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STR-Typing of Human DNA from Human Fecal Matter Using the QIAGEN QIAamp[®] Stool Mini Kit*

ABSTRACT: The purpose of this study was to compare the effectiveness of the QIAGEN QIAamp[®] Stool Mini Kit against a standard phenol-chloroform procedure for the extraction, quantitation, and STR-typing of human nuclear DNA from human feces. Stools from six subjects were sampled by swabbing and excision. Samples extracted with the QIAamp kit gave a wide range of DNA yields, whereas those extracted by the organic method yielded no DNA. DNA was not recovered from one subject's stools by either procedure. The QIAamp extracts were amplified with the Profiler Plus[™] and COfiler[™] kits, and PCR inhibition was observed with DNA extracts that were further concentrated. Substitution of water or TE-4 for the QIAamp elution buffer eliminated most, if not all, of the inhibition. A modified QIAamp procedure was used to extract thirty samples, which were subjected to one of five environmental conditions. DNA was recovered from all of these samples, and typing results were obtained on 93% of the samples.

KEYWORDS: forensic science, short tandem repeat typing, fecal matter, polymerase chain reaction inhibition, environmental conditions

Fecal matter is a less considered but potentially significant item of evidence. It is encountered in various casework situations, from trace quantities to entire stool deposits. In sexual assault cases, for example, small quantities of fecal material can be transferred to the sodomite's penis or other objects inserted anally. Contrastingly, a criminal may intentionally or unintentionally defecate at a crime scene, to leave an entire bowel movement. In many cases, the identification and individualization of fecal matter can establish the link between the victim and assailant.

Fecal matter is the end product of digestion. Digestion and absorption are functions of the gastrointestinal (GI) tract, which is basically a muscular tube that extends from the mouth to the anus. The GI tract is lined with epithelium (cell layer) and covered with peritoneum (membrane) along the majority of its length. The epithelial lining is regenerated every two to six days (4), and an estimated 17 billion cells are shed per day by the small intestine in humans (1). The intestinal lining is comprised of two distinct epithelial cell types: the columnar absorptive cell and the goblet cell. Defoliated cells that are not destroyed by the digestive process are excreted intact, although their morphology can be significantly altered (5). In addition, nucleated squamous epithelial cells can be transferred from the lining of the anal canal to the passing stool during the process of defecation (5).

Feces produced from an average diet is approximately 75% water and 25% solid material. The solid component includes bacteria (~30%); inorganic material, mainly calcium and phosphates

(~15%); and, fat or fat derivatives (~5%). The amount of cellulose and other indigestible fibers varies according to diet (1). Forensic protocols for the characterization of human feces have included the analysis of alkaline phosphatase, IgA immunoglobins, intestinal parasites, pancreatic α -amylase, urobilinogens and urobilins, and vegetable remnants (2,3). However, the presence of nucleated GI epithelial cells in stools offers the potential for nuclear DNA-based discrimination and CODIS comparison. This advantage has not been exploited because of problems involved in fecal DNA analysis. A major complication is the presence of degradative and inhibitory substances in feces that co-extract with the target DNA. Furthermore, these substances may interfere with the analysis, by standard methods, of other co-occurring physiological fluids. For example, lower-than-expected extraction yields and/or amplification success rates have been obtained from the semen component of semen-feces admixtures in casework conducted by one of the authors. Research indicates that bile salts and dietary plant polysaccharides can inhibit DNA amplification (6). Methods have been developed to remove fecal inhibitory substances, and have shown some success. In one procedure, DNA samples are diluted to reduce the concentration of the inhibitors (7). In other procedures, hexadecyltrimethyl ammonium bromide or chromatographic cellulose fiber powder is used in conjunction with an organic extraction (8,9).

The biotechnical company QIAGEN Inc. (Valencia, CA) has developed a commercially available kit for the extraction of nuclear DNA from human feces, by a relatively fast, silica-based extraction procedure. Included in the kit is a reagent that reportedly removes the PCR inhibitors innate to fecal matter. Additionally, the procedure enables for the preferential lysis of human cells over bacterial cells. The effectiveness of the kit was evaluated by Vandenberg and van Oorschot (10). However, the present study examined different parameters. Here, to evaluate the effects of different processing conditions, one excised sample from each stool specimen was extracted immediately as a fresh sample; a second was frozen for one week and processed after thawing; and a third was frozen for one week

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and processed without thawing. To simulate casework situations, a separate excised sample from each specimen was immersed in water for two hours at room temperature, and another was exposure to outdoor temperatures and indirect sunlight for one week. In addition to sampling by excision, swabbing as a collection method was evaluated in this study. Also, the effectiveness of the QIAGEN QIAamp[®] Stool Mini Kit was compared against a standard phenol-chloroform extraction procedure. Lastly, the DNA extraction yields and STR profiles were qualitatively compared and evaluated against the microscopic characteristics of the stool specimens.

