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EXTENDED SPECTRUM B-LACTAMASE-PRODUCING ESCHERICHIA COLI IN CLINICAL ISOLATES IN BENGHAZI, LIBYA: PHENOTYPIC DETECTION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERN

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SUMMARY: β-lactams are the most widely used group of antimicrobials; however, growing resistance to these invaluable drugs mediated by extended spectrum β-lactamase (ESBL) enzymes is a major concern. The present study was undertaken to determine the prevalence of these enzymes and their effect on antimicrobial susceptibility pattern by different phenotypic detection tests in clinical isolates of Escherichia coli in Benghazi, Libya. Antimicrobial susceptibility testing was carried out by Kirby-Bauer method. Ceftazidime and cefotaxime were used for screening potential ESBL producers. Confirmation was done by a combination of double disk synergy test (DDST) and phenotypic confirmatory disk diffusion tests (PCDDTs).

A total of 120 E. coli strains (40 urine, 20 sputum, 20 blood, and 40 wound swabs) from inpatients at different hospitals of Benghazi, Libya, were included in the study, of which, 24 (20%) isolates were ESBL producers. The resistance pattern to the tested antibiotics was as follows: ampicillin (80%), co-trimoxazole (60%), ciprofloxacine (40%), cefotaxime (30%), ceftazidime (30%),

Ceftriaxone (30%), gentamicin (30%), cefpirome (35%), ofloxacin (30%), imipenem (25%), and nitrofurantoin (40%).

All the isolates tested showed resistance to two or more drugs and were considered to be multidrug resistant. A higher rate of ESBL production and multidrug resistance was seen among isolates from pus swabs as compared to other sources. ESBL producers mediated high resistance to both β-lactams and non-β-lactams. Prolonged hospital stay and prior use of third-generation cephalosporins were identified as important risk factors for ESBL acquisition.

There is insufficient data regarding ESBL prevalence among E. coli strains from Benghazi, Libya. ESBLs not only pose a great threat to future of β-lactams, but they also endanger the utility of many non-β-lactams. To ensure rationale in antibiotic treatment, ESBL detection and reporting assumes a priority in near future in Benghazi, Libya.

Key words: Escherichia coli, multidrug resistance, extended spectrum β-lactamase (ESBL).

INTRODUCTION

With increasing antimicrobial and irrational use over the years, resistance to antimicrobial agents has emerged in a bacterium that poses new challenges for clinical management programs. Resistant bacteria are a big threat to

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the hospitalized patients as well as community (1). Among the wide array of antibiotics, lactams are the most commonly used agents accounting for over 50% antibiotics in use (2). Hence, production of *β-lactamase* appears to be more rapidly developing and clinically significant antimicrobial resistance mechanisms (3,4).

Plasmid-mediated extended spectrum *β-lactamase (ESBL)* enzymes confer resistance to third-generation cephalosporins and monobactams but are inactive against carbapenems and cephamycins (5). *ESBL*s are rapidly evolving group of *β-lactamases* that share the ability to hydrolyze third-generation cephalosporins and aztreonam but yet are inhibited by clavulanic acid (6).

*ESBL*s have emerged as a major problem in hospitalized patients worldwide and have been involved in epidemic outbreaks leading to proliferation of pencillin, cephalosporin, and aztreonam-resistant bacteria. Adequate laboratory detection is important to avoid treatment failures.

Since *ESBL-positive* isolates show false susceptibility spectrum to cephalosporins in standard disk diffusion tests, hence it is difficult to detect *ESBL* by this method. *ESBL*s are inhibited by *β-lactamase* inhibitors like clavulanic acid and tazobactam. This property of specific inhibition can be utilized for detection and confirmation of *ESBL*s (3).

Escherichia coli cause a number of diseases such as urinary tract infection, abscess, bacteremia, peritonitis, cholecystitis, chest infections, and, sometimes in severe cases, endotoxic shock and meningitis in neonates may occur.

