International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 1 (2014) pp. 30-37**
http://www.ijcmas.com

Original Research Article

Study of multidrug resistance and extended spectrum beta lactamases producing *Klebsiella pneumoniae* isolated from hospitalized patients

M.Mohamed Mahroop Raja[1*] and S.Ahmed John[2]

[1]Research and Development Centre, Bharathiar University,
Coimbatore – 641 046, Tamilnadu, India
[2]Jamal Mohamed College (Autonomous), Tiruchirappalli - 620 020 Tamilnadu, India.
*****Corresponding author

K e y w o r d s

Hospital acquired infection; Resistance; Beta lactamases; Outer membrane and Porin

A B S T R A C T

In the several decades, antibiotic resistance bacteria have been implemented a constant problem in hospital management. The emergence of multidrug resistance and extended spectrum of beta lactamase producing *Klebsiella pneumoniae* faces a serious illness in clinical setting and antibiotic resistant system. A total of 50 urine samples were collected from hospital acquired infectious patients. Among 50 samples, *Klebsiella pneumoniae* was found to be 18%, which was identified by the microbiologically experimental test under the guidelines of Bergey's manual of *determinative* Bacteriology. The isolates were highly resistant to various antibiotics such as Ampicillin, Aztreonam and Cephotaxime and sensitive to Ampicillin clavulnate, Cephotaxime clavulnate, Co- trimoxazole, Ertapenem and Imipenem. Among 18% of *Klebsiella pneumonia* samples, were analysed by various Extended spectrum beta lactamases screening test such as Double disk synergy test, Modified double disk method and Disk replacement method. *Klebsiella pneumoniae* isolate produced greater than 5mm zone of inhibition to various antibiotics such as Cephotaxime clavulnate, Cephotaxime alone, Ampicillin clavulnate and Ampicillin alone, which were indicated as positive result for extended spectrum of beta lactamases production. In SDS PAGE analysis, extended spectrum of beta lactamase producing *Klebsiella pneumoniae* associated with the loss of outer membrane protein. This is clearly demonstrated that Extended spectrum of beta lactamase producing *Klebsiella pneumoniae* had excellent reduced susceptibility to beta lactamases inhibitor antibiotic combination as a result of multidrug resistance towards the loss of outer membrane porin proteins.

Introduction

Extended spectrum beta lactamases producing organism pose unique challenges to clinical microbiologist, infection control professional and antibacterial discovery scientists. Extended spectrum beta lactamases are enzymes that inactivate beta lactam antibiotic such as Penicillin, Ampicillin, fist generation cephalosporin antibiotic Cephalothin, second generation

cephalosporin antibiotics Cefoxitin, cefuroxime, third generation cephalosporin antibiotics Ceftriaxone, Ceftazidime, Cefotaxime and Monobactam antibiotics and Aztreonam antibiotic (Livermore,1995). The emergence and spread of resistance is also threatening to create species resistance to all currently available agents. The rapidity of the development and spread of resistance is influenced by selective pressure, pre-existence of resistance gene and use of infection control measures. Resistance to beta lactam antibiotics among clinical isolates of gram negative bacilli is most often due to the production of beta lactamases (Samaha and Araj 2003). These enzymes are numerous and have lead to the development of extended spectrum beta lactamases and are widely distributed among the *Enterobacteriaceae* family (Braford, 2001; Thomson *et al*., 1996).

Enterobacteriaceae is an important pathogenic group in hospital acquired infection and most serious resistance patterns now emerging among gram negative rod *Klebsiella pneumoniae* are resistance to extended spectrum Penicillin, Cephalosporin groups and Aztreonam (Guzman *et al*.,2000). In addition, non-enzymatic mechanism that confer resistant to all cephalosporin antibiotics including cephamycins. These mechanism include the loss of particular protein within outermembrane. such a protein is called as porin that results in reduced permeability to the beta lactams (Nikaido,1989). It is a major role in the emergence of extended spectrum beta lactam resistant strain of *Klebsiella pneumoniae.* The present study is aimed to document the drug resistance pattern towards existence of extended spectrum of beta lactamases producing strain in *Klebsiella pneumoniae* due to the loss of outer membrane protein ompK35-40kDa.

