TECHNICAL NOTE

Fu Shyan Chiou,[1] M.S.; Chung Yen Pai,[2] M.S.; Yun Pung Paul Hsu,[2] Ph.D.; Chun Wei Tsai,[3] M.S.; and Chiou Herr Yang,[1] Ph.D.

Extraction of Human DNA for PCR from Chewed Residues of Betel Quid Using a Novel "PVP/CTAB" Method

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ABSTRACT: Residues of chewed betel quid (BQ) are often found on crime scenes in Taiwan and possibly some of the Southeast Asian countries. Although these residues are important biological evidences relating to the suspects, the forensic analysis of BQ evidence has been hindered by failures in extraction of human DNA for PCR analysis. Therefore, it is a prerequisite for relevant forensic casework to establish a reliable method for extracting DNA from chewed BQ residues.

Three conventional methods (salt/chloroform, 5% Chelex-100 resin, and QIAamp) were first tested for extraction of human DNA from 33 mock BQ samples, which had been stored for less than two months, and 50 four-year-old forensic BQ samples. PCR amplifications from the HLA-DQA1&PM and the STR loci were then used to test the quality of the extracted DNA. For the mock samples, three observations were made. First, PCR amplification of DNA extracted by using these conventional methods had low success rate. Second, the addition of extra Taq DNA polymerase could compensate the lost enzyme activities due to putative inhibitors and, thus, increase the yield. Third, using the Centricon™-100 column to remove putative inhibitors substantially improved the efficiency of PCR. However, for the four-year-old forensic BQ samples, none of the attempts for PCR were successful.

In order to solve the problem in PCR analysis of DNA from old BQ samples, we developed a DNA extraction method based on the use of polyvinyl pyrrolidone (PVP) and cetyltrimethylammonium bromide (CTAB), which bind to two common classes of PCR inhibitors in plants, polyphenols, and polysaccharides, respectively. The result showed that this "PVP/CTAB" method is completely successful for the mock BQ samples, and 92% (46 out of 50) successful for the four-year-old forensic BQ samples. To our best knowledge, this is the first report of a reliable method for the extraction of human DNA for PCR from chewed BQ residues. This method should provide a useful means for forensic identification in countries where betel chewing is common.

KEYWORDS: forensic science, betel quid residue, forensic identification, DNA extraction, PVP/CTAB, PCR amplification

[1] Department of Forensic Science, Central Police University, 56 Shu-Jen Road, Taoyuan Kueishan, Taiwan ROC.

[2] Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan ROC.

[3] Medical Jurisprudence Office, Criminal Investigation Bureau, National Police Administration Ministry of Interior Republic of China, Taipei, Taiwan ROC.

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For forensic purpose, genomic DNA has been extracted from saliva found on a variety of surfaces such as cigarette butts (1), postage stamps (2), and human skin (3). The residue of chewed betel quid (BQ) is one of the important biological evidences often found at crime scenes in countries where betel the chewing is prevalent. The spit-out BQ evidence left at the scene is usually in a form of chewed solids, which are red/brown in color and contain semi-dry BQ residue. Analysis of DNA from oral epithelial cells contained in chewed betel residues can lead to forensic identification. Unfortunately, the DNA analysis of BQ evidence has been hindered by the failure in DNA extraction and PCR amplification. Here we report a reliable method for the extraction of human DNA from the residue of chewed BQ.

Betel is originally grown in Malay, and is now widely used in the tropical zone and parts of the subtropical area. It can be estimated that about one hundred million people in the world chew betel quid, and in Taiwan alone, about two million out of the population of twenty-three millions do that. The commercial product of BQ in Taiwan is generally prepared by cutting an unripe betel nut into two halves and sandwiching with piper betel inflorescence, some Chinese herbs, and red slaked lime consisting mostly of oyster shell powder and orange rind. Betel chewing may generate sensations of cooling, inhibition of feeling cold, and stimulation of saliva production (4). These effects have drawn hundreds of millions of betel lovers, widespread in Taiwan, South Africa, India, and many Southeast Asian countries (5,6).

