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Prevalence of aminoglycoside and tetracycline resistance genes and their association with virulence factors in clinical isolates of *Acinetobacter baumannii*

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Abstract

High prevalence of antibiotic resistance in *Acinetobacter baumannii*(*A. baumannii*) is a serious threat to modern health care system due to its inherent and acquired resistance mechanism.It is difficult to treat and becoming resistant to various classes of antibiotics. The aim of the study was to investigate aminoglycoside and tetracyclineantibiotic resistancegenes in *A. baumannii* and their correlation with virulence factors. A total of 240 clinical *A. baumannii* isolates collected from Pakistan Institute of Medical Sciences (PIMS) were preceded for the present study. Initially isolates were identified by using standard microbiologicaltechniques. Antibiotic susceptibility assay was performed using Kirby Bauer disk diffusion method. Polymerase chain reaction (PCR) was done for screening of antibiotic resistance genesand virulence factors. PCR results showed that among aminoglycoside resistance genes20%, 8%, 1% and 0.42% of isolates were found positive for *aaC1*, *aadA1*, *aadB* and *aphA6*genes respectively. In case of tetracycline resistance genes, 13% isolates were positive for *tetB* and 1% for *tetA*. Among virulence factors, only one isolate harbored*csgA* gene and none of the isolates were positive for*iutA*, *cnf1* and *cvaC*. It was concluded that due to residing on same genetic elements antibiotic resistance genes suppress the effect of virulence factors.

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Introduction

Acinetobacter baumannii (*A. baumannii*) is a Gram-negative, catalase-positive, oxidase negative, non-motile, and aerobic in nature. It is worldwide reported as hospital-associated bacteria (Lin *et al.*, 2014). *A. baumannii* commonly causes bacteremia, pneumonia, meningitis and urinary tract infections (Al Anazi *et al.,* 2014). In 2009-2010 in United State, National Healthcare Safety Network (NHSN) discovered 1.8% infections caused by *A. baumannii* (Sievert *et al.*, 2013). *A. baumannii* is recognized as one of the important bacteria in ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*) pathogens by Infectious Diseases Society of America (IDSA) (Bush *et al.*, 2010). Due to multidrug resistance and long persistence in hospitals, *A. baumannii* became a serious threat for hospitalized patients. Nowadays in many hospitals carbapenem resistance (CR) is accounting as hallmark for extreme drug resistance (XDR) *A. baumannii*strains. Except some aminoglycosides, tigecycline and polymyxins these CR strains show resistance to all routinely used antibiotics. Colistin is used as a drug of choice to treat complicated *A. baumannii* infection but their efficacy is yet to be determined (Chang *et al.*, 2015).

Virulence factors play vital role in transmission, binding and invasion of the *A. baumannii* and cellular damages (Doi *et al.*, 2015). Pathogenesis of diseases caused by *A. baumannii*is derived from the presence of latent virulence genes. Some of the significant virulence factors of *A. baumannii* involved in human clinical infection are curli fibers (csg), cytotoxic necrotizing factor (cnf), colicin V production (cvaC), and aerobactin (iutA) (Eraç *et al.*, 2014;Eijkelkamp *et al.*, 2014). Screening of virulence factors in *A. baumannii* clinical isolates provides some great epidemiological outcomes to help medical practitioners and clinicians to control dissemination of infectious diseases caused by this bacterium. So it is mandatory to develop some control measures to overcome infections caused by this aggressive bacterium (Darvishi *et al.*, 2016).

According to the best of our knowledge, no previously published datais available about the virulence factors and antibiotic resistance genes (aminoglycoside, tetracycline) in clinical *A. baumannii*isolates from Pakistan. In Pakistan, limited studies reported phenotypic antibiotics resistance in*A. Baumannii* (Hassan *et al.*, 2014; Saba *et al.*, 2015).Therefore the present study focuses on the distribution of virulence factors and aminoglycosides, tetracycline resistance genes pattern in clinical. *Baumannii* isolates collected from tertiary care hospital PIMS Islamabad.

