IJAMHR

Int.J.Adv.Microbiol.Health.Res.2021;5(2):1-13

International Journal of Advanced Microbiology and Health Research
ISSN: 2457-077X. Volume 5, Issue 2 (April-June 2021), PP. 1-13
Available online at www.ijamhr.com

Original Research Article

Targeted Metagenomics using Next Generation Sequencing in Laboratory Diagnosis of Hospitalized Sepsis Patients

Deepanshi Mishra[1], Gita Satpathy[2], Naveet Wig[3], Daizy Paliwal[4] and Subrat Kumar Panda[4].

[1]Ocular Microbiology, Dr. R.P.Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi - 110029, India
[2]Department of Microbiology, All India Institute of Medical Sciences, New Delhi - 110029, India
[3]Department of Medicine, All India Institute of Medical Sciences, New Delhi - 110029, India
[4]Department of Pathology, All India Institute of Medical Sciences, New Delhi - 110029, India.

Article Info

Received 12th May, 2021
Revised 15th May, 2021
Accepted 17th May, 2021
Published online 22nd May, 2021

Keywords

- Sepsis
- Metagenomics
- Next generation sequencing
- Broad range PCR assay
- Operating Taxonomic Units

ABSTRACT

Introduction: Early knowledge of the causative agent in sepsis can provide early and accurate treatment with significantly better prognosis. **Aim and objectives:** Metagenomics for sepsis diagnosis is in the threshold for wider use in routine patient care. We evaluated targeted metagenomics in 14 hospitalized sepsis patients for bacterial detection along with broad range PCR assay. **Materials and Methods:** Targeted metagenomics by next generation sequencing (NGS) using ION Personal Genome Machine was done for 7 variable regions of 16S rRNA gene was done for bacterial detection in 14 hospitalized sepsis patients. NGS data was analyzed with Ion-Reporter software using QIIME and NCBI-BLSATN tools and NCBI-Genbank and Greengenes databases. A broad-range PCR-assay for 762bp region of 16S rRNA gene with Sanger's sequencing was also done. **Results and Conclusion:** Operating taxonomic units (OTU) of varying numbers for 2-6 bacterial species were detected in 12 of the 14 specimens by NGS, though OTUs of a single bacteria were in far excess numbers in each. Broad-range PCR for bacteria was positive in these 12 patients, identification was possible in 10. Bacteria in these 2 patients could be identified by NGS (Enhydrobacter aerosaccus). In all specimens maximum numbers of OTUs detected in NGS were for the same bacteria which was detected by broad-range PCR-assay. The culture positive specimen was positive in both molecular assays. Bacterial detection and identification up to species level was possible in 87.5% patients using matagenomics, though the method is currently expensive and needs careful interpretation.

INTRODUCTION

Blood stream infection leading to sepsis (a life-threatening organ dysfunction arising from a dysregulated host response to infection), is a leading cause of death worldwide and listed as a global health priority by WHO in 2017 [1]. Sepsis commonly results from a primary bacterial infection. Prompt and accurate identification of causative bacterial agent is essential for early administration of appropriate antibiotic treatment, which is life saving and increases survival chances of patients [2,3].

Till date culture isolation of bacteria and antimicrobial sensitivity testing remains the mainstay in microbiological diagnosis of sepsis in majority of health care facilities [4]. However culture isolation fails to isolate and identify bacteria in a significant proportion of cases [4,5]. This may be due to prior empiric antibiotics treatment, low microbial loads, presence of slow and fastidious causative agents etc. [3]. Use of molecular methods such as uniplex and multiplex PCR assays improved microbial diagnosis by increasing sensitivity and faster detection of organisms from blood specimens. A major inadequacy with these

assays was only pre-specified pathogens could be detected. Moreover the available commercial panels do not take into account the geographic variation between causative agents [6].

In recent time broad range PCR assay along with Sanger' sequencing method have found use in diagnosis of infections and has been found to be more useful than PCR assays targeted against specific pathogens. However in many instances broad range PCR assay fails to identify the exact species of the detected pathogen. There is a need for exact microbiological diagnosis for appropriate antimicrobial therapy [7].

In recent years, metagenomics has come in to use for detection of all microorganisms present in different environments including different body sites.16S metagenomics ,which is based on parallel deep sequencing of the 16S ribosomal RNA gene , provides an unbiased detection method for all bacteria present in a single assay [8,9]. Metagenomics with use of next generation sequencing can identify any microorganism to the species- or strain-level without any need for a prior knowledge or culture. Recently it has been evaluated in clinical diagnosis particularly in culture negative infections [10]. But till now, metagenomics has been used mainly in research laboratories. In a few studies, 16S metagenomics has been used for bacterial detection in blood stream infection and sepsis, but these were in research settings. There is a need for feasibility studies using 16S metagenomics for microbial diagnosis of sepsis in real life hospital settings [7,10].

The present study was conducted prospectively using 16S metagenomics deep sequencing of 7 of the 9 variable regions of 16S rRNA gene for detection of bacterial pathogens in 14 sepsis patients on Ion PGM platform in a tertiary care hospital. Broad range PCR assay for 762 bp region of 16S rRNA followed by Sanger's sequencing was also used in these specimens.

MATERIALS AND METHODS

Collection of clinical specimens and isolation in culture:

In this prospective study conducted between December 2016 and March 2018, 10 ml of blood was collected from 14 randomly selected clinically diagnosed (meeting Sepsis 3 clinical criteria guidelines [11]) and hospitalized sepsis patients by the treating physician after obtaining informed consent using standard sterile protocols.

