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Proteome profiling of carbapenem-resistant *K. pneumoniae* clinical isolate (NDM-4): Exploring the mechanism of resistance and potential drug targets

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ABSTRACT

The emergence of carbapenem resistance has become a major problem worldwide. This has made treatment of *K. pneumoniae* infections a difficult task. In this study, we have explored the whole proteome of the carbapenem-resistant *Klebsiella pneumonia* clinical isolate (NDM-4) under the meropenem stress. Proteomics (LC-MS/MS) and bioinformatics approaches were employed to uncover the novel mystery of the resistance over the existing mechanisms. Gene ontology, KEGG and STRING were used for functional annotation, pathway enrichment and protein–protein interaction (PPI) network respectively. LC-MS/MS analysis revealed that 52 proteins were overexpressed (≥10 log folds) under meropenem stress. These proteins belong to four major groups namely protein translational machinery complex, DNA/RNA modifying enzymes or proteins, proteins involved in carbapenems cleavage, modifications & transport and energy metabolism & intermediary metabolism-related proteins. Among the total 52 proteins 38 {matched to *Klebsiella pneumonia* subsp. pneumoniae (strain ATCC 700721/MGH 78578)} were used for functional annotation, pathways enrichment and protein–protein interaction. These were significantly enriched in the "intracellular" (14 of 38), "cytoplasm" (12 of 38) and "ribosome" (10 of 38). We suggest that these 52 over expressed proteins and their interactive proteins cumulatively contributed in survival of bacteria and meropenem resistance through various mechanisms or enriched pathways. These proteins targets and their pathways might be used for development of novel therapeutics against the resistance; therefore, the situation of the emergence of "bad-bugs" could be controlled.

1. Introduction

Worldwide resistance to broad-spectrum antimicrobials (extended-spectrum cephalosporins) is a well recognized problem among enterobacteriaceae, a family of Gram-negative bacteria [1,2]. *Klebsiella pneumoniae* is the most significant member of the enterobacteriaceae in the clinical setting. Carbapenems have served as an important antimicrobial class for the treatment of these drug resistant organisms, including those which producing extended spectrum beta-lactamases (ESBLs) [3,4]. However, due to production of carbapenemases, carbapenem resistant enterobacteriaceae (CRE) have been emerged and spread globally which is representing a serious threat to public health. Carbapenemases are specific beta-lactamases with the ability to hydrolyze carbapenems and their production is the most widespread cause of carbapenem resistance. An increasing number of class-A carbapenemases (KPC and GES enzymes), class-B metallo-beta-lactamases (VIM, IMP, and NDM beta-lactamases), class-C beta-lactamases (CMY-10 and PDC beta-lactamases) and class-D carbapenemases (OXA-23) have recently emerged. In addition, overproduction of class-C beta-lactamases (CMY-10 and PDC beta-lactamases) as well as loss of porins can also lead to carbapenem resistance [5–9]. Several explanations have been put forward to explain the mechanisms of carbapenem resistance but still our knowledge regarding resistance is fragmentary.

However less attention has been paid towards the transcriptome and proteome that might play an important role in the development of drug resistance. Proteome is the functional moiety of the cell and directly correlated to the external stimuli like drug stress and resistance [10]. During drug stress, internal harmony of the bacterial system disturbed and rapidly bacteria adopted alternative cellular functions mediated by proteome to overcome the effect. Proteomics coupled with bioinformatics are the potential strategy to explore the biological problems. Differential expression proteome analysis of drug resistant bacteria

Abbreviations: MIC, Minimum inhibitory concentration; ESBLs, Extended spectrum beta-lactamases; CLSI, Clinical and Laboratory Standards Institute; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; LB, Luria-Bertani

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under drug stress could unravel the novel mechanism of the bacteria to overcome the effects of drugs. Comparative proteomic studies addressing the whole cell proteome of drug resistant microbial strains with or without drug stress have been reported [11–16]. Through liquid chromatography coupled with mass spectrometry (LC-MS/MS) approach, we have analyzed the over expressed proteome of carbapenem resistant *K. pneumonia* (NDM-4) clinical isolate under the meropenem drug stress.

2. Materials and methods

2.1. Clinical bacterial strains and drug susceptibility testing

Carbapenems resistant *Klebsiella pneumonia* clinical isolate (NDM-4) was used in the present study. Drug susceptibility testing (DST) for the meropenem drug, against carbapenems resistant *Klebsiella pneumonia* clinical isolate was determined by micro-dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [17].

2.2. Culture and drug induction

K. pneumoniae (NDM-4) clinical isolate was grown in Luria Bertani broth (LB) at 37°C & 220 rpm. Sub-MIC (32 μg/ml) value of meropenem drug was added in one of the flask. Bacteria were grown up to the exponential phase (OD_{600} = 0.8), further cells were harvested by centrifugation at 8000 rpm for 8 min at 4°C and the cell pellet was stored at -80°C until required. All the experiments have replicated biologically.

2.3. Protein sample preparation

Cells were washed with normal saline and re-suspended in the lysis buffer (50 mMTris-HCl containing 10 mM $MgCl_2$, 0.1% sodium azide, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM (ethylene glycol tetraacetic acid) EGTA; pH 7.4) at the concentration of 1 g wet weight per 5 ml and then broken down by intermittent sonication by sonicator on power at 35% amplitude (Sonics & Materials Inc., Newtown, CT, USA for 10 min at 4 °C. Further homogenate was centrifuged at 12,000 *g* for 20 min at 4 °C and supernatant was stored at −80 °C [12–14]. The supernatant was precipitated overnight at -20°C by adding cold acetone to supernatant in access (1,4). The precipitated protein was collected by centrifugation (12,000 rpm, 20 min) and allowed to air dry and suspended in appropriate volume of protein dissolving buffer. Protein concentration was estimated using the Bradford assay [18]. Experiments have repeated technically.

