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Molecular epidemiology of Multidrug resistant Extended-Spectrum β-Lactamase Producing Klebsiella pneumoniae outbreak in a neonatal intensive care unit [*]

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Abstract

Background: Extended spectrum beta lactamase (ESBL)-producing Klebsiella pneumoniae have been occasionally reported as a cause of septicemia outbreak among pediatric patients in medical literature. We aimed to study the source of an outbreak of ESBL-producing K. pneumoniae in the Neonatal Intensive care Unit (NICU) at a tertiary care hospital in South India.

Methods: The outbreak was investigated by phenotypically typing the isolates followed by random amplified polymorphic DNA analysis (RAPD). A total of 31 K. pneumoniae, consisting of 27 blood isolates from neonates and 4 environmental isolates were studied. Antibiotic susceptibility patterns were determined using standard disc diffusion methods; ESBL production was tested both phenotypically and genotypically. The strains were typed using two primers AP4 and HLWL74.

Results: Except 2 environmental strains, all were found to be ESBL producers and of ESBL types TEM-1, SHV-12 and CTX-M- 15. Two different antibiotic resistance patterns were identified and the RAPD typing revealed two profiles. Phenotypic and genotypic analyses showed that 2 environmental strains had been responsible for the outbreak.

Conclusion: Safe clinical practices should be followed in neonatal wards to prevent spread of infection. This is the first report of blaCTX-M-15 producing K.pneumoniae and the first outbreak in our hospital due to CTX-M-15 producing K.pneumoniae.

Keywords: Extended spectrum β lactamase, Neonatal Intensive care Unit, Klebsiella pneumoniae, Outbreak

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Introduction

Klebsiella pneumoniae is an important nosocomial pathogen that has the potential to cause severe infections, particularly in intensive care units and amongst pediatric patients[1]. The increased use of extended spectrum cephalosporins has led to the emergence of resistant strains and outbreaks due to these organisms have been associated with higher morbidity and mortality[2]. Spreading of extended spectrum beta lactamase (ESBL) producing *K. pneumoniae* in a hospital may be a complex event involving several modes of spread, such as dissemination of several unrelated strains or the propagation of a single clone from patient to patient[3].

Mostly, ESBL-encoding genes are located within transposons or integrons, which strongly facilitates antibiotic-resistant gene transfer between bacterial species resulting in cross-transmission, thereby spreading resistance among related and unrelated gram-negative bacteria[4]. Investigation of a presumed outbreak by *Klebsiella* species often requires strain typing data to identify outbreak-related strains. Traditional techniques for typing *K. pneumoniae* are based on phenotypic characteristics and include biotyping, antibiogram typing, O-serotyping, bacteriocin and phage typing; but they all have poorly discriminatory power. Unfortunately, biochemical reaction patterns are usually invariable among clinical isolates[5]. Although, the molecular methods such as plasmid-profile analysis, ribotyping, small-fragment restriction endonuclease analysis, gene sequencing and pulsed-field gel electrophoresis are available, they cannot be used routinely in many laboratories in developing countries like India, due to high cost and poor resource settings.

Random Amplification of Polymorphic DNA (RAPD) is a molecular typing technique, which is based on PCR amplification of random DNA fragments with short primers 6-12 bp of arbitrary sequence. The resulting amplified fragments function as polymorphisms for DNA fingerprinting. In contrast to traditional target-specific PCR, no prior sequence information is required and the technique is potentially applicable to all bacteria. This technique has been applied successfully to epidemiological investigations of many bacterial and fungal species[6].

In this background, the aim of this study was to investigate the epidemiology and molecular characterization of multiresistant ESBL-producing *K pneumoniae* strains associated with an outbreak of bloodstream infection (BSI) in a busy neonatal intensive care unit (NICU) in a tertiary care hospital, South India.