Materials and Methods

Sample Preparation

Three adult males and three adult females participated in this study. Each subject defecated, without also urinating, into a clean bedpan, and sampled the bowel movement by collecting an intact section or liquid aliquot. A questionnaire was completed by each subject, which was submitted with the stool specimen. The questionnaire asked for information concerning diet, bowel habits, and general health, in addition to specific information on the tested bowel movement. Each subject also provided a buccal swabbing as a DNA reference sample. The stool specimens and buccal swabs were submitted to the laboratory, without prior refrigeration or freezing, between two and twelve hours after collection. At the laboratory, the specimens were processed immediately upon receipt. The specimens were first examined grossly, and their consistencies were graded according to the *Ivey scale* (11). The *Ivey scale* defines seven different consistencies: hard (resists puncture); formed (can be punctured); soft (can be cut); mushy (can be reshaped); loose (assumes shape of container); diarrhetic (flows); and watery (pours).

Following the gross examination, the specimens were sampled by two different methods: excision and swabbing. Six 180–220 mg samples of excised material were obtained from each specimen using a clean metal spatula. Repeated sampling was needed to obtain the target amount for each of the six samples. When the morphology of the specimen permitted, adjacent excisions were taken so as to include approximately the same amount of surface and underlying material. Three swab samples were also taken from each specimen. Each swabbing was performed so as to approximate a single spatula excision. Of the nine samples collected from each specimen, one excised sample was extracted immediately (fresh) with an organic procedure, whereas one excised sample and one swab sample were extracted fresh with the QIAGEN QIAamp[®] DNA Stool Mini Kit. The remaining four excised samples were subjected to one of the following treatments: 1) immersed in tap water for two hours at room temperature; 2) dried for one week under indirect natural light and outdoor temperatures, which ranged from ~5 to 20°C; 3) frozen (–20°C) for one week and kept frozen until the addition of the QIAamp ASL buffer; or 4) frozen (–20°C) for one week and allowed to thaw for two hours, at room temperature, before the addition of the QIAamp ASL buffer. After treatment, the four samples were extracted with the QIAamp[®] DNA Stool Mini Kit.

Blood and Microscopic Examination

One of the three swab samples from each specimen was tested for the presence of blood by the Kastle-Meyer (KM) test, whereas another swab was used to prepare two microscopic smears. One microscopic smear was stained with hematoxylin and eosin, and the other was mounted, unstained, with methyl salicylate. The smears were examined by brightfield and phase contrast microscopy at 200

and 400× magnification. The levels of bacteria, blood cells, columnar epithelial cells, muscle fibers (dietary meat), plant material, and squamous epithelial cells in each smear were graded, at 400× magnification, on a scale from zero to four. The grading system was as follows: 0, none identified; 1, rare or occasional observations, hard to find; 2, some in some fields, easy to find; 3, many in some or most fields; and 4, many in every field.

Organic Extraction

The reference buccal swabs and one excised sample from each stool were extracted by an organic procedure. The organic procedure included: 1) a cell lysis step (~12 h incubation at 56°C in 400 µL of stain extraction buffer [10 mM Tris, 10 mM EDTA, 100 mM NaCl, 39 mM dithiothreitol, 2% SDS, 20 µL of 10 mg/mL Proteinase K, and pH 8.0]), 2) a phenol/chloroform/isoamyl alcohol extraction step, and 3) a Centricon[®] YM-100 (Amicon) concentration and recovery step. The fecal DNA retentates were adjusted to a final volume of approximately 45 µL by vacuum-enhanced evaporation (Savant DNA SpeedVac[®] DNA110) and/or dilution with sterile water.

