

Original Research Article

Molecular identification of TEM-116 beta-lactamase gene in isolates of pathogenic *Pseudomonas aeruginosa*: A first report from Pakistan

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Abstract

Purpose: Purpose: To determine TEM-116 beta-lactamase gene prevalence in drug-resistant *Pseudomonas aeruginosa* isolates from Pakistan.

Methods: Sequence analysis of TEM beta-lactamase isolates and their antibiotic susceptibility patterns were carried out. Quantitative bacteriostatic concentrations for commonly used antibiotics were measured against TEM-116 beta-lactamase isolates.

Results: Among the 102 isolates of *P. aeruginosa*, 23 (22.5 %) were TEM beta-lactamase producers. Sequence analysis of TEM gene from selected isolates showed homology with TEM-116. Two mutations at positions 84 (p.Val84Ile) and 184 (p.Ala184Val) were documented. The TEM-116 isolates exhibited 100 % resistance to sulphamethoxazole/trimethoprim, amoxicillin/clavulanic acid and doxycycline, but showed sensitivity to levofloxacin, norfloxacin and amikacin. One TEM-116 *P. aeruginosa* (PA11) isolate was resistant to all available antibiotics.

Conclusion: These results reveal increased antibiotic resistance in the TEM-116 *P. aeruginosa* isolates studied, a phenomenon which will be helpful in understanding the molecular mechanisms of antibiotic resistance in *P. aeruginosa*.

Keywords: *P. aeruginosa*, Clinical isolates, Sequencing, TEM-116, Antibiotic susceptibility

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INTRODUCTION

P. aeruginosa is the leading cause of nosocomial infections in immuno-compromised patients in hospitals. The frequency of *P. aeruginosa* infections is high in burn patients due to easier access of pathogens [1]. Emergence of antibiotics resistance in *P. aeruginosa* is a major public health concern because the resistant organisms produce a wide range of beta lactamases. Production of extended spectrum beta lactamases (ESBLs) is the principle

mechanism involved in coping with antibiotics by *P. aeruginosa* [2]. The recent increase in ESBLs production by *P. aeruginosa* is alarming in developing countries due to non-judicious use/abuse of antibiotics. This practice engenders new modes of resistance while augmenting the prevalence of drug-resistant bacteria [3-5]. Among the ESBLs, TEM-1 beta-lactamase was first identified in *E. coli* and later-on in *P. aeruginosa* [6,7]. TEM-1 is the most commonly encountered beta-lactamase in Gram-negative bacteria. Up to 90 % of ampicillin resistance in *E.*

coli is due to the production of beta-lactamase by TEM-1 gene. TEM-2, the first derivative of TEM-1, has a single amino acid substitution from the original beta-lactamase [8,9]. Characterization of beta-lactamases in drug-resistant *P. aeruginosa* will be crucial in the study of mechanisms of molecular pathogenesis and virulence.

The present study was undertaken to screen and identify TEM beta lactamases among clinical isolates of *P. aeruginosa* in Pakistan. Moreover, antibiotic susceptibility was performed in TEM producing isolates.

EXPERIMENTAL

The study was conducted at the Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Kohat, Pakistan. Samples totalling 102 were collected from a tertiary care hospital, Peshawar, Khyber Pakhtunkhwa, Pakistan and aseptically transported to the research laboratory.

Pure culture of *P. aeruginosa* isolates

In our previous study, a total of 102 isolates were identified as *P. aeruginosa* on the basis of phenotypic and species specific PCR [10]. Pure cultures of *P. aeruginosa* were processed for this study. All the isolates were re-confirmed by biochemical assays using standard protocols.

Molecular detection of TEM beta-lactamase in *P. aeruginosa*

TEM gene amplification was checked in all 102 isolates of *P. aeruginosa*. Primers used were as follows: TEM forward 5'-TCGGGAAATGTGCG-3' and TEM reverse 5'-TGCTTAATCAGTGA GGCACC-3' primers [8]. Ready PCR mixture contained 10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.5 mM DDT, glycerol, Gelatin, NP-40, 0.05 mM Tween-20, 0.2 mM dNTPs and Taq DNA polymerase. 1 µL genomic DNA was used for every reaction in a total volume of 25 µL. The following PCR conditions were used for TEM gene amplification of *P. aeruginosa*: initial denaturation at 95 °C for 5min followed by 35 cycles, 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and final extension for 7 min. The amplified product was separated on agarose gel and visualized under UV trans-illuminator.

