4. Results

The present study was conducted in the Department of Microbiology, Assam University, Silchar, Assam, India. The duration of the study was November 2011 to April 2013. The bacterial strains were collected from Silchar Medical College and Hospital, Silchar, Assam. This tertiary referral hospital serves populations of Assam and neighbouring states like North Tripura, Mizoram, Meghalaya and Manipur.

A total of 663 consecutive non duplicate different clinical specimens have been studied during the period which comprises 373 isolates from indoor and 290 isolates from outdoor patients from different wards/OPDs from 643 clinical samples which included members of Enterobacteriaceae family and non fermenting Gram negative rods.

4.1 ESBL status among test isolates

4.1.1 Screening of ESBLs:

Among the 663 test isolates, a total of 494 (74.50%) isolates were suspected to be ESBL producers by screened agar dilution method which were further selected for phenotypic confirmatory test (Figure 17; Table 18).

Table 18: Screening of ESBL producing isolates

Organisms	**With *CTX**	**With **CAZ**	**Total** (n=)
***E. coli* (n=227)**	165	162	173
***Klebsiella pnuemoniae* (n=80)**	58	57	60
***Klebsiella oxytoca* (n=36)**	31	30	36
***Proteus vulgaris* (n=17)**	11	11	12
***Proteus mirabilis* (n=17)**	10	10	10
***Vibrio cholerae* (n=3)**	1	1	1
***Pseudomonas aeruginosa* (n=208)**	155	135	160
***Pseudomonas* Spp. (n=59)**	29	28	29
***Acinetobacter baumanii* (n=16)**	13	13	13
Total (n=663)	473	447	494

*CTX: cefotaxime; **CAZ: ceftazidime

4.1.2 Combined disc diffusion test:

Among all the screened positive isolates, a total of 360 (54.29%) isolates were confirmed as ESBL producing organism by combined disc diffusion method (Figure 18; Table 19). Prevalence rate was high in *K. oxytoca* (86.11%), followed by *E. coli* (55%), and *P. eruginosa* (62.01%) while it was lowest in K. *pneumonaie* (42.5%). The frequency of ESBL producers among hospital and community isolates were 61.08% (270/373) and 36% (90/290) respectively with their distribution ratio of 7:3 (Figure 19 and 20).

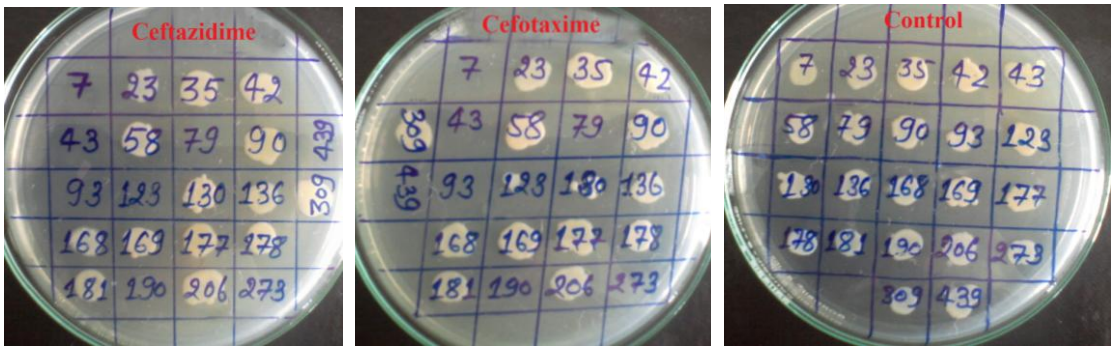


Figure 17: Screening of ESBLs on Mueller Hinton Agar by Agar dilution method (cefotaximeand ceftazidime at1µg/ml concentration and control plate without any antibiotic)

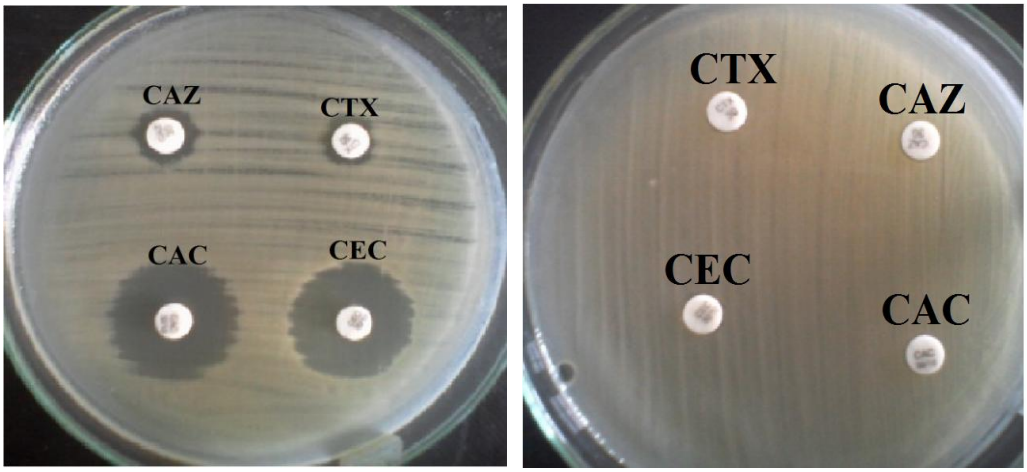


Figure 18: Figure showing the detection of ESBLs by Combined Disk Diffusion method. Cefotaxime (CTX) and ceftazidime (CAZ) in alone showed no zone of inhibition while CTX in combination with Clavulanic Acid (CEC) and CAZ in combination with clavulanic acid (CAC) showed good zone of inhibition (i.e. more than 17 mm) and in other plate showing ESBL negative isolates.

Table 19: confirmatory test of ESBL producing isolates

Organisms	**With *CTX**	**With **CAZ**	**Total** (n=)
***E. coli* (n=227)**	104	124	124
***Klebsiella pnuemoniae* (n=80)**	32	31	34
***Klebsiella oxytoca* (n=36)**	26	31	31
***Proteus vulgaris* (n=17)**	7	11	11
***Proteus mirabilis* (n=17)**	5	7	8
***Vibrio cholerae* (n=3)**	0	0	0
***Pseudomonas aeruginosa* (n=208)**	57	113	129
***Pseudomonas* Spp. (n=59)**	11	15	15
***Acinetobacter baumanii* (n=16)**	4	8	8
Total (n=663)	246	340	360

` *CTX: cefotaxime; **CAZ: ceftazidime

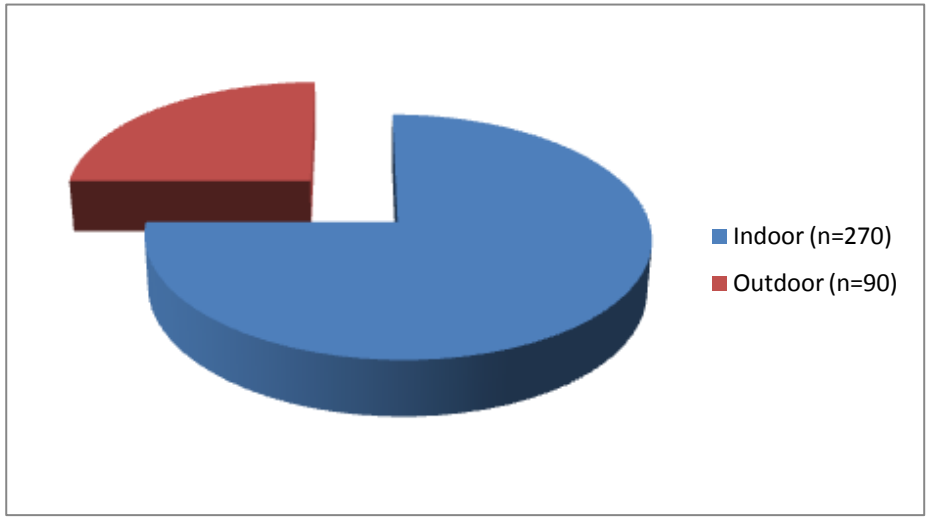


Figure 19: Proportion of ESBL producing organisms from indoor (n=270) and outdoor (n=90) patients

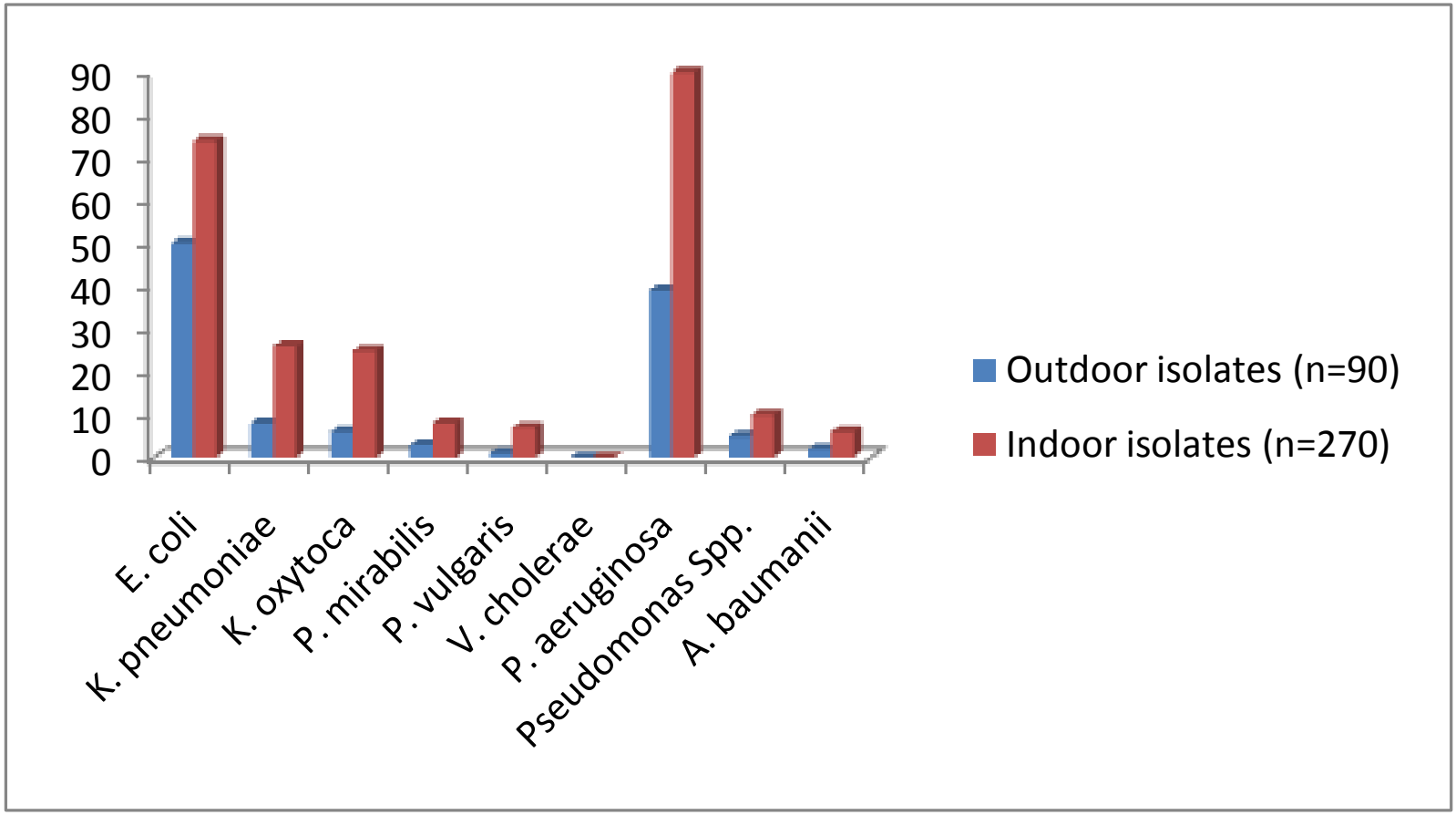


Figure 20: Production of ESBL positive indoor (n=279) and outdoor (n=90) isolates at species label

4.2 Antimicrobial susceptibility test

When tested the antimicrobial susceptibility profiling of all ESBL positive isolates, it was observed that among the ESBL producers, susceptibility against β-lactam antibiotics; imipenem showed the highest susceptibility 89.16% (n=321) followed by meropenem 86.55% (n=308) and cefoxitin 25% (n=90) (Table 20) while in case of non β-lactam antibiotics susceptibility was high against tigecycline 84.44% (n=304) followed by amikacin 55% (n=198) and gentamicin 42.22% (n=152) (Table 21) (Figure 21).

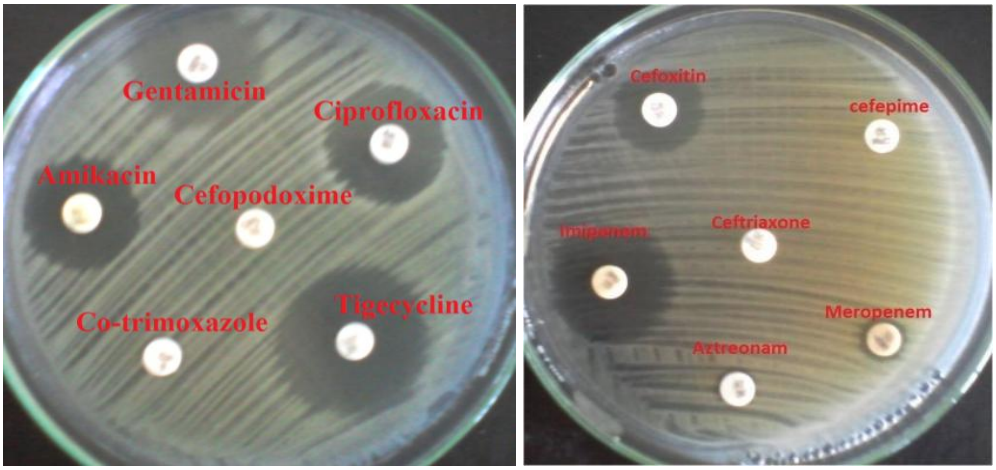


Figure 21: Antimicrobial susceptibility testing of ESBLs producers against commercially available β-lactam antibiotics and non β-lactam antibiotics

Table 20: Antimicrobial susceptibility patterns of ESBL producers against β-lactam antibiotics

Organisms	*FOX		*CPD		*CRO		*FEP		*IPM		*MEM		*ATM	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
***E. coli* (n=124)**	36	29.03	0	0	4	3.22	2	1.61	111	89.51	105	84.67	0	0
***Klebsiella pnuemoniae* (n=34)**	13	38.23	1	2.94	4	11.76	7	20.58	34	100	32	94.11	1	2.94
***Klebsiella oxytoca* (n=31)**	12	38.70	1	3.22	4	12.90	6	19.35	28	90.32	26	83.87	1	3.22
***Proteus vulgaris* (n=11)**	6	--	0	--	0	--	1	--	11	--	11	--	0	--
***Proteus mirabilis* (n=8)**	3	--	0	--	0	--	0	--	8	--	8	--	0	--
***Pseudomonas aeruginosa* (n=129)**	15	11.62	2	1.55	4	3.10	13	10.07	110	85.27	111	86.04	6	4.65
***Pseudomonas* spp. (n=15)**	5	--	0	--	2	--	12	--	13	--	11	--	2	--
***Acinetobacter baumanii* (n=8)**	0	--	0	--	0	--	0	--	6	--	5	--	0	--
TOTAL (n = 360)	90	25	4	1.11	18	5	41	11.38	321	89.16	308	85.55	10	2.77

***abbreviations**

FOX: cefoxitin; CPD: cefopodoxime; CRO: ceftriaxone; FEP: cefepime; IPM: imipenem; MEM: meropenem; ATM: aztreonam

Table 21: Antimicrobial susceptibility patterns of ESBL producers against commercially available non β- lactam antibiotics

Organisms	*AMK		*SXT		*GEN		*CIP		*TGC	
	N	%	N	%	N	%	N	%	N	%
***E. coli* (n =124)**	89	71.77	41	33.06	72	58.06	11	8.87	99	79.83
***Klebsiella pnuemoniae* (n=34)**	29	85.29	8	23.52	13	38.23	4	11.76	31	91.17
***Klebsiella oxytoca* (n=31)**	27	87.09	6	19.35	12	38.70	9	29.03	26	83.87
***Proteus vulgaris* (n=11)**	4	--	2	--	2	--	4	--	8	--
***Proteus mirabilis* (n=8)**	3	--	4	--	3	--	4	--	6	--
***Pseudomonas aeruginosa* (n=129)**	31	24.03	11	8.52	38	29.45	29	22.480	119	92.24
***Pseudomonas* spp. (n=15)**	11	--	6	--	9	--	3	--	8	--
***Acinetobacter baumanii* (n=8)**	4	--	6	--	3	--	3	--	7	--
TOTAL (n=360)	198	55	84	23.33	152	42.22	67	18.61	304	84.44

***abbreviations**
AMK: amikacin; SXT: trimithoprim/sulphamethoxazole; GEN: gentamicin; CIP: ciprofloxacin; TGC: tigecycline

4.3 Minimum inhibitory concentration

Among all the ESBL positive organisms; highest susceptibility was observed against; meropenem 53.61% (n=193) and ertapenem 43.61% (n=157) followed by imipenem 41.94% (n=151); ceftazidime 26.66% (n=96); cefepime 25.55% (n=92); aztreonam 17.77 % (n=64); ceftriaxone 12.22% (n=44); cefotaxime 11.11% (n=40) and rest of the isolates were found above the MIC break point (Table 22-29 and Figure 22).

Table 22: MIC of ESBL producers against imipenem

Organisms	**Antibiotic concentrations**								
	<4 µg/ml	**4 µg/ml**	**8 µg/ml**	**16 µg/ml**	**32 µg/ml**	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
***E. coli* (n =124)**	53	50	2	9	4	1	--	2	3
***Klebsiella pneumoniae* (n=34)**	17	8	1	--	1	2	1	1	3
***Klebsiella oxytoca* (n=31)**	18	7	--	--	1	1	2	1	1
***Proteus vulgaris* (n=11)**	5	2	--	--	1	1	--	1	1
***Proteus mirabilis* (n=8)**	3	3	--	--	--	1	--	--	1
***Pseudomonas aeruginosa* (n=129)**	46	11	9	14	5	23	2	1	18
***Pseudomonas* spp.(n=15)**	5	1	--	1	1	2	1	2	2
***Acinetobacter baumannii* (n=8)**	4	3	--	--	--	--	--	1	--
TOTAL (n=360)	151	85	12	24	13	31	6	9	29

Sensitive, Resistant

Table 23: MIC of ESBL producers against meropenem

Organisms	Antibiotic concentrations								
	<4 µg/ml	**4 µg/ml**	**8 µg/ml**	**16 µg/ml**	**32 µg/ml**	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
***E. coli* (n=124)**	77	25	1	8	5	--	--	2	6
***Klebsiella pneumoniae* (n=34)**	18	10	1	1	1	2	--	--	1
***Klebsiella oxytoca* (n=31)**	18	2	2	1	2	1	--	4	1
***Proteus vulgaris* (n=11)**	8	1	--	--	--	--	--	1	1
***Proteus mirabilis* (n=8)**	5	1	--	--	1	--	--	--	1
***Pseudomonas aeruginosa* (n=129)**	54	16	5	8	4	20	1	1	20
***Pseudomonas* spp. (n=15)**	8	1	1	1	1	1	--	1	1
***Acinetobacter baumannii* (n=8)**	5	2	--	--	--	--	--	1	--
TOTAL (n=360)	193	58	10	19	14	24	1	10	31

Sensitive, Resistant

Table 24: MIC of ESBL producers against ertapenem

Organisms	Antibiotic concentrations								
	<4 µg/ml	**4 µg/ml**	**8 µg/ml**	**16 µg/ml**	**32 µg/ml**	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
***E. coli* (n=124)**	62	50	7	--	--	--	--	2	3
***Klebsiella pneumoniae* (n=34)**	12	18	1	1	--	--	--	1	1
***Klebsiella oxytoca* (n=31)**	12	15	1	1	--	--	--	1	1
***Proteus vulgaris* (n=11)**	7	3	--	--	--	--	--	1	--
***Proteus mirabilis* (n=8)**	3	4	--	--	--	--	--	--	1
***Pseudomonas aeruginosa* (n=129)**	49	45	19	2	2	4	--	2	6
***Pseudomonas* spp. (n=15)**	5	--	1	2	2	1	1	1	2
***Acinetobacter baumannii* (n=8)**	**7**	1	--	--	--	--	--	--	--
TOTAL (n=360)	157	136	29	6	4	5	1	8	14

Sensitive, Resistant

Table 25: MIC of ESBL producers against aztreonam

Organisms	Antibiotic concentrations								
	<4 µg/ml	**4 µg/ml**	**8 µg/ml**	**16 µg/ml**	**32 µg/ml**	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
***E. coli* (n=124)**	12	10	4	11	8	9	15	13	42
***Klebsiella pneumoniae* (n=34)**	4	4	2	4	2	1	3	3	11
***Klebsiella oxytoca* (n=31)**	6	1	1	5	2	1	1	6	8
***Proteus vulgaris* (n=11)**	3	2	--	2	--	--	--	1	3
***Proteus mirabilis* (n=8)**	2	3	--	1	--	--	--	--	2
***Pseudomonas aeruginosa* (n=129)**	7	7	1	13	4	3	15	27	52
***Pseudomonas* spp. (n=15)**	2	--	--	2	1	3	2	5	--
***Acinetobacter baumannii* (n=8)**	1	--	--	--	--	--	--	--	7
TOTAL (n=360)	37	27	8	38	17	17	36	55	125

