

**Distribution of biofilm associated genes among *Acinetobacter baumannii* by *in-silico* PCR**

UNDER PEER REVIEW

## **ABSTRACT:**

### **Background:**

*Acinetobacter baumannii* is a coccobacillus that is gram negative, non motile, non fermentative and oxidase negative. It is the most common and successful nosocomial pathogen recognised by WHO. This dreadful pathogen causes urinary tract infections, ventilator associated pneumonia (VAP), bacteremia, etc., These infections are most common in hospital wards especially Intensive Care Unit (ICU). The infections are due to biofilm formation by the virulent genes of *A.baumannii*, and the common biofilm associated genes of *A.baumannii* were *bap*, *csuE*, *fimH*, *epsA*, *bfmS*, *ptk*, *pgaB*, *ompA*, *bla<sub>PER-1</sub>*. Among these, *bap*, *epsA* and *ompA* genes are highly prevalent among the clinical strains of *A.baumannii*.

### **Aim:**

To detect the three vital biofilm associated genes of *A.baumannii* by in-silico PCR analysis.

### **Materials and Methods:**

19 isolates of *A.baumannii* were selected and 3 target genes, namely *epsA*, *ompA* and *bap* gene were used for the amplification process through in-silico PCR simulation tools. Evolutionary analysis was done for the *ompA* gene.

### **Results:**

The *epsA* gene was expressed in 10.52% of the total strains selected with the highest occurrence of *ompA* gene as 57.89%. *bap* gene was not observed from the study strains included. From evolutionary analysis based on *ompA* distributed strains, the *Acinetobacter baumannii* SDF and *Acinetobacter baumannii* BJAB0715 might be the parental strains where the evolution of strains would have started. Through successive generations, the *Acinetobacter baumannii* MDR-ZJ06 and *Acinetobacter baumannii* TYTH-1 had become the multidrug resistant strains present in the environment.

**Conclusion:**

The findings of the study confirms the distribution of *epsA* and *ompA* genes among the 19 different strains of *A.baumannii*. The study suggests periodical monitoring of biofilm based virulence genes among the clinical strains and to curtail the *A.baumannii* infections.

**Keywords:** *Acinetobacter baumannii*; biofilm; innovative in-silico PCR; novel *ompA* gene; *epsA* gene ;*bap* gene; environmental strains

**Running title:** Distribution of biofilm genes among *Acinetobacter baumannii*

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## INTRODUCTION:

*Acinetobacter baumannii* is a coccobacillus that is gram negative, non motile, non fermentative and oxidase negative. It is the most common and successful nosocomial pathogen as recognised by the World Health Organization (WHO). This dreadful pathogen causes urinary tract infections, ventilator associated pneumonia (VAP), bacteremia and even soft tissue, abdominal, CNS infections. These microorganisms also develop infections in the urinary catheter and cause severe recalcitrant infections. These infections are most common in hospital wards especially in the Intensive Care Units (ICU) [1]. The infections are attributed to the biofilm formation by the virulent genes of *A.baumannii*, and the common biofilm associated genes of *A.baumannii* were *bap*, *csuE*, *fimH*, *epsA*, *bfmS*, *ptk*, *pgaB*, *ompA*, *bla<sub>PER-1</sub>* [2]. Among these, *bap*, *epsA* and *ompA* genes are highly prevalent among the clinical strains of *A.baumannii*. Biofilm formation occurs in three stages, early development, matrix formation and maturation where more iron uptake causes biofilm formation by *A.baumannii* [3].

*ompA* gene is the abundant surface protein responsible for serum resistance and biofilm formation. The AIS-0316 gene encodes *ompA* for putative transcription factor, they act as anti-repressor on the promoter region which inhibits AbH-NS protein for transcriptional regulation [4]. They have enhanced mortality due to abundant beta barrel protein which is encoded by the outer membrane and induces mitochondrial fragmentation for its pathogenesis [5]. Most of the gene belongs to the usher chaperone assembly system, where its products form pious bundle structure in the bacterium and forms an important factor in biofilm formation [6].

