CHAPTER-I

1. INTRODUCTION

Acinetobacter spp. usually considered to be opportunistic pathogens is emerging as a greater threat and challenge worldwide due to its high prevalence and ability to acquire antimicrobial drug resistance by all known modes of mechanisms that is variable in different countries, regions, hospitals and even different wards. Therefore, such type of local surveillance studies is found important in deciding the most adequate therapy for *Acinetobacter* infection (Chen et al., 2006).

Acinetobacter spp, usually considered to be opportunistic pathogen, is one of the most important notorious nosocomial health-care related pathogen especially in critically-ill hospitalized patients particularly in intensive care units and occasionally in other units too (Villegas & Hartstein, 2003; Fournier & Richet, 2006).They have been reported to cause nosocomial pneumonia and other respiratory tract infections, septicemia, wound sepsis, endocarditis, meningitis, urinary tract infection and other organ specific infections (Bergogne-Bérézin & Towner, 1996; Gaynes et al., 2005; Dijkshoorn et al., 2007). Community-acquired infections are also reported and they can cause suppurative infections in virtually every organ systems with a high fluid content and common in hot and humid temperature with a significance difference in behavior in various geographic locations (Houang et al., 2001).

In humans, *Acinetobacter* has been isolated from all culturable sites (Seifertet al., 1997) and are the most common Gram negative bacilli carried on the skin of hospital personnel and are, therefore, regular contaminants of the hospital environment (Mandell et al., 2000). Different modes of transmission of *A. baumannii* have been described including transmission via contaminated medical equipments, patient care items or the environment, mostly by cross-transmission via the hands of hospital personnel from colonized or infected patients (Bernards et al., 2004; Dijkshoorn et al., 2007).

Members of the genus *Acinetobacter* are ubiquitous, free living, strictly aerobic, short, often capsulated, non-motile, gram-negative (or gram variable) bacilli or coccobacilli (often diplococcobacilli) with a DNA G+C content of 39 to 47 mol% that grow on simple media and prefer moist environment and can be easily obtained from soil, water, food and sewage (Peleg et al., 2008).

Risk factors for *Acinetobacter baumannii* infection and/or colonization in epidemic settings have included host factors, specific procedures (Von Graevenitz et al., 1995), previous antimicrobial therapy, length of hospital stay and admission to wards with a high density of infected and/or colonized patients (Fournier & Richet, 2006). Invasive procedures involving endotracheal tube, central venous pressure (CVP) catheter, urinary catheter insertions, lumbar puncture, myelography, ventriculography and ventriculoperitoneal shunts are the leading risk factors for the infection, however, these may vary in different set-ups with epidemic outbreaks of infection or endemic colonization (Von Graevenitz et al., 1995).

At present, studies based on DNA/DNA hybridization have resulted in the description of 33 validated "genomic species", 17 of which have been given a valid species name (Peleg et al., 2008); and numbers have been assigned to the other genospecies. The most common important nosocomial *Acinetobacters* belong to the AC-AB (*Acinetobacter calcoaceticus- Acinetobacter baumannii*) complex and among AC-AB complex, *Acinetobacter baumannii* is the most common, dreadful, and successful pathogen in the hospital settings especially in intensive care units (ICUs). Different commercial phenotypic methods developed for species identification of this genus are: Vitek- 2, API- 20NE (BioMerieux, France), Phoenix and Microscan Walkaway systems. API-20NE, system is currently used in hospital laboratories but it requires complementation with other biochemical analysis such as growth at 44^{o}C to identify *Acinetobacter baumannii* (Bernard et al., 1996). Currently, ARDRA and AFLP are the most widely accepted and validated methodologies for identification of *Acinetobacter* to the species level (Peleg et al., 2008).

Susceptibilities to *Acinetobacter* spp. against antimicrobials is considerably different among countries, centers, and even among the wards of a given hospital and therefore, such type of local surveillance studies are found important in deciding the most adequate therapy for *Acinetobacter* infection (Chen et al.,2006). Multidrug-resistant *A.* baumannii particularly carbapenem-resistance has been recognized as an increasing threat in hospitals and as a global challenge (Dijkshoorn et al., 2007; Peleg et al., 2008). Because of the large variety of potential sources, its ability to rapidly acquire antimicrobial resistance and its propensity to persist in the environment, *A. baumannii* is difficult to control in the hospital setting. Numerous nosocomial outbreaks of *A. baumannii*, especially in intensive care units (ICUs), have been reported (Fournier & Richet, 2006).

Due to the presence of multiple resistance genes in large number of plasmids, integrons, and chromosomes and their easy transfer by conjugation, transformation and even transduction from resistant *Acinetobacters* to other *Acinetobacter* has played the significant role for the transfer of drug resistance. They have the ability to acquire the resistant genes from other resistant genera too. More than 80% of *Acinetobacter* isolates carry multiple indigenous resistant plasmids (R-plasmids) of variable sizes which can be readily transferred experimentally to other pathogenic bacteria and vice-versa by transformation and conjugation. Carbapenems were the choice of drugs to treat these bacteria in late 1990s, but carbapenem resistant clones have already been emerged (Go et al., 1994).The last resource antibiotics are rifampin, tigecycline, polymyxin B, and Colistin sulfate but resistance to these drugs has been also demonstrated (Hawley et al., 2007; Ko et al., 2007, Li et al., 2006).

In Kathmandu Medical College Teaching Hospital, *Acinetobacter* spp. is more frequently reported from different clinical specimens and most of these isolates are interestingly found to be susceptible against ceftriaxone, and co-trimoxazole in addition to carbapenems. Therefore, present study was designed to know the prevalence of *Acinetobacter* in various clinical samples, and their antibiotic susceptibility profile in Kathmandu Medical College, Sinamangal, Nepal.

CHAPTER-II

2. OBJECTIVES

2.1. GENERAL OBJECTIVES

To determine the prevalence and antimicrobial susceptibility profile of *Acinetobacter* spp. from various clinical specimens sent for bacterial culture.

2.2. SPECIFIC OBJECTIVES

1. To describe the bacteriological profiles a*nd Acinetobacter* spp. from various clinical specimens.
2. To describe the genderwise, age-wise and ward-wise distribution of isolated *Acinetobacter* spp. in these specimens.

3. To describe antimicrobial susceptibility profile of isolated *Acinetobacter* spp. from these specimens.

CHAPTER III

3. LITERATURE REVIEW

3.1. *Acinetobacter* spp.

Acinetobacter baumannii is emerging as a cause of numerous global outbreaks with increasing rates of resistance to antimicrobial agents. Multidrug resistant (MDR) strains have been isolated worldwide and it has been demonstrated that these strains can spread from areas with high rates of antimicrobial resistance to other areas with historically low rates (Perez et al., 2007). *Acinetobacter* spp. are ubiquitous in nature and have been recovered from soil, water, animals and humans (Droop, 1977), and have also been found in body lice collected from homeless people (La Scola and Raoult, 2004).

Acinetobacter can form part of the bacterial flora of the skin, particularly in moist regions such as the axillae, groin, and toe webs, and it has been suggested that at least 25% of normal individual carry *Acinetobacter* spp on their skin (Taplin et al., 1963; Somerville and Nobel, 1970) and occasionally in the oral cavity and respiratory tract of healthy adults (Rosenthal et al., 1974; Glew et al., 1977) with carriage rate much higher in hospitalized patients than non-hospitalized patient especially during outbreak of infection. Throat swabs (7-18%) and tracheostomy (45%) have been found to be positive (Rosenthal, 1974). Hospital acquired infections may derive more often from cross-contamination or hospital environmental sources rather than from endogenous sources in patients and a steady increase from 25 to 45% in the proportion of *Acinetobacter i*solates from superficial wounds has been recorded over the past decades (Joly-Guillou et al., 1990).

Various sources of hospital environment are responsible for the dissemination of nosocomial infection by *Acinetobacter* spp (Sherertz and Sullivan, 1985). *Acinetobacter* spp. (particularly *A. baumannii*) have been found in hospital sink trap (27%), floor swab culture (20%), air samples (11.5%) and bed rid cupboard (Crombach et al., 1989). The contamination of hospital environment by *Acinetobacter* spp. occurs more favorably in the vicinity of infected or

colonized patient (Cunha et al., 1980). *Acinetobacter* spp. can persist in the environment for many days or weeks, even in dry conditions on particles and dust (Jawad et al., 1996). Strains obtained from dry sources survive well than strains isolated from wet sources where *A. baumannii* survives desiccation better than other *Acinetobacter* spp. (Musa et al., 1990; Jawad et al., 1998; Perez et al., 2007) and is the genomic species more frequently implicated in hospital outbreaks. *A. lwoffii* has been reported to survive up to 7 days on dry surfaces while *A. baumannii* up to 90 days (Hirai, 1991).

During recent years, *Acinetobacter* spp. (Particularly *A baumannii*) has become a worldwide concern as the cause of many serious nosocomial infections and the majority of clinical isolates involved in hospital outbreaks belong to this species (Seifert et al., 1995). There is an increasing incidence of these infections in different intensive care units (ICU's) (Villers et al., 1998) often acquired from cross-infection which can be introduced initially by patients admitted from other hospitals (Bernards et al. 1998). Outbreaks are linked to contaminated respiratory tract equipment (Hartstein et al., 1998), intravenous access devices (Beck-Sague et al., 1990), bedding materials (Weernink et al., 1995), also on medical personnel hands (Patterson et al., 1991) or airborne transmission via aerosols (Simor et al., 2002).

The prevalence of *Acinetobacter* infections ranges from 2% to 10% of all gram negative bacterial infections in Europe (Hanberger et al., 1999) and about 2.5% of them in the United States (Jones et al., 2004). *Acinetobacter* spp. have been implicated in a variety of nosocomial and occasionally community-acquired infections including pneumonia, bloodstream infection (BSI), meningitis, urinary tract infection (UTI), skin and soft tissue infection, wound and burn infection, intravascular devices and implant related infection (Bergogne-Berezin and Towner, 1996; Wisplinghoff et al., 1999). Factors such as advance age, chronic lungs diseases, immunosuppression, surgery, use of antimicrobial therapy, presence of invasive devices, long time ICU stay, (Bergogne-Berezin and Towner, 1996), malignant disease, trauma, and burns (selender et al; 1986), leukaemia (Kelkar et al., 1989) and ambulatory peritoneal dialysis (Galvo et al., 1989; Valdez et al., 1991) seem to be among the most common

predisposing factors. The predisposing risk factors for septicemia are low birth weight, previous antibiotic therapy, mechanical ventilation, presence of neonatal convulsions and perenteral nutrition (Sakata et al, 1998; and Cisneros Rodriguez-Bano, 2002).

The most common *Acinetobacter* spp. causing significant bacteraemia is *A. baumannii*; either as a single pathogen or as part of polymicrobial bacteraemia predominantly in adults (Seifert et al., 1993). Neonates are the second important group of patients with *Acinetobacter* septicemia (Sakata et al; 1989). In adults, surgical wound infections and vascular catheter-bloodstream have been described to lead bacteraemia (Seifert et al; 1993). *Acinetobacter* spp; particularly *A. baumannii*, is an occasional cause of nosocomial UTI, in elderly debilitated patients those in the ICUs and in patients with permanent indwelling urinary catheters (Gaynes and Edwards, 2005). Most patients (80%) tend to be males, reflecting the higher prevalence of indwelling urinary catheters in this population as a result of prostate enlargement (Pedraza et al., 1993). *Acinetobacter* is responsible for just 1.6% of ICU- acquired UTIs in one study (Gaynes and Edwards, 2005). Secondary meningitis is the predominant form of *Acinetobacter* meningitis, although sporadic cases of primary meningitis have been reported particularly in adult males following neurological procedures such as myelopathy, lumbar puncture, ventriculopathy or head trauma (Berk et al., 2008). The predominant species is *A. bumannii* which is almost nosocomian.

A.baumannii caused 2.1% of ICU-acquired skin/soft tissue infections in one assessment (Gaynes and Edwards, 2005). It is a well-known pathogen in burn units and may be difficult to eradicate from such patients (Albrecht et al., 2006). *A. baumannii* is commonly isolated from wounds of combat casualties from Iraq or Afghanistan (Johnson et al., 2007; Petersen et al., 2007). A few cases of native – valve infective endocarditis (Gradon et al., 1992), peritonitis (Valdez et al., 1995), cholangitis and septic complications (Sacks-Berg et al., 1992), endophthalmitis or keratitis (Corrigan et al., 2001; Kau et al., 2002; Levy et al., 2005;) and bloody diarrhoea in a 3 month old infant (Grotiuz et al., 2006) have been reported.

Acinetobacter isolates involved in nosocomial infection frequently belong to glucose-acidifying variety but the majority of glucose-negative, non-hemolytic strains found in clinical specimens are mainly identified as *A. lwofii*, *A. johnsonii*, *Acinetobacter* genospecies 12; and most of the hemolytic isolates are identified as *A. hemolyticus* and *Acinetobacter* geneospecies 6 (Towner, 2006). Widespread dissemination of drug resistance genes and its pan-drug-resistant (PDR) potential which selects this pathogen or patients own flora on excessive use of antibiotics in hospital environment; high adaptability of these microorganisms to adverse environmental conditions, their spread and persistent in the hospital environment for many days, presence of these pathogens in the normal and diseased human skin and mucous membranes and increment of patients susceptible of acquiring these infections are factors involved in the spread and persistence of an epidemic of nosocomial infection caused by *A. baumannii* (Cisneros and Pachon, 2003).

3.1.1 Taxonomy and classification of *Acinetobacter*

The genus *Acinetobacter* was classified by the "Bergey's Mannual of Systematic Bacteriology" in the Family *Neisseriaceae* (Juni, 1984), with only *Acinetobacter calcoaceticus* as species and two subspecies that were *Acinetobacter anitratus* and *Acinetobacter lwoffii* (Bouvet and Grimont, 1986). Recent taxonomy developments have allowed the classification of the genus *Acinetobacter* in the family *Moraxellaceae* within the order *Gammaproteobacteria*, which includes the genus *Moraxella, Acinetobacter, Psychrobacter,* and related organisms (Rossau et al., 1991; Peleg et al., 2008) and which constitutes a discrete phylometric branch in superfamily II of the Proteobacteria on the basis of 16S rRNA studies and rRNA-DNA hybridization assays (Ingram et al., 1960).

Gram-negative bacteria from the genus *Acinetobacter* have been classified previously under at least 15 different "generic" names, the best known of which are *Bacterium anitratum* (Schaub et al., 1948); *Herellea vaginicola* and *Mima plymorpha* (Debord, 1939); *Achromobacter*, *Alcaligenes*, *Micrococcus calcoaceticus*, and "B5W" (Juni, 1978); and *Moraxella glucidolytica* and

Moraxella lwoffii (Piechaud et al., 1956; Brisou, 1957). However, in 1986, the taxonomy of the genus *Acinetobacter* was reorganized by combining the results of DNA-DNA hybridizations with the phenotypic characteristics (Barbe et al., 2004). The genus *Acinetobacter* is now defined as Gram-negative non-fermenting coccobacilli, with a DNA G+C content of 39 to 47 mol%, that are strictly aerobic, non- motile, catalase positive, and oxidase negative (Peleg et al., 2008).

At present, studies based on DNA/DNA hybridization have resulted in the description of 33 validated "genomic species", 17 of which have been given a valid species name (Peleg et al., 2008); numbers have been assigned to the other genospecies and some of the genomic species have been described independently by Bouvet and Jeanjean (1989) and Tjernberg and Ursing (1989). Due to minor discrepancies in the numbering system, the suffixes BJ or TU are added to the number of the genospecies to indicate which study they come from (Towner, 2006). In addition, there is a close relationship between the genomic species *A. calcoaceticus, A. baumnannii*, and the genospecies 3 and 13; therefore, as a result of the difficulties to differentiate the isolates according to their phenotypic characteristics, the term *Acinetobacter calcoaceticus-Acinetobacter baumannii* (AC-AB) complex is often used. Nevertheless, some authors still report these isolates as *A. calcoaceticus subspecies anitratus* (Fournier and Richet, 2006).

3.1.2 Identification of *Acinetobacter* in the laboratory

Acinetobacter is identified at a genus level as Gram-negative, strictly aerobic; non-fermenting, non-fastidious, non-motile, catalase-positive, indole negative and oxidase-negative coccobacilli with a DNA G+C content of 39-47 mol%. The oxidase test serves to differentiate the genus *Acinetobacter* from other related non-fermentative bacteria. Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay (Ingram et al., 1960; Towner, 2006).

Morphologically, *Acinetobacters* are short, plump, gram-negative rods (1.0 to 1.5 by 1.5 to 2.5 µm) in the logarithmic phage of growth and they generally adopt a more coccoid shape in the stationary phase with a tendency to group in pairs or also to form chains of variable lengths. Single pure cultures of *Acinetobacter* spp. often present variability in the Gram-stain together with variations in cell size and arrangement. These microorganisms are non-motile even if some "twitching": or gliding motility on semisolid media has occasionally been reported. In addition, they do not form spores, flagella are absent and generally encapsulated (Towner, 2006).

The *Acinetobacter* spp. generally form smooth, sometimes mucoid, grayish white or white to pale yellow colonies and can be grown in solid media like, sheep blood agar, MacConkey agar and Tryptic soy agar at 37^{o}C. Colonies of *AC-AB* complex are 1.5- 3 mm in diameter and resemble those of Enterobacteriaceae. Some environmental strains have been described to produce a diffusible brown pigment. *Acinetobacters* belonging to *AC-AB* complex never show hemolysis on sheep blood agar but other *Acinetobacter s*pecies *like A. hemolyticus*, *Acinetobacter* genomic species 6, 13BJ, 14BJ, 15BJ, 16 and 17 may show hemolysis (Ingram et al., 1960; Peleg et al., 2008). Growth occurs at a wide range of temperature, environmental isolates prefer incubation temperatures between 20-30^{o}C in contrast with the clinical isolates that normally grow at 37^{o}C and some of them even at 42^{o}C and 44 ^{0}C (Towner, 2006). They are non-fastidious microorganisms that easily grow in a simple mineral medium with single carbon and energy source; however, few strains can use glucose as carbon source. Isolation of *Acinetobacter* spp. can be achieved with standard laboratory media such as Trypticase soya agar (TSA) or Brain heart infusion agar (BHIA). The use of selective medium such as MacConkey may be helpful in recognizing colonies but it is preferable to use a selective medium that suppresses the growth of other microorganism (Ingram et al., 1960; Towner, 2006). A selective and differential medium containing bile salts, sugars and bromocresol purple and modified by addition of various antibiotics has been commercialized by Difco as Herellea agar (Holton, 1983). In addition, the Leeds *Acinetobacter* medium (LAM) is effective for the

recovery of most *Acinetobacter* genospecies from both clinical and environmental sources (Towner, 2006).

Bouvet and Grimont proposed phenotypic tests to identify each species in the genus *Acinetobacter* and this is widely used these days. This included 28 phenotypic tests initially and this scheme was revived and included growth at 37^{o}C, 41^{o}C and 44 ^{0}C, production of acid from glucose; gelatin hydrolysis; and assimilation of 14 different carbon sources. This test cannot identify recently named species but can differentiate 12/13 genomic species. *A. baumannii* and *Acinetobacter genospecies 13TU* couldn't be identified by this technique but *Acinetobacter genomic species 3* and *A. calcoaceticus* could be identified by monitoring growth at different temperatures. Finally, the proposed 22 tests comprised of growth at 37^{o}C, acidification of glucose, carbon sources utilization, hemolytic and two enzymatic tests. This system identified correctly the species at the probability level of 0.98 but genomic species 8/9 and 15TU, 4 and 5, and even 10 and 11 were not identified correctly. Phenotypic differentiation of genomic species within AC-AB complex was possible using the tests proposed by Kampfer P. in 1993.

