MOLECULAR DIAGNOSIS

The effect of human serum DNAases on the ability to detect antibiotic-killed *Escherichia coli* in blood by PCR

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PCR has proved superior to conventional blood culture for diagnosing bacteraemia in the presence of antibiotics. Nevertheless, even PCR might yield false-negative results if the template DNA were to be cleaved by serum DNAases after antibiotics had induced bacterial death. To evaluate the cleavage of bacterial template DNA by human serum DNAase I, serum samples inoculated with purified *Escherichia coli* DNA were incubated with increasing amounts of recombinant human DNAase (rhDNAase) and then examined by a PCR specific for *E. coli*. As a prerequisite of potential DNAase attack, the release of *E. coli* DNA after antibiotic-induced bacterial death was quantified by fluorescence microscopy and flow cytometry. Finally, the influence of rhDNAase on the PCR-based detection of antibiotic-killed *E. coli* in serum was assessed. The results indicated that purified *E. coli* DNA is remarkably stable in human serum; positive PCR results did not decrease significantly until the ratio of recombinant human DNAase I:*E. coli* rose to $10^6:1$. As only 14.8–28.4% of the total *E. coli* DNA was released after antibiotic killing, the PCR-based detection of *E. coli* fell by only 10% when cefotaxime-killed *E. coli* were incubated with rhDNAase. It was concluded that human serum DNAases and antibiotic killing do not compromise the reliability of PCR examinations for bacteraemia.

Introduction

Treatment of patients with broad-spectrum antibiotics increases the risk that bacteria present in the blood remain undetected because it may lead to false-negative blood cultures [1–3]. PCR technology has been shown to overcome some of the weaknesses of conventional culturing methods in such circumstances [2, 4, 5]. For instance, in a rat model of *Escherichia coli* bacteraemia, PCR was shown to yield a detection rate 80% higher than that of blood culture in cefotaxime-treated animals [2]. Nevertheless, it is still uncertain just how reliable PCR is and whether a negative result truly reflects the absence of bacteria in blood [4, 5], as mechanisms reducing the availability of the bacterial template DNA may cause false-negative PCR results. When bacteria are killed by antibiotics, DNA may be released and then degraded by human endogenous DNAases. However, it is not known to what extent

DNAases are active in human serum [6–8]. Furthermore, there are no data quantifying the release of DNA from bacteria after they have been killed by antibiotics. To clarify these points, the effect of human serum DNAase activity on the ability to detect purified *E. coli* DNA by PCR was investigated. Also, the release of *E. coli* DNA was assessed after incubation with antibiotics with different modes of action. Finally, this study tested whether DNAase compromises the detection of *E. coli* by PCR in serum when the bacteria have been killed by the antibiotic cefotaxime.

Materials and methods

E. coli and PCR

Unless otherwise indicated *E. coli* ATCC 11229 was cultured overnight in Tryptic Soy Broth (Sigma, Deisenhofen, Germany), centrifuged, washed three times in physiological saline and adjusted to a concentration of 10^8 cfu/ml, corresponding to an OD₆₀₀ of 0.12. The concentration was verified by direct plating of serial dilutions on blood agar and counting colonies after incubation for 24 h at 37°C.

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PCR of *E. coli* DNA was performed as described previously [2] with two pairs of nested primers derived from the *uidA* gene of *E. coli* encoding β -glucuronidase. The amplification product was visualised by gel electrophoresis and ethidium bromide staining.

Influence of human DNAases on the detection rate of purified E. coli DNA by PCR

Twenty samples of serum (0.1 ml) from a healthy individual were each inoculated with 30 pg of purified *E. coli* DNA (Amersham Pharmacia Biotech, Freiburg, Germany). Native human serum was prepared from venous blood as described for the measurement of DNAase I activity [9]. The reaction mixture was incubated for 2 h in a water bath at 37°C. DNAase activity in 20 control samples was inhibited by cooling on ice. To avoid loss of DNA the samples were heated for 10 min at 94°C and then centrifuged for 5 min at 14 000 rpm rather than being subjected to a separate DNA extraction procedure. A 10- μ l sample of each supernate was used for amplification of *E. coli* DNA by PCR. The minimum number of experiments needed to establish a difference of at least 30% between samples at 37°C and controls was calculated to be 20.

In a second set of experiments, the quantitative ratio of DNAase to DNA in the samples was varied by adding recombinant human DNAase (rhDNAase; Hoffmann-LaRoche, Grenzach-Wyhlen, Germany), which is considered to be equivalent to human serum DNAase I [7, 10]. Ten human serum samples were incubated with 30 pg of purified *E. coli* DNA and either 30 ng or 30 μ g of rhDNAase I (final volume 0.1 ml) and then processed as described above.

