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Improving molecular diagnosis of neonatal septicaemia: Comparative evaluation of different protocols for extraction of bacterial and fungal DNA from blood samples

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Abstract

Blood stream infections are important causes of morbidity and mortality. Early diagnosis and treatment with appropriate antibiotics are vital for better outcome. Equally important is avoiding treatment with broad spectrum antibiotics due to delay in diagnosis.

Nucleic acid amplification directly from blood samples (whole blood /plasma) has the potential of providing a faster method for the diagnosis of blood stream infections.

This study evaluated 5 protocols for extracting bacterial and fungal DNA from blood samples. DNA extracts were assessed for purity and concentration and analyzed for bacterial and fungal rDNA gene target using PCR. Protocol 2[spiked whole blood; blood cell lysis +ZR Fungal/Bacterial DNA kit], Protocol 3[spiked whole blood; blood cell lysis+QIAampDNA Mini kit] and Protocol 5[Spiked plasma; QIAampDNA Mini kit] yielded relatively pure DNA with median absorbance ratio of 1.78, 1.71and 1.73respectively.Protocol 2 and Protocol 3 yielded significantly higher mean DNA concentration than Protocol1, Protocol 4 and protocol 5 ($p<0.05$).Protocol 3 showed lowest detection limit for gram positive bacteria, gram negative bacteria and fungi.

In summary whole blood treated with blood cell lysis step followed by QIAamp DNA Mini kit proved to be the effective method for isolating bacterial and fungal DNA from whole blood in cases of blood stream infections.

Keywords: DNA extraction, Spin column, bead beating, bacteria, fungi, whole blood, plasma.

Introduction

Molecular identification of highly conserved bacterial (16S and 23S) and fungal (18S and 28S) ribosomal DNA targets can be used for diagnosis of blood stream infections.[1,2,3]

Standardizing extraction of bacterial and fungal DNA from blood samples is a crucial step for optimizing Nucleic acid amplification assays for the diagnosis of septicaemia. There are certain challenges in isolating bacterial /fungal DNA from blood.

These include presence of PCR inhibitors in blood like heme, lactoferrin, and IgG. The problem further increases due to high amount of human DNA versus the low amount of microbial DNA in whole blood. Blood in cases of septicaemia contain microorganisms of various cell wall structures, i.e. Gram-positive bacteria, Gram-negative bacteria, yeast or filamentous fungi. [4,5,6]

In scientific literature, methods for bacterial and fungal DNA isolation from blood are described, but many are enabling one to separately isolate either bacterial or fungal DNA from blood.[4,7]

It is necessary to compile several processes, for obtaining high-quality DNA matrix.

This study was done to establish sensitive, reproducible, cost effective and easy to use DNA extraction protocol that should be universal in its ability to extract bacterial and fungal DNA from blood samples. It should avoid possible cross contamination of samples. The final aim was to extract DNA with good quality (purity and intactness) and quantity.

Materials and Methods

The present study was conducted in the Department of Microbiology, Jawaharlal Nehru Medical College and Central Research Laboratory DattaMeghe Institute of Medical Sciences, Sawangi (Meghe), Wardha. A written permission from the Institutional Ethical Committee was obtained before starting the study.

It was experimental study using spiked blood and plasma samples

Spiked whole blood samples:

Blood of healthy volunteers was used for spiking experiment.

Following reference strains were used *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC10231.

10 fold serial dilutions were prepared from the bacterial suspension (Undiluted to 10^{-7}) with bacterial copy number of 1.5 x 10^{8} CFU/ml to 1.5 x 10^{1} CFU/ml.

10 fold serial dilutions were prepared from the fungal suspension (Undiluted to 10^{-7}) with fungal copy number of 5 x 10^{8} CFU/ml to 5 x 10^{1} CFU/ml.

For both the bacteria and fungi the above dilutions were prepared for 1ml of final volume. One milliliter aliquot was centrifuged at 4000 rpm for 10 minutes and supernatant was discarded. The pellets were suspended in 1 ml of whole blood or plasma. Such spiked samples were further used for DNA extraction.

