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Andrew J. Hopwood · Armando Mannucci
Kevin M. Sullivan

DNA typing from human faeces

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Abstract A method has been developed for the forensic analysis of faeces by DNA amplification and direct sequencing of a polymorphic segment of mitochondrial DNA. Starting from as little as 10 mg wet weight of faeces, DNA was extracted by a variety of protocols and amplified using primers specific to hypervariable region 1 of the mitochondrial control region. The resulting amplification products were sequenced in solid phase using an automated DNA sequencer. In total, mtDNA sequences were generated from the faeces of nine Caucasians and compared with sequences generated from their respective blood samples. Sequences of faeces and blood samples from the same individual were identical in every case, but a range of 1–10 nucleotide differences was observed between individuals, with an average sequence variation of approximately 4.88 per 400 bp. Of the various extraction protocols assessed in this study, greatest success rates were achieved using magnetisable beads to bind and purify the DNA. STR analysis of DNA extracted from faeces was not routinely possible.

Key words Faeces · Mitochondrial DNA · Extraction · Solid-phase sequencing · Forensic DNA typing

Introduction

The application of PCR to the analysis of human cellular material has greatly increased the speed and sensitivity of DNA analysis compared with multilocus and single-locus DNA profiling (Jeffreys et al. 1985; Gill et al. 1985; 1991). Such PCR-based tests include the amplification of variable number tandem repeat (VNTR) loci (Boerwinkle et al. 1989; Horn et al. 1989; Kasai et al. 1990; Tully et al. 1993) and short tandem repeats (STR) (Economou et al. 1990; Edwards et al. 1991; Morral and Estivill 1992; Kimpton et al. 1993). This technology allows consistent typing of as little as 1 ng of genomic DNA. Even greater sensitivity is afforded by the analysis of mitochondrial DNA which is present in copy numbers of 1000–10000 per cell (Bogenhausen and Clayton 1974), thereby enabling mtDNA typing results to be generated from certain evidential materials such as hair shafts (Higuchi et al. 1988; Wilson et al. 1995) and severely degraded human remains (Sullivan et al. 1992) which contain insufficient DNA for chromosomal typing. The most variable region of the human mitochondrial genome is the control region, which includes the origin of H-strand replication, both origins of transcription and the displacement (D)-loop. Polymorphisms are concentrated in two hypervariable regions (HV-1 and HV-2) on both sides of the origin of replication.

Human excrement is encountered as an evidential material in a small but significant proportion of burglary and sexual assault cases in the UK. Faeces comprises a complex mixture of diverse micro-organisms, digested and undigested food residues, mucus, soluble and insoluble products of the gastrointestinal tract and degradative enzymes derived from cells, food and bacteria (Iyengar et al. 1991; Sidransky et al. 1992). To date forensic analytical procedures which have been applied to faeces include Edelman's test for urobilinogen, which indicates the presence of faecal matter (Nicholls 1956); ABO blood grouping of rectal contents (Oshiro et al. 1984); and phosphoglucomutase (PGM) analysis of faecal stained anal swabs (Green and Sayce 1985), all of which are generally very limited in their evidential value.

MtDNA has been successfully extracted and analysed from the faeces of bears (Höss et al. 1992), but most developments in the DNA analysis of human faeces have been confined to the field of medical diagnostics, including the specific detection, by PCR, of strains of micro-organisms present in stools (Gumerlock et al. 1991; Abebe et al. 1992; Katzwinkel-Wladarsch et al. 1994) and the detection of colorectal tumours (Sidransky et al. 1992). How-

A. J. Hopwood (✉) · K. M. Sullivan
Forensic Science Service, Service Development,
Birmingham, B5 6QQ, UK

A. Mannucci
Istituto di Medicina Legale, Universita di Genova,
via di Toni 12, I-16132 Genoa, Italy

ever, little progress has been made towards identification of many of the factors present in faeces that are inhibitory to PCR. It is known that faecal constituents such as bilirubin and bile salts inhibit PCR even when present at low concentrations (Widjojoatmodjo et al. 1992). Therefore, for PCR analysis, DNA purification is required prior to amplification; alternatively, inhibitors can be reduced by dilution of the extract, but this is accompanied by a loss in sensitivity proportional to the dilution factor.

