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Comparison of DNA extraction methods for PCR amplification of mitochondrial cytochrome c oxidase subunit II (COII) DNA from primate fecal samples

Christopher A. Whittier[1], Arun K. Dhar[1,2], Chip Stem[3], Jane Goodall[4] & Acacia Alcivar-Warren[1,*]

[1]*Department of Environmental and Population Health and*
[3]*Section of International Medicine, Tufts University School of Veterinary Medicine, North Grafton, MA 01536, USA*
[4]*Jane Goodall Institute-USA, P.O. Box 599, Ridgfield, CT 06877, USA*
[2]*Present address: Super Shrimp Inc., 1545 Tidelands Ave., Suite J, National City, CA 91950, USA*
[*]*Author for correspondence (Fax: +1-508-839-7091; E-mail: aalcivar@opal.tufts.edu)*

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Abstract

Mitochondrial COII DNA was amplified by PCR from total DNA extracted from field collected primate fecal samples ($n = 24$) which had been stored without refrigeration for over 30 days. High molecular weight DNA total DNA was obtained from samples stored in 70% (v/v) ethanol, SDS lysis buffer (LB) and guanidine isothiocyanate buffer (GTB) than from samples stored in 10% formalin. Fecal DNA quality and COII amplification varied according to storage solution (formalin, ethanol, LB and GTB), extraction method (LB-based and GTB-based) and primate species (chimpanzee, baboon, human). It is recommended that fecal samples be collected in LB for DNA analysis. However, GTB-based protocols are suitable when total RNA is needed for epidemiological studies of viral diseases or gene expression analysis.

Introduction

The use of noninvasive sampling for genetic analysis has become increasingly popular since the introduction of polymerase chain reaction (PCR) (Kohn & Wayne 1997, Morin & Woodruff 1996, Ding *et al.* 1998 and references therein). Noninvasive tissue collection provides a quick, inexpensive, easy and safe alternative to traditional capturing and bleeding of wild animals, especially endangered species.

The recent findings regarding the origin of human immunodeficiency virus type 1 in West African chimpanzees has increased the interest and importance of using noninvasive primate samples for molecular analysis (Gao *et al.* 1999, Weiss & Wrangham 1999). DNA extracted from fresh and frozen fecal samples has been used for clinical diagnosis and epidemiologic elucidation, as well as sex determination and individual identification (Caldas *et al.* 1994, Kohn & Wayne 1997, Weiss *et al.* 1992). Most genetic studies using fecal DNA have benefited from the use of cold storage and have used only ethanol or methanol as storage solutions (Constable *et al.* 1995, Kohn & Wayne 1997, Reed *et al.* 1997). DNA has also been extracted from formalin-fixed fecal samples (Weiss *et al.* 1992, Acuna-Soto *et al.* 1993, Duflot *et al.* 1995, Balatbat *et al.* 1996, France & Kocher 1996, Rivera *et al.* 1996, Carville *et al.* 1997, DaSilva *et al.* 1997, Shedlock *et al.* 1997, Dowd *et al.* 1998, Zhu *et al.* 1998). The effects of formalin fixation on DNA extracted from various tissues have been well researched (DeGiorgi *et al.* 1994, Honma *et al.* 1993, Karlsen *et al.* 1994) and are believed to contribute to the generally low yield and low quality of fecal extracted DNA

(Shedlock *et al.* 1997, France & Kocher 1996). It is generally recognized that co-purified substances from fecal samples further contribute to the difficulty and inconsistency of PCR results (Deuter *et al.* 1995, Kohn & Wayne 1997). While various protocols have been developed to overcome these obstacles, few studies have compared preservatives, extraction protocols, or species differences for isolating PCR-amplifiable fecal DNA (Kongmuang *et al.* 1994, Reed *et al.* 1997, Wasser *et al.* 1997).

