

Adaptation of IncX3 plasmid encoding blaNDM-4 within broad host range: A study from India

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Research article

Keywords: BHR (Broad host range), NDM, plasmids, IncX3, NDM, Escherichia coli

Posted Date: June 2nd, 2019

DOI: <https://doi.org/10.21203/rs.2.9985/v1>

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Abstract

Different variants of blaNDM has been reported across Indian subcontinent within diverse host range of gram negative bacilli. Most of their transferability depends on types of plasmid that encodes resistance determinant. IncX type plasmid is more recently known for carrying carbapenem resistance and NDM-4 variant is considered to have better hydrolytic property. This study aims to investigate the maintenance and stability of blaNDM-4 within BHR and transcriptional response. E.coli of clinical origin that harbor blaNDM-4 within IncX3 plasmid were used. Six different hosts were selected for gene transfer experiment, two strains of E.coli, K.pneumoniae, P.mirabilis, A.baumannii and P.aeruginosa. All the transformants and parent isolate were subjected for stability within new host. IncX3 plasmid encoding blaNDM-4 was transferred in host when imipenem (0.5µg/ml) screen agar was used for selection of transformants which are coharbouring resistance for aminoglycosides and quinolone. Plasmid was successfully expanded within all the six recipients till 55th serial passages. Transcriptional expression with NDM gene was variable for parent isolates though for new hosts it was showing random increased pattern in Proteus, E.coli, and DH5α. This could highlight, external carbapenem pressure helps in maintenance and expression of blaNDM-4 within different host range and epidemiological significance and will help in tracking the genetic vehicle responsible for their transmission, restricting their spread.

Background

IncX type plasmids were previously known to be of narrow host range. Based on their restriction profile subtypes X1, X2 were formed (1). Later, based on phylogenetic differences, Johnsen et al., 2012 reported another two subtypes X3 and X4 (2). Inc X3 is better known for its ability to carry diverse types of resistance genes. Recently this subtype is more linked with *bla*_{NDM-4} and *bla*_{NDM-7} across the globe with a number of cases from India (3-5). Thus this plasmid type is probably emerging as a potential genetic vehicle for lateral expression of New Delhi Mettalo beta lactamases in this subcontinent. Although most reports of this plasmid type is within enterobacteriaceae, they may have potential chance to be disseminated to broader host range within hospital settings. The ability of plasmids to transfer, replicate and persist within a new host makes it most adapted and beneficial for the host. These adapted plasmids must help bacteria to survive against antibiotic exposure. Thus it would be interesting to know how these plasmids responds and when carbapenem therapy is initiated. Also how the resistance gene are expressed within different hosts when carbapenem stress is given. Therefore the present study was designed to investigate the transferability, stability, transcriptional response and copy number alteration of IncX3 type plasmids against carbapenem stress.

Methods

Bacterial sample:

Six isolates of *Escherichia coli*, harboring *bla*_{NDM-4}, isolated from hospital patients of Silchar Medical College and Hospital, India were selected for the study (Table1). The carriage of *bla*_{NDM-4} was confirmed

by PCR sequencing of whole gene. Plasmid incompatibility was determined by PCR assay (2). Presence of *bla*_{NDM} was determined by PCR assay using primers (NDM-F 5-GGGCAGTCGCTTCCAACGGT-3 and NDM-R 5-GTAGTGCTCAGTGTCGGCAT-3) (6). The isolates were identified by Gram staining method, standard biochemical characterization tests including IMViC test, urease test, triple sugar iron test, sugar fermentation test and nitrate reduction test and finally by 16s rDNA sequencing

Plasmid preparation and transmission assay:

Plasmids encoding *bla*_{NDM-4} were extracted by QIAprep Spin Miniprep Kit (Qiagen, Germany) as per manufacturer's instruction. Isolated plasmids were subjected to transformation assay. The recipient strain used was *E.coli* JM107, *E.coli* DH5 α , *Klebsiella pneumonia*, *proteus mirabilis*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* of clinical origin. Transformation was carried out by heat shock method (7). Transformants were selected on LB Agar plates containing ampicillin (100 μ g/ml). Conjugation experiment was performed using *bla*_{NDM-4} harbouring clinical strains as donors and azide resistant *E. coli* J53 as recipient and transconjugants were selected on medium containing either imipenem (0.5 μ g/ml) or ampicillin (100 μ g/ml) alongwith sodium azide (100 μ g/ml).

