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RESEARCH ARTICLE

PREVALENCE OF PLASMID MEDIATED AMINOGLYCOSIDE MODIFYING ENZYMES IN PSEUDOMONAS AERUGINOSA IN HOSPITALIZED PATIENTS AT A TERTIARY CARE CENTRE.

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Manuscript Info	*Abstract*
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Background:-Pseudomonas aeruginosa is an important cause of hospital-acquired infection. It is frequently recovered species from clinical specimens and multidrug-resistant are increasingly being reported worldwide. In spite of high resistance, aminoglycosides are still an important treatment option in infection with P. aeruginosa. The major factor responsible for aminoglycoside resistance is Aminoglycoside-modifying enzymes (AMEs), carried by mobile genetic elements.

Aim: The study was conducted to determine the prevalence of plasmid-mediated AME coding genes in *P. aeruginosa.*

Methods: One hundred and forty consecutive, non-repeat isolates of *P. aeruginosa* were collected from various clinical samples. Sensitivity to amikacin, gentamicin, netilmicin, and tobramycin were detected by Kirby-Bauer disc diffusion method. AMEs-coding genes were detected by the molecular method.

Result: Out of the 140 isolates, 62.14% were resistant to amikacin, 80% to gentamicin, 77.86% to netilmicin and 72.86% to tobramycin. 77.14% isolates were found to carry AMEs-coding genes. aac(6')-I was the most frequently encountered gene (58.57%) present either singly or in combinatios, followed by ant(2")-I (50%) and aph(3')-I (32.14%).

Conclusions: Markedly high resistance was observed against all aminoglycoside tested along with the high prevalence of AMEs-coding genes. These isolate may represent as potential reservoirs of aminoglycoside resistance in hospitals, having combinations of AMEs-coding genes on their plasmids. Good infection control practices and antibiotic stewardship programme are very important to prevent the spread of infections.

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Introduction:-

Hospital-acquired infections (HAI) are a major threat to patients admitted in the acute care units. It is one of the important public health problems around the world. In association with increased morbidity and mortality, HAI is an important cause of prolonged hospital stay, inconvenience to the patients and an economic burden on healthcare. [1] Decreased immunity, increasing numbers of invasive techniques, critical life-saving operations, and immunosuppressive therapies are frequently associated with transmission of drug-resistant bacteria among patients

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in hospitals with poor infection control practice. HAIs are most common in Intensive care units (ICU), acute surgical and orthopedic wards. The most frequent HAIs are infections of surgical wounds, urinary tract infections. Bloodstream infection and infections of lower respiratory tract including ventilator-associated pneumonia (VAP) are also very frequent in hospitalized patients. However, bloodstream infections and lower respiratory tract infections are the most lethal. [2] Enterobacteriaceae family being the most common identifiable groups overall, multidrug-resistant organisms, including *P. aeruginosa* are increasingly being reported worldwide and associated with a high mortality rate.[3, 4] *P. aeruginosa* are commonly associated with septicemia in burn patients, urinary tract infections in the catheterized patient, surgical site infections and chronic debilitating respiratory infections in cystic fibrosis patients. [2, 4, 5]

In addition being intrinsically resistant to several antimicrobial agents, *P. aeruginosa* can easily develop resistance to all conventional antipseudomonal antibiotics, limiting the choice of effective antibiotics and complicating treatment. [4, 6] The aminoglycosides are still very effective in the treatment of infections caused by aerobic gram-negative bacilli, including *P. aeruginosa*. Aminoglycosides act synergistically with other antibiotics active against the cell-wall like the β-lactam, thus facilitates cell killing and reduces the risk of development of resistance. [6] Infections with *P. aeruginosa* are commonly treated with an aminoglycoside and antipseudomonal penicillin (e.g., Piperacillin) or antipseudomonal cephalosporin (e.g., Ceftazidime). Three mechanisms are responsible for aminoglycoside resistance: alteration of outer membrane permeability or diminished inner membrane transport or active efflux leading to decreased intracellular accumulation; target modification by 16S rRNA mutation or methylation or ribosomal protein-coding gene mutation; enzyme-mediated drug modification [7, 8]. In the clinical setting, Aminoglycoside resistance is mainly due to AMEs causing modification of amino- or hydroxyl groups. This, in turn, reduces or inhibits binding of the aminoglycoside molecule to the ribosome and failure in trigging energy-dependent phase II. [8] The three main classes of AMEs are aminoglycoside N-acetyltransferases (AACs), aminoglycoside O-phosphotransferases (APHs), and aminoglycoside O-nucleotidyltransferases (ANTs). *aac(6')*–Ib is most frequently found in *P. aeruginosa* and a major contributor to aminoglycoside resistance. The genes coding for *aac(6')* is often part of mobile genetic elements. *aph (3')*-I and *aph (3')*-II are well documented in the clinical specimen of *P. aeruginosa*. [8] The genes encoding the APHs have been found on R − plasmid, transposons, integrons and responsible for multiple drug resistance. *ant (2")*-Ia is best known to confer resistance to gentamicin, kanamycin, and tobramycin. It is encoded on the plasmid or integrons in many Gram-negative bacteria, including *P. aeruginosa*. [8]

