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Antibiotic susceptibility pattern in Proteeae with special pattern of Amp-C β- lactamase

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Abstract

Amp C β -lactamases are reported from Enterobacteriaceae with increasing frequency. However, unlike Extended-spectrum β-lactamases (ESBLs), no screening and confirmatory tests have been uniformly established for strains that produce Amp C β -lactamases. They may not necessarily confer resistance to broad-spectrum cephalosporins when conventional CLSI breakpoints are used, although they may meet screening criteria for ESBLs. They typically have a negative confirmatory test for ESBLs and therefore laboratories may report Amp C producers as susceptible to broad-spectrum cephalosporins. This may have disastrous consequences if physicians erroneously use broad-spectrum cephalosporins to treat serious infections. Fortunately, several simple detection methods of class C β -lactamases have been reported recently. When ESBL production is suspected but the confirmatory tests are negative, the strains should be screened for the presence of class C β –lactamase. Reduced susceptibility to cefoxitin is a sensitive but not specific indicator of class C β -lactamase production. The 'AmpC disk test' may be incorporated into ESBL detection algorithms. The AmpC disk test was an easier, reliable and rapid method of detection of isolates that harbour AmpC β -lactamases. This suggests that AmpC disk test can be used for routine screening of the Amp C enzyme in the clinical laboratory.

Keywords: Amp C β -lactamases, extended spectrum beta lactamase (ESBL), Mueller Hinton Agar, Antibiogram.

Introduction

The genera Proteus, Providencia, and Morganella are related members of Family Enterobacteriaceae falling under the Tribe Proteeae. There are several species of Proteus but *Proteus mirabilis* and *Proteus vulgaris* account for vast majority of infections. Proteus species are common causes of Urinary tract infections and frequently isolated from wounds and rarely from blood stream infections. *Proteus mirabilis* strains are usually straightforward because most are sensitive to commonly used antibiotics except tetracycline whereas strains of *Proteus vulgaris* are generally more resistant (Michael S.Donnenberg.2005).

Providencia stuartii is the most common species of its genus isolated from clinical specimens but *Providencia rettgeri* is occasionally grown. It is a rather uncommon clinical isolate except from UTI. *Providencia rettgeri* & *Providencia stuartii* are resistant to multiple antibiotics including gentamicin, first generation cephalosporins and ampicillin (Michael S.Donnenberg.2005).

Morganella morganii is an infrequent nosocomial isolate usually from wounds or urine. *Morganella morganii* possess inducible Amp C β-lactamases and therefore intrinsically resistant to first

generation cephalosporins and ampicillin (Michael S.Donnenberg.2005).

These organisms are most commonly found in the human intestinal tract as part of normal human intestinal flora, along with *Escherichia coli* and *Klebsiella* species. They are also found in multiple environmental habitats, including long-term care facilities and hospitals. In hospital settings, it is not unusual for gram-negative bacilli to colonize both the skin and oral mucosa of both patients and hospital personnel. Infection primarily occurs from these reservoirs. However, *Proteus* species are not the most common cause of nosocomial infections.

Antibiotic resistance is increasing worldwide in both outpatients as well as hospitalized patients. Understanding the impact of drug resistance is of critical importance as the changing rate of antibiotic resistance has a large impact on the empirical therapy of infections.

The various mechanisms of drug resistance in Gram-negative bacilli include extended spectrum beta lactamase (ESBL) production, AmpC β-lactamase production, efflux mechanisms and porin deficiency. Amongst the mechanisms of resistance to third generation cephalosporins, production of ESBLs and AmpC β -lactamases are the most com mon. AmpC β -lactamases are clinically important because they confer resistance to narrow-, expanded-, and broad-spectrum cephalosporins, βlactam- β-lactamase inhibitor combinations and aztreonam. (Bauernfeind A *et al.,* 1998)

Many clinical laboratories currently test for production of ESBLs but do not attempt to detect plasmid mediated AmpC β -lactamases. These enzymes are typically associated with multiple antibiotic resistances, leaving a few therapeutic options. Since both ESBL and AmpC β-lactamase are encoded on plasmids and confer a selective advantage to strains harbouring these in a hospital setting.

