Phenotypic and genetic analysis of the antibiotic resistance patterns in uropathogenic *Escherichia coli* strains

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Abstract

The purpose of this work is to characterize by phenotypic and genotypic analysis the antibiotic resistance patterns in 118 Escherichia (E.) coli strains isolated from urinary tract infections in patients with neurogenic bladder. The E. coli strains have shown high level resistance to aminopenicillins, ampicillin + sulbactam, amoxicillin clavulanate, tetracycline, followed by quinolone, carboxipenicillins, aminoglycosides and cotrimoxazole. Multiple resistance to beta-lactams, aminoglycosides, fluoroquinolones and cotrimoxazol has been registered with high frequency among the studied strains. The phenotypic screening tests for the presence of beta-lactamases, i.e. cefinase and double disk synergism test (DDST) indicated that 65.2% of the tested strains are producing typical beta-lactamases. The synergy test was positive for the strains resistant to clavulanic acid, but susceptible to sulbactam, proving that this inhibitor is more sensitive for this test. Our study indicated a good correlation between the results obtained with DDST and E-test ESBL which confirmed the production of beta-lactamases in strains exhibiting synergism aspects in double disk diffusion test. The IEF analysis of the cellular sonicates revealed the presence of TEM pI 5.2; CTX-M pI 7.5; AmpC pI ≥ 8; SHV pI 7-8. The genotypic analysis by PCR and sequencing showed the presence of bla TEM and group 1 CTX-M genes in 75% of E. coli strains isolated from patients with neurogenic bladder.

Keywords: *Escherichia coli*, urinary tract infection, neurogenic bladder, resistance markers

Introduction

Urinary tract infections (ITU) are representing one of the most common infectious diseases encountered in all ages. Due to the large number of cases, with medical and economic considerable implications, the microbial strains involved in the aetiology of urinary infections are still a top priority of epidemiological and bacteriological studies (9). Enterobacterial strains and especially *Escherichia (E.) coli*, normally found in the microbiota of the gastrointestinal tract, are the most frequently aetiologic agents involved in the ITU pathology (1).

The extensive use of the antimicrobial substances led to the emergence of multiresistant strains, increasing the number of urinary infections and complicating their clinical picture. In neurological pathology, urinary infections are common because of the particular characteristics of these patients (2, 6). Extended bed immobilisation by motor deficit of hemiplegic or paraplegic type and associated mictional disorders are representing favoring conditions for the occurrence of urinary tract infections (8). As a particular result of neurological disease is the installation of neurogenic bladder, vesical residue and intestinal disbiosis (1, 8).
Material and Methods

**Microbial strains**

A number of 118 *E. coli* strains isolated from positive urine cultures taken from patients admitted to the Neuropsychiatric Clinical Hospital of Craiova during December 2006 and October 2007 were analyzed in the present study.

**Antibiotic susceptibility testing**

It was performed using standardized API ATB UR5 (ref. no. 14335, BIOMERIEUX), as well as disk diffusion method (12). The internal quality control was performed using *E. coli* ATCC 25922 reference strain (ref. no. 0335P, MicroBioLogics).

The following antibiotics were tested: **Piperacillin** (ref. SD066, HIMEDIA LAB., LMT), Amikacin (Ak 30µg ref. SD035), Ampicillin (AMP10µg ref. SD063), Ampicillin/Sulbactam (AS 10/1 µg ref. SD112), Amoxicillin/Clavulanic Acid (AMC 20/10µg SD063), Aztreonam (A0 30 µg ref. SD212), Cefazoline (Cz 30 µg ref. SD047), Cefepime (Cpm 30 µg ref. SD219), Cefazidime (Caz 30 µg ref. SD062), Ceftriaxone (Cro 30 µg ref. SD065), Cefuroxime (Cxm 30 µg ref. SD061), Cefalotin (Ch 30 µg ref. SD050), Cefotaxime (Ctx 30 µg ref. SD040), Cefoxitine (Fox 30 µg ref. SD041), Ciprofloxacin (Cf 5 µg ref. SD060), Co-Trimoxazole (Trimetoprim/sulfametaxazole) (Sxt 1,25/23,75 µg ref. SD010), Gentamycin (G 10 µg ref. 016), Imipenene (I 10 µg ref.073), Kanamycin (K 30 µg ref. SD017), Norfloxacine (Nx 10 µg ref. SD057), Piperacillin (Pc 100 µg ref. SD066), Tobramycin (Tb10 µg ref. SD044) HiMedia Lab. Lmt.

**Phenotypic tests for the confirmation of beta-lactamase production**

**b) Chromogenic tests**

The assessment of beta-lactamases was performed by the rapid cefinase test, consisting in the hydrolysis of nitrocephine, a chromogenic cephalosporin which in the presence of microbial beta-lactamases generates a colored product, evidenced after adding a loop of microbial culture taken from solid culture media.

