KPC-2-producing *Klebsiella pneumoniae* ST147 in a neonatal unit: Clonal isolates with differences in colistin susceptibility attributed to AcrAB-TolC pump.

Authors: Sharmi Naha[a], Kirsty Sands[b†], Subhankar Mukherjee[a†], Chayan Roy[c,ک], Moidu Jameela Rameez[c], Bijan Saha[d], Shanta Dutta[a], Timothy R Walsh[b] and Sulagna Basu[a*].

Affiliations:

[a]Division of Bacteriology, ICMR-National Institute of Cholera and Enteric Diseases, P33, CIT Road, Scheme XM, Beliaghata, Kolkata-700010, West Bengal, India;

[b]School of Medicine, Cardiff University, 6th Floor, Main Building, Heath Park Site, Cardiff, CF14 4XN, UK;

[c]Department of Microbiology, Bose Institute, P-1/12 CIT Road, Scheme VIIM, Kolkata-700054, West Bengal, India;

[d]Department of Neonatology, Institute of Post-Graduate Medical Education & Research and SSKM Hospital, Kolkata-700020, West Bengal, India.

[ک]Present Address: a) Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, South Dakota, USA.

b) South Dakota Center for Biologics Research and Commercialization, Brookings, South Dakota, USA.

† Both have contributed equally.

***Address for correspondence:**

Sulagna Basu

ICMR-National Institute of Cholera and Enteric Diseases, P33, CIT Road, Scheme XM, Beliaghata, Kolkata-700010, West Bengal, India

Email: supabasu@yahoo.co.in; basus.niced@gov.in

Telephone: +91-33-2353 7469/7470, 23705533/4478/0448; ext: 3055. Fax: +91-33-2363, 2370 5066

Running title: Differential colistin susceptibility in clonal *K.pneumoniae*

Abstract:-

This study describes the characterization of four KPC-2-producing *Klebsiella pneumoniae* strains from neonates belonging to a single sequence type 147 (ST147) in relation to carbapenem resistance and explores probable mechanisms of differential colistin resistance among the clonal cluster.

Whole Genome Sequencing revealed that the isolates were nearly 100% identical harbouring resistance genes (*bla*$_{KPC-2}$,OXA-9,CTX-M-15,SHV-11,OXA-1,TEM-1B, *oqxA, oqxB, qnrB1, fosA, arr-2, sul1, aacA4, aac(6')Ib-cr, aac(6')Ib*), and several virulence genes. *bla*$_{KPC-2}$ was the only carbapenem-resistant gene found, bracketed between IS*Kpn7* and IS*Kpn6* of Tn*4401*b on a non-conjugative IncFII plasmid. Remarkably, one of the clonal isolates was resistant to colistin, the mechanistic basis of which was not apparent from comparative genomics. The transmissible colistin resistance gene, *mcr*, was absent. Efflux pump inhibitor CCCP rendered 32-fold decrease in the minimum inhibitory concentration of colistin in the resistant isolate only. *acrB, tolC, ramA,* and *soxS* genes of the AcrAB-TolC pump system overexpressed exclusively in the colistin-resistant isolate, although the corresponding homologs of AcrAB-TolC pump, regulators and promoters were mutually identical. No change was observed in the expression of other efflux genes (KpnEF & KpnGH) or Two Component System genes (*phoP/phoQ*, *pmrA*/*pmrB*).

We postulate that colistin resistance in one of the clonal KPC-2-producing isolate was due to overexpression of AcrAB-TolC pump. This study is probably the first to report clinical clonal *K. pneumoniae* isolates with differences in colistin susceptibility. The presence of carbapenem-resistant isolates with differential behavior in the expression of genomically identical pump system indicates the nuances of the resistance mechanisms and the difficulty of treatment thereof.

Keywords: KPC-2, *Klebsiella pneumoniae*, ST147, Colistin resistance, AcrAB-TolC pump, Two Component System.

1. Introduction: -

Carbapenem resistance has challenged the medical system all around the globe. The repertoire of carbapenem-resistant genes (class A, B and D enzymes) and their spread via mobile genetic elements have compounded the problem. *Klebsiella pneumoniae* carbapenemases (KPCs), class A enzymes, confer decreased susceptibility to virtually all β-lactams and are mostly present in *Klebsiella pneumoniae* [1,2]. *K. pneumoniae* causes a wide range of infections, such as pneumonia, urinary tract infections, septicemia, liver abscesses etc., not only in immunocompromised as well as in immunosufficient ones [3]. According to the resistance map of CDDEP reports (2017) for India, 59% of *K. pneumoniae* are resistant to carbapenems, and this has increased from 56% in 2015 (https://resistancemap.cddep.org/).

Till now 46 types of KPCs have been reported worldwide (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/KPC). Though, the spread of KPC had been initiated by a single dominant clone sequence type 258 (ST258), it has now been reported among varied STs across the world [1,2].

With increased resistance towards carbapenems, colistin is being used as one of the last treatment options [1]. Isolates which are resistant to both colistin and carbapenem have been reported [4]. Such isolates are dreaded in clinical setups, particularly in neonatal intensive care units (NICU) as treatment options are already limited in the newborns. Colistin being a cationic peptide, acts by disrupting the negatively charged lipopolysaccharide (LPS). Resistance to colistin is generally attributed to upregulation of two-component system genes (TCSs) such as *phoP/phoQ* and *pmrA/pmrB*. Constitutive activation of these genes leads to modification of LPS, leaving colistin ineffective. Another important mechanism of resistance is by mutations/inactivation of *mgrB*, a

transmembrane protein, acting as a negative feedback loop for *phoP/phoQ* system. Nevertheless, many other mechanisms work simultaneously resulting in colistin resistance. With the emergence of mobilized colistin resistance gene, *mcr*, spread of resistance has increased several folds [4–6].