This paper describes the development of a reliable method for the DNA analysis of human faeces by mtDNA amplification and sequencing. Different extraction procedures were evaluated for their ability to yield amplifiable DNA template, and a number of non-human species were tested to show that the test was human-specific and unlikely to be affected by diet.

Materials and methods

Faeces samples from nine Caucasian donors were collected and stored at –20° C. Extraction of total DNA was carried out as described below. All samples were assayed by solid-phase DNA sequencing, and results were compared to the sequence results of control DNA extracted from blood.

In order to ensure no amplification was possible from faeces samples other than human, DNA samples from cat and dog were analysed to evaluate whether these would amplify with the primer sets under the given amplification conditions. As it is possible for incompletely digested foods to be passed in faeces (Höss et al. 1992), attempts were also made to amplify samples of DNA from common domestic species (cow, pig, sheep, red deer, rabbit, turkey and chicken) to determine whether undigested meat in faeces could theoretically interfere with the test.

Non-human DNA was extracted from blood by proteinase K digestion and organic extraction and quantified by UV spectrophotometry (Sambrook et al. 1989).

DNA extraction

Method 1

Extraction was carried out essentially as in Sidransky et al. (1992): 100 mg of faeces was suspended in 300 µl extraction buffer (500 m*M* Tris, 16 m*M* EDTA, 10 m*M* NaCl pH 9.0). Solid particles were removed by centrifugation. DNA in the supernatant was purified by SDS-proteinase K digestion, phenol chloroform extraction and ethanol precipitation as described by Sambrook et al. (1989). The DNA was further purified using the Geneclean II kit (Bio 101, La Jolla, Calif.)

Method 2

Between 10 and 100 mg of faeces was suspended in 300 µl extraction buffer (see Method 1) and spun at 10000 g in a microcentrifuge for 2 min. This pelleted the faecal solids, which were overlaid by a lighter-coloured layer of matter. The supernatant was carefully removed avoiding disturbance of the surface of the solids. After careful removal of the paler-coloured surface layer to a clean sterile tube, the faecal solids were discarded. The sample volume was made up to 300 µl with extraction buffer containing 0.5% w/v SDS and 400 µg/ml proteinase K and incubated at 56° C for 2 h. The sample was subsequently extracted twice with phenol, twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform. Following ethanol precipitation overnight at –20° C, the nucleic acids were collected by centrifugation at 10000 g for 30 min, washed in 70% ethanol and redissolved in 50 µl dH_2O.

Method 3

Samples of 10 and 100 mg of faeces were extracted as in method 2, but following proteinase K digestion, the extract was purified using the Geneclean II protocol, the DNA being eluted in a final volume of 50 µl water.

Method 4

Extraction was carried out using Amersham Nuclitips (Amersham, Buckinghamshire, UK) in accordance with the manufacturer's instructions. The Nuclitips comprise a pipette tip, the end of which is covered by a membrane. As a sample is passed through the membrane, DNA contained therein becomes bound to the membrane, which is added directly to a PCR cocktail. Samples of 100 mg faeces were suspended in 300 µl of lysis buffer 1 (320 m*M* sucrose, 10 m*M* Tris, 5 m*M* $MgCl_2$, 1% Triton X-100, pH 8.0). The suspension was spun at 100 *g* for 30 s to remove the solids and the majority of bacteria. The supernatant was set aside for 5 min to allow for lysis of the cells. Next, 100 µl of the supernatant was pipetted in and out of the tip until the tip became occluded. All the sample was expelled, and cell debris was removed from the tip by pipetting in and out in PBS until the membrane covering the tip was completely clean. The Nuclitip was removed to a 0.5-ml microfuge tube containing the PCR mix.