Materials and methods

Samples collection and processing

A total of 375isolates were collected Microbiology Laboratory at Pakistan Institute of Medical Sciences (PIMS), Islamabad Pakistan during January 2014-June 2015. The isolates were characterized by colony morphology on MacConkey and blood agar. Isolates were Gram-stained and later confirmed by biochemical tests using API 20NE Kit (biomeriuex, USA). Ethical approval was taken from bioethics committee Quaid-i-Azam University (QAU) Islamabad Pakistan.

Antibiotic susceptibility profiling

Antibiotic susceptibility assay was done on Muller Hinton agar by usingKirby Bauer disk diffusion method (Bauer *et al.*, 1966). Antibiotics susceptibility was checked for two groups of antibiotics including tetracycline (tigecycline (15µg), minocycline (30µg)) and aminoglycoside (amikacin (30µg), tobramycin (10µg) (Oxoid, UK). Result interpretation was done according to CLSI guidelines 2015. *E.coli* ATTCC 25922 was used as a quality control strain.

DNA extraction

DNA extraction was carried out using the boiling method according to the previously published protocol (Vaneechoutte *et al.*, 1995).

Polymerase Chain Reaction (PCR)

Primer sequences for antibiotic resistance genes and virulence genes used in this study were chosen from previously published data (Maleki *et al.*, 2013;

Aliakbarzade *et al.*, 2014; Darvishi *et al.*, 2017). Primers details are shown in Tables 1 and 2. PCR was carried out to detect antibiotic resistance genes, tetracycline resistance genes, *tet*B and *tet*A, aminoglycoside resistance genes such as, *aacC*1, *aadA*1, *aadB* and *aphA*6 and virulence genes such as *iutA*, *cvaC* and *cnf*1. The PCR parameter was set as; initial denaturation was carried out for 5 minutes at 95°C, 33 cycle each consisting of 30 seconds at 94°C second denaturation, annealing temperature for duplex PCRs for*tet*B, *tet*A (56°C), *aacC*1, *aadA*1(52°C), *aadB*, *aphA*6(55°C) and multiplex PCR for*iutA*, *cvaC, csgA,* and *cnf*1 was(58°C) respectively, initial extension was at 72°C for 1 minute and final extension at 72°C for 10 minutes. PCR product was run on 1% agarose gel and photographed using gel documentation system (Syngene, Germany).

Statistical analysis

Statistical analysis for determining the *p*-value was carried out to find a significant association between antibiotic resistance genes and their association with virulence factors.

This was done through chi-square analysis utilizing the scientific software GraphPad Prism version 7.03 to estimate statistical significance. *p*-value less than 0.05 was considered statistically significant.

Results

Isolates identification

Non pigmented colonies of *A. baumannii* appeared on MacConkey agar after incubation at 37˚C for 24 hours, as shown in Figure 1.

Table 1. Primers used for the detection of tetracycline and aminoglycoside resistance genes.

Primer	Primer sequence	Product size (bp)	Reference
tetA	F: GCG CGATCTGGTTCACTCG R: AGTCGACAGYRGCG CCGGC	164	MH Maliki *et al.*, 2013
tetB	F: CGTGAATTTATIGCTTCGG R: ATACAGCATCCAAAGCGCAC	206	
aphA6	F: ATGGAATTGCCCAATATTATTC R: TCAATTCAATTCATCAAGTTTTA	797	Aliakbarzade *et al.*, 2013
aadA1	F: ATGAGGGAAGCGGTGATCG R: TTATTTGCCGACTACCTTGGTG	792	
aadB	F: ATGGACACAACGCAGGTCGC R:TTAGGCCGCATATCGCGACC	534	
aacC1	F: ATGGGCATCATTCGCACATGTAGG R: TTAGGTGGCGGTACTTGGGTC	456	

Biochemical tests results

Out of 375 a total of 240 isolates were confirmed as *A. baumannii* after colony morphology and through biochemical identification tests.

