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Original scientific paper

Effect of Aminglycoside Administration on the Occurrence and Multiplication of Resistant Bacteria

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A B S T R A C T

In the prospective study the susceptibility of 41 Escherichia coli strains and 55 Pseudomonas aeruginosa strains to gentamicin, netilmicin and amikacin was tested at a 2-year interval (period I April 1998 to March 1999, and period II April to July 2001). Genotyping was performed by pulsed-field gel electrophoresis, and a clone based on 80% or 90% similarity was determined for each of the study bacteria. In 24 (32.0%) clones, strains showed no variation over 2-year interval, supporting the hypothesis on a priori susceptible strains. Transformation from susceptibility in period I to resistance in period II was demonstrated in 5 (6.7%) clones, a pattern consistent with the concept of bacterial development of resistance under the influence of antibiotics. However, there were 10 (13.3%) clones whose strains exhibited an inverse pattern. Accordingly, two-way transformation of susceptibility took place during the study period. The utilization of the study aminoglycosides had no major impact on the variation of microbial susceptibility. Changes in microbial susceptibility were found to follow some regular patterns, which were not influenced by the study aminoglycosides. Two phenomena were observed: (i) there were stable clones that did not develop resistance in spite of selective antibiotic challenge; and (ii) changes of susceptibility in isolated bacteria from both inpatient and outpatient strains of the same clone were two-way and reversible.

Key words: *aminoglycosides, antimicrobial resistance, change of susceptibility, clone, pulsed-field gel electrophoresis, Escherichia coli, Pseudomonas aeruginosa*

Introduction

Microbial resistance to aminoglycosides (or antibiotics in general) poses an increasing problem all over the world[1–3], especially in seriously ill patients[4–6]. Literature reports on the strategies used to prevent the spread of microbial resistance to various antibiotics (a multidisciplinary issue), including the three aminoglycosides tested in the present study (gentamicin, amikacin and netilmicin), have been almost exclusively focused on microbial resistance in inpatient settings[7]. These problems are pronounced in hospitals, as they involve hospitalized patients characterized by reduced defense responsiveness due to the underlying disease and administration of one or more antibiotics[8–17].

How do antibiotics promote resistance? The procedure of selection may only seem to be easy. Every time an antibiotic exerts its effect, the sensitive bacteria will gradually die. Bacteria as target organisms may survive the action of antibiotics if possessing any kind of resistance before their exposure to antibiotics or if they have acquired it later (by mutation or gene exchange). These bacteria should continue to multiply despite the presence of antibiotics, leaving resistant clones behind. Is it really going to happen? What are the present relations between the resistant and susceptible clones (strains) in the microbial population?

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Every time an antibiotic is administered, in addition to its effect on the target pathogenic microorganism it definitely has an effect on the normal bacterial flora of the body. The normal bacterial flora will have the very same response to the antibiotic and will develop resistance in the very same way as the target pathogenic microorganism. The increase in non-pathogenic but resistant bacteria is accompanied by the growth of the resistance reservoir in the microbial population, thus increasing the likelihood and rate of acquiring or transmitting resistance genes to various types of pathogenic and non-pathogenic bacteria. Besides, are we sure that antibiotics have an effect on resistance increase? Nowadays, biocides are often administered, posing the question of the extent of their effect on the occurrence of resistance[18]. In nature, there are residues of antibiotics that, in addition to other organic and inorganic compounds, definitely have an effect on the bacteria and their resistance. Are we in for the use of normal flora as an important factor in the combat against resistance[19,20]?

In veterinary medicine and agriculture, antibiotics are used not only for therapeutic purpose but also for prophylaxis and food improvement (as growth promoters). There is no doubt that antibiotics exist in our environment, wherefrom they may again influence the development of resistance[21]. Human medicine does not have continued antibiotic prevention, as is a common practice in veterinary medicine and agriculture. Yet, aren't human beings under constant influence of antibiotics, as certain quantities of various types of antibiotics are approved, e.g., in milk and meat? Physicians aiming only at preserving and improving human health cannot accept the fact that the occurrence of resistance is due to the use of antibiotics as growth promoters in food animals, merely to increase the profit of food industry. On the other hand, one cannot conceive the consequences that would be entailed by placing a ban, substantially reducing or stipulating strict conditions for the administration of antibiotics to food animals, as it would cause enormous financial losses[22–24]. In Europe, the use of antibiotics for alimentary purposes has been legally regulated since 1987. It has been clearly stated that antibiotics cannot and may not be a substitute for poor conditions and hygiene of animal keeping. Yet, no such regulations have been developed on the respective treatments and prophylaxis[25].