**b) DDST-(double disk synerity test)**

The extended spectrum beta-lactamase (ESBL) production is suspected when a synergic activity of the third generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone and aztreonam) is observed in the presence of the beta-lactamases inhibitor (i.e. clavulanic acid and/or sulbactam) (7, 10).

**b) Etest ESBL ceftazidime/ceftazidime + clavulanic ac. TZ/TZL** (Ref. 51003250, AB BIODISK Sweden).

The E-test ESBL strips are containing a double antibiotic gradient in each half, of a third generation cephalosporin and respectively third generation cephalosporin +clavulanic acid. The ratio between the minimal inhibitory concentration value (MIC) of ceftazidim and respectively ceftazidim + clavulanic acid greater or equal to 8 is an indicator for the ESBL production. Occasionally, an inhibition "blurred zone " or the deformation of the inhibition zone in the half containing the cephalosporin alone could occur, also indicating the ESBL production, the test exhibiting a better sensitivity that DDST (7, 10).

**b) Beta-lactamases separation by isoelectrofocusing (IEF)**

IEF constitutes only a presumptive method for the detection of the number and type of beta-lactamases based on their isoelectric point (pIs) after separation in polyacrylamide gel electrophoresis using a 111 Mini IEF cell (Bio Rad) apparatus.
4. Detection of resistance genes in *E. coli* strains

*a) PCR detection of bla-TEM and bla-CTX-M*  
(Bio Rad) (Jungmin Kim and Hoan-Jong Lee protocol for PCR TEM and respectively, Li Xu et al. for PCR CTX-M) (13, 14).

The microbial strain preserved on OMS medium was dispersed on Mac Conkey agar and after 24h incubation, one single colony was suspended in Brain Heart Infusion (BHI) and further incubated at 37°C, with stirring (200 rpm), for 18 h. After incubation, the culture was centrifuged, the sediment was washed three times in sterile distilled water and thereafter heated at 100°C for 10 minutes in order to release the DNA in the extracellular medium, the obtained lysate being further used in PCR.

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Results and Discussion

Out of the total of 291 positive urine culture, 118 isolates were *E. coli*, 93 *Klebsiella pneumoniae*, 54 *Proteus mirabilis*, 24 *Enterobacter cloacae*, and 2 *Pseudomonas aeruginosa*, thus *E. coli* proved to be the major etiology, these strains isolated from patients with different neurological disorders being chosen for further investigation (table no. 1).

Table no. 1. Distribution of the studied *Escherichia coli* strains in patients with different neurological disorders

<table>
<thead>
<tr>
<th>Sex</th>
<th>Neurological disorder</th>
<th>No. cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>Stroke</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Vertebro-medular trauma</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Multiple sclerosis</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Brain tumors</td>
<td>4</td>
</tr>
<tr>
<td>Man</td>
<td>Stroke</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Vertebro-medular trauma</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Multiple sclerosis</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Brain tumors</td>
<td>11</td>
</tr>
</tbody>
</table>

No significant difference between sexes was noticed in patients with stroke and vertebromedular trauma, while women with multiple sclerosis and the men with brain tumors proved to be more susceptible to urinary tract infections (table no. 1).

1) Antibiotic resistance phenotypes

The *E. coli* studied strains exhibited high resistance rates to aminopenicillins, ampicillin + sulbactam, amoxicillin + clavulanic acid, tetracyclines, followed by quinolones, carboxipenicillins, aminoglycosides and cotrimoxazole. It is to be mentioned the preserved susceptibility of 100% to imipenem and of 83.4% to amikacin. The interpretive reading of disk diffusion results allowed the inclusion of the tested strains in typical resistance phenotypes being simultaneously resistant to beta-lactams, aminoglycosides, fluoroquinolones and cotrimoxazole (Fig. 1).

![Fig. 1. The number of *E. coli* strains exhibiting different resistance phenotypes](image)

The resistance levels for aminopenicillins, cephalosporins and quinolones of the *E. coli* isolated in Romania are quite high as compared with other European countries (e.g. France, England etc.), that could be explained by the large scale use of these antibiotics without a real need (3, 4, 11).

Out of the total number of strains, 78.9% were cefinase positive. Surprisingly, 9 of the strains resistant to amoxicillin + clavulanic acid, were susceptible to ampicillin + sulbactam, demonstrating on one side, the substrate specificity of the beta-lactamases, and on the other side, the possible adaptation of *E. coli* strains to the clavulanic acid inhibitor, probably due to the high selective pressure in the hospital environment (Fig. 2).
Fig. 2. The representation of different susceptibility to sulbactam (UNZ) and clavulanic acid (AMO) for the same bacterial strain

DDST indicated that 65.2% of the tested strains are producing typical beta-lactamases, ceftazidime proving to be a better indicator for the presence of these enzymes than cefotaxime in this test. The synergy test was positive for the strains resistant to clavulanic acid, but susceptible to sulbactam, proving that this inhibitor is more sensitive in DDST (Fig. 3). Our study indicated a good correlation between the results obtained with DDST and E-test ESBL which confirmed the production of extended beta-lactamases in strains exhibiting synergism aspects in DDST (Fig. 4).

