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Community-Acquired Carbapenemase-Producing Uropathogenic Enterobacteria Strains

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Abstract Microbiological analysis of urine was conducted at 426 patients of private diagnostic center «Vitras» diagnosed with acute uncomplicated urinary tract infection (UTI). Microbial growth in diagnostic titers (10^3 and above CFU/ml) was accounted for 38,7%, 147 (87,7%) of isolates related to enterobacteria, 112 (78.3%) from them related to E. coli. 15 strains with various phenotypes of resistance in DDT to the 3rd base carbapenemase (imipenem, meropenem, ertapenem) were investigated for carbapenemase-producing by using NordmannP acid-chemical method in modification of Pires J. et al. - CARBA/BLU method and in parallel by genic-molecular PCR method in «real time» mode. Identification of genes encoding of carbopenemase-producing was conducted by using 2 sets of «AmpliSens® MDRMBL-FL»: to identify the genes of acquired carbapenemase-producing by metal-β-lactamase (MBL) of the VIM, IMP, NDM groups and «AmpliSense® MDR» to identify KPC/OXA-48-FL produced by Federal Budget Institution of Science «Central Research Institute of Epidemiology» of The Federal Service on Customers' Rights Protection and Human Well-being Surveillance. At community-acquired E.coli uropathogenic strains all genes detected using by PCR (5 out of 15, 33,3%) related to metal-β-lactamase class. The most frequent NDM-1 gene was detected - at 3 - just NDM-1 emerged, at one strain together with VIM and one more strain of E.coli possessed only the VIM gene. All these strains with NDM-1 and VIM genes were positive in the phenotypic CARBA/BLU method. The IMP genes MBL class, KPC genes and OXA-48 genes were not found in the studied isolates. An accumulation of amplification products of the VIM gene and the OXA-48 gene at separate isolates of Escherichia with a positive CARBA/BLU reaction was observed later (after the 31st cycle).

Keywords UPEC, MBL, NDM-1, OXA-48, VIM, IMP, CARBA/BLU, PCR

1. Introduction

The infections of urogenital tract infections (UTI) occupy the first place among all urological diseases, they have been found in both outpatient and hospital practice, and Escherichia coli is currently considered the most significant uropathogenic bacteria [11 2, 14]. The resistance of Escherichia, Klebsiella, and other opportunistic pathogenic Gram-negative bacteria to antibiotics is one of urgent problems of public health all over the world [14, 4, 10]. A particularly important role is played by the resistance of these microorganisms to beta-lactam antibiotics; at the same time leading by mechanism of resistance for E.coli and other enterobacteria is ability to produce enzymes that inactivate beta-lactams [10, 6, 5, 9]. In addition to the extended-spectrum β-Lactamases (ESBLs) capable to hydrolysis of all cephalosporin's, but not active on carbapenemase, all greater value is acquired carbapenemases active against all antibiotics of this class, excluding aztreonam [16, 10]. Carbapenems (imipenem, meropenem, ertapenem, doripenem) relate to reserve medicine; they have extensive coverage of action, low toxicity, good pharmacokinetic parameters, and the crucial - until recently a resistance to these medicine was rare enough [12, 13]. The occurrence and spread of carbapenemases-producing Enterobacteriaceae (CPE) and, in particular, Escherichia, is a significant negative factor for public health, therefore ensuring timely and reliable detection of CRE is the first crucial step in combating of this problem [4].

The study goal was to study of community-acquired carbapenemase-producing uropathogenic Escherichia strains by phenotypic and genotypic methods.

2. Materials and Methods

The study was carried out at the Department of Microbiology of the Tashkent Institute of Postgraduate Medical Education; patients were admitted for examination by private diagnostic center «Vitras» LLC

