Study on carbapenem resistance associated genes prevalent in India

THESIS

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By

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DECLARATION

I hereby declare that the work reported in the M. TECH. (integrated) thesis entitled **"Study on carbapenem resistance associated genes prevalent in India"** submitted at **Jaypee University of Information Technology, Waknaghat, India**, is an authentic record of work done by me (Shreeya Agrawal) for the period of July 2019-May 2020 carried out under the supervision of **Dr. Jitendraa Vashistt, Assistant**

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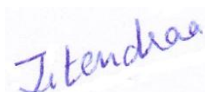
SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M. TECH. (integrated) thesis entitled **"Study on carbapenem resistance associated genes prevalent in India",** submitted by **ShreeyaAgrawal (161841) at Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out under my supervision, from July 2019 till May 2020. This work has not been submitted elsewhere for any other degree or diploma.

Supervisor



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Shreeya Agrawal

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APPENDIX

µg	-	Microgram
µl	-	Microliter
AST	-	Antibiotic susceptibility test
ATCC	-	American Type Culture Collection
BLAST	-	Basic local alignment search tool
LB	-	Luria broth
MDR	-	Multidrug resistance
MHA	-	mueller hinton agar
MIC	-	Minimum inhibitory concentration
min	-	Minutes
ml	-	Milli Litre
MSA	-	Multiple sequence alignment
ºC	-	degree Celsius
PCR	-	Polymerase chain reaction
UTI	-	Urinary tract infections
V/V	-	Volume/Volume
vol	-	Volume

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Abstract

Acinetobacter baumannii is one of the most concerned pathogens listed by WHO, which is in a need to be taken care off due to its increase in resistance property against many antibiotics which is known between us. The study has been done on the resistance found against the antibiotic carbapenem β-lactam. Various sites have been discovered in *Acinetobacter baumannii* where carbapenem antibiotic acts and blocks its activity. To counter-attack it, bacteria underwent mutations and made changes in its genome to block the particular antibiotic. So, to tackle this, carbapenem is given in various combinations. In this process of mutation in genome, various classes of OXA, IMP, VIM etc. genes have been identified. In this study, it was found that all these genes are mutants of one or another form of original gene. As a result, the same set of genes had been undergoing with number of mutations resulting it in a different set of genes in the same genome.

1. Introduction:

Acinetobacter baumannii is a gram negative, strictly aerobic, non-motile, coccobacillus, non-fermentative nosocomial bacterial pathogen with G+C content 39-47%. The optimum temperature for good growth of *Acinetobacter* genus is 35°C- 39°C[Jin*et al*, 2012]. *A. baumannii* is the only species which can grow efficiently till 45°C. Its name has been given after the name of bacteriologist Paul Baumann in 1986 [Antunwa *et al*, 2014].The history *A. baumannii* is little complicated. In 1911, *A. baumannii* was named as *Micrococcus calcoaceticus*. It was 1957, when the genus *Acinetobacter* was created but this strain of *Acinetobacter* kept under *Acinetobacter calcoaceticus* even till 1986.Nearly about for the next 50 years, it was still even kept under different groups like *Moraxella lwoffi, Alcaligenes hemolysans, Mirococcuscalco-aceticus,* and *Herellea vaginicola* as it was not still clearly and properly classified. The main problem prevalent in this genus was its classification among different strains due to similar phenotypic traits and chemotaxonomic m*et*hods.Later on, *A. baumannii* was classified on the basis on "ITS region" in 16S-23S rRNA [Lin*et al*, 2014;Evans*et al*, 2013].*A. baumannii* is a nosocomial pathogen, which can be defined as the micro-organisms found in the hospital environment and infect the already immuno-compromised patients admitted in the hospital. These pathogens are known to cause infections in the urinary tract, bloodstream and other parts of the body. They can also lead the infections with severe pneumonia.

Before 1970s, treatment of *A. baumannii* was possible with the wide range of antibiotics, like aminoglycosides, β-lactam, and T*et*racyclines. But after 1970s, it developed resistance to almost all class of antibiotics and emerged as MDR (multidrug resistant) bacteria. Mortality rates or death rate for *Acinetobacter* species varies b*et*ween 30% to 75% and that too with the highest rates with ventilator-dependent patients [Bergogne-Berezin*et al*, 1996].There are other organisms as well which are antibiotic resistant, and they are commonly known as ESKAPE pathogens. ESKAPE stands for *Enterococcus pneumoniae, Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii* and *Enterobacteriaceae*. ESKAPE has the capability of escaping common antibacterial treatments. ESKAPE pathogens are also responsible for nosocomial infections globally. Among these ESKAPE pathogens, *A. baumannii* tops the list and is known to be most challenging organism due to its antibiotic resistance nature. In 2017, WHO (World Health Organization) for the very first time has published a report entitling antibiotic resistant "priority pathogens" to direct and guide research and development related to new antibiotics for treating the infections caused by the pathogens, according to which *A. baumannii* tops the list and ranked 1 [Howard*et al*, 2012].

A. baumannii have the ability of forming biofilms which acts in favor of it and helps in surviving unfavorable environment like antimicrobial treatment, desiccation *et*c. Biofilms formation acts as thick slimy shield to micro-organisms colonies which protect them from external environment. This shield can be formed by either one type of bacterial colony or more than that. It can form biofilm on biotic surfaces like epithelial cells as well as abiotic surfaces like polystyrene, glass *et*c. Biofilms can be transferred and persist into human body through inert surfaces, which may include medical devices used internally or externally such

as prosth*et*ic heart valves, joint prosth*et*ics, cath*et*ers and pacemakers. Biofilms may also cause bloodstream and urinary tract infection, or may form emboli [Srivastava *et al*, 2016]. Emboli can be described as a clot which travels through the bloodstream, and blocks the blood vessels.

The treatment for *A. baumannii* infections has proved to be a major task in front of the world. Earlier carbapenem had been long regarded choice for this infection, but some cases have been reported against carbapenem resistance which made a great concern. Sulbactam was being used against this bacterial infection efficiently, but the activity of this as well reduced against carbapenem-resistant isolates. Apart from sulbactam, polymyxins also show sufficient antimicrobial activity against the infections caused by *A. baumannii* isolates. Minocycline or we can say particularly tigecycline, it's one of the derivatives, shows high antimicrobial against the infections caused by *A. baumannii*, though clinical evidences shows even resistance against them as well. Colistin remains the only antibiotic showing compl*et*e susceptibility to *A. baumannii* [Srivastava*et al*, 2016]. So, this implies that there is an urgent need of new antibiotics or drugs which can be used against the infections of *A. baumannii* as existing ones have already became resistant fully or partially.

Carbapenem drugs are still regarded as a choice for the treatment of infections against *A. baumannii.* But they cannot be used frequently to treat *A. baumannii* associated infection due to emergence of carbapenem resistant *A. baumannii* (CRAB). In present study I am trying to trace the similarities b*et*ween different carbapenem resistance encoding genes of *A. baumannii* using *in-silico* approach. In brief, different genes accounting CRAB were screened using text mining and multiple sequences alignment of proteins and genes FASTA sequences was performed.

2. Review of literature:

2.1 Global scenario

Acinetobacter baumannii is known to be most troublesome pathogen globally. This is so because infections caused by them proved to be a major threat to human soci*et*y and is leading to various number of deaths. The mortality rate due to *A. baumannii* is already b*et*ween 30% to 40% [Jin*et al*, 2012].

Acinetobacter baumannii is also known as "Iraqibacter" as it was used against Iraq and Afghanistan soldiers during Iraq war by US military. Its sudden emergence in military treatment facilities caused a great havoc among them. Its infection had spread to civilians as well in the hospital as infected soldiers were transported through various medical facilities. The interest thing about this is almost after 3 years, *i.e.,,* from 1 January 2002 to 31 August 2004), clinical isolates of *A. baumannii* was found in blood cultures obtained from 102 patients which were hospitalized at military medical facilities treating as service members injured in Iran-Kuwait and Afghanistan region [Giamarellou *et al*, 2008].

2.2 Habitat

Talking about natural habitat, *Acinetobacter* species are believed to be found everywhere in nature, not only in activated sludge, dump, sewage, hydrogen contaminated areas but also on veg*et*ables, humans and animals. This quality to be present in so many ecological niches led some authors to consider *Acinetobacter* as microbial weeds [Atrouni *et al*, 2016]. This understanding added to the misconception that *A. baumannii* is also omnipresent, while the fact is that not all Acinetobacter species find the natural environment as their habitat. *Acinetobacter baumannii* species commonly occurs in soil and water. They have the ability to survive on moist as well as dry surfaces. They can live on various common disinfectants, thus known as nosocomial pathogen.

The clinical isolates of *A. baumannii* shows that these have the ability of desiccation resistance, ability to remain alive in dry conditions, *i.e.,,* in water limited condition. Some isolates can survive for almost 100 days. *A. baylyi*, a non-pathogenic strain related to *A. baumannii* has capsular polysaccharide composed of repeating carbohydrate units and act as protective glycan shield against external threats, which ultimately results in desiccation resistance property. Although direct evidence for linking biosynth*et*ic pathways for capsular polysaccharides in *A. baylyi* and *A. baumannii* is still lacking, but the ability to r*et*ain water and to shield the cells in *A. baumannii* with polysaccharide capsules which help them to survive in dry conditions, gives the evidence that the capsules contributes to the resistance to desiccation in *A. baumannii*. Furthermore, studies have tried to link desiccation resistance with composition of the outer membrane. A particular mutant strain that produces under acylated lipo-oligosaccharide was unable to survive for long in desiccation environment. It was estimated that altered lipid composition of the outer membrane resulted in increased fluidity in this particular mutant had permitted leakage of water and hydrophilic nutrients out of the cell [Howard *et al*, 2012].

2.3 Infection by *A. baumannii*

Once *A. baumannii* is isolated from a hospital environment, it poses a higher risk for particularly ICU patients which are already immuno-compromised and spend a long period of time in hospital environment, are more prone to *A. baumannii* infections. Patients with dialysis or antimicrobial therapy, or those who are using medical devices like cath*et*ers, sutures, ventilators within last 3 months are at a higher risk of *A. baumannii* infections. Respiratory tract, blood, pleural fluid, urinary tract, surgical wounds, CNS, skin and eyes are the sites of infection caused by *A. baumannii*. As *A. baumannii* has the ability to form biofilms on the surface of the endo-tracheal tube, which may give rise to high level of population in the lower part of the respiratory tract, thus pneumonia patients who need mechanical ventilation are at higher risk of infection.

Talking about *A. baumannii*, it targ*et*s mainly moist tissues like mucous membranes, exposed skin areas due to some injury or accident. When any part of body, specifically skin or soft tissue is infected by *A. baumannii*, it initially looks like the skin of an orange, *i.e.,* peaud'orange. Eventually it leads to sandpaper-like appearance which gives the clear view of vesicles on the skin. This leads to appearance of hemorrhagic bullae, with a visible mortal process followed by bacterial infection in blood. If this is left unattended chemicals are released which trigger the inflammation throughout the body which ultimately leads to death [Longo*et al*, 2014].

