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EVALUATION OF MULTI DRUG RESISTANCE (MDR) IN CLINICAL GRAM NEGATIVE ISOLATES FROM KARACHI

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ABSTRACT

Total 83 gram negative bacteria were obtained from different clinical laboratories situated in Karachi during 2018-2019. Of these bacteria, 99% were found resistant against one or more of the following antibiotics: ampicillin, co-amoxiclav, gentamycin, neomycin, streptomycin and tetracycline. Frequency of resistance of bacterial isolates to individual antibiotics were found to be 92% for ampicillin, 94% for co-amoxiclav, 82% for gentamycin, 40% for neomycin, 90% for streptomycin and 53% for tetracycline. It was interesting to note that most of the isolates were MDR (multi-drug resistant) having resistance to three or more antibiotics at a time. Among the resistant bacteria, 11% were found to be resistant to three antibiotics, 24% were resistant to four antibiotics, 43% were resistant to five antibiotics and 17% were resistant to six antibiotics at a time. Some of the resistances were found transferable to sensitive bacteria in conjugation experiments showing the presence of conjugative R plasmids. The resistances were not completely cured by acridine orange showing the stability of these R plasmids in their bacterial hosts.

Key-words: antibiotics, multi drug resistant (MDR), clinical, conjugation, acridine orange

INTRODUCTION

Antibiotics belong to the class of antimicrobial agents that inhibit bacteria by various mechanisms including inhibition of nucleic acid synthesis, inhibition of protein synthesis, inhibition of cell wall synthesis and interference with folate metabolism (Basak *et al*., 2016). The choice of an antibiotic for the treatment of an infection has limitations as many antibiotics have their side effects, also some in high doses are associated with adverse reactions (Bernal *et al*., 2013). New antibiotics have been introduced on routine basis and the development of resistance against these have also been reported continuously, which is a global concern presently (Syed and Ravaoarinoro, 2012). Now a days, resistant pathogens contribute a major problem in the treatment of patients as it leads to increased mortality and morbidity and great economic loss to the patient and also to a nation (Alanis, 2005). In the 21st century the infections caused by resistant bacteria have become a major healthcare problem (Bradford, 2001; Memon *et al.,* 2022). The development of drug resistance observed in clinical, veterinary practices and agriculture is related to the indiscriminate use of antibiotics which is supported by various studies that have clearly correlated the resistance emergence and spread with the use of antibiotics (Davies, 1994; Pallecchi *et al*., 2007). Researchers seem that antibiotic resistance has emerged due to the selective pressure of antibiotics on the microorganisms (Gaur *et al*., 2006). Bacterial species specially from clinical sites have become resistant to conventional antibiotics and also the new and developmental antibiotics are few in number against Gram negative bacteria (Cornaglia, 2009; Baiden *et al*., 2010; Wise and Piddock, 2010). In Pakistan, *E. coli* isolates which were multidrug resistant to quinolones and third generation cephalosporin and quinolones were reported during 1980-1990, also there were reports of MDR *S. typhi* bearing R plasmids of epidemic typhoid fever in Pakistan and India (Khan *et al*., 1980; Pickering, 2004). These resistant superbugs are also a threat in the treatment nowadays (Memon *et al.,* 2022). The clinically important bacteria including *Pseudomonas, aeruginosa*, Methicillin Resistant *Staphylococcus aureus* (MRSA), and members of family *Enterobacteriaceae*, as *Klebsiella pneumoniae*, *E. coli*, and *Salmonella* sp., spread in the hospital environment by rapidly developing antibiotic resistance **(**Syed and Ravaoarinoro, 2012). The resistance is much more dangerous when it is transferable to other bacteria (horizontal gene transfer) by the process of conjugation (Arnold, 1980; Basak *et al*., 2016). In this era as we know the antibiotic resistance has been increasing day by day. Constant monitoring of susceptibility pattern is necessary on clinical isolates for selection of appropriate antimicrobial agents for therapy (Pickering, 2004). Efforts have been made to synthesize new antibiotics. The use of old antibiotics has also gained

interest because of the bacterial sensitivity (Chand *et al*. 2014). Now, it is required to use some noble (old) antibiotics to make it valuable, reusable through possible combinations with new antibiotics against clinical isolates. This would help to fight against more advanced form of microbes and also in the treatment of diseases caused by these microorganisms. The purpose of the current study was to find out the resistance profile of local clinical gram-negative bacteria against commonly used antibiotics and to check the transferability and stability of the antibiotic resistance in these bacteria.

