Urinary Tract Infections in Patients with Type 1 and Type 2 Diabetes: Etiology, Resistance to Antibacterial Chemicals and Virulence Features

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One of the most frequent chronic complications occurred in diabetes patients are the urinary tract infections (UTIs). This study aimed to investigate the incidence of UTIs in a cohort of 93 (47 males: 46 females) diabetic patients, the prevalence of different microbial species involved and their virulence and antibiotic resistance profiles. The identification of the uropathogenic strains in the positive urine samples was performed using conventional methods and API tests. After identification, the antibiotic susceptibility profiles were established by the standardized disk diffusion method and double disk diffusion test was performed for the confirmation of ESBL and inducible AmpC β –lactamase phenotypes. The isolated strains were tested for the production of different cell associated and soluble virulence factors, i.e.: bacterial adherence to cellular substrata (HeLa cells), hemolysins (hemolysis spot, CAMP-like), amylase, caseinase, aesculin hydrolysis, DNA-ase, lipase and lecithinase. In the analyzed group, the total prevalence of UTIs was of 46%, a higher incidence being observed in the female patients (64%). Similar to other studies, the etiology of UTI in the investigated diabetes patients was dominated by E. coli, followed by Klebsiella sp. strains. The isolated strains preserved good susceptibility rates to quinolones and aminoglycosides and revealed important virulence features, related to their capacity to colonize the cellular substratum and to produce soluble virulence factors involved in persistence, colonization and progression of the infectious process. The high percentage of beta-lactam resistant strains (including carbapenem-resistant ones) requires careful surveillance of the dynamics of susceptibility profiles for limiting the emergence of these strains in community.

Keywords: diabetes, urinary tract infections, resistance, virulence

Despite the huge research progress having made on diabetes, a disease mentioned more than five thousand years, there are still many unknowns concerning the etiology, prevalence, transmission, the possibility of preventing the disease or its complications and the therapeutic approaches. Therefore, diabetes still remains one of the most prevalent chronic diseases and the most common endocrine one, with a huge social impact due to its chronic complications, and the high morbidity occurring in varied age groups. Patients with diabetes present an increased risk for urinary tract infections due to immunosuppression and on the impaired kidney filtration system function, a condition known as diabetic nephropathy [1]. Diabetes patients with pathological urinary tract, urolithiasis and pregnant women patients with gestational diabetes are more prone to such infections, requiring special attention to the correct diagnosis and treatment, considering the high incidence of antibiotic resistant strains. Misuse of antibiotics can lead to recurrences of the infection and the emergence of resistant strains, that should be considered when prescribing antibiotics, and could lead to changes in first-line treatments in UT [2]. This study aimed to investigate the incidence of urinary tract infections in a cohort of diabetic patients, the prevalence of different microbial species involved and their virulence and antibiotic resistance profiles.

Experimental part
Materials and methods

The study was conducted during 2015-2016 on a cohort of 93 patients (47 males: 46 females), separated on three age group, i.e.: 20-40 years old (13M: 12F), 40-60 (16M: 16F) and 60-80 (18M: 18F). The blood sugar evaluation allowed the assessment of the status of carbohydrate metabolism in diabetic patients and glycated hemoglobin estimation provided a retrospective of glycemic status, independent of the circadian rhythm, diet and other transient fluctuations in blood glucose concentration. Immunoturbidimetric method, standardized by Diabetes Control and Complications Trial (DCCT) and certified by National Glycohemoglobin Standardization Program (NGSP) was used. Due to the high specificity of the anti-HbA1c antibody for a sequence of 4 amino acids from the N-terminus of the β chain, the test does not interfere with other conditions generating pathological hemoglobin, such as carbamyl hemoglobin seen in uremic patients or acetyl hemoglobin generated by aspirin therapy.

The concentration of the total hemoglobin was determined in a separate channel. In the hemolyzed blood

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All authors have equal contribution to the study .

samples, the released hemoglobin was converted into a derivative with a characteristic absorption spectrum, measured bichromatically; HbA1c percentage calculation was performed according to the DCCT / NGSP protocol, applying correction formulas:

$$\%HbA1c = (HbA1c/Hb) \times 91.5 + 2.157$$

The results interpretation was done according with American Diabetes Association (ADA) i.e.: normal: 4.8-5.6%; high risk of diabetes: 5.7-6.4%; diabetes: >=6.5%; diabetes patients needing treatment: >=7%.