2.4 Resistance mechanisms

Resistance means opposition. Major reason which allowed micro-organisms to excel so much is resistance only. This resistance has been developed against the medicines or antibiotics taken during any infection. Antibiotics are the drugs taken to stop the production of bacterial growth inside the body. They kill the bacteria and remove the ties which help them to proliferate inside any living body. But the major problem emerging in the world is the antibiotic resistance, *i.e.,* resistance developed by bacteria, not humans against the effects of antibiotics, which ultimately makes the treatment difficult than other non-resistant bacteria. This can be due to incomplete dosage or frequently use of antibiotics, that cause bacteria to mutant themselves. This leads to the emergence of multidrug resistant pathogens commonly found in hospitals and are critical threats for the patients nursing with catheters or ventilators. World Health Organization (WHO) has also recently updated the list of bacteria which need urgent attention and new antibiotics for their infection. The organization included ESKAPE pathogens in the list. They have differentiated the list in three categories of pathogens according to the urgency of need for new antibiotics, as critical, high and medium priority [Mulani*et al*, 2019].

A major quality which has allowed *A. baumannii* to survive under hospital environments is its multi drug resistance. It has inhabited several mechanisms to escape from various antibiotics; the main one is horizontal gene transfer from unrelated species of bacteria, in which resistance genes are transferred from resistant strains to the resistant strains of *A. baumannii*. Such resistance is known to be intrinsic resistance acquired by bacteria. In

addition to this, bacteria can develop or acquire resistance to antibiotics by several other ways, which can be explained by following 3 main groups:

1. By minimizing the intracellular concentrations of the antibiotic, which may be due to
   i. Less pen*et*ration into the bacterium.
   ii. Due to efflux pumps of antibiotics [Blair*et al*, 2015].

2. Modification of antibiotic targ*et*
   i. By gen*et*ic mutation.
   ii. By post-translational modification [Blair*et al*, 2015].

3. By inactivating the antibiotics
   i. By hydrolysis.
   ii. By modification [Blair*et al*, 2015].

In addition to these, there are some other factors as well, which are responsible for resistance against the development of bacterial infection of *A. baumannii.*

1. **AdeABC**- This is a virulence as well as resistance factor. It is an efflux pump present on the outer surface of bacteria and efficiently efflux out the antibiotics used for the treatment of *A. baumannii*. Antibiotics like Aminoglycosides, t*et*racyclines, chloramphenicol, erythromycin, chloramphenicol, fluoroquinolones, some b*et*a-lactams, trim*et*hoprim and also recently tigecycline, were found to be substrates for this pump [Wieczorek*et al*, 2008].

2. **AdeR**- The two-component regulatory system-AdeRS, controls the expression of multidrug resistance in *A. baumannii* involving *adeABC* efflux pump. AdeR consists of response regulator, as a C-terminal DNA-binding-domain and a N-terminal receiver domain. AdeR binds in the intercistronic region b*et*ween *adeR* and *adeABC* to a direct-repeat DNA [Lari*et al*, 2018].

2.4.1 Biofilms

Biofilms is one of the resistance mechanisms adopted by *A. baumannii* bacteria to protect itself from antibiotics. Biofilms are aggregate of micro-organisms in which cells that are frequently embedded within an extracellular polymeric substances (EPSs), which is self-produced which help them to adhere with each other or to a surface [figure 1]. Biofilms are being produced by most of the bacteria. Biofilm forming pathogens have a significant role in interaction with its host. There would be no harm if we say that bacteria (including *A. baumannii*) forming biofilms, have increased tolerance to external stresses. There are surface appendages, protective surface structures, adhesions like capsular polysaccharides that contribute in the maintenance and formation of biofilms [Longo *et al*, 2014].

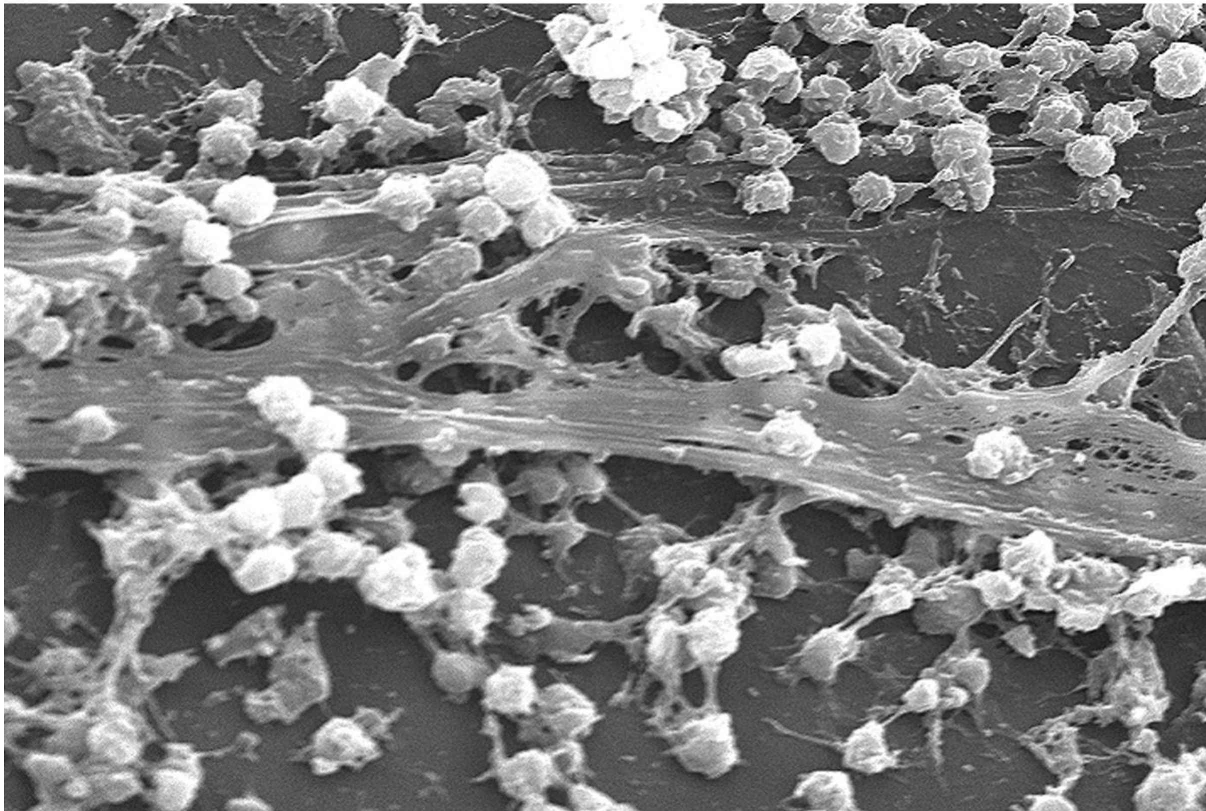


Figure 1. Representative of biofilms, taken from creative commons source- https://en.wikipedia.org/wiki/Biofilm#/media/File:Staphylococcus_aureus_biofilm_01.jpg

2.4.2 Mechanism of biofilm formation

The formation of biofilm is a process where micro-organisms attach as well as grow on the surface irreversibly. They can form this bond with an inert surface or to a tissue and then encased within a complex matrix. This attachment and complex matrix genesis, sprang the alteration in the growth rate and gene transcription in the organisms.

Biofilms can be formed by bacteria, fungi and protists. But there are some characteristics which are necessary to be there in these micro-organisms to form biofilm. Hydrophobicity, surface chemistry, roughness, surface free energy for the implants are some of those characteristics, without which biofilm cannot be formed by these micro-organisms. Among these, surface energy and hydrophobicity characters majorly favor biofilm formation [Nandakumar *et al*, 2013].

Type I chaperone-usher pilus system, also known as Csu pili produced and encoded by *A. baumannii* strains. This pili system is regulated by two-component regulatory system BfmRS [figure 2]. This two-component system is very important for biofilm formation and their maintenance on abiotic surfaces, but not required for their adhesion on biotic surfaces like human epithelial cells. Most *A. baumannii* strains carry the CsuA/BABCDE locus, but a subs*et* of clinical isolates has lost the csu cluster, which tells that these are not essential for biofilm formation and maintenance in all strains and other pilis can efficiently replace them.

There is an another two-component system, GacSA, its moderately control csu gene expression and thus indirectly regulates biofilm formation. There is an interesting fact that sub-inhibitory concentrations of trim*et*hoprim-sulfam*et*hoxazole have shown compl*et*e repression of expression of Csu pili in *A. baumannii*, which again showed that improper use of antibiotics can manipulate the population-level behavior and may promote planktonic lifestyle. *A. baumannii* also produce Bap, biofilm associated proteins, are a large surface-exposed protein, which mediates biofilm formation and maturation in *A. baumannii*. It has specific roles to perform. The first one is n cell-cell adhesion and secondly, it is required for the development of medically relevant material which are higher-order structures like polystyrene and titanium. Most of the sequenced strains of *A. baumannii* carry a bap gene, though many carries disrupted or shortened bap sequence. It is still not clear if it is due to sequence alignment errors or due to recombination events. Some strains of *A. baumannii* code for Bap proteins like BLP1 and BLP2, which contribute in the formation of mature biofilm same to BapAb. *A. baumannii* bacteria abundantly secr*et* an RTX, repeat in toxin like domain-containing proteins, also found in *Pseudomonas putida*, which also regulate biofilm development. There are some other factors as well which are crucial for formation of biofilm in *A. baumannii* like poly-β-1,6-N-ac*et*ylglucosamine (PNAG), produced by gram negative species. Antibodies used against PNAG, can eliminate *A. baumannii* in opsono-phagocytosis assays, which suggests that PNAG can act as a potential vaccine targ*et*. There are some other factors as well which contribute in the biofilm formation like auto transporter system, capsular polysaccharide and many others [Longo*et al*, 2014].

