

## Prevalence of AmpC and ESBL Producing *E. coli* and Antibacterial Effect of *Allium sativum* on Clinical Isolates Collected from Zahedan Hospitals

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Article information	Abstract
<p>Article history: Received: 3 Mar 2013 Accepted: 24 Apr 2013 Available online: 20 May 2013 ZJRMS 2014; 16(4): 6-10</p> <p>Keywords: AmpC <math>\beta</math> Lactamases <i>Allium sativum</i> Cefoxitin resistance <i>Escherichia coli</i></p> <p>*Corresponding author at: Infectious Disease and Tropical Medicine Research Center Zahedan University of Medical Sciences. E-mail: bokaeian.m@gmail.com</p>	<p><b>Background:</b> AmpC beta-lactamases are capable to hydrolyse all beta-lactam antibiotics except cefepime and carbapenems. Herbal medicines have been important sources of products for the developing countries in treating common infectious diseases and overcome the problems of resistance and side effects of the currently available antimicrobial agents.</p> <p><b>Materials and Methods:</b> Total 410 non repetitive clinical <i>E. coli</i> strains recovered during 7 month, were screened for AmpC production by disc diffusion test using cefoxitin (30 <math>\mu</math>g) discs and confirmed by combined disc diffusion test using phenyl boronic acid and the MIC and MBC of <i>Allium sativum</i> (<i>A. sativum</i>) alcoholic extract against AmpC positive <i>E. coli</i> isolates were determined.</p> <p><b>Results:</b> A total of 107 of 410 isolates (26%) were cefoxitin resistant and 13 (3.1%) isolates harboured AmpC enzymes. <i>A. sativum</i> alcoholic extract were effective against AmpC producing <i>E. coli</i> isolates.</p> <p><b>Conclusion:</b> There is need for a correct and reliable phenotypic test to identify AmpC beta lactamases and to discriminate between AmpC and ESBL producers and also these bioactive plants may help alleviate the problem of drug resistance.</p>

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### Introduction

Beta-lactam resistance in Gram-negative bacteria, especially *Escherichia coli*, is a main clinical problem. Evolution of resistance to beta lactam antibiotics in Gram negative, especially in *E. coli*, frequently results from the production of  $\beta$ -lactam ring [1]. Today, emerging newer  $\beta$ -lactamase enzymes including extended-spectrum  $\beta$ -lactamases (ESBLs) and AmpC  $\beta$ -lactamases are associated with misuse of  $\beta$ -lactam antibiotics. AmpC  $\beta$ -lactamase first reported in 1970s [1]. AmpC enzymes capable of hydrolyzing a wide variety of  $\beta$ -lactams, including aminopenicillins, cephalosporins, oxyimino-cephalosporins (e.g. ceftriaxone, cefotaxime, ceftazidime), cephamycins (e.g. cefoxitin, cefotetan) and monobactams, but they are susceptible to cloxacillin and 3-aminophenylboronic acid, while AmpC beta lactamases activity is not affected by the ESBL inhibitor clavulanic acid and in combination with porin loss, may also mediate resistance to carbapenems [2]. The plasmid-mediated AmpC genes are derived from including chosomal genes that have become mobilized and were transferred to organisms, which typically do not express chosomal  $\beta$ -lactamases such as klebsiella spp, or salmonella spp [3]. The increasing prevalence of plasmid-mediated AmpC  $\beta$ -lactamases and its role in resistance to many beta-lactam antibiotics in *E. coli* is becoming a serious worldwide problem. Medicinal plants have been considerable interest as potential sources of new compounds for drug design

and development [4]. *Allium sativum* (*A. sativum*) commonly known as Garlic belongs to the Amaryllidaceae family. The inhibitory and lethal activity of garlic extract against many pathogenic fungi and bacteria has been investigated by several researchers [5-6]. This survey was conducted to investigate the prevalence of AmpC-mediated resistance in clinical *E. coli* isolates by disc diffusion test and evaluation of the antibacterial activity of *A. sativum* extract against AmpC producing *E. coli* isolates.

