Research paper

Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study

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A R T I C L E   I N F O

Article history:
Received 12 February 2016
Received in revised form 12 April 2016
Accepted 14 June 2016
Available online 16 June 2016

Keywords:
Cell-free DNA
Half-life
Serum
Urine
Saliva

A B S T R A C T

Background: Cell-free DNA is naturally degraded in various bodily fluids. The aim of this study was to determine the degradation kinetics of DNA, with and without protein, in serum, urine and saliva.

Methods: Naked DNA and DNA-protein complex were prepared, added to the samples to be analysed and incubated at 37 °C and room temperature for various lengths of time. Alleles of 20 short tandem repeat loci were amplified from the incubated samples, and clearance models were generated from the mean peak areas.

Results: Plotting the natural logarithm of DNA concentration against the incubation time produced a linear relationship. The half-lives of DNA with and without protein in serum were 157.6 min and 30.8 min at 37 °C, 330.5 min and 70.5 min at room temperature, respectively. The half-lives of DNA with protein in saliva were 175.6 min and 251.3 min at 37 °C and room temperature, respectively. The half-lives of DNA in urine (both with and without protein) were too short to detect.

Conclusions: The kinetics of DNA degradation in serum and saliva followed a first-order clearance model. Urine had the strongest effect on DNA degradation, and the half-lives of DNA with protein were relatively longer than those of naked DNA.

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1. Introduction

Cell-free DNA (cfDNA) was first described by Mandel and Metais (1948), and its concentration was subsequently shown to be elevated in cancer patients (Leon et al., 1977). Apoptosis and necrosis are concerned with the main sources of cfDNA (Jahr et al., 2001). In the human body, cfDNA is primarily found in nucleosomes, mononucleosomes, oligonucleosomes, and virtosomes (Rykova et al., 2012), and it is generally complexed with lipids and proteins (Peters and Pretorius, 2011; Holdenrieder and Stieber, 2009).

The detection of cfDNA in serum, plasma, urine or saliva represents a new molecular diagnostic tool characterised by easy and non-invasive sampling. cfDNA has been studied in such diverse areas as cancer diagnosis and prognosis (Dawson et al., 2013; Begum et al., 2011; Nygaard et al., 2012), prenatal screening (Lau et al., 2002; Shea et al., 2013; Jensen et al., 2012), organ-transplant monitoring (Lo et al., 1998; Snyder et al., 2011) and acute medicine (Tsai et al., 2011; Cui et al., 2013).

Molecular biology tests include multiple processes, such as sample collection, storage, extraction and analysis; each step can greatly impact the final results. However, reports on the appropriate conditions and duration of DNA storage in vitro are limited and inconsistent. Jung et al. reported that the concentration of plasma DNA remained virtually unchanged after storage at 4 °C and room temperature for 24 h prior to centrifugation (Jung et al., 2003). Sozzi et al. found that plasma DNA stored at −80 °C degraded at a rate of 30.7% per year (Sozzi et al., 2005). Lo et al. showed that in 70% of subjects, the foetal plasma DNA concentration decreased to 31%–74% of the initial value after a 2 h incubation at 37 °C (Lo et al., 1999). Furthermore, the literature differs concerning the appropriate storage conditions and duration for urine samples. Urine samples with sodium azide can be stored for 20 days at room temperature (Vu et al., 1999), whereas the storage time for those with EDTA is ~72 days (Milde et al., 1999). van der Hel et al. reported that urine stored at −20 °C for 25 years without preservatives could still be used for molecular biology tests (van der Hel et al., 2002). Cannas et al. showed that the clearance rate of DNA in urine differed in different populations (Cannas et al., 2009). However, no study has addressed DNA degradation in saliva, and previous works have not systematically researched DNA degradation in serum, urine and saliva. In addition, the reported stability and degradation rates of DNA in bodily fluids vary significantly due to differences in extraction methods, PCR conditions and target sequences (Chiu et al., 2001; Fleischhacker et al., 2011; Su et al., 2004), and the nature of the nucleic acid measured (e.g., cell-free or cellular; mononucleosomes or oligonucleosomes).
To analyse cfDNA degradation in vitro, purified human genomic DNA without protein (henceforth called “naked DNA”) and DNA with protein (in the form of DNA-protein complex) were each added to serum and urine at 37 °C and room temperature. The added DNA was homologous to that in the corresponding bodily fluids to prevent allogeneic reactions. We performed DNA extraction with the MagCore HF16 Automated DNA/RNA Purification System and MagCore Genomic DNA Whole Blood Kit. The commercially available GoldenEye™ 20A Kit was used to amplify 20 short tandem repeat (STR) loci with target sequence lengths ranging from 77 bp to 446 bp. We quantified the amplified products using the ABI3130-Avent Genetic Analyzer. Following this approach, we analysed the temporal degradation patterns of naked DNA and DNA-protein complex in serum, urine and saliva.