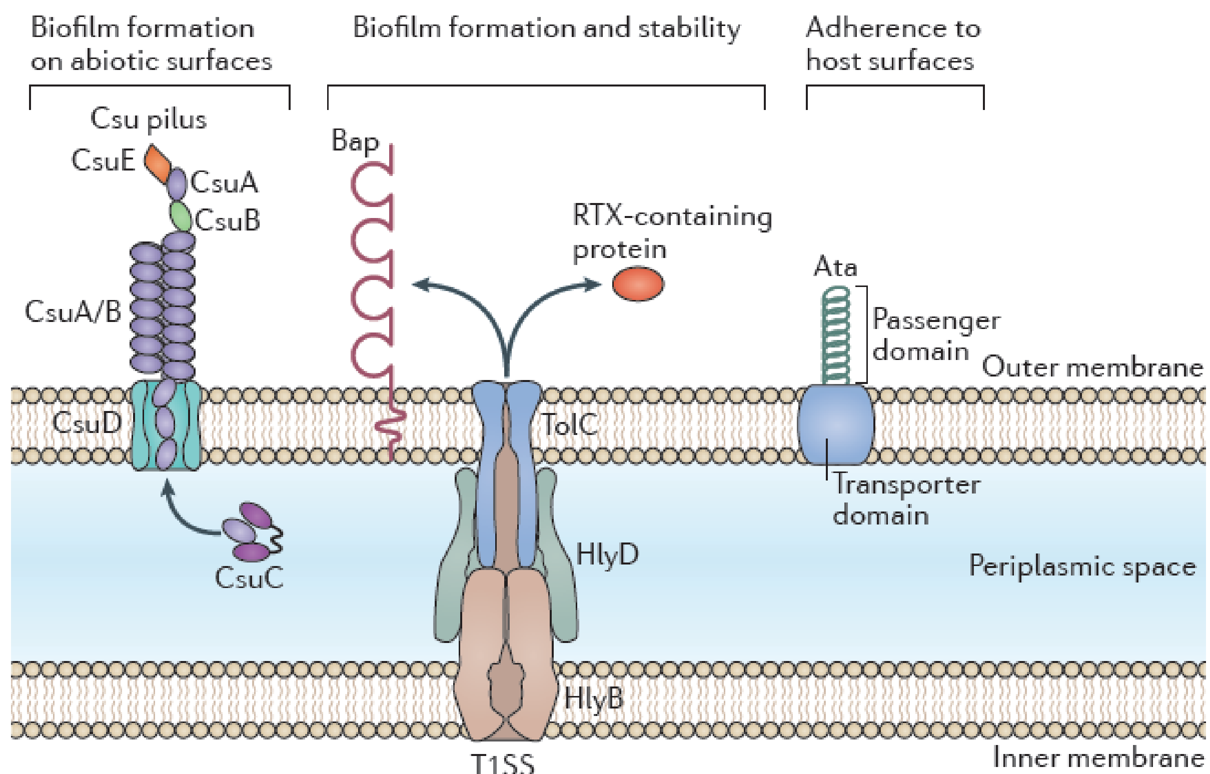


Figure 2. Biofilm formation by *A. baumannii* using csu pili [Longo*et al*, 2014].

2.4.3 Effects of biofilm on humans

As stated earlier, growth of biofilms on abiotic surfaces of *A. baumannii* is one the main cause for causing nosocomial infections. Due to its colonization on hospital equipments and connate medical devices, like urinary cath*et*ers, (CVSs) central nervous cath*et*ers, endotracheal tubes *et*c. When *A. baumannii* s*et*tles on the urinary cath*et*er, device placement can trigger the infection, cath*et*er- associated urinary tract infection due to presence of contamination is possible in ICU patients, at the insertion time, by one or more gram-positive or gram-negative bacterial species, giving rise to one or more often multiple species biofilms. The visual analysis of the inner surface of cath*et*er where biofilm was formed revealed a dense cell multilayer, formed of different shapes and sizes of bacteria, embedded in a rich exo-polysaccharide matrix.

Talking about CVC colonization, in these cath*et*er-related infections are caused in bloodstreams. The infections caused in this are less due to gram-negative bacteria (14%) than those infections caused by gram-positive bacteria (76%). The infections caused by gram-negative bacteria have been seen to be increased in the last decade, but was difficult to treat at the same time due to rise in antibiotic resistant gram-negative bacilli, which includes *A. baumannii* as well. In a forthcoming observational study performed on the patients of ICU who were kept in ventilation for more than 24 hours, out of 75 samples, 71 were reported to have biofilm formation and it was also found that *A. baumannii* and *Pseudomonas aeruginosa* were the most frequent bacterial species obtained [Longo*et al*, 2014].

It is strongly believed that two factors, drug resistance and environmental stickability have enabled *A. baumannii* to survive in nosocomial environment. Taking environmental conditions into account, like concentration of extracellular free iron, growth temperature, are known to be important for not only *A. baumannii* interaction with host, but also affect the quantity of biofilm which are being formed on abiotic surfaces. Indeed, *A. baumannii* when grown in the presence of iron- chelating compounds, showed a noteworthy reduction on the ability of biofilm formation, as well as its quality of being adherent to biotic and abiotic surfaces. There had been studies like these, like *A. baumannii* ATCC17978 strain when incubated under blue light, very little or no biofilm was observed on glass surfaces; while normal growth was observed when the same were incubated in dark environment. Such response was due to a photoreceptor protein, *i.e.,,* BlsA, this contains N-terminal blue-light-sensing-using flavin domain. There are mechanisms involved in which BlsA transduces the light signal which in turn controls gene expression are not known y*et*. However, it has been experimentally proven that the multiple transcription of BlsA at 28°C and 37°C of *A. baumannii* biofilm affects differentially to the response of light. This response is not limited here only, in fact it has a global effect on *A. baumannii* physiology, affecting motility and virulence along with biofilm formation. Supporting this with evidence, biofilm formation on abiotic surface is being affected by *et*hanol. In fact, anabolism of lipids and carbohydrates increased the level of proteins, which ultimately increased the presence of *et*hanol, which would be increasing carbohydrate content of biofilm, which would increase the biofilm formation and decrease bacterial motility [Longo *et al*, 2014].

2.5 Virulence factors of *A. baumannii*

Various studies have been done to study the virulence factors of *A. baumannii*, but still this are remains puzzled, especially when compared with other known gram-negative bacteria, and needs to be studied further. Some of the known factors are discussed below:

1. **OmpA (Outer membrane protein)**- This is virulence as well as resistance factor. It shows it's effect by combining with mitochondria. When it localizes with cell's mitochondria, apoptosis inducing factor and cytochrome c, both are released, which signals the cell to undergo apoptosis. This factor is also responsible for bacterial cell adhesion with the host body. Moreover, it provides resistance to the bacteria by increasing its persistence within the body's serum kills the complement mediated system of the serum [Longo *et al*, 2014].

2. **PBPs (Penicillin binding proteins)**- These play 2 roles within *A. baumannii*:
   i. They bind to the β-lactams and inactivate them
   ii. They also increase the cell stability. It does so as it is involved in synthesis of peptidoglycan, which composes a major portion of bacterial cell wall [Longo *et al*, 2014].

3. **Phospholipases**- Phospholipids forms the major portion of eukaryotic cell membrane. Bacterial cell produces this enzyme to destroy phospholipid and invade in eukaryotic host cells. As far as it has been studies, *A. baumannii* produces two phospholipids, phospholipid C and phospholipid D. It has also been studied that phospholipid D might be responsible for the spread of *A. baumannii* infection from lungs to the rest of the body [Wieczorek*et al*, 2008].

4. **Outer membrane vesicles**- They perform several roles, but after secr*et*ing out from the outer membrane of the bacteria.
   i. During the invasion of *A. baumannii* with the host cell, they deliver other virulent factors into the host cells. E.g. OmpA.
   ii. It allows the process of horizontal gene transfer to happen. [It has been observed that *OXA* 24 genes are transferred b*et*ween the cells, which provides resistance against the immune responses initiated by the host] [Longo *et al*, 2014].

5. **Capsular polysaccharides**- The capsular phenotype, made up of polysaccharides, was found to be an important component for the protection of the bacteria against the host's immune system [Longo *et al*, 2014].

6. **LPS (Lipopolysaccharides)**- Some level of resistance to the human serum was found to be involved by LPS present on the surface of cells, which are associated with antibacterial effects, along with increasing its resolution within soft tissues, during an

infection. This LPS also turns on the innate immune system by activating CD14 and toll like receptor [Longo *et al*, 2014].

2.6 Quorum sensing

Bacteria live in close alliance with eukaryotic hosts, as well as with other bacteria. They need constant monitoring and communication with their neighbors. To sense cell density and to activate adaptations, bacteria produce signals like hormones, auto inducers like molecules, by a process known as quorum sensing (QS). Autoinducers activate the gene expression in the organism, by binding to the transcriptional regulatory proteins.

QS is a complex process and mainly depend on the density of bacterial cells and typically involved with the genes which are associated with maintenance as well as maturation of biofilms. QS regulatory pathways comes into action at high cell density of bacterial culture. Thus, where local cell concentration is much higher than planktonic cultures, QS pathways are activated. QS has also the capability to control the virulence factors production in both gram positive as well as gram negative bacteria. Thus inhibitors used for inhibiting QS, will affect the bacterial pathogenecity as well [Landini *et al*, 2010].

Acyl Homoserine Lactone (AHL) mediated QS have linked with phenotypes that are beneficial to the AHL-producing community, including the production of motility, nodulation, virulence factors, plasmid transfer, antibiotic production, bio-emulsan (microbial polymeric emulsifiers) production, biofilm formation and bioluminescence. In gram-negative bacteria, AHL system is mediated by two proteins, which belong to LuxI and LuxR protein families. LuxI proteins interact directly with similar type of LuxR-type proteins with the help of AHLs. This complex then binds to a specific sequence of promoter known as lux-box, which ultimately regulates the QS targ*et* genes [Bhargava *et al*, 2010].

2.7 Mechanism of Carbapenems

Taking in account, Carbapenemase activity, it is related to penicillin. The accidental discovery of the first antibiotic, Penicillin by sir Alexander Fleming in 1928 is still remarkable and applaudable, which has no doubt found a new era in the field of medicines. These are several classes of penicillin which are currently in use. And the best part is very less % of population is found to be allergic to it. There are several classes of penicillin on the basis of varying side chains in thiazolidine and to β-lactam ring which enlisted as below [figure 3]:

i) Penicillin G- also known as Benzylpenicillin. This is a natural penicillin.
ii) Penicillin V- also known as Phenoxym*et*hylpenicillin or penicillin VK. It shares similar spectrum of activity with Penicillin G; thus, this is also natural penicillin.
iii) Aminopenicillins- as the name suggests, these are amino derivatives of natural penicillin. Eg. Ampicillin, amoxicillin *et*c.

iv) Carboxypenicillins- these are the penicillin with carboxyl acid group in the variable side chain. Ex. Carbenicillin, ticarcillin *et*c.

v) Ureidopenicillins and Piperazine- Penicillin consisting of ureido group along with piperazine produce this class of penicillin. Ex. Mezlocillin, azlocillin *et*c.