DNA Extraction:

For DNA extraction we used commercially available kits for whole blood and plasma samples. Different protocols used were

Protocol	Sample	Kit used	Blood cell lysis procedure
Protocol 1	Whole blood	Zymo kit	No
Protocol 2	Whole blood	Zymo kit	Yes
Protocol 3	Whole blood	Qiagen kit	Yes
Protocol 4	Plasma	Zymo kit	No
Protocol 5	Plasma	Qiagen kit	No

Blood cell lysis -In protocol 2 and protocol 3 blood cell lysis procedures was done on spiked blood samples before extraction using kits. 1ml spiked EDTA-blood was mixed with 3 ml red-cell lysis buffer (Sigma), incubated on ice for 10–15 min and Centrifuged at 3000 rpm for 10 min. Supernatant was discarded. Red-cell lysis buffer was added to the pellet and the same procedure is repeated twice or thrice. The cell pellet was resuspended in 300 µl white-cell lysis buffer (WCLB: RCLB containing200 µg/ml Proteinase K) and incubated at 65°C for 45 min. Centrifuged

at 5000 rpm for 10 min. Discarded the supernatant. Cell pellet suspended in 200 µl of PBS (phosphate buffered saline) was used for DNA extraction.

ZR Fungal/Bacterial DNA kit (Zymo kit) – 200 µl sample was taken in ZR bashing bead lysis tube. After adding 750 µl of lysis solution tubes were vortexed at the maximal speed for 5 minutes and subsequently centrifuged at 10,000 rpm for 1 minute. The supernatant was transferred to Zymo-Spin™ IV Spin filter and centrifuged at 7000 rpm for 1 minute. Filtrate after adding fungal/bacterial DNA binding buffer was transferred to Zymo-Spin™ IIC column. Centrifuged at 10,000 rpm for 1 minute. After washing with appropriate buffer DNA was eluted with 60 µl of DNA elution buffer.

QIAampDNA Mini kit(Qiagen kit)- 20 µl of Qiagen proteinase K was added to 200 µl sample along with 200 µl of buffer AL, and the sample was incubated for 30 minutes at 56^0C . 200 µl of 100% ethanol was added and the resulting lysate was loaded onto the QIAamp DNA mini kit column. The column was centrifuged at 6,000 rpm for 1 minute.and washed with 500 µl of buffers AW1 and AW2 successively. Purified nucleic acid was eluted with 100 µl of buffer AE.

Quality and quantity assessment of the extracted DNA

Characterisation of DNA extraction product for quality (purity) and quantity (concentration and extraction efficiency) was determined using a 1 µl extract analysed via spectrophotometer. Ratio of absorbance of light at 260nm and 280nm (A_{260}/A_{280})between 1.7&2 was taken as indicator for pure DNA.

Concentration by spectrophometry (µg/ml) = $(A_{260} – A_{320}) \times$ dilution factor × 50µg/ml

DNA concentration was also measured by Fluorometry (Qubit).

Sensitivity of DNA extraction protocols

Considering the results of quality and quantity estimation Protocol 2, Protocol 3, Protocol 4 and Protocol 5 were selected for further analysis. All DNA extracts were amplified via real time PCR using 16SrRNA(for bacterial) and ITS3,ITS4 (for fungal) primers.[1,8]This was done individually for reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans*ATCC10231) in each of their dilution. PCR was performed in a total volume of 25 uL PCR reaction mixture containing 2X SYBR(12.5 µl),10 pmol primer solution (2µl), Template DNA (5µl) ,ROX(0.50µl) and Nuclease Free water (5 µl). The procedure of amplification for bacterial PCR consisted of: 94°C for 1 minute and 40 cycles of 94°C for 5 sec., 57°C for 20 sec. and 72°C for 20 sec. The procedure of amplification for candidial PCR consisted of94°C for 1 minute and 40 cycles of 94°C for 5 sec., 59 °C for 20 sec. and 72°C for 20 sec. Positive and negative controls were included throughout the procedure. No template controls with nuclease free water were incorporated in each run.

In addition to amplification all the PCR products were tested for gel electrophoresis.Gel electrophoresis with 1.5% agarose gel was conducted with 1XTBE Buffer.2 ul of PCR products were analyzed in the electrophoresis at 60 volts for 30 minutes.

