Orginal article

Comparative Assessment of SepsiTest™ Platform to BactScreen™ and "in-house" MGB-based All Bacteria Assay for Detection of Bacteraemia in Whole Blood Samples

Huda H Al-Griw[1]*, Aisha M. Zaidi[2], Marwan M. Draid[3], and Ahmed El Marghani[4]

[1]Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli, Libya
[2]Department of Physiology, Biochemistry and Animal Nutrition, Faculty of Veterinary Medicine, University of Tripoli, Libya.
[3]Department of Pharmacology, Toxicology and Forensic Medicine, Faculty of Veterinary Medicine, University of Tripoli, Libya.
[4]Department of cell and tissue culture, Biotechnology Research centre (BTRC), Tripoli, Libya.

Received 10 September 2016 / Accepted 20 October 2016

Abstract
Several molecular techniques that are commercially described in the literature for early detection of blood stream infection in attempting to overcome the limitations of the gold standard blood culture. SepsiTest™ is aCE marked commercial platform that has been described in the literature. However, there were no studies addressing the accuracy of the test as a diagnostic platform for detection bacteraemia in whole blood samples. The study, conducted to investigate and discuss the strategy of SepsiTest™ in comparisonto our previously validated real-time PCR based assays (BactScreen™) and the previously published "in-house" minor groove binder (MGB)-based all bacteria assay. The three assays showed different sensitivity patterns for detecting bacteria in blood stream pathogens in favouring of using BactScreen™test. SepsiTest™ could be valuable but their lowest sensitivity in addition to their use of the unspecific SYBR Green fluoresce dye that question its diagnostic accuracy could be used as a last choice molecular diagnostic technique for detection of bacteraemia in whole blood samples. However, SepsiTest™ strategy rather, may provide useful diagnostic tool for detecting live pathogens in food technology.

Keywords: SepsiTest™, BactScreen™, PCR, Bloodstream pathogens

Introduction

Current infection diagnosis is based on standard blood culture techniques. However, microbiological culture has several limitations, not least that it takes several days to confirm infection and is therefore not useful in directing the early treatment with antibiotics. New techniques based on detection of pathogen DNA using real-time polymerase chain reaction (PCR) technology have the potential to address these limitations but their clinical utility is still to be proved. Studies have suggested that levels of bacteria in the bloodstream of patients with sepsis can be as little as 3-10 CFU/ml (Arpi *et al*., 1989;Beekmann *et al*., 2005;Peters et al., 2004). Therefore, maximising assay sensitivity is extremely important when developing effective pathogen DNA assays. The effectiveness of pathogen DNA extraction from blood is also an important determinant of assay sensitivity (Millar *et al*., 2000).

Septi Fast was reported by Lehmann, et al to have a detection limit between 3-100 CFU/ml depending on the pathogen(Lehmann *et al*., 2008).Septi Fast of which is so far the most studied platform proved to be valuable and highly sensitive for ruling in of 25 blood stream pathogens(Dark *et al*., 2015), but are expensive, laborious and complex platform for daily routine use(Leggieri *et al*., 2010).Therefore, finding simple cost less and highly sensitive pathogen detection system continued to be requisite. SepsiTest™ is an alternative commercial available PCR assays described in the literature for its ability to detect broad range of bacterial and fungal pathogens. Although, the assay has gained CE marked for European use but no proper study has addressed their accuracy as a diagnostic platform for detecting of bacteraemia from whole blood samples(Stevenson *et al*., 2016).SepsiTest™ technique is a broad range PCR using universal 16S Ribosomal DNA primer for bacteria and universal 18S ribosomal DNA primers for yeast, coupled with fluorescent detection of the products by SYBR® Green.

The presence of yeast or bacterial DNA is confirmed by melting curve analysis, although the technique does not allow species identification. "in-house" all-bacteria assay is a probe-based system that uses primers targeting the 16S Ribosomal DNA of bacteria and a minor groove binding (MGB) probe for detecting of the PCR products described originally by Ott and colleges (Ott *et al*., 2004). MGB-based tests are believed to be more sensitive than the SYBR Green detection based assays and may also produce less non-specific PCR products. Unfortunately, the MGB assay is unsuitable for melting curve analysis due to the hydrolysis nature of the probe during the reaction. The BactScreen ToolSet is hybridisation probe–based assay specifically adapted for amplification of eubacterial 16S rRNA and distinction of Gram-Negative and Gram-Positive species by Light Cycler PCR with melting curve analysis(Al-Griw, 2011).Our study was aimed to investigate the feasibility and the accuracy of

***Corresponding Author:** Huda AL- Griw. Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli. P. O. Box 13662 Tripoli, Libya.Tele.+218 924720640, Email: huda_algriw@hotmail.com

SepsiTest™ assay for detection of bacterial pathogens from blood samples compared to the "in-house" all-bacteria assay that utilised hydrolysis probe and BactScreen™ assay that utilise hybridised probe.

The present study addressed the quality and quantity of isolated DNA from blood samples spiked with purified bacterial DNA and/or intact organisms using two extraction protocols, namely MolYsis and modified High pure PCR template preparation techniques. The two methods were then used to assess, the performance of the commercially available SepsiTest™ platformon the LightCycler0.2in comparison to the previously evaluated BactScreen™ (Al-Griw, 2011)and "in house" all bacteria primers and MGB probe on the LightCycler480.

Materials and Methods

Spiking of Blood Samples with Intact Bacterial Cells

Blood samples were spiked with known amounts of intact bacterial. These were achieved by re-suspending 10^8 CFU bacterial pellets obtained from culture with 1ml EDTA treated blood samples proved to be clear from infection using Septi Fast assay. Using EDTA blood, 10-fold serial dilutions were prepared for each target bacterial and Genomic DNA was then extracted using different DNA extraction protocols as described below.