Method 5

Genereleaser (Cambio, Cambridge, UK) was assessed using two methods:

a) Samples of 100 mg faeces were suspended in 300 µl water. Aliquots of between 1 and 8 µl of the suspension were added to 20 µl of Genereleaser and the samples were heated as follows: 65° C for 30 s, 8° C for 30 s, 65° C for 90 s, 97° C for 180 s, 8° C for 60 s, 65° C for 180 s, 97° C for 60 s, 65° C for 60 s, 80° C for 5 min, in accordance with the manufacturer's instructions. The PCR reaction mix was added to the samples at 80° C, then heated at 95° C for 3 min followed by the thermal cycling programme for amplification of mtDNA.

b) Samples of 100 mg of faeces were suspended in 300 µl water. Solids were removed by centrifugation and the supernatant was discarded. Aliquots between 0.5 µl and 5 µl were added to 15 µl Genereleaser and placed in a microwave oven on full power for 5 min in accordance with the manufacturer's instructions. The amplification reaction was set up as described in 5 a.

Method 6

Samples of 10 and 100 mg of faeces were extracted as previously described by Sullivan et al. (1994). The sample was vortex mixed in 300 µl of water plus 100 µl G1 buffer from the Qiagen cell culture DNA kit (Qiagen, Dorking, UK) and incubated at 55° C for 20 min. To this was added 400 µl G2 buffer plus 0.4 mg proteinase K, followed by brief vortex mixing and 60 min incubation at 55° C. Undigested solids were removed by centrifugation in a microfuge at 10000 *g* for 10 min. The supernatant was decanted to a clean microcentrifuge tube, spun again at 10000 *g* for 10 min, and the supernatant removed to a clean microcentrifuge tube. DNA was extracted from the supernatant and resuspended in 40 µl dH_2O using the Geneclean II kit in accordance with the manufacturer's instructions.

Table 1 Sequence of primers used in mitochondrial amplification

Primer	Nucleotide sequence
L15933	5′-CAGTCTTGTAAACCGGAGATG
H00575	5′-TGAGGAGGTAAGCTACATAAACTG
5′Biotin L15997	5′Biotin-CACCATTAGCACCCAAAGCT
M13(-21) H16401	5′-TGTAAAACGACGGCCAGTTGATTTCACGGAGGATGGTG
5′Biotin H16401	5′Biotin-TGATTTCACGGAGGATGGTG
M13(-21) L15997	5′-TGTAAAACGACGGCCAGTCACCATTAGCACCCAAAGCT

Method 7

Samples of 100 mg faeces were suspended in 300 µl extraction buffer as in method 2, except that following removal of the pale-coloured surface layer to a clean sterile tube, the DNA was extracted using the DNA Direct kit (Dynal, Wirral, UK). Aliquots of 200 µl of beads in buffer A were added to the tube and left to stand for 5 min. This allowed lysis of the cells and binding of DNA to the surface of the beads. The tube was then placed in a MPC-E magnetic particle separator (MPC) (Dynal, UK) and the beads were allowed to migrate towards the magnet. The beads were washed 3 times in 200 µl of buffer B, each time allowing the beads to migrate towards the magnet. The beads were resuspended in 30 µl buffer C. This extract was used directly in the PCR reaction.

Quantification of human DNA from faeces

From each DNA extract 5 µl was quantified by direct comparison to standards of known concentration by the dot blot method, employing a biotinylated human-specific DNA probe to alpha satellite locus D17Z1, and ECL detection (Amersham) as described by Walsh et al. (1992). Quantitation was not carried out on extracts from the Genereleaser or Nuclitips methods, as DNA was not obtained as a solution from these protocols.

DNA amplification

Mitochondrial DNA for solid-phase sequencing was amplified as previously described by using two rounds of nested PCR (Sullivan et al. 1994) using the primers detailed in Table 1. In the first round, 1 µl of faeces extract or 100 pg of control DNA was amplified in a volume of 50 µl, utilising primers L15933 and H00575 in a 25-cycle reaction. A 1 µl aliquot of this product was added to two separate second-round reactions of 50 µl, with each primer pair comprising one 5′ biotinylated primer plus one chimeric primer including the M13 universal sequencing primer sequence at the 5′ end [5′ biotin L15997 with M13(-21) H16401, 5′ biotin H16401 with M13(-21) L15997]. This approach enabled both strands of HV-1 to be subsequently analysed and compared to verify the mtDNA sequence. If a sample did not give an amplification product, it was assessed for the presence of inhibitors by repeating the nested PCR with a fresh aliquot of extract to which had been added 1 ng of human cell line DNA prior to PCR. Amplification of non-human DNA was carried out as above, but 1 ng of DNA was added as a template. All PCR primers were synthesised by Oswel DNA Service, Southampton, UK.

