Exploring biofilm forming capacity of *A.baumannii* from different clinical sources and screening copper nanoparticles for antibiofilm activity.

By

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Project report completed under the supervision of

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June 2017

Submitted in partial fulfillment of the Degree of

Bachelor of Technology in Biotechnology

DEPARTMENT OF BIOTECHNOLOGYJAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, SOLAN

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Jaypee University of Information Technology

CERTIFICATE OF ORIGINALITY

This is to certify that the work titled " Exploring biofilm forming capacity of *A.baumannii* from different clinical sources and screening copper nanoparticles for antibiofilm activity" submitted by Chetansee Khanna in fractional completion of the necessities for the award of degree of Bachelors of Technology in Biotechnology, of Jaypee University of InformationTechnology, Solan, has been approved underneath the direction of Dr. Jitendraa Vashistt. This work has not been presented partially or completely to any other University or Institute for the grant of this or any other degree or diploma.

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ACKNOWLEDGEMENTS

I would seize this occasion to articulate my earnest thankfulness towards my guide, Dr. Jitendraa Vashistt, Assistant Professor (Senior Grade), Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, for his persistent support and unrivaled supervision, and most of all for his matchless endurance with which he chose to rear juvenile student like me at every step. His labors put in me far overshadow any of my efforts put in this project. I am truthfully thankful to him.

I am also thankful to Dr. Abhishekh Chaudhary for providing us with various nanoparticles synthesized by green synthesis, for using in my project.

I would also like to show my regards to Dr. Samir Dev Gupta, Director (Admin); Brig (Retd). K.K Marwah, Director (Academic affairs) and Dr. R.S Chauhan, Head of Biotechnology and Bioinformatics Department. I would also grab this chance to thank Ms. Nutan Thakur(PhD), Mr. Baleshwar, Mrs. Mamta, and Mr. Kamlesh for their assistance during the practical vocation at every possible step.

Signature and name of the student

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Date: 2017.

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Summary

A total of 48 *A. baumannii* strains were collected from Department of Microbiology (Bacteriology division), AIIMS, New Delhi during the study period. Clinical source of isolation of *A. baumannii* included patient's pus, respiratory and peritoneal fluid etc. Collected strains were characterized by standard biochemical assays (carbohydrate and amino acid utilization). *A. baumannii* (ATCC 19606) which is a type strain was also made in use as control against antimicrobial susceptibility analysis and biofilm production assay. Antimicrobial susceptibility test (AST) and minimum inhibitory concentration (MIC) of different antibiotics against isolates of *A. baumannii* were performed according to CLSI guidelines 2015 and ICMR standard operating procedure, bacteriology 2015. AST and MIC were done using Kirby Bauer's disk diffusion method and E- strips test, respectively.

Motility assays were performed which includes swarming and twitching to observe that if the pathogen is motile or not and to what extent motility is helping in dispersal of the pathogen in new environment. Antibiofilm activity of copper nanoparticles synthesized by green synthesis, amalgam with catharanthus-rosesus etc. was screened by antibiofilm assay.

Chapter 1: Introduction

Acinetobacter baumannii is a pleomorphic, aerobic, gram-negative coccobacillus bacterium. A health care related pathogen, which is also an opportunistic pathogen in human being, specially effecting people with immune-compromised systems due to any medical reasons [1]. These bacteria are normally correlated with water body environments [2], also it has been revealed to live and colonize on the skin as well as are now separated in huge amounts from the respiratory and oropharynx secretions of infected persons [3]. Now a days it is also exemplified as Multidrug-resistant and antimicrobial-resistant which is becoming the major reason for nosocomial (hospital derived) infections .Whereas to the scientists all over the world, its natural habitat is still indefinite [4]. From few years, it is renamed as "red alert" human pathogen, producing alertness among the medical alliance, occurring mainly due to wide and broad resistance spectrum of antibiotics [5]. With advancement in time Acinetobacter *baumannii* has appeared as a noteworthy hospital derived nosocomial pathogen, which is showing resistance to usually prescribed antimicrobials. It has newly expanded unsavory reputation as it causes some types of soft tissue infections in army men fighting and returning back from Iraq and Afghanistan. This occurrence of multidrug-resistant (MDR) pathogens has gradually emerged as solely the reason for various nosocomial and community-acquired infections [6]. The most common target of this organism is usually the pre-admitted patients in already immune-compromised state. Pneumonia which is derived from hospitals holds the most number of cases being reported due to this organism.

MDR characteristic is shown against many of the subsequent drug categories: antipseudomonal carbapenems (imipenem or meropenem), antipseudomonal cephalosporins (ceftazidime or cefepime), aminoglycosides (gentamicin, tobramycin, or amikacin), ampicillin-sulbactam and fluoroquinolones (ciprofloxacin or levofloxacin).

A Dutch microbiologist, named Martinus Willem Beigerinck, in 1911 revealed an aerobic, gram-negative, non-fermentative bacterium which we are acquainted with, is included in the genus Acinetobacter [7]. Acinetobacter started to be reported as a major nosocomial pathogen in the 1970s, though during those periods it was effortlessly treated due to its susceptibility towards prescribed antimicrobials. Whereas in the 1970s *A.baumannii* was listed to be sensitive to the majority antimicrobials, nowadays this organism displays broad spectrum resistance mechanism

to all most every first-line antibiotics [8]. Freshly, due to its apprehension in war regions, it has earned itself a label as “Iraqibacter”.

Usually, it sources outbreak of infection and health issues, together with wound infection, meningitis, pneumonia, urinary tract infection, and bacteremia [9]. Typically bacteria demonstrates motility by presenting twitching and swarming movements at times, other optional explanations for its motility is oozing of exopolysaccharide, which generates a film of elevated molecular weight sugar chains behind the bacterium to execute locomotion [10].

Chapter 2: Review of literature

Innate habitat:

Associates of the genus *Acinetobacter* are measured to be omnipresent organisms. Former verdicts have throw in to the frequent delusion that *A. baumannii* is also ever-present in nature [11]. The reality is, each kind of the genus *Acinetobacter* is not located in local environment but also majority of human extracted samples of *Acinetobacter* species always have function as pathogens [12, 13].

Epidemiology:-

Australia along with pacific islands

Primary reports of *A. baumannii* of this region showed community gained diseases, which have greatly diverse occurrence as compared to hospital-acquired illnesses. Indigenous racial setting, alcoholism, chronic obstructive airway disease, cigarette smoking and diabetes mellitus are being significant jeopardy issues. In addition to this, these mentioned strains are considerably vulnerable to antibiotics. Throat infections as well as micro-aspiration are implicated for pathogenecity of *Acinetobacter* caused infections [14, 15].

North America

There exist past cases of MDR reported happenings in this region. The organisms in this outburst were multidrug resistant, keeping hold of vulnerability only to polymyxins and ampicillin-sulbactam [16]. National supervision studies have established major method for development of MDR *Acinetobacter* species [17].

Africa

Statistics of resistance of this pathogen to various antimicrobials are usually for South Africa only, even though there are few reports from various countries [18]. Strains showing resistance are commonly found in few places (for an instance, burned regions, ICU wards etc.) which are being spread from one institution to other [19].

Latin America

Frequencies of non-susceptibility to various antibiotics like imipenem, gentamicin, ceftazidime, ciprofloxacin, meropenem and piperacillin-tazobactam in Latin America emerge to be amongst the uppermost issues [20]. A diversity of carbapenemases has also been recognized in *A. baumannii* isolates [21, 22, and 23].

Europe

Infections due *A.baumannii* have been a matter of concern in this country [24]. From 1980s, molecular typing methods specific to some regions are being used for detecting the hospital outbreaks of this pathogens infections [25]. Spreading of these strains is usually reported due to transfer of pre-infected patients from one hospital to another [26, 27].

Asia with the Middle East

Several eruption of pandrug-resistant species of *A.baumannii* are acknowledged from Asian along with some hospitals of Middle Eastern regions[28], also a range of carbapenemases are being depicted to be initiated from this region [29]. Resistance to tigecycline [30] along with polymyxin B [31] previously exists in this section.

Clinical manifestations of Acinetobacter baumannii infections

Pneumonia attained from hospital settings:

The majority of *A. baumannii* cases and the isolates derived from them are usually extracted from the respiratory routes of hospital admitted people. In most of the times, true pneumonia is difficult to be differentiated and characterized from other higher airway colonization infections. It also has been proved that ventilator-related pneumonia (VRP) is caused by this pathogen only. Classically, patients who have resided for long periods in ICU environment

are infected with *A. baumannii* infections [32], but it is contradictory in epidemic situations and conditions.

Community-gained Pneumonia

Many sultry sections of Australia as well as Asia have reported many cases of this type of pneumonia [33]. The disease most characteristically happens throughout the monsoon amid population with usual habit of alcoholism maltreatment which might cause necessary admittance in hospitals. This type of pneumonia is characterized by a lesser frequent route, lesser bloodstream related infections, and death rate ranging from 40 to 60% [34]. General cause of this infection would be throat infections, which happens in local population of around 10% with disproportionate alcoholism habits [35].

Meningitis

This type of disease is of nosocomial origin, which is also called as post-neurosurgical *A. baumannii* meningitis. More gram-negative pathogens are related in causing this disease in major of the cases [36], which makes it obvious for multidrug-resistant *A. baumannii* strains to be included in the list [37].

Traumatic Battlefield and Other Wounds

A. baumannii possibly, sporadically source either skin or soft tissue infections and diseases exterior of the military populace. These creatures caused 2.1% of ICU-acquired skin or soft tissue ailments in single evaluation [38]. It is a renowned pathogen in blaze units and could be tricky to eliminate from such patients [39]. *A. baumannii* is frequently cut off from wounds of war cases from Iraq as well as Afghanistan [40]. It was the mainly frequently secluded organism (32.5% of cases) in one evaluation of fight fatalities with usual and many tibia fractures.

Bloodstream Infection

A. baumannii was an additional reason of occurrence of ICU-derived blood infections as compared to non-ICU derived infection. This above mentioned *A.baumannii* bloodstream infection had the third peak rough mortality pace in the ICU. These types of infections were

the most recent of the entire cases of infections to happen throughout the medical environment [41]. Consequently it is not convinced whether the elevated death speed symbolizes its incidence in patients with continuing grave sickness or maybe this organism posses motility features which are not known.

Urinary Tract Infection

A. baumannii is an infrequent reason of UTI, which is accountable for just few cases of ICU-possessed UTIs [42]. Characteristically, *A.baumannii* is related with catheter-associated infection which is also commonly known as colonization. It is not standard for this organism to originate straightforward UTI in healthy individuals.

Other Manifestations

A little figure of occurrence of *Acinetobacter* endocarditis has also been reported [43]. The majority events have engaged prosthetic surgeries. *Acinetobacter*s might source endophthalmitis or keratitis, many a times, which is linked to contact lens utilization or subsequent to any sort of eye surgery [44].

Frequency

A.baumannii is also called as 'Iraqibacter' owing to its apparently sudden appearance in armed curative amenities throughout the Iraq combat. It has sustained to be a subject of worry for veterans and soldiers who doled out in Iraq as well as those in Afghanistan. Additional with transitory time, multidrug-resistant *A. baumannii* has extended to national hospitals in division owing to the transport of infected soldiers during diverse medical services [45]. From precedent years diverse surveys, it was affirmed that infection do not happen because of this bacteria at the instance of injury only, but is further probable nosocomially obtained, predictably because of the capacity of *A. baumannii* to continue on synthetic and abiotic facades for extensive stages, and the numerous conveniences to which wounded soldiers are uncovered throughout the casualty-clearance course and agenda [46, 47]. Risk reasons which adds to the possibilities of infection are protracted span of hospital wait, revelation to an intensive care unit (ICU), receiving of

perfunctory aeration, colonization strain, revelation to antimicrobial factors, existing operation, all-inclusive measures, and original severity of infection [47, 48].

Widespread of environmental infectivity is frequently illustrated, and outbursts of infection have been drawn to respiratory gears, injury treatment tasks, humidifiers, and patient concerned stuff [49, 50]. From diverse surveys the multi drug-resistance bacterial infections are normally established on a diversity of medical gears, curtains, patient cradle, area of iron, gate handles, respiratory concern equipments, tools and procedures implicated throughout wound care, brooms, keyboards etc. [51, 52, 53]. Its discovery and management of spreading is even more complex owed to its imitations, which coexists with extensive strains [54, 55]. *Acinetobacter* colonization is normally originated and noticed in patients who are protected in intensive care surroundings for extended times along with manifold intravenous lines, observing machines, surgical drains, indwelling urinary catheters .etc. in them. Yet no cultural fondness, sexual penchants and forecast for age of this bacteria is present [56].

Motility and morbidity

Mortality features and morbidity shown by *A.baumannii* infection causing strains narrate us the patient's fundamental cardiopulmonary immune position as compared to the intrinsic toxicity of the bacteria. These features and numbers in host showing various infects and illness are amplified mainly because of their own fundamental sickness inspite of the infection cause by this pathogen [56].

Antibiotics resistance mechanisms and methods

Quinolones

Alterations to DNA gyrase or topoisomerase IV throughout transmutations happening the *gyrA* gene as well as *parC* gene has been finely explained for *A. baumannii* [57]. These mutations obstruct the objective site binding. Several other important substrates are also known for multidrug efflux pumps [58], counting the pump AdeABC which is a type of RND [59] along with the AdeM which is a type of MATE pump [60].

Polymyxins

In spite of current reports on growing artificial laboratory built resistance along with hetero-resistance to the polymyxins class [61], machinery of this defense mechanism of *A.baumannii* is indefinite.

β-Lactams

Enzymatic mechanisms:

Nearly all widespread machinery of resistance of *A. baumannii* against β-lactams class is due to enzymatic dilapidation by enzymes known as β-lactamases. Though, in maintaining with the intricate scenery of this organism, several mechanisms frequently work in recital to create the similar phenotype [62].

AmpC cephalosporinases are intrinsically coded by chromosomes of *A. baumannii* strains [63] which are also recognized as *Acinetobacter*-extracted cephalosporinases (ADCs) [64].

Non-enzymatic methods:

Resistance against β-Lactam classes, together with resistance against carbapenem, alsocontributes to this methodology, containing variations in outer membrane proteins (OMPs) [65], and multidrug efflux channels [66] and variations in the similarity and appearance of penicillin-binding proteins [67].

Aminoglycosides

As described on top, the incidence of genes conventioning for aminoglycoside changing enzymes along with that of type-1 integron are extremely widespread in this pathogen [68]. This rising resistance apparatus damages combining of aminoglycoside to its intentiongenes and proteins and bestows elevated defense towards all such classes of antibiotics [69]. This class of antibiotics also plays the role of important substrates for AbeM pump, which is actually part of the multidrug and toxic amalgam extrusion (MATE) relatives [70].

Tetracyclines and Glycylcyclines

Defence mechanism against this class of antibiotics along with its imitative, are arbitrated through efflux ribosomal resistance [71]. Efflux pumps which are specific to this antibiotic class comprise regions encoded by the *tet*(A) to *tet*(E) predictors, frequently established within gram (-ve) bacteria. Separately from this type of efflux pumps, tetracylines along with glycyclines and their derivatives are vulnerable to efflux shown by various efflux schemes [72].

Remaining classes of Antibiotics

Presence of trimethoprim along with sulfamethoxazole resistance in *A. baumannii* is elevated in lots of regions worldwide [73]. 3′ end which is a conserved part of any integron normally have a *qac* gene merged to a *sul* gene, bestowing defence against many antibiotic classes [74]. As a result, resistance shown against sulfonamide has been revealed to be extremely prognostic of most of the isolates of this pathogen [75]. Likewise, resistance against trimethoprim (*dhfr*) and chloramphenicol (*cat*) are coded by some specific genes accounted in genetic structure of Acinetobacter [76]. Efflux might also supply to resistance alongside these agents [77].

