

Evaluation of Clinical Laboratory Methods for Plasma Cell-Free DNA Analysis in Suspected Septicaemia

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Abstract

Background: The amount and quality of plasma cell free DNA (cfDNA) changes in extreme physiological and pathological conditions. These changes could provide a basis for a novel prognostic biomarker in conditions as diverse as pregnancy, cancer, organ transplantation and septicaemia. Evaluation of current methods for cfDNA analysis is needed to identify the best practical approach to medical diagnostics.

Methods: Initially, cfDNA was extracted from plasma of nine patients with febrile illness using both QIAamp Circulating Nucleic Acid and QIAamp Mini Blood DNA kits. CfDNA concentration was determined by β -globin qPCR using PerfeCta and AmpliTaq mixes. Subsequently, Qubit fluorimetric and gel-on-a-chip assays were used to analyse plasma cfDNA from 64 additional patients with suspected septicaemia and positive blood cultures.

Results: Determination of CfDNA concentration by β -globin gene qPCR using AmpliTaq mix was superior to the PerfeCta qPCR. Moreover, unlike PerfeCta AmpliTaq qPCR determined similar genome equivalent copy numbers in cfDNA isolated by either DNA extraction method. QIAamp Mini Blood kit and AmpliTaq qPCR were subsequently used in a larger prospective study for cfDNA isolation and quantification, respectively. However, qPCR was less suited for the detection of high plasma cfDNA levels when compared to direct DNA measurement by Qubit (mean 2.23 ng/mL vs. 61.38 ng/mL, respectively) despite good correlation between the two methods. The DNA microfluidic chip method was then used to determine cfDNA fragment sizes and their relative concentrations revealing a presence of nucleosome-sized DNA fragments that were in strongly positive correlation with total cfDNA. Furthermore, apoptotic DNA was identified as a major DNA component in plasma with high cfDNA content.

Conclusions: β -globin qPCR is more suited to detection of low plasma cfDNA concentrations, while the Qubit assay is a better choice for rapid identification of high plasma concentrations. Apoptosis is a major source of cfDNA in plasma with high cfDNA concentrations based on DNA chip analysis.

Keywords: β -globin; qPCR; Circulatory cell-free DNA; Apoptosis

Introduction

Increased plasma levels of circulatory cell-free DNA occur in various medical conditions including sepsis [1]. Although the main source of cfDNA remains elusive, increased baseline levels of plasma cfDNA in severe sepsis were shown to have potential prognostic value for predicting disease outcome in several independent studies [2-4]. Moreover, the appearance of nucleosome-sized DNA fragments was also reported as an additional marker of prognostic value in plasma of septic patients [5].

Sepsis is a complex and potentially fatal condition triggered by local or systemic infections. It consists of chronological series of dysfunctional alterations in physiological networks. The most prominent changes occur in the immune system leading to an excessive loss of immune cells due to apoptosis [6,7]. In addition, changes in blood coagulation and in function of endothelial cells can result in fibrin deposition, endothelial impairment leading to multiple organ failure and fatality [8]. Besides their decisive role in the pathophysiology of septicaemia, both immune cell loss and organ failure may variously contribute to circulating cfDNA potentially providing an independent prognostic tool for predicting the severity and outcome of sepsis.

The major obstacle to using cfDNA as a clinical laboratory analyte of prognostic value in septicaemia is the lack of a standard laboratory approach to its isolation, detection and measurement. While plasma was found to be a more reliable source for cfDNA analysis than serum

[9,10], the quality of data varied according to the type of anticoagulant used, and conditions of blood storage and plasma processing applied [11-16]. Previous studies comparing efficiency of various plasma cfDNA isolation procedures indicated that in-house procedures were more efficient than the commercial ones though time consuming [17]. Different anticoagulants such as EDTA, citrate and heparin, in combination with varied durations and blood storage temperatures showed different effects on plasma cfDNA levels. EDTA was superior to the other two anticoagulants [14]. However, no comprehensive comparison of different DNA quantification methods has been performed to guide clinical laboratory investigation of patients with suspected septicaemia.

