BIOFILM FORMATION AND DRUG RESISTANCE PATTERN OF *ACINETOBACTER BAUMANNII* ISOLATED FROM CLINICAL SPECIMENS

A Dissertation Submitted to

Central Department of Microbiology,

Tribhuvan University, Kathmandu, Nepal,

in the partial fulfillment of the Requirements for the Award of Degree of Master of Science in Microbiology (Medical)

By

UPASANA GHIMIRE

Central Department of Microbiology,

Tribhuvan University

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RECOMMENDATION

This is to certify that **Ms. Upasana Ghimire** has completed this dissertation work entitled "**Biofilm formation and Drug resistance pattern of *Acinetobacter baumannii* isolated from clinical specimens**" as a partial fulfillment of M.Sc. Degree in Microbiology under our supervision. To our knowledge, this work has not been submitted for any other degree.

……………………… **……………………..**

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CERTIFICATE OF APPROVAL

On the recommendation of **Assoc. Prof. Dr. Dev Raj Joshi** and **Mr. Sanjit Shrestha,** this dissertation work of **Ms. Upasana Ghimire,** entitled "**Biofilm formation and Drug resistance pattern of** ***Acinetobacter baumannii*** **isolated from clinical specimens**" has been approved for the examination and is submitted to the Tribhuvan University in Partial fulfillment of the requirements for Master of Science Degree in Microbiology (Medical)

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ABSTRACT

Acinetobacter baumannii has emerged as a leading cause of nosocomial infections as they are capable to evolve resistance to various classes of antibiotics including carbapenems. The capacity of *A. baumannii* to form biofilm might be associated with increased antibiotic resistance and hence treatment failure. A cross-sectional study was carried with objective to associate the biofilm formation with the drug resistance pattern of *A. baumannii* and to detect *blaOXA-23*, *blaOXA-24* and *blaOXA-51* genes from phenotypic carbapenem resistant *A. baumannii.* A total of 180 clinical specimens including wound swab, pus, blood, urine, sputum, catheter tips,tissue, body fluidwere collected from study population, identified by microbiological procedures including Gram's staining, culture and various biochemical tests. The biofilm production was determined by using quantitative adherence assay. Antimicrobial susceptibility test were carried out by modified Kirby-Bauer disc diffusion method followingClinical and laboratoryStandards Insitiute CLSI guidelines 2019 while screening for Carbapenemase production was checked through Modified Hodge Test (MHT). All the phenotypically positive Carbapenem producers were subjected to molecular detection of the target resistant genes Polymerase chain reaction techniques. Out of 180 clinical specimens, 92 (51.11%) showed bacterial growth however,only 19 (20.65%) were identified as*A. baumannii*. Among all *A.baumannii*isolates 16 (84.21%) were multi-drug resistant and 12 (63.16%) were carbapenem resistant. The *blaOXA-51*gene was detected in all isolates whereas, *blaOXA-23* gene was detected in only 12 (63.16%) isolates but*blaOXA-24* gene was not detected in any of isolates. Among total, 17 (89.47%) *A.baumannii*producedbiofilm with 9 (47.37%) strong biofilm producers.The association between biofilm production and drug resistance was statistically significant.This study showed that most of *A. baumannii* isolates had a high level of antibiotic resistance and had a capacity to produce biofilm.This study provided valuable clue for management of*A. baumannii*infections in clinical settings.

Key words: *A. baumannii,* Wound, Cather tips, Tissue, Pus, Blood, Urine Sputum, BodyFluid, MDR, *blaOXA* genes,biofilm formation.

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ABBREVIATIONS

ADC	*Acinetobacter* Derived Cephalosporinses
AHL	N-acylhomoserine Lactones
AME	Aminoglycosides Modifying Enzymes
AST	Antibiotic Susceptibility Testing
ATCC	American Type Culture Collection
BAP	Biofilm Associated Protein
CDC	Centers for Disease Control and Prevention
COPD	Chronic Obstructive Pulmonary Disease
CLSI	Clinical and Laboratory Standards Institute
CVP	Central Venous Pressure
EDTA	EthyleneDiamine Tetracetic acid
EPS	Exopolymeric Substance
ESBL	Extended Spectrum Beta Lactamases
LRT	Lower Respiratory Tract
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MBL	Metallo-β-Lactamses
MDR	Multi-Drug Resistant
MHT	Modified Hodge Test
NDM	New Delhi Metallo- β-Lactamses
NHSN	National Healthcare Safety Network
OMP	Outer Membrane Proteins
PDR	Pandrug Resistant
PNAG	Poly-β-(1, 6)-N-Acetyl Glucosamine

QS	Quorum Sensing
UTI	Urinary Tract Infections
VAP	Ventilator Associated Pneumonia
XDR	Extensively Drug Resistant

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Genus *Acinetobacter* is identified as a Gram negative bacilli, ubiquitous, free living, strictly aerobic, short, often capsulated, non-motile, non-fermenting cocobacilli with a DNA G+C content of 39 to 47 mol% (Peleg et al 2008). Generally, *Acinetobacter* spp. form smooth, mucoid, grayish white or white to pale yellow colonies and can be grown in solid media like blood agar, MacConkey agar and Trypticase soy agar at 37°C. Itresembles Enterobacteriaceae and is of 1.5-3 mm in diameter (Constantiniu et al2004).

Acinetobacter calcoaceticus- Acinetobacter baumannii (AC-AB) complex comprises four species namely *A. calcoaceticus, A. baumannii*, and the genospecies 3 ("*Acinetobacter pittii"*) and genospecies 13TU ("*Acinetobacter nosocomialis*") which are genotypically and phenotypically highly related and difficult to distinguish phenotypically (Fournier and Richet 2006). These genomic species mainly associated with outbreaks of nosocomial infections. Fourth member, *A. calcoaceticus* is rarely involved in disease and is mostly environmental (Peleg et al 2008).

In this era, *A. baumannii* is an opportunistic pathogen which has great potential to acquire resistance determinants making it one of the alarm pathogen (Nowak et al 2012). At the beginning, it has little clinical significance but now it is being isolated more frequently, specifically in intensive care settings. It is an opportunistic nosocomial pathogen causing wound infections, peritonitis, endocarditis, cholangitis, ventilator associated pneumonia (VAP), bacteremia, ICU infections, and urinary tract infections (UTIs) particularly in patients with severe health condition (Abdalhamid et al 2014). Some rare cases of community acquired infections havealso been caused by *A. baumannii* such as endocarditis, peritonitis, cholangitis and septic complications (Manchanda et al 2010). The emergence of *Acinetobacter* as a significant nosocomial and opportunistic pathogen is

influence by its survival ability, ubiquitous presence, and rapid development of resistance to the commonly used antimicrobials (Dijkshoom et al 2007; Maragakis and Perl 2008).

In past few decades, particularly from 2005-2006, member of genus *Acinetobacter* have emerged from organisms of doubtful pathogenicity to pan-resistant nosocomial pathogens worldwide (Jaggy et al 2012). As a result of their ability to survive for long period on inanimate surfaces, they are commonly isolated from the hospital environment and are associated with skin colonization of inpatients and hospital personnel. It is assumed that its attribute to persist in these environments, as well as its virulence is effect of its capacity to form biofilms (Gaddy and Actis 2009). Biofilm is a relevant process due to its mechanism for antibiotic resistance, transfer of resistance plasmids and a medium for intracellular communication (Gurung et al 2013).

Biofilm is structured community of bacterial cells enclosed in a self-produced polymeric matrix adherent to an inert or living surface (Badave and Kulkarni 2015). Phenotypically biofilm formation is interrelated with exopolysaccharide (EPS) production, nutrient availability, bacterial surface components (outer membrane proteins, adhesins), quorum sensing and pilus formation (Gaddy and Actis 2009; Tomoras et al 2003). Biofilm can present on all types of surfaces such as plastic, metal, glass, soil particles, wood, medical implant materials, tissue and food products (Deibel 2001). Biofilm associated cell is discriminated from planktonic counter parts and by reduced growth rate, up and down regulation of gene and generation of extracellular polymeric matrix (Donlan and Costerton 2002; Kokare et al 2009).

A. baumannii were 73% MDR which was higher in biofilm producer in comparison to non-biofilm producers (Gurung et al 2013). Biofilm producing *A. baumannii* are far more resistant to antimicrobial agent than their planktonic counterparts that may be as a result of delayed penetration of antimicrobial agents through biofilm matrix, altered growth rate of biofilm organisms and other physiological changes through the biofilm mode of growth (Donlan and Costerton 2002).

For the treatment of *Acinetobacter* infections, protocol for antimicrobial therapy comprise of broad spectrum cephalosporins, the beta-lactamase inhibitor sulbactam, quinolones, carbapenems, amikacin, doxycycline, and minocycline.Tigcycline and colistin are recommended as the last therapeutic options either alone or in combination with therapy for infection caused by MDR strains (Talbot et al 2006).

A. baumannii have shown high degree of resistance to carbapenems, beta-lactam antibiotics, fluroqunilones and aminoglycosides. They have appeared as one of the most ambiguous pathogen to eradicate, using available antibiotics (Principe et al 2009). Against penicillin and third generation cephalosporins, extended spectrum beta-lactamases (ESBLs) encoded by *TEM*, *SHV* and *CTX* type genes perform important role in resistance (Safari et al 2015).

Infections caused by multidrug resistant *A. baumannii* are extremely difficult to treat. Carbapenems are most frequently used for treatment of infections. However, resistance to these agents is now World Health Organization's number one critical priority pathogen and has significantly limited treatment options (Shales and Bradford 2018). The underlying resistance mechanisms may be related to efflux pump overexpression, decreased permeability, and carbapenemase production. Among the carbapenemases, carbapenem-hydrolyzing class D β-lactamases (CHDLs) are considered the most prevalent cause of carbapenem resistance in *A. baumannii*(Heritier et al 2006). The CHDLs in *A. baumannii* can be grouped into six subclasses: intrinsic chromosomal OXA-51-like, acquired OXA-23-like, OXA24/40-like, OXA-58-like, OXA-143-like, and OXA235-like β-lactamases (Higgins et al 2010). The intrinsic *blaOXA-51* gene is characteristic of this species and is usually weakly expressed (Zavascki et al 2010). However, it can play a role in carbapenem resistance when an insertion sequence *ISAba1* precedes the gene, providing promoter sequences that increase its expression (Werneck et al 2011). The *blaOXA-23* gene, first characterized in Scotland, has been increasingly reported worldwide. *A. radioresistens* was recently identified as the progenitor of the *blaOXA-23*-like genes. From the different groups of

OXA-type enzymes defined on the basis of sequence comparisons, OXA-23, OXA-24, and OXA-58 (which can be plasmid- or chromosomally-encoded) have been most frequently associated to carbapenem-resistant clinical strains of *A. baumannii,* but OXA-23 has the highest dissemination worldwide (Poirel and Nordmann 2006).

Acinetobacter is especially troublesome in hospital setting where immunocompromised patient is at greater risk of nosocomial infection than the general patient. Among the *Acinetobacter* spp. biofilm-producing organisms are immensely resistant to antimicrobial agents than microorganisms which do not produce biofilm (Gurung et al 2013). Biofilm formation by these bacteria has been their protective weapon for continuing their survival in wide range of environmental condition. This increases a prominently therapeutic question for the treatment of nosocomial infections throughout the globe. The rising incidence of antibiotic resistance in *A. baumannii* is a matter of great hazard to mankind. If a necessary step is not taken to control this emerging pathogen then these drug resistant bacteria can damage many human lives with their infections (Eze et al 2018). This study is crucial for understanding the occurrence and distribution of the biofilm producing multidrug resistant *A. baumannii* in Nepalese clinical environment and the association between the molecular evolutionary mechanisms of carbapenem resistance *A. baumannii.*

1.2 Objective

1.2.1 General objective

To associate biofilm formation and antimicrobial susceptibility profile of *A. baumannii* isolated from different clinical specimens.

1.2.2 Specific objectives

- To determine the prevalence of *A. baumannii* in clinical set up.
- To test susceptibility of the*A. baumannii* isolates against selected array of antibiotics.
- To detect the *blaOXA-23*, *blaOXA-24*, and *blaOXA-51* gene in carbapenem resistant*A. baumannii.*
- To assess biofilm production in *A. baumannii* qualitatively and quantitatively.
- To correlate antimicrobial resistance and biofilm formation by *A. baumannii.*

CHAPTER II

LITERATURE REVIEW

2.1 History

In 1911, Beijerink a Dutch Microbiologist first isolated *Acinetobacter* spp. from soil in a calcium acetate containing mineral media and named *Micrococcus calcoaceticus* (Doughari et al 2011; Peleg et al 2008). Before 1950s, it had several names and now it is known as *Acinetobacter* (Munoz-Price and Weinstein 2008). The present genus *Acinetobacter* (from Greek 'akinetos', means nonmotile), was proposed by Brisou and Prevot in 1954 to identified the organisms depend on their motility in the tribe 'Achromobactereae' and was composed of non-pigmented Gram-negative saprophytic bacteria including both oxidase-negative and oxidase-positive species (Jung and Park 2015). This organism was previously grouped into various genera namely, *Achromobacter antitratus, Achromobacter mucosus, Bacterium antitratum, Moraxella lwoffii, Moraxella calcoaceticus, Nesseria winogradskyi* etc (Jung and Park 2015). Bergey's manual of systematic bacteriology in 1974 introduced the characteristics of *Acinetobacter calcoaceticus* and placed the bacteria in the family Neisseriaceae with two subspecies *Acinetobacter antiratum* and *Acinetobacter lwoffii* (Doughari et al 2011).

2.2 Taxonomy

Member of genus *Acinetobacter* is Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase positive, oxidase negative bacteria with a DNA G+C content of 39% to 47%. On current taxonomic data, it was admitted that the members of the genus *Acinetobacter* should be classified in the new family *Moraxellaceae* withinthe order*Gammaproteobacteria,* which includes the genera *Moraxella, Acinetobacter, Psychrobacter,* and related organisms. A vital development in the long and complicated history of the genus was achieved in 1986 by Bauvet and Grimont, who based on DNA-DNA hybridization studies distinguished 12

DNA (Hybridization) groups or genospecies, some of which were given formal species names, including *A. baumannii, A. calcoaceticus, A. haemolyticus, A. johnsonii, A. junii,* and *A. lwoffii* (Peleg et al 2008).

2.3 Species identification

Acinetobacter is identified to the genus as Gram-negative, catalase-positive, oxidase-negative, non-motile, non-fermenting coccobacilli. However, the organisms are often difficult to de-stain and falsely identified as Gram positive. There is no specific metabolic test that can distinguish *Acinetobacter* from other non-fermenting Gram-negative bacteria (Peleg et al 2008). A method which is often used to identify the genus depend on the ability of the mutant *A. baylyi* strain BD413 trpE27 to be transformed by crude DNA of any *Acinetobacter* species to a wild-type phenotype. While for species level identification, the 28 available phenotypic tests have proven to be 95.6% effective in identifying human skin-derived *Acinetobacter* (Morris et al 2019). However, phenotypic tests alone have proven to be ineffective in identifying more recently discovered genomic strains of *Acinetobacter*.

DNA-DNA hybridization is the standard method for species identification. Molecular methods including 16s RNA gene restriction analysis, high resolution fingerprint analysis by AFLP, ribotyping, tRNA spacer fingerprinting and sequencing of rpoB gene are useful for species identification (Peleg et al 2008). More recently, detection of *blaOXA-51* like Carbapenemase gene is the simple and convenient method of identifying *A. baumannii* which is easier than amplified rRNA gene restriction analysis and more reliable than biochemical identification (Turton et al 2006). Matrix Assisted laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry is also useful for identification of*A. baumannii* (Alvarez-Buylla et al 2012).

2.4 Habitat

Acinetobacter species are ubiquitous in nature, recovered from soil, water, animal and humans. They are normal flora of skin, mucous membrane, throat and respiratory tract of hospitalized patients. They can also be found in hospital foods. They are free living and fairly stable in the environment (Munoz-Prince and Weinstein 2008; Nazmul et al 2012).

Acinetobacter species are frequently isolated from animals like birds, fishes and foods like vegetables, milk, fruits etc. They are also found on medical equipment such as ventilator tubing, arterial pressure monitoring devices, humidifiers, wash basins, respirometers, dialysis machines etc. They are also isolated from skin of healthcare personnel, mattresses, pillows and wound of the hospitalized patients (Farid et al 2016; Kanafani and Kanj 2013). Digestive tract colonization is common in hospital environment with 41% rate in ICU patients (Fournier and Richet 2006). In healthy humans, *Acinetobacter baumannii* transiently colonizes at a low density on the warm and moist skin of axilla, groin, between toes, throat, nares and intestinal tract but it generally does not cause infection (Yong et al 2009). In the hospital environment, *Acinetobacter baumannii* can colonize the respiratory, urinary, gastrointestinal tract and wounds of the patient and cause infections in burn, trauma, mechanically ventilated and immune-compromised patients (Towner 2006). Epidemic of *Acinetobacter baumannii* infection on human lice was detected, that may act as source of human infections (Fournier and Richet 2006).

