CHAPTER-I

INTRODUCTION

Acute lower respiratory tract infection is a persistent and pervasive public health problem in both developed and developing countries. They cause a greater burden of disease worldwide than human immunodeficiency virus infection, malaria, cancer or heart attack (WHO, 2010).

Lower respiratory tract starts from the upper end of trachea and terminate at lungs. Under the normal functioning of the immune response, Lower respiratory tract unlike upper respiratory tract is free of normal microbial flora and potentially harmful microbes. The potential inhaled pathogens may be viruses, bacteria fungi and parasites. Lower respiratory tract infection may be the primary causes of acute illness such as pneumococcal pneumonia and Legionnaire's diseases or may be the result from the secondary bacterial invasion of lungs infected by respiratory viruses due to impaired immune defense normally sterile but can be the potential site of infection by different factors such as old age, pregnancy, steroid therapy, diabetes, cardiac diseases and HIV infection (Forbes et al., 2007).

LRTI is not a single form of disease but constitutes a group of specific infection each attributing a different epidemiology, pathogenesis, clinical features and outcome. The most common infections are acute bronchitis, chronic bronchitis, bronchiolitis, acute exacerbation of chronic bronchitis or chronic obstructive pulmonary disease (COPD) and pneumonia. Among the LRTIs, pneumonia presents the significant importance not only because of total episodes of cases but also due to its considerably high contribution to medical consultation, hospitalization, morbidity and mortality. Pneumonia and other lower respiratory tract infections are the leading cause of death worldwide (WHO, 2009).

Approximately 150 million new cases of pneumonia occur annually among children younger than 5 years worldwide, accounting for approximately 10-20 million hospitalizations. The WHO Child Health Epidemiology Reference Group estimated the median global incidence of clinical pneumonia to be 0.28 episodes per child-year. This equates to an annual incidence of 150.7 million new cases, of which 11-20 million (7-13%) are severe enough to require hospital admission. Ninety-five percent

of all episodes of clinical pneumonia in young children worldwide occur in developing countries.

A WHO Child Health Epidemiology Reference Group publication cited the incidence of community-acquired pneumonia among children younger than 5 years in developed countries as approximately 0.026 episodes per child-year, and a study conducted in the United Kingdom showed that 59% of deaths from pertusis are associated with pneumonia. Many children with ALRIs fail to receive adequate care, and overuse of antibiotics has led to an increase in drug-resistant bacteria (Yee-Wei Lim *et al.*, 2007).

The 2004 World Health Organization Global Burden of Disease Study estimated that lower respiratory tract infections (LRTIs), which include CAP, were responsible for 429.2 million episodes of illness worldwide and were the leading cause of disease burden measured in terms of disability-adjusted life years (DALYs) among all age groups. LRTIs accounted for the leading cause of disease in the developing countries and fourth position in the developed countries (4% of overall deaths in developed regions of the world) compared with the Middle East/North Africa (MENA) region, where overall mortality due to LRTIs has been reported to be as high as 10%.The burden of CAP (community acquired pneumonia) is of even greater concern for aging adults when considering that the number of persons aged over 60 years globally is projected to triple, from 759 million in 2010 to 2 billion by 2050 (Lipp et al., 2010).

Each year above 40,000 children aged below 5 years are estimated to die from pneumonia in Nepal. Infants are at the highest risk of deaths from pneumonia. ARI has a major impact on health services and household income, accounting up to 50% of visits by children to health centers. All this data are based on the inefficient and poor management system of diagnosis and reporting by health post and hospital to higher authority and finally to Ministry of Health. It emphasizes much has to be worked out in Nepal to reveal the actual current situation and gravity of problem mainly focusing adult population (MOH 2003).

Lower respiratory infections and tuberculosis (TB) remain a major cause of disability-adjusted life years (DALYS) worldwide. Tuberculosis is one of the world's deadliest diseases. WHO estimated that the largest number of new TB Cases in 2008 occurred in the South-East Asia Region, which accounted for 35% of incident cases globally. Statistics by the SAARC (South Asian Association for Regional Cooperation) TB

Centre in 2007 shows that about 50 % of the adults population in the region has already been infected with *Mycobacterium tuberculosis* and is at risk of developing tuberculosis disease. Furthermore, Tuberculosis causing the death of more adolescents and adults than any other single infection. Tuberculosis is a social disease with medical aspects (Park, 2007).

Mainly in the developing countries like Nepal, poverty, overcrowded, malnutrition, high population growth rate, lack of education, large families, early marriage and lack of early awareness of cause of illness etc. are the predominant and prominent social factors that account for the increasing prevalence of tuberculosis with the result that in most cases it has penetrated to all parts of the community and infection rates have reached a maximum. HIV is the single most important factor contributing to the increase in the incidence of TB and other lower respiratory tract infections mainly the CAP (WHO, 2007).

Since an empirical approach is nearly always necessary in the management of LRTI, greater emphasis must be placed on the decision of whether or not to prescribe an antibiotic. This decision should be based on an assessment of the severity of the disease, including underlying risk factors, and on markers for bacterial/parenchymal/invasive LRTI. The choice of empirical therapy must be based on the same data together with epidemiological information. One study found that patients took an average of 7 days to return to normal activities after an episode of LRTI. The choice of antibiotic must always cover *Streptococcus pneumoniae*, which remains the main pathogen of morbidity and mortality in CAP.

Empirical knowledge of likely causative agent and their antibiotic susceptibility pattern would help in selection of initial therapy. Moreover, the pathogens responsible for community acquired pneumonia are changing. Streptococcal pneumonia accounted for the majority of infection. But today, a wide range of community-acquired pathogens has been implicated as etiological agents including the bacterial types being well established. Multiple drug resistance (MDR) is defined as resistance to two or more of the antimicrobial agents belonging different structural classes pathogens has resulted CDC, 2006). Multidrug resistance among common bacterial) pathogens, into treatment failures and increased economic burden to contain these .(2009 ,thus dictating their early and reliable detection (ASM

In hospital, attempts should be made to obtain an etiological diagnosis in order to be able to switch to a specific treatment or to evaluate a failure of empirical therapy. Still, resistant strains may emerge during antibiotic therapy which is one of the contributing factors for the increase in the frequency of LRTI in recent years in the adult and children population of Nepal as well. As the incidence of antimicrobial resistance rises, so do costs associated with its consequences. Although many children do not receive adequate care for ALRI, others with similar symptoms but without antibiotic-treatable infection receive unnecessary therapy. Over prescription of antibiotics by both qualified and unqualified medical practitioners is common in developing countries, and self-medication through the purchase of antibiotics from drug vendors and pharmacies is also widespread. The overuse of antibiotics has increased resistance among common ALRI-causing bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b.

Several studies have been conducted in different countries throughout the world to find out the information about the etiologies of the lower respiratory tract infection and their antibiotic sensitivity pattern. Therefore, no single study can speculate exactly the condition of LRTI among the different population of different parts of world. Previously, there have been some studies done on LRTI in Nepal in 1994, 1997, 2004. But potential pathogens and their sensitivity pattern changes according to time and human activities. Furthermore, previous studies have not so far established the multi drug resistance and extended spectrum of beta lactamase pattern. Therefore, this study was conducted with the objectives of analyzing the etiological agents causing lower respiratory tract infection in all age groups and their antibiotic sensitivity pattern with special interest to MDR and ESBL.

CHAPTER-II

2. OBJECTIVE

2.1 GENERAL OBJECTIVE

To isolate and identify ESBL producing MDR bacteria from sputum samples of suspected LRTI patients visiting NPHL, Nepal.

2.2 SPECIFIC OBJECTIVES

1.To find the prevalence of Lower respiratory tract infection among the patients visiting NPHL.

2. To isolate and identify bacterial pathogens responsible for Lower respiratory tract infection.

3. To determine the distribution of *Mycobacterium tuberculosis* among the LRTI cases.

4. To detect ESBL producing MDR bacteria from sputum samples of suspected LRTI patients.

CHAPTER-III

3.1 LOWER RESPIRATORY TRACT INFECTION

Respiratory tract infection includes group of broad spectrum diseases from self limiting acute bronchitis to severe pneumonia. The term respiratory disease refers to a number of conditions that affect the lungs or their components; each of these conditions is characterized by some level of impairment of the lungs in performing a variety of different factors and other medical problems (which may or may not start in the lungs), are generally divided into two basic categories: acute respiratory infections and chronic respiratory disorders.

Respiratory tract begins with nasal or oropharynx to trachea, bronchea and into the lungs. Lower respiratory tract infection is the inflammation of the respiratory tract that begins from the trachea to the lungs alveoli secondary to entry and subsequent multiplication of an infectious agent. Generally URTIs are more common than LRTIs, and are usually caused by viruses. Common URTIs include acute nasopharyngitis, acute sinusitis, acute pharyngitis and acute tonsillitis. URTIs are generally mild and self-limiting in nature, and result in little or no mortality. In some cases, however, especially those caused by bacteria, URTIs can lead to serious complications. For example, epigloittitis caused by *Haemophilus influenzae* if left untreated, can be fatal and acute otitis media caused by S. pneumonia or *H. influenzae* can result in hearing loss and subsequent learning and speech impairments (Australian Indigenous Health/info Net, 2005)

LRTIs is less common than URTIs, account for most of the hospitalization and deaths associated with ARIs. As with URTIs, viruses are responsible for the majority of LRTIs, but the most serious forms are bacterial. LRTIs can result in hospitalization and death includes pneumonia, bronchopneumonia, acute bronchitis, and bronchiolitis.

3.2 Anatomy of lower respiratory tract

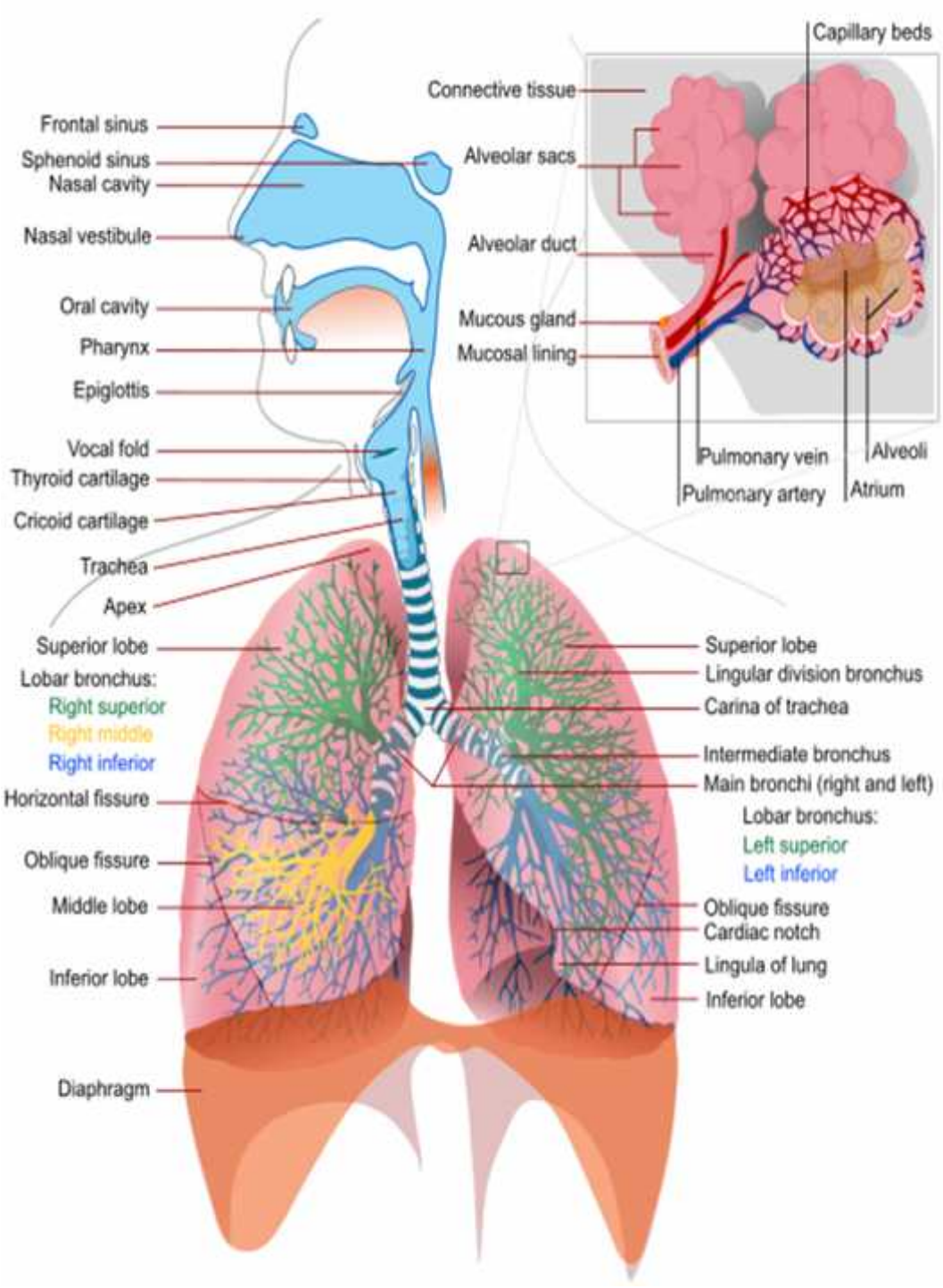
The respiratory tract is arbitrarily divided at the level of the lower border of cricoids cartilage into upper and lower respiratory tract. The major passages and structures of the upper respiratory tract include the nose or nostrils, nasal cavity, mouth, throat (pharynx), and voice box (larynx). Similarly, the major passages and structures of the lower respiratory tract include the trachea and within the lungs, the bronchi, bronchioles, and alveoli. Deep in the lungs, each bronchus divides into secondary and tertiary bronchi, which continue to branch to branch to similar airways called the bronchioles. The bronchioles end in air sacs called the alveoli. Alveoli are bunched together into clusters to from alveolar sacs. Gas exchange occurs on the surface of each alveolus by a capillaries carrying blood that has come through veins from other parts of the body.

3.2.1 Trachea

The trachea is made up of fibroelastic wall with c-shaped 16-20 rings of the cartilages is the continuation of the larynx and it bifurcates into right and left bronchi at the level of the 5^{th} thoracic vertebra. It is about 12 cm long and 2 cm wide. Humidifying, warming and filtering of air continues here and air reaches saturation and body temperature as it passes through trachea.

3.2.2 Bronchi and Bronchioles

The trachea has to divide for 23 generations to reach an alveolus. It first divides into two primary bronchi right and left which enters the right and the left lung respectively through their respective hilum. Each bronchus progressively divides into bronchioles, terminal bronchioles, respiratory bronchioles, alveolar ducts, atria, air saccules and finally pulmonary alveoli. The right principal bronchus is shorter (2.5 cm), wider and more in line with the trachea (vertical) than the left principal bronchus. Therefore inhaled particles tend to pass more frequently to the right lung; with the result than aspiration pneumonia is more common in the right side than the left. The bronchi have the same basic structure as the trachea.



Anatomy of Respiratory tract of Human

Source: From Wikipedia, the free encyclopedia

3.2.3 Lungs

Lungs are spongy in texture situated in thoracic cavity and each lung invaginates the corresponding pleural cavity. Right and left lungs are separated by mediastinum. Right lungs weigh 700gm and left lung weighs 600gm. The lungs consist of two sets of lobes: three lobes make up the right lung and two lobes make up the left lung. The left lung is smaller in order to accommodate the heart, which is situated under the left lung and behind the rib cage. Each lung has a branch of the bronchial tree that splits off the windpipe. The bronchi branches become smaller as they extend into the lung lobes, becoming bronchioles. The bronchi carry the air into and out of the lungs.

The functional units of the lung tissue are thin walled air sacs called alveoli, which are the ends of the smallest bronchioles. Each lung contains millions of these air sacs. The lungs inflate and deflate as air is transported into and out of the air sacs. Between the alveoli is elastic connective tissue, which is important to accommodate the expansion and collapse of the tissue. The pulmonary artery and pulmonary vein transport blood between the heart and lungs for gas exchange, with a network of capillaries surrounding each alveolus.

Inside the alveoli are macrophages, specialized white blood cells, which act as the first line of defense against foreign particles and pathogens that make it past the cilia in the bronchial tree and the defenses of the upper respiratory system.

3.2.4 Alveolar Cells and Surfactant

There are two types of alveolar, or pulmonary, cells, which are also called pneumocytes. Type 1 alveolar cells are flat, simple squamous epithelial cells that form the thin walls of the alveoli. Type II alveolar cells, also known as septal cells, secrete a lipoprotein called surfactant. Pulmonary surfactant prevents the lungs from collapsing by decreasing surface tension within the alveoli.

3.2.6 The Respiratory Muscle

The respiratory muscles are skeletal muscle controlled by the nervous system with some limited voluntary control. The muscles involved in respiration are:

Diaphragm – concave, or dome-shaped, muscle under the lungs and ribs, separating the chest cavity from the abdomen. When the diaphragm contracts, it flattens, allowing the lungs to expand and air is inhaled. When the diaphragm relaxes, air is expelled from the lungs. The diaphragm can be somewhat controlled with practice, such as the breathing exercises of singers.

Internal intercostals – between the ribs, inside the rib cage. The interosseous internal intercostals (actually between the bony parts of the ribs) pull the rib cage inward upon contraction for forced exhalation. The intercartilaginous internal intercostals help lift the ribcage when they contract, allowing the lungs to expand for inhalation.

External intercostals – between the ribs, on the outside of the rib cage. Like other muscles, the external intercostals shorten when they contract, pulling the rib cage up and out, aiding in inhalation

3.2.7 Pleura

Pleura are the outer covering of the lungs consisting of sac like double layered. Serous membrane. The inner visceral pleura envelop the lung itself and the outer parietal pleura lines the thoracic cavity. In between the two pleura membrane there is serous fluid which allows them to glide over each other, without friction during breathing. The small moisture-filled potential space between the visceral and parietal pleura is the pleural cavity.

3.2.8 Respiratory Epithelium

Respiratory Epithelium is a type of epithelium found lining the respiratory tract where it serves to moisten and protect the airways. It also functions as a barrier to potential pathogens and foreign particles, preventing infection and tissue injury by action of the mucociliary escalator. It is typified by pseudostratified columnar, ciliated epithelium with mucous secreting goblet cells. As bronchi progressively decrease in size, there is a gradual transition to ciliated simple columnar and finally simple cuboidal epithelium. There is also a gradual decrease in the number of goblet cells.

3.3 Physiology of respiratory tract

The main function of the respiratory system is to draw air into the lungs to allow the exchange of gases with blood circulating to the lungs. This blood supplies the cells of the body with oxygen and removes the waste products of metabolism. Tissues of the respiratory tract are thin and delicate, and become thinnest at the surfaces of the alveoli, where gaseous exchange occurs. The body has a number of mechanisms which protect these tissues and ensure that debris and bacteria do not reach them.

The respiratory system is situated in the thorax, and is responsible for gaseous exchange between the circulatory system and the outside world. Air is taken in via the upper airways (the nasal cavity, pharynx and larynx) through the lower airways (trachea, primary bronchi and bronchial tree) and into the small bronchioles and alveoli within the lung tissue. The respiratory tract is the pathway through which the body acquires fresh oxygen and removes unneeded carbon dioxide. Tissues of the respiratory tract are thin and delicate, and become thinnest at the surfaces of the alveoli, where gaseous exchange occurs. The body has a number of mechanisms which protect these tissues and ensure that debris and bacteria do not reach them.

3.3.1 Breathing and Lung Mechanics

Ventilation is the exchange of air between the external environment and the alveoli. Air moves by bulk flow from an area of high pressure to low pressure. All pressures in the respiratory system are relative to atmospheric pressure (760mmHg at sea level). Air will move in or out of the lungs depending on the pressure in the alveoli. The body changes the pressure in the alveoli by changing the volume of the lungs. As volume increases pressure decreases and as volume decreases pressure increases. There are two phases of ventilation; inspiration and expiration. During each phase the body changes the lung dimensions to produce a flow of air either in or out of the lungs. The body is able to change the dimensions of the lungs because of the relationship of the lungs to the thoracic wall. Each lung is completely enclosed in a sac called the pleural sac. Two structures contribute to the formation of this sac. The parietal pleura is attached to the thoracic wall where as the visceral pleura is attached to the lung itself.

In-between these two membranes is a thin layer of intrapleural fluid. The intrapleural fluid completely surrounds the lungs and lubricates the two surfaces so that they can slide across each other. Changing the pressure of this fluid also allows the lungs and the thoracic wall to move together during normal breathing. Much the way the skull that warm, moisten, and filter the air). The rhythm of ventilation is also controlled by the "Respiratory Center" which is located largely in the medulla oblongata of the brain stem. This is part of the autonomic system and as such is not controlled voluntarily (one can increase or decrease breathing rate voluntarily, but that involves a different part of the brain). While resting, the respiratory center sends out action potentials that travel along the phrenic nerves into the diaphragm and the external intercostal muscles of the rib cage, causing inhalation. Relaxed exhalation occurs between impulses when the muscles relax. Normal adults have a breathing rate of 12-20 respirations per minute.

3.3.2 The Pathway of Air

When one breathes air in at sea level, the inhalation is composed of different gases. These gases and their quantities are Oxygen which makes up 21%, Nitrogen which is 78%, carbon dioxide with 0.04% and others with significantly smaller portions. In the process of breathing, air enters into the nasal cavity through the nostrils and is filtered by coarse hairs (vibrissae) and mucous that is found there. The vibrissae filter macroparticles, which are particles of large size. Dust, pollen, smoke, and fine particles are trapped in the mucous that lines the nasal cavities (hollow spaces within the bones of projections inside the nasal cavity. The superior, middle, and inferior nasal conchae. Air passes between these conchae via the nasal meatuses. Air then travels past the nasopharynx, oropharynx, and laryngopharynx, which are the three portions that make up the pharynx. The tonsils which are part of the lymphatic system form a ring at the connection of the oral cavity and the pharynx. Here, they protect against foreign invasion of antigens. Therefore the respiratory tract aids the immune system through this protection. Then the air travels through the larynx. The larynx closes at the epiglottis to prevent the passage of food or drink as a protection to our trachea and lungs. The larynx is also our voice box; it contains vocal cords, in which it produces sound. Sound is produced from the vibration of the vocal cords when air passes through them. The trachea, which is also known as our windpipe,

has ciliated cells and mucous secreting cells lining it, and is held open by C-shaped cartilage rings. One of its functions is similar to the larynx and nasal cavity, by way of protection from dust and other particles. The dust will adhere to the sticky mucous and the cilia helps propel it back up the trachea, to where it is either swallowed or coughed up. The mucociliary escalator extends from the top of the trachea all the way down to the bronchioles, which we will discuss later. Through the trachea, the air is now able to pass into the bronchi.

