CHAPTER - 6
DISCUSSION

One of the most concerning emerging resistance traits among gram-negative bacteria is the ability of the organisms to produce carbapenem-hydrolyzing \( \beta \)-lactamases, which confer resistance to almost all \( \beta \)-lactams. Multidrug resistant carbapenem hydrolysing gram negative bacteria are increasing in prevalence worldwide. They have few treatment options and result in high mortality rates. The most clinically significant are KPC, MBLs namely VIM, IMP, NDM types and OXA carbapenemases. These genes are located on mobile genetic elements, allowing them to spread. Recognition of the presence of carbapenemase producers is of paramount importance for effective treatment and control.

**Carbapenem resistance in Gram negative bacteria**

Studies illustrating the prevalence of resistance among GNB are essential because wide regional differences exists, accentuating the need to take into account the local epidemiology (at the level of the country, the region, the hospital, and at times the individual hospital units/wards) when making decisions about empirical therapy for serious infections. While carbapenem resistance in *Pseudomonas* and *Acinetobacter* spp is well known, resistance among Enterobacteriaceae is increasing.

The overall carbapenem resistance in this study was 17.4%. The carbapenem resistance rate among GNB varies widely in the literature. While Taneja *et al.* reported it as 36.4% , Dutta *et al* documented it as 7.87%.[Taneja *et al.* 2003, Datta *et al.* 2012]. The incidence varies from as low as 1.8% to over 30% in India [Gupta *et al.* 2006, Gladstone *et al.* 2005, Shahid *et al.* 2012, Behera *et al.* 2011, Goel *et al.* 2009].
Hence there is a wide variation in carbapenem resistance rates among GNB in different parts of India.

In the Canadian infection surveillance program done in 2009-10 involving 20 acute care facilities across Canada, Mataseje et al, found that, of 58669 gram negative isolates, 444 were carbapenem resistant as determined by disc diffusion method. These 444 (0.76%) isolates were submitted to the National Microbiology laboratory for further study and analysis, 274 (0.55%) isolates only were found to be resistant to carbapenems as tested by E test method and Vitek 2. Hence in Canada, 3.3% (206/6260) of P. aeruginosa, 2.7% (9 /331) of A. baumannii were reported to be resistant and 0.1% (59/52078) of Enterobacteriaceae had reduced susceptibility to carbapenems. [Mataseje et al.2012]. Summary of data from the MYSTIC surveillance program carried over 10 years (1999-2008),showed that in USA, a gradual increase in resistance to the carbapenems was observed from 1999-2008. In 2008, resistance to imipenem and meropenem in A. baumannii was 47.9%, 7.5% respectively, 15.5% resistance to meropenem and imipenem resistance was reported in P aeruginosa and ≤ 2% imipenem and meropenem resistance in Enterobacteriaceae. [Rhomberg et al.2009]. The COMPACT (COMParative Activity of Carbapenem Testing) study surveyed the carbapenem susceptibility and MICs of doripenem, imipenem and meropenem against 1260 major Gram-negative pathogens isolated from hospitalised patients at 20 centres in five Asia-Pacific countries (New Zealand, The Philippines, Singapore, Thailand and Vietnam) during 2010. P. aeruginosa (625), Enterobacteriaceae (500), and other Gram-negative pathogens including A. baumannii (135) were collected from patients with bloodstream infection (32.2%), nosocomial pneumonia including ventilator-associated pneumonia (58.1%), and complicated intra-abdominal infection (9.7%), with 36.7% being isolated from patients in ICU. In
their study, 29.8% of *P. aeruginosa* and 73.0% of *A. baumannii* isolates were not susceptible to at least one carbapenem, whereas the majority of Enterobacteriaceae (97.2%) were susceptible to all carbapenems. [Kiratisin *et al.* 2012]. The COMPACT study report of Turkey reported that among 596 gram negative isolates obtained from 10 centres, 187 (31.4%) were resistant to at least one carbapenem based on the E-test.[Leblebicioglu *et al.* 2012]. Eighty centres in 16 countries from European, Middle Eastern and African countries participated in the COMPACT study and reported 21.7% (978/4498) of isolates to be non-susceptible to at least one carbapenem [Nordmann *et al.* 2011d]

**Carbapenem resistance in Enterobacteriaceae:** In this study among 2580 Enterobacteriaceae, 7% (181) were resistant to carbapenems. This resistance rate was comparable with several studies done in India and in other countries. Many authors have used one or more carbapenems as indicator drug for testing resistance to carbapenems by disc diffusion or MIC method. Resistance to carbapenems ranged from 2% to 22% in Indian studies and <1 % to 10.8% in studies conducted in other countries. [EARS net, Gupta *et al.* 2011, Hidron *et al.* 2008, Subbalaxmi *et al.*, 2010, Dutta *et al.* 2012]. Gupta *et al.* reported less resistance rate to imipenem than to meropenem.[Gupta *et al.* 2006]. In the present study, differential susceptibility between imipenem and meropenem was not encountered. In this study, carbapenem resistance was more common among Enterobacteriaceae isolates recovered from patients in the ICU. Similarly, studies done in tertiary care hospitals in Mumbai, New Delhi and Hyderabad also documented high rates of carbapenem resistance among ICU patients [Deshpande *et al* 2010, Datta *et al* 2012, Subbalaxmi *et al* 2010, Wattal *et al* 2010]
Carbapenem resistance in Enterobacteriaceae is a recent phenomenon and was uncommon in most countries before 1992. [Gupta et al. 2011]. Using data from the NNIS system from 1986 to 1990, Gaynes et al. found that only 2.3% of 1825 Enterobacter isolates tested non-susceptible to imipenem. [Gaynes et al. 1992]. However, over the last decade carbapenem resistant Enterobacteriaceae is on the increase. In the MYSTIC program, meropenem resistance among clinical isolates of K. pneumoniae increased significantly from 0.6% in 2004 to 5.6% in 2008. [Rhomberg et al. 2009]. Among isolates reported to the NHSN in 2006–2007, carbapenem resistance was reported in 4.0% of E. coli and 10.8% of K. pneumoniae isolates that were associated with certain device-related infections [Gupta et al. 2011, Hidron et al., 2008]. The CDC reported that, among health care–associated infections, 8% of Klebsiella spp. isolates were carbapenem resistant in 2007 compared with <1% in 2000 [CDC, 2009]. Sanchez et al. analyzed a total of 3,132,354 K. pneumoniae antimicrobial susceptibility results for 1998–2010 and reported that resistance to imipenem first appeared in The Surveillance Network (TSN) Database-USA in 2004 and rose gradually to 4.3% by the end of their study period [Sanchez et al. 2013]. Overall, carbapenem-resistant Enterobacteriaceae are still rare causes of human infections in most parts of Europe, except for Greece, Italy and Cyprus. According to the 2012 data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), the rates of carbapenem-resistance among invasive K. pneumoniae infections were: 60.5% in Greece, 28.8% in Italy, 9.2% in Cyprus, 6.3% in Solvakia, 3.6% in Malta, 2.9% in Hungary, 1.9% in Bulgaria and below 1% in the other 20 reporting countries. For E. coli the carbapenem resistance was not found or the rate was below 1% in all the reporting countries except Greece and Bulgaria which had 1.4% and 2.6% respectively. [EARSnet]. In the SENTRY antimicrobial surveillance
program (2007-09) from Europe and the Americas, Castanheira et al. reported 2% (329/15948) carbapenem resistance among *E. coli* and *K. pneumoniae*. [Castanheira et al. 2011b].

**Carbapenem resistance in NFGNB:** Of the NFGNB (1061), 608 were susceptible to carbapenems and 453 (42.7%) were resistant. While in *P. aeruginosa* isolates, it was 29.2% (143/482) in *Acinetobacter* isolates it was 50.5% (252/499).

**Carbapenem resistance in *A. baumannii***: In the present, study one half of the *Acinetobacter* species were resistant to carbapenems. Reports from other countries indicate that the resistance percentage is anywhere from 0% to 50% [Perez et al. 2007, Cisneros et al. 2002]. The European SENTRY study done from 2001 to 20 has documented the resistance rate in *Acinetobacter* spp. 26.3% and 29.6% to imipenem and meropenem respectively. [Turner et al. 2003, Gales et al. 2006]. Among the European countries Greece remains a “hotspot” for carbapenem resistant *A. baumannii*. A study from that country done in tertiary care centres reports the resistance rates to be 85% in ICUs, 60% in medical wards and 59% in surgical wards. [Manchandra et al. 2010]. An earlier MYSTIC Study found the prevalence to be around 43%. [Turner 2008]. A publication from USA, based on a study undertaken in burns unit found a alarming high rate of 87%. [Trottier et al. 2007, Manchandra et al. 2010]. In Mexican hospitals resistance rates have been reported to be 36.4% to imipenem and 37.4% to meropenem. [Morfin-Otero et al. 2012].

