KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

SCHOOL OF GRADUATE STUDIES

SCHOOL OF MEDICAL SCIENCES

DEPARTMENT OF CLINICAL MICROBIOLOGY

EPIDEMIOLOGY OF *KLEBSIELLA* INFECTIONS AT KOMFO ANOKYE TEACHING HOSPITAL.

BY DESMOND OMANE ACHEAMPONG

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A THESIS SUBMITTED TO THE DEPARTMENT OF CLINICAL MICROBIOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE MASTER OF SCIENCE DEGREE (MSC.) IN CLINICAL MICROBIOLOGY

BY

DESMOND OMANE ACHEAMPONG

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DECLARATION

I hereby declare that this submission is my own work towards the MSc (Clinical Microbiology) and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other Degree of the University, except where due acknowledgement has been made in the text.

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Finally, I wish to deeply thank my family for their support, love, care and prayers that have brought me this far. May God richly bless and keep you all. Amen.

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ABSTRACT

Different *Klebsiella species* may be responsible for various infections which may also differ with the site of the infection. These species may also present with different antimicrobial sensitivity patterns. However, the identification of *Klebsiella* to species level is not practiced in most of our hospitals, during bacteriological diagnosis, because of time constraints and labour. In view of these, antibiotics are normally administered to treat *Klebsiella* infections without considering the type of species responsible for that particular infection, or site of isolation of the *Klebsiella* species. The objective of this project was to determine *Klebsiella species* responsible for causing infections, their sensitivity patterns to the commonly used antimicrobial agents and the effectiveness of empirical treatment at KATH. Samples were collected from 2197 patients. These samples were cultured to isolate the *Klebsiella species.* Isolates were differentiated to the species level and their antimicrobial sensitivity patterns determined. The empirical treatment on 51 pediatric patients were monitored and compared with the laboratory sensitivity patterns of the isolates. Two hundred and five (205) *Klebsiella species* were isolated, these included *Klebsiella pneumoniae, K. oxytoca, K. rhinoscleromatis and K. ozaenae. K. pneumoniae* and *K. oxytoca* were the commonest isolates. Prevalence of *Klebsiella* infections in the commonest clinical specimens were sputum (14.1%), wound (12.6%), urine (10.7%) and blood (5.1%). The highest female cases were found in urine and the male cases were found in sputum. There was no significant difference between the out-patient and in-patient cases and among sexes of *Klebsiella* infections at Komfo Anokye Teaching Hospital. Mother Baby Unit (MBU) registered 21 *Klebsiella* isolates, Block A which accommodates pregnant women recorded 19 *Klebsiella* isolates, Block B which accommodates children registered 16 *Klebsiella* isolates and the pediatric emergency unit (PEU) recordered 15 *Klebsiella* isolates. Results also indicated that *K.*

pneumoniae was commonest species, but *K. oxytoca* was found to be more drug resistant than *K. pneumoniae*. The study also revealed that, aminoglycosides and third generation cephalosporins were the most effective invitro antimicrobials for managing *Klebsiella* infections at KATH. Results gathered from empirical treatment showed that, aminoglycosides and third generation cephalosporins were the most prescribed antimicrobials for *Klebsiella* infections. Although empirical treatment is very relevant, it cannot be relied upon completely, but should be combined with laboratory antibiotic sensitivity testing for effective *Klebsiella* infection treatment.

CHAPTER ONE

1.0 INTRODUCTION

Klebsiellae cause a variety of infections in humans. These include pneumonia, urinary tract infections (UTI), rhinoscleroma, ozena and other soft tissue infections. (Obiamiwe, 2006). They are usually opportunistic pathogens found in the environment and in mammalian mucosal surfaces, with the principal pathogenic reservoirs being the gastrointestinal tract of patients and the hands of hospital personnel (Podschun and Ulmann, 1998). *Klebsiella pneumoniae* and *K. oxytoca* are the commonest species responsible for human related *Klebsiella* infections, with *K. pneumoniae* accounting for 75 to 85% of *Klebsiella* infections reported worldwide (Farmer, 1999).

Klebsiella pneumoniae pneumonia is very fatal (Branger *et al.*, 2004). Most cases occur in middle-aged and older men with underlying debilitating diseases such as diabetes and chronic lung diseases (Liam *et al.*, 2001). The onset of *Klebsiella* pneumonia infection is usually sudden and symptoms associated with it are high fever, chills, malaise, body aches and productive cough with abundant, thick and blood-tinged sputum termed "currant jelly sputum" (Kobashi *et al.*, 2001). Mortality rates can be as high as 50%, and in the case of alcoholics, mortality can be almost 100% (Branger *et al.*, 2004).

Klebsiella pneumoniae is the commonest causative agent of urinary tract infections followed by *K. oxytoca* (Munro and Chambers, 2002) among the *Klebsiellae*. Characteristic symptoms include dysuria, frequent urination, low back pain and suprapubic discomfort. Systemic

symptoms such as fever and chills are usually indicative of underlying disorders like pyelonephritis and prostatitis (Munro and Chambers, 2002).

Klebsiella rhinoscleromatis and *K. ozaenae* cause rhinoscleroma and ozena, respectively and are rare (Zohar *et al*, 1990). They are associated with upper respiratory tract infections (Obiamiwe, 2006). Rhinoscleroma is chronic granulomatous infection with patients presenting with a purulent nasal discharge and nodule formation in the nose or sinuses that lead to respiratory obstruction (Obiamiwe, 2006). Ozena is a primary atrophic rhinitis. Common symptoms include nasal congestion and a constant nasal bad smell. Patients may also complain of headache and symptoms attributable to chronic sinusitis (Zohar *et al,* 1990)

Other diseases caused by *Klebsiellae* are bacteremia, wound infection, cholecystitis, cholangitis, diarrhea, meningitis, and endocarditis (Kobashi *et al.,* 2001). These infections are usually hospital-acquired (nosocomial infections) and occur among immunocompromised patients (Liam *et al.,* 2001).

Though culture and sensitivity testing results are required, initial therapy of patients with possible *Klebsiella* infection is empirical (Rice, 2001). Once culture and sensitivity results confirm infection and sensitivity result is released, treatment may be modified (Paterson, 2000). Usually, antimicrobial agents considered to have high intrinsic activity against *Klebsiella* are selected for severely ill patients (Paterson, 2000). Such antimicrobial agents include cefotaxime and ceftriaxone, which are third-generation cephalosporins, gentamicin and amikacin, which are aminoglycosides, imipenem and cilastatin which are carbapenems, and ciprofloxacin and

norfloxacin, which are quinolones (Segal-Maurer *et al.*, 1999). These agents may be used as monotherapy or in combination with other antimicrobials (Rice, 2001).

Antimicrobial therapy in Ghana is mainly empirical due to a relative lack of appropriate laboratory facilities for culture and sensitivity of bacteria in several health facilities (Newman *et al.*, 2006). Even, where laboratory facilities are available, culture and sensitivity tests take 48 to 72 hours to be ready (Newman *et al.*, 2006). In other cases it may not be requested at all due to the extra cost it constitutes for the patient (Ohene, 1997), giving way to empirical treatment. Empirical treatment, however, has its immediate and long term problems. The immediate problems may be not prescribing the appropriate drug leading to treatment failure, or the long term resulting in the evolution of multiple drug resistant strains of *Klebsiella,* making treatment of infections caused by such species and strains difficult. (Kaye *et al.*, 2000).

Length of hospital stay, performance of invasive procedures during treatment and improper use of antimicrobial agents are possible promoting factors for the acquisition of these multiple drug resistant *Klebsiellae* (Kaye *et al.*, 2000). In Ghana prevalence of drug resistance among the *Klebsiellae* is reported to be 57.65%, a value Newman *et al.*, (2006), considered to be high. This could account for the increase in the reported cases of *Klebsiella* infections in our hospitals in recent times (Newman *et al.*, 2006).

Podschun and Ullmann, (1998) reported on the percentage range of hospital-acquired *Klebsiella* infections worldwide, and quoted urinary tract infection to be 6-17%, pneumonia 7-14%, septicemia 4-15% and wound infection 2-4%. Also, Wen-Chien *et al*, (2002) reported that

Klebsiella accounts for 29% and 62% of all cases of community-acquired pneumonia in Taiwan and South Africa, respectively.

Reports from a research by Tulsi *et al.*, (2007) on the colonization and infections of neonates by *Klebsiella* in an intensive care unit of Kuwait hospital revealed that, coloniz ation increased from 10% on admission to 26% on day 3 and 39% on day 6. Carriage rate was 29% for intestine, 17% for umbilical stump, 13% for throat, 3% for skin and 1% for the external ear. Six babies out of 68 (8.8%) developed infection. They all had prior colonization with the same organism. Thirteen per cent of samples taken from ward environments were also positive for this organism.

A research in Nigeria on catheter associated urinary infection revealed that 36.6% were *Klebsiella* species and were among the commonest pathogens isolated (Taiwo and Aderoummu, 2006). Other studies on the prevalence of *Klebsiella* urinary tract infection in Nigeria by Aiyegoro *et al.*, (2007) and Ako-Nai *et al.*, (1993) recorded 25% and 22.8% respectively. Also according to a study carried out in 11 hospitals in 7 regions in Ghana, the percentages of *Klebsiella* isolates recorded in some selected clinical specimens were 11.7% in stool, 12.7% in urine, 8.5% in wound and 28.4% in sputum (Newman *et al.*, 2006). Another survey by Ohene, (1997) reported 10.2% as the prevalence of *Klebsiella* infections at Komfo Anokye Teaching Hospital. These relatively high figures support the fact that, *Klebsiella* infections are common and therefore require special attention.

According to Podschun and Ullmann (1998), different *Klebsiella species* may be responsible for the various *Klebsiella* infections. The different species may also differ with the site of infection.

The different species may present with different antimicrobial sensitivity pattern (Obiamiwe, 2006). However, the identification of *Klebsiella* to species level is not practiced in most of our hospitals, during bacteriological diagnosis, because of time constraints and labour. In view of these, antibiotics are normally administered to treat infections, including *Klebsiella* infections without considering the type of species responsible for that particular infection, or site of isolation of the *Klebsiella* species. This study therefore seeks to determine the *Klebsiella species* responsible for the various infections, their antimicrobial sensitivity patterns and the effectiveness of empirical treatment at KATH.

1.1 Aim of study

To investigate the epidemiology of *Klebsiella* infections at Komfo Anokye Teaching Hospital (KATH).

1.2 Specific Objectives

1. To determine *Klebsiella species* responsible for causing infections at KATH.
2. To determine the sensitivity of the *Klebsiella* isolates to the commonly used antimicrobial drugs at KATH.
3. To relate the *Klebsiella species* isolated to their antimicrobial sensitivity results and patients demographic data.
4. To determine the clinical effectiveness of empirical treatment for *Klebsiella* infections at KATH.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 CLASSIFICATION OF THE GENUS KLEBSIELLA

The genus *Klebsiella* is a member of the family Enterobacteriaceae (Ryan and Ray, 2004). It was identified in the 19th century and named after the discoverer Edwin Klebs (Ryan and Ray, 2004). The latest edition of *Bergey's Manual of Systematic* Bacteriology (Obiamiwe, 2006) classified the genus *Klebsiella* into five species, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella terrigena*, *Klebsiella planticola* and *Klebsiella ornithinolytica* with *K. pneumoniae* comprising three subspecies, *K. pneumoniae* subspecies *pneumoniae*, *K. pneumoniae* subspecies *ozaenae* and *K. pneumoniae* subspecies *rhinoscleromatis* but for simplicity they are normally referred to as *K. pneumoniae*, *K ozaenae* and *K rhinoscleromatis respectively* (Obiamiwe, 2006). *Klebsiella pneumoniae* and *K. oxytoca* are responsible for most of the *Klebsiella* infections associated with humans and are considered to be the clinically important species of the genus (Farmer, 1999).

2.2 HABITAT

Klebsiella species are ubiquitous in nature (Podschun and Ulmann, 1998). They have two common habitats, one being the environment, where they are found in surface water, sewage, soil and on plants. They are also found on the mucosal surfaces of mammals such as horses, swine, and humans (Podschun and Ulmann, 1998). In humans, *Klebsiella* species are present in the nasopharynx and in the intestinal tract (Obiamiwe, 2006).

2.3 PATHOGENECITY OF KLEBSIELLA

The *Klebsiellae* possess a capsule, pilli and the ability to produce siderophores, which enhance its capacity to cause infection in human (Podschun *et al.,* 2001).

2.3.1 Capsular Antigens

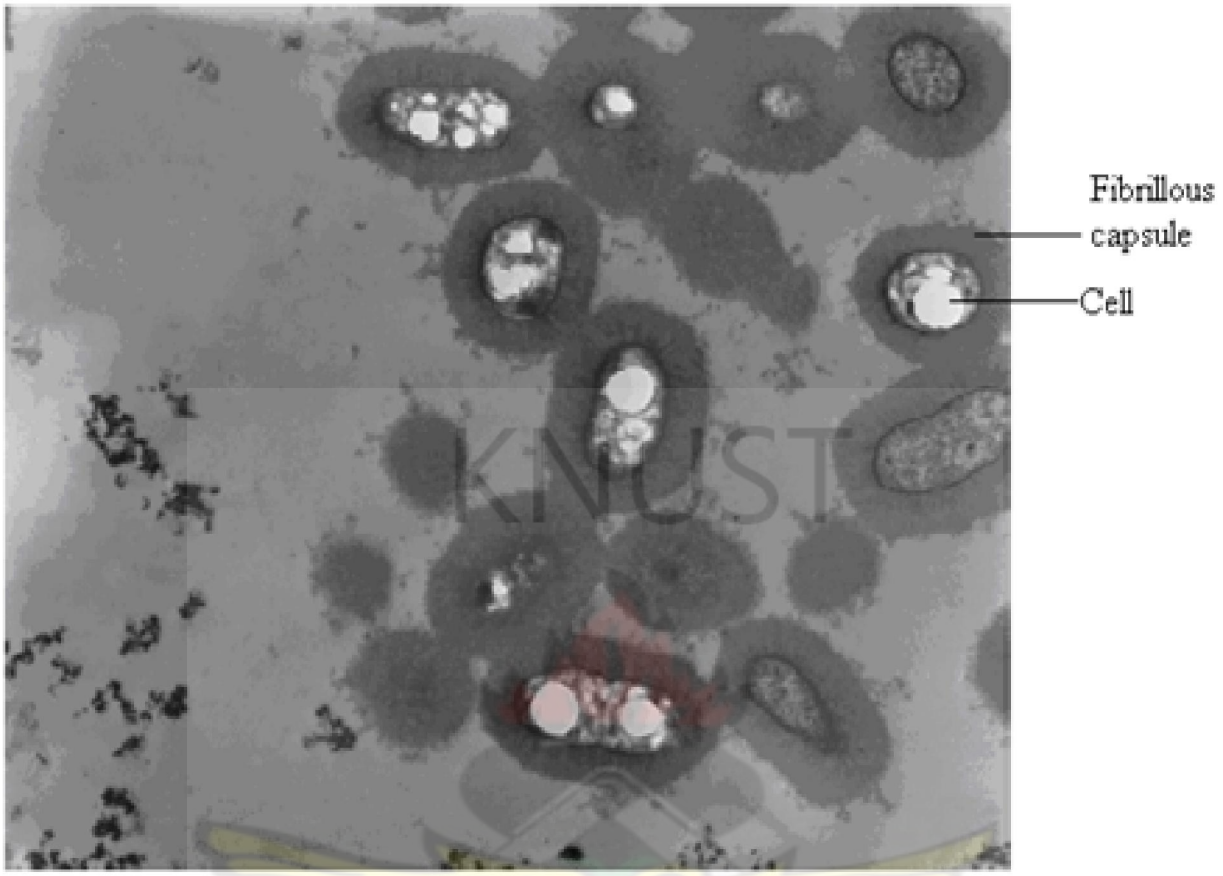
Klebsiellae possess prominent capsules which are repeating subunits, consisting of four to six sugars. In addition to the sugar, it also contains uronic acids which confer on it a negatively charged component (Campos *et al.*, 2004).

The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers as shown in Figure. 1 (Amako *et al.*, 1988). This protects the bacterium from phagocytosis by polymorphonuclear granulocytes, (Brisse *et al.*, 2004), and prevents killing of the bacteria by bactericidal serum factors, (Sahly *et al*, 2000).

2.3.2 Pili (Fimbriae)

Pili, otherwise known as fimbriae are nonflagellar, filamentous projections on the bacterial surface (Chung *et al.*, 2003). As a critical first step in the infectious process, microorganisms must come as close as possible to the host mucosal surfaces and maintain this proximity by attaching to the host cell (Schembri *et al.*, 2005). *Klebsiellae* attach to host mucosal surfaces using the pilli.

] Figure 1 Transmission electron micrograph of *K. pneumoniae* cells surrounded by thick layers of fibrillous capsular material. Courtesy of I. Ofek, Tel Aviv University, Israel. Reprinted from reference (Ofek and Doyle, 1994).



