Pak. J. Biotechnol. Vol. 20(1),1-9, 2023. http://doi.org/10.34016/pjbt.2023.20.01.773

Research Article

OPEN ACCESS

Available on http://www.pjbt.org
Pakistan Journal of Biotechnology
(PJBT)
(P-ISSN: 1812-1837 and E-ISSN: 2312-7791)

ESTIMATION OF MINIMUM INHIBITORY CONCENTRATION OF ANTIBIOTICS USING MICRO-TITER PLATE METHOD FOR NATIVE MULTI DRUG RESISTANT GRAM NEGATIVE BACTERIA

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Article Received 20-12-2022, Article Revised 11-02-2023, Article Accepted 10-03-2023.

ABSTRACT

Antibiotic resistance is considered as a global emergency due to its aggressive progression. Many factors contributing to this rise and one of the major factor in developing countries is indiscriminate use of antibiotics in clinics. Gram negative bacteria adding significant burden to clinical infections in native populations and therefore important to constantly monitor resistance pattern against different antibiotics. This study envisages to estimate the minimum inhibitory concentrations (MICs) for native prevalent multi drug resistance gram negative bacteria. Gram-negative bacteria were collected from different clinical labs situated in Karachi. Of these 83 isolates, 99% were found resistant against one or more of the following antibiotics: ampicillin, co-amoxiclav, gentamycin, neomycin, streptomycin and tetracycline. Most of the isolates were multi-drug resistant (MDR) having resistance to three or more antibiotics at a time. For ampicillin, 4.8% of the isolates were found Susceptible, 3.6% were Intermediate while 91.5% were found Resistant. For co-amoxiclav, 1.2% of the isolates were found Susceptible, 6% were Intermediate while 92.7% were found Resistant. For gentamicin, 3.6% of the isolates were found Susceptible, 10.8% were Intermediate while 86.7% were found Resistant. For neomycin, 21.6% of the isolates were found Susceptible, 40.9% were Intermediate while 43.4% were found Resistant. For streptomycin, 1.2% of the isolates were found Susceptible, 18% were Intermediate while 91.6% were found Resistant. For tetracycline, 15.7% of the isolates were found Susceptible, 45.7% were Intermediate while 53% were found Resistant. The study confirmed the presence of multidrug resistance in indigenous clinical gram-negative bacteria which is an alarming situation and needs development of effective alternative strategies for the treatment of infections.

Key words: gram-negative, resistant, MDR. antibiotics

INTRODUCTION

Antibiotics are used to inhibit or kill bacteria as remedy for different bacterial infections. However, since last decade and more it became a global challenge to maintain effectiveness of antibiotics due to rise in antibiotic resistance (Goossens, 2009). Bacteria have the ability to acquire different mechanisms to combat antibiotic action and it leads to resistance to particular antibiotic and the germs are no more affected and their growth continued (Reygaert., 2018).The treatment of infections caused by such clinically important resistant bacterial strains is not only difficult, but sometimes become impossible to cure (Founou *et al*., 2017). According to the report of Center for disease control and infection (CDC), the antimicrobial resistance is an urgent threat to global public health, killing at least 1.27 million people worldwide and associated with nearly 5 million deaths in only 2019. In the U.S., occurrence of more than 2.8 million resistant infections is reported each year. About 35,000 deaths occurred as a result of resistance. (2019 Antibiotic Resistance (AR) Threats Report). Antibiotic resistance is now considered as a very serious issue throughout the globe and specifically in under developing countries like Pakistan due to lack of awareness and resources. It is evident from the reports that both multi-drug resistant (MDR) and extensively drug resistant bacteria identified in Pakistan since last few years (Bilal, H., Khan *et al*., 2021). Pakistan faced outbreak of XDR Salmonella in 2016 and this strain showed 100% resistance to fluoroquinolones (Qamar, F. N *et al*., 2018). However, it is not limited to *Salmonella*. The number of clinical isolates posing global threats such as *Pseudomonas aeruginosa, Enterococci* especially Vancomycin Resistant *Enterococci* (VRE), Methicillin Resistant *Staphylococcus aureus* (MRSA) and members of family *Enterobacteriaceae*, including *Klebsiella pneumoniae*, *E. coli*, and *Psroteus sp*., with high level antibiotic resistance reported worldwide (Arias, C. A., & Murray, B. E. ,2009; Basak *et al*.,2016; de Oliveira Santos *et al*., 2022). One of the main cause of increase