In the Indian subcontinent, resistance to carbapenems in *A. baumannii* ranges from 14% to 59%. All the studies have been done on hospitalised patients. Notably, most resistant isolates were recovered from respiratory samples of patients in the ICU. [Gladstone et al. 2005, Taneja et al. 2003, Mahajan et al. 2011, Noyal et al. 2009, Gupta]
et al. 2006, Wattal et al. 2010]. From New Delhi, one tertiary care centre has documented high rates of resistance in both ICU (80%) and non ICU (57%) patients based on a one year study. [Wattal et al. 2010]. The rates of resistance observed in the present study is comparable to several other reports. The rates of resistance are higher among isolates from ICU patients and negligible in non ICU settings. This is in sharp contrast to the New Delhi based study. [Wattal et al. 2010]

**Carbapenem resistance in P. aeruginosa:** In this study among P. aeruginosa isolates, 29.2% (143/482) were resistant to carbapenems. P. aeruginosa has evolving virulence characteristics and antimicrobial resistance patterns which make it a difficult target for antibiotic therapy. Studies in many countries have also shown different rates of imipenem resistance in P. aeruginosa isolates. Similar to A. baumannii, carbapenem resistant P. aeruginosa were frequently isolated from patients in the ICU with lower airway infections. From South America, the MYSTIC study reported meropenem resistance in P. aeruginosa to be 64% and the SENTRY study documented a resistance of 49% to imipenem. [Pinheiro et al. 2008]. In the USA, the overall prevalence was 7% in 2002 and subsequently it was 17.7% in 2003 and 31% in 2006 among patients in ICU. [Pinheiro et al. 2008, Gaynes et al. 2005, Landman et al. 2007]. In Mexican hospitals resistance to imipenem was 17.8%. [Morfin-Otero et al. 2012]. According to the data of the SENTRY program, 5.1–8.4% of P. aeruginosa strains obtained from Canada were resistant to meropenem, 10.2–26.2% in Europe, and 7.6–9.0% in USA. [Gailiene et al. 2007, Gales et al. 2001 & 2006, Garcia-Rodriguez et al. 2002]. While reports from burns units in Pakistan documented 32.7% resistance to carbapenems in P. aeruginosa, Turkey and Tehran reported high rates of 69.9% and 94.7% respectively. [Naqvi et al. 2005, Moazami-Goudarzi et al. 2013, Ozkurt et al. 2005]. From among the East Asian Countries, Korea had a prevalence
of 52% [Song et al. 2001]. Rates of carbapenem resistance in the Arabian Peninsula over the last decade varied widely among the different countries but revealed a rising trend: 10.4-19% in 1994-95 from Kuwait; 22% in 1998 from Qatar; 63.3% in 2004-2007 from Oman; 16.2-91% in 2009-2010 from Saudi Arabia. [Zowawi et al. 2013]

In Indian studies, carbapenem resistance in P. aeruginosa has been reported from centres in Pondicherry, Vellore, Bangalore, Chandigarh, Mumbai, New Delhi, and Varanasi with the rates of resistance between 10.9% and 69%. [Shashikala et al. 2006, Taneja et al. 2003, Noyal et al. 2009, Navneeth et al. 2002, Behera et al. 2008, Kumar et al. 2011, Varaiya et al. 2008, Wattal et al. 2010, Kaul et al. 2007]. In a multicentric study including centres all over India conducted during 2005-07, 42.6% of P. aeruginosa were resistant to imipenem/meropenem. [Manoharan et al. 2010].

As a consequence of these trends and their geographical variability, it is important to consider current, local susceptibility patterns when selecting antimicrobial therapy in the treatment of each patient. These issues are also important considerations when determining which antibiotics to include in a formulary.

**Carbapenemases in Enterobacteriaceae**

Members of the family Enterobacteriaceae are among the most important bacterial human pathogens, accounting for the majority of the bacteria isolated from clinical samples. A major concern is that these gram negative bacilli are rapidly acquiring resistance to one or more antimicrobial agents traditionally used for treatment.
Metallobetalactamases in Enterobacteriaceae

Of the genes encoding for MBL, \textit{bla}_{NDM} and \textit{bla}_{VIM} were the only ones found in the study isolates. The other MBLs such as \textit{bla}_{IMP}, \textit{bla}_{GIM}, \textit{bla}_{SIM} and \textit{bla}_{SPM} were not detected. The first documented case of infection caused by bacteria producing NDM was in 2008, although retrospective analyses of stored cultures have identified the NDM genes in Enterobacteriaceae as early as 2006. Since its first description, there are a spate of reports from 40 countries worldwide, encompassing all continents except Antarctica. The spread of NDM involves a complex epidemiology encompassing a variety of species of NDM-positive bacteria and the inter-strain, inter-species and inter-genus transmission of diverse plasmids containing \textit{bla}_{NDM}, with the latter mechanism having played a more prominent role to date. Most reports suggest that the likely source of NDM-1 acquisition is the Indian subcontinent and is related to 'medical tourism'. A variant of NDM-1 (designated NDM-2) which differed by a single amino acid was reported in 2011 (Kaase \textit{et al.} 2011), and subsequently, a series of further variants (designated NDM-3–NDM-7) have been reported. [Johnson \textit{et al.} 2013, HPA 2009]

In this study, the gene encoding NDM was the most prevalent, being detected in 57.65\% (64) of which six coproduced \textit{bla}_{VIM}. Twenty seven isolates that produced NDM, had MIC to imipenem and meropenem in the susceptible range as per the CLSI criteria. The NDM producers were obtained from blood (17), respiratory secretions (9), exudative specimens (13) and urine (25). The majority of these isolates were \textit{K. pneumoniae} (30), followed by \textit{E. coli} (13), \textit{E. cloacae} (10), \textit{C. freundii} (10) and \textit{Providencia rettgeri} (1). The majority of NDM producers were from the specimens of patients in the ICU (59) and from non ICU there were 5 NDM producers. Identification of \textit{E. coli} producing NDM is a cause for concern as it a community
acquired pathogen with possible high rates of colonisation. Similarly, presence of NDM in *K. pneumoniae* is a threat in health-care settings as it is a common nosocomial pathogen and has a propensity to spread rapidly. These results of our study were comparable with several reported from India and other countries as many authors have isolated NDM producers mainly from patients in ICU, the most frequent source specimen being urine and the common organisms being *K. pneumoniae* and *E. coli*.

From India the first report of NDM-1 producing Enterobacteriaceae was published in 2010, in which 24 carbapenem resistant (by disc diffusion) Enterobacteriaceae exhibiting a positive MHT were collected in a period of 3 months and subjected to PCR for the detection of *bla*<sub>NDM</sub>. The gene was detected in 22 of 24 isolates with most isolates (14) from the ICU patients. Amongst the 22 NDM producing organisms 10 were *Klebsiella* spp, 9 were *E. coli*, 2 were of *Enterobacter* spp and one was *Morganella morganii*. The source specimens of the NDM producers were urine (11), sputum (4), blood (3) and one each from tracheal secretion, stool, bronchoalveolar lavage (BAL), swab, endotracheal secretion and pus.[Deshpande *et al*. 2010]. NDM producing Enterobacteriaceae has been subsequently reported from many centres across India. In Indian isolates, the prevalence of NDM-1 producers among carbapenem resistant Enterobacteriaceae ranges between 31.2% and 91.6%.[Nagaraj *et al*. 2012, Seema *et al*. 2012, Lascols *et al*. 2011, Castanheira *et al*. 2011a, Deshpande *et al*. 2010, Kumarasamy *et al*. 2010,]. More recently the northeastern region of India, has documented the prevalence of NDM to be relatively lower with 8.7% in *K. pneumoniae* and 5.2% in *E. coli*. [Bora *et al*. 2012, 2013].

A multicentric study done in 2009 using a collection of Enterobacteriaceae isolates from centres in Chennai, Haryana, Mumbai, Varanasi, Guwahati, Pakistan
and referral isolates in UK reference laboratory (between 2003-2009), analysed carbapenem resistant. From the Chennai centre, among 3521 Enterobacteriaceae, 141 exhibited carbapenem resistance. Of and 44 carried \textit{bla}_{NDM-1}. NDM production in Haryana was 13%. The NDM producing isolates from Chennai and Haryana were primarily from community acquired urinary tract infections, pneumonia, and bloodstream infections. [Kumarasamy \textit{et al.} 2010]. Castanheira M \textit{et al} postulated that the isolates producing NDM-1 were disseminated in the Indian health care facilities as early as 2006. In their investigation into the occurrence and characterization of carbapenem-resistant Enterobacteriaceae isolated from 14 Indian hospitals collected during 2006–2007 as part of SENTRY program, NDM-1 production was found in 38.5% (15 /39). These NDM-1 producers were from centres in New Delhi, Pune and Mumbai. The NDM-1 producers were associated with blood stream, skin and soft tissue and respiratory infections.[Castanheira \textit{et al.} 2011] International surveillance of intra-abdominal infections in 2009 (comprising centres in Europe, North America, Latin America, the South Pacific, the Middle East and Asia) undertaken as part of the Study for Monitoring Antimicrobial Resistance Trends programme (SMART) found NDM-1-positive Enterobacteriaceae only in Indian isolates. It revealed the presence of \textit{bla}_{NDM-1} in 33 isolates, of which 32 coproduced one or more β-lactamases such as ESBL, Amp C or OXA-48 and comprised five different species of Enterobacteriaceae. As in the other studies, the \textit{bla}_{NDM-1} gene was found in a range of species including \textit{E. coli}, \textit{K. pneumoniae}, \textit{E. cloacae}, \textit{Providencia rettgeri} and \textit{M. morganii} [Lascols \textit{et al.} 2011].