Several DNA extraction methods for saliva-stained material were reported. Word et al. (7) reported DNA extraction from fresh saliva and various saliva-stained materials such as envelopes, buccal swabs, gags, and cigarettes. They incubated the sample with a lysis buffer and proteinase *K*, and extracted DNA by using the "phenol/chloroform" method. Sweet et al. (8) reported an improved DNA extraction method for saliva stains deposited on human skin. They enhanced the yield of extracted DNA by using a modified "chelex" method (8). Their modifications included separation of the cells from the swab by treatment with proteinase K, and incubation at 56°C before the routine chelex DNA extraction. Fridez et al. (9) also evaluated the DNA extraction from saliva deposited on stamps, and they concluded that the phenol/chloroform method leads to much better PCR results than the chelex method. When DNA extracted by the chelex method was further purified with the Centricon™-100 microconcentrators (Amicon, Beverly,

MA, USA), the DNA could still be amplified by PCR. However, as far as we know, effective methods for the extraction and PCR of human DNA from chewed betel residue have not been reported.

The purpose of this study is to develop a reliable method for extracting human DNA from chewed BQ residue in forensic identification. Several common methods for the extraction of DNA from four-year BQ samples were first evaluated by PCR. Strategies for removing inhibitors of PCR were then evaluated. And eventually, a novel method based on the combined use of polyvinyl pyrrolidone (PVP) and cetyltrimethylammonium bromide (CTAB) was developed. Our result showed that this "PVP/CTAB" method is effective for the extraction of PCR-quality human DNA from chewed BQ samples, including four-year-old forensic BQ samples, which had been refractory to DNA analysis.

Methods and Materials

Chewed Residues of Betel Quid

Mock Samples—Commercial betel products were purchased and chewed for 5 min by one of twelve nonusers and 21 users who have been chewing BQ for 7–25 years. The residue after chewing was spit on a piece of filter paper (Whatman, 9 cm) and left to air-dry at room temperature for a period of time from two days to eight weeks. The weight of the dried betel residue ranged from 500 mg to 800 mg.

Four-year-old Forensic BQ Samples—Fifty BQ evidences were collected in a 1996 case, which was still under investigation. These samples were air-dried on filter papers and stored in the freezer (−20°C) after attempts for DNA extraction and PCR had failed.

Isolation of Buccal Cells from Chewed BQ Residues

Aliquots of 25 mg of the chewed BQ residue were put in 1.5 mL microtubes. The betel residue was suspended in 1 mL of sterile water for 40 min at room temperature, and the suspension was stirred for 2 min by using a toothpick. The betel residue was then picked out and discarded, and the remaining suspension was centrifuged at 12 000 × *g* for 2 min. The supernatant was withdrawn and discarded, and the pellet containing buccal cells was ready for DNA extraction.

DNA Extraction

The Salt/Chloroform Method—For each sample, 800 μL of SE buffer solution (75 mM NaCl, 25 mM EDTA, pH = 8.0), 1% SDS (final concentration), and 200 μg/mL proteinase *K* (final concentration) were added and incubated at 56°C overnight. A volume of 296 μL of 6 *M* NaCl solution was added to make the final volume 1192 μL, and the mixture was then divided equally into two 1.5 mL microtubes. For each tube, 596 μL of chloroform were added and the tube was agitated gently for 30 min. The rest of the procedures were done according to a published salt/chloroform protocol (10).

The QIAamp Method—This extraction procedure is performed by using the QIAamp DNA kit (Qiagen, Valencia, CA). For each sample, after addition of 400 μg of proteinase *K* and 200 μL of buffer AL (provided in the kit), the solution was incubated at 56°C for 1 h. DNA was extracted from the solution according to an instruction manual provided by the manufacturer (QIAGEN Inc. QIAamp DNA Mini Kit and QIAamp DNA Blood Min. Kit Handbook, Valencia, CA).

The 5% Chelex-100 Extraction Method [11]—For each sample, the pellet from the procedure of isolation of buccal cells was suspended in 300 μL of the 5% chelex-100 solution (pH 9.0). The solution was incubated at 56°C for 30 min and then heated at 100°C for 8 min. It was then centrifuged at 13 000 × *g* for 3 min; and the solution was ready for PCR.