This study characterizes four clonal *K. pneumoniae* collected from a NICU in terms of carbapenem resistance. The clonal isolates also showed differences in resistance to colistin. Given the fact that such difference in colistin resistance in genetically identical carbapenem-resistant isolates is rarely observed, and analysis of the mechanism of colistin resistance among these isolates was also carried out.

2. Materials and Methods: -

2.1. Identification of bacterial isolates, antimicrobial susceptibility testing and phenotypic assay for Extended Spectrum Beta-Lactamases (ESBLs) and Carbapenemases:

Bacterial isolates were collected from the blood of septicemic neonates from the neonatal intensive care unit of a tertiary care hospital of Kolkata, West Bengal by standard procedure. Isolation was done from the year 2013-2016, in which a total of 195 Enterobacteriaceae were recovered. Isolates were identified by in-house biochemical tests and further confirmed by VITEK® 2 Compact system (bioMérieux, Marcy-l'Étoile, France). Minimum inhibitory concentrations (MICs) were determined by E-test method (bioMérieux, Marcy-l'Étoile, France) for different antimicrobial agents. For colistin and fosfomycin (MP Biomedicals, California, USA), broth micro dilution and agar dilution were carried out respectively [7]. Results were analyzed according to Clinical & Laboratory Standards Institute (CLSI) for all antibiotics except for colistin & tigecycline which were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Since the breakpoint of tigecycline was not available for *K. pneumoniae* in EUCAST, thus the

breakpoint for *Escherichia coli* in EUCAST was used for *K. pneumoniae* (susceptible ≤ 0.5 mg/L; resistant > 0.5 mg/L) [8,9].

Isolates were tested for the production of ESBLs and carbapenemases with the ROSCO tablets according to manufacturer's instruction. Isolates exhibiting an increase of ≥5 mm in the inhibition zone of the combination disc with respective antibiotics were categorized as positive. [Rosco Diagnostica A/S, Taastrup, Denmark].

2.2. Genotypic determinants in K. pneumoniae isolates:

Based on the susceptibility pattern and phenotypic results, polymerase chain reaction (PCR) was done for the following β-lactamase genes ($bla_{\text{CTX-M,TEM,SHV,OXA-1}}$), AmpC genes ($bla_{\text{MOX,CMY,DHA,ACC, MIR/ACT,FOX}}$), carbapenemase genes ($bla_{\text{VIM,IMP,SPM-1,GIM-1,SIM-1,NDM-1,}}$ $bla_{\text{OXA-48,}}$ $bla_{\text{KPC,SME,IMI,GES,NMC}}$), 16S rRNA methylase encoding genes (*rmtA, rmtB, rmtC, rmtD & armA*), flouroquinolone genes (*qnr-A,B,S, qepA, aac(6')-Ib, aac(6')-Ib-cr*), addiction systems and virulence genes as described previously [10]. Plasmid types of the isolates were identified by PCR based replicon typing (PBRT) [11].

Primer walking was done to discern the genetic environment of the carbapenemase genes [2]. Primer sequences have been enlisted in Table S1.

2.3. Whole genome sequencing (WGS):

Total genomic DNA was extracted using the QIAamp DNA kit, and quantified using the Qubit 3.0 (Thermo Fisher Scientific). DNA libraries were prepared for paired end sequencing (2x301 cycles) using Nextera XT (Illumina Inc., San Diego, CA). Whole genome sequencing (WGS) was performed using the v3 chemistry on the Illumina MiSeq platform (Illumina Inc., San Diego, CA).

Quality control of raw reads included fastqc (0.11.2), and quality and adaptor trimming was performed using Trimgalore (0.4.3). Reads were assembled in contigs using the *de novo* assembler SPAdes (3.9.0) (.fasta) and were aligned to the original fastq reads using BWA aligner (0.7.15). Any assembly mapping errors in the contigs was corrected using Pilon (1.22). Assembly metrics were evaluated using Quast (2.1). Genomes (contigs) were annotated using Prokka (1.12) and the resulting .gbk file was viewed in Artemis (genome viewer, Sanger, UK). Resulting .gff files of Prokka were used to create a core genome alignment using Roary (v3.12.0). Fasttree was used to build the phylogenetic tree (.nwk file) and visualized in Interactive Tree Of Life (iTOL). BLAST Ring Image Generator (BRIG) [12] was used to evaluate genome wide sequence similarity using a KPC-2-producing ST11 *Klebsiella pneumoniae subsp. pneumoniae HS11286* as a reference genome. Single nucleotide polymorphism (SNP) analysis was done using kSNP3.0 using the file generated with Prokka [13].

ResFinder (3.0), PlasmidFinder (1.3) were used for genotypic characterization. Virulence genes were assessed by Institut Pasteur MLST and whole genome MLST databases (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) as well as by Kleborate pipeline (https://github.com/katholt/Kleborate) for more detailed analysis of the isolates. Promoter sequence analysis was done (http://www.fruitfly.org/seq_tools/promoter.html). Individual genes were BLAST searched against the four genomes along with MUMmer alignment. Genomes were visualized using SNAP Gene tool (4.3.8). INTEGRALL database (http://integrall.bio.ua.pt) was used to search the integron sequences. TETyper (https://omictools.com/tetyper-tool) was used with the suggested parameters, to type the transposable genetic element from paired-end sequencing data.

