J Forensic Sci, July 2003, Vol. 48, No. 4
Paper ID JFS2002347_484
Available online at: www.astm.org

TECHNICAL NOTE

Jazelyn M. Salvador,[1] *B.Sc. and Maria Corazon A. De Ungria,*[1] *Ph.D.*

Isolation of DNA from Saliva of Betel Quid Chewers Using Treated Cards*

ABSTRACT: Betel quid (BQ) chewing, a common tradition in tropical areas, often poses a problem during collection and DNA analysis of buccal samples from many indigenous communities for population genetic studies and in forensic analysis of chewed BQ residues. This study evaluated the use of FTA™ card, a chemically treated filter paper, in collecting buccal samples from long-term BQ users and subsequent PCR-based analysis using nine STR markers. A low overall success rate of amplification was observed in the samples extracted using a standard organic extraction procedure (7%) as compared with those prepared using the FTA™ card (89%). The presence of inhibitors in liquid DNA samples was verified when control DNA failed to amplify in the presence of an equal volume of liquid BQ samples. The use of the FTA™ card is more practical during field sampling than handling tubes containing buccal swabs.

KEYWORDS: forensic science, DNA isolation, betel quid, short tandem repeats, polymerase chain reaction, FTA™ card

Betel quid (BQ) chewing is a common tradition in tropical and subtropical areas, including India, Southeast Asia, East Africa, and New Guinea (1) and is reported to effect heightened alertness, sweating, and salivation (2). The practice of betel quid chewing poses a problem during collection and DNA analysis of buccal samples from many indigenous communities for population genetic studies and in forensic investigations (3). Areca nut (*Areca catechu*), slaked lime (often prepared from ground coral or seashells), and betel leaf (*Piper betle*) comprise the BQ mixture that produces bright red-colored saliva upon chewing (4). Complex reactions occur during chewing of BQ resulting in the formation of compounds (i.e., N-nitrosamines, reactive oxygen radicals) that cause DNA single strand breaks (5), oxidative DNA damage and DNA methylation (6). BQ extracts also contain several alkaloids (i.e., arecoline, arecaidine, guvacine, and guvacoline), and polyphenolic compounds, mainly hydroxychavicol and safrole (2), that are capable of inactivating enzymes, precipitating proteins, and cross-linking nucleic acids (7).

An alternative option for handling buccal swabs is to spread the sample on treated cards immediately after swabbing. Treated cards are practical for field purposes due to their compactness, light weight, and ease in handling. The present study evaluated the use of FTA™ cards (Whatman® BioSciences, MA) in collecting samples from long-term BQ users of an indigenous Filipino community during fieldwork and subsequent PCR-based analysis of buccal DNA using Short Tandem Repeat (STR) markers. STRs are tandemly repeated sequences, between 2 to 6 base pairs (bp) in length, that may be used to assess human genetic variations (8). The nine STR markers used were HUMF13A01, HUMFES/FPS, HUMvWA, D8S306, HUMDHFRP2, HUMTH01, HUMCSF1PO, HUMTPOX, and HUMFGA.

Materials and Methods

To examine the utility of treated cards, buccal cells were obtained from 11 long-term BQ chewers (BC) and four non-BQ chewers (NC) during fieldwork. Volunteers were asked to rinse their mouth with water to remove residual food debris and particles prior to sampling. Four oral swabs were obtained from each volunteer: two swabs were immediately spread on FTA™ Classic card which were air-dried for at least 2 h and stored in separate envelopes; the other two oral swabs were placed in 1.5 mL microcentrifuge tubes. The treated cards and oral swabs were stored at room temperature during the three-day sampling period. In the laboratory, 2 mm disks were prepared from the dried cards using a Harris MicroPunch™ apparatus (Life Technologies, Inc., Gaithesburg, MD) and processed following manufacturer's instructions (Whatman® BioSciences, MA) whereas DNA in the stored oral swabs was extracted using a standard organic extraction procedure (9).

