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Design Of Novel Sample Handling Tool To Facilitate Molecular Diagnosis Of Infection Without Blood Culturing

Bharath B R[1] and Alka Mehta[1]

Abstract

Bacterial infections can lead to many diseases, ranging from a simple food poisoning to severe arthritis. They are also the most common cause of healthcare linked infections. The Health care linked infections often known as Healthcare-associated infections (HAIs), have become one of the major public health threats. A prerequisite for successful treatment of any bacterial infection stems in the early yet sensitive diagnostic test that can detect the bacteria with high specificity. Point of care diagnostic underlying research outlined in this paper discusses at length the product development and proof of concept of one such point-of-care diagnostic test.

This proof of concept describes the method developed for sample processing and molecular diagnostics. The entire procedure takes only about 120 minutes, avoiding laborious blood cultures and provides a detection limit of ~1-10 CFU/mL bacteria. This diagnostic test can be used as a high throughput screening platform to diagnose a wide range of microorganisms from blood samples.

Keywords: *Blood Cell Lysis Reagent, PCR, Single Step DNA Isolation, Hospital Acquired Infection and Achromopeptidase.*

Introduction:

Healthcare-associated infections (HAIs) are one of the major public health threats nowadays[1,2]. HIA arises due to number of invasive procedures and the devices like catheters/ventilators being used t[1]o treat patients in the current healthcare system. HAI are prevalent in hospitals, especially in ICU[2,3]. Ninety percent of these infection caused by bacteria, and staphylococcus is most common infection of blood[1,4].

The HIAs mainly include central line associated bloodstream infections, catheter associated urinary tract infections, ventilator associated pneumonia and surgical site infections [3,5,6]. The etiological agents responsible for these infections are diverse and often resistant to antibiotics[7–9]. Different bacteria are able to assemble biofilms persisting in healthcare units, becoming more resistant to the antibiotic and being responsible for HAIs onset and spread[8,10]. A prerequisite for the successful treatment is early diagnosis for such bacterial infections with specific and sensitive methods for detecting microorganisms[11].

Even in the most extreme cases of infection, the pathogen load is very low and requires the blood samples to be cultured in order to increase the cell load for the diagnosis[11–13]. In addition, the cells are often heterogeneously distributed in the blood sample and are difficult to handle. This increases the time taken for the results to be obtained from the tests often leading to prescription of broad spectrum antibiotics[3,14–16]. The unchecked usage of broad spectrum antibiotics is a major cause of generation of multi drug resistant microorganisms[6,17,18].

[1]School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India.

For these reasons a fast, sensitive, and specific system is essential for the bacterial detection to enable early and specific intervention[12,19]. Here, we describe a test system based on PCR with sample processing steps that does not include blood culturing but allows the specific detection of small numbers (less than 10) of bacterial cells per millilitre in less than two hours.

This paper deals with the diagnosis of blood borne bacterial pathogens from hospital associated infections The objective of this project is to facilitate the molecular based detection methods for blood contaminants without affecting cell integrity of the microorganism during sample processing steps like blood lysing. This proof of concept shows the increase in sensitivity of the bacterial cell detection using blood samples spiked with bacteria. As an example we have used an avirulent bacterial strain of Staphylococcus aureus for spiking the blood samples in the lab setting. The paper further describes the lysing procedure used to selectively lyse the blood cells without compromising on the cellular integrity of the bacterium. Post the lysis of blood cells, the microorganism will be captured onto a membrane facilitating the nucleic acid extraction. Commercially available forward and reverse Oligonucleotide primers were used in polymerase chain reaction (PCR) to amplify and detect a specific gene FEM A responsible for encoding an enzyme amino acyltransferase in Staphylococcus aureus[20,21].

Material and Methods

Materials

Ammonium Chloride (254134), Potassium Bi-Carbonate (237205), Ethylene diamine tetra acetic acid (E9884), Achromopeptidase (A3547), Cellulose Acetate Filter Membrane, pore size 0.22 μm (CLS8160) were procured from Sigma Aldrich and Staphylococcus aureus with ATCC 25923 was used for the study.

