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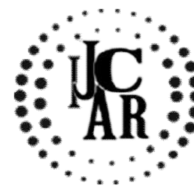

Research Article

OMP 36 MEDIATED MEROPENEM RESISTANCE IN CLINICAL ISOLATES OF *KLEBSIELLA PNEUMONIA*

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ABSTRACT

β-lactamase genes have evolved over the years with improved specificity and an extended array of antibiotic profile. The impact on resistance is also attributed to the intrinsic mechanisms of porin channel mediated transport of antibiotics. We here reported the intrinsic mechanism of porin channel mediated transport in meropenem resistant clinical isolates of *Klebsiella pneumoniae.* A total of 52 meropenem resistant *K. pneumoniae* isolates were selected and the antibiotic resistant gene status of TEM, CTX-M, SHV, NDM and OXA-48 and the omp 35 and 36 were detected through PCR. These isolates were selected on the basis of their phenotypic expression of β-lactamase production tested as per the Clinical & Laboratories Standards Institute (CLSI) guidelines, 2011. Four isolates based on the presence of the resistant genes were then selected as representatives to study the omp36 porin channel. Sequence analysis of all the representatives of omp36 from Kp08, Kp12, Kp13 and Kp36 plausibly suggested the presence of significant mutation. Structure analysis of all the representatives by *in silico* modelling, structure refinement and energy minimization showed that Kp12 has the abnormal organization of β-barrel omp36 porin channel where an extended β-sheet was blocking the channel core. Molecular docking analysis deciphered the blocking of meropenem antibiotics through the channel pore hindered by the mutant omp36. Variations in the gene sequence of omp36 have shown to affect meropenem resistance in *K. pneumoniae* which is observed to be just as sinister for treatment in clinical setting.

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INTRODUCTION

Resistance to antibiotics have been recognized since its discovery and along the timeline of developing new antibiotics coping mechanisms in bacteria also evolved (Silver, 2011). Oxacillin hydrolysing Class-D β-lactamase, OXA-48 is one such extrinsic coping mechanism with a remarkable activity against carbapenems. Variability in the resistance pattern in bacteria is a result of the coping mechanisms involved (Cho *et al*., 2015). OXA-48 hydrolyse carbapenems at a low level sparing expanded spectrum β-lactams and is not inhibited by EDTA or clavulanic acid (Livermore, 1995), however, the resistance to carbapenems is usually higher when associated with porin deficiencies or mutations that do not allow antibiotic entry (Zamana *et al*., 2014).

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This understanding emphasizes on the complete investigation of factors expressing themselves towards antibiotic resistance in clinical bacteria.

The outer membrane of gram-negative bacteria plays a significant role with a variety of functions; it serves as a diffusion barrier to extracellular solutes and also interacts with the environment. This membrane is composed of a bilayer containing phospholipids, lipopolysaccharide (LPS), and proteins. In some Gram-negative species such as *Enterobacter*, *Escherichia* and *Klebsiella*, β-lactam susceptibility has been shown to be closely related to the presence of non-specific porins belonging to the OmpC and OmpF groups (Ziervogel & Roux, 2012). The antibiotic resistant bacteria exhibit the ability to modify the porin profile where a shift in the type of porin expressed, reduction in porin expression level or presence of mutated porin play a critical role in resistance to β-lactamases and cephalosporinases. In most of these isolates, OmpK35, which belongs to the OmpF porin group and has a large channel size which is replaced with OmpK36, belonging

to the OmpC porin group and possesses a smaller channel size. This plays a major role in the existing antibiotic resistant trend exploring the mechanism and prevalence of its action against antibiotics has become important to understand the resistance breakout. (Blair *et al*., 2015)

With a chance detection of OXA-48 in a bacteria isolated from a patient diagnosed with acute aspiration pneumonia in a tertiary care centre in Assam, a retrospective study was conducted to detect the presence of this gene in other meropenem resistant *K. pneumoniae* and study the possible Therefore, through this study we aim to bring forth the co-occurring coping mechanisms related to antibiotic resistance in clinical bacteria.

MATERIALS AND METHODS

Bacterial isolates

K. pneumoniae isolates showing resistance to meropenem were included for this study. These isolates were obtained from various clinical specimens, such as sputum, pus, throat swab, urine, etc. of patients from a tertiary care centre in Assam. These isolates, a total of 52, were taken from a repository stored as glycerol stocks in -80°C. All isolates when collected were identified as *Klebsiella pneumoniae* by IMViC tests and Grams staining. PCR amplification of the housekeeping gene for malate dehydrogenase (*mdh*) was carried out as a confirmatory molecular detection test (Hæggman *et al*., 2004).