### Materials and Methods

In this descriptive research, a total number of 410 non repetitive clinical isolates *E. coli* were collected between May 2012 and December 2013 from hospitalized patient in three major hospitals in Zahedan, south-eastern Iran. The isolates were obtained from the cultures of urine (311), wound (89), blood (9), and unknown origin (1) (Fig. 1). Each sample was streaked on the blood and Mac Conkey agar medium and incubated at 37°C for 24 hour after incubation, *E. coli* isolates were detected by standard biochemical tests such as indole, methyl red, Voges-Proskauer, and citrate. Antibiotic susceptibility testing was performed by the Kirby Bauer method on Mueller-Hinton agar according to CLSI protocols [7]. The tested drugs (in  $\mu$ g) and their potencies as follow amoxicillin (25), azithromycin (15), cefexime (5), tetracylin (30),

erythromycin (15), nalidixic acid (30), difloxacin (25), trimethoprim-sulfamethoxazol (1.23+23.15), gentamicin (10). A 0.5 McFarland of test isolates was swabbed on Mueller-Hinton Agar plates and ceftazidime (30 µg) and ceftazidime-clavulanic acid (30/10 µg) discs were placed on medium at distance of 30 mm. Inoculated plates were incubated overnight at 35°C. An organism exhibiting 5 mm or greater zone size increase around the ceftazidime-clavulanic acid disc compared to the ceftazidime disc was considered indicative of ESBL production [2]. *E. coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 700603 were used as control strains. In accordance with the 2009 CLSI criteria, isolates with resistance to cefoxitin were selected for further study.

The boronic acid disk test was used for AmpC screening by inoculating Mueller-Hinton agar by the standard disk diffusion method and placing a disc containing 30 µg of cefoxitin and another containing 30 µg of cefoxitin and 400 µg of boronic acid onto the agar surface. Inoculated plates were incubated overnight at 35°C. The organism that demonstrated 5 mm or greater zone around the disc containing cefoxitin and boronic acid than the zone around the disc containing cefoxitin was considered as AmpC producer [8].

The minimum inhibitory concentrations (MICs) of cephalosporins including ceftazidime, cefpodoxime, cefteraxone and cefotaxime for all cefoxitin-resistance isolates detected in this study were determined by E-test (Table 1), and results were interpreted as CLSI guidelines. The fruit *Allium sativum* (*A. sativum*) was purchased from local market and kept in sterilized screw-cap glass container. Sample was crashed and transferred into glass container and preserved until extraction procedure was performed in the laboratory. Plant was properly dried and pulverized into a coarse powder as reported by Hanafy and Hatem [9]. Each of 20 g grinded powders was soaked in 60 ml ethanol 95%, separately for one day (shaking occasionally with a shaker). After one day of dissolving process, materials were filtered and the filtrates were evaporated using rotary evaporator. At last, 0.97 g of dried extracts were obtained and then stored at 4°C in air tight screw-cap tube [9].

Antibacterial activity of extract of *A. sativum* was tested using the agar well diffusion method [10]. The test inoculum (0.5 McFarland's turbidity) was spread onto Muller-Hinton agar by using a sterile cotton swab. Then the wells were made by a sterile well puncture. Twenty µl of extracts were added to each well and incubated at 37°C for 24 h. The presence of zones of inhibition was regarded as the presence of antimicrobial action. The diameter of zone of inhibition was measured in mm. From the inhibition zones seen, antimicrobial activity was expressed in terms of average diameter of the zones inhibition measured.

The broth microdilution method was used to determine MIC and MBC. All tests were performed in Mueller-Hinton broth supplemented with Tween 80 at a final concentration of 0.5%. Briefly, serial doubling dilutions of the extract were prepared in a 96-well microtiter plate ranged from 0.3 mg/ml to 10.0 mg/ml. To each well, 10

µl of indicator solution (prepared by dissolving a 10-mg extract in 2 ml of DMSO) and 10 µl of Mueller-Hinton Broth were added.

Finally, 10 µl of bacterial suspension ( $10^6$  CFU/ml) was added to each well to achieve a concentration of  $10^4$  CFU/ml. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates, and then they were placed in an incubator at 37°C for 18-24 hours.

The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate the visible growth. The lowest concentration at which the color and turbidity changes occurred was taken as the MIC value. The average of 3 values was calculated providing the MIC and MBC values for the tested extract. The MBC was defined as the lowest concentration of the extract at which the incubated microorganism was completely killed [11]. The results were expressed as mean and or ranked in order of importance as percent. The data were subjected to one-way analysis of variance (ANOVA), using the SPSS-17 software. *p*-value less than 0.05 was regarded as significant.

## Results

Among 410 isolates, 171 isolates were susceptible to all tested antibiotics. Of 239 remaining isolates, 107 isolates were resistant to cefoxitin and 132 were susceptible to it. ESBL phenotype was confirmed among all these 132 isolates by the combined disc diffusion (ceftazidime/ceftazidime-clavulanic acid). Also 40 of 107 cefoxitin resistant were ESBL positive (Fig. 2).

