Optimizing a reliable ex vivo human blood model to analyze expression of *Staphylococcus epidermidis* genes

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Human blood is often used as an ex vivo model to mimic the environment encountered by pathogens inside the host. A significant variety of experimental conditions has been reported. However, optimization strategies are often not described. This study aimed to evaluate key parameters that are expected to influence *Staphylococcus epidermidis* gene expression when using human blood ex vivo models. Our data confirmed that blood antimicrobial activity was dependent on initial bacterial concentration. Furthermore, blood degradation over time resulted in lower antimicrobial activity, with a 2% loss of leukocytes viability correlating with a 5-fold loss of antimicrobial activity against *S. epidermidis*. We further demonstrated that the volume of human blood could be reduced to as little as 0.18 mL without affecting the stability of gene expression of the tested genes. Overall, the data described herein highlight experimental parameters that should be considered when using a human blood ex vivo model for *S. epidermidis* gene expression analysis.
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**Abstract**

Human blood is often used as an *ex vivo* model to mimic the environment encountered by pathogens inside the host. A significant variety of experimental conditions has been reported. However, optimization strategies are often not described. This study aimed to evaluate key parameters that are expected to influence *Staphylococcus epidermidis* gene expression when using human blood *ex vivo* models. Our data confirmed that blood antimicrobial activity was dependent on initial bacterial concentration. Furthermore, blood degradation over time resulted in lower antimicrobial activity, with a 2% loss of leukocytes viability correlating with a 5-fold loss of antimicrobial activity against *S. epidermidis*. We further demonstrated that the volume of human blood could be reduced to as little as 0.18 mL without affecting the stability of gene expression of the tested genes. Overall, the data described herein highlight experimental parameters that should be considered when using a human blood *ex vivo* model for *S. epidermidis* gene expression analysis.

**Keywords**: Bacterial survival, *ex vivo* model, Gene expression, Human blood; *Staphylococcus epidermidis*, Volume of human blood.
Introduction

*Staphylococcus epidermidis* is a commensal inhabitant of healthy human skin and mucosae that can cause important infections, such as medical device-associated bloodstream infections (Otto 2009). Due to the clinical relevance of these infections, it is important to understand the strategies employed by *S. epidermidis* to evade the host immune system response. In order to better comprehend how *S. epidermidis* adapts to the host, gene expression studies are often conducted under conditions that try to mimic the *in vivo* environment (Loza-Correa. *et al.* 2019). Human blood ex vivo models have contributed to a better understanding of how pathogens survive in human blood, by evaluating the transcriptional response during incubation in human blood, as well as, by exploring host-pathogen interactions. These models are relatively easy to implement, have been reported in many clinically relevant microorganisms, including *S. epidermidis* (França *et al.* 2014; Qin *et al.* 2017), *Staphylococcus aureus* (Malachowa *et al.* 2011), *Neisseria meningitidis* (Echenique-Rivera *et al.* 2011; Nolte *et al.* 2002), *Streptococcus agalactiae* (Mereghetti *et al.* 2008), *Enterococcus faecalis* (Vebo *et al.* 2009) and *Candida albicans* (Fradin *et al.* 2005; Fradin *et al.* 2003; Hunniger *et al.* 2014).

However, the implementation of these models lacks often do not describe optimization steps and, not surprisingly, a significant variety of experimental set-up conditions have been reported (Fradin *et al.* 2003; França *et al.* 2014a; Hedman *et al.* 2012; Qin *et al.* 2017). Because different experimental designs can greatly influence experimental outcomes, we became interested in evaluating key parameters that can compromise *S. epidermidis* gene expression studies when using a human blood ex vivo model. To achieve this goal, we evaluated the initial bacterial concentration and the volume of human blood in the co-incubation assays, as well as leukocyte viability after blood collection, and assessed bacterial survivability and gene expression under a human ex vivo blood model.

Material and methods

Bacterial strains and growth conditions

*S. epidermidis* PT12003, isolated from a patient with a central catheter after stomach surgery (Freitas *et al.* 2018), was used in this study. One single colony was inoculated into 2 mL of Tryptic Soy Broth (TSB) (Liofilchem, Teramo, Italy) and incubated overnight at 37°C and at 120 rpm (ES-20 Shaker-Incubator, Biosan, Riga, Latvia). Planktonic cultures were started by adjusting the
optical density at 640 nm (OD⁶₄₀nm), to 0.050 (± 0.005) in 10 mL of TSB and grown, in a 25 mL flask, for 6h at 37°C and at 120 rpm. The suspension was then washed once and resuspended in 0.9% NaCl. Before further experiments, the OD⁶₄₀nm of bacterial suspension was adjusted to 2.7 in order to obtain 1×10⁹ CFU/mL.