3.3.3 Homeostasis and Gas Exchange

Homeostasis is maintained by the respiratory system in two ways: gas exchange and regulation of blood pH. Gas exchange is performed by the lungs by eliminating carbon dioxide, a waste product given off by cellular respiration. As carbon dioxide exits the body, oxygen needed for cellular respiration enters the body through the lungs. ATP, produced by cellular respiration, provides the energy for the body to perform many functions, including nerve conduction and muscle contraction. Lack of oxygen affects brain function, sense of judgment, and a host of other problems

3.3.4 Gas Exchange

Gas exchange in the lungs and in the alveoli is between the alveolar air and the blood in the pulmonary capillaries. This exchange is a result of increased concentration of oxygen, and a decrease of CO_2.

3.3.5 External Respiration

External respiration is the exchange of gas between the air in the alveoli and the blood within the pulmonary capillaries. A normal rate of respiration is 12-25 breaths per minute. In external respiration, gases diffuse in either direction across the walls of the alveoli. Oxygen diffuses from the air into the blood and carbon dioxide diffuses out of the blood into the air. Most of the carbon dioxide is carried to the lungs in plasma as bicarbonate ions (HCO_3^-). When blood enters the pulmonary capillaries, the bicarbonate ions and hydrogen ions are converted to carbonic acid (H_2CO_3) and then back into carbon dioxide (CO_2) and water. This chemical reaction also uses up hydrogen ions. The removal of these ions gives the blood a more neutral pH, allowing hemoglobin to bind up more oxygen. De-oxygenated blood

"blue blood" coming from the pulmonary arteries, generally has an oxygen partial pressure (pp) of 40 mmHg and CO pp of 45 mmHg. Oxygenated blood leaving the lungs via the pulmonary veins has O_2 pp of 100 mmHg and CO pp of 40 mmHg. It should be noted that alveolar O_2 pp is 105 mmHg, and not 100 mmHg. The reason why pulmonary venous return blood has a lower than expected O_2 pp can be explained by "Ventilation Perfusion Mismatch".

3.3.6 Internal Respiration

Internal respiration is the exchanging of gases at the cellular level. The Passage Way from the Trachea to the Bronchioles There is a point at the inferior portion of the trachea where it branches into two directions that form the right and left primary bronchus. This point is called the Carina which is the keel-like cartilage plate at the division point. We are now at the Bronchial Tree. It is named so because it has a series of respiratory tubes that branch off into smaller and smaller tubes as they run throughout the lungs.

3.3.7 Cellular Respiration

First the oxygen must diffuse from the alveolus into the capillaries. It is able to do this because the capillaries are permeable to oxygen. After it is in the capillary, about 5% will be dissolved in the blood plasma. The other oxygen will bind to red blood cells. The red blood cells contain hemoglobin that carries oxygen. Blood with hemoglobin is able to transport 26 times more oxygen than plasma without hemoglobin. Our bodies would have to work much harder pumping more blood to supply our cells with oxygen without the help of hemoglobin. Once it diffuses by osmosis it combines with the hemoglobin to form oxyhemoglobin. Now the blood carrying oxygen is pumped through the heart to the rest of the body. Oxygen will travel in the blood into arteries, arterioles, and eventually capillaries where it will be very close to body cells. Now with different conditions in temperature and pH (warmer and more acidic than in the lungs), and with pressure being exerted on the cells, the hemoglobin will give up the oxygen where it will diffuse to the cells to be used for cellular respiration, also called aerobic respiration. Cellular respiration is the process of moving energy from one chemical form (glucose) into another (ATP), since all cells use ATP for all metabolic reactions. It is in the mitochondria of the

cells where oxygen is actually consumed and carbon dioxide produced. Oxygen is produced as it combines with hydrogen ions to form water at the end of the electron transport chain (see chapter on cells). As cells take apart the carbon molecules from glucose, these get released as carbon dioxide. Each body cell releases carbon dioxide into nearby capillaries by diffusion, because the level of carbon dioxide is higher in the body cells than in the blood. In the capillaries, some of the carbon dioxide is dissolved in plasma and some is taken by the hemoglobin, but most enters the red blood cells where it binds with water to form carbonic acid. It travels to the capillaries surrounding the lung where a water molecule leaves, causing it to turn back into carbon dioxide. It then enters the lungs where it is exhaled into the atmosphere.

3.4 Pathophysiology of LRTI

An inhaled infectious organism must bypass the host's normal non immune and immune defense mechanisms in order to cause pneumonia. The non immune mechanisms include aerodynamic filtering of inhaled particles based on size, shape, and electrostatic charges; the cough reflex; mucociliary clearance; and several secreted substances (eg, lysozymes, complement, and defensins). Macrophages, neutrophils, lymphocytes, and eosinophils carry out the immune-mediated host defense.

3.4.1 Respiratory tract host defense

To prevent and minimize injury and invasion by microorganisms and foreign substances, various defense mechanisms have evolved, both systemically and within the respiratory tract. Some mechanisms are nonspecific and are directed against any invasive agent, whereas others are targeted against only microbes or substances with specific antigenic determinants. Many of the defenses are compromised in the fetus and newborn infant, resulting in more frequent breaches and consequent disruption of normal lung structure and function.

Nonspecific defenses include the glottis and vocal cords, ciliary escalator, airway secretions, migratory and fixed phagocytes, nonspecific antimicrobial proteins and opsonins, and the normal relatively nonpathogenic airway flora. Anatomic structures of the upper airway and associated reflexes discourage particulate material from

entering, whereas coordinated movement of the microscopic cilia on the tracheal and bronchial epithelia tends to sweep particles and mucus up the airway and away from the alveoli and distal respiratory structures (Howel et al., 2002).

Mucoid airway secretions provide a physical barrier that minimizes epithelial adhesion and subsequent invasion by microorganisms. These secretions typically contain complement components, fibronectin, and other proteins that bind to microbes and render them more susceptible to ingestion by phagocytes. Alveolar and distal airway secretions also include whole surfactant, which facilitates opsonization and phagocytosis of pathogens, as well as surfactant-associated proteins A (Sp-A) and D (Sp-D), both of which modulate phagocytosis, phagocyte production of oxyradicals, and cytokine elaboration (Richardson et al., 2003).

The secretions also contain directly inhibitory and microbicidal agents, such as iron-binding proteins, lysozymes, and defensins. Typical benign airway commensal, such as alpha-hemolytic streptococci and Coagulase-negative staphylococci, occupy mucosal sites and elaborate bacteriocins and other substances that prevent more pathogenic organisms from adhesion, replication, and possible opportunistic invasion.Most microorganisms that cause pneumonia and other LRTI are able to survive on airborne droplets. These droplets can float in the air for quite a long time and if still infectious can cause pneumonia (Park, 1994).

Newborns typically have sterile respiratory mucosa at birth, with subsequent uncontested colonization by microorganisms from the mother or environment. Accelerated access to distal respiratory structures and bypass of much of the ciliary escalator occur in infants who require endotracheal intubation. In these infants, increased physical disruption of epithelial and mucous barriers also occurs. In addition, interventional exposure to high oxygen concentrations, generous airway pressures, and large intrapulmonary gas volumes may interfere with ciliary function and mucosal integrity.

3.4.2 Systemic host defenses

Immunologic defense mechanisms targeted against particular pathogens typically emanate from specifically primed lymphocytes following presentation of processed antigens by macrophages. These mechanisms include cytotoxic, killer, suppressor, and memory functions; systemic and secretory antibodies; and consequent cascades of cytokines, complement, vasomotor regulatory molecules, hemostatic factors, and other agents. Secretary antibodies are typically multimeric and contain secretory component and J chains that render them more opsonic and more resistant to microbial proteases (Todar et al., 2011).

Many of the biochemical cascades triggered by specific immune responses serve to localize microbial invasion, amplify and focus recruitment of phagocytes to the affected sites, and directly disrupt the structural and metabolic integrity of the microbes. The role that these cascades play in triggering apoptosis (programmed cell death) in host and invader cells is still undergoing exploration.

Secretory antibodies and mucosal lymphoid tissue are absent or minimally functional for the first month of life postnatally. Systemic antibodies may enter pulmonary tissues but usually consist primarily of passively transmitted maternal antibodies, with reduced transplacental transport of maternal antibodies before 32 weeks' gestation. Specific systemic antibodies can be generated, but many components of the necessary immunologic machinery are relatively sluggish. Circulating complement components are present at approximately 50% of the concentration found in older children, although components of the alternative pathway are present in sufficient quantities to serve as effective opsonins.

3.5 Major Diseases of Lower Respiratory Tract

3.5.1 Bronchitis

Bronchitis is the inflammation of the membrane that lines the bronchial tubes resulting from extensive destruction of respiratory epithelium possible with some types of infection (such as influenza and repeated episodes of infection).

3.5.2 Acute bronchitis

Acute bronchitis is common in smokers and in patients with asthma, chronic bronchitis and emphysema. However, it also occurs in otherwise healthy individuals, usually due to viral infections. In both groups, when bacterial infection occurs, the bacteria most often incriminated are *S. pneumoniae* or *H. influenzae*. These infections cause an acute inflammation of the trachea and major airways, and as a consequence of this there is chest pain, commonly experienced as a raw feeling maximal on deep inspiration, as well as chest tightness. The patient may wheeze. In patients with pre-existing chronic airflow limitation the development of acute bronchitis can cause severe breathlessness.

Acute bronchitis causes an irritating, persistent dry cough, although after 1 or 2 days patients produce small amounts of mucoid thick sputum which subsequently becomes more plentiful and purulent. Appropriate antibiotic therapy is amoxicillin, erythromycin or tetracycline. In patients with chronic airflow limitation, aggressive therapies with bronchodilators are important, as acute bronchitis can precipitate a worsening of respiratory failure (Quinn et al., 2010).

3.5.3 Chronic Bronchitis

It is a disease predominantly of adults and is observed more frequently in middle aged men who are chronic heavy smokers. Chronic bronchitis is defined clinically by the presence of cough with sputum production for most days of at least 3 months a year for 2 consecutive years.

3.5.4 **Pneumonia**

Pneumonia is characterized by inflammation of the alveoli and terminal airspaces in response to invasion by an infectious agent introduced into the lungs through hematogenous spread or inhalation. The inflammatory cascade triggers the leakage of plasma and the loss of surfactant, resulting in air loss and consolidation.

The activated inflammatory response often results in targeted migration of phagocytes, with the release of toxic substances from granules and other microbicidal packages and the initiation of poorly regulated cascades (e.g., complement, coagulation, and cytokines). These cascades may directly injure host

tissues and adversely alter endothelial and epithelial integrity, vasomotor tone, intravascular hemostasis, and the activation state of fixed and migratory phagocytes at the inflammatory focus. The role of apoptosis (non inflammatory programmed cell death) in pneumonia is poorly understood.

In viral infections are characterized by the accumulation of mononuclear cells in the submucosa and perivascular space, resulting in partial obstruction of the airway. Patients with these infections present with wheezing and crackles (see Clinical Presentation). Disease progresses when the alveolar type II cells lose their structural integrity and surfactant production is diminished, a hyaline membrane forms, and pulmonary edema develops.

In bacterial infections, the alveoli fill with proteinaceous fluid, which triggers a brisk influx of red blood cells (RBCs) and polymorph nuclear (PMN) cells (red hepatization) followed by the deposition of fibrin and the degradation of inflammatory cells (gray hepatization). During resolution, intra-alveolar debris is ingested and removed by the alveolar macrophages. This consolidation leads to decreased air entry and dullness to percussion; inflammation in the small airways leads to crackles.

Four stages of lobar pneumonia have been described. In the first stage, which occurs within 24 hours of infection, the lung is characterized microscopically by vascular congestion and alveolar edema. Many bacteria and few neutrophils are present. The stage of red hepatization (2-3 d), so called because of its similarity to the consistency of liver, is characterized by the presence of many erythrocytes, neutrophils, desquamated epithelial cells, and fibrin within the alveoli. In the stage of gray hepatization (2-3 d), the lung is gray-brown to yellow because of fibrin purulent exudates, disintegration of RBCs, and hemosiderin. The final stage of resolution is characterized by resorption and restoration of the pulmonary architecture. Furious inflammation may lead to resolution or to organization and pleural adhesions.

Bronchopneumonia, a patchy consolidation involving one or more lobes, usually involves the dependent lung zones, a pattern attributable to aspiration of

oropharyngeal contents. The neutrophilic exudate is centered in bronchi and bronchioles, with centrifugal spread to the adjacent alveoli.

In interstitial pneumonia, patchy or diffuse inflammation involving the interstitium is characterized by infiltration of lymphocytes and macrophages. The alveoli do not contain significant exudates, but protein-rich hyaline membranes similar to those found in adult respiratory distress syndrome (ARDS) may line the alveolar spaces. Bacterial super infection of viral pneumonia can also produce a mixed pattern of interstitial and alveolar airspace inflammation. Miliary pneumonia is a term applied to multiple, discrete lesions resulting from the spread of the pathogen to the lungs via the bloodstream. The varying degrees of immunocompromised in military tuberculosis (TB), Histoplasmosis and Coccidioidomycosis may manifest as

Granulomas with caseous necrosis to foci of necrosis. Cytomegalovirus (CMV) Miliary herpesvirus, orvaricella-zoster virus infection in severely immuno-compromised patients results in numerous acute necrotizing hemorrhagic lesions.

3.5.4.1 Classification of Pneumonia

Distinction was in the past between typical and atypical pneumonias (typical being those caused by common pathogens and atypical being referred to those caused by Mycoplasma pneumoniae, Chlamydia pneumoniae, or Legionella pneumophila). But the clinical presentations were different and could help identify the etiologic organism and such classification has not been proven to be useful.

No categorization of pneumonia is satisfactory but for descriptive purpose the classification should be both anatomical and causal. As the setting in which a pneumonia develops has such major implications for the likely organisms and hence dictates the immediate choice of antibiotics, pneumonia are now classified as,

a) Hospital acquired
b) Community acquired
c) Recurrent pneumonia and
d) Pneumonia due to overt aspiration

3.5.4.2 Hospital-acquired (nosocomial) pneumonia

According to American Thoracic Society (ATS) guidelines, nosocomial pneumonia (also known as hospital-acquired pneumonia or health care–associated pneumonia) is defined as pneumonia that occurs more than 48 hours after hospital admission but that was not incubating at the time of admission (Cunha, 2011).

Up to 5% of patients admitted to hospital for other causes subsequently develop pneumonia (WHO, 2010). Particularly important predisposing factors are cigarette smoking, chroniclung disease, advanced age, obesity, the effects of anaesthesia and surgery, and prior use of broad-spectrum antibiotics. The causative organisms for hospital-acquired pneumonias are different from those responsible for community acquired pneumonia and, in particular, Gram-negative organisms are responsible for 50% of cases. Anaerobic infections are also important.

The prior use of broad-spectrum antibiotics and the consequent colonization of the oropharynx with Gram negative bacilli reduce the value of sputum culture and make it difficult to find the cause of hospital-acquired pneumonias. The management of these pneumonias involves close collaboration between clinical staff and microbiologists. Gram stain of the sputum may be more useful than culture. Blood cultures will be positive in up to 25% of cases (Fagon et al., 2003).

3.5.4.3 Community Acquired Pneumonia:

Community-acquired lower respiratory tract infection (LRTI) is a common cause of acute illness in adults. The spectrum of disease ranges from a mild mucosal colonisation or infection, acute bronchitis or acute exacerbation of chronic bronchitis/chronic obstructive pulmonary disease (AE-CB/COPD), to an overwhelming parenchymal infection with the patient presenting with severe community-acquired pneumonia (CAP).

Although the great majority of LRTIs are self-limiting viral infections, CAP is most often a bacterial disease with a substantial mortality. Thus, antibiotic treatment is rarely indicated for acute bronchitis and is only indicated for the more severe cases of AE-CB/COPD, but it is nearly always indicated for CAP, for which a delay in treatment may increase the risk of a fatal outcome. It may be difficult to differentiate

between a viral and a bacterial LRTI, or between bronchitis/AE-CB/COPD and CAP. This may be one reason why antibiotics are prescribed to more than two-thirds of patients with LRTIs in Europe and the USA. Considering the worldwide development of antibiotic resistance, this is not an acceptable situation.

Since an empirical approach is nearly always necessary in the management of LRTI, greater emphasis must be placed on the decision of whether or not to prescribe an antibiotic at all. This decision should be based on an assessment of the severity of the disease, including underlying risk factors, and on markers for bacterial/parenchymal/invasive LRTI. The choice of empirical therapy must be based on the same data together with epidemiological information. The choice of antibiotic must always cover Streptococcus pneumoniae, which remains the main pathogen of morbidity and mortality in CAP (Shibl et al., 2010).

In hospital, attempts should be made to obtain an etiological diagnosis in order to be able to switch to a specific treatment or to evaluate a failure of empirical therapy. Several guidelines for the management of community-acquired pneumonia have been published during the last 10 yrs. Some reports indicate that the implementation of such guidelines has resulted in lowered costs, length of stay in hospital and mortality. However, the results from these studies are not consistent and the evidence is still weak.

3.5.4.4 Recurrent pneumonia

Prior to making a diagnosis of recurrent pneumonia,the possibility of a non-infective cause of recurrent pulmonary problems should be considered. Alternative diagnose will include pulmonary infarction, pulmonary eosinophilia (including bronchopulmonary aspergillosis) and asthma.

3.5.4.5 Aspiration pneumonia

Infection is usually with anaerobic organisms derived from the upper respiratory tract. Aspiration pneumonia may be acute, extensive and progressive, or it may run a more subacute course and progress to lung abscess formation. When cavitating anaerobic pneumonias communicate with the bronchial tree the sputum is both copious and foul-smelling.

The site of the pneumonia or lung abscess will depend on the position of the patient at the time of aspiration. Aspirated material enters the right lung more easily than the left, and will enter the lower lobes when the subject is standing, and the apical segment of the lower lobes or the posterior segment of the upper lobes when supine. An important cause of anaerobic pneumonias and lung abscess is bronchial obstruction (e.g. with bronchogenic carcinoma), and if the diagnosis of aspiration is in doubt bronchoscopy is indicated. Radiological studies of swallowing will frequently be rewarding.

Anaerobic infection causes considerable tissue destruction, with abscess formation, and empyema and metastatic abscesses can also occur. Prompt treatment avoids such problems. Most upper respiratory tract anaerobes are sensitive to penicillin. Early aspiration pneumonias are adequately treated with amoxicillin, but more severe infections and lung abscesses require parenteral cefuroxime and metronidazole initially, followed by oral amoxycillin and metronidazole (depending on cultures). Postural drainage is important, and antibiotic therapy should be continued for 6 weeks or more to minimize lung destruction.

3.5.5 Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) refers to a combination of lung diseases that leads to a buildup of mucus or loss of elasticity in the lungs, resulting in restricted or blocked airflow that makes breathing increasingly difficult. Symptoms of COPD occur when the bronchial tubes become inflamed and thickened or the tiny air sacs in the lungs are less able to stretch, allowing less air to go in and out of the lungs. This limits not only the intake of oxygen but also the amount of carbon dioxide that can be exhaled. Most commonly the result of long-term smoking, COPD is one of the leading causes of illness or death in the world and is the fourth leading cause of death in the United States.

3.6 Pathogens of the lower respiratory tract infection

3.6.1 *Streptococcus pneumoniae*

Streptococcus pneumonia is a normal inhabitant of the human upper respiratory tract. The bacterium can cause pneumonia, usually of the lobar type, paranasal sinusitis and otitis media, or meningitis, which is usually secondary to one of the former infections. It also causes osteomyelitis, septic arthritis, endocarditis, peritonitis, cellulitis and brain abscesses. *Streptococcus pneumoniae* is currently the leading cause of invasive bacterial disease in children and the elderly. *Streptococcus pneumoniae* is known in medical microbiology as the pneumococcus, referring to its morphology and its consistent involvement in pneumococcal pneumonia.

S. pneumoniae is a transient member of the normal flora, colonizing the nasopharynx of up to 40% of healthy adults and children population. Children carry this pathogen in the nasopharynx asymptomatically for about 4-6 weeks, often several serotypes at a time. New serotypes are acquired approximately every 2 months. Serotypes 6, 14, 18, 19, and 23 are the most prevalent, accounting for 60-80% of infections depending on the area of the world. Pneumococcal infection accounts for more deaths than any other vaccine-preventable bacterial disease. Those most commonly at risk for pneumococcal infection are children between 6 months and 4 years of age and adults over 60 years of age. Virtually every child will experience pneumococcal otitis media before the age of 5 years. It is estimated that 25% of all community-acquired pneumonia is due to pneumococcus (Todar , 2011).

Until 2000, *S. pneumoniae* infections caused 100,000-135,000 hospitalizations for pneumonia, 6 million cases of otitis media, and 60,000 cases of invasive disease, including 3300 cases of meningitis. CDC reported 60,000 cases of invasive pneumococcal disease in 1997, resulting in approximately 6,000 deaths. The incidence of sterile-site infections has shown geographic variation from 21 to 33 cases per 100,000 populations. Disease figures are now changing due to conjugate vaccine introduction. In 2002, the rate of invasive disease was 13 cases per 100,000 in the United States. However, epidemics of disease have reappeared in settings such as chronic care facilities, military camps and day care centers, a situation not recognized since the pre-antibiotic era.

Also of concern, is the increased emergence of antibiotic resistance, especially in the past two decades. Multiple antibiotic resistant strains of *S. pneumoniae* that emerged in the early 1970s in Papua New Guinea and South Africa were thought to be a fluke, but multiple antibiotic resistances now covers the globe and has rapidly increased since 1995. Increases in penicillin resistance have been followed by resistance to cephalosporins and multidrug resistance. The incidence of resistance to penicillin increased from <0.02 in 1987 to 3% in 1994 to 30% in some communities in the United States and 80% in regions of some other countries in 1998. Resistance to other antibiotics has emerged simultaneously: 26% resistant to trimethoprim-sulfa, 9% resistant to cefotaxime, 30% resistant to macrolides, and 25% resistant to multiple drugs. Resistant organisms remain fully virulent but seem to have arisen in less than 10 serotypes. Serotypes 6A, 6B, 9V, 14, 19A and 23F are included in the vast majority of resistant strains ((Todar, 2011).

3.6.2 *Haemophilus influenza*

Haemophilus influenza, formerly called Pfeiffer's bacillus or *Bacillus influenzae*, Gram-negative, pleomorphic coccobacilli first described in 1892 by Richard Pfeiffer during an influenza pandemic. A member of the Pasteurellaceae family, it is generally aerobic, but can grow as a facultative anaerobe. *H. influenza* was mistakenly considered to be the cause of influenza until 1933, when the viral etiology of the flu became apparent.