Material and Methods

Source of the isolates:

During the month of June 2008, an increased frequency of *K pneumoniae* was isolated in the blood culture from NICU. Blood for culture was collected from these patients on clinical suspicion of neonatal sepsis. Blood cultures were done using biphasic

medium consisting of Brain Heart Infusion (BHI) agar and BHI broth with sodium polyanethol sulphonate as an anticoagulant.The clinical detail of the patients were recorded and environmental sampling, personal surveillance was done in NICU on mid June 2008.

A total of 31 isolates of *K.pneumoniae* were included. Twenty seven strains were isolated from neonates during the outbreak and four strains from environment sampling done in NICU. All the isolates were identified as per the standard bacteriological procedures[7] and they were stocked in 0.2% semi-solid agar tubes and stored under 4°C until further characterization.

Antimicrobial susceptibility testing:

Antimicrobial susceptibility of the isolates was done by the disk diffusion method on Mueller Hinton agar (Hi-Media, Mumbai) following the zone size criteria recommended by the Clinical Laboratory Standards Institute (CLSI) [8]. The antibiotic used were; ampicillin(10µg), amikacin(30µg), gentamicin(30µg), piperacillin(100µg), piperacillin/ tazobactum 100/10µg), cefoperazone/sulbactum (75/10µg), cefoxitin (30µg), cefotaxime (30µg), ceftazidime (30µg), ceftriaxone(30µg), ciprofloxacin(5µg) , cefepime (30µg), Trimethoprm-Sulfmethoxazole (7.5/2.5µg), meropenem(10µg), imipenem (10µg). The Minimum inhibitory concentration (MIC) of third generation cephalosporins (Hi-Media, Mumbai) and meropenem (Astra-Zeneka, UK) was done by agar dilution method as per CLSI guidelines[9]. Control strain *Escherichia coli* ATCC 25922 was included in each series.

Phenotypic detection of ESBL:

The isolates showing resistance to one or more third generation cephalosporins (3GCs) were tested for ESBL production by the combination disc method using cefotaxime(30µg), cefotaxime/clavulanic acid (30/10µg) and cefatzidime(30µg), ceftazidime/clavulanic acid (30/10 µg). A ≥5mm increase in diameter of the inhibition zone of the cephalosporin-plus-clavulanate disc, when compared to the cephalosporin disc alone, was interpreted as phenotypic evidence of ESBL production[8]. *K. pneumoniae* ATCC 700603 was used as positive control and *Escherichia coli* ATCC 25922 was used as negative control.

Molecular detection of ESBL-gene types:

For the molecular analysis, the template DNA was prepared from an overnight culture (18–24 h) on a Mueller–Hinton plate. Two colonies were suspended in 100 µl of distilled water and the cells were lysed by heating at 95°C for 10 min. Cellular debris was removed by centrifugation at 15,000 rpm for 2 min, and the supernatant was used

as a source for DNA for PCR. Isolates with the ESBL phenotype were examined for the presence of β -lactamases genes responsible for the resistance such as *bla*TEM, *bla*SHV, and *bla*CTX-M by PCR using the primers and conditions used elsewhere [10,11,12].

Typing by RAPD analysis:

K.pneumoniae isolates from the infected neonates and isolated from the environment were typed by RAPD technique to investigate the role of environment in transmission of infection. Two individual primers, AP4 (5'-TCA CGA TGC A-3'), HLWL74 (5'-ACG TAT CTG C- 3') [13] were used. The DNA was prepared by boiling method as above mentioned. Amplification was performed on a Corbett Research thermal cycler (HP, USA). The reaction mixtures comprised of 12.5 µl of red eye PCR master mix (Amplicon III), 2µl of lM AP4 and HLWL74 primers each individually, 7.5µl of sterile nano pure water and 3µl of template DNA. The final volume was 25µl. The amplification condition were initial denaturation for 7 min at 94°C; followed by 30cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C; and with a final extension for 10min at 72°C. The amplified products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The band patterns were visually interpretated and a difference of more than 2 bands were considered a given major type, one to two bands difference was considered a minor variant.