2.4. Multi-locus Sequence Typing (MLST) and Pulse Field Gel Electrophoresis (PFGE):

To identify the sequence types (ST), MLST database (https://cge.cbs.dtu.dk/services/MLST/) was used.

PFGE was performed using PulseNet standardized procedures with *XbaI* (50 U/plug) (http://www.cdc.gov/pulsenet/protocols.html) in a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA). *XbaI* macrorestriction patterns were visually interpreted according to Tenover criteria [14].

2.5. Transmissibility of carbapenemase carrying plasmid:

The transfer of carbapenemase gene into *E. coli* J53Az^{r} strain was performed by both solid- and liquid-mating conjugation techniques at varying temperatures. Electroporation was carried out with purified plasmid DNA [15] into *E. coli* DH10B (Invitrogen, California, USA) using a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA, USA). Transformants were selected on LB agar plates (BD BBL, MD, USA) supplemented with ertapenem (0.25mg/L) (Sigma-Aldrich, St Louis, MO, USA). Transformants retrieved were subjected to genotypic confirmation of carbapenemase and other β-lactamase genes followed by susceptibility testing.

2.6. ***Evaluating mechanisms of colistin resistance*** **-**

WGS data was evaluated using ResFinder for the presence of *mcr* or its variant genes.

2.6.1. Efflux pump activity**:**

MIC of colistin was determined in the presence of efflux pump inhibitors (EPIs) such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 10mg/L) and 1-(1-naphthylmethyl)-piperazine (NMP; 25mg/L) (Sigma-Aldrich, St Louis, MO, USA) to assess the role of efflux pump genes [16, 17]. MIC was done in triplicate in separate experiments.

2.6.2. Gene expression using real-time PCR (RT–PCR):

The mRNA expression level of TCS genes viz *phoP/phoQ, pmrA/pmrB* were analyzed [4,5]. Based on the phenotypic expression of EPIs, expression of transmembrane protein of AcrAB-TolC (*acrB*), *acrA, tolC* and their regulators (*soxS and ramA*) were studied [18]. Additionally, expression of KpnEF (small multidrug resistance family, SMR) and KpnGH (Major facilitator superfamily, MFS) efflux pumps [19, 20] were also analyzed.

Isolates were inoculated overnight in cation-adjusted Mueller-Hinton broth (BD BBL, MD, USA) in the presence of colistin sulphate (0.125mg/L) at 37°C with shaking. Overnight cultures were diluted (1/100) in cation-adjusted Mueller–Hinton broth containing colistin sulphate (0.125mg/L) and grown to mid-logarithmic phase at 37°C with vigorous shaking (230 rpm). RNA was harvested using NucleoSpin® RNA purification kit (Machery-Nagel, Germany) according to the manufacturer's instructions. RNA yield and quality were determined using a NanoDrop biophotometer plus (Eppendorf, Hamburg, Germany) followed by RNase-free DNase treatment (New England Biolabs, USA) and purity was assessed by PCR.

Two micrograms of the isolated total RNA were reverse transcribed to cDNA using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, Massachusetts, USA). Expression of the target genes were quantified using Power SYBR Green PCR Master Mix (Applied Biosystems, California, USA) in StepOne Plus Real-Time PCR system (Applied Biosystems, California, USA). The reactions were performed with 0.5 mM primer in 20 µL reaction volumes in duplicate in separate experiments. The relative RNA expression levels were calculated according to the ΔΔCt method, normalized with the ribosomal housekeeping genes (*rpsL* for TCS, KpnEF, KpnGH pumps, and *rrsE* for AcrAB pump). Relative expression of each target gene was then calibrated against the expression of a *K. pneumoniae* (EN5219) isolate susceptible to all antibiotics tested, which served as the reference strain (expression=1). A ≥2-fold increase in the expression of

genes when compared with the reference strain was considered as cut-off to denote significant overexpression [21].

2.6.3. Outer membrane protein profile:

Whole-cell extracts of the isolates were separated on 12% SDS–polyacrylamide gels and transferred to Immobilon-P membrane (Merck Millipore, Massachusetts, USA) following standard protocols. An isolate of *E. coli* (S205) which retained both the outer membrane proteins (OMPs) (OmpC/F) was used as a control for the western blots. Porins were detected using polyclonal anti-OmpC/F antibody with 1:3000 dilutions as described earlier [22].

3. Results and Discussion:

3.1. Identification of isolates and clinical presentation of neonates:

Of the different Enterobacteriaceae evaluated during the period of 2013-2016, only four isolates possessed bla_{KPC} and all four were *K. pneumoniae*. These four isolates were recovered during September, 2015 to December, 2015 indicating the emergence of this gene in this unit. There were no further cases of bla_{KPC}-harbouring isolates till end of 2016. The four isolates were named as *Kp1* (EN5226), *Kp2* (EN5261), *Kp3* (EN5269), and *Kp4* (EN5271) and analyzed in detail. Henceforth, isolates will be referred to as *Kp1, Kp2, Kp3* and *Kp4* isolated from neonates P1, P2, P3, and P4 respectively.