Based on monitoring and comparison of total antibiotic utilization with changes in antimicrobial resistance in different countries, it is quite clear that a reduction or increase in the utilization of antibiotics may but need not always be associated with a resistance decrease or increase. This is so because of the very complex relationship between the microorganisms and the use of antibiotics, not only in the field of health care but also in the fields of veterinary medicine and agriculture[26].

In the combat against the development of hospital resistance to antibiotics in general, including aminoglycosides, there are very similar strategies based on some common elements: (i) understanding the reasons and routes of occurrence and spread of antimicrobial resistance; (ii) reducing the »empiric« antibiotic utilization by accurate and rapid identification of the causative agents; (iii) withdrawing and reintroducing an antibiotic in therapeutic protocol at certain intervals (cycling), thus to actively influence the development of resistance; and (iv) administration of several antibiotics in individual patient therapy (rotation)[27].

Considering total resistance of microorganisms mentioned in the literature from this (limited) aspect, any evaluation of the antibiotic – bacterium relationship appears to be based on inpatient resistance. To our knowledge, there are no reports addressing the issues of microbial susceptibility or resistance through treatment of a part of the total microbial population as a temporally changeable (dynamic) entity in which all changes and influences happen that determine the bacterium as a living organism the attitude of which is in accordance with only some of the regularities observed. Therefore, the aim of this study was to observe the effect of aminoglycosides on the dynamics of change in microbial susceptibility at a two-year interval in inpatient and outpatient samples.

Material and Methods

Materials

In order to demonstrate the effect of study aminoglycosides (gentamicin, amikacin and netilmicin) on the development of resistance in *E. coli* and *P. aeruginosa* strains, samples were collected at a two-year interval. Samples of blood, urine, throat swabs and surgical wounds were obtained from patients hospitalized at Osijek University Hospital (OUH) (experimental group) and patients visiting primary health care (PHC) offices for low-grade infections (control group). First sampling was performed from April 1998 to March 1999 (period I), and second sampling from April to July 2001 (period II). PHC samples were obtained from patients that had not been hospitalized for the past five years. These samples served as a control group, since the study aminoglycosides are not used at PHC at all. Samples were stored in liquid nitrogen and revitalized immediately before submitting to pulsed-field electrophoresis. A questionnaire containing demographic data, date of sampling, OUH department or PHC office, type of sample, and results of susceptibility to study aminoglycosides was filled out for each sample.

Methods

The isolation, identification and determination of antibiotic susceptibility of bacteria were performed by the method of disk diffusion[28]. Bacterial susceptibility to study aminoglycosides (susceptible 3, resistant 2 or 0) was semi-qualitatively determined by disk diffusion in agar using standard methodology[29–30]. Strains were kept in liquid nitrogen until genotyping. Quantitative determination of susceptibility is based on dilution in bouillon. Strain genotyping was performed by pulsed-field gel electrophoresis (PFGE) on a CHEF III (BioRad) device[31].

Between-strain comparison was done by use of GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium).

Definitions

A clone represented 80% affinity or similarity of bacterial strains for each of the aminoglycosides tested (90% due to *P. aeruginosa* variability), based on genotyping, i.e. it represented the similarity between DNA fragments upon restriction. Clones were not produced on the basis of phylogenic similarity; the GelCompar II software was used to determine the strains belonging to a particular clone and the level (%) of similarity. The clones exerting the very same dynamics of susceptibility over two years yielded four pattern types: (a) stable clones detected in both periods, their susceptibility or resistance being unchanged, stable clones; (b) clones detected in only one period, their change of susceptibility could not be monitored; (c) clones showing transformation from resistance in period I to susceptibility in period II; and (d) clones showing transformation from susceptibility in period I to resistance in period II.

