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Short communication

Presence and potential of cell free DNA in different types of forensic samples

Mado Vandewoestyne, David Van Hoofstat, Aimée Franssen, Filip Van Nieuwerburgh, Dieter Deforce *

Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

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ABSTRACT

Extracellular or cell free DNA has been found to exist in many biological media such as blood and saliva. To check whether cell free DNA is present in the supernatant which is normally discarded during several DNA extraction processes, such as Chelex® extraction, DNA profiles of cell pellet and concentrated supernatant from 30 artificial case like samples and from 100 real forensic samples were compared. Presence of cell free DNA was shown in all investigated sample types. Moreover, in some samples additional alleles, not detected during analysis of the cell pellet, were detected, offering valuable information which would normally have been discarded together with the supernatant. The results presented here indicate that cell free DNA deserves further consideration since it has the potential to increase the DNA yield in forensic casework samples in general and in contact traces in particular.

1. Introduction

Extracellular or cell free nucleic acids (CNAs) were first reported in 1948 by Mandel and Metais, who discovered the presence of circulating DNA and RNA in the plasma of healthy and diseased individuals [1]. Since then, CNAs have been found to exist in many biological media, including blood [2,3], saliva [4], semen [5] and urine [6] and have been subject of research in oncology [7,8] and non-invasive prenatal diagnosis [9]. The origin of circulating CNAs remains obscure, although necrosis, apoptosis and active secretion have been suggested as potential mechanisms by which CNAs are released from cells [10,11].

To date, several studies have been performed on the potential of extracellular mRNA profiling in forensic science to identify the biological origin of forensic stains [12–14]. Less is known about the potential value of cell free DNA in forensic casework. Both Kita et al. and Linacre et al. have suggested that sweat contains extracellular DNA that might contribute to the DNA profiles obtained from touched surfaces [15,16]. The latter has recently been proven by Quinones and Daniel who detected cell free DNA in 80% of the healthy individuals who's sweat was analysed [17]. These authors suggest that this cell free DNA is a contributing factor to DNA recovered from touched items and state that it is likely that a substantial proportion of cell free DNA is being discarded with the supernatant during standard extraction processes such as Chelex® extraction. This would imply that potentially valuable information would be discarded as well.

Increasing the DNA yield would be of interest to all types of forensic trace samples. In the current study, the presence of cell free DNA was evaluated in 30 artificial samples and 100 samples from different origin (blood, cigarette buds, clothing, contact traces, nail cleaners, saliva, saliva (potentially with skin contact) and vomit) obtained from 78 forensic cases. To determine whether cell free DNA has an added value, DNA profiles from cell pellet were compared with DNA profiles from cell free concentrated supernatant.

2. Materials and methods

2.1. Sample selection

10 types of artificial case like samples were prepared in triplicate, using biological material (saliva, ejaculate from a fertile and from a vasectomized donor, blood, urine, vomit, faeces, perspiration and buccal cells) from healthy volunteers. An overview of these artificial case like samples is given in Supplementary Table 1. All artificial samples were single donor samples.

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100 samples were selected from 78 different forensic cases. The selection was based on sample type (blood, cigarette buds, clothing, contact traces, nail cleaners, saliva, saliva (potentially

* Corresponding author at: Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium. Tel.: +32 0 92648052; fax: +32 0 92206688.
E-mail addresses: mado.vandewoestyne@ugent.be (M. Vandewoestyne), david.vanhoofstat@ugent.be (D. Van Hoofstat), aimeefranssen@gmail.com (A. Franssen), filip.vannieuwerburgh@ugent.be (F. Van Nieuwerburgh), dieter.deforce@ugent.be (D. Deforce).

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with skin contact) and vomit) and on the type of profile obtained on the cell fraction.

2.2. Confirmatory tests for blood and saliva

Samples categorized as "blood" or "saliva" tested positive for benzidin or amylase test, respectively. For the benzidin test, a piece of sterile filter paper (Sigma–Aldrich, St. Louis, MO, United States of America) was rubbed gently on a small area of the stain. Subsequently, a drop of absolute EtOH (VWR International, Radnor, PA, United States of America), a drop of benzidin reagent (Merck, Darmstadt, Germany) and a drop of 30% H_2O_2 (Sigma–Aldrich) was added to the filter paper. A colour change to green/blue indicated the stain was positive for blood. For the amylase test Phadebas® paper (Phadebas, Lund, Sweden) was used according to manufacturer's instructions. Samples categorized as "saliva (potentially with skin contact)" were not subjected to an amylase test. This category consisted of samples where saliva was potentially present together with skin cells, such as bottle and can openings and samples taken from the presumptive mouth of balaclavas.

