

Molecular evaluations of extended spectrum β -lactamase producing strains of *Serratia* isolated from blood samples of the patients in Namazi Hospital, Shiraz, Southern Iran

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Received: December 2012, Accepted: September 2013.

ABSTRACT

Background and Objective: Nowadays, the presence of extended-spectrum β -lactamases (ESBLs) producing strains in *Serratia* genus causes the emergence of resistance to many antibiotics. So, the lack of proper diagnosis of ESBLs strains can lead to failure in the treatment. The objective of the present study was to investigate ESBLs production in *Serratia* strains isolated from the clinical blood samples in Shiraz, Iran.

Materials and Methods: In this study, 39 *Serratia* strains isolated from the patients referred to Namazi Hospital, during a 2 year period were tested. The antimicrobial resistance of the isolates to 21 antibiotics was evaluated using Kirby-Bauer disk diffusion method. Combination disk method was used to determine the ESBL phenotype among the isolates. PCR was performed to investigate the presence of ESBL genes of SHV, OXA and TEM types.

Results: The lowest antibiotic resistance rates belonged to meropenem (7.69%) and imipenem (5.12%). Overall, positive ESBL phenotype was identified in 69% (n = 27) of the isolates, 70.37% (n = 19) for *S. marcescens* and 29.62% (n = 8) for *S. liquefaciens*. Results obtained by PCR showed that only 20.51% carried OXA gene and 15.38% carried SHV-1 gene. TEM gene was detected in none of the isolates.

Conclusion: This study showed a high prevalence of the emerging ESBL producing strains among clinical isolates of *Serratia* that could lead to an increase in antibiotic resistance. However, ESBLs genes other than those tested here may be more responsible for the emergence of ESBL phenotype among *Serratia* clinical isolates in our region.

Keywords: *Serratia*, Extended-spectrum β -lactamases, OXA, SHV, TEM

INTRODUCTION

Extended spectrum β -lactamases (ESBLs) emerged among *Enterobacteriaceae* causes a major problem in the treatment of nosocomial infections, thanks to rendered resistance to oxyimino-cephalosporins

(e.g. ceftazidime and cefepime) and monobactams (e.g. aztreonam) (1). ESBLs are plasmid-mediated enzymes, mostly found in *Klebsiella pneumoniae* and *Escherichia coli*, but also described in bacteria of *Citrobacter*, *Enterobacter*, *Salmonella*, *Shigella*, *Proteus*, *Serratia*, and *Pseudomonas aeruginosa* (2-4). Non-ESBL producing organisms can be changed to ESBL-producing organisms mostly through mutations in parent β -lactamases, particularly TEM-1, TEM-2 and SHV-1. There are various genes of ESBLs worldwide, with TEM, OXA, CTX-M and SHV as the most common ones. The SHV, TEM and CTX-M enzymes are of Ambler molecular class A and Bush group 2be, but OXA-

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type β -lactamase belongs to class D and group 2d (5). First reports on ESBL-producing organisms were published in Germany in 1983 (6). In the next decade, the majority of reports came from France, and then ESBL-producing organisms spread across the world (7). In recent years, nosocomial infections caused by *Serratia* strains have become a growing concern. The ability of this opportunistic pathogen to acquire resistance to a broad spectrum of antibiotics has made the effective treatment more difficult. ESBL-producing enzymes rose up among *Serratia* genus and have increased multidrug resistance (8). Such β -lactamases are quickly transferable and have become a significant issue for human health conditions. The objectives of this study were to evaluate ESBL production and identify the types of such enzymes among *Serratia* strains, isolated from the blood samples of the patients in Shiraz Namazi Hospital, southern Iran.

MATERIALS AND METHODS

***Serratia* isolates.** This study was conducted at Professor Alborzi Clinical Microbiology Research Center (PACMRC) in Namazi Hospital, Shiraz, Iran, on 39 *Serratia* strains. The strains were isolated from BACTEC bottles of blood samples of the patients referred to the lab during a period of 2 years from March 2010 to March 2012. All isolates were identified based on the biochemical-microbial tests and then speciated using diagnostic Microgene Bioproducts kit (GN-ID A Panel U.K).

