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Integron-Borne Transmission of VEB-1 Extended-Spectrum β-Lactamase in Pseudomonas aeruginosa in a Tertiary Care Hospital in India

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Integron-Borne Transmission of VEB-1 Extended-Spectrum β-Lactamase in *Pseudomonas aeruginosa* in a Tertiary Care Hospital in India

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A total 14 clinical isolates of *Pseudomonas aeruginosa* that produced VEB-1 and were susceptible only to polymyxin B were recovered from hospitalized patients. VEB-1 was located within variable regions of the class 1 integron, flanked by resistant genes, and was horizontally transferable as well as carried within the IncP-type plasmid. We conclude that the IncP-type plasmid is responsible for the horizontal transmission of VEB-1-mediated expanded-spectrum cephalosporin resistance in this medical center.

VEB-type beta-lactamases are a rare type of enzymes responsible for conferring expanded-spectrum cephalosporin resistance, complicating therapeutic options (1). They are usually integron borne and horizontally transmitted at the intra- and interspecific levels (1). The present study investigates the genetic context of VEB-1 extended-spectrum beta-lactamases (ESBL) in nosocomial isolates of *Pseudomonas aeruginosa*.

A total of 136 isolates that were resistant to at least one of the expanded-spectrum cephalosporins (cefotaxime, ceftazidime, or ceftriaxone) by the agar dilution method and nonduplicates were selected for the study. The isolates were collected for a period of 1 year from January 2012 to December 2012. PCR assays were performed for detection of ESBL genes using bla_{GES}, bla_{OXA-10}, bla_{CTX-M}, bla_{SHV}, bla_{TEM}, and bla_{VEB} primers (2). Class 1 and class 2 integrons were characterized by integrase gene PCRs (3). Gene cassettes of the bla_{VEB} gene were amplified using 5′-CS and 3′-CS primers (3), Products were cloned on the pGEM-T vector (Promega, Madison, WI, USA) and sequenced. Plasmids from the parent strains were extracted using a GeneJET plasmid miniprep kit (Thermo Scientific, Lithuania). The transformation experiments were performed using *Escherichia coli* strain JM107 as the recipient. Transformants were selected on Luria-Bertani agar (Hi-Media, Mumbai, India) plates containing 1 μg/ml of cefotaxime, and all the transformants were subjected to PCR assays for the presence of bla_{VEB}. Plasmid incompatibility typing of all the transformants was done, targeting 18 different replicon types (4), and amplicons were sequenced. Restriction mapping of plasmids was performed using EcoRI, HindIII, and XbaI (Promega, Madison, WI, USA), and digested products were separated by pulse-field gel electrophoresis (CHEF DR III system, Bio-Rad, USA). Antimicrobial susceptibility (Kirby-Bauer disc diffusion) and MICs were determined, using the agar dilution method of the parent strains and transformants with various antibiotics including beta-lactams

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TABLE 1 Clinical and molecular history of bla_{VEB-1}

Serial no.	Sample identification	Age of patient (yr)	Sex of patient	Source (ward/clinic)	Type of clinical specimen	Integron arrangement/ genetic context	Location (Inc type)	Resistance profile[a]
1	153	20	Female	Surgery	Pus	2	P	CPD, AMK, CIP, GEN, CRO, ATM, FOX, FEP
2	182	23	Female	Surgery	Pus	1	P	CPD, AMK, CIP, FEP, CRO, ATM, FOX
3	264	70	Female	Surgery	Pus	2	P	CPD, AMK, CIP, GEN, CRO, ATM, FOX, FEP
4	303	65	Female	Orthopedic	Drain tip	4	P	CPD, AMK, GEN, CRO, ATM, FOX
5	306	55	Male	Surgery	Pus	3	P	CPD, CIP, CRO, ATM, FOX, FEP
6	310	35	Female	Surgery	Pus	1	P	CPD, AMK, CIP, CRO, ATM, FOX, FEP
7	361	40	Male	Surgery	Urine	3	P	CPD, CIP, CRO, ATM, FOX, FEP
8	517	20	Female	Surgery	Pus	2	P	CPD, AMK, CIP, GEN, CRO, ATM, FOX, FEP
9	518	60	Male	Surgery	Pus	3	P	CPD, AMK, CIP, FEP, ATM, CRO, FEP
10	543	80	Male	Medicine	Pus	Not found	P	CPD, FEP, ATM, FOX
11	544	19	Female	Surgery	Pus	2	P	CPD, AMK, CIP, GEN, CRO, ATM, FOX, FEP
12	545	20	Female	Surgery	Pus	2	P	CPD, AMK, CIP, GEN, CRO, ATM, FOX, FEP
13	571	56	Female	Medicine	Urine	Not found	P	CPD, AMK, CPM, ATM, FOX
14	584	17	Female	Medicine	Wound swab	2	P	CPD, AMK, CIP, GEN, CRO, ATM, CEP, FOX, FEP

[a] AMK, amikacin; CPD, cefpodoxime; CIP, ciprofloxacin; FEP, cefepime; CRO, ceftriaxone; ATM, aztreonam; GEN, gentamicin; FOX, cefoxitin.

