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Influence of antibiotic treatment on the detection of *S. aureus* in whole blood following pathogen enrichment

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Abstract

Background: Early pathogen detection and identification are crucial for an effective and targeted antibiotic therapy in patients suffering from blood stream infection. Molecular diagnostic methods can accelerate pathogen identification as compared to blood culture, but frequently suffer from the inhibition of polymerase chain reaction (PCR) by sample matrix components, such as host DNA, anticoagulants, or plasma proteins. To overcome this limitation, molecular diagnostic methods commonly rely on pathogen enrichment by selective lysis of blood cells and pelleting of intact pathogens prior to analysis.

Results: Here, we investigated the impact of antibiotic treatment on the recovery of pathogen DNA using an established pathogen enrichment protocol. Based on the hypothesis that induction of bacterial cell wall disintegration following antibiotic administration leads to incomplete pelleting of pathogen DNA, *S. aureus* was grown in human whole blood with or without addition of cell wall active (vancomycin, piperacillin) or non cell wall active (ciprofloxacin, clindamycin) antibiotics at clinically relevant concentrations. Pathogen detection remained unaffected by non cell wall active antibiotics or even increased in the presence of cell wall active antibiotics, indicating improved accessibility of pathogen DNA. Likewise, mechanical lysis of *S. aureus* prior to pathogen enrichment resulted in increased recovery of pathogen DNA. Quantification of pathogen and human DNA after selective lysis of blood cells and pathogen enrichment confirmed partial depletion of human DNA, leading to a net enrichment of pathogen DNA over human DNA.

Conclusion: Concurrent antibiotic administration does not reduce the recovery of pathogen DNA during pathogen enrichment by selective lysis and centrifugation.

Leads to a 10-fold human DNA depletion as compared to pathogen DNA. Moreover, we confirm that the recovery of pathogen DNA after pathogen enrichment is not negatively influenced by concurrent antibiotic administration.

Keywords: Molecular diagnostics, Pathogen detection, Blood stream infection, DNA extraction, Selective lysis, Antibiotics

Background

S. aureus is an opportunistic pathogen with the potential to cause community-associated and nosocomial infections [1]. It is the most common gram-positive pathogen associated with sepsis, with a prevalence of up to 20% of

all blood culture positive sepsis cases [2–4]. This high prevalence results from its ability to adapt to its environment by modulating the host immune response [5], to switch from a highly proliferative disseminative state into a slow growing, biofilm producing state [6], to grow intracellularly [7, 8], and to acquire antibiotic resistance [9].

Clinical guidelines for the management of sepsis [10] recommend immediate antibiotic therapy as well as pathogen identification, since early and targeted antibiotic treatment can significantly improve the survival of sepsis patients [11–14]. Blood culture is the current reference method for the identification of pathogens and

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for the characterization of their antibiotic susceptibility. It may, however, fail to detect slow-growing or intracellular pathogens and yield inconclusive results due to concurrent antibiotic treatment. Moreover, definitive results of culture are usually not available before 48 h [11, 13, 15]. Molecular diagnostic methods provide a time-to-result of 4–7 h, compatibility with antibiotic treatment, and do not depend on pre-selecting culture steps [13, 16, 17]. Still, the direct detection of pathogen DNA in blood samples (e.g. SeptiFast, Roche, Basel, Switzerland) is prone to interference of matrix components, such as host DNA [18, 19], heparin [20, 21], or plasma proteins [22–24]. To avoid inhibition of PCR and to increase the sensitivity of pathogen detection, virtually all current molecular diagnostic systems, including FAST ID BSI (QVella, Richmond Hill, Canada), Hybcell Pathogen Array (CubeDX, St. Valentin, Austria), T2 Bacteria (T2 Biosystems, Lexington, MA), SepsiTtest (Molzylm, Bremen, Germany), as well as Magicplex Sepsis Test (Seegene, Seoul, Korea), rely on pre-analytical pathogen enrichment [25]. Common to all pathogen enrichment protocols is the selective lysis of blood cells by addition of a hypotonic detergent solution. Pathogens withstand the osmotic pressure and are enriched by subsequent centrifugation prior to DNA quantification. We hypothesized that antibiotic treatment, by affecting bacterial cell wall integrity, might lead to incomplete pelleting of pathogens after selective lysis of the blood cells and centrifugation, and, consequently, result in a loss of pathogen DNA prior to analysis. Therefore, we investigated the impact of both, cell wall active and non cell wall active antibiotics on the pre-analytical enrichment of DNA of different *S. aureus* strains from human whole blood.