Antibiotic susceptibility profile of study isolates

All study isolates were evaluated for antibiotic susceptibility profiles, among which high resistance was examined against amikacin (93%), followed by tobramycin (65%), minocycline (59%) and tigecycline (50%). Isolates were highly susceptible to tigecycline (50%) followed by minocycline (41%) and tobramycin (17%) as shown in Figure 2. The data was highly significant with *p*-value <0.0001. Molecular detection of antibiotic resistance genes (tetracycline, aminoglycoside), and virulence genes. All the study isolates were screened for the presence of antibiotic resistance genes and virulence genes through PCR as shown in Figure 3.

Prevalence of tetracycline and aminoglycoside resistance genes

Among all study isolates, the prevalence of tetracycline resistance genes was 13% for *tet*B, 1% for

*tet*A, while in case of aminoglycoside resistance genes, 20% for *aacC*1, 8% for *aadA*1, and 0.42% isolates were found positive for *aphA*6 gene, as shown in Figure 4.

Prevalence of virulence factors

Among all the isolates none was positive for *iutA*, *cvaC* and *cnf1*. Only single isolate carried *csg*A virulence factor. Correlation between antibiotic resistance genes and virulence genes was highly significant (*p*-value <0.0001).

Table 2. Primers used for virulence factors (*cnf1*, *csga*,*cvaC*and*iutA*).

Primer	Primer sequence	Product size (bp)	Reference
Cnf1	F: AAGATGGAGTTTCCTATGCAGGAG R: CATTCAGAGTCCTGCCCTCATTATT	498	Darvishi *et al.*, 2017
csgA	F: ACTCTGACTTGACTATTACC R: AGATGCAGTCTGGTCAAC	200	
cvaC	F: CACACACAAACGGGAGCTGTT R: CTTCCCGCAGCATAGTTCCAT	680	
iutA	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	300	

Discussion

The current study reports high resistance against tetracycline and aminoglycoside in *A.baumannii* isolates and is the first report of tetracycline and aminoglycoside resistance genes associated with virulence factors in *A. baumannii*from Pakistan. In Pakistan, most of the studieshave been limited to phenotypic antibiotic resistance and no comprehensive study is available on the genetic basis of tetracycline and aminoglycoside resistance and its association with virulence factors.

*A.baumannii*has the ability to attain new function due to open pan-genome. Due to the excessive use of antibiotics *A. baumannii*infections remain persistent for long period of time in clinical settings. Moreover, this organism has evolved as a drug-resistant pathogen and some of its virulence factors facilitate its survival in hospital environment (Imperi *et al.*, 2011).In present study, among tetracycline, minocycline was highly resistant (59%) followed by tigecycline(50%) showing different resistance patterns from previously reported studies from Pakistan (Hassan *et al.*,2014; Begum *et al.*, 2013).In case of aminoglycoside, highest resistance was observed in 93% of isolates for amikacin followed by tobramycin 65% depicting close resemblance with other reports from Pakistan (Fakhuruddin*et al.*, 2017).

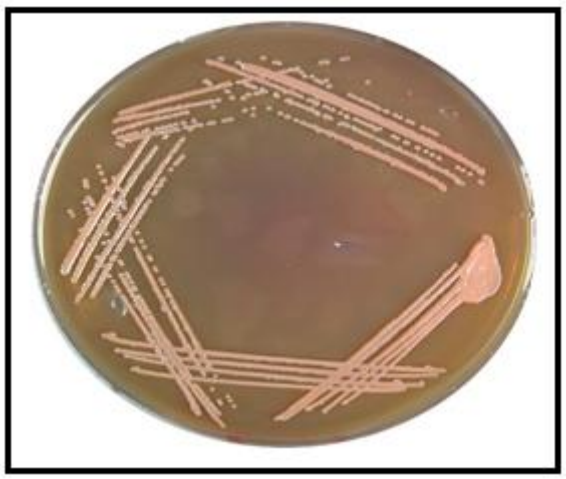

Fig. 1. Colony morphology of *A. baumannii* on MacConkey agar.

