In silico analysis of tetracycline resistance in *Acinetobacter baumannii* and its protein diversity

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Summary

Resistance towards antimicrobial drugs have posed a serious threat to mankind. *Acinetobacter baumannii*, a nosocomial pathogen have been spotted to hold a bunch of drug resistant genes which serves as an armour to escape from antimicrobial drugs commonly used for treatment. The present study describes the tetracycline resistant phenotype encountered in *A. baumannii* and its evolutionary relatedness with other bacterial species.

A small subset of genome sequence from *Acinetobacter sp.* was probed to assess the frequency of tetracycline-resistant phenotype by in *silico* amplification. Further, the protein sequences of PCR-positive genes were subjected to multiple sequence alignment, to deduce the phylogenetic relationship across several other bacterial species.

The *tet(B)* gene was found to occur in a greater frequency (42.1%) followed by *tet(A)* (10.5%),

among eleven different *tet* genes analysed. The *tet(A)* gene was found in strains harbouring plasmids and *tet(B)* was observed in plasmid-bearing strains as well as one strain devoid of plasmids. Multiple sequence alignment and phylogenetic analysis of the protein sequences revealed an evolutionary closeness of *tet*(A) and *tet*(B) from *A. baumanni*i to *E. coli* and *P. stuartii* respectively. A basic understanding on the molecular mechanisms underlying drug-resistant phenotype would eventually lead to the assessment of new drug targets. An evolutionary analysis of proteins encoding drug resistance might be useful to target closely related pathogens which enable the discovery of broad spectrum drugs targeting multiple pathogens.

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A.baumannii, tetracycline, PCR, phylogenetic analysis

Introduction

Acinetobacter baumannii, is one of the critically targeted red-list pathogen among the "ESKAPE" group of multidrug-resistant (MDR) organisms. The pathogen is charged with cassettes of genes encoding drug resistance which may pose a serious threat to mankind. Infamously known as the nosocomial pathogen, *A. baumannii* has been isolated from several other environmental niches which provide evidence on rapid dissemination and evolution of the species.[1-3] Resurgence of multidrug-resistant strains is the greatest challenge of this century. With limited number of antibiotics and most of them turning futile against MDR strains, newer drugs are to be designed to combat the threat posed by these pathogens. The usage of tetracycline for the treatment of multi-drug resistant *A. baumannii* has shown promising results in a cohort study conducted by Falagas[4] *et al.,* 2015. Such studies along with a basic understanding of the drug-resistant phenotypes and the molecular mechanisms underlying it would aid in finding new avenues to battle against these drug-resistant pathogens.

Numerous mechanisms on drug resistance[5-7] and virulence factors including biofilm formation and quorum sensing[8] have been identified and reported in this pathogen. Among all the pathways, drug efflux pumps play a major role is establishing the drug-resistant phenotype. Resistance to tetracycline is mediated either through efflux pumps or ribosomal protection system. These efflux pumps belong to a major facilitator superfamily (MFS) and are more specific to the drug. Several genes responsible for tet-resistant phenotype have been analyzed in *A. baumannii*. Among them *tet(A)* gene was first reported to be present in a Tn1721-like transposon, which can be transferred to other bacterial species via horizontal transfer.[9] *Tet(A)* gene was found to confer resistance to tetracycline and *tet(B)* conferred resistance to both tetracycline and minocycline.[10] A recent study substantiated the promising activity of cycline group of antibiotics against MDR strains of *A. baumannii.*[11] In this context, the present study was designed to analyze the most common tetracycline-resistant phenotype prevailing in *A. baumannii*. The study also adds up on the diversity of proteins encoded to exhibit the phenotype and compare the same with other important clinical bacterial species.

Materials and Methodology

Strains used in the study: A subset of eighteen whole genome sequences of Acinetobacter sp. was used in the present study (Table 1). Information about the genome including the plasmids and number of genes present in the strains was retrieved from National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/genome/genomes/2516).

