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Emergence of integron borne PER-1 mediated extended spectrum cephalosporin resistance among nosocomial isolates of Gram-negative bacilli

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***Background & objectives*: *Pseudomonas* extended resistant (PER) enzymes are rare type of extended-spectrum beta lactamases (ESBLs) that confer third generation cephalosporin resistance. These are often integron borne and laterally transmitted. The aim of the present study was to investigate the emergence of integron borne cephalosporin resistant PER-1 gene in diverse incompatibility (Inc) group plasmids among Gram-negative bacteria.**

***Methods*: A total of 613 consecutive, non-duplicate, Gram-negative bacteria of *Enterobacteriaceae* family and non-fermenting Gram-negative bacteria were isolated from different clinical specimens during a period of 18 months. For amplification and detection of $bla_{PER,}$ multiplex PCR was done. For understanding the genetic environment of $bla_{PER-1,}$ integrase gene PCR and cassette PCR (59 be) was performed. Gene transferability experiment was carried out and PCR based replicon typing was performed for incompatibility group typing of plasmids using 18 pairs of primers. An inhibitor based method was used for phenotypic detection of intrinsic resistance.**

***Results*: Multiplex PCR and sequencing confirmed that 45 isolates were harbouring $bla_{PER-1.}$ Both class 1 and class 2 integrons were observed among them. Integrase and cassette PCR (59 be) PCR results confirmed that the resistant determinant was located within class 1 integron. Transformation and conjugation experiments revealed that PER-1 was laterally transferable and disseminated through diverse Inc plasmid type. Efflux pump mediated carbapenem resistance was observed in all isolates. All isolates belonged to heterogenous groups.**

***Interpretation & conclusions*: This study demonstrates the dissemination of cephalosporins resistant, integron borne bla_{PER-1} in hospital setting in this part of the country and emphasizes on the rational use of third generation cephalosporins to slow down the expansion of this rare type of ESBL gene.**

Key words ESBLs - gene cassette - Gram-negative - Inc group - integron - PER - plasmids

Pseudomonas extended resistant (PER) beta-lactamase belongs to class A extended spectrum beta-lactamase (ESBL), which was first detected in France in *Pseudomonas aeruginosa* isolated from a Turkish patient in 1993[1]. The $bla_{\text{PER-1}}$ gene has been detected in *P. aeruginosa*[2], *Acinetobacter* spp.[3], *Proteus mirabilis*[4], and in *Salmonella enterica* serovar Typhimurium[5]. It has been reported that the genetic location of $bla_{\text{PER-1}}$ is either on the chromosome or on the plasmid[1,6]. Integrons also act as small mobile elements which carry and transfer antibiotics resistant genes. The prevalence of integron borne PER-1 is very high because these carry different antibiotics resistant genes in their gene cassettes[7-10]. This determinant is often carried by transposable elements which are responsible for their lateral spread among diverse group of bacterial pathogens[2].

This study was carried out to determine the occurrence of cephalosporin resistant, integron borne $bla_{\text{PER-1}}$ and its transmission dynamics in Gram-negative bacteria.

Material & Methods

Bacterial isolates: A total of 613 consecutive, non-duplicate, Gram-negative bacteria consisting of members of *Enterobacteriaceae* family and non-fermenting Gram-negative bacteria were isolated from different clinical specimens during a period of 18 months (November 2012 to April 2013) from different wards/clinics of Silchar Medical College and Hospital, Assam, India. The work was performed in the department of Microbiology, Assam University, Silchar, Assam. All the organisms were subcultured on MacConkey agar (Hi-Media, Mumbai, India).

Phenotypic detection of ESBL: All isolates were subjected to preliminary screening on Mueller-Hinton (M-H) agar plates containing two antibiotics namely cefotaxime and ceftazidime at 1 µg/ml[11]. All positive isolates were confirmed by combined disc diffusion method using cefotaxime (30 µg) and ceftazidime (30 µg) alone and in combination with clavulanic acid (10 µg) (Hi-Media, Mumbai, India) as per Clinical and Laboratory Standards Institute (CLSI) recommendation[11].

Molecular characterization bla_{ESBL} genes by multiplex PCR: For amplification and characterization of bla_{ESBL} genes, a set of five primers were used namely: bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV}, $bla_{\text{OXA-2}}$, and bla_{PER}[12]. Reactions were run under the following conditions: initial denaturation at 94°C for five min, 33 cycles of 94 °C for 35 sec, 51°C for one min, 72°C for one min and final extension at 72°C for seven min.

