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Study of Resistance to 82 Clinical Cases Enterobacteriaceae to Beta-lactam Antibiotics

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Abstract

Knowledge of antimicrobial resistance patterns in *E. coli*, the predominant pathogen associated with urinary tract infections (UTI) is important as a guide in selecting empirical antimicrobial therapy. To describe the antimicrobial susceptibility of *E. coli* associated with UTI in a major university hospital in Tehran (Iran), seventy-six clinical isolates of *E. coli* were studied for susceptibility to β-lactam antibiotics by the disc diffusion method and Minimal Inhibitory Concentrations determination. All isolates were resistant to ampicillin, amoxicillin and oxacillin. Resistance to the other tested antibiotics was shown to be 93.4% to cefradine, 76.3% to carbenicillin, 47.3% to cefazoline, 50% to cefalexin and 32.8% to cephalothin while 1.3% expressed resistance to cefoxitime, and 2.6% were resistant to ceftizoxime and ceftriaxone. Substrate hydrolysis by ultra violet spectroscopy showed that 87.4% harbored penicillinases, 9% produced cephlosporinases and 3.6% degraded both substrates. Clavulanic acid inhibited enzyme activity in 82.9%, of which 78.95% was penicillinases (group IIa) and 3.95% was cephalosporinases (group IIb) of the Bush classification system. These results indicate that *E. coli* can posses a variety of β-lactamases that are responsible for β-lactam resistance. Members of the family Enterobacteriaceae, particularly Escherichia coli is the most common causes of urinary tract infections in hospitals and societies. Beta-lactam antibiotics, particularly the third and fourth generation of cephalosporins are effective in treating these infections.

Keywords: Beta-lactams, Enterobacteriaceae, *Escherichia coli*

1. Introduction

Urinary Tract Infections (UTI) are the second most common infections present in community practice (Gonzalez & Schaeffer, 1999). Members of Enterobacteriaceae, specifically, *E. coli* are the main causes of urinary infections (Gupta, 2003). Extensive use of β-lactams in veterinary medicine and human practice is believed to be associated with selection of resistance in both pathogenic and nonpathogenic isolates of *E. coli* (Livermore, 1995). More than two hundred βlactamase enzymes are recognized which are classified into 4 main groups and 8 subgroups (Bush & Jacoby, 1996; Bush, 1989). The resistance of Enterobacter spp. to β-lactam antibiotics is most frequently mediated by production of TEM, SHV and AmpC β-lactamase (Barnaud et al., 2001). In the last decade, production of plasmidmediated ESBL which hydrolyzes a wide range of the most recently developed cephalosporins, has been recognized as an additional important emerging mechanism of resistance among members of the family Enterobacteriaceae including clinical isolates of *E. coli* (Bradford, 2001; Pitout et al., 1998). The first plasmid mediated βlactamases (TEM-1) was described in *E. coli* in 1960 and within a few years, it was found in many different genera of Gram-negative bacteria (Bradford, 2001). The AmpC family of β-lactamases occurs both chromosomally and plasmid-mediated in *E. coli* and plasmid encoded AmpC β-lactamases are found to be responsible for global outbreaks (Cudron, Moland, & Sanders, 1997; Eftekhar, 2005). We studied 76 urinary isolates of *E. coli* for their susceptibility to 12 β-lactam antibiotics. Preferred substrate hydrolysis was performed to determine the class of β-lactamases. DNA amplification of βlactamase types TEM, SHV and AmpC genes was carried out by PCR using type specific primers of blaTEM, ampC and SHV genes for all of the isolates.

