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Differential expression of Outer membrane proteins in early stages of meropenem-resistance in *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii has emerged as one of the six most important drug-resistant microbes in the world. Resistance by *A. baumannii* to β-lactams and in particular to meropenem is a serious concern. In this connection, it is essential to understand the changes in the outer membrane proteome of *A. baumannii* in the initial stages of resistance. For this we have chosen one low resistant strain with minimal inhibitory concentration (MIC) of 32 μg/ml and one intermediate strain with very low MIC of 0.8 μg/ml of meropenem and compared their outer membrane profiles with that of sensitive strain, ATCC 19606 of *A. baumannii*. Decreased expression of porins, transporters and increased production of metabolic enzymes like Succinyl-CoA synthetase, enoyl-CoA hydratase is a common feature in both intermediate strain and low resistant strains. Interestingly, the differential protein expression levels showed a direct relationship with increasing meropenem resistance. It is clear that initial exposure to meropenem resistance drives *A. baumannii* to restrict the production of CarO and transporters, while the upregulation of genes of altered CarO, metabolic enzymes, peroxidines and antioxidant protein assist in the survival of the bacterium. Because of these unique features of adaptation combined with high metabolic changes in response to antibiotic pressure, *A. baumannii* poses challenges in therapeutic strategies.

Keywords: *Acinetobacter baumannii*; meropenem-resistance; DIGE; Outer membrane protein .

1. Introduction

Bacterial infections are often treated by β-lactam group of antibiotics which is considered as the most effective against a number of negative bacteria including *Acinetobacter baumannii. A. baumannii*, a very common hospital pathogen in Intensive Care Units

(ICUs) and wards has been identified as one of the six important and highly drug resistant hospital pathogens by the "Infectious Disease Society of America"(IDSA) [1, 2]. Therefore, worldwide emergence of antibiotic resistance in *A. baumannii* poses a serious threat to human health. Presently, the latest analogs of β-lactam, prescribed to treat patients affected by these gram negative bacteria are meropenem and imipenem. However, increasing number of meropenem resistant isolates strongly restricts the effective therapy options [3, 4].

It is known that *A. baumannii* develops resistance using a number of ways including expression of β-lactamases, alterations in penicillin binding proteins (PBPs) [5, 6, 7], aminoglycoside-modifying enzymes in aminoglycosides resistant strains [8] etc. Bacterial membrane proteins are known to be key molecules in maintenance the permeability and efflux of antibiotic [9]. Differential expression of membrane proteins in susceptible and highly resistant strains of *A. baumannii* from different parts of the world clearly show a strong association with the emergence of the resistance phenotype [6, 10, 11, 12, 13, 14, 15]. Our recent report has clearly shown several altered outer membrane proteins in 'high β-lactam resistant strain' with minimal inhibitory concentration of 64 μg/ml of meropenem [16]. However, the profiling of outer membrane proteins in low resistant strains is not yet understood and is not defined. Keeping in view of the rise of the meropenem resistance, we have undertaken the proteomic analysis of outer membranes of *Acinetobacter baumannii* in the initial stages of the meropenem resistance.

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2. Material and methods

2.1 *Materials*

Muller Hinton agar, MacConkey agar, and Luria broth were purchased from Himedia laboratories Ltd, India and Pronadisa Conda Laboratories Canada, respectively. N-lauroyl-sarcosine and ammonium bicarbonate were from Sigma chemical co. USA. Immobilized dry strips, pharmalytes, Cy2, Cy3, Cy5 dyes, dithiotritol and iodoacetamide were purchased from GE Health Care, USA. Acrylamide, bisacrylamide, ammonium persulphate, TEMED, sodium dodecyl sulphate, EDTA, coomassie brilliant blue, and bromophenol blue were purchased from Bio-Rad laboratories, USA. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), acetonitrile and proteomic grade water were purchased from G. Biosciences, USA. Trypsin and dimethyl formamide were from Promega (USA) and Spectrochem (India), respectively. DNase was purchased from Promega, U.S.A. Acetic acid, glycerol, methanol were purchased from Qualigens, India and all other chemical were of analytical grade and purchased from Merck, India.

2.2 *Bacterial strains*

ATCC 19606 and forty four non-repetitive clinical strains of *A. baumannii* were collected from the Department of Microbiology, All India Institute of Medical Sciences, New Delhi (India). The clinical strains were confirmed as *A. baumannii* using standard biochemical [17].

