ijlpr 2022; doi 10.22376/ijpbs/lpr.2022.12.3.L150-158

International Journal of Life science and Pharma Research

ISSN 2250-0480

Research Article



# Molecular Determination of Virulence Factor Genes of *Acinetobacter baumannii* Isolates from Clinical Specimens

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**Abstract:** *Acinetobacter baumannii* is a gram negative coccobacilli present abundantly in nature, in soil and water. *Acinetobacter baumannii* is considered as a major cause of nosocomial infections affecting mainly ICU patients and other hospitalised patients. Both intrinsic and acquired antibiotic resistance of *A. baumannii* account for a significant cause of outbreaks. Significant levels of morbidity and mortality have been reported with outbreaks and common infections include ventilator associated pneumonia and bacteraemia. *A.baumannii* is also a common cause of bloodstream infections in the intensive care setting. Multiple virulence factors are required for the pathogenesis of infections by Gram negative bacteria including *A. baumannii*. Possession of specialized virulence genes enables pathogens to infect hosts efficiently. Virulence factors of *A. baumannii* were less identified compared to other Gram negative bacteria. Hence this study was done to identify the major virulence factor genes from the clinical isolates of multidrug resistant *A. baumannii* from a tertiary care hospital. A preliminary study was done to determine the prevalence of Acinetobacter infections in the region and then the isolates were subjected to determine the antibiotic sensitivity and to various molecular typing. Various clinical specimens like blood, urine, abscess, vaginal swab were analyzed and 15% of the isolates was confirmed and identified as to be resistant to carbapenems. A molecular typing was done to identify the genes conferring virulence factors. Presence of different genes like *Bap, Omp A, EpsA, ptk, AdeG* were screened from the isolates.

**Keywords:** *Acinetobacter baumannii*, Virulence factor, Multi drug resistance, Molecular typing, Efflux pump, Biofilm, Capsular Polysaccharide

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**Received On** 5 May, 2022
**Revised On** 9 May, 2022
**Accepted On** 16 May, 2022
**Published On** 18 May, 2022

**Funding** This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

**Citation** Fiji E, Anandharaj B and Jijo G Varghese , Molecular Determination of Virulence Factor Genes of Acinetobacter baumannii Isolates from Clinical Specimens.(2022).Int. J. Life Sci. Pharma Res.12(3), L150-158 http://dx.doi.org/10.22376/ijpbs/lpr.2022.12.3.L150-158

## 1. INTRODUCTION

*A. baumannii* is a Gram negative rods and it is short roads usually confused with cocci. It is named after the bacteriologist Paul Baumann[1]. *Acinetobacter baumannii* is identified as an ESKAPE pathogen by WHO (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* species), a group of pathogens with a high rate of antibiotic resistance that are responsible for Major nosocomial infections[2]. World Health Organisation (WHO) has included *Acinetobacter baumannii* in the 'critical' category list of pathogens for developing new antibiotics[3]. Members of *Acinetobacter* genus present abundantly in nature, in soil and water. In general, *Acinetobacter* spp. are found in wet environments, including moist soil/mud, wetlands, ponds, water treatment plants, fish farms, wastewater, and even seawater[4]. Some medically relevant species, such as *A. calcoaceticus*, *A. lwoffii*, *A. nosocomialis*, and *A. pittii*, have been found on vegetables, meat, dairy products, and human skin[5]. *Acinetobacter baumannii* is considered as a major cause of nosocomial infections affecting mainly ICU patients and other hospitalised patients[6].Matthieu *et al* 2013[7], listed many cases of community acquired infections of *Acinetobacter baumannii* around different parts of the world and extra hospital reservoirs like petroleum hydrocarbons, vegetables, slaughterhouses, agricultural soils.etc. Significant levels of morbidity and mortality have been reported with outbreaks[8] and common infections include ventilator associated pneumonia and bacteremia; less frequently burn wounds and urinary tract[9]. *A.baumannii* is also a common cause of bloodstream infections in the intensive care setting[10] and the lower respiratory tract infections and intravascular devices[11-,14] are reported to be the common sources. In addition, wound infections and urinary tract infections have also been reported as foci of infection[11]. Multiple virulence factors are required for the pathogenesis of infections by Gram negative bacteria including *A. baumannii* . Possession of specialized virulence genes enables pathogens to infect hosts efficiently. Virulence factors of *A. baumannii* were less identified compared to other Gram negative bacteria[15]. Hence this study was done to identify the major virulence factor genes from the clinical isolates of *A. baumannii* from a tertiary care hospital. Environmental survival of a bacteria is an important factor which influences the prevalence of nosocomial infections. Biofilm formation and biosynthesis of capsular polysaccharide facilitates the survival of *A. baumannii* on inanimate objects and act as a source of infections[16]. Microbial biofilm is a community of one or more organisms attached to sessile substrates or live organs[17] . Biofilm formation is thought to be an important pathogenic feature of *A. baumannii*, especially in cases of ventilator-associated pneumonias and catheter-related urinary tract infections, as it facilitates the survival of the bacterium in hostile environments[18]. The presence of OmpA gene facilitates *Acinetobacter* to produce biofilm, to induce apoptosis in host cells and to adhere the epithelial cells[19,20,21,22]. Hence OmpA is considered as a major virulence gene in *Acinetobacter* sp.. The presence of Biofilm associated protein (Bap) was also reported in *Acinetobacter*. Bap is a high molecular weight protein present in the bacterial surface which facilitates biofilm production[23]. The genes *ptk* and *epsA* are considered to be very important in *Acinetobacter* virulence as they confer capsular polysaccharides. Capsular polysaccharide confers pathogenicity by resisting complement activity and host phagocytosis. Evasion of the host immune response and growth in serum is facilitated by *ptk* and *epsA*. *Ptk* encodes a putative protein kinase and *EpsA* encodes a putative polysaccharide[24]. Efflux pumps contribute to intrinsic and acquired resistance in *A. baumannii* to a wide range of antibiotics and disinfectants[25]. Resistance nodulation efflux pumps are an important efflux pump family that contributes to drug resistance in *A. baumannii*. *AdeABC, Adeijk* & *AdeFGH* are the RND genes[26]. *AdeG* expression is regulated by LysR type transcriptional regulator (LTTR) and confers multidrug resistance[27]. Multiple virulence factors are required for the pathogenesis of infections by Gram negative bacteria including *A. baumannii*. Possession of specialized virulence genes enables pathogens to infect hosts efficiently. Virulence factors of *A. baumannii* were less identified compared to other Gram negative bacteria. Hence this study aimed to identify the major virulence factor genes from the clinical isolates of multidrug resistant *A. baumannii* from a tertiary care hospital. The Objective of the present study was to identify carbapenem resistant Acinetobacter species and to screen the genes encodes for various virulence factors.