2. Materials and Methods

Bacteria. Seventy-six clinical isolates of *E. coli* were selected from a collection of urinary Enterobacteriaceae from the Bacteriology Laboratory of Vali-E-Asr Hospital in Tehran (Iran) (Hosseini-Mazinani, Jafar-Nejad, & Ghandili, 2003). *E. coli* ATCC 25922 was used as a control for antibiotic susceptibility tests. K. pneumoniae 57-1 carrying

plasmid mediated SHV gene, *E. coli* MK148 carrying the ampC gene and *E. coli* harboring pTEM were used as positive controls for DNA amplification by PCR (Hosseini-Mazinani, 2996). Antibiotic susceptibility. The antibiotic susceptibility of bacteria was initially carried out by the disc diffusion method according to the NCCLS recommendations (National Committee for Clinical Laboratory Standards [NCCLS], 2000). The antibiotic discs were ampicillin (10 µg), amoxicillin (25 µg), carbenicillin (100 µg), cefalexin (30 µg), cephalothin (30 µg), cefazoline (30 µg), cefradine (30 µg), oxacillin (1µg), ceftazidime (30 µg), ceftriaxone (30 µg), ceftizoxime (30 µg) (Padtan Teb, Tehran, Iran) and amoxicillin-clavulonic acid (20/10 µg, Difco, USA). Minimum Inhibitory Concentrations (MIC) of the isolates was determined for ampicillin, ceftazidime, cefotaxime, ceftriaxone, cefepime and imipenem by the microdilution broth method using the NCCLS standard procedure ([NCCLS], 2000). Screening for ESBL production. The double disc synergy test was used to screen for ESBL production (Gonzalez and Schaeffer, 1999). Cefotaxime (30 µg), ceftriaxone (30 µg) and ceftizoxime (30 µg) were placed on Mueller Hinton agar plates adjacent to amoxicillin-clavulanic acid discs (20/10 µg). ESBL production was inferred when cephalosporin inhibition zones expanded by the clavulanate.

Substrate hydrolysis. Relative hydrolysis rates of benzylpenicillin and cephaloridine were evaluated by UV spectroscopy. β-lactamase activity was determined by measuring the decrease in optical density of a 0.1 mM solution of cephaloridine (255 nm) or benzylpenicillin (240 nm). Enzymes were called penicillinase if the relative rate of benzylpenicillin hydrolysis was approximately 30% higher than that of observed for cephaloridine, or cephalosporinase if cephaloridine was hydrolyzed at least 30 % faster than penicillin (Livermore,1 995; Ross & O'Callaghan, 1975).

DNA amplification. Plasmid DNA extraction was carried out using a rapid alkaline lysis method (Winokur, 2001). The oligonucleotide primers used for the PCR assays were; 5'-ATAAAATTCTTGAAGACGAAA3' and 5'-GTCAGTTACCAATGCTTAATC-3' for TEM, 5'-TGGTTATGCGTTATATTCGCC-3' and 5'GGTTAGCGTTGCCAGTGCT-3' for SHV and 5'ATGCAACAACGACAATCCATC-3' and 5'GTTGGGGTAGTTGCGATTGG-3' for AmpC βlactamases (Sutcliffe,1978 ; Bret, 1998). blaTEM and SHV primers were synthesized at the National Research Center for Genetic Engineering and Biotechnology, Iran and ampC primer was synthesized at Faza Pajooh (Tehran, Iran). Reactions were carried out in a Techne DNA thermocycler (Germany) in 25 µl mixtures containing 10 mM Tris-HCl (pH 8.3), 1 mM EDTA, 1.5 mM MgCl2, 200 µM of each deoxyribonucleoside triphosphate, 2-10 pM of oligonucleotide primers and 1 u of Taq DNA polymerase (Fermentas, Lithuania). Following a 4min incubation time at 94°C, 35 cycles were run with the following temperature profile for each cycle: 94°C for 1 min, the proper annealing temperature for each primer (58°C for blaTEM, 59°C for ampC and 52o C for SHV) for 1 min and 72°C for 1 min. An additional 5-10 min incubation time was also carried out at 72°C. PCR experiments for amplification of the SHV gene failed to produce a single DNA product regardless of numerous standardization strategies. Therefore, presence or absence of the desired fragment was determined on the basis of comparing the resulting bands with a positive control as well as DNA size markers.

3. Findings

3.1 Detection and Identification of Bacteria

Identifying bacteria by biochemical version and was confirmed by API 20E test.

