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Transcriptomic Insights into Differential Gene Expression in Acinetobacter baumannii Isolates from Colistin-Treated Patients Using Illumina Sequencing

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ABSTRACT

The rise of multidrug-resistant (MDR) *Acinetobacter baumannii* presents a critical threat to global health due to its remarkable antibiotic resistance. To explore its stress response mechanisms, RNA sequencing (RNA-seq) was conducted to compare the transcriptomes of *A. baumannii* ATCC19606 and a clinical isolate from a colistin-treated patient. Analysis identified 118 differentially expressed genes (DEGs) with thresholds of log2 fold change (Log2FC) > 1 or < −1 and FDR < 0.05.

Upregulated genes included those encoding twitching motility protein, p-aminobenzoate synthetase, pilin-like competence factor, and D-methionine-binding lipoprotein MetQ, with a 2-4 fold increase. Conversely, downregulated genes, such as ferredoxin, molecular chaperones GroES/GroL, and class C beta-lactamase ADC-158, suggested reduced metabolic activity.

These DEGs are implicated in stress response and metabolic transport pathways, providing critical insights into *A. baumannii's* adaptive mechanisms and potential vulnerabilities for therapeutic targeting.

Keywords: *A. baumannii, transcriptome sequencing, differential gene expression, down regulation, upregulation, illumina*

1. INTRODUCTION

Acinetobacter baumannii is a strictly aerobic, Gram-negative coccobacillus that has become a significant global pathogen, especially since the 1980s (Towner, 2009). This bacterium can cause opportunistic infections in the skin, urinary tract, lungs, and bloodstream, posing a serious threat to critically ill and immunocompromised patients and often being involved in hospital outbreaks. Notably, carbapenem-resistant A. baumannii is a critical concern, earning a priority 1 (critical) status on the WHO priority pathogens list for research and development of new antibiotics (Ashokan et al., 2019).Antepartum hemorrhage (APH) has been a leading cause of maternal mortality worldwide, especially in developing countries like India. Its early diagnosis and timely management can APH is defined as bleeding from the genital tract after 28 weeks of gestation to delivery of the baby.[1,2]

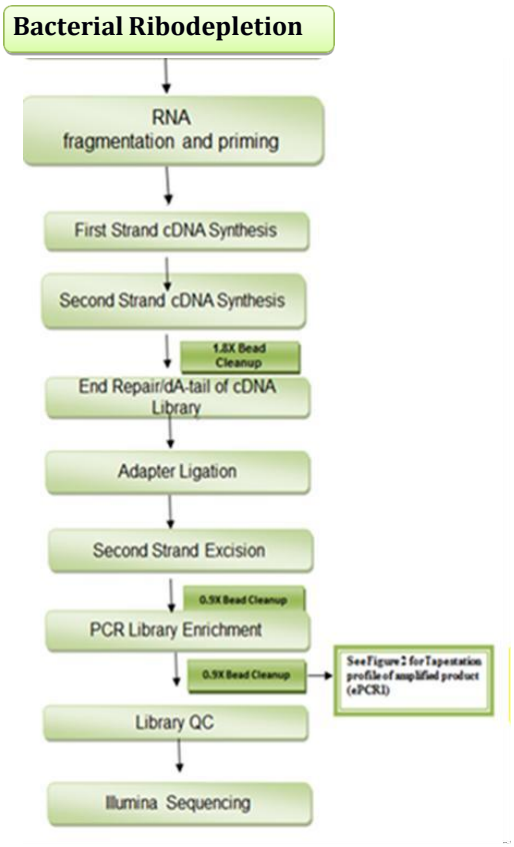
Members of the *Acinetobacter* genus, which are part of the Moraxellaceae family, include glucose non-fermenting, non-motile, non-fastidious, catalase-positive, and oxidase-negative bacteria (Howard et al., 2012). This genus comprises 59 species within the Gamma proteo bacteria class. The *Acinetobacter* calcoaceticus-*Acinetobacter baumannii* complex (ACB complex) includes *A. calcoaceticus, A. baumannii, A. pittii, A. nosocomialis, A. seifertii*, and *A. dijkshoorniae*, which are phenotypically similar (Mancilla-Rojano, 2020). Of these, *A. baumannii, A. pittii, A. nosocomialis, A. seifertii*, and *A. dijkshoorniae* are pathogenic to humans, whereas *A. calcoaceticus* has not been reported as an infectious agent (Nocera et al., 2021).

A. baumannii is highly resilient to various environmental conditions, capable of surviving for up to 100 days on dry surfaces (Shi et al., 2024). Some strains have developed genetic mechanisms that enhance catalase gene expression, increasing tolerance to oxidative stressors like hydrogen peroxide. Additionally, *A. baumannii* resists chlorhexidine due to the expression of efflux proteins that expel this chemical from the cell. Ethanol has also been reported to promote its growth and virulence. The bacterium can quickly adapt to changes in temperature and nutrient availability and has simple nutritional requirements (Shi et al., 2024). Its ability to form biofilms is a significant virulence factor, enhancing survival on various surfaces and persistence in hospital settings.

A major public health issue with *A. baumannii* is its ability to develop resistance to multiple antibiotic classes. This multidrug resistance involves several mechanisms, including aminoglycoside modification, β-lactamase production, modifying enzymes, permeability defects, target site alterations, and the upregulation of multidrug efflux pumps (Pagdepanichkit et al., 2016).

The strain ATCC19606 is one of the best-characterized strains of *A. baumannii*, which is widely used to study antimicrobial resistance and other stress (Zhu et al., 2020; Guo et al., 2022; Hui et al., 2021). It is resistant to sulfonamide but susceptible to a variety of other antibiotics, including β-lactams, aminoglycosides, quinolones, tetracyclines, and colistin (Hamidian & Hall, 2017; Yang et al., 2015). In the present study, to learn the adaptive response of *A. baumannii* to antibiotics, the transcriptome of ATCC19606 cells exposed to a subinhibitory concentration of minocycline was analyzed. Our results revealed differentially expressed genes involved in the ferredoxin, 50S ribosomal protein, Rhodonase domain-containing protein, DNA binding protein HU beta, translation initiation factor, UPF0391 membrane protein BDGL_002899, rubredoxin, probable $Fe^{(2+)}$ trafficking protein, molecular chaperone GroES, chaperonin GroL, class C beta-lactamase ADC-158, amino acid ABC transporter permease, and APC family permease, along with proteins conferring tolerance to group A colicins involved in acute regulation in response to colistin. These findings provide a basis for coistin resistance and target genes for potential therapies.

Figure 1: Work flow for NEBNext® Ultra™ II Directional RNA Library Preparation Kit



2. MATERIALS AND METHODS

2.1 Isolation and identification of *Acinetobacter baumannii*

In this study, we isolated the *A. baumannii* strain SO_10770_1 from an endotracheal aspirate of a patient in the critical care unit at NRI Medical College, Andhra Pradesh, India, in 2023. The bacterial strain was cultured on 5% sheep blood agar, chocolate agar, and MacConkey agar (all prepared by HI Media, India) and incubated for 18–24 hours at 35°C. The strain

was then identified through 16S rRNA Sanger sequencing.

2.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the BioMérieux Vitek 2 Automated Microbiology System (BioMérieux, France) with the Vitek 2 GN ID Test kit and Vitek 2 AST 407 Critical Care commercial test kit, following the manufacturer's guidelines. Minimum inhibitory concentration (MIC) breakpoints were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100, 27th Ed.). The antimicrobials tested included ticarcillin/clavulanic acid, ceftazidime, doripenem, cefepime, imipenem, meropenem, cefoperazone/sulbactam, ciprofloxacin, levofloxacin, gentamicin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, tigecycline, minocycline, and colistin.

