SELECTIVE AMPLIFICATION OF BACTERIAL 16S rDNA SEQUENCE FROM CLINICAL SAMPLES

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ABSTRACT

Background: Conventional blood culture method is time consuming and less sensitive; when fastidious or un-culturalable organisms are involved. The use of PCR targeting the 16S rRNA allows detection of bacteria; however, these primers have ability toco-amplifyhuman DNA. This Polymerase Chain Reaction (PCR) method is based on nucleic acid amplification test.

Objective of the study: This study determined a method for selective amplification of bacterial DNA from clinical samples without co-amplification of human DNA.

Materials and methods: Seventy one blood samples from clinically suspected cases of early onset neonatal sepsis were collected and analysed in parallel by culture and 16S rRNA amplification.DNA was extracted using commercial extraction QiAmp mini DNA kit and subjected to 16S rRNA amplification. The products were sequenced, analysed and comparedwith blood culture results. Positive and negative controls were used for extraction and amplification respectively.

Results: Out of 71 samples analysed, 5 (7.0%) samples by blood culture were equally positive for 16S rRNA PCR; the PCR was also able to identified 16 (22.5%) more positive samples which blood culture could not identify, but only 1 (1.4%) sample was identified positive using blood culture while PCR identified it as negative. During the study, 7 (9.9%) samples were identified positive by conventional blood culture but later found to be contaminants.

Conclusion: This study confirmed the presence of 16S rRNA among bacterial isolates and modification of PCR protocol with shorter denaturation temperature and time, leading to selective amplification of bacterial DNA. Therefore, there is need to carry-out this investigation on both culturable and unculturable specimens.

Keywords:*16SrRNA amplification, bacterial DNA, human DNA and Polymerase chain*

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INTRODUCTION

The diagnosis of bacterial infections can be difficult and time consuming especially the unculturable ones(Becker-Andre and Hahlbrock, 1989; Leser*et al.* 1995). Survival and activity of Pseudomonas sp. strain B13(FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacteria. Rapid and reliable molecular method is important in detection of potentially infected patients, particularly the young and the elderly. This would prevent unnecessary hospitalizations, empirical treatment and reduce associated medical costs as well as improve the quality of care. Polymerase chain reaction (PCR) amplification utilizing a universal bacterial primer pair, would allow rapid identification of the presence or absence of bacterial DNA, along with an identification of the bacterial species present. In several PCR, the template is a mixture of homologous genes. Examples are the amplification of several genes families from the DNA of a single species, the amplification of genes coding for rRNA (rDNA) from genomic DNA extracted from natural communities of microbesamplification of bacterial DNA from eukaryotes (Geert *et al.* 2008).The amplicon products are generated from template DNA by a process involving complex chemical kinetics, and the relative abundance of homologs among the final reaction products is usually a parameter of interest. Several sources of bias and error in a 16S rRNA gene amplification have been mentioned previously (Schloss, *et al.* 2011). We define biases as a misrepresentation of the relative abundances of microbial populations in a sample and errors as a misrepresentation of an actual sequence due to PCR amplification and sequencing. Errors can be introduced through several means. First, PCR polymerases typically have error rates of 1 substitution per 10^5–10^6 bases (Cline *et al.* 1996). Second, when amplifying DNA fragments from a heterogeneous template, there is a risk of chimera formation when incomplete PCR products serve as primers to amplify related fragments; the rate of chimerism is thought to range from 5 to 45% (Haas *et al.* 2011). Finally, errors are introduced in sequencing, regardless of the technology (Schloss, *et al.* 2011).Co-amplification of eukaryotic DNA with 16S rRNA gene-based PCR primers has been else reported as possible consequences for population fingerprinting of complex microbial communities(Geert*et al.,*2008). The cross-reactivity of 16S rRNA gene primers with non-target eukaryotic DNA has been reported previously (Rivas *et al*. 2004). This study has confirmed previous sporadic indications in literatures indicating that several commonly applied 16S rRNA gene primer sets lack specificity toward bacteria in the presence of eukaryotic DNA. Unless subsequent analysis of individual community ampliconscloning and sequencing is performed, the phenomenon of cross-reactivity remainsa potential source of systematic error in all 16SrRNA studies from eukaryotic samples. Amplification of high GC content genes by PCR is a major challenge during PCR this may be due to the difficulty in DNA denaturation (Nadia and Min, 2021).