Different commercial phenotypic methods such as Vitek- 2, API- 20NE (BioMerieux, France), Phoenix and Microscan Walkaway systems have been developed for species identification of this genus. These systems are unable to identify species among the AC-AB complex. *A. baumannii*, *Acinetobacter* genomic species *3*, and 13 TU are identified as *A. baumannii*. API-20NE, system is currently used in hospital laboratories but it requires complementation with other biochemical analysis such as growth at 44^{o}C to identify *A. baumannii* (Bernard et al., 1996). Biotyping methods are based on biochemical tests and can be used for comparative typing of strains (Bergogne-Berezin and Towner, 1996).Serological identification has been attempted with the analysis of capsular types (Traub and Spohr, 1994) and also with studies of lipopolysaccharide molecules (Pantophlet et al., 1999).Protein profiles have been used in epidemiological and taxonomic studies and have allowed to a successful identification of specific strains during endemic episodes and outbreaks in hospitals (Bergogne- Berezin and Towner, 1996).

3.1.3 Molecular Typing of *Acinetobacter*

Nitrocellulose filter method, S1 endonuclease method (Bouvet and Grimont in 1986), hydroxyapatite, and quantitative bacterial dot filter method (Tjenberg and Ursing, 1989) are DNA-DNA hybridization methods for molecular typing of *Acinetobacter*. ribotyping, Amplified 16s rRNA restriction Analysis (ARDRA), tRNA spacer fingerprinting, amplified fragment length polymorphism (AFLP), Pulse-field gel electrophoresis (PFGE), and PCR mediated DNA amplification such as REP-PCR (Peleg et al., 2008), specific gene sequencing especially with the 16s-23s rRNA gene intergenic spacer (ITS) regions, the recA gene and the rpoB gene, a microsphere based array (Lin et al, 2008), the detection of the blaoXA-51 gene encoding a carbapenemase, PCR- electrospray ionization mass spectrometry (PCR-ESI-MS), and a new PCR methodology (Peleg et al., 2008) are also molecular typing methods used for *Acinetobacter*.

3.1.4 Medically important *Acinetobacter* species

Four members of AC-AB Complex, *A. baumannii*, *Acinetobacter* genomic species 3, and 13 TU are the main genomic species associated with outbreaks of nosocomial infections. Fourth member, *A. calcoaceticus* is rarely involved in disease and is mostly environmental. Isolates belonging to the closely related DNA groups 3 and 13 TU, *A. johnsonii* and *A. lwoffii* have also been implicated in a number of outbreaks in ICUs (Ingram et al., 1960; Towner, 2006). *A. hemolyticus* has also been described as the causative agent of endocarditis (Castellanos et al., 1995). *Acinetobacter* genospecies 3 and *A. junii* have been found responsible for bacteraemia and sepsis in neonatal intensive care and paediatric oncology uhnits (de Beaufort et al., 1999; Kappstein et al., 2000). *A. johnsonii* has been isolated from patients with catheter-related blood-stream infection (Seifert et al., 1993). *A. lwoffi* has been isolated from patients with both nosocomial and community-acquired infections such as meningitis, peritonitis, endocarditis and endophthalmitis (Crawford et al., 1997; Valero et al., 1999).

3.2. Antibiotic Susceptibility Pattern of *Acinetobacter* spp

3.2.1. Emergence of antibiotic resistance in *Acinetobacter* spp

In the current review 'MDR *Acinetobacter* spp.' shall be defined as the isolate resistant to at least three classes of antimicrobial agents - all penicillins and cephalosporins (including inhibitor combination), fluoroquinolones, and aminoglycosides. 'XDR *Acinetobacter* spp.' shall be the *Acinetobacter* spp. isolate that is resistant to the three classes of antibiotics described above (MDR) and shall also be resistant to carbapenems; finally, 'PDR *Acinetobacter* spp.' shall be the XDR *Acinetobacter* spp. that is resistant to polymyxins and tigecycline (Falagas and Karageorgopoulos, 2008).

Acinetobacter spp. (and particularly, *A. baumannii*) have become resistant to many classes of antibiotics. *Acinetobacter* spp. appears to be well suited for genetic exchange and is among a unique class of gram negative bacteria that are described as "naturally transformable (Metzgar et al., 2004). *Acinetobacter* strains lacking mutS (part of the mismatch repair system that preserves genomic stability) exhibit increased mutation rates (Young and Ornston, 2001).The presence of competence genes comFECB and QLOMN allows the ready uptake of DNA from the environment (Link et al., 1998; Herzberg et al. 2000).

Most multi-resistant isolates of *Acinetobacter* spp. belong to the AC-AB complex, and many clinical isolates of *A. baumannii* are now resistant to all conventional antimicrobial agents, including carbapenems. Multidrug resistance typically results from the accumulation of multiple mutations and/or the acquisition of resistant genes from other bacterial genera, with the latter occurring by a variety of mechanisms, including the transfer of plasmids, transposons and integrons, carrying clusters of genes encoding resistance to several unrelated families of antibiotics simultaneously (Bergogne-Bérézin & Towner, 1996; Hartzell et al., 2007).The emergence of resistance among clinical isolates of *Acinetobacter* appears to be a combined effect of gene acquisition, following lateral gene transfer, and clonal spread of multiresistant clones (Towner, 2006). The emergence of antimicrobial-resistant

Acinetobacter species is due both to the selective pressure exerted by the use of broad-spectrum antimicrobials and transmission of strains among patients, although the relative contributions of these mechanisms are not yet known (Maragakis and Perl, 2008). In *Acinetobacter baumannii* resistance to several classes of antimicrobials can be observed when the mechanism of resistance is an efflux pump (McGowan, 2006).

A. baumannii is intrinsically resistant to commonly used antimicrobial agents such as aminopenicillins, first and second generation cephalosporin and chloramphenicol (Dijkshoorn et al., 2007). Antibiotic selective pressure selects a new opportunist pathogen that are multi-resistant to antibiotic and to date, some strains of *Acinetobacter* spp. have become resistant to almost all currently available antimicrobial agents, including carbapenems, which were considered the drug of choice for the treatment of infections caused by this microorganism (Fournier and Richet, 2006). Practices in the ICU contribute to the development of resistance because the use of antimicrobial agents per patient and surface area are significantly higher (Cisneros and Rodriguez - Bano, 2002). In addition, the difficulty to eradicate these bacteria has allowed them to colonize niches left vacant after the eradication of other more susceptible microorganisms (Van Looveren and Goossens, 2004).The antimicrobial resistance of *Acinetobacter* spp. varies among countries, centers and even among the different wards within the same hospital (Cisneros and Rodriguez-Bano, 2002). Factors related to the variation on resistance rates among hospitals would be the differences in antimicrobial usage, infection control practices and climate (Gale et al., 2001).

At present moment *Acinetobacter* infections are treated with carbapenems, polymyxins, cephalosporins (3^{rd} and 4^{th} generations) and tigecycline either single or in combination and the multidrug- , pandrug-, and extensive-drug resistant *A. baumannii* have already emerged. Rifampin was added in combination with carbapenems/polymyxins and had given promising results but rifampin resistant strains have already been noticed. So, at present situation there are no therapeutic options for the treatment of these bacteria. Despite its toxicity, colistin remains as a last resort antimicrobial agent to treat infection

caused by multi-resistant *A. baumannii* isolates. Unfortunately, the increment in the use of polymyxin has resulted in the emergence of colistin resistant pathogens in the order of 5% to 28% of the clinical isolate (Fulnecky et al., 2005). In addition, several authors have reported an increasing number of colistin heteroresistance and tolerance in the clinical environment (Li et al., 2006; Hawley et al., 2007).

All three major modes of chromosomal gene transfer have been demonstrated in *Acinetobacter* spp. but conjugation has so far been shown to play a significant role in the transfer of antibiotic resistance genes between members of this genus (Towner and Vivian, 1977). Both acquired and intrinsic resistant can contributes to multiresistance. A combination of several mechanisms may be present in the same microorganism as has also been observed in other gram-negative bacteria (Fernandez-Cuenca et al., 2003).

A. Resistance to beta- lactam agents

Acinetobacter species possess a wide array of beta-lactamases that hydrolyze and confer resistance to penicillins, cephalosporins, and carbapenems. The key determinant regulating over expression of this enzyme in *A. baumannii* is the presence of an upstream insertion sequence (IS) element known as ISAbal, which provides an efficient promoter (Heritier et al., 2006; Ruiz et al., 2007). Although TEM-1, TEM-2, and the carbenicillinase CRAB-5 beta-lactamase is known to occur in *A. baumannii*, Class A extended-spectrum beta-lactamases (ESBLS) have been found only more recently (Vila et al., 1993). In *A. baumannii*, blaPER-1, either plasmid or chromosomally encoded, was the first ESBL to be reported (Poirel et al., 2005). Also, PER- 2 (Peleg et al., 2008), blaVEB-1 ESBL (Poirel et al., 2003) are also found. TEM-1and TEM-2 are narrow spectrum penicillinases whereas CRAB-5 confers high level of resistance to aminopenicillins and carbenicillins. Moreover, TEM-92, TEM-116 and SHV-12 have been identified in *A. baumannii* (Queenan and Bush, 2007).

Class B metallo-beta-lactamases (MBLs) such as IMP, Verona integron-encoded MBLs (VIMs) and Seoul imipenemase (SIM-1) (mainly in *A. baumannii*) confer a high level of resistance to carbapenems as well as every

other beta-lactam antibiotics with the exception of aztreonam (Maragakis and Perl, 2008). Several MBLs have been described: IMP-1, IMP-2, IMP-4, IMP-5, IMP-6 and IMP-11, VIM-1, VIM-2 and SIM-1 (Lee et al., 2005; Walsh et al., 2005; Bonomo and Szabo, 2006).

Acinetobacter spp. has chromosomally encoded class C beta-lactamases represening a distinct family of beta-lactamases, the *Acinetobacter*-derived cephalosporinases (ADCs) (Vahaboglu et al., 1997). The bla genes code for class C cephalosporinases that hydrolyze penicillins and narrow-spectrum and extended-spectrum cephalosporins, but not cefepime or carbapenems. Thus, many clinical isolates are resistant to ceftazidime.

The main cause of carbapenem resistance in *A. baumannii* is class D carbapenemases (OXA-51/66 group) with several variants of these enzymes globally (Heritier et al., 2006; Brown and Amyes, 2006; Rice, 2006). OXA carbapenemases can be divided into following clusters: OXA-23-like (includes OXA-27 and OXA-49), OXA-(24)-40-like (includes OXA-25, OXA-26, and OXA-40), and OXA-58 (Poirel et al., 2007).Lack of outer-membrane proteins or altered porins are also responsible for carbapenem resistance (Livermore and Woodford, 2006).

Carbapenem resistance in *Acinetobacter* species has been linked to the loss of proteins thought to be through the outer membrane (Maragakis and Perl, 2008) and by point mutations. Beta-lactamases and outer-membrane alterations work together confering resistance to beta-lactam agents (Bonomo and Szabo, 2006).

B. Resistance to aminoglycosides

In *A. baumannii*, the over expression of the AdeABC efflux pumps, a member of the resistance-nodulation-cell division family may confer high-level resistance to carbapenems in conjuction with carbapenem-hydrolyzing oxacillinases (Marque et al., 2005). A single point mutation results in increased expression and hence in increased efflux (Marchand et al., 2004). In addition to AdeABC multidrug efflux pump, aminoglycoside resistance in *Acinetobacter baumannii* is mediated by plasmid or transposons-coded aminoglycoside -modifying enzxymes (AmEs) such as the adenylating, acetylating, and

phosphorylating AMEs (Nemec et al., 2004). Other mechanisms of resistance to aminoglycosides in *Acinetobacter* **s**pp. include alterations of the target ribosomal protein, and ineffective transportation of the antibiotic to the interior of bacteria (Vila et al., 1993).

C. Resistance to quinolones

Resistance of *Acinetobacter baumannii* to quinolones is often mediated by modifications in the structure of DNA gyrase secondary to mutations in the quinolone resistance-determining regions of the gyrA and parC genes (Vila et al., 1997; Seward and Towner, 1998). The other mechanism is by acquisition of mobile genetic elements or via efflux pumps. The mechanism involving modifications of lipopolysacharides is also seen in the resistance of *A. baumannii* to quinolone agents from mutations in both gyrA and parC topoisomerase enzymes (Cuenca et al., 2003).

D. Resistance to Chloramphenicol

Chromosomal and plasmid DNA associated chloramphenicol acetyltransferase I (CAT1) that might be transposon-encoded, is responsible for chloramphenicol resistance in clinical *Acinetobacter* isolates (Devaud et al 1982; Elisha, Steyn, 1991) and also from a change in permeability to the antibiotic or a mutation in the target protein (Vila et al., 1993).

E. Resistance to tetracyclines

Specific transposon-meidated efflux pumps TetA and Tet B are are responsible for tetracycline resistance in *A. baumannii* where TetB determines the efflux of both tetracycline and minocycline and TetA drives only the efflux of tetracycline (Guardabassi et al., 2000; Huys et al., 2005). The ribosomal protection protein (RPP) encoded by tet(M) gene shields the ribosome and protects the ribosome from the action of tetracycline, doxycycline, and minocycline (Ribera et al., 2003).

F. Resistance to Co-trimoxazole

The prevalence of trimethoprim-sulfamethoxazole (Co-trimoxazole) resistance in *A. baumannii* is high in many geographic regions (Van Looveren and Goossens, 2004; Gu et al, 2007). Integrons are very common among strains of *A. baumannii* that have a multidrug resistance phenotype. The 3-conserved region of an integron most commonly contains a qac gene fused to a sul gene, conferring resistance to antiseptics and sulfonamides, respectively (Walsh et al., 2005). Consequently, sulfonamide resistance has been shown to be highly predictive of integron-carrying strains of *A. baumannii* (Gu et al., 2007). Similarly, genes coding for trimethoprim (dhfr) resistance have also been reported within integron structures in *A. baumannii* and efflux pump may also contribute to resistance against these agents (Gu et al., 2007).

3.3 Global Epedemiology of *Acinetobacter* infection

Globally, *Acinetobacter* species is a major cause of hospital-acquired infection causing bacteraemia, urinary tract infections (UTI), and in particular nosocomial pneumonia, secondary meningitis, skin and soft-tissue infection with high mortality rate (Bergogne-Berezin and Towner, 2006).

The prevalence of *Acinetobacter* infections ranges from 2% to 10% of all gram negative bacterial infections in Europe (Hanberger et al., 1999) and about 2.5% of them in the United States (Jones et al., 2004). The prevalence of *Acinetobacter* from different clinical specimens was 8.4% (Oberoi et al., 2009) in which maximum number of isolates was from pus, 86.2 % followed by urine, 8.23% and blood 5.4%. The overall incidence of *Acinetobacter* was 15.2% - 19% in other independent studies (Roussel et al., 1996 and Sakata et al., 1998). The prevalence of *Acinetobacter* spp was 11.3% in a study conducted in Hong Kong (Siau et al., 1996), 9.5% (Joshi et al., 2006). In contrast, the prevalence among total bacterial isolates in Hong Kong was 7.4% (Siau et al., 1996). Similar study conducted showed the prevalence of *Acinetobacter* spp. ranging from 15.2% to 19% in different institutions (Roussel et al. 1996 and Sakata et al., 1998). In the study of Mishra and Bhujwala, 1986, maximum isolates were from pus (46.6%) followed by blood (21.3%) while in the study of Pedersen et al. 1970, maximum isolates were

from sputum (26.3%) and from urine (222%). Again, the prevalence of *Acinetobacter* spp. in Nepal was 5.77% (Ghimire et al; 2002), in India, 1.4% (Lahiri et al; 2004) and in different European countries 16.4% to 27.5% (Hanberger et al., 1999). In another study, 7-18% *Acinetobacter* was isolated from throat swabs, 45% from tracheostomy specimen and 25 to 45% from superficial wounds ((Rosenthal, 1974; Joly-Guillou et al., 1990).

A study conducted in Denmark showed the higher prevalence of *Acinetobacter* in both males (61.5%), and females (72.1%) of above 40 years (M=8/13, F=57/79), lower prevalence in age groups 21-40 years (M=30.75%, 4/13, F=25.3%, 20/79) and least (M=7.6%, F=2.5%) in age groups below 20 years. In this study, the overall prevalence of *Acinetobacter* was higher in females (85.87%) than in males (14.13%) out of total 92 isolates of *Acinetobacter* (Hoffmann et al. 1982).A study conducted in India showed about 80.2% of *Acinetobacter* spp. from hospital patients, whereas only 19.8% from community-acquired OPD cases (http://www.IndianJmedsci.org.2000).

The prevalence of *Acinetobacter* spp. in different respiratory tract specimens was 20.4% (Lahiri et al; 2004), 26.4% (Pedersen et al; 1970) and 32.2% (Ghimire et al; 2002). The prevalence of *Acinetobacter* spp. in Catheter tips from France showed a higher prevalence of 15.5% and from India showed a lower prevalence of 4.6% (Lahiri et al; 2004). In a review from the CDC, 7% of ICU-acquired pneumonias were due to *Acinetobacter* in 2003, compared to 4% in 1986 (Gaynes and Edwards. 2005). *Acinetobacter* is responsible for 3-5% of the nosocomial pneumonia and is emerging as an important complication of mechanical ventilation (Bergogne-Berezin and Towner, 1996; Martone et al., 1995). In ICU patients with mechanical ventilation, *Acinetobacter* is responsible of 15-24% of the pneumonia (Garcia-Garmendia et al., 1999; Gomez et al., 1999). In large surveillance studies from the United States, 5- 10% of cases of ICU-acquired pneumonia were due to *A. baumannii* (Gaynes and Edwards, 2005).

The prevalence of *Acinetobacter spp.* from blood specimens ranged 9.9% to 21.3% such as 21.3% (Mishra and Bhujwala, 1986), 9.9% (Lahiri et al., 2004), 10.2 % (Bang et al., 1998) and 18.0% (Garcia-Garmendia et al., 2001; Santucci et al., 2003) and 17.2% (Sharma, 2004), 6.69 % (Arora and Pushpa Devi, 2007) in India, 4.6% in Nepal (Khanal et al., 2004); 6.9% in Hong Kong (Siau et al., 1996); and1.5% in Japan (Nippon Rinsho and Iinuma, 2002). Again, the prevalence of BSI was found 1 to 9 % (Struelens et al, 1993; Cisneros et al., 1996; Sadar et al., 2002; wisplinghoff et al., 2004) and 10.2-18.0 % (Bang et al., 1998; Garcia-Garmendia et al., 2001; Santucci et al., 2003) in different studies. In a study conducted in Hongkong, only 22% of patients acquired *Acinetobacter* infection in ICUs (Siau et al., 1999). Among them, 56.9% males and 43.1% females were *Acinetobacter* positive from blood in the study conducted in Estonea. In United states from 1995-2002, *A. baumannii* was the 10th most common etiologic agent, being responsible for 1.3% of all monomicrobial nosocomial bloodstream infections which was a more common cause of 1.6% ICU- acquired BSI than of 0.9% of non- ICU BSI with crude mortality 34.0% to 43.4% in the ICU and 16.3% outside the ICU (Wisplinghoff et al; 2004).

