Improved extraction of PCR-quality community DNA from digesta and fecal samples

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Several DNA extraction methods have been reported for use with digesta or fecal samples, but problems are often encountered in terms of relatively low DNA yields and/or recovering DNA free of inhibitory substances. Here we report a modified method to extract PCR-quality microbial community DNA from these types of samples, which employs bead beating in the presence of high concentrations of sodium dodecyl sulfate (SDS), salt, and EDTA, and with subsequent DNA purification by QIAamp® columns [referred to as repeated bead beating plus column (RBB+C) method]. The RBB+C method resulted in a 1.5- to 6-fold increase in DNA yield when compared to three other widely used methods. The community DNA prepared with the RBB+C method was also free of inhibitory substances and resulted in improved denaturing gradient gel electrophoresis (DGGE) profiles, which is indicative of a more complete lysis and representation of microbial diversity present in such samples.

INTRODUCTION

There is an increasing interest in the microbial communities resident in the gastrointestinal tracts of vertebrates due to the recognition that these microbiomes play critical roles in the nutrition and health of their hosts (1). To overcome the bias and limitations inherent with cultivation-based methodologies, molecular techniques, such as *rrs*-targeted PCR-denaturing gradient gel electrophoresis (DGGE) analyses, are being increasingly used to better understand microbial diversity and community structure (2–4). For such studies, efficient extraction of community DNA of high purity from digesta, mucosal, and fecal samples is required. However, the microbiome present in such samples is complex and contains numerous hard-to-lyse bacteria. The physicochemical nature of these types of samples can also make DNA extraction particularly difficult (5). A number of methods have been used to extract community DNA from rumen digesta and fecal samples, such as enzymatic lysis and freeze-thaw (3,6), QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) (5,7–10), FastDNA® SPIN Kit (Qbiogene, Carlsbad, CA, USA) (11–13), bead beating (2,14–17), and liquid nitrogen grinding (18). These methods support the recovery of DNA in a form that is suitable for use in various PCR-based studies of microbial diversity. However, there is limited information concerning DNA yields using these different methods, and studies of microbial diversity and community structure will be best supported by DNA extraction methods that provide high yields of representative community DNA free of contaminating materials. Here we report the DNA yields obtained using four different DNA extraction methods: the QIAamp DNA Stool Mini Kit, the FastDNA SPIN Kit, a phenol-dependent bead-beating method (14), and a modified phenol-free bead-beating method [referred to as repeated bead beating plus column (RBB+C) method] developed in our laboratory. The new RBB+C method described here not only results in a 1.5- to 6-fold increase in DNA yield compared to the other methods, but it also produces superior results in PCR-based studies of diversity, as assessed by DGGE of the V3 region of *rrs* genes.

MATERIALS AND METHODS

DNA Extraction Procedures

The detailed procedures of the RBB+C method are described in Table 1. Cell lysis is achieved by bead beating in the presence of 4% (w/v) sodium dodecyl sulfate (SDS), 500 mM NaCl, and 50 mM EDTA. The buffer should also protect the released DNA from degradation by DNases, which are very active in rumen and gastrointestinal samples (19). After bead beating, most of the impurities and the SDS are removed by precipitation with ammonium acetate, and then the nucleic acids are recovered by precipitation with isopropanol. Genomic DNA can then be purified via sequential digestions with RNase and proteinase K, followed by the use of QIAamp columns.

Comparative Assessment of DNA Yield and Quality

A rumen digesta sample and a fecal sample were collected from a hay-fed dairy cow. Each sample was mixed well and divided into 0.25 g aliquots. Genomic DNA was extracted from these aliquots using the RBB+C method described earlier, the FastDNA SPIN Kit, the QIAamp DNA Stool Mini Kit, and the method reported by Whitford et al. [referred to as the bead beating plus phenol/chloroform (BB+P/C) method in this study] (14). DNA extraction was done in duplicates on each sample using each method. The quality of the community DNA was assessed by 0.8% agarose gel electrophoresis, and the percentage of DNA larger than 1.6 kb was estimated using the Spot Denso algorithm of the AlphaEaseFC™ software (Alpha Innotech, San Leandro, CA, USA). The DNA concentrations were quantified using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, USA), and the DNA yields were calculated from the average DNA concentrations of the duplicate extractions and the amount of samples used in the DNA extractions.