It occurs in both encapsulated and non encapsulated forms. Serologic typing is based on the antigenicity of the capsular polysaccharides. Of the 6 serotypes, type b causes most of the severe, invasive diseases such as meningitis and septicemia. Polyribosyl ribitol phosphate (PRP) capsule is an important virulence factor which renders type b *H influenzae* resistant to phagocytosis by PMNs in the absence of specific ant capsular antibody.

Haemophilus influenzae type b is a bacterium estimated to be responsible for some three million serious illnesses and an estimated 386 000 deaths per year, chiefly through meningitis and pneumonia. Almost all victims are children under the age of

five, with those between four and 18 months of age especially vulnerable. In developing countries, where the vast majority of Hib deaths occur, pneumonia accounts for a larger number of deaths than meningitis. However, Hib meningitis is also a serious problem in such countries with mortality rates several times higher than seen in developed countries; it leaves 15 to 35% of survivors with permanent disabilities such as mental retardation or deafness (CDC, 1997).

Haemophilus influenzae is part of the indigenous flora of the upper respiratory tract. Various percentages have been reported for asymptomatic carriers *of H influenza*, which vary from country to country. Encapsulated organisms type penetrates the epithelium of the nasopharynx and invades blood capillaries directly; non typable are less invasive, but they, as well as typable strains, induce an inflammatory response that cause disease. Antibody directed against PRP capsule of type b *H influenza* is primarily responsible for host resistace to infection (Baron, 1998).

Most strains of H. influenza are opportunistic pathogens; that is, they usually live in their host without causing disease, but cause problems only when other factors (such as a viral infection or reduced immune function) create an opportunity. Naturally-acquired disease caused by *H. influenzae* seems to occur in humans only. Non typable H influenza is a prominent cause of acute tracheobronchitis or pneumonia in patients who have underlying chronic bronchitis, emphysema, or obstructive pulmonary disease. Blood cultures are positive in 10 to 15 percent of patients with *Haemophilus pneumonia* (Barlett et al., 1998).

Hib is preventable highly effective vaccines have been available since the early 1990s. Yet hundreds of thousands of children die year after year from Hib disease. The two major obstacles to prevention of Hib disease are a shortage of information and a shortage of money. Eighty-nine countries offered infant immunization against Hib by the end of 2004, with two of those countries providing it in parts of their territories. Ninety-two percent of the populations of developed countries were vaccinated against Hib as of 2003. The vaccination coverage was 42% for developing countries, and 8% for least-developed countries (the majority of which are in sub-Saharan Africa). The incidence of Hib in Southeast Asia has been a matter of debate, and vaccine use there is very low. Malaysia, the first Asian country to introduce the vaccine, began a regular Hib vaccination programme in 2002 (CDC, 2005, WHO, 2011).

Treatment of Hib is through an intensive, sustained course of antibiotics, but this is not always accessible to poor populations in developing countries. Resistance of Hib to several of the more inexpensive but effective antibiotics is a growing cause of concern and provides additional impetus for expanding vaccine coverage.

3.6.3 *Staphylococcus aureus*

S aureus is a gram-positive coccus that is both catalase- and Coagulase-positive. Colonies are golden and strongly hemolytic on blood agar. They produce a range of toxins, including alpha-toxin, beta-toxin, gamma-toxin, delta-toxin, exfoliatin, enterotoxin, Panton-Valentine leukocidin (PVL), and toxic shock syndrome toxin–1 (TSST-1). The enterotoxins and TSST-1 are associated with toxic shock syndrome. PVL is associated with necrotic skin and lung infections and has been shown to be a major virulence factor for pneumonia and osteomyelitis.

Coagulase-negative staphylococci, particularly *S epidermidis*, produce an exopolysaccharide (slime) that promotes foreign-body adherence and resistance to phagocytosis. *S. aureus* can cause a range of illnesses from minor skin infections such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, and scalded skin syndrome, and abscesses, tolife-threateningdiseases such as pneumonia, meningitis, osteomyelitis, endocarditis, endocarditis, toxic shock syndrome (TSS), chest pain, bacteremia, and sepsis (Forbes et al., 2007).

Its incidence is from skin, soft tissue, respiratory, bone, joints, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections, often causing postsurgical wound infections. Each year, some 500,000 patients in American hospitals contract a staphylococcal infection.

It is less common as a cause of CAP, incidence being less than 1% but is very important because of its pathogenic potential. It can cause sudden devastating illness that culminates in death unless treated. There are two forms – encapsulated and un encapsulated. The encapsulated forms are better able to gain access the bloodstream and to produce bacteremia. Paradoxically, if unencapsulated forms do gain access to blood, they are more likely to produce the symptoms of shock or DIC, in the same way gram negative sepsis.

Staphylococcus aureus is even more important cause of hospital acquired pneumonia, being only second to *Pseudomonas aeruginosa*. *Staphylococcus aureus* solely account

for 20 to 30% of HAP. Methicillin-resistant *S. aureus* abbreviated MRSA and often pronounced "mer-sa", is one of a number of greatly-feared strains of *S. aureus* which have become resistant to most antibiotics. MRSA strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections. Over the past several decades, infections with Methicillin Resistant *Staphylococcus aureus* (MRSA) among hospitalized patients have become common. Recently, MRSA infections acquired outside of the hospital setting have been increasingly reported.

Staphylococcal pneumonia should be suspected in any severe pneumonia and in less severe pneumonia cases if response to anti pneumococcal antibiotics is unsatisfactory, evidence of progressive sepsis or radiological evidence of cavitation, abscess formation, pneumatocele or pneumothorax or if there is sign of metastatic abscess. Blood culture during high spikes of fever gives 25-33% yield on Staphylococcus pneumonia (Croften and Douglas, 2000).

Early and aggressive use of effective antimicrobial marks the cornerstone of treatment; despite treatment mortality of this form of pneumonia is still 25-50 %. Serious staph infections are more common in people with weak immune systems. This includes patients in hospitals and long-term care facilities and those receiving kidney dialysis (Croften and Douglas, 2000).

MRSA infections are grouped into two types:

Healthcare-associated MRSA (HA-MRSA) infections occur in people who are or have recently been in a hospital or other health-care facility. Those who have been hospitalized or had surgery within the past year are at increased risk. MRSA bacteria are responsible for a large percentage of hospital-acquired Staphylococcal infections.

Community-associated MRSA (CA-MRSA) infections occur in otherwise healthy people who have not recently been in the hospital. The infections have occurred among athletes who share equipment or personal items (such as towels or razors) and children in daycare facilities. Members of the military and those who get tattoos are also at risk. The number of CA-MRSA cases is increasing.

3.6.4 *Klebsiella pneumonia*

Klebsiella pneumoniae is among the most common gram-negative bacteria encountered by physicians worldwide. It is a common hospital-acquired pathogen, causing urinary tract infections, nosocomial pneumonia, and intra abdominal infections. *K. pneumoniae* is also a potential community-acquired pathogen. In this international collaborative study, we evaluated geographic differences and trends in three prominent presentations of community-acquired Klebsiella infection.

The genus *Klebsiella* belongs to the tribe Klebsiellae, a member of the family Enterobacteriaceae. The organisms are named after Edwin Klebs, a 19th century German microbiologist. *Klebsiella pneumoniae* are non motile, rod-shaped, gram-negative facultative anaerobic enteric bacteria with a prominent polysaccharide capsule. This capsule encases the entire cell surface, accounts for the large appearance of the organism on gram stain, and provides resistance against many host defense mechanisms. *Klebsiella spp* is the most common group of enteric gram negative bacilli to cause CAP and is also commonly implicated in nosocomial pneumonia (Croften and Douglas 2000).

Though it accounts for a small percentage of pneumonia cases, Klebsiella produces an extensive damage resulting in high case fatality rates which could reach up to 68% (Croften and Douglas 2000). This high mortality despite treatment is attributed at least in part to old age*; K pneumoniae* and *K oxytoca* are the 2 members of this genus responsible for most human infections. They are opportunistic pathogens found in the environment and in mammalian mucosal surfaces. The principal pathogenic reservoirs of infection are the gastrointestinal tract of patients and the hands of hospital personnel. Organisms can spread rapidly, often leading to nosocomial outbreaks.

Infection with *Klebsiella* organisms occurs in the lungs, where they cause destructive changes. Necrosis, inflammation, and hemorrhage occur within lung tissue, sometimes producing thick, bloody, mucoid sputum described as currant jelly sputum. The illness typically affects middle-aged and older men with debilitating diseases such as alcoholism, diabetes, or chronic bronchopulmonary disease. This patient population is believed to have impaired respiratory host defenses. The organisms gain access after the host aspirates colonizing oropharyngeal microbes into the lower respiratory tract.

Klebsiella spp. have also been incriminated in nosocomial infections. Common sites include the urinary tract, lower respiratory tract, biliary tract, and surgical wound sites. The spectrum of clinical syndromes includes pneumonia, bacteremia, thrombophlebitis, urinary tract infection (UTI), cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, and meningitis. The presence of invasive devices, contamination of respiratory support equipment, use of urinary catheters, and use of antibiotics are factors that increase the likelihood of nosocomial infection with Klebsiella species. Sepsis and septic shock may follow entry of organisms into the blood from a focal source.

K oxytoca has been implicated in neonatal bacteremia, especially among premature infants and in neonatal intensive care units. Increasingly, the organism is being isolated from patients with neonatal septicemia.

Extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of klebsiellae and, subsequently, the development of multidrug-resistant strains that produce extended-spectrum beta-lactamase (ESBL). These strains are highly virulent, show capsular type K55, and have an extraordinary ability to spread. Most outbreaks are due to a single clone or single gene; the bowel is the major site of colonization with infection of the urinary tract, respiratory tract, and wounds. Bacteremia and significant increased mortality have resulted from infection with these species.

In addition to prior antibiotic use, risk factors for infection include the presence of an indwelling catheter, feeding tube, or central venous catheter; poor health status; and treatment in an intensive care unit or nursing home. Acquisition of these species has become a major problem in most hospitals because of resistance to multiple antibiotics and potential transfer of plasmids to other organisms

Outbreaks of neonatal septicemia occur worldwide. Infection with *K pneumoniae* also has a worldwide distribution. *Klebsiellae pneumonia* cause as many as 14% of cases of primary bacteremia, second only to *Escherichia coli* as a cause of gram-negative sepsis. They may affect any body site, but respiratory infections and UTIs predominate. Of 145 reported epidemic outbreaks of nosocomial bacteremia during 1983-1991, 13 were attributed to Klebsiella organisms. The US Centers for Disease Control and Prevention report that Klebsiella strains were responsible for 3% of all pathogenic epidemic outbreaks.

*Klebsiella pneumonia*e is a necrotizing process with a predilection for debilitated people. It has a high mortality rate of approximately 50% even with antimicrobial therapy. The mortality rate approaches 100% for persons with alcoholism and bacteremia.

Klebsiella bacteremia and sepsis produce clinical manifestations similar to those caused by other gram-negative enteric organisms. Morbidity and mortality rates are comparable to those for other gram-negative organisms that cause sepsis and septic shock. In neonatal units, outbreaks caused by ESBL-producing strains present a more serious problem and may be associated with increased mortality.

3.6.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a common inhabitant of soil, water, and vegetation. It is found on the skin of some healthy persons and has been isolated from the throat (5 percent) and stool (3 percent) of non hospitalized patients. In some studies, gastrointestinal carriage rates increased in hospitalized patients to 20 percent within 72 hours of admission. Within the hospital, *P. aeruginosa* finds numerous reservoirs: disinfectants, respiratory equipment, food, sinks taps, toilets, showers and mops. Furthermore, it is constantly reintroduced into the hospital environment on fruits, plants, vegetables, as well by visitors and patients transferred from other facilities. Spread occurs from patient to patient on the hands of hospital personnel, by direct patient contact with contaminated reservoirs, and by the ingestion of contaminated foods and water.

The spread of *P. aeruginosa* can best be controlled by observing proper isolation procedures, aseptic technique, and careful cleaning and monitoring of respirators, catheters, and other instruments. Topical therapy of burn wounds with antibacterial agents such as silver sulfadiazine, coupled with surgical debridement, dramatically reduces the incidence of *P. aeruginosa* sepsis in burn patients. *Pseudomonas aeruginosa* is frequently resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and fluoroquinolins, resistant forms have developed.

The combination of gentamicin and carbenicillin is frequently used to treat severe Pseudomonas infections. Several types of vaccines are being tested, but none is currently available for general use. *Pseudomonas aeruginosa* as an etiologic agent of community-acquired pneumonia is rare. As a cause of CAP, *P aeruginosa*

typically affects patient who is immunocompromised by pre existing lower respiratory disease of a chronic nature (bronchiectasis, cystic fibrosis), blood dyscrasias or immunosuppressive therapy.

In contrast to the very low incidence of Pseudomonas pneumonia in community-acquired pneumonia, P pneumonia has been considered as an etiology in 21% to 38% of cases of hospital-acquired pneumonia. This is the most commonest cause of hospital acquired pneumonia (Croften and Douglas, 2000). Overall mortality rates for hospital-acquired pneumonia ranged from 42% to 75% (Arancibia F et al., 2002). The high mortality rate appears to be attributed to infection by *P aeruginosa*, in addition to underlying illness. In one small retrospective study, the estimated attributable mortality rate of ventilator-associated pneumonia caused by *P aeruginosa* was 40% to 50% (Brewer et al., 1996).

3.6.6 *Mycoplasma pneumoniae*

Mycoplasma pneumoniae is a type of atypical pneumonia. It is caused by the bacteria *M. pneumoniae.* This type of pneumonia usually affects people younger than 40. Various studies suggest that it makes up 15 - 50% of all pneumonia cases in adults and even more in school-aged children. People at highest risk for mycoplasma pneumonia include those living or working in crowded areas such as schools and homeless shelters, although many people who contract mycoplasma pneumonia have no identifiable risk factor. It usually produces mild upper respiratory tract disease; symptoms (fever, headache, malaise, cough) may develop weeks after infection and may persist for several weeks. Culture of *M pneumoniae* from sputum or a throat swab is possible, but can take 1-2 weeks and is insensitive.

Diagnosis is usually based on serologic tests, a fourfold increase in IgG titre is considered diagnostic of recent infection while sustained high levels may not be significant because they can persist for up to 1 year following infection.

3.6.7 *Chlamydia pneumoniae*

Chlamydial pneumoniae is non motile, gram negative coccoid bacteria.These are obligate intracellular parasites and exist in nature in two froms: an infectious particle called the elementary body (EB) and intracytoplasmic form called reticulate body

(RB) which take parts in replication and growth. *C. pneumoniae* is a common cause of pneumonia around the world. *C. pneumoniae* is typically acquired by otherwise healthy people and is a form of community-acquired pneumonia.

In addition to pneumonia, *C. pneumoniae* less commonly causes several other illnesses. Among these are meningoencephalitis, arthritis, myocarditis, and Guillain-Barré syndrome. Chlamydia causes approximately 5-15% of pneumonia cases and 6-9% of cases of CAP admitted to hospital. In most cases, people have 2-3 infections during their lifetime. C pneumoniae may cause approximately 5-15% of pneumonia cases (Kauppinen M et al., 1995).

Chlamydia pneumoniae is more strongly associated with the underlying chronic bronchitis. However, *Chlamydia pneumoniae* can cause a stubborn respiratory illness lasting several weeks or longer and tending to relapse after each course of antibiotics.

Diagnosis of *C pneumophila* is often difficult by culture method, Isolation of organism is made from the demonstration of a fourfold increase in IgG or IgM or a single IgM titre greater than 1:16 or IgG greater than 1:512.

Culture is positive in approximately 50% of cases. The specific LPS can be detected with the complement fixation (CF) test. PCR amplification is more sensitive than culture though not in commonly available

3.6.8 *Legionella pneumophila*

Legionella pneumophila is thin, pleomorphic Gram-negative bacilli, and is most commonly associated with pneumonia. *L pneumophila* has 15 serotypes In approximately decreasing order of clinical importance are *L pneumophila* serotype 1, about 50% of cases of legionnaire' disease (Forbes et al., 2007).

These are fastidious organisms, and require specific growth media. Organisms replicate in macrophages in the lower respiratory tract. *Legionella pneumophila* are ubiquitous and widely distributed in the environment. As a result, most individuals are exposed to *Legionella spp*; however, few develop few symptoms. Bacilli reside in surface and drinking water and are usually transmitted to humans in aerosols. Illness tends to affect middle aged and older people: with increased risk factor in COPD and with various types of immunosuppressant (Cancer, diabetes, dialysis patients, AIDS).

Bacteria bind to alveolar macrophages through the complement receptors and are engulfed into a phagosomal vacuole where they block phagolysosome fusion, thus preventing killing, and multiply freely in a high pH environment; eventually, the cell is destroyed, releasing a new generation of microbes (Forbes et al., 2007).

There are no reliable distinguishing clinical features present which cause the diagnosis in the lab difficult. Diagnosis is confirmed by culture, demonstration of bacterial antigen in body fluids, or serologic tests. Culture is preferred diagnostic method because it is sensitive and specific. Direct fluorescent antibody (DFA) is specific but has poor sensitivity. A commercially available radioimmunoassay for bacterial antigen in urine is satisfactory, but is available only for serogroup 1 of *L pneumophila.*

3.6.9 *Mycobacterium tuberculosis*

M tuberculosis is the etiologic agent of tuberculosis. Tuberculosis is the leading cause of death world from a single infectious disease. The disease affects nearly 2 billion yearly which is equal to one third of the population. The bacteria usually attack s the lungs but it can attack any part of the body such as kidney, spine, brain (extra-pulmonary TB). If not treated properly, TB disease can be fatal. Extra-pulmonary TB is much less common than the pulmonary, which occurs more frequently among persons with HIV. The tuberculosis crisis is likely to escalate since the HIV epidemic has triggered an even greater increase in the tuberculosis cases. The majority of tuberculosis patients are of age group between 15-45, persons in their most productive years of life. Tuberculosis kills over 2 million people worldwide yearly, more than any other single infectious disease including AIDS and malaria. Pulmonary tuberculosis is the form of disease with the highest rate of morbidity and mortality.

Based on surveillance and survey data, WHO estimates that 9.27 million new TB occurred in 2007 (139 per 100000 population), compared with 9.24 million new cases (140 per 100000 population) in 2006. Of these 9.27 million new cases, an estimated 44% or 4.1 million (61 per 100000 population) were new smear positive cases. Asia (South- East Asia and Western pacific regions) accounts for 55% of global cases, and Africa accounts for 31%, the other regions account for relatively small fractions of global cases.

An estimated 1.32 million HIV-negative people (19.7 per 100000 population) died from TB in 2007, and there were an additional 456000 (0.45 million) TB deaths among HIV-positive people. Deaths from TB among HIV-positive people account for 23% of the estimated 2 million HIV deaths that occurred in 2007.

There were an estimated 0.5 million cases of Multidrug Resistance TB (MDR-TB) cases in 2007.Of the 0.5 million cases of MDR-TB in 2007, 68% were smear-positive. Although the total number of incident cases of TB is increasing in absolute terms globally as a result of population growth, the three major indicators of impact-incidence, prevalence and mortality rate per 100000 populations are falling globally. Prevalence rate is falling at a faster rate than TB incidence.

3.7 Complication of LRTI

The complication of Lower respiratory tract infections are of two types .

3.7.1 Complication at acute phase of the infection

3.7.1.1 Pleural effusion

Pleural effusion is excess fluid that accumulates in the pleura, the fluid-filled space that surrounds the lungs. Excessive amounts of such fluid can impair breathing by limiting the expansion of the lungs during respiration.

3.7.1.2 Bronchiectasis:

Bronchiectasis is destruction and widening of the large airways. Bronchiectasis is often caused by recurrent inflammation or infection of the airways. It most often begins in childhood as a complication from infection or inhaling a foreign object. If the condition is present at birth, it is called congenital bronchiectasis. If it develops later in life, it is called acquired bronchiectasis. Bronchiectasis is an uncommon disease, most often secondary to an infectious process that results in the abnormal and permanent distortion of one or more of the conducting bronchi or airways. Bronchiectasis has undergone significant changes in regard to its prevalence, etiology, presentation, and treatment.

Bronchiectasis can be categorized as a chronic obstructive pulmonary disease manifested by airways that are inflamed and easily collapsible, resulting in air flow obstruction with shortness of breath, impaired clearance of secretions (often with

disabling cough), and occasionally hemoptysis. Severe cases can result in progressive impairment with respiratory failure.

Diagnosis is usually based on a compatible clinical history of chronic respiratory symptoms, such as a daily cough and viscid sputum production (see Clinical), and characteristic radiographic findings on CT scans, such as bronchial wall thickening and luminal dilatation.

Antibiotics and chest physiotherapy are the mainstay modalities. Additionally, management of underlying conditions, such as hypogammaglobulinemia or alpha1-antitrypsin deficiency, is essential to the overall treatment. Surgery is an important adjunct to therapy in some patients with advanced or complicated disease. Bronchiectasis is an abnormal dilation of the proximal and medium-sized bronchi (>2 mm in diameter) caused by weakening or destruction of the muscular and elastic components of the bronchial walls. Affected areas may show a variety of changes, including transmural inflammation, edema, scarring, and ulceration, among other findings. Distal lung parenchyma may also be damaged secondary to persistent microbial infection and frequent postobstructive pneumonia. Bronchiectasis can be congenital but is most often acquired. Congenital bronchiectasis usually affects infants and children.

Acquired forms occur in adults and older children and require an infectious insult, impairment of drainage, airway obstruction, and/or a defect in host defense. The tissue is also damaged in part by the host response of neutrophilic proteases, inflammatory cytokines, nitric oxide, and oxygen radicals. This results in damage to the muscular and elastic components of the bronchial wall. Additionally, peribronchial alveolar tissue may be damaged, resulting in diffuse peribronchial fibrosis.

The result is abnormal bronchial dilatation with bronchial wall destruction and transmural inflammation. The most important functional finding of altered airway anatomy is severely impaired clearance of secretions from the bronchial tree.

Impaired clearance of secretions causes colonization and infection with pathogenic organisms, contributing to the purulent expectoration commonly observed in patients with bronchiectasis. The result is further bronchial damage and a vicious cycle of

bronchial damage, bronchial dilation, and impaired clearance of secretions, recurrent infection, and more bronchial damage.

3.7.1.3 Lungs abscess:

Lung abscess is defined as necrosis of the pulmonary tissue and formation of cavities containing necrotic debris or fluid caused by microbial infection. The formation of multiple small (< 2 cm) abscesses is occasionally referred to as necrotizing pneumonia or lung gangrene. Both lung abscess and necrotizing pneumonia are manifestations of a similar pathologic process. Failure to recognize and treat lung abscess is associated with poor clinical outcome.