Sensitive, Intermediate, Resistant

Table 26: MIC of ESBL producers against cefotaxime

Organisms	Antibiotic concentrations								
	<4 µg/ml	**4 µg/ml**	**8 µg/ml**	**16 µg/ml**	**32 µg/ml**	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
***E. coli* (n=124)**	21	19	32	--	10	4	3	3	32
***Klebsiella pneumoniae* (n=34)**	3	2	--	11	2	1	3	5	7
***Klebsiella oxytoca* (n=31)**	2	2	--	5	3	4	2	5	8
***Proteus vulgaris* (n=11)**	3	1	--	1	1	--	2		3
***Proteus mirabilis* (n=8)**	3	1	--	1	--	--	--	1	2
***Pseudomonas aeruginosa* (n=129)**	6	8	4	40	6	9	7	8	41
***Pseudomonas* spp. (n=15)**	1	2	5	1	1	--	1	2	2
***Acinetobacter baumannii* (n=8)**	1	--	--	1	2	--	--	--	4
TOTAL (n=360)	40	35	41	60	25	18	18	24	99

Sensitive, Resistant

Table 27: MIC of ESBL producers against ceftazidime

Organisms	Antibiotic concentrations								
	<4 µg/ml	**4 µg/ml**	**8 µg/ml**	**16 µg/ml**	**32 µg/ml**	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
***E. coli* (n=124)**	21	10	5	14	24	8	12	9	21
***Klebsiella pneumoniae* (n=34)**	4	6	2	5	1	1	2	7	6
***Klebsiella oxytoca* (n=31)**	3	4	2	6	2	1	2	3	8
***Proteus vulgaris* (n=11)**	1	4	1	1	--	--	--	2	2
***Proteus mirabilis* (n=8)**	1	2	1	1	--	--	--	1	2
***Pseudomonas aeruginosa* (n=129)**	6	29	8	23	4	5	3	10	41
***Pseudomonas* spp. (n=15)**	1	1	--	2	--	--	4	3	4
***Acinetobacter baumannii* (n=8)**	1	2	--	1	--	--	--	2	2
TOTAL (n=360)	38	58	19	53	31	15	23	37	86

Sensitive, Intermediate, Resistant

Table 28: MIC of ESBL producers against ceftriaxone

Organisms	Antibiotic concentrations								
	<4 µg/ml	**4 µg/ml**	**8 µg/ml**	**16 µg/ml**	**32 µg/ml**	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
***E. coli* (n=124)**	14	13	7	16	9	7	11	15	32
***Klebsiella pneumoniae* (n=34)**	2	3	2	9	2	--	2	4	10
***Klebsiella oxytoca* (n=31)**	2	4	2	6	2	--	2	2	11
***Proteus vulgaris* (n=11)**	2	1	--	--	--	--	1	1	6
***Proteus mirabilis* (n=8)**	1	1	--	--	--	--	--	1	5
***Pseudomonas aeruginosa* (n=129)**	15	20	1	5	3	--	9	34	42
***Pseudomonas* spp. (n=15)**	5	2	1	3	1	--	1	1	1
***Acinetobacter baumannii* (n=8)**	3	--	--	1	--	--	1	--	3
TOTAL (n=360)	44	44	13	40	17	7	27	58	110

Sensitive, Resistant

Table 29: MIC of ESBL producers against cefepime

Organisms	Antibiotic concentrations								
	<4 µg/ml	**4 µg/ml**	**8 µg/ml**	**16 µg/ml**	**32 µg/ml**	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
***E. coli* (n=124)**	20	11	4	15	3	6	12	19	34
***Klebsiella pneumoniae* (n=34)**	5	2	1	8	2	1	6	2	7
***Klebsiella oxytoca* (n=31)**	1	1	--	7	1	1	6	2	12
***Proteus vulgaris* (n=11)**	3	4	--	1	--	--	--	--	3
***Proteus mirabilis* (n=8)**	2	1	--	1	--	--	--	1	3
***Pseudomonas aeruginosa* (n=129)**	14	11	6	18	3	3	11	19	44
***Pseudomonas* spp. (n=15)**	1	2	1	2	--	2	4	2	1
***Acinetobacter baumannii* (n=8)**	2	--	--	--	2	--	--	2	2
TOTAL (n=360)	48	32	12	52	11	13	39	47	106

Sensitive, Intermediate, Resistant

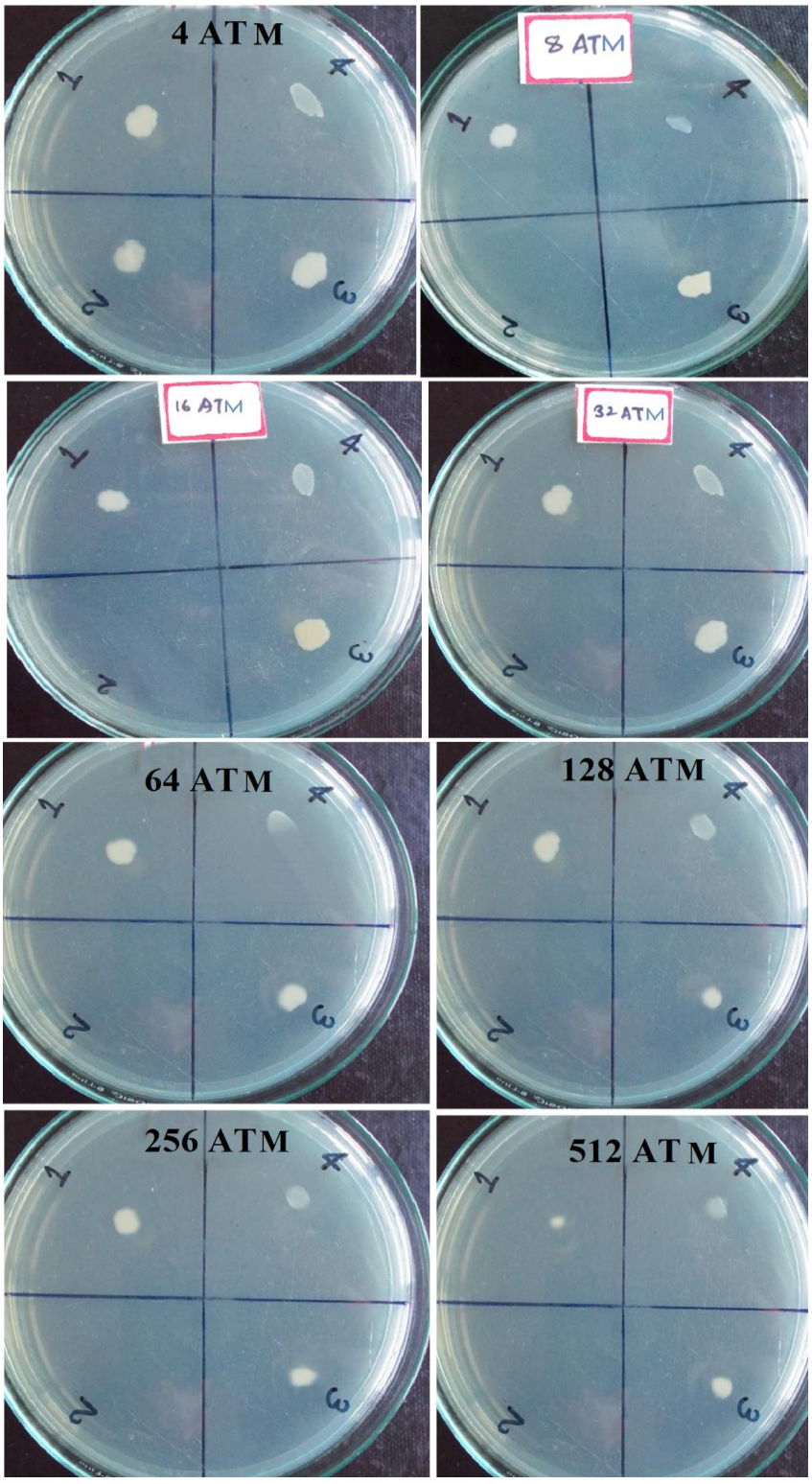


Figure 22: Minimum inhibitory concentration of ESBLs against Aztreonam at 4-512µg/ml concentration.

4.4 Genotypic characterization of ESBLs by Multiplex PCR:

Multiplex PCR results showed presence of different β-lactamase genes, among them CTX-M (n=131) type ESBL gene was most common among test isolates (n=360), followed by SHV (n=90), TEM (n=66), PER (n=45), OXA-10 (n=24), VEB (n=22), OXA-2 (n=18) and GES (n=2) (Table 30; Figure 23). A total of 83 isolates were harbouring single β-lactamase gene (Table 30; Figure 23), while in 126 isolates multiple β-lactamase genes were found (Table 31; Figure 24 and 25). There were 159 of isolates which did not show any amplification with target primers (Table 30).

Table 30: Distribution of ESBL gene among test isolates (harbouring single β-lactamase gene)

ESBL gene type / Organisms	**CTX-M**	**TEM**	**SHV**	**OXA-2**	**OXA-10**	**PER**	**VEB**	**GES**	**None**
***E. coli* (n=124)**	14	5	1	--	2	8	--	--	37
***Klebsiella pneumonia* (n=34)**	2	2	6	--	--	--	1	--	4
***Klebsiella oxytoca* (n=31)**	1	--	2	--	--	--	--	--	19
***Proteus vulgaris* (n=11)**	1	--	--	--	1	1	2	--	4
***Proteus mirabilis* (n=8)**	--	--	--	--	--	--	1	--	7
***Pseudomonas aeruginosa* (n=129)**	4	2	3	--	3	1	8	1	76
***Pseudomonas* Spp. (n=15)**	1	1	--	--	1	--	2	--	8
***Acinetobacter baumanii* (n=8)**	--	2	1	--	--	--	--	--	4
Total (n=360)	**23**	**12**	**13**	**0**	**7**	**10**	**14**	**1**	**159**

Table 31: Multiple combinations of β-lactamase genes (continued to next page)

Organisms	TEM +PER	CTX-M +SHV +PER	CTX-M +TEM +PER	OXA-10 +SHV +CTX-M	OXA-10 +SHV +CTX-M +TEM +PER	OXA-10 +SHV +OXA-2+ CTX-M + TEM +PER	SHV +CTX-M +TEM +PER	SHV +CTX-M +TEM +OXA-2	OXA-10 +SHV +VEB +PER	OXA-2 +CTX-M +PER	OXA-2 +CTX-M +TEM +SHV +PER	OXA-10 +OXA-2 +SHV +CTX-M	OXA-2 +CTX-M	OXA-2 +SHV	OXA-10 + OXA-2 + SHV
E. coli **(n=124)**	2	1	3	1	--	1	1	1	1	1	1	1	--	--	--
Klebsiella pneumonia **(n=34)**	--	--	--	1	--	--	--	1	--	--	--	--	--	2	--
Klebseilla oxytoca **(n=31)**	--	--	--	--	--	--	--	--	--	--	--	--	--	2	--
Proteus vulgaris **(n=11)**	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Proteus mirabilis **(n=8)**	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Pseudomonas aeruginosa **(n=129)**	--	2	1	1	--	--	3	--	--	--	1	1	1	--	1
Pseudomonas **Spp. (n= 144)**	--	1	1	--	1	--	1	--	--	--	--	--	--	--	--
Acinetobacter baumanii **(n=8)**	--	1	--	--	--	--	1	--	--	--	--	--	--	--	--
Total (n=360)	2	5	5	3	1	1	6	2	1	1	2	2	1	4	1

Table 31.1: Multiple combinations of β-lactamase genes

Organisms	CTX-M +TEM + VEB	CTX-M + PER	OXA-10 +CTX-M +TEM + PER	CTX-M + SHV + GES	PER + VEB	CTX-M + OXA-10 + VEB	SHV + CTX-M + VEB	OXA-10 + PER	CTX-M + TEM +SHV + VEB	OXA-2+ CTX-M+ SHV+ PER	CTX-M + TEM	SHV + CTX-M	SHV + CTX-M + TEM	CTX-M + OXA-2 + SHV	SHV + VEB	OXA-10 +OXA-2 +SHV +CTX-M +TEM
E. coli **(n=124)**	--	3	--	--	1	--	--	--	--	2	16	12	5	6	1	--
Klebsiella pneumonia **(n=34)**	--	--	--	--	1	--	--	--	--	--	2	5	3	1	--	1
Klebseilla oxytoca **(n=31)**	--	--	--	--	--	--	--	--	--	--	1	2	2	--	--	--
Proteus vulgaris **(n=11)**	--	--	--	--	--	--	--	--	--	--	1	--	--	--	--	--
Proteus mirabilis **(n=8)**	--	--	--	--	--	--	--	--	--	--	--	1	--	--	--	--
Pseudomonas aeruginosa **(n=129)**	1	1	1	1	--	1	1	1	--	--	4	5	1	--	--	--
Pseudomonas **Spp. (n= 144)**	--	1	--	--	--	--	--	--	1	--	1	2	1	--	--	--
Acinetobacter baumanii **(n=8)**	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Total (n=360)	1	5	1	1	2	1	1	1	1	2	25	27	12	7	1	1

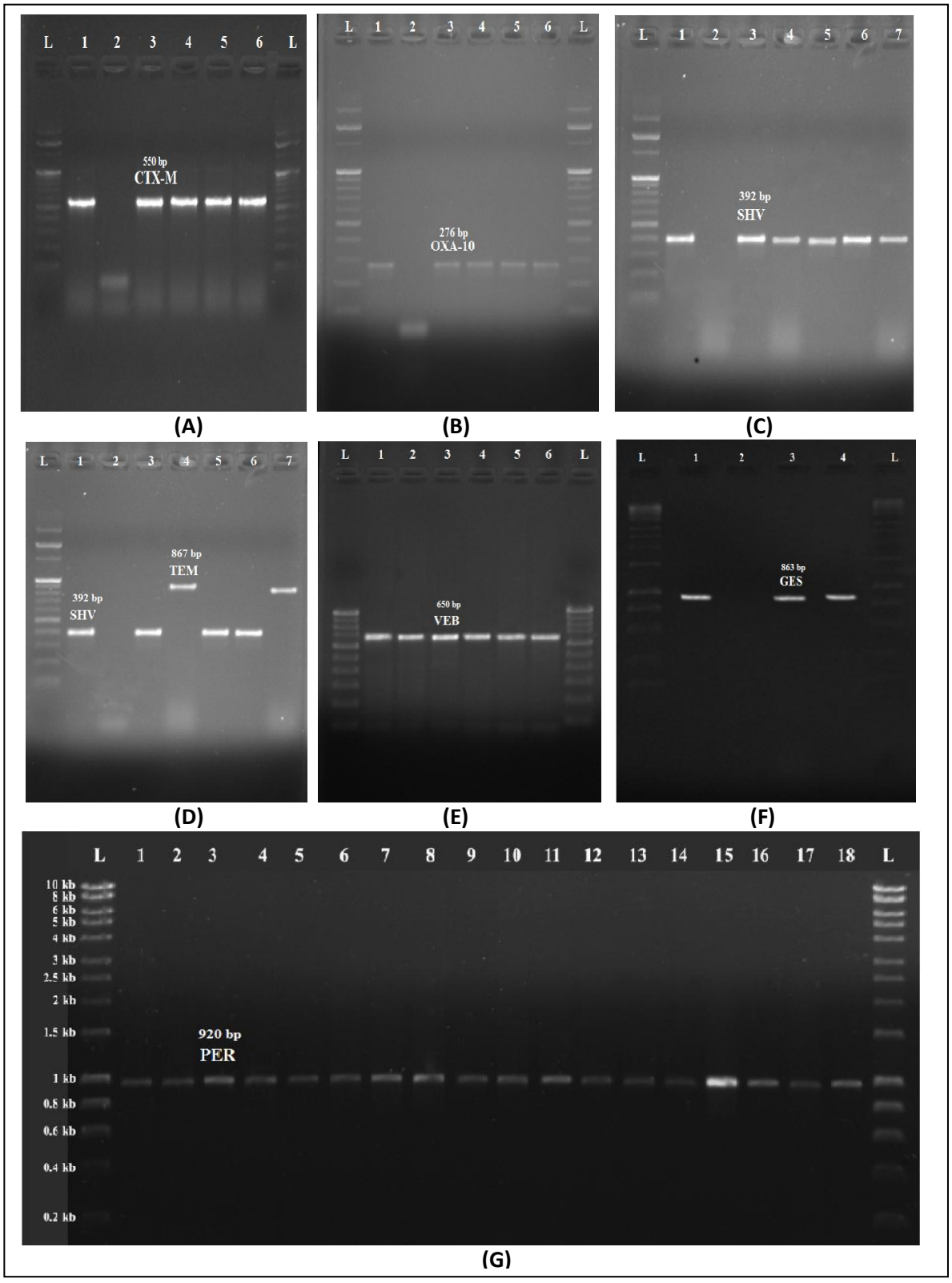


Figure 23: PCR amplification of *bla*$_{\text{ESBLs}}$. **(A)** 550bp CTX-M; **(B)** 276bp OXA-10; **(C)** 392bp SHV; **(D)** 867bp TEM; **(E)** 650bp VEB; **(F)** 863bp GES and **(G)** 920bp PER

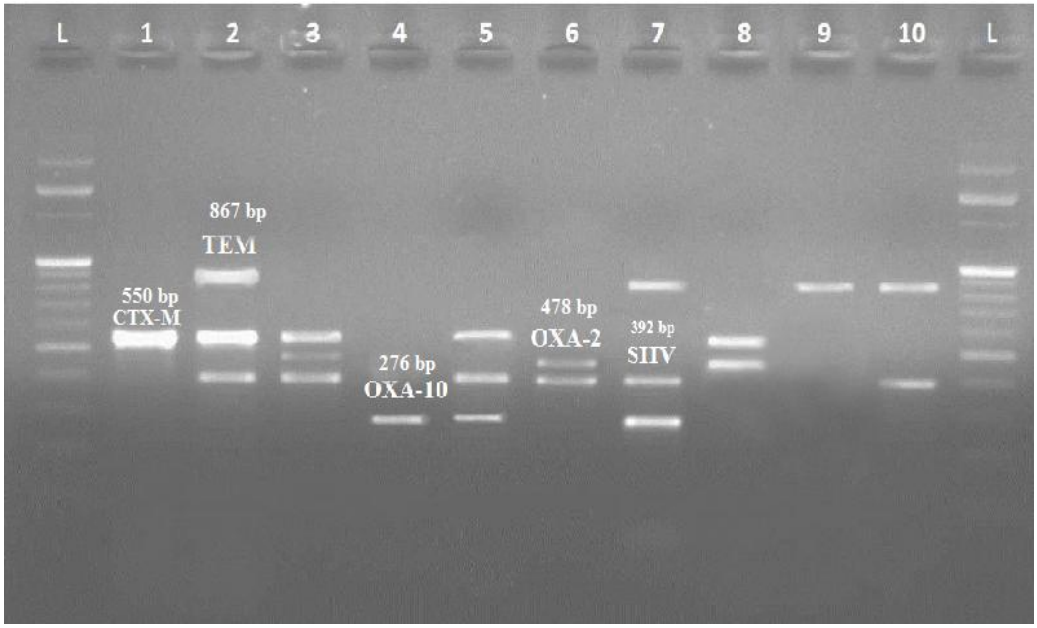


Figure 24: PCR amplification of multiple *bla* gene **Lane L:** 100 bp DNA ladder; **Lane 1- 12:** showing different beta lactamase gene of 276 bp, 550 bp, 392 bp, 478 bp and 867 bp showing OXA-10, CTX-M, SHV, OXA-2 and TEM β-lactamase gene respectively.

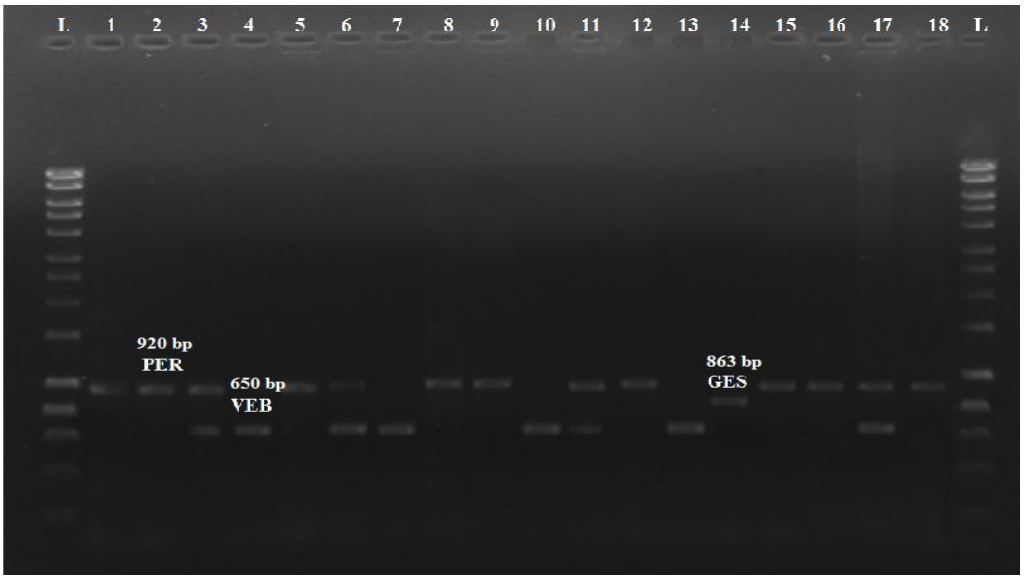


Figure 25: PCR amplification of PER, VEB and GES. **Lane L:** 10Kb DNA Hyper ladder; **lane 1-3, 5-6, 8-9, 11-12, 15-18:** PER (920bp); **lane 3-4, 6-7, 10-11, 13, 17:** VEB (650 bp); and **Lane 14 :** GES (863 bp).