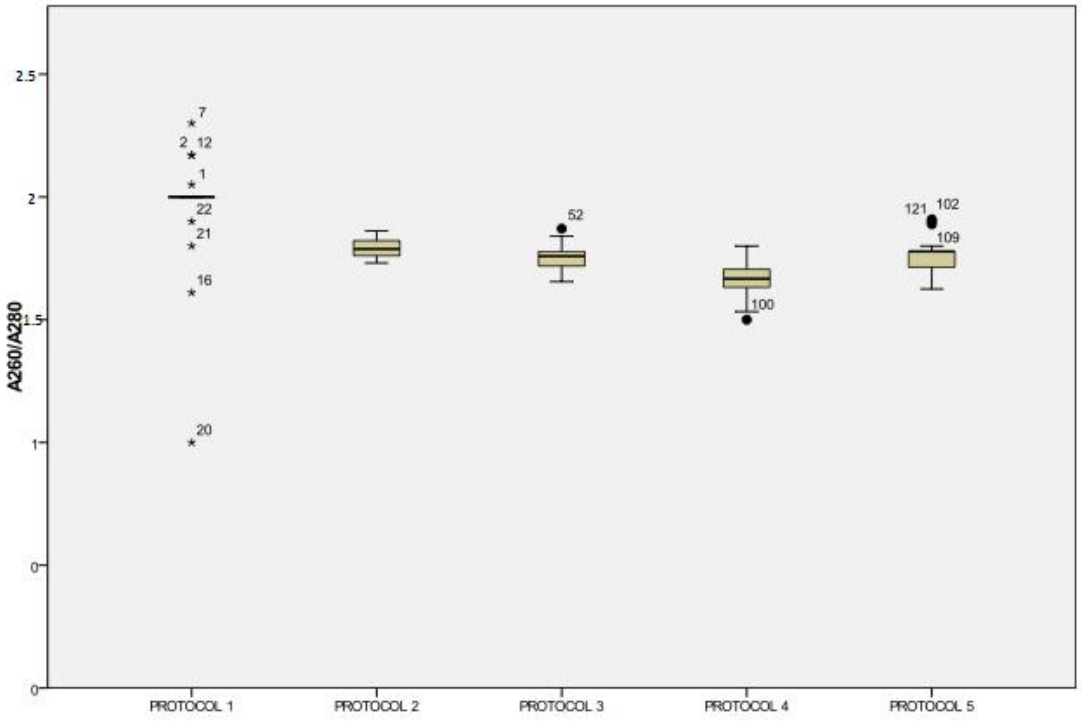
Results

Purity of extracted DNA

Box plot was plotted comparing absorbance ratios at 260nm and 280nm (A_{260}/A_{280})of the resulting DNA extracts as determined by 5 protocols. Each plot reveals the median (midline), inter-quartile range (IQR; box), 10th and 90th percentiles (error bars), and minimum/ maximum outliers.(Figure 1)

When compared against standard absorbance ratio of 1.8 representing pure double stranded DNA,Protocol 2, Protocol 3 and Protocol 5 yielded relatively pure DNA with median absorbance ratio of 1.78(IQR=1.62 – 1.93); 1.71(IQR =1.59 – 1.90) and 1.73(IQR=1.60 – 1.75) respectively.(Figure 1)

The median absorbance ratio of 2(IQR=1.9-2.1) in Protocol 1 and 1.58 (IQR=1.60-1.75) in Protocol 5 suggest RNA and protein contamination respectively.

