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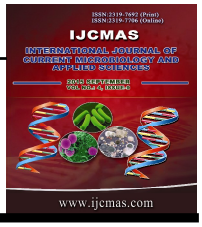

Original Research Article

A Study in Neonatal Sepsis Using Polymerase Chain Reaction-Based DNA Sequencing to Detect the Aetiological Agent in the Peripheral Blood of the Neonates with Sepsis

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Keywords

PCR, DNA sequencing, 16SrRNA region, Neonatal sepsis

A B S T R A C T

Blood culture, the gold standard for diagnosis of neonatal sepsis has low sensitivity and is time consuming. Therefore, objective is development and application of Polymerase chain reaction (PCR)-based DNA sequencing to detect and identify bacterium causing neonatal sepsis in culture negative specimens. Sixty three peripheral blood and 8 cerebrospinal fluid (CSF) referred from tertiary level Neonatal care Unit of the multi-specialty hospital from January 2012 to August 2013 were included. Specific Gram positive and Gram negative PCR followed by *16SrRNA* region DNA sequencing was carried out. Based on DNA homology with Genbank sequences bacterial species were identified. Clinical details of these neonates including risk factors for early and late onset sepsis, reports of septic screen, blood culture and short term outcome till discharge were collected by retrospective chart review. PCR based DNA sequencing revealed 56 bacterial genomes comprising of 55 single bacterial species and 1 mixed bacterial species. Among them, 47 (84%) were Gram negative, 5 (8.9%) were Gram positive and 4 (7.1%) were uncultured bacterium. *Escherichia coli* (n =16; 27.5%) was the predominant bacterium identified. This study revealed newer emerging bacterial pathogens- *Escherichia fergusonii*, *Cronobacter dublinensis*, *Enterobacter hormachei*, *Janibacter species*, *Kluyvera georgiana*, *Streptococcus pseudopneumoniae* as etiological agents of neonatal sepsis. To conclude, PCR based DNA sequencing is a rapid and specific molecular biological test to identify the causative agent of neonatal sepsis. Our study revealed novel bacterial pathogens as aetiological agents of neonatal sepsis.

Introduction

Neonatal sepsis is a common reason for admission in neonatal unit with an incidence of 30 per 1000 live births in India and is a major cause of mortality (Sankar et al. 2008;

Paul et al. 2011). Early diagnosis and early treatment of neonates with suspected sepsis are required to reduce sepsis related mortality. Clinical diagnosis of sepsis in new born infants is not easy because symptoms and signs are non-specific (Yaday et al. 2005). Blood culture is the gold standard for confirmation of diagnosis but the results of the test are available only after 48–72 hours. Presumptive management of neonatal sepsis with broad spectrum antibiotics is needed based on risk factors and clinical features (Benitz 2010; Richard 2012).

Molecular techniques like Polymerase Chain Reaction (PCR) have increasingly been used to detect and identify pathogenic organisms in clinical samples with high degrees of sensitivity and specificity (Klausegger et al. 1999; Jalava et al. 2000; Therese KL et al. 1998; Anand et al. 2000; Bagyalakshmi et al. 2006). Recent meta-analysis on molecular assays in neonatal sepsis concluded that, the present available methods do not have sufficient sensitivity to replace blood cultures and can be an adjunct test (Pammi et al. 2011). PCR targeting the conserved *16SrRNA* gene sequences followed by DNA sequencing enable the detection of bacterial species (Kattar et al. 2000; Kotilainen et al. 1998; Aarthi et al. 2011; Aarthi et al. 2012). The application of PCR based DNA sequencing to identify the bacterial aetiology from direct clinical specimens is limited (Kane et al. 1998, Laforgia et al. 1997, Shang et al. 2001). In order to establish rapid etiological diagnosis, reliable nucleic acid based amplification tests targeting infectious etiology is essential.

In earlier studies on neonatal sepsis, etiological agents like *Escherichia fergusonii* has been recovered from blood (Lia et al. 2011), *Cronobacter dublinensis* (Kleiman et al. 1981) and *Enterobacter hormachei* (Mshana et al. 2011), which can survive in infant formula were known to cause sepsis. *Janibacter* species (Elsayed et al. 2005) and *Klyuvera georgiana* (Carter et al. 2005) has been associated with bacteremia and *Streptococcus pseudopneumoniae* associated with sepsis (Harf-Monteil et al. 2006). We evaluated the identification of bacterial agents in neonatal sepsis by a newly developed rapid nucleic acid based amplification technique of Polymerase chain reaction (PCR) based DNA sequencing targeting *16SrRNA* region of bacteria in clinical specimens of suspect neonatal sepsis and their short term outcome.