2.3 *Minimal Inhibitory Concentration*

Agar dilution method was used to estimate the minimal inhibitory concentrations (MIC) of various β lactams for resistant strains of *A. baumannii* [18]. Concentrations of meropenem, in the range of 0.4 μg/ml to 64.0 μg/ml were used in estimating the MIC. The MICs of other antibiotics i.e. piperacillin, cefotaxime and ceftazidime were done up to 512 μg/ml. The plate without any antibiotic was inoculated in parallel, to serve as a control.

2.4 *Extraction of Outer Membrane proteins (Omps)*

Bacteria were grown in Luria-Bertani broth (250 ml) at 370C for 19.4 hours. Doubling time of *A. baumannii* ATCC19606 is 48min hence we grow bacteria for 25 generations. After 19.4 hours culture, bacteria were harvested by centrifugation. The pellet was suspended in 50mM Tris buffer, pH 7.5. Resuspended bacterial cells were sonicated using Misonix XL Ultrasonic processor under three pulses of 1min each at 50 Hz under cold conditions. Presence of nucleic acid (if any) can create vertical streaking in 2D PAGE and to avoid such interference from nucleic acid, lysed sample is conventionally treated with DNase [13]. Hence, the suspension containing the cell envelope was treated with DNase (10 μg/ml, for 15 min. at room temperature) and subjected to ultracentrifugation by Beckman Optima TL Ultracentrifuge for 30 min at 100000 g. After ultracentrifugation, total membrane fraction was obtained as pellet and this fraction was treated with 2% Sodium lauroyl sarcosinate for 30 min at room temperature which specifically solubilized inner membrane. The sample was further ultra- centrifuged for 30min. at 100000 g and outer membrane was obtained as pellet. The outer membrane fraction was stored at -700 C.

2.5 *Differential In Gel Electrophoresis (DIGE)*

Native, intermediate and low resistant strains of *A. baumannii* were grown three times for constant time period (19.4 hours) and in constant culture conditions (temperature 370C, 250 ml culture) and outer membrane proteins were isolated as above given protocol. The membrane pellets were solubilized in modified rehydration buffer (7M urea, 2M thio -urea, 2% CHAPS) at room temperature and quantified by using the 2D Quant Kit (GE Healthcare) following the manufacturer's protocol. Three sets of experiments were conducted according to our previous published experimental design [16]. In each set, proteins from native and resistant strain were labeled with the fluorescent dyes Cy 3 and Cy 5 separately [16]. An internal control of Cy 2 labeled proteins was also added in each experiment which constitutes a mixture of 1/6th protein fraction of native and 1/6th protein fraction of resistant strain.

For each labeling reaction, 50 μg of protein was incubated with 200 pmole of dye for 30 min in dark. To stop the reaction, 1 μl of 10 mM lysine was added and incubated for 10 min. All labeling incubations were carried out on ice. The final volume of reaction mixture was adjusted to 250 μl using rehydration buffer (7M urea, 2M thio-urea, 0.7 mg DTT, 2% CHAPS, 1.25 μl IPG buffer) and rehydrated for 16 hours in dark with 4-7pH immobilized pH gradient (IPG) strip.

2.6 *Two dimensional (2D) gel electrophoresis*

Isoelectric focusing of rehydrated IPG strip was carried out on Ettan IPGphor 3 IEF system (GE healthcare) using the step protocol: 150 V for 1 h, 500 V for 1 hour, 1500 V to 3 hour, 4500 V for 4 hour to a total of 24,000 volt-hours. After completion of the first dimension, strips were equilibrated for 15 minutes in 5 ml of SDS-equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30 % glycerol, 2 % SDS, 0.02 % bromophenol blue) and 0.05 % DTT in dark at room temperature [13]. After DTT treatment, the strips were treated with 1.25 % iodoacetamide solution prepared in SDS equilibration buffer for 15 min. in dark at room temperature. Second dimension was done on a 12 % polyacrylamide gel in SE 600 Ruby gel apparatus (GE healthcare). Gel was run at 15 mA for 30 min and then at 30 mA at 4°C till the bromophenol blue came out of the gel.