The details of the clinical status of the neonates are presented in Table S2. P1 had an extended stay of nearly a year and P2 was admitted for 2 months. P3 and P4 were in the unit for almost a month (Table S2). Since bla_{KPC} was never recovered from this unit before or after the aforesaid period, hence P1 carrying the bla_{KPC} was suspected to be the index case and all neonates had overlapping stay, indicating possibility of cross transmission. None of the neonates were treated

with colistin during their stay. Other studies have also reported nosocomial outbreaks [23] with KPC-producing strains including outbreaks in neonatal units [24].

3.2. Antibiotic susceptibility testing and phenotypic detection of ESBLs and carbapenemases:

Antibiotic susceptibility tests for the four isolates showed similar pattern of susceptibility for 14 antimicrobials except for colistin. *Kp1*, *Kp3*, and *Kp4* were susceptible to colistin whereas *Kp2* was resistant (MIC: 8mg/L). *Kp1-Kp4* was susceptible to gentamicin, cefoxitin, meropenem, and tigecyline only (Table 1).

ROSCO combination tablets indicated presence of ESBLs and KPC which were further verified by PCR/WGS.

3.3. Resistance and virulence determinants of K. pneumoniae:

Resistome analysis (≥98% identity and coverage) showed that the four strains possessed $bla_{\text{KPC-2}}$ as the only carbapenemase gene apart from the following genes: 5 β-lactamase genes $bla_{\text{OXA-9}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-11}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{TEM-1B}}$; *aacA4*, *aac(6')Ib* and *aadA1* (aminoglycoside resistance-encoding genes); *aac(6')Ib-cr*, *qnrB1*, *oqxA*, and *oqxB* (quinolone resistance); *fosA* for fosfomycin; *catB3* for phenicol; *arr-2* for rifampicin and *sul1* for sulphonamide (Table 2). Resistance determinants mentioned above were also detected by conventional PCRs to validate the results obtained from WGS analysis. *vagCD* was the only addiction system found in these isolates (Table 1). In accordance to earlier reports, our study also revealed association of KPC with resistance determinants conferring resistance to other antibiotics [1]. KPC (serine β-lactamases) are efficient in hydrolyzing cephalosporins, monobactams, and carbapenems (imipenem, and ertapenem). KPCs often evade detection by routine susceptibility screening as they are not as efficient as other carbapenemases, hence, leading to treatment failures and possess an exceptional potential for dissemination [1].

Different virulence genes, such as *fimH* (type-1 fimbrial adhesion protein), *wabG* & *uge* (biosynthesis of the outer core lipopolysaccharide; capsule and smooth lipopolysaccharide respectively), *kfuBC* (iron-uptake system), *ybt* (siderophore yersiniabactin cluster), *mrk* (type-3 fimbrial gene cluster), and *fyuA* (yersiniabactin receptor) were found within the genomes of the four isolates. Presence of these genes were also reported in *K. pneumoniae* from clinical setups [25, 26]. Isolates in this study harbored KL10 (K-loci), O3/O3a (O-loci) bearing *wiz420* and *wcz11* capsular genes. All the four strains belonged to yersiniabactin sequence type 135 (ybST135) and carried ybt14; ICE*Kp5* as the integrative conjugative element (Table 2). These virulence determinants provide the isolates with both adaptive advantages and offensive capabilities to persist and cause disease, thereby making *K. pneumoniae* an efficient nosocomial pathogen and good colonizer.

3.4. Transmissibility of KPC-bearing plasmids:

Transfer of KPC-2-bearing plasmid was unsuccessful by conjugation. Transformants (TF) were obtained on Luria agar plate supplemented with 0.25mg/L ertapenem upon electroporation. Presence of $bla_{\text{KPC-2}}$, *qnrB1*, *oqxA*, *oqxB*, *aac (6')-Ib-cr* were found in *Kp2-Kp4* whereas only $bla_{\text{KPC-2}}$ was found in transformants of *Kp1* (Table 1). MICs of transformants are presented in Table 1.

Plasmid-scaffold genes such as IncFIB, IncFII, and IncR were found by PBRT (Table 1) and an additional Col plasmid was found by PlasmidFinder tool (Table 2). Plasmid analysis revealed presence of bla_{KPC-2} on IncFII plasmid. $bla_{\text{KPC-2}}$ has been reported to reside on numerous plasmid types, such as IncF, IncI2, IncX, IncA/C, IncR, and ColE1. IncFII_{K} replicon is the preferred one [2]. IncF/ IncFII plasmids are generally conjugative [27] but in this study the IncFII plasmids were non-conjugative and could be transferred only by electroporation. This could also be a reason for its restrictive spread in the unit. Only four neonates were infected and spread of bla_{KPC-2} to other species was not observed (unpublished data).

Genetic environment of *bla*$_{KPC-2}$ was determined by primer walking method which was further validated by WGS data. *bla*$_{KPC-2}$ was found to be within a Tn*3*-based transposon, Tn*4401* and TETyper revealed it to be Tn*4401*b. It harbored two different types of IS elements. IS*Kpn7* belonging to IS*21* family, in the upstream of *bla*$_{KPC-2}$, and IS*Kpn6*, a member IS*1182* family, on downstream of the gene. Presence of transposase, *tnpA* and resolvase, *tnpR* of Tn*4401*b were found at the upstream of *bla*$_{KPC-2}$ right before IS*Kpn7* (Fig. 1). The results corroborate with other studies reporting the association of *bla*$_{KPC-2}$ with Tn*4401*b [2]. Presence of two IS elements each having its own transposase activity along with transposase of the Tn*3*-based transposon, itself initiate a strong transposition event. This facilitates movement of *bla*$_{KPC-2}$ among various plasmids as well as different organism leading to worldwide dissemination of the gene.

