Analysis of Biofilm forming ability and Antibiofilm activity of gold nanoparticles of *Acinetobacter baumannii* isolates from blood and urinary sources

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Submitted in partial fulfillment for the award of degree of Bachelor of Technology (B.Tech)

To,

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This is to certify that the work titled "Analysis of Biofilm forming ability and Antibiofilm activity of gold nanoparticles of *Acinetobacter baumannii* isolates from blood and urinary sources", submitted by "Nikhil Sahai", roll number- '131556' in partial fulfillment for the award of degree of Bachelor of Technology (B.Tech) of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor-

Date-

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Certificate of Originality

This is to certify that the work titled,"Analysis of Biofilm forming ability and Antibiofilm activity of gold nanoparticles of *Acinetobacter baumannii* isolates from blood and urinary sources", has been submitted and completed by me, 'Nikhil Sahai', roll number-'131556'in partial fulfillment for the award of degree of Bachelor of Technology (B.Tech) of Jaypee University of Information Technology, Waknaghat. I do so declare that a literature review and original research work has been carried out by me, in accordance with the academic rules and ethics. All unoriginal topics present in this work have been properly cited and referenced. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of the student

Name- Nikhil Sahai

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Nikhil Sahai

Date-

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Introduction

A. baumannii has evolved into one the most successful pathogens that exist today. While it was being researched in the 20th century as well, it was thrown into the spotlight during the military excursions of the USA military into the Middle East, where injured soldiers were reported to have their wounds infected by the bacteria. It can cause a host of diseases, majorly in hospital-settings where it spreads rapidly, leading to severe outbreaks of ventilator-associated pneumonia, bloodstream infection, wound infection, urinary tract infection, skin infection, and even meningitis [1]. However, its greatest threat stems from the multi drug resistance that this pathogen has acquired, since the 1970s, when it was more sensitive to antibiotics [2], leading it to be named as the number one threat, amongst hospital-related pathogens, by the World Health Organisation (WHO) [3]. Its virulence factors, pathogenesis, growth characteristics upon biotic and abiotic surfaces, as well as biofilm formation have recently been researched in great detail, giving us greater insights into the functioning of this organism. However, further research and effort needs to be put into developing methods of eradicating this bacteria from within patients and hospitals, and quickly, considering its rapid spread worldwide and high incidences of morbidity and mortality.

In light of this, the risk factors associated with contracting a multidrug resistant (MDR) *A. baumannii* infection were studied and the following were the findings: the longer the length of a hospital stay for a patient, the greater the risk; if the patient was exposed to an intensive care ward, his/her risk increases; the use of mechanical ventilation systems upon a patient increases his/her risk; as the severity of the patient's illness increases, his/her chances of contraction increase proportionally; the patient having undergone a surgery recently or any invasive procedure increases his/her risk; the contaminating presence of the bacteria in the environment increases the chances of infection; the greatest risk of contracting MDR *A. baumannii* infections lies in patients who have previously undergone antimicrobial therapy, especially therapies that involved the use of fluoroquinolones, third- generation cephalosporins, or carbapenems [4].

In India, MDR *A. baumannii* infection risk factors and MDR strain development has been studied. The risk factors for contracting an infection were found to be much the same as mentioned above. One study noted that more than 50% of the *A. baumannii* isolates, obtained from the hospital they had targeted, were MDR. Further, they observed a resistance to the most

commonly used antibiotics like amikacin, ciprofloxacin, etc. which has probably evolved due to the indiscriminate use of these antibiotics in India, with people often using them without a doctor's prescription. The researchers found an increasing resistance to carbapenams, which are generally the go-to drug for *A. baumannii* infections, while still displaying susceptibility to colistin and piperacillin/tazobactam [5]. In another study, the antibiotics, colistin and tigecyclin, were the only ones which could be deemed active against the MDR isolates, with some *A. baumannii* clinical isolates even displaying resistance against tigecyclin [6]. Colistin remains the only antibiotic to which the bacterium displays complete susceptibility to. This is an alarming trend since the future might hold *A. baumannii* infections with mechanisms to even resist this one remaining antibiotic.

Review of Literature

Acinetobacter baumannii is a gram negative, aerobic bacteria with the characteristics of being non-motile, non-fastidious, non fermenting, and giving negative results for oxidase presence and positive for catalase. The G+C content of the DNA of this bacteria ranges from 39% to 47%. The genus of this bacterium is often indistinguishable from other gram negatives which also display the non-fermenting phenotype, due to the difficulty occurred in destaining its members. Further, recently discovered *Acinetobacter* species are difficult to distinguish based upon phenotypic tests, and so, due to these two reasons, molecular techniques were formed to quicken/ease this process. There are currently several molecular methods to test and identify the species of acinetobacters, like amplifying the 16S rRNA region and consequently running it through a gene restriction analysis, analyzing the intergenic spacer region of the 16S-23S rRNA through a restriction analysis or analyzing the sequence of the gene spacer region of the same DNA region, etc [2].

Considering the habitat of *A. baumannii,* it is currently unknown whether this species is as ubiquitous as its relatives within the same genus, which can be isolated, through enrichment cultures, from soil and water samples. In humans, acinetobacters are a part of the human skin's flora, as well as being present on the mucous membranes of the body, even on non-hospitalised individuals, while hospitalized individuals had a much higher carrier rate. However, *A. baumannii's* presence was quite a rare occurrence as compared to other *Acinetobacter* species (like, *A. lwoffi*, *A. johnsonii*,etc.) and might be only an abnormal occurrence within human tissues during a diseased condition. An important correlation may have been obtained during such natural flora characterization studies, i.e., *Acinetobacter* species' carrier rate is dependent upon the season (seasonal variation), with the summer having a greater carrier rate than winter. This correlation is based upon a study conducted upon the students and nurses of a hospital in Hong Kong, where the carrier rate of acinetobacters upon the skin increased during the summer months and decreased during winter. This also explains the greater presence of *A. baumannii* in clinical samples during the summer months [1].

This bacterium can be found upon hospital devices and can resist desiccation, factors which have contributed greatly to its survival in hospitals and success as a pathogen. The various factors involved in persistence are still not completely known. However, some interesting findings do

exist: ethanol exposure can actually enhance the bacterium's growth and pathogenicity (ethanol actually induces about 49 genes within *A. baumannii*'s genome and can modulate its stress responses, indicating a major factor that might contribute to its growth in hospital environments; despite being a non-photo synthesizer, it can sense light and be affected by it (eg. blue light exposure inhibits *A. baumannii* biofilm formation) with even its virulence and motility being modulated [7]

Studies correlating *A. baumannii* persistence on inanimate objects/ environmental studies are scarce, however a study conducted in the UK reported the presence of *Acinetobacters* on vegetables, with *A. baumannii* being one of the species being predominantly found in such vegetables. A Hong Kong study reported a much higher presence of *Acinetobacter* species on vegetable surfaces along with a positive presence of *A. baumannii*. A few studies upon soil and water samples have also been conducted, giving varying results (larger number of occurrences of *A. baumannii* in soil samples from Hong Kong as compared to Europe). From these studies, we now know that acinetobacters are widely spread across a range of environments but *A. baumannii* is possibly not an organism commonly present in the environment, although more environment-wide studies need to be performed. This conclusion also continues to leave the question of whether environmental sources are responsible for *A. baumannii* outbreaks in communities present in tropical environments [1].

Acinetobacter baumannii is a major cause of several types of nosocomial infections, the most common of which is ventilator associated pneumonia. This disease is characterized by infection of the airway by *A. baumannii* via external sources and has a high mortality rate ranging from 40-70%. These can also be community acquired, with similarly high mortality rates of 40-60% [8].

Other commonly acquired *A. baumannii* infection types (from a hospital setting) are bloodstream infections. Its reservoirs are generally intravascular devices and any pre-existing respiratory tract infections the host may have. They may also, however, stem from open wound site infections and urinary tract infections and have mortality rates of 28% to 43%. Bloodstream infections of *A. baumannii* are more related to burn victims. If left untreated for long, the bacteria may enter the body through the wound site and cause systemic infections. Contrasting reports regarding its effect on mortality of burn victims with *A. baumannii* currently exist, which argue the

independence of the bacteria as an independent risk factor with regards to mortality of infected burn patients [8].

Soft tissue infections are another issue, especially found in wartime personnel serving in the Middle-East. It can cause severe issues in later stages (eg. Cellulitis), which then require surgical procedures and an antibiotic treatment course to treat. Another military related issue is the development of osteomyelitis in soldiers due to this bacterium [8].

In patients who have recently undergone neuro-surgical procedures, *A. baumannii* may cause meningitis with symptoms typical of bacterial meningitis. However, few studies studies have been conducted, with a large enough sample size, to provide a valid estimate of the mortality rate associated with *A. baumannii* associated meningitis [8].

Various studies have been conducted to study the virulence factors of *A. baumannii*, but this area still remains mysterious, especially when compared with the level of detail known for other gram negative bacteria, and requires further study. Some of the known factors are as follows:

1. Omp A- it travels and localizes to the host cell's mitochondria, where it causes cell death, which occurs since cytochrome c and apoptosis inducing factor, both, are released, which signal for the cell to undergo apoptosis. This factor also helps in the bacterial cell's adhesion and spread throughout the host organism. Further, it provides resistance to the bacteria against the complement mediated killing system of the serum, increasing its persistence within the body's serum [8].
2. Lipopolysaccharides (LPS) - the LPS present on the surface of cells were found to be involved in providing some level of resistance to the human serum associated antibacterial effects, along with increasing its persistence within soft tissues, during an infection. This LPS also turns on the innate immune system (since it activates CD14, as well as the Toll Like Receptor 4) [8].
3. Capsular polysaccharides- the capsular phenotype was found to be important for protection of the bacteria against the host's immune system, which is made up of polysaccharides [8].
4. Phospholipases – Bacteria produce these enzymes to enable host cell invasion by degrading the cell's phospholipids, which make up a eukaryotic cell's membrane. *A. baumannii* possesses two phospholipases, namely phospholipase D and phospholipase C. It was also

found that Phospholipase D might also play a role in the spread of *A. baumannii* from the lungs to the rest of the body [8].

5. Penicillin Binding Proteins (PBPs) – These proteins have a dual role within *A. baumannii*: they help inactivate β lactams after binding to them and increase cell stability. It does the latter since it is involved in peptidoglycan synthesis, which is a major part of the bacterial cell wall. Specifically, PBPs 7/8 showed possible effects on cell stability, when comparing mutants with wild type *A. baumannii* [8].
6. Outer Membrane Vesicles – These are secreted out of the outer membrane of the bacteria and perform several roles, namely: the delivery of other virulence factors into the insides of the host cells during the invasion process (eg. OmpA), it enables the process of horizontal gene transfer, as is evident by the observed transfer of the OXA 24 gene between cells, and finally, it provides resistance against the immune responses initiated by the host [8].

A major factor which has allowed *A. baumannii* to exist and thrive in hospital environments is its multi drug resistance. It has acquired several mechanisms to escape from various antibiotics, the predominant method of which is horizontal gene transfer from unrelated species of bacteria, where resistance genes are transferred from resistant strains to the sensitive *A. baumannii* (eg. acquirement of the AbaR1 resistance island by *A. baumannii* AYE). This bacterial mechanism allows *A. baumannii* to change its virulence factors (eg. adhesins, cell surface proteins, methods/modes of nutrient acquirement) [1].

The bacterium uses several resistance mechanisms to counter the effects of antibiotics. A few have been listed below:

1. Inactivating enzymes like beta lactamases (encoded for by the AmpC, PER, etc.) and carbapenamases (encoded for by the OXA genes, VIM, etc.) to grant resistance against beta lactams [8]
2. Efflux pumps (encoded for by AdeABC, Tet M, etc.) can provide resistance against fluoroquinolones, aminoglycosides, tetracyclines, and glycylcyclines [8]
3. Modifications of antibiotic targets are also utilized to render the drugs useless. Genes like gyrA and lipopolysaccharide synthesis genes can undergo mutations to modify the final product. This mechanism provides resistance against fluoroquinolones and polymixins [8]

In light of this bacteria's rising resistance to drugs, figuring out the effectiveness of/susceptibility of the bacteria to the existing drugs being used for treatment of an *A. baumannii* infection becomes extremely important. The usual method of doing so is through Antimicrobial Susceptibility Tests (AST). The commonly used ASTs are:

1. Broth Dilution- a range of antibiotic dilutions (generally two-fold) are placed within a 96-well plate, followed by inoculation of the bacterial culture (about 10 ^ 5 CFU/mL). An overnight incubation at 35° C later, the turbidity of the solutions are checked (turbidity indicated bacterial growth), and the lowest concentration of antibiotic that inhibited bacterial growth gives us a Minimum Inhibitory Concentration (MIC) for that antibiotic. The lower the MIC value, the greater the susceptibility of the bacteria to the antibiotic [9]
2. Antimicrobial gradient- an antibiotic gradient is created by placing plastic strips (E-strips) upon an agar media containing a lawn of the bacteria to be tested. The strips have an antibiotic gradient on its underside, with a scale printed upon the topside, displaying the concentration gradient to the tester. After overnight incubation, the MIC value can be determined by viewing the point at which the zone of inhibition intersects with the strip (the gradient at that point is the MIC) [9]
3. Disk diffusion- commercially prepared disks, containing different antibiotics (fixed concentrations) are placed upon a culture of the bacteria grown upon a Mueller-Hinton agar containing plate. Following this, the cultures are placed at 37° C for about 24 hours. Finally, the diameter of the zone of inhibition present about each antibiotic disk is measured using a scale or a caliper. This diameter indicates whether the bacteria is sensitive, resistant or displays intermediate susceptibility to a particular antibiotic concentration and can be determined by cross-checking the diameters with CLSI (Clinical and Laboratory Standards Institute) standards for the particular bacterial species and antibiotic in question. This test is qualitative, unlike the previous two [9]

As discussed earlier, *A. baumannii* can not only survive in a hospital setting but also thrive there. This is possibly due to its ability to interact with a variety of abiotic surfaces and persist on them for long stretches of time, all the while avoiding death by desiccation. Apart from general abiotic surfaces like furniture, linens, etc. it can colonise within medical

equipment like catheters and ventilators, and even biotic surfaces like the epithelial cells of humans [7].

Once the bacteria adheres to a surface, whether biotic or abiotic, it consequently forms biofilms, i.e., three dimensional, highly complex structures formed by cells in close contact with each other and producing an extra cellular matrix, which is comprised of nucleic acids, carbohydrates, proteins, etc. The three dimensional structure of these biofilms is quite variable and changes according to the surface it is being formed upon, with structures ranging from extremely complex, multilayered structures, to simple monolayers [7].