Biofilm by *A.baumannii*

Biofilm is colonization of microorganisms, mostly bacteria, attaching mutually in a tertiary arrangement and presentation burly observance to the facade. These fanatic cells are habitually well-established within a self-produced environment of extracellular polymeric substance (EPS) in accumulation submitted to as slime which is a polymeric accretion typically gathered of extracellular DNA, proteins, and polysaccharides. Biofilms may form on biotic (epithelial cells, fungal filaments) or abiotic surfaces (polystyrene, glass) and can be unbridled in natural, industrial and hospital environment [78, 79]. A.baumannii's aptitude to continue for extended periods in dissimilar environmental set up acts a major function in its Biofilm arrangement [80, 81]. The purpose of extracellular polymeric matrix is typically to defend the bacteria from environmental conditions, defend from the host and also used as a resistance machinery which usually stimulated throughout the time of infections [82]. It is pragmatic that the solitary clinical isolates of these bacteria which show vulnerability towards disinfectants, antimicrobials, various

environmental stresses etc. were the ones which unite to form Biofilms which in twist confirm to be multi drug resistant and antimicrobial resistant [83]. Biofilm beginning configuration and growth is an extremely synchronized and managed procedure by each of the films planktonic counterparts.

Mechanism of formation

The catalog of the aspects that can allegedly sway the configuration of the Biofilm includes nutrient accessibility, bacterial attachments (pili and flagella), bacterial surface gears (outer membrane proteins, adhesions), and quorum sensing and macromolecular discharges (polysaccharides, nucleic acids) [84]. *A. baumannii* shows adhesion equally to biotic surfaces, w improved through $bla_{\text{PER-1}}$ gene [85]. Fascinatingly, *A. baumannii* ATCC 17978 strains showed modest biofilm formation on glass exteriors when kept to grow under blue light, whilst usual biofilm creation was pragmatic when kept again in darkness for growth [86]. This observation is arbitrated by the Bls-A photo-receptor protein, which encloses a *N*-terminal blue-light-sensing. It has been stated about varied transcription of *bls-A* at different temperature profiles differentially influences biofilm formation in response to light. Actually it appears to show a worldwide consequence on this pathogens composition, upsetting biofilm structure as well as various other features [86]. Lately, ethanol has also been suggested to affect biofilm configuration on non-living surfaces. In detail, manufacturing of proteins occupied formation of carbohydrates along with lipids was also exposed to augment in ethanol presence, thus increasing biofilm type substance and therefore increasing its biofilm creation and lessening the motility [87]. *Acinetobacter spp*. accounted to create QS-signaling molecules because of which the glueyness and biofilm forming patterns of the bacteria was influenced due to the cell populace [88]. The *A. baumannii* species established *N*-acylhomoserine lactone (*i.e. N*-3-hy-droxydodecanoyl-homoserine lactone) which is a QS molecule that is significant for biofilm formation on various surfaces [89]. ATCC 19606 strain demonstrated that manufacturing of the pilus like proteins arbitrated by the CsuA/BABCDE usher-chaperone congregation scheme was necessary for overall biofilm development [90]. In addition to this, the appearance of the *csuA*/ *BABCDE* operon was originated to be synchronized by a two-component arrangement composed by the

sensory kinase, which is mainly encoded by *bfmS* and the other one is response regulator which is further encoded by *bfmR*. Suppression of the *bfmS* sensory kinase gene showed a reduction, though not full elimination [91]. A dissimilar contribution of a worldwide transcriptional repressor, which is also a homologue and family member of the histone-like nucleoid structures, have been pragmatic in biofilm creation [92]. Also the outer membrane protein, which is the OmpA, has a key place in the configuration of this pathogen on abiotic surfaces, and in the communication of this pathogen along with other cells [93]. SEM examination of biofilm have revealed that Bap (biofilm-associated protein), at cell surfaces is necessary for three-dimensional tower construction and water channel arrangement on medically applicable surfaces, together with polypropylene, polystyrene, and titanium [94]. Mutagenesis of locus *pglC* banned the fusion of glycoprotein with capsule, consequential in irregular biofilm arrangements. The *pglC* mutant, likewise to the wild kind strain, gave birth to thick cumulates on abiotic surfaces, whereas the study of the biofilm construction exposed an uneven and messy phenotype, signifying a unfocused attachment [95]. Biofilm expansion and maturation by *A. baumannii* clinical isolates depends on poly-β-1,6-*N*-acetylglucosamine (PNAG) production which is prearranged by a group of four genes (*pgaABCD*) [96].

Aims and Objectives:

1. To check the biofilm forming capacity of *A. baumannii* isolates from different clinical sources (sputum, pus and peritoneal fluid).
2. To compare the antibiotic resistance of the isolates with their biofilm forming capacity along with their clinical sources (sputum, pus and peritoneal fluid).
3. To compare the twitching motility phenotype of the isolates with their clinical sources (sputum, pus and peritoneal fluid) and biofilm forming capacity
4. To check for the viability of the usage of copper nanoparticles also copper nanoparticles along with amalgam of catharanthus-rosesus plant extract, as an antibiofilm compound.

Chapter 3: Materials and methods

A.baumannii identification and validation by colony PCR method:

PCR assays present the prospective for fast discovery and species recognition of pathogens [97]. A colony PCR-based assay was used for this project that can identify A. baumannii. Acinetobacter species recognition was done by a spacer region called as ITS region [98]. Primers ITS-F (59-CATTATCACGGT-AATTAGTG-39) along with ITS-R (59-AGAGCACTGTGCACTTAAG-39) were made in use for amplifying an portion of this mentioned region of size of around 208 bp. For the PCR assays of the *A.baumannii* (ATCC 19606 AND 1605) isolates, the DNA template was all set by boiling technique [99]. Momentarily, single colony of an untainted and pure culture was balanced in 20µl of nuclease free water and heated at 95°C for 10 min in a thermocycler machine. Following centrifugation in a micro centrifuge (bench top lab systems) at 6000g for three minutes, after which the superenatant collected was kept at -20ºC. PCR was performed with 2µl of template DNA (in duplicates) in a total reaction volume of 5µl consisting of 7.5µl of master mix, 0.5µl (F and R both) primer and 4.5µl of nuclease free water to make up the total volume. The PCR program consisted of an initial denaturation step done at 94°C for 5 min, which was repeated for about 35 cycles of final denaturation done at 94°C for 1 minute, annealing was performed at 58°C for 30 sec time, and an initial extension which was kept at 72°C for 30 sec time, with a final extension step as the last step at 72°C for 5 minutes. A thermocycler PCR was used.

Table 1: Reconstitution (1:10 dilution) of lyophilized primers:

Primer	Yield(nano-moles)	Working volume(µl)
ITS-F	40.7	407
ITS-R	25.7	257

The primers was synthesized and lyophilized by EUROFINS genomics for this project.

PCR gel electrophoresis:

The PCR amplified products with the ITS gene primers were seen and verified on 1.5% Agarose gel in 150 ml of 1X TAE buffer. To which 10µl of ETBR was added. 15µl of the above gained PCR samples was used in the well, run with a 100bp ladder (NEB) as a reference and standard.

Biofilm formation and quantification:

In totality 48 *A. baumannii* strains were gathered from Department of Microbiology (Bacteriology division), AIIMS, New Delhi during the examining stage. Clinical sources of isolation of *A. baumannii* incorporated patient's pus, respiratory and peritoneal fluid. Assembled strains were typified by typical biochemical assays (carbohydrate and amino acid utilization). Two standard strains of *A. baumannii* (ATCC 19606 and ATCC 1605) was also used as controls for antimicrobial susceptibility examination and biofilm creation assay [100].

Biofilm formation *in A. baumannii* is to be studied on two strictures:

- Time dependent quantification of biofilm (24 hours & 48 hours) {4 plates).
- Temperature dependent quantification of biofilm (24 hours at 37°C and 44°C, 48 hours at 37°C and 44°C) {8 plates}.

Culture Preparation:

Every *A.baumannii* strain was matured independently in Luria-Bertani medium (LB) overnight at 37°C and every sample from the above medium was again freshly-cultured for about 3X in LB for a time period of 24 h at 37º C along with shaking (200 rpm). These required cells were produced from above mentioned overnight grown bacterial cultures by the means of centrifugation performed at 3,600 *g* for 30 min at 4° C. Followed by washing of cells, which is performed 2 times in sterile 1x PBS [pH 7.2] and the bacterial cell pellet was then re-suspended in PBS again, which was further used as the inoculums. For the corroboration of the bacterial populace in the inoculums, McFarland Nephelometer Standards No. 0.5 was made and used [101]. Two hundred micro liters of the washed culture at 0.2 O.D. were used as the inoculums (6.0 logs CFU) (0.D. 0.1). Inoculation of sterile 96-wells polystyrene plates with 200 microL of each above prepared bacterial cell suspension (6.0 log CFU) was done.

Following incubation of the mentioned prepared culture plates at 37° C and 44° C for 24 and 48 hours, which is devoid of any agitation for biofilm production, the planktonic cells stuck on the sides of plate is washed properly, and with rigor, with already prepared PBS solution to detach these cells from the walls which was followed by keeping the plate upside down to dry followed by staining step with freshly prepared and filtered 1% crystal violet stain and kept for 15 min. The wells were washed once again with 200µl of freshly prepared ethanol-acetone solution (80:20 v/v) to solubilize the crystal violet dye complex and the biofilm produced in the wells. The optical density is then preferred to be taken at 570 nm (OD 570) which was determined using BIORAD spectrophotometer. Each step was performed three times and the average optical density was calculated for further use [102].

The following values were allocated for biofilm determination:

* Classify isolates as biofilm-forming if they capitulated the OD values that were at least twice those of the negative controls.

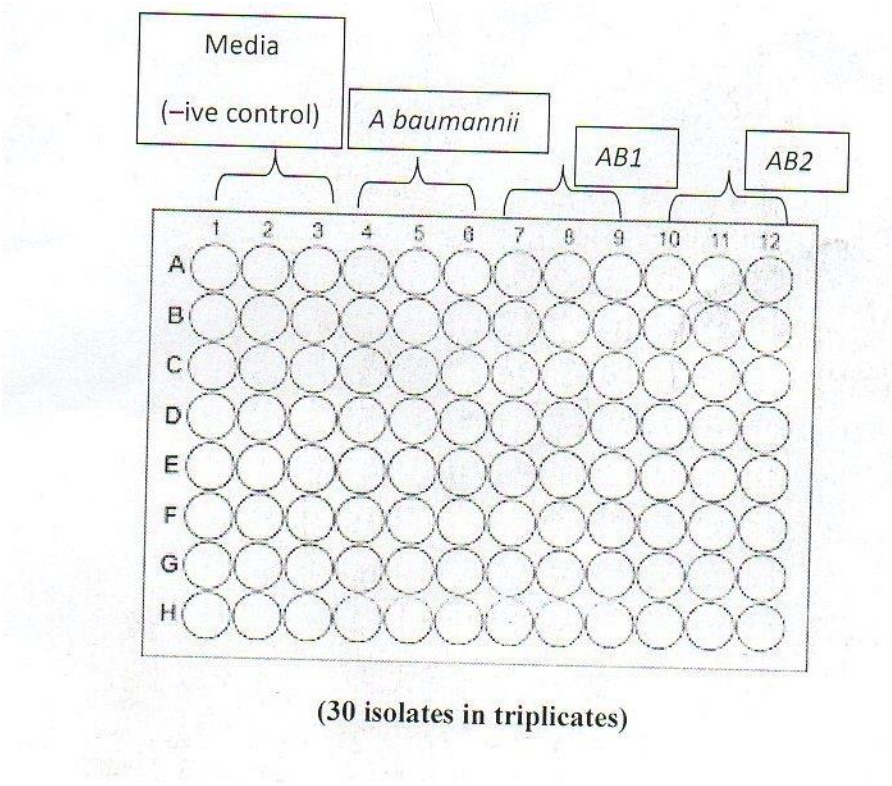
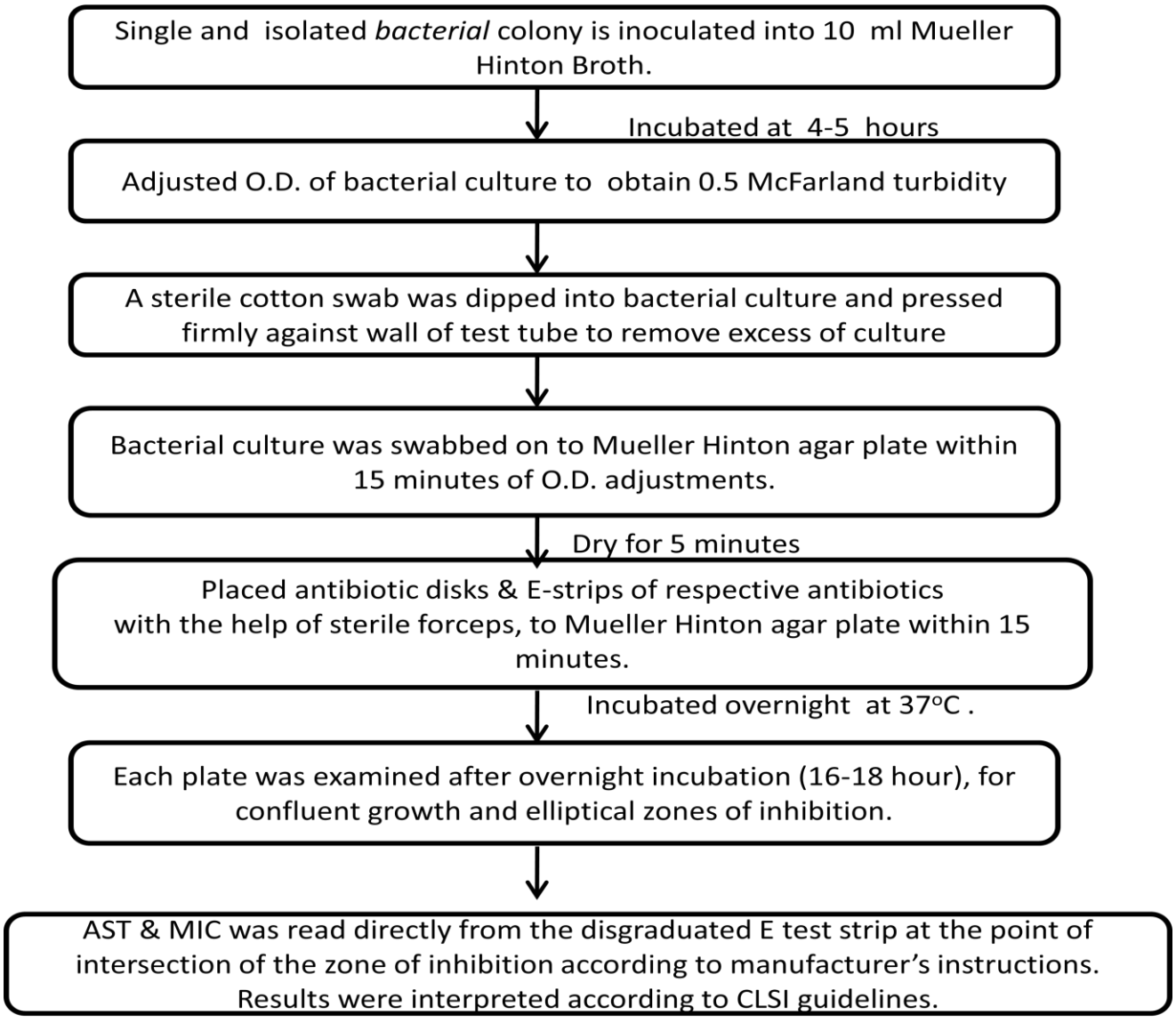


Fig 1: 96-well plate with allocation of isolates in triplicates

Biofilm creation capability in *A. baumannii* strains was also observed under bright field microscopy. Microscopic analysis also holds and authenticates the different capacities of biofilm producing isolates, qualitatively.