## 2. MATERIALS AND METHODS

### *2.1 Study Design and Area*

The study was conducted at Sunrise Institute of Medical Sciences (SIMS), Kerala, India. This study was reviewed and approved by the institutional ethical committee of Sunrise Institute of Medical Sciences (SIMS/IEC/03/2022).

### *2.2 Sample Collection and Isolation*

The samples were collected from patients admitted in the hospital in various departments as well as from OP patients. Various clinical specimens like urine, blood, pus, abscess and endo-tracheal aspirations were screened for the presence of *Acinetobacter* spp. All the samples were collected by aseptic methods[28].

### *2.3 Selective Culture and Biochemical Identification*

All samples were inoculated on to two enriched and selective agar media, 5% sheep blood agar (Biomerieux) and on Mac Conkey agar and incubated at 37 °C for 24 to 48 hours. All colonies resembling *Acinetobacter* were initially identified by standard morphological, cultural and biochemical characteristics. And further identification was done by Vitek 2 compact system from Biomerieux India pvt Ltd.

### *2.4 Susceptibility Testing*

Antibiotic susceptibility testing was done by disc diffusion on Mueller Hinton agar (Himedia) plates according to the guidelines of Clinical Laboratory Standards Institute[29]. Along with the disc diffusion method susceptibility was also analysed using AST N280 cards on Vitek 2 compact system. The antibiotics tested include Ampicillin sulbactam, Ceftazidime, Ciprofloxacin, Levofloxacin, Imepenem, Meropenem, Gentamycin, Tobramycin, Cotrimoxazole, Amikacin, Piperacillin tazobactam, Cefepime, Cefotaxim, Ceftriaxone, Doxycycline, Tetracycline, Colistin. The quality control of the antibiotic sensitivity was done with *Escherichia coli* ATCC25922.

### 2.5 Molecular Identification

A fragment of 16S rDNA resolved from the isolates was sequenced with 8F and 1492R primers using BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer. The resulting sequence was used to carry out BLAST with the nr database of NCBI genbank. The first ten similar sequences were aligned using Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA X.

### 2.6 Antimicrobial Susceptibility

Similarly, the Clinical and Laboratory Standards Institute[29] recommendations were followed to determine the antibiotic susceptibility of the confirmed isolates of *Acinetobacter* along with AST N280 cards on Vitek 2 compact system based determination. The antibiotics observed for susceptibility were Ampicillin sulbactam, Ceftazidime, Ciprofloxacin, Levofloxacin, Gentamycin, Amikacin, Tobramycin, Imepenem, Meropenem, Piperacillin tazobactam, Cefepime, Cefotaxim, Ceftriaxone, Tetracycline and Colistin.