2.7 *Image Acquisition and Analysis*

After second dimension, DIGE gels were scanned for Cy2, Cy3 and Cy5 fluorescence labeled proteins using a TyphoonTM TRIO Variable Mode Imager (GE Healthcare, USA). Cy2 images were scanned at an excitation of 488 nm using 520BP40 emission filter; Cy3 images were scanned at an excitation of 532 nm using 580BP30 emission filter; Cy5 images were scanned at an excitation of 633 nm using 670BP30 emission filter. All gels were scanned with a photomultiplier tube (PMT) setting of 600 volt. Images were cropped using Image-Quant™ version 6.5 (GE Healthcare, USA) to remove areas extraneous to the gel image. The final expression levels in the gels were determined by the DeCyder software version 7.0 (GE Healthcare, USA). All nine images (3 different experiments) were uploaded to the workspace using image loader module. Three differential analysis (DIA) sets were created by DIA module. In each set, protein spots on DIGE image pairs (Cy 3 and Cy 5 labeled) were co-detected automatically and each gel image was intrinsically link to its in-gel standard (Cy2 labeled image). The number of spots for co-detection procedure was set to 1500. Further, all three DIA workspaces were then imported to biological variance analysis (BVA) workspace. The experimental setup and relationship between samples were assigned in the BVA workspace. Each individual Cy3 or Cy5 gel image was assigned an experimental condition, either native or resistant according to the labeling and all Cy2 images were classified as standards; gel-to-gel matching of the standard spot maps from each gel. The gel with the highest spot count was assigned as the master gel. Matching between gels was performed utilizing the in-gel standard from each image pair. Land-marking was done by manually identifying well defined spots along with their neighboring spots and matching these spots across the standard images.

The degree of difference in standardized abundance between two protein spot groups is expressed as average ratio (fold change). A fold change with a threshold value of minimum two fold increase or decrease was used. Student t-test was performed for every matched spot-set, comparing the average and standard deviation of protein abundance for a given spot. As the threshold value of protein expression was set to minimum of 2 fold for all experiments, therefore the proteins which had more value than the threshold and had significant p value (≤ 0.05) of t-test were considered for identification [19].

2.8 *In-gel digestion and Electrospray ionization mass spectrometry (ESI-MS)*

After visualization with coomassie G-250, protein bands were excised from the 2D-SDS polyacrylamide gel and completely destained in 100µl destaining solution containing 1:1 100mM NH4HCO3 and 100% acetonitrile till the bands appear colourless. Finally gels were dehydrated in 100% ACN. Reduction of proteins was carried out in100µl of 10mM DTT in 50mM NH4HCO3 for 45 minutes at 560C. Alkylations of proteins were done in 100µl of 55mM Iodoacetamide (IAA) prepared in 50mM NH4HCO3. Again washing of gels were carried out in 100µl of destaining solution containing 1:1 ratio of 50mM NH4HCO3 and 100% ACN. Final dehydration of gels was done in 100% ACN for 15 minutes. Gels were centrifuged and supernatants were discarded and gels were completely dried in speed Vac for 20 min. Tryptic digest was started by the addition of 20 µl from a 12.5 ng/µl trypsin solution in 25 mM NH4HCO3 and kept on ice for 30 min for absorption. This was followed by further addition of 20 µl of 25mM NH4HCO3 solution in the reaction sample to overlay. The samples were kept at 370C for 16 hours for digestion. Digested peptides were solubilized in 10 µl 50% acetonitrile containing 0.1 % formic acid. 8 µl of the peptide solution was used to load in a silica capillary (Proxeon Biosystem, USA) which was then fixed to a QSTAR-XL QTOF mass spectrometer. The progress of each run was monitored by recording the total ion current (TIC) for positive ions as a function of time for ions in the m/z range of 400-1600 for MS and 140-1600 for MS/MS. Mass spectra were acquired using information-dependent acquisition (IDA) method. Nanospray ionization method was used with an ionspray voltage of 900. The other parameters are as follows: interface temperature = 50°C, curtain gas flow = 1.13 L/min, declustering potential 1 = 60 V, declustering potential 2 = 15 V focusing potential = 280 V.

Database searching was done using Mascot (Version 1.6b4 Matrix Science, UK). Modifications considered were oxidation of methionine and carbamidomethylation of cysteine as variable and fixed manner. Search was further considered upto charged state ranging from +2 to +3 and limited to Eubacteria. The peptide mass tolerance range was ± 1.0 Da and fragment mass tolerance was ± 0.3 Da. All the Mowse score values reported are significant, $p < 0.05$. All spectra were searched online in NCBInr database with 0-1 missed cleavage.