It is important to know the occurrence of ESBL and Amp C β -lactamases producing strains as well as their antibiotic susceptibilities to newer agents to guide empirical therapy for various infections. Although reported with increasing frequency, the true rate of occurrence of AmpC β -lactamases in different organisms, including members of Enterobacteriaceae, remains unknown.

The purpose of the present study was designed to provide a current scenario of Tribe Proteeae, its distribution in clinical specimens, the current antibiotic susceptibility pattern with an emphasis to increase awareness and demonstrate the need to detect the occurrence of Amp C enzymes.

Materials and Methods

The clinical material for the study was obtained from Sri Ramachandra Medical Centre and hospital, a tertiary care centre at Porur. The samples were obtained from patients admitted in both medical and surgical wards of various specialties like Medicine, Surgery Obstetrics, Gynaecology, Urology, Nephrology and Orthopaedics etc., and Intensive Care Units in Sri Ramachandra Medical Centre Porur in Chennai.

The Media and antibiotic discs for the study were procured from **Hi-media** (Chennai) and Standard procedures were followed in the preparation of both plating media and media for biochemical reactions. Quality check of the media and biochemicals was done using suitable control strains and were put into use only when found satisfactory.

Sugar fermentation:

Acid production from various carbohydrates (Inositol, Adonitol, Galactose, D-trehalose, Salicin, and Arabitol) was detected under aerobic conditions by adding 1gm of the sugar base to peptone water using bromothymol blue as indicator dispensed in 3-4 ml amounts in small sterile test tubes of 100mm to give a final concentration of 1% in the medium and autoclaved at 121 °C for 10 minutes. A loopful of the test strain is inoculated into the medium and incubated for 48-72 hours. A positive reaction is indicated by acid production in the medium, with a change in the initial colour to yellow. A green colour after 24 hour incubation indicates a negative result.

Ornithine and lysine decarboxylase

Ornithine and Lysine decarboxylase activity is determined using test of Moeller. Decarboxylase basal medium is prepared with 1 %(w/v) L-Ornithine dihydrochloride and the final medium is adjusted to pH 6. The medium is dispensed in 3-4 ml amounts in small (100 mm) test tubes and autoclaved at 121 °C for 10 minutes A loopful of an overnight culture of several well-isolated colonies are inoculated in the test medium, followed by overlaying each tube with 4-5 mm of sterile mineral oil. Inoculated tubes should be incubated at 37 °C for up to 24 hours. A positive reaction is indicated by alkalinisation of the medium, with a change in the initial colour to violet. A yellow colour after 24 hours incubation indicates a negative result.

Antibiogram: Media used: Mueller Hinton Agar; Method: Kirby-Bauer disk diffusion Method**:**

Antibiotic susceptibility testing was done on Mueller-Hinton Agar according to standard procedures. (CLSI, M07-A8 document, 2010) Colonies from an agar medium are touched with a wire loop and the growth is transferred to a test tube containing 1.5ml of sterile peptone broth and the tubes were incubated for two hours at 37º C to produce a bacterial suspension of moderate turbidity The density of the suspension is standardized by sterile broth to a density visually equivalent to the Barium sulphate standard, 0.5 McFarland Units.

A sterile cotton swab is dipped in to the suspension and surplus removed by rotation of the swab against the side of the tube above the fluid level. The Mueller Hinton agar medium was inoculated by even streaking of the swab over the entire surface of the plate in 3 directions. Then the appropriate antibiotic discs were applied onto the plate and incubated overnight at 37°C (Milles, and Amyes 1999).

ATCC 25922 *Escherichia Coli* were used as control strain and were included in each batch of susceptibility testing of the isolates under study. The sensitivity to first line and second line antibiotics was read after 24 hours incubation as per CLSI guidelines (MS100-S20, 2010). The following discs were used- ampicillin (10µg), amikacin (30µg), cefazolin (30µg), cefotaxime (30µg), ceftazidime (30µg), cefoxitin (30µg), cefipime (30µg), Co-trimaxazole (1.25/23.75µg), tobramycin (10µg), imipenem (10µg), meropenem (10µg), cefaperazone-sulbactum (75/30µg), tazobactum-piperacillin(10/100µg), Ciprofloxacin (5µg), and norfloxacin (10µg).