The critical role that annealing plays in efficient PCR amplification cannot be over emphasize, annealing can only be achieve when there is complete dissociation of double stranded DNA. High GC content in human genomecan pose an extra challenge to the amplification process because of the high melting temperature (Tm) due to the extra hydrogen bonding between cytosine and guanine base pairs(Borah, 2011). It is on this basis we introduce a strategy that aim at the selective amplification of bacterial DNA from clinical specimens without co-amplification of human DNA using any common DNA isolation kit available.

MATERIALS AND METHODS

This study was carried out at Neonatal intensive Care Units (NICUs) of Hospital Kuala Lumpur (HKL), Malysia. The Kuala Lumpur had a population of 32 million people with about 13000 health facilities both government and private as at 2010 (CEIC data. 2017); health facilities both government and private. The study population was made up of new-born babies up to 48 hours of birth who were admitted to the Neonatal Intensive Care Units (NICUs) of Hospital Kuala Lumpur (HKL), Malaysia. This hospital serves as a tertiary and referral hospital. It is located on 150 acres of prime land in the city with 84 wards and 2,300 beds, making it one of the largest hospitals in the world. More than 90 per cent of the beds in HKL are allocated for subsidized patients, providing access to an internationally established standard of affordable healthcare (MoHM, 2016). This study is cross-sectional prospective laboratory based study conducted on assented participants admitted in Neonatal Intensive Care Units (NICUs) of Hospital Kuala Lumpur (HKL), Malysiathose that showed signed and symptom of sepsis within a period of 6 months. Ethical approval to carry out this study was obtained from the hospital ethic committee of Hospital Kuala Lumpur (HKL), the study was performed according to Helsinkin, and questionnaire was administered to obtained demographic characteristic, factors associated with the disease in the study area.

The sample size in this study was calculated according to the formula described by Cochrane (1963) and Glenn (2010).

$n = [z^2 p (1- p)] \div [d^2]$

where: n = number of samples

z = statistical level of confidence at 95% = 1.96

p = prevalence = 4.92 (0.0492)

d = allowable error of 5% (0.05)

Therefore; $n = [(1.96)^2 \times 0.0492 (1- 0.0492)] \div [(0.05)^2] = 71$

n = 71 samples will be used in this study.

Specimen and Data Collection

Blood for laboratory investigation were drawn from seventy one (71)assented participants prior to antibiotic administration; 3mls of blood sample was collected from each neonate following standard procedure. One millilitre (1ml) of the blood sample was added into paediatric blood culture bottle and transported to microbiology laboratory for immediate incubation in automated BACTEC machine.

Positives culture were alerted after 72 hours period of incubation, the identified bottle was sub-cultured on chocolate, sheep blood agar and Mac-Conkey agar these pates were further incubated at 37^0C for the period of 24hours, some plates were cultured aerobically, facultative an aerobe and streak aerobe. Isolates were identified using biochemical.