4.5 Sequencing of *bla*$_{ESBLs}$ genes

Sequencing of the PCR products of *bla*$_{ESBLs}$ showed that isolates harboured TEM-1, SHV-148, CTX-M-15, OXA-10, PER-1, VEB-1 and GES-5 variants of ESBLs gene in this study area (Figure 26 to 32).

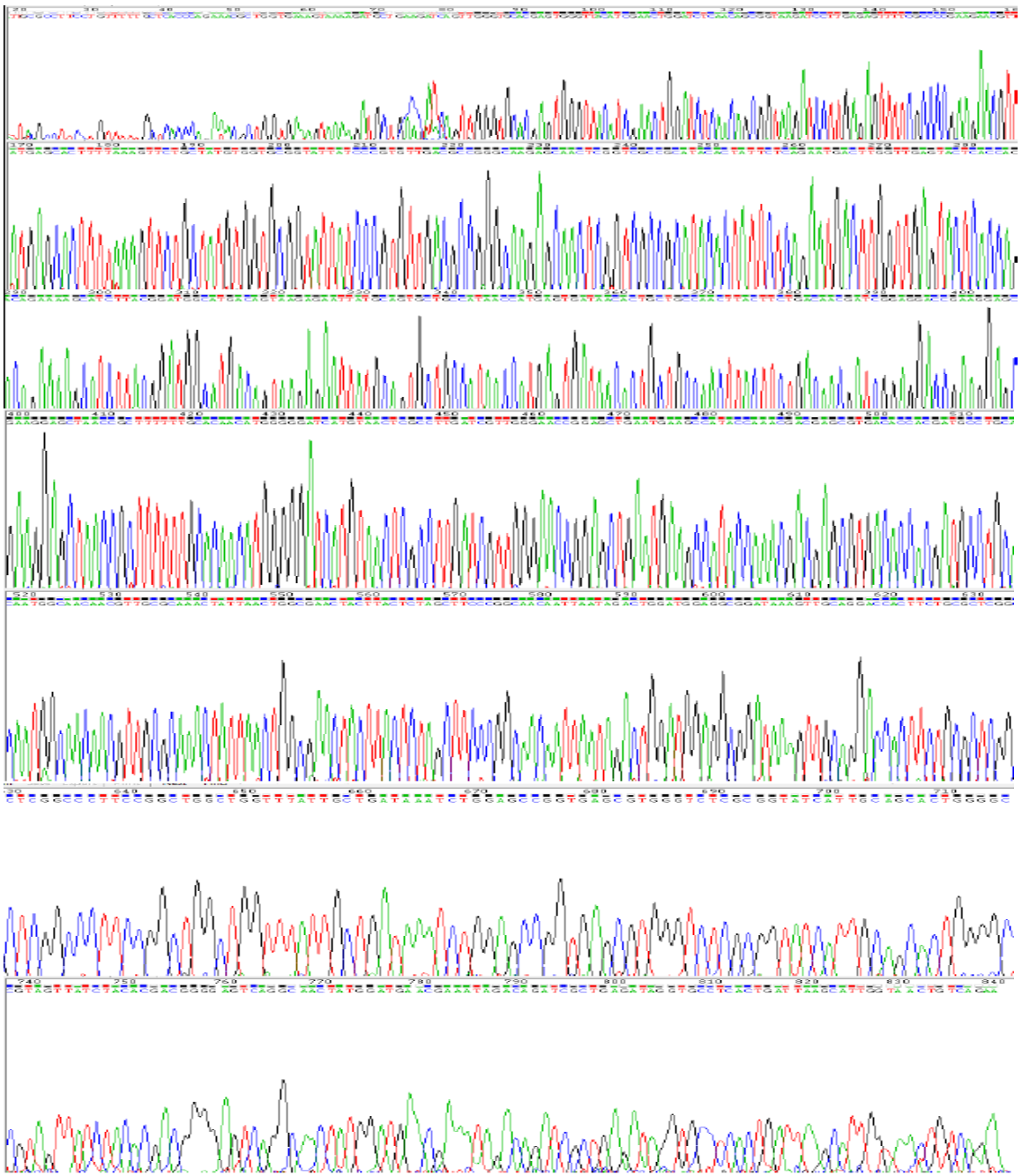


Figure 26: Electropherogram of TEM-1 PCR amplicon sequence

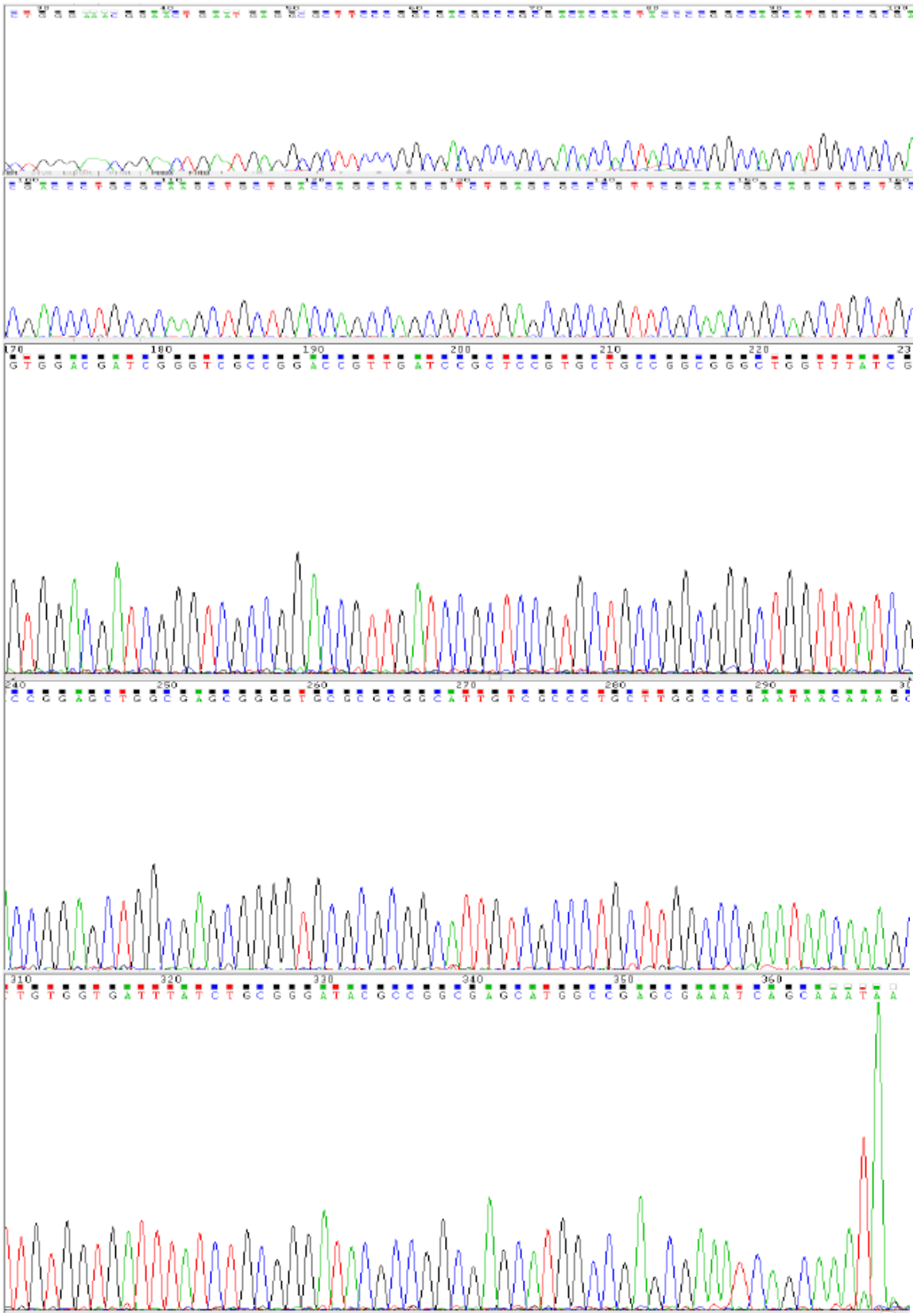


Figure 27: Electropherogram of SHV-148 PCR amplicon sequence

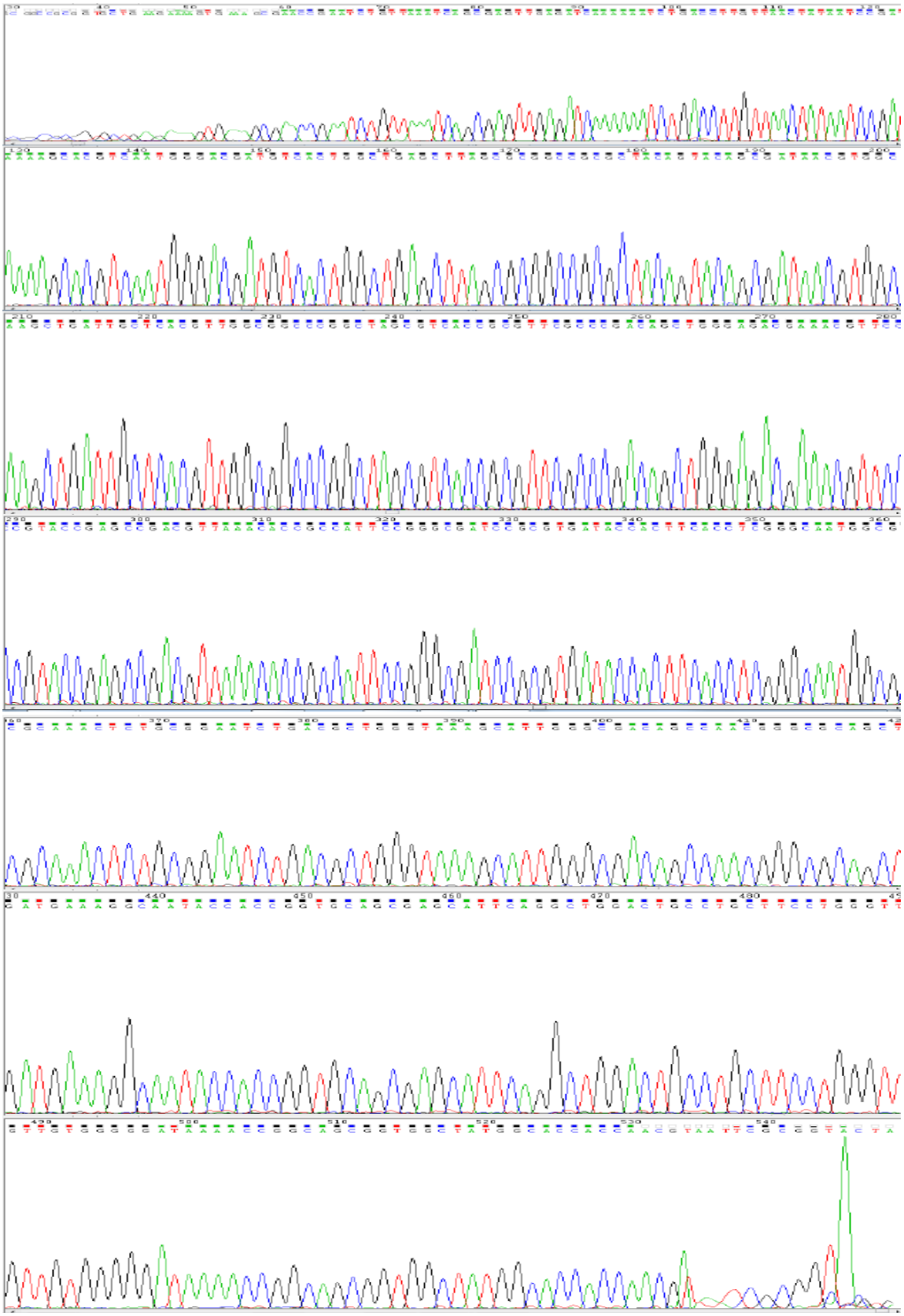


Figure 28: Electropherogram of CTX-M-15 PCR amplicon sequence

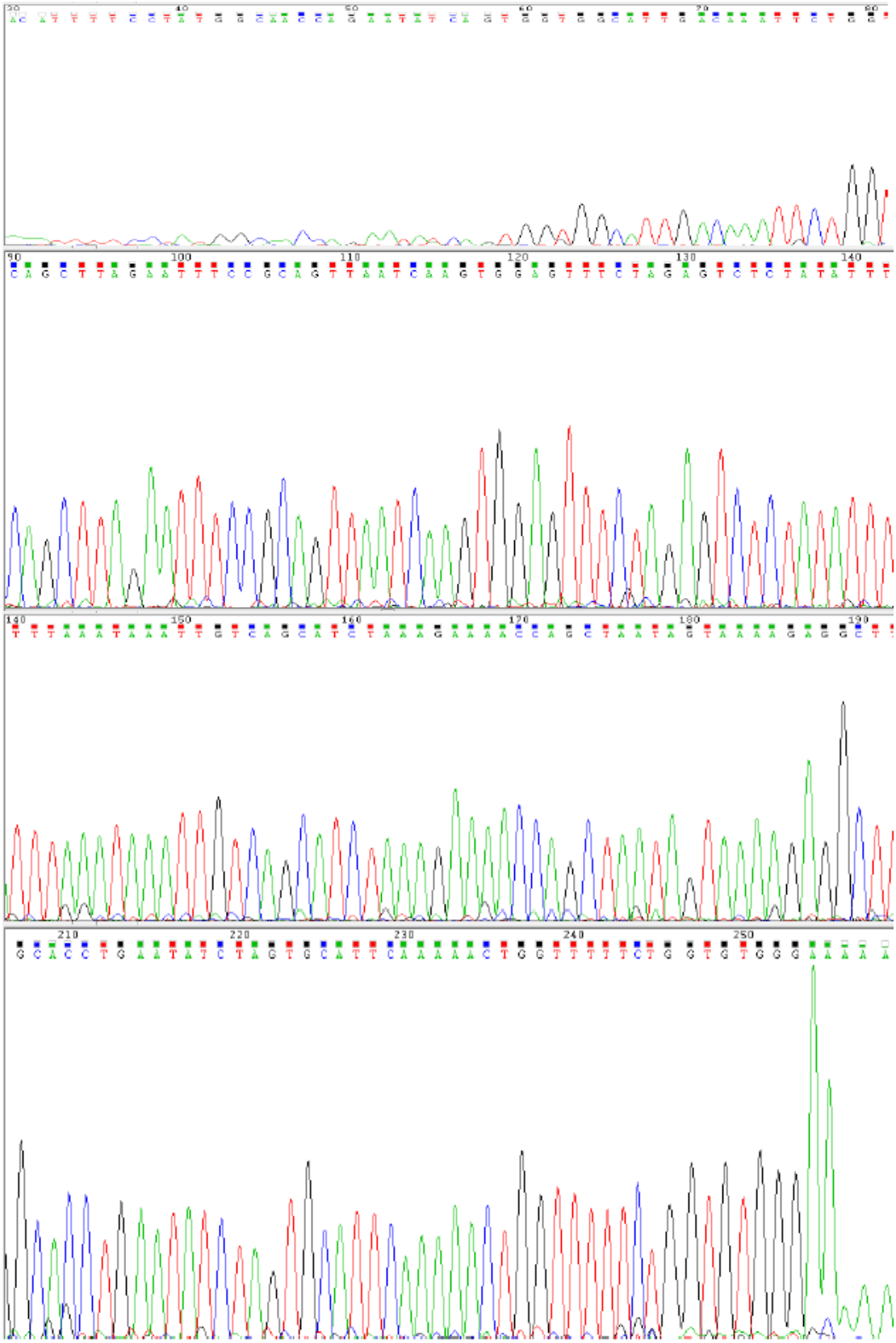


Figure 29: Electropherogram of OXA-10 PCR amplicon sequence

Figure 30: Electropherogram of PER-1 PCR amplicon sequence

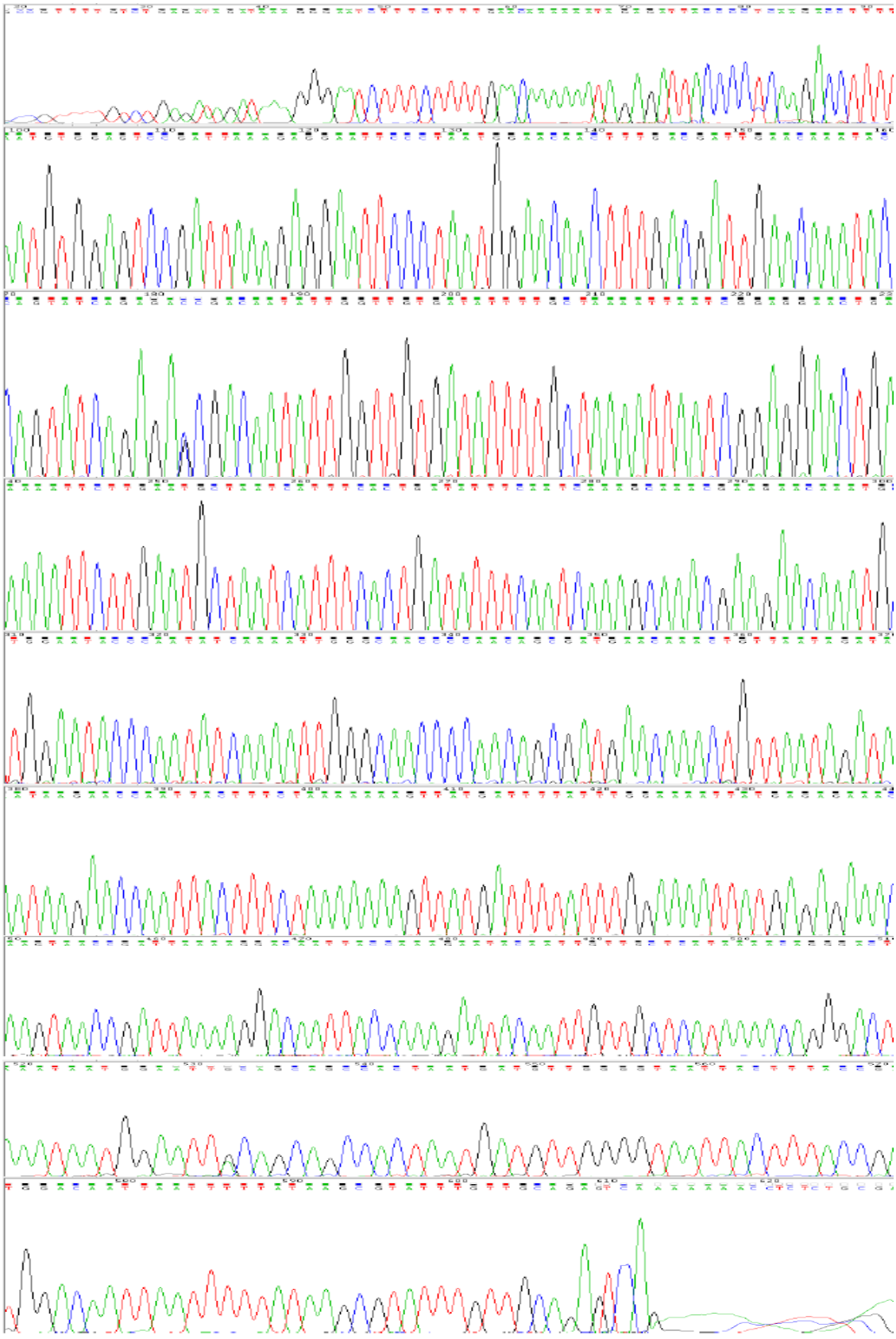


Figure 31: Electropherogram of VEB-1 PCR amplicon sequence

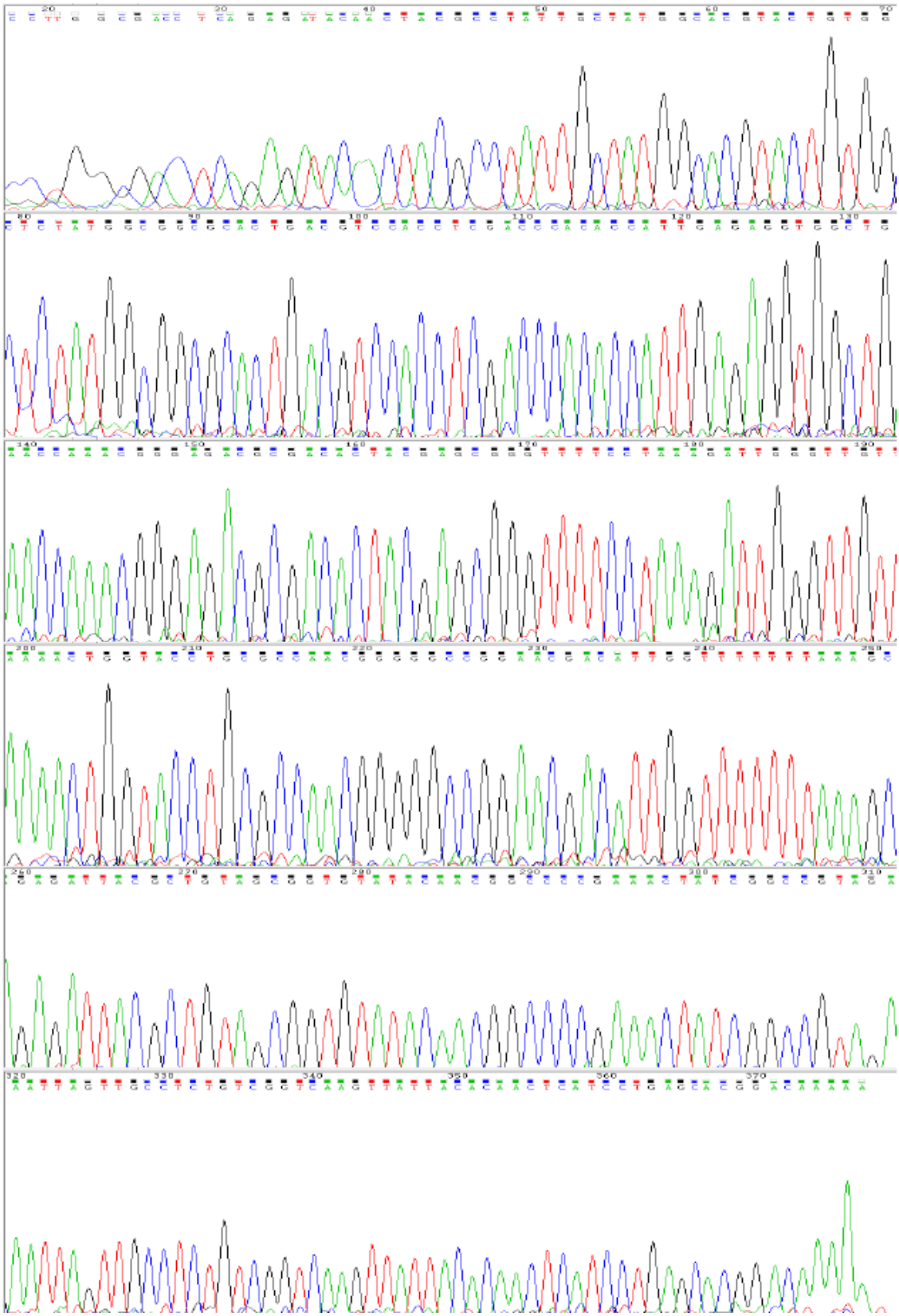


Figure 32: Electropherogram of GES-5 PCR amplicon sequence

4.6 PCR amplification and cloning of whole CTX-M-15 gene:

Whole gene including promoter region of *bla*$_{\text{CTX-M-15}}$ was amplified to give a product of 1212bp (Figure 33). The amplicon was successfully cloned in to pGEM-T vector and selected through blue white screening (Figure 34).