QIAamp[®] DNA Stool Mini Kit Extractions and Modifications

The remaining fecal samples from each specimen, after treatment, were extracted using the QIAGEN QIAamp[®] DNA Stool Mini Kit. Unless otherwise noted, all centrifugations were carried out in a Eppendorf Centrifuge 5417R at 14,000 rpm (20,000 × g) between 15 and 25°C. The extraction procedure basically followed the manufacturer's Protocol for Isolation of DNA from Stool for Human DNA Analysis (12); however, Steps 2 and 18 were modified for the purpose of this study. The modifications to Step 2 included: 1) a larger centrifuge tube (15 mL instead of 2 mL); 2) 2.6 mL of Buffer ASL (instead of 1.6 mL); and 3) an expanded homogenization procedure. The 2.6 mL volume of Buffer ASL was taken from the kit's stool tube protocol. The expanded homogenization procedure was as follows: the sample was vortexed for two minutes in Buffer ASL, then allowed to incubate at room temperature for about ten minutes. During this period, the material resistant to dissolution by vortexing was manually fragmented using wooden applicator sticks. The resultant suspension was then centrifuged at 5000 rpm using the IEC CENTRA[®] MP4R Centrifuge (International Equipment Company). Afterwards, 2.0 mL (instead of 1.4 mL) of the supernatant was withdrawn and processed as described in Step four of the handbook. The 2.0 mL volume was taken from the kit's stool tube protocol. In Step 18, a 0.1 mmol EDTA/Tris (TE-4) buffer (10 mM Tris base; 0.1 mM EDTA, Na₂-2H₂O; pH 7.5) was used in place of the QIAamp DNA elution buffer (Buffer AE).

In addition to aforementioned modifications, a Microcon[®] YM-100 (Amicon) concentration and recovery step was added at the end of the procedure. Samples were concentrated with the Microcon device by centrifugation at 500 × g for 20 min at 4°C. The final Microcon DNA retentates were raised to a volume of approximately 27 µL with sterile water.

DNA Quantitation

DNA samples were quantitated by slot blotting using the QuantiBlot Human DNA Quantitation Kit (Perkin Elmer, Foster City, CA), and the provided D17Z1 primate-specific probe, in combination with the SF Microfiltration Apparatus (Bio-Dot). The slot blot procedure that was used followed the manufacturer's recommendations. Equivalent sample volumes (1.0 µL and 5.0 µL of DNA

retentate) were used to compare the organic and QIAamp extraction procedures. Visualization of the bound DNA probe was achieved by chemiluminescent detection with ECL™ Western Blotting Detection Reagents (Amersham Pharmacia Biotech) and Kodak X-OMAT™ LS film.

STR Amplification

Each fecal DNA sample was amplified using the AmpFℓSTR® Profiler Plus™ PCR Amplification Kit (PE Biosystems, Foster City, CA). When sufficient quantities of DNA were recovered from the fecal sample, the DNA was additionally amplified by the AmpFℓSTR® COfiler™ PCR Amplification Kit (PE Biosystems, Foster City, CA). The buccal DNA samples were amplified by both kits. The amplification kits specify a DNA sample volume of 20 μL (13,14). A target concentration of 0.075 ng DNA/μL (1.5 ng template) was used in this study. DNA samples that were below this concentration were amplified neat (20 μL of the retentate). The DNA samples above 0.075 ng DNA/μL were diluted with TE-4 as necessary to obtain the targeted concentration. The Profiler Plus and COfiler amplifications were performed with a DNA Thermal Cycler 480 (Perkin Elmer) and GeneAmp Thin-Walled Reaction Tubes, following the manufacturer's protocol (13,14).

STR Typing

AmpFℓSTR® amplification products were analyzed by capillary electrophoresis and laser induced fluorescence using a ABI PRISM 310 Genetic Analyzer. Briefly, 1.5 μL of amplicon and 1 μL of GeneScan-500 [ROX] Internal Lane Size Standard were added to 24 μL of deionized formamide, denatured at 95°C for 3 min, then snap-cooled on ice for 2 min. The PCR products were then injected for 5 s at 15 kV, and electrophoresed for 24 min at 15 kV and 60°C. The electrophoretic capillary was 50 μm by 47 cm, and filled with Performance Optimized Polymer-4 (POP4) and 1X Genetic Analyzer Buffer. Allelic peaks were sized and typed using the GeneScan™ Version 3.1 and Genotyper™ Version 2.5 software, with a peak detection threshold of 75 relative fluorescence units (RFU).

Results

Questionnaire

The questionnaire results were used in this study for general comparisons; therefore, the data is presented here in summary form. The six subjects ranged in age from 26 to 53 years of age, and were reportedly healthy. Their diets included meat and vegetables at most or every meal. The subjects had defecated between 14 and 47 h prior to the tested bowel movement. None of the subjects reported the possibility of urine, blood, or semen in their stool specimen.