In this study we evaluated the efficiency of clinical laboratory methods for plasma cell-free DNA analysis in patients with suspected septicaemia. Initially, the efficiency of DNA extraction with two

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commercial DNA isolation kits was assessed by β -globin gene qPCR using two different DNA polymerase mixes (AmpliTaq vs. PerfeCta). The capacity to determine accurate plasma cfDNA concentrations was then evaluated for two methods, β -globin gene qPCR (AmpliTaq) and direct DNA measurement (Qubit). We also used microfluidic DNA chip to measure the size and amount of apoptotic DNA in plasma expanding further the prognostic capacity of plasma cfDNA. This study provides a strategy to accurate and reliable analysis of plasma cfDNA in patients with suspected septicemia, early upon admission prior to any other clinical studies.

Materials and Methods

Patients

EDTA plasma samples were obtained from samples routinely collected and analysed from a consecutive series of hospital patients with suspected septicemia who had also had at least one blood culture collection. No samples were collected specifically for this investigation. This study was registered as a clinical quality improvement project by the Sir Charles Gairdner Hospital Research Ethics Committee (Reference 2012-12).

Plasma cfDNA extraction and analysis

The blood was centrifuged at 800 g at room temperature, plasma collected, aliquoted and stored at -80°C following standard clinical laboratory procedure for plasma isolation and storage. A plasma-purifying step was used for the removal of cellular debris and high molecular weight cellular DNA contaminants released from necrotic cells [13,15]. Thawed plasma samples were centrifuged down for 5 min at room temperature (RT) at a maximum speed in a microcentrifuge (Eppendorf, DE) prior to DNA extraction. Plasma aliquots of 200 μL were collected and subjected to DNA extraction immediately. Two commercial kits were used for DNA extraction: QIAamp Circulating Nucleic Acid Kit and QIAamp DNA Blood Mini Kit (Qiagen, MD) according to the manufacturer's vacuum protocol. Final elution was performed with 50 μL ultra pure RNase/DNase-free deionized water. All assays were performed with cfDNA samples stored at 4°C .

Quantitative analysis of plasma cfDNA was performed with Qubit[®] dsDNA HS Assay Kit (Life Technologies, CA) according to the manufacturer's instructions. Samples were diluted 1 in 20 in a final volume of 200 μL of Qubit[®] working solution. After 10s vortexing and 2 min incubation at RT, DNA samples incorporated a fluorescent dye specific for dsDNA. Qubit[®] 2.0 Fluorometer (Life Technologies, CA) was used to measure the dsDNA concentration. Two replicate measurements were performed for 10 samples on consecutive days to confirm the consistency of Qubit readings.

Qualitative analysis of plasma cfDNA was done by microfluidic DNA chip gel electrophoresis using Agilent DNA 12000 Kit and Agilent 2100 Bioanalyzer (Agilent Technology, CA). DNA chips were run with two internal DNA markers of known size (50 bp and 17 kb, respectively) and a DNA ladder of standard concentrations that allowed automatic calculation of DNA fragment size and amount in each sample.

β -globin qPCR

Quantitative PCR was performed with β -globin gene primers: GLOB-524F (forward) 5'-GGCATGTGRAGACAGAGA- 3'; GLOB-579R (reverse) 5'-SASAGAGAGTCAGTGCCTAT- 3' and TaqMan probe: 5'-LightCycler-640-AGA AAC CCA AGA GTC-BHQ3- 3' (Roche, Australia), using AmpliTaq Gold PCR mix (Life Technologies,

CA) and PerfeCta[®] qPCR ToughMix[™] (Quanta BioSciences, MD). Duplicate 8 μL aliquots of each sample were loaded onto 36 or 72-RotorGene rings. PCR was performed as follows: one cycle for 10 min at 95°C followed by 50 cycles of 94°C for 12 sec, 55°C for 15 sec and 72°C for 20 sec on RotorGene (Corbett Life Science, Qiagen, MD). Serial dilutions of plasmid construct containing a fragment of human β -globin gene (pUCminus MCS: Major and Minor Group; 5.95 kb) were used to create a standard curve in the range of 3.4 to 3.4×10^3 genome equivalents (GE). Using a conversion of 1GE to 6.6 pg of human DNA, we extrapolated qPCR data into ng genomic DNA/ μL .