**Human Blood collection**

Peripheral blood was collected by venipuncture from Portuguese healthy adult volunteers with an age range between 25-40 (7 female and 4 male donors), not taking antibiotics or anti-inflammatory medication within the previous 14 days. Blood was drawn using one of the following anticoagulant tubes: K₂EDTA (Vacuette, Greiner Bio-one, Kremsmünster, Austria), sodium citrate (Vacuette, Greiner Bio-one, Kremsmünster, Austria) or lithium heparin (Becton Dickinson, NJ, USA). Blood was collected under a protocol approved by the Institutional Review Board of the University of Minho (SECVS 002/2014 (ADENDA)), which is in strict accordance with the Declaration of Helsinki and Oviedo Convention. All donors gave written informed consent to have blood taken.

**The influence of anticoagulants on bacterial growth**

In order to explore the effect of different anticoagulants on bacterial growth, TSB (Liofilchem, Teramo, Italy) was added to the different blood collection tubes and was gently inverted 5 to 8 times, before being transferred to a 25 mL flask. Planktonic bacteria obtained as described above was inoculated into TSB with the different anticoagulants, at a final concentration of 10⁷ CFU/mL. A negative control was included by inoculating bacteria in TSB without anticoagulants. Bacterial suspensions were incubated for 24h at 37°C and at 120 rpm (ES-20 Shaker-Incubator). Bacterial growth was determined by CFU quantification by taking aliquots every 2h. The aliquots were serially diluted and plated on TSB agar using the (Miles et al. 1938) and incubated at 37°C. At least two independent experiments with two technical replicates were performed.

**The ability of bacteria to survive in human blood**

For the evaluation of the ability of bacteria to survive in human blood, 0.1 mL of different concentrations of exponentially growing bacteria, were added to 0.9 mL of human blood in 2 mL tubes, to obtain final concentrations of 10⁸, 10⁷, 10⁶, 10⁵ or 10⁴ CFU/mL and incubated at 37°C, at 80 rpm, for up to 8h. Four and 8h after incubation, the enumeration of bacteria was determined by CFU counting, as described above. The number of CFU/mL immediately (i.e., less than 2 min)
after incubation with blood was used as a control to calculate the percentage of survival. This experiment was performed five independent times, using blood from five different donors.

Viability of human blood leukocytes over time

Human blood leukocytes viability after collection was used as an indicator of blood degradation over time. Whole blood was collected and incubated for up to 8h at 37°C and 80 rpm (PSU-10i, Biosan). At time points 0 (immediately after collection), 4h and 8h, 2 mL of whole blood was collected and incubated with 5 mL of red blood cells (RBC) lysis buffer (Alfa Aesar, Karlsruhe, Germany). The suspension was mixed by carefully inverting the tubes and then incubated at room temperature for 10 min. The reaction was stopped by adding 15 mL of phosphate buffered saline (PBS) (Gibco, MA, USA). Leukocytes were harvested by 10 min centrifugation at 300g and 4°C, and a new RBC lysis cycle was performed to lyse residual red blood cells. Leukocytes were then suspended in 0.5 mL of PBS and cells viability determined through flow cytometry (EC800, Sony Biotechnologies Inc, CA, USA), using propidium iodide staining (5µg/mL, Sigma, MO, USA).

This experiment was performed three independent times, using blood from different donors. Representative flow cytometry plots are presented in Supplementary Fig. S1.

The impact of time after blood collection on bacterial survival in human blood

Whole blood was collected and an aliquot (0.9 mL) was immediately taken and mixed in a 2 mL tube with 0.1 mL of S. epidermidis, to obtain a final bacterial concentration of \(10^5\) CFU/mL, and incubated at 37°C and 80 rpm for 4h. The remaining blood was kept under the same temperature and agitation conditions for 4h. After this time, a new 0.9 mL aliquot of blood was taken and a second incubation with 0.1 mL of S. epidermidis was performed for another 4h, in a new 2 mL tube. Bacteria was quantified by CFU counting, as described above. This experiment was performed three independent times, using blood from three different donors.