Plasmid stability within different hosts:

Plasmid stability analysis of *bla*_{NDM-4} producers in parent strain (*E.coli*) and transformants in different hosts i.e, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *E.coli* DH5 α) was performed by the serial passage method for consecutive 70 days at 1:1000 dilutions without any antibiotic pressure (8) After each passage, 1ml of the culture was diluted into 10³ dilution with normal saline, and 40 μ l of the diluted sample was spread on to the LB agar plate. After overnight incubation, 50 colonies from plates were randomly picked and subjected to phenotypic detection of MBL and further confirmed genotypically by PCR assay for the presence of *bla*_{NDM-4}.

Plasmid copy number alteration and transcriptional expression of *bla*_{NDM-4} within broad host range against concentration gradient imipenem stress:

Single colony of each host isolate was inoculated into LB broth with 1 μ g/ml, 2 μ g/ml, 4 μ g/ml and 8 μ g/ml of imipenem and also without any antibiotic (considered as a control for the reaction) and was incubated at 37°C for 4–7 hour till the OD reaches 0.9 at A₆₀₀. cDNA was extracted from each condition, the reaction was performed using 10 μ l of SYBR® Green PCR Master Mix (Applied Biosystem, Warrington, UK), 4ng plasmid DNA as template and 3 μ l of each primer (table 2) (10pmol) in a 20 μ l reaction and the relative fold change was measured by $\Delta\Delta$ CT method and was normalized against a housekeeping gene *rpsl* of *E. coli* (9). The each set of reaction was run in triplicate and the experiment was repeated thrice. Quantitative Real Time PCR was done to determine the level of alteration of the plasmid encoding *bla*_{NDM-4} using Step One Plus real time detection system (Applied biosystem, Warrington).

Susceptibility testing:

The antibiotic susceptibility was done by Kirby Bauer disc-diffusion method against antibiotics as piperacillin-tazobactam (100/10 µg), amikacin (30µg), gentamicin (10µg), ciprofloxacin (5µg), polymixin B (300units) ampicillin (30µg), cotrimoxazole (10µg) and carbenicillin (100µg) (Hi-Media, Mumbai, India). Minimum inhibitory concentration was performed by agar dilution method against imipenem, meropenem, cefepime & aztreonam, cotrimoxazole, ampicillin, ciprofloxacin and the results were compared with standard CLSI guidelines (10). The antibiotic susceptibility of the transformants was also determined

Result

Plasmids were obtained from 6 positive isolates which were showing IncX3 incompatible type in their genetic characteristics. Transformation assay was done with all those 6 isolates and found to be transferable in ampicillin screen agar as well as ciprofloxacin and gentamicin screen agar, and transformants were targeted to have the incompatible type i.e IncX3. Plasmid stability was checked by serial passage in 1:1000 dilution and was found that IncX3 type was carried till 55th passage.

Susceptibility pattern of cured hosts:

Susceptibility results of cured hosts revealed that all the hosts (*E.coli*, *Klebsiella pneumoniae*, DH5α, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) became susceptible to carbapenems (imipenem, meropenem), aminoglycosides (gentamicin, ampicillin) and quinolone (ciprofloxacin) group of antibiotics (Table 3 &4)

On analyzing the copy number for new hosts were variable under concentration gradient stress. On analyzing copy number of IncX₃ type plasmids it was observed that plasmid copy number increases with the increase of imipenem concentration in *Acinetobacter baumannii* but in all the hosts including the wild type expression level decreased with the increase in gradient antibiotic concentration of imipenem. The transcriptional expression with IncX₃ marker was random. There was a consistent level of transcriptional response for wild type with and without concentration gradient of imipenem stress (Fig1 & 2).

The effect of carbapenem (imipenem) antibiotic on the expression of *bla*_{NDM-4} gene, the transcriptional level of this gene was determined after exposing the harboring organism along with the new hosts (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) with carbapenem antibiotic. It has been observed that though the expression pattern of *bla*_{NDM-4} was showing no significant increase of expression. In case of wild type the transcriptional expression was variable whereas in case of new host, although expression level was random, the increased pattern was observed in all new host except *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Fig 3 & 4).