Phenotypic detection of carbapenemase production

Isolates resistant or intermediately resistant to any carbapenem were phenotypically detected for the production of carbapenemases by the Modified Hodge test (CLSI, 2011) and by comparing the zone diameter surrounding ertapenem discs supplemented with and without 0.5M EDTA (750 µg); an increase of zone diameter by ≥4mm suggested the production of metallo-carbapenemase (Hornsey, 2011).

Detection of β-lactamase and omp genes

Bacterial DNA isolation for all experiments was done by boiling lysis (Lal *et al*., 2007). 2 µL of this crude DNA extract was used as template for all the reactions. Primers for the detection of CTX-M (Jemima and Verghese, 2008), TEM and SHV (Lal *et al*., 2007), OXA-48 (Nordmann *et al*., 2011), and omp 35 and omp 36 (Zamana *et al*., 2014) were published earlier. The primer for NDM (Forward 5'-CACTTCCTATCTCGACATGC-3' and Reverse 5'-GGGCCGTATGAGTGATTG-3) were designed by creating a consensus sequences of all NDM variants as provided in Lahey.org/Studies/ (Bush *et al*., 2001). The reaction mixture for a uniplex PCR comprised 1.5 mM of $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM primers (forward and reverse each) with 0.5U AmpliTaq® DNA Polymerase (Applied Biosystems, California, USA) in a 1X PCR buffered reaction. Positive controls used were *K. pneumoniae* ATCC 700603 (SHV-18), J53 pMG298 (CTX-M-15), and clinical isolates each containing TEM, NDM and OXA-48 detected by uniplex PCR followed by sequence confirmation was used as a representative positive control and nuclease free molecular grade water (Himedia Pvt. India, Ltd, Mumbai) was used as negative control. The cycling conditions for amplifying the genes were as follows: initial denaturation at 95°C for 5 minutes and 30 cycles at 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, followed by a final extension of 3 min at 72°C. PCR products were analyzed in a 1.5% agarose gel. A molecular ladder of 100 bp was run with every gel.

Molecular modelling of omp36 protein

Four PCR amplicons of the omp36 gene of isolates Kp08, Kp12, Kp13 and Kp36 were selected (sequencing). All the four sequences were aligned in BLASTn (www.ncbi.nlm.nih.gov/BLASTn) individually and compared for sequence similarity with the members of their protein family. The nucleotide sequences were translated in six reading frames using EXPASY Sib tool (http://web.expasy.org/translate/) and the appropriate reading frames were selected for protein modelling. A protein modelling tool Modeller9v8 (Sali *et al*., 1995) was used to generate the 3-D model of all the four omp36 proteins. Modeller9v8 is a web-based service for protein structure prediction using the principles and techniques of homology modelling. The structures were further validated in Ramachandran Plot and additional loop refinement was done by using the loop model class in Modeller9v8 (Sali *et al*., 1995). The model with the lowest discrete optimized protein energy (DOPE) was chosen for further refinements.

Structure refinement and quality assessment

The structure of Omp36 with the lowest DOPE score obtained from Modeller9v8 was further improved by energy minimization at the YASARA Energy Minimization Server (www.YASARA.org/minimizationserver), in which molecular dynamics simulations of models were carried out in explicit solvent. It uses a new partly knowledge-based all atom force field derived from Amber. The presence of α-helix, β-sheet, turns, coils, etc was calculated was using PDB generate which provides an overview of the contents of each three dimensional structure (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html).

Multiple sequence analysis

To investigate these conserved amino acid residues, multiple sequence alignment was performed with representative members of omp 36 of all the sequences generated via ExPASY TRANSLATE saved in FASTA format. Alignment was performed using the ClustalW program, and final alignment was generated by ESpript (http://espript.ibcp.fr) for better understanding of conserved residues and structure. To investigate the conserved amino acid residues of the omp36 porin channel, multiple sequence alignment was performed with the four representative sequences which have substrate speficity to meropenem. The protein sequence was retrieved from PDB (http://www.rcsb.org/pdb/), saved in FASTA format. Alignment was performed using CLUSTALW program (Thompson *et al*., 1994) and final alignment was generated by ESpript (http://espript.ibcp.fr), for better understanding of conserved residues and structure.