Eight ml of blood from each patient was inoculated in to BD BACTEC Peds Plus /F inoculation medium and incubated in BD BACTEC 9050 Culture System (BD, USA) up to 5days at 37ºC. Positive cultures were sub-cultured on blood agar medium, MacConkey's agar medium and Sabouraud's agar medium. Bacterial identification was done using standard biochemical tests and MALDI-TOF (Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry) assay [Vitek2 Biomerix, France] using E.coli (ATCC 8739) as control.

DNA extraction for molecular analysis:

The total nucleic acid was extracted from the serum specimen separated from remaining blood samples using commercial QIAamp DNA Mini kit (Qiagen USA). Quantity and quality of nucleic acid extracted was checked using a Nanodrop spectrophotometer 8000 (Thermo Scientific, USA). The isolated DNA was used for metagenomics deep sequencing and broad range PCR assay.

Metagenomics deep sequencing:

Targeted 16S rRNA metagenomics deep sequencing was done using Ion Torrent PGM machine (USA) and Ion 16STM Metagenomics kits (Life technologies, USA) using the isolated DNA as per manufactures' instructions as follows briefly:

16S rRNA variable region gene amplification:

Multiplex PCR assay was done for amplification of 16S rRNA hypervariable regions 2, 4, and 8 in a single tube yielding amplicon fragments of ~250 bp, ~288 bp, and ~295 bp and hypervariable regions 3, 6-7, 9 in a second PCR tube yielding amplicon fragments of ~215 bp, ~260 bp, and ~209 bp, using primers and reagents and instructions provided with Ion 16S™ Metagenomics Kit (cat. no. A26216, Life technologies, USA).

PCR products were pooled in equimolar quantities and were purified using Agencourt Ampure reagent (Life technologies, USA). Fragment size and quantity of purified DNA were estimated as per manufactures' instructions.

Preparation of Library for next generation sequencing:
After end repair of the purified DNA fragments, these were ligated with adapter/ barcodes as per manufacturer's instructions. Further PCR amplification of these amplicons was done using primers and reagents provided with the library amplification kit (Cat no. 4471252) (Life technologies, USA).

Next generation sequencing:
Next generation sequencing of the amplified library was done after 5 cycles of emulsion PCR using Ion PGM™ HiTMQ™ OT2 KitTM 400 (Life technologies, USA) and next generation sequencing was done using Ion PGM™ HiTMQ™ sequencing reagents on a 318 (1000M.b.p.) micro-chip as per manufacturers' instructions.

Sequence analysis:
Base calling and adaptor trimming was performed using the computer software Torrent Suite (Life technologies, USA). The output reads were aligned and mapped using Ion Reporter™ software v5.10 with default parameters (Thermo Fisher Scientific, USA) for metagenome analysis including read mapping, annotation and reporting. 16S rRNA sequences were analyzed with the QIIME suite software tools (v1.8). The filtered sequence reads (Phred ≥ Q20) were binned to operational taxonomic units (OTUs), with an open-reference OTU picking method based on 97% identity to entries in the Greengenes database (v13.5) as per manufacturer's instructions.

Further, FASTQ files were processed for blast analysis for homology against available genes sequences in Genbank database using NCBI blast computer programme (http://www.mibi.nim.nih.gov.). The sequencing data was uploaded to Ion reporter database (https://ionreporter.thermofisher.com/ir/) and to Genbank NCBI database and accession numbers were obtained.

Broad range PCR assay:
Broad range PCR assay was performed in the isolated DNA for amplification of 762 bp of 16S rRNA gene using published primers [12] using standard strain of E.coli (ATCC 25922) as a positive control and sterile distilled water as a negative control. The PCR assays were done using the standardized parameters in a thermal cycler (Applied Bio system, USA) as described earlier [13]. Amplified PCR products were electrophoresed on 1.5% agarose gel and visualized under a Gel documentation system (UVP, USA). The amplified DNA fragments were purified from the gel using QIAquick Gel Extraction Kit (QIAgen, USA) as per the manufacturers' instructions.

Nucleotide sequencing was done directly on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA) as was discussed earlier [13].The nucleotide sequences were aligned using DNASTAR laser gene molecular biology suite software and aligned sequences were analyzed for homology in the Gene Bank database using NCBI BLAST computer programme (http://www.mibi.nim.nih.gov.).The nucleotide sequences of organisms determined as per CLSI MM18A document guidelines were deposited and accession numbers were obtained from NCBI databank [13].

RESULTS

Isolation in culture: Acinetobacter baumannii was isolated from the single culture positive specimen, fungus from none.

Metagenomics deep sequencing: The number of nucleotide bases obtained in NGS varied from 106,588,226 to 80,278,603 at Phred value of ≥Q20 (Table I). The number of reads obtained varied from 11,938 to 6,45,953 with average read length between 164bp-192bp (Table 1).

These nucleotide sequences binned to varying numbers operation taxonomic units (OTUs) at family, genus and species level for several bacteria in each of the 12 positive specimens (Table II).

In none of the specimens singleton OTUs was obtained. Only in 4 specimens (one culture positive and 3 culture negative), OTUs (varying from 11 to 9537) for 2 bacterial species were obtained. In rest 8 culture negative specimens OTUs belonging to 2-6 bacterial species were obtained, while OTUs for 2 of these bacterial species far out numbering others (Table II).

