

Molecular Characterization of Antibiotic Resistant Genes among Gram Negative Clinical Isolates

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Abstract

Increasing antibiotic resistance is a threat worldwide. Deep studies have to be done for correct and appropriate usage of antibiotics for treating infections efficiently and to curtail the spread of resistance. Hence, this study is aimed at understanding the prevalence of antibiotic resistance genes among 35 gram-negative bacterial isolates. By performing biochemical tests, the isolates were identified to be *Escherichia coli* (n = 15), *Klebsiella oxytoca* (n = 3), *Klebsiella pneumoniae* (n = 5), *Proteus mirabilis* (n = 4), *Proteus vulgaris* (n = 1) and *Pseudomonas aeruginosa* (n = 7). Antibiotic susceptibility tests showed that most *E. coli* isolates were resistant to ampicillin, levofloxacin and cefazolin. Ampicillin resistance was found in all of *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Proteus mirabilis* isolates. All *Proteus mirabilis* isolates were resistant to ciprofloxacin and co-trimoxazole. *Pseudomonas aeruginosa* showed maximum resistance to gentamycin and tobramycin. All 35 isolates were sensitive to amikacin, imipenem and meropenem. Polymerase Chain Reaction showed that AmpC and SHV genes were predominant in *E. coli* and *Klebsiella* species respectively. Among *Proteus mirabilis* and *Pseudomonas aeruginosa*, IntI gene was more prevailing. NDM1 gene was not detected in any of the isolates. This study also showed the prevalence of isolates resistant to multiple drugs due to the co-existence of resistant genes. Therefore, control of spread of infections, routine study in this field and proper guidelines for prescribing antibiotics is very necessary.

Keywords: Antibiotic resistance, ESBL, MBL, AmpC, Integrons

Introduction

20th century was a milestone in the field of medicine as antibiotics were discovered. Until then, even a minor bacterial infection, especially after a successful operation, was enough to kill a person. The discovery of antibiotics opened up a new gate in the field of medicine. The mass production of the antibiotic Penicillin, due to its effectiveness in treating infections, marked the dawn of the great antibiotic era [1]. The period between 1940 and 1960 was the golden age of antibiotics during which several antibiotics, which are used till now, were discovered.

But gradually, bacteria turned out to become resilient towards the antibiotics which were thought to have wholly conquered bacterial infections. Ever since the discovery of the first antibiotic, bacteria have always been looking for ways to act against antibiotics. Right from the 1940s, many strains of bacteria had started becoming resistant to the antibiotics used widely [2]. As predicted by Alexander Fleming, exposure of microbes to non-lethal quantities of antibiotics makes them resistant.

Acquiring resistance mechanisms is a natural process occurring in bacteria. But the factors that speed up the spread of resistance is entirely due to the carelessness and obliviousness of people which include failure to finish the entire course of medicine, self-medication and inappropriate or overuse of antibiotics. These factors result in improper dosage of the drug which will not be enough to kill the bacteria and instead, will make way for bacteria to develop resistance [3]. Over usage of antibiotics is also seen in fishing and agriculture [4]. Drug resistant bacteria can also be spread to humans from livestock or through waste water [5]. A comparative study done to check the prevalence of ESBL producing *Escherichia* species in livestock and humans showed that livestock animals like cattle were reservoirs of ESBL producing bacteria. Though transmission of these bacteria to humans is possible, the evidence for transmission of these bacteria was very less [6].

Bacteria gain resistance by mutations. It can also obtain resistance from the resistant cells in the vicinity. This is possible through transfer of genes through conjugation, transformation, transduction, or by transposons [7,8].

Bacteria confer resistance to antibiotics by modification of a drug target which is due to mutations at the site where the antibiotic targets the bacteria, molecular bypass- a mechanism by which bacteria prevent proper binding of the antibiotic, active efflux or by enzyme-catalyzed inactivation of antibiotics [9-11].