However, little is known about the prevalence of these AMEs-coding genes among *P. aeruginosa*. Our effort in this study was to explore the plasmid-mediated AMEs-coding genes responsible for aminoglycoside resistance in *P. aeruginosa* from clinical isolates.

Materials and Methods:-
Isolation & identification
This prospective study was carried out from December 2013 to November 2014. One hundred and forty consecutive, non-repeat clinical isolates of *P. aeruginosa* were collected from various clinical samples of hospitalized patients at a tertiary care hospital and were identified by conventional phenotypic methods.

Antimicrobial susceptibility testing
Isolates were tested for their susceptibility to four aminoglycosides; amikacin, gentamicin, tobramycin, and netilmicin by Kirby-Bauer disc diffusion method. Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines, 2014 (M100 – S24). [9]

Isolates resistant to any tested aminoglycosides; amikacin, gentamicin, tobramycin, and netilmicin were defined as aminoglycoside resistant. *P. aeruginosa* (ATCC 27853) served as a control.

Genotypic Detection of Aminoglycoside Modifying Enzymes
4-5 colonies of isolate were dissolved in 2 ml Luria Bertani broth. The broth was incubated for 48 hours at 37ºC. Cells were harvested, and Plasmid DNA was extracted by the spin column method (Hi-PURA Plasmid Miniprep, Hi-MEDIA).

Five AMEs – coding genes, *aac (6')*-I, *aph (3')*-I, *aac (3)*-I, *aac (3)*-II and *ant (2")*-I were selected for screening. PCR for detection of the AMEs – coding genes was carried out using five sets of primers, designed from sequence deposited in the GeneBank database   (Table 1).

The PCR assay was carried out in a final volume of 25 μl, containing 12.5 μl of 2X Dream TaqTM Green PCR Master Mix (Fermentas, USA), 1 μl (25 picomoles) of each primer (Integrated DNA Technologies, IDT), 1 μl of plasmid DNA and molecular grade water. Various amplification conditions were used for each primer set. Amplification products were detected by electrophoresis on 1% w/v agarose gel containing 0.5% ethidium bromide and visualized on UV transilluminator. Amplicon size was determined using ready-to-use 100 bp Plus DNA ladder (Fermentas, USA) run along with samples every time.

DNA Sequencing
Ten amplicons representative of the two sets of primers were verified by Sanger's capillary sequencing. The nucleotide sequences of the amplicons were analyzed using the open access software of National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Results:-
Out of 140 *P. aeruginosa*, 48 (34.29%) were isolated from pus, 26 (18.57%) from blood, 25 (17.85%) from tracheal aspirates, 16 (11.43%) from urine, 14 (10%) from tissues and 11 (7.86%) from other samples. (Figure 1)
73 (52.14%) isolates were obtained from different surgical wards including burn care unit and urology unit. 29 (20.71%) isolates were obtained from surgical ICU, 21 (15%) were from medical ICU and 17 (12.14%) from acute medical units. 117 (83.57%) isolates were obtained from male patients and 23 (16.43%) from female patients. (Figure 2)

87 (62.14%) isolates were resistant to amikacin, 112 (80%) to gentamicin, 109 (77.86%) to netilmicin and 102 (72.86%) to tobramycin (Table 2).