Gross and Microscopic Examination

The consistency of the stool specimens ranged from hard to loose. Blood was not detected by gross examination or the KM test in any specimen. (The KM test used here could detect blood diluted ten thousand times.) Erythrocytes (red blood cells) and leukocytes (white blood cells) were not identified in any of the microscopic smears. In reference to the microscopic findings, all fecal smears had high levels (graded as levels 3 and 4) of plant material and bacteria. Muscle fibers, blood cells, columnar epithelial cells, and spermatozoa were not identified in any preparation. Nucleated squamous epithelial cells were found in the fecal smears of two subjects;

however, their occurrence in these preparations was rare (graded as level 1).

Organic Extraction

DNA was recovered from the reference buccal samples by the organic procedure. In contrast, DNA was not obtained from any of the fresh fecal samples extracted organically, as quantified by the slot blot assay. The slot blot assay used here consistently detected a 0.039 ng DNA standard.

QIAamp® DNA Stool Mini Kit Extractions and Modifications

Initially, two problems were encountered with the QIAamp® DNA Stool Mini Kit. First, the recovery of DNA from the fresh fecal samples was intermittent. Sometimes, for example, one of a pair of samples would yield significant amounts of DNA, whereas its equivalent would yield no DNA. It was surmised that in some samples the human cells were not lysed in Step 2, because of poor homogenization of the sample with the lysis buffer (Buffer ASL). For the stool samples in this study, the problem was corrected by modifying Step 2 (see Materials and Methods). The modifications included the use of a larger centrifuge tube and a larger volume of Buffer ASL. Additionally, the instructions for Step 2 specify continuous vortexing for one minute or until the stool sample is thoroughly homogenized. Typically, the stool samples were not homogenized after two minutes of vortexing—sizable pieces still remained. To ensure complete homogenization, an expanded mixing procedure was implemented.

The second problem was PCR inhibition with Microcon concentrated, QIAamp extracts. The last step of the QIAamp procedure is to elute the bound DNA from the QIAamp silica-gel membrane with 200 μL of Buffer AE. Reportedly, this volume is necessary for effective elution. The final volume of the QIAamp DNA sample is ~200 μL. The Profiler Plus and COfiler amplification kits specify a DNA sample volume of 20 μL, and a target concentration of 0.075 ng DNA/μL (1.5 ng template) was used in this study. The initial quantitation studies indicated that some of the 200 μL QIAamp extracts had DNA concentrations below 0.075 ng DNA/μL. Therefore, a Microcon 100 concentration step was added at the end of the QIAamp procedure. This allowed for the production of 20 μL sample volumes. The Microcon concentrated, QIAamp extracts that had DNA concentrations above 0.075 ng/μL were diluted with TE-4 as necessary to obtain the target concentration in 20 μL. These samples amplified and typed without difficulty. However, the Microcon concentrated, QIAamp extracts that had < 1.5 ng template DNA in 20 μL were amplified neat, and demonstrated PCR inhibition. The suspected cause of the inhibition was high EDTA levels in the concentrated QIAamp DNA eluates. To test this supposition, three fecal samples from the same specimen were processed fresh using the QIAGEN kit, with aforementioned modifications of Step 2 and the Microcon step, under one of the following conditions: 1) elution with the QIAamp Buffer AE (0.5 mmol EDTA); 2) elution with QIAamp Buffer AE diluted 1:10 with sterile type 1 water; 3) elution with sterile type 1 water. All of the resultant DNA eluates had a DNA concentration of 156 pg/20 μL. A 20 μL aliquot of each eluate was amplified with the Profiler Plus kit. Therefore, the same amount of DNA was presented for amplification from a neat aliquot of each of the three eluates. This allowed for a direct comparison of the conditions. In this experiment, elution with the QIAamp Buffer AE did not produce allelic peaks above the 75 RFU threshold under GeneScan analysis. Elution with diluted Buffer AE produced four allelic peaks, whereas, elution with water produced eight allelic

TABLE 1—Fecal DNA yields per sample condition.

Fecal Sample Type	Average Starting Weight (mg)	Average DNA Yield (ng)	DNA Yield Range (ng)
Fresh excision	204.2	15.5	0.42 to 36.6
Fresh swab	78.1	0.88	0.34 to 1.5
Water immersed	207.0	9.0	0.42 to 33.7
Dried	204.7	0.83	0.21 to 1.7
Frozen	206.3	3.3	0.76 to 7.8
Frozen-thawed	208.7	3.6	1.0 to 5.1

peaks above threshold. Additionally, the four peaks seen by elution with dilute Buffer AE had approximately doubled in height by elution with water. Moreover, the results obtained for the water eluate were consistent with previous results obtained from routine samples that had ~156 pg of template DNA, which indicated that elution with water eliminated most, if not all, of the inhibition. Further research indicated that elution with 0.1 mmol EDTA/Tris (TE-4) gave results equivalent to that of water. TE-4 was subsequently chosen over water as the eluent, because of its buffering and chelating properties.