The objectives of this study were to: (1) assess different methods of total DNA extraction from preserved, field collected primate fecal samples stored without refrigeration (2) compare the quality of total DNA extracted from human, baboon and chimpanzee fecal samples by five different methods, and (3) amplify the mitochondrial cytochrome oxidase II (COII) gene from fecal total DNA using species-specific primers.

Methods

Collection of fecal samples

Baboon (*Papio anubis*) and chimpanzee (*Pan troglodytes*) fecal samples were collected within 10 min of defecation from identified individuals at Gombe National Park (GNP), Tanzania. Human fecal samples were obtained from GNP inhabitants. Within 6 h of collection, samples were divided into four aliquots and stored in the following preservatives/buffers: 10% buffered formalin, 70% (v/v) ethanol, guanidine isothiocyanate buffer (GTB, 5 M guanidine isothiocyanate, 1 M sodium citrate, 0.7% beta-mercaptoethanol, 10 mM EDTA, pH 7.2) and lysis buffer (LB, 0.02 M Tris/HCl, 0.01 M EDTA, 1% SDS, 0.15 M NaCl, and 0.01 M DTT, pH 8.0). Aliquots were collected in formalin and ethanol because of their routine use for parasitological studies. Formalin aliquots weighed from ∼0.5 g to 2.0 g, while all other aliquots weighed from ∼0.1 g to 0.8 g. All samples remained at ambient temperature in the field for 30 to 50 days and were then kept at 4 °C upon return to the United States until DNA extraction. For quality assessment, total DNA was extracted from eight fecal samples each of human, chimpanzee and baboon. DNA extracts from four individuals of each species were then chosen for PCR amplification of COII gene based on availability of DNA extracted (Tables 1, 2).

DNA extraction and quality assessment

Fecal DNA was isolated following protocols based on two buffer systems: guanidine isothiocyanate buffer, GTB (GT protocol) and lysis buffer, LB (Lysis protocol) (Alcivar *et al.* 1989). The combination of collection solution and extraction protocol defined five extraction methods. Since larger amounts of fecal material were collected in formalin, aliquots were processed by both GT and Lysis protocols. Ethanol preserved aliquots were processed only through GT protocol to extract both DNA and RNA. RNA will be used in the future to examine the expression of some host and parasite genes.The final five methods were: (1) samples aliquotted into GTB and processed through the GT protocol (GT method); (2) LB aliquots processed through the lysis protocol (Lysis method); (3) 10% formalin aliquots processed through the GT protocol (F–GT method), (4) 10% formalin aliquots processed through the lysis protocol (F–L method); and (5) 70% ETOH aliquots processed through the GT protocol (ethanol–GT method).

The lysis protocol entailed homogenization of fecal samples in 5 ml of lysis buffer, and incubation with 100 μg proteinase K ml^{-1} at 37 °C overnight before being brought to 1 M NaCl. One-tenth volume of 10% cetyl trimethylammonium bromide (CTAB)/0.7 M NaCl solution was added, and the samples were incubated at 65 °C for 10 min. Samples were then extracted twice with an equal volume of chloroform and once with phenol/chloroform/isoamyl alcohol (25:24:1 by vol.). DNA was precipitated with 2.5 volumes of 100% ethanol at −20 °C overnight, and the final pellet was resuspended in ultrapure distilled water (5′–3′ Inc., CO).

In the GT protocol, samples were homogenized in 5 ml of GTB and centrifuged at 2680 × *g* for 10 min to remove the fecal debris. N-laurylsarcosine (2%) and cesium chloride (0.15 g ml^{-1}) were added to the supernatant before layering on 5.7 M cesium chloride cushion. Samples were centrifuged at 23 °C for 14–18 h at 140 000 × *g* and the supernatant containing DNA was mixed with an equal volume of TE buffer (0.01 M Tris pH 7.5, 0.001 M EDTA pH 8.0) and 2.5 volumes of 100% ethanol and precipitated at −20 °C overnight. DNA was centrifuged at 5100 × *g* for 30 min at 4 °C, the pellet was rinsed with 70% ETOH and then processed according to the Lysis protocol as described above.