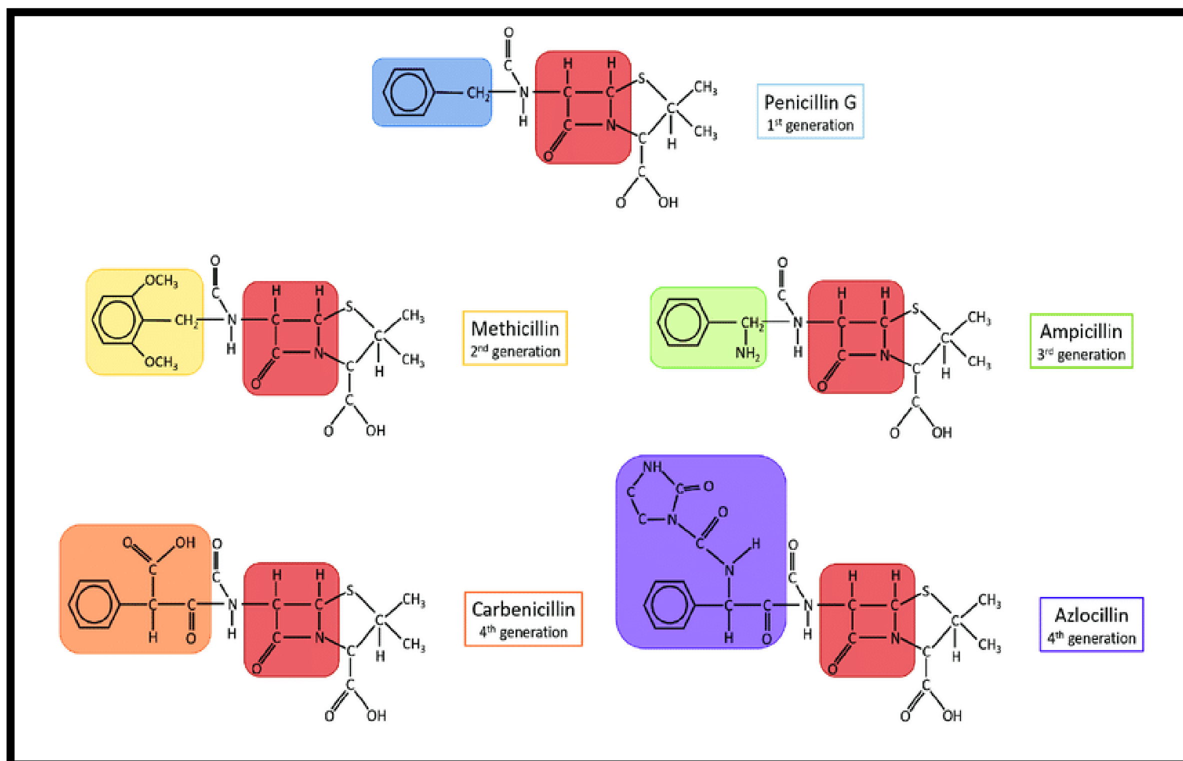


Figure 3. Different classes of penicillin with their structure[Lobanovska*et al*, 2017].

Penicillin attack on directly on the bacterial cell walls by binding directly with peptidoglycans. Likewise, carbapenems binds with PBPs, *i.e.,*, Penicillin Binding Proteins, which acts as a skel*et*on for bacterial cell wall, as it is a major part of peptidoglycans. Carbapenem binds with them, which makes bacterial cell wall week, and ultimately *get* ruptured leading to death of bacterial cell.

Carbapenem is a β-lactam antibiotic which is very effective antibiotic agent and frequent used to treat bacterial infection, wh*et*her it is severe or high risks of infections. It is used to treat infection against MDR *Acinetobacter baumannii*. Carbapenem includes many antibiotics like meropenem, imipenem, doripenem, ertapenem, doribex*et*c. Majorly used carbapenems are meropenem, imipenem, doripenem. But due to emerging of resistance against carbapenem activity in *Acinetobacter baumannii*, polymyxins are being utilized, polymyxin in use includes colistin or polymyxin B (PMB). Carbapenems and polymyxins combinations have also been reported. However, ertapenem in combination with PMB was seen to be least active, whereas imipenem, meropenem and doripenem when combined with PMBs, showed slight difference in their activities which was further influenced by *A. baumannii* strain as well as bacterial load [Lenhard*et al*, 2016].

2.8 β-lactamases

β-lactamases are the enzymes produced by bacteria in response to antibiotics taken by patient against the infections. Thus, this response by bacteria acts as a counteract to save itself from the l*et*hal actions of antibiotics.

These enzymes can be divided into 4 classes: A, B, C, D; based on amino acid sequence. Out of which class A, C and D uses catalytically active serine amino acid residue for the inactivation of various β-lactam drugs. While class B of β-lactamase includes m*et*allo-enzymes which requires zinc to show their catalytic activity, that too with a different mechanism compared to other 3 classes of β-lactamase.

We have focused on the class carbapenems, as it is a broad-spectrum antibiotic. And moreover, the antibiotics included in it like meropenem, imipenem *et*c. have the quality of resisting including extended spectrum β-lactamases as well as depressed chromosomal AmpC β-lactamases (class C), its hydrolysis by most of the β-lactamases. But most of the m*et*allo-β-lactamases along with some of the class A and D β-lactamases can hydrolyze these compounds as well [Walther-Rasmussen*et al*, 2006].

Class D β-lactamases

Earlier, class D β-lactamase was known as *OXA*cillinases as they commonly hydrolyze is*OXA*zolylpenicillin*OXA*cillin, that too much faster than benzylpenicillin which is a classical penicillin. Thus, the prefix, *OXA* of class D β—lactamases cites to their proposed penicillin substrate. Till now, class D β-lactamases have undersigned 150 different variants on protein level, out of which 45 of them exhibit carbapenem-hydrolyzing activities, which is the main contradiction to other class D β-lactamases [Lobanovska *et al*, 2016; Lenhard *et al*, 2016]. Till date, five main families of plasmid-encoded CHDLs *i.e.,*, cadherin-like domain have been pinpointed in *A. baumannii*, and they are *OXA*-23, *OXA*24/40, *OXA*-58, *OXA*-143, and *OXA*-235-like enzymes[Antunes *et al*, 2019]. Now, cadherins are actually calcium dependent adhesion which is highly important in the establishment of adherent junctions for binding of cells with each other.

Class C β-lactamase

This class includes the AmpC b*et*a lactamase, enzymes of cephalosporinases. They have been studied more active on cephalosporins than benzylpenicillin. Hyperproducing mutants produced by AmpC are resistant to aztreonam, penicillin, 3rd generation cephalosporins which encompass cefotaxime, ceftriaxone. Even it also includes cefepime, 4th generation cephalosporin which has broader spectrum activity compared to a poor inducer of AmpC of β-lactamase, ceftriaxone. Due to this, many organisms which produce AmpC are susceptible to cefepime. But still, the treatment with cefepime to AmpC producing organisms is debatable because of its inoculum effect (IE). IE is an effect where mic (minimal inhibitory concentration) of an antibiotic increase with the increase in the number of organisms

inoculated. In general, IE take place with β-lactam antibiotics in-tie up to β-lactamase producing bacteria.

Class B β-lactamase

This class of β-lactamase has been divided into 3 subclasses: B1, B2, B3. This class consists of m*et*alloenzymes containing one or two zinc ions. There are many families included in this, but the most common ones are IMP, VIM, NDM *et*c. They differ from other β-lactamases due to this demand of zinc ion at their active site. When compared to other classes of β-lactamases, these have poor hydrolytic capability and affinity for monobactams, plus cannot be inhibited by tazobactam or clavulanic acid.

Combinations consisting of cephalosporin and β-lactamase inhibitor includes “ceftolozane & tazobactam” and “ceftazidime & avibactam”, have been approved for the treatment of infections. But it has its own side effects including complicated intra-abdominal infections.

2.9Carbapenemase genes

There are several Carbapenemase genes like bla *OXA*-23 like (*OXA*-23,*OXA*-27,*OXA*-49, *OXA*-239), *OXA*-24 like (*OXA*-24, *OXA*-25, *OXA*-26, *OXA*-40, *OXA*-72), *OXA*-51 and mutants, *OXA*-58, *OXA*-143 like (*OXA*-143, *OXA*-231), *OXA*-235 like (*OXA*-235, *OXA*-236, *OXA*-237), *NDM-1, NDM-2, VIM-1, VIM-2, IMP-1, IMP-2et*c.[Hsu*et al*, 2017; Higgins*et al*, 2013] responsible for emerging *Acinetobacter baumannii* as a multidrug resistant bacterium. No doubt, these all genes are responsible for MDR *A. baumannii* but there are few genes which are prevalent in India as shown in table 1.

Table 1: Carbapenemase genes prevalent in India, and its impact over the world

s. No.	Carbapenemase gene common in India	Researched where in India	Significance over the world	Reference
1.	NDM-1	SRM Hospital, Tamil Nadu	UK, India*, Pakistan*, Bangladesh*, many countries of Europe, Asia, Africa, Austria, North America, Balkan states, Middle east (Oman & Iraq)	Khan*et al*, 2017;Vijayakumar*et al*, 2019;Kazi*et al*, 2015.

2.	bla-*OXA*-58	SRM Hospital, Tamil Nadu	Brazil, Europe (France), Balkans, Central Turkey, Spain, Italy, Greece, Romania	Routray*et al*, 2013;Vijayakumar*et al*, 2019; Kazi*et al*,2015.
3.	bla-*OXA*-23	SRM Hospital, Tamil Nadu	Columbia, Europe, France, Vi*et*nam, New Caledonia, Thailand, Australia, Tahiti, South Africa, United Arab, Emirates, Libya, Bahran, Egypt, Belgium, Algeria, Brazil	Routray*et al*; 2013;Vijayakumar*et al*, 2019; Kazi*et al*, 2015.
4.	bla-*OXA*-51	AIIMS, Delhi	Columbia, Iran	Tiwari*et al*, 2012;Kazi*et al*, 2015.
5.	bla-VIM-2	South India	Iran, North Korea	Amudhan*et al*; 2012;Kazi*et al*, 2015.
6.	bla-IMP-2	South India	Iran, Italy, Japan, North Korea	Amudhan*et al*; 2012.

Other than these, their mutants are also a common cause for carbapenem resistant *Acinetobacter baumannii*. Mutants like, *OXA*-23 like (*OXA*-23, *OXA* 27, *OXA* 49, *OXA*-239); *OXA*-51 like (*OXA*-51, *OXA*-64, *OXA*-65, *OXA*-66, *OXA*-68, *OXA*-70) [Hsu*et al*, 2017; Khan*et al*, 2017; Vijaykumar*et al*, 2019; Kazi*et al*, 2015; Routray*et al*, 2013; Tiwari*et al*, 2012; Amudhan*et al*, 2012; Marcoccia*et al*, 2016; Ou*et al*, 2014; Higgins*et al*, 2010; Pratap*et al*, 2016; Brown*et al*, 2005; Evans*et al*, 2014; Afzal-Shah*et al*, 2001; D'Andrea*et al*, 2009; Merino*et al*, 2014; Heritier*et al*, 2005; Smith*et al*, 2013]

Other than these there are various other carbapenemase genes which have been reported all over the world, like *OXA*-24 like (*OXA*-24, *OXA*-25, *OXA*-26, *OXA*-40, *OXA*-72); *OXA*58 like (*OXA*-58, *OXA*-69), SIM, *OXA*-134 *et*c. [Hsu*et al*, 2017; Khan*et al*, 2017; Vijaykumar*et al*, 2019; Kazi*et al*, 2015; Routray*et al*, 2013; Tiwari*et al*, 2012; Amudhan*et al*, 2012; Marcoccia*et al*, 2016; Ou*et al*, 2014; Higgins*et al*, 2010; Pratap*et al*, 2016; Brown*et al*, 2005; Evans*et al*, 2014; Afzal-Shah*et al*, 2001; D'Andrea*et al*, 2009; Merino*et al*, 2014; Heritier*et al*, 2005; Smith*et al*, 2013].

