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ORIGINAL ARTICLE

Basic Study

Tuf mRNA rather than 16S rRNA is associated with culturable *Staphylococcus aureus*

Anne JM Loonen, Petra FG Wolffs, Maikel de Bresser, Maurice Habraken, Cathrien A Bruggeman, Mirjam HA Hermans, Adriaan JC van den Brule

Anne JM Loonen, Maikel de Bresser, Maurice Habraken, Mirjam HA Hermans, Adriaan JC van den Brule, Laboratory for Molecular Diagnostics, Department of Medical Microbiology and Pathology, Jeroen Bosch Hospital, 5200 ME 's-Hertogenbosch, The Netherlands

Anne JM Loonen, Maikel de Bresser, Maurice Habraken, Department of Medical Molecular Diagnostics, Fontys University of Applied Sciences, 5600 AH Eindhoven, The Netherlands

Anne JM Loonen, Petra FG Wolffs, Cathrien A Burggeman, Department of Medical Microbiology, CAPHRI, Maastricht University Medical Centre, 6229 HX Maastricht, The Netherlands

Author contributions: Loonen AJM, Wolffs PFG and van den Brule AJC designed the research; Loonen AJM, de Bresser M and Habraken M performed the research; Loonen AJM, Wolffs PFG, Bruggeman CA, Hermans MHA and van den Brule AJC analyzed the data and wrote the paper.

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Correspondence to: Adriaan JC van den Brule, PhD, Laboratory for Molecular Diagnostics, Department of Medical Microbiology and Pathology, Jeroen Bosch Hospital, Box 90153, 5200 ME 's-Hertogenbosch,
The Netherlands. a.v.d.brule@jbz.nl
Telephone: +31-73-5538480
Fax: +31-73-5532136

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Abstract

AIM: To study the presence of various nucleic acids targets of *Staphylococcus aureus* (*S. aureus*) during bacterial growth and antibiotic induced killing in relation to viability.

METHODS: *S. aureus* was cultured to log phase and spiked in Todd Hewitt (TH) broth and whole blood of healthy human volunteers. Viability of *S. aureus* after flucloxacillin treatment (0, 1, 3 and 6 d) was assessed by culture on bloodagar plates. DNA and RNA were isolated from 200 μL. cDNA synthesis was performed by using random primers. The presence of *S. aureus* DNA, rRNA, and mRNA were determined by real-time polymerase chain reaction of the 16S rDNA and *tuf* gene (elongation factor Tu).

RESULTS: *S. aureus* spiked in TH broth without antibiotics grew from day 0-6 and DNA (*tuf* and 16S), and 16S rRNA remained detectable during this whole period. During flucloxacillin treatment *S. aureus* lost viability from day 3 onwards, while the 16S rRNA-gene and its RNA transcripts remained detectable. DNA and

rRNA can be detected in flucloxacillin treated *S. aureus* cultures that do not further contain culturable bacteria. However, *tuf* mRNA became undetectable from day 3 onwards. *Tuf* mRNA can only be detected from samples with culturable bacteria. When spiking *S. aureus* in whole blood instead of broth no bacterial growth was seen, neither in the absence nor in the presence of flucloxacillin. Accordingly, no increase in DNA and RNA levels of both 16S rDNA and the *tuf* gene were detected.

CONCLUSION: *Tuf* mRNA expression is associated with culturable *S. aureus* and might be used to monitor antibiotic effects.

Key words: Bloodstream infection; *Staphylococcus aureus*; Viability; mRNA; Polymerase chain reaction; Sepsis; Molecular diagnostics; Blood

Core tip: We report our first results from a proof-of-principle study where we show that *tuf* mRNA expression seems to correlate with active *Staphylococcus aureus* (*S. aureus*) infection. The commonly used target, 16S rRNA, seems unsuitable for viability measurements as it can be detected from samples containing unculturable bacteria. This study indicates that *tuf* mRNA expression is associated with viable *S. aureus*, as determined by culture.