As of now, NDM producing Enterobacteriaceae are reported from geographically diverse regions of the globe including Australia, Asia, the Far East, the USA, Canada, the Middle East and many countries in Europe. It is on the rise.
It is notable that in the present study, a significant number of NDM producers (27/64) had MIC to carbapenems within the susceptible range. Since their MIC ranged from 0.03 mg/L to 1 mg/L, it becomes mandatory that all Enterobacteriaceae, which exhibit resistance to one of the cephalosporins subclass III, are screened for the presence of NDM. Seema et al also observed in their study, that some of the blaNDM-1 harbouring isolates were found to be phenotypically susceptible to all three carbapenems (imipenem, meropenem and ertapenem) tested and hence suggested that, irrespective of antibiograms, all isolates should be routinely screened by molecular methods for NDM. [Seema et al, 2011].

In the present study, the NDM-1 producers were isolates from patients in the age group of age 4 days to 81 years with 48 being males and 16 being females. The majority of them were from the specimens of patients in the ICU (59/64) with high numbers in the multidisciplinary ICU (30) followed by the neurosurgery ICU (14), neonatal ICU (8), neurology (5), cardiology (1) and cardiothoracic surgery (1). In contrast to the present study, Bora et al observed higher incidence of NDM-1 producing *E. coli* in non ICU patients (64.28%, 9/14) than in ICU patients (35.72%, 5/14) [Bora et al. 2013]. In the neonatal ICU, there were 8 *K. pneumoniae* isolated from blood stream infection. Similarly, NDM producing Enterobacteriaceae have been observed in the neonatal setting in Indian hospitals, with two cases of neonatal
sepsis due to *K. pneumoniae* [Roy et al. 2011b] and a cluster of bloodstream infections due to *E. coli* in a neonatal unit.[Roy et al. 2011a]. Isolation of NDM-1 producing *E. coli* isolate from a sick newborn was also published recently. [Bora et al. 2013].

Therapeutic options against serious infections due to NDM-1 producers are limited to tigecycline and polymyxins. However, tigecycline may not reach desired serum levels to treat systemic infection leaving polymyxins as the last resort. In this study all the study isolates were resistant to cefotaxime, ceftazidime, aztreonam, cefepime, pipercillin-tazobactam and ciprofloxacin. Resistance to amikacin was detected in 76.6% (85). Susceptibility to tigecycline and colistin was universal. In the SENTRY study and the multicentric study by Kumarasamy *et al*, the NDM producers remained susceptible to both polymyxins and tigecycline  [Castanheira *et al*. 2011a, Kumarasamy *et al*. 2010]. However in the SMART study, only 51.5% of the isolates remained susceptible to both colistin and tigecycline.[Lascols *et al*. 2011]. Seema *et al* also reported that only 85.1% were susceptible to polymyxin B and 46.2% to tigecycline. [Seema *et al*. 2011]. The emerging resistance to polymyxins and tigecycline presents a significant clinical challenge.

**RAPD profile of NDM producers**

This study provides some insights into the complex molecular epidemiology of NDM-1 in this tertiary care centre. Genotyping of seven *E. coli* has revealed genetic diversity and heterogeneity. There were two *E. coli* isolates with similar RAPD pattern, but were from patients who were hospitalised at different points of time. Similarly, *K. pneumoniae* exhibits 12 different lineages of the 16 isolates analysed. Among them, 4 belonged to a single cluster. These were from neonates in
the ICU and cultured during the same period of time. This is perhaps indicative of a horizontal transfer. However, such transfer is not an important mechanism of dissemination of NDM-1 producers. Surveillance of water supplies and selective environmental sampling during the study period did not reveal a NDM-1 source. In addition, strict implementation of infection prevention and isolation protocols could have curtailed a possible outbreak.

Several studies have indicated extensive genotypic heterogeneity in the NDM-1 producing *E. coli* and *K. pneumoniae*. However, clonal spread leading to outbreak has been documented in only one centre from India [Kumarasamy *et al*. 2010]. This diversity of the NDM-1 producers is consistent with the findings of previous studies.[Kumarasamy *et al*.2010, Lascols *et al*.2011, Castanheira *et al*.2011a].

In the present study,4 NDM producing *K. pneumoniae* belonged to a single cluster in the neonatal ICU. A recent study described the first outbreak of NDM-1-producing *K. pneumoniae* in a neonatal unit in Colombia, South America and documented a clinical response to combined therapy with imipenem and ciprofloxacin. The subjects were hospitalized in the neonatal unit from birth. There was no contact with people from other countries. This suggested the possibility of horizontal transmission of plasmids from other species of bacteria harbouring NDM. The isolates belonged to the same clone suggesting that the bacteria were transmitted between patients who were concurrently hospitalized in the same room. This route was also strongly implied due to the fact that *K. pneumoniae* was not recovered from samples taken from hospital surfaces, eliminating the environment as a source of infection. Hence it was concluded that of NDM producing Enterobacteriaceae is not uncommon in newborn care settings.[Escobar Perez *et al*.2013]
Since the present study was carried out in a tertiary care teaching hospital, patients are transferred from a wide range of health-care facilities in the geographic region. Therefore, colonisation or infection with resistant strains is likely to occur before transfer, implying the import of such strains on admission. The wide diversity encountered in this study implies multiple source of origin. The genomic variability suggests strong selection pressure on bacterial population, underlining the necessity for proper management of antibiotic therapy, within health-care units. The epidemiology of NDM producers in this study is substantially representative of a divergent acquisition of multiple clones with limited dissemination between patients. Though an antibiotic policy with restriction of inducer drugs is implemented within this hospital, such measures are seldom implemented in other centers leading to an immense selection pressure. This calls for increased vigilance, continuous surveillance and strict enforcement of antibiotic policy with restricted use of inducer drugs.

**VIM and IMP**

In this study, VIM type MBL was produced by 7 study isolates of which six coproduced NDM; 3 of *K. pneumoniae* and 3 of *E. coli*. In one isolate of *K. pneumoniae* *bla*\text{VIM} was the only gene detected. *Bla*\text{IMP} and other MBLs (SIM, GIM, SPM) were not detected in any of the study isolates. There are very few reports of VIM/IMP type MBLs in Enterobacteriaceae from India.[Shahid et al. 2012, Nagaraj et al. 2012]. This is the first report of the coexistence of *bla*\text{VIM} and *bla*\text{NDM} in the same isolate.

Dwivedi et al., in 2009 reported the presence of MBL genes in 12 isolates obtained from patients with ventilator associated pneumonia and also concurrent
occurrence of multiple MBL genes in single isolate. In contrast to our study, the most common MBL subtype in their study was the bla\textsubscript{IMP}, followed by bla\textsubscript{VIM} and bla\textsubscript{SIM}. [Dwivedi \textit{et al.} 2009]. Subsequently another study from the same hospital reported the presence of bla\textsubscript{GIM} in addition to bla\textsubscript{IMP}, bla\textsubscript{VIM} and bla\textsubscript{SIM}. [Azim \textit{et al.} 2010].

**Producers of OXA-48**

In this study, bla\textsubscript{OXA-48} and its variant bla\textsubscript{OX A-181} were detected in two of the 111 isolates. These were \textit{K. pneumoniae} and \textit{C. freundii}, isolated from patients admitted to the ICU. The MHT was positive in both.

The class D, carbapenem hydrolyzing OXA-48 was first reported from Turkey in \textit{K. pneumoniae} [Carrer \textit{et al.} 2008]. Since then, outbreaks linked to OXA-48 have been described worldwide [Poirel \textit{et al.} 2012 a]. Variants of OXA-48 that differ by substitution of one or more amino acids were identified subsequently namely, OXA-163, OXA-181, OXA-204 and OXA-232. Of these, so far only OXA-181 variant possess significant carbapenemases activity and has been found to be associated with other carbapenemase encoding genes such as bla\textsubscript{NDM} and bla\textsubscript{VIM}. [Poirel \textit{et al.} 2012a, Castanheira \textit{et al.} 2011a]. [Poirel \textit{et al.} 2012a].

World over OXA-48 and its variant OXA-181 have been reported in several species of Enterobacteriaceae, particularly in \textit{K. pneumoniae}. Case reports referring to infections caused by OXA-181 producers have been described from the Netherlands, Singapore, France and Sultanate of Oman. The origin of most of these strains has been traced to the Indian subcontinent [Kalpoe \textit{et al.} 2011, Potron \textit{et al.} 2011, Koh \textit{et al.} 2012, Poirel \textit{et al.} 2011c]. More recently a Canadian publication has reported the occurrence of OXA-181 in patients from Dubai and Lebanon who had no contact with India. [Mataseje \textit{et al.} 2013] There are only two publications indicating the presence
of bla\textsubscript{OXA-48} / bla\textsubscript{OXA-181} in strains from India. [Lascols \textit{et al.} 2011, Castanheira \textit{et al.} 2011a]. So the magnitude of infections caused by of OXA-48 and OXA-181 producing Enterobacteriaceae in India is largely unknown.