Prevalence of AMEs-coding Genes
Among 140 isolates tested, 108 (77.14%) were found to carry AMEs-coding genes on their plasmid. They were found either singly or in different combinations. *aac(6')*-I was the most frequently encountered gene (58.57%) either singly or in combinations, followed by *ant(2")*-I (50%), *aph(3')*-I (32.14%) and *aac(3)*-II (8.57%). While *aac(3)*-I was not detected in any of the isolates. (Figure 3, 4, 5, 6) 33 (23.57%) isolates had only one AME-coding gene. *ant (2")*-I was the most common single AMEs-coding gene, detected in 19 isolates (13.57%) followed by *aac(6')*-I, in 10 (7.14%) isolates. 47 (33.57%) isolates were detected with carrying two genes. *ant (2")*-I and *aac(6')*-I combination were detected in 24 (17.14%) isolates, followed by *aph (3')*-I and *aac(6')*-I combination in 18 (12.86%) isolates. Coexistence of three AMEs-coding genes was detected in 27 (19.29%) isolates and 1 isolate (0.7%) with four AMEs-coding genes. The presence of AMEs-coding genes could not be detected on the plasmids of 13 resistance isolates (9.29%) despite showing resistance to at least one of the tested aminoglycoside antibiotics. (Table 3)

Discussion:-
P. aeruginosa is a major pathogenic species in the family Pseudomonadaceae. It is one of the most serious bacterial infection and is responsible for 10% of HAI. [1, 2] It has the considerable potency to become resistant to many antibiotics. Increasingly more antimicrobial resistant strains are being encountered in clinical practice, leaving the treating physician with limited treatment options and severe adverse outcome. [4, 8]

In the present study, 34.29% *P. aeruginosa* was isolated from pus, 18.57% from blood, 17.85% from tracheal aspirates, 11.43% from urine, 10% from tissues. In another study by Kalaivani R *et al*. showed the major source of *P. aeruginosa* was wound (43%) followed by endotracheal aspirate (19%), urine (16%) and blood (11%). [10] Srinivas B *et al*. also described the highest isolation rate from pus (55.35%) and urine (18.45%). [11] Study by Arora D *et al*. showed a major source of *P. aeruginosa* was urine (36%), followed by wound discharge (20%). [12]

52.14% *P. aeruginosa* was isolated from different surgical wards followed by surgical ICU (20.71%), medical ICU (15%) and other medical wards (12.14%). Raakhee T *et al.* showed the highest isolation of *P. aeruginosa* was from

surgical ICU (26.24%). [13] Anupurba S et al. also showed an important source of P. aeruginosa was surgical wards (29.9%) and burn care units (21.7%). [14]

Among aminoglycosides, 62.14% isolates were resistant to amikacin, 80% to gentamicin, 77.86% to netilmicin and 72.86% isolates were resistant to tobramycin. In a study done by Upadhyay S et al., resistance rate was 52.5% to amikacin, 60.9% to gentamicin and 58.9% to netilmicin. [15] Shahid M et al. showed high resistance to amikacin (55.1%) and tobramycin (83.6%). [16] Arora D et al. also found 41.5% resistance to amikacin and 79% to gentamicin. [12]

In present study, 76.43% isolates were found to carry AMEs. In another study by Chaudhary M et al., the prevalence of AMEs-carrying genes in P. aeruginosa was 68.92%. [17] aac(6')-I is the most prevalent AMEs –coding gene that specifies resistance to several aminoglycosides and shows high resistance against amikacin and gentamicin. [8] In the present study, 58.57% isolates were carrying aac(6')-I genes singly or in combination. Similar high prevalence of aac(6') genes in P. aeruginosa was reported by Shahid M et al. (42.8%) and Chaudhary M et al. (43.5%). [16, 17] The prevalence of ant (2")-I, aph (3')-I and aac (3)-II was 50%, 32.14%, and 8.57 % respectively. Chaudhary M et al. showed 18.9% and 9.4 % prevalence of ant (2") and aph (3) genes among P. aeruginosa. Shahid M et al. reported the prevalence of aph (3')-I and aac (3)-II were 16.3% and 20.4% isolates respectively. [16, 17]

