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Gentamicin Resistance in Clinical Strains of *Enterobacteriaceae* Associated with Reduced Gentamicin Uptake

K. ADWAN and N. ABU-HASAN

Department of Biological Sciences, An-Najah N. University, Nablus, Palestine
Fax 972 9 387 982
E-mail adwan@najah.edu

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ABSTRACT. Seven strains of *Enterobacteriaceae* resistant to gentamicin obtained as representatives of the predominant resistance profiles in the clinical laboratories of *Rafeidia and Al-Watani Hospitals* in Nablus (Palestine) were included. Five strains showed a broad aminoglycoside resistance profile but contained no evidence of gentamicin acetylation, adenylation, or phosphorylation. Gentamicin uptake in two tested strains was significantly reduced, compared to that of gentamicin-sensitive *E. coli* (MIC, 0.5 μg/mL). These strains are likely resistant due to a relative reduction of the amount of gentamicin and other aminoglycosides entering the bacterial cell. Two strains showed evidence of adenyltransferase ANT(2″)-I activity.

Resistance of most strains of *Enterobacteriaceae*, collected from the clinical laboratories of *Rafeidia and Al-Watani Hospitals* in Nablus in 1995, to gentamicin was predominantly due to enzymic adenylation or acetylation of gentamicin (Al-Asmar 1996). In the strains we examined, an R-plasmid and/or chromosome-mediated gentamicin resistance was proposed. During the period of 1996 to 1997, and mainly due to extensive and uncontrolled use of gentamicin in particular, has led to the emergence of strains resistant to gentamicin which did not contain detectable enzymic gentamicin modification. We present in this study a characterization of these strains and an examination of their significance in mediating clinically important gentamicin resistance.

MATERIALS AND METHODS

Bacteria. Seven strains of *Enterobacteriaceae* were selected as representatives of the predominant resistance profiles in the clinical laboratories of *Rafeidia and Al-Watani Hospitals* in Nablus between 1996 and 1997 (Table I). Patients admitted to these hospitals were from various regions of the northern parts of Palestine.

Susceptibility testing. Antimicrobial susceptibilities of the strains were determined by disk diffusion mainly as described by Bauer *et al.* (1966). Antibiotic disks (*Oxoid*) used were gentamicin (10 μg), tobramycin (10 μg), neomycin (30 μg), kanamycin (30 μg) and amikacin (30 μg). Minimum inhibitory concentration (MIC) for gentamicin (*Sigma*) was determined by agar dilution method (Ericsson and Sherris 1971) containing two-fold dilutions of the antibiotic ranging in concentration from 128 to 0.5 mg/L. Bacterial inocula were grown in Mueller–Hinton broth and were adjusted to contain approximately 10^5 CFU/mL. The plates were incubated at 37 °C for 18 h.

Enzyme assay. Crude extracts of bacteria were assayed for the presence of aminoglycoside-adenylating, acetylating, and phosphorylating enzymes as described by Ono *et al.* (1983). Assays were performed with gentamicin as substrate. Presence of aminoglycoside-modifying enzyme types was inferred according to Shannon and Phillips (1982).

Plasmid isolation. Plasmids were isolated according to Takahashi and Nagano (1984).

Plasmid curing. Overnight cultures were grown in Mueller–Hinton broth containing 5–10 mg/L ethidium bromide (*Sigma*) for 2 d. After dilution, the cultures were plated on Mueller–Hinton agar and then replicated onto plates supplemented with gentamicin.

Gentamicin uptake. A modification of the procedure of Hirai *et al.* (1986) was used. Organisms were grown in Nutrient broth (*Oxoid*) to mid-exponential phase, which is equivalent to absorbance of 0.5 at 650 nm. This was followed by addition of gentamicin to a final concentration of 8 mg/L. Aliquots (1 mL) were removed at 0, 1, 5, 10, 20 and 30 min. Cells were harvested by centrifugation, rapidly washed in 1 mL ice-cold 0.85 % NaCl, and resuspended in 0.5 mL 0.85 % NaCl. The cells were boiled for 7 min to elute gentamicin, the remnants were removed by centrifugation, and the gentamicin con-

tent of the supernatant was determined by bioassay. The zero time results, representing adsorbed gentamicin, were subtracted from those of the other time points. Results were expressed as mean ng of gentamicin per 10^8 of cells.