ESBL producing strains were identified based on their susceptibility to third generation cephalosporins as an initial screen. (CLSI guidelines, MS100-S20, 2010)

Screening of Amp C betalactamases

Amp C beta lactamases were screened by two different methods

1. Tris-EDTA / AmpC disk test

The test is based on use of Tris-EDTA to permeabilize a bacterial cell and release β-lactamases into the external environment. AmpC disks (i.e., filter paper disks containing Tris-EDTA), were prepared in-house by applying 20 µ1 of a 1:1 mixture of saline and Tris-EDTA to sterile filter paper disks, allowing the disks to dry, and storing them at 2 to 8°C. The surface of a Mueller-Hinton agar plate was inoculated with a lawn of cefoxitin-susceptible *E. coli* ATCC 25922 according to the standard disk diffusion method. Immediately prior to use, Amp C disks were rehydrated with 20 µl of saline and several colonies of each test organism were applied to a disk. A 30-µg cefoxitin disk was placed on the inoculated surface of the Mueller-Hinton agar. The inoculated Amp C disk was then placed almost touching the antibiotic disk with the inoculated disk face in contact with the agar surface. The plate was then inverted and incubated overnight at 35°C in ambient air. After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result) (Black *et al* 2004).

2. Cefoxitin Hodge Test

The surfaces of MacConkey agar plates were inoculated with a lawn of the indicator strain, ATCC *E coli* 25922, according to the CLSI disk-diffusion method. After the agar surface dried, a test strain was heavily streaked from the centre of the plate to the periphery and a Cefoxitin disk was placed at the centre. The plates were incubated overnight at 37°C, and the presence of definite growth of the indicator organism in the inhibition zone along with the test strain was interpreted as positive (Thomson *et al* 1992).

Results and Discussion

A total number of 100 clinical isolates of Tribe Proteeae were obtained from patients in a non consecutive non random manner. The samples were obtained from both in- patient and out-patient services. Hospitalized patients were from various surgical and medical wards and intensive care units. The 100 isolates were collected from various clinical samples like pus & wound swabs, broncho alveolar lavage and urine.

The various genus and species of Tribe Proteeae obtained in the study are depicted in Genus Proteus was the most common isolate (87%), followed by Genus Providencia (10%) and Genus Morganella (3%). *Proteus mirabilis* 47% was the most common species followed by *Proteus vulgaris* 41%, *Providencia stuartii* 7%, *Providencia rettgeri* and *Morganella morganii* 3% each.

Pus (52%) was the most common specimen followed by urine (47%) and Non- BAL (1%). *Proteus mirabilis* was the predominant species followed by *Proteus vulgaris.*

Tribe Proteeae are part of the Enterobacteriaceae family of gram-negative bacilli. *Proteus mirabilis* accounts for 90% of *Proteus* infections and can be considered a community-acquired infection. Whereas *Proteus vulgaris* and *Proteus penneri* are usually isolated from individuals in long-term care facilities and hospitals (Michael S.Donnenberg.2005).

In the present study, 87% of the infections were caused by Proteus species of which *Proteus mirabili*s contributed to 46% and *Proteus vulgaris* 41%. A similar pattern was shown by O' Hara *et al.* 2000 and Mitesh .H. The next common isolate was Genus Providencia (10%) of which *Providencia stuartii* was 7% and *Providencia rettgeri* was 3% followed by *Morganella morganii subsp. sibonii* 3%.

The most common source of Tribe Proteeae was wound infections (N=52) followed by urinary tract infection (N=47). Out of 47 urine isolates, 85% were Proteus species of which *Proteus mirabilis* and *Proteus vulgaris* contributed equally (N=20 each) similar to the studies conducted by Mitesh .H. Patel *et al.*,2010 and Khan MK *et al*.2008. *Providencia stuartii* and *Providencia rettgeri* contributed to 7.5% (N= 3) each. Only 1 isolate of *Morganella morganii subsp. sibonii* was isolated.