2.3. Chelex® DNA extraction and collection of supernatant

Samples were taken using a sterile cotton swab or a sterile scalpel. DNA was extracted as described earlier [18]. Samples were vortexed for 10 s in an Eppendorf tube filled with 1 ml of sterile water and incubated for 30 min at room temperature in a Thermomixer (Eppendorf, Hamburg, Germany). After incubation, sample remainders were removed using sterile tweezers and a centrifugation step (5 min at 14,100 × *g*) was performed. Supernatant was carefully transferred into a fresh Eppendorf tube. About 30 μl of supernatant was left in the Eppendorf tube containing the cell pellet. 200 μl 5% Chelex® (Bio-Rad, Hercules, CA, United States of America) was added to the cell pellet and samples were vortexed for 10 s before incubation at 56 °C for 30 min in a Thermomixer (Eppendorf). After vortexing for 10 s, samples were subsequently incubated in a boiling water bath for 8 min and vortexed for another 10 s. Finally, samples were centrifuged for 3 min at 14,100 × *g*.

2.4. Concentration of supernatant

From the cell free supernatant, recovered during Chelex® extraction, 500 μl was used for DNA concentration using Amicon Ultra 100k (Millipore, Billerica, MA, United States of America) sample reservoirs and centrifuged at 14,100 × *g* for 15 min. The sample reservoir was transferred in a fresh Amicon Ultra reservoir and centrifuged at 1550 g for 2 min. The concentrated supernatant was diluted with sterile water to an end volume of 30 μl.

2.5. DNA amplification and detection

All samples (cell pellet and concentrated supernatant) were amplified using a in house developed multiplex of 15 short tandem repeat (STR) loci (D3S1358, TH01, D21S11, D18S51, Amelogenin, vWA, D8S1179, TPOX, FGA, D5S818, D13S17, SE33, CD-4, D7S820 and D16S539) [19,20].

Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany) or Applied Biosystems (Carlsbad, CA, United States of America). Each reaction mix, with an end volume of 50 μl, contained 16.55 μM primer mix, 1× PCR buffer (Qiagen, Venlo, The Netherlands), 0.5 mM $MgCl_2$ (Qiagen), 200 μM dNTP (Applied Biosystems), 0.4 μg/μl albumin (Sigma–Aldrich), 5 U Hotstar Taq polymerase (Qiagen) and 30 μl cell pellet extract or concentrated supernatant. The samples were amplified on an Applied Biosystems GeneAmp 9700 60-well thermal cycler. Amplification parameters were: preincubation at 95 °C for 15 min, followed by 34 cycles of denaturation for 60 s at 94 °C, annealing for 60 s at 59 °C and extension for 80 s at 72 °C. This was followed by a final elongation step of 10 min at 72 °C. At the end of the PCR reaction the temperature was kept at 4 °C.

After PCR, the amplified fragments were separated and analysed by capillary electrophoresis using an ABI PRISM® 3100 Genetic Analyzer equipped with Genemapper ID v3.2 software (Applied Biosystems). Peak height minimum thresholds were set at 100 relative fluorescence units (RFU). When allelic drop-out (ADO) was expected for a profile due to low amount or bad quality DNA, homozygous loci were not taken into consideration. Probability of occurrence of the DNA profile was calculated using the random man not excluded (RMNE) method [21].

3. Results

To determine whether cell free DNA is present in forensic samples, DNA profiles from cell pellet and cell free concentrated supernatant from 30 artificial case like samples were compared.

Supplementary Table 1 shows how many alleles are detected in the concentrated supernatant of the artificial case like samples. For dried saliva samples and buccal swabs, all alleles detected in the cell pellet were also detected in the concentrated supernatant. For ejaculates from fertile donors and for dried urine, mainly partial profiles were obtained, whereas for ejaculates from vasectomized donors, vomit, faeces and perspiration samples no alleles were detected in the concentrated supernatant. When large blood stains (200 μl of dried blood) were analysed, no alleles could be detected in the concentrated supernatant, whereas for small blood stains (1 μl of dried blood), some donor alleles could be detected. This is most likely due to the fact that higher amounts of potential PCR inhibitors are present in the concentrated supernatant of larger blood stains. In none of the artificial case like samples allele drop ins were detected.