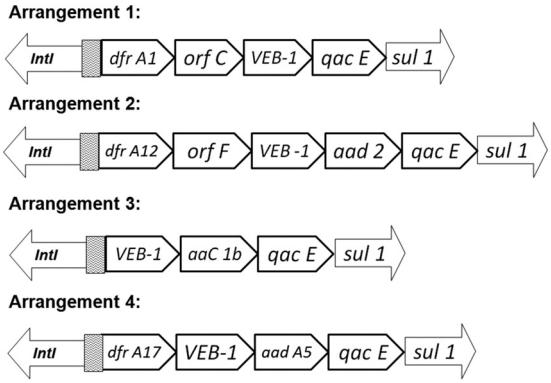

FIG 1 Structural variations of class 1 integron-carrying VEB-1.

and non-beta-lactams (Hi-Media), and results were interpreted according to the CLSI criteria (5). Isolates were typed by repetitive PCRs as described previously (6). All the VEB-1-positive isolates were further tested by the modified Hodge test and the imipenem-EDTA disk test (7) followed by PCR assays to detect production of class A (KPC), class B (NDM, VIM, and IMP), and class D (OXA-23, -24/40, and -58) carbapenemases (7–10).

A total of 14 isolates showed amplification with bla_{VEB} primers, which was further confirmed by sequencing as bla_{VEB-1}, and the rest of the isolates were harboring CTX-M-15 ($n = 5$), GES-5 ($n = 2$), OXA-10 ($n = 4$), SHV-148 ($n = 1$), and TEM ($n = 3$). Most of the VEB-bearing isolates were obtained from the surgery unit followed by the medicine and orthopedic wards and were recovered from pus, drain tip, urine, and wound swab samples collected from patients of different age groups (Table 1). We also confirmed that bla_{VEB-1} was located within the variable regions of class 1 integrons with four types of diverse arrangements (Fig. 1; Table 1). VEB-1 was flanked by *dfrA1* (arrangement 1), *dfrA12* (arrangement 2), and *dfrA17* (arrangement 4) in the upstream region, while in the downstream region *qacE* (arrangements 1, 2, 3, and 4), *aad2* (arrangement 2), and *aadA5* (arrangement 4) were found (Fig. 1). bla_{VEB-1} was successfully transferred on *E. coli* strain JM107 by transformation. PCR assays confirmed that bla_{VEB} was present in all the transformants and was found to be carried within the IncP-type plasmid according to the sequence results for all the transformants (Table 1). Restriction mapping of the plasmids showed similar banding patterns for all the transformants except two. The antimicrobial susceptibility results for the isolates showed that all were susceptible to polymyxin B. The MIC results showed that all the isolates and their transformants have high MIC values for oxyimino cephalosporins and monobactam, for which the inhibitory concentrations ranged from 8 to 512 μg/ml. However, high MIC values for carbapenem drugs were also observed in all the isolates and their transformants (Table 2). DNA fingerprinting with repetitive PCRs showed that 11 different clones harboring bla_{VEB-1} were distributed within the hospital. Carbapenemase activity was observed only by the modified Hodge test. However, none of the clones could be characterized by our target primers.

Horizontally transferable bla_{VEB-1}-mediated expanded-spectrum cephalosporin resistance has been reported from different parts of the world (1, 11–13) and carriage within the integron gene cassette is well established (11–14). However, in India, there has as yet been no report regarding their genetic context and transferability. The presence of this rare type of ESBL with diverse gene cassette arrangements within a single hospital setting signifies its epidemiological importance in terms of its origin, evolution, and source of acquisition and also its clinical implications for antimicrobial policy. In our study, the MIC data for carbapenem indicated that all the isolates and their transformants were not susceptible to carbapenems. Thus, a novel carbapenemase not identified by our primers may be coproduced and cotransferred with VEB-1 and could be contributing to both cephalosporin and carbapenem resistance in these strains.

Our study established transferability, within this hospital, of this resistant determinant through the IncP group plasmid. Thus, the present study is first report from this part of the world showing that the IncP-type plasmid acted as a genetic vehicle for horizontal transfer of VEB-1 cephalosporin resistance. We also established that the surgery ward was the origin for the dissemination of this rare type of enzyme in this hospital.