Antibiotic susceptibility test (AST) and minimum inhibitory concentrations determination (MIC):



Antibiotics were chosen according to ICMR guidelines: piperacillin tazobactum, cefepime, levofloxacin, tetracycline, amikacin, imipenem, meropenem, cefoperazone sulbactum, ceftazidime [103].

Motility Assay:

Modified LB broth in the company of 0.4% w/v agar was made in use, for the motility assays done in the project for *A.baumannii* strains. A single isolated colony of bacterium is inoculated into 10 ml Luria broth at 37ºc for overnight. Newly grown cultures were pierced to allow multiply and spreading of bacteria on the surface of the medium (which is 0.4% semisolid as mentioned above) that is only done for swarming type of motility and at the junction between the base of the Petriplate and L.B-medium (which is 0.8% semisolid) which is performed for twitching motility [104]. Plates were equipped on the similar day as on which inoculations are done. Subsequent to the above procedure, the plates were covered properly with parafilm and further incubated at 37°C for 48 hours.

For elucidation of isolates showing positive results, were announced to be the strains which demonstrated a region of >10 mm about the location of inoculation. For the twitching type of motility, after the inoculation, the agar was useless and thrown away, and the plates were tainted with freshly prepared 0.2% crystal violet previous to apparition and snapped. Assays were executed at least3X, for every possible isolate for confirming the results [105].

Antibiofilm activity of nanoparticles:

Antibiofilm activity of two nanoparticle solutions was perfomed by using a standard *A. baumannii* isolate, namely *A. baumannii* ATCC 1605 (multidrug resistant). Two nanoparticle solutions were taken in the study: sample 1 was nanoparticles synthesized from copper, plant extract and potassium iodide (KI) (0.5 M), sample 2 was nanoparticle made with copper combined only with potassium iodide (0.5 M). From this concentration working dilutions were made of 2mM, 1mM and 0.5mM for the test sample. Plain culture of ATCC 1605 was taken as negative control in a 96-well plate. Then the nanoparticles along with calculated volume of cultures to sum to 100µl are placed in the 96-well culture plates. After which plates were kept for incubation for biofilm production and their destruction due to antibiofilm particles, at 37ºc for the required time profiles. At the completion of incubation the plates were given 1X PBS

washing, twice, followed by staining each well with 1% crystal violet and keeping the stain for 30 minutes after which again single washing of PBS was given.

Biofilm formation restriction property of the above mentioned samples was checked by performing light microscopy and imaging of each well and plate. This was followed by dissolving the cells and biofilm with ethanol-acetone (80:20 v/v) solution and the O.D of the plates were taken at 570nm and recorded, to quantify the biofilm formation.

Table 2: required concentrations of nanoparticle sample 1 as well as for sample 2 with required *A.baumannii* (for ATCC 1605) culture volumes.

Molar concentration(mM)	Nanoparticles(µl)	Culture(µl)
2	0 (negative control)	100
2	51.2	48.8
2	25.6	74.4
2	12.8	87.2
2	6.4	93.6
2	3.2	96.8
2	1.6	98.4
2	0.8	99.2
2	0.4	99.6
1	0.2	99.8
0.5	0.2	99.8

The antibiofilm activity was studied for four different time profiles .i.e. is for 2 hours, 4 hours, 6 hours and 8 hours.

Chapter 4: Results

Colony boiling PCR method:

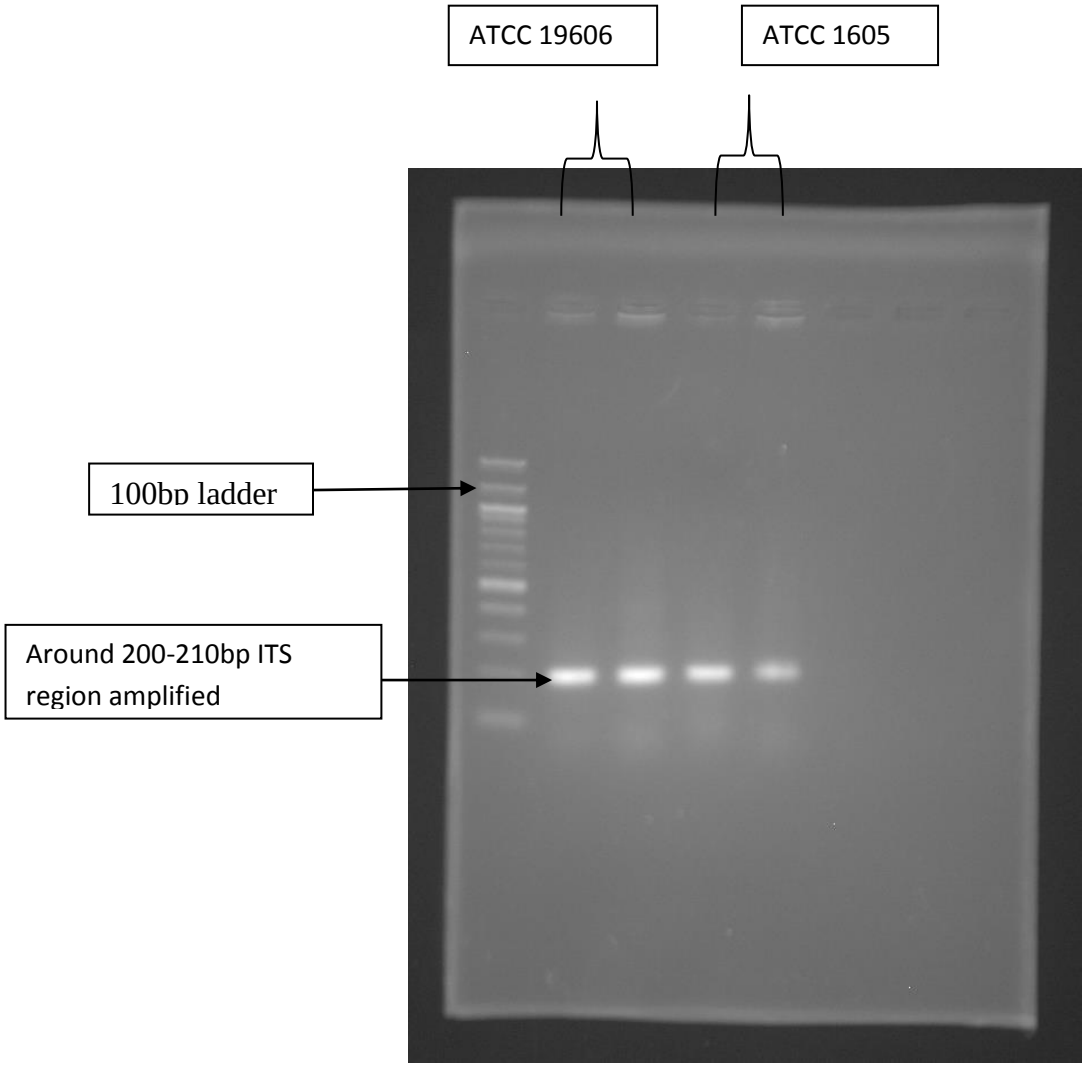
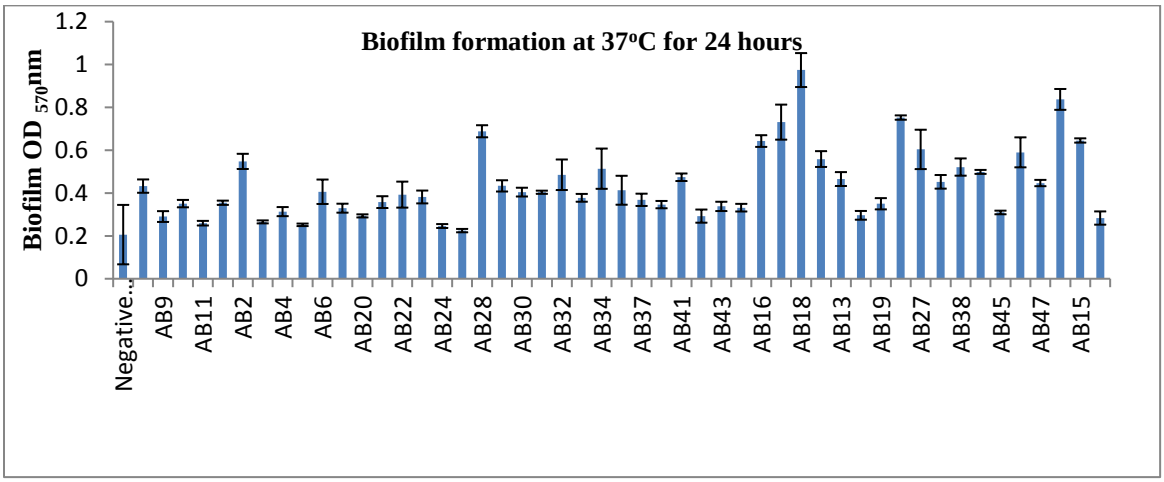


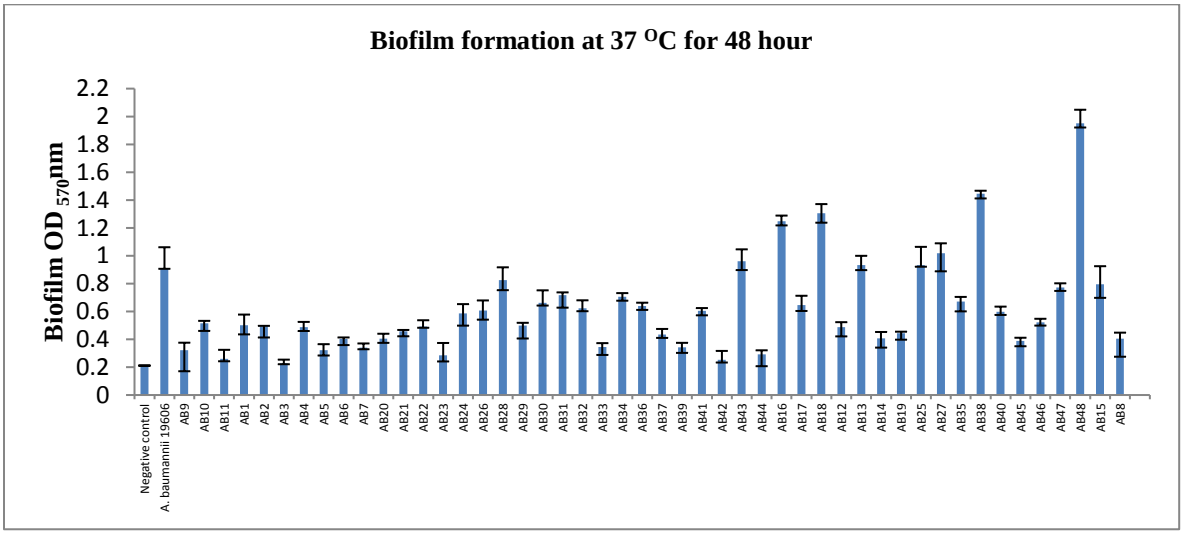
Fig 2: PCR gel electrophoresis with amplified ITS region bands of around 200bp size.

Biofilm formation and quantification:

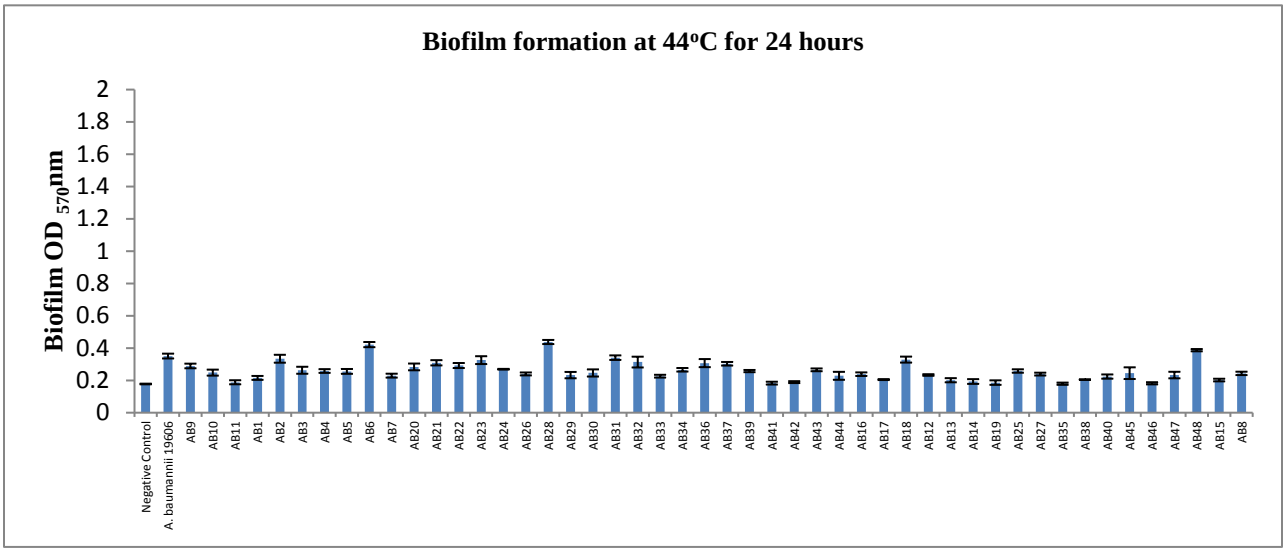
The graphs after plotting the observed O.D for the mean, standard deviation and standard error for triplicates of each strain and sample were:



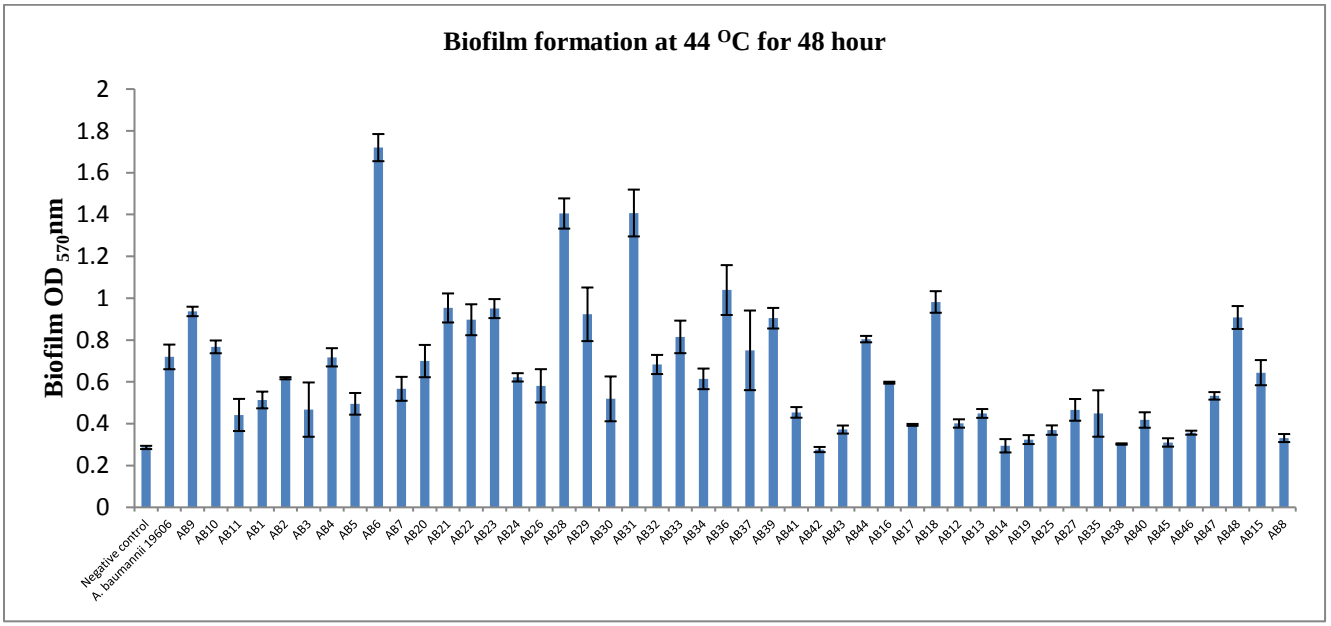
(A)