Docking Studies

The docking simulations were performed by using the AutoDock 4 software package (Morris *et al*., 2009). The antibiotic meropenem for docking study were obtained from pubchem (http://pubchem.ncbi.nlm.nih.gov). The omp36 modelled protein of the isolates Kp08, Kp12, Kp13 and Kp36 were also saved as a PDB file format after removing non-polar hydrogen atoms and adding their charges with the carbon atoms. The grid parameters were generated by using the

"autogrid" package present in AutoDock4 software suite. The grid maps were set with the dimensions of 126 x 126 x 126 in the xyz directions. In order to perform the final docking, the conformational search was performed by using the Lamarckian Genetic Algorithm, which was set to 40. Rest all other parameters were set to default values such as initial population size (150), and maximum number of generation (140). After 140 independent successful docking runs protein-ligand complex for each ligand having auto generated lowest free energy of binding (ΔG) confirmation were saved. Docking results were analyzed using PyMOL (www.pymol.org) and final figure was generated with the help of PyMOL.

RESULTS AND DISCUSSION

Phenotypic detection of carbapenemase production

Of the 52 tested *K. pneumoniae* isolates, 27 were identified as metallo-β-lactamase producers and 23 as carbapenemase producers. ESBL production was detected in 40 isolates from our previous experiments. The phenotypic expression of a β-lactamase trait is indicative of a possible antibiotic resistant trend meted upon by the presence of a representative gene. Each type of an ESBL or carbapenem resistant gene despite having different mechanism of activity, basically have a similar substrate profile. They hydrolyse the β-lactam antibiotics – mostly with a very high affinity towards 3rd generation cephalosporins and monobactams while the metallo-β-lactamases or other carbapenemases inactivate a much potent group of antibiotics – carbapenems. Phenotypic detection aids a clinician in strategizing the most appropriate mode of antibiotic therapy; however, it does not truly help understand the underlying genetic mechanism. Molecular tests become important for further analysis of resistance mechanism.

Detection of β-lactamase and omp genes

PCR standardized to detect the presence of the most common antibiotic resistant mechanisms included the CTX-M, TEM and SHV type which were detected in 16, 21 and 14 isolates respectively, of the total number of 40 isolates that were phenotypically detected. Only NDM was detected in these samples and in 15 of them while OXA-48 was detected in 8 of 23 phenotypically positive isolates (Supplementary Table). This data revealed the role of such β-lactamase gene(s) in mediating antibiotic resistance in bacteria harbouring these genes.

However, there are other mechanisms which mediate antibiotic resistance particularly to 3rd generation cephalosporins and carbapenems – porin mediated resistance. Porin protein serve as gateways to the entry of antibiotics in to the bacterial cell. Resistance mediated by porins would mean (i) deletion of the porin gene resulting in absence of the porin channel in the cellular membrane or (ii) mutations in the porin gene which alters the channel in a manner in which the antibiotic is not allowed entry into the cell. This porin mediated resistance can also be detected along with the presence of β-lactamase. (Blair *et al.*, 2015)

Using primers published earlier, the role of two porin channels - Omp 35 and Omp 36 were analysed. Both the porins have been touted to be responsible for antibiotic entry into the cell. Omp35 gene was detected in each of the tested bacterial isolate. Omp36 was detected in 47 of the 52 isolates tested and was PCR negative in 5 isolates.

Of these four isolates, Kp14 harboured CTX-M, TEM, NDM and OXA-48 genes and Kp36 harboured only NDM gene; these isolates showed the presence of extensive antibiotic resistance mechanism – intrinsic (loss of porin gene) and acquired (presence of β-lactamase genes) mechanism. While the remaining two isolates – Kp08, 12 tested positive to phenotypic detection of β-lactamase genes but were PCR negative (except Kp12 which carried TEM). The absence of omp36 warrants the prevailing antibiotic resistance profile (Table 2).

Structural characterization of the omp36 porin channel

The sequences from the four isolates - Kp08, Kp12, Kp14 and Kp36 were compared to the GenBank database using default parameters which displayed hits confirming the status as *Klebsiella pneumoniae* omp36 gene. While the protein sequences generated from using displayed hits belonging to Gram negative porins of the OM channel superfamily. For the comparison of the sequences generated, a control or reference sequence was selected from NCBI (http://www.ncbi.nlm.nih.gov/). All the four isolates that belong to the OM channel superfamily are rich in β-sheets.

