5.0 DISCUSSION

Bacteria from the Enterobacteriaceae family, as well as some non-fermenting Gram-negative such as *Pseudomonas aeruginosa* and *Acinetobacter* species are the most important causes of infections associated with health care worldwide. Antibiotic resistance among these pathogens has increased dramatically in recent years and spread worldwide. It is no longer uncommon to encounter Gram-negative infections that are resistant to all available antimicrobials (Mehrad et al., 2015).

Many antibiotic resistance genes are transmissible within and across bacterial species. In addition, many of these genes are clustered together on plasmids or transposons, resulting in the recipient bacteria acquiring resistance to entire panels of related and unrelated antimicrobial agents in a single transfer event. New resistance mechanisms are also being discovered on a regular basis (Blair et al., 2014). Therefore it is important to recognize them expeditiously to implement effective control measures.

Carbapenem resistance in GNB

The overall carbapenem resistance rate among GNB in the present study was 11.9 %. The carbapenem resistance rate among hospital-isolates of GNB has varied widely in the literature from 7.87% (Datta et al., 2012), through 12.2% (Gladstone et al., 2005), 17.32% (Gupta et al., 2006), 29% (Saini et al., 2016), and even as high as 36.4% (Taneja et al., 2003).

Carbapenem-resistance rates among GNB in India has varied from as low as 1.8% to as high as 30%, in different centres (Behera et al., 2011; Goel et al., 2009).

Carbapenem resistance in Enterobacteriaceae

The prevalence of carbapenem resistance in Enterobacteriaceae in our hospital was found to be 4.4% (49/1154). CRE rates as a percentage of all Enterobacteriaceae, has varied greatly in reports from other parts of India depending, at least partly, on the place and time of study. For example, Gupta et al. reported CRE prevalence rates between 17 and 22% in 2006. Wattal et al. also reported rates of CRE prevalence increased (13-51%) in a tertiary hospital in Delhi in 2010. Datta et al. reported a CRE-prevalence rate of 7.87% in tertiary hospital in northern India in 2012, while Nair & Vaz reported a resistance rate of 29% in Mumbai in 2013. Thus, there has been a significant variation in CRE prevalence rates reported from different parts of India.

The most common CRE in our study was *K. pneumoniae* (17/49; 34.6%), followed by *E. coli* (15/49; 30.6%). Similar results have been reported by Gupta et al. in 2006, Guh et al. in 2015, and Rohey et al. in 2017; in all these studies *K. pneumoniae* and *E. coli* were the commonest species of CRE.

Chauhan et al. reported higher carbapenem-resistance rates in *Klebsiella* spp. (29.69%) than in *E. coli* (14.64%), in a study published in 2015. A higher prevalence of carbapenem-resistance in *Klebsiella* spp. (31-51%) than in *E. coli* (2-13%) has also been reported from Delhi by Wattal et al. in 2010.

Carbapenem resistance in *Acinetobacter calcoaceticus-baumannii* complex (ACBC)

Our study revealed a substantially higher rate of carbapenem-resistance of 70% (104/179) in ACBC compared to Enterobacteriaceae and *P. aeruginosa*. This is similar to the finding of Shivaprasad et al. who reported a carbapenem-resistance rate of 50.59 in

ACBC in 2014. Similarly high rates of carbapenem-resistance have been reported in another Indian study by Kumar et al. in 2011.

Studies from Iran and Taiwan have reported carbapenem-resistance rates as high as 62% and 91.7% in *Acinetobacter* (Fallah et al., 2014; Su et al., 2012). However, Soo Koo et al. reported a much lower carbapenem-resistance rate of 8.3% among *Acinetobacter* spp. in 2007. The latter finding was possibly due to the stringent implementation of antimicrobial stewardship and effective healthcare-associated infection control practices.

Carbapenem resistance in *Pseudomonas aeruginosa*

Our study detected a carbapenem-resistance rate of 20.6% among all clinical isolates of *P. aeruginosa*. This is very similar to the rate of 21% reported by Singh et al. in 2009, and roughly similar to the rate of 10.2% found by Lin et al. in Taiwan in 2014. In other Indian studies, the rates of carbapenem resistance in *P. aeruginosa* has varied from 10.9% to 69% in different centres at Pondicherry, Chandigarh, Bangalore, New Delhi, Mumbai and Vellore, as reported by Shashikala et al., 2006; Taneja et al., 2003; Navneeth et al., 2002; Behera et al., 2008.; Varaiya et al., 2008; and Kaul et al., 2007.