The current study showed that among tetracycline resistance genes *tetB* (13%) had high prevalence rate than *tetA* which was 1% while from Pakistan no data is still available on genetic basis of these antibiotic resistance genes. In present study, a low prevalence of *tetA* and *tetB* genes were reported as compared to another study (Maleki *et al.*, 2014).Similar patterns of differences were observed in two other studies with

tetA in 95% and *tetB* in 62% isolates while another study reported 100% presence of*tetB* while none of the isolates carried *tetA* gene (Asadollahi *et al.*, 2012; Farsiani *et al.*, 2015). These discrepancies could be due to geography of the studied areas and variations between clinical samples. Low prevalence of resistance genes may be due to presence of non-enzymatic mechanisms (porins, efflux pumps) which has not been evaluated in current study.

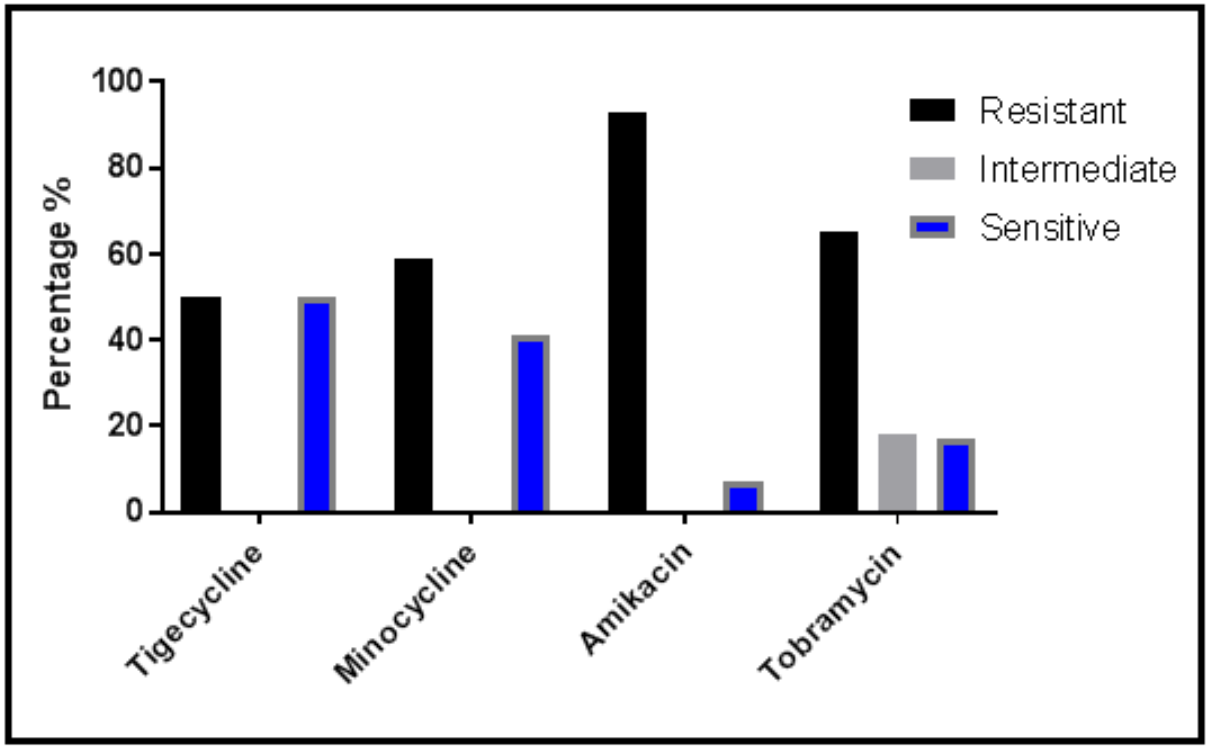

Fig. 2. Antibiotic susceptibility profile of study isolates.