Bacteriological culture

The current research has performed with a small scale broth culture and single colony isolation as per the standard protocol. ReaMetrix India Pvt Ltd., provided samples and strains of bacteria (Staphylococcus aureus, ATCC 25923, as explained by traditional microbiological approaches). In order to isolate a specific single colony the streak plate method used. Relationship between OD at 600 nm and CFU/mL was established by diluting the overnight broth bacteria cultures.

Collection of Blood

Blood samples were sourced from Jana Care Solution Pvt. Ltd. Bangalore with the ethical board approval number NHH/MEC-CL-2016-400. Blood samples used for the study are unidentified random samples without any patient details from persons not having any bacterial infection at the time of blood collection or in recent past. Bacterial cells were spiked at various concentrations to mimic the infected patient's sample.

Enzyme Solution for Bacterial Cell Lysis

Achromopeptidase was dissolved at a concentration 1 U/μL using Tris/EDTA buffer.

Blood Cell Lysis Reagent

Ammonium Chloride, Potassium Bi-Carbonate and Ethylene diamine tetra acetic acid (EDTA) was selected as a key chemical for the blood cell lysis reagent formulation. The concentration for Ammonium Chloride varied between 0.45 to 1.35%, Potassium Bi-Carbonate between 0.05 to 0.15% and EDTA between 0.0019 to 0.0056%. Each formulation was prepared using Milli Q water in 100 mL volumetric flask. The optimized lysis reagent was mixed with blood samples in 1:10 ratio and the degree of hemolysis and culture compatibility with the microorganism was studied.

Blood Smear Preparation and Imaging
The blood samples were diluted using 1X PBS (as negative control) and Optimized Blood Lysis Reagent (0.9% Ammonium Chloride, 0.1% Potassium Bi-Carbonate, 0.0037% Ethylenediaminetetraacetic acid, pH 7.4). After blood lysis, a drop of blood was applied on a glass slides and covered with coverslip, Upright research microscope Axio Imager 2 (Carl Zeiss) was used to capture the images at 40X lens.

DNA isolation on membrane
First, blood samples spiked with bacterial cells were treated with Blood Cell Lysis Reagent in proportion with a 1:10 ratio followed by incubation for 5 minutes. The mixtures was transferred onto a prefiltration step using glass frits with ~15 µm pore size and finally into a cellulose acetate filter membrane having pore size 0.22 µm. Later centrifuged at 16000 rpm for 1 minute at 25°C. RBC cell lysate flow through was discarded. The bacterial cells captured onto the cellulose acetate membrane were treated with Achromopeptidase enzyme solution for 10 minutes and centrifuged again at 16 000 ×rpm for 1 min. The DNA was collected as flow through and the time required to do this practice was noted ~15 minutes.

PCR and primer sets
In order to perform PCR, the current research has used T100™ Thermal Cycler (Bio-Rad) which was run under the following conditions: The initiation of the PCR started with denaturation for five minutes at the temperature 94°C followed by annealing for 1 minute at 61.6°C and amplification at 72°C for 1 minutes and repeated for 30 cycles. The final extension was allowed for 2 minutes at 72°C. The whole PCR procedure was completed for one hour and forty minutes.

The primers were selected based on literature survey to amplify and detect FEM A gene responsible for encoding an enzyme amino acyltransferase in Staphylococcus aureus. The forward primer 5′-AAACGGACGGCCCAATTCTAAACC-3′ and reverse primer 5′-CCACGTTTAAAGCGGTATACGCCA-3′ used as per manual present in the kit. The procured primer was dissolved by using Tris/EDTA buffer at a concentration of 100 pmol/µL. Later PCR mixture was prepared and carried out using 0.2 mL tubes containing a total of 25 µL of liquid which included 2.5 µL of 10× buffer, 0.5 µL dNTP, each (forward and reverse) primer, 0.33 µL Taq polymerase (upto 5 units required to make final concentration), 1 µL of isolated DNA, and double distilled H_2O. The PCR product was separated on 1% agarose gels. Later the product was scanned, and examined on trance illuminator (Bio-Rad). The expected 241 bp long PCR product was visualized on agarose gel.