The bacteria could be identified up to species level from each specimen. The 2 bacteria which could not be identified by broad range PCR assay could

be identified (Enhydrobacter aerosaccus in both) (Table II). The heat map figure for each specimen is given (Figure 1). The 2 broad range PCR negative specimens remained negative.
The bacterial species detected with highest OTUs among these 12 positive specimens were: Sphaerotilus natans (2032), Sphingobium xenophagum (1587), Acinetobacter baumannii (587), Pseudomonas aeruginosa (5731), Enhydrobacter aerosaccus (994), E.coli (511), S. aureus (4221), Enhydrobacter aerosaccus (4077), Pseudomonas duriflava (43587), Acinetobacter baumannii (9537), Streptococcus pneumoniae (713), Enhydrobacter aerosaccus (1882)
.

Broad range PCR assay: This was positive in 12 patients including the culture positive one. The identified bacteria from 10 of these 12 (with highest sequence match with available bacterial sequences in Gene Bank data base) are given in Table II these were the same for which highest number of OTUs were detected by NGS; in rest 2, bacteria could not be identified from the nucleotide sequence.

DISCUSSION

Sepsis is one of the most common causes of death among hospitalized patients, which is caused by dysregulated host response to infection. Blood culture/ microbial detection and identification of causative bacteria up to species level from blood remains the mainstay in laboratory diagnosis of sepsis. This helps in rapid administration of appropriate antibiotic. Prompt administration of appropriate antimicrobials within first 24 hours of sepsis diagnosis is life saving for the patient and prevents emergence of antibiotic resistance by avoiding use of empirical antibiotics [14].

Advanced genomics have the potential to provide faster, effective and precise clinical diagnoses and guide accurate treatment strategies in these situations [15].

In this study separated serum from patient's blood was directly used for metagenomics study. This was done because whole blood contains a lot of human DNA which can produce enough noise to overshadow the signals [16]. Majority of the studies on metagenomics have been done from inoculated blood culture bottles or culture isolates. Use of inoculated blood cultures for the assay was avoided, to save time [17]. Since culture positivity is usually low, bacterial isolates were not used.

We used targeted metagenomics using next generation sequencing of 16S rRNA gene of bacteria. Since 2014 metagenomics using NGS is gradually being used in diagnostics, such as outbreak investigation or genotyping of highly resistant microbes, molecular case finding, characterization and surveillance of pathogens etc. [18].

To get best results with highest resolution, in metagenomics preferably the entire 16S rRNA gene needs sequencing, while this may not be possible in clinical settings. However, the more numbers of variable regions are sequenced the better are the result [3, 19]. We used 7 of the 9 variable regions of 16S rRNA gene in this study. Previous studies have shown that, these 7 variable regions had given the best results for metagenomics [19, 20]. Different studies have used different regions of 16S rRNA for metagenomics. V3-V4 regions of 16S rRNA genes when used directly on whole blood of children with severe febrile illness; clinically significant bacteria could be detected in 29.3% of patients. From rest of the positive specimens bacteria could be identified up to genus level [10]. V3 region of 16S rRNA genes was also used in whole blood of 3 sepsis patients with suspected polymicrobial infection [21]. Metagenomics using V1/V2 and V3/V4 region were used for bacterial detection from female genital tract. V1/V2 region use failed to detect bacteria of importance in vagina while better result was obtained using V3/V4 hypervariable region [22].

In this studying NGS, quantitative read data was obtained in the form of number of sequence reads which were binned to bacterial OTUs with the help of computer software (Ion Reporter and QIIME). As reported earlier , a minimum of 10,000-15,000 reads in a specimen gives more accurate results.[15] In our study the lowest read numbers obtained in positive specimens was11,938 in specimen number 3 (culture negative, broad range PCR positive). In all the 12 specimens OTUs of varying numbers for multiple bacteria were present; in 3 of these only two bacterial species were detected. However, numbers of OTUs for one bacterium were always several logs higher than those for others. Very low numbers of reads (<100 and 262) were obtained from the 2 culture and PCR negative specimens.

Maximum numbers of OTUs detected in NGS were for the bacteria detected by broad range PCR assay in majority of the specimens. In this study serum specimens were also subjected to broad range PCR assay because it is not selective for any particular bacteria [23]. A 762bp fragment of 16S rRNA gene encasing the variable regions V2-V6 was used as the target, which has proven higher sensitivity while retaining the ability for identification of detected bacterial species [12]. In the present study all but 2 of the detected bacteria from sepsis could be identified by this method.

In this study , only 1 of the 14 blood samples (specimen number 11, *A baumannii)* from sepsis patients were culture positive where as bacterial genome was detected in 12 [85.7%, ($p<0.001$)] of these by both broad range PCR assay with Sanger's sequencing and 16S r RNA targeted metagenomics. This highlights the importance of molecular assays combined with nucleotide sequencing for organism detection. In 9 of the 12 positive specimens, Gram negative non fermenter (GNNF) bacteria were detected, with highest numbers of OTUs present. Though these GNNF bacteria are present ubiquitously in environment and were considered contaminants previously, in recent years their pathogenicity is beyond doubt especially in immunocompromised and other vulnerable individuals. These are emerging as important cause of blood stream infections [24]. As these are resistant to many of the commonly used antibiotics, these infections are especially difficult to treat.