2.5 Virulence factors of *Acinetobacter* spp

So far, only few factors of *Acinetobacter spp* has been known for their role to cause virulence, one factor in particular, OmpA, a member of the Outer membrane proteins (OMPs), has been determined to contribute significantly to the disease causing potential of the pathogen (Choi 2005). OmpA involves in adherence of *A. baumannii* to epithelial cells and mitochondria. It induces mitochondrial dysfunction and release of cytochrome C which leads to apoptosome formation that contributes to the apoptosis of the cell. It also

involves in resistance to complements and formation of biofilms (Howard et al 2012).

Acinetobacter spp has been demonstrated to exhibit cell surface hydrophobicity, an important determinant of bacterial adhesion. In *Acinetobacter* spp., the presence of protein protrusions, polysaccharide slimes on the cell surfaces receptors, fimbriae other cell wall components and cell surfaces enzyme confers hydrophobicity and facilitates the adhesion of bacterial cells to host cells (Camarena 2010). The hydrophobicity of a microorganism protects it from being phagocytized and appears to play role in attachment to various polymers. Hydrophobicity also confers the ability to adhere to plastic surfaces, such as catheters and prostheses (Doughari et al 2011).

Varieties of enzymes are produced by *Acinetobacter* spp. These include ureases, esterases, certain amino-peptidases, and acid phosphatases. Ureases help *Acinetobacter*spp., colonize the hypochlorhydric or achlorhydric human stomach including inflammation. The hydrolytic enzymes cause damage to lipid tissues. (Doughari et al 2011). Similarly, phospholipase D is associated with resistance to human serum, epithelial cell evasion and pathogenesis and phospholipase C enhances toxicity to epithelial cells(Camarena 2010).

The emergence of verotoxin-producing *A. hemolyticus* is worrisome given the high transformability of *Acinetobacter* spp. siderophores are the host iron binding protein structures responsible for iron uptake in bacteria (Eijkelkamp et al 2011). Toxic slime polysaccharides have also been reported. Outer membrane proteins have essential roles in pathogenesis and adaptation in host cells as well as in antibiotic resistance (Doughari et al 2011). Fimbriae expressed on the surfaces of the bacterial cell, contribute to the adhesion of pathogen to epithelia (Howard et al 2012).

The ability of *A. baumannii* to form biofilms allows it to grow persistently in unfavorable conditions and environments such as glass and equipment used in intensive care units, and/ or on biotic surfaces such as epithelial cells. Environmental signals, such as metal cations, play a role in controlling the

formation of biofilms, increasing the ability of *A. baumannii* to adhere to particular surfaces (Howard et al 2012). Biofilm formation is a relevant process because of being as a mechanism for antibiotic resistance, transfer of resistance plasmids, and a medium for intracellular communication (Gurung et al 2013). Outgrowth on mucosal surfaces and medical devices, such as intravascular catheters and endotracheal tubes can result in *A. baumannii* biofilm formation, which enhances the risk of infection of the bloodstream and airways (Tomoras et al 2003). *A. baumannii* causes significant bacteremia; either as a single pathogen or as part of polymicrobial bacteremia predominantly in adults (Seifert et al 1997).

2.6 Pathogenesis of *Acinetobacter*

The mechanisms involved in the establishment and progression of infections by *Acinetobacter* species are unclear. However, most through recent effort to analyze the sequence of genomic *A. baumannii* identifiedlarge amount of probable foreign DNA so called putative alien islands on the basis of differences in G plus C content and codon usage (Smith et al 2007). Homologous of virulence genes identified in other pathogens were found in 12 of these islands and largest islands contained genes for putative type IV secretion apparatus. Other islands harbor genes for antibiotic-resistance determinants, an indication that *A. baumannii* has high capacity to take in and incorporate foreign DNA. The comparison with *A. baylyi* was also illuminating since *A. baylyi* is noted for its remarkable natural transformability and ease with which it is able to express foreign genes. It was found that *A. baumannii* genome contains most of the gene involved in competence and homologs were found that may compensate for the two that it lacks. This finding may account for large amount of foreign DNA present. Although at same degree as *A. baylyi, A. baumannii is* also capable of natural transformation under certain conditions (Neilsen et al 2000).

2.7 Risk factors for acquisition of *Acinetobacter* infection

Though *Acinetobacter* spp, does not cause diseases among healthy individuals however, main sites of *Acinetobacter* infection are lower

respiratory tract (LRT) and urinary tract (UT). Risks factors for MDR *A. baumannii* infections are existence of *Acinetobacter* in multiple habitats, mechanical ventilation, previous antibiotic use, co-morbidity, neurologic impairment, chronic obstructive pulmonary disease (COPD) and diabetes mellitus (Dent et al 2010). Other risk factors include admission to ICU with complications, inappropriate antibiotic therapy and infection with resistant antibiotype. There is continuous occurrence and recurrence of *Acinetobacter* infections or colonization among patients in high risk wards and ICU (Prashanth and Badrinath 2006). Prolong hospitalization, elderly patients with underlying disease, endotracheal intubation, greater than eight days of hospital stay, intravenous lines, graft application and surgical interventions were significant risk factors for MBL positives strains (Garnacho-Montero et al 2005; Kumar et al 2012). Alcoholism, cigarette smoking, chronic lung disease and resistance in a tropical developing country are also associated with community acquired infection (Sinha et al 2014)

2.8 Clinical manifestations of *Acinetobacter* infections

Acinetobacter infections have emerged with increasing frequency. *Acinetobacter* spp., have emerged as particularly important organisms in ICUs and this is probably related, at least in part, to the increasingly invasive diagnostic and therapeutic procedures used in hospital ICUs over the last two decades (Bergogne-be`re`zin and Towner 1996). A 2008 report from US, the National Healthcare Safety Network (NHSN) reviewed the most frequent types of hospital-acquired infection in ICU due to gram negative bacteria like *Acinetobacter* spp. Infectious disease society of America has also included that *A. baumannii* as one of the six top-priority dangerous microorganisms responsible for the high morbidity and mortality (Cai et al 2012). This organism is well adapted to hospital environment, capable of spreading to new patients and making itself a nosocomial pathogen of particular concern and a public threat (Dikjshoom et al2007).

2.8.1 Hospital acquired pneumonia

Acinetobacter pneumonia occurs predominantly in intensive care unit (ICU) patients who require mechanical ventilation and tends to be characterized by a late onset. Other clinical manifestations of *Acinetobacter* pneumonia are similar to those reported for hospital-acquired pneumonia in general. Most cases of nosocomial *Acinetobacter* pneumonia occur in previously colonized patients. True *Acinetobacter* pneumonia must be distinguished from airway colonization in mechanically ventilated patients.*A. baumannii* was among the most common pathogens causing nosocomial pneumonia in a prospective observational study from 27 ICUs in nine different European countries; in Greece and Turkey, it was the most common isolate (Koulenti et al 2017). Nosocomial pneumonia secondary to *Acinetobacter* is associated with mortality rates of 35 to 70 percent, though attributedmortality is difficult to determine asmost patients have concurrent life-threatening conditions (Sunenshine et al 2007). Coexisting conditions appear to be a major predictor of outcome. One study noted higher mortality among patients with infection due to multidrug-resistant *Acinetobacter* than among patients with infection due to susceptible *Acinetobacter* strains or uninfectedpatients; however, when the severity of illness and underlying diseases were considered, the main difference was that patients with multidrug-resistant infection had longer hospital and ICU stays (Brotfain et al 2016).

Overall, positive blood cultures and signs of sepsis usually portend a bad prognosis. As an example, severe sepsis and septic shock were independent predictors or 30-day mortality in a study of patients with nosocomial *A. baumannii/calcoaceticus* complex pneumonia (Ozvatan et al 2016). Additionally, patients with pneumonia due to *Acinetobacter* spend more ventilator days in the ICU before detection of positive cultures than do patients with pneumonia due to other gram-negative bacilli or uninfected patients (Garnacho-Montero et al 2005).

2.8.2. Community acquired pneumonia

Community-acquired *Acinetobacter* pneumonia is typically characterized by a fulminant illness with an abrupt onset and rapid progression to respiratory failure and hemodynamic instability (Leung et al 2006). Septic shock ensues in around one-third of patients. This infection is recognized as rare but important in tropical area of Asia and Australia (Visca et al 2011).

2.8.3 Bloodstream infection

A. baumannii is also a common cause of nosocomial bloodstream infections in the ICUs. Main sources of *A. baumannii* bloodstream infection are lower respiratory tract infections and intravascular devices. It has mortality rate of 20% or more and characterized by fever, leukocytosis and positive blood culture (Latibeaudiere et al 2015). *Acinetobacter* species are the second most common pathogen to cause bloodstream infections acquired in hospitals (Ulu-Kilic et al 2013). *A. baumannii* represents 10-15% of bacteremia (Joly-Guillou 2005).It is responsible for the 1.3% of all monomicrobial nosocomial bloodstream infection in USA (Peleg et al 2008). About 30% of bloodstream infections attributed to *A. baumannii* were actually caused by *A. nosocomialis* and *A. pittii* but the organisms involved were misidentified by commercial identification systems (Visca et al 2011).

2.8.4 Burn infections

It is difficult to differentiate between infection and colonization of burn sites. Recent studies showed high incidence of *A. baumannii* in military burn units with 53% MDR. *Acinetobacter* burn infections can delay wound healing, failure of skin graft, infection of underlying tissue and systemic spread of bacteria (Maragakis and Perl 2008).

2.8.5 Endocarditis

Acinetobacter spp are a rare cause of infective endocarditis in native and prosthetic heart valves. In a study of 171 patients with prosthetic heart valve endocarditis resulting from nosocomial bacteremia, two cases were

attributable to *Acinetobacter* (Fang et al 2008). *Acinetobacter* endocarditis is typically characterized by acute onset with an aggressive course. Mortality tends to be higher in the setting of native valve endocarditis than prosthetic valve endocarditis, likely because of the low index of suspicion leading to delayed treatment in such cases.

2.8.6 Meningitis

Major cases of *Acinetobacter baumannii* meningitis occur in patients recovering from neurosurgical procedures. Clinical features of *Acinetobacter* meningitis resembles with those of other bacterial meningitis and includes fever, altered consciousness, headache and seizure (Maragakis and Perl 2008).

2.8.7 Skin and soft tissue, and bone infections

Acinetobacter may contaminate surgical and traumatic wounds, leading to severe soft tissue infections that can progress to osteomyelitis. Surgical wound infections with *Acinetobacter* are frequently related to the presence of prosthetic material and usually require extensive debridement (Song et al 2007). *Acinetobacter* has rarely been associated with community-acquired or hospital-acquired skin infections such as cellulitis and folliculitis as well as skin abscesses and necrotizing fasciitis. Traumatic wound infections due to multi-drug resistant *Acinetobacter* complex have been increasingly recognized after war injuries; environmental contamination of field hospitals appears to play an important role in these infections (Davis et al 2005).

2.8.8 Urinary tract infections

Urinary tract infection caused by *Acinetobacter baumannii* is often related to dwelling Foley catheters. These infections are usually benign and occur more frequently in rehabilitation centers than in ICU (Dijkshoom et al 2007). In a review of 5000 urinary tract infections in medical ICUs in the United States, 1.6% was due to *Acinetobacter* and, among them 95% of the infections was associated with urinary catheters (Gaynes and Edwards 2005).

2.9 Antibiotic resistance in *Acinetobacter baumannii*

Multi drug- resistant (MDR) *Acinetobacter spp.* is defined as the isolate resistant to at least three classes of antimicrobial agents mostly all penicillins and cephalosporins (including inhibitor combination), fluoroquinolones, and aminoglycosides. Extensively drug-resistant (XDR) *Acinetobacter* spp.is the *Acinetobacter* spp. isolate that is resistant to the three classes of antibiotics described above (MDR) and can also be resistant to carbapenems; finally, Pandrug -resistant (PDR) *Acinetobacter* spp. is the XDR *Acinetobacter* spp. that is resistant to polymyxins and tigecycline (Manchanda et al 2010).

Acinetobacter baumannii is intrinsically resistant to commonly used antimicrobial agents such as aminopenicillins, first and second generation cephalosporin and chloramphenicol (Dijkshoorn et al 2007). Microorganisms have developed the ability to make altered receptors for antimicrobial agents; have prevented agents from reaching their receptors within the bacterial cells, and now they have enzymes to destroy antibiotics and have resistant metabolic pathways (Okonko et al 2009).

The challenge of managing infections caused by *A. baumannii* has been complicated by an increase in antimicrobial resistance, especially multidrug resistance, with some strains now resistant to all or almost all commonly used antimicrobial agents (Blossom and Srinivasan 2008). Reduced susceptibility to β-lactams amongst biofilm bacteria is more likely to be a function of a diminished growth rate within the deeper recesses of the biofilm which causes the expression of penicillin-binding proteins that are unrepresentative of those normally targeted by these antibiotics (Gilbert and Brown 1998). Mutations in topoisomerase genes can provide fluoroquinolone resistance. Efflux pumps can expel various classes of antimicrobial agents including β-lactams, quinolones, tetracyclines, and aminoglycosides. *A. baumannii* also have several types of aminoglycoside-modifying enzymes (acetylating, adenylating, and phosphorylating). Production of AmpC cephalosporinases confers resistance to ceftazidime and other third-generation cephalosporins (Heritier et al 2006).

2.9.1 β-lactams

The most prevalent mechanism of β-lactam resistance in *A. baumannii* is enzymatic degradation by β-lactamases. However, in keeping with the complex nature of this organism, multiple mechanisms often work in concert to produce the same phenotype (Bou et al 2000).

Inherit to all *A. baumannii* strains are chromosomally encoded *AmpC* cephalosporinases, also known as *Acinetobacter*-derived cephalosporinses (ADCs). Unlike that of AmpC enzymes found in other Gram-negative organisms, inducible *AmpC* expression does not occur in *A. baumannii* (Bou and Martinez-Beltran 2000). The key determinant regulating overexpression of this enzyme in *A. baumannii* is the presence of an upstream IS element known as *ISAbal.* The presence of this element highly correlates with increased *AmpC* gene expression and resistance to extended-spectrum cephalosporins. Cefepime and carbapenems appear to be stable in response to these enzymes (Carvalho et al 2009).

The main cause of carbapenem resistance in *A. baumannii* is production of intrinsic or acquired carbapenemases. (Heritier et al 2006). The *blaOXA-51* is intrinsic gene of *A. baumannii* that mediate carbapenem resistance to this species. Other acquired OXA-group beta lactamases genes on plasmid are OXA-23,-40,-58,-143 and -235 groups. OXA-27 and OXA-49 are closely related enzymes that make up the *blaOXA-23* gene cluster in *A. baumannii.* Two other acquired OXA-type gene clusters with carbapenemase activity have been described, including the *blaOXA-24*-like (encoding OXA-24, -25, -26 and -40) and the *blaOXA-58*-like Carbapenemase genes (Brown and Amyes 2006).

Changes in outer membrane proteins (OMPs), multidrug efflux pumps and alterations in the affinity or expression of penicillin-binding proteins are non-enzymatic mechanisms for beta-lactam resistance, including carbapenem resistance (Peleg et al 2008). The outer membrane of *Acinetobacter* spp. acts as a substantial barrier against the penetration of antibiotics. One of the causes for the high antibiotic resistance is the presence of the small number of the

small size porins (Prashanth and Badrinath 2006). One of the studies suggested that porins of *Acinetobacter* spp. was less than 5% of the total outer membrane proteins (OMPs) while that of *E. coli* was reported to be about 60% that contributes to reduced permeability to antimicrobial agents (Rosenbusch 1974). Carbapenem resistance in *Acinetobacter* spp. has been thought to be linked to the loss of proteins through porin channels from outer membrane (Mussi et al 2005). Beta-lactamases and altered outer membrane work together to confer the resistance against beta-lactam agent (Bonomo and Szabo 2006).

2.9.2 Aminoglycosides

Aminoglycosides resistance in clinical isolates of *A. Baumannii* is mediated by plasmid or transposon-coded aminoglycosides modifying enzymes (AmES) such as adenylating, acetyling and phosphorylating AmEs) (Nemec et al 2001). In addition, 16SrRNA methylation, an emerging resistance mechanism for *A. baumannii* (armA) strains impairs the aminoglycoside binding to its target site and confers high-level resistance to all clinically useful aminoglycosides, including gentamicin, tobramycin and amikacin (Peleg et al 2008). Resistance to aminoglycosides is also through the use of efflux pumps such as AdeABC efflux pump. Other mechanism of resistance includes alternations of the target ribosomal protein, ineffective transportation of the antibiotic to the interior of the bacteria (Vila et al 2007).

