Isolation and Identification of bacterial species in neonatal sepsis using Polymerase Chain Reaction-Based 16S rRNA Sequencing

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Abstract

Neonatal Sepsis is a bacterial infection of the blood in a neonate and an infant younger than 4 weeks of age. Molecular methods may display usefulness due to the rapidity and the small sample volume required for analysis. These techniques, exhibitionist the presence of microbial DNA in the sample, which based on amplification or hybridization. The goal of this investigation to identify the pattern of organisms in neonatal sepsis using Polymerase Chain Reaction-Based 16S rRNA Sequencing in Al-Imammain Al-Kadhmain Teaching Hospital. Blood samples were inoculated into a blood culture bottle and incubated at 37°C under aerobic conditions, DNA extracted from blood samples then 16S rRNA PCR amplification and Sanger sequencing (PCR/Sanger-Seq) was used for detection of bacterial pathogens. Positive blood cultures were 41(82%) infants with sepsis, according to 16Sr RNA gene conventional PCR assay results improved that 40 (80%) out of 50 samples were PCR positive. These positive samples were analyzed using 16S rRNA gene sequencing, discordant identification results between API system and sequencing were found in 15 samples, and one sample was identified Genus/species achieved only by sequencing as a *Pantoea agglomerans*, sequencing was the only method that identified two bacterial strain belong *Achromobacter xylosoxidans* and three strains belong to *Enterobacter cloacae*. In conclusion, the use of PCR sequence analysis in diagnosis neonatal sepsis can be highlight rare types of bacteria that lead to this disease, which helps to focus on these species and to provide appropriate treatment.

Keywords: Early-onset sepsis, late-onset sepsis, 16S ribosomal RNA, API system.

INTRODUCTION:

Neonatal Sepsis is a bacterial infection of the blood in a neonate and an infant younger than 4 weeks of age. Babies with sepsis were listless, overly sleepy, floppy, weak, and pale [1]. Neonatal sepsis has been classified as either early onset sepsis (0-7 day of age) or late onset sepsis (7-30 days of age) [2, 3]. Early onset sepsis is mainly due to bacteria acquired before and during delivery. Transplacental infection or an ascending infection from the cervix might be caused by bacteria that harboring the mother's genitourinary tract, the second types of neonatal sepsis are late onset sepsis acquired after delivery (nosocomial or community sources) but it may occur via vertical transmission at birth [4].

Many bacteria implicated as a serious cause of neonatal sepsis but the most common type included Group B *Streptococcus* (GBS), Coagulase-negative *Staphylococcus*, *E. coli, Acinetobacter, Pseudomonas* and *Klebsiella* other organisms might be play role in neonatal sepsis such as *candida* [5]. Blood cultures are still the gold standard in the diagnosis of neonatal sepsis. However, obtaining cultures from neonates can be difficult as sample volumes are small and a fundamental number of cultures turn out to be contaminated or negative [6].

The culture results are not available until 24 to 48 hours after sampling and thus have no influence on the initial choice on whether to initiate or withhold antibiotic therapy [7].

Molecular methods may display usefulness due to the rapidity and the small sample volume required for analysis. These techniques, exhibitionist the presence of microbial DNA in the sample, which based on hybridization or amplification. Technical and analytical details about these methods have been already described for neonatal sepsis [8]. 16S ribosomal RNA (16S rRNA) gene has been exceedingly used to distinguish bacterial species and implement taxonomic studies [9]. Bacterial 16S rRNA genes generally have nine "hyper variable regions" that expound massive sequence diversity among different bacterial species and can be used for species identification [10]. Numerous studies have identified 16S rRNA hyper variable region sequences that identify a single bacterial species or differentiate among a limited number of different species or genera [11].

METHODS:

In this cross-sectional study (1.5-3 ml.) of venous blood was obtained from 50 neonates were attended and admitted to neonatal care unites of Al -Imamain Al-Kadhumain medical city hospital during the period from January to March 2017. Clinical manifestations including poor feeding, lethargy, temperature instability, respiratory distress, abdominal distention and seizures were determined by consultation of a pediatric specialist and verification of the information in the medical record. Inclusion criteria for selecting children were the age group (from zero time to 30 days), and diagnosed clinically with sepsis while exclusion criteria neonates with obvious congenital anomalies and neonates with neonatal respiratory distress syndrome (NRDS).

A consent letter was signed by each neonate parents and the study were approved by the Research Ethical Committee College Medicine of Al-Nahrain University. This cross-sectional study was conducted in the microbiology department at College Medicine of Al-Nahrain University. The following data were collected: gestational age, birthweight gender and whether the baby was born inside the hospital or outside the hospital and then transferred to the nursery.