Sequencing of bla_{ESBL}: PCR product was purified by Gene Jet PCR purification kit (Thermoscientific, Lithuania). In brief, 30 µl of purified products were used for sequencing along with 20 µl of bla_{ESBL} gene primers[12] (10 pmol each primers). Sequence results were analyzed using BLAST suite program of NCBI (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

Characterization of integrons by integrase gene PCR: For characterization of class 1 and class 2 integrons, integrase gene PCR assays were performed by using primers Int1F (CAG TGG ACA TAA GCC TGT TC), Int1R (CCC GAG GCA TAG ACT GTA) and Int2F (TTG CGA GTA TCC ATA ACC TG), Int2R (TTA CCT GCA CTG GAT TAA GC)[13]. Reactions were run under the following conditions: initial denaturation at 94°C for five min, 32 cycles of 94 °C for 35 sec, 51°C for one min, 72°C for one min and final extension at 72°C for seven min.

Association of bla_{ESBL} with gene cassette: For study of the association of gene cassette with bla_{ESBL} gene, 59 be cassette PCR was performed. Two PCR reactions were performed, one with HS287 and bla_{PER} reverse, another with HS286 and bla_{PER} forward[12,14]. The amplified PCR products were further sequenced. PCR amplification was performed using 30 µl of total reaction volume. PCR reactions were run under the following conditions: initial denaturation at 95°C for two min, 30 cycles of 95 °C for 20 sec, 52°C for 45 sec, 72°C for one min and final extension at 72°C for five min.

Plasmid preparation: All ESBL positive bacterial isolates were cultured in Luria-Bertani broth (Hi-Media, Mumbai, India) containing 1µg/ml of cefotaxime. Cultures were incubated on shaker incubator overnight at 37°C. Plasmids were purified by Gene Jet plasmid Miniprep kit (Thermoscientific, Lithuania).

Gene transferability of bla_{ESBL} gene by transformation and conjugation: Transformation was carried out using *Escherichia coli* JM107 (Fermentas, USA) as recipient[15]. Transformants were selected on cefotaxime (0.5 µg/ml) containing L-B agar plates. L-B agar control plate was used, with and without cefotaxime 0.5 µg/ml.

Conjugation experiments were carried out between clinical isolates as donors and an streptomycin resistant

E. coli recipient strain B (Bangalore Genei, Bengaluru), Overnight culture of the bacteria was diluted in Luria-Bertani broth and was grown at 37 °C till the OD of the recipient and donor culture reached 0.8-0.9 at A_{600}. Donor and recipient cells were mixed at 1:5 donor-to-recipient ratios and transconjugants were selected on cefotaxime (0.5 µg/ml) and streptomycin (800 µg/ml) agar plates.

Plasmid profiling and incompatibility (Inc) typing of ESBL producers: For plasmid profiling, 1.5 µl of each transformants was used and analyzed by agarose gel electrophoresis (1% agarose, Hi-Media, Mumbai, India).

For detection of incompatibility group plasmid in all bla_{PER} producing strains, PCR based replicon typing was carried out targeting 18 different replicon types, to perform five multiplex and three simplex PCRs to amplify the FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons[16].

Antimicrobial susceptibility and minimum inhibitory concentrations (MIC): Antimicrobial susceptibility was determined by Kirby Bauer disk diffusion method on M-H agar plates[17]. Antibiotics used in this study were listed in Table I**.** MICs of various antibiotics were also determined on Muller-Hinton agar plates containing 2, 4, 8, 16, 32, 64, 128, 256 µg/ml of antibiotics, by agar dilution method according to CLSI guidelines[11].

Table I. Antimicrobial agents used for the study

Antimicrobial agents	Concentration used (µg)
Amikacin	30
Ciprofloxacin	5
Gentamicin	10
Imipenem	10
Meropenem	10
Ertapenem	10
Trimethoprim/sulphomethoxazole	1.25/23.75
Cefopodoxime	10
Cefotaxime	30
Ceftazidime	30
Cefepime	30
Aztreonam	30

Detection of efflux pump activity: Efflux pump activity of the isolates were phenotypically detected by double disc synergy test using meropenem (10 µg) alone and in combination with CCCP (100 mM) (carbonyl cyanide m-chlorophenylhydrazone) (Hi-Media, Mumbai, India) as described earlier[18]. MIC reduction assay was performed with imipenem, meropenem and ertapenem alone and in combination with CCCP at a fixed concentration of 20 µg/ml[19].