52 strains were isolated from pus of which majority were *Proteus mirabilis* 48% (N=25) followed by *Proteus vulgaris* 45% (N=21). 8% (N=4) included *Providencia stuartii* and 4% (2) with *Morganella morganii subsp. sibonii.* Only 1 isolate of *Proteus mirabilis* was isolated from broncho alveolar lavage.

Proteus mirabilis and *Providencia stuartii* were the most common isolate from their respective genus. These organisms caused Urinary tract and wound infections equally but *Proteus mirabilis* was the most common isolate causing UTI. This is in accordance with other studies on Proteeae isolates (O' Hara *et al.*, 2000, Jensen *et al*., 1992).

All species of Tribe Proteeae are resistant to nitrofurantoin with varying degree of resistance to tetracycline. Strains of Proteus species have some unusual antibiotic sensitivity characteristics. All Proteus species are resistant to polymyxin B and colistin. In general *Proteus mirabilis* is the most sensitive species of the genus (Garrod *et al.*,1981).

Likewise in this study, Proteus *mirabilis* was more sensitive when compared to *Proteus vulgaris*. 35% of *Proteus mirabilis* were resistant to ampicillin and first generation cephalosporin,

16% for third generation cephalosporins while *Proteus vulgaris* exhibited 100 % & 43% respectively. Among the aminoglycosides, 7% & 14% of *Proteus vulgaris* were resistant to amikacin and tobramycin while with *Proteus mirabilis;* it was 3% and 6% respectively.

Most Proteus strains are sensitive to cefepime and cefpirome, quinolones and the β-lactamase producing strains are sensitive to carbapenems and aztreonam. However time to time they acquire plasmid encoding resistance to some of these antibiotics and cotrimaxazole to which they are normally sensitive. These resistance characteristics may be transferred to other Proteus strains and to Enterobacteriaeae and to *Pseudomonas aeruginosa* (Bernard.W.Senior, 2005).

In this study, 41% of Proteus strains were resistant to cotimaxozole, 28% to quinolones ciprofloxacin, and 3% to cefipime. 2% were resistant to carbapenems and β-lactam- β-lactamase inhibitor drugs like cefaperazone – sulbactum & tazobactum –piperacillin. Among ESBL producing strains of Proteus species, 24% were sensitive to cefipime, 95% to cefaperazone – sulbactum & tazobactum –piperacillin and 97% to carbapenems.

Providencia stuartii is one of the most antibiotic resistant species of Proteeae. They are resistant to penicillin, cephalosporins, aminoglycosides, but Providencia species is sensitive to ceftazidime, cephotaxime imipenem and aztreonam (Hawkey *et al*., 1984, Bernard W. Senior, 2003).

In this study, Providencia strains were universally resistant to ampicillin and first generation cephalosporins. 90% were resistant to third generation cephalosporins and 60% to fourth generation. Among the aminoglycosides, 90% were resistant to tobramycin and 70% to amikacin. 70% were resistant to both cotrimaxazole and quinolones, whereas 60% were susceptible to cefaperazone-sulbactum and Tazobactum-piperacillin. Least resistance was noted with carbapenems (30%).

In the present study, *Morganella morganii* isolates were universally resistant to β-lactam antibiotics, cotrimaxazole, quinolones and Aminoglycosides. But only 3 isolates were collected so the exact resistance pattern could not be derived. 33 % were sensitive to cefipime alone. Maximum sensitivity (100%) was noted with carbapenems and β-lactam- β-lactamase inhibitor combination drugs.

Altogether maximum susceptibility was seen with meropenem (97%) followed by imipenem (96%) and 94% were susceptible to both cefaperazone-sulbactum and Tazobactum-piperacillin. Maximum resistance (87%) was noted with ampicillin and first generation cephalosporins, 52% to third generation cephalosporins and 37% to fourth generation cefipime.