Total fecal DNA was electrophoresed in a 1% agarose gel containing 0.3 μg ethidium bromide ml^{-1}

Table 1. Evaluation of DNA quality of primate fecal samples extracted by five different methods.

Sample number	Species and sample ID	Extraction methods				
		F–GT	F–L	GT	Ethanol–GT	Lysis
Human						
1	BI-H-33	F	P	VG	F	VG
2	MM-H-49	G	P	VG	G	F
3	MM-H-52	G	P	G	F	VG
4	SH-H-56	VG	F	VG	G	VG
5	KA-H-31	F	G	VG	X	VG
6	AS-H-32	F	G	VG	X	G
7	AS-H-39	F	G	VG	G	G
8	MK-H-42	F	F	VG	G	G
Chimpanzee						
9	FF-K-19	VG	VG	F	G	VG
10	TZ-K-23	VG	VG	F	F	VG
11	GB-K-25	VG	VG	F	F	VG
12	BH-M-27	VG	VG	F	G	VG
13	FR-K-3	VG	G	P	X	G
14	PF-K-5	VG	VG	G	X	G
15	WL-K-9	VG	VG	P	P	F
16	SA-K-22	VG	VG	G	F	VG
Baboon						
17	MDA-B-63	VG	VG	P	G	VG
18	WBA-B-64	VG	F	P	F	VG
20	SF-AA-84	VG	VG	P	G	F
21	DR-DA-61	VG	VG	G	X	G
22	ARS-B-62	VG	VG	G	X	G
23	CN-B-66	P	VG	P	G	VG
24	DO-AA-68	VG	P	F	F	VG

VG = very good; mostly high molecular weight (MW) DNA; G = good; some high molecular and some sheared low molecular weight DNA; F = fair; mostly low MW DNA; P = poor; only low molecular weight DNA; X = not tested due to lack of DNA.

in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM sodium EDTA) at 40 V for ∼12 h. Total DNA quality was assessed subjectively as: very good (VG) = mostly high molecular weight (MW) DNA; good (G) = some high and some low MW DNA; fair (F) = mostly low MW DNA with very little high MW DNA; and poor (P) = only low MW DNA.

PCR amplification of mitochondrial COII

To design primers for mitochondrial COII amplification, the sequences of baboon (M74007), chimpanzee (M58009) and human (J01415) (Disotell *et al.* 1992, Ruvolo *et al.* 1993) were aligned using the PileUp program of GCG (University of Wisconsin, version 8.1). Sense (positions 78–99) and antisense (positions 472–495) primers for the ∼418 bp COII DNA were synthesized from sequences where maximum differences were observed among all three species:

Baboon (sense):	5′-AGCCCTTATAGCC ATATTTTTA-3′
Baboon (antisense):	5′-GATAGTTCATGAG TGTAGT-3′
Chimpanzee (sense):	5′-TGCCCTCATAATTA TCTTTCTC-3′
Chimpanzee (antisense):	5′-AACAGCTCATGAG TGTAGA-3′
Human (sense):	5′-CGCCCTCATAAT CATTTTCCTT-3′
Human (antisense):	5′-GACAGCTCATGA GTGCAAG-3′

A ∼245 bp COII DNA was amplified by using the same antisense primers and new sense primers overlapping the ∼418 bp DNA. The sequences for the ∼245 bp sense primers (positions 251–276) were:

Baboon (sense):	5′-TGTACATGACAGACGA AATCAATAAC-3′
Chimpanzee (sense):	5′-TTTACATAACAGACGA GGTCAACGAC-3′
Human (sense):	5′-TTTACATAACAGACGA GGTCAACGAT-3′