Determination of antimicrobial resistance. Antimicrobial resistance of the strains against 21 antibiotics was determined using standard Kirby-Bauer agar disk diffusion test, according to the CLSI protocol (Mast Co, U.K antimicrobial disk) (9). *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. The antibiotics and their respective amounts were as follows: gentamicin (10 μ g), cephalixin (30 μ g), ciprofloxacin (5 μ g), chloramphenicol (30 μ g), meropenem (10 μ g), cefuroxime (30 μ g), ceftriaxone (30 μ g), cefotaxime (30 μ g), imipenem (10 μ g), ampicillin (10 μ g), cefepime (30 μ g), ceftazidime (30 μ g), tetracycline (30 μ g), amikacin (30 μ g), ticarcillin (75 μ g), ceftizoxime (30 μ g), tobramycin (10 μ g), piperacillin (100 μ g), piperacillin/tazobactam (110 μ g), augmentin (30 μ g)

and aztreonam (30 μ g) (Mast Co, U.K.).

Phenotypic confirmatory test for ESBL. For phenotypic confirmation of the presence of ESBLs among the isolates, Combination-Disk Synergistic Test (CDST) was used as recommended by the Clinical and Laboratory Standard Institute (10). Antibiotic disks used in this method included ceftazidime (30 μ g), ceftazidime/clavulanate (40 μ g), cefotaxime (30 μ g) and cefotaxime/clavulanate (40 μ g) (Mast Co, UK). A difference of ≥ 5 mm in the zone of growth inhibition diameter between cephalosporin disks and their respective cephalosporin/clavulanate disk confirmed ESBL phenotype among the isolates.

PCR amplification for the detection of ESBL genes. To amplify ESBL genes by PCR method, initially bacterial genome was extracted using boiling water bath for 10 minutes. Briefly, a bacterial suspension in 1.5 ml of distilled water was prepared and incubated at 95°C for 10 minutes. The sample was centrifuged at 8000 rpm for 15 min and then, the supernatant was used as DNA template. The presence of *bla*_{OXA-1} and *bla*_{SHV-1} within bacterial genome was investigated by multiplex PCR and for *bla*_{TEM} gene by single PCR, using specific primers as shown in Table 1. The cycling conditions for the detection of *bla*_{OXA} and *bla*_{SHV} were as follows: initial denaturation at 95°C for 15 min, 30 cycles of 94°C for 30 sec, 62°C for 90 sec and 72°C for 60 sec, and a final extension at 72°C for 10 min. The cycling conditions for the detection of *bla*_{TEM} were initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 45 sec, and a final elongation at 72°C for 10 min. Finally, the PCR products were detected by electrophoresis in a 2% agarose gel with 1X TAE buffer. Gel staining was then performed using ethidium bromide (0.5 μ g/ml) and photographed under UV lamp (Gel docuenter Uvitec, EEC, England).

Escherichia coli ATCC 25922 and previously characterized *bla*_{OXA} and SHV positive *Klebsiella pneumoniae*, *bla*_{TEM}-positive *Salmonella enteritidis* and *Shigella sonnei* strains were used as controls for ESBL analysis (3, 4).

Statistical analysis. Phenotypic results and the data obtained from PCR assays were compared using SPSS statistical software, version 15 and Fisher's exact test. *P*-value equal or below 0.05 was considered as significant.

Table 1. Primers used for the detection of *bla*_{OXA}, *bla*_{SHV} and *bla*_{TEM} genes.