These biofilms have been hypothesized to be responsible for the adherence of the bacteria upon surfaces [7], resistance to desiccation [10], and even providing resistance against antimicrobial drugs[10]. In light of this, it has become ever important to study the various genes that are responsible for its formation and attachment to surfaces, a few of which have been discussed below:

1. The initial phase of biofilm formation, on an abiotic surface, requires the attachment of the cells to the stationary phase, which one is accomplished via pili. These pili are formed by cells through an assembly-chaperone system (CsuA/BABCDE). When interacting with epithelial cells, however, short and thin pili, which are independent of this system, are formed which allow the bacteria to adhere to the cells. The entire Csu operon is itself regulated by the products of genes bfmS and bfmR. BfmS encodes for a sensor kinase, and the gene's deactivation abolishes the bacteria's ability to attach to eukaryotic cells, while biofilm production on abiotic surfaces was merely decreased [7]
2. An outer membrane protein, OmpA, acts as a diffusion pore and is involved in both, abiotic surface attachment, as well as in *A. baumannii* interactions with eukaryotic cells. It can also be counted as a major virulence factor, as it can cause death of epithelial cells and affect dendritic cells via early onset apoptosis and delayed onset necrosis. The protein causes the mitochondria to create reactive oxygen species which induce the previously mentioned effects [7]
3. The biofilm associated protein (BAP) is a cell surface expressed, conserved adhesin, and act as an important component of biofilms. It is a required by *A. baumannii* cells to adhere to one another and to ensure the correct formation of three dimensional structures

like towers and water channels (required for biofilm formation upon abiotic surfaces). It also helps in the adhesion of the bacteria to eukaryotic cells(increases hydrophobic interaction potential with the host cells since BAP increases the bacteria's surface hydrophobicity) [7]

4. A glycosylation system also seems to be involved, since an O-glycosylation system is present in clinical isolates and was found to increase biofilm production. The O-pentasaccharide linked to glycoproteins was also discovered and its coding locus, pglC, obtained. If this locus was mutated, it caused abnormal biofilm formation as it prevented the formation of glycoproteins as well as the cell capsules. The mutation also led to non-specific attachment of the cells to abiotic surfaces [7]
5. The exopolysaccharide matrix is an important component of the biofilm. A major component of this matrix is the PNAG (poly-β-1,6-*N*-acetylglucosamine) which is required for the biofilm to mature and develop, making it an important cell secretory product [7]

These factors, being essential for the formation of biofilm, provide targets for drug design. Moreover, proteins/ other macromolecules that lead up to the formation of these factors can be targets for deletion/modification, such that the end products are no longer formed, disrupting biofilm formation.

Bacteria may be motile or non-motile, depending upon its available appendages (nozzles, flagella or pili). Motile bacteria may exhibit this phenotype through various mechanisms (sliding, gliding, swarming, twitching and swimming. Swimming and swarming motilities are best displayed within liquids and upon solid surfaces, respectively, and are enabled by bacterial flagella; the type IV pili's extension and retraction give rise to the twitching motility phenotype; if the cell extrudes cellular material out of the cell via nozzles, or if the cell surface has adhesins moving across its surface via protein motors, it exhibits gliding motility; if the growth of cells causes them to spread across a surface, it is referred to as sliding motility [11].

For more than 30 years, *Acinetobacter baumannii* was thought to be non-motile. This changed when it was discovered that the bacteria displays twitching motility. This motility was found to be affected greatly by iron concentrations/availability around the bacteria, the

density of cell population (since the autoinducer 3-OH-C12-HSL affects motility), and finally, by light (blue light had the greatest effects), although the mechanisms of control have not yet been elucidated. This motility phenotype is currently being studied, since its effect on the virulence of *A. baumannii* is still not clear and also since not all clinical isolates seem to display it [11].

Nanoparticles have quickly come to the forefront of medical sciences with several different compounds having already been established as possessors of antimicrobial activity. There are several advantages to using nanoparticles as antimicrobials, like having a large surface:volume ratio, the freedom to functionalize the nanoparticles with other compounds (eg. polymers) and its small size, which allows it to penetrate into cells. Among these, gold has been found to be advantageous to use due to the ease in preparing its nanoparticles, along with its lower toxicity, relative to nanoparticles of other compounds. Gold has already acquired a history of usage in various fields, such as diagnostics, gene therapies, etc [12]

The use of gold nanoparticles as an antimicrobial has been studied and found to be effective, and possibly possessing greater activity against gram negative bacteria. They were found to possess greater activity with increasing dosages and decreasing size of the nanoparticles [12]. Within bacteria, they exert their effects by two mechanisms:

1. They repress the microbe's metabolism by affecting the cell membrane's potential (thereby reducing Adenosine Triphosphate production) [12]
2. They prevent the binding of the rRNA subunits to tRNA, thereby repressing protein formation, within the cell (since translation is inhibited) [12]

Aims and Objectives:

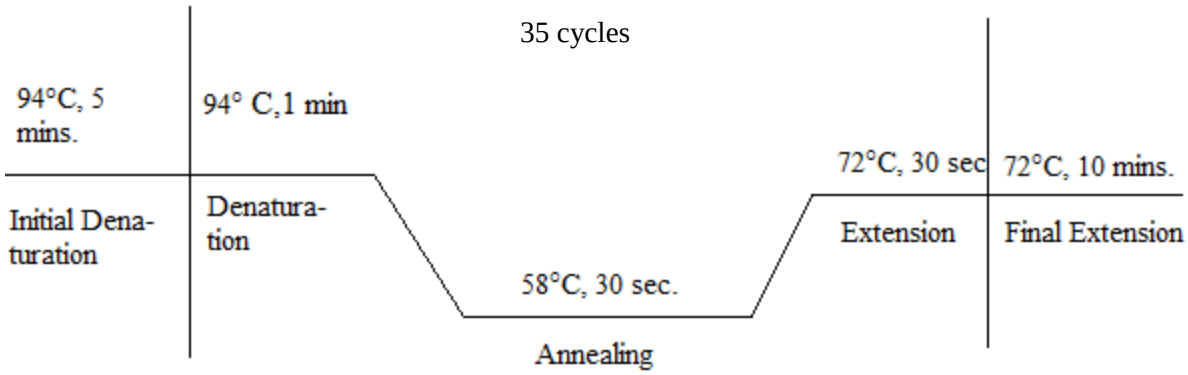
1. To check the biofilm forming capacity of *A. baumannii* isolates from different clinical sources (blood and urine).
2. To compare the antibiotic resistance of the isolates with their biofilm forming capacity along with their clinical sources (blood and urine).
3. To compare the twitching motility phenotype of the isolates with their clinical sources (blood and urine) and biofilm forming capacity.
4. To check for the viability of the usage of gold nanoparticles as an antibiofilm compound against *A. baumannii*

Materials and Methods:

Characterisation of the *Acinetobacter baumannii* isolates [13]

1. *A. baumannii* isolates were subcultured upon MacConkey agar media by streaking isolates upon its surface using an inoculating loop
2. Isolated colonies were picked using a micropipette tip
3. The tips were inserted into 20 µL of nuclease free, autoclaved, distilled water, each isolate within a Polymerase Chain Reaction (PCR) vial
4. The PCR vials were kept inside a thermal cycler and subjected to heat treatment (95°C for 10 minutes) to disrupt the cells
5. The vials were then centrifuged for 5 minutes
6. The supernatant in each vial was then used as the DNA containing sample for subsequent steps
7. The following reaction mixture was performed in new PCR vials for each sample:
    - DNA template- 2 µL
    - PCR master mix (GoTaq)- 7.5 µL
    - Forward primer- 0.5 µL
    - Reverse primer- 0.5 µL
    - Nuclease free water- 4.5 µL

    Where the forward and reverse primers have been constructed to amplify the 16S-23S rRNA intergenic spacer region (208 bp long amplicon) of *A. baumannii* and whose sequences are (5′-CATTATCACGGTAATTAGTG-3′) and (5′AGAGCACTGTGCACTTAAG-3′), respectively
8. The PCR vials were appropriately labeled and placed within a thermocycler with the following reaction conditions:



9. Following this, an electrophoresis chamber was prepared
10. 1.5 % agarose gel was prepared in 1X Tris Acetate EDTA (TAE) buffer and heated until the agarose was completely dissolved within the TAE buffer
11. After cooling the agarose solution to 60°C, ethidium bromide was added to make its concentration in the solution 0.5 µg/mL
12. The agarose solution was poured into a casting tray with a comb already in place and left to set
13. Once the gel has solidified, the casting tray, containing the gel, was placed within an electrophoretic tank with the comb near the anode
14. The tank was filled with 1X TAE buffer until the gel was covered with the buffer, following which, the comb was removed from the gel
15. 5 µL of each PCR product was loaded into the wells created within the gel along with a 1 kilo-bp DNA ladder
16. The electrophoresis process was started by applying a 100 V potential across the ends of the electrophoretic chamber
17. The run was continued until the DNA ladder completely opened (~30 minutes)
18. The gel was observed for any amplified bands under UV light

Antimicrobial Susceptibility Testing and Minimum Inhibitory concentrations [14]:

1. The bacterial isolates were subcultured in Mueller Hinton broth and incubated at 37°C for 4-5 hours
2. The ODs of the cultures were adjusted to 0.5 (if measured at 600 nm)
3. A sterile cotton swab was dipped into bacterial culture and pressed firmly against wall of test tube to remove excess of culture
4. Bacterial culture was swabbed on to Mueller Hinton agar plate within 15 minutes of O.D. adjustments
5. The plates were left to dry for 5 minutes
6. The antibiotic disks & E-strips of respective antibiotics was placed, with the help of sterile forceps, to Mueller Hinton agar plates within 15 minutes of the swabbing
7. The plates were then kept within an incubator, at 37°C, overnight
8. Each plate was examined after overnight incubation (16-18 hour), for confluent growth and elliptical zones of inhibition.
9. AST & MIC was read directly from the graduated E test strip at the point of intersection of the zone of inhibition according to manufacturer's instructions.
10. AST results were also read from the antibiotic discs
11. Results were interpreted according to CLSI (Clinical Laboratory and Standards Institute) guidelines.

Biofilm Formation Assay [15]:

1. The *A. baumannii* samples were inoculated into fresh media and incubated at 37°C until their OD reached 0.5 when measured at 600nm in a spectrophotometer.
2. 96- well plates were prepared for inoculation.
3. 100 µL of each sample was inoculated into a well, along with a negative and positive control, i.e., Luria Bertani broth and *A. baumannii* ATCC 19606, respectively.
4. 4 different sets of plates were thus created, with two sets being incubated at 37°C and two being incubated at 44°C.
5. From the plates kept at 37°C, one set was incubated for 24 hours, while the other was kept at that temperature for 48 hours. The same was done for the plates being incubated at 44°C, with regards to the time of incubation. The plates remained in static conditions.
6. After the respective time periods of incubation were completed, the plates were removed from the incubator and the culture from the wells was tapped out.
7. Each well was then washed twice with a 1X solution of Phosphate Buffer Saline (PBS).
8. This was followed by the staining of each well by a 1% crystal violet solution. The plates were then left to incubate at room temperature for 30 minutes.
9. The crystal violet solution was then tapped out of the wells and another washing with 1X PBS was administered to each well.
10. Each well was then observed under a bright field microscope at 400X magnification.
11. The dye in the wells was then solubilised using 100 µL of a 80:20 solution of ethanol:acetone in each stained well.
12. The Optical density of the contents of each well was then checked at a wavelength of 570 nm in a spectrophotometer.
13. The results for each time period and each temperature of incubation were then compiled.

Motility assay [16]:

1. Prepared cultures of the *A. baumannii* samples in Luria Bertani broth and incubated them at 37°C until they reached an optical density (OD) of 0.6 when measured at a wavelength of 600 nm in a spectrophotometer.
2. Petri plates containing 0.4% agar Luria Bertani media were prepared (semisolid media).
3. To check for twitching motility, 1µL of the *A. baumannii* culture was pipetted into the media interface which exists between its surface and the bottom of the plate.
4. The plates were labeled and left to incubate at 37°C for 24 hours.
5. The surface motility spread, if any, of the bacteria was observed after the incubation period and its diameter was measured.
6. The media was discarded from the plates.
7. The bottoms of the plates were stained with a 1% crystal violet solution.
8. The solution was left in the plates for 15 minutes, following which, the plates were drained and washed of any excess stain.
9. The diameters of the stained regions of the plates were recorded, if any were present.
10. The results for each sample were compiled.

Antibiofilm assay using Gold nanoparticles [17]:

1. Gold nanoparticle solution (coated with cetyl group and having a 10 nm diameter)r was obtained (40 nM solution)
2. Three washings were given to the nanoparticles solution to remove excess cetyl groups, using distilled water. This involved centrifuging the solution at 10000 rpm for 10 minutes, followed by discarding the supernatant and replacing it with an equal volume of autoclaved, distilled water.
3. Cultures of *A. baumannii* ATCC 19606 and ATCC 1605 were inoculated into fresh LB broth and incubated at 37°C until their ODs reached 0.6 when measured at a wavelength of 600 nm.
4. The nanoparticles effect on biofilm formation was checked by subjecting the two cultures to a range of nanoparticle concentrations: 2nM, 4nM, 8nM, 16nM, 32nM, along with a negative control (culture only) and blanks (LB broth only). The final volume in each well was 100 µL.
5. 4 such plates were set up, each being incubated at 37°C for different time periods: 2 hours, 4 hours, 6 hours and 8 hours.
6. Following the incubation period, the cultures were tapped out and wells washed twice using 1X PBS solution.
7. 1% w/v crystal violet solution was used to stain each well
8. After a 30 minute incubation, the wells were emptied of the excess stain and washed once more with 1X PBS.
9. Images of the stained cultures were obtained using bright field microscopy (400X magnification).
10. The adhered cells were solubilised using a 80:20 solution of ethanol:acetone.
11. The OD of the solubilised wells was obtained, at a wavelength of 570 nm.
12. The results were analysed to check for biofilm inhibition.