In a multicenter study, covering all of India in 2005-07, 42.6% of nosocomial P. aeruginosa isolates were resistant to carbapenems (Manoharan et al., 2010).

Minimum Inhibitory Concentration distribution in GNB

Carbapenem MICs of our resistant isolates were very high with mean values above 25 μg/ml for all organisms. Similar MIC ranges were also seen in the study done by Gaur et al. in 2008. In a study of A. baumannii isolates resistant to carbapenems from Khajuria et al. in the 2014, MIC values for imipenem and meropenem ranging from 16 to 64 mg / L. Rizek et al. in 2014 also noted high MIC values of imipenem (64 mg / ml and 256 mg / ml)

and meropenem (32 mg / ml and 256 mg / ml) in a study with carbapenem-resistant isolates of P. aeruginosa.

Colistin and Tigecycline resistance / sensitivity pattern

Susceptibility to tigecycline and colistin is retained by a majority of carbapenem-resistant GNB isolates; however, indiscriminate antibiotic use is leading to an increase of resistance rates against colistin and tigecycline too.

In our study, 12% and 14.2% of Enterobacteriaceae were resistant to tigecycline and colistin. The rate of resistance to tigecycline and colistin in ACBC in our study were 4.8% and 9.6% respectively. The resistance rate of *P. aeruginosa* to colistin was 9.6%; tigecycline was not tested as *P. aeruginosa* is intrinsically resistant to it.

Taneja et al. found 16% of their ACBC isolates to be carbapenem resistant in a study from northern India in 2011; most of these strains were resistant to colistin too. Wattal et al. noted 8% Pseudomonas spp. were to be resistant from a tertiary hospital in North India against colistin in ICU samples in 2010. Sweih et al. found resistance rates of 13.6% and 12% were towards tigecycline and colistin respectively in ACBC isolates in Kuwait in 2011.

However, a recent study by the United States by Lesho et al. in 2013 showing 14 of 28 isolates were colistin resistant. This is due to increased MDR Acinetobacter where in, colistin is the only choice for treatment. Therefore, even resistance to it incurred. Antibiotic resistance reflects the policy of antimicrobial use and circulation of drug-resistant clones in different countries

In the present study, the senstivity was 89 and 83% when compared to tigecycline and colistin in Enterobacteriaceae. Wesam, 2015 also reported 80.9% of Enterobacteriaceae isolates sensitive to colistin. The continued use of polymyxin,

especially as a selective digestive decontaminant resulted in the development of secondary resistance to colistin especially among Enterobacteriaceae (Lübbert et al., 2015). There are studies in different areas reported the resistance to this life saving antibiotic especially among Enterobacteriaceae (Chen et al., 2011; Garbati et al., 2013).

Susceptibility to tigecycline was 95 % in our study. Padmalakshmi et al. also reported 95.2% susceptibility in their study isolates in 2015.

Discrepancy in Results with Discs from Different Manufacturer

Antimicrobial susceptibility testing (AST) methodology has continuously developed throughout the antibiotic era, and this is true for the primary screening of carbapenem resistance too (Dalhoff et al., 2009). However, maintaining acceptable accuracy and precision continues to be a challenge.

In our study we used antimicrobial discs from two different manufacturers namely HiMedia and Rosco Diagnostica, while Etest strips were procured from bioMérieux for the confirmation. Categorical sensitivity results obtained with discs from Rosco Diagnostica showed complete agreement with sensitivity calculated from MICs obtained with bioMérieux Etest strips on the basis of CLSI 2015 criteria. However, 10.9% of the isolates, all of which incidentally belonged to family Enterobacteriaceae, were falsely categorised as carbapenem resistant when HiMedia discs were used but no such difference was found in *Acinetobacter calcoaceticus baumannii* complex and *P. aeruginosa.*

In a Philadelphia study, which has Kirby Bauer's disc diffusion compared to Agar dilution method for the imipenem resistance assay in P. aeruginosa, the agar dilution results and Kirby Bauer disc diffusion method showed a strong correlation with very few false susceptibilities (Fekete et al., 1994). In a recent study by Sinha et al. al., 2007, about 85% of the original as resistant meropenem isolates reported by the Kirby method was

subsequently found to meropenem MICs in sensitive area, thus improving the reliability of the disc diffusion method is questioned but, they used only 21 isolates to compare Kirby Bauer diffusions- and agar dilution methods what could have been responsible for the high error rates.