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We declare no conflicts of interest.

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TABLE 2 MICs of the parent strain versus those of transformants

Sample identification[a]	MIC of indicated antibiotic for the donor and transformants harboring VEB-1[b]:									Sample identification (*bla* gene)	MIC of indicated antibiotic for the strain and transformants with other known β-lactamases:								
	CTX	CAZ	CRO	FEP	IPM	MEM	ETP	ATM	PMB		CTX	CAZ	CRO	FEP	IPM	MEM	ETP	ATM	PMB
153 P	128	128	256	512	16	16	8	512	0.125	154 (OXA-10)	128	64	128	32	2	2	2	64	<0.125
153 TF	128	128	256	512	8	2	2	256	0.125	TF	128	64	128	32	2	2	2	64	<0.125
182 P	256	512	512	256	256	256	256	512	0.25	176 (OXA-10)	128	128	128	128	2	2	<2	256	0.125
182 TF	128	256	128	128	64	32	8	256	0.25	TF	128	128	128	128	2	<2	<2	256	<0.125
264 P	256	512	512	64	4	4	4	32	<0.125	188 (OXA-10)	256	128	256	128	4	2	2	512	0.125
264 TF	128	128	64	64	2	4	2	8	<0.125	TF	256	128	256	128	4	2	<2	512	0.125
303 P	256	256	512	256	256	32	4	512	0.125	283 (OXA-10)	256	256	256	256	4	2	<2	512	0.125
303 TF	128	256	512	256	256	8	2	512	0.125	TF	256	256	256	256	2	2	<2	512	0.125
306 P	16	32	256	256	8	64	8	256	0.125	270 (CTX-M-15)	64	128	128	256	<2	<2	<2	128	<0.125
306 TF	16	16	128	256	4	16	2	128	0.125	TF	64	128	128	256	<2	<2	<2	128	<0.125
310 P	512	512	512	512	512	512	512	512	0.25	430 (CTX-M-15)	64	64	64	128	2	2	<2	128	<0.125
310 TF	512	256	256	128	32	16	4	256	0.25	TF	64	64	64	128	2	2	<2	128	<0.125
361 P	128	16	32	256	64	8	8	64	<0.125	486 (CTX-M-15)	64	64	64	64	<2	<2	<2	128	0.125
361 TF	16	8	32	128	4	16	2	16	0.125	TF	64	64	64	64	<2	<2	<2	128	0.125
517 P	256	256	512	64	512	256	128	32	0.25	551 (CTX-M-15)	64	64	64	64	<2	<2	<2	32	<0.125
517 TF	128	128	256	32	8	16	4	32	0.25	TF	64	64	64	64	<2	<2	<2	32	<0.125
518 P	256	512	512	64	64	8	64	256	<0.125	562 (CTX-M-15)	32	64	256	256	2	<2	<2	128	<0.125
518 TF	64	128	256	16	8	4	2	32	<0.125	TF	32	64	256	256	<2	<2	<2	128	<0.125
543 P	128	16	128	16	128	8	16	32	<0.125	527 (SHV-148)	32	32	256	128	2	<2	<2	256	<0.125
543 TF	128	16	128	8	32	4	4	32	<0.125	TF	32	32	256	128	<2	<2	<2	256	<0.125
544 P	256	16	16	16	512	512	256	16	0.25	8 (TEM)	32	32	32	32	<2	<2	<2	64	<0.125
544 TF	128	16	16	16	16	8	4	16	0.25	TF	32	32	32	32	<2	<2	<2	64	<0.125
545 P	512	128	512	128	64	16	8	256	0.125	87 (TEM)	32	32	32	32	<2	<2	<2	64	<0.125
545 TF	128	64	128	64	8	4	2	16	0.125	TF	32	32	32	32	<2	<2	<2	64	<0.125
571 P	256	256	256	64	8	8	8	512	<0.125	95 (TEM)	32	32	32	128	<2	<2	<2	64	<0.125
571 TF	128	256	64	64	2	2	2	256	<0.125	TF	32	32	32	128	<2	<2	<2	64	<0.125
584 P	256	256	256	256	16	8	8	256	0.125	123 (GES-5)	256	256	256	256	2	2	<2	256	<0.125
584 TF	257	256	128	256	8	8	2	128	0.125	TF	256	256	256	256	2	<2	<2	256	<0.125
E. coli JM107 without plasmid	0.125	<0.125	0.125	<0.125	<0.125	<0.125	<0.125	0.125	<0.125	412 (GES-5)	256	256	512	256	4	2	<2	256	0.125
										TF	256	256	512	256	4	2	<2	256	0.125

[a] P, parent strain; TF, transformant.