2.3.3 Production of Siderophores

The growth of bacteria in host tissue is limited not only by the host defense mechanisms but also by the supply of available iron (Moore *et al.*, 2000). Iron is an essential factor in bacterial growth, functioning mainly as a redox catalyst in proteins participating in oxygen and electron transport processes (Ofek *et al.*, 2001). The supply of free iron available to bacteria in the host milieu is extremely low, since this element is bound intracellularly to proteins such as hemoglobin, ferritin, hemosiderin and myoglobin. They also bind extracellularly to high-affinity iron-binding proteins such as lactoferrin (Moore *et al.*, 2000). Many bacteria obtain their supply of iron in the host by secreting high-affinity, low-molecular-weight iron chelators, called

siderophores. Siderophores are capable of competitively taking up iron bound to host proteins (Sahly *et al.*, 2000).

2.4 CLINICAL PRESENTATION OF KLEBSIELLA INFECTIONS

Depending on whether *Klebsiella* infections occur inside hospital or outside hospital, they can be categorized into hospital-acquired or community-acquired infections (Liam *et al.*, 2001). The common infections caused by *Klebsiella species* outside the hospital also called community-acquired infections are pneumonia and urinary tract infection (Liam *et al.*, 2001). Community-acquired *Klebsiella* pneumonia affects people with underlying diseases, such as diabetes and chronic lung disease (Kaye *et al.*, 2000). *Klebsiella* pneumonia causes a severe, rapid-onset illness that often causes destruction in the affected areas of the lung (Kaye *et al.*, 2000). Infected persons generally present with high fever, chills and a cough productive of a lot of mucous. The mucous (or sputum) that is coughed up is often thick and blood tinged and has been referred to as "currant jelly" sputum due to its appearance (Kobashi *et al.*, 2001). There may also be pus surrounding the lung a condition known as empyema, which can be very irritating to the delicate lung tissue and can cause scar tissue to form. (Eisenstein and Zaleznik, 2000).

Klebsiella pneumoniae is the commonest causative agent of *Klebsiella* Community-acquired urinary tract infections followed by *K. oxytoca* (Munro and Chambers, 2002). Characteristic symptoms include dysuria, frequent urination, low back pain and suprapubic discomfort. Systemic symptoms such as fever and chills are usually indicative of pyelonephritis and prostatitis (Munro and Chambers, 2002).

K. rhinoscleromatis and *K. ozaenae* cause rhinoscleroma and ozena, respectively (Obiamiwe, 2006). Although they are rare, they are associated with upper respiratory infections and are mostly community-acquired (Obiamiwe, 2006). Rhinoscleroma is a form of chronic granulomatous nasal infection affecting the nasal passages and sinuses which can also include the pharynx and larynx (Van Renterghem *et al.*, 1993). Ozena is a chronic atrophic rhinitis (Eisenstein and Zaleznik, 2000). The condition can destroy the mucosa and is characterised by a chronic, purulent and often foul-smelling nasal discharge (Eisenstein and Zaleznik, 2000).

Hospital acquired infections caused by *Klebsiella* are bronchitis, urinary tract infections, surgical wound infections, pneumonia, diarrhea and infection of the blood (Lynch *et al.*, 1997). All of these infections can progress to shock and death if not treated early (Ryan and Ray, 2004).

2.5 BURDEN OF KLEBSIELLA INFECTIONS

Carpenter, (1990) reported that 10 to 50 cases of *Klebsiella* nosocomial pneumonia were recorded each year by large hospitals in the United States and also *Klebsiella pneumoniae* is considered as one of the commonest cause of community-acquired pneumonia in the United States (Eisenstein and Watkins, 1998 and Johanson, 1996).

Study by Wen-Chien *et al.*, (2002) has shown that, *K. pneumoniae* is commonly associated with community-acquired pneumonia in Africa and Asia. Twenty eight cases representing 29% of all community-acquired pneumonia cases were reported in Taiwan whereas 25 cases representing 65% of all community-acquired pneumonia cases were reported in South Africa.

Other studies from Taiwan (Chen *et al.*, 1992), Singapore (Lee *et al.*,. 1996), and South Africa (Feldmen *et al.*, 1995) corroborate these findings. In the Taiwanese study, *K. pneumoniae* accounted for 34% of 41 cases of community-acquired bacteremic pneumonia. *Klebsiella pneumoniae* was the cause of 15% of community-acquired pneumonia in Singapore. Also, *K. pneumoniae* was found to be the cause of pneumonia in 32% of patients with severe community-acquired pneumonia in Johannesburg and 11% of patients in Cape Town (Potgieter and Hammond, 1992).

In Europe, however, studies published since 1990 show that only 14 representing 2.3% of 621 patients admitted with severe community-acquired pneumonia had *K. pneumoniae* as the presumptive etiologic agent (Carpenter, 1990; Rello *et al.*, 1996; Moine *et al.*, 1995 and Torres *et al.*, 1991).

It has been estimated that symptomatic urinary tract infections result in as many as 7 million visits to outpatient clinics, 1 million visits to emergency departments and 100,000 hospitalizations annually in USA (Schappert, 1999). Report by Stamm, (2002) indicates that, in USA urinary tract infections have become common hospital-acquired infection, accounting for as many as 35% of nosocomial infections, and they are the second most common cause of bacteremia in hospitalized patients. According to Burke, (2006) in USA, *Klebsiella* prevalence rate was 14% and *was* the second commonest etiology after *E. coli*.

A study on the etiology of UTI in 1999 and in 2003 reports that the prevalence rates of *Klebsiella* were 7.3% for 1999 and 12.7% for 2003 (Orrett and Davis, 2006).

Raz *et al.,* (2000) carried out a research on community-acquired bacteriuria and reported that *Klebsiella* species accounted for 10.6% of all the cases and was the second commonest after *E. coli.* Also a study in Finland by Honkinen *et al.*, (1999) on bacterial causing urinary tract infection, *Klebsiella* species accounted for 55 cases representing 4.4%. Another study in Norway by Grude *et al.*, (2001) on percentage distribution of etiologic agent of UTI among outpatients and inpatients, reported that *Klebsiella* recorded 6-12% for outpatients and 6.2-15% for inpatients and was the second commonest after *E. coli.*

Nwanze *et al.*, (2007) worked on UTI in Okada village, Nigeria. They recorded *Klebsiella* as one of the commonest etiology with prevalence rate of 12.8%. In another study in Nigeria by Akerele *et al*, 2001, the prevalence rate of *Klebsiella* 21.5% was second to *E. coli.*

Klebsiella rhinoscleromatis which causes rhinoscleroma was first described by Von Frisch in 1882. Although this is considered rare (Obiamiwe, 2006), about 16,000 cases have been reported since 1960 (Van Renterghem *et al.*, 1993) and before this, 4000 cases were described (Andraeca *et al.*, 1993). Cases of rhinoscleroma have been reported from most parts of the world, mostly in developed countries in temperate zones (Andraeca *et al.*, 1993). Most European cases are reported from Central Europe (Poland, Hungary, Romania and Russia) (Van Renterghem *et al.*, 1993). The disease has also been reported from many countries of the Middle East, tropical Africa, India, South-east, central and south America (Andraeca *et al.*, 1993). Reports also show that, rhinoscleroma is found predominantly in rural areas and is common where socio- economic conditions are poor. Acquisition of the disease is facilitated by crowding, poor hygiene and poor nutrition. (Akhnoukh and Saad, 1987).

A study by Gorbacheva *et al.,* (1982) on the morbidity of ozena among the population of Minsk in Belarus from 1970 to 1980 found that ozena infection rate among the inhabitants of Minsk was 26.72% and was found to affect mainly children and women. They also found that the source of *K. ozaenae infection* is a sick person who begins to excrete the bacteria in the prodromal period of the disease. The excretion of the bacteria may continue for many years and may be spread by droplet or contact infection (Obiamiwe, 2006).

Klebsiellae have been recognized as cause of nosocomial infections which are important health problems throughout the world (Wenzel, 2003). They result in high morbidity and mortality, prolonged hospital stays, greater use of antibiotics, and increased costs (Wenzel, 2003). Studies have indicated that nosocomial infections occurred in 5%-10% of all hospitalizations in Europe and North America and in more than 40% of hospitalizations in parts of Asia, Latin America, and sub-Saharan Africa (Lynch *et al.*, 1997). In the United States, *Klebsiella* accounts for 3 to 7% of all nosocomial bacterial infections, placing them among the eight most important infectious pathogens in hospitals (Schaberg *et al.*,. 1991).

According to Podschun and Ullmann, (1998), hospital-acquired bacterial infections caused by *Klebsiella species* and their percentages worldwide are as follows: urinary tract infection 6-17%, pneumonia 7-14%, septicemia 4-15%, Neonatal septicemia 3-30% and wound infection 2-4%.

Prevalence of Nosocomial Infections in Kosovo was studied by Raka *et al.,* (2006).They reported that out of 167 patients surveyed, 29 had nosocomial infections with the overall prevalence rate of 17.4%. There were fewer male patients (41.9%) than female patients. The mean age of the patients was 45 years (range, 0-80 years). The commonest nosocomial infection was bloodstream

infection, which accounted for 62% of infections. The rest were surgical-site infection, nosocomial pneumonia, and nosocomial meningitis, each of which accounted for 10% of infections. The hospital wards with the highest prevalence observed were the intensive care units, both neonatal and adult accounting for 88.8% of infections, whereas the age group with the highest prevalence was newborns, who had 77.7% of all registered infections. *Klebsiella pneumoniae* was the main pathogen isolated from blood cultures accounting for 36.8% of infections.

A study in Nigeria by Oguntibeju and Nwobu, (2004) on post-operative wound infection revealed that, out of 60 cases recorded, 10 were caused by *Klebsiella species* representing 16.7%, which ranked it third only to *Pseudomonas aeruginosa* and *Staphylococcus aureus.* In another study on catheter associated urinary infection in Nigeria, *Klebsiella species* were reported to be the commonest pathogen isolated giving it a prevalence rate of 36.6% (Taiwo and Aderoummu, 2006).

Many hospital-acquired infections occur because of the invasive treatments that are often needed in hospitalized patients. For example, intravenous catheters used for fluid administration, catheters placed in the bladder for urine drainage and breathing tubes for people on a breathing machine can all increase the susceptibility to infection (Warren, 2001). While these devices may be needed in certain patients, they allow bacteria to bypass the natural barriers into a person's body.

2.6 LABORATORY DIAGNOSTIC CHARACTERISTICS OF KLEBSIELLA INFECTIONS

Klebsiella causes many types of infections, therefore the clinical symptoms only cannot be used during diagnosis. Culturing and identification are usually required for effective laboratory investigation and diagnosis (Virella, 1997).

This organism has simple nutritional requirements and grows well on basic media commonly used for members of the enterobacteriaceae making its isolation easy (Virella, 1997). Clinical specimens are plated for isolation on blood agar and a differential medium such as MacConkey or CLED (cysteine-lactose and electrolyte-deficient) agar (Alves *et al.*, 2006). *Klebsiella species* are strong lactose-fermenters (Virella, 1997). They therefore produce characteristic pigmented, large, mucoid colonies on lactose enriched media (Virella, 1997), as shown in Figure 2.

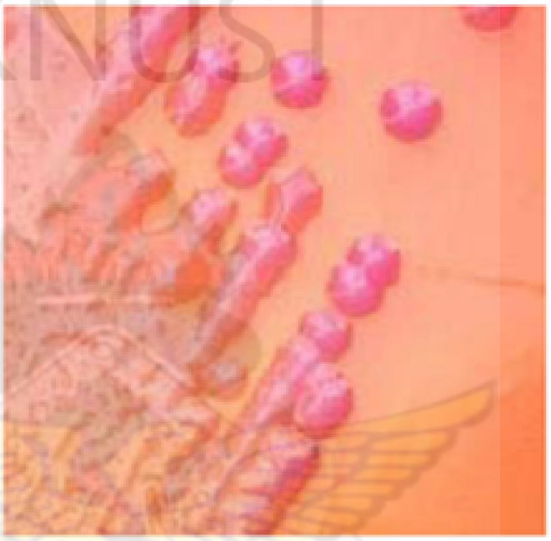
Klebsiella species are Gram negative, facultative anaerobic, non-spore- forming rods, which are nonmotile (Farmer, 1999). They are negative to oxidase test, but are able to reduce nitrates to nitrites (Alves *et al.*, 2006). All *Klebsiella species* ferment glucose, but fermentation of other carbohydrates varies (Alves *et al.*, 2006) . Lactose usually is fermented rapidly by most *Klebsiella* species (Alves *et al.*, 2006). *K ozaenae* is negative to malonate utilization test (Farmer, 1999). Some species of *Klebsiella* are positive to urease reaction, but they do so more slowly (Alves *et al.*, 2006). Also with the exception of *K. pneumoniae, K. rhinoscleromatis* and *K. ozaenae* all the other known species are indole positive (Alves *et al.*, 2006).

A battery of tests for biochemical properties is required to identify *Klebsiella* to the species level, these include indole test, methyl red test and malonate utilization test (Villera, 1997).

Commercial identification systems are now widely used by most US clinical laboratories and consist of 'kits' or miniaturized biochemical tests which are read manually (e.g., API-20E and BBL Crystal) or automatically (e.g., Vitek or MicroSCAN) (Farmer, 1999).

Recently, genotyping methods such as plasmid profiles (determined by agarose gel electrophoresis), RFLP (restriction fragment length polymorphism) of total DNA, pulsed-field gel electrophoresis, targeted analysis of DNA polymorphism, ribotype, and arbitrarily primed PCR (polymerase chain reaction) are employed in the definitive identification of the *Klebsiellae* and have been used in epidemiological studies (Lai *et al.*, 2000).

Figure 2. Distinctive mucoid colonies of Klebsilla on MacConkey agar.



2.6.1 Culture and isolation

Bacterial culture describes the isolation of bacterial species from patient specimens and the identification of those organisms by a combination of characteristics such as growth, biochemical profile etc. Culture requires a minimum of 18 hours of incubation before preliminary results are available, however they should always be attempted, particularly when the patient is severely ill, when the diagnosis is not clear, or when a highly contagious agent like

Klebsiella is suspected (Lai *et al.*, 2000) Clinical specimens for isolation of *Klebsiella species* include urine, blood, purulent material from wounds or abscesses, sputum, and sediment from cerebrospinal fluid (Lai *et al.*, 2000). Urine samples are inoculated on cystine-lactose-electrolyte-deficient agar (CLED) and sputum sample on blood and MacConkey agar. Also cerebrospinal fluid is inoculated on MacConkey and blood agar. The inoculated plates are incubated at 35-37^{o}C for 24hours or most often overnight. Blood samples are inoculated into brain heart infusion broth and incubated overnight before they are subcultured onto MacConkey and blood agar plates. Wound samples are transported to the laboratory in stuart transport medium, and incubated overnight in cooked meet broth before they are subcultured onto MacConkey and blood agar plates. These plates are incubated at 35-37^{o}C overnight. On blood agar, *Klebsiellae* produce large, grey-white mucoid colonies. On MacConkey they produce large pink mucoid colonies and on CLED they produce large yellow mucoid colonies (Virella, 1997).

2.6.2 Growth properties used in the Genus Identification

The colonies of *Klebsiellae* are unique and different morphologically from many lactose fermenting coliforms except *E. coli* and *Enterobacter*. Some strains of *E coli* and *Enterobacter* species sometimes produce colonies which may be similar to that of *Klebsiella*, so motility test is used in their identification and differentiation (Lai *et al.*, 2000). Whilst *E. coli* and *Enterobacter* species are motile, all the *Klebsiella species* are non-motile (Struve *et al.*, 2003).

2.6.3 Biochemical properties used in species differentiation of *Klebsiellae*

Klebsiella oxytoca is indole positive, whereas *K. pneumoniae, K. rhinoscleromatis* and *K. ozaenae* are indole negative. *Klebsiella pneumoniae* is negative to methyl red test, whereas *K.*

ozaenae and *K. rhinoscleromatis* are positive. In differentiating between *K. ozaenae and K. rhinoscleromatis,* the malonate utilization test is used. Whereas *K. ozaenae* is negative, *K. rhinoscleromatis* is positive in the malonate utilization test (Podschun and Ulmann, 1998).

2.7 ANTIMICROBIAL SUSCEPTIBILITY OF KLEBSIELLA SPECIES

Physicians not only wish to know the organism causing an infection but also the antibiotic that will control it. This is because antimicrobial susceptibities differ among the organisms and even species of the same genus may have different antimicrobial susceptibility patterns. The antibiotics most effective in inhibiting the growth of a causative bacterium are determined in vitro. Dilution sensitivity tests and disc diffusion sensitivity tests are the two common methods used in laboratory antimicrobial sensitivity testing (Struve *et al.*, 2003). Dilution sensitivity tests are performed in Microbiology Reference Laboratories for epidemiological purposes or when a patient does not respond to treatment thought to be adequate, relapses while being treated, or when there is immunosuppression. Disc diffusion sensitivity test is used by most laboratories to test routinely for antimicrobial sensitivity (Struve *et al.*, 2003). For clinical and surveillance purposes and to promote reproducibility and comparability of results between laboratories, WHO recommends the Kirby-Bauer disc diffusion technique (Struve *et al.*, 2003).