2.3 RNA Extraction and Quality Control

RNA was extracted from bacterial cells using Qiagen RNeasy mini kit (Cat No.74106). The lysate was thoroughly mixed with half volume of absolute alcohol, and loaded into RNeasy spin column placed in 2 ml collection tube. The tubes were centrifuged at 10,000 rpm for 1 min and flow through discarded. On column DNase I (Cat No.79254) treatment and subsequent column washes were performed according to manufacturer's instruction. RNA was eluted from the column using nuclease free water.

The purity and concentration of RNA was quantified using the Nanodrop Spectrophotometer (Thermo Scientific; 2000). The integrity of the samples was assessed on Tapestation (Agilent). RNA concentration was quantified using Qubit RNA HS assay kit (Q32855).

2.4 Library Preparation

RNA sequencing libraries were prepared with Illumina-compatible NEBNext® Ultra™ II Directional RNA Library Prep Kit (New England BioLabs, MA, USA) at Genotypic Technology Pvt. Ltd., Bangalore, India.

200 - 500 ng of Qubit-quantified total RNA was used for rRNA removal using the NEBNext rRNA Depletion Kit (BACTERIA) (Cat# E7850L). Ribodepletion was performed by following manufacturer's instructions. Briefly, hybridization of RNA to biotinylated rRNA-specific probes (DNA-based) was carried out at 95°C 2 minutes, followed by ramping down the complexes to 22°C, and then followed by RNAse H and DNase I digestion. Further the samples were subjected to 1.8X cleanup and eluted in 7ul NFW. The rRNA-free and cleaned-up RNA was quantified on Qubit fluorimeter.

The enriched RNA was subjected to fragmentation, first strand synthesis followed by second strand synthesis. The double stranded cDNA was purified with NEBNext purification beads (NEBNext, Cat # E7767S). The cDNA was end-repaired, adenylated and ligated to Illumina multiplex barcode adapters as per NEBNext® Ultra™ II Directional RNA Library Prep protocol followed by second strand excision using USER enzyme at 37 ˚C for 15mins.

The adapters used in the study were Illumina Universal Adapter:

5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC TTCCGATCT-3' and Index Adapter: 5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC [INDEX] ATCTCGTAT GCCGTCTTCTGCTTG-3'. [INDEX] – Unique sequence to identify sample-specific sequencing data (Table 1)

Table 1: **Estimated RNA Concentration and Purity**

RNA Concentration and Purity of samples estimated using Nanodrop Spectrophotometer and Qubit Flurometer

	NanoDrop QC					
S.No	**Sample**	**Ng/ml**	**260/80**	**260/230**	**Volume (µl)**	**Yield (ng)**
1	Control	1293	2.05	2.74	25	32325
2	Clinical isolate from Colistin treated patient	1134	2.13	1.23	25	28350

Qubit QC				
S.No	Sample	**Qubit Conc (ng/µl)**	**Volume (µl)**	**Yield (ng)**
1	Control	**1200**	**25**	**30000**
2	Clinical isolate from Colistin treated patient	**1146**	**25**	**28650**

Sample Quality Control					
S.No	Sample	**ND Purity Ratios**	**Qubit Yield**	**Tape RNA Integrity**	**Tape # RIN**
1	Control	**Optimal**	**Optimal**	**Optimal**	**9.1**
2	Clinical isolate from Colistin treated patient	**Optimal**	**Optimal**	**Optimal**	**9.2**

Adapter ligated cDNA was purified using NEBNext purification beads and was subjected to 12 cycles for Indexing- (98˚C for 30 sec, cycling (98˚C for 10sec, 65˚C for 75sec) and 65˚C for 5min) to enrich the adapter-ligated fragments. Final PCR product (sequencing library) was purified with NEBNext purification beads, followed by library quality control check. Illumina-compatible sequencing libraries were quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and its fragment size distribution was analyzed on Agilent 2200 Tape station.

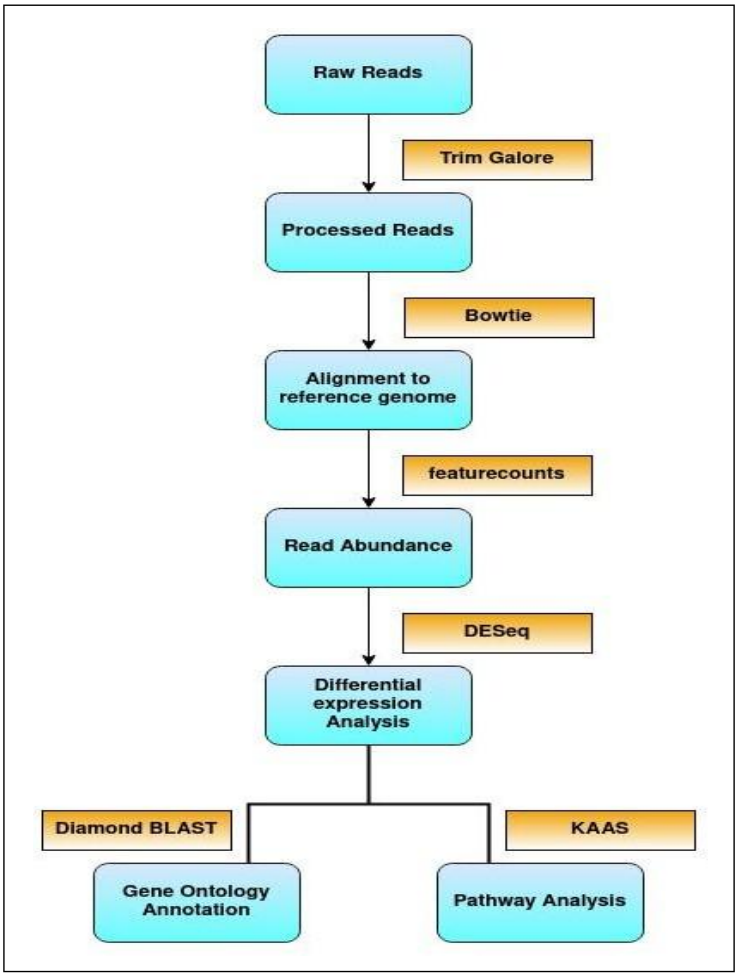
2.5 Illumina Sequencing

The libraries were sequenced on NovaSeq 6000 using PE150 read length and sequencing chemistry.

2.6 Data Analysis

Transcriptome analysis was performed by processing the raw data for removal of low quality reads and adapter sequences. The high quality reads were considered for alignment with reference genome and expression analysis was performed. The expression data was further used for downstream analysis such as GO annotation and pathway analysis to understand the functional role of each expressed transcript (Figure 2).

Figure 2: Transcriptome data analysis workflow

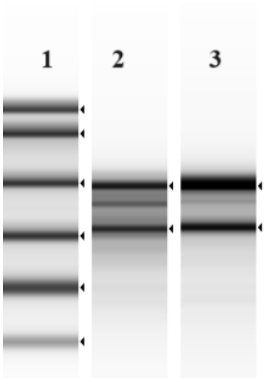


3. RESULTS

3.1 RNA Quality Control

The samples that passed quality assessment with optimal yield and concentration were deemed suitable for Illumina library preparation (Figure-3 and Table 1).

