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

**Nargis Alom Choudhury**
Assam University
**Deepjyoti Paul**
Assam University
**Bhaskar Jyoti Das**
Assam University
**Debadatta Dhar**
Silchar Medical College and Hospital
**Amitabha Bhattacharjee** ( ab0404@gmail.com )
Assam University

**Research article**

**Keywords:** BHR (Broad host range), NDM, Transcriptional expression

**Posted Date:** March 23rd, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-18649/v1

# Abstract

**Background:** Different variants of *bla*$_{NDM}$ have 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. These plasmids with external sub-inhibitory stress of antibiotics facilitate their propagation and maintenance within broad host range. IncX type plasmid is more recently known for carrying carbapenem resistance and NDM-4 variant is considered to have better hydrolytic property than other variants. Both of these factors interferes with therapeutic outcome in clinical setting. The current study was aimed to investigate the maintenance and stability of *bla*$_{NDM-4}$ within broad host range and transcriptional response.

**Results:** IncX3 plasmid encoding *bla*$_{NDM-4}$ was successfully transferred in six different host when imipenem (0.5µg/ml) screen agar was used for selection of transformants. It was also found to coharbour resistance for aminoglycosides and quinolone. When checked for stability, it was observed that the plasmid was successfully expanded within all the six recipients for 55$^{th}$ serial passages. Transcriptional expression with IncX3 was random but at consistent level for wild type and without concentration gradient stress of imipenem. Transcriptional expression with NDM gene was variable for parent isolates though for new hosts it was showing randomly increased pattern in proteus, *E.coli*, and DH5α

**Conclusion:** the present study could highlight that external carbapenem pressure helps in maintenance and expression of *bla*$_{NDM-4}$ within different host range. This study is of epidemiological significance and will help in tracking the genetic vehicle responsible for their transmission theirby 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 Metalo 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.

## Result

Plasmids were obtained from 6 positive isolates which were showing Inc X3 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 Inc X3. Plasmid stability was checked by serial passage in 1:1000 dilution and was found that inc X3 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 baumanni) became susceptible to carbapenems (imipenem, meropenem), aminoglycosides (gentamicin, ampicillin) and quinolone (ciprofloxacin) group of antibiotics (Table 3 &4)

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 |
|---|---|---|---|---|
| E.coli | 10 | 24 | $1.5 \times 10^8$ | Gentamicin, Ampicillin, Ciprofloxacin, Imipenem, Meropenem |
| Klebsiella spp. | 10 | 24 | $1.5 \times 10^8$ | |
| Proteus spp. | 10 | 24 | $1.5 \times 10^8$ | |
| Acinetobacter spp. | 10 | 24 | $1.5 \times 10^8$ | |
| Pseudomonas aeruginosa | 10 | 24 | $1.5 \times 10^8$ | |

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 |

On analyzing the copy number for new hosts were variable under concentration gradient stress. On analyzing copy number of $IncX_3$ 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 $IncX_3$ marker was random. There was a consistent level of transcriptional response for wild type with and without concentration gradient of imipenem stress (Figs. 1 & 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 (Figs. 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 Inc X5 subgroup (11). Till date Inc X 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 Inc X 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 Inc X3 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.

## Methodology

# 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 (Table 1). Samples were taken as a part of standard care and day to day routine sampling as suggested by clinicians. 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 16 s rDNA sequencing

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 |

# 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 DH5α, 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 µg/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 µg/ml) or ampicillin (100 µg/ml) alongwith sodium azide (100 µg/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 DH5α) was performed by the serial passage method for consecutive 70 days at 1:1000 dilutions without any antibiotic pressure (8) After each passage, 1 ml of the culture was diluted into $10^3$ dilution with normal saline, and 40 µl 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 µg/ml, 2 µg/ml, 4 µg/ml and 8 µg/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 $A_{600}$. cDNA was extracted from each condition, the reaction was performed using 10 µl of SYBR® Green PCR Master Mix (Applied Biosystem, Warrington, UK), 4 ng plasmid DNA as template and 3 µl of each primer (Table 2) (10 pmol) in a 20 µl reaction and the relative fold change was measured by ΔΔCT 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).