AmpC β-lactamase production was confirmed in 13 isolates (12.1%) of 107 cefoxitin resistant, and in the remaining (N=94) it was not detectable (Fig. 3). Of 239 isolates 54 isolates were negative for both enzymes. Antibiotic susceptibility of the AmpC producing *E. coli* isolates were as follow (Table 2) erythromycin (92.3%), tetracycline (92.2%) nalidixic acid (84.6%), cefixime (84.6%), difloxacin (84.6%) azithromycin (76.9%), amoxicillin (76.9%), trimethoprim-sulfamethoxazol (76.9%), gentamicin (76.9%). AmpC producing *E. coli* isolates were recovered from patients with urinary tract (N=12) and wound (N=1). Of these 76.9% were classified to be nosocomial in origin. Different inhibitory effect of alcoholic extract from *A. sativum* plant against most *E. coli* isolates were demonstrated in table 3, and alcoholic extract of *A. sativum* plant showed inhibitory effect against most AmpC positive *E. coli* isolates. Table 4 reveals the inhibitory effect of garlic extract on 13 AmpC producing *E. coli*. The diameter of the zones of inhibition around the discs varied from 17-35 mm, indicating that all 13 AmpC positive isolates (100%) were sensitive to garlic extract. One out of 13 *E. coli* isolates had MIC 2.5 mg/ml for alcoholic extract of *A. sativum*. The highest MIC and MBC value of alcoholic extract of *A. sativum* were 5 mg/ml and 10 mg/ml respectively. The highest and lowest MIC of AmpC positive *E. coli* isolates were determined >256 and 16 µg/ml respectively (Table 1).

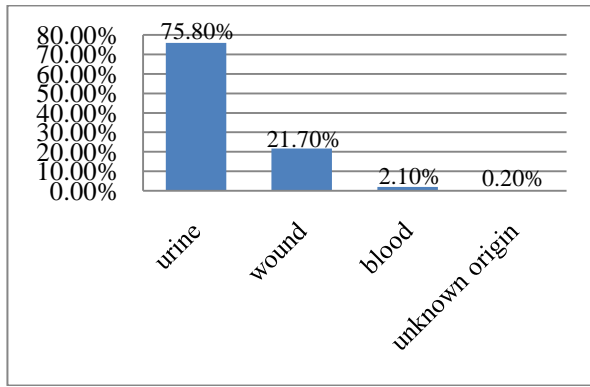


Figure 1. Distribution of samples among 410 E. coli

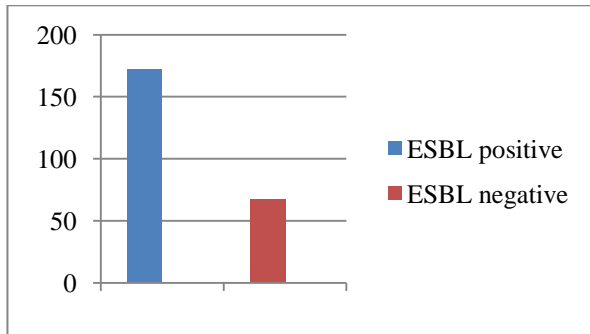


Figure 2. Prevalence of ESBL producing isolates

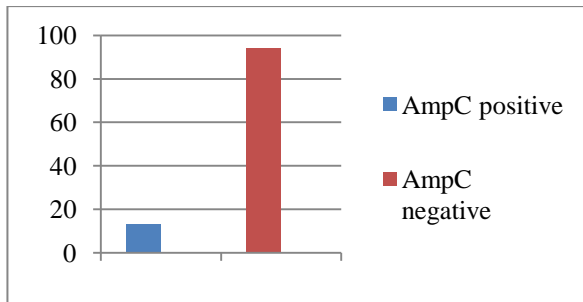


Figure 3. Prevalence of AmpC producing E. coli isolates

Discussion

Based on the result of this study, the prevalence of AmpC and ESBL producing *E. coli* was (3.1%), (32.1%) respectively by disc diffusion test. The MIC of AC ranged from 2.5 mg/ml to 10 mg/ml against the strains of *E. coli* and the most frequent numbers of isolates showing inhibitory effect in MIC of 5 mg/ml. AmpC producing *E. coli* isolates constitute a relevant epidemiological threat in hospitals [12-13]. The prevalence of AmpC producing *E. coli* isolates in Iran is not known, due to limited number of studies and difficulty that laboratories have in detecting this resistance mechanisms. In Iran AmpC prevalence has been reported in klebsiella spp (5.95%) and *E. coli* (5.7%) [14-15]. In another Iranian study, 10.2% of *E. coli* isolates produced AmpC  $\beta$ -lactamases [16]. Mansouri et al. reported the prevalence of ESBL and AmpC producing *K. pneumonia* (20%) and (2.6%) respectively [17]. In study