in antibiotic resistance particularly in developing countries is indiscriminate use due over the counter availability of antibiotics. The excessive use of antibiotics in agriculture and livestock also increasing the burden of resistance against antibiotics worldwide (Omoya and Ajayi, 2016). Presence of antibiotic resistance strains in hospitals further worsen the situation and nosocomial infections of resistant bacterial strains considered as a major cause of deaths in developing countries globally (Ducel *et al*., 2002). Gram-negative bacteria cause infections that lead to intensive care unit (ICU) with significantly high risk of morbidity and mortality therefore considered as most substantial health problem. Increase of antibiotic resistance in gram negative bacteria is therefore one of the major health problem (Oliveira, J., & Reygaert, W. C, 2019). Gram- negative bacteria have many ways to prevent the action of antibiotics including efflux pump, alteration in binding site and permeability of membrane and enzymes (Ruppé *et al*.,2015). However, outer membrane of gram negative bacteria is the main contributor to a resistance against extensive range of antibiotics. A small variation in outer membrane by gram negative bacteria such as alteration in hydrophobic characteristics can create resistance. As Gram positive bacteria do not possess outer membrane which makes it more resistant to antibiotics (Breijyeh *et al*.,2020). Various ecological studies have shown the association of increased antibiotic consumption with the emergence of resistance in various bacterial genera (Mevius *et al*., 2007; NethMap, 2008). Beta-lactamases are also considered as leading cause of resistance in gram-negative bacteria having worldwide spread and are found in many species of *Escherichia coli and Klebsiella pneumoniae* globally (Bradford, 2001; Jacoby and Munoz-Price, 2005; Queenan and Bush, 2007; Jacoby, 2009). They have also been found in strains of *Pseudomonas aeruginosa*. These strains are therapeutic challenges in the treatment of infections (Wood *et al*.,2023). The increase in the occurrence of Gram-negative MDR species is widely acknowledged by global and national organizations as a global threat including the WHO, European Centre for Disease Prevention and Control. Infectious Diseases Society of America (IDSA), and the US CDC, NIH Pakistan. The purpose of the current study is to find out the MICs of antibiotics against prevalent multi drug resistant (MDR) strains of the local clinical gram-negative bacteria.

MATERIALS AND METHODS

Collection of clinical isolates: Gram negative bacteria were obtained from the following pathological labs of Karachi,

1. Essa Lab s
2. Sindh Lab

The bacteria were collected and maintained on tryptone agar (Oxoid) slants and kept at 4°C.

Culture Identification: Gram negative bacteria collected from different labs were identified on the basis of microscopy (by using standard gram staining procedure), Colonial characteristics on Mackonkey's and different standard biochemical tests using Bergey's manual.

Media: Mackonkey's agar was used to differentiate lactose fermenters and non-lactose fermenters. Tryptone agar was used for maintaining the bacterial culture stock. For liquid inoculum preparation LB broth (Bactotryptone 10 g/L, Yeast Extract 1g/L, NaCl 8g/L) was used. For the determination of Minimal Inhibitory concentrations (MICs), Muller Hinton broth was used. All culture media were purchased from local oxoid distributor.

Antibiotics: Antibiotics used were ampicillin trihydrate (A), co–amoxiclav (C), gentamicin sulfate (G), neomycin sulfate (N), streptomycin sulfate (S) and tetracycline hydrochloride (T), these antibiotics were from Sigma, U.S.A. Antibiotic stock solutions (10 mg/ ml) were made in distilled water, sterilized by Millipore filters and kept at -20ºC.

Resazurin stain: Resazurin dye was purchased from Sigma Chemical Company, U.S.A. Stock solution (5 mg/ml) was prepared by dissolving 500mg Resazurin Sodium salt into 100ml PBS. Working solution (500 µg/ml) was prepared by 1:10 dilution in PBS.

Determination of Minimal Inhibitory Concentration (MIC) by broth micro-dilution method: All the cultures showed resistance to tested antibiotics on replica plate method for another study (under publishing process) were tested for Minimal inhibitory concentrations (MICs) in this study. MIC values of the six antibiotics were determined by using broth micro-dilution method in sterile 96 well micro-titer plates according tos the following protocol:

i. Antibiotic dilution preparations For each antibiotic, 10mg/ml of stock was diluted to 3mg/ml, 2mg/ml, 1mg/ml and 0.5 mg/ml. To achieve the desired concentration in micro-titer plate, 2X solution of each antibiotic was used.

ii. Preparation of bacterial suspensions

Bacteria were grown overnight in L.B. Broth at 37^0C, standardized by using 0.5 McFarland's Index, 10µl of bacterial suspension was dispensed into the wells.

iii. Preparation of micro-titer plates

To perform the assay 100µl of the MHB was dispensed into all wells of a micro-titer plate. 100µl of appropriate antibiotic (2X) was added in the wells in column 1. From the 1st column, 100µl was transferred into column 2, mixed well and 100µl was transferred serially till column 10 to make two-fold dilution, 100µl was discarded from column 10 (The concentration of antibiotic in each of the well after the two-fold dilution is presented in the Table#1). Last two wells were used as control. Initially, all the selected antibiotics tested against clinical isolates with starting concentration of 500 µg/ml. All those isolates showed resistance to set 1 (Table#1) concentrations were challenged with higher concentration. At the end 10ul of bacterial suspension was added to each well, except the 12th one which served as a negative control.