These preliminary results urged us to analyse whether cell free DNA could have an added value in forensic casework. For this aim, DNA profiles from cell pellet and concentrated supernatant of 100 samples from 78 different forensic cases were compared. As shown in Supplementary Table 2, cell free DNA was present in 90% of the samples. Overall, the concentrated supernatant contained less alleles than the cell pellet.

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In 16% of the samples, the cell free DNA had an added value, defined by a lower RMNE value of the combined DNA profile of cell pellet and concentrated supernatant versus the RMNE value of the cell pellet alone.

In the analysed blood samples, cigarette buds and nail cleaners, cell free DNA was present in 90.9% (20/22), 50.0% (6/12) and 100.0% (6/6) of the samples, respectively, but did not have an added value in any of these samples. In the saliva and saliva (potentially with skin contact) samples, cell free DNA was present in all samples and had an added value in 25.0% (1/4) and 21.4% (3/14) of the samples, respectively. In the clothing and contact trace samples, cell free DNA was present in most samples (92.3% (12/13) and 71.4% (24/28), respectively). Moreover the cell free DNA had an added value in 15.4% (2/13) of the clothing samples and in 32.1% (9/28) of the contact traces. The vomit sample (dried vomit on a cotton towel) showed no DNA profile in the cell pellet, whereas an almost full DNA profile was detected in the concentrated supernatant, clearly showing the presence and added value of cell free DNA.

Logically, in none of the samples where the cell pellet gave rise to a full DNA profile the cell free DNA had an added value. In the mixed cell pellet samples, the cell free DNA had an added value in

12.5% (4/32) of the samples. For samples with a partial profile from a single contributor this number increased to 61.5% (8/13). In 25.0% (4/16) of the samples where no profile was obtained from the cell pellet, cell free DNA was present in the concentrated supernatant, leading to additional information.

The potential value of cell free DNA in some forensic cases is exemplified in Fig. 1. This figure shows six loci of the cell pellet and the corresponding concentrated supernatant of a bottle opening. The cell pellet was less informative than the concentrated supernatant with only one locus showing alleles with an RFU > 100 and no allelic drop out expected (i.e. heterozygous loci). The RMNE value of the cell pellet was 1/4.1 E + 02 while the concentrated supernatant combined with the cell pellet had an RMNE value of 1/3.5 E + 13. In the concentrated supernatant peak heights were higher and 8 loci showed heterozygous peaks with an RFU > 100. 4 loci showed homozygous peaks with an RFU > 100. These were not taken into account for the calculation of the match probability, but were confirmed by comparison with the DNA profile obtained from a reference sample in the same forensic case (data not shown).