S. epidermidis gene expression assays

Three unrelated genes were selected as probes for assessing gene expression stability: SERP_RS11970, SERP_RS10985 and SERP_RS08870. Two different experimental conditions were tested: (i) the utilization of different anticoagulants on the collection tubes and (ii) the
reduction of the volume of blood used during the co-incubation assays (total incubation volume of
1 mL, 0.6 mL, 0.5 mL, and 0.2 mL). Blood samples were transferred into 2 mL tubes and, then,
bacteria were added to each tube to obtain a final concentration of $10^8$ CFU/mL. The tubes were
incubated for 2h at 37°C and 80 rpm (PSU-10i). After the co-incubation period, samples were
sonicated for 5s at 33% amplitude (Cole-Parmer 750- Watt Ultrasonic Homogenizer 230 VAC,
IL, USA) to lyse eukaryotic cells. Total RNA isolation, complementary DNA synthesis (cDNA)
and quantitative PCR (qPCR) were performed as previously optimized (França et al. 2012), with
minor modifications. In brief, after mechanical and chemical lysis of bacterial cells, total RNA
was purified using EZNA total RNA kit (Omega Biotek, GA, USA). Genomic DNA was degraded
by DNase I (Thermo Scientific, MA, USA) and cDNA synthesized, from 200 ng of total RNA, by
RevertAid M-MuLV reverse transcriptase (Thermo Fisher Scientific) and using random primers
(NZYTech, Lisboa, Portugal) as priming strategy. Finally, qPCR was prepared by mixing 2 µL of
1:100 diluted cDNA with 5 µL of Xpert Fast SYBR (Grisp, Porto, Portugal), 0.5 µL of each
forward and reverse primers at 0.5 µM and 2 µL of nuclease-free water. The run was completed
in a CFX96™ thermal cycler (Bio-Rad, CA, USA) with the following cycling parameters: 3 min
at 95°C followed by 40 cycles of 5s at 95°C and 25s at 60°C. The primers used were designed
using Primer3 software (Koressaar & Remm 2007; Untergasser et al. 2012) and synthesized at
Metabion (Steinkirchen, Germany). Primers sequences, size of the amplicon and reaction
efficiency are presented in Table 1. The quantification of the transcripts for each gene under study
was determined using 16S rRNA as reference gene and by applying the delta $C_q$ method ($E^{\Delta C_q}$), a
variation of the Livak method (Livak & Schmittgen 2001), where $\Delta C_q = C_q$ (reference gene) - $C_q$
target gene and E is the experimentally determined reaction efficiency. Reaction efficiencies
were determined using the dilution method (Pfaffl 2004) at 60°C.

**Statistical analysis**

Statistical analysis was carried out with GraphPad Prism Version 6 Trial (CA, USA). For
comparisons among different groups one-way or two-away ANOVA, with Tukey’s comparisons
test, were used when appropriate (the tests used are detailed in the figure caption). $P < 0.05$ was
considered significant.
Results and discussion

Human blood *ex vivo* models have been developed to mimic bloodstream infections as an affordable alternative to *in vivo* models (Echenique-Rivera *et al.* 2011; Malachowa *et al.* 2011; Qin *et al.* 2017). During human blood collection, anticoagulants need to be used to prevent blood clotting. It has been pointed out that different anticoagulants may influence the experimental outcome (Freitas *et al.* 2008; Strobel & Johswich 2018). The most commonly used anticoagulants are heparin, citrate and EDTA (Engstad *et al.* 1997). Citrate and EDTA prevent blood from clotting through binding free calcium ions (Strobel & Johswich 2018), while heparin inhibits coagulation by enhancing the activity of antithrombin III (Engstad *et al.* 1997). Herein, pilot experiments were initially performed to determine the best anticoagulant for *S. epidermidis* gene expression analysis. Thus, the influence of heparin, citrate, and EDTA was evaluated on bacterial growth and transcription levels of the selected genes. As shown in Supplementary Fig.S2, EDTA, but not the other tested anticoagulants, inhibited bacterial growth in the first 8h, eventually leading to bacterial death, after 24h of incubation. A similar effect was reported before in some strains of *N. meningitidis*, when using citrate as anticoagulant (Strobel & Johswich 2018) but in our experimental setup, we did not observe any inhibitory effect regarding *S. epidermidis* growth. Interestingly, when analyzing bacterial gene expression no significant differences were found among the different anticoagulants tested. Taken into consideration (i) these pilot results, (ii) the availability and (iii) price difference between the anticoagulants tested, and previous experimental results (França *et al.* 2014; Franca *et al.* 2016) we selected heparin for the remaining the experiments.