### 2.7 Screening for Virulence Associated Genes

Four Carbapenem resistant *Acinetobacter baumannii* isolates were subjected to screening for various virulence factor coding genes. The extraction of total genomic DNA using Mag Genome DNA isolation kit procedure as per the manufacturer instructions. Quality of the genomic DNA was assessed using 0.7 % agarose gel along with 1kb DNA ladder as size standard and the quantity of the genomic DNA was assessed in UV-Vis Spectrometer. Amplification of Virulence genes were carried out for the sample using primers and the details of the genes and primers used are mentioned in the table. Presence or absence of expected band was considered as +ve or –ve genotype of the strains. PCR-generated amplicon was confirmed and purified using GeneJET PCR purification kit (Thermo Scientific, EU-Lithuania) to remove the primer dimer and other carryover contaminations. The quality of the product was assessed using 2% agarose gel along with 100bp DNA ladder as size standard. The primer sequence used and corresponding genes are listed in table1

**Table 1: Primers used for gene excretions.**

| VIRULENCE FACTOR | GENE | PRIMER | REFERENCE |
|---|---|---|---|
| **Efflux Pump** | *adeG* | F-CTCCGTCCATTCCATAAACCA<br>R-AGATGGCAGACAGTGCAAAC | 30 |
| **Biofilm** | *Bap* | F:TACTTCCAATCCAATGCTAGGGAGGGTACCAATGCAG<br>R:TTATCCACTTCCAATGATCAGCAACCAAACCGCTAC | 31 |
| **Biofilm** | *ompA* | F- GTTAAAGGCGACGTAGACG<br>R - CCAGTGTTATCTGTGTGACC | 31 |
| **Capsular Polysaccharide** | *ptk* | F - ATTTCAGGGCTTATTGGTC<br>R - TCATAAGCAGCAACGGCAG | 32 |
| **Capsular Polysaccharide** | *epsA* | F- ACAAACTTCTTCTGTAGCACC<br>R -AAAAATACTCTGCCATAGGG | 32 |

**Primer sequence used for screening of virulence genes.**

## 3. RESULTS

### 3.1 Sample collection & isolation:

A total of 500 clinical samples were collected aseptically from various patients from Sunrise Institute of Medical Sciences, Kochi and a total of 39 isolates of *Acinetobacter* species were identified. Most of the isolates are from urine samples (49%), followed by pus samples (18%), Blood samples (13%), Sputum samples (10%), Endo tracheal secretion (5%), vaginal swab (3%) and semen samples (3%) (figure1).

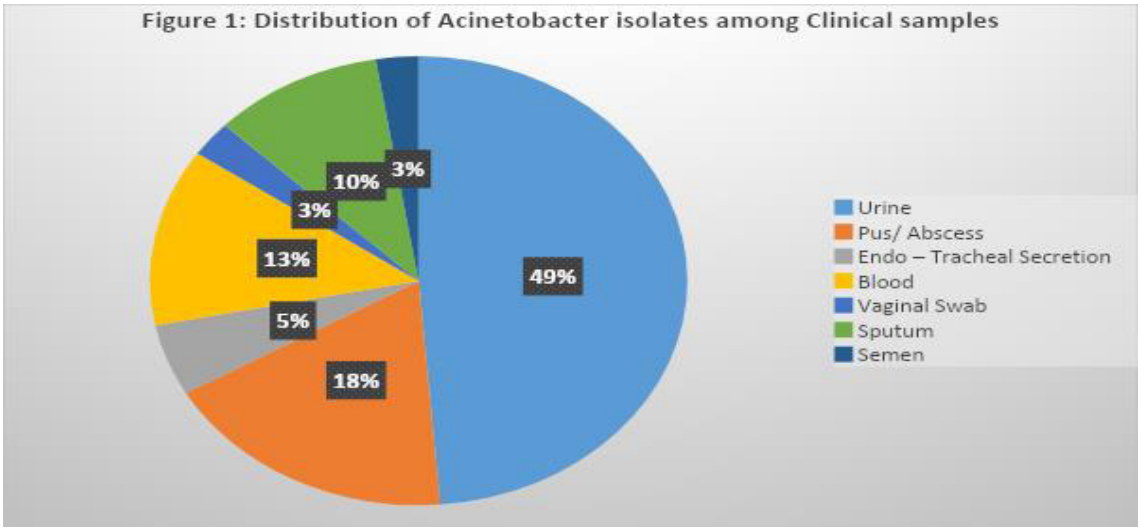


Fig1: Percentage of *Acinetobacter* isolates from various specimens.