Methods

Bacteria and reagents

Antibiotics (Table 1), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, pH 7.0, cell culture grade) and adenine were purchased from Sigma-Aldrich (St. Louis, MO). D-Glucose was obtained from Merck (Darmstadt, Germany). *S. aureus* culture strains (ATCC 12600, 29213, and 29737) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Wild-type strains (WT32217, 32237, and 32248) were isolated from patient material at the Division of

Hygiene and Medical Microbiology, Medical University of Innsbruck, Austria. Strains were cultivated on lysogeny broth (LB, Lennox formulation) agar plates (Carl Roth, Karlsruhe, Germany) at 37 °C. Overnight cultures were obtained by inoculating single colonies into LB medium. To determine the minimal inhibitory concentration for individual antibiotics used in this study, overnight cultures were diluted 1:5,000 in LB medium in 96-well polystyrene microwell plates (CELLSTAR®, Greiner Bio-One GmbH, Frickenhausen, Germany), serial dilutions of antibiotics were added, and incubation was performed for 24 h at 37 °C.

Human whole blood

Venous human whole blood was collected from healthy adult volunteers into tubes (Vacuette, Greiner Bio-One, Kremsmuenster, Austria) containing sodium heparin or EDTA. Blood collection was approved by the Ethical Review Board of Danube University Krems, and written informed consent was obtained from all donors.

Cultivation of *S. aureus* in human whole blood

Freshly drawn human whole blood anticoagulated with heparin was buffered with 1/50 volume of 1 M HEPES and supplemented with 2 mg/L glucose and 48 µg/L adenine per hour. Overnight cultures of each strain were diluted 1:1000 in LB medium, spiked into supplemented whole blood (typically 4 mL) at a ratio of 1:2560 (ATCC 12600), 1:205 (ATCC 29213), 1:1700 (ATCC 29737), 1:730 (WT32217), 1:1700 (WT32237), and 1:128 (WT32248), and incubated at 37 °C with gentle shaking. These spiking ratios were chosen based on growth curves for the individual strains to obtain pathogen concentrations of approximately 5000 colony forming units (CFU) per mL after 4 h. For each strain, growth was assessed in whole blood from six donors ($n = 3$ per donor) for up to 8 h.

Antibiotic pretreatment of *S. aureus*

To investigate whether weakening or disruption of bacterial cell walls by antibiotic pretreatment would influence subsequent pathogen enrichment and, consequently, PCR-based quantification of pathogen DNA, *S. aureus* was spiked into whole blood and incubated for 4 h as described above to ensure logarithmic growth. Subsequently,

Table 1 Antibiotics used in this study and their mechanism of action

Antibiotic	Cell Wall Active	Mechanism of Action
Vancomycin (VAN)	+	interferes with cell wall synthesis by preventing formation and crosslinking of peptidoglycan strands [26–28]
Piperacillin (PIP)	+	interferes with cell wall synthesis by binding to enzymes required for the extracytoplasmatic stage of cell wall formation [28, 29]
Ciprofloxacin (CIP)	–	prevents replication of bacterial DNA by inhibiting DNA gyrase [30, 31]
Clindamycin (CLI)	–	inhibits bacterial protein synthesis by binding to 50S ribosomal subunits [32]

final concentrations of 15 µg/mL vancomycin (VAN), 20 µg/mL piperacillin (PIP), 1 µg/mL ciprofloxacin (CIP), or 2 µg/mL clindamycin (CLI), respectively (Table 1), were added, and incubation was continued for another 90 min at 37 °C with gentle agitation. Subsequent pathogen enrichment and DNA extraction were performed as described below. Spiked blood without antibiotic treatment served as control.

Strain ATCC 29213 was additionally grown in the presence of CIP and VAN for up to 72 h to investigate potential differences between blood culture based and qPCR based pathogen detection.