In875 class 1 integron was found in *Kp1-Kp3*. In *Kp4*, the 3′CS region could not be retrieved, though it carried class 1 integron. In all of the isolates, insertion of *S.ma.I2* group IIc intron has been found within the *attC* site of the *arr2* gene cassette (Fig. 2).

3.5. Molecular typing of K. pneumoniae:

BRIG analysis revealed that the four genomes had significant levels of similarities with the reference strain (Fig. 3). Nearly 100% identity across the genomes was evident, though the four genomes also have minute differences among themselves which is common between clonal populations. The core genome alignment and resulting phylogenetic tree shows that the four isolates group in 2 clusters. *Kp2* and *Kp4* are more closely related, as are isolates *Kp1* and *Kp3* (Fig. 4A). MLST revealed that all the four isolates belonged to a single sequence type 147 (ST147) (Table 2). This finding matched with indistinguishable band patterns in PFGE (Fig. 4B).

Presence of *bla*$_{KPC}$ has been reported in almost 115 different STs worldwide of which, ST258 is the leading one [2,23]. KPC-2 was found in ST258 in Europe and USA, and in ST37, ST392, ST395, and ST11 in Asia-Pacific regions [2]. In our study, *bla*$_{KPC-2}$ was harbored in ST147 *K. pneumoniae*. Interestingly, the ST147 *K. pneumoniae* clone has been associated with different carbapenemases including NDM, KPC, OXA-48, OXA-181 or OXA-204 enzymes; making this ST a globally high-risk clone [28-30]. Presence of ST147 *K. pneumoniae* has been reported from India previously in association with colistin resistance [31] but presence of *bla*$_{KPC-2}$ was never encountered in ST147 from India.

3.6. Analysis of colistin resistance:

Of the four clonal isolates, one (*Kp2*) demonstrated raised MIC value (8mg/L) for colistin. Clonal isolate with difference in susceptibility is rarely encountered. Though an earlier study had reported variations in susceptibility to meropenem in clonal *K. pneumoniae* [32] but to the best of our knowledge, no such variation in terms of colistin has been reported yet.

WGS data was extensively explored in search of recently reported *mcr* genes and its variants. No such gene was found to be present within the genome of the colistin-resistant strain. SNP analysis showed a total of 12 SNPs within the core genome of *Kp2* which differ with the other 3 strains (*Kp1, Kp3, Kp4*). But none of these genes have been reported to be associated with colistin resistance. Details of the SNPs have been included in Table S3.

Colistin resistance is chiefly mediated by covalent modification of the lipid A moiety of lipopolysaccharide (LPS) by 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN), resulting in reduced binding ability of colistin to the LPS [33]. Based on the established mechanisms of colistin resistance, expression of TCSs was studied by realtime RT-PCR. *phoP,*

phoQ, *pmrA*, and *pmrB* did not show significant change among the isolates when compared with reference sample EN5219 (Table 3). Sequences of these genes along with other genes known to play a significant role in colistin resistance such as *mgrB*, *pmrC*, *pmrD* (responsible for downstream modification of LPS), *crrAB* (two-component regulator of *pmrAB*), and *arnBCADTEF-pmrE* (operon that mediate synthesis and transfer of L-Ara4N to LPS) were analyzed from the WGS data but no change was observed in their sequences, which ruled out the involvement of the TCSs and other modifying genes for colistin resistance in *Kp2*.

A decrease in MIC of colistin from 8mg/L to 0.25mg/L (32-fold) was found in case of *Kp2* in the presence of CCCP (Table 3). No such change was encountered for other isolates either with CCCP or with any other EPI. CCCP is a nonspecific EPI and acts on the energy source of several efflux pumps as well as on other membrane proteins. Decreased MIC of colistin in the presence of CCCP might indicate a probable role of efflux pump in colistin resistance in *Kp2* [17, 34].

RT-PCR showed overexpression of regulators and efflux pump genes *viz. ramA* (5.5-fold), *soxS* (2.7-fold), *acrB* (4.5-fold), and *tolC* (2-fold), in *Kp2* only when compared with the colistin-susceptible *K. pneumoniae* (EN5219) (Table 3) and *Kp1, Kp3, and Kp4*. This further substantiated the change in MIC value for colistin in presence of CCCP in *Kp2*. This indicated role of an active AcrAB-TolC efflux pump conferring colistin resistance in *Kp2*. Padilla *et al*. (2010) also reported the presence of an energy-dependent efflux of colistin by efflux pump gene, *acrB* [17] similar to our observation.