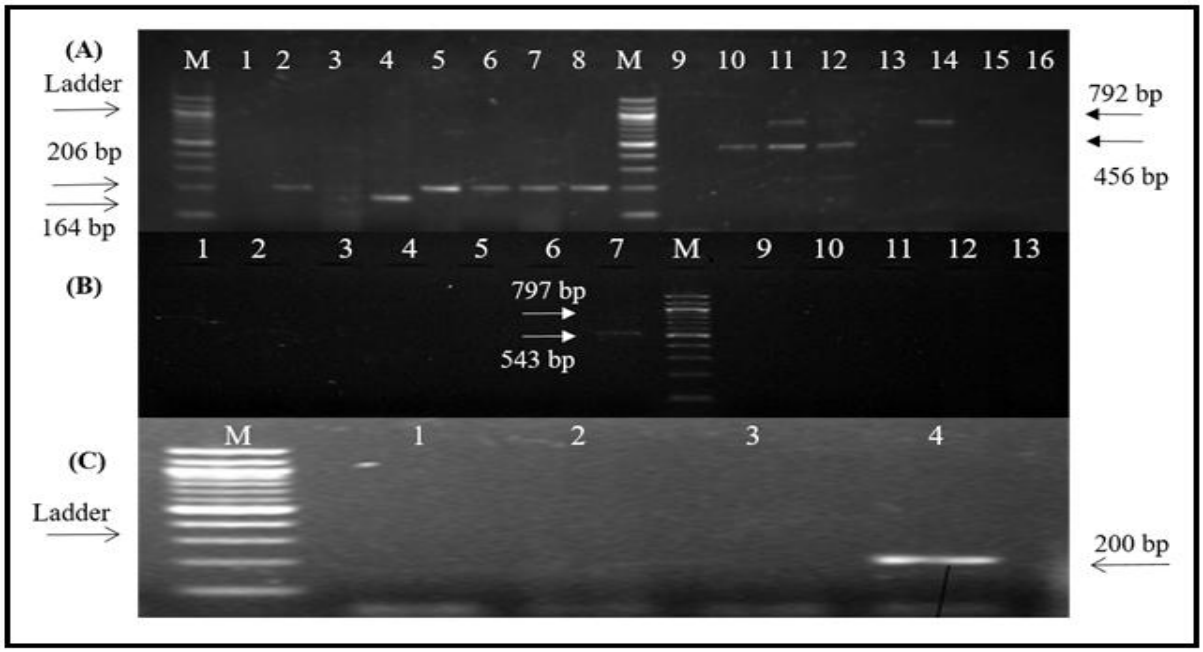

Fig. 3. Representative gel images showing PCR products for detection of Antibiotic resistance genes (A) *tet*B gene (206bp), *tet*A (164bp gene), aadA1 gene (792bp), aacC1(456bp) (B)*aph*A6 gene (797bp), *aad*B gene (543bp) (C)*csg*A gene (200bp).

A. *baumannii* develops resistance against aminoglycosides by producing aminoglycoside modifying enzymes (AME). Present study reported aminoglycoside resistance genes *aacC1* in 20%, *aadA1* in 8%, *aadB* in 1% and *aphA6* in 0.42% isolates of *A. baumannii*. Prevalence rate detected in our study were not in agreement with the previous study conducted byHujar *et al* (Hujer *et al.,* 2006).Another study reported aminoglycoside resistance genes in clinical isolates of *A. baumanniiaph6, aacC1, aadA1* and *aadB*with65%, 63.3%, 41.7% and 3.3% prevalence rate (Moniri *et al.*, 2010). A study conducted by Asadollahi*et al* in Iran reported*aphA6*, *aadA1* and *aadB* in 17.3%, 17.3% and 4.3% isolates respectively (Asadollahi *et al.*, 2012). Farsiani *et al* in 2015 also reported aminoglycoside resistance genes with different prevalence rate as compared to the present study (Farsiani *et al.,* 2015*)*.The contradictions found between present study and all the previous studies may be due to variations in clinical samples, handling, and geographical conditions. Differences may be caused due to the loss of resistance genes residing on plasmids.