Plate was incubated at 37^0C for 24 hours. Next day each well was added by 10µl of resazurin stain (500 µg/ml) to observe the viability of bacteria. The lowest concentration of the antibiotic that inhibited the growth was considered as MIC of that antibiotic. (Elshikh *et al*., 2016)

RESULTS AND DISCUSSION

Collection, Purification and Characterization of bacterial isolates from clinical sources: On the basis of various biochemical tests, the clinical isolates were identified as *Escherichia coli* (total 29), *Klebsiella* (total 17), *Salmonella typhi* (total 11), *Pseudomonas* (total 9), *Proteus* (total 6), *Aeromonas* (total 6), *Enterobacter* (total 3), *Shigella* (total 1) and *Morganella* (total 1), (Table#2). Minimum Inhibitory Concentration of antibiotics: MICs of resistant isolates were obtained against 83 clinical isolates to know their existing resistant levels. The results are presented in Table #3. According to CLSI standards of MIC, each antibiotics was characterized into 'Susceptible, Intermediate and Resistant' (Kosikowska *et al*., 2020). For ampicillin, 4.8% of the isolates were found Susceptible, 3.6% were Intermediate while 91.5% were found Resistant. For co-amoxiclav, 1.2% of the isolates were found Susceptible, 6% were Intermediate while 92.7% were found Resistant. For gentamicin, 3.6% of the isolates were found Susceptible, 10.8% were Intermediate while 86.7% were found Resistant. For neomycin, 21.6% of the isolates were found Susceptible, 40.9% were Intermediate while 43.4% were found Resistant. For streptomycin, 1.2% of the isolates were found Susceptible, 18% were Intermediate while 91.6% were found Resistant. For tetracycline, 15.7% of the isolates were found Susceptible, 45.7% were Intermediate while 53% were found Resistant (Figure 1.1 to 1.6). Of these bacteria, 99% were found resistant to one or more of the following antibiotics: ampicillin, co-amoxiclav, gentamycin, neomycin, streptomycin and tetracycline. This high percentage reflects the indiscriminate use of antibiotics (Al-Tawfiq *et al.*, 2020) . It was interested to note that most of the isolates were multi drug resistant (MDR) having resistance to three or more antibiotics at a time. Among the resistant bacteria, 11% were found to resistant to three antibiotics, 24% were resistant to four, 43% were resistant to five and 17% were resistant to all the six antibiotics at a time. The results of this study confirmed the presence of multidrug resistance in local population of clinical gram-negative bacteria. As far as the MIC values are concerned, most of the strains were found 'Resistant' against all the six antibiotics except for neomycin which showed relatively lesser amount of resistance by bacteria. These MDR strains are the global challenge due to the lack of effective treatment options (Wise and Piddock, 2010; Norouzi *et al*., 2014; Memon *et al*., 2022). The resistant strains are not only found in clinical bacteria but also has emerged in increase number in opportunistic and commensal bacteria (Chopra and Roberts, 2001). The drug resistance emergence is related to the indiscriminate use of antibiotics in clinical and veterinary practices and in agriculture and it is supported by different studies (Alsaedi *et al*., 2022). There is thus an emergent need for both novel classes of antibiotics and novel approaches for the treatment.

Table 01. Concentrations of all tested antibiotics achieved in a micro-titer plate (column 1-10) for MIC determination using this table

Concentrations of all the antibiotics used in µg/ml										
Set 1	500	250	125	62	31	15	7	3.5	1.75	0.87
Set 2	1000	500	250	125	62	31	15.5	7.75	3.87	1.93
Set 3	2000	1000	500	250	125	62	31	15.5	7.75	3.87
Set 4	3000	1500	750	375	187.5	93.75	46.8	23.4	11.7	5.8

Table#2: Biochemical characterization of clinical isolates collected from diagnostic laboratories

Triple Sugar Iron Test				**Lac**	**Cat**	**Oxi**	**Urease**	**Ind**	**MR**	**VP**	**Cit**
S	**B**	**H_2S**	**G**								
Y	Y	-	+	+	+	-	-	+	+	-	-
Culture codes identified as: *Escherichia coli*				KI-1, KI-2, KI-3, KI-4, KI-5, KI-6, KI-7, KI-8, KI-18,KI-20, KI-21, KI-22, KI-25,KI-28, K-30, K-31, K-32,K-37, K-38, K-60, K-61, K-62, K-65, K-66, K-69, K-71, K-73, K-74, KI-88.							
R	Y	-	-	-	+	-	+	-	-	+	+
Culture codes identified as: *Pseudomans spp*				KI-12, K-15, K-27, KI-45, KI-52, KI-54, KI-57, KI-76, KI-83.							
Y	Y	-	V	+/ -	+	-	+	+/-	-	+	+
Culture codes identified as: *Klebsiella spp*				KI-9, KI-23, KI-33, KI-34, KI-36,KI-39,KI-42, KI-47, KI-53, KI-56, KI-58, KI-59, KI-63, KI-67, KI-70, KI-81, KI,87.							
Y	Y	+	+	-	+	+	-	+	-	+	-