Statistical analysis

All statistics were performed with Prism 6.0 (GraphPad, San Diego, CA) operating under MAC OS 10.2. The following calculations were performed on the experimental data: descriptive column statistics, Mann-Witney U test, Wilcoxon Ranked Sum test, Spearman's non-parametric correlation coefficient and regression curve analysis. Prism 6.0 was also used to generate box and whisker plots, distribution histograms of individual data sets and XY cartesian plots for correlation analysis.

Results

Method selection for plasma cfDNA isolation

The efficiency of two commercial DNA extraction kits was assessed using β -globin gene qPCR for DNA quantification as a first step. Plasma cfDNA was extracted from 9 patients with QIAamp Mini Blood DNA and Circulatory Nucleic Acid kits in parallel. DNA concentration was determined by β -globin gene qPCR using two different DNA polymerase mixes, PerfeCta and AmpliTaq (Figure 1). When total plasma was used directly in qPCR, only the PerfeCta mix produced a positive signal while the qPCR reaction with AmpliTaq mix was consistently negative (Figure 1). The PerfeCta mix performed much better in qPCR with DNA samples obtained by the Circulatory Nucleic Acid kit than with those extracted by Mini Blood DNA kit (Figure 1). However, this difference in the qPCR assay efficiency between the two DNA extraction methods disappeared completely when qPCR was performed with AmpliTaq enzyme mix (Figure 1). Moreover, qPCR

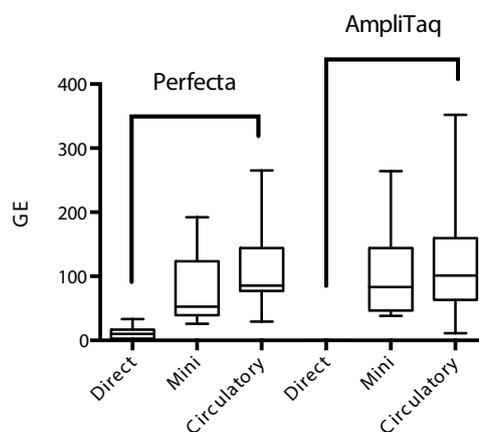


Figure 1: Quantitative analysis of cfDNA by qPCR. Two different qPCR systems, PerfeCta and AmpliTaq, were used to quantify cfDNA directly in plasma or following purification by QIAamp mini blood DNA and circulating nucleic acid isolation kits, respectively. β -globin gene copy numbers extrapolated into genome equivalents (GE) are plotted on the y-axis.

on purified plasma cfDNA obtained by either DNA extraction method was more efficient using AmpliTaq enzyme mix than with PerfeCta. Despite the fact that the PerfeCta qPCR mix overcame PCR inhibitors unlike AmpliTaq, it turned out to be less efficient when used on purified cfDNA. From these results (Figure 1), we concluded that the Mini Blood DNA kit in combination with β -globin gene AmpliTaq qPCR was suitable for plasma cfDNA detection in these hospital patients.

Evaluation of cfDNA quantitation methods

Sixty-seven cfDNA samples from 61 consecutive patients obtained with Mini Blood DNA isolation kit (Qiagen) were subjected in parallel to two different DNA quantification methods, direct fluorimetric double-strand DNA (dsDNA) measurement (Qubit) and β -globin qPCR. Median values of DNA concentrations obtained by these two methods differed considerably (Figure 2). The direct DNA measurement method (Qubit) performed much better than qPCR as indicated by significantly higher median values (Wilcoxon matched-pairs signed-rank test; $p < 0.0001$). In addition, there was a wider distribution of values by direct dsDNA assay and a more accurate estimate in the high range of DNA concentrations than by qPCR (Table 1). In contrast, qPCR provided a more sensitive estimate of DNA concentrations in samples containing lower cfDNA levels than the direct dsDNA assay although it underestimated DNA concentrations in samples with higher cfDNA levels (Table 1). Despite such significant differences, there was a broadly linear positive correlation between paired results obtained by these two methods (Spearman coefficient $\rho = 0.81$; $p < 0.0001$). We also used direct light absorbance of purified cfDNA at 260 nm and 280 nm (NanoDrop, NanoDrop Technologies, Wilmington, USA) to measure plasma cfDNA, but this indirect method produced consistently low 260/280 nm ratios suggesting a low ratio of DNA to other blood derived products (data

not shown). The NanoDrop method was not used further. Similarly, when we used the fluorimetric method of dsDNA measurement on untreated plasma, we were not able to detect cfDNA (data not shown).