3. Results

MICs of 44 clinical strains of *A. baumannii* were done for the routinely used β-lactams and also for the less common meropenem. Based on their MIC values, the clinical strains of *A. baumannii* were divided into two groups: intermediate (IR) strain with MIC 8.0 µg/ml and low resistant (LR) with MIC ≥16µg/ml. The MIC of various β-lactams for the low resistant *A. baumannii* (LR122) obtained are: piperacillin and cefotaxime, 128µg/ml; ceftazidime, 64 µg/ml and meropenem, 0.8 µg/ml. However, MIC of same group of β-lactam for intermediate resistant strain (IR259) of *A. baumannii* showed much higher MICs as follows: piperacillin and cefotaxime, >512µg/ml ; ceftazidime, 128µg/ml and meropenem, 0.8µg/ml.

3.1 *Differential in gel electrophoresis (DIGE) and Mass spectrometry*

Two representative strains RS 259 and RS 122 of *A. bau-*

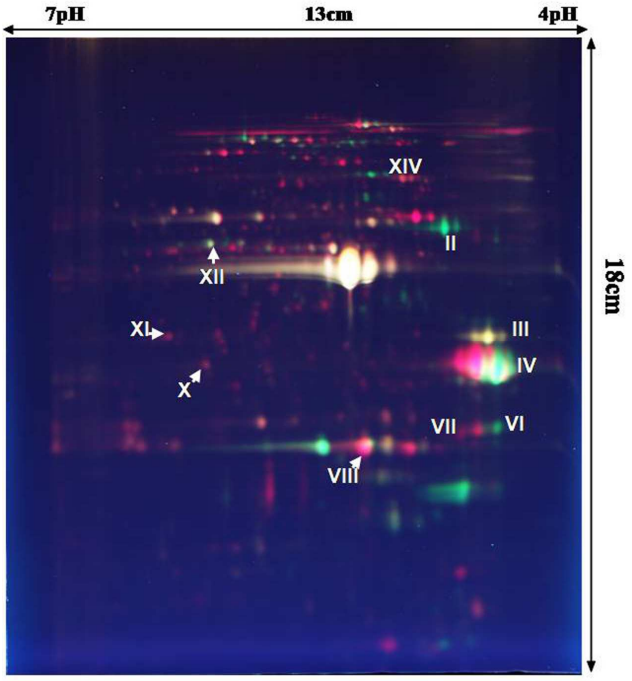

Figure 1a. Differential In Gel Electrophoresis (DIGE) of outer membrane fraction of *Acinetobacter baumannii* ATCC19606 (50 µg), intermediate strain RS 259 (50 µg) and pooled fraction (50 µg) using Cy3, Cy5 and Cy 2 dye, respectively. Isoelectric focusing (IEF) was done using 13 cm, 4-7 pH range IPG strip and second dimension was done on 16X18 cm gel- electrophoresis. Gels were analyzed by Typhoon Imager.

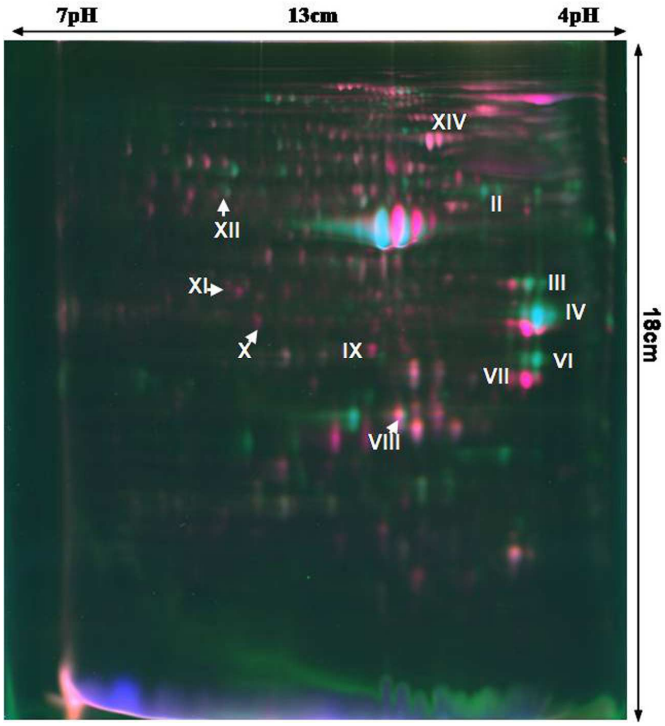

Figure 1b. Differential In Gel Electrophoresis (DIGE) of outer membrane fraction of *A. baumannii* ATCC19606 (50 µg) and low resistant strain RS 122 (50 µg) and pooled (50 µg) using Cy3, Cy5 and Cy2 dye, respectively. The experimental conditions and labeling are same as in figure 1a.