DNA extraction

All samples handling and genomic DNA extraction was performed under HEPA bio-safety cabinets class II using aseptic techniques for minimise the risk of contamination. Using MolYsis technique, Blood samples were processed according to the manufacturer's instructions of the SepsiTest™ blood pathogen Detection Kit (Molzym GmbH, Bremen, and Germany). Briefly, 1ml of each spiked whole blood samples were subjected to human DNA depletion protocol following by microbial DNA extraction. The adsorbed pathogenic DNA is then eluted into1.5ml DNA-free reagent tubes with 300 µl of preheated (70°C) DNA-free deionised water. DNA from the same spiked blood samples were extracted using high Pure PCR Template Preparation Kit combined with Red Cell Lysis Buffer (HP-RCL).

This extraction involves lysis and removal of red blood cells before pathogen DNA extraction. Briefly, 1 ml of each spiked whole blood samples were added to 2ml of red cell lysis buffer in a 15ml sterile Falcon tube. The contents were mixed by inverting the tubes several times, placed on a rocking platform for 10 min at room temperature and centrifuged at 2,500 rpm for 5 min. The clear red supernatant was then removed by pipetting and the lysis steps were repeated for another time when required. This washing step removes any remaining lysate, leaving pellets that contain white blood cells and any intact pathogen cells. RCL pellets were then re-suspended into 200µl PBS and transferred into nuclease-free 1.5 ml micro-centrifuge tubes and processed according to the manufacturer's instructions of High Pure PCR template preparation kit(Roche Diagnostics GmbH) with the following modifications, use of 500µl of binding buffer, 80µl of proteinase K and 200µl of isopropanol. The isolated DNA from each tube was eluted in 200µl of pre-warmed elution buffer and stored at -80^{o}C for further use.

Molecular diagnostics

The three PCR assays SepsiTest™, BactScreen™ and "in-house" MGB-based all bacteria assay were carried out using the reagents provided with their kits. SepsiTest™ uses universal primers specific for 16srRNA bacterial sequences covering gram negative and gram-positive bacteria. Each reaction mixture contained; 8 µl DNA-free water, 8 µl 2.5x Mastermix, 2 µl 10x DNA staining solution, 0.8 µl MolTaq 16S enzyme and 2 µl of template DNA.After centrifugation; the PCR amplification was carried out in 20 µl glass capillary tubes in the LightCycler®2.0 instrument according to the following parameters; Pre- incubation cycle at 95°C for 1min, followed by 40 amplification cycles (denaturation at 95°C for 5 seconds, annealing at 55°C for 5 sec and extension at 72°C for 25 sec), a T_m analysis program for one cycle (95 °C, 65°C, 95 °C continuous and then a cooling period to 40 °C for 30 sec.

The methodology of "in-house" MGB-based all bacteria assay was described by Ott and co-workers using universal primers combined with minor groove binder (MGB) fluorescent probes to increase specificity and sensitivity (Ott et al., 2004). PCR amplification was carried out in 96-well PCR plates on the Light Cycler® 480 instrument. In this reaction, conserved primers that bind and amplify the full length of 16SrRNA gene were used (forward TPU1 5'-AGAGTTTGATCMTGGC TCAG; (reverse RTU8 5'-AAGGAGGTGATCCANCCRCA).

The reaction mix contained 5 µM of each primer, 2 µM VIC-labeled MGB universal probe (ACTGAGACACGGTCCA), 10 µl ready to use PCR Master mix (Applied Biosystems) and 2 µl of template DNA in a final volume of 20 µl. The run protocol on the Light Cycler 480 was adapted to the following parameters: pre-incubation program for activation of Taq polymerase (95°C for 10 min), 45 amplification cycles (denaturation at 95°C for 30 secs, annealing at 60°C for 1 min, extension at 72°C for 1.5 min with single fluorescence measurement), Followed by a period of 1 min for cooling the reaction block to 40°C. Fluorescence was detected at 528-564 nm. BactScreen real-time PCR was carried out in 20 µl reactions on the LightCycler®480 instrument. The reaction mix and PCR parameters was prepared as described by Al-griw (2011).

Results

The limit of detection (LOD) of SepsiTest™ assay was determined using purified DNA from Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacterial strains. The assay detected from 50ng to 25fg *S.aureus* DNA/µl with Ct

values increasing as DNA levels decreased. Negative controls containing DNA-free water in place of *S. Aureus* DNA has also generate a significant fluorescence signal. However, in melting curve analysis, the lowest concentration showed expected T_m values (87.63 ±0.1°C) for a Gram-positive species was 500fg/ µl only whereas, reactions; 50 fg/ µl, 25fg/µl of gram positive *S.aureus* DNA and the negative control were observed under T_m values (77.82 ± 0.72 °C) indicating primer dimer or unspecific amplification. Similarly, the fluorescence amplification curves from *E.coli* DNA in the range 1ng/µl to 10fg/µl was also detected by SepsiTest™.