PCR amplification: About 11 genes encoding tetracycline resistance were opted for the study (table 2). *In silico* amplification of *tet* genes against 18 genomes were performed using *in silico* simulation tools for molecular biology experiments.[12-13] Primers for the amplification of *tet* genes were selected from the literature source.[14-15]

Protein sequences: Protein sequences for the genes which returned positive result in PCR were retrieved from NCBI database. These sequences were subjected to BLAST analysis. Non-redundant and clinically associated genus of bacteria showing 100% query coverage with 95-99% identity was selected for multiple

Table 1 Genome sequences of *Acinetobacter* sp. used for *in silico* amplification in the present study

Ref Seq	Species of *Acinetobacter*	Associated infection	Origin of genomes	Genome size (Mb)	Genes	Plasmids
NC_014259	*Acinetobacter* spp. *DR1*	-	Soil	4.12	3999	-
NC_005966	*Acinetobacter* spp. *ADP1*	-	Soil	3.59	3359	*
NC_017387	*Acinetobacter baumannii TCDC-AB0715*	Bacteremia	Taiwan	4.13	3956	p1ABTCDC0715 p2ABTCDC0715
NC_017162	*Acinetobacter baumannii 1656-2*	Nosocomial infection	South Korea	3.9	3922	ABKp1 ABKp2
NC_010611	*Acinetobacter baumannii ACICU*	Nosocomial Pneumonia	Rome	3.9	3839	pACICU1 pACICU2
NC_017847	*Acinetobacter baumannii MDR-TJ*	Hospital strain	China	3.96	4071	pABTJ1 pABTJ2
NC_021726	*Acinetobacter baumannii BJAB07104*	Bacteremia	China	3.95	3910	p1BJAB07104 p2BJAB07104
NC_017171	*Acinetobacter baumannii MDR-ZJ06*	Bacteremia	China	3.99	3882	pMDR-ZJ06
NC_021729	*Acinetobacter baumannii BJAB0868*	Ascites isolate	China	3.90	3861	p1BJAB0868 p2BJAB0868
NC_018706	*Acinetobacter baumannii TYTH-1*	Bacteremia	Taiwan	3.95	3795	-
NC_021733	*Acinetobacter baumannii BJAB0715*	Spinal fluid	China	4.00	3918	pBJAB0715
NC_009085	*Acinetobacter baumannii ATCC 17978*	Wild-type	ATCC	3.97	3905	pAB1 pAB2
NC_010410	*Acinetobacter baumannii AYE*	Nosocomial infections	France	3.93	3900	p1ABAYE p2ABAYE
NC_011595	*Acinetobacter baumannii AB307-0294*	Bacteremia	USA	3.76	3544	-
NC_011586	*Acinetobacter baumannii AB0057*	Bacteremia	USA	4.05	3971	pAB0057
NC_023028	*Acinetobacter baumannii ZW85-1*	Diarrhea	China	3.76	3712	ZW85-1p pAbNDM-1
NC_020547	*Acinetobacter baumannii D1279779*	Bacteremia	Australia	3.70	3564	pD1279779
NC_016603	*Acinetobacter calcoaceticus PHEA-2*	-	Industry waste water	3.86	3674	-

*Data not available

Table 2

List of primers used to amplify Tet efflux pumps

Gene	Primers (5'-3')	Amplicon size (bp)
Tet A	F-GCGCGATCTGGTTCACTCG R- AGTCGACAGYRGCGCCGGC	164
Tet B	F-TACGTGAATTTATTGCTTCGG R-ATACAGCATCCAAAGCGCAC	206
Tet C	F-GCGGGATATCGTCCATTCCG R-GCGTAGAGGATCCACAGGACG	207
Tet D	F-GGAATATCTCCCGGAAGCGG R-CACATTGGACAGTGCCAGCAG	187
Tet E	F-GTTATTACGGGAGTTTGTTGG R-AATACAACACCCACACTACGC	199
Tet G	F-GCAGAGCAGGTCGCTGG R-CCYGCAAGAGAAGCCAGAAG	134
Tet H	F-CAGTGAAAATTCACTGGCAAC R-ATCCAAAGTGTGGTTGAGAAT	185
Tet J	F-CGAAAACAGACTCGCCAATC R-TCCATAATGAGGTGGGGC	184
Tet Y	F-ATTTGTACCGGCAGAGCAAAC R-GGCGCTGCCGCCATTATGC	181
Tet Z	F-CCTTCTCGACCAGGTCGG R-ACCCACAGCGTGTCCGTC	204
Tet 30	F-CATCTTGGTCGAGGTGACTGG R-ACGAGCACCCAGCCGAGC	210
Tet 39	F- CTCCTTCTCTATTGTGGCTA R- CACTAATACCTCTGGACATCA	701

sequence alignment (MSA). The subcellular localization of the protein was analysed using CELLO v2.5.[16-17]

Multiple sequence alignment (MSA): Pairwise alignment of the protein sequences retrieved from NCBI database was performed using Clustal W (https://www.genome.jp/tools-bin/clustalw). MSA was performed using MEGA 7.0 (Molecular Evolutionary Genetics Analysis) software.[18] The programme MUSCLE was used to align multiple sequences of proteins followed by which a phylogenetic tree was reconstructed by applying maximum likelihood statistical and bootstrap method with 500 replications[19-21] (Figure 1a and 1b).