Lung abscesses can be classified based on the duration and the likely etiology. Acute abscesses are less than 4-6 weeks old, whereas chronic abscesses are of longer duration. Primary abscess is infectious in origin, caused by aspiration or pneumonia in the healthy host; secondary abscess is caused by a preexisting condition (eg, obstruction), spread from an extra pulmonary site, bronchiectasis, and/or an immunocompromised state. Lung abscesses can be further characterized by the responsible pathogen, such as Staphylococcus lung abscess and anaerobic or Aspergillus lung abscess.

Most frequently, the lung abscess arises as a complication of aspiration pneumonia caused by mouth anaerobes. The patients who develop lung abscess are predisposed to aspiration and commonly have periodontal disease. Bacterial inoculums from the gingival crevice reaches the lower airways, and infection is initiated because the bacteria are not cleared by the patient's host defense mechanism. This results in aspiration pneumonitis and progression to tissue necrosis 7-14 days later, resulting in formation of lung abscess.

Published reports since the beginning of the antibiotic area have established that anaerobic bacteria are the most significant pathogens in lung abscess. In a study by Bartlett et al in 1974, 46% of patients with lung abscesses had only anaerobes isolated from sputum cultures, while 43% of patients had a mixture of anaerobes and aerobes. Aerobic bacteria that may infrequently cause lung abscess include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* (rarely), *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Actinomyces species*, *Nocardia species*, and

gram-negative bacilli. Aerobic organisms, frequently hospital acquired, are associated with poor outcomes. A retrospective study reported the overall mortality rate of lung abscesses caused by mixed gram-positive and gram-negative bacteria at approximately 20%.

Challenging current expert opinion, a study by Wang et al suggested that the bacteriologic characteristics of lung abscess have changed. In a series of 90 patients with community-acquired lung abscess in Taiwan, anaerobes were recovered from just 28 patients (31%); the predominant bacterium was *K pneumoniae*, in 30 patients (33%). Another significant finding was that the rate of resistance of anaerobes and *Streptococcus milleri* to clindamycin and penicillin increased compared with previous reports.

Nonbacterial and atypical bacterial pathogens may also cause lung abscesses, usually in the immunocompromised host. These microorganisms include parasites (eg, *Paragonimus* and *Entamoeba species*), fungi (eg, Aspergillus, Cryptococcus, Histoplasma, Blastomyces, and Coccidioidesspecies), and Mycobacterium species.

Most patients with primary lung abscess improve with antibiotics, with cure rates documented at 90-95%. Host factors associated with a poor prognosis include advanced age, debilitation, malnutrition, human immunodeficiency virus infection or other forms of immune suppression, malignancy, and duration of symptoms greater than 8 weeks. The mortality rate for patients with underlying immunocompromised status or bronchial obstruction who develop lung abscess may be as high as 75%.

3.7.1.4 Empyema thoracis

The lungs abscess may rupture into pleural space and the effusion in pleural space may become grossly purulent (contains bacteria and white blood cells) thereby leading to empyema thoracis.

3.6.1.5 Pneumothorax

The sub pleural emphysematous bullae may rupture into the pleural space resulting in the formation of spontaneous pneumothorax. Other complications are,

-Fluid and electrolyte hypoatremia

-Anemia, thrombocytopenia

-Hepatic jaundice

-Azotemia (nitrogenous products in the blood)

-Septicemia, Meningitis, Osteomyelitis and Metastatic abscess in different parts of body etc.

3.6.2 Chronic complication

Chronic cases usually manifested as a decrease in arterial $PO_{2.}$ due to thickening of alveolar membrane and widening of interstitial space due to fibrosis. Blood vessels become entrapped in the fibrotic tissue which leads to development of pulmonary hypertension. Non affected arterioles may also constrict due to persisting hypoxia and hypercapnia. Respiratory failure that leads to the decrease of partial pressure of oxygen in blood less than 60 mm of Hg with or without hypercarpnia. Bronchial carcinoma may develop from cicatrized (fibrosed) tissue.

3.6.3 Clinical Symptoms of LRTI

The presenting symptoms of respiratory diseases are few, but the structural and functional disturbances which these symptoms reflect are numerous. Dysponea (shortness of breath) and cough are the primary presenting symptoms for patients with respiratory system disease. Less common symptoms include haemoptysis (the coughing up of blood) and chest pain, often with a pleuritic quality.

3.6.3.1 Dysponea

Difficulty in breathing or breathlessness is a subjective symptom and is related to undue consciousness for respiratory effort, typically associated with some form of heart or lung disease, also known as air hunger. The clinical analysis of dysponea relies on characteristics of breathlessness: its timing, and the circumstances which precipitate or relieve it.

3.6.3.2 Coughing

Some features of cough are of diagnostic significance. Persistent cough is most commonly observed in cigarette smoking adult population. In non- smoking

population persistent cough is usually due to asthma. Exposure to dusts and fumes at work is now well recognized as a cause of persistent cough, even in non- smokers. Tuberculosis, Bronchiectasis, Cystic fibrosis, sinus and laryngeal disease, lymphoma, pulmonary fibrosis and pulmonary oedema are other causes of persistent cough (Seaton, 2000).

3.6.3.3 Haemoptysis

It is defined as expectoration of blood. Amount of blood in haemoptysis may vary from slight streaking of sputum with blood to massive haemorrhage. It can originate from disease of the airways, the pulmonary parenchyma, or the vasculature. The most common causes in most series are chronic bronchitis, bronchiectasis, and bronchogenic carcinoma followed by tuberculosis, fungal infections, bacterial pneumonia and abscess and pulmonary infection (Wilson et al., 2000).

3.6.3.4 Chest pain

Chest pain in disease of the respiratory system usually originates from involvement of the parietal pleura. As a result, the pain is accentuated by respiratory motion and is often referred to as pleuritic. Most conditions giving rise to pleuritic pain are acute and inflammatory in origin: either infective when there is usually associated pneumonia (plerisy is particularly common in pneumococcal pneumonia). Recurrent pleurisy at the same site should suggest bronchiectasis. If pleurisy progress to pleural effusion; the sharp pain largely disappears and is replaced by a dull and more constant ache or heaviness, quantitatively roughly proportional to the amount of fluid (Oxford Text book of Medicine, 3rd edition).

3.8 Diagnosis of LRTI

Nosocomial or hospital acquired pneumonia is a lower respiratory tract infection that appears during or after hospitalization in a patient who was not incubating the infection on admission. It is diagnosed by the following: clinical signs, pyrexia, usually purulent sputum, relevant X-ray changes and preferably microbiological diagnosis from bronchial lavage, transtracheal aspirate or protected brush culture.

3.8.1 Radiological Diagnosis

Chest radiograph can confirm the presence and location of the pulmonary infiltrates; assess the extent of the pulmonary infection; detect pleural involvement, pulmonary cavitation, or hilar lymphodenopathy in LRTI. However, chest radiographs may be normal when the patient is unable to mount an inflammatory response (eg in agranulocytosis). Findings vary with the type of LRTI.

3.8.2 Microbiological Diagnosis

A wide range of bacteria, viruses, fungi, and parasites colonize or infect the airways, lungs, and pleura. The choice and interpretation of laboratory tests for the isolation or detection of these organisms present clinicians with a demanding challenge. The role of the microbiology laboratory is to provide some clues to the likely causative organism. Unfortunately, in many cases, the causative organism is never isolated with certainty.

3.8.3 Choice of samples

3.8.3.1 Bronchoscopy specimens: Bronchoscopy specimens can be obtained by bronchialveolar lavage, protected specimen brush, or biopsy of lung tissue. This procedure is complicated to perform and expensive but mostly free from nasopharyngeal contamination. There is risk from bleeding and hypoxia which can sometimes require mechanical ventilation of a patient.

3.8.3.2 Ezpectorated sputum: Expectorated sputum is the most readily available sample of respiratory infection. It is a particularly attractive specimen for analysis as it can be obtained simply, at low cost, and without risk to the patient. However, it is liable to be contaminated with upper respiratory tract colonizers. Induced sputum is considered a valuable sample for the detection of some respiratory pathogens in patients who otherwise fail to expectorate sputum.

3.8.3.3 Transtracheal aspiration: It provides samples of respiratory secretions which are largely free from nasopharyngeal contamination. This procedure is helpful when bronchoscopy is not available, but is often unpleasant for the patient.

3.8.3.4 Lung fluid: It can be aspirated from the lung parenchyma for examination using a fine needle. This technique is conventionally used to aspirate fluid from peripherally located intrapulmonary cavities (abscess). It is often complicated and not common sample type.

3.8.3.5 Open-Lung biopsy: Samples provide the most informative specimens, but biopsy is too invasive to be performed routinely.

3.8.3.6 Pleural fluid or pleural biopsy: Specimen obtained by percutaneous needle provides reliable samples free from contamination with nasopharyngeal colonizing organisms.

3.8.3.7 Pleural fluid: Pleural fluid is obtained from chronically draining chest tube are not reliable, since chest drains rapidly become colonized with potential pathogens after insertion.

3.8.3.8 Blood: Blood culture provides highly reliable results and should be performed in ill patients suspected of infection. Organisms that cause pneumonia, such as *S pneumoniae, H influenzae, S aureus, Klebsiella pneumoniae and others* can be cultured from the blood of patients.

3.8.4 Sputum Microscopy

Hipporrates in the 15^{th} century BC. Was mentioned the importance of sputum examination. His observation of the sputum sample included color, taste, and smell for diagnostic criteria in the treatment of LRTI patients. In case of Pneumococcal pneumonia, the character of the sputum varies with the stage of the disease. In the early stage of typical lobar pneumonia, the sputum is scanty and transparent, with occasional blood flecks. As the disease progress to the red hepatization stage, the sputum becomes rust red, very tenacious and mucopurulent. In *Staphylococcal pneumoniae*, yellow, purulent, voluminous sputum is present.

3.8.5 Homogenization

If the received sputum is very thick, it will be difficult to make the smear on slides for microscopic examination and culture because pathogenic organisms are likely to be

trapped in the purulent part and escape from our diagnosis. So in such specimens, mucolytic agent can be used which consist of 0.1% dithiotheritol that hydrolyzes the bond between the glycoprotein in sputum. This sputum is lysed, homogenized and diluted. The method's hypothesis is that the organisms causing infection is presented in significant number than any other superficial contaminating organisms and overcome the effect of dilution than the contaminating ones.

3.8.6 Direct macroscopic examination of stained samples

Examination of the sputum remains the mainstay of the evolution of a patient with acute bacterial pneumonia. Unfortunately, expectorated material is frequently contaminated by potentially pathogenic bacteria that colonize the upper respiratory tract without actually causing disease. This contamination reduces the diagnostic specificity of any lower respiratory tract specimens. In addition, certain common pulmonary pathogens, such as anaerobes, mycoplasmas, Chlamydia, Pneumocystis. Mycobacteria, fungi, and legionellae, cannot be cultured by routine methods (Barlet et al., 1987).

Again, microscopic examination of sputum serves as an important guide to assess the suitability of the sample for the cultural studies. This is based on the cellular composition of the sputum. Presence of less than 10 epithelial cells and more than 25 leucocytes per low power field in the samples makes it a true representative of bronchial secretions and thereby improves the diagnostic value of culture (ASM criteria). Gram negative pneumonias are hard to diagnose initially on sputum examination. Gram stains of sputum may be confusing, since morphologically similar organisms are present in normal throat flora. Almost any of the gram negative aerobic organisms have the potential to cause disease of the lower respiratory tract, but the more common ones are Haemophilus, Klebsiella, Pseudomonas, Enterobacter, *E coli* etc. With the exception of the foul green sputum seen in Pseudomonas infection, no classic macroscopic findings are present in the sputum. Haemophilus being particularly important as a pathogen in adults with a diagnosis of chronic bronchitis or bronchiectasis.

3.9 Culture

After homogenization and microscopic examination the appropriate specimen is cultured in Chocolate agar, Blood agar, and MacConkey agar. Microscopic examination should be performed to determine if sputum is acceptable for culture. Numerous studies have shown that approximately 50% of submitted specimen are contaminated with or are oropharyngeal secretions.

In the laboratory, each specimen is routinely plated in Chocolate agar, Blood agar, and MacConkey agar. Chocolate agar plate is added with the 10 U Bacitracin and 5 mcg Optochin in primary and secondary inoculums respectively. Then plates are incubated at 37^0 C overnight with the chocolate agar plates in a 5% CO_2 atmosphere. The hypothesis underlying the streaking method is that the pathogenic organism will be present in greater numbers than any other superficial contaminating organisms. Specific identification of all pathogen is performed by standard methods. If no pathogens are present, the predominating organism or normal flora is reported. The culture should be correlated with the previous Gram stain. If many organism seen on Gram stain, but no growth or only scanty growth obtained on culture, then either the culture method was inadequate or the flora was suppressed by antibiotics giving special attention to anaerobes.

3.10 Antibiotic Sensitivity test

The disk- diffusion method also called Kirby-Bauer Disk-Diffusion Method is common routine test in a clinical laboratory where a large number of isolates are tested of susceptibility to numerous antibiotics. An agar plate is uniformly inoculated with the test organism and a paper disk impregnated with a fixed concentration of an antibiotic is placed on the agar surface. Growth of the organism and diffusion of the antibiotic commence simultaneously resulting in a circular zone of inhibition in which the amount of antibiotic exceeds inhibitory concentrations. The diameter of the inhibition zone is a function of the amount of drug in the disk and susceptibility of the microorganism.

Standardization of this test also depends on the size of inoculums, composition of medium, temperature of incubation, degree of moisture and thickness of the agar. If these conditions are uniform, reproducible tests can be obtained and zone diameter is

only a function of the susceptibility of the test organism. Zone diameter can be correlated with susceptibility as measured by the dilution method. Further correlations using zone diameter allow the designation of an organism as “susceptible”, “intermediate”, or “resistant” to concentrations of an antibiotic which can be attained in the blood or other body fluids of patients requiring chemotherapy.

3.11 Identification of isolated organism

Standard biochemical tests are performed for proper identification of causal organism. In cases with difficult diagnosis, serologic tests can be performed.

3.12 Serological tests

Serological tests can be used for pulmonary infection by a range of pathogens late in the infection. This test is more useful in testing for mycoplasma and cytomegalovirus infection and other herpes viral infection. The fourfold rise in antibody levels between an acute and convalescent serum sample is the usual approach to making a serological diagnosis. This form of diagnosis is useful for viral, mycoplasmal, chlamydial, Q fever and *legionella spp*. infections. In some other infection like Histoplasmosis, Coccidioidomycosis, Filariasis, and Echinococcus have been used. In these infections, the presence of antibody suggests the presence of active infection by these pathogens.

3.13 Molecular Technique

The amplification of species-specific DNA sequences using the polymerase chain reaction offers an attractive new approach to detecting the presence of a pathogen. Therefore, when used as a test of respiratory infection, its potential is limited to infections caused by organisms which are not associated with colonization of the upper respiratory tract. Also, slow growing or non-cultivable organisms may be detected more rapidly with the use of this test. However, it may be many years before the polymerase chain reaction or other DNA amplification techniques are routinely used in the diagnosis of respiratory infectious disease.

3.14 MDR and ESBL production

Though the drug resistance happens even with the appropriate use of antibiotics, but their inappropriate use makes the problem much worse. According to the US centre for Disease Control and Prevention (CDC) estimated that more than 40% of antimicrobial courses prescribed in by physician's offices were inappropriate. According to the clinical practice guideline, antimicrobial treatment of adults with nonspecific respiratory tract infection neither resolves nor prevents complications and widespread use of broad-spectrum antibiotics and the emergence of multidrug-resistant bacterial pathogens have become a vicious cycle so it should be avoided. The emergence of resistance to antimicrobial agents is a global public health problem. Resistance to antibiotics may develop by means of chromosomal mutation, by the acquisition of a transferable resistance plasmid or transposon, by the capture by a an integron of antibiotic-resistance genes that are parts of discrete mobile cassettes, or possibly by interspecies genetic transformation, as when the DNA of closely related Streptococcal species generates the mosaic penicillin-binding protein genes of penicillin-resistant *pneumococci*. Many of these genes were found in nature before the introduction of antibiotics. Extensive use of antimicrobial agents favors the resistant strains by eliminating more susceptible competitors. Antibacterial drugs are powerful weapons when used reasonably against infectious targets, but when they are improperly used for nonspecific symptoms or infection that is probably viral; their use may only contribute to bacterial resistance. The defining criteria for MDR in this study were resistance to ≥ 3 of the antimicrobial agents (Antimicrobial Agents and Chemotherapy, August 2002).

3.15 Extended Spectrum of Beta Lactamase (ESBL)

The term refers to beta lactamase enzymes produced mainly by some species of Gram negative bacteria mainly *Escherichia coli* and *Klebsiella pneumoniae* that encode for resistance to broad-spectrum beta lactam antibiotics that normally have activity against Gram-negative bacilli. These antibiotics are the third generation ciphalosporins like cefotaxime, ceftriaxone, ceftadime, and aztreonam and cefpodoxime. These enzymes are not active against the cephamycins, and are inhibited by clavulanic acid. Microorganisms which produce ESBLs often carry resistance to other classes of antibiotics as well. All ESBL producing organisms

should be considered resistant to all penicillins, cephalosporins and aztreonam. β lactamase is the enzyme hydrolyse the β lactam ring of penicillins, cephalosporins, and related antimicrobial drugs, rendering them inactive. There are a number of β-lactamases, which vary in substrate specificity and host range (Bushet et al., 1995).

Genes encoding these extended-spectrum β- lactamases were typically carried on self-transferable plasmisds that often carried other determinants of antibiotic resistance. Since these genes may be located on transposable elements, they may move into various plasmids, permitting the dissemination of extended-spectrum β- lactamases among gram-negative bacilli. Studies of outbreaks of nosocomial infections with Enterobacteriaceae that produce extended-spectrum β- lactamases suggest that these strains arose in response to the selective pressure created by the use of extended-spectrum cephalosporins. The first hospital outbreak of an ESBL-containing Gram-negative organism was reported in Germany in 1983 (Knothe et al., 1983). Nosocomial outbreaks caused by multi drug resistant Klebsiella clone carrying a TEM-3 gene were described within a year in France (Brun-Buisson, et al., 1987). Over the past decades, ESBL containing Enterobacteriaceae have emerged as serious nosocmial pathogens throughout world mainly Europe and United states.

3.16 Detection of ESBL

Clinical Laboratory Standards Institute (CLSI) has developed broth microdilution and disk diffusion screening tests using selected antimicrobial agents (1). Each *Klebsiella pneumoniae*, *K. oxytoca*, or *Escherichia coli* isolate should be considered a potential ESBL-producer if the test results are as follows:

The sensitivity of screening for ESBLs in enteric organisms can vary depending on which antimicrobial agents are tested. The use of more than one of the five antimicrobial agents suggested for screening will improve the sensitivity of detection. Cefpodoxime and ceftazidime show the highest sensitivity for ESBL detection. CLSI recommends performing phenotypic confirmation of potential ESBL-producing isolates of *K. pneumoniae*, *K. oxytoca*, or *E. coli* by testing both cefotaxime and ceftazidime, alone and in combination with clavulanic acid (1). Testing can be performed by the broth microdilution method or by disk diffusion. For MIC testing, a decrease of $\geq$ 3 doubling dilutions in an MIC for either cefotaxime or ceftazidime

tested in combination with 4 µg/ml clavulanic acid, versus its MIC when tested alone, confirms an ESBL-producing organism. For disk diffusion testing, a $\geq$ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL-producing organism (CLSI, 2009).

CHAPTER IV

4. MATERIALS AND METHODS

The descriptive study was done to determine the bacterial etiology of acute Lower Respiratory Tract Infection in the patients visiting NPHL (National Public Health Laboratory). The laboratory works was carried out in the Microbiological laboratory of NPHL. The specimen was collected from the patients visiting the NPHL and the study was conducted from first of Jesth to 15th of Poush last 2067.

4.1 Specimen collection and transport

Specimen is collected with the supervision by professional health personnel familiar with the methods for obtaining proper clinical correlations. Specimens labeled "sputum" seldom contained lower respiratory tract secretions only. Saliva, nasopharyngeal secretion, commensal bacteria of upper respiratory tract and food particles often contaminated the specimen. For the best examinations, first morning specimen was preferred. Sputum specimen submitted stringently for AFB staining was collected for the study by mutual consent with patients. Those sputum specimens requested strictly for AFB staining were considered for the study. A specimen was collected in a sterile, disposable, impermeable container with a screw cap or tightly fitting cap or cork or lid. After the patient expectorated the sputum into the container, a routine care was taken to avoid any sputum that has been smeared by the patient on the outside surface of the container. The patient's clinical history was taken during the time of sample collection. The samples were collected only from the patient who did not have any treatment during that time. The data collection form is given in appendix I. After receiving the sample in the sample collection site, it was immediately transported to the microbiological laboratory and processed further following standard bacteriological procedures within 4 hours.

4.2. Inclusion Criteria

The inclusion criteria for a case were sputum fulfilling the criteria of American Society for Microbiology as:

Any person of any age and sex complaining of chest pain; and/or shortness of breath with or without wheezing; and /or coughing; and /or haemoptysis with one or more constitutional symptoms including fever, sweating, weight loss etc.

Patients diagnosed as acute exacerbation of chronic obstructive pulmonary disease, bronchiectasis and those having radiological evidence of consolidation, pleural effusion, empyema, and chronic debilitated patient from intensive care unit suspected of aspiration and other type of pneumonia were also included in our study. According to the ASM criteria a reliable lower respiratory tract specimen has more than 25 leucocytes and fewer than 10 epithelial cells per low power field.Specimen collected for the study was only the sputum. During the study period, all together 250 samples were collected from the LRTI suspected cases age range from 9 to 84 years.

