CERTIFICATION

This is to certify that the dissertation entitled, "Detection of Oxacillinase genes that confers carbapenem resistance in *Acinetobacter baumannii*, in hospital acquired infections" is the bonafide work of **Dr.Sudhir Babji**, toward the M.D. Branch-IV (Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R. Medical University, to be conducted in March 2009.

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DETECTION OF OXACILLINASE GENES THAT CONFER CARBAPENEM RESISTANCE IN *ACINETOBACTER BAUMANNII*, IN HOPSITAL ACQUIRED INFECTIONS.

Dissertation submitted as part of fulfillment for the M.D. (Branch IV Microbiology) Degree examination of The Tamil Nadu Dr.M.G.R. Medical University to be held in March 2009.

CONTENTS

INTRODUCTION

Acinetobacter calcoaceticus-baumannii is a non fermenting gram negative bacilli or cocco bacilli belonging to the family *Moraxellaceae.* It is a ubiquitous organism found in soil, sewage, and dry surfaces (1, 2). They can be readily isolated from areas of moist skin such as toe webs, groin, and the axilla. The organism can be identified from hospital equipment such as ventilators, curtains, telephones, door handles(2). It was until recently, dismissed as a non pathogen, contaminating clinical specimens. Of late the organism has been recognized as a nosocomial pathogen and has been, increasingly so, in patients admitted in intensive care units, on mechanical ventilation and on central venous catheters. It has emerged as a leading cause of Ventilator Associated pneumonia (VAP) (3, 4). The organism has low virulence potential, but with the emergence of multidrug and pan drug resistant strains, this organism has emerged as a leading cause of mortality, morbidity, and increased hospital cost and duration of hospitalization (5, 6). In the year 1984, *Acinetobacter spp.* was classified in the family *Neisseriaceae,* but more recently the molecular taxonomic studies have resulted in the reclassification of the organism in the new family of *Moraxellaceae* in 1991(7).

The gold standard for definitive identification of the species of these bacteria is by DNA-DNA hybridization. This is available only in reference laboratories. Most of the clinical laboratories report the organisms as belonging to '*Acinetobacter calcoaceticus-A.baumannii complex*' or the sacchrolytic *Acinetobacter* and includes *A.baumannii, A.calcoaceticus*, *Acinetobacter* genomospecies 3, and *Acinetobacter* genomospecies 13TU and as the non sacchrolytic *Acinetobacter spp* based on bio-chemical reactions.

A.baumannii is the most commonly isolated from the sacchrolytic group and *A.lwoffii* is the most common isolated from the non sacchrolytic group(7).

The drugs most effective in the early 80's, were the common anti pseudomonal cephalosporin, namely ceftazidime and cefotaxime. This was later replaced by the carbapenem group of drugs- imipenem and meropenem. By the early 1990s, there was resistance reported to this class of antibiotics as well. Over the years the, resistance to various class of anti microbial agents, has shown an upward trend across various studies done across the world and thus this organism has emerged as an important pathogen amongst the nosocomial infections. The resistance rate varies from 14% to as high as 30%. A study conducted in Christian Medical College, Vellore has shown an incidence of 14.2%(8).

This has prompted a search for effective drugs for treating infections caused by carbapenem resistant *A. baumannii*. They may be classified as

1. Drugs of renewed interest-Polymyxins and Sulbactam.
2. Newer drugs-Tigecycline.

Polymyxins act by disrupting the cell membrane. These drugs, though discovered in the 1980's, had fallen into disrepute due to severe nephrotoxicity. With the increased isolation of pan drug resistant strains, the use of this drug has been evaluated. The various in vitro and clinical use of this drug has shown the efficacy of this drug in combating the Multidrug and Pan drug resistant strains (9, 10).

Sulbactam has shown intrinsic activity against *Acinetobacter spp*., and has been shown to be effective as a mono therapeutic agent in clinical trials conducted in Spain and

Germany(11). Though not available as a single drug, it is available in combination with Cefeperazone and Ampicillin.

Tigecycline belonging to the tetracycline group of drugs acts via the inhibition of protein synthesis. The susceptibility to Tigecycline has varied in various reports across the world. No studies have been done in India, evaluating the efficacy of this drug in our isolates

Various mechanisms have been described by which *Acinetobacter spp.* mediates resistance to carbapenem group of drugs. This includes efflux pumps, loss of porin channel, and the most important being the production of various β-lactamases(4). Various classes of these enzymes have been detected in *Acinetobacter spp*., but the most widespread of them all belong to the class D group in the Ambler classification-the Oxacillinases. The oxacillinases are classified into nine major groups and each of these groups has many subtypes in them. The most wide spread of these enzymes is the OXA-51 enzyme, which is found to be present in all isolates of *A. baumannii*. This enzyme has been evaluated to be a molecular marker for rapid identification of this organism, from clinical isolates. The gene for this enzyme is present on the chromosome of *A.baumannii(12)*. The other most wide spread and well documented enzyme is the OXA-23. This enzyme is mediated via transposons. Also integrons have been described in *Acinetobacter spp*., the most important class found in *Acinetobacter spp.* is Class I. These integrons encode for resistance against different class of antimicrobial agents. Thus dissemination of these genes has made this organism resistant to all commonly available antibiotics, making the treatment options severely limited.

AIM & OBJECTIVES

Aim

To detect the various genes those encodes for carbapenem resistance in clinical isolates of *A.baumannii* and ascertain the molecular marker for rapid detection for the same.

Objectives

1. To identify *A.baumannii* phenotypically.
2. To evaluate OXA-51 for identification of *A.baumannii.*
3. To determine the gene encoding for carbapenem resistance (OXA-51 and OXA-23).
4. To determine the prevalent antimicrobial susceptibility profile among our study isolates.
5. To determine the susceptibility of carbapenem resistant *A.baumannii* to Tigecycline by disc diffusion method.
6. To determine the baseline MIC value for drugs of renewed interest (Polymyxins, Sulbactam) against carbapenem resistant nosocomial *A.baumannii*
7. To investigate for presence of other resistance genes in representative isolates.
8. To correlate the outcome in patients infected with carbapenem and Tigecycline resistant strains.
9. To correlate clinically the outcome using Clinical Pulmonary Infection Score (CPIS) and Charlson Co-morbidity Index (CCI).

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Acinetobacter species are gram negative non fermenting coccobacillary organism which has been increasingly implicated in various hospital acquired infections, leading to increased hospital stay, morbidity, mortality.

3.1 The organism

The *Acinetobacter* species are common free living saprophytes found in soil, water, sewage, and food products like pasteurized milk. They are known to survive for weeks on clothing, dry surfaces such as bed rails, ventilators, and curtains (1, 13).

3.1.1 Classification

The organism is encapsulated, aerobic, non fermentative gram negative short rods and on isolation from clinical materials can be frankly coccobacillary. The organism was classified(7, 13), first as *Diplococcus*, and then has been subsequently name as *Micrococcus, Mima*, and *Achromobacter.* The first strains of *Acinetobacter spp* were isolated by M.W. Beijerinck in 1911 from soil and were named as *Micrococcus calcoaceticus*. In 1954, J.Brisou and A.R. Prevot created the genus *Acinetobacter.* The word '*Acinetobacter*' derived from Greek meaning non motile, bringing together Gram negative saprophytes which do not produce pigments-the Tribe *Achromobactereae*, and also those which are non motile. In the year 1971, the *Subcommittee on the Taxonomy of Moraxella and Allied Bacteria* proposed that the genus *Acinetobacter* should include only the oxidase negative strains. In the year 1984, Bergey's Manual of Systematic Bacteriology classified *Acinetobacter* in the family *Neisseriaceae,* but more recently the molecular taxonomic studies have resulted in the reclassification of the organism in the

new family of *Moraxellaceae* in 1991. This family also includes *Moraxella, Psychrobacter* and related organisms. The organism based on 16S rRNA and rDNA-DNA hybridization assays, is hence classified in the Super family II of the Proteobacteria, Family *Moraxellaceae,* Genus *Acinetobacter* (13).

The genus was further subdivided into 12 genomospecies, by Bouvet and Grimont in 1986 based on DNA-DNA hybridization and nutritional characteristics(7). Further in the year 1989, the number of genomospecies was increased to 17. But some of the same genomospecies had different phenotypic characters; hence different DNA groups had same number. This lead to lot of confusion regarding the nomenclature of the organism at the species level. The type species is genomospecies 1 – *A.calcoaceticus* and this is primarily isolated from soil. The genomospecies 2 is *A.baumannii* and includes those species which were formerly classified as *Herella vaginicola* and then as *A.calcoaceticus var. anitratus.* Other organisms are *A.hemolyticus* (genomospecies 4), *A.junii* (genomospecies 5), *A.johnsonii* (genomospecies 7), *and A.lwoffii* (genomospecies 8). The rest of the species remained unnamed. Majority of these are non pathogenic to man, and hence most of the clinical laboratories report the organisms as belonging to *'Acinetobacter calcoaceticus-baumannii complex'* or the 'sacchrolytic *Acinetobacter*' and the non sacchrolytic organisms as the 'non sacchrolytic *Acinetobacter*'. *A baumannii* is the most commonly isolated from the sacchrolytic group and *A.lwoffii* is the most common isolated from the non sacchrolytic group.

3.1.2. Morphology and cultural characteristics

The organism is described as being short, plump, Gram negative rods typically 0.9-1.6X1.5-2.5μm in the logarithmic phase of growth, but often becoming more coccoid in

the stationary phase. The cell wall structure is typical to that of the gram negative bacteria, but the cells are difficult to destain. The cells occur in pairs, but also in chains of variable length. No spores are formed and flagella are absent. Although generally considered as non motile, ”twitching” or “gliding” motility has been described for some strains, especially on semisolid agar. Many of the strains are encapsulated, and this can be readily seen on India Ink preparations(13).

The organism is non fastidious in its growth requirements and will grow freely on ordinary simple media without any enrichment. The members of this genus are strictly aerobic and can grow at a wide range of temperatures. Most strains will grow at 33ºC, but some environmental isolates will grow 20-30ºC. The clinical isolates of *Acinetobacter* will normally grow at 37ºC, and some strains even at 42ºC. After 24 hours, the colonies are between 0.5-2mm in diameter; opaque to translucent, usually non pigmented, but some strains form white to cream colored colonies, which vary in consistency from butyrous to smooth and mucoid. The organism grows well on MacConkey agar, and produce faint pink colonies.

Some specific media have been described for selective enrichment, from soil and water- liquid enrichment culture (Baumann’s Enrichment Medium), and differential isolation can be done on an antibiotic- containing selective solid media. The most commonly used is Leeds *Acinetobacter* Medium, which has been found to be the best for isolating *Acinetobacter* species from environmental and clinical sources. It contains Vancomycin, Cefsulodin and Cephradine(13).

3.1.3 Biochemical characteristics(7)

Organism	Genomo sp.	Urease	Nitrate reduced	Growth at 37ºC	Growth at 44ºC	Hemolysis	OF Glucose	Arginine hydrolysis	Malonate utilized
A.johnsonii	7	-	-	-	-	-	-	V	V

A.baumannii	2	-	-	+	+	-	+	+	+
A.haemolyticus	4	-	-	+	-	+	V	+	-
A.calcoaceticus	1	-	-	+	-	-	+	+	+
A.junii	5	-	-	+	-	-	-	+	-
A.lwoffii	8/9	-	-	+	-	-	-	-	-

The organism is Oxidase negative, showing the absence of cytochrome C and catalase positive. The sacchrolytic (*A.calocaceticus-baumannii complex*) group acidifies most OF carbohydrates; in particular, definitive identification is made by demonstrating the rapid production of acid from lactose (1% and 10%). In contrast, *A.lwoffii* is assachrolytic. The bio chemicals commonly utilized, in clinical laboratory, are OF glucose, Malonate utilization, Arginine dihydrolysis, and Nitrate reduction.

3.1.4 Virulence factors

Although the organism is considered a low grade pathogen, certain characteristics which it possess, enhance the virulence of this organism in clinical infections (4).

1. The presence of a polysaccharide capsule made of L-rahmnose, D- glucose, D-glucuronic acid and D- mannose which protects the organism against phagocytosis and also makes it more hydrophilic.
2. The property of adhesion, to human epithelial surfaces due to capsule and fimbriae.
3. The presence of enzymes which damage host lipids.
4. The presence of lipopolysacchride which like other gram negative organisms is lethal to mice, pyrogenic in rabbits, and a positive Limulus ameobocyte lysate test.
5. The production of slime by some strains is important in mixed infection.
6. The production of iron containing siderophores such as acinetobactin, which chelates iron and helps the organism to survive ,under conditions of low iron

supply, in humans points to the role of iron in the virulence mechanisms of *Acinetobacter.*

7. These organisms are frequently resistant to multiple antibiotics used to treat nosocomial infection.

The experimental work done on mice has proven that the organism has low virulence 10^6 to 10^8 is the 50% lethal dose, even in neutropenic mice (14).

3.1.5 Habitat

Certain *Acinetobacter* species are found as normal constituents of the human skin. They can be readily isolated from areas of moist skin such as toe webs, groin and the axilla(2, 4). The organism can be identified from hospital equipment such as ventilators, curtains, telephones, door handlers(1). The organisms are ubiquitous and are found in soil, water, and sewage. Estimates have shown that *Acinetobacter spp.* may constitute as much as 0.001% of the total aerobic population of soil and water. In fresh water ecosystems, they have been found to be about 10^4 organisms per 100 ml(13).

Acinetobacter spp. has also been isolated from a variety of food stuffs, which include various poultry, milk products, and vegetables.

3.1.6 Industrial use of *Acinetobacter* species

Various species of *Acinetobacter* have been found to elaborate and excrete polymers which have found wide ranging utilities in the industry. Some of these polymers are “emulsan”, “biodispersan”, “alasan”. The various uses in the industry are

1. Bioremediation of waste waters and effluents.
2. Production of biopolymers and bio surfactants- For prevention of dental plaque.
3. Biomass production- Production of immune adjuvant.

4. Clinical uses- Production of glutaminase- asparaginase, Production of L-carnitine.

3.2 Risk factors for acquiring *Acinetobacter* species infection

The species which are implicated in clinical disease belong to the sacchrolytic group that is the *Acinetobacter calcoaceticus-baumannii complex*. The other main isolated *Acinetobacter* species from clinical specimens is *A.lwoffii*.

Risk factors for acquiring for infections can be divided as community acquired and hospital acquired (7).

The risk for community acquired *Acinetobacter spp.* infection are alcoholism, cigarette smoking, chronic lung disease, diabetes mellitus(2).

The risk factors for nosocomial infection include length of hospital stay, surgery, wounds, previous infections (independent of antibiotic usage), Fecal colonization with *Acinetobacter spp.* ,treatment with broad spectrum antibiotics, indwelling central intravenous or urinary catheters, admission to a burns unit or intensive care unit, parenteral nutrition and mechanical ventilation(2).

Outbreaks are frequently located in intensive care units and burn units involving patients on mechanical ventilation. Sources of transmission which have been identified include respiratory equipments such as resuscitator bags, valves, ventilator circuits, suction catheters. Other sources include humidifiers, pillows, mattresses, bedpans(2). The patients on mechanical ventilation and infected with resistant strains of *Acinetobacter spp.* were at a 21.8% attributable mortality, higher hospitalization costs, and longer ICU and hospital stays(15). *Acinetobacter* pneumonias are characterized by a late onset and

though still not clearly implicated in an increased mortality, were definitely linked with a longer hospital stay.

A study reported in India shows that, mechanical ventilation and a particular antimicrobial pattern, i.e. is resistance to ceftazidime, cefotaxime, amikacin, ciprofloxcin, ofloxacin contributed to increased mortality in hospitalized patients, infected with *Acinetobacter spp*(16).