The prevalence of *Acinetobacter* in urine specimens ranged 8.23% to 51.97% in a study conducted by different researchers at different places (Lahiri et al., 2004; Oberoi et al., 2009). *Acinetobacter* was responsible for just 1.6% of ICU-acquired UTIs in one study, 2.1% of ICU-acquired skin/soft tissue infections (Gaynes and Edwards, 2005) and 32.5% of combat victims with open tibial fractures (Johnson et al., 2007).

The prevalence of *Acinetobacter* spp. in different pus specimens was 86.2% (Oberoi et al; 2009), 46.6%((Mishra and Bhujwala, 1986), 11.18% (Lahiri et al; 2004), 42.0%(Ghimire et al; 2002), 21.5% in USA (Jones et al., 2004) and 6.8% in ICU patients to 32.8% in general ward patients in Hong Kong (Siau et al., 1996). Body fluids in Hong Kong showed the prevalence rate of 5.8% in general ward to 11.1% in ICU (Siau et al., 1996). The study at the Division of Nephrology, National University Hospital, Singapore revealed 13 episodes of

acute peritonitis (AP) in eleven patients over an 18 month period accounting for 14.3% of the total number of peritonitis episodes (Lye et al., 1989).

Since 1975, *Acinetobacter* showed increasing resistance to older antibiotics including penicillins, cephalosporins (first and second generation), cephamycins such as cefpodoxin (Garcia et al., 1983), and almost all aminoglycosides, chloramphenicol, and tetracyclines (Dowding, 1997) and during late 1980s and1990s, worldwide emergence and spread of *Acinetobacter* strains resistant to imipenem emerged (Fournier and Richet, 2006; Montefour et al., 2008). Pan-drug resistance *A. baumanni* susceptible to polymyxin and ampicillin - sulbactam were reported as early as 1991 and 1992 in United States (Go et al., 1994).

In a surveillance study of the antibiotic susceptibility patterns of the isolates from the ICUs of five European countries (1999), the prevalence of resistance in *Acinetobacter* spp. to gentamicin was 0 – 81%, amikacin 10 – 51%, ciprofloxacin 19 – 81%, ceftazidime 0 – 81%, piperacillin-tazobactam 36 – 75%, and imipenem 5 – 19% (Hanberger et al., 1999). Subsequent data from 40 centers in 12 countries participating in the MYSTIC program (2006) revealed a substantial increase in resistance rates for meropenem (43.4%) and imipenem (42.5%). The resistance of *Acinetobacter* spp. to imipenem from 1991– 2004 was in the range of no resistance to 50% (Cisneros and Rodríguez-Baño, 2002; Perez et al., 2007). Among *Acinetobacter* spp. derived from 30 European centers from the worldwide collection of SENTRY from 2001 to 2004, the proportion of strains resistant to imipenem and meropenem was: 26.3% and 29.6% respectively (Turner and Greenhalgh, 2003; Gales et al., 2006).

In an industry supported surveillance report (MYSTIC) from 48 European hospitals for the period 2002–2004, just 73.1% of isolates were susceptible to meropenem and 69.8% were susceptible to imipenem (Unal and Garcia-Rodriguez, 2005), with 32.4%, 34.0%, and 47.6% being susceptible to ceftazidime, ciprofloxacin, and gentamicin, respectively (Unal and Garcia-Rodriguez, 2005).

The prevalence of imipenem resistance in *Acinetobacter baumannii* isolated from a burns unit of USA was found to be as high as 87% (Trottier et al., 2007).

Numerous outbreaks of pandrug-resistant *A. baumannii* have been documented in Asian and Middle Eastern hospitals, and a variety of carbapenemases have been described to originate there (Abbo et al., 2005; Chan et al., 2007; Jeong et al., 2006; Ko et al.,2007). Rates of non-susceptibility in SENTRY isolates (2001–2004) exceeded 25% for imipenem and meropenem, 40% for ceftazidime, 35% for amikacin, and 45% for ciprofloxacin (Gales et al., 2006). Different studies in India from 2005- 2006 reported a prevalence of 14%-35% carbapenem resistance in *Acinetobacter* spp. isolated from different clinical samples (Gladstone et al., 2005; Sinha and Srinivasa, 2007). In Hong Kong imipenem was most susceptible (94.2%) followed by amikacin (72.7%), ofloxcin (66.7%), netilmicin (66.4%), ceftazidime (55.4%), cotrimoxazole (49.1%) and gentamicin (47.9%) (Siau et al., 1996).In another study conducted in India, the resistance pattern was observed as follws: piperacillin= 97.9%, carbenicillin= 68.8%, cefotaxime= 80.0%, ceftazidime= 80.0%, cefoperazone= 82.3%, imipenem=9.1%, meropenem= 9.8%, gentamicin= 85.8%, tobramycin= 84.2%, smikacin= 74.6%, netilmicin= 80.4%, ciprofloxacin= 80.8%, norfloxacin= 78.1% and cefoperazone-sulbactam= 31.2% (Gaur et al., 2008). In a study, 95.6% *Acinetobacter* spp. were sensitive to the combination of drug cefoperazone-sulbactam and 94.6% sensitive to meropenem (Capoor et al., 2005).

Data from the National Nosocomial Infection Surveillance system collected from 1986 to 2003, involving many hospitals throughout the United States, showed significant increases in *Acinetobacter* strains resistant to amikacin (5% to 20%), ceftazidime (25% to 68%), and imipenem (0% to 20%) (Gaynes and Edwards, 2005).Study between 2004 and 2005 from 76 centers throughout the United States showed only 60.2% *Acinetobacter* spp. susceptible to imipenem (Trottier et al.,2007).In another study *Acinetobacter* spp. were 10% to 15% susceptible for carbapenems, 35% to 40% for ceftazidime, 10% to 30% for aminoglycosides, and 35% to 40% for ciprofloxacin (Rhomberg and Jones,

2007). 71% of *Acinetobacter* isolates were susceptible to meropenem or imipenem in an assessment from a surveillance program in the period 2002–2004(Unal and Garcia-Rodriguez, 2005).

Acinetobacter is resistant to most β-lactam antibiotics, particularly penicillins and cephalosporins, especially in ICU patients where ceftazidime, piperacillin and carbapenems are among the β-lactam antibiotics most active against *A. baumannii* (Seifert et al., 1993; Vila et al., 1993 and Shi et al., 1996). 97% susceptibility rate of *A. baumannii* to imipenem was reported from Saudi Arabia and Japan (Al-Tawfig, 2007; Ishii et al., 2005) and 100% from patients in eight Dutch hospitals and Germany in 1990 (Buirma et al., 1991). In a study from Turkey and Spain, the rate of carbapenem resistance in *Acinetobacter* spp. ranged from 9.6 to 43.7% (Karsligil et al., 2004; and Cisners et al., 2005). In 1990, 70 *Acinetobacter* spp. from ICU patients in 16 Belgian hospitals showed susceptibilities to ceftazidime and ceftriaxone of 86% and 74%, respectively (Verbist, 1991). Amoxy-clav was 22.3% susceptible in Hong Kong (Siau et al., 1996), 52.3-67.0% susceptible in India (Lahiri et al. 2004 and Sharma et al., 2004) and 91.0% susceptible in 1990 from patients in eight Dutch hospitals (Buirma et al., 1991).

Higher (88.5-94%) susceptibility to sulbactam-cefoperazone against *Acinetobacter* was seen in India (Oberoi et al., 2004; Gaur et al., 2008) and 98% susceptible in China (Wang et al., 2000). Similar multicenter study in China showed both imipenem and cefoperazone/ sulbactum with 97%, and 89% susceptible respectively (Zhonghua et al.; 2000).Moreover, another study Conducted in India showed 4.4% to 46% resistance to cefoperazone-sulbactam against *Acinetobacters* where ESBL production was seen in 6% and IBL (Inducible Beta Lactamase) production was seen in 7% of *Acinetobacter spp.* (Kucukates and Kocazeybek, 2002; Capoor et al., 2005). In study conducted at different places, more than 71% of the cefoperazone-resistant *Acinetobacter* species strains were susceptible to the cefoperazone-sulbactam combination due to the in-vitro intrinsic activity of sulbactam against *Acinetobacter* species (Traub and Spohr, 1989; Urban et al., 1993).

In a Spanish study published in 1993, 63% of 54 *A. baumannii* isolates tested were susceptible to trimethoprim–sulphamethoxazole (Vila et al. 1993). Similar low resistance of co-trimoxazole among *Acinetobacter spp*. was also observed in a study in Itali (Capone et al., 2008). A moderate susceptible isolates were obtained in Hong Kong, 48.1% (Siau et al., 1996), India, 61.9% (Sharma, 2004). In 1999, 43.8% of 32 *A. baumannii* isolates from the ICUs of four different hospitals in Turkey were susceptible to trimethoprim–sulphamethoxazole (Kocazeybek et al., 1999), while in a Slovakian study published in 2002, 58% of 50 *Acinetobacter* spp. isolates were resistant to trimethoprim–sulphamethoxazole (Hostacka and Klokocnikova, 2002).

Higher amikacin and gentamicin reistance have also been observed in different places at different times (Hoffmann et al. 1982). In Turkey, only 8.7% of 80 isolates from ICUs in 1996 were susceptible to gentamicin and only 29.1% to amikacin (Günseren et al. 1999). In 1997, of 164 isolates of *Acinetobacter* spp., 17.1% were susceptible to gentamicin and 34.8% to amikacin (Aksaray et al., 2000). In contrast, Chang et al. and Oberoy et al. reported higher susceptibility rates of 74.5% and 64.7% respectively among *Acinetobacter spp.* strains for amikacin (Chang et al. 1995 and Oberoi et al. 2009). 62.5% *Acinetobacter baumannii* isolates were susceptible to amikacin and 15.6% to gentamicin from Turkish ICUs in 1999 (Kocazeybek, 2001). In a Spanish study during the early 1990s, 50% of 54 *A. baumannii* isolates tested were susceptible to tobramycin, 33% to gentamicin, 66% to netilmicin, and 72% to amikacin (Vila et al. 1993). Between 1991 and 1996, an increase in aminoglycoside resistance among clinical isolates of *Acinetobacter* spp. was noticed in Spain, rising from 33.0% to 71.8% for tobramycin, and from 21.0% to 83.7% for amikacin (Ruiz et al., 1999).

In 1996 - 1998 in Greece, 92.4% -92.6% of the ICU isolates were resistant to ciprofloxacin (Kocazeybek et al., 1999; Maniatis et al., 2003). In 1996- 1999 in Turkey, 26.4%-32.9% of *Acinetobacter spp*. isolates from ICUs were susceptible to ciprofloxacin (Günseren et al., 1999; Kocazeybeket al., 1999; and Aksaray et al., 2000). In a Slovakian study published in 2002, 68% of the 50 tested *Acinetobacter* spp. isolates were resistant to ciprofloxacin (Hostacka

and Klokocnikova, 2002).In Spain, Vila et al., 1993 found ciprofloxacin (70%) and ofloxacin (72%) to be more active against clinical isolates of *A. baumannii* than norfloxacin (18%), but in a separate study, ciprofloxacin resistance in clinical isolates of *Acinetobacter* increased in Spain from 54.4% in 1991 to 90.4% in 1996 (Ruiz et al., 1999).

3.4. Scenario of *Acinetobacter* spp. in Nepal.

Although authentic data regarding *Acinetobacter* are not available, *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., 2004). The Prevalence of *Acinetobacter* spp. in urine from Man Mohan Memorial Community Hospital was 2.3% (Basnet et al., 2009). In this study, the *Acinetobacter* spp. was the third most common isolate among total GNB isolates.

In Nepal almost MDR *Acinetobacter* spp. isolates were resistant to ceftazidime (Bomjan, 2005; Shrestha et al., 2007; Baral, 2008; Basnet et al., 2009; Kattel et al., 2008). In a study conducted in Nepal, 80% ESBL negetive *Acinetobacter* spp. were susceptible to ceftazidime but higher degree of carbapenem resistance for imipenem (40.3%) and meropenon (19.2%) was seen among these EBSL negative *Acinetobacter spp.* (Kandel, 2010). In studies conducted in Nepal, *Acinetobacter spp*. were 88.04-96.6%, 40.0- 96.6% and 98.4% resistant to amikacin, gentamicin and ceftazidime respectively (Ghimire et al., 2002; khanal et al., 2008; Basnet et al., 2009; and Kandel, 2010). In Nepal ciprofloxacin and ofloxacin were susceptible in 35.9% and 37.9% of the isolates respectively (Kandel, 2010). Ciprofloxacin was found 96.7% resistant (Ghimire et al., 2002) and 76.0% susceptible (Basnet et al., 2009) in the different studies conducted in eastern Nepal. Lower rates of susceptibility against netilmicin (10.6%) and tobramycin (4.1%) were found in a study conducted in eastern Nepal (Ghimire et al., 2002).

CHAPTER –IV

4. MATERIALS AND METHODS

4.1 Materials

The materials, equipment and various reagents used in different stages of this study are listed in Appendix IV.

4.2. Methodology

4.2.1 Study site and study period

After taking consent from the Hospital Ethical Committee**,** the study was conducted in the Department of Microbiology at Kathmandu Medical College (KMC), Sinamangal, Kathmandu, Nepal; a 700-beded tertiary care hospital; from October 2009 to March 2010 (6 month duration).

4.2.2 Study population

This study included patients of all age groups and both sexes visiting KMC, from whom the samples were sent for routine culture and antibiotic susceptibility testing. The demographic parameters, clinical history, prior antibiotic use, etc. were recorded.

4.2.3 Sample size and sample types

A total of 5965 different samples including urine (2794), blood (1707), sputum and other respiratory specimens, different body fluids such as peritoneal, ascitic, pleural, semen, bile, cerebrospinal fluid (CSF),tips (catheter, CVP, Foley), DJ-stunt, tissue sent for routine culture and antibiotic susceptibility testing were processed during the study period. Samples obtained in a sterile, clean, and wide-mouthed and leak proof container with no visible signs of contamination and labeled and transported properly with demographic information of patients were accepted, otherwise repeat sample was requested.

4.2.4 Study design

The study is a cross-sectional study determining the prevalence of *Acinetobacter* spp. in different samples sent to microbiology department of KMC and evaluating the antimicrobial susceptibility pattern of the isolates.

4.2.5 Data collection

The demographic parameters, brief clinical history, prior antibiotic use, if yes duration and type, were recorded using a standard questionnaire.

4.2.6 Collection and transportation of specimens

4.2.6.1 Urine samples

Patients were asked to collect 10-20 ml of clean voided (clean- catch) first morning mid-stream urine in a sterile, dry, wide- necked, leak- proof plastic container (ready- made and sealed); instructing the patient not to halt and restart the urinary system for a mid-stream urine collection but preferably move the container into the path of already voiding urine. Patients were advised not to touch the inside part of the container by hands or any other body surfaces. The container was then 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. This specimen was processed without delay (i.e. within two hours).

4.2.6.2 Sputum, throat swabs 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 was advised not to reuse or gargle the 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 was suggested to collect after drinking hot water for the patient difficult for deep-cough. The container was labeled properly and immediately delivered to the laboratory as soon as possible for further

processing. For collecting throat swab, the head of the patient was leaned in a backward position as far as possible and the swab was inserted into the mouth with the aid of tounge depressor. The swab was rubbed over the inflamed tonsillar area and posterior pharynx or areas that had lesions, or were membranous. The swab was rotated over all of the affected areas and withdrawn avoiding the touch to the tongue or other areas of the mouth. Other respiratory specimens such as bronchio-alveolar lavage (BAL), endo-tracheal and intra-tracheal aspirations were sent in the laboratory after collecting by clinicians. These specimens were labeled properly with required demographic information and laboratory numbers then processed without delay.

4.2.6.3 Exudates

Exudates samples were usually obtained from wounds or abscesses that are clinically infected or deteriorating or that fail to heal over a long period. For closed wounds and aspirates, 2% cholorohexidine followed by an iodine solution was used for disinfection whereas for open wounds it was debrided then rinsed thoroughly with sterile saline prior to collection of pus sample. Pus samples contained the deepest portion of the lesions or exudates were collected rather than superficial debris. Swab collection was avoided as long as aspirates or biopsy samples could be obtained. If swab was the only option, it was obtained by gentle rolling of cotton wool over the surface of the wound 4 to 5 times, focusing on area where there was evidence of pus or inflamed tissue applying aseptic techniques. The pus sample was collected by clinicians and the required information regarding collection was provided by microbiology department. The pus/swabs thus collected were transported immediately to the laboratory after proper labeling with demographic information, type of specimen and anatomic location. For Fine Needle Aspiration Cytology (F N A C) and other aspirated specimens, the syringe was properly capped, labeled and processed immediately.

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

These specimens were obtained with the help of trained physicians by percutaneous aspiration taking care to avoid contamination with commensal microbiota. The needle puncture site was cleansed with alcohol and disinfected

with iodine solution to prevent the specimen contamination or infection of patient. About 3-5ml of the sample was drawn and transported to the laboratory, after proper labeling.

4.2.6.5 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. In case of delay in processing, the specimen was kept at room temperature rather than refrigeration.

4.2.6.6 Eye and Ear specimens

Eye specimens consisted of conjunctival swab, conjunctival /corneal scrapings and vitreous taps. Conjunctival swab was collected after removing the excess debris from the outside then cleaning the eye with normal saline and gauze pad; wiped from inner to outer canthus. Then one or two drops of topical anaesthetic was instilled and a moistened swab with sterile physiologic saline was taken and carefully rubbed over the lower conjunctiva to collect epithelial cells without touching other surfaces. The swab was held parallel to the eye rather than pointed directly to it to avoid irritation. One swab per eye was collected. Conjunctival or corneal scrapings were collected by ophthalmologist using a wire culture loop to get epithelial cells. The conjunctival swab or wire culture loop was streaked directly on the proper culture media provided by microbiology laboratory. After proper labeling, the media were transported to the laboratory immediately. Vitrous taps were collected by ophthalmologist by needle aspiration (0.1-0.3 ml).

In case of external ear specimens, the excess debris was cleaned from the patient's ear by using sterile normal saline and gauze pads. The sterile swab was inserted into the ear canal and rotated gently against the walls of the canal avoiding damage to the eardrum. The swab was drawn out without touching the other surfaces to prevent contamination, then labeled properly and transported to the laboratory immediately.

4.2.6.8 Blood and Bone-marrow collection

The blood samples were collected aseptically and diluted with Brain Heart Infusion (BHI) broth in 1:10 ratio (i.e. 1 part blood and 9 parts broth). In neonates and children one ml blood was mixed with nine ml of broth whereas in adults two ml blood was mixed with eighteen ml broth. Then the broth bottle was labeled properly and incubated at 370C for up to 96 hours aerobically. For bone-marrow culture, the BHI broth was sent to the wards with required information and then the specimen was collected by trained clinicians. The specimen was labeled and incubated up to 97 hours at 370C**.**

4.2.6.9 Other miscellaneous specimens

Other specimens such as bile, DJ-stunt, catheter tips, CVP-tips, Foley's tip, tissue, high vaginal swabs etc were collected and sent to the laboratory applying aseptic techniques in a sterile container or tubes after proper labeling and without delay.