*bap* gene codes for the development of three dimensional biofilm towers and channels on both biotic and abiotic surfaces [7]. *fimH* gene was the most virulent gene detected in 74% of blood samples which was collected from hospital acquired infections [8]. *A.baumannii* produces exopolysaccharide or EPS encoded by *epsA* which accumulates on the cell surface providing protection against harsh cell environment [9]. *epsA* gene from EPS is a sticky cell formed in the biofilm which is located in the extracellular matrix of the mitochondria. It is responsible for 50 to 90% of total organic carbon found in biofilm, which helps in bacterial survival and resistance. The production of EPS plays a vital role in aggregation of bacteria in the biofilm formation, in which the P1-P8 genome decreases the expression of genes in the amplification process. *bfmS*

regulates cell envelope structure for virulence and biofilm formation, acts as sensor kinase and is one of the most important virulence genes [10]. The distribution of *ptk* gene was found to be 95% which was collected from 100 isolates [11] from immunocompromised patients in ICU. The most common pattern of presence of biofilm genes was found to be *bfmS-csuE-epsA-bap-kpsMT-ompA-ptk-pgaB*. The previous work for drug resistant *A.baumannii* was conducted as clinical studies from urine samples collected from urinary tract infected patients [12–14] and through conventional analytical tests and also by PCR [15].

Through most of the research works, it is confirmed that *A.baumannii* isolates colonise more on the respiratory tract, urinary tract causing severe infections. Many studies documenting the drug resistant isolates, not many studies are related to the co-occurrence of biofilm genes among the drug resistant strains. In-silico PCR analysis is a useful tool for ensuring primer specificity and identifies mismatches in primer binding sites. It avoids amplification of unwanted amplicons and is useful in detection of any pathogens, gene discovery and molecular diagnosis [16,17]. So, the aim of the study was to detect the three vital biofilm associated genes of *A.baumannii* by in-silico PCR analysis.

#### **MATERIALS AND METHODS:**

**Study Setting :** The present study was an observational *in-silico* study done in the Department of Microbiology, Saveetha Dental College and Hospital. Institutional approval for the research was obtained and the SRB approval number is IHEC/SDC/UG-1906/21/147.

#### **Strains used in the present study:**

Genome sequences of the *Acinetobacter baumannii* strains used in the study are provided under table 1. The analysis of genomic strains was done in in-silico simulation tools where 19 isolates of *A.baumannii* were selected from a select default tool and used for distribution of the biofilm associated genes [18].

### **Primers used in the study:**

3 most common biofilm associated genes, namely *ompA*, *epsA*, *bap* gene were opted for the study [19] and the primers used for detection of selected biofilm associated genes are given in table 2.

### **In-silico PCR amplification:**

The amplification of biofilm associated genes which was characterised were performed using in-silico PCR simulation tools. There are a high variety of web servers useful in designing primer sequences and computational optimisation which is used for conditioning the PCR. The primers are validated successfully for GC clamp, self annealing and hairpin formation using the 'PCR Primer stat' program. The web server used was *insilico.ehh.es* and the genus was selected as *Acinetobacter*. Selected primers were given as input in the forward and reverse primers column and the command of amplify was performed. The bands generated were analyzed for the frequency of its presence among the selected strains. The presence of genes in different strains of *A.baumannii* were provided through this tool in a few seconds [20,21].

### **Evolutionary analysis by Maximum Likelihood method:**

The evolutionary history was concluded through the Maximum Likelihood method, using the Tamura Nei model. The evolutionary analysis included 14 nucleotide sequences. It was conducted in Mega X software. The bands from in-silico PCR amplification were used and placed in this software. The evolutionary analysis was done for all the three biofilm genes, where only *ompA* gene associated strains was selected for phylogenetic analysis and was given in figure 3.

### **RESULTS:**

From the results analysed from in-silico PCR simulation tool, it is inferred that *epsA* gene was present in 2 strains, *ompA* gene was present in 11 strains and *bap* gene was not present in any of the strains. From figure 1, the *epsA* gene was present in strains namely *A.baumannii* AB307-0294, *A.baumannii* AYE and not present in other strains. The *epsA* gene showed the highest annealing temperature of 60°C with the DNA amplicon size as 451bp. There was no need for

phylogenetic analysis for *epsA* associated strains as it was present in only 2 strains. The *epsA* gene was expressed in 10.52% of the total genome of *A.baumannii* which is shown in figure 4.