Mechanical lysis of *S. aureus*

To achieve complete pathogen disintegration, fresh *S. aureus* overnight cultures (ATCC 29213) were diluted 1:5000 in LB medium, incubated for 2 h at 37 °C, mixed with an equal volume of 0.1 mm zirconium beads (Biozym, Hessisch Oldendorf, Germany) in a 0.3 mL PCR tube (Bio-Rad, Hercules, CA), and vortexed using a regular benchtop vortex (VortexGenie2, Carl Roth) at maximum speed for up to 120 min. Samples drawn after 30 s, 5 min, and 120 min were characterized by scanning electron microscopy, and bacterial lysis was confirmed by quantification of viable bacteria (CFU) as described below. Suspensions of lysed bacteria obtained at the indicated time points were spiked into freshly drawn whole blood anticoagulated with EDTA at a ratio of 1:100, and pathogen enrichment as well as isolation and quantification of pathogen DNA were performed as described below.

Quantification of viable bacteria

To quantify viable bacteria, spiked blood samples were diluted 1:5 in 0.9% NaCl (Fresenius Kabi, Bad Homburg, Germany), and 100 µL of the dilutions were plated onto LB agar (Carl Roth, $n = 3$). Plates were incubated overnight at 37 °C, and colonies were counted manually on the next day.

Pathogen enrichment and extraction of pathogen DNA

Pathogen DNA was isolated from spiked blood samples using a commercial pathogen enrichment system (GINA Pathogen Enrichment, CubeDX, St. Valentin, Austria) according to the instructions of the manufacturer. The enrichment protocol relies on the selective lysis of blood cells by detergent buffer, followed by centrifugation to pellet intact pathogens, lysis of pelleted pathogens by heating in alkaline buffer, and extraction of pathogen DNA using MiniSpin columns. For comparison, total DNA was extracted without previous pathogen enrichment using a total DNA extraction kit (MagMAX DNA Multi-Sample Kit, Thermo Fisher Scientific, Waltham, MA) according to the protocol of the manufacturer.

Quantification of DNA

qPCR was performed using *Staphylococcus* spp. specific 16S rRNA gene primers and primers specific for the beta-actin gene as control for human DNA. All primers are specified in Additional file 1: Table S1. Samples were mixed with an equal volume of SsoAdvanced SYBR Green Master Mix (Bio-Rad, Hercules, CA) containing 0.5 µM of each primer to yield a total reaction volume of 20 µL. qPCR was performed using a Roche LightCycler 96 (Basel, Switzerland) and comprised a 2 min preheating step at 94 °C, followed by 45 cycles of denaturation at 94 °C for 5 s, annealing at 53 °C for 10 s, and extension at 72 °C for 15 s. Correct amplification was verified by melting curve analysis. cT values were calculated using the Roche LightCycler96 Software version 1.1.0.1320.

Scanning electron microscopy

Sample preparation for scanning electron microscopy included filtration of bacterial suspensions through 0.22 µm polycarbonate isopore membranes (Merck Millipore, Darmstadt, Germany), followed by fixation with 2.5% glutaraldehyde (Carl-Roth) in 0.9% NaCl for 2 h at room temperature and dehydration in a graded alcohol series (30–100%). Samples were sputtered with gold using a Q150R (Quorum Technologies Ltd., Laughton, UK) at 30 kV in DC mode for 60 s, and images were acquired using a FlexSEM 1000 scanning electron microscope (Hitachi, Mannheim, Germany) at an accelerated voltage of 20 kV.