Antibiotic resistance is really a serious issue. Treatment of infections caused by bacteria which are resistant to more than 3 drugs (termed as 'Multi Drug Resistant' bacteria), is very tedious and may involve the use of more than 6 drugs [12]. Some strains are resistant even to the latest generation antibiotics. Doctors find treating these MDR bacterial infections very challenging [13,14]. Drug resistant bacterial infections prolong the time taken for treatment in hospitals [15]. There is significant relationship between drug resistance and mortality [16,17]. A report by WHO confirms that infections due to antibiotic resistant bacteria possess twice the chance of causing death compared to the infections caused by non-resistant bacteria [18]. MDR gram-negative bacteria commonly appear in cases of infections in the urinary tract, skin and blood stream. Multi drug resistance increases the severity in treating people with burns [19,20]. Development of resistance has also complicated urological practices [21]. The most prevalent bacteria in Urinary tract infections are *E. coli*, *Klebsiella species*, *Proteus species* and *Pseudomonas species* [22]. Though many strains of bacteria have become resistant to almost all beta lactam antibiotics, fosfomycin and nitrofurantoin are still effective in treating urinary tract infections [23]. Thus, MDR gram-negative bacterial infections can be managed by giving antibiotics to which they are susceptible.

The common genes conferring resistance to these bacteria which are reported in many countries are associated with beta lactamase which includes ESBL genes (TEM, SHV), MBL genes (NDM) and AmpC genes [24-26]. Apart from beta lactamase production, bacteria have integrons as another important mechanism that promote the spread of resistance genes from one bacterium to another [27,28]. Integrons are gene capture systems that contain an *IntI* gene encoding for integrase enzyme which helps in the integration of gene cassette containing resistance genes [29]. Three classes of integrons are commonly associated with resistance. Class 1 is more frequently found. Integrons can also be present in commensal organisms [30].

There is a serious necessity for research in this field to control the factors responsible for the spread of resistance. Also, there is a constant need to study the prevalence of MDR gram-negative bacterial infections in order to bring about effective treatment by giving antibiotics to which they are susceptible. Hence this study is aimed at understanding the diversity of drug resistant gram-negative bacteria isolated from clinical samples obtained from hospitals and laboratories in and around Chennai.

In this study, we have isolated 35 gram-negative bacteria. Biochemical tests were carried out and these bacteria were identified to be of the species *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Antibiotic susceptibility tests were done to determine if the organisms were sensitive or resistant to the antibiotics used. Further, the presence of 5 resistance genes (TEM, SHV, NDM, AmpC and *IntI*) was screened using Polymerase Chain Reaction.

Materials and methods

Isolation of gram-negative bacteria

50 bacterial isolates were collected from hospitals and laboratories in and around Chennai during July and August 2019. The isolates derived from clinical samples which included urine, blood and pus alone were included in the collection. The bacteria were cultured by streaking onto MacConkey's agar and incubated for 24 h. Then, gram's staining was done to differentiate gram positive and negative bacteria [31]. Bacterial smear was made and it was heat fixed. Crystal violet was added and after 1 min it was washed. Gram's iodine was added and washed after 1 min. Decolorizer was added followed by immediate washing. The smear was covered with safranin and washed after 1 min. Microscopic observation was done. Bacteria which were gram negative were isolated and pure culture was made for those cultures by quadrant streak method.

Catalase test

A few drops of H₂O₂ was taken on a slide. Using a nichrome loop, the test organism was taken and mixed with H₂O₂. The slide was observed for the formation of bubbles [32].

Oxidase test

A colony of the test organism was placed on an oxidase disc and was observed for color change. Oxidase positive organisms show a color change to purple color within 10 s. Oxidase negative organisms do not show any color change [32].

Biochemical tests

The following biochemical tests were performed to identify the isolated bacteria using Bergey's manual [32,33].

Indole test

Organisms were inoculated in peptone broth. After 24 h incubation at 37 °C, a few drops of Kovac's reagent were added. The test tube was observed for the appearance of red ring, which indicates the production of indole.