The PVP/CTAB Extraction Method—Twenty-five mg of the residue from the control samples or the four-year old samples were put into 1.5 mL microcentrifuge tubes. Five hundred μL of the extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA, 2% PVP), 56 μL of 10% SDS (1% final concentration), and 200 μg of proteinase *K* (20 mg/mL) were added into the tube. The solution was incubated at 56°C for 2 h, and then at 100°C for 2 min. A solution of 6 *M* NaCl was added to give a final NaCl concentration of 1 *M*. The residue was discarded, and a solution containing 10% CTAB/0.7 *M* NaCl, and a solution of bovine serum albumin (10 mg/mL) were added to give a final concentration of 1% CTAB and 2% BSA. The solution was incubated at 65°C for 10–20 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added into the solution, mixed thoroughly and centrifuged at 15 000 × g for 15 min at room temperature. The aqueous phase was withdrawn and transferred to a new microcentrifuge tube. Then another volume of the 10% CTAB/0.7 *M* NaCl solution was added into the solution to give a final concentration of 1% CTAB. The solution was incubated at 65°C for 5 min. The aqueous solution was thoroughly mixed with an equal volume of chloroform/isoamyl alcohol (24:1) and was centrifuged at 15 000 × *g* for 15 min. The aqueous phase was then withdrawn and transferred to a new microcentrifuge tube. An equal volume of isopropanol was added to the aqueous solution. The tube was kept at room temperature for 10 min, and then centrifuged at 15 000 × *g* for 15 min. The solution was discarded and the precipitate was dissolved in 500 μL of the TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0). The procedures of incubation in 10% CTAB/0.7 *M* NaCl (final CTAB concentration 1%), extraction with chloroform/isoamyl alcohol (24:1), and precipitation in isopropanol were repeated twice more. The precipitate was then washed twice with 700 μL of a solution containing 76% ethanol and 10 mM ammonium acetate, and then collected at the bottom of the tube by centrifugation at 15 000 × *g* for 5 min. The DNA pellet was dried in a 37°C oven for 1 h and then dissolved in 70 μL of ddH_2O.

Tests of Quantity and Quality of Extracted DNA

The amount of extracted human DNA was quantified by using a slot blot hybridization procedure described by Waye et al. (12). The quality of the extracted human DNA (using the salt/chloroform method) from three kinds of samples (chewed betel residue, oral swabs, and bloodstains) of the same individual was tested by PCR at the STR loci (described below).

For the extraction of DNA from chewed BQ residue, the commercial betel product was chewed for 5 min, and the residue containing the oral epithelial cells was collected and processed as described above.

For the extraction of DNA from oral swabs, the mouth of the subject was vigorously cleaned three times by rinsing with mineral water, and a cotton swab was used to collect the oral epithelial cells. The cotton swab was suspended in 1 mL of autoclaved distilled water in a sterile 1.5 mL microcentrifuge tube, and incubated at room temperature for 1 h. The swab was twirled vigorously for 2 min with an autoclaved toothpick to release the oral epithelial cells, and then the swab was removed. The sample was centrifuged for 5 min at 13 000 × *g* in room temperature, and all but about 50 μL of the supernatant was discarded.

For the extraction of DNA from bloodstains, two drops of whole blood were spotted on a piece of autoclaved cotton cloth, dried at room temperature, and stored at −20°C. One cm^2 area of the

bloodstained cloth was cut out and placed in a sterile 1.5 mL microcentrifuge tube. Five hundred μL of a digestion buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 1% SDS, pH 7.5), and 15 μL of proteinase *K* (final concentration: 0.3 mg/mL) were added. The solution was mixed gently and incubated at 56°C overnight. The bloodstain substrate was removed with an autoclaved disposable pipette tip. The solution was ready for DNA extraction.

PCR Analysis of Extracted DNA

The STR Loci—The Amp*ℓ*STR Profiler™ PCR amplification kit (Perkin-Elmer Corporation, Foster City, CA, USA) was used to amplify STR at nine loci: D3S1358, VWA, FGA, TPOX, TH01, CSF1PO, D5S818, D13S317, and D7S820. The PCR mixture contained 10.5 μL of the reaction buffer, 0.5 μL of AmpliTaq Gold DNA polymerase (2.5 units), 5.5 μL of the primers set solution, 3 ng of template DNA, and 5.5 μL ddH_2O. The thermal-cycling profile was: incubation at 95°C for 11 min, 28 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min, and then hold at 60°C for 45 min. The PCR products were genotyped using the ABI PRISM™ GeneScan^R Analysis 3.1 software (Perkin-Elmer Corporation, Foster City, CA).