To test primer specificity, amplification was performed using variable concentrations of $MgCl_2$ (2.0 mM, 1.5 mM and 1.0 mM) and DNA (100 ng, 10 ng and 1 ng) extracted by the Lysis method from one individual of each species with each of three primer pairs. After initial optimization, PCR was performed using DNA from three additional individuals of each species extracted by all five methods. The final reaction mixtures contained 10 ng or 1 ng DNA, 1× PCR buffer (Promega, WI), 0.2 mM dNTPs, 2.5 U *Taq* (Promega), 1.5 mM $MgCl_2$, 10 μM of sense and antisense primers in a 100 μl reaction volume. The PCR temperature profile was 93 °C for 1 min, 50 °C 1 min and 72 °C for 2 min for 40 cycles in a thermocycler (PTC-100^{TM}, MJ Research Inc., MA). Negative controls with *Giardia* DNA and no DNA were used in all PCR reactions. We included *Giardia* DNA as negative control for PCR because some of these samples were positive for *Giardia* by ELISA and microscopic inspection (C.A. Whittier *et al.* unpublished). Amplification results were subjectively scored based on intensity of DNA bands (Table 2).

Results

Quality of total fecal DNA

The quality of total fecal DNA varied depending on the preservative, extraction method and primate species (Table 1, Figure 1). For formalin-preserved aliquots processed through GT or Lysis methods, very good quality DNA was obtained for chimpanzee and baboon extracts, whereas the quality of human fecal DNA was not as good. For the GT method, very good quality DNA was observed for most of the human samples whereas the quality of chimpanzee and baboon DNA was not as good. For the ethanol–GT method, the DNA quality was mostly fair or good for all three species. Samples extracted by the Lysis method provided mostly very good quality DNA for all three species (Table 1, Figure 1). In summary, both GT and Lysis methods provided best quality of DNA for human samples whereas the F–GT, F–L and Lysis methods provided best quality DNA for chimpanzee and baboon.

Comparison of extraction methods for detection of host COII DNA

The presence of primate-specific DNA in fecal samples was tested by amplifying ∼418 bp and ∼245 bp of mitochondrial COII DNA using species-specific primers. Successful amplifications were obtained only when primers were used with template DNA from corresponding species. Amplification varied depending on extraction method, species, amount of template DNA and the size of amplified product (Table 2 and Figure 2). Better PCR amplifications were obtained for all samples when DNA was processed by GT, ethanol–GT and Lysis methods compared to F–GT and F–L methods. Amplifications from GT, ethanol–GT and Lysis methods were less consistent for chimpanzee and baboon DNA than human. Amplification products were also obtained from two formalin preserved human aliquots and one chimpanzee aliquot using both extraction protocols. Attempts to amplify ∼245 bp DNA did not improve overall results compared to ∼418 bp DNA amplifications. There were multiple aliquots which amplified only the ∼245 bp band as well as those that amplified only the ∼418 bp band. Amplification of ∼245 bp DNA for most of the baboon ethanol–GT and Lysis aliquots failed in spite of multiple attempts. Some aliquots were not consistent in independent amplifications (Table 2). In summary, amplification products were obtained from GT, ethanol–GT and Lysis methods for all three species, albeit at different DNA concentrations.

Discussion

Comparison of fecal DNA extraction methods showed considerable variation in the quality of total fecal DNA and PCR amplification depending on the preservative, extraction protocol and primate species. This could be due to variability in proportion of DNA from microbes and food contaminants to target DNA in fecal samples. The GT method provided the best quality of total DNA for human samples whereas for chimpanzee and baboon samples equivalent quality of total

Table 2. PCR amplification of ~418 and ~245 bp mitochondrial cytochrome C oxidase subunit II DNA from primate fecal samples.

