Chapter 1

Introduction and Review of Literature
Chapter 1: Introduction & Review of literature

Contents

1.1 Introduction of review literature. 7
1.2 Urinary Tract Infections (UTI) by Acinetobacter. 8
   1.2.1 Urinary tract infection. 9
   1.2.2 Acinetobacter infections. 10
   1.2.3 Significance of Acinetobacter in UTI infections. 11
1.3 Biofilms in pathogenic microorganisms. 12
   1.3.1 Biofilms- General view. 13
   1.3.2 Biofilms in pathogenesis. 14
   1.3.3 Factors affecting biofilm formation.
      1.3.3.1 Surfaces. 15
      1.3.3.2 Nutrients. 16
      1.3.3.3 Environmental cues. 16
      1.3.3.4 Gene regulation. 17
1.4 Plasmids in Acinetobacter. 18
   1.4.1 Acinetobacter plasmids. 18
   1.4.2 Role of plasmids. 19
   1.4.3 Implications in antibiotic resistance transfer.
      1.4.3.1 β-Lactams. 19
      1.4.3.2 Aminoglycosides. 20
      1.4.3.3 Quinolones. 21
      1.4.3.4 Other Antibiotics. 21
   1.4.4 Plasmid curing. 22
1.5 VEG-media for isolation of microorganisms. 23
   1.5.1 Routine media. 23
   1.5.2 Vegetable media. 24
1.6 Conclusion. 25
1.7 References. 26
1.8 Genesis of work. 27
1.9 Hypothesis. 28
1.10 Objectives. 29
1.1 **Introduction of review literature:**

After getting Ph.D. admission in 2004, detailed literature search was carried out according to the topic of the research work. Some of the most important aspects studied were the general information about *Acinetobacter* strains, new media, isolation of *Acinetobacter* spp. from urinary tract infections (UTI) and urinary catheters, antibiotic resistance in *Acinetobacter* spp., adhesion of *Acinetobacter* strains to the abiotic surfaces and biofilm in urinary catheters, characterization of plasmids in *Acinetobacter* spp. isolated from UTI and Gene transfer from *A. baumannii* to other bacteria.

Literature survey was performed in various National libraries as well as National Medical Library (NML), New Delhi; Jaykar Library, University of Pune, Pune; National Chemical Library (NCL), Pune; National Institute of Virology (NIV), Pune; Bioinformatics Center, University of Pune, Pune. Accordingly, numerous review articles and research papers were collected.

In order to get relevant information about thesis topic, different data bases were searched. Literature search on preparation of new media, biofilm formation and other related topics was done by engines like www.google.com, www.Yahoo.com, pub med.com and the internet facility of University of Pune. More than 500 references have been collected from different libraries and internet scientific sources which provided a ground work for methodology as well as discussion used in this research study.

**Based on the collected papers in this subject, one review with following contents has been written:**

- Urinary Tract Infections (UTI) by *Acinetobacter*.
- Plasmids in *Acinetobacter*.
- Biofilms in pathogenic microorganisms.
- VEG-media for isolation of microorganisms.

According to different papers and review articles, hypothesis and objectives of this research work were constructed.
1.2 Urinary Tract Infections (UTI) by *Acinetobacter*:

*Acinetobacter* species are capable of causing infection. Most of these bacteria were isolated from hospitalized patients, particularly those recovered from urine (Patwardhan, 2008). *Acinetobacter* infections usually involve organ systems that have a high fluid content (e.g., urinary tract) or catheter-associated bacteruria (Lahiri et al, 2004). This part of study is focused on the role of non-fermenter *Acinetobacter* strains in urinary tract infections (UTI).

1.2.1 Urinary tract infection:

Urine contains a variety of fluids, salts and waste products but it is normally sterile. If bacteria penetrate into bladder or kidney and multiply in the urine, they may cause urinary tract infection (Narchi and Hamdani, 2010). The diagnosis of UTI is based on a quantitative urine culture yielding greater than 100,000 colony-forming units ($10^5$ CFU) per milliliter of urine, which was termed "significant bacteriuria." This value was chosen because of its high specificity for the diagnosis of true infection, even in asymptomatic persons. However, several studies have established that one third or more of symptomatic women have CFU counts below this level as low-coliform-count infections (Franz & Horl, 1999).

The most common symptoms of a bladder infection are dysuria, frequency of urination, an urge to urinate. An upper urinary tract infection or pyelonephritis may also present with flank pain and a fever (Wan et al, 2007). The symptoms of urinary tract infections may vary with age and the part of the urinary system that was affected (Grover et al, 2009). Lower urinary tract infections in adults may manifest with symptoms including hematuria (blood in the urine), inability to urinate despite the urge, and malaise. Other signs of UTI include foul-smelling urine and cloudy urine. Whereas in newborns the condition may cause jaundice and hypothermia, in the elderly, symptoms of urinary tract infections may include lethargy and a change in mental status, signs that are otherwise nonspecific (Khassawneh & Hayajneh, 2010). Urinary catheters are a risk factor for urinary tract infections. The risk of an associated infection can be decreased by only catheterizing when necessary, using aseptic technique for insertion and maintaining unobstructed closed drainage of the catheter (Bergogne et al, 1993).

