5. DISCUSSION

*Pseudomonas aeruginosa* is amongst the leading cause of nosocomial infections responsible for 10-15% of these infections worldwide. These infections include: pneumonia, urinary tract infections and bacteraemia. Infections due to this organism are often difficult to treat due to its resistance to various antibiotics. β-lactam antibiotics are the most widely used agents to treat *P. aeruginosa* infections. The resistance to these antibiotics is reported from all over the world. The selective pressure exerted by the misuse and overuse of these antibiotics in the hospitals leads to the emergence of multidrug resistant strains. The inability to detect the relevant mechanism of resistance has been responsible for the dissemination of resistant strains throughout the world. The most important mechanism of resistance to β-lactam antibiotics is the production of an enzyme β-lactamase, which destroys their β-lactam ring. The genes responsible for different β-lactamases are present either on the chromosomal DNA or other accessory part of the genome or on the transferable segments located on the plasmids. These enzymes were initially commonly found in *Klebsiella* spp. and *E. coli* species but later on in all other members of the family *Enterobacteriaceae* as well as other gram negative bacilli such as *Pseudomonas aeruginosa* and *Acinetobacter baumanii* (Arora and Bal, 2005). The prevalence of multi drug resistant *P. aeruginosa* (MDRPA) strains that produce various β-lactamase enzymes is increasing tremendously throughout the world. The options for treating such strains are limited and complicated because of different susceptibility patterns to different antibiotics which may sometimes result in treatment failures consequently causing significant morbidity and mortality (Itokazu *et al.*, 1996). The emergence of multidrug resistance in *P. aeruginosa* not only makes the treatment expensive but may increase the complexity as well as duration of treatment. The management of infections caused by MDRPA is a major challenge to the clinicians and healthcare workers because such strains offer resistance to most of the routinely used antibiotics. The continuous surveillance and molecular characterization of *P. aeruginosa* strains in the hospital settings as well as in the communities is therefore of vital importance. The determination of their susceptibility patterns against different classes of antibiotics, investigation of the mechanisms of virulence and to study the drug resistance
mechanisms and epidemiological studies are some of the very important aspects that are required to be studied. Clinical studies together with the improved methods of antimicrobial susceptibility testing are needed to identify risk factors associated with the development of MDR strains. Also, the most appropriate antimicrobial regimens and duration of treatment are also to be worked out on regular basis in order to manage \textit{P. aeruginosa} infections effectively.