Typical Tm values (88.89± 0.1) were obtained in melting curve analysis for DNA samples for the concentrations down to 500fg/µl -0.001ng/µl though reactions; 100 fg/µl, 50 fg/µl, 25 fg/µl, 10 fg/µl of gram negative *E.coli* DNA and the negative control were observed under T_m values (78.17 ± 0.28 °C) indicating primer dimer or unspecific amplification. The performance of "in house" all bacteria primers and MGB probe on the LightCycler480 in detecting a range of concentrations of purified *E. coli* DNA and *S.aureus* DNA showed a great sensitivity compared than SepsiTest™ and was able to detect down to at least 25fg/µl (50fg/PCR) of *E. coli* DNA or *S.aureus* DNA without any significant amplification of the negative control. Limit of Detection (LOD) of BactScreen™ for DNA from Gram Negative and Gram-Positive Species has been previously evaluated(Al-Griw, 2011).The minimum analytic sensitivity of the assay as confirmed with the melting curve analysis was comparable to the "in house" all bacteria primers and MGB probe assay - 25fg/µl (50fg/PCR) of bacterial DNA.

MolYsis extraction was included as a part of SepsiTest™ protocol. To address whether the sensitivity of SepsiTest™ could be affected with the use of MolYsis extraction protocol for samples prepare, a second extraction technique namely high pure purification kit in conjugation to red cell lysis buffer was used. Both extraction techniques were used for extraction of *E. coli* DNA from spiked blood sample with different concentration of *E. coli* cells. All DNA samples extracted by both methods then assayed using the three real-time PCR assays; SepsiTest™, Hybridized probe-based 16Sr DNA assay (Bactscreen™), and "in house" 16Sr DNA all bacteria and MBG probe. Figure (1) shows summary of the lower detection limits obtained by the three real-time PCR assays. When a MolYsis extraction technique was used, The lower detection limit of samples amplifying using SepsiTest™ assay as confirmed by the correct melting point (Tm values = 88.89 ± 0.1) in the melting curve analysis was only 10^5 CFU/ml Figure (2) (MB). The lower detection limit was raised to 10^3 – 10^4 CFU/ml figure (3) (A). When the same spiked samples were assayed using "in house" all bacteria primers and MGB probe on the LightCycler480, while hybridisation probe–based 16S rDNA assay (BactScreen) for the same samples gave better detection limit (10^3 CFU/ml) figure (4) (MB).By using the second extraction protocol for the same spiked samples, similar detection limit were obtained for SepsiTest™ (10^5 CFU/ml)Fig.1, while samples assayed using all bacteria primers and MGB probe on the LightCycler480 revealed a detection limit of 10^3 CFU/ml, amplifying the same samples with hybridisation probe–based 16S rDNA assay (BactScreen) gave even better detection limit (10^1- 10^2 CFU/ml) Figure (1).

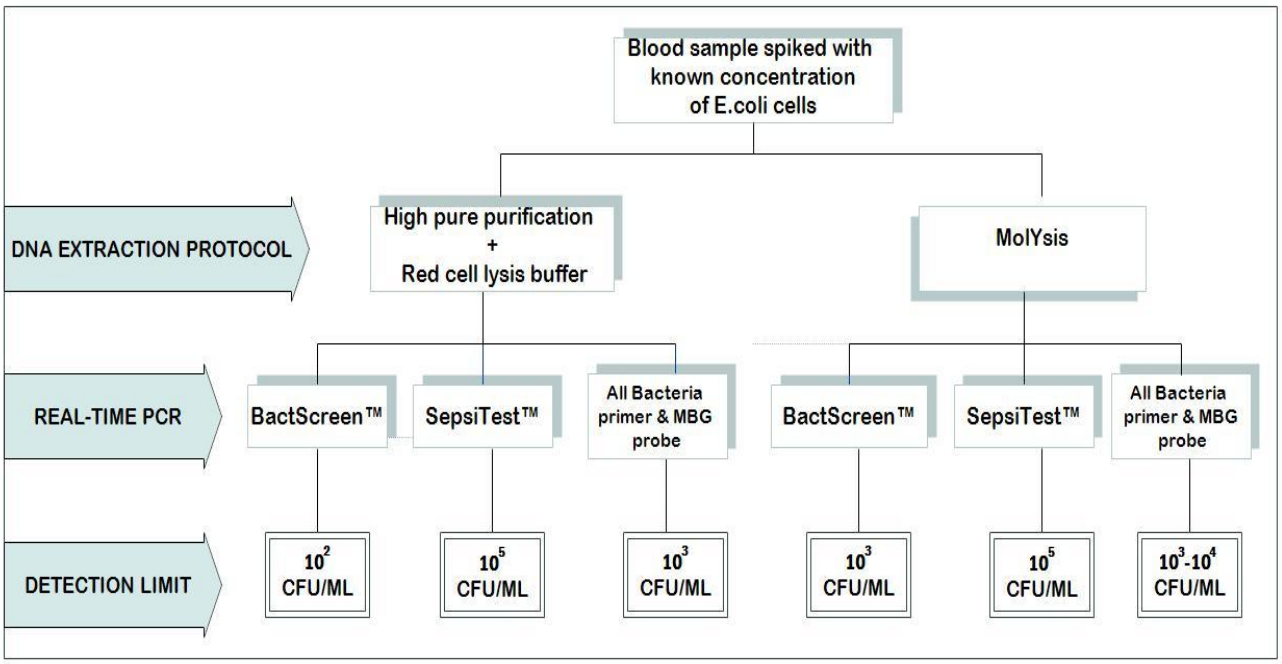

Figure 1. Shows the lower detection limit of the three real-time PCR assays, SepsiTest™, Hybridized probe-based 16Sr DNA assay (Bactscreen™), and "in house" 16Sr DNA all bacteria and MBG probe using either MolYsis or high pure purification technique for extraction *E. coli* DNA from spiked blood sample with different concentration of *E. coli* cells.