The HLA-DQA1 and PM Loci—The "AmpliType^R DQA1-&PM" PCR-amplification and typing kit (Perkin Elmer/Roche Molecular System, Branchburg, New Jersey.) was used to evaluate the quality of the extracted DNA. The PCR mixture contained 20 μL of reaction buffer, 20 μL of the primer set solution, and 3 ng of the template DNA. For the PCR of template DNA extracted by the conventional methods, 0.5 units to 2.0 units of extra AmpliType^R Taq DNA polymerase (one unit of enzyme is defined as the amount that will incorporate 10 n moles of dNTP's into acid-insoluble material per 30 min in a 10 min incubation at 74°C) were added before the PCR was carried out. The program for the PCR was: 32 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 30 s, followed by incubation at 72°C for 7 min at the end of the cycles. The genotypes were analyzed by using a reverse dot-blot hybridization method included in the kit.

Results and Discussion

For the mock samples from nonusers, at least 200 ng of DNA were obtained from 25 mg of three-week-old betel residues using all the extraction methods tested. In general, the longer the storage time, the less DNA could be obtained. For example, the amount of DNA extracted from 25 mg of a nonuser sample decreased from 225 ng (day 1) to 112 ng in 8th weeks. The amount of DNA varied for the mock samples from the BQ users. For example, it ranged from 12.5 ng to 500 ng when the PVP/CTAB method was used. The variation was apparently independent of the BQ-chewing history, but could probably depend on the amount of buccal cells released into the BQ residues. In contrast to the mock samples, when all four of the extraction methods described were applied to the fifty-four-year-old forensic BQ samples, only 2 ng of DNA in average were obtained from 25 mg of BQ residue. Generally speaking, the "salt/chloroform" method, the "chelex-100" method, and the "PVP/CTAB" method yielded more DNA in comparison to the "QIAamp" method.

Although at least 100 ng of DNA could usually be obtained from the mock samples within the first eight weeks, the efficiencies of PCR for DNA extracted from chewed BQ residues were generally lower than those from the bloodstains or the oral swabs, when samples from the same individual were compared (Fig. 1). For the