Determination of HbA1c antibodies enables estimation of plasma glucose concentrations and approximate averages can be calculated according following equation [3] eAG (mg/dL) = 28.7 × HbA1C - 46.7; eAG(mmol/l) = 1.59 × HbA1C - 2.59.

The identification of pathogenic agents in the positive urine samples was performed using conventional methods and API tests. After identification, the antibiotic susceptibility profiles were established by the standardized disk diffusion method (using Mueller-Hinton medium) [4, 5]. For results interpretation and defining the categories of sensitivity, the CLSI 2016 was used.

For detection of ESBL producing strains double diffusion test was performed, for evidencing the synergism between amoxicillin - clavulanic acid (AMC) and third generation cephalosporins, i.e., ceftazidime (CAZ) and cefotaxime (CTX). For detection of inducible AmpC β-lactamase, double diffusion test evidenced the antagonism between imipenem (IMP) and CAZ / CTX [6, 7].

The isolated strains were tested for the production of different soluble virulence factors, i.e.: hemolysins (hemolysis spot, CAMP-like), amylase, caseinase, aesculin hydrolysis, DNA-ase, lipase and lecithinase, using specific media with incorporated biochemical substrata for the tested enzymes, i.e. sheep blood agar, amidon, casein, aesculin, DNA, Tween 80 agar and lecithin [8, 9].

Evaluation of the bacterial adherence to cellular substrata (HeLa cells) was performed using the Cravioto's adapted method, Briefly, the HeLa cells monolayers were incubated for 2h at 37°C with the microbial strains suspensions and then Giemsa stained and examined microscopically to evaluate the adherence index and patterns. The adherence index was expressed as the ratio between the number of the eukaryotic cells with adhered bacteria and 100 eukaryotic cells counted on the microscopic field. The adherence patterns were defined as: localized adherence (LA) when tight clusters of microorganisms were noticed on the HeLa cell surface, aggregative adherence (AA) when a microbial stacked brick pattern characterize the attachment, diffuse adherence (DA) when the bacteria adhered diffusely, covering the whole surface of the cell [10].

Results and discussions

The analyzed cohort exhibited a uniform discrete binomial distribution according to the type of diabetes, i.e. 43% had type I and 57% type two diabetes.

Our results revealed that blood glucose levels increase with age in both types of diabetes, being slightly higher in case of type 1 diabetes (fig. 1). Also, according to figure 1b, an effective control of blood glucose levels was not observed in any type of diabetes and it seems that control is becoming more difficult with age. Better control was still observed in type 2 diabetes, with values closer to 8%, with an average of 9.15 (± 1.07) % (typical values for a controlled diabetes).
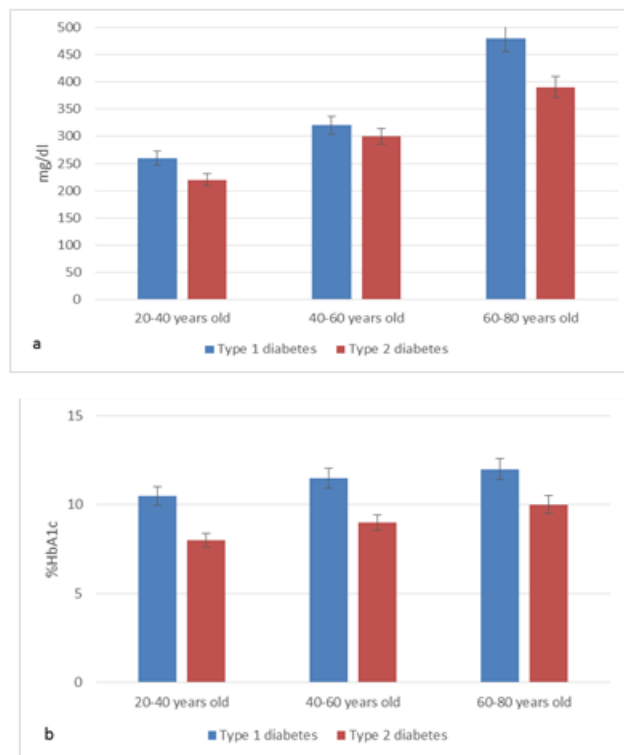

Fig. 1. The mean values of blood glucose (a) and glycated hemoglobin (b), depending on the age

A midstream urine count ≥10^5 CFU/mL is considered a positive diagnostic for UTI [11]. From the 93 patients with diabetes, 45 of them (46%), 29 women and 16 men have developed UTIs. The incidence of UTIs in the analyzed cohort is much more higher than that reported in other studies performed in Romania and abroad. A recent study performed in Romania on 2,465 adult patients with diabetes mellitus (DM) found a prevalence of UTIs of 12.0%, being higher in females than in males and higher in patients with type 2 DM compared with patients with type 1 DM [12].