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INTRODUCTION

Bacteremia is defined as the presence of viable bacteria in the bloodstream[1]. The current gold standard method for the detection of microorganisms in the bloodstream is blood culture and subsequent identification of the bacteria by conventional (sometimes automated) biochemical techniques or MALDI-TOF MS[2-4]. An important advantage of this method is that only viable microorganisms are detected. A major disadvantage of the method is that the time-to-results is long (24-72 h) due to the involvement of culturing steps. Because fast and accurate diagnosis is of crucial importance for patients suffering from bloodstream infection (BSI), molecular (real-time) polymerase chain reaction (PCR) applications are increasingly being applied to decrease time to pathogen identification, thereby improving patient outcome[5-8]. However, all commercially available sepsis tests [SeptiFAST (Roche), SepsiTest (Molzym), and MagicPlex Sepsis Test (Seegene)] are based on DNA detection. DNA is a stable molecule and the presence of DNA of a certain pathogen does not provide information about the viability status of that pathogen as the DNA can originate from either living or dead pathogens[9-12]. In contrast to DNA, bacterial messenger RNA molecules have a half-live of only minutes[13]. For that reason, several studies have evaluated the detection of mRNA as a marker for the presence of actively growing bacteria[9,11,14-18]. Some of these studies have focused on detection of viable pathogens from food and environmental samples[14], while other studies focused on human disease and viability of pathogens from spiked culture broths (*i.e.*, *Borrelia burgdorferi, Escherichia coli, Salmonella typhi, Shigella sonnei, Mycobacterium smegmatis*)[10,11,17,19]. Few studies used clinical specimens (*Mycobacterium tuberculosis* from sputum samples, *Aspergillus* spp. from blood samples, *Chlamydia trachomatis* from cervical smears and urine)[12,15,16]. If RNA markers can be used to assess pathogen viability for BSI, the application of PCR based methods on RNA (cDNA) would be of great significance.

BSI can be caused by numerous pathogens[20]. In this study, the most commonly detected Gram-positive bacterium; *Staphylococcus aureus* (*S. aureus*) was chosen for reconstruction experiments. To investigate which nucleic acid molecule most favourably correlates to the viability status of *S. aureus*, DNA and rRNA of 16S rRNA gene, and DNA and mRNA levels of the *tuf* gene (elongation factor Tu) were measured in response to antibiotic therapy. Both the 16S rRNA and *tuf* gene are household genes with relatively high expression levels and therefore indicative of protein expression and thus most likely bacterial viability[21]. The aim of this work was to find a suitable marker for *S. aureus* viability to be able to improve BSI diagnostics.

MATERIALS AND METHODS

Bacterial strain and growth conditions

S. aureus (ATCC 25923) was used for reconstruction (spiking) experiments. Todd Hewitt (TH) broth was inoculated with *S. aureus* and cultured overnight at 35 ℃. Subsequently, a 1:100 dilution was made in fresh TH broth (5 mL) for additional culturing to exponential phase (optical density 0.2 at 600 nm; approximately 1 × 10^7 cells/mL).

Spiking of S. aureus in TH broth and whole blood

See Figure 1 for an overview of the experimental setup. *S. aureus* bacteria grown in exponential phase were diluted in either TH broth or pooled (of similar blood type, *i.e.*, O+), 1 d old, residual whole blood from healthy volunteers. The 100 and 10 colony forming units (CFU)/mL dilutions were made in 2 tubes with an end volume of 10 mL TH broth or whole blood, respectively. To one tube an overdose of flucloxacillin (floxapen 5 μg/mL, 1 mL, Actavis, Baarn, the Netherlands) was added (to kill the bacteria) and to the other 1 mL physiological

