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Research paper

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

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ABSTRACT

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. However, 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.

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 widely considered to be 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).

Abbreviations: cfDNA, cell-free DNA; STR, short tandem repeat.

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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 complexe) 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

Serum, urine and saliva samples were obtained from healthy volunteers exclusive of related diseases, aging from 20 to 30, at Dalian Medical University.

To prevent the contamination of other DNA sequences, PCR inhibitors and other substances, the saliva samples were collected in the morning after a fast of at least 6 h, and the volunteers were asked to rinse their mouths with water before each collection. All the samples were collected and stored in the sterile tubes, and the visually observed unusual specimens were abandoned. The serum, saliva 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.

2.1. 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 (Soares-Vieira et al., 2001). A total of 1.2 ml saliva was collected in a sterile tube and was immediately mixed and distributed into 200 μl aliquots. Genomic DNA (DNA without protein, naked DNA) was extracted using the E.Z.N.A. Blood DNA Kit (OMEGA Bio-tek, Inc., Norcross, GA, USA) with an elution volume of 100 μl per column, according to the "Blood and Body Fluid DNA Spin Protocol" provided by the manufacturer.

The concentrations of the isolated DNA samples were measured using a micro-spectrophotometer (Vastech Inc., Wilmington, USA) according to the manufacturer's instructions.

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 EDTA. The blood samples were centrifuged at 1600*g* for 10 min at 4 °C to produce three layers, with the buffy coat in the middle layer. The plasma was discarded, and the buffy coat was carefully removed, placed in a 2 ml tube and completely mixed with 800 μl sterile water by vortexing. The DNA-protein complex suspension was generated by sonication (KS-500F, Ningbo, China) in an ice bath with a 1/4″ microtip at 40% amplitude for 30 cycles (run: 3 s; pause: 2 s) for cell breaking. The sonicated product was centrifuged at 12,000*g* for 8 min at 4 °C, and the supernatant was collected for further use.

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 1600*g* 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 16,000*g* 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.

2.4. 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 1600*g* for 10 min at 4 °C. The supernatant was transferred to another sterile tube and was centrifuged at 16,000*g* 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 until 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*, *D7S820*, *D8S1179*, *D13S317*, *D16S539*, *D18S51*, *D21S11*, *D19S433*, *D6S1043*, *D12S391*, *D2S1338*, *PentaD*, *PentaE* 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.