The bladder wall, in common with most epithelia is coated with a variety of cationic antimicrobial peptides such as the defensins and cathelicidin which disrupt the integrity of
bacterial cell walls (Yang et al, 2004). In addition, there are also mannosylated proteins present, such as Tamm-Horsfall proteins (THP), which interfere with the binding of bacteria to the uroepithelium (Raffi et al, 2009). Since bacterial binding to urinary epithelial cells is an important factor in establishing pathogenicity for these organisms, its disruption results in reduced capacity for invasion of the tissues. Moreover, the unbound bacteria are more easily removed when voiding. The use of urinary catheters (or other physical trauma) may physically disturb this protective lining, thereby allowing bacteria to invade the exposed epithelium (Nicolle, 2005). During cystitis, uropathogens subvert innate defenses by invading superficial umbrella cells and rapidly increasing in numbers to form intracellular bacterial communities (IBCs). By working together, bacteria in biofilms build themselves into structures that are more firmly anchored in infected cells and are more resistant to immune-system assaults and antibiotic treatments. This is often the cause of chronic urinary tract infections (Hatt & Rather, 2008; Wilson & Gaido, 2004).

1.2.2 Acinetobacter infections:

The genus *Acinetobacter* is now defined as gram negative (but sometimes difficult to destain) coccobacilli, with a DNA G+C content of 39-47 mol%, that are strictly aerobic, nonmotile, catalase positive, and oxidase negative (Towner, 2006). Good growth occurs on complex media between 20 and 30°C without growth factor requirements, while nitrates are rarely reduced (Dhakephalkar and Chopade, 1994). Crucially, extracted DNA is able to transform mutant strain BD413 *trpE27* to the wild-type phenotype (Juni, 1972; Pardesi et al, 2007). Most *Acinetobacter* strains can grow in a simple mineral medium containing ammonium or nitrate salts and a single carbon and energy source such as acetate, lactate, or pyruvate (Yavankar et al, 2007). Members of the genus are classified in the family *Moraxellaceae*, which includes *Moraxella, Acinetobacter, Psychrobacter*, and related organisms (Rossau et al, 1991) 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 (Rossau et al, 1989; Van Landschoot et al, 1986).

Infections of the urinary tract are among the most common infectious diseases in humans (Williams, 1996; McCaig et al, 1995) and intestine is usually the source of organisms producing UTI (Cattel et al, 1974; Senewiratne et al, 1973). Excluding enterobacteriaceae, *Acinetobacter* species and *Stenotrophomonas maltophilia* are the second and third most common gram negative bacilli respectively encountered in clinical specimens (Forbes et al,
Bacteria of the genus *Acinetobacter* are increasingly being implicated in numerous outbreaks and have become a growing concern in hospitals (Biendo et al, 1999; Wroblewska et al, 2004; Pimentel et al, 2005). Numerous studies have now supported the original observation (Bouvet & Grimont, 1987) that *A. baumannii* is the main genomic species associated with outbreaks of nosocomial infection (Seifert et al, 1993). However, many other *Acinetobacter* species are also responsible for nosocomial infections (Seifert et al, 1993; Domingo et al, 1995; Prashanth et al, 2000; Van Dessel et al, 2002). These organisms are usually commensal, but they are emerging as important opportunistic pathogens because they are rapidly evolving toward multidrug resistance and are often involved in various nosocomial infections that can be severe, such as bacteremia, meningitis, or pneumonia (Bergogne & Towner, 1996; Seifert et al, 1994; Go et al, 1994). Although many outbreaks of *A. baumannii* infection or colonization in medical, surgical, neonatal, and burn intensive care units have been reported, the epidemiology of these infections remains unclear because *A. baumannii* is ubiquitous and infections may occur on either a sporadic or an epidemic basis (Lortholary et al, 1995; Horrevorts et al, 1995; Scerpella et al, 1995). The main sites of infection are the lower respiratory tract and urinary tract, and these distribution sites are very similar to that of other nosocomial gram-negative bacteria (Bergogne & Towner, 1996). *A. baumannii* and DNA group13 have emerged as important organisms in ICU settings; in particular they may be related to the advanced invasive diagnostic and therapeutic procedures adopted in ICUs in the last decade (Bergogne & Towner, 1996). *A. baumannii* is now recognized as a major pathogen involved in nosocomial infections causing epidemic outbreaks or endemic occurrence with a documented high mortality rates (Cisneros et al, 1996; Gomez et al, 1999). Recently a few *A. baumannii* outbreaks have also been reported from India (Mittal et al, 2003; Kapil et al, 1998).

Diagnosis of infection with “unusual” *Acinetobacter* genospecies therefore often depends on clinical indications and repeated isolation of the same strain from a single patient. *Acinetobacter* genomic species 3 and 13TU (Tjernberg & Ursing, 1989) have been implicated in nosocomial outbreaks of infection (Dijkshoorn et al, 1993), while *A. johnsonii* has been associated with catheter-related bacteremia (Seifert et al, 1993). It is worth re-emphasizing the close relationship between genomic species 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3, and 13TU. This *A. calcoaceticus*-*A. baumannii* complex (Gerner-Smidt et al, 1991; Kampfer et al, 1993) contains isolates that are mostly glucose acidifying, and the complex therefore corresponds quite well to the *A. calcoaceticus* subsp. *anitratus* designation.
that is still used in some new reports. The majority of glucose-negative, nonhemolytic strains found in clinical specimens seem to be identified mainly as A. lwaffii, A. johnsonii, or Acinetobacter genomic species 12, and it seems that these species are also natural inhabitants of human skin. Most hemolytic isolates are identified as A. haemolyticus or Acinetobacter genomic species 6. Other groups seem to be implicated only occasionally in human infections. Although A. baumannii appears to be the Acinetobacter genomic species of greatest clinical importance, repeated isolation of another genomic species (particularly one belonging to the A. calcoaceticus-A. baumannii complex) from a patient should be a cause for suspicion of infection, especially if clinical symptoms are also present (Bergogne-Berezin & Towner, 1996).