Despite the discovery of ESBLs and AmpC β-lactamases at least a decade ago, there remains a low level of awareness of their importance and many clinical laboratories have problems in detecting ESBLs and especially AmpC β – lactamases . Confusion exists about the importance of these resistance mechanisms, optimal test methods, and appropriate reporting conventions. Failure to detect these enzymes has contributed to their uncontrolled spread and sometimes to therapeutic failures (Thomson *et al.*,2001).

The objective of this work was to obtain some experimentally based prediction on the possible emergence of 'extended spectrum' Amp C β -lactamases and further to compare two phenotypic Amp C detecting methods. However, few studies (Manchanda, *et al.,* 2003, Khan *et al*., 2008) have assessed the occurrence of Amp C β -lactamases among these species in India.

Hodge test and Tris-EDTA (AmpC) disk test were used for detection of Amp C enzymes. The Hodge test showed 60 of the strains to be positive and Amp C disk test showed 56 to be positive. 52 isolates were identified by both the methods as AmpC producers. Though Hodge test showed more number of strains to be positive, many showed indeterminate results and were repeated thrice to confirm the result, whereas Tris –EDTA test showed better and consistent results than Hodge test.

Figure 1: Distribution of Proteeae isolates in clinical specimens

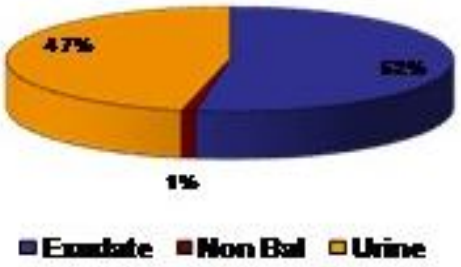


Figure 2: Distribution of Tribe Proteeae isolates

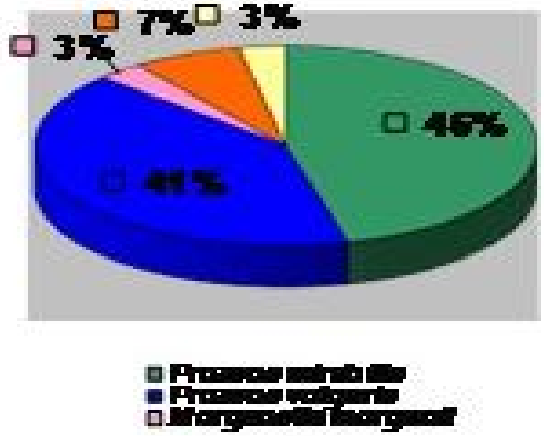


Table 1: Distribution of species in clinical specimens

Species	Exudates	Non-BAL	Urine	Total
Proteus mirabilis	25	1	20	**46**
Proteus vulgaris	21	0	20	**41**
Morganella morganii subspp.sibonii	2	0	1	**3**
Providencia rettgeri	0	0	3	**3**
Providencia stuartii	4	0	3	**7**
Total	**52**	**1**	**47**	**100**