PCR Analysis

DNA extraction

DNA was extracted according the method described by QIAmp DNA mini kit (Qiagen, Germany). The blood samples sent to the research laboratory were stored in a refrigerator at 4^0C for 72 hours or until there was a clear separation into plasma and red cell layers. The plasma was carefully removed into a sterile 1.5ml tube and stored at -20^0C until further analysis. DNA was extracted from 200µl of the resulting plasma using QIAmp DNA mini kit (Qiagen, Germany).The final DNA was eluted in 100µl elution buffer and stored in -20^0C until PCR analysis.Blood samplefromproven septicaemia confirmed by blood culture and PCR was used as positive control

DNA amplification

DNA amplification was carried out in Biometrathermocycler (Biometra GmbH, Germany). PCR reaction comprised of two oligonucleotide universal primers 5'-TGAAGAGTTTGATCATGGCTCAG-3' and 5'-TCGTTGCGGGACTTAACC-3 (IDT, Singapore). The 50µl reaction consisted of 0.25 units of High-fidelity DNA polymerase (New England biolabsinc.) in the presence of 100µM dNTPs, and 0.25µM of each primer in Q5 reaction buffer. The thermocycling condition included an initial denaturation at 98^0C for 5 seconds, 35 cycles of 98^0C for 10 seconds, 58^0C for 30seconds, 72^0C for 30 seconds and a final extension at 72^0C for 2 minutes. Same protocol was repeated with 80^0C, 2 minutes initialdenaturation and 10 seconds of denaturation respectively. The PCR products were electrophoresed in 1.2% gel at 80V, for 90 minutes.

The gel was visualized using gel documentation system (Bio Rad Laboratory Inc.) Products at the expected band size of 1100bp were considered positive.

Purification of PCR products

Purification was performed to obtain a clean amplicon for sequencing reaction. It was carried out using PCR purification kit (Vivantis® Technologies, SdnBhd) as per manufacturer's instruction

Sequencing

Purified products were sequenced commercially (DNA Sequencer ABI 3730-XL, 1st BASE Company, Singapore). The sequences obtained were checked for sequence quality using ABI sequence scanner Version 1.0. The good quality sequences were aligned using alignment tool in the SDSC Biology work bench 3.2. The aligned sequences were blasted against a quality controlled 16S rRNA gene library (Sepsis test-blast.net) to identify the organism. Genus and species identification was presumed to be correct for the isolate with sequence identity of ≥97% to reference strain in the data base.

RESULTS

Blood culture and PCR results

A total of 71 blood samples were collected from consented / assented participants, the samples were analysed using PCR and blood culture as a gold standard, 27 (38%) samples were found positive using both methods [6 (8.5%) blood culture and 21 (29.6%) PCR].Out thepositive samples5 (7.0%) samples by blood culture were equally positive for 16S rRNA PCR; while PCR was able to identified 16 (22.5%) more positive samples which blood culture could not identify, but only 1 (1.4%) sample was identified positive using blood culture while PCR identified it as negative as shown in table 1 below. During the study, 7 (9.9%) samples were identified positive by conventional blood culture but later found to be contaminants.

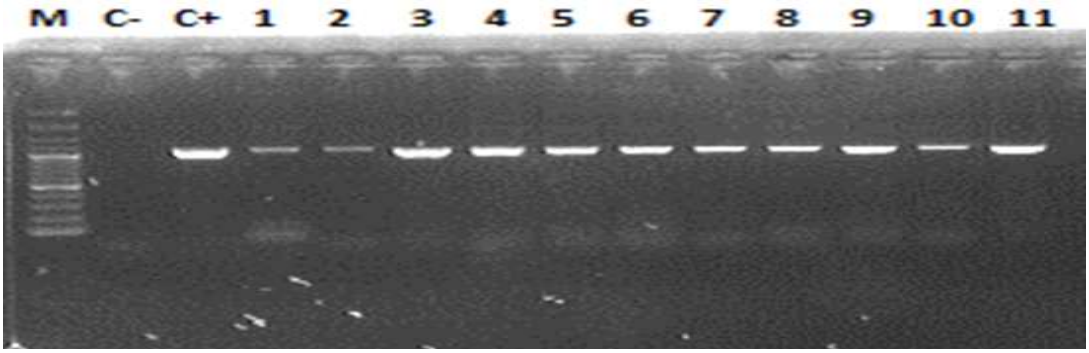

Figure 1: A representation of the PCR assay for detection of 16S rRNA from neonatal blood sampleM: 100bp ladder, Lane 1: Control negative (Primer, master mix and distilled water no template DNA), Lane 2: Positive control (S. agalactiae from septis neonates), Lane 3-13 Samples.