2. Materials and methods

2.1. Human genomic DNA purification and quantitation

Saliva samples were collected in the morning after a fast of at least 6 h. To minimise DNA contamination and PCR inhibitors, the volunteer rinsed his mouth with water 30 min before each collection. All the samples were collected and stored in the sterile tubes, and the visually observed unusual specimens were abandoned. The saliva, serum and urine samples' processing and DNA amplification were separated from each other, there were no cross contaminations among samples. The whole process was conducted under the sterile condition, and the secondary pollution was eliminated. The repeated tests were performed 3 repetitions within one month.

This study was approved by the Ethics Committee of Dalian Medical University.

2.2. Preparation of DNA-protein complex suspension

In the preparation of DNA-protein complex, protease was not added in the process. Using venipuncture, we collected 2 ml blood from the volunteer into vacuum tubes containing fibrin ferment. The blood samples were centrifuged at 1600g for 10 min at 4 °C. The serum was carefully removed and transferred into plain polypropylene tubes without disturbing the blood clot, which was then centrifuged at 16,000g for 5 min. The supernatants were collected into fresh polypropylene tubes.

A total of 5000 ng DNA-protein complex suspension and 5000 ng naked DNA were placed into two sterile tubes containing 4 ml serum each, and the tubes were vortexed thoroughly to mix. The mixture was divided into 200 μl aliquots labelled S0, S1, S2, S3, S4 and S5; G0, G1, G2, G3 and G4. “S” represents serum with naked DNA added, while “G” represents serum with DNA-protein complex added. S0 and G0 were stored at −20 °C immediately after separation. S1–S5 were stored at −20 °C after incubations at 37 °C for 20, 40, 60, 80 and 120 min in sequence; G1–G4 were incubated at 37 °C for 40, 180, 240 and 360 min, respectively, prior to storage at −20 °C. The samples were incubated at room temperature (22–25 °C) with the same procedure as mentioned above for a group of room temperature.

2.3. DNA degradation in serum

Using venipuncture, 20 ml blood was collected into vacuum tubes containing fibrin ferment. The blood samples were centrifuged at 1600g for 10 min at 4 °C. The serum was carefully removed and transferred into plain polypropylene tubes without disturbing the blood clot, which was then centrifuged at 16,000g for 5 min. The supernatants were collected into fresh polypropylene tubes.

A total of 5000 ng DNA-protein complex suspension and 5000 ng naked DNA were placed into two sterile tubes containing 4 ml serum each, and the tubes were vortexed thoroughly to mix. The mixture was divided into 200 μl aliquots labelled T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, and T12. T0 was immediately stored at −20 °C. T1–T6 and T7–T12 were stored at −20 °C following incubations at 37 °C and room temperature for 3 h, 6 h, 9 h, 12 h, 15 h and 18 h, respectively.

2.4. DNA degradation in saliva

Because relatively high quantity of DNA in saliva, it was unnecessary to add DNA to saliva. A total of 3 ml of saliva was collected into a sterile tube under the conditions described above. The saliva was mixed thoroughly and divided into 200 μl aliquots labelled T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, and T12. T0 was immediately stored at −20 °C. T1–T6 and T7–T12 were stored at −20 °C following incubations at 37 °C and room temperature for 3 h, 6 h, 9 h, 12 h, 15 h and 18 h, respectively.