Methyl Red (MR) test

Organisms were inoculated in MR-VP broth and incubated for 24 h at 37 °C. A few drops of methyl red indicator were added. Reddish pink color indicates a positive reaction for MR test and a yellow colour indicates negative reaction.

Voges Proskauer (VP) test

Organisms were inoculated in MR-VP broth. After 24 h incubation at 37 °C, a few drops of Barrit reagent A followed by Barrit reagent B were added. The color change after 15 min is observed. Pink color indicates a positive reaction.

Citrate test

Organisms were streaked onto Simmon's citrate agar and incubated for 24 h at 37 °C. Blue color indicates a positive reaction and green color indicates a negative reaction.

Urease test

Urea agar medium was prepared and autoclaved. To 950 mL of this, 50 mL of 40 % urea solution was added. The media was dispensed in tubes and allowed to solidify in slanting position and organisms were streaked in this Urea agar. After 24 h incubation, a color change to pink indicates a positive result.

Triple sugar iron test

Organisms were inoculated in TSI agar by stabbing onto the butt and streaking on the slant. Color change to yellow (acidic -A), pink (alkaline-K), black (H₂S production) and production of gas was observed.

Antibiotic susceptibility test (AST)

AST was performed for all the 35 samples using Kirby- Bauer disc diffusion technique. It was done according to the guidelines of CLSI [34]. MacFarland (0.5) standard was used to compare the density of inoculum to be used in AST. The organisms were uniformly inoculated onto Muller Hinton Agar and exposed to Antibiotic discs. The plates were incubated for 18 h at 37 °C. The diameter of zone of inhibition was measured. Amikacin, amoxicillin-clavulanic acid, ampicillin, aztreonam, cefazolin, cefepime, cefotaxime, ceftazidime, cefuroxime, ciprofloxacin, co-trimoxazole, fosfomycin, gentamycin, imipenem, levofloxacin, meropenem, nitrofurantoin, piperacillin-tazobactam and tobramycin were used for isolates of *Enterobacteriaceae*. For *Pseudomonas* isolates, amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, gentamycin, imipenem, levofloxacin, meropenem, piperacillin-tazobactam and tobramycin were used.

Polymerase chain reaction (PCR)**DNA isolation**

1.5 mL of bacterial culture was taken in a microfuge tube and centrifuged at 10,000 rpm for 2 min. The pellet was resuspended in digestion buffer and incubated for an hour at 50 °C. To this, equal volume of Phenol-Chloroform-Isoamyl alcohol was added (25:24:1) and centrifuged at 10,000 rpm for 10 min. The top aqueous layer was transferred into fresh tubes and ammonium acetate and ice-cold ethanol were added. It was centrifuged at 5,000 rpm for 5 min. The pellet was washed with 70 % ethanol, air dried and re-suspended in TE buffer [35].

PCR

PCR was carried out for the detection of the 5 genes using primers and cycle conditions described in **Table 1** (Agilent technologies) [36-38]. Primers were obtained from Eurofins, Bangalore. After completion of PCR, the PCR products were loaded on 2 % agarose gel. A 1,500 bp DNA marker (Takara) was used for detecting the size of the bands obtained in the gel. Electrophoresis was carried out and Medicare Gelstan UV illuminator was used to document the gel after staining with ethidium bromide.

Table 1 PCR primers and cycle conditions.

