"QUANTITATIVE CULTURES OF AEROBIC BACTERIA AND THEIR ANTIBIOTIC SUSCEPTIBILITY PATTERNS FROM ENDOTRACHEAL ASPIRATES OF PATIENTS WITH VENTILATOR ASSOCIATED PNEUMONIA. A ONE YEAR CROSS-SECTIONAL STUDY."

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This is to certify that the dissertation entitled **"QUANTITATIVE CULTURES OF AEROBIC BACTERIA AND THEIR ANTIBIOTIC SUSCEPTIBILITY PATTERNS FROM ENDOTRACHEAL ASPIRATES OF PATIENTS WITH VENTILATOR ASSOCIATED PNEUMONIA. A ONE YEAR CROSS-SECTIONAL STUDY."** is a bonafide research work done by the candidate Reg. No. BI0109002.

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LIST OF ABBREVIATIONS

ARDS	:	Acute respiratory distress syndrome
BAL	:	Bronchoalveolar lavage
CAP	:	Community acquired pneumonia
CDC	:	Centre for disease control
COPD	:	Chronic obstructive pulmonary disease
CPIS	:	Clinical pulmonary infection score
EDTA	:	Ethylene diamine tetra acetic acid
ETA	:	Endotracheal aspirate
HAP	:	Hospital acquired pneumonia
ICU	:	Intensive care unit
LRT	:	Lower respiratory tract
MBL	:	Metallo-beta lactamase
MDR	:	Multidrug resistance
MV	:	Mechanical ventilation
MICU	:	Medical intensive care unit
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
PTC	:	Protected telescoping catheter
QEA	:	Quantitative cultures of ETAs
TLC	:	Total leukocyte count
VAP	:	Ventilator-associated pneumonia

ABSTRACT

Background: Ventilator-associated pneumonia (VAP) is an important intensive care unit (ICU) infection in mechanically ventilated patients. VAP occurs approximately in 9-27% of all intubated patients. Samples collected by invasive bronchoscopic methods have been shown to be highly specific in diagnosing VAP. However, bronchoscopy is not accessible in many settings and because of the invasive nature, cost of the bronchoscopy and need of a bronchoscopist for the procedure; samples obtained by non bronchoscopic methods may be useful alternative to bronchoscopy in the diagnosis of VAP.

Aim of the Study: The present study was undertaken

1. To isolate and identify aerobic organisms associated with ventilator-associated pneumonia.
2. To study the antibiotic susceptibility pattern of the isolates by Kirby Bauer disc diffusion method.
3. To detect metallo-beta lactamase producing *Pseudomonas aeruginosa* isolates.

Materials and Methods: The present study was undertaken at the Department of Microbiology, J.N. Medical College, Belgaum during the period from 01-01-09 to 01-01-10. Endotracheal aspirates (ETA) were collected from patients with suspected VAP, and direct gram's stain criteria was used to accept the sample. Quantitative cultures of ETA were performed with the threshold for microbiological diagnosis of VAP taken as 10^5 colony forming units (cfu)/ml. Isolates showing reduced susceptibility to Imipenem were selected for detection of MBLs enzymes by Imipenem-EDTA combined disk method.

Results: Out of 53 cases, 2 (3.77%) were polymicrobial. *Acinetobacter baumannii* 49.09% (27/55) and *Pseudomonas aeruginosa* 30.91% (17/55) were the most common pathogens isolated. Most of the organisms were highly resistant to the commonly used antibiotics. Piperacillin-tazobactam and doxycycline were found to be most effective against *Acinetobacter baumannii.* MBL was produced by 47.06% (8/17) of *Pseudomonas aeruginosa*.

Conclusion: The bacteriological approach for the management of VAP helps the clinicians in choosing the appropriate antibiotics. This study showed that quantitative cultures of endotracheal aspirate at a cut off point of 10^5 cfu/ml is a practical diagnostic method in clinically suspected ventilator associated pneumonia.

Key words: Ventilator-associated pneumonia, Endotracheal aspirate, Quantitative cultures, *Acinetobacter baumannii*

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INTRODUCTION

Ventilator-associated pneumonia (VAP) is defined as pneumonia occurring more than 48 hours after patients have been intubated and received mechanical ventilation[1]. VAP is the most common nosocomial infection in the intensive care unit (ICU) with an incidence ranging from 8 to 28 % in intubated mechanically ventilated patients[2, 3, 4].

The pathogenesis for VAP is related to host and treatment related colonization factors. Aspiration of oropharangeal pathogens and the leakage of secretions containing bacteria around the endotracheal tube are principal factors for the development of VAP[4,5]. The progression from colonization to tracheobronchitis to VAP is a dynamic equilibrium. Diagnosing VAP requires a high clinical suspicion combined with bedside examination, radiographic examination, and microbiologic analysis of respiratory secretions[1]. Diagnostic testing in VAP is necessary as it allows one to define whether a patient has pneumonia as the reason for a set of signs and symptoms and to find out the etiologic pattern when pneumonia is present. Due to increasing incidence of multidrug-resistant (MDR) organisms in ICUs, early and correct diagnosis of VAP is an urgent challenge for optimal antibiotic treatment[2].

Ventilator-associated pneumonia complicates the course of patients receiving mechanical ventilation inspite of major advances in techniques for its diagnosis and treatment. In the absence of a gold standard, VAP is assumed to be diagnosed more accurately by bronchoscopic sampling and microbiological cultures of the lower respiratory tract[6]. Bronchoscopy, being invasive, is not uncommonly associated with complications, especially in patients on high respiratory supports. This has paved the way for less invasive tests such as endotracheal aspirates (ETA)

and quantitative ETA cultures with a threshold of 10^5 to 10^6 bacteria per milliliter of exudates that is considered as optimal for the microbiological confirmation of VAP[6,7,8].

More importantly, recent small trials have repeatedly shown that there is no advantage of bronchoscopic cultures over quantitative ETA cultures when mortality was considered as the end-point further strengthening the case for quantitative ETA as a diagnostic tool [8,9,10,11].

Detection of causative organisms and their antibiotic susceptibility is crucial for diagnosis of VAP in order to initiate the appropriate antibiotic treatment thereby reducing the adverse effects of inadequate antibiotic treatment on the patient prognosis[3].

Hence the present study is undertaken to isolate, identify and quantitate the aerobic bacteria and to perform the antibiotic susceptibility testing from the endotracheal aspirates of the clinically suspected patients of VAP.

OBJECTIVES

1. To isolate and identify aerobic organisms associated with ventilator associated pneumonia.
2. To study the antibiotic susceptibility pattern of the isolates by Kirby Bauer disc diffusion method.
3. To detect metallo-beta lactamase producing *Pseudomonas aeruginosa* isolates.

REVIEW OF LITERATURE

Pneumonia represents a spectrum of disease that ranges from community-acquired pneumonia (CAP) to hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP). HAP is defined as pneumonia that occurs 48 hours or more after hospital admission, which was not incubating at the time of admission while VAP refers to pneumonia occurring more than 48 hours after endotracheal intubation.[12]

DEFINING PNEUMONIA BY LOCATION IN THE LUNG

Pneumonia may be defined according to its location in the lung:

• Lobar pneumonia occurs in one part, or lobe, of the lung.

• Bronchopneumonia tends to be scattered throughout the lung[13].

DEFINING PNEUMONIA BY ORIGIN OF INFECTION

COMMUNITY ACQUIRED PNEUMONIA (CAP) People with this type of pneumonia contracted the infection outside a hospital setting. It is one of the most common infectious diseases. It often follows a viral respiratory infection, such as the flu. Commonest organism causing C0AP is Streptococcus pneumoniae. Other pathogens include Haemophilus influenzae, Mycoplasma, and Chlamydia.

HOSPITAL ACQUIRED PNEUMONIA (HAP) Hospital-acquired pneumonia is an infection of the lungs contracted during a hospital stay. This type of pneumonia tends to be more serious, because hospital patients already have weakened defense mechanisms, and the infecting organisms are usually more dangerous than those

encountered in the community. Hospital acquired pneumonia is also called nosocomial pneumonia. A subgroup of hospital-acquired pneumonia is ventilator-associated pneumonia (VAP), a highly lethal form contracted by hospitalized patients on ventilators occurring more than 48 hours after intubation [12].

VENTILATOR-ASSOCIATED PNEUMONIA (VAP) Ventilator-associated pneumonia is defined as pneumonia occurring more than 48 hours after patients have been intubated and received mechanical ventilation.

CLASSIFICATION

Onset of ventilator-associated pneumonia is of 2 types; early and late. Early onset ventilator-associated pneumonia occurs 48 hours to 96 hours after intubation and is associated with antibiotic susceptible organisms. Late onset ventilator associated pneumonia occurs more than 96 hours after intubation and is associated with antibiotic resistant organisms. Interventions to prevent ventilator associated pneumonia should begin at the time of or if possible, before intubation.

This categorization helps to predict the implicated pathogens and guides us in the initial empiric therapy with antibiotics, which is known as the epidemiological approach[14].

Incidence

Most studies have suggested that VAP develops in 9-27% of mechanically ventilated patients. In a recent retrospective cohort study in which data from 9080 patients who were mechanically ventilated for >24 hrs were reviewed, VAP developed in 9.3%[14]. The risk of VAP increases with the duration of mechanical

ventilation. However, at least one study has suggested that the incremental risk of VAP remains constant at ~1% per day[15].

In a study conducted by Cook DJ et al, daily risk of developing VAP increased until day 5, then decreased over the duration of stay in the intensive care unit[16].

In an Indian study by Dey A et al, incidence of VAP was found to be 45.4% among mechanically ventilated patients.[3]

Prevalence estimates vary between 6 and 52 cases per 100 patients, depending on the population studied. On any given day in the ICU, an average of 10% of patients will have pneumonia with VAP in the overwhelming majority of cases [17].

A prospective investigation of VAP in 724 critically ill patients who had received prolonged ventilatory assistance after admission found a mean rate of 23%; the frequency rose from 5% for patients receiving mechanical ventilation for 1 day to 69% for those receiving MV for more than 30 days [2].

Mortality and Morbidity

In contrast to other nosocomial infections, for which mortality is low, ranging from 1% to 4%, the morality rate for VAP ranges from 24% to 50% and can reach more than 75% in some specific settings or when lung infection is caused by high risk pathogens.[2]

Attributable mortality

Because the risk factors for VAP and death are directly related, the severity of the underlying disease can influence both events. Thus, it is difficult to determine whether such patients would have survival if VAP had not occurred.

The concept of "attributable mortality" defined as the percentage of deaths that would not have occurred in the absence of this infection, has been developed. Crude ICU mortality rates of 24-76% have been reported for VAP at various institutions. ICU ventilated patients with VAP appear to have a two to 10 fold higher risk of death compared to patients without pneumonia. The results of several studies conducted between 1986 and 2005 have confirmed the following observation despite variations among studies that partly reflect the population considered, overall mortality rates for patients with or without VAP were : 71% Vs 29% [15], 50% Vs 30%[18], 70.3% Vs 35.5% [19] 33% Vs 19% [20], and 29.4% Vs 30.6% [21] respectively.

Measuring the impact of VAP on health care resources has been difficult. In one study, VAP prolonged the duration of mechanical ventilation from 10 to 32 days. In another, the median length of stay in the ICU for patients who developed VAP was 21 days versus a median of 15 days for paired control subjects[2].

Pathogenesis

Pathogenesis and routes of entry: For any infection to occur, there must be an interplay of 3 factors ie; impaired host defence, access of pathogenic bacteria in sufficient numbers to the lower respiratory tract and the virulence of the organism[22].

Pathogens causing VAP may be part of the host's endogenous flora at the time of hospitalization or may be acquired exogenously after admission to the health care institution, from the hands, apparel, or equipment of health care workers, hospital environment, and use of invasive devices.[4] The organism may gain access into the lungs by one of several routes ie; microaspiration of oropharyngeal secretions, aspiration of gastric contents, inhalation, hematogenous spread, direct inoculation and exogenous penetration (eg pleural space). Out of these microaspiration is the most common.

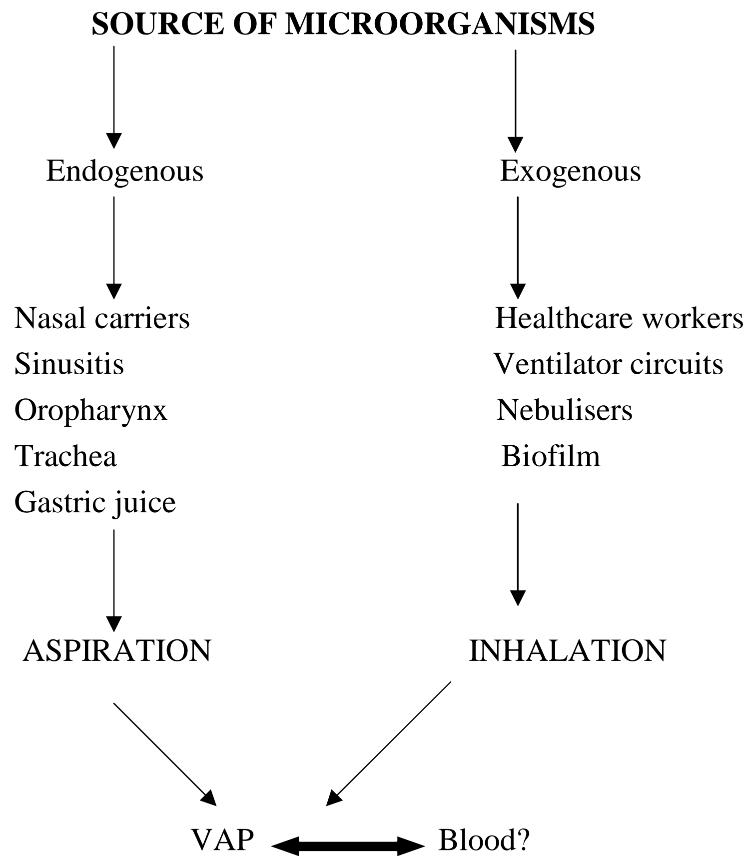

Fig-1 : Pathogenesis of ventilator-associated pneumonia. Micro- organisms can reach lung parenchyma from different reservoirs[23].

Routes of bacterial entry[5,23]

a) Microaspiration of oropharyngeal secretions colonised with pathogenic bacteria.

b) Aspiration of esophageal / gastric contents.

c) Inhalation of an infected aerosol.

d) Hematogenous spread of infection from a distant site of infection.

e) Exogenous penetration from an infected site (i.e. pleural space).

f) Direct inoculation into the airway of incubated patients from ICU personnel.

Oropharyngeal Colonization:

The oropharynx appears to be the initial site of colonization in most cases of VAP[24].Colonization of the upper respiratory tract by gram negative bacilli is mediated by alterations in the surface properties of the epithelial cells. In healthy individuals a film of fibronectin covers the epithelium lining the mucosa of the mouth and oropharynx, and prevents the Gram-negative bacteria from adhering to the epithelial cells. This protective coating is lost in very ill individuals, so that pathogenic Gram-negative organisms adhere to receptors present on epithelial cells of the mucosa and soon colonize it. The number of these bacterial receptors on both upper and lower airway epithelial cells is increased in many illnesses. The risk factors responsible for oropharyngeal colonization with gram negative bacteria include neutropenia, prior antibiotic therapy, alcoholism, azotemia, coma, diabetes, serious illness, hypotension, intubation, smoking, surgery and neutralisation of gastric acid. [4,24]

Aspiration :

The potential routes of infection, microaspiration of a small volume of oropharyngeal secretions previously colonized with pathogenic bacteria is most common. Microaspiration is reported even in healthy people during sleep, but it is the presence of pathogenic bacteria which are able to overcome the lower respiratory tract defenses that is most important in the development of pneumonia. The incidence of aspiration increases when the gag reflex is impaired, if there is an alteration is level of consciousness, when certain devices such as nasogastric tube or endotracheal tubes (ET) are used or if esophageal disease is present.

Among mechanically ventilated patients additional routes of entry exist. The ET tubes by pass host defenses above the vocal cords and impair lower respiratory tract defenses such as cough and mucocilliary clearance. Contaminated secretions can

pool above the inflated ET cuff and are not easily removed by suctioning. The secretions can leak around the ET cuff and directly enter the lower respiratory tract when there are changes in airway caliber during swallowing and breathing.[4,24]

Gastric Colonization:

The acid within the stomach serves as a major deterrent to bacteria swallowed in the saliva. If gastric acidity is suppressed by the use of antacids and H2-antagonists, the bacteria within the stomach survive, multiply and soon colonize the upper gastrointestinal tract. Gastric contents laden with Gram-negative bacteria could easily regurgitate and be aspirated into the lungs, causing aspiration pneumonia. This is particularly frequent in obtunded patients, sedated patients or following a large vomit. Prophylaxis and treatment of bleeding stress ulcers with sucralfate which does not significantly increase gastric pH in most patients, has resulted in a reduced incidence of nosocomial pneumonias.

Haematogenous spread:

Bacteraemia is not frequently considered a source of microorganisms producing VAP. Blood cultures in patients with VAP are clearly useful if there is a suspicion of another probable infectious condition, but the isolation of a microorganism in the blood does not confirm that microorganism as the pathogen causing VAP. Rarely infected emboli from a septic thrombophlebitis can, lead to septic infarcts within the lung. Catheter related sepsis or any other source of sepsis can cause bacteraemia with haematogenous spread of infection into the lungs, causing pneumonia[23].

Inhalation :

Contaminated respiratory equipment (nebulizers, humidifiers, ventilator tubing etc) are a source of infected aerosols. Infected particles 3-5 microns in size can be deposited into the terminal bronchioles and alveoli, thereby causing a lower respiratory tract infection.

Iatrogenic Causes:

Lack of aseptic precaution during suction of tracheobronchial secretions, either through an endotracheal tube or tracheostomy is an important cause of lower respiratory tract infection. Medical staff or respiratory therapy equipments harbour pathogenic flora that can be directly inoculated into tracheobronchial tree.[4,23]

Risk factors

Several authors have examined risk factors for VAP, and a summary of independent risk factors from the literature was published in a recent review by Chastre and Fagon (Table 1) [2]

Table 1: Independent risk factors for ventilator associated pneumonia identified by Multivariate analysis from selected studies.

