First Report of OXA-4, an ESBL Isolated from *Pseudomonas aeruginosa* a South Indian Strain

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Abstract The OXA-type β -lactamases are so named because of their oxacillin-hydrolyzing abilities. In this study we characterize an extended spectrum β -lactamase, designated OXA-4, produced by a clinical isolate of *Pseudomonas aeruginosa*. ESBL production was detected by double disk synergy test. The *P. aeruginosa* isolate was obtained from endotracheal suction tip of 84 years old male patient diagnosed with CVA and hypertension. ESBL producing OXA β -lactamases was detected by PCR with primers specific to the conserved regions of the coding genes. Iso electric focusing was done to confirm the significance, sequencing the amplified product was also done. In the phenotypic identification, the strain was highly resistant to third generation cephalosporins and also to imipenem. The PCR amplified product for OXA β -lactamase was viewed at 919 bp. The pI point for the same was identified at 7.2. With the help of sequencing the amplified OXA β -lactamase was identified as OXA-4 gene. Here we report *P. aeruginosa* producing OXA-4 ESBL for the first time in the Indian subcontinent.

Keywords OXA-4 · *P. aeruginosa* · ESBL

Introduction

The OXA-type β -lactamases are so named because of their oxacillin-hydrolyzing abilities. These β -lactamases (group 2d) are characterized by hydrolysis rates for cloxacillin and oxacillin greater than 50 % than that for benzyl penicillin. They predominantly occur in *Pseudomonas aeruginosa* but have been detected in many other Gram-negative bacteria [1].

Pseudomonas aeruginosa is a leading cause of nosocomial infections, including pneumonia, urinary tract infections and bacteremia. They have the intrinsic resistance to the extended-spectrum cephalosporin, have an outer membrane with selective permeability to β -lactams and by modification of outer membrane porins diminish permeability to other antibiotics.

β -lactamase enzymes destroy the β -lactam ring by two major mechanisms of action. Firstly, the most common β -lactamases have a serine based mechanism of action. They are divided into three major classes (A, C, and D) on the basis of the amino acid sequences. They contain an active site consisting of a narrow longitudinal groove, with a cavity on its floor (the oxyanion pocket), which was loosely constructed in order to have conformational flexibility in terms of substrate binding. Close to this lies the serine residue that irreversibly reacts with the carbonyl carbon of the β -lactam ring, resulting in an open ring (inactive β -lactam) and regenerating the β -lactamase. These enzymes are active against many penicillins, cephalosporins, and monobactams.

Secondly, a less commonly encountered group of β -lactamases are the metallo β -lactamases or class B β -lactamases. They used a divalent transition metal ion, most often zinc, linked to a histidine or cysteine residue or both, to react with the carbonyl group of the amide bond

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for most of the penicillins, cephalosporins, and carbapenems, but not monobactams [2].

In fact, the most common OXA-type β -lactamase, OXA-1 has been found in 1–10 % of *Escherichia coli* isolates. Most OXA-type β -lactamases do not hydrolyze the extended-spectrum cephalosporin to a significant degree and are not regarded as extended spectrum β -lactamases (ESBLs). Increased and extended resistance to β -lactams in *P. aeruginosa* is generally imputed to the over production of β -lactamase due to point mutations in the promoter region.

The incidence of carbapenem resistant strains and the OXA-type β -lactamases associated with them has continued to increase. Six novel OXA-type enzymes were characterized from carbapenem resistant strains collected worldwide from 1995 onwards [3]. In France, derivative of OXA-10 (numbered OXA-28) was identified in a *P. aeruginosa* isolate [4]. OXA-18 ESBL which is an extended-spectrum derivative of the narrow-spectrum OXA-13 β -lactamase (numbered OXA-19) were also discovered in France in *P. aeruginosa* isolates [1]. OXA-16, the third ESBL mutant of OXA-10 enzyme was isolated from *P. aeruginosa* in the year 1998 [5]. The evolution of OXA-type β -lactamases from parent enzymes with narrower spectra has many parallels with the evolution of SHV- and TEM-type ESBLs. Unfortunately there are very few epidemiologic data on the geographical spread of OXA-type ESBLs.

In this study we characterize an extended spectrum β -lactamase, designated OXA-4, which was produced by a clinical isolate of *P. aeruginosa*. This isolate was multi drug resistant to all third generation cephalosporin and also to carbapenems.