3.3 Antimicrobial Susceptibility pattern for *Acinetobacter*

Acinetobacter spp. is inherently resistant to the β-lactam group of drugs. Till 1984-85, the drugs most effective against *Acinetobacter* infections were ceftazidime (80%) and imipenem (100%). Data available from the early 1990's, showed an increased resistance to the cephalosporin and carbapenem group. This trend continued, through the years and the latest data shows that the resistance to ceftazidime is about 50-60% and to imipenem is in the range of 20%-30%, thus continues to show an upward trend (17). Many different definitions are present in literature for Multidrug and Pan drug resistant *Acinetobacter* species. Multidrug resistance is resistance to more than two of the following five drug classes:

- antipseudomonal cephalosporins (ceftazidime or cefepime),
- antipseudomonal carbapemens (Imipenem or Meropenem),
- ampicillin-sulbactam,
- fluoroquinolones (ciprofloxacin or levofloxacin),
- amino glycosides (gentamicin, tobramycin, or Amikacin).

Despite "pan-" meaning "all," pan drug resistance is often defined as resistance to all antimicrobials that undergo first-line susceptibility testing that have therapeutic

potential against *A. baumannii*. This would include all β-lactams (including carbapemens and sulbactam, fluoroquinolones, and amino glycosides. However, with the increased use of the Polymyxins and possibly Tigecycline, this definition will have to encompass the newer and renewed drugs(4).

3.4 Epidemiology of *Acinetobacter* infections

Acinetobacter spp. are implicated in nosocomial infections and are increasingly difficult to treat as majority of the isolates are multidrug or pan drug resistant. The infections are being documented with increased frequency over the last few years from centers all over the world including India.

Burden of disease –World wide

Various studies have shown the isolation of multidrug resistant *Acinetobacter spp.* especially *Acinetobacter calcoaceticus-baumannii*. The occurrence of imipenem resistant ACB complex was first described in the year 1991 in a New York centre and has then been increasingly reported from across the continents and has of late become the second most common isolate from critically ill patients admitted in the intensive care unit, second only to *Ps. aeruginosa*.

3.4.1 The Americas

The imipenem susceptibility was determined in a surveillance network carried out in the USA from January 1994 to June 1998 and it was found that, of 976,927 total isolates collected over the study period, there was a year by year increment in the intermediate susceptible/completely resistant strains as determined by the NCCLS guidelines. Starting from 2.76% there has been an increment to about 5.8% by June 1998(17). The National Nosocomial Infection Surveillance system collected data from 1986 to 2003, showed

resistance to Imipenem increase from 0% to 20%(4). Another study based in 76 centers across USA has revealed a resistance of 39.8%(4). One interesting fact which has come to the fore is the rate of *Acinetobacter spp.* infections in army personnel returning from war in Iraq and Afghanistan (3, 15). It was found that war wound infected with *Acinetobacter spp* showed an imipenem resistance of 37%. In other countries like Guatemala, *Acinetobacter spp* as a cause of ventilator associated pneumonia is described to the tune of 17%, second only to Pseudomonas (3). In the Latin American countries a resistance of about 39% was estimated in a surveillance program conducted during 2004-2005(4).

3.4.2 South Africa

In South Africa the incidence of Imipenem resistant *Acinetobacter spp* isolated from blood stream infections, was to the tune of 30%.

3.4.3 European scenario

It was in Scotland, in the year 1986 that the first carbapenemase enzyme was identified. Till the early years of this decade, carbapenem resistance was not a major health problem in Europe, with a resistance ranging from 0% in Germany to 2.2% in England (18, 19). Since the year 2003, there has been an increase in the isolation of resistant strains which has increased over the years. The European isolates are unique in that the various isolates are clonally related. There is a southeast clone prevalent in England, and a single clone isolated from 55 hospitals in France. In addition to this, there are 3 international *A.baumannii* clones –called as European clones I, II and III, which are prevalent across Europe. Carbapenem resistance seems to be highest in the countries of Turkey, Greece, Italy, and England and the rates appear to be the least in countries of Germany and The

Netherlands. A study published states that *Acinetobacter spp* infections most frequently involve the respiratory tract of patients on ventilators and *Acinetobacter* pneumonia is more common in critically ill patients in Asian (range 4–44%) and European (0–35%) hospitals than in United States hospitals (6–11%). In Europe there is a difference seen in different countries regarding the proportion of ICU-acquired pneumonias, caused by *Acinetobacter spp* with low numbers in Scandinavia, and gradually rising in Central and Southern Europe. A higher proportion of *Acinetobacter spp* isolates were resistant to amino glycosides and piperacillin/tazobactam in Asian and European countries than in the United States(20).

3.4.4 Asia

Even in the Far East, there is an increase in *Acinetobacter spp* infections and those caused by carbapenem resistant strains have been documented. In Korea, the incidence of resistance has been documented to be 17% (21). The SENTRY study has revealed a rate of > 25% for this region in a survey done in 2001-2004.

3.4.5 Indian Scenario

The Indian scenario is not very different from the world scenario. Sporadic reports appear in literature as case reports of infections caused by multidrug resistance *Acinetobacter* species. A study done in Amritsar has revealed an incidence of *Acinetobacter* septicemia to be 12.3%. A study published from Christian Medical College, Vellore has documented an incidence of 14.2% *Acinetobacter* strains resistant to carbapenem class of drugs(8). A study published from All India Institute of Medical Sciences, New Delhi in 2005 has given a prevalence of 34.7% resistance to meropenem and 27.2% resistance in their institute(22). A study published from St. John's Medical College, Bangalore has shown a

resistance of 14%(23). Another study from Chandigarh in 2003 also has shown a resistance of 20% (19).

Author	Year	In patient/ out patient	No. of isolates tested	Major drugs tested	% resistance
Gupta *et al*	2006(22)	In patient	2626	CZD,CZD+CA, CTX,CTX+CA	27.2%
Arora *et al*	2006(24)	In patient	108	β-lactams, AG	12.3%
Gladstone *et al*	2005(8)	In patient	606	β-lactams, AG	14.2%
Sinha *et al*	2007(23)	Not mentioned	150	β-lactams, AG	14%
Taneja *et al*	2003(25)	In patients	85	β-lactams, AG	20%

β-lactam includes penicillin group, β-lactam + β-lactamase inhibitors, Carbapenem, Cephalosporins, CTX- Cefotaxime CZD- Ceftazidime, CA- Clavulanic acid, AG- amino glycosides.

3.5 Mechanisms of resistance

The presence of various mechanisms conferring resistance to the different class of antibiotics has been well documented. The various mechanisms are mediated by genes which are either chromosomally or plasmid mediated. Various integrons and transposons have been detected which encode for various proteins which confer resistance in these organisms (26).

3.5.1 Resistance to Aminoglycosides

The resistance to amino glycosides is mediated via modifying enzymes which have all been found to be located within the class I integrons. The major class of enzymes found is acetyltransferases, nucleotidytransferases and phosphotransferases. Also efflux pumps have been documented which throw out the antibiotic from within the cell. The pumps

which have been implicated in *Acinetobacter spp.* are AdeABC from the RND class and also AbeM from the MATE class. The newest addition is the methylation of 16S r RNA from Korea and Japan which confers high level resistance to all amino glycosides.

3.5.2 Resistance to Quinolones

Modification of DNA gyrase or Topoisomerase IV through mutations in the gyrA gene is well documented in *Acinetobacter calcoaceticus-baumannii complex*. Also the efflux pumps AdeABC and AdeM are also implicated, in the same.

3.5.3 Resistance to Other antibiotics

Resistance to tetracycline is mediated by the ribosomal protection, conferred by the *tet*(A) and *tet*(B) d genes and also by efflux pumps. Resistance to sulfamethoxazole is mediated by sul gene which is found on the 3' conserved region of an integron(4). The presence of sulfa resistance is highly predictive of the isolate carrying integrons. Resistance to chloramphenicol and trimethoprim is mediated by cat and dhfr genes respectively, all of which are present on integrons.

3.5.4 Resistance to β- lactam group

The resistance to beta lactam group of drugs includes both methods which involve the presence of enzymes which hydrolyze the beta lactam ring as well as mechanisms which lead to decreased concentrations of the drug, i.e. via the efflux pumps The beta lactamase or enzymes which cause hydrolysis of the beta lactam ring are classified by two systems.- Ambler's classification which is based on amino acid sequences and Bush-Jacoby-Medeiros classification, which is a functional classification.

Ambler's classification

- Class A- Penicillinases [TEM, SHV].
- Class B- Metallo-ß-lactamases [IMP, VIM].-These need zinc as a moiety for its functioning.
- Class C- Cephalosporinases [AmpC].
- Class D- Oxacillinases [OXA-23.OXA-58].

Classes A.C, D have a serine moiety at the active site.

Bush –Jacoby-Medeiros classification(27)

Group	Enzyme	Mol. Class	Inhibited by Clavulanic Acid.	Example
1	Cephalosporinase	C	No	P99,MIR-1
2a	Penicillinase	A	Yes	*S.aureus*[B]
2b	Broad spectrum	A	Yes	SHV-1
2be	Extended spectrum	A	Yes	TEM-3
2br	Inhibitor resistant	A	Diminished	TEM-30
2c	Carbenicillinase	A	Yes	CARB-3
2d	Cloxacillinase	D or A	Yes	OXA-1
2e	Cephalosporinase	A	Yes	FEC-1
2f	Carbapenemase	A	Yes	IMC-1
3	Carbapenemase	A	No	IMP-1
4	Penicillinase		No	SAR-2

The beta lactamase enzymes are both chromosomally as well as plasmid mediated. The AmpC cephalosporinases are chromosomally mediated and are found inherently in all *A. baumannii* strains. These enzymes are active against all beta lactams except cefepime and the carbapenem. They have an upstream regulator which is termed as ISAba1 which regulates the over expression of this gene(28).

Extended spectrum beta lactamase (ESBL) belonging to the Ambler Class A group have been described in these organisms. The various enzymes described in this organism are VEB-1 from France, PER-1 from Turkey, Belgium, TEM-92, and TEM-116 from Italy and SHV-12 from China. The most concerning of the enzymes described so far are the carbapenem hydrolyzing enzymes. These include 2 main class of enzymes- Ambler class D OXA type (group 2d of the Bush classification), which are the serine oxacillinases and are the most widespread and the metallo beta lactamses from the Ambler class B.

The first oxacillinase to be described with carbapenem hydrolyzing property was from Edinburgh, Scotland. It was named as ARI-1(for *Acinetobacter* resistant to Imipenem) and was found to of plasmid origin, was transferable, and was later renamed as $bla_{\text{OXA-23}}$. It was found to be of 273 amino acid length showing sequence homology to Ambler class D, oxacillin–hydrolyzing enzymes. Two highly conserved motifs, S-T-F-K and K-T-G (positions 79 to 82 and 216 to 218), believed to contribute to the function of the serine active site, and were also defined. A third motif, F-G-N at positions 152 to 154, which differs from the corresponding motif in all other OXA enzymes by the presence of phenylalanine instead of tyrosine, was identified. This unique substitution was postulated to have significant biochemical effects and was possibly a factor in the evolution of carbapenem resistance in *Acinetobacter*(29). Its biochemical characterization revealed a β-lactamase with a pI value of 6.65 and was poorly inhibited by clavulanic acid and EDTA. It has been recently determined that the gene for this has been transferred from another *Acinetobacter* species –*Acinetobacter radioresistens*. It is a commensal found commonly on the skin of humans, especially hospitalized patients. The gene was found to be chromosomal in this organism and it is hypothesized that *A.baumannii* first inserted an

ISAba-1 element into the genome of the *A.radioresistens*, and from there it got the *bla*$_{\text{OXA-23}}$ gene on the plasmid (30). Now more than 100 oxacillinase type enzymes have been identified in *Acinetobacter spp*. They are classified as those that occur chromosomally mediated and those that are mediated from plasmids. Nine main clusters of OXA type enzymes have been identified, which in their own groups have a sequence homology of about 92.5 to 99% to each other and 40-70% homology with the enzymes from other enzymes (31). Out of the listed below the first four groups, are the most implicated in clinical disease. Of them the first three are plasmid mediated, and the fourth is intrinsic that is chromosomally mediated. The nine groups are

1. *bla*$_{\text{OXA-23}}$ which has 2 other OXA types OXA-27,OXA-49.
2. *bla*$_{\text{OXA-24}}$ which has OXA24,25,26,40.
3. *bla*$_{\text{OXA-58}}$.,which is the only one in its group
4. The fourth group is chromosomally mediated and consists of *bla*$_{\text{OXA-51}}$ which encodes for OXA-51, OXA-64,-65,-66,-69.
5. OXA-55
6. OXA-48 which includes OXA-54,-SAR-2
7. OXA-50 which also includes OXA-50a to OXA-50d,-PoxB
8. OXA-60a to OXA-60d
9. OXA-62.

Of this OXA-51 is of special interest as this has been proposed to be ubiquitous in all *A. baumannii* isolates and is being touted as a molecular marker for the identification of *A. baumannii*(12). It was first reported from Argentina in the year 2004 and its analysis revealed a molecular mass of 35.5 kDa. Ampicillin was found to be the best substrate, and slow hydrolysis of imipenem was detected. It was also determined, that the enzyme had highest affinity for imipenem as a substrate (32). A study done in England was able

to identify the gene in all isolates studied(12). Of further concern is the emergence of two distinct clones within the OXA-23 group. A study done in England, has identified 2 clones which have a different susceptibility profile, clone 2 being, susceptible to amikacin whereas clone 1 was resistant. Minocycline also had only borderline activity against clone1 but good activity against clone 2 (33).

The expression of the above mentioned genes, is dependent on various insertion sequences, which act as upstream regulators, the presence of which causes the over expression of the genes leading to clinical carbapenem resistance. The insertion sequences have a two fold action; firstly they encode for a transposase and hence are mobile: secondly they can contain promoter regions that lead to the over expression of downstream resistance elements (28). This is especially true about the OXA-23 and OXA-58 and may regulate the expression of OXA-51. This also mediates tetracycline resistance.

The other classes of enzymes, important for carbapenem resistance are the metallo beta-lactamase group of enzymes. These are found within class I integrons in *A. baumannii* and though less described in *Acinetobacter spp.* isolates are about 100-1000 times more potent than OXA type carbapenemases in hydrolysis of carbapemens. The 3 types of MBL found in *A. baumannii* are IMP, VIM, SIM(4).

Another important mechanism of multidrug resistance in *Acinetobacter* spp is integrons. Integrons are assembly platforms that incorporate exogenous open reading frames (ORFs) by site-specific recombination and convert them to functional genes by ensuring their correct expression. All integrons have three key elements necessary for the capture of exogenous genes: a gene (*intI*) encoding an integrase belonging to the tyrosine-

recombinase family; a primary recombination site (*attI*); and an outward- orientated promoter (Pc) that directs transcription of the captured genes. Integron-encoded integrases can recombine discrete units of circularized DNA known as gene cassettes. Integration occurs downstream of the resident Pc promoter at the *attI* site, allowing expression of the genes in the cassette. All integron-inserted gene cassettes identified share specific structural characteristics and generally contain a single gene and an imperfect inverted repeat at the 3′ end of the gene called an *attC* site. The *attC* sites are a diverse family of nucleotide sequences that function as recognition sites for the site-specific integrase. They vary in length from 57 bp to 141 bp, and their nucleotide sequence similarities are primarily restricted to the boundaries, which contain conserved sequences known as the R″ sequence and the R′ sequence. At present, five classes of mobile integrons are known to have a role in the dissemination of antibiotic-resistance genes. These classes have been historically defined based on the sequence of the encoded integrases, which show 40–58% identity. All five classes are physically linked to mobile DNA elements, such as insertion sequences (ISs), transposons and conjugative plasmids, all of which can serve as vehicles for the intraspecies and interspecies transmission of genetic material. Three classes of mobile integrons are historical classes that are involved in the multiple-antibiotic-resistance phenotype. Class 1 integrons are associated with functional and non-functional transposons derived from Tn*402* that can be embedded in larger transposons, such as Tn*21*. Class 2 integrons are exclusively associated with Tn*7* derivatives, and class 3 integrons are thought to be located in a transposon inserted in as-yet-uncharacterized plasmids. Class 1 integrons are found extensively in clinical isolates, and most of the known antibiotic-resistance gene cassettes belong to this class. Over 80

different gene cassettes from class 1 integrons have been described. Between them, these elements confer resistance to all known β-lactams, all aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampin, erythromycin, fosfomycin, lincomycin and antiseptics of the quaternary-ammonium-compound family (34). Study done by Turton *et al* has shown that the Class I integrase was found in all strains in an epidemic outbreak in England and have proposed it as a marker for detecting epidemic outbreaks of *Acinetobacter* (26).