To assess a potential inhibition of rhDNAase activity by compounds present in human serum, including sodium, the experiments were repeated in a buffer solution providing optimal conditions for the enzymic activity of rhDNAase (50 mM Tris-HCl buffer, pH 7.5, supplemented with 10 mM MgCl₂ and 1 mM CaCl₂) [8]. Then 67 μ l of the Tris-HCl buffer, pH 7.5, were spiked with 30 pg of *E. coli* DNA and incubated with rhDNAase (3 pg, 30 pg, 300 pg, 3 ng, 300 ng) in a final volume of 0.1 ml for 2 h at 37°C. Ten μ l of the reaction mixture were used for amplification of *E. coli* DNA by PCR. It was determined that a minimum of 10 experiments would be necessary to establish a relevant difference of at least 80% between samples spiked with rhDNAase and controls.

Assessment of DNA release from E. coli after treatment with antibiotics

Samples of an *E. coli* suspension (1 ml) in physiological saline (10⁸ cfu/ml) were incubated with 100 μ l of cefotaxime, ciprofloxacin, imipenem or gentamicin (final antimicrobial concentrations: 5, 0.2, 5 and 4 mg/L, respectively) at 4°C for 2 h. Complete bacterial killing was verified after 15 min by plating

100 μ l on to blood agar and counting colonies after incubating the plates for 24 h at 37°C. Controls contained *E. coli* in 1100 μ l of physiological saline without antibiotics. After 2 h, 10 μ l of the *E. coli* suspension were heat-fixed on microscope slides and the DNA was stained with 25 μ l of the nucleic acid dye 4',6-diamidino-2-phenylindole (DAPI; Sigma) 2 μ l/ml for 5 min in the dark. Bacteria were embedded in Dako fluorescent mounting medium (Dako, Copenhagen, Denmark). For each antibiotic and the respective controls, 10 visual fields were randomly chosen and evaluated with an Axioplan microscope (Zeiss, Oberkochen, Germany) in both the fluorescence and phase-contrast modes for each visual field. Pictures were taken at a magnification of 1000 and digitalised with the KS 300 digital image processing system (Kontron Electronic GmbH, Echting, Germany). The bacterial count was performed by two independent observers; the number of bacteria obtained in the phase-contrast mode was taken as the total bacterial count and set at 100%. The percentage of bacteria that had released DNA was calculated as the difference between the total bacterial count and the number of bacteria which remained visible in the fluorescence mode.

In addition, flow cytometry was used to assess the release of DNA from *E. coli* after antibiotic treatment. *E. coli* grown overnight on tryptic soy agar plates was suspended to 10⁹ cfu/ml (OD₆₅₀ 0.75) in 10 ml of phosphate-buffered saline (PBS). DAPI (10 μ l of a 3 mM suspension in water) was added to 1-ml samples of the bacterial suspension to yield a final DAPI concentration of 30 μ M. The resulting suspension was mixed and incubated at room temperature for 60 min in the dark. Bacteria were centrifuged eight times (8000 rpm in a microfuge for 15 min at room temperature); after each centrifugation the bacteria were washed in PBS. The pellets of stained bacteria from the last centrifugation were suspended to a final volume of 1 ml in PBS containing cefotaxime 5 mg/L (for treated samples) or PBS alone (for untreated samples). These samples were incubated at 4°C. After 30 min, 10- μ l samples were plated on blood agar and incubated overnight at 37°C to monitor bacterial killing. Control samples contained bacteria not stained with DAPI and either with or without exposure to cefotaxime. After another 90 min (a total of 2 h after exposure to cefotaxime), bacterial fluorescence was measured by flow cytometry (Coulter Elite ESP flow cytometer) with a UV-enhanced argon laser for excitation and a 405-nm bandpass filter set for emission. Approximately 5000 events were recorded for each sample. The percentage of bacteria with fluorescence lower than the mean fluorescence of the untreated controls was used as a measure of DNA loss.

Influence of human DNAases on the detection of cefotaxime-killed E. coli by PCR

E. coli were incubated with 20 μ g of cefotaxime for 15 min at 37°C in 0.9 ml of Tris-HCl buffer or human

serum (final volume 1 ml; final bacterial concentration 10 cfu/ml). Then 20 buffer and 30 serum samples were each incubated with either 50 ng or 50 μ g of rhDNAase (final volume 1.5 ml) for 2 h at 37°C, resulting in ratios of rhDNAase to total *E. coli* DNA content of 10^6 :1 and 10^9 :1. As controls, 40 buffer and 60 serum samples were incubated with 0.5 ml of buffer instead of rhDNAase. After incubation, *E. coli* DNA was extracted with the Purgene DNA isolation kit for gram-negative bacteria (Biozym Diagnostik GmbH, Hessisch Oldendorf, Germany) according to the manufacturer's protocol; the total DNA was used for PCR. It was calculated that the numbers of experiments necessary to determine a 30% decrease of positive PCR results in buffer and serum were 20 and 30, respectively.