Table 1 Primers and probes used in this study

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Probe 5'-3'	Ref.
tuf	tcctggttcaattacaccacatactg	ggaaatagaattgtggacgatagtttga	FAM-tgataatacrtawacttctgc-BHQ1	[24]
16S	acggtcttgctgtcactta	tacacatatgttcttccctaataa	VIC-gtaacggcttaccaaggc-BHQ1	[22]

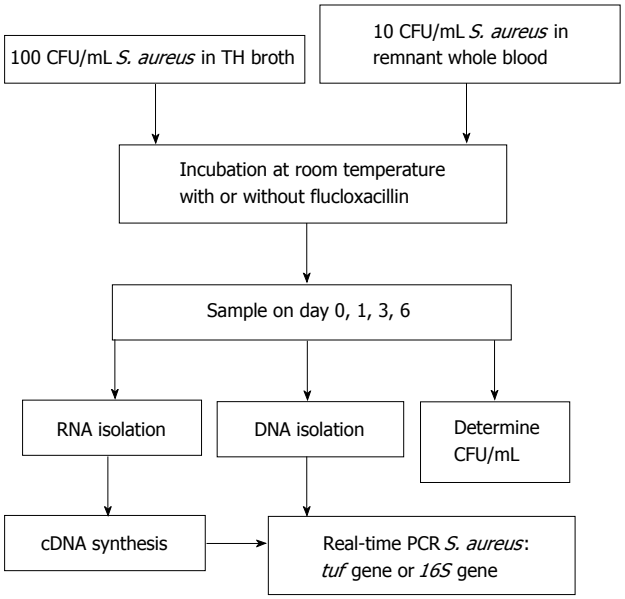


Figure 1 Overview of the experimental setup. CFU: Colony forming unit; *S. aureus*: *Staphylococcus aureus*; TH: Todd Hewitt; PCR: Polymerase chain reaction.

salt solution, this was used as a control. Both tubes were placed on a shaker at room temperature. On days 0, 1, 3, and 6, 200 μL samples were taken from both tubes (flucloxacillin treated and untreated) for DNA and RNA isolation. Additionally, 100 μL was taken to determine CFU/mL on blood agar plates (Tryptone Soya Agar with sheep blood, Oxoid Deutschland GmbH, Wesel, Germany). Bacterial death was defined as the inability of producing colonies on bloodagar.

DNA and RNA isolation

The obtained 200 μL samples (TH broth and whole blood) were centrifuged at 14000 rpm for 2 min. The supernatant was removed and the pellet was washed once with 200 μL ultra-pure water and centrifuged for 2 min at 14000 rpm. The obtained pellet was resuspended in 20 μL lysozym (12.5%) and 75 μL lysostaphin (100 μg/mL) and incubated for 30 min at 37 ℃ while shaking (1000 rpm). RLT buffer with β-mercaptoethanol (1:100) (Qiagen RNA blood mini kit) was added and the samples were stored at -80 ℃ until all time points were collected. The EasyMAG (BioMérieux, Marcy L'Etoile, France) was used for DNA isolation by using the specific B protocol. RNA was isolated by using the RNA blood mini kit (Qiagen), according to manufacturer's instructions. DNAse treatment was performed as described in the manual provided (Qiagen RNA blood mini kit) using columns to degrade the DNA in the samples.

cDNA synthesis with random primers

Reverse transcription was performed on RNA samples using the SuperScript™ Ⅱ First-Strand Synthesis System for real-time-PCR (Invitrogen, Carlsbad, CA, United States, according to manufacturer's protocol). Each sample was split in two for the plus and minus reverse transcriptase reaction to check DNA degradation (DNAse treatment on column).