Not all carbapenem resistance is mediated via the various enzymes described, but also due to the presence of efflux pumps which actively pump out the antibiotic from the internal milieu of the cell. Of significance in carbapenem resistance are two efflux pumps- AdeABC from the RND (Resistance –nodulation-division) family and the AbeM pump of the MATE (Multidrug and toxic compound extrusion) family (4, 35). In addition, the loss of a porin channel termed as CarO porin which is a 29-kDa protein was implicated in resistance to carbapemens. This protein is a novel Outer Membrane Protein, which is unique to the *Moraxellaceae* family (4, 36).

All the above stated mechanisms can occur singly or in conjugation with each other. The most worrisome of this is the fact that the majority of the resistance genes are found on transposons and also because of the insertion sequences which carry the genetic information for drug resistance across various classes of drugs. Indiscriminate utilization of one class of drugs can lead to expression of resistance to another class of drug. For example the presence of genes encoding for MBLs, also has genes encoding for amino glycoside resistance so, the over use of carbapemens, can lead to the development of resistance to both carbapemens and amino glycosides.

3.6 Laboratory Methods to determine Resistance:

3.6.1 Phenotypic Methods

Screening methods

1.According to the CLSI (Clinical and laboratory Standards Institute), break points have been laid down to determine the resistance or susceptibility of *Acinetobacter spp* for various antibiotics by the disc diffusion (DD) and minimum inhibitory concentration (MIC)(37). According to the January, 2008 document, for a disc strength of 10µg for Imipenem and Meropenem, a zone diameter of <13mm as determined by disc diffusion is categorized, as resistant. MIC as determined by broth dilution or agar dilution method using cation adjusted Mueller-Hinton broth /agar, was interpreted as resistant for values of >16µg/ml.

2. Various automated systems have been evaluated for their efficacy in detecting carbapenem resistance. Their pick up rate ranged from 6.7 to 87%. Also E test (Epsilometer Test), gave inconsistent results due to presence of colonies in the zone of inhibition(4).

3. A clue for the presence of MBL's can be got by a susceptibility pattern of a strain resistant to all antibiotics except Aztreonam. This is because the action of the MBLs is inhibited by Aztreonam.

4. The presence of the efflux pumps can be determined by the growth of organism in the presence of reserpine or xylene.

Confirmatory tests

a. A method of detection for the presence of MBLs and carbapenemases is the Modified Hodge's test and the double disc synergy using EDTA instead of a β-lactamase inhibitor.

The modified Hodge test uses *Escherichia coli* ATCC 25922 and 10 µg imipenem disc instead of *Staphylococcus aureus* ATCC 25923 and a 10 µg penicillin disc, respectively(38).

Modified Hodge test is carried out on Mueller-Hinton agar. The plate is inoculated, as a lawn culture, with a 1 in 10 dilution of 0.5McFarland adjusted, overnight culture suspension of *Esc.coli* ATCC 25922. After allowing the plate to dry for a while, 10 µg Imipenem disc is placed at the center of the plate and test strains are streaked from the edge of the disc to the periphery of the plate in four different directions. After overnight incubation the plates are observed for the presence of a 'cloverleaf shaped' zone of inhibition. The plates with such zones are interpreted as modified Hodge test positive(38).

For the EDTA-disk diffusion synergy test an overnight broth culture of the test strain, (opacity adjusted to 0.5 McFarland opacity standards) is used to inoculate a plate of Mueller-Hinton agar. After drying, a 10 µg imipenem disc and a blank filter paper disk, 6mm in diameter made from Whatmann filter paper number 2 are placed 10mm apart from edge to edge. 10 µl of 0.5 *M* EDTA solution is then applied to the blank disc, which resulted in approximately 1.5 mg/disc. After overnight incubation, the presence of an enlarged zone of inhibition is interpreted as EDTA synergy positive(38). The sensitivity of this test is 95.7%. Also instead of EDTA, 2-mercatopropionic acid can be used.

b. E test strips for metallo β-lactamase production are available as imipenem and imipenem-EDTA combinations. A positive test for metallo β-lactamase production is interpreted as a three fold or decrease in MIC of the imipenem in the presence of EDTA

c. Carbapenem inactivation assays are a fast way of detecting carbapenemase activity, especially for weakly hydrolyzing enzymes like OXA-23.

d. The cloverleaf test, where suspensions of whole cells or and/or an extract of the suspect isolate are tested against Imipenem on an agar plate. Altered growth of an indicator strain (*Esc.coli* ATCC 25922) around an imipenem disk is a positive result (31).

e. Iso electric focusing (IEF), is used to separate out proteins, by charge and the detection of β-lactamses can be done by using the chromogenic cephalosporin nitrocefin. Overlaying of the gel with EDTA, clavulanic acid, or Aztreonam can detect sensitivity of the enzyme to these potential inhibitors. IEF is an excellent modality to detect multiple β-lactamases in one isolate. IEF can also be used as an bioassay by using an agar overlay with Imipenem and a second overlay with a susceptible indicator organism .Growth over an enzyme band indicates a potential carbapenemases. This is especially effective in detecting those enzymes which have a poor hydrolyzing rate with nitrocefin (31).

f. Imipenem hydrolysis can be most reliably detected by spectrophotometric measurement using crude cell extracts or purified β-lactamases. If a metallo β-lactamase is suspected, brief pre incubation with EDTA prior to analysis results in a lower hydrolysis rate (31).

g. Mass spectrometry can be utilized to determine the loss of the 29-kDa porin channel

3.6.2 Molecular Methods

Various studies towards the determination of genes responsible for the carbapenem resistance have been published. Polymerase Chain Reaction (PCR) is a laboratory technique which amplifies a DNA target region, so as to obtain a million or more copies which can then be easily visualized by using DNA staining techniques. Many of the

genes have been fully sequenced and have been expressed in *Esc.coli* as part of transformation experiments, proving beyond doubt, the role of these enzymes in resistance. There are various multiplex polymerase chain reactions, which at the same time can detect for different groups of β-lactamase in the same reaction (39). PCR's have been developed which determine the presence of different groups of OXA enzymes in the same reaction. Also PCR's to detect the integrons have been developed (26). PCR is the fastest way to determine, which family the beta-lactamase belongs to.

Enzyme family	Primers	Primer sequence (5'-3')	Fragment size (bp)	Reference
Class D oxacillinases				
Subgroup 1 (OXA-23)	P5	AAGCATGATGAGCGCAAAG	1,066	Donald *et al*(29)
	P6	AAAAGGCCCATTTATCTCAAA		
Subgroup 2 (OXA-24)	Forward	GTACTAATCAAAGTTGTGAA	1,023	Afzal *et al*(40)
	Reverse	TTCCCCTAACATGAATTTGT		
Subgroup 3 (OXA-69)	OXA-69A	CTAATAATTGATCTACTCAAG	975	Heritier *et al*(41)
	OXA-69B	CCAGTGGATGGATGGATAGATTATC		
Subgroup 4 (OXA-58)	Pre-OXA-58prom+	TTATCAAAATCCAATCGGC	934	Heritier *et al*(42)
	PreOXA-58B	TAACCTCAAACTTCTAATTC		
Multiplex PCR for OXAs in *A. baumannii*	OXA-51-like	TAATGCTTTGATCGGCCTTG	353	Woodford *et al*(39)
		TGGATTGCACTTCATCTTGG		
	OXA-23-like	GATCGGATTGGAGAACCAGA	501	
		ATTTCTGACCGCATTTCCAT		
	OXA-24-like	GGTTAGTTGGCCCCCTTAAA	246	
		AGTTGAGCGAAAAGGGGATT		
	OXA-58-like	AAGTATTGGGGCTTGTGCTG	599	
		CCCCTCTGCGCTCTACATAC		
Class B metalloenzymes				
SIM-1	SIM1-F	TACAAGGGATTCGGCATCG	571	Lee *et al*(43)
	SIM1-R	TAATGGCCTGTTCCCATGTG		
Integron PCR	5' CS	GGCATCCAAGCAGCAAG	Variable	Turton *et al*(26)
	3' CS	AAGCAGACTTGACCTGA		

3.7 Treatment modalities for Multi drug resistant and Pan drug resistant *Acinetobacter* infection

With the ever increasing isolation of carbapenem resistant strains, the treatment options left with the clinician have been severely compromised. This is also due to the fact that the isolates tend to be resistant to many of the other commonly prescribed antibiotics. As determined by the Infectious Diseases Society of America, *A.baumannii* is one of the "red alert" pathogens that greatly threatens the utility of the currently available antibacterial agents(4). The presence of integrons carrying multiple resistance genes on them, render many of the available antibiotics useless, in spite of them not being utilized in the particular case.

The drugs being advocated for resistant *Acinetobacter* infections are

1. **Tigecycline-** This is a novel glycycline antibiotic, and has a bacteriostatic effect on MDR *Acinetobacter spp*. It is a 9-t- butylglycylamido semi synthetic derivative of Minocycline. It also acts by binding to the 30S ribosomal subunit(4). The main advantage for this drug is that it evades the resistance mediated by the tet(A) and tet(E) determinants which have made the organism completely resistant to the action of tetracycline. Though this drug is being marketed as an alternative treatment for MDR *Acinetobacter* infection, reports of resistance have already started to emerge from various parts of the globe. Reports of resistance have come in from Germany, Spain, and Israel. In Israel as high as 80% resistance is reported. It also has been found not to provide any benefits when given as combination therapy(44). Mechanisms have been elucidated, by which the organism has developed resistance to this antibiotic. The organism uses the AdeABC efflux pump, to pump out the antibiotic. It has been clearly

demonstrated in studies, that the adeB gene is over expressed in isolates, showing an increased MIC to Tigecycline. The presence of resistance to the antibiotic is already present in the Middle East(45). The problem is further compounded by the lack of standardized guidelines, thus making the interpretation of a zone diameter or MIC value not reproducible across testing centers. No break points have been set by the CLSI. According to the product information for Tigecycline, a fresh medium (not less than 12 hours old) and a paper disc impregnated with 15µg/ml should be used. Some authors have utilized the break points set for Enterobacteriaceae, which is not recommended as the cut off are based on extensive studies done on the wild type, infection causing isolates and the drug properties in vitro. Comparison of E test with Broth dilution method of MIC has not been very satisfactory, with some authors finding excellent concordance between the two methods, whereas some have reported a four fold difference(4).

2. **Polymyxins**- Belong to a group of polycationic peptides naturally synthesized by *Bacillus polymyxa*, a non actinomycete bacterium. Five chemicals were identified of which only two compounds were commercially developed –Polymyxin B and Polymyxin E or colistin. They act primarily on gram negative cell wall, leading to rapid permeability changes in the cytoplasmic membrane and ultimately cell death. These drugs cross the bacterial outer membrane by a self promoted pathway by competitive divalent ion displacement by the bulky poly cations, which non- covalently cross the bridge adjacent to the polysaccharide components. Consequently, the bacterial outer membrane becomes distorted and more permeable, permitting increased uptake of the permeabilizing compounds.

These drugs till the late 1980's were the only option for treatment of multidrug resistant *Ps. aeruginosa* infections(46). These drugs were deemed to be too toxic for routine use, because of the severe nephrotoxicity and neurotoxicity associated with these drugs, with the advent of the cephalosporin class of drugs in the 80's, these drugs were more or less forgotten and their use was restricted to only topical use. With the emergence of MDR and Pan drug resistant *Acinetobacter* infections, has led to renewed interest in this class of drugs. It has also been increasingly seen that the nephrotoxicity attributed to the administration of these drugs, was in fact partly due to the sepsis and the inflammatory response mounted by the patient himself. Many trials conducted in vitro have proven the efficacy of these drugs in the bactericidal action against these infections. Also clinical trials conducted in the United States as well as in centers in Europe, have proven beyond doubt the efficacy of these drugs, used in combination therapy especially with rifampicin, tobramycin(47). Also in vitro testing, by using Fractional Inhibitory Concentration Indices (FICIs), has proven that rifampicin, azithromycin and imipenem MICs fell when used in combination with Polymyxin B. FICIs are calculated as MIC of drug X (in combination)/ MIC of drug X alone + MIC of drug Y(in combination)/MIC of drug Y alone(10, 48). Other in-vitro studies have shown a 100% synergistic action against MDR *A.baumannii* using rifampicin and colistin in combination (49, 50). Also a study done has shown 92% synergy between colistin and minocycline(51). The better monitoring facilities for renal functions have led to the reuse of this drug much more than before. The curative rate on treatment with these drugs have been estimated to range from 57 to >80%

(4). Studies have proven the use of Colistin in neublized form, for ventilator associated pneumonia has proven much more beneficial, without the accompanying, nephrotoxicity and neurotoxicity. Only complication noticed was broncho spasm. This problem has been solved by using bronchodilators before administering the dose of colistin(4). The problem, which is becoming apparent, is the super infection with organisms which are inherently resistant to colistin like Serratia *spp*, Proteus *spp*. The use of intra thecal or intra ventricular colistin for nosocomial meningitis caused by *Acinetobacter* is now documented(4). The CLSI has not laid down guidelines for the interpreting the disc diffusion zones(37). This is attributed to the fact that the antibiotic is a very large molecule and the diffusion into the agar medium is not very satisfactory. It, though has given interpretations for MIC's and studies done have given a good co-relation with disc diffusion data(46). Resistance has been documented for this antibiotic as well, though the susceptibility for Polymyxin B is 95% and colistin is 96.7%(46). Hetero resistance is documented, that is the same sample will have both resistant and sensitive strains. The exact mechanism of resistance has not been worked out but is attributed to a change in the cell wall permeability, with decreased binding to the LPS, or a change in the OMPs(52).

3. **β- lactam and β-lactamase inhibitors**- One of the drugs, which holds some promise as an alternate for treating *Acinetobacter* infections is Sulbactam. This is a β –lactamase inhibitor and is like clavulanic acid and tazobactam. It is marketed in combination with ampicillin or cefeperazone. These β- lactamase inhibitors are β-lactam ring containing compounds, which did not have very strong bactericidal

effect, when they were discovered. It was also discovered, that these compounds, bind to the β-lactamase enzymes, thus keeping the drug safe from the hydrolytic action of these enzymes, and enabling the drug to act. The efficacy of these drug combinations was assessed and it was found that sulbactam had an intrinsic activity against *Acinetobacter* infections (53); tazobactam had intermediate effect whereas clavulanic acid had no activity against them (54). In vitro studies have proven conclusively that the, sulbactam component of the combination is the effective agent against these infections. This was conclusively proven when the MICs of β-lactam alone was compared with that of sulbactam and the combination also, and it was seen that there was a reduction of MICs when used in combination as compared to the β-lactam drug alone, which was usually completely resistant(54). In vivo studies also have proven the usefulness of this drug. Studies have documented, an improvement in 95.6% patients started on ampicillin-sulbactam combination and 94.4% improvement on Sulbactam only therapy(11). Ampicillin-sulbactam is marketed in a ratio of 2:1. Sulbactam now is marketed as a solo drug in countries like Germany, Spain, and France. The CLSI also has laid down guidelines for the interpretation of ampicillin-sulbactam MICs and Disc diffusion testing. A study also proved that the efficacy of a cefeperazone-sulbactam combination as compared to imipenem (55). The epidemiology of sulbactam sensitivity across the globe is different, with a trend towards increased resistance as well. One of the other problematic areas is in the treatment of *Acinetobacter* meningitis, as sulbactam has very poor penetrability

into the meninges, thus making this a non viable option for the treatment of meningitis (4, 11).