2.5. DNA degradation in urine

A 10 ml aliquot of fresh urine obtained from the volunteer was centrifuged at 1600g for 10 min at 4 °C. The supernatant was transferred to another sterile tube and was centrifuged at 16,000g for 5 min at 4 °C. The supernatant was transferred to a new tube, and sterile water was added for dilutions of 1:2, 1:4 and 1:8. We placed 200 μl of each diluted sample into a new tube and added either 250 ng DNA-protein complex suspension or 250 ng naked DNA. As a control, the same volume of water underwent the same procedures. All the processes were performed at 4 °C. Then the samples were immediately stored at −20 °C before subsequent steps.

2.6. Automatic DNA extraction from prepared serum, urine and saliva samples

Each prepared serum, urine and saliva sample was thawed once. DNA was immediately extracted using the MagCore HF16 Automated DNA/RNA Purification System (RBC Bioscience Corp., Taiwan) with the MagCore Genomic DNA Whole Blood Kit (RBC Bioscience Corp., Taiwan).

2.7. STR amplification and fragment analysis

The extracted DNA samples were amplified via PCR using commercially available GoldenEye™ 20A Kit (Beijing PeopleSpot Inc., Beijing, China). The following 20 STR loci were amplified: CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S800, D8S1179, D13S317, D16S539, D18S51, D21S11, D19S433, D6S1043, D22S139, D25S138, PetitA and the amelogenin locus (Table 1).

Each PCR amplification was performed in a 10 μl reaction volume, according to the manufacturer's instructions. The amplification conditions were 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 60 °C for 1 min and 70 °C for 1 min; and a final elongation of 30 min at 60 °C.
were obtained using the following equation:

\[ \text{Mean peak area (RFU)} = (25,658 + 23,771 + 15,095 + \ldots + 314) / 148 \]

After the amplification, 1.5 μl of the PCR product was loaded onto an ABL 3130-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA) for capillary electrophoresis, and the collected data was automatically analysed with the GeneMapper® ID Software v3.2 (Applied Biosystems, Foster City, CA). Allele peaks were labelled if the peak area was equal to or > 50 relative fluorescence units (RFU). The relative concentration of degraded DNA was calculated according to the STR profiles and peak areas produced automatically by the software.

2.8. Statistical analysis

The relative concentration of degraded DNA was expressed as the mean peak area, calculated by dividing the sum of the interpreted peak areas by the total peak number of the full STR profile. As an example, Table 2 illustrates the calculation of the mean peak areas from the data obtained from the T0 amplification.

In the serum and saliva samples, the DNA concentration decreased with increasing incubation time. For a linear regression analysis, the natural logarithms of the peak areas were plotted against the incubation time, and the regression equation (y = −0.0259x + 8.1826) is obtained, the slope (k) = −0.0259. So according to the formula \( T_{1/2} = \ln 2 / |k| \), the half-life (\( T_{1/2} \)) is 26.8 min.

3. Results

3.1. STR profiles and analysis

The STR profiles and peak areas of each sample were obtained automatically using the GeneMapper® ID Software v3.2. The saliva and serum samples with added DNA (including naked DNA and the DNA-protein complex suspension) that were stored immediately at −20 °C exhibited the predicted alleles (Fig. 1). The alleles were also consistent with those in the profiles obtained from the diluted urine samples with added DNA.

3.2. DNA degradation in serum and saliva

Dynamic degradation curves were generated by plotting peak areas against incubation times (Fig. 2A, C and E). To determine the degradation model, a linear regression analysis was performed by plotting the natural logarithms of the peak areas against the incubation times; at 37 °C for example, the R² values for naked DNA degradation in serum, DNA-protein complex degradation in serum and DNA degradation in saliva were 0.98, 0.91 and 0.92, respectively (Fig. 2B, D and F). These data indicate that the natural logarithms of the peak areas exhibited a linear relationship with time. Correspondingly, linear relationship was also obtained by the above-mentioned DNA at room temperature. In other words, DNA degradation followed a first-order clearance model in these instances (Lau et al., 2002). The half-lives of naked DNA in serum, DNA-protein complex in serum and DNA in saliva are shown in Table 3.