Real-time PCR for tuf and 16S rRNA

Table 1 for an overview of the used primers and probes (*tuf* and 16S rDNA). The 16S rDNA primers, specific for most clinically relevant staphylococci, were described by Matsuda *et al*[22]. However, the 16S rDNA forward primer was slightly modified to adapt to proper annealing temperature. An XS- probe (Biolegio, Nijmegen, The Netherlands) for *Staphylococcus* spp. detection based on 16S was specifically designed. The PCR mix used has been described previously[23]. Additionally, *tuf* or 16S primers (900 nmol/L), *tuf* or 16S probe (200 nmol/L), and 5 μL sample were added to obtain an final volume of 20 μL. A positive and negative control (nuclease free water) were added in each PCR run. The *tuf* PCR and program used were described previously[24]. Both PCRs were run in white plates on the LightCycler 480 Ⅱ (Roche Diagnostics).

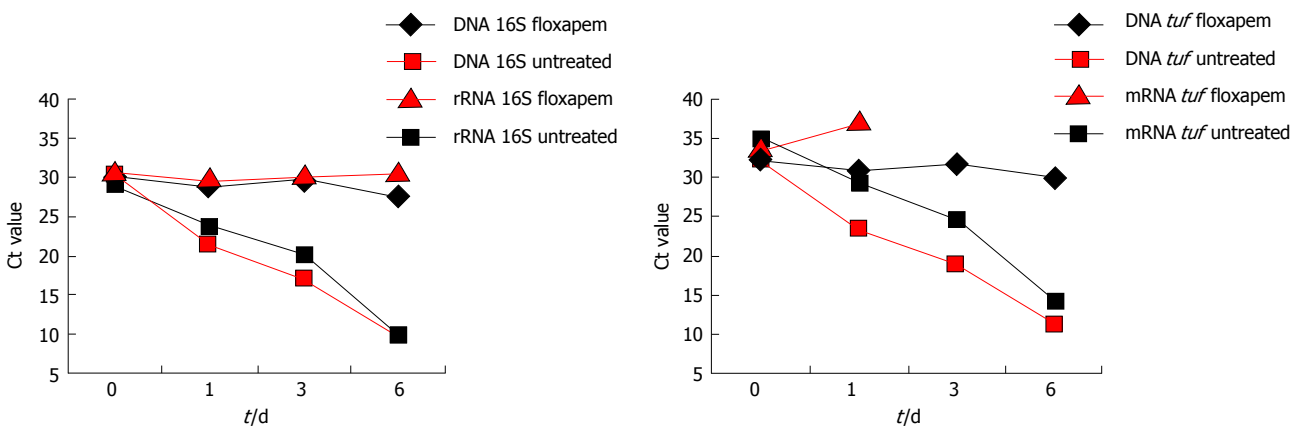
RESULTS

Detection of S. aureus DNA, rRNA, and mRNA after antibiotic treatment from TH broth

In absence of flucloxacillin the *S. aureus* bacteria continued to grow. At day 0, on average 330 ± 28 (average ± SD) CFU/mL were detected on bloodagar plates (Table 2). At days 1, 3, and 6 the CFU/mL increased to > 1000 CFU/mL. In contrast, bacterial growth was arrested in flucloxacillin (antibiotic) treated samples and no colonies were detected on bloodagar at days 3 and 6.

Simultaneously, samples were taken for DNA and RNA isolation. In the absence of flucloxacillin, Ct values of both 16S (DNA and rRNA) and *tuf* (DNA and mRNA) decreased in time (Figure 2). In the presence of flucloxacillin, DNA of the 16S rDNA gene and the *tuf* gene were detected until day 6, while bloodagar plates indicated absence of culturable *S. aureus* on day 3. 16S rRNA also remained detectable up to 6 d of treatment. However, *tuf* mRNA could not be detected on days 3 and 6. The data indicate that *S. aureus* DNA and rRNA can still persist in the absence of viable bacteria as demonstrated using culture.