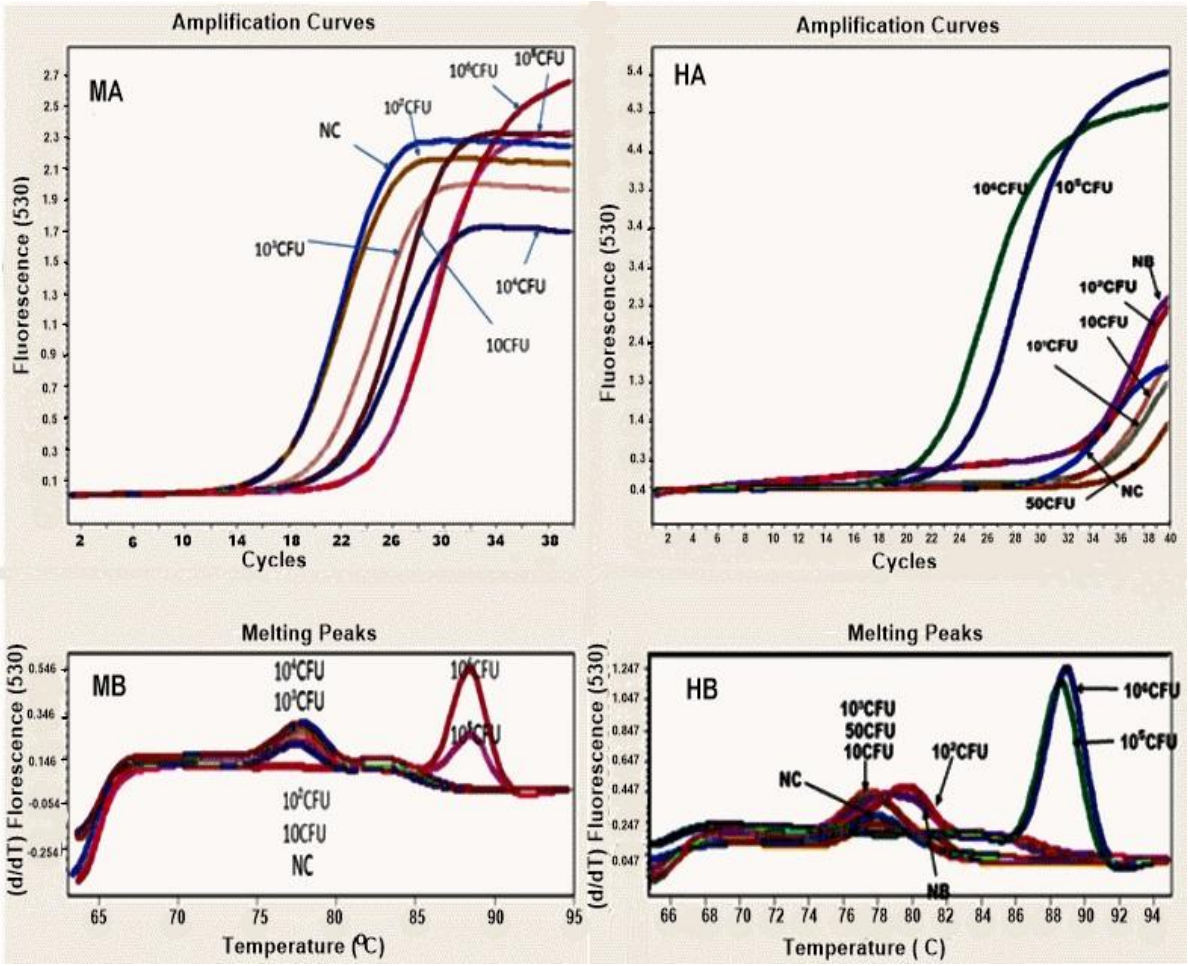

Figure 2. Analysis of spiking blood sample with different concentration of *E. coli* cells extracted either with MolYsis extraction technology (MA& MB) or High pure DNA purification technique (HA and HB), using SepsiTest™. Data shows representative results of real-time SepsiTest™ PCR. Amplification curves (Panel A) and melting curves (Panel B) are shown for spiking with (106- 101CFU/ml) of E. coli un-spiked blood sample (NB) and Negative control (NC).