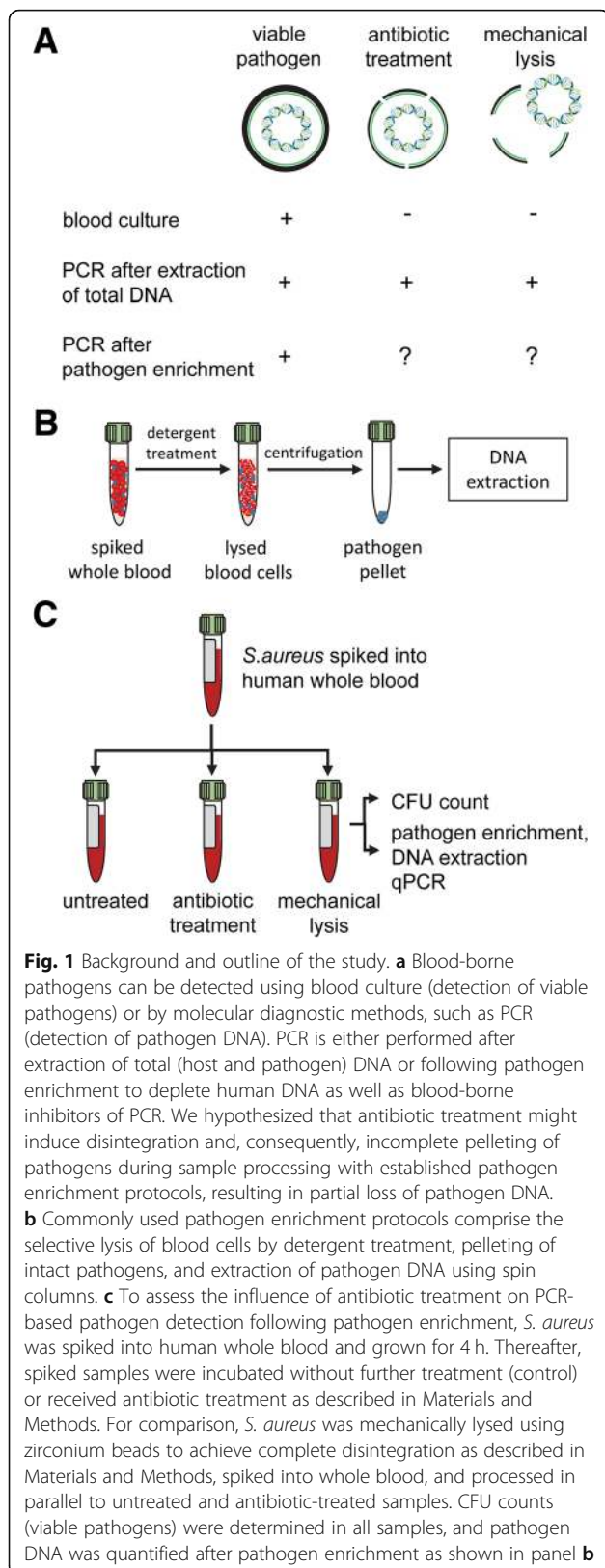
Statistical analysis

Statistical analysis was performed using SigmaPlot 13.0 (Systat Software, Erkrath, Germany) and graphs were plotted using GraphPad Prism version 7.02 (La Jolla, CA). At least three replicates were performed for each experiment. Data are presented as mean \pm standard deviation or as mean and 95% confidence intervals (CI). The significance of differences was calculated using paired t-test for comparison of a sample to a reference value, or, in case of multiple time points, by Friedman repeated measures analysis of variance on ranks, followed by multiple comparisons versus reference using Dunnett's Method. P -values < 0.05 were considered as statistically significant.

Results

Growth of *S. aureus* in human whole blood and antibiotic susceptibility

The experimental setup of this study is outlined in Fig. 1. All *S. aureus* strains exhibited logarithmic growth in human whole blood supplemented with glucose and adenine after a lag phase of 2–4 h (Fig. 2). All strains were susceptible to VAN, PIP, CIP and CLI. Minimal



inhibitory concentrations for individual antibiotics are listed in Additional file 1: Table S2.

Influence of antibiotic treatment on the recovery of pathogen DNA

To assess whether the presence of antibiotics would influence the recovery of *S. aureus* during pathogen enrichment, spiked whole blood was treated with either cell wall active (VAN, PIP) or non cell wall active (CIP, CLI) antibiotics (Table 1). cT values remained constant for CIP and CLI (ΔcT -0.5 ± 0.9 and 0.4 ± 0.9 as compared to the reference taken prior to the addition of antibiotics), or decreased significantly for the cell wall active antibiotics PIP and VAN (ΔcT -2.9 ± 1.4 and -2.4 ± 1.3) (Fig. 3a), suggesting enhanced accessibility of pathogen DNA in the presence of cell wall active antibiotics. VAN, PIP, and CIP, which exhibit bactericidal activity [33–35], induced a reduction of CFU counts as compared to the reference, while CLI exerted bacteriostatic effects with stable CFU counts (Fig. 3b). No significant differences were detected between individual *S. aureus* strains (data not shown). Upon incubation with VAN and CIP for up to 72 h, pathogen DNA remained constant in the presence of VAN or even increased in the presence of CIP, providing further evidence that antibiotic treatment does not induce a loss of pathogen DNA during pathogen enrichment (Fig. 3c). CFU counts decreased continuously over time, and no viable bacteria were detected beyond 48 h (Fig. 3d). Scanning electron microscopy did not provide evidence for cell wall degradation or reduced density of culture (Fig. 3e).