Samples were amplified in a 25-μL reaction volume consisting of 1X PCR buffer (50 mM KCl, 20mM Tris HCl, 1.5 mM $MgCl_2$), 0.36 μM of each primer—unlabeled primer (Gibco-BRL, Life Technologies, Inc., Gaithesburg, MD) and Cy5-labeled primer (Genset Oligos, Singapore), 200 μM of each dNTP, 60 ng/μL bovine serum albumin (BSA), and 0.02 U/μL Taq polymerase (Gibco-BRL Life Technologies, Inc., Gaithesburg, MD) at HUMF13A01, HUMFES/FPS, HUMvWA, D8S306, HUMDHFRP2 and HUMTHO1 loci. The same PCR mixture was used for the amplification of HUMCSF1PO, HUMTPOX, and HUMFGA loci except for the primer concentration, which was 0.25 μM each.

[1] DNA Analysis Laboratory, Natural Sciences Research Institute, Miranda Hall, University of the Philippines, Diliman, Quezon City 1101, Philippines.

* This study was supported by the Natural Sciences Research Institute (NSRI) and the European Commission.

Received 1 Oct. 2002; and in revised form 25 Jan. 2003; accepted 25 Jan. 2003; published 1 May 2003.

PCR was carried out in a Biometra UNO (Biometra, Germany) thermal cycler using thermocycling conditions reported previously (10,11). PCR products were detected and analyzed using an automated fluorescence based system, ALF Express™, ALFwin™ and Allelelinks™ version 1.01 software (Amersham-Pharmacia Biotech, Sweden), according to manufacturer's instructions. Sizes of PCR products were scored using in-house allelic ladders described previously (10,11). Alleles were assigned when peak areas are greater than 70% of the highest allele (8) and measure at least 50 relative fluorescent units (r.f.u.) (12).

To test for the presence of PCR inhibitors in DNA prepared from liquid samples of BQ chewers, 2.5 μL of the DNA sample was mixed with an equal volume of control DNA obtained from a non-BQ user, and subsequently amplified and analyzed at two STR loci (HUMvWA and HUMFES/FPS).

Results and Discussion

The success rates of PCR amplification of template DNA extracted from BQ chewers (BC) and non-BQ chewers (NC) using FTA™ card and standard organic extraction procedures are summarized in Table 1. Liquid and FTA™ card samples of all four control non-BQ users were successfully amplified in 9 STR markers (100%). On the other hand, a low overall success rate of amplification in 9 STR markers (7%) was observed in liquid samples of BQ chewers as compared with those prepared using FTA™ card (89%). Peak signals of the genotypes of BQ chewers generated from DNA prepared with FTA™ card were distinct and comparable to those of control non-BQ users (Fig. 1). In addition, absence of PCR product (BC06) and dropout of an allele (BC09-HUMTPOX) were observed in BC samples extracted using standard organic procedure.

Alkaloids and polyphenolic compounds present in BQ components and by-products of BQ mixture reactions, such as N-nitrosamines, are capable of damaging DNA (5,6) as well as inhibiting protein activity (7). Thus, data obtained suggest possible degradation of the template DNA upon exposure to BQ extracts after cell lysis and/or presence of putative PCR inhibitors in liquid DNA samples of BQ users that result in decreased or no amplification (Fig. 1).