Solid-phase sequencing

Aliquots of 20 µl of streptavidin-coated Dynabeads (Dynal) were prewashed in ten volumes of beading-washing buffer (BW; 2 *M* NaCl, 10 m*M* Tris, 1 m*M* EDTA, pH 7.5) and resuspended in 40 µl BW. Then, 40 µl of the second-round PCR product was added and incubated at 48° C for 15 min. The DNA/bead complex was concentrated on the side of the tube with an MPC and washed once in 50 µl BW and once in sterile distilled water (SDW). The DNA was made single-stranded by denaturing with 8 µl 0.15 *M* NaOH for 7 min. The supernatant was removed and the beads were washed with 50 µl 0.15 *M* NaOH. This was followed by subsequent washes in 60 µl BW, 60 µl 1 × TE (10 m*M* Tris, 1 m*M* EDTA pH 7.5) and 60 µl SDW. The beads were resuspended in 16 µl SDW. Sequencing using a Prism dye primer sequenase single-stranded DNA sequencing kit (Perkin Elmer) was in accordance with the manufacturer's instructions, except that primer annealing was carried out at 65° C for 2 min and the temperature reduced to 25° C over 35 min. When the reaction was complete, 15 µl of stop salt (1 *M* NaAc, 20 m*M* EDTA, pH 8.0) was added to the T tube followed by addition of the A, C and G reactions. The beads were then washed twice in 50 µl Tris-Tween (250 m*M* Tris, 0.1% Tween-20, pH 8.0) and once in 1 × TE. The beads were finally resuspended in 4 µl deionised formamide. The samples were denatured at 37° C for 4 min, placed on ice, and the supernatant transferred to a new tube prior to loading on to a 6% acrylamide, 7 *M* urea gel with a well-to-read length of 36 cm. Data was collected on an Applied Biosystems 373A stretch sequencer running at 30 W and analysed using Applied Biosystems programs Sequence Analysis version 2.1.0 and Sequence Navigator version 1.0.

STR analysis

Amplification of the locus HUMTH01 was attempted on samples which gave measurable results in the genomic DNA quantitation assay. Amplification parameters were essentially as described by Kimpton et al. (1993), except that the number of amplification cycles was increased from 30 to 35.

Results and discussion

Evaluation of extraction methodologies

Method 1

MtDNA amplification was successful from one-third of the extracts. All the remaining extracts failed to amplify due to the inhibition of the PCR, assessed by the addition of DNA to the extract prior to amplification. All extracts lacked quantifiable yields of nuclear DNA.

Method 2

Samples extracted using this method produced amplification products from mtDNA in 90% of cases. The majority of samples that did not amplify were from larger amounts (100 mg) of starting material, and these extracts showed inhibition of the PCR. On sequence analysis, good-quality results for the full length of the PCR product were generated. The sequence from faeces was identical to that obtained from the control DNA in each individual. A typical

