

## COMPARATIVE STUDY OF GELATINASE ACTIVITY AND PELLICLE FORMATION AMONG EXTENDED-SPECTRUM BETA-LACTAMASE AND NON-EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING *ACINETOBACTER BAUMANNII* FROM DIABETIC FOOT ULCER INFECTIONS

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### ABSTRACT

**Objective:** The aim of the study was to assess and compare the gelatinase activity and pellicle formation in extended-spectrum beta-lactamase (ESBL) and non-ESBL producing *Acinetobacter baumannii* isolates from diabetic foot ulcer infection (DFI).

**Methods:** A total of 42 isolates of *A. baumannii* recovered from patients of DFI from September 2016 to February 2018. Isolates were identified by the standard microbiological method and confirmed by the BD Phoenix 100 system. The antimicrobial susceptibility test was performed by the Kirby-Bauer disk diffusion method and ESBL was detected by double disk diffusion synergy test method. Gelatinase production was determined by the Luria Bertani agar supplemented with 30 g/L gelatin, and pellicle formation was determined by the Mueller-Hinton broth which is incubated at two different temperatures.

**Results:** A total of 42 *A. baumannii* isolates were multidrug resistant. Among 21 isolates, each was ESBL and non-ESBL producers. Pellicle formation at 25°C in ESBL and non-ESBL producer isolates was 47.61% (10/21) and 28.57% (06/21). Pellicle formation at 37°C in ESBL and non-ESBL producer isolates was 57.14% (12/21) and 42.85% (09/21), respectively. Gelatinase production was present in 38.09% ESBL and 28.57% in non-ESBL producers. ESBL strains were more virulent compared to non-ESBL producers among patients of DFIs.

**Conclusion:** This study showed that pellicle formation at 37°C was highly virulent due to ESBL producers. Gelatinase production was elevated in ESBL compared to non-ESBL producer isolates. This attribute of the isolates could render ESBL positive more pathogenic. Colistin and polymyxin B are the only choices of treatment for multidrug-resistant *Acinetobacter baumannii* infections.

**Keywords:** *Acinetobacter baumannii*, Diabetic foot ulcer, Gelatinase, Multidrug resistance, Pellicle.

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### INTRODUCTION

*Acinetobacter baumannii* is a Gram-negative Coccobacillus that causes nosocomial infections such as urinary tract infections, respiratory infections, bacteremia, secondary meningitis, and wound infections in immunodeficiency individuals including diabetic patients [1].

Among diabetic foot ulcer infection (DFI) and 14.28% *A. baumannii* were found symptomatic with necrotizing fasciitis, osteomyelitis, pus discharge, leukocytosis, and low immune, so it can be considered as pathogen not to be colonizer due to the threatening of limb amputation [2,3]. *A. baumannii* is the second most commonly found pathogen among non-fermented bacteria in DFIs. Peripheral arterial diseases are often present in patients of DFIs and may lead to the poor penetration of antibiotics into the lower-limb tissues, and thereby, this promotes growth of resistant bacteria [4]. Few virulence factors of *A. baumannii* have been recognized; these are biofilm formation, siderophore production, cell surface hydrophobicity, gelatinase production, and pellicle formation. Gelatinase is a protease enzyme produced by bacteria in ulcers/wound lead to break down the collagen in subcutaneous tissue, casein, hemoglobin, and other bioactive peptides and also are involved in inflammation, and contribute to virulence [5]. Pellicle formation in *A. baumannii* allows them to survive on less or nutrient-limited surfaces for several days [6]. The pellicle formation needs different proteins that are associated with pili formation such as chaperone-usher system pili and the putative type III pili [7]. The matrix of *A. baumannii* pellicles is complex, containing different extracellular polymeric substances, that is lipopolysaccharide,