Sequencing:

Representative amplified products of TEM, SHV, CTX-M group- 1 PCRs from each cluster were outsourced to Macrogen, Korea for sequencing by the Sanger method using an ABI 373A DNA sequencer, using the primers described previously[10,11,12].

Results

Antimicrobial susceptibility testing:

As determined by disc-diffusion antibiotic susceptibility testing, all the 27 clinical isolates and 2 environmental isolates exhibited the same pattern of resistance to β-lactam agents, demonstrating resistance to penicillins (ampicillin and piperacillin), extended-spectrum cephalosporins (ceftriaxone, ceftazidime and cefotaxime) and cefepime. In addition, they exhibited resistance to gentamicin, chloramphenicol, piperacillin/tazobactam and trimethoprim/sulfmethoxazole. Based on the susceptibility to other aminoglycoside (amikacin), carbapenem (meropenem) and cefperazone/sulbacatam combinations, 2 antibiotypes were defined for the 29 isolates (Table 1). Nine isolates belonged to resistance pattern I, which was characterized by

resistance to amikacin, meropenem, and cefperazone/sulbactam. The 20 isolates belonged to antibiotype pattern II, which differed from I by susceptibility to amikacin, meropenem, and cefperazone/sulbactam .All isolates tested remained susceptible to imipenem. The MIC of 3GCs for both antibiotype I and II were >256 μg/ml, and the MIC of meropenem for antibiotype I and II were 16 μg/ml and <2 μg/ml respectively. The two environmental isolates E3 and E4 were susceptible to all drugs except ampicillin (Table 1).

ESBL types:

All the 27 isolates from neonates and 2 environmental isolates were ESBL positive phenotypically and they carried TEM, SHV and CTX-M group I genes by PCR. Sequencing reveals that TEM-1, SHV-12 and CTX-M 15 type of ESBLs were present in both clinical and environmental strains.

RAPD typing:

In RAPD analysis, two distinct band patters were generated individually by primers AP4 and HLWL74. An average of 2-4 bands per pattern was generated with AP4 primer and with HLWL74 an average of 9-11 bands was generated, thus two RAPD clusters was recorded among the outbreak strains. All the strains from each RAPD clusters shows 100% similarity to the environmental isolates and the two clusters showed a major pattern difference (Figure 1a, 1b). The environmental source of cluster I was found to be the oxygen circuit (at patient end) and for cluster II, the suction tube. The susceptible environmental E3, E4 strains showed varied RADP patterns (Figure 2).

The mean age of the infected patients is 4.8 days and standard error is (.508), the male distribution is 51.9% and female distribution is 48.1%. The distribution of antibiotype II is higher 66.7% and the RAPD type B is 18%. The observed mortality rate among the infected patients is (.67).

Discussion

ESBL producing *K. pneumoniae* are usually resistant to the first line of antibiotics. Additionally, their ability to spread rapidly amongst patients often leads to nosocomial outbreaks constitutes a persistent problem in many parts of the world, especially in intensive care units. Moreover, these bacteria are increasing sources of resistance to other group of antibiotics also[13,14]. In the hospital environment, spreading of these organisms may be a complex event involving several modes of epidemic spread, such as dissemination of several epidemic strains or the propagation of a single clone from patient to patient[15].

K.pneumoniae that produces ESBL has been associated with infection acquired in NICU. A major risk factor for colonization or infection with ESBL producing bacteria is long term antibiotic exposure and frequent use of antimicrobial agents in NICUs. It is a common practice to use aminogycosides and 3GC in a neonatal septicemic cases[16,17]. Usually prescribed antimicrobials fail to inhibit these pathogens, and the most effective antimicrobials against ESBL producing *K.pneumoniae* are carbapenems. Meropenem and imipenem are the two carbapenem available in India; their prescription is based on individual physician criterion that could lead to inappropriate use of antibiotics favoring selection of resistant strains.