Gene	Primer	Sequences	Reference
<i>bla</i> _{TEM}	forward	5'-ATAAAATTCTTGAAGACGAAA-3'	11
<i>bla</i> _{TEM}	reverse	5'-GACAGTTACCAATGCTTAATCA-3'	
<i>bla</i> _{SHV}	forward	5'-CTTTATCGGCCCTCACTCAA-3'	12
<i>bla</i> _{SHV}	reverse	5'-AGGTGCTCATCATGGGAAAAG-3'	
<i>bla</i> _{OXA}	forward	5'-ACACAATACATATCAACTTCGC-3'	13
<i>bla</i> _{OXA}	reverse	5'-AGTGTGTTTAGAATGGTGATC-3'	

RESULTS

Serratia isolates. Using diagnostic Microgene kit, from 39 *Serratia* clinical isolates, 30 (77%) were identified as *Serratia marcescens* and 9 (23%) as *Serratia liquefaciens*.

Antimicrobial susceptibility testing. The data revealed that; 56.41%, 48.71%, 46.15%, 43.58%, 41.02%, 33.33%, 33.33%, 33.33%, 33.33%, 33.33%, 30.76% and 25.64% of the *Serratia* strains were susceptible to ceftazidime, ceftizoxime, gentamicin, cefepime, aztreonam, chloramphenicol, amikacin, piperacillin, ticarcillin, ceftriaxone, cefotaxime and tobramycin, respectively. Overall, these strains were found to be more susceptible to imipenem (94.87%), meropenem (92.30%), ciprofloxacin (87.17%) and piperacillin/tazobactam (84.61%). The least susceptibility was observed for augmentin (7.69%) and tetracycline, ampicillin, cefuroxime (5.12%) but none of the isolates was susceptible to cephalixin.

Multidrug resistant strains. The strains exhibiting resistance to more than 2 or 3 classes of antibiotics were considered as multidrug resistant (MDR) strains. The most frequent patterns of MDR among the 39 *Serratia* isolates were as cephalixin-chloramphenicol-cefuroxime-ampicillin-tetracycline-augmentin and gentamicin-cephalexin-chloramphenicol-cefuroxime-ceftriaxone-cefotaxime-ampicillin-cefepime-ceftazidime-tetracycline-amikacin-ticarcillin-ceftizoxime-tobramycin-piperacillin-augmentin-aztreonam with frequencies of 15.38% and 12.82%, respectively (Table 2).

Determination of ESBL phenotype. The results of combination disk assay showed that from all the strains, 69% (n = 27) were identified as positive ESBL phenotype. Moreover, it was also indicated that a high percentage of *Serratia* samples were fully resistant (R = 100%) to cefotaxime (CTX), but none was completely resistant to ceftazidime (CAZ).

Detection of *bla*_{OXA}, *bla*_{SHV} and *bla*_{TEM} genes by PCR. Of 39 *Serratia* isolates, 10 samples (25.64%) demonstrated the presence of either OXA or SHV or both genes at the same time (Fig. 1a). The OXA-1 gene was observed in 20.51% (n = 8) of the strains but SHV gene in 15.38% (n = 6). Moreover, *bla*_{TEM} gene was not detected in any of the strains (Fig. 1b).

Statistical analysis. To determine the relationship between the presence of ESBL gene and ESBL phenotype among the *Serratia* isolates, Fisher's exact test was performed and *p*-value was determined for each of the genes. *P*-value represents the reliability of the target gene for displaying the ESBL phenotype. The Statistical analysis showed that *p*-value for *bla*_{OXA} and *bla*_{SHV} genes were 0.042 and 0.151, respectively. However, due to the absence of *bla*_{TEM} among the examined isolates, statistical analysis was not performed for this gene. The results were reliable for the *bla*_{OXA} gene but it was not for SHV gene.

DISCUSSION

The *Serratia* strains are opportunistic pathogens

Table 2. Frequency of MDR patterns among the *Serratia* isolates.

Pattern of MDR	% of MDR
CFX-C-CXM-AP-T-AUG	15.38%
GM-CFX-C-CXM-CRO-CTX-AP-CPM-CAZ-T-AK-TC-CZX-TN-PRL-AUG-ATM	12.82%
GM-CFX-C-CXM-CRO-CTX-AP-CPM-CAZ-T-AK-TC-CZX-TN-PRL-PTZ-AUG-ATM	7.69%

CFX, cephalixin; C, chloramphenicol; CXM, cefuroxime; AP, ampicillin; T, tetracycline; AUG, augmentin; GM, gentamicin; CRO, ceftriaxone; CTX, cefotaxime; CPM, cefepime; CAZ, ceftazidime; AK, amikacin; TC, ticarcillin; CZX, ceftizoxime; TN, tobramycin; PRL, piperacillin; ATM, aztreonam; PTZ, piperacillin/tazobactam.

