Evaluation of Antibiotic Tolerance in *Pseudomonas aeruginosa* for Aminoglycosides and its Prediction of Resistance Development Through In-silico Transcriptomic Analysis

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Running title: *In vitro* study of amikacin, gentamicin, and tobramycin

Abstract

Pseudomonas aeruginosa causes severe life-threatening infections and are difficult to treat. The lack of antibiotic response in *P. aeruginosa* is due to adaptive resistance, which prevents the entry of antibiotics into cytosol of the cell. Among different groups of antibiotics, aminoglycosides show superior antibiotic response and are used as a parental antibiotic for treatment. This study aims to determine the kinetics of adaptive resistance development and gene expression changes in *P. aeruginosa* exposed to amikacin, gentamicin, and tobramycin. *In vitro* antibiotic exposure to *P. aeruginosa* was performed and optical density of the cells were

monitored for every 12 hours until 72 hours. The growth pattern plotted in graph represents the kinetics of adaptive resistance developed to respective antibiotics. The transcriptomic profile of *P. aeruginosa* PA14 to post exposed antibiotic was taken from Gene Expression Omnibus (GEO), NCBI. The gene expressions of two datasets were analyzed by case-control study. Tobramycin exposed *P. aeruginosa* failed to develop adaptive resistance in 0.5ug/mL, 1ug/mL and 1.5ug/mL of its MIC. Whereas, amikacin and gentamicin treated *P. aeruginosa* developed tolerance in the inhibitory concentrations of the antibiotics. This depicts the superior *in vitro* response of tobramycin over the gentamicin and amikacin. Furthermore, tobramycin treated *P. aeruginosa* microarray analysis resulted in low expression of catalytic enzyme 16s rRNA Methyltransferase E, B & L, alginate biosynthesis genes and several proteins of Type 2 Secretory System (T2SS) and Type 3 Secretory System (T3SS). The Differentially Expressed Genes (DEGs) of alginate biosynthesis, and RNA Methyltransferases suggests increased antibiotic response and low probability of developing resistance. The use of tobramycin as a parental antibiotic with its synergistic combination might combat *P. aeruginosa* with increased response.

Keywords

Adaptive resistance, *in vitro* exposure, aminoglycoside, *Pseudomonas aeruginosa*, Differentially Expressed Genes (DEGs), transcriptomic analysis.

Introduction

P. aeruginosa is an opportunistic pathogen, causes severe life-threatening infections and are difficult to treat because of the limited susceptibility to antimicrobials and high frequency of emergence of antibiotic resistance during therapy[1]. Multi-drug resistance (MDR) in *P. aeruginosa* is increasing due to over-exposure to antibiotics[2] developed by various physiological and genetic mechanisms which includes multidrug efflux pumps, beta-lactamase production, outer membrane protein (porin) loss and target mutations. In the hospitals, MDR *P. aeruginosa* are common, often with simultaneous resistance to ciprofloxacin, imipenem, ceftazidime and piperacillin[3].

Aminoglycosides are major group of antibiotics with potential bacteriocidic effect for the treatment of *Pseudomonas* infections in hospitals. Aminoglycosides used alone or with its synergistic combination is used to treat various infections to overcome drug resistance, particularly in cystic fibrosis patients[4], and infective endocarditis[5]. In the face of systemic infection with shock/sepsis, antimicrobial therapy should consist of two intravenous (IV) antimicrobial agents, with one of these being an aminoglycoside[6] because it exhibits concentration-dependent bactericidal activity and produce prolonged post-antibiotic effects[7].

β-lactam antibiotic plus an aminoglycoside is the commonly used synergistic combinations for treatment of clinical infections. Other combinations are fluoroquinolone & aminoglycosides and

tetracycline & aminoglycosides. Clinical isolates show high percent of susceptibility to aminoglycosides than the other first-line antibiotics[8]. Despite high susceptibility to aminoglycosides in clinical isolates, *P. aeruginosa* exhibits physiological adaptations to the antibiotics which results in less response to its synergistic combinations.

P. aeruginosa thrives in the inhibitory concentration of antibiotics gradually and acquires adaptive resistance, which makes the treatment more complicated[9]. This resistance mechanism was characterized by modification of the cytoplasmic membrane, condensing of membrane proteins and reduction of phospholipid content[10] which reduces penetration of antibiotics into the plasma membrane. Studies shows that the adaptive resistance can also develop by up-regulation of efflux pumps especially, MexXY-OprM[11].

Among immunocompromised patients, *P. aeruginosa* are favored to adapt the administered antibiotics and enable better survival of the bacterial generation by emerge as physiologically resistant groups[12]. Adaptive resistance is developed due to rapid transcriptomic alteration in response to antibiotic[13]. Better understanding of the kinetics of adaptive resistance and transcriptomic changes during antibiotic exposure can develop scientific insight on the adaptive resistance mechanism in *P. aeruginosa*[13]. On this background our study was designed for better understanding of adaptive resistance in *P. aeruginosa* for gentamicin, amikacin and tobramycin which are commonly used aminoglycosides as first line antibiotics.

Materials and Methods

Broth Dilution Method: Minimum Inhibitory Concentration (MIC) of *P. aeruginosa ATCC* 27853 was determined by broth dilution method as per the Clinical and Laboratory Standards Institute (CLSI) guidelines. MIC assay was performed for gentamicin, amikacin and tobramycin (purchased from Sigma Aldrich) with log phase culture (5×10^8 CFU/mL) in Mueller Hinton Broth (MHB) using 96-well microtiter plate. The final optical density (OD) was determined in Epoch™ Microplate spectrophotometer at 600nm.

***In vitro* exposure of antibiotics to *P. aeruginosa*:** From the recorded MIC values, *P. aeruginosa* was inoculated in 10 mL of MHB in a Tarsons tube with corresponding antibiotic concentrations and cell density were adjusted to OD_{600} 0.26 (in log phase). The experiment set up was observed for 72 hours. *P. aeruginosa* was inoculated in antibiotic concentrations 0.5µg/mL, 1µg/mL & 1.5µg/mL for gentamicin & tobramycin. For amikacin, 1µg/mL, 2µg/mL & 3µg/mL antibiotic concentrations were taken. All antibiotic concentrations taken were based on MIC determined for *P. aeruginosa ATCC* 27853. The experimental condition was incubated at 37^{o}C with optimal shaking of 74rpm. At every 12 hours the turbidity was monitored by measuring OD_{600} in microtiter plate reader (Epoch). The results were graphically presented. The tube with growth were sub-cultured in nutrient agar and the colonies were conformed for *P.*

aeruginosa by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) identification from BioMérieux.