Materials and Methods

Sample Collection and Handling

This study was performed at a hospital who admitted hospital in at after 24 hours. Mid stream urine specimens were collected from K.A.P.Vishwanatham government medical college, Tiruchirappalli, Tamil nadu, India. A total of 50 clinical urine samples were collected from hospital admitted patient at study enrollment. All the specimens were processed within 24 hours of collection.

Identification of Isolates

Morphological and biochemical analysis were carried out by various microbiologically significant examinations such as Gram staining, Motility, Cultural characteristic of growth and various important biochemical methods were used to identify the growth of the bacteria.

Disc Diffusion Method

According to the guidelines of the Clinical Laboratory Standard Institute, Multi drug resistance was detected by using Disk Diffusion test which was performed on Muller- Hinton agar medium. The plates were incubated at 37^0C for 24 hours. Any growth with less than 12 mm in diameter zone around the disk was considered indicative of drug resistance to the bacterial growth.

Screening of Extended Spectrum Beta Lactamases

Double Disk Synergy Test

As per Clinical and Laboratory Standard

Institute (CLSI) guidelines, the combined disk method depends on comparing the zone of inhibition around disks containing an indicator cephalosporin with and without clavulanic acid. Disk containing Ceftazidime (30mg) alone and Ceftazidime along with an inhibitor cephalosporin such as clavulanate (30/10mg) were placed on the inoculated Muller Hinton agar plates.

Modified Double Disk Diffusion Method

The standardized test strains were inoculated in Muller-Hinton agar plates. Disk containing expanded-spectrum Cephalosporins Cefepime (30mg), Cephotaxime 30mg), Ceftazidime (30mg), Cefpirome (30mg) are placed 30 mm (center to center) from an amoxicillin-Clavulanate (Ac 30/10mg) disk. After overnight incubation at 37^0C, the detection of an Extended spectrum of beta lactamases by the test organism is inferred by the presence of characteristic distortion or expansion of the inhibition zones towards the Amoxicillin-Clavulanate disk

Disk Replacement Method for Extended Spectrum Beta Lactamases Confirmation

The standardized test strains were inoculated in Muller-Hinton agar plates**.** Two disk containing Amoxyclav were placed, after 1 hour at room temperature, the disc were removed and replaced with Ceftazidime and Cefuroxime. After overnight incubation at 37^0C, the production of an ESBL by the test organism was detected.

SDS- PAGE Analysis

In SDS PAGE, the proteins are wrapped around by SDS molecules that cleave the disulphide bond and the β mercapto ethanol acts as a sulphahydryl reducing reagent. When heating at 100^0C for 3 minutes, it leaves a linear protein with a diameter of 1.6nm and the length of band depends on the number of aminoacids. Due to the discontinuous buffering system during ionization the chloride ions acts as a leading ion. Because of its mobility is faster than the fastest moving protein whereas glycine is poorly ionized due to its pH it moves slowly and act as a tailing ion. Its mobility is slower than the slowest protein. This ionization thus creates a local high voltage gradient in the stocking gel. When the glycine reaches separating gel the localized voltage gradient disappears and it migrates faster than the protein are migrated based on their size. SDS –PAGE was performed with 10% separating gel and 4% stacking gels. After electrophoresis, gels were stained with 0.05% coomassive brilliant blue and destained

Results and Discussion

The present study was used to carry out the detection of extended spectrum of beta lactamases producing *Klebsiella pneumoniae*. A total of 50 urine samples were collected from hospital acquired infectious patients. According to the Bergey's manual of determinative bacteriology, the isolates identified positively by Voges proskauer, Citrate utilization, Catalase test, TSI agar test and also produced brown dark centered mucoid colonies on EMB agar and ferment lactose on Macconkey agar and were detected as *Klebsiella pneumoniae.* Among 50 samples, *Klebsiella pneumoniae* was found in 9 samples (Table1). Totally 9(18%) *Klebsiella pneumoniae* samples were isolated from the various clinically associated urine sample.