The culture negativity in these specimens may be due to prior antibiotic therapy, (this being a tertiary care hospital, most patients had received some form of therapy before coming here) or due to genuine difficulty in culture of these organisms in laboratory.

In recent years, GNNF bacterial infections of the blood stream are being increasingly reported from all over the world [24]. Many of these ubiquitous environmental GNNF bacteria though were considered previously as contaminants, in recent years their pathogenic potential has been recognized. They are being considered as genuine blood stream infection causing organisms [24]. Detection and identification of these organisms up to species level from sepsis patient is of further importance due to their intrinsic and extrinsic resistance to commonly used antibiotics.

In this study, *E coli, S pneumoniae* and *Staphylococcus aureus* were detected in 3 specimens by broad range PCR assay and NGS results for highest number of OTUs at both genus and species level. Of the 9 specimens from which GNNF bacteria were detected, in 3 *A. baumannii* (2) and *Pseudomonas aeruginosa* were detected and identified unambiguously by broad range PCR assay and NGS results for highest OTU numbers at both genus and species levels. However in specimen number 10, *Pseudomonas aeruginosa* was detected by broad range PCR assay. But from NGS results highest numbers of OTUs were for *Pseudomonas duriflava* species.

In specimen number 1, broad range PCR results suggested presence of *Sphingomonas paucimobilis*, however from NGS results, highest number of OTUs at genus level were for unclassified Burkholderia and at species level were for *Sphaerotilus natans*. *S. natans* is a sewage inhabiting bacteria. To our knowledge, no human infection has yet been assigned to *S. natans*. Burkholderia spp. was considered previously as part of the Pseudomonas group of organisms. *Sphingomonas spp.* is also related to Pseudomonas group , sharing major virulence factors such as adherence , anti phagocytic, iron uptake, quorum sensing and protease activities etc. [25]. Therefore the organism may either belong to Burkholderia or *Sphingomonas spp.* Maturation of software and database for bioinformatics analysis of NGS data may resolve this issue in future. Burkholderia is known to cause blood stream infections. *Sphingomonas paucimobilis* is emerging as an important opportunistic pathogen. It is widely found in nature in soil, water etc. and has been isolated from hospital environment and equipment etc. It has been reported from human infections like bacteremia/ blood stream infection, bone and soft tissue infections, arthritis and from CSF and has been associated with outbreaks [25].

In specimen number 2, *Sphingomonas paucimobilis* was detected by broad range PCR; NGS results showed highest number of OTUs at genus level for Sphingomonas at genus level and *Sphingobium xenophagum* at species level. Both Sphingomonas and Sphingobium are related genera belonging to a single family *Sphingomonadaceae* [26]. Since the

highest number of OTUs at both genus and species level in NGS results were for Sphingobium spp., the infecting agent may well be *Sphingobium xenophagum. Sphingobium* spp. are widely present in environment like soil. *Sphingobium olei* has been reported from human peritonitis [25, 26].

The organism which could not be identified from broad range PCR assay product in specimen number 14, however the NGS results showed highest number of OTUs at genus level for Acinetobacter and highest number of OTUs at species level for *Enhydrobacter aerosaccus* (previously *Moraxella osloensis*). Since blood stream infection has been reported by both *Acinetobacter* spp. and *Enhydrobacter aerosaccus* it is difficult to pin point the causative agent in this case.

Enhydrobacter aerosaccus was reported earlier using 16S rRNA next generation sequencing by from blood of patient with acute myeloid leukemia [27]. In another study, *E. aerosaccus* was reported from patient of brain abscess using metagenomics analysis [28].

Acinetobacter baumannii which was detected by culture (1) and molecular methods (2) in this study, is a well documented blood stream infection causing agent. It was documented from blood of transfusion-associated sepsis patients using metagenomics next generation sequencing [29]; *Acinetobacter baumannii* was also reported in bone and joint infections using culture and metagenomics [30]; in nasal microbiota of sepsis patients using NGS and 16S rRNA gene profiling [31].

The organism could not be identified from broad range PCR assay result in specimen number 5. NGS results noted highest number of OTUs at genus level for Pseudomonas and highest number of OTUs at species level for *Enhydrobacter aerosaccus.* These are unrelated bacteria and both can cause blood stream infection.

The specimen's number 3 and 12, showed concordant results for *A baumannii* in broad range PCR assay and NGS result at both genus and species level. The specimen number 9 showed concordant results for *Enhydrobacter aerosaccus* in all, though broad range PCR assay sequences also had 100% match with *Sphingomonas paucimobilis*.

CONCLUSION

O In this study, by broad range PCR assay and Sanger's sequencing we could detect bacterial DNA in 12 of the 14 (85.7%) of the blood specimens, however 2 could not be identified from this sequence data. Gradually next generation sequencing platforms are becoming compact, cheaper thereby becoming affordable by clinical microbiology laboratories. Use of targeted metagenomics in these 14 specimens could detect bacteria in the same 12 specimens and all 12 could be identified. Bacterial identification results in broad range PCR assay and targeted metagenomics were identical up to species level in 7 specimens. In rest 5 specimens, though related Genera/ species were identified by both the methods, the exact species identified were different. Since in 16S metagenomics results, the bacterial OTU numbers were obtained quantitatively, we assumed that the may be the bacterial species identified in this method was the infecting species. Further work is needed to bring clarity.

ACKNOWLEDGEMENTS

The authors thank all the clinical faculties and residents for the clinical specimens.