Culture codes identified as: *Aeromonas spp*				KI-10, KI-35, KI-46, KI-64, KI-75, KI-84.							
R	Y	+	+	-	+	-	+	-	-	+	V
Culture codes identified as: *Proteus spp*				KI-13, KI-44,KI-48, KI-80,KI-85,KI-86.							
Y	Y	-	+	-	+	-	-	-	-	+	+
Culture codes identified as: *Enterobacter spp*				KI-16, KI-24, KI-29.							
R	Y	-	-	-	+	-	-	-	+	-	-
Culture codes identified as: *Shigella spp*				KI-17							
R	Y	-	V	-	+	-	+	+	+	-	-
Culture codes identified as: *Morgenella spp*				KI-19							
R	Y	+	V	-	+	-	-	-	-	+	-
Culture codes identified as: *Salmonella typhi*				KI-40, KI-41, KI-43, KI-49, KI-50, KI-51, KI-55, KI-68, KI-72, KI-79, KI-82.							

Key for Table#2:
Triple sugar iron test: S = slant, B= But, Y= Yellow (acidic), R = Red (Alkaline), G= gas production, H2S = Production of H2S gas, V= variable results (+/-) while Lac= Lactose broth fermentation, Cat = Catalase, Oxi = Oxidase, Ind= Indole, MR= Methyl Red, VP = Voges Praoskauer test and Cit = Citrate utilization test. Symbol (+) = presence of activity and (–) absence of activity.

Table#3: MIC Values for the clinical isolates (µg/ml) using 96-well plate

S.No.	Culture code	A	C	G	N	S	T
1	*Escherichia coli* KI-1	500	375	125	5.8	375	3
2	*Escherichia coli* KI-2	1500	125	125	1.9	250	31
3	*Escherichia coli* KI-3	2000	1000	1000	7	125	125
4	*Escherichia coli* KI-4	93.75	375	187.5	5.8	11.7	31
5	*Escherichia coli* KI-5	3000	187.5	187.5	3	500	31
6	*Escherichia coli* KI-6	1000	3000	500	375	31	7
7	*Escherichia coli* KI-7	500	125	125	5.8	7	500
8	*Escherichia coli* KI-8	3000	500	500	500	2000	31
9	*Klebsiella* KI-9	2000	2000	250	15	500	62
10	*Aeromonas* KI-10	125	1000	250	62	1000	31
11	*Pseudomonas* KI-12	375	750	250	93.75	93.75	500
12	*Proteus* KI-13	500	375	62	5.8	375	187.5
13	*Pseudomonas* KI-15	375	2000	250	7	250	31
14	*Enterobacter* KI-16	23.4	750	500	3	250	31
15	*Shigella* KI-17	1500	500	250	3	62	15
16	*Escherichia coli* KI-18	500	500	31	11.75	62	250
17	*Morgenella* KI-19	1.9	750	1000	7	500	15
18	*Escherichia coli* KI-20	500	750	2000	62	2000	93.75
19	*Escherichia coli* KI-21	3000	500	500	62	1000	62
20	*Escherichia coli* KI-22	2000	750	500	62	93.75	250
21	*Klebsiella* KI-23	500	187.5	15	7	31	31
22	*Enterobacter* KI-24	500	7	1500	500	250	15
23	*Escherichia coli* KI-25	3000	93.75	125	3	125	7
24	*Pseudomonas* KI-27	1500	500	500	5.8	125	125
25	*Escherichia coli* KI-28	2000	250	187.5	5.8	1000	31
26	*Enterobacter* KI-29	500	750	750	5.8	375	93.75
27	*Escherichia coli* KI-30	62	500	250	31	125	3
28	*Escherichia coli* KI-31	750	500	125	500	31	3
29	*Escherichia coli* KI-32	2000	250	62	62	500	3
30	*Klebsiella* KI-33	3000	500	2000	250	2000	15
31	*Klebsiella* KI-34	250	500	1000	500	187.5	31
32	*Aeromonas* KI-35	3000	1500	500	7	250	62
33	*Klebsiella* KI-36	375	187.5	11.7	375	11.7	46.8
34	*Escherichia coli* KI-37	2000	1000	750	23.4	250	7
35	*Escherichia coli* KI-38	2000	5.8	2000	125	750	3
36	*Klebsiella* KI-39	187.5	500	500	250	31	7
37	*Salmonella typhi* KI-40	500	500	31	500	250	15
38	*Salmonella typhi* KI-41	750	1000	375	15	1000	62
39	*Klebsiella* KI-42	375	500	11.7	11.7	93.75	93.75
40	*Salmonella typhi* KI-43	500	500	125	3	125	62