Host Factors	Intervention Factors	Other Factors
Serum albumin <2.2g/dl	H2 blockers ± antacids	Season fall, winter
Age >60 years	Paralytic agents, continuous intravenous sedation	
ARDS	>4 units of blood products	
COPD, pulmonary disease	Intracranial pressure monitoring	
Coma or impaired consciousness	MV >2D	
Burns, trauma	Positive end expiratory pressure	
Systemic Organ failure	Frequent ventilator circuit changes	
Severity of illness	Reintubation	
Large volume gastric aspiration	Nasogastric tube	
Gastric colonization	Supine head position	
Upper respiratory tract colonization	Transport out of ICU	
Sinusitis	Prior antibiotic or no antibiotic therapy	

ETIOLOGY

The main cause of ventilator-associated pneumonia (VAP) is the aspiration of the bacteria that colonize the upper respiratory tract or upper digestive tract into the lower respiratory tract.[2,5] Some of these bacteria are scarcely pathogenic for the lung, such as most species of the genus *Corynebacterium, Streptococcus pneumoniae, Enterococcus* and *Bacillus* species and coagulase-negative staphylococci. Their isolation from the lower respiratory tract (LRT) in patients subjected to mechanical ventilation has generally no pathological significance. In contrast, the microorganisms most frequently responsible for VAP are *Staphylococcus aureus*, most microorganisms of the Enterobacteriaceae family and some Gram-negative, nonglucose-fermenting bacilli such as *Pseudomonas aeruginosa* and *Acinetobacter baumanii*. Hence, the search carried out by the microbiology laboratory is preferentially geared towards identifying these pathogens.

Potential etiologic agents of VAP include both multidrug resistant (MDR) and non-multidrug resistant (non-MDR) bacterial pathogens. The relative frequency of individual MDR pathogens can vary significantly from hospital to hospital and even between critical care units within same institution.

Many hospitals have problems with *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA), but other multidrug resistant (MDR) pathogens are often institution specific[17].

Non-MDR pathogens	MDR pathogens
Streptococcus pneumoniae	*Pseudomonas aeruginosa*
Hemophilus influenzae	MRSA
MSSA	*Acinetobacter spp.*
Antibiotic-sensitive Enterobacteriaceae	Antibiotic-resistant Enterobacteriaceae
Escherichia coli	*Enterobacter spp.*
Klebsiella pneumoniae	ESBL-positive strains
Proteus spp.	*Klebsiella spp.*
Enterobacter spp.	*Legionella pneumophilia*
Serratia marcescens	*Burkholderia cepaia*
	Aspergillus spp.

Common pathogens currently associated withVAP[26]

A] **Early onset VAP**

1) Gram-positive cocci

Methicilin-sensitive *Staphylococcus aureus* (MSSA)

Streptococcus pneumoniae

2) Gram negative bacilli

Escherichia coli

Klebsiella species

Serratia marcescens

B] **<u>Late onset VAP:</u>**

1) <u>Gram-positive cocci</u>

Methicilin-resistant *Staphylococcus aureus* (MRSA)

2) <u>Gram negative bacilli</u>

Enterobacteriaceae species

Acinetobacter baumannii

Pseudomonas aeruginosa

On many occasions, VAP is polymicrobial, although this in itself is not an aggravating factor[27]. The more resistant microorganisms appear in the so-called late onset form of VAP, which occurs 5-7 days into mechanical ventilation or hospital stay[28].

In one Indian study[3] multiresistant bacteria, mainly *Acinetobacter spp.* (47.9%) and *Pseudomonas aeruginosa* (27%) were the most commonly isolated pathogens in both types of VAP. No anaerobic organism as VAP pathogen could be isolated and signifies that anaerobes are not the usual pathogens causing VAP. Only one *Candida albicans* was isolated from one elderly longstanding diabetic patient, and the colony count was also very less ($<10^3$ cfu/ml), which determines that *Candida* was a tracheal colonizer only.

There is also some evidence of anaerobic bacteria playing a role as causal agents of VAP although, so far, this evidence can only be considered as anecdotal[29].

Fungi as the causal agents of VAP are rare, except in a small subgroup of patients with severe immunodeficiency associated mainly with neutropenia or the use of high doses of corticosteroids[30]. Isolates of the genus *Candida* obtained from LRT secretions can be mostly attributed to simple colonization and do not often indicate

pneumonia[4,30,31]. The same may be said of *Aspergillus* and other filamentous fungi, whose appearance in LRT samples should be interpreted in the light of the patient's immune status. *Aspergillus* may cause VAP in patients under steroid treatment, with neutropenia or with other serious forms of immunodeficiency[4].

Similarly, viruses are not a usual cause of VAP, yet some herpes viruses are often isolated from respiratory secretions in patients with and without VAP.[36,37] Their presence seems to be an indicator of disease severity but to date there is insufficient data on the potential role of these viruses as a cause of VAP. The occurrence of influenza in patients subjected to mechanical ventilation is rare[23,32].

Sampling Techniques

The latest recommendations of the American Thoracic Society and the Infectious Diseases Society of America include taking LRT specimens. It is essential that the laboratory is informed of the type of sample submitted to adequately process the sample and interpret the results[12].

In a survey of different sampling techniques Ruiz et al,[10] found no differences in rates of diagnoses, length of Intensive Care Unit (ICU) stay, days on mechanical ventilation and crude 30-day or adjusted mortality.

Routine Surveillance Cultures

Bacterial colonization of the trachea and bronchi preceding VAP is well recognized as a risk factor and as part of the pathogenic mechanisms [5,17]. The pathogenesis of VAP may be exogenous (the patient's airways or digestive tract is not colonized before the clinical manifestations of VAP; the microorganism is directly transmitted from outside) or secondary endogenous (the oropharyngeal mucosa, the stomach and the upper airways had acquired "nosocomial" microorganisms;

aspiration and failure of the defense mechanisms allows infection to take place). According to this classification, at least theoretically, the pathogens start to grow before they become responsible for the infection. By checking bacteria growing in the colonized patients, it could be possible to start the best antimicrobial (with a narrow spectrum) immediately as VAP becomes a clinically relevant problem. The role of surveillance cultures is obviously limited in the secondary endogenous pathway. However, the use of a routine surveillance program for bacterial colonization is still controversial [33].

Hayon et al showed the uselessness of surveillance samples in the prediction of microorganisms causing VAP. In this study, only 35% of the respiratory samples found all of the microorganisms that were isolated by BAL culture. Therefore, it is not recommended that routine LRT cultures (neither endotracheal aspirates nor protected specimen brush (PSB) samples) are taken in patients lacking criteria that could raise a suspicion of VAP [23,33].

Specimens to be collected on the suspicion of pneumonia

The type of specimen that should be obtained for microbiologic processing as soon as VAP is suspected is another issue that has generated a large amount of medical literature. There is no doubt that the quickest, easiest and cheapest sample to obtain is the endotracheal aspirate. The doubt that arises is whether bronchoscope-directed sampling methods (plugged telescoping catheter or BAL, etc.) are sufficiently more efficient than simple endotracheal aspiration to warrant their higher cost and, mainly, the delay that these more complex procedures often entail[34].

Quantitative culture techniques:

Owing to inevitable oropharyngeal bacterial contamination of all respiratory secretion samples, the use of techniques that quantify cultures is the *key issue* for a microbiology laboratory, despite this is not always undertaken in many hospitals today[35].

In view of the unreliability of qualitative microbiological evaluation of ETA and lower respiratory tract secretions, quantitative culture techniques were developed. Since there is a bacterial burden in the respiratory tract even in the absence of active infection, cutoff values for bacterial concentration to separate infection from colonization were defined. Threshold values are difficult to determine as there is a large overlap between bacterial counts in the presence and absence of histologically proven pneumonia.

Marquette et al have shown that quantitative culture of ETA secretions may have an acceptable diagnostic accuracy, when histological diagnosis was used as a "gold standard"[36].

Some studies have shown that quantitative ETA cultures correlate well with the result of invasive diagnostic techniques [8,9,10,56] .

Lambert R et al [37] showed that quantitative cultures of endotracheal aspirate (QEA) may avoid false positive results, but also provide controversial results, depending on the bacterial load, duration of ventilation and prior antibiotic treatment. The sensitivity ranges from 38- 100%, while specificity ranges from 14- 100% using a threshold 10^5- 10^6 cfu/ml, the sensitivity appears to have narrow range 50- 70% as well as specificity 70-85%.

Numerous factors can influence the results of quantitative cultures, including the timing of the pneumonia, the skill and experience of the operator, the adequacy of the specimen, technical aspects such as appropriate processing and delays in transport to the laboratory, special populations such as those with chronic obstructive pulmonary disease (who may have relatively high bacterial counts without pneumonia), and prior or concurrent antibiotic therapy.

Because of these potential limitations, it is important to bear in mind that a

quantitative culture that exceeds a threshold value is not diagnostic of VAP by itself. False-positive quantitative cultures could be secondary to bronchiolitis, colonization, or oropharyngeal contamination. Likewise, a result below these threshold values does not rule out the presence of pneumonia, particularly in the setting of prior antibiotic therapy. While higher bacterial counts correlate with a higher likelihood of VAP, lower counts are associated with a lower probability. Consequently, rather than interpreting a quantitative culture as either "positive" or "negative," it is clinically more useful to utilize the exact number of cfu/ml[1].

The fact that the different laboratory specimens have different bacterial count thresholds assigned to them is related to the dilution of the respiratory secretions with which the microbiologist has to work.

Bronchoscopic versus Nonbronchoscopic Sampling Procedures

Samples collected by invasive bronchoscopic methods have been shown to be highly specific in diagnosing VAP. However, bronchoscopy is not accessible in many settings and because of the invasive nature, cost of the bronchoscopy and need of a bronchoscopist for the procedure; samples obtained by non bronchoscopic methods may be useful alternative to bronchoscopy in the diagnosis of VAP. The use of the bronchoscope also carries risks, such as inducing cardiac arrhythmia, hypoxemia, bleeding, pneumothorax, along with greater costs both in terms of time and resources. The overall sensitivity provided by bronchoscopic and non-bronchoscopic sampling techniques is comparable[38].

Inherent advantages of nonbronchoscopic techniques include less invasiveness; less compromise of oxygenation, ventilation, and respiratory mechanics during the procedure; less likelihood of increasing intracranial pressure;

less likelihood of inducing arrhythmias; availability where there is no bronchoscopist; lack of contamination presented by the bronchoscopic channel; availability to patients with small endotracheal tubes; and lower cost. Of the quantitative techniques, QEA is least invasive, most readily available, and least expensive, and it requires the least experience and is easily repeatable[1].

Rajasekhar T et al [9] in an Indian study have reported on comparison of the results of quantitative cultures of blind bronchial sampling (BBS) and endotracheal aspirate (ETA), the agreement between the results of BBS and ETA was 83.3%.

In study conducted by M. Ruiz et al, length of ICU stay and mechanical ventilation as well as mortality was not significantly influenced by bronchoscopic or non-bronchoscopic diagnostic techniques used for microbial investigation[39].

Endotracheal Aspiration

Endotracheal aspiration is the simplest method of obtaining airway secretions in the mechanically ventilated patient. The aspirate obtained is undiluted or only slightly diluted and a bacterial count 10^5 cfu/mL indicates a positive culture result. Qualitatively treated at the microbiology laboratory, this type of specimen shows high sensitivity (80-100%), although its specificity is unacceptably low (14-47%)[40].

In contrast, the quantitative culture of endotracheal aspirates provides results that are comparable to those obtained using the plugged telescoping catheter or by BAL[1,8,9,41,42,43].

Protected Specimen Brush (PSB)

The cut-off value recommended for this method is 10^3 cfu/mL for a sample to be considered clinically significant. The mean sensitivity of PSB is estimated at 73±18% (SD) and its mean specificity is 82±19% (SD)[44].

In general, it is accepted that the probability that a positive result reflects the presence of VAP is very high. On the contrary, the rate of false negatives runs from 0 to as much as 40%[45]. False negatives have been attributed to a lack of standardization of the technique, obtaining specimens too early, unilateral or blind sampling and to previous antibiotic treatment[46,47].

Herer B, Fuhrman C, Demontrond D et al showed that, repeating the protected specimen brush method in the same patients and circumstances is not very reproducible[48].

The rate of false positives is around 30% and can be ascribed to the contamination of samples with microorganisms from the upper respiratory tract or to the collection of microorganisms colonizing the lower tract, which could be promoted by accompanying diseases, such as chronic obstructive pulmonary disease.

Bronchoalveolar Lavage (BAL)

Broncho alveolar lavage is performed by advancing the bronchoscope as far as a subsegmental bronchus (generally a 3rd or 4th generation bronchus), until its lumen becomes occluded. The next step consists of distally installing 20-50mL aliquots of sterile saline solution and resuctioning the contents of the distal bronchus. Although the volume recovered usually ranges from 5-70% of the volume installed, 5mL is generally sufficient for microbiological analysis.

Quantitative bacterial cultures are essential to distinguish between colonization and infection and it is currently accepted that the differentiation threshold is 10^4-10^5 cfu/mL.

Blood Cultures

Blood cultures show low sensitivity and low specificity for diagnosing VAP. A positive result is obtained in only 10-30% of cases. On the contrary, the lungs are not the source of bacteraemia in approximately half the patients with positive blood cultures[49].

Lung Biopsy as the Final Reference

A lung biopsy specimen is not routinely used to diagnose VAP due to obvious risks. This procedure is generally employed in post-mortem studies to validate other techniques and to gain knowledge on the physiopathology of VAP[50].

Transport to the Laboratory

Baselski VS et al showed that collected specimens in patients with suspected ventilator-associated pneumonia should ideally be transported to the microbiology laboratory in under 30 minutes for immediate processing to avoid bacterial overgrowth[51].

In a study conducted by deLassence A et al, the impact of refrigerating BAL specimens for 24 hours before processing was assessed. At 24 hours, the sensitivity, specificity, positive and negative predictive values were 77, 100, 100 and 93% when compared to cultures performed instantly. Thus, this delay can cause a reduction in the sensitivity of the technique of over 20%, which is clearly unacceptable. Even if no substantial losses in the qualitative or quantitative recovery of microorganisms are assumed, the delay in providing information will have clinically devastating consequences[52].

Rapid Information Provided by the Gram Stain

Blot et al[53] assessed the value of the Gram stain in patients with suspected VAP, on respiratory secretions obtained by endotracheal aspiration and the plugged telescoping catheter. They found a high microbiologically proven sensitivity in the use of endotracheal aspirates for the diagnosis of VAP (91%) and a high NPV in the test (94%) in patients not undergoing recent changes in antibiotic treatment. Blot et al. argued that a negative Gram stain in an endotracheal aspirate has high negative predictive value for a diagnosis of VAP and justifies the decision to not start antibiotic treatment.

Criteria used to reject ETAs from adult patients include greater than 10 squamous epithelial cells per low power field or no organisms seen under oil immersion in the entire field [54,55].

The clinical diagnosis of ventilator associated pneumonia (VAP)

The diagnosis of VAP is an unsettled and controversial area, with no agreement about whether the decision to start antibiotic therapy, in the setting of suspected infection, should be guided by clinical criteria or by microbiologic data from quantitative samples of lower airway secretions. This controversy exists because the clinical definition of pneumonia although sensitive, is not very specific.

Even though the use of clinical definition of pneumonia may be overly inclusive, a large body of data has demonstrated that mortality in VAP is reduced when patients receive prompt and accurate empiric therapy. Thus, when faced with a patient who, on clinical grounds, may have pneumonia, the clinician must often initiate broad spectrum empiric therapy, accounting for all pathogens that are likely to be causing infection, in an effort to "protect" the at risk patient.

For all these reasons, some investigators have proposed that whenever VAP is suspected, the patients should have a sampling of lower respiratory tract secretions (by bronchoscopic protected brush, bronchoalveolar lavage, blind brush or lavage, or endotracheal aspirate) which is then cultured quantitatively and the results used for several purposes[57].

Clinical diagnostic criteria of VAP

VAP is defined as new pulmonary infiltrate (or radiologically confirmed worsening of new pre-existing infiltrate), together with atleast two of the following criteria:

Leucocytosis (>12,000/mm^3),

Leucopenia (<4000/mm^3),

Fever (38ºC),

Hypothermia (35ºC) or

Purulent tracheal secretions

Pugin J, Auckenthaler R et al[58]showed body temperature, white blood cell count, volume and appearance of tracheal secretion, oxygenation (PaO_2/FiO_2), chest X ray and tracheal aspirate cultures into a clinical pulmonary infection score as a diagnostic tool for pneumonia. They found that clinical pulmonary infection score of more than six was associated with sensitivity 93% and a specificity of 100%.

Singh N, Rogers P et al[59] used modified clinical pulmonary infection score with in a clinical management algorithm in an attempt to reduce unnecessary antibiotic use in patients in whom ventilator associated pneumonia was suspected. In this series of patients, modified score remaining less than six at 3 days safely allowed stopping antibiotics. However, the diagnostic value of the clinical pulmonary infection score has yet to be confirmed. In addition, clinical utility of such a score would be higher if it helped clinicians in their decision to initiate or with hold antibiotic therapy in patients clinically suspected of ventilator associated pneumonia rather than only to confirm or exclude pneumonia after 2-3 days when tracheal aspirate culture results are available.

Preventive strategies

The two important processes involved in the pathogenesis of ventilator-acquired pneumonia are

1. Bacterial colonization of the aerodigestive tract and

2. Aspiration of the contaminated secretions into the lower airway.

Therefore, preventive strategies for ventilator-acquired pneumonia are directed at reducing the bacterial burden colonizing the aerodigestive tract, and decreasing the occurrence of aspiration[60].

Reducing the bacterial burden colonizing the aerodigestive tract:

1. Reducing the use of antibiotics :

VAP occurring late during mechanical ventilation are more likely to be due to the high risk multidrug resistant bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus*[60]. VAP due to antibiotic resistant pathogens are associated with greater hospital mortality and longer lengths of hospital stay than those due to antibiotic sensitive bacteria. Therefore, reducing the unnecessary use of antibiotics should be the primary objective in order to prevent the more serious forms of VAP. The recent trends in treatment strategies reflect this approach. Changing or rotating antibiotic class periodically in an ICU may also reduce incidence of antibiotic resistance[61].

2. Stress ulcer prophylaxis:

Critically ill patient on ventilatory support frequently receives antacids and H2 blockers to prevent the occurrence of stress ulcers and gastrointestinal bleeding. Bacterial colonization of the stomach is encouraged by agents that increase the gastric pH and thereby promote the occurrence of VAP. Sucralfate may be less likely to increase the risk of pneumonia, but may, however be less efficacious in preventing hemorrhage[62].The routine use of H2 blockers should be avoided, except where the risk of gastrointestinal bleeding is high.

3. Selective digestive decontamination (SDD):

Most studies have shown that SDD reduces the risk of pneumonia. Sanchez GM et al[63] showed that SDD reduced the incidence of VAP (11% vs 29%, $p<0.001$).

However, there are the potential risks of antibiotics resistance and toxicity, and the absence of demonstratable effect on mortality has prevented widespread acceptance[61].

4. Chlorhexidine oral rinse:

The use of oral washes may be appropriate in certain selected high-risk patients, but there is potential for bacterial selection and colonization[61].

5. Handwashing:

The oldest measure to prevent nosocomial infection in health care institutions is hand hygiene but compliance by ICU staff is inadequate[21,64]. Use of hand washing disinfectants may achieve better compliance and prevention. Disposable gloves and gowns may also prevent cross infection.

Interventions to prevent aspiration:

1. Semi recumbent positioning to reduce the occurrence of aspiration.:

Patients should be placed in a semi recumbent position rather than supine. Accidental self extubation with consequent reintubation should be avoided through appropriate analgesia/sedation and physical restraints. Reintubation performed with the patient in semi recumbent supine position may also be beneficial[61].