CONTRIBUTORS

Deepanshi Mishra did the experimental work. Gita Satpathya planned the work, arranged the funds, and reviewed the data and the manuscript. Naveet Wig did the clinical work and provided patient specimens. Daizy Paliwal helped in experimental work. Subrat Kumar Panda planned the work, arranged the funds and reviewed the data.

REFERENCES

1. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study. Lancet (London, England) 2020;395(10219):200-11.
2. Zengler K. To Grow or Not To Grow: Isolation and cultivation procedures in the genomic age. In: Fredricks DN, editor. The Human Microbiota: How Microbial Communities Affect Health and Disease: Wiley Blackwell2013; pp. 289±302.
3. Watanabe N, Kryukov K, Nakagawa S, Takeuchi JS, Takeshita M, Kirimura Y ,

Mitsuhashi S et al. Detection of pathogenic bacteria in the blood from sepsis patients using 16S rRNA gene amplicon sequencing analysis. PLOS ONE 2018; 13(8): e0202049.
4. Jordana-Lluch E, Giménez M, Quesada MD. Improving the diagnosis of bloodstream infections: PCR coupled with mass spectrometry. Biomed Res Int. 2014; 501214.
5. Deurenberg RH, Bathoorna E, Chlebowicz MA, et al., (2017). Application of next generation sequencing in clinical microbiology and infection prevention. Journal of Biotechnology 2017; 243:16–24.
6. Carrara L, Navarro F, Turbau M, et al. Molecular diagnosis of bloodstream infections with a new dual-priming oligonucleotide-based multiplex PCR assay. J Med Microbiol 2013; 62(11):1673–1679.
7. Rutanga JP and Nyirahabimana T. Clinical significance of molecular diagnostic tools for bacterial bloodstream infections. a systematic review. Inter discip Perspect Infect Dis 2016; 6412085.
8. Kuczynski, Lauber, Walters, Parfrey, Clemente, et al. Experimental and analytical tools for studying the human microbiome. Nat Rev Genet 2012;13: 47–58.
9. Weinstock GM. Genomic approaches to studying the human microbiota. Nature 2012; 489:250- 6; PMID:22972298; http://dx.doi.org/10.1038/ nature11553.
10. Decuypere S, Meehan CJ, Van Puyvelde S, De Block T, Maltha J, Palpouguini L, et al. Diagnosis of bacterial bloodstream infections: a 16S metagenomics approach. PLoS Negl Trop Dis 2016;10:1e12.https://doi.org/10.1371/journal.pntd.0004470.
11. Singer M, Duetschman CS and Seymour CW. The third international consensus definitions for sepsis and septic shock (Sepsis-3).JAMA 2016; 315(8):801-810.
12. Xu J, Smyth CL, Buchanan JA, Dolan A, Rooney PJ, Millar BC, et al. Employment of 16 S rDNA gene sequencing techniques to identify culturable environmental eubacteria in a tertiary referral hospital. J Hosp Infect 2004; 57, 52–58.
13. Mishra D, Satpathy G, Chawla R, Venkatesh P, Ahmed NH, Panda SK. Utility of broad-range 16S rRNA PCR assay versus conventional methods for laboratory diagnosis of bacterial endophthalmitis in a tertiary care hospital. Br J Ophthalmol. 2018;0:1–5.
14. Rogina P, Skvarc M, Stubljar D, Kofol R, Kaasch A. Diagnostic Utility of Broad Range Bacterial 16S rRNA Gene PCR with Degradation of Human and Free Bacterial DNA in Bloodstream Infection Is More Sensitive Than an In-House Developed PCR without Degradation of Human and Free Bacterial DNA. Mediators of Inflam 2014; 10.1155/108592.
15. Liesenfeld O, Lehman L, Hunfeld KP, Kost G. Molecular diagnosis of Sepsis: New Aspects and recent developments. Eur Journal of Micro and Immuno. 2014; 1, pp. 1–25.
16. Radstrom P, Knutsson R, Wolffs P, Lovenklev ML, Lofstrom C. Pre-PCR processing: strategies to generate PCR- compatible samples. Molecular Biotechnol. 2004; 26:133–146.
17. Hassan RM, Enany MGE, Rizk H. Evaluation of broad range 16S rRNA PCR for the diagnosis of bloodstream infections: two years of experience. J Infect Dev Ctries 2014; 8(10): 1252-1258.
18. Deurenberg RH, Bathoorna E, Chlebowicz MA, et al., (2017). Application of next generation sequencing in clinical microbiology and infection prevention. Journal of Biotechnology 2017;243:16–24.
19. Bukin YS, Galachyants YP, Morozov IV, Bukin SV, Zakharenko AS and Zemskaya TI. The effect of 16S rRNA region choice on bacterial community metabarcoding results: Scientific Data, 5 February 2019.
20. An integrated research solution for bacterial identification using 16S rRNA sequencing on the Ion PGM™ System with Ion Reporter™ Software. Ion Torrent application note, Thermo fisher scientific.
21. Faria MM, Conly JM, Surette MG. The development and application of a molecular community profiling strategy to identify polymicrobial bacterial DNA in the whole blood of septic patients. BMC Microbiol. 2015;15:215.
22. Graspeuntner S, Loeper N, Künzel S, Baines JF & Rupp J. Selection of validated hypervariable regions is crucial in 16S-based microbiota studies of the female genital tract. Scientific Reports 2018; 8:9678.
23. Jenkins C, Ling CL, Ciesielczuk HL, et al. Detection and identification of bacteria in

clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. J Med Microbiol 2012;61(Pt 4):483–8.
24. Whistler T, Sangwichian O, Jorakate P, Sawatwong P, Surin U, Piralam B, et al. Identification of Gram negative non-fermentative bacteria: How hard can it be? PLoS Negl Trop Dis 2019; 13(9): e0007729.
25. Saeb ATM, David SK and Brahim HA. In Silico Detection of Virulence Gene Homologues in the Human Pathogen Sphingomonas spp. Evolutionary Bioinformatics 2014;10:229-238.
26. Glaesar SP and Kamfer P. The family Sphingomonadaceae. Springer 2014: The prokaryotes pp 641-707.
27. Sung JY, Hong SK, and Kim EC. The First Korean Case of Moraxella osloensis Bacteremia in a Patient with Acute Myeloid Leukemia. Ann Lab Med 2014;34:256-258.
28. Masalma M Al, Lonjon M, Richet H, Dufour H, Roche P-H ,et al. Metagenomic Analysis of Brain Abscesses Identifies Specific Bacterial Associations. CID 2012;54.
29. Crawford E. The Temporal and Geographic Reach of International Humanitarian Law (Forthcoming). In Ben Saul, Dapo Akande (Eds.), Oxford Guide to International Humanitarian Law (Forthcoming). United Kingdom: Oxford University Press 2019.
30. Ruppé E, Woerther P-L and Barbier F. Mechanisms of antimicrobial resistance in Gram-negative bacilli. Ann. Intensive Care 2015; 5:21.
31. Tan X-l‡, Liu H-y‡, Long J, Jiang Z, Yuemei Luo et al. Septic patients in the intensive care unit present different nasal microbiotas. Future Microbiol. 2019; 14(5), 383–395.

Table I: Next generation sequencing run information for serum specimens

S. No.	No. of reads	No. of bases Q20	Average read length	Average coverage depth
1	31,492	46,72,568	170bp	81.2X (Run 1)
2	60,673	95,28,213	181 bp	81.2X (Run 1)
3	11,938	19,13,019	187 bp	81.2X (Run 1)
4	81,147	1,25,85,931	177 bp	81.2X (Run 1)
5	22,446	32,22,181	164 bp	81.2X (Run 1)
6	262	37,381	167 bp	81.2X (Run 1)
7	5,62,597	80,278,603	167 bp	81.2X (Run 1)
8	6,45,953	106,588,226	186 bp	81.2X (Run 1)
9	5,24,873	76,456,850	167 bp	81.2X (Run 1)
10	2,70,326	45,595,264	192 bp	81.2X (Run 1)
11	45,315	66,14,097	176 bp	41.2X (Run 2)
12	1,05,395	1,70,08,930	190 bp	41.0X (Run 2)
13	93	13,705	178 bp	41.0X (Run 2)
14	2,13,123	3,24,10,700	180 bp	41.0X (Run 2)

Q20- Phred score

Table II: Bacteria detected and identified using broad range PCR assay and next generation sequencing from serum specimens with their accession numbers

S. No.	Results of Broad range PCR assay/Sanger sequencing (100% identity)	Results of next generation sequencing (OTU reads Genus level)	Results of next generation sequencing (OTU reads species level)	Accession number for 16S Metagenomics
1	*S. paucimobilis*	Unclassified Burkholderia 2077, Burkholderia 29, Limnobacter 29, Sphingobium 27, Acinetobacter 15, Cloacibacterium 14, Enterococcus 10, Streptococcus 10	*Sphaerotilus natans* 2032, *Limnobacter thiooxidans* (29), *Sphingobium xenophagum* (27), *Cloacibacterium normanense* (14)	MN044722, MN044723
2	*S. paucimobilis*	Sphingobium 1728, Pseudomonas 377, Paracoccus 109, Aerococcus 97, Micrococcus 249, Limnobacter 172, Staphylococcus 126, Sphingomonas 59, xiguobacterium 28, Rothia 18, Sphingopyxis 30, Pelomonas 31, Actinomycetospora 18, Corynebacterium 17, Methylophilus 11, Brachybacterium 10, Tabrizicola 7	*Sphingobium xenophagum* (1587), *Limnobacter thiooxidans* (172), *Aerococcus urinaeequi* (97), *Pseudomonas duriflava* (86), *Acinetobacter lwoffii(59), Staphylococcus aureus (25)*	MN044747-MN044750
3	*A. baumannii*	Acinetobacter 711, Luteimonas 50	*Acinetobacter baumannii* (587), *Luteimonas sp.* (50), *Acinetobacter soli* (21), *Acinetobacter haemolyticus*(13)	MN044726-MN044728, MN044742, MN044743

