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Comparison of four DNA extraction methods to extract DNA from cigarette butts collected in Lebanon

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ABSTRACT

Cigarette butts collected from crime scenes represent valuable sources of DNA. However the extraction of the genetic material may deem challenging especially when different contaminants may compromise the integrity, quality, and quantity of DNA obtained. This study aims at comparing four extraction methods (Chelex-100, soaking + Chelex-100, Chelex-100 + PK, and DNA IQ™ System) with the intention of identifying the one with maximal recovery rate and profiling success. DNA was extracted using aforementioned four methods from 70 cigarette butts collected from sites across Lebanon. DNA was quantified by qPCR using TaqMan Quantifiler Kit on an Applied Biosystems 7300 SDS instrument and genotypes were obtained using the PowerPlex® 21 kit on an Applied Biosystems 3130 Genetic Analyser. The findings of this work showed that DNA extraction with Chelex-100 + PK is preferred to the other three methods when seeking both, a high yield and the generation of maximal numbers of full profiles. The Chelex-100 + PK method is simple, cost effective, and therefore suitable for routine cigarette butts case studies.

1. Introduction

DNA extracted from cigarette butts collected from crime scenes represents crucial evidence in criminal investigations. In a country like Lebanon where 45.6% [1] of the population (highest in the Middle East) are cigarette smokers, cigarette butts represent an important source of evidence and are repeatedly found and collected. Different standardised extraction methods can be used to extract DNA from cigarettes. However, these methods have shown a variation in the amount of DNA extracted and the quality of DNA profiles generated. Chelex-100 is a commonly used, easy, and affordable method, however PCR inhibitors are commonly carried over in the extracts [2]. To improve its performance, modifications to this method have been attempted like the addition of proteinase K [3], or soaking the sample in pure water [4]. Another commercially available kit, DNA IQ System is validated for processing a range of samples including cigarette butts [5]. The aim of this study was to establish a comparison between four commonly used DNA extraction methods and to identify the most suitable and efficient for the extraction of DNA from crime scene collected cigarettes.

2. Material and methods

2.1. Samples

For the purpose of this study, 70 cigarette butts were collected from different sites across Lebanon over a period of eight months. In addition to that, two cigarette butts were obtained from two of the authors of this manuscript to be used as positive controls. All samples were stored in separate paper bags and were kept at ambient room temperature until the day of analysis. The temperature in the storage room is checked using a digital thermometer and logged daily.

2.2. Sampling

Each of the collected cigarette butts was handled with sterile forceps while four cross-sectional slices were cut using sterile scalpel blades. Each of the four cross-sections had a width of 4 mm. The outer paper cover of each cross-section was removed and placed in a 1.5 ml Eppendorf tube for DNA extraction.

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2.3. DNA extraction

Each of the four cross-sections of each cigarette butt was assigned to be processed with one of the four DNA extraction method stated previously in this paper and described in details here below.

2.3.1. Chelex-100 (5%)

Two hundred microlitres of 5% Chelex (Bio-Rad Laboratories, CA, USA) were added to each tube and vortexed vigorously for 5 s. Following an incubation step of 60 min at 56 °C, all tubes were vortexed for 10 s. This step was followed by a second incubation step of 8 min at 100 °C. All tubes were then vortex-mixed for 10 s and centrifuged at maximum speed (13,000 rpm) for 3 min. Twenty microlitres of the supernatant containing DNA were moved to a second tube and stored at 4 °C until the time of quantification and analysis.

2.3.2. Soaking + Chelex 100 (5%)

The second DNA extraction method involved a slight modification of the Chelex-100 protocol by allowing the cigarette butt cross-sections to soak in 500 µl nuclease-free water at room temperature for a period of 30 min and vortexing every 1–2 min. These tubes were later spun at maximum speed for 3 min. The supernatant was then discarded, and no > 20 µl of residual supernatant were left in the tube. There onwards, the procedure followed the exact protocol of Chelex 100 described above.

2.3.3. Chelex-100 (5%) + proteinase K

The third DNA extraction method consisted of adding 5 µl of proteinase K (20 mg/ml) along with 200 µl of 5% Chelex at the first step of the protocol and then carried on with the Chelex 100 protocol as described above.