2.4. Separation and identification of proteome by nanoLC-Triple TOF5600-MS

Equal amount of protein samples were digested using trypsin. Further, digested protein samples were analyzed using a Triple TOF 5600 mass-spectrometer (AB-Sciex, USA) equipped with Eskigent MicroLC 200 system (Eskigent, Dublin, CA) having an Eskigent C18-reverse phase column (150 × 0.3 mm, 3 μm, 120 Å). In order to generate spectral library for protein identification experiment, Data dependent analysis (DDA) was performed for the individual samples, to generate high quality spectral ion libraries for SWATH analysis, by operating the mass spectrometer with specific parameters. Spectral library were generated using information dependent acquisition (IDA) mode after injecting tryptic digest of 2 g on column using Eksigent nano LC-Ultra™ 2D plus system coupled with SCIEX Triple TOF® 5600 system fitted with nano spray III source. The samples were loaded on the trap (Eksigent Chrom XP 350 μm × 0.5 mm, 3 μm 120 Å) and washed for 30 min at 3 μL/min. A 120 min gradient in multiple steps (ranging from 5 to 50% Acetonitrile in water containing 0.1% formic acid) was set up to elute the peptides from the ChromXP 3-C18, 0.075 × 150 mm, 3 μm, 120 Å analytical column. Experiment has done in technical replicates.

2.5. Information dependent acquisition (IDA) parameters

In IDA ion library generation method was used where maximum 20 most intense multiple charged ions per MS cycle were selected to perform MS/MS fragmentation. A dynamic exclusion criterion was applied to each of the ions for 10 s. The accumulation time for each MS/MS experiment was set to 70 ms.

2.6. SWATH parameters for label free quantification

In SWATH acquisition method Q1 transmission window was set to 12 Da from the mass range for 350-1250 Da. Total 75 windows was acquired independently with an accumulation time of 62 ms, along with three technical replicates for each of the sets. Total cycle time was kept constant at < 5 s. To generate spectral library Protein Pilot™ v. 5.0 was used. For Label free quantification the peak extraction and spectral alignment were performed using PeakView® 2.2 Software with the specific parameters such as; number of peptides as 2, number of transitions as 5, peptide confidence as 95%, XIC width as 30 ppm, XIC extraction window as 3 min. The data was further subjected to Marker View software V 1.3 (AB Sciex) to get statistical data interpretation. In Marker View, Peak area under the curve for the selected peptides was normalized by internal standard protein (beta galactosidase) spike during the SWATH accumulation. The results were shown as three output files containing AUC of the ions, the summed intensity of peptides for protein and the summed intensity of ions for the peptide. All SWATH Acquisition data were processed using the SWATH Acquisition MicroApp 2.0 in PeakView® Software.

2.7. Data analysis

Data was processed with Protein Pilot Software v. 5.0 (AB SCIEX, Foster City, CA) utilizing the Paragon and Progroup Algorithm. Analysis was also done using the integrated tools in Protein Pilot at the 1% false discovery rate (FDR).

2.8. Gene ontology and pathway enrichment

In this study *Klebsiella pneumonia* subsp. pneumoniae (strain ATCC 700721 / MGH 78578) was used as a reference strain to carry out all functional annotation studies. The proteins that showed an alignment identity ≥50% over 80% of sequence length were assigned as its homolog and annotated with UniProtKB accession number which have used for the subsequent functional studies. For functional annotation, we used slim version of Gene ontology (GO) term; obtained from Gene Ontology Consortium (http://www.geneontology.org/) [19]. KEGG database was used to assign the metabolic pathway to the proteins [20].

2.9. Integration of a protein–protein interaction (PPI) network

To find the interaction partners, protein–protein interaction (PPI) information obtained by STRING database v10.0 (http://www.string-db.org/) [21–26] STRING based PPI established by experimental studies as well as by genomic analysis like domain fusion, phylogenetic profiling high-throughput experiments, co-expression studies and gene neighborhood. In the present study interactions having score ≥0.4 were considered as significant.

3. Results

3.1. LC-MS/MS based proteins identification

In this study we have grown the carbapenem resistant isolate under meropenem drug stress (Sub-MIC: 32). Further we have identified the proteome of the resistant bacteria by LC-MS/MS and using SWATH workflow; 1156 proteins were quantified at 1% FDR. Among them, 52

Table 1
Details of the over expressed proteome (> 10 folds) under meropenem stress in *Klebsiella pneumonia* clinical isolates (NDM-4).