The results showed that among 82 cases of *Escherichia coli* cases, 76 cases were *Citrobacter freundii*, a case was *Enterobacter*, a case was *Klebsiella pneumoniae*, a case was *Klebsiella oxytoca*, and a case was *Hafnia*.

3.1.1 Antibiotic Susceptibility Results

A) by disk diffusion method:

All tested bacteria were resistant to antibiotics like amoxicillin, ampicillin and Oxacillin and only 9% of cases were resistant to coamoxiclave. As it is shown in Figure 1 and Figure 2, resistance to Cefradine and carbenicillin was 97.5% and 79.20%; and resistance to other antibiotics was 20.7% Cephalothin, 26.8% cefalexin and 31.7% cefazoline, 1.2% to Cefoxitime, 1.2% Ceftizoxime 1.2%, and Ceftiaxime.

B) The method MIC:

The results of the MIC are shown in Figure 3. MIC test showed susceptibility to the samples.

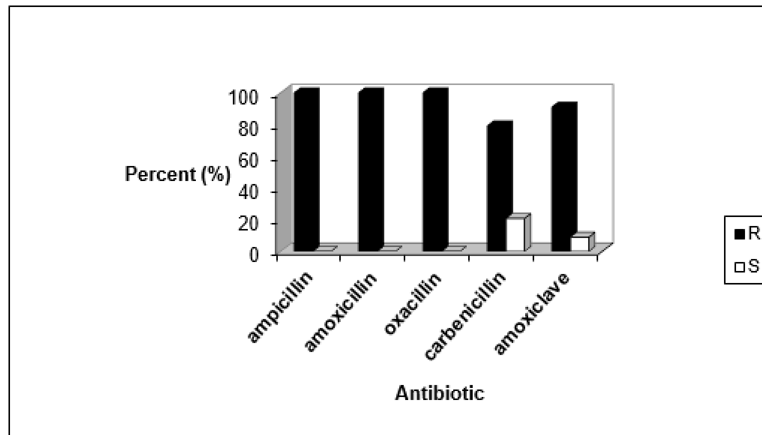


Figure 1. Results of Penicillin antibiotics