Samples were immediately transferred to Brain heart infusion medium vial which prepared specially for bacterial cultivation, and incubated in 37°C, then samples were subcultured in MacConkey and blood media aerobically while chocolate agar media incubated under CO_2 conditions, the initial reading was recorded after 24 hours and the results continued to be recorded for 72 hours. The result was considered negative after this time, if there is no growth. Api20E system for Enterobacteriaceae, Api Staph System, Api 20 Strep System according to the procedure suggested by the manufacturing company (bio-Merieux) were used to recognize the bacterial species level. Seconded parts of blood were used in extraction of bacterial DNA using ready kit (Geneaid)® Total DNA Extraction.

16S rRNA PCR amplification and Sanger sequencing (PCR/Sanger-Seq):

Two primers sequence to 16S rRNA (5' to3') 27F AGAGTTTGATCCTGGCTCAG 1492R GGGTTACCTTGTTACGACTT(Bio Corp Canada) to produce a DNA fragment of (1500) base pair were used in conventional

PCR, PCR mixture without DNA template (non-template negative control) were used as negative control The thermocycling conditions with a cleaver scientific thermal cyclers (TC 32/ 80-UK) were as follows: After initial denaturation at 94°C for 5 min, the 30-cycle amplification profile consisted of 95 °C for 30 s, 60°C for 30 s and 72°C for 1 min. Final elongation was at 72 °C for 5 min. 10 µl of each PCR product was subjected to 1.5% (wt/vol) agarose gel electrophoresis with ethidium bromide (0.5 µg /ml; Sigma), 10 microliters of the 1500bp DNA ladder was subjected to electrophoresis in a single lane, served as marker during PCR products electrophoresis amplicon visualization was performed using an UV light transilluminator. The Sanger sequencing was carried out by using BigDye™ in the Macrogen; Inc. is a South Korea public biotechnology company.

Results of Nucleotide sequences are analysis and determined on both strands of PCR amplification products by using Macrogen sequencing facility (Macrogen Inc., Seoul, Korea) and the data obtained were matched with the online database using BLAST [12].

Statistical Analysis:
Statistical analysis was performed with Graph Pad Prism version 6 software, percentages were used for the comparison between samples of the study. Data analysis was done using Chi-square for the comparison of categorical data.

RESULTS:
Distribution of pathogens:
Positive blood cultures were detected in 41(82%) infants with sepsis, Gram negative was the major causative pathogen 53.6% (22/41) followed by Gram positive 43.9% (18/41) and one sample showed growth of candida species 2.4%(1/41), **Table** [1].

16Sr RNA gene conventional PCR assay was used for the detections of bacterial neonatal sepsis with amplicon product 1500 bp, results improved that 40 (80%) out of 50 samples were PCR positive. The remainder 10(20 %) were PCR negative. These positive samples were analyzed using the ABI prism 3730XL analyzer according to the Macrogen, Inc. Instructions for 16S rRNA gene sequencing **figure** [1] In general, mismatched identifications were observed in the characteristics and types of the bacterial isolated they has assumed a proof by API system

and sequencing , Discordant identification results with the two methods were found in 15 samples, and one sample was identified Genus/species achieved only by sequencing as a *Pantoea agglomerans* , sequencing was the only method that identified two bacterial strain belong *Achromobacter xylosoxidans* and three strains belong to *Enterobacter cloacae* as shown in **Table** [2].

Table (1): Pathogens isolated from neonatal sepsis by blood culture.

Blood culture	Total (%)
Gram negative	22 (53.6)
Gram positive	18(43.9)
Candida species	1(2.4)
Total positive	41(82)
No growth	9 (18)

Table (2) Comparison between bacterial identification by API system and sequencing