Sequencing of amplified TEM gene and bioinformatics analysis

Amplified PCR product was sequenced using forward primer for the TEM gene. Sequencing

reactions were performed by MacroGen (Seoul) Korea. Online available Bioedit software was used for sequence analysis. Amino acid sequence of TEM was analysed on translation tool (www.expasy.org/) and was matched with all previously reported TEM genes [11].

Antibiotic susceptibility assay of TEM-116 beta-lactamase-producing *P. aeruginosa*

Antibiotic sensitivity pattern of TEM-116 beta-lactamase producing *P. aeruginosa* isolates were determined by modified Kirby-Bauer method [12]. A 0.5 McFarland dilution of refreshed bacterial isolate was inoculated on Mueller Hinton agar (MHA). Antibiotic discs (Oxoid, UK) including amoxicillin/clavulanic acid (AMC), aztreonam (ATM), ceftazidime (CAZ), cefepime (FEP), imipenem (IPM) ceftriaxone (CRO), amikacin (AK), doxycycline (DO), gentamicin (CN), ciprofloxacin (CIP), norfloxacin (NOR), levofloxacin (LEV), sulphamethoxazole/trimethoprim (SXT), sulzone (SCF) and chloramphenicol (C) were placed on MHA plates and then incubated overnight. After incubation, zones of inhibition were measured and interpreted in accordance with Clinical Laboratory Standard Institute (CLSI) guidelines [13].

E-test

Quantitative minimum inhibitory concentrations (MICs) of the selected antibiotics (including ciprofloxacin, ceftriaxone, gentamicin and amikacin) against TEM 116 beta-lactamase-producing *P. aeruginosa* were evaluated by E-test in accordance with manufacturer's instructions (AB Biodisk, Solna, Sweden).

RESULTS

Among the 102 *P. aeruginosa* isolates, the amplified product of 972 bp corresponding to TEM gene size was found in 23 isolates (22.5 %) (Figure 1).

TEM amplified gene product was processed for sequencing. When nucleotide sequence was translated into amino acids, it showed 100 % amino acid sequence homology with TEM 116. In chromatogram the nucleotides at position c.250G>A and c.551C>T, refer to the substituted nucleotides and p.Val84Ile and p.Ala184Val refer to the amino acids substitutions at these two positions in comparison to the original TEM 1 gene sequence (Figure 2).