3.3. Effects of urine on DNA stability

Although the STR profile of the naked DNA in urine could not be obtained, full profiles were generated from the urine with added
DNA-protein complex and the diluted urine with each form of DNA. Full profiles were also obtained from the negative control groups. A regression analysis of the urine with added naked DNA and the urine with added DNA-protein complex was performed by plotting the dilution factors against the natural logarithms of the peak areas (Table 4). The linear regression equations were obtained with using natural logarithms of the mean peak areas as independent variables, and the dilution factors as dependent variables. The mean peak areas obtained from the negative controls (sterile water with added DNA) represented the initial concentrations. Half of the added naked DNA and DNA-protein complex became degraded at dilution factors could be calculated from regression equations as shown in Table 5.

4. Discussion

The proteins wrapped up DNA have significant effect on DNA protection; therefore this research takes observation on naked DNA and DNA-protein complex, respectively. cfDNA is released from nucleus into blood and other bodily fluids at 37 °C, and samples will be placed at room temperature for a long time after in vitro, thus DNA degradation at 37 °C and room temperature was observed, respectively. Serum, urine and saliva are easy sampling body fluids for DNA extraction, so the degradation kinetics of DNA in these body fluids was observed in this study. The majority involve manual extraction using time-consuming procedures that are highly prone to inconsistencies caused by human handling, which ultimately lead to high interobserver variability (Fleischhacker et al., 2011; Johnson et al., 2004). Here, we isolated DNA using the MagCore HF16 Automated DNA/RNA Purification System, which can isolate 16 samples within 45 min, with consistent yields and levels of purity. The reagent was used for DNA extraction from EDTA anticoagulant blood, the OD value was stably between 1.8 and 1.9; good linear relationship was shown for the DNA yields with the used blood volume of 20 μl, 50 μl, 80 μl, 100 μl, 150 μl and 200 μl. This purification system is widely used for nucleic acid detection (Tai et al., 2012).

The STR amplification and analysis were performed using a paternity test system in a recognised judicial appraising laboratory, and the entire process was highly automated and commercialised. The 20 STR loci amplified include 13 core CODIS (Combined DNA Index System) loci (CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51 and D21S11) and D19S433, D6S1043, D12S391, D2S1338, PentaD, PentaE and amelogenin for sex determination. The 20 STR loci were amplified in this study because these loci were relatively small fragments (77 bp to 446 bp) for PCR amplification and the data of degradation kinetics can be directly used for sample collection, storage and preparation for PCR use.

The DNA concentration was quantified using the peak area, which was obtained automatically through genotyping with GeneMapper®.
ID Software v3.2. The peak area was chosen to represent the DNA degradation in vitro for two reasons. First, because peak shapes vary, the peak area is a better indicator of DNA quantity (Perlin & Szabady, 2001; Gill et al., 1998). Second, using the peak area in the capillary zone electrophoresis with laser-induced fluorescence (CZE-LIF) assay to quantify DNA concentration in serum and plasma is as accurate and sensitive as the widely used real-time PCR (Zhang et al., 2004; Sang & Ren, 2006).

The linear relationship that was found between the natural logarithm of the DNA concentration and the incubation time suggests that DNA degradation in serum and saliva follows a first-order clearance model. The $R^2$ values for the degradation of naked DNA and DNA-protein complex in serum and the degradation of naturally existing DNA in saliva were 0.98, 0.91 and 0.92, respectively. The stored isolated DNA and the DNA in plasma were calculated in the same way (Sozzi et al., 2005). Because first-order clearance had been demonstrated, the half-lives could be calculated using the equation from Section 2, and they were not dependent on the initial DNA concentrations. In this study, the quantity of DNA added did not affect the calculated

### Table 3

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Test</th>
<th>Serum</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>22–25 °C</td>
<td>1</td>
<td>346.5</td>
<td>71.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>315.0</td>
<td>72.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>330.0</td>
<td>67.3</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
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<td>70.5</td>
</tr>
<tr>
<td>37 °C</td>
<td>1</td>
<td>157.5</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>161.2</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>154.0</td>
<td>34.3</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>157.6</td>
<td>30.8</td>
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</table>

### Table 4

<table>
<thead>
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<th>Dilution</th>
<th>Mean peak area (RFU)</th>
</tr>
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<tbody>
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<td>DNA with protein</td>
<td>“Naked DNA”</td>
</tr>
<tr>
<td>2×</td>
<td>8681.9</td>
</tr>
<tr>
<td>4×</td>
<td>17,243.9</td>
</tr>
<tr>
<td>8×</td>
<td>30,162.9</td>
</tr>
<tr>
<td>Control</td>
<td>31,380.0</td>
</tr>
<tr>
<td>Half-dilution factor</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Half-dilution factor: the dilution at which half the quantity of added DNA was degraded. The linear regression equations for the naked DNA group and the DNA-protein complex group were $y = 2.21x - 15.29$ ($R^2 = 0.96$) and $y = 4.75x - 41.44$ ($R^2 = 0.94$), respectively.