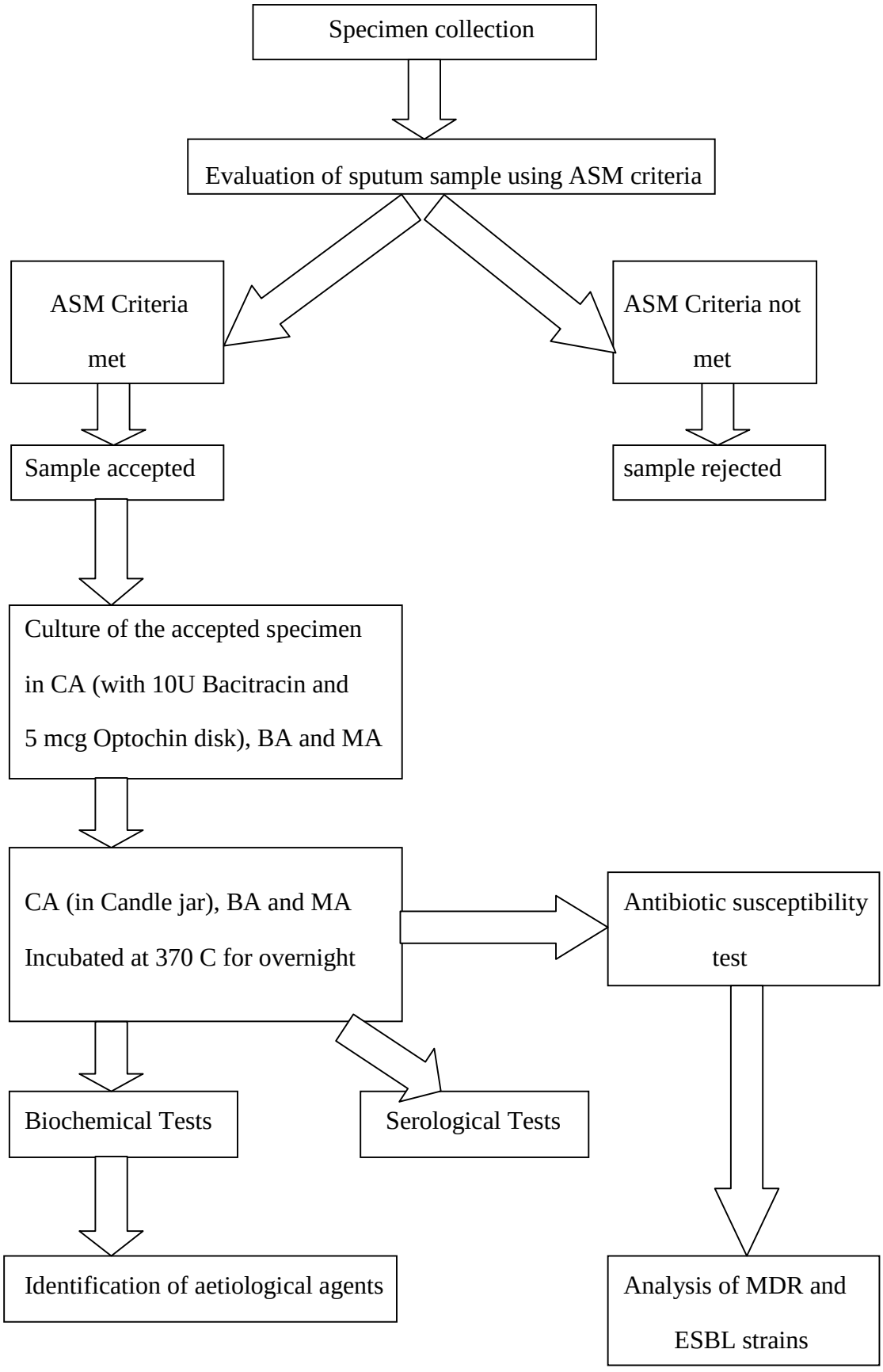
4.3 Processing of Specimen

The specimen that was received in laboratory was examined macrospically to check the contamination of saliva. If in case it was watery specimen, it was discarded and the report was sent to the physician stating that specimen was mainly saliva, or unsuitable for examination. Subsequently, the color, consistency, presence of blood, pus was noted.

4.4 Homogenization of the specimen

Specimens which were very thick and mucoid were first homogenized with commercially available sputasol containing 0.01% dithiotheritol. Non homogenous specimen contains non uniform distribution of organisms, but after homogenization every part of it will contain equal distribution of organisms. To homogenize, 5ml of sterile distilled water was added to the given vial containing sputalysin, dithiotheritol powder and mixed gently to dissolve the powder completely. The content was then added with aseptic precaution to 95 ml of sterile distilled water which makes 1:20 dilution. Then equal volume of diluted sputasol was added to the sputum sample aseptically, then mixed gently and was incubated at 37 degree Celsius for 30 minutes for complete homogenization of sputum.

METHODOLOGY



4.5 Microscopic Examination of sputum

4.5.1 Gram Staining

Purulent part of sputum was transferred to a clean, grease free glass slide. It was air dried: heat fixed and stained using Gram's stain technique, and observed under microscopy. The Gram stained sputum smear was especially observed to distinguish between gram-positive and gram-negative bacteria, which have distinct, consistent constituent difference in their cell walls.

In the Gram stain, the smear was first heat fixed and then stained with basic dye, crystal violet, which is taken up in similar amounts by all bacteria. The slides are then treated with an iodine mixture (mordant) to fix the stain, washed briefly with acetone decolorizer, and finally counterstained with a dye of different color (safranin). Gram-positive organisms retain the initial violet stain, while gram-negative organisms are decolorized by the organic solvent and hence show the pink counter stain. The difference between gram-positive and gram-negative bacteria lies in the ability of the cell wall of the organism to retain the crystal violet.

Examination of Gram stained smear of the specimen frequently reveals whether the specimen is satisfactory or not. According to the ASM criteria a reliable specimen has more than 25 leucocytes and fewer than 10 epithelial cells per low power field. If the guidelines were met then smear was examined under 100X magnification and various fields were examined. During observation various factors including morphology of the organism, stain taken by the organism, uniformity of the staining, pure or mixed form of organism, arrangement of the organism, number of the organism whether plenty or scanty etc. were noted.

4.5.2 AFB Staining

A thin sputum smear was made on a clean, grease free glass slides taking purulent portion of the specimen. It was air dried, alcohol fixed and stained with Ziehl-Neelsen acid fast stain. Thus prepared sputum slide was examined under high power microscopy for the presence of any acid fast bacilli.

4.6 Sputum Culture

The media used in our purpose to isolate the commonly sought etiological agents of lower respiratory tract infections were Chocolate agar, Blood agar and MacConkey agar. With the help of the wire loop, the homogenized specimen was inoculated in these three media. In the chocolate agar plate, 10U Bacitracin disk and 5mcg Optochin disk were placed in primary and secondary zone respectively to screen out *Haemophilus influenza* and *Streptococcus pneumoniae* then was incubated at 37 degree Celsius for overnight in candle jar. MacConkey and blood agar on the other hand were incubated in aerobic condition.

4.7 Isolation of the pathogens

Culture plates after overnight incubation were observed for the presence of growth. Any significant growth obtained on the primary plates was first sub-cultured to obtain pure culture for further processing.

4.8 Identification of the sputum isolates

The isolates were identified according to standard microbiology procedures. Biochemical tests were performed for identification of medically important bacteria, and serological tests in some cases.

4.9 Biochemical tests used for the identification of pathogens

Different set of biochemical tests were done for the identification of the isolated bacteria. The pure form of the culture was obtained from the primary culture medium and then it was proceeded for biochemical tests detail of which is described in the appendix. Catalase test, Oxidase test, Indole production test, Methyl red test, Voges Proskauer (VP) test, Citrate test, Triple Sugar Iron Agar (TSI) test were performed and the pathogens were identified.

4.10 Bile Solubility test

A solution of 10% sodium deoxycholate was prepared in distilled water, was autoclaved and stored at room temperature. Emulsification of several colonies of suspected pneumococci from the blood or chocolate agar were inoculated into two test tubes containing 5 ml nutrient broth and was incubated at 37 degree Celsius for overnight. Then 0.5 ml (two drops) of bile salts solution was added in one tube while another was taken as control. Incubation was continued for 15 to 30 minutes. In the positive test turbidity was cleared when compared with the control tube (Monica, 2000).

4.11 Optochin Sensitivity test

A paper disk containing 5mcg of optochin was placed in the primary inoculums of a chocolate agar plate streaked with the pure culture of suspected pneumococci and the plate was incubated at 37 degree Celsius in candle jar for overnight. After incubation the plate was observed for zone of inhibition surrounding the disk.

4.12 Bacitracin Sensitivity test

A paper disk containing 10U of bacitracin was placed in the primary inoculums of a chocolate agar plate streaked with the material from the specimen and the plate was incubated at 37 ºC in candle jar for overnight, after incubation the plate was observed for the colonies of *H. influenza* growing near the bacitracin disk.

4.13 Satellitism Test

With the help of straight wire the pure culture of Haemophilus colonies were streaked horizontally on a blood agar plate. Then, from pure culture of *Staphylococcus aureus*, few colonies were streaked vertically and the plate was incubated at 37ºC for overnight. After incubation, the culture was examined for growth and satellite colonies in the neighbourhoods of Staphylococcal colonies. Growth of *Haemophilus influenzae* depend upon X and V factor. Both X and V factors are present in blood agar prepared with defibrinated horse blood. Growth, however, is greatly improved on

chocolate agar as more V factor is available. V factor is released from the red cells when the blood is heated at 75ºC.

4.14 Antibiotic Sensitivity testing of isolates

The antibiotic sensitivity testing was done by CLSI (Clinical And Laboratory Standard Institute) recommended Kirby- Bauer Sensitivity testing method. Sensitivity testing was done for all the 163 isolates. Mueller and Hinton agar was maintained at 4mm in Petri dishes. Using a sterile wire loop, a single isolated colony of which the sensitivity pattern was to be determined was touched and inoculated into a nutrient broth and was incubated for 2-4 hours. After incubation in light source, turbidity of the suspension was matched with the turbidity standard, Mac Farland 0.5. Using a sterile swab, a plate of Mueller and Hinton agar was inoculated using carpet culture technique. Then it was left for 3-5 minutes to let surface of the agar to dry.

After that using sterile forceps, appropriate antimicrobial discs were placed, evenly distributed on the inoculated plate. Within 30 minutes of applying the discs, the plate was inverted and then incubated aerobically at 35 ºC. for overnight. After overnight incubation, the test plates were examined to ensure confluent growth. Using a scale the diameter of each zone of inhibition was measured.

4.15 Purity Plate

To check for the purity of inoculums used for biochemical tests, the same inoculums was simultaneously subcultured in respective medium and incubated. Pure growth of organisms both in pre inoculation and post inoculation portion of the medium is the indication of aseptic condition.

4.16 ESBL (Extended Spectrum of Beta Lactamase) Detection

The isolates suspected as ESBL were all preceded for confirmation by using combined disc diffusion method. Guidelines for phenotypic confirmatory testing using ceftriaxone (30ug) versus cefriaxone/clavulanic acid (30/10ug) and cefotaxime (30ug) versus cefotaxime/clavulanic acid (30/10ug) were issued by CLSI. Suspected strain was inoculated in a MHA broth and was incubated for 4-6 hours. Then it was

carpet cultured in MHA plates. After few minutes, it was incorporated with separate ceftriaxone and cefotaxime disc and other combination of ceftriaxzone and clavulanic acid and cefotaxime and clavulanic acid. After overnight incubation, the result was read according to the CLSI guidelines. 5 mm increase in a zone diameter for an antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone, indicates probable ESBL production.

4.17 Quality Control for test

Quality of each test was maintained by using standard procedures in order to obtain results that were both reliable and desirable. So quality control was applied at different levels during the study. The quality of each agar plates prepared was tested by incubating one plate of each lot on the incubator. During identification of organism, for each test ATCC (*E.coli* ATCC 25922, *K. pneumoniae* ATCC 700603) control positives and control negative were taken simultaneously. For sensitivity test, Mueller Hinton agar was prepared carefully maintaining 4mm thickness. The pH of the medium was checked. Disc containing the correct amount of antimicrobial were used. For the antibiotics discs also the antibacterial quality was checked. During the experiment, all the procedures were carried out in strict aseptic condition.

CHAPTER V

5. RESULT

5.1 Sputum Culture Positivity

During the study conducted for 8 months, all together 250 sputum specimens were received in the laboratory and processed according to the standard microbiological methods. Out of the total specimens, 211 specimens were further processed as they meet criteria while the remaining 39 specimens that did not meet the criteria were rejected. Rejection of the specimens were based upon the standard guidelines according to which direct smear of sputum sample should contain more than 25 pus cells and less than 10 epithelial cells. Those sputum specimens that do not meet the above criteria indicate the upper respiratory tract and oral contamination. These specimens were not further processed.

Of 211 sputum specimens, 82 showed the significant growth (38.86%) while in 119 (56.39%) specimens, the growth was not significant and in 10 (4.73%) specimens, no growth was noted. The results are shown in the Table 1.

Table 1: Sputum Culture Positivity that met ASM Criteria

Specimen	Accepted Specimen			Rejected Specimen
	Non Significant Growth	Significant Growth	No Growth	
Sputum	119 (56.39%)	82(38.86%)	10 (4.73%)	39
Total	**211(84%)**			**39(15.6%)**

5.2 Occurrence and Distribution of Bacteria in Sputum Specimen

Among the 82 isolates, all together 12 different types of bacterial isolates were identified. Of the total isolates, 51 (62.2%) were Gram negative bacteria and 31 (37.8%) were Gram positive bacteria. Of all the isolates, *Klebsiella pneumoniae* was the most frequently encountered 21(25.6%) followed by *Staphylococcus aureus* (24.39%), *Haemophilus influenza*e (15.85%), *Streptococcus pyogenes* (7.31%), *Streptococcus pneumoniae* (6.09%), *Pseudomonas aeruginosa* (6.09%), *Escherichia coli* (54.87%), *Citrobacter freundi* (2.44%), *Klebsiella oxytoca* (2.44%), *Proteus mirabilis* (2.44%), *Moraxella catarrhalis* (1.22%) and *Acinetobacter calcoaceticus (*1.22%). The result is shown in the able 2.

Table 2: Distribution of bacteria pathogens of Positive LRTI cases:

Organisms	**Sputum positive cases**	**%**	**Total %**
Gram Positive Bacteria			
Staphylococcus aureus	20	64.51	24.39
Streptococcus pyogenes	6	19.35	7.31
Streptococcus pneumoniae	5	16.12	6.09
Total	**31**	**100**	

Gram Negative Bacteria			
Klebsiella pneumoniae	21	41.17	25.6
Haemophilus influenza	13	25.49	15.85
Pseudomonas aeruginosa	5	9.80	6.09
Escherichia coli	4	7.84	4.87
Citrobacter freundii	2	3.92	2.43
Klebsiella oxytoca	2	3.92	2.43
Proteus mirabilis	2	3.92	2.43
Acinetobacter calcoaceticus	1	1.96	1.21
Moraxell catarrhalis	1	1.96	1.21
Total	**82**	**100%**	**100%**

5.3 Age and gender wise distribution of LRTI Cases

The age and gender was recorded from all the LRTI cases included in the study. The gender and age wise distribution of cases were presented in the following table.

5.4 Prevalence of LRTI by age and gender:

In the study, positive cases of the LRTI were observed highest among the age group 51-60 (55%) followed by <20 (20.73%) and so on. Relatively lower frequency (28.81%) was belonging to age group 31-40. However the statistical analysis showed that there is no significant difference in age and LRTI cases. The results are shown in Table 3.

Table 3: Age and gender wise distribution of total cases:

Age Groups (Years)	Male		Female		Total Cases	Total positive cases
	No. of cases studied	Positive cases	No. of cases studied	Positive cases	No.	No. %
<20	15	7	8	4	23	11 (47.82%)

21-30	26	9	13	4	39	13 (33.33%)
31-40	45	10	14	7	59	17 (28.81%)
41-50	29	11	13	7	42	18 (42.85%)
51-60	13	6	7	5	20	11 (55%)
>60	16	6	12	6	28	12 (42.85%)
Total %	**144**	**49 (34.02%)**	**67**	**33 (49.2%)**	**211**	**82 (38.86%)**

5.5 Age Group distribution of LRTI pathogen:

K. pneumoniae was found predominant in patients of age group 41-50 and in the age group less than 20. *H influenza* was found predominant in patients of age less than 20, *S. pneumoniae* was found predominantly in patients of age group 51-60, and other gram negative bacteria were found as shown in the Table 4.

Table 4: Age Group distribution of LRTI pathogens

Organism	Age Group						Total
	<20	21-30	31-40	41-50	51-60	>60	
Gram Positive							
Staphylococcus aureus	1	4	5	6	4	0	20
Beta hemolytic *Streptococci*	1	0	3	1	1	0	6
Streptococcus pneumoniae	2	0	0	1	0	2	5
Total	**4**	**4**	**8**	**8**	**5**	**2**	**31**
Gram Negative							
Klebsiella pneumonia	5	1	3	7	3	2	21
Haemophilus influenza	3	1	0	1	3	5	13
Pseudomonas aeruginosa	0	0	3	0	1	1	5
Escherichia coli	0	0	1	0	1	2	4
Citrobacter freundii	0	0	1	0	1	0	2
Klebsiella oxytoca	0	1	0	1	0	0	2
Proteus mirabilis	0	0	1	0	1	0	2
Moraxella catarrhalis	0	1	0	0	0	0	1
Acinetobacter calcoaceticus	0	0	0	0	1	0	1
Total	**12**	**9**	**17**	**17**	**16**	**12**	**82**

5.6 Distribution of Acid Fast Bacilli in studied LRTI cases:

Among the 211 cases screened for Acid Fast Bacilli, AFB was found in 18(8.53%) of them. The study showed higher prevalence of suspected pulmonary tuberculosis in males

(11/18, 61.11%), than in females (7/18, 38.88%) in all age groups except age group 31-40 years, where greater female population (3/14, 21.42%) compare to male (2/45, 4.44%) were AFB positive. AFB was frequently detected (16/18, 88.88%) in majority of the patients below 50 years, however, 2 (11.11%) positive cases were recorded in patients 60 years and older as depicted in Table 5.

Table 5 : Distribution of AFB in studied LRTI Cases :

Age group (Years)	Male		Female		Total Cases studied	Total AFB Positive cases
	No. of cases studied	No. of AFB positive	No. of cases studied	No. of AFB positive		
<20	15	1	8	1	23	2(11.11%)
21-30	26	3	13	1	39	4(22.22%)
31-40	45	2	14	3	59	5(27.77%)
41-50	29	2	13	-	42	2(11.11%)
51-60	13	2	7	1	20	3(16.66%)
61-70	9	1	4	-	13	1(5.55%)
71-80	3	-	6	1	9	1(5.55%)
>80	4	-	2	-	6	-
Total	**144**	**11**	**67**	**7**	**211**	**18(100%)**

5.7 Antibiotic susceptibility pattern of bacterial isolates.

Antibiotic susceptibility pattern of bacterial isolates towards antibiotics was tested using Kirby-Bauer agar discs diffusion method and the zone diameter. Each antibiotic disc, were compared with those in zone interpretative chart and reported as sensitive, intermediate and resistant. All the isolated strains were tested against antibiotics listed below in Table no.6.

Table 6: Results of Antibiotic sensitivity test by Disc diffusion method for isolates of LRTI.

S. No	Sample code	Antibiotics									
		Amox	Cip	Ts	CRO	Ofx	Nft	C	Oxa	Atz	Pg
1	NKp01	S	S	S	R	S	R	-	-	R	-
2	NKp02	R	R	S	S	R	S	-	-	S	-
3	NKp03	R	S	S	IMD	S	S	-	-	R	-
4	NKp04	R	S	S	S	S	R	-	-	S	-
5	NKp05	S	S	S	S	S	S	-	-	S	-
6	NKp06	R	S	S	S	S	S	-	-	IMD	-
7	NKp07	R	R	S	R	R	R	-	-	S	-
8	NKp08	S	R	R	S	S	S	-	-	R	-
9	NKp09	R	R	R	R	R	R	-	-	S	-
10	NKp10	S	S	R	S	R	R	-	-	R	-
11	NKp11	R	R	S	S	R	R	-	-	S	-
12	NKp12	R	R	R	S	R	S	-	-	R	-
13	NKp13	S	R	S	S	S	R	-	-	S	-
14	NKp14	R	S	IMD	S	R	S	-	-	S	-
15	NKp15	R	R	R	R	R	S	-	-	S	-
16	NKp16	S	S	S	S	IMD	S	-	-	S	-
17	NKp17	R	R	R	R	R	R	-	-	R	-
18	NKp18	IMD	S	S	S	R	S	-	-	S	-
19	NKp19	R	R	S	S	S	R	-	-	S	-
20	NKp20	R	S	R	S	S	S	-	-	S	-
21	NKp21	R	R	S	R	R	R	-	-	R	-
22	NHi01	S	S	S	S	S	-	-	-	S	-
23	NHi02	S	S	S	R	S	-	-	-	S	-
24	NHi03	R	S	S	S	S	-	-	-	R	-
25	NHi04	S	S	R	R	R	-	-	-	S	-
26	NHi05	S	S	R	S	S	-	-	-	S	-
27	NHi06	S	S	R	S	S	-	-	-	S	-
28	NHi07	S	S	R	IMD	S	-	-	-	S	-
29	NHi08	S	S	S	R	S	-	-	-	S	-
30	NHi09	S	S	S	S	S	-	-	-	S	-
31	NHi10	S	S	R	S	S	-	-	-	S	-
32	NHi11	S	S	R	R	S	-	-	-	S	-
33	NHi12	S	S	S	S	IMD	-	-	-	S	-
34	NHi13	S	S	R	S	S		-	-	S	-
35	NPa01	R	S	R	R	R	R	-	-	S	-
36	NPa02	R	R	S	R	R	R	-	-	S	-
37	NPa03	R	S	R	S	S	R	-	-	R	-
38	NPa04	R	R	R	R	R	R	-	-	R	-
39	NPa05	R	R	S	R	S	R	-	-	S	-
40	NEc01	R	R	S	R	R	S	-	-	R	-
41	NEc02	R	S	S	S	S	S	-	-	R	-
42	NEc03	R	R	S	R	R	R	-	-	S	-
43	NEc04	R	R	R	R	S	S	-	-	IMD	-
44	NCf01	R	S	S	S	R	-	-	-	S	-
45	NCfo2	R	R	R	R	S	-	-	-	S	-
46	NKo01	R	R	S	R	R	-	-	-	R	-
47	NKo02	R	S	R	R	S	-	-	-	R	-
48	NPm01	S	R	S	S	R	-	-	-	R	-

