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Prevalence of Extended Spectrum β-lactamase *SHV* and *OXA* Producing Gram Negative Bacteria at Tertiary Care Hospital of Lahore, Pakistan

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AW performed the experiments. MS and AW wrote the manuscript. SJ and SS designed the study. JA contributed in data analysing. IJ and FR helped in sample collection. NS reviewed the manuscript.

Key words
Gram negative rods, Extended spectrum β-lactamase, Cephalosporin, bla_{SHV}, bla_{OXA}

ABSTRACT

The present study was planned to detect drug resistant genes like "bla_{SHV}" and "bla_{OXA}" among gram negative rods (GNRs) isolated from different clinical specimens, collected from Jinnah Hospital Lahore during January-December 2015. Majority of the GNRs were isolated from urine samples (55%) followed by pus (28%), blood (7%), sputum (6%), Broncho-alveolar lavage (2%), fluid (1%) and from tips (1%). The isolated organisms were *Escherichia coli* (n=60), *Klebsiella pneumoniae* (n= 20), *Enterobacter cloaca* (n= 09), *Enterobacter aerogenes* (n= 02), *Proteus mirabilis* (n= 05), *Proteus vulgaris* (n=02) and *Citrobacter freundii* (n= 02). Extended spectrum beta lactamases (ESBL) production was screened by double disc synergy test (DDST) and combination disc method as per Clinical Laboratory Standard Institute (CLSI) guidelines 2015. Presence of "bla_{SHV}" and "bla_{OXA}" genes was tested by PCR technique. Every isolate was ESBL positive and resistant against third generation cephalosporin and Aztreonam. Notably, 15% of ESBL producing strains harbored bla_{SHV} gene while 43% were positive for bla_{OXA} gene. In a nutshell, diverse variety of ESBL-positive GNRs is distributed, the findings of the present study should be considered for planning strategies to treat and prevent the further spread of ESBL-producing gram-negative rods infections.

INTRODUCTION

Emergence of drug resistant pathogens is precarious global issue in current medicine. Improper use of antimicrobials induces a selective pressure on microorganisms, which results in the development of antimicrobial resistance (Akhtar *et al*., 2018). Bacteria use variety of strategies to acquire drug resistance which include enzymes production, extended spectrum beta lactamases (ESBL), metallo beta lactamases (MBL) and Carbapenamases, reduced uptake of drug, efflux of antibiotics and cell target modifications (Fluit *et al*., 2001).

The Gram negative rods (GNRs) are most common source of infection in developing countries. High mortality and morbidity has been associated with GNRs associated infections. A very high death rate 42% to 100% has been reported in patients infected with ESBL producing bacteria (Ehlers *et al*., 2009; Kaftandzieva *et al*., 2011; Zaniani *et al*., 2012; Colodner, 2005). Drug resistance leads to prolonged hospital stay of patients and increase in health expenditures which ultimately effect the economy of country as well as rate of morbidity and mortality (Maragakis *et al*., 2008). ESBL enzymes production is most potent mechanism, which inactivate beta-lactam antibiotics by destroying the amide bond of beta-lactam ring (Gelinski *et al*., 2014).

ESBL enzymes producing organisms have been isolated from various clinical samples all over the world.

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In Pakistan, Ali *et al.* (2016) reported that ESBL were found in 59.2% of 250 isolates of *E. coli*. Another study done by Rahman *et al.* (2016) reported that 44.2% of 355 isolates were ESBL producer. Recently, resistance against carbapenems has also been reported all over the world. This is mainly attributed to special type of beta lactamases called metallo beta lactamases (MBL). These types of enzymes possess capability to break down the carbapenems (Akhtar *et al*., 2018).