Statistical analysis

In all experiments, Fisher's exact test was applied for statistical analysis with the SAS software system, version 6.12 (Cary, USA). The level of significance was set to 0.05 [11].

Results

Influence of human DNAases on the detection rate of purified *E. coli* DNA by PCR

When purified *E. coli* DNA was incubated in human serum for 2 h at 37°C and then put into the nested PCR, 18 of 20 reactions were positive. In the control experiment in which DNAase activity was inhibited by holding at 4°C, 18 of 20 reactions were positive, indicating that endogenous DNAase activity does not degrade the *E. coli* template DNA to a degree sufficient to impair the PCR reaction. To determine the threshold at which the detection rate drops noticeably, increasing amounts of rhDNAase were added to serum spiked with 30 pg of purified bacterial DNA. The *E. coli* DNA was detected by PCR in all 10 samples tested when the ratio of DNAase to *E. coli* DNA was 10^3 :1; detection was impaired significantly (2 of 10 samples positive) at a weight ratio of rhDNAase I:DNA of 10^6 :1 (Fig. 1a). When the experiments were performed in Tris-HCl buffer the threshold value for significant PCR impairment was reached at a rhDNAase:DNA ratio of 10^4 :1 (Fig. 1b).

DNA release from *E. coli* after antibiotic treatment

Killing *E. coli* with cefotaxime, ciprofloxacin, imipenem and gentamicin caused a loss of DAPI-positive *E. coli* cells in the range 15–28% (Fig. 2, Table 1). Based on a mean DNA content of 5 fg/*E. coli* cell [12], the observed loss of DAPI-positive *E. coli* corresponds to an antibiotic-induced loss of DNA between 14.2 fg and 7.4 fg per sign 10 micro-organisms. By flow cytometry, the mean fluorescence of DAPI-stained organisms decreased by 16% after treatment with cefotaxime for

Fig. 1. Detection of purified *E. coli* DNA by PCR in human serum and buffer and influence of recombinant human DNAase. Human serum (a) or buffer (b) was inoculated with 30 pg of *E. coli* DNA and incubated with increasing amounts of rhDNAase (■) or buffer for control (□) for 2 h at 37°C (final volume: 1.1 ml), resulting in weight proportions of rhDNAase I:*E. coli* DNA as indicated on the horizontal axis. Thereafter, 10- μ l samples were used for amplification of a 486-bp fragment of the *uidA* gene of *E. coli* by PCR. * $p < 0.05$.

2 h (Fig. 3). Unstained *E. coli* with and without cefotaxime treatment showed no appreciable autofluorescence (data not shown). These results show that although bactericidal concentrations of antibiotics had been used, which led to complete cell death within 15 min, a remarkable amount of DNA was still present within the cells after 2 h.

Influence of human DNAases on the detection of cefotaxime-killed *E. coli* by PCR

When cefotaxime-killed *E. coli* were incubated with large amounts of rhDNAase in serum and the samples were then examined by PCR, the *E. coli* detection rate did not drop significantly. At a calculated weight ratio of rhDNAase to total *E. coli* DNA content of 10^6 :1, *E. coli* DNA was detected in 17 of 30 samples by PCR. In controls without the addition of rhDNAase, the presence of *E. coli* DNA was detected in 17 of 30 serum samples. At a rhDNAase:DNA ratio of 10^9 :1, 20 (66%) of 30 PCR results were positive; in the controls, 23 (76%) of 30 reactions were positive. Even when the reaction was performed in buffer in which rhDNAase activity was $>10^2$ times higher than in serum, no significant differences were seen between the PCR detection rates of rhDNAase-treated and untreated

Fig. 2. Release of DNA from *E. coli* after antibiotic treatment. *E. coli* were incubated with buffer (A, B) or bactericidal concentrations of cefotaxime (C, D) or imipenem (E, F) at 4°C for 2 h and 15 min, then stained with DAPI and analysed by fluorescence (A, C, E) and phase-contrast (B, D, F) microscopy. Magnification $\times 1000$; bar 5 μm . Arrows mark bacteria visible in phase-contrast and not visible by fluorescence.