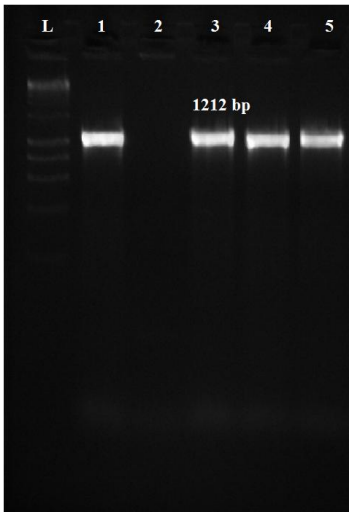


Figure 33: Detection of CTX-M-15 whole gene. **Lane L:** showing the 10 Kb DNA Hyper ladder; **Lane 1:** CTX-M-15 whole gene positive control; **Lane 2:** CTX-M-15 negative control; **Lane 3-5:** showing CTX-M-15 whole gene (1212bp)

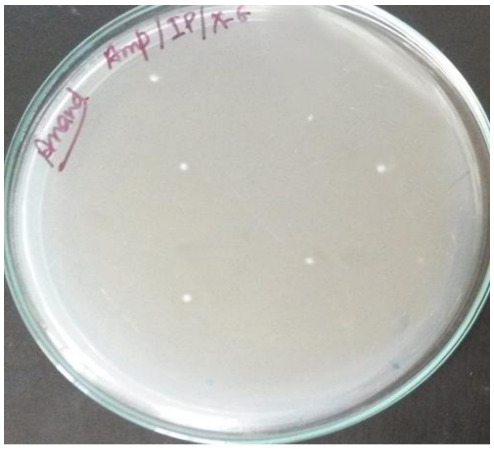


Figure 34: Clone of CTX-M-15 whole gene as white colonies

4.6.1 Minimum inhibitory concentration of the clone:

Both MIC_{50} and MIC_{90} value of the clones were found to be above the break point for expanded spectrum cephalosporins and monobactams (Table 32).

Table 32: MIC_{50} and MIC_{90} of clones

Antibiotics	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Aztreonam	64	256
Cefotaxime	32	128
Cftazidime	64	128
Ceftriaxone	64	128
Cefepime	64	256

4.7 Determination of genetic variants of CTX-M and OXA-10 by Denaturing gradient gel electrophoresis:

After performing DGGE, all the CTX-M (Figure 35) and OXA-10 (Figure 36) types were found to be of same variant.

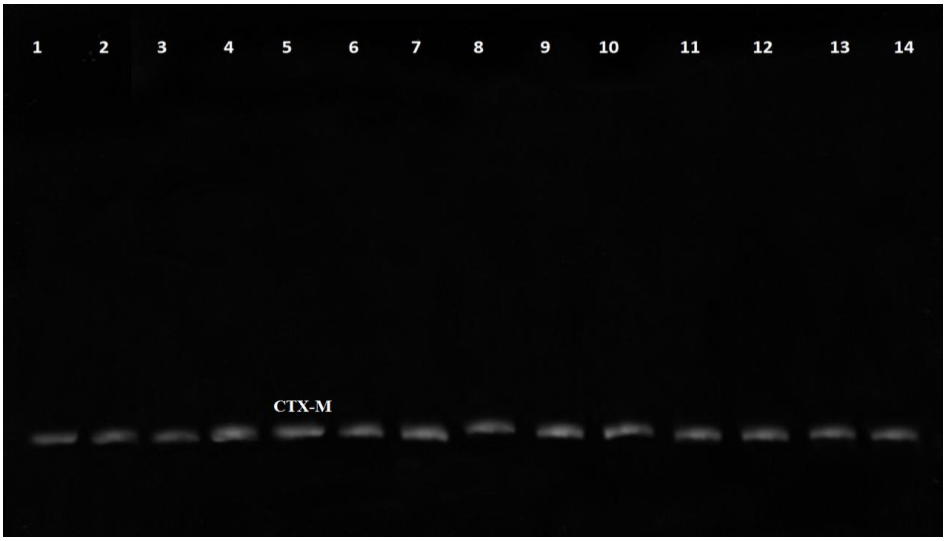


Figure 35: Denaturing gradient gel electrophoresis pattern of CTX-M gene. Lane1-14: There was no genetic variation among CTX-M gene.

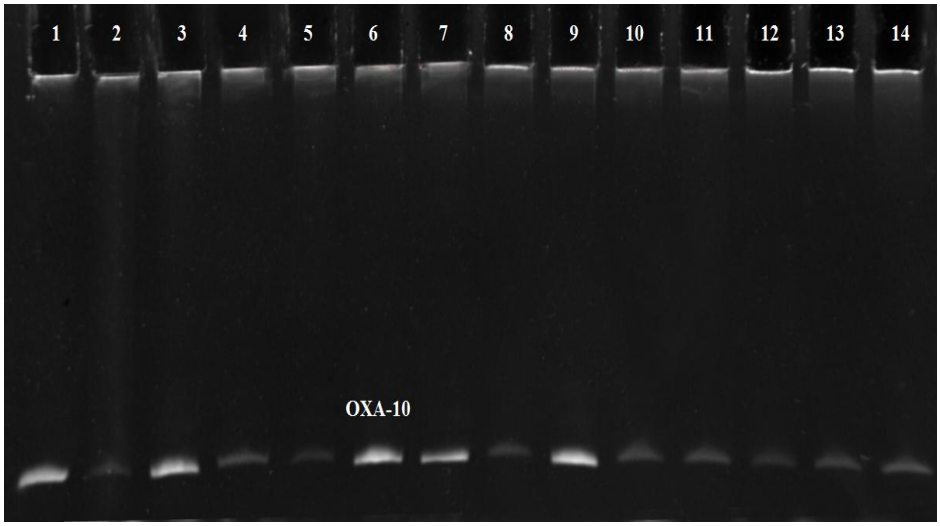


Figure 36: Denaturing gradient gel electrophoresis pattern of OXA-10 gene type. Lane: 1-14: OXA-10 gene was of same DGGE types.

4.8 DNA finger printing of ESBL producers

4.8.1 DNA finger printing of CTX-M-15 producers by PFGE

PFGE was performed for clonal dissemination/diversity of ESBLs harbouring isolates and it was found that ESBLs producing *E. coli* isolates harbouring CTX-M-15 gene disseminated through the twenty one clonal types (n=21) within the species in this study area (Figure 37).

4.8.2 DNA fingerprinting of ESBL producing isolates by ERIC PCR

After performing ERIC PCR, there were 41 types of *E. coli*, 9 types *Klebsiella pnemoniae*, 5 types *klebsiella oxytoca*, 3 types *Proteus mirabilis*, and 2 types *Proteus vulgaris* were found in this geographical part of the world (Figure 38-40).

4.8.3 DNA fingerprinting of ESBL producing non fermenting gram negative rods by REP PCR

DNA fingerprinting of non-fermenters was analysed after performing REP PCR and it was observed that there were 14 types *P. aeruginosa*, and 6 types *Acinetobacter baumanii* (Figure 41 and 42).

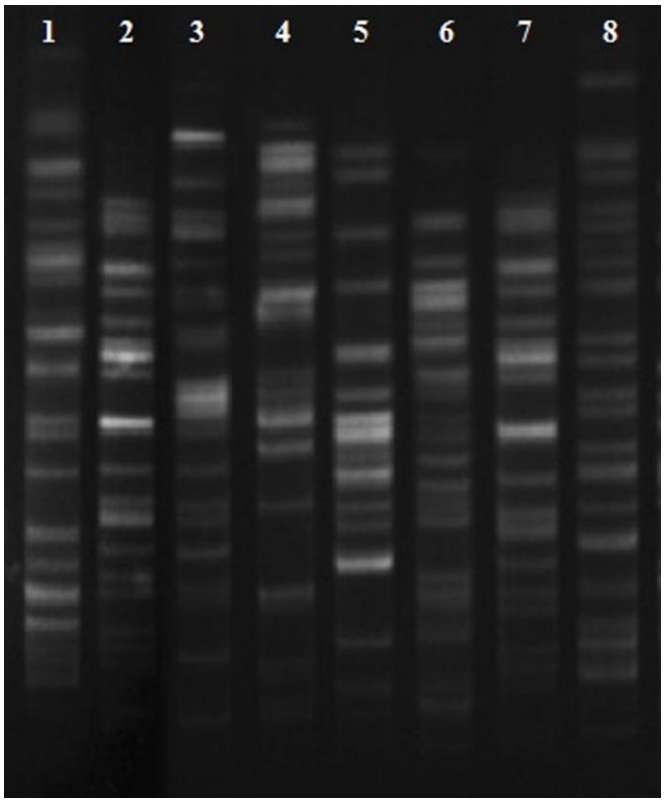


Figure 37: PFGE pattern of *E. coli* isolates harbouring CTX-M-15 gene digested by *Xba*I, Lane 1-8: *E. coli* PFGE Type1-8.

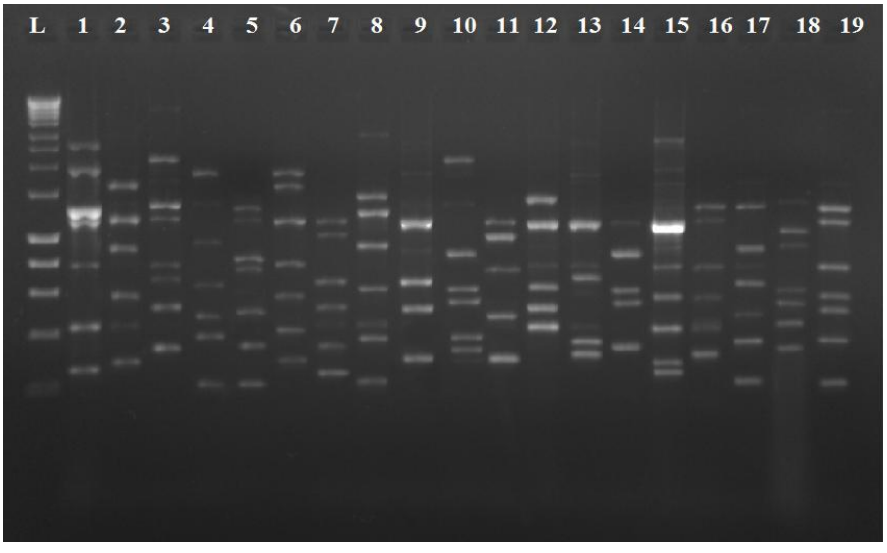


Figure 38: DNA finger printing of *E. coli* by ERIC PCR, **Lane 1-19**: *E. coli* ERIC Type 1-19**.**

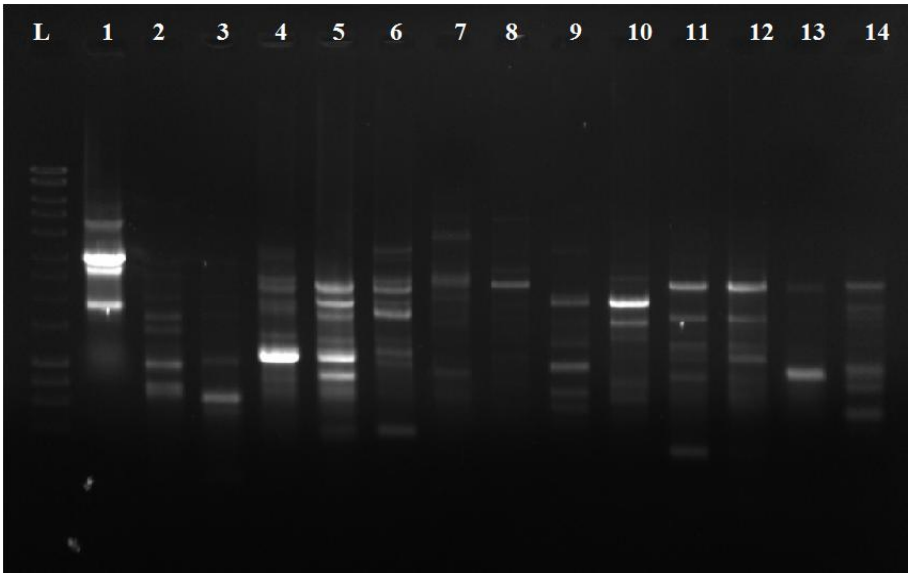


Figure 39: DNA finger printing of *K. Pneumonia* and *K. oxytoca* by ERIC PCR. **Lane L:** 10Kb DNA hyper ladder I; **Lane 1-9:** ERIC pattern of *K. Pneumonia* Type 1-9; **Lane 10-14:** ERIC pattern of *K. Oxytoca* Type 1-5.

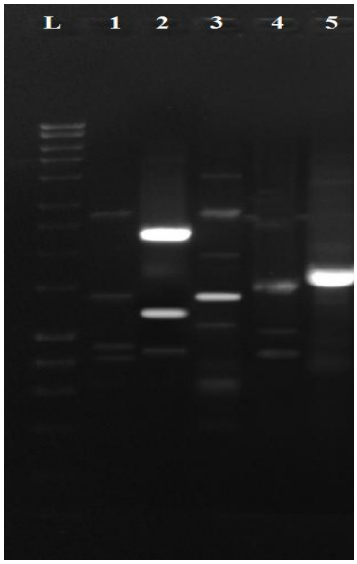


Figure 40: DNA finger printing of *Proteus mirabilis* and *Proteus vulgaris* by ERIC PCR**. Lane L:** 10Kb DNA hyper ladder I; **Lane 1-3:** ERIC pattern of *Proteus mirabilis* Type 1-3; **Lane 4-5:** ERIC pattern of *Proteus vulgaris* Type 1-2.

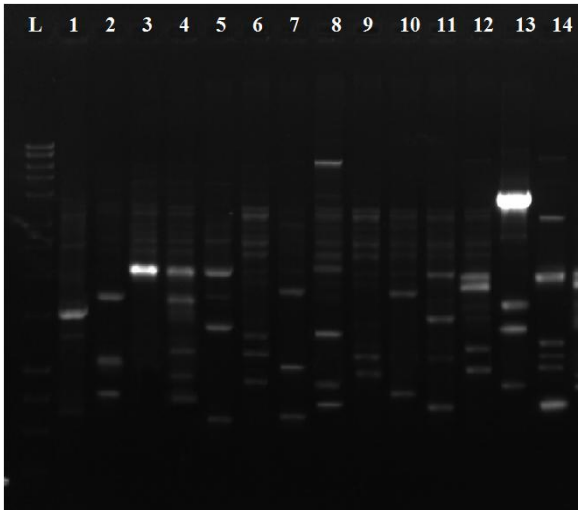


Figure 41: DNA finger printing of *P. aeruginosa* by REP PCR, *P. aeruginosa* Rep Type 1-14

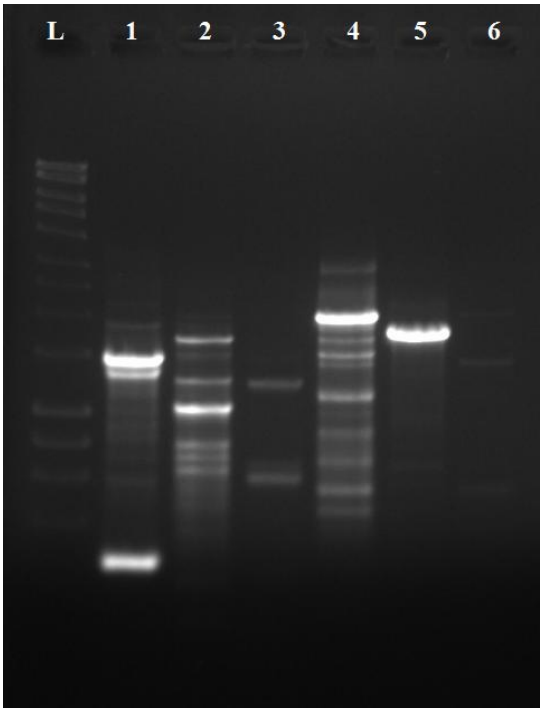


Figure 42: DNA finger printing of *Acinetobacter baumanii* by REP PCR, *A. Baumanii* Rep Type 1-6.

4.9 Genetic environment of *bla*$_{\text{ESBLs}}$

4.9.1 Detection of class 1 and class 2 integrons by integrase gene PCR:

Integrons were characterized among all the ESBL positive organisms and it was found that 255 isolates were harbouring integrons (70.83%). Among them 218 (60.05%) were class 1 integron, and 17 (4.72%) were carrying class 2 integron (Table 33, Figure 43) while absence of integron was observed in 105 isolates (29.16%) (Table 33). In 20 isolates (5.55%) both class 1 and class 2 integrons were present.

Table 33: Distribution of integrons in ESBL positive isolates

Organisms	**Integron types**			**None**
	Class 1	**Class 2**	**Class 1 & 2**	
***E. coli* (n=124)**	78	7	6	33
***Klebsiella pneumonia* (n=34)**	26	1	1	6
***Klebsiella oxytoca*(n=31)**	22	--	--	9
***Proteus vulgaris* (n=11)**	7	1	1	2
***Proteus mirabilis* (n=8)**	5	--	1	2
***Pseudomonas aeruginosa* (n=129)**	70	5	10	44
***Pseudomonas* Spp. (n=15)**	7	2	1	5
***Acinetobacter baumanii* (n=8)**	3	1	--	4
Total (n=360)	**218**	**17**	**20**	**105**

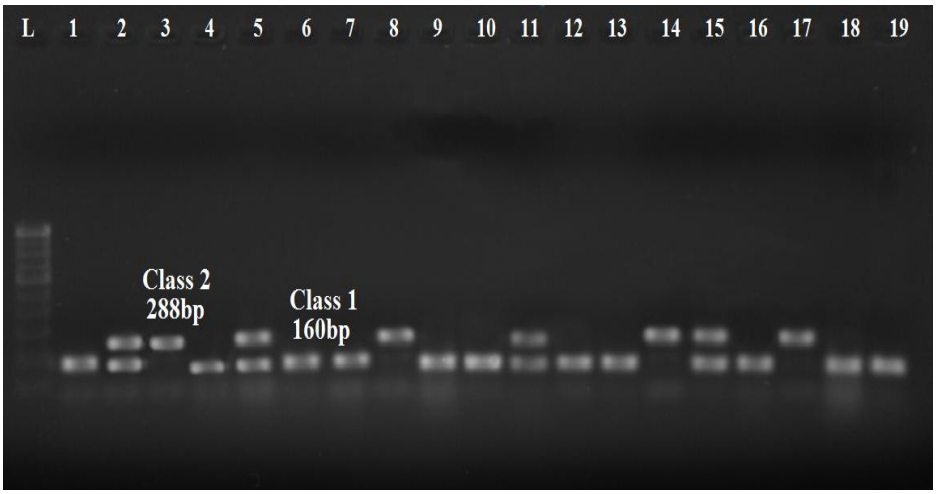


Figure 43: PCR amplification of integrase gene, **Lane L:** 100bp ladder; **Lane 1-2, 4-7, 9-13, 15-16, 18-19:** 160 bp class 1 integron; **Lane 2-3, 5, 8, 11, 14, 15, 17:** 288 bp class 2 integron.

4.10 Genetic linkage of *bla*$_{\text{ESBLs}}$:

PCR amplification results confirmed that 239 *bla*$_{\text{ESBLs}}$ were present within the variable region of the integrons. Of which *bla*$_{\text{OXA-10}}$ (n=18), *bla*$_{\text{OXA-2}}$ (n=12), *bla*$_{\text{SHV}}$ (n=48), *bla*$_{\text{PER}}$ (n=28), *bla*$_{\text{VEB}}$ (n=18) and *bla*$_{\text{GES}}$ (n=2) were linked within integron gene cassettes. However *bla*$_{\text{CTX-M}}$ (n=51) was found to be linked with integron gene cassettes only in *P. aeruginosa*.