[b] CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; IPM, imipenem; MEM, meropenem; ETP, ertapenem; ATM, aztreonam; PMB, polymyxin B.

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Correspondence

A report on the presence of GES-5 extended spectrum beta-lactamase producing *Pseudomonas aeruginosa* associated with urinary tract infection from north-east India

Sir,

Guiana extended spectrum (GES) beta lactamase belongs to molecular class A[1] frequently found in Gram-negative rods by and large in *Pseudomonas aeruginosa* in addition to other members of *Enterobacteriacae*[2]. Till date, only a few studies on epidemiology and environmental burden of GES-type ESBLs have been published[1,2].

We present the occurrence of bla_{GES-5} harbouring *Pseudomonas aeruginosa* isolated from human urine specimen from north-east India.

The study was conducted in the department of Microbiology, Assam University, Silchar, Assam, India, from January to December 2012. The first isolate (AM 328) was obtained from the urine sample of a 4 month old female in May 2012 and the second isolate (AM 438) was recovered from the urine of a 39-year-old male in July 2012 (Table). These patients attended the Out Patient Department of Silchar Medical College and Hospital, Silchar, Assam and diagnosed with urinary tract infection. The selection of the samples was based on the initial screening of isolates for the presence of ESBL[3]. Antimicrobial susceptibility was determined by Kirby Bauer disc diffusion method on Muller-Hinton agar plates[3]. The following antibiotics were used for antimicrobial susceptibility: cefopodoxime (10 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (30 µg), trimithoprim/dulphamethoxazole (1.25/23.75 µg), tigecycline (15 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), ceftriaxone (30 µg), aztreonam (30 µg) and cefoxitin (30 µg). Minimum inhibitory concentrations (MIC) of various antibiotics [cefotaxime, ceftazidime, Ceftriazone, cefepime, imipenem, meropenem, ertapenem and aztreonam (Hi-Media, Mumbai, India] were determined on Muller Hinton agar plates containing 2,4, 8, 16, 32, 64, 128, 256 mg/l of antibiotics, by agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines[3].

Table. Clinical information and molecular details of bla_{GES-5} carrying *P. aeruginosa* urinary isolates

ID. No.	Male/ Female	Age (y)	Integron	Replicon type
AM-328	Female	4	Class-1	I1/Iγ, FIA, FIB, W, FIC, FrepB and K
AM-438	Male	39	Class-1	I1/Iγ, FIA, FIB, W, FIC and FrepB

For amplification and characterization of bla_{ESBL} genes, a set of six primers were used: bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{OXA-2}, bla_{OXA-10} and bla_{GES} as described previously[1]. Reactions were run under the following conditions: initial denaturation 94°C for 5 min, 33 cycles at 94°C for 35 sec, 51°C for one min, 72°C for one min and final extension at 72°C for seven min. PCR product was purified by Gene Jet PCR purification Kit (Thermo Scientific, Lithuania); 30 µl of purified products were used for sequencing along with bla_{GES}. Efflux pump activity of the isolates was phenotypically detected by double disc synergy test[4] using imipenem (10 µg), ertapenem (10 µg) and CCCP (100mM) (carbonyl cyanide m-chlorophenylhydrazone) (Hi-Media, Mumbai, India) as described earlier[4]. MIC reduction assay was performed using imipenem and ertapenem alone and in combination with CCCP at a concentration 20 µg/ml[5]. For detection of class 1 and class 2 integron, integrase gene PCR were performed as described previously[6]. For detection of association of gene cassette with bla_{ESBL} gene, two PCR reactions were carried out, one with HS287 and $bla_{GES-1-B}$, another with HS286 and $bla_{GES-1-B}$[1,7]. The amplified products were further sequenced. Transformation

was carried out using *Escherichia coli* JM107 as recipient. Transformants were selected on Luria-Bertani (L-B) agar (Hi-Media, Mumbai, India) plates containing 0.5 mg/l cefotaxime. L-B agar with and without cefotaxime 0.5 mg/l control plate was used. Conjugation experiments were performed between clinical isolates as donors and a streptomycin resistant *E. coli* recipient strain B (Genei, Banglore). Overnight culture of the bacteria was diluted in L-B broth and was grown at 37 °C till the optical density (O.D.) of the reciepient and donor culture reached 0.8-0.9 at A_{600} absorbance. Donor and recipient cells were mixed at 1:5 donor-to-recipient ratios and transconjugants were selected on cefotaxime (0.5 mg/l) and streptomycin (800 mg/l) agar plates; 1.0 µl of each sample was used for plasmid profiling and analysed by agarose gel electrophoresis (1% agarose, Hi-Media, Mumbai, India). For the detection of incompatibility group type of plasmid in all bla_{GES-5} 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/Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons as described previously[8]. Typing of bla_{OXA-2} harbouring isolates was done by Enterobacterial repetitive intergenic consensus sequences PCR[9].