Figure: 3 Antimicrobial susceptibility pattern of Tribe Proteeae

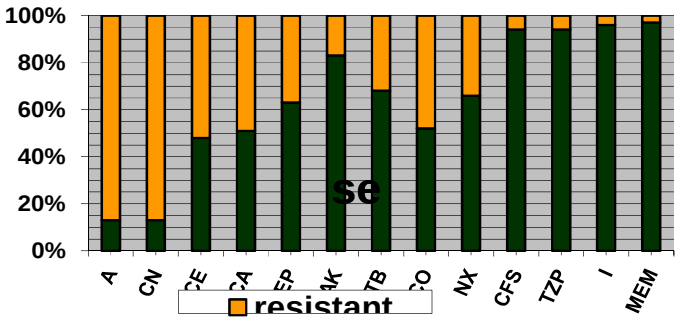


Figure 4: Amp C detection β-lactamase producers – Comparison by two methods

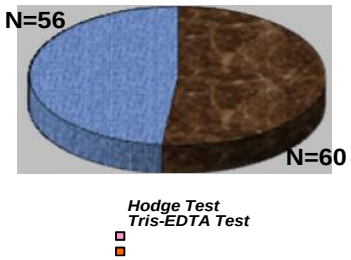


Figure: 5 Distribution of drug resistance pattern

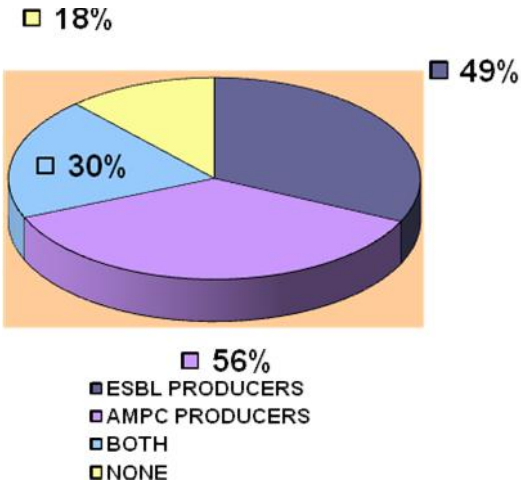


Figure 6: Antibiotic Susceptibility pattern of Amp C β-lactamase producers

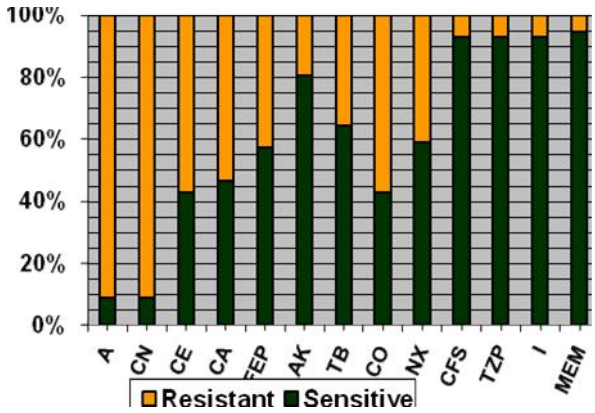


Figure 7: Antibiotic Susceptibility pattern of ESBL producers

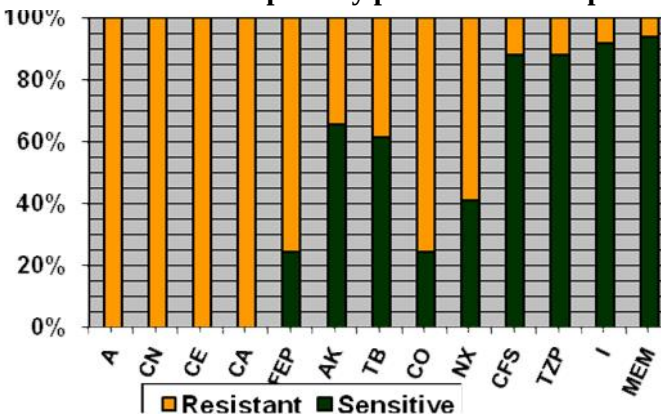


Figure 8: Antibiotic Susceptibility pattern of Proteus species

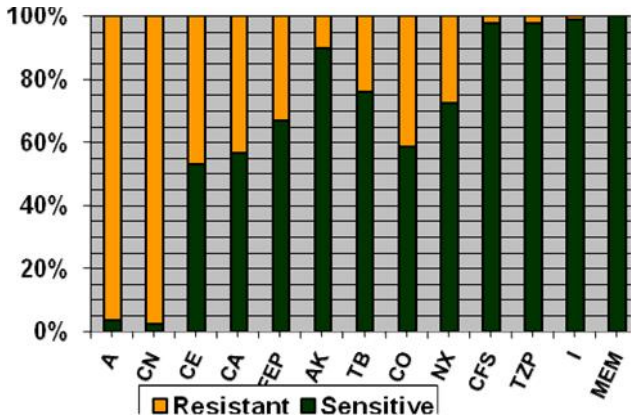
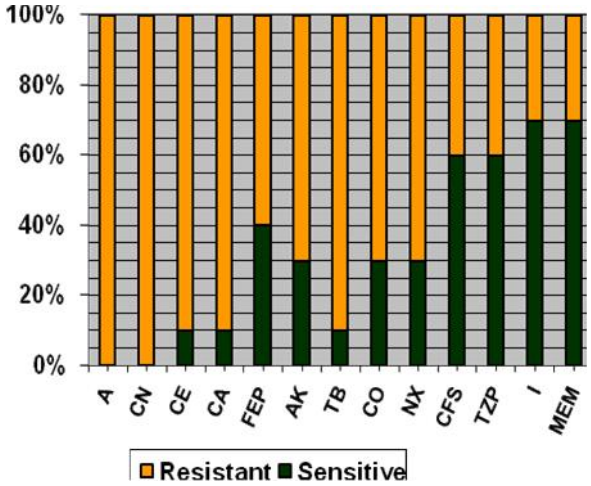