The success rates in amplifying HUMTH01 (18%) and HUMF13A01 (82%) support possible degradation of DNA in samples from BQ chewers even when bound on treated cards. HUMTH01 is particularly sensitive to DNA degradation resulting in the non-amplification of an allele (allelic drop-out) and false homozygotes are typed instead of heterozygotes when handling environmentally challenged samples (13,14). In the HUMTH01 marker, besides the two samples that showed distinct peaks, small peaks were detected in three additional samples. These peaks were below the minimum requirement of 50 r.f.u. even after repeated amplifications and hence were scored as negative. Likewise, another sample possessed a peak area less than 50 r.f.u at the HUMF13A01 marker and was not considered as positive. HUMF13A01 consists of larger alleles (179 to 235 bp) compared with other markers, e.g., HUMTPOX (102 to 138 bp) and HUMvWA (122 to 182 bp) (11,15). The effect of DNA degradation is more pronounced on larger markers such as HUMF13A01. Notably, the largest marker HUMCSF1PO (291 to 331 bp) was successfully typed thus, other factors besides size may have contributed to the variable success in typing HUMF13A01.

Moreover, amplification of control DNA (30 ng in 25 μL PCR reaction volume) obtained from a non-BQ user at two STR loci (HUMvWA and HUMFES/FPS) was unsuccessful (data not shown) when an equal volume of liquid DNA from BQ chewers was added in the PCR mixture. This result confirms the presence of PCR inhibitors in the liquid DNA which were not completely removed in DNA preparations of buccal samples from BQ chewers using standard organic extraction procedure. Notably, amplification of buccal DNA from residues of BQ isolated using salt/chloroform, 5% chelex-100 resin, and QIAamp methods also had low success rates (3,16).

The problem of the presence of PCR inhibitors during DNA extraction from BQ chewers was overcome by using FTA™ card that resulted in successful amplifications (89%). The efficiency and sensitivity of the use of FTA™ card for DNA isolation lies on the dry solid matrix, impregnated with chelators, denaturants, and free-radical traps, which binds and protects the DNA against substantial chemical and/or biological degradation (17). Since released DNA is sequestered and preserved intact in the FTA™ card matrix (17), inhibitors derived from BQ mixtures were removed during the series of brief washes of the treated cards. DNA integrity was preserved and further degradation of the DNA, normally associated with conventional methods, was prevented by using FTA™ cards.

The use of FTA™ cards provided a means of preparing DNA templates without the need for extensive isolation and purification steps, which are usually necessary when dealing with environmentally challenged samples. We have shown in this study that a sufficient amount of template DNA, free of PCR inhibitors, was obtained from long-term betel quid chewers with FTA™ card for PCR-based STR analysis. Moreover, the use of FTA™ card was found to be simple, rapid, and efficient method for sample collection and storage during fieldwork.

TABLE 1—*Rate of successful PCR amplification of DNA from saliva of betel quid chewers (BC) and non-chewers (NC) at nine short tandem repeat (STR) loci.*

SAMPLES	Extraction Procedure	Rate of Successful Amplifications at Nine Short Tandem Repeat (STR) Markers (%)								
		vWA	TH01	FES/FPS	TPOX	FGA	CSF1PO	D8S306	DHFRP2	F13A01
Betel quid chewers	Organic	0	0	0	27	0	18	0	18	0
(BC) (*n*=11*)	FTA™ card	100	18	100	100	100	100	100	100	82
Non-chewers (NC)	Organic	100	100	100	100	100	100	100	100	100
(*n=4*)	FTA™ card	100	100	100	100	100	100	100	100	100