49	**NPm02**	**R**	**S**	**S**	**S**	**S**	**-**	**-**	**-**	**R**	**-**
50	**NMc01**	**R**	**R**	**S**	**R**	**R**	**-**	**-**	**-**	**R**	**-**
51	**NAc01**	**R**	**R**	**R**	**R**	**R**	**-**	**-**	**-**	**R**	**-**
52	**NSa01**	**R**	**S**	**S**	**-**	**-**	**-**	**S**	**R**	**S**	**-**
53	**NSa02**	**S**	**R**	**R**	**-**	**-**	**-**	**S**	**S**	**S**	**-**
54	**NSa03**	**R**	**S**	**S**	**-**	**-**	**-**	**R**	**R**	**S**	**-**
55	**NSa04**	**R**	**R**	**S**	**-**	**-**	**-**	**S**	**S**	**S**	**-**
56	**NSa05**	**R**	**R**	**R**	**-**	**-**	**-**	**R**	**R**	**S**	**-**
57	**NSa06**	**S**	**S**	**R**	**-**	**-**	**-**	**S**	**S**	**S**	**-**
58	**NSa07**	**R**	**IMD**	**S**	**-**	**-**	**-**	**S**	**R**	**IMD**	**-**
59	**NSa08**	**R**	**S**	**S**	**-**	**-**	**-**	**S**	**R**	**S**	**-**
60	**NSa09**	**R**	**R**	**R**	**-**	**-**	**-**	**R**	**R**	**R**	**-**
61	**NSa10**	**IMD**	**R**	**S**	**-**	**-**	**-**	**S**	**S**	**S**	**-**
62	**NSa11**	**S**	**S**	**R**	**-**	**-**	**-**	**S**	**S**	**S**	**-**
63	**NSa12**	**S**	**S**	**R**	**-**	**-**	**-**	**R**	**R**	**S**	**-**
64	**NSa13**	**R**	**R**	**R**	**-**	**-**	**-**	**R**	**S**	**IMD**	**-**
65	**NSa14**	**R**	**IMD**	**S**	**-**	**-**	**-**	**S**	**S**	**S**	**-**
66	**NSa15**	**S**	**S**	**R**	**-**	**-**	**-**	**IMD**	**S**	**S**	**-**
67	**NSa16**	**S**	**R**	**S**	**-**	**-**	**-**	**S**	**R**	**R**	**-**
68	**NSa17**	**R**	**R**	**S**	**-**	**-**	**-**	**S**	**S**	**R**	**-**
69	**NSa18**	**R**	**S**	**R**	**-**	**-**	**-**	**S**	**S**	**S**	**-**
70	**NSa19**	**S**	**S**	**S**	**-**	**-**	**-**	**S**	**R**	**S**	**-**
71	**NSa20**	**S**	**R**	**R**	**-**	**-**	**-**	**R**	**S**	**S**	**-**
72	**NSp01**	**S**	**S**	**S**	**-**	**-**	**-**	**R**	**R**	**S**	**-**
73	**NSp02**	**S**	**S**	**S**	**-**	**-**	**-**	**S**	**R**	**R**	**-**
74	**NSp03**	**R**	**S**	**S**	**-**	**-**	**-**	**S**	**S**	**R**	**-**
75	**NSp04**	**S**	**S**	**R**	**-**	**-**	**-**	**R**	**S**	**S**	**-**
76	**NSp05**	**S**	**S**	**R**	**-**	**-**	**-**	**R**	**S**	**R**	**-**
77	**NSpy01**	**S**	**S**	**R**	**S**	**-**	**-**	**S**	**S**	**S**	**S**
78	**NSpy02**	**S**	**R**	**R**	**S**	**-**	**-**	**R**	**S**	**S**	**S**
79	**NSpy03**	**R**	**S**	**R**	**S**	**-**	**-**	**R**	**S**	**R**	**S**
80	**NSpy04**	**R**	**S**	**S**	**S**	**-**	**-**	**S**	**S**	**R**	**S**
81	**NSpy05**	**S**	**R**	**R**	**S**	**-**	**-**	**R**	**S**	**R**	**S**
82	**NSpyo06**	**S**	**S**	**S**	**R**	**-**	**-**	**R**	**S**	**R**	**S**

NKp01-NKp21 – NPHL *K pneumoniae* strain 01-21, NHi01-13 – NPHL *H influenzae* 01-13, NPa01-05 – NPHL *P aeruginosa* 01-05, NEc01-04 – NPHL *E coli* 01-04, NCf01-02 – NPHL *C fruendii* 01-02, NKo01-02 – NPHL *K oxytoca* 01-02, NPm01-02 – NPHL *P mirabilis* 01-02, NAc01 – NPHL *A calcoaceticus*01, NMc01 – NPHL *M catarrhalis* 01, NSa01-20 – NPHL *S aureus* 01-20, NSp01-06 – NPHL *S pyogenes* 01-06, NSp01-05 – *S pneumoniae* 01-05, R – Resistant, IMD – Intermediate, S – Sensitive, Amox – Amoxicillin, Cip – Ciprofloxacin, Ts – Cotrimoxazole, CRO – Ceftrixone, Oft – Ofloxacin, Nft – Nitrofurantoin, C – Chloramphenicol, Oxa – Oxacilin, Atz – Azithromycin, Pg - Penicillin.

Of the total Gram Negative isolates, *Klebsiella pneumoniae* showed more sensitivity to Ceftriaxone (67%) followed by Cotrimoxazole (62%), Nitrofurantoin (52%), Ciprofloxacin (48%) and showing least sensitivity towards Amoxicillin (28%). Similarly, *Haemophilus influenzae* showed highest sensitivity towards Ciprofloxacin (100%) and least towards Cotrimoxazole (46%). All isolates of *Pseudomonas aeruginosa* showed 100 % resistance towards Amoxicillin and Nitrofurantoin. *Escherichia coli* were found more sensitive towards Nitrofurantoin and Cotrimoxazole (75%) and showed 100% resistance towards Amoxicillin. Antibiotic susceptibility pattern of Other Gram Negative bacterial isolates shown in the table below. Among the antibiotics used, Cotrimoxazole showed highest sensitivity (55%) against the total Gram negative isolates and least sensitivity showed against Amoxicillin (35%) which are shown in the Table 7.

Table 7: Antibiotic susceptibility pattern of Gram negative bacterial isolates:

Antibiotics used / Isolates	Amox			Cip			Ts			CRO			Nft		
	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
K.pneumoniae	6	14	1	10	11	0	13	7	1	14	6	1	11	10	0
n = 21	28	67	5	48	52	0	62	33	5	67	28	5	52	48	0
H .influenzae	12	1	0	13	0	0	6	7	0	8	4	1	11	1	1
n = 13	92	8	0	100	0	0	46	54	0	61	31	8	84	8	8
P. aeruginosa	0	5	0	2	3	0	2	3	0	1	4	0	0	5	0
n = 5	0	100	0	40	60	0	40	60	0	20	80	0	0	100	0
E.coli	0	4	0	1	3	0	3	1	0	1	3	0	3	1	0
n = 4	0	100	0	25	75	0	75	25	0	25	75	0	75	25	0
C. fruendii	0	2	0	0	2	0	1	1	0	0	2	0	2	0	0
n = 2	0	100	0	0	100	0	50	50	0	0	100	0	100	0	0
K . oxytoca	0	2	0	0	2	0	1	1	0	0	2	0	1	1	0
n =2	0	100	0	0	100	0	50	50	0	0	100	0	50	50	0
P. mirabilis	0	2	0	1	1	0	1	1	0	2	0	0	0	2	0
n = 2	0	100	0	50	50	0	50	50	0	100	0	0	0	100	0
M. catarrhalis	0	1	0	0	1	0	1	0	1	0	1	0	0	1	0
n = 1	0	100	0	0	100	0	100	0	100	0	100	0	0	100	0
A.calcoacetics	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
n = 1	0	100	0	0	100	0	0	100	0	0	100	0	100	0	0
Total	18	32	1	27	24	0	28	22	1	26	23	2	29	21	1
N= 51	35	63	2	53	47	0	55	43	2	51	45	4	57	41	2

Among the total isolates of Gram positive bacteria, 75% *Staphylococcus aureus* were found sensitive towards Chloramphenicol and Azithromycin respectively.50% *S aureus* were found resistant towards Ciprofloxacin followed Cotrimoxazole i.e. 45% and least

sensitivity towards Amoxicillin i.e. 40%. *Streptococcus pneumonia* were found 100% sensitive towards Ciprofloxacin followed by Amoxicillin i.e. 80%. *Beta hemolytic Streptococci* were found 67% sensitive towards Amoxicillin and Ciprofloxacin respectively and least sensitivity (33%) towards Chloramphenicol and Azithromycin. Among the antibiotics used against the total Gram positive isolates, Azithromycin showed highest sensitivity (62%) and least sensitivity towards Cotrimoxazole (48%) which are shown below Table 8.

Table 8: Antibiotic susceptibility pattern of Gram Positive bacterial isolates:

Antibiotics used / Isolates	Amox			Cip			Ts			C			Atz		
	S %	R %	I %	S %	R %	I %	S %	R %	I %	S %	R %	I %	S %	R %	I %
S. aureus	**8 40**	**11 55**	**1 5**	**9 45**	**9 45**	**2 10**	**10 50**	**10 50**	**0 0**	**13 65**	**6 30**	**1 5**	**15 75**	**3 15**	**2 10**
S pneumoniae	**4 80**	**1 20**	**0 0**	**5 100**	**0 0**	**0 0**	**3 60**	**2 40**	**0 0**	**2 40**	**3 60**	**0 0**	**2 40**	**3 60**	**0 0**
S. pyogenes	**4 67**	**2 33**	**0 0**	**4 67**	**2 33**	**0 0**	**2 33**	**4 67**	**0 0**	**2 33**	**4 67**	**0 0**	**2 33**	**4 67**	**0 0**
Total	**16 52**	**14 45**	**1 3**	**18 58**	**11 36**	**2 6**	**15 48**	**16 52**	**0 0**	**17 55**	**13 42**	**1 3**	**19 62**	**10 32**	**2 6**

5.7 Multiple drug resistant patterns of the isolates

Among the 82 isolates, 36 (43.9%) isolates showed the multi drug resistant pattern.15 isolates showed resistance to 3 drugs, 9 isolates showed resistance to 4 drugs, 6 isolates showed resistance to 5 drugs and 6 showed resistance to more than 6 isolates.MDR pattern was shown in Table 9.

MDR Pattern / Isolates No.	3 Drugs	4 Drugs	5 Drugs	>5Drug	Total MDR
K pneumoniae	3	2	3	2	10
S aureus	5	1	1	1	8
H influenzae	1	0	0	0	1
S pyogenes	2	0	0	0	2
S pneumoniae	1	0	0	0	1
P aeruginosa	2	2	0	1	5

Escherichia coli	0	2	1	0	3
Citrobacter freundii	0	1	0	0	1
Klebsiella oxytoca	0	1	0	1	2
Proteus mirabilis	1	0	0	0	1
M catarrhalis	0	0	1	0	1
A calcoaceticus	0	0	0	1	1
Total	**15**	**9**	**6**	**6**	**36**

Table 9: Multiple drug resistant pattern of the isolates:

Among the isolates, *Pseudomonas aeruginosa, Klebsiella oxytoca, Moraxella catarrhalis* and *Acinetobacter calcoaceticus* showed 100% MDR pattern. 47.61 % isolates of *Klebsiella pneumoniae* showed MDR pattern. Similarly, 40% isolates of *Staphylococcus aureus* were found MDR strains. The least percentage i.e. 7.69% *Haemophilus isolates* were found to be MDR strains. Other isolates showing the MDR percentage are shown below in Table 9.

5.8 Detection of ESBL in bacterial isolates

Extended Spectrum of Beta Lactamase (ESBL) was screened for all the potential multi drug resistant strains in the study. Among 10 MDR isolates *of K pneumoniae*, ESBL was detected in 3 (30%) and of 3 MDR isolates of *E. coli*, ESBL was detected in 1(33.33%). The result was shown in table 10.

Table 10: Detection of ESBL in bacterial isolates

S.N	Bacteria	No. of isolates	No. of MDR	No. of ESBL producing MDR strains	% of ESBL
1.	*K. pneumoniae*	21	10	3	30%
2.	*E . coli*	4	3	1	25%
3.	**Total**	25	13	4	30.77%

CHAPTERNVI

DISCUSSION

The present study was conducted to have better understanding on the current trend of the microbial spectrum causing acute LRTI and the efficacy of antibiotics being used against them. Besides, the study also included descriptive studies on the incidence of MDR organisms and ESBL producing strains.

Out of 211cases presenting with signs and symptoms of LRTI, 144 were male and 67 were female i.e. 2.14:1. The age of cases ranged from 9 to 84 years. Subgroup based on age unveiled the prevalence of positive LRTI cases in age group 51-60 (55%) and minimum being in age group 31-40 (28.81%). Comparatively low prevalence in age group above 60 could be due to short average life expectancy of Nepalese population (61years) that have reflected in this study.

Sputum samples were most frequently processed specimen in LRTI. Up to 90% of the investigated sputum had been found positive for microbial isolates in various studies conducted in the past. Out of the total 211 cases, 82 (38.86%) positive cases of LRTI was estimated based on the yield of sputum culture in the study. Few similar studies were

carried out in Nepal. One study carried out by Shrestha et al., 1994, which showed significant growth in 34.55% of cases. A study carried out by Joshi et al., 1997, showed significant growth in 57.35% of cases. In the study carried out by Hosker et al., 1994, significant growth was obtained in about 60% of the cases. Furthermore, Woodhead in 1992 reported to have pathogens involved in three quarters of the cases. Relatively lower yield i.e. 38.86% in this study may be attributed to various factors. NPHL being the reference laboratory so most of the cases were from the community acquired type and in some cases natural history of infectious disease in the patients has already been modified by use of different type of antibiotics by health professionals at different level before undergoing diagnosis in NPHL.

The use of antibiotics without appropriate diagnosis might have played a significant role in culture negativity. Again, viruses like adenovirus, rhinovirus, respiratory syncytial virus and parainfluenzae virus which are significant contributors of LRTI were not included in this study due to resource limitation. Besides that, certain very common respiratory pathogens such as *Mycoplasmas, Chlamydia, Legionellae, Mycobacteria*, Fungi, *Pneumocystis* and anaerobes could not be cultured by routine methods. Despite the continued identification of new pathogens and increasingly sophisticated diagnostic methods, a specific causative microbial pathogen cannot be identified in 30-50% cases.

Each cases studied was screened for tuberculosis. Presence of AFB on their sputum smear was very helpful or, rather mandatory for this study. Sputum smear processed during the study were stringently those submitted for AFB staining for two reasons. First this study included the AFB screening and secondly to rule out any specimen contaminated with saliva.

About 72 % Microscopic examination of Gram stain correlated positively with culture results (in 59 cases). However, Correlation between Gram staining and culture results was 80% of LRTI cases studied by Dodannavar et al., 1985. Sputum culture is no doubt to be the most rewarding investigations in identifying respiratory pathogens. Forbes et al., 2007 mentioned that Gram staining of sputum and blood culture as gold standard method for the identification of pneumonia in patients. Moreover, Gram staining has been found equally efficient for screening respiratory pathogens which can be helpful in establishing

LRTI in places where there is no proper culture facility but Gram stained sputum smear is not infalliable guide to direct antibiotic therapy in adults with community acquired bacterial pneumonia though it has certain degree of reliability as warned by Gleckman et al., (1988).

In the study carried out by Coffey et al (1995). *Streptococcus pneumoniae* was the primary bacterial cause of LRTI in adult population followed by *H. influenzae* and others. According to the study carried out by Shibl et al., that published in Lippincott Williams & Wilkins, Inc 2010 *Pneumococcal pneumoniae* was the most common pathogen implicated in adult CAP. By going through some review studies published from North America, over three decades, Barlett et al.,(1995), concluded that *Streptococcus pneumoniae* was the most common cause of CAP(up to 60% cases), followed by *Haemophilus influenza* (3-10%), *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Other etiologies CAP were *Staphylococcus aureus, M Pneumoniae, Legionella, C pneumoniae,* and some viruses.

In this study *Klebsiella pneumoniae* was isolated as a primary bacterial cause of LRTI which differed from the other works. The reason of this could be as our study was Public health laboratory based, there was intermixing of community acquired and hospital acquired LRTI cases and there is universal preponderance of gram negative bacteria in both hospital and community. Although *Streptococcus pneumonia* remains the most prevalent or frequently isolated etiologic agent in most cases of CAP, other organisms such as *Haemophilus influenzae* and *Moraxella catarrhalis, Klebsiella peumoniae* and other gram negative bacteria as well as the so-called atypical pathogens, including *L pneumonphila*, *C pneumoniae, M pneumoniae* and are now being reported more frequently than in the past. Even in patients with bacteremic *S pneumoniae* pneumonia, it has been estimated that the usual laboratory methods cannot detect the pathogen in more than 50% of expectorated sputum specimens. This low sensitivity may be due to misidentification of the alpha hemolytic colonies *S pneumoniae* as nonpathogenic alpha-hemolytic streptococci(normal viridian streptococci), overgrowth of the cultures by hardier colonizing organisms, or loss of fastidious organisms due to slow transport or improper laboratory processing (Harrison's Med. 15th Ed , 2001). Among the Gram

positive bacteria, *Staphylococcus aureus* was predominant accounting for 24.4% of cases followed by beta hemolytic Streptococci (7.31%) and *Streptococcus pneumonia* (6.09%).In case of gram negative bacteria,

Klebsiella pneumoniae was the most encountered not only among the gram negative group but among all 21 isolates. Majority of them, 7 (33.33%) were detected among the elderly age group above 40 years, 12 (57.14%). Finding of *Klebsiella pneumoniae* as the bacterial isolate from the most of the reported cases of CAP is 1-2% but it dramatically increase in elderly, alcoholics, diabetics, liver patients and HIV infection. The prevalence CAP by *Klebsiella pneumoniae* in the study carried out in Taiwan was 28%, 57 of 202 cases (Wen-Chien et al., 2000).

Klebsiella pneumoniae is followed by *Haemophilus influenzae* (15.85%) which is commonest isolate not only among the gram negative bacteria but also among the total isolates. Surveys of the incidence of *H influenzae* in community-acquired respiratory infections indicate that this pathogen accounts for between 2% and 11% of cases of CAP and for more than half of all bacterial cases of AECB (Robert Guthrie et al., 2001). Out of total 13 isolates of *H influenzae*, 5(38.46%) were seen in patients in age group above 60 years followed by 3 (23%) of cases in age group below 20 and 51-60 each. In adults, *H influenzae* pneumonia most frequently occurs in middle age LRTI patients with compromised pulmonary function more during an acute exacerbation and alcoholism. According to Royal Brompton et al., 2000, *H influenzae* accounts for almost half of the isolates among the bacterial isolates from the sputum specimens of patient with acute exacerbation of COPD.

Pseudomonas aeruginosa was the third major Gram negative bacteria and fourth most common bacteria identified in LRTI (5, 6.09%). According to CDC, *P aeruginosa* is the fourth most common cause of hospital acquired pneumonia but in the other hand*; P aeruginosa* is an opportunistic pathogen that causes respiratory infection and variety of systemic infections, particularly in patients with severe burn, diabetics, cancer, AIDS and immune suppression. Ventilator associated pneumonia is the most common nosocomial infection in ICU patients. In the USA, 69 per cent of pathogens associated with ventilator associated pneumonia were Gram negative pathogens (Fridkin, 1997). *Pseudomonas*

aeruginosa and Enterobacter spp. were the most common Gram negative pathogens. *P aeruginosa* has been considered as an etiology in 21%- 38% of cases of hospital-acquired pneumonia. Bacteremic pneumonia commonly occurs in neutropenic cancer patients undergoing chemotherapy. Lower respiratory tract colonization of cystic fibrosis patients by mucoid strains of *Pseudomonas aeruginosa* is common and difficult to eradicate (Todar's Online Textbook of Bacteriology 2011). *P aeruginosa* is the epitome of an opportunistic pathogen and never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect, if the tissue defenses are compromised in some way. Infection due to *P aeruginosa* are seldom encountered in healthy adults but in the last two decades the organism has become increasingly recognized in hospitalized patients with impaired immune system (Neu, 1983) Since the organism is inherently resistant to many classes of drugs and is able to acquire resistance to all effective antimicrobial drugs, so the infection by *Pseudomonas aeruginosa* is often problematic (Livermoner et al., 2002).

Esherichia coli was another isolates diagnosed mostly from older patients. *E coli* being the member of Enterobacteriaceae family which cause infection in older patients due to prior gastrointestinal or urinary tract infection which then disseminated to lungs secondary to bacteremia (Berk et al.,1985). Most of the important factor determining the pattern of microbial isolates was the setup where the infection was contracted. *E coli* pneumonia is also common among the children. According to study carried out in china, *Escherichia coli* was the fourth major etiology among the children (Hua Cz et al., 2002).

However, the most important variable that determines the pattern of microbial isolates in most of the cases the setup where the infection was occurred. Gram negative bacteria contributed 62.2%.of total isolates, this finding is not according to the general pattern of community-acquired pneumonias showing possible reason behind could be intermingling of the hospital and community acquired pathogens and study set up.

Antimicrobial chemotherapy has been one of the great areas of therapeutic success in clinical medicine, particularly in the second half of the 20th century. Its use has resulted in dramatic reductions in both morbidity and mortality from infectious disease and has made possible many treatments that were previously either complicated or limited by the

risk of infection. However, bacterial resistance to the effects of antibiotics is an increasing problem due to indiscriminate use that threatens the utility of these therapies and compromises patient care. Bacterial resistance to antibiotics in LRTI is a serious problem and is increasing in prevalence world-wide at an alarming rate. Bacteria are among the most adaptable and educable microbes on this planet. These microbes have learned how to survive for centuries under the most adversarial conditions created by man and nature. They have managed to elude our constant pharmacologic innovations through their own ingenious mechanisms of mutation and genetic transformation. The unforgiving rise in the frequency of multidrug resistance among leading pathogens should cause great concern and incite a commitment to act responsibly; otherwise we have to pay a big price both in terms of money and lives.

According to WHO, for every 100 respiratory infections, only 20% of cases require antibiotic therapy which strongly indicated the unnecessary use of drugs. Globally, community-acquired respiratory tract infections account for a large proportion of antibiotic prescriptions and visits to family practitioners. Increases in the number of newly identified or previously unrecognized pathogens, the availability of new antimicrobial agents, and the evolution of bacterial resistance mechanisms have contributed to changes in the epidemiology and treatment of respiratory tract infections. In current clinical practice, etiological pathogens are seldom identified either before or after initiation of therapy, and objective criteria decreasing the level of uncertainty in choosing effective antimicrobial agents are lacking. It is also important to take into account that the relationship between the pathogens and the susceptible host is highly complexes and shaped by conflicting forces. As consequences, the spread of microbial resistance has further complicated this already complex arena. However, the key factor appears to be whether or not microbial resistance is able to affect the clinical outcomes of antibiotic treatment. Most importantly, a variety of characteristics including etiology and individual risk factors appear to be associated with mortality and other medical outcomes of LRTI (G.Di Maria et al., 2010).

Since the respiratory tract infection attributes to the significant burden of morbidity and mortality and about three quarters of antibiotics are prescribed for treating RTIs, in this

context of consideration for reducing resistance emergence, prevalence, cost minimization and making appropriate therapeutic decision, respiratory tract infection including LRTI are appropriate area of action. This is the prime objective to study the present situation of drug resistance among the LRTI pathogens.