Transcriptomic analysis and protein functional regulations: The differential gene expression analysis was performed by exploring Microarray datasets published in Gene Expression Omnibus (GEO) NCBI, available as accessible series no. GSE9991 and GSE9989. Two datasets with different conditions, 1.) Tobramycin treated planktonic culture of *P. aeruginosa* (GSE9991) and 2.) Tobramycin treated *P. aeruginosa* biofilm (GSE9989) were analyzed. In GSE9991, the data of 4 samples was analyzed by case-control study. Planktonic culture of *P. aeruginosa* PA14 exposed to 5 µg/mL tobramycin for 30 minutes at 37^0C was the condition of the culture. Two samples GSM252561 and GSM252562 of tobramycin treated PA14 planktonic culture was taken as test, which was compared to 2 samples GSM252559 and GSM252560 of unexposed PA14 planktonic culture as control. GSE9989 consist of 6 samples, in which 3 samples GSM252496, GSM252501 and GSM252505 of unexposed *P. aeruginosa* biofilm were taken as control and 3 samples GSM252506, GSM252507 and GSM252508 of Tobramycin exposed *P. aeruginosa* biofilm was taken as test. Biofilms were grown on CFBE41o- cells in culture for 9 hours in MEM/0.4% arginine. Replicate samples were then incubated in the presence or absence of 500 µg/mL tobramycin for 30 minutes.

Differential expression of genes was compared between test and controls groups. The top 250 DEGs were included. The raw data were normalized by log2 transformation in NetworkAnalyst 3.0 (https://www.networkanalyst.ca/) and significant DEGs with p-value of ≤0.05 were taken for functional analysis. Limma statistics was employed for the microarray datasets. Gene Ontology (GO) and pathway enrichment analysis was performed in PANTHER Classification System (http://pantherdb.org/) and DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/). The targeted genes were annotated functionally using Pseudomonas Genome DB (http://pseudomonas.com/) and PseudoCyc (http://www.pseudomonas.com:1555/). Protein and molecular interactions of DEGs were determined.

Results

MICs of gentamicin, amikacin and tobramycin for *P. aeruginosa* (ATCC 27853) were 0.5 µg/mL, 1.5 µg/mL and 0.5 µg/mL.

The initial OD_{600} of the bacterial culture was ~0.26. After 12 hours the OD fell to ~0.13. In 0.5 µg/mL & 1 µg/mL of gentamicin (Figure 1) and 1 µg/mL & 2 µg/mL of amikacin (Figure 3) tubes OD_{600} increased after 24 hours.

The OD_{600} after 48 hours, reached the initial OD ~0.26 for gentamicin and amikacin. The cells attained log phase and the growth were exponential in the next following hours (Figures 1 and 3). In tobramycin and higher concentrations of gentamicin and amikacin, growth had declined,

and no growth were observed till 72 hours (Figures 1 and 2). The growth in 1 µg/mL & 2 µg/mL of amikacin and 0.5 µg/mL & 1 µg/mL of gentamicin tubes were confirmed as *Pseudomonas aeruginosa* by MALDI-TOF identification system.

In GSE9991 among 125 of DEGs 53 genes were upregulated and 72 were downregulated. In GSE9989, a total of 307 genes were differentially expressed in which 52 genes were upregulated and 255 genes were downregulated. Variations in expression of DEGs were represented in volcano plot (Figure 4). Functionally studied DEGs included in the study were 17 from GSE9991 and 22 from GSE9989 (Table 1&2).

Gene Ontology: The DEGs in the study were involved in molecular functions like catalytic activity, binding, transcription regulation and transporter activity. Biological process includes biological regulations, cellular process, localization, metabolic process. Protein classes includes Hydrolase, transcription factors, transferase, and transporter proteins.

Functional enrichment analysis: The DEGs included in the study were involve in Type II Secretary Proteins, Type II Transport Domains, Alginate Biosynthesis, Repressors for alginate synthesis, Transcriptional repressor of SOS response, RNA Methyltransferases, 16S rRNA 7-methylguanosine methyltransferase, Translocation protein in type III secretion and Type III Export protein. Other functional annotation clusters (Table 3) and enriched groups (Figure 5) from DAVID Bioinformatics Resources 6.8 were represented in tables and graph.

Discussion

The experimental setup of *in vitro* exposure of antibiotic to *P. aeruginosa* reflects the antibiotic tolerance in chronic infections, where the response to antimicrobials diminishes due to development of adaptive resistance. Among amikacin, gentamicin and tobramycin evaluated for antibiotic tolerance in *P. aeruginosa*, tobramycin was observed to be more effective at its MIC by suppressing the antibiotic tolerance mechanism. Functional annotations of DEGs in Microarray datasets mimicking our experimental condition exposed the possible effectiveness of tobramycin in antibiotic tolerance.

Among the functional enrichments observed in GSE9991 and GSE9989 datasets, the following enrichments play a significant role in antibiotic tolerance and virulence of *P. aeruginosa.* Methylation of 16s rRNA by Methyltransferase[14] is a common mechanism of resistance to aminoglycosides leading to loss of affinity of the drug to the target. In GSE9991, **RNA Methyltransferases:** PA0419, a Ribosomal RNA small subunit methyltransferase E which methylates 16s rRNA bases in 30s subunit was downregulated than the control. PA0017 and PA3680 genes of class B and J methyltransferase also lags expression. In GSE9989, **16S rRNA 7-methylguanosine methyltransferase:** gidB belongs to Methyltransferase G, which involves in methylation of 7^{th} nucleotide guanosine, confers resistance to aminoglycoside by decreasing the binding affinity to its target[15]. Log Fc -3.32 times low expression of gidB were observed.

Following gene expression profile of Methyltransferases suggest low incidence of resistance development during tobramycin exposure.

In chronic infections caused by *P. aeruginosa*, biofilm formation is reported[16], which confers additional resistance from host defenses and antibiotics[17]. Some antibiotics are involved in the up-regulation of genes that are responsible for induction of alginate production of a mucopolysaccharide, with an altered LPS and lipid A, which results in reduced antigen presentation to the immune system[18]. In GSE9991, **Alginate Biosynthesis:** algL a lyase precursor, participating in catabolic activity of alginic acid leading to deconstruction[19] was expressed high in log fold of 0.64. algR is regulatory protein of alginate biosynthesis genes[20] declined in expression by Log Fc -0.35. pslF was low expressed by log -1.03 which is one among glycosyltransferase family, involved in extracellular polysaccharide biosynthetic pathway[21]. In GSE9989, **Repressors for alginate synthesis:** mucA is an anti-sigma factor and mucB is a negative regulator of algU, which is sigma factor of alginate synthesis[22]. High expression of these genes leads to low alginate production. The following transcriptional changes observed would possibly affect the alginate production to significant level in the presence of tobramycin treatment.

Some of the DEGs were linked to suppress proteins of type II secretion system and type III secretion system, which participates in virulence activity of *P. aeruginosa*. In GSE9991, **Type II**

Secretary Proteins: PA2677, PA2672 and PA0687 participates in catalytic and transporter protein activity was down-regulated. Other transporter domains xcpR, xcpU, xcpV, xcpX, xcpY, xcpZ, tadB and tadD were also under expressed[23], affecting the T2SS. In GSE9989, **Type II Transport Domains:** xcpQ, xcpS, xcpT, xcpU, xcpV, xcpW, xcpX and xcpY which involves in effluxion the toxin from xcpR, a cytosolic domain was down-regulated, affecting the export of T2SS[24]. **Translocation protein in type III secretion:** pscQ, pscP and pscR are translocation protein of type III secretion system, which translocate the toxin across the host cell cytoplasmic membrane. Downregulation of pscQ negatively impact the toxin delivery to cytosol of host cell. **Type III Export protein:** pscE, pscF, pscG, pscH, pscI, pscJ, pscK are the export proteins of type III secretion system present in cytoplasmic membrane which transfers the toxin from cytosolic domain to MS ring of basal body[24]. Low expression of all these proteins, prevents toxins to reach filament, from where it is translocated into host cell. These transcriptomic changes suggest the suppression of toxin port systems (T2SS and T3SS) which may lead to decrease in the virulence of the organism during tobramycin treatment.