In another similar study by Joseph et al. in 2011, noted that the good performance of Kirby Bauer's diffusion method in testing the meropenem resistance in Acinetobacter spp. As there was no huge error and only 1.8% large errors noted in A. lwoffii, which was well within the acceptable range.

But we have not found any recent study which has focused on comparison of Kirby Bauer disc diffusion method using discs from different manufacturer with other reference methods. So we recommend the laboratory should perform one alternate primary screening test especially for carbapenem antibiotics according to their capabilities.

Phenotypic characterization

The Modified Hodge Test (MHT) is most commonly used for detecting carbapenemases in Enterobacteriaceae. However, only 28.5% of our carbapenem-resistant Enterobacteriaceae isolates were positive on MHT (using ertapenem as the indicator), even though all strains carried at least one carbapenemase (blaNDM-1) gene, the action of which was potentiated in anticipation by the addition of zinc sulphate in the testing medium (Mueller-Hinton II Agar; Becton-Dickinson, USA).

MHT (using imipenem instead of the usual ertapenem), had an even poorer sensitivity when applied to carbapenem-resistant strains of *P. aeruginosa* and ACBC and 3.2% and 6.7% respectively.

In a study by Castanheira et al. published in 2011, 73.3% (11/15) of NDM-1 producing isolates were negative or weakly positive for MHT. MHT may therefore be

unsatisfactory only for the detection of blaNDM-1 carbapenemase. This is possibly one of the reasons for the low positivity of our isolates on MHT, as all produced blaNDM-1.

This very low sensitivity of MHT in the detection of NDM is also mentioned in the most recent guidelines of the Clinical Institute and Laboratory Standards (NCCLS). (CLSI M100-23). A weak positive and false negative results for Enterobacteriaceae especially blaNDM -1 production also reported by Birgy et al. in 2011.

However, according to Thomson, 2010, the MHT does not have the ability to differentiate between carbapenemase types. Furthermore, Ribeiro et al. in 2014 explained that even though the MHT has >90% sensitivity, it was difficult to interpret and is prone to subjectivity. This was evident in the present study, since all test isolates that produced even the slightest indentation was taken as positive. However, according to Centre for Disease Control and Prevention (CDC), some isolates may produce a slight indentation but still lack carbapenemases.

In the literature, the specificity of the modified Hodge test differs because some of the authors analyzed data for different groups of bacteria including non-fermenters and Enterobacteriaceae, and blaNDM-1 was detected with other carbapenemases (Girlisch et al. 2012 a).

The other reasons may be the killing of the *E. coli* lawn by many *P. aeruginosa* test isolates, resulting in the common occurrence of indeterminate results that introduce a strong subjective element in the interpretation of tests (Pasteran et al., 2011).

Girlich et al. in 2011 reported Class A and class D carbapenemase producers were detected by the MHT. False-negative results were obtained for 7 out of 14 blaNDM-1 producing Enterobacteriaceae.

According to our results, it is obvious that the detection of these threats through MHT is problematic (ie false-negative results may occur), delay the implementation of infection control measures that resulting in continued propagation of strains.

KPC/MBL detection kit

A large variety of carbapenem hydrolyzing enzymes has been identified in Gram-negative bacilli (Canton et al., 2012). Out of these, NDM, VIM, OXA-48, KPC have been reported the most often and still continue to spread (Potron et al., 2013).

Inhibition assays using specific inhibitors allows discrimination among different types of carbapenemases. This may be recommended for routine diagnostic applications due to its low cost, reliability and good discriminatory potential. The Rosco KPC / MBL kit is one commercial identification assistance assay that has the combination of meropenem and three inhibitors (boronic acid, dipicolinic acid and cloxacillin) for three variants of carbapenemases.

The KPC/MBL kit was used to test our 49 CRE isolates. The kit identified 42 (85.7%) of these strains as MBL-positive, even though all carried the blaNDM-1 gene. This finding is comparable to that of Jeremiah et al. who reported in 2014 an almost identical sensitivity of 80% for detecting MBL in their CRE isolates, all of which were positive for blaNDM-1. The same kit yielded a sensitivity of 91% for MBL detection in the hands of Creighton & Jayawardane in 2015, while Doyle et al. had reported 100% sensitivity and specificity for NDM-producers earlier in 2012. A similar pattern has been reported by Pantel et al. too in 2015.