Results

Optimisation of Blood Lysis Reagent
Ammonium Chloride, Potassium Bi-Carbonate and Ethylenediaminetetraacetic acid (EDTA) varied between 0.45 to 1.35%, 0.05 to 0.15% and 0.0019 to 0.0056% respectively. Each formulation was tested on blood samples with various levels of hemoglobin between 6 g/dL to 23 g/dL. Hemoglobin estimation was performed using HemoCue Hb 201+ analyzer in duplicates. Blood samples were mixed with each of the prepared formulations in 1:10 ratio and observed for visual hemolysis and compared against the negative control 1X PBS. Each formulation was tested on 40 blood samples in 5 replicates for lysis as shown in Figure 1.

Blood samples treated with optimised Blood Lysis Reagent (1.1% Ammonium Chloride, 0.12% Potassium Bi-Carbonate, 0.0027% Ethylenediaminetetraacetic acid, pH 7.4) was observed under the upright research microscope Axio Imager 2 (Carl Zeiss) 40X lens and

compared before and after hemolysis as shown in the Figure 1b. Based on the images Figure 1 and 2, the blood sample was completely hemolyzed within 10 minutes of incubation with the optimised Blood Lysis Reagent.

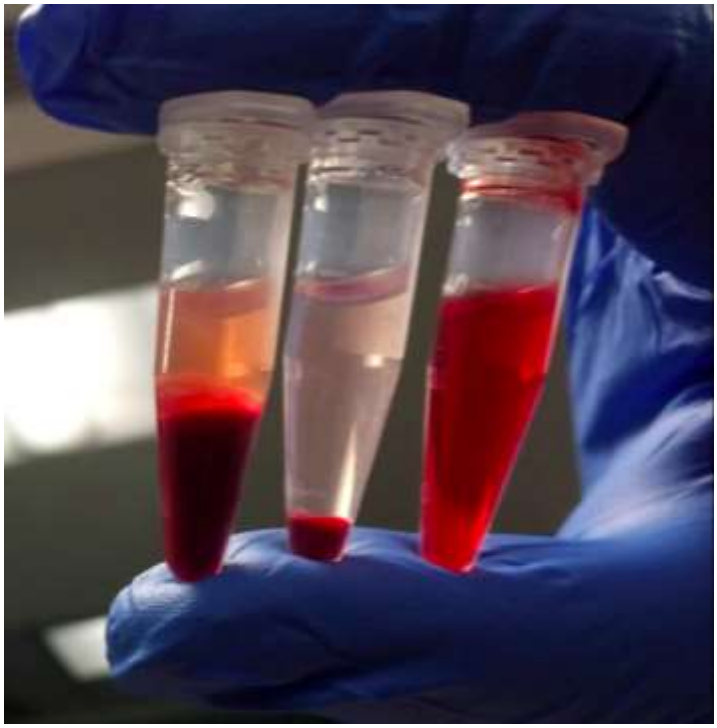

Figure 1 : Blood cell lysis with newly formulated hemolysis reagent Tube 1-Blood Sample, Tube 2-Blood Sample in 1X PBS, Tube 3-Blood Sample with Blood Lysis Reagent