When characterized genotypically, the first isolate AM 328 possessed multiple beta lactamase genes, harboured *CTX-M*, *SHV* and *GES* while the second isolate AM 438 harboured only single bla_{GES} gene. Sequencing of the PCR product with the GES primers revealed that both the isolates harboured a bla_{GES-5} variant gene. Efflux pump mediated carbapenem resistance was also noticed in both isolates. A sharp reduction in MIC was observed against ertapenem when CCCP was added at a fixed concentration of 20 µg/ml. Class 1 integron was found in both isolates (Table). Sequencing results confirmed that bla_{GES-5} was class 1 integron borne. Both isolates were carrying multiple plasmids. A plasmid of ~ 40 Kb was common in both. Incompatibility typing of the first isolate showed that there was diverse Incompatibility groups Inc groups: I1/Iγ, FIA, FIB, W, FIC, FrepB and K while in the second isolate group I1/Iγ, FIA, FIB, W, FIC and FrepB were found (Table). Transformation was successful with AM-438 where it was found that bla_{GES-5} was located within W Inc type plasmid. However, these were not conjugatively transferable to *E. coli*. The first isolate was found to be susceptible to imipenem and cefoxitin and the second isolate showed suceptibility against imipenem, meropenem and gentamicin. While the first isolate showed MIC for all tested antibiotics as >256 mg/l, the second isolate showed 64 mg/l to carbapenems and monobactam and 32 mg/l to cephalosporins. ERIC PCR result showed that both isolates belonged to diverse clonal types.

The presence of *GES-5* gene has been frequently detected in *E. coli*[10, 11], *Klesbsiella pneumoniae*[12] and *P. aeruginosa*[13,14].

In agreement with the current study, integron mediated GES-5 has been earlier reported from other parts of the Asia[11]. However, in our study, presence of bla_{GES-5} was traced from the community isolate which emphasized the need for epidemiological investigation, origin and evolution of this resistant determinant from this geographical location. Unlike the previous concept[15], the studied isolates were phenotypically susceptible to carbapenem, whereas presence of efflux pump activity was noticed with ertapenem.

In conclusion, the presence of bla_{GES-5} in *P. aeruginosa* is perhaps the first report from this part of India. Presence of this rare type ESBL in community and their presence within plasmid require further investigation for potential transmission dynamics and proper therapeutic alternatives.

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संख्या/No.: 2013-1354(1150-13) 1532

दिनांक/ Dated: 21-3-14

Dear Dr. Bhattacharjee,

Kindly refer to your online submission dated 22.01.14.

Your modified article entitled "**Emergence of integron brone PER-1 mediated extended spectrum cephalosporin resistance among nosocomial isolates of gram negative bacilli"** was considered by the Editorial Board of our Journal and was found suitable for publication subject to editorial corrections and will be scheduled for publication in one of the forthcoming issues of the IJMR.

With kind regards,

Yours sincerely,

(ANJU SHARMA)

Dr. Amitabha Bhattacharjee,
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Assam University,
SILCHAR – 788 011 (Assam).

इंडियन जर्नल ऑफ मेडिकल रिसर्च

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संख्या/No:.....

दिनांक/ Dated:.....22.11.13.

Dear Dr. Bhattacharjee,

Kindly refer to your online submission dated 18.11.13.

Your modified Letter-to-Editor entitled "**First Description of GES-5 extended spectrum beta-lactamase producing *Pseudomonas aeruginosa* associated with urinary tract infection from India"** was considered by the Editorial Board of our Journal and was found suitable in principle for publication subject to editorial corrections and will be scheduled for publication in one of the forthcoming issues of the IJMR.

With kind regards,

Yours sincerely,

(ANJU SHARMA)

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संख्या/No:.....

दिनांक/ Dated:.....27.1.14

Dear Dr. Bhattacharjee,

Kindly refer to your online submission dated 19.11.13.

Your modified article entitled "**Genetic environment of OXA-2 beta-lactamase producing gram negative bacilli from a tertiary referral hospital of India"** was considered by the Editorial Board of our Journal and was found suitable in principle for publication subject to editorial corrections and will be scheduled for publication in one of the forthcoming issues of the IJMR.

With kind regards,

Yours sincerely,

(ANJU SHARMA)

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