Overexpression of soxRS has been found to induce colistin hetero-resistance in *Enterobacter* sp. [35] which triggered upregulation of *acrA* and *tolC*. Though upregulation of *soxS* has been found in the study, yet no change in the sequences of *soxS* and its regulator, *soxR* have been noticed among the strains. As there was overexpression of *acrB*, *tolC* and its regulators *ramA*, their sequences were

analyzed but no differences were noticed in the WGS data of the four isolates. Since, *ramA* is the global regulator of *acrB,* the promoter sequences of *ramA, soxS*, *acrB,* and *tolC* were also assessed with no differences to be identified from the genomic data. Further, sequences of local repressor of *ramA, acrA,* and *acrB*, (*ramR*, and *acrR*, respectively) were investigated, but no variation was noticed in these gene sequences. Similar observations for the overexpressed AcrAB-TolC pump and *ramA* have been made in other studies, with no genetic background to substantiate such findings, thereby indicating presence of an alternate mechanism regulating expression of these genes [17,36]. *ramA* mediated alteration of lipid A moiety in *K. pneumoniae* has also been reported to result in altered susceptibility to colistin [37]. Hence, increased *ramA* levels can also mediate LPS alterations, which along with the AcrAB-TolC likely contributed towards increased survival in presence of colistin. In this study also, *Kp2* had increased expression of *ramA.* Therefore, it is likely that a functional AcrAB-TolC pump and *ramA* are crucial in mediating decreased susceptibility towards colistin in *Kp2*.

KpnEF can efficiently efflux colistin whereas KpnGH acts as an intrinsic resistance determinant in *K. pneumoniae* [19, 20]. However, in our study, the four isolates showed similar level of expression for both the SMR and MFS type efflux pumps implying no role of these efflux pumps towards colistin resistance.

Apart from these, presence of a new transmembrane protein, *Enterobacter* colistin resistance gene (*ecr*) corresponding to colistin resistance has been reported [38]. But no such homologue was found to be present in the genome of *Kp2*.

Additionally, resistance to colistin is also attributed to change in porin profile among Enterobacteriaceae [39]. All the isolates showed similar pattern of porins, loss of *OmpK35* but intact *OmpK36*. With no observed difference in the profile of porins in the colistin-resistant and

susceptible isolates, it was concluded that OMPs had no role to play towards colistin resistance in this study.

4. Conclusion-

This study reports the emergence of KPC-2 in ST147 *K. pneumoniae* isolated from septicemic neonates. The strains showed 100% mutual identities and possessed a repertoire of resistance and virulence determinants. A difference in colistin susceptibility among the indistinguishable strains was attributed to AcrAB-TolC pump overexpression. The present study is probably the first to report such differences in colistin susceptibility for clonal *K. pneumoniae* isolates and similar occurrence has never been demonstrated in clinical isolates. WGS data did not indicate any change in the homologs known to be involved in the expression of the AcrAB-TolC pump system. This differential expression may be attributed to hitherto unknown regulatory mechanism of AcrAB-TolC pump. This phenomenon of variation in expression of genetically identical pump in isolates which are nearly similar, enhances our perspective on the versatility of resistance mechanisms in gram-negative bacteria.

Acknowledgments-

We extend our thanks to AM. Queenan, GA. Jacoby, O. Moquet, K. Lee and S. Brisse for providing PCR controls, H. Schwarz & HI. Zgurskaya for the antibodies and Thomas Jove for curating integron sequences. We would like to thank Wriddhiman Ghosh for his scientific advice. We also thank the staff of the Department of Neonatology, who cared for the neonates and Mr. Subhadeep De for his laboratory assistance.

Accession numbers-

All genomes sequences have been submitted to NCBI database with SRA accession number SRP136309, BioProject accession number PRJNA445372 and GenBank accession numbers PYWD00000000.1 (EN5226), QCZB00000000.1 (EN5261), QCZC00000000.1 (EN5269), and PYWE00000000.1 (EN5271).

Declarations-

Funding: The study was supported by Indian Council of Medical Research (ICMR), India, intramural funding. S.N was recipient of fellowships from Department of Science & Technology, West Bengal & later from ICMR. S.M was recipient of fellowship from ICMR. The funding agency did not play role in the study design, data collection, analysis and interpretation, writing of the report, or the decision to submit the work for publication.

Competing Interests: None to declare. The authors declare no conflict of interest.

Ethic Approval: The study protocol was approved by the Institutional Ethics Committee of the ICMR-National Institute of Cholera and Enteric Diseases (No. A-1/2016-IEC). Patient information was anonymized and de-identified prior to analysis.

References-

[1] Arnold RS, Thom KA, Sharma S, Phillips M, Kristie Johnson J, Morgan DJ. Emergence of *Klebsiella pneumoniae* carbapenemase-producing bacteria. South Med J 2011;104:40–5. doi:10.1097/SMJ.0b013e3181fd7d5a.

[2] Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: Epidemiology, genetic context, treatment

options, and detection methods. Front Microbiol 2016;7:895. doi:10.3389/fmicb.2016.00895.

[3] Paczosa MK, Mecsas J. *Klebsiella pneumoniae*: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev 2016. doi:10.1128/MMBR.00078-15.

[4] Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P RG. In Vivo Emergence of Colistin Resistance in *Klebsiella pneumoniae* Producing KPC-Type Carbapenemases Mediated by Insertional Inactivation of the *PhoQ/PhoP mgrB* Regulator. Antimicrob Agents Chemother 2013;57:5521–6. doi:10.1128/AAC.01480-13.

[5] Cannatelli A, Di Pilato V, Giani T, Arena F, Ambretti S, Gaibani P, *et al*. *In Vivo* Evolution to Colistin Resistance by PmrB Sensor Kinase Mutation in KPC-Producing *Klebsiella pneumoniae* Is Associated with Low-Dosage Colistin Treatment. Antimicrob Agents Chemother 2014;58:4399–403. doi:10.1128/aac.02555-14.

[6] Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, *et al*. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 2016;16:161–8. doi:10.1016/S1473-3099(15)00424-7.