Table 2
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 | |

# Susceptibility Testing:

The antibiotic susceptibility was done by Kirby Bauer disc-diffusion method against antibiotics as piperacillin-tazobactam (100/10 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), polymixin B (300units) ampicillin (30 µg), cotrimoxazole (10 µg) and carbenicillin (100 µg) (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

# Abbreviations

Inc- Incompatibility, IncX- Incompatibility X type, IncX3- Incompatibility X3, blaNDM –new delhi metallo betalactamase, NDM- new delhi metallo beta lactamase, MBL- metallo beta lactamase, PCR- polymerase chain reaction, E.coli- Escherichia coli, BHR- broad host range, NDM F primer- new delhi metallobetalacta,mase 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 the datas are available in the manuscript.

Competing interests:  No competing interest exists.

Funding information

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.

# References

1. Jones CS, Osborne DJ, Stanley J. Molecular comparison of the IncX plasmids allows division into IncX1 and IncX2 subgroups. J Gen Microbiol 1993; 139: 735–41
2. Johnson TJ, Bielak EM, Fortini D et al. Expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant Enterobacteriaceae. Plasmid 2012; 68: 43–50
3. Khan AU, Nordmann P. NDM-1-producing Enterobacter cloacae and Klebsiella pneumoniae from diabetic foot ulcers in India. Journal of medical microbiology. 2012 Mar 1;61(3):454-6.
4. Paul D, Bhattacharjee A, Ingti B, Choudhury NA, Maurya AP, Dhar D, Chakravarty A. Occurrence of blaNDM-7 within IncX3-type plasmid of Escherichia coli from India. Journal of Infection and Chemotherapy. 2017 Apr 1;23(4):206-10.
5. Choudhury NA, Paul D, Chakravarty A, Bhattacharjee A, Chanda DD. IncX3 plasmid mediated occurrence of blaNDM-4 within Escherichia coli ST448 from India. Journal of infection and public health. 2018 Jan 1;11(1):111-4.
6. Paul D, Dhar Chanda D, Maurya AP, Mishra S, Chakravarty A, SharmaGD, et al. Co-carriage of blaKPC-2and blaNDM-1in clinical isolates of Pseu-domonas aeruginosa associated with hospital infections from India. PLOS ONE2015;10(12):e0145823, http://dx.doi.org/10.1371/journal.pone.0145823

7. Paul D, Dhar D, Maurya AP, Mishra S, Sharma GD, Chakravarty A, et al. Occurrence of co-existing blaVIM-2 and blaNDM-1 in clinical isolates of Pseudomonas aeruginosa from India. Ann Clin Microbiol Antimicrob 2016;15:31.
8. Locke JB, Rahawi S, LaMarre J, Mankin AS, Shaw KJ. Genetic environment and stability of cfr in methicillin-resistant Staphylococcus aureus CM05. Antimicrobial agents and chemotherapy. 2011 Oct 24:AAC-05420.
9. Swick MC, Morgan-Linnell SK, Carlson KM, Zechiedrich L. Expression of multidrug efflux pump genes acrAB-tolC, mdfA and norE in Escherichia coli clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrob Agents Chemother 2011;55:921–4.
10. Clinical and Laboratory Standard Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement. 2011; M100-S21, Wayne, USA.
11. Chen L, Chavda KD, Fraimow HS et al. Complete nucleotide sequences of blaKPC-4- and blaKPC-5-harboring IncN and IncX plasmids from Klebsiella pneumoniae strains isolated in New Jersey. Antimicrob Agents Chemother 2013; 57: 269–76.
12. Du XX, Wang JF, Fu Y et al. Genetic characteristics of blaNDM-1-positive plasmid in Citrobacter freundii isolate separated from a clinical infectious patient. J Med Microbiol 2013; 62: 1332–7.
13. Albrechtova K, Dolejska M, Cizek A et al. Dogs of nomadic pastoralists in northern Kenya are reservoirs of plasmid-mediated cephalosporinand quinolone-resistant Escherichia coli, including pandemic clone B2-O25-ST131. Antimicrob Agents Chemother 2012; 56: 4013–7
14. Agnes Sonnevend, Amna Al Baloushi, Akela Ghazawi, Rayhan Hashmey, Safinaz Girgis, Mohammed Baraa Hamadeh, Mohammed Al Haj and Tibor Pa, Emergence and spread of NDM-1 producer Enterobacteriaceae with contribution of IncX3 plasmids in the United Arab Emirates Journal of Medical Microbiology (2013), 62, 1044–1050
15. Dobiasova H, Dolejska M; Prevalence and diversity of IncX plasmids carrying fluoroquinolone and β-lactam resistance genes in *Escherichia coli*originating from diverse sources and geographical areas, *Journal of Antimicrobial Chemotherapy*, Volume 71, Issue 8, 1 August 2016, Pages 2118–2124
16. Das N, Valjavec-Gratian M, Basuray AN, Fekete RA, Papp PP, Paulsson J, Chattoraj DK. Multiple homeostatic mechanisms in the control of P1 plasmid replication. Proceedings of the National Academy of Sciences. 2005 Feb 22;102(8):2856-61.