Observations

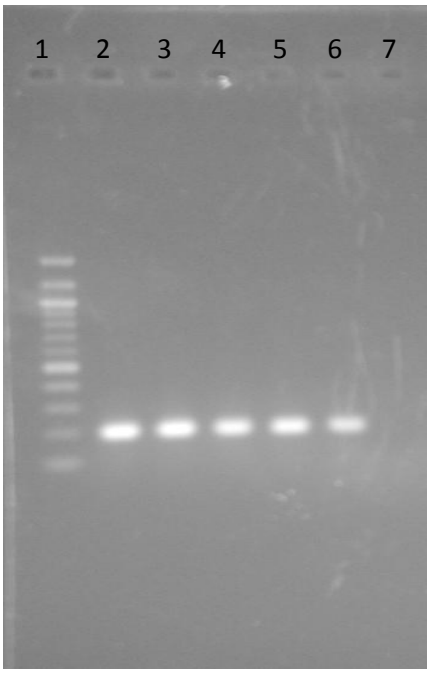


Figure 1: Agarose gel electrophoresis image of *A. baumannii* isolates using ITS region specific primers (208 bp amplicon). Lane 1 100 bp DNA ladder. Lanes 2-6 - amplification at 208 bp. Lane 7 - negative control

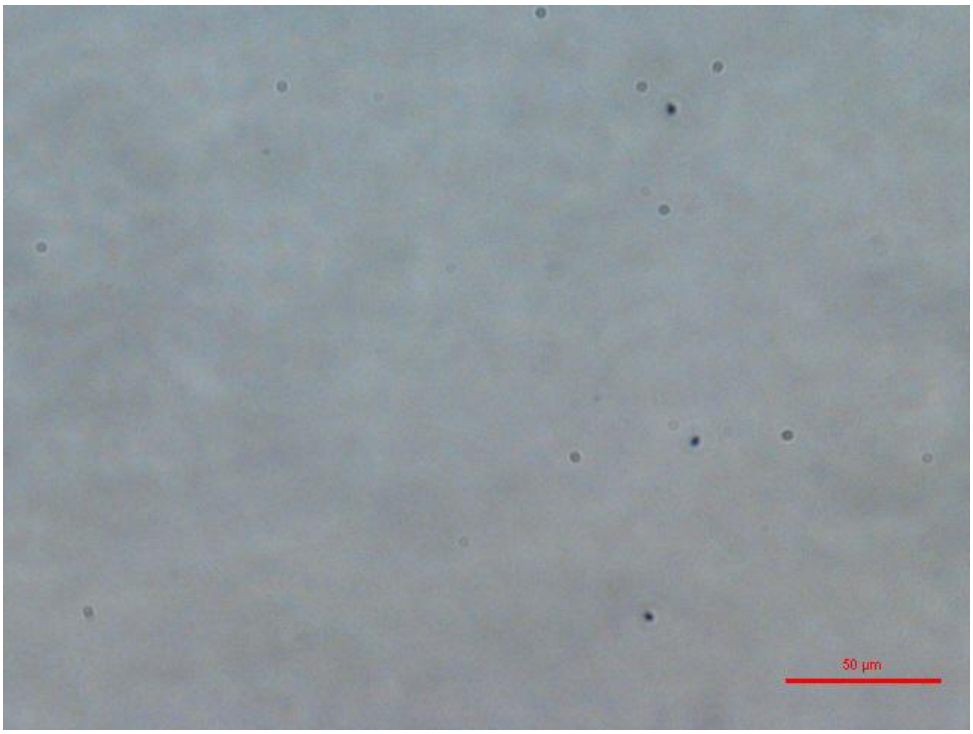


Figure 2: Negative control for *A. baumannii* isolates grown at 37°C, analysed by bright field microscopy (400X).

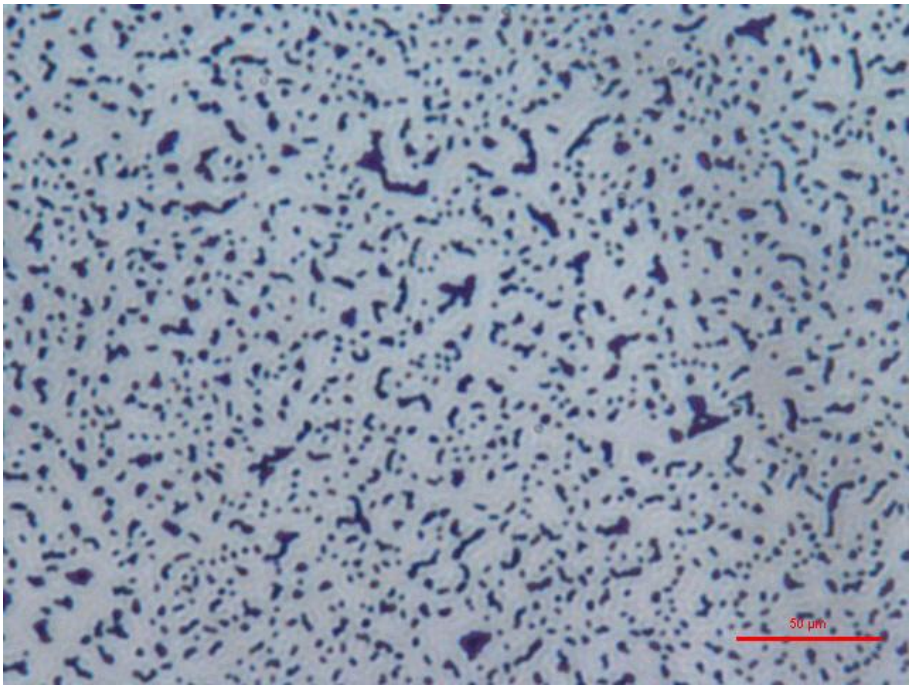


Figure 3: Biofilms of *A. baumannii* grown at 37°C, for 24 hours, analysed by bright field microscopy (400X)

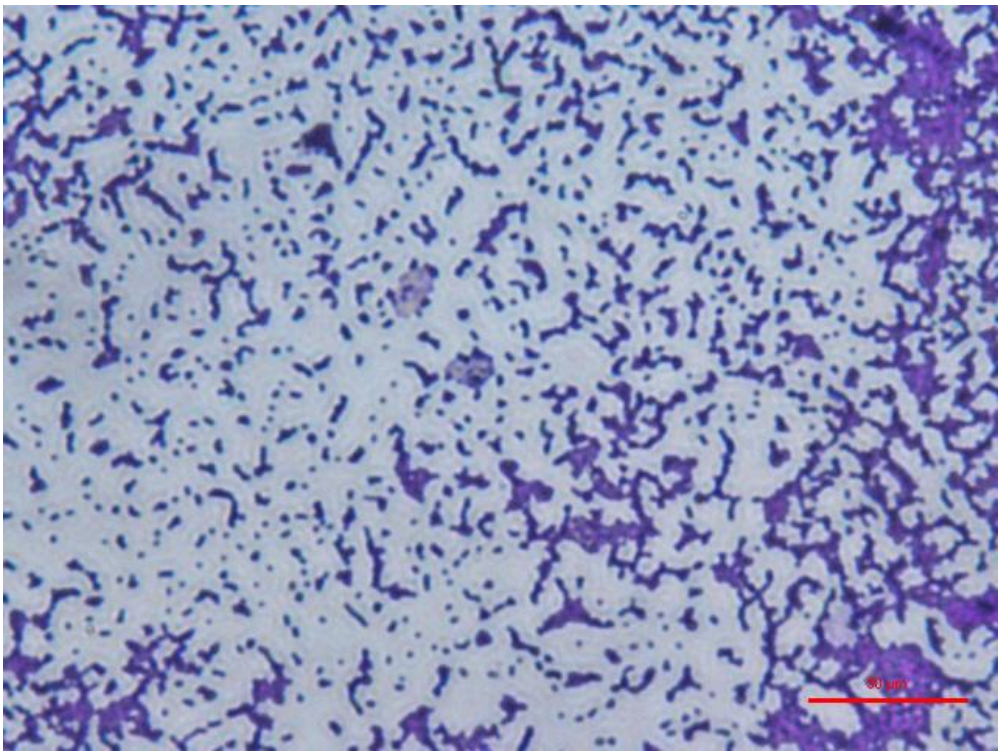


Figure 4: Biofilms of *A. baumannii* grown at 37°C, for 48 hours, analysed by bright field microscopy (400X).

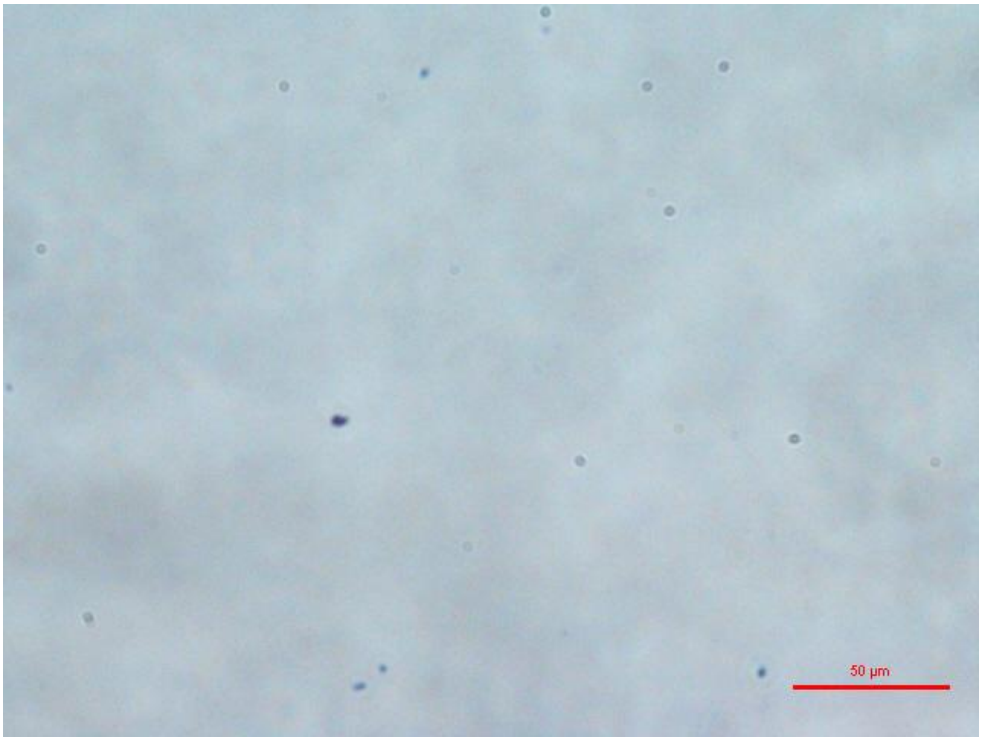


Figure 5: Negative control for *A. baumannii* isolates grown at 48°C, analysed by bright field microscopy (400X)

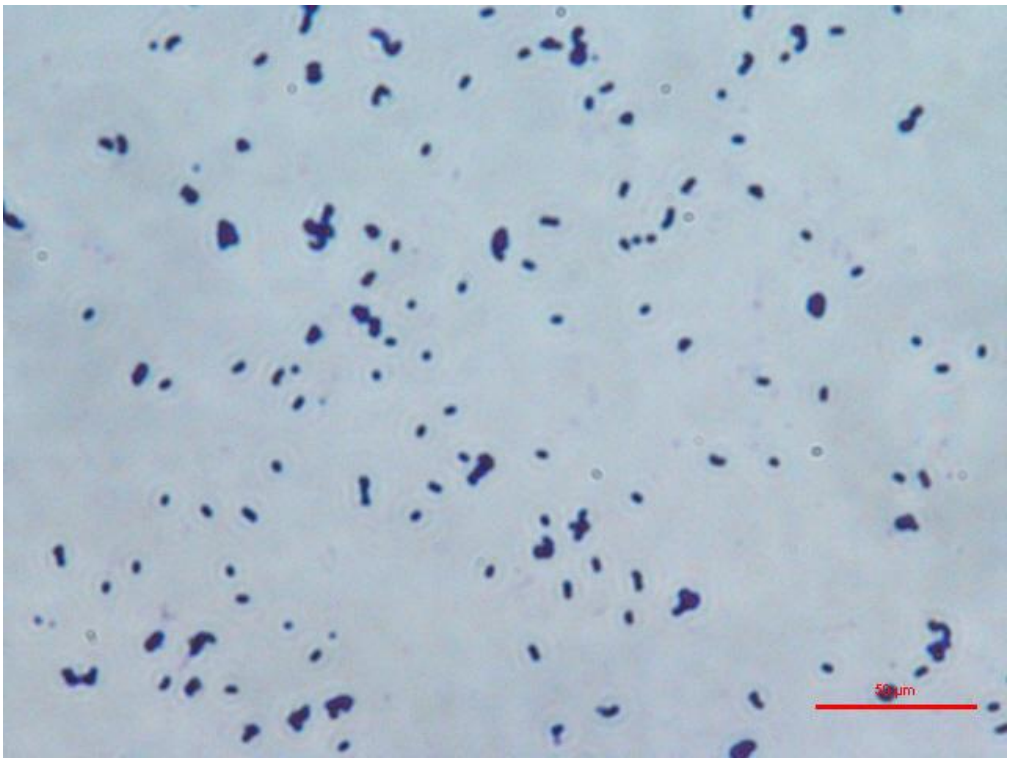


Figure 6: Biofilms of *A. baumannii* grown at 44°C, for 24 hours, analysed by bright field microscopy (400X).

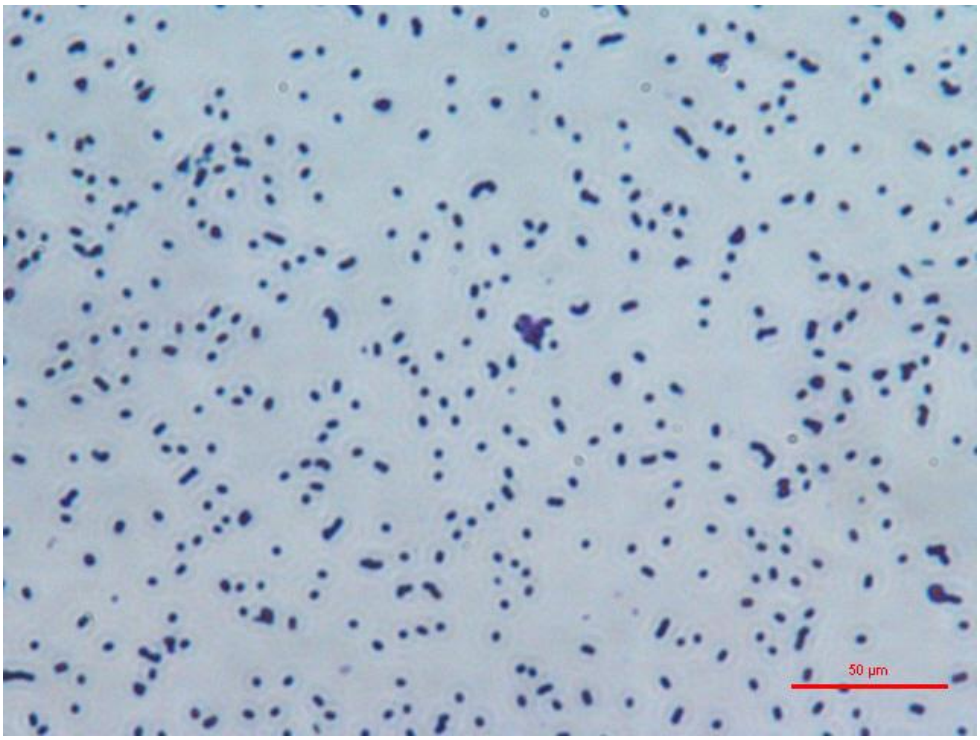


Figure 7: Biofilms of *A. baumannii* grown at 44°C, for 48 hours, analysed by bright field microscopy (400X).