Miller *et al*. reported that in Europe *aac(6')*-II was the most prevalent followed by *ant (2")*-I. [18] Whereas, Kim JY et al. from Korea nationwide study on *P. aeruginosa* observed that *aac(6')*-I and *ant (2")*-I were prevalent but none harboured *aac(6')*-II. [19]

Conclusion:-

This study provided information about resistance pattern of different aminoglycosides against *P. aeruginosa* and resistance determinants providing useful comparative data for future study. Because of synergism with other drugs and post antibactericidal effect, aminoglycosides remain useful antipseudomonal agents. However, the high prevalence of plasmid-mediated aminoglycoside resistance in *P. aeruginosa* among hospitalized patients was observed which could easily spread and be disseminated among other bacteria. Proper infection control practices and periodic monitoring of resistance to aminoglycoside antibiotics are required to minimize further dissemination and preserve the usefulness of this important class of antibiotics for the treatment of complicated infection.

Ethical approval:
Not required

Legends for figures:-
   Figure 1:-Sample wise distribution of isolates.
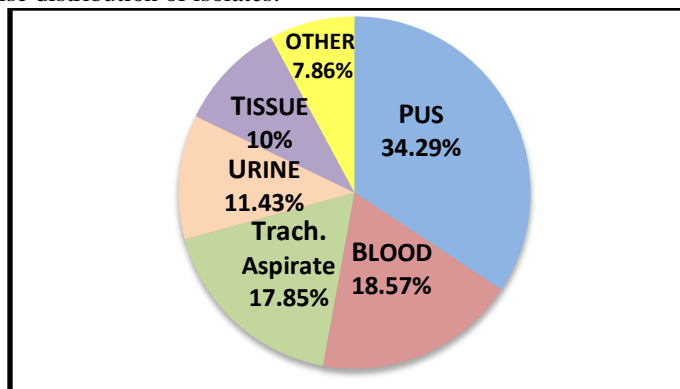
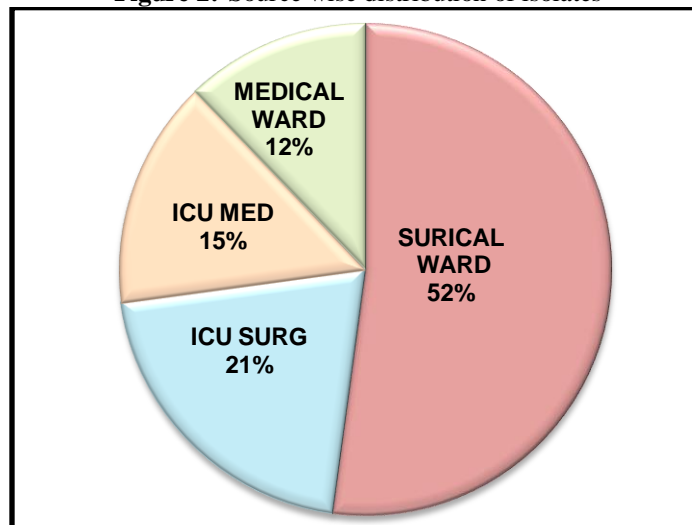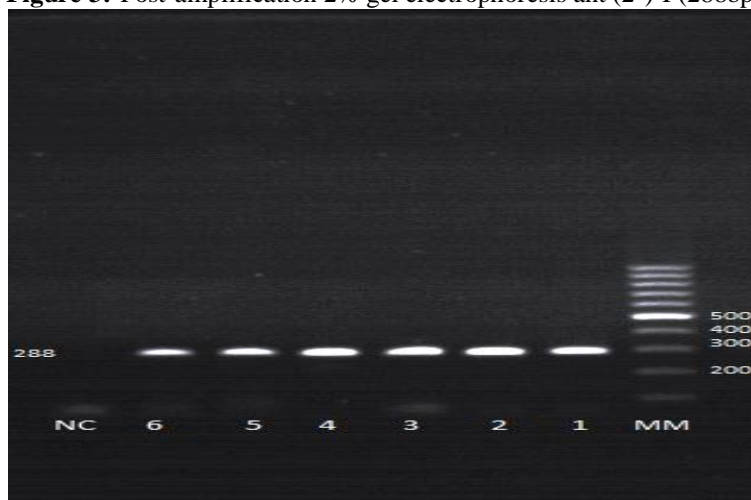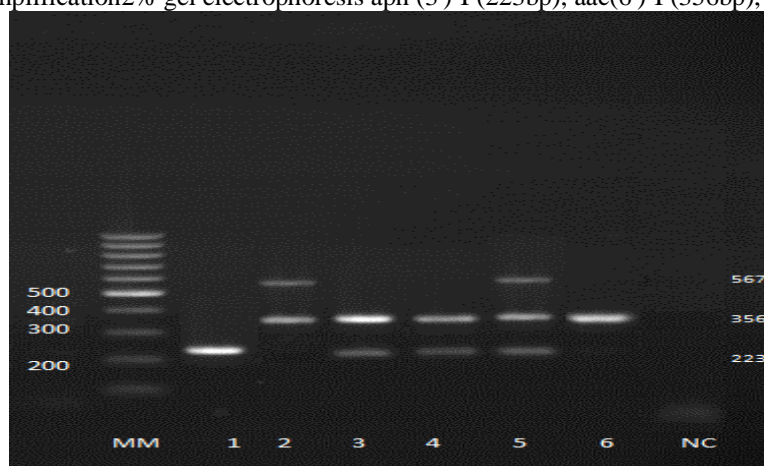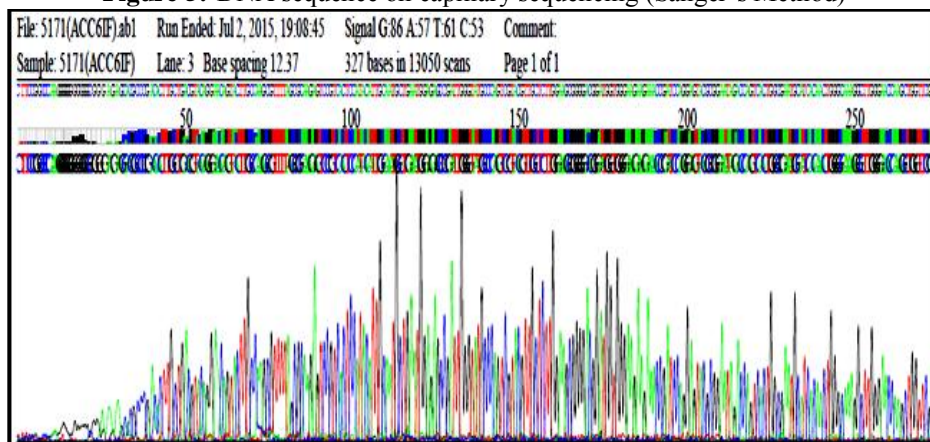**Figure 2:-**Source wise distribution of isolates