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**Fig. 2.** DNA degradation and kinetics analysis in serum and saliva. A, B: Dynamic degradation curves for naked DNA in serum before and after natural logarithm transformation. C, D: Dynamic degradation curves for DNA-protein complex in serum before and after natural logarithm transformation. E, F: Dynamic degradation curves for DNA in saliva (DNA naturally exists in saliva) before and after natural logarithm transformation.
half-lives, and the added DNA was much more abundant than the naturally occurring cfDNA in the serum (Bryzgunova et al., 2006) and urine (Edgar, 1992) of healthy subjects. Therefore, whether sonication was used for DNA extraction, the degradation of the naturally existing cfDNA in the serum and urine could be neglected.

The report by Lo et al. that 31%–74% of foetal plasma DNA remained after a 2 h incubation at 37 °C in 70% of subjects (Lo et al., 1999) prompted us to perform incubations in the order of hours. Previous studies have reported the storage time and clearance of cfDNA in plasma or serum at 4 °C, 37 °C, −80 °C and room temperature. However, these studies were mainly restricted to the clearance of one or two target gene sequences (Jung et al., 2003; Sozzi et al., 2005; Lo et al., 2009). In the present work, the mean peak areas of 20 STR loci (36 target gene sequences) were successfully obtained from these samples. A plot of the dilution factors against the natural logarithm of peak areas produced a linear regression. The degradation of both forms of DNA in urine is clearly rapid. Transrenal DNA is reportedly composed of low-molecular-weight DNA fragments ranging from 150 to 200 bp (Botetatu et al., 2000). Our observations produced a reasonable conclusion: the cfDNA had already degraded into small fragments before detection due to the strong effects of urine. The urine components are very complex; therefore, in order to understand why the DNA degrades so quickly in the urine, what really matters for its degradation, and what the influence is, further research is needed.

In conclusion, our results indicate that the effect of urine on DNA is stronger than that of serum or saliva. The degradation of DNA-protein complex in serum is quite similar to that in saliva; they both follow a first-order clearance model. Furthermore, when the PCR target sequences range from 77 bp to 446 bp, the calculated half-life of DNA-protein complex in serum is 5 times longer than that of “naked DNA”, which implies a protection effect of protein on DNA in circulation (Kragh-Hansen et al., 2002). What’s more, the DNA degradation gets faster with the temperature increases. According to these findings, we suggest the immediate isolation of DNA from serum and saliva because their half-lives are <3 h. Furthermore, urine is not an appropriate specimen for quantitative molecular diagnostic analysis due to its rapid degradation of DNA.

Gene therapy tries to inject the designed exogenous DNA sequences into the target cells in vivo, change and regulate the original gene expression state of the target cells. In this process the exogenous DNA sequences has to overcome the degradation effects of the bodily fluids. Therefore, the data in this study also has a vital value for the implementation of the gene therapy.

The limitation of this study majors on the analysis of the degradation effects of serum, urine and saliva without considering the interindividual differences. However, vital reference values exit in this research for the basic estimation that interindividual differences won’t have significant effects on the DNA degradation kinetics in body fluids. In addition, individual researchers should perform nucleic acid stability analysis on their own samples due to the variability of subjects and detection conditions, including PCR conditions and target sequence lengths.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

Acknowledgements

We would like to give our sincere thanks to He Min, Dai Xiaomeng, Wu Yingtao and Zhou Ying for concordance studies. We are grateful to the whole workers from Dalian Blood Center for their help in DNA isolation and STR analysis. We also thank Xu Qinghan and Pan Jie for statistical analysis and figure making.

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