4	*Pseudomonas aeruginosa*	Pseudomonas 5731, Acinetobacter 11	*Pseudomonas aeruginosa* (5731)*, Acinetobacter baumannii* (11)	MN044724, MN044725
5	No significant match found	Pseudomonas 2901, Acinetobacter 2704, Paracoccus 1448, Enhydrobacter 994, Staphylococcus 663, Roseomonas 573, Corynebacterium 523, Microbacterium 394, Cellvibrio 321, Micrococcus 299, Ignatzschineria 272, Rheinheimera 270, Chryseobacterium 259,Aeromonas 225, Kocuria 238, Lysinibacillus 222, Sphingomonas 211, Dietzia 203, Bacillus 151, Jonesia 132, Nocardioides 106, Pontibacter 104, Methylobacterium 113,Filomicrobium 100, Phenylobacterium 98, Georgenia 89, Piscicoccus 88, Ornithinimicrobium 82, Facklamia 81, Moraxella 67, Propionibacterium 65, Azospirillum 63, Flavobacterium 62, Mycobacterium 62, Herbaspirillum 60, Exiguobacterium 58, Agrococcus 57, Brachybacterium 55, Brevundimonas 55, Blastococcus 53, Gordonia 41, Oscillatoria 40, Weissella 37, Streptococcus 33, Devosia 31, Cloacibacterium 24, Rothia 24, Microvirga 52, Enterococcus 40, Comamonas 40, Rubellimicrobium 38, Hydrogenophaga 37, Nocardiopsis 37, Actinophytocola 33, Pelagibacterium 33, Tetrasphaera 33, Novosphingobium 30, Empedobacter 29, Dermacoccus 28, Oceanobacillus 28, Asticcacaulis 25	*Enhydrobacter aerosaccus* (994), *Roseomonas pecuniae*(420), *Acinetobacter junii* (287), *Ignatzchineria indica* (261), *Microbacterium ginsengisoli* (248), *Corneybacterium* (151), Kocuria palustris 105, Corynebacterium glutamicum 101, Rheinheimera sp. 89, Moraxella osloensis 67, Jonesiaquin ghaiensis 65, Paracoccus aminovorans 61, Pseudomonas sagittaria 56, Sphingomonas hankookensis 50, Piscicoccus intestinalis 88, Propionibacterium acnes 65, Brevundimonas viscosa 55, Azospirillum sp. 46, Kocuria flava 43, Comamonas aquatica 40, Sphingomonas koreensis 33, Blastococcu saggregatus 31, Acinetobacter haemolyticus 30, Mycobacterium smegmatis 30, Vasilyevaeaenhydra 26, Devosia glacialis 31, Rhodocista sp. 22, Nocardioidesoleivorans 26, Rothiadentocariosa 24, Gordoniaaraii 22, Dechloromonasagitata 20, Oleispira antarctica 20, Bacillus longiquaesitum 19, Aerococcus urinaeequi 17, Agrococcus casei 17, Microbacterium profundi 16, Agrococcus versicolor 15, Geminicoccus roseus 14, Staphylococcus hominis 13, Brachybacterium squillarum 13, Corynebacterium nuruki 12, Massiliatimonae 12, Bacillus sp. 12, Weissella paramesenteroides 12, Methylobacterium goesingense 11, Acinetobacter baylyi 11, Agrococcus jejuensis 11, Pontibacamethyl aminivorans 10, Staphylococcus haemolyticus 10, Massilia consociata 10, Corynebacterium efficiens 10, Paracoccus tibetensis 10	MN049885-MN049896

6	Negative	Negative	*Paracoccus sphaerophysae* 138, *Micrococcus lylae* 120,	-
7	*E. coli*	Enterobacter 33, Shigella 17, Escherichia 689	*E.coli* (511), *S. dysenteriae* (11)	MN044733, MN044734
8	*S. aureus*	*Staphylococcus* 46635	*S. aureus* (4221), *S. auricularis* (121), *S. haemolyticus* (81)	MN044745, MN044746
9	*Enhydrobacter aerosaccus* (100%), *S. paucimobilis* (100%)	Enhydrobacter 4077, Micrococcus 1522, Sphingomonas 1071, Moraxella 817, Paracoccus735, Acinetobacter 382, Rheinheimera 444, Gordonia169, Pseudomonas167, Exiguobacterium 116, Geodermatophilus 105, Rothia 89, Kocuria 74, Cloacibacterium 73, Rhodococcus 49, Dietzia 57, Microbacterium 43, Stenotrophomonas 21, Sphingobium 29, Acidovorax 38, Methylobacterium 42	*Enhydrobacter aerosaccus* (4077), *Moraxella osloensis* (817), *Micrococcus endophyticus* (495), *Sphingomonas* panni (166), *Acinetobacter baylyi* (102), *Paracoccus tibetensis* (100)	MN121589, MN044736-MN044738
10	*Pseudomonas aeruginosa*	Pseudomonas 65549, Micrococcus 489, Paracoccus 358, Acinetobacter 248, Aerococcus 107, Corynebacterium 99, Streptococcus 80, Microbacterium 73, Staphylococcus 55, Dietzia 25, Sphingomonas 32, Brachybacterium 28, Pleomorphomonas 25, Rheinheimera 41, Hydrogenophilus 36, Neisseria 33	*Pseudomonas duriflava* (43587), *Pseudomonas sp.* (4206), *Pseudomonas uteola* (422), *Pseudomonas stutzeri* (184), *Aerococcus urinaeequi* (107), *Acinetobacter schindleri* (80)	MN044739-MN044741