β-lactam antibacterial agents consist of penicillins, cephalosporins, monobactams, and carbapenems. They all have β-lactam ring, which is hydrolyzed by *β-lactamases*. *β-lactamase*-producing bacteria are increasing in number and cause more severe infections because of their mutation (7).

Increasing β-lactam resistance in *E. coli* has become a worrying threat worldwide.

Most of the mechanisms involved in the β-lactam resistance are linked to the production of *β-lactamases*, including clavulanic-acid inhibited *ESBL*s and carbapenemases that are the most powerful enzymes able to degrade most β-lactams. The most common *ESBL*s circulating in Enterobacteriaceae are TEM-, SHV-, and CTX-M-types. There are other emerging enzymes that are mostly plasmid encoded and are distributed in a large variety of species. The *ESBL* VEB-1 is known to be largely distributed in Southeast Asia; PER-1 in Turkey, Italy, Korea, and also South America; and GES/IBC-type enzymes in Greece and Japan mostly. All these *ESBL*s are known to efficiently hydrolyze *ESBL*s, and some variants of the GES family also hydrolyze imipenem at a low level. Other enzymes of the KPC family have been also reported in *E. coli*. KPC-2 has been shown to be prevalent in *E. coli* in Israel, and KPC-3 has been reported sporadically in the USA. These latter enzymes are considered as *ESBL*s since they are inhibited by clavulanic acid and their hydrolytic efficiencies toward imipenem are very high, thus giving rise to high-level resistance to carbapenems in those *E. coli* isolates.

In addition to these *ESBL* determinants, metallo *β-lactamases* (MBL) have been identified in *E. coli*. These enzymes that are not inhibited by clavulanic acid and hydrolyze carbapenems very efficiently belong to the IMP and VIM groups. In *E. coli*, IMP-1 has been identified in Japan and VIM-2 in Greece, but the most worrying observation is that related to the spread of VIM-1 in Greece. Indeed, this determinant has been identified in clinical isolates also harboring *ESBL*-encoding genes, thus leading to panresistance in those strains. Plasmids are located into class 1 integron structures, thus facilitating the occurrence of multidrug resistance via the colocalization of other antibiotic resistance genes.

MATERIALS AND METHODS

Study Design

A cross-sectional, prospective, in vitro surveillance study of antibiotic resistance pattern among *E. coli* isolated from inpatients in and around Benghazi was conducted between July 2009 and March 2010 from the referral centers providing specialized clinical services to the people of the area. The study population constituted 120 clinical isolates of *E. coli* from inpatients from July 2009 to March 2010.

Sample Size and Inclusion Criteria

E. coli isolates from urine, sputum, blood, and wound-swab of inpatients of different referral hospitals of Benghazi, Libya, were included in the study. A total of 120 *E. coli* strains (40 urine,

20 sputum, 20 blood, and 40 wound swabs) constituted the study group.

Sample Collection and Handling

E. coli isolates with a complete patient history were transported to the study laboratory in the Department of Medical Microbiology on nutrient agar slopes and MacConkey agar, and isolates were processed directly after confirmation.

Sample Preparation

E. coli isolates were again confirmed by their characteristic features (morphologies) from the primary cultures on MacConkey agar medium and confirmed by standard biochemical tests (Triple Sugar Iron Agar reaction, motility, urease, citrate utilization, and indol tests).

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method as per CLSI guidelines (8) with the following set of antibiotics: ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) cefpirome (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), gentamicin 10 µg), ampicillin (10 µg), imipenem (30 µg), and nitrofurantoin. Disks of the antibiotics were impregnated onto an inoculated, dried surface of a 150-mm Muller Hinton agar plate, and incubated at 37⑤C for 24 hours.

ESBL Screening

Ceftazidime and cefotaxime were included in the primary panel for screening potential *ESBL* producers. Isolates with inhibition zone diameter of 22 mm for ceftazidime and 27 mm for cefotaxime were considered as potential *ESBL* producers as per the CLSI/NCCLS guidelines (8,9) and put to confirmatory testing by a double disk synergy test (DDST) and two phenotypic confirmatory disk diffusion tests (PCDDTA and PCDDT).