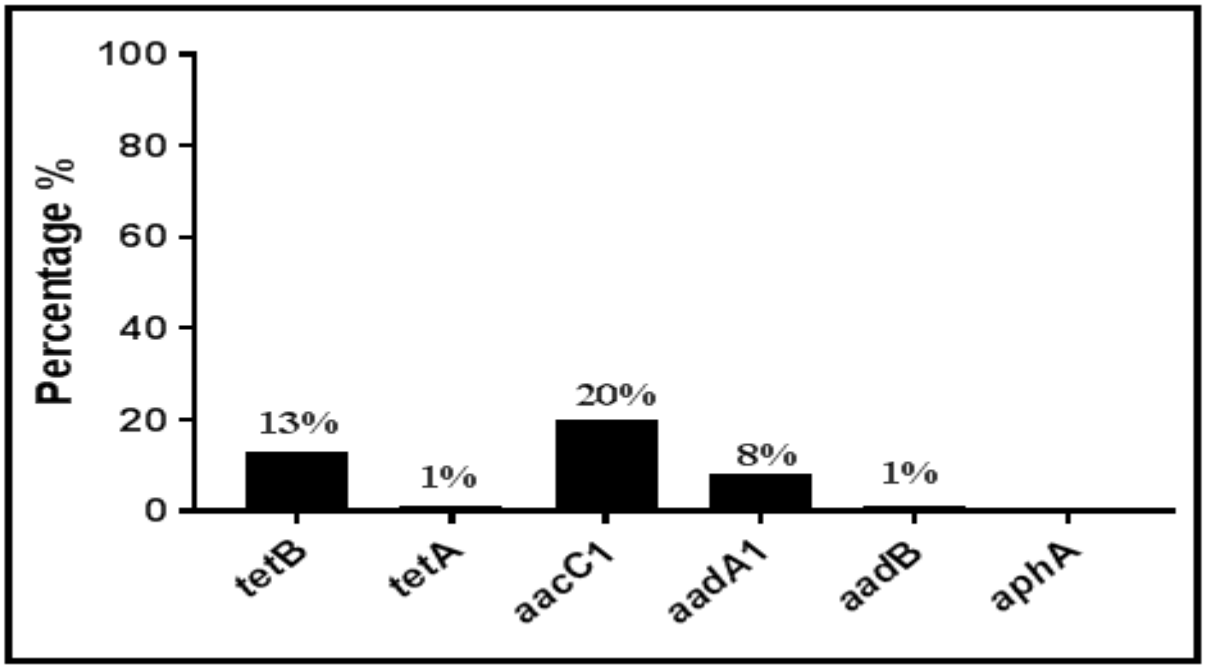

Fig. 4. Prevalence of Antibiotic resistance genes.

Cnf1, *csgA*, *iutA* and *cvaC*are significant virulence factors that arefound in *A. baumannii.*

The present study reported that only single isolate carried *csgA* gene, while all of the isolates were negative for the remaining genes.In 2015 studies conducted by Daryanavard *et al* and Momtaz *et al* reported virulence factors with high prevalence rate in clinical strains of *A. baumannii*. Daryanavard *et al* detected *csgA*, *iutA*, *cnf1* and *cvaC* with a prevalence rate of 55%, 30%, 40% and 10% respectively (Daryanavard *et al.*, 2015). Likewise Momtaz *et al* also found all these genes with high prevalence rate as compared to our finding. They reported *cnf1* in 35%, *iutA* in 19%, *cvaC*in 21.48% and *csgA* in 12.39% clinical isolates of *A. baumannii* (Momtaz *et al.*, 2015). Variations in prevalence of antibiotics resistance genes with virulence factors may involve the suppression of virulence genes due to high antibiotic resistance.

Conclusion

It was concluded that antibiotic resistance genes and virulence factors are in a complex relationship with each other. Antibiotic resistance genes can suppress the expression of virulence factors if both reside on the same genetic element.

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