Influence of mechanical lysis on the recovery of pathogen DNA

Next, we performed mechanical lysis of *S. aureus* and spiked the lysates into whole blood to assess the impact of pathogen disintegration on the recovery of pathogen DNA during subsequent pathogen enrichment. Culture as well as scanning electron microscopy confirmed the efficient lysis of *S. aureus* (Fig. 4a). The recovery of pathogen DNA increased with progressing pathogen disintegration, confirming the efficient enrichment of pathogen DNA despite the absence of intact bacteria (Fig. 4b).

Selectivity of DNA isolation: pathogen versus host DNA

Based on the enhanced recovery of pathogen DNA following pathogen disintegration by mechanical lysis, we went on to assess the ability of the pathogen enrichment protocol to selectively isolate *S. aureus* DNA, i.e. to deplete human DNA. *S. aureus* DNA and human DNA were quantified following DNA isolation from spiked whole blood using (i) pathogen enrichment (protocol A) or (ii) total DNA extraction without previous pathogen enrichment (protocol B). qPCR (Table 2)

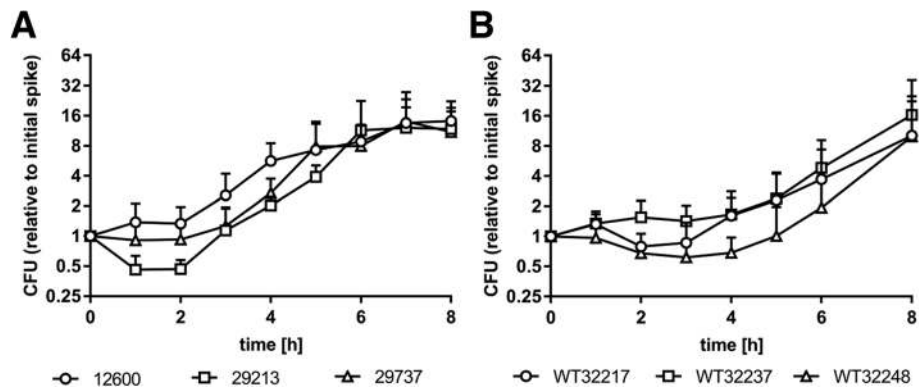


Fig. 2 Growth of *S. aureus* in supplemented human whole blood. **a** *S. aureus* strains obtained from ATCC and **b** wild-type strains isolated from patient material reached exponential growth (log phase) after 2–4 h of incubation in whole blood supplemented with glucose and adenine as described in Materials and Methods. CFU counts are indicated relative to the initial spiking concentration of 10^3 – 10^4 CFU/mL ($n = 6$)

revealed the presence of residual human DNA in samples isolated with the pathogen enrichment protocol, however, samples obtained by total DNA extraction contained significantly higher amounts of human DNA ($\Delta\text{cT } 6.1 \pm 0.3$). Pathogen DNA was nearly equally well enriched with both protocols ($\Delta\text{cT } 2.1 \pm 0.4$), resulting in an approximately 16-fold enrichment of pathogen DNA over human DNA using the pathogen enrichment protocol.

Discussion

Early adequate antimicrobial therapy is crucial in the management of blood stream infection, where broad-spectrum antibiotics are administered upon the first suspicion of infection [10], followed by targeted antimicrobial treatment after identification of the causative pathogens by blood culture or molecular diagnostic methods [36, 37]. While there is ample evidence that antimicrobial treatment can interfere with blood culture [38, 39], the impact of antibiotic therapy on molecular diagnostic pathogen detection remains incompletely defined. The only prospective study published to date in this context revealed a significantly higher rate of positive results with SeptiFast multiplex PCR as compared to blood culture in children under concurrent antimicrobial therapy [40].

Since PCR-based pathogen detection in whole blood is, however, susceptible to inhibition by blood-borne factors and host DNA, pathogens are commonly enriched from whole blood prior to analysis. Established enrichment protocols rely on the selective lysis of blood cells in hypotonic detergent and subsequent pelleting of intact pathogens, followed by extraction of pathogen DNA and PCR. We hypothesized that antimicrobial therapy, in particular administration of cell wall active antibiotics, might result in a release of DNA from damaged bacteria and, consequently, in decreased DNA recovery during

pathogen enrichment. To test this hypothesis, we chose *S. aureus* as model pathogen, since it is a leading cause of blood stream infection worldwide.