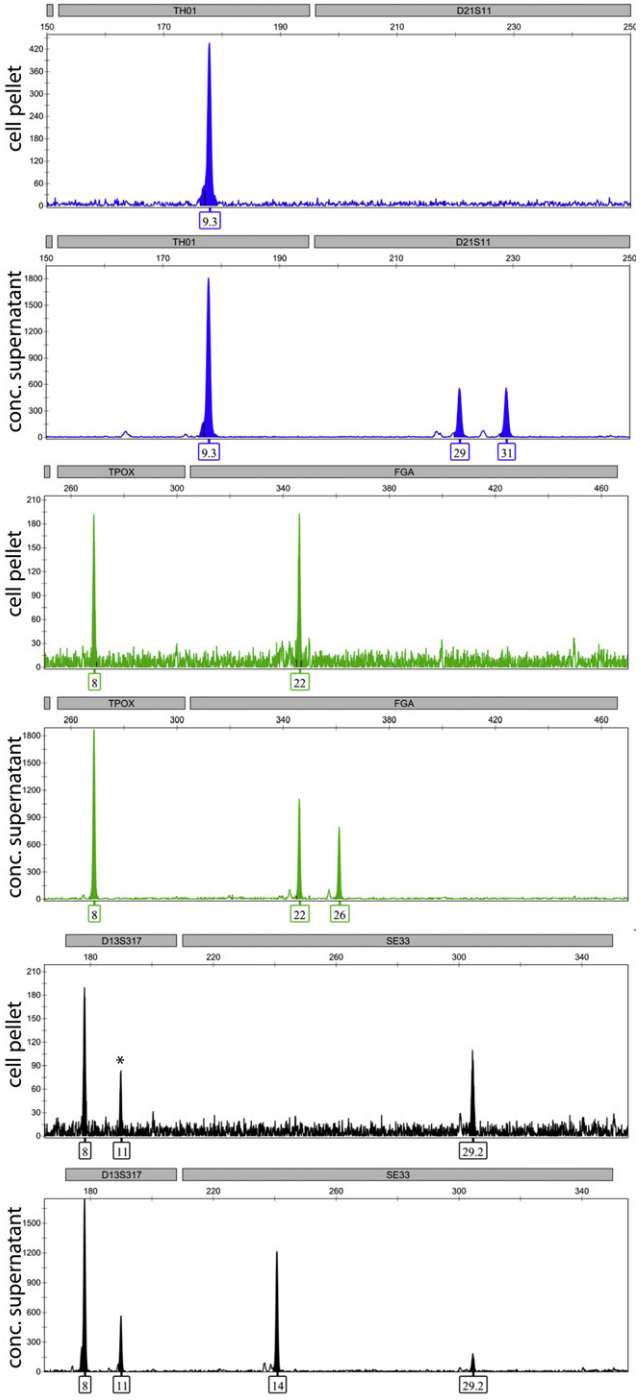

Fig. 1. Comparison of cell pellet and concentrated cell free supernatant for six different loci (TH01, D21S11, TPOX, FGA, D13S317 and SE33): the concentrated supernatant contains valuable additional information. Allele 11 of locus D13S317 falls below detection limit in the cell pellet (*) but not in the concentrated supernatant.

4. Discussion

The results described above clearly prove the potential presence of cell free DNA in the supernatant which is normally discarded during most DNA extraction procedures, such as Chelex® extraction. It can be assumed that this cell free DNA is a combination of DNA which is already present extracellularly within the stain and DNA that is freed from cells during the pre-extraction process when the biological material is soaked off from its support. Cell membranes may rupture due to osmotic movement of water into the cells, this way releasing the cellular DNA. The origin of the cell free DNA has however no influence on its potential value.

A comparison between the DNA profiles of cell pellet and concentrated supernatant and their probability of occurrence showed that not all alleles present in the concentrated supernatant were present in the cell pellet or vice versa. This indicates that information can be lost when supernatant is discarded during extraction. In this study, about half of the supernatant was used for DNA concentration. It can be assumed that, by concentrating the entire supernatant, an even higher DNA yield could be obtained.

Contact traces benefit the most from the analysis of concentrated supernatant with an added value of the cell free DNA in almost one third of the samples. This is most likely due to cell free DNA, originating from cornified layers of skin, present in sweat deposited on objects upon touch [15–17]. In most cases, cell pellets from contact traces result in partial or mixed profiles. Not surprisingly, it was shown that analysis of concentrated supernatant is more likely to be informative for these types of profiles, than when the cell pellet results in a full profile from a single contributor.

For clothing, where the contact between the fabric and the skin is supposed to be more intense and prolonged than in contact traces, cell free DNA was present in all samples but had less added value than in contact traces. This is mainly due to the fact that most cell pellets of clothing samples resulted in either full or mixed DNA profiles.

Analysis of artificial case like samples indicated that cell free DNA was present in the ejaculate of fertile but not in that of vasectomized men. This is in accordance with the findings of Chou et al. [22] who state that the presence of cell free DNA in semen is correlated to parameters linked to normal sperm function and suggest the use of cell free DNA as a marker for semen quality. These authors state that the source of cell free DNA in ejaculates are cells undergoing spermatogenesis (e.g. secondary spermatocytes and spermatides), which are obviously not present in the ejaculates of vasectomized men.