S.No.	Protein name	Accession number	Log fold change vs. *P*-value
1	Elongation factor Tu	A6TEX7	66.69
2	Branched-chain alpha-keto acid dehydrogenase subunit E2	A0A2A5PUJ8	36.43
3	Elongation factor G	A6TEX8	34.35
4	Methionine adenosyltransferase	A6TDV1	26.71
5	Type I restriction-modification system,DNA-methyltransferase subunit M	W1BCK1	26.65
6	Beta-hexosaminidase	A6T7G6	26.48
7	Alpha-ketoacid dehydrogenase subunit beta	A0A2A5PUI0	26.36
8	30S ribosomal protein S11	A6TEV0	24.57
9	Beta-lactamase	A6TIL8	22.72
10	Type I restriction-modification system,specificity subunit S	W1BEE5	21.73
11	Metallo-beta-lactamase NDM-5	U5TTL2	20.55
12	Outer membrane protein C	A6TBT2	19.39
13	50S ribosomal protein L5	A6TEW0	19.20
14	Carbamoyl-phosphate synthase (glutamine-hydrolyzing)	A6T4I0	18.94
15	30S ribosomal protein S4	A6TEU9	18.43
16	DNA-binding protein	A6TAK8	17.94
17	30S ribosomal protein S13 (Fragment)	A6TEV1	17.22
18	UDP-glucose 6-dehydrogenase	A6TBE0	16.98
19	30S ribosomal protein S7	A6TEX9	16.68
20	Formate *C*-acetyltransferase	A6T6Z6	16.47
21	50S ribosomal protein L14	A6TEW2	16.22
22	tRNA (cytidine/uridine-2-O-)-methyltransferase TrmJ	A6TCF3	15.71
23	30S ribosomal protein S17	A6TEW3	15.68
24	Acetate kinase	A6TBY3	15.43
25	Metallo-beta-lactamase	A0A0K2DRS9	15.36
26	50S ribosomal protein L2	A6TEW9	15.15
27	Iron-sulfur cluster carrier protein	W1GPR1	14.65
28	Bifunctionalpolymyxin resistance protein ArnA	A6TF98	14.40
29	30S ribosomal protein S13 (Fragment)	A6TEV1	14.30
30	Catabolite control protein A	A0A2A5PY42	13.76
31	Elongation factor G	W1BCD5	13.64
32	50S ribosomal protein L15	A6TEV3	13.63
33	Biotin carboxylase of acetyl-CoA carboxylase	A6TES3	13.54
34	tRNA (guanine-N(7)-)-methyltransferase	A6TDW8	13.14
35	RibonucleaseVapC	A6TIM9	12.95
36	mRNA interferase	W1 AM39	12.88
37	50S ribosomal protein L17	A6TEU7	12.81
38	Transaldolase	A6T4E8	12.65
39	Exoribonuclease II	A6T7Z4	12.46
40	3-deoxy-manno-octulosonate cytidylyltransferase	A6T710	12.16
41	CTP synthase	A6TD54	11.95
42	Carbamoyl-phosphate synthase large chain	W1BCF0	11.79
43	NADH oxidoreductase	A6T6X1	11.67
44	Dihydropteroate synthase	A6TIZ0	11.65
45	Chromosome (Plasmid) partitioning protein ParB	A6TIC7	11.37
46	Glycine–tRNA ligase beta subunit	A6TFH4	10.85
47	GTP-binding protein TypA/BipA	A6TG74	10.83
48	Catabolite repressor-activator	A6T4M3	10.59
49	Protein RecA	A6TCW1	10.32
50	DNA-binding protein	W1AQL9	10.10
51	Exoribonuclease 2	W1B6L8	10.06
52	Inositol-1-monophosphatase	A6TCF4	10.05

proteins were over expressed up to ten log folds change vs. *p*-value and tabulated in the Table 1. The level of over-expression has been represented as the log folds change vs. p-value ratio. In-depth analysis of over expressed 52 proteins suggested that these belong to four major groups. These proteins involved in process of protein translational machinery complex, DNA/RNA modifying enzymes or proteins, proteins involved in intermediary metabolism & respiration and proteins involved in carbapenems drugs cleavage, modifications and transport.

Table 2
List of 38 DEGs in response to meropenem in *Klebsiella pneumoniae* subsp. *pneumonia* (strain ATCC 700721 / MGH 78578).

S. No.	Gene name	Protein name	Accession number	Log fold change vs. P-value
1	tufA	Elongation factor Tu	A6TEX7	66.69
2	fusA	Elongation factor G	A6TEX8	34.35
3	metK	Methionine adenosyltransferase	A6TDV1	26.71
4	nagZ	Beta-hexosaminidase	A6T7G6	26.48
5	rpsK	30S ribosomal protein S11	A6TEV0	24.57
6	bla	Beta-lactamase	A6TIL8	22.72
7	ompC	Outer membrane protein C	A6TBT2	19.39
8	rplE	50S ribosomal protein L5	A6TEW0	19.2
9	carB	Carbamoyl-phosphate synthase (glutamine-hydrolyzing)	A6T4I0	18.94
10	rpsD	30S ribosomal protein S4	A6TEU9	18.43
11	hns	DNA-binding protein	A6TAK8	17.94
12	rpsM	30S ribosomal protein S13 (Fragment)	A6TEV1	17.22
13	ugd	UDP-glucose 6-dehydrogenase	A6TBE0	16.98
14	rpsG	30S ribosomal protein S7	A6TEX9	16.68
15	pflB	Formate *C*-acetyltransferase	A6T6Z6	16.47
16	rplN	50S ribosomal protein L14	A6TEW2	16.22
17	trmJ	tRNA (cytidine/uridine-2-O-)-methyltransferase, TrmJ	A6TCF3	15.71
18	rpsQ	30S ribosomal protein S17	A6TEW3	15.68
19	ackA	Acetate kinase	A6TBY3	15.43
20	rplB	50S ribosomal protein L2	A6TEW9	15.15
21	arnA	Bifunctionalpolymyxin resistance protein ArnA	A6TF98	14.4
22	rplO	50S ribosomal protein L15	A6TEV3	13.63
23	accC	Biotin carboxylase of acetyl-CoA carboxylase	A6TES3	13.54
24	trmB	tRNA (guanine-N(7)-)-methyltransferase	A6TDW8	13.14
25	mck	RibonucleaseVapC	A6TIM9	12.95
26	rplQ	50S ribosomal protein L17	A6TEU7	12.81
27	talB	Transaldolase	A6T4E8	12.65
28	rnb	Exoribonuclease II	A6T7Z4	12.46
29	kdsB	3-deoxy-manno-octulosonate cytidylyltransferase	A6T710	12.16
30	pyrG	CTP synthase	A6TD54	11.95
31	hcr	NADH oxidoreductase	A6T6X1	11.67
32	sul	Dihydropteroate synthase	A6TIZ0	11.65
33	sopB	Chromosome (Plasmid) partitioning protein ParB	A6TIC7	11.37
34	glyS	Glycine–tRNA ligase beta subunit	A6TFH4	10.85
35	bipA	GTP-binding protein TypA/BipA	A6TG74	10.83
36	fruR	Catabolite repressor-activator	A6T4M3	10.59
37	recA	Protein RecA	A6TCW1	10.32
38	suhB	Inositol-1-monophosphatase	A6TCF4	10.05

3.2. Functional analysis of meropenem- responsive proteins

Among 52 over expressed proteins 38 proteins were enriched by using the *Klebsiella pneumonia* subsp. pneumoniae (strain ATCC 700721/MGH 78578) as a reference strain to carry out all functional annotation studies (Table 2). Analysis of functional enrichment revealed that the most abundant biological process (BP), among the up-regulated proteins, was “translation”. 14 of 38 up-regulated proteins were found to be involved in translation. Other major processes in decreasing order of representation were biosynthetic process, cellular nitrogen compound metabolic process, and carbohydrate metabolic process. 11, 11 and 7 proteins were part of these processes respectively (Fig. 1a). In molecular function (MF) category, the upregulated proteins