After an adaptation phase of several hours, both, *S. aureus* culture and wild-type strains reached stable growth in supplemented heparinized whole blood under our experimental conditions, while earlier studies with MRSA had observed rapid uptake of spiked *S. aureus* by neutrophils [7], with constant or decreasing CFU counts over time. The use of a methicillin-susceptible strain in our study could explain these divergent findings, since carriage of antibiotic resistance genes can reduce bacterial growth rates [41, 42]. Furthermore, we performed experiments for up to 72 h, after 4 h of adaptation of *S. aureus* to its environment. This required buffering as well as supplementation of whole blood with glucose and adenine, as commonly used for the storage of blood products [43]. Since wild type strains required a lag phase of 4 h for adaptation, we chose to incubate all strains for 4 h in supplemented whole blood prior to antibiotic administration, and we adjusted initial spiking concentrations to achieve a density of about 5000 CFU/mL after this adaptation phase. While this bacterial load was clearly higher than in septic patients, where pathogen loads of typically 1–10 CFU/mL have been reported [13], it allowed for the quantification of *S. aureus* using both, microbiological cultivation and qPCR and enabled the reliable determination of changes in qPCR signal intensity, which would not have been feasible at lower CFU counts.

To assess the influence of concurrent antibiotic treatment on pathogen enrichment, we used PIP and VAN according to clinical guidelines for the treatment of *S. aureus* infections [44, 45]. For comparison, we chose CIP, which is widely prescribed by general practitioners for the treatment of pneumonia, suspected gastrointestinal infections, or infections of the genitourinary tract