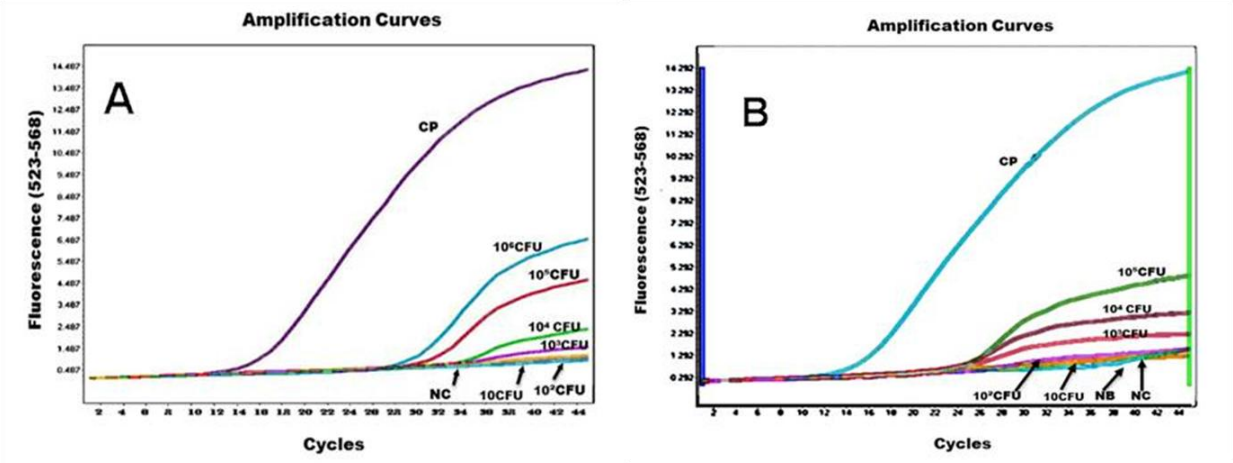

Figure 3. Analysis of spiking blood sample with different concentration of *E. coli* cells using either SepsiTest™™ extraction technique (MolYsis) panel (A) or High pure purification DNA template in conjugation with the use of red cell lysis buffer Panel (B) and assayed using all bacteria primer and probes in house assay on LC480. (NC) Negative control, (NB) none spiked blood sample and (CP) control positive.

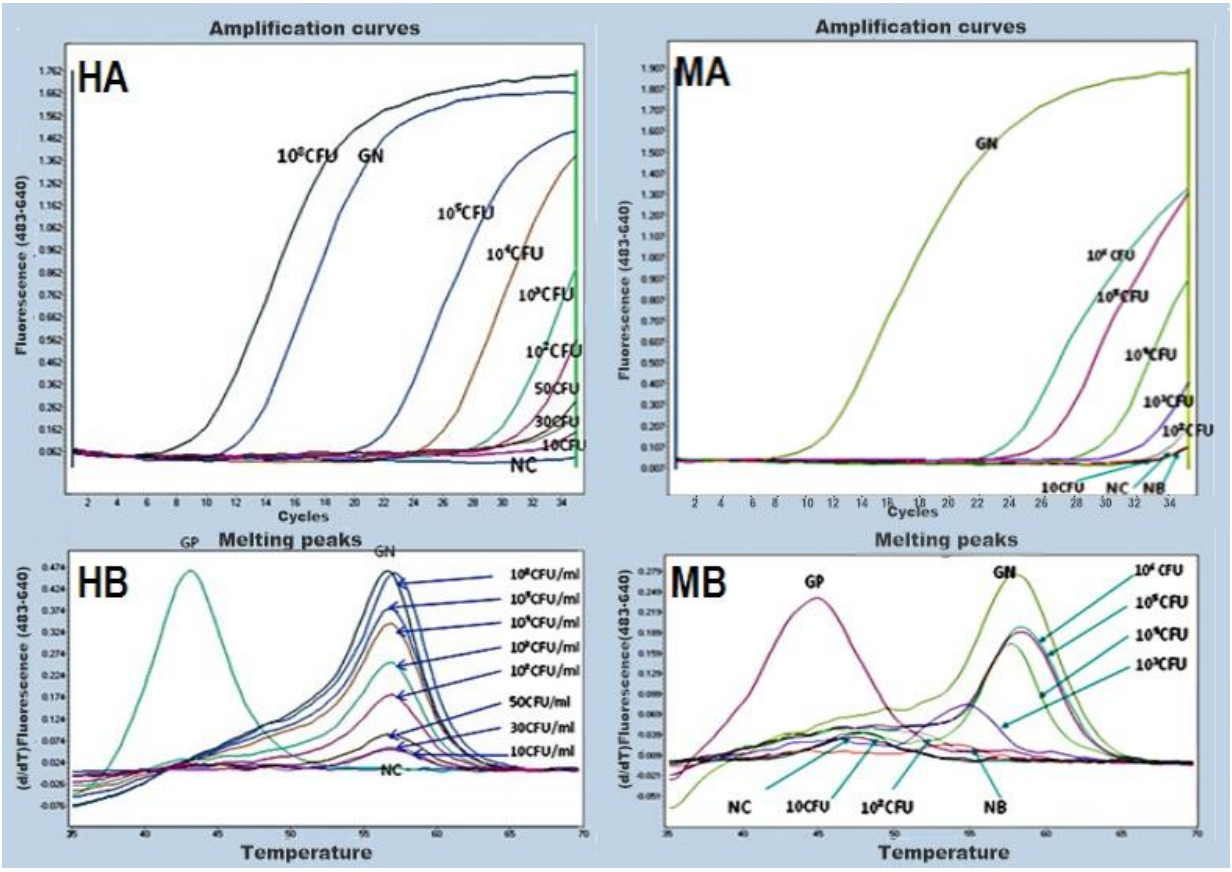

Figure 4. Analysis of spiking blood sample with different concentration of *E.coli* cells extracted with MolYsis (MA and MB) or High pure DNA purification technique (HA and HB) using BactScreen toolset. Data shows representative results of real-time BactScreen PCR. Amplification curves (Panel A) and melting curves (Panel B) are shown for spiking with (108- 101 CFU/ml) of *E.coli*, Negative control (NC),Gram positive control (GP) and Gram negative control (GN).

Discussion

The objective of this study is to address the performance of the commercially available SepsiTest™ against another two real time PCR assays; "in house" all bacteria primer and MGB probe (hydrolysis probe) and Bactscreen (hybridized probe). SepsiTest™ is a complete kit, it uses a MolYsis technology for DNA extraction and use a 16 S Ribosomal RNA primer and a SYBR Green as a florescence dye for the PCR. In the first step of assessment, we aimed to investigate the PCR of SepsiTest™ assay, excluding the effect the extraction technique use to prepare the DNA templet for the assay, meaning to check whether the primer was designed to amplifies the target region as was intended without amplifying of any non-specific products. SepsiTest™ was able to detect only upto500fg/µlto 0.001ng/µl or 0.001-0.002ng/PCR of purified *E.coli* DNA and *S.aureus* DNA equivalent to 2-4 x 10^2 CFU/PCR according to Nadkarni and colleagues(Nadkarni et al., 2002).When the same samples assayed using "in-house" all-bacteria assay, we obtained better detection limit(25fg/µl or 50fg/ PCR equivalent to 10 CFU/PCR for both gram negative and positive bacterial DNA with constant increase to the Ct values as DNA level decreased.