Figure-3: Determining the RNA purity through Agarose Electrophoresis

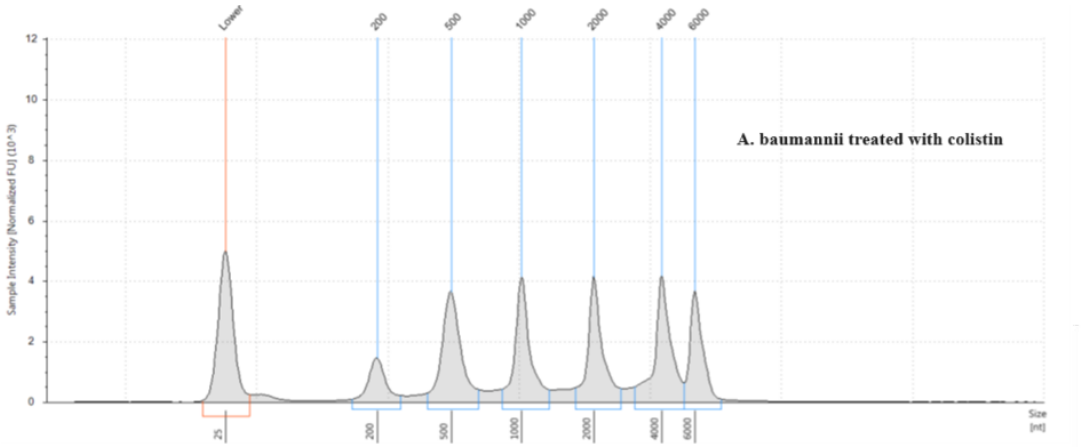


Lane 1-RNA ladder
Lane 2-A. baumannii (control)
Lane 3-A. baumannii (clinical isolate)

3.2 Library Preparation

The Illumina-compatible sequencing libraries showed an average fragment length of 416 bp and sufficient concentration to obtain the desired amount of sequencing data. Table 2 lists the concentration of libraries obtained and indices used. Figure 4 is a TapeStation profile of a representative sequencing library

Figure 4a: TapeStation Profiles of A. baumannii sps



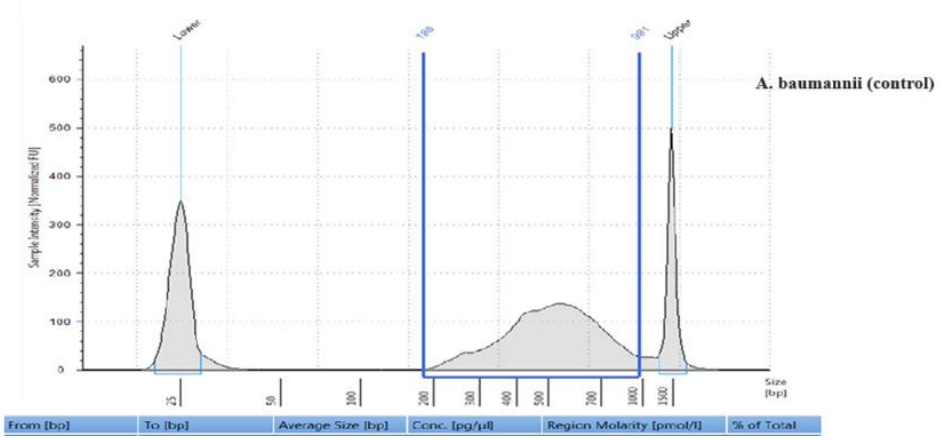


Table 2: Description of Libraries

Sl.No.	Sample ID	Qubit Conc. (ng/ul)	Vol (ul)	Yield (ng)	Barcode ID	Index Sequence
1	Control	4.96	10	49.6	NEB46	TCCCGA
2	Clinical isolate from Colistin treated patient	1.18	10	11.8	NEB45	TCATTC

3.3 Primary Analysis

3.3.1 Illumina Sequencing

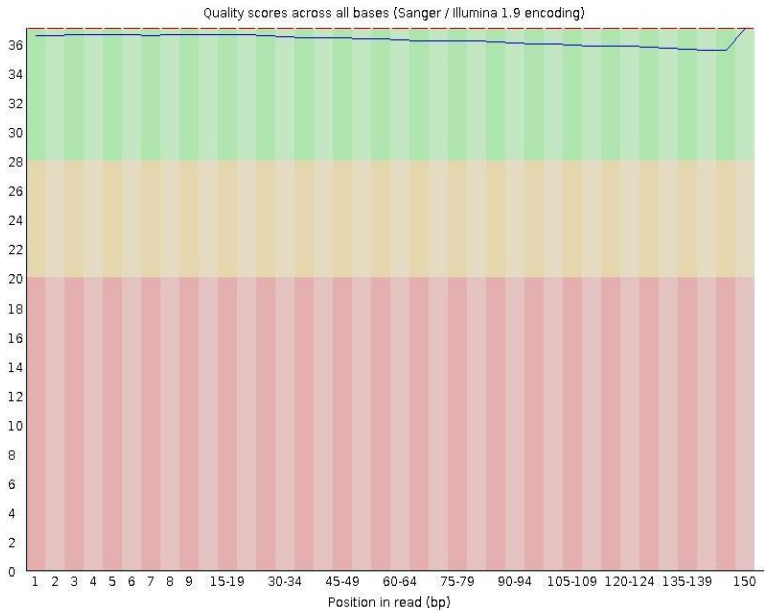
The data obtained from the sequencing run was de-multiplexed using Bcl2fastq software v2.20 and Fastq files were generated based on the unique dual barcode sequences. The sequencing quality was assessed using FastQC v0.11.8 software. The adapter sequences were trimmed and bases above q30 were considered. Low quality bases were filtered off during read pre-processing and used for downstream analysis.

3.3.2 Raw Data QC

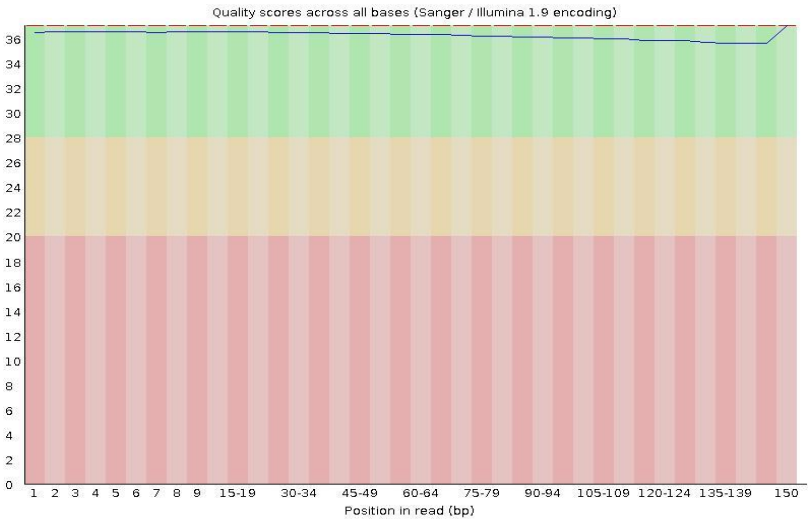
The raw data sequencing quality was assessed. The adapter sequences were trimmed and low quality bases were filtered off during read pre-processing. The reads with Phred

score >q30 were used in downstream analysis. Representative quality scores at each base position along the length of read were plotted and the graphs are shown below (figure 5).

Figure 5: Average base quality



R1:



R2:

Figure 5: Average base quality: The position in the read is plotted on the x- axis and the q-score is plotted on the y-axis. The red line is the median value q-score. The dark blue line is the mean value q-score. A q-score above 30 (>99.9% correct base called) is considered as high quality data.

3.3.3 Data Statistics

Using Illumina sequencing technology, we generated an average of 19.05 million paired end raw data where an average of 18.62 million paired end reads were retained as high quality (>q30) data. Nearly 97.87% (Table 3) of total reads were retained as high quality (>q30) data.