**n*, number of samples.

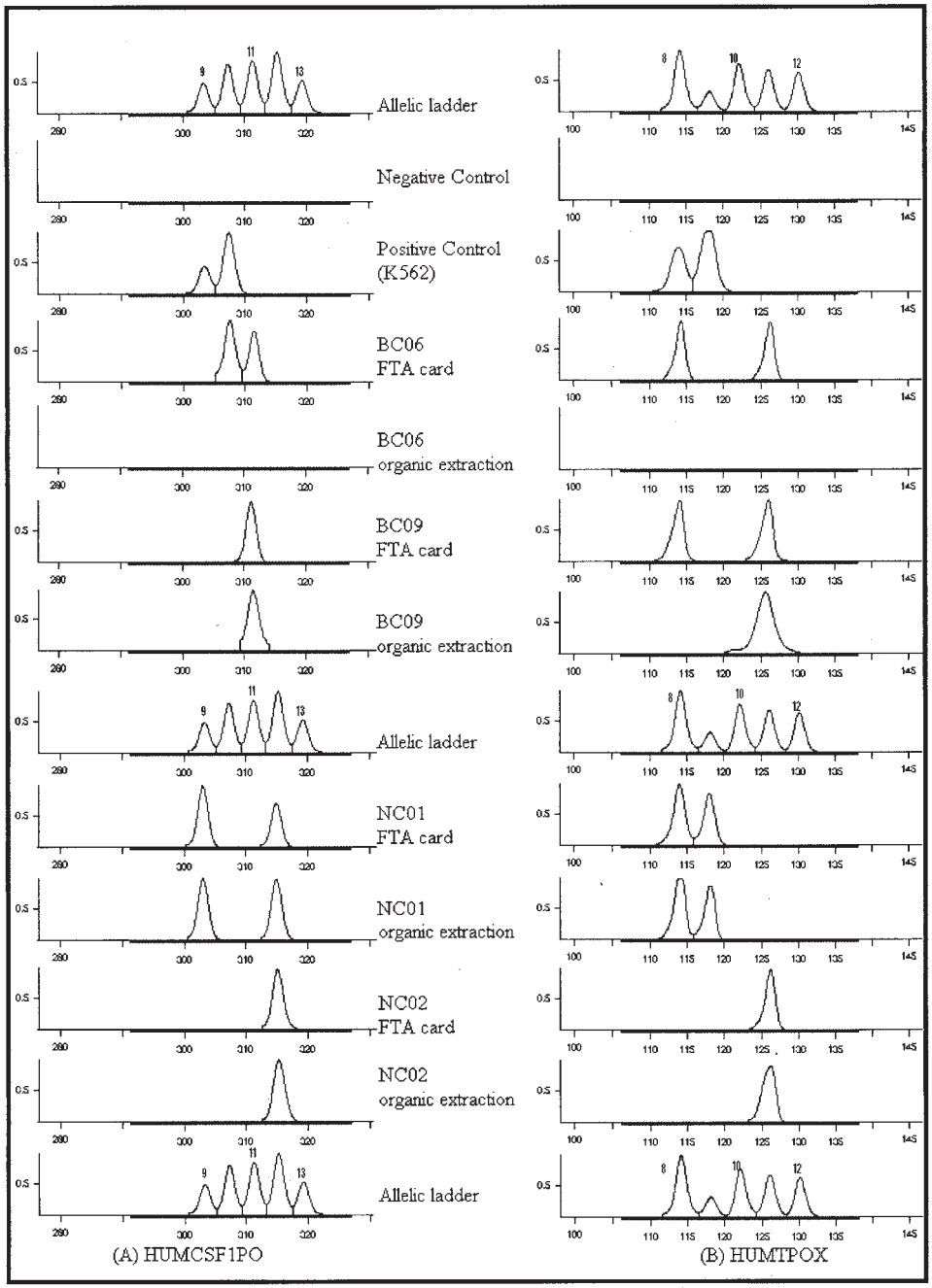

FIG. 1—*Electropherogram of DNA extracted from two betel quid chewers (BC) and non-chewers (NC) amplified at HUMCSF1PO (A) and HUMTPOX (B) loci. Absence of detectable PCR products (BC06) and dropout of an allele (BC09-TPOX) were evident in BC samples extracted using the standard organic procedure. The x-axis is scaled into number of bases and the y-axis represent the peak area of the curve relative to the largest peak area within the lane.*

Acknowledgments

The authors would like to acknowledge the excellent technical assistance of Ms. Michelle Music M. Tan, Ms. Gayvelline C. Calacal and Mr. Frederick C. Delfin during DNA fragment analysis, Mr. Henry B. Perdigon during sample collection, Ms. Miriam Ruth M. Dalet for editing the manuscript and to Dr. Saturnina C. Halos for her interesting insights. We also wish to thank the New Tribes Mission—Philippines for their valuable assistance during collection of samples from a tribal community.