MATERIALS AND METHODS

Sources of gram-negative bacteria

Gram negative bacteria were obtained from the following pathological laboratories of Karachi,

1. Essa Lab
2. Sindh Lab

The bacteria were collected and maintained on tryptone agar slants and kept at 4°C before which these were purified twice on MacConkey's agar.

Standard bacterial strain

The standard bacterial strain used in the study was *E.coli* 40MD obtained from M. Dubow of Canada. It was used as a recipient strain in conjugation experiments having high level of chromosomal streptomycin resistance. The genotype is indicated below:

F $^-$ Δ pro lac *trp$^-$ str^r*

Media

Tryptone agar consisted of bactotryptone 17g/L, agar 6g/L (pH 7.0). Resistance determinations were made on MacConkey's Agar (Oxoid), to which single antibiotics were added at desired concentrations (25 µg/mL, 50 µg/mL and100 µg/mL). Minimal Inhibitory concentrations (MICs) of the standard bacterial strain, used as a recipient in conjugal crosses, was determined as described by Jahan (1991) and Saeed (2003). The standard strain was inhibited at the concentration of 30 µg of antibiotic per mL of the medium, for all antibiotics. The *E. coli* 40 MD had high level, chromosomal streptomycin resistance and could resist more than 500 µg/mL.

For conjugation experiments, bacterial cultures were grown in L.B. broth (Bactotryptone 10 g/L, yeast extract 1 g/L, NaCl 8 g/L). The transconjugants were selected on Minimal Agar or on MacConkey's Agar, depending on the nature of the conjugal cross. Minimal Agar had the same composition as that of Davis Minimal Agar (Difco).

Antibiotics

Following antibiotics were used: 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.

Amino acids

Amino acids proline and tryptophane were from Sigma, U.S.A. Stocks of 2 mg/mL were prepared in distilled water, sterilized by Millipore filters and kept at – 20ºC.

Determination of Antibiotic Resistance and Sensitivity of clinical isolates

For the determination of the antibiotic resistance or sensitivity of the indigenous bacteria collected from various clinical sources, the procedure of velvet replication as used by Jahan (1991) was adopted. The replication was carried out on MacConkey's agar plates containing 25 µg/mL, 50 µg/mL and100 µg/mL of each of the test antibiotics.

Conjugal transfer of Antibiotic resistance to Sensitive bacteria

For Conjugation experiments, cultures of donor (resistant) and standard recipient bacteria (*E. coli* 40MD) were grown in L.B. broth for overnight at 37ºC. Next morning 1000 µl of both donor and recipient bacteria were added into fresh10mL L.B. broth and grown with shaking at 37 ^{0}C for about 1 hour to obtain

the log phase culture. Donor and recipient cultures were mixed in 1:10 ratio and incubated at 37ºC for two hours. Recipient and donor controls were also prepared and incubated simultaneously. After two hours the control and conjugation mixture tubes were centrifuged at 3500 R.P.M. for 30 minutes. Supernatant was discarded and sediment was streaked on selectively prepared MacConkey's agar medium (mixed with antibiotics). Plates were at 37ºC for overnight and next day observed for any transconjugants (bacteria acquiring resistance or R plasmid).

Transconjugants of wild type donors were selected on the basis of streptomycin resistance and lactose fermentation character. Most of the donors used in the conjugation experiment were streptomycin sensitive (or having very low-level resistance as confirmed by MICs). On the other hand, the recipient (*E. coli* 40MD) carried high level chromosomal resistance to streptomycin. Transconjugants could then be selected on MacConkey's agar plates carrying an appropriate antibiotic at 50µg/mL (to which the recipient was sensitive) and 200µg/mL of streptomycin (to which the donor was sensitive). The markers acquired during conjugation (resistance to antibiotics) were then confirmed by replication, for which individual antibiotic plates were prepared using MacConkey's agar (A_{50}µg/mL, C_{50}µg/mL, G_{50}µg/mL, N_{50}µg/mL S_{200}µg/mL, T_{50}µg/mL). The minimal medium with or without supplements of amino acids (tryptophan and proline) was also prepared (to confirm the auxotroph recipient).