41	*Proteus* KI-44	500	2000	62	3	500	3
42	*Pseudomonas* KI-45	500	2000	2000	3	2000	3
43	*Aeromonas* KI-46	15	500	2000	3	62	3
44	*Klebsiella* KI-47	1.9	93.75	23.4	5.8	62	23.4
45	*Proteus* KI-48	1500	500	500	3	250	375
46	*Salmonella typhi* KI-49	93.75	2000	750	1000	1000	3
47	*Salmonella typhi* KI-50	187.5	1500	46.8	31	250	62
48	*Salmonella typhi* KI-51	500	500	500	7	62	7
49	*Pseudomonas* KI-52	23.4	7	3	3	31	3
50	*Klebsiella* KI-53	187.5	375	375	5.8	1500	1.9
51	*Pseudomonas* KI-54	2000	500	1000	250	500	31
52	*Salmonella typhi* KI-55	3000	2000	2000	7	250	62
53	*Klebsiella* KI-56	2000	1000	1000	3	31	3
54	*Pseudomonas* KI-57	1000	375	93.75	250	500	3
55	*Klebsiella* KI-58	3000	1500	250	500	2000	250
56	*Klebsiella* KI-59	375	375	125	500	500	15
57	*Escherichia coli* KI-60	3000	1500	1000	3	187.5	93.75
58	*Escherichia coli* KI-61	500	375	5.8	5.8	93.75	11.7
59	*Escherichia coli* KI-62	187.5	1500	46.8	31	250	62
60	*Klebsiella* KI-63	3000	250	62	31	1000	3
61	*Aeromonas* KI-64	3000	3000	2000	2000	2000	125
62	*Escherichia coli* KI-65	2000	1500	125	7	500	31
63	*Escherichia coli* KI-66	93.75	62	15	7	31	15
64	*Klebsiella* KI-67	93.75	1000	125	3	1000	125
65	*Salmonella typhi* KI-68	375	750	2000	1000	500	250
66	*Escherichia coli* KI-69	3000	125	23.4	5.8	1.9	31
67	*Klebsiella* KI-70	750	750	1500	15	1000	125
68	*Escherichia coli* KI-71	3000	1500	11.7	11.7	375	11.7
69	*Salmonella typhi* KI-72	46.8	250	62	3	125	3
70	*Escherichia coli* KI-73	1500	250	125	3	125	15
71	*Escherichia coli* KI-74	3	500	250	7	1000	31
72	*Aeromonas* KI-75	375	125	500	3000	1500	15
73	*Pseudomonas* KI-76	2000	1500	750	3	250	7
74	*Salmonella typhi* KI-79	0.95	3	1.5	5.8	5.8	11.7
75	*Proteus* KI-80	500	500	250	31	31	3
76	*Klebsiella* KI-81	1500	2000	500	7	31	62
77	*Salmonella typhi* KI-82	93.75	1000	500	1000	500	62
78	*Pseudomonas* KI-83	1000	1000	250	500	500	15
79	*Aeromonas* KI-84	500	7	1000	62	46.8	3
80	*Proteus* KI-85	3000	1000	500	250	7	15
81	*Proteus* KI-86	2000	2000	1000	187.5	250	7
82	*Klebsiella* KI-87	500	15	11.7	11.7	1500	187.5
83	*Escherichia coli* KI- 88	2000	93.75	7	3	125	7

KEY for Table #3: A = Ampicillin, C = Co- amoxiclav, G = Gentamicin, N = Neomycin, S = Streptomycin, T = Tetracycline