Statistical analysis

Statistical analyses were performed with the Statistical Package for the Social Sciences, version 15.0 (SPSS Inc., Chicago, IL, USA). χ^2 test (df=1) was used for the analysis of differences between strains of each pattern. The level of significance was set at $P<0.05$. E. coli and P. aeruginosa resist types (gentamicin-amikacin-netilmicin, GAN) are presented as absolute and relative frequencies. A statistical model of 2x2 contingency tables was used on data processing[32].

Results

A total of 320 (158 inpatient and 162 outpatient) samples were collected, of which 221 *E. coli* strains (89 inpatient and 132 outpatient) and 99 *P. aeruginosa* strains (69 inpatient and 30 outpatient) were isolated during the two study periods. Of these, 41 *E. coli* strains and 55 *P. aeruginosa* strains were retrieved by revitalization.

Throughout the study periods, the highest proportion of *E. coli* resist types susceptible to all three study aminoglycosides (gentamicin, amikacin and netilmicin, GAN-1, n=28) and a low proportion of resistant resist types were detected (GAN-5, n=4) (Table 1). Other resist types as transient forms between GAN-1 and GAN-5 were rarely recorded. Most frequently GAN-1 resist type was isolated from both inpatient and outpatient samples. Of the aminoglycosides tested, *E. coli* showed highest rate of resistance to gentamicin (31.7%), followed by netilmicin (22%) and amikacin (12.2%).

P. aeruginosa showed quite a different distribution of resist types, predominated by GAN-5 resist type (n=19), followed by GAN-1 resist type (n=18) (Table 2). It should be noted that GAN-3 resist type resistant to gentamicin and netilmicin (n=17) was ranked between GAN-1 and GAN-2. Of the three aminoglycosides tested, *P. aeruginosa* showed highest rate of resistance to gentamicin (67.3%), followed by netilmicin (65.5%) and amikacin (34.5%).

Utilization of aminoglycosides

Between 1997 and 2001, the utilization of amikacin was on a constant decline, however, a significant decrease was only recorded between 1998 and 1999 ($\chi^2 = 322.115$, $P<0.005$). The utilization of gentamicin increased, with an abrupt one-year rise in 1999. The utilization of gentamicin showed considerable oscillation, i.e. an increase in 1998–1999 ($\chi^2=5359.170$, $P<0.005$), a decrease in 1999–2000 ($\chi^2=1716.843$, $P<0.005$), to rise again in 2000–2001 ($\chi^2=3350.033$, $P<0.005$). The utilization of netilmicin during the five-year study period showed a substantial rise in the first two years, to fall abruptly in 1999 ($\chi^2=1840{,}211$, $P<0.005$), and rise in 2000 ($\chi^2=7755{,}331$, $P<0.005$) (Figure 1).

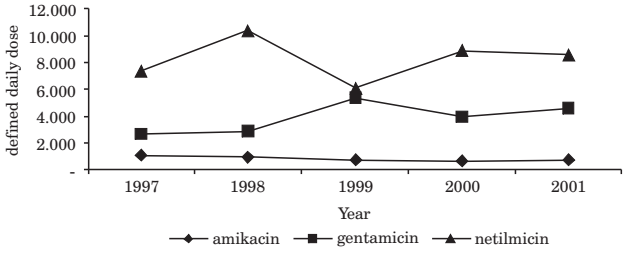

Fig. 1. Aminoglycoside utilization at Osijek University Hospital 1997–2001.