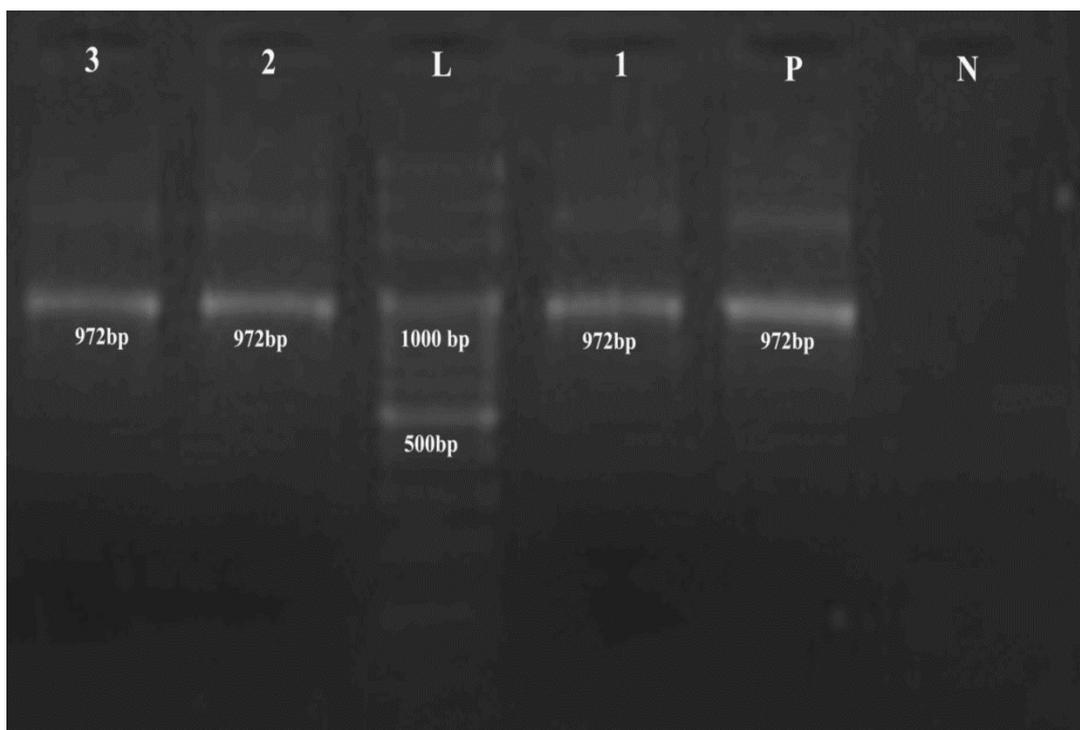


Figure 1: Detection of TEM beta-lactamase gene in *P. aeruginosa*: TEM gene (972bp) was specifically amplified and resolved on 1.8 % agarose gel. 1, 2 and 3 in the gel indicated TEM gene amplification. L: Lader, P: Positive control, N: Negative control

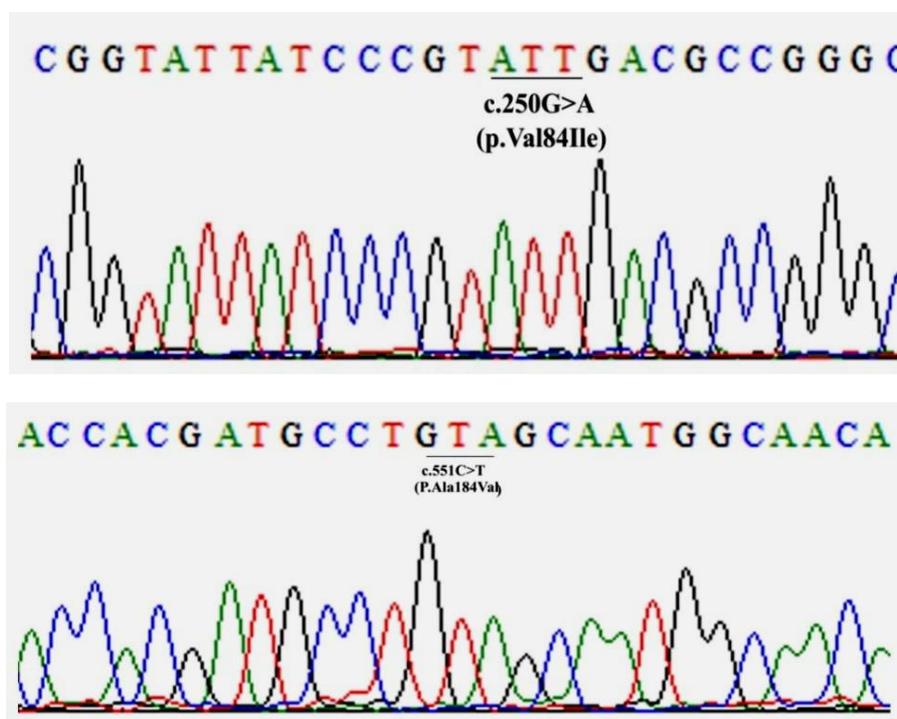


Figure 2: Chromatogram of the sequenced data showing mutation in nucleotides at positions 250 and 551 for the corresponding amino acids Ile and Val in TEM 116

BLAST analysis of the translated amino acids sequence with TEM 1, showed two mutations that include replacement of valine by isoleucine at position 84, and replacement of alanine by valine at position 184 (Table 1). The two

mutations at positions 84 and 184 confirmed the presence of TEM-116 when used online tool (<http://www.laced.uni-stuttgart.de>).