Results

In silico analysis of the *Acinetobacter* spp. selected for the present study revealed the presence of *tet(A)* and *tet(B)* in 10.5% and 42.1% of the strains. The majority of strains harboured plasmids (72.2%), which is responsible for the horizontal transfer of drug-resistant gene cassettes among other pathogens. Interestingly, *tet(A)* was found in strains with plasmids and *tet(B)*, although with a close connection with plasmid bearing strains, was also found in a strain devoid of plasmids. There were no evidence of co-occurrence of both *tet(A)* and *tet(B)* gene in any of the strains analysed. The subcellular localization of *tet(A)* and *tet(B)* protein was found to be the inner membrane with a reliability score of 4.960 and 4.993 respectively. Pairwise sequence alignment of Tet(A) protein revealed conserved regions among several species of bacteria with a score ranging from 98.2-99.7. Similarly, the pairwise alignment of tet(B) protein also produced scores in the range of 98-99.7.

Discussion

The outer membrane of Gram-negative bacteria restricts the amount of antimicrobials entering the cell, parallel efflux pumps actively exports several specific classes of antimicrobials out of the bacteria.[22] The efflux system has been classified into six families, such as (a) ATP binding cassette, (b) major facilitator superfamily (MFS), the resistance modulation division family, the MATE (multi-drug and toxic compound extrusion) family, drug or metabolite transporter (DMT) superfa-

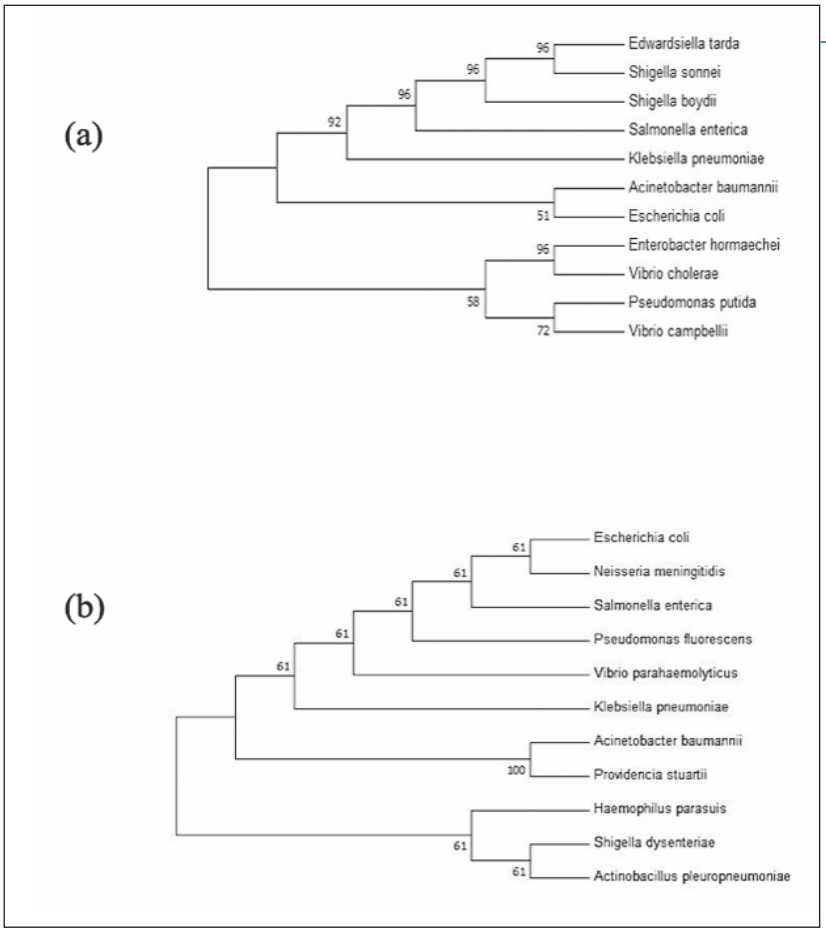

Figure 1

(a) Phylogenetic tree showing diversity in (a) Tet-A and (b) Tet-B protein of *Acinetobacter baumannii*

mily and SMR (small multi-drug resistance) family. Major efflux pumps encoding genes associated with tetracycline resistance in *A. baumanni* are *tet(A)* and *tet(B)*. An epidemiological study carried out by Marti and collegues[10] reported a frequency of 66% for *tet(B)* and 13.6% for *tet(A)* gene. This report was in concordance with the findings of the present study.