2.10 Text mining of carbapenemase genes& associated mutations corresponding to carbapenem resistance.

1. Mutations in NDM-1 gene.

Few mutations have been reported in NDM-1 gene by various authors, that has been enlisted below [Khan*et al*, 2017; Marcoccia*et al*, 2016]. NDM-1. Gene sequence was taken from NCBI with accession id >NC_020818.1:8964-9880 [Ou*et al*, 2014].

Table 2. mutations in NDM-1 in *Acinetobacter baumannii* with their positions at gene and protein level

Position	Mutation	Reference
82	C to G	Khan*et al*, 2017.
94	C to A	
107	G to A	
205	G to A	
220	G to A	
262	Gto T/ G to C	
283	G to A	
388	G to A	
389	A to G	
454	G to A	
460	A to C/A to G	
508	G to A	
598	G to C	
665	G to A	
698	C to T/G to T	
35	Isoleucine to threonine	Marcoccia*et al*, 2016
35	Isoleucine to serine	Marcoccia*et al*, 2016

2. Mutation of carbapenemase *OXA*-58
   Mutation has been reported in *OXA*-58 gene, which gives rise to new variant, which is known to be as *OXA*-69 [Higgins*et al*, 2010]. Gene sequence was taken from NCBI with accession id NC_010481.1 [Pratap*et al*, 2016].
   Table 3. mutations in *OXA*-58 in *Acinetobacter baumannii* with their positions at gene and protein level.

Position	Mutations	Mutation makes the variant	Reference
342	A to T	69	Higgins PG*et al*, 2010
114	Leu to Phe	69	

3. Mutations in *OXA*-51 gene.

Mutation has been reported in *OXA*-51 gene, which gives rise to new various variants depending on the position of mutations, which are known to be as *OXA*-64, *OXA*-65, *OXA*-66, *OXA*-68, *OXA*-69, *OXA*-70, *OXA*-71. All these have been listed below in the table [33-34]. The protein sequence was taken from NCBI with accession no. >AOR84133.1

Table 4. mutations in *OXA*-51 in *Acinetobacter baumannii* with their positions at protein level

Position	Mutation	Mutation makes the variant	Reference
5	Thr to Ala	*OXA* 64	Brown S *et al*, 2005; Evans BA *et al*, 2014.
	ACA to GCA		
38	Ala to Gly		
	GCA to GGA		
48	Val to Ala		
	GTA to GCA		
5	Thr to Ala	*OXA* 65	
	ACA to GCA		

48	Val to Ala		
	GTA to GCA		
107	Gln to Lys		
	CAA to AAA		
194	Pro to Gln		
	CCA to CAA		
225	Asp to Asn		
	GAC to AAC		
5	Thr to Ala	*OXA* 66	
	ACA to GCA		
36	Glu to Val		
	GAA to GTA		
48	Val to Ala		
	GTA to GCA		
107	Gln to Lys		
	CAA to AAA		
194	Pro to Gln		
	CCA to CAA		
225	Asp to Asn		
	GAC to AAC		
5	Thr to Ala	*OXA* 68	
	ACA to GCA		
24	Thr to Ser		
	ACT to TCT		

48	Val to Ala		
	GTA to GCA		
117	Asp to Asn		
	GAC to AAC		
146	Lys to Asn		
	AAG to AAT		
194	Pro to Gln		
	CCA to CAA		
195	Lys to Glu		
	AAA to GAA		
225	Asp to Asn		
	GAC to AAC		
5	Thr to Ala	*OXA* 69	
	ACA to GCA		
36	Glu to Asp		
	GAA to GAC		
48	Val to Ala		
	GTA to GCA		
57	Gln to His		
	CAA to CAT		
107	Gln to Glu		
	CAA to GAA		
117	Asp to Asn		
	GAC to AAC		

194	Pro to Gln		
	CCA to CAA		
225	Asp to Asn		
	GAC to AAC		
5	Thr to Ala	*OXA* 70	
	ACA to GCA		
36	Glu to Lys		
	GAA to AAA		
48	Val to Ala		
	GTA to GCA		
105	Asp to Asn		
	GAC to AAC		
146	Lys to Asn		
	AAG to AAT		
194	Pro to Gln		
	CCA to CAA		
198	Asp to His		
	GAT to CAT		
5	Thr to Ala	*OXA* 71	
	ACA to GCA		
48	Val to Ala		
	GTA to GCA		
96	Ala to Thr		
	GCA to ACA		

4. Evolution of *OXA*-27 from *OXA*-23

Mutation has been reported in *OXA*-23 gene, which gives rise to new variant, which is known to be as *OXA*-27 [Alfal-Shah*et al*, 2001]. Gene sequence has been taken from NCBI with accession no. >NC_025109.1 [3D'Andrea*et al*, 2009].

Table 5. mutations in *OXA*-23 in *Acinetobacter baumannii* with their positions at gene and protein level

Position	Mutations	Mutation makes the variant	Reference
162	T to C	*OXA*-27	Afzal- shah M *et al*, 2001
283	A to G		
95	threonine to alanine		
741	T to A		
247	asparagine to lysine		

5. Evolution OF *OXA*-25from*OXA*-24.

OXA-25 is believed to be evolved from *OXA*-24. Few mutations have been noted in *OXA*-24 gene which makes *OXA*-25 as one of its variants [Alfal-Shah*et al*, 2001]. The gene sequence has been taken from NCBi with accession number >NC_012813.1 [Merino*et al*, 2014].

Table 6. mutations in *OXA*-24 in *Acinetobacter baumannii* with their positions at gene and protein level

Position	Mutations	Mutations makes the variant	Reference
624	A to C	*OXA*-25	Afzal- shah M *et al*, 2001
142	isoleucine to leucine		
424	A to G		

268	serine to leucine		
604	A to G		
202	lysine to glutamate		
Addition			
199 & 200	Glumates		

2.11 Minimum Inhibitory Concentration **(MIC):** MIC is the concentration of antibiotics used against bacterial infection where no visible growth is been observed. Various studies have been done to analyze the effect of mutations on the carbapenemase genes on the MICs of various antibiotics used against *A. baumannii* bacterial infection. The first isolate for carbapenem resistant *A. baumannii* was *OXA*-23, 1985 with the MIC 16mg/l. The resistance gene was found to be located on plasmid, as it was transferable. The breakpoint, the point which is marked as a border to decide wh*et*her any concentration of antibiotic to be considered resistant or susceptible, was decided to be 16µg/ml for *A. baumannii* [Evans*et al*, 2014].

It is not necessary that resistance would come or MIC would increase only when the resistance gene mutants, other mechanisms can also contribute. Like activation of AdeABC efflux pump escalate the MIC to 32µg/ml [Evans*et al*, 2014]. Likewise, there are many other strains of *A. baumannii* which have undergone changes during this transition and study period. Some of them have been listed below, this paper has been taken from [Evans *et al,* 2014].

Table 7 Change in MIC recorded in different strains of *A. baumannii*.

Strain	**Enzyme group**	**MIC (µg ml^{-1})**		**Reference**
		Imi	**Mer**	
A. baumannii CIP 70.10		0.25	0.25	Héritier*et al*, 2005
A. baumannii BM4547		0.5	0.5	Héritier*et al*, 2005
A. baumannii ATCC		0.125	0.5	Smith*et al*, 2013

Strain	Enzyme group	MIC (µg ml^{-1})		Reference
		Imi	Mer	
17978				
A. baumannii CIP 70.10 + *OXA*-23	23	16	16	Héritier*et al*, 2005
A. baumannii BM2.47 + *OXA*-23	23	>32	>32	Héritier*et al*, 2005
A. baumannii ATCC 17978 + *OXA*-23	23	16	64	Smith*et al*, 2013
A. baumannii CLA-1 Δ*OXA*-40		2	4	Héritier*et al*, 2005
A. baumannii 17978		0.25	0.5	Higgins*et al*, 2013
A. baumannii CLA-1 *OXA*-40	40	>32	>32	Héritier*et al*, 2005
A. baumannii 17978 + *OXA*-40	40	>32	>32	Higgins*et al*, 2013
A. baumannii CIP 70.10 + *OXA*-40	40	4	4	Héritier*et al*, 2005
A. baumannii BM4547 + *OXA*-40	40	8	8	Héritier*et al*, 2005
A. baumannii ATCC 15151		0.5		Chen*et al*, 2010

Strain	Enzyme group	MIC (µg ml^{-1})		Reference
		Imi	**Mer**	
A. baumannii ATCC 15151 + *OXA*-82	51	32		Chen*et al*, 2010
A. baumannii CIP 70.10 + *OXA*-58	58	2	2	Héritier*et al*, 2005
A. baumannii BM4547 + *OXA*-58	58	32	32	Héritier*et al*, 2005
A. baumannii CIP 70.10 + *OXA*-97	58	2	2	Poirel*et al*, 2008
A. baumannii ATCC 19606		0.19	0.19	Higgins*et al*, 2009
A. baumannii ATCC 19606 + *OXA*-143	143	32	32	Higgins*et al*, 2009
A. baumannii 17978 + *OXA*-235	235	3	4	Higgins*et al*, 2013

3. Objectives:

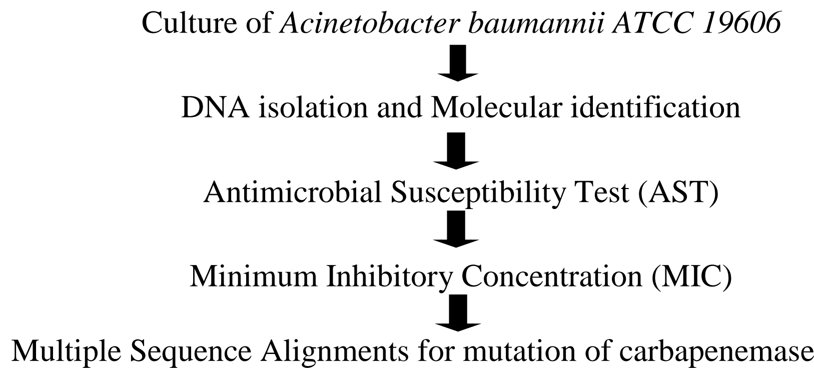
Present study has following objectives:

a) Molecular Identification and characterization of *Acinetobacter baumannii.*
b) Differentiation of bacteria on basis of b*et*a lactam resistance.
c) Bioinformatics approach for association of
    (i) B*et*a lactam antibiotic and b*et*a lactamase enzyme.
    (ii) virulent factors of *Acinetobacter baumannii* with human cells.

- To evaluate the further results with *in-vitro* experiments.

4. Materials and m*et*hodology

4.1 Work flow chart



4.2 Materials utilized

Luria broth, MacConkey agar, and Mueller-Hinton agar purchased from Hi-media Mumbai, India. PCR master mix purchased from takara, and primers were used from Eurofins scientific USA. Antibiotic discs and strips (T*et*racycline, Imipenem, ceftazidime, ceftotaxime, netilmicin, ciprofloxacin).