2.3.4. DNA IQ system

The fourth DNA extraction method was the DNA IQ System (Promega, USA) which was followed according to the manufacturer's protocol and the final elution volume was 25 µl.

2.4. DNA quantification

All DNA extracts were quantified using the fluorescence based TaqMan Quantifiler kit (Applied Biosystems, Foster City, CA, USA). Synthetic DNA was included in the master mix to act as an internal control which allows the detection of any inhibition. DNA quantification was performed according to the following protocol: a 200 ng/µl DNA stock solution was diluted with Quantifiler Duo DNA dilution buffer to give eight DNA standard solutions, ranging from 50 ng/µl to 23 pg/µl. The standards were mixed thoroughly. A master mix was then prepared by mixing 10.5 µl of Quantifiler Human Primer Mix and 12.5 µl of Quantifiler PCR Reaction. A volume of 2 µl of each sample or 2 µl of standard buffer (for blank) was mixed with 13 µl of the master mix. The qPCR was conducted on an Applied Biosystems 7300 SDS instrument with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of (95 °C for 15 s), then 60 °C for 60 s. After thermal cycling was completed, data analysis was performed by the SDS software to generate a standard curve for the quantification of the unknown samples.

2.5. PCR

After quantification, the 288 (4 × 72) extracted DNA samples of all cigarette butts were amplified and genotyped. The DNA amplification was performed in a 12.5 µl total reaction volume using the PowerPlex® 21 System (Promega, USA). Each reaction contained 2.5 µl PCR mix, 2.5 µl primer pair mix, and 4 µl molecular grade water, in addition to 3.5 µl of template DNA. The reaction was conducted on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) according to the following cycling conditions: initial denaturation at 96 °C for 1 min; 30 cycles of (denaturation at 94 °C for 10 s, annealing at 59 °C for 1 min and extension at 72 °C for 30 s) followed by a final extension step at 60 °C for 10 min. All amplified samples were stored at −20 °C in a light-protected box until the time of genotyping.

2.6. Capillary electrophoresis and genotyping

A master mix containing 1 µl of CC5 Internal Lane Standard 500 and 10 µl of Hi-Di formamide was prepared for each sample. After vortexing, 11 µl of master mix were aliquoted into each well of 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA, USA). For each reaction, 1 µl of Allelic ladder, 1 µl of PCR positive and PCR negative control were loaded separately. Furthermore, 1 µl of PCR product from each sample was loaded into the respective well. The plate was sealed with Plate Septa 96-well (Applied Biosystems, Foster City, CA, USA) before being placed on GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and heated at 95 °C for three min before the temperature was rapidly dropped down to 4 °C and held for 3 min. Then, the plate was placed on plate base and covered with plate retainer. Capillary electrophoresis was performed on an Applied Biosystems 3130 Genetic Analyser under the following parameters: 5 s injection time, 3 kV injection voltage, and 1500 s run time. The resulting data was analysed using GeneMapper ID software v. 3.2 where the detection threshold was placed at 200 RFU. Profiles for which we obtained a genotype for all loci of the kit will be referred to, throughout the manuscript, as full profiles.

2.7. Statistical analysis

The data was analysed using SPSS v22 (IBM SPSS, IBM Corp, Somers, NY, USA). Normally distributed data was analysed using a paired *t*-test whereas a Wilcoxon signed rank test was used for non-parametric data. Furthermore, a Chi Squared test was used to analyse difference between categorical data. The data is presented as means followed by standard deviations and significance was accepted when $p < .05$.

3. Results

The distribution of the quantities of DNA extracted from the 280 (4 × 70) samples using the four extraction methods are presented in Fig. 1. Soaking the sections of cigarette butts in 500 µl before proceeding with Chelex-100 protocol has resulted in the acquisition of a significantly ($p < .001$) lower quantity of DNA than the other three methods (Tables 1 and 2). The results show that a higher quantity of