Qualitative analysis of plasma cfDNA

We used Agilent microfluidic DNA chip to determine the presence of nucleosome-sized DNA fragments (average of 165-180 base pairs) in the samples (Figure 3A). In addition, Agilent software was used to calculate the concentrations of apoptotic DNA in each sample from the electropherograms (Figure 3B). Subsequently, the concentration of apoptotic DNA was plotted against total cfDNA previously determined by dsDNA assay (Qubit). The amount of apoptotic DNA in plasma increased consistently from below 10% to over 90% corresponding to a rise in total plasma cfDNA from below 100 to over a 1,000 ng/mL (Figure 4). There was a significant positive correlation between total cfDNA and apoptotic DNA (Spearman's test: $r = 0.879$; $p < 0.000$). Paired analysis revealed significant differences in concentrations between total cfDNA and apoptotic DNA in 76 plasma samples (Wilcoxon matched-pairs signed-rank test; $p < 0.0001$). However, the non-logarithmic plot showed a non-linear regression line cutting the cfDNA axis below 100 ng/mL, indicating a possible lower threshold for apoptotic DNA appearance (Figure 5).

The association between total plasma cfDNA amount and the appearance of apoptotic DNA was examined further in two groups of plasma samples: one ($n = 42$) that did not show any presence of apoptotic DNA and another ($n = 76$) in which apoptotic DNA was evident (Table 1). The cfDNA concentrations in these two groups of plasma samples were significantly different (Mann Whitney test; $p < 0.0001$), while analysis of paired data sets showed a lack of correlation (Spearman coefficient: $r = -0.038$). In addition, the lower threshold of plasma DNA concentration critical for the appearance of apoptotic DNA was further defined to be between 39 and 146.5 ng/mL, derived from the lowest DNA concentration in the group of samples containing apoptotic DNA and the highest DNA concentration in the group of samples without apoptotic DNA, respectively (Table 1).

Discussion

Plasma cfDNA may increase during septicaemia to reach very high concentrations. The estimated cut-off value for predicting mortality among ICU patients is 2,350 ng/mL [4,18]. While the very high plasma cfDNA concentration may serve as an independent predictor of mortality among the ICU patients [2], the situation with other hospital patients is different; hospital non-survivors have lower plasma cfDNA values than the ICU non-survivors at admission and 72 h later [3]. This finding is possibly influenced by the fact that hospital patients are a more heterogeneous group with a variety of underlying co-morbidities and other risk factors. In a separate study of hospital bacteremia non-survivors, a distinct cut-off value of plasma cfDNA concentration of 1,520 ng/mL in the first four days following blood culture was identified as an independent risk factor for case fatality [5]. In addition, apoptotic DNA correlated with poor clinical outcomes in bacteraemic patients [5].

The current study of patients with suspected septicaemia compared the efficacy of DNA extraction and quantification methods. Based on our results, standard QIAamp Mini Blood DNA extraction procedure provided a more satisfactory method for isolating cfDNA from small volumes of blood than the Circulatory Nucleic Acid extraction kit. This result is consistent with the intended use of the Mini Blood DNA kit which is designed for small plasma volumes (up to 200 μ L), unlike