Antimicrobial resistance is reported from all over the world, and resistance levels are increasing yearly, making the resistant strains new pathogens, capable of causing outbreaks. A publication on the outbreak of nosocomial infections due to *Klebsiella pneumoniae* by Alert *et al.,* (2005) reported one hundred and fifty-four clinical isolates of *Klebsiella pneumoniae* resistant to broad-spectrum cephalosporins, aztreonam and amikacin. These resistant strains were responsible for an outbreak of nosocomial infections in a university hospital in Paris. This outbreak affected 39

patients in the intensive care unit, 8 patients in haematology units and 11 patients in surgical and medical units. These antibiotic resistant strains were responsible for 48% urinary tract infections, 21% wound and drainage fluids infection, 14% respiratory tract infections, 12% infection of blood and 5% of stool infections.

According to a study carried out on the antimicrobial susceptibility patterns of *Klebsiella* species from the Lagos University Teaching Hospital by Abe-Aibinu (2000), isolates were obtained from urine, swabs of wounds, ear, throat and eye. Identification was carried out by conventional methods and antimicrobial susceptibility was investigated by a disk diffusion method. Seventy percent of the isolates were susceptible to amikacin, ceftazidime, ceftriaxone, aztreonam and nalidixic acid. However, ofloxacin and norfloxacin were observed to be more effective with 90-93% sensitivity. Sixty-three percent of isolates were susceptible to gentamicin. The gentamicin-resistant *Klebsiella species* were mostly from urine samples. Isolates were in general, highly resistant (80%) to cotrimoxazole, tetracycline and amoxicillin-clavulanic acid.

A study by Gangoue-Pieboji *et al.,* (2006) on antimicrobial activity against gram negative bacilli from Yaounde Central Hospital, Cameroon reported that, high rates of resistance were found in most of the bacteria studied. Resistance to all isolates was mostly observed to amoxicillin (87%), 74% for piperacillin and 73% for trimethoprim/sulfamethoxazole. Susceptibilities to third generation cephalosporins (cefotaxime, ceftazidime) and monobactam (aztreonam) were 71% for *Klebsiella species.* Imipenem (98%) sensitivity was the most active antibiotic followed by the ofloxacin (88%). Susceptibility of *Klebsiella species* for gentamicin was 67%.

A study carried out in 11 hospitals in 7 regions in Ghana on the resistance to antimicrobial drugs by Newman *et al.,* (2006) reported that, 309 (57.6%) of 536 *Klebsiella species* isolated, showed

antimicrobial resistance. They concluded that the prevalence of drug resistance among the *Klebsiella species* was high. The report also revealed that, 82 % of isolates were resistant to tetracycline, 76 % to ampicillin, 75 % to chloramphenicol, and 73 % to cotrimoxazole. Lower prevalence of resistance was found for gentamicin, cefuroxime and cefotaxime. Gentamicin recorded 28% resistant isolates with cefuroxime recording 27%, (Newman *et al.*, 2006).

Infections caused by multiresistant strains of *Klebsiella* species are fatal (Kurupati *et al.*, 2007). In the 1970s, these strains were chiefly aminoglycoside-resistant *Klebsiella* strains (Fisman and Kaye, 2000). Since 1982, strains that produce extended spectrum betalactamase, which render them resistant to extended-spectrum cephalosporins, have evolved (Anderson *et al.*, 2006). These strains are observed in both *K. pneumoniae* and *K. oxytoca* isolates (Decre *et al.*, 2004).

2.8 TREATMENT OF KLEBSIELLA INFECTIONS

Treatment of infection caused by the *Klebsiella species* depends largely on their antimicrobial susceptibility. However, empirical therapy is often required. In community-acquired pneumonia, for example, effective treatment consists of empirical coverage for Gram-negative organisms, aggressive ventilation, and supportive care (Liam *et al.*, 2001). Third-generation cephalosporins or quinolones provide coverage for community-acquired *K pneumoniae* infection (Fisman and Kaye, 2000).

In nosocomial *K. pneumoniae* pneumonia, antibiotics with high intrinsic activity such as imipenem, third-generation cephalosporins, quinolones, or aminoglycosides may be chosen and used alone or in combination (Kobashi *et al.*, 2001).

Cases of UTI caused by susceptible strains of *Klebsilla species* may be treated with oral quinolones or with intravenous aminoglycosides, imipenem, aztreonam, or third-generation cephalosporins. Other measures may include correction of an anatomical abnormality or removal of a urinary catheter (Warren, 2001).

Klebsiella meningitis may be treated with third-generation cephalosporins which are the drugs of choice because of their superior central nervous system penetration (Stock and Wiedemann, 2001). Other measures include removal of infected shunts (Kobashi *et al.,* 2001). The suggested duration of treatment is 3 weeks because higher relapse rates have been noted in patients treated with shorter courses of therapy (Babini and Livermore, 2000).

Klebsiella endophthalmitis and endocarditis are rare. Therapy for endophthalmitis may be intravitreal, intravenous, or both (Anderson and Janoff, 1998). Intravenous ceftazidime and aminoglycosides are the recommended drugs (Fisman and Kaye, 2000).

Endocarditis has been treated with a combination of an intravenous aminoglycoside and a beta-lactam antibiotic (Fisman and Kaye, 2000). Treatment duration of 6 weeks with antibiotic therapy such as third-generation cephalosporins and aminoglycosides is considered reasonable to cure the patient (Anderson and Janoff, 1998).

With other *Klebsiella* species such as *K oxytoca*, antibiotic susceptibility and treatment guidelines are virtually identical to those for *K pneumoniae* (Decre *et al.,* 2004*)*. Rhinoscleroma is treated with combination antimicrobial therapy for 6-8 weeks. Therapeutic choices include aminoglycosides, tetracycline, sulfonamides, rifampin, and quinolones (Gamea and el- Tatawi, 1990). Ozena may be treated with a 3-month course of ciprofloxacin (Stock and Wiedemann,

2001). Intravenous aminoglycosides and trimethoprim are also useful in the treatment of these conditions (Gamea and el- Tatawi, 1990). Susceptibility testing is usually required to guide antibiotic choice (Fisman and Kaye, 2000).

Surgery is required if drainage or debridement is necessary (Anderson and Janoff, 1998). Surgery may also be needed to correct underlying anatomical abnormalities that predispose patients to *Klebsiella* infection. An example is correction of posterior urethral valves in patients with recurrent UTIs (Sedor and Mulholland, 1999).

2.9 EMPIRICAL TREATMENT OF KLEBSIELLA INFECTIONS

Empirical treatment, although has its own challenges may be necessary in the sense that certain infections require prompt attention but laboratory investigations may take an average of 7 days, and at least 3days to complete (Kristensen *et al.*,2001).

Kristensen *et al.,* (2001) carried out a study on empirical treatment in Aalborg hospital, Denmark. Eight hundred and thirteen (813) patients with bacteremia were recruited. The sources of infection were the urinary tract, abdomen and lungs with the following percentages; 28%, 23% and 13% respectively. The antibiotic treatment prescribed by the physician was appropriate in 78% of patients when compared with the laboratory antimicrobial sensitivity results.

In another study on the benefit of appropriate empirical antibiotic treatment in patients with bloodstream infection at Tel-Aviv, Isreal by Leibovici *et al.,* (1998) involving 3431 patients, second-generation cephalosporins were prescribed for 34% of patients, third-generation cephalosporin were prescribed for 15% of patients, an aminoglycoside was prescribed for 28% of

patients, ampicillin for 6% of patients, vancomycin for 6% and metronidazole for 5% of patients. Appropriate antibiotic treatment was given to 2158 (63%.)

2.10 CONTROL OF KLEBSIELLA INFECTION IN THE HEALTH CARE CENTRE

Healthcare associated *Klebsiella* infections are *Klebsiella* infections transmitted to patients and healthcare personnel as a result of healthcare procedures in hospital and other healthcare settings. A good infection control programme for these healthcare associated infections should aim at protecting patients, protecting healthcare personnel, and promotion of safety, quality, and value in the healthcare delivery system (Nicolle, 2001).

Recent reports indicate that, *Klebsiella* infections in the hospital are associated with high mortality rates due to antimicrobial resistance (Scheckler, 1998). Antimicrobial resistance is a predictable outcome of antimicrobial use. The rapidity with which resistance emerges and its extent are proportional to the intensity of antimicrobial use (Nicolle, 2001). Resistance first emerges in populations with a high frequency of infection, due to either underlying patient status or interventions compromising host defences (Goldman, 1996). Where patients at risk are in close proximity, the transmission of organisms between them will be facilitated, and the opportunity for a single strain to disseminate widely is enhanced (Scheckler, 1998). All these features are present in health care facilities, both acute and chronic care facilities and areas such as intensive care units. Thus, health care facilities, particularly those which are large and care for the most complex patients are focal points in the emergence of antimicrobial resistance (Goldman, 1996). KATH is an example of such facility.

Multifaceted proposals to address the problem of antimicrobial resistance have uniformly stated that optimal infection control programmes in health care facilities are an essential component (Nicolle, 2001). The WHO Global Strategy for Containment of Antimicrobial Resistance recommends that hospital management "establish infection control programmes with responsibility for effective management of antimicrobial resistance in hospitals and ensure that all hospitals have access to such a programme" (Nicolle, 2001). The essential features and appropriate resources for an optimal infection control programme have been identified as surveillance of nosocomial infections; outbreak investigation and control; policy development, review and compliance monitoring isolation practices, hand hygiene, sterilization or disinfection of equipment and supplies, housekeeping, laundry and food; employee health relevant to infections and education of staff, patients and visitors (Scheckler, 1998)

This study seeks to determine *Klebsiella species* responsible for causing infections at KATH. It also aims at determining the sensitivity of the *Klebsiella* isolates to the commonly used antimicrobial drugs at KATH. The result will enable medical practitioners relate the *Klebsiella species* isolates to their antimicrobial sensitivity results and patients demographic data. Furthermore, the study aims at determining the effectiveness of empirical treatment of *Klebsiella* infections at KATH.

CHAPTER THREE

3.0 PROJECT DESIGN AND METHODOLOGY

3.1 Sample size

Two hundred and five (205) *Klebsiella species* were isolated from 2197 patients suspected of bacterial infections who reported for medical diagnosis and treatment at the Komfo Anokye Teaching Hospital (KATH). Clinical specimens were collected from both out-patients and in-patients. Samples collected were blood, sputum, urine, wound and aspirates (drainage fluids) for culture to isolate the organism. Detailed demographic data including age and gender were also taken from these patients.

3.2 Culture and isolation

The various clinical samples received were cultured on blood agar and MacConkey agar. The urine samples were cultured on CLED agar. The composition and preparation of these media are presented in appendix 5.

3.2.1 Urine Culture

Patients were given sterile, dry, wide-necked, leak-proof containers and requested 10-20ml midstream urine (MSU). Patients were educated on how to take midstream urine with as little contamination as possible. To do this they were asked to pass urine, discarding the first part and then collect the midstream urine. In-patients were assisted by the hospital personnel in the collection and delivering of the specimens to the laboratory. The delivered urine specimens were then labeled with patient's information on the laboratory request form. Microscopy was done immediately to establish the extent of infection by looking out for significant pyuria (5cell/ul of

urine). That is, 5 or more white blood cells per field. This was followed by urine culture immediately. Using a calibrated loop the urine samples were inoculated onto CLED agar. The loop was calibrated to hold 0.002ml of urine, so colony counts of 20 colonies which are equivalent to 10^4 bacteria/ml is considered significant and diagnostic for urinary tract infection. Culture was repeated for samples which produced mixed bacteria growth by requesting new samples from the patients.

3.2.2 Blood Culture

Culture bottles of volume 20ml containing 15ml of brain heart infusion broth (Appendix 4) was labeled with the patients name, age, date and laboratory number. Using a pressure cuff, a suitable vein in the lower arm was located. About 50mm diameter of the venepunture site was then disinfected with 70% ethanol and allowed to air dry. Using a 5ml sterile syringe and a 21G x $^1/_2$ inches needle, about 5ml and 2ml of blood were drawn from the adult patients and children respectively. The plaster on the culture bottle cap was removed and the cap disinfected with 70%. The needle was then inserted through the cap to dispense the blood into the culture bottle. The top of the culture bottle was disinfected again with 70% ethanol and the tape or protective cover replaced. Without delay, the blood was mixed with the broth and immediately incubated overnight before they were subcultured on MacConkey and blood agar. A cooled sterile bacteriological loopful of the inoculum was taken and spread on the MacConkey and blood agar plates and the blood bottles were reincubated. The plates were also incubated at 35-37^{o}C for 24 hours (overnight) and then inspected for growth. Blood specimens which did not show growth in the first subculture were subcultured the third time before discarded. This whole process takes about 7 days.

3.2.3 Culture of Wound swabs and Drainage Fluids

Aspirates such as synovial fluid, pleural fluids and pus from boils were collected with sterile needle and syringe and deposited into a sterile leak-proof container by Medical Officers and submitted to the laboratory. Where the pus was scanty, it was collected with a sterile cotton-wool swab from the wound. The swab was then immersed in a bottle containing Stuart transport medium (Appendix 4) and sent immediately to the laboratory. These swabs were then cultured on MacConkey and blood agar plates. The swab was applied to a small area of the plates, a cooled sterile bacteriological loop was then used to spread the inoculum. The plates were then incubated at 35-37^{o}C.

3.2.4 Culture of Ear Discharge

Ear swabs taken from individuals with ear discharge were sent to the laboratory through the Stuart transport medium labeled with patients information. The swab of the specimen was applied to a small area of the MacConkey, blood agar and chocolate agar plates, and a cooled sterile bacteriological loop was used to spread the inoculum. The plates were then incubated at 35-37^{o}C for 24 hour. A smear from the swab was prepared on the slide for Gram stain (Appendix 4).

3.2.5 Culture of Sputum

The sputum sample was collected in a sterile leak-proof container labeled with patient's name, age, sex, ward and laboratory number. It was then delivered immediately to the laboratory. The sample was plated on blood, chocolate and MacConkey agar using a sterile loop. The plates were

then incubated overnight at 35-37^{o}C. A loopful of sample was smeared onto a glass slide for Gram stain (Appendix 4).

The suspected bacteria produced large yellow mucoid colonies on CLED, large grey white colonies on blood agar and chocolate agar, and large pink mucoid colonies on MacConkey agar. A Gram stain showed that the organism was large Gram negative rod bacteria, therefore *Klebsiella species* was suspected. Motility test was also done.

Bacterial colonial morphology characteristic of *Klebsiella* were harvested and inoculated onto nutrient agar to prepare a purity plate and incubated overnight. From the purity plate few identical colonies were picked and inoculated in a sterilized glycerol broth and stored in the refrigerator for later use for the biochemical tests.

The following biochemical tests; indole test, methyl red test and malonate utilization test, and growth at 10^{0}C were used to identify the organism to species level.

3.3 BIOCHEMICAL TESTS

The biochemical tests used include the following; indole test, methyl red test and malonate utilization test.

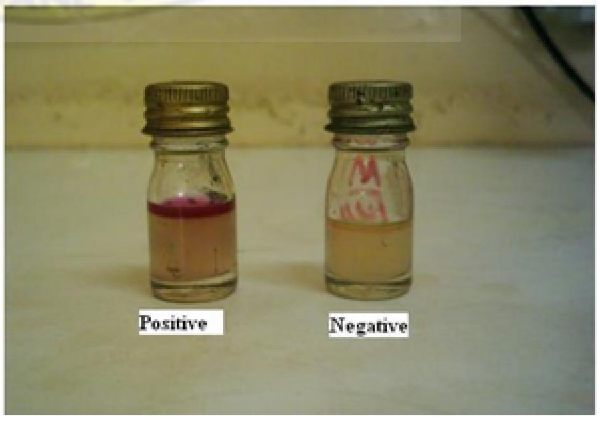
Table 1. Biochemical Reactions of *Klebsiella* species

Species	Characteristics			
	Indole	Utilization of Malonate	Methyl red test	Growth at 10^0C
K. pneumoniae	negative	positive	negative	negative
K. oxytoca	positive	positive	negative	positive
K. rhinoscleromatis	negative	positive	positive	negative
K. ozaenae	negative	negative	positive	negative
K. terrigena	negative	positive	positive	positive
K. planticola	negative	positive	negative	positive
K. ornithinolytica	positive	positive	positive	positive

3.3.1 Indole Test

The indole test was done by growing pure *Klebsiella* culture in a sterile peptone broth (Appendix 4) for about 18-24 hours. Following overnight incubation, a few drops of Kovacs'reagent (Appendix 4) were added to the culture broth, using a

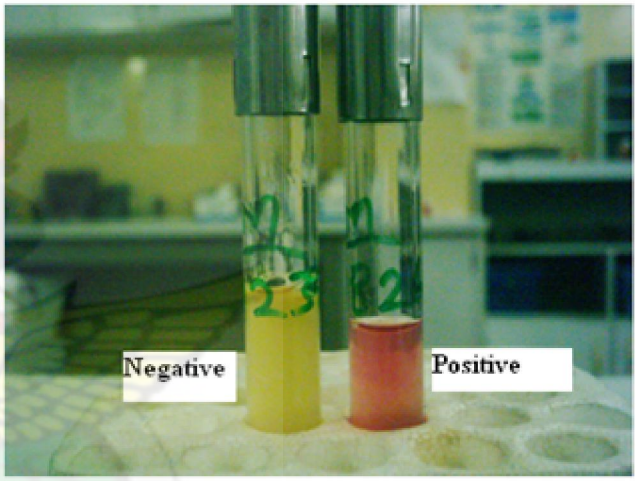
Plate 1. Indole test



Pasteur pipette. As presented in Plate 1, appearance of a red layer indicated that the test was positive whereas yellow layer indicated that the test was negative. The indole test was used to differentiate between *Klebsiella pneumonia, K. rhinoscleromatis, K. ozaenae* and *Klebsiella oxytoca*. *K. oxytoca* was positive whereas the rest were negative. *Escherichia coli* and *Enterobacter cloacae* were used as positive control and negative control respectively.