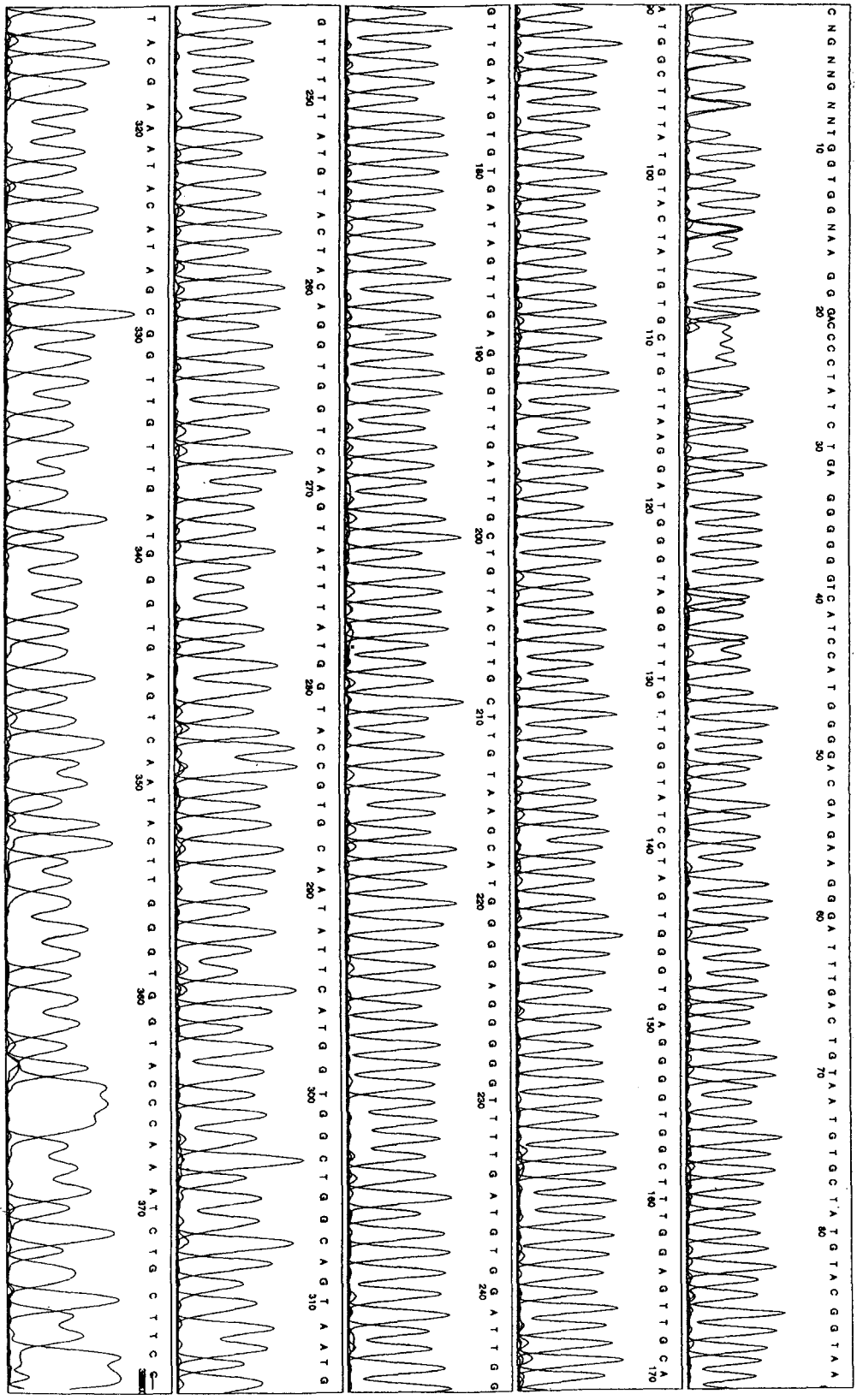

Fig. 1 Sequence of mtDNA purified from faeces. The PCR product was amplified from DNA extracted by method 2 using primers L15933 and H00575 in the first round and 5′ biotin H16401 and M13(-21) L15997 in the second round. The product was captured onto a streptavidin-coated paramagnetic bead and sequenced using the Prism dye-primer sequenase single-stranded DNA sequencing kit (Perkin Elmer). The resulting reaction was run on an Applied Biosystems 373A Stretch sequencer and analysed using Applied Biosystems Sequence Analysis version 2.1.0 and Sequence Navigator version 1.0

sequencing result is shown in Fig. 1. All extracts lacked quantifiable yields of DNA apart from one sample for which 200 ng/g of genomic DNA was detected. This sample gave a weak but reproducible result upon amplification and analysis of locus HUMTH01.

Method 3

This extraction technique was more rapid than method 2 and gave mitochondrial sequence results of comparable quality. However, mtDNA amplification was successful from only two-thirds of the samples. The remaining third showed inhibition. No extracts produced quantifiable yields of genomic DNA.

Methods 4 and 5

No amplification products were generated from extracts using either Nuclitips or Genereleaser. Inhibition was apparent in all extracts.