The Ct values obtained on day zero are similar for



flucloxacillin	Yes		No		Yes		No		Yes		No		Yes		No	
(floxapen)	DNA 16S		DNA 16S		rRNA 16S		rRNA 16S		DNA *Tuf*		DNA *Tuf*		mRNA *Tuf*		mRNA *Tuf*	
Days	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
0	30.09	0.34	30.57	1.65	30.64	4.49	29.09	3.87	32.01	0.42	32.41	0.98	33.41	3.66	34.95	5.48
1	28.61	0.40	21.46	0.65	29.73	0.30	23.84	1.72	30.80	0.59	23.39	0.99	37.02	0	29.42	1.71
3	29.62	0.62	16.95	0.04	30.00	0.71	20.17	0.93	31.72	0.46	18.95	0.29			24.77	2.95
6	27.72	1.11	9.62	0.16	30.49	2.08	10.00	0.00	29.83	1.38	11.31	0.46			14.35	0.06

Figure 2 *Staphylococcus aureus* polymerase chain reaction results with and without flucloxacillin treatment in Todd Hewitt broth. *S. aureus* bacteria (100 CFU/mL) continue to grow in absence of flucloxacillin and as a result DNA and RNA levels of both tuf and 16S increase in time (Ct values decrease). When flucloxacillin is added 16S DNA, 16S rRNA, and *tuf* DNA levels remain relatively stable in time. Whereas *tuf* DNA is still detectable, *tuf* mRNA is not detectable on day 3 and 6 after flucloxacillin treatment. The experiment was performed twice. Ct values are depicted. CFU: Colony forming unit; *S. aureus*: *Staphylococcus aureus*.

Table 2 Plate counts of *Staphylococcus aureus* samples with or without flucloxacillin treatment from Todd Hewitt broth

	Flucloxacillin	Yes		No	
		Average	SD	Average	SD
Days	0	290	14	330	28
	1	255	7	Infinity	ND
	3	0	0	Infinity	ND
	6	0	0	Infinity	ND

Numbers represent colony forming units/mL. Infinity: Uncountable plate due to large amount of colonies; ND: Not determined.

both 16S DNA and rRNA with(out) flucloxacillin. This is not true for *tuf* DNA and mRNA. Ct values obtained for *tuf* mRNA are on average 2 Ct higher as compared to *tuf* DNA (day 0).

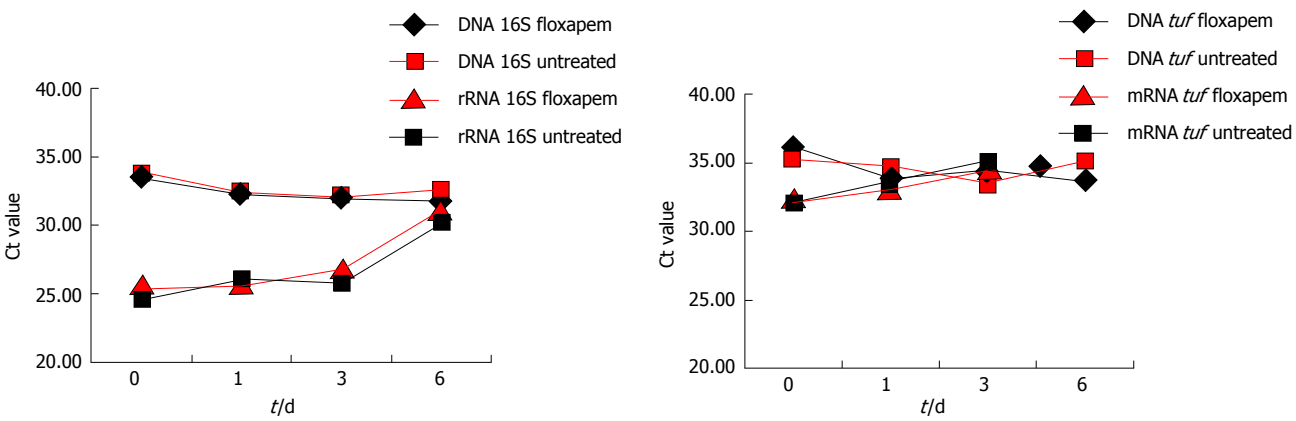
These results demonstrate that *tuf* mRNA is the nucleic acid target that could only be detected from samples which contain culturable bacteria. DNA and rRNA targets could be detected in flucloxacillin treated *S. aureus* cultures that do not further contain culturable bacteria. This experiment was performed twice on independent days, and showed similar results.