This study describes an outbreak due to multiresistant ESBL-producing *K pneumoniae* in a neonatal ICU. Apparently, the outbreak started at the end of may reached peak on June and subsided on July (Figure 3). We suggest that successive small outbreaks, as reported here, may be partly due to changes in the multiplication rate of strains. The micro-organisms generally multiply at a low rate but this may increase for some reason, resulting in the spread of a micro-organism to several patients and possible infections. After transmission, the organisms may regress, leaving patients susceptible to colonization and infection by other strains of *K. pneumoniae.*

In the present study, all the 27 clinical isolates and the 2 environmental isolates were multidrug resistance and ESBL producers nine meropenem-resistant isolates were detected from the patients during the same outbreak period, which suggests that exposure to meropenem induced expression of resistant strains. This resistance may be associated with a modification of a major outer membrane protein or changes in lipopolysaccharide [18]. The widespread use of meropenem in ICUs is of concern as this selects meropenem-resistant isolates. Since both meropenem sensitive and resistant strains caused the outbreak at the same time, this raises the question whether multiple colonization involved sequential exposure to different strains or whether genotypic variations within strains were induced *in vivo* in response to changes in therapy. It is difficult to determine the role, if any, of antibiotic therapy in the evolution of different isolates of *K.pneumoniae.*

Antibiotic-resistant Gram-negative organisms are a significant risk to severely ill children in ICUs and in many instances these are imported into the unit or rapidly acquired from environmental reservoirs[19]. In our study, the isolates from two environmental sites such as oxygen circuit (at patient end) and the suction tube were found to be the cause for the outbreak by the horizontal transfer of these strains. The other 2 RAPD types III and IV from the environment were the susceptible wild strains.

Prior to testing of the multiresistant *K. pneumoniae* strains, RAPD was performed with a random of 6 epidemiologically unrelated strains of *K. pneumoniae* that had the usual susceptibility pattern, showing resistance to ampicillin alone. The RAPD patterns produced were all dissimilar to those seen with the strains from NICU. Typing of the strains using AP4 and HLWL74 gave good discriminatory bands and we suggest that both can be used individually or simultaneously for better interpretation in typing a large number of strains.

The high level of mortality (Table 1) arising from intra hospital infections with multi-drug resistant strains of *K.pneumoniae*, as seen, in this study, is alarming. We did not find any statistically significant associations between patient mortality and any of the factors like sex, age, or antibiotype. The P value is (>0.05). However, this negative result still may be worth mentioning. It appears that the lack of control of contamination sources and hygiene has caused the dissemination if the pathogen among the patients. Simple hygienic measures, such as hand-washing practices, the use of sterile equipment (particularly for intravenous access and when possible), and patient cohorting (i.e., grouping patients with similar infections in the same location) can help prevent the further spread of these resistance traits. Also, the empirical use of carbapenems for nosocomial sepsis where ESBL *Klebsiella* is prevalent should be evaluated and parameters to prevent over usage should be placed.

K. pneumoniae infections have been caused by a variety of strain genotypes that could be transmitted from one patient to another in different ways, and it is important to monitor such strains closely to prevent their spread. In this situation, the design of rational infection control measures that require the adoption of new antibiotic policies in addition to improving hospital hygiene becomes even more challenging[20].

Nosocomial infections in neonatal ICUs are the most difficult and tedious to manage and control. The outcome of neonatal infections can be improved if illness is recognized early and appropriate agents are promptly administered. Knowledge of epidemiological and anti-microbial susceptibility pattern of common pathogens in a given area helps to inform the choice of antibiotics. Epidemiological surveillance studies such as the current one should provide useful information base to guide practice and policies on rational use of anti-infective agents and to eradicate the source of environmental reservoir.

In conclusion, this is the one of the first reports from our hospital in which the causative organism of an outbreak of infection with multi-drug resistant CTX-M-15 producing *K.pneumoniae* associated with a high mortality has been characterized at the molecular level. The RAPD typing is good and can be used as a screening, rapid and inexpensive test for ESBL producing *K. pneumoniae* during investigation of outbreaks.