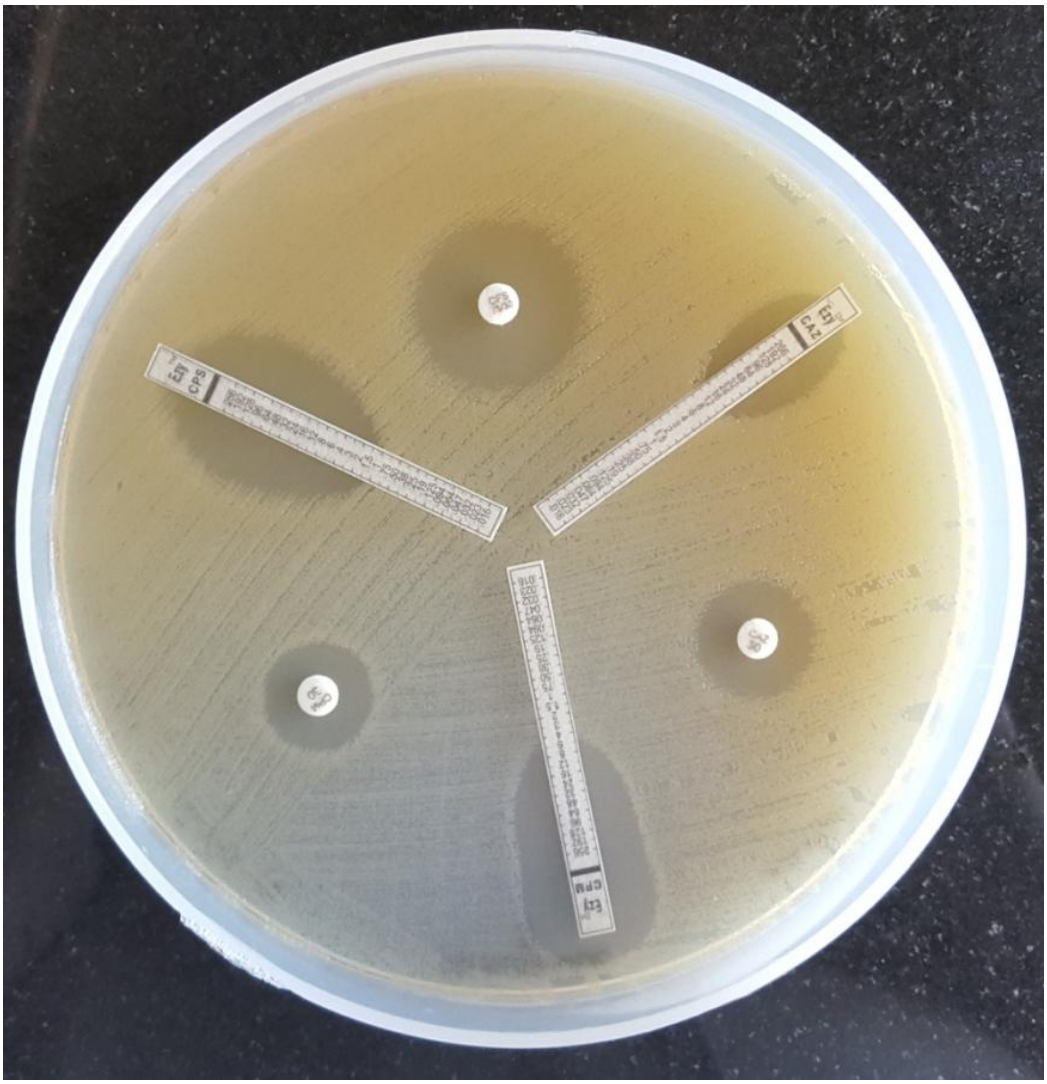


Figure 8: AST/MIC testing of *A. baumannii* ATCC 19606. The antibiotic discs and strips were of [CAZ] ceftazidime, [CPM] cefepime, and [CPS] cefoperazone sulbactum.

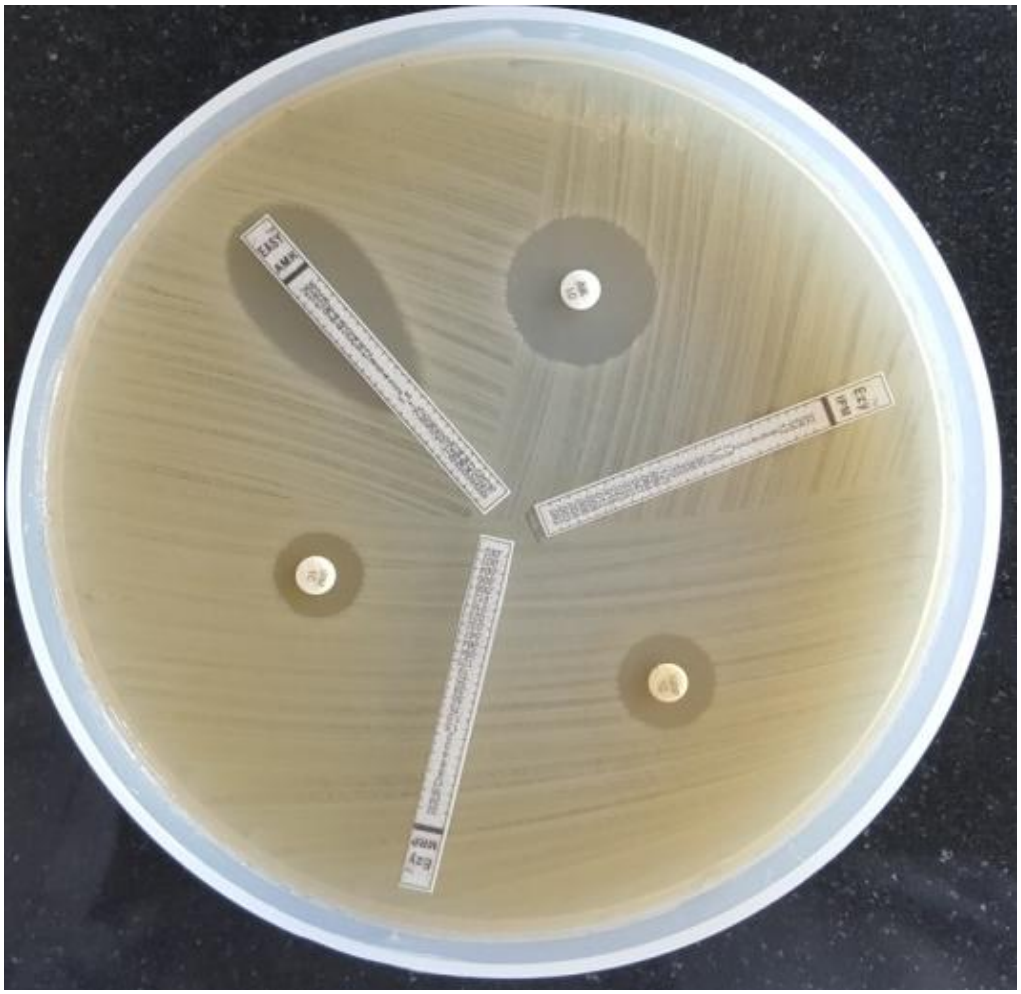


Figure 9: AST/MIC of an *A. baumannii* blood isolated, resistant strain, using antibiotic disks of: AMK [imipenem], IPM [imipenem] and MRP [meropenem].

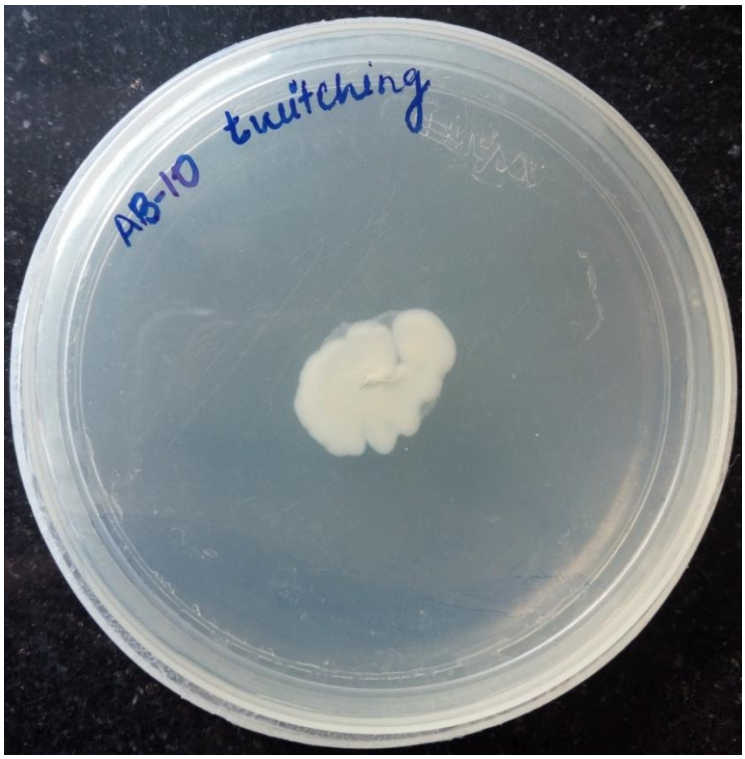


Figure 10: Plate displaying Twitching motility assay after the 24 hour incubation period, before staining.

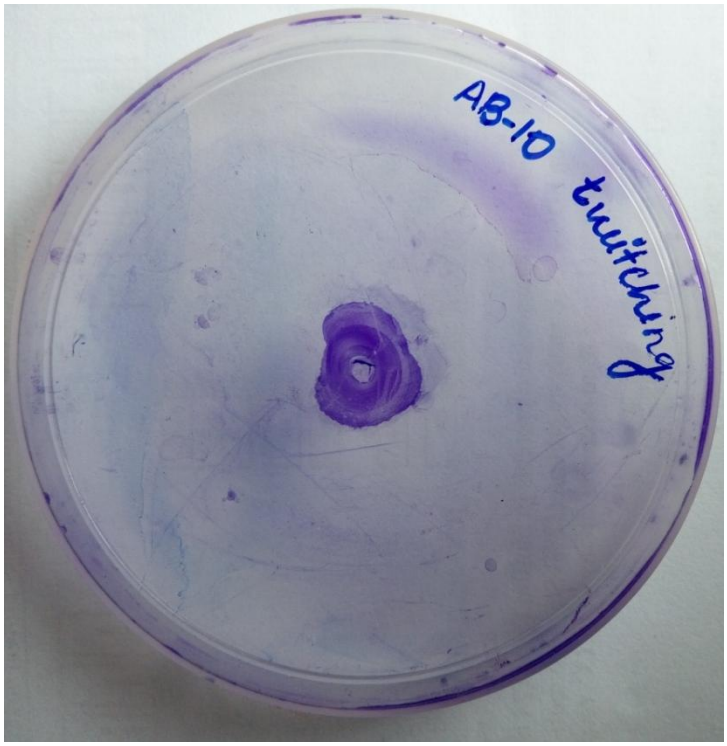


Figure 11: Plate displaying Twitching motility assay after the 24 hour incubation period, after removal of gel and staining.

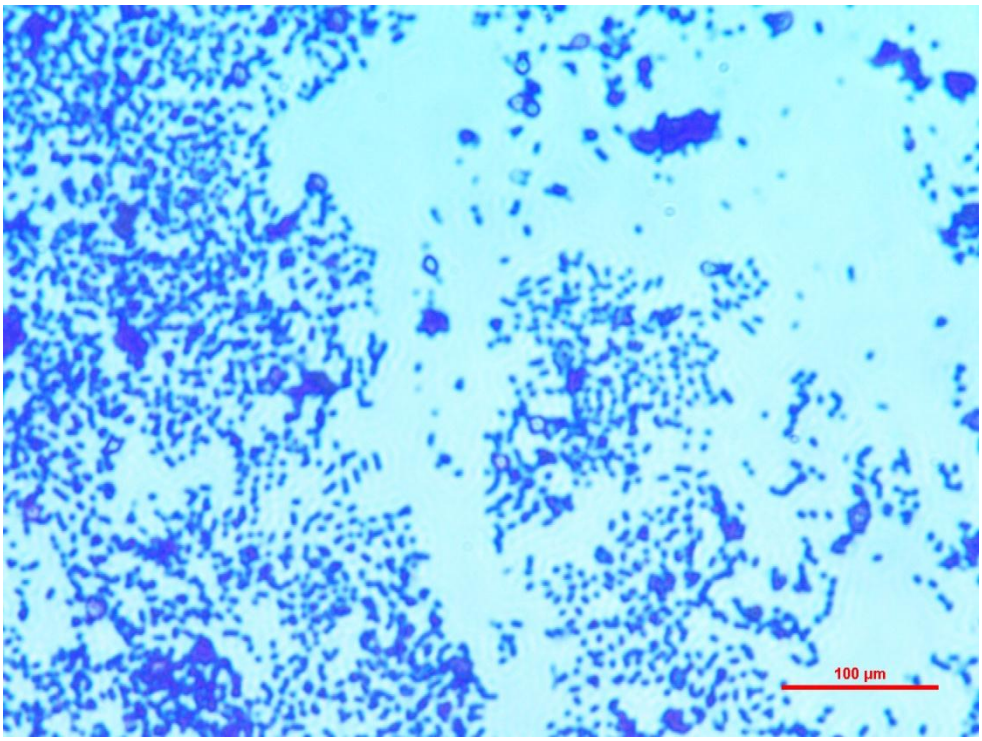


Figure 12- Representing microscopic analysis of *A. baumannii* ATCC 1605 without gold nanoparticles at 37°C after 4 hours.