1.2.3 Significance of Acinetobacter in UTI infections:

A. baumannii does not have fastidious growth requirements and is able to grow at various temperatures and pH conditions (Bergogne-Berezin & Towner, 1996). The versatile organism exploits a variety of both carbon and energy sources. These properties explain the ability of Acinetobacter species to persist in either moist or dry conditions in the hospital environment, thereby contributing for transmission (Getchell-White et al, 1989; Wendt et al, 1997). This hardiness, combined with its intrinsic resistance to many antimicrobial agents, contributes to the organism's fitness and enables it to spread in the hospital setting. Acinetobacter was identified from 20% of hospital cases. The occurrence of species from Acinetobacter genus was reported in clinical material as five species: A. junii, A. baumannii, A. haemolyticus, A. lwaffii and A. johnsonii. Most frequently isolated species were present in purulent materials and in samples from respiratory tract infections and urinary tract infections (Gospodarke & Kania, 1992).

Nosocomial urinary tract infection is caused only infrequently by Acinetobacter spp. It occurs most commonly in elderly debilitated patients, in patients confined to ICUs, and in patients with permanent indwelling urinary catheters. Most patients (80%) tend to be men (Pedraza et al, 1993), perhaps reflecting the higher prevalence of indwelling urinary catheters in this population as a result of prostatic enlargement (Hoffmann et al, 1982). Studies on Acinetobacter in various countries have shown a predominance of isolation from UTIs (2-61) per cent (Towner, 1997; Glew et al, 1997; Bergogne-Berezin & Towner, 1996). Urinary tract infection to compare hospital infections with those in outpatients identified from samples sent to laboratories by the general practitioners and A.baumannii (70.39%) were present in
UTIs (De Stefano et al, 1992). The likelihood of isolation of *A. baumannii* from a hospitalized patients is related to temporospatial (extrinsic, ecologic characteristics) factors such as colonization pressure, nurse-to-patient ratio, and other ward characteristics and to individual patient risk factors (Bonten et al, 1998; Aharon et al, 2005).

### 1.3 Biofilms in pathogenic microorganisms:

#### 1.3.1 Biofilms-General view:

A biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix. Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces and can be credited with the discovery of microbial biofilms. The “bottle effect” has been reported for marine microorganisms, i.e., bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach (Heukelekian & Heller, 1940). It was observed that the number of bacteria on surfaces was dramatically higher than in the surrounding medium (in this case, seawater). However, a detailed examination of biofilms would await the electron microscope, which allowed high-resolution photomicroscopy at much higher magnifications than did the light microscope (Zobell, 1943). After many years, scanning and transmission electron microscopy were used to examine biofilms on trickling filters in a wastewater treatment plant to explain them to be composed of a variety of organisms (based on cell morphology). By using a specific polysaccharide- stain called Ruthenium red and coupling this with osmium tetroxide fixative, these researchers were also able to show that the matrix material surrounding and enclosing cells in these biofilms was polysaccharide (Jones et al, 1969).

During 1973, microbial slimes were studied in industrial water systems and it was found that they were not only very tenacious but also highly resistant to disinfectants such as chlorine (Characklis, 1973). Based on observations of dental plaque and sessile communities in mountain streams, some researchers put forth a theory of biofilms that explained the mechanisms whereby microorganisms adhere to living and nonliving materials and the benefits accrued by this ecologic niche (Costerton et al. 1978; Donlan, 2002).

For the medical community, the first surprise is about the bacteria that form biofilms preferentially in very high shear environments. Planktonic bacteria can adhere to surfaces
and initiate biofilm formation in the presence of shear forces that dwarf those of heart valves and exceed Reynolds numbers of 5,000 (Characklis & Marshall, 1990). The Reynolds number is a dimensionless number describing the turbulent flow of a liquid; if this number is high, turbulent flow exists; if it is low, laminar flow conditions prevail. Engineers speculate that turbulent flow enhances bacterial adhesion and biofilm formation by impinging the planktonic cells on the surface, but whatever the mechanism, biofilms form preferentially at high-shear locations in natural and industrial systems (Characklis & Marshall, 1990).

Studies of bacterial adhesion with laboratory strains of bacteria, many of which had been transferred thousands of times and lost their ability to adhere, first indicated that very smooth surfaces might escape bacterial colonization. Subsequent studies with “wild” and fully adherent bacterial strains showed that smooth surfaces are colonized as easily as rough surfaces and that the physical characteristics of a surface influence bacterial adhesion to only a minor extent (Costerton et al, 1999). Once a biofilm has formed and the exopolysaccharide matrix has been secreted by the sessile cells, the resultant structure is highly viscoelastic and behaves in a rubbery manner (Stoodley et al, 1998). When biofilms are formed in low-shear environments, they have a low tensile strength and break easily, but biofilms formed at high shear are remarkably strong and resistant to mechanical breakage (Donlan & Costerton, 2002).

1.3.2 Biofilms in pathogenesis:

Koch’s postulates state that (i) the organism is regularly found in the lesions of the disease, (ii) it can be isolated in pure culture on artificial media, (iii) inoculation of this culture produces a similar disease in experimental animals, and (iv) the organism can be recovered from the lesions of these animals (Davis, 1980). The question of whether biofilms are etiological agents of disease in many cases cannot be proven according to Koch’s postulates.

