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Validation of Two Extraction Methods for Human DNA from Cigarette Butts

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ABSTRACT

Cigarette butts found in crime scenes may be used to identify persons and link them to a crime through DNA profiling of epithelial cells from saliva stains on these materials. Downstream analysis of cigarette butts poses some challenges because these are often exposed to chemical contaminants and environmental conditions which lead to DNA degradation. In this study, several factors were tested to compare the amount and quality of DNA obtained from cigarette butts extracted using an organic procedure and the QIAamp® DNA Micro Kit (QIAGEN). Results show that exposure to an outside environment had a significant effect on DNA yield and amplifiability for both extraction procedures. Prolonged storage of cigarette butts of up to six months affected the amount of DNA that can be extracted using the QIAamp® DNA Micro Kit. However, complete DNA profiles can be generated from cigarette butts stored for six months provided that these samples are stored indoors under controlled temperature conditions and with minimal exposure to contaminants.

Keywords: Cigarette butts, DNA typing, DNA yield, forensic science

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INTRODUCTION

DNA analysis of evidentiary materials is a powerful tool for linking an individual to a victim or a crime scene (Hochmeister et al. 1991; Walsh et al. 1992). Proper crime scene collection and handling are essential to preserve the integrity of the evidence.

Saliva may be found on various surfaces found at the crime scene, such as cigarette butts (Hochmeister et al. 1991; Watanabe et al. 2003), leftover food (Sweet and Hilderbrandt 1999; Abaz et al. 2002), and bite marks on human skin (Pretty and Sweet 2001; Chávez-Briones et al. 2015). DNA from epithelial cells present in saliva may be used for DNA analysis. However, saliva stains on most surfaces, once dry, are invisible to the naked eye. In addition, DNA preparations can potentially contain co-extracted inhibitors that could affect downstream DNA analysis. In a report by Watanabe et al. (2003), certain kinds of dyes in cigarette butts inhibit PCR amplification. Thus, a key determining step in the success or failure of obtaining a DNA profile relies on the extraction method used to recover amplifiable DNA. With the availability of multiplex Short Tandem Repeat (STR) DNA marker systems targeting autosomal chromosomes (aSTRs) or gonosomal chromosomes (XSTRs and YSTRs), generating DNA profiles with a high capacity to differentiate human sources was made possible (Hochmeister et al. 1991; Balogh et al. 2003).

In a 2009 survey, about 28.3% of Filipinos aged 15 years and older, or about 17.3 million individuals smoked cigarettes in the Philippines (WHO 2009). Hence, cigarette butts are likely to be present in crime scenes that may contain DNA from (1) the perpetrator who smoked while waiting for the opportune moment to commit the crime; (2) the victim/s, including those whose location remains unknown; and (3) the witness who could provide vital information for the investigation.

Many crimes in the Philippines are located outdoors where the average temperature is 28.3°C, and the humidity ranges from 71 - 85% (PAGASA 2016). When DNA contained in crime scene samples are not recovered soon after a crime, these outdoor conditions promote microbial growth which leads to the degradation of DNA.

In the present study, we compared the utility of an organic procedure and a silica-based DNA extraction method for handling cigarette butt samples. We also tested the effect of storage time and conditions, and cigarette type, on the generation of human DNA profiles from these samples.

MATERIALS AND METHODS

Institutional Review

Clearance to conduct the study was provided by the Natural Sciences Research Institute, University of the Philippines Diliman.

Sample Collection

Samples were collected from two volunteers who are regular smokers. Both volunteers were asked to smoke three types of cigarettes, namely (1) regular-type; (2) light or less nicotine; and (3) light with menthol, following their normal routines.

For the 24-hour stored samples, three regular-type cigarette butts from each volunteer were collected and stored for 24 hours indoors at room temperature (25°C - 27°C). Volunteers were asked to smoke three cigarettes in one day and each cigarette was smoked at a different time of the day. Each cigarette butt was split into two slices with the cut parallel to the seam of the cigarette. Each half was placed in separate sterile tubes, suspended, and incubated in 12 mL phosphate buffered saline (PBS) solution for three hours with shaking. For every cigarette butt, one half was subjected to organic DNA extraction, while the other half was subjected to DNA extraction using the QIAamp® DNA Micro Kit.