The topology of final modelled structure was generated by PDBsum (http://www.ebi.ac.uk/pdbsum/) displayed 15 β-strands with 3 α-helices and 18 random coils for Kp08 (Figure - 1), 15 β-sheets with 7 α-helices and 23 random coils in Kp12, 17 β-s with 5 α-helices and 25 random coils in Kp14 and 17 β-s with 4 α-helices and 22 random coils in Kp36.

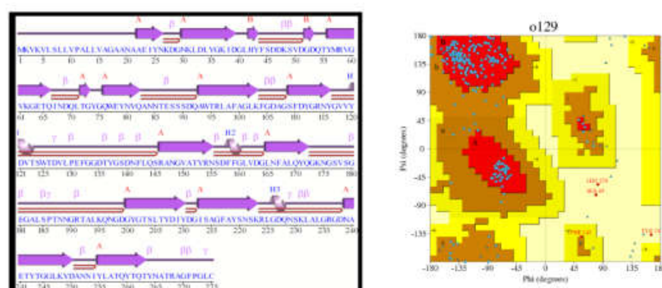

Figure 1 Secondary structure and Ramachandran plot omp36 sequenced from isolate Kp08.

Structure validation using Ramachandran plot after energy minimization exhibited 81.9%, 82.4%, 84.4% and 84.9% in the favoured region, 16.4%, 15.6%, 13.9% and 13.2% in additional allowed regions and 0.4%, 1.6%, 1.6% and 1.2% in generously allowed regions, respectively.

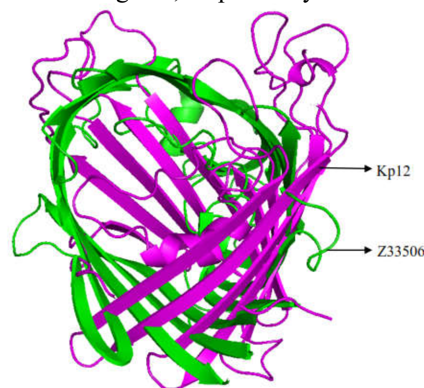

Figure 2 Overlapping structures of omp36 porin protein channel of isolate Kp12 (Purple) and *K. pneumoniae* ATC 13883 (Z33506).

Therefore, the predicted models can be considered as of good quality and were adopted for further docking studies.

Supplementary Table Antibiotic resistance, gene status and phenotypic confirmation of all 52 isolates