.In this study, antibiotic susceptibility pattern of *S pneumoniae* showed that Ciprofloxacin (100%) and Amoxicillin (80%) were the most effective drugs of choice followed by Chloramphenicol (60%) and the least effectives are Azithromycin and Cotrimoxazole. The antibiotic resistance patterns of S pneumoniae were found to be considerably increased to most of the previously susceptible drugs. Resistance to tetracycline, macrolides and other drugs were reported. According to one study carried out in Portugal, resistance pattern of *S pneumoniae* to macrolides especially erythromycin had been increased to six fold from 1993 to 2003. Resistance to erythromycin, clarithromycin, and azithromycin was higher in pediatric patients than in adults. The overwhelming majority (82.3%) of macrolide-resistant isolates were multidrug resistant, although 44.9% were fully susceptible to penicillin. Multiple antibiotic resistant strains of *S. pneumoniae* that emerged in the early 1970s in Papua New Guinea and South Africa were thought to be a fluke, but multiple antibiotic resistance now covers the globe and has rapidly increased since 1995. Increases in penicillin resistance have been followed by resistance to cephalosporins and multidrug resistance.

The incidence of resistance to penicillin increased from <0.02 in 1987 to 3% in 1994 to 30% in some communities in the United States and 80% in regions of some other countries in 1998. Resistance to other antibiotics has emerged simultaneously: 26% resistant to trimethoprim-sulfa, 9% resistant to cefotaxime, 30% resistant to macrolides, and 25% resistant to multiple drugs. Resistant organisms remain fully virulent but seem to have arisen in less than 10 serotypes. Serotypes 6A, 6B, 9V, 14, 19A and 23F are included in the vast majority of resistant strains. When *S pneumoniae* develops resistance to penicillin, it typically manifests resistance to most other antibiotics, including cephalosporins, macrolide, and trimethoprim-sulfamethoxazole. Penicillin resistance in *S pneumoniae* results from a genetic alteration of penicillin-binding proteins (Coffey et al., 1995).

In the era of emerging antimicrobial resistance, prevention of pneumococcal infections is paramount; vaccination strategies offer an important approach to controlling DRSP. An existing pneumococcal polysaccharide vaccine that can prevent a substantial number of pneumococcal infections, including those caused by DRSP, is underutilized. The vaccine is recommended by the Advisory Committee for Immunization Practices (ACIP) for use in persons older than 2 years of age who have certain underlying medical conditions and for all persons older than 65 years of age. It is not recommended for routine use among children under 2 years of age because it does not provide immunity consistently in this age group. An effective vaccine is needed to prevent pneumococcal infections in this population, which has the highest risk for otitis media and meningitis caused by DRSP. If the prevalence of pneumococcal infection (and therefore antimicrobial use) can be substantially reduced by vaccination, the impact of DRSP may diminish. Novel vaccine demonstration projects supported by federal and state health agencies are under way to explore means of increasing coverage with the effective 23-valent pneumococcal polysaccharide vaccine. This vaccine contains most of the serotypes of S. pneumoniae that have developed penicillin resistance. Investigators have begun studying a more effective protein-conjugate pneumococcal vaccine.

Out of 20 isolates of *S aureus*, 13 (65%) isolates were MDR and 5 isolates (25%) were MRSA strains. The growing and urgency surrounding the problem of antibiotic resistance was heightened recently by alarming reports of community-onset infection caused by strains of Methicillin-resistant *Staphylococcus aureus* (MRSA) throughout the world (CDC, 2001). Early in the antibiotic era, *S. aureus* was recognized as capable of developing resistance to penicillin. By the end of the 1950s, many clinical studies revealed staphylococcus with multiple drug resistance. In 1960, the introduction of semi synthetic anti staphylococcal penicillins such as Methicillin increased the therapeutic choices as did the introduction of first-generation cephalosporins several years later. MRSA is now prevalent in more than 15% of nosocomial strains (Sigler & Hessen, 1993) and the effectiveness of first-generation cephalosporins has waned creating the necessity for three new generations of multiple cephalosporins. Vancomycin, a glycopeptides antibiotic, has become the treatment of choice for MRSA; a last antimicrobial agent

within the clinician's armamentarium. The mechanism of resistance was the production of a plasmid-mediated beta-lactamase enzyme (penicillinase) which hydrolyze penicillin's beta lactam ring. Methicillin was among the first of these agents to be introduced into clinical practice, but strains of MRSA were identified as early as 1961. Since then, MRSA has become increasingly prevalent in many countries around the world.

In the past two decades, the prevalence of MRSA strains has steadily increased in hospitals in the United States and abroad. National Nosocomial Infections Surveillance (NNIS) data collected by the Centers for Disease Control in the early to mid-1980s indicated that MRSA were limited mainly to relatively large urban medical centers and that rates were 5% to 10%. Smaller, non referral centers were relatively free of MRSA, with prevalence rates well below 5%. By the 1990s, rates among these smaller (<200-bed) community hospitals had increased to 20%, and twice that rate was found in the larger urban centers. More recent surveillance data from NNIS indicate that rates have continued to rise, with the prevalence of MRSA isolates from intensive care units approaching 50% by the end of 1998. Unless this upward trend has reversed, the prevalence rate of MRSA in U.S. hospitals likely has reached 50%. At these high rates, the emergence of correspondingly high rates of MRSA strains in the community can be anticipated. Because no systematic, population-based surveillance of community isolates of *S. aureus* exists, the true prevalence of MRSA cannot be determined.

Community-acquired strains are feral or freestanding. Regardless of the origins, which are likely to become obscured as clones move back and forth between hospital and community over time, emergence of MRSA within the community is a major threat with several important clinical implications: treatment failure with accompanying complications or death may result if an anti staphylococcal beta-lactam antibiotic is used and the infecting strain proves to be resistant; infections caused by Methicillin-resistant strains may be more difficult to manage or more expensive to treat, perhaps because Vancomycin is inherently less efficacious and the increasing prevalence of MRSA will inevitably increase Vancomycin use, adding further to the problem of antibiotic-resistant gram-positive bacteria.

Antimicrobial resistance to penicillin, Methicillin, or Vancomycin is an unavoidable consequence of the selective pressure of antibiotic exposure. Although the details of the epidemiology of staphylococcal drug resistance may change, the fundamental forces driving it are similar. The question is not whether resistance will occur, but how prevalent resistance will become. Minimizing the antibiotic pressure that favors the selection of resistant strains is essential to controlling the emergence of these strains in the hospital and the community, regardless of their origins.

Antimicrobial resistance of *Klebsiella pneumoniae* was found considerably increasing. About 76% of *K pneumoniae* were found resistant to amoxicillin, followed by Nitrofurantoin, ESBL conformation test was performed for the ceftazidime resistant strains and 3(14.28%) of the total isolated *K pneumoniae* were confirmed as ESBL strains. In a survey conducted in Czech Republic hospitals in September and October 2004, the prevalence of extended-spectrum β-lactamase (ESBL) production by Klebsiella pneumoniae was estimated to be 25.6 % and the prevalence of ESBL-positive strains was 38.5 % in ICUs and 15.8 % in standard wards (M Kolár et la.,2004) But the prevalence of extended-spectrum β-lactamase (ESBL) production by *Klebsiella pneumonia* approaches 50% in some countries, with particularly high rates in eastern Europe and Latin America (David L. Paterson et al., 2004).

All most all strains of *K pneumoniae* isolated from LRTI cases were found to be multiple drug resistance and were resistant to all classes of antibiotics. Although ESBLs have been detected in a wide variety of gram-negative bacteria, *Klebsiella pneumoniae* has been found to be the most common species to produce ESBLs. In a 1997–1998 survey of Klebsiella isolates from ICUs in southern and Western Europe, 25% possessed ESBLs. However, in a 2000 survey of eastern European centers (e.g., Russia, Poland, and Turkey), almost 50% of *K. pneumoniae* isolates produced ESBLs. Similarly high rates of ESBL production have been observed in some parts of Asia and Central and South America. Despite the high prevalence of ESBL-producing organisms in many parts of the world, data on the treatment of serious infections due to such organisms remain sparse.

Treatment of serious infection with ESBL-producing *K. pneumoniae* is difficult because the organisms are frequently resistant to multiple antibiotics. However, in vitro, ESBL-producing organisms may sometimes appear to be susceptible to combination therapy with β-lactams/β-lactamase inhibitors, third- and fourth-generation cephalosporins, aminoglycosides, and quinolones. Susceptibility rates for these antibiotics are 0%–80%, depending on the geographical location of the study site (David L. Paterson et al., 2004). Factors that predispose patients to colonization and infection with these organisms are use of invasive medical devices such as urinary catheters, endo-tracheal tubes and central lines for long periods of time and use of broad spectrum antibiotics.

Among all the isolates, *Psuedomonas aeruginosa* infection demonstrates the most alarming condition showing very high drugs resistance pattern. *Pseudomonas aeruginosa* is frequently resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and fluoroquinolins, resistant forms have developed. The combination of gentamicin and carbenicillin is frequently used to treat severe Pseudomonas infections *Pseudomonal aeruginosa* was found to be most susceptible to fluoroquinolins, Ciprofloxacin (60%). All the isolates of Pseudomonas were MDR showing the resistance to 2 or more antibiotics.

Most patients with Cystic fibrosis and invasive hospital procedures acquire chron*ic P. aeruginosa* infections early in life; these infections afflict patients for decades and are responsible for much of the morbidity and mortality of people with this disease. Most of these infections are clonal and infections are acquired independently by each patient, presumably from diverse environmental reservoirs (Laura Martínez-Solano et al., 2008).

In the isolated 4 strains of *Escherichia coli*, 1 strain was found to be ESBL strain while 3 of them were MDR strains. Remaining 8 isolates, *C freundii (2*), *Klebsiella oxytoca* (2), *Proteus mirabilis* (2), *Acinetobacter calcoaceticus (*1) and *Moraxella catarrhalis* (1) showed considerable resistance pattern.

Of all 211 sputum screened for TB, AFB was detected in 18(8.53%).An acid-fast bacillus (AFB) smear is used to look for AFB in a sample from the site of suspected infection. This is a relatively quick way to determine if an infection may be due to one of the acid-

fast bacilli, the most common of which is *M. tuberculosis*. An AFB smear may be done in conjunction with a molecular test for TB termed nucleic acid amplification test (NAAT). These tests are not diagnostic, but they can provide a presumptive diagnosis, which can aid in the decision of whether to begin treatment before culture results are available. Results of an AFB smear (and NAAT) are typically available several hours after a sample is collected, while an AFB culture typically takes days to weeks. A negative AFB smear means that no infection is present, that symptoms are caused by something other than Mycobacteria, or that the Mycobacteria were not present in sufficient numbers to be seen under the microscope.

Usually at least three samples are collected to increase the probability that the organisms will be detected. Nevertheless, if AFB smears are negative and there is still a strong suspicion of a Mycobacterial infection, then additional samples may be collected and tested on different days. A smear negative sample may still grow Mycobacteria since the culture media allows low numbers of bacteria to multiply and be detected. If Acid Fast Bacilli are detected by AFB staining, then the patient is said to have smear positive tuberculosis and it is important to carry out smear microscopy because it correctly and efficiently identifies the cases which are infectious and therefore have the highest priority for care (WHO, 2010).

Positive AFB smears indicate a probable Mycobacterial infection. However, a culture must be performed to confirm a diagnosis.

VI Conclusion

Appropriate prescribing of antibiotics can effectively slow the development of bacterial resistance. Before selecting an antibiotic for a patient with a community-acquired RTI, primary care clinicians should first evaluate whether such treatment is even necessary. Treatment of ABS, ABECB, or CAP is compromised by increasing pathogen resistance to the currently used antibiotics. Dramatic changes in the epidemiology and susceptibility of bacterial strains during the last decade have mandated new approaches to managing many infections. Overzealous use of antibiotics has been a large part of the problem. Widespread use of broad-spectrum antibiotics and the emergence of multidrug-resistant bacterial pathogens have become a vicious cycle. Newer and more ingenious antibacterial agents are urgently required to meet the challenges posed by these epidemiologic trends. Curbing inappropriate use of antimicrobial agents will be as important as learning the nuances between each new agent.

The current therapy for lower respiratory tract infections is often empiric, usually involving administration of a β -lactam or macrolide. However, the increasing prevalence of antibiotic resistance in frequently isolated respiratory tract pathogens has complicated the antimicrobial selection process. The selection of appropriate antibiotic therapy for the treatment of lower respiratory tract infections must evolve based on the changing patterns of isolated organisms and emerging resistance to conventional therapies.

VII SUMMARY AND RECOMMENDATION

7.1 SUMMARY

1. LRTI is the one of the major cause of morbidity and mortality.
2. Out of the 82 positive cases, 51(62.2%) were due to Gram Negative Bacteria and 31 (37.8%) were due to Gram Positive Bacteria.
3. Among the total isolates, *Klebsiella pneumoniae* was the most common isolate (25.6%), followed by *S aureus* (24.39%), *Haemophilus influenzae* (15.85%), *S pyogenes* (7.31%), *S pneumonia* (6.09%) and *P aeruginosa* (6.09%).
4. Out 211 cases of LRTI screened for AFB, 18 cases showed the positive result. Of which, male (11, 61.11%) and female (7, 38.88%).
5. The prevalence of MDR was 40% (8/2) in *S aureus*, 33.33% (2/6) in *S pyogenes*, 20% (1/5) in *S pneumonia*. In case of GNB, all isolates of *P aeruginosa* (5), *K oxytoca* (2), *A calcoaceticus* (1), *and M catarrhalis* (1) showed MDR in in-vitro experiment.
6. Three isolates of *K pneumoniae* (14.28%) and one isolate of *E coli* (25%) were detected as ESBL.
7. The most effective drug for *S aureus* was Chloramphenicol and Azithromycin with sensitivity 80% each. In case of *S pneumoniae*, Ciprofloxacin (100%) was the most effective.
8. The most effective drug for GNB was Ciprofloxacin (62.74%) and the least effective was Amoxicillin (33.3%).

7.2 RECOMMENDATIONS

1. The current study is only a base-line survey based on laboratory findings and prospective study. In depth study including more representative population might be helpful in developing control measures to check increasing morbidity and mortality due to respiratory infection each year.

2. Etiology diagnosis could be established in 38.86%. The reason could be the association of other agents like Virus, Fungi, Legionella, Mycoplasma, Mycobacterium and parasites etc. Further investigation should be carried out to unveil those etiological agents since this study could not include these agents.

3. The need of active case findings for Tuberculosis and gender based study seem necessary for effective monitoring of the disease,

4. Antibiotic should be prescribed whenever possible based on culture sensitivity test results. If not possible at least Gram stain and AFB stain of the sample should be performed to take the guidelines for the prompt treatment. Widely used drugs were gradually become ineffective. So effective formulation of treatment system is suggested to tackle the treatment failure and increase frequency of drug resistance.

5. MDR and ESBL strains should be subjected to further genetic study in order to acquire more information regarding genetic makeup of these modified strains.

6. There should be the effective network system to monitor laboratory practice in order to meet high quality outcomes and prudent use of antibiotics.

7. Dissemination of this type of research outcome should be made available to all the health professionals.

CHAPTER VIII

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APPENDIX I

Clinical and microbiological profile of LRTI cases

Name of the patient: …… Date: …….

Age: …… Sex: ……

Specimen type: ……

Lab No.: …….

Brief Clinical History: ……

Patient on antibiotics: Yes. No.

If yes, Antibiotics taken: 1)…….. 2)……....

Duration of treatment: ……

Microbiological Investigation:

Day 1

1. Collection of the sample
   Time of sample collection: ……..
   Mode of collection: ……..
   Receiving time at the laboratory: …….

2. Microscopy
   Gram staining…….. AFB staining ……

3. Culture of specimen on: 1)……2)……..3)……..
   Incubation type: 1) Aerobic 2) Anaerobic 3) Microaerophilic
   Incubated at: …… Incubation time: ……

Day 2

1. Observation of the primary culture plate and enrichment media if left.

Media	Feature	Size	Shape	Color	Texture	opacity	Consistency
CA							
BA							
MA							

2. Gram staining of the desired colony: ……..
3. Oxidase test: …… Catalase test: ……
4. Subculture of the colony on the nutrient agar

5. Biochemical test

6. Test performed Result

   …… ……..

Day 3

1. Observation of secondary plates
2. Results of biochemical test

   Test performed Result
   TSIA ……..
   SIM ……...
   Citrate ……
   Urea ……
   Decarboxylase test:
   Lysine : ……...……
   Arginine : …….…..
   Ornithine: ……

   Organism identified: ……..

   Antibiotic Susceptibility pattern by Kirby-Bauer Method

S.N.	Antibiotic used	Zone of inhibition	Result
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

Performed by
……..

Checked by
……..

APPENDIX II

Equipments and Materials used during the study

Equipments

Autoclave: Sternite, Japan

Centrifuge: Heltich, Japan

Distillation plant: India

Refrigerator: Sanyo Japan, LG Korea

Hot Air Oven: Memmert, Germany

Incubator: Sakura, Japan

Water bath: Boekel 148003, Japan

Weighing balance: Choyo MP, Japan

Microscope: Olympus, Japan

Media used

MacConkey agar

Blood agar

Chocolate agar

Nutrient agar and nutrient broth

Nutrient broth

Mueller Hinton agar and Mueller Hinton broth

MR-VP medium

Simon citrate agar

Urea broth

Triple sugar iron agar

Sulfide indole motility agar

The entire medium used was from

1. Mast Company Limited UK
2. Oxoid Unipath Limited Hampshire UK

Reagents

NaCl

Crystal violate

Gram's iodine

Absolute alcohol

Safranin

3% H2O2

Paraffin oil

Normal saline

Barritt's reagent

Kovac's reagent

All of these reagents were brought from the local suppliers.

Antibiotic discs

Amoxicillin (30 µg)

Chloramphenicol (30 µg)

Ciprofloxacin (5 µg)

Ofloxacin (5 µg)

Nalidixic acid (30 µg)

Ceftriaxone (30 µg)

Cefotaxime (30 µg)

Tetracycline (30 µg)

Furazolidone (100 µg)

Cotrimoxazole (25 µg)

Erythromycin (15 µg)

Amikacin (15 µg)

Gentamicin (15 µg)

Polymyxin B (300 U)

These entire antibiotic discs used were brought from Mast Co. Ltd. U.K.

APPENDIX III

A. Composition and preparation of different culture media

All the culture media used were from the Mast co. ltd. U.K.

1. Nutrient agar

Composition	gm/ltr
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH at 25ºC	7.4±0.2

28 gram of medium was suspended in 1000 ml of the distilled water and boiled to dissolve completely. Then medium was autoclaved at 121 ºC (15lbs pressure) for 15 min. the sterilized medium was then poured in to sterilized petridishes and then was allowed to cool.

2. Blood agar base (Infusion agar)

Composition	gm/ltr
Beef heart infusion form	500
Tryptose	10.00
Sodium chloride	5.00
Agar	15.00
Final pH at 25ºC	7.3±0.2

42.5 gram of the medium was suspended in 1000 ml of distilled water, dissolved by boiling and sterilized by autoclaved at 121ºC for 15 mins. After cooling to about 50-55 ºC, 5% v/v defibrinated sheep blood was added aseptically, then mixed with gentle rotation and poured in to sterilized petridishes and was allowed to cool.

3. Chocolate agar

The streilized blood agar was poured in petri plates and allowed to solidify and was heated at 75 degree Celsius in an oven for 30 minutes. By the time the color changes to chocolate brown.

4. MacConkey agar

Composition	gm/ltr
Peptic digest of animal tissue	17.00
Proteose peptone	3.00
Lactose	10.00
Bile salt	1.50
Sodium chloride	5.00
Neutral red	0.03
Agar	15.00
Final pH at 25ºC	7.1±0.2

51.3 gram of the medium was dissolved in 1000 ml of distilled water and then boiled to dissolve completely. The media was autoclaved at 121ºC for 15 mins. Sterilized medium was then poured in to sterile petridishes and was allowed to cool.

5 . Nutrient broth (NB)

Composition	gm/ltr
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Final pH at 25ºC	7.4±0.2

13 gram of the medium was dissolved in 1000ml of water and then boiled to dissolve completely. The medium was then dispensed in to the tubes about 3ml in each and

autoclaved at 121ºC for 15 minutes. The sterilized medium was then cooled to room temperature.

6. Mueller Hinton agar (MHA)

Composition	gm/ltr
Beef Infusion	300.00
Casein Acid Hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH at 25ºC	7.3±0.2

38 gram of the medium was dissolved in 1000ml of distilled water and then boil to dissolve completely. The medium was autoclaved at 121ºC for 15 mins. The sterilized medium was then poured in sterilized petridishes and was allowed to cool.

B. Composition and preparation of different biochemical test media

1. Simon citrate agar

Composition	gm/ltr
Magnesium sulfate	0.20
Mono ammonium dihydrogen phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Bromothymol blue	0.08
Agar	15.00
Final pH at 25ºC	6.8±0.5

24.2 gram of the medium was dissolved in 1000 ml of distilled water and boiled to dissolved completely.3 ml of medium was dispensed in each tube and autoclaved

at 121ºC for 15 minutes. The sterilized mediums are allowed to settle at slant forming position.

2. Urea agar base (Christensen urea agar)

Composition	gm/ltr
Peptic digest of animal tissues	1.00
Dextrose	1.00
Monopotassium phosphate	0.8
Dipotassiu phosphate	1.20
Sodium chloride	5.00
Agar	15.00
Phenol red	0.012
Final pH at 25ºC	6.8±0.2

24 gram of the medium was suspended in 950 ml of water and dissolved by boiling and autoclaved at 121ºC for 15 minutes. After cooling to 50 ºC, 50 ml of sterile 40% urea solution was poured in to the medium and mixed with gentle rotation. Then 5 ml of the medium was dispensed in each tube and slant was prepared.

3. Sulfide indole motility (SIM) agar

Composition	gm/ltr
Peptic digest of animal	30.00
Beef extract	3.00
Peptonized iron	0.20
Sodium thiosulfate	0.025
Agar	3.00
Final pH at 25ºC	7.3±0.2

36.23 gram of the medium was dissolved in 1000 ml of distilled water and boiled to dissolve completely. Then it was dispensed in the test tube about 4 ml and autoclaved at 121 ºC for 15 minutes. Then it was cool down.

4. MR-VP medium

Composition	gm/ltr
Buffered peptone	7.00
Dextrose	5.00
Di- potassium phosphate	5.00
Final pH at 25ºC	6.9±0.2

17 gram of medium was dissolved in 1000 ml of distilled water and boiled to dissolve completely. 3 ml of medium was dispensed in each tube and autoclaved at 121ºC for 15 minutes.

5. Triple sugar iron (TSI) agar

Composition	gm/ltr
Peptic digest of animal tissue	10.00
Casein Enzymatic Hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulfate	0.30
Agar	12.00
Phenol red	0.024
Final pH at 25ºC	7.4±0.2

65 gram of the medium was dissolved in 1000ml of distilled water and dissolved completely. Then it was dispensed in to the tubes and autoclaved at 121ºC for 15 minutes. The sterilized medium in the test tube was then allowed to set in slant with a butt of 1inch thickness.