It is known that bla OXA-181 coexists with other carbapenemases. [Lascols \textit{et al.} 2011, Castanheira \textit{et al.} 2011a]. However, in this study the bla\textsubscript{OXA-181} did not coexist with other carbapenemases. In the SENTRY study conducted between 2006-07, out of a total of 1443, 39 were carbapenem resistant and of them 10 were found to harbor OXA-181 alone and one \textit{K. pneumoniae} isolate carried both bla\textsubscript{OXA-181} and bla\textsubscript{VIM-5}. [Castanheira \textit{et al.} 2011a] Data from the SMART study 2009 on 235 Indian isolates that were non susceptible to ertapenem, indicated that bla\textsubscript{OXA-48} existed alone (3) or in combination with bla\textsubscript{NDM-1} in \textit{Enterobacter cloacae} (2). [Lascols \textit{et al.} 2011]

The suspicion of presence of bla\textsubscript{OXA-48} is often very complicated because some of them may be carbapenem resistant but remain susceptible to cephalosporins subclass III which may be regarded as the potential treatment of choice. However in the Indian scenario this phenomenon may be encountered very rarely because of the high prevalence of ESBL among Enterobacteriaceae. Thus the resistance pattern becomes broader leaving only limited therapeutic options such as polymyxins and tigecycline. [Dimou \textit{et al.} 2012]. The OXA-48/ OXA-181 producers in this study were ESBL screen test positive. Unlike the observation that the OXA-48/ OXA-181 have only low level resistance to carbapenems, these isolates had MIC in the range of 16-128 mg/L thus exhibiting frank resistance. This may be attributed to the coexistence of ESBL in combination with porin loss. [Mataseje \textit{et al.} 2013]

In this study, the OXA-48/ OXA-181 producers remained susceptible only to colistin and tigecycline in-vitro. Both patients were treated with colistin. While the
patient with *Klebsiella pneumoniae* septicaemia recovered, the other patient expired. Many patients with infections caused by OXA-48 producers have significant comorbidity and prolonged hospital stay as was encountered in these two patients [Paño-Pardo *et al.* 2013].

It can be reasonably assumed from the study that production of OXA-48 / OXA-181 is not a major mechanism of carbapenem resistance. Out of 111 Enterobacteriaceae screened, only 2 (1.8 %) were found to be OXA-181 producers. This is in sharp contrast to the study by Castanheira *et al* where of 39 Enterobacteriaceae, 10 were OXA-181 producers [Castanheira *et al.* 2011a]. In the SMART study among 66 isolates with a carbapenemase encoding gene, 5 were *bla*<sub>OXA-48</sub> positive [Lascols *et al.* 2011]. Additionally, *bla*<sub>OXA-48</sub> / *bla*<sub>OXA-181</sub> in this study did not coexist with other carbapenemase encoding genes as reported in the earlier studies.[Castanheira *et al.* 2011a, Lascols *et al.* 2011]. Notably, the carbapenem MICs were high. Since there are no specific phenotypic tests for the detection of this enzyme, PCR is the gold standard for their identification in the clinical microbiology laboratory.

**Producers of KPC**

In the present study *bla*<sub>KPC</sub> was not detected in any of the study isolate. Similarly Nagaraj *et al* also pointed to the absence of KPC in carbapenem resistant Enterobacteriaceae. [Nagaraj *et al.* 2012]. The SMART study and SENTRY study also did not find *bla*<sub>KPC</sub> in the Indian isolates [Castanheira *et al.* 2011a, Lascols *et al.* 2011]. More recently, researchers from Varanasi did find the presence of *bla*<sub>KPC</sub> in three Enterobacteriaceae isolates which included 2 *E. coli* and one *K. pneumoniae*. The *E. coli* isolates were from blood and urine and *K. pneumoniae* was from urine
Overall, it is noteworthy that KPC enzymes are not prevalent in the Indian subcontinent as is in the western countries.

The present study screened for KPC using PBA inhibition test. Even though 36 isolates were screen test positive which included *K. pneumoniae* (15), *C. freundii* (10), *E. coli* (6) and *E. cloacae* (5), PCR did not detect *bla* KPC in any of them. The attributable reasons can be false positivity due to high-level of Amp C-type cephalosporinases and porin alterations.[Miriagou *et al*. 2010, Giske *et al*. 2011]. Since PBA inhibit class C β-lactamases also, Birgy *et al* recommended the testing of strains in parallel on both PBA agar and cloxacillin agar. If the increases in the inhibition zones are similar with the two agents, the presence of a KPC can be ruled out. In contrast, with KPC-producing strains, they observed no difference in the inhibition zones in the presence and absence of cloxacillin and therefore inferred that the β-lactamase was a class A carbapenemase. [Birgy *et al*.,2012]. In the present study cloxacillin was not used for this differentiation.

The *E. coli, C. freundii* and *E. cloacae* isolates exhibited a positive Amp C screen test. Since these organism are known to possess chromosomal Amp C, it may be assumed to be the reason for the false positivity exhibited in the KPC screen test. The distinction between plasmid and chromosomal Amp C is possible only by performing isoelectric focusing or by transfer experiments. [Jacoby *et al*. 2009]. This study did not employ these tests. Carbapenem resistance in these isolates is possibly due to the Amp C production along with porin loss. In the case of *K. pneumoniae* (15) isolates that exhibited positive KPC screen test, four isolates carried plasmid Amp C genes which may be assumed as the reason for false positivity of KPC screen test. Since this species does not possess chromosomal Amp C, detection of Amp C is presumed as plasmid borne. One isolate produced OXA-48/OXA-181. It has been
noted that OXA-48 producing isolate may exhibit false positive KPC screen test. [Miriagou et al. 2010, Giske et al.2011]. The remaining 10 isolates were cefoxitin resistant and exhibited positive Amp C screen test using boronic acid. In these the reason for a false positive KPC screen test could not be ascertained.

**Plasmid Amp C in *E. coli* and *K. pneumoniae***

In this study screening for Amp C producers was done by disc diffusion and MIC determination using cefoxitin and results of both were found to be comparable. Hence it may be assumed that cefoxitin MIC determination is not mandatory for Amp C screening. These isolates were then subjected to phenotypic tests namely cefoxitin Hodge test and boronic acid inhibitor method. Presence of Amp C genes was confirmed by multiplex PCR. Considering PCR as the gold standard, the sensitivity and specificity of the phenotypic tests employed were compared. The Hodge test fared better in terms of sensitivity and specificity when compared to the inhibitor based test (78.2% and 59.2% Vs 65.2% and 25.9%). Both the tests had better negative predictive values.

Overall, plasmid mediated Amp C was detected in 29.8% (23) of the study isolates, which included 11/25 *E. coli* and 12/52 *K. pneumoniae*. The prevalence of plasmid mediated Amp C varies widely in different parts of the world from 2% to 46%. [Jacoby 2009, Tan et al.2009] In Indian studies, the prevalence of Amp C ranged from 8% to 47%. [Hemalatha et al. 2007, Sinha et al. 2008].

The following plasmid Amp C types were looked for - CIT, DHA, ACC, EBC, MOX and FOX of which the latter two were not detected. Coexistence of multiple types was observed. The most common were the CIT family (LAT-1 to LAT-4, CMY-2 to CMY-7 and BIL-1), followed by the DHA which was found alone
in 3 isolates and together with EBC in four. ACC was detected in one isolate only. Worldwide, $\text{bla}_{\text{CMY-2}}$ is the most prevalent plasmid mediated Amp C. The other commonly reported Amp C is the DHA [Jacoby 2009]. Outbreaks of infections have been traced to strains harbouring CMY-2, MIR-1, BIL-1, ACT-1, ACC-1, etc. FOX enzymes have been described in Spain, Argentina and Italy, while MOX has been reported mainly from Japan and France.[Jacoby 2009, Lee et al.2007]. There are very few reports on the prevalence of different plasmid Amp C genes in Indian isolates. In one study, out of 455 E coli isolates, multiplex PCR detected 103 (22.6%) isolates harbouring different families of Amp C gene with $\text{bla}_{\text{CIT}}$ being the predominant gene.[Upadhyay et al.2011]

**Comparison of phenotypic tests and PCR results**

The overall distribution of carbapenemases in Enterobacteriaceae (67/111) is as follows: NDM alone in 58, NDM+ VIM in 6, VIM alone in 1 and OXA-48/OXA-181 in 2. The most common carbapenemase among Enterobacteriaceae is NDM, followed by VIM. OXA-48/OXA-181. Notably KPC and other MBL types namely IMP, SIM, SPM, GIM were not produced. The OXA-48/OXA-181 and the VIM producers exhibited resistance to carbapenems. $\text{Bla}_{\text{NDM}}$ was detected in 27 carbapenem susceptible isolates in which MBL screen was positive in 9 isolates and negative in 18 isolates.