Strain #	Phenotypic detection of ESBL	Genetic basis of resistance			Phenotypic detection of MBL	Genetic basis of resistance	Phenotypic detection of Carbapenemase	Genetic basis of resistance	Genetic basis of Porin mediated resistance[a]	
	DDST	CTX-M	TEM	SHV	CDDT	NDM	MHT	OXA-48	omp 35	omp 36
Kp01	P	N	N	N	N	N	N	N	P	P
Kp02	P	N	N	N	N	N	N	N	P	P
Kp03	P	N	N	N	P	N	N	N	P	P
Kp04	P	N	P	P	P	P	P	N	P	P
Kp05	P	P	N	P	P	P	P	P	P	P
Kp06	N	N	N	N	P	N	P	N	P	P
Kp07	N	N	N	N	N	N	N	N	P	P
Kp08	P	N	N	N	N	N	N	N	P	N
Kp09	P	P	N	N	P	P	P	P	P	P
Kp10	P	P	P	P	P	P	P	P	P	P
Kp11	N	N	N	N	N	N	N	N	P	P
Kp12	P	N	P	N	N	N	N	N	P	N
Kp13	P	N	N	N	P	N	N	N	P	P
Kp14	P	P	P	N	P	P	P	P	P	N
Kp15	P	P	P	N	P	P	N	N	P	P
Kp16	N	N	N	N	N	N	N	N	P	P
Kp17	P	N	N	P	N	N	N	N	P	P
Kp18	P	P	P	P	N	N	P	N	P	P
Kp19	P	N	P	P	N	N	P	N	P	P
Kp20	N	N	N	N	N	N	N	N	P	P
Kp21	P	N	N	N	P	P	P	N	P	P
Kp22	N	N	N	N	N	N	P	N	P	P
Kp23	P	P	P	P	P	N	N	N	P	P
Kp24	P	P	P	N	P	N	P	N	P	P
Kp25	P	N	N	P	P	P	P	N	P	P
Kp26	P	N	N	N	P	P	P	N	P	P
Kp27	P	P	P	P	P	N	P	N	P	P
Kp28	P	N	N	N	N	N	N	N	P	P
Kp29	P	N	N	N	P	P	P	N	P	P
Kp30	P	P	P	P	P	P	P	N	P	P
Kp31	P	N	N	N	P	P	N	N	P	P
Kp32	P	N	N	N	N	N	N	N	P	P
Kp33	P	P	P	N	P	N	N	P	P	P
Kp34	P	P	P	N	N	N	N	N	P	P
Kp35	N	N	N	N	N	N	N	N	P	P
Kp36	P	N	N	N	P	P	P	N	P	N
Kp37	P	P	P	P	P	P	P	N	P	P
Kp38	P	P	P	P	P	N	N	N	P	P
Kp39	P	N	P	N	N	N	N	N	P	P
Kp40	P	N	P	N	N	N	N	N	P	P
Kp41	P	N	P	N	N	N	N	N	P	P
Kp42	P	N	P	N	P	N	P	P	P	P
Kp43	N	N	N	N	N	N	N	N	P	P
Kp44	N	N	N	N	P	N	P	N	P	P
Kp45	N	N	P	N	P	N	P	P	P	P
Kp46	N	N	N	P	P	P	P	N	P	P
Kp47	N	N	N	N	N	N	N	N	P	P
Kp48	P	P	N	N	N	N	N	N	P	P
Kp49	P	N	N	N	N	N	N	N	P	P
Kp50	P	N	N	N	N	N	N	N	P	N
Kp51	P	N	N	P	P	N	P	N	P	P
Kp52	P	P	P	N	N	N	N	N	P	P
Total number	40	16	21	14	27	15	0	0	23	8
Percent Prevalence	76.92	30.76	40.38	26.92	51.92	28.84	0	0	44.23	90.38

Key – P: Positive, N: Negative, aDetected by PCR of omp 35/36

The superimposition of the modelled omp36 porin channel of Kp12 over *K. pneumoniae* ATC 13883 (Z33506) shows the complete structural arrangement of the clinical isolate blocking the open channel from entry of meropenem or any carbapenem (Figure-2). These overlapping structures allowed understanding how intrinsic mechanisms affect antibiotic resistance.

Multiple sequence analysis

MSA results showed that the amino acid residues are conserved within the selected representatives as compared to the wild type strain, *K. pneumoniae* ATC 13883 (Z33506). However, all isolates showed mutation from position 58-61 were the wild type sequence read -ETRL- while the clinical isolates showed the deletion of TR and changes in the amino acid from E to R and L to V. Similar changes were observed at position 186-188 wherein Kp08 had an addition of LSP in the amino acid sequence, greatly altering the fold of the omp36 porin protein. Deletions after 290th amino acid were observed in all sequences. Espript analysis showed the deletion of prevalent β-sheets (17, 18 and 19) in all the isolates out of 20 β-sheets as compared with the wild type omp36.