Keeping all this in view, the present study has been designed to determine the susceptibility patterns of \textit{P. aeruginosa} strains to different antibiotic classes. This would give a clue about the multi drug resistant (MDR) and multi drug sensitive (MDS) phenotypes. We used the criterion of multidrug resistance i.e. resistance to 3 or more groups of antimicrobials to regard \textit{P. aeruginosa} isolates as MDR, as used by earlier workers (Souli \textit{et al.}, 2008 and Gill \textit{et al.}, 2011). The same criterion to regard an isolate as MDR has also been selected by other workers. However, some researchers have used different criteria: an isolate which is resistant to two or more drugs or drug classes of therapeutic relevance has been regarded as MDR (Irfan \textit{et al.}, 2008 and Saderi \textit{et al.}, 2010) while others regard those strains as MDR which are resistant to four or more classes of antimicrobial agents (Goyal \textit{et al.}, 2010). With the criterion followed by us, 30/87 (34.48\%) \textit{P. aeruginosa} isolates were recorded as MDR. A very high proportion (84.7\%) of MDR \textit{P. aeruginosa} strains from Odisha has been reported by Dash \textit{et al.} (2014). In an earlier study, Chauhan and Sharma (2013) from this region recorded 69.5\% \textit{P. aeruginosa} isolates as multidrug-resistant. A frequency of 71\% MDRPA strains has been reported from Tamil Nadu by Mohanasoundaram (2011). Lower frequencies have however been reported from other countries by different research groups: 22.7\% and 29\% from Pakistan respectively by Gill \textit{et al.} (2011) and Farhatullah \textit{et al.} (2009), 14\% in Houston, US (Tam \textit{et al.}, 2010). Very low prevalence of 14/10,000 hospital admissions in Israel has been reported from Israel by Aloush \textit{et al} (2006). A study conducted in Japan, reports an incidence of 1.1\% MDR strains of \textit{P. aeruginosa} (Kirikae \textit{et al.}, 2008). These workers defined MDR \textit{P. aeruginosa} isolates as those which were resistant to carbapenems, amikacin and fluoroquinolones.
The antimicrobial susceptibility patterns observed in the present study revealed 91.95% isolates susceptible to levofloxacin, 89.65% to piperacillin/tazobactum followed by 86.21% to meropenem, 82.76% to amikacin, 79.31% to cefepime, 74.71% to piperacillin, 70.11% to gentamicin, 67.82% to cefoperazone and ceftazidime, 66.67% to imipenem, 60.92% of the isolates were susceptible to ciprofloxacin and aztreonam. It may be inferred from these results that moderate numbers of isolates were resistant to cephalosporins, carbapenems, fluoroquinolones and aminoglycosides. However, a lower frequency to other groups of antibiotics has been recorded. Amutha et al., (2009) reported a high frequency of resistant strains to ampicillin (85%) followed by amikacin (62.2%), gentamicin (48%), imipenem (5%), meropenem (17%) and ciprofloxacin (50.9%). Dash et al., 2014 observed 77.7% strains resistant to ceftazidime, followed by cefepime (64.8%), piperacillin (45%), ciprofloxacin (38.9%), levofloxacin (36.1%), gentamicin (37.3%) and amikacin (30%). Similar resistance patterns of *P. aeruginosa* have been found by other workers in India (Javiya et al., 2008 and Prakash et al., 2012). A very high frequency (100%) of *P. aeruginosa* strains resistant to carbapenem and quinolones and 91% against penicillin/cephalosporins, 21% against aminoglycosides has been observed in US by Tam et al., (2010). However, these workers reported 97% strains susceptible to colistin. Similar observation has been made regarding the effectiveness of this drug by Timurkaynak et al., (2006) from Turkey. Gul et al., (2007) reported more than 90% strains sensitive to ciprofloxacin. However, this comparative evaluation between antimicrobials was impaired because there was variation in the choice of antibiotic tested in different studies.

The present study also aims at comparing the role of virulence factors of MDR and multidrug sensitive (MDS) *P. aeruginosa* isolates as the pathogenesis of *P. aeruginosa* infections depends upon several virulence factors of this organism. Several extracellular products are produced by *P. aeruginosa* after colonization of the organism which can lead to extensive tissue damage, bloodstream invasion and dissemination. The *in vitro* phenotypic expression of all the six virulence factors studied was demonstrable by majority of the isolates. Proteases are assumed to play a major role during acute *P. aeruginosa* infections. Proteases such as LasB elastase,
LasA elastase and alkaline protease are able to destroy the protein elastin. The later forms a bigger constituent of human lung tissue that is responsible for lung expansion and contraction. We observed protease production by 70% MDR isolates and 90% MDS isolates. Lipase is another virulence factor that was produced by 66.67% MDR and 56.67% MDS isolates. Biofilm formation is another protective mechanism of the organism which confers a mucoid consistency to *P. aeruginosa* isolates and acting as a protecting niche for the bacterium against the recognition of the immune system and the action of antibiotics. Biofilm is a layer of extracellular polymeric substances (EPS) which adheres to a solid surface. It is well established that the bacteria growing in biofilms are more resistant to antimicrobial agents than their planktonic counterparts. We observed the biofilm formation by all the MDR as well as MDS isolates excepting a very few MDS as non producers. Very low frequencies of 9.3% and 8% respectively of MDS and MDR have been reported as biofilm producers by some workers (Deptula and Gospodarek, 2010). In the present study, the frequencies of hemolysin and gelatinase were comparable in respect of MDS and MDR phenotypes. Stehling *et al.* (2008) compared the virulence traits of nonmucoid and mucoid isolates and did not observe any significant difference in the production of hemolysin and gelatinase. Jacome *et al.* (2012) observed 93.4%, 72.1%, 34.4% strains of *P. aeruginosa* having gelatinase, hemolysin and biofilm production activities respectively. In conclusion, we did not observe significant differences between MDR and MDS *P. aeruginosa* phenotypes with regard to virulence factors expressed by them.