Overuse of antibiotics hiked up transcriptional regulation, favoring adaptive resistance leading to decline of *in vivo* activity over time[25]. The study suggests the use of tobramycin for treatment of chronic *pseudomonas* infection, as tobramycin failed to favor adaptive resistance in *P. aeruginosa* and positive transcriptomic regulations for antibiotic response. Tobramycin is restricted for systemic use due rise of creatinine level during initial days of therapy. Intensity of

nephrotoxicity between aminoglycosides is poorly understood. Recent cohort study on, nephrotoxicity suggested that, tobramycin has less comparative toxicity over Gentamicin[26]. Considering the *in vivo* drug response and predisposing factors, tobramycin one among the option might enable a better treatment alternative from the current drug combinations.

Transcriptional alterations in microbes are dynamic event triggered by environmental changes, which out-turns the increase in adaptive resistance. Although, adaptive resistance involves in hike of baseline MIC of the bacteria over time, genetic resistance is a function of time. It takes several generations of the bacteria to achieve genotypic resistance. The methodology of constantly switching antibiotics through *in vitro* exposure of antibiotics would enable us in deciphering which clinical isolates would be physiologically resistant, leading to alternative aminoglycoside treatment for combating chronic infections.

Figures and Tables

Genes	Log Fc
PA2677	-2.21809
PA0687	-1.44816
PA2672	-1.23836
xcpR	-0.28436
xcpU	-0.50274
xcpV	-0.19849
xcpY	-0.22214
xcpZ	-0.21626
tadB	-0.16973
tadD	-0.25359
algR	-0.35921
algL	0.640009
pslF	-1.03452
PA1839	2.200892
PA0419	-0.92439
PA0017	-0.33728
PA3680	-0.34165

Table 1. Test-control analysis of DEGs in Tobramycin treated planktonic *P. aeruginosa* (GSE9991)

Genes	Log Fc
mucA	4.094536
mucB	1.155318
lexA	3.42962
gidB	-3.31909
pscQ	-2.35821
pscP	-3.64432
pscR	-3.11905
pscG	-1.75324
pscH	-1.52118
pscE	-3.84799
pscF	-1.3676
pscI	-2.27261
pscJ	-1.43567
pscK	-1.6767
xcpQ	-2.31778
xcpS	-1.40397
xcpT	-0.72049
xcpU	-1.33575
xcpV	-3.11543
xcpW	-0.69783
xcpX	-0.42221
xcpY	-0.5616

Table 2. Test-control analysis of DEGs in Tobramycin treated *P. aeruginosa* biofilm (GSE9989)

Clusters	% Enriched	P-Value	Fold Enrichment	FDR
Cluster 1				
Bacterial secretion system	50.00	9.75E-21	16.68	2.93E-20
Protein secretion by type II secretion system	30.56	4.38E-15	43.27	8.77E-14
Protein transporter activity	27.78	1.65E-14	52.90	2.65E-13
Protein transport	27.78	4.30E-12	33.91	1.89E-10
Type II protein secretion system complex	22.22	1.21E-10	42.90	1.33E-09
Transport	27.78	2.72E-04	4.31	0.003997
			Cluster 1 Enrichment Score: 12.16	
Cluster 2				
Type II protein secretion system complex	22.22	1.21E-10	42.90	1.33E-09
Prokaryotic N-terminal methylation site	13.89	5.95E-06	38.23	4.58E-04
Methylation	11.11	1.52E-04	35.91	0.003334
			Cluster 2 Enrichment Score: 6.32	
Cluster 3				
Methyltransferase	13.89	7.52E-04	11.56	0.007267
rRNA processing	11.11	9.42E-04	19.69	0.007267
S-adenosyl-L-methionine	13.89	9.91E-04	10.75	0.007267
rRNA base methylation	8.33	0.002519	36.88	0.010077
Cytoplasm	22.22	0.009652	3.16	0.047188
Transferase	13.89	0.396132	1.53	0.977778
Cytoplasm	19.44	0.486036	1.22	1
			Cluster 3 Enrichment Score: 2.06	

Table 3: Functional annotations of the DEGs clustered into various groups with significant enrichment score. Derived from the source DAVID Bioinformatics Resources 6.8.

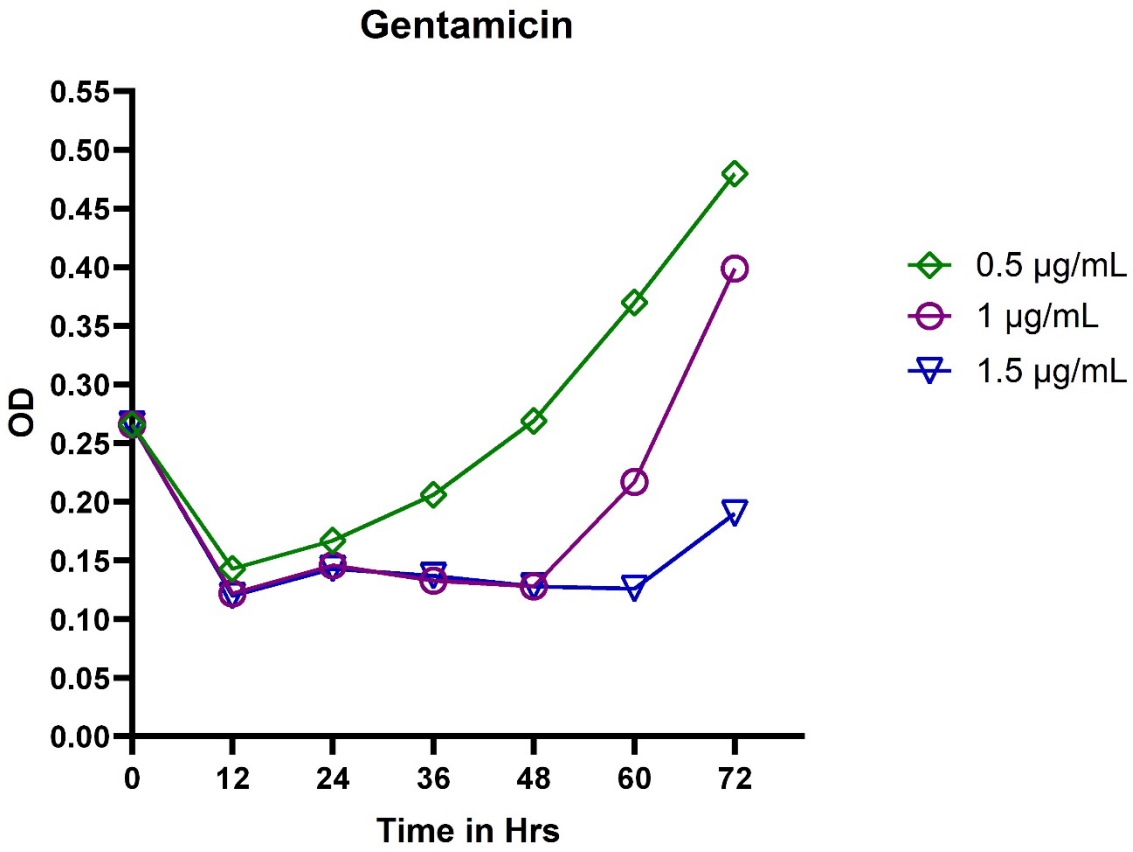

Figure 1: *In vitro* exposure of 0.5µg/mL, 1µg/mL, and 1.5µg/mL MIC concentrations of gentamicin to *P. aeruginosa*. The OD values depict the kinetics of adaptive resistance in the cell.