exopolysaccharides, and cell surface appendages that may be contributing factors for the occurrence and prevalence of *A. baumannii* in the clinical environment which exhibits different cell properties in wound/ulcers [8]. In the hospital ward environment, pellicles could be formed in small droplets, and therefore, may involve in bacterial colonization to pathogenic for immunodeficiency or diabetic foot ulcer patients. Bacterial bunch may eventually be detached from the pellicle and move freely to cause infection [9]. Pellicle formation is more readily formed at 25°C and 37°C. Bacteria in the pellicle could revert to the abiotic surface and cause infection. Due to this reason, hospital ward disinfectants and antiseptics should target this biofilm structure and protect bacteria from external hazards [6]. Such variations and differences could pose a challenge in the development of strategies that counter biofilm formation as a convenient and general therapeutic target to treat *A. baumannii* infections in patients of diabetic foot ulcer. This study was conducted to understand the virulence factors of the ESBL, non-ESBL producers, pellicle formation, and gelatinase production by *A. baumannii* in DFI patients, which may lead to the development of secondary infections and non-healing ulcer and lead to amputation of lower limb extremities. The purpose of the study was to assess and compare the gelatinase activity and pellicle formation in ESBL and non-ESBL producing *A. baumannii* isolates from DFI.

### METHODS

A prospective 1-year study was carried out in the Department of General Surgery, Microbiology, and Yenepoya Research Center, Yenepoya Medical College, Mangalore, India. A total of 42 non-duplicate ESBL and non-ESBL

producing *A. baumannii* isolates were collected from September 2016 to September 2017 after obtaining Ethical Clearance from Institutional Ethics Committee (Reg.No.- YU172/2016). *A. baumannii* was identified using standard methods based on Gram staining, cultured on 5% sheep blood, and MacConkey agar media, and identification was done using the BD Phoenix 100 system [10]. Antimicrobial susceptibility testing of *A. baumannii* was done by the Kirby-Bauer disk diffusion method in accordance with the CLSI guidelines 2016 [11].

#### Gelatinase assay

The isolate was grown in brain heart infusion broth and incubated at 37°C for 18 h. One loopful of culture was inoculated on to Luria Bertani Agar containing gelatin (30 g/L). The plates were incubated overnight at 37°C and then cooled for 5 h at 4°C. The positive result was indicated by the appearance of a turbid halo around colonies on the medium for gelatinase production [5].

#### Pellicle or air-liquid interface biofilm (ALI) assay

Pellicle assay or ALI assay was performed by inoculating 5 ml of Mueller-Hinton (MH) broth taken two in polystyrene tubes with a single colony of isolates of *A. baumannii* with an initial OD 600 to 0.01. The tubes were then incubated for 3 days at 25°C or 37°C without shaking. Pellicle formation if any; appeared as a white layer at the surface of MH broth [7]. The negative control was used by *A. baumannii* ATCC 19606, and positive control of *Pseudomonas aeruginosa* (PA01) was maintained simultaneously.

#### Statistical analysis

Statistical analysis was performed using SPSS 23.0 version software (SPSS Inc., Chicago, IL). Descriptive statistics like frequency (%) were used for analysis. Variables were expressed as percentages. All the data were expressed as table diagrams.

### RESULTS

A total of 42 isolates of *A. baumannii*; 21 each from ESBL and non-ESBL producers from DFI were assessed in the study. All the patients were typed 2 diabetes mellitus and comorbidities with hypertension, nephropathy, neuropathy, retinopathy, and peripheral vascular diseases. The age group of these patients ranged from 30 to 80 years, and the maximum number of patients was in the age group of 44 to 64 years. Male mean age (55.55±10.25) and female mean age (52.56±10.38) were male predominant compared to female.