Method 6

All extracts generated from 100 mg starting material failed to yield mtDNA PCR products. However one-third of extracts starting from 10 mg of faeces were successfully amplified using the mtDNA protocol and gave good sequence data. No extract gave quantifiable genomic DNA.

Method 7

Extraction of DNA using the DNA Direct kit was both the fastest and the most reliable of the methods tried. DNA could be extracted from a batch of six samples in a form suitable for amplification in under 20 min. MtDNA amplification products and sequence results were generated from 100% of faecal extracts. It was possible to amplify mtDNA extracted directly from faecal slurries with a 100% success rate, showing that the beading and washing method employed by this protocol is very efficient at separating DNA from inhibitors during extraction. Although all samples gave an apparent yield of between 40 ng and 136 ng of human nuclear DNA per gram of faeces, all attempts to amplify locus HUMTH01 failed. No inhibition was apparent, suggesting that the major part of the nuclear DNA in the extracts may have been degraded to such an extent that it was not a suitable template for amplification. The DNA extracted by this method was readily visible and evident as a discrete band of high molecular weight on an ethidium bromide-stained 1% agarose gel (data not shown).

Overall, method 7 is the procedure of choice for mtDNA analysis as it provides a simple protocol for the consistent extraction of mtDNA suitable for amplification and subsequent sequencing. If necessary, DNA can be extracted from faeces in less than 20 min without the need for organic extraction or any precipitation steps. The kit is also capable of extracting DNA from faecal samples in amounts sufficient for quantification by a human-specific probe. However, no positive results for STR amplification were gained from these extracts, although a large quantity of DNA was present as viewed by agarose gel electrophoresis. The dot-blot quantitation assay employed may have given false positives by non-specific binding of the DNA probe to excessive amounts of bacterial nucleic acids present in the samples. It is more likely, however, that the nuclear DNA in the extracts was in the main part degraded to fragments of a size which, although suitable for quantification using the probe to D17Z1, were too small to act as a template for PCR.

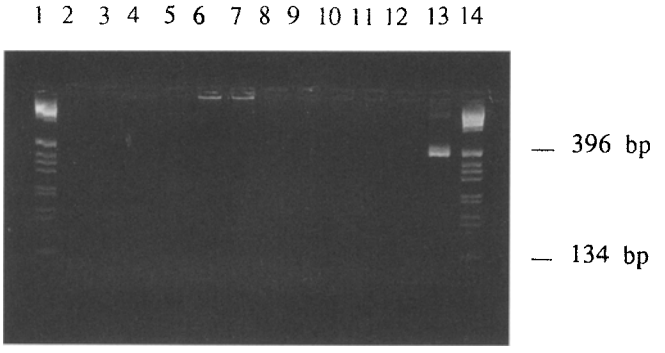

Fig. 2 Ethidium bromide-stained agarose gel showing results of attempted mtDNA amplification from common animal species. *Lanes 1, 14* 1-kbp ladder (DNA molecular weight marker), *lanes 2–10* 8-µl aliquots of second-round PCR products from cat, dog, rabbit, sheep, pig, cow, chicken, turkey and red deer, respectively, *lanes 11, 12* negative control water blanks, *lane 13* human DNA yielding a 442-bp product from 100 pg starting material

MtDNA sequencing results

A typical sequence electropherogram of DNA amplified from a faecal extract is shown in Fig. 1. There was no difference in the quality of mitochondrial sequence from the amplification products of DNA from faecal or blood extracts, and there was no evidence of interference with the sequence data from dietary content of faeces. This was confirmed by the failure to amplify DNA samples from common meat products under the defined PCR conditions. Similarly, DNA from cat and dog, which are a source of faeces in the domestic environment, did not yield PCR products under the standard amplification conditions (Fig. 2). Pairwise comparisons of mitochondrial sequence data from the nine samples yielded from 1 to 10 differences between the nine Caucasian subjects with an average sequence variation of approximately 4.88 per 400 bp. The sequencing results are summarised as differences to the reference sequence (Anderson et al. 1981) in Table 2.