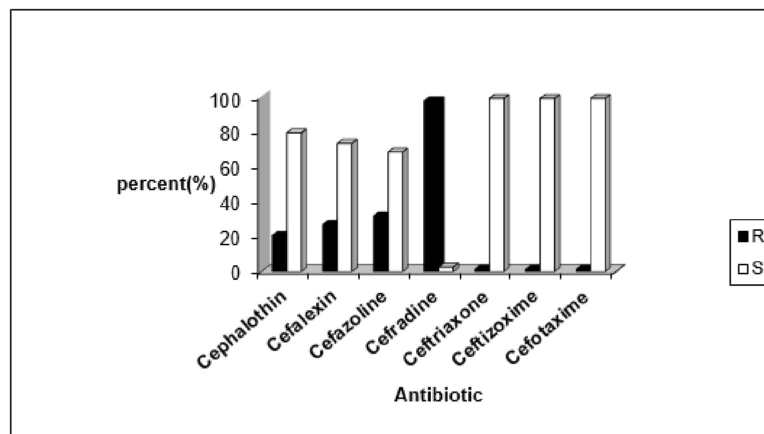


Figure 2. Results of Cephalosporin antibiotics

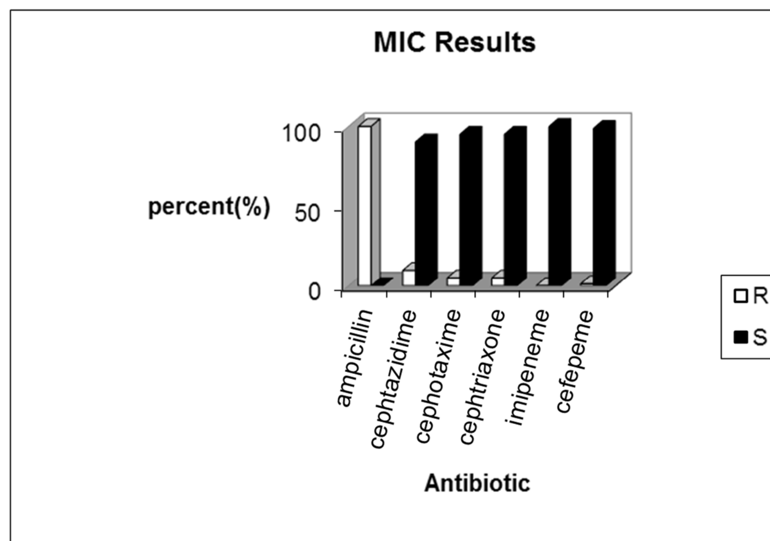
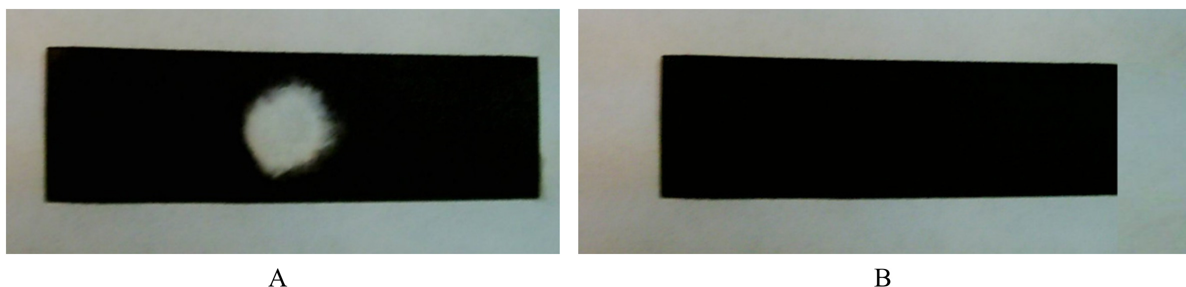


Figure 3. The results of the MIC

3.1.2 Results of colonies Test with iodometer

Iodometer test showed the presence of enzyme in all bacteria.

As it is seen in Figure 4, colonies of bacteria with beta-lactamase enzyme have a white halo.



A B

Figure 4. The results of the colony by Iodometer. A) Bacteria containing beta-lactamase B) bacteria lacking the enzyme beta-lactamase

3.1.3 The Results of Substrate Hydrolysis

As it is shown in Figure 5, depending on the rate of hydrolysis of benzyl penicillin and Cefaloridine to each other (spectrophotometry) 87.4% organisms contain enzyme penicillinase, 9% organisms contain enzymes Cephalosprinase and 3.6 percent contain both enzymes equally.