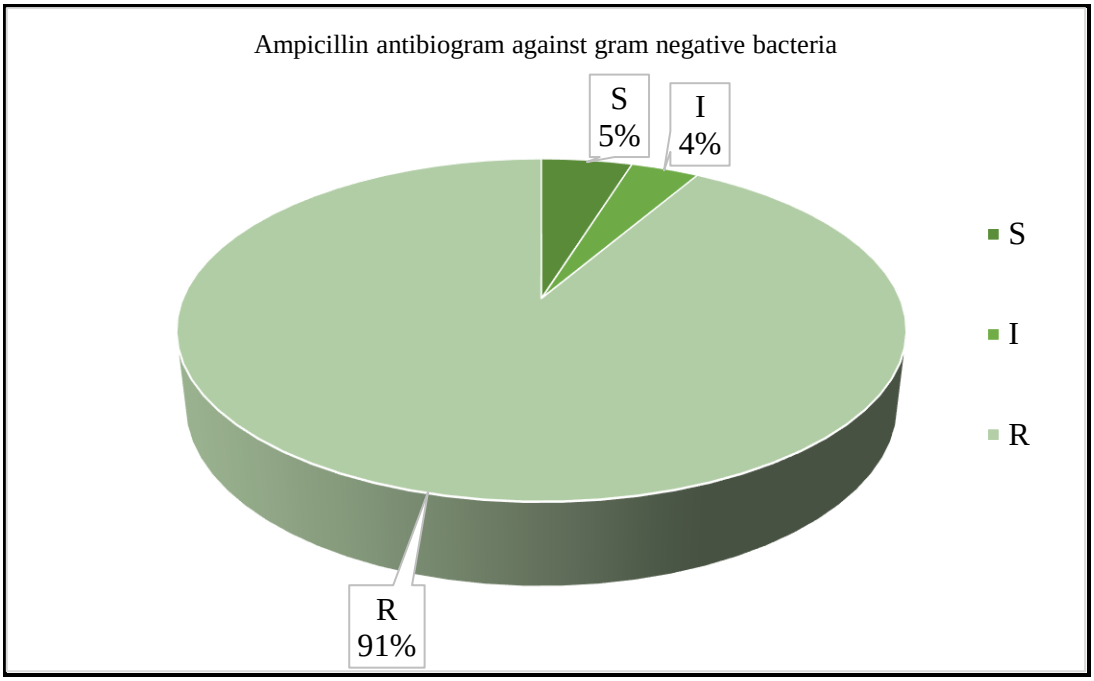

Fig. 1.1: MIC of Ampicillin against Gram -negative bacteria

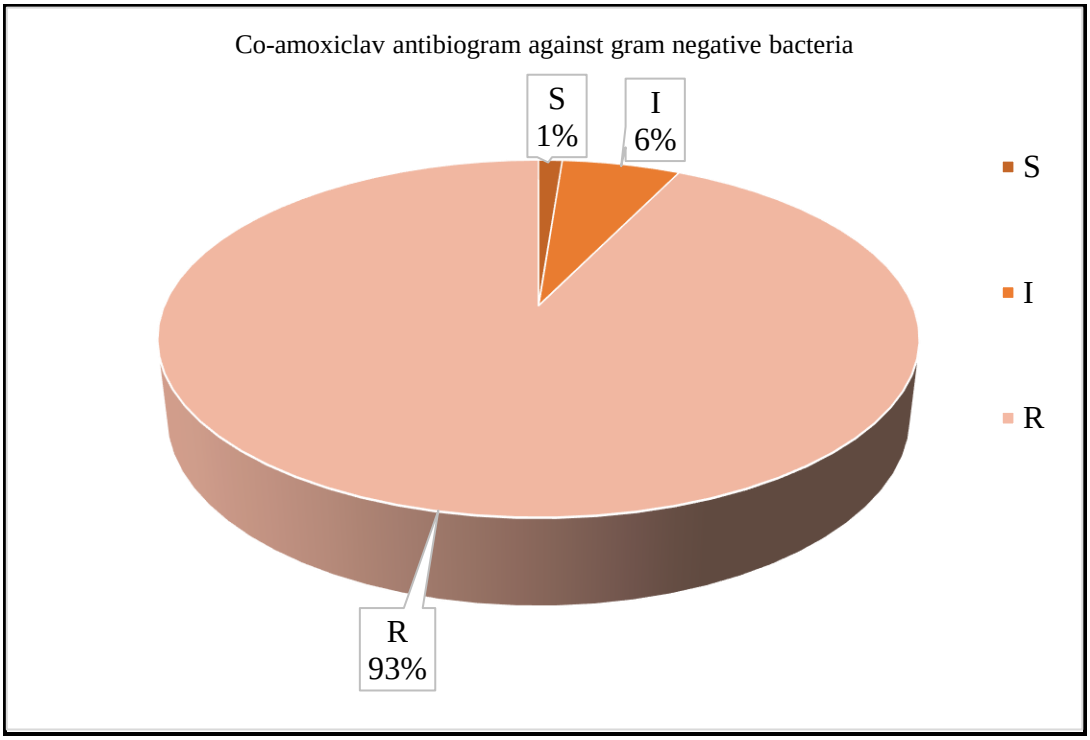

Fig. 1.2: MIC of Co-amoxiclav against Gram - negative bacteria

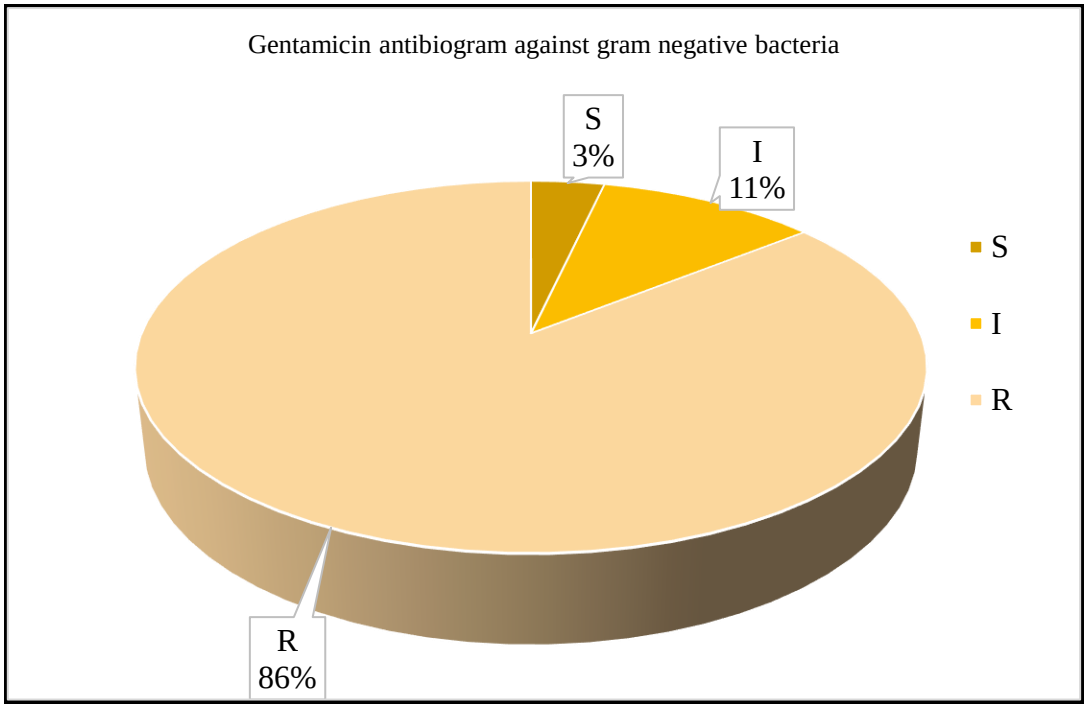