Primers	Primer sequence	Product size(bp)	Cycle conditions
TEM (F) TEM (R)	5'-ATGAGTATTCAACATTTCCG-3' 5'-CCAATGCTTAATCAGTGAGG-3'	850	Initial denaturation at 95 °C for 5 min 35 cycles of: Denaturation at 95 °C for 1 min Annealing at 60 °C for 2 min Extension at 72 °C for 1 min Final extension at 72 °C for 10 min
SHV (F) SHV (R)	5'-TCAGCGAAAAACACCTTG-3' 5'-TCCCAGATAAATCACCA-3'	475	Initial denaturation at 95 °C for 5 min 35 cycles of: Denaturation at 95 °C for 1 min Annealing at 60 °C for 2 min Extension at 72 °C for 1 min Final extension at 72 °C for 10 min
NDM (F) NDM (R)	5'-GGTTTGGCGATCTGGTTTTTC-3' 5'-CGGAATGGCTCATCACGATC-3'	621	Initial denaturation at 95 °C for 10 min 35 cycles of: Denaturation at 95 °C for 30 s Annealing at 52 °C for 40 s Extension at 72 °C for 50 s Final extension at 72 °C for 10 min
AmpC (F) AmpC (R)	5'-CCCCGCTTATAGAGCAACAA-3' 5'-TCAATGGTCGACTTCACACC-3'	634	Initial denaturation at 95 °C for 5 min 35 cycles of: Denaturation at 95 °C for 1 min Annealing at 60 °C for 2 min Extension at 72 °C for 1 min Final extension at 72 °C for 10 min
IntI (F) IntI (R)	5'-GCCACTGCGCCGTTACCACC-3' 5'-GGCCGAGCAGATCCTGCACG-3'	898	Initial denaturation at 95 °C for 5 min 35 cycles of: Denaturation at 95 °C for 2 min Annealing at 60 °C for 1 min Extension at 72 °C for 1 min Final extension at 72 °C for 10 min

Results and discussion

Among the 50 isolates collected, there were 35 gram-negative bacteria which originated from urine (n = 27), pus (n = 5) and blood (n = 3) samples. Catalase and oxidase tests were carried out. Catalase positive organisms showed the formation of bubbles and oxidase positive organisms turned purple on the oxidase disc. The number of organisms positive and negative for Gram's staining, catalase tests and oxidase tests are given in **Table 2**.

Table 2 Gram's staining, Catalase and Oxidase tests of the clinical isolates.

Method	No. of positive isolates	No. of negative isolates
Gram's staining	0	35
Catalase test	35	0
Oxidase test	7	28

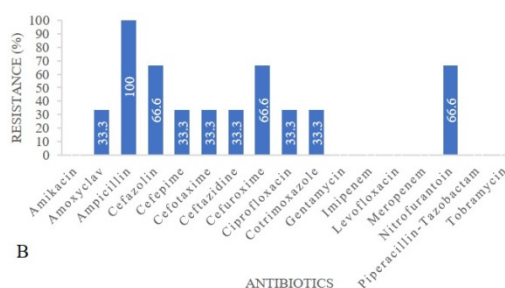
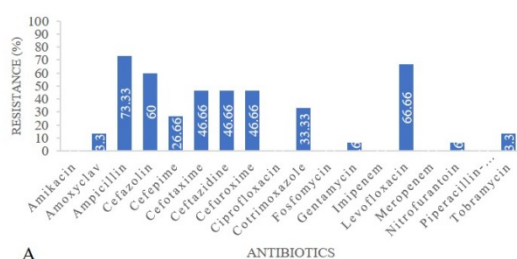
The isolates were identified using biochemical tests. Out of the 35 isolates, 15 were identified to be *E. coli*, 3 were *Klebsiella oxytoca*, 5 were *Klebsiella pneumoniae*, 4 were *Proteus mirabilis*, 1 was *Proteus vulgaris* and 7 were *Pseudomonas aeruginosa*. The biochemical characterization of the bacteria is given in **Table 3**.

Table 3 Biochemical characterization of the isolates.

Organisms	Indole	MR	VP	Citrate	Urease	TSI
<i>Escherichia coli</i> (n = 15)	+	+	-	-	-	A/A, Gas+, H ₂ S-
<i>Klebsiella oxytoca</i> (n = 3)	+	-	+	+	+	A/A, Gas+, H ₂ S-
<i>Klebsiella pneumoniae</i> (n = 5)	-	-	+	+	+	A/A, Gas+, H ₂ S-
<i>Proteus mirabilis</i> (n = 4)	-	-	-	+	+	K/A, Gas+, H ₂ S+
<i>Proteus vulgaris</i> (n = 1)	+	-	-	+	+	K/A, Gas+, H ₂ S+
<i>Pseudomonas aeruginosa</i> (n = 7)	-	-	-	+	-	K/K, Gas-, H ₂ S-

(+) indicates positive reaction, (-) indicates negative reaction, A and K indicate acidic and alkaline pH change, respectively.