These results have point out that the Real-time PCR is affected by the type of dye or florescence probes used. "In house" all bacteria primers and MGB probe utilize Taq man probe a hydrolysis probe which can monitor the reaction much specifically than SYBR Green dye. However, as it is mechanism of detection relays on the amplification curves while unsuitable for melting curve analysis, any inhibition in the florescence could result in inhibition of the assay itself. The detection limit of BactScreen was previously studied and reported as 62fg/PCR or 12CFU/PCR of purified DNA(Al-Griw, 2011). This result is important because bacteremia is often associated with low numbers of circulating organisms. Kreger et al (1980) Showed that 73% of patients with gram-negative clinical significance bacteremia had less than 10 CFU per ml of blood culture (Kreger *et al*., 1980).

The second step aimed to examine the proposed extraction methods for purified pathogenic DNA and removing the inhibitory substance thus increases the assays sensitivity. These was done by determine the efficiency of the three assays for amplifying DNA that has been extracted from spiking blood samples (thus mimicking true clinical samples) using different methods of DNA extraction. We could only achieve positive results from spiking blood samples with as little

as 10^5 CFU per ml of *E. coli* cells that have been extracted using MolYsis technique and assayed using SepsiTest™ Fig. 3 (MB).Smaller amounts, 10^3- 10^4 and 10^3CFU per ml could be detect when the same DNA samples were assayed either using "in house" All bacteria primers - MGB probe or BactScreen respectively Fig. 2 (A and B). These results suggested that MolYsis technique was not efficient as would expect for removing PCR inhibitions thus increasing assays sensitivity. For more investigation, another extraction protocols named High pure template purification technique was used. These techniques are not intended to remove human or free DNA as suggested by MolYsis. The results indicated that SepsiTest™ PCR continued to have low sensitivity (10^5CFU/ml) even with substituting it is extraction protocol (MolYsis) with high pure template purification techniqueFig.3 (HM). The lower sensitivity of SepsiTest™ may attributed to other blood component rather than human DNA that caused such inhibition specially when using SYBR Green1 as fluorescence detection dye. Moreover, when the same samples assayed using "in house" all bacteria assay,10-foldextra sensitivity was gained with the use of high pure purification technique. However, the inhibition was more prominent with the use of high pure than when MolYsis where used. BactScreen gave sensitivity of 10^3 CFU/ml when MolYsis extraction technique was usedFig.4 (MB) and much more with High pure DNA purification technique (10^1–10^2CFU/ml) Figure (4) (HB).

These results emphasise that with MolYsis extraction technique we not only lose human DNA but a reasonable amount of bacterial DNA could be also removed while the extraction. Moreover, if the primer designed to be highly sensitive, present or absent of human DNA would not affect the sensitivity of the assay. In a comparative study between 5 primer pairs to determine the most sensitive primer for diagnosis of *Brucella* from blood in present or absent of human DNA, one primer pair was found to be most sensitive and promising tool for diagnosis of *Brucella* although it is sensitivity was affected with the present of human DNA (Navarro *et al*., 2002).

The MolYsis DNA isolation kit as a part of SepsiTest™ kit, proved to be effective in removing PCR inhibitors, this was obvious when DNA extracted from spiked blood samples assayed using the all bacteria primers and MGB probe on the LightCycler480 while DNA extracted from spiked blood samples using other extraction technique such high pure purification fail to amplify with the same efficiency Figure (2) (A and B). Gebert *et al*., 2008compared the efficiency of using MolYsis complete DNA isolation kit with QIAamp DNA Mini Kit (Qiagen) and High Pure PCR Template Preparation Kit (Roche) in conjunction with a universal Gram-differential -PCR to monitor the time to positive signalling in spiked blood cultures. He confirmed the efficiency of MolYsis DNA isolation kit for removing the PCR inhibitors from blood cultures comparing with the other studied techniques. However, they reported a lower detection limit of 10-20 CFU/ml for Gram-positive bacteria and 200-400 CFU/ml for Gram-negative bacteria(Gebert *et al*., 2008).

Similarly, MÜhln (2010) reported a lower detection limit of110-460CFU/ml for Gram-negative and 20-40CFU/ml for Gram-positive using the same extraction technique (Muhl *et al*., 2010). Although, the PCR of those two studies were different from each other and from our study, the detection limit difference between Gram-positive and Gram negative suggested that the sensitivity of those assays has been dramatically affected by the extraction techniques.

Gram-positive bacteria have rigid cell walls, which to some extant can stand the lysis action of MolDNase enzyme, in contrast to Gram-negative bacteria that have relatively weaker cell wall and higher fat content, making it more susceptible to the diffusion of MolDNase inside the cells and fragile to the effect of residual DNAase. Indeed, the application of MolDNase as a first step to remove human DNA can cause loss of some bacterial cells specially those affected with the treatment of antibiotic. The loss of bacterial DNA in the extraction could be related to three points. First, diffusion of the MolDNase through the thin labile bacterial cell wall (e.g. genus *treponema*) or fragile bacterial cell wall such as those affected with antibiotic or attacked with body defense mechanism (Horz *et al*., 2008). Second, the protocol of the extraction lacks the heat inactivation step thus the effect of residual DNAase would be continued on the finally released template DNA following the washing step. Residual DNAase activity has been documented even with incubation on 95 °C for 50 min of heat treatment(Hanaki et al., 2000),(Silkie et al., 2008). Finally, our previous study suggested that at least 90% of free DNA could be removed with the supernatant following the centrifugation step without of application of MolDNase (Al-Griw, 2011). This is particularly important in case of low grad bacteremia where the amount of bacteria DNA fall under the detection limit of the assay.