DNA Yields with the Modified QIAamp® DNA Stool Mini Kit Procedure

The modifications to the QIAGEN procedure resulted in the recovery of DNA from all samples, under the five environmental conditions, for five out of the six subjects. The exception was Subject 2. None of the fresh samples from this subject's multiple specimens yielded DNA. Subject 2 was excluded from the remainder of the study because the excised fresh samples were assumed to be the optimal samples for DNA recovery in these experiments. Table 1 summarizes the DNA quantitation results from the other five participants. Table 1 also provides the average starting weight for samples under each condition. The starting weight is the wet weight of the fecal sample prior to any treatment.

STR Typing

The Profiler Plus kit amplifies ten genetic loci; whereas, the COfiler kit amplifies seven genetic loci. Partial (<10 Profiler Plus loci; <7 COfiler loci) or complete Profiler Plus and COfiler profiles were obtained from 28 of the 30 fecal samples processed by the modified QIAamp procedure. Tables 2–4 summarize the results for each subject and sample condition. Listed in the tables are the

TABLE 2—Amount of template DNA and Profiler Plus/COfiler results per sample condition for subjects 1 and 3.

Subject	Sample Type	Profiler Plus			COfiler	
		Input DNA (ng)	# Loci Detected	Allelic Dropout	# Loci Detected	Allelic Dropout
1	Fresh excision	1.53	10	No	7	No
	Fresh swab	1.07	10	No	NT	...
	Water immersed	1.53	9	Yes	6	No
	Dried	0.94	5	Yes	NT	...
	Frozen	1.50	10	No	7	No
	Frozen-thawed	1.50	10	No	7	No
3	Fresh excision	1.50	10	No	NT	...
	Fresh swab	0.94	9	No	NT	...
	Water immersed	0.94	10	Yes	NT	...
	Dried	0.16	4	Yes	NT	...
	Frozen	0.70	0	No	NT	...
	Frozen-thawed	0.70	4	No	NT	...

NT = Not tested.

... = No data.

TABLE 3—Amount of template DNA and Profiler Plus/COfiler results per sample condition for subjects 4 and 5.

Subject	Sample Type	Profiler Plus			COfiler	
		Input DNA (ng)	# Loci Detected	Allelic Dropout	# Loci Detected	Allelic Dropout
4	Fresh excision	0.31	3	No	NT	...
	Fresh swab	0.31	8	Yes	NT	...
	Water immersed	0.31	3	No	NT	...
	Dried	0.16	3	No	NT	...
	Frozen	0.55	4	No	NT	...
	Frozen-thawed	0.55	3	No	NT	...
5	Fresh excision	1.53	10	No	7	No
	Fresh swab	0.24	0	No	NT	...
	Water immersed	1.51	10	No	7	No
	Dried	0.94	10	No	NT	...
	Frozen	1.50	10	No	7	No
	Frozen-thawed	1.50	10	No	7	No

NT = Not tested.

... = No data.

TABLE 4—Amount of template DNA and Profiler Plus/COfiler results per sample condition for subject 6.

Subject	Sample Type	Profiler Plus			COfiler	
		Input DNA (ng)	# Loci Detected	Allelic Dropout	# Loci Detected	Allelic Dropout
6	Fresh excision	1.25	2	No	NT	...
	Fresh swab	0.55	2	Yes	NT	...
	Water immersed	1.25	3	Yes	NT	...
	Dried	0.51	2	Yes	NT	...
	Frozen	1.64	4	No	NT	...
	Frozen-thawed	1.64	2	No	NT	...

NT = Not tested.

... = No data.

number of loci detected for each sample, and the occurrence of allelic dropout at one or more heterozygous loci is indicated by a *yes* or *no*. The tables additionally provide the amount of template DNA used for each amplification kit.

Typing results were not obtained from the frozen sample of subject 3 and swab sample of subject 5, and these samples contained 0.70 ng and 0.24 ng of template DNA, respectively. Partial profiles were obtained from samples with less DNA; however, the consumption of the two samples in analysis prevented further investigation as to the cause.

