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Short communication

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

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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

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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

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