4.3 Macroscopic examination of specimens

The urine sample obtained was observed for its color and turbidity and reported accordingly. Similarly, the sputum sample was macroscopically examined to see whether it consisted of only saliva or real sputum. In case if it was found only to be watery, it was reported as 'unsuitable for microbiological examination and another specimen were requested. Other specimens such as pus swabs were observed whether they were sent in proper transportation media or not. Body fluids, CSF were observed for turbidity, blood stains, clot etc.

4.4 Culture of Specimens

4.4.1 Urine Culture

The urine samples were cultured onto the MacConkey agar and Blood agar plates by the semi-quantitative culture technique using a standard calibrated loop. A calibrated loop was immersed vertically just below the surface of well-mixed uncentrifuged urine specimen. A loopful of urine was then streaked on the plate to make straight line inoculums down the center of the plate and the urine was streaked by making series of passes at 90^{o} angle throughout the

inoculums. The plates were then incubated at 37^{o}C over night. Colony count was performed so as to calculate the number of CFU per ml of urine and the bacterial count was reported as: Less than 10^4 CFU / ml organisms: not significant growth; 10^4–10^5 $_{CFU}$ / ml organisms: doubtful significance (suggested repeat specimen) and more than 10^5 $_{CFU}$ / ml organisms: significant bacteriuria. If the culture indicated presence of two uropathogens both showing significant growth, definitive identification and antimicrobial susceptibility testing of both were performed whereas in case of ≥ 3 pathogens, it was reported as multiple bacterial morphotypes and asked for appropriate recollection with timely delivery to laboratory (Isenberg, 2004).

4.4.2 Sputum, throat swab and other respiratory specimens

The sputum samples were inoculated into the blood agar, chocolate agar and MacConkey agar plates. For, sputum, in chocolate agar plate a 5 mg Optochin disc and a 10U Bacitracin disc were added to screen out *Streptococcus pneumoniae* and *Haemophilus influenzae* respectively, whereas for throat swab, 0.05U Bacitracin disc was added to the plate to screen *Streptococcus pyogenes*. The chocolate agar and blood agar plates were incubated at 37^{o}C for overnight in 5 – 10% CO_2 environment whereas the MacConkey agar plate was incubated at 37^{o}C in an aerobic condition.

4.4.3 Pus, Body Fluids, CSF and Semen

These samples were inoculated into Blood Agar, chocolate agar and MacConkey agar plates. The blood agar and chocolate agar plates were incubated in a 5 – 10% CO_2 enriched atmosphere at 37^{o}c and MacConkey plates were incubated aerobically at 37^{o}c overnight. Additionally, these samples were inoculated into Mueller Hinton Broth (MHB) for enrichment and incubated aerobically at 37^{o}c overnight. In case no growth was observed from primary inoculation, the MHB thus inoculated was used to re-inoculate the plates.

4.4.4 Blood Samples

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^{o}C for up to 96 hours sub-culturing at every 24h of incubation if visible turbidity or hemolysis was observed. It was sub-cultured in Blood agar plate and MacConkey agar plates then incubated aerobically at 37^{o}C for 24 hours. After 96 hour of incubation, if no growth was seen then the broth was discarded in a proper manner.

4.4.5 Other miscellaneous specimens

Other specimens such as catheter tips, CVP-tips, Foley's tip, DJ-stunt, tissue, endo-tracheal and intra-tracheal tubes, etc were inoculated in Blood agar and MacConkey agar plates directly and enriched in the BHI broth first then sub-cultured in the Blood and MacConkey agar plates and incubated at 37ºC aerobically for 24 hours or overnight. Eye swabs and ear swabs were inoculated into Chocolate agar also and incubated in 5-10% CO_2 atmosphere.

4.5 Identification of *Acinetobacter* spp.

The identification of *Acinetobacter* spp. was performed by following standard diagnostic procedures. All clinical specimens were initially processed by the routine microbiology laboratory tests to separate the non-fermenters from other gram negative bacilli and eventually identified as *Acinetobacters*. Typical colonies were enumerated, picked, and examined further. *Acinetobacter* were identified by gram-staining, cell and colony morphology, activity in the oxidation / fermentation (O-F) test, absence of motility, and negative oxidase and positive catalase tests and other various biochemical tests according to "Bergey's Manual of Systematic Bacteriology." At first , the bacteria were screened from the primary culture plate then isolated pure culture colonies was obtained in the MacConkey Agar (MA) and Sheep Blood Agar (SBA) plates and all the biochemical tests were performed. The ability of each isolate to oxidize glucose in Hugh and Leifson's medium was tested. The isolates were reported as glucose-oxidizing or non-glucose-oxidizing strains of *Acinetobacter* spp. The incubation of the culture plates was done at 37^{0}C and

all non-hemolytic glucose-oxidizing isolates were tested for growth at 44^0C.These temperature- tolerant isolates were identified as AC-AB complex. There was no further attempt to differentiate the isolates by other specific methods such as DNA-hybridization or extensive substrate assimilation tests. The various conventional biochemical tests used are described in Appendix IV.

4.6 Antimicrobial susceptibility tests of *Acinetobacter* isolates

Antimicrobial susceptibility testing of clinical isolates of *Acinetobacter* was performed by the Modified Kirby-Bauer disc-diffusion technique (CLSI M02-A9) using Mueller Hinton Agar (MHA, Hi-Media Laboratories, and Mumbai, India). Altogether 24 antimicrobial agents were tested in 4 MHA plates for each organism i.e. 6 different antimicrobial discs per plate. The following antimicrobial agents with their concentrations given in parenthesis were used: Amoxycillin (30μg), Amoxy-clave (30 μg), Imipenem (10 μg) Meropenem (10 μg), Piperacillin (30 μg), Carbenicillin (100 μg), cefoperazone/ Sulbactam (30/75μg), Cephalexin (30 μg), Ceftriaxone (30 μg), Ceftazidime (30 μg), Cefuroxime (30 μg), Cepfodoxime (10 μg), Cefoxitin (30 μg), Cephalothin (30 μg), Gentamicin (10 μg), Amikacin (30 μg), Tobramycin (10 μg), Netilmicin (30 μg), Kanamycin (30 μg), Tetracyeline (30 μg), Chloramphenicol (30 μg), Ciprofloxacin (5 μg), Ofloxacin (5 μg) and Co-trimoxazole (25 μg).The detailed account of the test procedures are explained in Appendix V.

4.7 Preservation of the *Acinetobacter* isolates

The *Acinetobacter* isolates in pure culture, after performing the biochemical and antimicrobial susceptibility testing, were preserved in Tryptic Soya broth (TSB) containing 20.0 % Glycerol and kept at -70 ^{0}C until further tests were required**.**

4.8 Clinical features of cases of *Acinetobacter* infection

The following clinical characteristics were recorded: Patient's age, sex, duration of hospitalization, admission to ICUs, mechanical ventilation, urinary and intravenous catheterization, presence of underlying disease or condition, days on previous antibiotic therapy and surgery if any. Standard definitions as given by Center for Disease Control and Prevention were used to differentiate

categories of infection and infection versus colonization etc. The infection acquired upon hospitalization for 72 hr or more was defined as hospital-acquired; but it was community-acquired if otherwise.

4.9 Data analysis

All the results obtained were entered into the worksheet of statistical package for social science (SPSS) software (verson 17.0) and Microsoft 2007 spread sheets then analyzed.

4.10 Quality control

4.10.1 Monitoring and regular evaluation of laboratory equipments, reagents, and media

Laboratory equipments like incubator, refrigerator, and autoclave and hot-air oven were regularly monitored for their efficiency. The temperature of the incubator and refrigerator was monitored twice a day. Reagents and media were regularly monitored for their manufacture and expiry date and a proper storage conditions. After preparation, they were properly labeled with preparation date and self-life. The quality of media prepared was checked by subjecting one plate of each batch for sterility and performance testing.

4.10.2 Purity plate

The purity plate was used to ensure that the inoculation used for biochemical tests was pure culture and also to check maintenance of aseptic conditions. Thus, while performing biochemical tests, the same inoculums was sub-cultured in respective medium and incubated. The media were then checked for the appearance of pure growth of organisms.

4.10.3 Quality control during antibiotic susceptibility testing

MHA and the antibiotic discs were checked for their lot numbers, manufacturing dates, expiry dates, and storage conditions. For the standardization of Kirby-Bauer test and for performance testing of antibiotics and MHA, control strains of *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were tested primarily. The thickness of MHA was

maintained at 4 mm and the pH at 7.2 - 7.4. Similarly antibiotic discs containing the correct amount as required by the tests were used.

4.11 Limitations of the study

Inability to include large number of the specimens from different territories, no determination of MICs and resistance mechanisms of the antibiotics used, no availability of API 20-NE system and other batteries of biochemical tests, then no genetic analysis of the isolates to find out the exact genospecies prevalent for the nosocomial infections in this hospital remained the major limitations of this study.

4.12 Flow-chart of Methodology

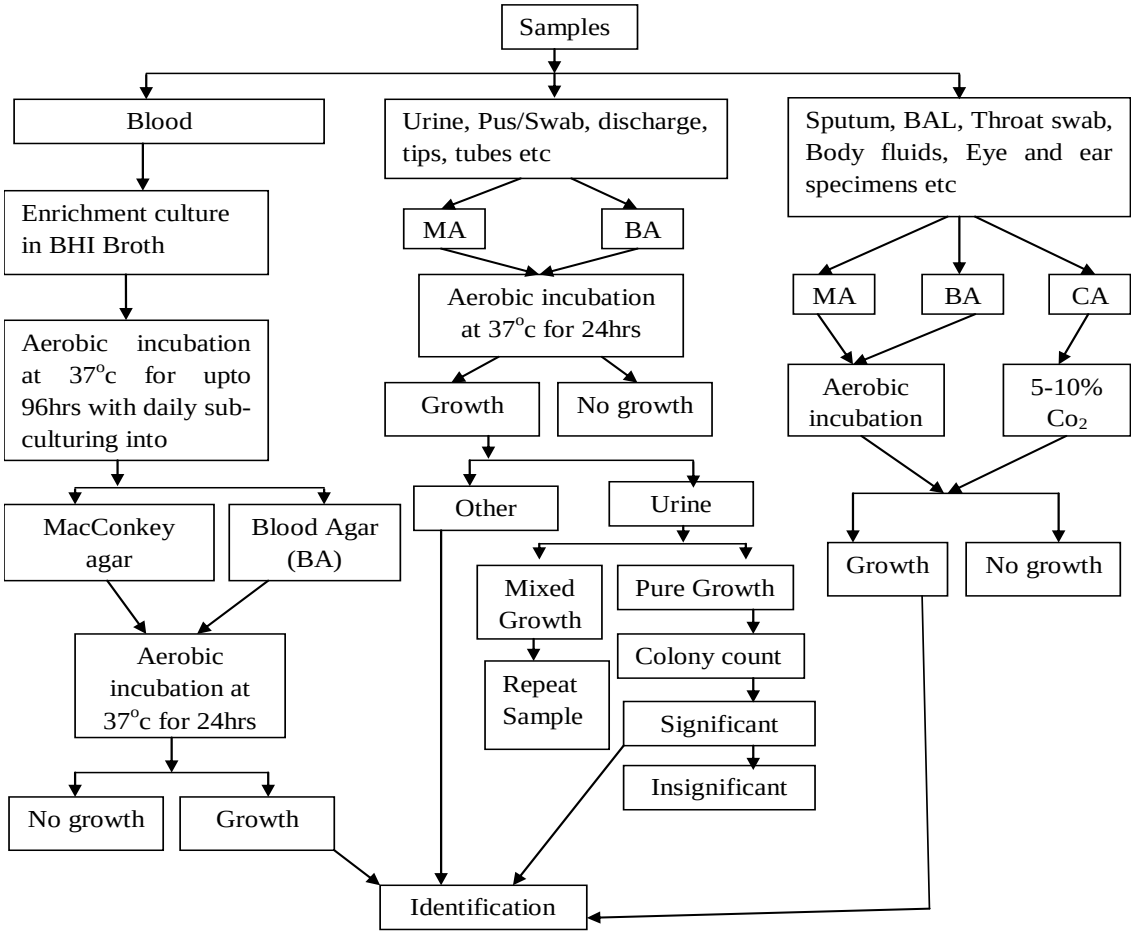

Fig.1 Flow Chart of Isolation of *Acinetobacter* spp.

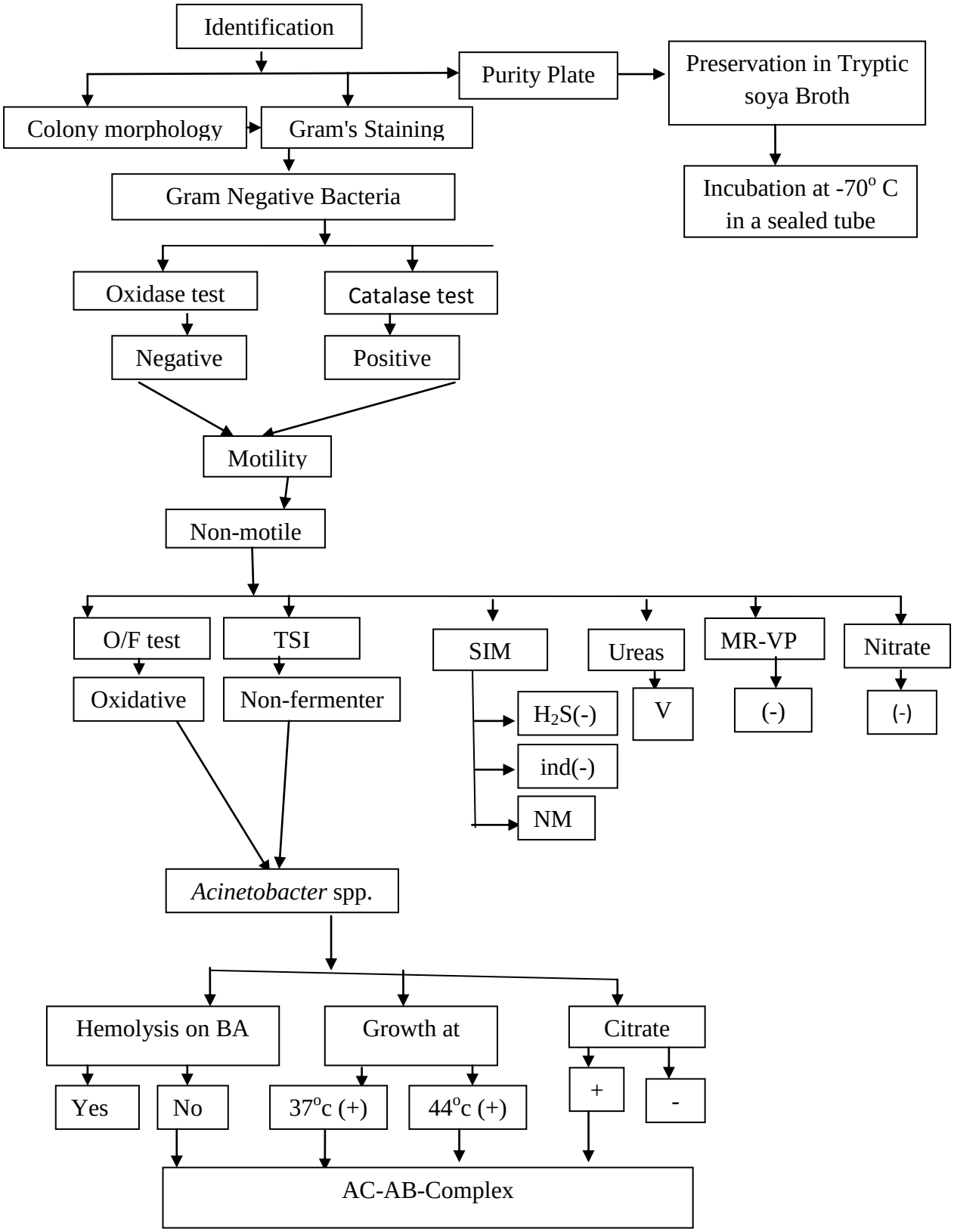

Fig.2 Flow Chart of Identification of *Acinetobacter* spp.

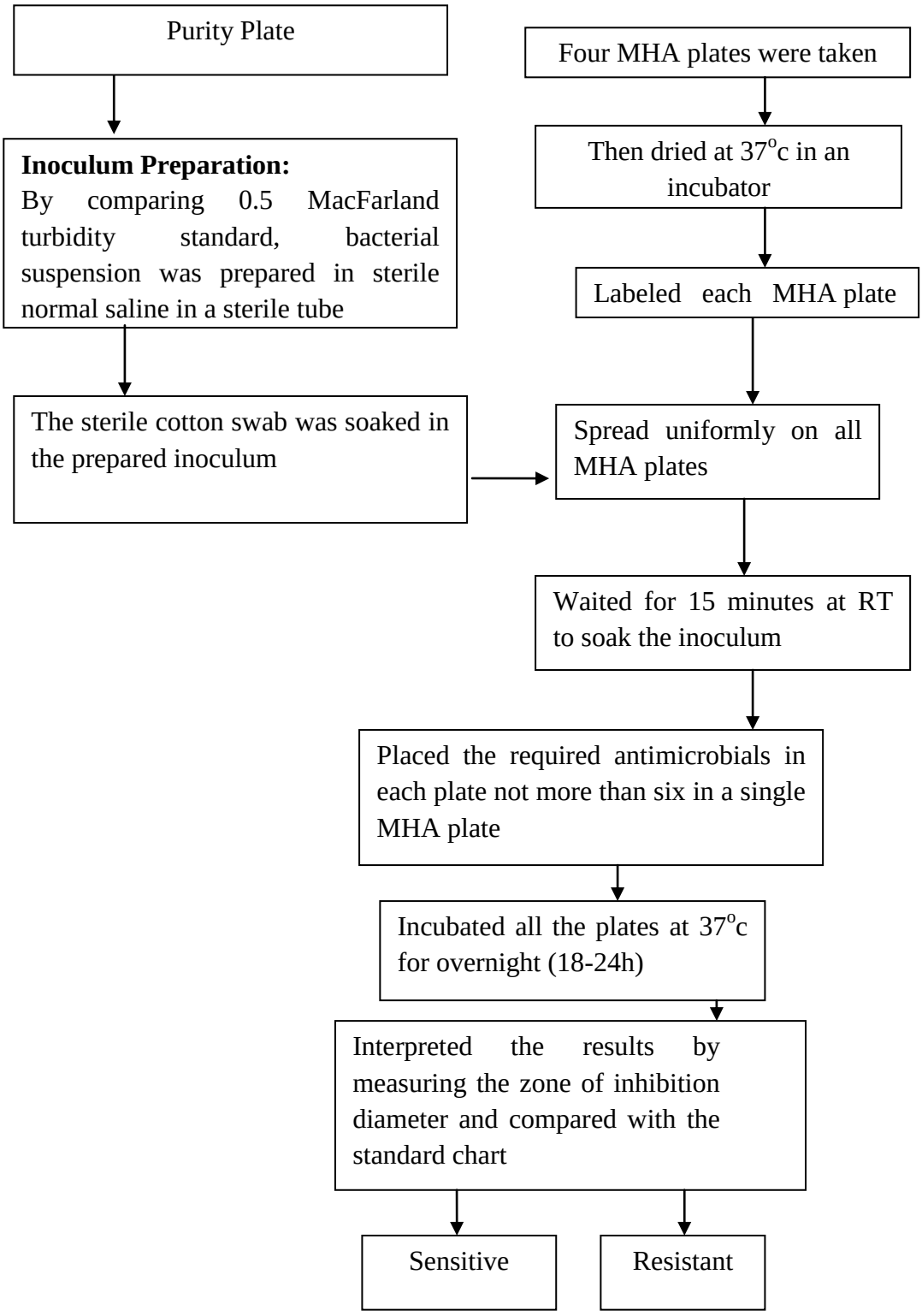

Fig.3 Flow Chart of antimicrobial susceptibility testing of *Acinetobacter* spp.