Discussion

Subject 2

Human DNA was recovered, by the modified QIAamp[®] procedure, from all fecal samples for five of the six subjects. The exception was subject 2. Subject 2 provided four, reportedly normal, stool specimens over a period of two months; the shortest interval between the submissions was one week. Fresh samples from each specimen were collected and processed as that of the other subjects; however, DNA was not recovered from any of the specimens by the organic, unmodified QIAamp, and modified QIAamp procedures. Subject 2 reported to be of good health and without a history of significant gastrointestinal problems. Nucleated cells were not identified in this subject's microscopic smears; however, this was true for three subjects whose samples did yield DNA. Subject 2 showed levels of plant material and bacteria similar to that of the other subjects. The results from subject 2 are inexplicable within the context of this study. Further investigation is needed to determine if this represents normal human variation.

Source of Fecal DNA

The human alimentary tract is lined with a columnar epithelium from the cardiac orifice of the stomach to the anal canal. However, intact cells were found in the microscopic preparations of only two of the five subjects whose samples gave DNA. The cells identified in these cases (one male and one female subject) were nucleated squamous epithelial cells, which were consistent in appearance with those of the anal canal at the ano-cutaneous junction. Defoliated cells of the anal canal retain their in situ appearance in feces (5). Columnar epithelial cells, which are the predominate cell type of the GI tract, were not identified in any specimen. However, the morphology of these cells are significantly altered by the digestive process (5), and may have been unrecognized. Leukocytes, although present in the intestinal lining (4), are typically not found in feces (5). Blood is not normally present in stools (15), and was not detected in any specimen. It is therefore presumed that GI epithelial cells are source of the human DNA recovered from the fecal samples, and supportive evidence is pro-

vided by the finding of nucleated squamous epithelial cells, in the absence of detectable blood and blood cells, in some of the samples.

Cell Distribution and Sampling

Each stool specimen was sampled with a swab, which was subsequently processed in parallel with the corresponding fresh, excised sample. The DNA yield of the fresh swab and the fresh excision were compared to assess the effectiveness of swabbing as a sampling method. However, as a result of these comparisons, the distribution of human cells in stools came into question. For example, the fresh swab samples of subjects 4 and 6 gave greater DNA yields (pg DNA/mg stool) than the corresponding fresh excised samples. With both subjects, the swab sample was contiguous with the fresh excised sample, and the two samples were extracted concurrently. The swab samples contained a fraction of the surface and underlying areas of the stool as included in the excised samples. According, the swab samples had 75% less fecal matter by weight than the corresponding excised sample; however, the swab samples gave two to four times the DNA yield. This finding suggests that human cells may not be uniformly distributed along the length of a stool in some cases. A non-uniform distribution is also suggested by the poor representation of cells in some of the smears as compared to the DNA yields of adjacent samples. Moreover, there may be differences in cell content between the surface and core of a stool. Reportedly, the muscular contractions of the large intestine produce mixing movements, which gradually expose all of the fecal material to the epithelial surface of the colon (16). However, the mixing of fecal material is probably less in the rectum and anal canal with the process of defecation, and this may result in higher concentrations of cells on the surface of the stool. A cell enriched surface is suggested by the research of Vandenberg and van Oorschot (10). Further research is needed to fully evaluate the distribution of cells in stools.

As previously stated, a swab sample was collected with each stool specimen for the purpose of developing a sampling protocol. To evaluate the minimal sampling requirements, a single cotton swab was inserted into each stool specimen so as to contact approximately the same amount of surface and underlying material as would be included in a single spatula excision. The resultant swabs were basically covered with fecal matter. DNA yields of 1 ng or greater were obtained from only two of the five swabs. The results indicate that the sampling of a stool with a single swab by the above method would be generally insufficient given, in part, the recommended 1.0–2.5 ng of input DNA for both the Profiler Plus and COfiler PCR amplification kits (13,14). Therefore, in consideration of the collective results of this study, the authors recommend a sampling strategy that includes multiple excised samples and surface swabbings, when the morphology of the stool permits. The samples would be extracted separately; however, the QIAamp

DNA eluates can be subsequently combined and concentrated, if individual samples are insufficient.

Organic and QIAamp® Extractions

The phenol-chloroform extraction method is a successful, general purpose procedure in forensic casework. However, the organic procedure used in this study failed to recover human DNA from the fecal samples. This has also been witnessed in casework. Fecal samples require a special approach because of the endogenous materials that can degrade human cells and nuclear DNA.