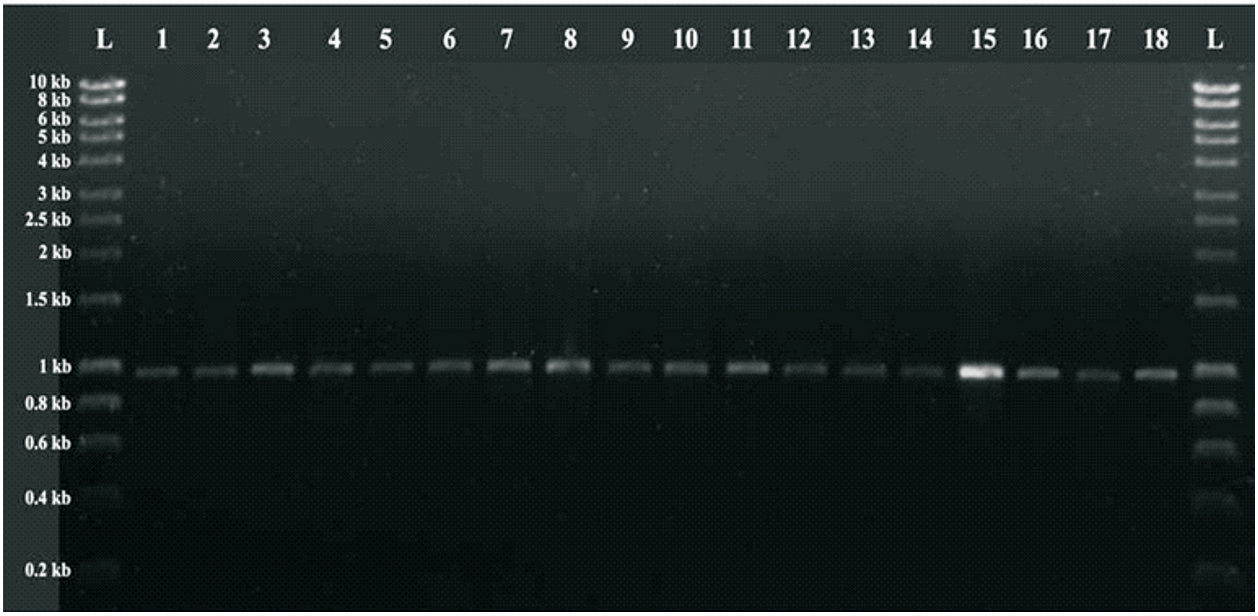

Figure. PCR amplified product of bla_{PER-1}. L: Hyper ladder I (Bioline; UK); 1-18: 920 bp PCR amplified product of bla_{PER-1}.

