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Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces

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Abstract

Feces contain intestinal bacteria and exfoliated epithelial cells that may provide useful information concerning gastrointestinal tract health. Intestinal bacteria that synthesize or metabolize potential carcinogens and produce anti-tumorigenic products may have relevance to colorectal cancer, the second most common cause of cancer deaths in the USA. To facilitate epidemiological studies relating bacterial and epithelial cell DNA and RNA markers, preservative/extraction methods suitable for self-collection and shipping of fecal samples at room temperature were tested. Purification and PCR amplification of fecal DNA were compared after preservation of stool samples in RNAlater (R) or Paxgene (P), or after drying over silica gel (S) or on Whatman FTA cards (W). Comparisons were made to samples frozen in liquid nitrogen (N2). DNA purification methods included Whatman (accompanying FTA cards), Mo-Bio Fecal (MB), Qiagen Stool (QS), and others. Extraction methods were compared for amount of DNA extracted, DNA amplifiable in a real-time SYBR-Green quantitative PCR format, and the presence of PCR inhibitors. DNA can be extracted after room temperature storage for five days from W, R, S and P, and from N2 frozen samples. High amounts of total DNA and PCR-amplifiable *Bacteroides* spp. DNA (34%±9% of total DNA) with relatively little PCR inhibition were especially obtained with QS extraction applied to R preserved samples (method QS-R). DNA for human reduced folate carrier (SLC19A1) genomic sequence was also detected in 90% of the QS-R extracts. Thus, fecal DNA is well preserved by methods suitable for self-collection that may be useful in future molecular epidemiological studies of intestinal bacteria and human cancer markers.

Keywords: *Bacteroides*; DNA extraction; DNA preservation; Enteric bacteria; Feces; Stool

1. Introduction

Feces contain intestinal bacteria and exfoliated epithelial cells that may provide useful information concerning gastrointestinal tract health. For example, bacteria activate or metabolize potential carcinogens (Blaut et al., 2006; Knasmuller et al., 2001; Vanhaecke et al., 2006) or can have anti-tumor effects (Fukui et al., 2001) that may have relevance to colorectal cancer, the second most common cause of cancer deaths in the USA. With the gastrointestinal tract being the largest area of the body that is constantly exposed to ingested/digested food and microorganisms, it is conceivable that luminal exposure may play a significant role in the development of colorectal cancer.

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Epithelial cells in feces represent a potential source of early biomarkers of gastrointestinal tract cancers. Although a variety of biomarkers have been utilized in epidemiological studies on colorectal cancer, most previous markers have been blood-based. However, markers analyzed from intestinal samples may be more relevant to the onset and detection of colon cancer. While approximately 55% of dry fecal weight is attributed to bacteria, Nair and co-workers report that approximately 1.5 million colonic epithelial cells can also be isolated per gram of stool (Desilets et al., 1999; Iyengar et al., 1991). Thus, exfoliated gastrointestinal tract cells in feces may be an alternative for evaluating colon cancer biomarkers.

Stool sample analysis offers a non-invasive opportunity to evaluate both luminal exposure to different types of bacteria as well as exfoliated epithelial cell markers for colorectal cancer risk. However, one of the major obstacles to introducing fecal markers in population studies has been the difficulty in collecting adequate samples for assays from a large number of subjects. This difficulty is exacerbated by the fact that standard fecal collection procedures require fresh or frozen samples, which limits its application in a community-based setting. As a result, epidemiological studies utilizing fecal specimens have often been limited in the number of study subjects and in controlling potential confounders. Fecal self-collection kits have recently been used in large-scale epidemiological studies involving the diagnosis of food-borne illnesses, but these kits lacked any DNA/RNA preservation method, potentially limiting their full usefulness (Jones et al., 2004). Since new technologies have become available to preserve tissue DNA and RNA for a period of time at room temperature, application of such technologies to fecal samples may have great potential for epidemiological studies.

In the present feasibility study, multiple methods for fecal preservation and DNA extraction were tested. Since a major problem with complex samples such as feces is the presence of PCR inhibitors, analytical methods were designed to detect, quantify, and identify conditions under which PCR inhibition was minimal. While this paper focuses on DNA preservation, extraction, and quality, the methods studied were also chosen for their likely suitability for preserving RNA as well. Altogether, several ambient temperature preservation and extraction combinations were capable of yielding usable DNA; however, one combination of ambient preservation and extraction methods gave the most consistent yield of relatively inhibitor-free DNA.