S. aureus viability measurements from spiked whole blood

In order to mimic a bloodstream infection, whole blood samples instead of TH broth were spiked with log phase *S. aureus* and growth was measured both in presence and absence of flucloxacillin. Furthermore, a more clinical significant initial bacterial load was used of 10 CFU/mL (instead of 100 CFU/mL). The plate counts (Figure 3) show that in both presence and absence of flucloxacillin bacterial numbers decrease. DNA and RNA measurements of 16S and *tuf* also showed growth arrest, both in presence and absence of flucloxacillin, as no decrease in Ct values was observed in time (0-6 d) (Figure 3), this in contrast to the results obtained by using spiked TH broth in absence of flucloxacillin (Figure 2).

The Ct values obtained by detecting 16S DNA and rRNA are lower as compared to the Ct values for *tuf* DNA and mRNA. A difference of at least 6 Ct was observed when comparing 16S rRNA levels to *tuf* mRNA levels. When comparing Ct values for DNA detection of both genes the differences were less pronounced, but still significant (approximately 3 Ct). A clear difference was observed between the spiked TH broth samples (Figure 2) and the spiked whole blood samples at day zero (Figure 3). Ct values for 16S DNA and rRNA were comparable in TH broth (day 0), but not in whole blood. For *tuf* DNA and mRNA this phenomenon was also observed, in whole blood the Ct values for *tuf* mRNA were lower than for *tuf* DNA (day 0). Furthermore, there seemed to be a trend towards higher Ct values from day 0-6, independent of flucloxacillin, for both 16S rRNA and *tuf* mRNA in whole blood. This confirms the culture results obtained from whole blood.

In addition, it was investigated whether fresh (max 1 h) and residual (1 d old) whole blood differed in their performances as medium for bacterial culture. TH broth was used as a control: In the absence of flucloxacillin large amounts of colonies were detected up to 6 d of culture, while in the presence of flucloxacillin colonies were detected on days 0 and 1, and no colonies were



flucloxacillin	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
(floxapen)	DNA 16S	DNA 16S	rRNA 16S	rRNA 16S	DNA *Tuf*	DNA *Tuf*	mRNA *Tuf*	mRNA *Tuf*	plate counts	
Days										
0	33.43	33.67	25.17	24.62	36.09	35.27	31.95	32.12	12	6
1	32.15	32.35	25.55	26.15	33.82	34.57	32.89	33.60	5	7
3	32.10	32.06	26.69	25.86	34.30	33.55	34.29	35.04	7	2
6	31.56	32.54	31.13	30.28	33.72	35.16			1	0

Figure 3 *Staphylococcus aureus* polymerase chain reaction results with and without flucloxacillin treatment in whole blood. Both 16S and *tuf* DNA levels remain relatively stable in time in presence and absence of flucloxacillin. 16S rRNA and *tuf* mRNA show an increase in Ct values (reduction in RNA level). *Tuf* mRNA was not detectable on day 6. Ct values and CFU/mL (plate counts) are depicted.

Table 3 Plate counts of *Staphylococcus aureus* with or without flucloxacillin treatment from Todd Hewitt broth, fresh whole blood (1 h) and remnant whole blood (1 d)

		TH broth		Fresh whole blood		Remnant whole blood	
Flucloxacillin		Yes	No	Yes	No	Yes	No
Days	0	20	22	10	4	3	6
	1	14	Infinity	2	3	1	1
	3	0	Infinity	2	1	0	3
	6	0	Infinity	1	0	0	0

Numbers represent colony forming units/mL. Infinity: Uncountable plate due to large amount of colonies; TH: Todd Hewitt.

detected on bloodagar plates on days 3 and 6 (Table 3). Plate counts from spiked blood samples did not resemble those obtained from spiked TH broth samples. The initial plate count on day 0, while initiated from the same log phase culture, was half or less when compared to the count on day 0 for TH broth samples. However, no significant differences were observed between fresh and residual blood samples both in presence or absence of flucloxacillin.