The production of Metallo β-lactamase (MBL) is one of the important mechanisms of resistance exhibited by *P. aeruginosa*. Prevalence of MBL producing clinical isolates of *P. aeruginosa* have been continuously reported globally with increasing frequency over the past few years and the strains producing these enzymes have been responsible for prolonged nosocomial outbreaks followed by serious infections. MBL producing *P. aeruginosa* strains pose a therapeutic challenge, the mechanism and spread of such strains should be properly understood in order to achieve proper diagnosis and infection control management (Angadi *et al.*, 2012). In the present study, we observed 72.5% (29/40) MBL positive *P. aeruginosa* isolates which is even higher as compared to earlier study (46.55%) in this region (Chauhan and Sharma, 2013). Prevalence rates
ranging from 7-65% of MBL producing *P. aeruginosa* have been reported from different parts of India: Bangalore (12%) (Navneeth *et al.*, 2002), Kolkata (8.2%) (Mendiratta *et al.*, 2005), Chennai (14%) (Hemlatha *et al.*, 2005), Mumbai (20.8%) (Varaiya *et al.*, 2008), Nagpur (8.05%) (Aggarwal *et al.*, 2008), Maharashtra (11.4%) (Attal *et al.*, 2010).

Carbapenems are the only reliable antibiotics for the treatment of infections due to gram negative MDR strains particularly those which produce extended spectrum β-lactamases (ESBLs) and AmpC enzymes. Widespread use of these antibiotics increases the problem of resistance to this group of antibiotic. We observed, 33.89% isolates resistant to imipenem, 23.89% to ertapenem followed by meropenem (13.89%) and doripenem (6.67%), while lower rates 9% (Troillet *et al.*, 1997), 9.8% (Brown and Izundu, 2004), 9.9% (Raja and Singh, 2007) of resistance to imipenem have been reported by other groups. Higher frequencies have however been reported by other researchers: 32% (Nagaveni *et al.*, 2010), 71.4% (Murugan *et al.*, 2010), 59% (Prajapati *et al.*, 2011), 69% (Saderi *et al.*, 2008) and 55% (Awari and Nighute, 2012). Disconcordance between the combined disc diffusion test and E-strip test for screening MBL producers among the isolates resistant to imipenem or meropenem or both the antibiotics has been observed. 45% and 50% isolates respectively were confirmed as MBL producers by these tests. However, only 10% of the isolates were positive by both the tests (Table 4.6). The disconcordance may be due to the concentration of the antibiotic and inhibitor combination, particularly in the E-test there is antibiotic gradient along the strip. Further the screening agents in the two tests also vary (the screening agent in the combined disc test is imipenem whereas it is meropenem in E-strip test). Also, the phenotypic expression of MBLs depends upon the environmental conditions of the assay. In order to ascertain the role of MBL, it would be essential to characterize the isolates at genetic level to confirm the presence of MBL genes which has been undertaken in the present study. Other workers have reported the frequencies of 28.89% and 22.22% isolates of *P. aeruginosa* respectively for MBL production by combined disc test and MBL E-strip test (Patwardhan *et al.*, 2013).
We observed 48.14% (108/180) AmpC positive *P. aeruginosa* isolates among cefoxitin resistant isolates following the CLSI guidelines. Of these, 50/108 (46.29%) isolates were positive by disc antagonism test whereas 2/30 (6.66%) positive by E-strip test. The disc antagonism test thus proved more sensitive as compared to E-strip test. The sensitivity of the latter can be enhanced by increasing the concentration of the drug in the strip. Higher frequencies of AmpC producing *P. aeruginosa* (55.5%) from South India (Mohamudha *et al.*, 2010) and 59.4% from Uttar Pradesh (Upadhyay *et al.*, 2010) have been reported. However, other researchers from different parts of the country have reported lower frequencies: 20% from Aligarh (Shahid *et al.*, 2003), Kolkata (17.3%) (Arora and Bal, 2005), 22% from a tertiary care hospital in northern India (Bhattacharjee *et al.*, 2008) and Pondicherry (16.4%) (Umadevi *et al.*, 2011).

