Quantitative data of the SeptiFast real-time PCR is associated to disease severity in patients with sepsis

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ABSTRACT

Background

The commercial test SeptiFast is designed to detect DNA from bacterial and fungal pathogens in whole blood. The method has been found to be specific and of high rule-in value for early detection of septic patients. For positive results, the software gives automatically information about the identified pathogen, without quantification. However, it is possible to manually derive Crossing point (Cp) values, i.e. the PCR cycle when DNA is significantly amplified. We aimed to study if Cp values are correlated to disease severity.

Methods

We used a study cohort with patients with positive SeptiFast test for bacteria (coagulase-negative staphylococci excluded), from a recent study where patients with suspected sepsis at the Emergency Department were included. Cp values were compared with disease severity, classified as severe sepsis/septic shock or non-severe sepsis, according to the criteria of American College of Chest Physicians/Society of Critical Care Medicine.

Results

Ninety-four patients with a median age of 74 years (range 14-96 years) were included. The prevalence of severe sepsis/septic shock in the study was 29% (27/94). SeptiFast positive blood samples from patients with severe sepsis/septic shock had significantly lower Cp median values compared with those from patients with non-severe sepsis, i.e 16.9 (range: 7.3-24.3) versus 20.9 (range: 8.5-25.0), p<0.001. Positive predictive values of the SeptiFast test for detection of severe sepsis/septic shock were 34% at SeptiFast Cp cut-off <25.0, 35% at Cp cut-off <22.5, 50% at Cp cut-off <20.0, and 73% at Cp cut-off <17.5. Patients with positive Septifast test with a Cp value <17.5 had significantly more severe sepsis (73% versus 15%, p<0.001), developed more septic shock (14% versus 0%, p=0.010), were more often admitted to the Intensive Care Unit (23% versus 4%, p=0.016), had more frequently positive BC (100% versus 32%, p<0.001)
and had longer hospital stays (in median 19.5 [range: 4-78] days versus 5 [range: 0-75] days, p<0.001) compared with those with a Cp value >17.5.

Conclusions

Our results suggest that introduction of quantitative data to the SeptiFast test could be of value in the assessment of sepsis severity. Moreover, it might also be used to predict a positive BC result.

BACKGROUND

Bloodstream infections are associated with high morbidity and mortality rates worldwide [1, 2]. Prompt administration of adequate antibiotics is crucial for the successful outcome of severe sepsis and septic shock [3, 4]. Blood culture (BC) is the current ‘gold standard’ for diagnosing bloodstream infections, but the method has several limitations. The sensitivity is low for slow-growing and fastidious organisms[5], and when antibiotics have been given prior to culture [6, 7]. It has been shown that a positive BC is found only in about 30% of patients with severe sepsis and septic shock [8]. Furthermore it takes about 24-72 hours before the pathogen is identified. With the implementation of new techniques, such as matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), the time to identification can be significantly shortened, and it is shown that this reduction has a significant effect on the time to adequate antibiotic treatment [9, 10].

For better initial antibiotic appropriateness, techniques that can confirm the infectious aetiology as soon as possible are needed. Polymerase chain reaction (PCR) analysis for detection of bacterial DNA on blood samples can be made within hours and is virtually unaffected by the prior use of antibiotics [5]. An additional advantage of PCR is the possibility of quantification of bacterial DNA.
For broad routine use of PCR techniques in sepsis diagnostics, there is a need for validated commercial assays. There are already a few commercial tests available for molecular identification of pathogens directly on blood samples [11-13]. Among such tests, the Light Cycler SeptiFast Test (Roche Diagnostics) is, to date, the most used and investigated in clinical studies [14], and it is approved for clinical use in Europe. The method has been evaluated in a systematic review [15], and found to be specific, and of high rule-in value for early detection of septic patients. The SeptiFast test is used for pathogen identification, and the result does not include any quantitative information.

The diagnosis of sepsis is sometimes difficult, and for the clinician it is a major challenge to early identify patients at risk of septic shock and death. In addition to the clinical evaluation, laboratory tests are the main diagnostic tools in the initial assessment. Thus, a rapid molecular test that can contribute to predict the risk of developing severe sepsis or septic shock would be of significant clinical value. Previous studies have shown that a high bacterial DNA load in blood is related to a more severe disease and an increased risk of development of septic shock, and consequently, to poor prognosis [16-20]. In line with this, we aimed to study whether quantitative data of the commercial SeptiFast test could provide information regarding disease severity.