DISCUSSION

For patient survival it is important to provide fast and accurate identification of BSI causing pathogens. Only viable microorganisms can be cultured, and fastidious or damaged organisms can be present in whole blood but often remain culture negative. Molecular diagnostics might provide solutions for these problems, as pathogens in antibiotic treated patients who remain culture negative (due to presence of antibiotics in the bloodstream) can be identified by PCR[25,26]. As BSI is defined as the presence of viable pathogens in the bloodstream, it might be important for a molecular assay to allow pathogen viability measurements from whole blood.

The results that were obtained after spiking *S. aureus* in TH broth, with and without flucloxacillin, indicated that *tuf* mRNA might be a more promising marker to measure viability than DNA and rRNA. *Tuf* mRNA levels correlated with the culture results from both TH broth and whole blood, whereas 16S DNA, 16S rRNA and *tuf* DNA levels did not. These data confirm results from previous studies[9,10,15,18].

The *S. aureus* bacteria used in this study seemed to die in whole blood (growth reduction on agar plates in absence of flucloxacillin) or enter a state in which they are viable but non-culturable (VBNC)[27-29]. Bacteria enter the VBNC state in response to stress, such as starvation, incubation outside the growth temperature range, or oxygen concentration[29]. In this study, several stressful conditions might have been present. *S. aureus* was cultured in whole blood in which white cells might inhibit bacterial growth[30]. Additionally, incubation took place at room temperature (RT) for 6 d, and waste products were not removed from the culture tube. In future studies, it might be useful to remove the white blood cells from whole blood before spiking (buffy coat), and incubate the samples at 35 ℃ instead of RT to create better growth conditions. Furthermore, different *S. aureus* strains need to be tested to confirm our results.

In this study, bacteria were considered dead when they were unable to produce colonies on bloodagar. However, as mentioned before, bacteria can enter a VBNC under stressful conditions[27,31]. Bacteria that are not culturable can potentially still be viable and infective.

A limitation of this study is that bacterial viability was only measured by colony formation on bloodagar plates. In future studies additional methods to assess bacterial viability might be included, for instance the Live/Dead BacLight Bacterial Viability Kit (Invitrogen). This kit provides two nucleic acid stains [green-fluorescent SYTO 9 dye and red-fluorescent propidium iodide (PI)] to be able distinguish live bacteria (intact membranes) from dead bacteria (compromised membranes). PI is a cell membrane impermeable dye and can only enter compromised pathogens[32]. Another option to differentiate live from dead pathogens is exposure to the dye propidium monoazide (PMA) followed by real-time PCR. PMA cannot penetrate viable cells with intact cytoplasmic membranes[33]. The PMA dye can enter dead pathogens and bind DNA, thereby inhibiting PCR amplification.

The Ct values obtained for *S. aureus* spiked in TH broth are different from those in the whole blood. Because a lower amount of *S. aureus* bacteria (10 CFU/mL) was spiked in whole blood, as compared to TH broth (100 CFU/mL), one would expect the Ct value to be 3,3 (1 log) higher in whole blood samples. This difference of approximately 3 Ct was seen in whole blood as compared to TH broth for DNA (both 16S and *tuf*). However, the Ct values obtained for *tuf* mRNA and 16S rRNA (t = 0) were higher in TH broth as compared to whole blood (approximately 2 and 5 Ct, respectively). Both RNA targets (*tuf* and 16S) seem to be expressed at a higher level in whole blood. This unexpected phenomenon might be a result of the difference in environment (blood *vs* broth). This confirms findings reported by Cenciarini *et al*[14] who showed that it is difficult to compare RNA viability markers for one pathogen kept in different conditions.