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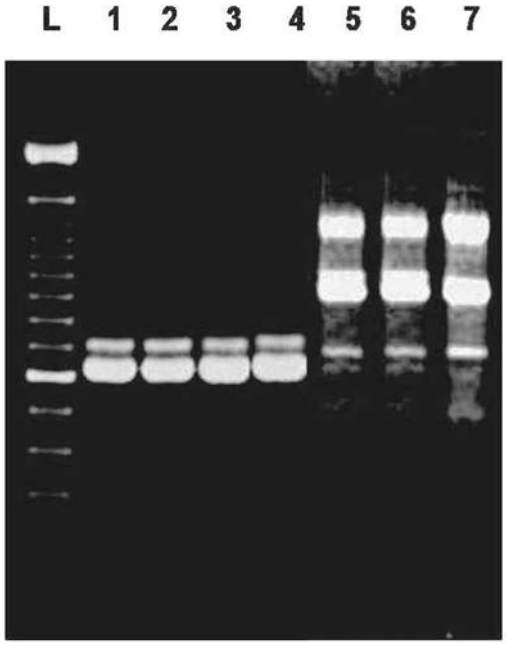
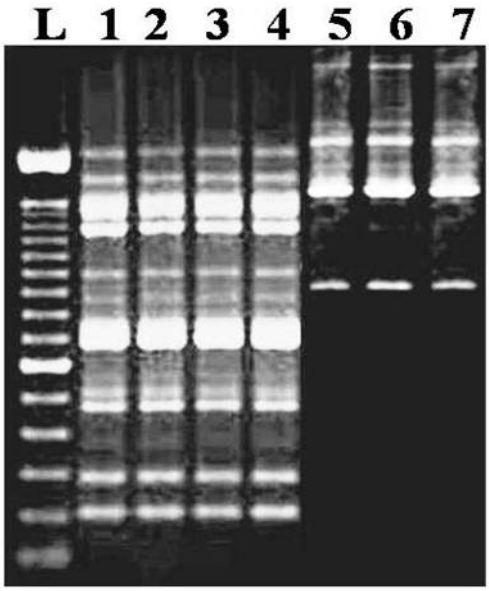
Table 1: Characteristics of 27 neonates with septicemia caused by ESBL-producing *Klebsiella pneumoniae* and 4 Environmental K. *pneumoniae* isolates from NICU

S. no.	Date of collection	Age(in days)/ sex	Antibiotype	RAPD cluster	Patient outcome
1	2.6.08	D1/M	I	A	Expired
2	5.6.08	11/M	I	A	Improved
3	6.6.08	3/F	I	A	Expired
4	17.6.08	2/M	I	A	Improved
5	17.6.08	4/M	I	A	Expired
6	6.6.08	6/F	I	A	Expired
7	19.6.08	1/M	I	A	Expired
8	28.6.08	7/M	I	A	Expired
9	29.6.08	6/F	I	A	Improved
10	3.6.08	5/M	II	B	Improved
11	3.6.08	7/F	II	B	Expired
12	9.6.08	3/M	II	B	Expired
13	10.6.08	5/M	II	B	Expired
14	10.6.08	9/M	II	B	Improved
15	12.6.08	8/F	II	B	Improved
16	12.6.08	5/F	II	B	Expired
17	13.6.08	8/F	II	B	Expired
18	15.6.08	4/M	II	B	Expired
19	15.6.08	5/F	II	B	Expired
20	18.6.08	2/M	II	B	Improved
21	20.6.08	7/M	II	B	Expired
22	20.6.08	3/F	II	B	Expired
23	26.6.08	1/F	II	B	Expired
24	24.6.08	6/F	II	B	Improved
25	26.6.08	5/F	II	B	Expired
26	26.6.08	1/F	II	B	Improved
27	28.6.08	6/M	II	B	Expired
28	16.6.08	Oxygen ciruit (Patient end)	I	A	-
29	16.6.08	Suction tube	II	B	-
30	16.6.08	Feeding area	III	C	-
31	16.6.08	Weighing scale	IV	D	-