PCR and DGGE

The V3 region of the *rrs* gene was amplified by PCR using primers 357f (5′-CCTACGGGAGGCAGCAG-3′) and 519r (5′-ATTACCGCGGCKGCTGG-3′) (20). The 357f primer has a 40-base GC clamp attached to its 5′ end. All PCR amplifications were performed using a PTC-100® Peltier Ther-

mal Cycler (MJ Research, Waltham, MA, USA) in 50-µL volumes containing 1× PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 200 µM dNTP, 500 nM each primer, 1.75 mM $MgCl_2$, 670 ng/µL bovine serum albumin (BSA) (note that successful PCR amplification does not require BSA, however, the inclusion of BSA is recommended to ensure robust amplification), and 1.25 U Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), which allows for hot-start PCR. After an initial denaturation at 94ºC for 4 min, 10 cycles of touchdown PCR were performed (94ºC for 30 s, 61ºC for 30 s, with a 0.5ºC per cycle decrement, and 72ºC for 1 min), followed by 25 cycles of PCR (94ºC for 30 s, 56ºC for 30 s, and 72ºC for 1 min), and a final extension step at 72ºC for 7 min. Negative controls, containing all the components except DNA templates, were included in parallel. Prior to DGGE, 5 µL of each PCR product were subjected to 1.5% (w/v) agarose gel electrophoresis to confirm successful amplification of the V3 region. Then, 15 µL aliquots were resolved in a 7.5% polyacrylamide gel (37.5:1) containing a 40%–60% gradient of denaturants [100% denaturants consisting of 40% (v/v) formamide and 7 M urea]. The DGGE gel was run at 60ºC and 82 V for 15 h using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The DGGE gel was then stained with GelStar® (Cambrex, Rockland, ME, USA), and the gel images were captured using a FluorChem® Imager (Alpha Innotech).

Table 1. Protocol of the Repeated Bead Beating Plus Column (RBB+C) Method

I. Cell lysis:

1. Transfer 0.25 g of sample into a fresh 2-mL screw-cap tube. Add 1 mL of lysis buffer [500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate (SDS)] and 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm).
2. Homogenize for 3 min at maximum speed on a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK, USA).
3. Incubate at 70°C for 15 min, with gentle shaking by hand every 5 min.
4. Centrifuge at 4°C for 5 min at 16,000× *g*. Transfer the supernatant to a fresh 2-mL Eppendorf® tube.
5. Add 300 µL of fresh lysis buffer to the lysis tube and repeat steps 2–4, and then pool the supernatant.

II. Precipitation of nucleic acids:

6. Add 260 µL of 10 M ammonium acetate to each lysate tube, mix well, and incubate on ice for 5 min.
7. Centrifuge at 4°C for 10 min at 16,000× *g*.
8. Transfer the supernatant to two 1.5-mL Eppendorf tubes, add one volume of isopropanol and mix well, and incubate on ice for 30 min.
9. Centrifuge at 4°C for 15 min at 16,000× *g*, remove the supernatant using aspiration, wash the nucleic acids pellet with 70% ethanol, and dry the pellet under vacuum for 3 min.
10. Dissolve the nucleic acid pellet in 100 µL of TE (Tris-EDTA) buffer and pool the two aliquots.