For the six-month storage samples, four cigarettes for each cigarette type (regular, lights, and lights-menthol) were collected from each volunteer. For each cigarette type, volunteers were asked to smoke four cigarettes per day with two cigarettes smoked in the morning and two cigarettes smoked in the afternoon. Volunteers were also asked to leave some time before smoking the second cigarette. Samples were stored at room temperature prior to extraction. Each cigarette butt was split into two slices with the cut parallel to the seam of the cigarette. One half was subjected to organic DNA extraction, while the other half was subjected to DNA extraction using the QIAamp® DNA Micro Kit.

For the indoor storage set-up, four cigarettes for each cigarette type (regular, lights, and lights-menthol) were collected from each volunteer and stored for six months indoors. As for the outdoor storage set-up, four cigarettes for each cigarette type (regular, lights, and lights-menthol) were collected from each volunteer and left outdoors for six months. Volunteers smoked each cigarette in intervals.

Cigarettes left outdoors were deposited directly on soil and were exposed to external elements, such as direct sunlight and rain. Cigarettes were also exposed to any animal, insect, and microbial activity that may occur in the area the cigarettes were deposited on.

DNA Extraction

QIAamp® DNA Micro Kit extraction was carried out using the manufacturer's protocol with some modifications. Modifications include the use of 640 µL of lysis solution to submerge the cigarette butt samples and incubating these in lysis solution for two hours.

Organic extraction was performed using the protocol described by Budowle et al. (2000) with some modifications. The modified protocol made use of a 1000-µL cell lysis solution containing 835 µL of TEN buffer (10 mM Tris, 10 mM EDTA, and 100 mM NaCl), 100 µL of 20% SDS (Invitrogen), 40 µL of 1 M DTT (Roche), and 25 µL of 20 mg/mL Proteinase K (Novagen). Samples were incubated with shaking at 56°C for ~24 hours in an Eppendorf Thermomixer® (Eppendorf). An equal volume of 25:24:1 mix of phenol (Invitrogen), chloroform (Merck Millipore), isoamyl alcohol (J.T.Baker), and a MaXtract® Low Density (QIAGEN) phase-lock gel was used to separate organic and aqueous layers. DNA extracts were purified using a Microcon® 100 centrifugal filter (Merck Millipore) and was eluted using 40 µL of TE^{-4} buffer.

DNA Amplification

DNA was quantified using the PowerQuant® System (Promega Corporation) (Ewing et al. 2016), and quantitation results were analyzed using the PowerQuant® Analysis Tool following manufacturer's instructions. A 0.50-ng DNA template was amplified using half-volume reactions (12.5 µL) of the PowerPlex® 16 HS System (Promega Corporation) kit. Polymerase Chain Reaction (PCR) conditions were as follows: initial denaturation at 96°C for 2 minutes, followed by 10 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 70°C for 45 seconds. This was followed by 22 cycles of denaturation at 90°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 70°C for 45 seconds. PCR amplification was completed with a final extension at 60°C for 30 minutes and a final hold at 4°C.

Fragment Detection and Profile Analysis

Detection of the amplified DNA fragments was carried out in the Applied Biosystems® 3500 Genetic Analyzer (Thermo Fisher Scientific). Generated profiles were analyzed using the GeneMapper® ID-X Software version 1.2 (Thermo Fisher Scientific). An analytical threshold of 50 relative fluorescence units (RFU) was used to distinguish true peaks from background noise.

Allele Recovery Computation

Blood samples from volunteers were collected and were used to generate their reference DNA profiles for comparison with the DNA profiles generated from the cigarette butts. The number of alleles in a volunteer's reference profile was considered as the volunteer's "expected" number of alleles. The number of alleles in the DNA profile from the cigarette butt replicate was considered as the "observed" number of alleles for that sample replicate. Allele drop-ins detected in cigarette butt samples were not considered part of the "observed" alleles.

To determine the percentage allele recovery for each sample replicate, the observed allele number was divided by the expected number of alleles, then multiplied by 100.

Statistical Analysis

Data in the comparison of DNA yield from the two DNA extraction methods, storage environment, and storage time were analyzed using the unpaired Student's *t*-test. Data from the comparison of DNA yield from different cigarette types were analyzed using one-way Analysis of Variance (ANOVA). All statistical analyses were performed using the GraphPad Prism 7 software (GraphPad Software, Inc.).

RESULTS AND DISCUSSION

Comparison of DNA yield and allele recovery between the standard organic procedure and the QIAamp® DNA Micro Kit method for DNA extraction

For regular-type cigarettes extracted after a 24-hour storage period, there was no significant difference (p=0.078) between the DNA yields of the two extraction methods ($mean_{org}$=719.97±303.16; $mean_{QIA}$=1188.33±500.35) (Table 1). Complete DNA profiles were generated from all DNA extracts of 24-hour stored samples.