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VITROSDIAGNOSTICS. 426 patients were diagnosed with a diagnosis of acute uncomplicated urinary tract infection (UTI) from October 2017 through March 2018. Cultivation of urine samples was carried out on Endo medium with selection of typical lacto-positive colonies. The study was carried out using by sectoral Gould method, microorganisms growing in urine dilution of 10^3 CFU/ml and above in accordance with International Standard [2]. Microbial growth in diagnostic titers was 38,7% (165 of 426), 147 (87,7%) of isolates related to enterobacteria, of which 112 (78.3%) related to E.coli. A sensitivity of the isolated Escherichia to various classes of antibiotics was determined by disk-diffusion test (DDT) according to recommendations of the EUCAST [15]. Internal quality control of determining the sensitivity to antibiotics was carried out with the verificatory Reference strain E.coli ATCC 25922. 15 strains of Escherichia with various resistance phenotypes in DDT to the 3rd basic carbapenems (imipenem, meropenem, ertapenem) were studied for carbapenemase-producing by NordmannP acid-chemical method in modification of Pires J. et al. by CARBA / BLU method and in parallel by genic-molecular PCR method. Genic-molecular testing was conducted in reference PCR laboratory of Diagnostics Research Institute of Virology of the Ministry of Health of Republic of Uzbekistan (Uzbekistan, Tashkent), in accordance with manufacturer's instructions of the set. Identification of genes encoding carbapenemase-producing was conducted using by 2 sets: «AmpliSens® MDRMBL-FL»: 1) to identify the genes of acquired carbapenemase-producing by metal-β-lactamase (MBL) of the VIM, IMP, NDM groups; 2) «AmpliSense® MDR» to identify KPC/OXA-48-FL produced by Federal Budget Institution of Science «Central Research Institute of Epidemiology» of The Federal Service on Customers' Rights Protection and Human Well-being Surveillance (Russia). Both sets are designed for PCR with hybridization-fluorescence detection of amplification products in "real time" mode. The PCR material was DNA samples obtained by extraction from samples of pure E.coli culture isolated from urine's patients with community-acquired UTI in titers of 103 / CFU / ml and above. Extraction of bacterial DNA was performed using by Ampli Prime DNA Sorb sets (Central Research Institute of Epidemiology, Russia) according to attached instructions. Amplification was carried out according to program in the tablet-type device.

Table 1. Temperature, time and cycles' number at «AmpliSense-1» program

Tablet-type devices			
Cycles	**Temperature, °C**	**Time**	**Cycles' number**
	95	15 min	1
1	95	5 s	5
2	60	20 s	
	72	15 s	
	95	5 s	40
3	60	30 s fluorescence detection signal	
	72	15 s	

3. Results and Discussion

Considering the importance of antibiotic resistance problem, in order to determine the resistance of modern pathogens to antibiotics, great attention is paid to not only genic-molecular, but also to phenotypic methods that are available for implementation in routine practice. It is recommended (EUCAST) at the first stage to identify "suspicious" bacteria for the production of extended-spectrum β-Lactamases (ESBLs or carbapenemases) by ordinary DDT with 3 basic antibiotics of each group. When screening for carbapenemases, if at least one of the recommended carbapenems is stable (imipenem, meropenem and ertapenem), confirming the carbapenemase-producing can be validated by several phenotypic methods, including CARBA/BLU method. Earlier, we have found that local uropathogenic E.coli resistance to semisynthetic and inhibitor-protected penicillins and cephlosporins of the 2nd and 3rd generations was high, and majority (83,1%) of the studied cultures were classified as "suspicious" to ESBLs products, which was confirmed by disk combined method with clavulanic acid. It was also shown that resistance to carbapenems in screening with imipenem, meropenem and ertapenem in DDT in out-of-hospital E.coli averaged 59,1%, but phenotypes were different, the most frequent combination being sensitivity to imipenem, moderate resistance to meropenem and insensitivity to meropenem ertapenem.

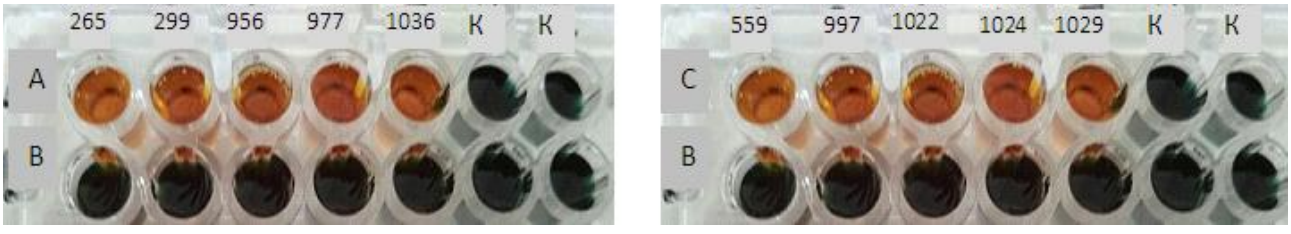

Figure 1. The study results of 10 community-acquired E.coli urogenic strains at CARBA/BLU test: Legend: A, C - experimental samples (culture+imipnem+indicator); B-control samples (E.coli culture ATCC+indicator)

15 strains were selected from total number of E. coli strains suspicious for carbapenemase-producing in DDT, and CARBA / BLU method was used as a confirmatory phenotypic method. These results presented in figure 1.

The picture shows that 10 strains of Escherichia were positive at CARBA/BLU test, which manifested itself as bright yellow staining of wells (from 1st to 5th) in A and C row. The contents did not change color at all wells that testifies about negative result (control samples).