Organisms over expressing AmpC β-lactamases are a major clinical concern because the strains are usually resistant to β-lactam drugs except cefepime, ceftirome and carbapenems. Failure to detect AmpC β-lactamase producing strains has contributed to therapeutic failures and uncontrolled spread of such strains. It becomes therefore necessary to detect them as early as possible particularly in the hospital settings where antibiotic therapies are initiated immediately. The adoption of such practice would be helpful in deciding the appropriate therapy which in turn would prevent further spread of such strains.

Resistance of *P. aeruginosa* strains to carbapenems has emerged as an important problem worldwide since early 2000s and the number of bacterial species that produce metallo β-lactamases (MBLs) is continuously rising. There have been several reports from India and abroad on gram negative bacteria producing carbapenemases. The most common among them are imipenem hydrolysing enzyme (IMP), Verona integron encoded metallo β-lactamase (VIM), *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo betalactamase (NDM) encoded by beta lactamase genes; *blaIMP*, *blaVIM*, *blaKPC* and *blaNDM* genes, respectively.

In the present study, segments of selective β-lactamase genes (*blaNDM*, *blaGIM*, *blaFDC* and *blaCMY*) of the MBL and AmpC resistant isolates of *P. aeruginosa* were amplified in PCR assays. The primers were designed using Primer3Plus tool out of the reference sequences of *bla* genes downloaded from Gene Bank. The primer sequences of all the
bla genes matched with the respective gene precursors of reported P. aeruginosa strains by BLASTn analysis. Since the results of phenotypic tests are variable because of different environmental conditions of the tests and sometimes false positive results may be recorded. The determination of the prevalent genotypes would be helpful in studying the epidemiology of P. aeruginosa strains in this geographic region. Further, the genotypic methods are quite accurate and specific.

The New Delhi metallo β-lactamase (NDM-1) is a novel type of MBL named after the city of origin. It was first reported in 2009 in a Klebsiella isolate obtained from a Swedish patient who had been previously hospitalized in New Delhi. The blaNDM-1 gene is flanked by insertion sequence ISAbα125 and located on the transposon Tn125 and a truncated bleomycin resistance gene which regulates the RecA-dependent mutation rate, and therefore plays a role in the stabilization of blaNDM positive isolates. This blaNDM-1 region is a part of the variable region of a new complex class 1 integron bearing insertion sequence common region 1 (ISCR1) and localized in the bacterial chromosome. The transposon Tn125 carrying blaNDM has also been described in the bacterial chromosome, supporting the ability of this gene to move between both bacterial DNA molecules (Diene and Rolain, 2014; Janvier et al., 2013).

We recorded 11/29 (37.93%) isolates of P. aeruginosa positive for the blaNDM gene by PCR amplification and band size of 475bp was observed on agarose gel electrophoresis while remaining eighteen isolates were non-producers of NDM encoding genes. However, higher frequencies 70.96%, 53.4%, and 45.94% of this gene respectively have been reported from Puducherry (Bhaskar et al., 2013), Chandigarh (Mohan et al., 2015) and Bangalore (Anjana et al., 2014). Lower frequency (6.55%) of this gene has been reported from Chennai (Shanthi et al., 2014). The blaNDM gene has also been reported from other gram negative bacteria. Deshpande et al. (2010) have reported a frequency of 9/24 (37.5%) carbapenem resistant E. coli from a tertiary care unit in Mumbai. Samant et al. (2015) reported a high prevalence of blaNDM-1 in 67.3% MDRGNB (Multi Drug Resistant Gram Negative Bacteria) isolates. Maximum MDRGNBs were isolated from pus and urine samples in which P. aeruginosa and E. coli were the predominant NDM-1 producers. A high frequency of
\textit{bla\textsubscript{NDM-1}} in many genera and species of Gram-negative bacteria indicated that this gene can spread at a high rate (Shanthi \textit{et al.}, 2014).