For this purpose, we used a study cohort with positive Septifast test results from a recent prospective study [21]. The manufacturer gave us access to Crossing point (Cp) values for all positive PCR results. A Cp value represents the PCR cycle when DNA is significantly amplified, and it is generally accepted as an indirect and inverse marker of the DNA load. In the present study, we wanted to investigate whether the level of the Cp values could differentiate cases with severe sepsis/septic shock from those with non-severe sepsis.
MATERIALS AND METHODS

Setting and patients
The department of Infectious Diseases, Örebro University Hospital, Sweden, provides service for a population of 275,000 inhabitants in the county of Örebro, and is organised into an outpatient clinic and a ward for 30 adult patients.

In the prospective study [21], all patients who were subjected to BC at the department and gave their informed consent during one full year, from October 2007 to September 2008, were enrolled. If the patient was unable to provide consent due to illness, a next-of-kin could give the consent, or it could be given the next day. This study approach was approved by the regional ethical review board in Uppsala, Sweden. Blood samples for PCR analysis were taken at the same time as BC from all included patients. The results of the PCR assays were unknown to the clinicians until the closure of the study. In total 1,093 patients were included in the study, and 113 positive SeptiFast PCR were found in 107 patients. In 50 of them the same pathogen was also found in the BC. These 107 patients form the study population in the present study. As we aimed to study bacteria, we excluded four cases positive for fungi. Ten cases positive for coagulase-negative staphylococci (CoNS) were excluded, as they had no microbiological or clinical support for infection with CoNS, and were considered to represent skin contamination. Finally, 99 positive PCR tests from 94 patients were included in the study.

Laboratory analyses
BC was performed with the Bactec system (Becton Dickinson and Company, Sparks, MD, USA), with a Bactec 9240 incubator, and culture duration time of 7 days. For each BC a volume of 8-10 ml of venous blood was inoculated in one Bactec PlusAerobic/F bottle and the same volume in one Bactec Plus Anaerobic/F bottle.
Whole blood was collected in sterile EDTA tubes through the same venepuncture as the blood samples for BC were taken. The whole blood was then stored for a maximum of 4 h at room temperature, or up to 3 days at +4°C, or 3 months at −70°C prior to DNA preparation. DNA was extracted manually from 1.5 mL of the EDTA blood, using the SeptiFast Prep KitMGRADE (Roche Diagnostics GmnH). In the extraction step, an internal control was added to each sample. A negative control supplied by the manufacturer was included in each extraction series, and reagent controls were used as a positive control of the PCR reactions. Quantitative PCR was performed using the SeptiFast method, which is described in details elsewhere [21]. In the SeptiFast test, the internal transcribed spacer (ITS) region is used as the target to specifically distinguish 25 different bacterial and fungal pathogens (Table 1). For the Gram-positive assay, the analysis is based on melting peaks and Cp values. To reduce false-positive results by assumed contaminants, the software includes Cp cut-off values, e.g., for Streptococcus species, this cut-off value is at 20 cycles, and for other Gram-positives at 25 cycles. We have no access to data from samples with Cp values above cut-off, as the test results then were reported as negative. The assay for Gram-negative bacteria is solely based on melting-peaks, and there are no Cp cut-off values. However, for test results positive for Gram-negatives with a Cp value above 25 cycles, the software reports only Cp >25, and no continuous variable. In this study 12 cases with PCR positive for Gram-negatives with Cp values above 25 are included. In the statistical analyses we have given them a Cp value of 25.0.

**Crossing point (Cp)**

During PCR, the amount of PCR product formed is measured at each cycle and reported in fluorescence units. In the SeptiFast test, this starts after cycle six, as the first cycles only focus on annealing at different temperatures. The more target DNA present in a sample, the more
quickly a significant PCR product is generated. A sample is positive if the amount of
fluorescence produced rises above a defined threshold level. A crossing point (Cp) value is
created, showing how many PCR cycles are required to reach the threshold level. Thus, the
more target DNA present in a sample, the lower the Cp value will be. The Cp value is the term
used in LightCycler, and is the same as the threshold cycle (Ct) value in other PCR assays.