Nickel and Costerton studied coagulase-negative staphylococci (CoNS) in chronic prostatitis and were able to detect these organisms in biopsies from infected individuals. For several of the diseases such as periodontitis, native valve endocarditis, cystic fibrosis and UTI, this association is stronger than the other diseases (Nickel & Costerton, 1992).

Evidences for biofilm formation have come from a number of studies, using both scanning electron microscopy (Murdoch-Kinch et al, 1997; Santiago et al, 1994; Whitehouse et al, 1991) and viable plating of organisms isolated from the dental unit components (Tall et al,
A variety of bacteria was observed which embedded in an apparent polysaccharide matrix and they also could be cultured by the same organisms from both tubing biofilm samples and water samples, and numbers were similar in both types of samples (Whitehouse et al, 1991). The ability of Acinetobacter baumannii to adhere to and persist on surfaces as biofilms could be central to its pathogenicity. Production of pili and a biofilm-associated protein and the expression of antibiotic resistance are needed for robust biofilm formation on abiotic and biotic surfaces. This multi-step process also depends on the expression of transcriptional regulatory functions, some of which could sense nutrients available to cells (Gaddy et al, 2009).

All types of Foley catheters, including silvercoated and nitrofurazone impregnated catheters are vulnerable to colonization by crystalline biofilms (Morris, 1997; Stickler & Sabbuba, 2007). At present, no effective technique is available to prevent the problem (Liedl, 2001; Capewell & Morris, 1993; Stickler, 1996). Catheters are colonized by single species. As the catheter remains in place, the number and diversity of organisms can be increased and mixed communities develop. Most reports about biofilm formation in UTI and urinary catheters are related to the different bacteria such as Acinetobacter baumannii, Enterobacter aerogenes, S. epidermidis, Enterococcus faecalis, E. coli, Proteus mirabilis, Providencia stuartii, P. aeruginosa, Proteus mirabilis, Klebsiella pneumonia and M. morganii (Stickler, 1996). It should be noted that only a small percentage of the different morphological types observed by scanning electron microscopy and transmission electron microscopy could be grown by culturing. It is possible that at least a percentage of the organisms in these biofilms may not be culturable or cannot compete with the more rapidly growing organisms commonly isolated on complex media (Nickel et al. 1989).

1.3.3 Factors affecting biofilm formation:

1.3.3.1 Surfaces:

The surface could be a dead or living tissue, or any inert surface. The attachment of bacteria to one surface is a complex process, with many variables affecting the outcome. Further, growth requires a complex developmental pathway involving a series of events that are regulated in response to environmental- and bacterial-derived signals (Pratt et al, 1998).
There are many organics in nature, formed within minutes of exposure, and continue to grow for several hours (Loeb & Neihof, 1975). A prime example may be the proteinaceous conditioning film called ‘acquired pellicle’, which develops on tooth enamel surfaces in the oral cavity. A number of host-produced conditioning films such as blood, tears, saliva, intervascular fluid, respiratory secretions and urine influence the attachment of bacteria to biomaterials (Mittelman, 1996).

The surface may have several characteristics that are important in the attachment process. Microbial colonization appears to increase as the surface roughness increases (Characklis et al, 1990). This is because shear forces are less and surface area is more on rougher surfaces. Most investigators have found that bacteria attach more rapidly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals (Fletcher & Loeb, 1979; Pringle & Fletcher, 1983). A material surface exposed in an aqueous medium becomes conditioned or coated by polymers from that medium, and the resulting chemical modification will affect the rate and extent of microbial attachment (Bendinger et al, 1993).

1.3.3.2 Nutrients:

Increase in nutrient concentration correlated with an increase in the number of attached bacterial cells. However, low nutrient concentrations to measure are sufficient for biofilm growth. Biofilm bacteria acquire nutrients by concentrating trace organics on surfaces by the extracellular polymer, using the waste products from their neighbours and secondary colonizers, and by pooling their biochemical resources with different enzymes to break down food supplies. Because the biofilm matrix is often negatively charged, many nutrients (particulary cations) are attracted to the biofilm surface. Besides, nutrients with negative charge can exchange with ions on the surface. This provides bacterial cells within the biofilm with plenty of food compared to the surrounding water (Cowan, 1991).

1.3.3.3 Environmental cues:

Other characteristics of the aqueous medium, such as pH, nutrient levels, iron, oxygen, ionic strength and temperature, may also play a role in the rate of microbial attachment to a substratum. Several studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems (Donlan et al, 1994; Fera et al, 1989). This effect
may be due to water temperature or other unmeasured, seasonally affected parameters. For example, an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the attachment of biofilm forming bacteria to glass surfaces, presumably by reducing the repulsive forces between the negatively-charged bacterial cells and the glass surfaces (Fletcher, 1998).

1.3.3.4 Gene regulation:

There are mounting evidences to show that both up- and down-regulation of a number of genes occurs in the attaching cells upon initial interaction with the substratum. For example, 22% of the genes were upregulated and 16% down-regulated in biofilm-forming *P. aeruginosa* (Combaret et al, 1999). It was demonstrated that *algC* upregulation expressed within minutes of attachment to a surface in a flow cell system. Genes encoding for enzymes involved in glycolysis or fermentation (phosphoglycerate mutase, triosephosphate isomerase, and alcohol dehydrogenase) are up-regulated in most biofilm-forming bacteria (Davies & Geesey, 1995). The researchers surmised that the up-regulation of these genes could be due to oxygen limitation in the developed biofilm, favouring fermentation (Becker et al, 2001). A recent study also showed that *algD, algU, rpoS* and genes controlling polyphosphokinase synthesis were up-regulated in biofilm formation of *P. aeruginosa* (Pulcini, 2001).