The amplicons of \textit{bla\textsubscript{NDM}} gene were sequenced for their nucleotides and compared using BLAST\textsubscript{n} analysis with previously reported bacterial strains possessing this gene. The isolate PA126 showed 99\% homology with \textit{bla\textsubscript{NDM-1}} variant of different standard NCBI sequences of \textit{E. coli} strains V308 (LC095548.1), V266 (LC095524.1) and \textit{Pseudomonas spp.} strains NF81 (KP772171.1), NF117 (KP772196.1) (Fig-4.11 and 4.12). The isolate PaIg20 had 99\% homology with \textit{bla\textsubscript{NDM-7}} variant of different standard NCBI \textit{E. coli} strains V46 (LC095463.1), V5 (LC095457.1), V4 (LC095455.1) and V2 (LC095452.1) (Fig-4.13 and 4.14). However, isolate PaIg53 showed 99\% homology with different standard NCBI strains of \textit{E. coli} NF92 (KP772213.1), strain 15 (JQ348841.1), strain CR53 (KP826711.1) and \textit{Pseudomonas spp.} strain NF91 (KP772212.1) (Fig-4.15 and 4.16). However, this isolate could not be assigned to a particular variant type as it had homology to different variants of different bacterial spp. while the other two were identified as variants NDM-1 and NDM-7. The multiple alignment of the amplicons of the three isolates (PA126, PaIg20 and PaIg53) by CLUSTAL OMEGA analysis tool revealed a sequence homology ranging between 86\% to 98.77\% among the isolates (Fig-4.17 and 4.18). The ability of NDM producing \textit{P. aeruginosa} to survive under a wide range of environmental conditions and potential to spread in hospital settings make them unique. Though NDM is not as widespread as other MBLs such as IMP and VIM, strict awareness and continuous surveillance of NDM is crucial considering the difficulties in therapeutic management and control. Further studies are required at regional and national level in order to determine the frequency of NDM variants of \textit{P. aeruginosa}.

The GIM (German imipenemase) has been reported to occur in clinical isolates of Germany and recently it has been found to occur on a single isolate of \textit{Serratia marcescens} (Rieber \textit{et al.}, 2012). The \textit{bla\textsubscript{GIM-1}} gene cassette was found to be embedded in a class 1 integron structure, together with the ESBL gene \textit{bla\textsubscript{OXA-2}}, and the aminoglycoside resistance genes aacA4 and aadA1(Castanheira \textit{et al.}, 2004). We did not record the presence of \textit{bla\textsubscript{GIM}} in the 29 isolates tested. However, in a previous study from our laboratory did record 22.2\% isolates of \textit{P. aeruginosa} carrying \textit{bla\textsubscript{VIM}}.
gene (Chauhan & Sharma, 2015). Initially, MBL genes such as bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM} and bla_{SIM} were confined to their countries of origin, but with the passage of time incidence of VIM and IMP were detected worldwide, spreading from \textit{P. aeruginosa} to \textit{Enterobacteriaceae} (Bose et al., 2012). Ours is perhaps the first report of occurrence of bla_{NDM-1} and bla_{NDM-7} variants in this geographic region. Other β-lactamase genes may be present in the isolates that did not carry bla_{NDM} gene or alternatively other mechanisms of resistance might have been operating in these isolates such as hyperproduction of AmpC or other β-lactamases, porin defect or up-regulation of efflux pumps etc. In this study, however, the presence of these mechanisms was not evaluated.