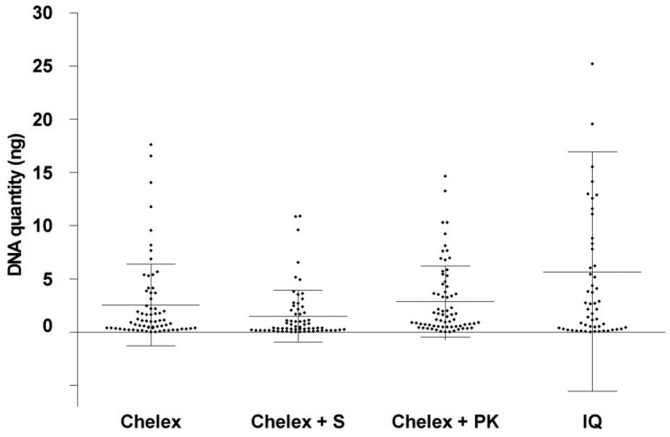

Fig. 1. Column scatter graph showing the mean and standard deviation of the quantities of DNA obtained from cigarette butts using the different extraction methods.

Table 1
Mean quantity of DNA extracted from cigarette butts between four selected methods.

Method	Mean quantity (ng)
Chelex	2.559 ± 3.845 (68)
Chelex + Soaking	1.505 ± 2.430 (65)
Chelex + PK	2.881 ± 3.331 (69)
IQ	5.680 ± 11.249 (53)

Values are expressed as mean ± SD. The number of samples (n) included in the analysis is in parenthesis.

Table 2
Comparison of the four selected DNA extraction methods to each other.

Method 1	Method 2	P value
Chelex	Chelex + Soaking	< **0.001**
Chelex	Chelex + PK	**0.048**
Chelex	IQ	0.070
Chelex + Soaking	Chelex + PK	< **0.001**
Chelex + Soaking	IQ	< **0.001**
Chelex + PK	IQ	0.263

Bold indicates p-values < .05

Table 3
Frequencies of full and partial genetic profiles obtained using the four extraction methods.

Method	Full Profiles	Partial Profiles
Chelex-100	57.4 (39)	42.6 (29)
Chelex-100 + Soaking	44.1 (30)	55.9 (38)
Chelex-100 + PK[a]	74.6 (50)	25.4 (17)
IQ	29.6 (16)	70.4 (38)
P value	< 0.001	

Values are expressed as frequencies with the number of samples (n) in parenthesis.

[a] Chelex + PK vs (Chelex-100, Chelex + Soaking, IQ): p < .001, OR = 3.6, 95% CI 1.95–6.75.

DNA was isolated using the IQ System. Furthermore, Chelex-100 + PK succeeded in extracting a greater amount of DNA than Chelex-100 (p = .048), and Chelex-100 + Soaking (p < .001). We also report no differences in the amounts of DNA extracted from white or red cigarette butts except when extracted with Chelex-100 + PK where significantly (p = .012) more DNA was obtained from white cigarette butts (Table 3).

Furthermore, the results show that there is a significant difference (p < .001) in the ability of the examined four methods in generating full DNA profiles (Table 4). In specific, greater success in obtaining full profiles was reported from samples extracted using the Chelex-100 + PK method when compared to the remaining methods combined (p < .001; OR 3.6; 95% CI 1.95–6.75). In relation to that, we would like to mention that there was a consistency in the behaviour of the internal positive control (IPC) across all four extraction methods. This was determined by examining the Ct values which were clustered around 30 with very small standard deviations: Chelex-100 (Ct: 30.72 ± 0.21), Chelex-100 + Soaking (30.84 ± 0.38), Chelex-100 + PK (30.77 ± 0.15), and IQ (31.20 ± 1.78).

Table 4
Comparison of the quantity of DNA extracted by each method between white and red cigarette butts.

Method	Mean quantity from white cigarettes (ng)	Mean quantity from red cigarettes (ng)	P value
Chelex	2.735 ± 3.925 (46)	2.190 ± 3.734 (22)	0.345
Chelex + Soaking	1.605 ± 2.488 (45)	1.279 ± 2.339 (20)	0.139
Chelex + PK	3.407 ± 3.536 (47)	1.758 ± 2.576 (22)	**0.012**
IQ	4.751 ± 5.790 (36)	7.645 ± 18.217 (17)	0.391

Bold indicates p-values < .05. Values are expressed as mean ± SD. The number of samples (n) included in the analysis is in parenthesis.