Extraction method	F–GT				F–L				GT				Ethanol–GT				Lysis			
DNA amount/ reaction	10 ng		1 ng		10 ng		1 ng		10 ng		1 ng		10 ng		1 ng		10 ng		1 ng	
COII DNA (bp)	418	245	418	245	418	245	418	245	418	245	418	245	418	245	418	245	418	245	418	245
Human																				
1. B1-H-33	−	−	−	−	+	+	−	−	+++	+++	++	+	+++	+++	++	+++	++	++	−	−
2. MM-H-49	+++	−	++	++	−	NT	−	NT	++	++	++	++	−	−	+++	+++	−	−	+	++
3. MM-H-52	−	−	−	−	+	+	−	−	−	−	++	++	+++	+++	+++	++	++	−	−	+
4. SH-H-56	−	+	−	−	++	+	−	−	++	+++	++	++	+++	+++	++	+++	+++	+	−	+
Chimpanzee																				
9. FF-K-19	−	−	−	−	−	−	−	−	−	−[a]	+	−[a]	−	−	++	+	−	−[a]	−	−[a]
10.TZ-K-23	−	−	−	−	−	−	−	−	+++	−[a]	+++	++[a]	−	−	+++	++	+++	+[a]	+	+/−[c]
11. GB-K-25	−	−	+	−	−	−	−	+	−	−[a]	−	−[a]	−	−	−	−	++	−[a]	+	−[a]
12. BH-M-27	−	−	−	−	−	−	−	−	+++	−/+[c]	+++	++/−[c]	−	−	+++	+	+++	+[a]	+	−[a]
Baboon																				
17. MDA-B-63	−	−	−	−	−	−	−	−	−	−	−	+++	−	−[a]	+	−[b]	+	−[b]	−	−[b]
18. WBA-B-64	−	−	−	−	−	−	−	−	−	+	−	−	−	−[a]	++	−[b]	+	−[b]	−	−[b]
19. SO-DA-65	−	−	−	−	−	−	−	−	+++	+++	+++	+++	−	−[a]	+	−[b]	+	−[b]	−	−[b]
20. SF-AA-84	−	−	−	−	−	−	−	−	−	−	+	++	−	−[a]	+++	−/+/+[c]	−	−[b]	+++	−/+/+[c]

− = no amplification; + = faint band; ++ = bright band; +++ = very bright band; NT = not tested.
When considerable differences were observed between ~245 bp and ~418 bp DNA amplifications for chimpanzees (GT and Lysis) and baboon (ethanol–GT and Lysis) samples, they were repeated 2 or 3 times.
[a,b] = samples repeated two ([b]) or three ([c]) times and similar results were obtained.
[c] = discrepancy in independent amplification.