4.3 Procedure

4.3.1 Culturing of *Acinetobacter baumannii* strain and confirmation with PCR amplification

1. Overnight grown culture of *A. baumannii* ATCC 19606 was inoculated into 10ml of Luria broth.
2. Cultures were incubated for overnight at 37°c at 120 rpm.
3. Strain was streaked on MacConkey agar plates and incubated at 37°c for overnight.
4. Isolated colony was picked and dissolved in 10µl nuclease free water.
5. The culture was boiled at 95°c for 10 min in order to disrupt bacterial cells.
6. The content was centrifuged at 3000 rpm for 3 minutes.
7. 1 µl of supernatant contains DNA and used for the PCR reaction
8. PCR reaction mixture was prepared with the addition of following components in table 2:

Table 8: Different components and their amount used in PCR reaction

Contents	Amount
Nuclease free water	4.5 µl
DNA template	2 µl
PCR master mix (takara)	7.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl

9. The PCR product was amplified in Applied Biosystem thermocycler for 30 cycles with conditions mentioned in figure 4.

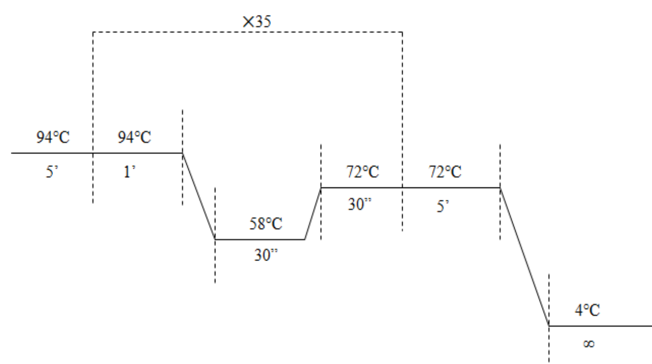
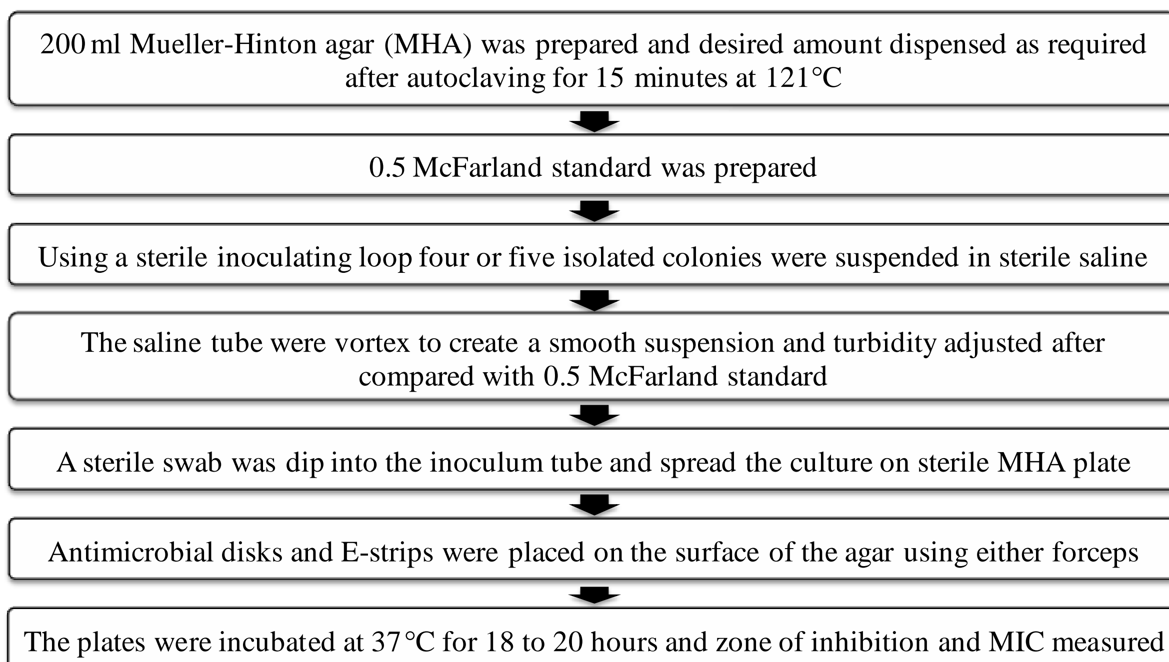


Figure 4- PCR conditions for the amplification of 23s-16s inter transcribed region (ITS) region. The reaction was allowed to run 30 cycles.

4.3.2 AST (Antibiotic Susceptibility Test) and MIC (Minimum Inhibitory Concentration)- [Hudzicki*et al*]



4.4 Basic Local Alignment Search tool (**BLAST):**

If we elaborate the term BLAST, it would stand for Basic Local Alignment Search tool. It takes into account biological sequences, wh*et*her it's nucleotide sequence or protein sequence. This tool is used to find the regions of similarity b*et*ween the above-defined biological sequences. It calculates its statistical significance too. There are 2 terms used in BLAST, query sequence and database. Query sequence means the sequence about which we need to gather the information. While database consists of sequences already reported and stored in its bank. There are different types of BLAST:

i) BLASTN-Both, query and database are DNA sequences.
ii) BLASTP-Both, query and database are protein sequences.
iii) BLASTX- Here the query is DNA and proteins are the databases. Comparison is done by converting query sequence to the possible frames, and then compares each with the database.
iv) TBLASTN- Query consisting of protein sequence is compared with database of DNA.
v) TBLASTX- Both, query and database are the protein encoded in a DNA.
vi) BLAST2- This takes into account gaps as well in the alignments, thus known as advanced BLAST.
vii) PSI-BLAST- This stand for Position Specific Iterated BLAST. As the name suggests, it does it does iterative database searches. Iteration means the process of rep*et*ition to generate a sequence as an outcome.

4.5 Multiple sequence alignment:

Multiple sequence alignment or MSA is the alignment of 2, 3 or more sequences which can be of any protein, DNA or RNA sequence of similar lengths. It is done to figure out the homology and evolutionary relationships b*et*ween the sequences entered, with the help of the phylogenetic trees constructed [figure 5]. Through MSA, every minute d*et*ailing can be figured out easily. It can d*et*ect point mutations, insertion and del*et*ion within minutes. Through MSA, individual nucleotides or amino acids or even protein domains with secondary and tertiary structures sequences can be accessed with an ease.

Aligning sequence would be much difficult if we go manually. So, we use the computational algorithms to analyze the sequences. We have many softwares for doing so. For example, T-coffee, ClustalW, MUSCLE *et*c. We do use some symbols to ease the analyzation task like:

i) "." for similar residue.
ii) ".." for highly similar residue.
iii) "*" for identical residues.
iv) "-" for the gaps.

MUSCLE software has been used in this project for the alignment where Clustal-omega (clustal ω). It is considered above than other options for MSA like T-Coffee or MUSCLE because it has better speed and average accuracy when compared, depending on the options we opt. Moreover, it also provides the alignment results in different colors.

The main requirement of MSA are sequences. The sequences have been taken from NCBI and the research papers which have reported the mutants of those sequences.

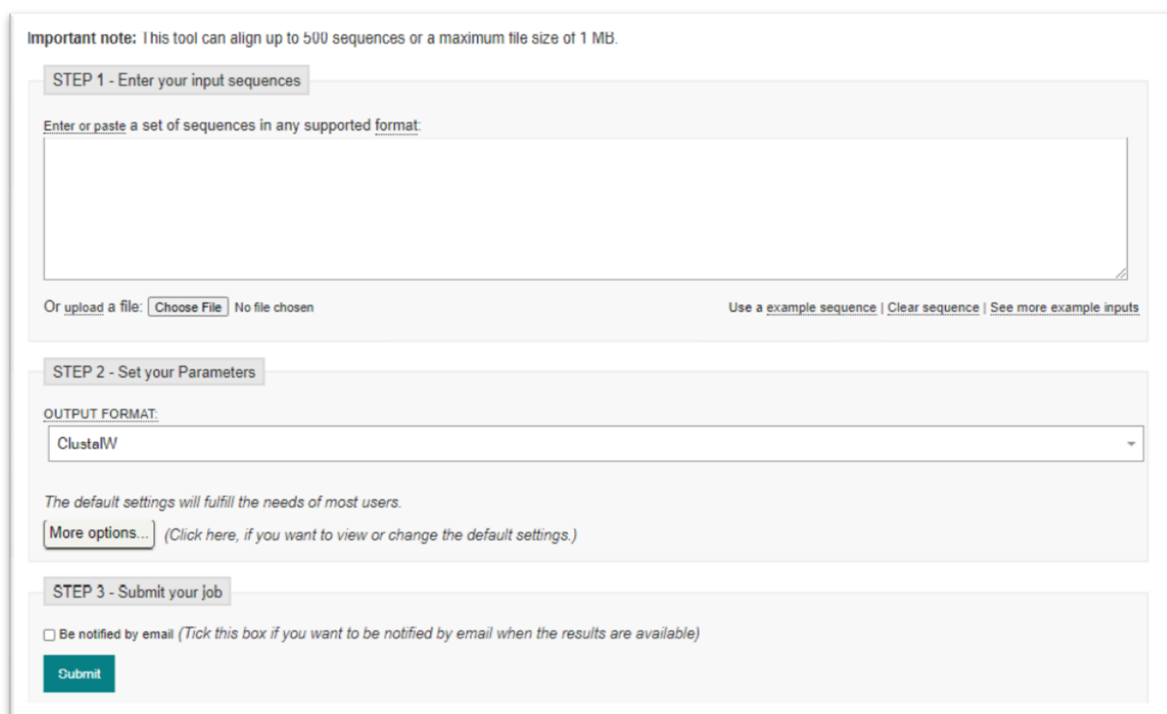


Figure 5. Window of MUSCLE MSA

4.6 Phylogenetic tree analysis

An online tool has been used to analyze the pyrogenicity of various *OXA*s studied. This helped to know about their origin and characterization. It also helped to see which *OXA* belonged to which family.

5. Results

5.1 Confirmation of *A. baumannii* strain.

Acinetobacter baumannii strain ATCC 19606 was taken for the purpose of study, and was gown in McConky agar as shown in figure 6.

Figure 6. Culture of ATCC 19606

Antimicrobial susceptibly and minimum inhibitory concentration for *A. baumannii* isolates was d*et*ermined for different antibiotics including meropenem and imipenem. Type strain ATCC 19606 was used as a control for d*et*ermination of AST and MIC. The zone of inhibitions and MIC value of two carbapenems listed in table 8 and representative figure 7.