(B)



(C)

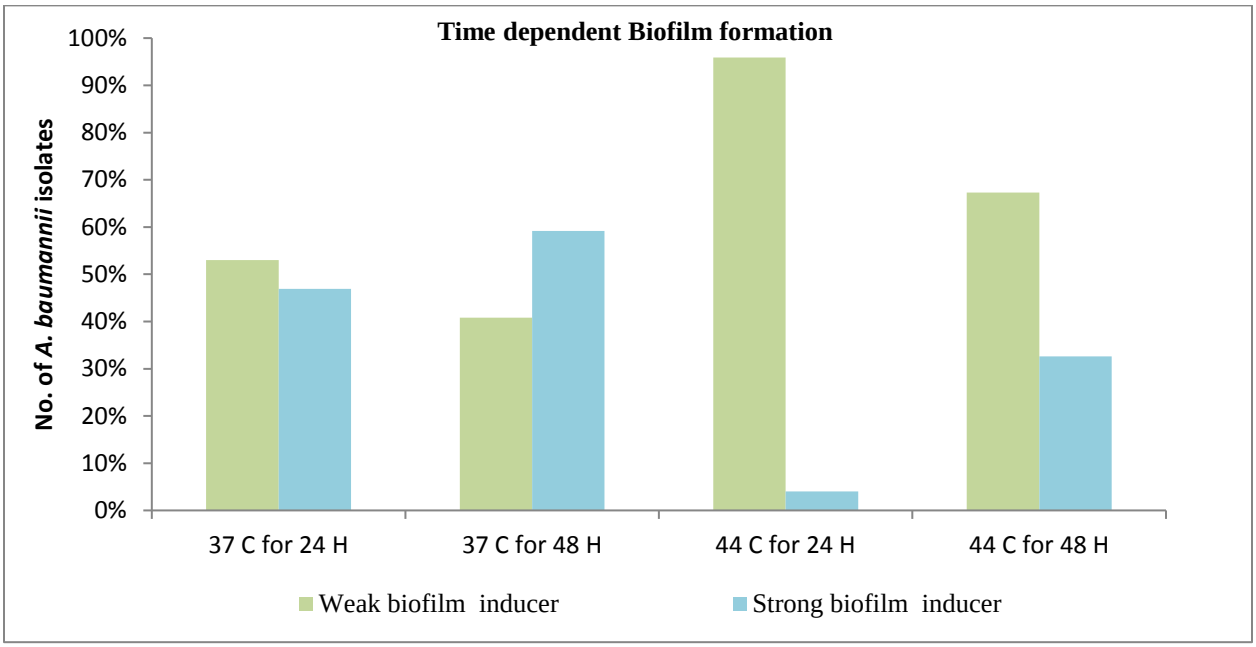


(D)

Fig 3: A, B, C and D: Quantification of biofilm mass by crystal violet assay. Bar diagram shows OD_{570} mean ± SE (y-axis) against different *A. baumannii* isolates on (x-axis). Each value represents the triplicate of experiments. Panel A& B represents biofilms production at 37^0C (24 h and 48 hours) while C&D shows the biofilm expression at 44^0C for 24 and 48 hours.

	No of *A. baumannii* Strains			
	37 C for 24 H	37 C for 48 H	44 C for 24 H	44 C for 48 H
Weak biofilm inducer	53%	40.80%	95.90%	67.30%
Strong biofilm inducer	46.90%	59.18%	4%	32.60%

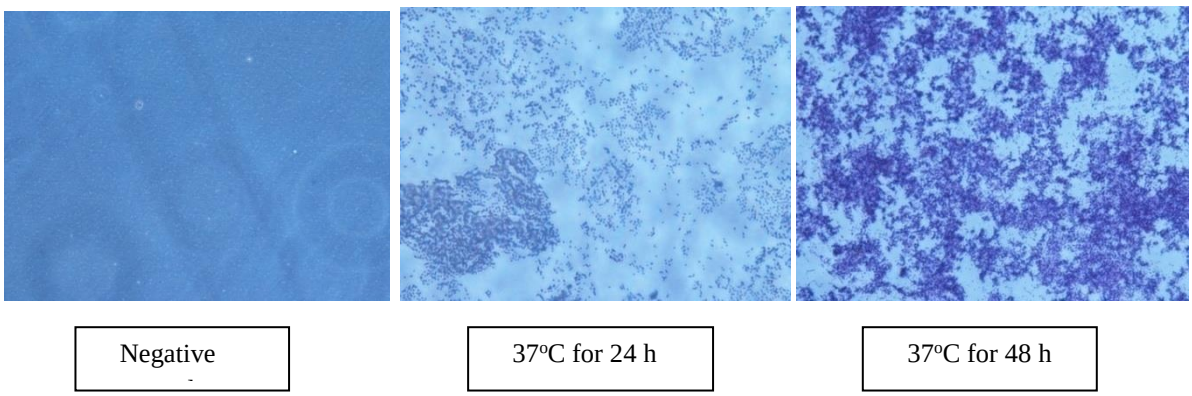
Table 3: Comparison of percentages of weak and strong biofilm formers with respect to time.



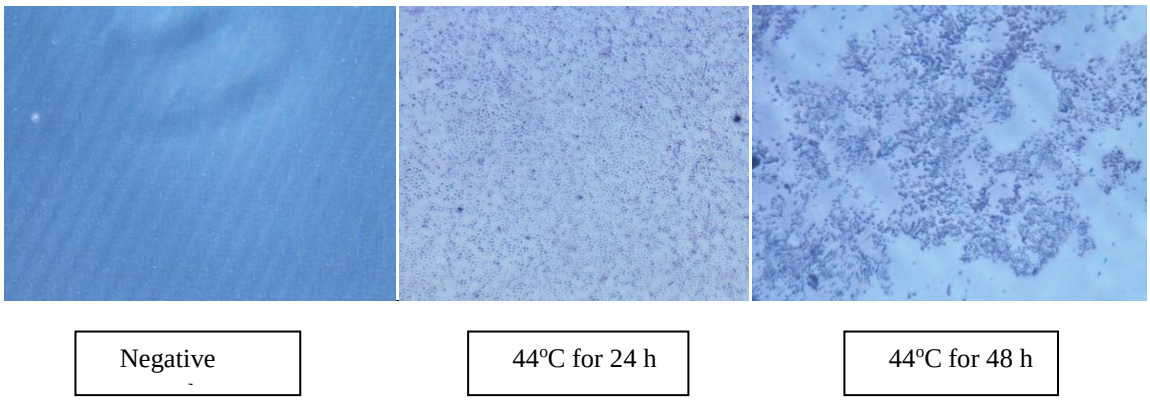
(E)

Fig 4: image E is Time dependent analysis of biofilm producing ability of isolates of *Acinetobacter baumannii***.**

Ability to form biofilm at elevated temperature combined with multidrug resistance, might contribute to the survival of these organisms and their dissemination in the hospital environment.



Panel A.



Panel B.

Fig 5: Panel A and B are time and temperature dependent bright field microscopy analysis (400X) of *A. baumannii* isolates attached to polystyrene surfaces with crystal violet staining (1% w/v for 10 minutes). Panel (A) represents biofilms grown at 37^0C for 24 and 48h and negative control while, panel (B) shows biofilms grown at 44ºC for 24 and 48h and negative control.

AST and MIC results:

Table 4: Antimicrobial susceptibility patterns of *A. baumannii* isolated from different sources.

Source	Antibiotics	PTZ	LEV	TE	CPM	CAZ	CPS	MRP	IPM	AMK
Respiratory	S	4%	8%	20%	8%	16%	8%	12%	4%	24%
	R	81%	78%	78%	81%	81%	74%	67%	85%	78%
	I	16%	12%	4%	12%	4%	20%	20%	8%	0%
Pus	S	33%	0%	33%	0%	0%	33%	33%	0%	33%
	R	33%	100%	33%	100%	100%	33%	33%	100%	67%
	I	33%	0%	33%	0%	0%	33%	33%	0%	0%
Peritoneal fluid	S	50%	50%	50%	50%	50%	50%	50%	50%	50%
	R	50%	50%	50%	50%	50%	50%	50%	50%	50%
	I	0%	0%	0%	0%	0%	0%	0%	0%	0%

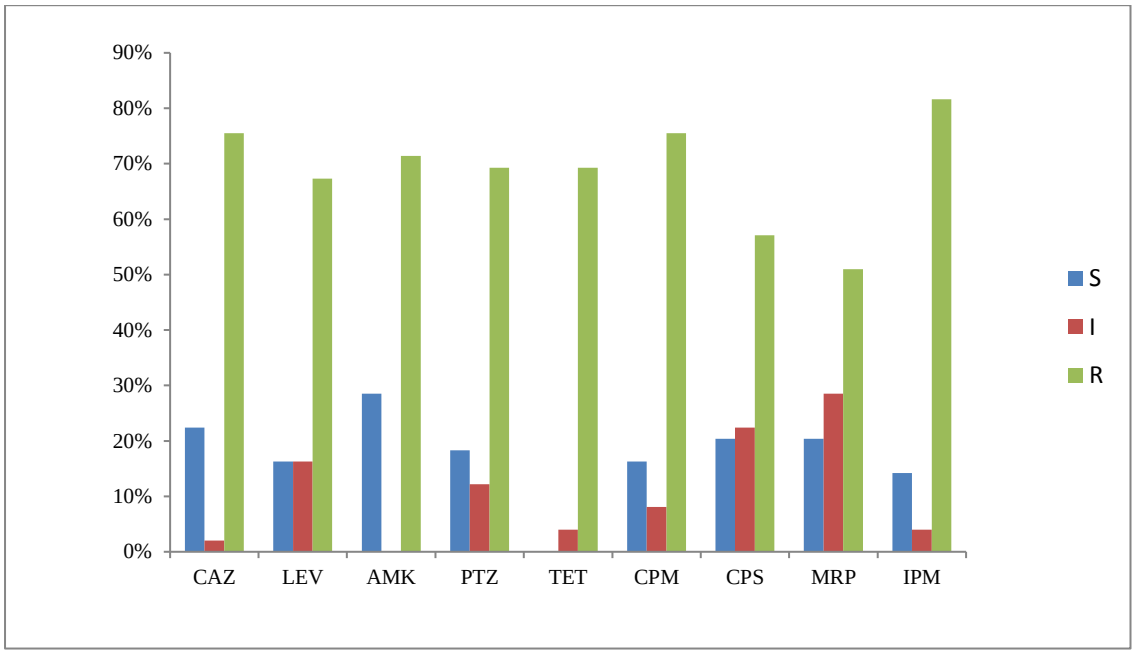
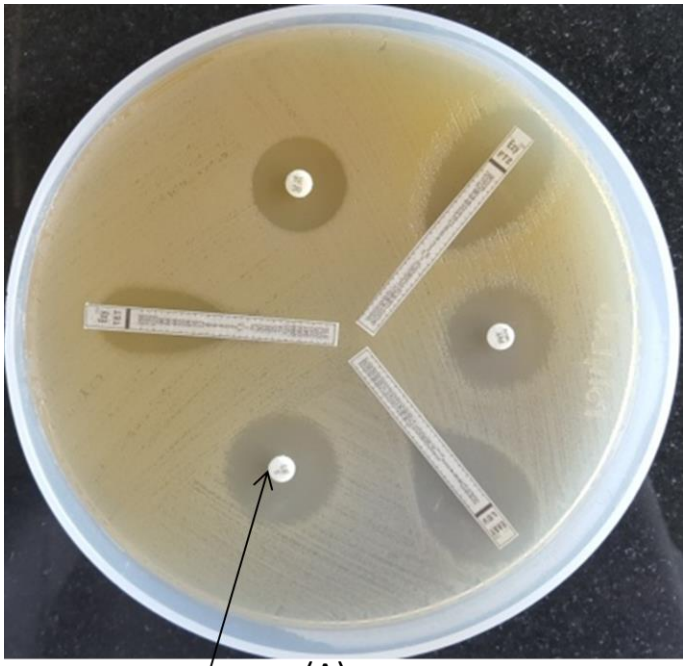


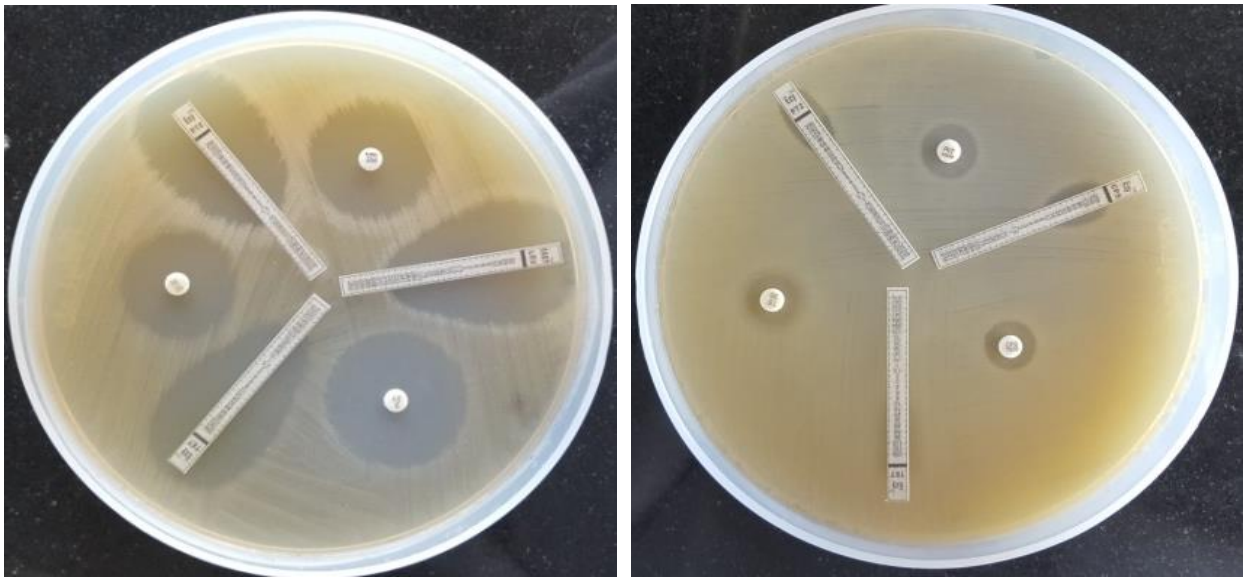
Fig 6: Antibiotic resistance phenotypes of *A.baumannii* isolates. X-axis represents different *A. baumannii* isolates and Y- axis represents percentage of isolates S=Susceptible, I=Intermediate and R= Resistant.