Bacteria and other microorganisms, such as *Candida*, inhabit the human gut as part of its normal flora. Brooks et al. (17) estimate a bacterial concentration in the sigmoid colon and rectum of 10^{11} cells per gram of intestinal contents. High levels of bacteria were noted in the subject's samples of this study. Given the admixture of human cells and those of other organisms in feces, one advantage of the QIAGEN procedure may lie in the cell lysis step. The QIAamp® DNA Stool Mini Kit handbook reports that human cells are efficiently lysed in Buffer ASL at room temperature, which was the incubation temperature used in this study (12). In contrast, bacterial cells and parasites are effectively lysed in Buffer ASL at 70–95°C. The preferential lysis of human cells in a short period of time may subsequently limit the exposure of the DNA to degrading substances, and thus contribute to the success of this method. Furthermore, the preferential lysis of human cells may reduce the amount of co-extracted microbial DNA, which can interfere with downstream applications.

An additional advantage of the QIAamp® DNA Stool Mini Kit lies in the treatment of DNA-damaging substances and PCR inhibitors native to fecal samples. Bilirubin, bile salts, and plant polysaccharides present in feces can co-extract with human DNA and inhibit PCR (8,18). The QIAGEN kit includes an adsorptive reagent, InhibitEX, which is in the form of a tablet. The chemical composition of the reagent is proprietary. Reportedly, the DNA-damaging substances and PCR inhibitors are adsorbed by the InhibitEX matrix, which is subsequently separated from the DNA extract by centrifugation. The present study did not test for the presence of PCR inhibitors in the fecal samples. High levels of plant material were noted in the subject's samples; however, all samples processed by the QIAamp procedure included the InhibitEX reagent. Therefore, the effectiveness of the reagent cannot be directly evaluated. However, indirect evidence is provided by the authors' experience with the few casework examples where DNA was recovered by the organic procedure, albeit in low levels, from neat fecal matter. Amplified products were not obtained in any of these cases.

QIAamp® DNA Stool Mini Kit Modifications

Two problems were encountered with the QIAamp® DNA Stool Mini Kit which required deviating from the stated protocol. The first problem was the lack of cell lysis because of the intermittent problem of incomplete homogenization of samples with Buffer ASL. The user's manual emphasizes the importance of thorough homogenization. The problem was corrected by simply using a larger centrifuge tube, a larger volume of Buffer ASL, and an expanded homogenization procedure.

The second problem was PCR inhibition with Microcon concentrated, QIAamp extracts. Reportedly, the QIAamp eluent, Buffer AE, contains 0.5 mmol EDTA. This quantity of EDTA in the eluates, it was surmised, could have removed Mg^{+2} ions from the polymerase reaction and thus cause the inhibition. The Microcon procedure followed in this study functioned to reduce sample vol-

ume, while concentrating molecules above 100,000 Daltons. To exchange buffers or remove low molecular weight contaminants, the manufacturer recommends repeated concentration and reconstitution steps (19). This procedure was not evaluated as a method for correcting the inhibition. Instead, the problem was solved here by substituting Buffer AE with water or TE-4.

Environmental Conditions and DNA Yields

Analysis of the five environmental conditions on DNA yields is limited by the small number of subjects and samples, in addition to the possibility of inter-sample differences in cell concentrations with each stool specimen. Therefore, only general observations and qualitative comparisons will be made.

The use of the modified QIAamp extraction procedure resulted in the recovery of human DNA from each of the six fecal samples of the five subjects. However, the amount of DNA recovered from the 30 fecal samples varied considerably. Of the five sample conditions, the fresh extractions produced the greatest DNA yields for all of the subjects. However, there were considerable differences in yields between the subjects, which presumably represents, in part, individual differences in GI physiology. Inter-individual variation in stool DNA content was additionally observed by Vandenberg and van Oorschot (10).

In the present study, the fresh excision samples had an average starting weight of 204.2 mg feces, and gave an average yield of 15.5 ng DNA (Table 1). The fresh excision yields ranged from 0.42 to 36.6 ng DNA for the five subjects. Vandenberg and van Oorschot (10) reported an average DNA yield of 2.6 ng, and a range of 0.6 to 8.0 ng DNA, from 200 mg fecal samples from ten subjects. In consideration of both studies, greater yields might be expected based on the estimated daily loss of enterocytes from the small intestine, not including cells shed from other segments of the GI tract. Many factors may effect fecal DNA concentrations, such as, the mass and interval of the bowel movement, the subsequent degradation of exfoliated cells and DNA, and the efficiency of the extraction procedure. Nevertheless, the fresh extractions did overall produce the best results.