Among the 50 carbapenem resistant isolates, carbapenemase encoding genes were detected in 40 which included NDM (31), NDM+ VIM (6), VIM (1) and OXA-48/OXA-181 (2). In 10 carbapenem resistant isolates, none of the genes included in the study were detected. Both MHT and MBL screen tests were positive in 7 isolates, which is suggestive of production of MBLs other than the ones looked for in the
study. In three isolates, MHT was positive and MBL was negative. In these, the probable mediating mechanism is CTX-M /Amp C production along with porin loss as they exhibited a positive ESBL and Amp C screen test.[Miriagou et al. 2010, Giske et al. 2011].

On comparing the phenotypic (MBL screen) tests results with PCR, it was that found among the MBL producers (65), MBL screen test was positive in 44 isolates only. In 21 MBL producers (NDM), the MBL screen test was negative indicating false negativity. Therefore, the sensitivity of MBL screen test is 67.7% and specificity is 78.3%.

OXA-48/OXA-181 producing isolates (2) were MHT positive. The MBL screen test was also positive in them. The reason could be a false positive MBL screen test or presence of MBLs other than what was looked for in the study. An association between OXA carbapenemase production and a false positive EDTA test result has been described previously [Giske et al. 2010]

In the PCR negative isolates (44), both MHT and MBL screen test were positive in 8 isolates. Of them, 7 were resistant to carbapenems as determined by MIC. The possible explanation is the production of MBLs other than those looked for in the study or the production of other carbapenemases. In one isolate, even though the phenotypic tests were positive, it remained susceptible to both imipenem and meropenem suggesting perhaps a false positivity. In 36 isolates MHT was positive but MBL screen test was negative. Of these 33 were carbapenem susceptible indicating false positive MHT due to CTX-M production. Three isolates were resistant to carbapenems. They exhibited positive ESBL and Amp C screen production thus thus suggesting that the possible operating mechanisms mediating can be ESBL production
or hyper production of Amp C together with porin loss. [Miriagou et al. 2010, Giske et al. 2011]. Presence of other beta-lactamases namely, ESBLs and/or Amp C renders the phenotypic tests unreliable since many of these mechanisms mask each other.

In this study MHT was positive in all isolates but carbapenemase encoding genes were detected in 67 isolates only. Though this test is said to be sensitive for detection of carbapenemase, it has a low specificity as it cannot differentiate between the different classes of carbapenemases. [Miriagou et al. 2010, Giske et al. 2011]. In the present study the sensitivity and specificity were not ascertained. The finding of false positive MHT in 44 isolates is notable, because MHT has been the recommended test by the CLSI for the phenotypic detection of carbapenemase producing bacteria in the clinical microbiology laboratory. False positive results with MHT may be obtained due to production of CTX-M with reduced outer membrane permeability. The prevalence of CTX-M being very high in India, the value of this test may be undermined. Therefore, PCR remains the gold standard for the detection of carbapenemase producers. Regarding the MBL screen test, it detected all the VIM producers when VIM was alone or in combination with NDM. Only 37 out of 58 NDM-1 producers were MBL screen test positive indicating that the test may not be very sensitive for NDM detection.

Analysis of risk factors influencing the acquisition of carbapenemase producing Enterobacteriaceae

While several authors have focused on the epidemiology, laboratory detection methods and molecular characterization of carbapenemase-producing bacteria, only a few have analysed the risk factors for acquiring the infections and their clinical outcomes. Identification of risk factors associated with carbapenem-resistant infection
assists in the empiric therapeutic decision-making process and also allows for early implementation of appropriate infection prevention measures. Compared with infections caused by susceptible strains of the same species, infections caused by several antibiotic-resistant bacteria have been associated with worse outcomes, including longer hospitalizations and high rates of morbidity and mortality. [Patel et al. 2008, Gupta et al. 2011]

Considering these facts, this study attempted to analyze the factors influencing the acquisition of carbapenemase producing Enterobacteriaceae and the clinical outcomes analyzed. Univariate analysis revealed that gender (males), stay in the ICU, mechanical ventilation, presence of multiple indwelling device, presence of diabetes mellitus, presence of focal infection or sepsis, surgical interventions, and usage of multiple antimicrobial agents and carbapenems were significant risk factors influencing the acquisition of carbapenemase producing Enterobacteriaceae. The mean duration of hospital stay was 26.4 days in patients with carbapenemase positive Enterobacteriaceae infections and 22.98 in those with carbapenemase negative Enterobacteriaceae infections. The duration of stay was prolonged in both the groups but it was not a significant risk factor. Presence of other comorbid underlying disease and the outcome of the infection were not significant. However, on subjecting the variables to multivariate analysis, none of these factors assessed were significantly associated with infections caused by carbapenamase producing Enterobacteriaceae.

In various studies from different countries, organ/stem cell transplantation, stay in ICU, mechanical ventilation, prolonged hospital stay, multiple indwelling device, severity of underlying illness, presence of comorbid conditions, recent surgical procedures were identified as risk factors for acquiring carbapenemase producing Enterobacteriaceae infection [Patel et al. 2008, Schwaber et al. 2008, Hussein...

In this study, patients with carbapenem-resistant Enterobacteriaceae infection had serious comorbid conditions such as chronic renal failure and multiple injuries due to road traffic accidents. Most patients were in the ICU, required mechanical ventilation and were exposed to broad-spectrum antibiotics such as betalactams, aminoglycosides and fluoroquinolones. Invasive procedures and multiple medical devices commonly play a more important role in increasing susceptibility to nosocomial infections and likely provided a portal of entry of the organism.

The impact of antibiotic resistance on the outcome of patients with nosocomial infections is controversial. Although it is generally accepted that drug resistance is associated with increased morbidity and mortality some studies found no such relationship. Underlying co-morbidities, delay in the initiation of appropriate antimicrobial therapy, and the severity of illness of the patients infected by the multidrug-resistant pathogens, may be important confounders. Therefore, appropriate adjustment for these confounding factors is essential in studies that determine the impact of antimicrobial resistance. [David et al.2012, Correa et al.2013]
It is well known that carbapenem resistant Enterobacteriaceae infections are associated with both high morbidity and mortality [Borer et al. 2012, Bratu et al. 2005, Neuner et al. 2011, Daikos et al. 2009]. In this study, the mortality rate was 56.7% in patients infected with carbapenemase producing Enterobacteriaceae and 47.7% in patients infected with carbapenemase non producers. This was statistically not significant. Moreover, it is difficult to assess the attributable mortality when both groups had high overall mortality. It may be assumed that greater disease severity and poor patient condition contributed to the poor outcomes, not necessarily the infection itself.

Previous studies have suggested that removal of the focus of infection, such as a catheter, debridement, or drainage, is an effective way of improving survival among patients with carbapenem-resistant K. pneumoniae infections [Patel et al. 2008]. However, these adjunctive therapies were not evaluated in the present study.

The primary objective of the present study was not the analysis of the risk factors for acquiring carbapenem resistant Enterobacteriaceae infections or the influence of such infections on mortality. Moreover the sample size may not be adequate to assess these individual factors objectively. Assessment of the severity of the underlying illness by APACHE scoring was not done and therefore it was not possible to attribute the mortality in relation to the infection or to the comorbid conditions. Prospective case controlled studies are needed to have a better understanding of the risk factors for infection.
Carbapenemases in *Pseudomonas* and *Acinetobacter* Species

**Class A**: *Bla*$_{KPC}$ was not detected in the study isolates. Reports of KPC production in *Pseudomonas* and *Acinetobacter* is scarce.[Rasmussen et al. 2007, Robledo et al. 2010,2011]

**MBL**: Infections caused by MBL producing NFGNB are associated with high rates of mortality, morbidity and rising health-care costs. MBL producing *Pseudomonas aeruginosa* was first reported in Japan in 1991 and since then, they have disseminated worldwide [Kaleem *et al.*2010]. The detection of MBL producing GNB is therefore necessary to aid in appropriate treatment and infection control measures, and to prevent their dissemination. The most common MBLs in NFGNB include the VIM, IMP,GIM, SPM, SIM enzymes and the recently identified NDM-1. In particular, *bla*$_{VIM-2}$ has emerged as a dominant MBL variant worldwide [Samuelson *et al.* 2010, Cornaglia *et al.* 2011].

Several phenotypic methods are available for the detection of MBL producing bacteria. All these methods are based on the ability of a metal chelator such as EDTA and thiol-based compounds to inhibit the activity of MBL. [Lee *et al.*2003, Miriagou *et al.*2010, Hemalatha *et al.* 2005,Pitout *et al.*2005]. Though several methods are advocated in many studies, CLSI does not recommend a standardised method for the detection of MBL producing isolates. In this study, screening for carbapenamases production was done by MHT and MBL production by inhibitor based method using EDTA as inhibitor.

