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ANTIBIOTIC RESISTANCE IN COMMENSAL *Escherichia coli* IN ARMENIA

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ABSTRACT

In this study, we investigated the frequency of antibiotic resistance among human commensal *Escherichia coli* strains isolated from several cohorts, healthy and diseased, in Armenian populations during 2002-2005. Also, the antibiotic resistance profile of selected gut microbiota was studied in people taking the commercial probiotic preparation Okarin. There was a gradual significant increase in the number of multi-resistant (resistant to two and more classes of antibiotics) commensal *E. coli* strains in general populations from 3% in 2002 to 17% in 2005. Comparative analysis of the general population cohort with the cohorts with Familial Mediterranean Fever, chronic colitis, gastritis, and peptic ulcers in 2004-2005 revealed that the diseased populations carry a significantly higher load of antibiotic resistant *E. coli.*

Keywords: *gut microbiota, commensal E. coli, antibiotic resistance*

INTRODUCTION

The emergence and rapid dissemination of antibiotic resistant pathogens is a significant problem in clinical medicine and agriculture [5]. The processes and mechanisms underlying this phenomenon are still poorly understood. Recently, the attention has turned into the gut commensal bacteria that may serve as reservoirs, from which the transient bacteria may acquire and transmit antibiotic resistance genes to pathogens [8]. Not only commensal microbiota may serve as a reservoir of these genes but it could also produce recombinant versions of antibiotic resistance genes that confer the higher level of antibiotic resistance than the original genes [9]. *A priori* the gut ecosystem can be considered as a site potentially conducive for horizontal gene transfer. The constant temperature and nutrient supply are maintained in the system and it has a high density of bacterial populations, frequently forming biofilm-like structures on the surface of food particles and host tissues [7], thus resembling the bacterial conjugation conditions in a laboratory. One of such reservoirs could be commensal *Escherichia coli* populations, from which antibiotic resistance genes can be acquired by pathogenic members of the *Enterobacteriaceae* under ileal conditions [1].
The goal of this study was to evaluate the antibiotic resistance profiles and dynamic of commensal *E. coli* isolated from several cohorts in Armenia, including communities and patients with gastrointestinal disorders. We also studied the effect of probiotic usage on antibiotic resistance profile of gut microflora.

MATERIALS AND METHODS

Sampling. The cohorts investigated in this study were the patients with Familiar Mediterranean Fever (FMF) disease (n=27), with chronic colitis, gastritis, and peptic ulcers (n=12). The community cohorts were 186 healthy controls from the next regions in Armenia: Yerevan (n=114), Kotayk (n=22), Armavir (n=22), and Gegharkunik (n=28). Fecal *E. coli* isolates were also collected from patients with FMF, chronic colitis, gastritis, and breast cancer (mean age 24,4 years) taking either the commercial probiotic Okarin (n=21) or placebo (n=16). All patients were at the Republican Clinical Hospital, and Fanarjyans Oncology Centre, Yerevan, Armenia. The lyophilized probiotic preparation Okarin in ampoules (IMBIO, Ukraine) contained ca. $2.5\text{-}^{0.25}0.25\text{-}2.5\text{x}10^{10}$ viable cells and was taken once or twice daily, for 30 consecutive days. The gut microbiota was analyzed 4-6 months after the discontinuation of probiotic or placebo administration.
None of the healthy controls and patients has been treated with antibiotics, hormones, radiotherapy or any other immunosuppressive or chemotherapeutic agents for 2-3 weeks before sampling.
Freshly voided faeces were collected in sterile plastic bags and transported to laboratory on ice. Faecal material (1*g*) was mixed with 9*ml* of PBS and vortexed for 2 min. The debris was removed by low-speed centrifugation and the supernatant was serially diluted in PBS. The dilutions were plated on MacConkey agar for preliminary identification of *E. coli*, with further analysis using the selective media and conventional biochemical testing [3, 6]. Taxonomically

defined *E. coli* isolates were grown aerobically at 37˚C in LB medium (10*g* tryptone; 5*g* yeast extract; 10*g* NaCl per litre; pH=7,5), solidified with 1.8% agar when necessary.

Antibiotic resistance profiling. At least 5 *E. coli* isolates from each person were tested for susceptibility to the next antibiotics: tetracycline (15*μg/ml*), doxycycline (15*μg/ml*), amoxicillin (25*μg/ml*), ampicillin (35*μg/ml*), cefoperazone (75*μg/ml*), cefoxitin (50*μg/ml*), kanamycin (50*μg/ml*), gentamicin (50*μg/ml*), chloramphenicol (30*μg/ml*), and streptomycin (50*μg/ml*). The cells were plated on LB-agar with corresponding antibiotics and inspected for growth after incubation for 24 h at 37˚*C*.