# Figures

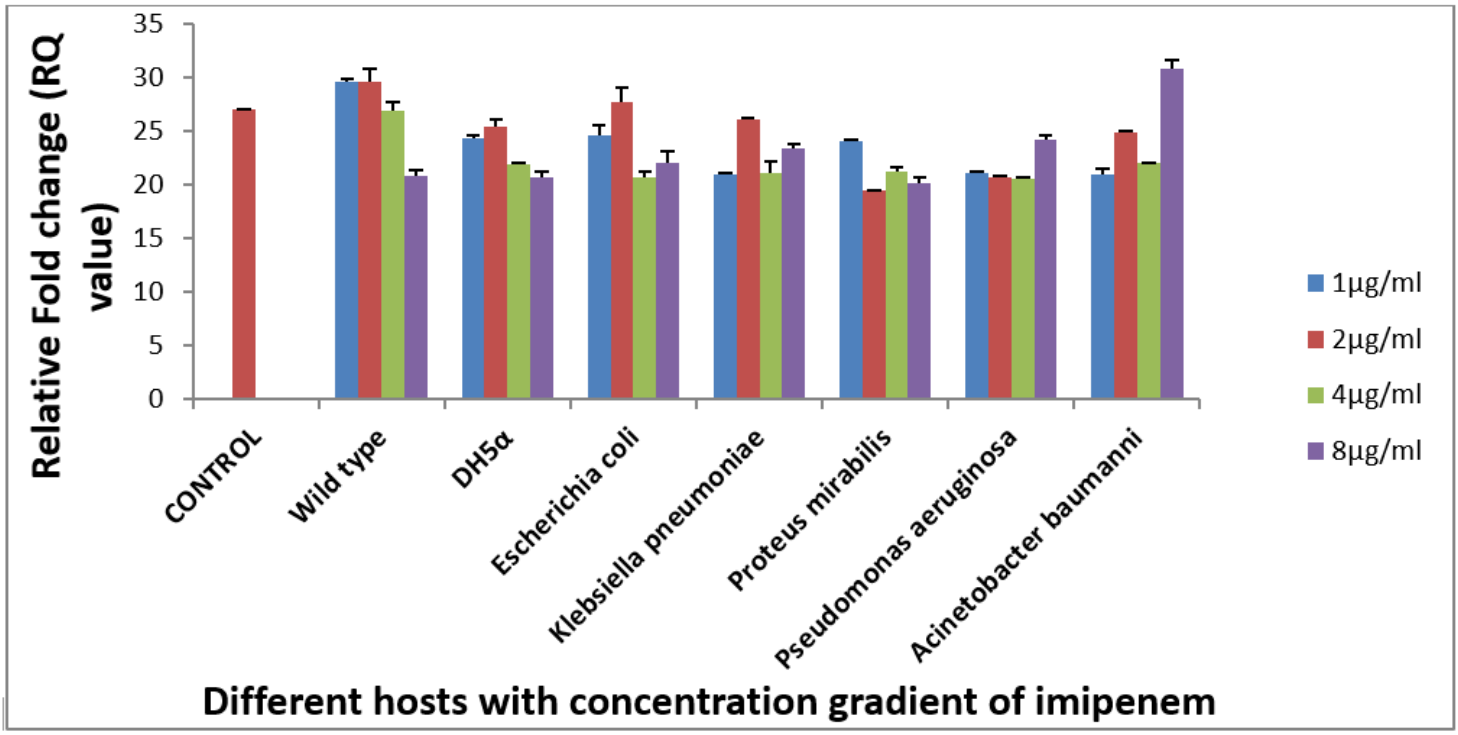


**Figure 1**

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

