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RESEARCH ARTICLE

Adaptation of IncX3 Plasmid Encoding *bla*$_{NDM-4}$ within A Broad Host Range

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ABSTRACT

Objectives: The current study was aimed to investigate the adaptability and stability of *bla*$_{NDM-4}$ within a broad host range and transcriptional response.

Methods: Six isolates of *Escherichia coli,* harboring *bla*$_{NDM-4}$ were confirmed by PCR sequencing of the whole gene. Transformation and conjugation assay were carried out and plasmid incompatibility was determined by PCR assay. The serial passage was done for consecutive 70 days without any antibiotic pressure for both parent strain and transformants. Transcriptional expression of *bla*$_{NDM-4}$ within a broad host range against concentration gradient imipenem stress was studied.

Results: IncX3 plasmid encoding *bla*$_{NDM-4}$ was successfully transferred in six different hosts when imipenem (0.5 µg/ml) screen agar was used for the selection of transformants. It was also found to harbor resistance for aminoglycosides and quinolone. When checked for stability, it was observed that the plasmid was successfully expanded within all six recipients for 55th serial passages. Transcriptional expression with IncX3 was random but at a 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 patterns in Proteus, *E. coli*, and DH5α.

Conclusion: The present study could highlight that external carbapenem pressure helps in the 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 by restricting their spread. *J Microbiol Infect Dis 2021; 11(2):75-81.*

Keywords: *Broad host range, NDM, Transcriptional expression*

INTRODUCTION

IncX type plasmids were previously known to be of narrow host range. Based on their restriction profile subtypes X1, X2 was 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 the lateral expression of New Delhi Metallo beta-lactamases in this subcontinent. Although most reports of this plasmid type are within Enterobacteriaceae, they may have a potential chance to be disseminated to a 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 respond and when carbapenem therapy is initiated. Also, how the resistance gene is expressed within different hosts i.e. *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Proteus*

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mirabilis, *Escherichia coli* DH5α 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 samples

Six isolates of *E. 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. Consent from participants was taken regarding the study. The carriage of *bla$_{NDM-4}$* was confirmed by PCR sequencing of the whole gene. Plasmid incompatibility was determined by PCR assay [2]. The 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 the 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.

Table 1. *Escherichia coli* isolates used for this study.

No	Sample ID	Age (year)	Specimen	Ward
1	NH-19	12	Stool	Medicine
2	NH-36	32	Urine	Medicine
3	NH-28	27	Surgical wound	Surgery
4	NH-31	62	Stool	Outpatien
5	NH-56	48	Urine	Outpatient
6	NH-39	10	Urine	Gynecology

Plasmid preparation and Horizontal gene transfer experiment

Plasmids encoding *bla$_{NDM-4}$* were extracted by QIAprep Spin Miniprep Kit (Qiagen, Germany) as per the manufacturer's instruction. Isolated plasmids were subjected to transformation assay. The recipient strain used was *E. coli* JM107, *E. coli* DH5α, *K. pneumonia*, *P. mirabilis*, *P. aeruginosa*, and *Acinetobacter baumannii* of clinical origin. The transformation was carried out by the heat shock method [7]. Transformants were selected on Luria Bertani agar plates containing ampicillin (100 µg/ml). A conjugation experiment was performed using *bla$_{NDM-4}$* harboring clinical strains as donors and sodium 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) along with 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. *K. pneumonia*, *A. baumannii*, *P. aeruginosa*, *P. 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 dilutions with normal saline, and 40 µl of the diluted sample was spread on to the Luria Bertani agar plate. After overnight incubation, 50 colonies from plates were randomly picked and subjected to phenotypic detection of Metallo Beta-Lactamase 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:

A single colony of each host isolate was inoculated into Luria Bertani 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 ^{0}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), 4ng plasmid DNA as a template, and 3 µl of each primer (Table 2) (10 pmol) in a 20 µl 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]. Each set of reactions 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 (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), polymixin B (300 units) 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. Flow diagram of the whole procedure carried out is given in Figure 1.

Ethical approval

Ethics approval and consent to participate: Written informed consent was obtained from the patients. The study was approved by Assam University Institutional Ethical Committee.