In this study, detection of mRNA and rRNA was performed by using reverse-transcription real-time PCR. Birch *et al*[34] investigated the use of PCR, real-time-PCR and nucleic acid sequence based amplification (NASBA) for assessment of bacterial viability. They found that NASBA offered the highest sensitivity of the three methods tested. However, presence of residual fliC DNA and mRNA could be detected by NASBA 30 h post-death (culture negative). Other studies have shown that RNA detection by NASBA could be used to monitor infections after antibiotic treatment[12,19]. These contradictory findings again demonstrate that it is important to thoroughly investigate which RNA target is suitable for viability measurement of a certain pathogen.

In this study, results were obtained from as little as 200 μL whole blood. Larger volumes of blood are needed to be able to detect clinical relevant bacterial loads[35]. As bacterial enrichment is a prerequisite to be able to detect bacteria from whole blood, RNA isolation methods should include such an approach. Both Polaris (Biocartis, Mechelen, Belgium) and MolYsis (Molzym GmbH, Bremen, Germany) have developed suitable techniques for pathogen DNA enrichment from large volumes of blood[35]. However, these enrichment strategies are not suitable for RNA isolation. A small pilot study indicated that the first steps, of both the MolYsis and the Polaris pathogen enrichment methods, in which human cells and DNA were removed, did not kill the pathogens present in the whole blood samples as shown by positive cultures (data not shown).

In conclusion, this study clearly demonstrated that detection of *S. aureus tuf* mRNA, in contrast to DNA and rRNA, correlates to bacterial viability status as determined by culture. Therefore, *tuf* mRNA might be a promising marker to measure active *S. aureus* bloodstream infection. After development of RNA isolation procedures from large volumes of whole blood, future clinical studies are needed to validate the preliminary findings obtained in this study.

COMMENTS

Background

Bloodstream infections (BSIs) are characterized by high morbidity and mortality and can be caused by a broad variety of microorganisms. Bacteremia is defined as the presence of viable bacteria in the bloodstream. Currently, blood cultures are still the gold standard to detect pathogens from the bloodstream. However, cultures are very time-consuming (24-72 h) and patients need to be treated immediately. Molecular assays (detection of pathogen DNA) can provide results within hours, but the clinical value of DNA detection is still unclear. It might be useful to be able to assess viability of the bacteria in blood samples of patients. Molecular assays that detect the presence of DNA of a specific pathogen can be positive even after viable organisms have been eradicated. The clinical value of pathogen DNA, rRNA and mRNA detection from whole blood needs further investigation.

Research frontiers

Molecular diagnostics can provide improved detection and identification of pathogens causing BSI. Implementation of these methods reduces time-to results, offers high sensitivity and specificity, and overall improve the laboratory process for BSI. Detection of DNA by polymerase chain reaction does not provide information about the viability status of a pathogen as the DNA can originate from either living or dead pathogens. The authors attempted to find a marker that enabled their to measure *Staphylococcus aureus* (*S. aureus*) viability to further improve BSI diagnostics.

Innovations and breakthroughs

The authors describe a novel approach in molecular diagnostics based on the need to assess bacterial viability and not only detect the presence of bacterial DNA. Clinical relevance of bacterial DNA detection can be limited due to the longer half life of DNA. Antibiotic treatment does not result in the break-down of pathogen DNA. It is important to note that the frequently used 16S rDNA/rRNA target cannot be used to monitor viability of *S. aureus* bacteria. More research is needed to confirm them data and to find suitable mRNA targets to be able to detect the broad variety of bacteria that are commonly detected in patients.

Applications

The study results suggest that *Tuf* mRNA may represent a suitable marker for the detection of viable *S. aureus.*

Peer-review

This manuscript describes a novel approach in molecular diagnostics based on the need to monitor viability and not only DNA presence of a microorganism.

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