4.11 Sequencing of integron borne *bla*$_{\text{ESBLs}}$:

Sequencing of the PCR product of 5'-CS and CTX-M-R'; 5'-CS and OXA-10-R'; 3'-CS and OXA-10-R'; as well as 5'-CS and VEB-1-R' primers revealed that all of the isolates were located within variable regions of class 1 integron with diverse types of genetic arrangements.

4.12 Genetic mapping of integron gene cassettes carrying ESBL genes

Sequencing results confirmed that OXA-10 was located within class I integron with five different types of arrangement (Figure 44). Upstream region of $bla_{\text{OXA-10}}$ was flanked by *dfrA12* (Type 1), *dfrA17* (Type 5), *dfrA1* (Type 3 and Type 2), *dfrA7* (Type 4), *arr2* (Type 2), *aac A4* (Type 2), *aad A5* (Type 4 and Type 5), while in downstream regions *aad A2* (Type 1), *aad A1* (Type 2 and Type 3), *aac (6')1b* (Type 4), and *qacE* (Type 1-5) genes were present (Figure 44).

$bla_{\text{VEB-1}}$ was located within variable regions of class 1 integron with four types of diverse arrangements (Figure 45). VEB-1 was flanked by *dfr A1* (Arrangement 1), *dfr A12* (Arrangement 2), and *dfr A17* (Arrangement 4), in the upstream region while in the downstream region *qacE* (Arrangement 1, 2, 3, and 4), *aad2* (Arrangement 2), and *aadA5* (Arrangement 4) were found (Figure 45).

In *P. aeruginosa* harbouring CTX-M-15, it was observed that $bla_{\text{CTX-M-15}}$ was located within class 1 integron (Figure 46). In the upstream regions of CTX-M-15 was flanked by *dfrA17* and *aadA5* genes while in the downstream regions *qacE* and *sul1* gene were found (Figure 46).

It has been also observed that *P. aeruginosa* isolates carrying $bla_{\text{GES-5}}$ was found to be located within variable regions of class 1 integron with two different types of genetic arrangement (Figure 47). Arrangement 1: upstream region flanked by *attI1*, followed by GES-5 and in downstream region *qacE* and *sul1* gene were present (Arrangement 1) while in arrangement 2: upstream region flanked by *attI1, GES-5* followed by truncated portion of 59 base element genes was found to be present while in the downstream region *aacA4*, *qacE* and *sul1* genes were found (Arrangement 2) (Figure 47).

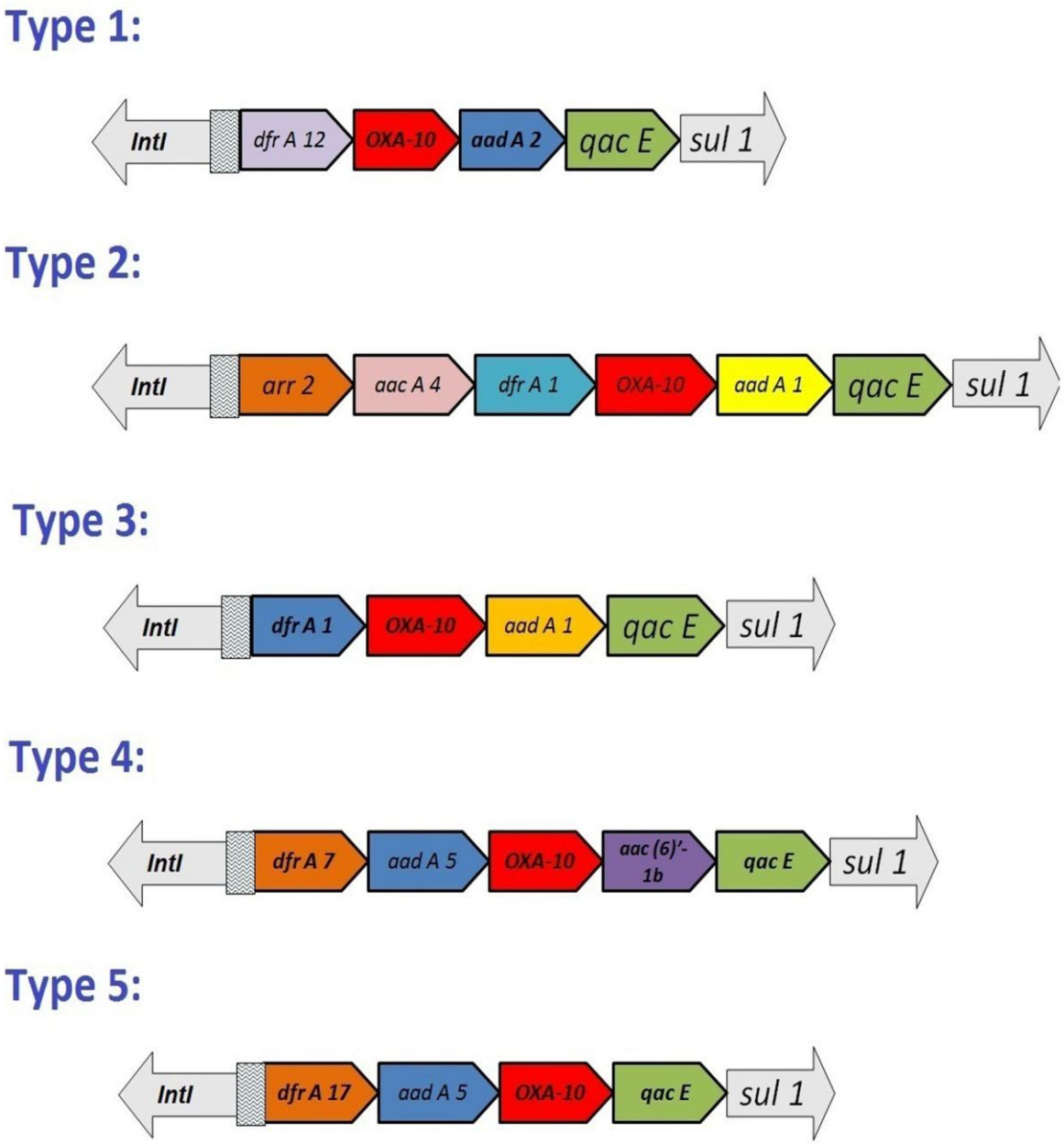


Figure 44: Schematic representation of variable region of class 1 integron types in OXA-10 producing gram negative bacilli

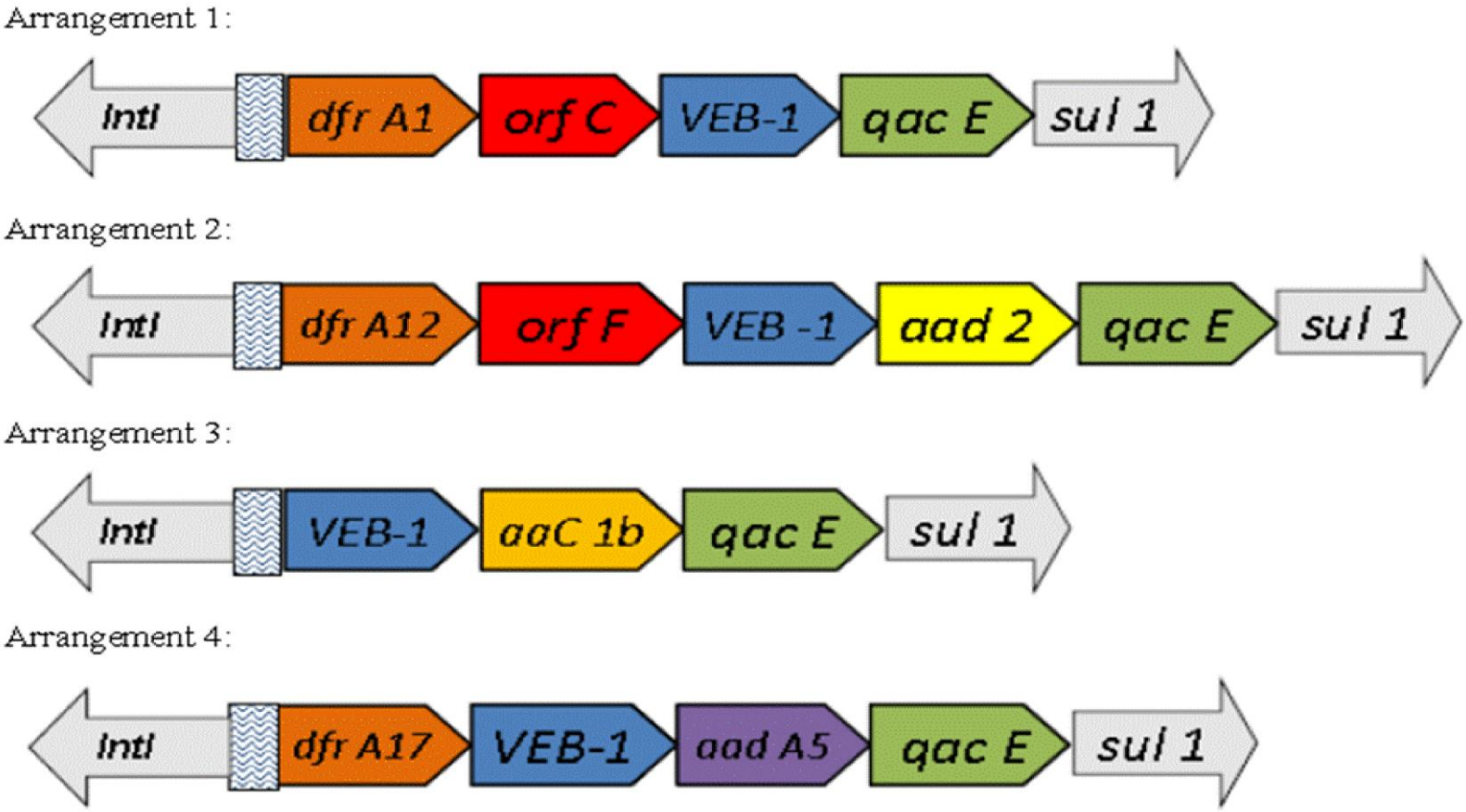


Figure 45: Structural variation of class 1 integron carrying VEB-1

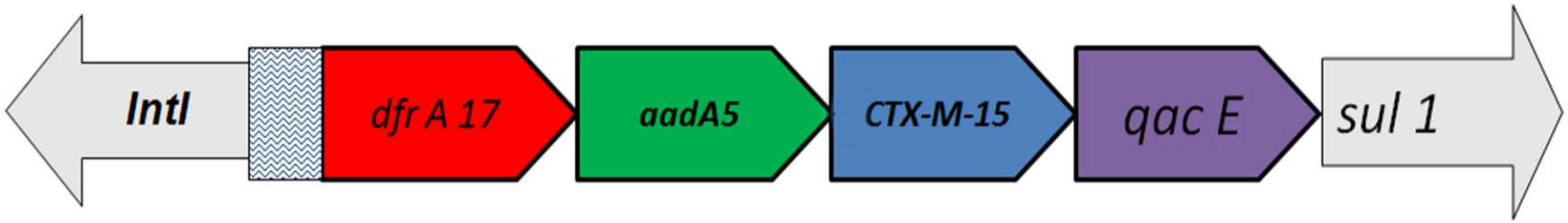


Figure 46: Schematic representation of variable regions of class 1 integron carrying CTX-M-15 in *P. aeruginosa* isolates

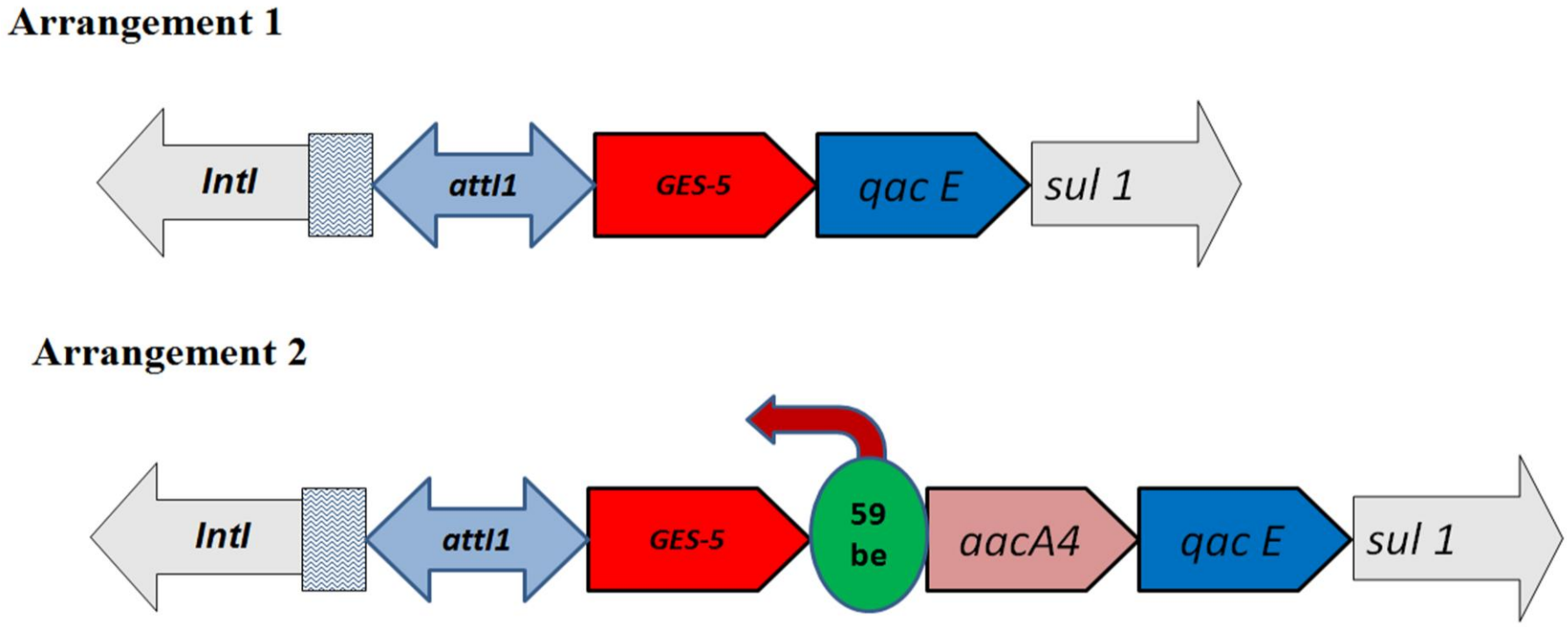


Figure 47: Structural variations of class 1 integron carrying GES-5 ESBL gene

4.13 Association of ESBL gene with mobile genetic elements:

PCR results showed that most CTX-M-15 genes were associated with *tnpISEcp1; tnpIS26;* and *IS26* and SHV-148 associated with *IS26* insertion sequence only. Sequencing results suggested that $bla_{\text{CTX-M-15}}$ was arranged with mobile genetic elements with ten different types of array (Figure 48).

4.14 Genetic array of $bla_{\text{CTX-M-15}}$ genes with mobile genetic elements:

Ten different types of genetic were observed after analysing of sequencing results (Figure 48).

In genetic arrangement **Type 1**; *tnpA, IS26, ISEcp1*, and *ORF477* were associated with *CTX-M-15*. In this arrangement *tnpA* with *IS26* present in reverse orientation in upstream portion followed by *tnpA* with *ISEcp1* gene while in downstream regions *tnpA* followed by *CTX-M-15* was found to be present in reverse orientation with *ORF477* (Figure 48).

In genetic arrangement **Type 2**; *tnpA, IS26, ISEcp1*, and *CTX-M-15* with *ORF477*. Here in upstream regions *tnpA* with reverse orientation of *IS26* followed by *tnpA* in reverse oreientation pursued by *ISEcp1* while in downstream regions *tnpA* was found to be present with *CTX-M-15* and *ORF477* (Figure 48).

In genetic arrangement **Type 3**; *tnpA, ISEcp1, CTX-M-15* and *ORF477* was found to be present. In this insertion gene cassette *tnpA* with *ISEcp1* present in upward direction and *tnpA* with *CTX-M-15* and *ORF477* present in downstream regions (Figure 48).

In genetic arrangement **Type 4**; *tnpA, IS26, ISEcp1, CTX-M-15* and *ORF477* present in association with resolvase gene *tnpR*. In upstream region *tnpA* with *IS26*; followed by *tnpA* in reverse orientation with *tnpR* and *tnpA* with *ISEcp1* while *tnpA* and *CTX-M-15* in reverse orientation with *ORF477* was found to be present in downstream region (Figure 48).

In genetic arrangement **Type 5**; *tnpA, IS26, ISEcp1, CTX-M-15* and *ORF477* was found to be present in the insertion gene cassette. Here *tnpA* with *IS26* and *tnpA* with *ISEcp1* in

reverse orientation in upstream region however *CTX-M-15* with *ORF477* was found to be present in downstream region of insertion gene cassette (Figure 48).

In the genetic arrangement **Type 6**; *tnpA*, *IS26*, *ISEcp1*, *CTX-M-15* and *ORF477* were present. In this type of arrangement *tnpA* with *IS26* and *tnpA* with *ISEcp1* in upstream region followed by *tnpA* with *CTX-M-15* and ORF 477 were present in downstream regions of this arrangement (Figure 48).

In arrangement **Type 7**; *tnpA, IS26, ISEcp1, CTX-M-15* with *ORF477* were present. Here *tnpA* with *IS26* followed by *tnpA* with reverse orientation of *ISEcp1* in upstream region however in downstream region *tnpA* in reverse orientation was found to be present with *CTX-M-15* and *ORF477* (Figure 48).

In genetic arrangement **Type 8**; *IS26, tnpA, ISEcp1, CTX-M-15* and *ORF477* were present in gene cassette. In this arrangement *IS26* followed by reverse orientation of *tnpA* after that *ISEcp1* in upstream part while reverse orientation of *tnpA* followed by *CTX-M-15* and *ORF477* was found to be present (Figure 48).

In the genetic arramgement **Type 9**; *ISEcp1*, *tnpA*, *CTX-M-15* and *ORF477* were found to be present. In upstream region *ISEcp1* followed by *tnpA* while *CTX-M-15* with *ORF477* was present in downstream region (Figure 48).

In the genetic arrangement **Type 10**; *IS26, tnpA, ISEcp1, CTX-M-15* and *ORF477* was present in insertion gene cassette. In the upstream region of this arrangement *IS26* with *tnpA* followed by *ISEcp1* with *tnpA* while reverse orientation of *CTX-M-15* with *ORF477* gene was present in this type of arrangement (Figure 48).

In case of SHV-148 harbouring *E. coli* isolates, $bla_{\text{SHV-148}}$ was found to be associated with mobile gene elements *IS26*.

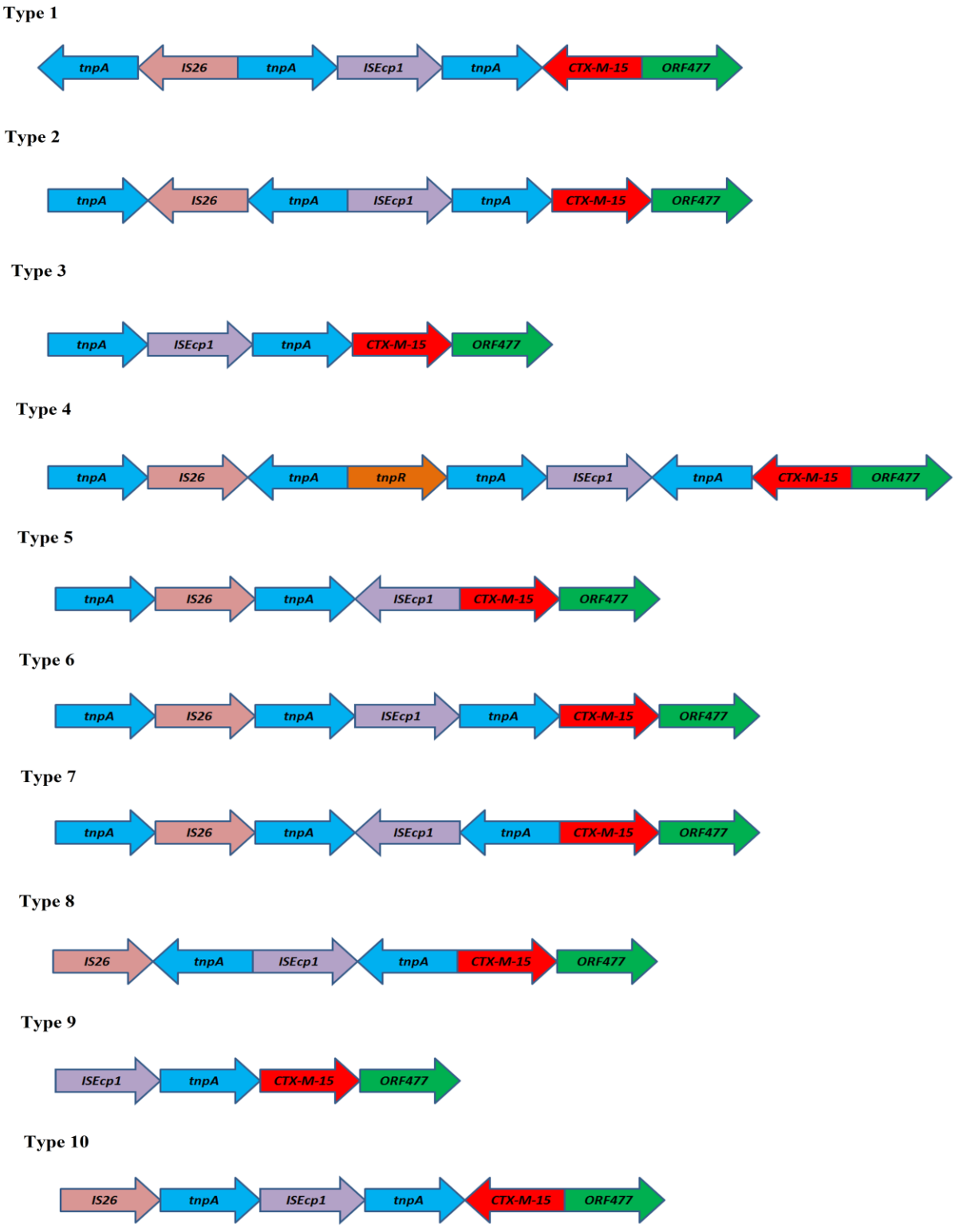


Figure 48: Schematic representation of diverse genetic arrangements of CTX-M-15 genes with mobile genetic elements

4.15 Transferability of *bla*$_{\text{ESBLs}}$

4.15.1 PCR detection of β-lactamase genes in transformants:

A total number of 192 isolates were used for transformation. Of which transformation was successful with 181 isolates (Figure 49A). PCR was performed in 181 transformants and results were indicative that all the ESBL genes could be transformed successfully to the recipient strain. However in 105 transformants, plasmid harboured more than one type of *bla*$_{\text{ESBL}}$ genes would be transferred.

