MULTIDRUG RESISTANT GRAM-NEGATIVE BACTERIA IN CLINICAL ISOLATES FROM KARACHI

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ABSTRACT

A total of 54 gram-negative bacteria obtained from various pathological labs and hospitals of Karachi were screened for their resistance to ampicillin, chloramphenicol, gentamycin, kanamycin, neomycin, streptomycin and tetracycline antibiotics. Of the 54 bacteria, 50 were resistant to one or more antibiotics. Among the resistant bacteria, 13 out of 28 were found to transfer their resistances by conjugation. This indicates that at least 46% of clinical gram-negative bacteria in Karachi possess various types of transferable R plasmids, such as pAK5, pAK9, pAK10, pAK11, pAK12, pAK13, pAK14, pAK15, pAK16, pAK17, pAK18, pAK19, pAK20 and pAK21. The non-conjugative R plasmids included pMT14 and pZ26. Only pAK15 showed 26% segregation even after 20 consecutive transfers in plain broth (spontaneous segregation) whereas only pAK15 and pAK16 showed any significant loss of their markers in curing by acridine orange. The stability of R plasmids is more dangerous from clinical point of view.

Keywords: R plasmids, multi-drug resistance, gram-negative bacteria.

INTRODUCTION

Transmissibility of drug resistance among different species of Enterobacteriaceae by cell-to-cell contact or conjugation was first reported in Japan in 1959 by Ochiai *et al*. (1959). Watanabe (1967) reported that multiple antibiotic resistance spreads among clinical bacteria because of the indiscriminate use of antibiotics in clinical practice. Later on, various workers described the incidence of antibiotic resistance to have a worldwide occurrence (Mitsuhashi *et al.*, 1969; Pocurull *et al.*, 1971; Khatoon, 1971; Anderson and Smith 1972; Davies, 1981 and Krcmery *et al*., 1985). Gram-negative bacteria carrying R plasmids are the most serious problem among antibiotic resistant organisms because their resistance to multiple drugs can spread in epidemic proportion throughout hospitals and whole communities. Non pathogenic bacteria bearing R plasmids are equally dangerous because they can transfer their R plasmids (along with resistances borne by them) to the pathogenic bacteria. Transferable or infectious drug resistance, therefore, constitutes a serious threat to public health.

Multidrug-resistant gram-negative bacteria have lately become more prevalent (Holt *et al.*, 2007; McGawan, 2006 and Wroblewska *et al.*, 2006) and are causing great problems in chemotherapy.

Extra chromosomal elements, including R plasmids may be lost spontaneously from the host cell because of some errors in replication or segregation (Novick, 1969). These losses (elimination or curing) can be increased by treating the host cells with certain chemical agents like acridine orange (Novick, 1969; Ansari and Khatoon, 1996). However, some plasmids are very stable and are not lost either spontaneously or under the action of chemical agents (Bounchand *et al.* 1969; Derylo and Lorkiewicz, 1970; Khatoon and Ali Mohammad, 1986; Khatoon and Jahan, 1995). The latter are more dangerous from the view-point of chemotherapy.

The study was designed to investigate (a) the drug resistance pattern of the clinical gram-negative bacteria (b) the transferability of their resistances by conjugation and (c) stability/instability of R plasmids by spontaneous segregation and curing with acridine orange.

MATERIALS AND METHODS

Sources of gram-negative bacteria

Gram-negative bacteria were obtained from hospitals or pathological labs of Karachi. The bacteria were collected on tryptone agar slants, and were purified twice on MacConkey's agar. They were later maintained on tryptone agar slants. Most bacteria were already identified at source, however if needed, identification was carried out by biochemical reactions and by the reaction on T.S.I medium.

Standard bacterial strains

The standard bacterial strains used in the study as recipients of R plasmids, included *Escherichia coli* 13-6a, *E. coli* 40MD, *E. coli* FPL5014, and *E. coli* AB712 with the following genotypes:

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E. coli 13-6a : *F*$^-$ *lac*$^+$ *proA*$^-$ *ade*$^-$ *trp*$^-$ *met*$^-$ *str*^{r} *T6*^{r}
E. coli 40 MD: *F*$^-$ Δ*pro lac* *trp*$^-$ *str*^{r}
E. coli FPL5014: *F′ pro*$^+$*lac*$^+$ / Δ*pro lac* *thi*$^-$ *str*^{s}
E. coli AB712 : *F*$^-$ *thr*$^-$ *leu*$^-$ *thi*$^-$ *pro*$^-$ *lac*$^-$ *str*^{r}

Antibiotics

The antibiotic used were: Ampicillin trihydrate (A), Chloramphenicol (C), Gentamycin sulfate (G), Kanamycin sulfate (K), Neomycin sulfate (N), Streptomycin sulfate (S) and Tetracycline hydrochloride (T). All the antibiotics were obtained from Sigma Chemical Company, U.S.A.