Antibiotic Susceptibility Test was done for the 35 gram-negative isolates. Percentage of antibiotic resistance for all the 6 species is given in **Figure 1**. It was seen that 73.33 % of *E. coli* are resistant to ampicillin and 66.6 % of *E. coli* were resistant to levofloxacin. Cefazolin was resisted by 60 % of *E. coli* isolates. All of *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Proteus mirabilis* were resistant to ampicillin. *Proteus mirabilis* also showed 100 % resistance to ciprofloxacin and co-trimoxazole.



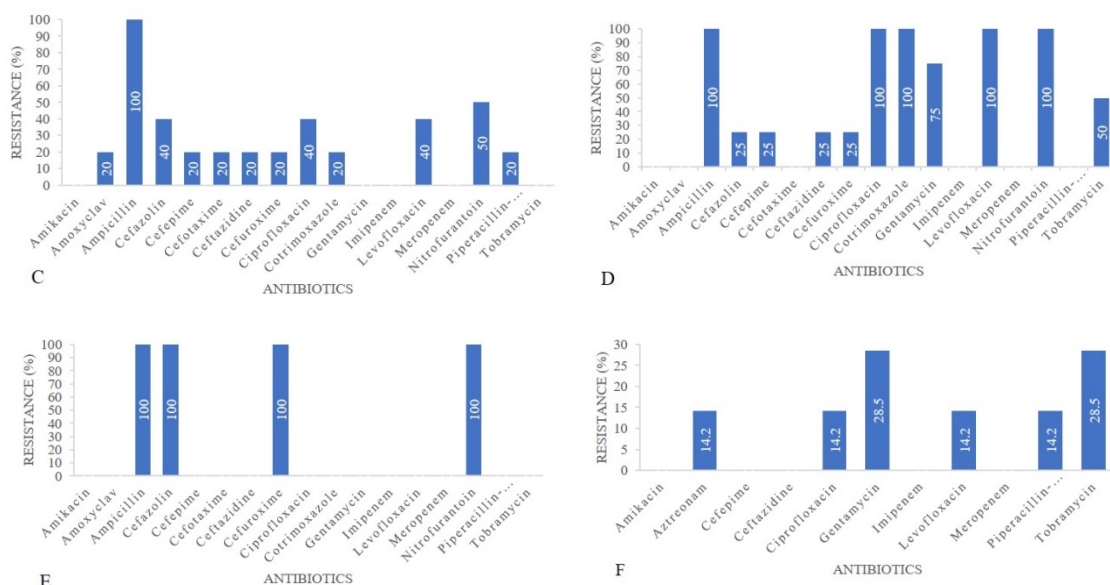


Figure 1 Percentage of antibiotic resistance in A) *E. coli*, B) *Klebsiella oxytoca*, C) *Klebsiella pneumoniae*, D) *Proteus mirabilis*, E) *Proteus vulgaris* and F) *Pseudomonas aeruginosa*.

It is apparent from the antibiotic susceptibility tests that maximum of the isolates is resistant to penicillins, cephalosporins and fluoroquinolones. The data are comparable to a study made in North India, where *Klebsiella* isolates from a riverine environment showed maximum resistance to ampicillin, cefazolin, cefotaxime and ceftazidime [39]. Another study in North India has shown high resistance of *K. pneumoniae* to aminoglycosides (77 %) which is contrary to our study, where all *K. pneumoniae* isolates are sensitive to them [40]. Similar to ours, a study in Iran has reported high resistance of *E. coli* to cephalosporins and high sensitivity to meropenem and amikacin [41]. Also, in Europe, there has been a noteworthy increase in the resistance of *E. coli* and *Klebsiella* spp. towards aminoglycosides, third generation cephalosporins, carbapenems and fluoroquinolones [42].