2. Avoidance of large gastric volumes:

Nutritional support should be provided in a manner that gastric overdistension does not take place. The residual volume in the stomach prior to a feed should be monitored. Prokinetic agents such as metoclopramide, mosapride should be used when necessary. Small bore tubes placed in the jejunum may reduce the incidence of aspiration, but it awaits further validation[61].

3. Subglottic drainage:

Continuous subglottic drainage of secretions that pool above the endotracheal cuff has been shown to reduce the incidence of VAP[65]. But these more expensive,

specialized endotracheal tubes should be a part of an organized approach in preventing VAP.

4. Oral intubation:

Prolonged (>48 hrs) nasal intubation should be avoided as an association was demonstrated between nosocomial sinusitis and ventilator-associated pneumonia. Oral intubation is the preferred option.

5. Ventilator-related factors:

Routine changing of ventilator circuits has not proven to be beneficial in reducing the incidence of VAP[66]. This lack of benefit is attributable to the rapid bacterial colonization of the circuit tubing. More frequent circuit changes are indicated when there is overt soilage from blood or vomitus. Accumulated condensate should also be watched for and removed. Heat moisture exchange (HME) may decrease the incidence of VAP perhaps by minimizing the condensate. They are also easy to use and less expensive. Certain HME filters can be left in place for upto one week, further increasing their cost effectiveness[67]. There are studies that do not confirm that HMEs reduce the rate of VAP. Moreover, HME filters may impose resistive load and difficulties in weaning[68].

6. Infection control programmes employing several interventions:

Interventions aimed at controlling both colonization of the aerodigestive tract with pathogenic bacteria and aspiration have been successful in preventing VAP and are also cost effective[69]. Chemicals that block genes in bacteria that form biofilms, antibodies that block fibronectin-building protein adhesion bacteria, and the use of specialized coating that block bacterial adherence are the future directions in the area of prevention.

In a study by Mulin et al found that converting from an open unit to single ICU rooms greatly reduced colonization by *A. baumannii*[24].

Management of ventilator associated pneumonia.

Management of VAP needs to balance the avoidance of unnecessary antibiotic overuse, and the provision of adequate initial empiric therapy. A rational strategy starts with immediate initiation of adequate antibiotics and collection of respiratory secretions to evaluate the causative organisms. As a minimum, an endotracheal aspirate with direct staining and quantitative cultures should be obtained. Overall, the need to choose adequate antibiotics correctly and expeditiously calls for the use of broad-spectrum antibiotics, but the choice should be narrowed quickly in the light of microbiologic information.

Initial empiric antibiotic therapy

The key decision in initial empirical therapy is whether the patient has risk factors for multi-drug resistant (MDR) organisms. Patients admitted after a recent hospitalization or from a healthcare associated facility (nursing home, dialysis centre etc) should be classified as at risk for MDR pathogens. In studies of HAP and VAP, hospitalization of at least 5 days is required to increase the risk of infection with these organism[60].One consequence of increasing antimicrobial resistance is an increased probability of inappropriate initial empiric antimicrobial treatment of infections.[70]

The most common pathogens include *Pseudomonas aeruginosa*, *Acinetobacter* species, *Klebsiella pneumoniae*, *Enterobacter* species, and MRSA. Patients at risk of infection with these organisms should initially receive a combination of agents that can provide a broad spectrum of coverage to minimize the potential for inappropriate antibiotic treatment. In the therapy of suspected pseudomonal infection, therapy should involve a selected beta-lactam plus either an antipseudomonal quinolone or an aminoglycoside. The choice should be based on local patterns of antimicrobial susceptibility, and anticipated side effects, and should

also take into account which therapies patients have recently received (within the past 2 weeks), striving not to repeat the same antimicrobial class if possible. Each ICU should collect similar data to establish its own "best empiric therapy regimen" tailored to the antibiotic susceptibility patterns of the local flora.

Specific common VAP pathogens

The choice of specific agents will be dictated by the results of sensitivity testing, the availability of these agents, and issues of cost and formulary restriction. Four multidrug resistant pathogens merit special discussion.

Genus *Acinetobacter*

The genus *Acinetobacter* is currently classified in the family *Moraxellaceae* and consists of bacteria that are non-motile, oxidase-negative, gram-negative coccobacilli. Currently there are at least 25 genomospecies described within the genus *Acinetobacter*. Genomospecies 1 is the type species and is named *A.calcoaceticus*. It is isolated principally from soil. Genomospecies 2 is named *Acinetobacter baumannii*; it includes isolates previously referred to as *A.calcoaceticus var. anitratus*. Genomospecies 4 is named *A.haemolyticus*; genomospecies 5 is named *A.junii*; genomospecies 7 is named *A.johnsonii*; genomospecies 8 is named *A.lwoffi*; and genomospecies 12 is named *A.radioresistens*. Most of the remaining genomospecies are unnamed.

Acinetobacter baumannii is the second most frequent nonfermenter encountered in clinical laboratories, but with only about one tenth the frequency of *Pseudomonas aeruginosa*. The following are the characteristics by which a definitive identification of *Acinetobacter baumannii* can be made:

- Appear as cocci or coccobacilli on gram stain
- Grow well on MacConkey agar (colonies may have a slightly pinkish tint, a helpful characteristic when present)
- Do not produce cytochrome oxidase
- Exhibit rapid utilization of glucose, with production of acid
- Exhibit rapid utilization of 10% lactose, with production of acid
- Are nonmotile
- Are penicillin-resistant
- Growth at 42ºC [82,87]

The increase of *A. baumannii* infections is due to its great resistance to the environment which enables it to spread, its limited virulence and its extraordinary ability to develop resistance to all antimicrobials [23].

The simplicity of the nutritional requirements of *A. baumannii*, the variety of sources of carbohydrates that it uses and its ability to grow in different temperatures and pH values, explain its prolonged survival in inert environmental elements, which is similar to Staphylococcus aureus and much greater than other Gram-negative bacteria. These qualities facilitate the intense contamination of hospital equipment, which is typical of outbreaks caused by this pathogen.

The virulence factors of *A. baumannii*, epithelial cell adhesion, the production of lipolytic enzymes and lipopolysaccharide of the cell wall are scarce compared to other Gram-negative bacteria[5].

Risk factors for VAP due to Acinetobacter included neurosurgery, ARDS, head trauma, and gross aspiration in one series, and prior ceftazidime therapy and poor hand-washing in another[5]. *A. baumannii* is one of the most common etiologies of hospital-acquired pneumonia (HAP), especially in late onset ventilator-associated

pneumonia (VAP).Crude mortality for patients with ventilator-associated A. baumannii pneumonia is high, ranging from 33–70 % [23].

Multidrug resistance increasingly reported in these pathogens is posing a threat to hospitalized patients due to the limitation of therapeutic options. The acquisition of multidrug resistance is related to environmental contamination and contact with transiently colonized health care providers. Carbapenems have been the drug of choice for treatment of infections caused by *A. baumannii*. However, in recent years, the number of isolates showing resistance to carbapenems has increased worldwide. This is mediated by the lack of drug penetration (i.e. porin mutations and efflux pumps) and/or carbapenem-hydrolyzing beta-lactamase enzymes such as OXA carbapenemases and metallo-beta-lactamases.

The first identified OXA-type enzyme with carbapenem-hydrolyzing activity was from an *A. baumannii* strain isolated in 1985 from Scotland and was originally named ARI-1, but was renamed as bla_{OXA-23} .The bla_{OXA-23} cluster ($bla_{OXA-23,27,49}$) now contributes to carbapenem resistance in *A. baumannii* globally. Two other plasmid-encoded acquired OXA-type clusters with a carbapenamase activity have been described, which are bla_{OXA-24} -like ($bla_{OXA-24,25,26,40}$) and bla_{OXA-58} -like genes. The bla_{OXA-23} , bla_{OXA-24} and bla_{OXA-58} like enzymes are plasmid/chromosomally encoded which explains their widespread distribution. The bla_{OXA-51} like gene cluster is unique in that it naturally occurs in *A. baumannii*. Therefore it is chromosomally located and is widely prevalent. Similar to other class D enzymes, they have a greater affinity for imipenem than meropenem. Their role in carbapenem resistance is related to the presence of an insertion sequence ISAba1, situated upstream, possibly providing a promoter for the hyperproduction of beta-lactamase genes. Strains that harbour multiple OXA encoding genes have been reported from several geographical

areas. The antibiotic treatment of *Acinetobacter* spp. is limited because of native resistance to many class of antibiotics[87].

Since the acquisition of multidrug resistant *A. baumannii* is related to environmental contamination and colonization in health care providers, control measures should address the source of infection. Continued careful attention to hand hygiene, contact isolation, barrier precautions, adequate environmental cleaning, and careful disinfection of patient care equipments along with surveillance are essential to prevent the outbreak of infections caused by these multidrug resistant strains[87].

The most consistently effective antibiotics are the carbapenems, the sulbactum component of ampicillin-sulbactum, and the polymyxins[71,72].

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an aerobic nonfermenting Gram-negative bacillus and is intrinsically resistant to many classes of antibiotics.. Pseudomonas has numerous virulence factors, including many that appear to facilitate lung infection. The most important are a family of secreted exotoxins (ExoS, ExoT,ExoU [PepA], and ExoY) that are injected directly into the cytoplasm of host cells, using the so-called type III secretion system[5,23].

Pneumonia is generally diffuse and bilateral, with minimum pleural effusion, although sometimes empyema can be developed. Generally, the findings in the chest X-ray are non-specific, but a few differences exist. The major distinguishing radiographic factor is development of a lung abscess. Nodular bilateral lesions, predominantly in inferior lobes, and it is such a characteristic image that it should make the presence of *Pseudomonas aeruginosa* suspected.

Pseudomonas aeruginosa has the capacity to readily develop resistance to all known classes of antibiotics, and resistance can develop in 30-50% of patients

currently receiving monotherapy, but no data show that this problem can be avoided by the use of combination therapy[73,74]. A prospective study of an aminoglycoside added to a carbapenem did not show improved outcome or a difference in the rate of developing resistance during therapy, when compared with monotherapy with a carbapenem[74]. Where as a quinolone could be an alternative to aminoglycoside, with the theoretic advantage of improved respiratory penetration, no prospective study has compared a fluoroquinolone based combination therapy with -lactam monotherapy.

Enterobacteriaceae

The Enterobacteriaceae, or enteric Gram-negative bacilli, are a group of aerobic lactose-fermenting Gram-negative bacilli that normally reside in the lower gastrointestinal tract. Antibiotic therapy and critical illness can suppress the normal bacterial flora and lead to an overgrowth of Enterobacteriaceae in the gut and colonization of the skin and the upper gastrointestinal and respiratory tracts. Individual members of this genus have unique intrinsic antimicrobial susceptibility patterns, but the most concerning development has been the acquisition of extended-spectrum -lactamases that render the bacteria resistant to penicillin and cephalosporin antibiotics[5].

The hallmark of ESBL producing Enterobacteriaceae is a variable response to cephalosporins and thus third-generation agents should be avoided as monotherapy when these pathogens are suspected or isolated[75]. A reliable choice is carbapenem, which is generally active against these organisms[76]. Because these microorganisms are also likely to demonstrate resistance to aminoglycosides and fluoroquinolones, the benefit of combination therapy is uncertain. Piperacillin-tazobactam has been used for the treatment of VAP, but its efficacy against ESBL-organisms is uncertain and should be used with caution and at adequate doses[77].

Methicillin resistant *Staphylococcus aureus*:

Staphylococcus aureus is a gram-positive cocci that frequently colonizes the anterior nares and is consistently one of the most important causes of nosocomial infection and of VAP. Staphylococci cause VAP throughout the course of critical illness. Proven risk factors for VAP caused by methicillin-sensitive *S. aureus* include younger age, traumatic coma, and neurosurgical problems. Risk factors for VAP caused by MRSA include COPD, longer duration of mechanical ventilation, prior antibiotic therapy, prior steroid treatment, and prior bronchoscopy[5].

Although vancomycin has been accepted as standard therapy for this pathogen, both industry-sponsored clinical trials and studies from individual centers have consistently reported clinical failure rates of 40% or greater with a standard dose (1 g every 12 hourly) of vancomycin for MRSA pneumonia[78]. Combination therapy with other agents, such as rifampin[79], aminoglycosides, and others, has been tried but no prospective clinical data have documented the value of this approach.

Two large multicentre trials of linezolid demonstrated equivalence to vancomycin in patients with hospital acquired pneumonia[80]. When the two studies were combined and analyzed by multivariate techniques, linezolid was found to have a significant association with both clinical cure and lower mortality, especially for patients with ventilator associated pneumonia due to MRSA[81].

Metallo- -lactamase producing *Pseudomonas aeruginosa*

The emergence of multidrug resistance among gram-negative bacteria is a notable threat. Clinically relevant species of gram negative bacilli are often resistant to -lactam antibiotics, including extended spectrum cephalosporins, but rarely to carbapenems. Carbapenems are often used as last resort antibiotics for treating

infections caused by multidrug-resistant gram-negative bacilli, as they are stable and respond only to extended spectrum and AmpC -lactamases.

However, emergence of acquired carbapenemases, particularly Ambler class B metallo- -lactamases (MBLs), IMP and VIM, have been increasingly reported in Asia, Europe, Canada and in many geographical locations. Another type (SPM-1) has been reported in South America. Five enzymes have been identified (IMP, VIM, SPM, GIM and SIM types) in various host organisms, the most common ones being found in *Pseudomonas* spp. and *Acinetobacter* spp. *Pseudomonas aeruginosa* producing metallo- -lactamases (MBLs), was first reported from Japan in 1991 and then the resistance spread to other species[96].

Gram-negative bacilli can develop resistance to beta-lactam antibiotics by three mechanisms: 1) alteration of the antimicrobial target receptor molecule in the bacteria; 2) decreasing the accessibility of the antimicrobial to the target by altering the entry of the antimicrobial into the cell or increasing the removal of the antimicrobial from the cell; 3) destruction or inactivation of the antimicrobial. The ability of bacteria to produce beta-lactamase enzymes is the major cause of resistance to beta-lactam antibiotics. These enzymes hydrolyze four-membered beta-lactam rings, converting them into beta-amino acids, that unlike active members within this class of antibiotics, no longer interfere with bacterial cell wall synthesis and growth.

The -lactamase enzymes fall into four classes on the basis of their sequence homology, or on the basis of their substrate spectrum and responses to inhibitors into a larger number of functional groups. In the Ambler classification, class A, C and D enzymes employ serine as the reactive site to attack the -lactam bond of penicillins, cephalosporins and carbapenems. These enzymes cleave the amide bond of the -lactam ring thus inactivating the antibiotic, while class B (metallo- -lactamases)

requiring zinc ions for their activity. Metallo- -lactamases (MBLs) catalyze the identical (as serine enzymes) chemical reaction, using one or two divalent cations (Zn^{2+}) coordinated to two water molecules as the reactive nucleophiles (Figure 2).

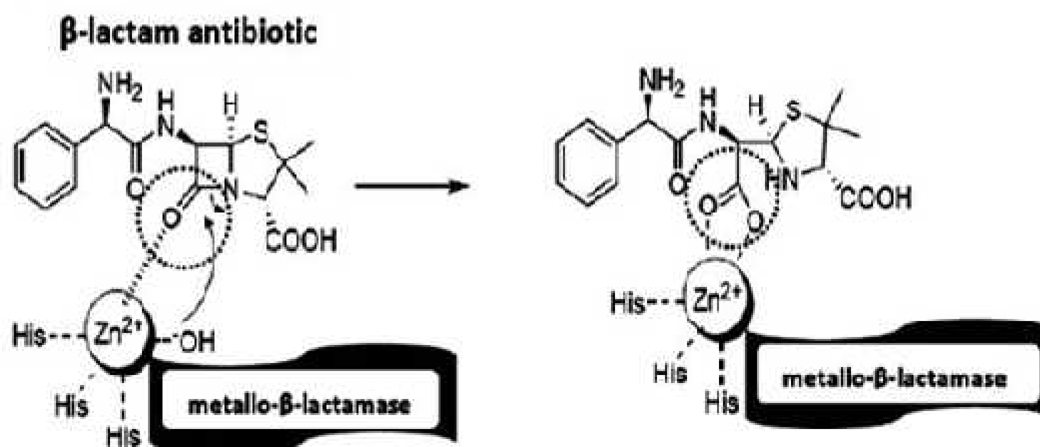

Figure 2 : Mechanism of the hydrolysis of -lactam antibiotics through metallo- -lactamases[98]

MBLs are produced by bacteria as extracellular or periplasmatic enzymes. All known representatives' posses conserved metal binding sites and require zinc ions as enzymatic cofactors. These enzymes can degrade all of -lactam antibiotics except monobactams and are special constant and efficient carbapenemases activity. Moreover, metallo- -lactamases are not susceptible to therapeutic -lactamase inhibitors. Their increasing emergence in pathogenic bacterial strains (particularly *P. aeruginosa* and Enterobacteriaceae) due to a rapid dissemination by horizontal gene transfer induced a growing interest in this enzyme family because of the lack of efficient therapies to treat infected patients [98].

Acquired drug resistance is frequent in nosocomial isolates of *P. aeruginosa* and often involves more than one antimicrobial class. Acquired metallo- lactamases (MBL) have recently emerged as one of the most worrisome resistance mechanisms

owing to their capacity to hydrolyze with the exception of aztreonam, all -lactams including carbapenems and also because their genes are carried on highly mobile elements, allowing easy dissemination. Such strains are not susceptible to therapeutic serine lactamase inhibitors (such as clavulanate and sulfones)[82,83,84].

MBL producing isolates are also associated with a higher morbidity and mortality. Moreover, given that MBLs will hydrolyze virtually all classes of -lactams and that we are several years away from the development of a safe therapeutic inhibitor; their continued spread would be a clinical disaster.

Resistance to carbapenems in *P.aeruginosa* may develop due to impermeability, which occurs due to the loss of the opr D porin, the up regulation of an active efflux system present in these organisms, or the production of MBLs. Carbapenem hydrolysing MBLs have been reported in several countries and have emerged as the most important mechanism of carbapenem resistance[82,84].

Currently, no standardized method for MBL detection has been proposed. Several nonmolecular techniques have been studied, all taking advantage of the zinc dependence of the enzymes, by using chelating agents such as EDTA or 2-mercaptopropionic acid to inhibit their activities.

Screening for MBL production is done in imipenem resistant isolates by the following methods[84,86,88]:

Imipenem (IMP)-EDTA combined disk test

The IMP-EDTA combined disk test is performed as described by Yong *et al* . Test organisms are inoculated on to plates with Mueller Hinton agar as recommended by the CLSI. Two 10 µg imipenem disks are placed on the plate, and appropriate amounts of 10 µL of EDTA solution were added to one of them to obtain the desired concentration (750 µg). The inhibition zones of the imipenem and

imipenem-EDTA disks are compared after 16 to 18 hours of incubation in air at 35°C. In the combined disk test, if the increase in inhibition zone with the Imipenem and EDTA disk was 7 mm than the Imipenem disk alone, it is considered as MBL positive.