The KPC/MBL kit identified two (4%) of our isolates as positive for both blaKPC and MBL. However, molecular testing could not detect the blaKPC gene in these isolates, all of which were positive for the metallo-beta-lactamase, blaNDM-1. This is similar to the

finding of Creighton & Jayawardane who reported blaNDM producers misclassified as KPC/NDM co-producers by this kit in 2015. Doyle et al. also reported 33% of blaNDM producers getting identified as being positive for both blaNDM and blaKPC in 2012.

The overall usefulness of the KPC/MBL kit was reduced as this kit format does not allow identification of blaOXA-producers, which are increasing in importance as mediators of carbapenem resistance. In these circumstances, temocillin may be used as a substitute marker for similar blaOXA carbapenemases as high resistance to temocillin is a exclusive feature of blaOXA-like carbapenemase producing bacteria. Based on this property, manufacturers have now added a fifth temocillin tablet to the existing package. This new package is now able to identify all types of carbapenemases immediately.

Commercial kits

As carbapenemase-positive GNB are increasingly reported worldwide, there is a need to detect them by simple and rapid means. Rosco Diagnostica has developed two such kits, i.e., the Rapid CARB Blue and Neo-Rapid CARB, based on the colour change of pH indicators consequent to acidification of the test medium by hydrolysis of the β-lactam ring.

In our hands, the Rapid CARB Blue Kit showed a low sensitivity overall, with the highest figure seen in Enterobacteriaceae (79.5%) followed by 41.9% and 41.3% in *P. aeruginosa* and ACBC respectively. Novais et al. also reported a lower sensitivity of the test for *P. aeruginosa* and *A. baumannii* in 2015. Similar results were reported by Huang et al. in 2014 and Gallagher et al. in 2015. Novais et al. attributed the lower sensitivity of the kit to the smaller inocula used, and / or to the instability of imipenem in tablets in their study reported in 2015. In addition, they found that the tablets were hard to emulsify, and that the white, limestone tablet material, cloud the tube and may lighten the reaction color

when the tubes were disturbed during incubation or reading, making it difficult to read the results. A similar phenomenon was observed by Gallagher et al. in 2015.

A modified form of this kit named the Neo rapid CARB kit was therefore developed by incorporating three changes as follow: 1) Increasing the quantity of imipenem in the tablets, 2) Changing the pH indicator from bromothymol blue to phenol red, and 3) Using a larger test inoculum, to get over the problems associated with the earlier version of the kit. On using this modified kit, our results showed a modest increase in sensitivity for carbapenemase detection in *P. aeruginosa* from 41.9% to 51.6%.

AbdelGhani et al. tested 189 isolates consisted of Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* with the new kit and reported an overall sensitivity of 98% in 2015. Huber et al. in their study of 2016, interpreted Neo-Rapid CARB kit results by colorimetry, and achieved a sensitivity of 98% in Enterobacteriaceae.

'In-House' or improvised colorimetric tests

Rapid existing colorimetric tests for carbapenemases (Carba NP, Blue Carba & CarbAcineto NP) were placed in their original and modified forms in Enterobacteriaceae, P. aeruginosa and Acinetobacter spp. in the present study.

In spite of its ease of use, the Carba NP was not found to be sufficiently sensitive for *Acinetobacter* spp., so a modified form called the CarbAcineto NP test was proposed by Dortet et al. in 2014. The bacterial inoculum used in the CarbAcineto NP was doubled compared to that in the Carba NP test, leading to an increased amount of enzyme released in the revealing solution. Secondly, the lysis buffer used for the Carba NP test was replaced by a hyper-osmotic solution of 5 M NaCl which does not interfere with the slight pH changes seen in organisms producing small amounts of carbapenemases.

Continuing with the aim of simplifying the original test, Pires et al. proposed the Blue Carba test for Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* in 2013. Blue carba was validated for the detection of the production of carbapenemases directly from primary colonies in sample dish. In the Blue-carba-test version, bromothymol blue as indicator was used as its pH range of 6.0 to 7.6 covers the pH 6.8, the optimal pH for most β-lactamases.

Several past studies have tested the Carba NP, CarbAcineto NP, Blue Carba tests individually (Dortet et al., 2014; Vasoo et al., 2013; Rao et al., 2016); however, ours is the first to compare the Carba NP test directly with the Blue Carba test in Enterobacteriaceae and *Pseudomonas aeruginosa*, and the CarbAcineto NP test with the Blue Carba test in *Acinetobacter calcoaceticus baumannii* complex. It is noteworthy that the Blue Carba test did not perform better than the older tests in our hands, despite claims of its being an improvement over the Carba NP and CarbAcineto NP tests.