Curing of the Conjugative R plasmids

In order to study the curing of resistant markers by acridine orange, the transconjugant bearing R plasmid was inoculated in L.B. broth and kept at 37°C for overnight. Next morning, the overnight culture is used to inoculate fresh L.B. bearing concentration, 100µg/mL of acridine orange (100-fold dilution). Because of the sensitivity of acridine orange towards light, all of the work was carried out in the dark. The test and control cultures (with and without acridine orange respectively) were incubated over night at 37 ^{0}C. Next morning, both the control and the test cultures were diluted and plated on MacConkey's Agar and incubated to get the isolated colonies. Some 100 colonies from test plates were picked onto MacConkey's agar plates along with the original host and *E. coli* 40MD as control. After overnight incubation at 37^{0}C, the colonies were replicated on MacConkey's agar plates containing each of the test antibiotics. Plain MacConkey's agar plates were used as controls in the beginning and at the end of each replication. Presence of growth was taken as resistance and absence of growth was taken as sensitivity (loss of resistance) of the culture.

RESULTS AND DISCUSSION

Collection, Purification and Characterization of bacterial isolates from clinical sources

Total 83 gram-negative bacteria were obtained from different hospitals and labs of Karachi. On the basis of various biochemical tests, the isolates were identified as *Escherichia coli* (Total 29), *Klebsiella* (Total 17), *Salmonella typhi* (Total 11), *Pseudomonas* (Total 9), *Proteus* (Total 6), *Enterobacter* (Total 3), *Shigella* (Total 1) and *Morganella* (Total 1), while 14 remained unidentified (Table 1).

Determination of Resistance Pattern

Resistant profile was determined for all the isolates after screening to the commonly used antibiotics: ampicillin, co-amoxiclav, gentamycin, neomycin, streptomycin and tetracycline. The resistance was tested at three different levels i.e., 100µg/mL, 50µg/mL and 25 µg/mL of each antibiotic. A resistance pattern was determined for each bacterium after screening it against all the six antibiotics (Table 1). Of the Total 83 bacteria collected, 82 (99%) were found resistant to one, two or more antibiotics. Frequency of resistance of bacterial isolates to individual antibiotics were found to be 92% for ampicillin, 94% for co-amoxiclav, 82% for gentamycin, 40% for neomycin, 90% for streptomycin and 53% for tetracycline. The high frequency of antibiotic resistance among clinical bacteria is alarming as it causes hazards in the treatment. This high percentage reflects the indiscriminate use of antibiotics.

It was an interesting finding that most of the isolates were resistant to more than one antibiotic (multi drug resistant). Among the resistant bacteria, 11% were found to be resistant to three antibiotics, 24% were resistant to four antibiotics, 43% were resistant to five antibiotics and 17% were resistant to six antibiotics at a time. The findings reported here confirmed the presence of multidrug resistance in local population of clinical gram-negative bacteria.

Table 1. Gram negative bacteria and their resistance pattern.