Gram-negative bacteria respond to nutrient limitation and other environmental stresses by synthesizing sigma factors. In *E.coli*, those sigma factors that are under the control of the *rpoS* regulon regulate the transcription of genes whose products mitigate the effects of stress. By studying *E. coli* biofilms formed by strains with and without the *rpoS* gene, it was found that the *rpoS* *E. coli* biofilms had higher densities and a higher number of viable organisms. Since *rpoS* is activated during slow growth of this organism, it appears that conditions that elicit the slowing of bacterial growth, such as nutrient limitation or build-up of toxic metabolites, favor the formation of biofilms (Adams & McLean, 1999). Nutrient limitation and increases in toxic metabolite concentrations might be particularly acute within the depths of established biofilms. Agar-entrapped *E. coli* cells were more resistant to an aminoglycoside as oxygen tensions were decreased. this effect was due to lowered uptake of the antibiotic by the oxygen-starved cells (Tresse et al, 1995). The initial bacterial association with a surface may result in the repression or induction of genes, which in turn results in a number of physiological responses (Dagostino et al, 1991).
1.4 Plasmids in *Acinetobacter*:

1.4.1 *Acinetobacter* Plasmids:

Several reports have revealed that 80% of *Acinetobacter* isolates carry multiple plasmids of variable molecular size (Gerner-Smidt et al., 1989; Seifert et al., 1994). Most indigenous plasmids from *Acinetobacter* strains are relatively small (<23 kb) without any conjugative functions. A special interest for members of this genus also relies on the ability of some strains, i.e. those belonging to the species *A. baylyi*, to undergo natural transformation (Davison, 1999; De Vries & Wackernagl, 2002). This attribute has made the *A. baylyi* strain ADP1 (also named BD413) an exceptional tool for genetic analysis and engineering (Young et al., 2005; Fondi et al., 2010).

1.4.2 Role of plasmids:

Plasmids play an important role in the biology of most prokaryotic organisms, and *Acinetobacter* spp. appear to be no exception to this generalization (Towner, 1991). Several studies have reported that most *Acinetobacter* isolates carry multiple indigenous plasmids of variable molecular size (Gerner-Smidt, 1989; Seifert et al, 1994), although other scientists report problems in isolating plasmid DNA from *Acinetobacter* spp., often because of unappreciated difficulties in lysing the cell wall of these organisms. Although many clinical isolates of *A. baumannii* show widespread and increasing resistance to a whole range of antibiotics, there have been only a few studies in which plasmid-mediated transfer of resistance genes has been demonstrated.

However, as postulated by Towner (Towner, 1991), failure to observe transfer of resistance may simply reflect the absence of a suitable test system for detecting such transfer. For historical reasons, scientists attempting to transfer plasmids from clinical isolates of any gram-negative species have tended to use *E. coli* K-12 as a recipient strain. Complex and varied transfer frequencies of standard plasmids belonging to different incompatibility groups have been observed between *Acinetobacter* strain EBF 65/65 and *E. coli* K-12, and a number of these plasmids required an additional mobilizing plasmid for retransfer to occur (Chopade et al, 1985). Accordingly, it is not surprising that most reported cases of indigenous transmissible antibiotic resistance from *Acinetobacter* spp. have been associated with plasmids belonging to broad-host-range incompatibility groups (Towner, 1991).
There remains a need to vigorously assess the potential for mobilization of antibiotic resistance genes to and from the different genomic *Acinetobacter* species that have now been delineated. Transposons probably play an important role, in conjunction with integrons (Collis et al, 1993), in ensuring that particular novel genes can become established in a new gene pool, even if the plasmid vectors that transferred them are unstable, and there have been several reports of chromosomally located transposons carrying multiple antibiotic resistance genes in clinical isolates of *Acinetobacter* spp. (Towner, 1991).

### 1.4.3 Implications in antibiotic resistance transfer:

*Acinetobacter* is a genus that appears to have a propensity to develop antibiotic resistance extremely rapidly, perhaps as a consequence of its long-term evolutionary exposure to antibiotic-producing organisms in soil environment. This is in contrast to more “traditional” clinical bacteria, which seem to require more time to acquire highly effective resistance mechanisms in response to the introduction of modern radical therapeutic strategies; indeed, it may be their ability to respond rapidly to challenge with antibiotics, coupled with widespread use of antibiotics in the hospital environment, that is responsible for the recent success of *Acinetobacter* spp. as nosocomial pathogens. However, although all three of the major modes of chromosomal gene transfer have been demonstrated in *Acinetobacter* spp. (Herman & Juni, 1974; Towner & Vivian, 1976), only conjugation has so far been shown to play a significant role in the transfer of antibiotic resistance genes between members of this genus (Chopade et al, 1985; Towner & Vivian, 1977). The following sections summarize the known biochemical and genetic mechanisms of resistance of *Acinetobacter* spp. to the major groups of antibiotics.