Discussion And Conclusion

Expansion of IncX plasmid family was first proposed by Johnson et al based on phylogenetic differences within conserved regions of 18 sequenced IncX plasmids into another two subtypes, IncX3 and IncX4 (2). *Klebsiella pneumonia* ST429 isolate was found to carry *bla*_{KpC-5} which harbours plasmid assigned to have IncX5 subgroup (11). Till date IncX plasmids were found to carry various antibiotic resistance genes, e.g. Pmqr genes (qnrS1,oqxAB), ESBL genes (*bla*_{TEM-52}, *bla*_{CTX-M}, *bla*_{SHV-12}), carbapenemase genes (*bla*_{NDM}) and others (12-13). The findings as indicate that this plasmid type can carry diverse range of resistance genes in enterobacteriaceae.

IncX3 type is linked encoding of different NDM variants across the globe (3-4,14). This study also reports the presence of *bla*_{NDM4} within IncX3 type plasmid. Thus, analysis of this plasmid by determining the copy number alteration is of utmost importance. The study could highlight that plasmid copy number of IncX3 type is maintained under carbapenem stress in diverse host range. The finding is quite unique to the earlier studies where IncX is regarded as having narrow host range (15).

Plasmid copy number is dependent on the type of organism which acts as host of that plasmid and the origin of replication. It is also reported that mutation can bring high copy number (16). The current study showed antibiotic pressure helps in maintenance and adaptation of IncX type plasmid within diverse host range although there was no significant alteration of plasmid copy number. The study establishes a linkage among selection pressure, stability and copy number of plasmids encoding resistance genes.

The study also investigates analysis of transcriptional expression of *bla*_{NDM} encoded within IncX3 type within different hosts and it was observed that the gene was transcriptionally expressed in all the host ranges. This could be due to the adaptation of this plasmid in an unknown host machinery. Thus, this finding is of significant importance with respect to future infectious diseases risk assessment, evaluating and minimizing the selective pressure in clinical settings thereby, slowing down the horizontal transmission of multidrug resistance.

List Of Abbreviations

Inc- Incompatibility, IncX- Incompatibility X type, IncX3- Incompatibility X3, *bla*_{NDM} –new delhi metallo betalactamase, NDM- ew delhi metallo beta lactamase, PCR- polymerase chain reaction, *E.coli*- *Escherichia coli*, BHR- broad host range, NDM F primer- new delhi metallo betalactamase forward primer, NDM R primer- new delhi metallo beta lactamase reverse primer, IMViC test- Indole Methyl red Voges proskauer Catalase test, rDNA- recombinant Deoxyribo Nucleic Acid, LB agar- Luria Bertany agar, LB broth- Luria Bertany broth, OD- optical density, cDNA- complementary deoxy ribo nucleic acid, DNA- deoxy ribo nucleic acid, CT- threshold cycle, ESBL- extended spectrum beta lactamases, CLSI- clinical and laboratory standard institute

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication:

All the authors have gone through the manuscript and agreed to publish the paper in esteemed journal

Availability of data and material

All supporting data are provided in the manuscript. If any secondary data required corresponding author will provide it to the readers

Competing interests

No other conflicts of interest exist.

Funding

The authors received no specific grant from any funding agency.

Authors' contributions

NAC has conceived the study plan and performed all the experimental work and prepared manuscript. DP and BJD has participated in real time based experiment and helped in manuscript preparation. DDC has provided the samples for study and helped in data analysis and preparation of the manuscript. AB has designed study protocol and corrected the manuscript and overall supervised the work.

Acknowledgements

The authors would like to acknowledge HOD, Department of Microbiology, Assam University, Silchar. The authors sincerely acknowledge the financial support provided by Maulana Azad National Fellowship wide memo number F1-17.1/2012-13/MANF-2012-13-MUS-ASS-14254 /(SAIII/ Website) to carry out the work.