A. *baumannii* was the predominant genus in the isolates which confers 85% of the total isolates. Three other genus were also identified, *Acinetobacter lowffii* (8%), *Acinetobacter junii* (5%) and *Acinetobacter ursingii* (3%). (Table2)

| Table 2: Prevalence of different Acinetobacter species from the clinical specimens | |
|---|---|
| Genus of *Acinetobacter* | **No of isolates** |
| ***A baumanii*** | 33 |
| ***A junii*** | 2 |
| ***A lowfii*** | 3 |
| ***A ursingii*** | 1 |

### *3.2 Antibiotic Sensitivity*

Out of the 39 isolates, 6 isolates were observed to be multidrug resistant and carbapenem resistant strains. The most susceptible antibiotic was colistin; i.e., 83% of the Carbapenem resistant *Acinetobacter baumanii* (CRAB) isolates were susceptible to colistin. The observed susceptibility pattern of the CRAB isolated showed that tobramycin has the second highest percentage (77%). Most of the other tested antibiotics were found to be resistant and the pattern of resistance of the isolates were shown in figure 2.

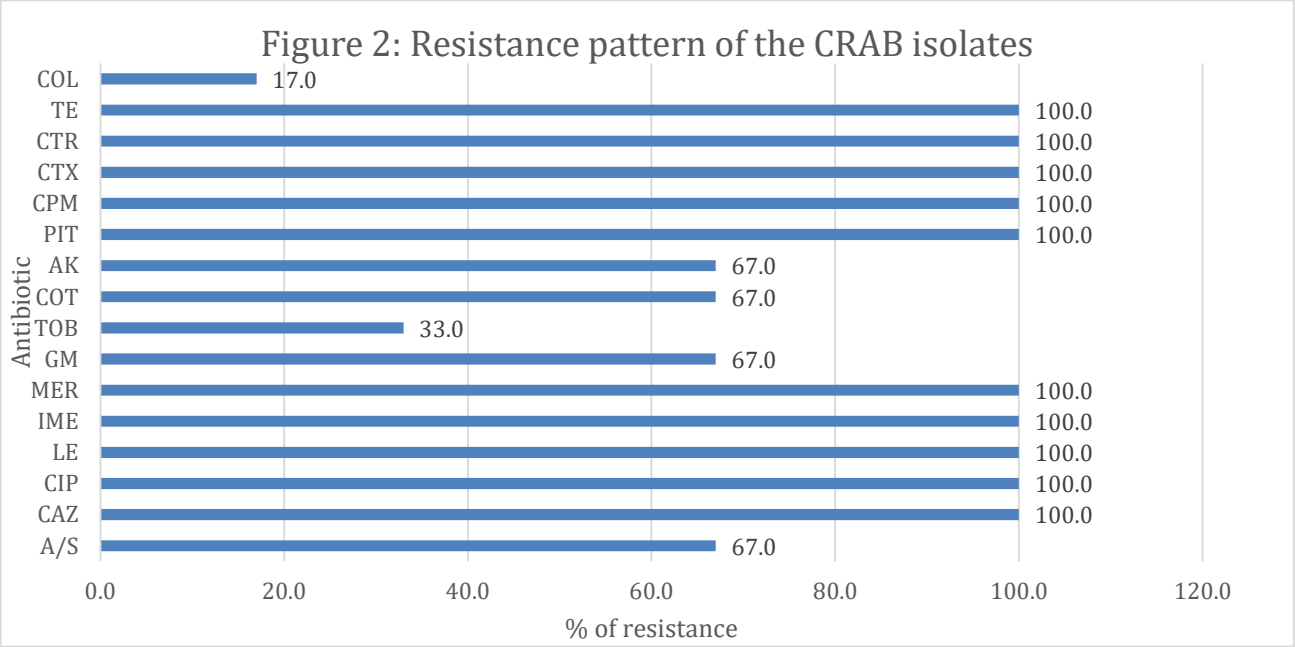


Fig 2: Antibiogram of the CRAB isolates. COL - Colistin; TE – Tetracyclin; A/S - Ampicillin sulbactam; CAZ – Ceftazidime; CIP - Ciprofloxacin, LE- Levofloxacin; GM – Gentamycin; AK –Amikacin; TOB – Tobramycin; IME – Imepenem; MER – Meropenem; PIT - Piperacillin tazobactam; CPM – Cefepime; CTX - Cefotaxim, CTR – Ceftriaxone.