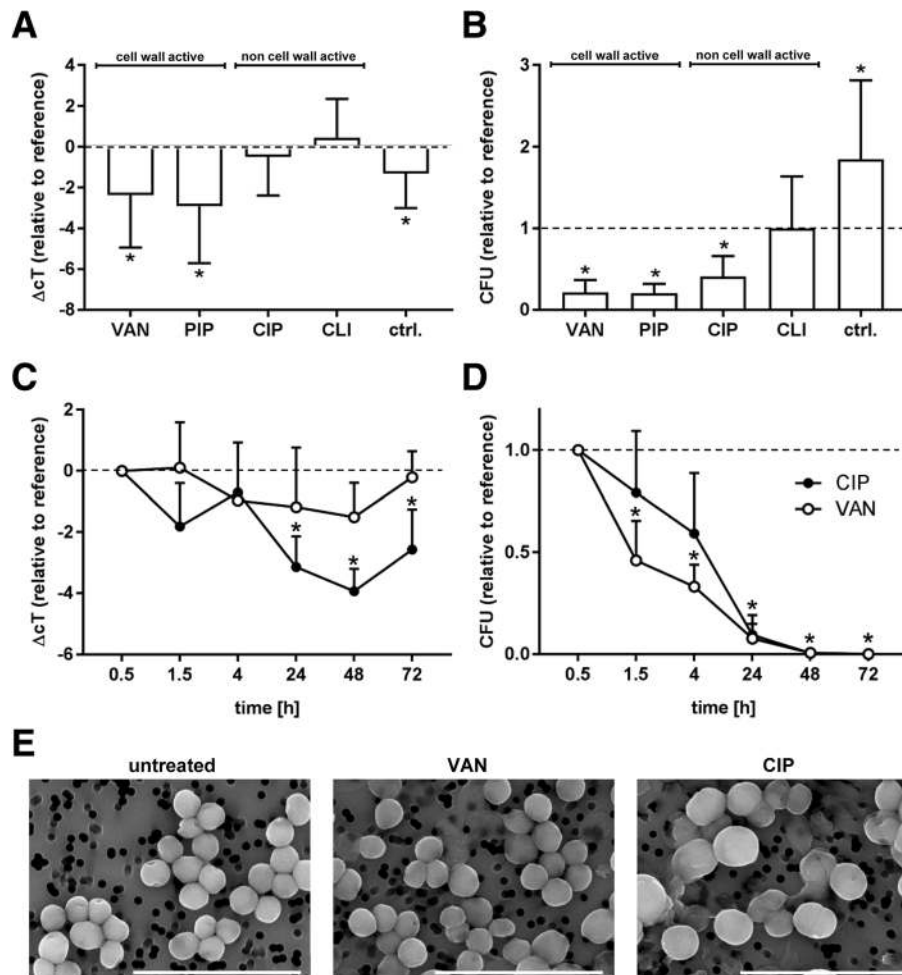


Fig. 3 Influence of antibiotic treatment on the recovery of pathogen DNA following pathogen enrichment. **a-b** *S. aureus* was grown in human whole blood, followed by a 90 min incubation in the presence of antibiotics, and CFU counts as well as pathogen DNA were quantified as described in Materials and Methods. Spiked whole blood without adsorbent treatment served as control (ctrl). ΔcT values and CFU counts are given relative to the reference (sample taken prior to the addition of antibiotics). Recovery of pathogen DNA was not influenced by CIP and CLI, while DNA recovery was increased after treatment with the cell wall active antibiotics VAN and PIP. CFU counts were significantly reduced for PIP, VAN, and CIP (bactericidal), or remained constant for CLI (bacteriostatic), confirming efficacy of antibiotic treatment. Data are presented as mean \pm standard deviation ($n = 18$; paired t -test). **c-d** Upon incubation of spiked blood samples with VAN and CIP over 72 h, the recovery of pathogen DNA remained stable (VAN) or even increased (CIP), while no viable bacteria were detectable beyond 48 h ($n = 4$; Friedman repeated measures and Dunnett's Method). **e** Scanning electron micrographs of *S. aureus* incubated for 24 h in the presence of VAN and CIP failed to provide evidence for cell wall degradation or reduced culture density, as compared to the untreated control. Scale bar, 5 μ m. Data are given as mean \pm standard deviation

[46, 47], as well as CLI, which is commonly used for the treatment of skin and soft tissue infections in outpatient care [48]. All antibiotics were applied to achieve concentrations equivalent to mean plasma levels reported in the literature [26, 27, 29–32]. We used an established manual pathogen enrichment kit, since it allows for intervention at multiple steps of the pre-analytical protocol, while protocols provided by other suppliers are fully integrated systems, which process blood samples automatically and do not allow for pathogen quantification at individual stages of the pre-analytical protocol. Still, our findings are likely to be valid for most other current pathogen enrichment protocols, as these systems differ

only insignificantly regarding the composition of lysis reagents.

Regardless of the antibiotic used, *S. aureus* DNA was not lost during the pre-analytical pathogen enrichment and was still detectable after 3 days of incubation in supplemented whole blood. Scanning electron microscopy revealed that *S. aureus* stayed largely intact over the course of the experiment despite antibiotic treatment, suggesting that pathogen DNA remained encapsulated by the bacterial cell wall and was protected from blood-borne degradation factors such as DNase [49]. Notably, the administration of cell wall active antibiotics resulted in a significant increase in the qPCR signal. It is highly