Materials and Methods

The study was carried out in a referral microbiological laboratory in South India. Clinical specimens of peripheral blood (collected in BD vacutainers) and CSF, referred for evaluation of neonatal sepsis from a tertiary level neonatal unit of a multi-specialty hospital during the period from January 2012 to August 2013 were included in the study. Institutional Ethical clearance was granted to conduct the study informed consent was obtained before collection of peripheral blood. Peripheral blood sample for molecular analysis was sent from the referral hospital, if the culture did not show any bacterial growth after 48 hours of incubation period and there was a strong clinical suspicion of sepsis. CSF samples were sent for molecular analysis if CSF analysis based on cell counts and biochemistry was suggestive of meningitis and cultures were sterile. To evaluate PCR based DNA sequencing, a total of 14 control specimens obtained from adult septicemic patients which were culture positive were tested The list of blood culture positive control specimens used is given in Table 1.

Concordant results were obtained between PCR based DNA sequencing and culture results. The performance of this diagnostic test was approved by our Institutional Ethical Board (IRB).

Polymerase chain reaction targeting *16SrRNA* for detection of bacteria

First round of PCR targeting *16SrRNA* for detection of bacteria was carried out as per the method of Therese et al. 1998. Nested amplification was carried out using first round of bacterial amplified product with primers differentiating Gram positive and negative bacteria as per the method of Aarthi et al 2013. The reaction mixture consisted of 50 µl reaction with 25 µl Milli Q water, 5 µl 10X *Taq* buffer, 8 µl dNTPs (100 µM each),1 µl (100 picomoles) of each primer Gram positive forward primer 5' AAGGGGATAACTGGGAAACG 3' Reverse primer 5' TCCGAAACCTTCAT CCTCAC 3' and Gram negative forward primer 5' ACCGCGAAAAGCACCGGCTA 3' and gram negative reverse primer 5' TCC CCACGCTTTCGCGCCTA 3' and 0.3 µl (1 unit) *Taq* polymerase and 5 µl first round amplified product. The thermal profile consisted of initial denaturation at 94ºC for 5 minutes followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 60ºC for 1 minute, extension at 72ºC for 2 minutes and final extension at 72ºC for 7 minutes. The second round of amplification was carried out with the same thermal profile for 25 cycles with annealing of 55ºC for both gram positive and gram negative bacteria. The amplified product of Gram positive and Gram negative PCR were subjected to DNA sequencing to determine the identity of bacteria.

PCR based DNA sequencing

The PCR amplified products were further subjected to cycle sequencing reaction, consisting of 4µl of big dye terminator, 2µl of amplified product, 2 picomoles /µl of forward or reverse primer and 2µl of deionized water. The PCR profile consisted of denaturation at 96°C for 1min, followed by 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min and final extension of 4°C. The cycle sequenced products were further purified according to the standard protocol, loaded onto ABI 3100 Genetic Analyzer (Applied Biosystems, USA) with polymer POP6 and sequenced. The sequences were analyzed using BIO EDIT, (downloaded from *http://www.mbio. ncsu.edu/BioEdit/bioedit.html*), and finally blasted with NCBI Blast website *http://blast.ncbi.nlm.nih.gov/Blast.cgi* to identify the species based on DNA homology. Further confirmation of the bacterial identity was performed using multalin analysis software and construction of phylogenetic tree. The sequences of the identified bacterium were compared along with the Genbank bacterial sequences using multalin analysis and a phylogenetic tree was constructed (Pubmed nucleotide search). Based on the results of BLAST, multalin analysis and phylogeny, bacterial species were identified.

Collection of Neonatal Variables

Neonatal details were collected by retrospective chart analysis. Early onset sepsis (EOS) and Late onset sepsis (LOS) was defined when the onset was before 72 hours of age and after 72 hours of age respectively. Birth weight, gestational age, mode of delivery, reason for suspicion of sepsis, risk factors for early onset sepsis such as spontaneous preterm birth (premature rupture of membranes, spontaneous preterm labor) and other risk factors were collected. The age at the onset of late onset sepsis, risk factors as requirement of long lines, umbilical lines, ventilation, total parenteral nutrition, feeding

pattern and previous treatment with antibiotics were recorded. Results of blood cultures done by BACTEC 9120 or brain heart infusion broth, septic screening reports such as C-reactive protein, total count, differential count levels and also the short term outcome till discharge of the neonates were collected. For study purpose short term outcome was defined as death, abnormal neurological examination at discharge and favorable outcome if there was no neurological abnormality by clinical examination at discharge.