Table 1 Bacterial species identified by both blood culture and PCR analysis (n=71)

Microorganism	**Cases identified by blood culture**	**(%)**	**Cases identified by PCR**	**(%)**
Janthinobacteriumlividum	0	0.0	7	33.3
Streptococcus agalactiae	2	33.3	4	19.0
Pseudomonas species	0	0.0	2	9.5
Escherichia coli	2	33.3	4	19.0
Burkholderaeles specie	0	0.0	1	4.76
Corynebacteriumspp	1	16.6	0	0.0
Acinetobacterbaumanii	0	0.0	1	4.8
Achidovoraxspp	0	0.0	1	4.8
Micrococcus spp	1	16.6	1	4.76
Total	**6**	**100**	**21(100)**	**100**

Sequencing

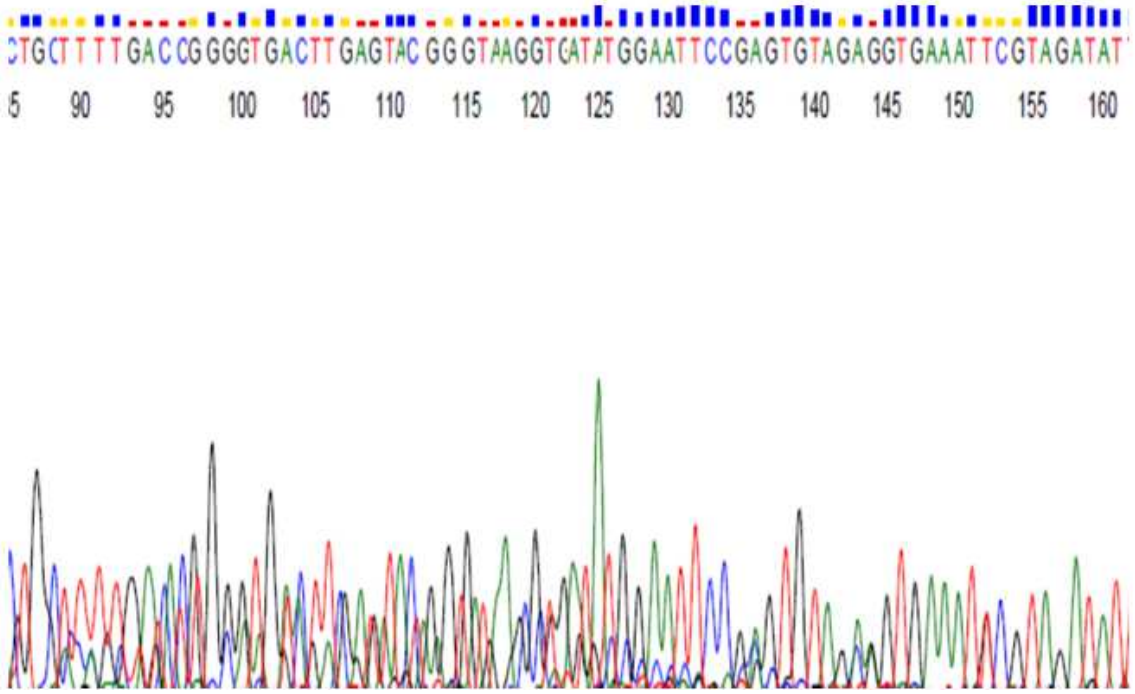

Figure 2:5minutesDNA initial denaturation at 95^0C

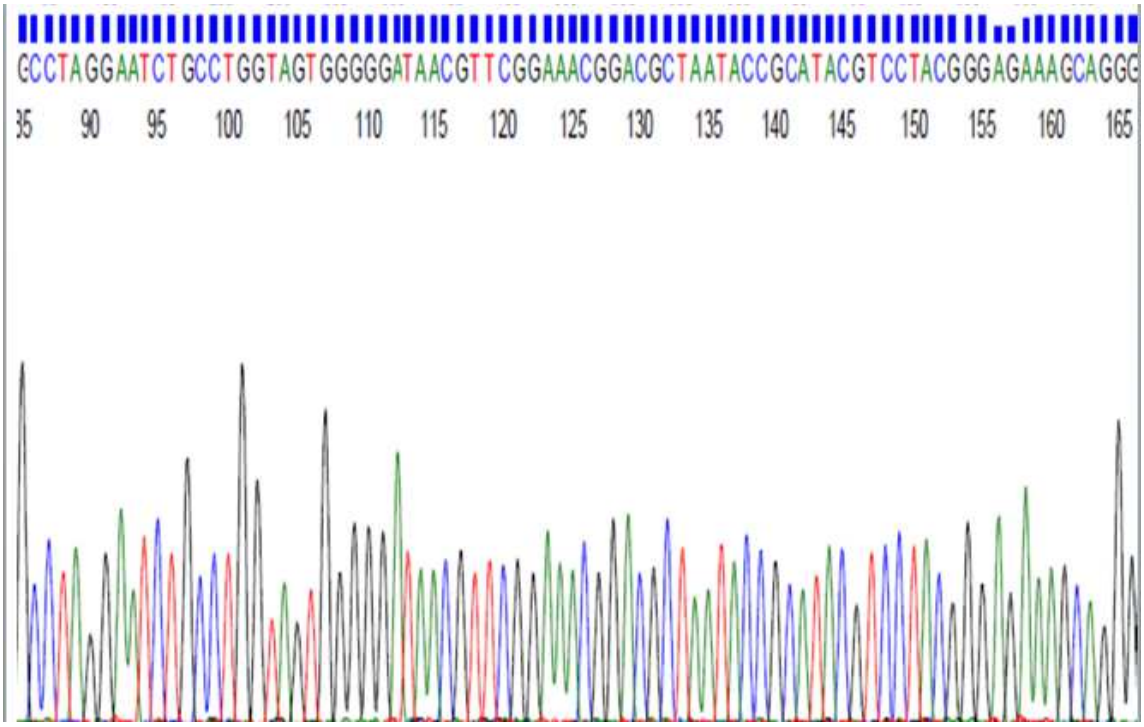

Figure 2:15 seconds initial denaturation at 80^0C

DISCUSSION

16S rDNA sequencing has become a reference method for bacterial taxonomy and identification. Many microbial infections are characterised by low pathogen loads in the presence of high amount of human DNA. However, in many diseases such as neonatal sepsis microbial DNA is shaded by human DNA (Horz, *et al.* 2008). In this study, 27 (38%) samples were found positive using both methods [6 (8.5%) blood culture and 21 (29.6%) PCR].This finding is lower than that of Michael *et al.,* (2007); that stated that,16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%), with from 1 to 14% of the isolates remaining unidentified after testing this could be as a result of small sample size. This study was also similar to the findings of MignardandFlandrois, (2007) in Pierre Bénite, France whose findings were 568 (83.1%) isolates to the species level and 108 (15.6%) to the genus level, with 7 (1%) isolates remaining unidentified even to the genus level. Therefore, using the criteria defined for sequence analysis, 16S rDNA sequencing made it possible to identify bacterial isolates to the species level and genus level while some isolates remaining unidentified even to the genus level.

CONCLUSION

This study confirmed the presence of 16S rRNA among bacterial isolates; this showed modification of PCR protocol with shorter denaturation temperature and time leading to selective amplification of bacterial DNA.Therefore, there is need to carry-out this investigation on both culturable and unculturable samples and make services available, affordable, and accessible to the patients who need them.

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