mannii were chosen from intermediate and low resistant strain groups and are referred as IR and LR, respectively. 2D -DIGE profiles are given in figure 1a and figure 1b with respect to ATCC 19606.

In general, there is a high similarity of the outer membrane profiling amongst all the three strains ATCC, IR and LR (Figure 1a and b) which can be clearly seen from the superimposed images. The similarity profiles of *A. baumannii* native, intermediate and low resistant can be easily understandable as the bacteria do not go through a major shift in the protein profile in the early stages of resistance. However, a couple of new proteins infact can be located for example like spot IX (Figure 1b). Biological variation analysis (BVA) of native and IR strain revealed 11 differentially expressed protein spots of which three were downregulated and five more were upregulated. Protein spots IV, XII and X are highlighted with *. The proteins with non-significant expression in one strain (LR or IR) are reported as N.S. While in the case of LR strain, five proteins were down-regulated and five proteins were upregulated. Comparative differential expression of specific proteins of native ATCC is presented in Table 1.

3.2.1. *Downregulated proteins*

CarO protein and their altered forms: Two isoforms (protein spots IV and VI) were identified as CarO. In case of intermediate and low resistant strains, there is a clear decrease in the CarO proteins. The expression level of spot IV (29 kDa, 4.5 pI) showed a down regulation (-6.8 fold), while in case of comparison between the low resistant strain and ATCC the expression loss of the same protein had increased upto the 38 fold (Figure 1a and 1b). Similarly, down- regulation of spot VI was also noted in a sequential manner (-13 fold in IR and -57 fold in low resistant) (Table 1).

We also found the migratory differences in CarO isoforms of spot IV and VI (Figure 1a and 1b). Another major spot i.e. Spot VII was identified as the hypothetical protein of *A. baumannii* (gi|301512444) and the upregulation of spot VII is more than 5 folds in IR and LR strains (table 1, Figure 1a and 1b).

34 kDa transporter: Spot III showed a significant down-regulation only in low resistant strain (-6.5 fold) (Figure 1b) and later identified as 34 kDa Outer membrane protein of *A. baumannii*.

Lipid transporter: Protein spot II was down-regulated in both resistant strains in a similar manner. The down regulation of this protein was seen more in intermediate strain (-38 fold) as compared to low resistant strain (-22 fold) (Figure 1a and 1b). This protein was identified as outer membrane transporter protein of *A. baumannii*.

OprD porin: We also found spot XII as basic protein OprD homologous protein of *Pseudomonas spp*. OprD. An equal expression loss is seen in intermediate strain (-2.9 fold) and low resistant strain (-3.1 fold) (Figure 1a, 1b).

Metabolic enzymes: Two upregulated protein spots X and

Table 1. Identification of differentially expressed proteins of *Acinetobacter baumannii* intermediate strain (RS 259) and low resistant strain (RS 122) with reference to ATCC 19606. Differential expression is shown as fold change (minimum 2 fold, p-value ≤0.05).