Clinical data and definitions
A retrospective chart review was performed by a specialist in Infectious Diseases (I.Z.) for
evaluation of severity of illness, without knowledge of SeptiFast data. Collected data included
demographic details, underlying diseases, source of infection, antibiotic treatment, length of
hospital stay, Intensive care unit (ICU) admission and mortality. Laboratory parameters and
clinical observations (heart rate, blood pressure, temperature, respiratory rate, pulse oximetry)
from the time of admission were recorded. The patient’s clinical condition on admission was
classified as systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis or septic
shock by using the criteria published by the American College of Chest Physicians/Society of
Critical Care Medicine (ACCP/SCCM) [22], see Table 2.

Statistics
Medians and range were used for descriptive statistics of continuous variables, and percentages
for categorical variables. Comparison of groups was performed using the Mann-Whitney-U test
for continuous variables and the Chi-square or Fischer’s exact test for categorical variables. A
p-value of < 0.05 was regarded as significant. Positive predictive values were calculated from
cross-tabulations. The IBM SPSS Statistics, version 21, New York, USA, was used for
calculations.
RESULTS

Study population

In table 3, demographic data and clinical characteristics are shown for the 94 patients with positive SeptiFast PCR. The main foci of infection were urinary tract (n=27, 29%), gastrointestinal/biliary tract (n=11, 12%), skin (n=11, 12%), orthopaedic (n=10, 11%) and chest (n=9, 10%). Six (6%) patients had an unknown focus of sepsis and 15 (16%) had no evidence of bacterial infection.

The most frequently detected pathogens with SeptiFast test in the study were *Escherichia coli* (n=33), *Staphylococcus aureus* (n=24), *Streptococcus* species (n=13) and *Klebsiella pneumoniae/oxytoca* (n=13). Four patients were PCR positive for more than one pathogen (Table 4). Noteworthy is that only 2 cases with *Streptococcus pneumoniae* were detected. This is due to a very low sensitivity for *S. pneumoniae* by SeptiFast test in the prospective study, only 12% (PCR positive/ BC positive =2/16) [21].

Among all 94 patients with positive SeptiFast PCR, 27 patients had severe sepsis or septic shock upon arrival, according to the criteria of ACCP/SCCM. Thirty out of 99 positive PCR results were found in patients with severe sepsis/septic shock.

Cp values in relation to severity of disease

An overview of all bacteria found by SeptiFast PCR in the study, divided into samples from patients with severe sepsis/septic shock or non-severe sepsis, is shown in Table 5. The Cp values were significantly lower in the group with severe sepsis/septic shock compared with the group with non-severe sepsis in the study population. This was shown also in the sub-groups with positive PCR test for *S. aureus*, *Streptococcus* species and *E. coli* (Table 5).
Table 6 and 7 demonstrate positive predictive values (PPV) with different cut-off limits for
detection of severe sepsis/septic shock for the most frequently found pathogens and for all
Gram-positive and Gram-negative bacteria. Positive predictive values of the SeptiFast test
for detection of severe sepsis/septic shock in the entire study population were 34% at SeptiFast
Cp cut-off <25, 35% at Cp cut-off <22.5, 50% at Cp cut-off <20, and 73% at Cp cut-off <17.5.
In table 3 clinical characteristics for patients with Cp <17.5 and >17.5 are presented separately.
Patients with Cp <17.5 do not differ in demographics compared to those with Cp>17.5, but
have significantly more severe sepsis, develop more often septic shock, demand more often
ICU treatment, and have longer hospital stays. We have not calculated on negative predictive
values (NPV), sensitivities and specificities, as we have no data over the number of patients
with severe sepsis/septic shock and negative SeptiFast test.

Eight patients with severe sepsis/septic shock attended the ICU with a median Cp value of 16.3
(range: 7.3-25.0). Among them, 3 patients were subjected to advanced intensive care, including
inotrop drugs and mechanical ventilation; all three were BC positive. One of them, an elderly
man with spondylitis and SeptiFast test positive for S. aureus, with a Cp value of 14.6, died.
The other two patients survived; a young girl with epidural abscess and SeptiFast test positive
for Streptococcus species, Cp 16.6, and an elderly lady with lung abscess and SeptiFast test
positive for S. pneumoniae, Cp 7.3.