2.9.3 Quinolones

Mutation in *gyA* and *parC* genes that cause phenotypic modification in DNA gyrase and topoisomerase IV may lead to quinolone resistance. Drug influx and efflux system encoded by chromosomal DNA mediates reduced expression of OMPs involved in drug influx and increased expression of efflux proteins resulting in active drug expulsion: these are also responsible for quinolone resistance. Plasmid encoded quinolone resistance determinants *qnrA, qnrB,* and *qnrS* have also been identified in *A. baumannii* that protect DNA by inhibiting binding of quinolones to DNA gyrase and topoisomerase (Yang et al 2016).

2.9.4 Tetracyclines

The main mechanisms responsible for tetracycline resistance in *A. baumannii* are expression of efflux pump and ribosomal protection. *Tet A* and *Tet B* are the most extensively characterized genes responsible for ribosomal protection (Maleki et al 2014). The efflux determinant *Tet A* confers resistance to tetracycline whereas *Tet B* confers resistance to tetracycline and minocycline (Vila et al 2007). These efflux pumps do not affect the new tetracyclines such as glycylcyclines (Vila et al 2007). Ribosomal protection is mediated by the *Tet M* and *Tet O* determinants, with *Tet M* being described rarely for A. *baumannnii* (Ribera et al 2003). In addition, the resistance to tetracycline is also mediated by multidrug AdeABC efflux pump (Magent et al 2001).

2.9.5 Cephalosporins

Majority of *Acinetobacter* species are resistant to third and fourth generation cephalosporins by producing natural *AmpC* beta-lactamase called ADC beta-lactamase. *AmpC* cephalosporinases are chromosomally encoded and placed in subgroup among the class C beta lactamases. ESBL production also leads to cephalosporin resistance (Doi et al 2015).

2.9.6 Polymyxins

Polymyxins are the group of polycationic peptide antibiotics that were discovered more than 60 years ago and exhibit potent efficacy against most Gram-negative bacteria. Among all five polymyxins (A-E), only polymyxin B and polymyxin E (colistin) with one amino acid difference are used clinically. Colistin is a key component of combination therapies used to treat MDR *A. baumannii* infections (Cai et al 2012). The mechanisms of colistin resistance include loss of LPS (Song et al 2007) and the addition of phosphoethanolamine to LPS by the pmrAB two-component system (Adams et al 2009). Mutations in pmrA and pmrB acitivate pmrC, which adds phosphoethanolamine to the hepta-acylated form of lipid A (Beceiro et al 2011). However, an investigation of the in vivo activities of various antimicrobial combinations against colistin resistant *A. baumannnii* showed that the most effective combinations against colistin resistant *A. baumannnii*

are colistin-rifampin and colistin-teicoplanin, indicating that colistin is most common constituent of antimicrobial combinations even against colistin resistant *A. baumannnii* (Bae et al 2016).

2.10 Biofilm formation in *Acinetobacter*

Biofilm is a sessile community of microorganisms in which cell are irreversibly attached to a substratum or interface or to each other and are embedded in a matrix of extracellular polymeric substances produced by them (Deibel 2001). It is composed of microorganisms (+15% by volume) attached to either each other or to living or abiotic surfaces and may be embedded within a complex extracellular matrix (+85% by volume) comprised of polysaccharides, proteins, nucleic acids and glycoproteins (Donlan and Costerton 2002; Kokare et al 2009). Production of biofilm in *Acinetobacter* is controlled by various cell density dependent, environmental and surface expressed factors. It is inhibited by a chelating agent i.e. EDTA, which has the ability to bind cations that may be required to stabilize the negatively charged molecules of the extracellular polymeric substances (Longo et al 2014). The medical devices such as catheters, artificial valves areeffectively and efficiently used often compromised by the formation of biofilms. (Tomaras et al 2003). In human diseases, biofilm have been increasingly recognized as important. Biofilm producers account 80% of microbial infections in the human body (Deibel 2001). *Acinetobacter* biofilm play a significant role in infectious diseases such as cystic fibrosis, periodontitis, bloodstream infection and UTI because of their ability to indwell medical devices (Pour et al 2011).

Biofilm is highly regulated by a series of molecular event (Gaddy and Actis 2009). They require intracellular signaling and transcribe different set of genes different from, planktonic counterpart (Kokare et al 2009). *CsuA/BABCDE* mediated pilli formation played a role in initial step of biofilm formation by allowing bacterial cell to adhere to abiotic surfaces resulting in initiation of microcolony formation that preceded the full development of biofilm structures. *CsuE* coded for tip adhesion, pili production as well as biofilm formation (Bhargava et al 2010). *A. baumannii* contain *pgaABCD* locus that encode a protein which synthesizes a cell-associated poly-β-(1, 6)-

N-acetyl glucosamine (PNAG). PNAG helps in the development and maturation of biofilm (Gaddy and Actis 2009). In addition to PNAG, *Acinetobacter* contains biofilm-associated protein (BAP), homologous of *Staphylococcus* BAP for stabilization and maturation of biofilm. Cell adhesiveness and biofilm formation in plastic surfaces is higher in strain harboring *blaPER-1* gene than those that do not harbor this genetic trait (Rao et al 2008).

2.10.1 Steps of biofilm formation

Biofilm formation is complex and multistep process which requires special type of signaling between cells of microorganism (quorum sensing) and transcription of different sets of genes (Jamal et al 2018).

A. Attachment to the surface

Attachment to the host surface occurs in two stages i.e. reversible and irreversible attachment (Armbruster and Parsek 2018). Reversible attachment is aided by microbial factors pilli and physical forces like bacterial cellular surface charge, surface hydrophobicity, pathogenic and nutritional conditions of the host. Vander Waals force, electrostatic force and bacteria to bacteria interactions may also help in attachment of bacteria to a surface (Palmer et al 2007).

B. Quorum-sensing (QS) signal

Quorum sensing is regulation of gene expression in response to fluation of population density (Miller and Bassler 2001). *A. baumannii* has quorum-sensing AbaR receptor protein and N-(-3-hydroxydodeanonly)-1-homoserine lactone acts as virulence factors and regulate surface motility (Subhadra et al 2016)

C. Exopolymeric substances Formation

The exopolymeric substance (EPS) include polysaccharides, proteins, glycoproteins, glycolipids, extra cellular DNA, metal ions, divalent cations and other surface active components (Yadav et al 2012). EPS provide

protection against environmental stress i.e. antibiotics, disinfectants and irradiation (Kokare et al 2009). It facilitates adherence to biotic or abiotic surfaces, microcolony formation and three dimensional surfaces to mature biofilm (Van and Michiels 2010).

D. Microcolony formation

When the attachment of microorganism to the biotic or abiotic surfaces become stable, microbial cells divide and start producing biofilm matrix components as a result of chemical signals to form microcolonies (Armbrustera and Parsek 2018).

E. Colonization and maturation

In this stage microbial cells communicate with one another to regulate their activities and physiological processes through releasing, sensing and responding to small diffusible signal molecules called auto inducers (Saleh 2014). Auto inducers facilitate quorum sensing that allows microbial cells to regulate expression in cell density dependent manner (Jamal et al 2018). As a result of gene expression EPS gives three dimensional structures to the biofilm along with water-filled channels are formed for transport of nutrients within the biofilm (Jamal et al 2018; Toyofuku et al 2016).

F. Detachment

Some bacterial cell transfer from mature biofilm to planktonic growth and attach again to a new surface. Dispersion occurs by two ways namely active dispersal and passive dispersal (Jamal et al 2018). Active dispersal depends on cell motility or degradation of EPS which is triggered by changes in environmental conditions such as temperature change, starvation, oxygen deficiency, and metabolite accumulation. Whereas, passive dispersal depends on physical factors such as shearing force under liquid flow conditions (Toyofuku et al 2016). During infection, biofilm detachment is most important step as it may lead to the dissemination of biofilm-associated infection (Joo and Otto 2012).

2.10.2 Biofilms and antimicrobial resistance

Most important advantages of biofilm formation in bacterial population is antimicrobial resistance. The antibiotic concentration required to eradicate bacterial population within biofilm is thousand times higher than required to eradicate planktonic bacteria of same species (Fatima et al 2015). EPS of biofilm prevent antimicrobial agent by acting as an anionic exchanger. Sometimes, time required for antimicrobial agent to penetrate biofilm is longer than duration of treatment or antibiotic lifetime which increases risk of treatment failure in many cases (Kokare et al 2009). The growth rate and metabolic activity of bacteria within biofilms is affected by availability of nutrients and oxygen. There is limited amount of nutrient and oxygen in deep layers of biofilm. So the bacteria inside biofilms have slow metabolic growth rate and division rate which make bacteria resistance to antimicrobial drugs targeting in dividing cells. (Singh et al 2017). Horizontal gene transfer through mobile genetic element such as plasmid, transposons, or bacteriophages can occur between cells forming biofilm by their close relationship thus, spreading resistance markers. Elevated expression of efflux pump in biofilm than planktonic cell also confers high level of resistance to antimicrobial agent (Kinikar et al 2014). In addition, mutation frequency in biofilm is significantly higher than planktonic counterparts, enabling traditional resistance to antimicrobial agents.

2.11 Epidemiology of infections caused by Acinetobacter spp.

In 1970s first infections caused by *Acinetobacter* spp. was reported and was considered as pathogenic commensal opportunist of minimal clinical significance (Glew et al 1997). Since then, these organisms have emerged as important nosocomial pathogens. It has been estimated that 45,000 US and 1 million global cases of Acinetobacter infections occur per year (Wong et al 2017).

*Acinetobacter baumannii*is classified by the Infectious Diseases Society of America as one of the six most important multidrug resistant (MDR) microorganisms in hospitals worldwide (Talbot et

al2006).Today, *Acinetobacter* infections have spread rapidly through hospitals across the globe. The highest density of infections occurs in intensive care units (ICUs). U.S. National Healthcare Safety Network (NHSN) 2009-2010 surveillance data found that *Acinetobacter* spp. caused 1.8% of all health care-associated infections (Sievert et al 2013). Based on surveillance studies from hospital networks, the frequency is similar in ICUs across Europe and Latin America (Villar et al 2014). However, in China, Thailand, Taiwan, Vietnam, and some countries in South America, *Acinetobacter* causes a much higher proportion of nosocomial infections and may be the predominant nosocomial pathogen. It is also becoming a predominant nosocomial pathogen in India (Golia et al 2013). In Asian and certain Latin American countries, *Acinetobacter* is one of the three most common causes of bacteremia and nosocomial pneumonia (Luna et al 2014). There are an estimated 45,000 (range, 41,400 to 83,000) cases of *Acinetobacter* infections per year in the United States and 1 million (range 600,000 to 1,400,000) cases globally per year (Spellberg and Rex 2014).

Carbapenemase have been increasingly reported in *Acinetobacter* species over the past 10 years worldwide. Carbapenem resistance in *Acinetobacter baumannii* is now an emerging issue worldwide. Surveillance studies indicate that the percentage of carbapenem-resistant isolates gradually increased over the last ten years in Europe, North America and Latin America (Peleg et al 2008). A study conducted in Greece between 1996 and 2007 showed that carbapenem resistance rate increased from 0 to 85.1% (Souli et al 2008) whereas in UK resistance rate increased from 0 to 55% between 1998 and 2006 (Wareham et al 2008). In another study conducted in ICUs patient of a Turkish hospital, *A. baumannii* isolates showed resistance rate of 80.3% and 71.2% for imipenem and meropenem respectively (Dizbay et al 2008).

Similarly, *Acinetobacter* species comprise 8.4% of ventilator-associated pneumonia and 2.2% of central-line-associated bloodstream infections in the USA. Carbapenem resistance accounts for 65% of *A. baumannii* pneumonia in USA and Europe (Farrell et al 2014) and clonal complex 92 was the most frequently identified worldwide (Zarrilli et al 2013). Clonal complex 92,

corresponding to global clone 2, were most prevalent, and OXA-23 oxacillinase was responsible for the majority of carbapenem resistance in USA and Europe (Kim et al 2013). Likewise, a recent study showed that >60% of *A. baumannii* isolates causing hospital acquired pneumonia in Asian countries were PDR and carbapenem resistant. (Kim et al 2013). Carbapenem resistance rates are high among Gram-negative bacteria in the hospitals of Southeast Asia. Carbapenem resistance rates in *A. baumannii* have generally exceeded by 40% throughout all of India and OXA enzymes are the predominant Carbapenemase in *A. baumannii* in India (Kazi et al 2015).

Similarly, many studies have found that strong survival activity of *A. baumannii* in strict environments and highly resistant to various antibiotics is due to biofilm formation (Gaddy and Actis 2009).The type of association that exists between biofilm formation and antibiotic resistance phenotypes remains controversial. According to some studies, the type and the reservoir of resistance determinants harbored by *Acinetobacter* spp. can influence its capacity to form biofilm. Biofilm formation is more strongly associated with MDR. *A. baumannii* strains than with the susceptible strains (Longo et al 2014). A study performed on clinical isolates from patients with nosocomial infections in three hospitals in Tehran showed that at least 92% of the biofilm-forming isolates were MDR (Babapour et al 2016). A significant correlation between multidrug resistance and biofilm formation of clinical and environmental isolates was established i.e., clinical isolates had a higher ability to form strong biofilm than environmental isolates (Bardbari et al 2017). It was also found that 25% of the 72 clinical isolates of *A. baumannii* isolated from India were resistant to ampicillin-sulbactam, 36.1% to imipenem, 66.6% to ceftazidime, 72.2% to ciprofloxacin, 80.5% to amikacin, and 84.7% to piperacillin while 62.5% (45/72) of isolates produced biofilm (Badave and Kulkarni 2015).The majority of the clinical *Acinetobacter* spp. isolates from Bangladesh were reported as MDR and biofilm producers, especially those isolated from ICU samples (Nahar et al 2013). More than 86% of the isolates from a tertiary care hospital in Mexico were resistant to ciprofloxacin, ceftazidime, and cefotaxime, and 50.7% and 35.5% of the isolates were resistant to imipenem and meropenem, respectively. Of these

isolates, 28.3% and 25.7% were positive for the *blaOXA-58* and *blaOXA-72* genes, respectively (Bocanegra-Ibarias 2015).Also, in a study conducted in India, all isolates were also able to form biofilms (Vijayakumar et al 2016). The frequency of genes involved in biofilm formation was largely similar to other studies (Azizi et al 2016; Bardbariet al 2017).

2.12Antibiotic resistant *Acinetobacter* spp. in Nepal

Acinetobacter infection is increasing rapidly in Nepal. A study conducted at BPKIHS, over a period of one year (January 2002 - December 2002) showed 13.4% *Acinetobacter* isolates from different clinical specimens. Among these, 42.5% were from pus/aspirates, 32.2% from endotracheal tube (ETT), 13.0% from blood, 3.4% from CSF, and 8.9% from other miscellaneous specimens (Ghimire et al 2005). The Prevalence of *Acinetobacter* spp. in urine from Man Mohan Memorial Community Hospital was 2.3% (Basnet et al 2009).

In Nepal almost MDR *Acinetobacter* spp. isolates were resistant to ceftazidime (Shrestha et al 2015; Baral, 2008; Basnet et al 2009). *Acinetobacter* spp. was 88.04-96.6%, 40.0- 96.6% and 98.4% resistant to amikacin, gentamicin and ceftazidime respectively (Ghimire et al 2002; Basnet et al 2009). Ciprofloxacin were resistant to 96.7% isolates and susceptible to 76.0% isolates (Basnet et al 2009; Ghimire et al 2002) in the different studies conducted in eastern Nepal. Similarly, from Shrestha et al 2015, of the 246 *Acinetobacter* spp. isolates, 122 (49.6%) were MDR *A. baumannii*, with the majority being resistant to aminoglycosides, carbapenems and fluoroquinolones but not to colistin and tigecycline. These isolates harboured the 16S rRNA methylase gene *armA* as well as *bla*NDM-1, *blaOXA-23*or*blaOXA-58*. Likewise, Joshi et al 2017 reported that of total 44 analyzed 97.7% (*n*=43)isolates were carbapenem-resistant *A. baumannii* (CR-AB) and 97.7% (*n*=43) were multidrug resistant *A. baumannii* (MDR-AB). All isolates were susceptible to colistin. The *blaOXA-23* gene was also detected in all isolates.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The materials, equipments and reagents that were used in various stages of this study are listed in Appendix I.

3.2 Methods

3.2.1 Research design

The present study was hospital based cross-sectional study.