The sensitivity of the Carba NP and Blue Carba tests in our hands was 91.8% (45/49) in Enterobacteriaceae; this is in tune with other studies. The same tests were significantly less sensitive (61.2%; 19/31) in *P. aeruginosa*. This is similar to the rate of 37 % reported by Pragasam et al. in 2016, and is also supported by the CLSI guideline (CLSI M100-S25) which mentions a lower sensitivity of the Carba NP test in *P. aeruginosa*.

The CarbAcineto NP and Blue Carba tests had an identical sensitivity of 84.6% (88/104) for detecting carbapenemase enzymes in ACBC strains, yielding positive results in the same strains in all cases. This is similar to the finding of Vijayakumar et al. who reported 91% positivity by CarbAcineto NP test in 2016.

Substituting analytical reagent grade imipenem monohydrate with pharmaceutical grade imipenem-cilastatin made no difference to test performance, provided the

concentration of imipenem was doubled when using imipenem-cilastatin. Similar results were reported by Hartl et al. in 2017.

Harvesting the adjacent bacterial mass near toi imipenem neo sensitabs improved the performance of the carba NP and blue carba by 6.25% in Enterobacteriaceae. We are the first study report an improvement in test performance on enzyme induction by substrate exposure. Interestingly, the same effect was not observed in ACBC or Pseudomonas aeruginosa.

A limitation of all the 'in house' tests is that the revealing solution containing pH indicator and imipenem, has to be prepared every time just before testing to avoid the spontaneous degradation of the antibiotic In addition, the colour change of red to orange-yellow is very subtle and its interpretation is often observer-dependent. These factors should be taken into consideration when interpreting test results.

Carbapenem inactivation method (CIM)

Carbapenem inactivation methods (CIM) are promising because they do not require special material inputs, and are therefore useful in small laboratories with limited budgets. They also allow the distinction between the two important mechanisms of carbapenem-resistance, namely β-lactamase activity and reduced permeability.

Overall, 73 (70.1%) *Acinetobacter calcoaceticus baumannii*. 15 (48.3%) *P. aeruginosa* and 38 (77.5%) Enterobacteriaceae isolates showed carbapenemase activity in our study. All isolates positive on CIM were positive for at least one carbapenemase gene on testing by PCR. However, we did not find 100% sensitivity as claimed by van der Zwaluw et al., in 2015. False-negative results were obtained especially with blaNDM-1 producing strains. A study from Turkey reported a significantly higher sensitivity of 100%; it must, however, be noted that only one of their study strains were blaNDM-1 positive

(Bayramoglu et al., 2016). The study of Song et al., in 2016 found the sensitivity of CIM to be 95.8% and 100% in Enterobacteriaceae and *P. aeruginosa* respectively; but, his study did not cover *Acinetobacter* spp.

Carbapenem-inactivation methods proved to be neither rapid (results were available only the next day) nor user-friendly as the test required the handling of highly concentrated bacterial suspensions and the manipulation of antibiotic discs.

Carbapenemases genes

All our isolates, which included *Acinetobacter calcoaceticus baumannii* complex, *P. aeruginosa*, and different species of family Enterobacteriaceae, were positive for the blaNDM-1 gene. In a study by Bashir et al. from 2014, nine of the 15 metallo β-lactamase carrying isolates were found to be NDM-1 producers. The latter strains included *Citrobacter freundii* (3), *Klebsiella pneumoniae* (2), *Escherichia coli* (2), *Pseudomonas aeruginosa* (1) and *Acinetobacter* spp. (1). This highlights the tremendous potential of the *bla*NDM-1 gene for dissemination.

The prevalence of blaNDM-1 producers among carbapenem-resistant Enterobacteriaceae isolates in different centres in India has ranged between 31.2% and 91.6%. (Nagaraj et al., 2012; Seema et al., 2012; Lascols et al., 2011; Castanheira et al., 2011; Deshpande et al., 2010; Kumarasamy et al., 2010). Shanti et al. found the NDM gene to be the most prevalent, being detected in 57.65% of all Enterobacteriaceae isolates in 2014. Another Indian report by Deshpande et al. detected the NDM-1 gene in 22 out of 24 carbapenem-resistant isolates in 2010. The 22 NDM-1 positive organisms included *Klebsiella* spp. (10), *E. coli* (09), *Enterobacter* spp. (02) and *Morganella morganii* (01). One another study from 2014 reported the presence of bla_{NDM-1} in all (13) carbapenem-resistant *K. pneumoniae* isolates in Sharjah, UAE, where many patients travel frequently to

South Asia (Dash et al., 2014). Sultan et al. reported blaNDM-1 positivity in 93% of their carbapenem-resistant isolates from neighbouring Pakistan too in 2013. However, the prevalence of the NDM gene has been significantly lower in the north-eastern region of India at 8.7% in *K. pneumoniae* and 5.2% in *E. coli* (Bora et al., 2013).