RESULTS

Two strains, 1 and 2, possess the enzyme profile of ANT(2")-I (Table I). No evidence of gentamicin-acetylating or adenylating activity was detected in any of the other strains (3 to 7); moreover, these strains were examined and found to be negative for gentamicin phosphorylation. This form of modification had been previously detected although it does not cause strains to be gentamicin-resistant. The R-plasmid of 16–18 kbp was detected in strains 1, 2 and 6. When these strains were subjected to plasmid curing, strains 1 and 2 lost gentamicin resistance and adenylating activity. However, no loss of any resistance marker was observed in strain 6.

Table I. Antibiotic susceptibility, plasmid analysis, plasmid curing and aminoglycoside-modifying enzyme assay of the strains[a]

Isolate no.	Strain	Strain characteristics	MIC to gentamicin mg/L	Plasmid size (kbp)		Lost resistance markers	Aminoglycoside modifying enzyme
				content	lost		
1	*Proteus* sp.	Gen^r, Kan^r, Tob^r	64	18	18	Gen,Kan,Tob	ANT(2")-I
2	*Klebsiella* sp.	Gen^r, Kan^r, Tob^r	128	17	17	Gen,Kan,Tob	ANT(2")-I
3	*Providencia* sp.	$Gen^r, Neo^r, Kan^r, Tob^r, Ami^r$	128	none	–	–	none
4	*Proteus* sp.	$Gen^r, Neo^r, Kan^r, Tob^r, Ami^r$	64	none	–	–	none
5	*Klebsiella* sp.	$Gen^r, Neo^r, Kan^r, Tob^r, Ami^r$	>128	none	–	–	none
6	*E. coli*	$Gen^r, Neo^r, Kan^r, Tob^r, Ami^r$	128	16	16	none	none
7	*E. coli*	$Gen^r, Neo^r, Kan^r, Tob^r, Ami^r$	32	none	–	–	none

[a]Gen – gentamicin, Neo – neomycin, Kan – kanamycin, Tob – tobramycin, Ami – amikacin.

DISCUSSION

In our bacterial collection, resistance to two or more of the clinically important deoxystreptamines was observed (Table I). This probably reflects the fact that gentamicin (and/or partly tobramycin, neomycin and kanamycin) have been exclusively used in Palestine. Resistance to gentamicin, kanamycin and tobramycin appeared in two strains. This resistance profile was related to the presence of adenyltransferase ANT(2")-I activity. The occurrence of the ANT(2")-I mechanism in gentamicin-resistant *Enterobacteriaceae* was previously reported in the same hospitals (Al-Asmar 1996).

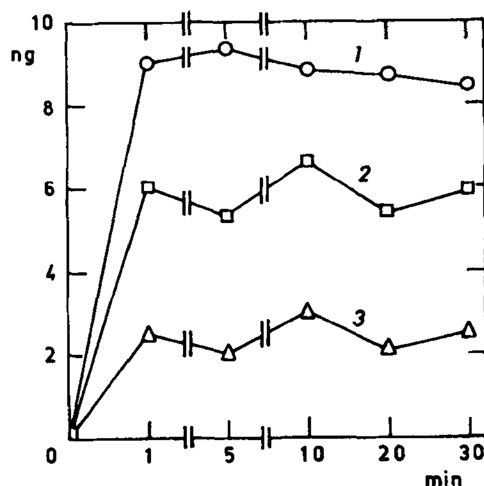

Fig. 1. Gentamicin uptake (ng per 10^8 cells) of *E. coli* isolates exposed to 8 mg/L; *1* – gentamicin-sensitive, *2* – strain no. 7, *3* – strain no. 6.

The results suggest a nonconjugative R-plasmid localization of resistance genes in strains 1 and 2, since when these strains are subjected to plasmid curing, gentamicin resistance and adenylating activity is lost (Table I).

The broad aminoglycoside resistance pattern shown by strains 3 to 7 suggest chromosomally reduced uptake of antibiotics. Aminoglycoside cross resistance, including gentamicin, tobramycin, neomycin and amikacin, in various clinical settings was mainly attributed to permeability, a condition which excludes further use of any other aminoglycosides (Kallova *et al.* 1995; McNeill *et al.* 1984). This observation was further supported by the reduced uptake of gentamicin in isolates 6 and 7 and the absence

of gentamicin modifying activity. Thus it seems reasonable to conclude that these isolates are resistant because of a relative reduction in the amount of gentamicin and other aminoglycosides. Such resistance could be due to a permeability barrier provided by the cell wall or the inner membrane. The difference in the capability of strains 6 and 7 to accumulate gentamicin is related to their sensitivity to gentamicin.

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