3.2.2 Study site and period

The study period was from Marchto September 2019. The clinical specimensincluding wound swab, pus, blood, urine, sputum, catheter tips,tissue, and body fluid were collected and processed at a tertiary health care center, the phect-Nepal Kirtipur Hospital, Kirtipur, Kathmandu. Further experiments were conducted at Central Department of Microbiology, Tribhuvan University.

3.2.3 Study Population

The study population included the patients of all age groups and both genders visiting phect-Nepal Kirtipur hospital whosespecimens were requested for routine bacterial culture and antibiotic susceptibility testing.The specimens were collected from Post- Anesthesia Care Unit, Plastic Surgery Ward, Intensive Care Unit and New General ward) and from outpatient department. The consent (Appendix –II) was taken from each patient/guardian prior to interviewing them to fill up structured questionare (Appendix – III)

3.2.4 Sample size

A total of 180 samples of indwelling medical devices that were sent to laboratory for routine culture and antibiotic susceptibility testing were processed and analyzed during the study period. The prevalence of *Acinetobacter* spp. was found to be 12.7% in Nepal (Amayta and Acharya 2015).

Therefore,

Prevalence rate (p) = 0.127, allowable error (e) = 0.04 (absolute error)

Sample size (N) = $(Z\alpha)^2 p (1-p)/e^2$, where,

Z= standard normal deviation set at 95% confidence level q= 1-p

Hence,

$$N = (1.6)^2 \times 0.127 \times 0.873 / 0.04^2$$

$$= 177.40 \sim 180$$

3.2.5 Criteria for sample collection

3.2.5.1 Inclusion criteria

Clinical samples (non-duplicate) obtained from different specimens of phect-Nepal Kirtipur hospitals of Kathmandu valley processed at the hospital laboratory were included in this study.

3.2.5.2 Exclusion criteria

Unlabeled, improperly transported, contaminated samples were not included in this study. Additionally duplicated organisms from the same samples were also excluded.

3.2.6 Ethical Approval

Ethical approval for this study was obtained from the Institutional Review Committee (IRC), Phect-Nepal (004-2019). The letter of approval is presented in Appendix IV.

3.2.7 Sample collection and transportation

All the clinical spemens including wound swab,pus, blood, urine, sputum, catheter tips,tissue, and body fluid were collected by experienced medical personnel in a clean, leak proof container according to the specimen type as per the guidelines of the hospital.Thus collected specimens were immediately transferredto the microbiology laboratory for routine culture and sensitivity testing. All the samples were processed immediately without delay (Cheesbrough 2006).

3.2.7.1 Pus and wound swabs

Pus samples were collected on a sterile cotton swab or aspirated in syringe. For the closed wounds and aspirates, 2% cholorohexidine followed by an iodine solution was used for the disinfectantion whereas for the open wounds, it was debrided then rinsed thoroughly with sterile saline prior to collection of pus samples. Pus samples contains deepest portion of the lesion or exudates. Swab collection should be avoided as long as aspirates or biopsy sample can be obtained. If the swab is the only the option, then sterile cotton wool swab was gently rolled over the surface of the wound approximately five times, focusing on area where there is evidence of pus or inflamed tissue and labelled with date, time, and the patient's information. Two samples were taken from each patient, one for culture and for direct Gram stain.

3.2.7.2 Urine samples:

Patients were asked to collect 10-20 mLof clean voided (clean- catch) first morning mid-stream urine in a sterile, dry, wide- necked, leak- proof plastic container (ready- made and sealed). Patients were advised not to touch the inside part of the container by hands or any other body surfaces. The container

was labeled properly and immediately delivered to the laboratory with the requisition form with patient's clinical history as soon as possible for further processing. Catheterized specimens or supra-pubic aspirates were collected with the assistance of a clinician from infants and patients who were unable to produce clean-catch mid-stream urine specimens because of urologic or neurologic problems including impaired consciousness. The specimens were processed without delay within two hours.

3.2.7.3 Sputum and other respiratory specimens

The Sputum sample was collected in a wide-mouthed leak- proof, disposable plastic container under the supervision of health care worker. The patient were advised not to rinse or garglingthe mouth with non-sterile water or mouthwash prior to sample collection and also instructed to collect specimen resulting from deep cough but not the saliva or post-nasal discharge. Early morning sample before tooth-brush were suggested to collect after drinking hot water for the patient difficult for deep-cough. The container were labeled properly and immediately delivered to the laboratory as soon as possible for further processing. Other respiratory specimens such as bronchio-alveolar lavage (BAL), endo-tracheal and intra-tracheal aspirations were sent in the laboratory after collection by clinicians.

3.2.7.4 Cerebrospinal Fluid (CSF)

This is a medical procedure that is performed by a trained physician guided by appropriate precautions. Two to three samples were collected either by lumbar puncture or ventricular shunt into sterile leak- proof, vials or tubes after proper labelling and dispatched to the laboratory immediately(Doherty and Forbes 2014).

3.2.7.5 Blood collection

The blood samples were collected aseptically and diluted with Brain Heart Infusion (BHI) broth in 1:10 ratio (1-part blood and 9- parts broth). In neonates and children one mLblood was mixed with nine mLof broth whereas in adults two mLblood was mixed with eighteen mLbroth. Then the broth

bottle was labeled properly and incubated at 37 °C for up to 96 hours aerobically.

3.2.7.6 Body fluid Specimens (Pleural, Peritoneal and Synovial fluids)

These specimens were obtained with the help of trained physicians. About 3-5 mL of the sample were drawn and transported to the laboratory after proper labeling.

3.2.7.7 Miscellaneous specimens

Other specimens such as bile, catheter tips, endo-tracheal aspiration, bronchoalveolar lavage, high vaginal swabs etc. were collected and sent to the laboratory in a sterile container or tubes after proper labeling and without delay.

3.2.8 Processing of the specimens

3.2.8.1 Culture of specimens

Urine, catheter tips, Foley's tips, pus, wound swab, were inoculated into Brain Heart Infusion Broth, Blood Agar, and MacConkey agar plates where assputum, CSF, pleural fluids, synovial fluid, endo-tracheal tubes, intra-tracheal tubes were inoculated into Blood agar plate, and MacConkey agar plate. Blood samples after aseptic collection were poured into BHI broth in 1:10 ratio immediately and mixed well. The BHI broth was incubated at 37°C for up to 96 hours sub-culturing at every 24 hours. The Blood agar plate and Chocolate agar plates were incubated in 5- 10% CO_2 for 24 hours (Cheesbrough 2006). The composition and preparation of all microbiological media used in this study are given in Appendix – V.

3.2.9 Identification of *A. baumannii*

Identification of *Acinetobacter* spp. was performed by following standard diagnostic procedures. After overnight incubation, typical non-fermenting colonies of *Acinetobacter* spp. were isolated. These colonies were then subjected to further processing via Gram staining (appendix – VI) and other

biochemical tests (Appendix – VII). *A. baumannii* was identified on the basis of various characteristics such as positive catalase test, negative oxidase test, non-motile, indole negative, citrate positive, urease negative, Alk/alk H_2S^- G^- in TSIA medium, oxidative in Hugh and Leifson`s medium, negative gelatin hydrolysis test, acid production from glucose, lactose, xylose, galactose, mannose, but not from sucrose and mannitol(variable in case of maltose) and its ability to grow at 37°C and 44°C (Constantiniu et al 2004).

3.2.10 Antimicrobial susceptibility testing

Antibiotic susceptibility testing of all confirmed isolates was performed by using the Kirby-Bauer disk diffusion method (Appendix – VIII)recommended by Clinical Laboratory Standard Institute (CLSI) of guidelines (CLSI 2019) using commercial antibiotics discs of HI Media Company. Briefly, organisms were grown overnight at 37°C in 5 ml nutrient broth. The cultures were adjusted to match a McFarland 0.5 standard and spread on Mueller-Hinton agar plates using sterile swabs. The plates were dried at room temperature for 30 minutes before placing the antibiotic discs at equidistance. The plates were incubated at 37°C for 24 hours, and the zone of inhibition was measured in terms of diameter (mm). Organisms were classified as sensitive or resistant by comparing the diameter of inhibition zone with zone interpretative chart. Control organisms were used to assess the quality of Antibiotic discs. Antibiotic discs and control used are mentioned in Appendix III.

3.3 Method for the detection of Biofilm production

All the 180 isolates strains of *A.baumannii* were subjected for biofilm detection by tube method and microtiter plate method.

3.3.1 Tube method

This was qualitative assay for biofilm formation was performed according to the method described by the Christensen et al (1982). Borosilicate glass tubes containing 10 mL of tryptone soy broth (TSB) with 1% glucose (Hi media, Mumbai) were inoculated with a loopful of a pure culture of ACB complex grown overnight from nutrient agar plate, Tubes containing only

tryptone soy broth (TSB) with 1% glucose were included in the test as negative controls whereas tube containing A. baumannii (ATCC 1906) as positive control. After 24 hours of incubation at 37°C, the content of each tube was decanted and then washed with phosphate buffer saline (PH 7.3) for 2-3 times. The washed tubes were then stained with 0.1% crystal violet for 15 minutes. Then the tubes were again washed with de-ionized water to remove the excess stain and dried at 37°C for half to 1 hour. A positive result was indicated by the presence of an adherent film of stained material on the inner surface of the tube. Tubes were examined, and the amount of biofilm formation were scored as 0-absent, 1-weak, 2-moderate or 3-strong. Experiments were performed in triplicate and repeated three times.

3.3.2 Microtiter Plate method

This quantitative test was performed as described by Christensen et al (1982). A loopful of test organism isolated from fresh agar plates were inoculated in 1 mL of tryptone soya broth (TSB) with 1% glucose. Broths were incubated at 37°C for 24 hours which were then diluted 1:100 with fresh TSB. Individual well of 96 well microtiter plate were filled with 200µLof dilutes cultures broths. TSB with 1% glucose was used as the negative control on one lane of the microtiter plate and *A. baumannii* ATCC 19606 as a positive control in another lane of the microtiter plate. The plate was then incubated at 37°C for 24 hours. After the incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 Ml PBS (PH 7.3) four times to remove the free floating bacteria. Biofilm formed by the bacteria adherent to the wells were fixed by 2% sodium acetate and then stained by the 100µLof 0.1% crystal violent for 15 minute at room temperature. Excess stain was removed by the washing the plate with de-ionized water and biofilm was quantified by measuring the absorbance at 630nm following solubilization of attached biofilm in 95% ethanol (Sanchez et al 2013). The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic et al (2000).

3.3.2.1 Interpretation of biofilm production

OD value	Biofilm Production
OD ≤ ODc < OD ≤ 2 * ODc	Non/Weak
2 * ODc < OD ≤ 4 * ODc	Moderate
4 * ODc < OD	Strong

The cut – off optical density (ODc) was setas three standard deviationsbe above the mean OD of the negative control.

Table 1: Interpretation of biofilm production

3.4Carbapenemase production testing

Modified Hodge Test (MHT) is a simple phenotypic test for detection of carbapenemase enzyme in bacteria. It is based on the inactivation of a carbapenem by carbapenemase-producing strains that enable a carbapenem-susceptible indicator strain *(E. coli* ATCC 25922) to extend growth towards a carbapenem-containing disc along the streak of inoculum of the test strain. Positive result gives cloverleaf-like indentation (Wong et al 2015).

For the test, 0.5 McFarland dilution of the *E. coli* ATCC 25922 in 5 mLbroth or saline was prepared. Then it was diluted 1:10 by adding 0.5 mLof the 0.5 McFarland to 4.5 mLof MHB or saline. A lawn of diluent was streaked on MHA and allowed to dry 3-5 minutes. 10µg meropenem or ertapenem susceptibility disc was placed in the center of the test area. In a straight line, *A. baumannii* was streaked from the edge of the disc to the edge of the plate at 3 different places. Plate was incubated overnight at 35°C in ambient air for 16-24 hours. After 16-24 hours of incubation the plate for a clover leaf-type indentation at the intersection of the *A. baumannii* and the *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disc were examined.

3.5 Molecular examination

3.5.1 Crude plasmid DNA extraction

Carbapenemase resistant *Acinetobacter baumannii* was preserved on tryptic soya broth for DNA extraction process. DNA was extracted by Alkaline

hydrolysis method using molecular grades reagents (Appendix – IX). For plasmid extraction*A. baumannii* strains cultured in Luria Bertani broth at 37°Covernight. 1.5 mLof bacterial culture was placed in a 2.5 mLEppendrof tube. Cell was harvested by centrifugation at 11000 rpm for 5 minutes at room temperature. Immediately supernatant was decanted and residual broth was blotted away. Second 1.5 mLof culture was transferred to microfuge tube and centrifuged as above. Bacterial pellet was resuspended in 100 µLof solution I and it was shaken then placed in the water bath for 5 minutes. 200 µLof solution II was added and mixed well by inverting gently 4 to 5 times. It was also placed on water bath for 3-5 minutes. Immediately 150 µLof Solution III was added and well by inverting gently. The tube was again kept in ice-bath for 5 minutes without shaking. Suspension was centrifuged at 13000 rpm for 10 minutes and 100-200 µlL micropipette supernatant was transferred to a new Eppendrof tube. Equal volume of phenol: chloroform was added and shaken vigorously to form an emulsion. Further the tube was centrifuged at 13000 rpm for 2-3 minutes. Following the centrifugation upper aqueous phase was collected avoiding lower organic phase into new microfuge tube. Two volume of 95% ethanol was added and mixed well. Immediately tube was placed on the water bath for 15 minutes. The preparation was centrifuged at 13000 rpm for 5 minutes. Then the supernatant was poured off. The pellet was washed by adding 100 µLcold 70% ethanol and the inverting the tube well onto the paper towel. The tube was allowed to sit at room temperature with cap open for 5-10 minutes to permit evaporate away. 40 µLof 1X TE buffer was added to the tube and it was shaken gently to dissolve the pellet. Finally, the plasmid preparation was stored at-4°Cor -20°C. (Wilson 2001)

3.5.2 Amplification of carbapenem resistance gene (*blaOXA-23*, *blaOXA-24* and *blaOXA-51*) by PCR.

The reaction mixture for the PCR was prepared as follows:

a) For the blank: 11 µLPCR water, 13 µLMaster mix, 0.5 µLForward primer and 0.5 µLReverse primer.

b) For sample: 8 μLPCR water, 13 μLMaster mix, 0.5 μLForward primer, 0.5 μLReverse primer, 3 μLPlasmid DNA.

All these mixtures were added on individual amplification tube making up the total volume to 25 μL. The template for PCR amplification in clinical isolates was a plasmid DNA preparation. Amplification reaction was carried out under the following thermal cycling conditions:

3.5.2.1 Specific primers used in this study for the amplification of target gene

Primers	Target	Amplicon size	Sequence	Reference
OXA-23 F	*bla OXA-23*	501 bp	5'-GATCGGATTGGAGAACCAGA-3'	Woodford et al (2006)
OXA-23 R			5'-ATTTCTGACCGCATTTCCAT-3'	
OXA-24 F	*bla OXA-24*	246 bp	5'-GGTTAGTTGGCCCCCTTAAA-3'	Woodford et al (2006)
OXA-24 R			5'-AGTTGAGCGAAAAGGGGATT-3'	
OXA-51 F	*bla OXA-51*	353 bp	5-TAATGCTTTGATCGGCCTTG-3'	Woodford et al (2006)
OXA-51 R			5-TGGATTGCACTTCATCTTGG-3'	

The details of all primers are given in Appendix – X.

Table 2: Specific primers for the amplification of target gene

35.2.2 Amplication reaction under thermal cycling conditions:

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final extension
blaOXA-23	94°C for 5 minutes	94°C for 30 second	52°C for 40 second	72°C for 50 second	72°C for 10 minutes

blaOXA-24	94°C for 5 minutes	94°C for 30 second	52°C for 40 second	72°C for 50 second	72°C for 10 minutes
blaOXA-51	94°C for 5 minutes	94°C for 30 second	57°C for 40 second	72°C for 50 second	72°C for 10 minutes

The detailed PCR protocols are described in Appendix – XI.

Table 3: Amplication reaction under thermal cycling condition

3.5.3 Detection of PCR products by electrophoresis

The amplified products were characterized by performing gel electrophoresis with 1.5% agarose gel made in w/v tris-acetate-EDTA. 0.5 µLof Ethidium bromide was used in the gel as tracking dye. The gel was allowed to solidify on the plastic cast with comb. After proper solidification, 1 µLof 100bp DNA ladder was loaded, and 3 µLnegative control or blank was loaded on separate well. The sample 3 µLof PCR amplicons were respectively loaded on the wells.

3.6 Quality Control

For the proper result interpretation quality control plays important role. It is must for the error free result without any biasness. This leads to obtain of reliable microbiological results. Thus, during the study quality control and check were applied in various areas to obtain specific and sensitive results.