S.no.	Bacterial Strains	Resistant pattern* (µg/mL)
1.	*Escherichia coli* KI-1	$A_{100}C_{100}G_{100}S_{100}$
2.	*Escherichia coli* KI-2	$A_{100}C_{100}G_{100}S_{100}T_{25}$
3.	*Escherichia coli* KI-3	$A_{100}C_{100}G_{100}S_{100}T_{100}$
4.	*Escherichia coli* KI-4	$A_{50}C_{100}G_{100}T_{25}$
5.	*Escherichia coli* KI-5	$A_{100}C_{100}G_{100}S_{100}T_{25}$
6.	*Escherichia coli* KI-6	$A_{100}C_{100}G_{100}N_{100}S_{25}$
7.	*Escherichia coli* KI-7	$A_{100}C_{100}G_{100}T_{25}$
8.	*Escherichia coli* KI-8	$A_{100}C_{100}G_{100}N_{100}S_{100}T_{25}$
9.	*Klebsiella* KI-9	$A_{100}C_{100}G_{100}S_{100}$
10.	*Aeromonas* KI-10	$A_{100}C_{100}G_{100}N_{100}S_{100}T_{25}$
11.	*Pseudomonas* KI-12	$A_{100}C_{100}G_{100}N_{50}S_{50}T_{100}$
12.	*Proteus* KI-13	$A_{100}C_{100}G_{50}S_{100}T_{100}$
13.	*Pseudomonas* KI-15	$A_{100}C_{100}G_{100}S_{100}T_{25}$
14.	*Enterobacter* KI-16	$C_{100}G_{100}S_{100}T_{25}$
15.	*Shigella* KI-17	$A_{100}C_{100}G_{100}S_{100}T_{25}$
16.	*Escherichia coli* KI-18	$A_{100}C_{100}G_{25}S_{50}T_{100}$
17.	*Morgenella* KI-19	$C_{100}G_{100}S_{100}$
18.	*Escherichia coli* KI-20	$A_{100}C_{100}G_{100}N_{50}S_{100}T_{50}$
19.	*Escherichia coli* KI-21	$A_{100}C_{100}G_{100}N_{50}S_{100}T_{50}$
20.	*Escherichia coli* KI-22	$A_{100}C_{100}G_{100}N_{50}S_{100}T_{100}$
21.	*Klebsiella* KI-23	$A_{100}C_{100}S_{25}T_{25}$
22.	*Enterobacter* KI-24	$A_{100}G_{100}N_{100}S_{100}$
23.	*Escherichia coli* KI-25	$A_{100}C_{50}G_{100}S_{100}$
24.	*Pseudomonas* KI-27	$A_{100}C_{100}G_{100}S_{100}T_{100}$
25.	*Escherichia coli* KI-28	$A_{100}C_{100}G_{100}S_{100}T_{25}$
26.	*Enterobacter* KI-29	$A_{100}C_{100}G_{100}S_{100}T_{50}$
27.	*Escherichia coli* KI-30	$A_{50}C_{100}G_{100}N_{25}S_{100}$
28.	*Escherichia coli* KI-31	$A_{100}C_{100}G_{100}N_{100}S_{25}$
29.	*Escherichia coli* KI-32	$A_{100}C_{100}G_{100}N_{100}S_{25}$
30.	*Klebsiella* KI-33	$A_{100}C_{100}G_{100}N_{100}S_{100}$
31.	*Klebsiella* KI-34	$A_{100}C_{100}G_{100}N_{100}S_{100}T_{25}$
32.	*Aeromonas* KI-35	$A_{100}C_{100}G_{100}S_{100}T_{50}$
33.	*Klebsiella* KI-36	$A_{100}C_{100}N_{100}T_{25}$
34.	*Escherichia coli* KI-37	$A_{100}C_{100}G_{100}S_{100}$
35.	*Escherichia coli* KI-38	$A_{100}G_{100}N_{100}S_{100}$
36.	*Klebsiella* KI-39	$A_{100}C_{100}G_{100}N_{100}S_{25}$ T_{25}
37.	*Salmonella typhi* KI-40	$A_{100}C_{100}G_{25}N_{100}S_{100}$
38.	*Salmonella typhi* KI-41	$A_{100}C_{100}G_{100}S_{100}T_{50}$
39.	*Klebsiella* KI-42	$A_{100}C_{100}S_{50}T_{50}$
40.	*Salmonella typhi* KI-43	$A_{100}C_{100}G_{100}S_{100}T_{50}$
41.	*Proteus* KI-44	$A_{100}C_{100}G_{50}S_{100}$
42.	*Pseudomonas* KI-45	$A_{100}C_{100}G_{100}S_{100}$
43.	*Aeromonas* KI-46	$C_{100}G_{100}T_{25}$
44.	*Klebsiella* KI-47	$C_{50}S_{50}$
45.	*Proteus* KI-48	$A_{100}C_{100}G_{100}S_{100}T_{100}$
46.	*Salmonella typhi* KI-49	$A_{50}C_{100}G_{100}N_{100}S_{100}$
47.	*Salmonella typhi* KI-50	$A_{100}C_{100}G_{25}N_{25}S_{100}T_{50}$
48.	*Salmonella typhi* KI-51	$A_{100}C_{100}S_{50}$