Imipenem-EDTA double disk synergy test (DDST)

The IMP-EDTA double disk synergy test is performed as described by Lee *et al*. Test organisms are inoculated on to plates with Mueller Hinton agar as recommended by the CLSI. An imipenem (10 µg) disk is placed 20 mm centre to centre from a blank disk containing 10 µL of 0.5 M EDTA (750 µg). Enhancement of the zone of inhibition in the area between imipenem and the EDTA disk in comparison with the zone of inhibition on the far side of the drug is interpreted as a positive result.

EDTA disk potentiation using cephalosporins (e.g. ceftazidime, ceftizoxime, cefepime and cefotaxime)

Test organisms are inoculated on to plates with Mueller Hinton agar as described for the standard disk diffusion test. A filter paper (Whatmann No. 2) blank disk is placed and the following disks [ceftazidime (30 µg), ceftizoxime (30 µg), cefotaxime (30 µg), cefepime (30 µg)] are placed 25mm center to center from the blank disk. Ten microlitre of 0.5 M EDTA solution is added to the blank disk and the plate are incubated overnight at 35º C. Enhancement of the zone of inhibition in the area between the EDTA disk and any one of the four cephalosporin disks in comparison with the zone of inhibition on the far side of the drug is interpreted as a positive result.

Modified Hodge test[88]

10 µg meropenem disk is placed in the centre of the plate and 10 µl of 50mM zinc sulfate solution is added to the disk along with EDTA. Carbapenem resistant isolates is streaked with good inoculum of 0.5cm around the meropenem starting from the disk to outwards in four different directions. The plates are incubated at 37°C overnight; zone around the meropenem disk with clover leaf indent is taken as positive test.

MBL E-test

The E Test MBL strip containing a double sided seven-dilution range of IPM (4 to 256 µg/mL) and IPM (1 to 64 µg/mL) in combination with a fixed concentration of EDTA has been reported to be the most sensitive format for MBL detection. The E-test is done according to manufacturer's instructions. MIC ratio of IP (Imipenem)/IPI (Imipenem-EDTA) of >8 or >3 $\log_2$ dilutions indicates MBL production. The commercially available MBL-E test is simple to perform, but is highly insensitive at detecting carbapenem-susceptible MBL carrying organisms and is costly.

Molecular detection

The genetic techniques used to detect MBLs like PCR and DNA probing, while sensitive, are based on the presumption that the clinical isolates produce a related MBL gene, which may or may not be the case. These methods will not indicate the type of variant that is present, which will require sequencing. As most MBLs are strongly linked to class 1 integrons, the genetic elements themselves could be amplified and sequenced, thereby giving information on the structural gene and its adjacent DNA. However, these methods, rather like gene cloning, are highly specialized and beyond most clinical laboratories[83].

In a study by Behera B et al, Imipenem-EDTA combined disk test and imipenem-EDTA MBL E test were found to be equally effective for MBL detection, however, given the cost-constraints, Imipenem-EDTA combined disk test could be used as a convenient screening method in the clinical microbiology laboratories[84].

In an Indian study by Dey et al, Metallo-beta lactamases (MBLs) were produced by 50% of *Pseudomonas aeruginosa* and 21.74% of Acinetobacter spp[3].

In another study by Yong D et al, Imipenem-EDTA combined disc test differentiated all MBL-producing pseudomonads, and the sensitivity and specificity for Acinetobacters were 95.7 and 91.9% respectively[86].

METHODOLOGY

The present study was conducted at the Department of Microbiology, JNMC, Belgaum.

Source of Data : Patients with clinical suspicion of ventilator-associated pneumonia in MICU at KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum.

Study design : A one year cross sectional study.

Sample size : Sample size: **:** 53

The formula used was $n = 4pq/d^2$; where n = sample size

P = prevalence = 52%

q = 100-p

d = 15% of error

Statistical methods used for analyzing the data:

1. Percentage of isolate of aerobic bacteria in patient were compounded.

2. Percentage of patients susceptible for different antibiotics were compounded

Inclusion criteria: All the patients 18 years and above that were under mechanical ventilation for more than 48 hours and clinically suspected of having contracted VAP ; were included in this study.

VAP is defined as new pulmonary infiltrate (or radiologically confirmed worsening of new pre-existing infiltrate), together with atleast two of the following criteria:

Leucocytosis (>12,000/mm3)

Leucopenia (<4000/mm3)

Fever (38ºC)

Hypothermia (35ºC)

Purulent tracheal secretions

Exclusion criteria: All patients with clinical and radiological signs suggestive of pneumonia on admission.

DATA COLLECTION:-

Clinically diagnosed ventilator-associated pneumonia were observed and data such as age, gender, date of admission into and discharge from the intensive care unit, risk factors involved, underlying diseases, date of intubation/ tracheostomy, duration of mechanical ventilation etc. (copy of Proforma enclosed) were obtained. Time period of intensive care unit stay prior to initiation of ventilation, duration of intensive care unit and hospital stays, were also recorded. Days of antibiotic therapy and a short description of radiological findings were recorded.

Collection of endotracheal aspirates (ETA)

Endotracheal aspirate (≥1ml) was collected under aseptic precaution in patients with clinical suspicion of ventilator-associated pneumonia in MICU. The ETA was collected using a 22-inch Ramson's 12 F suction catheter with a mucus extractor, which was gently introduced through the endotracheal tube for a distance of approximately 25-26 cm. Chest vibration or percussion for 10 min was used to increase the retrieved volume (1 mL) in case the patient produced very little secretions.

DIRECT SMEAR EXAMINATION:

Smear was made on a clear glass side and was fixed by flaming over the Bunsen burner. After fixing, Gram staining was done for the smear and was examined under low power and oil immersion objective. Criteria used to reject endotracheal aspirate from patients

1) >10 squamous epithelial cells per low power field or

2) No organisms seen under oil immersion in the entire field.

Days **Procedure**

Day 1

Gram staining done

↓

Homogenize by vortexing for 1 minute with glass beads

↓

Centrifuge at 3000rpm for 10 minutes

↓

Serially dilute in 0.9% sterile saline solution with final dilutions of 10^{-1} ,10^{-2}, 10^{-3}, 10^{-4},10^{-5},10^{-6}

↓

10µl of diluted sample inoculated on Blood agar, Chocolate agar and MacConkey agar medium

↓

Incubate at 37ºC for 18-24 hours
(Chocolate agar plate will be incubated in CO_2)

↓ Observed for growth

↓ Number of colonies counted, Multiplied by Dilution factor, Expressed as CFU/ml.

Day 2

1) Colony characters observed
2) Smear for gram stain done
3) Hanging drop for motility
4) Tests for enzymes like Catalase, Oxidase, Coagulase
5) Biochemical tests done
6) Sugar fermentation test- glucose, lactose, sucrose ,maltose, mannose, arabinose, xylose

Antibiotic susceptibility testing done by using Kirby Bauer disc diffusion method on Mueller-Hinton agar plate.

Day 3 Observed the biochemical tests, sugar fermentation and sensitivity pattern were read.

Metallo- -lactamase producing *Pseudomonas aeruginosa* is detected by Imipenem-EDTA combined disc test.

GRAM NEGATIVE BACILLI: For lactose fermenting and non-lactose fermenting colonies, further Gram staining, Hanging drop for motility and catalase and oxidase tests were done. After which the following biochemical tests were done. They include-

- Indole test
- Methyl Red
- Voges Proskauer
- Citrate
- Urease
- Nitrate reduction
- Phenylalanine dehydrogenase
- Mannitol motility
- Triple sugar iron
- Oxidative-Fermentative glucose
- Oxidative-Fermentative lactose (1% and 10%) concentration
- Sugar fermentation

GRAM POSITIVE COCCI: For gram positive bacteria that are gram-positive cocci-catalase test, oxidase tests were done. For cocci in clusters oxidase negative, catalase positive, slide and tube coagulase tests were done. The colonies which were seen on the aerobic culture plates were examined in detail by the methods specified by Mackie and McCartney[25].

INTERPRETATION OF QUANTITATIVE CULTURE RESULTS

The diagnostic threshold for ETA was taken as 10^5 cfu/ml. Growth below the threshold was assumed to be due to colonization or contamination.

1. Gram's stain : A thin smear from a single colony was made on a glass slide and fixed by flaming over the Bunsen burner. After fixing crystal violet was poured over the smear, care was taken to completely cover the smear, it was allowed to stand for 1min, washed with tap water. Then Gram's iodine was poured over the slide and kept for one minute, washed with water and was decolorized by 95% alcohol till the blue color of crystal violet disappeared. The slide was washed with water and counter stained by safranine for 1min. Then the slide was washed with water, dried and was observed under oil immersion objective. In all those smears, gram negative bacilli were seen, further motility of these organism were noted by hanging drop method[25].

Quality control :

Positive control: *Staphylococcus aureus* ATCC 25923

Negative control: *Escherichia coli* ATCC 25922

2. Hanging drop: For this, a small amount of paraffin wax was placed around the lip of the well on the concavity slide. A smooth saline suspension of an individual colony was prepared with the help of a clean sterile loop and loopful of the material was placed on a cover slip. The slide was inverted and pressed over the cover slip, the

guiding the drop of bacterial suspension in to the centre of the well. The slide was carefully examined by hanging drop method to know the motility of the different gram negative bacilli. In doubtful cases, motility was confirmed by inoculating onto semisolid media.

One peptone water tube was inoculated with the growth. Care was taken to select individual discrete colonies. The tube was kept at 37° for 2 hours and turbidity was compared with 0.5 McFarland standards. It was used for sugar fermentation tests, biochemical tests and also for antibiotic sensitivity[25].

BIOCHEMICAL TESTS:

3. Indole test:

Principle: This test was done to demonstrate the ability of certain bacteria to decompose the amino acid tryptophan in to indole. Here tryptophan rich medium was used**.**

Procedure: Kovac's reagent method was employed.

Kovac's reagent- preparation,

Amyl or isoamyl alcohol	150ml,
-dimethylaminobenzaldehyde	10gm,
concentrated HCL	50ml.

Individual colonies were inoculated on to tryptophan broth were incubated at 37°C for 18-24hrs. To this 0.5ml of Kovac's reagent was added and gently shaken.[98]

Interpretation : Appearance of red color was taken as indole producer.

Quality control :

Positive control: *Escherichia coli* ATCC 25922

Negative control: *Klebsiella pneumoniae*.

4. Urease test :

Principle : This was done to determine the ability of bacteria to decompose urea in ammonia. Here Christensen's urea agar medium was used.

Procedure: Inoculate heavily over the entire surface of Christensen's urea agar medium with the peptone water culture and incubate at 37°C. Examine after 4hrs and then overnight incubation[82].

Interpretation: Positive: when the indicator turned to purple-pink

Negative: no change in colour.

Quality control:

Positive control: *Proteus species*,

Negative control: *Escherichia coli.*

5. Citrate utilization test:

Principle: To determine the ability of bacteria to utilize citrate as sole source of carbon for its growth. Simmon's citrate medium was used to know the utilization of citrate.

Procedure: The citrate slant was inoculated with the suspected single colony and medium was incubated at 37°C for 24 to 48hrs. A positive reaction was indicated by blue colour and streak of growth. A negative reaction is indicated if original green color and no growth is observed.

Quality control:

Positive control: *Enterobacter aerogenes*,

Negative control*: Escherichia coli.*

6. Triple sugar iron agar test:

Principle: This was done to determine the ability of bacteria to ferment carbohydrates incorporated in a growth medium and production of hydrogen sulfide**.** Triple sugar iron (TSI) agar medium contains 10 parts Lactose, 10 parts sucrose, 1 part glucose

and peptone. Phenol red and ferrous sulphate serve as indicators of acidification and H_2S production respectively. With a sterile straight inoculating wire, touch the top of a well-isolated colony.

Procedure: Inoculate TSI by first stabbing through the centre of the medium to the bottom of the tube and then streaking the surface of the agar slant. Incubate the tube at 37°C for 18 to 24 hours the results were interpreted as follows.

Interpretation :

Slant/butt	**Color**	**Utilization**
Alkaline slant/No change in butt (K/No change)	Red/No Change	Glucose, lactose, sucrose non-utilizers.
Alkaline slant/Acid butt(K/A)	Red/Yellow	Glucose only fermented; peptones utilized
Acid slant/Acid butt(A/A)	Yellow/Yellow	Glucose fermented, lactose and/or sucrose fermented
Alkaline slant/Alkaline butt (K/K)	Red/Red	No fermentation of glucose, lactose or sucrose. Peptones utilized

A black precipitate in the butt indicates production of ferrous sulphide and H_2S gas (H_2S^+). Bubbles or cracks in the media indicate the production of CO_2 or H_2[82].

7. Methyl Red test :

Principle: To determine the ability of bacteria to produce and maintain the low pH after prolonged incubation.

Procedure: Inoculate the glucose phosphate peptone water medium with a young culture and incubate at 37°C for 48 hrs. To this, add about five drops of the methyl red reagent. Mix and read immediately. Positive tests are bright red and negative tests are yellow.

Quality control:

Positive control: *Escherichia coli* .

Negative control: *Enterobacter aerogenes*

8. Voges Proskauer test :

Principle: This test was done to determine the ability of an organism to produce neutral end products like acetyl methyl carbinol or it reduction product 2,3 butylene glycol from glucose fermentation.

Medium: Glucose phosphate peptone water.

Procedure: Inoculate the glucose phosphate peptone water medium with a young culture, and incubate at 37°C for 48hrs, to this, add 1ml of 40% potassium hydroxide and 3ml of solution of -naphthol in absolute ethanol.

Interpretation : A positive reaction is indicate by the development of a pink color in 2-5min, becoming crimson in 30 min.

Quality control:

Positive control: *Enterobacter aerogenes,*

Negative control: *Escherichia coli.*

9. Sugar fermentation test:

Principle: This test was done to determine the ability of an organism to ferment a specific carbohydrate that is incorporated in a basal medium, thereby producing acid with or without visible gas.

Procedure: The test was performed on conventional culture media with test sugar. The common sugar fermentation media used for present study were glucose, sucrose, lactose, maltose, mannose, arabinose and xylose. From the peptone water tube (which was incubated for 2hrs. after inoculation) all the sugar fermentation media were inoculated with the help of a clean sterile loop. Care was taken to sterilize the loop

every time, after dipping in different sugars. After different media were inoculated, these were incubated at 37°C for 18-24hrs. After 24hrs, the sugar media were examined for the production of acid indicated by pink color and gas (presence of an air bubble inside the durham's tube).

Interpretation: Positive test in indicated by change in color to pink with or without gas formation in durham's tube. Negative test is indicated by growth, but no change in colour[25].

10. Oxidation/ Fermentation test (Modified Hugh and Leifson) :

Principle: This test was done to know of the organism uses carbohydrate substrate to produce acid byproducts either oxidative or fermentative.

Procedure: Hugh-Liefson's basal medium prepared and carbohydrate to be added was sterilized separately and added to give final concentration of 1%. The medium was then tubed to a depth of about 4cm.

Duplicate tubes of medium were inoculated by stabbing. One tube was promptly covered with a liquid paraffin to a depth of 1cm and were incubated at 37°C for 18-24hrs[82].

Interpretation:

- Fermenting organisms produce an acid reaction throughout the medium is covered (anaerobic) as well as the open (aerobic) tube.
- Oxidizer produces an acid reaction only in the open tube.
- Organisms that cannot breakdown the carbohydrate aerobically or anaerobically produce an alkaline reaction in the open tube and change in covered tube.

11. Nitrate Reduction test:

Principle : The test is used to determine the ability of an organism to reduce nitrate. The reduction of nitrate to nitrite is determined by adding sulfanilic acid and alpha-napthylamine. The sulfanilic acid and nitrate react to form a diazonium salt. The diazonium salt then couples with -napthylamine to produce a red, water soluble azo dye[82].

Procedure : This liquid medium was inoculated with the suspected single colony and the medium was incubated for 18-24hrs.

Add 0.1ml of the test reagent to the test culture. The test reagent was prepared by mixing equal volumes of solution A (8.0gm of sulphanilic acid in 1liter of acetic acid 5mol/liter) and solution B (5.0gm of -napthylamine in 1 liter of acetic acid 5mol/liter). A red color developing within a few minutes indicates the presence of a nitrate. No colour indicated that nitrate have not been reduced or reduced to product other than nitrites, such as ammonia, molecular nitrogen, nitric oxide or nitrous oxide and hydroxylamine. Addition of zinc dust to all negative tests was necessary. Zinc ions reduced nitrates to nitrites and development of red colour after addition of zinc dust indicated the presence of residual nitrates[82].

Quality control:

Positive control: *Escherichia coli*

Negative control: *Acinetobacter baumannii*

12. Phenylalnine Deaminase test (PPA) :

Principle : To determine the ability of bacteria to deaminate phenylalanine to phenyl pyruvic acid.

Procedure : This test was done to know the ability of the organism to deaminate phenylalanine with the production of phenyl pyruvic acid, which reacts with ferric

salts to give green color. Inoculated the agar slope medium containing DL-phenylalanine with a fairly heavy inoculum and incubate 37°C for 18-24hrs. After incubation, allow a few drops of 10% solution of ferric chloride to run down over the growth on the slope if the test is positive, a green color will develop in the fluid and in the slope.

Quality control:

Positive control: *Proteus species.*

Negative control: *Escherichia coli.*

13. Amino acid decarboxylase and Arginine dihydrolase test:

Principle: Decarboxyases are a group of substrate- specific enzymes that are capable of reacting with the carboxyl portion of amino acids, forming alkaline-reacting amines. The conversion of arginine to citrullin is a dehydrolase, in which an NH_2 group is removed from arginine.

Procedure : Here Moellar decarboxylase broth base was used. Test organism was inoculated in the medium with straight wire then overlay tubes with sterile mineral oil to cover about 1 cm of the surface. Incubate and read daily for 4 days. The medium first becomes yellow due to acid production during glucose fermentation later if decarboxylation occurs, the medium turns to violet colour.

Quality control :

Positive control: *Pseudomonas aeruginosa.* ATCC 27853

Negative control: *Klebsiella pneumoniae*.

14. Catalase test :

Principle: The enzyme catalase mediates the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water. The presence of the enzyme in bacterial isolates is

evident when a small inoculum is introduced into H_2O_2, and the rapid elaboration of oxygen bubbles occur. The lack of catalase is evident by a lack of or weak bubble.

Procedure: Presence of catalase was demonstrated by test tube method. A small amount of the culture to be tested was picked from a nutrient agar plate with a clean sterile platinum loop or a clean, thin glass rod and was inserted in to 3% hydrogen peroxide solution held in a small, clean tube.

Interpretation : The production of gas bubbles from the surface of the solid culture material indicates a positive reaction and negative reaction when there were no gas bubbles[82].