3.8 Factors affecting outcome in patient's infected with resistant isolates

Various factors affect the outcome of patients infected with multidrug and pan drug resistant *Acinetobacter* isolates. Some of the most important factors to be assessed in predicting mortality are the co-morbid conditions which the patient has in addition to the disease process. These factors include chronic and acute conditions. The chronic conditions include diabetes, hypertension, and immunosuppressive conditions like HIV etc. The acute conditions include dyselectrolytemia, cardiovascular compromise etc. All these factors affect the outcome of the patient. In studies done in evaluate the outcome of a disease or interventional strategy it is important to account for these factors as well.

The acute scoring systems include APACHEII scoring, SOFA scoring. For chronic comorbidities the scoring systems are Charlsons scoring index and CDS (chronic disease score). To get rid of these confounding factors in studies many scoring systems have been in place to remove the bias in studies. These scores initially were developed for non infectious diseases, but of late they have been applied to infectious disease processes as well. Charlson scoring index gives differential weight age to different chronic com morbid conditions like diabetes with or without renal failure, chronic liver disease, HIV, malignancies etc.(ANNEXURE I). A study done to assess the predictive value of this score for risk of infection was found to be inadequate (56, 57).

In patients on artificial ventilation the risk of acquiring a nosocomial infection especially pneumonia increases manifold. This is due to the impaired mucociliary action, suppression of cough reflex, pooling of secretions. To assess whether a patient has truly

developed ventilator associated pneumonia or is at an increased risk for the same a scoring system was developed known as the Clinical Pulmonary Infection Score (CPIS). The CPIS system has been developed as a score to predict the development of pneumonia in intensive care unit patients especially those on ventilators. This scoring system developed by Pugin *et al* in 1991 has proven useful in predicting the development of pneumonia in ICU patients. The score has the following components

- Tracheal secretions
- Chest X-ray Infiltrates
- Temperature
- Leukocyte count
- $P_{Ao2/ Fio2}$ mmHg
- Microbiology

All of the above are given a score of 0 to 2 with a maximum score of 16. It has been proven statistically that a score of 6 or >6 at the time of admission and at three days after admission to an intensive unit is a strong predictor of VAP (Ventilator Associated Pneumonia). This has a sensitivity of 93% and specificity of 100%. The sample to be collected for checking the microbiology of the respiratory tract is broncho alveolar lavage (BAL)(58, 59).

This scoring system has been evaluated for prediction of mortality in ICU patients and has been compared with APACHE II score and SOFA score. The study revealed that CPIS system was not able to predict mortality as compared to APACHEII (60).

3.9 Trends towards care and treatment of MDR *Acinetobacter* infections

With the increased recognition of *Acinetobacter* as a leading cause of nosocomial infections especially in patients who are on mechanical ventilation and in intensive care

units, leading to increased hospital stay and mortality, the management of these infections has become an issue.

Spread from one patient to another has prompted towards isolation procedures being employed, as in segregation of patients, and the restriction of carbapenems as empiric therapy agents for ICU admissions. Studies have comprehensively proved the efficacy of a sulbactam based therapy, to be as efficacious as an imipenem based therapy, thus providing with an alternative antibiotic option, decreasing selection pressure on the organism. In addition, surveillance procedures involving the environment and health personnel, implementation of Standard Precautions and the search for newer drugs are warranted.

Materials and methods

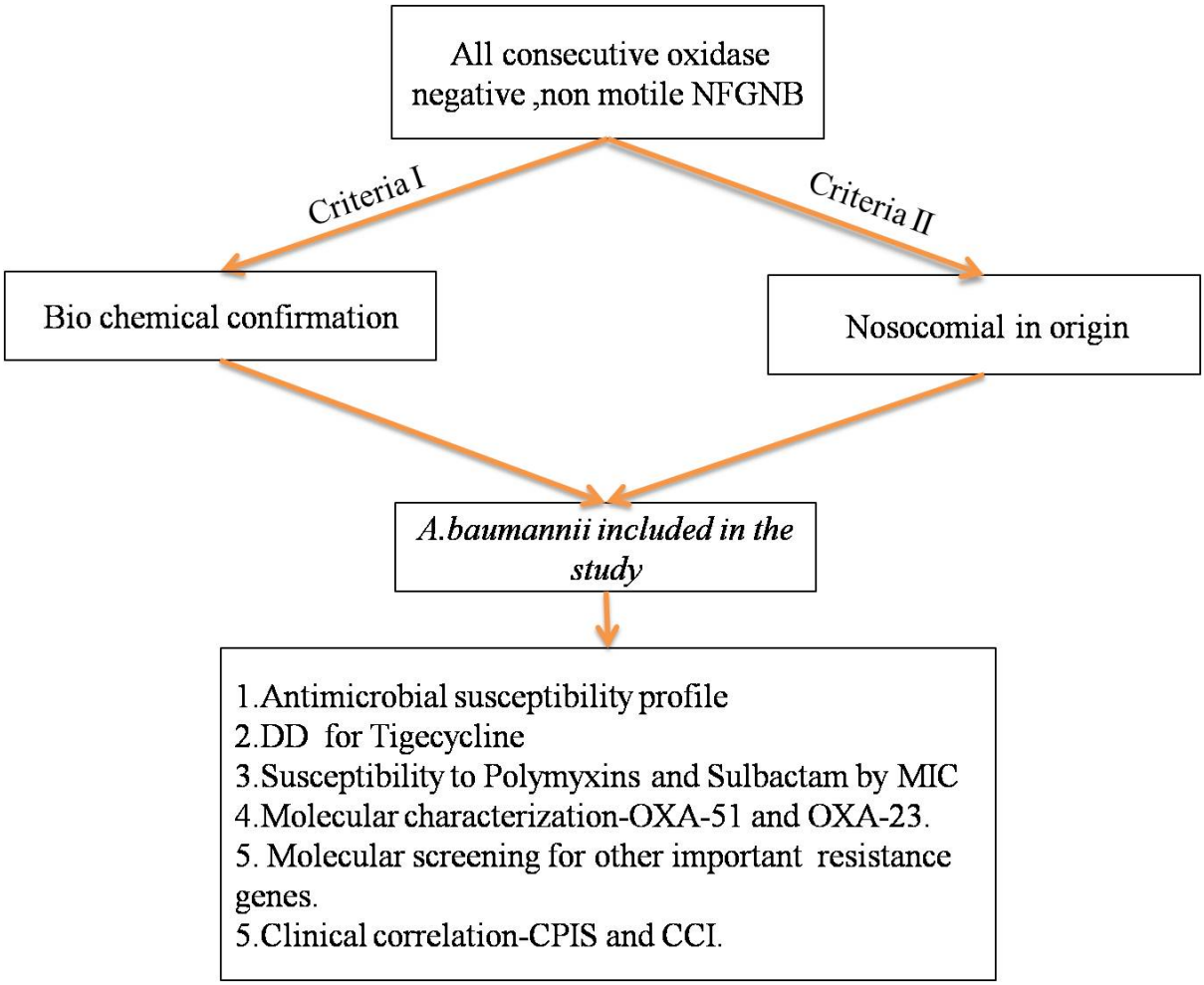
Type of Study-Descriptive

Materials- A total of 100 isolates of *Acinetobacter calcoaceticus-baumannii* complex were enrolled in this study. The isolates were from various clinical materials sent to the Clinical Microbiology department for bacteriological culture, biochemical identification, and antibiotic susceptibility testing. Isolates included in this study were obtained from blood, sputum, Endo tracheal aspirate, tracheal aspirate, central lines, cerebrospinal fluid, wound swabs, infected tissue.

Inclusion criteria

The isolates were collected form patients, who were diagnosed to have nosocomial infections defined as infections developing 48 hours after hospital admission. Samples collected for a period of 1 year, i.e. samples coming during the time period from, December 2006 to December 2007 were included in this study. As the main objective was to detect for OXA-51, using the formula to calculate for prevalence-

$$n = \frac{z^2 P(1-P)}{d^2}$$ (Where Z is 95% point of the std. normal distribution and d is the precision of the prevalence estimate), the sample size was found to be 73. Hence one hundred nosocomial *A.baumannii* isolates was included in this study.



Samples were collected from patients on mechanical ventilation, having central venous catheters, indwelling urinary catheters, post surgical patients being subjected to dressing or drainage of surgical wounds, including burns. Samples, send from various clinical units, were processed according to standard protocol followed in the laboratory(61). All samples excluding blood cultures, were inoculated onto 7% sheep blood agar, chocolate agar and Mac Conkey agar, streaked by three streak method, and incubated at 37ºC over night. The next day, gram negative bacilli colonies were further characterized by using preliminary biochemical screening. The blood cultures in the department are processed in semi –automated system Bact/Alert 240. Based on a colorimetric method, the system detects the presence of carbon dioxide produced during the metabolic processes of the

organism. Once the carbon dioxide reaches a minimum thresh hold the automation machine flags positive. The bottle is then removed from the machine-Gram stain is done from the blood culture supernatant -if there is presence of gram negative organism, the following preliminary biochemical reactions are put up- Mannitol motility medium, Triple sugar iron agar medium, Indole production from peptone water, and Citrate utilization on Simmons Citrate Medium(61).

Organisms giving the following reaction, was further taken up in the study-

- Mannitol motility medium- mannitol not fermented, non motile.
- Triple sugar iron agar- Alkaline slant/alkaline butt, no gas or hydrogen sulphide production, demonstrating the inability of the organism to ferment either of the three sugars present- glucose, sucrose, and lactose. Also the organism is unable to produce any gas or hydrogen sulphide.
- Indole production- The absence of a pink ring on adding the indole reagent (Ehrlich's reagent- paramethylaminobenzaldehyde in ethyl alcohol), denotes a negative reaction.
- Citrate utilization- Absence of any blue color denotes absence of increased alkalinity, produced as a result of citrate utilization.

The motility was further confirmed by inoculating the organism in nutrient broth, incubating it 37ºC for two hours, and then checking for motility by hanging drop(61).

A. PHENOTYPIC CHARACTERIZATION

Table I: Preliminary Bio-chemical identification

Bio chemicals (Preliminary Screening Media)	Reaction for *Acinetobacter calcoaceticus-baumannii complex*
Oxidase production	Negative**(-)**
Mannitol motility medium	Not fermented ,Non motile **(-,-)**
Triple sugar iron agar	Alkaline slant/Alkaline butt**(-/-)**
Indole production	Negative**(-)**
Citrate utilization	Negative**(-)**

1. Biochemical identification of Acb complex

All Oxidase negative non motile non fermenting gram negative bacilli were biochemically characterized, to identify the strains of *A.* calcoaceticus-baumannii complex(7). The following biochemical reactions were set up.

Table II: Biochemicals used to identify Acb Complex

Reactions	*A.calocaceticus-baumannii* complex(expected result)(7)
Oxidase	Negative
Motility	Negative
Gram stain	Gram negative cocco bacilli or bacilli
OF Dextrose	Oxidative utilization only
Nitrate reduction	Negative
Malonate utilization	Positive
Arginine utilization	Positive

All the isolates were biochemically characterized using the following bio chemicals – oxidase reaction of the organism was determined by using a Whatmann filter paper No. 2 soaked in Oxidase reagent(1% aqueous solution of tetra methyl-para-phenylene diamine dihydrochloride), Hugh Leifson medium for determination of oxidative utilization of glucose, malonate broth for determination of malonate utilization, Moeller's decarboxylase medium for determination of arginine dihydrolase production ,and potassium nitrate broth for determination of nitrate reduction(7).

B. Anti microbial profile

2a. Disc diffusion method

Antibiotic sensitivity was assessed for all the strains, by disc diffusion method, using the modified Kirby –Bauer method(61). Medium used was Mueller-Hinton agar (Obtained from Difco) poured in an 85 mm plastic plate. The organism was sub cultured on sheep blood agar, to obtain it in pure culture. Three to four colonies were suspended, in nutrient broth and incubated for two hours at 37ºC, so as to get the organism in the logarithmic phase. The density of the suspension was standardized with nutrient broth, visually equivalent to the Barium Sulphate standard, 0.5 McFarland units. Within, fifteen minutes of preparation of the suspension, a sterile cotton-wool swab was dipped into the suspension and the surplus removed by rotating the swab against the side of the test tube. With this swab, the agar plate was inoculated by even stroking of the swab over the entire surface of the plate in three directions so as to obtain a lawn culture. After brief drying, the antibiotic disc was placed, 6 on each plate(61). With each batch of tests, a control for each antibiotic was also set up. The control strains were obtained from American Type Culture Collection (ATCC). The control strains were included as per the CLSI guidelines.

Table III: Source and control strains of Antimicrobial agents used

Antibiotic	Source	Control strain used(37)
Cefotaxime(30µg)	Sigma	*Ps.aeruginosa* ATCC 27853
Ceftazidime(30µg)	Sigma	*Ps.aeruginosa* ATCC 27853
Cefepime(30µg)	Becton Dickinson	*Ps.aeruginosa* ATCC 27853
Amikacin(30µg)	Hi Media	*Ps.aeruginosa* ATCC 27853
Gentamicin(10µg)	Sigma	*Ps.aeruginosa* ATCC 27853
Netilmicin (30µg)	United Biotech	*Ps.aeruginosa* ATCC 27853
Levofloxacin(5µg)	Cipla	*Ps.aeruginosa* ATCC 27853
Ciprofloxacin(5µg)	Sigma	*Ps.aeruginosa* ATCC 27853
Chloramphenicol (30µg)	Oxoid	*S.aureus* ATCC 25923
Ticarcillin/clavulanicacid(75/10µg)	Becton Dickinson	*Ps.aeruginosa* ATCC 27853
Piperacillin/tazobactam (100/10µg)	Becton Dickinson	*Ps.aeruginosa* ATCC 27853
Cefeperazone/sulbactam (75/10µg)	Becton Dickinson	*Ps.aeruginosa* ATCC 27853
Trimethoprim /Sulfamethoxazole (1.25/23.75µg)	Hi Media	*S.aureus* ATCC 25923
Tigecycline(15µg)	Oxoid	*Ps.aeruginosa* ATCC 27853
Colistin(10µg)	Hi Media	*Ps.aeruginosa* ATCC 27853
Polymyxin B (300U)	Sigma	*Ps.aeruginosa* ATCC 27853
Imipenem(10µg)	Ranbaxy	*Ps.aeruginosa* ATCC 27853
Meropenem(10µg)	Astra-Zeneca	*Ps.aeruginosa* ATCC 27853

Interpretations were made using the Clinical and Laboratory Standards Institute, USA guidelines (January 2008, M100-S18- Volume 28 No.1, Table 3, Page 78/79)(37) and reference was used for Polymyxin B Disc diffusion as no CLSI guidelines exist for the same(46).

For Tigecycline the guidelines laid down by F.D.A. were used.

(http://www.wyeth.com/content/showlabeling.asp?id=474).

Tigecycline DD	**Control-** *Escherichia.coli* ATCC 25922 (22-28mm)		
	Susceptible (mm)	**Intermediate (mm)**	**Resistant (mm)**
For *Acinetobacter*	>19	15-18	<14

Using the CLSI guidelines, 80 isolates which were resistant to imipenem and meropenem and 20 susceptible isolates were included. The cut of for disc diffusion, as given by CLSI is-Imipenem (10µg-<13mm to >15mm{R-S}, and Meropenem 10µg-<13mm to >15mm{R to S})(37)..