Fig. 1.3: MIC of Gentamicin against Gram- negative bacteria

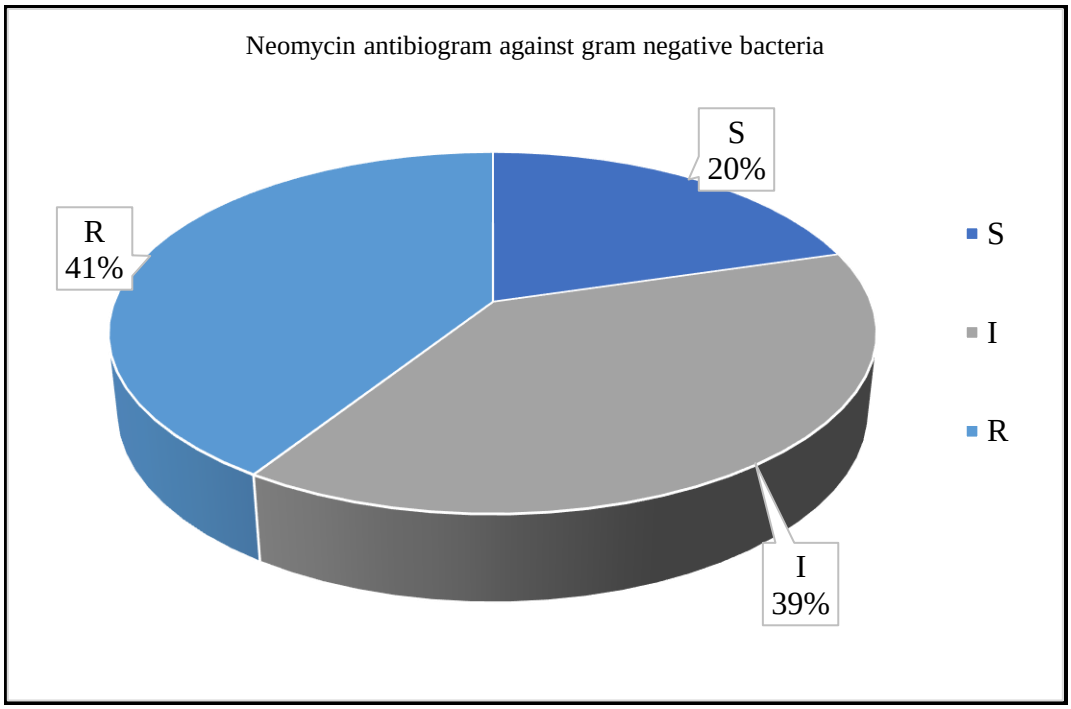

Fig. 1.4: MIC of Neomycin against Gram- negative bacteria

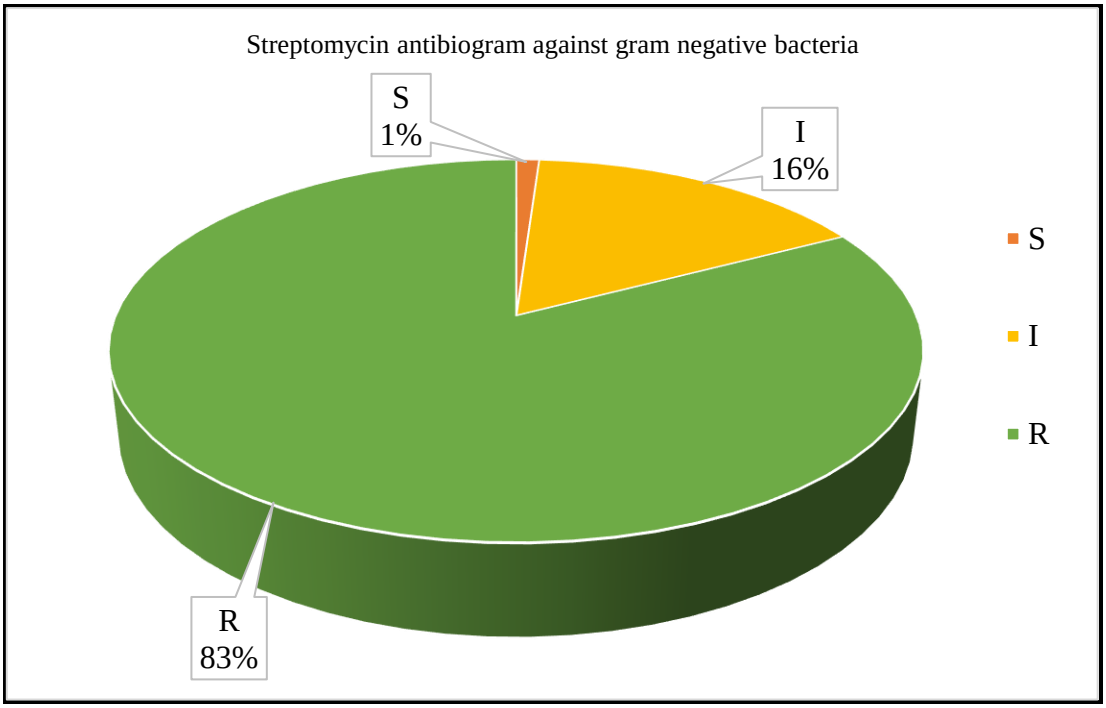

Fig. 1.5: MIC of Streptomycin against Gram -negative bacteria

