods. M, 100 bp DNA ladder marker; K, blank.

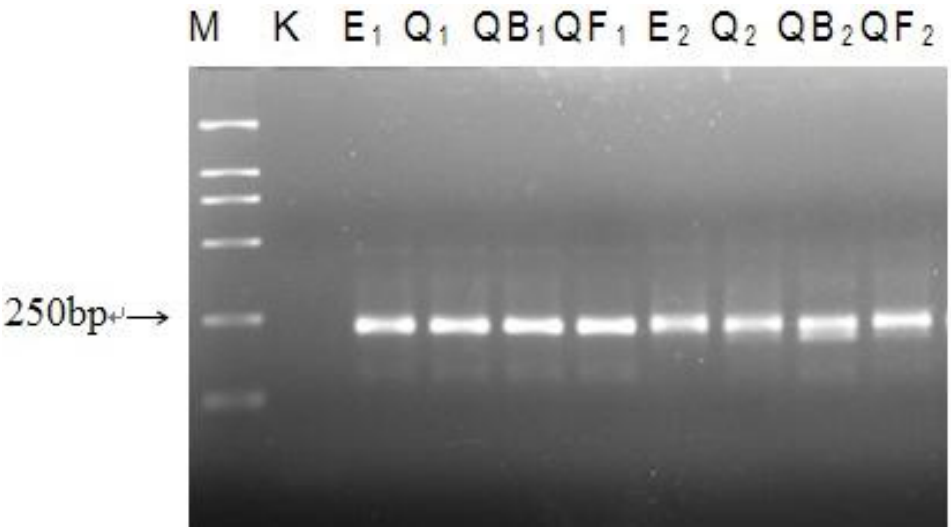

Figure 3. Electrophoresis of amplified product of V3 variable region of infant fecal DNA with different extraction methods. M, DL2000 DNA marker; K, Blank.

method E; these bands should be representative dominant bacterial strain in infant feces. This suggested that the different DNA extraction methods had different sensitivity for the different bacteria. In addition, the number of bands obtained by the different methods was also different.

The clustering analysis of the DGGE profiles is shown in Figure 5. The similar coefficient of the DGGE profile bands obtained by the four DNA extraction methods was ranged from 0.70 to 0.86, but the similarity between the QF method and the other three methods was relatively low, which indicated that the difference between the obtained DGGE spectrum using the QF method and the three methods was large. The similar coefficient of the obtained DGGE spectrum from the Q method and QB method was above 0.80, which indicated that the similarity of the DGGE spectrum obtained from the two methods was high.

The number of bands obtained by the different methods is shown in Figure 6. The number of bands obtained by the methods E, Q, QB and QF in No. 1 sample was 13, 17, 12 and 13, but the number in No. 2 sample was 24, 28, 24, and 23 respectively, which was mainly due to the influence of the different feeding patterns. For the four methods, the number of detected bands in the same sample was different, and the number of bands detected by the Q method was more than the other three methods ($P<0.05$). Shannon-Weaver Index of the four methods in

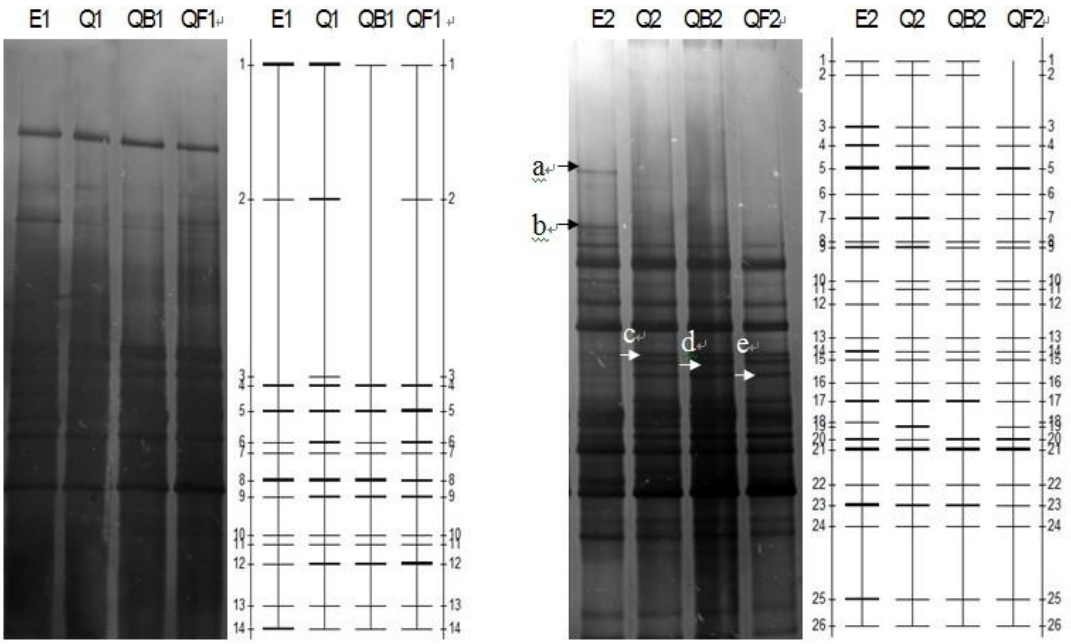

Figure 4. DGGE profiles and schematic diagram of 1 and 2 samples with four DNA extraction methods.