Table 1: Gram-negative bacteria and their resistance patterns

S. No.	Bacterial Strain Number*	Resistance Pattern**
1	*E.coli* (AS-46)	AS
2	*E.coli* (AS-47)	A
3	*E.coli* (AS-48)	AGST
4	*E.coli* (AS-49)	AGK^{rest}ST
5	*Enterobacter* (AS-50)	AS$^{v.rest}$T
6	*E.coli* (AS-51)	ACGT
7	*E.coli* (AS-52)	ACGKNST
8	*E.coli* (AS-53)	AG$^{v.rest}$K$^{v.rest}$ST
9	*E.coli* (AS-54)	AC^{rest}G$^{v.rest}$KNT
10	*Enterobacter* (AS-55)	ACST
11	*Klebsiella* (AS-56)	ACG^{rest}ST
12	*E.coli* (AS-57)	ACST
13	*E.coli* (AS-58)	AG$^{v.rest}$T
14	*E.coli* (AS-59)	ACGKST
15	*E.coli* (AS-60)	CGKNT
16	*E.coli* (AS-61)	Sensitive
17	*Enterobacter* (AS-62)	AG^{rest}T
18	*Enterobacter* (AS-63)	AG^{rest}T
19	*Klebsiella* (AS-64)	ACGKNST
20	*Klebsiella* (AS-65)	AC^{rest}GKNST
21	*E.coli* (AS-66)	AGST
22	*E.coli* (AS-67)	ACGKNST
23	*Klebsiella* (AS-68)	AGK^{rest}T
24	*Shigella* (AS-69)	AKNST
25	*Klebsiella* (AS-70)	AGK^{rest}T
26	*E.coli* (AS-71)	KNT
27	*E.coli* (AS-72)	AS
28	*E.coli* (AS-73)	AS
29	*E.coli* (AS-74)	A
30	U.I. (AS-75)	ACGKNST
31	*Enterobacter* (AS-76)	A
32	*Klebsiella* (AS-77)	AGK^{rest}T
33	*E.coli* (AS-78)	AST
34	*Enterobacter* (AS-79)	AGK$^{v.rest}$T
35	*Klebsiella* (AS-80)	ACGKNST
36	*Klebsiella* (AS-81)	A
37	*Enterobacter* (AS-82)	AGK^{rest}ST
38	*E.coli* (AS-83)	AST
39	*E.coli* (AS-84)	AGK^{rest}T
40	*Enterobacter* (AS-85)	ACGKNT
41	*E.coli* (AS-86)	AGST
42	*E.coli* (AS-87)	G $^{v.rest}$KN
43	*Klebsiella* (AS-88)	AST
44	*E.coli* (AS-89)	ACST
45	*E.coli* (AS-90)	AGK^{rest}T
46	*E.coli* (AS-91)	ACGK$^{v.rest}$N$^{v.rest}$T
47	*E.coli* (AS-92)	ACG$^{v.rest}$ST
48	*E.coli* (AS-93)	Sensitive
49	*Enterobacter* (AS-94)	ACST
50	*Klebsiella* (AS-95)	AGK^{rest}T
51	*E.coli* (AS-96)	Sensitive
52	*E.coli* (AS-97)	ACGKST
53	*S.typhi* (Z-26)	AS ^{rest}
54	*E.coli* (MT-14)	AST

**E. coli = Escherichia coli*, *S.typhi = Salmonella typhosa*, U.I.= Unidentified.
**A = ampicillin, C = chloramphenicol, G = gentamycin, K = kanamycin, N = neomycin, S = streptomycin, T = tetracycline. rest = restricted growth, v. rest = very restricted growth.

Media

Tryptone agar consisted of: bactotryptone 17 grams, agar 6 grams, distilled water 1000 ml (pH 7.0). Triple Sugar Iron Agar (T.S.I) was obtained from Difco. Resistance determinations were made on MacConkey's Agar (Difco), to which single antibiotics were added at desired concentrations (usually 100µg/ml) as has been described earlier (Amir Ali and Khatoon, 1976; Khatoon, 1976). Minimal Inhibitory concentrations (MICs) of the standard bacterial strains, used as recipients in conjugal crosses, were determined as described by Amir Ali and Khatoon (1976) and Jahan (1991). All the standard strains were inhibited at the concentration of 30µg of antibiotic per ml of the medium, for all antibiotics. The *E. coli* strains 13-6a, 40 MD and AB712 had high level, chromosomal streptomycin resistance and could resist more than 500µg/ml.