TABLE 1
NUMBER OF ESCHERICHIA COLI RESIST TYPES STRAINS ACCORDING TO STUDY PERIODS (N=41)

	GAN-1		GAN-2		GAN-3		GAN-4		GAN-5	
	+++		–++		–+–		—+		——	
Period	I	II	I	II	I	II	I	II	I	II
Inpatient	3	13	0	2	2	1	0	0	2	1
Outpatient	8	4	1	0	0	2	0	1	1	0
Total	28		3		5		1		4	

*GAN-1... GAN-5 – gentamicin-amikacin-netilmicin resist types
*»–« – resistant to aminoglycosides, »+« susceptible to aminoglycosides

TABLE 2
NUMBER OF PSEUDOMONAS AERUGINOSA RESIST TYPES STRAINS ACCORDING TO STUDY PERIODS (N=55)

	GAN-1		GAN-2		GAN-3		GAN-5	
	+++		–++		–+–		——	
Period	I	II	I	II	I	II	I	II
Inpatient	10	4	1	0	10	0	5	8
Outpatient	2	2	0	0	6	1	4	2
Total	18		1		17		19	

*GAN-1... GAN-5 – gentamicin-amikacin-netilmicin resist types
*»-« – resistant to aminoglycosides, »+« susceptible to aminoglycosides

PFGE

Genotyping was performed in 41 *E. coli* strains and 55 *P. aeruginosa* strains that were successfully revitalized upon removal from liquid nitrogen. All 41 *E. coli* strains were classified into 20 clones at a similarity level of 80%. Of these, 12/20 (60%) clones had at least two or more strains, 8/12 (66.7%) clones contained strains from both inpatient and outpatient samples. 8/20 (40%) clones had only one strain (Figure 2).

P. aeruginosa strains were arranged in the form of 18 clones at a similarity level of 90% because they would form nearly one clone at the 80% level of similarity; 13/18 (72.2%) clones contained at least 2 strains, and 5/18 (27.8%) clones only one strain; 4/13 (30.8%) clones contained strains from both inpatient and outpatient samples (Figure 3).

The clones containing one strain were excluded from further analysis. The change of susceptibility in strains of a clone between the two study periods was assessed for each of the study bacteria and antibiotics. The clones showing the same pattern of susceptibility change irrespective of the bacterium and antibiotic involved formed the same pattern. Considering the change of susceptibility of each clone to each of the study antibiotics over the two-year period, each clone (Figure 3) additionally formed three new clones to each of the three antibiotics.

Thus, there were 75 clones in total, which yielded four different patterns (Table 3). In pattern A (24/75, 32%), the strains found in 24/24 clones (100%) exhibited permanent (unchanged) susceptibility to the study aminoglycosides in both study periods, irrespective of the inpatient or outpatient sample origin. None of the pattern A clones contained strains resistant to study aminoglycosides in both study periods.

The predominant pattern B (36/75, 48%) contained various clones with strains susceptible and/or resistant to study aminoglycosides, however, only detected in one of the study periods, thus precluding determination of their pattern of susceptibility dynamics.

In pattern C, transformation from resistance to susceptibility (10/75, 13.3%) between periods I and II was recorded in 9 clones (12.2%; $\chi^2=14.845$; $p<0.005$), whereas pattern D (5/75, 6.7%) showed inverse transformation from susceptibility to resistance in 4 clones (5.4%).

Comparison of the parameters of antibiotic susceptibility between inpatient and outpatient strains pointed