4.15.2 PCR detection of Integron in transformants:

After performing integrase gene PCR, 181 isolates were found to carry integron in their plasmid. Among these, class 1 integron was detected in 141 transformants and class 2 integron in 31 transformants, among which 9 transformants showed both the classes of integrons.

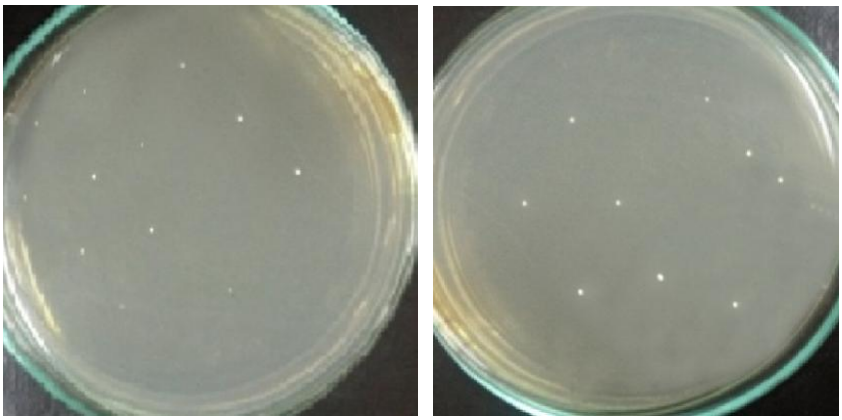
4.15.3 Antibiogram study of transformants (Co-resistance study):

Antimicrobial susceptibility was done after performing transformation for 181 transformants and the following results were obtained (Figure 50).

- 181 transformants were resistant to cefopodoxime.
- 175 transformants were resistant to ceftriaxone.
- 169 transformants were resistant to cefepime.
- 160 transformants were resistant to Aztreonam.
- 111 transformants were resistant to Amikacin.
- 101 transformants were resistant to Ciprofloxacin
- 103 transformants were resistant to Gentamicin.

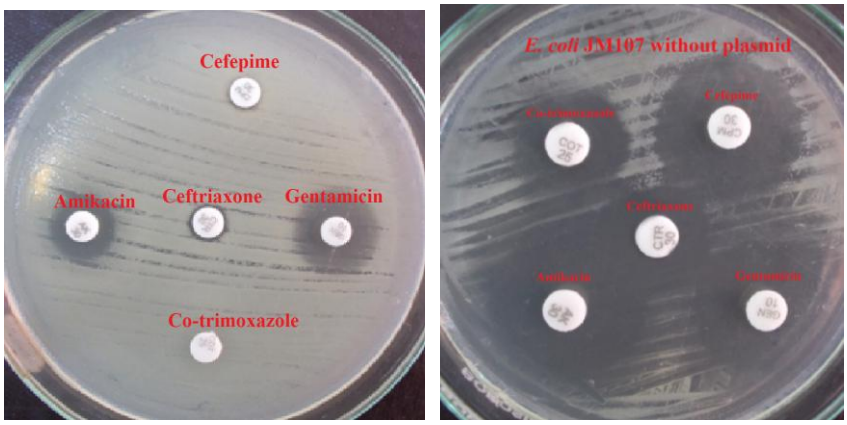
4.15.4 MIC of transformants:

High MIC_{50} and MIC_{90} was observed against all tested cephalosporins in transformants of members of enterobacteriacae and *P. aeruginosa* isolates harbouring single as well as multiple ESBL genes (Table 34-44).



(A) **(B)**

Figure 49 (A): Transformants in *E. coli* JM107 recepient strain; **(B)** Transconjugants in *E. coli* F^- strain (small white colonies)



(A) **(B)**

Figure 50 (A): Antibiotic susceptibility of transformants against β-lactam and non β-lactam antibiotics; **(B)** Antibiotic susceptibility of *E. coli* JM107 without plasmid against β-lactam and non β-lactam antibiotics

Table 34: MIC_{50} and MIC_{90} of *bla*$_{VEB-1}$ harbouring transformants in members of *Enterobacteriacae*

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	4	4	2	4
Meropenem	4	4	2	2
Ertapenem	2	4	2	2
Aztreonam	128	256	128	256
Cefotaxime	128	256	256	256
Ceftazidime	128	256<	256	256<
Ceftriaxone	256	256<	256	256<
Cefepime	128	256	256	256<

Table 35: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{VEB-1}$ in *Pseudomonas aeruginosa* isolates

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	4	4	4	8
Meropenem	4	4	2	4
Ertapenem	2	2	2	2
Aztreonam	256<	256<	256	256
Cefotaxime	256	256	128	128
Ceftazidime	256<	256<	128	256
Ceftriaxone	256<	256<	256	256<
Cefepime	128	256	256	256<

Table 36: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{OXA-10}$ in members of *Enterobacteriacae*

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	4	4	4	4
Meropenem	2	2	4	4
Ertapenem	2	2	2	2
Aztreonam	128	256	32	128
Cefotaxime	32	32	64	64
Ceftazidime	64	64	128	256<
Ceftriaxone	64	64	128	256<
Cefepime	64	128	256	256<

Table 37: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{PER-1}$ in members of *Enterobacteriacae*

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	4	4	4
Meropenem	2	2	4	4
Ertapenem	2	2	2	4
Aztreonam	128	256	128	256<
Cefotaxime	32	128	128	256
Ceftazidime	64	128	256	256<
Ceftriaxone	256	256	256	256
Cefepime	128	256	256	256<

Table 38: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{PER-1}$ in *Pseudomonas aeruginosa* isolates

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	4	4	4
Meropenem	2	2	4	4
Ertapenem	2	2	2	2
Aztreonam	128	256	128	256<
Cefotaxime	64	128	128	256
Ceftazidime	64	128	128	256
Ceftriaxone	128	256<	256	256<
Cefepime	128	256	256	256

Table 39: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{OXA-2}$ in members of *Enterobacteriacae*

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	4	4	4
Meropenem	2	2	2	4
Ertapenem	2	2	2	2
Aztreonam	256	256<	256<	256<
Cefotaxime	128	256	256	256
Ceftazidime	128	128	256	256
Ceftriaxone	256	256	256<	256<
Cefepime	128	256	256	256<

Table 40: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{OXA-2}$ in *Pseudomonas aeruginosa* isolates

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	2	2	8
Meropenem	2	2	2	4
Ertapenem	2	2	2	2
Aztreonam	256	256<	256<	256<
Cefotaxime	128	256	128	256
Ceftazidime	64	128	256	256<
Ceftriaxone	64	256<	256	256<
Cefepime	256	256<	256	256<

Table 41: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{SHV}$ in members of *Enterobacteriacae*

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	2	2	2
Meropenem	2	2	2	2
Ertapenem	2	2	2	2
Aztreonam	16	32	64	128
Cefotaxime	32	64	32	64
Ceftazidime	32	128	32	128
Ceftriaxone	64	256	64	128
Cefepime	16	64	64	128

Table 42: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{SHV}$ in *Pseudomonas aeruginosa* isolates

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	4	2	4
Meropenem	2	2	2	2
Ertapenem	2	2	2	2
Aztreonam	16	256	64	256
Cefotaxime	32	128	128	256
Ceftazidime	64	256	128	256
Ceftriaxone	64	128	256	256<
Cefepime	64	128	128	256

Table 43: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{CTX\text{-}M\text{-}15}$ in members of *Enterobacteriacae*

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	2	2	4
Meropenem	2	2	2	4
Ertapenem	2	2	2	2
Aztreonam	64	128	128	256<
Cefotaxime	32	128	64	128
Ceftazidime	32	128	64	128
Ceftriaxone	32	64	128	256<
Cefepime	32	64	128	256<

Table 44: MIC_{50} and MIC_{90} of transformants harbouring *bla*$_{CTX\text{-}M\text{-}15}$ in *Pseudomonas aeruginosa* isolates

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	4	2	4
Meropenem	2	2	2	4
Ertapenem	2	2	2	2
Aztreonam	64	128	256	256<
Cefotaxime	16	128	256	256<
Ceftazidime	16	128	256	256<
Ceftriaxone	32	256	256	256<
Cefepime	32	256	256	256<

4.16 Analysis of plasmids:

Plasmid was analysed from the transformants carrying ESBL genes of each type and the observation was as following: A ~25 Kb plasmid was found in the isolates carrying OXA-10; ~15 Kb, 18 Kb and 20 Kb plasmid was carrying CTX-M, ~15Kb and 10Kb plasmid was found carrying SHV, ~20 and 30Kb plasmid was found carrying VEB, and ~30 Kb and 40Kb plasmid was found carrying PER genes (Figure 51 A-E). However ~8Kb, 10 Kb, 12Kb, 15Kb, 20Kb, 35Kb, and 40Kb plasmid were common in the isolates harbouring multiple ESBL genes (Figure 51 F).

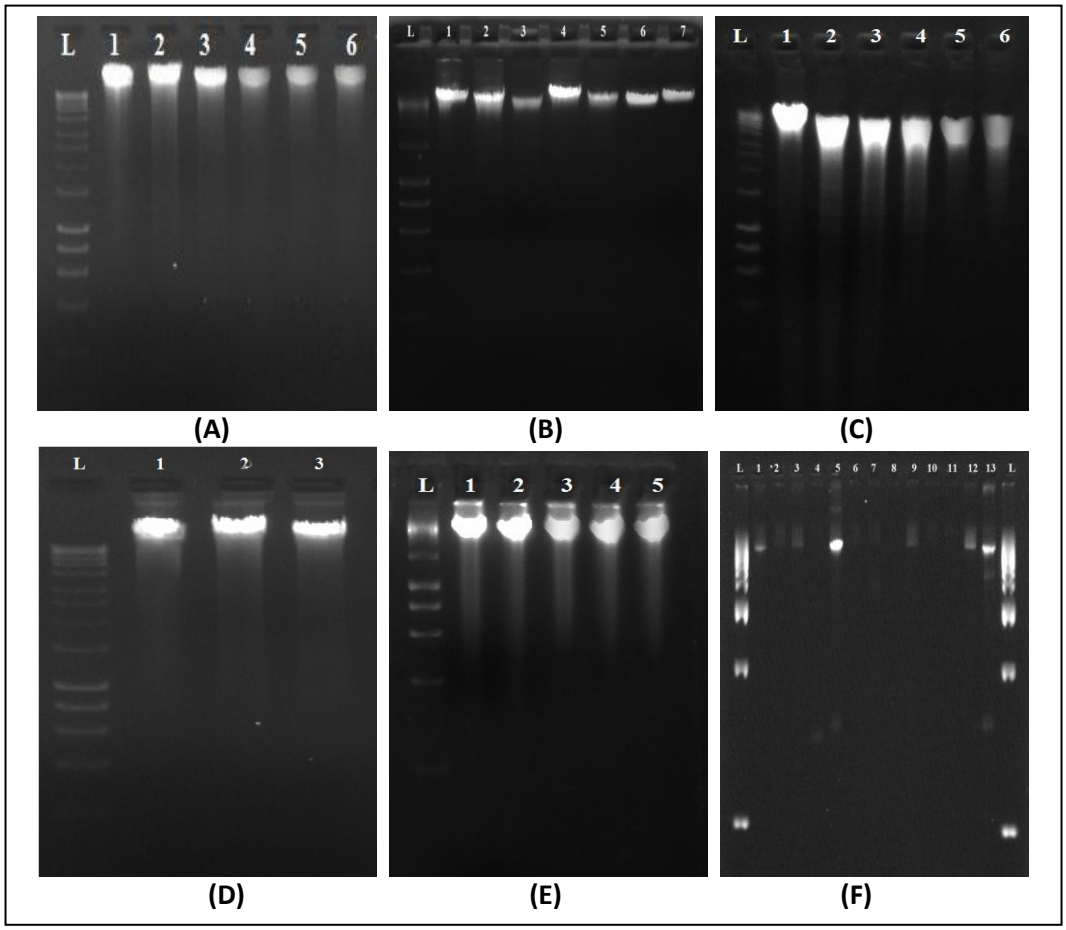


Figure 51: Analysis of plasmid of transformants harbouring ESBL genes.**(A)** plasmid in OXA-10; **(B)** Plasmid in CTX-M; **(C)** plasmid in SHV; **(D)** plasmid in VEB; **(E)** plasmid in PER; and **(F)** plasmid in transformant harbouring multiple ESBL genes.

4.17 Analysis of conjugative transferability of plasmids with ESBL genes:

4.17.1 ESBL production and PCR detection of bla_{ESBL} among transconjugants:

Conjugation experiment was performed with 192 isolates and it was found that 161 isolates were conjugatively transferable (Figure 49 B). All the transconjugants showed ESBL production by combined disk diffusion method and PCR results were indicative that β-lactamase genes were present in all the transconjugants plasmid.

4.17.2 PCR detection of Integrons in transconjugants:

On integrase gene PCR, 161 isolates were found to carry integron in their plasmid. Among these, class 1 integron was detected in 135 transconjugants and class 2 integron in 16 transconjugants, among which 10 transconjugants showed both the classes of integrons.

4.17.3 Resistance transfer and susceptibility testing of transconjugants:

Further susceptibility testing was done after performing conjugation for 161 transconjugants and following results were obtained:

- 161 transformants were resistant to cefopodoxime.
- 151 transformants were resistant to ceftriaxone.
- 145 transformants were resistant to cefepime.
- 140 transformants were resistant to Aztreonam.
- 109 transformants were resistant to Amikacin.
- 101 transformants were resistant to Ciprofloxacin
- 98 transformants were resistant to Gentamicin.

4.17.4 MIC of transconjugants:

High MIC_{50} and MIC_{90} was observed in all transconjugants against all tested cephalosporins (16-256<µg/ml) while in case of carbapenem drug MIC_{50} and MIC_{90} was 2-8µg/ml (Table 45-50).

Table 45: MIC_{50} and MIC_{90} of transconjugants harbouring *bla*$_{CTX-M-15}$

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	MIC_{90}(µg/ml)	MIC_{50}(µg/ml)	MIC_{90}(µg/ml)
Imipenem	2	2	4	4
Meropenem	2	2	2	4
Ertapenem	2	2	2	2
Aztreonam	32	64	256	256<
Cefotaxime	16	32	32	128
Ceftazidime	16	32	32	128
Ceftriaxone	64	32	32	128
Cefepime	32	32	64	256

Table 46: MIC_{50} and MIC_{90} of transconjugants harbouring *bla*$_{OXA-10}$

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	MIC_{90}(µg/ml)	MIC_{50}(µg/ml)	MIC_{90}(µg/ml)
Imipenem	4	4	4	8
Meropenem	4	4	4	4
Ertapenem	4	4	4	4
Aztreonam	256	256<	256<	256<
Cefotaxime	256	256	256<	256<
Ceftazidime	256	256	256<	256<
Ceftriaxone	128	256	256<	256<
Cefepime	128	256	256<	256<

Table 47: MIC_{50} and MIC_{90} of transconjugants harbouring *bla*$_{SHV-148}$

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	2	2	2
Meropenem	2	2	2	2
Ertapenem	2	2	2	2
Aztreonam	32	64	64	256
Cefotaxime	32	64	128	256
Ceftazidime	64	128	256	256
Ceftriaxone	64	128	128	256
Cefepime	64	128	128	256

Table 48: MIC_{50} and MIC_{90} of transconjugants harbouring *bla*$_{OXA-2}$

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	4	4	4	4
Meropenem	4	4	2	4
Ertapenem	2	4	2	4
Aztreonam	64	128	256	256<
Cefotaxime	32	64	256	256
Ceftazidime	32	64	256	256<
Ceftriaxone	32	64	256	256<
Cefepime	64	128	256	256

Tabe 49: MIC_{50} and MIC_{90} of transconjugants harbouring *bla*$_{PER-1}$

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	2	4	4	4
Meropenem	2	2	4	4
Ertapenem	2	2	2	2
Aztreonam	256<	256<	256<	256<
Cefotaxime	128	256	256	256<
Ceftazidime	256	256	256	256<
Ceftriaxone	256	256	128	256<
Cefepime	256	256	256	256<

Table 50: MIC_{50} and MIC_{90} of transconjugants harbouring *bla*$_{VEB-1}$

Antibiotics	Isolates harbouring single ESBL genes		Isolates harbouring multiple ESBL genes	
	MIC_{50}(µg/ml)	**MIC_{90}(µg/ml)**	**MIC_{50}(µg/ml)**	**MIC_{90}(µg/ml)**
Imipenem	4	8	4	8
Meropenem	4	4	4	8
Ertapenem	4	4	4	4
Aztreonam	128	256	256	256<
Cefotaxime	128	256	256	256<
Ceftazidime	256	256<	256	256<
Ceftriaxone	256	256<	256	256<
Cefepime	256	256<	256	256<

4.18 Plasmid Incompatibility typing:

Plasmid incompatibility group typing in transformants suggests that *bla*$_{VEB-1}$ was located within P and F Inc group; OXA-10 within Y Inc group however multiple Inc groups like I1, P, W, N, FIIS, FrepB, FIA, FIB, K, B/o was found to carry CTX-M-15, OXA-2, SHV-148, PER-1 and GES-5 (Figure 52; Table 51-58). Isolates harbouring multiple ESBL genes were originated through diverse Inc group types *viz:* I1, FIA, FIB, W, Y, P, FIC, FIIs, FrepB, K and B/o (Table 59).

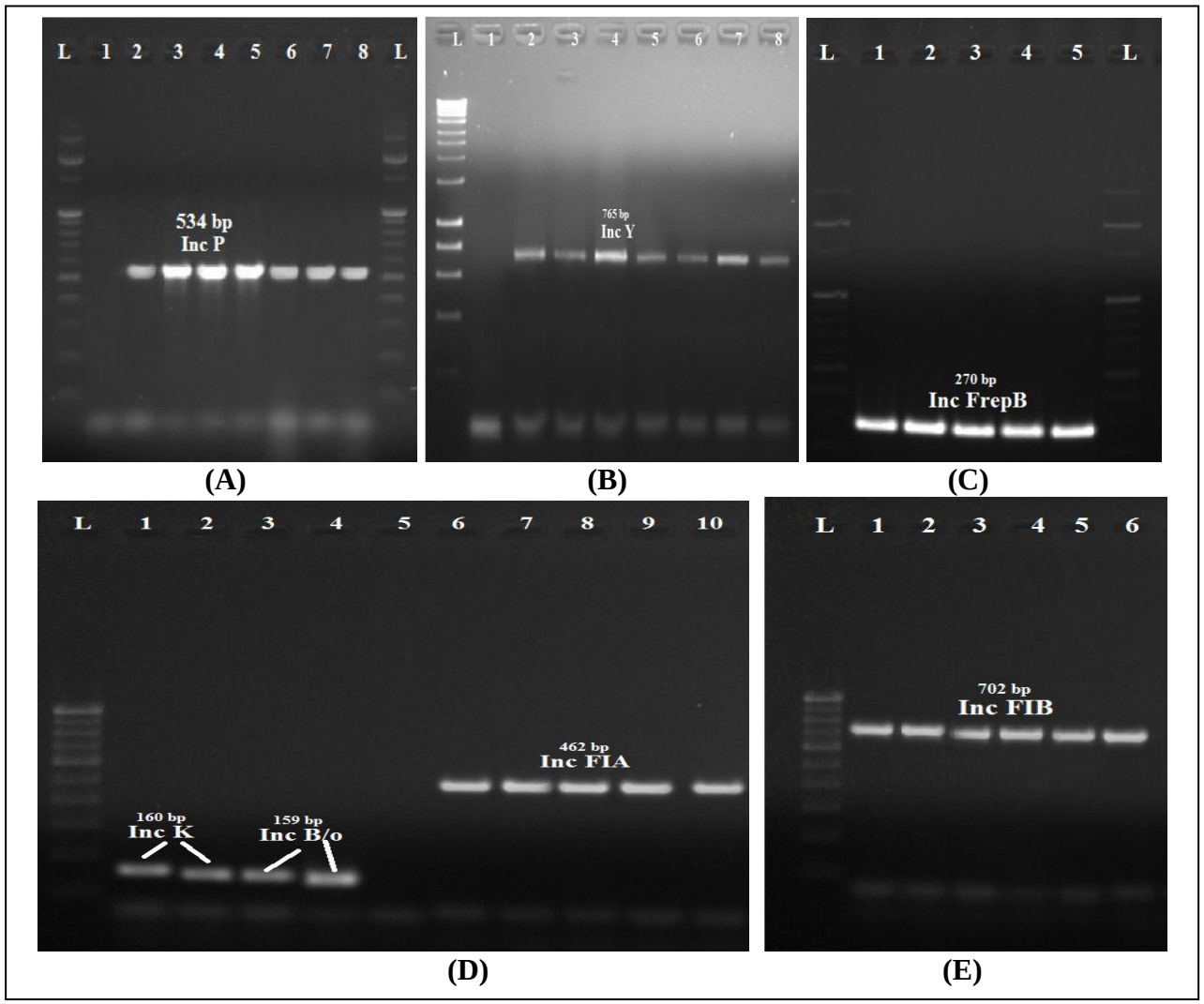


Figure 52: PCR detection of Inc groups in transformants **(A)** 534 bp Inc P in plasmid harbouring *VEB-1*; **(B)** 765 bp Inc Y in plasmid harbouring *OXA-10*; **(C)** 270 bp Inc FrepB; **(D)**160 bp Inc K, 159 bp Inc B/o, 462 bp Inc FIA; and **(E)** 702 bp Inc FIB