Figure 3:-Post-amplification 2% gel electrophoresis ant (2")-I (288bp)

Figure 4:-Post-amplification2% gel electrophoresis aph (3')-I (223bp), aac(6')-I (356bp), aac (3)-II (567 bp).

Figure 5:-DNA sequence on capillary sequencing (Sanger's Method)

Figure 6:-Nucleotide sequence BLAST on NCBI site.

Table 1:-List of Primers

Primer	Primer sequences (5' – 3')	Target genes [a]	Amplicon size (bp)
APH(3')I-f	TTATGCCTCTTCCGACCATC	aph (3')-Ia/ aph (3')-Ic	223
APH(3')I-r	GCCTGAGCGAGACGAAATAC		
AAC(6')I-f	TTGCGATGCTCTATGAGTGG	aac (6')-Ib/ (aacA4)	356
AAC(6')I-r	CGTTTGGATCTTGGTGACCT		
AAC(3)I-f	CCACCTACTCCCAACATCA	aac (3)-I/ (aacC1)	329
AAC(3)I-r	TTCCCGTATGCCCAACTTT		
AAC(3)II -f	GCAGAAGGCAATAACGGAG	aac (3)-IIa/ aac (3)-IIc	567
AAC(3)II-r	CCAGGCATCGGCATCTCATA		
ANT(2")I-f	AAGCACGATGATATTGATCTG	ant (2")-Ia/ (aadB)	288
ANT(2")I-r	GGCATAGTAAAAGTAATCCCA		

f: forward primer, r: reverse primer

[a] Alternate names of target genes as described by Ramirez et al [8]