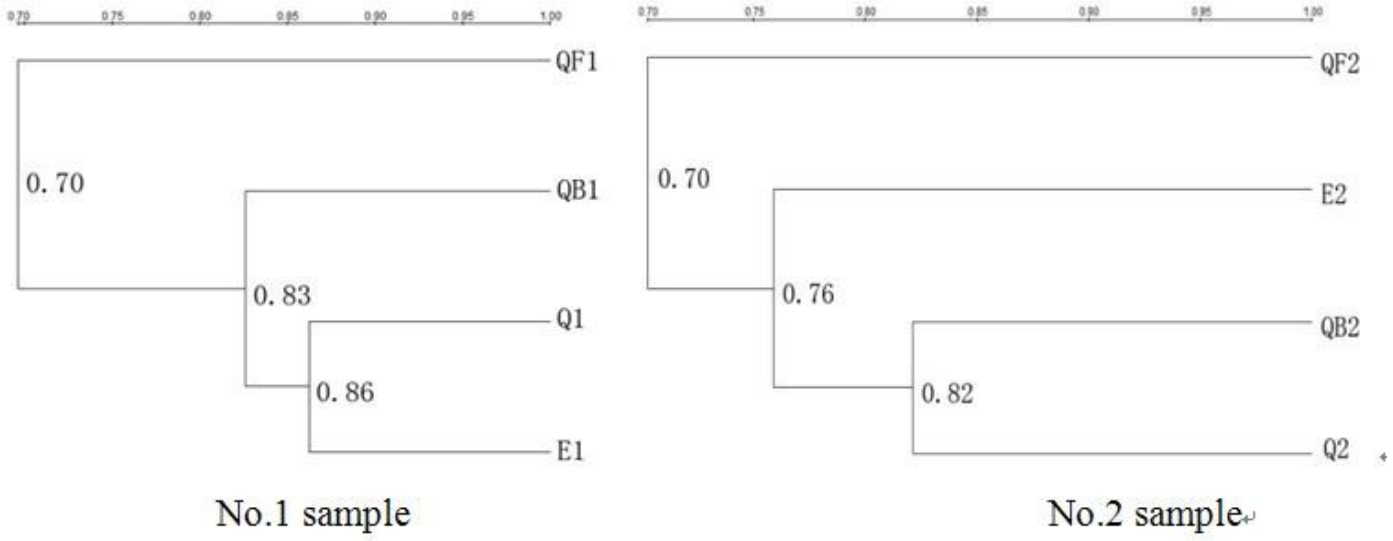

Figure 5. Cluster analysis of DGGE profiles.

1 and 2 samples could well reflect that the infant intestinal flora had a high diversity. While the Shannon-Weaver index (2.87 and 3.23 respectively) in the method Q in 1 and 2 samples was higher than the other three methods, which indicated the DNA extracted by the method Q represented the highest diversity of the infant gut flora ($P<0.05$).

DISCUSSION

The methods for the evaluation of the effect of DNA extraction includes cell microscopy counting method, calculating the recovery of the added alien bacteria and DGGE diversity analysis. In this study, PCR-DGGE was used to evaluate the effect of the DNA extraction from the infant intestinal microorganisms. The DGGE method is a qualitative and semi-quantitative molecular biological method; the abundance of microorganisms can be reflected by DGGE profile. The number of bands reflected the abundance of the infant intestinal flora, and carried semi-quantitative analysis of bacteria by the brightness of the bands.