For conjugation experiments, bacterial cultures were grown in L.B. broth (Khatoon 1976; Khatoon and Ali Muhammad 1986) or Antibiotic Medium No.3, Oxoid. Conjugal crosses were carried out by the broth method as described earlier (Khatoon, 1976). 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). For segregation and curing, bacteria were grown in L.B. broth and the experiments were performed as described earlier (Saeed *et al.*, 2003).

Table 2: R plasmids isolated from gram-negative bacteria **

S. No.	R Plasmid Designation	Original Host (Donor)	Resistance Pattern of R Plasmid
1	pAK5	*Escherichia coli* (Z-18)	S_{700}(?)*T_{30}
2	pAK9	*Escherichia coli* (MT-12)	$A_{50}S_{500}$(?)*T_{30}^{rest}
3	pAK10	*Escherichia coli* (MT-16)	$A_{50}S_{500}$(?)*T_{30}
4	pAK11	*Escherichia coli* (MT-44)	$A_{50}S_{500}$(?)*
5	pAK12	*Escherichia coli* (MT-45)	$A_{50}C_{30}S_{500}$(?)*T_{30}
6	pAK13	Unidentified (MT-37)	$A_{50}S_{500}$(?)*
7	pAK14	*Escherichia coli* (MT-55)	T_{30}
8	pAK15	*Escherichia coli* (MT-1)	T_{30}
9	pAK16	*Escherichia coli* (MT-2)	T_{30}
10	pAK17	*Escherichia coli* (AS-71)	$K_{50}N_{50}T_{30}$
11	pAK18	*Escherichia coli* (AS-47)	A_{50}
12	pAK19	*Enterobacter* (AS-62)	$A_{50}G_{30}K_{50}T_{30}$
13	pAK20	*Enterobacter* (AS-62)	A_{50}
14	pAK21	*Escherichia coli* (AS-84)	$A_{50}G_{30}K_{50}$
15	pMT14	*Escherichia coli* (MT-14)	$A_{50}S_{500}$(?)*T_{30}
16	PZ26	*Salmonella typhi* (Z-26)	$A_{50}S_{500}^{rest}$(?)*

**The recipients used in the conjugal crosses were either *E. coli* 40MD, *E. coli* 13-6a or *E. coli* AB712, all of which carried high level chromosomal streptomycin resistance
*It is not clear whether this resistance is due to the R plasmid or it is the chromosomal resistance of the recipient.
A = ampicillin, C = chloramphenicol, G = gentamycin, K = kanamycin, N = neomycin, S = streptomycin, T = tetracycline, rest = restricted growth.

Table 3: Spontaneous segregation of the resistance determinants of R plasmids

S. No.	R Plasmid	Antibiotic* Resistance Determinants	% of colonies with lost markers	Markers lost
1	pAK9	$A_{50}T_{30}^{rest}$	0	-
2	pAK10	$A_{50}T_{30}$	2 18	A T
3	pAK11	A_{50}	0	-
4	pAK12	$A_{50}C_{30}T_{30}$	6	T
5	pAK13	A_{50}	0	-
6	pAK14	T_{30}	6	T
7	pAK15	T_{30}	26	T
8	pAK16	T_{30}	18	T
9	pAK17	$K_{50}N_{30}T_{30}$	2 16	N T
10	pAK18	A_{50}	0	-
11	pAK19	$A_{50}G_{30}K_{50}T_{30}$	6	T
12	pAK20	A_{50}	0	-
13	pAK21	$A_{50}G_{30}K_{50}$	0	-

*A = ampicillin, C = chloramphenicol, G = gentamycin, K = kanamycin, N = neomycin, T = tetracycline

Isolation of R plasmids

The isolation of R plasmids was genetically made by conjugating the wild type, antibiotic resistant bacteria, obtained from clinical sources, to any of the standard *Escherichia coli* strains: 40MD, AB712 or 13-6a, mentioned above. As all the standard strains (40MD, AB 712 and 13-6a) were sensitive to 30 μg/ml of each antibiotic except for streptomycin, the selection of transconjugants was made on MacConkey's or Minimal Agar plates supplemented with 30μg/ml of the antibiotic of which the transmission was to be observed along with high level of streptomycin (500 μg/ml) to which the donor was sensitive. Any growth on the plates indicated the conjugal transmission of resistance(s) from the donor to the recipient involving the presence of conjugative R plasmid in the donor. The transmission of streptomycin