### *3.3 Molecular Identification*

5 carbapenem resistant isolates were further subjected to 16s rRNA sequencing and fragment of 16S rDNA gene was amplified by PCR. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel (Figure 3 ). Consensus sequence of 1200 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. Figure 3 : Gel Image of 16s rRNA

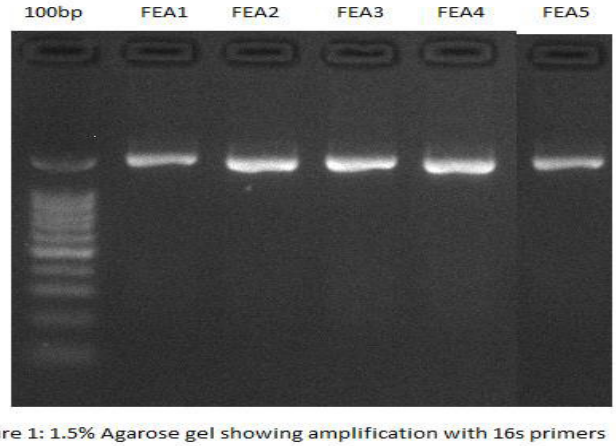


Fig 3: Gel Image of 16s rRNA. Lane 1 - 100bp ladder scale; Lane 2 - isolate 1; Lane 3 - Isolate2; Lane 4 – Isolate3; Lane 5 – Isolate4; Lane 6 – Isolate5

The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score, first ten sequences were selected and aligned using multiple alignment software program Clustal W. A distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA X (figure 4 ) and all the five strains were identified and confirmed as *Acinetobacter baumanii* based on sequencing and phylogenetic studies. Figure 4 : Evolutionary relationships of taxa

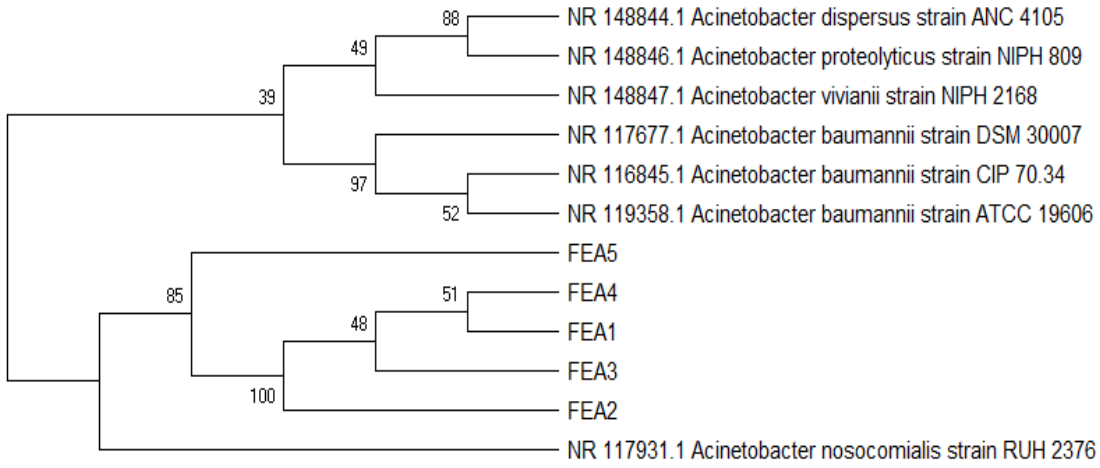


Fig 4: Phylogenetic tree of the isolates. FEA1 – isolate 1; FEA2 – isolate2; FEA3 – isolate3; FEA4 – isolate4; FEA5 – isolate5.

### 3.4 Virulence gene determination

Four Carbapenem resistant isolates (FEA1, FEA2, FEA3 and FEA4) of *Acinetobacter baumanii* were subjected to molecular level analysis for the presence of virulence associated genes. Screening for the presence of five genes (*adeG, Bap, OmpA, ptk & epsA*) were carried out in all four CRAB isolates (Table 3).

Table 3: Virulence genes screened in the CRAB isolates

| Gene | FEA 1 | FEA2 | FEA3 | FEA4 |
|---|---|---|---|---|
| ***Bap*** | + | + | + | + |
| ***OmpA*** | + | + | + | + |
| ***adeG*** | + | + | + | + |
| ***ptk*** | - | + | - | + |
| ***epsA*** | - | - | - | + |

**Presence of genes from the isolates number 1-4 were explained. (+) is the gene present in the curresponding isolate**

Biofilm production in *Acinetobacter* leads to colonization on hospital environments and act as a source of nosocomial infections. A gene which confers Biofilm associated protein (*Bap*) and *OmpA* gene were screened in all four isolates. All the isolates were positive for the presence of these biofilm associated genes (figure 5 & 6 ).