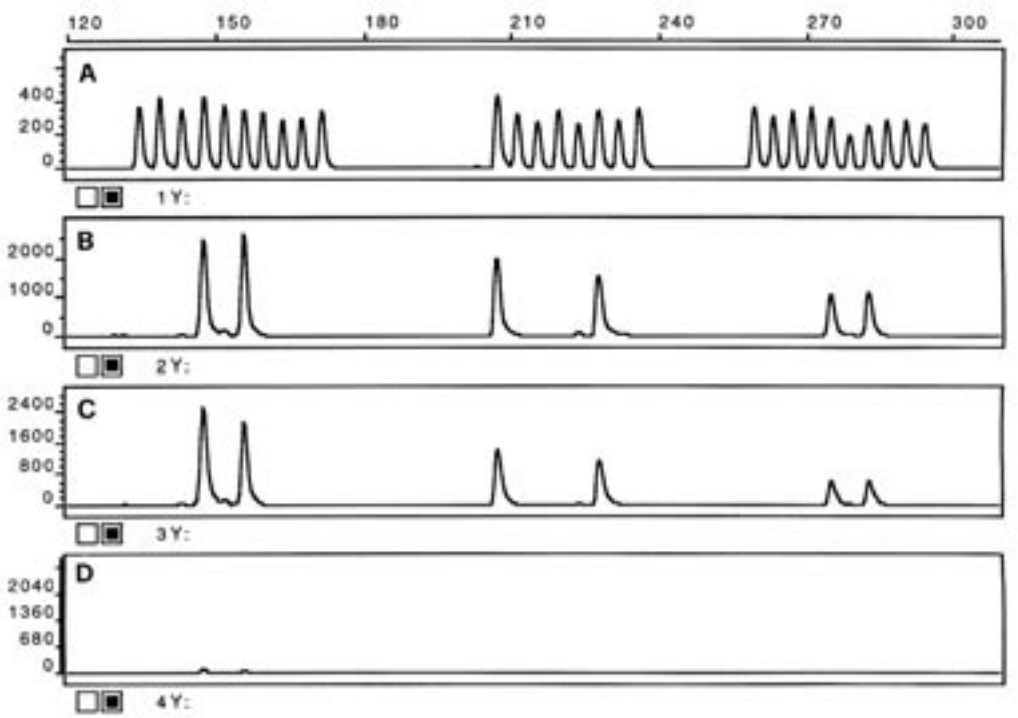

FIG. 1—*PCR of three STR loci (D5S818, D13S317, and D7S820) using DNAs extracted by conventional extraction methods from bloodstains, oral swabs, and chewed BQ residues of the same individual. Panel (A) shows the DNA ladders for markers D5S818, D13S317, and D7S820. Panels (B), (C), and (D) represent allele patterns of the marker loci for DNAs extracted from blood, buccal cells, and betel residue samples, respectively. For DNA from chewed BQ residue (Panel D), the PCR result would be significantly influenced by extraction procedure, and thus only two of the nine genes loci (D3S1358, VWA, FGA, TPOX, TH01, CSF1PO, D5S818, D13S317, and D7S820) were successfully amplified.*

mock samples that had been stored for up to eight weeks, PCR success rates (evaluated using the HLA-DQA1&PM markers) were very low for the salt/chloroform, the chelex-100, and the QIAamp methods (Table 1). In contrast, for DNA extracted using the PVP/CTAB method, the PCR amplifications were completely successful (Table 1). Moreover, genotyping results were clear and complete (Fig. 2).

The low success rates for the conventional methods were probably due to the presence of PCR inhibitors in the extracted DNA. To show this, a mixing experiment was done as follows. The BQ materials were ground into fine pieces without chewing, and then the conventional DNA extraction procedures were performed. When 2 μL of the final extract were added into 25 μL of a PCR mixture containing 5 ng of pure template DNA, the PCR always failed (data not shown). Even when only one μL of the extract was added, the PCR efficiency diminished significantly (i.e., some loci could not be amplified). In contrast, when the BQ materials were ground and extracted with the newly developed PVP/CTAB method (see below), and then the extract was added to the test PCR, no inhibition was observed (data not shown).

Several strategies, including filtration of DNA solutions, addition of extra amount of Taq DNA polymerase, and addition of BSA were tried to improve the PCR. For DNA extracted with the QIAamp method, the addition of an extra amount of AmpliType^R Taq DNA polymerase (1.5 units into 50 μL of PCR mixture containing 3 ng of DNA template), resulted in the appearance of PCR products (data not shown). For DNA extracted with the salt/chloroform method, additional Taq DNA polymerase increased the yield. However, for DNA extracted with the chelex method, addition of extra Taq polymerase did not help at all. However, when the extracted DNA was further purified with the Centricon-100 filtration unit and washed four times with 2 mL of ddH_2O, and extra Taq polymerase (0.5 units) was added, the success rate increased remarkably. It is noteworthy that for the DNA extracted using the PVP/CTAB method, PCR was 100% successful for the mock samples from both the users and the nonusers, even without the addition of extra Taq DNA polymerase (Table 1 and Fig. 2).

Fifty forensic samples of chewed BQ residues were collected four years ago. These BQ evidences have been stored at −20°C since then. Our initial attempts to extract DNA for PCR by using the three conventional methods also failed. However, when we applied the newly developed PVP/CTAB method to extract DNA from these forensic samples, which have been stored for almost four years, the PCR success rate was more than 90% (46 out of 50) (Table 1). The guideline rules proposed by Gill et al. (13) were applied when we handled these forensic samples. Figure 3 shows an electropherogram after using the PVP/CTAB method to extract DNA from an eight-week-old mock BQ sample, for which the traditional extraction methods had failed. These modifications resulted in colorless DNA pellets with yields similar to those obtained using the salt/chloroform method and the chelex method, but with qualities for PCR far superior to those from the conventional methods. The high success rate can be attributed to the fact that PVP and CTAB bind to polyphenols and polysaccharides respectively, which are known to be present in different concentrations in the leaves, barks, and fruits of most plants.