Table 4: Curing of the resistance determinants of R plasmids by acridine orange*

S. No.	R Plasmid	Antibiotic** Resistance Determinants	% of colonies with lost markers	Markers lost
1	pAK9	$A_{50}T_{30}^{rest}$	0	–
2	pAK10	$A_{50}T_{30}$	0	–
3	pAK11	A_{50}	0	–
4	pAK12	$A_{50}C_{30}T_{30}$	2	ACT
5	pAK13	A_{50}	0	–
6	pAK14	T_{30}	20	T
7	pAK15	T_{30}	52	T
8	pAK16	T_{30}	34	T
9	pAK17	$K_{50}N_{50}T_{30}$	2 10	KNT T
10	pAK18	A_{50}	0	–
11	pAK19	$A_{50}G_{30}K_{50}T_{30}$	0	–
12	pAK20	A_{50}	0	–
13	pAK21	$A_{50}G_{30}K_{50}$	0	–

*Acridine Orange was used in the concentration of 120 μg/ml for curing experiments.
**A = ampicillin, C = chloramphenicol, G = gentamycin, K = kanamycin, N = neomycin, T = tetracycline.

resistance could later be ascertained by further conjugating the resulting transconjugants to the standard *E. coli* strain FPL5014 (mentioned above) that was sensitive to 30 μg/ml of all the antibiotics including streptomycin. By this method, total 13 out of 28 wild type clinical strains showed presence of R plasmids. Details on the conjugation experiments have been described earlier (Saeed, 2003).

Curing of plasmids

For studying curing by acridine orange, the bacterium bearing R plasmid was grown overnight in L.B. broth. Next morning, fresh L.B. broth containing 120 μg/ml of acridine orange was inoculated with the overnight culture to give a 100 fold dilution. All this work was carried out in the dark because of the light sensitivity of acridine orange. A control tube lacking acridine orange was always included. The test & the control cultures were incubated overnight at 37°C. The test culture & the control culture were then diluted & plated on MacConkey's Agar plates, to obtain isolated colonies. Some 100 colonies from each plating were grided onto MacConkey's Agar plates. After overnight incubation at 37°C, these were replicated on antibiotic containing plates to check for the loss (or its absence) of antibiotic resistance determinants.

RESULTS

Screening for antibiotic resistance

A number of 54 gram negative bacteria, collected from clinical sources were screened for their resistance to ampicillin(A), chloramphenicol (C), gentamycin(G), kanamycin(K), neomycin(N), streptomycin(S) and tetracycline (T). Of the 54 bacterial strains, 50 were found resistant to one or more antibiotics. The resistances were tested at a level of 100 μg/ml medium. The bacteria included species of *Salmonella, Shigella, Klebsiella, Enterobacter*, *Escherichia,* & some other unidentified organisms (table 1).

Conjugal transfer of drug- resistance(s)

The resistant bacteria (potential R plasmid donors) were conjugated to standard *E. coli* K-12 recipients: such as 13-6a, 40MD or AB712 for the conjugal transfer of their resistances. Resistance to streptomycin could only be ascertained after transfer of the R plasmid to a streptomycin sensitive *E.coli* FPL5014 strain. Total 16 R Plasmids were isolated (table 2).

Spontaneous loss and curing of conjugative R plasmids

The conjugative R plasmids pAK9, pAK10, pAK11, pAK12, pAK13, pAK14, pAK15, pAK16, pAK17, pAK18, pAK19, pAK20 and pAK21 were studied for spontaneous loss and curing with acridine orange (tables 3 and 4 respectively). The presence of non-conjugative R plasmids, pMT14 and pZ26, was detectable by their mobilization with the co-existing, conjugative plasmid pSK1a and KR61-KNST (Saeed, 2003).

DISCUSSION

From the results reported here, it appears that more than 92% of clinical gram-negative bacteria of Karachi are resistant to one or more antibiotics. From among them, at least 78% are multidrug-resistant and more than 46% possess conjugative R plasmids.

The R plasmids were quite stable even after 20 consecutive transfers in plain broth (spontaneous segregation) and did not show any significant loss of their resistance markers. Only pAK15 showed 26% loss of its resistance. Similarly only pAK15 and pAK16 showed a significant loss of their markers in curing by acridine orange. The other plasmids were quite stable. These results seem very similar to those reported by Khatoon (1971) and Ansari and Khatoon (1996).

Conjugal transferability and stability of R plasmid is very dangerous from the viewpoint of chemotherapy. Hence antibiotics should be administered with great care in clinical practice because their indiscriminate use may lead to the development and spread of drug resistant or multidrug-resistant bacteria.

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