Quality control :

Positive control: *Staphylococcus aureus*

Negative control: *Streptococcus pyogenes*

This test was used to differentiate *Staphylococcus spp.* from *Streptococcus spp*.

15. Oxidase test :

Principle : To determine the presence of an enzyme oxidase, which catalyse the transport of electrons between electron donors in the bacilli and redox dye. The dye is oxidized to indophenol blue producing deep purple colour.

Procedure : Wet filter paper method was used for this test. Strips of Whatman's No. 1 filter paper was soaked with a little freshly made 1% solution of tetramethyl-para-phenylene-diamine dihydrochloride and then with a help of sterile glass rod a single colony from the medium was rubbed over the strip.

Interpretation: A positive reaction was indicated by an intense deep purple blue, appearing within 5-10 seconds and a negative reaction by absence of colouration or by colouration later than 60seconds.

Quality control:

Positive control: *Pseudomonas aeruginosa ATCC 27583*

Negative control: *Escherichia coli ATCC 25922*

16. Coagulase test:

This was done to differentiate between the *Staphylococcus* species.

Both slide coagulase test and tube coagulase test was done.

a) Slide Coagulase test :

Principle : Staphylococcal coagulase is a protein that has prothrombin like activity which can convert fibrinogen to fibrin. A visible clot will result. Slide coagulase test detects bond coagulase which is attached to the bacterial cell wall and is not present in culture filtrate. Fibrin strands are formed between the bacterial cells when suspended in plasma (fibrinogen), causing them to clump into visible aggregates.

Procedure : A smooth milky suspension of the growth was made in normal saline over a clean glass slide. Make similar suspension of control positive and negative strains to confirm the proper reactivity of the plasma. To the test suspension a loop full of undiluted human plasma was added and the suspension was observed for the appearance of coarse clumps.

Interpretation : Read as positive when a course clumping of cocci was visible to the naked eye within10 seconds. Read as negative when there was absence of clumping of any reaction within 10 seconds or slow reaction was seen after 10 seconds. Negative and slow reacting stains were re-examined by the tube test.

b) Tube Coagulase test :

Principle: Detects free coagulase, a thrombin like substance, present in culture filtrate. Free coagulase reacts with serum substance (coagulase reacting factor) to form a complex that, in turn, reacts with fibrinogen to produce the fibrin clot.

Procedure : Prepare a 1 in 6 dilution of the plasma in saline (0.85% NaCl) place 1ml volumes of the diluted plasma in a small tube. Emulsify a colony of the *Staphylococcus* under test in a tube of the diluted plasma. With each batch of test include positive and negative control, a tube of unseeded diluted plasma to confirm than it does not clot spontaneously. Incubate the tube at 37°C preferable in a water bath, for upto 4hrs. Examine at 1, 2 and 4hrs for clot formation by tilting the tube through 90°C. Leave the negative tubes at room temperature over night and reexamine[82].

Interpretation:

Positive: Any degree of visible clot formation or stiff gel formation or if clots was seen floating in the medium.

Negative: When plasma remains wholly liquid or showed only a flocculent or ropy precipitate.

Quality control:

Positive control: *Staphylococcus aureus*

Negative control: Coagulase negative *Staphylococcus.*

Based on these tests, the following individual organisms were identified with characteristics mentioned below :

Staphylococcus aureus :

They are gram positive, spherical cocci of about 1 micrometer in diameter, arranged in grape like clusters or singly, in pairs and short chains. They are aerobes and facultative anaerobes, with temperature range of 10-42°C, the optimum being 37°C and pH 7.4-7.6. They grow readily on ordinary media forming colonies of 2-4mm diameter, circular, convex, smooth, shiny, opaque and easily emulsifiable. They produce non-diffusible golden yellow pigment. Most of strains are hemolytic.

They are catalase positive and oxidase negative, slide and tube coagulase test positive. They ferment number of sugars, producing acid but no gas. Mannitol is fermented anaerobically.

Klebsiella pneumoniae:

They are gram negative, short, plump, straight rods, about 1-2 x 0.5-0.6 µm in size. They are non-motile, non-sporing and capsulated. They grow well on ordinary media large, opaque, dome shaped mucoid colonies of varying degree of stickiness. They produce bright pink colonies on MacConkey agar due to lactose fermentation. They are catalase positive, oxidase negative, reduce nitrates to nitrites. Indole, MR are negative and VP, citrate are positive. They produce acid/acid on TSI with abundant gas. They are urease positive. They ferment a wide range of carbohydrates including glucose, lactose, sucrose, mannitol, xylose, adonitol and inositol with production of acid and abundant gas. Lysine decarboxylase is positive but ornithine decarboxylase is negative.

Citrobacter freundii:

They are gram negative straight rods, 1 micrometer x 2-6micrometer in size, occur singly or in pairs. They are motile, non-capsulated and non-sporing. They produce small, circular, opaque or translucent colonies on nutrient agar. They produce light pink coloured on MacConkey agar due to late lactose fermentation.

They are catalase positive, oxidase negative, reduce nitrates to nitrites. Indole negative, MR positive, VP negative, citrate positive, H_2S is produced. They ferment glucose, lactose (late), sucrose, maltose, xylose, mannitol with production of acid and gas. They are lysine decarboxylase negative.

Pseudomonas aeruginosa:

They are gram negative, slender bacilli 1.5-3micrometer x 0.5 micrometer in size, actively motile, non-sporing, non-capsulated and non-fermentative.

They are obligate aerobes. Growth occurs at a wide range of temperatures 6-42°C, the optimum being 37°C and the optimum pH is 7.4-7.6. They grow well on ordinary media, producing large, opaque, irregular colonies, with a distinctive, musty or earthy smell with bluish green water soluble pigment. They produce diffuse hemolysis on blood agar.

They are catalase positive, oxidase negative, reduce nitrates to nitrites, indole, MR positive, VP negative and citrate positive. Produce alkaline/no change reaction in TSI medium. Arginine decarboxylase test is positive but lysine and ornithine decarboxylase tests are negative. They are oxidative but not fermentative on O-F test.

Acinetobacter spp.:

They are gram negative or gram variable bacilli or coccobacilli, aerobic, short, stout, non-motile, non-sporing and often capsulated. They grow well on simple media producing colonies which are white or cream coloured, smooth, circular and opaque. They are non-hemolytic on blood agar. On MacConkey agar they produce a faint pink tint. The following are the characteristics by which a definitive identification of *Acinetobacter baumannii* can be made:

- Appear as cocci or coccobacilli on gram stain
- Grow well on MacConkey agar (colonies may have a slightly pinkish tint, a helpful characteristic when present)
- Do not produce cytochrome oxidase
- Exhibit rapid utilization of glucose, with production of acid

- Exhibit rapid utilization of 10% lactose, with production of acid
- Are nonmotile
- Are penicillin-resistant
- Growth at 42ºC [82]

They are catalase positive, oxidase negative, do not reduce nitrates to nitrites, indole negative, produce alkaline/ no change in TSI medium. They utilize citrate. They do not ferment sugars. They are only oxidative on O-F test.

Further these bacterial antibiotic sensitivity patterns were examined.

Antibiogram testing:

Antibiotic sensitivity was tested by Kirby-Bauer’s disk diffusion method. Mueller-Hinton agar plate was used. One-two colonies from the culture plate were inoculated into 2 ml of peptone water and incubated at 37°C for 2hours. Turbidity was compared to that of 0.5 McFarland’s standard (1.5 x 10^{8} CFU/ml). A cotton swab was immersed rotated in this inoculum, the swab was then pressed to the sides of the tube so on to remove excess inoculum. The swab was then used to inoculate the plate of Mueller-Hinton agar, in three different directions to ensure an even and complete distribution of the inoculum over the entire plate. The antibiotic disks were applied within 15 minutes of inoculation of plate and the plate inverted for incubated for 18-24 hrs. at 37° .

Commercially obtained Hi-media disks were used. The strength of disks used and their zone size interpretative standards were according to guidelines by CLSI guidelines standards.

If these organisms were not sensitive to any of the drugs, then a second line of antibiotics was put up using the same procedure as observed. The drugs used for gram positive organisms were-

- Penicillin (Pn) (10 Units)
- Erythromycin (E) (15microgram)
- Clindamycin (Cd) (10microgram)
- Cotrimoxazole(CO) (25microgram)
- Cephalexin (Cp) (5microgram)
- Linezolid (Lz) (30microgram)
- Doxycycline (Do) (30microgram)
- Ciprofloxacin (Cip) (5microgram)
- Ceftazidime (Caz) (30microgram)
- Amoxyclav (Amc) (30microgram)
- Vancomycin (Va) (30microgram)

The drugs used for gram negative organisms were :

- Amikacin (Ak) (30microgram)
- Cephalexin (Cp) (5microgram)
- Erythromycin (E) (15microgram)
- Ciprofloxacin (Cip) (5microgram)
- Ceftazidime (Caz) (30microgram)
- Imipenem (I) (10microgram)
- Cotrimoxazole(CO) (25microgram)
- Cefotaxime (Ce) (30microgram)
- Amoxyclav (Amc) (30microgram)
- Piperacillin-tazobactam (Pt) (100 microgram/10microgram)
- Meropenem (Mr) (10microgram)

Methicillin Resistant *Staphylococus aureus* (MRSA):

Disc diffusion test: This test was done to know the methicillin resistant *Staphylococcus* strains. A direct colony suspension of each *S. aureus* isolated was prepared to a 0.5 McFarland standards and placed on Mueller-Hinton agar containing

2-4% NaCl. An Oxacillin (1microgram) disc was placed on the surface and incubated at 33-35°C for 24 hours after incubation, the zone of inhibition was recorded. Zone diameter of lesser or to equal 10mm was considered as resistant, greater or equal to 13mm as susceptible whereas 11-12mm was considered an intermediate.

Detection of metallo- -lactamase producing *Pseudomonas aeruginosa*

MBL screening

Screening for MBL production was done in imipenem resistant isolates by the following method:

Imipenem(IMP)-EDTA combined disk test [84,86]

The IMP-EDTA combined disk test was performed as described by Yong *et al* . Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by the CLSI.

Two 10 µg imipenem disks (Hi-Media) were placed on the plate, and appropriate amounts of 10 µL of EDTA solution were added to one of them to obtain the desired concentration (750 µg). The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 hours of incubation in air at 35°C.

In the combined disk test, if the increase in inhibition zone with the Imipenem and EDTA disk was 7 mm than the Imipenem disk alone, it was considered as MBL positive.

RESULTS

Total number of (Endotracheal aspirate) samples processed – 54

Rejected (endotracheal aspirate) sample -1

One endotracheal aspirate sample was rejected by following Gram stain criteria. (>10 squamous epithelial cells per low power field or no organisms seen under oil immersion field in the entire field).

Table 1: Distribution of cases according to gender (n=53)

Cases	VAP	Percentage (%)
Female	16	30.19
Male	37	69.81
Total	53	100.00

Table shows the sex wise distribution of patients. Out of 53 cases of ventilator- associated pneumonia, 16(30.19%) were female and 37(69.81%) were male.

Table 2: Age-wise distribution of cases (n=53)

Age(Years)	Number of patients	Percentage (%)
18-28	14	26.41
29-38	6	11.32
39-48	8	15.09
49-58	10	18.87
59	15	28.30

Older patients (59 years) were found to be more susceptible (28.30%) to ventilator-associated pneumonia than younger patients.

Table 3: Distribution of cases according to clinical conditions (n=53)

Sl. no.	Clinical conditions	Number of patients	Percentage (%)
1	OP Poisoning	6	11.32
2	Stroke	6	11.32
3	Sepsis (Urosepsis, Postpartum)	4	7.55
4	Congestive cardiac failure	4	7.55
5	COPD/Pulmonary disease	4	7.55
6	Complicated malaria	4	7.55
7	Alcoholic liver disease	3	5.66
8	GB syndrome	3	5.66
9	Viral fever	3	5.66
10	Partial hanging (Suicidal attempts)	2	3.77
11	Malignancy	2	3.77
12	Acute pancreatitis/ARF	2	3.77
13	Hepatic encephalopathy	2	3.77
14	Viral fever with encephalitis	2	3.77
15	Metabolic encephalopathy	2	3.77
16	Snake bite	1	1.89
17	Right lower limb encephalitis	1	1.89
18	Epilepsy	1	1.89
19	Myasthenia gravis	1	1.89

Table 3 shows the clinical spectrum of our cases that includes maximum 6 cases of organophophorus poisoning and stroke.

Table 4: Distribution of cases according to the onset

VAP	No. of patients (n=53)
Early onset VAP	21
Late onset VAP	32

Out of 53 cases, 39.62 % (21/53) were categorized under the early-onset VAP and the remaining 60.38 % (32/53) late-onset VAP.

Table 5: Risk factors identified in VAP patients

Sl. No.	Risk factors	No of patients	Percentage
1	Enteral feeds	20	37.74
2	Alcohol	15	28.30
3	Advanced age	15	28.30
4	Smoking	12	22.64
5	Steroids	12	22.64
6	Reintubation	8	15.09
7	Sepsis	6	11.32
8	Malignancy	1	1.89

Significant risk factors were enteral feeds, alcohol, advanced age, smoking, steroids, reintubation and sepsis.

Table 6: Polymicrobial isolations (n=2)

Sl. No.	Organisms
1	*Citrobacter freundii* (1.8x 10^{7} CFU/ml) and Methicillin-resistant *Staphylococcus aureus* (2.1x10^{6} CFU/ml)
2	*Acinetobacter baumannii* (2.2x10^{6} CFU/ml) and *Pseudomonas aeruginosa* (2.0x10^{5} CFU/ml)

Only two cases out of 53 showed mixed culture isolation

Table 7: Organisms isolated from endotracheal aspirate (n=55)

Sl. No	Organisms	No. of isolates	Percentage (%)
1	*Acinetobacter baumannii*	27	49.09
2	*Acinetobacter* species	1	1.82
3	*Pseudomonas aeruginosa*	17	30.91
4	*Klebsiella pneumoniae*	3	5.45
5	*Escherichia coli*	2	3.64
6	*Citrobacter freundii*	1	1.82
7	*Staphylococcus aureus*	4	7.27
	Total	55	100.00

Overall, most common organism isolated was *Acinetobacter baumannii* (49.09%)

Table 8: Correlation of organism isolated in early and late onset VAP (n=55)

Sl. no	Organisms	No. of isolates	Early	Late
			No. (%)	No. (%)
1	*Acinetobacter baumannii*	27	10(37.04)	17(62.96)
2	*Acinetobacter* species	1	1(100)	0(0)
3	*Pseudomonas aeruginosa*	17	5(29.41)	12(70.59)
4	*Klebsiella pneumoniae*	3	2(66.67)	1(33.33)
5	*Escherichia coli*	2	1(50)	1(50)
6	*Citrobacter freundii*	1	1(100)	0(0)
7	*Staphylococcus aureus*	4	2(50.00)	2(50.00)
	Total	55	22(40)	33(60)

GRAPH 1 :

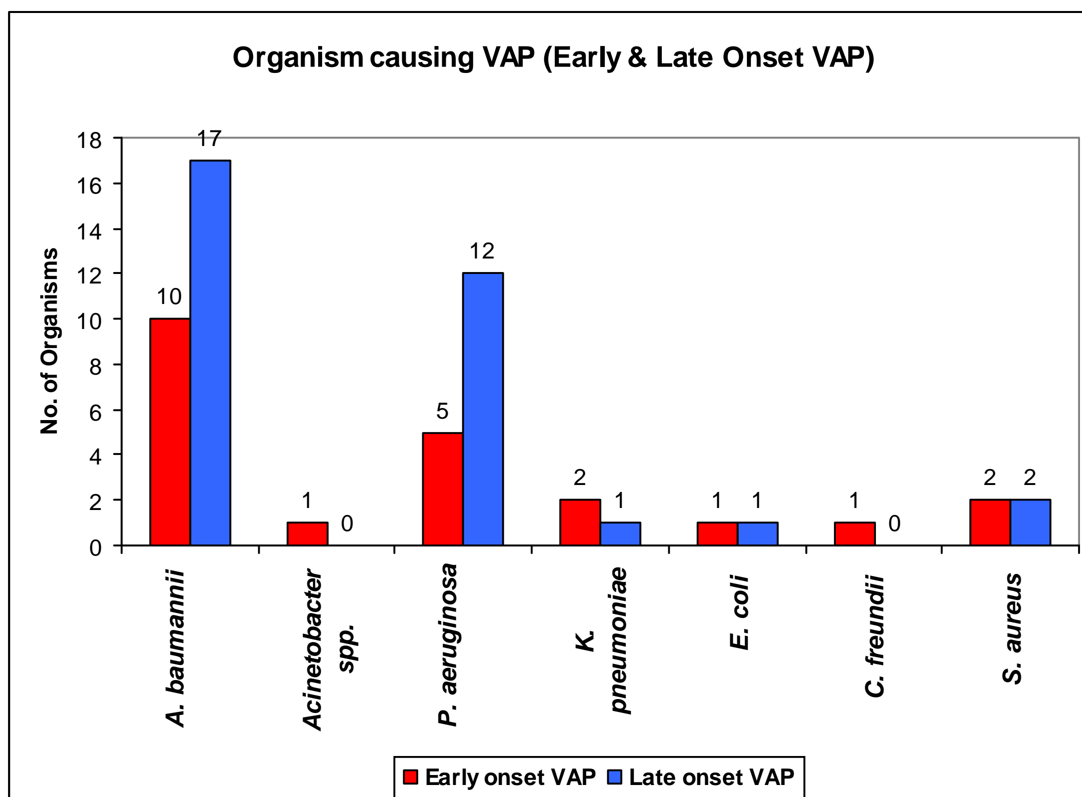

Table 9a : Antibiotic susceptibility pattern of Gram negative isolates:

Isolates	No. of isolates	Cip			CO			Ak			Ca		
		S	I	R	S	I	R	S	I	R	S	I	R
Acinetobacter baumannii	27			**27**	1		**26**	1		**26**	4		**23**
Acinetobacter species	1			**1**			**1**			**1**			**1**
P. aeruginosa	17			**14**			**17**	3		**14**	10	1	**6**
K.pneumoniae	3			**3**			**3**			**3**		1	**2**
E.coli	2			**2**			**2**	1		**1**	1		**1**
C. freundii	1		1				**1**			**1**	1		

*__Cip__ - Ciprofloxacin, **CO**-Cotrimoxazole , **Ak**-Amikacin, **Ca**-Ceftazidime

Table 9b : Antibiotic susceptibility pattern of Gram negative isolates :

Isolates	No. of isolates	I			Cp			Pt			Ao		
		S	I	R	S	I	R	S	I	R	S	I	R
Acinetobacter baumannii	27	2		**25**				17	2	**8**	2		**25**
Acinetobacter species	1			**1**						**1**			**1**
P. aeruginosa	17	7	2	**8**				13		**4**	2		**15**
K.pneumoniae	3	3					**3**	3				1	**2**
E.coli	2	1		**1**			**2**	1		**1**	1		**1**
C. freundii	1	1					**1**	1					**1**

I-Imipenem , **Cp**-Cephalexin, **Pt**-Piperacillin-tazobactam , **Ao**-Aztreonam

Table 9c : Antibiotic susceptibility pattern of Gram negative isolates :

Isolates	No. of isolates	Amc			Do			Ce			Mr		
		S	I	R	S	I	R	S	I	R	S	I	R
Acinetobacter baumannii	27			**1**	15	1	**11**			**1**			**1**
Acinetobacter species	1				1								
P. aeruginosa	17												
K.pneumoniae	3	1	1	**1**									
E.coli	2			**2**									
C. freundii	1			**1**									

Amc-Amoxyclav, **Do**-Doxycycline , **Ce**- Cefotaxime , **Mr**- Meropenem

Table 10a : Antibiotic susceptibility pattern of Gram positive isolates:

Isolates	No. of isolates	Pn		Er		Cp		CO		Cip		Cd	
		S	R	S	R	S	R	S	R	S	R	S	R
S.aureus	4		**4**		**4**	1	**3**		**4**		**4**	1	**3**

***Pn**- Penicillin **, Er**- Erythromycin, **Cp**- Cephalexin, **CO**- Cotrimoxazole , **Cip**- Ciprofloxacin , **Cd**- Clindamycin

Table 10b: Antibiotic susceptibility pattern of Gram positive isolates:

Isolates	Lz		Ac		Ce		Do		Va	
	S	R	S	R	S	R	S	R	S	R
S.aureus	1	**2**		**3**	3		3		3	

Lz-Linezolid, **Amc**- Amoxyclav , **Ce**-Cefotaxime, **Do**- Doxycycline, **Va** - Vancomycin

Table 11: MRSA producing *S.aureus*:

Total no. of *S.aureus* isolated	MRSA isolated	MSSA isolated
4	3	1

Table12: Metallo-beta lactamase producing *Pseudomonas aeruginosa* isolates:

Total number of *Pseudomonas aeruginosa* isolates	MBL producers	Non MBL producers
17	8(47.06%)	9(52.94%)

Eight isolates (47.06%) of *Pseudomonas aeruginosa* were plasmid-mediated metallo-beta lactamases enzyme producers, which is detected by Imipenem-EDTA combined disk method.