CAZ –ceftazidime, LEV-levofloxacin, AMK- amikacin, PTZ- piperacillin tazobactum, TET-tetracycline, CPM-cefepime, CPS-cefoperazone sulbactum, MRP- meropenem, IPM- imipenem.

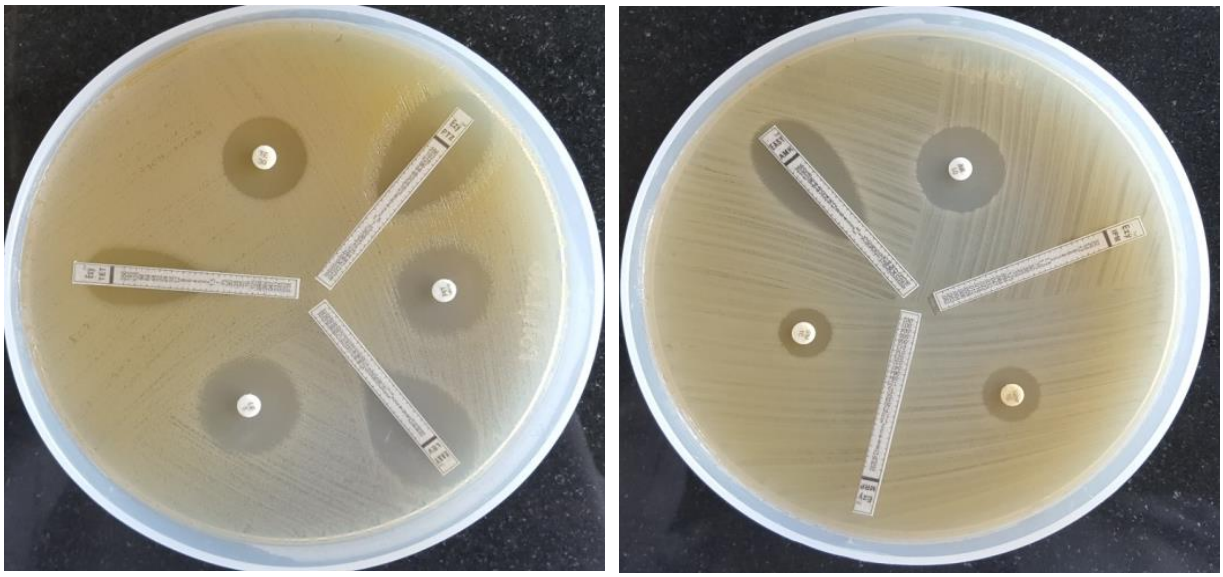


(A)

Zone of lysis



(B)



(C)

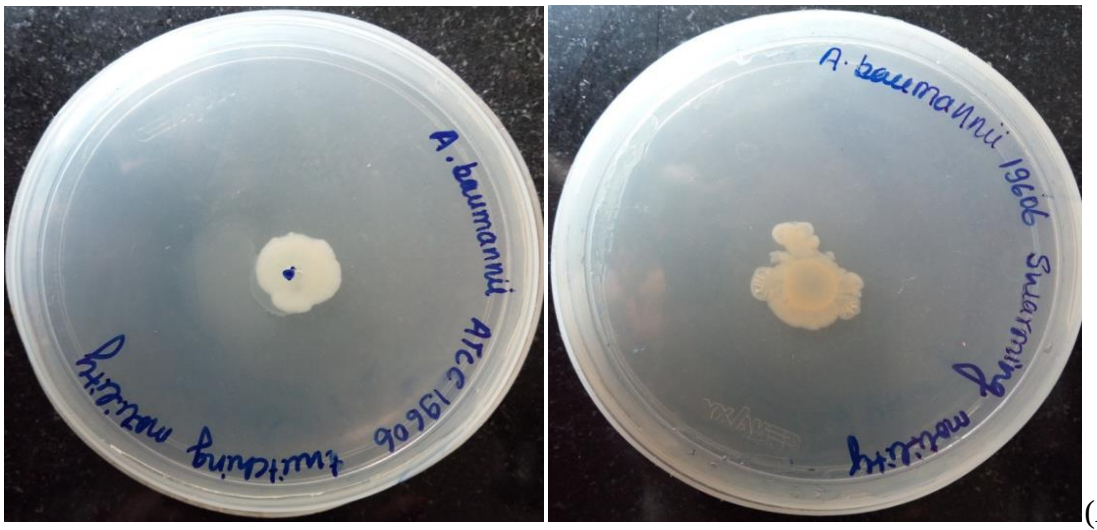
Fig 7**:** Comparative analysis of antimicrobial susceptibility patterns and minimum inhibitory concentrations of *Acinetobacter baumannii*

(A) Type strain ATCC 19606;

(B) Drug susceptible strain AB41 & drug resistant strains AB32 from Respiratory isolates.

(C) Drug susceptible AB17 and drug resistant AB18 strains from Pus isolates.

Motility assay:



(A)

(B)

Fig 8: Representing twitching and swarming motility assays performed in semisolid 0.4% modified LB agar.

Panel A: represent non motile multidrug resistant control strain *A. baumannii* ATCC 1605.

Panel B: represent *motile A. baumannii* isolate from respiratory source.

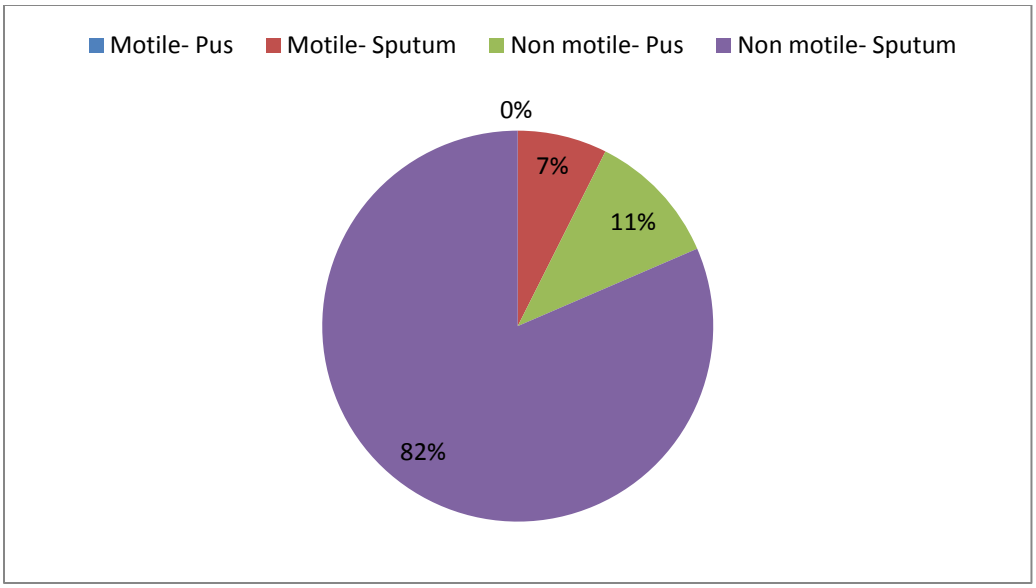


Fig 9: pie chart showing comparison of motility percentages of *A.baumannii* isolates of pus and sputum sources post-staining.

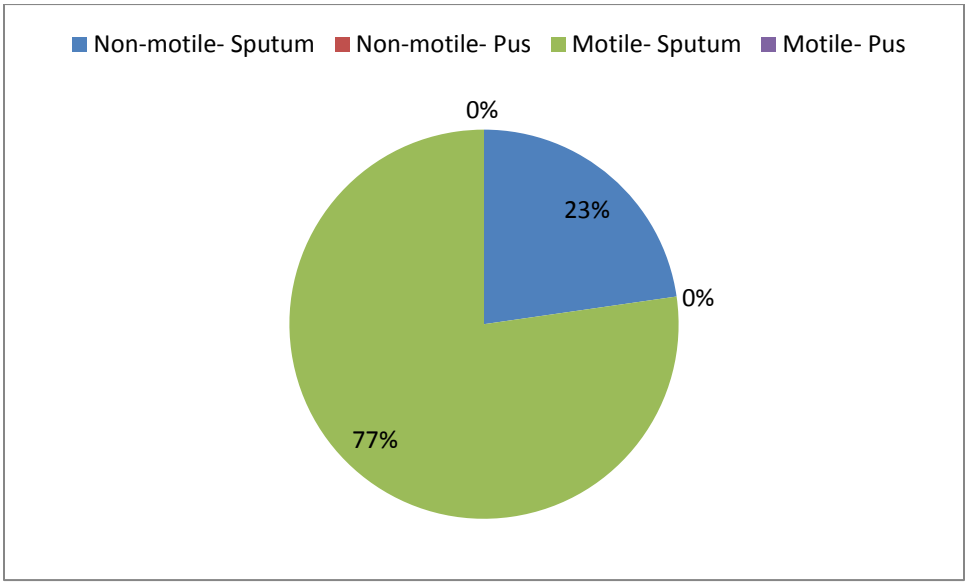


Fig 10: comparison of motility of different *A.baumannii* isolates from different sources before staining step.

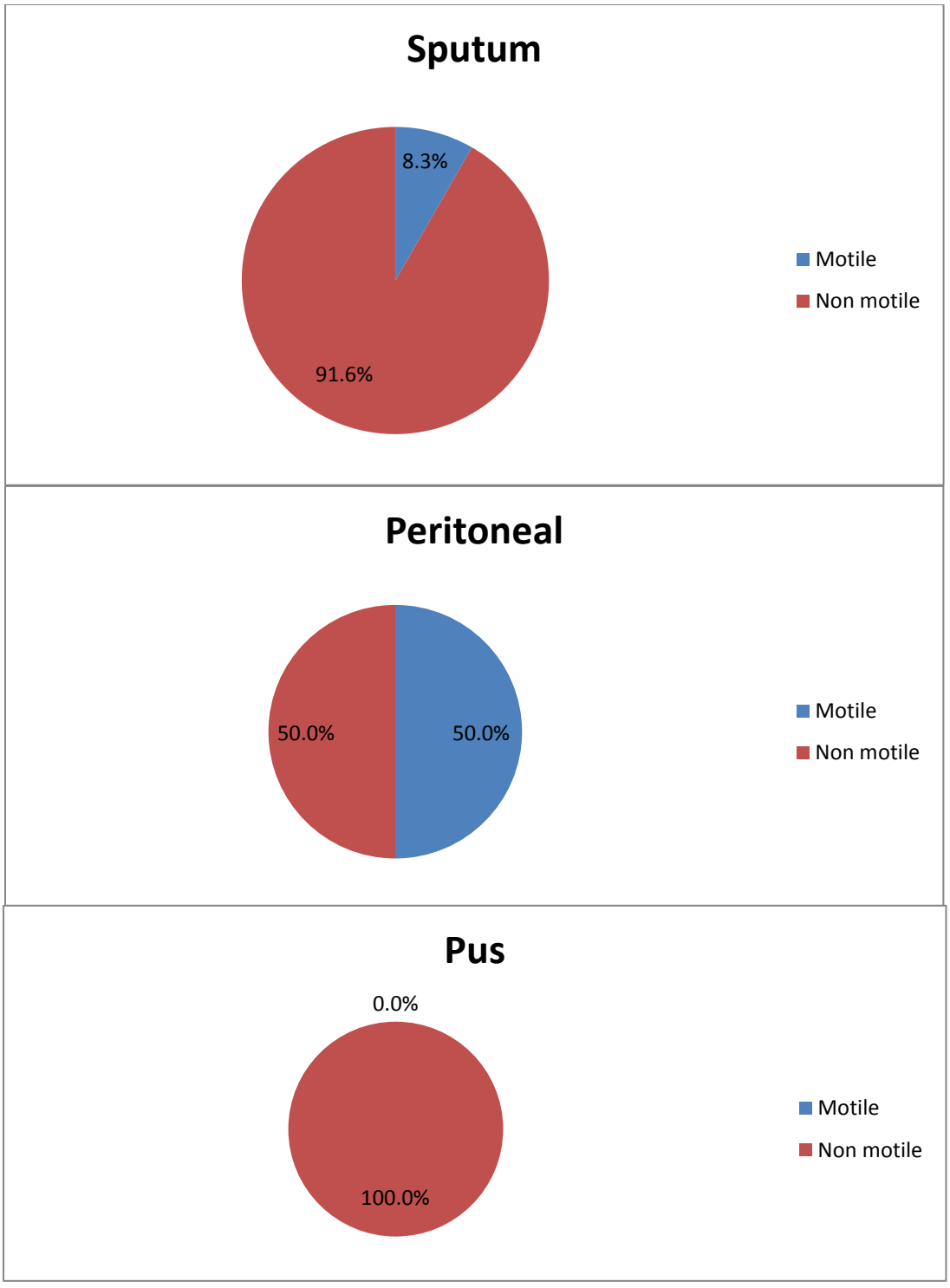


Fig 11: individual pie charts showing motility of isolates from various sources.

sources	Motile (%)	Non-motile (%)
sputum	8.3	91.6
pus	0	100
Peritoneal fluid	50	50

Table 5: percentage representation of motility results of different sources isolates.

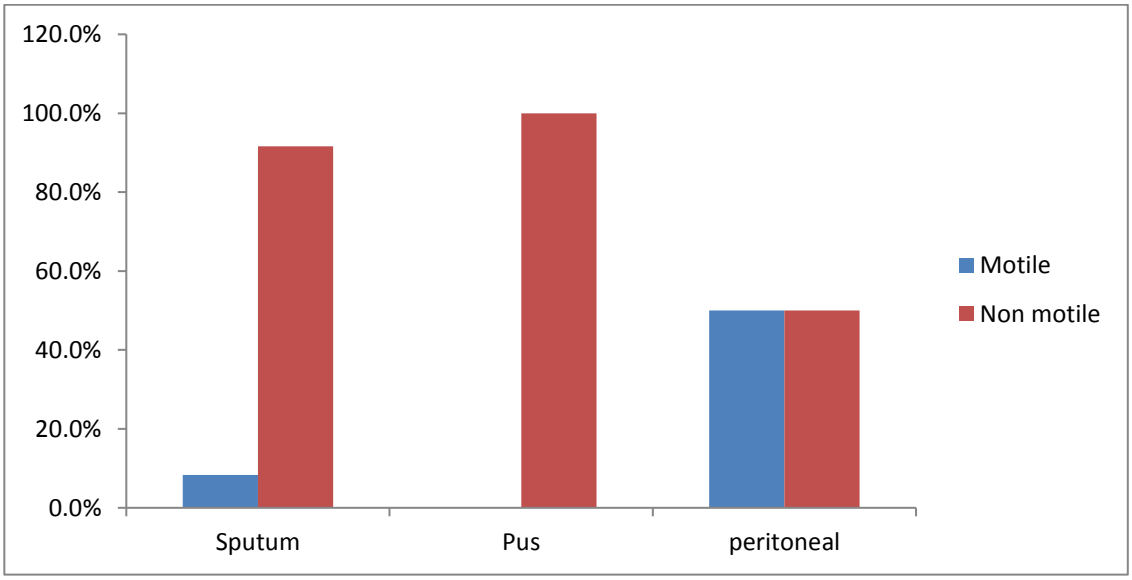


Fig 12: comparison of motility of different sources isolates of *A.baumannii* in single bar graph

Antibiofilm assay:

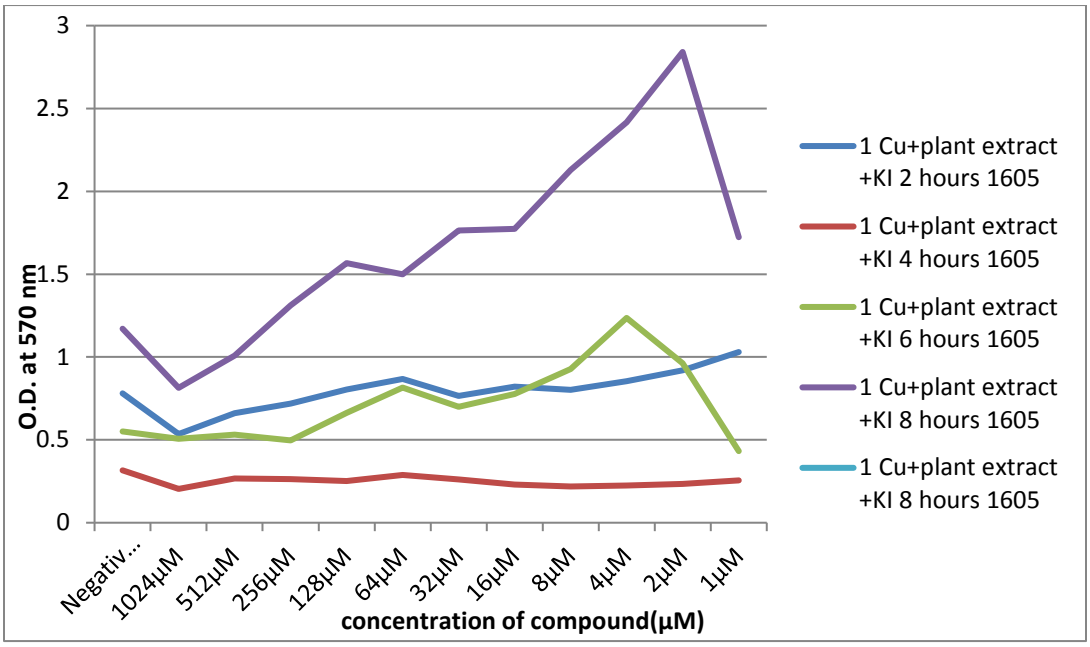


Fig 13: quantification of antibiofilm assay of sample 1(copper + catharanthus-rosesus plant extract + potassium iodide) at three variant temperate profiles i.e. at 2hours, 4hours, 8hours.

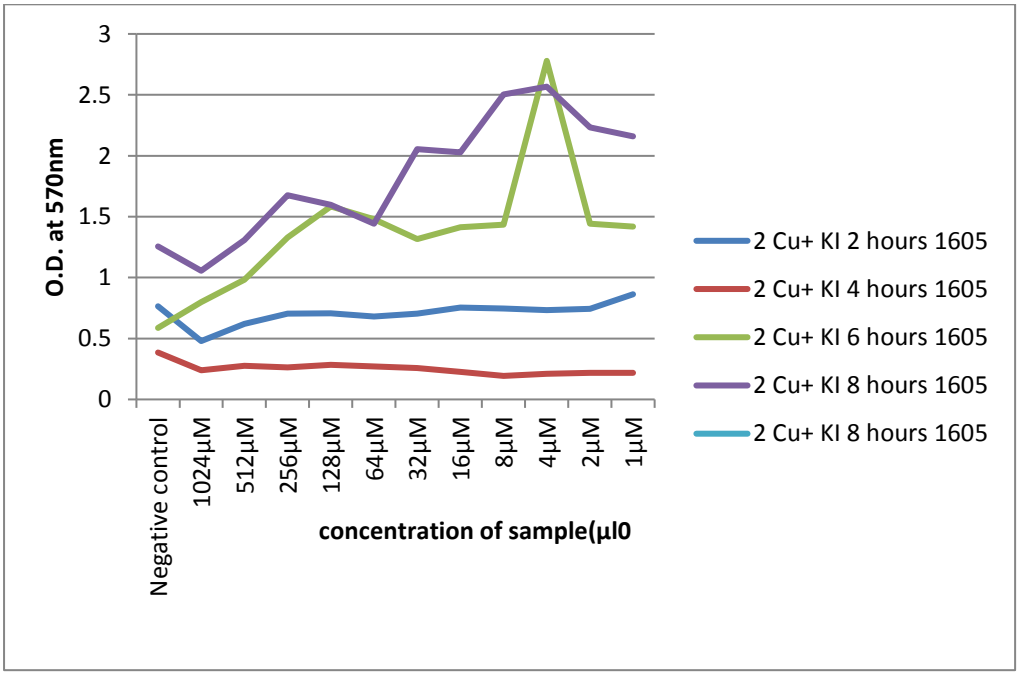
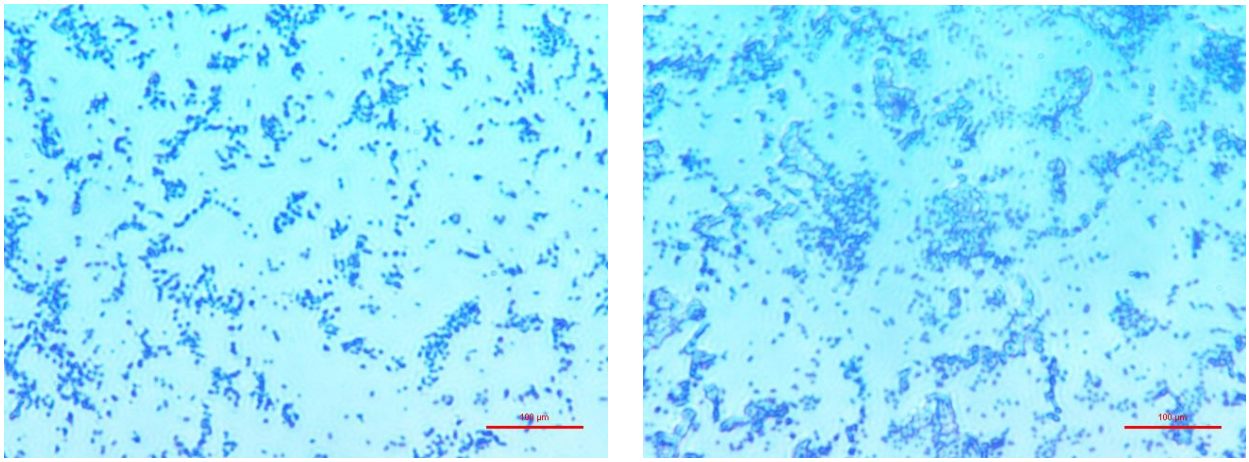
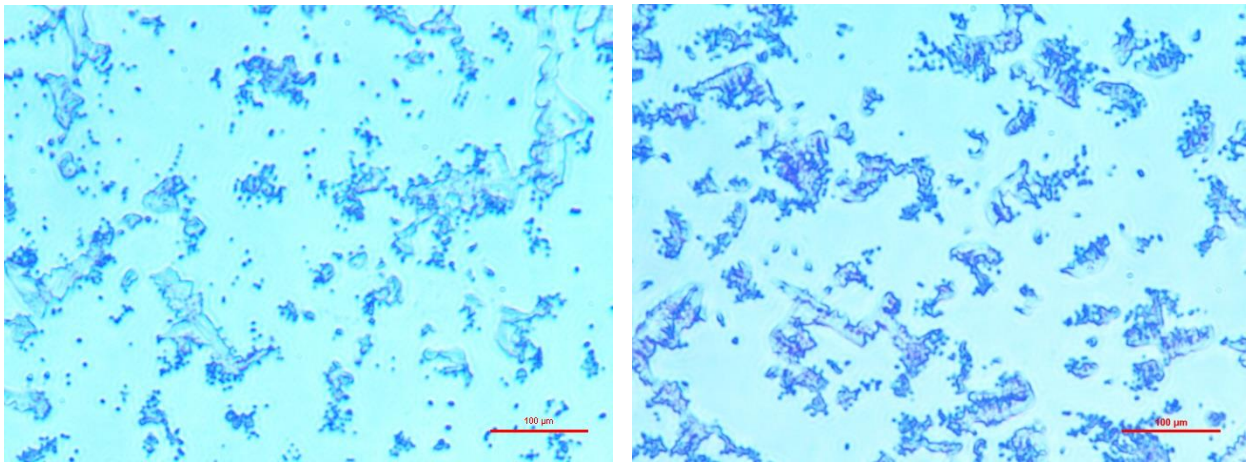


Fig 14: quantification of antibiofilm assay of sample 1(copper + potassium iodide) at three variant temperate profiles i.e. at 2hours, 4hours, 8hours.



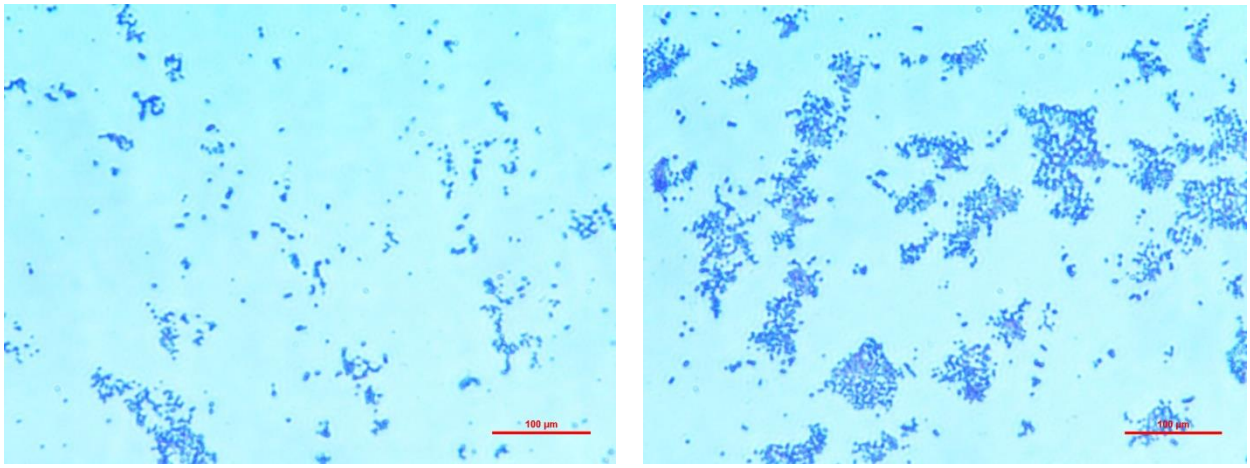
(A) (B)

Fig 15: A and B are time dependent bright field microscopy analysis (400X) of antibiofilm activity of sample-2 against *A. baumannii* isolates attached to polystyrene surfaces with crystal violet staining (1% w/v for 30 minutes) for 2 hours. Panel (A) represents biofilm formation at 1024µM concentration of sample 2 while, panel (B) shows biofilm formation at 256µM conc. of the sample 2.



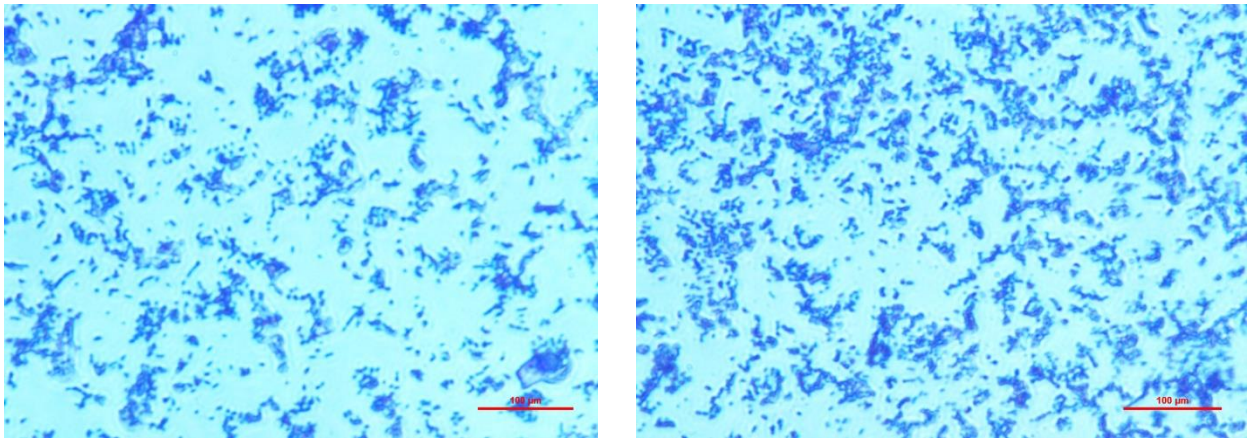
(C) (D)

Fig 16: C and D are time dependent bright field microscopy analysis (400X) of antibiofilm activity of sample-1 against *A. baumannii* isolates attached to polystyrene surfaces with crystal violet staining (1% w/v for 30 minutes) for 4 hours. Panel (C) represents biofilm formation at 1024µM concentration of sample 1 while; panel (D) shows biofilm formation at 0.5µM conc. of the sample 1.



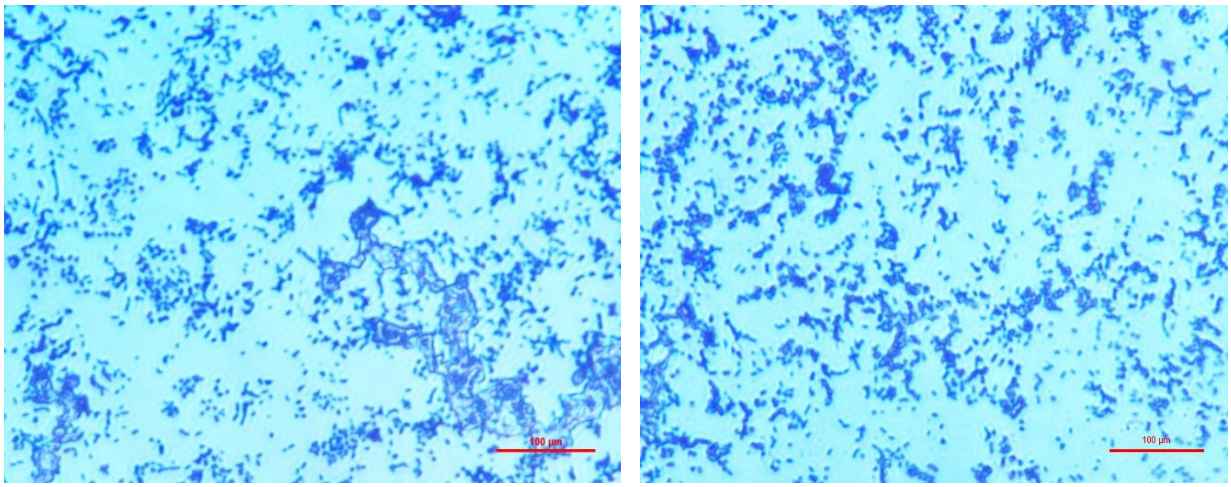
(E) (F)

Fig 17: E and F are time dependent bright field microscopy analysis (400X) of antibiofilm activity of sample-2 against *A. baumannii* isolates attached to polystyrene surfaces with crystal violet staining (1% w/v for 30 minutes) for 4 hours. Panel (E) represents biofilm formation at 256µM concentration of sample 2 while, panel (F) shows biofilm formation at 32µM conc. of the sample 2.



(G) (H)

Fig 18: G and H are time dependent bright field microscopy analysis (400X) of antibiofilm activity of sample-1 against *A. baumannii* isolates attached to polystyrene surfaces with crystal violet staining (1% w/v for 30 minutes) for 8 hours. Panel (G) represents biofilm formation at 1024µM concentration of sample 1 while; panel (H) shows biofilm formation at 0.5µM conc. of the sample 1.