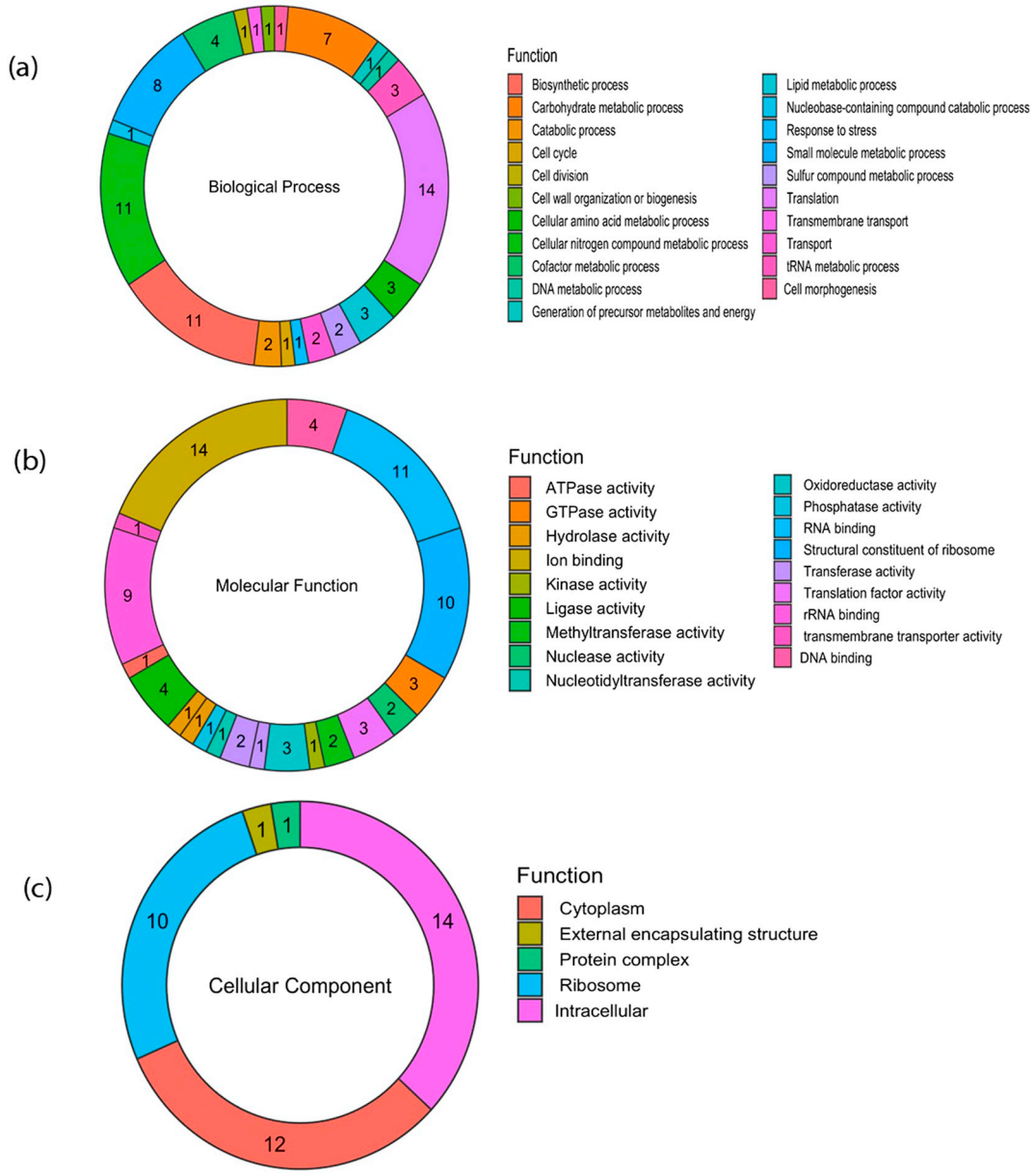

Fig. 1. Functional categories of the upregulated proteins obtained from MS/MS experiments. The 38 proteins were broadly classified based on GO analysis: (a) biological process (b) molecular function, and (c) cellular component.

were enriched in “ion binding” (14 of 38), “RNA binding” (11 of 38), “structural constituent of ribosome” (10 of 38) and “rRNA binding” (9 of 38) (Fig. 1b). Cell component (CC) enrichment analysis showed that the up-regulated genes were significantly enriched in the “intracellular” (14 of 38), “cytoplasm” (12 of 38) and “ribosome” (10 of 38) (Fig. 1c).

3.3. KEGG pathway analysis

The most significantly enriched pathways, in which the over expressed proteins belong, was analyzed by KEGG shown in Fig. 2 and Table 3. The up-regulated proteins were enriched in ribosome (10 of 38), microbial metabolism in diverse environments (4 of 38), beta-lactam resistance (3 of 38), biosynthesis of secondary metabolites (3 of 38), propanoate metabolism (3 of 38), amino sugar and nucleotide sugar metabolism (3 of 38), carbon metabolism (3 of 38), and pyruvate metabolism (3 of 38).

3.4. Protein-protein interaction network (PPIs)

Among the 38 proteins, four plasmid encoded proteins namely ribonuclease VapC (RNaseVapC), dihydropteroate synthase (DHPS), beta-lactamase (bla) and RepFiA replicon did not show any interaction. Moreover, quantitative interaction network was created for remaining 34 up-regulated genes and total of 1430 nodes and 4099 edges related to these proteins were obtained from the Cytoscape (Fig. 3).