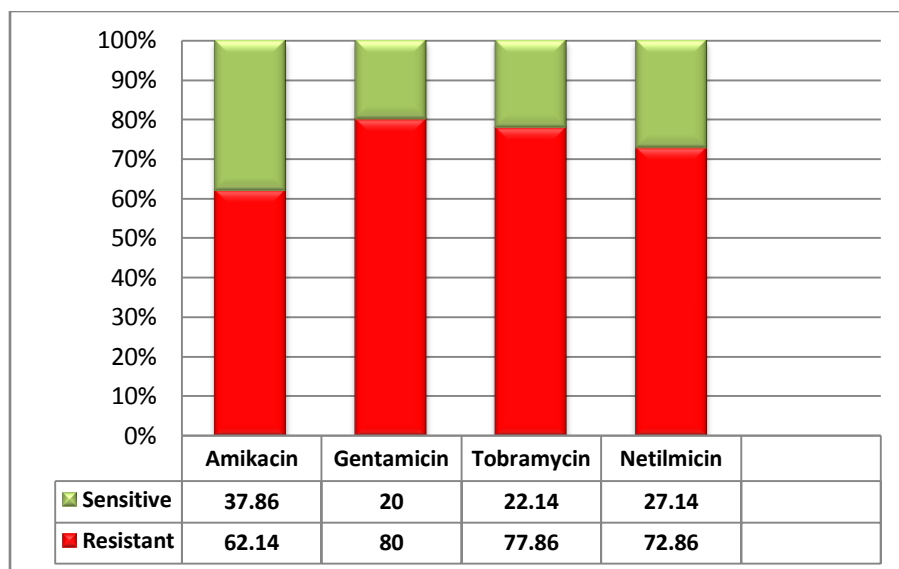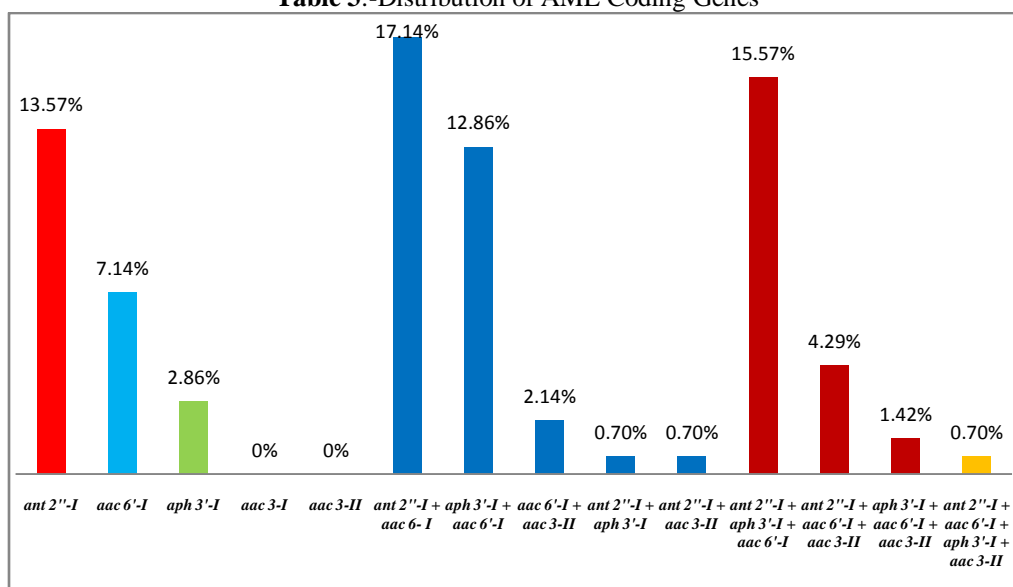**Table 2:-**Antimicrobial sensitivity pattern against aminoglycosides.

	Amikacin	Gentamicin	Tobramycin	Netilmicin
Sensitive	37.86	20	22.14	27.14
Resistant	62.14	80	77.86	72.86

Table 3:-Distribution of AME Coding Genes

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