Among the 24 patients with findings of S. aureus in PCR, four patients had endocarditis, all of
them were BC positive and two of them had severe sepsis/septic shock. The median Cp value
for patients with endocarditis was 13.6 (range: 10.4-18.6), compared with 20.2 (range: 12.1-
22.2) for the other cases with positive S. aureus PCR (p=0.007).

**BC results in relation to severity of disease and Cp values**
The frequency of severe sepsis/septic shock was higher among BC positive cases, 47% (23/49) compared with BC negative ones, 14% (7/50) in the whole study cohort (p<0.001).

Median Cp values were lower in samples from patients with positive BC compared with those from BC negative patients, i.e. 17.2 (range:7.3-25) versus 21.5 (range:18.6-25.0) in the whole study cohort (p<0.001), 16.4 (range:10.4-21.7) vs 20.9 (range:20.0-22.2) in samples positive for S. aureus (p<0.001), 16.9 (range:14.7-19.9) vs 19.6 (range:18.6-19.8) in samples positive for Streptococcus species (p=0.049), and 19.6 (range:13.7-25) vs 21.9 (range:19.8-25.0) in samples positive for E. coli (p=0.008).

DISCUSSION

To our knowledge, this is the first study to evaluate quantitative data of a commercial PCR assay for bacterial species in blood samples. The results of the study indicate that SeptiFast Cp values correlate to disease severity in community onset bloodstream infections. When the SeptiFast test is used for pathogen identification and PCR positivity is reported, Cp values could probably be useful in identifying patients with severe sepsis/septic shock at an early stage of the disease. A low Cp value, with a cut-off level at 17.5 cycles as proposed in this study, might be used as a predictive tool in the clinical assessment. In addition to rapid information about the aetiology, a positive Septifast test with a Cp value <17.5, could give an early signal about a risk of a more severe disease and indicate that the BC probably also will be positive later on.

Concordance between BC and SeptiFast PCR has been shown to be moderate [21, 23-27], with a high frequency of PCR positive results not confirmed by a positive BC, and vice versa. A
positive PCR but a negative BC can occur due to fastidious microbes, sub-optimal culture conditions or antibiotic treatment before sampling. False-positive PCR results can occur due to contamination from sampling tubes or reagents. To minimize such risk, the EDTA tubes used in the study were endotoxin and DNA-free, and high-quality PCR reagents, free of bacterial or fungal DNA were used in the SeptiFast test, as recommended by the manufacturer.

PCR positivity, even when BC remains negative, was recently found to be correlated to disease severity [27]. This suggests that such a result probably often represents a relevant bloodstream infection that BC has failed to detect.

In the present study there were several positive PCR tests with high Cp values and without microbiological verification, and it is difficult to determine their clinical significance. Many of the BC negative cases had other microbiological or clinical support, but not all of them [21]. A study cohort where all PCR positive samples were supported by a positive BC, or other microbiological or clinical verification, would have been optimal.

A possible explanation to a negative PCR despite a positive BC might be that the sample volume of about one mL, compared with 20 mL in BC, is not always enough to detect bacterial DNA, when the bacterial load is low. This is a limitation with SeptiFast and other PCR assays for bacterial identification directly on blood samples, leading to suboptimal sensitivities. Other reasons to a false-negative SeptiFast test might be competition of the PCR by human DNA in the blood, problems with the selection of species-specific targets in the test, or, for Gram-positive bacteria, especially Streptococcus species, a detection level set to high.

The most apparent advantage of molecular methods compared with BC is the possibility of a rapid diagnosis of aetiology with results available within hours. The time required to conduct a SeptiFast analysis is about 6 hours, but this assumes optimal laboratory conditions, which is difficult to obtain in everyday clinical routine.
A disadvantage with PCR techniques compared with BC is the lack of drug susceptibility testing. However, genetic determinants of drug resistance can be found by PCR. SeptiFast has an optional step where samples positive for *S. aureus* are tested for the presence of the *mecA* gene in a subsequent run. We did not use this step in our study as the frequency of methicillin resistant *S. aureus* (MRSA) is low in Sweden, and all BC positive cases with *S. aureus* were methicillin sensitive in the present study.