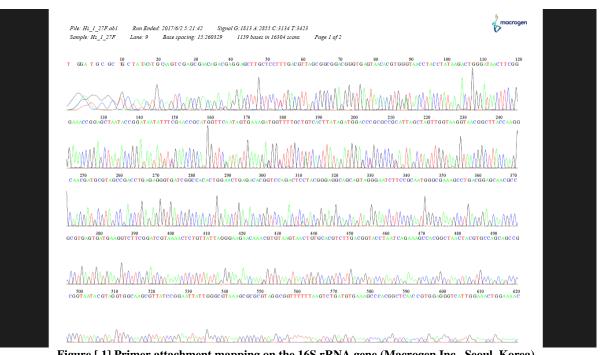| Bacterial API system	Bacterial sequencing
Staphylococcus aureus	*Staphylococcus epidermidis*
Staphylococcus aureus	*Staphylococcus heamolyticus*
Citrobacter freundii	*Achromobacter xylosoxidans*
Aeromonas hydrophilia	*Serratia marsecnce*
Pseudomonas cepacia	*pseudomonas aeruginosa*
klebsiella pneumoniae	*Acinetobacter bumanii*
Veridanis streptococcus	*Aerococcus veridanis*
Citrobacter freundii	*Acinetobacter bumanii*
Pseudomonas aeruginosa	*Achromobacter xylosoxidans*
Escherichia coli	*Klebsiella pneumonia*
Citrobacter freundii	*Enterobacter cloacae*
Serratia marsecnce	*Enterobacter cloacae*
Streptococcus agalactia	*Staphylococcus aureus*
Streptococcus agalactia	*Staphylococcus hominins*
Enterobacter	*Enterobacter cloacae*
Pantoea	*Pantoea agglomerans*

Figure [1] Primer attachment mapping on the 16S rRNA gene (Macrogen Inc., Seoul, Korea)

DISCUSSION:

Based on the current data, the percentage of neonatal sepsis was 82% out of 50 patients enrolled in this study, this observation disagreement with a study done in Iraq byAlbahadle and Abdul Abass [13] where sepsis was constituted 89.76% of the studied neonate, another study conducted by Ibrahim and Rahma [14] stated that neonatal sepsis was 12.4% However, a study by Al-Hamadani. found that the sepsis was 58 % [15].

This discrepancy in such results may be due to different reasons such as blood culture technique, administration of antibiotic by mother, difficulty in sample collection, development of much more antibiotics resistant bacterial strains.

The sequencing method in this investigation gave different results from the API system assay with regard to the types of bacteria causing the neonatal sepsis. Out of (40) bacterial species set for sequence method, the result of nucleotide sequence data for 15 bacterial species were completely differ from identification by API system while the *Pantoea* species which were isolated and diagnosed by the API system was identified in this methods as *Pantoea agglomerans*, that suggest this method useful for the identification of rare bacterial species and for those isolates not readily identified by bacteriological tests.

This compatible with results of Woo *et al.* who reported that the 16SrRNA sequencing has played a very important role in the accurate identification of bacterial isolates and in the discovery of novel bacteria in clinical microbiology laboratories. [16].

In current study sequencing method revealed that two bacterial strain belong *Achromobacter xylosoxidans* which is an opportunistic pathogen that can cause severe infections, especially in immunocompromised patients, it has been found in neonates and in patients with cancer, neutropenia, can be mistaken for other non-lactose fermenting gram negative bacteria mainly *pseudomonas* species. *Achromobacter xylosoxidans* already resistance to different kind of antibiotic such as first-and second-generation cephalosporins, ampicillin, Aztreonam, aminoglycosides tetracyclines, and Rifampin [17,18]

These findings are compatible with many studies that reported increase prevalence of bacteremia due to *A. xylosoxidans* and paediatricians ought to know about these bacteria while treating neonates or immunocompromised patients due to resistant pattern to many antimicrobial agents [19].

Furthermore, other three strain belong to *Enterobacter cloacae* were reported in this study by sequencing methods, *E. cloacae* is rising as a neonatal bacterial pathogen, causes various invasive disease such as bacteraemia, endocarditis and respiratory tract infections and its related to sever mortality [20] and this finding in harmony with study by Mahapatra who found that *Enterobacter cloacae* a leading cause in neonatal sepsis [21].

This organism has been reported to be isolated in varying percentages ranging from 1.5 to 14.6 by various authors Kuruvilla *et al.* [22] Sinha *et al.* [23] *E.cloacae* septicaemia should be emphasised and taken into account for its rising incidence.

Another interesting point in this study is the detection by sequencing of a single infection resulting from *Aerococcus veridanis.*

This finding in similar with Jay and Park who reported that physicians and researcher ought to know that *Aerococcus veridanis* infection may occur neonates and may cause neonatal infection underlining the potential invasiveness of this pathogen [24,25].

CONCLUSION:

According to our study, the use of PCR sequence analysis in diagnosis neonatal sepsis can be highlight rare types of bacteria that lead to this disease, which helps doctors to focus on these species and to provide appropriate treatment, which reduces the forgetfulness of deaths and also reduce the complications of neonatal sepsis.