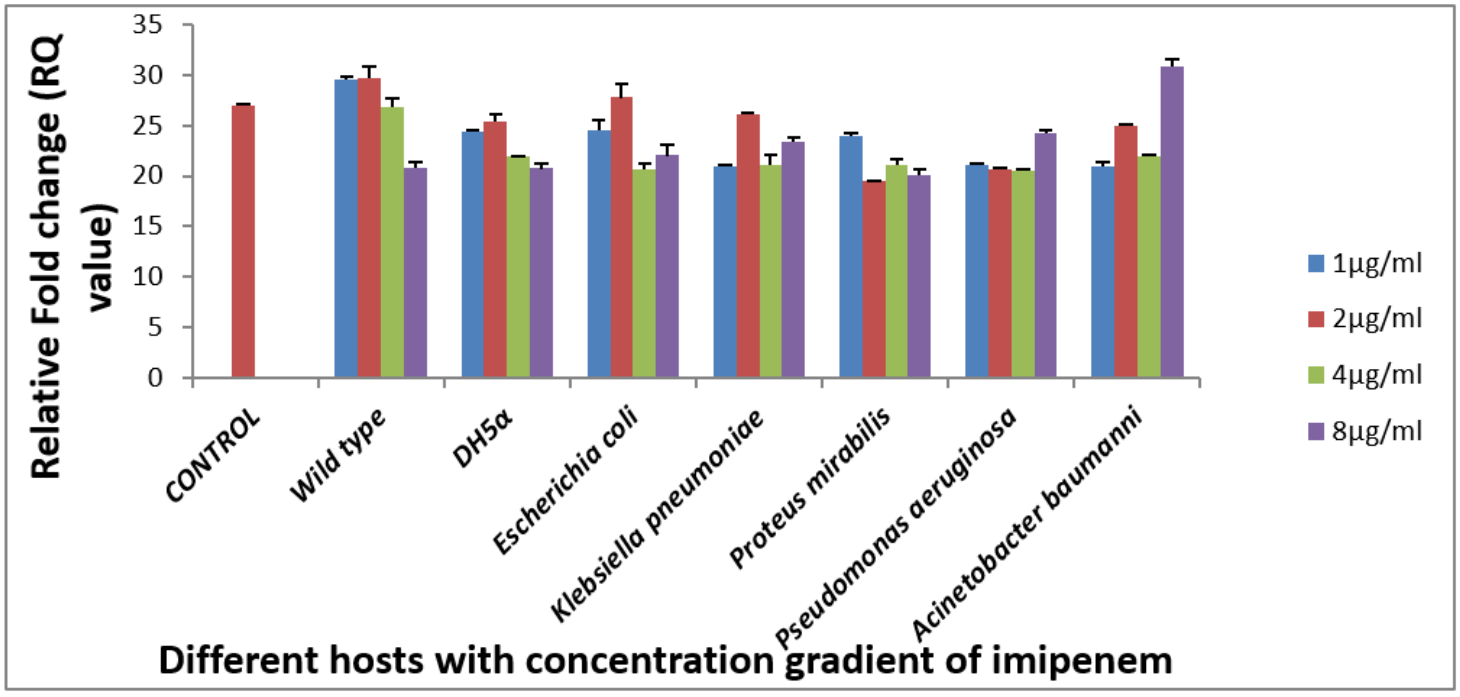


**Figure 2**

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