Until today, little is known about the bacterial DNA load in *S. aureus* bacteraemia [19]. Concerning the focus of *S. aureus* bloodstream infection, a correlation has been shown between time to positivity in BC, used as a surrogate marker for bacterial load, and endovascular infection sources, such as endocarditis [28]. Recently it was shown that persistently positive SeptiFast test can predict endovascular complications in patients with *S. aureus* bloodstream infections [29]. The endocarditis group in our study was very small, only four patients. Despite this, a correlation was found showing that Cp values seem to be lower in endocarditis than in other *S. aureus* bloodstream infections. Such correlation might be of significant clinical value as there is always a considerable risk of endocarditis in *S. aureus* blood stream infections. The Cp value could be an indicator when endocarditis should be suspected, and distinguish which patients should be subjected to further diagnostics.

Our study has several limitations, whereof the most important is the limited size of the study cohort. Further studies are needed to confirm the correlation between Cp values and disease severity, preferable with larger study cohorts and a higher proportion of patients with severe sepsis/septic shock. For certain pathogens the sample size was too small to enable any statistical analyses for the specific species. We have chosen to present results for the largest groups of specific pathogens, for Gram-positive and Gram-negative pathogens, and for the
entire study cohort. The manufacturer of the SeptiFast test uses different Cp cut-off for
different pathogens, and pathogenicity and growth manner differ between species.
Consequently, the associations we found for groups of pathogens put together are less reliable
than the pathogen-specific associations.
The positive predictive values are calculated on this study cohort where the prevalence of
severe sepsis was about 30% and cannot be generalized. The suggested cut-off level at 17.5
cycles is calculated on this specific study population and needs further validation in other
studies before it can be applicable in a clinical context. This value is also lower compared to
other real-time PCR tests since the fluorescence in the SeptiFast test is not measured the first
six cycles.
The Cp values of the SeptiFast test can only serve as a positive predictive tool, as high Cp
values cannot rule out severe sepsis/septic shock. In a real scenario, there would certainly be a
number of cases with severe sepsis/septic shock and negative PCR, due to the limited
sensitivity of the SeptiFast test [21]. As we lack a control group with such patients, we have not
been able to calculate on negative predictive values, which is a limitation.
Concerning the 12 samples positive for Gram-negative bacteria with Cp values >25, we have
unfortunately no access to continuous variables. We have given them a Cp value of 25.0, which
is a statistical concern. However, this should not affect the found associations, as data is
presented in medians, and all calculations are comparing median values and not means.

Conclusion
Studies have shown that PCR identification by the SeptiFast test has the potential to become a
cost-effective component for managing sepsis [30-32]. The possible benefits of a more rapid
diagnosis of sepsis aetiology would be earlier adequate antimicrobial treatment and reduced
mortality. Still, there is a need to optimize the commercial assays for this purpose, where the
most important issue is to obtain better sensitivities. In addition to this, we want to emphasize the need for further evaluation of quantitative data and its clinical implications. Today there is no information about the amount of DNA of the identified pathogen in the results of commercial PCR tests for sepsis diagnostics. Our results indicate that quantification data would give valuable information to the user concerning the clinical significance of a positive test. As an improvement of such tests, we suggest introduction of automatic presentation of Cp values, (or Ct values when other PCR platforms than the LightCycler are used), as a part of the test result. The manufacturer of The SeptiFast test can easily derive Cp values, and it should be possible to routinely share this information with the user. Even better, for easier interpretation, would be if a calculation of the bacterial DNA load, measured in copies/mL, was included in the test result.

COMPETING INTERESTS
Reagents and means for technical assistance were provided by Roche Diagnostics. All authors declare no conflicts of interest.

AUTHORS’ CONTRIBUTIONS
Conceived and designed the project: IZ, KS, PO, PM. Conducted the prospective study: PJ. Performed the retrospective chart review: IZ. Performed the laboratory experiments: PM, PO. PJ. Analysed the data: IZ, KS, PM. Wrote the paper: IZ. Reviewed the paper: PJ, PM, PO, KS.

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Figure legends

Figure 1

**Title:** Number of samples from patients with severe sepsis/septic shock in relation to positive predictive values.

**Detailed legend:** Bars in blue are showing cumulative numbers of SeptiFast positive samples from patients with severe sepsis/septic shock in the study population at different Cp cut-off values. The green line is showing positive predictive values for detection of severe sepsis/septic shock at different Cp cut-off values.
Additional files provided with this submission:

Additional file 1: Tables140203.docx, 40K
http://www.biomedcentral.com/imedia/1070841900120617/supp1.docx