Table 13: Chest X-ray finding in relation to the organism isolated

Chest X-ray findings					
Sl. No	**Organisms**	**No. of isolates**	**Left No. (%)**	**Right No. (%)**	**Bilateral No. (%)**
1	*Acinetobacter baumannii*	27	6(22.2)	10(37.03)	11(40.74)
2	*Acinetobacter* species	1	0	1(100)	0
3	*P. aeruginosa*	17	2(11.76)	5(29.41)	10(58.82)
4	*K. pneumoniae*	3	1(33.33)	2(66.67)	0
5	*E.coli*	2	1(50)	1(50)	0
6	*C. freundii*	1	1(100)	0	0
7	*S.aureus*	4	2(50)	1(25)	1(25)
	Total	55	13	20	22

Table 14: Mortality as per organism isolated (n=55)

Causative agent	Total isolates	Early		Late	
		Survivors (%)	Non-Survivors (%)	Survivors (%)	Non-Survivors (%)
Acinetobacter baumannii	27	6	4	6	11
Pseudomonas aeruginosa	17	5	0	6	6
Klebsiella pneumoniae	3	2	0	0	1
Acinetobacter species	1	1	0	0	0
E.coli	2	1	0	1	0
C. freundii	1	1	0	0	0
Staphlococcus aureus	4	2	0	0	2
	55	18	4	13	20

Table 15: Outcome of Ventilator associated pneumonia as according to cases (n=53)

	Early	Late
Recovered	16(76.19)	13(40.62)
Expired	5(23.80)	19(59.37)
Total	21	32

Table 15 depicts that the mortality was higher in late onset ventilator associated pneumonia group (59.37%) as compared to early onset ventilator associated pneumonia group (23.80%).

DISCUSSION

The present study was carried out in Department of Microbiology, J.N.M.C., Belgaum on 54 patients with clinical suspicion of ventilator-associated pneumonia. We have collected 54 endotracheal aspirate samples from patients admitted to Medical Intensive Care Unit at KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum.

To avoid the risk of sample being contaminated with oropharangeal secretion, we have followed the Gram stain criteria for rejecting the Endotracheal aspirate sample which states that >10 squamous epithelial cells/Low power field or no organism seen in Oil immersion field in the entire field [54,55]. Based on this criteria one endotracheal aspirate sample was rejected.

Out of 53 samples processed it was found that 69.8% of the cases belonged to males and 30.19% were to females. Thus the infection rate was found to be more common in males than in females. Similarly Rello J et al[14] (64%) and Gupta A et al[89] (63.54%) also showed male gender dominance in ventilator-associated pneumonia.

The mean ± SD age of patients was 46.4± 18.45 years (range 18 to 86 years). Bimodal distribution of age was observed with the first peak between 18-28 years of age group (admission diagnosis comprising mainly of organophophorus poisoning, postpartum sepsis and viral fever) and second peak at around 59years of age(28.89%). The findings of our study regarding the patient profile were almost similar to the study conducted in 2003 by Apostolopoulou E et al[90]and Rello J et al[14].

The clinical spectrum of cases indicates that the maximum number of cases enrolled in the study involved organophophorus poisoning and stroke (6 cases each) followed by sepsis, congestive cardiac failure, pulmonary disease and complicated malaria 4 cases each.

Distribution of total number of cases was done depending on the duration of mechanical ventilation into “early-onset VAP” developing within 4 days of intubation and “late-onset VAP” developing after 4 days of intubation. The percentage of patients with early onset VAP was 39.62% and late onset VAP was 60.38%. This is because probability of getting VAP increases with the duration of mechanical ventilation[97].

In one study it was found the risk rises between 1% and 3% for each day the patient requires endotracheal intubation and mechanical ventilation[91].

A study conducted in 23 ICU’s which included 724 critically ill patients found that the frequency of VAP rose from 5% for patients receiving MV for day 1 to 69% for those receiving MV for more than 30 days[2].

The common risk factors for VAP were enteral feeds, alcohol, smoking, steroids, advanced age, reintubation and sepsis.

Samples collected by invasive bronchoscopic methods have been shown to be highly specific in diagnosing VAP. However, bronchoscopy is not accessible in many settings and because of the invasive nature, cost of the bronchoscopy and need of a bronchoscopist for the procedure; samples obtained by non bronchoscopic methods may be useful alternative to bronchoscopy in the diagnosis of VAP.

We have performed quantitative cultures on the endotracheal aspirates with a threshold value of 10^5cfu/ml of QEA. Out of 53 endotracheal aspirate samples processed, 51 showed monomicrobial pathogens. All samples showed two or more different types of colonies but on applying quantitative culture thresholds of 10^5 cfu/ml only significant one type of colony was processed in 51(96.23%) samples. In only two cases (3.77%) we got polymicrobial isolates.

In another similar study 172 episodes of bacteremic nosocomial pneumonia were studied out of which 22 (13%) episodes of lung infections were caused by multiple pathogens[2].

Total 51(92.72%) gram negative organisms and 4(7.27%) gram positive organisms were isolated from 53 endotracheal aspirate samples. Among the gram negative isolates, *Acinetobacter* species (50.91%) was the most commonly isolated pathogen followed by *Pseudomonas aeruginosa* (30.91%). Other gram negative organisms isolated were *Klebsiella pneumoniae* (5.45%), *Escherichia coli* (3.64%) and *Citrobacter freundii* (1.82%). Out of 28 organisms belonging to the genus *Acinetobacter*, 27 isolates were of *Acinetobacter baumannii*. Among gram positive organisms *Staphylococcus aureus* accounted for 7.27%.

Microbial flora of hospitalized and critically ill patients becomes drastically altered within days after admission. In these patients, usually low virulent mixed flora of orophryanx and anaerobic flora of colon become overgrown by endogenous aerobic gram negative bacilli, which can then colonize the airway and lead to lung infection. This may be the reason for increased incidence of gram negative organisms[4]. Earlier reports show that among gram negative organisms, *Pseudomonas aeruginosa* [2,92,97] and *Acinetobacter baumannii* [3] were the commonest causative agent of VAP.

Etiological agents widely differ according to the population of the patients in the intensive care unit, duration of hospital stay and prior antimicrobial therapy. The increase of *Acinetobacter baumannii* infections is due to its great resistance to the environment which enables it to spread, its limited virulence and its extraordinary ability to develop resistance to all the antimicrobials. In addition, it has also been recorded that it can be spread by aerosols [23,93].

Among the early onset VAP common organisms isolated were *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Citrobacter freundii* and *Staphylococcus aureus* while in case of late onset VAP common organisms were *Acinetobacter baumannii, Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* in accordance to other studies [3,12].

Two isolates of *Staphylococcus aureus* were isolated from early-onset VAP. One of them was methicillin-resistant *Staphylococcus aureus*, but MRSA is generally associated with late onset VAP[29].This may be due the fact that this case showed mixed infection with predominant organism being *Citrobacter freundii* (1.8×10^7 cfu/ml) while MRSA was showing 2.1×10^6 cfu/ml in quantitative cultures. Secondly, the patient had history of prior antibiotic therapy which increases risk of MRSA.

Multidrug-resistant (MDR) organisms are a major threat to VAP patients. Among the 27 isolates of *Acinetobacter baumannii*, 1(3.70%) was resistant to all group of antibiotics tested, including carbapenems. Among the 27 isolates of *Acinetobacter baumannii*, 26(96.29%) were resistant to Cotrimoxazole and Amikacin, 25(92.59%) to Imipenem, 23(85.16%) to Ceftazidime, 11(40.74%) to Doxycycline, 8(29.63%) to Piperacillin-tazobactam and 27(100%) resistant to Ciprofloxacin.

The antibiotic resistance patterns of nonfermenters were almost the same in both early- and late- onset VAP. Many of the early-onset VAP cases had the risk factors such as prior antibiotic therapy and current hospitalization for five days or more for infection with MDR pathogens. That could be the reason for almost similar susceptibility pattern of the isolates from early- and late-onset VAP. Even the American Thoracic Society guidelines support the same reasoning by suggesting that patients with early-onset VAP who have received prior antibiotics or who had the prior hospitalization within the past 90 days are at greater risk for colonization and infection with MDR pathogens and should be treated similarly to patients with late-onset VAP[95].

Pseudomonas aeruginosa was resistant to Cotrimoxazole (100%), Aztreonam (88.23%), Ciprofloxacin and Amikacin (82.35%), Imipenem (47.06%), Ceftazidime (35.29%) and Piperacillin-tazobactam (23.53%). Increased resistance might be due to various factors like prolonged usage of antibiotics, prolonged hospital stay or by the liberation of either IMP- type metalloenzymes or carapenemases by *Pseudomonas aeruginosa*.

Out of three isolates of *Klebsiella pneumoniae*, all were sensitive to Imipenem and Piperacillin-tazobactam and were resistant to Ciprofloxacin, Cotrimoxazole and Amikacin while two isolates were resistant to Ceftazidime.

Among four isolates of Staphylococcus aureus, three were methicillin-resistant *Staphylococcus aureus*.

Presently, there is concern about the acquisition of plasmid-mediated metallo-beta lactamases active against carbapenems, penicillins and cephalosporins. In our

study, eight isolates (47.06%) of *Pseudomonas aeruginosa* were plasmid-mediated metallo-beta lactamases enzyme producing strains detected by imipenem-EDTA disc method which corresponds to a study were metallo-beta lactamases were produced by 50% of *Pseudomonas aeruginosa*[3].

In our study, *Acinetobacter baumannii* caused predominantly bilateral and right sided pneumonia whereas *Pseudomonas aeruginosa* caused more of bilateral bronchopneumonia.

Husni et al [94] found X-ray pattern in *Acinetobacter baumannii* VAP is non-specific. In half of the cases it causes lung infiltration and a diffuse bilateral pattern is seen in other half of the cases.

Some enzymes of *Pseudomonas aeruginosa* have invasive properties, causing thrombosis of pulmonary vessels and pulmonary infarction; this produces nodular bilateral lesions, predominantly in inferior lobes, and it is such a characteristic image that it should make the presence of *Pseudomonas aeruginosa* suspected[23].

In the present study it was found that the mortality rate was 45.28% which was in accordance with previous studies such as those by Gupta A et al[89] (46.67%). Mortality of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in comparison to rest of organisms was statistically non-significant ($_{2}^{2}$ =3.238; df=2; p=0.198).

Mortality was influenced by type of organism isolated being highest for infections caused by *Acinetobacter baumannii* (55.56%) and *Staphylococcus aureus* (50%).Gupta A et al [89] documented that mortality was highest for infections caused by *Acinetobacter baumannii* (83.33%) and *Klebsiella pneumoniae* (71.42%).

The rate of mortality was less in early-onset VAP (23.80%) as compared to late onset VAP group (59.37%) and the difference was found to be statistically significant ($_{1}^{2}$=6.473; df=1; p=0.011) which was in accordance to other similar studies [92].

CONCLUSION

A study on microbial etiology of ventilator-associated pneumonia conducted at KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum and Department of Microbiology, JNMC, Belgaum during the period from January 2010 to December 2010, revealed the following findings:

- VAP was found to be more common in males than in females.
- Bimodal distribution of age was observed with the first peak between 18-28 years of age group and second peak at around 59years of age.
- Most of endotracheal aspirate samples showed monomicrobial pathogens at a quantitative cultures threshold of 10^5 cfu/ml.
- The most frequently isolated microorganisms were *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.
- Late-onset VAP was more common than early-onset VAP.
- Among the early onset VAP common organisms isolated were *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Citrobacter freundii* and *Staphylococcus aureus.*
- In case of late onset VAP common organisms were *Acinetobacter baumannii, Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*.
- *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were highly resistant for multiple drugs.
- Almost half *Pseudomonas aeruginosa* isolates were metallo-betalactamases enzyme producing strains detected by Imipenem-EDTA combined disk test.

SUMMARY

54 patients with of ventilator associated pneumonia in MICU at KLES Dr. Prabhakar Kore Hospital and Medical Research Centre admitted over a period of one year between January 2010 to December 2010 were studied.

Late-onset VAP was more common than early-onset VAP. The most common risk factor for VAP was enteral feeds (37.74%) followed by alcohol (28.30%), advanced age (28.30%), smoking (22.64%), steroids (22.64%), reintubation (15.09%) and sepsis (11.32%). The most frequently identified causative organism was *Acinetobacter* species (50.91%) followed by *Pseudomonas aeruginosa* (30.91%), *Staphylococcus aureus* (7.27%), *Klebsiella pneumoniae* (5.45%), *Escherichia coli* (3.64%) and *Citrobacter freundii* (1.82%).

Among the early onset VAP common organisms isolated were *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Citrobacter freundii* and *Staphylococcus aureus* while in case of late onset VAP common organisms were *Acinetobacter baumannii, Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus.*

Among the 17 isolates of *Pseudomonas aeruginosa*, 8 (47.06%) of the carbapenem-resistant strains were MBLs-producing strains.

The bacteriological approach for the management of VAP avoids the problem of overtreatment by separating colonizers from infecting pathogens. A quantitative culture of endotracheal aspirate at a cutoff point of 10^5 cfu /ml of QEA is a practical diagnostic method in clinically suspected ventilator associated pneumonia. It can be easily and repeatedly performed to help clinician in decision making regarding antibiotic use.

Microorganisms responsible for VAP may differ according to the population of patients in the ICU, the duration on mechanical ventilatory support, the duration of hospital and ICU stays. The causative microorganisms of VAP varied markedly across treatment sites, resulting in the need for large-scale variations in antimicrobial prescribing practices. Instead of following general recommendations, antimicrobial prescribing practices for VAP should be based on up-to-date information of the pattern of multiresistant isolates from each institution.

Knowledge of risk factors of VAP and causative microbial flora in a local setting would be important to ensure more effective utilization of antibiotics and thereby, a better outcome. It would also allow formulation of strategies to decrease the incidence of VAP.

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PROFORMA

TOPIC : QUANTITATIVE CULTURES OF AEROBIC BACTERIA AND THEIR ANTIBIOTIC SUSCEPTIBILITY PATTERNS FROM ENDOTRACHEAL ASPIRATES OF PATIENTS WITH VENTILATOR ASSOCIATED PNEUMONIA. A ONE YEAR CROSS-SECTIONAL STUDY.

Case No. : IP No. :

Name: Unit:

Age: Occupation:

Sex: Date of sample collection:

Address: Specimen:

Date of admission: Date of discharge:

Chief complaints:

Present history:

Past history:

Personal history:

Family history:

Treatment history:

Physical examination:

Systemic examination:

CVS

CNS

RS

P/A

Diagnosis at admission:

Days in ICU:

Days with mechanical ventilator:

Investigations:

1. Chest x ray

2. Leukocyte count

3. Temperature

4. pao_2/fio_2

5. Renal function tests

6. Liver function tests

7. Microbiological investigations:

Macroscopic examination:

Direct Smear (Gram stain):

Culture:

Blood agar:

Chocolate agar

MacConkey agar:

Gram staining of smear from colony:

Motility:
Catalase :
Oxidase :

A. Gram positive cocci :
Coagulase :
Mannitol fermentation:
Urease test:
Relevant special investigation:

Identification of isolates:
Antibiogram :

Penicillin G (10U):	Oxacillin(5mg) :
Erythromycin(15µg):	Cephalexin(30µg) :
Vancomycin(30µg) :	Ciprofloxacin (5µg):
Trimethoprim-sulphamethazole(25µg):	Clindamycin(10µg) :
Linezolid(30µg):	Ceftriaxone(30µg) :

MRSA producers – Oxacillin / Methicillin incubated at 30^0C.

B. Gram Negative bacilli :
Nitrate reduction test:
IMViC test :
Urease test :
TSI test :
Sugar fermentation :

Glu	Lac	Suc	Malt	Man	Arab	Xyl

Lysine Decarboxylation
Arginine Decarboxylation
Ornithine Decarboxylation
Relevant special investigation:

Identification of isolates :
Report :
Antibiogram :
Amikacin (30µg):
Cefotaxime (30µg):
Ciprofloxacin (5µg) :
Trimethoprim-sulphamethazole (25µg):
Piperacillin/tazobactam (100µg/10µg) :
Doxycycline (30µg) :
Imipenem (10µg):
Amoxyclav (30µg):
Levofloxacin (5µg):
Aztreonam (30µg):
Ceftazidime (30µg):
Cefoxitin (30µg):

Tests for detection of Extended Spectrum Beta Lactamase:

Potential Disc Diffusion Test:

Tests for detection of AmpC -Lactamase:

AmpC disk test

Tests for detection of Metallo- -lactamase (MBL):

Imipenem-EDTA combined disk test

In hospital mortality:

CONSENT FOR PARTICIPATION IN RESEARCH

We are requesting you to enroll yourself in study titled "**QUANTITATIVE CULTURES OF AEROBIC BACTERIA AND THEIR ANTIBIOTIC SUSCEPTIBILITY PATTERNS FROM ENDOTRACHEAL ASPIRATES OF PATIENTS WITH VENTILATOR ASSOCIATED PNEUMONIA. A ONE YEAR CROSS-SECTIONAL STUDY."** conducted by PG student in Microbiology, Belgaum KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, KLE University, Belgaum, bearing **Reg. No. BI0109002**

You have been requested to participate in research because you are into the study group. During the study you will be asked some questions and you are supposed to answer to the best of you knowledge.