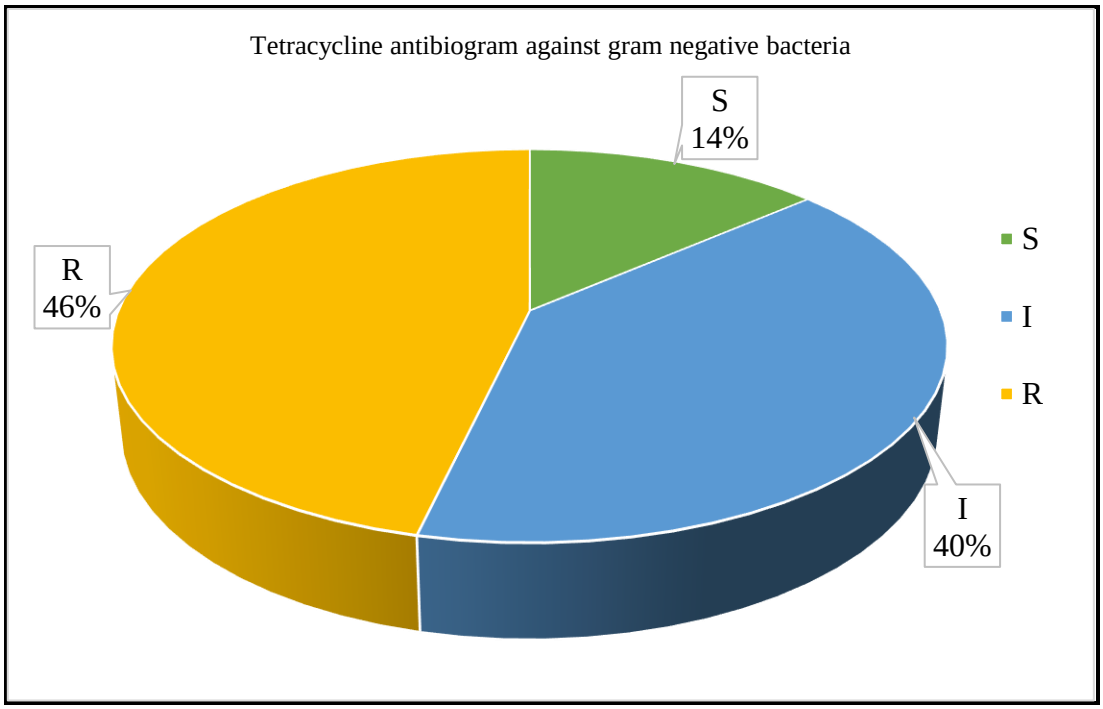

Fig. 1.6: MIC of Tetracycline against Gram -negative bacteria

ACKNOWLEDGEMENTS

The study was supported by the Dean research grant, faculty of Science, University of Karachi.

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