Materials and Methods

Case History

The patient was 84 years old male diagnosed with cerebrovascular accident and hypertension. An isolate of *P. aeruginosa* was obtained from endotracheal suction tip of the patient, who was on a ventilator.

MIC Testing and Confirmation of ESBL Activity

Organisms were tested by agar dilution method as described in CLSI standard M100-S15. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used for quality control. The double disk synergy test for the detection of ESBL activity was performed essentially as recommended by CLSI M100-S15 by screening for synergism between clavulanate (represented by a disk of amoxicillin-clavulanic

acid) and ceftazidime (CTZ), cefotaxime (CTX) [6]. The disks were placed at distance of 15–20 mm (center to center). A potentiation of the inhibitory zones of any of the expanded spectrum β -lactams by clavulanate was considered suggestive of ESBL production.

Minimal inhibitory concentration (MIC) testing was done with CTZ, CTX, ticarcillin alone and in combination with clavulanic acid. MIC was also done with piperacillin, piperacillin–tazobactam, cefoparazone and cefoparazone sulbactam. A reduction in the MIC of three times doubling dilutions was used as a cut-off.

Isoelectric Focusing

Bacteria exponentially growing at 37 °C in Luria–Bertani medium were harvested, and cell-free lysate was prepared by lysozyme and EDTA treatment method [7]. Analytical isoelectric focusing was performed using broad range soluble ampholytes (Biorad) covering a pH range 3.5–10. β -Lactamases were visualized by overlaying gels with filter paper moistened with a 0.25 mg/mL solution of nitrocefin (Oxoid) in 0.1 M sodium phosphate buffer (pH 7.0) containing dimethylsulphoxide (1 % v/v). The intact substrate molecule is yellow, but becomes pink when β -lactam bond is broken, so that the focused bands with β -lactamase activity appear pink on a yellow background.

Molecular Analysis

The presence of *bla* OXA resistance genes was detected by PCR. The oligonucleotide primers used for the PCR assays were as follows, [8].

OXA-F-5'-AGC CGT TAA AAT TAA GCC C-3'
OXA-R-5'-CTT GAT TGA AGG GTT GGG CG-3'.

The product was amplified in 100 μ L reaction mixtures containing 1 μ L of chromosomal DNA, 50 mM KCl, 1.5 mM MgCl₂, 0.1 μ M oligonucleotide primers, 200 μ M deoxynucleoside triphosphates, and 1.25 U of Taq DNA polymerase (Medox). The PCR conditions used were an initial denaturation at 94 °C for 3 min, followed by 32 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for 45 s, elongation at 72 °C for 6 min and it is followed by the final extension at 72 °C for 5 min. The PCR products were run at 1.2 % agarose containing ethidium bromide and visualized under UV trans illuminator.

Direct Sequencing of PCR Products

PCR products were used as templates for nucleotide sequence determination. A fragment of 919 bp of *bla*_{OXA} was amplified. The amplification products were purified with the Ultra Clean PCR purification kit (Mobio

Laboratories) and their nucleotide sequences were sequenced in an automated sequencing machine of ABI3100 Avant (Applied Biosystems, Foster City, CA, USA) at the Medical Research Foundation, Tamilnadu, India. Nucleotide sequence determinations were performed on both DNA strands and on two independently generated amplimers.

Nucleotide Sequence Accession Number

The DNA sequence and deduced amino acid sequence of OXA-4 has been deposited in GenBank and assigned the accession number EU380316.

Results and Discussions

Nosocomial infections with *Enterobacteriaceae* expressing ESBLs have become widespread. It is important to detect *Pseudomonas* strains producing ESBLs in a clinical laboratory and to differentiate them from depressed mutants, because they can be responsible for the spread of resistance genes in hospital. However, difficulties have been noted in trying to detect ESBL production in *P. aeruginosa* over-expressing chromosomally mediated cephalosporinase.

Strains of *P. aeruginosa* producing OXA-4 ESBL has been reported in very few area throughout the world. This study demonstrates the presence of extended-spectrum β -lactamase resistance strain of *P. aeruginosa* recovered from endotracheal aspirate obtained from a patient on a ventilator. The isolate was resistant to oxyimino-cephalosporins and cephamycins. The patient had received several courses of CTZ and CTX therapy that provided the selective pressure for the emergence of resistance. The increase in CTZ MIC's and the MIC's of other cephalosporins was due to the acquisition of OXA gene.