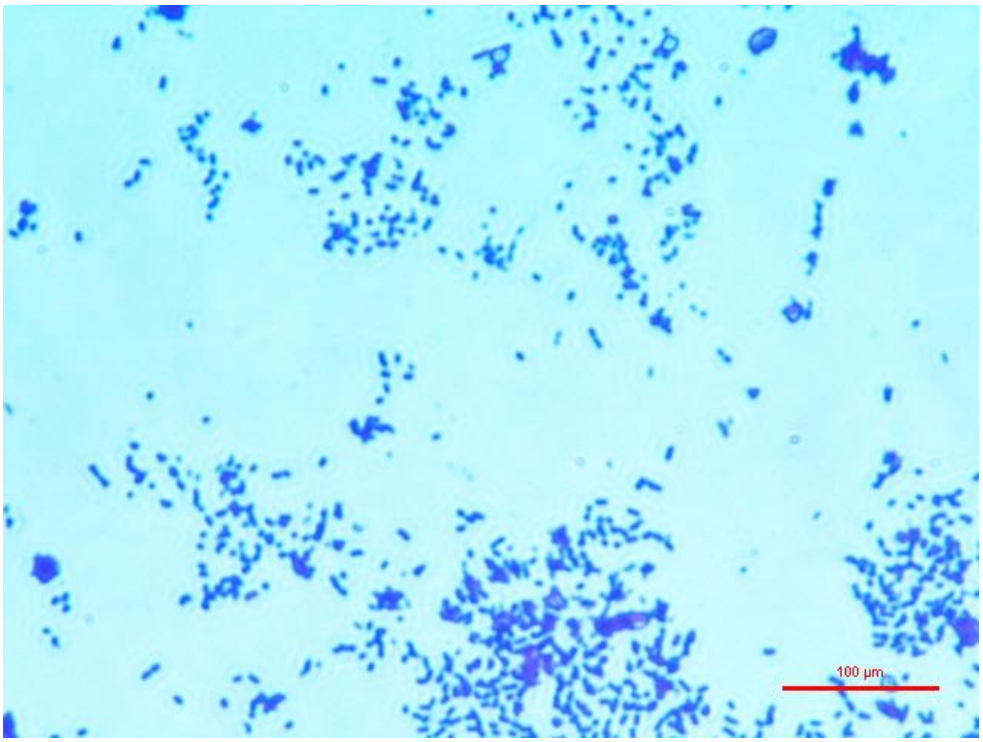


Figure 13- Representing microscopic analysis of *A. baumannii* ATCC 1605 with gold nanoparticles at 37°C after 4 hours.

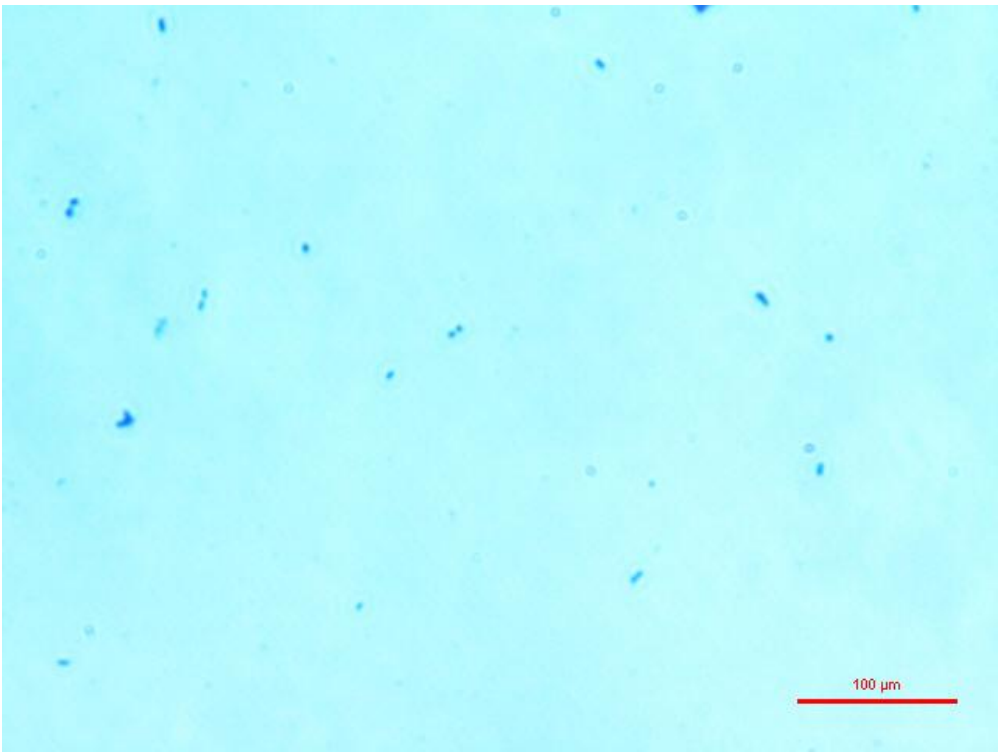


Figure 14 - Representing microscopic analysis of *A. baumannii* ATCC 1605 with 32nM gold nanoparticles at 37°C after 4 hours.

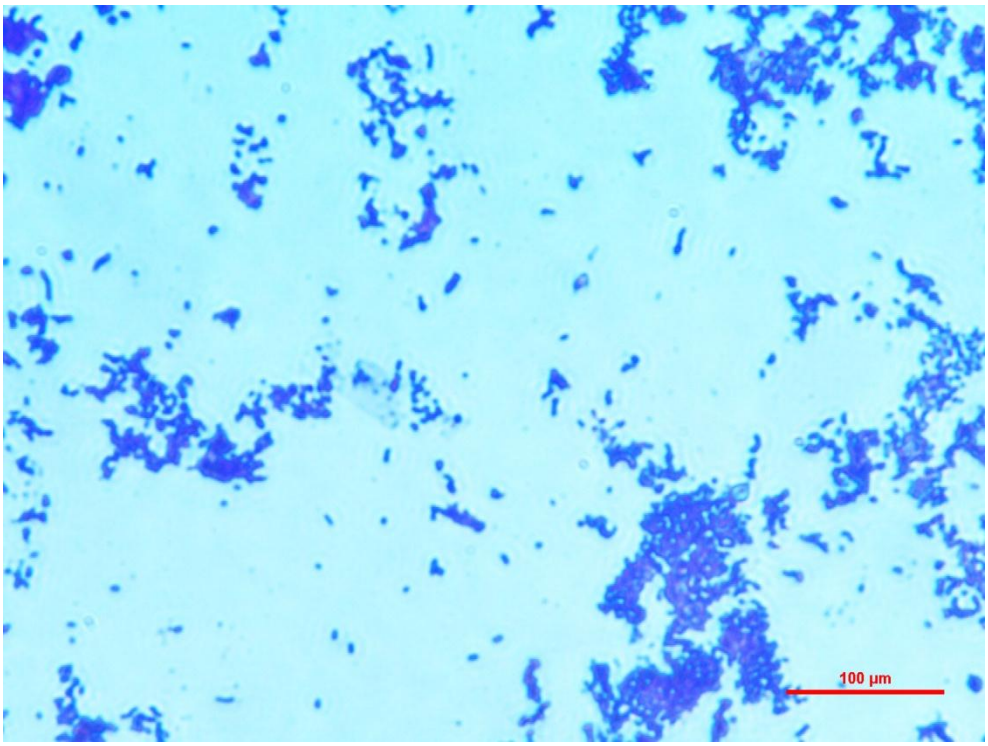


Figure 15 – Representing microscopic analysis of *A. baumannii* ATCC 19606 without gold nanoparticles at 37°C after 4 hours.

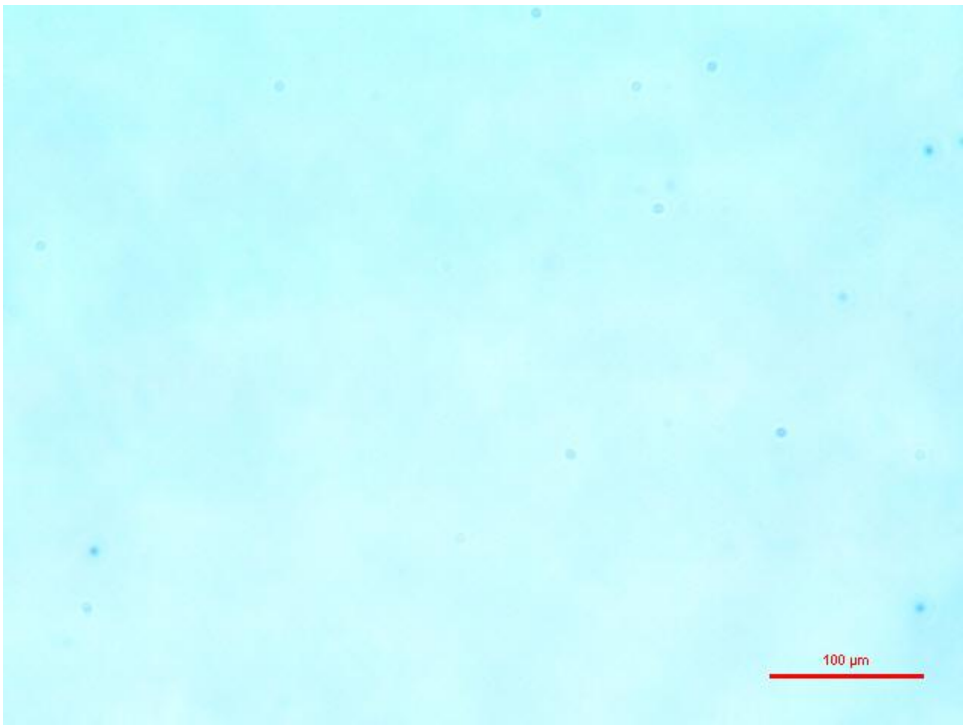


Figure 16 –Antibiofilm assay , *A. baumannii* ATCC 19606, 8nM gold nanoparticle concentration, 4 hours, 37°C

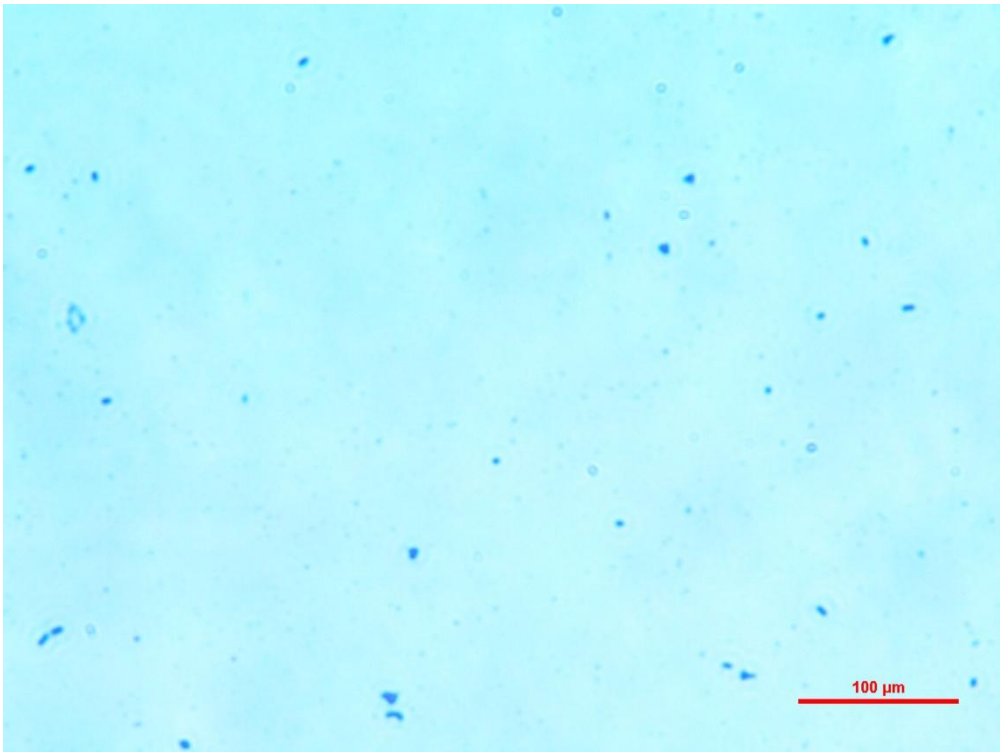


Figure 17 –Antibiofilm assay, *A. baumannii* ATCC 19606, 32nM gold nanoparticle concentration, 4 hours, 37°C

Results:

Charcterization of *A. baumannii* isolates:

To characterize the obtained samples, representative strains were put through a colony PCR procedure. The primers used in the PCR reaction amplified a segment of the 16S-23S rRNA ITS sequence which would give an amplicon of 208 bp.

As was evident from the gel run results, the bands displayed in the lanes with the DNA templates samples were at the same location as the 200 bp band of the DNA ladder, confirming the identity of the samples as being those of *A. baumannii*.

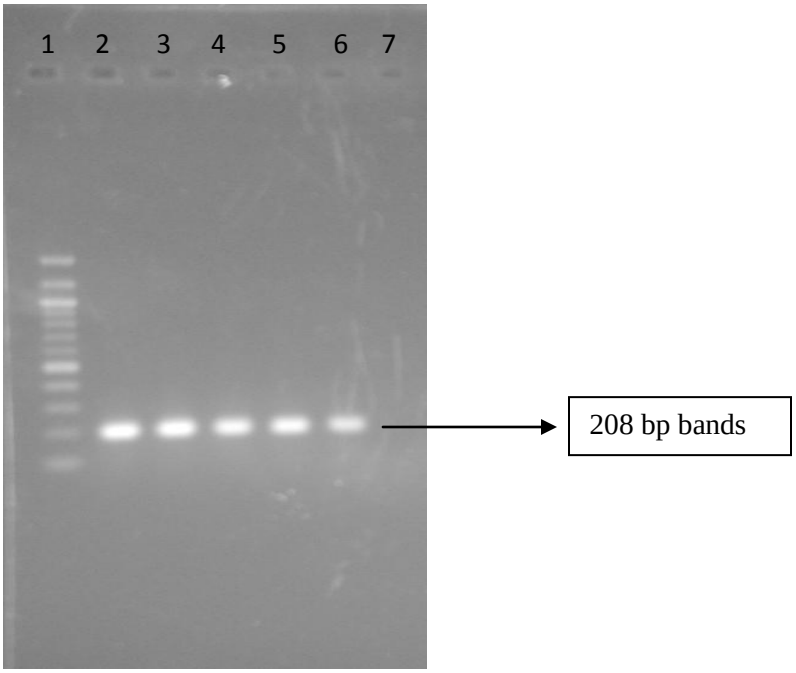


Figure 18 -Agarose gel electrophoresis image of *A. baumannii* samples using ITS region specific primers (208 bp amplicon). Lane 1 - 100 bp DNA ladder. Wells 2-6 - bacterial isolates to be tested. Lane 7 - negative control.