Results and Discussion

Among the 61 infants with suspect sepsis, in whom blood cultures showed no growth at 48 hours incubation period, 71 clinical specimens were referred from the neonatal unit in the study period. This included 63 peripheral blood and 8 CSF specimens. The results of PCR targeting *16SrRNA* region of bacteria is shown in Figure 1.

PCR based DNA sequencing revealed 56 bacterial genomes comprising of 55 single bacterial species and 1 mixed bacterial species in peripheral blood specimens. Gram negative bacteria was detected in 47 (84%), Gram positive in 5 (8.9%) and uncultured bacterium in 4 (7.1%) peripheral blood specimens respectively.

The results of PCR based DNA sequencing performed on the *16SrRNA* amplified products are listed in Table 2 with *GENBANK Accession* numbers. *Escherichia coli* (n=16; 25%) was the commonest organism identified in the clinical specimens followed by *Escherichia fergusoni* (n=12; 19%). The application of PCR based DNA sequencing revealed newer bacterial pathogens causing neonatal sepsis. This includes *Escherichia fergusonii, Cronobacter dublinensis, Enterobacter hormachei, Janibacter species, Kluyvera georgiana, Streptococcus pseudopneumoniae, Raoultella planticola, Herbaspirillum species.*

Median birth weight and gestational age at birth of the infants (n=61) was 1880 (600-4180) grams and 33 weeks (26-40 weeks) respectively. Among the peripheral blood specimens 29 specimens (29 infants) had suspect EOS and 34 specimens (32 infants) had suspect LOS. Final blood culture by BACTEC 9120 (55 specimens) or brain heart infusion broth (8 specimens) were negative in 61 peripheral blood specimens. Comparative analysis of the onset of sepsis and bacteria identified by PCR based DNA sequencing targeting *16SrRNA* region are provided in Table 2.

Among the 29 infants with suspect EOS antenatal risk factors such as spontaneous preterm birth (preterm rupture of membranes or spontaneous preterm labor), abruption, clinical chorioamnionitis and meconium stained amniotic fluid were seen in 15, 3, 2 and 2 specimens respectively. The commonest clinical symptom in these neonates was respiratory distress in 18 infants, followed by lethargy apnea, requirement of inotropes, seizures. Septic screen was negative in 11 infants. The commonest organism isolated by PCR in early onset sepsis was *Escherichia coli*, followed by *Escherichia fergusonii*. Only one gram positive organism was isolated in EOS (*Staphylococcus hemolyticus*) and PCR was also negative in 3 infants. The median age of suspect LOS in 32 infants (34 peripheral blood specimens) was 7 (range 5-82) days. A majority of them were very low birth weight (n=15) and below 32 weeks gestation at birth (n=14). Risk factors such as presence of long lines and umbilical lines, use of parenteral nutrition, invasive ventilation and hospital stay more than 2 weeks were seen in 13, 6, 15, 11 and 7 infants respectively.

The commonest clinical symptom was lethargy, followed by apnea, respiratory distress, severe hyperbilirubinemia, fever and abdominal distension. Septic screen was positive in 28 clinical specimens. The commonest organism isolated by PCR in late onset sepsis specimens was Escherichia coli followed by *Escherichia fergusoni*, *Cronobacter dublinensis*, and *Janibacter species*. There were 3 Gram positive organisms isolated in LOS and PCR was negative in 5 specimens. The details of bacterial pathogens are shown in Table 2.

Short term outcome showed 3 deaths and 3 infants had abnormal neurological examination at the time of discharge. PCR was positive in all 6 blood specimens, despite blood cultures showed no growth. *Escherichia coli* and *Cronobacter dublinensis* sepsis was cause of death in two infants and one had vein of Galen malformation. *Streptococcus pseudopneumonia* sepsis and meningitis was the cause for neurologic abnormality in one infant. Among the other 2 infants one had cerebral malformation and the other was an extreme preterm baby with *Candida* sepsis.