In antibiotic disk diffusion, totally 33 various concentration of antibiotics were used against *Klebsiella pneumoniae* (Table 2).Ampicillin (100%), Aztreonam (100%) and Cephotaxime (100%) antibiotics were highly resistant to *Klebsiella pneumoniae*, followed by Cefpirome and Cefoxitin (Table3).Antibiotic Imipenem (88.8%), followed by Ampicillin clavulnate(77.7%), Cephotaxime clavulnate (77.7%), Co-trimoxazole (77.7%), Ertapenem (77.7%) and Meropenem (77.7%) were highly sensitive (Table 4).

According to the Clinical and Laboratory Standard Institute published guidelines for performing an Extended Spectrum of Beta lactamase confirmatory test. In *Klebsiella pneumoniae* isolate produced Extended Spectrum of Beta lactamase activity and it was observed by using various screening test. In Double disk synergy test, Cephotaxime (30mg) and Cephotaxime clavulnate (20/10 mg) were placed on the Mueller Hinton Agar plates. The zone of inhibition around the Cephotaxime disk was expanded by the Cephotaxime clavulnate on *Klebsiella pneumoniae* .The zone of inhibition was measured as greater than 5mm between two antibiotics such as Cephotaxime and Cephotaxime clavulnate were detected as Extended Spectrum of Beta Lactamases production.
In Modified Double Disk Diffusion, Zone of inhibition around the disk such as Cefepime, Cephotaxime, Ceftazidime and Cefpirome were increased towards the disk containing Amoxycillin Clavulanic acid as an indicative of Extended spectrum beta lactamases production by *Klebsiella pneumoniae*. In Disk Replacement Method on *Klebsiella pneumoniae*, two Amoxycillin Clavulnate disk were placed and were removed after 1 hour at room temperature and replaced with Ceftazidime and Cefuroxime. In replacement disk method, zone of inhibition around the disk greater than 5mm which were indicated as positive result for extended spectrum beta lactamases detection.

In SDS PAGE analysis of *Klebsiella pneumoniae* isolates revealed several protein bands in the range between 70kDa to 30kDa. The results indicated that the strain *Klebsiella pneumoniae* lost outer membrane protein, called as porin is found in the range of 35-40 kDa

Klebsiella pneumoniae is a successful opportunistic pathogen and has been associated with the outbreak of hospital acquired infection in worldwide and generally associated with high morbidity and mortality (,Lopes *et al*.,2005& Cohen,2000). *Klebsiella pneumoniae* strain is determined as multidrug resistance if it displays resistance to antibiotic from three or more classes. Our data supports about often difficult therapy for hospital acquired infection caused by extended spectrum beta lactamases producing strain *Klebsiella pneumoniae.* Frequently this organism not only resistance to Penicillin, Cephalosporin groups, Monobactam and Aztreonam. but often are characterized by associated resistance to other classes of antimicrobials (Luzzaro ,2006).but do not affect Cephamycin antibiotic such as Cefoxitin and Cefotetan or Carbapenems such as Imipenem or Meropenem(1 NCCLS ,1999). Extended spectrum beta lactamases that mediate resistance to some

Table.1 Source of the specimens examined from hospital acquired infectious patients

S.No	Age	Sex	Source of specimens	Organism
1.	45	F	Painful urination	*Klebsiella pneumoniae*
2.	37	M	Fever with diarrhoea	*Klebsiella pneumoniae*
3.	45	M	ICU patient	*Klebsiella pneumoniae*
4	12	F	Fever	*Klebsiella pneumoniae*
5	11	F	Frequent urination	*Klebsiellapneumoniae*
6	35	M	Hospital staff	*Klebsiellapneumoniae*
7	35	F	Pregnancy	*Klebsiella pneumoniae*
8.	58	F	Blood in urine	*Klebsiella pneumoniae*
9.	52	F	Post menopausal women	*Klebsiella pneumoniae*