CHAPTER-V

5. RESULTS

A total of 5965 clinical specimens received at Department of Microbiology of Kathmandu Medical College, Sinamangal were processed for the isolation and identification of different bacteria from October 2009 to March 2010. Bacterial isolates resembling non-fermenters were screened for *Acinetobacter* spp. by oxidase test. The screened *Acinetobacter* spp. were sub-cultured in Sheep Blood agar and MacConkey agar to obtain pure colonies and confirmed by gram's staining, oxidase test, motility test and other different routine biochemical tests. Then the prevalence of *Acinetobacter* spp was determined. The age-wise, gender-wise, and ward-wise distribution of the isolated *Acinetobacter* spp was also determined along with the record of demographic parameters, clinical history, prior antibiotic use, etc.

5.1. Gender wise distribution of various specimens

A total 5965 specimens received were processed containing various types of specimens, among these, 2846 (47.7%) were from males and 3119 (52.3%) were from females. (Table5.1)

Table5.1. Gender wise distribution of various specimens

Types of Specimens	Number of specimens N, (%)		
	Males	Females	Total
Urine	1240 (43.6)	1554 (49.8)	2794 (46.8)
Blood	960 (33.7)	747 (23.9)	1707(28.6)
Exudates	318 (11.2)	497 (15.9)	815 (13.7)
Respiratory specimens	143 (5.0)	159 (5.1)	302 (5.1)
Body Fluids	131 (4.6)	117 (3.4)	248 (4.2)
Catheter tips	30(1.1)	28 (0.9)	58(1.0)
Miscellaneous	24 (0.8)	17 (0.5)	41(0.7)
Total	2846 (47.7)	3119 (52.3)	5965

5.2. Bacteriological profiles of various specimens

Out of total 5965 clinical specimens received and processed, 810 (13.6%) specimens were growth positives of which 713 (88%) bacterial isolates were gram negative and 97 (12.0%) isolates were gram positive bacteria. (Table5.2)

Table5.2. Bacteriological profiles of various clinical specimens

Types of Bacteria	Number of isolates in different specimens, n/(%)							Total number of isolates N (%)
Gram Negative Bacteria:	Urine	Blood	Exudates	Respiratory	Body Fluids	Catheter tips	Misce-llaneous	
Escherichia coli	370 (78.1)	8 (17.4)	54 (54)	5 (11.4)	8 (61.5)	7 (23.3)	2 (33.3)	454 (63.7)
Acinetobacter spp.	31 (6.5)	10 (21.7)	6 (6)	13 (29.5)	2 (15.4)	9 (30.0)	3 (50.0)	74 (10.4)
Klebsiella spp.	34 (7.2)	6 (13.0)	15 (15)	9 (20.5)	1 (7.7)	8 (26.7)	0	73 (10.2)
Citrobacter freundii	10 (2.1)	1 (2.2)	14 (14)	6 (13.6)	1 (7.7)	4 (13.3)	1 (16.7)	37 (5.2)
Proteus spp.	16 (3.4)	2 (4.4)	7 (7)	0	1 (7.7)	2 (6.6)	0	28 (3.9)
P. aeruginosa	8 (1.7)	0	2 (2)	8 (18.2)	0	0	0	18 (2.5)
Salmonella spp.	0	17 (36.9)	0	0	0	0	0	17 (2.4)
Enterobacter spp.	4 (o.8)	1 (2.2)	2 (2)	3 (6.8)	0	0	0	10 (1.4)
Serratia marcescens	1 (0.1)	0	0	0	0	0	0	1 (0.1)
Stenotrophomonas maltophila	0	1 (2.2)	0	0	0	0	0	1 (0.1)
Total	**474 (95.6)**	**46 (92.0)**	**100 (60.6)**	**44 (97.8)**	**13 (81.3)**	**30 (100)**	**6 (75.0)**	**713 (88.0)**
Gram Positive Bacteria:								
Staphylococcus aureus	10 (45.5)	2 (50)	59 (91.8)	1 (100)	3 (100)	0	2 (100)	77 (79.4)
Staph. saprophyticus	8 (36.4)	0	0	0	0	0	0	8 (8.2)
CoNS	0	2 (50)	6 (9.2)	0	0	0	0	8 (8.2)
Enterococcus faecalis	4 (18.2)	0	0	0	0	0	0	4 (4.1)
Total	**22 (4.4)**	**4 (8.0)**	**65 (39.4)**	**1 (2.2)**	**3 (18.7)**	**0**	**2 (25.0)**	**97 (12.0)**
Grand Total	**496 (61.2)**	**50 (6.2)**	**165 (20.4)**	**45 (5.6)**	**16 (1.9)**	**30 (3.7)**	**8 (0.9)**	**810**

5.3. Prevalence of *Acinetobacter* spp. in different specimens

The prevalence of *Acinetobacter* spp. was 9.1% out of total 810 bacterial isolates and 10.4% out of 713 total gram negative bacterial isolates. Among total 74 *Acinetobacter* spp isolated, the highest prevalence (41.9%) was found in urine specimens and least (2.7%) in body fluids. The highest prevalence was found in miscellaneous specimens (50%) and least in pus/swabs (6%) among gram negative bacterial isolates from different specimens. (Table5.3)

Table5.3. Prevalence of *Acinetobacter* spp. in different specimens

Types of Specimens	Number of total Bacterial Isolates	Number of total GNB Isolates	Number of *Acinetobacter* Isolates	Prevalence of *Acinetobacter spp.* in percentages		
				Among total bacterial isolates	Among total GNB isolates	Among total *Acinetobacter* isolates (n=74)
Urine	496	474	31	6.2	6.5	41.9
Blood	50	46	10	20.0	21.74	13.5
Exudates	165	100	6	3.64	6.0	8.1
Respiratory	45	44	13	28.9	29.5	17.6
Body fluids	16	13	2	12.5	15.4	2.7
Catheter tips	30	30	9	30	30	12.2
Miscellaneous	8	6	3	37.5	50	4.1
Total	**810**	**713**	**74**	**9.1**	**10.4**	**100.0**

5.4. Gender- wise distribution of *Acinetobacter* spp in various specimens (n=74)

In our study, the distribution of isolated *Acinetobacter* spp in different specimens was higher in males than in females except in urine and exudates. In cases of body fluids and miscellaneous specimens *Acinetobacter* spp were found only from males. (Table5.4)

Table5.4. Gender- wise distribution of *Acinetobacter* spp in various specimens

Types of Specimens	Number of specimens N, (%)		
	Males	Females	Total
Urine	12(38.7)	19(61.3)	**31(41.9)**
Blood	6(60)	4(40)	**10(13.5)**
Exudates	3(50)	3(50)	6(8.1)
Respiratory	8(61.5)	5(38.5)	13(17.6)
Body Fluids	2(100)	0	2(2.7)
Catheter tips	5(55.6)	4(44.4)	9(12.2)
Miscellaneous	3(100)	0	3(4.1)
Total	**39(52.7**)	**35(47.3)**	74

5.5. Distribution of *Acinetobacter* spp in different age-groups from different specimens

Out of 74 total isolates of *Acinetobactor* spp. 39 (52.7%) were from male patients whereas 35 (47.3%) were from females and the distribution was found to be highest, 24 (32.5%) in age groups (21-30) years and least, 5 (6.7%) in age groups below 10. (Table5.5)

Table5.5 Distribution of *Acinetobacter* spp in different age-groups from different specimens (n=74)

Age group in years	No. of *Acinetobacter* isolates in different specimens							No. of *Acinetobacter* isolates in		
	urine	blood	Respiratory	exudate	Catheter tips	body fluids	miscellaneous	Males n (%)	Females n(%)	Total N (%)
≤10	0	5	0	0	0	0	0	3(7.7)	2(5.7)	5(6.7)
11-20	0	2	0	0	1	0	0	2(5.1)	1(2.8)	3(4.0)
21-30	**15**	**2**	**1**	**0**	**2**	**1**	**2**	**11(28.2)**	**13(37.1)**	**24(32.4)**
31-40	7	1	0	0	0	0	0	4(10.2)	4(11.4)	8(10.8)
41-50	4	0	2	0	1	0	1	4(10.2)	8(22.8)	12(16.2)
51-60	1	0	6	1	3	0	0	6(15.4)	2(5.7)	8(10.8)
≥60	4	0	4	5	2	1	0	9(23.1)	5(14.3)	14(17.5)
Total	31	10	13	6	9	2	3	39(52.7)	35(47.3)	74

5.6 Distribution of *Acinetobacter isolates* in various wards

Among different wards, the distribution of *Acinetobacter* was found to be highest in general ICU with 19 (25.7%) isolates and least in surgical wards with only 2 (2.7%) isolates. In total, the prevalence of *Acinetobacter spp.* was higher in different intensive care units (ICU) than in other wards. (Table.5.6)

Table.5.6 Gender-wise distribution of *Acinetobacter* isolates in various wards (n=74)

Different Wards	No of *Acinetobacter* isolates in			Percentage (%) of *Acinetobacter* isolates		
	Male	Female	Total	Male	Female	Total
MICU	11	5	16	14.86	6.75	21.62
NICU	3	1	4	4.05	1.35	5.40
General ICU	10	9	19	13.51	12.16	25.67
SHCU	4	9	13	5.40	12.16	17.56
Post-operative	4	3	7	5.40	4.05	9.45
Orthopedic	3	0	3	4.05	0	4.05
Medical Words	1	4	5	1.35	5.40	6.75
Surgery	1	1	2	1.35	1.35	2.70
Plastic Surgery/ Burn ward	1	1	2	1.35	1.35	2.70
Other wards	1	2	3	1.35	2.70	4.05
Total	39	35	74	52.70	47.30	100

5.7 Clinical characterization of patients infected with *Acinetobacter* spp.

Among total 74 isolates of *Acinetobacter* spp. all isolates were hospital acquired. Among all 74 *Acinetobacter* positive patients, 52 (70.27%) patients were admitted to ICUs, 46 (60.18%) needed mechanical ventilation, 30 (40.54%) used urinary and IV catheters, 13 (17.56%) patients had endotracheal intubations, 39 (53.7%) patients had existing chronic illness, and 35 (47.29%) had surgery. (Table.5.7)

Table.5.7 Clinical characterization of patients infected with *Acinetobacter* spp. *(n=74)*

Characteristics	No. of Cases/74	Total Percentage %
Hospital Stay (days):		
1 - 7	23	31.08 %
More than 7	51	68.92 %
Risk Factor Distribution:		
Admission to ICU	52	70.3%
Mechanical Ventilation	45	60.8 %
Urinary and IV catheterization	30	40.5 %
Endotracheal Intubations	13	17.6 %
Existing Chronic Ilness	39	52.7 %
Surgery	35	47.3%

5.8. Antibiotic susceptibility pattern in 74 totals *Acinetobacter* isolates from different clinical specimens

Among 24 different antimicrobials tested, Meropenem, Imipenem, Co-trimoxazole, Cefoperazone/Sulbactam, Ceftriaxone and Chloramphenicol showed highest susceptibility as 94.6%, 89.2%, 83.8%, 73.3%, 86.5% and 78.4% respectively (Table5.8.1) but other antimicrobials showed poor susceptibility and no susceptibility at all. (Table5.8.2 and Table.5.8.3)

Table 5.8.1 Susceptibility pattern of *Acinetobacter* isolates against different antimicrobials from different specimens in percentage (n=74)

Antibiotics used and their symbols	Conc. (μg/ml)	Susceptibility pattern of *Acinetobacter* isolates in Percentages (%) to different antimicrobials from different clinical isolates							
		Urine (31)	Blood (10)	Resp-ratory (13)	Exud-ates (6)	Cath-eter Tips (9)	Body fluids (2)	Other (3)	Total isolates (74)
Imipenem (I)	10	100.0	100.0	69.2	83.3	66.7	100.0	100.0	**89.2**
Meropenem (MR)	10	100.0	100.0	84.6	100.0	77.8	100.0	100.0	**94.6**
Cefoperazone-Sulbactam (CPS)	100	80.6	60.0	53.8	50.0	22.2	100.0	0.0	**70.3**
Ceftriaxone (CI)	30	93.5	100.0	69.2	83.3	66.7	100.0	100.0	**86.5**
Chloramphenicol (C)	30	83.9	80.0	53.8	66.7	88.9	100.0	100.0	**78.4**
Co-trimoxazole(Co)	25	90.3	100.0	61.5	66.7	77.7	100.0	100.0	**83.8**
Carbenicillin(Cb)	100	16.1	10.0	23.0	16.7	22.2	0.0	0.0	17.6
Piperacillin(PC)	100	16.1	30.0	23.0	16.7	22.2	0.0	0.0	18.9
Ceftazidime (Ca)	30	19.4	40.0	23.0	33.3	22.2	50.0	33.3	25.7
Gentamicin (G)	10	25.8	40.0	30.8	33.3	33.3	50.0	33.3	31.1
Amikacin(Ak)	30	32.3	40.0	30.8	33.3	33.3	50.0	33.3	33.8
Tobramycin (Tb)	10	6.5	20.0	15.4	0.0	11.1	50.0	0.0	10.8
Netilmicin (Nt)	30	22.6	20.0	15.4	16.7	11.1	50.0	0.0	18.9
Tetracycline (T)	30	9.7	60.0	15.4	16.7	22.2	0.0	0.0	18.9
Ciprofloxacin(Cf)	05	32.3	30.0	23.0	33.3	22.2	50.0	0.0	28.4
Ofloxacin(Of)	05	19.4	20.0	15.4	16.7	22.2	50.0	0.0	18.9

Table.5.8.2. Antimicrobials not susceptible at all to all isolates of *Acinetobacter* spp from different clinical specimens

S. N.	Antibiotics used and their symbols	Concentration (μg/ml)	Susceptibility profile of *Acinetobacter* isolates in Percentages (%) to different antimicrobials
1.	Amoxycillin (Am)	30	0
2.	Amoxy-clave (Ac)	30	0
3.	Cephalexin (Cp)	30	0
4.	Cefuroxime (Cu)	30	0
5.	Cepfodoxime (Cep)	10	0
6.	Cefoxitin (Fox)	30	0
7.	Cephalothin (Kz)	30	0
8.	Kanamycin (K)	30	0

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

The incidence of Nosocomial infection caused by *Acinetobacter* is rarely reported in Nepal, as compared to other South Asian countries and if reported there is not uniformity in the reporting pattern. In KMC hospital, *Acinetobacter* spp. are more frequently reported from different clinical specimens and most of these isolates are interestingly found to be susceptible against ceftriaxone, and co-trimoxazole in addition to carbapenems. Therefore, this study was designed to determine the prevalence and search for the antimicrobials most suitable for the therapeutic option in KMC, Sinamangal Kathmandu, Nepal (A 700-bedded tertiary care hospital situated at the heart of Kathmandu Valley).

Of the total 5965 specimens received, 810(13.6%) specimens were growth positives of which gram negative bacteria predominated with 713 (88.0%) isolates. Same type of result was obtained in previous studies conducted in Nepal (Shrestha et al., 2007; Baral, 2008; Basnet et al., 2009) and in other countries (Blomberg et al., 2005).

In this study, the prevalence of *Acinetobacter* spp. from different clinical specimens was 10.4% among 713 gram negative bacterial isolates which was similar (11.3%) in a study conducted in Hong Kong (Siau et al., 1996). Among these 713 gram negative bacterial isolates the highest prevalence was found in miscellaneous specimens (50%) followed by different tip specimens (30%), respiratory tract specimens (29.5%), blood specimens (21.7%), urine (6.5%) and lowest in different pus/swab specimens (6.0%).The prevalence of *Acinetobacter* ranged from 2% to 10% of all gram negative bacterial infections in Europe (Hanberger et al., 1999), 2.5% in the United States (Jones et al., 2004) and 8.4% in CMC hospital Ludhiana-141008, Punjab,India (Oberoi et al., 2009).

In our study, the overall prevalence of *Acinetobacter* spp. was 9.1% (74/810). This was comparable with the similar study done in India and Hong Kong where the prevalence of *Acinetobacter* spp was in between 7.4% to 9.5% (Siau et al., 1996; Joshi et al., 2006). Among 74 isolates of *Acinetobacter* in our study, the prevalence was found to be highest from urine specimens, (41.9%) followed by respiratory specimens (17.6%), blood specimens (13.5%), tip specimens (12.2%), and least (2.7%) from different body fluids. Similar study conducted in different institutions showed the prevalence of *Acinetobacter* spp. ranging from 15.2% to 19% (Roussel et al. 1996 and Sakata et al., 1998). In the study of Mishra and Bhujwala (1986), maximum isolates were from pus (46.6%) followed by blood (21.3%) out of total 75 isolates of *Acinetobacter* spp. while in the study of Pedersen (1970), maximum isolates were from sputum (26.3%) and from urine (21.3%) out of total 72 isolates of *Acinetobacter* spp.The variable prevalence of *Acinetobacter* spp. in different studies might be due to local situation, arrangement of specimens in the study groups, type of institution, local use of antibiotics, sanitary conditions, the number of specimens processed and the rates of bacterial isolation and the study period involved etc. In our study, urine specimens were predominantly received for culture as compared to other specimens and hence the high figure of prevalence (41.9%) was obtained.

In our study, the distribution of *Acinetobacter* from different wards was the highest in intensive care units (70.27%) which were also similar with the study conducted in Hong Kong and India (Siau et al., 1996). In our study, a slightly higher incidence was found in males (52.7%) than in females (47.3%) which were also comparable with other such type of study (Siau et al., 1996). This might be due to large number of males admitted to the intensive care units; more necessicity of medical instruments to the males; and more males suffered from road-traffic accidents etc. However, no correlation studies have been done till now.

In our study, the prevalence of *Acinetobacter* was found to be highest (32.5%) in age groups 21-30 yrs and it was the least (5.4%) in age groups below 20 yrs. Among 39 *Acinetobacter* isolates from males, the prevalence was the highest

(28.2%) in age groups (21-30) yrs and least (7.7%) in age groups below 10 years. Again, among 35 isolates of *Acinetobacter* from females, the prevalence was highest (37.1%) in age groups (21-30) years and least (2.8%) in age groups below 10.The olders above 40 years were ranked in the second position by the infection of *Acinetobacter* spp. in both males and females. Our report was in favour of the previous reports conducted in different places from 1957-1979 (Glew et al. 1977; Lothe and Griffin, 1965; Rocha and Guze, 1957; Rosenthal et al., 1977 and Wallace et al., 1976). Similar type of study conducted in Denmark showed the higher prevalence of *Acinetobacter* in both males and females of above 40 years, lower prevalence in age groups 21-40 years and least in age groups below 20 years (Hoffmann et al. 1982).