#### 1.4.3.1 β-Lactams:

As with other gram-negative organisms, most resistance to β-lactams in *Acinetobacter* spp. is associated with the production of β-lactamases, including the widely distributed TEM-1 and TEM-2 enzymes (Devaud et al, 1982; Joly-guillou et al, 1988; Philippon et al, 1980. An analysis of 76 ticarcillin-resistant (MIC, >256 mg/liter) *Acinetobacter* strains for their β-lactamase content found penicillinase activity in only 41% of the resistant strains (Joly-guillou et al, 1988), of which the majority produced an enzyme with a pI of 5.4 (TEM-1 like), although a few had an enzyme with a pI of 6.3, which is characteristic of the β-lactamase CARB-5. Some β-lactamase activity was also identified with a pI above 8.0. These
were undefined enzymes that were presumed to be chromosomally encoded cephalosporinases because of their high pI. A separate study identified cephalosporinase activity in 98% of the clinical isolates of *A. baumannii* studied (Vila, 1993), and it therefore seems that cephalosporinases are the predominant β-lactamases in this species. Four such enzymes, designated ACE-1 to ACE-4, have been studied in detail (Hood & Amyes, 1999). All four enzymes were identified as cephalosporinases, although some possessed a little activity against penicillins and none had detectable hydrolyzing activity against aztreonam or the broad-spectrum cephalosporins, ceftazidime or cefotaxime. All four enzymes showed their maximum activity against cephaloridine and, except for ACE-4, showed good activity against cephradine. ACE-1 showed the broadest spectrum of activity with some hydrolysis of cefuroxime. The contribution of these chromosomal β-lactamases appears to be important in the expression of β-lactam resistance but may work in concert with a permeability reduction and altered penicillin-binding proteins that may already confer some inherent resistance (Obara & Nakae, 1991; Sato & Nakae, 1991). The acquisition of plasmid-encoded penicillinases does not seem to have been of paramount importance in the long-term β-lactam resistance of this genus (Joly-Guillou & Bergogne-Berezin, 1990).

A particularly worrying development is the identification of a novel β-lactamase, designated ARI-1, in an imipenem-resistant strain of *A. baumannii* isolated from a blood culture (Paton et al, 1993). This enzyme hydrolyzes both imipenem and azlocillin but not cefuroxime, ceftazidime, or cefotaxime. Direct conjugative transfer of the ARI-1 gene from its original *A. baumannii* host to an *A. junii* recipient has been demonstrated (Scaife et al, 1995), and the same plasmid can be visualized in the donor and recipient strains. These last observations suggest strongly that ARI-1 is a plasmid-encoded carbapenemase, a development that may have extremely serious long-term consequences.

### 1.4.3.2 Aminoglycosides:

Aminoglycosides are used widely for the treatment of *Acinetobacter* infections and increasing numbers of highly resistant strains have been reported since the late 1970s. Few published studies have been devoted to investigating the genetic nature of aminoglycoside resistance in *Acinetobacter* spp., but both plasmid and transposon locations for aminoglycoside resistance genes have been demonstrated (Devaud et al, 1982; Goldstein et al, 1983; Lambert et al, 1990).
1.4.3.3 Quinolones:

The emergence of *Acinetobacter* spp. as important hospital pathogens has occurred at the same time as increased reliance on 4-quinolones for the treatment of serious infection. The development of 4-quinolone resistance is often quite difficult to demonstrate in the laboratory and this finding has been extrapolated to suggest that resistance will be rare in the clinical situation. This is true for bacteria such as *E. coli* but does not seem to be the case for nonfermentative gram-negative bacteria such as *Acinetobacter* spp. Although the precise mechanism is virtually unknown, it is clear that *Acinetobacter* spp. can develop 4-quinolone resistance readily. Bacterial resistance to 4-quinolones in other genera has often been attributed to changes in the structure of the DNA gyrase subunits, usually by *gyrA* mutations (Vila et al, 1994).

*Acinetobacter* strains are less permeable to antibacterial agents than are many gram-negative organisms, and 4-quinolone resistance can also be conferred by outer membrane changes that result in decreased uptake. Selection of resistance by 4-quinolones can result in cross-resistance to β-lactams in *E. coli* and *P. aeruginosa* (Neu, 1988). This suggests that resistance results from alterations in the outer membrane, leading to decreased uptake. Studies on the development of 4-quinolone resistance in *P. aeruginosa* suggest that outer membrane protein changes are an early development in the buildup of resistance genes contributing to 4-quinolone resistance (Quibell et al, 1993), and this is also likely to be true for *Acinetobacter* spp. In support of this hypothesis, an increase in the proportion of *Acinetobacter* isolates with combined resistance to all β-lactams, all aminoglycosides, and quinolones has been demonstrated (Muller-Serieys et al, 1989).

1.4.3.4 Other Antibiotics:

Although it is known that various antibiotic resistance genes carried on plasmids of different incompatibility groups can be transferred into *Acinetobacter* spp. from *E. coli* (Chopade et al, 1985), there have been few other studies of antibiotic resistance mechanisms in clinical isolates of *Acinetobacter* spp. High-level (MIC, >1,000 mg/liter) trimethoprim resistance has been reported (Muller-Serieys et al, 1989), and the genes encoding such resistance are often associated with multiple other resistance genes in transposon structures on large conjugative plasmids. Similarly, the chloramphenicol acetyltransferase I (CAT1) gene has been
associated with both chromosomal and plasmid DNA in a clinical *Acinetobacter* isolate, suggesting that the CAT1 gene might be transposon encoded and had improved its survival potential by locating in both replicons (Elisha & Steyn, 1991).