The lower sensitivity of SepsiTest™ especially for gram-negative bacteria raises some concern and uncertainty about the effectiveness of using this test for diagnosis of gram-negative sepsis. Horz *et al* (2008) raise very important question about how much removing of interfering human DNA is necessary and how much reduction in bacterial DNA would be acceptable(Horz *et al*., 2008).Moreover, It has been reported that the 16S rDNA gene codes for the 16S ribosomal is found only in bacteria phylum and therefore it is unlikely that 16S ribosomal PCR-based assay could be affected by mammalian DNA contamination(Sleigh *et al*., 2001), the more likely is that other blood components are responsible of such interfering. In fact, losing of 90% of bacterial DNA could be compensate by addition of 3 or 4 cycles or concentrate the bacterial DNA by reducing the finally amount of elution buffer (e.g. 30µl).

Unfortunately, SepsiTest™ uses SYBR Green as fluorescence detection that would gave unspecific

amplification and primer dimer particularly with low copies of pathogenic DNA.

In fact, MolYsis DNA extraction technique for elimination of human DNA may become useful if larger volume of blood samples is used i.e.≥ 5ml, although the efficiency of the extraction may still be affected when high amount of free DNA in the sample. In normal blood sample the amount of human DNA ~ 15ng/µl (Al-Griw, 2011)and using 5ml of blood sample could raise the amount of human DNA to 75ng/ µl and even more in septic patient, thus removing of 90% of human DNA meaning at least 7.5ng/µl would be remaining.

The strategy of removing free DNA using molYsis extraction techniques incorporated in SepsiTest™ may improve the PCR amplification but would be more useful to be used in food technology. Similar strategy were developed by Mukhopadhyay *et al*., 2002, to eliminate the free extraneous DNA released in the media following death and lysis of pathogenic cells to predict the possible live pathogen present in the food(Mukhopadhyay and Mukhopadhyay, 2002).

Although SepsiTest™ has gained CE mark for European use; no comprehensive evaluation studies are available in the literature up to date. Wellinghausen *et al* ., (2009) evaluated SepsiTest™ performance for rapid detection of blood stream infection compared to blood culture on 342 clinical blood sample taken from 187 patients whom exhibit SIRS, sepsis or neutropenic fever(Wellinghausen *et al*., 2009). Although, the sensitivity and specificity of the assay was 87% and 85% in respectively, the result was rather difficult to interpret. Considering 41 samples in which PCR was positive and blood culture negative, only 11 samples who classified as probable to true bacteremia were confirmed from other body cultures in that, 5 samples were Coagulase-negative *staphylococci* (CNS). Moreover, in at least three episodes of PCR positive in which the result classified as indeterminate PCR results, non-pathogenic soil and water bacterial species were found.

These results raise a big concern about the accuracy of the sampling methods particularly that most blood samples taken from peripheral catheter systems.CNS in most cases represent contaminations from the skin flora although they can be aetiologically relevant (Beekmann *et al*., 2005). Contaminant bacteria DNAmay get access into PCR reactions from plastic ware, anti-coagulant, or skin flora itself (Sontakke *et al*., 2009). Unfortunately, SepsiTest™ has not adjusted for acceptable level of contamination with CNS as was done with Septi Fast (Haag *et al*., 2013). The problem of contamination and PCR-culture discrepant results was reported by Haag H *et al*., (2013). In their study SepsiTest™ was evaluated for their use in routine diagnostics. Typical 96 specimens comprised tissue from the heart in case of infective endocarditis (IE), synovial tissue from suspected prosthetic joint infections, or blood and blood cultures from patients with suspected blood stream infection were PCR analysed and compared to culture results. Focusing on the14-blood sample among the samples studied, two whole blood (WB) samples were invalid and thus excluded from the analyses due to sample cross contamination. Four cases where concordant negative and concordant true positive approved infections in two cases both with *S. aureus* pathogens. Among four cases that classified as culture-negative while PCR analyses was positive, one case was considered to be false positive because the relevance of the PCR finding is not clear. Interestingly, three blood cultures were taken from the same patient, two-yielded growth of *Enterococcus faecium* while the third remained negative.

The PCR analyses of the third blood culture identified *A. johnsonii and Corynebacterium* Spp. This PCR was classified as likely contaminant and false negative. Presence of *A. johnsonii* and *Corynebacterium* reflects presumably a contamination event during blood drawing or during routine culture processing in the bacteriology unit (Haag *et al*., 2013).In general, the promising sensitivity and specificity of SepsiTest™ assayreported by Wellinghausen *et al*., 2009 and the study results of Haag *et al*., (2013) did not reflect the quality of the test. Detection of soil, water or irrelevant bacteria species confirming the unspecific amplification as indicates by the use of SYBR Green dyeand raise important question of whether the melting curve analysis were usedin these studies as a primary analysis for positive cases otherwise sequencing of such samples are misleading, costly and time consuming. Keeping in mind the overall actual cost for each specimen (180€), hands-on time and the highly demand for molecular diagnostics skilled technician to perform the test, we believe that SepsiTest™ assay was not robust enough to be used alongside blood culture for identification of infectious agent present in the blood.