ESBL producing bacteria have usually large plasmids and carry drug resistance genes against various antimicrobial agents. ESBL were first identified in *Klebsiella* in 1980 (Bush and Jacoby, 2010). Later on it was also reported in various Gram negative rods of Enterobacteriaceae (Abrar *et al*., 2018). ESBL producing bacteria are difficult to treat with routine antibiotics and they may cause serious infections in surgical and intensive care unit patients. They frequently cause pneumonia, meningitis, peritonitis, intra-abdominal abscess and urinary tract infections (Dhillon *et al*., 2012). The frequency of ESBL production varies among various GNRs. In Pakistan, high prevalence of ESBL particularly in *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* has been reported (Sana *et al*., 2011). More than 1300 clinically significant β lactamases have been isolated up to date. There are four major types of ESBLs which include $bla_{TEM,}$ bla_{CTX-M}, bla_{SHV}, and bla_{OXA}. More than 172 bla_{TEM}, 400 bla_{OXA}, 193 bla_{SHV} and 130 bla_{CTX-M} have been discovered (Lahey Clinic Foundation, 2011; Bush and Jacoby, 2010).

SHV-type beta lactamases derive their name from sulfhydryl variable (Paterson and Bonomo, 2005). According to classification SHV-1 belongs to group A (Chaves *et al*., 2001). SHV type β lactamases are one of most important types of ESBL found in clinical isolates (Paterson and Bonomo, 2005). They can hydrolyze ceftazidime and cefotaxime efficiently (Bradford, 2001).

OXA type β lactamases are classified in to group D according to functional classification (Poirel *et al*., 2010). More than 400 variants of OXA type β lactamases have been discovered up to date (Antunes and Fisher, 2014). Wild type OXA-1was involved in hydrolysis of Oxacillin and Cloxacillin that is why they were named OXA (Oxacillin hydrolyzing) (Héritier *et al*., 2005). These enzymes are very diverse and are capable of posing drug resistance of various spectrums. They can be narrow spectrum or broad spectrum. In Pakistan very, little data is available on identification of extended spectrum β lactamase producing genes. Therefore, present study was planned to detect drug resistant genes like "bla_{SHV}" and "bla_{OXA}" among Gram negative rods isolated from different clinical specimens.

MATERIALS AND METHODS

Collection of bacterial isolates

Clinical samples comprising of pus, urine, blood, sputum, broncho-alveolar lavage (BAL) and tips from various patients hospitalized at Jinnah Hospital Lahore during the period of January to December 2015, were cultured for the isolation of gram-negative bacterial strains.

Identification of bacterial isolates

The bacterial isolates were identified by conventional morphology and biochemical based tests. Bacterial strains were sub-cultured on blood and MacConkey agar and plates were incubated at 37°C overnight aerobically (Akhtar *et al*., 2018). The colony morphology and culture characteristics were studied to identify bacterial species (Akhtar *et al*., 2018). The isolates were further confirmed employing Analytical Profile Index 20 (API 20 system) (Bio Merieux, France).

Antimicrobial susceptibility determination

Antimicrobial resistance of the study isolates against cephalosporin antibiotics (ceftazidime, ceftriaxone, cefotaxime and aztreonam) was performed by Kirby-Bauer disc diffusion method according to Clinical Laboratory Standards Institute (CLSI) 2015 guidelines. For this, bacterial strains were grown on Mueller-Hinton agar (Oxoid UK) and zone of inhibition was measured around the ceftazidime (30μg), cefotaxime (30μg), ceftriaxone (30μg) and aztreonam (30μg) antibiotic disk. The isolates were classified into susceptible, intermediate or resistant according to the CLSI guidelines. *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603) were used as control strains.

Phenotypic detection of ESBL

Double disc synergy test (DDST)

Clavulanic acid, present in the co-amoxiclav disc, acts synergistically with the third-generation cephalosporin and/or aztreonam and resulted in the increase of zone size of these antimicrobials or elliptical zone between the tested antibiotic disc and co-amoxiclav disc. This was considered as a positive result for ESBL production.