1.4.4 Plasmid curing:

Various methods for elimination of antibiotic resistance markers have been reported which use physical agents such as heat (Groves 1979; Trevors, 1985; Bhattacharya et al., 1988) and chemical agents such as plumbagin (Lakshmi & Thomas, 1996), ethidium bromide (Patwardhan et al., 2008), sodium dodecyl sulphate (Tomoeda et al., 1968) and acridine orange (Patwardhan et al., 2008). Several curing agent have been used to cure bacterial plasmids. Curing of plasmids of *Acinetobacter* spp. by various curing agents has been done plumbagin, (5 hydroxy 2 methyl 1,4 napthoquinone), a compound derived from roots of *Plumbago zeylinica* has been reported to have antibacterial activity that eliminated multidrug resistant R plasmid from *Acinetobacter* spp. (Dhakephalkar, 1993). Elimination of ampicillin antibiotic marker from wild strain *A. haemolyticus* TA 34 as well as transformant *E.coli* DH5α using different curing agents such as acriflavin, acridine orange, ethidium bromide and plumbagin confirmed its plasmid encoded nature.

One research revealed that elimination of plasmid from antibiotic resistant *A. baumannii* and antibiotic sensitivity of *A. baumannii* cured isolates confirmed plasmid borne nature of antibiotic resistance markers. In three isolates of *A. baumannii*, plasmid elimination was observed by using conventional curing agents such as acridine orange and ethidium bromide. The cured isolates showed very low MIC values as compared to original isolates. Physical loss of plasmid from cured strains showed plasmid borne nature of antibiotic resistance markers (Patwardhan et al., 2008).

Another research indicated that the pUTI89 plasmid has characteristics of both F plasmids and other known virulence plasmids. Both *in vitro* and *in vivo* assays were used to examine the function of pUTI89 using plasmid-cured UTI89. No differences were observed between UTI89 and plasmid-cured UTI89 based on growth, type 1 pilus expression, or biofilm formation. However, in a mouse model of UTI, a significant decrease in bacterial invasion, CFU and IBC formation of the pUTI89-cured strain was observed at early time points postinfection compared to the wild type. Through directed deletions of specific operons on
Chapter 1: Introduction & Review of literature

pUTI89, the cjr operon was partially implicated in this observed defect. Our findings implicate pUTI89 in the early aspects of infection (Cusumano et al, 2010).

1.5 VEG-media for isolation of microorganisms:

1.5.1 Routine media

Several selective and differential media are currently in use for the isolation of *Acinetobacter* spp. The first such medium used for the isolation of *Acinetobacter* spp. was reported by Mandel et al. (Mandel et al, 1964) in 1964. This medium contained agar, soy peptone, pancreatic digest of casein, sodium chloride, lactose, maltose, bile salts, and bromocresol purple. The medium was modified to Herellea agar by substituting sucrose for maltose (MacFaddin, 1985). Another selective and differential medium was described by Holton (Holton, 1983); that medium contained desiccated ox bile instead of the bile salts (Mandel et al, 1994) in the original formulation and the antibiotics ampicillin, cefsulodin, and vancomycin. Holton (Holton, 1983) excluded lactose and bromocresol purple from his medium and substituted instead fructose, mannitol, phenylalanine, and phenol red.

Other selective media for the isolation of *Acinetobacters* have been described but are not widely used (Garrison, 1963; Grehn & Vongraevenitz, 1978). Previous mentioned media were either not sufficiently selective (because of the absence of appropriate inhibitory agents for other organisms) or too inhibitory (because of the presence of a high concentration of agents inhibitory for *Acinetobacter* spp., particularly ampicillin). So, the other prepared medium could be used to isolate *Acinetobacter* spp. of DNA groups 1, 2, 3, and 13 from clinical and environmental sources. Ampicillin was excluded from this medium, and the concentrations of the other antibiotics were adjusted after determining the MICs of a range of potentially useful antibiotics for a diverse collection of *Acinetobacter* spp. This medium was named as Leeds *Acinetobacter* Medium (LAM) and it was tested for inhibitory action by using a large collection of *Acinetobacter* spp. from environmental and clinical sources. Evaluation in use was carried out in two wards of a large tertiary-care referral hospital involving a semiquantitative comparison with other existing media (Jawad et al, 1994).

*Acinetobacter* agar was another selective medium used ethanol as a carbon source and an inorganic nitrogen source. Bile salts, ethanol, tetracycline and cycloheximide acted as selective agents. Cultures of *Acinetobacter* isolates produced small, convex, yellow colonies

22
on this medium after 48h incubation at 30°C. In contrast, type cultures of *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii*, *Pseudomonas fluorescens* and *Enterobacter cloacae* did not grow on the *Acinetobacter* agar plates under these conditions. When *Acinetobacter* agar was inoculated with samples to which *Acinetobacter*, *E. coli*, *C. freundii* and *K. pneumoniae* had been added and incubated at 30°C for 48 h, all colonies were typical of *Acinetobacter* in appearance and five random colonies were confirmed as *A. calcoaceticus*. Three 1-ml samples of water taken from natural sources spread onto *Acinetobacter* agar and incubated at 30°C for 48 h produced only colonies typical of *Acinetobacter* in appearance and were confirmed as *Acinetobacter*. When *Acinetobacter* agar was used to test 39 samples of butter, only one sample was shown to contain *Acinetobacter* and none of the other eight types of bacteria independently isolated from these butters grew on the *Acinetobacter* agar (Flint & Hartley, 1994).