[7] Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008;3:163–75. doi:10.1038/nprot.2007.521.

[8] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty seventh Informational Supplement *M100-S27*. CLSI, Wayne, PA, USA, 2005.

[9] The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone Version 9.0, 2019. 2018:0–99. doi:10.1111/j.0021-8782.2005.00369.x.

[10] Mukherjee S, Bhattacharjee A, Naha S, Majumdar T, Debbarma SK, Kaur H, *et al*. Molecular characterization of NDM-1-producing *Klebsiella pneumoniae* ST29, ST347, ST1224, and ST2558 causing sepsis in neonates in a tertiary care hospital of North-East India. Infect Genet Evol 2019;69:166–75. doi:10.1016/j.meegid.2019.01.024.

[11] Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 2005;63:219–28. doi:10.1016/j.mimet.2005.03.018.

[12] Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA. BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons. BMC Genomics 2011;12:402. doi:10.1186/1471-2164-12-402.

[13] Gardner SN, Slezak T, Hall BG. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. Bioinformatics 2015;31:2877–8. doi:10.1093/bioinformatics/btv271.

[14] Tenover FC, Arbeit RD, Goering R V, Mickelsen PA, Murray BE, Persing DH, *et al*. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233–9.

[15] Kado CI L ST. Rapid Procedure for Detection and Isolation of Large and Small Plasmids. J Bacteriol 1981;145:1365–73.

[16] Ni W, Li Y, Guan J, Zhao J, Cui J, Wang R LY. Effects of Efflux Pump Inhibitors on Colistin Resistance in Multidrug-Resistant Gram-Negative Bacteria. Antimicrob Agents Chemother 2016;60:3215–8. doi:10.1128/AAC.00248-16.

[17] Padilla E, Llobet E, Doménech-Sánchez A, Martínez-Martínez L, Bengoechea JA, Albertí S. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. Antimicrob Agents Chemother 2010;54:177–83. doi:10.1128/AAC.00715-09.

[18] Roy S, Datta S, Viswanathan R, Singh AK, Basu S. Tigecycline susceptibility in *Klebsiella pneumoniae* and *Escherichia coli* causing neonatal septicaemia (2007-10) and role of an efflux pump in tigecycline non-susceptibility. J Antimicrob Chemother 2013;68:1036–42. doi:10.1093/jac/dks535.

[19] Srinivasan VB, Rajamohan G. KpnEF, a New Member of the *Klebsiella pneumoniae* Cell Envelope Stress Response Regulon, Is an SMR-Type Efflux Pump Involved in Broad-Spectrum Antimicrobial Resistance. Antimicrob Agents Chemother 2013;57:4449–62. doi:10.1128/aac.02284-12.

[20] Srinivasan VB, Singh BB, Priyadarshi N, Chauhan NK, Rajamohan G. Role of novel multidrug efflux pump involved in drug resistance in *Klebsiella pneumoniae*. PLoS One 2014;9. doi:10.1371/journal.pone.0096288.

[21] Keeney D, Ruzin A, McAleese F, Murphy E, Bradford PA. MarA-mediated overexpression of the AcrAB efflux pump results in decreased susceptibility to tigecycline in *Escherichia coli*. J Antimicrob Chemother. 2008;61:46-53. doi:10.1093/jac/dkm397.

[22] Datta S, Roy S, Chatterjee S, Saha A, Sen B, Pal T, *et al*. A five-year experience of

carbapenem resistance in Enterobacteriaceae causing neonatal septicaemia: Predominance of NDM-1. PLoS One 2014;9. doi:10.1371/journal.pone.0112101.

[23] Chen L, Mathema B, Chavda KD, DeLeo FR, Bonomo RA KB. Carbapenemase-producing *Klebsiella pneumoniae*: molecular and genetic decoding. Trends Microbiol 2014;22:686–96. doi:10.1097/CCM.0b013e31823da96d.Hydrogen.

[24] Giuffrè M, Bonura C, Geraci DM, Saporito L, Catalano R, Di Noto S, *et al*. Successful control of an outbreak of colonization by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* sequence type 258 in a neonatal intensive care unit, Italy. J Hosp Infect 2013;85:233–6. doi:10.1016/j.jhin.2013.08.004.

[25] Wasfi R, Elkhatib WF, Ashour HM. Molecular typing and virulence analysis of multidrug resistant *Klebsiella pneumoniae* clinical isolates recovered from Egyptian hospitals. Sci Rep 2016;6:38929. doi:10.1038/srep38929.

[26] Shon AS, Bajwa RPS, Russo TA. Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*: A new and dangerous breed. Virulence 2013;4:107–18. doi:10.4161/viru.22718.

[27] Ravi A, Valdés-Varela L, Gueimonde M, Rudi K. Transmission and persistence of incF conjugative plasmids in the gut microbiota of full-term infants. FEMS Microbiol Ecol 2018;94. doi:10.1093/femsec/fix158.

[28] Messaoudi A, Haenni M, Mansour W, Saras E, Khalifa ABH, Chaouch C, *et al*. ST147 NDM-1-producing *Klebsiella pneumoniae* spread in two Tunisian hospitals. J Antimicrob Chemother 2017;72:315–6. doi:10.1093/jac/dkw401.

[29] Giakkoupi P, Papagiannitsis CC, Miriagou V, Pappa O, Polemis M, Tryfinopoulou K, *et al*.