CSF analysis was done in 51 neonates and features of meningitis as evidenced by high cell counts and hypoglycorrhachia or high protein levels were seen in 13 infants (3 EOS, 10 LOS). Although CSF cultures showed no growth in all 13 infants, PCR analysis done in 8 CSF specimens identified the presence of single bacteria in 3 specimens. The organisms isolated were *Streptococcus pseudopneumoniae* in one infant, *Escherichia coli* in one infant and in both infants PCR from peripheral blood and CSF showed the same organism. *Staphylococcus aureus* was isolated from the CSF of third infant, but the peripheral blood specimen was negative for PCR.

Gram negative bacteria belonging to *Enterobacteriaceae* were predominan etiological agents of early onset and late onset sepsis. Newer bacterial pathogens namely *Escherichia fergusonii, Janibacter species, Cronobacter dublinensis, Kluyvera georgiana, Raoultella planticola, Herbaspirillum* species, *Escherichia alberti*, *Enterobacter cowanii*, and *Streptococcus pseudopneumoniae* were detected in neonatal sepsis. PCR based DNA sequencing performed on amplified products revealed *Escherichia fergusonii* in 2 neonates, 1 each of *Herbaspirillum* species *Janibacter limosus* and *Streptococcus pseudopneumoniae* in other 3 neonates.

PCR based DNA sequencing performed on the paired specimens of blood and cerebrospinal fluid revealed similar etiological agents of *Streptococcus pseudopneumoniae.* The multalin analysis performed on *16SrNA* sequences of *Streptococcus pseudopneumoniae* detected from the peripheral blood and CSF specimen is shown in Figure 2.

Multalin analysis of *Streptococcus pseudopneumoniae* from peripheral blood and CSF specimens revealed high sequence similarity with that of *Streptococcus pseudopneumoniae* (Genbank Accession number: NR_074987). The multalin analysis performed on the*16SrRNA* sequences of *Streptococcus pseudopneumoniae* reveled nucleotide polymorphisms at position 55 (G to A); 295 (G to C); 300 (G to C); 401 (G to C) in both peripheral blood and CSF specimens. The *16SrRNA* sequences of *Streptococcus pseudopneumoniae* detected from CSF revealed nucleotide polymorphisms at positions 231, 232(CG to TT) and 247 (Deletion of A). The comparative analysis of the onset of sepsis vs bacteria identified by PCR based DNA sequencing targeting 16SrRNA region is provided in Table 3. The emerging bacterial pathogens of neonatal sepsis detected in the

study and their clinical significance is provided in Supplementary file -Table 4.

Pipercillin and tazobactum with amikacin was the commonest antibiotic used for treatment in 41 infants, followed by Imipenem with amikacin, Meropenem and Colistin. Vancomycin was used in treatment of gram positive organism and infants with *Staphylococcus hemolyticus* and *Streptococcus thermophilus* was treated with Ampicillin. In clinical management of neonatal sepsis, Piperacillin- tazobactum and amikacin was administered in 43 neonatal sepsis and good prognosis obtained with the same.

The distribution of clinical diagnosis and the distribution of antibiotics administered for clinical management of neonatal sepsis are provided in Figure 3 and Figure 4 respectively. Further analysis on Gestation, Onset of sepsis and Mode of delivery revealed no statistical significance.

Neonatal mortality rate of India in 2010 is 34 per 1000 live births and accounts for 75% of infant deaths (Sankar et al. 2008**).** Neonatal sepsis is a leading cause of death and bacterial pathogen distribution is different in developing countries with a predominance of Gram negative bacteria and a higher mortality. Blood culture is the gold standard for neonatal sepsis, but has low sensitivity (Behera et al. 2010).

In a study conducted by Reir Nilson et al 2009, the diagnosis of bacterial sepsis in the newborn, a pathogenic bacterium was detected in the blood culture in only 19.4% of these patients. With the molecular method of broad range 16S rDNA PCR, the detection of bacteria improved to 29.0%. *16SrRNA* sequencing has a role in bacterial detection at clinical or public health setting, due to its presence in all bacteria (multigene family - operons). The function of *16SrRNA* gene has not changed over time, suggesting that random sequence changes are accurate measure of evolution; and *16SrRNA* gene (1,500 bp) is large enough for species level bacterial identification (Janda et al. 2007).