Figure 9: Antibiotic pattern of Providencia species



In this study, AmpC β -lactamases was seen mainly in *Proteus vulgaris* 52% (N=29) followed by *Proteus mirabilis* 30% (N=17), *Providencia stuartii* 11% (N=6), *Providencia rettgeri* and *Morganella morganii* 3.5% (N=2) each. Not many studies have been done exclusively on Proteeae isolates. Mitesh .H. Patel *et al*., 2010 have shown 28% of Proteeae isolates to be Amp C β -lactamases producers.

Higher resistance pattern was seen in Amp C β-lactamase producing strains. 91% were resistant to both ampicillin and first generation cephalosporin cefazolin. 57% and 54% were resistant to cephotaxime and ceftazidime respectively. Only 57% of the strains were susceptible to cefipime whereas 96% were susceptible to both β-lactamase- β-lactamase inhibitor combination drugs. Maximum sensitivity 99% was noted with carbapenems.

Most of the studies are done on Gram negative bacilli as such with the predominant organisms being *Escherichia coli, Klebsiella pneumoniae* and a few isolates of *Proteus mirabilis* as they are considered indicator organisms for plasmid mediated Amp C β -lactamases producers. Therefore not much of comparative studies are available.

In this study, there were more Amp C β-lactamase producers(56%) when compared to ESBL producers (49%, N=62).30% of the strains were both ESBL and Amp C β-lactamase producers and only 18% of them were negative for both. Out of the 38 Non ESBL producers, 20 were Amp C β-lactamase producers about 53%. Failure to detect these enzymes could lead to treatment failures and therefore there is an absolute necessity to detect these enzymes. In this study the Amp C disk test provided a simple, convenient, and accurate means of detection of plasmid-mediated Amp C β -lactamases in organisms.

Limitations of the study

Of the 123 scapula included, 64 belonged to right and 59 to left side. The shape of the GC was found as inverted comma, pear, triangular and oval. The most common shape observed was of pear shaped GC in 69 (56.09%) of 123 scapula. 43 (34.95%) were of inverted comma shape, 8 (6.5%) of oval shape and 3 (2.4%) were triangular.

Summary and Conclusion

- A total of 100 Proteeae strains were isolated from urine, pus and bronchoaveolar lavage.
- They were differentiated into Genus Proteus , Providencia and Morganella
- The most common Genus was Proteus followed by Providencia and Morganella
- The most common species in Genus Proteus was *Proteus mirabilis* followed by *Proteus vulgaris*
- The most common species in Genus Providencia was *Providencia stuartii* followed by *Providencia rettgeri*
- *Morganella morganii* is the only species isolated with subspecies *sibonii.*
- *Proteus mirabilis* was the most common isolate from urine and pus followed by *Proteus vulgaris*
- *Proteus mirabilis* was the only single isolate of bronchoalveolar lavage
- Out of 100 isolates, 16% were multidrug resistant.
- ESBL screen by third generation cephalosporins yielded a resistance of 49%
- Amp C β- lactamase enzymes were detected in 56% of the isolates by both Hodge test and Tris-EDTA test
- The most dominant species to be multidrug resistant was *Providencia stuartii* followed by *Morganella morganii* which also showed resistance to carbapenems
- Among the Amp C β- lactamase strains 57% were susceptible to cefipime whereas 96% were susceptible to both β-lactamase- β-lactamase inhibitor combination drugs and 99% to carbapenems.Amp C β- lactamase strains seen among non ESBL producers were 53%

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