When splitting for the colour of the cigarette butt, Chelex-100 + PK method displayed a great potential at generating full DNA profiles when compared to the other methods combined. This was applicable for both white (p = .006; OR 2.67; 95% CI 1.30–5.48) and red cigarettes (p < .001; OR 8.31; 95% CI 2.21–31.17). Moreover, when comparing the effectiveness of each method in generating a full profile between both colours, no differences were observed for Soaking + Chelex-100, Chelex-100 + PK, or IQ System Method. However, the unmodified Chelex-100 method generated significantly more full profiles from white cigarettes than red cigarettes (p = .030; OR 3.15; 95% CI 1.08–9.16).

4. Discussion

This study has established a comparison of different DNA extraction methods for the isolation of DNA from cigarette butts obtained from different sites across the Lebanese territories. The Chelex-100 method when modified by the addition of a proteinase K step [3] proved to be a better method than the remaining three methods since it resulted in the acquisition of the largest quantities of DNA when compared to other Chelex methods, and also resulted in the generation of a greater number of full genetic profiles.

Chelex-100 (Bio-Rad Laboratories, CA, USA) is a chelating resin which uses ion exchange to bind transition metal ions [6]. During extraction, the chelating agents protect the nuclear material from degradation by binding to the cellular components which were released following the boiling step. The Chelex-100 method is a widely chosen extraction method which does not require a phenol-chlorophorm step, nor the use of multiple tubes [7]. However Chelex-100 is weak at removing inhibitors which can interfere with DNA amplification through PCR [2]. Chelex-100 was previously compared to other extraction methods and displayed on all occasions reduced extraction abilities [2,8,9].

The PK solution, a broad spectrum serine protease, is added to DNA extraction protocols to protect the nucleic acid by digesting nucleases (DNases, and RNases) and other contaminating proteins present in the samples. Such role explains the improved results observed in the Chelex-100 + PK extraction method. In fact, Chelex-100 + PK was previously compared to Chelex-100 and other methods and was found to produce higher molecular weight DNA molecules which subsequently generated large PCR amplicons [4].

We report that DNA IQ is a powerful method for the recovery of DNA as it resulted in the extraction of the largest mean amount of DNA from all samples. Our findings agree with the results published in a recent study which also compared DNA IQ and Chelex-100 to other extraction methods [9]. In their study, Ip et al. (2015) found that IQ yielded a higher amount of DNA and full genetic profiles than Chelex-100. However, it is surprising that, in our study, the DNA extracted using DNA IQ resulted in a smaller percentage of full profiles than the other examined methods (Table 4).

We also reported that the Chelex + PK method was significantly better than the other methods in recovering DNA from both white and red cigarette butts. Additionally, more DNA was extracted from white cigarettes than red cigarettes using Chelex-100. This could be due to the light nature of tobacco introduced in white cigarettes which could deposit less inhibitors and DNA degrading agents than the tobacco found in red cigarettes. Furthermore, the presence of colouring materials on red cigarette butts, which would be absent in white cigarette butts, could be also be acting as PCR inhibitors.

It is worth highlighting that all cigarettes used in this study were collected from different sites around Lebanon over a period of

8 months. We do not believe that the storage time would have contributed to difference in DNA yield and quality between the four examined extraction methods since all methods were used on the butts at the same time. Since smokers have got different smoking habits, this could result in different contact area between their lips and the cigarette. Therefore to highlight some limitations of this study, one can argue that differences in yields are due to the distance of the cross sections introduced in the experiments from the optimal contact area between the lips and the cigarette butts. Moreover, it was not possible to control for the surface from which the cigarettes were collected and therefore different environmental inhibitors could have been collected. Furthermore, variations in the amount of DNA extracted and the number of full genetic profiles between cigarettes could be attributed to the time that passed before the butts were removed from their respective sites.

5. Conclusions

In conclusion this study highlights the importance of conducting DNA extraction methods comparison studies on different types of forensic samples. Here we have reported the success of extracting DNA from cigarette butts using Chelex-100 + PK which results in the acquisition of the second largest DNA yield and the greatest numbers of full genetic profiles. When combined with the ease of use, a low time and financial cost, we can conclude that it is a more suitable method for routine case-work involving cigarette butts.

Declaration of interest

Authors declare no conflict of interest.

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