MIC Testing and Confirmation of ESBL Activity

When disks were placed 15–20 mm apart, a synergistic effect was observed, due to complete resistant of the isolate to β -lactam antibiotics alone and in combination with β -lactam inhibitor like clavulanic acid (Fig. 1). In addition to β -lactam resistance, the isolate was resistant to aminoglycosides including gentamycin, amikacin and netilmycin. The isolate was also found to be resistant to cefepime, a fourth generation cephalosporin and imipenem, carbapenem. To confirm the significance, MIC was also done for the same. In this study, MIC was done with CTZ, CTX, TIC, PIP and CFP alone as well as in combination with β -lactam inhibitor. Bert et al. [9] in 2005 have also reported MICs with a similar set of antibiotics. Even though there are no specific guidelines for these antibiotics in the CLSI

Fig. 1 Detection of ESBL production using disc method. Increase in zone towards the clavulanic acid was observed

document, (which deals only with CTX, CTZ alone and in combination with clavulanic acid) we have taken the reading as >3-fold drop in MIC with the combination antibiotics as ESBL producers.

The MIC's of ceftazidime (>1,024 $\mu\text{g/mL}$) was not much higher than those of cefotaxime (1,024 $\mu\text{g/mL}$). The MIC's for both the third generation cephalosporin with combination of clavulanic acid doesn't show much difference. The MIC readings are 1,024 and 512 $\mu\text{g/mL}$. The presence of clavulanic acid has not much lowered the MIC's of ticarcillin and piperacillin, susceptibility of the isolate to these antibiotics was not fully restored. The MIC's of cefoparazone and cefoparazone-sulbactam are >1,024 and 1,024 $\mu\text{g/mL}$, respectively. These results showed in association with the production of OXA ESBL and the chromosomal group β -lactamase was over produced.

Most of the authors in India have used phenotypic methods like disk diffusion, MIC for reporting or identifying the ESBL producers. In South India, Subha and Ananthan [10] of Chennai in 2002 have reported 6.6 % ESBL prevalence among *Klebsiella pneumoniae* from children using DDST method. Jain et al. [11] from North India reported that 86.6 % of *Klebsiella* spp., 73.4 % of *Enterobacter* spp., and 63.6 % of *Escherichia coli* strains from cases of neonatal septicemia to be ESBL producers in 2003 using DDST method. Menon et al. [12] of Chennai in 2006 reported the occurrence of ESBL producers in the *Enterobacteriaceae* isolates as 28.5 % among *Enterobacter* spp., 21.2 % among *Klebsiella* spp., and 19.2 % among *E. coli* using double disc and three dimensional methods. Taneja et al. [13] reported on a selected group of uropathogens and identified 36.5 % of isolates, as ESBL producers using DDST method.

Iso-Electric Focusing

The different β -lactamases produced by R (resistant) factors can be easily recognized by analytical isoelectric focusing, this feature can be used in epidemiological studies for identifying the enzyme responsible for the acquired resistance of a strain [14]. But with isoelectric focusing it was impossible to say whether an enzyme was chromosomally or plasmid mediated unless the necessary genetic experiments had also been done to prove this. In our study the isolate producing OXA-4 ESBL was analyzed for its β -lactamase content. The pink band was produced at 7.2 (OXA-4). No additional band of activity was detected with chromogenic cephalosporin as substrate.

Identification of OXA-4-Encoding Gene by PCR and DNA Sequencing

By using OXA specific primers, a DNA fragment of 919 bp was amplified from the genomic DNA sample of the isolate. Nucleotide sequencing of the OXA PCR products of the strain with the pI 6.8 showed that they represent the amplified genes coding for the OXA-4 enzyme as described by Medeiros et al. [15] (Figs. 2, 3). In our results sequence analysis revealed that the OXA gene showed 100 % similarities (Fig. 4) with OXA-4 which was previously reported from *E. coli* in Brazil, in the year 1985.

Sequence Analysis of the OXA-4 β -Lactamase Gene in *P. aeruginosa*

The OXA-4 gene comprises an initiation codon ATG (positions 51 to 53) and the stop codon TAA (positions

Fig. 2 Agarose gel picture for the amplified OXA β -lactamase gene. Lane M 1 kb marker, Lane 1 *P. aeruginosa* isolate positive for OXA gene, Lane 2–5 clinical isolate negative for OXA gene

Fig. 3 Zymogram developed after IEF of crude extracts from ESBL positive OXA strain. Presence of *pink spots* indicates the beta-lactamase activity. (Color figure online)

871–873), which translates into a protein of 275 amino acids (Fig. 4) showing the homology to Ambler class D (Bush group 2d) oxacillin-hydrolyzing enzymes. Within this protein, two highly conserved motifs, serine–threonine–phenylalanine–lysine tetrad (S–T–F–K) was found at positions 72–75; and lysine–threonine–arginine (K–T–G) was found at positions 216–218; it included the conserved serine and lysine residues characteristic of β -lactamases possessing a serine active site. The third motif was tyrosine–glycine–asparagine (Y–G–N) at positions 145–147.