Table II. Clinical and molecular details of organisms harbouring bla_{PER-1}

Strain ID	Age (yr)	Sex	Wards/OPD	Type of specimen	Organisms	Genotype of beta lactamase	Integron	Replicon type
1.	71	M	Medicine	Urine	*Escherichia coli*	*PER-1*	Class 1	FrepB, N, FIA, FIB, FIC, P
2.	47	M	ICU	Sputum	*Acinetobacter* spp.	*SHV-148, CTX-M-15, TEM-15, PER-1*	Class 1	FrepB, FIA, FIB
3.	32	F	Gynaecology	Urine	*Pseudomonas aeruginosa*	*PER-1*	Class 1	Frep B, FIC, Y, P
4.	20	F	Surgery	Pus	*E. coli*	*TEM-1, PER-1*	Class 1	FrepB, FIA, FIB
5.	70	F	Female Burn Unit	Urine	*E. coli*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 2	I1/Iγ, FIB, Y, FrepB, B/o
6.	17	M	Surgery	Urine	*E. coli*	*CTX-M-15, TEM-1, PER-1*	Class 1	FrepB, FIC
7.	24	M	Medicine	Stool	*E. coli*	*CTX-M-15, PER-1*	Class 1	FrepB, FIA, B/o
8.	45	F	Female Burn Unit	Pus	*Pseudomonas aeruginosa*	*CTX-M-15, SHV-148, PER-1*	Class 1&2	FrepB, FIC, B/o
9.	70	F	Gynaecology	Urine	*Pseudomonas aeruginosa*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 1	FrepB
10.	2	M	Paediatrics	Urine	*E. coli*	*CTX-M-15, TEM-1, PER-1*	--	FIA, FIC, Y, B/o
11.	3 months	F	Paediatrics	Urine	*Pseudomonas aeruginosa*	*CTX-M-15, TEM-1, PER-1*	Class 1	FrepB, FIA, FIB
12.	43	F	Surgery	Pus	*E. coli*	*CTX-M-15, TEM-1, PER-1*	Class 1	FrepB, Y, B/o
13.	55	F	Surgery	Pus	*E. coli*	*CTX-M-15, PER-1*	Class 1	FIB, FrepB, K, B/o
14.	5	F	ENT	Oral swab	*E. coli*	*SHV-148 ,CTX-M-15, PER-1*	Class1	FIA, FIC, FrepB
15.	30 months	M	Paediatrics	Urine	*Pseudomonas aeruginosa*	*CTX-M-15, TEM-1, PER-1*	Class 1	FIA, FIB, FrepB, B/o
16.	50	F	Surgery	Urine	*E. coli*	*TEM-1, PER-1*	Class 1	FIA, FIB, FIC, Y, B/o
17.	34	M	Medicine	Stool	*Proteus mirabilis*	*PER-1*	Class 1	FIA, FIB, FIC, Y, K
18.	40	M	Medicine	Urine	*E. coli*	*CTX-M-15, PER-1*	Class 1	FIB, FrepB, K
19.	70	F	Surgery	Pus	*Pseudomonas aeruginosa*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 2	FIA, FrepB
20.	40	F	Gynaecology	Urine	*E. coli*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 1&2	None with target primers
21.	28	F	Medicine	Urine	*Pseudomonas aeruginosa*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 1	FIA, B/o
22.	10	F	Paediarics	Urine	*Pseudomonas aeruginosa*	*CTX-M-15, TEM-1, PER-1*	Class 1	FrepB, FIA, FIB, P
23.	19	M	Surgery	Urine	*Pseudomonas aeruginosa*	*CTX-M-15, PER-1*	Class 1	FIB, FrepB
24.	48	M	Medicine	Sputum	*E. coli*	*SHV-148, CTX-M-15, PER-1*	Class 1	FrepB
25.	30	F	Medicine	Stool	*E. coli*	*CTX-M-15, PER-1*	Class 1	FrepB, FIA, FIB, FIC, K

Contd...

Strain ID	Age (yr)	Sex	Wards/OPD	Type of specimen	Organisms	Genotype of beta lactamase	Integron	Replicon type
26.	72	M	Medicine	Urine	*E. coli*	*CTX-M-15, PER-1*	--	FIB, FIC, K,
27.	45	M	Medicine	Stool	*E. coli*	*PER-1*	Class 1	None with target primers
28.	25	M	Medicine	Stool	*E. coli*	*PER-1*	Class 2	FIA, FIC, Y, B/o
29.	2 months	M	Paediatrics	Urine	*Pseudomonas aeruginosa*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 1	None with target primers
30.	25	M	ICU	Sputum	*Pseudomonas aeruginosa*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 1	FrepB, FIA, FIC, Y, B/o
31.	3	F	Paediatrics	Urine	*E. coli*	*SHV-148, CTX-M-15, PER-1*	Class 2	FIA, FIB, FIC, Y, B/o
32.	25	M	Orthopedics	Pus	*E. coli*	*PER-1*	Class 1	FrepB, N, FIA, FIB, FIC, Y
33.	23	M	Medicine	Stool	*Pseudomonas aeruginosa*	*CTX-M-15, TEM-1, PER-1*	Class 1&2	FrepB, FIA, FIC, Y, B/o
34.	33	F	Medicine	Stool	*Pseudomonas aeruginosa*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 1	FrepB, FIA, FIC, Y, K, B/o
35.	52	M	Dermatology	Urine	*E. coli*	*PER-1*	Class 2	FIB, FIC, Y,
36.	28 months	F	Paediatrics	Urine	*E. coli*	*PER-1*	Class 1	FrepB, FIB, FIC, Y
37.	55	F	Surgery	Pus	*E. coli*	*SHV-148, PER-1*	Class 1	FIB, K, B/o
38.	10	F	Paediatrics	Urine	*Klebsiella pneumoniae*	*PER-1*	Class 1	FrepB, N, FIA, FIB, W, FIC, Y
39.	29	M	Medicine	Urine	*E. coli*	*SHV-148, CTX-M-15, TEM-1, PER-1*	Class 2	FrepB, Y, B/o
40.	3	F	Paediatrics	Urine	*E. coli*	*PER-1*	--	FrepB, FIA, FIB, FIC
41.	3	M	Paediatrics	Urine	*E. coli*	*PER-1*	Class 1	FIA, FIB, FIC, K,
42.	27	F	Gynaecology	Urine	*Pseudomonas aeruginosa*	*PER-1*	Class 1	FrepB, K, B/o
43.	30	F	Gynaecology	Pus	*E. coli*	*CTX-M-15, TEM-1, PER-1*	--	FrepB, FIA, FIB, FIC, Y, B/o
44.	47	F	Gynaecology	Urine	*Pseudomonas aeruginosa*	*CTX-M-15, PER-1*	Class 1	FrepB, FIA, FIB, FIC, Y, B/o
45.	10 months	F	Paediatrics	Urine	*E. coli*	*PER-1*	Class 1	FrepB, FIA, Y, K