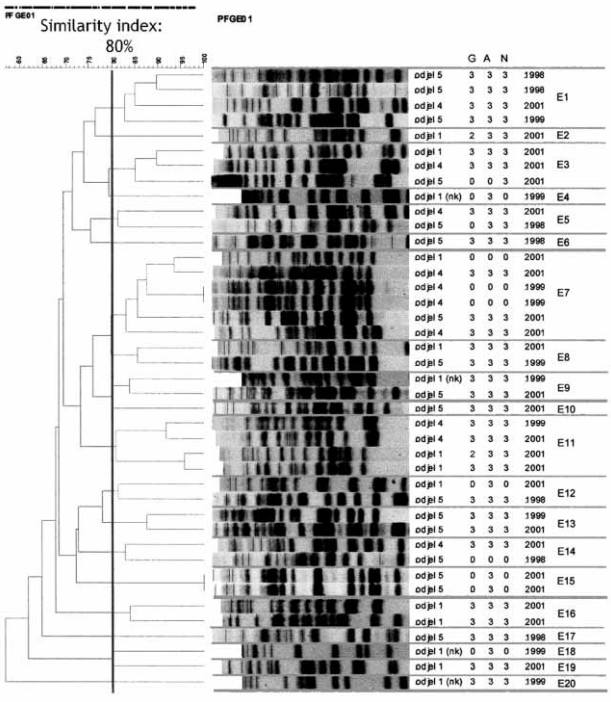

Fig. 2. PFGE clones of E. coli at 80% level of strain similarity. Dendrogram generated by GelCompar II software showing the PFGE clonal groups identified among E. coli isolates from inpatient and outpatient. Vertical bold line indicate similarity index. Horizontal bold lines distinguish different clones. Odjel 1 – 4 – Osijek University Hospital departments (inpatient), Odjel 5 – Primary Health Care (outpatient), E1–E20 – clones of E. coli.

TABLE 3
NUMBER OF E. COLI AND P. AERUGINOSA PATTERNS ACCORDING TO SUSCEPTIBILITY TO STUDY AMINOGLICOSIDES

			Sampling period			
			1998/1999	–2001		
Pattern	Number of clones	Susceptibili	N	N		Pattern direction
A	24	"3"	34	28	↔	stable clones, no changes
		"0"	–	–		
B	36	"3"	21	21	–	clones in only one period
		"0"	30	26		
C	10	"3"	13	16	↗	changing toward susceptibility
		"0"	33	3		
D	5	"3"	7	2	↘	changing toward resistance
		"0"	–	5		

* »3« – susceptibility, »0« – resistance

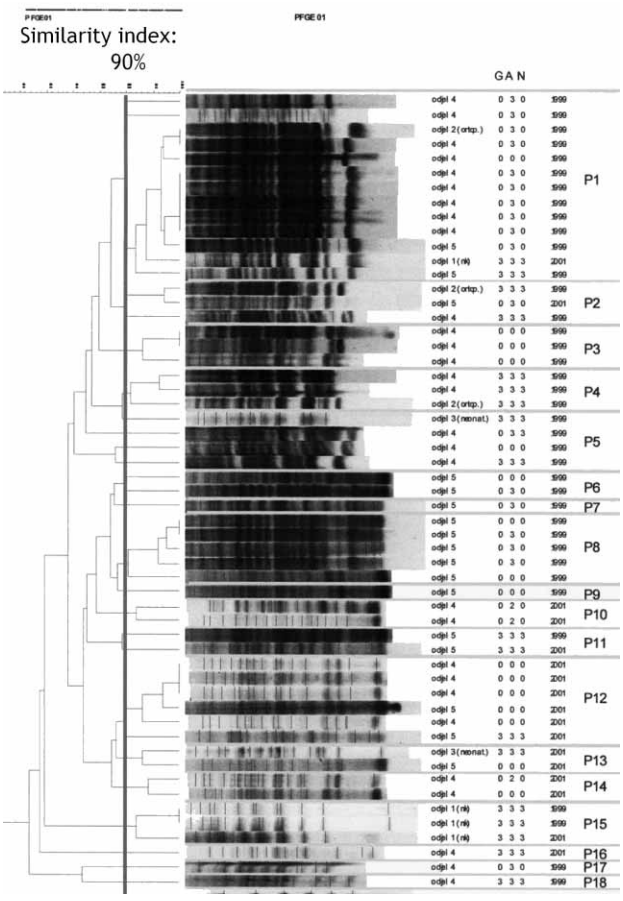

Fig. 3. PFGE clones of P. aeruginosa at 90% level of strain similarity. Dendrogram generated by GelCompar II software showing the PFGE clonal groups identified among P. aeruginosa isolates from inpatient and outpatient. Vertical bold line indicate similarity index. Horizontal bold lines distinguish different clones. Odjel 1–4 – Osijek University Hospital departments (inpatient), Odjel 5 – Primary Health Care (outpatient), P1–P18 – clones of P. aeruginosa.

to the following conclusions applying to all strains of the study bacteria: 1) the coverage of susceptible strains in period II ranged from 44% to 68%; 2) support was slightly higher in inpatient than in outpatient strains; 3) the strength of transformation to resistant strains was higher in inpatient strains; and 4) the lift by approximately 1 indicated a weak intensity of transformation of susceptible strains in period I to resistant strains in period II (Table 4).