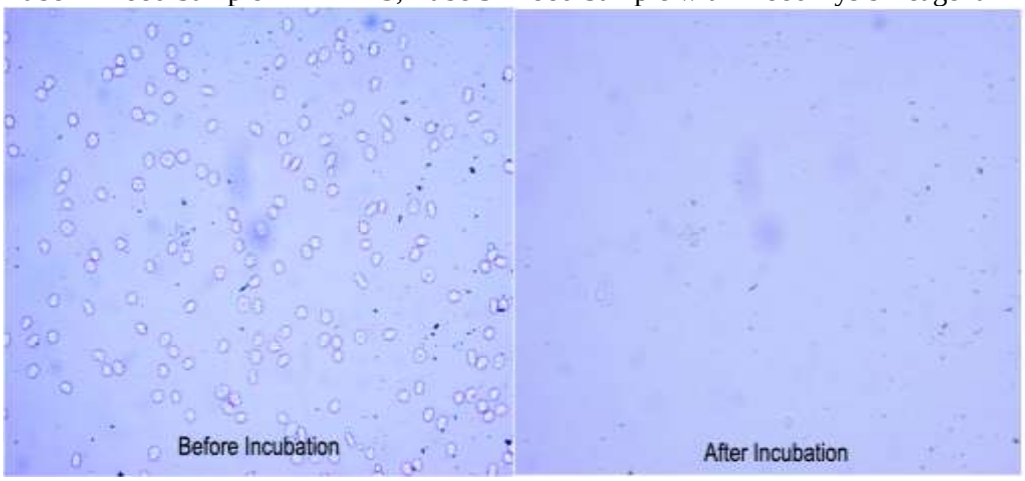

Figure 2 : Blood samples under microscope showing blood cells Before and After the Hemolysis with lysis buffer

Culture Compatibility

Overnight cultured cells with OD 0.6 were treated with the Blood Cell Lysis Reagent in the ratio 1:1 and incubated for 60 minutes at room temperature. Post incubation the mixture was diluted 10 times using 1X PBS and 100 µL was plated onto the LB Agar media. Overnight cultured cells without the treatment of Blood Cell Lysis Reagent was considered as positive control. The plate was incubated at 37°C overnight for the observation of colonies and compared against the positive control as shown in Figure 3. No difference in colony formation indicating that blood lysis formulation does not lyse or damage bacterial cells.

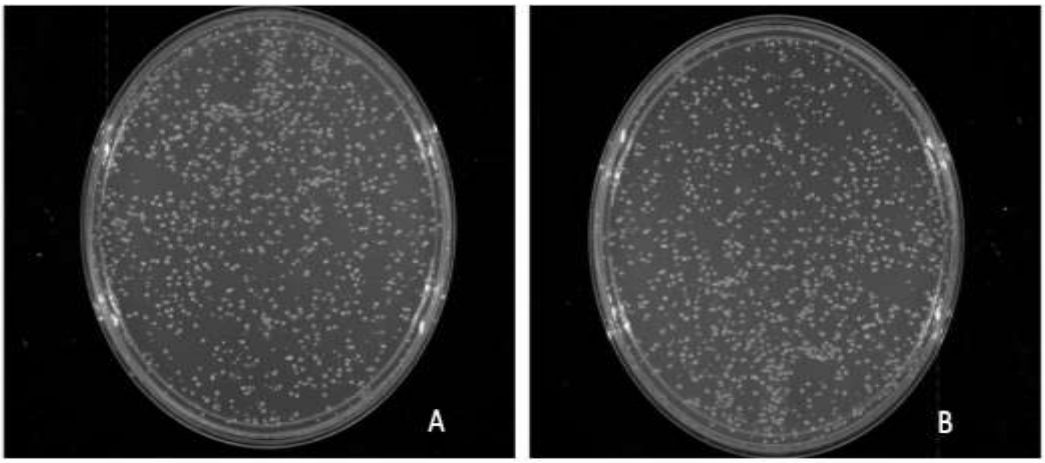

Figure 3: Effect of blood lysis reagent on the viability of Staphylococcus aureas studied by colony formation assay on LB agar medium. A- culture treated with hemolysis formulation, B- treated with equal volume of PBS

Limit of Detection:

Bacterial cells with known concentration were spiked to the blood sample. The sample was processed as described in the material sections, and the limit of detection was evaluated. Figure 4 displays the data in a demonstrative way where DNA was isolated from 10^3, 10^2, 10 and 1 CFU/mL (S aureus) and PCR amplified. As shown in Figure 4, 1 CFU/mL bacteria can be detected.