The study materials of urogenic E.coli strains for presence of carbapenemal antibiotic resistance genes in comparison with the CARBA / BLU-test was present below. It was noted that genes IMP (related to MBL), and KPC genes encoding serine carbapenemases (molecular class A) were not found in the studied isolates. All identified genes related to molecular class of metall-β-lactamase (MBL). The figure of accumulation of fluorescent signal of accumulation of products of amplification of fragments of NDM-1 and VIM genes are presented below.

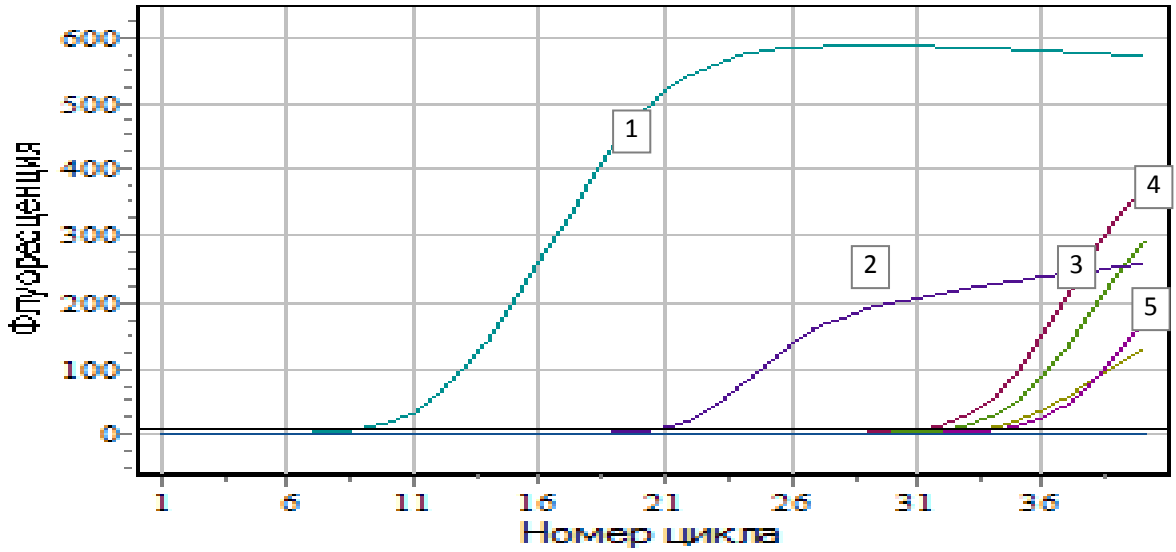

Figure 2. Figure of accumulation of amplification products of NDM-1 genes: Legend: 1 - E.coli №956; 2 - K+; 3 - E.coli №1036; 4 - E.coli №977; 5- E.coli №265

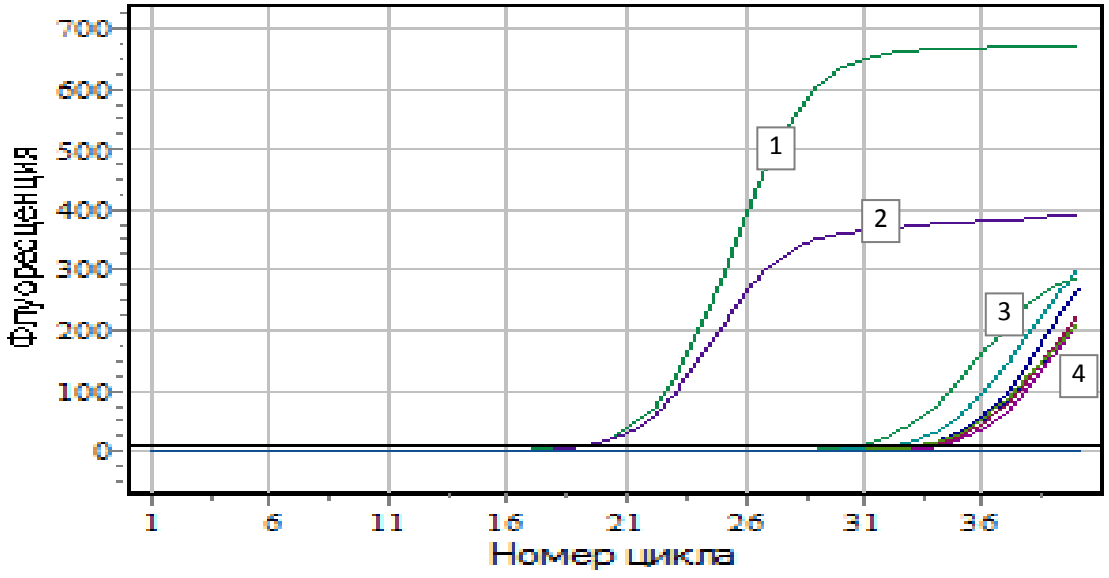

Figure 3. Figure of accumulation of amplification products of VIM genes: Legend: 1 - K+; 2 - K+; 3 - E.coli №299; 4 - E.coli №956