4. Discussion

Emergence of carbapenem resistant *K. pneumoniae* has continuously risen and significantly threatens the world. Over expression of carbapenemases and loss of porins are the well known phenomena of carbapenem resistance although these elucidated resistome mechanisms are still in fragmentary phase. In the present study we have found the over expression of a panel of proteins (52 proteins) in the meropenem induced culture (sub-MIC) which might play a pivotal role in

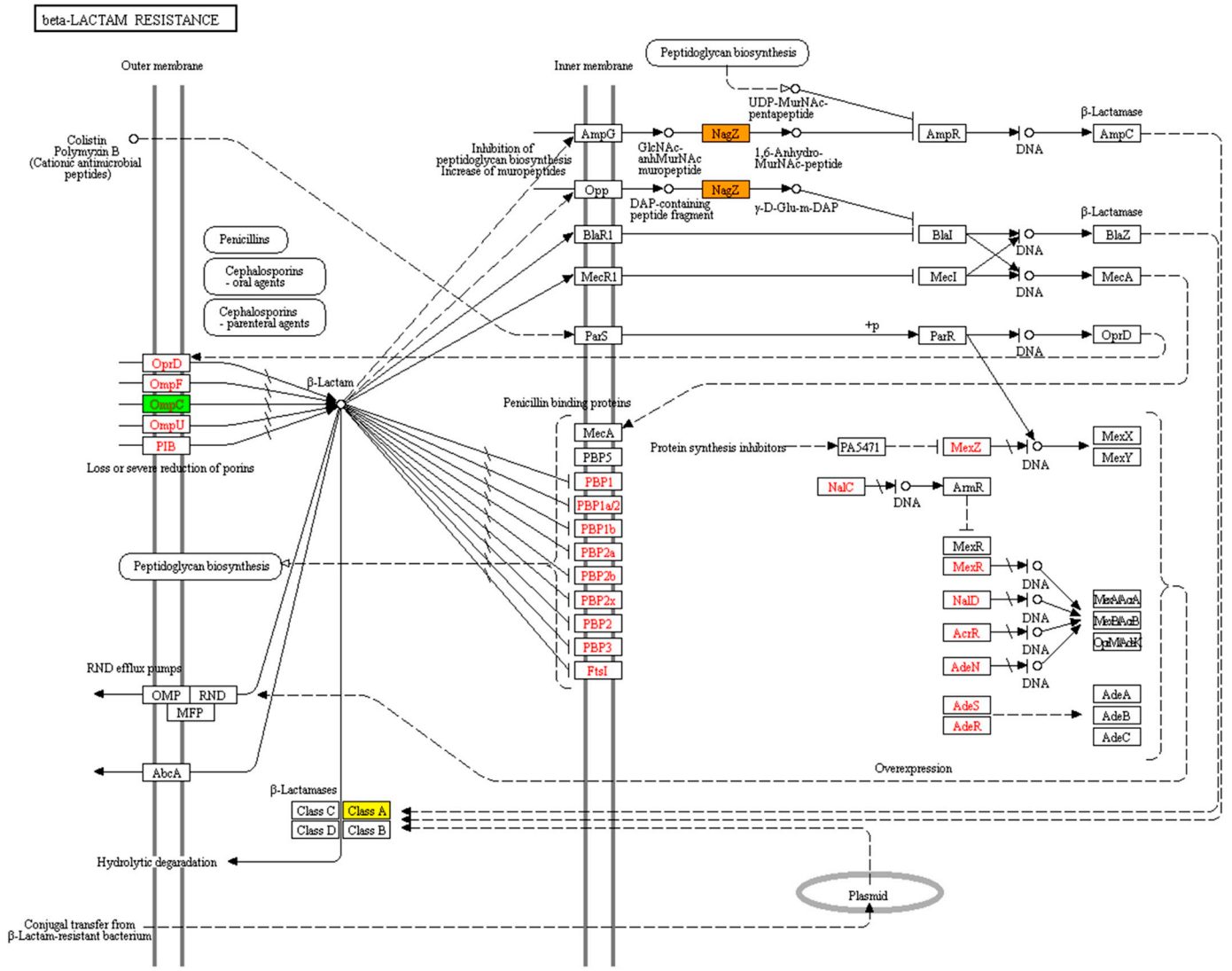

Fig. 2. beta-lactam resistance pathway manually curated using KEGG. Three over expressed genes in nagZ (Orange), bla (yellow) and ompC (green) were successfully mapped on to this pathway. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

carbapenem resistance of *K. pneumoniae* (NDM-4). These 52 proteins belong to four major classes such as protein translational machinery complex, DNA/RNA modifying enzymes or proteins, proteins involved in energy metabolism & intermediary metabolism and proteins involved in carbapenems drugs cleavage, modifications and transport.

4.1. Ribosomal protein translational machinery complex formation and regulation

A panel of proteins involved in the ribosomal proteins translational machinery complex formation and its regulation process were found to be over expressed in carbapenem resistant *K. pneumoniae* clinical strains induced with the meropanam. These proteins are Elongation factor Tu, Elongation factor G, 30S ribosomal protein S4, 30S ribosomal protein S7, 30S ribosomal protein S11, 30S ribosomal protein S13, 30S ribosomal protein S17, 50S ribosomal protein L2, 50S ribosomal protein L5, 50S ribosomal protein L14, 50S ribosomal protein L15 and 50S ribosomal protein L17. Elongation factor Tu (EF-Tu) is a GTP binding protein, involved in elongation of peptide chain during protein translation [27,28]. Elongation factor G (EF-G) regulates the peptide elongation as well as ribosome recycling which are the crucial steps of translation [29]. We also reported the over expressions of EF-Tu and EF-G in the drug resistant tuberculosis [13,30]. Remaining 30S, 50S ribosomal proteins along with EF-Tu and EF-G cumulatively make a ribosomal protein translational machinery complex which involved in proteins biosynthesis.