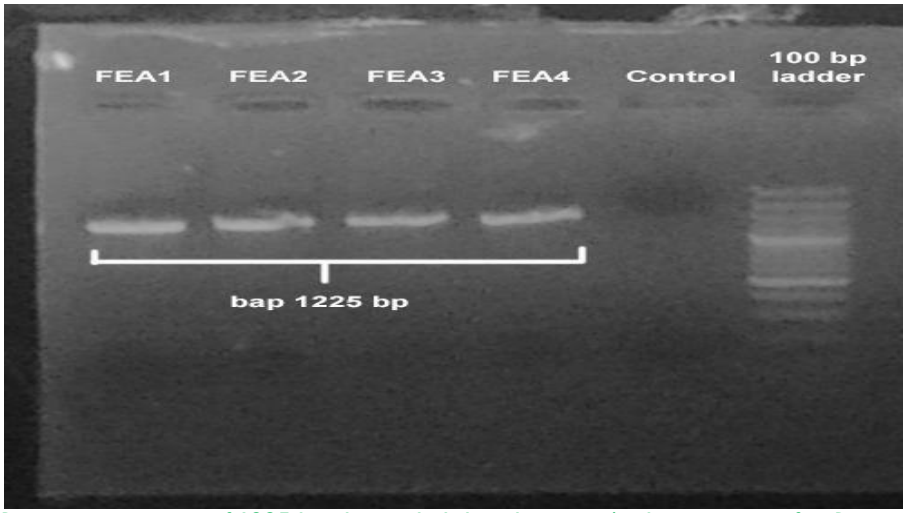


Fig 5: Gel image of *Bap* gene presence of 1225 band revealed that the stains/isolates are +ve for *Bap* genotype lane 1 to 4: isolates 1 to 4; lane 5: -ve control; lane 6: 100 bp ladder.

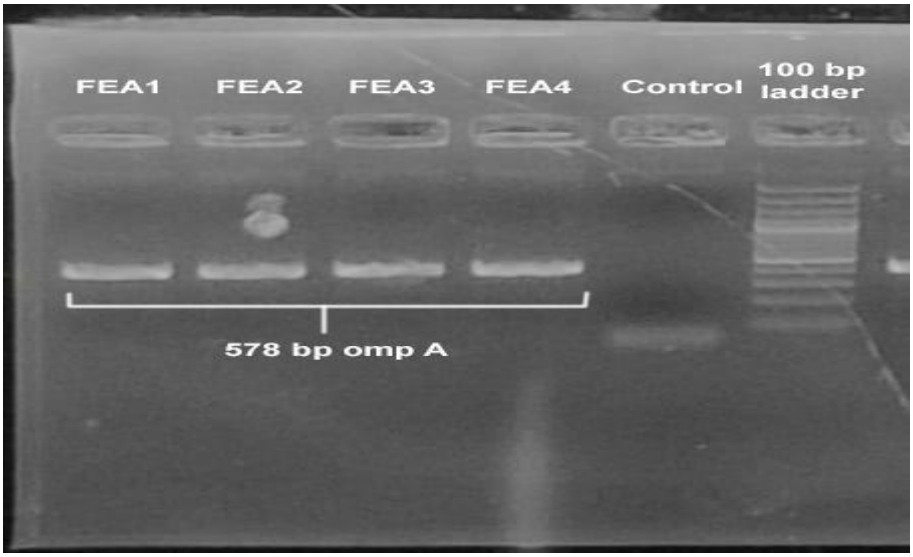


Fig 6: Gel image of ompA gene presence of 578 band revealed that the stains/isolates are +ve for ompA genotype lane 1 to 4: isolates 1 to 4; lane 5: -ve control; lane 6: 100 bp ladder.

*adeG* gene which encodes for the efflux pump in *Acinetobacter baumanii* was detected in all four isolates tested (figure 7).

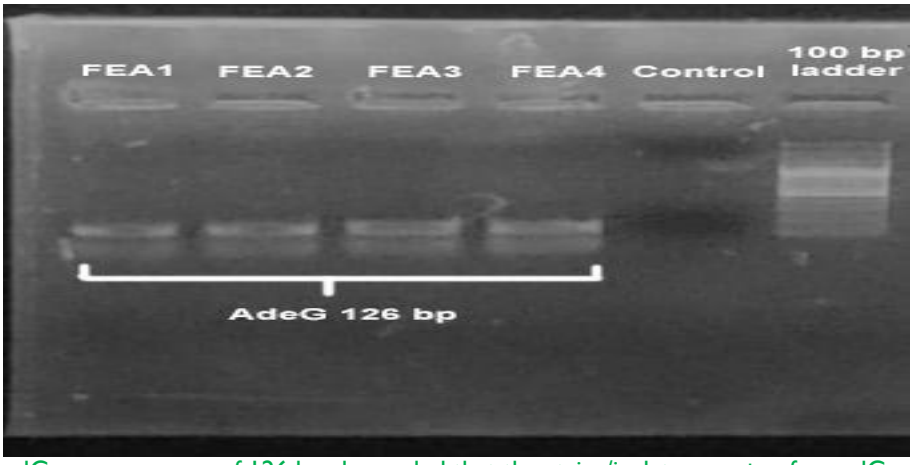


Fig 7: Gel image of aedG gene presence of 126 band revealed that the stains/isolates are +ve for aedG genotype lane 1 to 4: isolates 1 to 4; lane 5: -ve control; lane 6 :100 bp ladder.