Table 51: Incompatibility typing of transformants harbouring $bla_{\text{VEB-1}}$

Replicon Types	Organisms							
	E. coli	***K. pneumoniae***	***K. oxytoca***	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	--	--	--	--	--	--	--	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	--	--	--	--	--	--	--	--
FIA	3	1	1	1	2	--	--	--
FIB	3	1	1	1	2	--	--	--
W	--	--	--	--	--	--	--	--
Y	--	--	--	--	--	--	--	--
P	3	--	--	--	--	14	--	--
FIC	2	--	--	--	--	--	--	--
A/C	--	--	--	--	--	--	--	--
T	--	--	--	--	--	--	--	--
FII_S	--	--	--	--	--	--	--	--
FrepB	2	1	1	--	--	--	--	--
K	--	--	--	--	--	--	--	--
B/O	--	--	--	--	--	--	--	--

Table 52: Incompatibility group typing of transformants harbouring *bla*$_{\text{OXA-10}}$

Replicon Types	Organisms							
	E. coli	***K. pneumoniae***	***K. oxytoca***	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	--	--	--	--	--	--	--	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	--	--	--	--	--	--	--	--
FIA	--	--	--	--	--	--	--	--
FIB	--	--	--	--	--	--	--	--
W	--	--	--	--	--	--	--	--
Y	9	3	--	1	--	--	--	--
P	--	--	--	--	--	--	--	--
FIC	--	--	--	--	--	--	--	--
A/C	--	--	--	--	--	--	--	--
T	--	--	--	--	--	--	--	--
FII$_S$	--	--	--	--	--	--	--	--
FrepB	--	--	--	--	--	--	--	--
K	--	--	--	--	--	--	--	--
B/O	--	--	--	--	--	--	--	--

Table 53: Incompatibility typing of transformants harbouring *bla*$_{\text{CTX-M-15}}$

Replicon Types	Organisms							
	E. coli	***K. pneumoniae***	***K. oxytoca***	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	4	2	2	1	--	2	--	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	2	--	--	--	--	--	--	--
FIA	54	15	15	2	2	29	2	1
FIB	58	17	18	1	1	25	4	1
W	23	--	--	--	--	--	--	--
Y	6	3	1	1	1	5	--	--
P	3	--	--	--	--	3	2	--
FIC	49	28	26	-	-	30	3	--
A/C	--	--	--	--	--	--	--	--
T	--	--	--	--	--	--	--	--
FII$_S$	15	13	14	--	--	14	4	--
FrepB	29	15	17	3	3	30	2	1
K	17	3	4	--	--	18	1	--
B/O	27	10	9	--	--	13	3	--

Table 54: Incompatibility typing of transformants harbouring *bla*$_{\text{OXA-2}}$

Replicon Types	**Organisms**							
	E. coli	***K. pneumoniae***	***K. oxytoca***	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	2	--	--	--	--	--	--	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	--	--	--	--	--	--	--	--
FIA	17	2	2	3	5	2	1	--
FIB	16	6	4	2	3	3	1	--
W	--	--	--	--	--	--	--	--
Y	1	--	--	--	--	--	--	--
P	--	--	--	--	--	--	--	--
FIC	15	10	12	3	4	15	11	--
A/C	--	--	--	--	--	--	--	--
T	--	--	--	--	--	--	--	--
FII$_S$	--	--	--	--	--	--	--	--
FrepB	14	3	3	6	8	2	1	1
K	8	2	1	--	--	--	--	--
B/O	12	5	2	--	--	--	--	--

Table 55: Incompatibility typing of transformants harbouring *bla*$_{\text{SHV-148}}$

Replicon Types	**Organisms**							
	E. coli	***K. pneumonia***	***K. oxytoca***	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	--	--	--	--	--	--	--	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	--	--	--	--	--	--	--	--
FIA	29	19	21	1	1	14	5	1
FIB	32	21	23	1	1	19	2	1
W	--	--	--	--	--	--	--	--
Y	--	--	--	--	--	--	--	--
P	--	--	--	--	--	1	--	--
FIC	27	18	22	--	--	10	3	--
A/C	--	--	--	--	--	--	---	--
T	--	--	--	--	--	--	--	--
FII$_S$	3	2	4	--	1	8	1	--
FrepB	31	18	18	1	1	14	3	2
K	1	1	2	--	--	2	--	--
B/O	1	--	3	--	--	4	1	--

Table 56: Incompatibility typing of transformants harbouring *bla*$_{\text{PER-1}}$

Replicon Types	Organisms							
	E. coli	***K. pneumoniae***	***K. oxytoca***	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	1	--	--	--	--	--	--	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	2	1	--	1	--	--	--	--
FIA	15	1	--	1	--	7	2	1
FIB	15	1	--	--	--	4	1	1
W	--	1	--	--	--	--	--	--
Y	12	1	--	1	--	4	1	--
P	1	--	--	1	--	2	--	--
FIC	15	1	--	--	--	4	2	--
A/C	--	--	--	--	--	--	--	--
T	--	--	--	--	--	--	--	--
FII$_S$	--	--	--	--	--	--	--	--
FrepB	16	1	--	--	--	10	3	1
K	6	--	--	1	--	--	--	--
B/O	10	--	--	--	--	6	2	--

Table 57: Incompatibility typing of transformants harbouring *bla*$_{\text{GES-5}}$

Replicon Types	Organisms							
	E. coli	***K. pneumoniae***	***K. oxytoca***	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	--	--	--	--	--	1	--	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	--	--	--	--	--	--	--	--
FIA	--	--	--	--	--	1	--	--
FIB	--	--	--	--	--	1	--	--
W	--	--	--	--	--	1	--	--
Y	--	--	--	--	--	--	--	--
P	--	--	--	--	--	--	--	--
FIC	--	--	--	--	--	1	--	--
A/C	--	--	--	--	--	--	--	--
T	--	--	--	--	--	--	--	--
FII$_S$	--	--	--	--	--	--	--	--
FrepB	--	--	--	--	--	1	--	--
K	--	--	--	--	--	1	--	--
B/O	--	--	--	--	--	--	--	--

Table 58: Incompatibility typing of transformants harbouring single ESBL genes

Replicon Types	Organisms							
	E. coli	***K. pneumoniae***	***K. oxytoca***	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	2	1	--	1	1	2	2	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	--	--	--	--	--	--	--	--
FIA	21	9	10	5	2	18	2	2
FIB	19	4	7	3	2	15	3	1
W	15	1	--	1	--	--	--	--
Y	4	--	--	--	--	3	2	--
P	4	--	1	--	--	12	7	--
FIC	23	13	11	1	1	17	12	--
A/C	--	--	--	--	--	--	--	--
T	--	--	--	--	--	--	--	--
FII_S	6	6	9	2	1	12	2	--
FrepB	25	12	13	4	3	20	3	3
K	9	3	4	--	1	17	2	--
B/O	14	--	7	1	--	11	2	1

Table 59: Incompatibility typing of transformants harbouring multiple ESBL genes

Replicon Types	Organisms							
	E. coli	***K. pneumoniae***	***K.* oxytoca**	***Proteus mirabilis***	***Proteus vulgaris***	***P. aeruginosa***	***Pseudomonas* Spp.**	***A. baumanii***
HI1	--	--	--	--	--	--	--	--
HI2	--	--	--	--	--	--	--	--
I1	6	1	2	1	-	4	2	--
X	--	--	--	--	--	--	--	--
L/M	--	--	--	--	--	--	--	--
N	--	--	--	--	--	--	--	--
FIA	46	19	22	2	1	19	4	1
FIB	49	17	20	2	1	12	3	2
W	16	2	2	--	1	5	4	--
Y	9	--	--	--	--	2	1	--
P	8	--	--	--	--	2	2	--
FIC	43	11	13	1	--	24	4	2
A/C	--	--	--	--	--	--	--	--
T	--	--	--	--	--	--	--	--
FII_S	33	15	15	4	3	19	5	--
FrepB	52	18	19	2	--	30	1	1
K	38	9	9	--	--	22	1	--
B/O	29	7	6	1	--	15	3	--

4.19 Plasmid stability analysis:

Plasmid stability of *bla*$_{ESBL}$ genes was analyzed and observed that single as well as multiple ESBL genes were highly stable (Table 60 and 61). Following results were observed for each type (Table 60):

- *bla*$_{CTX-M-15}$ were stable after 115 serial passages,
- *bla*$_{OXA-10}$ were stable after 115 serial passages,
- *bla*$_{VEB-1}$ were stable after 115 serial passages,
- *bla*$_{GES-5}$ were stable after 115 serial passages;
- *bla*$_{PER-1}$ were stable till 110 serial passages,
- *bla*$_{SHV-148}$ were stable till 91 passages,
- *bla*$_{OXA-2}$ were stable till 89 serial passages.

However multiple ESBL genes were found highly stable and following results were observed (Table 61):

- Combination of OXA-10 + VEB-1 + SHV-148 were stable after 115 serial passages (OXA-10 for 115; VEB-1 for 115; and SHV-148 for 115 passages),
- Combination of OXA-10 + OXA-2 + SHV-148 + CTX-M-15 + PER-1 were stable after 115 serial passages (OXA-10 for 115; OXA-2 for 115; SHV-148 for 115 CTX-M-15 for 115; and PER-1 for 115 passages),
- Combination of OXA-2 + PER-1 were stable after 115 serial passages (OXA-2 for 115 and PER-1 for 115 passages),
- Combination of OXA-10 + OXA-2 + SHV-148 were stable after 115 serial passages (OXA-10 for 115; OXA-2 for 115 and SHV-148 for 115 passsages),
- Combination of OXA-10 + CTX-M-15 + VEB-1 were stable after 115 serial passages (OXA-10 for 115; CTX-M-15 for 115; and VEB-1 for 115 passages),

- Combination of CTX-M-15 + SHV-148 + VEB-1 were stable after 115 serial passages (CTX-M-15 for 115; SHV-148 for 115 and VEB-1 for 115 passages),
- Combination of GES-5 + SHV-148 + CTX-M-15 were stable after 115 serial passages (GES-5 for 115; SHV-148 for 115; and CTX-M-15 for 115 passages).

Table 60: Plasmid stability analysis of organisms harbouring single β-lactamase genes

***bla*$_{ESBLs}$ type**	**Stability (days)**											
	1-10	**11-20**	**21-30**	**31-40**	**41-50**	**51-60**	**61-70**	**71-80**	**81-90**	**91-100**	**101-110**	**111-115**
CTX-M-15	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
SHV-148	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	--	--	--
OXA-10	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
OXA-2	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	--	--	--	--
PER-1	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	--
VEB-1	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
GES-5	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable

Table 61: Plasmid stability analysis of organisms harbouring multiple β-lactamase genes

Multiple *bla* genes	Stability (days)											
	1-10	**11-20**	**21-30**	**31-40**	**41-50**	**51-60**	**61-70**	**71-80**	**81-90**	**91-100**	**101-110**	**111-115**
OXA-10 + VEB-1 + SHV-148	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
OXA-10 + OXA-2 + SHV-148 + CTX-M-15 + PER-1	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
OXA-2 + PER-1	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
OXA-10 + OXA-2 + SHV-148	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
OXA-10 + CTX-M-15 + VEB-1	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
CTX-M-15 + SHV-148 + VEB-1	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
GES-5 + SHV-148 + CTX-M-15	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable

4.20 PCR mapping of integron carrying multiple ESBL genes:

Sequencing results confirmed that in *P. aeruginosa* and *E. coli* isolates multiple ESBL genes were arranged in a linear fashion within class 1 integron in six diverse types of arrangements. Arrangement 1 (Figure 53) was observed in *P. aeruginosa* isolated from surgery ward, harboured OXA-10, OXA-2 and SHV-148 genes. Arrangement 2 (Figure 53) was found in *E. coli*, isolated from surgery ward, harboured three ESBL genes OXA-10, VEB-1 and SHV-148 in integron gene cassette where SHV-148 was in reverse orientation. Arrangement 3 (Figure 53) was observed in *P. aeruginosa* isolated from surgery ward. This isolate was found to be carrying CTX-M-15, OXA-10 and VEB-1 genes. Arrangement 4 (Figure 53) was found in *P. aeruginosa* from Medicine clinics. In this arrangement CTX-M-15, SHV-148 and VEB-1 gene was found to be present. Arrangement 5 (Figure 53) was found in *P. aeruginosa* from paediatrics ward. This isolate was found to be carrying GES-5, SHV-148 and CTX-M-15 genes. Arrangement 6 (Figure 53) was found in *E. coli* isolate from female burn unit. This isolate was harbouring PER-1, OXA-2, SHV-148, CTX-M-15 and OXA-10 genes. In this arrangement all these genes were found in the reverse orientation within the class 1 integron gene cassettes.

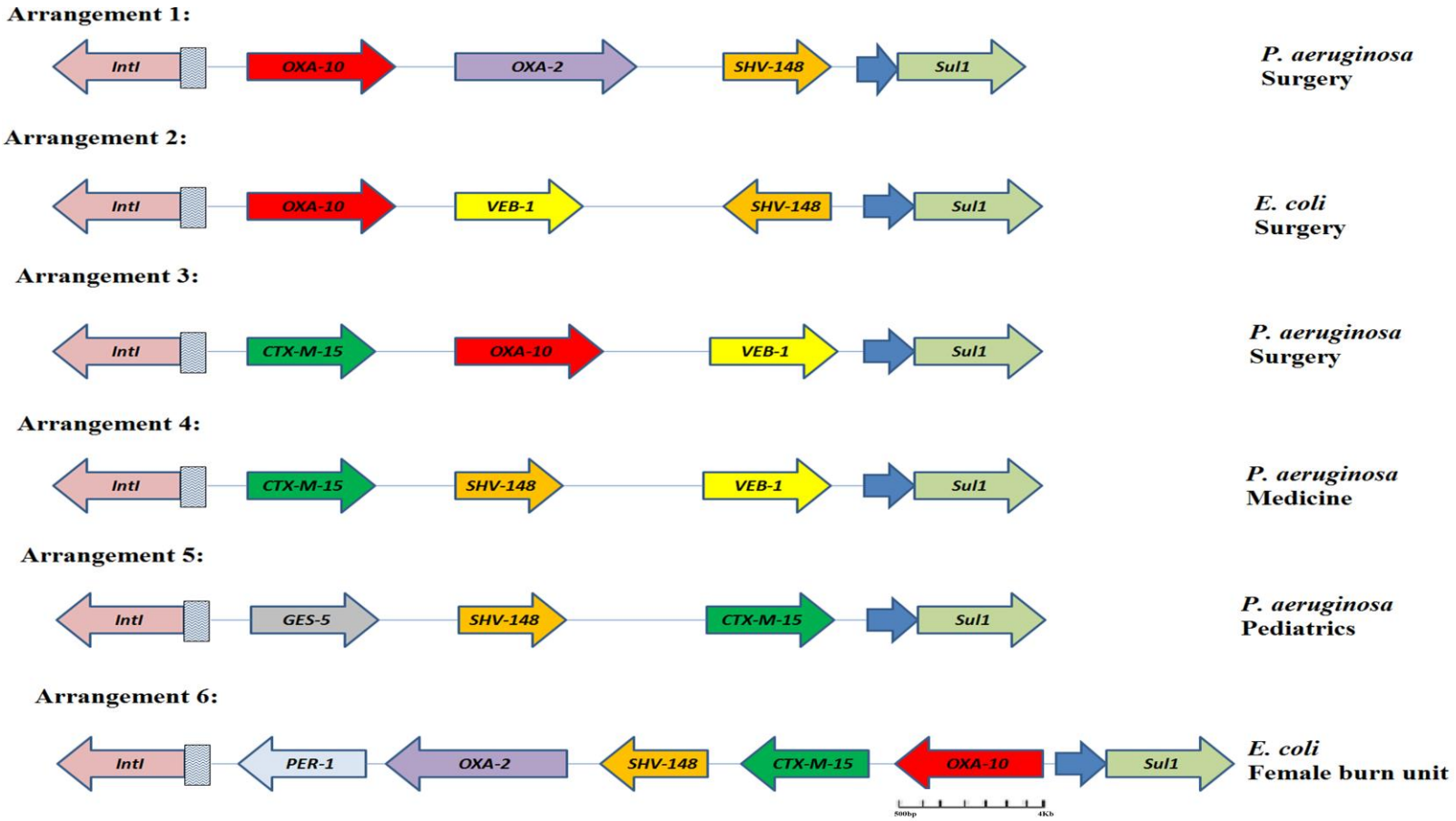


Figure 53: Schematic diagram of integron mapping of *P. aeruginosa* and *E. coli* isolates harbouring multiple ESBL genes arranged within six types of genetic arrangements

4.21 Detection of transcription level of multiple ESBL genes

While performing quantitative Real Time PCR, it was observed that SHV-148 was expressed more compared to other ESBL genes when the organism was grown without any antibiotics (Figure 54). In CTX-M-15 transcription level was high when induced with ceftriaxone (CRO) (Figure 55), however transcription of this gene was reduced significantly below the basal level when ceftazidime (CAZ) was used in the growing culture. In case of SHV-148 transcription was increased in the presence of ceftriaxone (CRO) and significant increase was also noticed with cefotaxime (CTX) and ceftazidime (CAZ) (Figure 56). For PER-1, ceftazidime (CAZ) could enhance the expression of this gene more compared to ceftriaxone (CRO) and cefotaxime (CTX) (Figure 57). But in case of OXA-10 and OXA-2, there was no transcription under the induced and non induced condition (Figure 58 and 59). By statistical analysis with Graph prism software using One way ANOVA, all the data was found in significant level ($P<0.0001$) and by Turkey's multiple comparison test data was found in significant and P value was $P<0.0001$.