In our isolate, the DNA sequencing product was compared with the previously identified known OXA-4 β -lactamase sequences [15]. After analyzing, the DNA sequence was translated into protein sequence by BLAST to identify the similarities. In the DNA sequence analysis at 48th position “A” was present in the previously identified OXA-4. But in our isolate instead of “A”-purine “G”-pyrimidine was present (Fig. 4). The change took place in the area followed by the forward primer sequence. Even though a purine was present instead of pyrimidine in the DNA sequence, it doesn’t play a significant role. Because,

Fig. 4 Nucleotide and protein sequence of the coding region of OXA-4 β -lactamase in *Pseudomonas aeruginosa* in comparison to those of previously identified OXA-4 in *E. coli*, OXA-31, OXA-30. Nucleotide differences in other *bla*_{OXA-4} was marked and indicated above. The underlined nucleotide sequence represents the primers OXA-F and OXA-R used for the PCR amplification and DNA sequencing

OXA-4	1	<u>ag ccg tta aaa tta agc cct</u>	tta cca aac caa tac aaa cca	41
42		ata ctt gtt atg	aaa aac aca ata cat atc aac ttc gct att	83
			M K N T I H I N F A I	11
84		ttt tta ata att gca aat att atc tac agc agc gcc agt gca		125
		F L I I A N I I V S S A S A		25
126		tca aca gat atc tct act gtt gca tct cca tta ttt gaa gga		167
		S T D I S T V A S P L F E G		39
168		act gaa ggt tgt ttt tta ctt tac gat gta	tcc aca aac gct	209
		T E G C F L L Y D V	S T N A	53
210		gaa att gct caa ttc aat aaa gca aag tgt gca acg caa atg		251
		E I A Q F N K A K C A T Q M		67
252		c (OXA-31) gca cca gat tca act ttc aag atc gca tta tca ctt atg gca		293
		A P D S T F K I A L S L M A		81
294		ttt gat gcg gaa ata ata gat cag aaa acc ata ttc aaa tgg		335
		F D A E I I D Q K T I F K W		95
336		gat aaa acc ccc aaa gga atg gag atc tgg aac agc aat cat		377
		D K T P K G M E I W N S N H		109
378		aca cca aag acg tgg atg caa ttt tct gtt gtt tgg gtt tcg		419
		T P K T W M Q F S V V W V S		123
420		caa gaa ata acc caa aaa att gga tta aat aaa atc aag aat		461
		Q E I T Q K I G L N K I K N		137
462		tat ctc aaa gat ttt gat tat gga aat caa gac ttc tct gga		503
		Y L K D F D Y G N Q D F S G		151
504		gat aaa gaa aga aac aac gga tta aca gaa gca tgg ctc gaa		545
		D K E R N N G L T E A W L E		165
546		agt agc tta aaa att tca cca gaa gaa caa att caa ttc ctg		587
		S S L K I S P E E Q I Q F L		179
588		cgt aaa att att aat cac aat ctc cca gtt aaa aac tca gcc		629
		R K I I N H N L P V K N S A		193
630		ata gaa aac acc ata gag aac atg tat cta caa gat ctg gag		671
		I E N T I E N M Y L Q D L E		207
672		aat agt aca aaa ctg tat ggg aaa act ggt gca gga ttc aca		713
		N S T K L Y G K T G A G F T		221
714		gca aat aga acc tta caa aac gga tgg ttt gaa ggg ttt att		755
		A N R T L Q N G W F E G F I		235
756		ata agc aaa tca gga cat aaa tat gtt ttt gtg tcc gca ctt		797
		I S K S G H K Y V F V S A L		249
798		aca gga aac ttg ggg tcg aat tta aca tca agc ata aaa gcc		839
		T G N L G S N L T S S I K A		263
840		aag aaa aat gcg atc acc att cta aac aca cta aat tta taa		881
		K K N A I T I L N T L N L		276
882		aaa atc taa tgg caa aat <u>cgc cca acc ctt caa tca ag</u>		919

Since OXA-4 is a β -lactamase enzyme, the DNA sequence was translated into protein to confirm its significance. The translated protein was compared with the known previously identified OXA-4 sequence. The similarities between our sequence and previously identified sequence showed 100 % similarities. Even though there was a mutational change at the 48th position, the translation starts only from 50th position. The start codon ATG was present at 50th to 52nd position. So the protein identity was 100 % similar to the previously identified OXA-4 sequence.