An important issue related to human blood *ex vivo* models is the bacterial concentration used. Although the quantity of microbes present in human blood during bacteremia is estimated to be up to $10^4$ CFU/mL (Opota *et al.* 2015), often higher concentrations of bacteria have been used in human blood *ex vivo* models (Askarian *et al.* 2017; França *et al.* 2014; Qin *et al.* 2017), mainly due to the lack of sensitivity of many experimental methods to assess lower bacterial concentrations (Bacconi; *et al.* 2014; Machado *et al.* 2013). For instance, it is known that for the analysis of the transcriptomic response of bacteria, the initial bacterial concentration needs to be significantly higher, to ensure a sufficient amount of RNA for downstream applications (Hedman *et al.* 2012). To investigate the effect of different initial bacterial concentration on the ability of *S. epidermidis* to survive in human blood, different bacterial concentrations ($10^4$ to $10^8$ CFU/mL)
were used for the bacterial survival assays. After 4h and 8h of co-incubation, the percentage of bacterial survival was determined (Figure 1). Since it is known that there is a significant source of experimental variability when working with human samples, due to the inherent traits of the donors such as age (Eady *et al.* 2005), gender (Whitney *et al.* 2003) and the proportion of different blood cell populations (Cobb *et al.* 2005), this experiment was performed with blood from five different donors, to increase the significance of our results. Not surprisingly, the ability of *S. epidermidis* to survive in human blood was cell concentration dependent: the lower the inoculum, the higher the percentage of bacterial killing by human blood (Figure 1). Interestingly, after 4h of co-incubation, cell death was observed in all tested bacterial concentrations but after 8h of co-incubation, the higher concentration inocula presented higher cell density than at time zero. This suggests that over time, and when the bacterial inoculum was $10^8$ CFU/mL, blood lost some antimicrobial activity leading to bacteria growth.

To confirm that longer blood incubation periods would result in lower antimicrobial activity, a second experiment was performed. For this assay, we selected a bacterial concentration of $10^5$ CFU/mL, taken in consideration the significant killing observed after 4h of incubation (Figure 1). As shown in Figure 2A, the co-incubation of *S. epidermidis* with human blood after 4h, resulted in different bacterial killing rates, depending if the blood was used immediately or 4h after collection: while 97% of bacterial was killed if blood was used immediately, only 84% of death was observed if we used blood 4h after collection. It is well described that blood antimicrobial activity against *S. epidermidis* involves important components such as complement and leukocytes (Le *et al.* 2018). As such, to evaluate blood degradation, we assessed leukocytes viability right after blood collection, and also 4 and 8h after collection. As shown in Figure 2B, right after blood collection, 3% of leukocytes were already dead. This fact may be related to the process of collection and processing time of human blood (Ferrante & Thong 1980). The results also showed that 4h after blood collection, cell death rate was slightly increased to 5% and then to 15% after 8h. Interestingly, a 2% reduction in leukocyte viability (from 3 to 5%) was correlated with 13% reduction in antimicrobial activity (from 97% to 84%). However, it should be noted that blood degradation over time will likely affect other important components that were not quantified herein, such as complement (Nordahl *et al.* 2004). As such, based on these results, it’s not possible to determine which exact mechanism involved in blood degradation contributed to the effective
loss of antimicrobial activity over time. Nevertheless, our data clearly confirms that longer waiting
times after blood collection will contribute to blood degradation. This fact is important to consider
when planning ex vivo experiments with blood, as it may have substantial consequences on the
results obtained, especially if a higher bacterial inoculum is needed, such as when performing
RNA-sequencing (França et al. 2014; Franca et al. 2016; Malachowa et al. 2011; Qin et al. 2017).
Some studies have used human blood ex vivo model with incubation periods up to 24h (Nolte et
al. 2002). However, as shown by our results, 24h is an extensive period of incubation which may
yield higher blood degradation and, as such, should be avoided when determining bacterial
survival studies in human blood. Blood degradation can occur when research laboratories are not
physically close to human blood collecting centers, and this should also be taken into consideration
when planning experiments using human blood. A simple way to reduce this obstacle is to maintain
the blood using gentle agitation (Afonso et al. 2010).