antimicrobial and are commonly inhibited by beta lactamases inhibitor such as Clavulnic acid, Sulbactam and Tazobactam (Paterson and Bonomo, 2005).Clinical Microbiology laboratories should take into account this changing epidemiology and adjust accordingly the screening and confirmatory procedures for extended spectrum beta lactamases production especially for *Klebsiella pneumoniae* strain. Among *Klebsiella pneumoniae*, the emergence of resistance to extended spectrum cephalosporin has been a major concern, initially in limited number of bacterial species and now expanding rapidly. This resistance mechanism has been found around the world and it cause hospital acquired infection outbreak, appears to be increasing in prevalence and merits further study to define the best option for detection and treatment (Philippon *et al*., 2002).Our finding demonstrates an increasing incidence of hospital acquired infection with multi drugs resistance Extended spectrum beta lactamases producing strain of *Klebsiella pneumoniae.*

SDS PAGE analysis has been clearly demonstrated that porin deficiency contributes to increasing the level of resistance to extended spectrum beta lactamases producing strain. Generally, *Klebsiella pneumonia* will not only possess a beta lactamases but also exhibit porin deletion and thus resistance derives from synergy between reduced permeability and beta lactamases activity (Hernandez *et al*.,1999) Moreover, the outer membrane porin proteins are medically significant because it constituents play a major role in the permeability of antimicrobial agents ,substrates and in interaction with host defense mechanism(Turner, 2005).

Table.2 Disk diffusion pattern of *Klebsiella pneumoniae*

Sl.No	Antimicrobial agent	Conc.	R	I	S
1.	Amikacin	30	-	3	6
2.	Ampicillin	10	9	-	-
3.	Ampicillin/clav	30	-	2	7
4	Ampicillin/sulbactam	10/10	-	4	5
5.	Amoxy/clav	30	-	5	4
6.	Aztreonam	30	9	-	-
7.	Cefepime	30	7	1	1
8.	Cefepime/Tazobactam	30/10	2	4	3
9.	Cefixime	5	5	3	1
10.	Cefoxitin	30	6	2	1
11.	Cefpirome	30	8	1	-
12.	Cefpodoxime	10	4	3	2
13.	Ceftazidime	30	5	1	3
14.	Ceftazidime/Tazobactam	30/10	-	5	4
15.	Ceftizoxime	30	2	3	4
16.	Cefuroxime	30	4	3	2
17.	Cephalothin	30	5	3	1
18.	Cephotaxime	10	9	-	-
19.	Cephotaxime/clav	30	-	6	12
20.	Ciprofloxacin	5	1	3	5
21.	Co Trimoxazole	25	-	2	7
24.	Ertapenem	10	-	1	8
25.	Gentamycin	10	-	3	6
24.	Imipenem	10	-	2	7
25.	Levofloxacin	5	1	5	3
26.	Meropenem	10	-	2	7
27.	Moxifloxacin	5	1	6	2
28.	Nitrofurantoin	300	1	5	3
29.	Norfloxacin	10	2	4	3
30.	Ofloxacin	5	2	5	2
31.	Piper/Tazobactam	100/10	-	3	6
32.	Tetracycline	30	-	1	8
33.	Trimethoprim	25	4	1	4

R – Resistant, I – Intermediate sensitive, S- Sensitive

Table.3 Resistance pattern of *Klebsiella pneumoniae*

S.No	Antimicrobial agent	Concentration (mg)	No. of resistance sample	Percentage (%)
1.	Ampicillin	10	9	100
2.	Aztreonam	30	9	100
3.	Cefixime	5	5	55.5
4.	Cefoxitin	30	6	66.6
5.	Cefpirome	30	8	88.8
6.	Ceftazidime	30	5	55.5
7.	Cefuroxime	30	4	44.4
8.	Cephotaxime	10	9	100