As of now, NDM producing Enterobacteriaceae have been reported from geographically diverse regions of the globe (Struelens et al., 2010; Johnson et al. 2013). Countries reporting NDM include Australia (Poirel et al., 2010), Canada (Kus et al. 2011), China (Ho et al., 2012), France (Arpin et al., 2012), Guatemala (Pasteran et al., 2012), Oman (Poirel et al., 2011, Dortet et al., 2012 b), Kenya (Poirel et al., 2011 a), Kuwait (Jamal et al., 2012), South Africa (Brink et al., 2012), Spain (Sole et al., 2011), South Korea (Kim et al., 2012) and Thailand (Rimrang et al., 2012).

In a study by Shanti et al. in 2014, only four out of 61 carbapenem-resistant strains of *P. aeruginosa* were found to harbour the blaNDM-1 gene. *Pseudomonas aeruginosa* positive for blaNDM-1 have been reported from Serbia too; interestingly none of the source patients had a history of travel to the Indian subcontinent (Jovcic et al., 2011, Flateau et al., 2012). *P. aeruginosa* positive for blaNDM-1 have recently been reported from India as well (Khajuria et al., 2013).

blaNDM-1 production in *A. baumannii* has serious implications since it is an important nosocomial pathogen. blaNDM-1 positive *Acinetobacter* species has been reported from Pune (Bharadwaj et al., 2012), Chennai (Karthikeyan et al., 2010) and very recently from CMC, Vellore too (Pragasam et al., 2016).

In a study of China blaNDM-1 gene was observed in only four of 2109 (0.18%),the isolates of Acinetobacter spp. (Chen et al., 2011) *A. baumannii* isolates expressing blaNDM-1 MBL have been isolated in Germany and Serbia too (Pfeifer et al., 2011, Poirel et al., 2012). 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).

VIM-type carbapenemases

In our study only one Enterobacteriaceae isolate (*Enterobacter cloacae*) was found to have the VIM gene. There are very few reports of VIM MBLs in Enterobacteriaceae from India (Shahid et al., 2012; Nagaraj et al., 2012). Dwivedi et al. reported the presence of VIM genes in 12 isolates obtained from patients with ventilator associated pneumonia and also concurrent occurrence of multiple MBL genes in a single isolate in 2009.

In our study, 20 strains of *P. aeruginosa* were positive for blaVIM genes. The prevalence of this carbapenemase in India has ranged from 7% to 65% among carbapenem-resistant *P. aeruginosa* (Arunagiri et al., 2012; Manoharan et al., 2010). In one study, the rate of MBL production was 24.5% among 61 *P. aeruginosa* isolates, and blaVIM type was the commonest (Manoharan et al., 2010). Another study from India also reported blaVIM-2 from *P. aeruginosa* (Toleman et al., 2007). In a national survey, conducted to characterize 301 MBL producing Pseudomonas species from 10 medical centers in India, MBL genes were detected in 18.9% of isolates and 5 VIM variants were reported with VIM-2 the most common.

The others were VIM-6, VIM-11, VIM-5 and VIM-18 (Castanheira et al., 2009). Khorvash et al. reported a blaVIM gene prevalence of 14.6% in carbapenem-resistant *P. aeruginosa* in 2015. In another study by Rajabnia et al., 18% of carbapenem-resistant *P. aeruginosa* isolates carried a VIM-1 gene in 2015.

In our study three ACBC isolates were found to carry VIM genes. Nordmann & Poirel reported in 2008 that VIM enzymes were not common in *A. baumannii*. Kock et al. reported a similar situation with only one strain of VIM-positive ACBC among 97 carbapenem-resistant isolates tested in 2015. However, VIM was found to be much

commoner in two other studies from Greece and Korea where it was the commonest MBL gene (Tsakris et al., 2006; Yum et al., 2002).

Purohit et al. found the blaVIM gene in 7 (16.28%) of 43 carbapenem-resistant ACBC isolates in 2012. El-Agerry et al. screened 48 carbapenem-resistant *A. baumannii* isolates for MBL production by Etest in a study published in 2014. Thirteen out of 48 isolates were phenotypically positive for MBL, and 8 of these were found to be positive for the blaVIM-1 gene on PCR. Interestingly, the blaVIM-1 gene was also found in five out of 35 isolates that were MBL-negative on phenotypic testing.