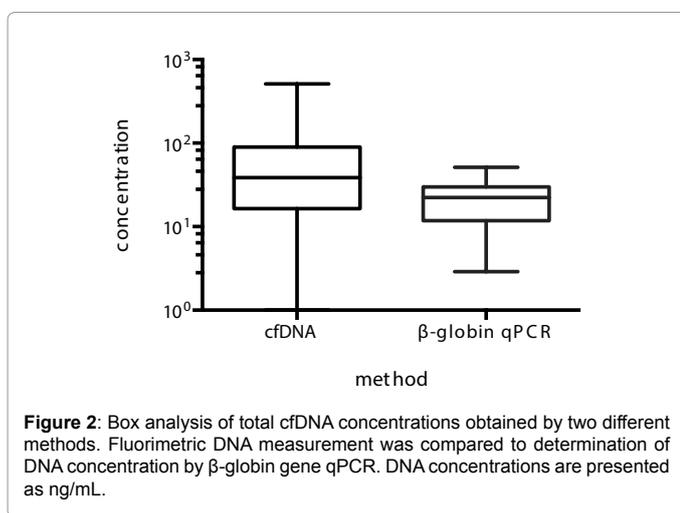


Figure 2: Box analysis of total cfDNA concentrations obtained by two different methods. Fluorimetric DNA measurement was compared to determination of DNA concentration by β -globin gene qPCR. DNA concentrations are presented as ng/mL.

Method	Units	n	Min	Max	Median	Mean	SD	SEM	L95%	U95%	P
Qubit	ng/mL	67	0	510	38.5	61.4	71.6	8.7	43.9	78.8	WRST* <0.0001
qPCR	ng/mL	67	2.9	51	22.3	22.2	11.5	1.4	19.4	25.0	
Qubit Apop +	ng/mL	76	39	1245	118	117	186	21	135	220	MWUT** <0.0001
Qubit Apop -	ng/mL	42	4	146	39	41	31	5	31	51	

WRST* - Wilcoxon matched-pairs signed rank test
MWUT** - Mann Whitney test

Table 1: Summary of quantitative cfDNA analyses data.