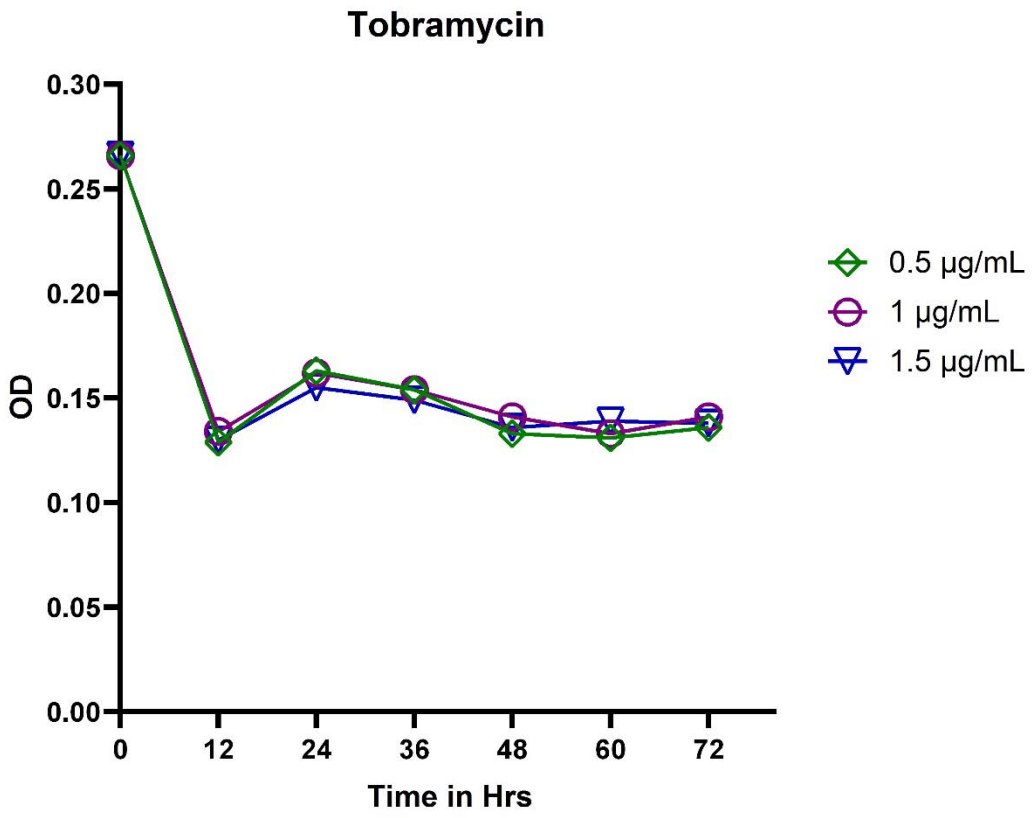

Figure 2: *In vitro* exposure of 0.5 µg/mL, 1 µg/mL, and 1.5 µg/mL MIC concentrations of tobramycin to *P. aeruginosa*. The OD values depict the kinetics of adaptive resistance in the cell.

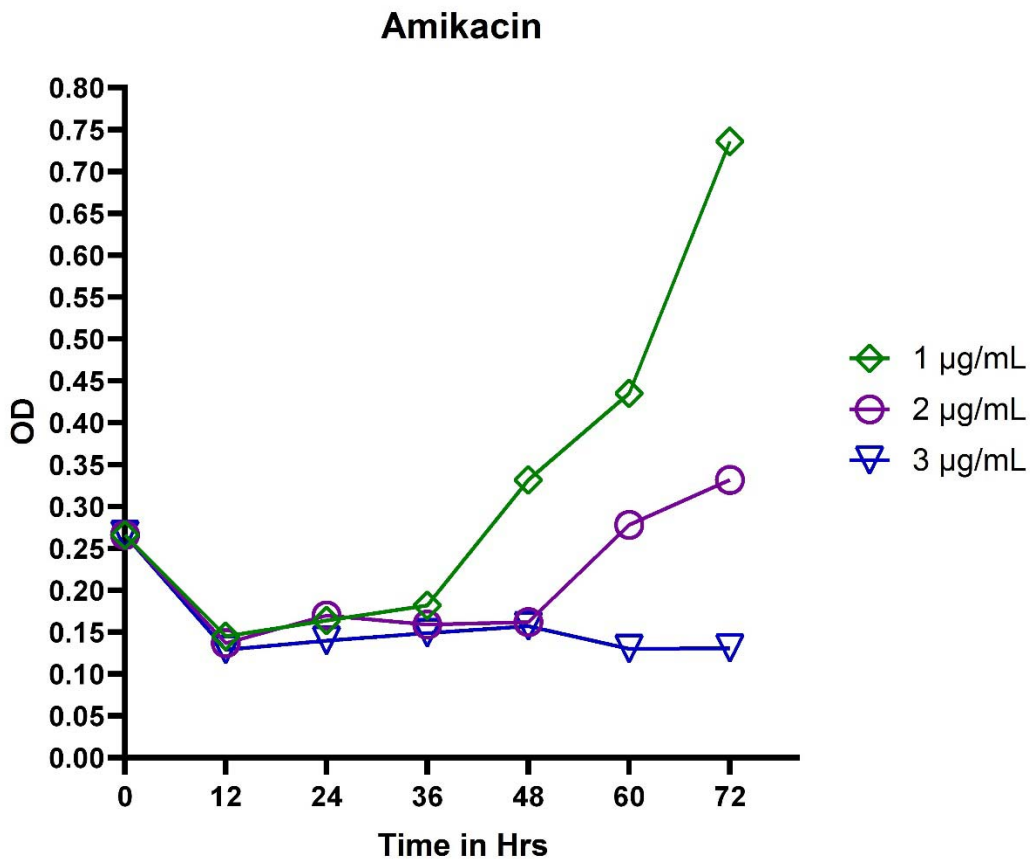

Figure 3: *In vitro* exposure of 1 µg/mL, 2 µg/mL, and 3 µg/mL MIC concentrations of amikacin to *P. aeruginosa*. The OD values depict the kinetics of adaptive resistance in the cell.