An update of the evolving epidemic of blaKPC-2-carrying *Klebsiella pneumoniae* in Greece (2009-10). J Antimicrob Chemother 2011;66:1510–3. doi:10.1093/jac/dkr166.

[30] Nahid F, Zahra R, Sandegren L. A blaOXA-181-harbouring multi-resistant ST147 *Klebsiella pneumoniae* isolate from Pakistan that represent an intermediate stage towards pan-drug resistance. PLoS One 2017;12:e0189438. doi:10.1371/journal.pone.0189438.

[31] Pragasam AK, Shankar C, Veeraraghavan B, Biswas I, Nabarro LEB, Inbanathan FY, *et al*. Molecular mechanisms of colistin resistance in *Klebsiella pneumoniae* causing bacteremia from India-A first report. Front Microbiol 2017;7:2135. doi:10.3389/fmicb.2016.02135.

[32] Pournaras S, Kristo I, Vrioni G, Ikonomidis A, Poulou A, Petropoulou D, *et al*. Characteristics of meropenem heteroresistance in *Klebsiella pneumoniae* carbapenemase (KPC)-producing clinical isolates of *K. pneumoniae*. J Clin Microbiol 2010;48:2601–4. doi:10.1128/JCM.02134-09.

[33] Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: Acquired and intrinsic resistance in bacteria. Front Microbiol 2014;5:1–18. doi:10.3389/fmicb.2014.00643.

[34] Baron SA, Rolain JM. Efflux pump inhibitor CCCP to rescue colistin susceptibility in mcr-1 plasmid-mediated colistin-resistant strains and Gram-negative bacteria. J Antimicrob Chemother. 2018;73:1862-1871. doi: 10.1093/jac/dky134.

[35] Telke AA, Olaitan AO, Morand S, Rolain JM. soxRS induces colistin hetero-resistance in *Enterobacter asburiae* and *Enterobacter cloacae* by regulating the acrAB-tolC efflux pump. J Antimicrob Chemother. 2017;72:2715-2721. doi: 10.1093/jac/dkx215.

[36] Rosenblum R, Khan E, Gonzalez G, Hasan R, Schneiders T. Genetic regulation of the *ramA*

locus and its expression in clinical isolates of *Klebsiella pneumoniae*. Int J Antimicrob Agents 2011;38:39–45. doi:10.1016/j.ijantimicag.2011.02.012.

[37] De Majumdar S, Yu J, Fookes M, McAteer SP, Llobet E, Finn S, *et al*. Elucidation of the *RamA* regulon in *Klebsiella pneumoniae* reveals a role in LPS regulation. PLoS Pathog 2015;11:1–22. doi:10.1371/journal.ppat.1004627.

[38] Huang L, Feng Y, Zong Z. Heterogeneous resistance to colistin in *Enterobacter cloacae* complex due to a new small transmembrane protein. J Antimicrob Chemother. 2019;74:2551-2558. doi: 10.1093/jac/dkz236.

[39] Kádár B, Kocsis B, Tóth Á, Kristóf K, Felső P, Kocsis B, *et al*. Colistin resistance associated with outer membrane protein change in *Klebsiella pneumoniae* and *Enterobacter asburiae*. Acta Microbiol Immunol Hung 2017;64:217–27. doi:10.1556/030.64.2017.017.

Figure Legend

Fig. 1. Schematic representation of Tn*4401*b bearing ***bla*$_{KPC-2}$**.

Genes and their corresponding transcription orientations are indicated by horizontal arrows. Tn*4401b* is delimited by two inverted repeat sequences, IRR and IRL (grey triangles). Small open triangles represent the inverted repeats of IS*Kpn6* and IS*Kpn7*.

Fig. 2. Diagrammatic representation of class 1 integron found in KPC-2 bearing isolates.

arr-2 (ADP-ribosyl transferase), *S.ma.I2* (group IIc intron), ***qacEΔ11*** (quaternary ammonium compound resistance protein), ***sul1 (***sulphonamide resistant dihydropteroate synthase***),* orf5** (an open reading frame of unknown function).

Fig. 3. BRIG analysis of KPC-2-producing *K. pneumoniae*.

Comparative genome map of KPC-2-producing *K. pneumoniae* using *Klebsiella pneumoniae subsp. pneumoniae HS11286* as a reference. From the inside out, circle 1 represents the mean centered G+C content; circle 2 shows GC skew calculated as (G − C) / (G + C); circle 3 to 6 represent the genome sequence similarity of EN5269 (*Kp3*), EN5226 (*Kp1*), EN5271 (*Kp4*), and EN5261 (*Kp2*) based on color gradient against the reference genome. The color gradients indicate the degree matching in the BLAST result for a shared region, as given to the top right of the ring.

Fig. 4. Molecular typing and relatedness of KPC-2-producing *K. pneumoniae* isolates. (**A)** Phylogenetic tree analysis among the *bla*$_{KPC-2}$ containing isolates under study using iTOL. Branch length has been depicted showing evolutionary differences between the isolates. *Kp1* & *Kp3* are closely related so as *Kp2* & *Kp4*. (**B)** PFGE of *XbaI*-digested genomic DNA of KPC-2-producing *K.*

pneumoniae, isolated from blood of 4 septicemic neonates. Lane 1: *Kp2*; lane 2: *Kp1*; lane 3: *Kp3*; lane 4: *Kp4*, and lanes M: *Salmonella* serotype *Braenderup H9812* as reference standard.