OXA-4 is a plasmid mediated enzyme which was reported by Medeiros et al. in 1985 in *E. coli*. [15] They reported OXA-4 which resembled OXA-1 in substrate profile and molecular weight but was recognized by a 0.1 pH unit difference in iso-electric point. It also differs in reactions with inhibitors and in the linearity of its penicillin hydrolysis rate. OXA-4 and OXA-7 was reported in *E. coli* strains from Sao Paulo, Brazil, as was OXA-30, an OXA-1 derivative originally isolated in Hong Kong [16].

The OXA-4 type β -lactamase has been detected in *P. aeruginosa* isolates in 1999 by Marumo et al. [17]. They reported that OXA-4 β -lactamase is functionally very similar to the OXA-1 β -lactamase, since the two amino acid differences between these two enzymes are not related to substrate specificity. In Argentina in the year 2003, only one of the 65 isolates harbored a novel orf513-bearing class 1 integron that was characterized in a *Citrobacter freundii* isolate and was termed In38, with the *aacA4-bla_{OXA-4}* cassette array within the variable region [18]. In 2005 *qnr* sequence gene was reported in In4 family class 1 integrons of original structure, down stream of orf513 and upstream from another resistance gene (*dfrA3b*) [19]. The presence of OXA-4 in our isolate is not plasmid mediated enzyme. PCR was done with the chromosomal DNA.

Five distinct groups of oxacillinases have been described by Sanschgrin et al. [20]. Among that OXA group III β -lactamases include OXA-1 and its derivatives OXA-4, OXA-30 and OXA-31. In this study, the deduced amino acid sequence belongs to Ambler class D β -lactamases, since an active-site serine and two other structural elements characteristic of class D β -lactamases were found. Protein alignments with other class D β -lactamase amino acid sequences showed that this ESBL had greatest percentage of sequence identity with two oxacillinase, OXA-31 and OXA-30. In OXA-31 proline was present instead of alanine at the position of 68 and in OXA-30 Alanine was reported at the position 49 instead of valine at OXA-4.

OXA enzymes, which are the only ESBLs of class D, represent another relatively prevalent ESBL family. TEM, SHV and OXA ESBLs are structural derivatives of acquired, so-called broad-spectrum β -lactamases, such as TEM-1, TEM-2 and SHV-1 (all Bush subgroup 2b), and OXA-2 and OXA-10 (subgroup 2d), respectively. The

presence of OXA-type enzymes in *Enterobacteriaceae* has been described infrequently. OXA-1, OXA-2, OXA-10, and OXA-17 were previously reported in some parts of the world [21].

First report describing a VEB-1 like gene in *P. aeruginosa* was reported from the Indian subcontinent by Aubert et al. [22] in the year 2004. The first SHV-11 ESBL-producing *S. dysenteriae* strain was recorded at New Delhi in India during 1999 [23]. A total of 23 clinical isolates (15 *E. coli* and 8 *K. pneumoniae*) recovered during 2002 and 2003 were found to be resistant to CTX, CTZ. They were further investigated for CTX-M genotyping by PCR. PCR revealed the presence of CTX-M type ESBLs in 19 isolates. Sequencing of 4 isolates revealed the presence of CTX-M-15. This was the first report identifying CTX-M type ESBL from clinical isolates of *E. coli* and *K. pneumoniae* from South India [24]. Grover et al. [25] of New Delhi in 2006 reported the prevalence of TEM and SHV in ESBL producing Gram-negative bacteria to be up to 88.8 %. Sekar et al. [26] of Chennai reported that 44.4 % of *E. coli* and 35.29 % *K. pneumoniae* strains were found to be positive for *bla_{CTX-M}* gene by PCR in 2006. Lal et al. [27] of New Delhi (2007) stated that TEM gene was present in 19 (20 %), SHV in 8 (8.4 %) and both TEM and SHV genes were present in 67.3 % (64) of the selected isolates.

As of date there are no reports of OXA type beta lactamases from the Indian subcontinent. This is the first report in India describing OXA-4 ESBL from *P. aeruginosa*. *Emphasis>*

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