DNA fingerprinting by enterobacterial repetitive intergenic consensus (ERIC) sequences PCR: Typing of all ESBL producing isolates were done by ERIC-PCR, using two primers: ERIC-F and ERIC-R[20].

Results & Discussion

Forty five isolates showed amplification with bla_{PER} primers (Figure, Table II) which were further confirmed by sequencing as bla_{PER-1}. However, 12 isolates were found to carry only $bla_{PER,}$ while in the remaining 33 co-existence of CTX-M-15, SHV-148 and TEM-1 was observed along with bla_{PER-1} (Table II).

Integrase gene PCR results demonstrated that 32 isolates harboured class I, and six isolates carried class II integron. Both class I and class II integrons were found in three isolates (Table II), four isolates were negative for integron. bla_{PER-1} was found to be class 1 integron borne that was further confirmed by sequencing.

Transformation experiment established the fact that bla_{PER-1} was carried within plasmid and was conjugatively transferable in *E. coli* JM 107. PER-1 could be amplified by PCR in both transformants and tranconjugants. Plasmid incompatibility typing of all bla_{PER-1} producers illustrated that there were diverse

Inc groups: N, FIA, FIB, W, Y, P, FIC, F and K were present in all bla_{PER-1} positive isolates (Table II).

All isolates were susceptible to tigecycline (100%) followed by imipenem (95.55%), and less susceptible to meropenem (75%). Other antibiotics like amikacin, gentamicin, ciprofloxacin, co-trimoxazole, cefepime, ceftriaxone and cefoxitin showed moderate to lower activity. A high MIC was noticed against all tested cephalosporins (≥256 µg/ml), and monobactam (≥256 µg/ml). Efflux pump mediated carbapenem resistance was noticed in all 45 isolates. A reduction in MIC was observed in all 45 isolates against imipenem, ertapenem and meropenem when CCCP was added at a fixed concentration of 20 µg/ml. All isolates were clonally non related as observed in ERIC-PCR.

In the current study bla_{PER-1} was found to be disseminated among members of *Enterobacteriacae* and in non-fermenting Gram-negative bacteria that were resistant to multiple groups of antibiotics. In Iran, the prevalence rate of PER-1 was 27.5 per cent in *P. aeruginosa* whereas in *A. baumannii* prevalence rate was 51 per cent due to indiscriminate consumption of antibiotics by patients[7,8].

The present study gives an insight in to horizontal transferability of this resistance determinant through integron gene cassettes in Gram-negative bacteria which has also been reported from other parts of the world[7,9,10]. The most important finding of this study was carriage of bla_{PER-1} within different incompatibility (Inc) groups of plasmids which revealed that multiple carrier vehicles were responsible for expansion of this determinant and probably had diverse source of acquisition. To complicate the situation further, carbapenem therapy is restricted due to efflux pump activity, leaving little therapeutic alternatives. The study highlights the presence of this rare type of ESBL in this part of the country and deserves immediate awareness and controlled use of broad spectrum antibiotics to slow down the maintenance and persistence of this resistant determinant within hospital environment.

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