Statistical analysis. Statistical analysis was performed using the CHITEST (null hypothesis). The probability of $p<0.05$ was to reject the null hypothesis.

RESULTS AND DISCUSSION

Antibiotic resistance in community. Results presented in *Tab. 1* revealed that there was a steady decrease of antibiotic susceptible commensal *E. coli* populations in the Yerevan community in Armenia, from 95% in 2002 to 75% in 2005. The number of isolates with resistance to one antibiotic increased four-fold but the number of multi-resistant isolates (defined as resistant to two and more classes of antibiotics) increased almost six-fold. The data collected at one point from three other distant communities demonstrated the similar antibiotic resistance carriage rates (*Tab. 1*). Thus the geographical location had little impact on antibiotic resistance profiles of commensal *E. coli.* The reason for the increase of the antibiotic resistance carriage rate among commensal *E. coli* isolates in the communities remains unclear. The use of antibiotics in agricultural settings in Armenia is negligible and presumably the major driving force for the selection of antibiotic resistance among gut commensals must be the human consumption of antibiotics. It's not possible to tell, however, whether this is the consequence of the increased antibiotic usage in hospitals with further dissemination to the communities or it is due to the increase of antibiotic use in communities since no data on the level of antibiotic use during these years in hospitals and in the communities were available.

Table 1. The dynamics of antibiotic resistance in commensal *E. coli* isolated from communities in Armenia during January 2002 - July 2005.

Resistance of commensal *E. coli* isolates to antibiotics (%)	2002 Yerevan N=18 n=109	2003 Yerevan [a] N=25 n=125	2004				2005 Yerevan[a] N=22 n=114
			Yerevan [a] N=25 n=127	Kotayk [NS] N=22 n=111	Armavir [NS] N=22 n=113	Gegharkunik [a] N=28 n=144	
Sensitive to all antibiotics tested	95	89	83	79	83	78	75
Single resistance	2	5	5	8	6	12	8
Multiple resistance**	3	6	12	13	11	10	17

N – the number of people; n - the number of isolates; ** - resistant at least to two classes of antibiotics; [a] - $P< 0.05$; [NS] – not significant compared with data from Yerevan (2004).

Antibiotic resistance in patients with gastrointestinal disorders. In a comparative study, we collected and analyzed commensal *E. coli* isolates from a community control population and from patients suffering from gastrointestinal disorders such as FMF, chronic colitis, gastritis, and peptic ulcers in Yerevan during January 2004 – July 2005. The proportion of antibiotic susceptible *E. coli* in these patients was almost two-fold lower than in the community (34% to 43% vs. 78%) (*Tab. 2*). Interestingly, the carriage of multi-resistant *E. coli* strains was very similar in all cohorts, suggesting that the dissemination of this trait does not dependent on the profile of antibiotic usage or the health status. At the same time, resistance to a single antibiotic was more prevalent in *E. coli* isolates from the diseased persons, exceeding the corresponding numbers of the healthy control almost seven-fold when combined together (*Tab. 2*). Resistance to the second generation tetracycline (doxycycline), the second generation of cephalosporins (cefoperazone and cefoxitin), and chloramphenicol was not encountered in 115 isolates from the healthy controls while people with chronic diseases carried the sizeable populations of these bacteria. The absence of resistance to the second generation tetracycline and cephalosporins in the general population suggests that the community cannot be the source of resistance to newer antibiotics; these resistances are selected in hospital settings. Remarkably, the patient cohorts carried chloramphenicol-resistant *E. coli* in the range of 8% to 11% (*Tab. 2*). The use of this antibiotic in human health care is extremely rare because of its toxicity and side effects and most probably this trait was co-selected through the therapy by other, commonly used, drugs. The most prevalent resistance encountered in all cohorts was resistance to penicillins, followed by resistance to tetracyclines (*Tab. 2*). Hospital patients had two-fold higher level of resistance to these two antibiotics than the general population. In addition, *E. coli* isolates from the former cohort carried resistances to cephalosporins and chloramphenicol, which were not encountered in the community.

In summary, there are two points that need to be emphasized. First, the multi-resistant commensal *E. coli* carriage rates are very similar in the community and hospitals, thus not depending on the antibiotic use regimen. No studies of molecular mechanisms behind this phenomenon were attempted in this work and we hypothesize that this may be the case of circulation of a particular mobile genetic element such as a conjugative plasmid or transposon, which is

independent of selection by antibiotics. These elements may circulate in a broader environment and include the bacteria of diverse origin and taxonomic position [4]. Second, the isolates from the hospital cohorts demonstrated much higher single antibiotic resistance carriage rates than controls. These isolates were also resistant to newer antibiotics, the trait that was never encountered in the community. The most plausible explanation for these differences is that there is a strong selective pressure of antibiotics in hospital environments, which selects for resistant commensal microbiota. There is a trend for the increase of antibiotic resistance among gram-negative nosocomial infections [2], this may involve commensal bacteria as well.