In our study, among total 74 isolates of *Acinetobacter* spp. all isolates were hospital acquired , among these 31.08% were isolated from the patients within 1-7 days of hospital stay and 68.92% isolates were isolated from patients with more than 7 days of hospital stay (7 to 120 days). But similar study conducted in India showed about 80.2% of *Acinetobacter* spp. from hospital patients and 19.8% from community-acquired OPD cases. (http://www.IndianJmedsci.org.2000). A stay in a high-risk unit has been identified as a risk-factor in previous studies (Prashantanath and Badrinath, 2006; Cisneros and Rodriguez-Bano, 2002).

In our study, the prevalence of *Acinetobacter* among 474 total GNB isolates from urine was 6.5% of which 38.7% were from males and 61.3% were from females. The prevalence of *Acinetobacter* in urine specimens ranged 8.23% to 51.97% in a similar study conducted by different researchers at different places (Pedersen et al., 1970; Lahiri et al., 2004; Oberoi et al., 2009).The higher percentage of females in our study corresponds to the predominance of women among patients in the hospital with UTI and the females get infected more than the males due to anatomical structure of the female urogenital structures. In our study, the high prevalence of *Acinetobacter* infection causing UTI was highest in age groups (21-30) years and least in age groups below 10 years. This is in favour of the fact that UTI is more common in sexually active adult and least in children before puberty. The high prevalence of *Acinetobacter* in urine

specimens in our study might be due to large number of urine specimens processed and large number of bacterial isolates from these specimens. Factors predisposing persons to urinary tract infections caused by *Acinetobacter* are chronic pyelonephritis, diabetes mellitus, malformation of the urinary tract, urethral obstruction with hydronephrosis, neurologic bladder disorders, calculus, urological surgery, bladder catheterization, instrumental examination of urinary tract, incontinence due to a gynecological disorder, residence in an intensive care unit and prior antibiotic therapy (Rocha and Guze , 1957; Reynolds and Cluff 1963; Robinson et al., 1964;Gardner et al., 1970; Thong , 1975). But in our study, no information on such all factors was available.

In our study, the prevalence of *Acinetobacter* spp. from blood specimens was 13.5% out of 74 isolates. In the similar study conducted by different researcher at different institutions the prevalence ranged 9.9% to 21.3% (Mishra and Bhujwala, 1986; Garcia-Garmendia et al., 2001; Santucci et al., 2003; Lahiri et al., 2004;). In our study, the isolation rate of bacteria was low from blood specimens and isolation of *Acinetobacter* was also low but it seemed to be noticeable for the awareness for the clinicians and microbiologists in the future. Again, in our study, the prevalence of *Acinetobacter* was 20.0% among 50 total blood isolates and 21.7% among 46 total 46 GNB isolates from blood which was almost similar (17.2%) with the study conducted in India (Sharma et al., 2004). But the prevalence of *Acinetobacter* in blood specimens was low (1.5% to 6.9%) in the similar study conducted in Nepal, Hong Kong, India, and Japan (Nippon Rinsho and Iinuma, 2002; Khanal et al., 2004; Arora and Pushpa Devi, 2007). In our study, *Acinetobacter* spp. was the second most common gram negative isolate from blood specimens after Salmonella spp. which was also Nippon Rinsho and Iinuma, 2002 comparable with the previous report (Zakuan et al., 2009). According to the SENTRY antimicrobial resistance surveillance program *Acinetobacter* spp. was among the ten most frequently isolated pathogens causing blood stream infection (BSI) in 14 European countries particularly in the program from 1997-2002 (Biedenbach et al., 2004).

In our study, among 10 isolates of *Acinetobacter* from blood, 60% isolates were from males and 40% were from females. This finding was comparable with the similar study conducted in Estonea where 56.9% were males and 43.1% females. A longer stay at high risk unit had been identified as risk factor in several studies (Koeleman et al., 1997; Lee et al; 2004).The use of broad spectrum antimicrobial agents have been identified as risk factors for acquisition of *Acinetobacter* infection in several other studies (Lortholary et al., 1995, Koeleman et al., 1997; Garcia- Garmendia et al. 2001). The second most common risk factor identified in case control studies was mechanical ventilation (Falgas and Kopterides, 2006).

The prevalence of *Acinetobacter* spp. in different pus specimens was 8.1% Out of total 74 isolates of *Acinetobacter*. Similar studies conducted by different researchers showed 6.8% to 86.2% prevalence of *Acinetobacter* spp in different pus specimens (Mishra and Bhujwala; 1986; Siau et al., 1996; Ghimire et al; 2002; Lahiri et al; 2004; Jones et al., 2004; Oberoi et al; 2009). In our study, the low prevalence of *Acinetobacter* spp. in different pus specimens might be due to less number of pus specimens and less number of bacterial isolates from these specimens. The patients from whom specimens were received might be from outpatient department (OPD) or other general wards where predisposing factors such as ICU stay, administration of third generation cephalosporins, use of ventilator, catheter and other devices were minimum.

In our study, the prevalence of *Acinetobacter* spp. in different respiratory tract specimens was 17.6% among 74 total isolates of *Acinetobacter* spp. This result was slightly lower than the similar studies conducted by different researchers such as 20.4% (Lahiri et al; 2004), 26.4% (Pedersen et al; 1970) and 32.2% (Ghimire et al; 2002). Again, in our study, the prevalence of *Acinetobacter* among 810 total bacterial isolates was 1.6% (13/810) which was almost similar with the study conducted in India, 2.6% (Lahiri et al., 2004) and slightly lower than that of the study conducted in BPKIHS, Nepal (4.3%)(Ghimire et al., 2002). *Acinetobacters* cause nosocomial pneumonia in ICU with a frequency of 3 to 5% even higher in patients with mechanical ventilation with crude mortality rate of 30 to 75% (Torres et al., 1990).

In our study, the prevalence of *Acinetobacter* spp. in catheter tips was 12.2% (9/74) among 74 total *Acinetobacter* isolates. Studies from India and France showed 4.6% to 15.5% prevalence of *Acinetobacter* spp. in Catheter tips (Lahiri et al; 2004). The prevalence was higher 55.6% in males than in females, 44.4 %. This may be due to predominant need of catheterization for males due to enlarged prostate or other causes than for females. Catheterization; due to colonization of *Acinetobacter* more frequently, is the common risk factor for *Acinetobacter* infection (Lortholary et al., 1995).

In our study, among 74 total isolates of *Acinetobacter*, 2.7% isolates were from body fluids. Similar study conducted in Hong Kong showed the prevalence rate of 5.8% in general ward to 11.1% in ICU which was much higher than in our study (Siau et al., 1996). The study at the Division of Nephrology, National University Hospital, Singapore revealed 13 episodes of acute peritonitis (AP) in eleven patients over an 18 month period accounting for 14.3% of the total number of peritonitis episodes (Lye et al., 1989).The prevalence of *Acinetobacter* was 12.5% and15.4% among total 16 bacterial isolates and 13 total GNB isolates from body fluids respectively. The number of fluid specimens processed was less than the other specimens in our study, and as a result the isolation rate and prevalence of *Acinetobacter* spp. was lower. There are very few reports of *Acinetobacter* infection in body fluids. *Acinetobacter* infections involve every organ systems with high fluids content such as respiratory tract, blood, CSF, peritoneal fluids, urinary tract etc (Bernards et al., 2004). *Acinetobacter* can cause peritonitis and pleuritis in patients undergoing continuous ambulatory peritoneal dialysis where technique failure and diabetes mellitus are the main underlying risk factors (Galvo et al., 1989). Peritonitis is still the most important complication leading to increased morbidity and patient dropout from the CAPD program (Nolph et al., 1980; Prowant et al., 1983). The prevalence range differs depending on the number of the specimens processed in different institutions. So, our prevalence data may differ with other prevalence data in different institutions conducted by different researchers. So we recommend conducting the study regarding the

prevalence of *Acinetobacter* in different body fluids at different climates and seasons.

In our study, the prevalence of *Acinetobacter* spp. in miscellaneous specimens such as Dj- shunt, different tissues and pseudocyst was 4.1% among total 74 *Acintetobacter* isolates and 37.5% and 50% of the total bacterial isolates and total GNB isolates from these specimens respectively. This result was within the range of 1.97% to 18% obtained by different researchers at different times in different countries such as in Hong Kong, India, USA, France and Belgium (Siau et al., 1996; Lahiri et al., 2004). The lower prevalence in our study might be due to small number of specimens and low number of total bacterial isolates. The isolation rate of *Acinetobacter* in DJ-shunt was maximum 37.5% (3/8). The possible reason behind variable results in different studies might be due to type of specimens included under miscellaneous specimens. In our study, all isolates of *Acinetobacter* were from DJ-shunt alone which is vulnerable medical device to *Acinetobacter* colonization and is found to be important risk factor for the infection of *Acinetobacter* (Lortholary et al., 1995). It has been shown that *Acinetobacter* spp. can form biofilm on the surface of various implants and also in the environment (Bano et al., 2008).

In this study, all isolates from different clinical specimens were tested against 24 antimicrobial drugs by the Modified Kirby-Bauer disc-diffusion technique (CLSI M02-A9) using Mueller Hinton Agar (MHA, Hi-Media Laboratories, and Mumbai, India). Among 24 different antimicrobials tested, Meropenem, Imipenem, Co-trimoxazole, Cefoperazone/Sulbactam, Ceftriaxone and Chloramphenicol showed more than 70% susceptibility as 94.6%, 89.2%, 83.8%, 73.3%, 86.5% and 78.4% respectively. Similar type of antimicrobial susceptibility pattern was obtained in Hong Kong and India (Siau et al., 1996; Gaur et al., 2008). However, the variable reports of different antimicrobials against *Acinetobacter* in various researches conducted at different places are seen (Ghimire et al. 2002; Jones et al. 2004; Khanal et al. 2004; Lahiri et al. 2004; Basnet et al. 2009; and Oberoi et al. 2009).

In our study, out of five antimicrobials of penicillin-group used, amoxycillin, piperacillin and carbenicillin were (81.1% to 100%)) resistance to all isolates of *Acinetobacter* whereas imipenem and meropenems were most susceptible at 89.2% and 94.6% respectively. *Acinetobacter* is resistant to most β-lactam antibiotics, particularly penicillins and cephalosporins, especially in ICU patients and ceftazidime, piperacillin and carbapenems are among the β-lactam antibiotics most active against *A. baumannii* (Seifert et al., 1993; Vila et al., 1993 and Shi et al., 1996). The main mechanism of resistance to β-lactam antibiotics in *Acinetobacter* spp. is the production of β-lactamases encoded either by the chromosome or by plasmids (Amyes and Young, 1996), the low permeability of the outer-membrane of *Acinetobacter* resulting from the small outer-membrane pore size and/or limited porin production as well as alterations in the affinity of penicillin-binding proteins (Sato and Nakae, 1991) and over-expression of the chromosomal cephalosporinase AmpC (Danes et al., 2002) has been implicated in the resistance of *Acinetobacter* to these antibiotics. Resistance to ampicillin, carbenicillin and piperacillin has been attributed to the production of TEM-1 (Ruiz et al. 1999), TEM-2 (Devaud et al., 1982), OXA-21 (Danes et al., 2002) β-lactamase. The non-TEM, non-SHV extended-spectrum β-lactamases PER-1 and VEB-1 are the only extended-spectrum β-lactamases reported to date in *A. baumannii* (Vahaboglu et al., 1997; Poirel et al., 2003).

In our study, carbapenems such as imipenem and meropenems were most susceptible at 89.2% and 94.6% respectively and were found to be the drug of choice. Similar high (97%) susceptibility rate of *A. baumannii* to imipenem were reported from Saudi Arabia and Japan (Ishii et al., 2005; Al-Tawfig and Mohandhas, 2007). In our study, the sensitivity of carbapenem is higher than in other studies because of its lower usage. The lower resistance rate of *Acinetobacter* spp. to imipenem in our study might be due to its recent introduction for use in this hospital and hence low selective pressure created. Higher degree of carbapenem resistance for imipenem (40.3%) and meropenon (19.2%) was seen among EBSL negative *Acinetobacter* spp (Kandel, 2010). In contrast to our study, 9.6 to 49.3% resistance to imipenem was observed in Teharan, Turkey, Spain, Israel and Italy (Karsligil et al., 2004; Cisners et al.,

2005; Navon-venezia et al., 2007; Baran et al., 2008; Capone et al., 2008 and Feizabadi et al., 2008). Carbapenem-resistant *Acinetobacter baumannii* has emerged in many parts of the world which is due to reduced permeability by porin channel down regulation (porin loss), unregulated efflux of the drug, PBP with reduced affinity and most importantly the production of plasmid borne AmpC like β-lactamases, different class B (metallo-β-lactamases) and class D ß-lactamases (oxacillinases) and *Klebsiella pneumoniae* carbapenemases (Livermore, 2002; Poirel and Nordmann, 2002). Beta-lactamases and outer membrane alterations work together to confer resistance to beta-lactam agents (Bonomo et al., 2006).The main mechanism of carbapenem resistance is through the acquition of B (IMP and VIM types) and D class (members of the OXA-23- and OXA-24-related families) carbapenemases (Go et al., 1994; Merz et al., 2004; Ishii et al., 2005) and AmpC (Sinha and Srinivasa, 2007). Moreover, co-existence of blaAmpC and blaCTX-M genes in bacteria with deceased outer membrane permeability may lead to carbapenem resistance (Shahid et al., 2007; Wang et al. 2009). Studies have shown a high incidence of resistance to carbapenem among *Acinetobacters* from patients in intensive care units, suggesting that intensive care units are the epicenter for carbapenem-resistant *Acinetobacters* (Manikal et al., 2000; Corbella et al., 2000).

In our study, Co-amoxyclav, sulbactam-cefoperazone and Co-trimoxazole were tested against all isolates of *Acinetobacter* spp. as combined drug tests. Among these drugs Co-amoxyclav was not susceptible at all whereas sulbactam-cefoperazone and Co-trimoxazole were susceptible in 73.3% and 78.4% of the total isolates respectively. Amoxy-clav was 22.3% susceptible in Hong Kong (Siau et al., 1996), 52.3-67.0% susceptible in India (Sharma, 2004 and Lahiri et al. 2004) and 91.0% susceptible in 1990 from patients in eight Dutch hospitals (Buirma et al., 1991).The main mechanisms of resistance to amoxicillin/clavulanic acid in our study might be due to inactivation by those bacterial beta-lactamases that are not themselves inhibited by clavulanic acid, including class B, C and D; alteration of PBPs, which reduce the affinity of the antibacterial agent for the target and impermeability of bacteria or efflux pump mechanisms.

In our study, 73.3% isolates of *Acinetobacters* were sensitive to cefoperazone-sulbactam combination which was almost similar with the observation conducted in India and China where the susceptibility ranged from 89% to 98% (Wang et al., 2000; Zhonghua et al.; 2000; Oberoi et al., 2004; Gaur et al., 2008). In study conducted at different places, more than 71% of the cefoperazone-resistant *Acinetobacter* species were susceptible to the cefoperazone-sulbactam combination due to the in-vitro intrinsic activity of sulbactam against *Acinetobacter* species (Traub and Spohr, 1989; Urban et al., 1993). Hence, in our study too, the synergistic effect of sulbactam was seen to cefoperazone and we recommend performing combined-drug tests to find the synergistic effect of sulbactam and other beta-lactam antibiotics regularly in KMC Hospital. This will be valuable and more informative to the clinicians as an option for treatment in *Acinetobacter* infection.

In our study, Co-trimoxazole was found to be susceptible 78.4% of the total *Acinetobacter* isolates which was also similar with the study conducted in Spain and Itali (Vila et al. 1993; Capone et al., 2008). A moderate susceptible isolates were obtained India (Sharma, 2004). The studies conducted in Spain, Turkey, Slovakia and Russia from 1997 to 2002, 56.2%- 88% isolates of *Acinetobacter* spp. were resistant to trimethoprim–sulphamethoxazole (Martin-Lozano et al., 1996; Kocazeybek et al., 1999; Hostacka and Klokocnikova, 2002 and Stratchounski et al., 2002). The prevalence of trimethoprim-sulfamethoxazole (Co-trimoxazole) resistance in *A. baumannii* is high in many geographic regions (Van Looveren and Goossens**,** 2004; Gu et al., 2007). Genes coding for trimethoprim (dhfr) resistance have been reported within integron structures in *A. baumannii* (Houang et al., 2003; Gu et al., 2007). Co-trimoxazole resistance occurs by (i) overproduction of host DHFR, (ii) mutation in the structural gene for DHFR and (iii) acquisition of the dfhr gene encoding a resistant form. In our study, the high susceptibility of Co-trimoxazole might be due to its rare use in recent days in treating various infections resulting in reduced selective pressure on bacteria. This observation was also notable information to the clinicians and microbiologists as an alternative option for the treatment of patients. However, we recommend evaluating in-vitro as well as in- vivo susceptibility tests of Co-trimoxazole by

other CLSI recommended methods in this hospital and other institutions in Nepal to find out the actual scenario.

In our study, almost all *Acinetobacter* spp. isolates were resistant to cephalosporins ranging from 10% to 100%. Among them, cephalexin, cefuroxime, cefpodoxime, cofoxitin and cephalothin were completely resistant to all isolates.This might be due to overuse, misuse and most frequent use of cephalosporins randomly by patients themselves, pharmacy and even by the clinicians too. Similar results were obtained in different clinical centers in Nepal and in Italy where almost MDR *Acinetobacter* spp. isolates were resistant to cefepime, ceftazidime, and aztreonam (Shrestha et al., 2007; Baral, 2008; Capone et al., 2008; Kattel et al., 2008 and Basnet et al., 2009). In a study conducted in Nepal, 80% ESBL negative *Acinetobacter* spp. were susceptible to ceftazidime (Kandel, 2010). This higher cephalosporin resistance among these organisms might be due to hyper production of AmpC- beta lactamase rather than ESBLS. In our study, among all cephalosporins tested, ceftriaxone was the most susceptible (86.5%) antimicrobial but high level (74.3%) of resistance was noticed for ceftazidime. Similar high resistant *Acinetobacter* spp. to ceftazidime was obtained in a study conducted in Nepal (Ghimire et al, 2002).This is in contrast to the report obtained in India and Nepal where ceftazidime was 88-90% sensitive (Oberoi et al. 2009 and Basnet et al, 2009). The resistance of cephalosorins occurs by a distinct family of beta-lactamases, the *Acinetobacter*-derived cephalosporinases (ADCs) (Vahaboglu et al., 1997). In our study, among the cephalosporin-group of antimicrobial tested the drug of choice was ceftriaxone which is a notable thing to treat the infections caused by *Acinetobacter* spp. in this threatful era of the pan-drug resistance *Acinetobacter* infections.