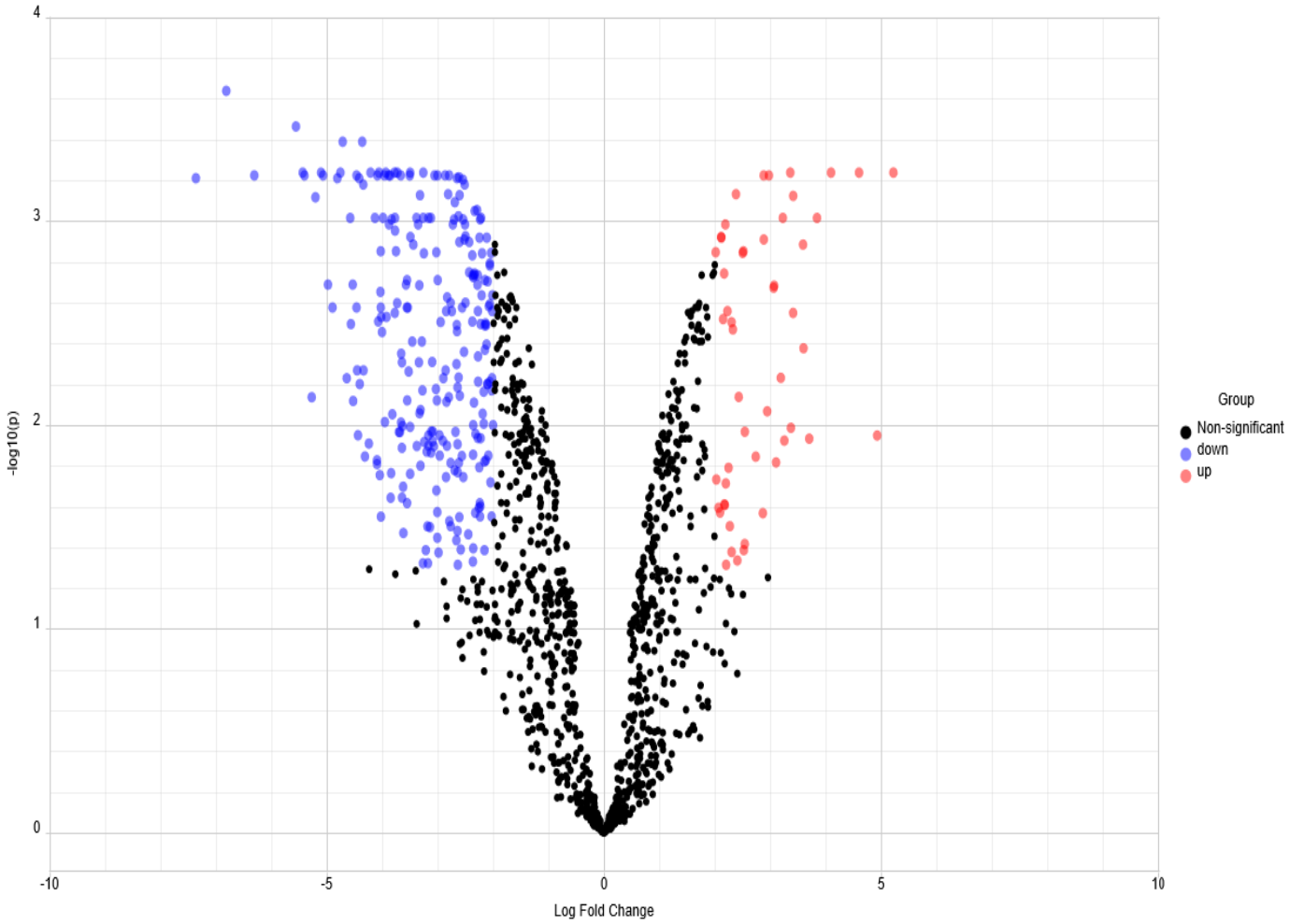

Figure 4: Distribution of DEGs in *P. aeruginosa* exposed to tobramycin using volcano plot, built form the source NetworkAnalyst 3.0

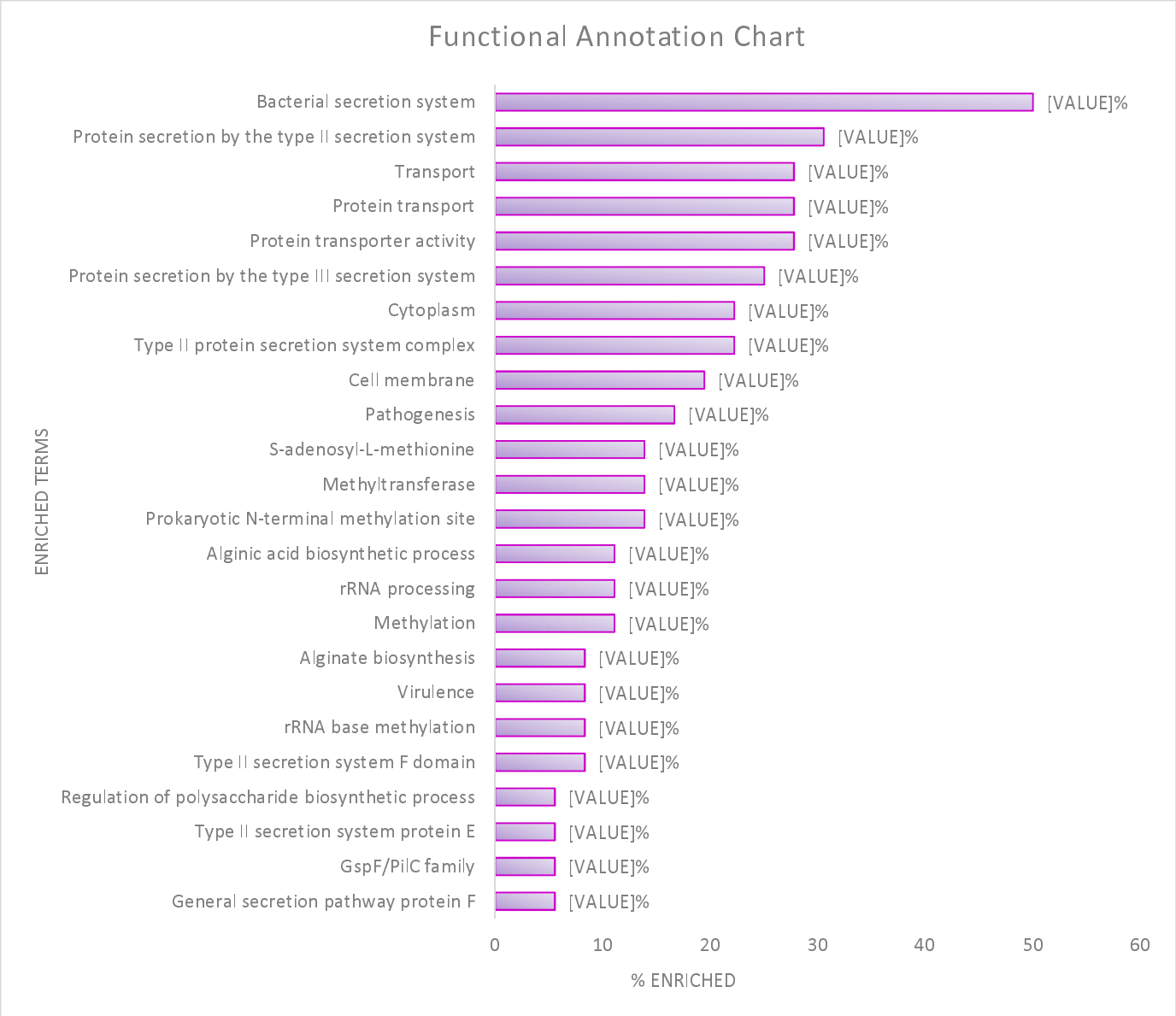

Figure 5: Functional enrichment graph of DEGs. Enriched terms were taken from DAVID Bioinformatics Resources 6.8

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Author Contributions

All the authors had contributed for the above study in several ways.