3.3.2 Methyl Red Test

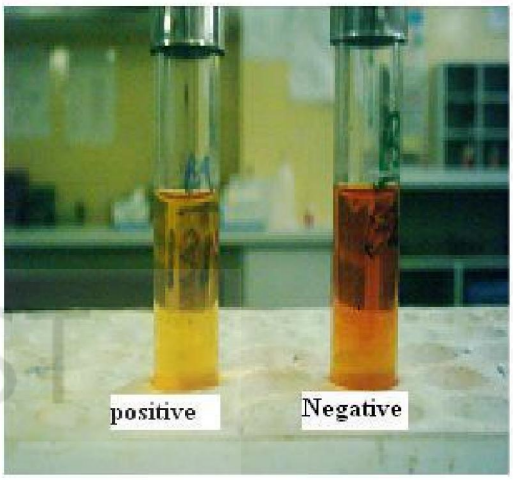
Plate 2. Methyl red test



The methyl red test was done by inoculating a pure culture of *Klebsiella* into a labeled Methyl Red and Voges-Proskauer (MR-VP) broth media tubes (Appendix 4) by means of a sterile loop. The test tubes were then incubated at 37^{o}C for 48 hours. Following incubation, a dropperful of methyl red indicator was added to the test tubes of MR-VP broth. As in dicated in Plate 2, a red colour or yellow to orange appeared immediately. Red colour showed that the test was positive and the yellow to orange colour indicated that the test was negative. This test was used to confirm or rule out both *Klebsiella pneumoniae* and *Klebsiella oxytoca.* Both are negative whereas *K. rhinoscleromatis* and *K. ozaenae* are positive to the test. *Escherichia coli* and *Enterobacter cloacae* were used as positive and negative control respectively.

3.3.3 Malonate Utilization Test

Plate 3. **Malonate utilization test**



The malonate utilization test was done by adding few drops of methyl orange indicator to the prepared malonate broth (Appendix 4). A pure culture of *Klebsiella species* was inoculated into the broth and incubated for 48 hours. As presented in Plate 3, a colour change resulted from change in pH, from acid to base due to the utilization of malonate which changed the methyl orange indicator from orange to yellow indicated that the test was positive. This test was used in the differentiation of *K. ozaenae and K. rhinoscleromatis*. *K. ozaenae* was negative whereas *K. rhinoscleromatis* was positive. *Enterobacter aerogenes* and *Escherichia coli* were used as positive and negative control respectively.

3.4 GROWTH AT 10^{o}C

Figure 3. Labcon low temperature incubator.

This test was used to differentiate between *K. rhinoscleromatis* and *K. terrigena,* since they react similarly to the various biochemical tests. The test was done by plating the suspected *K. rhinoscleromatis* and *K. terrigena* isolate on nutrient agar and incubated at temperature of 10^{o}C overnight using the Labcon low temperature incubator, shown in Figure 3 (at the

Clinical Microbiology Department, KNUST). Whereas *K. terrigena* grow at 10^0C, *K. rhinoscleromatis* do not grow. *K. oxytoca* and *K pneumoniae* were used as positive and negative control respectively.

3.5 MOTILITY TEST

A drop of peptone water was placed on a slide. A colony of *Klebsiella* was emulsified in the peptone water. The cover slip was inverted carefully and placed over the concave portion of a cavity slide. Motility was then observed at x400 magnification on the microscope. This test was used in differentiating between *Klebsiella pnuemoniae* and *Enterobacter species*. *Klebsiella pneumoniae* was non-motile whereas *Enterobacter species* was motile. Care was taken to observe true locomotion but not passive drifting and Brownian motion. *Escherichia coli* was used as control.

Figure 4 FLOW CHART FOR ISOLATION AND IDENTIFICATION OF KLEBSIELLA SPECIES

3.6 ANTIBIOTICS SENSITIVITY

Common antimicrobial agents used for managing infections of coliforms at KATH were used. These are ampicillin (10ug) which is broad-spectrum penicillin, cefuroxime (30ug) which is 2nd generation cephalosporin, ceftriaxone (30ug) and cefotaxime (30ug) which are 3rd generation cephalosporin, gentamicin (10ug) and amikacin (30ug) which are aminoglycosides and co-trimoxazole (25ug) which is 1:5 mixture of trimethoprim and sulphamethoxazole, tetracycline (10ug) and chloramphenicol (10ug). The disk diffusion test method by Kirby-Bauer was used.

3.6.1 The disk diffusion test (Kirby-Bauer method)

The Kirby-Bauer method of determining bacterial susceptibility to antimicrobials is a standardized filter paper disk-agar diffusion test. The test is standardized to avoid variations in results. (Struve *et al*, 2003).

A sterile microbiological loop was used to pick 3-5 well-isolated colonies of similar appearance to the *Klebsiella* colonies on the culture plate and was emulsified in 3-4 ml of sterile peptone water in a test tube. In a good light, the turbidity of the suspension (*Klebsiella* in peptone water) was compared with the turbidity standard (Appendix 4) which is a mixture of 0.5 ml of barium chloride and 99.5 ml of the sulphuric acid solution in a similar test tube. Using a sterile swab, a plate of Mueller Hinton agar (Appendix 5) of pH 7.2-74 was inoculated with the suspension. Excess fluid was removed by pressing and rotating the swab against the side of the test tube above the level of the suspension. The inoculum was evenly spread over the surface of the medium in three directions, rotating the plates approximately 60^0 to ensure even distribution. With the Petri dish lid in place, 3-5 minutes was allowed for the surface of the agar to dry.

A pair of foreseps was used to deposit the filter paper disks containing the above mentioned antimicrobial agents onto the medium. The Petri dish was then incubated overnight at 35-37^{o}C. Following the incubation overnight, the diameters of the zones of inhibition were measured in mm and recorded. The values obtained were compared with the interpretative chart values provided by National Committee for Clinical Laboratory Standards (NCCLS) presented in Appendix 3, was used to interpret the zone size of each antibiotic, reporting the organism as resistant, intermediate resistant and sensitive (NCCLS, 2003). *Escherichia coli* ATCC 25922 was used as control.

Table 2. The interpretative chart of the zone of inhibition of the antibiotics used

Antibiotics	Diameter of zone of inhibition (mm)		
	Susceptible	Intermediate resistant	Resistant
Ampicilin	≥ 17	14-16	≤13
Cefotaxime	≥23	15-22	≤14
Ceftriaxone	≥23	15-22	≤14
Cefuroxime	≥23	15-22	≤14
Chloramphenicol	≥18	13-17	≤12
Tetracycline	≥19	15-18	≤14
Gentamicin	≥15	13-14	≤12
Amikacine	≥15	13-14	≤12
Co-triamoxazole	≥16	11-15	≤10

3.7 EMPIRICAL TREATMENT

Empirical treatments of 51 pediatric patients were monitored and compared with their antibiotics sensitivity in vitro. Patients who had positive cultures were traced to their wards and the records department, and their folders retrieved. Records of antimicrobials prescribed before laboratory results were submitted were compiled. Lab results were compared with the records obtained from the patients folders. Results were then compared. The age range of patients was 1day to 9years.

3.8 DATA ANALYSIS

Analysis of variance (ANOVA) was used to show whether the difference in the resistant patterns of *K. pneumoniae* and *K. oxytoca* was significant or not. Also the difference between reported cases of in-patients and out-patients of *K. pneumoniae* infections was analysed with (ANOVA). A 95% confidence interval was used. These formulae were used in the calculation of the F-value;

SSb= Sum of squares between groups

SSw= Sum of squares within groups

MSb= Means of squares between groups

MSw= Means of squares within groups

K= Number of groups

N= $n_1+n_2+\ldots\ldots\ldots..+n_k$= Sum of the sample sizes for the groups

$$MS_b = \frac{SSb}{K-1}$$

$$MSw = \frac{SSw}{N-K}$$

$$F = \frac{MSb}{MSw}$$

If the F-value is bigger or equal to the P-value, then the claim that ‘‘there is no significant difference’’ is rejected but if the F-value is less then the claim is accepted.

CHAPTER FOUR

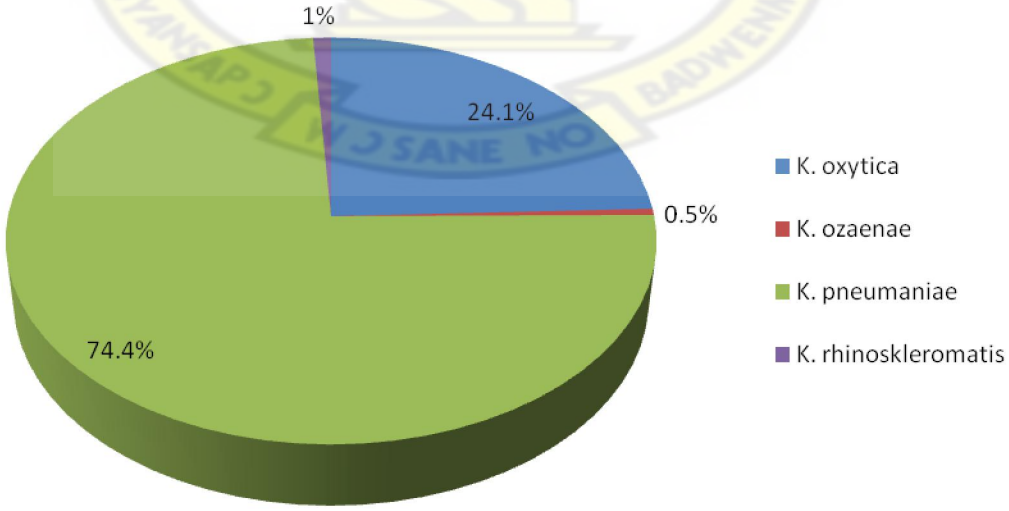
4.0 **RESULTS**

4.1 ***KLEBSIELLA* SPECIES ISOLATED**

From 20th December, 2006 to 20th July, 2007, a total of 205 *Klebsiella* species were isolated from various clinical specimens of 2197 patients (non-repeat) who were referred to the microbiology laboratory at Komfo Anokye Teaching Hospital for routine diagnosis.

The *Klebsiella* isolates were identified to the species level, where four species were identified namely *Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella ozaenae* and *Klebsiella rhinoscleromatis. Klebsiella pneumoniae* was the commonest species isolated among the *Klebsiellae* representing 74.4%, followed by *Klebsiella oxytoca* representing 24.1%. There were 2 *Klebsiella rhinoscleromatis* isolates representing 1% and *K. ozaenae* had only one isolate, representing 0.5% as presented in Figure 5 below.

Figure 5 Percentage representation of *Klebsilla* species Isolated



4.2 CLINICAL SPECIMENS ENCOUNTERED

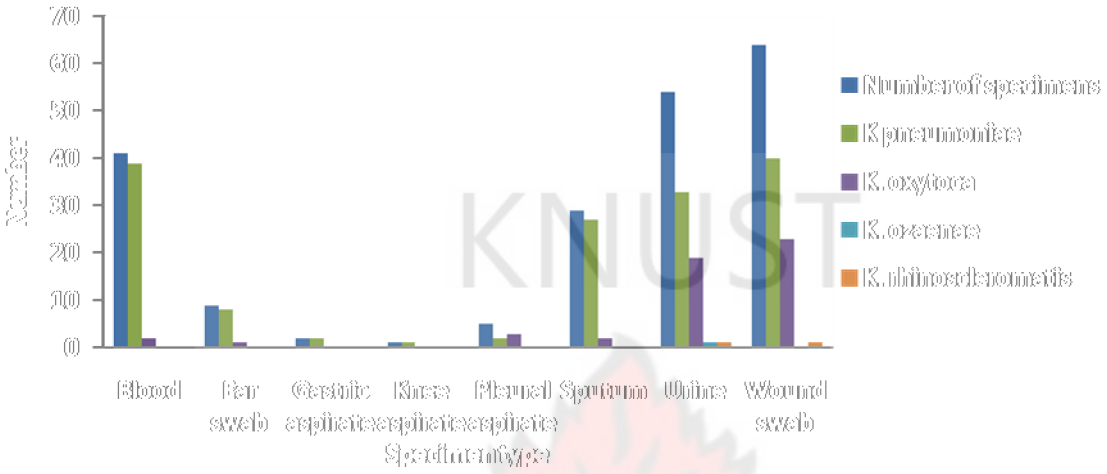
As presented in appendix 3 (Table 2) and Figure 6, the isolates were obtained from 8 different specimens namely wound, urine, blood, sputum, ear swab, pleural aspirates, gastric aspirate and knee aspirate. Wound swab gave the highest *Klebsiella* isolates of 64 representing 31.2% followed by urine with 54 isolates representing 26.3%. Blood had 41 isolates representing 20% whereas isolates from sputum were 29 representing 14.1%. Isolates from ear swabs were 9 representing 4.4%, pleural aspirate isolates were 5 representing 2.4%, whereas gastric aspirate had 2 isolates representing 1% and only one *Klebsiella* isolate was obtained from knee aspirates. These isolates were from a total of 507 wound samples,

502 urine samples, 802 blood samples, 206 sputum samples, 73 ear swabs, 54 pleural aspirates, 31 gastric aspirates and 22 knee aspirates examined.

4.2.1 *Klebsiella* species obtained from the specimens analyzed

As shown in appendix 3, *K. pneumoniae* was the commonest species isolated in all the specimens with the exception of pleural aspirate where it has 2 isolates as against 3 of the *K. oxytoca*. The highest isolates were from wound with 40, blood with 39, urine with 33 and sputum with 27. *Klebsiella oxytoca* recorded 23 isolates in wound and happens to be the highest followed by urine with 19. The only *K. ozaenae* isolate encountered was from urine and the two isolates of *K. rhinoscleromatis* were recorded in urine and wound.

Figure 6. Representation of Klebsiella species in the various specimens



4.3 TYPE OF PATIENTS

There were a total of 103 out-patients and 102 in-patients. The in-patient cases were from seven wards in the hospital. Mothers babies unit (MBU) registered 21 cases which happened to be the highest followed by Block A (pregnant women) which had 19 cases. Block B (accommodate children) registered 16 cases, Pediatric emergency unit (PEU) had 15 cases, Block C (accident unit) registered 14 cases, and Block D (medical unit) had 13 cases, with MEU (men emergency unit) registering 2 cases which was the least as shown in Table 4 below.

4.3.1 *Klebsiella* species in relation to patient type

Out of the 152 *Klebsiella pneumoniae* isolated, 58.6% were from in-patients whereas 41.4% were from out-patients. *Klebsiella oxytoca* on the other hand had 50% for both in-patients and out-patients. Also, all the isolates of both *K. rhinoscleromatis* and *K. ozaenae* were from out-patients as presented in Table 3 above.

The analysis of variance (ANOVA) was used to test for significant difference between out-patient and in-patient cases of *K. pneumoniae*. The Excel output for the calculation of ANOVA is presented in appendix 2. F-value of 0.52 was recorded for the analysis with a corresponding P-value of 0.59. This indicates that the difference between out-patients and in-patient cases of *K. pneumoniae* is statistically not significant.