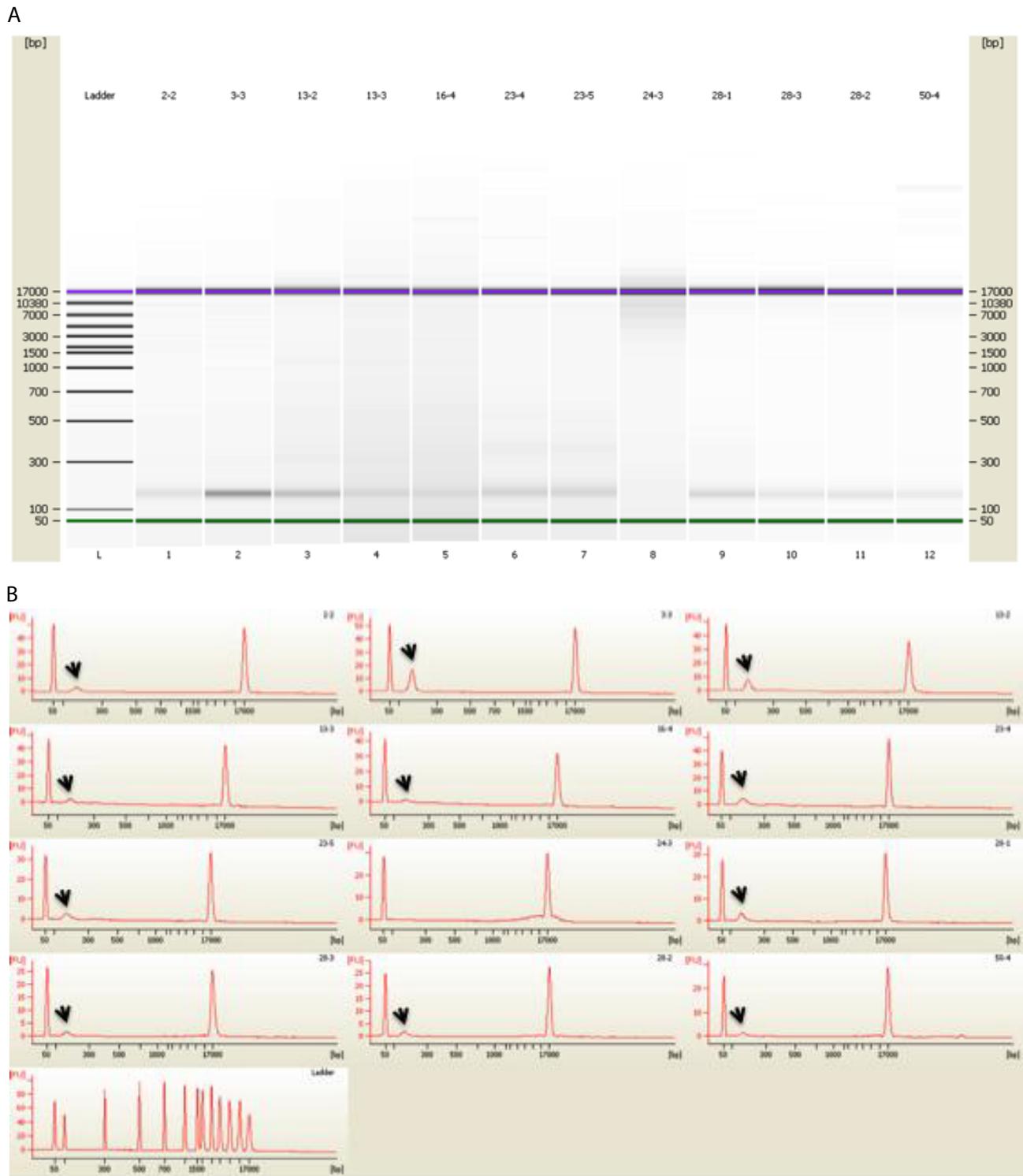


Figure 3: Microfluidic DNA gel electrophoresis of plasma cfDNA. **A** – Gel image of individual plasma samples. DNA ladder is shown on the left with fragment sizes in base pairs (bp). The arrow on the right indicates the position of apoptotic DNA. The low and high-weight DNA markers are presented by green and purple lines, respectively. **B** – Electropherograms of individual cfDNA samples and of DNA ladder. The arrows indicate apoptotic DNA peaks. The area below the peak is used to calculate the amount of apoptotic DNA in plasma.

the Circulatory Nucleic Acid Kit which is optimized for a larger 1 mL volume (Qiagen). In lower plasma volumes ranging up to 200 μ L, the two DNA extraction kits evaluated here were similarly effective.

In a previous study, QIAamp DNA extraction columns performed satisfactorily in conjunction with 7 different cfDNA isolation methods for 2mL pooled serum samples [17]. QIAamp Midi Blood DNA Kit

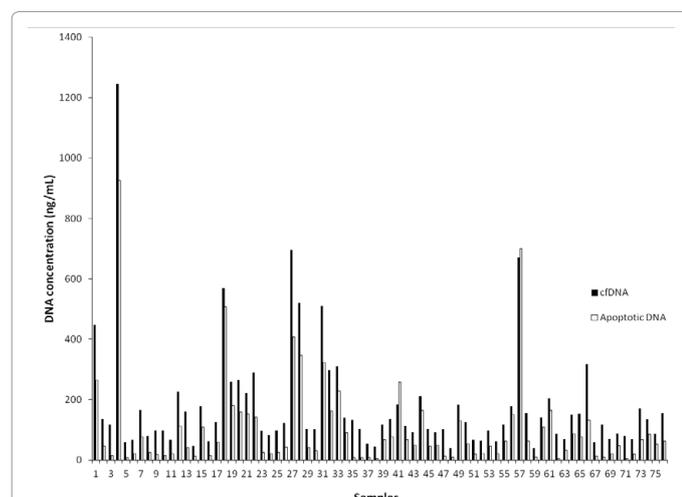


Figure 4: Contribution of apoptotic DNA to total plasma cfDNA. Parallel analysis of total cfDNA (black columns) and apoptotic DNA (white columns) in 76 plasma samples obtained from hospital patients is presented.

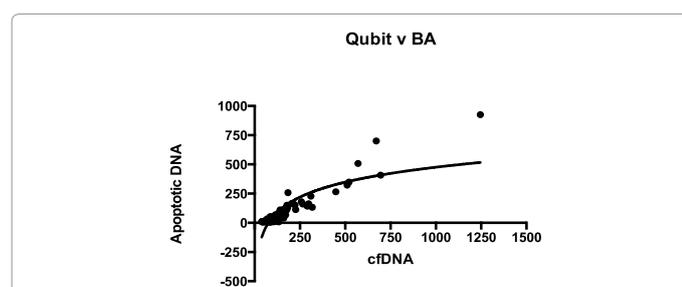


Figure 5: Non-logarithmic regression analysis. Apoptotic DNA concentrations were plotted against total plasma cfDNA concentrations on a non-logarithmic scale. A non-linear regression line cuts cfDNA axis below 100ng/mL determining probable lower threshold for apoptotic DNA generation.