Table 3: Illumina paired-end read statistics

Sample name	Raw_Read Count	Processed_Count	% of high quality data
Control	21482441	20182283	93.95
Clinical isolate from Colistin treated patient	16048465	15802089	98.46

Note: Read count mentioned is for R1 only, equal number of reads will be there in R2. Total reads generated are: R1 Count * 2

3.3.4 Genome Mapping

Reference genome: ***Acinetobacter baumannii*** reference genome downloaded from NCBI database.

[https://www.ncbi.nlm.nih.gov/datasets/genomes/?acc=GCF_002116925.1,GCF_00211692 5.1&utm_source=gquery]

On aligning all processed reads to the ***Acinetobacter baumannii*** reference, an average of 85.06% of the reads showed complete mappability to the reference [Table 4]. In a typical experiment, it is possible to see alignment percent in the range of 70-95% to the reference genome. However, the number depends on multiple factors like sample quality, RNA quality, coverage of genome, and relatedness of the genome and alignment parameters. The alignment (BAM) files can be viewed and inspected in any standard genome viewer such as the IGV browser (Robinson et al., 2011) and (Thorvaldsdóttir et al., 2012) can be downloaded from :

Table 4: Sample wise alignment statistics to reference genome

Sample name	% Alignment [*Acinetobacter baumannii* reference]	% Rfam Alignment
Control	80.61%	9.08%
Clinical isolate from Colistin treated patient	88.64%	11.16%

Link: https://software.broadinstitute.org/software/igv/

3.4 Secondary Analysis

3.4.1 Expression analysis

DESeq normalized expression values were used to calculate fold change for a given transcript/gene. The regulation for each transcript/gene was assigned based on log2fold change. The transcripts /genes that show log2fold change less than -1 are represented as down regulated, the values greater than 1 are represented as up regulated and in between -1 to 1 are termed as neutrally regulated.

An example DGE transcript and interpretation is given in (Table 5). Heatmap was generated for top 20 transcripts expressed (Figure 6).

Table 5: Up and down regulated transcripts

Genes upregulated in Clinical isolate from Colistin treated patient in comparision to control

Transcript_ID	Protein names	Gene names	Pathway	Molecular Function	Fold Change
cds-WP_000355489.1	Twitching motility protein	pilT BDGL_000161	Bacterial motility proteins [02035]; Secretion system [02044]	GO:0005524~ATP binding	2.23975255
cds-WP_100223356.1	p-aminobenzoate synthetase (EC 4.1.3.)	pabB ABSDF2837	Folate biosynthesis [00790]	GO:0016829~lyase activity	2.03073539
cds-WP_001046417.1	Pilin like competence factor	comE BDGL_002630	Bacterial motility proteins [02035]; Secretion system [02044]	NA	2.03899042
cds-WP_000079421.1	D-methionine-binding lipoprotein MetQ	BDGL_000868	ABC transporters [02010]; Transporters [02000]	NA	2.02692538
cds-WP_000094278.1	Uncharacterized protein	ABSDF1794	NA	NA	2.58794937
cds-WP_001140931.1	Uncharacterized protein	ABSDF0868	NA	GO:0003824~catalytic activity;GO:0046872~metal ion binding	2.11741312
cds-WP_000792951.1	HTH tetR-type domain-containing protein	BDGL_002727	NA	GO:0003677~DNA binding	2.258574
cds-WP_000048916.1	Transcriptional regulator	B9T35_08140	NA	GO:0003677~DNA binding	2.258574

cds-WP_000995631.1	Hydrolase	B9T35_11080	NA	GO:0016787~hydrolase activity	2.66637208
cds-WP_001030690.1	Putative Phosphopantetheine binding protein	ppsA BDGL_003041	NA	GO:0031177~phosphopantetheine binding	3.76429
cds-WP_000835166.1	Uncharacterized protein	ABSDF1807	NA	NA	3.05848562
cds-WP_000770268.1	Uncharacterized protein	BDGL_001286	NA	NA	3.52902187
cds-WP_002135133.1	SASA domain-containing protein	A8B73_17770	NA	GO:0016787~hydrolase activity	2.35268125
cds-WP_002017921.1	NA	NA	NA	NA	4.23482625
cds-WP_000864073.1	Adenylylsulfate reductase, beta subunit	BDGL_001081	NA	NA	4.23482625
cds-WP_000041515.1	Cation efflux system permease, putative	Ajs_3474	NA	GO:0008324~cation transmembrane transporter activity; GO:0046872~metal ion binding	2.8232175
cds-WP_001133073.1	HTH tetR-type domain-containing protein	BDGL_000698	NA	GO:0003677~DNA binding	2.8232175
cds-WP_000729387.1	Uncharacterized protein	F945_00937	NA	NA	4.7053625

Genes down regulated in Clinical isolate from Colistin treated patient in comparision to control

Transcript_ID	Protein names	Gene names	Pathway	Molecular Function	FoldChange
cds-WP_000457358.1	GtrA domain-containing protein	ABSDF0864	NA	NA	0.117634062
cds-WP_001129188.1	Uncharacterized protein	BDGL_001429	NA	NA	0.144780385
cds-WP_002134788.1	Uncharacterized protein	ABSDF1792	NA	NA	0.156845417
cds-WP_001180453.1	HTH tetR-type domain-containing	BDGL_001292	NA	GO:0003677~DNA binding	0.1882145

	protein				
cds-WP_000934372.1	Uncharacterized protein	BDGL_000707	NA	NA	0.235268125
cds-WP_000100162.1	DUF559 domain-containing protein	ABSDF1332	NA	NA	0.235268125
cds-WP_001034582.1	Putative pilus assembly protein (FilF)	ABSDF2830	NA	NA	0.235268125
cds-A4U85_RS03690	NA	NA	NA	NA	0.247319722
cds-WP_000013652.1	30S ribosomal protein S20	rpsT ABSDF1833	Ribosome [03011]; Ribosome [03010]	GO:0003735~structural constituent of ribosome; GO:0019843~rRNA binding	0.272876041
cds-WP_000830584.1	Alpha/beta hydrolase	BEN71_12875	NA	GO:0004806~triglyceride lipase activity; GO:0008236~serine-type peptidase activity	0.28232175
cds-WP_000289091.1	Putative regulatory or redox component complexing with Bfr, in iron storage and mobility (Bfd)	ABSDF0318	Others [99994]	NA	0.287940093
cds-WP_000011679.1	Uncharacterized protein	BDGL_000914	NA	NA	0.29564667
cds-WP_000462893.1	Uncharacterized protein	ABSDF0483	NA	NA	0.305814808
cds-WP_000524488.1	NA	NA	NA	NA	0.313690833
cds-WP_001019739.1	PG_binding_3 domain-containing protein	ABSDF2454	NA	NA	0.313690833
cds-WP_002011	NA	NA	NA	NA	0.313690833

867.1					
cds-WP_000192619.1	Uncharacterized protein	ABSDF2123	NA	NA	0.31996465
cds-WP_000401163.1	Uncharacterized protein	BJI46_03165 F4V57_01075	NA	NA	0.332143235
cds-WP_000061134.1	Uncharacterized protein	BDGL_000197	NA	NA	0.332143235
cds-WP_001049818.1	Uncharacterized protein	ABSDF1711	NA	NA	0.336097321
cds-WP_001025680.1	Uncharacterized protein	BDGL_000778	Function unknown [99997]	NA	0.337510815
cds-WP_000949688.1	Uncharacterized protein	F967_01270	NA	NA	0.338845287
cds-WP_000761577.1	Uncharacterized protein	BDGL_000211	NA	NA	0.34437798
cds-WP_000108365.1	NA	NA	NA	NA	0.345783123
cds-WP_000138830.1	Glutaredoxin domain-containing protein	ABSDF2089	Chaperones and folding catalysts [03110]; Mitochondrial biogenesis [03029]	GO:0046872~metal ion binding; GO:0051536~iron-sulfur cluster binding; GO:0097573~glutathione oxidoreductase activity	0.347375755
cds-WP_000490267.1	UPF0391 membrane protein BDGL_002899	BDGL_002899	NA	NA	0.348600783
cds-WP_000564581.1	Uncharacterized protein	ABSDF1906	NA	NA	0.349315783
cds-WP_001133277.1	HTH tetR-type domain-containing protein	BDGL_000698	NA	GO:0003677~DNA binding	0.352902187
cds-WP_001094391.1	Uncharacterized protein	BDGL_000505	NA	NA	0.360371017
cds-WP_000575	Uncharacterized	BDGL_000916	NA	NA	0.36195