4.2. DNA/RNA modifying/degradation enzymes and related proteins

Over expression of the DNA/RNA modifying enzymes and its related proteins were observed in the study. These proteins are Type I restriction-modification system-DNA-methyltransferase subunit M, Type I restriction-modification system-specificity subunit S, RecA protein, DNA-binding protein, CTP synthase, Exoribonuclease-2, tRNA (cytidine/uridine-2'-*O*-)-methyltransferase (TrmJ), tRNA (guanine-N (7)-)-methyltransferase, Glycine–tRNA ligase beta subunit, Ribonuclease (VapC) and mRNA interferase (pemK). Meropenam pressure induced stress and activates the repairs systems (SOS system) for DNA and RNA repair. RecA protein involved in repair processes of damaged DNA, DNA recombination, and SOS response [31] which trigger the cells to re-enter into the cell cycle. Methylation of the DNA and RNA is another mechanism of defence through which bacteria save themselves by any stress and drug stress. Therefore, the over expression of these tRNA (cytidine/uridine-2'-O-)-methyltransferase (TrmJ) and tRNA (guanine-N (7)-)-methyltransferase might be contributed to resistance. Ribonuclease (VapC) is the toxin VapC of the two component toxin-antitoxin

Table 3
KEGG pathway analysis of DEGs associated with *Klebsiella pneumonia* subsp. *pneumoniae* (strain ATCC 700721/MGH 78578).

Category	KEGG Team	Count	Protein list
Up-regulated	Ribosome	10	rplQ,rpsD,rpsK,rpsM,rplO,rplE,rplN,rpsQ,rplB,rpsG
	Microbial metabolism in diverse environments	4	talB,pflB,ackA,accC
	beta-Lactam resistance	3	nagZ,ompC,bla
	Biosynthesis of secondary metabolites	3	talB,metK,accC
	Propanoate metabolism	3	pflB,ackA,accC
	Amino sugar and nucleotide sugar metabolism	3	nagZ,ugd,yfbG
	Carbon metabolism	3	talB,ackA,accC
	Pyruvate metabolism	3	pflB,ackA,accC
	Biosynthesis of amino acids	2	talB,metK
	Pyrimidine metabolism	2	carB,pyrG
	Biosynthesis of antibiotics	2	talB,accC
	Lipopolysaccharide biosynthesis	1	kdsB
	Aminoacyl-tRNA biosynthesis	1	glyS
	Methane metabolism	1	ackA
	Inositol phosphate metabolism	1	suhB
	Pentose and glucuronateinterconversions	1	ugd
	Cationic antimicrobial peptide (CAMP) resistance	1	yfbG
	Taurine and hypotaurine metabolism	1	ackA
	Alanine, aspartate and glutamate metabolism	1	carB
	Folate biosynthesis	1	sul
	Two-component system	1	ompC
	Ascorbate and aldarate metabolism	1	ugd
	Pentose phosphate pathway	1	talB
	Homologous recombination	1	recA
	Cysteine and methionine metabolism	1	metK
	Streptomycin biosynthesis	1	suhB
	Fatty acid biosynthesis	1	accC
	Fatty acid metabolism	1	accC
	Butanoate metabolism	1	pflB

(TA)/VapBC system in which toxins inhibit the cell growth and antitoxins repress translation of the toxin genes. In Gram positive and Gram negative bacteria, VapC ectopic expression inhibits cell growth by inhibiting the global rate of translation [32,33]. STRING analysis revealed that toxin VapC directly interact to VapB antitoxins which may neutralized the cognate VapCs through protein-protein interaction. The mRNA interferase/pemK is also the toxin of a type II toxin-antitoxin (TA) system, encoded by plasmid R100 and it is the homologous of MazF, an *E.coli* toxin [34]. They inhibit the translation by mRNA cleavage. Exoribonuclease-2 (rnb) also involved in mRNA degradation. It selectively hydrolyzes single-stranded polyribonucleotides processively in the 3′ to 5′ direction. In the present study over expression of methyltransferase, RecA proteins, VapC, mRNA interferase/pemK and exoribonuclease-2 might be involved in DNA/RNA repair, maturation and turnover.

4.3. Proteins involved in drugs modifications or drugs degradation and resistance

Higher level enzymes production (AmpC Beta-lactamase, penicillinase, Extended Spectrum Beta-Lactamase (ESBL), carbapenemase, Beta-lactamase, and Metallo-beta-lactamase) have reported to contribute in the carbapenem resistance [35,36]. ArnA/Bifunctional polymyxin resistance protein is the first enzyme specific to the lipid A-Ara4N pathway. It modifies the lipid-A through 4-amino-4-deoxy-L-arabinose (Ara4N) and makes the bacteria to resist the antibiotics such as polymyxin and other [37]. This sub-pathway is part of the pathway UDP-4-deoxy-4-formamido-beta-L-arabinose biosynthesis, nucleotide-sugar biosynthesis, lipopolysaccharide biosynthesis and bacterial outer membrane biogenesis. Outer membrane proteins played an important role in the carbapenem resistance. Brinkworth et al., 2015 reported a panel of outer membrane proteins and exportins by 2DE and LC-MS/MS, however outer membrane protein C was not reported [38]. In our study outer membrane protein C was over expressed, sequence similarity reveled that it matches 100% to porin OmpK35 which is involved in resistance [39]. Therefore, we hypothesized that over expression of beta lactamases degrade the drug inside the cell so that cell unable to get signal for the repression of ompC. This study revealed the cumulative effect of beta lactamases, bifunctional polymyxin resistance protein ArnA, and ompC which might be contributed into carbapenems resistance through different mechanisms and may generate the clue for future research.

4.4. Proteins involved in energy metabolism and intermediary metabolism

In this study we have identified a penal of proteins belong to energy and intermediary metabolism. These are branched-chain alpha-keto acid dehydrogenase subunit E2, 3-deoxy-manno-octulosonate cytidylyltransferase, Methionine adenosyltransferase, Beta-hexosaminidase, Alpha-ketoacid dehydrogenase subunit beta, Carbamoyl-phosphate synthase (glutamine-hydrolyzing), UDP-glucose 6-dehydrogenase, Acetate kinase, Catabolite control protein A, Biotin carboxylase of acetyl-CoA carboxylase, Transaldolase, 3-deoxy-manno-octulosonate cytidylyltransferase, CTP synthase, Carbamoyl-phosphate synthase large chain, NADH oxidoreductase, Dihydropteroate synthase, GTP-binding protein TypA/BipA, Catabolite repressor-activator, Carbamoyl-phosphate synthase large chain and Inositol-1-monophosphatase. These proteins involved in generation of various intermediate metabolites of many metabolic pathways such as amino acid metabolism, lipid metabolism, nucleotide metabolism, tetrahydrofolate biosynthesis, carbon catabolism and glucose or energy metabolism. CTP synthase is an essential enzyme involved in formation of CTP from UTP and ATP using glutamate as nitrogen source [40]. Methionine adenosyltransferase catalyzes the formation of S-adenosylmethionine (AdoMet) from methionine and ATP.