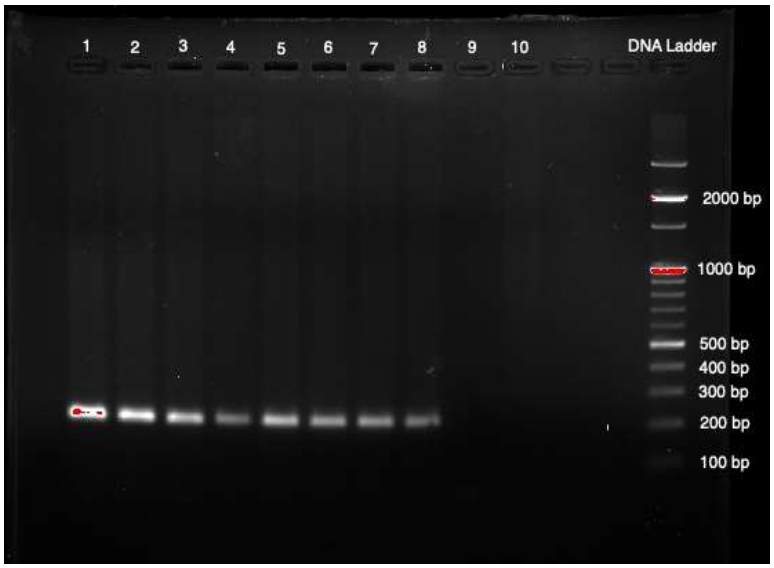

Figure 4: PCR Results. The DNA was isolated from bacteria (S aureus) using Achromopeptidase.

As shown in Figure 4, the lane 1-8 shows DNA amplification. The lane 1-8 had amplification at high to low end. In this sense lane 1-2 had cell load 10^3 where lane 3 and 4 had 10^2. The lane 5 and 6 was having cell load of 10 whereas lane 7 and 8 had 1 cell load. The cell load unit was in CFU/mL (Colony Forming Unit). The lane 9 and 10 had no bacterial DNA hence served as negative control while the last lane served as molecular size marker.

Discussion and Conclusion:

Diagnostics in infectious diseases are in the midst of a technological revolution[22]. Bacterial infections are of serious concern for health care professionals[2]. For more than a century,

confirming a bacterial infection involved isolating a pathogen by growing it from culture from a sterile body sample. This process was slow, imprecise, and subject to errors introduced by collection conditions and growth media requirements of fastidious organisms[23,24].

A prerequisite for the successful treatment of bacterial infections is a specific and sensitive method of detecting microorganisms[11]. Some methods to detect bacteria are time consuming like culturing techniques, whereas others are faster but lack specificity and/or sensitivity like microscopy techniques[11,12,25]. Even in the most extreme cases of infection, the pathogen load is very low. The cells are often heterogeneously distributed, and the sample is difficult to handle. This leads to a high rate of false positivity during diagnosis. Isolation and concentration of the cells of interest increases the sensitivity of the diagnostic assay. Sample processing is often required prior to the cell concentration depending on the properties of the biological sample. Blood have a nominal cell load that is many folds higher than that of the pathogenic microorganism. Hence, prior to concentration of these pathogens, there is a need to isolate them from the clinical matrix[20]. A size-exclusion based filter can be used to eliminate the accumulation of unwanted cell debris onto capture membrane. Capture membrane concentrate the cells which can be detected by staining, culture and/or molecular based detection techniques[12,25].

An attempt was made to show a proof-of-concept by overcoming the need of culturing the blood samples to increase the cell load of the microorganism. In the current research the presence of the bacteria spiked at various levels to the blood samples was able to detect the presence using PCR technique with a detection limit of ~1-10 CFU/mL. The proposed approach was performed in an extremely short time window and has shown promising results over reducing the crucial diagnosis time around 2 hours from the time of sample processing. Considering the sensitivity with the proposed approach and in addition with the PCR assay using specific primers, one can develop any novel molecular tests for routine clinical diagnosis[19,26]. In this sense the current research has demonstrated a proof of concept for a sample handling tool focusing on the blood contaminants to facilitate molecular based testing without culturing the sample.

Future prospects: There is always a gap between invention and engineering mostly in the development of a methodology which is faster, effective and act in an efficient way[22,27]. The current research has proven an effective shortened period of time for diagnosis. The incorporating of novel methodologies and associated results can effectively be used at larger scale by IVD (Invitro Diagnostics) manufacturers[27]. The current research hasn't worked on building any specific platform for a particular disease but gives certain key components like Blood Lysis Reagent without impacting the cellular integrity and single step DNA isolation procedure. These concepts can be integrated to achieve the best turnaround time for diagnosis[11]. The idea of this proof of concept has to be validated at various levels. Taking advantage of well-established molecular platforms while keeping an eye on new emerging components may provide the best mix of novelty and scalability for IVD manufacturers to create a new diagnostic test.

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