In our study, out of 5 aminoglycosides tested, the overall susceptibility pattern ranged between (0%) to 33.8%. In other studies conducted in Nepal, *Acinetobacter* spp. were 88.04-96.6% and 40.0- 96.6% resistant to amikacin and gentamicin respectively (Ghimire et al., 2002; khanal et al., 2008; Basnet et al., 2009; and Kandel, 2010). Higher amikacin and gentamicin reistance have also been observed in other places at different times (Elston and Hoffman,

1966; Crues et al. 1979 and Hoffmann et al. 1982). Only 8.7% of 80 isolates from ICUs in 1996 were susceptible to gentamicin and only 29.1% to amikacin (Günseren et al. 1999*)*. In Turkey 17.1% *Acinetobacter* spp were susceptible to gentamicin and 34.8% to amikacin in 1997 (Aksaray et al., 2000*)*. In contrast Chang et al. and Oberoy et al. reported higher susceptibility rates of 74.5% and 64.7% respectively among *Acinetobacter* spp. strains for Amikacin (Chang et al. 1995 and Oberoi et al. 2009).In our study, Netilmicin and Tobramycin were susceptible in 18.9% isolates and 10.8% isolates respectively which were comparable with a study conducted in eastern Nepal (Ghimire et al., 2002). In a Spanish study during the early 1990s, 50% *A. baumannii* isolates tested were susceptible to tobramycin, 33% to gentamicin, 66% to netilmicin, and 72% to amikacin (Vila et al. 1993). Between 1991 and 1996, an increase in aminoglycoside resistance among clinical isolates of *Acinetobacter* spp. was noticed in Spain (Ruiz et al., 1999). The presence of intergrons containing aminoglycoside-modifying enzymes (AMEs) is associated with resistance to gentamicin and tobramycin but not with resistance to amikacin among *Acinetobacter* isolates (Lin et al., 2010).The higher aminoglycoside resistance among *Acinetobacter* isolates might be due to reduced uptake or decreased permeability, altered ribosomal binding site and most importantly the production of aminoglycoside modifying enzymes viz. AACs, ANTs and APHs (Shaw et al., 1993; Mingeot-Leclercq et al., 1999).In our study kanamycin was completely resistant to all *Acinetobacter* isolates. The highest resistance rate against kanamycin may be due to excessive production of ANT(4')(4")I, APH(2")/AAC(6), APH(3') ,and ACC(3') III and to Tobramycin may be due to excessive production of ANT(4')(4")I, APH(2")/AAC(6') (Livermore et al., 2001). Aminoglycoside resistance was more predominant among ESBL negative *Acinetobacter* spp. in a study conducted in Nepal (Kandel, 2010). In our study, the higher resistant rates of *Acinetobacter* to all aminoglycosides might be due to above mentioned resistance mechanisms and increased selective pressure created by this antimicrobials.

In our study, susceptibility against chloramphenicol and tetracycline was 78.4% and 18.9% respectively. In a Spanish study published in 1993, all 54 *A. baumannii* isolates tested were susceptible to chloramphenicol (Vila et al., 1993) which was in favor of our study. The susceptibility pattern of chloramphenicol ranged from 53.8% to 100% in different clinical specimens where this was the most susceptible in different body fluids and least in respiratory isolates. Tetracycline susceptibility ranged from 0 to 60 % in which it was found to be most susceptible in blood isolates (60%) and not susceptible in isolates from different body fluids and DJ-shunts. However, the average susceptibility pattern of Tetracycline was 18.9%. In our hospital, chloramphenicol and Tetracycline were commonly used drugs previously against blood isolates. Nowadays, these drugs are not frequently used to treat *Acinetobacter* infection and other bacterial infections in this hospital and other hospitals in Nepal due to their side-effects in patients. Therefore, due to their low selective pressure these drugs might have susceptibile against *Acinetobacter* spp. in our study. Chromosomal and plasmid DNA associated chloramphenicol acetyltransferase I (CAT1) that might be transposon-encoded, is responsible for chloramphenicol resistance in clinical *Acinetobacter* isolates (Devaud et al 1982***;*** Elisha and Steyn, 1991) and also from a change in permeability to the antibiotic or a mutation in the target protein (Vila et al., 1993). Specific transposon-meidated efflux pumps TetA and Tet B are responsible for tetracycline resistance in *A. baumannii* where TetB determines the efflux of both tetracycline and minocycline and TetA drives only the efflux of tetracycline (Guardabassi et al., 2002; Huys et al., 2005). The ribosomal protection protein (RPP) encoded by tet M) gene shields the ribosome and protects the ribosome from the action of tetracycline, doxycycline, and minocycline (Ribera et al., 2003).

Out of two fluoroquinolones tested, ciprofloxacin and ofloxacin were almost resistant to *Acinetobacter* isolates. These drugs were only susceptible in 28.4% of isolates and 18.9% of isolates respectively. Similar type of result was obtained in a study in Nepal (Kandel, 2010). Ciprofloxacin was found 2.3% to 76% susceptible in the different studies conducted in eastern Nepal (Ghimire et al., 2002; Basnet et al., 2009). In 1996 - 2000 in Greece, Turkey, and Slovakia

67.1% to 92.6% of the ICU isolates were resistant to ciprofloxacin (Aksaray et al., 2000; Hostacka and Klokocnikova, 2002; Maniatis et al., 2003). The resistance pattern of ofloxacin is higher because of more frequent use of this drug than ciprofloxacin in this KMC hospital. The resistance patterns of *Acinetobacter* to floroquinolones showed variability in different institutions in Nepal and other countries due to variability of the local situations. Resistance of *Acinetobacter baumannii* to quinolones is often mediated by modifications in the structure of DNA gyrase secondary to mutations in the quinolone resistance-determining regions of the gyrA and parC genes (Vila et al., 1997; Seward and Towner, 1998), by aquisiton of mobile genetic elements or via efflux pumps and modifications of lipopolysaccharides from mutations in both gyrA and parC topoisomarese enzymes (Cuenca et al., 2003).

6.2 Conclusion

In conclusion, the prevalence of *Acinetobacter* spp in Kathmandu Medical College Teaching Hospital was high (9.1%) with all the isolates being hospital-acquired and predominant in ICUs, higher in male patients than in female patients of age groups (21-30) years and highest in miscellaneous specimens followed by catheter tips and respiratory tract specimens among the total isolates from each type of specimens. Carbapenems, Ceftriaxone, and Co-trimazole were the most effective antimicrobials against *Acinetobacter* spp. in this hospital. A high rate of susceptibility of *Acinetobacter* spp. to Ceftriaxone and Co-trimoxazole was the noticeable thing in this study.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

1. The prevalence of *Acinetobacter* spp out of 810 total bacterial isolates was 9.1% and out of 713 total gram negative bacterial isolates *was* 10.3%.

2. Among 74 isolates of *Acinetobacter* spp, 39(52.7%) were from male patients and 35 (47.3%) were from females and the prevalence was found to be highest (32.5%) in age groups (21-30) years and least (5.4%) in age groups below 20 years.

3. Out of 74 total *Acinetobacter* isolates, the isolation rate was found to be highest in urine specimens, (41.9%) followed by respiratory tract specimens, (17.6%), blood (13.5%), catheter tips (12.2%), exudates (8.1%), miscellaneous specimens, (4.1%) and body fluids (2.7%) .

4. The prevalence of *Acinetobacter* spp was found to be highest in miscellaneous specimens followed by catheter tips, respiratory tract specimens, and least in exudates among total bacterial isolates and among total gram-negative bacterial isolates from each type of specimens.
5. All isolates of *Acinetobacter* were hospital-acquired and among them predominant isolates (70.2) % were from intensive care units.
6. Among 24 different antimicrobials tested to all *Acinetobacter* isolated, Meropenem, Imipenem, Co-trimoxazole, Salbactam/Cefoperazone, Ceftriaxone and Chloramplenicol showed susceptibility to 94.6%, 89.2%, 83.8%, 73.3%, 86.5% and 78.4% of the isolates respectively.

7.2. Recommendations

1. Carbapenems, Ceftriaxone, Chloramphenicol, Co-trimoxazole and Cefoperazone/Sulbactam antimicrobials are recommended to treat the patients infected by *Acinetobacter* spp. and to test these antimicrobials routenly in all clinical laboratories in Nepal and other countries.

2. More extensive study regarding the sources of *Acinetobacter* infection or colonization, the predisposing factors related to the infection, the mechanisms of resistance to extended spectrum beta-lactams and current carbapenems and effect of antibiotic cycling is recommended to find the actual scenario of *Acinetobacter* in this hospital.

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APPENDIX-I

PATIENT'S REQUEST FORM

Name :... ... ...

Age/ Gender :... ... ...

Address :... ... ...

Brief Clinical History:

Culture requested for :.. … … … .. … .. ..

Patient under antibiotic treatment : a). Yes … … b) No… …

If yes, Antibiotic (s) taken: 1) ... ... 2) …….

Duration of treatment :... ... ...

MICROBIOLOGICAL PROCEDURES

Day 1

Date :…….

Specimen type:... ... . Time of specimen collection:... ... ...

Method of specimen collection:... ... ...

Time of specimen receipt at the laboratory … … … … …

Condition of specimen… … … … … … …

Culture on: 1) … … … 2) … … … 3) … … …

Incubation: 1) Aerobic 2) Microaerophilic

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

Day2

Date : … … … …

Observation for colony characteristics, Gram-staining reaction and preliminary tests on primary culture plates:

Isolate ID	Colony characteristics									
	Medium	shape	size	color	texture	margin	elevation	opacity	consistency	Hemolysis

Gram's reaction:……..

Catalase:… … … … … … Oxidase :… … … … … … … …

Motility:… … … … … … … … … … … … … … … …

Provisional identification of organism :… … … … … … …

Inoculation on different biochemical test media

1) … … … .. 2) … … … .. 3) … … … … 4) … … … 5) … … …

Day3

Date : … … … …

A. Observation of Biochemical tests

Sample ID	Isolate ID	SIM	MR	VP	TSI	Citrate	Urease	O-F test	Growth at 44˚C	Identified Organism

Note: SIM,Sulfide Indole Motility test; MR, Methyl Red; VP Voges Proskauer; TSI, Triple Sugar Iron agar test; O-F, Oxidation-Fermentation test

B. Antibiotic susceptibility Test: Kirby-Bauer Method

Date :… … … …

Disks Manufacturer:… … Manufacture date:… …… Expiry date :… … … ..

Medium used/Manufacturer:.. … … … Medium Manufacture date:…

Medium Expiry date:… … … .. Medium preparation date : … … …

Isolate ID	Organism	Antibiotic disks used and Disk contents								

Note: S= Susceptible, R= Resistant

APPENDIX-II

1. Composition and preparation of different culture media

The culture media used were manufactured by following companies, viz. Hi-Media Laboratories Pvt. Limited Bombay, India, Oxoid Unipath Ltd., Basingstoke, Hampshire, England, and mast Diagnostics, Mast house Derby Road, Bootle.

1. Blood agar (Hi-Media, M073)

Blood agar base (infusion agar) +5-10% sheep blood

Ingredients	**gm/liter**
Beef heart infusion	500.00
Tryptose	10.00
Sodium Chloride	5.00
Agar	15.00

Final pH (at 25^0C): 7.3± 0.2

Preparation : As directed by the manufacture, 42.5 grams of the blood agar base medium was suspended in 1000ml distilled water and sterilized by autoclaving at 121^{0F}C (15 lbs pressure) for 15 minutes . After cooling to 40-50^{0F}C, 50ml sterile defibrinated sheep blood was added aseptically and mixed well before pouring.

2. Chocolate agar (CA)

The sterilized blood agar was poured in petri plates and was allowed to solidify and was heated at 80 FCin an oven for 30 minutes. By this time, the color changed to chocolate brown.

3. MacConkey agar (Hi-Media, M008)

Ingredients	gm/liter
Peptone	20.00
Lactose	10.00
Sodium chloride	5.00
Neural red	0.04
Agar	20.00

Final pH (at 25^0C): 7.4± 0.2

Preparation: As directed by the manufacturer, 55 grams of the medium was suspended in 1000ml of distilled water and then boiled to dissolve completely. Them the medium was sterilized by autoclaving at 121^{0F}C (15 Ibs pressure) for 15 minutes.

4. Mueller Hinton Agar (Mast, DM 170D)

Ingredients	**gm/liter**
Beef, infusion form	300.00
Casein Acid Hyrolysate	17.50
Starch	1.50
Agar	17.00

Final pH (at 25^0C): 7.4± 0.2

Preparation : As directed by the manufacturer,38 grams of the medium was suspended in 1000ml distilled water and the medium was warmed to dissolve, then the medium was sterilized by autoclaving at 121^0C (15 Ibs pressure) for 15 minutes. The sterilized medium was then poured into sterile petri plates and allowed to cool.

5. Nutrient agar (Hi-Media M001)

Ingredients	**gm/liter**
Peptone	10.00
Sodium Chloride	5.00
Beef, Extract	10.00
Yeast Extract	1.50
Agar	12.0

Final pH (at 25^0C): 7.4± 0.2

Preparation: As directed by the manufacturer, 37 grams of the medium was suspended in 1000ml of distilled water and then boiled to dissolve completely. The medium was sterilized by autoclaving at 121^0C (15 Ibs pressure) for 15 minutes and poured into sterile Petri plates.

6. Nutrient Broth (Hi-Media,M002)

Ingredients	**gm/liter**
Peptone	5.00
Sodium Chloride	5.00
Beef, Extract	1.50
Yeast Extract	1.50

Final pH (at 25^0C): 7.4± 0.2

Preparation: As directed by the manufacturer, 37 grams of the medium was dissolved in 1000ml distilled water then the medium was dispensed in test tube in amount of 3ml in each and autoclaves at 121^0C (15 Ibs pressure) for 15 minutes. The sterilized medium was then cooled to room temperature.

7. Mueller Hinton Broth (Oxid, CM 0405)

Ingredients	**gm/liter**
Beef	300.00
Casein Hyrolysate	17.50
Starch	1.50
Calcium	0.0036
Magnesium	6.29

Final pH (at 25^0C): 7.4± 0.2

Preparation: As directed by the manufacturer, 37 grams of the medium was dissolved in 1000ml distilled water, mixed well to dissolve, dispensed in screw-capped bottles in amount of 3ml in each and sterilized by autoclaving at 121^0C (15 Ibs pressure) for 15 minutes.

8. Tryptone soy broth+20% Glycerol

Ingredients	**gm/liter**
Pancreatic Digest of Casein	15.00
Enzymatic Digest of Soybean Meal	5.00
Sodium Chloride	5.00
Glycerol	200 ml

Preparation: As directed by the manufacturer, 37 grams of the medium was suspended in 1000ml of distilled water containing 200ml glycerol and mixed thoroughly. It was boiled completely and autoclaved at 121^0C for 15 minutes.

II. Biochemical test Media

1. MR-VP Medium (Hi-Media, M 070I)

Ingredients	**gm/liter**
Buffered Peptone	7.00
Dextrose	5.00
Dipotassium Phosphate	5.00

Final pH (at 25^0C): 6.9± 0.2

Preparation: As directed by the manufacturer, 17 grams of the medium was dissolved in 1000ml distilled water, heated to dissolve completely, dispensed in the amount of 3 ml in each test tube and autoclaved at 121^0C (15 Ibs pressure) for 15 minutes. The tubes were then allowed to cool.

2. Hugh and Leifson's Medium (Hi-Media, M 826)

Ingredients	**gm/liter**
Tryptone	2.00
Sodium Chloride	5.00
Dipotasssium Phosphate	0.30
Bromothymol Blue	0.08
Agar	2.00

Final pH (at 25^0C): 6.8± 0.2

Preparation: As directed by the manufacturer, 19.40 grams of the medium was dissolved in 1000ml distilled water and then heated to boiling to dissolve

completely. The medium was then sterilized by autoclaving for 15 minutes at 15 Ibs pressure (121^0C) to 1000ml sterile medium 100ml of sterile Dextrose was added aseptically, mixed thoroughly and dispensed in 5ml quantities into sterile culture tubes.

3. Sulphide Indole Motility (SIM) medium (HI-Media, M 181)

Ingredients	**gm/liter**
Beef Extract	3.00
Peptone	30.00
Patronized Iron	0.20
Sodium thiosulphate	0.025
Agar	3.00

Final pH (at 25^0C): 7.3±0.2

Preparation: As directed by the manufacturer, 36.23 grams of the medium was suspended in 1000ml distilled water and dissolved completely. It was dispensed into test tubes to a depth of about 3 inches and autoclaved for 15 minutes at 15 Ibs pressure (121 ^{0}C).

4. Simmon's Citrate Agar (Hi-Media, M099)

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

Final pH (at 25^0C): 6.8±0.2

Preparation: As directed by the manufacturer, 24.2 grams of the medium was dissolved in 1000ml distilled water. Then 3ml of the medium was dispensed in each tubes and sterilized by autoclaving at 121^0C (15 Ibs pressure) for 15 minutes. After autoclaving, tubes containing media were tilted to form slants during cooling.

5. Triple sugar iron (TSI) agar (Hi-Media, M 021)

Ingredients	**gm/liter**
Peptic digest of animal tissue	10.00
Casein enzynmatic hydrolysate	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 25^0C): 7.4±0.2

Preparation: As directed by the manufacturer, 24.2 grams of the medium was dissolved in 1000ml distilled water and sterilized by autoclaving at 121^0C (15 Ibs pressure) for 15 minutes. The medium was allowed to set in sloped form with a butt of about 1 inch thickness.

6. Christensen urea broth (Hi-Media, M 112)

Ingredients	**gm/liter**
Peptic digest of animal tissue	1.00
Dextrose	1.00
Sodium Chloride	5.00
Dipotassium Phosphate	1.20
Monopotassium Phosphate	0.80
Phenol Red	0.012

Final pH (at 25^0C):7.4±0.2

Preparation: As directed by the manufacturer, 24.0 grams of the medium was dissolved in 950 ml distilled water and sterilized by autoclaving at 121^0C (15 Ibs pressure) for 15 minutes. After cooling to about 45^0C, 50 ml of sterile 40.0% urea was added aseptically and mixed well. Then 5 ml in each tube was dispensed and cooled to room temperature.

III. Staining and test reagents

1. Preparation of Gram staining reagents

(a) Hucker's Crystal Violet stain: In a clean piece of paper, 40 gm of crystal violet (90.095% dye content) was weighed, dissolved in 400 ml of ethanol (95.5%), filtered and stored in a clean brown bottle at room temperature. For the preparation of working solution of crystal violet, 40 ml of stock solution was added to 160 ml of filtered ammonium oxalate solution (1.0%).

(b) Lugol's Iodine: To prepare stock solution of Lugol's iodine, 25 gm of iodine crystals and 50 gm of potassium iodide crystals were dissolved in 500 ml of distilled water in a brown glass bottle. For the preparation of working solution, 60 ml of Lugol's iodine stock solution was mixed with 220 ml of distilled water and 60 ml of 5.0% sodium bicarbonate solution.

(c) Acetone-Alcohol Decolorizer (1:1): To 50 ml of ethanol (9.0%) 50 ml of acetone was mixed well and transferred into a clean bottle. The preparation was labeled with data and stored at room temperature.

(d) Safranin (Counter-stain): The stock solution was prepared by dissolving 5gm of safranine in 200 ml of 95.0% ethanol and the working solution was prepared by mixing 20 ml of stock solution with 180 ml of distilled water.

2. Normal saline

Preparation: in 100 ml of distilled water, 0.85gm of sodium chloride was dissolved well in a leak-proof bottle. The bottle was then labeled and stored at room temperature.

3. Test Reagents

A. For Catalase test

To make 100ml Catalase Reagent (3%H_2O_2)

Hydrogen peroxide	3.00 ml
Distilled Water	97.00 ml

Preparation: To 97 ml of distilled water, 3 ml of hydrogen peroxide was added and mixed well.

B. For Oxidase test

To make 100 ml oxidase reagent (impregnated in Whatman's No.1 filter paper)

Tetramethyl p-phenylene diamine dihydrochloride (TPDD)	1.00 gm
Distilled Water	100.00 ml

Preparation: In 100 ml of distilled water 1gm of TPDD was dissolved. To that solution strips of Whatman's No.1 filter paper were soaked and drained for about 30 seconds. Then those strips were freeze-dried and stored in a dark bottle tightly sealed with a screw cap.