AmpC β-lactamases are located on the chromosome of gram-negative bacteria viz. \textit{Serratia spp.}, \textit{Pseudomonas spp.}, \textit{Acinetobacter spp.}, \textit{Citrobacter spp.} and \textit{Enterobacter spp.} These genes may also be carried on the plasmids, as this gene is located on a transposon which is easily mobilized from plasmid to chromosome and vice-versa. The enzymes coded by these genes present a threat since these enzymes confer resistance to cephemycins (cefoxitin) and are not affected by β-lactamase inhibitors. Also, they can provide resistance to carbapenems in strains with loss of outer membrane porins. AmpC enzymes are inducible in many bacteria and can be expressed at high levels by mutation. Overexpression of such enzymes confers resistance to broad spectrum cephalosporins.

AmpC-type (ACT) variants derived from \textit{P. aeruginosa} are also known as \textit{Pseudomonas} derived cephalosporinase (PDC). Likewise ADC is named as Acinetobacter derived cephalosporinase (Rodriguez-Martinez et al., 2009). In the present study, a frequency of 11/52 (21.16%) AmpC producing \textit{P. aeruginosa} have been recorded by PCR amplification of AmpC-type gene (bla_{PDC}). The BLASTn analysis of the nucleotide sequences of bla_{PDC} gene amplicons of three isolates PA41, PaIg20 and PA91 were 99% homologous to the published sequences of \textit{P. aeruginosa} strains. The details of which are given under the results section. On alignment of the nucleotide sequences of the amplicons of the three isolates by CLUSTAL OMEGA analysis tool revealed a sequence homology ranging from 97.04% to 97.1% (Fig- 4.25 and 4.26)
The cephamycins (CMY) gene are other resistance genes which confer resistance to broad spectrum $\beta$-lactam antibiotics. Currently 43 CMY alleles of this gene are so far known of which CMY-2 is the commonest having the broadest geographic spread. CMY-1, 8, 9, 10, 11, and 19 are related to chromosomally determined AmpC enzymes, while the remainder are plasmid-mediated AmpC $\beta$-lactamases (Jacoby, 2009). We did not observe any $bla_{CMY}$ type in the present study. There is a possibility that other AmpC genes or $\beta$-lactamase genes could be present. However, the occurrence of AmpC-type genes in $P. aeruginosa$ strains have been reported with different frequencies from other parts of the country: Delhi (20%), Kolkata (17.3%) (Mohamudha et al., 2010). Other mechanisms of cefoxitin resistance in AmpC non-producers have been reported such as lack of permeation of porins or it may be due to production of carbapenemases (Mohamudha et al., 2010). However, some types of this resistance gene have been reported by other workers in India: In 1998, CMY-4 was reported from India from a strain of $K. pneumonia$ (Philippon et al., 2002) and CMY-6 type was reported from Uttar Pradesh in 2009 (Shahid et al., 2009). A low incidence of $bla_{CMY}$ gene was reported in 3/18 (16.6%) $P. aeruginosa$ isolates from Iraq (Al-Jubori et al., 2014).

In the present study, we did observe the co-occurrence of MBL and AmpC-type beta-lactamases in six isolates (PaIg53, PA41, PaIg20, PA126, PA141 and PaIg70) (Tables 4.13 & 4.14). However, the co-expression of these classes of genes was detected in a total of 14 (20.9%) isolates as detected by phenotypic tests (Table 4.12). Higher frequencies of co-producers of MBL and AmpC-type enzymes in $P. aeruginosa$ have been reported from Varanasi by Upadhyay et al., 2010. Higher frequencies have been reported from other countries as well: 43.1% from Egypt (Abd El-Baky et al., 2013) and 41.9% from Iran (Rafiee et al., 2014).

The coexistence of MBLs and AmpC $\beta$-lactamases suggests that these may be important contributing factors for carbapenem and cephalosporin resistance. It might be difficult to treat isolates having such combination because one is left with limited therapeutic options to treat infections caused by such strains. It is therefore, essential to look for these classes of genes on routine basis in the clinical laboratories.