Figure 1: RAPD analysis of representative *K. pneumoniae* outbreak isolates



1a) L- 100bp molecular DNA marker, lanes 1-3 clinical isolates & lane 4- environmental isolate showing same RAPD pattern (cluster I), lanes 5-6 clinical isolates& lane 7- environmental isolate showing same RAPD pattern (cluster II) with primer AP4**.**



1b) L- 100bp molecular DNA marker, lanes 1-3 clinical isolates & lane 4- environmental isolate showing same RAPD pattern (cluster I), lanes 5-6 clinical isolates& lane 7- environmental isolate showing same RAPD pattern (cluster II) with primer HLWL74.

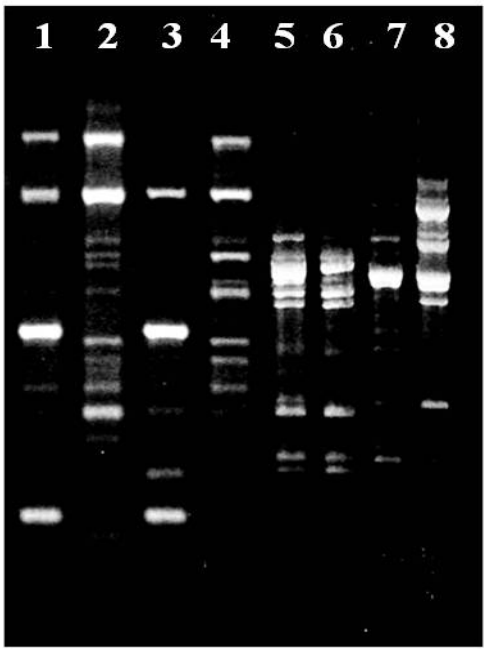


Figure 2: RAPD patterns of susceptible isolates with primer HLWL74. Lane 1&2, environmental isolates during the outbreak. Lane 3-8, epidemiologically unrelated strains

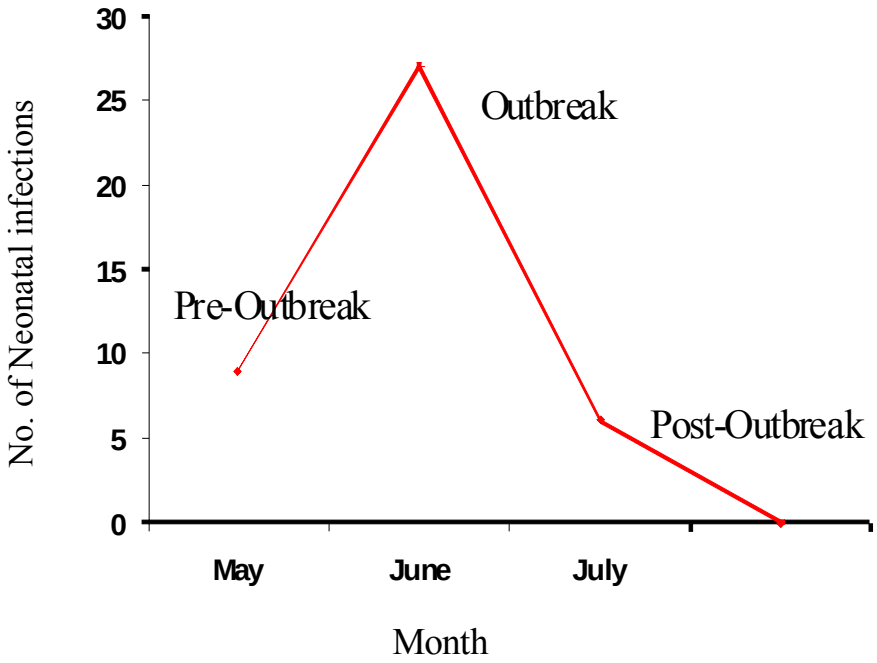


Figure 3: Number of blood stream infections cases due to *Klebsiella pneumoniae* during the month of May- July 2008 in the Neonatal Intensive Care Unit