Table 1. DNA recovery from regular-type cigarette butt samples extracted using organic QIAamp® DNA Micro Kit extraction methods after a 24-hour indoor storage period.

DNA Extraction Method	DNA Yield (ng DNA/g cigarette butt) Mean ± SD
Organic Extraction	719.97 ± 303.16
QIAamp® DNA Micro Kit	1188.33 ± 500.35

In samples stored indoors for six months prior to processing, difference in DNA yield (p=0.008) was significant ($mean_{org}$=1013.55±565.03; $mean_{QIA}$=139.45±229.31) (Figure 1a and Table 2). Allele recovery across extraction methods (p=0.073) was not significant.

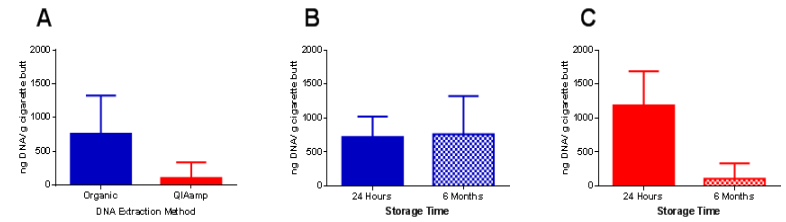

Figure 1. Comparisons of DNA yield across different factors. (a) Comparison of DNA yields between the organic and the QIAamp® DNA Micro Kit extraction methods from regular-type cigarette butt samples stored indoors for six months prior to extraction. Difference in DNA yields between the two extraction methods is significant (p=0.008). (b) Comparison of DNA yields from cigarette butt samples extracted using the organic method with respect to indoor storage times. Difference in DNA yield (p=0.877) between the two storage durations is not significant. (c) Comparison of DNA yields from cigarette butt samples extracted using the QIAamp® DNA Micro Kit with respect to indoor storage times. Difference in DNA yield between the two storage durations is significant (p=0.0001).

Table 2. DNA recovery from regular-type cigarette butt samples extracted using organic and QIAamp DNA Micro Kit extraction methods after a 6-month indoor storage period

DNA Extraction Method	DNA Yield (ng DNA/g cigarette butt) Mean ± SD
Organic Extraction	1013.55 ± 565.03
QIAamp® DNA Micro Kit	139.45 ± 229.31

After six months of storage prior to processing, DNA extracts from the QIAamp® DNA Micro Kit have lower DNA yields compared to those obtained via the organic extraction procedure. This observation illustrates the limitation of a silica membrane-based DNA extraction method, such as the QIAamp® DNA Micro Kit. When the lysis solution is transferred to the QIAamp® MinElute Column, DNA binds to the silica particles in the membrane while other molecules remain in the lysis solution. Since the amount of silica particles of the QIAamp® MinElute Column is limited, the amount of DNA that may be recovered from the lysis solution is also limited.

Competitive binding among human DNA, non-human DNA, and other substances occurring in the silica membrane contributes to the decrease in human DNA yield using silica membrane-based DNA extraction. Competitive binding occurs when negatively-charged non-human DNA or other molecules bind to the positively-charged silica surface instead of the target human DNA. Furthermore, human DNA bound to the silica surface could be displaced by molecules with a stronger affinity to the silica surface. Mundorff and Davoren (2014) noted that PCR inhibitors and non-human DNA may also co-isolate with human DNA during extraction.

Allele recovery data show that the amount of amplifiable DNA recovered during silica column extraction generated partial (>80% alleles recovered) to full profiles.

Effect of storage environment on DNA yield and allele recovery

DNA yield in cigarette butt extracts exposed outdoors for six months was lower compared to samples stored indoors at room temperature. Samples extracted using the organic extraction method exhibited a significant difference in DNA yield with respect to storage environments (p=0.0001). Similarly, the difference in DNA yield between samples stored in different environments and extracted using the QIAamp® DNA Micro Kit was significant (p=0.001). Poor allele recovery was observed in samples exposed outdoors (0 – 17%), while better allele recovery was remarked in

samples stored indoors (27 – 100%). Poor allele recovery of samples left outdoors was supported by DNA quantitation results, wherein the PowerQuant® Analysis Tool flagged 33% of samples as degraded, while 54% had a zero quantitation value.