Several research groups (Rupenthal et al. 2005, Jordan et al. 2006, Brozanski et al. 2006) have focused on the application of *16SrRNA* sequencing to rapidly identify the bacterial aetiology of neonatal sepsis. Although the recent meta-analysis on molecular diagnosis in neonatal sepsis concluded that presently available methods lack adequate sensitivity to replace blood cultures, present study showed that PCR based DNA sequencing targeting 16SrRNA region of bacteria detected newer bacterial pathogens causing neonatal sepsis.

These bacteria are uncultivable or have highly exacting nutrient requirements. *Klebsiella pneumoniae* is the commonest cause of neonatal sepsis in India (Anita et al. 2006). But in this case series *Escherichia coli* (27.5%) is the commonest isolate from peripheral blood specimens and blood culture revealed no growth at 48 hours incubation period. The reported incidence of *Escherichia coli* neonatal sepsis in studies are 13%, (Bizzarro et al. 2008), 18.9% (Bizzarro et al. 2003), 33% (Frantz et al. 2011), Das et al. 2011 and MacFie et al. 1999 have reported on the pattern of colonization of the neonatal gut by aerobic Gram-negative bacilli (GNB) and evaluated the association between gut colonization and sepsis in the developing countries. The study revealed that colonization was influenced by a stay in the neonatal intensive care unit and prolonged use of a feeding tube; and also an association between gut colonization and neonatal sepsis was observed.

Table.1 List of blood culture positive control specimens identified by PCR targeting 16SrRNA

Positive control specimens	Blood culture results	PCR based DNA sequencing
Pseudomonas aeruginosa	Positive	Correlated
Salmonella paratyphi A	Positive	Correlated
Escherichia coli	Positive	Correlated
Salmonella typhi	Positive	Correlated
Klebsiella pneumoniae	Positive	Correlated
Klebsiella oxytoca	Positive	Correlated
Acinetobacter species	Positive	Correlated
Enterobacter species	Positive	Correlated
Burkholderia cepacia	Positive	Correlated
Enterobacter species	Positive	Correlated
Staphylococcus aureus	Positive	Correlated
Coagulase negative Staphylococcus	Positive	Correlated
Alpha hemolytic Streptococci	Positive	Correlated
Enterococcus faecalis	Positive	Correlated

Table.2 Bacterial pathogens identified in peripheral blood specimens by PCR targeting 16SrRNA amplified products

Bacterial pathogen identified by PCR	EOS (n = 26)	LOS (n=29)	Genbank Accession numbers (n)
Escherichia coli	8	8	JQ661175 (2), JX195712 (1), JN654456 (1), NR074891 (8), NR024570 (2), KC138802 (1), EF064807 (1)
Escherichia fergusonii	6	6	NR074902(3), NR027549 (9)
Cronobacter dublinensis	2	3	NR044062 (5)
Enterobacter hormachei	2	2	NR042154 (4)
Janibacter species	2	1	NR0263362 (1),NR383745 (1),NR024570 (1)
Kluyvera georgiana	0	2	NR024883 (2)
Herbaspirillum species	0	1	JF990839 (1)
Escherichia alberti	1	0	NR025569 (1)
Enterobacter cowanii	1	0	NR025566 (1)
Burkholderia cepacia,	1	0	JN800445 (1)
Raoultella planticola,	1	0	NR024996 (1)
Acinetobacter calcoaceticus	0	1[#]	AB650603 (1)
Staphylococcus haemolyticus	1	1[#]	KC329826 (2)
Streptococcus pseudopneumoniae	0	1	NR074987 (1)
Streptococcus thermophilus	0	1	NR042778 (1)
Untypable bacteria	1	3	JQ436076(1), HM283810 (1), JF200819 (1), GU362968 (1)