Capsular polysaccharide is a very important virulence factor as it helps in resisting host immune response, survival inside the host etc. *ptk* and *epsA* are two important virulence genes which encode capsular polysaccharide were screened. Two out of four isolates were positive for the presence of *epsA* and only one strain was positive for *ptk*. (Figure 8 & 9)

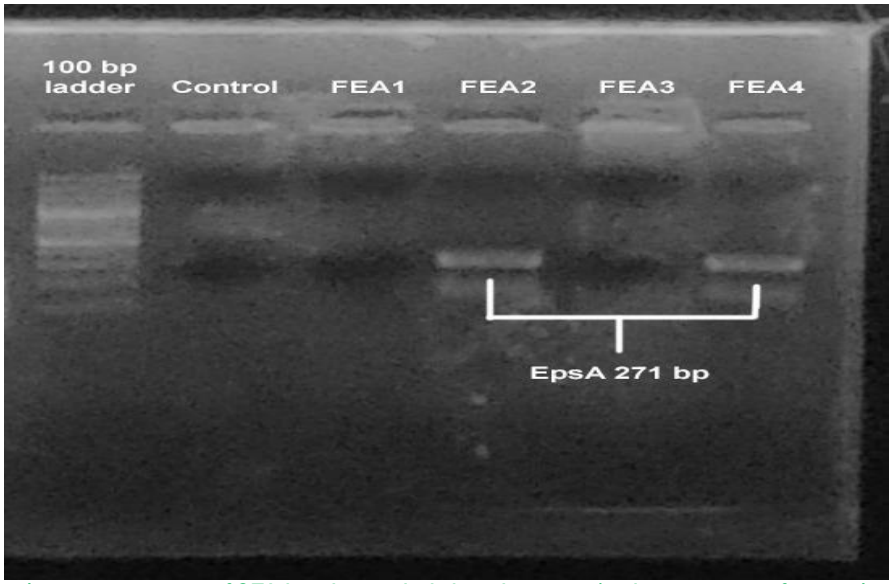


Fig 8: Gel image of espA gene presence of 271 band revealed that the stains/isolates are +ve for espA genotype lane 3 to 5: isolates 1 to 4; lane 2: -ve control; lane 1 :100 bp ladder.

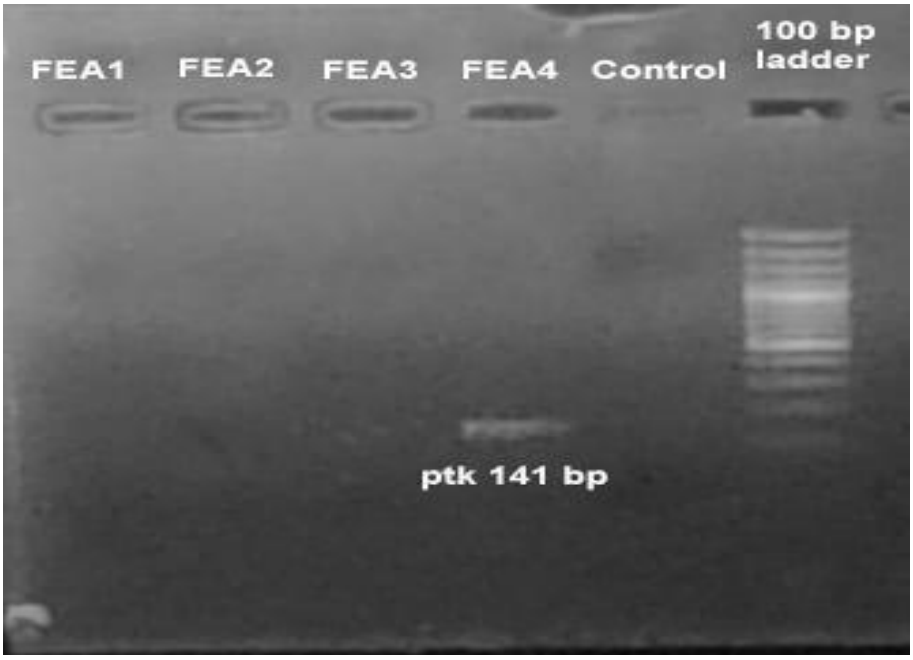


Fig 9: Gel image of ptk gene presence of 141 band revealed that the stains/isolates are +ve for ptk genotype lane 1 to 4: isolates 1 to 4; lane 5: -ve control; lane 6 :100 bp ladder.