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Tables

Table 1 Escherichia coli isolates obtained for this study

1	NH-19	Female	12.5 years	Stool	Medicine
2	NH-36	Male	32 years	Urine	Medicine
3	NH-28	Male	27 years	Surgical wound	Surgery
4	NH-31	Male	62 years	Stool	OPD
5	NH-56	Female	48 years	Urine	OPD
6	NH-39	Female	10 years	Urine	Genital ward

Table2. Oligonucleotides used as primer for the amplification of carbapenamase gene

Name of target genes	Primer Sequence(5’-3’)	Amplification size (bp)
Inc X3 F	5'-GTTTTCTCCACGCCCTTGTTCA-3'	351
Inc X3 R	5'-CTTTGTGCTTGGCTATCATAA-3'	
NDM F	GGGCAGTCGCTTCCAACGGT	476
NDM R	CGACCGGCAGGTTGATCTCC	

Table 3: Elimination analysis of plasmid carrying Inc X type treated with SDS for broad host range experiment

Organisms	SDS concentration (%)	Incubation time (hrs)	Inoculums size (CFU/ml)	Susceptible profile
<i>E.coli</i>	10	24	1.5x10 ⁸	Gentamicin, Ampicillin, Ciprofloxacin, Imipenem, Meropenem
<i>Klebsiella</i> spp.	10	24	1.5x10 ⁸	
<i>Proteus</i> spp.	10	24	1.5x10 ⁸	
<i>Acinetobacter</i> spp.	10	24	1.5x10 ⁸	
<i>Pseudomonas aeruginosa</i>	10	24	1.5x10 ⁸	

Table 4: Curing of plasmids harboring different Incompatibility types with SDS

Incompatibility types	Concentration of SDS curing agent (%)	Selection marker	Number of colonies grown with antibiotic/ no. of colonies grown without antibiotic	Number of colonies PCR positive with antibiotic/ no. of colonies without antibiotic
Inc I	10	Imipenem	0/105	0/0
Inc FIA	10	Imipenem	0/86	0/0
Inc FREP	10	Imipenem	0/109	0/0
Inc K/B	10	Imipenem	0/89	0/0
Inc X3	10	Imipenem	0/193	0/0

Figures

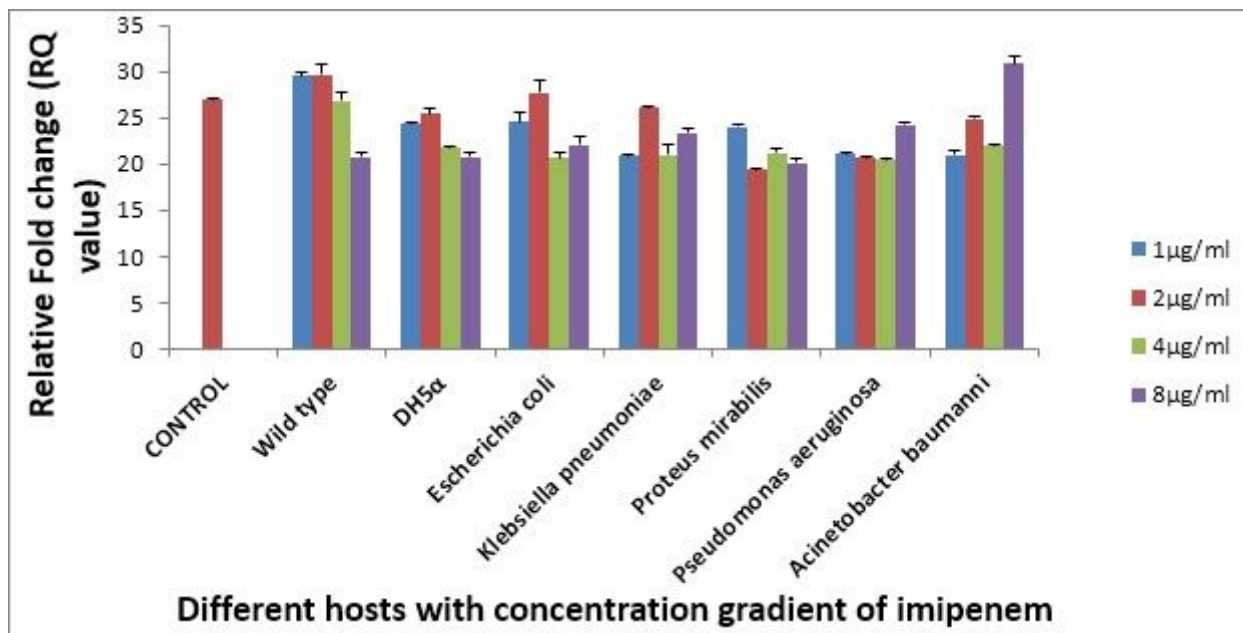


Figure 1

Change in plasmid copy number of blaNDM-4 under exposure of concentration gradient carbapenems and error bars represent the standard deviation