References

1. Deng JF, Ger J, Tsa WJ, Kao WF, Yang CC. Acute toxicities of betel nut: rare but probably overlooked events. Clinical Toxicology 2001; 39(4):355–60.
2. Chu NS. Effects of betel chewing in the central and autonomic nervous systems. J Biomed Sci 2001;8:229–36.
3. Chiou FS, Pai CY, Hsu YPP, Tsai CW, Yang CH. Extraction of human DNA for PCR from chewed residues of betel quid using a novel "PVP/CTAB" method. J Forensic Sci 2001;46(5):166–71.
4. Marshall M. Betel chewing incompletely understood in journal supplement. Am J Public Health 1994;84:1523.

5. Sundqvist K, Liu Y, Nair J, Bartsch H, Arvidson K, Grafstrom RC. Cytotoxic and genotoxic effects of areca-nut related compounds in cultured human buccal epithelial cells. Cancer Res 1989;49: 5294–8.
6. Friesen M, Maru G, Bussachini V, Bartsch H, Nair U, Floyd RA. Formation of reactive oxygen species and of 8-hydroxy-2-deoxyguanosine in DNA *in vitro* with betel-quid ingredients. In: International agency for research on cancers, editors. IARC Monographs on the evaluation of carcinogenic risks to humans. France: International Agency for Research on Cancer, 1988;417–21.
7. Khanuja SPS, Shasany AK, Darokar MP, Kumar S. Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Mol Biol Reporter 1999;17:1–7.
8. Butler JM. Forensic DNA typing: biology and technology behind STR markers. San Diego, CA USA: Academic Press, 2001
9. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York, USA: Cold Spring Harbor Laboratory Press, 1989.
10. Halos SC, Chu JY, Ferreon ACM, Magno MMF. Philippine population database at nine microsatellite loci for forensic and paternity applications. Forensic Sci Int 1999;101:27–32.
11. Urquhart A, Oldroyd NJ, Kimpton CP, Gill P. Highly discriminating heptaplex short tandem repeat PCR system for forensic identification. Biotechniques 1995;18(1):116–20.
12. Tomsey CS, Kutz M, Flowers B, Furnea J, Giles B, Kucherer S. Case work guidelines and interpretation of short tandem repeat complex mixture analysis. Croat Med J 2001;42:276–80.
13. Schmerer WM, Hummel S, Herrmann B. Optimized DNA extraction to improve reproducibility of short tandem repeat genotyping with highly degraded DNA as target. Electrophoresis 1999;20: 1712–6.
14. Lygo JE, Johnson PE, Holdaway DJ, Woodroffe S, Whitaker JP, Clayton TM, et al. The validation of short tandem repeat (STR) loci for use in forensic casework. Int J Leg Med 1994;107:77–89.
15. *http://www.cstl.nist.gov/biotech/strbase/str_fact.htm*
16. Pai CY, Hsieh LL, Tsai CW, Chiou FS, Yang CH, Hsu BD. Allelic alterations at the STR markers in the buccal tissue cells of oral cancer patients and the oral epithelial cells of healthy betel-quid-chewers: an evaluation of forensic applicability. Forensic Sci Int 2002;129:158–67.
17. Burgoyne L, inventor. Flinders Technologies Pty, Ltd., assignee. Dry solid medium for storage and analysis of genetic material. US patent 5,972,386. 1999 October 26.

Additional information and reprint requests:
Dr. Maria Corazon A. De Ungria
DNA Analysis Laboratory, Natural Sciences Research Institute
Miranda Hall, University of the Philippines, Diliman
Quezon City 1101, Philippines
E-mail address: mcadu@uplink.com.ph