Of the 179 *Pseudomonas* and *Acinetobacter* isolates, MHT was positive in 167, indicating the production of carbapenamases. For the 12 isolates that were carbapenem resistant but MHT negative, the test was performed on MHA
supplemented with zinc sulphate (70mg/L). Out of this, the test turned out to be positive in 2 isolates of *P. aeruginosa*. Hence MHT was positive in 169 isolates (94.4%). The remaining 10 were MHT negative, thereby suggestive of other mechanisms such as loss of porins or upregulation of efflux pumps. [Karthika *et al.*2009, Miriagou *et al.*2010]. MBL screen test was positive in 80.4% (144).

PCR detected the MBL genes, *bla*$_{VIM}$/*bla*$_{IMP}$ in 51.4% (92). In 52 isolates that were MBL screen test positive though *bla*$_{VIM}$/*bla*$_{IMP}$ were not found. Therefore we performed multiplex PCR to detect all the MBL genes including *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{SPM}$, *bla*$_{SIM}$, *bla*$_{GIM}$. The results of the multiplex PCR correlated with the results of PCR done using consensus primers for *bla*$_{VIM}$/*bla*$_{IMP}$. Still, SIM, GIM, SPM were not detected. Furthermore, we performed PCR to detect the presence of *bla*$_{NDM}$. *Bla*$_{NDM}$ was detected in 18 isolates which included 8 isolates which carried *bla*$_{VIM}$ also. Hence overall, in 42 isolates that were MBL screen tests positive, none of the MBL encoding genes were found. Despite the good performance of inhibitor based methods for the detection of MBL by using EDTA, it is not a specific test. False positive results have been reported in *P. aeruginosa* as EDTA acts on the membrane of the bacterial cell and increase the cell permeability. Presence of OXA carbapenamases in *A. baumannii* may also lead to false positive results. [Miriagou *et al.*2010, Wirth *et al.*2009]. Hence the results of the MBL phenotypic tests must be interpreted cautiously.

The overall *bla*$_{VIM}$/*bla*$_{IMP}$ production among the study isolates was 51.4%. Of the 61 *P. aeruginosa* isolates, 36 produced the above enzymes. Out of the 116 *A. baumannii* isolates, 54 produced VIM/IMP. These results indicate that carbapenem resistance in *P. aeruginosa* is mainly due to MBL production whereas in *A. baumannii* it is due to the presence of multiple beta lactamases, which may include
the OXA carbapenamases. The common MBL genotype was the bla\textsubscript{VIM} (89). Bla\textsubscript{IMP} was found in three isolates of which one carried both.

In Asia, bla\textsubscript{IMP} and bla\textsubscript{VIM} are prevalent. Bla\textsubscript{IMP} is found mainly in Japan, Korea, China, Taiwan, and Iran [Fang et al. 2008, Peymani et al.2011, Franco et al. 2010,Kim et al. 2013]

The prevalence of MBL in India has ranged from 7% to 65% among carbapenem-resistant \textit{P. aeruginosa}. [Arunagiri et al.2012, Manoharan et al. 2010]. In one study, the rate of MBL production was 24.5% among 61 \textit{P. aeruginosa} isolates, and bla\textsubscript{VIM} type was the most common [Manoharan et al. 2010]. Another study from India also reported bla\textsubscript{VIM-2} from \textit{P. aeruginosa} [Toleman et al. 2007]. In a nationwide survey conducted to characterise 301 MBL producing \textit{Pseudomonas} species in 10 medical centres from India, MBL genes were detected in 18.9% of the isolates and 5 VIM variants were reported with VIM-2 being the most common. The others were VIM-6, VIM-11, VIM-5 and VIM-18. [Castanheira et al 2009].

In India, MBL production among \textit{A. baumannii} isolates has been reported as 42%. The most prevalent MBL gene was bla\textsubscript{IMP-1} [Karthika et al. 2009]. There is limited data on the prevalence and distribution of MBLs in \textit{A. baumannii} among Indian isolates.

Regarding resistance profiles, all isolates were resistant to other classes of antimicrobial agents such as aminoglycosides and fluoroquinolones. All the \textit{P. aeruginosa} and the lone \textit{P. stutzeri} were resistant to aztreonam, indicating the concomitant presence of other beta lactamases. Among \textit{P. aeruginosa} 91.8% remained susceptible to colistin. Susceptibility to tigecycline was seen in 93.1% (108) of \textit{A. baumannii} isolates. In this study, bla\textsubscript{VIM} | bla\textsubscript{IMP} production contributes to
51.4% of carbapenem resistance. Hence early detection of MBL producing organisms is important to guide in the treatment of infections caused by them and also to arrest their spread. In the clinical microbiology laboratory, all clinical isolates that are resistant to carbapenems must be screened for carbapenamase and MBL production by using simple phenotypic tests. To conclude, carbapenem resistance in \textit{P. aeruginosa} is chiefly mediated by MBL production. The common MBL gene is \textit{bla VIM}. The development of simple and inexpensive screening methods to detect carbapenamases and MBL production in Microbiology Laboratories is crucial for optimal treatment of patients, particularly critically ill and hospitalized patients, and to control the spread of resistance.

\textit{NDM in P. aeruginosa}

Identification of \textit{Bla\textsubscript{NDM-1}} gene in many genera and species of Gram negative bacteria indicate that this gene can spread at a high rate. The genes encoding NDM are heterogeneous on basis of molecular size and location. In Enterobacteriaceae it is plasmid -borne, while in \textit{A. baumannii} both chromosomal and plasmid location is reported. Till date reports of NDM-1 in \textit{P. aeruginosa} is scarce. There are only three reports in the medical literature of its occurrence, two reports from Serbia and interestingly none of them had a history of travel to the Indian subcontinent and one recent report from India. [Jovcic \textit{et al}. 2011, Flateau \textit{et al}. 2012, Khajuria \textit{et al}. 2013]. Though there are only sporadic reports, knowledge of its prevalence is essential because \textit{P. aeruginosa} is an environmental pathogen with intense colonization capacity and ability to persist for indefinite periods in the hospital environment.[Johnson \textit{et al}.2013, Lister \textit{et al}.2009].Thus far, no other country or region in the world has reported NDM-1 in \textit{P. aeruginosa} [Johnson \textit{et al}. 2013].At the Military Medical Academy in Serbia, routine analysis of carbapenemase producing
bacterial isolates, revealed NDM-1 in seven clinical isolates of *P. aeruginosa*. The source patients were hospitalized in Serbia and had no history of travel to any other country. Subsequently, in 2012 France reported recurrent pyelonephritis due to NDM-1 producing *P. aeruginosa*. This patient had history of prior hospitalization in Serbia and gave rise to the hypothesis that the Balkan states may be endemic for NDM-1 producers. In both the reports there was no complete documentation of plasmid-borne or chromosomal localization of *bla*<sub>NDM-1</sub> in *P. aeruginosa*. Both the reports emphasized the use of PCR for detection of *bla*<sub>NDM</sub> [Jovicic et al. 2011, Flateau et al. 2012]. The Indian report was from a tertiary care centre in which *bla*<sub>NDM-1</sub> was detected in four isolates among 40 carbapenem resistant *P. aeruginosa*. [Khajuri et al. 2013]

This study looked for the presence of *bla*<sub>NDM-1</sub> in carbapenem resistant *P. aeruginosa*. Considering that out of 61 carbapenem resistant *P. aeruginosa* only 4 were found to harbor NDM-1, it can be reasonably assumed that NDM-1 is not a major mechanism mediating carbapenem resistance in *P. aeruginosa* in this hospital. This being only a single centre report, further studies are required at national or regional levels to understand the magnitude and prevalence of NDM-1 in *P. aeruginosa*. Additionally coexistence with other carbapenemase encoding genes is evident in a single isolate. To the best of our knowledge, this is the first report of the presence of both *bla*<sub>VIM</sub> and *bla*<sub>NDM-1</sub> in a single clinical isolate of *P aeruginosa*.

The clinical history of the source patients was perused and the outcomes were followed up. On receipt of the microbiology culture reports all the patients were initiated on polymyxin B therapy. One patient responded to the therapy while the others succumbed to the infection. The patient who survived was followed up till discharge after recovery. Of the previously reported Serbian patients infected with
NDM-1 *P. aeruginosa*, two expired and the one with pyelonephritis recovered from infection on treatment with colistin. In the study from India, two isolates were from central venous catheter tip cultures in patients with septicaemia and one from the pus sample in a patient with surgical site infection. All the 3 patients were successfully treated with colistin. One Isolate was recovered from the urine sample of a patient who had pyelonephritis with pyuria who was later treated successfully with a combination of colistin and amikacin [Jovcic *et al.* 2011, Flateau *et al.* 2012, Khajuria *et al.* 2013].

NDM-1 producing *P. aeruginosa* are undoubtedly challenging. Their ability to survive under a wide range of environmental conditions and potential to spread in hospital settings make them unique. Though not as prevalent as other MBLs such as IMP and VIM, a strict vigilance and continuous surveillance of NDM-1 is essential considering the difficulties in therapeutic management and control. In the present study, though NDM was detected in 2.8% (4/143) of carbapenem resistant *P. aeruginosa*, these are associated with high mortality. Therefore their identification is crucial for appropriate treatment of patients and also to implement infection prevention measures to curtail their dissemination.