The polyphenolic molecules are usually retained in plant vacuoles, and they have been shown to inactivate enzymes, precipitate

TABLE 1—*Success rates of PCR of template DNAs extracted from chewed BQ residues using four kinds of extraction methods. The extracted DNA samples were PCR-amplified and genotyped at nine of the STR loci, the HLA DQA1 & PM loci, or the ABO gene locus. The PCR for each sample was counted as a success when at least one of these loci was successfully amplified.*

	Chelex	QIAgen	Salt/Chloroform	PVP/CTAB
BQ within 8 weeks	0% (0/36)	16% (6/36)	27% (10/36)	100% (26/26)
Four-year-old samples	0% (0/10)	0% (0/10)	0% (0/10)	92% (46/50)

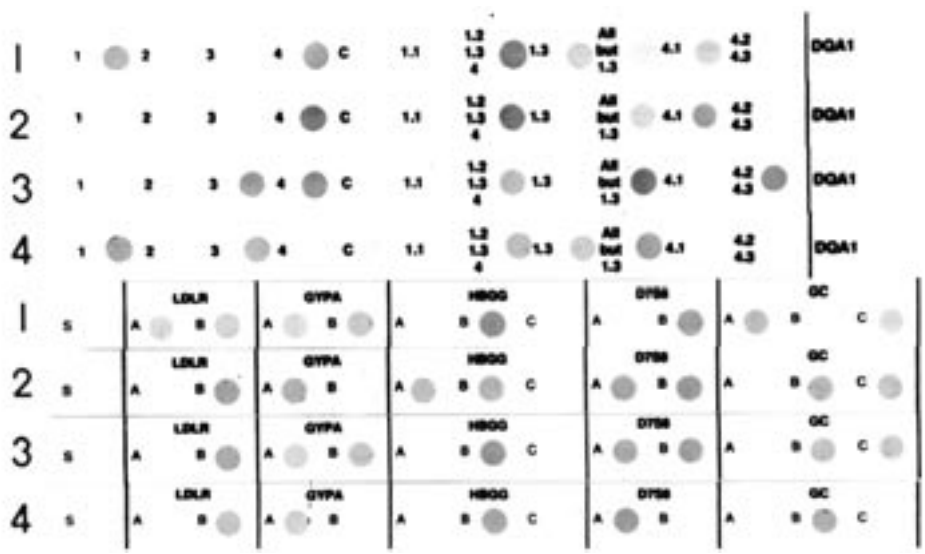

FIG. 2—*Genotyping results of DNA extracted from four mock BQ samples (eight-week-old) using the PVP/CTAB method. PCR products from the DQA1 and PM loci were analyzed by the reverse-dot-blot technique using the "AmpliType^R DQA1&PM" PCR-amplification and typing kit (Perkin Elmer/Roche Molecular System, Branchburg, New Jersey). The corresponding genotypes at the DQA1, GC, D7S8, HBGG, GYPA, and LDLR loci are: 1.3/4.1, A/C, B/B, B/B, A/B, and A/B for Subject 1; 4.1/4.1, B/C, A/B, A/B, A/A, and B/B for Subject 2; 3/4.2(4.3), B/C, A/B, B/B, A/B, and B/B for Subject 3; 1.3/3, B/B, A/A, B/B, A/A, and B/B for Subject 4.*