2. Materials and methods

2.1. Stool samples

Fifteen fresh stool samples, obtained from patients being evaluated at the vascular clinic of the John D. Dingell VA Medical Center (Detroit, MI), were collected in plastic containers that were immediately put on ice. The vascular clinic was used for recruitment as it would not be expected that such patients would be more likely than the general population to

surgery clinic. This research protocol was approved by the Wayne State University and VA Medical Center Human Investigation Committees and written informed consent was obtained from each study participant. Samples were further processed or transferred to preservative (see below) within 1 h. Although only ten stool samples were needed, fifteen were collected since five samples were inadequate for further processing due to poor consistency (i.e., too watery) or inadequate quantity and were not used in the study. In addition to the above samples collected at the VA Medical Center (referred to, collectively, in this paper as "VA Samples"), preliminary tests of various methods (prior to the above 15 samples) were conducted with anonymously provided stool samples collected by the Ram laboratory, by methods approved by the Wayne State University Human Investigation Committee.

2.2. Sample preparation, preservation, and storage

For each VA sample, 0.2 g aliquots (at least five for each preservative method) were removed by taking cores of the stool sample with a cut-off 1 ml syringe, where 0.2 ml is ≈0.2 g. Each 0.2 g core received one of the preservative treatments, which included spreading and drying on a Whatman FTA card (W; Whatman, Florham Park, NJ.), drying over silica gel beads (S), submersion in 1.0 ml *RNAlater*™ (R; Ambion, Austin, TX.), immersion in 1.0 ml *Paxgene*™ (P; PreAnalytiX, Hombrechtikon, Switzerland), and refrigerator storage (F). Except as noted for pilot tests, the W, S, R, and P preservation methods incorporated a five-day "hold" period at ambient temperature to mimic the likely delay between self-collection of a sample and receipt by an analytical laboratory, for comparison to alternative storage procedures utilizing 24 h refrigeration or immediate freezing in liquid nitrogen.

For W samples, the 0.2 g of feces was spread over two of the four quadrants of the FTA card, allowed to dry approximately 2 h at room temperature, and then placed in a protective barrier pouch with silica gel desiccant packet. For S samples, 0.2 g of feces was placed over silica gel beads (~10 ml) and ~1 cm of glass wool in a 50 ml tightly sealed sterile polypropylene tube. R and P samples were stored in 2 ml sterile polypropylene tubes. After five days storage at room temperature, W and S samples were transferred to −80 °C. Also, after five days, R and P samples were centrifuged (2 min at 10,000 × g), the supernatant was removed, and the pellet was stored at −80 °C. For F samples, 0.2 g of feces was sealed in a sterile 50 ml polypropylene tube and placed in a 4 °C refrigerator for 24 h and then transferred to −80 °C. On the day of collection, remaining portions of each stool sample (designated N2) were placed in paper-lined aluminum foil wrappers, flash-frozen in liquid N_2, and immediately stored at −80 °C. The above methods, along with their associated extraction methods (next section) are summarized in Table 1.

2.3. Sample extraction

DNA extraction procedures included MoBio Fecal (MB;

Table 1
Summary of stool sample preservation and DNA extraction methods examined in this study