This result is consistent with the study of Casey et al. (2013) who reported a lower count of nucleated cells from cigarette butts left outdoors compared to those stored indoors.

Effect of storage time on DNA yield and allele recovery

DNA extracts obtained using the QIAamp® DNA Micro Kit exhibited a significant difference in DNA yield with respect to storage times (p=0.0001). For these samples, higher DNA yield was observed after storage for 24-hours compared to samples stored for six months.

No significant difference was observed in the DNA yields of samples extracted using the organic method and stored for 24 hours versus those stored for six months (p=0.877) (Figure 1b). By contrast, the DNA yields of 24-hour and 6-month samples extracted using the QIAamp® DNA Micro Kit were significantly different (p=0.0001) (Figure 1c).

DNA left on cigarette butts is highly prone to oxidation from reactive oxygen species (ROS), such as nicotine present in cigarette smoke (Ginzkey et al. 2012). Exposure of dehydrated (Matsuo et al. 1995) and lyophilized (Molina and Anchordoquy 2008; Bonnet et al. 2010) DNA to atmospheric oxygen at room temperature can also lead to DNA oxidation. In the oxidized state of DNA, the DNA molecule becomes less negatively charged, which in turn makes the binding of DNA to silica weaker. Thus, the prolonged exposure to oxidative stresses during long-term storage can decrease the adsorption efficiency of silica columns in DNA extraction. It is therefore recommended that evidentiary samples that may have been exposed to harsh environmental conditions, such as cigarette butts left in a crime scene, must be processed immediately to decrease DNA loss during storage.

No significant difference in allele recovery with respect to storage durations was observed in both organic (p=0.408) and QIAamp® DNA Micro Kit (p=0.051) extraction methods.

Effect of cigarette type on DNA yield and allele recovery

Smoking habits differ from person to person. Smokers would have preferences on the type of cigarette based on the amount of nicotine and presence of other ingredients (e.g., menthol).

Three cigarette types, namely regular, less nicotine (lights), and less nicotine with menthol (lights-menthol), were tested to determine the effect of cigarette type on the amount and quality of DNA recovered. For both extraction procedures, the results showed that cigarette type had no significant effect on DNA yield (p_{org} = 0.465; p_{QIA} = 0.748) nor on allele recovery (p_{org} = 0.613; p_{QIA} = 0.297) (Tables 3 and 4).

Table 3. DNA yield and allele recovery from cigarette butt samples of three types of cigarettes extracted using the organic extraction method after a six-month indoor storage period.

Cigarette Type	DNA Yield (ng DNA/g cigarette butt) Mean ± SD	% Allele Recovery Mean ± SD
Regular	760.17 ± 528.53	99.58 ± 1.18
Light	1653.89 ± 2003.18	99.58 ± 1.18
Light with Menthol	1272.32 ± 1013.93	100.00 ± 0

Table 4. DNA yield and alele recovery from cigarette butt samples of three types of cigarettes extracted using the QIAamp® DNA Micro Kit after a six-month indoor storage period.

Cigarette Type	DNA Yield (ng DNA/g cigarette butt Mean ± SD	% Allele Recovery Mean ± SD
Regular	104.59 ± 214.50	81.64 ± 26.17
Light	176.64 ± 245.51	88.33 ± 20.39
Light with Menthol	178.21 ± 141.56	97.32 ± 7.58

CONCLUSIONS

This study shows that the amount and quality of human DNA on cigarette butts deteriorate over time, and the extent of DNA loss is more pronounced outdoors under warm and humid conditions. A suitable extraction method for a certain type of cigarette butt sample would differ on a case-to-case basis. For samples collected

indoors and stored for a short period of time, it is more practical to use the QIAamp® DNA Micro Kit because the process is faster, makes use of less hazardous chemicals, and involves less tube transfers, which in turn decreases the risk of contamination during processing. If possible, extraction of DNA from cigarette butts that are submitted as evidence should be processed immediately to maximize DNA recovery. For samples collected outdoors and stored indoors for a long period of time, the use of the organic extraction method is advised, since it can yield more DNA, allowing increased number of analysis despite low copies or low quality of DNA. Storage of cigarette butt samples in laboratories with controlled temperature ranging from 22°C to 25°C and with reduced humidity slows but does not completely stop DNA degradation. This is particularly important in the Philippines where delays in the collection and submission of evidence to DNA laboratories result in the prolonged outdoor exposure of samples, including cigarette butt samples, in locations that are subject to warm and humid conditions.

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