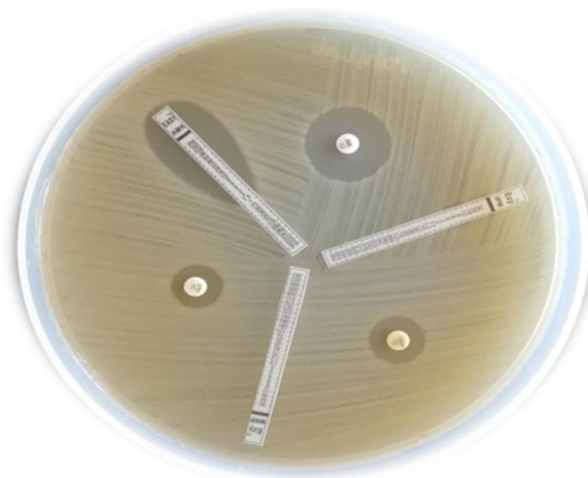


Figure 7. AST and MIC tests on ATCC 19606 culture

Large zone of inhibition was observed in strain ATCC 19606 and antibiotics were effective at very less concentration *i.e.,* less than µg/ml. The isolates AB1 and AB2 found resistant for meropenem and imipenem upto 32 µg/ml concentration.

Table 8- **Zone of inhibition and MIC values of different carbapenems against *A. baumannii* isolates**

	Meropenem		**Imipenem**	
Bacterial isolates	**Zone of inhibition (mm)**	**MIC value (µg/ml)**	**Zone of inhibition (mm)**	**MIC value (µg/ml)**
ATCC 19606	26	0.75	32	0.2
AB1	13	>32	14	>32
AB2	12	>32	13	>32

5.2 Results of PCR reaction

PCR reaction for the amplication of 'ITS' region for all the strains was done. PCR product was run on 1.2% agarose gel along with 100bp ladder [figure 8].

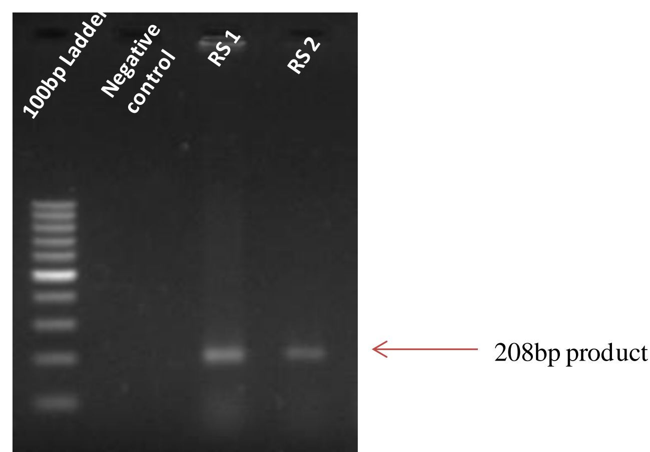


Figure 8. PCR product (ITS region) of various strains of *A. baumannii*separated on 1.2% agarose gel.

5.3 MSA of genes reponsible for carbapenem resistance in India

```
OXA-51     ----------TTATAACAAGCGCT------------ATTTTTATTTCAGCC-----TGCT     55
OXA-69     ----------TTATAACAAGCGCT------------ATTTTTATTTCAGCC-----TGCT     55
OXA-23     ATGAATAAATATTTTACTTGCTATGTGGTTGCTTCTCTTTTTCTTTCTGGTTGTACGGTT     144
OXA-27     ATGAATAAATATTTTACTTGCTATGTGGTTGCTTCTCTTTTTCTTTCTGGTTGTACGGTT     175
OXA-49     ATGAATAAATATTTTACTTGCTATGTGGTTGCTTCTCTTTTTCTTTCTGGTTGTACGGTT     60
OXA-239    ATGAATAAATATTTTACTTGCTATGTGGTTGCTTCTCTTTTTCTTTCTGGTTGTACGGTT     60
                     :*:*:**::** .*            .*****.****:*       * *

OXA-51     CACCTTA-TATAGTGACTGCTAATCCAAATCACAGCGC-----TTCAAAATCTGATGAAA     109
OXA-69     CACCTTA-TATAGTGACTGCTAATCCAAATCACAGTGC-----TTCAAAATCTGATGACA     109
OXA-23     CAGCATAATTTAATAAATGAAACCCCGAGTCAGATTGTTCAAGGACATAATCAGGTGATT     204
OXA-27     CAGCATAATTTAATAAATGAAACCCCGAGTCAGATTGTTCAAGGACATAATCAGGTGATT     235
OXA-49     CAGCATAATTTAATAAATGAAACCCCGAGTCAGATTGTTCAAGGACATAATCAGGTGATT     120
OXA-239    CAGCATAATTTAATAAATGAAACCCCGAGTCAGATTGTTCAAGGACATAATCAGGTGATT     120
           ** *:** *:**.*.*.**.:*. **.*.*** *  *      :**:****:*.***  :

OXA-51     AAGCAGAGAAAATTAAAAATTTATTTAACGAAGTACACACTACGGGTGTTTTAGTTATCC     169
OXA-69     AAGCAGAGAAAATTAAAAATTTATTTAACGAAGCACACACTACGGGTGTTTTAGTTATCC     169
OXA-23     CA--------------TCAATACTTTGATGAAAAAAACACCTCAGGTGTGCTGGTTATTC     250
OXA-27     CA--------------TCAATACTTTGATGAAAAAAACACCTCAGGTGTGCTGGTCATTC     281
OXA-49     CA--------------TCAATACTTTGATGAAAAAAACACCTCAGGTGTGCTGGTTATTC     166
OXA-239    CA--------------TCAATACTTTGATGAAAAAAACACCTCAGGTGTGCTGGTTATTC     166
           .*              :.*:*:.***.* ***. *.**** :*.*****  *.** ** *

OXA-51     AACAAGGCCAAACTCAACAAAGCTATGGTAATGATCTTGCTCGTGCTTCGACCGAGTATG     229
OXA-69     ATCAAGGTCAAACTCAACAAAGCTATGGTAATGATCTTGCTCGTGCTTCGACCGAGTATG     229
OXA-23     AAACAGATAAAAAAATTAATCTATATGGTAATGCTCTAAGCCGCGCAAATACAGAATATG     310
OXA-27     AAACAGATAAAAAAATTAATCTATATGGTAATGCTCTAAGCCGCGCAAATACAGAATATG     341
OXA-49     AAACAGATAAAAAAATTAATCTATATGGTAATGCTCTAAGCCGCGCAAATACAGAATATG     226
OXA-239    AAACAGATAAAAAAATTAATCTATATGGTAATGCTCTAAGCCGCGCAAATACAGAATATG     226
           *:..**. .***.:.::.*:. .**********.***:.  ** **::. **.**.****

OXA-51     TACCTGCTTCGACCTTCAAAATGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAA     289
OXA-69     TACCTGCTTCGACCTTCAAAATGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAA     289
OXA-23     TGCCAGCCTCTACATTTAAAATGTTGAATGCCCTGATCGGATTGGAGAACCAGAAAACGG     370
OXA-27     TGCCAGCCTCTACATTTAAAATGTTGAATGCCCTGATCGGATTGGAGAACCAGAAAGCGG     401
OXA-49     TGCCAGCCTCTACATTTAAAATGTTGAATGCCCTGATCGGATTGGAGAACCAGAAAACGG     286
OXA-239    TGCCAGCCTCTACATTTAAAATGTTGAATGCCCTGATCGGATTGGAGAACCAGAAAACGG     286
           *.**:** ** **.** ****** * *****  *******. * ***.**** **..*..

OXA-51     CCACCACAGAAGTATTTAAGTGGGACGGGCAA-AAAAGGCTATTCCCAGAATGGGAAAAG     348
```

Figure 9: MSA of the gene sequences responsible for carbapenem resistance prevalent in India

The comparison of different oxa genes, accounting carbapenem resistance to *A. baumannii* in India showed conserved regions except deletion mutations at certain positions (figure 9). Further the phylogenetic analysis revealed that OXA 51 and OXA 69 are more related in comparison to OXA 49, OXA 27 and OXA 239 (figure 10).

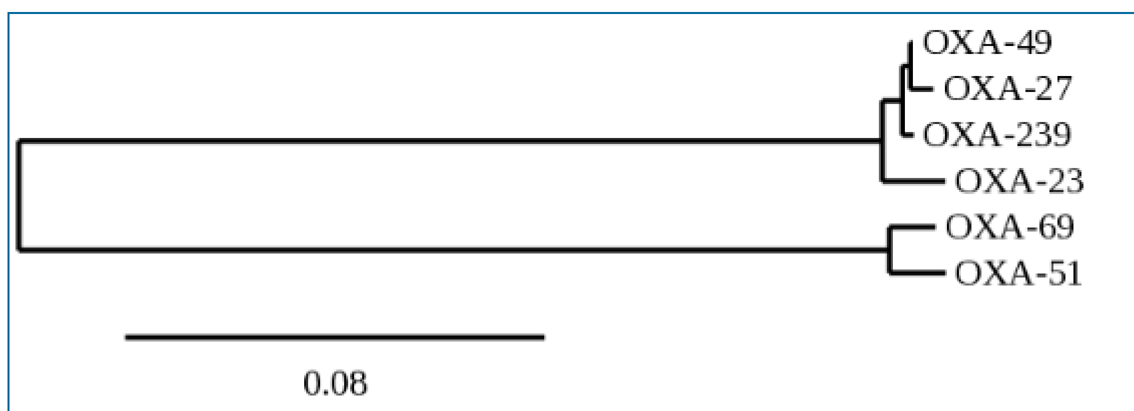


Figure 10: Phylogen*et*ic tree analysis for carbapenem resistance genes prevalent in India.

5.4 MSA of genes reponsible for carbapenem resistance in the world.

```
OXA-103     GTTTAA----------------TTAAGGAAGAGGCTTTT---------------GCCTC-     69
OXA-24      ------------------------------------------------------------     0
OXA-25      ------------------------------------------------------------     0
OXA-72      ------------------------------------------------------------     0
OXA-26      ------------------------------------------------------------     0
OXA-40      ------------------------------------------------------------     0
OXA-143     GTTAACTTTCAATAA-TTGAATTAAAATATTATACTTTA---------------GGCAC-     72
OXA-231     GTTAACTTTCAATAA-TTGAATTAAAATATTATACTTTA---------------GGCAC-     72
OXA-164     ------------------------------------------------------------     0
OXA-97      -------------------CGGCTTT---TTC---TTCAGCATACTTTTTGA--AACAC-     76
OXA-58      ------------------------------------------------------------     0
OXA-96      -------------------CGGCTTT---TTC---TTCAGCATACTTTTTGA--AACAC-     38
OXA-235     GATATAACTCATTGAGATGTGTCATAGTATTCGTCGTTAGAAAACAATTATTATGACATT     540
OXA-236     ------------------------------------------------------------     0
OXA-237     ------------------------------------------------------------     0
OXA-134     ------------------------------------------------------------     0

OXA-103     --------------TTTTTTATTTTC-----TATT---GATCTGGTGTTTAAAATGAATA     107
OXA-24      ------------------------------------------------------------     0
OXA-25      ---------------------TTCCC-----CT------AACATGAATTTGTAATGAAAA     28
OXA-72      -----------------------------------------------------ATGAAAA     7
OXA-26      ---------------------TTTCC-----CT------AACATGAATTTGTAATGAAAA     28
OXA-40      -----------------------------------------------------ATGAAAA     7
OXA-143     --------------TCAAAACTTTCC-----CT------AACATAAATCTGTAATGAAAA     107
OXA-231     --------------TCAAAACTTTCC-----CT------AACATAAATCTGTAATGAAAA     107
OXA-164     ------------------------------------------ATGAAATTA---TTAAAA     15
OXA-97      -T-----------ACCAAATTTTAA------AGTTGTATATCATGAAATTA---TTAAAA     115
OXA-58      ------------------------------------------------------------     0
OXA-96      -T-----------ACCAAATTTTAA------AGTTGTATATCATGAAATTA---TTAAAA     77
OXA-235     ATTTCAATGAGTTATCTATTTTTGTCGTGTACAGAGAATATCCTGAACTTA---TGAAAA     597
OXA-236     --------------------------------------------------A---TGAAAA     7
OXA-237     --------------------------------------------------A---TGAAAA     7
OXA-134     --------------------------------------------------A---TGAAAA     7

OXA-103     AATATTTTACTTGCTATGT----GGTTGC-TTCTCTTTTTC-----------TTTCTGGT     151
OXA-24      ------------------------------------------------------------     0
```

Figure 11: MSA of the gene sequences responsible for carbapenem resistance prevalent in the world.