REFERENCES

1- Goldstein, B., Giroir, B., Randolph, A., International pediatric sepsis consensus conference: Definitions for sepsis and organ dysfunction in pediatrics. Pediatr Crit Care Med. 2005; 6:2–8. doi.org/10.1097/01.PCC.0000149131. 72248.E6

2- Kari, A., Ann, L., Anderson-Berry, Delair,.S.F., *et al*. Early-Onset Neonatal Sepsis, Clin Microbiol Rev. 2014 Jan; 27(1): 21–47. doi: 10.1128/CMR.00031-13

3- Van den Hoogen, A., Gerards, L.J., Verboon, M.A., *et al*. Long-term trends in the epidemiology of neonatal sepsis and antibiotic susceptibility of causative agents. Neonatology 2010. 97(1):22-8. [Medline]. doi:10.1159/000226604

4- Wax, J.R, Lucas, F.L., Lamont, M., *et al*., Maternal and Newborn Outcomes in Planned Home Birth vs Planned Hospital Births: A Meta-analysis. Am JObstet Gynecol. 2010; 203:243. e1-243.e8.

5- Kaufman, D., Fairchild, K.D., Clinical microbiology of bacterial and fungal sepsis in very-low-birth-weight infants. Clin Microbiol Rev 2004; 17:(638–680).

6- Michael, C., Cotton, A., Duration of empirical antibiotic therapy for infants suspected of early-onset sepsis, Curr Opin Pediat J. 2013; 25(2): 167–171.

7- Escobar, G.J, what have we learned from observational studies on neonatal sepsis? Pediatr. Crit. Care Med. 2005, 6 (3 Suppl): S138–145.

8- Andries, W.D., Blood Culture Systems: from patient to result in critical care and Emergency Medicine "Sepsis – An ongoing and significant challenge" Dreyer, licensee InTech: 2012: chapter 15, 456-460.

9- Keshaav, K.P., Vishnu,V.P., MSA(Multiple sequence alignment) and phylogenetic relationship of 16 s rRNA in bacteria causing infective endocarditis J. Pharm. Sci. & Res., 2017, (12)Vol. 9:2444-2446

10- Becker K, Harmsen D, Mellmann A, *et al*. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of Staphylococcus species. J Clin Microbiol 2004:42(4988–95).

11- Bertilsson S, Cavanaugh CM, Polz MF. Sequencing-independent method to generate oligonucleotide probes targeting a variable region in bacterial 16S rRNA by PCR with detachable primers. Appl Environ Microbiol 2002:68(86-6077).

12- https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch.

13- Albahadle A. and Abdul Abase A, Common causes of neonatal sepsis in Kadhimiya Teaching Hospital, QMJ,2010:6(10).

14- Ibrahim S., Rahma S, Microbiological Profile of Neonatal Septicemia. the Iraqi postgraduate medical J. 2012:11(1).

15- Al-Hamadani A, Sheeh A, Neonatal septicemia in AL_Najaf AL_Ashref governerate : bacteriological profile and antimicrobial sensitivity QMJ. 2008, 4(5).

16- Woo PC, Lau SK, Teng JL, *et al*, "Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories" J. Clin. Microbiol. Infect., 2008, 14: (2).908-934.

17- Rachel RP, Anandan Sh, Ebenezer K, *et al*. Achromobacter xylosoxidans septicaemia in a neonate paediatric infectious disease 6 (2 0 1 4) 9 9 e1 0 1.

18- Turel O, Kavuncuoglu S, Hosaf E, *et al*. Bacteremia due to Achromobacter xylosoxidans in neonates: Clinical features and outcome. Braz J Infect Dis, 2013; 17(4): 450–54.

19- Kim S, Kim VH, Monique L, *et al*. Sepsis Caused by Achromobacter Xylosoxidans in a Child with Cystic Fibrosis and Severe Lung Disease Am J Case Rep. 2016; 17: 562–566.

20- Fraser SL, Arnett M, Sinave CP. Enterobacter infections: overview-emedicine infectious disease http://emedicine.mdscape.com/article/216845.overview [Updated: Sep 05, 2017]

21- Mahapatra A, Ghosh S K, Mishra S, *et al*. Enterobacter cloacae: A predominant pathogen in neonatal septicaemia. Indian J Med Microbiol 2002; 20:110-2.

22- Kuruvilla KA, Pillai. S, Jesudasan M, et al. Bacterial profile of sepsis in a neonatal unit in South India. Indian Pediatr 1989; 35:851-585.

23- Sinha N, Dev A, Mukherjee AK, Septicemia in neonates and early infancy. Indian J Pediatr 1986; 53:249-256.

24- Jay W, Park MD, Grossman O, Aerococcus viridans Infection Case Report and Review, clinical pediatrics,1990,29(9) :225-226

25- Rasmussen M. Aerococcus: an increasingly acknowledged human pathogen Clinical Microbiology and Infection Volume 22, Issue 1, January 2016, Pages 22-27