AST and MIC testing:

	PTZ-D	PTZ-M	LEV-D	LEV-M	TE-D	TE-M	CPM-D	CPM-M	CAZ-D	CAZ-M	CPS-D	CPS-M	MRP-D	MRP-M	IPM-D	IPM-M	AMK-D	AMK-M
AB12	16/R	128µg/R	10/R	>32µg/R	0/R	>256 µg/R	0/R	>256 µg/R	0/R	>256 µg/R	13/R	>256 µg/R	14/R	>32µg/R	12/R	>32µg/R	0/R	>256 µg/R
AB13	31/S	1.5µg/S	36/S	0.032 µg/S	29/S	0.125 µg/S	31/S	0.047 µg/S	33/S	0.25µg/S	27/S	1.5µg/S	38/S	0.125 µg/S	39/S	1.5µg/S	26/S	1.5µg/S
AB14	30/S	1.5µg/S	34/S	0.094 µg/S	31/S	0.19µg/S	34/S	0.064 µg/S	33/S	0.50µg/S	32/S	1.5µg/S	40/S	0.047 µg/S	39/S	0.125 µg/S	29/S	0.875 µg/S
AB19	10/R	>256 µg/R	0/R	>32µg/R	9/R	>256 µg/R	9/R	>256 µg/R	0/R	>256 µg/R	11/R	>256 µg/R	9/R	>32µg/R	12/R	>32µg/R	12/R	64µg/R
AB25	14/R	40µg/l	11/R	>32µg/R	0/R	>256 µg/R	15/I	12µg/l	18/S	16µg/l	18/I	48µg/l	16/I	>32µg/R	14/R	>32µg/R	24/S	1.5µg/S
AB27	21/S	12µg/S	15/I	6µg/I	21/S	12µg/l	0/R	>256 µg/R	0/R	>256 µg/R	17/I	48µg/l	16/I	>32µg/R	16/R	>32µg/R	14/R	64µg/R
AB35	31/S	2µg/S	35/S	0.125 µg/S	22/S	1.5µg/S	34/S	0.19µg/S	30/S	0.75µg/S	25/S	4µg/S	36/S	0.5µg/S	34/S	0.125 µg/S	30/S	1.5µg/S
AB38	34/S	0.75µg/S	38/S	0.016 /S	28/S	0.125 µg/S	38/S	0.064 µg/S	32/S	0.25µg/S	29/S	0.75µg/S	37/S	0.064 µg/S	32/S	0.125 µg/S	28/S	0.75µg/S
AB40	0/R	>256 µg/R	9/R	>32µg/R	9/R	>256 µg/R	10/R	>256 µg/R	0/R	>256 µg/R	13/R	>256 µg/R	10/R	>32µg/R	9/R	>32µg/R	0/R	>256 µg/R
AB45	13/R	192µg/R	11/R	>32µg/R	0/R	>256 µg/R	10/R	>256 µg/R	0/R	>256 µg/R	14/R	>256 µg/R	16/I	>32µg/R	14/R	>32µg/R	10/R	>256 µg/R
AB46	20/I	32µg/l	11/R	>32µg/R	0/R	>256 µg/R	14/R	>256 µg/R	0/R	>256 µg/R	16/I	32µg/l	15/I	>32µg/R	12/R	>32µg/R	0/R	>256 µg/R
AB47	10/R	>256 µg/R	15/I	>32µg/R	11/R	>256 µg/R	0/R	>256 µg/R	0/R	>256 µg/R	15/I	>256 µg/R	16/I	>32µg/R	16/R	>32µg/R	0/R	>256 µg/R
AB48	12/R	>256 µg/R	13/R	12µg/R	0/R	>256 µg/R	0/R	>256 µg/R	0/R	>256 µg/R	18/I	48µg/l	16/I	>32µg/R	11/R	>32µg/R	0/R	>256 µg/R

Table 1

AB9	13/R	>256µg/R	9/R	>32µg/R	0/R	>256µg/R	9/R	>256µg/R	9/R	>256µg/R	12/R	>256µg/R	11/R	>32µg/R	11/R	>32µg/R	0/R	>256µg/R
AB10	10/R	>256µg/R	0/R	>32µg/R	0/R	>256µg/R	11/R	>256µg/R	0/R	>256µg/R	13/R	>256µg/R	10/R	>32µg/R	13/R	>32µg/R	0/R	>256µg/R
AB11	11/R	>256µg/R	16/I	>32µg/R	0/R	>256µg/R	12/R	>256µg/R	0/R	>256µg/R	13/R	>256µg/R	17/I	>32µg/R	12/R	>32µg/R	0/R	>256µg/R

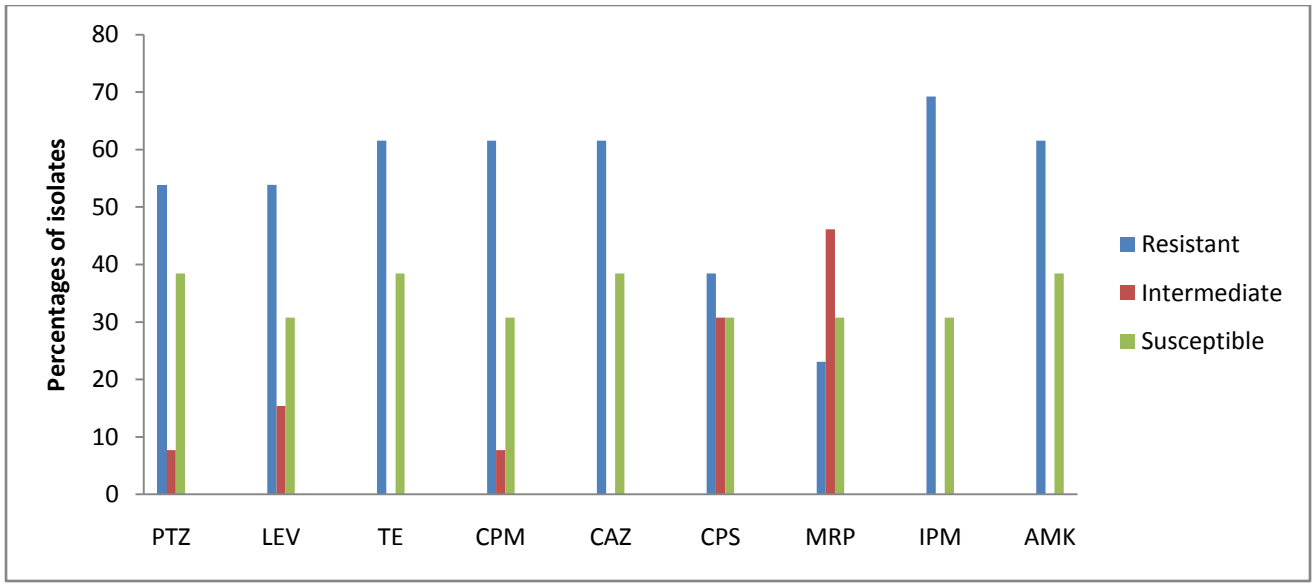
Table 2

Table 1- AST/MIC results of blood isolates of *A.baumannii.*

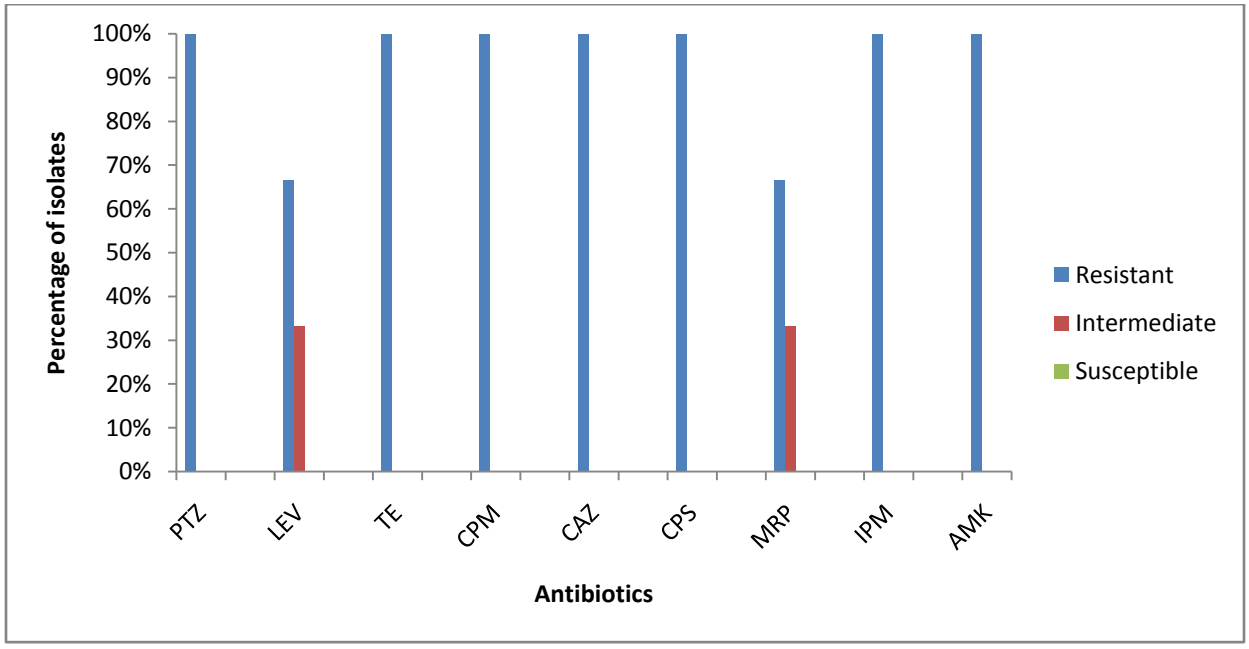
Table 2- AST/MIC results of urine isolates

S=Susceptible, I=Intermediate, R= Resistant.CAZ –ceftazidime, LEV-levofloxacin, AMK- amikacin, PTZ-piperacillin tazobactum, TET- tetracycline, CPM-cefepime, CPS-cefoperazone sulbactum, MRP- meropenem, IPM-imipenem

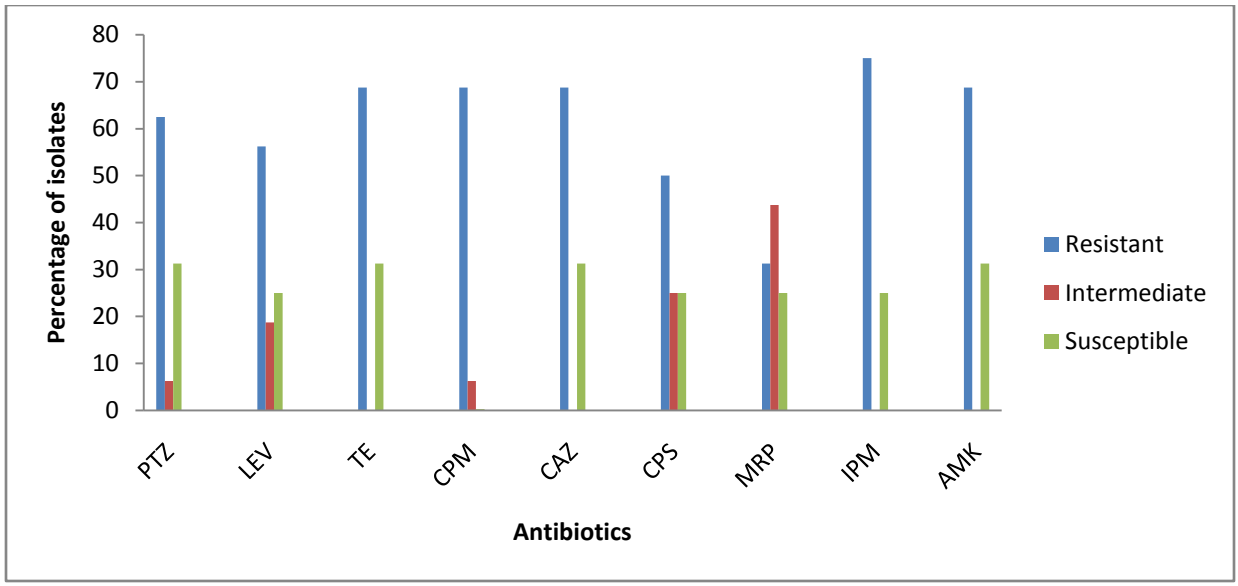
D-Disc diffusion, M- MIC strips (E-strips)



Graph 1- Antibiogram patterns of *A. baumannii* blood isolates. CAZ –ceftazidime, LEV-levofloxacin, AMK- amikacin, PTZ- piperacillin tazobactum, TET- tetracycline, CPM-cefepime, CPS-cefoperazone sulbactum, MRP- meropenem, IPM- imipenem



Graph 2- Antibiotic resistance phenotypes of *A. baumannii* urine isolates. CAZ –ceftazidime, LEV-levofloxacin, AMK- amikacin, PTZ- piperacillin tazobactum, TET- tetracycline, CPM-cefepime, CPS-cefoperazone sulbactum, MRP- meropenem, IPM- imipenem



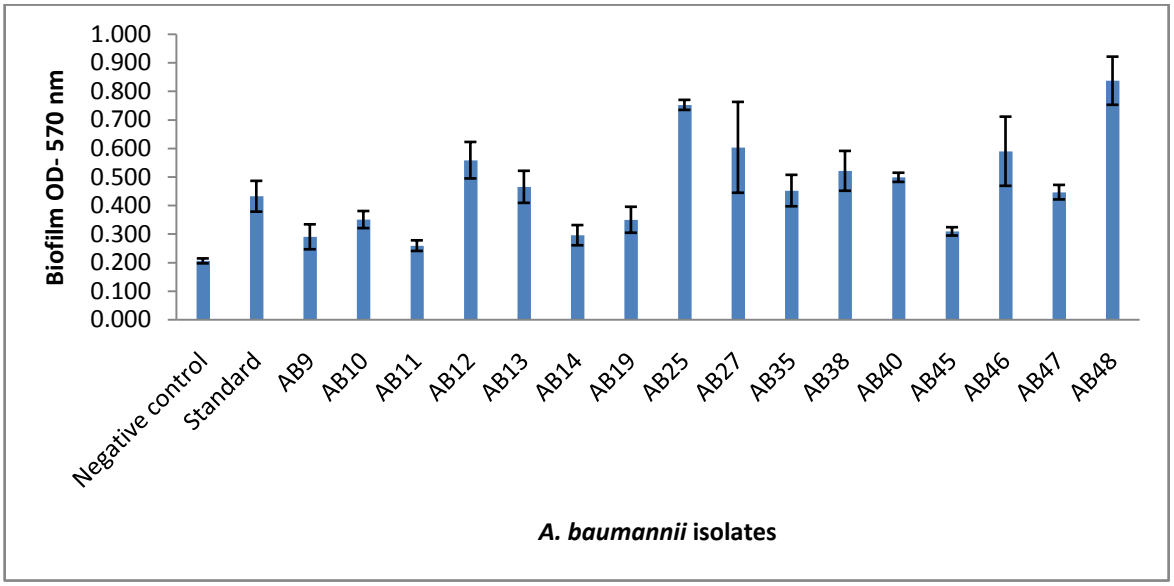
Graph 3-Antibiotic resistance phenotypes of all *A. baumannii* isolates. CAZ –ceftazidime, LEV-levofloxacin, AMK- amikacin, PTZ- piperacillin tazobactum, TET- tetracycline, CPM-cefepime, CPS-cefoperazone sulbactum, MRP- meropenem, IPM- imipenem

The proportions of isolates which were tested as sensitive, against all antibiotics remained below 40%, whether checking by isolate source, or altogether.