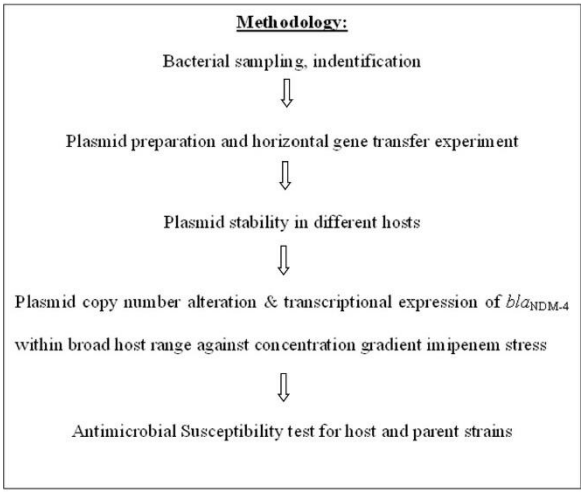

Figure 1. Flow diagram of the procedure carried out.

RESULTS

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 dilutions 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*, *K. pneumoniae*, DH5α, *P. mirabilis*, *P. aeruginosa*, and *A. baumanni*) became susceptible to imipenem, meropenem, gentamicin, ampicillin, and ciprofloxacin (Table 3). All the study hosts were having a resistant phenotype before curing.

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 *A. baumannii* but in all the hosts including the wild type expression level decreased with the increase in gradient antibiotic concentration of imipenem (Figure 2). The transcriptional expression with the IncX3 marker was random. There was a consistent level of transcriptional response for wild type with and without concentration gradient of imipenem stress (Figure 3).

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 (*E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, and *A. 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 the case of wild-type the transcriptional expression was variable whereas in the case of a new host, although expression level was random, the increased pattern was observed in all-new hosts except for *K. pneumoniae*, *P. aeruginosa,* and *A. baumannii* (Figure 4).