49.	*Pseudomonas* KI-52	S_{25}
50.	*Klebsiella* KI-53	$A_{100}C_{100}G_{100}S_{50}$
51.	*Pseudomonas* KI-54	$A_{100}C_{100}G_{100}N_{100}S_{100}T_{25}$
52.	*Salmonella typhi* KI-55	$A_{100}C_{100}G_{100}S_{100}T_{50}$
53.	*Klebsiella* KI-56	$A_{100}C_{100}G_{100}S_{25}T_{50}$
54.	*Pseudomonas* KI-57	$A_{100}C_{100}G_{50}N_{100}S_{100}$
55.	*Klebsiella* KI-58	$A_{100}C_{100}G_{50}N_{100}S_{100}T_{25}$
56.	*Klebsiella* KI-59	$A_{100}C_{100}G_{100}N_{100}S_{100}$
57.	*Escherichia coli* KI-60	$A_{100}C_{100}G_{100}S_{100}T_{50}$
58.	*Escherichia coli* KI-61	$A_{100}C_{100}S_{50}$
59.	*Escherichia coli* KI-62	$A_{100}C_{100}G_{25}N_{25}S_{100}T_{50}$
60.	*Klebsiella* KI-63	$A_{100}C_{100}G_{50}N_{25}S_{100}$
61.	*Aeromonas* KI-64	$A_{100}C_{100}G_{100}N_{100}S_{100}T_{100}$
62.	*Escherichia coli* KI-65	$A_{100}C_{100}G_{100}S_{100}T_{50}$
63.	*Escherichia coli* KI-66	$A_{50}C_{50}S_{25}$
64.	*Klebsiella* KI-67	$A_{50}C_{100}G_{100}S_{100}T_{100}$
65.	*Salmonella typhi* KI-68	$A_{100}C_{100}G_{100}N_{100}S_{100}T_{100}$
66.	*Escherichia coli* KI-69	$A_{100}C_{100}T_{25}$
67.	*Klebsiella* KI-70	$A_{100}C_{100}G_{100}S_{100}T_{100}$
68.	*Escherichia coli* KI-71	$A_{100}C_{100}S_{100}$
69.	*Salmonella typhi* KI-72	$A_{25}C_{100}G_{50}S_{100}$
70.	*Escherichia coli* KI-73	$A_{100}C_{100}G_{100}S_{100}$
71.	*Escherichia coli* KI-74	C_{100}
72.	*Aeromonas* KI-75	$A_{100}C_{100}G_{100}N_{100}S_{100}$
73.	*Pseudomonas* KI-76	$A_{100}C_{100}G_{100}S_{100}$
74.	*Salmonella typhi* KI-79	Nil
75.	*Proteus* KI-80	$A_{100}C_{100}G_{100}N_{25}S_{25}$
76.	*Klebsiella* KI-81	$A_{100}C_{100}G_{100}S_{25}T_{50}$
77.	*Salmonella typhi* KI-82	$A_{50}C_{100}G_{100}N_{100}S_{100}T_{50}$
78.	*Pseudomonas* KI-83	$A_{100}C_{100}G_{100}N_{100}S_{100}$
79.	*Aeromonas* KI-84	$A_{100}G_{100}N_{50}S_{25}$
80.	*Proteus* KI-85	$A_{100}C_{100}G_{100}N_{100}$
81.	*Proteus* KI-86	$A_{100}C_{100}G_{100}N_{100}S_{100}$
82.	*Klebsiella* KI-87	$A_{100}S_{100}T_{100}$
83.	*Escherichia coli* KI- 88	$A_{100}C_{50}S_{100}$

***** A = Ampicillin, C = Co- amoxiclav, G = Gentamicin, N = Neomycin, S = Streptomycin, T = Tetracycline

Conjugal transfer of Antibiotic Resistance to Sensitive bacteria

The resistant bacteria (potential R plasmid donors) were conjugated to standard *E. coli* 40MD in a Total 56 crosses. Total 7 conjugative R plasmids were isolated by this method (Table 2).