11	*Acinetobacter baumannii*	Acinetobacter 25345	*Acinetobacter baumannii* (9537), *Acinetobacter soli* (544)	MN044721
12	*Streptococcus pneumoniae*	Streptococcus 9128	*Streptococcus pneumoniae* (713), *Streptococcus infantis* (21)	MN044744
13	Negative	Negative	Negative (No reads)	-
14	Uncultured bacterium	Acinetobacter 6821, Brevundimonas 5835, Sphingomonas 4526, Enhydrobacter 1882, Micrococcus 2203, Paracoccus 1600, Pseudomonas 783, Chryseobacterium 456, Microbacterium 395, Kocuria 336, Rhizobium 365,Staphylococcus 357, Pseudonocardia 245, Sphingobium 210, Corynebacterium 234, Pelomonas 138, Propionibacterium 131, Alishewanella 128, Massilia 242, Bacillus 157, Ornithinimicrobium 100, Moraxella 92, Pseudoxanthomonas 87, Methyloversatilis 73, Nocardioides 73, Clostridium 66, Rheinheimera 60, Skermanella 58, Stenotrophomonas 50, Salinicoccus 49, Streptococcus 48, Exiguobacterium 45, Nocardiopsis 44, Rhodobacter 43, Actinomycetospora 42, Klebsiella 41, Macrococcus 10	*Enhydrobacter aerosaccus* (1882), *Brevundimonas faecalis* (1437), *Acinetobacter junii* (1264), *Brevundimonas terrae* (1236), *Sphingomonas hankookensis* 947, *Acinetobacter lwoffii* 813, *Brevundimonas diminuta* 689, *Sphingomonas mucosissima* 502, *Microbacterium ginsengisoli* 336, *Sphingobium aromaticiconvertens 210, Sphingomonas pseudosanguinis 199, Micrococcus endophyticus 194, Sphingomonas yabuuchiae 170, Sphingomonas dokdonensis 167, Kocuria turfanensis 159, Sphingomonas panni 150, Pelomonas puraquae 138, Propionibacterium acnes 131, Micrococcus lylae 116, Staphylococcus gallinarum 104, Acinetobacter baylyi 101, Pseudomonas stutzeri 100, Moraxella osloensis 92, Massilia alkali tolerans85, Acinetobacter towneri 70, Acinetobacter johnsonii 71, Clostridium hiranonis 66, Microbacterium mitrae 59, Acinetobacter sp. 57, Staphylococcus auricularis 50, Staphylococcus kloosii 43, Corynebacterium coyleae 41, Brevundimonas sp. 40, Pseudomonas psychrotolerans 40, Nocardioides pyridinolyticu 32*	MN044729-MN044732

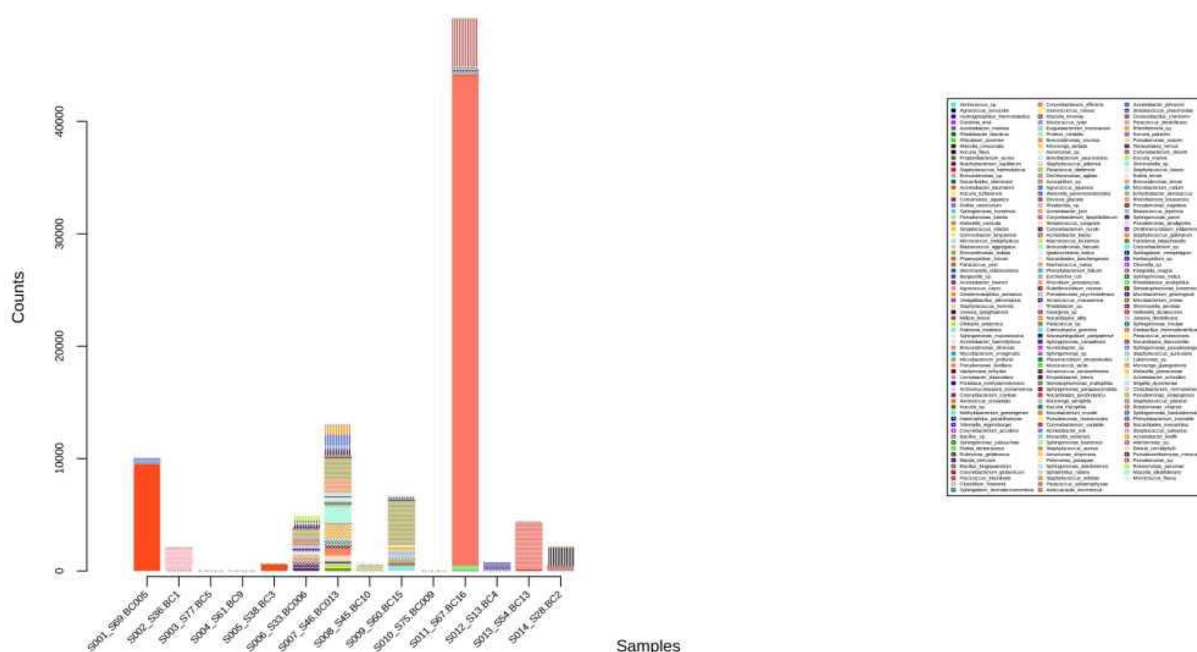

Figure 1: Heat map representing species level identification of bacteria in serum

Corresponding Author: Dr. Gita Satpathy
Ex. Professor and Head, Department of Microbiology &
Ex. Prof. Incharge, Ocular Microbiology Dr. R.P.C. for Ophthalmic Sciences, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-110029.

E-mail: gita.satpathy@gmail.com

How to cite this article:
Mishra D, Satpathya G, Wig N, Paliwal D and Panda SK. Targeted Metagenomics using Next Generation Sequencing in Laboratory Diagnosis of Hospitalized Sepsis Patients. Int.J.Adv.Microbiol.Health.Res., 2021; 5(2):1-13.

Source of Financial Support: Internal funding from All India Institute of Medical Sciences, New Delhi
Conflict of interest: Nil.