Table 1: Amino acid substitutions of TEM-116 beta-lactamase-producing *P. aeruginosa*

Beta lactamase	Residue (triplet code) at amino acid:	
	84	184
TEM-1	Val (GTT)	Ala (GCA)
TEM-116	Ile (ATT)	Val (GTA)

After analysis of sequence homology, the TEM-116 beta-lactamase producing isolates were processed for antibiotic susceptibility pattern. The TEM-116 isolates showed 100 % resistance (n = 7) to sulphamethoxazole/trimethoprim, amoxicillin/clavulanic acid and doxycycline. However they showed increased sensitivity to levofloxacin, norfloxacin and amikacin. One isolate, PA11 showed resistance to all the antibiotics used in the study (Table 2).

MICs were determined to assess the quantitative inhibitory concentration of commonly used antibiotics against TEM producing *P. aeruginosa*. It was found that MIC of ceftriaxone was >512 µg/ml against three isolates (PA5, PA9 and PA11) while PA11 isolate was found to be the most resistant among all TEM-116 producing isolates (Table 3).

DISCUSSION

Resistance to beta-lactam antibiotics in communities and hospitals has created a major health problem leading to increased mortality, morbidity and high health care expenses.

Production of extended spectrum beta-lactamases (ESBLs) in bacteria is the principle mechanism of resistance against beta-lactam antibiotics. Among ESBLs, TEM beta-lactamase family has variable diversity, prevalence and global distribution [11]. In TEM family, TEM-116 was first reported among Enterobacteriaceae in Korea [14] and in *P. fluorescens* [15]. One study has documented co-production of TEM-116 and SHV-2a in *P. aeruginosa* isolates in France [16]. In the present study, TEM-116 showed two point mutations which cause amino acid substitution from valine to isoleucine at position 84 and alanine to valine at position 184. Similar mutations in TEM 116 were also reported in *P. fluorescens* [15].

When antibiotic susceptibility pattern was checked, it was found that TEM 116-producing *P. aeruginosa* isolates exhibited maximum resistance to sulphamethoxazole/trimethoprim, amoxicillin/clavulanic acid and doxycycline. In several studies, ESBLs and TEM 116 isolates were highly resistant to commonly available antibiotics [16-18]. These reports are in line with our findings. Moreover TEM-116 producers in the current study exhibited sensitivities to levofloxacin, norfloxacin and amikacin, which indicate effective bacteriostatic activity against the TEM-116 isolates. When bacteriostatic activities of selected antibiotics including ciprofloxacin, ceftriaxone, gentamicin and amikacin were checked, they showed similar variable activities as reported earlier [17].

Table 2: Antibiotic susceptibility pattern of TEM 116 beta-lactamase-producing *P. aeruginosa*

Isolates	LEV	IPM	CRO	CAZ	C	C	CIP	SX	SC	AMC	NOR	AK	DO	FE	ATM
					N	N		T	F				P		
PA1	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R
PA2	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
PA3	S	S	S	S	R	S	S	R	S	R	S	S	R	S	S
PA5	R	S	R	R	R	R	R	R	S	R	R	S	R	R	R
PA9	S	R	R	R	R	S	S	R	I	R	S	S	R	R	R
PA11	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
PA20	S	I	R	R	R	R	R	R	R	R	S	R	R	R	R

R; resistance, I; intermediate, S; sensitive

Table 3: MIC of selected antimicrobial agents against TEM 116 producing *Pseudomonas aeruginosa* isolates

Isolate	Antibiotic MIC (µg/ml)			
	Ciprofloxacin	Ceftriaxone	Gentamicin	Amikacin
PA1	≤8	16	128	64
PA2	32	64	128	128
PA3	1	16	≤8	≤8
PA5	128	>512	>512	256
PA9	32	>512	≤8	≤8
PA11	>512	>512	>512	>512
PA20	≤0.5	32	128	16

Among these isolates (PA11) showed resistance to all the antibiotics used in the study. The possible reason might be the presence of full spectrum of beta lactamases and other virulence factors in this isolate (PA11).

CONCLUSION

In the present study, increased antibiotic resistance was reported in the TEM-116 *P. aeruginosa* isolates from Pakistan. These findings are considered beneficial in understanding the mechanism(s) involved in development of antibiotic resistance in *P. aeruginosa*.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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