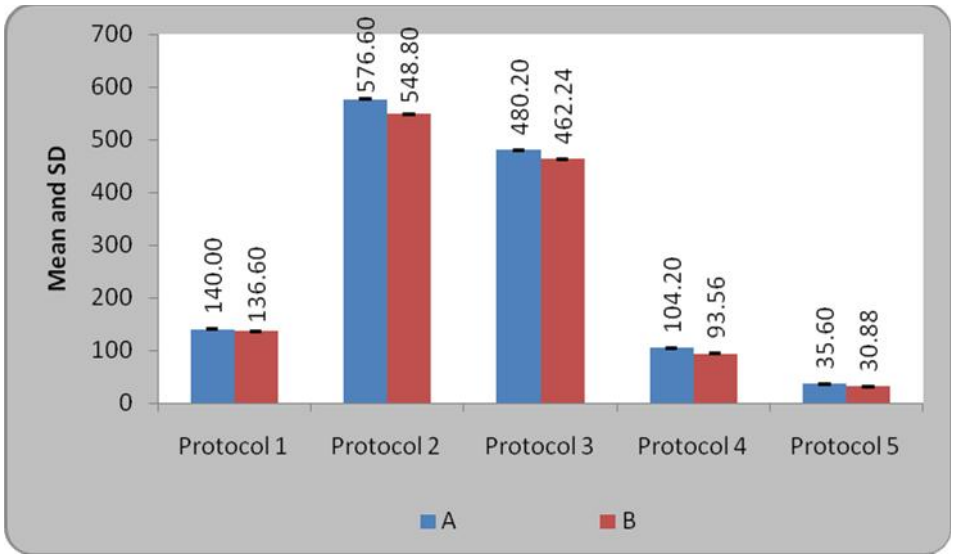


. = min, . = max, * extreme skewed values

Figure1- Box plot of A_{260}/A_{280} by different DNA extraction protocols

Concentration (yield) of extracted DNA

The calculated value of DNA concentration from spectrophotometric absorbance as well as reading from Qubitflurometer revealed that protocol 2 and Protocol 3 yielded significantly higher mean DNA concentration than Protocol1, Protocol 4 and protocol 5.[Figure 2]



A-Concentration by spectrophotometer; B- Concentration by Qubit

Figure 2- Comparison of mean DNA concentration of DNA extracts generated from 5 protocols

Sensitivity of DNA extraction protocols

The limit of detection of various protocols for detection of three species by real time SYBR green PCR is depicted in Table 1.

Table 1. Sensitivity of DNA extraction protocols

Protocol	Limit of detection		
	***Escherichia coli* ATCC 25922**	***Staphylococcus aureus* ATCC 25923**	***Candida albicans* ATCC10231**
Protocol 2	1.5 x10 ^{1}CFU/ml	1.5 x10 ^{2}CFU/ml	5x10 ^{3}CFU/ml
Protocol 3	1.5 x10 ^{1}CFU/ml	1.5 x10 ^{2}CFU/ml	5x10 ^{2}CFU/ml
Protocol 4	1.5 x10 ^{2}CFU/ml	1.5 x10 ^{2}CFU/ml	5x10^{3}CFU/ml
Protocol 5	1.5 x10 ^{2}CFU/ml	1.5 x10 ^{2}CFU/ml	5x10 ^{2}CFU/ml

Protocol 3 showed lowest detection limit for all the reference strains tested.

For fungi Qiagen method DNA samples showed lower Ct values and high intensity bands on gel electrophoresis of the PCR product as compared to ZR bio kit method. Qiagen method is found to be better as compared to ZR bio method due to reproducible and consistent results.

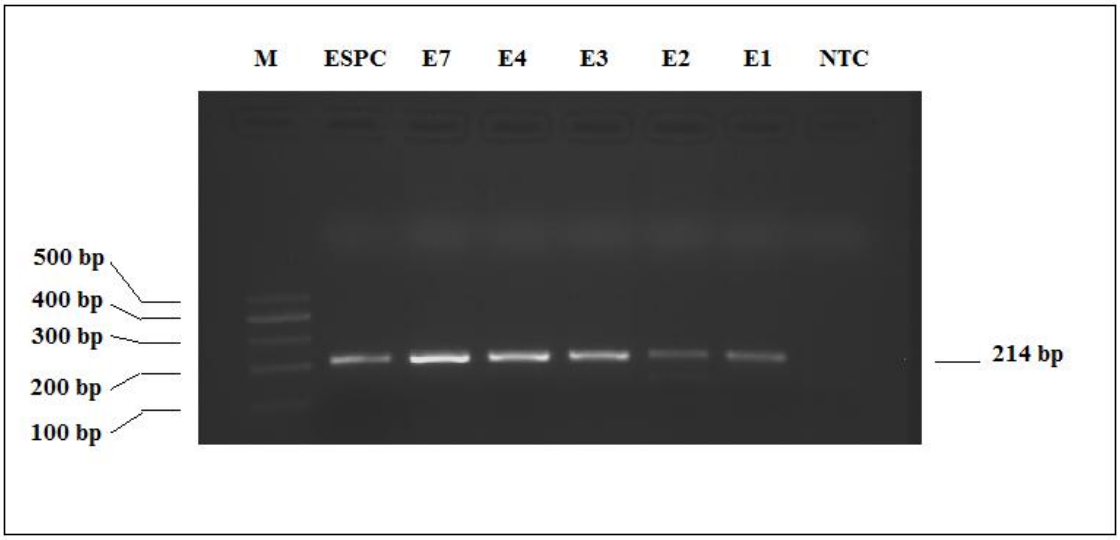


Figure 3: Bacterial PCR loaded on 1.5% agarose gel with Molecular Marker 100-500bp