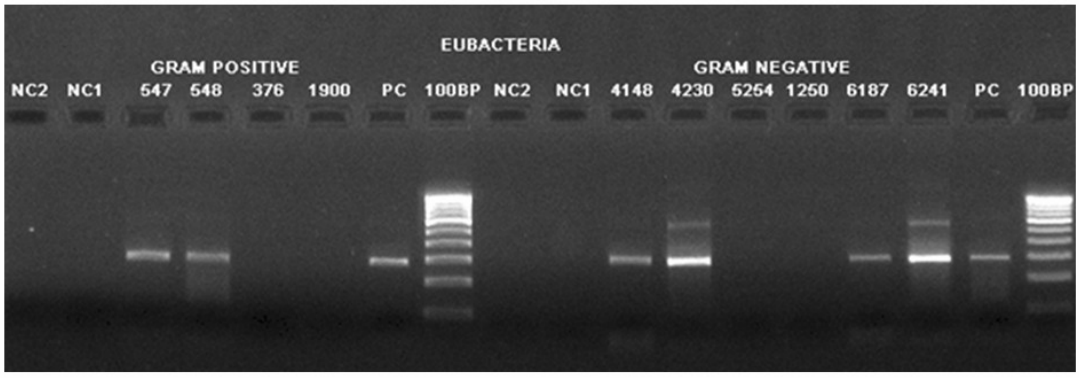
Note: # Mixed growth; EOS – Early onset sepsis; LOS – Late onset sepsis

Table.3 Comparative analysis of the onset of sepsis vs bacteria identified by PCR based DNA sequencing targeting 16SrRNA region

Onset of sepsis	Clinical Specimen (N =71)		PCR targeting 16SrRNA region positive N = 57	Bacteria identified by PCR based DNA sequencing targeting 16SrRNA region
	Blood N = 63	CSF N = 8		
Early onset < 3 days N = 4	4	0	4	*Escherichia coli* (3) *Burkholderia cepacia* (1)
Late onset sepsis > 3 -10 days N = 43	38	5*	34	*Escherichiacoli* (9) *Escherichia fergusonii* (8) *Janibacter species* (3) *Cronobacter dublinensis* (2) *Uncultured bacterium* (3) *Kluyvera georgiana* (2) *Raoultella planticola* (1) *Escherichia albertii* (1) *Enterobacter cowanii* (1) *Enterobacter hormachei* (2) *Streptococcus pseudopneumoniae* (2)*
Late onset sepsis > 10 days N = 24	21	3	19	*Escherichia coli* (4) *Escherichia fergusonii* (5) *Cronobacter dublinensis* (3) *Enterobacter hormachei* (2) *Uncultured bacterium* (1) *Herbaspirillum* species (1) *Staphylococcus haemolyticus* (1) *Streptococcus thermophilus* (1) *Staphylococcus haemolyticus* # (1)+ *Acinetobacter calcoaceticus*

NOTE : CSF : Cerebrospinal fluid; *Among the 5 CSF specimens subjected to PCR based DNA sequencing bacteria was detected only in one specimen. *Streptococcus pseudopneumoniae* was detected from the peripheral blood and CSF of the same neonate.; # Mixed bacterial aetiology of *Acinetobacter calcoaceticus* and *Staphylococcus haemolyticus* were detected in one peripheral blood specimen.

Figure.1 Results of specific Gram positive and Gram negative PCR targeting 16SrRNA region of bacteria



Gram positive

NC2 :Negative control II round
NC1 :Negative control I round
547 : Peripheral blood positive
548 : Cerebropsinal fluid positive
376 : Peripheral blood negative
1900 : Peripheral blood negative
PC: Positive control *S. aureus* ATCC 25923
100 bp : Molecular weight marker 100 bp ladder

Gram negative

NC2 : Negative control II round
NC1 : Negative control I round
4148 : Peripheral blood positive
4230 : Peripheral blood positive
5254 : Peripheral blood negative
1254 : Peripheral blood negative
6187 : Peripheral blood positive
6241 : Peripheral blood positive
PC: Positive control *E. coli* ATCC 25922

Figure.2 Multalin analysis performed on the *16SrRNA* sequences of bacteria detected from peripheral blood and cerebrospinal fluid specimen with the sequences of *Streptococcus pseudopneumoniae* (Genbank Accession number : NR_074987.1)