2b. Minimum Inhibitory Concentration Estimation

The Minimum Inhibitory concentration for Polymyxin B was estimated by agar dilution method for the 80 imipenem, meropenem resistant strains(61). The medium used was Mueller Hinton agar with serial dilutions of the antibiotic, 18-20 ml of the medium was prepared in test tubes and allowed to cool in 50ºC water bath. Serial dilutions of the antibiotic Polymyxin B were made in sterile saline. Doubling dilutions were made by weighing 1 mg of pure substance of Polymyxin B, dissolving it in 5.64 ml of sterile saline and so as to obtain a concentration of 640µg/ml, and diluting it serially so as to obtain a dilution of 0.31µg/ml in the last tube. The diluted antibiotic was added to the cooled medium, in a ratio of 2 ml of the Polymyxin B solution with 18 ml of the agar so as to obtain a maximum concentration of 64µg/ml and a least concentration of 0.031µg/ml. After adding the Polymyxin B solution, the agar was mixed well, and poured into the Petri dish. After allowing the medium to settle and solidify, the plates were dried. One control plate containing only the agar without the antibiotic solution was also made. The inoculum was standardized by adjusting the suspension made in nutrient broth, to 0.5 Mc Farland units, and then taking 0.001 ml of the broth and diluting in 5 ml of saline. The

control strain used was *Ps.aeruginosa* ATCC 27853. The test inoculum was spot inoculated with the help of a platinum loop, so as to deliver 0.001 ml of the inoculum. The plates were incubated at 37ºC for 18 hours, and the reading was taken as presence of growth or absence of growth at the spot of inoculation, in the serially diluted antibiotic plates. Interpretations were based on the CLSI guidelines, January 2008, volume 28, no.1, M7-A7 page number 143, and page No.104. For obtaining the MIC for sulbactam, a similar procedure was carried out so as to obtain a highest dilution of 32µg/ml and a lowest concentration of 0.5µg/ml on the plate. Control used was *Esc.coli* ATCC 25922. The interpretations were made as per CLSI 2008 guidelines(37).

For Polymyxin B

	Sensitive (µg/mL)	**Resistant (µg/mL)**
Pseudomonas aeruginosa ATCC 27853	<0.25	>2
Acinetobacter calcoaceticus-baumannii complex	<2	>4

For Sulbactam

	Sensitive (µg/mL)	**Intermediate (µg/mL)**	**Resistant (µg/mL)**
Escherichia.coli ATCC 25922	<1	-	>4
Acinetobacter calcoaceticus-baumannii complex	<4	8	>16

3. Molecular characterization of strains.

The presence of OXA-51 and OXA-23 was detected in using modified, Multiplex Polymerase Chain Reaction protocol reported by Turton *et al.* (26).

DNA extraction- The organism was grown in pure, on sheep blood agar, at 37ºC overnight. Three colonies of the organism were suspended in 100µl of sterile saline and were kept in water bath at 100ºC for half an hour. Following this the suspension was centrifuged at 6,000 rotations per minute for 2 minutes, and 3µl of the supernatant was used as DNA template.

Table IV: Molecular reagents used and their source

	Company
10XPCR buffer	Fermentas Life Sciences, USA
1.5 nM $MgCl_2$	Fermentas Life Sciences, USA
1.5 Taq DNA Polymerase	Fermentas Life Sciences, USA
Deoxy Nucleotide Tri Phosphate(200µM)	Fermentas Life Sciences, USA
Primer OXA-23 Forward(12.5 pmol)	Sigma Aldrich Chemicals Pvt. Ltd, Bangalore
Primer OXA-23 Reverse(12.5 pmol)	Sigma Aldrich Chemicals Pvt. Ltd, Bangalore
Primer OXA-51 Forward(12.5 pmol)	Sigma Aldrich Chemicals Pvt. Ltd, Bangalore
Primer OXA-51 Reverse(12.5 pmol)	Sigma Aldrich Chemicals Pvt. Ltd, Bangalore
Sterile water for injection	Nirilife, Gujarat.

The primers for the Polymerase chain reaction were obtained from Sigma Aldrich Chemicals Pvt. Ltd, Bangalore. The oligonucleotides were provided as a desalted base and, the primers were reconstituted as per the manufacturer's instructions in 0.5X TE buffer. As per manufacturer's instructions, stocks were stored at -20ºC and aliquots were stored at 4ºC.

The Primer sequences were:

Oxacillinase 23(26)

	Target	Base pair size	Author	Year of publication
OXA-23	$bla_{OXA\text{-}23like}$	501	Turton *et al*	August 2006

[F]-5′-GAT GTG TCA TAG TAT TCG TCG T-3′
[R]-5′-TCA CAA CAA CTA AAA GCA CTG T-3’

Oxacillinase 51(26)

	Target	Base pair size	Author	Year of publication
OXA-51	$bla_{OXA\text{-}51like}$	353	Turton *et al*	August 2006

[F]-5'-TAA TGC TTT GAT CGG CCT TG-3'
[R]-5'-TGG ATT GCA CTT CAT CTT GG-3'

Table V: Volume of each reagent used for one PCR reaction

The total reaction volume was 25µl. Each component of the Master Mix was as follows.

	In micro liters
10XPCR buffer	2.5
1.5 nM $MgCl_2$	1.5
1.5 Taq DNA Polymerase	0.3
Deoxy Nucleotide Tri Phosphate(200µM)	0.5
Primer OXA-23 Forward(12.5 pmol)	0.25
Primer OXA-23 Reverse(12.5 pmol)	0.25
Primer OXA-51 Forward(12.5 pmol)	0.25
Primer OXA-51 Reverse(12.5 pmol)	0.25
Sterile water	15.7
Total	22

To this, 3µl of extracted DNA was added.

The Multiplex PCR (26), cycling settings were as follows

1. Initial denaturation was done at 94ºC for 3 minutes followed by
2. 35 cycles of
Denaturation at 94ºC for 45 seconds
Annealing at 57ºC for 45 seconds
3. Extension at 72ºC for 1 minute followed by
4. Extension maintenance at 72ºC for 5 minutes.

Detection of the specific amplicons was done by performing electrophoresis on 2% Agarose gel (Obtained from Sigma). The gel was prepared by adding 2 mg agarose in

100 ml of 1XTBE buffer, heating it, so as to melt it and then pouring in gel tray and allowing it to set. Blank reaction was also run so as to check for the purity of the reaction. Control strains (Courtesy: David W. Wareham, Centre for Infectious Disease, Institute of Cell and Molecular Science, Barts and The London, Queen Mary's School of Medicine and Dentistry, London, UK) were included in every run. The controls were- one strain of *Acinetobacter baumannii-calcoaceticus* complex positive for OXA-51 only, one control strain positive for both OXA-23 and OXA-51 and the third strain negative for both OXA-51, OXA-23.

Control strains	Bands expected
Ab11	OXA-23 and OXA-51 positive
Ab14	OXA-51 only
Ab36	No band

Also a, molecular weight ladder, a 50 base pair DNA ladder (O' Range Ruler, obtained from Fermentas Life Sciences, USA) was included in the gel electrophoresis so as to detect the base pair weight of the amplicons obtained. The electrophoresis was run at 120V for one hour, in 0.5XTBE buffer.

This gel was then stained in 0.5% Ethidium bromide (obtained from Sigma Aldrich) so as to stain the DNA products which were then visualized using the Universal Hood II gel documentation machine form BIO RAD laboratories. The molecular weight of the product expected was, for OXA-51- 353 bp and for OXA-23- 501 bp.

Eighteen randomly selected strains were assessed fro the presence of other genes encoding for resistance (Courtesy: David W. Wareham, Centre for Infectious Disease, Institute of Cell and Molecular Science, Barts and The London, Queen Mary's School of Medicine and Dentistry, London, UK).

Isolates were tested for the following genes:

1.**ESBLs**

- TEM,
- SHV-like,
- CTX-M3 like,
- CTX-M14 like
- PER
- VEB

2.**AmpC**

- MOX-like,
- DHA-like
- ACC-like
- EBC-like

3.**Carbapenemases**

- Class D-OXA 58and OXA-24
- Class A-KPC
- Class B- IMP, GIM, VIM, SPM,SIM

4.**Quinolone resistance determinants**

- qnrA, qnrB, qnrS

5**. Integrons**

- Class I,II,III

- FOX-like

B. Clinical correlation

4a. Clinical Pulmonary Infection Score (CPIS)

Also the clinical pulmonary score was calculated for the patients suffering nosocomial pneumonia, using the Clinical Pulmonary Infection Score.

Table VI: CPIS (58)

CPIS POINTS	0	1	2
Tracheal Secretions	Rare	Abundant	Abundant +purulent
Chest X-ray infiltrate	No infiltrate	Diffused	Localized
Temperature	>97.4 and <101.2	>101.2 and<102.2	>102.2 or <96.8
Leukocytes/mm^3	>4000and<11000	<4000or>11000	<4000or>11000+band
$P_{Ao2/ Fio2,mmHg}$	>240or ARDS	-	<240 or no ARDS
Microbiology	Negative	-	Positive

Total score=

4b. Charlson Chronic Co-Morbidity Index

The chronic co morbidity scoring was done using the Charlson Co-morbidity Index. Co-morbid conditions such as diabetes and vascular disease are among the many factors that contribute to the risk of infection with antibiotic-resistant bacteria. The co-morbidity index developed by Charlson *et al*. is a validated method of classifying co-morbidity to predict short- and long-term mortality. It replaces direct measures of the severity of an illness, which require a prospective data collection. The Charlson index assigns weights for a number of major conditions present among secondary diagnoses. The index score is the total of assigned weights, and represents a measure of the burden of co-morbid disease. It has been successfully used as a risk-adjustment approach conjunction with laboratory and clinical data. The Charlson Co-morbidity Index was originally designed as a measure of the risk of 1-year mortality attributable to co-morbidity in a longitudinal study of general hospitalized patients. This scoring system though not developed for assessing infectious pathologies has been

successfully adapted to the same and studies have shown that it can be used as a predictor of infection with antibiotic resistant organisms. (ANNEXURE I).

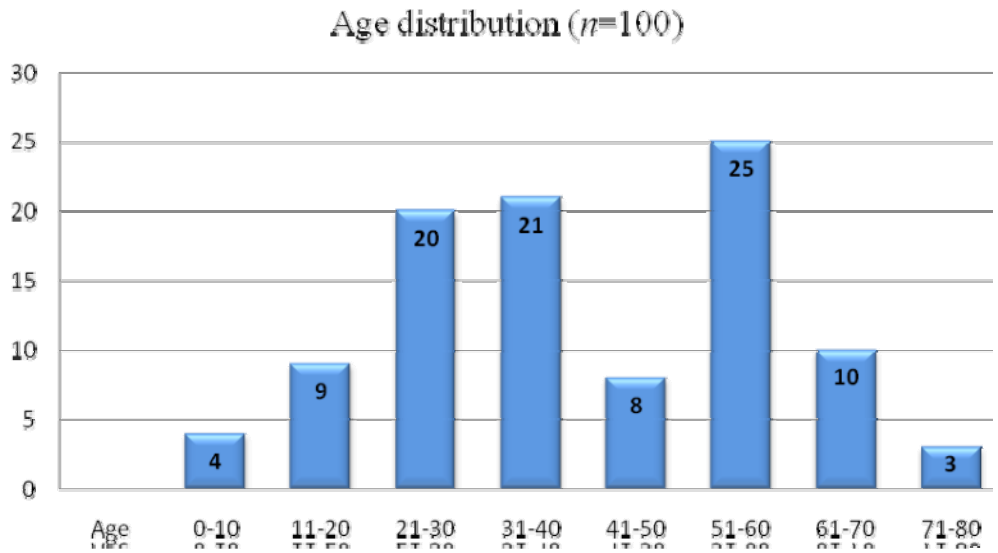
4c. Outcome in patient infected with nosocomial *A.baumannii* resistant to Carbapenems and Tigecycline.

An attempt was made to correlate the clinical outcome of patient infected with a carbapenem resistant and Tigecycline resistant isolate. A 2X2 table was constructed and the p value was calculated, to assess if infection with the resistant isolate significantly influenced the mortality rate in patients.

RESULTS

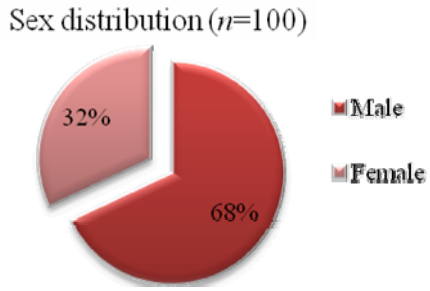
A. Materials – One hundred clinical isolates of *Acinetobacter baumannii* obtained from patients suffering from nosocomial infections were included in this study.

Fig.1: Age distribution of the patients with nosocomial *A.baumannii* infection



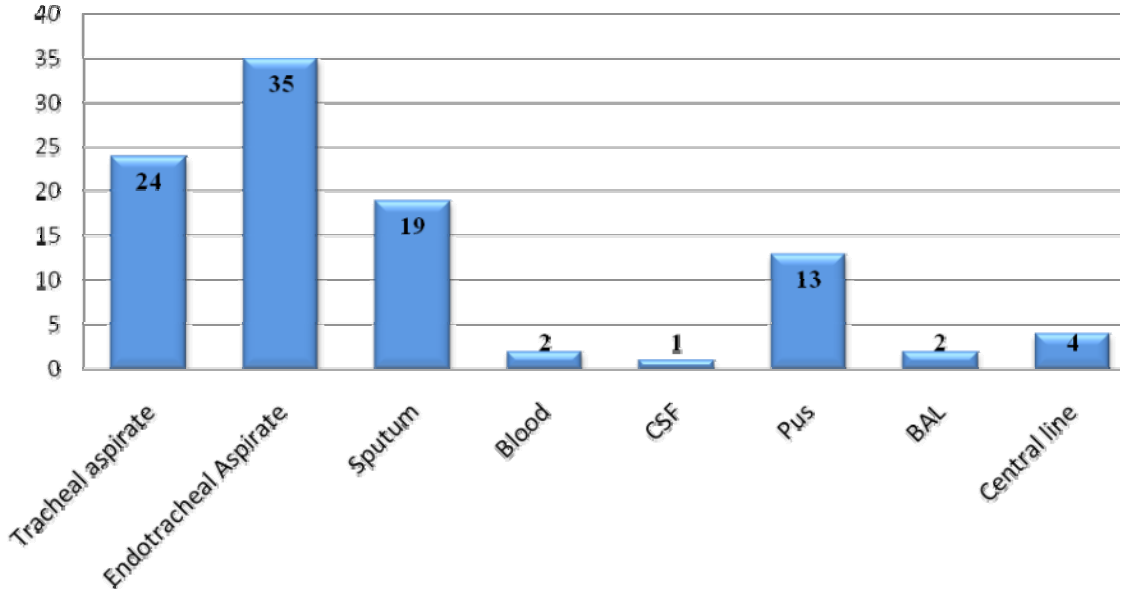
The bar diagram shows the age wise distribution of the patients, whose isolates were included in the study. Majority of the isolates were in the 50-60 year age group, and the Inter Quartile Range (IQR: 31 yrs) , with the youngest being aged 12 days to the oldest being 77 years.

Fig.2: Sex Distribution of the patients with *A.baumannii* nosocomial infection



The pie diagram depicts the sex distribution of the isolates investigated in the study, 68 of which were isolated from male patients and the rest from female patients.

Fig.3: Nosocomial *A.baumannii* isolates included from various clinical samples.