An American study from 2014 found that a UTI diagnosis was more common in men and women with diabetes than in those without diabetes (9.4% vs 5.7%, respectively) among 89,790 matched pairs of patients with and without type 2 diabetes mellitus [13]. The microbial etiology was represented by members of *Enterobacteriaceae* family, i.e. *E. coli* (78%) and *Klebsiella* spp. (22%) strains. Results obtained in this study were similar to those in other studies [14, 15]. Indeed, no marked difference in the type of microorganisms causing the UTI in the diabetic and nondiabetic patients was observed, *E. coli* being predominant pathogen species isolated also from diabetic patients [16].

Regarding the antibiotic resistance profiles, the results demonstrated that the tested strains were resistant to beta-lactam antibiotics, with the following resistance rates: AMC-82.22%, CAZ-34.78 %, FOX-23.91%% and CXM-19.57% (figure 2). From all beta-lactams tested, the tested strains were most sensitive to IMP (only 15.22% of them being resistant). Resistance to the third generation cephalosporins (CAZ) was mediated in these strains by the presence of broad-spectrum beta-lactamases (ESBL) or the simultaneous presence of ESBLs and AmpC enzymes, the two mechanisms of resistance to beta-lactam antibiotics generating high levels of resistance to cephalosporins.

Regarding other classes of tested antibiotics, *Enterobacteriaceae* strains had relatively high levels of resistance to trimethoprim/sulfamethoxazole (30.43%),
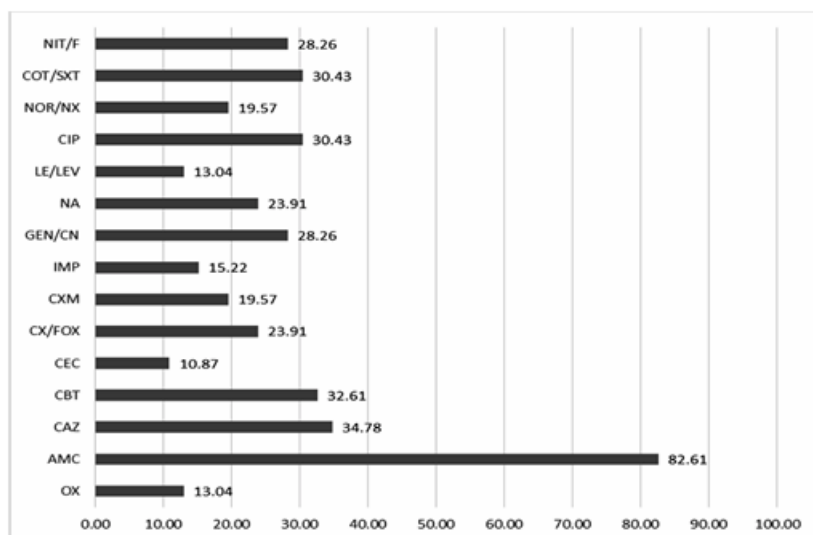
Fig.2. Antibiotic resistance rates in the tested strains

Antibiotic class	Antibiotic	Resistant	Intermediary	Susceptible
penicillins	Oxacillin (OX)	6	17	23
B-lactam/b-lactamase inhitors	Amoxicillin + clavu-lanic acid (AMC)	38	0	8
cephems	Ceftazidim (CAZ)	16	8	22
	Ceftibuten (CBT)	15	18	13
	Cefaclor (CEC)	5	21	20
	Cefoxitin (FOX)	11	20	15
	Cefuroxim (CXM)	9	17	20
carbapenems	Imipenem (IMP)	7	15	24
aminoglycosides	Gentamicin (GEN)	13	14	19
quinolones	Nalidixic acid (NA)	11	18	17
fluoroquinolones	Levofloxacin (LEV)	6	23	17
	Ciprofloxacin (CIP)	14	16	16
	Norflaxacin (NOR)	9	28	9
folate pathway in-hitors	Sulphametoxazole (SXT)	14	16	16
nitrofurantoin	Nitrofurantoin (NIT)	13	10	23