Double Disk Synergy Test

In the DDST, ceftazidime, cefotaxime, and ceftriaxone 30 µg each were placed at a distance of 15 mm edge to edge from a centrally placed augmentin disk containing 20 µg of amoxicillin + 10 µg of clavulanic acid. Result was inferred if the inhibition zone around these antibiotic disks increased toward the disk containing clavulanate, i.e., augmentin (10).

Phenotypic Confirmatory Disk Diffusion Tests

Ceftazidime and cefotaxime 30 µg each were used alone and in combination with 10 µg of clavulanic acid in the PCDDT A and B, respectively. Individual disks were placed at least 3 cm center to center apart. An increase in zone diameter of either ceftazidime or cefotaxime by 5 mm with clavulanic acid versus its diameter when tested alone was considered as *ESBL* positive (8). Susceptibility or resistance to Cefoxitin 30 µg disk was used to differentiate *ESBL*s from AmpC *β-lactamases.* Isolates were considered as *ESBL* producers when they showed synergy with clavulanate in any one or more of the confirmatory tests and tested sensitive to both cefoxitin and Imipenem. Isolates were considered as AmpC *β-lactamase* producers when they failed to show synergy with clavulanate in any of the confirmatory tests and tested sensitive to imipenem but resistant to cefoxitin (11). If the test isolate showed synergy with clavulanate in any one or more of the confirmatory tests but tested resistant to cefoxitin it was considered as producing both *ESBL*s and AmpC *β-lactamases* simultaneously.

Quality controls Standard laboratory procedures were strictly followed. Sample collection and handling was carefully performed, and control strain of *E. coli* (ATCC 25922) was used in the study.

RESULTS

Out of a total of 120 *E. coli* strains (40 urine, 20 sputum, 20 blood, and 40 wound swabs) from inpatients at different hospitals of Benghazi, Libya, 24 (20%) isolates were positive for *ESBL*.

The resistance pattern to the tested antibiotics was as follows: ampicillin (80%) ciprofloxacine (40%), cefotaxime (30%), ceftazidime (30%), ceftriaxone (30%), gentamicin (30%), cefpirome (35%), ofloxacin (30%), imipenum (25%), and nitrofurantoin (40%).

All the isolates tested showed resistance to two or more drugs and were considered to be multidrug resistant. A higher rate of *ESBL* (30%) production was seen among isolates from pus swabs followed by blood (20%), urine (15%), andsputum (10%). *ESBL* producers mediated resistance to third-generation cephalosporins as a result of prolonged hospital stay and prior use of third-generation cephalosporins that are documented as important risk factors for *ESBL* acquisition. The overall prevalence of *ESBL*-producing *E. coli* was 20% (n = 24/120), and most of the patients had hospital stay of more than a week and with a history of prior third-generation cephalosporin use in the preceding two weeks. Majority of *ESBL*-producing *E. coli* were recovered from the surgical wound site (n = 12/40, 30%) followed by blood (n = 4/20, 20%), urine (n = 6/40,

Table 1: Distribution of ESBL-positive Escherichia coli isolates.

Nature of specimen		Positive		Negative	
		No	%	No	%
Wound	Total-40	12	30	28	70
Sputum	Total-20	4	20	16	80
Blood	Total 1-20	2	10	18	80
Urine	Total-40	6	15	34	85

15%), and sputum (n = 2/20, 10%). Maximum numbers of *ESBL*-producing *E. coli* were obtained from postoperative ward followed by SICU, neonatology, dialysis section, and oncology wards. Patients with sepsis yielded highest number of *ESBL* producers followed by patients admitted in SICU. The distribution of *ESBL*-producing isolates among the different specimens, sensitivity and resistance pattern of antibiotics, and sites of infection are shown in Table 1, 2, and 3 respectively.

Table 3 shows the maximum percentage of *ESBL* producers, i.e., 30% belonged to pus swabs from wound infections.

Table 2: Antibiotic sensitivity % resistance pattern of Escherchia coli isolates.