III. Removal of RNA, protein, and purification:

11. Add 2 µL of DNase-free RNase (10 mg/mL) and incubate at 37°C for 15 min.
12. Add 15 µL of proteinase K and 200 µL of Buffer AL (from the QIAamp DNA Stool Mini Kit), mix well, and incubate at 70°C for 10 min.
13. Add 200 µL of ethanol and mix well. Transfer to a QIAamp column and centrifuge at 16,000× *g* for 1 min.
14. Discard the flow through, add 500 µL of Buffer AW1 (Qiagen), and centrifuge for 1 min at room temperature.
15. Discard the flow through, add 500 µL of Buffer AW2 (Qiagen), and centrifuge for 1 min at room temperature.
16. Dry the column by centrifugation at room temperature for 1 min.
17. Add 200 µL of Buffer AE (Qiagen) and incubate at room temperature for 2 min.
18. Centrifuge at room temperature for 1 min to elute the DNA.
19. Aliquot the DNA solution into four tubes. Run 2 µL on a 0.8% gel to check the DNA quality.
20. Store the DNA solutions at -20°C.

RESULTS AND DISCUSSION

We compared four microbial DNA extraction methods to identify a high-yield technique to obtain PCR-quality community DNA from rumen digesta and fecal samples. The techniques assessed included two commercial column-based methods, the FastDNA SPIN Kit and the QIAamp DNA Stool Mini Kit, which rely on bead beating or a proprietary buffer for cell lysis, respectively. A previously published phenol-dependent bead-beating method (BB+P/C; Reference 14) was also used, as well as a modification of this method that uses different DNA extraction conditions and column-based purification (RBB+C; Table 1). The RBB+C method recovered 164.2 µg of community DNA per gram of rumen digesta sample, while the FastDNA SPIN Kit, the QIAamp DNA Stool Mini Kit, and the BB+P/C method yielded only 25.1, 31.6, and 114 µg, respectively. Similar results were obtained with fecal samples. The RBB+C method recovered 82.5 µg of community DNA per gram of sample, whereas the other three methods yielded 15.4, 16.1, and 58.5 µg, respectively. The duplicate DNA extractions produced very similar DNA yields, with the standard deviations ranging from 0.5%–4.3% of the mean. These improvements represent a 1.5- to 6-fold increase in DNA recovery relative to other methods. There are few studies that report DNA yields per gram of digesta sample. One study reported a yield of 25–30 µg of community DNA per gram of rumen digesta, by grinding cells in the presence of liquid nitrogen and cetyltrimethylammonium bromide (CTAB) extraction (18) and a second method employing lysozyme plus freeze-thaw and phenol/chloroform extraction recovered 28.6–30 µg of com-

munity DNA per milliliter of rumen fluid (6). The modifications described here do not add a significant amount of time compared to these other methods but improve DNA yields substantially.

The quality of the recovered DNA is shown in Figure 1. As expected, all the bead-beating methods caused some shearing of community DNA. The BB+P/C method resulted in the worst DNA shearing, probably because of the extended time of bead beating relative to the other methods. The RBB+C method has two important modifications compared to the other procedures. First, an incubation step at 70ºC is included after the bead-beating step to enhance bacterial cell lysis in the presence of SDS (21). Second, the bead-beating step is repeated after the lysate containing the released DNA is collected and replaced with fresh lysis buffer. As such, our modifications both increase yield and minimize damage to community DNA compared to other bead-beating methods. Based on densitometry measurements, about 85% of the DNA recovered by the RBB+C method is larger than 1.6 kb (the length of the entire *rrs* gene), making it suitable for PCR-based analyses of microbiomes.