Discussion

Although employing microbiological methods, the present study was primarily focused on the bacterium – aminoglycosides inter-relationship from the epidemiological rather than microbiological point of view. During the study period, the utilization of gentamicin and netilmicin was on a considerable increase, while the utilization of amikacin showed a substantial decline due to financial reasons. However, these changes in aminoglycosides utilization were not accompanied by respective changes in the susceptibility of the tested *E. coli* and *P. aeruginosa* strains to gentamicin, amikacin and netilmicin. Changes in microbial susceptibility were found to follow some regular patterns, which were not influenced by the study aminoglycosides. Two phenomena were observed: (i) there were stable clones that did not develop resistance in spite of selective antibiotic challenge; and (ii) changes of susceptibility in isolated bacteria from both inpatient and outpatient strains of the same clone were two-way and reversible.

Considering the study aminoglycosides, 2/3 *E. coli* strains were susceptible to gentamicin, which was a substantially lower rate as compared with other aminoglycosides, although the utilization of gentamicin was several times lower than the utilization of netilmicin in particular years of the study period. Accordingly, it appears that the utilization of study aminoglycosides alone had no major role in the development of *E. coli* resistance, and probably gained in importance only in conjunction with other factors.

Despite the decline recorded in the utilization of amikacin over several years, there were no substantial changes in the susceptibility pattern of *P. aeruginosa*. The finding of 36 clones belonging to pattern B (Table 3) may suggest that a considerably longer time than two years is needed for the reversible, two-way changes of *P. aeru-*

TABLE 4
TABLE OF CONTIGENCY ON STUDY BACTERIA AND AMINOGLYCOSIDES

	Sampling site	Coverage (%)	Support (%)	Strength (%)	Lift
	Inpatient	44	20	45	0.99
Gentamici	Outpatient	47	24	50	1.00
	Average	45	21	47	0.99
	Inpatient	65	28	65	1,05
Amikacin	Outpatient	68	29	68	1,06
	Average	66	29	66	1,05
	Inpatient	46	19	42	0,99
Netilmicin	Outpatient	50	24	47	1,00
	Average	47	21	44	0,99

ginosa (unlike *E. coli*) susceptibility to be observed. Considering *P. aeruginosa* susceptibility to gentamicin and netilmicin, resistant strains were found to prevail, which was consistent with the utilization of these antibiotics.

Four patterns could be formed of 75 clones of the study bacteria and aminoglycosides. Pattern A consisted of clones that did not change their susceptibility over the two-year period (n=24, 32.4%). These data speak for the fact that there are strains that do not change their susceptibility at all, irrespective of the selective aminoglycosides challenge. The most common pattern B contained various clones (36, 48%) with strains that were either susceptible or resistant to the study aminoglycosides, but were only detected in one of the study periods. Therefore, the pattern of susceptibility modification could not be determined in these clones. The clones of this particular pattern would require the number of sampling to increase, or reduce (for *E. coli*) or the interval between sampling procedures to increase (*P. aeruginosa*).

The clones of pattern D (n=5, 6.7%) developed or otherwise acquired resistance genes under the influence of antibiotics, which was consistent with the current state-of-the-art on the issue.

The clones of pattern C (n=10, 13.3%) showed strain transformation from resistance in period I towards susceptibility to aminoglycosides in period II, indicating the occurrence of an inverse change of susceptibility, to our knowledge not described to date. Thus, sharply opposed, inverse processes of changing resistance to aminoglycosides took place in the bacterial population over a certain period of time irrespective of the utilization of the study aminoglycosides. So, the hypothesis on a minor proportion of resistant clones found in period I and expected to substantially increase in period II due to the effect of aminoglycosides was only in part confirmed by these observations. In other words, the behavior of pattern C clones shed some new light upon the bacterium – antibiotic relations, where a resistant bacterium of a clone need not maintain antibiotic resistance permanently or over a prolonged period of time, therefore the resistant strain of the clone need not turn predominant with time. This will only be possible to demonstrate in additional studies that should include at least three samplings at three different time intervals. The impact of aminoglycosides on the development of resistance of the study bacteria was statistically non-significant, as also confirmed by the low intensity of the lift of susceptible strains in period I to resistant strains in period II, which was ~1. (Table 4)