The bar diagram shows the various clinical samples from which the isolates were included in the study. Majority of the specimens were received from patients on ventilators and this included Endotracheal aspirate (patients with an endotracheal tube in situ) and tracheal aspirate (patients with a tracheostomy).

Fig.4: Nosocomial *A. baumannii* from various systemic/local infections.

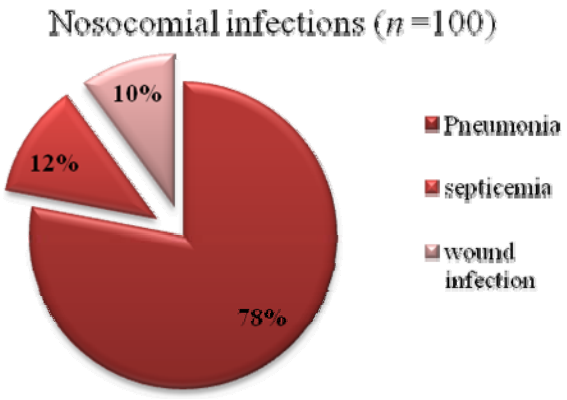


Figure 4 shows the number of patients with nosocomial infections, which were grouped according to the clincial diagnosis.

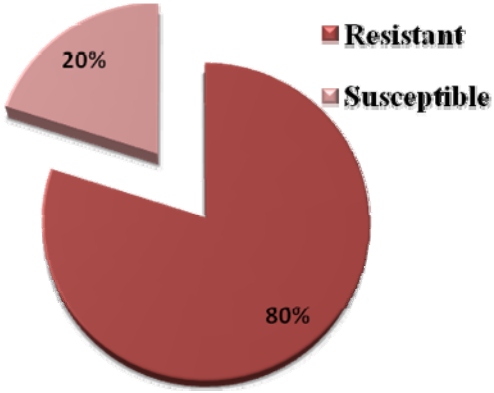


Fig.5: Number of carbapenem resistant and susceptible nosocomial *A.baumannii* isolates studied(*n*=100).

The diagram depicts the number of strains that were resistant (*n*=80) and susceptible (*n*=20) in this study.

B. Biochemical identification.

All the clinical isolates were characterized phenotypically and identified as *A.baumannii*. The preliminary screening was based on the following bio chemical profile

- Non motile,
- Oxidase negative
- Triple sugar iron showing alkaline slant with alkaline butt with no gas production.
- Indole negative.

These were further characterized by extended biochemical tests as follows

- Oxidative utilization of glucose,
- Nitrate reduction,
- Arginine dihydrolysis
- Malonate utilization.

Six isolates of the one hundred isolates tested, had variable biochemical reactions. Three isolates were negative for Arginine dihydrolysis and three were negative for Malonate utilization(7).These variable bio chemical reactions are expected for *A.baumannii*.

C. Anti microbial Susceptibility profile.

C.1 By Disc Diffusion test

Table I: Antimicrobial susceptibility profile of nosocomial *A.baumannii* isolates.

***n*=100**	**R**	**S**	**I**
CTX	**100**	**0**	**0**
CZD	100	0	0
CPI	98	2	0
GENT	85	15	0
AK	84	16	0
LEVO	80	19	1
CIPRO	85	15	0
CEF+SULB	74	18	8
TIM	99	1	0
P/T	99	1	0
SXT	76	24	0
CHLO	89	11	0
TIG	73	20	7
COLISITN	4	96	0
PB300	4	96	0

R= Resistant, S= Susceptible, I= Intermediate susceptible

CTX-cefotaxime, LEVO- levofloxacin, SXT-Sulfamethaxzole+ Trimethoprim,
CZD-ceftazidime, CIPRO- Ciprofloxacin, CHLO-Chloramphenicol,
CPI- Cefipime, CEF+SULB-cefaperazone+sulbactam, PB300- Polymyxin B
GENT- Gentamicin, Tim-Ticarcillin+Clavulanic acid ,Colistin
AK-Amikacin, P/T- Piperacilin+ Tazobacatm, TIG-Tigecycline

Fig7: Antimicrobial profile of nosocomial *A.baumannii* ioslates.(*n* = 100)

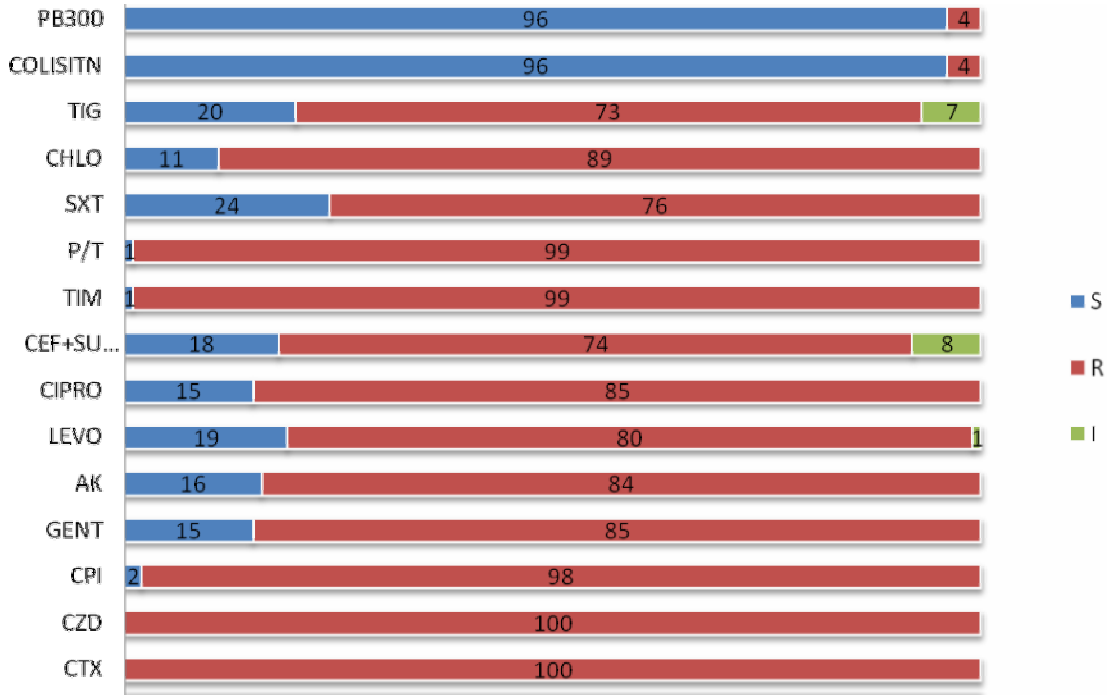
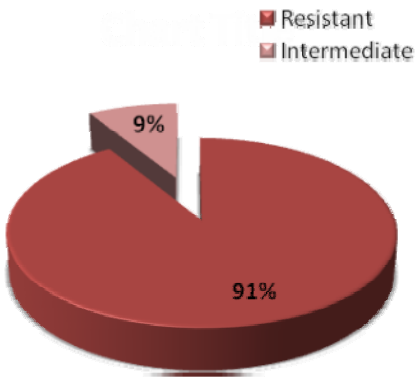


Figure 7 depicts the antimicrobial profile for as determined by the disc diffusion test, using the modified Kirby-Bauer method. The interpretations were done using the CLSI January, 2008 guidelines and published references for PB 300, Colistin and Tigecycline. There was 100% resistance detected to CTX,CZD and very high levels of resistance to β-lactam+β-lactamase inhibitor combination. Also the level of resistance was more than 80% to the fluoroquinolone and amino glycoside groups. The most effective drugs seem to be the Polymyxins (PB300 and Colistin) which showed 96% susceptibility.

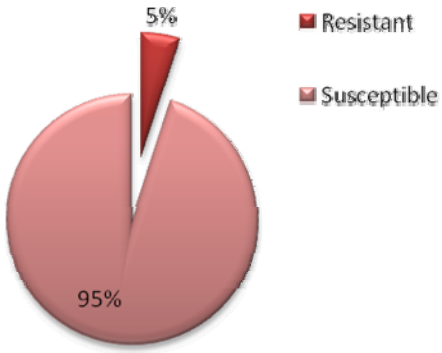
Fig.8: Tigecycline susceptibility among nosocomial *A.baumannii* resistant to carbapenems.(*n* = 80)



Tigecycline is advocated as a alternate drug for treatment of carbapenem resistant isolates. Disc diffusion carried out showed that all of the resistant strains,none were susceptible to Tigecycline (9% showing intermediate susecptibility to Tigecycline).

C.2 Minimum inhibitory concentration (MIC)

Fig.9: Polymyxin B susceptibility pattern of nosocomial *A.baumannii* resistant to carbapenems determined by Agar dilution method(*n* = 80).



The MIC was determined for Polymyxin B by the agar dilution method and interpretations were made using the CLSI 2008 guidelines. The figure depicts 95% of the isolates were inhibited by Polymyxin B, making this an effective alternative therapeutic agent.

Table II: MIC values obtained for Polymyxin B nosocomial *A.baumannii* resistant to carbapenems.

MIC dilutions for Polymyxin B (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
A.baumannii (*n*=80)			3	1		5	61	15			
Ps.aeruginosa ATCC 27853							1				

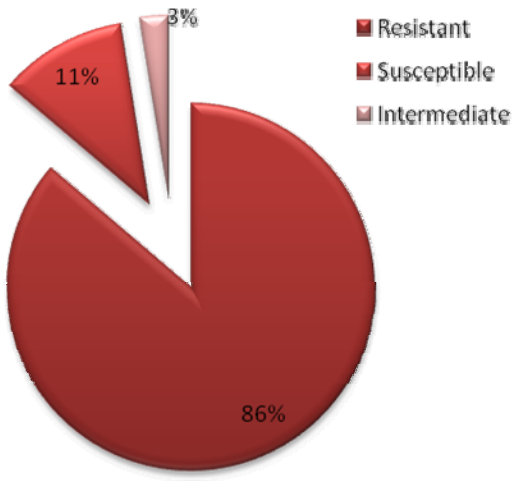
The minimum inhibitory concentration was determined for Polymyxin B was determined using agar dilution technique. This revealed the MIC's of majority of the susceptible isolates to be around 1µg/ml. The control used *Ps.aeruginosa* ATCC 27853, which was satisfactory. Four isolates were found to be resistant to Polymyxin B.

Table III: Details of nosocomial *A.baumannii* resistant to Polymyxin B.

Sample I.D.	Source	MIC value (µg/ml)	Patient outcome
SP2176	Endotracheal Aspirate	16	Cured
SP1400	Endotracheal Aspirate	16	Expired
SP4151	Endotracheal Aspirate	16	Expired
SP4051	Endotracheal Aspirate	8	Expired

There were however four isolates with an MIC in the resistant range. Three of these had an MIC of 16 whereas one had an MIC of 8. Three of these pateints died.

Fig.10: Sulbactam susceptibility pattern of nosocomial *A.baumannii* resistant to carbapenems.



MIC for Sulbactam was performed by the agar dilution method and interpreted by using the CLSI

guidelines, showed that only 11% of the isolates were being inhibited by Sulbactam.

Table IV: Sulbactam MIC values for nosocomial *A.baumannii* resistant to carbapenems.

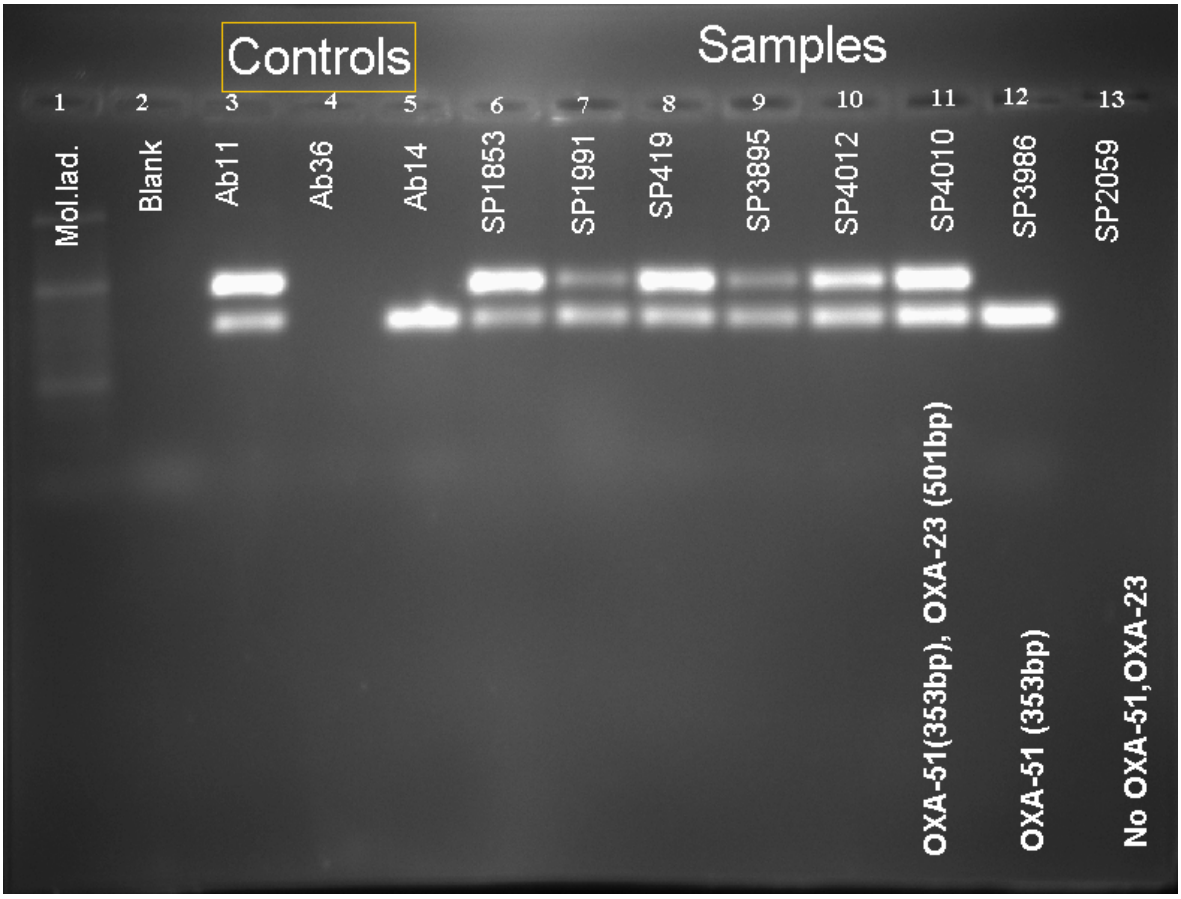
MIC dilutions for Sulbactam (µg/ml)	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125
***A.baumannii* (*n*=80)**				10	58	2	1	3	4					
***Esc.coli* ATCC 25922**								1						

Table four depicts the Sulbactam MIC values obtained for the isolates tested. Sixty nine of these isolates had an MIC in the resistant range and majority of the isolates were found to be inhibited only at a drug concentration of 16µg/ml. Clearly this shows that the drug is not effective in the carbapenem resistant isolates in this study.

D. Molecular determination of resistance.

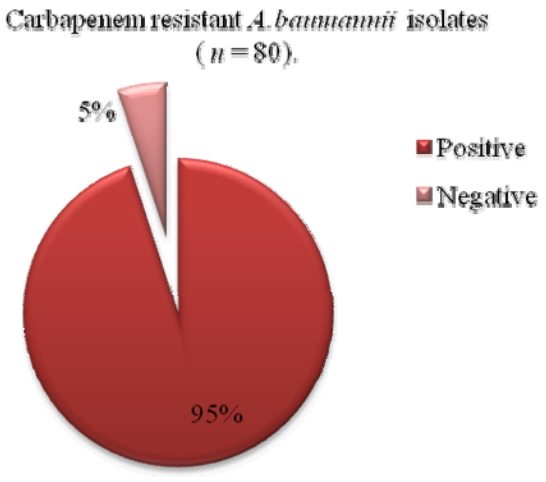
A modified multiplex PCR was standardized using published protocol(Turton *et al* 2006). The amount of Magnesium chloride added was increased from 1µl to 1.5µl per 25µl reaction as compared to the published protocol.