Combination disc method

This method is referred to phenotypic confirmatory method by CLSI guidelines. ESBL detection was done by combined disc method using cefotaxime (30ug) versus cefotaxime plus clavulanate (30+10 μg) and ceftazidime (30μg) versus ceftazidime plus clavulanate (30+10 μg) (Oxoid) according CLSI 2015 guidelines. Isolates which

showed an enlarged zone of inhibition greater than 5 mm on the side using combined disc compared to the results seen on the side without clavulanic acid combination were confirmed as ESBL producer.

Molecular identification of ESBL genes

The genomic DNAs were extracted from all phenotypically ESBL producing strains by using TIANamp Genomic DNA extraction (TIANGEN Biotech Beijing, Co., Ltd.). For bla_{SHV} and bla_{OXA} detection, the extracted DNAs were subjected to PCR using primer sequences already described in the literature (Sana *et al*., 2011). Briefly, forward (5'CACTCAAGGATGTATTGT G-3') and reverse (5'-TTAGCGTTGCCAGTGCTCG-3') primers were used for bla_{SHV} detection. Whereas, bla_{OXA} was detected by using forward (5'ACACAATACATATCAACTTCGC3') and reverse (5'AGTGTGTTTAGAATGGTGATC3') primers. The PCR conditions were used as follows; initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 40sec, primary extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplified PCR products were separated on 1.5% agarose gel by gel electrophoresis and visualized under UV light for the evaluation of molecular size of amplified products.

Statistical analysis

Results were analyzed with the SPSS (version 20.0, SPSS Inc). The detection of bla_{SHV} and bla_{OXA} genes in isolates was presented as percentages in the Tables.

RESULTS

In the present study, altogether 100 gram-negative rods: *E. coli* (n=60), *Klebsiella pneumoniae* (n= 20), *Enterobacter cloaca* (n= 09), *Enterobacter aerogenes* (n= 02), *Proteus mirabilis* (n= 05), *Proteus vulgaris* (n=02) and *Citrobacter freundii* (n= 02), were isolated from clinical samples. It is worth mentioning here that majority of the clinical isolates (55%) were recovered from urine followed by 28% from pus, 7% from blood, 6% from sputum, 2% from broncho-alveolar lavage (BAL), 1% from fluid and 1% from tips. The distribution of bacterial isolates in clinical specimens is enlisted in Table I. All of these isolates manifested resistance against third generation cephalosporin and aztreonam antibiotics.

The combination disc method and double disc synergy test for the phenotypic detection of ESBL production revealed that all isolate was positive for ESBL production. The representative ESBL producing isolates by Combination disc method and Double disc synergy test are shown in Figure 1.

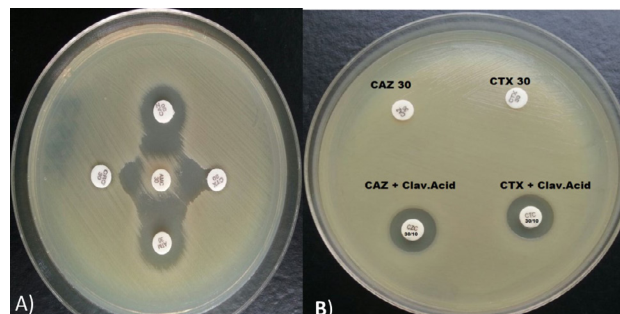

Fig. 1. Phenotypic detection of ESBL producers by Combination disc method and Double disc synergy test respectively; A) The presence of elliptical inhibitory zone between cefotaxime and augmentin indicate the ESBL positive organism; B) Positive results are shown by increase in ≥ 5-mm zone diameter for either antimicrobial agent tested in combination with clavulanate vs the zone diameter of the agent when tested alone; CAZ, Ceftazidime disc; CTX, Cefotaxime disc.