Hospitalization and increased use of fluoroquinolones might probably be the reasons for high resistance to levofloxacin [43]. Since 2000, third generation cephalosporins were prescribed empirically as there was increasing resistance to quinolones and penicillins. Besides, there has been a widespread availability of third generation cephalosporins than narrow spectrum antibiotics. In order to curtail drug resistance, it is necessary that narrow spectrum antibiotics are manufactured and made available commonly and also, they should be used wherever possible instead of broad spectrum antibiotics [44].

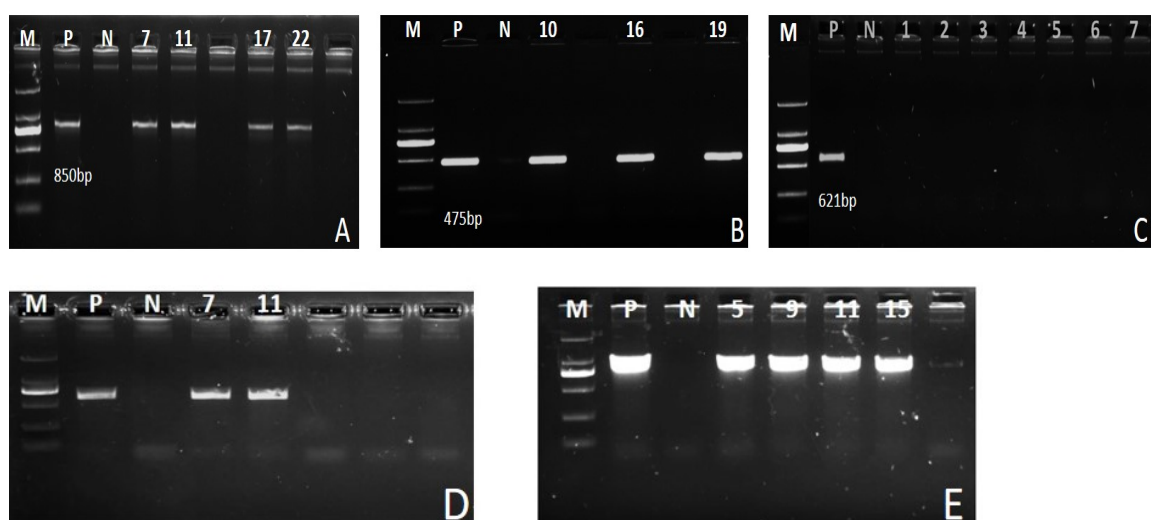
Pseudomonas aeruginosa showed maximum resistance to gentamycin (28.5 % isolates) and tobramycin (28.5 % isolates) which are aminoglycosides. However, higher resistance has been reported on aminoglycosides to *Pseudomonas aeruginosa* in other studies [45,46].

Carbapenem resistance is a growing threat. Treatment of bacterial infections caused by bacteria that are resistant to carbapenems is highly challenging [47]. Interestingly, all our isolates were 100 % sensitive to carbapenems (imipenem and meropenem), unlike several studies reported all over the country [46,48,49]. Another study in Tamil Nadu done with *E. coli* isolates has also showed complete susceptibility to carbapenems [50]. All of those isolates were sensitive to amikacin as well.

PCR was carried out and the 5 genes were visualized using Medicare Gelstan UV illuminator (**Figure 2**). The distribution of the 5 genes among the 6 species of bacteria isolated is given in **Table 4**.

Table 4 Number of isolates positive for the antibiotic resistant genes.