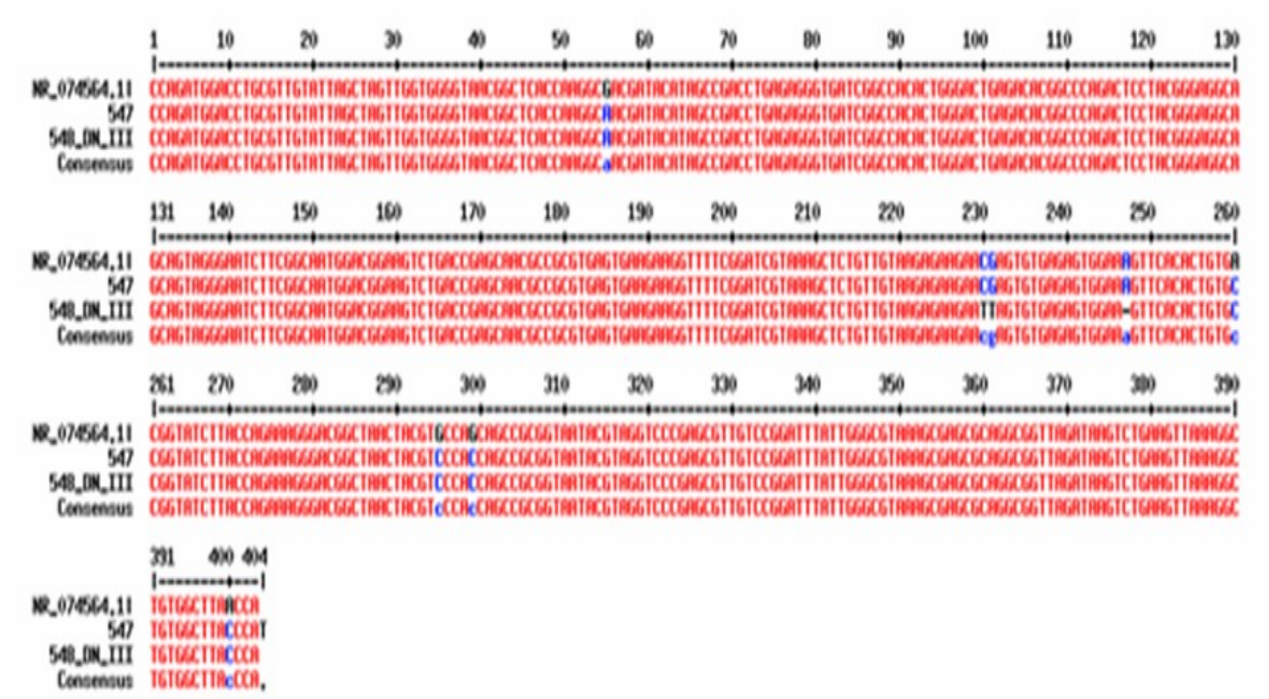


Figure.3 Distribution of clinical presentation

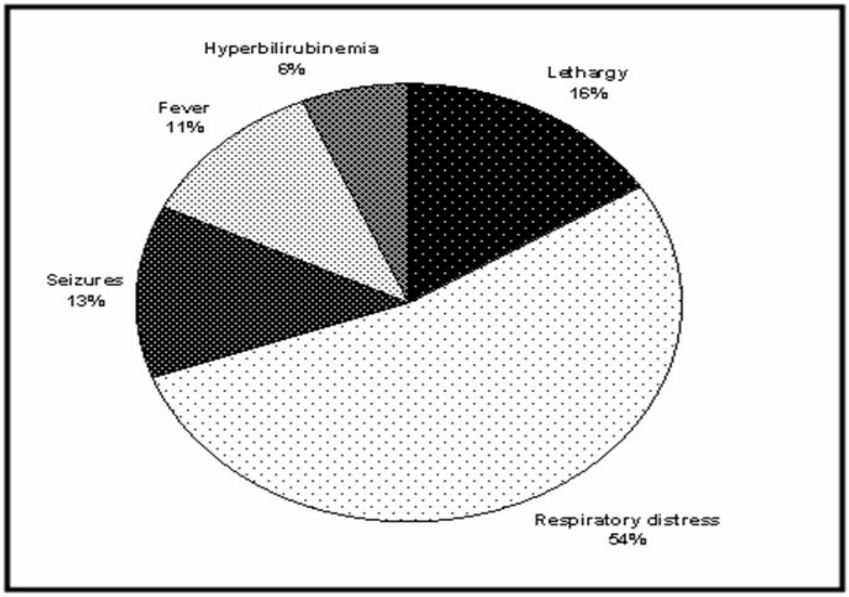
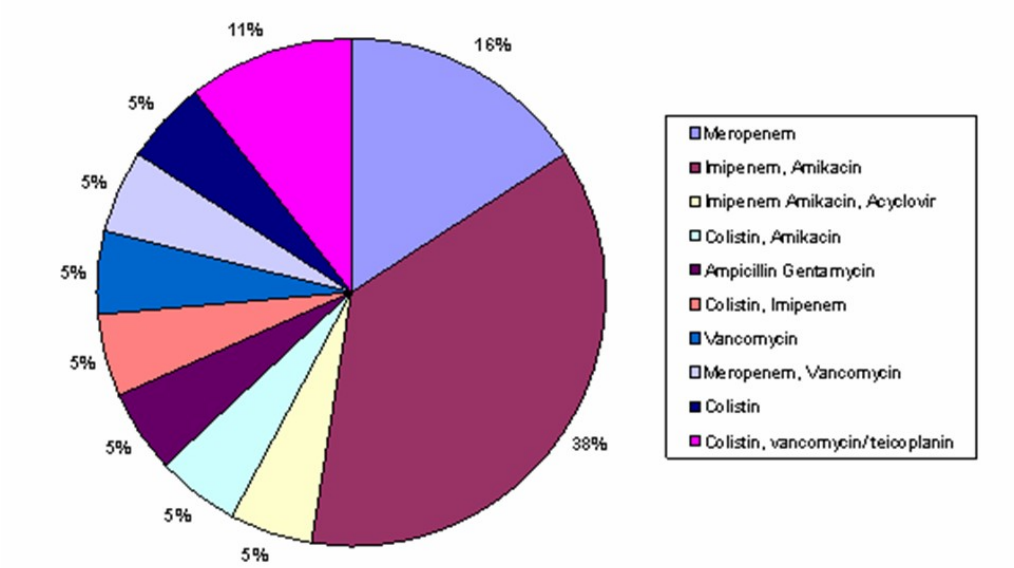