Your participation in research is voluntary. Your decision whether or not to participate in the study will not affect your relationship with Jawaharlal Nehru Medical College. If you decide to participate you are free to withdraw at any time.

The purpose of research is to isolate and identify the aerobic bacteria from clinically suspected cases of ventilator-associated pneumonia and to carry out antibiotic susceptibility testing.

PROCEDURE INVOLVED:

Microbiological study of the material obtained from Endotracheal aspirate will be done to detect the aerobic bacteria causing ventilator-associated pneumonia.

RISKS AND BENEFITS

There are no extra risks involved and benefits are to be evaluated.

PRIVACY AND CONFIDENTIALITY

The only people to know that you are a research subject are members of the research team. No information about you or provided by you during research will be disclosed to others without your written permission, except:

1. In emergency to protect your rights and welfare.
2. If required by law.

AUTHORIZATION TO PUBLISH RESULTS

When the results of the research are published or discussed, in a conference no information will be displayed that would disclose your identity. Any information that is obtained in connection with this study and that can be identified with you will remain confidential.

FINANCIAL INCENTIVES FOR PARTICIPATION

You will not paid / offered any free gifts for participating in the research. You will not be reimbursed for expenses.

I undersigned ____________________________ have been explained in my vernacular language about the study and my participation in the study is voluntary. If I want, I can withdraw at any time. Also I have been given enough time to clear my doubts and rights as study participant.

In case you have any questions about your rights as a study participant, you can contact Dr. V. D. Patil.

CONSENT STATEMENT

Signature or left hand thumb print of participant or legally authorized representative.

Participants Name __________________________ Signature ______________

Witness Name Signature __________________________ Signature ______________

Experimenters Name Signature __________________________ Signature ______________

Date :

Place :

EDTA-added Imipenem disk preparation

A 0.5 M Ethylenediamine tetraacetic acid (EDTA) solution was prepared by dissolving 186.1 g of disodium EDTA.$2H_2O$ (Merck specialties, Worli, Mumbai) in 1000 ml of distilled water and adjusted it to pH of 8.0 by Sodium hydroxide (NaOH). The mixture was sterilized by autoclaving.

10µl of 0.5 M EDTA solution was added to 10µg Imipenem disks (Hi-Media) to obtain a concentration of 750µg. The disks were dried immediately in an incubator and stored at 4°C for 12 weeks in airtight vials without desiccant[86].

MASTER CHART

Sl. No.	IP NO.	Age (Years)	Sex	Direct Gram stain	ETA isolate	DO VAP	Penicillin	Oxacillin	Erythromycin	Clindamycin	Cefotaxime	Linezolid	Vancomycin	Cephalexin	Ciprofloxacin	Cotrimoxazole	Amoxyclav	Imipenem	Amikacin	Aztreonam	Pipercillin-tazobactam	Ceftazidime	Doxycycline	Meropenem	MRSA	MBL	Risk factors	Intubation (No. of times)	Outcome
1	349027	34	M	Ac.	A. bau	Early									R	R		R	R	R	R	S	S				Alc	1	D
2	351205	58	M	Ac.	A. bau	Early									R	R		R	R	R	R	S	S				Alc	1	D
3	352110	55	F	Ac.	MRSA	Early	R	R	R	R	S	R	S	R	R	R	R						S		Pos		EF	1	D
					C. freu									R	I	R	S	S	R	R	S	S						1	
4	361988	84	M	Ac.	A. bau	Late									R	R		R	R	R	S	R	R				Alc	1	E
5	370941	50	M	Ac.	A. bau	Early									R	R		R	R	R	S	R	R				Alc	1	E
6	359460	75	F	Ac.	P. areg	Late									S	R		I	R	R	S	S				P	EF	1	E
7	373058	47	M	Ac.	A. bau	Late									R	R		R	R	R	I	R	S				EF	1	D
8	372725	26	F	Ac.	A. bau	Late									S	S		S	S	S	S	S	S				Negative	1	E
					P.areg										R	R		R	R	R	R	R				NP		1	
9	373942	46	M	**Rej.**																									
10	372508	30	M	Ac.	A. bau	Late									R	S		S	S	R	R	R	S				EF	1	D
11	374738	27	F	Ac.	P.areg	Early									R	R		R	R	R	S	S				P	Negative	1	D
12	370372	54	M	Ac.	K. pneu.	Early								R	R	R	S	S	R	I	S	R					Ster,Alc,Smok	2	D
13	379707	18	F	Ac.	K. pneu.	Late								R	R	R	I	S	R	R	S	I					Ster	2	E
14	381738	45	M	Ac.	E.coli	Early								R	R	R	R	S	R	R	R	S					Alc	1	D
15	383546	24	M	Ac.	P.areg	Early									R	R		S	S	S	S	S				NP	EF	1	D
16	384676	35	F	Ac.	P.areg	Early									R	R		S	R	R	S	S				NP	Negative	1	D
17	384281	48	M	Ac.	S.aureus	Early	R	S	R	S				S	R	R									Neg		Ster	1	D
18	385724	35	M	Ac.	A. bau	Early									R	R		R	R	R	R	S	S				Negative	1	D
19	0387924	68	M	Ac.	P.areg	Late									S	R		R	S	R	S	I				P	Negative	2	E
20	387212	63	F	Ac.	A. bau	Late									R	R		R	R	R	S	R	R				EF	1	E
21	0387924	68	M	Ac.	A. bau	Late									R	R		R	R	R	S	R	R				Alc,Smok	1	E
22	0389167	70	F	Ac.	A. bau	Late									R	R		R	R	R	S	R	R				Negative	1	E
23	0391536	76	F	Ac.	A. bau	Early									R	R		R	R	R	S	R	R				Negative	1	D
24	0390909	56	M	Ac.	A. bau	Late									I	R		R	R	S	S	R	R				Negative	1	E
25	391314	63	M	Ac.	P.areg	Early									S	R		I	R	R	S	R				NP	Alc,Smok,EF	2	D
26	390900	23	M	Ac.	MRSA	Late	R	R	R	R	S	S	S	R	R	R	R						S		Pos		Ster,EF	1	E
27	393378	21	M	Ac.	A. bau	Late									R	R		R	R	R	R	R	S				Ster	1	E

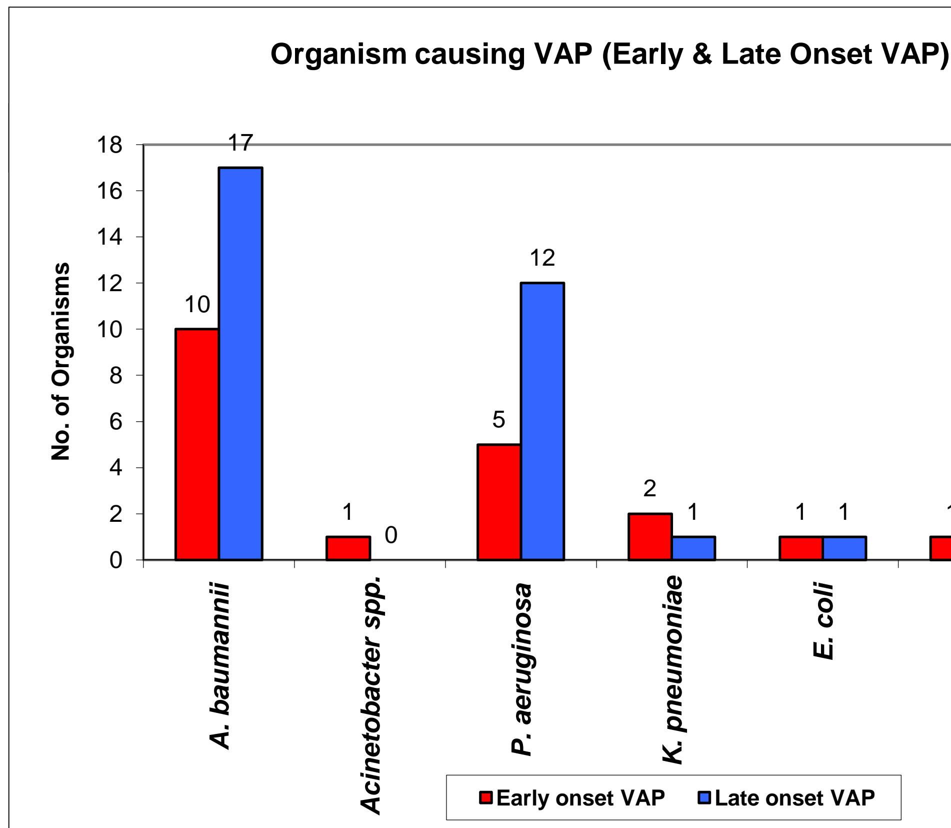
Sl. No.	IP NO.	Age (Years)	Sex	Direct Gram stain	ETA isolate	DO VAP	Penicillin	Oxacillin	Erythromycin	Clindamycin	Cefotaxime	Linezolid	Vancomycin	Cephalexin	Ciprofloxacin	Cotrimoxazole	Amoxyclav	Imipenem	Amikacin	Aztreonam	Pipercillin-tazobactam	Ceftazidime	Doxycycline	Meropenem	MRSA	MBL	Risk factors	Intubation (No. of times)	Outcome
28	395187	35	F	Ac.	A. bau	Early									S	R		S	R	R	S	R	S				Ster,EF	1	E
29	395384	23	M	Ac.	A. bau	Early					R				R	R	R	R	R	R	R	R	R	R			Alc,EF	1	D
30	395189	55	F	Ac.	P.areg	Late									R	R		R	R	S	S	R				P	EF	1	D
31	396126	48	M	Ac.	A. spp.	Early									R	R		R	R	R	R	R	S				Smok	1	D
32	396436	60	M	Ac.	A. bau	Late									R	R		R	R	R	I	R	S				Ster,Alc,Smok,EF	1	E
33	397240	26	M	Ac.	P.areg	Late									R	R		S	S	R	S	R				NP	Negative	1	D
34	3977312	59	F	Ac.	A. bau	Late									R	R		R	R	R	S	R	S				Ster,EF	1	D
35	398331	75	M	Ac.	A. bau	Early									R	R		R	R	R	S	R	S				Ster,Alc,Smok	2	E
36	397396	68	M	Ac.	E.coli	Late								R	R	R	R	R	S	S	S	R					Smok,EF	2	D
37	398247	27	M	Ac.	A. bau	Late									R	R		R	R	R	S	R	R				Smok	1	D
38	398748	60	M	Ac.	A. bau	Early									R	R		R	R	R	S	R	R				Negative	1	D
39	399266	25	F	Ac.	P.areg	Late									R	R		S	R	R	S	S				NP	Ster,EF	1	E
40	400749	48	F	Ac.	A. bau	Early									R	R		R	R	R	S	R	S				Negative	1	E
41	400884	49	M	Ac.	P.areg	Late									R	R		R	R	R	S	S				P	EF	1	D
42	401809	49	F	Ac.	P.areg	Late									R	R		R	R	R	S	S				P	EF	1	D
43	402727	42	M	Ac.	A. bau	Late									R	R		R	R	R	S	R	S				Alc,EF	3	E
44	402761	19	M	Ac.	P.areg	Early									R	R		R	R	R	S	S				NP	EF	1	D
45	404292	62	M	Ac.	A. bau	Late									R	R		R	R	R	S	R	R				Ster,Alc,Smok	1	E
46	403790	18	M	Ac.	P.areg	Late									R	R		S	R	R	S	R				NP	Negative	2	D
47	411755	39	F	Ac.	P.areg	Late									R	R		S	R	R	S	S				NP	Negative	1	D
48	425671	42	M	Ac.	K. pneu.	Early								R	R	R	R	S	R	R	S	I					Ster,Alc,EF	1	D
49	416689	25	M	Ac.	A. bau	Late									R	R		R	R	R	R	R	S				Negative	1	E
50	418932	66	M	Ac.	MRSA	Late	R	R	R	R	S	R	S	R	R	R	R						S		Pos		Alc	1	E
51	427273	53	M	Ac.	P.areg	Late									R	R		R	R	R	S	S				P	Alc, Smok	1	E
52	340001	64	M	Ac.	P.areg	Late									R	R		R	R	R	S	R				P	Negative	1	E
53	362126	30	M	Ac.	A. bau	Late									R	R		R	R	R	S	R	S				Smok	1	D
54	362253	25	M	Ac.	A. bau	Late									R	R		R	R	R	R	R	S				Negative	1	D

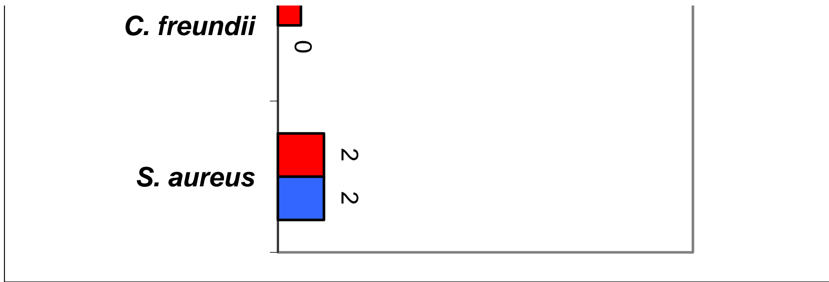
Sl. No.	IP NO.	Age (Years)	Sex	Direct Gram stain	ETA isolate	DO VAP	Penicillin	Oxacillin	Erythromycin	Clindamycin	Cefotaxime	Linezolid	Vancomycin	Cephalexin	Ciprofloxacin	Cotrimoxazole	Amoxyclav	Imipenem	Amikacin	Aztreonam	Pipercillin-tazobactam	Ceftazidime	Doxycycline	Meropenem	MRSA	MBL	Risk factors	Intubation (No. of times)	Outcome

KEY TO MASTER CHART

A.bau	:	*Acinetobacter baumannii*
A.spp.	:	*Acinetobacter* species
P.aeru	:	*Pseudomonas aeruginosa*
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
E.coli	:	*Escherichia coli*
C.freu	:	*Citrobacter freundii*
K.pneu	:	*Klebsiella pneumoniae*
M	:	Male
F	:	Female
Ac.	:	Accepted
Rej.	:	Rejected
VAP	:	Ventilator-associated pneumonia
DO	:	Duration
MBL	:	Metallo-beta lactamase
P	:	Producer
NP	:	Non-producer
D	:	Discharged
E	:	Expired
R	:	Resistant
I	:	Intermediate
S	:	Sensitive
Pos	:	Positive
Neg	:	Negative
EF	:	Enteral feeds
Alc		Alcohol
Smok	:	Smoking
Ster	:	Steroids

		Early onset	Late onset VAP
1	*A. baumannii*	10	17
2	*Acinetobacter* spp.	1	0
3	*P. aeruginosa*	5	12
4	*K. pneumoniae*	2	1
5	*E. coli*	1	1
6	*C. freundii*	1	0
7	*S. aureus*	2	2





C. freundii
0
S. aureus
2
2

PHOTOGRAPHS

Photo 1: An ICU unit

Photo 2: Mucus Extractor

Photo 3: Serial dilution of endotracheal aspirate in 0.9% sterile saline solution with final dilutions of 10^1, 10^2, 10^3, 10^4, 10^5, 10^6

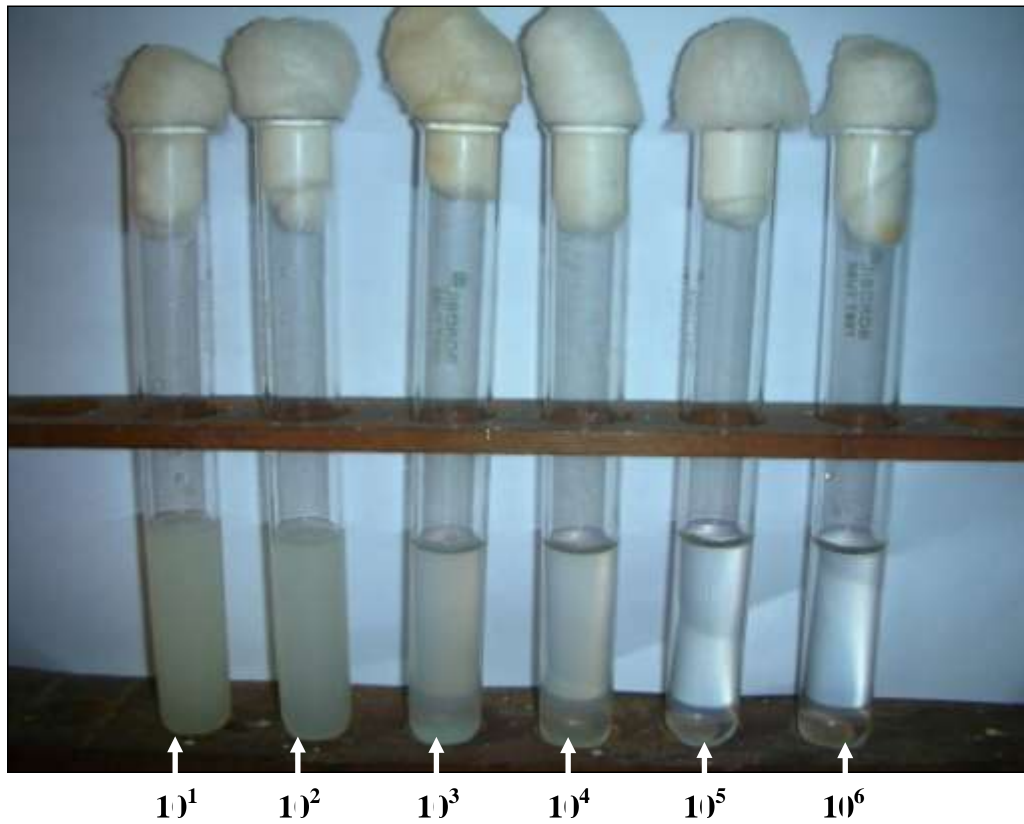


Photo 4: Photo micrograph of Gram staining of ETA (Showing < 10 squamous epithelial cells/ low power field)