**NDM-1 in A. baumannii**

Although the most widespread carbapenemases in *A. baumannii* are the Class D OXA types and the MBLs namely VIM and IMP, more recently NDM-1 is being reported from many countries. NDM-1 production in *A. baumannii* has serious implications since it is an important nosocomial pathogen. Following their occurrence in Enterobacteriaceae, there were reports of NDM-1-positive *Acinetobacter* spp. in a hospital in Pune [Bharadwaj *et al.* 2012], and in a hospital in Chennai [Karthikeyan *et
One study reported co-existence of NDM-1 with other carbapenamases such as the OXA [Karthikeyan et al. 2010]. Local and global surveillance data on the prevalence of NDM-1 producing A. baumannii is scarce. With limited therapeutic options, NDM-1 in Acinetobacter species is a cause for concern in critically ill patients with life threatening infections. Adequate detection of carbapenamase producing A. baumannii is crucial for infection control measures and appropriate a choice of antimicrobial therapy.

In the present study, 13 of 116 (11.2%) carbapenem resistant A. baumannii harboured the bla\textsubscript{NDM} gene. Amongst them, 6 also had the bla\textsubscript{VIM}, bla\textsubscript{OXA-23} like and bla\textsubscript{OXA-51} like genes, while six others had bla\textsubscript{OXA-23} and bla\textsubscript{OXA-51} without VIM along with the bla\textsubscript{NDM}. One isolate harboured bla\textsubscript{NDM} alone. All the 13 isolates exhibited a positive result with MHT and inhibitor based disc test for the presence of carbapenemases and MBL. Their MIC\textsubscript{90} to imipenem and meropenem were 32mg/l and 64 mg/l respectively. These NDM producers were obtained from respiratory secretions (9), blood (3) and cerebrospinal fluid (1). The majority of the isolates were from one multidisciplinary intensive care unit of the hospital.

Bla\textsubscript{NDM} was detected in the lone Acinetobacter lwoffii isolate which also carried the bla\textsubscript{VIM}. It exhibited a positive MHT and MBL screen tests. This isolate was from the blood stream infection of a 4 years old child who underwent a cardiac surgery for closure of ventricular septal defect and was in the cardiac ICU on ventilator support. The child responded to treatment with colistin.

In a study from China, the prevalence of the bla\textsubscript{NDM-1} gene was extremely low (4/2109 isolates-0.18%). [Chen et al. 2011]. A. baumannii isolates expressing NDM-1 MBL have been isolated in Germany, Serbia [Pfeifer et al. 2011, Poirel et al.]
Recently, clonal spread of NDM-2 producing *A. baumannii* strains have been described in a rehabilitation ward in Israel and in the United Arab Emirates [Espinal et al. 2011, Ghazawi et al. 2012].

While *bla*$_{NDM}$ has commonly been found on plasmids in Enterobacteriaceae, it is notable that there has only been one report of plasmid-mediated NDM in *A. baumannii* [Chen et al. JAC 2011], although diverse plasmids encoding NDM have been found in other species of *Acinetobacter* [Fu et al. 2012, Yang et al. 2012]. In all other reported isolates of *A. baumannii*, the *bla*$_{NDM}$ gene was located on the chromosome [Bogaerts et al. 2012, Bonnin et al. 2012, Boulanger et al. 2012, Espinal et al. 2011, Hrabák et al. 2012, Kaase et al. 2011, Pfeifer et al. 2011].

Though not as prevalent as other MBLs such as IMP and VIM, a strict vigilance and continuous surveillance of NDM is essential considering the difficulties in therapeutic management and control. Since acquisition of multidrug resistant *A. baumannii* is related to environmental contamination and carried in the hands of health care providers, control measures should address the source of infection. Continued careful attention to hand hygiene, contact isolation, barrier precautions, adequate environmental cleaning and careful disinfection of patient care equipments along with surveillance is essential to prevent outbreak of infections caused by these multidrug resistant strains.

**OXA carbapenemases in *A. baumannii***

Carbapenem-hydrolyzing *bla*$_{OXA-23}$ was first reported in *A. baumannii* in 1985. Since then several of them have been reported worldwide. Four families of OXA genes have been identified in *A. baumannii*: *bla*$_{OXA-23}$ like (*bla*$_{OXA-23}$, *bla*$_{OXA-27}$ and *bla*$_{OXA-49}$); *bla*$_{OXA-24}$ like (*bla*$_{OXA-24}$, *bla*$_{OXA-25}$, *bla*$_{OXA-26}$ and *bla*$_{OXA-40}$); *bla*
and \textit{bla}\ OXA-51 like. The last group constitutes a family of chromosomal enzymes typically present in \textit{A. baumannii} \cite{Peleg et al 2008, Poirel et al 2006, Mostachio et al 2009}. Though the presence of \textit{bla}\ OXA genes in \textit{A. baumannii} is widely known, there is a paucity of information on the distribution of different types of OXA carbapenemases in isolates from the Indian subcontinent. \cite{Karunasagar et al 2011, Roy et al 2011c, Karthikeyan et al 2010}.

In this study, overall OXA carbapenemases were detected in 91.3\% (106) of carbapenem resistant \textit{A. baumannii}. \textit{Bla}\ OXA-51 (99) and \textit{bla}\ OXA-23 (95) were the most common OXA carbapenemases and they coexisted in 89 isolates. \textit{Bla}\ OXA-51 was found alone in nine isolates and \textit{bla}\ OXA-23 alone in six isolates. \textit{Bla}\ OXA-24 was detected only in two isolates, of which one also carried \textit{bla}\ OXA-51 and \textit{bla}\ OXA-58.

Numerous studies have reported that \textit{bla}\ OXA-23 is the most frequent type of carbapenemases identified among the carbapenem resistant \textit{A. baumannii} \cite{Mostachio et al 2009, Andriamanantera et al 2010, Koh et al 2007}. The coexistence of \textit{bla}\ OXA-23 like, \textit{bla}\ OXA-24 like, \textit{bla}\ OXA-51 like and \textit{bla}\ OXA-58 like genes has been reported, especially that of \textit{bla}\ OXA-23 and \textit{bla}\ OXA-51. \cite{Sung et al 2008, Mendes et al 2009}. \textit{Bla}\ OXA-24 has been reported from Spain, Belgium, France and United States while \textit{bla}\ OXA-58 has been identified in many countries. \cite{Peleg et al 2008}. In one study on 62 isolates of the \textit{Acinetobacter} species, \textit{bla}\ OXA-23 was the most common. \cite{Karunasagar et al 2011} In another study on four carbapenem resistant \textit{A. baumannii} blood stream isolates, \textit{bla}\ OXA-23 was found to be the most prevalent. \cite{Roy et al 2011c}.

In this study, \textit{bla}\ VIM coexisted with \textit{bla}\ OXA-23 and \textit{bla}\ OXA-51 in 49 isolates and in 1 isolate \textit{bla}\ VIM, \textit{bla}\ IMP, \textit{bla}\ OXA-23 and \textit{bla}\ OXA-51 were found. The simultaneous existence of \textit{bla}\ OXA and MBL encoding genes has been reported from several

Among the 116 study isolates, 97.4% (113) were susceptible to polymyxin and 93.1% (108) to tigecycline. The lack of universal susceptibility to these two drugs is a cause for concern. All the study isolates were uniformly resistant to aminoglycosides and fluoroquinolones. Though carbapenems are the drug of choice to treat A. baumannii infections, such resistance profiles limits therapeutic options to polymyxins and tigecycline. Easy and simple phenotypic tests are required for the early identification of carbapenamases in the clinical laboratories to notify the treating physicians and also devise methods to contain their spread.

To sum up, \textit{bla}_{OXA-23} and \textit{bla}_{OXA-51} are the most common types of OXA carbapenemases in \textit{A. baumannii}. This type of resistance is a factor with a significant threat in hospitals. It should be addressed with alternative and newer therapeutic strategies, strict infection control measures and continuous surveillance. A simultaneous existence of different classes of carbapenemases is a problem to reckon with and hence detection methods are required for each of these. In outbreak settings, an initial screening of the putative carbapenamase producers will help to organize intervention and early directed therapy.

**Comparison of phenotypic tests with PCR results**

\textit{P. aeruginosa}: Among 61 carbapenem resistant \textit{P. aeruginosa}, MBL genes were detected in 39 isolates. The MBL genes found included \textit{bla}_{VIM}, \textit{bla}_{IMP} and \textit{bla}_{NDM}. \textit{Bla}_{VIM} alone was detected in 33 isolates, \textit{bla}_{NDM} alone in 3, \textit{bla}_{IMP} alone in 1. \textit{Bla}_{VIM} coexisted with \textit{bla}_{NDM} in one isolate. \textit{Bla}_{VIM} and \textit{bla}_{IMP} were found together in a single isolate. MHT was positive in all the PCR positive (39) isolates. MBL screen
test was positive in 38 isolates and negative in one isolate. The sensitivity of MHT and MBL screen test were 100% and 97.4% respectively. Both tests had low specificity.