Organisms	TEM	SHV	NDM	AmpC	IntI
<i>Escherichia coli</i> (n = 15)	5	0	0	13	3
<i>Klebsiella oxytoca</i> (n = 3)	0	1	0	1	1
<i>Klebsiella pneumoniae</i> (n = 5)	0	3	0	2	1
<i>Proteus mirabilis</i> (n = 4)	2	0	0	0	3
<i>Proteus vulgaris</i> (n = 1)	0	0	0	0	0
<i>Pseudomonas aeruginosa</i> (n = 7)	0	0	0	0	1

**Figure 2** Agarose gel showing some PCR amplified samples of A) TEM gene B) SHV gene C) NDM gene D) AmpC gene and E) IntI gene. (M-Marker, P- Positive control, N-Negative control, Numbers above lanes indicate some of the isolates positive for those genes).

The bacteria which possess genes encoding beta lactamase have the ability to resist beta lactam antibiotics. Beta lactamases are commonly encoded by ESBL genes, MBL genes and AmpC genes [51]. Extended Spectrum Beta Lactamases (ESBL) are enzymes produced by some strains of bacteria that act against beta lactam antibiotics. Extended Spectrum Beta Lactamases are commonly encoded in genes like TEM, SHV and CTX-M. ESBL genes have been reported widely in gram-negative bacteria like *E. coli*, *Klebsiella*, *Proteus* and *Pseudomonas* spp. [52,53]. Studies show that multi drug resistance is common among bacteria having ESBL genes [54]. Carbapenems are a class of antibiotics for which much resistance is not reported compared to the other classes. Carbapenem resistance is encoded by Metallo-beta lactamase genes. AmpC beta lactamases are cephalosporinases. *Pseudomonas aeruginosa* and *E. coli* are frequently harbored with AmpC genes [55].

Molecular characterization showed that 86.6 % of *E. coli* had AmpC gene in them. This was followed by TEM gene (33.3 %) and IntI gene (20 %). 13.3 % of *E. coli* had both TEM and AmpC genes. All the 3 genes were found in 13.3 % of *E. coli*. 33.33 % of *Klebsiella oxytoca* had all 3 SHV, AmpC and IntI genes. 60 % of *Klebsiella pneumoniae* had SHV gene, 40 % had AmpC gene and 20 % had IntI gene. 20 % of *Klebsiella pneumoniae* had both AmpC and IntI genes. Among *Proteus mirabilis*, 75 % had IntI gene and 50 % had TEM gene. 50 % of *Proteus mirabilis* had both IntI and TEM genes. *Proteus vulgaris* had no resistance genes at all. 14.28 % of *Pseudomonas aeruginosa* had only IntI gene.

To summarize, the predominant gene found in the 35 isolates was AmpC (45.71 %). This was followed by ESBL genes, either TEM or SHV (31.2 %). IntI gene was present in 25.7 % of the isolates. The presence of IntI gene (belonging to class 1 integrons) also gives chance for bacteria to develop resistance to various antibiotics as it helps in integrating gene cassettes with resistance genes [29]. 13.3 % of *E. coli* isolates and 33.33 % of *Klebsiella oxytoca* had ESBL, AmpC and IntI genes in them. The co-existence of various ESBL genes and AmpC genes is the reason for multi drug resistance of bacteria to many antibiotics like penicillins, cephalosporins and beta lactamase inhibitors [56].

NDM is a frequently reported gene for carbapenem resistance [57]. No isolates were found to have NDM. This is relatable with the 100 % sensitivity to imipenem and meropenem in the AST. As a result, we can conclude that carbapenems could probably be a better drug of choice in this area.

Conclusions

The prevalence of genes with antibiotic resistance was studied among 35 gram negative clinical isolates and it has been observed that most of the isolates had antibiotic resistance genes with some of them being multi drug resistant. High resistance to penicillin and cephalosporin was observed while there was no resistance to carbapenems by any of the isolates. Bacterial infections are difficult to treat since bacteria have become resistant to almost all the classes of antibiotics that are currently used. The high prevalence of resistance genes is a risk factor which extends the time taken for healing and it may also lead to death in some cases. Thus, there is a serious need for research in the field of drug resistance as this will help us monitor the prevalence of resistant bacteria and thereby improve guidelines in the usage of antibiotics for the treatment of bacterial infections. People should become aware and avoid using antibiotics inappropriately.

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