OXA-48

The blaOXA-48 gene was found only in *K. pneumoniae* isolates in our study. This corresponds to the findings of Sharma et al., 2016 who reported 32 % of *K. pneumoniae* isolates positive for the blaOXA-48 genes. This data also corresponds to the findings of a study done in Riyadh by Shibl et al. who studied 60 carbapenem-resistant isolates of *K. pneumoniae* and detected the blaOXA-48 gene in 47 of these in 2013.

KPC-type Carbapenemase

None of our study isolates were found to carry the blaKPC gene. The SMART study and SENTRY study did not find blaKPC in their Indian isolates either (Castanheira et al., 2011; Lascols et al., 2011). More recently, researchers from Varanasi did find blaKPC in three Enterobacteriaceae isolates, of which two were *E. coli* and one was *K. pneumoniae* (Upadhyay et al., 2012). Reports of KPC production in *Pseudomonas* and *Acinetobacter* is also scarce (Rasmussen et al., 2007, Robledo et al., 2011). In contrast, Nordmann et al. observed that KPC made the greatest contribution to carbapenem resistance, especially in sporadic outbreaks, in their review article in 2014.

Efflux pump detection

The ability of pathogenic bacteria to prevent the accumulation of drugs inside the cytoplasm with the help of membrane embedded efflux pumps, is now well recognized as a significant mechanism for antibiotic resistance.

These efflux pumps often have wide substrate ranges that recognition of their activity is through its effect on the concentration of the various compounds (Webber & Coldham, 2010). Several methods have been published to assess efflux in living cells in a dynamic way making use of fluorescent probes such as Ethidium bromide or Hoechst-33342 (bisbenzimide) or lipophilic dyes such as Nile Red (Amaral et al., 2011; Bohnert et al., 2010; Viveiros et al., 2010).

In our study we used ethidium bromide as an indicator of efflux activity. The principle of this assay is the passage of ethidium bromide across the cytoplasmic membrane and its subsequent accumulation inside bacterial cells. Ethidium bromide traverses the bacterial cell wall, and accumulates there to the point of producing fluorescence visible to the naked eye when excited by ultraviolet (UV) light. Most efflux pumps for resistant bacteria may extrude ethidium bromide from the bacterial cytoplasm, thus reducing its concentration in the bacterial cytoplasm to the point, to make detectable fluorescence (Martinsa et al. 2013)

In our study, the ethidium bromide test was positive in 14.67% (27/184) isolates, as indicated by the lack of fluorescence under UV light. Suresh et al. reported 20% (25/123) positivity by this method in Gram-negative MDR strains in 2016.

All isolates were also tested for the contribution of efflux pump to their carbapenem resistance, as determined by a decrease in their carbapenem MIC in the presence of an efflux pump inhibitor. In this study we used reserpine as the efflux pump inhibitor and meropenem as the pump substrate. Only 20 (10.8%) of our 184 isolates

showed a decrease in the MIC of meropenem in the presence of reserpine. This percentage has varied among different studies from 20% (Saxena et al., 2015) through 22% (Sinha et al., 2007) to 36% (Belgode et al., 2016).

Reserpine does not inhibit all types of efflux pumps. Therefore it is possible that more strains may have turned out to possess functioning efflux pumps if other inhibitor has been used. Of course it is also possible that the other strains truly lacked functioning efflux pumps.

Limitations of the study

- Not all carbapenemase genes could be looked for because of financial limitations. The genomic environment of the carbapenemase genes, e.g., their location in chromosomal DNA, plasmids or transposons were not studied, again because of resource limitations.
- The role of overproduction of AmpC-type beta-lactamases in carbapenemases was not explored and also the role of porin loss in carbapenem-resistance was not investigated at all, again because of resource constraints.
- Along with phenotypic tests, efflux pumps should have been detected by genotypic methods because not all efflux pumps are detectable with chromogenic substrates or pump inhibitors.