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	

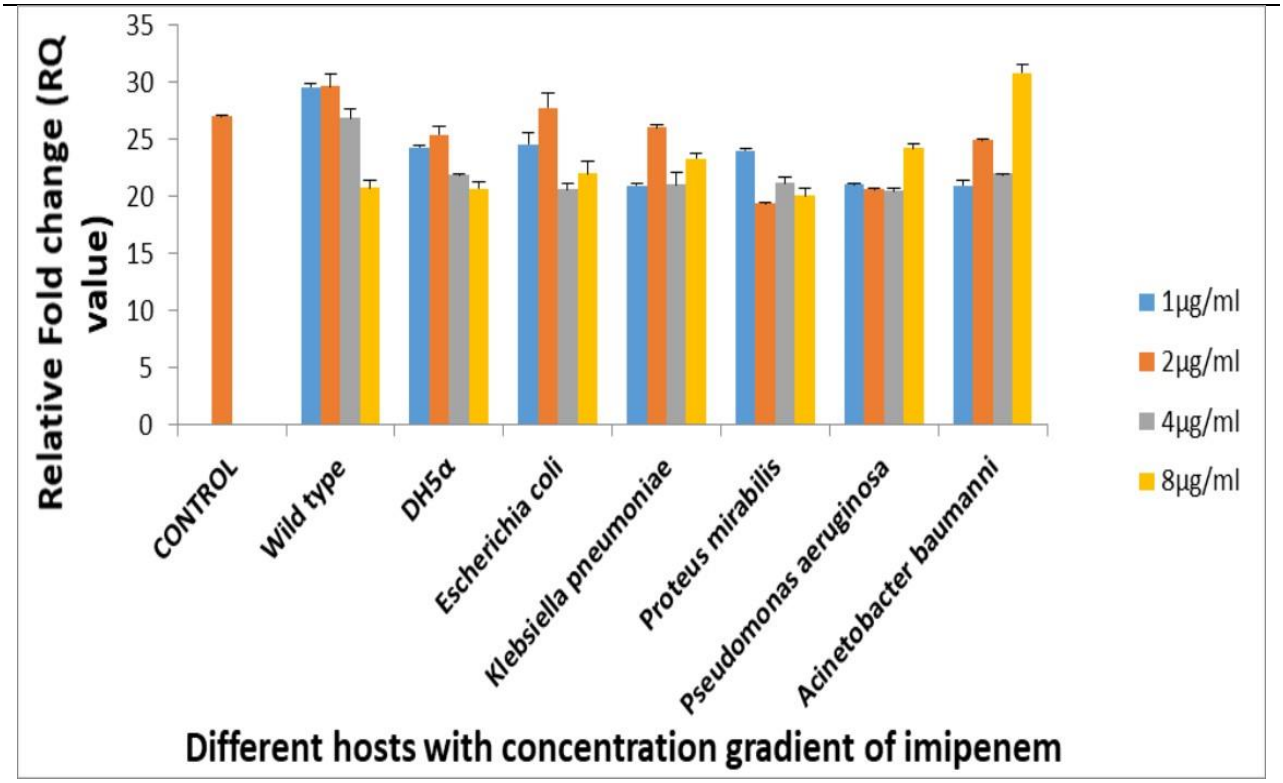

Figure 2. Change in plasmid copy number of *bla$_{NDM-4}$* under exposure of concentration gradient carbapenems and error bars represent the standard deviation.

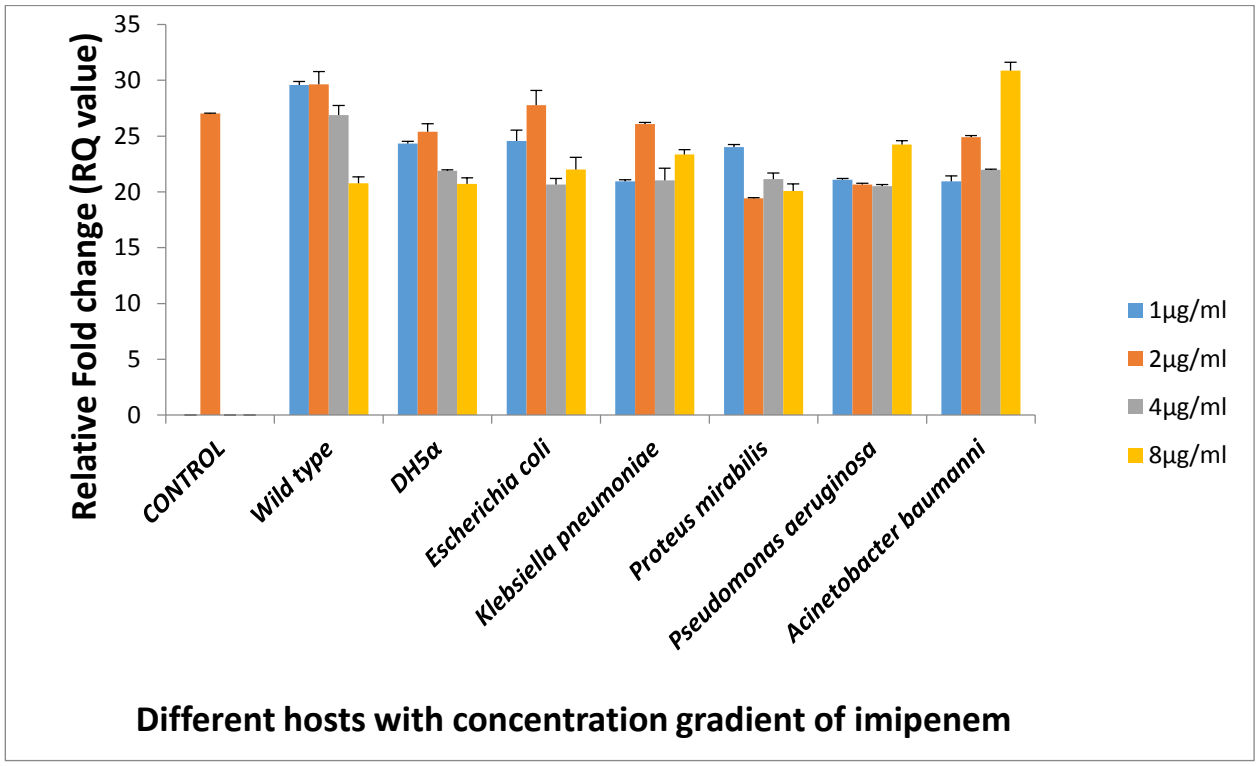

Figure 3. Change in plasmid copy number of IncX$_3$ under exposure of concentration gradient carbapenems and error bars represent the standard deviation.

