Original Article

Zahedan Journal of Research in Medical Sciences

Journal homepage: www.zjrms.ir

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

Sara Shayan,[1] Mohammad Bokaeian,*[2] Shahram Shahraki,[1] Saeide Saeidi[3]

1. Department of Microbiology, Zahedan University of Medical Sciences, Zahedan, Iran
2. Infectious Diseasec and Tropical Medicin Research Center Zahedan University of Medical Scienes
3. Department of Biology, Faculty of Sciences, Islamic Azad University, Kerman, Iran

Article information

Article history:
Received: 3 Mar 2013
Accepted: 24 Apr 2013
Available online: 20 May 2013
ZJRMS 2014; 16(4): 6-10

Keywords:
AmpC β Lactamases
Allim sativum
Cefoxitin resistance
Escherichia coli

*Corresponding author at:
Infectious Diseasec and Tropical Medicin Research Center Zahedan University of Medical Scienes.
E-mail:
bokaeian.m@gmail.com

Abstract

***Background*:** 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.

***Materials and Methods*:** Total 410 non repetitive clinical *E. coli* strains recovered during 7 month, were screened for AmpC production by disc diffusion test using cefoxitin (30 µg) discs and confirmed by combined disc diffusion test using phenyl boronic acid and the MIC and MBC of *Allim sativum* (*A. sativum*) alcoholic extract against AmpC positive *E. coli* isolates were determined.

***Results*:** A total of 107 of 410 isolates (26%) were cefoxitin resistant and 13 (3.1%) isolates harboured AmpC enzymes. *A. sativum* alcoholic extract were effective against AmpC producing *E. coli* isolates.

***Conclusion*:** 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.

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 β-lactam ring [1]. Today, emerging newer β-lactamase enzymes including extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases are associated with misuse of β-lactam antibiotics. AmpC β-lactamase first reported in 1970s [1]. AmpC enzymes capable of hydrolyzing a wide variety of β-lactames, 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 chrosomal genes that have become mobilized and were transferred to organisms, which typically do not express chrosomal β-lactamases such as klebsiella spp, or salmonella spp [3]. The increasing prevalence of plasmid-mediated AmpC β-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 µg) and their potencies as follow amoxicillin (25), azithromycin (15), cefexime (5), tetracylin (30),

erythromycin (15), nalidixic acid (30), difloxain (25), trimethoprim-sulfumethoxazol (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* ATTCC 25922 and *Klebsiella pneumonia* ATTCC 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 containg cefoxitin was considered as AmpC producer [8].

The minimum inhibitory concentrations (MICs) of cephalosporins including ceftazidime, cefpodoxime, cefteriaxone 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%), difloxacain (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).

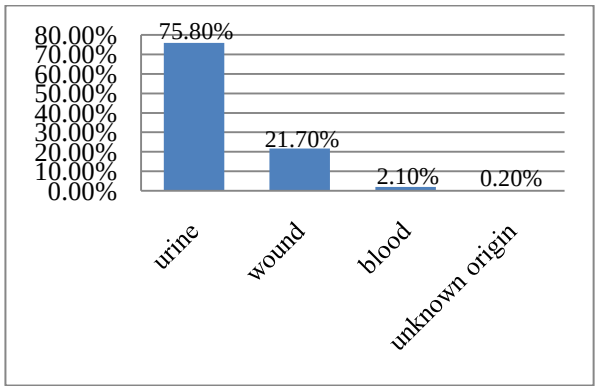

Figure 1. Distribution of samples among 410 E. coli

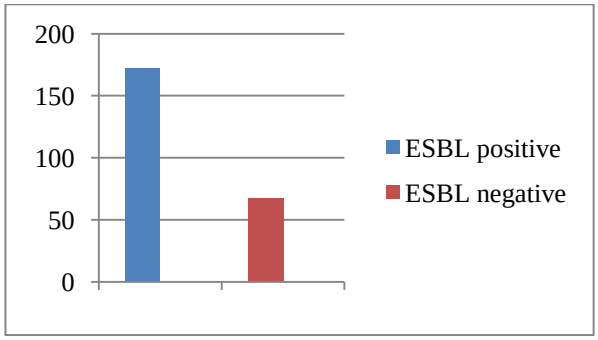

Figure 2. Prevalence of ESBL producing isolates

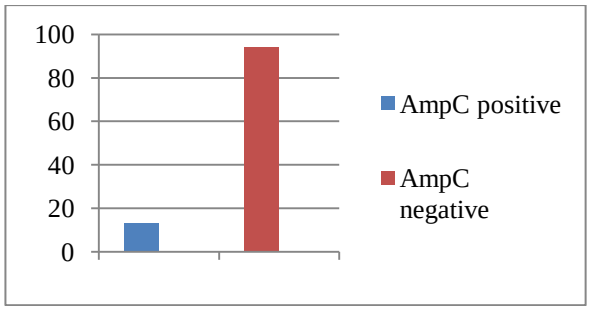

Figure 3. Prevalence of AmpC producing E. coli isolates

Table 1. MIC (µg/ml) values of several β-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)
Difloxacain	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)
Trimetoprime-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

A. sativum 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

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 β-lactamases [16]. Mansouri et al. reported the prevalence of ESBL and AmpC producing *K. pneumonia* (20%) and (2.6%) respectively [17]. In study 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 β-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 β-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 chrosomal 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. savitum*) cloves. It is produced by the interaction of the non-protein amino acid aliin 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.

Acknowledgements

This paper has been extracted from project No. 5653 (Performer: Mohammad Bokaeian)

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.

Funding/Support

Zahedan University of Medical Sciences.