Fig. 3. Positive DDST

Fig. 4. Positive E-test ESBL
Out of the total number of the tested strains, 20% were suspected for the production of CTX-M ESBL and 18.3% exhibited resistance to one third generation cephalosporin and cefoxitin, but were negative in DDST, probably due to the simultaneous production of other type of beta-lactamase that could mask the presence of the ESBL (AmpC beta-lactamases or inhibitor resistant cephalosporinases - IRT).

Eventhough from clinical point of view, the differentiation between these enzymes is not critical because the therapeutic options among beta-lactam antibiotics are limited in both cases, however the detection of hidden ESBL is of great epidemiological importance, especially in the hospital environment.

The isoelectric analysis of the beta-lactamase type

The IEF analysis of the cellular sonicates in polyacrylamide gel was performed in the presence of an IEF standard (represented by a mix of 9 natural proteins with isoelectric points from 4.45 to 9.6) (Bio Rad) after nitrocephin staining and revealed the presence of TEM pI 5.2; CTX-M pI 7.5; AmpC pI ≥ 8; SHV pI 7-8.

PCR bla-TEM identified the TEM presence in 75% of beta-lactamase producing strains (Fig. 5), four of these strains being resistant to both tested inhibitors (i.e. clavulanic acid and sulbactam).

![Image](Molecular size marker 100bp)

![Image](Bla-TEM 972 bp)

![Image](Positive control)

**Fig. 5.** Gel electrophoresis showing five *E. coli* strains positive for bla-TEM gene, as revealed by the specific 972 bp *bla*-TEM amplicons

PCR bla-CTX-M indicated that 20% of the tested strains are producing this enzyme, the molecular analysis being totally correlated with the phenotypic results (Fig. 6). Concerning the extended spectrum betalactamases groups (CTX-M 1,-3,-10,-11,-12,-15,-22,-23,-27,-28,-29,-30,-34,-36,-37 și -42), our strains belonged to CTX-M group 1.
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Fig. 6. Gel electrophoresis showing three *E. coli* strains positive for CTX-M gene, as revealed by the specific 260 bp amplicons.

The RFLP analysis performed with Dde 1 and Pst 1 confirmed the presence of *bla-TEM* gene, the expected restriction fragments being obtained (Fig. 7).

Fig. 7. Gel electrophoresis of *bla-TEM* amplicon restriction fragments performed for *E. coli* strains harboring the *bla-TEM* gene.

The molecular sequencind detected only *bla-TEM* 1 (Fig. 8), but no ESBL of TEM type, despite the phenotypic tests results indicating the presence of extended spectrum beta-lactamases.
The nine strains exhibiting sensitivity to the beta-lactamase inhibitor sulbactam and simultaneous resistance to clavulanate, is confirming other results cited in the literature, namely that the frequent use of clavulanate both in laboratory tests, as well as in the antimicrobial therapy, in association with amoxicillin, resulted in an increase of bacterial resistance to this inhibitor. Thus, bacteria have evolved adaptive mechanisms and they are no more responding to the inhibitor. The excessive use of clavulanate, but also of sulbactam in the laboratory practice and in therapy renders tazobactam the beta-lactamase inhibitor with the greatest efficiency in the beta-lactamase detection tests (5, 7).

The phenotypic testing indicated a small percentage of \textit{E. coli} strains positive for the presence of AmpC beta-lactamases (the eleven suspected strains exhibiting resistance to cefoxitin, ceftazidime and ceftriaxone, but negative for the DDST), but the presence of the suspected AmpC enzyme could not be confirmed by molecular means.

**Conclusion**

Our results have revealed that \textit{E. coli} strains are holding the top of ITU etiology in patients with motor deficit, along with \textit{Klebsiella pneumoniae}, \textit{Proteus mirabilis}, \textit{Enterobacter cloacae} and \textit{Pseudomonas aeruginosa}. The \textit{E. coli} strains have shown high level resistance to aminopenicillins, ampicillin + sulbactam, amoxicillin clavulanate, tetracycline, followed by quinolone, carboxipenicillins, aminoglycosides and cotrimoxazole, the multiple resistance to beta-lactams, aminoglycosides, fluoroquinolones and cotrimoxazol being registered with high frequency among the studied strains. The phenotypic screening tests revealed the presence of beta-lactamases sensitive or resistant to inhibitors and AmpC. Phenotypic testing revealed also atypical phenotypes consisting of resistance to amoxicillin clavulanate but susceptible to the ampicillin + sulbactam. Phenotypic analysis carried out by sinergism tests and E-Test ESBL and genotypic analysis by PCR and sequencing showed the presence of \textit{bla TEM} and group 1 CTX-M genes in 75% of \textit{E. coli} strains isolated from patients with neurogenic bladder. Since selective pressure exhibited by resistant bacteria has become and is still a public health problem, a good collaboration between laboratory and clinician is absolutely needed in the future in order to enable the implementation of good therapeutic policies able to prevent the emergence of multiresistant strains.

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