Discussion

In order to optimize high sensitivity broad range real time PCR assay for the early diagnosis of neonatal septicaemia we evaluated different protocols of DNA extraction.

High sensitive PCR requires DNA extraction method with high efficiency and removal of PCR inhibitors. Many commercial kits were developed and evaluated. In the present study we evaluated different DNA extraction protocols using whole blood/plasma samples and kits with enzyme+spincolumn/spin column+bead beating for extraction of bacterial/fungal DNA from blood samples. We also assessed the use of blood cell lysis for extracting microbial DNA from whole blood samples.

In the present study protocol 2 and protocol 3 with preliminary blood cell lysis was found helpful in getting pure, intact DNA in high concentration compared to protocol 1 without blood cell lysis. This finding was in concordance to other studies using preliminary blood cell lysis step. [5]

We found protocol 2 using whole blood samples, blood cell lysis + step ZR Fungal/Bacterial DNA kit(bead beating+spin column)best in terms of purity and concentration of DNA obtained. The use of bead beating was also found useful by some researchers as a method for extraction of pathogenic nucleic acids from microorganisms specially fungi. [5,11]There are many advantages of bead beating like it is faster as well as cheaper than enzymatic treatments, permits safe handling of Samples, allows all the steps to be carried out with a single tube and thus can avoid cross contamination among samples.

Protocol 3 using whole blood samples, blood cell lysis step and QIAampDNA Mini kit (enzymiclysis+spin column) was found best in terms of limit of detection for bacterial and fungal DNA. YeaStaranother kit on the principle of Enzymiclysis, and spin column was also quoted as optimal for extraction of Candida DNA from whole blood.[11,12]

Spin column based kits with some modified protocols were successfully used in past for extraction of bacterial and fungal DNA from blood, plasma, serum.[1,4,9]These kits have low costs and they are easy to use. The standard equipment required for their use is available in most routine laboratories. Most protocols take about 45 minutes to 1 hour to complete, producing high yields of DNA with minimum contamination.

When tested for sensitivity using real time PCR overall Protocol 3 showed lowest values with limit of detection (LOD) for *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* 15 CFU/ml, 150 CFU/ml and 500 CFU/ml respectively.LOD for gram positive and gram negative bacteria was less than fungal LOD which may be due to the fact that their cell wall lyses more easily so it is possible to get larger amount of DNA from the same number of cells in comparison to fungi.

Protocol 2 was found equally sensitive for the *Escherichia coli* and *Staphylococcus aureus*. But for *Candida albicans* it showed high LOD of 5000 CFU/ml.

In earlier report of DNA of spiked whole blood was extracted by using preanalysis sample treatment with enzymes (lysozyme, lysostaphin, lyticase) and glass beads followed by commercial columns, LOD was 3CFU /ml for *Escherichia coli*, 30 CFU /ml for *Staphylococcus aureus* and 300 CFU/ml for *Candida albicans* and *Aspergillus fumigatus*.[5] In another study it was 10^3 CFU/ml for gram positive and gram negative bacteria and 10^4 CFU/mL, respectively for fungi.[12] In another study using Taqman based broad range PCR using 16SrRNA gene it was 10^3 CFU/ml for *Escherichia coli* and *Staphylococcus aureus*.[13]Lowest sensitivity reported was 10^1 CFU/ml for each of the four studied species of microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus fumigatus*).[14]

This difference in LOD may be due to various factors like method of DNA extraction, primers-probes used, reagents and PCR reaction used.

In this study Assessing DNA extract quantity and quality should be applied when developing and validating DNA extraction method for microbial detection as no single extraction method was optimal for all organisms. Similarly single method cannot be applied to all samples.

To validate the results there is need to assess these protocols for quality and quantity using blood samples from blood culture positive cases.

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