1.5.2 Vegetable media

In a time of animal diseases, like bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) or foot and mouth disease, it is important to have a good alternative possibility. 100% vegetable protein source that means no risk of BSE/TSE, no problems with certificates and regulations. But it is not the only reason for a change, because several test showed that it is possible to get a better growth or yield by using Plant Peptone (Clelanda et al, 2007).

Many workers have suggested vegetable media for the cultivation of various organisms, although none of these has been found entirely satisfactory as a general-purpose medium. From vegetable material alone we should prepare media to have equal or more satisfactory than, meat-infusion peptone broth or agar as general culture media. The vegetable materials have been cotton-seed meal, peanut meal, soy-bean meal, various whole and sprouted grains, beans and seeds (Brewer, 1943).

One of the fundamental performance parameters of a bacteriological culture medium is its ability to promote the early and rapid growth of micro-organisms. Peptone, protein hydrolysates, infusions and extracts are the major sources of nitrogen and vitamins in culture media. Peptones are water-soluble ingredients derived from proteins by hydrolysis or digestion of the source material. Vegetable origin peptones are widely used in culture media
for cultivation. These are excellent source of vitamins and are available in the following types: Soy peptone GMO-Free, animal-free is a specially designed product, soy peptone is a papaic digest of defatted soybean meal. The growth characteristics of a range of bacteria that are routinely used in quality control practices should be compared for representative vegetable- based tryptic soy formulations. All of the representative microorganisms grew well on the vegetable-based media and the media provided suitable recoveries of the organisms following simulated storage (Clelanda et al, 2007).

1.6 Conclusion:

A large number of reports describe the outbreaks of *Acinetobacter* associated nosocomial infections such as secondary meningitis, pneumonia, wound, burn and urinary tract infections. These bacteria have been associated with opportunistic infections that were rare and of modest severity but during last two decades, an increase has been observed in both incidence and severity of their infection. Recently, *A. baumannii* are emerging as important cause of urinary tract infections and it appeared to be highly resistant to *in vitro* antibiotics in general. *Acinetobacter* is known to show resistance to a majority of commercially available antibiotics (penicillins, aminoglycosides, cephalosporins and quinolones) and therefore raises an important therapeutic problem. Biofilm formation is an important feature of most clinical isolates of *Acinetobacter* spp. especially in urinary tract and urinary catheters. Mixed infections also enhance the ability of these isolates to transfer resistance markers to the other clinical strains by transformation or conjugation. So, the removal of biofilms made up of two or more bacterial communities is thus very critical to decrease the incidences of gene transfer between bacteria. This may significantly decrease the formation of new multiple antibiotic resistant strains. A control of the spread of these infections thus demands the removal of *Acinetobacter* sp. from medical settings. The ability of *Acinetobacter* species to adhere to the surfaces, form biofilms, display antibiotic resistance and gene transfer demands an urgent need to study the responsible factors for their spread. On the other hand, most of available culture media are not reliable for isolation and differentiation of *Acinetobacter* spp. and the usual media were used in the past for this purpose was originated from animal protein. New media should be aimed to be prepared totally from plant sources without peptone and any antibiotics. These media should be cheap, easy in preparation with maximum capability for identification of *Acinetobacter* spp. among the other Gram negative in comparison to other media.
1.7 References:


Enterococci: an important infection control variable Arch Intern Med 158:1127-1132.


Chapter 1: Introduction & Review of literature


Chapter 1: Introduction & Review of literature


Chapter 1: Introduction & Review of literature


Chapter 1: Introduction & Review of literature


1.8 Genesis of work:
Clinical as well as environmental Acinetobacter spp. have been observed to be highly resistant to commonly used antibiotics and drugs (Dhakephakar & Chopade 1994; Shakibaie et al. 1999). Complex and varied transfer frequencies of standard plasmids belonging to different incompatibility groups have been observed between Acinetobacter strain EBF 65/65 and E. coli K-12, and a number of these plasmids required an additional mobilizing plasmid for retransfer to occur (Chopade et al. 1985). A. baumannii causes severe infections in compromised patients, survives on abiotic surface in hospital environments and colonizes different medical devices. Analysis of the processes involved in surface attachment and biofilm by the prototype strain 19606 was initiated (Tomaras et al 2003). Nosocomial urinary tract infection is caused only infrequently by Acinetobacter spp. (Pedraza et al. 1993). Thirteen A. baumannii strains isolated from urine of hospitalized and non hospitalized patients with different ages were investigated for the presence of virulence factors (Braun & Vidotto 2005). Studies on Acinetobacter spp. from upper respiratory tract and some other parts of the body from healthy human and patients have been studied at Microbiology Department in Pune University and this study will provide a good base line for detailed studies on Acinetobacter from urinary tract infections.

1.9 Hypothesis:
Acinetobacter spp. from urinary tract infection exhibit properties like antibiotic resistance, biofilm formation; they may contain naturally occurring plasmids and may provide the clue of acquisition as well as transfer of antibiotic resistance genes by Acinetobacter spp. to other bacteria.

1.10 Objectives:
1. Preparation of new media from plant materials for isolation of Acinetobacter spp.
2. Isolation, Identification and characterization of different species of Acinetobacter from UTI and antibiotic resistance in Acinetobacter spp. isolated from UTI.
3. Studies on adhesion by Acinetobacter spp. to the abiotic surfaces and biofilm in urinary catheters.