#### Gelatinase assay

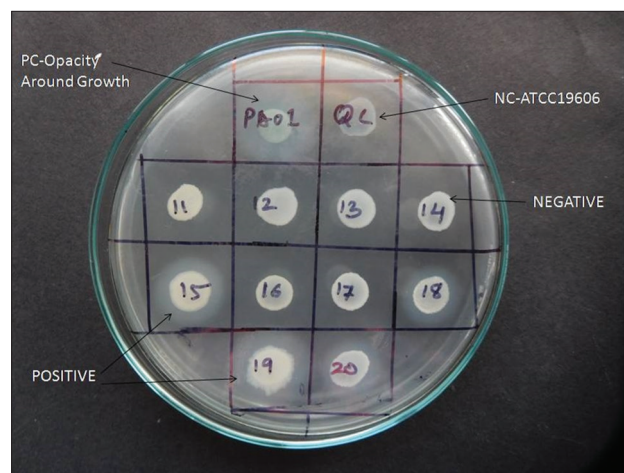
All the isolates were multidrug resistant among ESBL and non-ESBL producer in *A. baumannii* (n=21 each), positive gelatinase producers were observed 8/21(38.09%) and 6/21 (28.57%), respectively (Fig. 1 and Table 1). Gelatinase production was absent in 13/21 (61.90%) of ESBL and 15/21 (71.43%) of non-ESBL producers. ESBL producers were more gelatinase producers compared to non-ESBL producers.

#### Pellicle formation or ALI assay

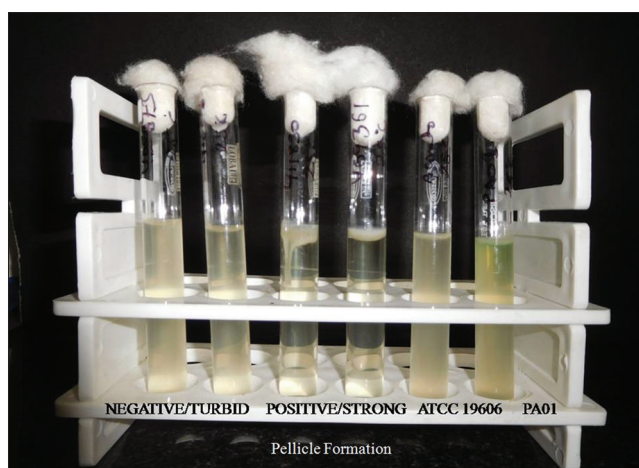
After 24 h of incubation; a thin pellicle began to form at the surface of the liquid media; which further grew in thickness and by the end of the third day; an opaque and solid structure covered the entire liquid surface. 42 *A. baumannii* from diabetic foot ulcer isolates was visually analyzed for pellicle formation, and 16 (38.09%) isolates tested positive. Among pellicle forming isolates, ESBL and non-ESBL were 10/21 (47.61%) and 6/21 (28.57%), respectively (Fig. 2 and Table 2).

### DISCUSSION

Nearly 15–25% of type 2 diabetes mellitus patients experience DFI during their lifetime. The predominance is more in males compared to female patients [12]. DFIs occur mainly due to polymicrobial and monomicrobial infections [13]. Multidrug resistance and production of ESBL by microbes and their infections are associated with the increased length of hospital stay, with higher morbidity, mortality rate, and cost burden to the patients. The virulent factors were more in ESBL isolates compared to non-ESBL isolates. *A. baumannii*, considered to be a low-



**Fig. 1:** Gelatinase production in *Acinetobacter baumannii* from extended-spectrum beta-lactamase producers isolates. DFI: Diabetic foot ulcer infection; NC: Negative control (*A. baumannii* ATCC19606); PC: Positive control (PA01=*Pseudomonas aeruginosa* 01)



**Fig. 2:** Pellicle formation or air-liquid interface biofilm in *Acinetobacter baumannii* from Extended-spectrum beta-lactamase producers isolates. PA01: *Pseudomonas aeruginosa* 01

**Table 1:** Association of *A. baumannii* in ESBL isolates among pellicle formation and gelatinase production from DFI

<i>Acinetobacter baumannii</i> ESBL (n=21)	Present (%)	Absent (%)
Pellicle or ALI at 25°C for 3 days	10 (47.61)	11 (52.38)
Pellicle or ALI at 37°C for 3 days	12 (57.14)	9 (42.86)
Both 25°C and 37°C for 3 days incubation	10 (47.61)	8 (38.09)
Gelatinase production	8 (38.09)	13 (61.90)

ESBL: Extended-spectrum beta-lactamase, ALI: Air-liquid interface biofilm, DFI: Diabetic foot ulcer infection

grade pathogen indeed has many putative virulence factors. It causes a significant major problem in immunodeficiency or diabetic patients.