In dried blood samples, the analysis of the concentrated supernatant did not have an added value. This type of samples mostly results in single contributor full profiles, hence, the obtained results are in accordance with the fact that samples resulting in a full cell pellet profile do not benefit from the analysis of the concentrated supernatant. Although it is known that blood serum contains cell free circulating DNA [23], the presence of PCR-inhibitory substances in the supernatant of blood traces might

interfere with the DNA polymerase and degrade or capture target nucleic acids. Inhibitors that have been identified in blood are in most cases natural components of blood, mainly haeme [24] and immunoglobulin G [25]. Since the inhibitors are inherent to the samples, avoidance of this phenomenon is not possible. Moreover, not all cell free DNA in the bloodstream is present as naked DNA. It can be associated with histones in nucleosomes, bound to other plasma proteins or packed in apoptotic bodies [26], hence this cell free DNA is centrifuged down together with the cell pellet during extraction and contributes to the DNA profile of the cell pellet. For these reasons, analysis of supernatant of blood traces has no added value.

Contrary to the artificial vomit samples, where none of the donor alleles were detected in the concentrated supernatant, almost a full DNA profile was obtained in a real forensic case sample with dried vomit on a cotton towel. The cell pellet from this sample had given no profile, again proving the potential added value of cell free DNA in specific settings. Since cell free DNA is associated with cell degradation, the detected cell free DNA in this sample is most likely derived from columnal epithelial cells that form the lining of the stomach and intestines. These cells have a rapid turnover time owing to the harsh acid environment of the stomach [27].

As proven by the example in Fig. 1, the saliva samples and the artificial case like samples, high amounts of cell free DNA can be present in saliva stains. In accordance, Anoruo et al. measured 66 ng of DNA per μl of saliva, roughly corresponding to 10,000 cells. However, only ~100 buccal cells were microscopically observed per μl of saliva [28]. This large inconsistency between expected and observed cell count suggests the presence of high amounts of cell free DNA in saliva. Moreover, the background of inhibitory substances is much lower and less complex in saliva than in blood [29]. Nevertheless, the cell free DNA only had an added value in about one fourth of the saliva and the saliva (potentially with skin contact) samples. Samples that had benefit from the analysis of the cell free DNA fraction all showed partial profiles in the cell pellet.

Cell free DNA was either not present or was shown not to have an added value for dried faeces samples, dried perspiration, cigarette buds and nail cleaners. The fact that no cell free DNA was detected in the dried perspiration samples is in contrast with the findings of Quinones and Daniel who detected cell free DNA in the perspiration of 80% of the analysed individuals [17].

Whilst Chelex® DNA extraction is still widely used, many labs, for various reasons, now use alternative DNA extraction methods such as DNA IQ™ System (Promega), PrepFiler (Applied Biosystems), QIAamp DNA Investigator Kit (Qiagen) and similar ones. These alternative DNA extraction methods use different mechanisms and processes that may significantly limit the observed loss of cell free DNA compared to Chelex® DNA extraction. After cell lysis, both DNA released from the cells and cell free DNA are adsorbed to either silica or magnetic silica particles. DNA is eluted after different wash steps.

Recently, Phillips et al. compared the extraction efficiency of Chelex-100® and QIAamp DNA Investigator Kit [30]. Despite the small data set, the highest median DNA quantities were observed with the QIAamp DNA Investigator Kit. The authors state that pipetting errors and transfer of PCR-inhibiting Chelex® resin are likely to explain the lower quantification results of the Chelex® extraction. To our opinion, the fact that the Chelex® extraction method does not take into account the cell free DNA is one possible explanation for the lower DNA yield obtained with this extraction method. Using the QIAamp DNA Investigator Kit the sample is dissolved in a proteinase K containing lysis buffer. After incubation, the lysate contains both cellular and cell free DNA, which could explain the higher DNA yield.

5. Conclusion

Considering the results described here, it is without doubt that cell free DNA is present in the supernatant which is usually discarded during Chelex® extraction. Moreover, this cell free DNA can contain information which is not detected when only the cell pellet is analysed. In some cases, discarding the cell free DNA containing supernatant implies discarding valuable information. This is mainly the case for contact traces. Therefore, to our opinion, the supernatant should not be analysed at the same time as the cell pellet, but should be stored for potential additional analysis in case the cell pellet would not result in a useful DNA profile. Alternatively, the concentrated supernatant could be added immediately to the extract of the cell pellet before PCR. For samples other than contact traces the additional workload induced by concentrating all supernatants might however not be justified.

In conclusion, the presence of cell free DNA in forensic casework samples deserves further consideration since it has the potential to increase the DNA yield in forensic casework samples in general and in contact traces in particular.

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