	R	%	S	%
Ampicillin	96	80	24	20
Cefotaxime	36	30	84	70
Ceftazidime	36	30	84	70
Ceftriaxone	36	30	84	70
Gentamicin	36	30	84	70
Ciprofloxacin	48	40	72	60
Cefpirome	42	35	78	65
Ofloxacin	36	30	84	70
Imipenem	30	25	90	75
Nitrofurantoin	48	40	72	60

Among the total of 120 isolates screened 20% (n = 24/120) were screen positive as well as PCDDT positive using either ceftazidime or cefotaxime. DDST was able to detect all the 24 strains; PCDDT using ceftazidime/ceftazidime + clavulanate and PCDDT using cefotaxime/cefotaxime + clavulanate also detected all the 24 isolates as *ESBL* producers. Both PCDDTs together increased the detection rate. All the 24 representative *ESBL*-positive isolates identified by the screening method tested also positive by DDST and PCDDT.

All the 24 isolates were screened for *ESBL* production, and showed inhibition zone of <22 mm and <27 mm for ceftazidim and cefotaxim, respectively (Figures 1, 2, and 3).

The resistance pattern was also assessed between *ESBL* producers and nonproducers, and it was found that the degree of resistance was greater for the isolates that were *ESBL* producers. MDR was also found to be greater among *ESBL* producers (data not shown). MDR was also found to be greater among these *ESBL* producers.

DISCUSSION

Faced with the global emergence of antimicrobial resistance, several studies have been undertaken to assess the susceptibility of bacterial pathogens to different antibiotics (12, 13). These findings revealed that there is a widely spreading resistance to most of the available antibiotics. A susceptibility study conducted in California University on prospectively collected 255 *E. coli* isolates showed 22% resistance to trimetoprim-sulfamethoxazole (14).

Table 3: Nature of infection in ESBL positive Escherichia coli.

Site	Positive (n)	%	Negative (n)	%
Wound	12/40	30	28/40	70
Blood	4/20	20	16/20	88
Urine	6/40	15	34/40	85
Sputum	2/2	10	18/20	90

In a separate study in the USA, antimicrobial susceptibility testing on 123,691 *E. coli* isolates showed similar results. Resistant patterns were observed to nitrofurantoin (1%), ciprofloxacin (3.7%), sulfamethoxazol (18.6%), and ampicillin (39.1%). In comparison to most of the prospective surveillance studies, a retrospective in vitro surveillance study was conducted in Saudi Arabia to assess antibiotic susceptibility patterns among *E. coli* isolates. Inpatients isolates were more likely to be antibiotic resistant than outpatient isolates, as observed in the resistance patterns to ampicillin (63% in inpatients, 50% in outpatients), sulfamethoxazol (44% in inpatients, 30% in outpatients), and ciprofloxacin (33% in inpatients, 14% in outpatients) (15).

In a separate study, a double disk diffusion (Synergy test) was employed to study the prevalence of *ESBL* in nosocomial and outpatients in Pakistan, delineating 70% of Klebsiella pneumoninae, 33.33% of Enterobacter cloacae, and 28.57% of *E. coli* with this property (16).

In India, in a tertiary care hospital, a significant, overall resistance to carbapenims was reported 22.16% to meropenem and 17.32% to imipenem. *E. coli* isolates showed lower resistant patterns as compared to the more resistant pseudomonas species (3.3% versus 37.6% to meropenem; 2.1% versus 30% to imipenem) (17).

Present study at Benghazi, Libya, was targeted to determine the production of *ESBL* among clinical isolates of *E. coli* and also assess the in vitro susceptibility of routinely used antibiotics. Role of *ESBL*-positive *E. coli* in producing aggressive biofilms especially in diarrheagenic strains has also been studied extensively (18-23) and may pose bigger challenges in future. It is concluded that inpatients are the main source of *ESBL*-producing *E. coli*, and efforts should be made to asses its burden to community as well.