C. Kovac's reagent for Indole test

To prepare 40ml reagent

Isoamyl alcohol	30.00 ml
4-dimethylaminobezaldehyde	2.00 gm
Conc.hydrochloric acid	10.00 ml

Preparation: In 30 ml Isoamylalcohol, 2gm of 4-dimethylaminobezaldehyde was dissolved in a clean brown bottle. Then, 10 ml of conc. HCL was added to that and mixed well.

D. Methyl Red Solution for Methyl Red test

To make 50ml solution

Methyl red	0.05 gm
Ethyl alcohol (absolute)	28.00 ml
Distilled Water	22.00 ml

Preparation: To 28ml ethanol, 0.05gm of methyl red was dissolved in a clean brown bottle. Then, 22ml distilled water was added to that bottle and mixed well.

E. Barritt's reagent for Voges-Proskauer test

Voges-Proskauer reagent A:

To make 100ml

α-napthol	5.00 gm
Ethyl alcohol (absolute)	100.00 ml

Preparation: To 25ml distilled water, 5gm of α-napthol was dissolved in a clean brown bottle. Then the final volume was made 100 ml by adding distilled water.

Voges-Proskauer reagent B (100 ml)

Potassium Hydroxide	40.0 gm
Distilled water	100.0 ml

Preparation: To 25 ml distilled water, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding distilled water.

The three parts of reagent A (50% α-napthol) was mixed with one part of reagent B (40% KOH) to get the working solution of the Barritt's reagent.

APPENDIX-III

I. List of equipments, materials and supplies

A.General microbiology laboratory equipments

a. Autoclave (Stermite, Japan) b. Incubator (Sakura, Japan) c. Hot air Oven (Memmert, Germany and GallenKamp) d. Microscope (Olympus, Japan) e. Refrigerator, 4-8 degree Celsius (Sanyo, Japan); -20 degree Celsius (Videocon, India); -75 degree Celsius (Sanyo, Japan) f. Weighing machine (Ohaus Corporation, USA) g. Water bath (Boekel Scientific, USA) h. Gas burner i. Glasswares j. Inoculating loops and wires k. Sterile cotton swabs l. Sterile Petri plates m. McFarland 0.5 turbidity standard n. Vortex mixer o. Ruler p. Forceps.

B. Microbiological media

(a)Blood Agar (b) Chocolate Agar (c) MacConkey Agar (d) Mueller Hinton Agar (e) Mueller Hinton Broth (f) Hugh and Leifson Medium (g. Sulfide Indole Motility medium (h) MR-VP medium (i) Triple Sugar Iron Agar (j) Urea broth (k) Simmon's Citrate Agar (l) Nutrient Agar (m) Tryptone Soya Broth

C. Chemicals and reagents

Catalase reagent ($3\%H_20_2$), Oxidase reagent (1% Tetramethyl p-phenylene diamine dihydrochloride), Kovac's reagent, Barritt's reagent (40% KOH, 5% α-naphtol in a ratio of 1:3), Barium Chloride, Conc H_2SO_4, Glycerol, Gram's reagent, etc

D. Antibiotic disks

The following antibiotic disks were used for the antibiotic susceptibility tests which were from Mast Group Ltd., Mast House, Derby Road, Bottle, Merseyside, L201EA, UK and Oxoid Unipath Ltd., Basingstoke, Hampshire, England: Amoxycillin, Amoxy-clave, Imipenem, Meropenem, Cefoperazone/ Salbactam, Carbenicillin, Piperacillin, Cephalexin, Cefuroxime, Cepfodoxime, Cefoxitin, Cephalothin, Ceftriaxone, Ceftazidime, Gentamicin, Amikacin, Kanamycin, Tobramycin, Netilmicin, Tetracycline, Chloramphenicol, Ciprofloxacin, Ofloxacin, and Co-trimoxazole.

APPENDIX-IV

A. Gram-Staining

The method was first devised by Sir Hans Christian during the late 19th century. This method can be used effectively to divide all bacterial species into two major groups: Gram-positive (those that take up and retain the basic dye Crystal violet even on washing) and Gram-negative (those that allow the crystal violet dye wash out easily with the decolorizer of alcohol or acetone). The following steps were performed for Gram-staining:

1. A thin oval smear of the material to be examined was prepared on a clean grease free glass slide and air dried. The material on the slide was then heat fixed and allowed to cool before staining.
2. The slide was flooded with Gram's iodine solution and allowed to remain without drying for 30-45 seconds. Then the slide was rinsed with tap water to remove the excess stain.
3. Then the slide was flooded with Lugol's iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface. Again the slide was rinsed with tap water to remove excess iodine.
4. The slide was flooded with acetone/alcohol decolorizer for 10 seconds and rinsed immediately with sufficient tap water until no further color flowed from the slide with the decolorizer.
5. The slide was flooded with counter stain (Safranin) for 2 minutes and washed off with tap water.
6. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

Quality Control

The laboratory staining procedure and reagents were tested prior to use of new lots of each staining and decolorizing agents and at least weekly thereafter, using Escherichia coli ATCC 25922 (Gram negative) and Staphylococcus aureus ATCC 25923 (Gram positive).

B. Biochemical tests for bacterial identification

Catalase Test

Microorganisms produce hydrogen peroxide (H_2O_2) during aerobic respiration, which is lethally toxic to the cell itself. Catalase enzyme breaks down hydrogen peroxide into water and oxygen. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, with major exception being Streptococcus species.

About 2-3 drops of 3.0% H_2O_2 was put on the surface of a clean glass slide and a small amount of a culture from Nutrient Agar plate was applied with sterile wooden or glass stick. Presence of effervescence indicated the positive test. A false positive reaction was prevented by avoiding the use of the culture of the culture medium with catalase (e.g. Blood Agar) or an iron loop.

Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye, Tetramethyl-p-phenylene diamine dihydrochlorde (TPDD), the cytochrome oxidase oxidizes it into a deep purple colored end product, Indophenol, which is detected in the test.

A small aliquot of colony of the test organism was rubbed on the filter paper soaked in Oxidase reagent with the help of sterile glass rod. The positive test was indicated by the appearance of blue-purple color within 10 seconds.

Oxidation-Fermentation test

This test is done to determine the oxidative or fermentative metabolism of carbohydrate resulting in production of various organic acids as end products. Some bacteria are capable of metabolizing carbohydrates (as exhibited by acid production) only under aerobic conditions, while others produce acid both aerobically and anaerobically. Most medically important bacteria are facultative anaerobes.

The test organism was stabbed into the bottom of two sets of tubes with Hugh and Leifson's media, containing bromothymol blue as the pH indicator. The inoculated medium in one of the tubes was covered with a 10 mm deep layer of sterile paraffin oil. The tubes were then incubated at 37 degree Celsius for 24 hrs. After incubation, the tubes were examined for carbohydrate utilization as shown by acid production. Fermentative organisms utilized the carbohydrate both in the open and sealed tubes as shown by a change in colour of the medium from green to yellow. However, oxidative were able to use the carbohydrate only in the open tube.

Indole production test

This test detects the ability of the organism to produce an enzyme "trytophanase" which oxidizes tryptophan to form indolic metabolites; Indole, Skatole (Methyl Indole) and Indoleacetic acid. Indole, if present, combines with the Aldehyde present in the reagent to produce red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in Indole.

A smooth bacterial colony was stabbed on Sulfide Indole Motility (SIM) medium by a sterile inoculating wire and the inoculated media was incubated at 37 degree celsius for 24 hours. After 24 hours incubation, 0.5 ml of Kovac's reagent was added. Appearance of red color on the top of media indicated the Indole positive reaction.

Motility test

Motile organisms migrate from the stab line and diffuse into the medium causing turbidity whereas non-motile bacteria show the growth only along the stab-line, and the surrounding medium remains colorless and clear. This test was macroscopically observed in semisolid Sulfide Indole Motility (SIM) medium.

Methyl Red Test

This test is performed to test the ability of an organism to produce sufficient acid from the fermentation of glucose. The degree of acidity is denoted by intensity of color change of methyl red indicator over pH range of 4.4-6.0.

A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37 degree Celsius for 24 hours. After incubation, about 5 drops of Methyl Red reagent was added and shaken well for maximum aeration and kept for 15 minutes. Positive test was indicated by the development of pink red colour.

Citrate utilization test

This test detects whether an organism can utilize citrate as a sole source of carbon for metabolism with resulting alkalinity. Oganisms capable of utilizing citrate as sole carbon source also utilizes the ammonium salts present in the medium as sole nitrogen source. Production of ammonia imparts alkalinity, thus changing the color of indicator Bromothymol blue from green (pH 3.0-7.6) to blue (pH $\geq$ 7.6).

A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37 degree Celsius for 24 hrs. A positive test was indicated by the growth of organism and change of media by green to blue.

Triple Sugar Iron (TSI) Agar test

The TSI Agar is used to determine the ability of an organism to utilize carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium).

The test organism was stabbed on the butt and streaked on the surface of TSI slant and incubated at for 24hrs. Acid production limited only to the butt region of the tube was indicative of glucose utilization, while acid production in slant and butt indicated sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow reaction at acidic pH and red reaction at alkaline condition.

Urease test

This test demonstrates the ability of bacteria to produce urease which decomposes urea, releasing Ammonia and Carbon Dioxide, Ammonia thus imparts alkalinity and changes the color of phenol red indicator incorporated in the medium pink.

The test organism was inoculated in a medium containing urea and the indicator phenol red and incubated at 37^0C overnight. Change in color of the medium to deep pink red indicated positive reaction, i.e. urease production.

APPENDIX-V

1. Antimicrobial Susceptibility Testing by Disk-Diffusion Method

A. Principle: A standardized inoculum of bacteria was swabbed onto the surface of MHA plate. Filter paper disks impregnated with antimicrobial agents were placed flat on the agar surface. After overnight incubation, the diameter of the zone of inhibition was measured around each disk. By referring to the tables in the CLSI disk diffusion standard, a qualitative report of susceptible, intermediate or resistant was obtained.

B. Procedures

1. Preparation of 0.5 McFarland Standard turbidity solutions: To 0.5ml of 0.048M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$), 99.5ml of 0.81M H_2SO_4 (1% v/v) was added with constant stirring. The solution was then distributed into test tubes, well screw capped, wrapped with aluminium foil and stored in dark till use.The agar plates and antibiotic disk cartridges were brought to room temperature before use. Agar plates were made to contain no excess moisture on their surface by placing them in the 35 degree Celsius ambient air incubator with lids ajar for evaporation.

2. Inoculum preparation: By touching 2-3 morphologically similar colonies not more than 24 hrs old with sterile loop on nonselective agar plate, MHB or NB was inoculated and incubated at 37 degree Celsius until turbidity matched with that of the 0.5 McFarland Standard turbidity solutions.

3. Inoculation of agar plates: Within 15 minutes of adjusting turbidity, a sterile cotton swab was dipped into the inoculum and rotated against the wall of the tube above the liquid to remove excess inoculums. The entire surface of the Mueller Hinton agar plate was swabbed by rotating plates approximately 60º between successive swab strokes to ensure even distribution. Any touching to sides of the plates and aerosol formation was avoided. Finally, the swab was run around the edge of the agar to remove any excess moisture. The plates were left for about 10-15 minutes to let the agar surface dry before the application of the disks.

4. Application of disks and Incubation: Appropriate antibiotic disks were placed evenly (not closer than 24 mm from center to center) on the inoculated agar surface either with dispenser or manually with sterile forceps and a gentle pressure was applied on each disk with sterile forceps to ensure complete contact of disk with agar. Not more than six disks were placed on a 90 mm Petri plate.Within 30 minutes of disk application, the plates were incubated in inverted position at 35ºC in an ambient air incubator for 16 to 18 hours.

5. Reading, interpretation and reporting: Only the plates with lawn of confluent or nearly confluent growth were read.The diameter of zones of inhibition was measured in nearest whole millimeter with a measuring scale by observing the plate against nonreflecting surface. In case of sulfonamides, tri-methoprim and co-trimoxazole, light growth (<20.0% of lawn growth) was disregarded and zone of inhibition was measured by using the edge of the more obvious margin of the zone. In case of development of discrete colonies within the zone of inhibition (which may represent mixed culture or resistant variants), each morphologically different single colonies were sub cultured, re-identified and retested for antibiotic susceptibility. If they were still present, the colony-free inner zone was measured. Criteria specified by the CLSI/BSAC/EUCAST were used unambiguously to interpret the zone of inhibition for each antibiotic agent and the categorical results were reported as either susceptible (S), or resistant (R).

6. Quality control:

a. QC strains: i) Escherichia coli, ATCC25922 ii).Staphylococcus aureus, ATCC 25923

b. Monitoring Accuracy

i. QC strains were tested daily and weekly by following routine procedures and results were recorded. Also the lot number and expiry date of the antibiotic disks and MHA were noted.

ii) The obtained results were compared to expected results (CLSI QC tables) and in case of any aberrant result; the procedure was continued with corrective action.

APPENDIX-VI

Clinical and demographic details of different clinical specimens

Table of demographic and bacteriological profiles of urine specimens

Types of Bacteria	Number of bacterial isolates in Urine speimens		Total number of bacterial isolates in Urine specimens, (%)
	Males (%)	Females (%)	
GNB:			
Escherichia coli	118 (31.9)	252 (68.1)	370 (78.1)
Klebsiella spp.	12 (35.3)	22 (64.7)	34 (7.2)
Acinetobacter spp.	12 (38.7)	19 (61.3)	31 (6.5)
Proteus spp.	5 (31.2)	11 (68.8)	16 (3.4)
Citrobacter spp.	4 (40.0)	6 (60.0)	10 (2.1)
P. aeruginosa	3 (37.5)	5 (62.5)	8 (1.7)
Enterobacter spp.	1(25.0)	3 (75.0)	4 (0.8)
Serratia marcescens	0 (0)	1 (100)	1 (0.2)
Total	155 (32.7)	319 (67.3)	474 (95.6)
GPB:			
Staph. aureus	3 (30.0)	7 (70.0)	10 (45.5)
Staph. saprophyticus	2 (25.0)	6 (75.0)	8 (36.4)
E. faecalis	1 (25.0)	3 (37.0)	4 (18.2)
Total	6 (27.3)	16 (72.7)	22 (4.4)
Grand total	161 (32.5)	335 (67.5)	496 (100)

Table of demographic and bacteriological profiles of blood specimens

Types of Bacteria	Number of isolates in Blood speimens		Total no of isolates in Blood specimens (%)
	Males (%)	Females (%)	
GNB:			
Salmonella spp.	12 (70.58)	5 (29.42)	17 (36.95)
Acinetobacter spp.	6 (60.00)	4 (40.00)	10 (21.74)
Escherichia coli.	3 (37.50)	5 (62.50)	8 (17.39)
Klebsiella spp.	1 (16.66)	5 (83.34)	6 (13.04)
Proteus spp.	2 (100.00)	0 (0)	2 (435)
Citrobacter spp.	0 (0)	1 (100)	1 (2.17)
Enterobacter spp.	0 (0)	1 (100)	1 (12.17)
S. maltophila	0 (0)	1 (100)	1 (12.17)
Total	24 (52.18)	22 (47.82)	46 (92.00)
GPB:			
Staph. aureus	2 (100)	0(0)	2 (50.00)
Coagulase negarive staphylococci (CoNS)	2 (100)	0 (0)	2 (50.00)
Total	4 (100)	0 (0)	4 (8.00)
Grand Total	28 (56.00)	22 (44.0)	50 (100)

Table of demographic and bacteriological profiles of Catheter tip specimens

Types of Orgainisms	Number of bacterial isolates in catheter tips		
	Males, n (%)	Females, n (%)	Total isolates N (%)
Acinetobacter spp.	5 (55.6)	4 (44.4)	9 (30.0)
Klebsiella spp.	3 (37.5)	5 (62.5)	8 (26.7)
Escherichia coli	1 (16.6)	6 (85.7)	7 (23.3)
Citrobacter freundii	0 (0)	4 (100)	4 (13.3)
Proteus spp.	0 (0)	2 (100)	2 (6.6)
Total	9 (30)	21 (70)	30

Table of demographic and bacteriological profiles of different exudates

Types organisms	Number of isolates in						Total number of isolates, N (%)
	pus	HVS	Umbilicus swab	Eye swab	Wound swab	Discharges	
GNB:							
Escherichia coli.	26	18	3	1	5	1	54(54.0)
Klebsiella spp.	7	1	1	0	5	1	15 (15.0)
Citrobacter freundii	7	0	2	1	4	0	14 (14.0)
Proteus spp.	3	0	1	1	2	0	7 (7.0)
Acinetobacter spp.	3	0	0	0	3	0	6(6.0)
P. aeruginosa	1	0	0	0	1	0	2 (2.0)
Enterobacter spp.	2	0	0	0	0	0	2(2.0)
Total	49	19	7	3	20	2	100 (60.6)
GPB:							
Staph. aureus	45	0	2	3	7	0	57(87)
CONS	4	0	0	0	2	0	6 (9.2)
MRSA	1	0	0	0	1	0	2(3.1)
Total	50	0	2	3	10	0	65(39.4)

Table of demographic and bacteriological profiles of miscellaneous specimens

Types of Bacteria	Number of isolates in			Total Males n (%)	Total Females n (%)
	DJ- Shunt	Tissues	Pseudocyst		
Acinetobacter spp.	3	0	0	3 (100)	0 (0)
Escherichia coli	0	2	0	1 (50.0)	1 (50.0)
Citrobacter freundii	0	1	0	1 (100)	0 (0)
Staph aureus	0	1	1	0 (0.0)	2 (100)
Total	3	4	1	5 (62.50)	3 (37.5)

Table of demographic and bacteriological profiles of different body fluids

Types of Bacteria	Number of isolates in					Total isolates in	
	Bile	CSF	Peritoneal	Pleural	Others	males, (%)	females, (%)
Escherchia Coli	3	1	1	0	3	5 (62.5)	3(37.50)
Acinetobacter spp.	0	0	1	1	0	2(100)	0 (0)
Klebsiella spp.	1	0	0	0	0	0 (0)	1 (100)
Citrobacter freundii	0	1	0	0	0	1 (100)	0(0)
Proteus spp.	0	1	0	0	0	1 (100)	0(0)
Staph aureus	2	0	1	0	0	1(33.3)	2(66.66)
Total	6	3	3	1	3	10(62.5)	6(37.50)

Table of demographic and bacteriological profiles of respiratory tract specimens

Types of Microorganisms	Number of isolates in					Total Males n, (%)	Total Females n, (%)
	Sputum	BAL	ET-Tube	IT-Tube	Bronchial Aspirate		
Acinetobacter spp.	6	4	2	1	0	8 (61.5)	5 (38.5)
Klebsiella spp.	8	0	1	0	0	4 (44.4)	5 (55.6)
P. aeruginosa	4	2	1	0	1	6(75)	2 (25)
Citrobacter freundii	4	1	1	0	0	3 (50)	3(50)
Escherichia coli	3	0	2	0	0	1 (20)	4(80)
Enterobacter spp.	3	0	0	0	0	2 (66.7)	1 (33.3)
Staph. aureus	0	0	1	0	0	1 (100)	0 (0)
Total	28	7	8	1	1	25(55.6)	20(44.4)