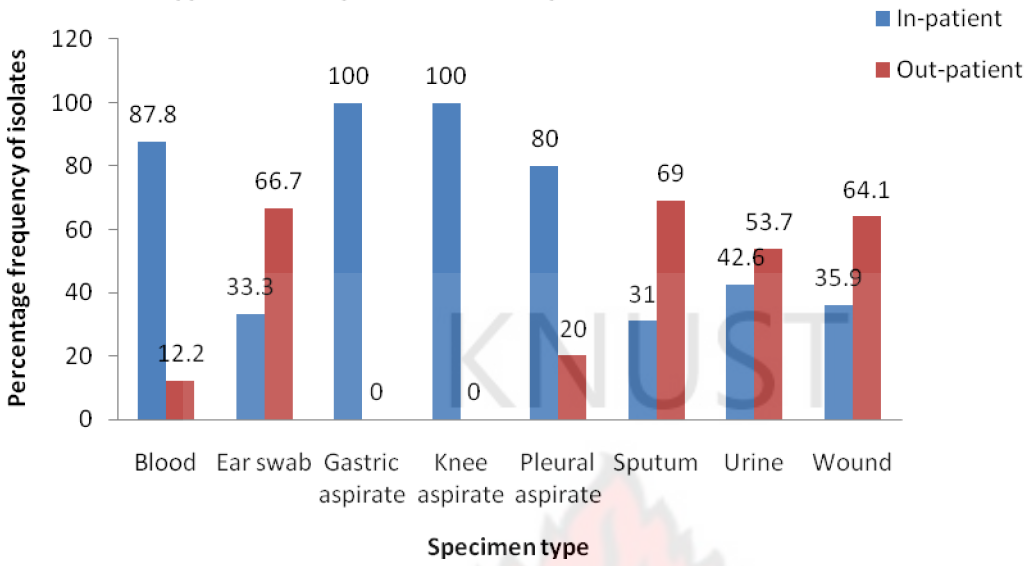
Table 3. *Klebsiella* isolated in relation to sample type and ward

Wards	Blood	Urine	Wound	Sputum	Knee aspirate	Gastric aspirate	Ear swab	Pleural aspirate
Block A	2	10	1	1	1	2	0	2
Block B	1	4	11	1	0	0	1	0
Block C	0	6	4	3	0	0	1	0
Block D	2	0	5	4	0	0	1	1
PEU	10	3	2	0	0	0	0	0
MBU	21	0	0	0	0	0	0	0
MEU	0	1	0	0	0	0	0	1
OPD	5	30	41	20	0	0	6	1
Total	41	54	64	29	1	2	9	5

Legend: Block A (accommodates preegnant women), Block B (accommodates children), Block C (accident unit), Block D (medical unit), PEU (Pediatric emergency unit), MBU (mother baby unit), MEU (medical emergency unit) and OPD (out patient department).

As indicated in Figure 7 and Table 3, the highest in-patient cases were from blood with 90% whereas ear swab with 66.7 and wound with 64.1% were the highest values for out-patients cases.

Figure 7. Isolates of *Klebsiella* obtained in the various specimen types for out-patients and in-patients



4.4 *KLEBSIELLA* SPECIES IN RELATION TO GENDER OF PATIENTS

The were a total of 101 males and 104 females. As presented in Table 4 , *Klebsiella pneumoniae* infection was detected among 74 females representing 48.7% and 76 males representing 51.3%. *Klebsiella oxytoca* on the other hand was isolated from 27 females representing 54% and 23 males representing 46% of the isolates. The two isolates of *K. rhinoscleromatis* and the only *K. ozaenae* isolate were from females.

Table 4. *Klebsiella* species isolated in relation to the gender of patients

Species	Female		Male	
	Number	%	Number	%
K. pneumoniae	74	48.7	76	51.3
K.oxytoca	27	54	23	46
K. rhinoscleromatis	2	100	0	0
K.ozaenae	1	100	0	0

4.5 *KLEBSIELLA* SPECIES IN RELATION TO AGE OF PATIENTS

The ages of patients ranged between 1day to , 85 years. The highest *Klebsiella* infection was detected in the age groups 1-9 and 30-39 years whereas age group 80-89 years was the least infected as presented in appendix 3. As indicated in Table 5 below, the highest *K. pneumoniae* infection was detected in individuals in the age group 40-49 years whereas the highest *K. oxytoca* infection was found in the age group 30-39 years. The analysis of variance (ANOVA) was used to test for significant difference between the cases of *K. pneumoniae* infections and cases of *K. oxytoca* infections in terms of age. The Excel output for the calculation of ANOVA is presented in appendix 2. F-value of 17.68051 was recorded for the analysis with a corresponding P-value of 0.000532. This indicates that there was significant difference between cases of *K. pneumoniae* infections and infections *K. oxytoca* in terms of age.

Table 5. *Klebsiella* species isolated in relation to age of patients

Age group	*K. pneumoniae*		*K. oxytoca*		*K. ozaenae*		*K. rhinoscleromatis*	
	No. of Isolates	%	No. of Isolates	%	No. of Isolates	%	No. of Isolates	%
< 1	21	13.8	1	2	0	0	0	0
1-9	24	15.8	8	16	0	0	0	0
10-19	15	9.9	6	12	0	0	0	0
20-29	16	10.5	7	14	0	0	0	0
30-39	22	14.5	9	18	0	0	0	0
40-49	23	15.1	5	10	0	0	0	0
50-59	11	7.2	4	8	0	0	1	50
60-69	6	3.9	2	4	0	0	1	50
70-79	9	5.9	6	12	0	0	0	0
80-89	5	3.3	2	4	1	100	0	0

4.5.1 Age distribution among the clinical specimens encountered

As indicated in Table 6 below, age group 10-19 years had the highest wound isolates of 18.8%. The highest urine isolates of 20.4% were from the age group 1-9 years, the highest blood isolates were from the population under one (<1) year with 43.9%. The age groups 1-9 and 10-19 recorded the highest ear isolates with 33.3%, the highest pleural isolates were obtained by the age group 60-69 years with 40% whereas the highest sputum isolates were from the age group 40-49 years with 40%. The analysis of variance (ANOVA) was used to test for significant difference among the various clinical specimens used in terms of age. The Excel output for the calculation of ANOVA is presented in appendix 2. F-value of 5.922216 was recorded for the analysis with a corresponding P-value of 1.86×10^{-5}. This indicates that there was significant difference among the various clinical specimens used in terms of age.

Table 6. Age distribution among the clinical specimens encountered

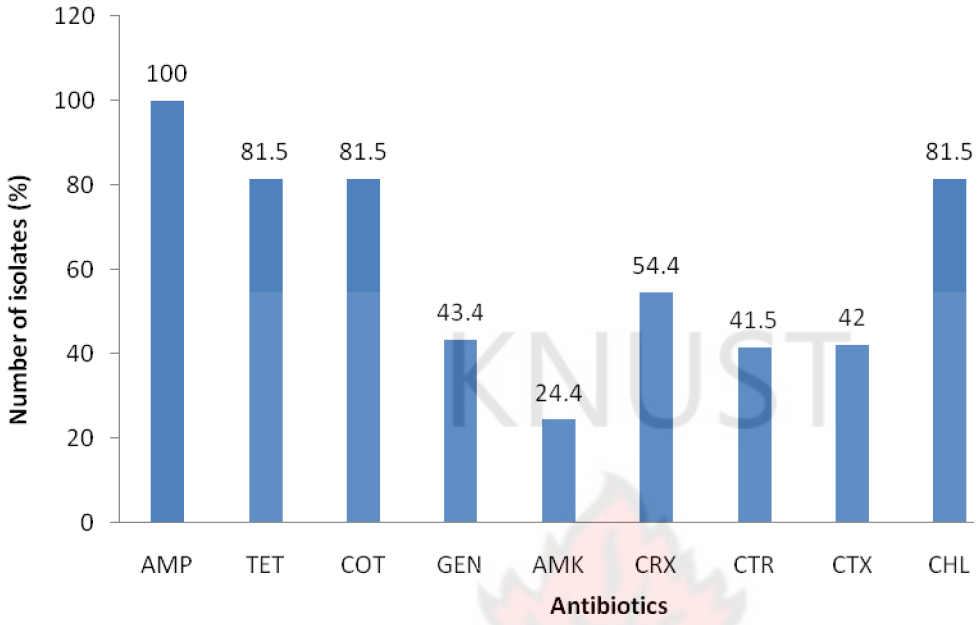
Age group	W/S		Urine		Blood		E/S		P/A		G/A		K/A		Sputum	
	No.	%	No. %	%	No. %	%	No.	%	No.	%	No.	%	No.	%	No.	%
< 1	2	3.1	1	1.9	18	43.9	0	0	0	0	0	0	0	0	0	0
1-9	6	9.4	11	20.4	11	26.8	3	33.3	0	0	1	50	0	0	0	0
10-19	13	20.3	2	3.7	1	2.4	3	33.3	0	0	1	50	0	0	1	3.4
20-29	12	18.8	7	13	3	7.3	0	0	0	0	0	0	0	0	1	3.4
30-39	12	18.8	9	16.7	3	7.3	1	11.1	0	0	0	0	0	0	7	24
40-49	5	7.8	10	18.5	2	4.9	0	0	0	0	0	0	1	100	11	40
50-59	4	6.3	4	7.4	0	0	0	0	1	20	0	0	0	0	6	21
60-69	1	1.6	3	5.5	2	4.9	1	11.1	2	40	0	0	0	0	0	0
70-79	6	9.4	5	9.3	0	0	0	0	1	20	0	0	0	0	3	10
80-89	3	4.7	2	3.7	0	0	1	11.1	1	20	0	0	0	0	0	0

W/S-wound swab, E/S-ear swab, P/A- Pleural aspirate, G/A-gastric aspirate, K/A-knee aspirate, No.-number

4.6 ANTIBIOTIC SENSITIVITY TEST RESULTS

All the isolates were resistant to ampicillin, 81.5% of the isolates were resistant to tetracycline, co-trimoxazole and chloramphenicol. Resistance was shown to amikacin, ceftriaxone, cefotaxime, gentamicin and cefuroxime by 24.4%, 41.5%, 42%, 43.4% and 54.7% of the isolates respectively as indicated in Figure 8. The antibiotics and percentages of *Klebsiella* isolates which showed intermediate resistance to them are as follows; tetracycline (10.2%), co-triamoxazole (6.8%), gentamicin (26.8%), amikacin (19.5%), cefuroxime (39.0%), ceftriaxone (10.2%), cefotaxime (9.8%) and chloramphenicol (8.3%). This is shown in Table 8 below.

Figure 8. Antimicrobial Resistance Patterns of the Isolates



Legend: AMP-Ampicillin, TET-Tetracycline, COT-Co-trimoxazole, GEN-Gentamicin, AMK-Amikacin, CRX-Cefuroxime, CTR-Ceftriaxone, CTX-Cefotaxime, CHL-Chloramphenicpl.

4.6.1 Antibiotic susceptibility patterns of *K. pneumoniae* and *K. oxytoca* compared

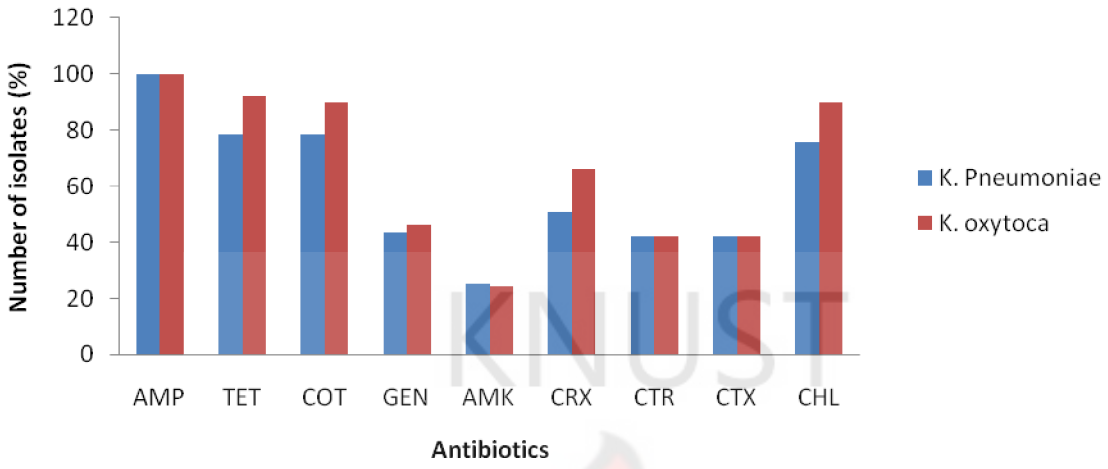
As shown in Figure 9, 70.4% of the *K. pneumoniae* isolates were susceptible to amikacin which happens to be the highest susceptible antibiotic. No susceptibility was shown to ampicillin. High levels of resistance were shown to tetracycline and cot-trimoxazole by 78.3% of the isolates, and chloramphenicol by 75.7% of the isolates. *Klebsiella oxytoca* isolates which were susceptible to Amikacin were 74%, 54% were susceptible to ceftriaxone and cefotaxime, and 50% were to gentamicin. All the isolates were resistant to ampicillin, 92% were resistant to tetracycline, whereas 90% were resistant to cot-trimoxazole and chloramphenicol.

Table 7. *Klebsiella* species and their antimicrobial sensitivity patterns

Antibiotics	Sensitivity	K. oxytoca	K. pneumoniae	K. ozaenae	K. rhinoscleromatis
AMP	Intermediate	0	0	0	0
	Resistance	50	152	1	2
	Susceptible	0	0	0	0
TET	Intermediate	2	18	1	0
	Resistance	46	119	0	2
	Susceptible	2	15	0	0
COT	Intermediate	1	11	1	1
	Resistance	45	119	0	0
	Susceptible	4	22	0	1
GEN	Intermediate	17	37	0	1
	Resistance	23	66	1	0
	Susceptible	10	49	0	1
AMK	Intermediate	11	29	0	1
	Resistance	12	38	0	0
	Susceptible	27	85	1	1
CRX	Intermediate	15	62	1	2
	Resistance	33	77	0	0
	Susceptible	2	13	0	0
CTR	Intermediate	2	19	0	0
	Resistance	21	64	0	0
	Susceptible	27	69	1	2
CTX	Intermediate	1	19	0	0
	Resistance	22	64	0	0
	Susceptible	27	69	1	2
CHL	Intermediate	4	12	1	0
	Resistance	45	115	0	2
	Susceptible	1	25	0	0

Legend: Intermediate-Intermediate resistance, Resistance-Full resistance.

Figure 9. Antimicrobial Resistant patterns for *K. pneumoniae* and *K. oxytoca* compared



The analysis of variance (ANOVA) was used to test for significant difference between resistant patterns of *K. pneumoniae* and *K. oxytoca* to the nine antibiotics. The Excel output for the calculation of ANOVA is presented in appendix 2. F-value of 0.26 was recorded for the analysis with a corresponding P-value of 0.62. This indicates that the difference between their resistant patterns is not statistically significant.

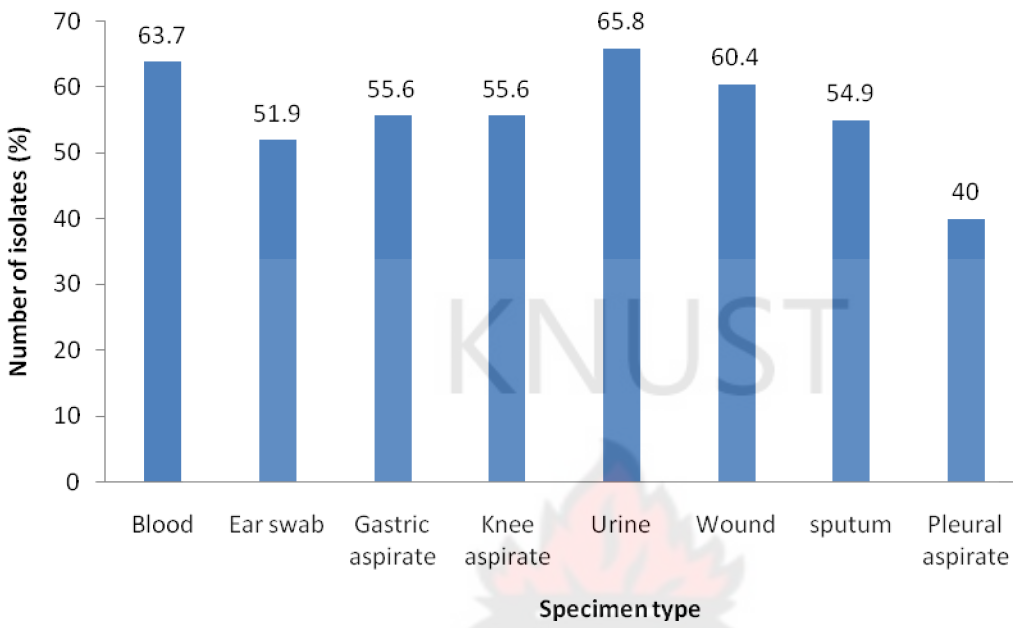
4.6.2 Levels of antibiotic susceptibilities in the various specimen

As indicated in Figure 10 and Table 8, the highest antimicrobial resistant isolates were found in urine with a percentage of 65.8% followed by blood with 63.7%. Ear swab, gastric aspirate, knee aspirate, sputum and wound had 51.9%, 55.6%, 55.6%, 40%, 55.6% and 60.4% resistant isolates respectively.