A recent clinical study reports the efficacy of tigecycline against MDR *A. baumannii*, where all the strains tested sensitive for the drug. The frequency of resistance exhibited by the pathogen against tetracycline, minocycline and doxycycline was 89%, 35% and 25% respectively. Molecular analysis revealed a frequency of 2.2% for *tet(A)* gene and 87.6% for *tet(B)* gene. Interestingly, co-occurrence of *tet* genes were observed in about 1.1% of the isolates.[11] A similar study conducted in Iranian population revealed high frequency of *tet(B)* gene (70.4%) in burn-associated cases.[23] An interesting fact about *tet(B)* gene and its association with ISCR elements conferring the ability to transpose into other species was elucidated by Vilacoba[24] *et al.* Several epidemiological studies substantiates the prevalence of *tet(A)* and *tet(B)* genes in *A. baumannii,* with *tet(B)* scoring high in terms of frequency. An Algerian study also confirms the frequency of *tet(B)* to be 65.92% in the strains analysed.[25] The authors also report the frequency of *tet(B)* gene to be the highest when compared to *tet(A)*[26] employing *in silico* techniques. Increasing number of drug resistance encoding genes in *A. baumannii*[26,27] and their dissemination[34,35] in different geographical locations have put immense pressure on the surveillance mechanisms operating in different parts of the world.

The present study reveals the most common tetracycline resistant genes which may be prevalent in the community. The protein analysis of *tet(A)* and *tet(B)* provides an insight into the diversity and evolutionary relationship of the proteins among different bacterial pathogens. Although there are certain limitations of the study such as, (a) the strains selected for the study may not be a representative population of all the strains present globally, (b) since the sample size is small, further analysis on a larger sample size would be helpful to arrive at a precise conclusion about the prevalence of tetracycline resistant genes and (c) the study focuses only on a few vital genes reported to be

associated with tetracycline resistant phenotype. There could be several other mechanisms by which the pathogen resists the antibiotic, which has be revealed by further *in vitro* studies.

Conclusion

Drug resistance is developing into a global threat due to increasing number of drug resistant pathogens. They are not only confined to the hospital environments where the selective pressure due to antibiotic usage is high, but also get disseminated to other sources and finally enter the community. The presence of drug resistant species turns treatment refractory and also builds complications due to longer survivability in the host system. A thorough knowledge on the molecular mechanisms underlying the resistance development process, will definitely aid in the enhancement of drugs targeted towards the resistome of pathogens. Further studies on the mutations associated with the efflux pumps will open up new avenues into the drug development sector.

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Table 3

Frequency distribution of *tet* genes among different strains of *A. baumannii* and related species

Gene	Frequency (N=19)	Percentage (%)
Tet A	2	10.53
Tet B	8	42.11
Tet C	0	0
Tet D	0	0
Tet E	0	0
Tet G	0	0
Tet H	0	0
Tet J	0	0
Tet Y	0	0
Tet Z	0	0
Tet 30	0	0
Tet 39	0	0