Method abbreviation	Preservation method	Hold time [a]	Extraction method	Stool mass extracted	Time needed for extraction [b]
Q2N-P	Paxgene, 1 ml	5 days	Qiagen RNA/DNA Mini [c]	0.2 g	Two 8 h days
Q2N-R	RNAlater, 1 ml	5 days	Qiagen RNA/DNA Mini[c]	0.2 g	Two 8 h days
Q2N-S	Silica gel beads, 10 ml	5 days	Qiagen RNA/DNA Mini[c]	0.2 g	Two 8 h days
Q2N-W	Whatman FTA card	5 days	Qiagen RNA/DNA Mini[c]	~0.01 g	Two 8 h days
Q2N-F	Refrigeration	1 day	Qiagen RNA/DNA Mini[c]	0.2 g	Two 8 h days
Q2N-N2	Liquid nitrogen	Immediate	Qiagen RNA/DNA Mini[c]	0.2 g	Two 8 h days
QS-P	Paxgene, 1 ml	5 days	Qiagen QIAamp DNA Stool Mini	0.2 g	3–5 h
QS-R	RNAlater, 1 ml	5 days	Qiagen QIAamp DNA Stool Mini	0.2 g	3–5 h
QS-S	Silica gel beads, 10 ml	5 days	Qiagen QIAamp DNA Stool Mini	0.2 g	3–5 h
QS-W	Whatman FTA card	5 days	Qiagen QIAamp DNA Stool Mini	~0.01 g	3–5 h
QS-F	Refrigeration	1 day	Qiagen QIAamp DNA Stool Mini	0.2 g	3–5 h
QS-N2	Liquid nitrogen	Immediate	Qiagen QIAamp DNA Stool Mini	0.2 g	3–5 h
Q2L-P	Paxgene, 1 ml	5 days	Lysozyme; then Qiagen RNA/DNA Mini[c]	0.2 g	Two 8 h days
Q2L-R	RNAlater, 1 ml	5 days	Lysozyme; then Qiagen RNA/DNA Mini[c]	0.2 g	Two 8 h days
Q2L-S	Silica gel beads, 10 ml	5 days	Lysozyme; then Qiagen RNA/DNA Mini[c]	0.2 g	Two 8 h days
Q2L-W	Whatman FTA card	5 days	Lysozyme; then Qiagen RNA/DNA Mini[c]	~0.01 g	Two 8 h days
Q2L-F	Refrigeration	1 day	Lysozyme; then Qiagen RNA/DNA Mini[c]	0.2 g	Two 8 h days
MB-P	Paxgene, 1 ml	5 days	Mo-Bio Fecal	0.2 g	2–3 h
MB-R	RNAlater, 1 ml	5 days	Mo-Bio Fecal	0.2 g	2–3 h
MB-S	Silica gel beads, 10 ml	5 days	Mo-Bio Fecal	0.2 g	2–3 h
MB-W	Whatman FTA card	5 days	Mo-Bio Fecal	~0.01 g	2–3 h
MB-F	Refrigeration	1 day	Mo-Bio Fecal	0.2 g	2–3 h

[a] The hold time is the amount of time the sample is held in or with the preservative prior to transfer to the −80 °C freezer.
[b] The range of time needed for extractions depends on the number of samples (up to 10) processed simultaneously.
[c] The Qiagen RNA/DNA Mini kit also results in the purification of RNA in another step of the two day procedure.

(QS; Qiagen, catalogue number 51504, Hilden, Germany), and modified 2-day Qiagen RNA/DNA Mini (Q2L/N, where 2 stands for "two-day method" and L/N stands for Lysozyme/No lysozyme treatment; Qiagen, catalogue number 14123). In pilot tests, a DNA extraction method accompanying Whatman FTA cards failed to extract DNA effectively from our sample types. This study therefore evaluated MB, QS, Q2N, and Q2L procedures as alternatives for extracting DNA from the Whatman FTA cards. For samples preserved by R, P, S, and F, full aliquots originally weighing 0.2 g were extracted by each method. N2 samples were extracted only by QS and Q2N procedures. For W samples, 20 FTA card-punches (using the Whatman 2.0 mm card punch and giving a total of ~0.01 g of the original fecal sample) were extracted by each method. Accordingly, this study analyzed a total of 220 DNA extracts: 4 extraction methods per each of 5 preservative methods and 2 extraction methods for the N2 method, for each of the 10 VA samples).

All extraction procedures followed original manufacturers' standard procedures for fecal DNA extraction except for the modified Qiagen 2-day procedure and the previously noted alternative to Whatman's procedure. Modifications to the Qiagen RNA/DNA Mini kit included the addition of (or lack of) lysozyme (5 mg/μl, Sigma L-7651) in 200 μl TE buffer (pH 8.0) for an initial room temperature incubation period of 10 min (Q2L method). Samples that were not treated with lysozyme (Q2N=no lysozyme method) were incubated on ice for 10 min with 200 μl TE added to them. Following the incubations, 0.2 g of sterile DNase-free sand and 1 ml of GITC and 1 mM EDTA [pH 7.0], 0.5% 2-mercaptoethanol) were added to both lysozyme and non-lysozyme samples, and samples homogenized for 20 min at maximum speed on a vortex, using a horizontal tube adaptor. Q2N/L samples were then centrifuged at 10,000 ×g for 20 min and supernatant transferred to new tubes. Following centrifugation, 0.5 ml of Qiagen solution QRL-1 buffer was added to each sample and the new solutions passed through an 18 G needle/syringe 10 times. Next, 0.5 ml of Qiagen solution QRV-1 was added to the samples, mixed well, and samples centrifuged (10,000 ×g) at 4 °C for 20 min. The supernatant was then transferred to a new tube, 0.8 volumes of ice-cold isopropanol added, and tubes placed at −80 °C overnight. Day 2 of the 2-day procedure began with step #6 of the manufacturer's instructions, under the animal cell protocol.