References

1. Jacoby G, Medeiros A. AmpC beta-lactamases. Clin Microbiol Rev 2009; 22(1): 161-82.
2. Peter-Getzlaff S, Polsfuss S, Poledica M, et al. Detection of AmpC beta-lactamase in Escherichia coli: Comparison of three phenotypic confirmation assays and genetic analysis. J Clin Microbiol 2011; 49(8): 2924-32.
3. Mocktar C, Govinden U, Sturm AW and Essack S. The effect of mutations in the AmpC promoter region on β-lactam resistance from an Escherichia coli clinical isolate in a public sector hospital in KwaZulu-Natal, South Africa. Afr J Biotechnol 2008; 7(15): 2547-2550.
4. Thulasi G, Amsaveni V. Antibacterial activity of Cassia auriculata against ESBL producing E. coli from UTI patients. Int J Microbiol Res 2011; 2(3): 267-272.
5. Adetumbi MA, Lau BHS. (garlic)-A natural antibiotic. Med Hypotheses 1983; 12(3): 227-237.
6. Uzodike EB, Igwe IC. Efficacy of garlic (Allium sativum) on Staphylococcus aureus conjuctivities. JNOA 2005; 12: 20-22.
7. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing, 19th information supplement. M100-S19. Wayne, PA: Clinical and Laboratoy Standards Institute; 2009.
8. Coudron PE. Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in Klebsiella spp, Escherichia coli, and Proteus mirabilis. J Clin Microbial 2005; 43(8): 4163-4167.
9. Hanafy MS, Hatem ME. Studies on the antimicrobial activity of Nigella sativa seed (black cumin). J Ethnopharmacol 1991; 34(2-3): 275-8.
10. Anushia C, Sampathkamur P, Ramkumar L. Antibacterial and antioxidant activities in Cassia auriculata. Glob J Pharmacol 2009; 3(3): 127-130
11. Yu J, Lei J, Yu H, et al. Chemical composition and antimicrobial activity of the essential oil of Scutellaria barbata. Phytochemistry 2004; 65(7): 881-4.
12. Papanicolaou GA, Medeiros AA, Jacoby GA. Novel plasmid mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of Klebsiella pneumonia. Antimicrob Agents Chemother 1990; 34(11): 2200-2209.
13. Bauernfeind A, Chong Y, Schweighart S. Extended broadspectrumβ-lactamase in Klebsiella pneumoniae including resistance to cephamycins. Infection 1989; 17(5): 316-321.
14. Mansouri S, Kalantar D, Asadollahi P, et al. Characterization of Escherishia coli strains producing extended spectrum beta lactamases and AmpC type beta lactamases isolates from hospitalized patients in kerman, Iran. Roum Arch Microbiol Immunol 2012; 71(2): 81-6.
15. Niakan M, Chitsaz M, Metvaei AR. Prevalence of AmpC type extended sp ectrum beta genes in clinical, isolates of Klebsiella pneumoniae. J Med Microbial 2008; 2(2): 1-8.
16. Sultan-Dallal M, Sabaghi A, Molla-Aghamirzaeie H, et al. Prevalence of AmpC and SHV β-lactamases in clinical isolates of Ecsherichia coli from Tehran hospitals. Jundishapur J Microbiol 2012; 6(2): 176-18.
17. Mansouri S, Kalantar D, Asadolahi P, et al. Characterization of Klebsiella pneumonia strains producing extended spectrum beta lactamases and AmpC type beta lactamases isolates from hospitalized patient in Kerman, Iran. Roum Arch Microbial Immunol 2012; 71(2): 81-6.
18. Hussain M, Hasan F, Shah AA, et al. Prevalence of class A and AmpC beta lactamases in clinical Ecsherichia coli isolates from Pakistan, Islamabad. Jpn J Infect Dis 2011; 64(3): 249-52.
19. Pitout JD, Gregson DB, Church DL and Laupland KB. Population based laboratory surveillance for AmpC beta lactamases producing Ecsherichia coli. Emerg Infect Dis 2007; 13(3): 443-8.
20. Beesley T, Knott-Hunziker V, Petursson S, et al. The inhibition of class C beta-lactamases by boronic acids. Biochem J 1982; 209(1): 229-33.
21. Yeh YY, Liu L. Cholesterol-lowering effect of garlic extracts and organosulfur compounds: Human and animal studies. J Nutr 2001; 131(3s): 989S-93S.

22. Kim YK, Pai H, Lee HJ, et al. Bloodstream infections by extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in children: Epidemiology and clinical outcome. Antimicrob Agents Chemother 2002; 46(5): 1481-91.
23. Gardner CD, Messina M, Lawson LD and Farquhar JW. Soy garlic and ginkgo biloba: Their potential role in cardiovascular disease prevention and treatment. Atheroscler Rep 2003; 5(6): 468-475.
24. Lawson LD, Bauer R. Phytomedicines of Europe: Chemistry and biological activity (Acs symposium series). 1st ed. USA: American Chemical Society; 1998: 176-80.
25. Daka D. Antibacterial effect of garlic (Allium sativum) on Staphyloccus aureus: An in vitro study. Afr J Biotechnol 2011; 10(4): 666-669.
26. Afamefuna L. In vitro determination of bactericidal effects of garlic (Allium sativum) on Staphylococcus aureus and Escherichi coli. Niger J Sci Technol Environ Educ 2010; 3(1): 152-156 .

Please cite this article as: Shayan S, Bokaeian M, Shahraki S, Saeidi S. Prevalence of AmpC and ESBL producing E. coli and antibacterial effect of Allim sativum on clinical isolates collected from Zahedan hospitals. Zahedan J Res Med Sci (ZJRMS) 2014; 16(4): 6-10.