3.6.1 Monitoring and regular evaluation of laboratory equipment, reagents and media

Laboratory equipment like incubator, refrigerator, and autoclave and hot air oven were regularly monitored for their efficacy. The temperature of the incubator and the refrigerator was monitored for their efficiency. The temperature of the incubator and the refrigerator was monitored for their expiry date and proper storage conditions. After preparation they were properly labelled with preparation date and self-life. The quality of media prepared was checked by subjecting one plate of each batch for sterility and performance test.

3.6.2 Purity plate

Purity plate was used to ensure that the inoculum used for the biochemical tests was pure culture and also to see whether the biochemical tests were performed in an aseptic condition or not. Hence, while performing biochemical tests, the same inoculum was sub cultured in respective media and incubated. Pure growth of the organisms both in pre and post inoculation portion of the medium was considered as the indication of aseptic condition.

3.6.3 Quality control during antimicrobial susceptibility testing

Muller Hinton agar and the antibiotic discs were checked for their lot, number, manufacture and expiry date, and proper storage. For the standardization of Kirby-Bauer test and for the performance testing of antibiotics and MHA, control strains of *E. coli* (ATCC25922), *S. aureus* (ATCC25923), and *A. baumannii* (ATCC19606) were tested primarily. Sterility of the test plates were determined by incubating plates for 48 hours at 37˚C only those plates which showed no growth were utilized.

3.7 Data analysis

All the data obtained were analyzed using statistical programming Statistical Package of Social Sciences (SPSS version 21.0). Chi-square test was used to determine the association of independent variables. Chi-square is very useful test which can be applied to find the significant in the same type of data. It is most commonly used when data are in frequencies such as in the number of response in two or more categories. A value of $\alpha \leq 0.04$was assumed wherever applicable and 95% confidence intervals along with the exact p-values were considered.

3.8 Validity and reliability

Validity of the test was maintained by checking the expiry dates of the regents, media used and performing pre-testing of the tools. Furthermore, quality assessment of each laboratory equipment and procedure at the regular basis were performed. Reliability of the study depended on the different

literature reviews, expert's opinion and guidance from the respective supervisors.

3.9 Limitation of the study

This study is primarily limited by short time for survey and its evaluation**.** This study is confined to diagnose only clinical specimen but not hospital environment and community isolates. Due to time limitation 16s rRNA sequencing was not done in this study. The study was only done in vitro not in vivo. The molecular mechanism of biofilm production is beyond the scope of this study.

CHAPTER IV

RESULTS

A total of 180 clinical specimens from in-patients (Post- Anesthesia Care Unit, Plastic Surgery Ward, Intensive Care Unitand New General ward) and from outpatient department were received and processed in the microbiological laboratory of phect-Nepal Kirtipur Hospital during the study period. Among 180 clinical samples 92 (51.11 %) sample showed growth while 88(48.89%) showedno growth.

4.1 Distribution of *A.baumannii* in cuture positive samples

From 92(51.11%) culture positive samples, 19(20.65%) isolates (figure 1) were identified as *A.baumannii* on the basis of various biochemical test and growth temperatures.

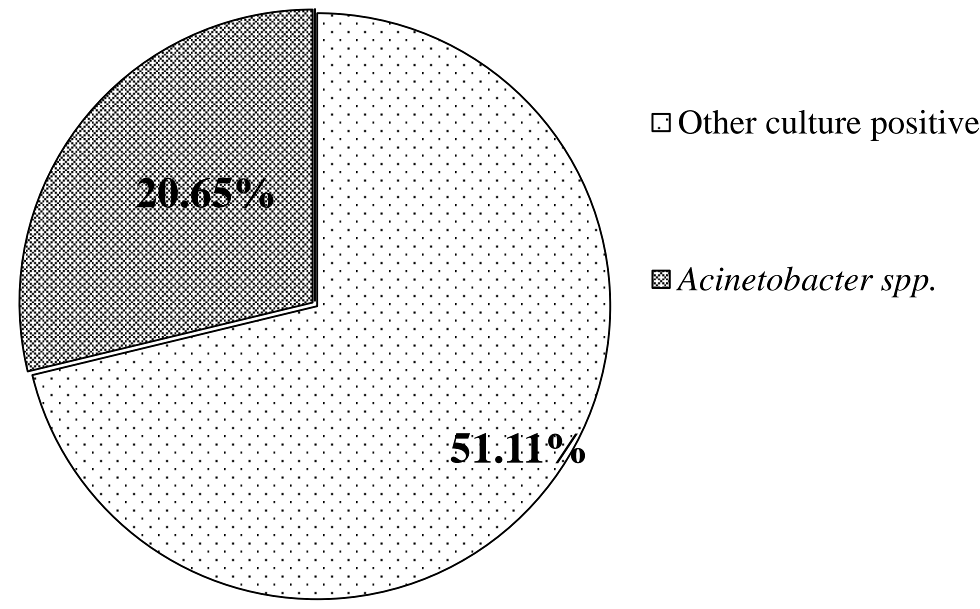


Figure 1: Distribution of *A.baumannii* in culture positive samples

4.2 Prevalence of *A. baumannii* among different clinical specimens

Out of 19 isolates of *A. baumannii,* majority of *A. baumannii* was found in wound swab 6 (31.58%) followed by tissue 4 (21.05%), sputum 3(15.79%) as shown in Figure 2.

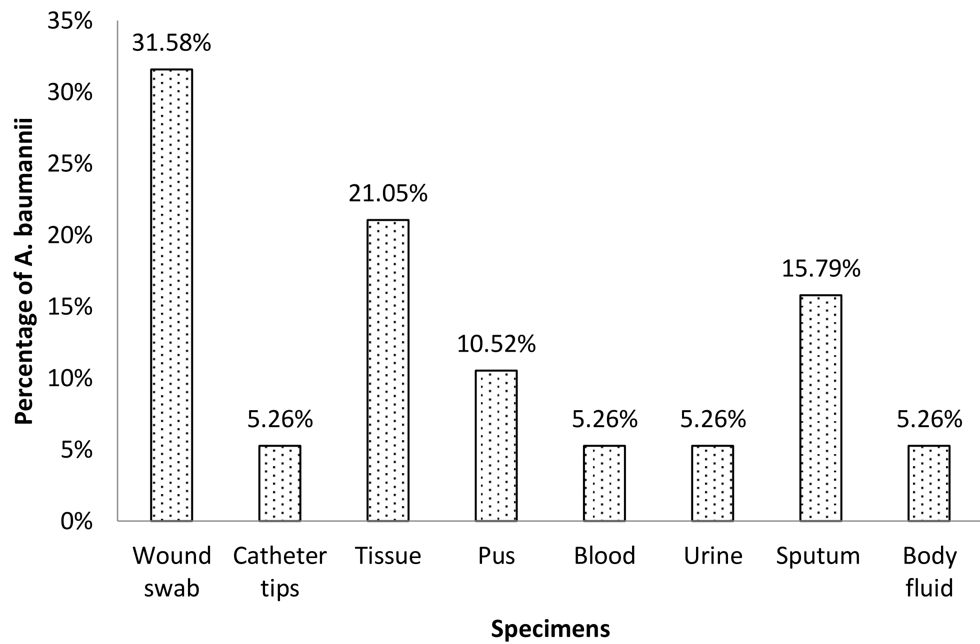


Figure 2: Distribution of *A. baumannii* among different clinical specimens

4.3Distribution of the *A. baumannii* isolates among different wards

As shown in table 4, out of 19 isolates of *A. baumannii*, majority of isolates were obtained from post-anesthesia care unit 5 (20%), and least was found in outpatient department 3 (4.19%).

Table 4: Distribution of theA.baumannii isolates among different wards

Ward	Total	Numbers of *A. bumannii*	Percentage (%)
Post-Anesthesia Care Unit (PACU)	25	5	20%
Emergency	29	4	13.80%
Plastic Surgery Ward(PSW)	38	5	13.16%
Intensive Care Unit (ICU)	8	1	12.5%
New General Ward(NGW)	10	1	10%
Out Patient Department (OPD)	70	3	4.19%
Total	**180**	**19**	**10.55%**

4.4 Distribution of *A. baumannii* among different age groups

Out of 19 isolates of *A.baumannii*, highest prevalence was found in female 11(57.90%) than male 8(42.10%). Highest prevalence was seen in age group of 21-30yrs as shown in table 5.

Table 5: Distribution of *A. baumannii* among different age groups of male and female patients

Age group (years)	Gender		Total
	Male	Female	
1-10	2	1	3(15.80%)
11-20	1	1	2 (10.52%)
21-30	3	2	5 (26.31%)
31-40	1	2	3(15.8%)
41-50	0	4	4 (21.05%)
51-60	1	1	2(10.52%)
Total	**8 (42.10%)**	**11 (57.90%)**	**19(100%)**

4.5 Antibiotic susceptibility pattern of *A. baumannii*

Out of Fourteen antibiotics tested, all 19 *A. baumannii* was found susceptible to colistin and tetracycline and more resistance was seen against cefotaxime (100%), ceftazidime (100%), Cefepime (73.68%), imipenem (89.48%), meropenem (89.48%), gentamicin (89.48%), cotrimoxazole (89.48%) amikacin (84.21%), ciprofloxacin (83.6%) as shown in table 6.

Table 6: Antibiotic susceptibility pattern of *A. baumannii*

Antibiotics	Sensitive (%)	Resistance (%)
Amoxicillin/calvulanic acid	1(5.26%)	18(94.74%)
Cefotaxime	0	19(100%)
Ceftazidime	0	19 (100%)
Cefepime	5(26%)	14(73.68%)
Cotrimoxazole	2 (10.52%)	17(89.48%)
Ciprofloxacin	3(15.4%)	16(83.6%)
Amikacin	3(15.79%)	16(84.21%)
Gentamicin	3(15.79%)	16 (84.21%)
Imipenem	2 (10.52%)	17 (89.48%)
Meropenem	2 (10.52%)	17 (89.48%)
Piperacillin-tazobactam	6(31.58%)	13 (68.42%)
Tetracycline	19 (100%)	0
Polymyxin B	14 (73.69%)	5(26.31%)
Colistin	19 (100%)	0
Doxycycline	2 (10.52%)	17(89.48%)

4.6 Prevalence of MDR *A. baumannii* among total isolates

Out of 19 isolates of *A. baumannii,* 84.21% were found to be MDR and 15.79% were found to be non-MDR (Figure 3).

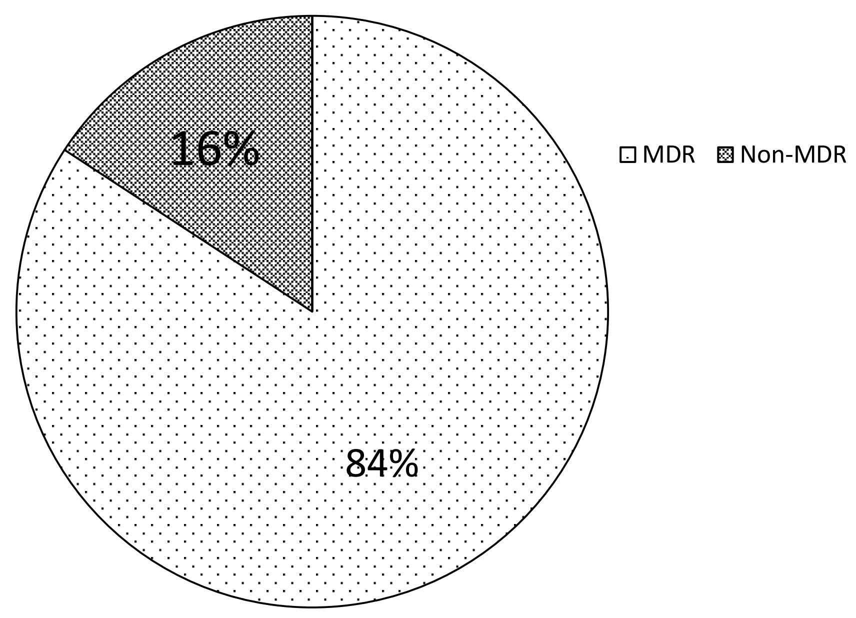


Figure 3: Prevalence of MDR *A. baumannii* among total isolates

4.7 Comparison of two method for biofilm detection in *A. baumannii*

19 isolates were tested for biofilm production by two methods i.e. tube culture method and microtiter plate method. Out of 19 isolates of *A. baumannii* from microtiter plate method, 9 (47.37%) shows strong biofilm producers, while tube method shows 11(57.90%) strong biofilm producers as shown in table7.

Table 7: Biofilm detection by microtiter plate method and tube method

Method	Biofilm producers (Number)			Non-producers	Total
	Strong	Moderate	Weak		
Microtiter plate method	9	5	3	2	**19**
Tube method	11	6	2	0	**19**

4.8 Association between biofilm formation and MDR *Acinetobacter* isolates

Out of 16(84.21%) MDR strains, 9(47.37%) were strong biofilm producers. There was significant relationship between biofilm producers and MDR strains (p <0.04) as shown in table 8.

Table 8: Association between biofilm formation and MDR *Acinetobacter* isolates

		Multidrug resistance		Total	P-value*
		MDR	Non-MDR		
Biofilm producer	**Strong**	9(47.37%)	0	**9 (47.37%)**	0.005
	Moderate	4(21.05%)	1 (5.26%)	**5 (26.31%)**	
	Weak	3 (15.79%)	0	**3 (15.79%)**	
	Non-producer	0	2 (10.53%)	**2(10.53%)**	
Total		**16(84.21%)**	**3(15.79%)**	**19(100%)**	

*chi square test

4.9 Association between biofilm formation and carbapenem producer *Acinetobacter* isolates

Out of 12(63.15%) carbapenem producer, 8(42.10%) were strong biofilm producers. There was significant relationship between biofilm producers and carbapenem (p <0.04) as shown in table 9.

Table 9: Association between biofilm formation and carbapenem producer *Acinetobacter* isolates

		Carbapenem producer		Total	P-value
		Producer	Non-producer		
Biofilm producer	Strong	8(42.10%)	0	**9 (47.37%)**	0.036
	Moderate	4(21.05%)	1 (5.26%)	**5 (26.31%)**	
	Weak	0	3(15.79%)	**3 (15.79%)**	
Non-producer		0	2 (10.53%)	**2(10.53%)**	
Total		**12(63.15%)**	**7(36.83%)**	**19(100%)**	

4.10 Distribution of biofilm producers among different clinical specimens

Among different sample, biofilm producers were found to be highest in wound swab 6(31.58%). There is no association between site of infection and biofilm producer (P>0.04) as shown in table 10.

Table 10: Sample wise distribution of biofilm producers

Sample Type	Biofilm producer		Total	P-value (Chi square test)
	Positive	Negative		
Wound swab	6	0	6	0.75
Catheter tips	1	0	1	
Tissue	3	1	4	
Pus	2	0	2	
Blood	1	0	1	
Urine	1	0	1	
Sputum	2	1	3	
Body fluid	1	0	1	
Total	**17**	**2**	**19**	

4.11 Evaluation of carbapenemase production

Among 19 isolates *A. baumannii* isolates, 17 were screened positive for Carbapenemase production; of those Modified Hodge test confirmed the 12 carbapenemase producing *A. baumannii* (table 11).

Table 11: Evaluation of Carbapenemase production

Phenotypic tests	Total	Carbapenemase producers	
		Number	Percent
AST by Kirby-Bauer method	19	17	89.47%
MHT	19	12	63.15%

4.12 Concurrence of OXA genes in *A. baumannii* isolates

As shown in figure 4, of total 19 *A. baumannii,* all isolates habour *blaOXA-51*gene however, among them only 63.2% isolates harbor *blaOXA-23* gene but none of the isolate carry *blaOXA-24*. 7 carbapenem sensitive phenotypes were also contained. *blaOXA-51* and *blaOXA-23* gene were co-occur in 63.2% isolates. However, all 3 genes didn't co-occur.

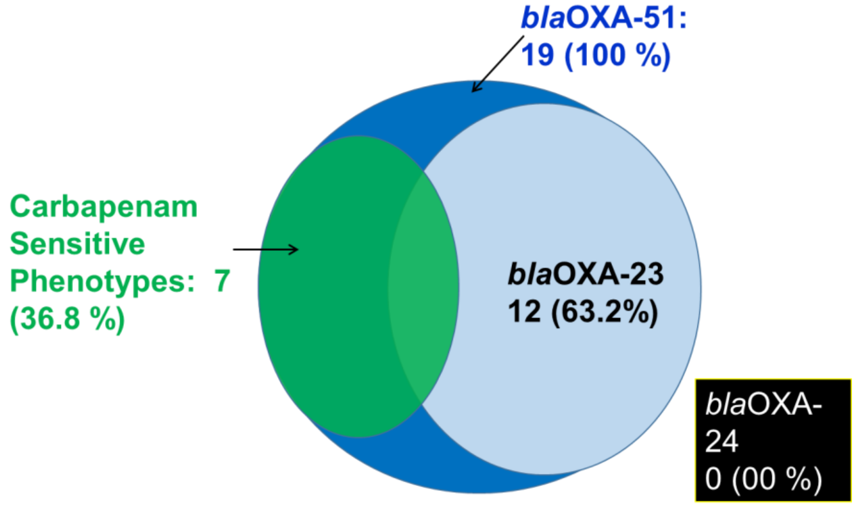


Figure 4: Venn diagram showing concurrence of OXA genes in *A. baumannii* isolates

4.9 Comparison of Modified Hodge test with *blaOXA-23*

Out of 12 MHT positive *A. baumannii,* all isolates harbored *blaOXA-23* gene. Significant association between occurrence of *blaOXA-23* gene and Modified Hodge Test ($p<0.04$) as shown in table 12.