As can be seen, all the identified genes were found in strains positive at CARBA/BLU test. All of them related to molecular class of metal-β-lactamase, total amount of such strains were 5/33,3%. NDM-1 genes were found in 4 strains of Escherichia - at 3 (265, 977, 1036) – only NDM-1, at one (956) in combination with VIM gene. The VIM gene was detected only at one culture (299) without combining with other genes. 5 E.coli urogenic strains were positive in

CARBA/BLU, but in PCR at 3 of them (599, 997, 1022) the VIM gene amplification products were manifested only after the 31st amplification cycle (picture 3). The same situation was observed with the OHA-48 gene (related to serine carbapenemases of D group), which, according to literature, is one of the most common carbapenem resistance genes [13]. In 3 strains of E.coli positive in CARBA/BLU test (1036, 299, 265) the accumulation of amplification products of this gene was later (picture 4) and exceeded boundary values of threshold cycle (Ct more than 31), which according to manufacturer's instructions It is not possible to attribute these samples to positive findings.

The sets of primers and proof sticks used by us were designed in accordance with the genes strains circulating in Russia, which is quite far from Uzbekistan. The fact that the prevalence of microorganism resistance genes in different countries and territories can vary significantly is indicated by many publications, for example, Nordmann P et al. [6] demonstrated this for ESBLs - 3-5% in France, >80% in India. The negative results of PCR with a positive CARBA/BLU result might be due to: or absence of primers for known but rarely encountered carbapenemase genes (for example, SPM, GIM or others) and not included in multiplex set used by us; or circulation in our territory of previously not described genes of this group.

As it was noted, genetic analysis was also conducted as well with 5th strains E.coli, which in CARBA/BLU phenotypic method were negative. Preliminary screening of these strains in DDT found various resistances to phenotypes among them to 3 carbapenems. Specific attention attracts strain 1060: total absence of growth inhibition zones to 3 carbapenems neither in CARBA/BLU, nor in PCR revealed of positive results. Most likely, resistance of this strain to carbapenems is caused not by enzymes production, and other mechanisms of resistance, for example - partial either total loss of porins, activation of efflux, or combination of both mechanisms.

Despite few number of cultures studied, the detection of MBL producers among the out-of-hospital E.coli urogenic strains is quite worrying sign, which may indirectly indicate a wide circulation of microorganisms resistant to the most active reserve antibiotics in the region - carbapenems. According to many authors, MBL and beta-lactamase type OXA (B and D classes) are currently the biggest problem. In our region, the prevalence of community-acquired carbapenemase-producing uropathogenic enterobacteria strains has not been studied previously, and this problem requires further extensive researches. Since most carbapenemase genes have plasmid localization and can quickly spread in population by horizontal way, the problem of carbapenem-resistance of bacteria becomes particularly acute.

According to ECDC (European Center for Disease Prevention and Control) in 2013, the highest level of resistance to carbapenem antibiotics was recorded at K.pneumoniae - 8,3%, but at E. coli this indicator was much lower and was just 0,2%, which correlated with data from St.Petersburg. It is also known, that countries which have an increase in frequency of carbapenem-resistant E.coli are epidemiologically unfavorable [12]. It is suggested that rapid changes in resistance of microorganisms to antibiotics occur differently in various regions and territories of the world and depend on many factors. Therefore, local monitoring of antibiotic resistance is a key factor for increasing effectiveness of antimicrobial therapy. Determination of CPE using PCR, which we used, is a reference standard for accurate identification of carbapenemases genes. The review by J. Hrabak, et al. [3] highlights the possibilities, advantages and disadvantages of pheno- and genotypic methods for detection of carbapenemase-producing enterobacteria. It is noted that a large number of well-known genes of carbapenemases makes their routine screening difficult because of need to set up a large number of PCR reactions, and this method does not allow detecting the new genes of carbapenemases. With a detailed analysis of existing phenotypic and molecular methods for detecting carbapenemases today (sensitivity, specificity, accessibility, price, etc.) J.D.Lutgring, et al. [4] come to a fair conclusion - there is no single method that is ideal for all situations.

4. Conclusions

1. In community-acquired uropathogenic E.coli strains, in 33,3% (5/15), metal-beta-lactamase genes were found. The most frequently detected NDM-1 gene - at 3 - only NDM-1; one strain in combination with VIM and one isolate - only VIM.

2. All strains with the NDM-1 and VIM genes were positive in CARBA/BLU phenotypic method.

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