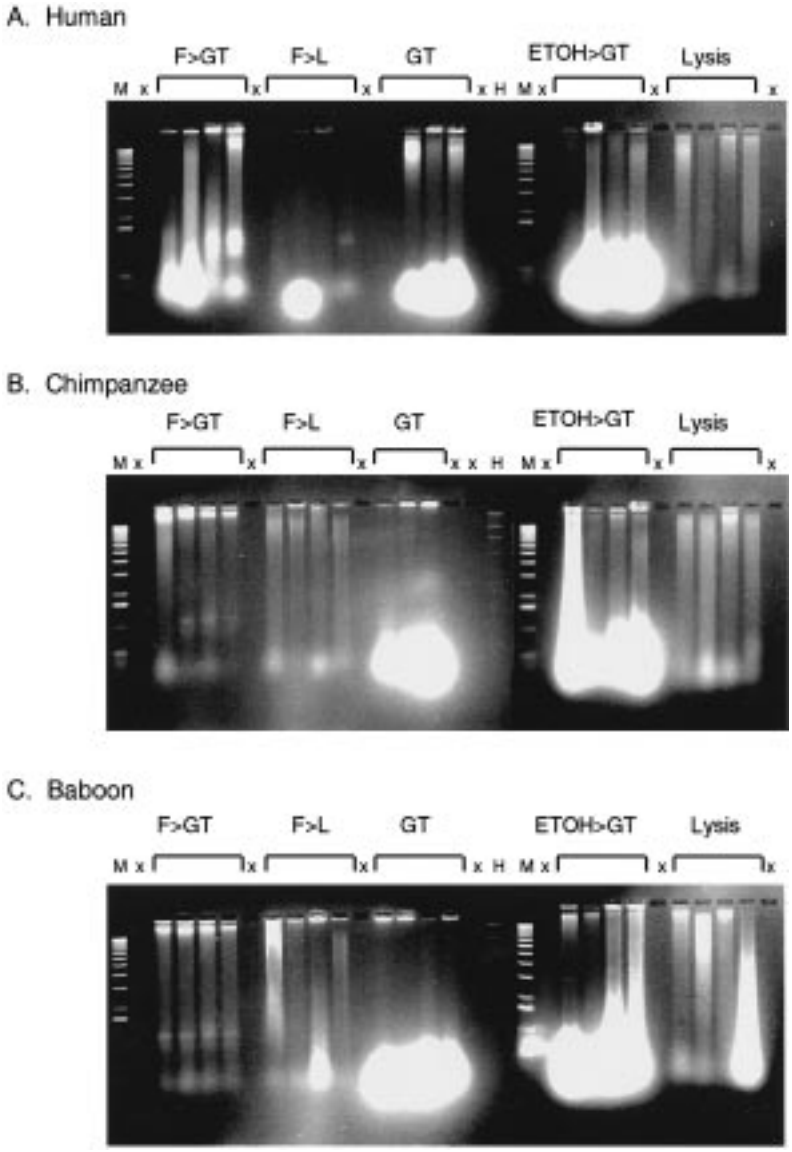

Fig. 1. Computer scanned composite figure of DNA quality control gels from (A) human samples #1, 2, 3 and 4 (B) chimpanzee samples #9, 10, 11, and 12 (C) baboon samples #17, 18, 19 and 20 extracted by five methods. DNA samples were run in a 1% agarose gel in TAE buffer at 40 V for ~12 h. M = 1 kb molecular weight marker; H = Lambda DNA cut with *Hin*dIII; *x* = empty lane; F>GT, F>L, GT, ethanol>GT and Lysis refer to different extraction methods (see Materials and methods). (A) Human samples extracted by F>GT, GT, ethanol>GT and Lysis methods contain ~0.5–1.0 μg of DNA. All F>L lanes contain ~0.1 μg of DNA. (B) Chimpanzee samples extracted by F>GT and Lysis methods contain ~1.0 μg of DNA, GT samples contain ~0.1 μg of DNA, F>L and ethanol>GT samples contain ~0.25–0.2 μg of DNA. (C) Baboon samples extracted by F>GT, F>L, and Lysis methods each contain ~1.0 μg of DNA except for F>L (sample #20) which contains only ~0.15 μg. GT samples contain ~0.05–0.1 μg of DNA and ethanol>GT samples contain ~0.5 μg of DNA.

DNA was obtained by F–GT, F–L and Lysis methods. Although it has been suggested that DNA from formalin-fixed samples are generally of low quality (Shedlock *et al.* 1997), the extraction protocols described here provided good quality total DNA from formalin-preserved samples.

In this study, formalin-preserved fecal DNA of chimpanzees and baboons could not be amplified very successfully, probably due to the inhibitory effects of formalin on PCR (De Giorgi *et al.* 1994, France & Kocher 1996, Greer *et al.* 1991). It has been suggested that formaldehyde causes hydroxymethylation of ex-