Table 2. Antibiotic resistance profiles of gut commensal *E. coli* in community and hospitals in Yerevan, Armenia (January 2004 – July 2005).

Resistance of commensal *E. coli* isolates to antibiotics (%)	Community control N=23; n=115	Patients	
		FMF patients [a] N=27; n=137	Patients with chronic colitis, gastritis, and peptic ulcers [a] N=12; n=60
Sensitive to all antibiotics tested	78	34	43
Tetracyclines Tetracycline Doxycycline	14 14 0	28 26 10	27 25 8
Penicillins Amoxicillin Ampicillin	25 22 12	50 50 26	45 45 42
Cephalosporins Cefoperazone Cefoxitin	0	12 8 67	15 13 3
Chloramphenicol	0	11	8
Streptomycin	1	-	-
Resistant strains	7	49	40
Multi-resistant strains**,	15	18	17

- - not determined; N – the number of people; n - the number of isolates; * - resistant to at least two classes of antibiotics;
[a] - P< 0.005

Effect of probiotic consumption on antibiotic resistance. There has been a considerable controversy surrounding the antibiotic resistance dissemination possibility by probiotic bacteria, especially of vancomycin resistance by enterococci [2]. In East Europe, the antibiotic resistance trait of probiotics is considered to be beneficial because it allows to treat the antibiotic-caused bacteremia as early as possibly, even during the antibiotic treatment phase. At the same time, the EU experts propose as safety criteria for probiotics the elimination of resistances or at least the lack of transferability (http://europa.eu.int/comm/food/fs/sc/scan/out108_en.pdf). In our preliminary experiments we characterized antibiotic resistance profiles of two *E. coli* strains, which are the components of probiotic formulation Okarin. The strains appeared to be resistant to tetracyclines and, in addition, one of them was also resistant to ampicillin (our unpublished data). In healthy volunteers that were taking this probiotic for 30 days and discontinued it 4-6 months before the sampling (see Materials and Methods), the number of antibiotic susceptible *E. coli* isolates dropped significantly in comparison with the placebo-taking control (*Tab. 3*). This was due to the increase of numbers of single antibiotic resistant commensal *E. coli,* because the number of multi-resistant isolates remained unchanged. It is not apparent, however, whether the changed antibiotic resistance profile reflects the successful colonization by one of the probiotic strains (with a single resistance) or the transfer of antibiotic resistance to the indigenous *E. coli* population in the gastrointestinal tract of volunteers. In the patient cohort, where the antibiotic resistance was already prevalent, no significant effect of the previous probiotic intake on the antibiotic resistance profile of commensal *E. coli* was seen (*Tab. 3*). The results suggest that even not prolonged intake of an antibiotic resistant probiotic by healthy individuals may contribute to the rise of antibiotic resistant *E. coli* flora in the gut. It remains to be ascertained; however, what mechanisms were involved in this phenomenon, successful colonization by antibiotic resistant probiotic strain or this was the consequence of antibiotic resistance gene dissemination by the transient probiotic strain.

CONCLUSIONS

Resistance and multi-resistance to the commonly used antibiotics in commensal *E.coli* is on the rise in Armenian communities, independent of geographical location. And it is even higher in patients with various gastrointestinal disorders. Since the antibiotic use in agriculture in this country is negligible, the major driving force for the rise of antibiotic resistance is the human consumption, in communities and hospitals. Intake of a probiotic preparation with antibiotic resistant bacterial components may represent a risk factor for antibiotic resistance increase in healthy subjects.

Table 3. Antibiotic resistance of gut commensal *E. coli* from healthy and diseased subjects taking probiotic Okarin

Resistance of commensal *E. coli* isolates to antibiotics (%)	Community control* N=23 n=115	Community control taking placebo [a] N=13 n=65	Community control taking Okarin [b] N=11 n=57	Patients with FMF, chronic colitis, gastritis, peptic ulcers, and breast cancer taking placebo; N=16; n=84	Patients with FMF, chronic colitis, gastritis, peptic ulcers, and breast cancer taking Okarin [NS]; N=21; n=105
Sensitive to all antibiotics tested	78	78	50	44	43
Resistant to one antibiotic	7	8	37	13	13
Multiple resistance**	15	14	13	43	44

N – the number of people; n - the number of isolates; * - January 2004 – July 2005, Yerevan; ** - resistant to at least two classes of antibiotics; [a] - P< 0.005, compared with the healthy control group; [b] - P< 0.005, compared with the healthy control group taking placebo; [NS] – Not significant in comparison with the patients taking placebo

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