In the present study we also used system biology approach for integration of identified proteins. Among 52 over expressed proteins, 38 DEGs are successfully mapped on *Klebsiella pneumoniasubsp. pneumoniae* (strain ATCC 700721 / MGH 78578) strain representative proteome. Among 38 upregulated proteins, a major chunk (> 18%) were ribosomal protein encoding genes (rplB, rplE, rpsM, rplN, rpsD, rpsK, rplO, rpsQ, rpsG, rplQ) and genes involved in tRNA metabolism (trmB, trmJ

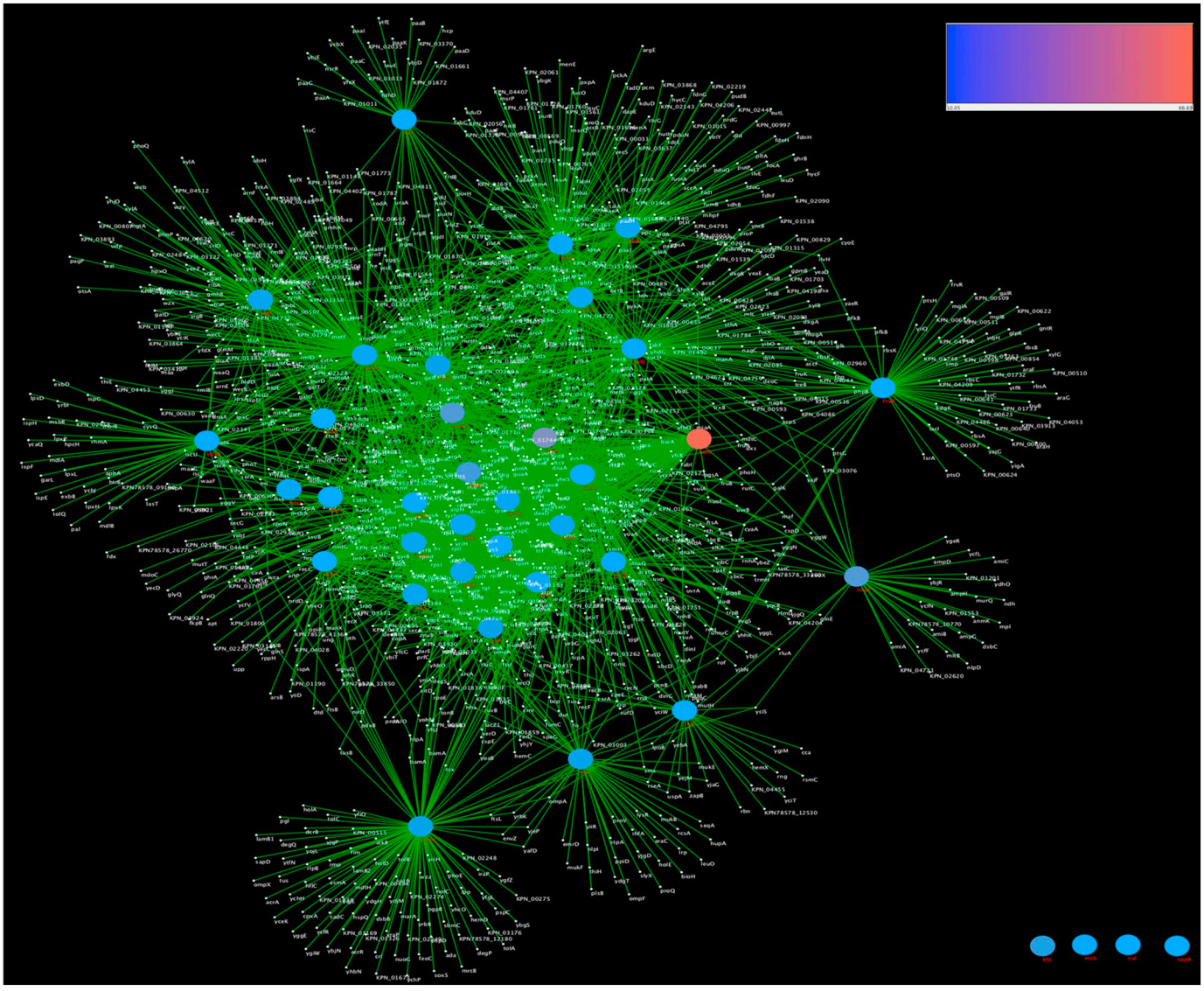

Fig. 3. The interaction networks for over expressed genes in *K. pneumonia.* Shown are changes of gene expression under meropenem stress condition, data derived from MS/MS and visualized in Cytoscape [45]. Color scale denotes log fold vs. *p*-value in gene expression.