MB extraction resulted in 50 μl of DNA solution, while QS and Q2L/N extractions each resulted in 200 μl DNA (the Q2N/L methods also resulted in the subsequent extraction of RNA). The above extraction methods varied considerably in time to complete, as summarized in Table 1, and this factor may also be a consideration in choosing which method to use. Resultant DNA samples were stored at −80 °C until quantitation and characterization could be performed.

2.4. Picogreen assay and DNA quantitation

DNA was measured by a fluorometric *Quant-iT™ Picogreen®* (Molecular Probes, Eugene, OR) assay using the *Bio-Rad MyiQ®* real-time single color PCR detection system (Bio-Rad, Hercules

(RFU) of DNA standard and fecal DNA samples. Phage λ DNA was used as the calibration standard in a dilution series ranging from 0 ng/μl to 200 ng/μl. Fecal-extracted DNA was measured in 2.0 μl of duplicate undiluted (designated 1:1), 1:10, and 1:100 dilutions. *Nanodrop*® (Nanodrop Technologies, Wilmington, DE) spectrophotometer (A260/280) measurements of DNA were also performed on most samples, but often indicated variably higher levels of absorbance than the fluorometric method would have predicted, possibly due to non-DNA contaminants (data not shown), some of which may be PCR inhibitors. Picogreen, with its high affinity and specificity for dsDNA, provided a more reliable measure of DNA.

2.5. Real-time PCR assay

2.5.1. PCR primers and thermocycle conditions

Preliminary PCR experiments involved testing primers for multiple groups of bacterial species, using cycle conditions described in each reference (see list of primers, Table 2.). *Bacteroides* DNA was chosen as the primary target in the VA samples due to its high abundance and consistent presence. Real-time *SYBR®-Green* (Molecular Probes, Eugene, OR) PCR of the VA DNA samples, was accomplished using a 16S rDNA *Bacteroides* target (Bac32F/708R) and a "touch-down" protocol (Don et al., 1991). PCR supermix was made using 12.5 μl SYBR-Green II master mix (containing Taq polymerase, dNTP's, $MgCl_2$, SYBR-Green fluorescent dye, flourescein (for signal normalization), and Tris buffer), 11.0 μl water, 0.25 μl each of 20 pmol/μl Bac32F (5′-AACG CTAG CTAC AGGC TT-3′) and 708R (5′-CAAT CGGA GTTC TTCG TG-3′) primers, which yields a 676 bp amplicon as initially described by Bernhard and Field (2000), and 1.0 μl of the template DNA. The touch-down *Bacteroides* PCR was performed in duplicate on undiluted DNA (1:1) and on dilutions of 1:10, 1:100, and 1:1000. *Bacteroides fragilis* (ATCC 25285) DNA, at a concentration of 20 ng/μl, served as a positive control. The PCR protocol began with an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 94 °C denaturation for 20 s, 62 °C primer annealing for 20 s (decreasing in decrements of 0.3 °C per cycle), and 72 °C extension for 45 s; and a final 72 °C elongation step for 10 min. PCR products were verified via agarose gel(s) and/or melt-curve analysis.

Table 2
Primers used for various bacterial groups

Bacterial group/species	Primer set	Primer reference
Bacteroides	Bac32F/Bac708R	Bernhard and Field (2000)
Clostridium	Ccoc477/Ccoc916R	Matsuki et al. (1996)
Desulfovibrio	Dsv691F/Dsv826R	Matsuki et al. (1996)
Lactobacillus	Lacto157F/Lacto379R	Byun et al. (2004)
Escherichia coli	16E1F/16E2R/16E3R	Tsen, Lin and Chi (1998)
Enterococcus	Efs130F/Efs490R	Matsuki et al. (1996)
Fusobacterium	FPR-1/FPR-2	Wang, Cao and Cerniglia (1996)
Bifidobacterium	Bif164F/Bif601R	Matsuki et al. (1996)