Table.4 Sensitive pattern of *Klebsiella pneumoniae*

S.No	Antimicrobial agent	Concentration (mg)	No. of sensitive sample	Percentage (%)
1.	Amikacin	30	6	66.6
2.	Amoxy/clav	30	4	44.4
3.	Ampicillin/clav	30	7	77.7
4.	Ampicillin/sulbactam	10/10	5	55.5
5.	Cefepime/Tazobactam	30/10	3	33.3
6.	Ceftazidime/Tazobactam	30/10	4	44.4
7.	Cephotaxime/clav	30	7	77.7
8.	Ciprofloxacin	5	5	55.5
9.	Co trimoxazole	25	7	77.7
10.	Ertapenem	10	7	77.7
11.	Imipenem	10	8	88.8
12.	Meropenem	10	7	77.7
13.	Piper/Tazobactam	100/10	6	66.6

In recent years, there has been an emerging phenomenon of the beta lactam resistance determinants involved in *Klebsiella pneumoniae* may pose a challenge for detection and selection of new therapy for reducing these resistances to prevent clinically complicated disease.

Monitoring of Extended spectrum beta lactamases production of *Klebsiella pneumoniae* and antimicrobial patterns are necessary to avoid treatment failure condition in patient with hospital acquired infection. Strict antibiotic policy should be adopted in hospital to estimate the impact of higher resistance in *Klebsiella pneumoniae* bacteria and to take steps for reducing this drug resistance.

Acknowledgement

We thank the Department of Microbiology, Jamal Mohamed College (Autonomous), Tiruchirappalli-620020, for supporting and fulfilling all the needs to carry out this work.

References

Livermore, D.M.,1995. β-lactamases in laboratory and clinical resistance. Clinical Microbiol. Rev. 8:557-584.

Samaha-K foury, J.N., and Araj, G.F. 2003.Recent developments in beta lactamases and extended spectrum beta lactamases. BMJ.327:1209-1213.

Braford, P., 2001. Extended spectrum beta lactamases in the 21st century: Characterization, epidemiology and detection of this importance resistance threat. Clinical Microbiol. Rev. 14:933-51.

Thomson, K.S., Prevan, A.M. and Lagrange, P.H.1996. Novel plasmid mediated beta lactamases in Enterobactericeae:emerging problems for new beta lactam antibiotics. Curr.Clinical topics. Infect.Dis.16:151-163.

Guzman-Blanco, M., Casellas ,J.M. and Sader, H.S.2000. Bacterial resistance to antimicrobial agents in Latin America: The giant is awakening. Infect. Dis. Clinics. North America. 14:67-81.

Nikaido, H., 1989. Outermembrane barrier as a mechanism of antimicrobial resistance. Antimicro. Agents Chemothera. 33:1831-36.

Lopes, A.C.S., Rodrigues, J.F. and Junior, M.A.M.2005. Molecular typing of *Klebsiella pneumoniae* isolates from hospital in Recife, Brazil. Microbiol. Res.160:37-45.

Cohen, M.L., 2000. Changing patterns of infectious disease. Nature. 406:762-767.

Luzzaro, F. , Mezzatesta, M. and Mugnaioli, C.2006. Trends in production of extended-spectrum (β)-lactamases among enterobacteria of medical interest: Report of the second Italian nationwide survey. J. Clinical Microbiol.. 44:1659-1664.

National committee for clinical laboratory standards. Performance standards for antimicrobial susceptibility testing. NCCLS approved standard 1999.M100-S9, M100-S10,M7-A5,M7-A4. National committee for clinical laboratory standards, Wayne, PA.

Paterson, D.L., and Bonomo, R.A. 2005. Extended spectrum beta lactamases: A clinical updates. Clinical Microbiol. Rev. 18:657-686.

Philippon ,A., Guillaume ,A., George, A. and Jacoby, G.A.2002. Plasmid determined AmpC-Type β-lactamases, Antimicro. Agents Chemothera. 46:1-11.

Hernandez Allez , S., Alberti, S., Alvarez, D., Dosmenech Sanchez ,A., Martinez, L., Gil, J., Tomas, J.M. and Benedi, V.J.1999. Porin expression in clinical isolates of *Klebsiella pneumoniae*. Brazilian. J. Microbiol. 145: 673 -679.

Turner, P.J., 2005. Extended Spectrum beta lactamases. Clinical Infect. Dis. 41: 273 – 275.