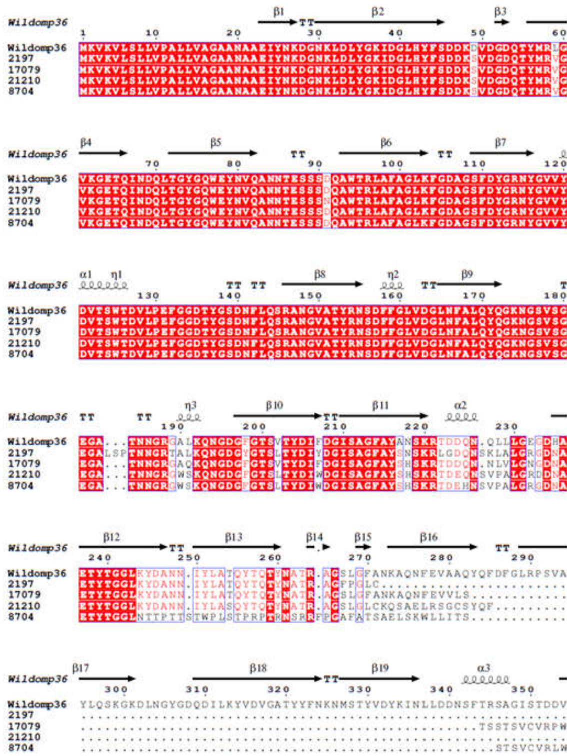

Figure 3

Interaction studies based on molecular docking

The ligand binding sites were identified in the structures of all the omp36 modelled proteins obtained from the isolates Kp08, Kp12, Kp13 and Kp36 by using the AutoLigand module [16] present in the autodock4 software package. The meropenem was docked in the ligand binding sites identified in all the omp36 proteins (Figure 4). The docking studies between omp36 obtained from the Kp08 isolates and meropenem showed the free energy of binding of -4.48 Kcal/mol and inhibition constant of 515.86 µM. The meropenem was found to be interacted with Asp140, Leu194, Gln196, Phe271 and Cys275 of Kp08. Similarly with Kp12, it was showing interaction with Ser141, Asn143, Lys194, His227, Leu233, Arg268 and Pro270 with binding energy of -4.89 Kcal/mol as well as the inhibition constant of 260.57 µM.

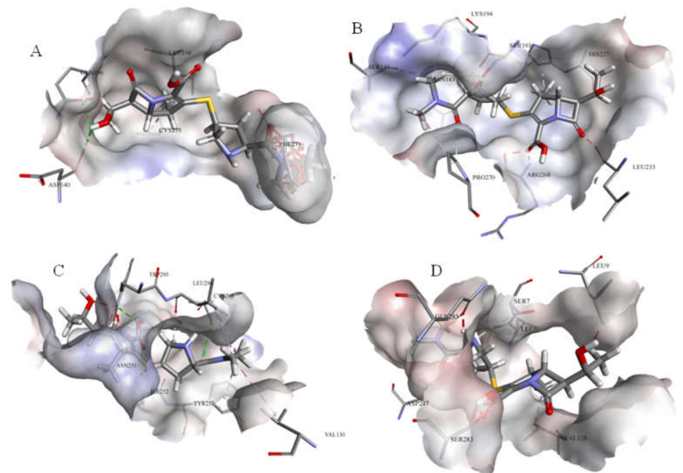

Figure 4: The interaction profile of meropenem with opm36 of (A) Kp08, (B) Kp12, (C) Kp13 and (D) Kp 36

Furthermore, with Kp13 and Kp36 we have observed the binding energy of -3.06 Kcal/mol and -3.89 Kcal/mol respectively. The residues Val130, Asn251, Ile252, Tyr253, Tpr295, Leu296 and Cys298 of Kp13 were involved in interactions, whereas for Kp36 the Leu6, Ser7, Leu9, Val128, Ser283 and Gln285 form the binding sites (Table - 1).

Table 1 The docking simulations generated parameters showing the interaction behaviors of different strains isolated omp36 proteins and meropenem

S. No	Complex name	Free energy of Binding (kcal/mol)	Ligand Efficiency	Inhibition constant (µM)	vdW + Hbond + desolv Energy (kcal/mol)	Intermolecular energy (kcal/mol)	Total internal (kcal/mol)	Torsional energy (kcal/mol)	Interacting Residues
1.	Kp08	-4.48	-0.17	515.86	-5.16	-6.57	-3.16	2.09	Asp140, Leu194, Gln196, Phe271, Cys275
2.	Kp12	-4.89	-0.19	260.57	-4.61	-6.98	-4.15	2.09	Ser141, Asn143, Lys194, His227, Leu233, Arg268, Pro270
3.	Kp13	-3.06	-0.12	5.72	-3.7	-5.15	-4.99	2.09	Val130, Asn251, Ile252, Tyr253, Tpr295, Leu296, Cys298
4.	Kp36	-3.89	-0.15	1.45	-5.57	-5.96	-5.08	2.09	Leu6, Ser7, Leu9, Val128, Ser283, Gln285

CONCLUSION

Despite screening for antibiotic resistance genes in clinically relevant bacteria it becomes important to consider intrinsic factors [6]. The outer membrane porin channels plays an integral role in the development of resistance even without the expression of commonly occurring β-lactamase genes as shown in the results (by isolates Kp08, Kp12 and Kp13).

Phenotypic expression of antibiotic resistance but absence of genetic factors thus could be attributed to non-enzymatic factors.

Ethical statement

Study methodology was approved by Institutional Ethical Committee of Gauhati University (GU/ACA/Ethics/2012/3993 dated 10/1/12).

Conflict of Interest

We (authors) state that there are neither conflicts of interest nor any competing financial interests.

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