The ESBL producing isolates were further subjected to PCR for the detection of bla_{SHV} and bla_{OXA} genes the presence of which is considered as one of foremost mechanism of cephalosporin resistance among gram-negative rods. The bla_{SHV} positive samples showed amplification products of 885 bp as shown in Supplementary Figure 1A. Similarly, the amplification of 813 bp PCR product from carbapenem resistant isolates indicated that those isolates harbor bla_{OXA} gene. A representative gel showing the PCR product of bla_{OXA} gene is presented in Supplementary Figure 1B. In the nutshell, PCR data showed that only 15 (15%) of ESBL producing strains harbor bla_{SHV} gene. Among those bla_{SHV} positive isolates, twelve strains were of *K. pneumoniae* and three of *E. coli*. However, none of *E. cloaca, E. aerogenes*, *P. mirabilis, P. vulgaris* and *C. freundii* strain was found harboring bla_{SHV} gene. Unlike bla_{SHV}, elevated bla_{OXA} gene positivity 43 (43%) was observed among ESBL producing clinical isolates. Among those 43 bla_{OXA} positive isolates, 30 (30%) strains were of *E. coli,* 8 (08%) of *K. pneumoniae,* 3 (3%) of *E. cloaca* and 2 (2%) of *P. mirabilis.* The Table II briefly describes the distribution of bla_{SHV} and bla_{OXA} genes in ESBL producing gram negative rods under this study.

DISCUSSION

The most dominant ESBL producer in present study was *E. coli* (n=60) followed by *K. pneumoniae* (n= 20). A study was conducted by Khalid *et al*. (2013) in Pakistan reported *E. coli* as leading ESBL producer followed by *K. pneumoniae*. These results are similar to our study results.

Table I. Distribution of bacterial isolates in clinical specimens.

Organism	Sources of sample							
	Urine	Pus	Blood	Sputum	BAL	Fluid	TIP	
Escherichia coli	34 (56.6%)	19 (31.6%)	05 (8.3%)	0	0	01 (1.6%)	01 (1.6%)	n=60
Klebsiella pneumoniae	07 (35%)	04 (20%)	01 (5%)	6 (30%)	2 (10%)	0	0	N=20
Enterobacter cloacae	07 (77.7%)	01 (11%)	1 (11%)	0	0	0	0	N=9
Enterobacter aerogenes	01 (50%)	01 (50%)	0	0	0	0	0	N=2
Proteus mirabilis	04 (80%)	01 (20%)	0	0	0	0	0	N=5
Proteus vulgaris	01 (50%)	01 (50%)	0	0	0	0	0	N=2
Citrobacter freundii	01 (50%)	01 (50%)	0	0	0	0	0	N=2
Total.	55 (55 %)	28 (28 %)	07 (7 %)	06 (6%)	2 (2%)	01 (01%)	01 (01%)	100

Table II. Distribution of *bla*$_{SHV}$ and *bla*$_{OXA}$ in ESBL producing gram-negative rods.

Organism	bla$_{SHV}$ (N=15)	bla$_{OXA}$ (N=43)
Escherichia coli (n=60)	03 (5%)	30 (50%)
Klebsiella pneumoniae (n=20)	12 (60%)	08 (40%)
Enterobacter cloaca (n=09)	0 (0%)	03 (33.3%)
Enterobacter aerogenes (n=02)	0 (0%)	0 (0%)
Proteus mirabilis (n=05)	0 (0%)	02 (40%)
Proteus vulgaris (n= 02)	0 (0%)	0(0%)
Citrobacter freundii (n= 02)	0(0%)	0(0%)
Total:100	15%	43%

Urinary tract infections are severe health affecting problems all over the world. The most common organism associated with urinary tract infections are *E. coli*, *K. pneumoniae, and P. mirabilis.* The *E. coli* is responsible for nearly 85% of community acquired urinary tract infections and approximately 50% of hospital acquired urinary tract infections. The *E. coli* is also one of most common organisms which are isolated from catheter associated urinary tract infections (Sabir *et al*., 2014).