The comparison of different *OXA* genes, accounting carbapenem resistance to *A. baumannii* worldwide showed conserved insertion mutations in the sequence of OXA 103, OXA 143, OXA 231, OXA 96, OXA 97, and OXA 235 (figure 11). Further the phylogenetic analysis revealed that OXA 58, OXA 96, OXA 97 and OXA 164 are more

related. Similarly, OXA 134, OXA 237, OXA 236 and OXA 235 are conserved, and OXA 26, OXA 25, OXA 24, OXA 40, OXA 72, OXA 231 and OXA 143 are evolved from mutations in single gene (figure 12).

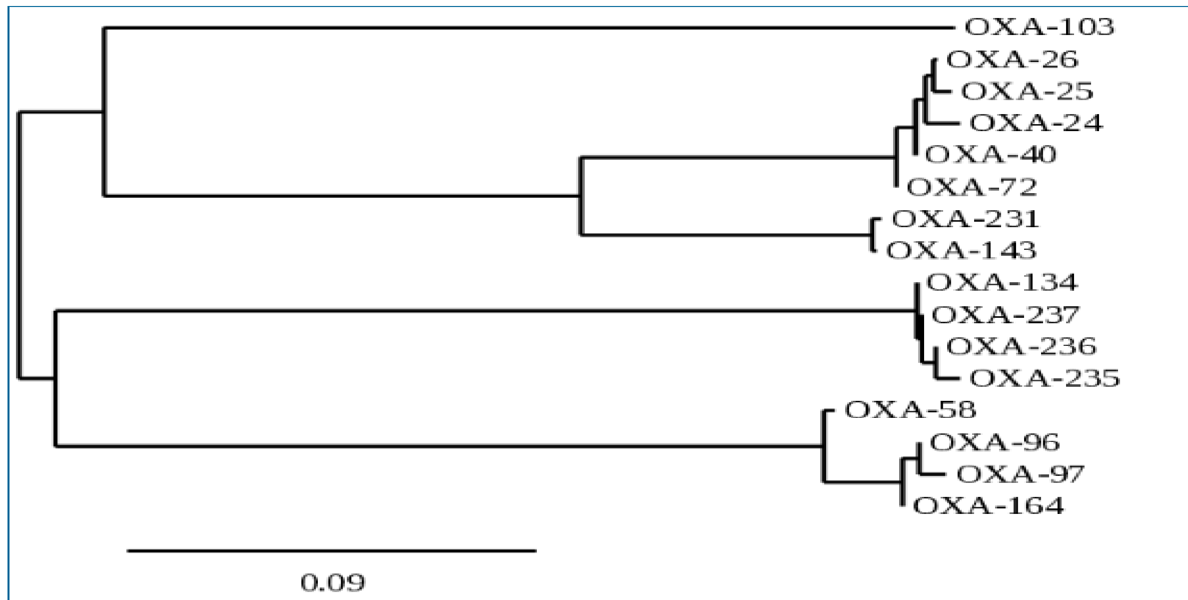


Figure 12: Phylogen*et*ic tree analysis for carbapenem resistance genes prevalent in the world.

5.5 Association among the genes found commonly in India and the world.

```
OXA-103     CTCTTTTTTATTTTCTATTGATCTGGTGTTTAAAATGAATAAATATTTTACTTGCTATGT     126
OXA-23      CTCTTTTTTATTTTCTATTGATCTGGTGTTTAAAATGAATAAATATTTTACTTGCTATGT     110
OXA-27      CTCTTTTTTATTTTCTATTGATCTGGTGTTTAAAATGAATAAATATTTTACTTGCTATGT     141
OXA-49      ----------------------------------ATGAATAAATATTTTACTTGCTATGT     26
OXA-239     ----------------------------------ATGAATAAATATTTTACTTGCTATGT     26
OXA-51      CACTC--------------TTACTTATAAC------------------------------     29
OXA-69      CACTC--------------TTACTTATAAC------------------------------     29
OXA-24      ------------------------------------------------------------     0
OXA-25      ----------TTCCCC---TAACATGAATTTGTAATGAAAAA-ATTTATACTTCCTATAT     46
OXA-72      ----------------------------------ATGAAAAA-ATTTATACTTCCTATAT     25
OXA-26      ----------TTTCCC---TAACATGAATTTGTAATGAAAAA-ATTTATACTTCCTATAT     46
OXA-40      ----------------------------------ATGAAAAA-ATTTATACTTCCTATAT     25
OXA-143     CACTCAAAACTTTCCC---TAACATAAATCTGTAATGAAAAA-ATTTATACTTCCTATTC     125
OXA-231     CACTCAAAACTTTCCC---TAACATAAATCTGTAATGAAAAA-ATTTATACTTCCTATTC     125
OXA-164     AAATTATTAAA--------AATATTGAGTTTA----------------GTTTGCTTAAGC     39
OXA-97      AAATTATTAAA--------AATATTGAGTTTA----------------GTTTGCTTAAGC     139
OXA-58      ------------------------------------------------------------     0
OXA-96      AAATTATTAAA--------AATATTGAGTTTA----------------GTTTGCTTAAGC     101
OXA-235     AACTTATGAAA--------ACTCTT--ATTTT-----------------GTTGCC-----     613
OXA-236     -----ATGAAA--------ACTCTT--ATTTT-----------------GTTGCC-----     23
OXA-237     -----ATGAAA--------ACTCTT--ATTTT-----------------GTTGCC-----     23
OXA-134     -----ATGAAA--------ACTCTT--ATTTT-----------------GTTGCC-----     23

OXA-103     GGTTGCT--TCTCTTTTTCTTTCTGGTTGTAC-GGTTCAGCATAATTTA--ATAAATGAA     181
OXA-23      GGTTGCT--TCTCTTTTTCTTTCTGGTTGTAC-GGTTCAGCATAATTTA--ATAAATGAA     165
OXA-27      GGTTGCT--TCTCTTTTTCTTTCTGGTTGTAC-GGTTCAGCATAATTTA--ATAAATGAA     196
OXA-49      GGTTGCT--TCTCTTTTTCTTTCTGGTTGTAC-GGTTCAGCATAATTTA--ATAAATGAA     81
OXA-239     GGTTGCT--TCTCTTTTTCTTTCTGGTTGTAC-GGTTCAGCATAATTTA--ATAAATGAA     81
OXA-51      --AAGCG---CTATTTTTATTTC------AGCCTGCTCACCTTATATAGT---GACTGCT     75
OXA-69      --AAGCG---CTATTTTTATTTC------AGCCTGCTCACCTTATATAGT---GACTGCT     75
OXA-24      ------------------------------------------------------------     0
OXA-25      -TCAGCATTTCTATTCTAGTTTCTCTCAGTGCATGTTCATCTATTAAAACTAAATCTGAA     105
OXA-72      -TCAGCATTTCTATTCTAGTTTCTCTCAGTGCATGTTCATCTATTAAAACTAAATCTGAA     84
```

Figure 13 MSA of different sequences of *OXA*s found in *Acientobacter baumannii.*

The comparison of different oxa genes, accounting carbapenem resistance to *A. baumannii* in India and worldwide showed the genes mutations prevalent in India are more conserved (figure 13). Further the phylogenetic analysis classified different mutated genes into conserved groups (figure 14). This shows how *OXA*s are related, for eg. *OXA*-25 and *OXA*-26 are the mutants of *OXA*-24, and thus belong to the *OXA*-24 family.

5.6 Phylogen*et*ic tree analysis:

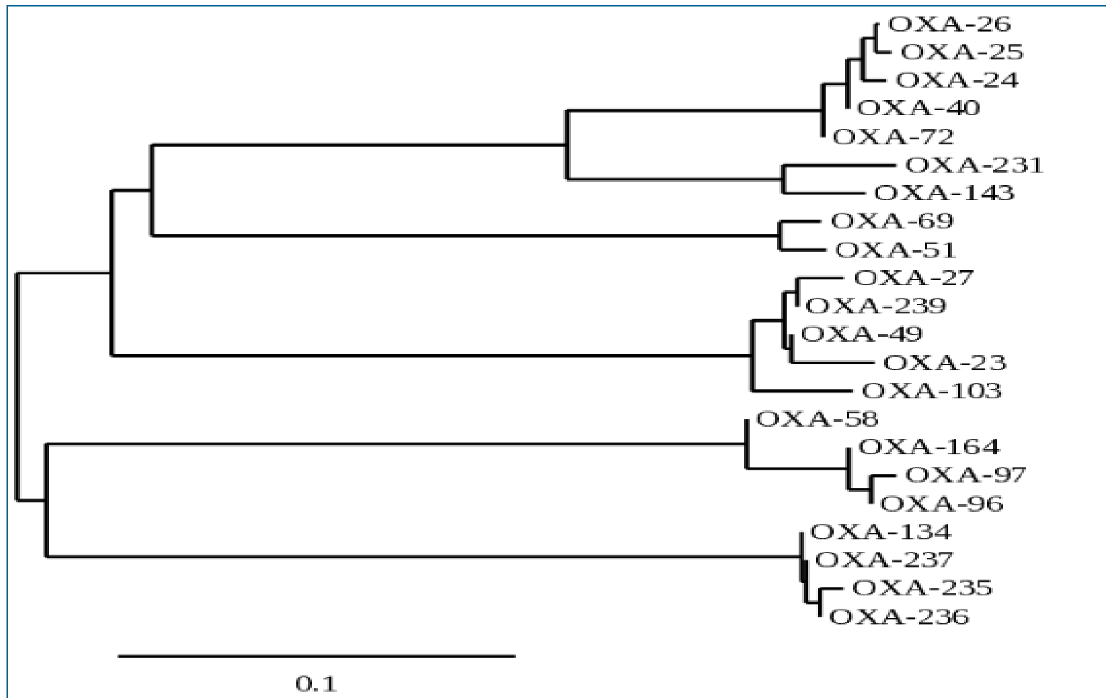


Figure 14: Phylogen*et*ic tree analysis of various sequences of *OXA*s.

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