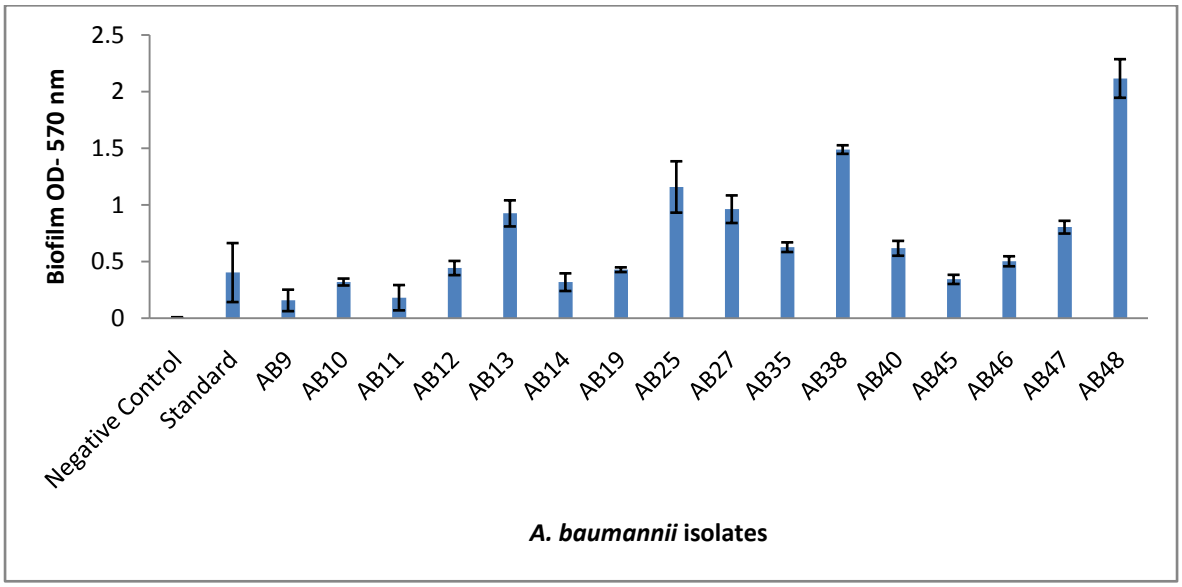
The isolates derived from urine were found to be highly resistant (60-100% were resistant) to nearly all the tested antibiotics, while the blood isolates displayed a greater sensitivity to the antibiotics (30-70% were resistant).

Biofilm formation:

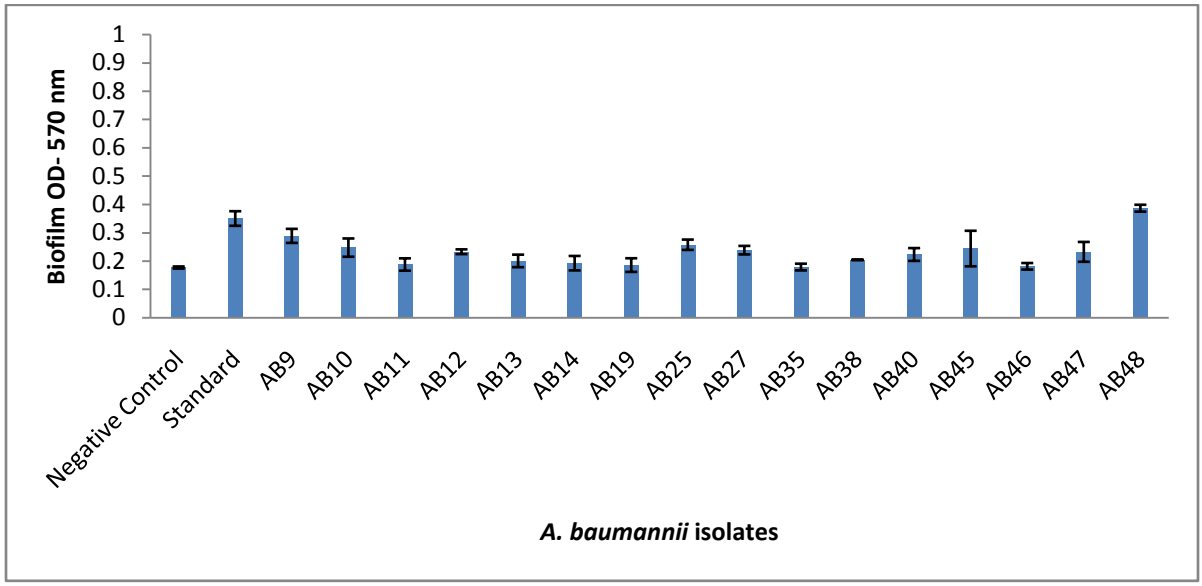
The biofilm formation characteristics of the isolates was studied under two different temperatures, and was time dependent, i.e, the effect of time upon the biofilm formation at both temperatures was studied as well.



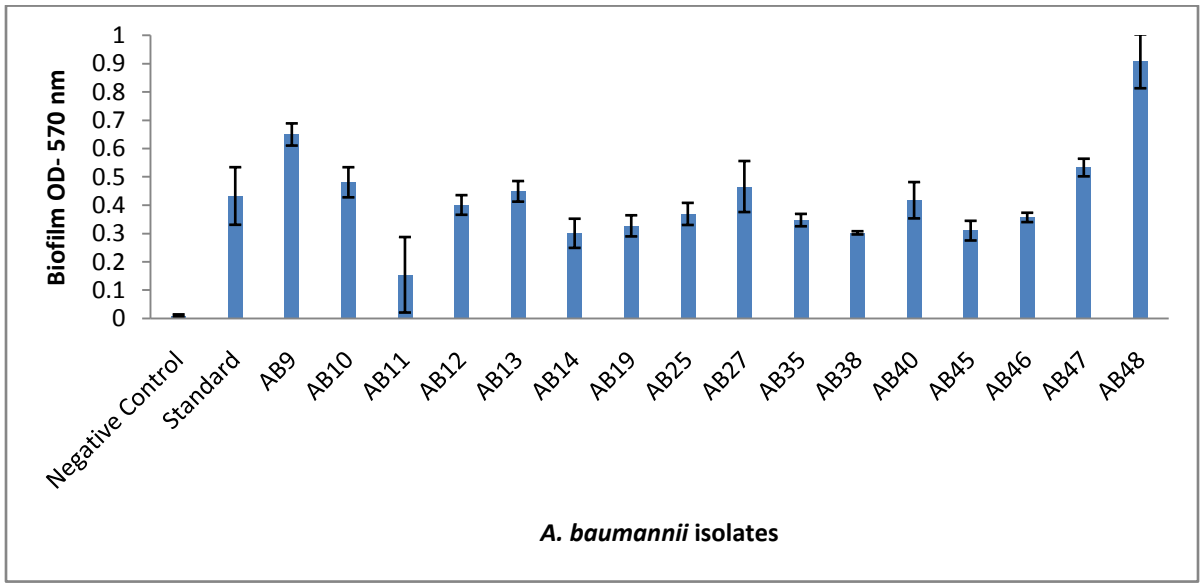
Graph 4-Biofilm formation levels of the different isolates incubated for 24 hours at 37°C. The standard strain is *A. baumannii* ATCC 19606



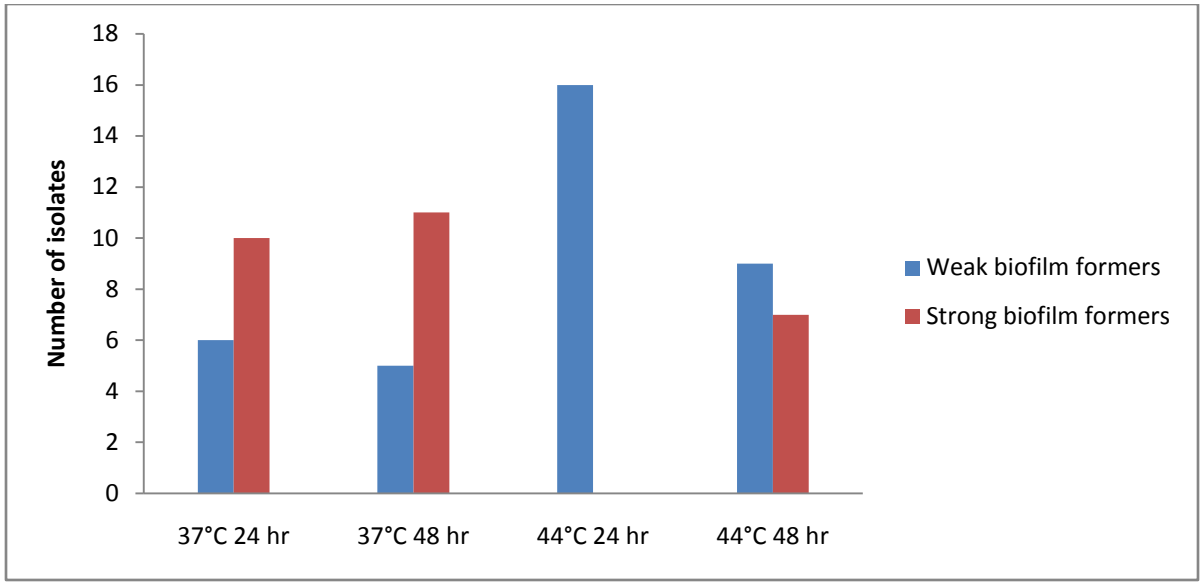
Graph 5- Biofilm formation levels of the isolates when incubated for 48 hrs at 37°C. The standard strain is *A. baumannii* ATCC 19606



Graph 6-Biofilm formation levels of isolates when grown for 24 hours at 44°C. The standard strain is *A. baumannii* ATCC 19606



Graph 7-Biofilm formation levels of isolates grown for 48 hrs. at 44°C. The standard strain is *A. baumannii* ATCC 19606



Graph 8-Time and temperature dependent study of biofilm formation ability of various *A. baumannii* isolates

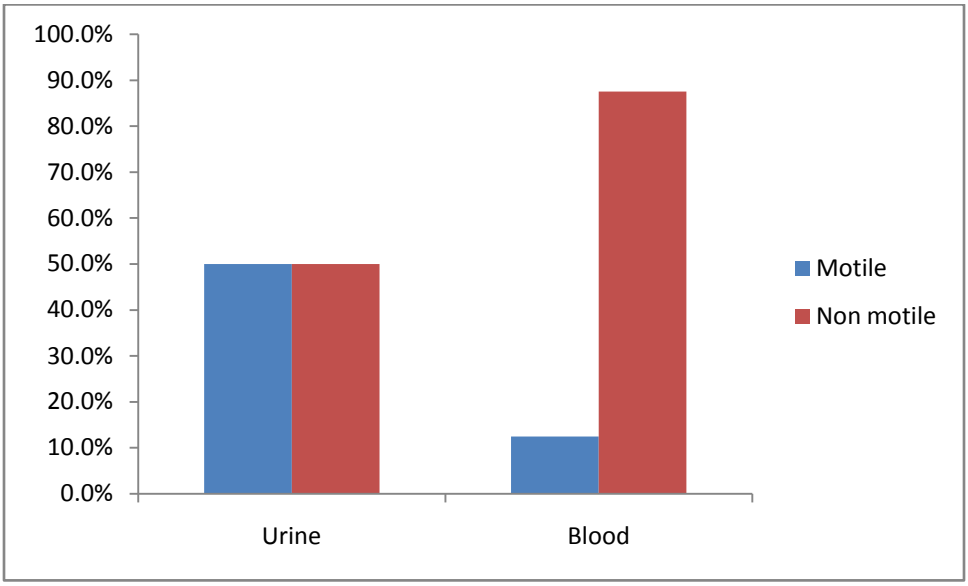
From these bar charts, it was found that biofilm forming capability of *A. baumannii* isolates remains high at 37°C while becoming weak when incubated at 44°C. This phenotype, regarding the low biofilm formation at 44°C, changes if the incubation time increases, as is evident by the results obtained from the isolates incubated for 48 hours.

Another strange observation is the increase in biofilm forming capability of urine isolates if incubated for 48 hours at 44°C (66.7% strong biofilm formers). The urine isolates were otherwise weak biofilm formers at all other temperatures (0% strong biofilm formers).

The blood isolates displayed mostly high biofilm formation at both periods of incubation at 37°C (77% and 84.6% strong biofilm formers after 24 hours and 48 hours incuvation, respectively), while displaying weak biofilm formation capability at 44°C (0% strong biofilm formers), when incubated for 24 hours, which then increases if given more time for incubation, i.e., 48 hours (38.5% strong biofilm formers).