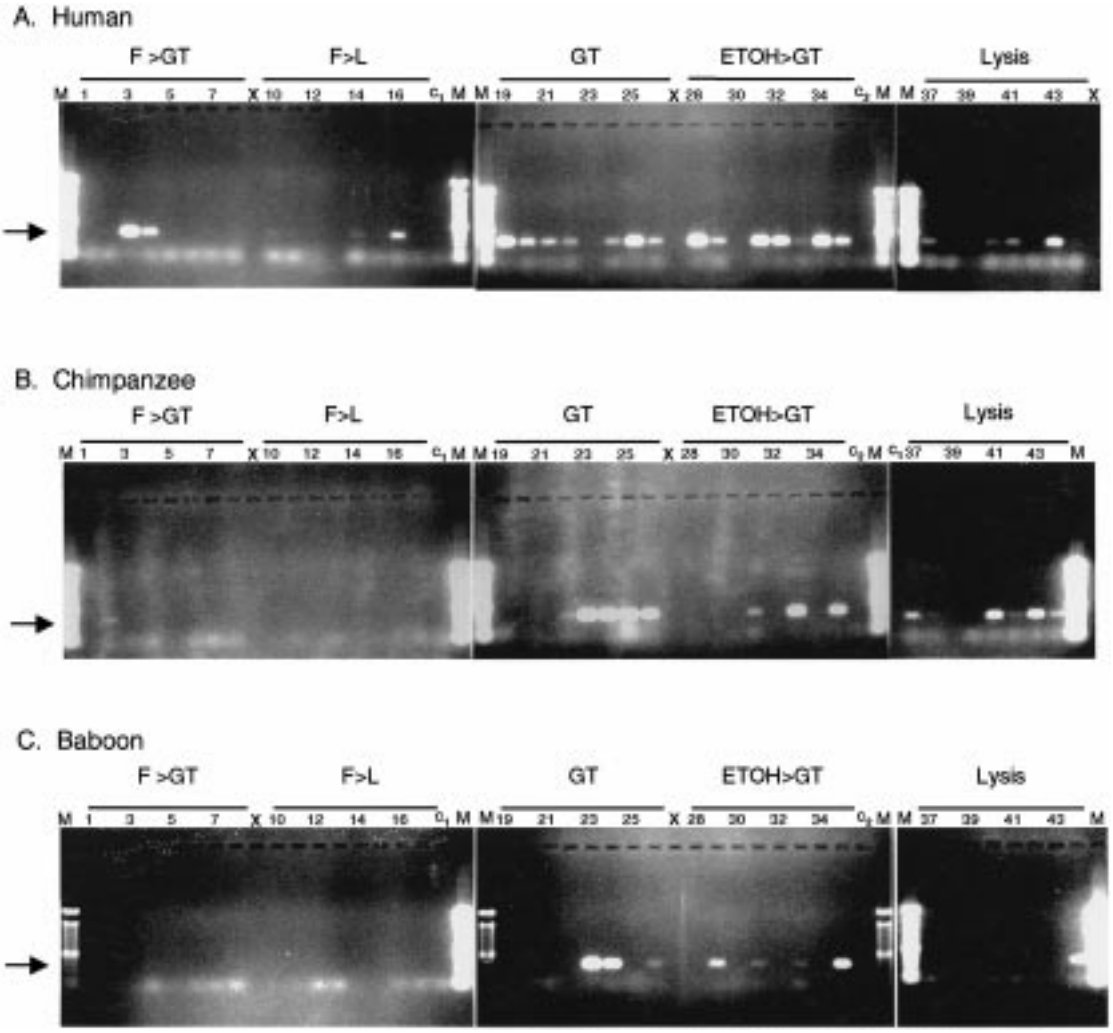

Fig. 2. Computer scanned composite figure of PCR amplification of mitochondrial COII gene using fecal DNA extracted by five methods using species-specific primers. Four samples of each species were used for PCR amplification. Sequential samples from (A) human #1, 2, 3 and 4 (B) chimpanzee #11, 9, 10 and 12, and (C) baboon #18, 17, 19 and 20 are shown. For each method and each sample the first lane contained 10 ng of DNA and the second lane contained 1 ng DNA. Lanes with 10 ng DNA are numbered. Amplified DNA was run in a 4% agarose gel at 25–40 V overnight. M = 100 bp molecular weight marker (Gibco); x = empty lane; C_1 = negative control (without DNA); C_2 = negative control with *Giardia* DNA. Arrows indicate ~418 bp COII DNA.