823.1	protein				0961
cds-WP_001984826.1	Bacteriolytic lipoprotein entericidin B	ecnB ABSDF0648	Transporters [02000]	NA	0.36305335
cds-WP_000897685.1	NA	NA	NA	NA	0.370191344
cds-WP_086223981.1	NA	NA	NA	NA	0.376429
cds-WP_000760495.1	Rubredoxin	rubA BDGL_000276	NA	GO:0005506~iron ion binding; GO:0009055~electron carrier activity	0.379683429
cds-WP_000859008.1	Uncharacterized protein	BDGL_001671	NA	NA	0.379968432
cds-WP_001284370.1	Translation initiation factor IF-1	infA CDG61_15075	Translation factors [03012]	GO:0003743~translation initiation factor activity; GO:0019843~rRNA binding; GO:0043022~ribosome binding	0.38073359
cds-WP_000420545.1	Uncharacterized protein	ABSDF1646	NA	NA	0.383910329
cds-WP_000735804.1	Uncharacterized protein	ABSDF2687	NA	NA	0.387212122
cds-WP_000277448.1	Putative bacteriophage protein	ABSDF1805	NA	NA	0.392113542
cds-WP_001043034.1	DNA-binding protein HU-beta	hup BDGL_001006	DNA repair and recombination proteins [03400]; Chromosome and associated proteins [03036]; DNA replication proteins [03032]	GO:0003677~DNA binding	0.393316344
cds-WP_085916973.1	Uncharacterized protein	BDGL_001982	NA	NA	0.398146058
cds-WP_001205031.1	50S ribosomal protein L33	rpmG CDG61_14930	Ribosome [03010]; Ribosome [03011]	GO:0003735~structural constituent of ribosome	0.401619324
cds-WP_000736	Uncharacterized	ABSDF2168	NA	NA	0.406854652

703.1	protein				
cds-WP_000993415.1	Uncharacterized protein	BDGL_001779	NA	NA	0.408389953
cds-WP_001218560.1	Uncharacterized protein	ABSDF2558	NA	NA	0.409161956
cds-WP_000738603.1	Uncharacterized protein	ABSDF3239	NA	NA	0.418838599
cds-WP_000269732.1	Uncharacterized protein	ABSDF3675	NA	NA	0.419667466
cds-WP_000831329.1	50S ribosomal protein L34	rpmH CDG61_17410	Ribosome [03010]; Ribosome [03011]	GO:0003735~structural constituent of ribosome	0.420775029
cds-WP_000616034.1	Uncharacterized protein	ABSDF2084	NA	NA	0.422481484
cds-WP_000749814.1	Uncharacterized protein	BDGL_000817	NA	NA	0.423204201
cds-WP_000047695.1	Uncharacterized protein	BDGL_001028	NA	NA	0.423482625
cds-WP_001232531.1	Uncharacterized protein	ABSDF1620	NA	NA	0.428710805
cds-WP_000251637.1	NA	NA	NA	NA	0.431324896
cds-WP_000724539.1	Uncharacterized protein	ABSDF0041	Transport [99977]	NA	0.433871087
cds-WP_002010078.1	Uncharacterized protein	ABSDF2215	NA	NA	0.434029127
cds-WP_000244900.1	DUF2061 domain-containing protein	C3941_30215	NA	NA	0.434341154
cds-A4U85_RS19115	Uncharacterized protein	BDGL_002639	NA	NA	0.434341154
cds-WP_000201632.1	50S ribosomal protein L27	rpmA ABSDF0744	Ribosome [03010]; Ribosome [03011]	GO:0003735~structural constituent of ribosome	0.436027552

cds-WP_000135049.1	DNA-directed RNA polymerase subunit omega (RNAP omega subunit) (EC 2.7.7.6) (RNA polymerase omega subunit) (Transcriptase subunit omega)	rpoZ ABSDF0321	RNA polymerase [03020]; Transcription machinery [03021]; DNA repair and recombination proteins [03400]	GO:0003677~DNA binding; GO:0003899~DNA-directed RNA polymerase activity	0.437243591
cds-WP_001279871.1	Acyl carrier protein (ACP)	acpP BDGL_000089	PATHWAY: Glycolipid biosynthesis; KDO(2)-lipid A biosynthesis. {ECO:0000256|ARBA:ARBA00024328}.; PATHWAY: Lipid metabolism; fatty acid biosynthesis. {ECO:0000256|HAMAP-Rule:MF_01217, ECO:0000256|RuleBase:RU003545}.; Fatty acid biosynthesis [00061]; Biosynthesis of various secondary metabolites - part 2[00998]	GO:0000036~ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process	0.439382023
cds-WP_000138016.1	Arsenate reductase (EC 1.20.4.1)	arsC BDGL_000835	Enzymes with EC numbers[99980]	GO:0008794~arsenate reductase (glutaredoxin) activity	0.445328951
cds-WP_001280161.1	Putative 3-methyladenine DNA glycosylase (EC 3.2.2.-)	ABSDF0617	Base excision repair [03410]; DNA repair and recombination proteins [03400]	GO:0003677~DNA binding;GO:0003905~alkylbase DNA N-glycosylase activity	0.447995591
cds-WP_000867907.1	Deleted.	NA	Ribosome [03011]; Ribosome [03010]	NA	0.453324814
cds-WP_000619820.1	Rhodanese domain-containing protein	ABSDF0504	NA	NA	0.453888765
cds-WP_000927115.1	Uncharacterized protein	BDGL_000221	NA	NA	0.454310862

cds-WP_000182506.1	Uncharacterized protein	ABSDF1204	NA	NA	0.454851708
cds-WP_001170994.1	Outer membrane protein assembly factor BamE	smpA bamE ABSDF2539	Transporters [02000]	NA	0.455024066
cds-WP_000800035.1	Tolerance to group A colicins, single-stranded filamentous DNA phage, required for OM integrity	tolA BDGL_001562	NA	NA	0.455598591
cds-WP_001097630.1	Uncharacterized protein	ABSDF3650	NA	NA	0.455831992
cds-WP_001047855.1	Uncharacterized protein	ABSDF2717	NA	NA	0.463451639
cds-WP_000089996.1	Probable Fe(2+)-trafficking protein	BDGL_003169	NA	GO:0005506~iron ion binding	0.466138715
cds-WP_000913165.1	Uncharacterized protein	ABSDF3482	NA	NA	0.47053625
cds-WP_001288210.1	FAD assembly factor SdhE	ABSDF0452	Prokaryotic defense system [02048]	NA	0.47053625
cds-WP_001288624.1	NA	NA	NA	NA	0.47053625
cds-WP_000097955.1	Putative pseudouridylate synthase (EC 4.2.1.70)	ABSDF1621	Transfer RNA biogenesis [03016]; Ribosome biogenesis [03009]	GO:0003723~RNA binding;GO:0004730~pseudouridylate synthase activity;GO:0009982~pseudouridine synthase activity	0.47053625
cds-WP_000656405.1	Uncharacterized protein	ABSDF2508	NA	NA	0.47053625
cds-WP_000756788.1	Haloacid dehalogenase	CL42_12145	General function prediction only [99996]	GO:0016787~hydrolase activity	0.47053625