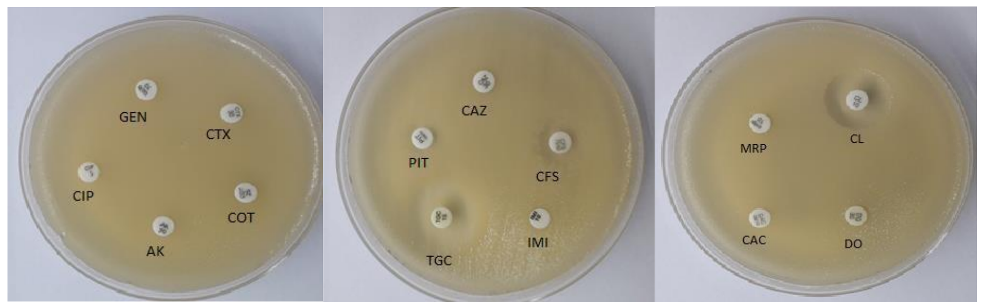
Table 12: Comparison of Modified Hodge Test with *blaOXA-23*

Modified Hodge Test	*blaOXA- 23* gene		Total	P- value
	Positive	Negative		
Positive	12	0	12	0.000
Negative	0	7	7	
Total	**12**	**7**	**19**	

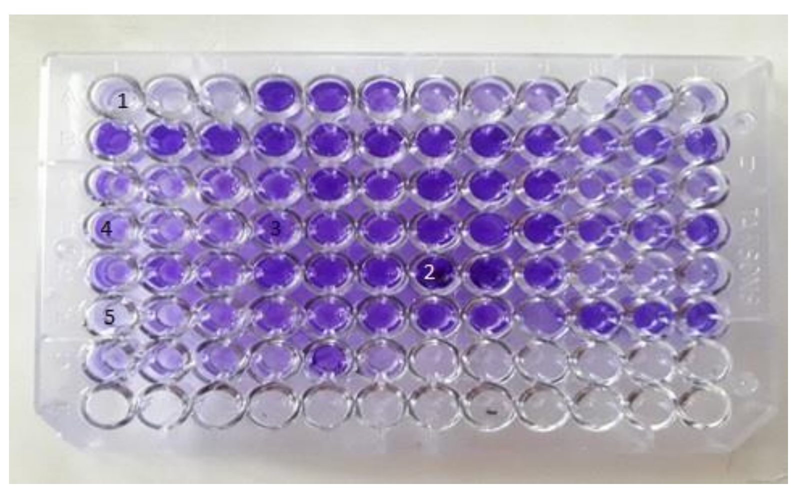
Photograph 1: *A. baumannii* on

Macconkey agar (isolate number 1657)

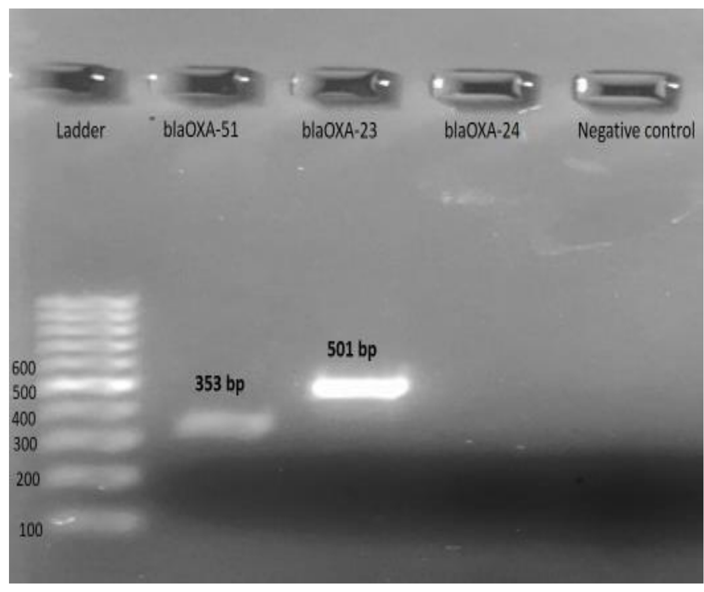
Photograph 2: Biochemical test of *A. baumannii* complex (834)
From left to right: (Triple Sugar Iron agar- ALK/ALK, H2S -ve, Gas -ve, Citrate- +ve, Urease - -ve, Methyl red (MR) test- -ve, Voges-Proskauer (VP) test- -ve, Indole test- -ve)



Photograph 3: Antibiotic susceptibility test of *A. baumannii*(2586). Sensitive to CL- Colistin, TGC-Tigcycline and resistance to AK-Amikacin, CAC- Cefotaxime, CAZceftazidime, CFS- Cefepime, PIT- piperacillin/Tazobactam, CIP- Ciprofloxacin, GEN- Gentamicin, IMI- Imipenem, MEM-Meropenem, COT-Cotrimoxazole , PB- Polymyxin B, DO- Doxcycline



Photograph 4: Microtiter plate method for detection of biofilm production. (1= negative control, 2= strong producer, 3= moderate producer, 4= weak producer, 5
= non producer)



Photograph 5: Gel Electrophoresis of PCR amplicons of blaOXA-51, blaOXA-
23, and blaOXA-24 gene. (From Left to right)

CHAPTER V

DISCUSSION

The key objective of this study was to associate biofilm formation and antibiotic resistance with emphasis on carbapenam reistsance and cooccurance of three OXA genes in clinical *A. baumannii* isolates. For this, a total of 180 non-duplicatevarious specimens from in-patients and out-patients were processed;out of which 92 specimens were bacterial culture positive with 19 isolates of*A. baumannii* asidentified bystandard microbiological procedures. Thus, the prevalence of *A. baumannii* inthis study was found to be 20.65% among the growth positive clinical specimens.Similar prevalence was also reported by Joshi et al (2017)study from Nepal.

Out of 19 isolates maximum number of *A. baumannii* were isolated from wound swab (31.58%) followed by tissue (21.5%), sputum(15.79%), pus (10.52%), catheter tips (5.26%), urine (5.26%) and body fluid (5.26%). In the study by Chaudhary et al (2017) isolated *A. baumannii* from pus (31.53%), tracheal aspirate (28.46%), sputum (17.69%), wound swab (7.69%), urine (7.69%), blood (5.38%), ET tips (0.76%), bronchial wash (0.76%). In agreement to the present findings,Joshi et al (2017) also reported*A. baumannii* from sputum (59.1%), tracheal aspirates (20.4%), catheter tip, (9.1%), pus (9.1%) and urine (2.3%).

In this study, maximum number of isolates from post-anesthesia care unit (20%), followed by emergency (13.80%), new general ward (10%), outpatient department (4.19%). Hou and Yang (2015) isolated the pathogen from ICU, pneumology, emergency and cerebral surgery, accounting for 33.67%, 17.86%, 16.33% and 32.14%, respectively. According to Joshi et al (2017), maximum numbers of the isolates were from ICU (61.7%). Another report from Mishra et al (2013) also reported 85% of the isolates of *A. baumannii* from in-patients who were immunocompromised. The risk factors associated with *A. baumannii* are longer stay in ICU, malignancy, multiorgan system failure, mechanical ventilation, prior antibiotic used and invasive procedures such as tracheostomy, intravenous and urinary catheter (Bachmeyer et al

2005). *A. baumannii* has become important hospital pathogen especially in burn and ICU wards and is the second most common nosocomial pathogen leading to 10-43% mortality rates in patients admitted to ICU wards (AmirMoezi et al 2016).

Likewise, prevalence of *A. baumannii* was found to be highest (26.31%) in age group between 21-30 years which is similarby Bhandari (2012), Thapa (2014) and Ghimire (2014) studies. According to Vaze et al (2013) patients working age (18-60 years) have a significantly higher chance of *A. baumannii* infection.

From past decades, A. *baumannii* are highly resistant to antibiotics, and therefore therapeutic options are becoming increasingly limited (Turton et al 2006). In this study, all 19 isolates of *A. baumannii* showed that the organism was 90 % resistant to amoxicillin, amikacin, cephotaxime, ciprofloxacin, cotrimoxazole and piperacillin-tazobactam. Resistant rate of 80% was observed for meropenem and imipenem. Similarly, for chloramphenicol 85% was seen. Similar resistant pattern was observed for imipenem and meropenem in the study by Sohail et al (2016); Guven et al (2014); Safari et al (2007); Shrestha et al (2013). A study from Turkey in 2014 by Gundeslionglu et al (2014) showed 91.3% carbapenem resistant among *Acinetobacter* isolates.

Acinetobacter is notorious for its multidrug resistance (MDR). The rapid emergence and spread of MDR strains may be due to the combined effect of up regulation of its innate resistance mechanism coupled with gene acquisition following lateral gene transfer and clonal spread of MDR clones (Howard et al 2012; Towner 2006). Treatment of infections caused by MDR *A. baumannii* is complicated in Asian countries such as Turkey, India and Iran (Shirmohammadlou et al 2018). In this study, 84.21% of *A. baumannii* isolates were multidrug resistant which is similar with the result of Khanal et al (2013) and Mishra et al (2013), where the prevalence of MDR was 85.4% and 95%. The frequency of MDR *A. baumannii* isolates ranged from 32.7 to 93% was also found in Iran (Vahdani et al 2011).The high frequency of

antibiotic resistance in this survey is the most probably due to the extensive misuse of antimicrobial agents in Nepal.

Carbapenem were the main pillar of antimicrobial therapy. Nowadays, the drug of choice as a carbapenems has increasingly being ineffective due to higher resistance rate in *A. baumannii* (Asif et al 2018). The resistance of *A. baumannii* to carbapenem is mediated by one of the resistance mechanism including enzymatic inactivation, active efflux of drugs and modification of target sites (Mussi et al 2005). A study in UK for carbapenem resistant in *Acinetobacter* of two teaching hospitals from 2010 to 2012 was found to be 47 to 77 % (Freeman et al 2015). Similarly, froma study in USA reported for the carbapenem resistant was 48% (Zilberberg et al 2016). Recklessly, from review study the frequency of carbapenem resistant in *Acinetobacter* in Singapore, India and Pakistan were 50%, 85% and 62-100% respectively. Correspondingly, from Chile, Korea and Portugal resistance rate was found to be 70%, 92%, and 100% respectively (Tal-Jasper et al 2016).

Acinetobacter baumannii showed 100% sensitive to colistin. This study is in favor of study carried out by Sarada et al (2014) in which 99.2% of sensitive have been reported for the colistin. After all, different rates of colistin resistance have been reported by different literatures as 2.9%, 1 % (Guven et al 2014; Jaggy et al 2012). Recently, there are limited options for treatment of carbapenem-resistant *A. baumannii* infections and colistin and tigecycline are considered the last choice to infection control (Shirmohammadlou et al 2018).

Among Meropenem resistant *A. baumannii* isolates, 12 (63.16%) showed Carbapenemase production MHT. Similarly, in the study carried out by Hafa et al (2019) showed that 69.6% were positive for the Carbapenemase production MHT. Carbapenemase production is determined by the MHT which is originally has been recommended by the Center for Disease Control and Prevention (CDC), and it has revealed appropriate efficiency for Carbapenemase detection (Collins et al 2009). This study showed that, MHT could be used in clinical laboratories for monitoring emergence carbapenemase in *A. baumannii,* especially MDR isolates.

Molecular assays such as PCR which are relatively fast and cheap can check the presence of carbapenems-resistant genes from each DNA samples. The PCR reaction included 19 isolates, which processed to detect the set of *bla*OXA genes. Production of class D carbapenemases consists of OXA-type β-lactamases which can hydrolyze carbapenem antibiotics and considered as the main factors that caused a multi-drug resistance of *A. baumannii* (Al-Samaree and Al-Khafaji 2016). *Bla*OXA-51 genes are factors of carbapenem-resistance in *Acinetobacter* which are inherently existed in all *A. baumannii* strains (Bonnin et al 2012). The *bla*OXA-51-like gene was found in all 19 (100%) clinical isolates of *A. baumannii*, and according to these findings; all the previous isolates were identified as *A. baumannii.* The current results demonstrated high specificity of this primer for *A. baumannii*; therefore, it was used for the identification of *A. baumannii* at the species level by PCR (Shali and AK 2012).Since *bla*OXA-51 gene is present in all strains of *A. baumannii*, this gene can be used as a positive control (Brown et al 2005). Therefore, all isolates in this study were confirmed as *A.baumannii* by genotypic (*blaOXA-51*) detection, in line with thefindings of worldwide studies (Koh et al 2007; Warner et al 2016).

The presence of *blaOXA-51*-like gene is not related to the level of carbapenem resistance in *A. baumannii* isolates, because it controlled by the insertion sequence *ISAba1*upstream of this gene which provides a promoter for the expression of *blaOXA-51*-like gene (Musafer 2007). The high rates of *blaOXA-51*-like gene are back to the fact that this gene is a ubiquitous, an intrinsic, and chromosomally located gene in *A. baumannii*. Furthermore, the G+C content of *blaOXA-51* is compatible with the genomic content of *A. baumannii*, that’s why it's completely intrinsic to this bacterium (Mugnier et al 2010).

Upon the current results, the production of *blaOXA-23*-like gene is the dominant carbapenems resistance gene in the local isolates of *A. baumannii* isolates. The wide spread of *blaOXA-23*-like gene may be attributed to the ability of these bacteria to acquire resistance against many antibiotics which contributes in the distribution of this gene in hospitals all over the world. In

comparison with the different studies, the present results were in agreement with the results of Hussein (2013) and Ghaima (2016) who established very close results. Hussein (2013) found that the *blaOXA-23*-like gene was the dominant among carbapenem resistance genes that existed in *A. baumannii* isolates collected from Medical City hospitals in Baghdad and achieved 71 (91.03%) out of 78 Imipenem resistant *A. baumannii* (IRAB) isolates while Ghaima (2016) determined 60 (71.4%) of 84 multidrug resistant *A. baumannii* (MDRAB) isolates (Al-Warid and Al-Thahab 2014). Relative findings of *blaOXA-23* prevalence were recorded in other parts of the Middle East such as Bahrain, United Arab Emirates, and Saudi Arabia (Alsultan 2015). The prevalence of OXA-23-like producing *A. baumannii* isolates was reported in many countries, such as Korea, Singapore, Thailand (Azimi et al 2015), Bulgaria, China, Brazil and Afghanistan (Niumsup et al 2009). Additionally, high percentage of *blaOXA-23*-like gene (83%) was found in burn patients in Tehran hospitals (Özcan et al2015). Moreover, the *blaOXA 23*-like was a common gene in MDR *A. baumannii* isolates in southeast of Turkey and the presence of this gene was found in 48 (60%) isolates (Jal'oot et al 2016). Similarly, high prevalence of *blaOXA-23* carrying *A. baumannii* strains has been reported from Nepalese patients (Shrestha et al 2015). In this study, 12 out of 19 isolates were resistant to carbapenem, 100% (12/12) of which carried *blaOXA-23* gene. Likewise, the study carried out by Joshi et al (2017) hasreported highest distribution showing 100% *blaOXA-23* genes in carbapenem resistant isolates. However, Khorsi et al (2015) and Chang et al (2005) have reported 67.02% and 80.60%, respectively of *blaOXA-23* carbapenemase resistant gene amongimipenem resistant isolates.

Furthermore, in this study, *blaOXA-24* were not detected in any isolates, indicated that carbapenemases resistance could possibly not due to these genes. This result is similar with study conducted in Brazil (Carvalho et al 2009) and the United States of America (Warner et al 2016). Likewise Nirwati et al (2018) also did not detect *bla*OXA-24 gene in Indonesia. Previous study from Nepal including Joshi et al (2017) and Shrestha et al (2015) didn't detect this gene too. However, *blaOXA-24*gene was reported in some studies in Taiwan (Kuo et al 2010), Iran (Azizi et al 2015), Poland (Nowak et al 2012)

and France (Jeannot et al 2014). Altogether, these results suggest a specific distribution of *blaOXA* gene variants in different regions.