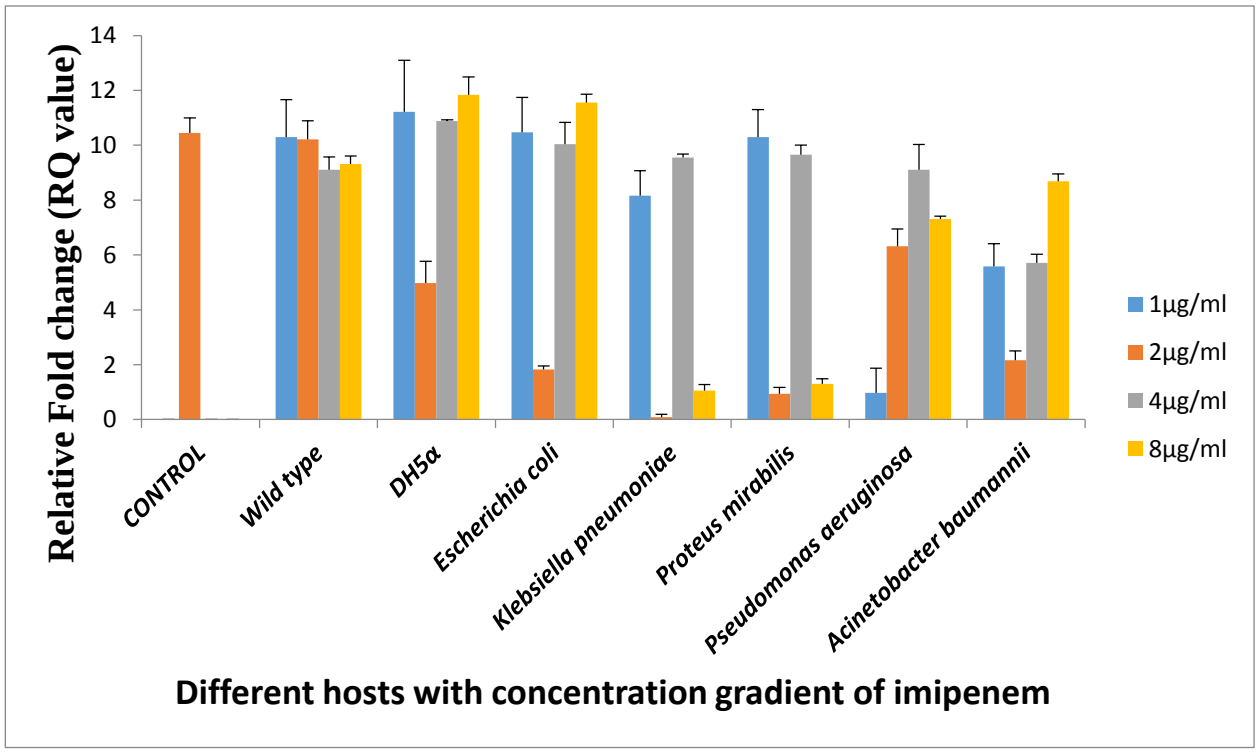

Figure 4: Transcriptional expression of *bla$_{NDM-4}$* under concentration gradient carbapenem (imipenem) exposure.

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)	Susceptibility profile
E. coli	10	24	$1.5x10^8$	Gentamicin, Ampicillin, Ciprofloxacin, Imipenem, Meropenem
Klebsiella spp.	10	24	$1.5x10^8$	
Proteus spp.	10	24	$1.5x10^8$	
Acinetobacter spp.	10	24	$1.5x10^8$	
Pseudomonas aeruginosa	10	24	$1.5x10^8$	

DISCUSSION

The present study underscores how bacteria respond when carbapenem therapy is initiated to a patient having an infection of carbapenem-resistant bacteria. IncX3 type is linked encoding of different NDM variants across the globe ranging from *bla$_{CTX}$*-M-15 as well as Genetic analysis of *bla$_{NDM}$*-7 showing association of IS*Aba125*, IS5 and a truncated portion of IS*Aba125* in the upstream region and bleMBL gene. IncX3 type was identical with flanking regions harboring the IS5-interrupted ISAba125 [3,4,14]. The 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]. *K. pneumonia* ST429 isolate was found to carry *bla$_{KpC}$*-5 which harbors 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 indicate that this plasmid type can carry a diverse range of resistance genes in Enterobacteriaceae.

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 a narrow host range [15).

Plasmid copy number is dependent on the type of organism which acts as the host of that plasmid and the origin of replication. It is also reported that mutation can bring high copy numbers [16]. The current study showed antibiotic pressure helps in the 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 an association 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 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.

Conclusion

The present study could highlight that external carbapenem pressure helps in the 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 by restricting their spread.

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