2.5.2. Assessment of PCR inhibition

Since the presence of PCR inhibitors in DNA extracts could affect the accuracy of real-time PCR measurements of DNA concentration, the amount of inhibition, if any, was estimated by two methods: In the first method, the change in the average Ct (Ct is the cycle at which the baseline or threshold RFU value is exceeded,) for a 10-fold DNA dilution series ranging from 1:1 to 1:1000 was determined. In the absence of PCR inhibition, the expected result is that higher amounts of starting DNA will result in a lower value of Ct. At 100% PCR efficiency (i.e., a doubling of the amplicon concentration each cycle), each 10-fold dilution would be expected to produce a change of Ct (ΔCt) of Log(10)/Log(2)=~3.32 cycles. By comparing average shifts in Ct with this theoretical performance in the absence of inhibition, the influence of significant concentrations of PCR inhibitors could be estimated.

A second measure of the presence of PCR inhibitors compared the relative fluorescence (RFU) produced by the final PCR product of the undiluted DNA sample to the final RFU for diluted, potentially less inhibited samples. The RFU of the final PCR product is a measure of the total amount of DNA produced, possibly modified by quenching or autofluorescence. A lower final RFU for the undiluted DNA sample, compared to that obtained at 1:10 or 1:100 would indicate the presence of PCR inhibition.

2.5.3. Calculation of DNA concentration

The amount of *Bacteroides* DNA was calculated based on the relative Ct values, using the formula [Cal]*2^(Ctcal-Ctu)*dil, where [Cal] is the concentration of a known reference DNA measured in a PCR reaction run at the same time, Ctcal is the Ct obtained for the reference DNA sample, Ctu is the Ct obtained for the sample with unknown concentration of the target DNA, and dil is the dilution factor of the sample compared to the solution for which the concentration is being calculated. This calculation assumes a doubling of the amount of DNA for each additional cycle of Ct, an assumption that is justified if no PCR inhibition is occurring. In the present experiments, this calculation was applied to extracts that had been diluted 1:100 (i.e., dil=100), for which data will be presented showing no inhibition.

2.6. Amplification of human genomic DNA

Aliquots of DNA were also analyzed for a specific human target DNA, human reduced folate carrier (SLC19A1) genomic sequence (Genbank accession number U19720), using a nested PCR procedure capable of detecting small amounts of human DNA. In the primary PCR, reactions contained 5 μl GeneAmp® 10× PCR Buffer II (Applied Biosystems, N8080130), 4 μl dNTPs (Applied Biosystems, N8080007), 3 μl 25 mM $MgCl_2$ (Applied Biosystems N8080130), 2.5 μl dimethylsulfoxide, 1.0 μl of each primer (10 pmol/μl each of hRFC2308R (5′-AAGA GCAC CAAG GATG ACCA GCAA TGTC-3′) and hRFC1525F (5′-AGGA GAAG GCAG CACA GGCA CTAG)-3′, 0.5 μl 5 U/μl Taq DNA polymerase (Promega, PR-M8291)

the final reaction volume to 50 µl. Second round PCR mixture was the same, but utilized 2.0 µl of first round PCR product for the DNA template and used as primers hRFC1857R (5′-GCGC CCGA GAAT CACT TGGT TTCA CATT-3′) and hRFC1643F (5′-GGAG CAGA GACA GAGC GACC CATA CCTG-3′). The primary PCR thermocycle consisted of 94 °C for 3 min initial denaturation, 35 cycles of amplification (30 s 94 °C, 45 s 64 °C primer annealing, 1 min 72 °C elongation), and 7 min final 72 °C elongation. Second round PCR was identical except only 32 cycles were used and the annealing step was at 62 °C. PCR products were separated on 2% agarose gels stained with ethidium bromide.

3. Results

3.1. DNA yield

Total amounts of DNA extracted with different preservative and extraction combinations varied considerably (Fig. 1), with some combinations being significantly different from others (One Way ANOVA, $p<0.001$). The highest yields tended to be obtained for DNA preserved with either R or P; viz., the top four average yields were for QS-P, QS-R, Q2L-R, and Q2N-R, with yields of 12–25 µg total DNA from the 0.2 g (wet weight) fecal starting material. The QS extraction method accounted for 4 of the top 6 average DNA yields. The MB method consistently gave lower yields than the other methods.