Table 8. Antimicrobial sensitivity patterns in the specimen types

Antibiotics	Sensitivity	Blood	Ear swab	Gastric aspirate	Knee aspirate	Pleural aspirate	Sputum	Urine	Wound	Total
AMP	Intermediate	0	0	0	0	0	0	0	0	0
	Resistance	41	9	2	1	5	29	54	64	205
	Susceptible	0	0	0	0	0	0	0	0	0
TET	Intermediate	6	2	0	0	2	3	3	5	21
	Resistance	32	7	1	0	2	23	46	55	166
	Susceptible	3	0	1	1	1	3	5	4	18
COT	Intermediate	4	0	0	0	2	2	2	4	14
	Resistance	30	8	2	1	1	24	44	54	164
	Susceptible	7	1	0	0	2	3	8	6	27
GEN	Intermediate	8	5	1	0	2	11	10	18	55
	Resistance	20	3	1	1	1	11	27	25	89
	Susceptible	13	1	0	0	2	7	17	20	60
AMK	Intermediate	8	4	0	1	0	8	13	19	53
	Resistance	10	0	1	0	1	5	16	17	50
	Susceptible	23	5	1	0	4	16	25	37	111
CRX	Intermediate	15	4	1	0	2	13	17	27	79
	Resistance	23	4	1	1	2	13	34	31	109
	Susceptible	3	1	0	0	1	3	3	5	16
CTR	Intermediate	5	3	0	0	2	3	4	7	24
	Resistance	19	2	1	1	0	10	28	23	84
	Susceptible	17	4	1	0	3	16	22	34	97
CTX	Intermediate	5	3	0	0	1	3	3	6	21
	Resistance	20	2	1	1	1	9	28	24	86
	Susceptible	16	4	1	0	3	17	23	34	98
CHL	Intermediate	12	0	1	0	0	5	6	4	28
	Resistance	20	7	0	0	5	21	43	54	150
	Susceptible	9	2	1	1	0	3	5	6	27

Figure 10. Percentage representation of Antimicrobial Resistant isolates in the body fluids encountered



4.7 EMPIRICAL TREATMENT

An attempt was made to compare the treatment patterns of 51 pediatric patients before the laboratory antimicrobial sensitivity results were ready (empirical treatment) with the laboratory generated sensitivity results for the isolates of these patients (invitro). As presented in Table 9, gentamicin was prescribed for 38.7% of patients, amikacin for 10.7% of patients, ceftriaxone for 8% of patients, cefotaxime for 9.3% of patients, cefuroxime for 4% of patients and chloramphenicol for 5.3% of patients. Ampicillin was prescribed for 1.3% of patients, Cot-trimoxazole for 5.3% of patients and Tetracycline for 2.6% patients. These antibiotics were prescribed either as monotherapy or in combination with other antibiotics as indicated in appendix 1.

Table 9. Number of times prescribed and the sensitivity patterns of the antibiotics used in the empirical treatment

Antibiotics	No. of times antibiotic was prescribed	%	Sensitivity pattern against *Klebsiella*		
			R	I	S
AMP	1	1.3	1	0	0
TET	2	2.6	2	0	0
COT	4	5.3	2	2	0
GEN	29	38.7	14	2	13
AMK	8	10.7	2	1	5
CRX	3	4	2	1	0
CTR	6	8	2	2	2
CTX	7	9.3	2	1	4
CHL	4	5.3	2	2	0
CHL	4	5.3	2	2	0
CIP	3	4	NT	NT	NT
FLO	2	2.6	NT	NT	NT
FLU	6	8	NT	NT	NT

Legend: R-Full resistance, I-Intermediate resistance, S-Susceptible, NT-Not tested.

As indicated in Table 10, intermediate antibiotic susceptibility pattern was shown by 9.8% of patient's isolates. Also, 37.3% of patient's isolates showed resistance to all the antibiotics used in the antibiotic susceptibility test. Antimicrobial susceptibility was shown by 36 of patient's isolates representing 52.9%. This percentage may be higher since three antibiotics; ciprofloxacin (CIP), flucloxacillin (FLU), and floxacillin (FLO) were prescribed for 4%, 2.6% and 8% of patients respectively outside the ones whose susceptibility patterns were tested in the laboratory.

In most cases, they were prescribed in combination with the antibiotics used in this research as indicated in appendix 1.

Table 10. Sensitivity patterns of the 51 isolates for which empirical treatment was given.

Sensitivity	Number of isolates	%
Susceptible	27	52.9
Intermediate	5	9.8
Resistant	19	37.3
Total	51	100

CHAPTER FIVE

5.1 DISCUSSION

Species identification and antibiotic sensitivity testing are critical in the diagnosis and treatment of persons infected with *Klebsiella*. It is also required in disease prevention, patient management, and surveillance of infection. However, these practices are usually ignored in most of our hospitals mainly due to limited resources, time and labour. This problem can therefore be addressed by carrying out frequent research work in that direction, and making the research findings available to the health practitioners in these less resourced hospitals. The aim of this study was to investigate the epidemiology of *Klebsiella* infections at Komfo Anokye Teaching Hospital (KATH).

5.1.1 *Klebsiella species* isolated

Four species of *Klebsiella* were isolated, these included *K. pneumoniae, K. oxytoca*, *K. rhinoscleromatis* and *K. ozaenae. Klebsiella pneumoniae* was the commonest followed by *K. oxytoca*. This finding is supported by Farmer, (1999), who reports that *K. pneumoniae* and *K. oxytoca* are the commonest species responsible for human related *Klebsiella* infections with *K. pneumoniae* accounting for 75 to 85% of *Klebsiella* infections worldwide. *Klebsiella rhinoscleromatis* and *K. ozaenae* which recorded 1% and 0.5% respectively are reported to be rare in our part of the world (Obiamiwe, 2006). The other species of *Klebsiella* such as *Klebsiella terrigena*, *Klebsiella planticola* and *Klebsiella ornithinolytica* were not encountered in this study and may be due to the fact that, they are clinically uncommon (Farmer, 1999).

5.1.2 Clinical specimens from which *Klebsiellae* were isolated

Out of the eight (8) specimens from which *Klebsiella* were isolated, wound, urine, blood and sputum were the commonest. It can therefore be inferred that wound infections, UTI, septicemia and pneumonia are the common *Klebsiella* infections at KATH which is consistent with what was reported by Podschun and Ullmann, (1998) as the commonly encountered *Klebsiella* infections worldwide.

Prevalence of *Klebsiella* infections in the specimens encountered at KATH are 12.6% in wound swab, 10.7% in urine, 5.1% in blood, 14.1% in sputum, 12.3% in ear swab, 9.3% in pleural aspirate, 6.5% in gastric aspirate and 4.5% in knee aspirate. Again, Podschun and Ullmann, (1998) reported on the prevalence of the following *Klebsiella* infections worldwide as urinary tract infection (urine) 6-7%, pneumonia (sputum) 7-14%, septicemia (blood) 4-15%, neonatal septicemia (blood) 3-30% and wound infection (wound) 2-4%. The prevalence rates recorded in this study are relatively higher than what was reported worldwide by Podschun and Ullmann, (1998). These relatively high figures may be due to the reason that, KATH is a regional and a teaching hospital and most of the cases which come here are usually referral cases from either the district or the private hospitals. In addition most patients prefer KATH to the district hospitals in the region. Also it may be due to the fact that *Klebsiella* infections are gradually becoming common and difficult to manage with time.

5.1.2.1 Species recorded in the various specimens analysed

Klebsiella pneumoniae was the commonest species isolated in all the specimens with the exception of pleural aspirate where *K. oxytoca* had the highest isolates of 3 as against 2 isolates of *K. pneumoniae*. This may be due to the reason that *K. pneumoniae* is the most virulent among all the *Klebsiella* species, as reported by Farmer, (1999). Many research works have also corroborated this finding. According to Obiamiwe, (2006) *K. pneumoniae* is the leading cause of urinary tract infections among the *Klebsiella species.* Also Ryan and Ray, (2004) reported that *K. pneumoniae* is the main causative agent of *Klebsiella* nosocomial infections followed by *K. oxytoca*. Again Raka *et al.,* (2006) reported in their work that *K. pneumoniae was* the main pathogen isolated from blood culture accounting for 36.8% of infections. And as the name implies *Klebsilla pneumoniae* is the leading cause of *Klebsiella* pneumonia (Liam *et al.*, 2001). Although *K. pneumoniae* is considered the commonest etiological agent of *Klebsiella* pneumonia (Liam *et al.*, 2001), *K. oxytoca* was responsible for two cases of *Klebsiella* pneumonia, which confirmed the fact that any of the *Klebsiella* species can produce any of the *Klebsiella* infections when the suitable conditions are available (Obiamiwe, 2006).

5.1.3 *Klebsiella* species in relation to type of patient

There was a marginal difference betweem in-patients and out-patients cases recorded in the study, indicating that *Klebsiella* infections are common in both the community and the hospital. *Klebsiella pneumoniae* infections in in-patients were higher than in out-patients, but a statistical analysis showed that the difference between them was not significant ($p< 0.005$). *Klebsiella oxytoca* on the other hand had equal cases for both *in*-patients and out-patients, whereas all the infections of *K. ozaenae* and *K. rhinoscleromatis* were out-patient cases.

The highest out-patient cases were registered in ear swab and wound swab, therefore ear infection and wound infection may be considered as the commonest community-acquired *Klebsiella* infections report at KATH. Also the highest in-patient case was blood. Septicemia (blood) may therefore be considered as the commonest hospital-acquired *Klebsiella* infection reports at KATH.

5.1.3.1 *Klebsiella* infections in relation to the wards of the hospital

The Mother baby unit (MBU), Block A, Block B and Pediatric emergency unit (PEU) registered the highest *Klebsiella* infections. Mothers babies unit (MBU) and Pediatric emergency unit (PEU) which accommodate babies, expectedly had most of the isolates from blood samples since babies are usually prone to sepsis (Wegner *et al.*, 1997). Block A which houses pregnant women registered most of the isolates from urine samples. This may be due to the reason that pregnant women are usually prone to urinary tract infections (Wegner *et al.*, 1997). Also Block B which accommodates children had most of the isolates from wound samples. These wards may therefore be considered as *Klebsiella* infection prone wards and may be sources of *Klebsiella* nosocomial infections. This conclusion was drawn from the figures recorded in this study and the fact that these wards have consistently being recording large numbers of *Klebsiella* infections according to the statistical records at the Microbiology Department of KATH.

5.1.4 *Klebsiella* species in relation to gender of patient

The total number of females who were infected with *Klebsiella* was marginally higher than males. These figures show clearly that, *Klebsiella* infection is common among all sexes. This is corroborated by a research by Raka *et al.*, (2006) in Kosovo where there were fewer male

patients (41.9%) than female patients. Male cases of *K. pneumoniae* were higher than females whereas more females were infected with *K. oxytoca* than males.

The highest female cases were found in urine. This may be explained by the reason that women are 30 times more likely than men to develop urinary tract infections, partly owing to anatomic factors that facilitate contamination of the perineal area and upward invasion of the bladder by the intestinal flora (Wegner *et al.*, 1999). The highest male cases were from sputum. This may be due to certain habits such as alcoholism and smoking which are common with men and usually increase the susceptibility of such men to the pathogen (Liam *et al.*, 2001).

5.1.5 *Klebsiella* species in relation to age of patients

The age groups 1-9 and 30-39 years were the most infected and may therefore be considered as the most prone to *Klebsiella* infections. The least prone age group to *Klebsiella* infections was 80-89 years. The highest *K. pneumoniae* and *K. oxytoca* infections were detected in age groups 40-49 and 30-39 years respectively. The difference between infections of *K. pneumoniae* and infections of *K. oxytoca* in terms of age was highly significant ($p>0.005$).

The highest urine isolates were registered by the age group 1-9 years and this may be due to the reason that UTI is very common among school age children especially girls (Wegner *et al.*, 1997). Also the population under one (<1) year had the highest blood infections and this might be due to *Klebsiella* strains responsible for nosocomial infections since most of the patients were in-patients and babies who are usually immunocompromised and therefore susceptible to nosocomial infections and that, babies are usually prone to sepsis (Wegner *et al.*, 1997). The

highest wound isolates were from the age groups 10-19 years and since most of the patients were out-patients (64.1%) and the age groups in question are very active group and are usually involved in injurious activities, they are prone to injuries and easily exposed to *Klebsiella* infections. Also the highest sputum isolates were recorded by the age group 40-49 years and as reported by Liam *et al.*, (2001) most cases of *Klebsiella* pneumonia occur in middle-aged and older men.

5.1.6 Antimicrobial sensitivity patterns

Klebsiella showed high resistance to ampicillin, tetracycline, co-trimoxazole and chloramphenicol. Hundred percent of the isolates were resistant to ampicillin and this may be due to the production of extended-spectrum betalactamase which renders ampicillin ineffective (Anderson *et al.*, 2006). Low resistance was shown to amikacin, ceftriaxone, cefotaxime and gentamicin. The highest susceptibility of isolates was found for amikacin followed by gentamicin, ceftriaxone and cefotaxime.

These results are consistent with the report from a study which was carried out in Ghana by Newman *et a.l*, (2006). In their study, 82% of the *Klebsiella* isolates were resistant to tetracycline, 76% were resistant to ampicillin, 75% were resistant to chloramphenicol and 73% were resistant to co-trimoxazole. Therefore ampicillin, tetracycline, chloramphenicol and co-trimoxazole may not be suitable antibiotics for managing *Klebsiella* infections. The suitable antibiotics for treating *Klebsiella* infections according to this research and reports from other studies are amikacin, gentamicin, ceftriaxone and cefotaxime. The potency of these antibiotics has been confirmed again by a research on antimicrobial susceptibility patterns of *Klebsiella* in

Lagos by Abe-Aibinu, (2000). In this study, 70% of the isolates were highly susceptible to amikacin and ceftriaxone with 63% of isolates being susceptible to gentamicin.

The seemingly increase in the number of resistant isolates of *Klebsiella* to aminoglycosides and third generation cephalosporins which are considered suitable antibiotics for managing *Klebsiella* infections in this study could be due to aminoglycosides-resistant *Klebsiella* strains (Fisman and Kaye, 2000) and also the production of extended spectrum betalactamase, which render them resistant to extended spectrum cephalosporins (Anderson, *et al.*, 2006).

5.1.6.1 Antibiotic susceptibility patterns of *K. pneumoniae* and *K. oxytoca* compared

Both *K. pneumoniae* and *K. oxytoca* isolates were highly susceptible to amikacin, although *K. oxytoca* recorded the highest percentage. Relatively, high levels of resistance were exhibited by *K. oxytoca* than *K. pnuemoniae* to the following antibiotics; tetracycline, chloramphenicol, and co-trimoxazole, but from the statistical analysis (ANOVA) shown in appendix 2, there was no significant difference between the resistant patterns of *K. pneumoniae* and *K. oxytoca* ($p>0.005$). This is corroborated by a report by Decre *et al.*, (2004) to the effect that, antibiotic sensitivity for *K. oxytoca* is virtually identical to *K. pneumoniae*.

5.1.6.2 Levels of antibiotic susceptibility in the various specimens

The highest antimicrobial resistant isolates were from urine followed by blood and wound. In the case of wound infections, since most of the cases were out-patient cases as indicated in Table 8, it may be due to the reason that patients usually undergo self medication before they report to the hospital because of the subtle nature by which these infections start (Obiamiwe, 2006). This practice could results in an increase in the antibiotics resistance level of the pathogen, since the

usage of the appropriate antibiotics in their correct dosage is always a problem (Goldman, 1996). In the case of UTI and blood infection (septicemia), since most of the cases were in-patient cases as shown in Figure 11, it could be due to antimicrobial resistant strains of *Klebsiella* in the hospital environment responsible for *Klebsiella* nosocomial infections (Alert *et al.*, 2005).

According to a study by Abe-Aibinu, (2000) most antibiotic resistant strains of *Klebsiella* were from urine samples, confirming the results obtained in this study. In view of this, antibiotic sensitivity testing should be done at all cost when urine, blood and wound samples are involved. Even in situations where empirical treatment is inevitable, a switch in medication must be done, when the laboratory antibiotic sensitivity results are ready and proved relevant.

5.1.7 Empirical treatment

Gentamicin was the most prescribed antibiotic followed by amikacin, cefotaxime and ceftriaxone. The reason may be that, aminoglycosides and third generation cephalosporins are the commonest suitable antibiotics for managing *Klebsiella* infections at KATH. In the case of aminoglycosides, although amikacin is very potent, it is very expensive compared to gentamicin.

The isolates of 19 out of the 51 patients recruited, showed resistance to the antibiotics prescribed by physician in vitro. This puts the prevalence of resistance to antibiotics prescribed at 37.3% which is relatively reasonable compared to other research works by Leibovici *et al.*, (1998) and Kristensen *et al.*, (2001), which reported 37% and 22% respectively as prevalence of resistance.