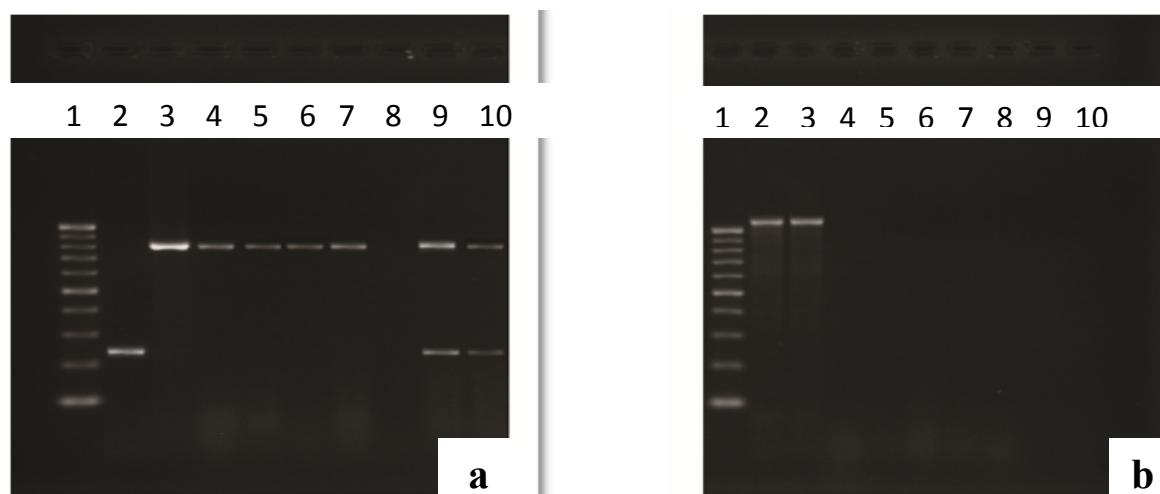


Fig. 1. (a): Multiplex PCR assay for bla_{OXA} and bla_{SHV} . Lane 1, 100-bp DNA ladder; lane 2, positive control for SHV gene (237 bp); lane 3, positive control for OXA gene (813 bp); lanes 4,5,6,7 clinical isolates of ESBL positive (bla_{OXA}); lanes 9,10, clinical isolates of ESBL positive for both bla_{SHV} and bla_{OXA} . **(b):** Single PCR assay for bla_{TEM} . Lane 1, 100-bp DNA ladder; lanes 2,3, positive control for TEM gene (1080 bp); lanes 4-10, clinical isolates of ESBL negative (bla_{TEM}).

and responsible for 2% of nosocomial infections that are often caused by *S. marcescens* species, however, *Serratia liquefaciens* infections are rare and mostly due to poor hygiene. These organisms are involved in the development of some diseases such as meningitis, urinary tract infections, sepsis, bacteremia and endocarditis. *Serratia* strains are resistant to a range of antibiotics used to treat bacterial infections. ESBL genes are highly frequent in the majority of bacteria, especially *Enterobacteriaceae* family. Their presence among the bacteria makes them ESBL expressing organisms. Such bacteria become resistant to third and fourth generation cephalosporins and monobactams, in addition to the majority of antibiotics (14). ESBL producing organisms often colonized in the patients hospitalized for a long time, especially those who use urinary catheters or central venous lines. Different reports indicated the presence of ESBL producing strains among the *Serratia* genus (15,16). The treatment of diseases caused by ESBL organisms must be based on the results of new antibiogram tests but not empirical therapy because they have potential resistance to multiple antibiotics (17,18). In this study, phenotypic evaluation by combination disk method was indicative of a prevalence of 69% for ESBL producing strains in *Serratia* species. We diagnosed only two species of *marcescens* and *liquefaciens* among the *Serratia* isolates in our study. The prevalence of positive ESBL among these strains was 70.37% (n = 19) and 29.62% (n = 8), respectively. However, the rate of ESBL production in the *Serratia*