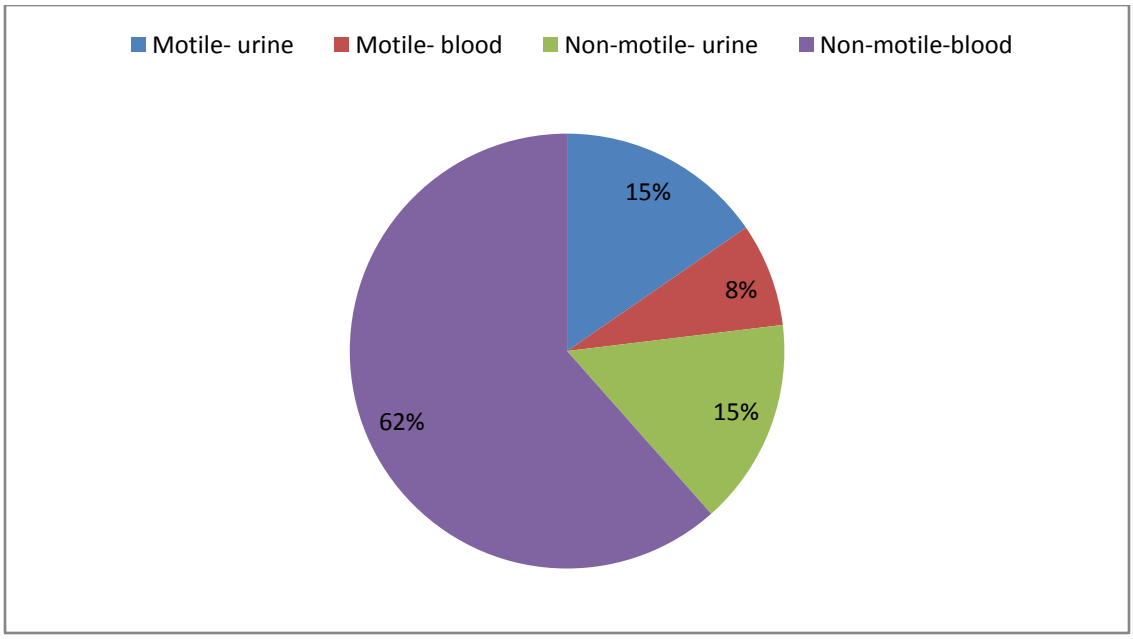
Motility Testing (Twitching)-

The motility of *A. baumannii* clinical isolates from two sources was checked (blood and urine). The results were found to be variable.



Graph 9- Bar graph displaying the comparisons between the number of motile and non- motile isolates found from the two sources- blood and urine.

It was found that the isolates from urine possessed a greater proportion of motile *A. baumannii* strains (50%) while the blood isolates only had 10% of the cultures displaying twitching motility. Looking at an overall picture, 77% of all isolates were non-motile, with urine isolates dominating the number of motile samples. The subset of isolates that did display motility, leave themselves open to correlation with their source and other characteristics studied at present. These studies maintain their importance due to how little is known about the motility phenotype in *A. baumannii.*

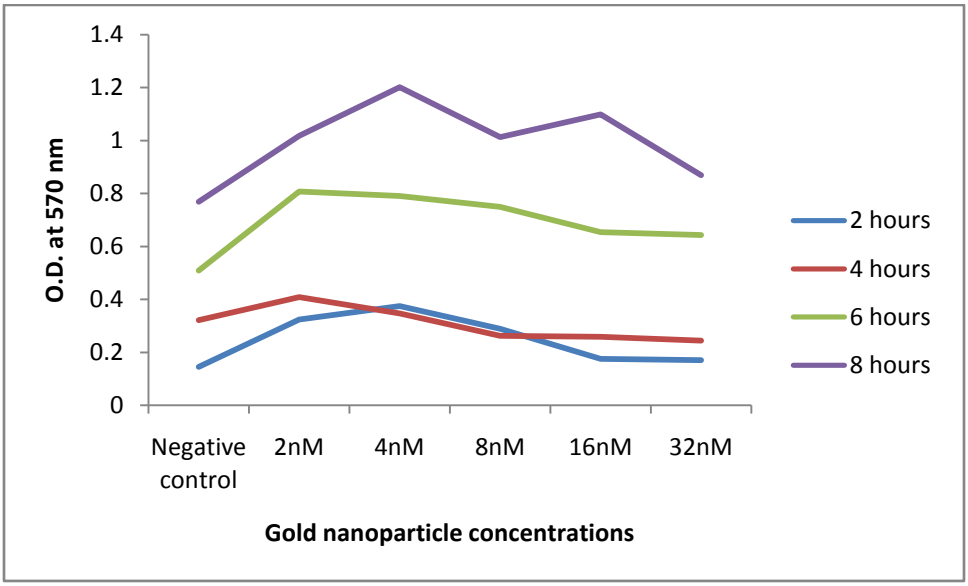


Graph 10- Pie chart representation of the contribution of the differently sourced isolates to the overall proportion of motile samples and non-motile samples

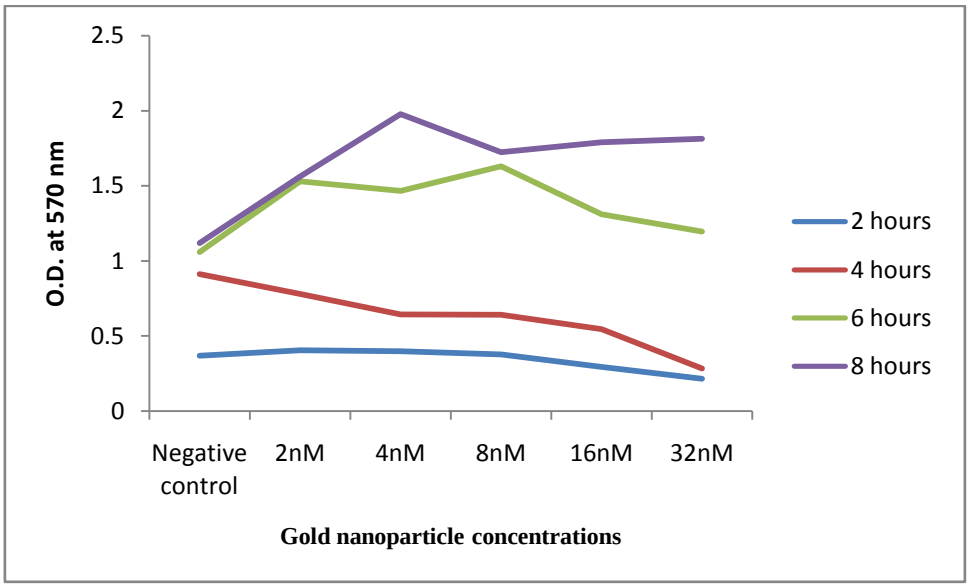
Antibiofilm assay:

Gold nano particles (size, morphology)

The effect of various concentrations of gold nanoparticles upon biofilm formation of *A. baumannii* strains- ATCC 19606 and ATCC 1605, was studied in a time dependent fashion. The anti-biofilm activity readings were taken after 2 hours, 4 hours, 6 hours and 8 hours. The following graphs display the results obtained:



Graph 11-Effect of gold nanoparticles, at different concentrations, upon *A. baumannii* strain ATCC 19606. Readings taken at different time points



Graph 12-Effect of gold nanoparticles, at different concentrations, upon *A. baumannii* strain ATCC 1605. Readings taken at different time points

From these results, it is clear that the nanoparticles display antibiofilm effects for upto 4 hours, after which biofilm growth seems to increase, and reached levels even higher than the negative control which contained only the culture, and no nanoparticles.

For the ATCC 19606 strain, an 8 nM concentration of gold nanoparticles was found to decrease growth, i.e, 8 nM was the minimum concentration required to inhibit biofilm formation, for upto 4 hours, in *A. baumannii* strain ATCC 19606.

For the ATCC 1605 strain, a concentration of 16 nM was found to be enough to produce an anti-biofilm effect, after 2 hours. However, if checked after 4 hours, even a 2nM concentration was enough to cause biofilm formation inhibition in *A. baumannii* strain ATCC 1605.

Discussion:

Acinetobacter baumannii biofilm formation has frequently been a part of studies and research being performed upon it due to its apparent association with the bacteria's hardiness, i.e., its ability to survive under harsh conditions, aiding it in avoiding antimicrobials from invading cells, and even in colonizing blood vessels in the human body. Through our research however, we have also studied the differential effect that the source of the *A. baumannii* isolate can have on its biofilm forming capability.

Starting with an overview, it was found that the isolates were mostly strong biofilm formers at 37°C and continued to be so even when checked after 48 hours of incubation at the same temperature, indicating this to be a part of the optimal growth temperature range for the growth of this organism. At an incubation temperature of 44°C, biofilm growth seemed to be inhibited when checked after 24 hours, but showed considerable improvement when checked again after a 48 hour incubation period. This shows the ability of *A. baumannii* to grow at elevated temperatures, with a slightly elongated growth phase where it possibly synthesizes heat shock proteins/other macromolecules required for its survival at elevated temperatures. The proteins in the DnaK machine and the GroE machine could possibly be involved with the adaptation mechanism (as these were found to be the primary proteins which are produced by the cell on exposure to a heat shock) [18]. This shows the great adaptability this bacteria has to higher temperatures, which is important especially when considering the raise in temperature it will encounter when shifting from an external environment to within a host's body [18].

On differentiating biofilm formation capability based upon the source of the isolates being tested, it was found that the urine isolates mostly contained weak biofilm formers, as compared to the blood isolates. However, even the urine isolates displayed a strong biofilm formation phenotype after the 48 hour incubation under 44°C. These results, combined with the above discussion might be indicative of biofilm production as a stress reaction, wherein the biofilm provides some sort of defense against external conditions, be it desiccation or the host organism's immune system.

The twitching motility phenotype of *A. baumannii* isolates was studied and the results displayed its positive presence in the isolates. The motility phenotype was found to be in a higher

proportion amongst the urine isolates, as compared to the cultures isolated from blood. This is new territory as motility characteristics have only recently begun to be studied based upon the source of isolation of the bacteria and also since its role in virulence is still under research. A possible reason for the urine isolates being more motile might be the need of motility for colonization of the urinary tract, by bacteria, as has been previously studied[19]. Further, the blood isolates while being more motile, also possess the characteristic of being higher biofilm formers, while the urine isolates, while being more frequently motile, are weak biofilm formers, giving us a possible link between motility and biofilm formation. An exact reason for more motile isolates being weak biofilm formers is not currently known, leaving more scope for future research.

The antimicrobial susceptibility testing and minimum inhibitory concentration tests carried forward an increasingly worried trend, i.e., multi drug resistance in *A. baumannii* clinical isolates. The isolates were, generally, highly resistant to the antibiotics that are suggested for use, in case of an *A. baumannii* infection, by the Indian Council of Medical Research (ICMR), thus displaying resistance to all the generally used antibiotics (since these are often indiscriminately used by patients against a variety of diseases). Moving forward, the use of combined therapies against *A. baumannii* must be considered more frequently, as they have been found to be effective in the past and also help reduce the chances of the bacteria developing resistance to any single antibiotic used in the therapy. Further, colistin might be the only antibiotic that is currently effective against *A. baumannii*(not studied here) since the other major antibiotics are found to be ineffective now, making it important to reduce its indiscriminate use by the general public, and even when used, to be utilized in combination with other antibiotic classes, like fluorquinolones and carbapenems [5, 6].

Stratifying the results according to the *A. baumannii* sources, we find that the urine isolates display a high amount of resistance against all the antibiotics tested. The blood isolates, while being majorly resistant, also had some susceptible isolates and showed the lowest resistance to meropenem. These results display the importance of studying antibiotic resistance patterns according to sources since differently sourced isolates seem to display different antibiotic susceptibility patterns (as is evident by our results). Once generalized, by studying a large

number of isolates differentiated based upon source, antibiotic regimes specific to the site of infection can be developed.

The antibiofilm effects of the gold nanoparticles seemed to be evident by our results. Its success is possibly due to its small size, due to which it can penetrate into the exo-polysaccharide matrix of the biofilm and be engulfed by the bacterial cells held within and then proceed to kill them through mechanisms already discussed in a previous section.

The antibiofilm action, however, seemed to wean off after 4 hours, as could be seen through the results of the biofilm estimation at the 6 hour and 8 hour marks of incubation periods, after nanomaterials were added. This could possibly be caused due to either a loss in morphology of the nanoparticles after a time point(due to external factors) or due to a decrease in free nanoparticles, since a majority of the nanoparticles had already been engulfed by the bacteria, leaving a small number of bacteria, that had been initially left alive, to resume biofilm formation.

Further, it could be seen that biofilm formation had increased significantly, as compared to the negative controls, after 6 and 8 hours of incubation, which could possibly be because of a stress response of the bacteria where it responded to an environmental threat by increasing its biofilm production.

The time-dependent antibiofilm phenotype however, allows for the gold nanoparticles to be incorporated into a hospital's cleaning routine, since a cleaning schedule maintains a cleanup round every 3 hours, a length of time during which the nanoparticles are at their highest effectiveness.

These gold nanoparticles may also be used as an effective drug delivery system to target biofilms which form within a human's blood vessels, since it has been found to be less toxic (relative to nanoparticles of other compounds) [12] while retaining its ability to penetrate through biofilms.

Conclusion and Future Prospects:

Acinetobacter baumannii displays itself as a major threat, especially within hospitals with a high density of patients (since the chances of an outbreak increases). There needs to be a greater impetus placed within research institutions to find new antibiotics against this bacterium as its .multidrug resistance phenotype continues to expand to the most commonly used ones. There is also scope for creation of an antibiotic regime specific to different infection sites. In relation to this, further source stratified studies of multi drug resistant isolates need to be conducted with an even wider variety of sources from which *A. baumannii* has been isolated.

This bacteria's biofilm forming capability increases and is part of *A. baumannii's* response to external stress (like elevated temperature conditions). Hospitals need to maintain high levels of sanitation to prevent its growth on various surfaces. Antibiofilm compounds may be added to the list of chemicals being used, already, by hospital cleaning crews to aid in this endeavor. Gold nanoparticles can be proposed as a potential solution, after some further standardization of the minimum concentration required to inhibit biofilm formation.

Another future area for study is the effect of disrupting biofilm formation (through molecular methods like gene silencing or gene knockouts) and then studying the effect this has upon the persistence of *A. baumannii* at higher temperatures.

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