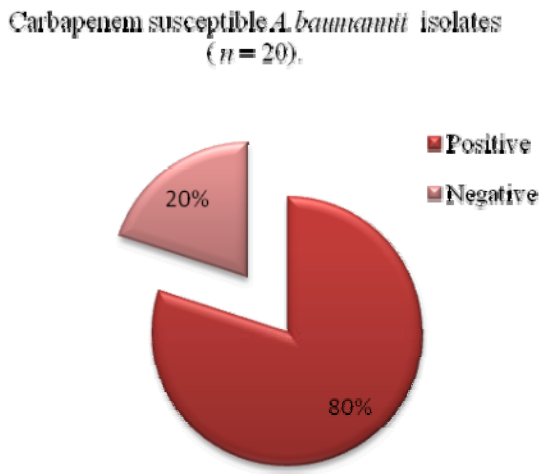
Fig: 11 Ethidium bromide stained gel showing bands for OXA-51 and OXA-23.



This picture depicts the standard multiplex PCR including controls provided. Lane 3,4,5 are the controls included and the rest of the lanes are a clinical isolates of *A.baumannii*.

Fig.12: OXA-51 in nosocomial *A.baumannii* isolates.





Of the tested 80 carbapenem resistant isolates, 95% of them were positive for the gene encoding for OXA-51. Of the twenty susceptible isolates tested, 80% were positive for the gene. This favours the hypothesis that OXA-51 may be a molecular marker for identification of *A.baumannii*.

Fig.13: OXA-23 in nosocomial *A.baumannii* isolates.

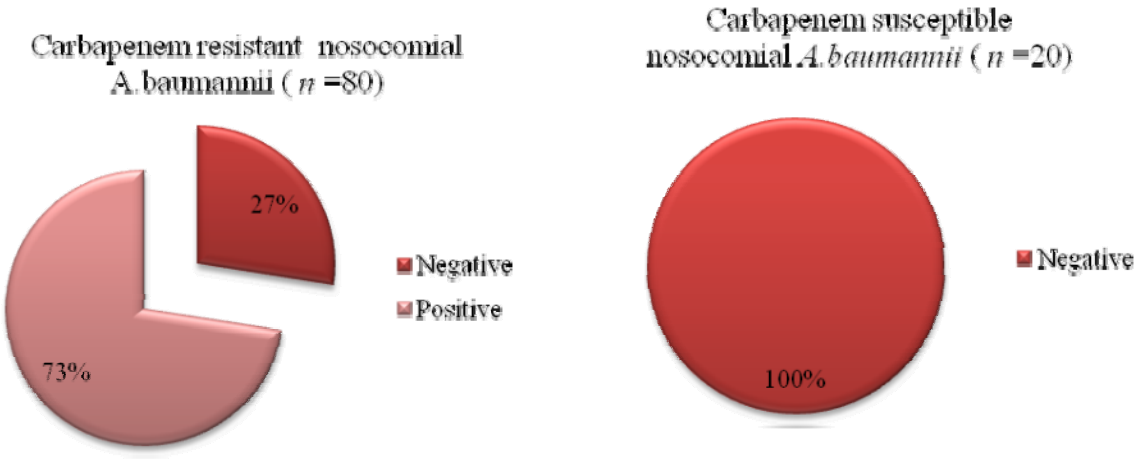


Figure 13 shows the number of isolates which were positive for the gene encoding for OXA-23 enzyme. It was found to be positive in 58 of the tested 80 carbapenem resistant isolates. The susceptible strains were all negative for OXA-23.

TableV: Comparative table showing the nosocomial *A.baumannii* isolates positve for OXA-51 and OXA-23

	OXA-51		OXA-23	
	Carbapenem Susceptible(*n*=20)	**Carbapenem Resistant(*n*=80)**	**Carbapenem Susceptible(*n*=20)**	**Carbapenem Resistant(*n*=80)**
Positive	16	76	0	58
Negative	4	4	20	22

Table V shows the number of *A.baumannii* isolates which were positive for OXA-51 and OXA-23. It shows the presence of OXA-51 in both susceptible and resistant strains. Thus the OXA-51 may be used a molecular marker. The number of carbapenem resistant *A.baumannii* isolates positive for OXA-23 is 58. None of the susceptible isolates were positive for OXA-23. Thus OXA-23 seems to play a major role in determination of carbapenem resistance. The carbapenem resistant isolates negative for OXA-23, could have become resistant due to other mechansims or even with only OXA-51.

Table VI: The other resistance determinants identified in the randomly selected eighteen isolates.

Other genes tested	
ESBL	
TEM	**4**
SHV-like	0
CTX-M3 like	0
CTX-M14 Like	0
PER	**1**
VEB	0
Transmissible Amp-C	
MOX	0
CIT-like	0
DHA-like	0
ACC-like	0
EBC-like	0
FOX-like	0
Class D carbapenemases	
OXA-58	0
OXA-24	0
Class A carbapenemases	
KPC	0
Class B carbapenemases	
IMP	0
VIM	0
GIM	0
SPM	0
SIM	0
Quinolone resistance	
qnrA	0
qnrB	0
qnrS	0
Integrons	
Class I	**11**
Class II	0
Class III	0

Table six outlines the various molecular determinants of resistance which were looked for in the randomly selected carbapenem resistant *A.baumannii* isolates. All the isolates were negative for other classes of carbapenemases. Of interest is the fact that eleven of the eighteen isolates were positive for class I integrons. These Class I integrons encode resistance for various classes of anti microbial agents at the same time and explain the resistance of tested isolates to so many classes of anti microbial agents. .(Courtesy: David W. Wareham, UK).

Fig.14: Number among the randomly chosen *A.baumannii* isolates found to carry other genes encoding for resistance (*n* =18).

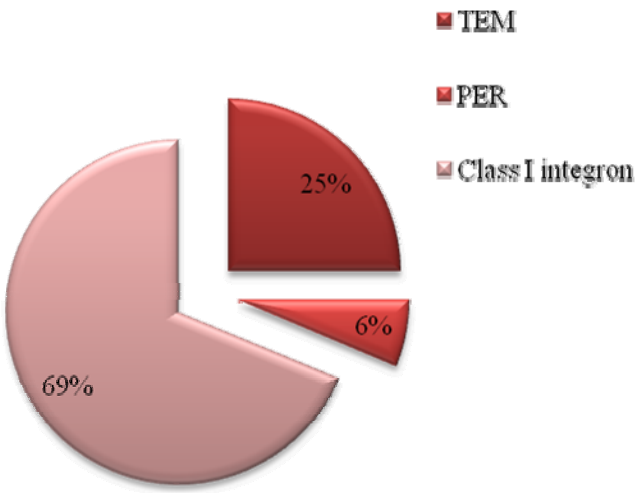
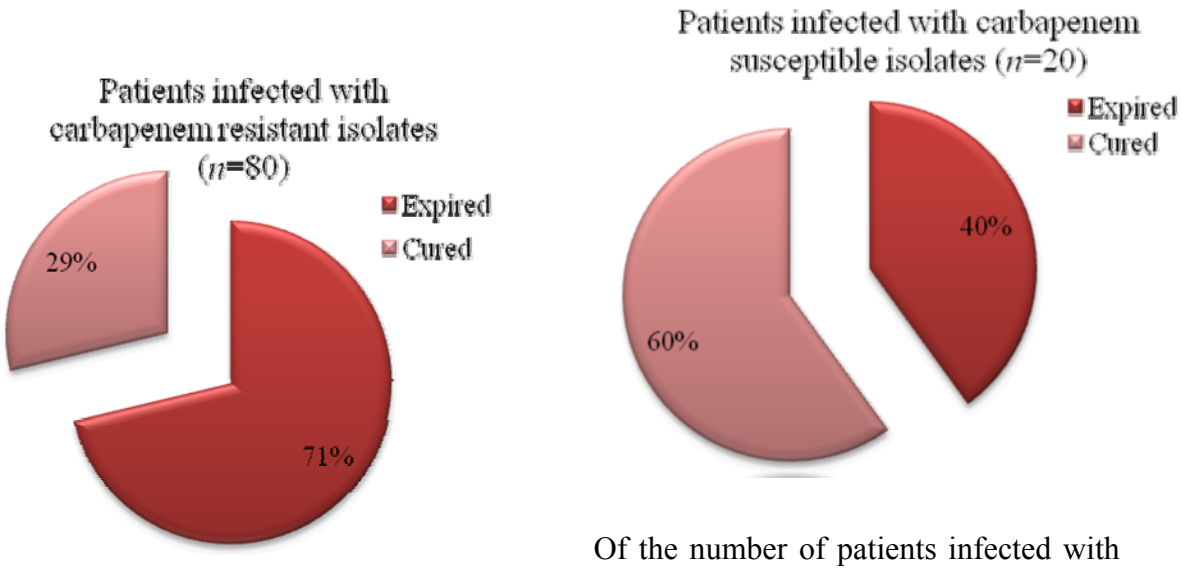


Figure 14 shows the number of isolates which were identified to be carrying the genes tested for. One of the isolate was positive for PER gene, four positive for the TEM gene and eleven of the isolates were positive for Class I Integron. Rest of the genes tested for were negative in all the 18 isolates.

Fig.15: Outcome of the patients infected with *A.baumannii* isolates.



Of the number of patients infected with a carbapenem resistant isolate (*n*=80), 57 of them expired. Of the number of pateints infected with carbapenem susceptible isolate (*n*=20), eight died.

Table VII: Comparative table showing outcome of nosocomial *A.baumannii* infected patients with carbapenem resistant/susceptible strains.

Carbapenem AST	Mortality	No mortality
Resistant (*n*=80)	57	23
Susceptible (*n*=20)	8	12

p value=0.018 significant.

The above table compares, the mortality in patients infected with a carbapenem resistant strain as compared to infected with a susceptible strain. The calculated p value is highly

significant and shows that the infection with a carbapenem resistant strain predisposes to a higher mortality.

Table VIII: Comparative table showing outcome of carbapenem resistant nosocomial *A.baumannii* infected patients with Tigecycline resistant/susceptible strains.

Tigecycline AST	Mortality	No mortality
Resistant (*n*=73)	41	32
Moderately susceptible (*n*=7)	6	1

p value= 0.23

The outcome of patients infected with tigecycline resistant *A.baumannii* was assessed. Infection with a tigecycline resistant strain did not signifcantly effect the outcome of the patient.

Table IX:Comparative table showing outcome of nosocomial *A.baumannii* infected patients with Pan drug resistant strains.

Resistance	Mortality	No mortality
Pan drug resistant (*n*=57)	37	20
Not pan drug resistant (*n*=43)	24	21

p value=0.36 not significant

The above table compares the mortality in patients infected with a pan drug resistant isolate(isolate resistant to all except Polymyxins) as compared to infection with a non-pan drug resistant isolate. No significant increase in the mortality was observed in patients who were infected with pan drug resistant *A.baumannii* isolates.

Table X: CPIS (Clinical Pulmonary Infection Score)

Score	Expired	Cured	Total
0 to 6	2	8	10
7 to 9	5	6	11
10 to 12	36	21	57

CPIS	Mortality	No mortality
<6	2	8
>6	41	27

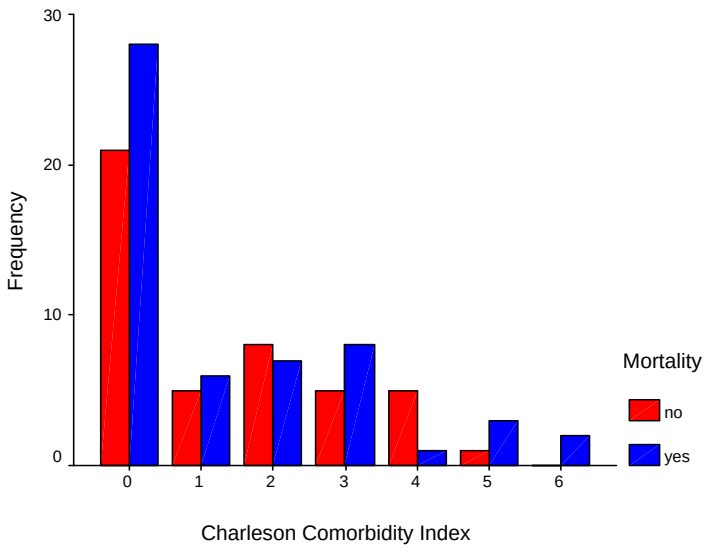
The clinical pulmonary infection score was calculated for patients suffering from nosocomial pneumonia(*n*=78). As per recommendations assuming 6 as cut off for the clinical diagnosis of VAP, we found that there was an increase in death rate with an increase in CPIS.

Fig.16: Charlson Comorbidity Scoring index

Charleson Comorbidity Index * Mortality Crosstabulation

			Mortality		
			no	yes	Total
Charleson Comorbidity Index	0	Count	21	28	49
		% within Charleson Comorbidity Index	42.9%	57.1%	100.0%
	1	Count	5	6	11
		% within Charleson Comorbidity Index	45.5%	54.5%	100.0%
	2	Count	8	7	15
		% within Charleson Comorbidity Index	53.3%	46.7%	100.0%
	3	Count	5	8	13
		% within Charleson Comorbidity Index	38.5%	61.5%	100.0%
	4	Count	5	1	6
		% within Charleson Comorbidity Index	83.3%	16.7%	100.0%
	5	Count	1	3	4
		% within Charleson Comorbidity Index	25.0%	75.0%	100.0%
	6	Count		2	2
		% within Charleson Comorbidity Index		100.0%	100.0%
Total		Count	45	55	100
		% within Charleson Comorbidity Index	45.0%	55.0%	100.0%

*the chronic co-morbid entities list is given as annexure I.



It was found that there was no significant association between mortality and any chronic comorbid condition.

DISCUSSION

Discussion

Acinetobacter calcoaceticus-baumannii complex has emerged as the leading cause of mortality and morbidity in hospital acquired infections. The organism is mainly isolated from the respiratory tract, in patients on artificial ventilation. A study published from CMC Vellore, has shown an incidence of 14.2% for carbapenem resistant *Acinetobacter spp*. isolated from endo tracheal aspirates (8). A study done in JIPMER, Pondicherry has shown an incidence of 48.8% of *Acinetobacter spp*. (16). *Acinetobacter* infections most frequently involve the respiratory tract of intubated patients and *Acinetobacter*

pneumonia has been more common in critically ill patients in Asian (range 4–44%) and European (0–35%) hospitals than in United States hospitals (6–11%) (20)). 78% of isolates included in this study had come from patients suffering from pneumonia.
The organism has become increasingly difficult to treat with the increasing number of isolates being resistant to many of the different class of drugs. The definition of multidrug and pan drug resistant has varied in various studies. Various studies done in India have failed to clearly define the definition used for classifying an isolate as being multidrug or pan drug resistant ((62, 63). Some authors have termed isolates as being pan drug resistant without testing for Polymyxins. Kuo *et al*. (2003) from Taiwan defined PDR as resistance to all antibiotics commercially available (ceftazidime, ciprofloxacin, cefepime, aztreonam, ciprofloxacin, piperacillin–tazobactam, ticarcillin–clavulanate, ofloxacin, amikacin, imipenem and meropenem). No testing was done for colistin or ampicillin–sulbactam (64)Wang *et al*. (2003) from Taiwan defined PDR as resistance to all currently available antimicrobials (including carbapenems), except colistin and Polymyxin B(65). PDR in this study was defined as isolates resistant to all antibiotics except colistin. The same problem plagues the definition of Multi drug resistant *Acinetobacter*. Ruiz *et al*. (2003) from Spain have described isolates resistant to at least amikacin, amoxycillin–clavulanic acid, ampicillin, ceftazidime, and chloramphenicol(66). Smolyakov *et al*. (2003) from Israel described isolates resistant to all antibiotics tested routinely but susceptible only to ampicillin–sulbactam and colistin(67). Urban *et al*. (2003) from USA have described isolates resistant to cefepime, ceftazidime, or imipenem, or sensitive only to polymyxins and sulbactam(68). In this study MDR was defined as those isolates showing resistance to more than two of the

following classes of antibiotics (Anti pseudomonal cephalosporins (ceftazidime or cefepime), antipseudomonal carbapenems (imipenem or meropenem), ampicillin-sulbactam, fluoroquinolones (ciprofloxacin or levofloxacin), and aminoglycosides (gentamicin, tobramycin, or amikacin).