cds-WP_000368364.1	Putative bacteriophage protein	ABSDF1806	NA	NA	0.47053625
cds-WP_001090544.1	DUF1989 domain-containing protein	BDGL_000611	Function unknown [99997]	NA	0.47053625
cds-WP_001077693.1	HTH cro/C1-type domain-containing protein	CL42_06435	NA	GO:0003677~DNA binding	0.47053625
cds-WP_000774579.1	Uncharacterized protein	ABSDF2226	NA	NA	0.47053625
cds-WP_000540685.1	Uncharacterized protein	BKE30_13730	NA	NA	0.47053625
cds-WP_000114563.1	NA	NA	NA	NA	0.47053625
cds-WP_085940424.1	Uncharacterized protein	BDGL_002695	NA	NA	0.47053625
cds-WP_000018791.1	NA	NA	NA	NA	0.47053625
cds-WP_000042163.1	MarR family transcriptional regulator	B9T35_07270	Transcription factors [03000]	GO:0003700~sequence-specific DNA binding transcription factor activity	0.47053625
cds-WP_000784267.1	Uncharacterized protein	ABSDF0550	Chaperones and folding catalysts [03110]	NA	0.475883253
cds-WP_000678928.1	Uncharacterized protein	ABSDF0646	NA	NA	0.481230255
cds-WP_000144889.1	Ferredoxin	fdxA ABSDF1115	Energy metabolism[99982]	GO:0009055~electron carrier activity;GO:0046872~metal ion binding;GO:0051538~3 iron, 4 sulfur cluster binding;GO:0051539~4 iron, 4 sulfur cluster binding	0.48190186
cds-WP_000161	Uncharacterized	BDGL_001552	NA	NA	0.482012744

254.1	protein				
cds-WP_000379022.1	Diacylglycerol kinase (EC 2.7.1.107)	dgkA ABSDF0819	Glycerolipid metabolism [00561]; Glycerophospholipid metabolism [00564]	GO:0004143~diacylglycerol kinase activity;GO:0005524~ATP binding;GO:0046872~metal ion binding	0.483253446
cds-WP_000524329.1	Uncharacterized protein	BDGL_003409	NA	NA	0.486168683
cds-WP_000795915.1	Uncharacterized protein	BDGL_000771	NA	NA	0.492253307
cds-WP_000609117.1	Diamine N-acetyltransferase	SAMN05444165_1312	Arginine and proline metabolism [00330]	GO:0008080~N-acetyltransferase activity	0.492942738
cds-WP_000733015.1	Uncharacterized protein	BDGL_002399	NA	NA	0.494045776
cds-WP_001274776.1	NA	NA	NA	NA	0.494063062
cds-WP_000490937.1	Uncharacterized protein	ABSDF0667	NA	NA	0.498687991
cds-WP_000024050.1	KTSC domain-containing protein	BDGL_000456	NA	NA	0.499227485
cds-WP_000887099.1	Uncharacterized protein	ABSDF0760	Function unknown[99997]	NA	0.499526635
cds-WP_000039916.1	Putative nitrate transport protein (NasF)	nasF BDGL_001495	Two-component system [02022]	NA	0.499944765

.

Genes not expressed/silenced in Clinical isolate

Transcript_ID	Sample_3_397 Expression	Protein names	Gene names	Pathway	Molecular Function	Biological Process
cds-WP_000183811.1	2.915636996	Site-specific DNA-methyltransferase (adenine-specific) (EC 2.1.1.72)	BEN71_05830	Prokaryotic defense system [02048]	GO:0003677~DNA binding;GO:0008170~N-methyltransferase activity;GO:0009007~site-specific DNA-methyltransferase (adenine-specific) activity	GO:0009307~DNA restriction-modification system
cds-WP_001243392.1	2.915636996	Uncharacterized protein	ABSDF3358	NA	NA	NA
cds-WP_000679991.1	1.457818498	DNA polymerase V	F945_01386	DNA repair and recombination proteins [03400]	GO:0003684~damaged DNA binding	GO:0006281~DNA repair
cds-WP_000470079.1	1.457818498	Vanillate O-demethylase oxygenase subunit (4-hydroxy-3-methoxybenzoate demethylase)	vanA BDGL_000396	Aminobenzoate degradation [00627]	GO:0005506~iron ion binding;GO:0008168~methyltransferase activity;GO:0016491~oxidoreductase activity;GO:0051537~2 iron, 2 sulfur cluster binding	GO:0032259~methylation
cds-WP_001098394.1	1.457818498	Uncharacterized protein	DJ533_00885	NA	NA	NA

New Genes expressed in clinical isolate

Transcript_ID	3A_397T Expression	Protein names	Gene names	Pathway	Molecular Function	Biological Process
cds-WP_100743362.1	2.057869347	NA	NA	NA	NA	NA
cds-A4U85_RS13025	6.17360804	ISL3 family transposase (Fragment)	EF099_20305	NA	NA	NA
cds-WP_002061576.1	0.685956449	Putative transcriptional regulator	ABSDF2741	NA	GO:0003677~DNA binding	NA

		(TetR family)				
cds-WP_000438219.1	1.371912898	Aldehyde dehydrogenase	calB BDGL_000375	Enzymes with EC numbers[99980]	GO:0016620~oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	GO:0006081~cellular aldehyde metabolic process
cds-WP_001983635.1	1.371912898	Two-component system sensor protein	colS BDGL_001704	NA	GO:0000155~phosphorelay sensor kinase activity	NA
cds-WP_000701381.1	1.371912898	Integrase catalytic domain-containing protein	P256_00233	NA	GO:0003676~nucleic acid binding	GO:0015074~DNA integration
cds-WP_001017483.1	1.371912898	Uncharacterized protein	ABSDF3352	NA	NA	NA
cds-WP_000938194.1	0.685956449	Uncharacterized protein	AMD27_12655	NA	NA	NA
cds-WP_000041313.1	2.057869347	Uncharacterized protein	BDGL_000387	NA	NA	NA
cds-WP_001219640.1	0.685956449	MerR family transcriptional regulator	AGRI_13945	NA	GO:0003677~DNA binding;GO:0003700~sequence-specific DNA binding transcription factor activity;GO:0046872~metal ion binding	GO:0045893~positive regulation of transcription, DNA-templated
cds-WP_001984959.1	0.685956449	PG_binding_3 domain-containing protein	ABSDF2454	NA	NA	NA
cds-WP_000190710.1	0.685956449	Short chain dehydrogenase	dhrS4 BDGL_001300	NA	GO:0016491~oxidoreductase activity	NA
cds-WP_000990621.1	0.685956449	Putative transcriptional regulator (TetR family)	ABSDF2195	NA	GO:0003677~DNA binding	NA