(I) (J)

Fig 19: I and J are time dependent bright field microscopy analysis (400X) of antibiofilm activity of sample-2 against *A. baumannii* isolates attached to polystyrene surfaces with crystal violet staining (1% w/v for 30 minutes) for 8 hours. Panel (I) represents biofilm formation at 1024µM concentration of sample 2 while, panel (J) shows biofilm formation at 0.5µM conc. of the sample 2.

Chapter 5: Discussion

Colony boiling PCR validation:

The ITS region is a conserved region used for identification of *A.baumannii* [d3], as these ITS genes have lower rates of variations in different strains and are mostly the conserved regions having housekeeping genes.

Preciseness of this ITS region amplification which is used for identification of the *A. baumannii* strains was further assured by Agarose gel electrophoresis which showed amplified bands of the *A.baumannii* ATCC 19606 and 1605 after using the specific ITS region primers. The gel picture viewed under GELDOC verified the results of PCR.

Biofilm formation and quantification:

Our results showed that 37°C is more appropriate temperature for biofilm formation as compared to 44°C. Mature biofilm formation occurred at prolonged incubation periods i.e. at 48 hours. However at elevated temperature conditions (44°C) more than 30% *A. baumannii* isolates were capable of forming mature biofilms following incubation for 48 hours.

MDR isolates of *A. baumannii* from respiratory and pus showed higher biofilm formation at 37^{o}C for 24 hours to 48 hours. However, there was no significant association found between susceptible phenotype with high production of biofilm.

At 24 hours of incubation period, >50% of isolates produced biofilm at 37^{o}C, while, few isolates produced biofilm at 44^{o}C (~4%). After prolonged incubation periods (48 hours), all *A baumannii* strains were capable of forming biofilms *in-vitro* at 44^{o}C, which makes this pathogen as a succesful survivor in adverse conditions of higher temperatures in hospital environments.

Biofilm forming capacity varied among different clinical sources which showed respiratory isolates as strong biofilm former followed by pus then peritoneal fluid.

AST and MIC result discussion:

Majority of strains were associated with respiratory infections followed by blood, pus urine and peritoneal fluid. Antibiotics included for AST and MIC against bacterial isolates are based on their usage as conventional antimicrobial regimen prescribed by medical practitioners for treating *A. baumannii* infections. Therefore, levofloxacin, tetracycline, amikacin, ceftazidime, cefepime, imipenem, meropenem, piperacillin-tazobactm and cefoperazone-sulbactum were utilized in theexperiment. AST and MIC results of antibiotics are interpreted according to CLSI guidelines 2015 through visible circular and elliptical zone of lysis around susceptible isolates against antibiotic disk and E strips.

Proportions of resistance among strains isolated from respiratory secretions are in the range between 68%-85%. While, highest proportions of sensitive *A. baumannii* isolates against all tested drugs are originated from pus and peritoneal fluid. *A. baumannii* susceptibility rates observed for different antibiotics were Levofloxacin (16.3%), Tetracycline (26.5%), Amikacin (28.5%), Meropenem (20.4%), Imipenem (14.2%), Ceftazidime (22.4%), Cefepime (16.3%), Piperacillin-tazobactum (18.3%), and Cefoperazone-subactum (20.4%).

Resistance rates were high for most antimicrobial agents with the exception of amikacin and to a lower extent to ceftazidime, meropenem and cefoperazone-sulbactum. Respiratory isolates were found most sensitive against amikacin & ceftazidime. Isolates from pus and peritoneal fluid showed highest level of resistance against all antibiotics (50-100%).

Proportions of sensitive isolates against all tested antibiotics were found between 15%-30%. Amikacin, ceftazidime, cefoperazone sulbactum showed highest sensitivity against *A. baumannii* isolates more than 80% resistance levels were observed against imipenem. Overall, antibiotic resistant strains from different sources expressed more ability to form biofilms as compared to antibiotic sensitive strains.

Motility assay:

While studying about motility features in biofilm-forming strains of *A. baumannii*, though very less is known about the motility for many of the strains, isolates demonstrating elevated motility might be due to in excess of expression of type IV pili-related genes as contrasted to non motile.

Otherwise non motile isolates that exhibit small or nix motility may be deficient of type IV biogenesis genes. 50% of peritoneal fluid isolated showed high motility, followed by respiratory sources i.e. 8.3%. Least or null motility was shown by pus derived strains may be due to lack of pili in their structures. These results contradict the initial facts about *A.baumannii* that it is a non-motile pathogen. These results also contribute towards the fact that motility of these organisms help them in colonization and biofilm formation on biotic and abiotic surfaces.

Antibiofilm activity of nanoparticles:

For 2 hours: sample 1 showed no effect on the biofilm capability of ATCC 1605 at any concentration.

Sample 2 showed decrease in biofilm formation at concentration of 1024µM, showing antibiofilm activity but showed comparitively high biofilm formation at a lower concentration of 256µM.

For 4 hours: sample 1 also showed decrease in biofilm formation at concentration of 1024µM, showing antibiofilm activity but showed high biofilm formation at a lower concentration of 0.5µM.

Sample 2 showed lesser biofilm production at concentration of 256µM as compared to biofilm formation at lesser concentration of 32µM, of the sample.

For 8 hours: both sample 1 and sample 2 had null or less effect on the biofilm forming capacity of ATCC 1605 and showed no positive results.

It can be concluded that by increasing the incubation time periods of the antibiofilm compounds their antibiofilm activity is reduced and deteriorated.

Chapter 6: References

1. Eliopoulos GM, Maragakis LL, Perl TM. Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options. Clinical infectious diseases. 2008 Apr 15;46(8):1254-63.

2. Howard A, O'Donoghue M, Feeney A, Sleator RD. Acinetobacter baumannii: an emerging opportunistic pathogen. Virulence. 2012 May 1;3(3):243-50.
3. Sebeny PJ, Riddle MS, Petersen K. Acinetobacter baumannii skin and soft-tissue infection associated with war trauma. Clinical Infectious Diseases. 2008 Aug 15;47(4):444-9.
4. Antunes LC, Visca P, Towner KJ. Acinetobacter baumannii: evolution of a global pathogen. Pathogens and disease. 2014 Aug 1;71(3):292-301.
5. Cerqueira GM, Peleg AY. Insights into Acinetobacter baumannii pathogenicity. IUBMB life. 2011 Dec 1;63(12):1055-60.
6. Peleg AY, Seifert H, Paterson DL. Acinetobacter baumannii: emergence of a successful pathogen. Clinical microbiology reviews. 2008 Jul 1;21(3):538-82.
7. Nemec A, Musilek M, Maixnerova M, De Baere T, van der Reijden TJ, Vaneechoutte M, Dijkshoorn L. Acinetobacter beijerinckii sp. nov. and Acinetobacter gyllenbergii sp. nov., haemolytic organisms isolated from humans. International journal of systematic and evolutionary microbiology. 2009 Jan 1;59(1):118-24.
8. Fournier PE, Vallenet D, Barbe V, Audic S, Ogata H, Poirel L, Richet H, Robert C, Mangenot S, Abergel C, Nordmann P. Comparative genomics of multidrug resistance in Acinetobacter baumannii. PLoS Genet. 2006 Jan 13;2(1):e7
9. Eliopoulos GM, Maragakis LL, Perl TM. Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options. Clinical infectious diseases. 2008 Apr 15;46(8):1254-63.
10. McQueary CN, Kirkup BC, Si Y, Barlow M, Actis LA, Craft DW, Zurawski DV. Extracellular stress and lipopolysaccharide modulate Acinetobacter baumannii surface-associated motility. Journal of microbiology. 2012 Jun 1;50(3):434-43.
11. Peleg AY, Seifert H, Paterson DL. Acinetobacter baumannii: emergence of a successful pathogen. Clinical microbiology reviews. 2008 Jul 1;21(3):538-82.

12. Seifert H, Baginski R, Schulze A, Pulverer G. The distribution of Acinetobacter species in clinical culture materials. Zentralblatt für Bakteriologie. 1993 Nov 1;279(4):544-52.
13. Seifert H, Schulze A, Baginski R, Pulverer G. Plasmid DNA fingerprinting of Acinetobacter species other than Acinetobacter baumannii. Journal of clinical microbiology. 1994 Jan 1;32(1):82-6.
14. Anstey NM, Currie BJ, Hassell M, Palmer D, Dwyer B, Seifert H. Community-acquired bacteremic Acinetobacter pneumonia in tropical Australia is caused by diverse strains of Acinetobacter baumannii, with carriage in the throat in at-risk groups. Journal of clinical microbiology. 2002 Feb 1;40(2):685-6.
15. Anstey NM, Currie BJ, Withnall KM. Community-acquired Acinetobacter pneumonia in the Northern Territory of Australia. Clinical Infectious Diseases. 1992 Jan 1;14(1):83-91.
16. Go ES, Urban C, Burns J, Mariano N, Mosinka-Snipas K, Rahal JJ, Kreiswirth B, Eisner W. Clinical and molecular epidemiology of Acinetobacter infections sensitive only to polymyxin B and sulbactam. The Lancet. 1994 Nov 12;344(8933):1329-32.
17. Weinstein RA, Gaynes R, Edwards JR, National Nosocomial Infections Surveillance System. Overview of nosocomial infections caused by gram-negative bacilli. Clinical Infectious Diseases. 2005 Sep 15;41(6):848-54.
18. Iregbu KC, Ogunsola FT, Odugbemi TO. Infections caused by Acinetobacter species and their susceptibility to 14 antibiotics in Lagos University Teaching Hospital, Lagos. West African journal of medicine. 2002;21(3):226-9.
19. Marais E, de Jong G, Ferraz V, Maloba B, Dusé AG. Interhospital transfer of pan-resistant Acinetobacter strains in Johannesburg, South Africa. American journal of infection control. 2004 Aug 31;32(5):278-81.
20. Unal S, Garcia-Rodriguez JA. Activity of meropenem and comparators against Pseudomonas aeruginosa and Acinetobacter spp. isolated in the MYSTIC Program, 2002–2004. Diagnostic Microbiology and infectious disease. 2005 Dec 31;53(4):265-71.
21. Peleg AY, Seifert H, Paterson DL. Acinetobacter baumannii: emergence of a successful pathogen. Clinical microbiology reviews. 2008 Jul 1;21(3):538-82.

22. Villegas MV, Kattan JN, Correa A, Lolans K, Guzman AM, Woodford N, Livermore D, Quinn JP, Colombian Nosocomial Bacterial Resistance Study Group. Dissemination of Acinetobacter baumannii clones with OXA-23 carbapenemase in Colombian hospitals. Antimicrobial agents and chemotherapy. 2007 Jun 1;51(6):2001-4.
23. Coelho J, Woodford N, Afzal-Shah M, Livermore D. Occurrence of OXA-58-like carbapenemases in Acinetobacter spp. collected over 10 years in three continents. Antimicrobial agents and chemotherapy. 2006 Feb 1;50(2):756-8.
24. Van Looveren M, Goossens H. Antimicrobial resistance of Acinetobacter spp. in Europe. Clinical microbiology and infection. 2004 Aug 1;10(8):684-704.
25. Bergogne-Berezin E, Towner KJ. Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clinical microbiology reviews. 1996 Apr;9(2):148.
26. Da Silva G, Dijkshoorn L, Van Der Reijden T, Van Strijen B, Duarte A. Identification of widespread, closely related Acinetobacter baumannii isolates in Portugal as a subgroup of European clone II. Clinical microbiology and infection. 2007 Feb 1;13(2):190-5.
27. Turton JF, Kaufmann ME, Warner M, Coelho J, Dijkshoorn L, Van Der Reijden T, Pitt TL. A prevalent, multiresistant clone of Acinetobacter baumannii in Southeast England. Journal of Hospital Infection. 2004 Nov 30;58(3):170-9.
28. Marchaim D, Navon-Venezia S, Schwartz D, Tarabeia J, Fefer I, Schwaber MJ, Carmeli Y. Surveillance cultures and duration of carriage of multidrug-resistant Acinetobacter baumannii. Journal of clinical microbiology. 2007 May 1;45(5):1551-5.
29. Afzal-Shah M, Woodford N, Livermore DM. Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β-lactamases associated with carbapenem resistance in clinical isolates of Acinetobacter baumannii. Antimicrobial agents and chemotherapy. 2001 Feb 1;45(2):583-8.
30. Navon-Venezia S, Leavitt A, Carmeli Y. High tigecycline resistance in multidrug-resistant Acinetobacter baumannii. Journal of Antimicrobial Chemotherapy. 2007 Apr 1;59(4):772-4.

31. Gales AC, Jones RN, Sader HS. Global assessment of the antimicrobial activity of polymyxin B against 54 731 clinical isolates of gram-negative bacilli: report from the SENTRY antimicrobial surveillance programme (2001–2004). Clinical microbiology and infection. 2006 Apr 1;12(4):315-21.
32. Garnacho-Montero J, Ortiz-Leyba C, Fernández-Hinojosa E, Aldabó-Pallás T, Cayuela A, Marquez-Vácaro JA, Garcia-Curiel A, Jiménez-Jiménez FJ. Acinetobacter baumannii ventilator-associated pneumonia: epidemiological and clinical findings. Intensive care medicine. 2005 May 1;31(5):649-55.
33. Bick JA, Semel JD. Fulminant community-acquired Acinetobacter pneumonia in a healthy woman. Clinical infectious diseases. 1993 Oct 1;17(4):820-1.
34. Durand ML, Calderwood SB, Weber DJ, Miller SI, Southwick FS, Caviness Jr VS, Swartz MN. Acute Bacterial Meningitis in Adults--A Review of 493 Episodes. New England Journal of Medicine. 1993 Jan 7;328(1):21-8.
35. Núñez ML, Martínez-Toldos MC, Bru M, Simarro E, Segovia M, Ruiz J. Appearance of resistance to meropenem during the treatment of a patient with meningitis by Acinetobacter. Scandinavian journal of infectious diseases. 1998 Jan 1;30(4):421-3.
36. . Weinstein RA, Gaynes R, Edwards JR, National Nosocomial Infections Surveillance System. Overview of nosocomial infections caused by gram-negative bacilli. Clinical Infectious Diseases. 2005 Sep 15;41(6):848-54.
37. Trottier V, Segura PG, Namias N, King D, Pizano LR, Schulman CI. Outcomes of Acinetobacter baumannii infection in critically ill burned patients. Journal of burn care & research. 2007 Mar 1;28(2):248-54.
38. . Whitman TJ. Infection control challenges related to war wound infections in the ICU setting. Journal of Trauma and Acute Care Surgery. 2007 Jun 1;62(6):S53.
39. . Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases. 2004 Aug 1;39(3):309-17.