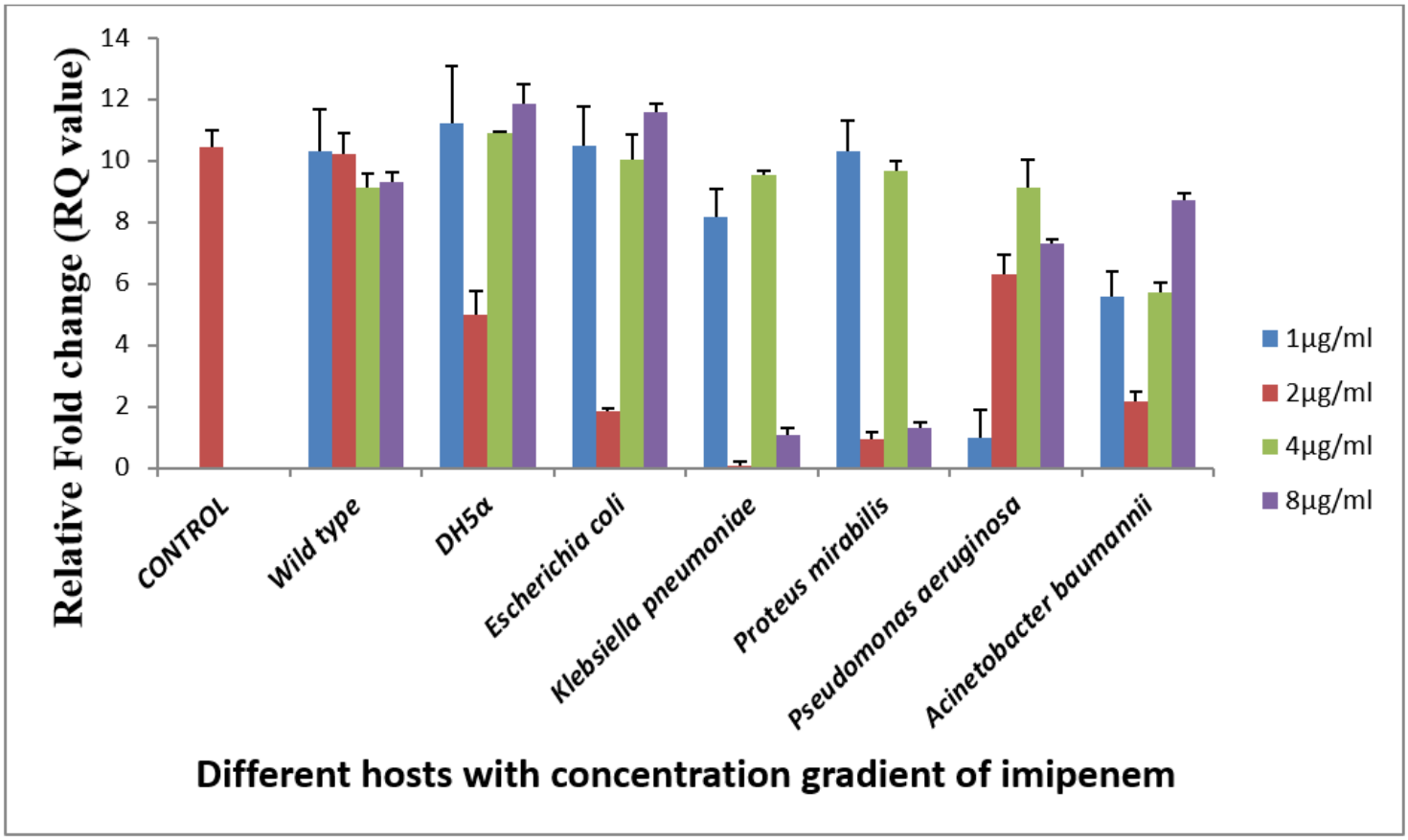


Figure 3

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