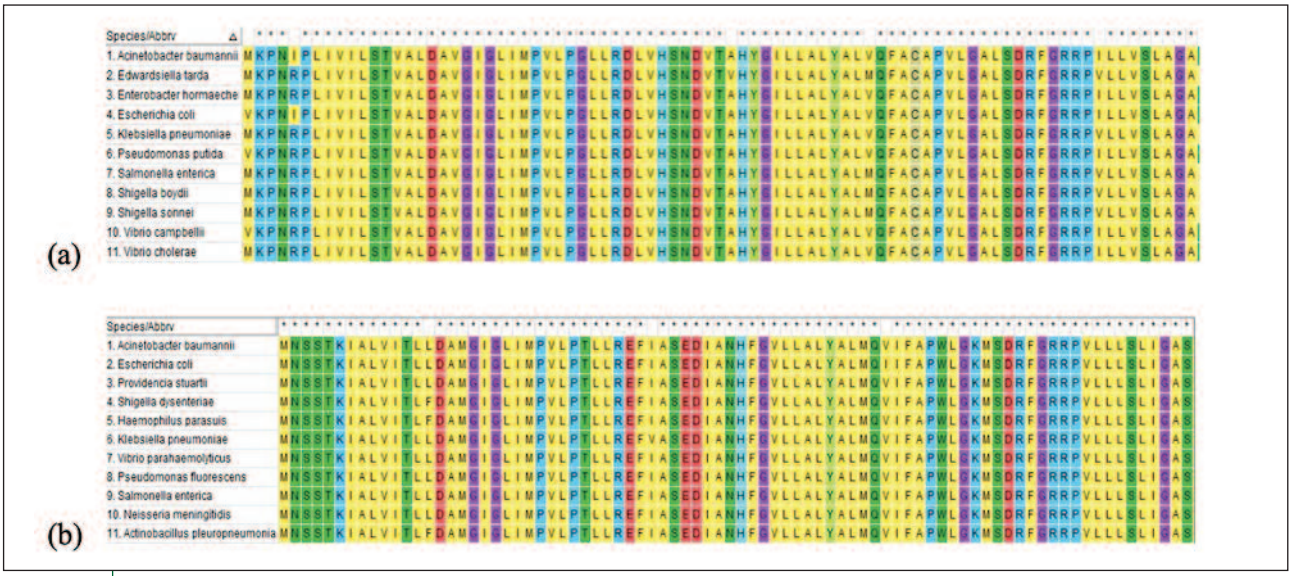

Figure 2 Multiple sequence alignment of TetA and TetB protein of *Acinetobacter baumannii*

Περίληψη

Ανάλυση *in silico* γονιδίων σχετιζόμενων με αντοχή στην τετρακυκλίνη στελεχών *Acinetobacter baumannii* και της ποικιλομορφίας της πρωτεϊνης έκφρασής τους

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Τα αυξανόμενα ποσοστά αντοχής στα αντιβιοτικά αποτελεί σοβαρή απειλή για τη δημόσια υγεία. Το *Acinetobacter baumannii* αποτελεί νοσοκομειακό παθογόνο το οποίο φέρει ποικιλία γονιδίων αντοχής, με αποτέλεσμα να καταφέρνει να διαφεύγει των συνηθισμένων χρησιμοποιούμενων για θεραπεία αντιμικροβιακών. Στην παρούσα μελέτη γίνεται γονοτυπική ανάλυση του γονιδίου αντοχής στην τετρακυκλίνη στελεχών *A. baumannii* με αντοχή στο συγκεκριμένο αντιβιοτικό, καθώς και εύρεση τυχόν φυλογενετική συσχέτισή του με αντίστοιχο άλλων βακτηριακών ειδών. Υλικό αποτέλεσε μικρή νουκλεοτιδική αλληλουχία γενετικού υλικού από στελέχη *Acinetobacter* spp. η οποία εκφράζει την αντοχή στην τετρακυκλίνη (*tet* γονίδιο). Αυτή συνδέθηκε με ειδικούς ανιχνευτές (probes) ακινητοποιημένους σε μεμβράνη από πυρίτιο και ακολούθησε πολλαπλασιασμός της (*in silico* amplification). Το προϊόν πολλαπλασιασμού αλληλουχήθηκε και συγκρίθηκε με ανάλογο γονίδιο άλλων βακτηριακών ειδών.
Κατά την ανάλυση 11 διαφορετικών *tet* γονιδίων, το *tet(B)* γονίδιο βρέθηκε σε μεγαλύτερο ποσοστό (42.1%), ακολουθούμενο από το *tet(A)* γονίδιο (10.5%). Το *tet(A)* γονίδιο βρέθηκε σε στελέχη που φέρουν πλασμίδια, ενώ αντίθετα το *tet(B)* γονίδιο βρέθηκε τόσο σε στελέχη που φέρουν, όσο και σε στελέχη που στερούνται πλασμιδίων. Η μελέτη των συγκεκριμένων γονιδίων, καθώς και η φυλογενετική ανάλυση των προκυπτουσών από αυτά πρωτεϊνικών αλληλουχιών αποκάλυψαν εξελικτική συσχέτιση των *tet (A)* και *tet (B)* του *A. baumannii* με τα των βακτηρίων *Escherichia coli* και *Providencia stuartii* αντίστοιχα. Η κατανόηση των μοριακών μηχανισμών της μικροβιακής αντοχής θα μπορέσει να οδηγήσει στην εύρεση νέων φαρμακευτικών στόχων. Παράλληλα η εξελικτική ανάλυση των πρωτεϊνών που εκφράζουν αντοχή στα αντιβιοτικά μπορεί να αποβεί χρήσιμη στην ανακάλυψη ευρέως φάσματος αντιβιοτικών με δράση έναντι πολλών, φυλογενετικά σχετιζόμενων μεταξύ τους, παθογόνων.

Λέξεις κλειδιά
A. baumannii, τετρακυκλίνη, PCR, φυλογενετική ανάλυση

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