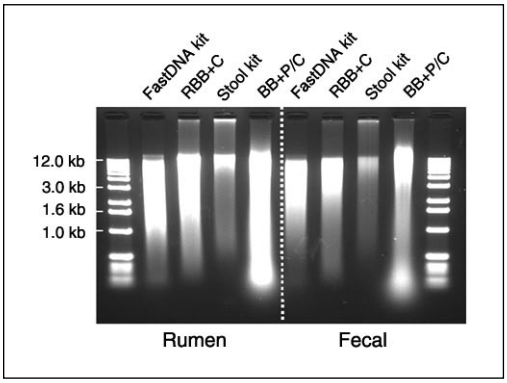

Figure 1. Agarose gel electrophoresis of the community DNA extracted from the rumen digesta and the fecal samples using the four extraction methods. The methods used were the FastDNA SPIN Kit (FastDNA kit), repeated bead beating plus column (RBB+C), QIAamp DNA Stool Mini Kit (Stool kit), and bead beating followed by phenol/chloroform (BB+P/C) extractions. The mobility of the loading dyes, and perhaps the DNA also, in the BB+P/C lanes was noticeably retarded. Rumen, rumen digesta samples; fecal, fecal samples.

The quality of the community DNA recovered by the RBB+C method was further validated by the PCR-DGGE profiles produced from the different community DNA samples (Figure 2). Although DGGE is not a quantitative method, the number of PCR products and their respec-

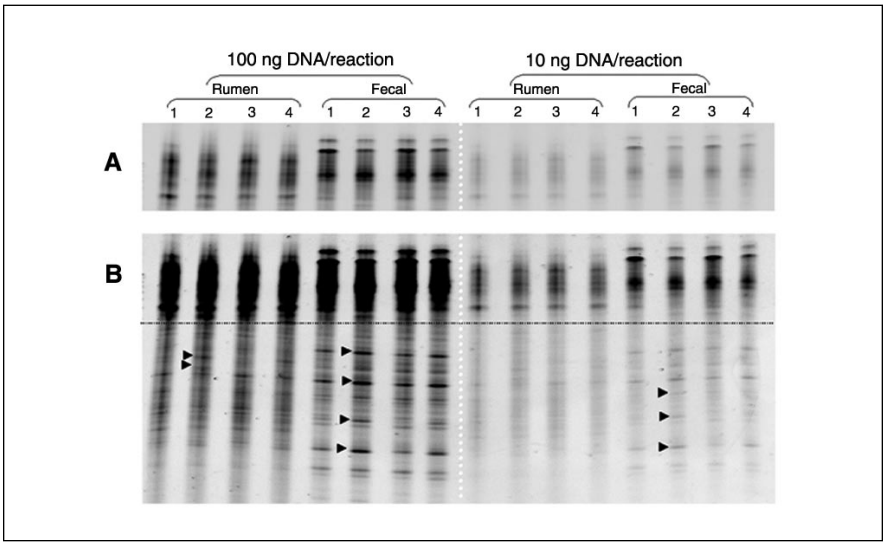

Figure 2. Denaturing gradient gel electrophoresis (DGGE) gel profiles of the V3 region amplified by PCR from the DNA extracted from the rumen digesta and fecal samples using the four methods. (A) The top portion of the gel with reduced exposure. (B) The entire gel. Lane 1, FastDNA SPIN Kit; lane 2, the repeated bead beating plus column (RBB+C) method; lane 3, QIAamp DNA Stool Mini Kit; lane 4, the bead beating followed by phenol/chloroform (BB+P/C) method. The arrows indicate bands with increased intensity or additional bands derived from the DNA extracted with the RBB+C method. Rumen, rumen digesta samples; fecal, fecal samples.

tive intensities are routinely used in a descriptive and comparative manner. The community DNA recovered with our method afforded the detection of extra PCR products, and several other bands in the DGGE profile were also more intense, both of which should improve the resolving power for this type of analysis and maximize the opportunities for effective band excision and reamplification.

Our RBB+C method of DNA extraction adds 20–30 min hand-on time to the process of DNA extraction but results in dramatic improvements in both DNA yield and quality, relative to other bead-beating methods. Additionally, the content and quality of information arising from PCR-DGGE methods of analysis are also improved with DNA extracted using this new RBB+C method. This method could also be used with other types of samples, such as soil and aquatic samples, especially when PCR-based analysis is the preferred method of examining microbial diversity and community structure.

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