posed exocyclic amino groups of adenine, guanine and cytosine (Chang & Loew 1994). AT-rich regions, being more abundant in mitochondrial DNA, may be highly susceptible for hydroxymethylation and inhibition during PCR amplification. The differences in PCR amplification success between human samples versus chimpanzee and baboon samples may be attributable to increased metabolic (digestive enzymes, bile salts, etc.) and/or dietary (plant polysaccharides) PCR inhibitors in the non human primates (Kohn & Wayne 1997).

The amplification success of DNA from formalin-fixed tissues is shown to vary depending on storage time and the size of the amplified product (Greer *et al.* 1991, Honma *et al.* 1993). In the present study, decreasing the PCR product sizes from ~418 to ~245 bp did not improve the overall amplification results. Although isolated DNA was stored frozen, amplification of ~245 bp DNA were done ~12 months after the ~418 bp DNAs were amplified. It is possible that with increasing storage time, even smaller DNA fragments will be difficult to amplify. Recently, Wasser *et al.* (1997) reported that some sun bear fecal samples pro-

vided better results with ~398 bp than with ~246 bp DNA of mitochondrial D-loop (see Table 1 in Wasser *et al.* 1997). Discrepancies between quality of total DNA and PCR amplification have also been reported for other species (France & Kocher 1996, Reed *et al.* 1997).

Most fecal genetic studies have used cold storage for preserving fecal samples and only a few have preserved samples at ambient temperature (Gerloff *et al.* 1997, Sugiyama *et al.* 1993, Wasser *et al.* 1997), like the present study. The methods described here, especially the Lysis method, have proven successful in isolating a high yield of total DNA (data not shown) for PCR amplification without any need for immediate cold storage. This has application in noninvasive sampling of free ranging animals for genetic analysis. We also observed that time gap (0–6 h in our study) between collection of samples and placing them in preserving solution and the number of days (32–372 days) between sample collection and DNA isolation appeared to have no effect on quality and quantity of DNA (data not shown).

Although assessment of fecal total RNA was not an objective of this study, the GT based extraction method can provide total RNA along with DNA. The quality of total RNA was evaluated in a subset of samples extracted by the GT protocol. Samples stored in both GTB and ethanol processed by the GT extraction protocol (GT method and ethanol–GT method, respectively) did provide total RNA of fairly good quality as shown by the relative ratio of 28 s to 18 s rRNA (data not shown).

Though the GTB-based protocols are time consuming, they were used on primate fecal samples because they have proven successful for obtaining good quality total DNA and RNA from a variety of species (i.e., crustaceans, birds and mammals), thereby allowing for comparison of storage conditions and optimal DNA yield. From fecal samples which weighed less than one gram, up to an estimated 800 ng of total RNA was obtained. This RNA quantity could be increased with larger sample volumes. With the recent interest in noninvasive epidemiological HIV-1 research in chimpanzees (Gao *et al.* 1999, Weiss & Wrangham 1999), an immediate application of GTB based methods for primate fecal samples could be for RNA analysis of chimpanzee fecal samples.

This study demonstrated that good quality DNA can be extracted from field collected primate fecal samples stored without refrigeration for over 30 days. Based on DNA quality and ease of sample processing, this study indicates that the Lysis method is the best for DNA extraction from primate fecal samples, particularly when a large number of samples have to be processed. If RNA is needed for gene expression analysis for epidemiological purposes, and sufficient quantities of fecal material are collected, a GT based extraction protocol is most suitable. It is also noteworthy that the widely available, inexpensive, and safe solution, ethanol, provided comparable quality of total DNA, PCR amplifiable DNA and fairly good quality total RNA when processed through the GT extraction protocol. For all these methods, optimization for amount of template DNA in PCR amplifications should be a prerequisite.

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