The overall prevalence of *bla*$_{SHV}$ and *bla*$_{OXA}$ in present study was 15% and 43% respectively. Similarly, a study was conducted by Moosavian *et al*. (2012) on various members of ESBL producing enterobacteriaceae reported 15 % of *bla*$_{SHV}$ gene prevalence. Another similar study was carried out by Zaniani *et al*. (2012) in Iran who reported 14.4% *bla*$_{SHV}$ prevalence among ESBL producing *K. pneumoniae* and *E.* coli. Another study, carried out by Ramazanzadeh *et al*. (2010) in Iran showed 18.7% *bla*$_{SHV}$ prevalence in ESBL producing *K. pneumoniae*. The findings of this study are in accordance with our study results. A very little data is available on ESBL genes in Pakistan. Hussain *et al*. (2011) conducted a study in Islamabad, Pakistan and they reported 15.4% *bla*$_{SHV}$ gene. Zongo *et al*. (2015) reported 18.7% *bla*$_{SHV}$ prevalence in West Africa. Yesmin *et al*. (2015) reported 18.69% of *bla*$_{SHV}$ prevalence from Bangladesh in various members of Enterobactericeae and *Pseudomonas* species. These results are in accordance to the results of current study.

In current study the *bla*$_{SHV}$ was most commonly found in *K. pneumoniae*. About 12 (60%) from 20 isolates were positive for *bla*$_{SHV}$ gene. A study was conducted by Ahmed *et al* (2013) revealed 63.1% *bla*$_{SHV}$ gene positive among ESBL producing *K. pneumoniae.* Similar study conducted by Shahcheraghi *et al*. (2007) in Iran and described 69% prevalence of *bla*$_{SHV}$ in *K. pneumoniae*. In another study Feizabadi *et al*. (2010) so reported similar high prevalence of *bla*$_{SHV}$ (67.4%) in *K. pneumoniae*.

In our study *bla*$_{OXA}$ gene was detected in 43% of isolates. A study was carried out by Farzana *et al*. (2013) in Dhaka, Bangladesh and reported 43% *bla*$_{OXA}$ gene in ESBL producing GNRs isolated from pus and wound samples. Sana *et al*. (2011) conducted a study in Lebanon and they reported 45.2% isolates were positive for *bla*$_{OXA}$ gene. Another study was conducted by Moubareck *et al*. (2005) in Lebanon they reported alike results for *bla*$_{OXA}$ gene in extended spectrum β lactamase producing enterobacteriaceae.

Mostatabi *et al*. (2013) showed 20.51% prevalence of *bla*$_{OXA}$ gene from clinical isolates of *Serratia*. In Pakistan, few studies were done to identify ESBL producing genes. A study was done in Faisalabad, Pakistan and prevalence of *bla*$_{OXA}$ gene was found to be 17.2% in ESBL producing *E. coli* (Saeed *et al*., 2009). These results are different from the current study. The geographical variations could explain this disparity in results. Moreover, discrepancy in results may also be due to different sample sizes and difference in time which the bacterial isolates were collected (Al-Agamy *et al*., 2009).

There are few limitations in our study. The sample size was not large enough to describe the exact burden of beta lactamases genes in our region. Because, history of the patient was not collected so we were unable to discriminate between nosocomial and community acquired strains of pathogens.

Appropriate infection control practices and barriers are crucial to avoid spread and outbreaks of ESBL producing gram negative rods. Furthermore, policy of antibiotic rotation should be adopted to stop the further spread and selection of extended spectrum β lactamases producing bacteria. The other genes that are associated extended spectrum β lactamases production should be explored to find out the genes that are more common in Pakistan.

CONCLUSION

The bla_{SHV} and bla_{OXA} genes are also present in our population. bla_{OXA} is more prevalent in ESBL producing gram negative rods in locality of Lahore, Pakistan. In this study we concluded that bla_{OXA} is more dominant type of ESBL in *E. coli* than other gram negative rods in our area. While bla_{SHV} is principal type of ESBL in *Klebsiella pneumoniae*. In this study it was also concluded that most of the ESBL producing organism were isolated from urine and pus.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2019.51.6.2345.2351

Statement of conflict of interests

The authors declare there is no conflict of interest.

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