From figure 2 analysed, *ompA* gene was present in 11 strains from 19 selected isolates, namely *Acinetobacter baumannii* 1656-2 chromosome, *Acinetobacter baumannii* ACICU, *Acinetobacter baumannii* ATCC 17978, *Acinetobacter baumannii* BJAB07104, *Acinetobacter baumannii* BJAB0715, *Acinetobacter baumannii* BJAB0868, *Acinetobacter baumannii* MDR-TJ, *Acinetobacter baumannii* MDR-ZJ06, *Acinetobacter baumannii* TYTH-1, *Acinetobacter baumannii* ZW85-1, *Acinetobacter baumannii* SDF. The *ompA* gene showed an annealing temperature of 58°C and DNA amplicon size of 531bp. The phylogenetic tree for *ompA* associated strains was provided in figure 3. The *ompA* gene was expressed in 57.89% of the total genome of *A.baumannii* which is shown in graph 1. *bap* gene was not associated with these 19 selected strains as there may be presence of other virulent genes found in them.

From figure 3, the phylogenetic tree shows that the *Acinetobacter baumannii* SDF and *Acinetobacter baumannii* BJAB0715 might be the parental strains where the evolution of strains would have started. Through successive generations, the *Acinetobacter baumannii* MDR-ZJ06 and *Acinetobacter baumannii* TYTH-1 had become the multidrug resistant strains present in the environment. The isolates possessed *ompA* gene in higher frequency which might be the reason for multidrug resistance, biofilm formation and causative of severe infections.

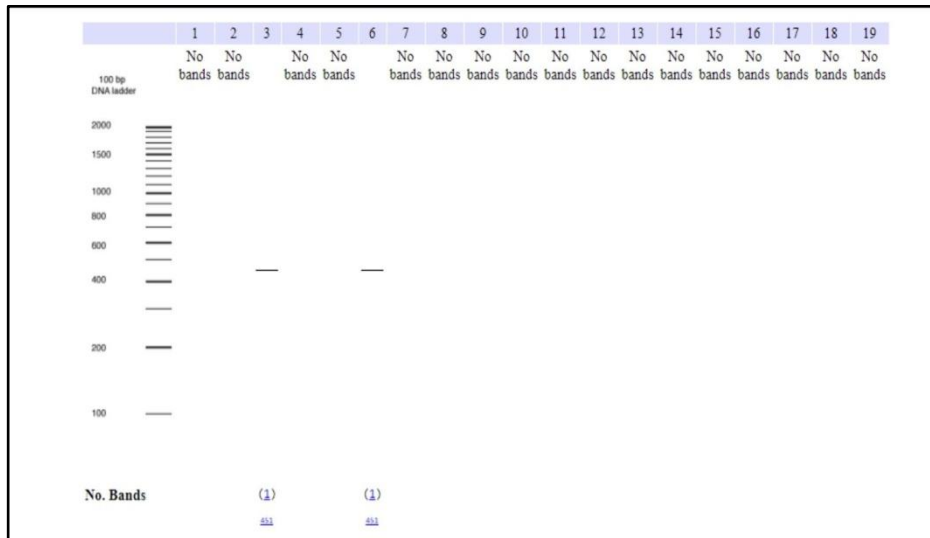
**Table 1:** Strains used for the in-silico PCR analysis and the detection of *epsA*, *OmpA* and *bap* genes

S.No	Strains of <i>Acinetobacter</i>	<i>epsA</i> gene	<i>ompA</i> gene	<i>bap</i> gene
1	<i>Acinetobacter</i> spp. DR1	-	-	-
2	<i>Acinetobacter</i> spp. ADP1	-	-	-
3	<i>Acinetobacter baumannii</i> SDF	-	+	-
4	<i>Acinetobacter baumannii</i> TCDC- AB0715	-	-	-
5	<i>Acinetobacter baumannii</i> 1656-2 chromosome	-	+	-
6	<i>Acinetobacter baumannii</i> ACICU	-	+	-
7	<i>Acinetobacter baumannii</i> MDR-TJ	-	+	-
8	<i>Acinetobacter baumannii</i> BJAB07104	-	+	-
9	<i>Acinetobacter baumannii</i> MDR- ZJ06	-	+	-
10	<i>Acinetobacter baumannii</i> BJAB0868	-	+	-
11	<i>Acinetobacter baumannii</i> TYTH-1	-	+	-
12	<i>Acinetobacter baumannii</i> BJAB0715	-	+	-
13	<i>Acinetobacter baumannii</i> ATCC 17978	-	+	-
14	<i>Acinetobacter baumannii</i> AYE	+	-	-
15	<i>Acinetobacter baumannii</i> AB307- 0294	+	-	-