The carbapenem resistant *A.baumannii* isolates were also tested for their susceptibility towards Tigecycline by the disc diffusion method. Also the susceptibility to Polymyxin B and Sulbactam was assessed performing MIC using the agar dilution method. Of the tested resistant isolates none were completely susceptible to Tigecycline. Only 9% of the tested isolates were intermediately susceptible to the drug (n=80). The resistance to Tigecycline has varied in different studies. This is because there are no universal prescribed guidelines for the in vitro testing for Tigecycline. No studies have been performed in India. Souli *et al* (69), have shown 100% susceptibility to Tigecycline (n=100). 26% isolates from a study in Argentina have been reported to be resistant(70). A study published from Israel has shown a resistance rate of 66% with 12% being intermediate susceptible (n=82)(71) . Interpreting the data generated from this study, it becomes clear that there is a definite difference in the susceptibility pattern of *Acinetobacter* across different geographical areas. This can partly be explained based on the fact that the cut of guidelines given by BSAC is different as compared to FDA guidelines. A better picture would emerge if there were uniform guidelines developed for the interpretation of Tigecycline susceptibility. Another potential point of error is the content of manganese present in the Mueller-Hinton agar used for sensitivity testing, with a higher manganese content giving more isolates as resistant as compared to a lesser amount of manganese(72).

Various studies have proven the efficacy of Sulbactam as an alternate drug. Studies done in France and Spain (*n*=47 patients) (53) have proven the efficacy of this drug as mono therapy to treat carbapenem resistant *Acinetobacter* (11). This agent is not available as a pure substance in India. The drug is available as combination with ampicillin and cefeperazone. Various studies done in India have revealed a resistance range of 5.3% to 48.2% to these combination drugs (62, 73). A study done in 2003 has shown a resistance rate of 54.3% in Turkey.(n=121)(74). A study done in Chile has shown an increase over time in the resistance rates towards Sulbactam, from 30.8%in 1990-1992 to 51.7% in 1997-1998(75). Another study has shown a resistance rate of 68.2% (9). Surprisingly of the isolates tested for susceptibility to Sulbactam, only 11% were susceptible and another 3% were intermediately susceptible.

Of the eighty strains subjected to susceptibility testing for Polymyxin B in this study, 95% were found to be susceptible. This is in accordance to the various studies published having proven. Polymyxins have been proven to be an effective therapeutic agent against carbapenem resistant *Acinetobacter* spp. No studies have been performed in India to specifically look at the in vitro activity of polymyxins against carbapenem resistant *Acinetobacter* spp. Studies done in various parts of the world have shown 95.8% to 100% of the isolates to be susceptible (*n*=48, Azap Kurt *et al* 2006, *n*=66, Dizbay 2008)(9, 76). Use of polymyxins has also gained popularity due to the better monitoring facilities of renal functions and dialysis.

The presence of four isolates which were resistant to Polymyxin B is of concern. Though no reports have been published from India regarding resistance to Polymyxins, but reports of colistin resistance has emerged from Australia(77). Report of Polymyxin B

resistance ranging from 2.1% to 88.9% has been published from a study in Korea.(78). One of the potential problems with testing for Polymyxin B susceptibility is there are no standard guidelines for interpreting disc diffusion values. CLSI does not give guidelines for the same. Values used in this study are from the work done by Gales *et al*((46). This is because the Polymyxin molecule is of very large molecular weight and does not diffuse uniformly in the agar medium and hence the standard recommendations is to report an isolate as being resistant only after performing an MIC on the suspect isolate.

The gold standard for identification of this organism is DNA-DNA hybridization as described by Bouvet *et al*. This is an elaborate and cumbersome procedure and is done only in select reference laboratories. The bio chemical identification system cannot differentiate between *A. baumannii*, *A.calcoaceticus, Acinetobacter* genomospecies 3 and *Acinetobacter* genomospecies 13TU. This is the reason, that for conventional identification systems, *Acinetobacter* is reported as *A. calcoaceticus-baumannii complex* or sacchrolytic *Acinetobacter*. The search for alternate identification markers has lead to the identification of OXA-51 as a molecular marker for identification of *Acinetobacter baumannii*. A study done by Turton *et al* in 2006 had shown the presence of this gene only in *A.baumannii* (*n*=141). The isolates were also further confirmed by using DNA-DNA hybridization technique. The gene was absent in *A.calcoaceticus*, *A.lwoffii*.(12). A study done in Turkey showed 77.8% of the isolates to be carrying the OXA-51(*n*=72) (79). Confirmation of isolates was not done in this study. Another study done in Iran detected the presence of OXA-51 in all the isolates tested (*n*=108) (80). No such studies have been carried out in India till date. Of the isolates tested in this study (*n*=100), 92 showed the presence of OXA-51. This shows the presence of OXA-51 in a large number

of our isolates. The rest of the eight isolates which does not show the presence of OXA-51 gene might be belonging to the other members of this complex.

The other gene investigated in this study was for the OXA-23 enzyme. This is one of the most widespread genes, encoding for resistance(4). No studies to detect these genes in Indian isolates have been carried out. A study done in Argentina showed the presence of OXA-23 in 63.4% (*n*=41)(81) of the carbapenem resistant isolates. Another study done in China showed the presence of OXA-23 in 94.15% of the isolates (*n*=342) (82). In this study 73% of the tested isolates (*n*=80), were positive for OXA-23.

There were four carbapenem resistant isolates which were negative for both OXA-51 and OXA-23. The resistance mechanism in these isolates could be due to presence of other mechanisms described such as presence of carbapenemases, efflux pumps, and loss of outer membrane porin channel.

Eighteen of the isolates were randomly chosen and subjected to analysis for detection of other genes which encode for resistance to other classes of anti microbial agents. This revealed the presence of class I integrons in eleven of the tested isolates. No studies have been carried out in India to identify integrons in the isolates here. Turton *et al* have found Class I integrons to be present in all isolates tested by them (26). Another study done in Spain showed 84% (*n*=70), of the strains to be positive for Class I integrons (83).

The mortality in the patients infected with carbapenem resistant strains was determined in this study. There was a significant association noted between the two and was found to be statistically significant. A study done in 2007 had proven that infection with MDR *Acinetobacter* predisposed to longer hospital stay and higher mortality (84). Another study done in Korea has proven that the thirty day mortality rate in patients suffering

from imipenem resistant *Acinetobacter* bacteraemia was 57.5% as compared to27.5% in those infected with susceptible strains (85).

Impact of tigecycline resistance on mortality was assessed in the carbapenem resistant isolates. No significant association was found in our patients. Studies linking infection with a tigecycline resistant strain to outcome of the disease process in the patients has not been carried out any where else in the world.

Also the mortality of the patients infected with pan drug resistant isolates was determined. It was found that there was no correlation between the two. This may be explained on the basis of the other co morbid processes going on the patient- hemodynamic instability, renal or cardiovascular compromise. No studies to correlate pan drug resistance with mortality have been done.

Clinical Pulmonary Infection Score (CPIS) was calculated for the patients who had nosocomial pneumonia. This was further correlated with the outcome of the patient as in mortality or cure. It was found that with an increase in the CPIS, there was an increase in mortality, which was found to be statistically significant. No studies relating the CPIS with mortality have been undertaken in India. A study done in Ankara, Turkey had shown no correlation between CPIS and mortality (60).

The Charlson co morbidity index was calculated for the patients in this study. This index takes into account a host of chronic co-morbid conditions which can influence the outcome of the disease process. The index was compared with the mortality of the patients, so as to gauge the significance of the chronic conditions in our patients. It was found that the chronic co-morbid conditions did not influence the outcome in our patients, thus indicating that the infection with *A.baumannii* in a nosocomial set up was

an independent predictor of mortality and not statistically associated with the chronic com-morbid conditions. No studies have so far been done to assess the effect of chronic co-morbid conditions on patients infected with *A.baumannii*.

CONCLUSIONS

Conclusions and summary

The total number of nosocomial *A.baumannii* isolates included in this study was one hundred. 78% of the isolates included in this study were isolated from nosocomial pneumonia. This indicates that *A. baumannii* is a leading cause of nosocomial pneumonia.

The resistance to β-lactam agents was 100%, to amino glycosides and fluoroquinolones was 85% and to the β-lactam +β-lactamase inhibitor combination was 79%. The number of PDR *A.baumannii* was 57% and MDR *A.baumannii* was 43%. The number of isolates being resistant to so many anti microbial agents is indeed concerning.

Efficacy of the newer drug being advocated as a treatment option for carbapenem resistant *A.baumannii* - Tigecycline was evaluated in our study isolates. None of the isolates tested, were susceptible to Tigecycline with only seven of the isolates being intermediately susceptible. The lack of standard break points for defining susceptibility or resistance has also partially lead to varied results across the world.

Susceptibility to the drugs of renewed interest (Polymyxins and Sulbactam) was assessed by determining the MIC using the agar dilution method. Susceptibility to Polymyxins was 95% of the carbapenem resistant isolates (*n*=80). This was in accordance to the

reports published world wide. Surprisingly the susceptibility to Sulbactam in the carbapenem resistant isolates was only 11%. This high level of resistance is an area of concern.

Of the one hundred isolates tested in this study, OXA-51 was present in 92% of the isolates. This further supports the hypothesis of using OXA-51 as a molecular marker for the identification of *A.baumannii.* The other gene encoding for resistance OXA-23 was present in 73% of the carbapenem resistant isolates tested. This proves the resistance mechanisms in our isolates are similar to those reported across the world. The presence of Class I integrons in eleven of the eighteen representative isolates tested, gives a preliminary idea with regard to the other mechanisms encoding for resistance across various classes of anti-microbial agents. Further studies need to be done on a larger sample size.

The randomly tested isolates were negative for the other ESBL's, transmissible Amp C genes, Class A, B, and other Class D (OXA-58 and OXA-24) carbapenemases. Also they were negative for quinolone resistance determinants.

Infection with carbapenem resistant *A.baumannii* significantly increased the risk of mortality in the patients included in the study. Thus it proves the fact that infections with a carbapenem resistance isolate do affect the clinical outcome of the patient. Increase in CPIS was significantly associated with increase in mortality in the patients suffering from nosocomial pneumonia. The chronic co-morbid conditions did not significantly increase the risk of mortality in the patients included in this study.

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ANNEXTURE 1

Charlson Comorbidity Index Score Calculator

Condition

Myocardial Infarction	Hemiplegia	Mod-Severe Liver Disease	Metastatic Solid Tumor
Congestive Heart Failure	Mod-Severe Renal Disease		AIDS
Peripheral Vascular Disease	Diabetes with Organ Damage		
Cerebrovascular Disease	Any Tumor (within last 5 years)		
Dementia	Lymphoma		
Chronic Obstructive Pulmonary Disease	Leukemia		
Connective Tissue Disease			
Peptic Ulcer Disease			
Mild Liver Disease			
Diabetes			

Age by Decade | 0-49 | 50-59 | 60-69 | 70-79 | 80-89 | 90-99 | 100+ |

Age Unadjusted CCI Score is 2 **Age Not Selected**

Reset CCI Calculator

ANNEXTURE 2

PROFORMA FOR CARBAPENEM RESISTANT HOSPITAL ACQUIRED *ACINETOBACTER BAUMANNII* STUDY

1. **NAME** **AGE** **SEX**
2. **HOSPITAL NUMBER** **WARD** **UNIT**
3. **OCCUPATION**
4. **DATE OF ADMISSION** **DATE OF DISCHARGE/DEATH**
5. **DATE OF NOSOCOMIAL INFECTION DEVELOPING:**
6. **DATE OF SURGICAL PROCEDURE IF ANY:**
7. **DATE OF TRANSFER TO ICU IF ANY:**
8. **ADMISSION DIAGNOSIS:**
9. **OUTCOME- CURED EXPIRED DISCHARGED**
10. **CLINCAL DATA**

1. FEVER AT ADMISSION: **YES/NO**

2. WHAT NOSOCOMIAL INFECTION DOES THE PATIENT HAVE:

DOES THE PATIENT FULFILL THE FOLLOWING DEFINITIONS?IF SO CIRCLE THE FEATURES THAT THE PATIENT HAS

Nosocomial urinary tract infection:

Patients having at least one of the following signs and symptoms with no other recognizable cause of fever, with fever > 38°C,urgency,frequency,dysuria or suprapubic tenderness and a positive urine culture ie > 10^5 organisms/cm^3 of urine with no more than 2 microorganisms grown on culture with symptoms occurring more than 48 hrs after hospital stay.

Nosocomial Septicemia

Patients who develop fever, hypotension, tachycardia, tachypnoea after 48 hours after admission to the hospital, the pathogen being cultured in one or more blood cultures and having no relation to an infection at another site.

Nosocomial meningitis
Patients who develop fever, headache, neck pain, vomiting, altered sensorium after a neurosurgical procedure or any procedure that could have led to Nosocomial meningitis, with no other cause for fever.

Nosocomial deep abscesses
Patients who develop fever, deep abscesses and pain after any surgical procedure or any procedure that could have led to a Nosocomial deep abscess.

IV Catheter tips and Post operative pyogenic isolates
Patients who develop fever, thrombophlebitis ,evidence of sepsis following the use of an IV catheter or post operative infections that have developed 48 hrs after admission.

Nosocomial Pneumonia:
Patients who develop new onset pneumonia more than 48 hours after admission to the hospital. The clinical features include fever, leucocytosis,new onset purulent sputum/respiratory secretions, new onset or worsening cough or breathlessness, bronchial breath sounds, or worsening gas exchange, a chest radiograph with new or progressive infiltrate ,consolidation or cavitation (1).

CPIS(MODIFIED SCORE)

CPIS POINTS	**0**	**1**	**2**
Tracheal Secretions	Rare	Abundant	Abundant+purulent
Chest X-ray infiltrates	No infiltrate	Diffused	Localized
Temperature	>97.4 and <101.2	>101.2 and<102.2	>102.2 or <96.8
Leukocytes/mm^3	>4000and<11000	<4000or>11000	<4000or>11000+band
$P_{Ao2/ Fio2,mmHg}$	>240or ARDS		<240 or no ARDS
Microbiology	Negative		Positive

Total score=

11. MICROBIOLOGY SECTION

SOURCE

12. AST DATA OF THE ORGANISM

	RESULTS
CTX	
CZD	
CPI	
IMP	
MERO	
GENTA	
AK	
LEVO	
CIPRO	
MAGNEX	
TIMENTIN	
PIP/TAZO	
SXT	
CHLORO	
TIGE	
COLISITIN	
PB300	

MIC
PB300

SULBACTUM

13. BIOCHEMICAL SPECIATION

OF GLUCOSE	
NITRATE REDUCTION	
ARGININE DIHYDROLASE	
MALONATE UTILISATION	

14. MULTIPLEX P.C.R RESULTS

OXA 23	
OXA 51	