Our study showed gelatinase production by 38.09% ESBL and 28.57% non-ESBL isolates of *A. baumannii*. Surprisingly, none of the earlier studies have reported ESBL and non-ESBL types in DFI isolates of *A. baumannii*. Some studies reported gelatinase production in *A. baumannii*, but it is not clear whether specimens/sample were taken from the patient with DFI or not. Hence, this study is the first report ESBL and non-ESBL production in *A. baumannii* from DFI patients.

**Table 2: Association of *A. baumannii* in non-ESBL isolates among pellicle formation and gelatinase production from DFI**

Non-ESBL (n=21)	Present (%)	Absent (5)
Pellicle or ALI at 25°C for 3 days	6 (28.57)	15 (71.43)
Pellicle or ALI at 37°C for 3 days	9 (42.85)	12 (57.14)
Both 25°C and 37°C for 3 days incubation	4 (19.04)	9 (42.86)
Gelatinase production	6 (28.57)	15 (71.43)

ALI: Air-liquid interface biofilm, ESBL: Extended-spectrum beta-lactamase, DFI: Diabetic foot ulcer infection

Cevahir *et al.* [14] reported that gelatinase activity occurred in 14% *A. baumannii* infections. Rasha *et al.* [15] reported 100% gelatinase production by *A. baumannii* isolates.

Pellicle formation assay in our study showed that among 42 *A. baumannii* from diabetic foot ulcer isolates; 38.09% pellicle forming isolates were found. Pellicle formation at 25°C and 37°C in ESBL and non-ESBL each was 47.61% and 28.57%; 57.14% and 42.85%, respectively. Similarly, Marti *et al.* [7] reported that pellicle formation at 25°C and 37°C was 35.9% and 12.2%, respectively. Another study by Chabane *et al.* [6] reported pellicle formation or air-liquid interface biofilm formation at 25°C at 30.32% in *A. baumannii* isolates. Sara *et al.* [16] reported that the *A. baumannii* group has a higher ability to form pellicle than other than *A. baumannii* and this feature could be connected to the higher pathogenic rate of *A. baumannii* and probably is contributing factor to the increased risk of clinical infection. In our study, colistin and polymyxin B only effective drug for multidrug-resistant isolates of diabetic foot ulcer patients. Similarly, Sadia *et al.* [17] reported, sensitive was 98% colistin and polymyxin B to stop the rapid spreading of ESBL producers. *A. baumannii* has a higher ability to form a pellicle or air-liquid interface biofilm than other species, it could be connected to the higher colonization rate of patients by pathogenic *A. baumannii*, and probably contribute to the increased risk of DFI. The Gelatinase is a proteolytic enzyme that is capable of hydrolyzing gelatin, which can traverse the cell membrane and hydrolyze collagen in subcutaneous tissues during wound infections. It's linked to inflammation, therefore, to contribute to the virulence on a human.

## CONCLUSION

Pellicle formation or air-liquid interface biofilm formation and gelatinase production by *A. baumannii* was high in ESBL producers compared to non-ESBL producer isolate from DFIs. Virulence factors play a significant role in multidrug-resistant *A. baumannii* infections. On that point numerous factors were studied in which the pellicle contribution was maximized to the infection from diabetic foot ulcers in patients. Understanding, these elements that influence the virulence of this organism will help in recognition of these pathogenic isolates with high or low potential virulence. Colistin and polymyxin B are the only choices of the drug against multidrug-resistant *A. baumannii* infection.

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## AUTHOR'S CONTRIBUTION

Diwan Mahmood Khan has contributed to the study conception, design, sample collection, analysis and interpretation of the data, and manuscript preparation. Dr. I. Venkatakrishna Rao and Dr. M. S. Moosabba have contributed to the study conception and critical revision of the manuscript. All the authors have contributed equally to the communication of the study, writing, and editing the article.

## CONFLICTS OF INTEREST

None of the authors have any conflicts of interest to be declared.

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