Future research areas

- Carbapenemase gene detection by reverse-transcription PCR for mRNA, for the definitive proof of gene expression.
- Genomic environment of the carbapenemase genes, e.g., their location in chromosomal DNA, plasmids or transposons
- Role of overproduction of AmpC-type β-lactamases in carbapenemases and also the role of porin loss in carbapenem-resistance
- Molecular characterization of efflux pump and attempts of developing efflux inhibitors as possible development of new agents

6.0 SUMMARY

A total of 1544 isolates of Gram negative bacilli were studied, of which 184 were resistant to one or more carbapenem drugs. *Acinetobacter calcoaceticus baumannii* complex (104 isolates; 52%) was the dominant species, followed by *Pseudomonas aeruginosa* (31 isolates; 15.5%). Among the 49 isolates of Enterobacteriaceae, *K. pneumoniae* was the commonest with 17 (34.6 %) isolates, followed by *E. coli* with 15 (30.6 %) isolates.

Most carbapenem-resistant GNB came from the intensive care units and the neurosurgery ward. The largest numbers of carbapenem-resistant isolates were isolated from pus, urine, and endotracheal tube aspirates.

Carbapenem-resistant isolates were generally also resistant to most other antimicrobials except tigecycline and colistin.

False-positive carbapenem-resistance

Six (10.9%) Enterobacteriaceae isolates appeared to be carbapenem-resistant upon testing with the Kirby-Bauer disc diffusion technique with discs procured from HiMedia. These isolates later turned out to be sensitive when retested with the Kirby-Bauer disc diffusion technique with discs procured from Rosco Diagnostica, Denmark, and also by the Etest method, resulting in a false-positivity rate of 10.9% for disc diffusion.

In addition, four isolates of *Stenotrophomonas maltophilia* and six isolates of *Elizabethkingia meningoseptica* had to be left out of the study because they are intrinsically resistant to carbapenem.

Minimum Inhibitory Concentration

The Etest method for minimum inhibitory concentration (MIC) showed that the carbapenem MICs of most resistant isolates were ≥32 μg/ml.

The remaining 184 isolates were then tested for carbapenemase enzymes and efflux pumps by phenotypic and genotypic methods.

PHENOTYPIC METHODS

Modified Hodge Test (MHT)

Of the 184 isolates tested by MHT, only 22 tested positive.

Rosco KPC and MBL detection kit

Of the 49 Enterobacteriaceae isolates, 40 were MBL positive, 2 were both MBL and KPC positive and the remainder did not produce any of the enzymes and were presumed to be resistant by other mechanisms.

Rosco Rapid CARB Blue kit

43 (41.3%) *Acinetobacte*r, 13 (41.9%) *P. aeruginosa* & 39 (79.5%) Enterobacteriaceae isolates showed the presence of carbapenemases by this kit.

Rosco Neo Rapid CARB kit

The Enterobacteriaceae and *Pseudomonas* isolates were then tested by Rosco neo rapid CARB kit. 40 (81.6%) *Enterobacteriaceae* isolates and 16 (51.6 %) *P. aeruginosa* isolates were tested positive as carbapenemase producers

Carba NP test, CarbAcineto NP and Blue Carba tests with modifications

Overall 45 (91.8%) Enterobacteriaceae and 19 (61.2%) *P. aeruginosa* isolates were found to be positive for carbapenemase production by Carba NP and Blue Carba tests

Overall 84.6% *Acinetobacter calcoaceticus baumannii* were positive by CarbAcineto NP and Blue Carba test for the carbapenemase detection**.**

In terms of modifications, no difference in results was found regarding the source of imipenem while in three (6.25 %) Enterobacteriaceae isolates carbapenemase production was found to be inducible by imipenem

Carbapenem inactivation method (CIM)

Of the resistant isolates, 73 (70.1%) *Acinetobacter* spp., 15 (48.3%) *P. aeruginosa* and 38 (77.5%) Enterobacteriaceae isolates were tested positive for carbapenemase activity by this test.

GENOTYPIC METHODS

Detection of different carbapenemase genes

Majority of the isolates showed the presence of NDM-1 carbapenemase gene. In addition to NDM-1gene, the isolates also showed the presence of VIM and OXA-48 genes. Majority (83.3 %) of the VIM positive were *P. aeruginosa*.

OXA-48 was detected in only *K. pneumoniae* isolates. None of the study isolate found to be positive for KPC gene.

EFFLUX PUMP ACTIVITY

The presence of efflux was detected by two methods namely ethidium bromide cartwheel method and agar dilution method using reserpine as efflux inhibitor

Ethidium bromide cartwheel method

On performing the efflux protocol 27 isolates were found to be positive. i.e. (the strains showing little or no fluorescens under UV light)

Reserpine as efflux pump inhibitor

Only 20 isolates showed a decrease in the MIC of meropenem when reserpine was added.