	Contributor 1	Contributor 2	Contributor 3	Contributor 4
Concepts	✓			
Design	✓	✓		
Definition of intellectual content			✓	✓
Literature search	✓	✓		
Experimental studies	✓	✓		
Data acquisition	✓			
Data analysis	✓			
Statistical analysis	✓			
Manuscript preparation	✓	✓		
Manuscript editing	✓	✓	✓	✓
Manuscript review		✓	✓	✓
Guarantor			✓	

Conflict of Interest

The authors of the study have no conflict of interest.

Ethical Statement

The study did not involve any human or animal experimentations.

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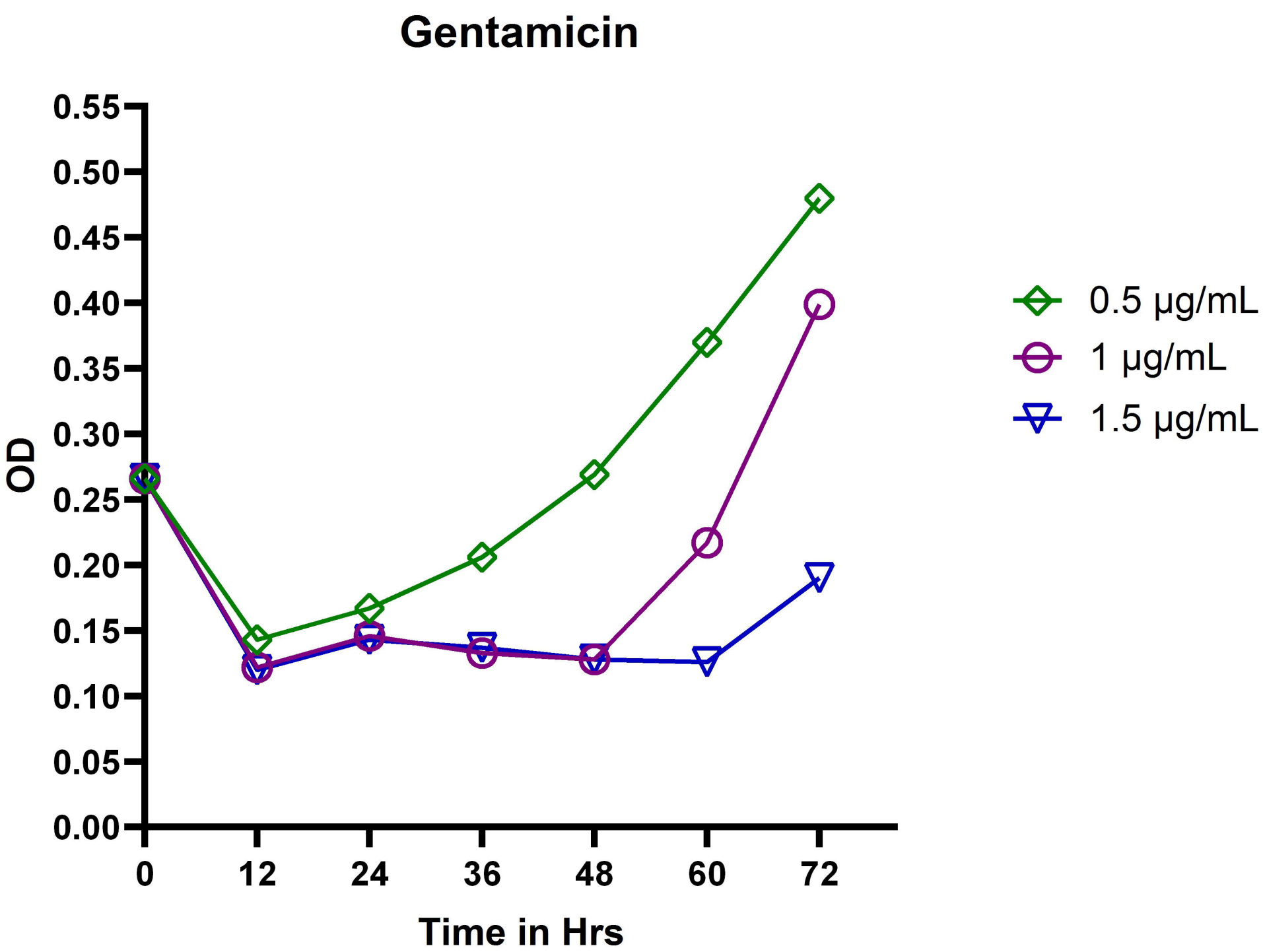
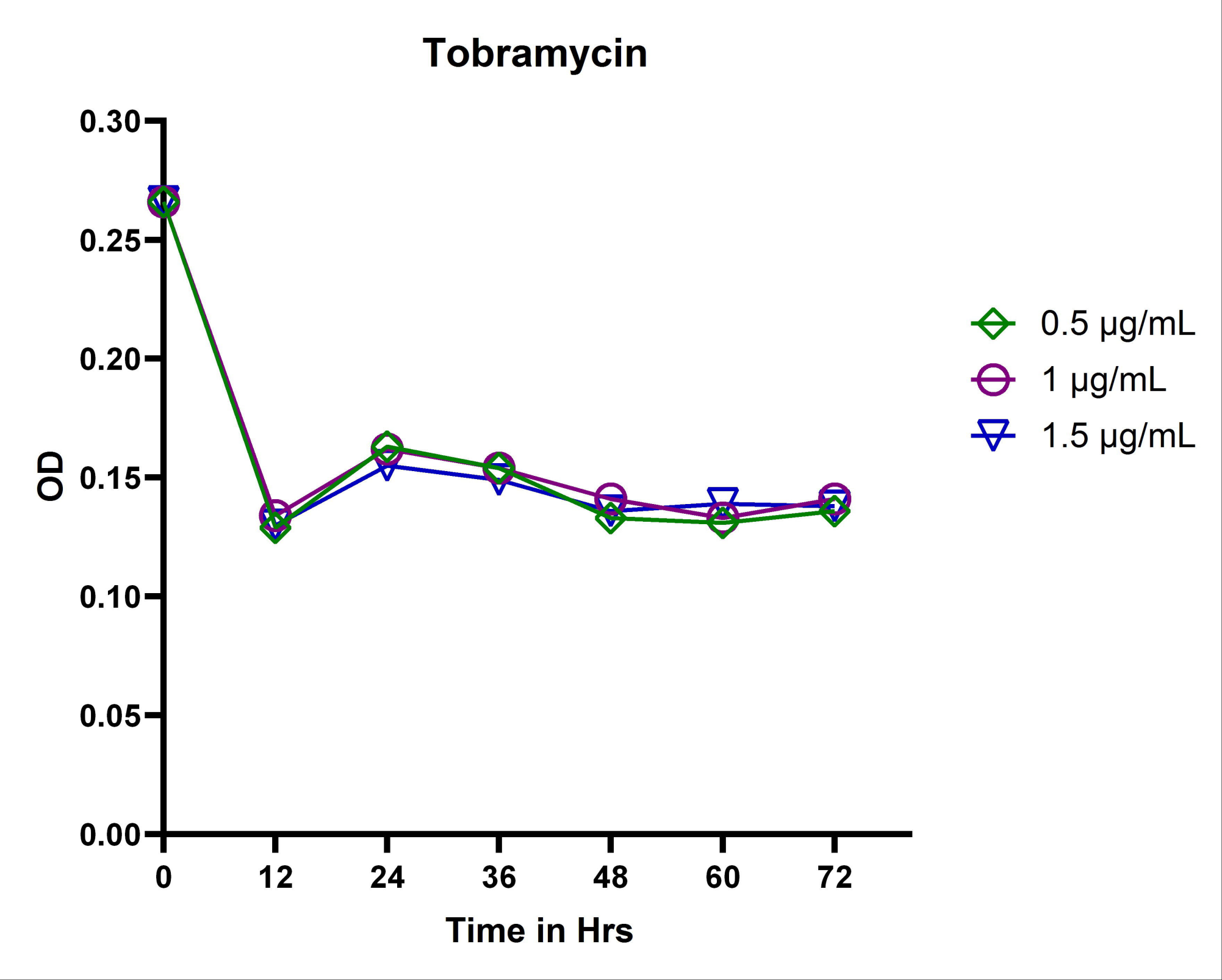
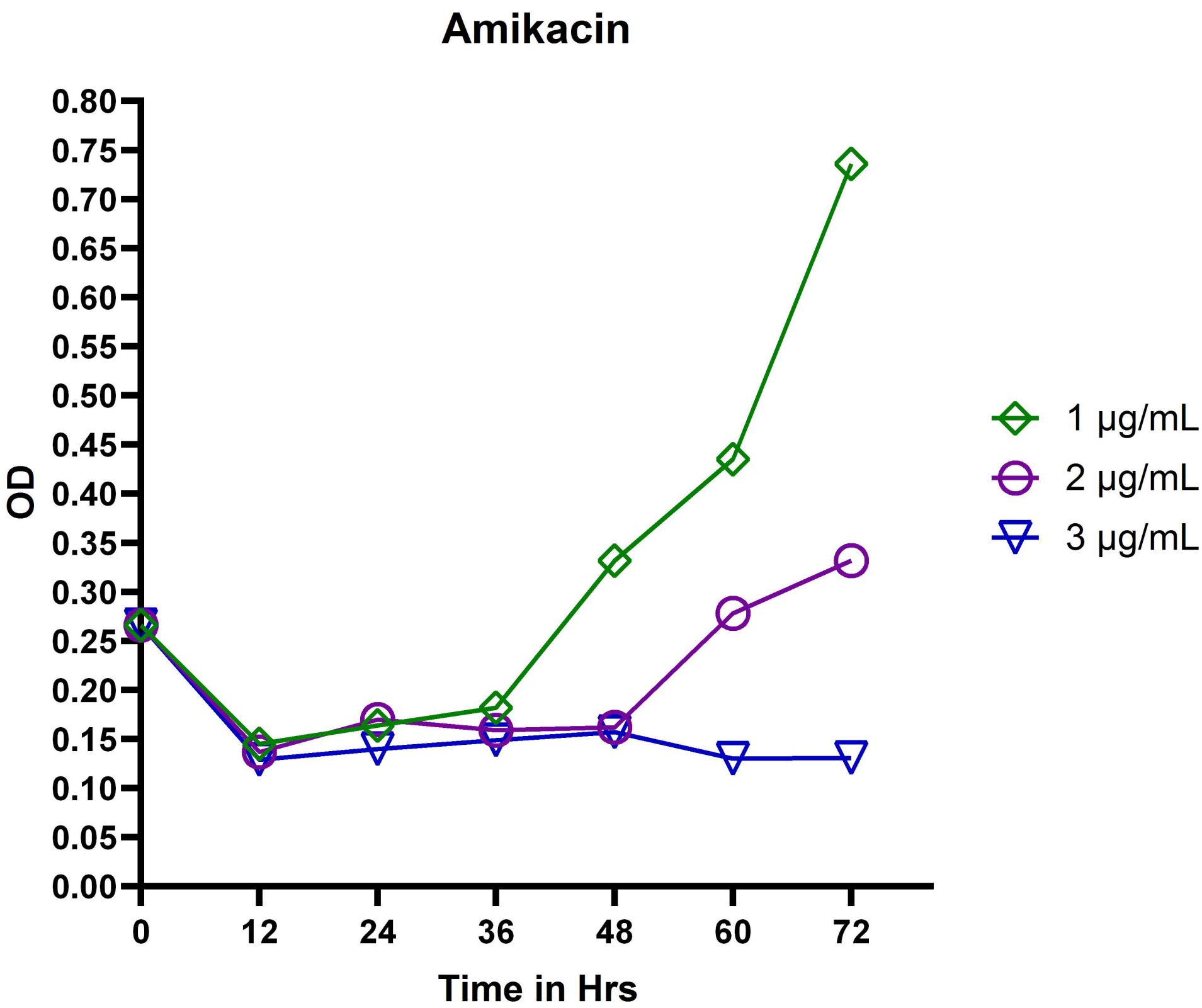
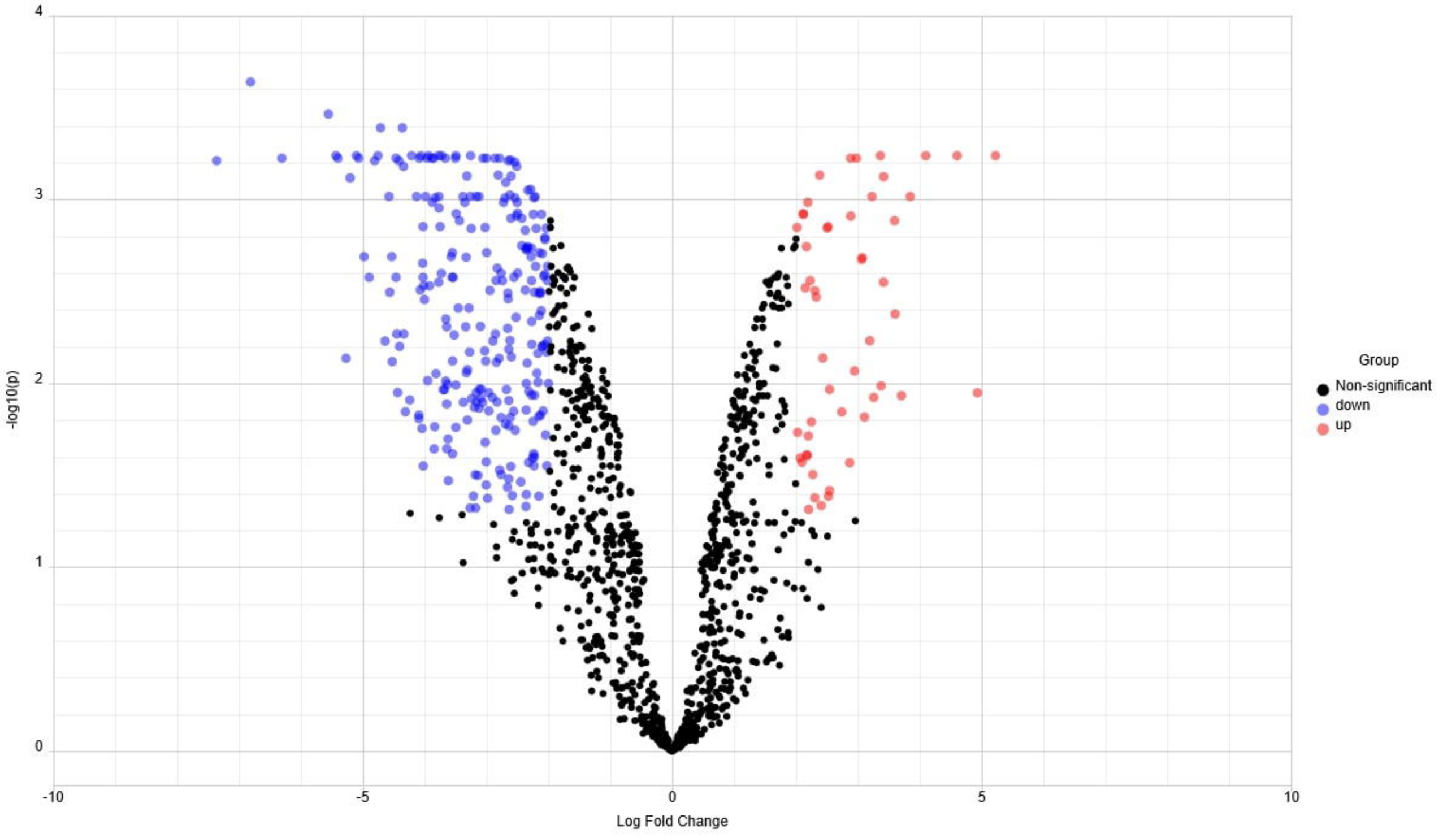
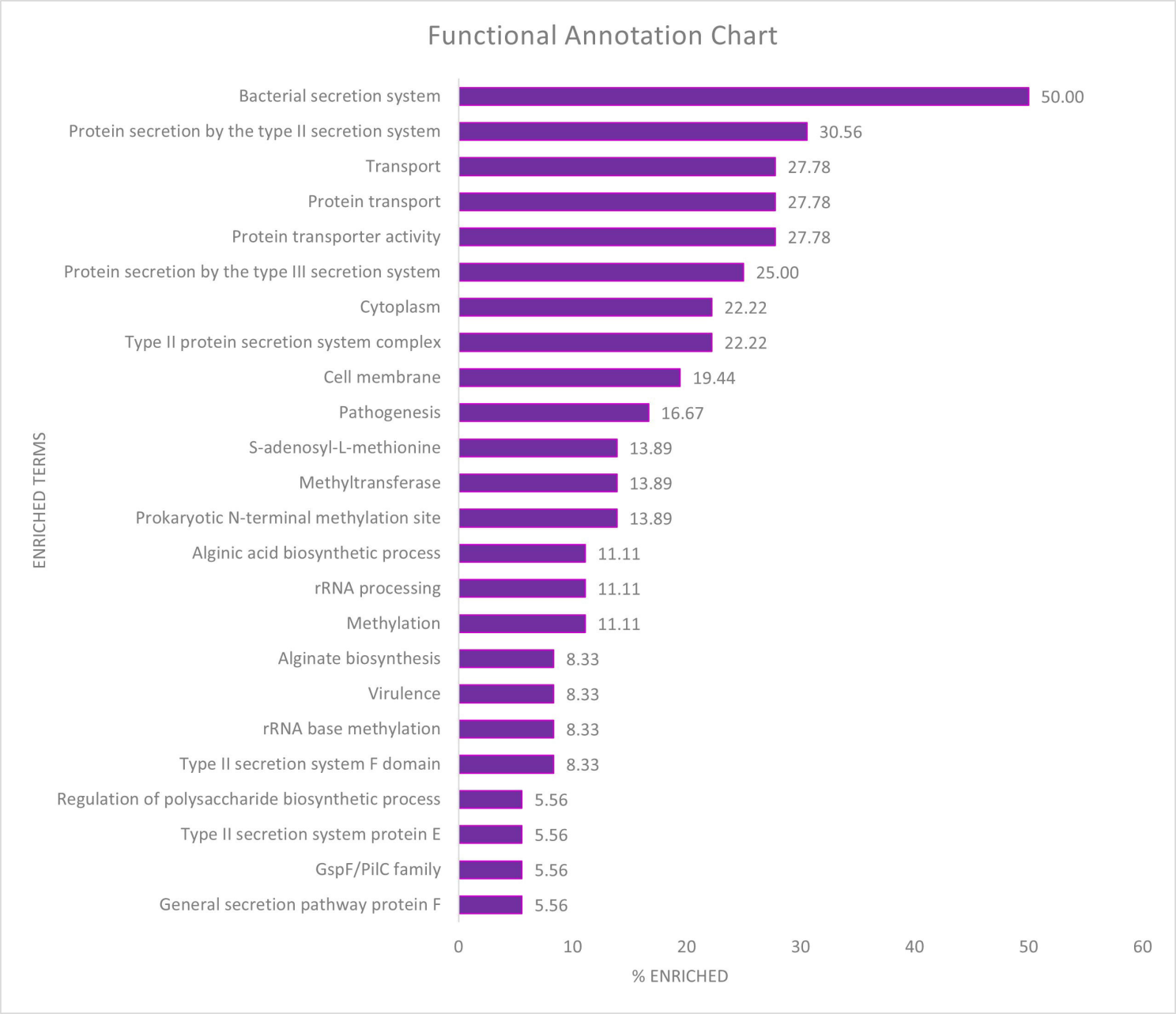