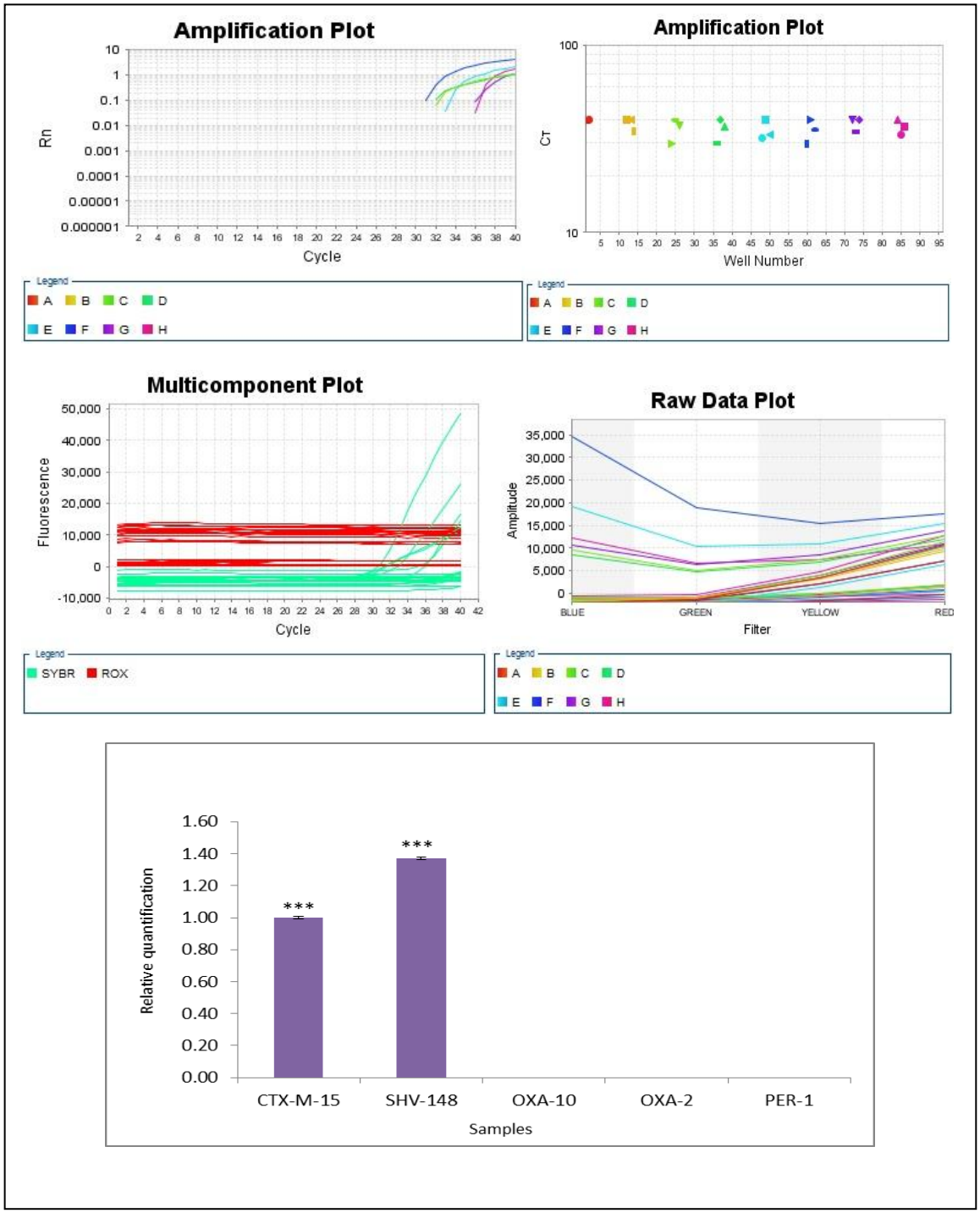


Figure 54: Expression of multiple ESBL genes without any antibiotic pressure

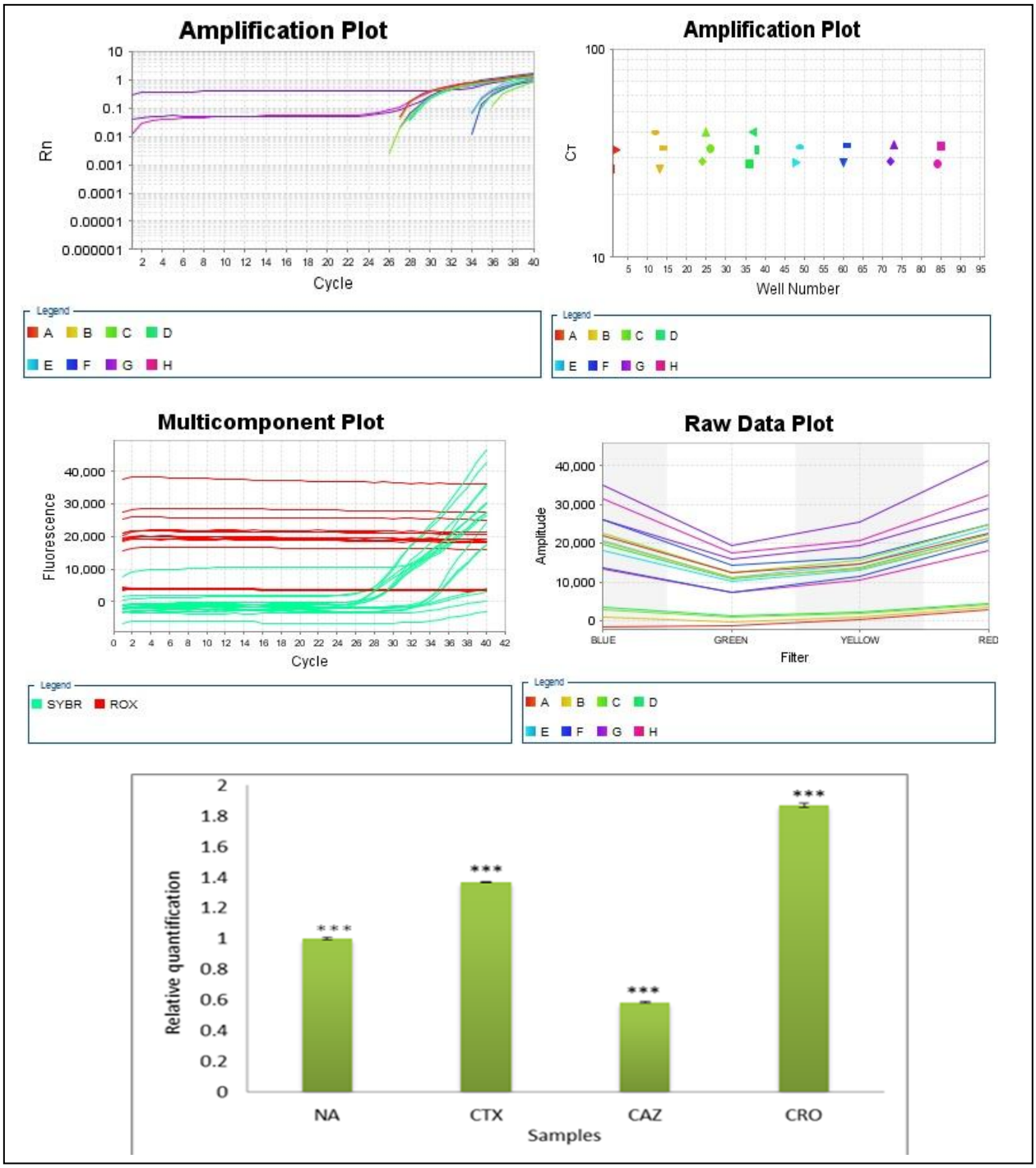


Figure 55: Expression of CTX-M-15 under inducing condition

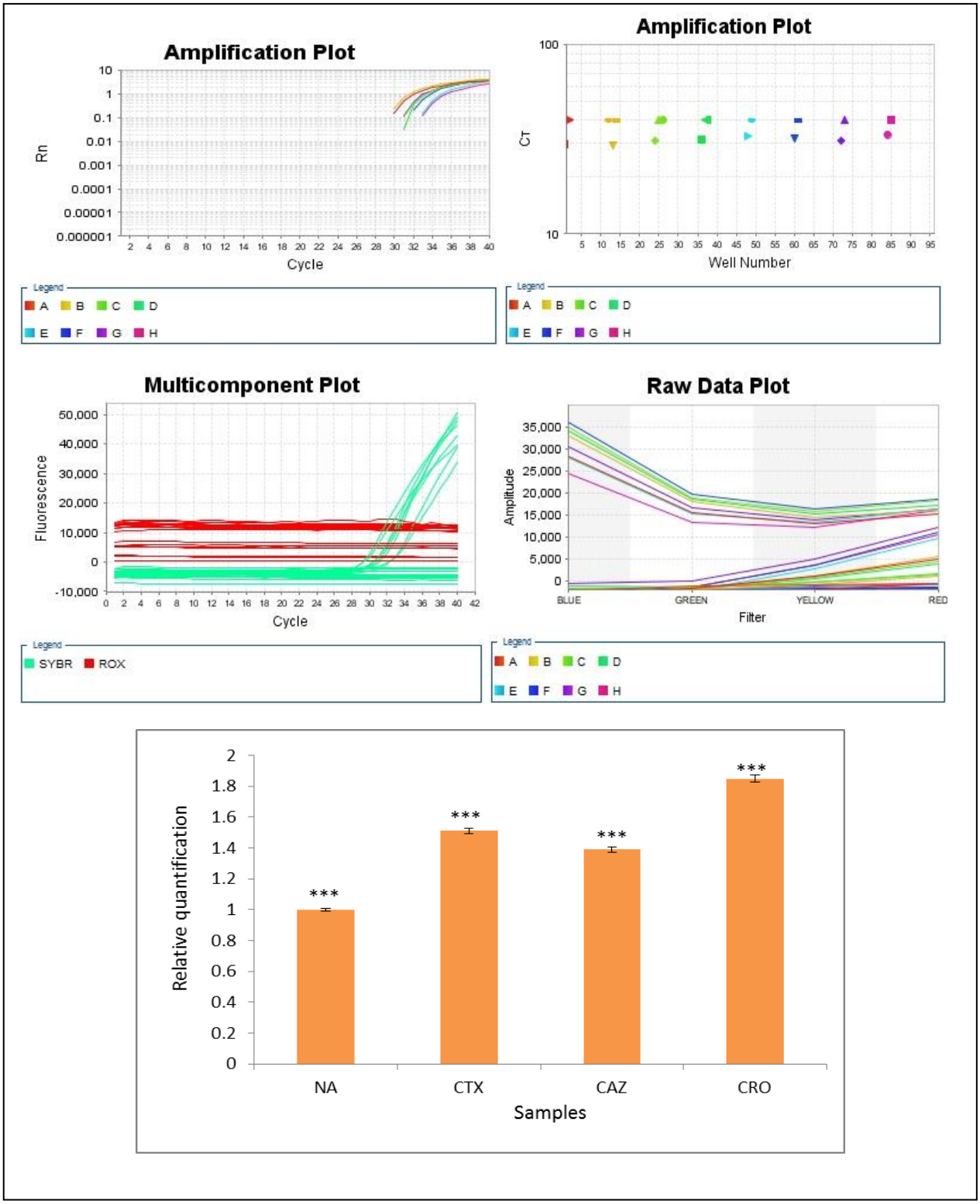


Figure 56: Expression of SHV-148 under inducing condition

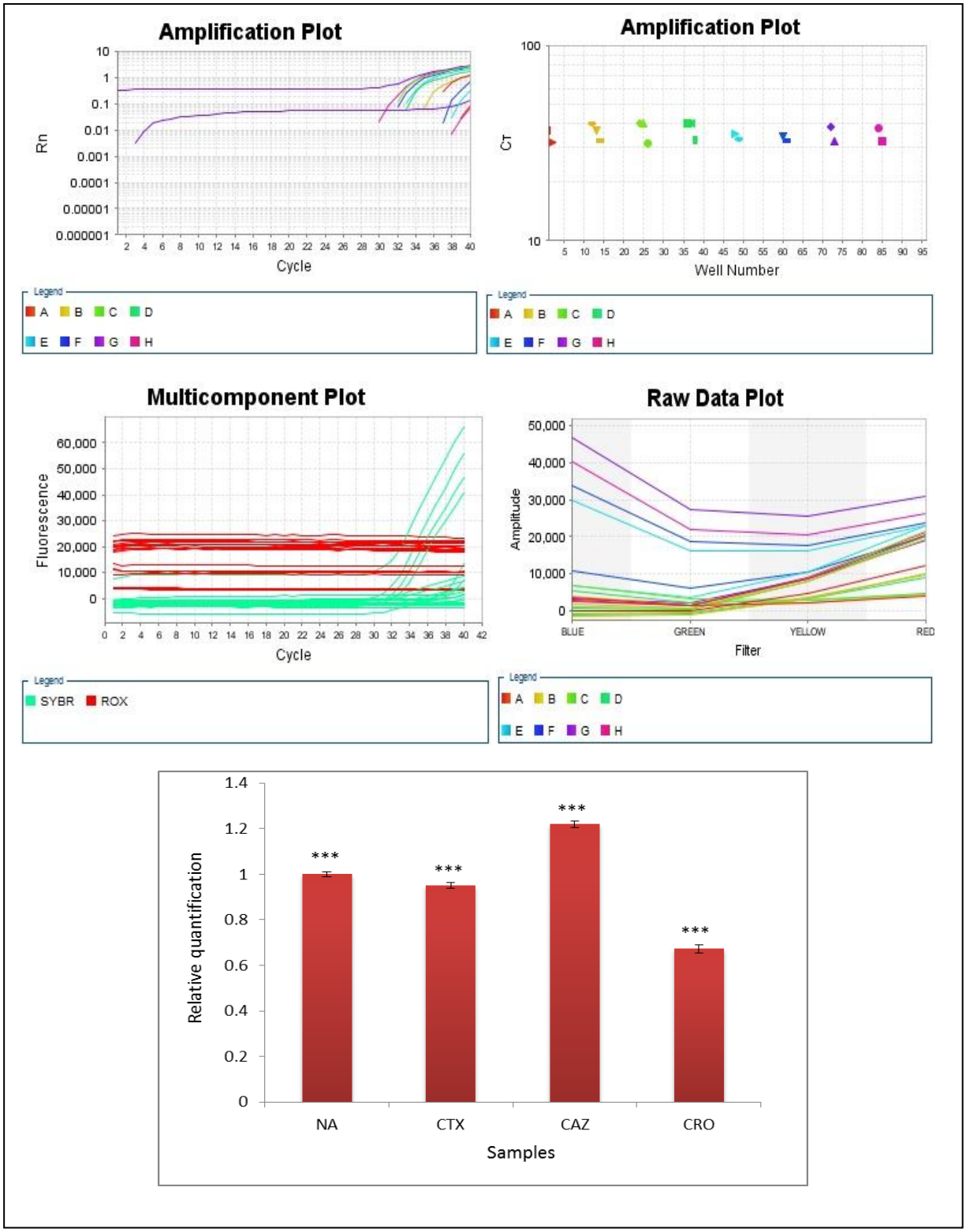


Figure 57: Expression of PER-1 under inducing condition

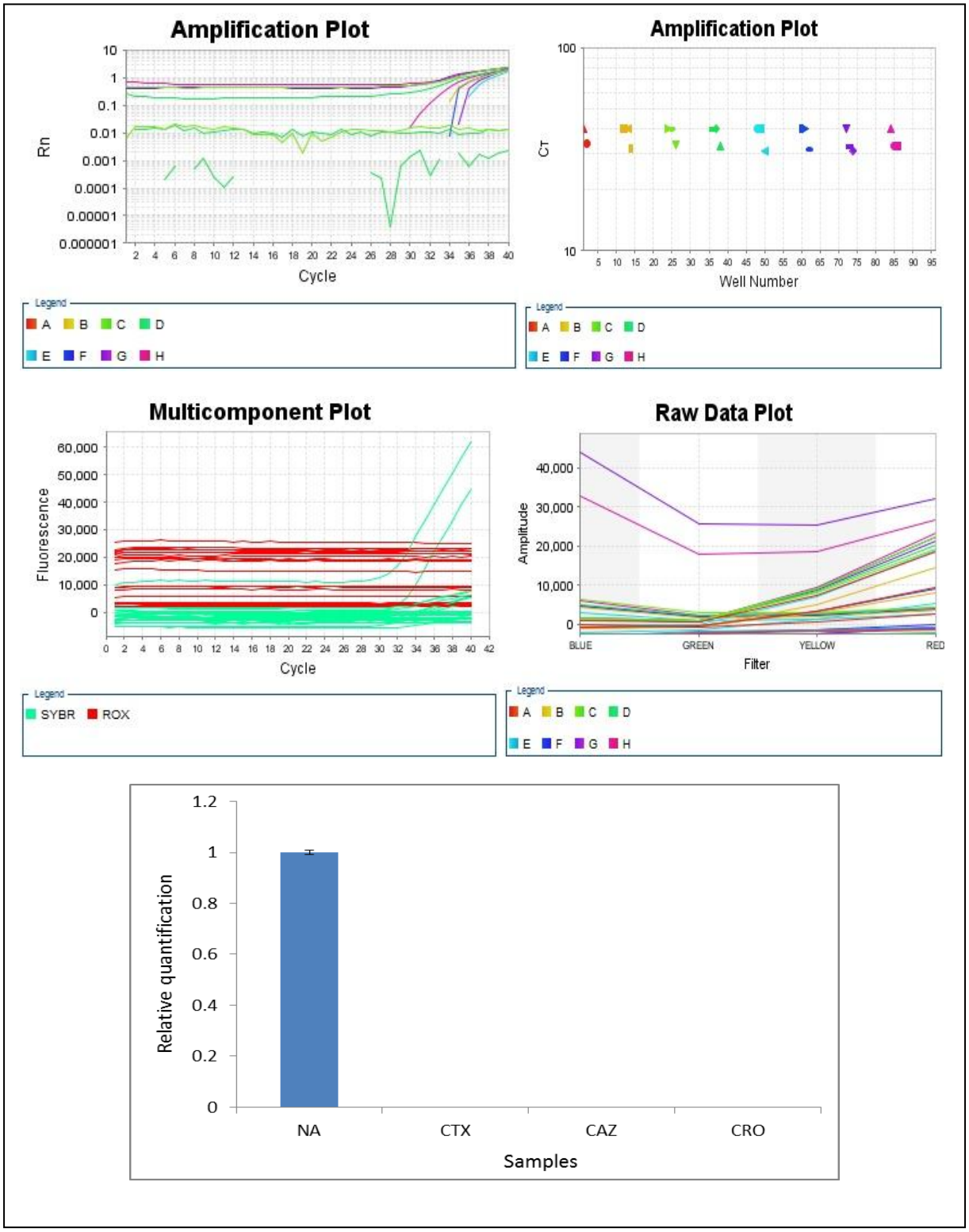


Figure 58: Expression of OXA-10 under inducing condition

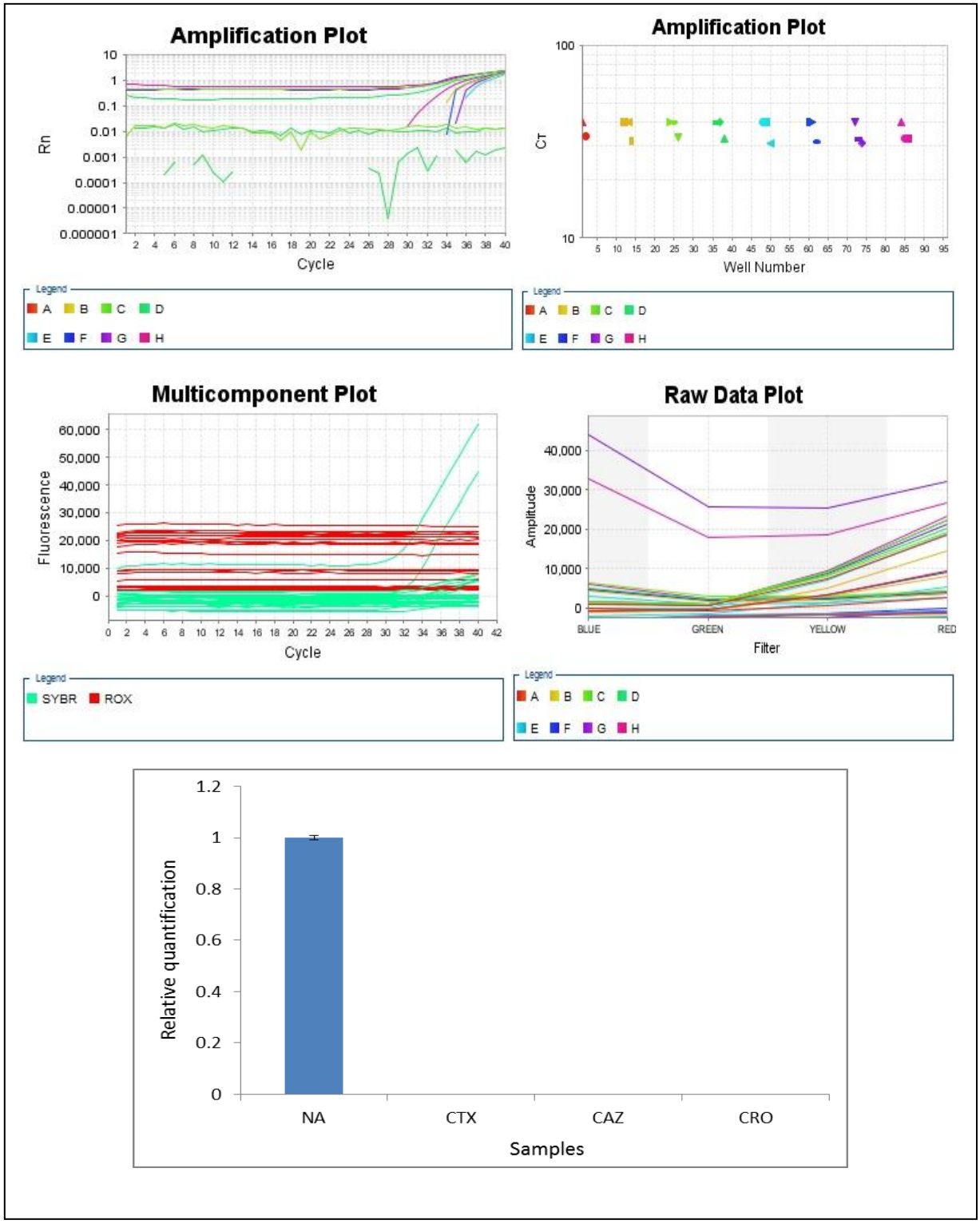


Figure 59: Expression of OXA-2 under inducing condition

Statistical analysis of CTX-M-15

Table Analyzed	
t test data	
One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	7341
R squared	0.9996

ANOVA Table	SS	df	MS
Treatment (between columns)	2.661	3	0.887
Residual (within columns)	0.000967	8	0.000121
Total	2.662	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
A vs B	-0.3693	58.2	P < 0.001	-0.3981 to -0.3406
A vs C	0.408	64.29	P < 0.001	0.3793 to 0.4367
A vs D	-0.87	137.1	P < 0.001	-0.8987 to -0.8413
B vs C	0.7773	122.5	P < 0.001	0.7486 to 0.8061
B vs D	-0.5007	78.89	P < 0.001	-0.5294 to -0.4719
C vs D	-1.278	201.4	P < 0.001	-1.307 to -1.249

Statistical analysis of SHV-148:

Table Analyzed	
t test data	
One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	477.4
R squared	0.9944

ANOVA Table	SS	df	MS
Treatment (between columns)	1.098	3	0.366
Residual (within columns)	0.006133	8	0.000767
Total	1.104	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
A vs B	-0.3933	24.6	P < 0.001	-0.4657 to -0.3209
A vs C	-0.51	31.9	P < 0.001	-0.5824 to -0.4376
A vs D	-0.8467	52.96	P < 0.001	-0.9191 to -0.7743
B vs C	-0.1167	7.298	P < 0.001	-0.1891 to -0.04427
B vs D	-0.4533	28.36	P < 0.001	-0.5257 to -0.3809
C vs D	-0.3367	21.06	P < 0.001	-0.4091 to -0.2643

Statistical analysis of PER-1

Table Analyzed	
t test data	
One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	306.6
R squared	0.9914

ANOVA Table	SS	df	MS
Treatment (between columns)	0.4599	3	0.1533
Residual (within columns)	0.004	8	0.0005
Total	0.4639	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
A vs B	0.05333	4.131	P > 0.001	-0.005136 to 0.1118
A vs C	-0.2233	17.3	P < 0.001	-0.2818 to -0.1649
A vs D	0.3267	25.3	P < 0.001	0.2682 to 0.3851
B vs C	-0.2767	21.43	P < 0.001	-0.3351 to -0.2182
B vs D	0.2733	21.17	P < 0.001	0.2149 to 0.3318
C vs D	0.55	42.6	P < 0.001	0.4915 to 0.6085