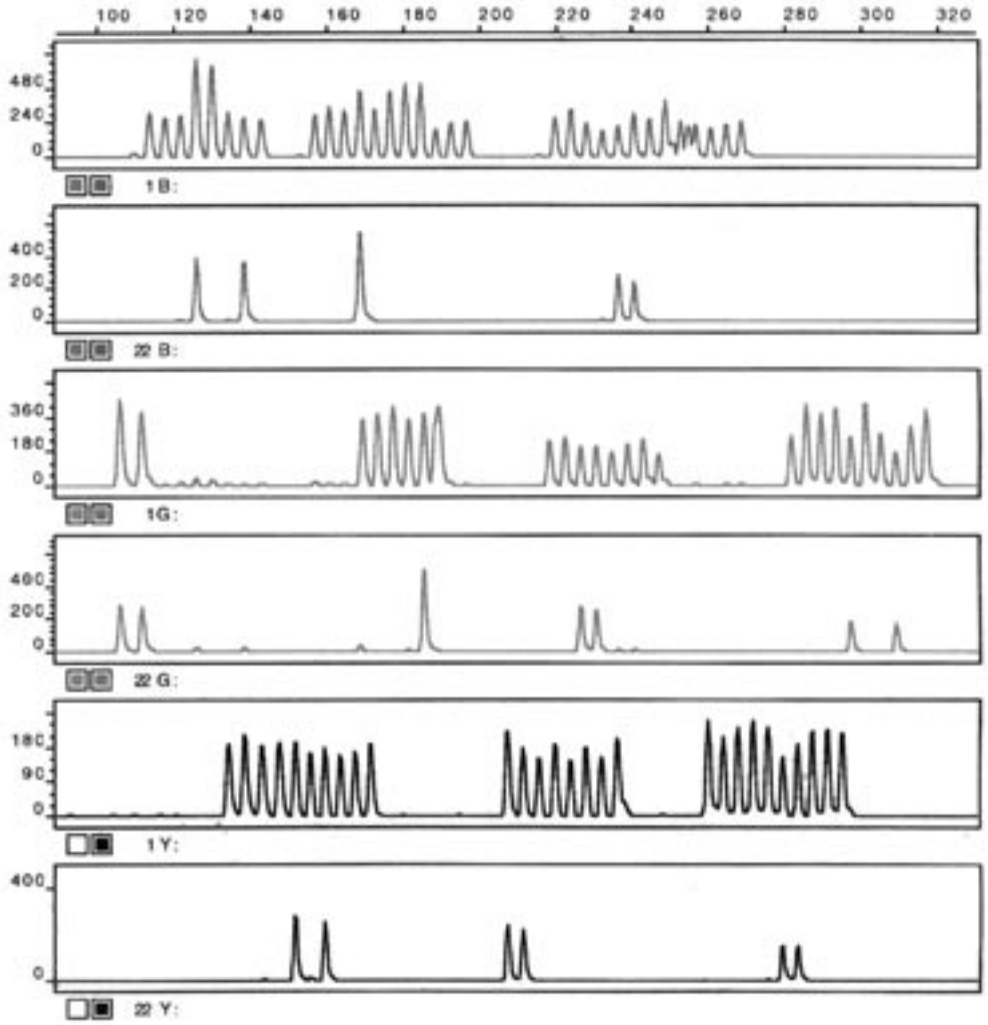

FIG. 3—*An electropherogram of AmpFℓSTR Profiler™ (Perkin Elmer-Applied Biosystems, Foster City, CA) for an eight-week-old BQ sample processed by PVP/CTAB method. This sample had failed by using the traditional extraction methods. Duplicate analysis of old or forensic samples was always carried out to check the reproducibility of artifact bands. The parameters given in the user's manual of AmpFℓSTR Profiler kit, especially the amount of DNA template and PCR cycles, were necessarily applied to amplify the STR loci in forensic sample DNA. The guideline rules proposed by P. Gill et al. (13) should be applied when processing forensic samples.*

proteins, and form cross-links with nucleic acids (14). PVP binds to polyphenols, and it has been used to enhance the yield of PCR from plant DNA (14). The polyphenol-PVP complex in the water-soluble layer can be removed after alcohol precipitation of DNA. The CTAB procedure was originally developed for the precipitation of polysaccarides and the extraction of DNA from fungi and plants (15). In our PVP/CTAB method, the addition of proteinase *K* before the CTAB extraction was to help lyse the buccal cells and help release the DNA from the histone proteins, and the incubation in CTAB/NaCl at 65°C for four times was to help precipitate the polysaccharides thoroughly. However, the exact reaction mechanisms between these reagents and cellular components such as histone proteins and the putative PCR inhibitors remain to be elucidated. We conclude that an effective method for the extraction and PCR analysis of DNA from chewed residues of BQ has been established, and its application to forensic BQ evidences should facilitate the investigation of criminal cases in countries where betel-chewing is prevalent.

Acknowledgments

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Additional information and reprint requests:
Chiou-Herr Yang, Ph.D.
Professor
Department of Forensic Science
Central Police University
56 Shu-Jen Road
Taoyuan Kueishan
Taiwan ROC 33334
Fax: 011-886-33275907
E-mail: una033@sun4.cpu.edu.tw