and glyS) (Table 2). The GO-term analysis of over-expressed proteins showed that they were mainly involved in carbohydrate metabolic process, translation, biosynthetic process, cellular nitrogen compound metabolic process and small molecule metabolic process. At level of molecular function these proteins carry out RNA binding, structural constituent of ribosome, r-RNA and ion binding. The analysis also showed that these proteins were part of ribosome; cytoplasm and intracellular space (Fig. 1).The previous report suggested that antibiotics stress mostly influences the metabolic pathways and ribosomal subunits expression [41]. Interestingly, our results from high throughput LC-MS/MS confirm the assumption that the over expression of these proteins may be a compensative mechanism for bacteria to defence antibiotics. In the present work we tried to characterize the current expression profile in response to *K. pneumoniae* in presence of meropenem. We mapped the metabolic pathways of carbapenem resistant clinical strain (NDM-4) through over expressed proteins to understand the mystery of beta-lactam resistance (Fig. 2). Analysis of metabolic pathways using KEGG has also concurred with the GO-term analysis. We found meropenem influence certain pathways namely, ribosome component, microbial metabolism in diverse environments, beta-lactam resistance, biosynthesis of secondary metabolites, propanoate metabolism, amino sugar and nucleotide sugar metabolism, carbon metabolism and pyruvate metabolism (Table 3). These metabolic pathways contribute to a complex biological process (for e.g. pyruvate metabolism) that promotes some of antibiotics uptake [41]. Here, we hypothesized that overexpressed proteins were mostly affected those metabolic pathways which involved in antibiotic-resistant mechanism while it is not an antibiotic-mediated cell death process. We also constructed a PPI network of 38 over expressed proteins (Fig. 3). Our analysis revealed that some upregulated proteins were considered hub protein which indicates their important role in translation, metabolism and beta-lactam drug resistance [42]. One protein, namely recA, had degree of connectivity 320 (Fig. 3) and contributes in the homologous recombination pathway (Table 2). Earlier studies also reported the role of RecA in increased tolerance to antibiotic treatment by enhancing DNA repair that occurs either directly by antibiotic-induced DNA damage or indirectly from metabolic and oxidative stress [43]. Furthermore, our experimental results showed that recA was also highly expressed in meropenem stress condition. Therefore, we can hypothesize that this protein might contribute to the progression of antibiotic resistance with drug treatment. There are several genes that are involved in regulation of ampC expression. nagZ is one of the important gene which produces β-*N*-acetylglucosaminidase. Degradation product of peptidoglycan cell wall, GlcNAc-1,6-anhydromuropeptides are generated in periplasm.

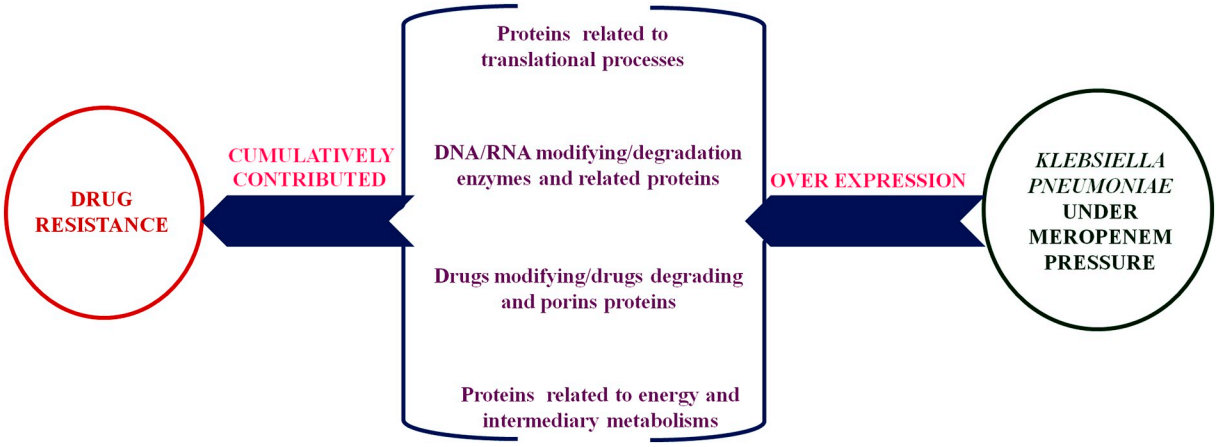

Fig. 4. Representative model shown the summarised mechanism based on the outcome of the study.

They are internalized into cytoplasm by ampG encoded inner membrane permease. In the cytoplasm NagZ transforms GlcNAc-1,6-anhydromuropeptides into 1,6-anhydromuropeptides, which in-turn induces AmpC synthesis by interacting with the LysR-type transcriptional regulator AmpR [44]. Under normal condition, 1, 6-anhydromuropeptides are processed by the *N*-acetyl-anhydromuramyl-L-alanine amidase AmpD, blocking *ampC* induction. Whereas under drug stress or beta-lactamase inducers (for e.g. meropenem), large amounts of muropeptides are generated and collected into the cytoplasm, which leads to the AmpR-mediated initiation of *ampC* expression [44]. Hence, we can foresee that three genes viz. nagZ, ompC and bla have vital role in antibiotic resistance and can be used to exploit its resistance mechanism. On the basis of these over expressed proteins, we proposed a model (Fig. 4) which may explore the new mechanism of carbapenem resistance. In the present study we suggest that cumulative effect of over expressed proteins, mapped pathways and interactive proteins partner of the over expressed proteins might be involved in carbapenem drugs cleavage, modifications and transport which may contribute in the carbapenem resistance.

5. Conclusion and future prospects

To concise, present study focused the whole proteome of carbapenem resistant *K. pneumoniae* clinical isolate (NDM-4) under meropenem stress through proteomic and bioinformatic approaches. These 52 over expressed proteins belong to four major groups such as protein translational machinery complex, DNA/RNA modifying enzymes or proteins, proteins involved in carbapenems cleavage, modifications, transport, energy metabolism and intermediary metabolism related proteins which suggested their direct or indirect role in the carbapenem resistance. Pathways analysis (GO and KEGG) and proteins-proteins interaction suggested that these 52 over expressed proteins, pathways and their interactive proteins cumulatively contributed to the survival of bacteria and meropenem resistance through various mechanisms. These proteins targets and their pathways might open a new vistas of drug resistance and could be used for the development of novel therapeutics against the resistance. Therefore the situation of emergence of bad-bugs could be prevented.

Authors' contribution

DS design the concept, experimented and wrote the manuscript. AG and MK have done the system biology analysis. AUK designed the problem and guided the study and finalized the manuscript.

Conflict of interest

Authors declare no conflict of interest.

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