Table 1. MIC ( $\mu$ g/ml) values of several  $\beta$ -lactams for *E. coli* clinical isolates

No. of isolates	Ceftazidime	Ceftriaxone	Cefpodoxime	Cefotaxime
8	0.0125	4	2	8
12	128	4	2	2
11	8	16	4	16
15	64	128	64	32
10	128	64	256	256
7	4	8	2	8
12	64	32	256	256
20	32	64	128	128
12	128	128	256	>256
1	>32	64	256	256

Table 2. Antimicrobial susceptibility of AmpC producing *E. coli*

Antimicrobial agent tested	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Nalidixic acid	0 (0)	2 (15.3)	11 (84.6)
Cefixime	0 (0)	2 (15.3)	11 (84.6)
Azithromycin	1 (7.6)	2 (15.3)	10 (76.9)
Difloxacin	1 (7.6)	1 (7.6)	11 (84.6)
Tetracyclin	1 (7.6)	0 (0)	12 (92.2)
Amoxicillin	2 (15.3)	1 (7.6)	10 (76.9)
Trimetoprim-Sulfamethoxazol	2 (15.3)	1 (7.6)	10 (76.9)
Gentamicin	1 (7.6)	2 (15.3)	10 (76.9)
Erythromycin	0 (0)	1 (7.6)	12 (92.3)

Table 3. Minimum inhibitory concentration of *A. sativum* extract against AmpC producing *E. coli*

<i>A. sativum</i> concentration (mg/ml)	0.3	0.62	1.25	2.5	5	10
MIC (%)	0	0	0	7.6	69.2	15.3
MBC (%)	0	0	0	0	15.3	23

Table 4. Inhibitory effect of garlic extract on the AmpC producing *E. coli* isolates by disc diffusion tests

No. of isolates (%)	Range of the zone of inhibition (mm)
2 (15.3)	17-22
3 (23)	24-27
4 (30.7)	27-32
4 (30.7)	35

for Pakistan, among 121 clinical isolates of *E. coli*, 78 and 43 were identified as ESBL and AmpC producers, respectively. The highest resistance (89%) was observed against cefotaxime, followed by ciprofloxacin (87.6%) and cefepime (87%) [18]. In study for Canada a total of 369 AmpC  $\beta$ -lactamase-producing *E. coli* isolates were identified [1]. Annual incidence rates were 1.7, 4.3, 11.2, and 15 per 100,000 residents for each year, respectively [19]. Based on criteria of CLSI the use of cefoxitin resistance is a marker for detection of AmpC producing isolates but in our study, significant numbers of cefoxitin resistant isolates were not positive for AmpC production, hence other mechanisms of resistance should be considered [1]. Among gram negative bacteria, the emergence of resistance to extended-spectrum cephalosporins has been a major concern, initially in a

limited number of bacterial species and now expanding rapidly [1]. There is a need for a correct and reliable phenotypic test to identify AmpC  $\beta$ -lactamases and to discriminate between AmpC and ESBL producers. Inhibitor based method using boronic acid appears to be effective in discriminating this type of resistant isolates [8, 20]. In all these AmpC producers, we were not able to distinguish between the chromosomal derepressed and plasmid mediated enzymes as this requires genotypic confirmatory test. It also showed that the alcoholic extracts of *A. sativum* had potent antimicrobial activity against *E. coli* isolates [21]. Garlic (Genus *Allium*, Family Alliaceae) is one of the oldest cultivated plants to have been an integral part of human health and diet. Garlic has been in used since ancient times in India and China for a valuable effect on the heart, blood circulation and cardiovascular disease [20-23]. Allicin is the main biologically active component of freshly crushed garlic (*A. sativum*) cloves. It is produced by the interaction of the non-protein amino acid alliin with the enzyme alliinase [24]. Medicinal plants could be sources of compounds which might be useful in managing beta-lactam resistant bacteria. In another study the MIC of extract on

*Staphylococcus aureus* was determined to be greater than 7.5 mg/ml [25]. The potency of garlic extracts were shown by susceptibility of *S. aureus* and *E. coli* to it, Zone diameters of 17-35 mm was obtained from garlic inhibition against *E. coli* while that of 16-30 mm of same were obtained against *S. aureus*. The mean zone of inhibition for *E. coli* was 29 mm and that of *S. aureus* was 20 mm [26]. However, further studies about the isolation of active compounds and the absence of toxicity of plant extracts are necessary to propose these plants as alternative approaches to resistance management.

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### Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

### Conflict of Interest

The authors declare no conflict of interest.

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