Contrastingly, the lowest DNA yields were obtained from the dried samples for the majority of the subjects (Table 1). These preparations were not smears, but excised pieces that were allowed to dry in bulk. On drying, the samples were reduced to small hard masses, which were particularly difficult to homogenize with the ASL buffer. In addition to degradation, a less-than-optimal homogenization process may have contributed to the lower DNA yields from these samples. In the environmental impact study of Vandenberg and van Oorschot (10), 200 mg samples of a stool were exposed to natural lighting and room temperature conditions for 1 to 91 days. The DNA yields from these samples were not specified; however, full Profiler Plus profiles were obtained from each. It is noteworthy that Vandenberg and van Oorschot mixed the stool specimen prior to sampling. The effects of specimen mixing on DNA yields, as compared to our sampling procedure, requires further investigation.

In contrast to the dried samples, the water-treated samples of the present study were readily homogenized with the ASL buffer (the buffer was added after the water was decanted). In three cases, the water-immersed sample gave a DNA yield equivalent to that of the corresponding fresh sample; whereas in two cases, the yield from the water-treated sample was about three-quarters less than that of the corresponding fresh sample. Overall, the data suggests the possibility of recovering nuclear DNA from stools found in toilets, however, the extent to which contamination is a problem in these cases remains to be determined.

Reportedly, the QIAmp procedure can be performed on fresh and frozen fecal samples (12). However, the manufacturer cautions that frozen samples should not be allowed to thaw before the addition of the ASL buffer, because the DNA in the samples may degrade. The frozen and frozen-thawed samples gave similar DNA yields in four of the five subjects, and of the four, two subjects showed frozen and frozen-thawed sample yields that were similar to the fresh sample. With the two other subjects, however, the fresh sample gave five to seven times the yield of the frozen and frozen-thawed samples. In reference to the fifth subject, the yield from the frozen-thawed sample was five times greater than that of the frozen sample, and was equivalent to that of the fresh sample. The data does not qualitatively demonstrate an appreciable reduction in DNA yields with frozen-thawed samples, under the present testing conditions. The interpretation of the data is complicated by the possibility of variation in DNA concentrations within a stool specimen as suggested in this study. Possibly a larger sample pool or an extended thawing period may show significant differences between the two conditions. In the absence of additional data, however, the authors here recommend adherence to the manufacturer's protocol.

STR Typing

STR-typing results were obtained from 28 of the 30 QIAmp extracts, and, reflective of DNA yields, the degree of profiling varied considerably (see Tables 2–5). The results ranged from a profile (0.51 ng template DNA) that had an incomplete amelogenin result and a concordant D8S1179 result, to entire Profiler Plus and Cofiler profiles (0.94–1.53 ng template DNA). Overall, the quantity of template DNA was the primary determinant of the quality of the STR profiles in this study. Notable exceptions include two samples from subject 6, where 1.64 ng DNA were amplified from both, but only two and four loci profiles were produced. These profiles showed the typical pattern of degraded samples, with the loss of larger loci.

Complete Profiler Plus or Profiler Plus-Cofiler profiles were obtained from 60% of the fresh samples, 40% of the frozen and frozen-thawed samples, and 20% of the swab, water-treated, and dried samples. Contrastingly, Vandenberg and van Oorschot (10) obtained eight complete and two partial (four or more loci) Profiler Plus profiles from the 200 mg fresh samples of ten individuals. Their use of a longer electrokinetic injection time (ten versus five seconds) may have contributed to their success.

In the present study, heterozygous allelic balance (peak height ratio > 0.67) was demonstrated at all loci of the complete profiles. However, allelic imbalance, allelic dropout, and absent loci were evident in the partial profiles of other samples. These problems generally affected the larger alleles and loci. In cases of allelic dropout, the height of the detected heterozygous peak was near threshold. Allelic dropout and absent loci were demonstrated in 44% and 94% of the partial profiles, respectively. In 6% of the partial profile cases, typing results were obtained for all Profiler Plus loci, but allelic dropout occurred at one or more of the loci. In forensic casework, these partial profiles may provide useful information, but care must be exercised in their interpretation.

Significantly, all alleles identified in the fecal samples matched those of the subjects' reference samples. Discordant extraneous peaks were not detected, above the 75 RFU threshold, in any of the 28 samples. This study did not find direct evidence of the mistyping of non-human DNA in the fecal samples.

Conclusion

The individualization of human feces has been a long-standing problem in forensic casework. The development of the QIAamp[®]

DNA Stool Mini Kit significantly advances our analytical and investigative capabilities. Although the kit may require modifications to interface with specific downstream applications—its cost, ease-of-use, and final product makes it an efficient and effective forensic tool.

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