Table 1. DNA release from *E. coli* after antibiotic treatment *in vitro*

Treatment*	Bacterial numbers [†]			Calculated amount of lost DNA [‡] (fg/10 <i>E. coli</i>)
	Phase-contrast	Fluorescence	Difference (%)	
None	44.7 (30.7)	42.3 (29.2)	4.7 (4.1)	2.3
Cefotaxime	38.9 (11.2)	28.0 (14.6)	28.4 (9.1)	14.2
Ciprofloxacin	58.3 (33.1)	46.8 (29.8)	20.4 (7.1)	10.2
Imipenem	42.6 (28.2)	35.1 (22.1)	17.6 (3.3)	8.8
Gentamicin	36.4 (19.1)	30.8 (16.0)	14.8 (9.5)	7.4

**E. coli* were incubated with buffer, or bactericidal concentrations of cefotaxime, ciprofloxacin, imipenem or gentamicin at 4°C for 2 h and 15 min, then stained with DAPI.

[†]The numbers of bacterial cells were analysed by fluorescence and phase-contrast microscopy in 10 randomly chosen identical visual fields. The number of bacteria in the phase contrast was set at 100%. The percentage of bacteria that had lost DNA was quantified by counting DAPI-stained bacteria in the fluorescence mode and comparison with the bacterial number in the phase contrast mode. Values represent means (SD).

[‡]The loss of DNA from 10 *E. coli* cells was calculated from the difference between DAPI-stained cells and cells visible by phase-contrast microscopy based on a mean DNA content of 5 fg/cell.

controls. At a calculated ratio of rhDNAase: *E. coli* DNA of 10^6 :1, PCR demonstrated the presence of *E. coli* in 17 (85%) of 20 samples, which did not differ from the control experiments. At a rhDNAase:*E. coli* DNA ratio of 10^9 :1 the PCR detection rate for *E. coli* was 16 (80%) of 20; in controls it was 18 (90%) of 20. This observation suggests that the DNA content of *E. coli* is protected from DNAase attack even after the bacteria have been killed by antibiotics.

Discussion

The ability to detect bacteria independently of

propagation makes the PCR technique attractive for diagnosing bacteraemia during antibiotic treatment. The results of the present study further confirm the reliability of the PCR method for this purpose. This reliability can be explained by the findings that antibiotic-killed *E. coli* do not release a large proportion of their DNA into the bloodstream and that the released bacterial DNA is not readily degraded by blood DNAases.

Surprisingly little is known about the release of bacterial DNA after antibiotic treatment. Gentamicin treatment of *E. coli* was reported to cause leakage of most of the DNA, but exact data were not given [13].

Fig. 3. Release of DNA from *E. coli* after cefotaxime treatment measured by flow cytometry. DAPI-stained *E. coli* were incubated with bactericidal concentrations of cefotaxime (–) or with PBS alone (–) at 4°C for 2 h and analysed by flow cytometry. Unstained *E. coli* (with or without cefotaxime) had minimal fluorescence (data not shown).

Legionella pneumophila released 50% of its radiolabelled DNA 3 h after treatment with penicillin G [14]. In the present study, it was observed by phase-contrast and fluorescence microscopy that 15–30% of the *E. coli* DNA was released after treatment with cefotaxime, ciprofloxacin, imipenem or gentamicin. Similarly, flow cytometry showed that the mean amount of DAPI-stained DNA decreased by 16% after treatment with cefotaxime. This moderate difference might be explained by the different methods used to assess the DNA release and by different modes of action of the antibiotics used.

Human serum DNAases are known to digest double-stranded DNA down to tri- or tetra-oligodeoxynucleotides *in vitro* [8, 15] and to digest therapeutically applied synthetic oligodeoxynucleotides in mammalian serum [16, 17]. In contrast, in the present study it was observed that cleavage of purified *E. coli* DNA to a degree sufficient to influence the PCR results occurred only at high weight ratios of DNAase to DNA. This contradiction might be explained by three facts. Firstly, because of the high sensitivity of the PCR method even minimal residues of uncleaved DNA yield positive results. Secondly, it has been suggested that the sodium concentration in human serum might decrease human DNAase activity [7, 18]. Finally, a protective effect of histone-like proteins against degradation by DNAases has been described in genomic *E. coli* DNA sequences [19], whereas synthetic oligodeoxynucleotides lack these protective compounds.

The observation that even large amounts of rhDNAase reduced the PCR-based detection of 10 *E. coli* per ml of serum by 10% at most suggests that the detection of higher bacterial concentrations would not be compromised by either antibiotic treatment or plasma DNAases, as the amount of DNA still available for amplification far exceeds the sensitivity limit. Thus, in bacteraemia of children – where bacterial concentrations of 10–100 micro-organisms/ml of blood are reported [20] – the use of the PCR technique might be particularly promising for achieving a diagnosis during antibiotic treatment.

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