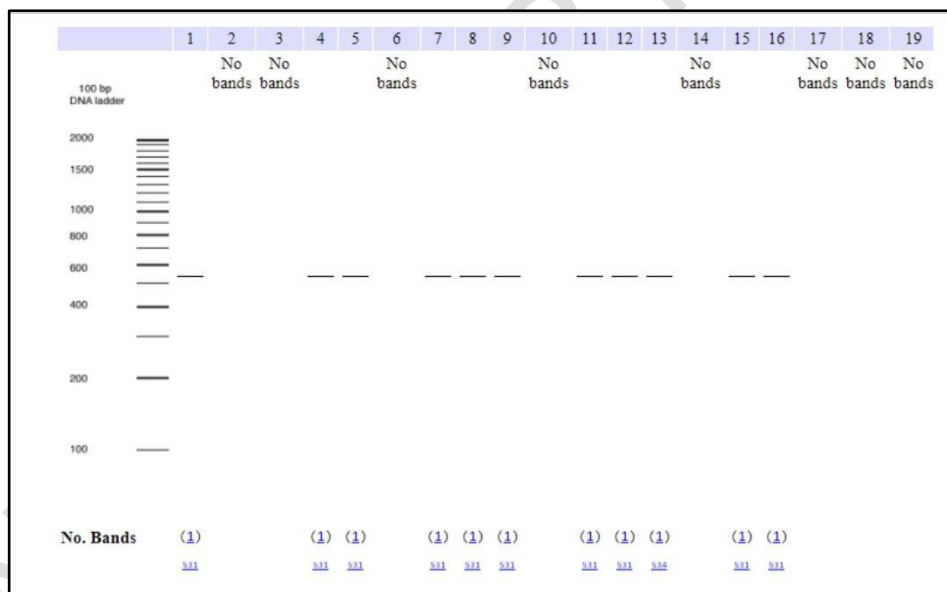
16	<i>Acinetobacter baumannii</i> AB0057	-	-	-
17	<i>Acinetobacter baumannii</i> ZW85-1	-	+	-
18	<i>Acinetobacter baumannii</i> D1279779	-	-	-
19	<i>Acinetobacter pittii</i> PHEA-2	-	-	-

**Table 2:** Primers and PCR conditions used for the detection of the three target genetic determinants

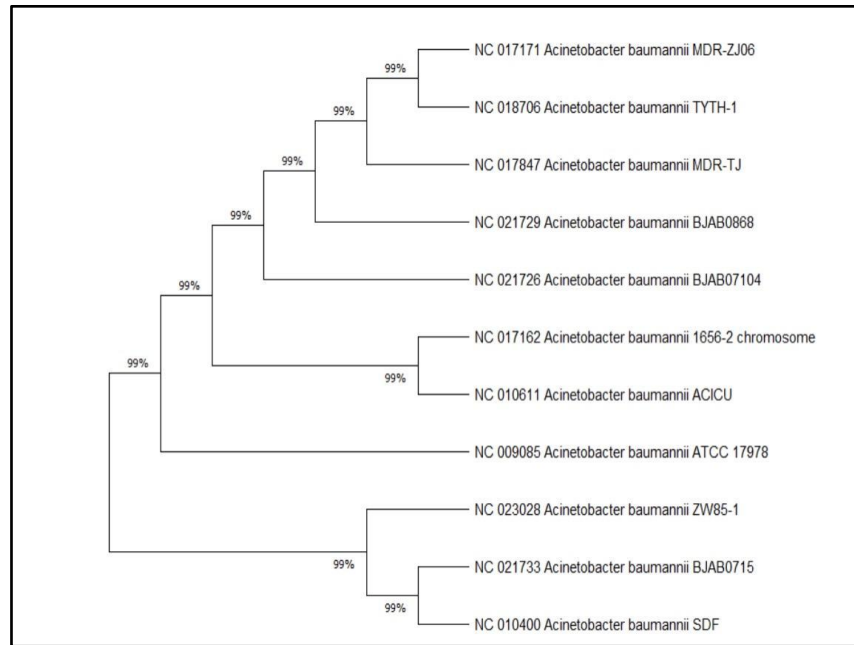
Target Genes	Primers sequences (5'-3')	Annealing Temperature(°C)	DNA amplicon Size (bp)
<i>epsA</i>	AGCAAGTGGTTATCCAATCG ACCAGACTCACCCATTACAT	60	451
<i>ompA</i>	CGCTTCTGCTGGTGCTGAAT CGTGCAGTAGCGTTAGGGTA	58	531
<i>bap</i>	TACTTCCAATCCAATGCTAGG GAGGGTACCAATGCAG TTATCCACTTCCAATGATCAG CAACCAAACCGCTAC	55	1225