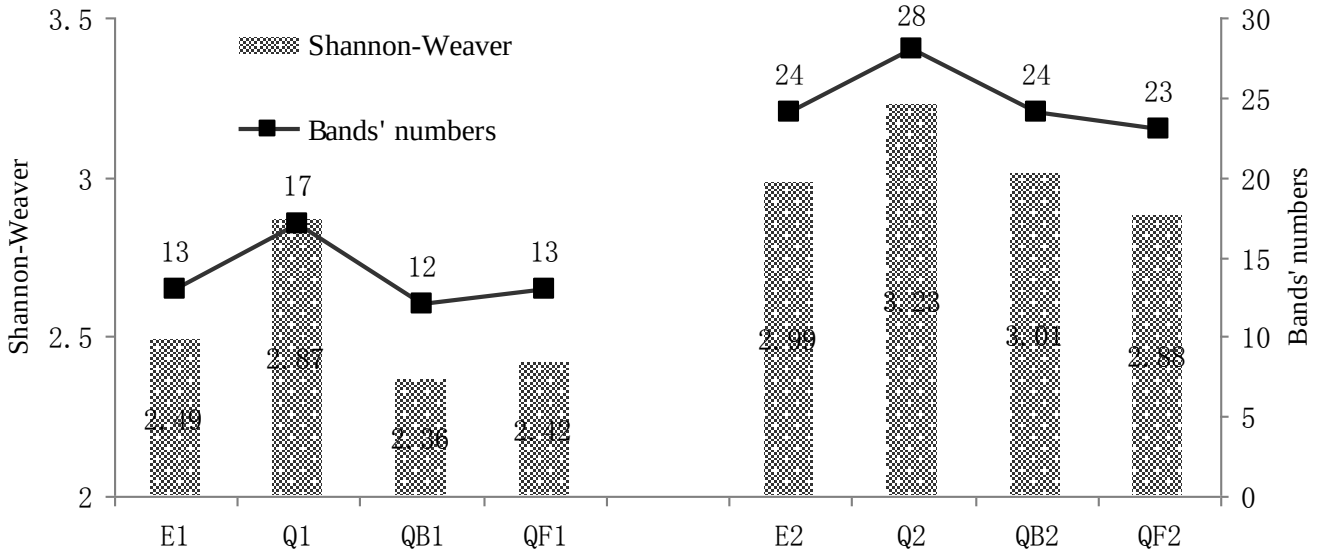

Figure 6. Shannon-Weaver index of 1 and 2 samples with the different DNA extraction methods. Means with the Q method compared within the other three methods are significantly different ($P < 0.05$).

The QIAamp DNA stool mini kit method, QIAamp DNA stool mini kit+Bead beating method, QIAamp DNA stool mini kit+ Frozen thawed method and E.Z.N.A. Stool DNA Kit method are the DNA extraction methods commonly used in the study of gut microflora. The concentration of DNA extracted directly by using the commercialized QIAamp DNA stool mini kit was low that it could not be detected. On this basis, we improved the efficiency of DNA extraction and increased the extracted DNA concentration, then found the suitable DNA extraction kit.

The similarities of DGGE profile bands acquired by four DNA extraction methods were different, which were mainly reflected in the brightness and number of the trips. Previous study showed that the bead beating method had a higher cracking rate of bacterial cells, which was widely used in the intestinal microbial Molecular Ecology (Xiang et al., 2006). Some researchers united the Bead beating method and QIAamp DNA stool mini kit method to increase the efficiency of bacterial lysis (Ben-Amor et al., 2005). The biodiversity of extracted microorganisms from the QB and QF method was slightly lower than the Q method, because vigorous shaking or repeated freezing and thawing could degrade the extracted DNA fragment, and affect the PCR amplification; thereby the abundance of microorganism was reduced. Furthermore, the QB and QF method increased the tedious extraction step and operating time; the result was prone to bias and the cost was increased. So the QB and QF method did not improve the effect of the infant gut microbial DNA extraction. Compared with the Q method, the microbial diversity from the E method was slightly lower, the cost of EZNA Stool DNA Kit was high, and had more steps. The brightness of the bands in the DGGE profiles from the different DNA extraction methods had some difference, which indicated that the proportion of the dominant bacteria in the infant faces was different. This was due to the different degree of bacterial broken, DNA extraction and purification mechanism. In addition, the stool sample micro flora's composition and the sensitivity for the extraction method was different, therefore there were also differences in the extracted DNA (Ariefdjohan et al., 2010).

The QIAamp DNA stool mini kit's bacteria broken wall was moderate, and had been widely used for fecal flora DNA extraction. It was the effective method for the molecular analysis of the gut micro flora. Higher extraction efficiency allowed for better recovery of DNA from an environmental sample; it could provide a more comprehendsive profile of the bacterial community in the sample (Santos and Bicalho, 2012; de Lipthay et al., 2004; Martin-Laurent et al., 2001). Previous study (Li et al., 2003) evaluated the efficiency of the DNA extraction from the QIAamp DNA stool mini kit method by using PCR-DGGE technology and microscope counting method, and found that the bacteria cracking efficiency of the kit was above 95%. From quantitative detection, Mcorist et al. (2002) found that the QIAamp DNA stool mini kit method was the most effective method for extracting the fecal flora DNA. In the present study, this kit was used usually for the infant stool total microbial DNA extraction, but it was found that the concentration of the extracted DNA was relatively low and not detected by agarose gel electrophoresis. To enhance the DNA extraction rate, we increased the cracking temperature to 95°C. After agarose gel electrophoresis, we found that the concentration of the extracted DNA was high, and the microbial diversity was higher.

From comparative analysis and the differences of the infant fecal DGGE profile, we proved that the DNA extraction method would affect the infant intestinal flora's diversity. Compared to QB, QF and E methods, the Q method

was simple, time-short, low-cost, and could reflect the higher diversity of the infant gut flora, so we concluded that the QIAamp DNA stool mini kit method was suitable for the infant fecal DNA extraction. At the same time, we deduced that PCR-DGGE could be an important tool for the DNA studies.

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