cds-WP_000154590.1	1.37191 2898	FAD dependent oxidoreductase	choB BDGL_000403	Steroid degradation[00984]	GO:0016614~oxidoreductase activity, acting on CH-OH group of donors;GO:0050660~flavin adenine dinucleotide binding	NA
cds-WP_000800799.1	1.37191 2898	Putative outer membrane protein	ABSDF0898	NA	NA	NA
cds-WP_002134769.1	1.37191 2898	Uncharacterized protein	BJL95_22590	Cationic antimicrobial peptide (CAMP) resistance [01503]; Peptidases and inhibitors [01002]	GO:0005509~calcium ion binding	NA
cds-WP_000163911.1	0.68595 6449	Tyr recombinase domain-containing protein	BOW53_10210	NA	GO:0003677~DNA binding	GO:0006310~DNA recombination;GO:0015074~DNA integration
cds-WP_001110159.1	1.37191 2898	Uncharacterized protein	F945_03289	NA	NA	NA
cds-WP_000564784.1	2.05786 9347	Glutamine--scyllo-inositol transaminase	degT BDGL_002974	Amino acid related enzymes [01007]; Amino sugar and nucleotide sugar metabolism [00520]; O-Antigen nucleotide sugar biosynthesis [00541]	GO:0003824~catalytic activity	NA
cds-WP_001022433.1	1.37191 2898	DUF927 domain-containing protein	AMQ28_04300	Replication and repair[99976]	NA	NA
cds-WP_002134405.1	0.68595 6449	ATP-binding protein	DJ533_03325	NA	GO:0005524~ATP binding	NA
cds-WP_001126090.1	1.37191 2898	Helix-turn-helix protein, CopG	copG BDGL_000618	Transcription factors [03000]	NA	GO:0006355~regulation of transcription, DNA-templated
cds-WP_001089573.1	0.68595 6449	SMI1_KNR4 domain-containing	KPC_2390	NA	NA	NA

		protein				
cds-WP_000589668.1	0.685956449	Hydrolase or metal-binding protein	CDG61_05810	NA	GO:0016787~hydrolase activity	NA
cds-WP_001087992.1	1.371912898	LysR family transcriptional regulator	L861_13720	Transcription factors [03000]	GO:0003700~sequence-specific DNA binding transcription factor activity	NA
cds-WP_000759831.1	0.685956449	Uncharacterized protein	KPC_3056	NA	NA	NA
cds-WP_000905420.1	1.371912898	Lipoprotein signal peptidase (EC 3.4.23.36) (Prolipoprotein signal peptidase) (Signal peptidase II) (SPase II)	lspA Ajs_3473	PATHWAY: Protein modification; lipoprotein biosynthesis (signal peptide cleavage). {ECO:0000256|HAMAP-Rule:MF_00161}.; Peptidases and inhibitors [01002]; Protein export [03060]	GO:0004190~aspartic-type endopeptidase activity	NA
cds-WP_085940550.1	6.173600804	Uncharacterized protein	BDGL_000271	NA	NA	NA
cds-WP_000917503.1	0.685956449	Uncharacterized protein	BDGL_002720	NA	NA	NA
cds-WP_000008459.1	2.743825796	Uncharacterized protein	BFG52_07515	NA	NA	NA
cds-WP_001046789.1	1.371912898	Type I site-specific deoxyribonuclease (EC 3.1.21.3)	BEN71_05840	Prokaryotic defense system [02048]	GO:0003677~DNA binding;GO:0009035~Type I site-specific deoxyribonuclease activity	GO:0006304~DNA modification
cds-WP_001166630.1	3.429782245	Deleted.	NA	Transcription factors [03000]	NA	NA
cds-WP_001984934.1	1.371912898	Putative transcritional regulator	ABSDF1415	NA	GO:0003700~sequence-specific DNA binding transcription factor activity;GO:0043565~sequence-specific DNA binding	NA

Declaration: Studies involving human subjects adhered to ethical guidelines, with informed consent obtained from all participants.

Conflict of Interest: None of the authors have conflict of interest.

Figure 6: Read statistics (raw and processed reads)

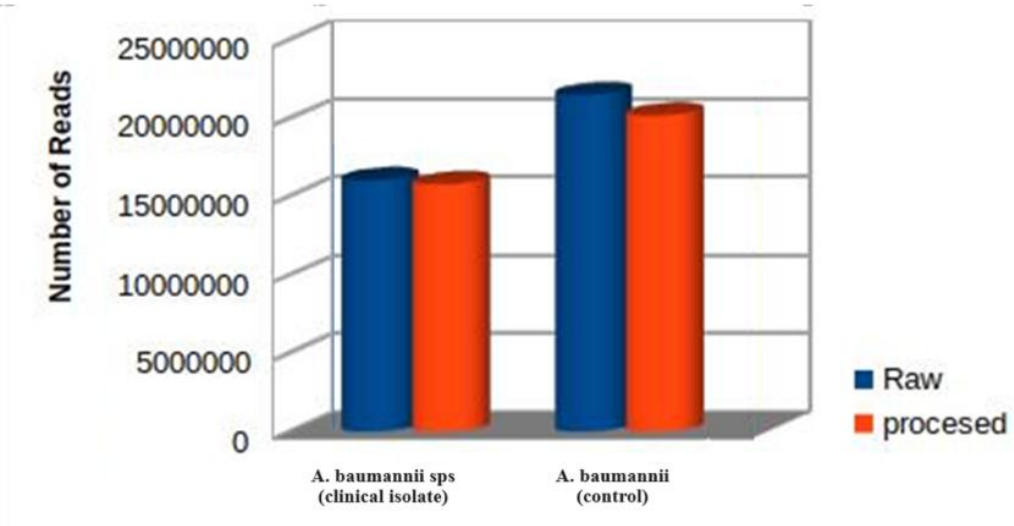


Figure 6: Read statistics (raw and processed reads) representing the total number of reads generated (in blue color) and high quality reads used for downstream analysis (in orange color). An average of 97.87% of high quality reads were retained for the downstream analysis.

Transcript cds-WP_000085212.1 is 1.70 times up regulated in 6A sample [Treated] compared to Sample_3_397 sample [Control]. Similarly, Transcript cds-WP_000863328.1 is 1.71 times down regulated in 6A sample [Treated] compared to Sample_3_397 sample [Control].

The sample wise expression values at transcript/gene level were obtained after normalization between control and treatment samples. These normalized values were further used for log2FC calculations. The details have been provided in the DGE reports.

Normalized expression of a given transcript/gene may change across different comparisons depending on the size factor for all DGE combinations. Fold changes are calculated based on "Expression of test sample / Expression of control sample". Below mentioned tool can be used for heatmap generation for your data of interest.

The sample-wise normalized (RPKM) values provided in expression matrix "GT_SO_10857_A_Read_Count_Matrix.xlsx [RPKM_Matrix]" are not same as the normalized values provided in DGE reports.

Clustvis: https://bio.tools/clustvis

3.4.2 Gene ontology (GO) and pathway analysis

Transcripts/Genes were assigned with a homolog protein (Uniprot) from other organisms with known functions, if the match was found at e-value less than e^{-5} and minimum similarity greater than 30%.

For pathway analysis, the representative reference organism [***Acinetobacter baumannii***] was considered as reference for pathway identification. Compiled pathways per transcript/gene were mapped to the DE genes.

3.4.3 Functional Annotation of Differentially Expressed Genes

A COG (clusters of orthologous groups of proteins) analysis was carried out to evaluate the functional categorization. The results showed that the differentially expressed genes were classified into 11 COG categories. The 18 upregulated genes were involved in secondary metabolite biosynthesis, transport and catabolism, translation, ribosomal structure and biogenesis, lipid transport and metabolism, energy production and conversion, inorganic ion transport and metabolism, function unknown, and general function prediction. The 100 downregulated genes were involved in post-translational modification, protein turnover, chaperones, defense mechanisms, amino acid transport and metabolism, cell wall/membrane/envelope biogenesis, lipid transport and metabolism, general function prediction, energy production and conversion, and inorganic ion transport and metabolism (Figure 2).