40. Olut AI, Erkek E. Early prosthetic valve endocarditis due to Acinetobacter baumannii: a case report and brief review of the literature. Scandinavian journal of infectious diseases. 2005 Jan 1;37(11-12):919-21.
41. Levy J, Oshry T, Rabinowitz R, Lifshitz T. Acinetobacter corneal graft ulcer and endophthalmitis: report of two cases. Canadian Journal of Ophthalmology/Journal Canadien d'Ophtalmologie. 2005 Feb 1;40(1):79-82.
42. Omer MI, Gumaa SA, Hassan AA, Idris KH, Ali OA, Osman MM, Saleh MS, Mohamed NA, Khaled MM. Prevalence and Resistance Profile of Acinetobacter baumannii Clinical Isolates from a Private Hospital in Khartoum, Sudan. American Journal of Microbiological Research. 2015 Jan 23;3(2):76-9.
43. McQueary CN, Kirkup BC, Si Y, Barlow M, Actis LA, Craft DW, Zurawski DV. Extracellular stress and lipopolysaccharide modulate Acinetobacter baumannii surface-associated motility. Journal of microbiology. 2012 Jun 1;50(3):434-43..
44. Fournier PE, Richet H, Weinstein RA. The epidemiology and control of Acinetobacter baumannii in health care facilities. Clinical infectious diseases. 2006 Mar 1;42(5):692-9.
45. Playford EG, Craig JC, Iredell JR. Carbapenem-resistant Acinetobacter baumannii in intensive care unit patients: risk factors for acquisition, infection and their consequences. Journal of Hospital Infection. 2007 Mar 31;65(3):204-11.
46. Bernards AT, Harinck HI, Dijkshoorn L, Van der Reijden TJ, Van den Broek PJ. Persistent Acinetobacter baumannii? Look inside your medical equipment. Infection Control & Hospital Epidemiology. 2004 Nov 1;25(11):1002-4.
47. Zanetti, G., Blanc, D.S., Federli, I., Raffoul, W., Petignat, C., Maravic, P., Francioli, P. and Berger, M.M., 2007. Importation of Acinetobacter baumannii into a burn unit: a recurrent outbreak of infection associated with widespread environmental contamination. *Infection Control & Hospital Epidemiology*, *28*(06), pp.723-725.
48. Maragakis LL, Cosgrove SE, Song X, Kim D, Rosenbaum P, Ciesla N, Srinivasan A, Ross T, Carroll K, Perl TM. An outbreak of multidrug-resistant Acinetobacter baumannii associated with pulsatile lavage wound treatment. Jama. 2004 Dec 22;292(24):3006-11.

49. Villegas MV, Hartstein AI. Acinetobacter Outbreaks, 1977–2000. Infection Control & Hospital Epidemiology. 2003 Apr 1;24(04):284-95.
50. Marchaim D, Navon-Venezia S, Leavitt A, Chmelnitsky I, Schwaber MJ, Carmeli Y. Molecular and epidemiologic study of polyclonal outbreaks of multidrug-resistant Acinetobacter baumannii infection in an Israeli hospital. Infection Control & Hospital Epidemiology. 2007 Aug 1;28(08):945-50.
51. Oteo J, García-Estébanez C, Migueláñez S, Campos J, Martí S, Vila J, Domínguez MÁ, Docobo F, Larrosa N, Pascual Á, Pintado V. Genotypic diversity of imipenem resistant isolates of Acinetobacter baumannii in Spain. Journal of Infection. 2007 Sep 30;55(3):260-6.
52. Arnold KE, Leggiadro RJ, Breiman RF, Lipman HB, Schwartz B, Appleton MA, Cleveland KO, Szeto HC, Hill BC, Tenover FC, Elliott JA. Risk factors for carriage of drug-resistant Streptococcus pneumoniae among children in Memphis, Tennessee. The Journal of pediatrics. 1996 Jun 30;128(6):757-64.
53. Hamouda A, Amyes SG. Novel gyrA and parC point mutations in two strains of Acinetobacter baumannii resistant to ciprofloxacin. Journal of Antimicrobial Chemotherapy. 2004 Sep 1;54(3):695-6.
54. Ribera A, Ruiz J, de Anta MT, Vila J. Effect of an efflux pump inhibitor on the MIC of nalidixic acid for Acinetobacter baumannii and Stenotrophomonas maltophilia clinical isolates. Journal of Antimicrobial Chemotherapy. 2002 Apr 1;49(4):697-8.
55. Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454. Antimicrobial agents and chemotherapy. 2001 Dec 1;45(12):3375-80.
56. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H+-coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters. Antimicrobial agents and chemotherapy. 2005 Oct 1;49(10):4362-4.
57. Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L. Heteroresistance to colistin in multidrug-resistant Acinetobacter baumannii. Antimicrobial agents and chemotherapy. 2006 Sep 1;50(9):2946-50.

58. Quale J, Bratu S, Landman D, Heddurshetti R. Molecular epidemiology and mechanisms of carbapenem resistance in Acinetobacter baumannii endemic in New York City. Clinical infectious diseases. 2003 Jul 15;37(2):214-20.
59. Ruiz M, Marti S, Fernandez-Cuenca F, Pascual A, Vila J. Prevalence of ISAba1 in epidemiologically unrelated Acinetobacter baumannii clinical isolates. FEMS microbiology letters. 2007 Sep 1;274(1):63-6.
60. Hujer KM, Hamza NS, Hujer AM, Perez F, Helfand MS, Bethel CR, Thomson JM, Anderson VE, Barlow M, Rice LB, Tenover FC. Identification of a new allelic variant of the Acinetobacter baumannii cephalosporinase, ADC-7 β-lactamase: defining a unique family of class C enzymes. Antimicrobial agents and chemotherapy. 2005 Jul 1;49(7):2941-8.
61. Costa SF, Woodcock J, Gill M, Wise R, Barone AA, Caiaffa H, Levin AS. Outer-membrane proteins pattern and detection of β-lactamases in clinical isolates of imipenem-resistant Acinetobacter baumannii from Brazil. International journal of antimicrobial agents. 2000 Jan 31;13(3):175-82.
62. Héritier C, Poirel L, Lambert T, Nordmann P. Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in Acinetobacter baumannii. Antimicrobial agents and chemotherapy. 2005 Aug 1;49(8):3198-202.
63. Obara M, Nakae T. Mechanisms of resistance to β-lactam antibiotics in Acinetobacter calcoaceticus. Journal of Antimicrobial Chemotherapy. 1991 Dec 1;28(6):791-800.
64. Seward RJ, Lambert T, Towner KJ. Molecular epidemiology of aminoglycoside resistance in Acinetobacter spp. Journal of medical microbiology. 1998 May 1;47(5):455-62.
65. Doi Y, Arakawa Y. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clinical Infectious Diseases. 2007 Jul 1;45(1):88-94.
66. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H+-coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters. Antimicrobial agents and chemotherapy. 2005 Oct 1;49(10):4362-4.

67. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nature reviews microbiology. 2004 Feb 1;2(2):95-108.
68. Lear G, Lewis GD, editors. Microbial biofilms: current research and applications. Horizon Scientific Press; 2012.
69. Bahador A, Bazargani A, Taheri M, Hashemizadeh Z, Khaledi A, Rostami H, Esmaili D. Clonal lineages and virulence factors among Acinetobacter baumannii isolated from Southwest of Iran. J. Pure Appl. Micribiol. 2013 Sep 1;7:1559-66.
70. de Breij A, Dijkshoorn L, Lagendijk E, van der Meer J, Koster A, Bloemberg G, Wolterbeek R, van den Broek P, Nibbering P. Do biofilm formation and interactions with human cells explain the clinical success of Acinetobacter baumannii?. PloS one. 2010 May 20;5(5):e10732.
71. Donlan RM. Biofilms: microbial life on surfaces. Emerg Infect Dis. 2002 Sep 1;8(9).
72. Fluit AC, Florijn A, Verhoef J, Milatovic D. Presence of tetracycline resistance determinants and susceptibility to tigecycline and minocycline. Antimicrobial agents and chemotherapy. 2005 Apr 1;49(4):1636-8.
73. Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454. Antimicrobial agents and chemotherapy. 2001 Dec 1;45(12):3375-80.
74. Van Looveren M, Goossens H. Antimicrobial resistance of Acinetobacter spp. in Europe. Clinical microbiology and infection. 2004 Aug 1;10(8):684-704.
75. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-β-lactamases: the quiet before the storm?. Clinical microbiology reviews. 2005 Apr 1;18(2):306-25.
76. Chen CH, Young TG, Huang CC. Predictive biomarkers for drug-resistant Acinetobacter baumannii isolates with bla. J Microbiol Immunol Infect. 2006;39:372-9.
77. Loli A, Tzouvelekis LS, Gianneli D, Tzelepi E, Miriagou V. Outbreak of Acinetobacter baumannii with chromosomally encoded VIM-1 undetectable by imipenem-EDTA synergy tests. Antimicrobial agents and chemotherapy. 2008 May 1;52(5):1894-6.

78. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H+-coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters. Antimicrobial agents and chemotherapy. 2005 Oct 1;49(10):4362-4.
79. Hönig A. Linkage of Ifo Survey and Balance-Sheet Data: The EBDC Business Expectations Panel & the EBDC Business Investment Panel. Schmollers Jahrbuch. 2010 Oct 1;130(4):635-42.
80. Toutain CM, Caiazza NC, O'Toole GA. Molecular basis of biofilm development by pseudomonads. InMicrobial biofilms 2004 Jan 1 (pp. 43-63). American Society of Microbiology.
81. Chen CH, Young TG, Huang CC. Predictive biomarkers for drug-resistant Acinetobacter baumannii isolates with bla. J Microbiol Immunol Infect. 2006;39:372-9.
82. Loli A, Tzouvelekis LS, Gianneli D, Tzelepi E, Miriagou V. Outbreak of Acinetobacter baumannii with chromosomally encoded VIM-1 undetectable by imipenem-EDTA synergy tests. Antimicrobial agents and chemotherapy. 2008 May 1;52(5):1894-6.
83. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H+-coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters. Antimicrobial agents and chemotherapy. 2005 Oct 1;49(10):4362-4.
84. Lee HW, Koh YM, Kim J, Lee JC, Lee YC, Seol SY, Cho DT. Capacity of multidrug-resistant clinical isolates of Acinetobacter baumannii to form biofilm and adhere to epithelial cell surfaces. Clinical microbiology and infection. 2008 Jan 1;14(1):49-54.
85. Mussi MA, Gaddy JA, Cabruja M, Arivett BA, Viale AM, Rasia R, Actis LA. The opportunistic human pathogen Acinetobacter baumannii senses and responds to light. Journal of bacteriology. 2010 Dec 15;192(24):6336-45.
86. Nwugo CC, Arivett BA, Zimbler DL, Gaddy JA, Richards AM, Actis LA. Effect of ethanol on differential protein production and expression of potential virulence functions in the opportunistic pathogen Acinetobacter baumannii. PLoS One. 2012 Dec 20;7(12):e51936.

87. Bhargava N, Sharma P, Capalash N. Quorum sensing in Acinetobacter: an emerging pathogen. Critical reviews in microbiology. 2010 Nov 1;36(4):349-60.
88. Niu C, Clemmer KM, Bonomo RA, Rather PN. Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii. Journal of bacteriology. 2008 May 1;190(9):3386-92.
89. de Breij, A., Gaddy, J., van der Meer, J., Koning, R., Koster, A., van den Broek, P., Actis, L., Nibbering, P. and Dijkshoorn, L., 2009. CsuA/BABCDE-dependent pili are not involved in the adherence of Acinetobacter baumannii ATCC19606 T to human airway epithelial cells and their inflammatory response. *Research in microbiology*, *160*(3), pp.213-218.
90. Tomaras AP, Flagler MJ, Dorsey CW, Gaddy JA, Actis LA. Characterization of a two-component regulatory system from Acinetobacter baumannii that controls biofilm formation and cellular morphology. Microbiology. 2008 Nov 1;154(11):3398-409.
91. Eijkelkamp BA, Stroeher UH, Hassan KA, Papadimitrious MS, Paulsen IT, Brown MH, Lo R. Adherence and motility characteristics of clinical Acinetobacter baumannii isolates. FEMS microbiology letters. 2011 Oct 1;323(1):44-51.
92. Choi C.H., Lee E.Y., Lee Y.C., Park T.I., Kim H.J., Hyun S.H., Kim S.A., Lee S.K., Lee J.C. (2005). Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and in-duces apoptosis of epithelial cells. *Cell. Microbiol.* **7**, 1127-1138
93. Brossard KA, Campagnari AA. The Acinetobacter baumannii biofilm-associated protein plays a role in adherence to human epithelial cells. Infection and immunity. 2012 Jan 1;80(1):228-33.
94. Lees-Miller RG, Iwashkiw JA, Scott NE, Seper A, Vinogradov E, Schild S, Feldman MF. A common pathway for O-linked protein-glycosylation and synthesis of capsule in Acinetobacter baumannii. Molecular microbiology. 2013 Sep 1;89(5):816-30.
95. Choi AH, Slamti L, Avci FY, Pier GB, Maira-Litrán T. The pgaABCD locus of Acinetobacter baumannii encodes the production of poly-β-1-6-N-

acetylglucosamine, which is critical for biofilm formation. Journal of bacteriology. 2009 Oct 1;191(19):5953-63.

96. Bouvet PJ, Grimont PA. Taxonomy of the genus Acinetobacter with the recognition of Acinetobacter baumannii sp. nov., Acinetobacter haemolyticus sp. nov., Acinetobacter johnsonii sp. nov., and Acinetobacter junii sp. nov. and emended descriptions of Acinetobacter calcoaceticus and Acinetobacter lwoffii. International Journal of Systematic and Evolutionary Microbiology. 1986 Apr 1;36(2):228-40.
97. Surendran Nair M, Prasad DK, Baskaran SA, Venkitanarayanan K, Amalaradjou MA. Inactivation of Acinetobacter baumannii Biofilms on Polystyrene, Stainless Steel and Urinary Catheters by Octenidine Dihydrochloride. Frontiers in Microbiology. 2016;7:847.
98. Badave GK, Kulkarni D. Biofilm producing multidrug resistant Acinetobacter baumannii: an emerging challenge. J. Clin. Diagn. Res. 2015 Jan;9:8-10.
99. Fredricks DN, Relman DA. Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor sodium polyanetholesulfonate. Journal of Clinical Microbiology. 1998 Oct 1;36(10):2810-6.
100. Chang, H.C., Wei, Y.F., Dijkshoorn, L., Vaneechoutte, M., Tang, C.T. and Chang, T.C., 2005. Species-level identification of isolates of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex by sequence analysis of the 16S-23S rRNA gene spacer region. *Journal of clinical microbiology*, *43*(4), pp.1632-1639.
101. Vaneechoutte M, Dijkshoorn L, Tjernberg I, Elaichouni A, de Vos PA, Claeys G, Verschraegen G. Identification of Acinetobacter genomic species by amplified ribosomal DNA restriction analysis. Journal of Clinical Microbiology. 1995 Jan 1;33(1):11-5.
102. Barry T, Colleran G, Glennon M, Dunican LK, Gannon F. The 16s/23s ribosomal spacer region as a target for DNA probes to identify eubacteria. Genome Research. 1991 Aug 1;1(1):51-6.
103. ICMR Standard operating procedure Bacteriology 2015 and CLSI guidelines 2015.

104. Clemmer KM, Bonomo RA, Rather PN. Genetic analysis of surface motility in Acinetobacter baumannii. Microbiology. 2011 Sep 1;157(9):2534-44.

105. Vijayakumar S, Rajenderan S, Laishram S, Anandan S, Balaji V, Biswas I. Biofilm formation and motility depend on the nature of the Acinetobacter baumannii clinical isolates. Frontiers in public health. 2016;4.

Chapter 7: Appendix

- 1x PBS [pH 7.2]: composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4.
- Modified luria broth: tryptone – 10 g/l; NaCl – 5 g/l; yeast extract – 5 g/l; agar- 0.4%.
- Ethanol:acetone :80ml of ethanol +20ml of acetone

- Luria broth: 20gm for 1000ml of distilled water.
- Mc farland reagent:

Mc Farland standard no.	0.5	1	2
1% barium chloride(ml)	0.05	0.1	0.2
1% sulphuric acid(ml)	9.95	9.9	9.8