Spot No.	Mw. (in KDa)	pI	Fold change (IR)	Fold change (LR)	Identification & (Accession No.)	Score & % coverage	Function
Downregulated Proteins							
II	50	4.9	(-) 38.9	(-) 22.9	O M transporter *A. baumannii* ATCC 19606 gi|260557248	116/12	Lipid Transport
III	34	4.7	N.S.	(-) 6.5	Putative 34 kDa Omp, *A baumannii* ACICU gi|184159810	476/46	Non specific Transport
IV*	29	4.5	N.S.	(-) 38.6	Putative Omp, *A. baumannii.* gi|72535025	697/52	Non specific Transport
VI	26	4.5	(-) 13.6	(-) 57.9	Putative Omp, *A. baumannii.* gi|72535025	614/52	Non specific Transport
XII*	47	5.9	(-) 2.9	(-)3.1	OprD, *A. geno. spp. 3* gi|193735465	203/11	Basic amino acid Transport
Upregulated Proteins							
VII	25	4.7	6.7	5.4	hypothetical protein AbauAB05_12702, *A. baumannii* AB058 gi|301512444	189/25	Transport
VIII	20	4.9	3.0	1.5	Peroxiredoxin, *A. baumannii* AB058 gi|301513596	241/40	Antioxidation
IX	24	5.1	-	2.56	Putative antioxidant protein, *A baumannii AYE* gi|169794796	317/32	Antioxidation
X*	30	5.6	1.8	2.7	Succinyl-CoA synthetase α-subunit, *A. baumannii SDF* gi|169632628	442/29	Metabolism
XI	29	5.7	3.5	3.1	enoyl-CoA hydratase, phenylacetic acid degradation, *A. baumannii AYE* gi|169796408	153/13	Metabolism
XIV	57	4.9	3.8	N.S.	Chaperonin GroEL, *A. baumannii* SDF gi|169632653	362/18	Protein folding

* Proteins were also found in high resistant strain in our earlier report [16]. Non-significant protein spots are shown by N.S.

XI were identified as enzymes integrated with energy producing reactions. Spot X had shown a sequential upregulation in both, intermediate (1.8 fold) and low resistant (2.7 fold) strains and identified as Succinyl-CoA synthetase protein [20]. Similar to Succinyl CoA synthetase, a threefold increment was seen in the expression level of enol-CoA hydratase enzyme (Protein spot XI).

Chaperonin GroEL and antioxidant proteins: Chaperonin GroEL protein was found in four fold elevated level in intermediate strain (spot XIV). Spot VIII protein had an upregulation of 3 fold and 1.5 fold in intermediate and low resistant strain, respectively. However, spot IX had a significant differential expression in low resistant strain only, and intermediate had not shown the differential expression of this protein.

4. Discussion

It is essential to identify the outer membrane proteins of *A. baumannii* which show their altered expression against different analogs of β-lactams. In the preceding years, a gradual increase in the resistance patterns of *A. baumannii* has been noticed in India as well as several other countries. The frequency of *A. baumannii* five year back was only 8-10%

while presently the presence of *A. baumannii* in our hospital has increased upto 30% (our unpublished data). MIC data clearly indicates a high resistance shown in all the 44 resistant strains for cefotaxime, piperacillin, and ceftazidime (MIC range up to 512 μg/ml). It is good to note that potency of meropenem is still high in these strains (MIC range 0.1 μg/ml to 8 μg/ml, n = 34) of *A. baumannii*. However, the resistance is slowly emerging out even to meropenem (MIC range 16-32μg/ml, n = 9). *A. baumannii* is rapidly emerging out as highly resistant organism and it will be very difficult to treat the infections as no high effective antibiotic is presently available.

4.1 *Down-regulation of porins as first defense in resistance*

It is well evident that the intrinsic level of antibiotic resistance in gram-negative bacteria is directly regulated by porins [21]. Porins have the ability to diffuse even the large size molecules like antibiotics [22, 23]. Therefore, the bacterium, as a first step decreases the production of porins in order to control the entry of the antibiotic inside the bacterial system. This method of increasing resistance is a first line of defense and exerts a profound influence on the entry of the hydrophilic antibiotics like beta-lactams [24]. This feature has been described in majority of gram-negative bacteria like *E. coli* and *Pseudomonas*. In this category of porin, the first member is the CarO. The sequential downregulation in the expression clearly indicates the response of the antibiotic and it appears to be the initial step of the bacterium to resist against antibiotic load. CarO protein has got a special attention in the current decade and majority of the studies published in resistance mechanisms of *A. baumannii* stated its loss of expression or its alteration [10, 12, 13]. Based on the decreased expression more than 50 fold, it can be easily concluded that CarO isoform of 26 kDa is more susceptible for antibiotic load (Figure 1b). CarO isoforms is indeed responsible for nonspecific diffusion channel formation and plays important role in carbapenem resistance [12, 25].

Anomalous expression profile of 34 kDa protein is noticed in our study. Low resistant strain shows the significant down -regulation of 34 kDa protein while, neither IR strain nor high resistant strain showed a significant down-regulation. However, earlier reports suggested its potential role in β-lactam resistance [26]. A lipid transporter has been found to be downregulated in intermediate and low resistant strains. It is well known that β-lactams are hydrophilic in nature, however, the downregulation of this lipid transporter is due to resistance for antibiotics other than lactams.