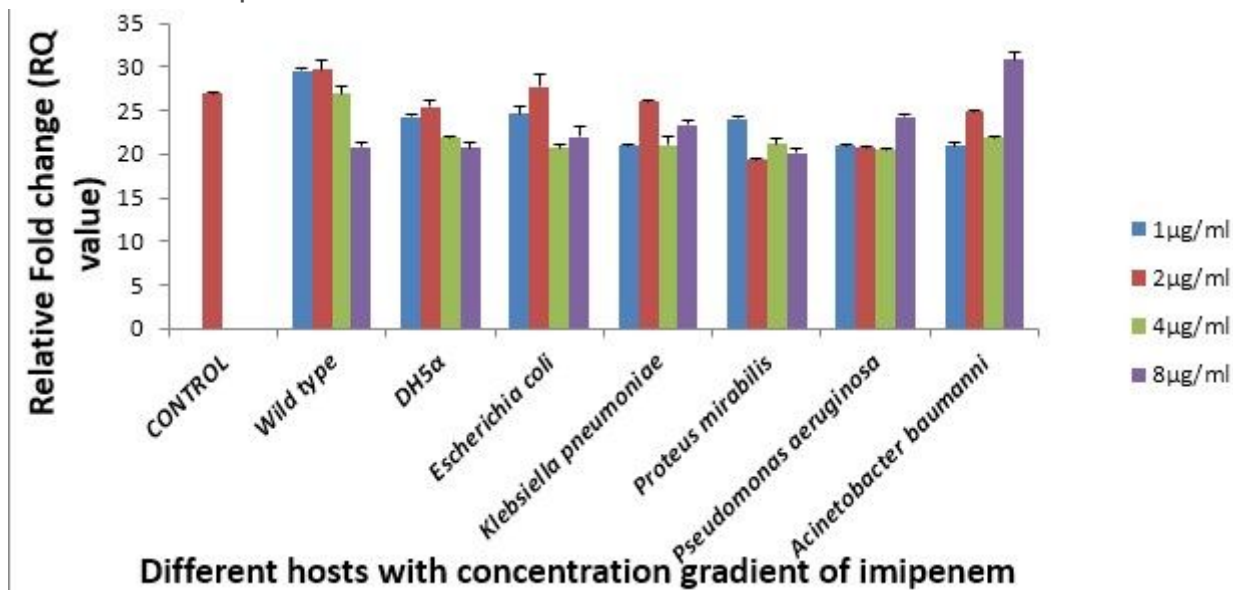


Figure 2

Change in plasmid copy number of IncX3 under exposure of concentration gradient carbapenems and error bars represent the standard deviation