outperformed four other commercial kits and was surpassed by two in-house methods, Sodium-iodide (NaI) and phenol-chloroform (PC). Although the phenol method was independently confirmed to be superior [13], its potential hazard remains an important obstacle to its extensive use in clinical laboratory settings. Consequently, QIAamp DNA extraction columns remain an important tool for plasma cfDNA extraction in clinical laboratory setting. However, the choice between Mini Blood DNA vs. Circulatory Nucleic Acid chemistry will remain optional. As reported here, Mini Blood DNA kit provides satisfactory results with suspected septicaemia, while Circulatory Nucleic Acid Kit may be better suited to study chronic non-infective diseases with much lower plasma cfDNA outputs (data not shown).

Against prediction, β -globin qPCR performed better with the AmpliTaq PCR mix than with PerfeCta despite the fact that the PerfeCta system was optimized to overcome known blood PCR inhibitors. While PerfeCta performed better with whole plasma, it was not observed with purified plasma cfDNA. An alternative approach to removal of PCR inhibitors by bisulfite treatment has been reported although no beneficial effect on qPCR results was found [17].

Fluorimetric dsDNA assay determines real DNA concentrations in ng/ μ L of physically present DNA molecules, while qPCR determines the copy numbers of β -globin gene and corresponding genome equivalents (GE) in cfDNA relative to serial dilutions of an external

DNA standard. So, by extrapolation qPCR values represent an estimate of genome DNA based on the β -globin gene amplification. The most practical findings from our study are an evaluation of the efficiency and sensitivity of cfDNA analysis by comparing quantification methods. Our approach compared cfDNA concentrations calculated from qPCR with the dsDNA concentrations obtained by direct measurement. The size of the PCR product used in our study was 55 bp. According to a previous report, this size should be satisfactory for accurate detection of gene copy numbers in fragmented cfDNA [19]. This attempt to design a calibrated duplex real-time PCR for unbiased detection of Y chromosome versus autosomal loci in a mixture of male/female DNAs gave optimal results with short PCR products of about 66-67bp, while larger or unequal PCR products resulted in underestimation of a particular target sequence. Despite calculation of genome equivalents from qPCR endpoints, the β -globin qPCR method underestimated the amount of cfDNA in the sample in comparison with the direct fluorimetric dsDNA assay. β -globin qPCR is therefore less suited to clinical applications in septicaemia, in which qPCR appears to underestimate very high cfDNA concentration, better demonstrated by fluorimetric measurement of dsDNA. Accordingly, direct fluorimetric method appears to be a better choice for measuring the full range of cfDNA concentrations in septicaemia. In contrast, qPCR is a more sensitive method for detection of low plasma cfDNA levels and is more applicable to single gene/locus detection such as in pre-natal, cancer and organ-transplantation applications that target particular chromosomal loci, oncogenes or SNPs.

Another key finding from our study was the measurement of nucleosome-sized apoptotic DNA. A previous study used a subjective, visually-assessed approach to estimate apoptotic DNA [5]. Here we calculated apoptotic cfDNA relative to the proprietary DNA standards and internal size markers. This approach determined the concentration of apoptotic DNA in each cfDNA sample and indicated a direct positive correlation between the total cfDNA and apoptotic DNA. The dominant type of DNA in samples with the highest cfDNA concentrations was apoptotic DNA, suggesting that apoptosis was the predominant contributor to the rise of cfDNA concentrations in these samples. The association between plasma cfDNA, other analytes and symptoms in these patients during disease progression remains to be determined.

In conclusion, silica-membrane-based DNA purification appears to be the method of choice for plasma cfDNA isolation combined with a direct fluorimetric assay for quantification of cfDNA in plasma from patients with suspected septicaemia. In addition, we demonstrated the use of a microfluidic DNA chip to detect and measure apoptotic DNA, confirming that apoptotic DNA was the major cfDNA component in patients with a high plasma cfDNA. We are currently applying the approach described here to study a possible association between plasma cfDNA and other clinical parameters in suspected septicaemia.

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