strains was consistent with that in a previous study by Wu LT, et al in 2004 (65% positive ESBL phenotype) (15). Based on the results of disk diffusion method, the isolates showed a high degree of resistance to the third generation cephalosporins (cefotaxime, 69.23%; ceftriaxone, 66.66%; ceftizoxime, 51.28%; ceftazidime, 43.58%) and the fourth generation cephalosporins (cefepime, 56.41%). The findings also indicate that due to the emergence of ESBL strains, the third and fourth generation cephalosporins could not be reliable for the treatment of the diseases caused by *Serratia* strains. In this study, the effects of ciprofloxacin, one of the fluoroquinolones, on the *Serratia* isolates were examined and resistance of 12.82% was observed.

However, it has been demonstrated that from 21 antibiotics tested, imipenem (5.12%) and meropenem (7.69) can be considered as the most effective drug for the treatment of infection caused by ESBL strains of *Serratia*, followed by fluoroquinolones (19). Among the strains under our study, 94.87% were MDR. This is probably due to the presence of mobile resistance elements carrying resistance genes to multiple antibiotics among the isolates. The PCR results obtained from this study showed that of the three genes under investigation for ESBL producing strains, bla_{OXA} gene (20.51%) was the most frequent one among *Serratia* isolates. The frequency of bla_{SHV} gene was 15.38%, but none of the isolates had bla_{TEM} gene. Overall, the frequency of ESBL strains based on PCR results was 25.64% but 69.23% by phenotypic

evaluation. The exact prevalence of ESBL genes among the bacteria is not clear, maybe due to the lack of sufficient studies in this field or improper identification of the genes. However, it is clear that ESBL organisms are widely spread worldwide among *Enterobacteriaceae* family. The most frequent types of ESBL enzymes include SHV, TEM, OXA and CTX-M, but there are other ESBL enzymes with different frequencies among the bacteria (VEB, PER, GES) (20, 2). The differences between the results of this study and the others could be because of the presence of other ESBL genes within bacterial total genome that were not considered here. In two previous studies, *bla*_{SHV} and *bla*_{CTX-M-3} were identified as the most common ESBL genes in *Serratia marcescens* (21, 22). However, it should be considered that the frequency and type of ESBL genes vary in different geographical areas and even from region to region. The results of statistical analysis indicated a significant relationship between the presence of *bla*_{OXA} gene and ESBL phenotype ($p = 0.042$), but there was no such a relationship between SHV and ESBL phenotype ($p = 0.151$). This might be due to the limited number of samples in this study or the lack of SHV beta-lactamase in these ESBL strains.

In conclusion, these data showed that carbapenems followed by ciprofloxacin and piperacillin/tazobactam are the most effective antibiotics against the *Serratia* strains isolated in our region, while cephalosporins showed no effects on them. Moreover, according to the present phenotypic and genotypic data it seems that about 44% of the present ESBL producing strains carry beta-lactamase genes other than the genes under investigation in this study. More genetic studies for detection of other ESBL genes are being pursued in our lab. Regarding the important role of *Serratia* as a causative agent in nosocomial infections and widespread presence of ESBL strains among them, developing and performing tests for the detection of ESBL producing *Serratia* strains as a routine test in clinical laboratories is recommended.

ACKNOWLEDGMENTS

This study has been financially supported by grant#90-25, provided by Prof. Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences. We are grateful to Sareh Roosta in the Center for Development of Clinical Research of Namazi Hospital for her assistance in data analysis

and Mrs Marziyeh Hosseini in Professor Alborzi Clinical Microbiology Research Center for her critical assistance in performing the molecular diagnostic methods. Our thanks are also due to Hassan Khajehhei, Ph.D, for copy editing of the manuscript.

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