OprD is a characteristic protein of porin family and was shown to be responsible for β-lactam resistance in gram negative bacteria [27, 11]. In the present study, the protein spot XII was found to be down regulated in both resistant strains (LR and IR). The spot XII was identified as OprD of *A. baumannii* (Figure 1a and 1b). However, a four fold upregulation was reported in high resistant *A. baumannii* [16]. Siroy et al. 2006 found no change in expression of OprD in multidrug resistant strain of *A. baumannii* [13]. We can say that there is no clear picture of direct involvement of OprD in resistance of *A. baumannii* and other gram negative bacteria because of conflicting reports [11,13,16,27]. Therefore, it may be speculated that *A. baumannii* during the initial stages of resistance, makes use several porins including OprD while in high resistance it uses other porins at the cost of OprD.

It is noticed that the down-regulation of porins is sequential and greater loss of expression has been noticed in low resistant strain as compared to intermediate strain. Therefore, it can be easily hypothesized that during the development of resistance, the bacterium uses downregulation or alterations in porins as a first step and these proteins gets downregulation in a sequential manner to combat the bactericidal effects of hydrophilic antibiotics.

Besides downregulated proteins, several Omps are upregulated as a means of adaptation of resistant bacteria in adverse conditions of antibiotic exposure. One such group of proteins is CarO isoforms.

Altered CarO isoforms: In LR strain CarO protein shows two isoforms with molecular weight 25 kDa and 26 kDa represented as the spot VII and spot VI, respectively. We would like to emphasize that this observation is not an artifact as the comparative DIGE study between the native and resistant bacterium was done three times and for each experiment set bacteria were grown. The anomalous migration of these proteins may be due to the change in the primary level of the protein which suggests modification in the primary role of this protein i.e. diffusion pathway may be modified. It appears that due to the meropenem load there is a modification or alteration of the internal residues of the protein spot VI which resulted in the shifting of these protein spots [13]. The presence of altered CarO isoforms is thus justifying the importance of porins in uplifting the resistance in pathogenic bacteria.

Metabolic enzymes upregulation: The previous comparative studies carried out between susceptible and resistant isolates of *A. baumannii* revealed high expression of metabolic enzymes and due to which a versatile metabolism is found in the resistant strains [28]. Concurrently, our data also found high expression of enzymes related to metabolism. In this regard, Succinyl-CoA synthetase and enol-CoA hydratase enzyme upregulation, clearly indicates that resistant bacteria have versatile metabolism as compared to susceptible bacterium.

Majority of the bacteria have multiple copies of the groEL gene which are active under different environmental conditions. It may be hypothesized that the antibiotic stress leads to the production of more production of this enzyme complex for proper folding of proteins in stress environment. It has been demonstrated that bactericidal antibiotics like beta-lactams induce the production of reactive oxygen species (ROS) for bacterial cell death [29]. Also, it was recently demonstrated that an up-regulation of several antioxidant proteins enhances the ability of bacteria to survive against ROS

and RNI damage [30]. The antioxidant proteins and peroxidines are evolved to combat the adverse effect of high antibiotic pressure on resistant bacteria [31]. Low resistant strain may use more of these proteins to defend itself against oxidative damage from human immune cells, such as macrophages. In fact, it supports the hypothesis that the presence of more than one differentially expressed protein influences the ability to infect and to spread in the population.

All these upregulated proteins of resistant bacteria support the fact of versatile metabolism of resistant *A. baumannii* which can survive in extreme conditions and have high adaptations as compared to the susceptible bacteria.

5. Conclusions

The transformation efficiency of *A. baumannii* is exceptionally high due to which it can acquire resistance in its early phases. Therefore, it is very important to monitor changes in the level of antibiotic susceptibility among clinical isolates. However, at present, it is unknown how the early phases of resistance in *A. baumannii* correspond to change in different protein expression profiles. The results described in this study permit to hypothesize that compensation for the meropenem-resistant phenotype may be responsible for the different protein expression in *A. baumannii*. Results appear to reveal that an interplay mechanism is present between antibiotic resistance, biological fitness and virulence. It can be concluded that porins, metabolic enzymes and antioxidative proteins have a potential role in the meropenem-resistance as most of the resistant strains harbor these specific proteins in *A. baumannii.*

6. Supplementary Material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/67/0

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