References

Al-Griw H (2011). Molecular detection of bloodstream pathogens in critical illness. PhD, The University of Manchester, UK.

Arpi M, Bentzon M W, Jensen J and Frederiksen W (1989). Importance of blood volume cultured in the detection of bacteremia. *Eur J Clin Microbiol Infect Dis*. 8**:**838-842.

Beekmann S E, Diekema D J and Doern G V (2005). Determining the clinical significance of coagulase-negative staphylococci isolated from blood cultures. *Infect Control Hosp Epidemiol*. 26**:**559-566.

Dark P, Blackwood B, Gates S, McAuley D, Perkins GD, McMullan R, Wilson C, Graham D, Timms K and Warhurst G (2015). Accuracy of lightcycler ((r)) septi*fast* for the detection and identification of pathogens in the blood of patients with suspected sepsis: A systematic review and meta-analysis. *Intensive Care Med*. 41**:**21-33.

Gebert S, Siegel D and Wellinghausen N (2008). Rapid detection of pathogens in blood culture bottles by real-time pcr in conjunction with the pre-analytic tool molysis. *J. Infect*, 57**,** 307-316.

Haag H, Locher F and Nolte O (2013). Molecular diagnosis of microbial aetiologies using sepsitest™ in the daily routine of a diagnostic laboratory. *Diagn Microbiol Infect Dis*. 76**:**413-418.

Hanaki K, Nakatake H, Yamamoto K, Odawara T and Yoshikura H (2000). Dnase i activity retained after heat inactivation in standard buffer. *Biotechniques*,.29**:**38-40, 42.

Horz H P, Scheer S, Huenger F, Vianna M E and Conrads G (2008). Selective isolation of bacterial DNA from human clinical specimens. *J Microbiol Methods*. 72**:**98-102.

Kreger B E, Craven D E, Carling PC and McCabe WR (1980). Gram-negative bacteremia. Iii. Reassessment of etiology, epidemiology and ecology in 612 patients. *Am J Med*. 68**:** 332-343.

Leggieri N, Rida A, Francois P and Schrenzel J (2010). Molecular diagnosis of bloodstream infections: Planning to (physically) reach the bedside. *Curr Opin Infect Dis*. 23**:**311-319.

Lehmann L E, Hunfeld K P, Emrich T, Haberhausen G, Wissing H, Hoeft A and Stuber F (2008). A multiplex real-time pcr assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples. *Med Microbiol Immunol*. 197**:**313-324.

Millar B C, Jiru X, Moore J E and Earle J A (2000). A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material. *J Microbiol Methods*. 42**:**139-147.

Muhl H, Kochem A J, Disque C and Sakka S G (2010). Activity and DNA contamination of commercial polymerase chain reaction reagents for the universal 16s rdna real-time polymerase chain reaction detection of bacterial pathogens in blood. *Diagn Microbiol Infect Dis.* 66**:**41-49.

Mukhopadhyay U K and Mukhopadhyay A (2002). A low-cost, rapid, sensitive and reliable pcr-based alternative method for predicting the presence of possible live microbial contaminants in food. *Current Sci*. 83**:**53-56.

Nadkarni M A, Martin F E, Jacques N A and Hunter N (2002). Determination of bacterial load by real-time pcr using a broad-range (universal) probe and primers set. *Microbiol.* 148**:**257-266.

Navarro E, Escribano J, Fernandez J and Solera J (2002). Comparison of three different pcr methods for detection of brucella spp in human blood samples. *FEMS Immunol Med Microbiol*. 34**:**147-151.

Ott S J, Musfeldt M, Ullmann U, Hampe J and Schreiber S (2004). Quantification of intestinal bacterial populations by real-time pcr with a universal primer set and minor groove binder probes: A global approach to the enteric flora. *J Clin Microbiol*. 42**:**2566-2572.

Peters R P, Mohammadi T, Vandenbroucke-Grauls C M, Danner S A, van Agtmael M A and Savelkoul PH (2004). Detection of bacterial DNA in blood samples from febrile patients: Underestimated infection or emerging contamination. *FEMS Immunol Med Microbiol*. 42**:**249-253.

Silkie S S, Tolcher M P and Nelson K L (2008). Reagent decontamination to eliminate false-positives in escherichia coli qpcr. *J Microbiol Methods*. 72**:**275-282.

Sleigh J, Cursons R and La PM (2001). Detection of bacteraemia in critically ill patients using 16s rdna polymerase chain reaction and DNA sequencing. *Intensive Care Med*. 27**:**1269-1273.

Sontakke S, Cadenas M B, Maggi R G, Diniz P P and Breitschwerdt E B (2009). Use of broad range16s rdna pcr in clinical microbiology. *J Microbiol Methods*. 76**:**217-225.

Stevenson M, Pandor A, Martyn-St James M, Rafia R, Uttley L, Stevens J, Sanderson J, Wong R, Perkins G D, McMullan R and Dark P (2016). Sepsis: The lightcycler septi*fast* test mgrade(r), sepsitest™ and iridica bac bsi assay for rapidly identifying bloodstream bacteria and fungi - a systematic review and economic evaluation. *Health technology assessment (Winchester, England)*, 20**:**1-246.

Wellinghausen N, Kochem A J, Disque C, Muhl H, Gebert S, Winter J, Matten J and Sakka SG (2009). Diagnosis of bacteremia in whole-blood samples by use of a commercial universal 16s rrna gene-based pcr and sequence analysis. *J Clin Microbiol*. 47**:**2759-2765.