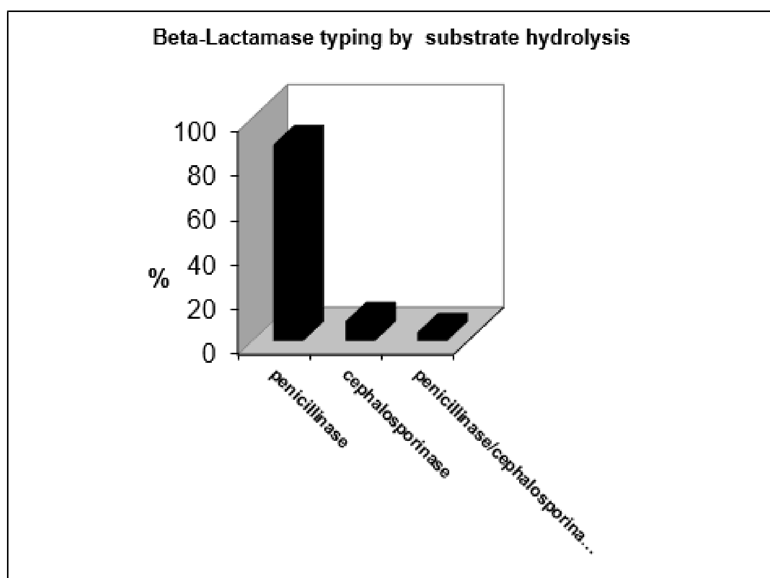


Figure 5. The results of hydrolysis of the substrate by Spectrophotometer

3.1.4 Lactamase Classification Results

None of the beta-lactamase enzymes tested showed no inhibition with EDTA. This does not mean that Group III was among the beta-lactamase. However, 91% enzymes were inhibited by clavulanic acid, and taking into account the results of hydrolysis of the substrate (Figure 5) showed that 87.4% organisms were penicillinase that were resistant to clavulanic acid, and this class of beta-lactamase belonged to Group 2a, 3.6% organisms equally showed penicillinase and cephalosporinase and can be inhibited by clavulanic acid and belonged to the Group 2b, and 9% were penicillinase that cannot be inhibited by clavulanic acid which belonged to Group 4. Among the studied organisms, Group I, i.e. cephalosporinases were not resistant to clavulanic acid (Figure 6).

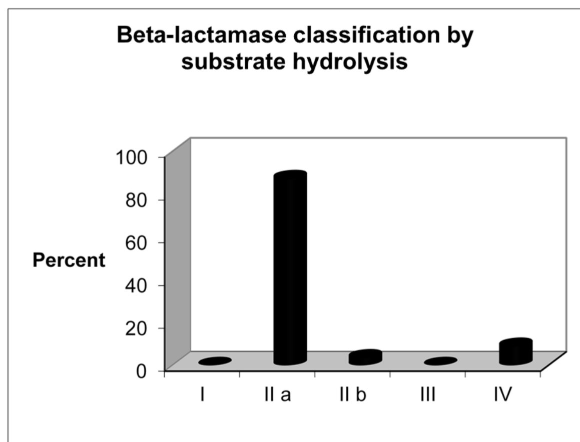


Figure 6. The results of the classification of beta-lactamases

3.1.5 Results of E. Test

The results of 82 clinical cases revealed a broad spectrum beta-lactamase enzymes (ESBL) in three samples of bacteria (17,23a, b29). As it is seen in Figure 7, clover leaf form indicates the presence of ESBL, and creating a clover leaf form in these cases because of resistant to third generation cephalosporins and enzyme containing beta-lactamase (ESBL) are inhibited with clavulanic acid. Lack of beta-lactamase enzyme (ESBL) is shown in figure A.