Those which showed only intermediate susceptibility to one or more of the antibiotics used were 9.8%% and usually, in the absence of susceptible antibiotics, intermediate susceptible antibiotic is the preferred option. A marginal increase in the dosage may produce the same effect as a susceptible antibiotic. Also 27 of the patients representing 52.9% had their isolates being susceptible to one or more of the antibiotics used in vitro which could be higher, since three antibiotics namely ciprofloxacin (CIP), flucloxacillin (FLU), floxacillin (FLO) were prescribed for patients outside the laboratory used ones and the fact that intermediate susceptibility antibiotics might produce the required results in the isolates which showed intermediate susceptibility to the antibiotics used.

Other studies on empirical treatment didn't show much departure from the results obtained in this study. A study on empirical antibiotic treatment at Tel-Aviv, Isreal by Leibovici *et al*, (1998) revealed that, second generation cephalosporin was prescribed for 34% of patients, third generation cephalosporin for 15% of patients and 63% of the isolates were susceptible to antibiotics used. Another empirical treatment study in Denmark by Kristensen *et al*, (2001) had high susceptible isolates of 78%.

5.2 CONCLUSION AND RECOMMENDATION

Klebsiella pneumoniae, K. oxytoca, K. rhinoscleromatis and K. ozaenae were responsible for the various *Klebsiella* infections at KATH. *K. pneumoniae* was the commonest followed by *K. oxytoca*, and these may be the most virulent *Klebsiella* species. The commonest clinical specimens encountered were wound samples, urine, blood and sputum samples. The highest female and male cases were from urine and sputum samples respectively. Also the highest out-

patients and in-patients cases were from wound sample and blood respectively. Prevalence of *Klebsiella* infections in these commonly encountered clinical specimens; wound, urine, blood and sputum were 12.6%, 10.7%, 5.1% and 14.1% respectively.

There was no significant difference between the out-patient and in-patient cases. The most susceptible age groups to *Klebsiella* infections were 1-9 and 30-39 years and the least infected group was 80-89 years. Mother baby unit (MBU), Block A, Block B and the pediatric emergency unit (PEU) were the most *Klebsiella* infection prone wards, recording the highest cases of *Klebsiella* infections in that order. Also the infection was common among all sexes.

The research also revealed that, aminoglycosides such as amikacin and gentamicin, and third generation cephalosporins such as ceftriaxone and cefotaxime were the most effective antibiotics for managing the various *Klebsiella* infections at KATH. The use of ampicillin, tetracycline, chloramphenicol and co-trimoxazole should not be encouraged in the treatment of *Klebsiella* infections at KATH, since most isolates showed high resistance to these antibiotics.

Results gathered from empirical treatment showed that, aminoglycosides and third generation cephalosporins were the most prescribed antibiotics. And although empirical treatment is very relevant, it cannot be relied upon completely, but should be combined with laboratory antibiotic sensitivity testing for effective *Klebsiella* infection treatment.

REFERENCES

Abe-Aibinu I. E., (2000). A Comparative Study on the Antimicrobial Susceptibility patterns of *Klebsiella* and Enterobacter species from the Lagos University Teaching Hospital. Journal of the Nigerian Infection Control Association: **(3)2**: 14-17.

Aiyegoro O. A., Igbinosa O. O., Ogunmwonyi I. N., Odjadjare E. E., Igbinosa O. E. and Okoh A. I., (2007). Incidence of urinary tract infections among children and adolescents in Ile-Ife, Nigeria. African Journal of Microbiology Research. Pp.13-19. http://www.academicjournals.org/ajmr

Akhnoukh S. and Saad E. F., (1987). Iron-deficiency in atrophic rhinitis and Scleroma. Ind J Med Res: **85**:576-579.

Ako-Nai A.,K., Kassim O. O., Adeniran M. O. and Taiwo O. O., (1993). A study of urinary tract infections at Ile-Ife, Nigeria. East Afr Med J. **70(1),** 10-14.

Alert G., Sanson-le Pors M. J., Rouveau M., Fournier G., Marie O., Schlemmer B. and Phillippon A., (2005). Outbreak of nosocomial infections due to *Klebsiella pneumoniae* producing SHV-4 beta-lactamase. European Journal of Clinical Microbiology and Infections Diseases. **(9)11**; 779-803.

Alves M. S., Dias, R. C. d. S., de Castro, A. C. D., Riley, L. W. and Moreira, B. M. (2006). Identification of Clinical Isolates of Indole-Positive and Indole-Negative *Klebsiella* spp.. *J. Clin. Microbiol.* **44**: 3640-3646.

Amako, K., Meno Y. and Takade A., (1998). Fine structures of the capsules of *Klebsiella pneumoniae* and *Escherichia coli* K1. J. Bacteriol. **170**:4960– 4962.

Anderson D. J., Engemann J. J., Harrell L. J., Carmeli Y., Reller L. B. and Kaye, K. S., (2006). Predictors of mortality in patients with bloodstream infection due to Ceftazidime-resistant *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **50**: 1715-1720.

Anderson M.J. and Janoff E.N., (1998). *Klebsiella* endocarditis: report of two cases and review. Clin Infect Dis; **26(2)**: 468-474.

Andraeca R., Edson R. S. and Kern E. B., (1993). Rhinoscleroma: a growing concern in the United States? Mayo clinic experience. Mayo Clin Proc; **68**:1151-1157.

Babini G. S. and Livermore D. M., (2000). Antimicrobial resistance amongst *Klebsiella* species. collected from intensive care units in Southern and Western Europe in 1997-1998. *J Antimicrob Chemother* **45**: 183-189.

Branger J., Knapp S., Weijer S., Leemans J. C., Pater J. M., Speelman P., Florquin S. and van der Poll T., (2004). Role of Toll-Like Receptor 4 in Gram-positive and Gram-negative pneumonia in mice. *Infect. Immun.* **72**: 788-794.

Brisse S., Issenhuth-Jeanjean S. and Grimont P. A. D., (2004). Molecular serotyping of *Klebsiella* species isolates by restriction of the amplified capsular antigen gene cluster. *J. Clin. Microbiol.* **42**: 3388-3398.

Burke A. C., (2006). Urinary tract infection, female. Professor of medicine, state university of New York school of medicine at Stony Brook; Chief, infectious division, Winthrop-university hospital. htt://www.emedicine.com/uti/uti2.htm. (08/28/2007).

Campos M. A., Vargas M. A., Regueiro V., Llompart C. M., Alberti S. and Bengoechea J. A., (2004). Capsule Polysaccharide Mediates Bacterial Resistance to Antimicrobial Peptides. *Infect. Immun.* **72**: 7107-7114.

Carpenter J.L., (1990). *Klebsiella* pulmonary infections: occurrence at one medical center and review. Rev Infect Dis; **12**:672-682.

Chen C.W., Jong G.M. and Shiau J.J., (1992). Adult bacteremic pneumonia: bacteriology and prognostic factors. J Formos Med Assoc; **91**:754-759.

Chung J. W., Altman E., Beveridge T. J. and Speert, D. P., (2003). Colonial Morphology of Burkholderia cepacia Complex Genomovar III: Implications in Exopolysaccharide Production, Pilus Expression, and Persistence in the Mouse. *Infect. Immun.* **71**: 904-909.

Decre D., Burghoffer B., Gautier V., Petit J.C. and Arlet G., (2004). Outbreak of multi-resistant *Klebsiella oxytoca* involving strains with extended-spectrum beta-lactamases and strains with

extended-spectrum activity of the chromosomal beta-lactamase. *J Antimicrob Chemother* **54**: 881-888.

Eisenstein B.I. and Watkins V., (1998). Diseases caused by Gram-negative bacilli. In: Fauci AS, Braunwald E, Isselbacher KJ, Kasper DL, Hauser SL, Longo DL, et al., editors. Harrison's principles of internal medicine. 14th Ed. New York: McGraw-Hill, p. 120.

Eisenstein B.I. and Zaleznik D.F., (2000). Enterobacteriaceae. In: Mandell GL, Bennett JE, Dolin R, editors. Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases. Edinburgh: Churchill Livingstone; p. 2302.

Farmer J.J., (1999): Enterobacteriaceae: Introduction and identification. In: Murray PR, Baron, EJ, Pfaller MA, eds. Manual of Clinical Microbiology. 7th ed. Washington, DC: American Society for Microbiology; 438-447.

Feldman C., Ross S., Mahomed A.G., Omar J. and Smith C., (1995). The aetiology of severe community-acquired pneumonia and its impact on initial empiric, antimicrobial chemotherapy. Respir Med; **89**:187-92.

Fisman D.N. and Kaye K.M., (2000). Once-daily dosing of aminoglycoside antibiotics. Infect Dis Clin North Am Jun; **14(2**): 475-487.

Gamea A.M., and el-Tatawi F.A., (1990). The effect of rifampicin on rhinoscleroma: an electron microscopic study. J Laryngol Otol; **104(10):** 772-777.

Gangoue-Pieboji J., Koulla-Shiro S., Ngassam P., Adiogo D. and Ndumbe P., (2006). Antimicrobial activity against gram negative bacilli from Yaounde central Hospital, Cameroon. Afr Health Sci; **6(4):**232-235.

Goldman D.A., (1996). Strategies to prevent and control the emergence and spread of antimicrobialresistant microorganisms in hospitals. *JAMA*; **275:**234–240.

Gorbacheva V. N., Lebedev N. I., Miakinnikova M. V.and Ovcharenko T. M., (1982). Epidemiological problems of ozena and the means for controlling this infection. Zh Mikrobiol Epidemiol Immunobiol, **(9):**42-45.

Grude N., Tveten Y. and Kristiansen B.E., (2001). Urinary tract infections in Norway: bacterial etiology and susceptibility. A retrospective study of clinical isolates. Clin Microbiol Infect; **7**:543–547.

Honkinen O., Lehtonen O. P., Ruuskanen O., Huovinen P. and Mertsola J., (1999). Cohort Study of bacteria species causing urinary tract infection and urinary tract abnormalities in children. BMJ. **318(7186**): 770-771.

Johanson W.G., (1996). Pneumonia caused by aerobic Gram-negative bacilli. In: Bennett JC, Plum F, editors. Cecil textbook of medicine. 20th ed. Philadelphia: WB Saunders Co. p. 54-58.

Kaye K.S., Fraimow H.S. and Abrutyn E., (2000). Pathogens resistant to antimicrobial agents. Epidemiology, molecular mechanisms, and clinical management. Infect Dis Clin North Am; **14(2):** 293-319.

Kobashi Y., Ohba H. and Yoneyama H., (2001). Clinical analysis of patients with community-acquired pneumonia requiring hospitalization classified by age group. Kansenshogaku Zasshi; **75(3):** 193-200.

Kristensen B., Larsen S., Schoenheyder H. C., Leibovici L., Paul M., Frank U. and Andreassen S., (2001). Dicision support system (DSS) for antibiotic treatment improves empirical treatment and reduces costs. Abstr Intersci Conf Antimicrob Agents Chemother, abstract no. L-2063.

Kurupati, P., Tan K. S. W., Kumarasinghe, G. and Poh, C. L., (2007). Inhibition of Gene Expression and Growth by Antisense Peptide Nucleic Acids in a Multiresistant {beta}-Lactamase-Producing *Klebsiella pneumoniae* strain. *Antimicrob. Agents Chemother.* **51**: 805-811.

Lai Y.C., Yang S.L. Peng H.L. and Chang, H.Y., (2000). Identification of Genes Present Specifically in a Virulent Strain of *Klebsiella pneumoniae*. *Infect. Immun.* **68**: 7149-7151.

Lee K.H., Hui K.P., Tan W.C. and Lim T.K., (1996). Severe community-acquired pneumonia in Singapore. Singapore Med J; **37**:374-377.

Leibovici L., Shraga I., Ducker M., Konigsberger H., Samra Z. and Pitlik S. D., (1998). The benefit of appropriate empirical antibiotic treatment in patients with bloodstream infection. Journal of Internal Medicine, **244**: 379-386.

Liam C.K., Lim K.H. and Wong C.M., (2001). Community-acquired pneumonia in patients requiring hospitalization. Respirology; **6(3)**: 259-264.

Lynch P., Jackson M., Preston G.A. and Soule B.M.,(1997). *Infection Prevention With Limited Resources.* Chicago: ETNA Communications.

Moine P., Vercken J.B., Chevret S. and Gajdos P., (1995). Severe community-acquired pneumococcal pneumonia. Scand J Infect Dis; **27**:201-206.

Moore T. A., Moore B. B., Newstead M. W. and Standiford T. J., (2000). {gamma}{delta}-T Cells Are Critical for Survival and Early Proinflammatory Cytokine Gene Expression During Murine *Klebsiella* Pneumonia. *J. Immunol.* **165**: 2643-2650.

Munro R. and Chambers I., (2002). Laboratory Diagnosis of Urinary Tract Infection: Common sense Pathology. Australian Doctor. **5**: 1-12.

NCCLS. (2003). Performance Standards for Antimicrobial Disc Susceptibility Test Approved Standard, M2-A5. Villanova Pan, USA: National Ehinmidu Antibiotics susceptibility of urine bacteria isolates Trop J Pharm Res. Committee for Clinical Laboratory Standards. **2**:228-232.

Newman M.J., Frimpong E., Asamoah-Adu A. and Sampane-Donkor E., (2006). Resistance to antimicrbial drugs in Ghana. The Ghanaian – Dutch collaboration for Health Research and Development pp 1-6.

Nicolle L.E., (1999). Regional dissemination and control of epidemic methicillin-resistant *Staphylococcus aureus*. *Infect Control Hosp Epidemiol*;**20**:202– 205.

Nwanze P. I., Nwaru L. M., Oranusi S., Dimkpa U., Okwu M. U., Babatunde B. B., Anake T. A., Jatto W.and Asagwara C. E., (2007). Urinary tract infection in Okada village: Prevalence and antimicrobial susceptibility pattern. Scientific Research and Essay **(2)4**:112-116.

Obiamiwe U. M.B.B.S., (2006). *Klebsiella* infection. Fellow, Center for AIDS Research and Education, David Geffen School of Medicine at UCLA http://www.emedicine.com/med/topic1237.htm (07/04/2007).

Ofek I., Mesika A., Kalina M., Keisari Y., Podschun R., Sahly H., Chang D., McGregor D. and Crouch E., (2001). Surfactant Protein D Enhances Phagocytosis and Killing of Unencapsulated Phase Variants of *Klebsiella pneumoniae*. *Infect. Immun.* **69**: 24-33.

Oguntibeju O. O. and Nwobu R. A. U., (2004). Occurrence of Pseudomonas aeruginosa in post-operative wound infection. Pak J Med Sci. **20(3)**:187-191.

Ohene A., (1997). Bacterial pathogens and their antimicrobial susceptibility in Kumasi, Ghana. East Afr Med J. **74(7)** 450-455.

Orrett F. A. and Divis G. K., (2006). A comparison of antimicrobial susceptibility profile of urinary pathogens for the years, 1999 and 2003. West Indian med. J. **(55)2**.

Paterson D.L., (2000). Recommendation for treatment of severe infections caused by Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs). Clin Microbiol Infect; **6(9)**: 460-463.

Podschun R. and Ullmann U., (1998). *Klebsiella* species as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev; **11(4):** 589-603.

Podschun R., Pietsch S., Holler C. and Ullmann, U., (2001). Incidence of *Klebsiella* Species in Surface Waters and Their Expression of Virulence Factors. *Appl. Environ. Microbiol.* **67:** 3325-3327.

Potgieter P.D. and Hammond J.M.J., (1992). Etiology and diagnosis of pneumonia requiring ICU admission. Chest; **101**:199-203

Raka L., Zoutman D., Mulliqi G., Krasniqi S., Dedushij I., Raka N., Ahmeti S., Shala M., Vishaj A. and Elezi Y., (2006). Prevalence of Nosocomial infections in High-Risk Units in the University Clinical Center of Kosovo. Infection Control and Hospital Epidemiology, **(27)4:** 421-423.

Raz R,, Okev N., Kennes Y., Gilboa A., Lavi I. and Bisharat N., (2000). Demographic Characteristics of Patients with Community-Acquired Bacteriuria and Susceptibility of Urinary Pathogens to Antimicrobials in Northern Israel. IMAJ. **2.** 426-429.

Rello J., Rodriguez R., Jubert P. and Alvarez B., (1996). Severe community-acquired pneumonia in the elderly: epidemiology and prognosis. Clin Infect Dis;**23**:723-728.

Rice L., (2001). Evolution and clinical importance of extended-spectrum beta-lactamases. Chest; 119(2 Suppl): **391**S-396S.

Ryan K.J. and Ray C.G., (2004). *Sherris Medical Microbiology*, 4th ed., McGraw Hill, p. 370.

Sahly H., Podschun R., Oelschlaeger T. A., Greiwe M., Parolis H., Hasty D., Kekow J., Ullmann U., Ofek I. and Sela, S., (2000). Capsule Impedes Adhesion to and Invasion of Epithelial Cells by *Klebsiella pneumoniae*. *Infect. Immun.* **68**: 6744-6749.