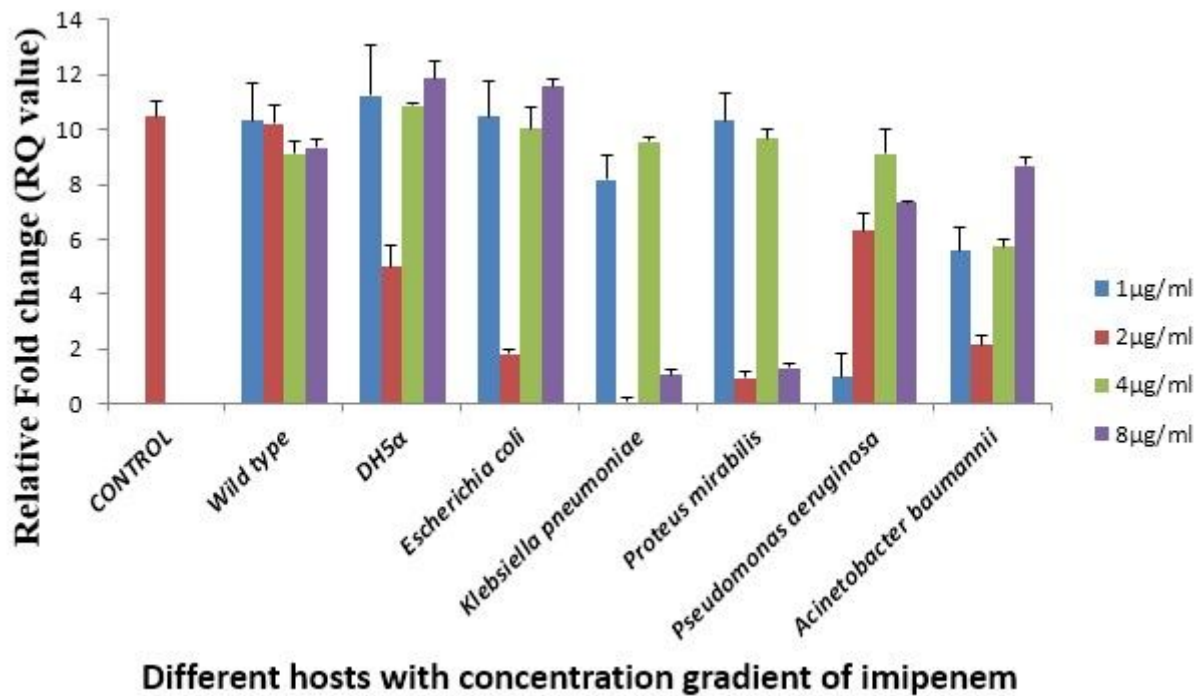


Figure 3

Transcriptional expression of blaNDM-4 under concentration gradient carbapenem (imipenem) exposure

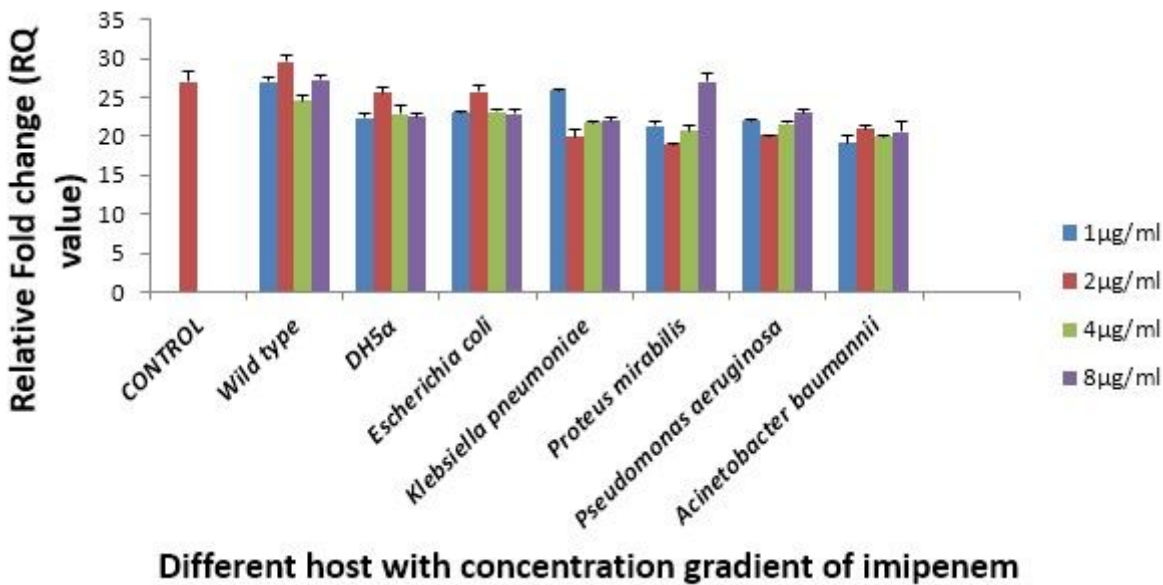


Figure 4

Transcriptional expression of IncX3 type plasmids under concentration gradient carbapenem (imipenem) exposure