Resistant strains of a clone were not only detected in inpatient samples, characterized by seriously ill patients, large-scale use of antibiotics, and conditions favoring the development of resistant strains, but also in outpatient samples collected from individuals visiting PHC offices. This finding showed that resistant strains of the study bacteria were as widely spread in inpatient and outpatient microbiological settings. Appropriate choice and monitoring of a consistent strategy of aminoglycosides utilization (e.g., antibiotic rotation, cycling, etc.) appear to play a more important role in the management of patients with resistant causative agents (nosocomial infection) than in the control of the spread of resistance in the study bacteria (or microbial resistance in general). Hospital system is not a closed system; it may only be easier or simpler to identify microbial resistance due to the disease severity and patient treatment. That is why the issue of microbial resistance is currently perceived and interpreted exclusively from the hospital view, thus obviating the fact that the hospital microbiological setting is just a segment of the overall integral microbiological system in which resistant strains are probably impossible to confine within the site of their generation. In healthy or less severely ill carriers of resistant clones from the outpatient population, their health is generally normal or only slightly impaired, thus the problem of microbial resistance being by far less pronounced as compared with the inpatient population. In the present study, there was no significant susceptibility difference between inpatient and outpatient isolates, suggesting a high and rapid strain circulation between these two settings. If it were exclusively the antibiotics that determine the development and spread of microbial resistance (taking decades of their administration into account), the question arises why resistant strains have not yet spread so as to predominate the microbial population at large, after decades of the use of antibiotics?

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UTJECAJ UPORABE AMINOGLIKOZIDA NA NASTANAK I ŠIRENJE OTPORNIH BAKTERIJA

S A Ž E T A K

U razmaku od 2 godine (1998/1999 i 2001) ispitana je osjetljivost 41 soja *Escerichie coli* (*E. coli*), 55 sojeva *Pseudomonas aeruginosa* (*P. aeruginosa*) na gentamicin, netilmicin i amikacin. Za genotipizaciju korištena je pulsfieldgelektroforeza (PFGE). Klonovi su za svaku bakteriju određeni na temelju sličnosti od 80%, odnosno 90%. Cilj je ispitivanja promatrati djelovanje aminoglikozida na dinamiku promjena osjetljivosti sojeva nekog klona u razmaku od 2 godine u navedenim bakterijama i iz bolničkih i iz izvanbolničkih uzoraka. Najveći je broj klonova čiji su sojevi pronađeni samo u jednom ispitanom razdoblju (36, 48%) te se za njih nije mogla odrediti promjena osjetljivosti između dva razdoblja od 2 godine. U 24 klona (32%) sojevi u razmaku od 2 godine nisu mijenjali osjetljivost. Ovi podaci govore u prilog hipotezi da *a priori* postoje osjetljivi i otporni sojevi. U 5 (6,7%) klonova dokazana je promjena od osjetljivosti sojeva u prvom razdoblju prema otpornosti u drugom razdoblju. Ovakvo ponašanje je sukladno shvaćanju da bakterije razvijaju otpornost pod djelovanjem antibiotika. Međutim, dokazano je i postojanje 10 (13,3%) klonova čiji sojevi pokazuju suprotno: otporni sojevi iz prvog razdoblja »gube« otpornost i postaju osjetljivi u drugom razdoblju. Dakle, u promatranom su vremenu promjene osjetljivosti dvosmjerne. 88% klonova sadrže sojeve i iz bolničkih i iz izvanbolničkih uzoraka što ukazuje da nema više zatvorenih sredina. Potrošnja ispitanih aminoglikozida ne utječu na promjenu osjetljivosti.