The above percentage of *ESBL* isolates in accordance with various studies varies slightly among different geographical areas, institutions, and countries. Frequency of *ESBL*-producing *E. coli* in Europe, North America, and western pacific is reported as 1-8% and more, i.e., 20% in South Africa (24). The lower prevalence of *ESBL* in Western countries can be explained by their vigorous infection control measures, judicious use of third-generation cephalosporins, appropriate *ESBL* detection, enforcement of strict hand hygiene, and isolation of *ESBL*-infected patients. Also once an *ESBL*-producing strain is detected, it should be reported as resistant to all pencillins, cephalosporins, and aztreonam even if they test as susceptible.

Kader, *et al.* (25) observed resistance of 92% to meropenum in *E. coli ESBL* producers, which is in accordance with our study. Faith reported high resistance to ciprofloxacin at 54.2% and 45% to gentamicin, which agreed with the current study (26). Yu Yunsong has also similarly reported 72% resistance to ciprofloxacin in his study from Zegiang province of china in *ESBL*-producing *E. coli* (27). Test sensitivity and specificity of confirmatory test (PCDDT) in our study is in accordance with the study of Linscot who has reported it as 96% and 100%, respectively (28). Rafay *et al.* (29) all from Oman have reported 100% resistance to third-generation cephalosporins, such as cefotaxime, ceftazidime, and ceftriaxone, which is contrary to the current study. Jain *et al.* (30) from India have reported 59.5% resistance to ceftazidime, which is higher as compared to our current study.

*ESBL*s are most often associated with *E. coli* and K. pneumonae and get transferred to genera of other enteric bacilli including Proteus mirabilis, Citrobacter, and Serratia. Global prevalence of *ESBL*-producing organisms presently varies from <1% to 74% (31,32). Initially the *β-lactamases* produced were active against only a few β-lactams, but over the years microorganisms have learnt to elaborate newer *β-lactamases* with extended substrate profile, and such important group of enzymes are active against virtually all β-lactams except the carbapenems (33).

ESBL production varies from hospital to hospital because of variation in selection of type of antibiotics and the antibiotic selection pressure thereof. Patients having infections by *ESBL*-producing organisms are at higher risk of treatment failures with third-generation cephalosporins, thus making it mandatory for all microbiology laboratories to screen such bacterial strains for *ESBL* production using reliable tests recommended by NCCLS (National Committee for Clinical Laboratory Standards) or as per new CLSI (Clinical and Laboratory Standards Institute) guidelines. And if an isolate is confirmed to be *ESBL* producer, third-generation cephalosporins should be prescribed with caution in such cases. Incorporation of screening and confirmatory methodologies for *ESBL* detection vis-à-vis Klebsiella and *E. coli* species in our systems is necessary for rationalizing third-generation cephalosporins usage. Resistance to such commonly used antibiotics leads to treatment failures. This public health risk has become a global problem, with some countries seriously affected (34, 35). Members of the family Enterobacteriaceae, including *E. coli*, are among the most important human pathogens accounting for the majority of bacterial strains isolated from clinical patient samples. Natural or acquired mutations in bacteria after human involvement are caused by the uptake of foreign genetic material, either incorporated into the bacterial chromosome or existing in an independent, stable form (36, 37). *ESBL* production by bacteria is an important threat to clinical therapeutics. These organisms elaborate plasmid-encoded *β-lactamases*, a variety of which have been described among members of the family Enterobacteriaceae. First described in 1983, *ESBL* producers have contributed to the dramatic increase in recent years to resistance among gram-negative bacteria to β-lactam agents. Plasmid-borne genes code for enzymes that hydrolyze penicillins, cephalosporins, and aztreonam, and are inhibited by clavulanic acid.

Reporting of *ESBL* production from microbiology laboratory has to be interpreted scientifically, as in vitro sensitivity to third-generation cephalosporins in *ESBL*-positive organisms amounts to in vivo resistance. These *ESBL*-producing resistant strains that emerge out of irrational use of antimicrobials are difficult to treat and thus pose a big challenge for the future especially in tertiary care setups.

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