**Figure 1:** In-silico PCR amplification showing the amplicons of *epsA* with an amplicon size of 451 bp



**Figure 2:** In-silico PCR amplification showing the amplicons of *ompA* with an amplicon size of 531bp



**Figure 3:** Phylogenetic tree constructed based on the *ompA* gene identified in different strains of *A.baumannii*

## DISCUSSION:

The present investigation documents the distribution of biofilm associated genes among the virulent strains of *A.baumannii*. Higher frequency was observed with *ompA* and *epsA* that correlates with the earlier studies [22]. *ompA* was present in 11 strains and *epsA* gene was present in 2 strains. The previous study analysed 17 genomes where the current study used 19 genomes and genotype 2 and 3 encoded for 1 to 15 and 6 to 10 genes for virulence factors. The *ompA* gene was present in 57.89% and *epsA* gene was present in 10.52% of the total strains. Another work by Emmanuel C Eze, et al, 2018 [23] concluded that out of 24 clinical isolates selected, 22 expressed *bap* gene which was 91.66% but in our study, *bap* gene was not associated with the selected strains. The *ompA* gene was expressed in 100% of the strains in the previous study but in the present study, it was expressed in 11 out of 19 selected strains which should be 57.89% and *epsA* gene was 10.52%.

The work by Thomas A Russo, et al, 2010 [24] found that *A.baumannii* AB 307-0294 strain possessed an *epsA* gene which was similar to present study where *epsA* gene was present in that strain of *A.baumannii*. The *bap* gene expressed none of the strains which is in contrast with the

earlier study where *bap* gene was present in about 66% of total strains. The previous study also found that annealing temperature for *ompA* gene was 56°C where in present study it was 58°C. The DNA amplicon size for the genes was found to be decreased when compared with previous author's work.

Another research by Rapee Thummeepak, et al, 2016 [25] concluded that *ompA* gene was present in 84.4% of total genomic strains, *bap* gene for 48% but in the present study there is a lesser distribution of selected biofilm genes. It may be due to the presence of other genes like *fimH*, *csuE*, *pgaB*, etc., which makes the difference in distribution. Our study selected 3 target genes for 19 strains but in previous work, it was seven target genes. Yet in another study *bap* gene was present in 92% of total strains but in the current study, no distribution was found [26].

This type of research is the need of the hour in all the laboratories for epidemiological surveillance. The present study did not undergo any experimental verification which may be considered as the limitation of the same. In recent years, multidrug resistant *A.baumannii* has become more prevalent in hospital wards, ICU and it has become a more dreadful pathogen causing severe infections. These genes could be further targeted for advanced therapeutic strategies such as gene therapy, as WHO had recognised this pathogen to be a critical priority for the need of antibiotics through research and development, and this study forms a platform for future researchers and can be taken up by geneticists for their research to provide an efficient treatment strategy for *A.baumannii* associated infections. Though there is much research undergoing in the microbial field [27–35], [36–40], [41], [42], [43], there should be importance given to this pathogen otherwise within a few decades, it will be the irresistible pathogen and mortality rate will increase in folds. The limitation of the study is that it was carried out as a computational approach, and thus the future prospects are set to evaluate the same using the clinical strains for the frequency of the genetic determinants.

## **CONCLUSION:**

The present investigation documents the role of various genetic determinants that contribute to the pathogenesis of *A.baumannii* in health-care settings. Among the various virulence factors, the findings of the study confirms the distribution of *epsA* and *ompA* genes among the 19 different

strains of *A.baumannii*. In addition, the study recommends the *in-silico* PCR tool as an efficient methodology for the preliminary selection of the virulence determinants in the pathogenic bacteria. It also suggests the need of periodical monitoring of biofilm based virulence genes in all the health care settings to curtail the *A.baumannii* infections.

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