In 22 carbapenem resistant isolates none of the genes looked for were detected. Among them both MHT and MBL screen test were negative in 5 isolates suggestive of non carbapenemase mediated mechanisms such as porin loss or upregulated efflux pumps. Both the tests were positive in 10 isolates, which is indicative of the presence of novel MBLs other than the ones looked for in the study. In 5 isolates MHT was positive and MBL screen test negative, the probable mechanism may be the presence of other carbapenemase or a false positive MHT result. In isolates with a positive MBL screen test and MHT negative (2), it was assumed that carbapenem resistance is due to the novel MBLs. In this case a false positive MBL screen result may also be a possibility and hence the mediating mechanism may be porin loss or upregulated efflux pumps. [Miriagou et al. 2010]

**A. baumannii:** Among 116 A. baumannii, 111 carried one or more carbapenemase encoding gene. The sensitivity of MHT and MBL screen test was 100%. In 5 isolates, none of the genes were found. In three of these isolates both MHT and MBL screen test were negative and the probable mediating mechanism may be porin loss or upregulated efflux pumps. In the case of two isolates that exhibited positive results for both MHT and MBL screen tests, the possible mechanisms may be assumed as presence of novel carbapenemase encoding genes including MBL [Miriagou et al. 2010, Karthika et al. 2009]
RAPD of VIM producing *A. baumannii* and *P. aeruginosa*

The acquisition of resistant bacteria in hospitals may be a consequence of selective pressure exerted by the use of antibiotics and/or horizontal dissemination. The hospital environment remarkably promotes selection and quick distribution of resistant strains. It is of crucial importance to carry out epidemiological surveys including a detailed characteristic and relationship among strains isolated in particular environment and time, as well as to become aware of risk factors, sources and ways of infection distribution.[Katarzyna et al. 2012]. Often the sporadic isolation of multiresistant strains does not draw the attention in time to determine the nosocomial spread of one clone. The collection of multi resistant, nosocomial pathogens for typing may help to discover hidden reservoirs, and the circulation of outbreak clones in different wards.

Genomic relatedness *P. aeruginosa* (10) isolates was tested by RAPD profiling using 208 primer and 272 primers. The dendrogram was constructed according to the banding pattern using UPGMA. None of the isolates were clonally related.

In case of *A. baumannii* (10) isolates selected for RAPD profiling using DAF4 primer and ERIC primers, the dendrogram was constructed according to the banding pattern using UPGMA. Five different genotype diversities were exhibited by 10 isolates. Of them, 3 isolates showed diverse clonality; 5 isolates showed similar clonality and were grouped together and two other isolates exhibited clonal relatedness among them. Results of dendrogram constructed by both primers showed 100% distance coefficient for the 5 isolates and two isolates that possessed similar clonality. These five isolates were obtained from patients admitted to the
multidisciplinary adult ICU admitted at the same time, indicating a possible horizontal transmission. Two isolates which exhibited relatedness among themselves were from patients in two different ICU: one from multidisciplinary ICU and the other from neurosurgery ICU. There was no transfer of the patient or the health care provider between both ICU during that period.

Presence of diverse clones in this study is comparable with many studies from different parts of the world. In the SENTRY surveillance program, among 57 MBL producing Pseudomonas isolates collected at 10 Indian hospitals, the isolates were clustered in 33 groups and were not genetically related. [Castanheira et al. 2009]. Recently in Spain the MBL producing P. aeruginosa isolates exhibited high degree of diversity with 56 isolates being grouped into 32 different sequence types [Gomila et al. 2013]. In Singapore also Koh et al report a that MBL producing P. aeruginosa were diverse [Koh et al. 2010]

A. baumannii isolates had a high genetic diversity, no clonality was observed, which corresponds to reports from USA, China, Iran, Brazil and Taiwan. [Srinivasan et al. 2009, Wang et al. 2013, Shahcheraghi et al. 2011, Lin et al. 2011, Sader et al. 2005]. Two South Indian studies also documented genetic heterogeneity among Acinetobacter species. [Karthika et al. 2009, Prashanth et al. 2005]

With the exception of a few sporadic cases of possible horizontal transmission, there were no highly epidemic strain isolated, which is expected to be a result of the good infection control measures taken by the clinicians and the health care providers in our hospital.

The diversity of VIM producing A. baumannii and P. aeruginosa isolates in our settings could provide useful information for infection control. The clonal
relatedness of the five *A. baumannii* isolates in the multidisciplinary ICU and the fact that they may be transmitted horizontally highlight that infection control measures such as environmental cleaning and hand hygiene must be reinforced to reduce the further spread of *A. baumannii*. Since the study was retrospective, environmental sampling and screening of the hands of the health care providers were not done during the study period to find the source of infection and mode of transfer. Our centre being a tertiary care teaching hospital, patients are transferred from a wide range of health-care facilities in the geographic region. Therefore, colonisation or infection with resistant strains is likely to occur before transfer, implying the import of such strains on admission.

The genomic variability suggests strong selection pressure on bacterial population, underlining the necessity for proper management of antibiotic therapy, within health-care units.

**Assessment of risk factors for mortality in NFGNB**

Elucidation of the risk factors for carbapenem resistant gram negative infection or colonisation is vital if efforts are to be designed to curb resistance. Furthermore, identifying the effect of resistance on clinical and economic outcomes is critical in prioritizing future interventions to optimize therapy for these types of drug-resistant infections. A high endemic incidence rate of carbapenem resistant *Pseudomonas* and *Acinetobacter* infection has been observed at our setting in the past years, mainly in the ICU.[Shanthi et al.2009] This fact prompted us to want to better understand the risk factors associated with such infection in hospitalised patients particularly in the critically ill. Even though our study was not intended to look at the clinical variables, since the study was conducted in a tertiary care University teaching
In this study mortality rate was 55.8% in patients infected with carbapenemase producing NFGNB. The mortality rate was 44.1% in patients with carbapenemase negative NFGNB infections. Univariate analysis demonstrated that prolonged stay in hospital (>20 days), stay in ICU, mechanical ventilation, presence of indwelling device, being a diabetic, presence of focal infection or sepsis, usage of multiple antimicrobial agents including carbapenem were all significant factors influencing mortality in patients infected with carbapenem resistant NFGNB. These factors portray a severely ill patient who requires intensive contact with caregivers and for whom the disease, treatment, and invasive devices compromise protective barriers. The intensive nursing care given to these patients and the placement of several invasive devices introduce multiple opportunities for failure of infection prevention measures. Moreover, the intensity of selection pressure by broad-spectrum antibiotics is high in the ICU. The care of bedridden patients also requires contact with healthcare providers who serve as the vector for transmission of these resistant bacteria. Treatment with multiple antibiotic agents with broad-spectrum is the norm in such patients and this particularly eradicates the competitive flora. Gender, surgical intervention, presence of comorbid conditions were not significant factors influencing mortality.

Subsequently onn subjecting the variables to multivariate logistic regression analysis there was a significant association with presence of multiple indwelling
device, diabetes mellitus and focal or generalized sepsis. Similar observations have been made and documented by several authors [Lautenbach et al. 2006, 2009, 2010, Onguru et al. 2008, Carmeli et al. 2011, Furtado et al. 2009, Munoz-Price et al. 2010, Tuon et al. 2012].

In addition to being a retrospective analysis of the patient clinical data, this study is limited by the lack of severity scores such as Acute Physiology and Chronic Health Evaluation (APACHE) score, possible bias from unadjusted confounding variables, such as baseline severity of illness/comorbidities, and different timing of bacterial culture/isolation and therapy initiation. The true impact of carbapenem resistant infection on patient outcomes remains controversial. The conventional thought that NFGNB are associated with reduced virulence has been challenged. Available clinical data suggest that the infections caused by NFGNB may be associated with worse outcomes such as mortality, morbidity, requirement for surgical intervention, prolonged length of hospital stay and so on. However, these clinical investigations are often confounded by inconsistency such as the presence of multiple mechanisms of resistance in a single isolate and retrospective study design. Well-designed prospective class controlled studies addressing these drawbacks should be performed with adequate sample size.

**Limitations of this study**

Characterisation of other non-enzymatic mechanisms mediating carbapenem resistance such as upregulated efflux pumps, porin defects and hyper production of Amp C beta lactamase were not performed in this study. Prospective case controlled study with adequate sample size and a primary objective to identify the risk factors for infection with carbapenemase producing GNB and the outcome of such infections are
needed to have a clear understanding of this problem. Molecular investigations must be used to detect the association of various mechanisms of resistance and expression of virulence factors.

**Future directions**

- Characterisation of other non-enzymatic mechanisms mediating carbapenem resistance such as upregulated efflux pumps and porin defects
- Strict enforcement of antibiotic stewardship programs and infection prevention measures
- Evaluation of novel Inhibitors of carbapenemase enzymes compatible with human tissues
- Evaluation of efflux pump inhibitors for therapeutic use