Table 1
ANTIBIOTIC RESISTANCE/ SENSITIVITY PROFILE OF TESTED STRAINS

aminoglycoside antibiotics (GEN-28. 26%) and some quinolones (CIP-30.43%, NA-23.91%, NOR-19.57%), excepting LEV, for which the resistance levels were relatively lower 13.04%) (table 1, fig. 2). This fact proved that, in case of complicated urinary tract infections caused by *K. pneumoniae* and *E. coli*, derivatives of fluoro-quinolones (levofloxacin) still remain the best therapeutic option. According to our data, *E. coli* strains resistant to trimethoprim + sulfamethoxazole were sensitive to the other classes of antibiotics, while the ESBL-producing strains were sensitive to amikacin and imipenem.

Another result sustaining the high frequency of UTI in diabetic patients is increased adherence of the bacteria to uroepithelial cells [17]. In our study, the isolated *Enterobacteriaceae* tested strains showed the adherence capacity to cellular substrate (represented by HeLa cells) in 40% of the cases, reflecting their potential to initiate an infectious process. The adherence patterns observed were: aggregative (20%) and diffuse (18%) adherence. After colonization of the urinary bladder and reaching a critical density bacterial activity is switched on exoenzymes synthesis. The enzymes from the invasins family (hemolysins, proteases and lipases) accmulate in the host organism causing bacterial dissemination and infection progression. The results highlighted that the most of the strains presented at least one of the seven tested virulence factors. The presence of all these enzymes is proving the pathogenic potential of these strains. Most strains were positive for lecithinase (98%), that together
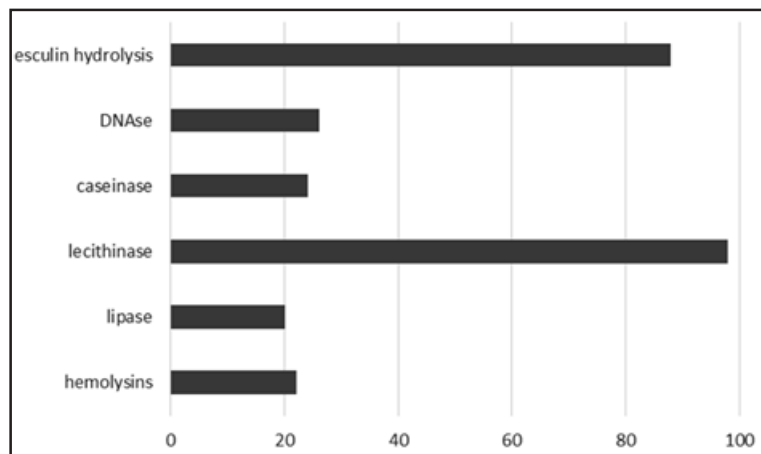
Fig. 3. The expression rate of different virulence factors expressed by *Enterobacteriaceae* strains

with hemolysins and lipases (22%, respectively 20%) are pore forming toxins that cause pores formation in the cell membrane, allowing the dissemination of infection (fig. 3). The tested strains presented also a high capacity to hydrolyze esculin (88%). Iron is an essential compound for microbial growth and virulence. In the extracellular medium iron is found in a non assimilable form, the bacteria requiring siderophores to acquire it. It was proved that iron may be fixed by esculetol with high affinty, therefore esculinase has an important role in ensuring Fe uptake required for the activation of bacterial genes and expression of some virulence factors [18]. Caseinase (24% of the tested strains) is a proteolytic enzyme that hydrolyses casein, a protein abundant in milk. Numerous studies have shown that proteases produced by pathogenic organisms may contribute to the severe symptoms of infection.

Conclusions

Research conducted over the past decade has shown that an increasing sedentary lifestyle and obesity rates have led to increased incidence of diabetes. One of the most frequent chronic complications occurred in diabetes patients are the urinary tract infections. In the analyzed group, the total prevalence of UTI was of 46%, a higher incidence being observed in female patients (64%). Similar to other studies, the results obtained concerning the etiology of UTI have revealed *E. coli* being the most commonly isolated bacteria, followed by *Klebsiella* sp. The isolated strains preserved good susceptibility rates only to some fluoroquinolones and revealed important virulence features, related to their capacity to colonize the cellular substratum and to produce soluble virulence factors involved in persistence, colonization and progression of the infectious process. The high percentage of beta-lactam (including carbapenem)-resistant strains requires careful surveillance of the dynamics of susceptibility profiles for limiting the emergence of these strains in community.

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