The resistances if found on plasmids are more detrimental as these could be conjugally transferred to non-resistant strains making them resistant (Desai *et al*., 1981; Cooper *et al*., 1983; Sayah *et al*., 2005; Toroglu *et al*., 2005) and not only pose the problem in the treatment of disease but also responsible for the spread of these resistant bacteria.

Curing with Acridine Orange (A.O.)

All the R plasmids listed in Table 2 were studied for curing by acridine orange at the level of 100 µg/mL of the medium. All of these were isolated from clinical bacterial strains. The R plasmids pKA1 and pKA6 did not show curing of any resistance markers. pKA2, pKA3, pKA4, pKA5 and pKA7 showed 95, 92, 96, 98 and 51% loss, respectively of only ampicillin resistance marker, which shows that this marker is considerably curable with acridine orange. pKA2 and pKA7 also showed 81% and 46% loss of co-amoxiclav and tetracycline resistances respectively. It therefore appears that majority of resistances are quite stable in their host cell as far as curing experiments are concerned. Since all the R plasmids were originated from clinical

bacterial strains, these studies are very important and indicate that these R plasmids can create hazards in the treatment of diseases and can spread in bacterial populations, most of them are stable, without even showing loss of their resistances.

The study was useful to obtain the current status of antimicrobial resistance in local clinical isolates and also guide towards the control mechanisms which can be used to limit the emergence and spread of these drug resistant pathogens. The awareness of antimicrobial resistance patterns is very important for the selection of appropriate antibiotic therapy in order to improve treatment outcomes. Emerging antibiotic resistance can be reduced by the reuse of some noble antibiotics, alone and in combination with the new ones.

Table 2. Transconjugants isolated from gram-negative bacteria in Conjugation*.

S.No.	Transconjugants	Original Host (Donor)	Resistance pattern **
1	16-C	*E. coli* (KI-50)	$\mathbf{A_{50}C_{50}\,S_{500}T_{20}}$
2	24-C	*E. coli* (KI-18)	$\mathbf{A_{50}C_{50}\,S_{500}}$
3	31-C	*E. coli* (KI-30)	$\mathbf{A_{50}C_{50}\,S_{500}}$
4	32-C	*Proteus* sp. (KI-80)	$\mathbf{A_{50}C_{50}\,S_{500}\,T_{20}}$
5	49-C	*Proteus* sp.(KI-85)	$\mathbf{A_{50}C_{50}\,S_{500}\,T_{20}}$
6	52-C	*Klebsiella* (KI-39)	$\mathbf{A_{50}C_{50}\,S_{500}T_{20}}$
7	54-C	*E. coli* (KI-84)	$\mathbf{A_{50}C_{50}\,S_{500}T_{20}}$

*The recipient used in the conjugal crosses was *E.coli* 40MD which carried high level chromosomal streptomycin resistance; **A = Ampicillin, C = Co- amoxiclav, S = Streptomycin, T = Tetracycline

Table 3. Curing of resistance determinants of R plasmids by Acridine Orange.

S. No.	R plasmid	Antibiotic Resistance Pattern	Markers Lost	% Loss
1	pKA1	$A_{50}C_{50}\,\mathbf{S_{200}}T_{20}$	Nil	0
2	pKA2	$A_{50}C_{50}\,\mathbf{S_{200}}$	A and C	A= 95 C=81
3	pKA3	$A_{50}C_{50}\,\mathbf{S_{200}}$	A	A= 92
4	pKA4	$A_{50}C_{50}\,\mathbf{S_{200}}T_{20}$	A	A=96
5	pKA5	$A_{50}C_{50}\,\mathbf{S_{200}}T_{20}$	A	A= 98
6	pKA6	$A_{50}C_{50}\,\mathbf{S_{200}}T_{20}$	Nil	0
7	pKA7	$A_{50}C_{50}\,\mathbf{S_{200}}T_{20}$	A and T	A= 51 T=46

A = Ampicillin, C = Co- amoxiclav, S = Streptomycin, T = Tetracycline

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