Figure 7: Heatmap representing up and down regulated genes

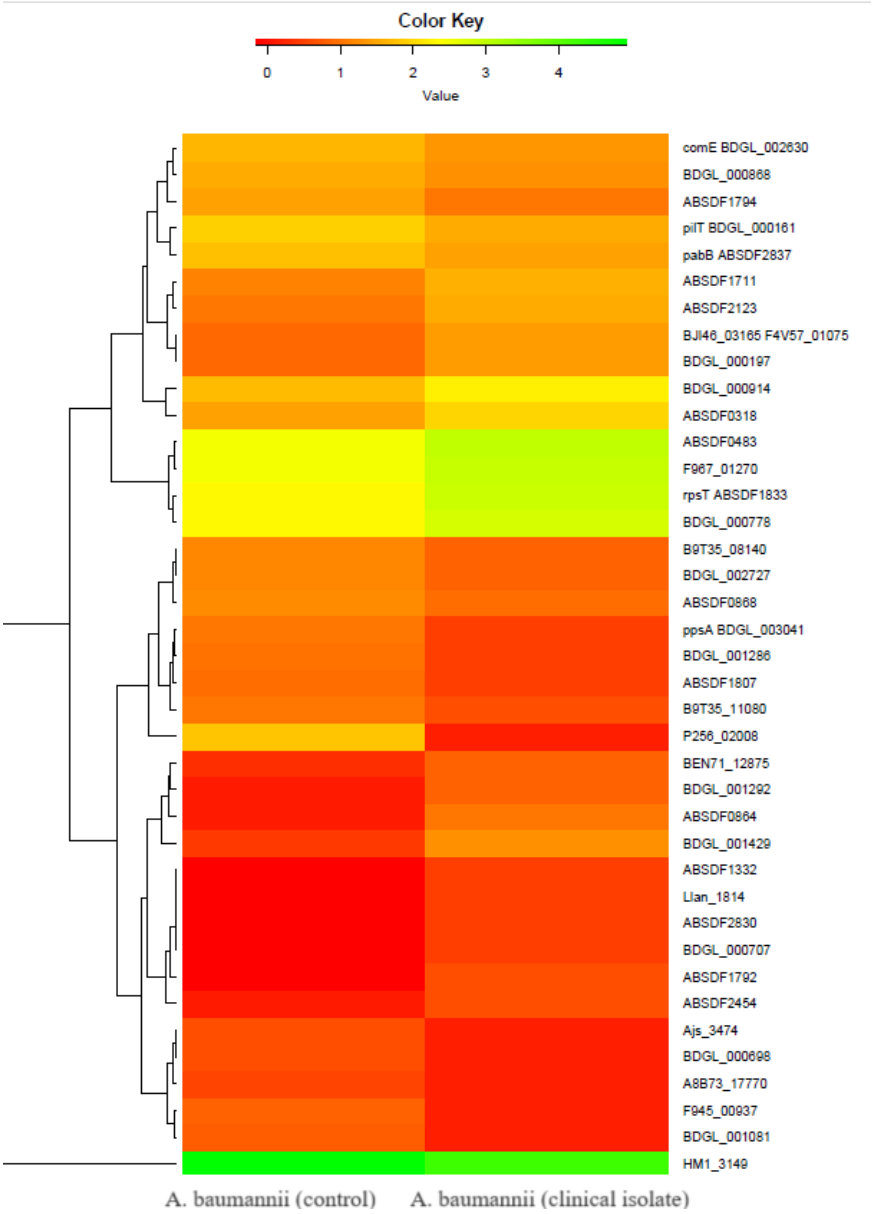


Figure 7: Heatmap representing up and down regulated genes for in A. baumannii control sps and A. baumannii treated with colistin.

Note: For heatmap generation log2fold change is sorted in descending order. Expression values of both A. baumannii control sps and A. baumannii treated with colistin samples are considered along with the corresponding gene names. The dendrogram on y-axis of the heatmap represents the relatedness or how similar 2/more transcripts are to each other. The transcripts that cluster together are similar to each other based on expression values.

4. DISCUSSION

The widespread use of antibiotics in both human and veterinary medicine has led to the detection of subinhibitory concentrations of antibiotics in various environments, including hospital effluent, municipal sewage, sewage treatment plant effluent, surface water, and groundwater (Kümmerer, 2003; Brady and Jamal, 2013). This exposure to low levels of antibiotics is believed to accelerate the evolution of bacteria, contributing to the development of resistant strains. Colistin has been extensively used since the middle of the last century in animals, particularly in swine, for the control of enteric infections. Colistin is presently considered the last line of defense against human infections caused by multidrug-resistant Gram-negative organisms such as carbapenemase-producer *Enterobacterales*, *Acinetobacter baumanni*, and *Pseudomonas aeruginosa* (Andrade et al., 2020). This study investigates the rapid response of A. baumannii to minocycline exposure by

conducting RNA-seq analysis when the bacterium was exposed to half the minimum inhibitory concentration (MIC) of 2mg/ml colistin.

The down regulated genes and their expressed proteins are- GtrA domain-containing protein, HTH tetR-type domain-containing protein, DUF559 domain-containing protein, Putative pilus assembly protein (FilF), 30S ribosomal protein S20, Alpha/beta hydrolase, Putative regulatory or redox component complexing with Bfr, in iron storage and mobility (Bfd), PG_binding_3 domain-containing protein, Glutaredoxin domain-containing protein, UPF0391 membrane protein BDGL_002899, Bacteriolytic lipoprotein entericidin B, Rubredoxin, Translation initiation factor IF-1, Putative bacteriophage protein, DNA-binding protein HU-beta, DUF2061 domain-containing protein, DNA-directed RNA polymerase subunit omega (RNAP omega subunit) (EC 2.7.7.6) (RNA polymerase omega subunit) (Transcriptase subunit omega), Acyl carrier protein (ACP), Arsenate reductase (EC 1.20.4.1), Putative 3-methyladenine DNA glycosylase (EC 3.2.2.-), Rhodanese domain-containing protein, Outer membrane protein assembly factor BamE, Tolerance to group A colicins, single-stranded filamentous DNA phage, required for OM integrity, Probable Fe^{2+}-trafficking protein, FAD assembly factor SdhE, Putative pseudouridylate synthase (EC 4.2.1.70), Haloacid dehalogenase, Putative bacteriophage protein, DUF1989 domain-containing protein, HTH cro/C1-type domain-containing protein, MarR family transcriptional regulator, Ferredoxin, Diacylglycerol kinase (EC 2.7.1.107), Diamine N-acetyltransferase, KTSC domain-containing protein, Putative nitrate transport protein (NasF).

Of the 18 upregulated genes, the following proteins are known to exist: Twitching motility protein, p-aminobenzoate synthetase (EC 4.1.3.-), Pilin like competence factor, D-methionine-binding lipoprotein MetQ, HTH tetR-type domain-containing protein, Transcriptional regulator, Hydrolase, Putative Phosphopantetheine binding protein, SASA domain-containing protein, Adenylylsulfate reductase, beta subunit, Cation efflux system permease, putative, HTH tetR-type domain-containing protein. Further research is needed to explore the roles of the genes that were differentially expressed in the clinical isolate.

5. SUMMARY

Transcriptome sequencing and analysis was carried out for 2 ***Acinetobacter baumannii*** samples. RNA sequencing libraries were prepared using NEBNext Ultra II Directional RNA library preparation reagents and workflow. The libraries were paired-end sequenced on Illumina NovaSeq 6000 sequencer for 150 cycles.

The raw data were pre-processed by trimming adapters, and removing low-quality reads. The high quality reads obtained after pre-processing ranged from 15-20 million reads. The pre-processed data was aligned to ***Acinetobacter baumannii*** reference. The alignment percentage to the reference ranged from 80-88% for both the samples. Number of expressed transcripts ranged between 3213-3255 for both the samples.

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