## 4. DISCUSSION

This study was conducted in a tertiary care hospital at Kochi, Kerala and revealed that the prevalence of *Acinetobacter baumanii* infections are comparatively low in the region when compared to previous reports from other regions. Ibrahim 2019, [33]reported a very high prevalence of *Acinetobacter baumanii* related nosocomial infections in Saudi Arabia. The prevalent sample source in the present study was also in contrast with other authors. blood sample was reported as the predominant source of *Acinetobacter* infections in a study conducted by[34] Manik et al.,2000. They[34] reported that 17% of 4180 isolates were from blood samples. But in our study, the predominant source of *Acinetobacter* was urine samples (48%) and only 12.8 % of isolates were from blood samples. *Acinetobacter baumanii* was reported as the predominant species isolated by[35] Rit et al, 2012. *Acinetobacter baumanii* conferred 74.02% of total isolates and other types of *Acinetobacter* were only 25.98%. 85% of the total isolates were *Acinetobacter baumanii* in our study which is in accordance with[35] Rit et al., 2012. Similarly, 30% and 27% of susceptibilities were reported by[35] Rit et al., 2012 against gentamycin and trimethoprim sulfamethoxazole in accordance to the 33% of susceptible isolates of the present study. In 2015[38], it as reported as 53.5% of the isolates were resistant to amikacin, 83.7% to tetracyclin, 86% to ceftazidime, 90.7% to trimethoprim sulfametoxazol, 93% to cefepime, imipenem, meropenem, and ampicillin–sulbactam. Most of the CRAB isolates showed similar resistance pattern in our study also. In previous studies[36,37], maximum resistance was seen against cefotaxim, cefepime,ciprofloxacin, piperacillin tazobactam, ceftazidime, Imepenem and meropenem. The resistance pattern in our study was also similar to these studies. Persistence and survival ability of the bacteria in hospital environments made it a major cause of nosocomial infections. One of the major factors contributing to survival of *Acinetobacter baumanii* on the abiotic surfaces is the ability to form biofilm.[19,40]. Previous studies have reported a higher biofilm formation rate (80-91%) in *Acinetobacter baumanii*[41].It was also reported that antibiotic resistance and biofilm production have a positive relationship[42].These inferences were in accordance with the findings of the present study. Both the tested genes associated with biofilm production, *Bap* & *OmpA*, were present in all the four isolates of multidrug resistant *Acinetobacter baumanii*. Efflux pumps contribute to a wide range of resistance in *Acinetobacter baumanii* against antibiotics and disinfectants. Resistance against the disinfectants helps them to survive in hospital settings and resistance against antibiotics helps them in in-vivo survival[43]. Rosenfeld, 2011 [44]reported that there will be an increased expression of efflux pump genes in multi drug resistant *Acinetobacter baumanii.* Similarly all four carbapenem resistant isolates were screened positive for efflux pump coding gene, *AdeG*, in the presence study. It was indicated that *adeF*, *adeG* and *adeH* were very prevalent in *Acinetobacter baumanii* isolated from humans[45]. Approximately 90% of the *Acinetobacter baumanii* isolated had the adeg operon in a study[46], which was in accordance with results of our study. 100% of the isolates were positive for *AdeG* gene. Russo et al., 2010 [32]reported that two genes *ptk* and *epsA* were required for capsule polymerization and assembly in *Acinetobacter baumanii* which in turn helps the organism to resist host immune mechanism. *Ptk* encodes a putative protein tyrosine kinase (PTK) and *epsA* encodes a putative polysaccharide export outer membrane protein (EpsA). In accordance with these studies, in the present study two isolates were positive for the presence of the *EpsA* gene and one isolate was positive for the *ptk* gene.

## 5. CONCLUSION

The spread of Acinetobacter infections are related to several factors like duration of antibiotic usage, patient co-morbidities, virulence of bacteria, etc. The genomic studies revealed important factors of Acinetobacter which are contributing to the survival on abiotic settings and pathogenecity of *A. baumannii.* Knowledge regarding such factors are important in preparing proper prevention protocols. A local antibiogram pattern database preparation and implementation of antibiotic stewardship based on the antibiogram might help in prevention of development of antibiotic resistance. Proper disinfection protocol

development based on the virulence mechanism of the bacteria can help to restrict the Acinetobacter infection spread. Hence this study strongly recommends implementation of proper sterilization protocol and antibiotic stewardship along with the continuous surveillance of spread.

## 6. ACKNOWLEDGMENT

The authors acknowledge the management and staffs of Sunrise Institute of Medical Sciences and staff members of MR Government Arts College, Mannargudi, Thiruvarur District, Tamil Nadu, India.

## 7. AUTHOR CONTRIBUTION STATEMENT

Ms. Fiji E conceived and planned the experiments. Fiji E and Mr. Jijo G Vaghese carried out the experiments with the support from Dr. B. Anandharaj. All authors discussed the results and contributed to the final manuscript. Dr. B. Anandharaj supervised the entire project.

## 8. CONFLICT OF INTEREST

Conflict of interest declared none.

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