3.2. PCR amplification of bacterial DNA

3.2.1. Qualitative survey of bacterial groups

In preliminary experiments, fecal samples that had been directly frozen in a −80 °C freezer prior to extraction were tested with a variety of primer sets (Table 2) that target various bacterial groups expected to be present in fecal samples. In pilot tests of the various DNA extraction techniques on 5–10 stool samples each, the relative amounts of PCR products, as judged by lower Ct values, for the various bacterial groups was *Bacteroides* > *Clostridium* ~ *Desulfovibrio* ~ *Fusobacterium* > *Lactobacillus* > *Bifidobacterium* >> *Escherichia coli* and *Enterococcus*. The qualitative results identified *Bacteroides* spp. as being reliably present and at a generally higher level than other targeted bacterial groups. Accordingly, subsequent quantitative studies on the VA samples focused on *Bacteroides* spp. Before presenting the quantitative results, however, we consider the presence of PCR inhibitors, which can affect PCR-based detection and quantitation.

3.2.2. PCR inhibition

Undiluted DNA extracts sometimes produced less PCR product than extracts that had been diluted 10-fold, providing clear evidence of the presence of PCR inhibition. The amount of inhibition was estimated by two methods in order to compare the efficacy of various methods at removing the inhibitors and also to determine conditions under which comparatively little inhibition was present. Fig. 2 shows results of the first method, in which ΔCt, the shift in Ct for each 10-fold dilution of the sample, was compared to 3.32, the theoretical shift in the absence of inhibition. For some samples, such as Q2L-R and QS-S, ΔCt is negative, i.e., the average Ct for the undiluted sample, 1:1, is higher than the average Ct for the 1:10 dilution, clearly indicating the presence of PCR inhibitors. By this standard, QS-R, Q2N-W, and Q2N-F samples had the least amount of PCR inhibition, comparing ΔCt values determined for undiluted (1:1) v. 1:10 samples. Also, by this criterion, no PCR inhibition occurred for any DNA sample diluted to 1:100, which showed ΔCt values >3 for all methods (Fig. 2B).

The second measure to assess the influence of PCR inhibitors compared the relative fluorescence (RFU) produced by

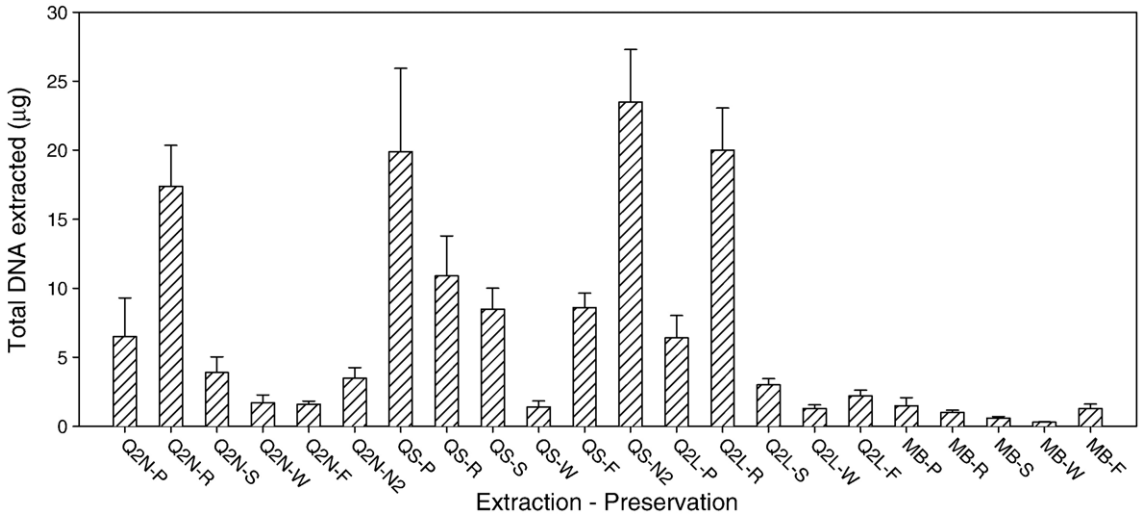

Fig. 1. Amounts of DNA extracted from feces preserved and extracted by various methods. Labels are of the form X–Y, where X is the DNA extraction method and Y is the preservative method. Abbreviations for the methods are identified in the text. Starting material in each case is 0.2 g feces, except for W samples, which were

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