STR analysis

Although an estimated 10^{10} cells are lost per day from the gastrointestinal tract (Sidransky et al. 1992), routine STR

Table 2 Summary of mtDNA sequence differences compared to the Anderson reference sequence

Nucleotide position	16051	16069	16093	16126	16189	16192	16207	16218	16224	16274	16294	16298	16304	16311	16314	16356	16390
Reference sequence (Anderson et al.)	A	C	T	T	T	C	A	C	T	G	C	T	T	T	A	T	G
1	–	–	–	C	–	T	G	–	–	A	T	–	C	–	–	–	–
2	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–
3	G	–	C	–	–	–	–	–	C	–	–	–	–	C	–	–	–
4	–	–		C	–	–	–	–	–	–	T	–	–	–	C	–	–
5	–	–		–	–	–	–	–	–	–	–	–	–	–	–	C	–
6	–	–	C	–	–	–		–	C	–	–	–	–	C	–	–	–
7	–	T	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	T	–	–	–	C	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	A

analysis was not possible following extraction by any of the methods described. Only a single sample extracted by method 2 gave a reproducible result with a TH01 singleplex amplification. Assuming each cell contains approximately 6 pg of genomic DNA, this corresponds to 600 mg of DNA lost daily via the intestine. Assuming the average mass of stool per day is approximately 100 g (Goy et al. 1976; Burkitt et al. 1972) and degradation of DNA is minimal, a stool sample might contain 6 mg of DNA per gram. However, the amount of quantifiable genomic DNA extracted from faeces (200 ng/g from one sample with method 2, 40–136 ng/g from method 7) was much lower than the theoretical yield. This is probably due to the action of degradative enzymes derived from cells, food and bacteria on the DNA.

The success rate of STR analysis was very low. Mitochondrial DNA was successfully amplified from the same extracts, suggesting that failure of STR analysis was probably a consequence of degradation of genomic DNA rather than inhibition of the PCR. The quality of the genomic DNA extracted may be poor due to the presence of degradative enzymes and harsh conditions in the colon. It is possible that, while the cellular and nuclear membranes are disrupted in the gastrointestinal tract, the mitochondrial envelope is somehow not lysed, thus affording mtDNA some protection against degradation. MtDNA may also be protected to a certain extent from exonuclease digestion by its conformation as a covalently closed circular DNA molecule. The success of mitochondrial analysis, however, is more likely to be due to the mtDNA being present in much higher copy number than nuclear DNA. The high copy number of mtDNA also allows analysis in samples where inhibitors of PCR are present: following dilution of a sample to a level where inhibition is minimised, the nuclear DNA concentration may be too low to generate reliable results but mtDNA is more likely to be present in a sufficient quantity to allow reliable amplification.

This paper demonstrates the utility of mtDNA sequencing for the DNA typing of human faecal samples. For forensic casework we presently restrict mtDNA sequencing analysis to discrete samples. However, mixtures of DNA can be analysed provided that all possible interpretations of the data are considered. Scenarios include mixtures, experimental contamination and heteroplasmy. Previous work in our laboratory (Sullivan et al. 1994) has shown that the combined sequence of a 10:1 mixture corresponds to the sequence of the major component. However, if the background noise is low enough, the sequence of the minor component can be identified by visualising the smaller peaks under the peaks of the majority species. With more even ratios of templates, if the sample is known to be a mixture and the sequence of one component is known, the sequence of the second may be deduced. Heteroplasmy cannot be ruled out unless a mixture is known to be present and both contributing sequences of that mixture have been separately analysed.

The protocol detailed above enabled all nine Caucasians in this study to be distinguished from each other. Overall, within HV-1 the probability of a chance match (pM) between 100 unrelated British Caucasians has been estimated to be 0.034 (Piercy et al. 1993, using $p = \Sigma x 2$ where x is the frequency of mitochondrial genotype). If this is combined with second-round amplification and sequencing of HV-2 the combined pM for both regions is 0.026. This compares favourably with analysis of a single STR locus, for example HUMTH01, which has a pM of 0.086 (Urquhart et al. 1995), but is far less discriminating than multiplex STR systems. However, at present we have not found a reliable protocol for the extraction and subsequent multiplex STR amplification of genomic DNA from faeces. This problem is the subject of further research.

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