Gentamicin
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD
0
12
24
36
48
60
72
Time in Hrs
0.5 µg/mL
1 µg/mL
1.5 µg/mL


Tobramycin
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD
0
12
24
36
48
60
72
Time in Hrs
0.5 µg/mL
1 µg/mL
1.5 µg/mL


Amikacin
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD
0
12
24
36
48
60
72
Time in Hrs
1 µg/mL
2 µg/mL
3 µg/mL


4
3
2
1
0
-log10(p)
-10
-5
0
5
10
Log Fold Change
Group
Non-significant
down
up


Functional Annotation Chart
Bacterial secretion system
Protein secretion by the type II secretion system
Transport
Protein transport
Protein transporter activity
Protein secretion by the type III secretion system
Cytoplasm
Type II protein secretion system complex
Cell membrane
Pathogenesis
S-adenosyl-L-methionine
Methyltransferase
Prokaryotic N-terminal methylation site
Alginic acid biosynthetic process
rRNA processing
Methylation
Alginate biosynthesis
Virulence
rRNA base methylation
Type II secretion system F domain
Regulation of polysaccharide biosynthetic process
Type II secretion system protein E
GspF/PilC family
General secretion pathway protein F
50.00
30.56
27.78
27.78
27.78
25.00
22.22
22.22
19.44
16.67
13.89
13.89
13.89
11.11
11.11
11.11
8.33
8.33
8.33
8.33
5.56
5.56
5.56
5.56
ENRICHED TERMS
0
10
20
30
40
50
60
% ENRICHED