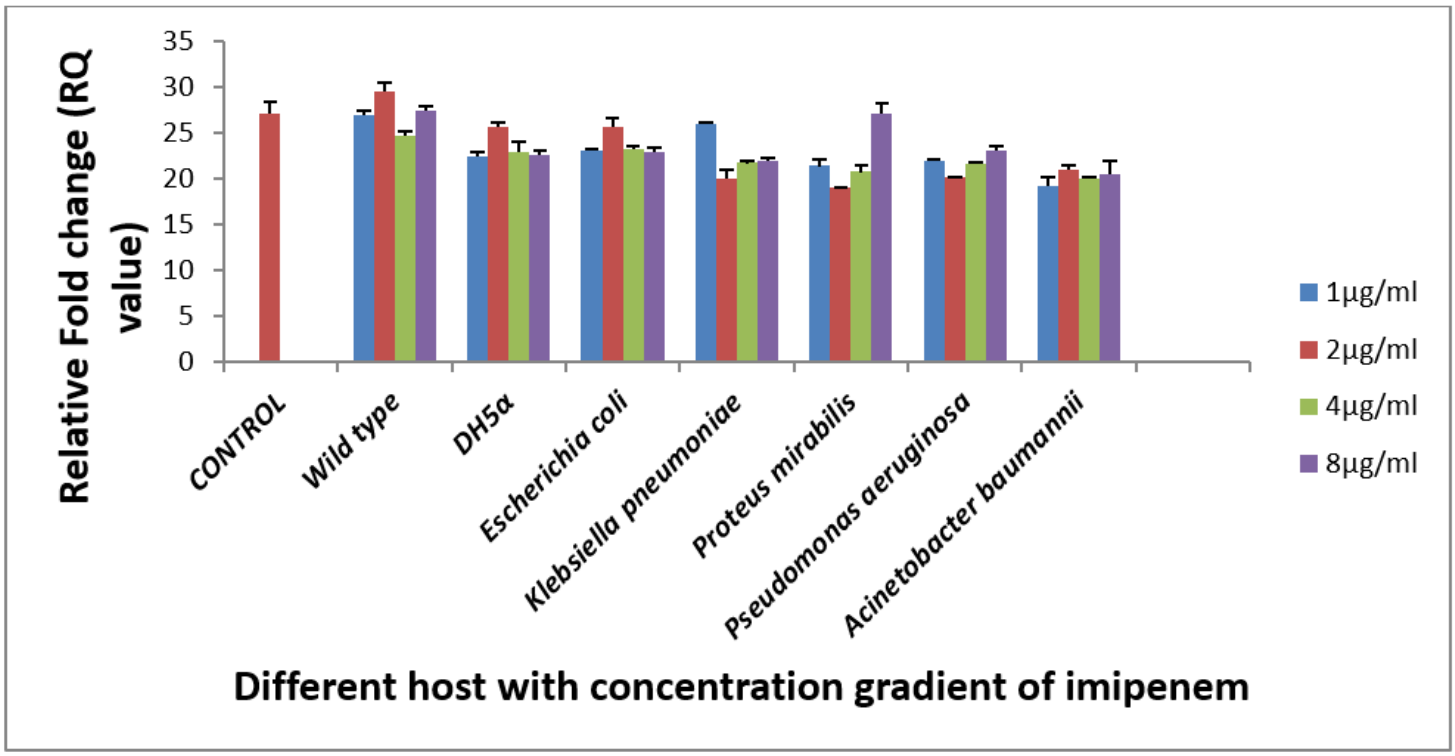


Figure 4

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