In this study, it was found that *blaOXA-23* gene was detected in most of meropenem resistant *A. baumannii*, but not detected in any meropenem sensitive isolates. These results support the data that, in addition to the antimicrobial-inactivating enzyme production, there are other mechanisms of antimicrobial resistance in *A.baumannii*. Such mechanisms include the reduced access to the bacterial targets (due to the decreased outer membrane permeability as a result of the loss or reduced expression of porins and overexpression of multidrug efflux pumps) and mutations that change the cellular targets (alterations in penicillin-binding proteins) (Manchanda et al 2010).

To the bestknowledge thisstudy revealed the highest co-existence of *blaOXA-51/23* in *A. baumannii* isolates from Nepal and also demonstrated the increase of co-existence of *blaOXA-51/23* over the time in Nepal. The results of thus studyare consistent with the previous studies that report high carbapenem resistance in *A. baumannii* strains from Nepal with OXA-type carbapenemases predominancy. It seems that the increasing combination of these genes and element may lead to an increase in resistance rate against carbapenems among *A. baumannii* isolates.

During antibiotic treatment, biofilm production is effective strategy for the survival (Rao et al 2008). Biofilm producing-microorganisms can develop antimicrobial resistance by preventing the penetration of the antimicrobial agents through the biofilm matrix. Other mechanisms include altered growth rate and physiological conditions of biofilm organisms (Donlan and Costerton 2002). Biofilm formation is suspected of being one of the key pathogenic features of *A. baumannii*, particularly with device-related infections. In the present study, the qualitative tube method and quantitative microtiter plate method showed 11 isolates (57.90%) and 9 isolates (47.37%) as strong biofilm producers. Corroborating with these results a study conducted in India is also reported 77% biofilm production by qualitative method and 69% by quantitative method (Abdi-Ali 2014). Although qualitative method is simple

and easy method, reading of the results may be complicated. So that observers have different interpretation. In this study, 89.47% are biofilm producer. Similar results of 100%, 77% 100% and 98.5% of biofilm production have been reported by Chaskar et al (2014), Kim et al (2015), M'hamedi et al (2014) and Mostafa (2015) showed higher prevalence of biofilm formation.

In the present study, out of totalbiofilm producers were from wound swab, followed by, pus, catheter tips, blood, urine body fluid whereas non-biofilm producers were from tissue and sputum. A study by Cevahir et al (2008) showed that maximum isolates from wound swab followed by blood, sputum, pleural fluid, urine cerebrospinal fluid. Likewise, the study by Babapour et al (2016) showed that biofilm were produced from sputum followed by urine.

Out of 16 isolates in the standard microtiter plate method with moderate to strong biofilm formation, 14 (87.5%) were MDR. This bacterium, especially MDR and biofilm producing strains can cause serious infectious diseases in hospitalized patients. There was statistically significant relationship between biofilm formation and antibiotic resistance. The result is in accordance with other study (Babapour et al 2016). Most of the *Acinetobacter* species have ability to produce biofilm. This could potentially increase colonization of antibiotic resistant bacteria in hospital environment. This phenomenon was also observed in *S. aureus* where biofilm production was mostly found in MSSA isolates as compared to MRSA isolates (Nuryastuti et al 2015).In contrast, Gurung et al (2013) reported that the antibiotic resistance was significantly higher among biofilm producer *A. baumannii* than that of nonproducer. The result can be used as a caution to increase awareness of biofilm producing *A. baumannii.*

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The prevalence of *A. baumannii* among the patients attending phect-Nepal Kritipur Hospital was 12.5%. The results of the study provide a higher antibiotic resistance among clinical isolates of *A.baumannii* including multidrug resistance and resistance towards carbapenems which represents a serious therapeutic challenge in hospitalized patients. *blaOXA-51* gene was detected in all *A. baumannii* isolates, while *blaOXA-24* was not detected in any of the isolates. Most of *A. baumannii* carried *blaOXA-23* gene. SinceOXA genes can be efficiently transmitted by plasmid, it isimportant to prevent the spread of *blaOXA-23* gene to other bacteria to suppress the level of carbapenem resistance.Furthermore, biofilm formation was significantly associated with drug resistance among *A. baumannii* and making it difficult to establish a consensus on the relationships that exist between biofilm formation and environmental triggers, antibiotic resistance, virulence mechanisms as well as the contributions of the source of the bacterial isolates. Though future studies are anticipated to understand the influence of bacterial source, environmental factors, antibiotic resistance, and virulence determinants on biofilm formation, this study provides clues that resistance of *A. baumannii* to meropenem might be related to the biofilm production.

6.2 Recommendations

1. Assuming that misuse or overuse of antibiotics would be one of reasons for high frequency of antibiotic resistant *Acinetobacter baumannii* observed in this study, prescription of antibiotics based on antibiogram obtained from microbiological analysis is recommended.

2. This study reported the carbapenemase producing *A. baumannii* were sensitive towards colistin and tigecycline, hence these are recommended as potential drugs to treat the infections caused by carbapenem resistance *Acinetobacter* species.

3. As biofilm production by *Acinetobacter* was associated with antibiotic resistance in this study, the screening tests for biofilm production in the laboratory may be useful to predict the drug resistance among the isolates.

4. In general, biofilm contributes to antibiotic resistance as a physical barrier. However, it is not clear whether any underlying mechanisms correlate biofilm production and antibiotic resistance. Therefore future studies are recommended to understand coexpression of biofilm and resistance related genes.

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APPENDIX I

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A. EQUIPMENTS:

Autoclave (Sakura, Japan), Microtiter plate reader (Tarsons, India),

Incubator (Ambala Cantt., India), Petriplates,

Microscope (Olympus, China),Weighing machine (Phoenix, USA),

Hot air oven (Tarsons, India),Centifuge (Tarsons, India),

Micropipette (Tarsons, India), Microtiter plate (Tarsons, India),

Thermocycler (GeneiTM), Gel Documentation (Uvietch Cambridge)

Electrophoresis tank (mupid j, Cosmobio Co).

B. MICROBIOLOGICALMEDIA:

All the microbiological media were from Hi-media Laboratories Pvt. Limited, Bombay, India:

Nutrient Agar (NA),Blood Agar (BA),

MacConkey Agar (MA), Muller Hinton Agar (MHA),

Simmons Citrate Media,Hugh and Leifson`s Media,

Urease Media, Gelatin agar,

Sulfide Indole Motility Media (SIM),

Trypticase Soy Broth (TSB) with 1% Glucose,

Sugar media (glucose, sucrose, etc.).

C. CHEMICALS AND REAGENTS:

I. For Microbiological test:

Crystal violet, Gram's Iodine,

95% alcohol, Safranin,

Kovac's reagent, 3% Hydrogen peroxide,

Barritt' reagent (α-napthanol and KOH).

. II. For PCR and Gel Electrophoresis:

10X Buffer,Distilled water,

dNTPs,DNA polymerase,

Primers, Agarose,

Loading dye, Ethidium bromide,

DNA ladder,TAE.

D. ANTIBIOTIC DISC:

All the antibiotics discs used for susceptibility were from Hi-media Laboratories Pvt. Limited, Bombay, India:

Piperacillin (100µg), Pipercillin-tazobactum (100/10 µg),

Ampicillin(10 µg), Ampicillin-sulbactam (10/10 µg),

Ceftazidime (30 µg), Ceftriaxone (µg),

Cefepime (30 µg), Meropenem (10 µg),

Imipenem (10 µg), Gentamicin (30 µg),

Amikacin (10 µg), Tetracycline (30 µg),

Ciprofloxacin (5 µg), Polymyxin B (300 units),

Colistin (20 µg).

E. Miscellaneous

Glass slides, Test tubes,

Conical flasks, Cotton,

Droppers, Forceps,

Immersion oil, Inoculating loop,

Inoculating wire, Lysol,

Measuring cylinder, Petri dishes,

Pipettes, Plastic containers,

Spatula, PCR tubes,

Microfuse tubes(1.5 mL)

APPENDIX –II

Consent form

Name of the study: BIOFILM FORMATION AND DRUG RESISTANCE PATTERN OF ACINETOBACTER BAUMANNII ISOLATED FROM CLINICAL SPECIMENS

Name of organization: ………………………………………………………

I was requested to participate in the research study named above. I have been provided information and had the opportunity to ask questions about the study, and I found the answers satisfactory. I hereby give the consent to participate in this study by providing my sample.

I understand that I can quit my participation at any time.

Name of participant: ……………………………

Signature: ……………………………

Date: ……………………………..

Note: For illiterate, participant must give their stamp of right hand thumb in the box provided after understanding the contents written above.

I hereby verify that the investigator has explained this form in clear way and provided the opportunity to question about the study. The participant has given consent freely.

Name of witness: ………………….

Signature: ………………….

Date: …………………….

I have clearly explained the study to the participant. I hereby verify that the participant was not forced to participate in this study and this consent was given freely.

Investigator Name: …………………….

Signature: …………………

Date: …………………

APPENDIX –III

Questionnaire

Clinical and Microbiological profile of patient

Clinical profile

Name: …………..   Lab no: ……….

Age/sex: ………..   Date: …………

Bed no: …………

Address: ………..

Brief Clinical History

Date of admission: ………….

Date of Operation: ………….

Patient on antibiotics   yes   no

If Yes, Antibiotic(S) taken 1)………   2) …………

Duration of treatment

Microbiology profile

Day 1 (……/……/……)

Specimen: …………..

Time of sample collection: ……….

Mode of collection: …………..

Receiving time at the laboratory: ………..Inoculation on 1………2 ………...

Incubation temperature…………..   Incubation time ……….

Day 2 (……/……/……)

Observation of culture plates: No Growth / Growth

Number of Colonies:

Colony Characteristics:

Media used feature	Shape	Size	Color	Consistency	Opacity

Gram staining results: ……. Oxidase: …………..

Catalase test: ………

Coagulase test: ……….

Provisional identification of the organism

Inoculation on 1 ………. 2 ………..

Day 3 (……. /…… /……)

Biochemical test

SIM ….. TSI ……..

Citrate ….. Urease ……

Others ……

Organism identified as:

1. Biofilm production test

Isotate (ID)	Tube test method	Microtitre plate method
Result		

2. Antibiotic susceptibility testing by Kirby Bauer disc diffusion method

Antibiotics used	Conc of antibiotics	Zones of inhibition (mm)	Interpretation

APPENDIX –IV

ETHICAL APPROVAL

Institutional Review Committee

Date: March 15, 2019

To,
Ms. Upasana Ghimire
Central Department of Microbiology
Tribhuvan University, Kirtipur, Kathmandu

Dear Ms. Ghimire,

I am pleased to inform you that the Institutional Review Committee (IRC) of the Public Health Concern Trust, Nepal (phect-NEPAL) has approved the following research project to conduct at Kirtipur Hospital of phect-NEPAL.

Project title: Biofilm Formation and Drug Resistance Pattern of *Acinetobacter baumannii* from clinical sample

IRC No.: 004 - 2019

Responsible supervisor:
Mr. Sanjit Shrestha, Chief, Pathology Department, Kirtipur Hospital, phect-NEPAL

Other researcher/supervisor:
Dr. Dev Raj Joshi, Central Department of Microbiology, Tribhuvan University, Kirtipur
Dr. Basant Maharjan, Director, CHDP, phect-NEPAL

Student researcher:
Ms. Upasana Ghimire, M. Sc. Microbiology, Central Department of Microbiology, Tribhuvan University, Kirtipur

Period (dates) of approval: March 15, 2019 - September 14, 2019

Projects may be renewed for up to a total of five years by submitting a request for an extension 45 days prior to the above date of expiration. A new application for research is needed if the research is to continue for more than five years.

The following conditions apply to your approval. Failure to abide by these conditions may result in suspension/termination of your project and/or disciplinary action.

Limit of approval: Approval is strictly limited to the research as described in the submitted ethics application.

Amendments to project: Any changes to the research project must have prior approval from the IRC. Upon submission of the amendment form, if the changes are found to be significant, a new full application for approval of the revised project may be required.

Unanticipated or adverse events: The IRC must receive a written report of any unanticipated and/or adverse events encountered during the course of the research within 14 days of the

v1.3

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event occurring. After such an event, the research must be halted until the report is reviewed by the IRC and written approval to resume is granted. Failure to do so may lead to the suspension or termination of the project.

Monitoring and auditing: All projects may be subject to monitoring and/or auditing at any time by the IRC or a designated third party.

Progress report(s): Researchers must submit to the IRC a progress report on completion of data collection and also an annual report if the project was approved for more than one year. Failure to submit a progress/annual report may lead to the suspension or termination of the project.

Final report: Researchers must submit a final report to the IRC upon completion of the research project.

If you have any queries, please contact the IRC.

On behalf of the Institutional Review Committee, I wish you all the best.

Ganesh Dangal

....

Prof. Dr. Ganesh Dangal
Chairperson
Institutional Review Committee (IRC)
Public Health Concern Trust (phect-NEPAL)
Email: irc@phectnepal.org

(Project title: Biofilm Formation and Drug Resistance Pattern of *Acinetobacter baumannii* from clinical sample)

Student researcher: Ms. Upasana Ghimire, M. Sc. Microbiology, Central Department of Microbiology, Kirtipur

APPENDIX –V

COMPOSITION AND PREPARATION OF DIFFERENTAND BIOCHEMICAL MEDIA, STAINING AND TEST REAGENTS

A. COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

1. MacConkey Agar

Ingredients	gm/litre
Peptic digest of animal tissue	1.50
Pancreatic digest of gelatin	17.00
Casein enzymatic hydrolysate	5.00
Lactose	10.00
Bile salts	1.50
Sodium chloride	5.00
Crystal violet	0.001
Neutral red	0.03
Agar	15.00

Final Ph (at 25°C) 7.1 +/-0.2

51.53 grams of medium was suspended in 1000 ml distilled water and heated till boiling to dissolve the medium completely. It was sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes and after cooling to 40-50°C poured into sterile petriplates.

2. Blood agar

Ingredients	gm/ltr
Beef heart infusion	500.00
Sodium chloride	5.00
Tryptose	10.00
Agar	15.00

Final Ph (at 25°C) 7.3+/-0.2

40 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was sterilized by autoclaving at 15

lbs pressure (121°C) for 15 minutes and after cooling to 40- 50°C, 5% sterile defrinated sheep blood was added aseptically, then mixed with gentle rotation and immediately poured in sterile petriplates.

3. Muller Hinton Agar

Ingredients	gm/ltr
Beef infusion form	300.00
Casein acid hydrolysate	17.50
Starch	17.50
Agar	17.00

Final pH (at 25°C) 7.3+/-0.2

38 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and after cooling to 40- 50°C, poured in sterile petriplates.

4. Nutrient agar

Ingredients	gm/ltr
Peptone	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Agar	12.00

Final pH (at 25°C) 7.3+/-0.2

13 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and after cooling to 40- 50°C, poured in sterile petriplates.

5. Nutrient broth

Ingredients	gm/ltr
Peptone	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50

Final pH (at 25°C) 7.3+/-0.2

13 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

6. Trytone Soy broth +20% Glycerol

Ingredients	**gm/ltr**
Pancreatic digest of casein	15.00
Enzymatic digest of soyabean meal	5.00
Sodium chloride	5.00
Glycerol	200 ml

Final pH (at 25°C) 7.3+/-0.2

13 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

A. COMPOSITION OF DIFFERENT BIOCHEMICAL TEST MEDIA

1. Sulphide Indole Motility (SIM) medium

Ingredients	**gm/ltr**
Beef extract	3.00
Peptone	30.00
Peptonized Iron	0.20
Sodium thiosulphate	0.025
Agar	3.00

Final pH (at 25°C) 7.3+/-0.2

36 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

2. MR-VP medium

Ingredients	**gm/ltr**
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00

Final pH (at 25°C) 6.9+/-0.2

17 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3. Simmon's citrate agar

Ingredients	**gm/ltr**
Magnesium sulphate	0.20
Mono ammonium phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Agar	15.00
Bromothymol blue	0.08

Final pH (at 25°C) 6.8+/-0.2

24.2 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was distributed to clean test tubes to a depth of about 3 inch and sterilized by autoclaving at 151 lbs pressure (121°C) for 15 minutes. After autoclaving tubes with medium were tilted form slant.

4. Triple sugar iron (TSI) agar

Ingredients	**gm/ltr**
Peptone	10.00
Tryptone	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Ferrous sulphate	0.20
Sodium chloride	5.00
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.00

Final pH (at 25°C) 6.8+/-0.2

65 grams of medium was suspended in 100 ml distilled water and heated till boiling to dissolve the medium completely. It was distributed to clean test tubes to a depth of about 3 inch and sterilized by autoclaving at 151 lbs pressure (121°C) for 15 minutes. After autoclaving tubes with medium were tilted form slant and butt of about 1 inch.