Deoxyribonuclease (Dnase) Agar medium

Composition	gm/ltr
Tryptose	20
Deoxyribonuclease	2
Sodium chloride	5
Agar	12
Final pH	7.3

Preparation: As directed by the manufacturing company, 3.9 gram of the medium was dissolved in 1000 ml of distilled water and heatedto dissolve in media. The medium was sterilized by autoclaving at 15lbs pressure at 121°C for 15 minutes.

Hugh-Leifson Medium (O/F basal medium)

Composition	gm/ltr
Tryptone	2
Sodium chloride	5
Dipotassium phosphate	0.3
Bromothymol blue	0.08
Agar	2

Preparation: As directed by the manufacturing company, 0.94 gram of the medium was dissolved in 1000 ml of distilled water and heated to dissolve the media. The medium was dispensed in test tube and sterilized by autoclaving at 15 lbs pressure at 12°C for 15 minutes.

C. Composition and preparation of different staining reagent

1. **Gram stain**

a. Crystal violet solution

Crystal violet	20.00
Ammonium oxalate	9.00
Ethanol or Methanol	95.00ml
Distilled water	1000ml

Preparation: 20 grams of crystal violet was weighed in a clean piece of paper and transferred to a clean brown bottle. Then 95 ml of ethanol was added and mixed until the dye is completely dissolved. To the mixture, 9 grams of ammonium oxalate dissolved in 200ml of distilled water was added. Finally the volume was made 1000ml by addition of distilled water.

b. Lugol's Iodine

Composition	gm/ltr
Potassium iodide	20 gm
Iodine	10gm
Distilled water	1000 ml

Preparation: to 250 ml of distilled water, 20 gm of potassium iodide was dissolved and 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1000mlby addition of distilled water.

c. Acetone alcohol decolorizer

Composition	gm/ltr
Acetone	500ml
Ethanol (Absolute)	475ml
Distilled water	25ml

Preparation: 475 ml of ethanol was added to 25 ml of distilled water and mixed and kept in a clean bottle. Then immediately 500ml of acetone was added to the bottle and mixed well.

d. Safranin (Counter stain)

Composition	gm/ltr
Safranin (2.5% in 95% ethanol)	10.00 ml
Distilled water	100 ml

Preparation: 2.5% of Safranin solution was prepared in 95% ethanol and 10 ml of prepared suspension was mixed in 100 ml of distilled water.

2. Ziehl-Neelsen stain

a. Carbol fuschin

Composition	gm/ltr
Basic fuschin (powder)	10
Phenol (crystalline)	50
Alcohol (95% or 100% ethanol)	100ml
Distilled water	1000ml

Direction: Basic fuschin powder and phenol crystals were weighed and dispensed in a 1 litre flask over a boiling water bath for about 5 minutes, shaking the contents from time to time. When solution was complete, the alcohol was added and mixed throughly. Then distilled water was added. The mixture was filtered before use.

b. Acid alcohol decolorizer (3% v/v)

Composition	gm/ltr
Concerntrated hydrochloric acid	75 ml
Industrialized methylated spirit	2425 ml

Direction: The methylated spirit was poured into a large flask. The flask was placed in 5-8 cm of water in the sink. The hydrochloric acid was added and the top of the flask was covered to stop fumes escaping.

c. Malachite green

Composition	gm/ltr
Malachite green	5 gm
Distilled water	500 ml

Direction: The dye was added to the distilled water in a bottle and shaked to dissolve. The1% stock solution was diluted to make the working solution.

1% stock solution	40 ml
Distilled water	360 ml

3. Normal saline

Composition	gm/ltr
Sodium chloride	0.85gm
Distilled water	100ml

Preparation: o.85 gram of sodium chloride was weighed and added to a bottle containing 100ml of distilled water and mixed well to dissolve the salt completely and autoclaved. Then it was stored.

4. Biochemical Test Reagents

a. For catalase test

Catalase reagent (3% H_2O_2)

Hydrogen peroxide	1ml
Distilled water	9ml

Preparation: To the 9ml of distilled water, 1ml of hydrogen peroxide was added and mixed well so as to make 3% solution of hydrogen peroxide.

b. For oxidase test

Oxidase strip soaked in oxidase reagent

Tetra methyl para-phenylene diamine dihydrochloride(TPD)	1gm
Distilled water	100ml

Preparation: 1 gram of TPD was dissolved in 100 ml of distilled water and strips of Whatmann no. 1 paper was soaked and drained for anout 30seconds. Then the strip was freeze dried and stored in dark bottle tightly.

b. **For indole test**

Kovac's indole reagent

Para Dimethyl amino benzaldehyde	2.00gm
Isoamyl alcohol	30.00ml
Concentrated hydrochloric acid	10.00ml

Preparation: in 30 ml of isoamyl alcohol, 2 gram of para amino bemzaldehyde was dissolved and transferred to clean brown bottle. Then to this solution,10 ml of concentrated hydrochloric acid was added and mixed well.

c. For methyl red test

Methyl red solution

Methyl red	0.05gm
Ethyl alcohol	28.0ml
Distilled water	22.0ml

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

d. For Voges Proskauer test

Barritt's reagent

Solution A

Alpha-Napthol	5.0gm
Ethyl alcohol	100ml

Preparation: 5gm of α-Napthol was dissolved in 25 ml ethanol and transferred in to clean bottle. Then final volume was made 100ml by adding ethanol.

Solution B

Potassium hydroxide (KOH)	40.0gm
Distilled water	100ml

Preparation: 40 gram of KOH was dissolved in 25 ml of distilled water and transferred in to the clean bottle and final volume was made 100ml by adding distilled water.

e. **Bile salt solution:**

Composition	gm/ltr
Commercially available sodium deoxycholate	10g
Distilled water	100ml

Preparation: A 10% solution of sodium deoxycholate was prepared by adding 10gm sodium deoxycholate powder in 100 mll distilled water and transferred into a clean bottle and was autoclaved.

f. Name of Sputasol

"Dithiotheritol"
CODE-DR 89 Basingstoke
Hampshire, England.

g. Turbidity standard equivalent to McFarland 0.5

1% V/V solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99 ml of distilled water. 1% W/V solution of barium chloride was prepared by dissolving 0.5 gram of dehydrate barium chloride in 50 ml of distilled water. Then to the 99.5ml of 1% sulphuric acid solution, 0.5 ml of barium chloride solution was mixed and stirred continuously. Then the solution was transferred in to the clean screw capped tube and stored at dark place until use. The test tube for the broth preparation should be of same size as of McFarland tube. The tubes can be stored and used for six months.

APPENDIX IV

A. Procedure for gram staining (Forbes et al., 2007)

Gram staining is differential staining that differentiates all the bacterial species in to two large groups: gram positive and gram negative. Following steps are involved during gram staining.

1. A thin film of material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 second.
4. The slide was rinsed with tap water, shaking off excess.
5. Then the slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with acetone alcohol decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear require more aggressive decolorization.
8. The slide was flooded with counter stain Safranin for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

B. Procedure for Antibiotic Sensitivity Testing (AST) by Disc Diffusion Method.

In the treatment and control of infectious disease, AST is done to select effective antimicrobial drugs against suspected organisms. Disc diffusion method is widely used technique for susceptibility testing and done by Kirby Bauer disc diffusion method.

The following steps are involved in AST by Kirby Bauer disc diffusion method.

1. An isolated colony of organism was suspended in the nutrient broth and incubated at 37ºC for 4 hours. The turbidity was matched with 0.5 McFarland turbidity standards.
2. A sterile cotton swab was taken and introduced in to the tube taken out the organism and swabbed uniformly on the surface of Mueller Hinton agar medium.

3. The plate was allowed to dry and antibiotic disc were placed on the agar surface and incubated for 18-24 hours.
4. After incubation the zone size was measured and results were interpreted according to the standard guidelines.

C. Procedure for Ziehl-Neelsen staining (Forbes et al., 2007)

It was performed for the screening of Mycobacterium tuberculosis and other Acid fast bacilli in sputum samples. Due to presence of mycolic acids in their cell walls Acid fast bacilli retains the basic dye despite acid-alcohol treatment which differentiates it from other bacterium. Following steps are involved during Ziehl-Neelsen staining.

1. Fix smears on heated surface (60°C for at least 10 minutes).
2. Flood smears with carbolfuschin (primary stain) and heat to almost boiling by performing the procedure on an electrically heated platform or by passing the flame of a Bunsen burner underneath the slides on a metal rack. The stain on the slides should steam. Allow slides to sit for 5 minutes after heating; do not allow them to dry out.
3. Wash the slides in distilled water (note: tap water may contain acid-fast bacilli). Drain off the excess liquid.
4. Floold slides with 3% HCL in 95% ethanol (decolorizer) for approximately 1 minute. Check to see that no more red color runs off the surface when the slide is tapped. Add a bit more decolorizer for every thick slides or those that contains to “bleed” red dye. Wash throughly with water and remove the excess.
5. Flood slides with methylene blue (counter stain) and allow to remain on surface of slides for 1 minute. Wash with distilled water and stand slides upright on paper towels to air dry.

APPENDIX V

Interpretation charts for antibiotics

A. Zone size interpretative chart (CLSI interpretation)

Antibiotic used	Concentration (µg)	Diameter of zone size(mm)			ATCC culture *E.coli* 25922 target zone size (mm)
		Resistant	intermediate	Sensitive	
Ampicillin	10	≤ 13	14-16	≥ 17	16-22
Ciprofloxacin	5	≤ 19	20-21	≥ 22	30-40
Ofloxacin	5	≤ 19	20-21	≥ 22	29-33
Nalidixic acid	30	≤ 13	14-18	≥ 19	22-28
Chloramphenicol	30	≤ 12	13-17	≥ 18	21-27
Ceftriaxone	30	≤ 13	14-20	≥ 21	29-35
Cefotaxime	30	≤ 14	15-22	≥ 23	29-35
Erythromycin	15	≤ 13	14-22	≥ 23	22-30
Amikacin	15	≤ 13	14-15	≥ 16	19-26
Gentamicin	15	≤ 14	15-16	≥ 17	19-26
Furazolidone	100	≤ 18		≥ 18	22-26
Cotrimoxazole	25	≤ 10	11-15	≥ 16	24-32
Tetracycline	30	≤ 14	13-18	≥ 19	18-25
Polymyxin B	300	≤ 11		≥ 11	13-19

B. Zone size interpretative chart (EUCAST interpretation)

Antibiotic used	Concentration (µg)	Diameter of zone size(mm)			ATCC culture *E.coli* 25922 target zone size (mm)
		Resistant	intermediate	Sensitive	
Ampicillin	10	≤ 14			16-22
Ciprofloxacin	5	≤ 19	20-21	≥ 22	30-40
Ofloxacin	5	≤ 19	20-21	≥ 22	29-33
Nalidixic acid	30				
Chloramphenicol	30	≤ 17		≥ 17	21-27
Ceftriaxone	30	≤ 20	21-22	≥ 23	29-35
Cefotaxime	30	≤ 18	19-20	≥ 21	29-35
Erythromycin	15				
Amikacin	15	≤ 13	14-15	≥ 16	19-26
Gentamicin	15	≤ 14	15-16	≥ 17	19-26
Furazolidone	100				22-26
Cotrimoxazole	25	≤ 13	11-15	≥ 16	24-32
Tetracycline	30				
Polymyxin B	300				

APPENXIX VI

a) Catalase test :

The catalase test facilitates the detection of the enzyme catalase in bacteria. It is essential for differentiating catalase-positive Micrococcaceae from catalase-negative Streptococcaceae. While it is primarily useful in differentiating between genera, it is also valuable in speciation of certain gram positives such as Aerococcus urinae (positive) from Aerococcus viridians (negative) and gram-negative organisms such as Campylobacter fetus, Campylobacter jejuni, and Campylobacter coli (all positive) from other Campylobacter species. Some have reported its value in the presumptive differentiation among certain Enterobacteriaceae . The catalase test is also valuable in differentiating aerobic and obligate anaerobic bacteria, as anaerobes are generally known to lack the enzyme. In this context, the catalase test is valuable in differentiating aerotolerant strains of Clostridium, which are catalase negative, from Bacillus, which are catalase positive.

The enzyme catalase is present in most of cytochrome containing aerobic and facultative anaerobic bacteria, the main exception is Streptococcus spp. Usually organisms which lack the cytochrome system also lack the catalase enzyme and therefore are unable to breakdown hydrogen peroxide. Catalase is hemeprotein. The prosthetic group is made up of four atoms of trivalent iron (ferric) per molecule, which retains its oxidized state during enzyme activity. Hydrogen peroxide is reduced flavoprotein reacts directly with gaseous oxygen by way of electron reduction to form hydrogen peroxide and not by direct action between hydrogen and molecular oxygen.

For the test, 2-3 ml of hydrogen peroxide solution was poured in a test tube and then using a sterile glass rod several colonies of the test organisms were immersed in hydrogen peroxide solution. Bubbling of the gas was noted. The lack of catalase was evident by lack of or weak bubble production. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide (13). Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2$). This reaction is evident by the rapid formation of bubbles.

b) Oxidase Test:

The oxidase test is a test used to determine if a bacterium produces certain cytochrome c oxidases. It uses disks impregnated with a reagent such as N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) or N,N-Dimethyl-p-phenylenediamine (DMPD), which is also a redox indicator. The reagent is a dark blue to maroon color when oxidized, and colorless when reduced. Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidize (an iron containing haemoprotein). These both catalyse the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The test reagent, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol blue. The cytochrome system is usually only present in aerobic organisms which are capable of utilising oxygen as the final hydrogen receptor. The end product of this metabolism is either water or hydrogen peroxide (broken down by catalase). The oxidase test is based on the bacterial production of an oxidase enzyme.

The oxidase papers moistened with distilled water and a colony from the fresh culture was picked up with a sterile glass rod and smeared on the paper. Within 10 seconds the change in color was noted.All Pseudomonas and Neisseria spp. Produce and enzyme called oxidase which in the presence of atmospheric oxygen, and cytochrome c, oxidize the oxidase reagent to form a colored compound.

C) Methyl red test:

Methyl red is used in the Methyl Red (MR) Test, used to identify bacteria producing stable acids by mechanisms of mixed acid fermentation of glucose. The methyl red test is the "M" portion of the four IMViC tests used to characterize enteric bacteria. The methyl red test is used to identify enteric bacteria based on their pattern of glucose metabolism. All enterics initially produce pyruvic acid from glucose metabolism. Some enteric subsequently use the mixed acid pathway to metabolize pyruvic acid to other acids, such as lactic, acetic, and formic acids. These bacteria are called methyl-red positive and include Escherichia coli and Proteus vulgaris. Other enterics subsequently use the buytylene glycol pathway to metabolize pyruvic acid to

neutral end-products. These bacteria are called methyl-red-negative and include Serratia marcescens and Enterobacter aerogenes. The methyl red test uses a pH indicator in form of methyl red, to determine the hydrogen ion concentration (pH) arising out of fermentation of glucose by various organism. The hydrogen ion concentration depends on the gas ratio (CO2 & H2) which in turn is an index to the different pathways of glucose metabolism exhibited by various organisms. The different fermentation patterns are due to various in enzymes concerned with pyruvic acid metabolism present in the organism.

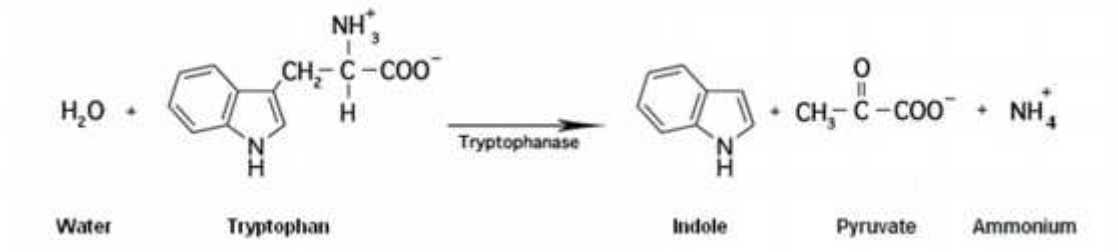
Methyl red positive organisms produce stable acids, maintaining a high concentration of hydrogen ions until a sudden concentration is reached. The validity of methyl red test depends upon a sufficient incubation period in oder to permit the difference in glucose metabolism to occur. The organisms to be tested should be incubated at least two days at 35-37^0 Celsius.

The culture is incubated in Clark and Lubb's medium (MR/VP, pH 6.9). Half of this medium is then used for the methyl red test and the other half for a Voges-Proskauer test. Methyl red indicator is added to the medium. Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4-6.0. For this test, the organism from nutrient broth was incubated and was incubated at 37^0 Celsius for 24 hours. After incubation 2-3 drops of Methyl red was added and color change was noted.

d) Indole production test:

Amino acid Tryptophan is oxidized by certain bacteria to form three major indolic metabolites; indole, skatole (methyl iodole), and indoleacetic acid. Intracellular enzymes involved in this deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole. The enzyme is Tryptophanase. The organisms are inoculated (stabbed) into SIM (Sulphide Indole Motility) medium from the nutrient broth and incubated at 37^0 Celsius for 24 hours. When 2-3 drops of Kovac's reagent (p-dimethylaminobenzaldehyde in acid ethanol) is added to a medium with indole in it, a dark pink color develops. If indole id present combines with the aldehyde present in Kovacs reagent to give a red color in the alcohol layer. The indole test must be read by 48 hours of incubation because the

indole can be further degraded if prolonged incubation occurs. The acidic pH produced by Escherichia coli limits its growth. The color reaction is based on the presence of the pyrrole structure present in the indole.

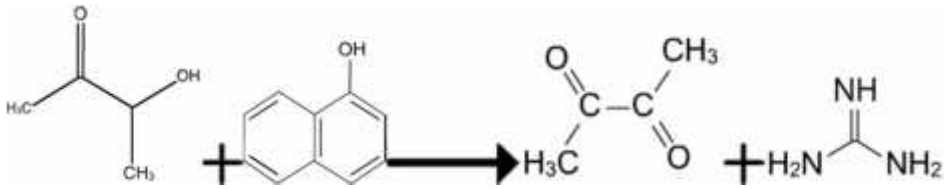


e) Voges Proskauer (VP) test:

The purpose of this test is to determine whether an organism can produce acetylmethylcarbinol (acetoin) from fermentation of glucose. The culture is incubated in Clark and Lubb's medium. Half of this medium is then used for the methyl red test and the other half for a Voges-Proskauer test. Alpha-napthol (5%) and potassium hydroxide (40%) are added to the medium. If acetoin is present the medium will turn pink-reddish in color.

The Voges-Proskauer test determines the capability of some organisms to produce non acidic or neutral end products, such as acetyl methyl carbinol, from the organic acids that result from glucose metabolism.

The reagent used in this test is Barritt's reagent, consists of a mixture of alcoholic a-naphthol and 40% potassium hydroxide solution. Detection of acetyl methyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of the a-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium. As a result, a pink complex is formed, imparting a rose color to the medium. Development of a deep rose color in the culture 15 minutes following the addition of Barritt's reagent is indicative of the presence of acetyl methyl carbinol and represents a positive result. The absence of rose color is a negative result.



Actyl methyl carbinol Alpha naphthol diacetyl Guanidine group of peptone (pink) complex)

To differentiate among the enteric organisms such as Escherichia coli, Enterobacter aerogenes and Klebsiella pneumoniae.

Bacteria like Enterobacter hafniea, are VP variable at 37 c but positive at 25c to 35c. Cowan and Steel found that incubation at 30c for 5 days is the minimum time period to detect the VP positive organisms among the Enterobacteriaceae using Barrits reagent (alpha napthol in ethanol and 40% KOH). However for most of the VP positive organisms of Enterobacteriaceae, about 18 to 24 hours of incubation is sufficient. The positive test is indicated by development of Pinkish red color and yellow color at the surface of the medium is obtained for the negative test. For the test, the organisms from nutrient broth was inoculated and was incubated at 37 c for 24 hours. After incubation the broth was added with Barrits reagent and tube was incubated for 10-15 minutes to note the color change.

f) Citrate test:

Simmons citrate agar tests the ability of organisms to utilize citrate as a carbon source. Simmons citrate agar contains sodium citrate as the sole source of carbon, ammonium dihydrogen phosphate as the sole source of nitrogen, other nutrients, and the pH indicator bromthymol blue. This test is part of the IMViC tests and is helpful in differentiating the Enterobacteriaceae. Organisms which can utilize citrate as their sole carbon source use the enzyme citrase or citrate-permease to transport the citrate into the cell. These organisms also convert the ammonium dihydrogen phosphate to ammonia and ammonium hydroxide, which creates an alkaline environment in the medium. At pH 7.5 or above, bromthymol blue turns royal blue. At a neutral pH, bromthymol blue is green, as evidenced by the uninoculated media.

If the medium turns blue, the organism is citrate positive. If there is no color change, the organism is citrate negative. Some citrate negative organisms may grow weakly on the surface of the slant, but they will not produce a color change. In bacteria the cleavage of citrate involves an enzyme system without the intervention of the coenzyme A. This enzyme is called citrate or citrate demolase. The medium used is for the citrate fermentation test is "Simmons citrate medium" also contains inorganic ammonium salts. Organism capable of utilizing citrate as its sole carbon source also utilizes the ammonium salt as its sole nitrogen source. Ammonium source is broken down to ammonia with resulting alkalinity.

g) Triple Sugar Iron (TSI) Agar

Triple sugar iron agar (TSI) is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and the pH indicator phenol red. It is used to differentiate enterics based on the ability to reduce sulfur and ferment carbohydrates. Triple Sugar Iron Agar is recommended for differentiation of enteric, Gram-negative bacilli from clinical specimens, dairy samples, and food products. The medium measures a bacterium's ability to utilize three sugars, glucose, sucrose and lactose, and their concentration are 0.1%, 1.0%, and 1.0 respectively. A pH indicator included in the medium can detect acid production from fermentation of these carbohydrates. Fermentation of glucose alone will show as a yellow color in the butt of the medium, fermentation of sucrose and/or lactose will cause both butt and slant to be yellow. Bacteria that attack glucose by oxidation or not at all are often obligate aerobes and will only grow on the slant of the agar and often cause an alkaline reaction there due to the utilization of peptones. Production of hydrogen sulphide leads to blackening. Results are given as slant/butt/gas production/hydrogen sulphide production. It is due to the reduction of sodium thiosulphate to hydrogen sulphide. Production of other gases was marked by craks in the agar as well as an air gap at the bottom of the test tube.

The organism to be tested is cultured in a medium which contains urea and the indicator phenol red. When the strain is urease producing, the enzyme will hydrolyze the urea to give ammonia and carbon dioxide. With the release of ammonia, the

medium becomes alkaline as shown by a change in color of the indicator to pink red. Using a straight wire, a pure colony of test organism was streaked over the slope of urease agar slant and incubated at 37c for 24 hours. After incubation, urease production was examined by a red-pink color of the medium.