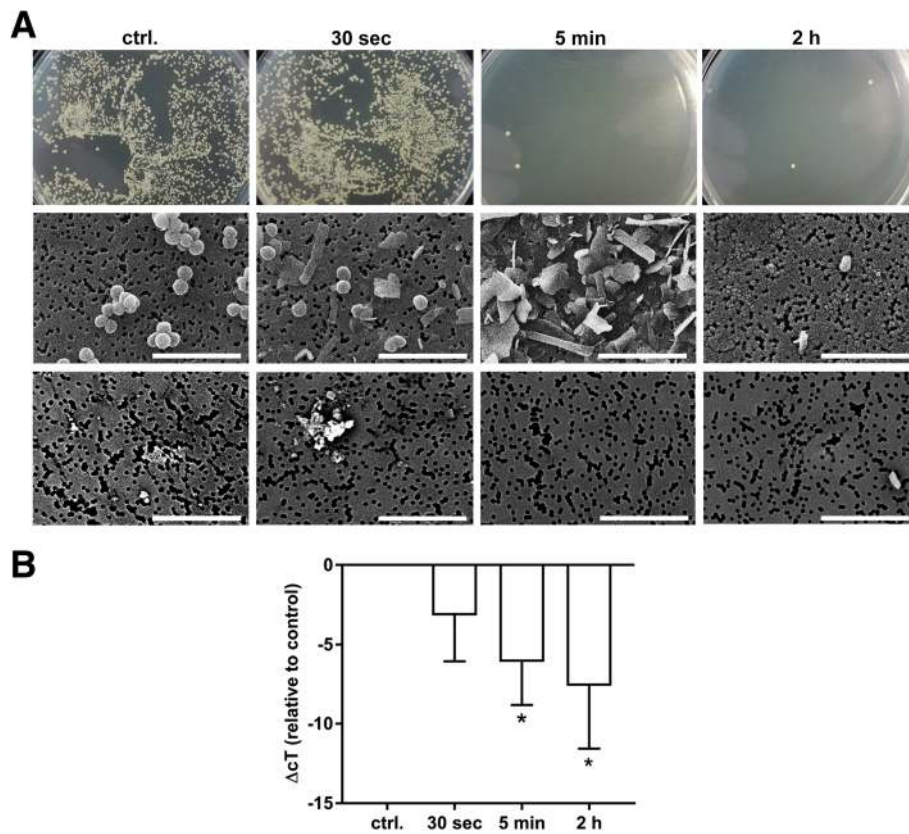


Fig. 4 Influence of mechanical lysis on the viability and recovery of *S. aureus* DNA after pathogen enrichment. **a** *S. aureus* was mechanically lysed using zirconium beads and spiked into human whole blood as described in Materials and Methods. Culture confirmed the absence of viable bacteria after 5 min of lysis. Fragmentation of bacteria was confirmed by scanning electron microscopy. **b** Lysed *S. aureus* was spiked into whole blood, and DNA was quantified after pathogen enrichment as described in Materials and Methods. DNA recovery increased in parallel with pathogen disintegration. Data are given as mean ± standard deviation (*n* = 3; Friedman repeated measures and Dunnett’s Method). Scale bar, 5 μm

probable that this effect was not related to the process of pathogen enrichment as such, but was rather due to incomplete lysis of gram-positive bacteria in alkaline solution after the actual pathogen enrichment step, and we assume that cell-wall active antibiotics support the release of pathogen DNA by reducing the thickness of the peptidoglycan layer of gram-positive bacteria [50]. Even

mechanical disintegration of *S. aureus* prior to pathogen enrichment resulted in enhanced detection of pathogen DNA. This further confirms previous reports on the incomplete solubilization of bacterial DNA, which was particularly reported for gram-positive bacteria [51], most likely due to its association with the cell walls [45].

Table 2 Selective depletion of human DNA by pathogen enrichment

	qPCR after	cT value	
		Human DNA	<i>S. aureus</i> DNA
spiked whole blood	protocol A	23.15 ± 0.38	25.98 ± 0.75
	protocol B	17.06 ± 0.08	23.85 ± 0.64

Human whole blood was spiked with *S. aureus* and processed in parallel with an established pathogen enrichment protocol (protocol A) and by total (host and pathogen) DNA extraction with magnetic beads (protocol B) as described in Materials and Methods. qPCR revealed the presence of residual human DNA (positive PCR for beta-actin) in samples isolated with the pathogen enrichment protocol, but samples obtained by total DNA extraction contained significantly higher amounts of human DNA. Pathogen DNA was nearly equally well enriched with both protocols, resulting in a net enrichment of pathogen DNA over host DNA using the pathogen enrichment protocol. Data are presented as mean ± standard error of the mean (*n* = 4)

While a comparison of pathogen DNA recovery from whole blood, either after selective lysis and pathogen enrichment or after extraction of total DNA, revealed that pre-analytical pathogen enrichment was not associated with enhanced detection of pathogen DNA, human DNA was depleted by 4–5 cT values, which might be relevant for the pre-analytical depletion of blood-borne PCR inhibitors. Likewise, the reduction of human DNA background may be crucial for emerging analytical approaches including next-generation sequencing [52].

Conclusion

In conclusion, our study provides evidence that concurrent antibiotic administration is not associated with decreased recovery of pathogen DNA after pathogen enrichment by selective lysis and centrifugation. On the

contrary, cell wall active antibiotics seem to increase the yield of pathogen DNA, presumably by supporting pre-analytical DNA solubilization. Moreover, we confirmed a depletion of human DNA as compared to pathogen DNA by at least a factor of 10 during pathogen enrichment.

Additional file

Additional file 1: Table S1. Primers for qPCR used in this study. **Table S2.** Minimal inhibitory concentrations of VAN, PIP, CIP, and CLI for the *S. aureus* strains used in this study. (PDF 292 kb)

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Authors' contributions

MP compiled the manuscript and performed the experimental work. AS supported with experimental planning, data analysis, writing, as well as preparation of the figures. LK isolated wild type strains, tested for antibiotic susceptibility, reviewed the draft, and provided input from a medical perspective together with DOH. VW extensively reviewed and critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data are presented in the manuscript and in the additional supporting material.

Ethics approval and consent to participate

Blood collection was approved by the Review Board of Danube University Krems, and written informed consent was obtained from all donors.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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