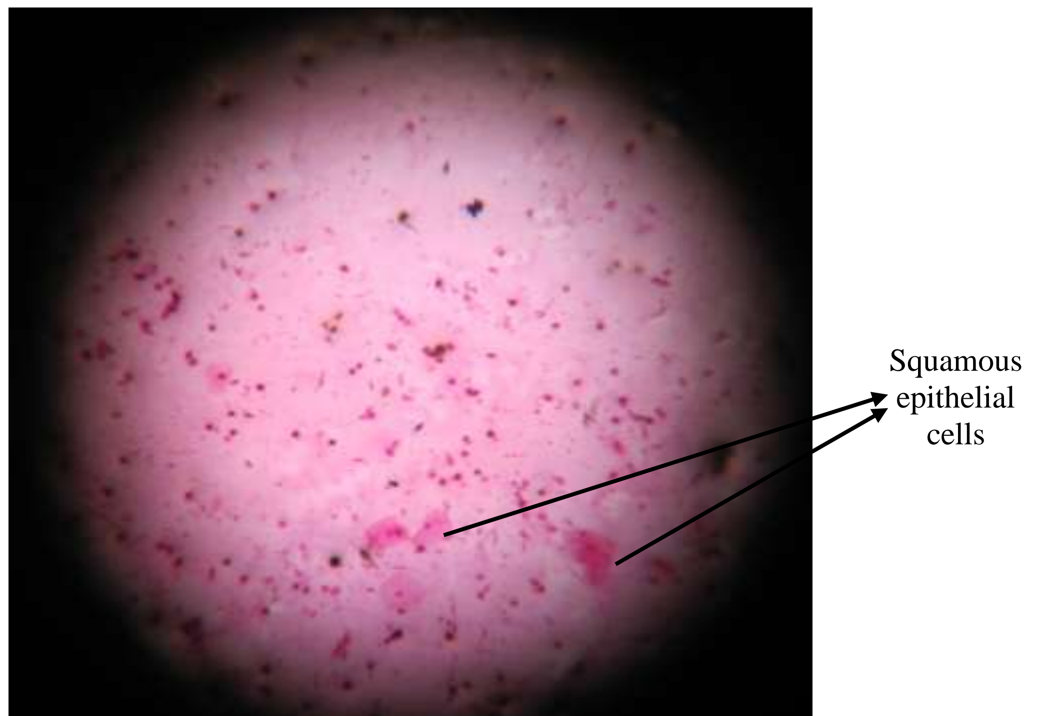


Photo 5: Blood agar plate 1

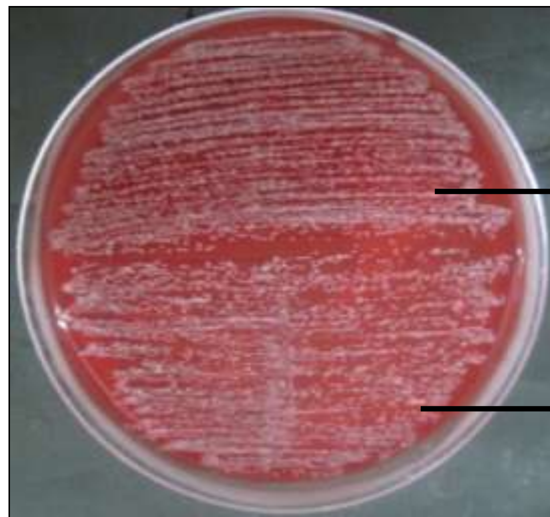


Photo 6: Blood agar plate 2

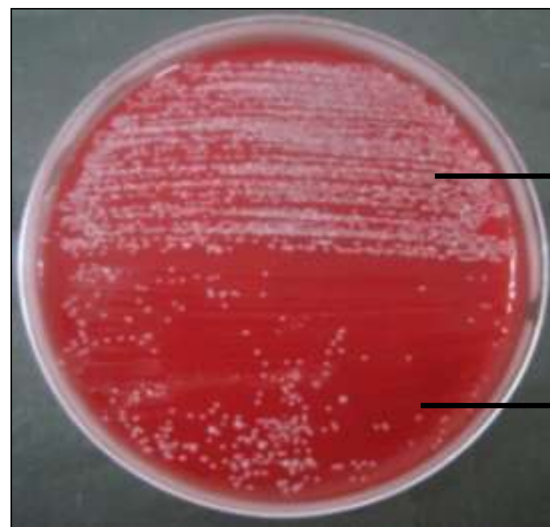


Photo 7 : Blood agar plate 3

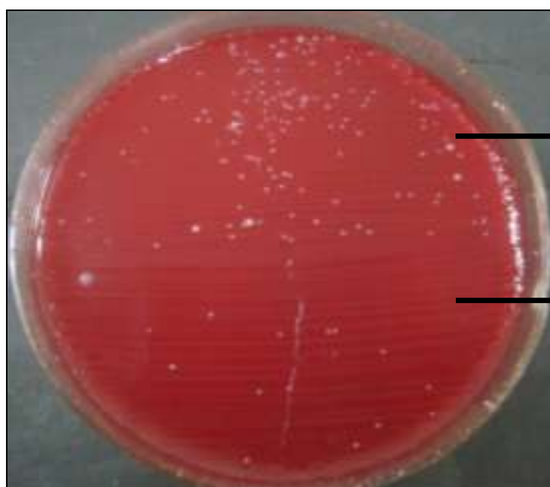


Photo 8 : MacConkey agar plate 1

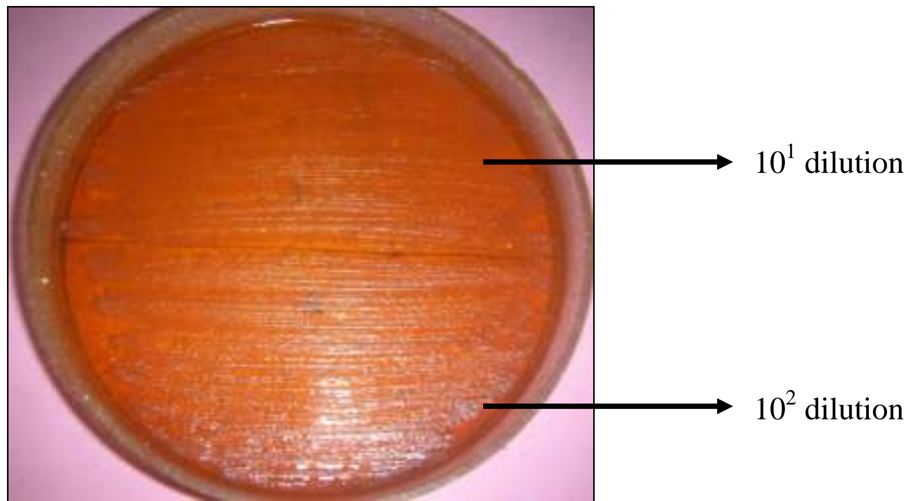


Photo 9 : MacConkey agar plate 2

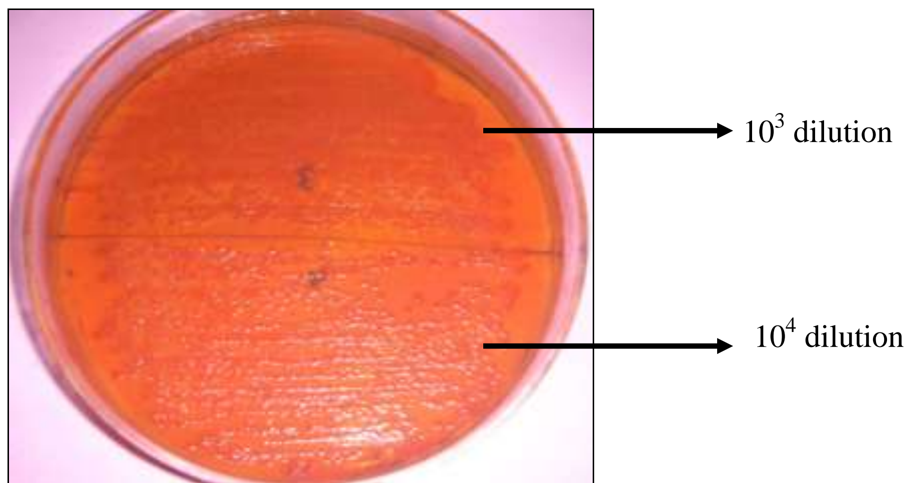


Photo 10 : MacConkey agar plate 3

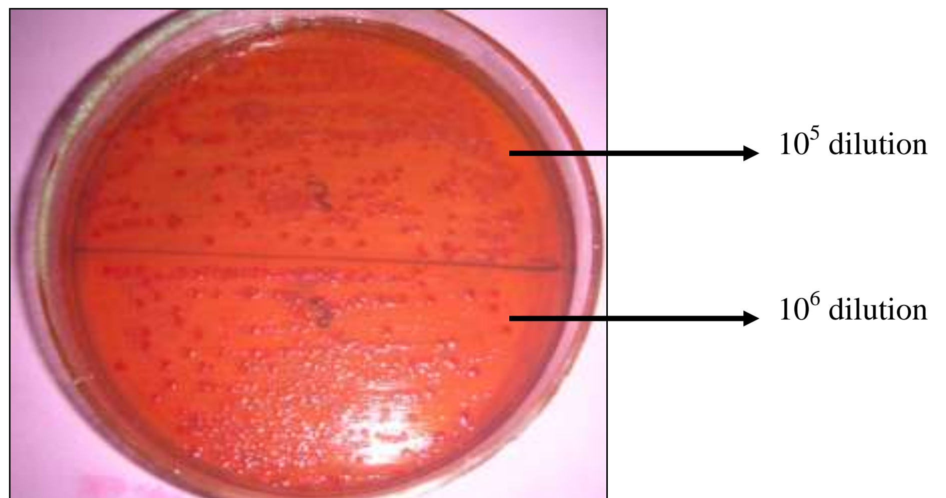


Photo 11: Test showing OF reactions of *Acinetobacter baumannii*

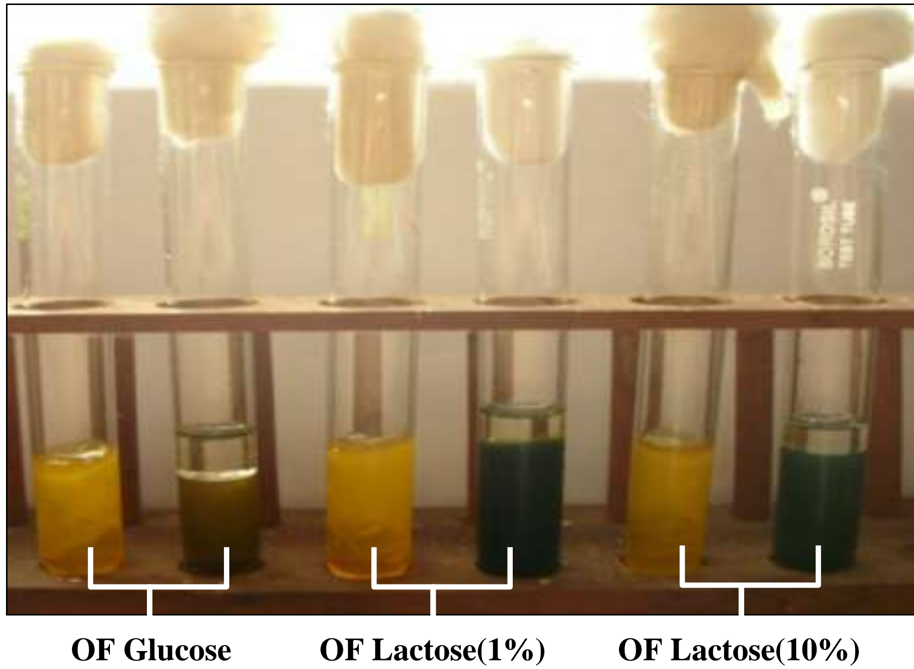


Photo 12 : Methicillin-resistant *Staphylococcus aureus*

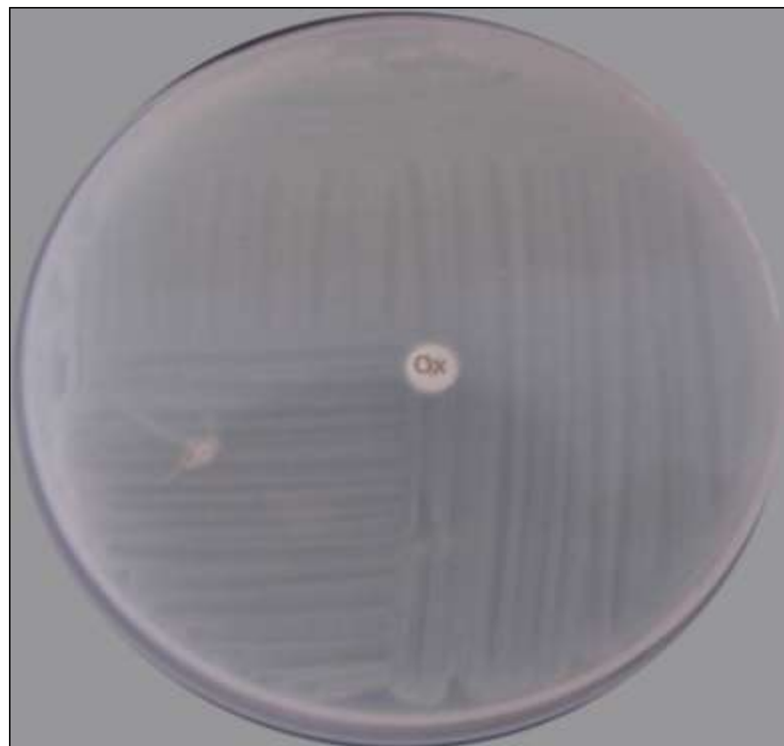
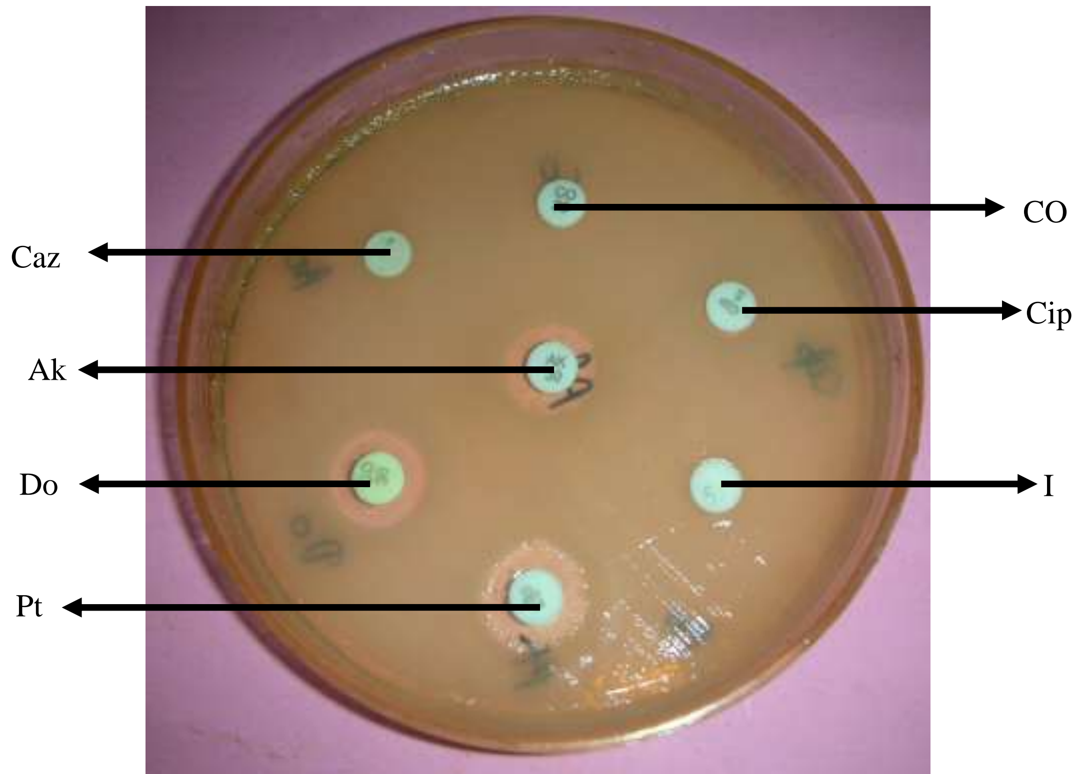
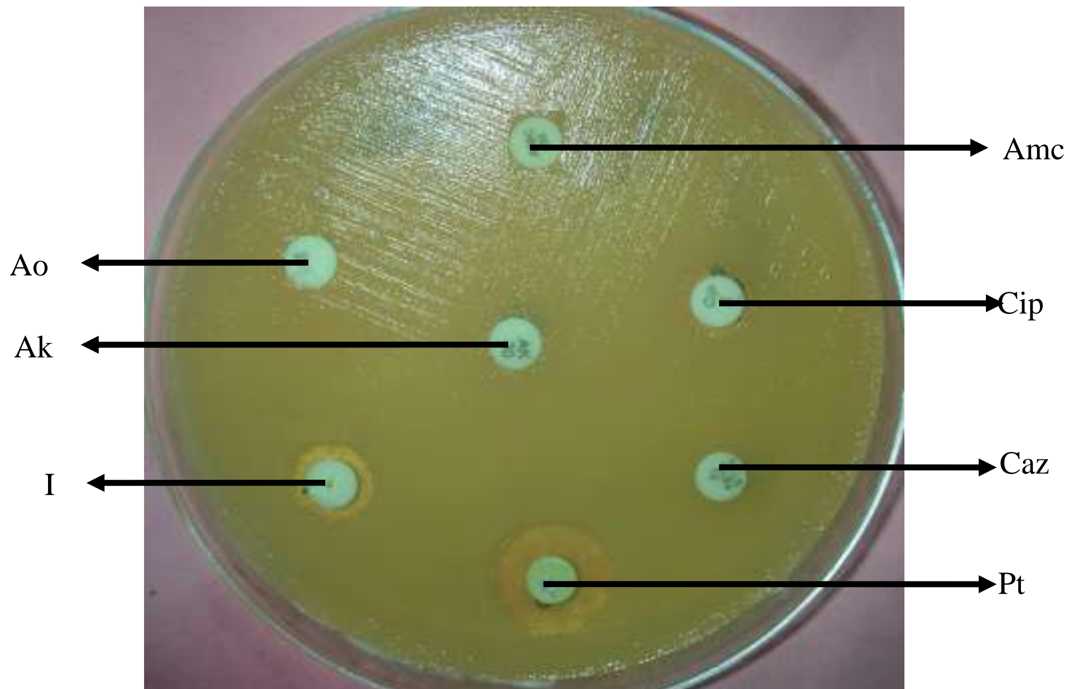


Photo 13 : Antibiogram of *Acinetobacter baumannii*



(Caz-Ceftazidime, Ak-Amikacin, Do-Doxycycline, Pt-Piperacillin-tazobactam, CO-Cotrimoxazole, Cip- Ciprofloxacin, I-Imipenem)

Photo 14 : Antibiogram of *Pseudomonas aeruginosa*



(I-Imipenem, Ao-Aztreonam, Pt-Piperacillin-tazobactam, Caz-Ceftazidime, Cip-Ciprofloxacin, Ak-Amikacin, Amc-Amoxyclav,)

Photo 15 : Metallo-β-lactamase producing *Pseudomonas aeruginosa* detection by Imipenem-EDTA combined disc test