B. COMPOSITION AND PREPARATION OF DIFFERENT STAINING AND TEST REAGENTS

1. For Gram's staining

a. Crystal violet solution

Requirements	Weight
Crystal violet	20 gm
Ammonium oxalate	9 gm
Ethanol or methanol	95 ml
Distilled water (D/W)	1000 ml

Direction: in a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then 95 ml of ethanol was added and mixed until the dye was completely dissolve. To the mixture, 9 gm of ammonium oxalate dissolved in 100ml of D/W was added. Final volume was made 1 L by adding D/W

b. Lugol's iodine

Requirements	Weight
Potassium iodide	20 gm
Iodine	10 gm
Distilled water (D/W)	100 ml

Direction: in a clean piece of paper, 20 gm of potassium iodide was weighed and dissolved in 250 ml of D/W. Then 10 gm of iodine was added and mixed until completely dissolved. Final volume was made 1 L by adding D/W.

c. Acetone alcohol decolorizer

Requirements	Weight
Acetone	500 ml

Ethanol (Absolute)	475 ml
Distilled water (D/W)	25 ml

Direction: to a 25 ml D/W, 475 ml of absolute was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

d. Safranin

Requirements	Weight
Safranin	10 gm
Distilled water (D/W)	1000 ml

Direction: in a clean piece of paper, 10 gm of safranin was weighed and transferred to clean bottle. Then 10 L of D/W was added and mixed until completely dissolved.

2. Normal saline

Requirements	Weight
Sodium chloride	0.85 gm
Distilled water	100 Ml

Direction: in a clean piece of paper, 0.85 gm of sodium chloride was weighed and transferred to clean bottle. Then 100 ml of D/W was added and mixed well until completely dissolved.

3. Biochemical tests reagents

a. Catalase reagents

Requirements	Weight
Hydrogen peroxide	3 ml
Distilled water (D/W)	97 ml

Direction: to a 97 ml of D/W. 3 ml of H_2O_2 was added and mixed well.

b. Oxidase reagent (impregnated in a Whatman's No.1 filter paper)

Requirements	Weight
Tetramethyl p-phenylene diamine dihyrochloride (TPD)	1 gm
Distilled water (D/W)	100 gm

Direction: to a 100 ml of D/W, 1 gm of TPD was added and mixed well. To a solution, a strip of Whatman's No. 1 filter paper was soaked for about 30 seconds. Then these strips were freeze dried and stored in dark bottle tightly sealed with screw cap.

c. Kovac's reagent

Requirements	**Weight**
Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2 gm
Conc. Hydrochloric acid	10 ml

Direction: 2 gm of p-dimethyl aminobenzaldehyde was dissolved in 30 ml of isomyl alcohol and transferred to a clean brown bottle. Then 10 ml of conc. HCL was added and mixed well.

d. Methy red solution

Requirements	**Weight**
Methyl red	0.05 gm
Ethyl alcohol	28 ml
Distilled water (D/W)	22 ml

Direction: 0.05 gm of methy red was dissolved in 28 ml ethanol and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

e. Barritt's reagents

Solution A (α- napthanol)

Requirements	**Weight**
α- napthanol	5 gm
Ethyl alcohol	25 ml
Distilled water (D/W)	100 ml

Direction: to 25 ml of ethanol, 5 gm of α- napthanol was dissolved and transferred into a clean brown bottle. Then the final volume was made to 100 ml by adding D/W.

Solution B (KOH)

Requirements	**Weight**
Potassium hydroxide	40 gm

Distilled water (D/W)	100 gm

Direction: to 25 ml of D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made to 100 ml by adding D/W.

4. McFarland Tube (0.5)

0.5 ml of 0.048 M $BaCL_2$ (1.17% w/v $BaCL_2.H2O$) was added to 99.5 ml of 0.18 M $H_2.SO_2$ (1% w/v) with constant stirring. The McFarland standard was thoroughly mixed with to ensure that it is evenly suspended. Using matched cuvettes with a 1 cm light path and water as blank standard, the absorbance was measured in a spectrophotometer at a wavelength of 625 nm. The acceptance range for the turbidity standard is 0.08-0.13. The standard was distributed into screw-cap tubes of the same size and volume as those used to prepare the test inoculum. The tubes were sealed tightly to prevent loss by evaporation and stored protected from light at room temperature. The turbidity standard was vigorously agitated on a vortex mixer before use. Standards may be stored for up to 6 months, after which time they should be discarded.

C. COMPOSITION AND METHOD OF PREPARATION OF DIFFERENT REAGENTS FOR GEL ELECTROPHORESIS

1. 50X TAE Buffer

242 grams of Tris base was dissolved in 500 ml distilled water. The mixture was thoroughly mixed and then 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.3) was added. Final volume was adjusted to 1 liter. The solution was stored at room temperature.

2. 2% Agarose gel

0.50 grams of agarose was dissolved in 25 ml 1X TAE buffer. The mixture was thoroughly mixed and then boiled till clear solution was obtained. 0.7 µLEtBr was added to the solution and then left at room temperature to cool for a while. When the temperature reached lukewarm, the solution was poured in an electrophoresis plate with comb and left to solidify.

APPENDIX- VI

PROCUDURS OF GRAM'S STAINING

Procedure:

A. Clean, grease free slide was taken.
B. Smear of suspension on slide with loopful of sample was prepared and air dried.
C. The smear was flooded with crystal violet for 1 minute.
D. It was washed.
E. Smear was flooded with Gram's iodine and it was allowed to stand for 1 minute.
F. It was washed with water.
G. 95% ethyl alcohol was used as decolorizer for 10 seconds. It was added drop by drop until crystal violet fails to wash from smear.
H. The slide was washed with water.
I. It was then counterstained with safranin for 45 seconds.
J. It was washed with water.
K. The slide was blot dried with bibulous paper and examined under oil immersion.

APPENDIX- VII

PROCEDURESOF BIOCHEMICAL TEST USED FOR IDENTIFICATION OF BACTERIA

A. Catalase test:

A small amount of a culture from nutrient agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on a surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood agar) or if an iron wire loop is used.

B. Oxidase test

A piece of filter paper was soaked with few drops of oxidase reagents (Whatman's No.1filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydocholride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue purple color within 10 seconds.

C. Indole production test

Smooth bacterial colony was swabbed on SIM (Sulphide Indole Motility) medium at 37°C for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in the indole.

D. Methly Red test:

A pure colony of the test organism was inoculated into 2 ml of MR-VP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by development of bright red color, indicating acidity and negative by yellow color.

E. Voges-Proskaur test

A pure colony of the test organism was inoculated into 2 ml of MR-VP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeratin and kept for 15 minutes, positive test is indicated by development of pink red color.

F. Citrate utilization test:

A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and indicated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above Ph 7.6; a blue color develops due to alkalinity of the medium.

G. Triple sugar iron (TSI) agar test:

The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

1. Yellow (Acid) / Yellow (Acid), Gas, H_2S → Lactose / Sucrose fermenter, H_2S producer.
2. Red (Alkaline) / Yellow (Acid), No Gas, No H_2S → Glucose, Lactose and Sucrose non-fermenter
3. Red (Alkaline) / No change → Glucose, Lactose and Sucrose non-fermenter
4. Yellow (Acid) / No change → Glucose-Oxidiser
5. No change / No change → Non- fermenter

H. Urease test:

Urease test is performed on urea broth or agar medium containing pH indicator phenol red. During incubation, microorganisms possessing urease enzyme is produced which cleaves urea to NH_3 that raises the pH of medium such that phenol red changes from yellow (pH 6.8) to a red or deep pink (cerise) color. Failure to produce this color is evidence of a lack of urease enzyme in the test organism. In the procedure, the test organism was inoculated into urea broth. The tube was incubated at 37°C for 18-24 hours and the tube was examined for the color change.

APPENDIX- VIII

ANTIBIOTIC SUSCEPTIBILITY TESTING AND MODIFIED HODGE TEST

A. Antibiotic susceptibility testing:

a) Preparation of 0.5 Mc Farland standard:

Add 0.5 ml of 0.048 M $BaCL_2$ (1.17% w/v $BaCL_22H_20$) to 99.5 ml of 0.18 M H_2SO_4 (1% w/v) with constant stirring. Solution is kept in dark by wrapping with aluminum foil.

b) Preparation of inoculum:

By touching 2-3 morphologically similar colonies with sterile loop, inoculate into MHB or NB and incubate at 37°C until turbidity matches with that of 0.5 Mc Farland Standards. Direct colony suspension method can also be used.

c) Inoculating into agar plates:

1. MHA agar plate is used for the purpose, which was brought to room temperature before use. All the moisture should be removed before use.
2. Using sterile swab, a plate of bacterial suspension is inoculated using carpet culture technique. The plate is left for about 5 minutes to let the agar surface dry.
3. Using sterile forceps, appropriate antimicrobial disc (6mm diameter) is placed evenly distributed on the inoculated plate, not more than 5 discs are placed on the 90 mm diameter petri plate.
4. Within 30 minutes of applying the discs, the plate is incubated at 37°C for 16-18 hrs.
5. After overnight incubation, the plates are examined to ensure confluent growth. Using a measuring scale, the diameter of each zone of inhibition in mm is measured and results interpreted accordingly.

B. Modified Hodge test:

0.5Mc Farland dilution of the *E. coli* ATCC 25922 in 5ml of broth dilution or saline was prepared. Then it was diluted 1:10 by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of MHB or saline. A lawn of diluent was streaked on MHA and allowed to dry 3-5 minutes. 10µg meropenem or ertapenem susceptibility disc was placed in the center of the test area. In a straight line, *A. baumannii* was

streaked from the edge of the disk to the edge of the plate at 3 different places. Plates was incubated overnight at 37°C in ambient air for 16-24 hours. After 16-24 hours of incubation the plate for a clover leaf-type indentation at the intersection of the *A. baumannii* and the *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disc were examined.

APPENDIX- IX

COMPOSITION AND PREPARATION OF REAGENTS USED IN PLASMID EXTRACTION

1. TE buffer (10X and 1X, Ph 8):

Requirements

1M Tris-HCL, pH 8.0

0.5M EDTA, pH 8.010mM EDTA

Composition of 10X buffer:

100mM Tris Cl

Composition of 1X buffer

10mM Tris Cl

1mM EDTA

Procedure:

To prepare 100 ml of 10X Tris-EDTA solution, take 88 ml deionized / Milli-Q water in 250 ml beaker / conical flask. Add 10 ml of 1M Tris Cl (pH 8.0) and 2 ml of 0.5 M EDTA (pH 8.0). Mix it.

Check the pH. It should be 8.0. If it is not 8.0 then adjust by keeping HCL and NaOH. Sterilize the solution by autoclaving.

To prepare 1X solution, take 1 volume of 10X solution, add 9 volume of water deionized / Milli-Q. Mix it.

Store at room temperature.

2. Phenol:Chloroform (1:1)

Phenol equilibration

Remove the crystalline phenol from -20°C freezer and thaw it at 60-65°C. Add desired volume of the phenol to an appropriate sized bottle. Add an equal volume of 10X TE buffer to phenol. Shake vigorously. Allow the layer to separate which may take few minutes. Aspirate the top layer perform similar process with second 10X TE buffer. Add an equal volume of 1X buffer to phenol and also repeat with second volume of 1X TE buffer. Leave a small volume of 1X TE buffer to phenol and also repeat with second volume of 1X TE buffer. Leave a small volume of 1X TE buffer above phenol after final aspiration. Check the pH which should be 8.0. If it is still too high perform equilibration step again.

For phenol: chloroform (1:1) preparation mix equal volume of the both equilibrated phenol and chloroform.

3. Solution I (Glucose-Tris-Cl-EDTA buffer pH 8)

Requirements:Composition:

1 M Glucose solution 50 mM Glucose

1M Tris Cl (pH 8.0) 25 mM Tris.Cl (pH 8.0)

Deionized / Milli-Q water10mM EDTA (pH 8.0)

Measuring cylinder, conical flask,

beaker, magnetic stirrer

Procedure:

To prepare 100 ml of suspension buffer, take 80 ml of deionized water in a 100 ml measuring cylinder. Add 5 ml of 1 M Glucose solution, 2.5 ml of 1M Tris Cl (pH 8.0). Adjust the volume to 100 ml with deionized water. Mix the solution and autoclave. Store at 4°C.

4. Solution II (0.2 N NaOH + 2% SDS)

Requirements:

10 N Sodium hydroxide (NaOH) solutions

10% sodium dodecyl sulfate (SDS)

Deionized / Milli-Q-water

Measuring cylinder, conical flask, beaker, magnetic stirrer

Composition:

0.2 N Sodium hydroxide (NaOH)

1 %(w/v) sodium dodecyl sulfate (SDS)

Procedure:

To prepare 100 ml of lysis solution, take 80 ml of deionized water in a 100 ml measuring cylinder. Add 2 ml of 10 N NaOH solution and 10 ml of SDS. Adjust the volume to 100 ml with deionized water. Mix the solution. Prepare solution II freshly and use at room temperature.

5. Solution III (Potassium acetate buffer)

Requirements: **Composition:**

5M Potassium acetate solutions3M Potassium

Glacial acetic acid 5M Acetate

Deionized / Milli-Q-water

Measuring cylinder, conical flask, beaker, magnetic stirrer

Procedure:

To prepare 100 ml of neutralization solution, take 28.5 ml of deionized water in a 100 ml measuring cylinder. Add 60 ml of 5M potassium acetate solutions and 11.5 ml of Glacial acetic acid. Mix the solution. Store the solution at room temperature.

APPENDIX- X

DETAILS ABOUT PRIMER OXA-23, OXA-24 AND OXA-51

All the primers were from Macrogen, Korea:

1. OXA-23

Oligo	OXA-23-like-1					
Sequence	5’-GAT CGG ATT GGA GAA CCA GA -3' (20 mer)					
GC%	MW		Yield		Scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
50.0	6215.0	6258.8	7.2	30.0	0.05	58.4

Oligo	OXA-23-like-2					
Sequence	5’-ATT TCT GAC CGC ATT TCC AT -3' (20 mer)					
GC%	MW		Yield		Scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
40.0	6018.0	6051.0	6.1	30.0	0.05	54.3

2. OXA-24

Oligo	OXA-23-like-1					
Sequence	5’-GGT TAG TTG GCC CCC TTA AA -3' (20 mer)					
GC%	MW		Yield		Scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
50.0	6018.0	6095.1	6.4	30.0	0.05	58.4

Oligo	OXA-23-like-2					
Sequence	5’- AGT TGA GCG AAA AGG GGA -3' (20 mer)					
GC%	MW		Yield		Scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
45.0	6270.0	6219.0	7.4	30.0	0.05	56.4

3. OXA-51

Oligo	OXA-51-like-1					
Sequence	5’-TTA TGC TTT GAT CGG CCT TG -3' (20 mer)					
GC%	MW		Yield		Scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
45.0	6114.0	6140.5	6.3	30.0	0.05	56.4

Oligo	OXA-51-like-2					
Sequence	5’-TGG ATT GCA CTT CAT CTT GG-3' (20 mer)					
GC%	MW		Yield		Scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
45.0	6114.0	6120.9	6.3	30.0	0.05	56.4

APPENDIX- XI

PCR PROTOCOLS

Step I: Choosing target substrates and PCR primers:

The choice of the target DNA is, of course, dictated by the specific experiment. However, one thing that is common to all substrate DNAs is that they must be as clean as possible and uncontaminated with other DNAs. Primer can be prepared by the researchers themselves but that always may not be possible for all researchers so choosing specific PCR primers available in the market is an important issue in any PCR amplification.

Step II: Reaction set up:

Table 15: Basic reaction components for PCR

Components	**25µL reaction mixture**
PCR master mix (1X Reaction buffer, $MgCL_2$, dNTPs and polymerase enzymes)	13µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
Target DNA	3 µL
Nuclease Free Water	8 µL

Note: Prepare the reaction mixture in PCR tube. Gently mix the reaction mixture and collect all the liquid to the bottom of the tubes by a quick spin if necessary. Then transfer the PCR tubes to the thermocycler.

Step III: Thermocycling conditions for a routine PCR:

Table: Thermocycling conditions for a routine PCR

Steps	**Temperature**	**Time**
Initial denaturation	94°C	10 min
Denaturation	94°C	30 sec
Annealing	52°C	40 sec
Extension	72°C	50 sec
Final extension	72°C	5 min

Hold 4°C

Note: The thermocycling conditions may vary depending on number of cycles, primers used and type of PCR.

Step IV: Validating the reaction:

Once the PCR reaction has run, there are two ways of determing success or failure. The first is to simply take some of the final reaction and run it out on an agarose gel with an apporiate molecular weight marker to make sure that the reaction was successful and the second and ultimate validation of a PCR reaction is to directly sequence the amplicons.