Figure.4 Chart representing the distribution of administered for clinical management of neonatal sepsis



Emergence of *Escherichia fergusoni* in clinical specimens has been described from Orissa (Mahapatra et al. 2005), India and in this study it was the second common bacteria isolated from clinical specimen. Isolation in *Enterobacter hormachei* (Townsend et al. 2008), has been described as a causative agent of neonatal sepsis and this study revealed a resurgence of this pathogen. In the present study *Enterobacter cowanii, and Burkholderia cepacia* have been described as aetiological agents of sepsis.

We presume the novel bacteria detected in the study as the etiological agents of sepsis and not mere colonizers as we correlated the presence of bacteria in peripheral blood with that of clinical signs and symptoms of neonatal sepsis. In order to rule out these bacteria as etiological agents of sepsis, it is essential to assess the state of colonization of the mother's genital tract. The state of colonization of their ability to cause sepsis can be detected by developing nucleic acid based amplification techniques targeting the genes coding for virulence factors of these novel bacteria. In the present study, *16SrRNA* PCR was able to detect bacterial pathogens in culture negative suspect EOS and LOS; hence decisions on antibiotics duration and diagnosis was possible. The main limitation is non-availability of drug sensitivity, but limiting the antibiotics only for gram positive or gram negative bacteria was possible. Similarly the detection of bacteria from CSF specimens has changed the management of these infants. Among the 32 infants with suspect LOS, 17 infants had received antibiotics within 5 days prior to the onset of symptoms. Due to retrospective collection of neonatal data, details of peripartum antibiotics usage in mothers are incomplete.

The cost of PCR based DNA sequencing per test is Rs 3210 which is inclusive of Rs 1950 for eubacterial PCR and Rs 1260 for PCR based DNA sequencing. In order to apply this test for diagnostic use, a tertiary care centre should be equipped with a DNA sequencer. The significance of this test lies on not only identifying the etiological agent of neonatal sepsis but also to rule out any bacterial cause and accordingly initiate appropriate treatment.

The utility of this test in a tertiary care

hospital is that initially PCR technique at least can be employed in healthcare settings to rule out the bacterial cause of neonatal sepsis. Application of PCR targeting the *16SrRNA* of bacteria would prove useful in avoiding the unnecessary use of antibiotics.

The present study showed that PCR based DNA sequencing is a sensitive method for detecting etiological agents of neonatal bacterial infections in culture-negative specimens. . To overcome this, future assays have to be developed targeting the organism and antibiotic panel. The identification of the etiology by PCR based DNA sequencing serve as a guide promoting the rationale use of antibiotics. This study revealed newer bacterial pathogens as etiological agents of neonatal sepsis.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Ethical Approval: Submitted to institutional ethics sub-committees (IRB)

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