Another important practical aspect when considering using human blood as an ex-vivo model is
the limitation of blood availability. As such, the ability to reduce the volume of blood per
experiment, without compromising the results, is of interest. When analyzing several published
gene expression studies, it was observed that different volumes of human blood, ranging from 0.2
mL to 80 mL per experiment, have been reported (Echenique-Rivera et al. 2011; Hedman et al.
2012; Hunniger et al. 2014; Mereghetti et al. 2008; Qin et al. 2017). As the long-term goal of our
research group is to assess global transcriptomic changes occurring with S. epidermidis gene
expression, using ex vivo blood models, there was interest in determining if reducing the volume
of blood in the co-incubation assays down to 0.18 mL had a detrimental effect on the stability of
gene transcription. The starting volume was 0.9 mL, in order to compare with our previous data
obtained using RNA-seq (França et al. 2014). For this assay, we selected a bacterial concentration
of 10^8 CFU/mL to ensure a sufficient amount RNA that would be needed for future RNA-
sequencing studies (Hedman et al. 2012). The transcription of three unrelated genes was assessed.
One of the selected genes was sepA (SERP_RS08870), which codifies a protease that plays an
important role in bacterial immune invasion through the degradation of antimicrobial peptides
produced by the host (Lai et al. 2007). The two other selected genes were SERP_RS11970, a gene
that codifies a major facilitator superfamily and SERP_RS10985, a universal stress protein. As
shown in Figure 3, no significant differences were found in the expression of the selected genes
using, in any of the different volumes of human blood tested. Noteworthy, a volume of blood as low as 0.18 ml per reaction could be used without impacting the transcription of the selected genes. Nevertheless, we acknowledge that a limitation of this study was the fact that the selected genes had relative expressions (to the 16S ribosomal RNA) between ~10^-3 and ~10^-7. While this is a very large dynamic range, we can’t exclude the possibility that very low expressing genes (≤10^-8) could potentially be affected differently.

Conclusion

There remains a great deal of work to be done in clarifying the factors that contribute to *S. epidermidis* survival and adaptation to blood, which contribute to the evasion from the host immune system. The improvement of human blood *ex vivo* models will contribute to standardization of experimental conditions and produce a more reliable experimental setup and, consequently, contribute to obtain results with higher clinical relevance. The findings from the study are of technical importance for future studies since it highlights key parameters that should be considered when using human blood as an *ex vivo* model for the analysis of the gene expression of *S. epidermidis*, in particular, the possibility of using low volume of blood per reaction, without compromising the experimental results, at least in regard to the parameters tested herein.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 (on next page)

List of primers used for the quantification of gene expression by qPCR
| Gene          | Primer sequence (5´-3’)                     | Product size (base pair) | Efficiency (%) |
|--------------|---------------------------------------------|--------------------------|---------------|
| SERP_RS00125 | Fw: GGGCTACACACGTGCTACAA                    | 176                      | 97            |
|              | Rv: GTACAAGACCCGGGAACGTA                    |                          |               |
|              | Fw: CAGGCATTGAACCTCCCAAT                    |                          |               |
| SERP_RS11970 | Rv: AATTCGGGGGCGATATTTAGG                   | 109                      | 103           |
|              | Fw: ATGATTTTAGTGTATCCCTGACT                 |                          |               |
| SERP_RS10985 | Rv: CACTAATTGCAAGATCATTTTCTG                | 102                      | 110           |
|              | Fw: TCTTAAGGCGATCTCCGCCTA                   |                          |               |
| SERP_RS08870 | Rv: GTCTGGTGCGAATGATGTTG                    | 196                      | 97            |

Table 1. List of primers used for the quantification of gene expression by qPCR
Figure 1

The effect of initial bacterial concentrations on the ability of *S. epidermidis* to survive in human blood after 4h and 8h of incubation.

The bars represent the mean plus standard deviation of five independent experiments, performed with five different donors. Statistical analysis was performed using two-way ANOVA and Tukey´s multiple comparisons test.

Significant differences between $10^8$ and the other bacterial concentrations are depicted with **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. 

![Graph showing percentage of survival over time and bacterial concentration](image)
Figure 2

The influence of time after blood collection on bacterial survivability and leukocyte viability.

(A) **Bacterial survivability after 4h of co-incubation with blood.** Bacteria was added to blood immediately after collection or 4h post-blood collection. Bacterial survivability was determined by CFU counting. The bars represent the mean plus standard deviation of three independent experiments, performed using three different donors.

(B) **Viability of human blood leukocytes after collection.** Leukocytes viability was assessed by flow cytometry immediately, 4 and 8h after blood collection, before utilization in the co-incubation assays. The bars represent the mean plus standard deviation of three independent experiments, performed using three different donors. Statistical analysis was performed using one-way ANOVA and Tukey’s multiple comparison test. *p < 0.05. **p < 0.01.
Figure 3

The influence of using different volumes of human blood in co-incubations assays on the stability of transcription levels of SERP_RS11970, SERP_RS10985 and SERP_RS08870 genes.

Bacteria was added to each tube to obtain a final concentration of $10^8$ CFU/mL and then the tubes were incubated for 2h at 37°C. The bars represent the mean plus standard deviation of normalized expression of three independent experiments, performed with three different donors. Statistical analysis was performed, respectively, using one-way ANOVA and Tukey´s multiple comparisons test.