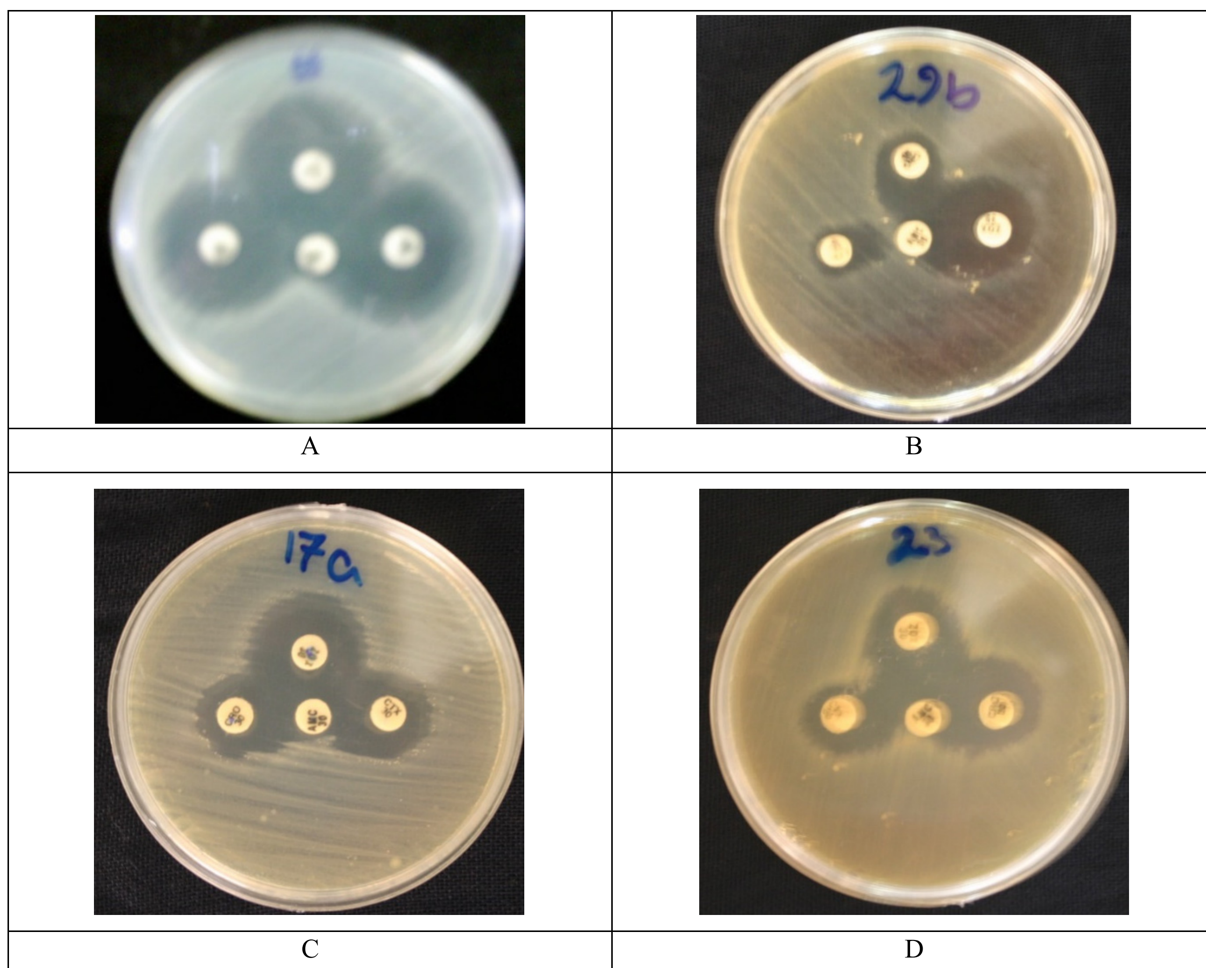


Figure 7. The results of A: ESBLS= bacterial non-ESBL, C, B and D= bacteria with ESBL

3.2 The Plasmid DNA Extraction

Plasmid DNA extraction on 20% of the samples (21 samples) by agarose gel 0.8% is shown in Figure 8.

As it can be seen in the figure, plasmid samples have different bands.

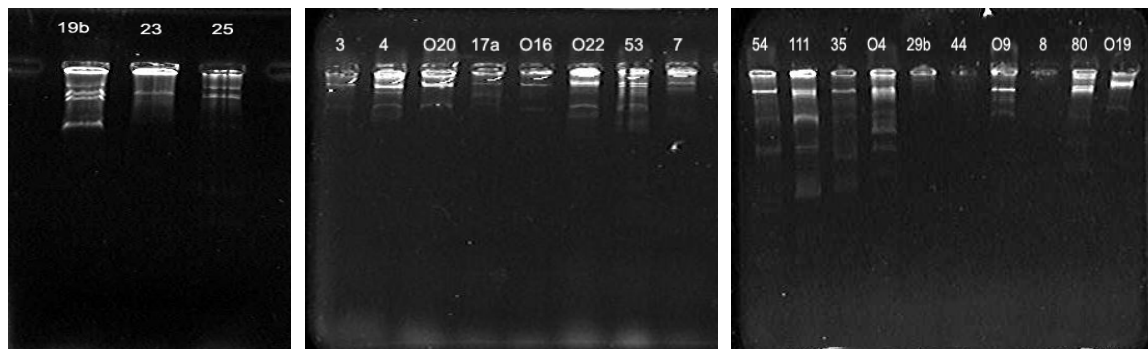


Figure 8. Results of the extraction of plasmid DNA (agarose gel 0.8%)

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