Schaberg D. R., Culver D. H. and Gaynes R. P., (1991). Major trends in the microbial etiology of nosocomial infection. Am. J. Med**. 91**:72S-75S

Schappert S.M., (1999) Ambulatory care visits to physician offices, hospital outpatient departments, and emergency departments: United States, 1997. Vital Health Stat 13; **143**: i-iv, 1–39.

Scheckler W.E., (1998). Requirements for infrastructure and essential activities of infection control and epidemiology in hospitals: a consensus panel report. Society of Healthcare Epidemiology of America. *Infect Control Hosp Epidemiol*; **19**:114–124.

Schembri M. A., Blom J., Krogfelt K. A., and Klemm P., (2005). Capsule and Fimbria Interaction in *Klebsiella pneumoniae*. *Infect. Immun.* **73:** 4626-4633.

Sedor J. and Mulholland S.G., (1999). Hospital-acquired urinary tract infections associated with the indwelling catheter. Urol Clin North Am ; **26(4)**: 821-828.

Segal-Maurer S., Mariano N. and Qavi A., (1999). Successful treatment of ceftazidime-resistant *Klebsiella pneumoniae* ventriculitis with intravenous meropenem and intraventricular polymyxin B: case report and review. Clin Infect Dis May; **28(5)**: 1134-1138.

Stamm W.E., (2002). Scientific and clinical challenges in the management of urinary tract infections. Am J Med; **113**:1S–4S.

Stock I. and Wiedemann B., (2001). Natural antibiotic susceptibility of *Klebsiella pneumoniae*, K. *oxytoca*, K. planticola, K. ornithinolytica and K. terrigena strains. *J Med Microbiol* **50**: 396-406.

Struve C., Forestier C. and Krogfelt K. A. (2003). Application of a novel multi-screening signature-tagged mutagenesis assay for identification of *Klebsiella pneumoniae* genes essential in colonization and infection. *Microbiology* **149**: 167-176.

Taiwo S. S. and Aderounmu A. O. A., (2006). Catheter Associated Urinary Tract Infection: Aetiological Agents and Antimicrobial Susceptibility patterns in Ladeke Akintola University Teaching Hospital, Osogbo, Nigeria. **9**:141-148.

Torres A., Serra-Batlles J., Ferrer A., Jimenez P., Celis R. and Cobo E., (1991). Severe community-acquired pneumonia. Epidemiology and prognostic factors. Am Rev Respir Dis; **144**:312-318.

Tulsi C. D., Siham A., Ghaffoor M. B., Abraham C.and Eiman A. (2007). Colonization and Infections of Neonates by *Klebsiella* pnuemoniae in an Intensive care Unit. Journal of Tropical pediatrics; **31(4):**200-203.

Van Renterghem L., Joly B. and Carbaret V., (1993). Sclerome et rhinosclerome, un diagnostic difficile: nevue de la literature a propos dune nouvelle observation. Cahier d'otol Rhinol Laryngol: **10**:419-424.

Virella, G. (1997). Gram-negative rods III, opportunistic and zoonotic bacteria. Chapter 17. In: Microbiology and infectious diseases. 2nd ed. Williams and Wilkins: 159-166.

Warren J.W., (2001). Catheter-associated urinary tract infections. Int J Antimicrob Agents; **17(4)**: 299-303.

Wegner G. E., Virella G. and Del Bene V., (1997). Urinary tract infection. Chapter 51. In: Microbiology and infectious diseases. 2nd ed. Williams and Wilkins: 449-453.

Wegner G. E., Virella G. and Del Bene V., (1997). Bacteremia and Sepsis. Chapter 47. In: Microbiology and infectious diseases. 2nd ed. Williams and Wilkins: 409-417.

Wen-Chien K., David L. P., Anthanasia J. S., Dennis S. H., Anne V. G., Sunita M., Jose M. C., Herman G., Lutfiye M., Gordon T., Keith P. K. Joseph G. M., and Victor L., (2002). Community-Acquired *Klebsiella* pnuenoniae Bacteremia: Global Differences in Clinical Patterns. Emerging Infectious deseases. **(8) 2**. P.61-64

Wenzel R.P., (2003). *Prevention and Control of Nosocomial Infections,* 4th ed. Philadelphia: Lippincott, Williams & Wilkins; 14-33.

Zohar Y., Talmi Y.P. and Strauss M., (1990). Ozena revisited. J Otolaryngol; **19(5)**: 345-349

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APPENDIX 3

Table 1. Age distribution of patients

Age	Number	Percentage distribution
< 1	22	10.7
1-9	32	15.6
10-19	21	10.2
20-29	23	11.2
30-39	32	15.6
40-49	29	14.1
50-59	15	7.3
60-69	9	4.4
70-79	15	7.3
80-89	7	3.4

Table 2. *Klebsiella* species isolated from various specimens in relation to in-patients and out-patients

Specimens Type	Number Of Specimens	Species												
		K. pneumaniae			*K. oxytoca*			*K. ozaenae*			*K. rhinoscleromatis*			Total
		In-patient	Out-patient	Total	In-patient	Out-patient	Total	In-patient	Out-patient	Total	In-patient	Out-patient	Total	
Blood sample	802	3	36	39	2	0	2	0	0	0	0	0	0	41
Ear swab	73	6	2	8	1	0	1	0	0	0	0	0	0	9
Gastric aspirate	31	2	0	2	0	0	0	0	0	0	0	0	0	2
Knee aspirate	22	1	0	1	0	0	0	0	0	0	0	0	0	1
Pleural aspirate	54	2	0	2	2	1	3	0	0	0	0	0	0	5
Sputum sample	206	11	16	27	0	2	2	0	0	0	0	0	0	29
Urine sample	502	23	10	33	10	9	19	0	1	1	0	1	1	54
Wound swab	507	13	27	40	10	13	23	0	0	0	0	1	1	64
Total	2197	61	91	152	25	25	50	0	1	1	0	2	2	205

APPENDIX 4

Liquid Media

A. Peptone Water

Peptone water may be used as a growth medium or as the basis of carbohydrate fermentation media, whilst pure culture peptone water is a convenient inoculum for a series of fermentation tubes or other diagnostic media.

Composition

Peptone water contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Gram/litre
Peptone	10.0
Sodium chloride	5.0
pH 7.0-7.4	

Preparation

The medium was prepared by dissolving 15 grams in 1 litre of distilled water. It was mixed well and distributed into bijoux bottles in 5ml volumes, and then sterilized by autoclaving at 121^0C for 15 minutes.

Mode of Action

The amino acid tryptophan is found in nearly all proteins. Bacteria that contain the enzyme tryptophanase can hydrolyse tryptophan to its metabolic products namely; indole, pyruvic acid and ammonia. The bacteria use the pyruvic acid and ammonia to satisfy nutritional needs. Indole is not used and accumulates in the medium. The presence of indole can be detected by the addition of Kovacs' reagent. Kovacs' reagent reacts with the indole, producing a bright red compound on the surface of the medium. Bacteria producing a red layer following addition of Kovacs' reagent are indole positive, the absence of a red colour indicates tryptophan was not hydrolyzed, and the bacteria are indole negative (Downes and Ito, 2001).

Kovac's Reagent

This reagent is used for the detection of indole. It is prepared by dissolving 10 grams of 4-dimethylamino-benzaldehyde in 150ml of iso-amyl alcohol. After dissolution 50ml of concentrated hydrochloric acid is added to it. It is then stored in a refrigerator in an amber bottle.

B. Methyl Red and Voges-Proskauer (MR-VP) medium

The medium is recommended for the Methyl Red and Voges-Proskauer tests for the differentiation of the coli-aerogenes group.

Composition

Methyl Red and Voges-Proskauer medium contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Gram/litre
Peptone	7.0
Glucose	5.0
Phosphate buffer	5.0

pH 6.7-7.1

Preparation

Seventeen grams was dissolved in 1 litre of distilled water. The solution was then distributed into the final containers and sterilized by autoclaving at 121^0C for 15 minutes.

Mode of Action

Methyl Red and Voges-Proskauer (MR-VP) medium contain peptone, dextrose (glucose) and dipotassium phosphate. Dextrose is a significant ingredient that determines what types of end products an organism forms from degrading glucose. Certain bacteria ferment carbohydrates, including glucose, and produce a variety of acids as by-products or end products. After 48 hours of growth of such bacteria, the pH of the culture reaches 4.5 or below (because of these acid end products), which changes the methyl red indicator to its acid colour (red), proving positive to methyl red test. In another vain, other bacteria utilizing the same quantity of glucose do produce ethyl alcohol, acetoin, and acetyl methyl carbinol which are nonacidic. Because the pH does not

reach 4.5, the methyl red does not display its acid colour (red), proving negative to methyl red test (Downes and Ito, 2001).

C. Cooked Meat Broth

An excellent medium for the primary growth and maintenance of aerobic and anaerobic organisms.

Composition

Cooked Meat Broth contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Gram/litre
Heart muscle	454.0
Peptone	10.0
'Lab-Lemco' powder	10.0
Sodium chloride	5.0
Glucose	2.0

pH 7.0-7.4

Preparation

To prepare the medium 10 grams of powder was dissolved in 100 ml of distilled water. This was allowed to stand for 15 minutes until the meat particles are thoroughly wetted. This was then sterilized by autoclaving at 121^0C for 15 minutes.

Mode of Action

Cooked Meat Broth prepared from heart tissue is a well established medium for the cultivation of anaerobic and aerobic organisms. It has the ability to initiate bacterial growth from very small inocula and to maintain the viability of cultures over long periods of time. Mixed cultures of bacteria survive in Cooked Meat Medium without displacing the slower growing organisms. The products of growth do not rapidly destroy the inoculated organisms and therefore it is an excellent medium for the storage of aerobic and anaerobic bacteria. The addition of glucose to

the formulation allows rapid, heavy growth of anaerobic bacteria in a short time and leads to a more rapid identification of important anaerobes.

D. Brain Heart Infusion Broth

A highly nutritious infusion medium recommended for the cultivation of streptococci, pneumococci, meningococci and other fastidious organisms. Suitable for blood culture work.

Composition

The Brain Heart Infusion Broth contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Grams/litre
Calf brain infusion solids	2.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5

pH 7.2-7.6

Preparation

To prepare this medium 37 grams in 1 litre of distilled water. It was mixed well and distributed into final containers. Sterilisation was done by autoclaving at 121^0C for 15 minutes

Mode of Action

Brain Heart Infusion is essentially a buffered infusion broth giving similar results to the brain dextrose broths originally employed for cultivation of streptococci, and for the cultivation of dental pathogens.

E. Malonate Broth

Malonate broth is used for differentiation among the *Enterobacteriaceae* based on malonate utilization.

Composition

Malonate broth contains the following in grams per litre according to Downes and Ito (2001).

Composition	Grams/litre
Ammonium sulfate	2.0
Dipotassium phosphate	0.6
Monopatassium phosphate	0.4
Sodium chloride	2.0
Sodium Malonate	3.0
pH 6.9-7.3	

Preparation

Eight grams of the powder was dissolved in 1 litre of distilled water. It was then boiled for 1 minute to completely dissolve the powder. It was then Autoclaved at 121^0C for 15 minutes.

Mode of Action

Malonate Broth contains ammonium sulfate, which is the sole source of nitrogen in the medium; sodium malonate is the sole source of carbon. Dipotassium phosphate and monopotassium phosphates provide buffering capability. Sodium chloride maintains the osmotic balance of the medium. Increased alkalinity resulting from malonate utilization will cause a colour change in acid base indicator.

F. Stuart Transport Medium

This is a transport medium for fastidious pathogenic organisms.

Composition

Stuart transport medium contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Gram/litre
Sodium glycerophosphate	10.0
Sodium thioglycollate	0.5
Cysteine hydrochloride	0.5
Calcium chloride	0.1
Methylene blue	0.001
Agar	5.0
pH 7.2-7.6	

Preparation

To prepare this medium, 16grams of the powder was dissolved in 1 litre of distilled water. It was then boiled shortly to dissolve the medium completely. It was sterilized by autoclaving at 121^0C for 15 minutes.

Mode of Action

This is a non-nutritional semi solid substrate for the preservation of *Neisserria* species and other fastidious organisms during their transport from clinic to laboratory. It may also be used for transport of other bacteriological specimens.

G. Turbidity Standard Solution

This is a barium sulphate standard against which the turbidity of the test and control inocula can be compared. When matched with standard, the inocula should give confluent or almost confluent growth.

Preparation

One percent solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of distilled water. Also, 1% solution of barium chloride was prepared by dissolving

0.5 grams of dehydrated barium chloride ($BaCl_2.2H_2O$) in 50ml of distilled water. Zero point six millitres of the barium chloride solution was added to 99.4ml of the sulphuric acid solution and mixed. Small volume was then transferred to a capped tube same as the type used for preparing the test and control inocula.

H. Sample Storage

Glycerol broth was used to store the samples.

Preparation

The broth was prepared by weighing 20% of brain-heart infusion broth. Distilled water and glycerol was added using the ratio of 4:1 distilled water to glycerol. The mixture was stirred until a uniform solution was obtained. A micropipette was used to pipette 1ml of the solution into Eppendorf tubes. The broth was then sterilized at 121^{o}C for 15 minutes.

APPENDIX 5

Solid Media

A. MacConkey Agar

This is a differential medium for the differentiation and isolation of the *Enterobacteriaceae*. This medium also supports the growth of *Staphylococci* and *Enterococci*, but inhibits the growth of *Streptococci* and the *Haemophilus*. Swarming of *Proteus* is prevented.

Composition

It is formulated to contain the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England.

Composition	Grams/litre
Peptone	20.0
Lactose	10.0
Bile salts No. 3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
pH 6.9-7.3	

Preparation

This was prepared by suspending 51.5 grams in 1 litre of distilled water. It was then boiled shortly to dissolve completely. Sterilization was done by autoclaving at 121^0C for 15 minutes.

Mode of Action

This medium is used as a differentiated medium, differentiating organisms into lactose and non-lactose fermenting organisms. The lactose-fermenting organisms, by the fermenting of lactose, produce acids which act upon the bile salt and absorb the neutral red, giving red colonies. The non-lactose fermenting colonies give an alkaline reaction, do not absorb the neutral red and produce colourless colonies.

B. Cystine-Lactose-Electrolyte Deficient (CLED)

This medium is recommended for diagnostic urinary bacteriology. The medium supports the growth of all urinary potential pathogens giving good colonial differentiation and clear diagnostic characteristics.

Composition

CLED contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Grams/litre
Peptone	4.0
'Lab-Lemco' powder	3.0
Tryptone	3.0
Lactose	10.0
L-cystine	0.128
Bromothylmol blue	0.02
Agar	15.0
pH 7.0-7.4	

Preparation

To prepare this medium 36.2 grams of the powder was suspended in 1 litre distilled water. It was boiled shortly to dissolve completely. Sterilization was done by autoclaving at 121^0C for 15 minutes.

Mode of Action

This medium supports the growth of all urinary pathogens and gives good colonial differentiations and clear diagnostic characteristics. The presence of important contaminants such as diphtheroids, lactobacilli and micrococci is also clearly elicited, giving an indication of the degree of contamination. In the laboratory CLED medium provides a valuable non-inhibitory diagnostic agar for plate culture of urinary organisms. It is electrolyte deficient to prevent the swarming of *Proteus* species.

C. Blood Agar

This is a non-selective general purpose medium which may be enriched with blood or serum.

Composition

Blood agar contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Grams/litre
'Lab-Lemco' powder	10.0
Peptone neutralized	10.0
Sodium chloride	5.0
Agar	15.0
pH 7.1-7.5	

Preparation

Forty grams of the powder was suspended in 1 litre of distilled water. It was boiled shortly to dissolve completely. Sterilization was done by autoclaving at 121^0C for 15 minutes. The solution was then cooled to 50^0C and 7% of human blood added. It was mixed with gentle rotation and poured into Petri dishes.

Mode of Action

This is a non-selective general purpose medium widely employed for the growth of pathogenic and non-pathogenic bacteria. Without additions, the medium may be employed as a nutrient agar or as a medium for the short-term maintenance of stock cultures. With added serum or other enrichments, the medium becomes suitable for the cultivation of many fastidious organisms. With added blood, the medium is not only enriched, but becomes suitable for the determination of the typical haemolytic reactions which are important diagnostic criteria for streptococci, staphylococci, and other organisms.

D. Mueller-Hinton Agar

This is an antimicrobial susceptibility testing medium which may be used in internationally recognized standard procedures.

Composition

Mueller-Hinton Agar contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Grams/litre
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.2-7.4	

Preparation

To prepare the medium 38 grams of the powder was dissolved in 1 litre of distilled water. It was then boiled shortly to dissolve the medium completely. It was sterilized by autoclaving at 121^0C for 15 minutes.

Mode of Action

Mueller-Hinton Agar was designed to be a reproducible culture medium for the isolation of pathogenic *Neisseria* species. The inclusion of starch ensures that toxic factors found during growth will be absorbed and its presence is often essential to establish growth from very small inocula.

E. Nutrient Agar

This is a general purpose medium which may be enriched with 10% or other biological fluid.

Composition

Nutrient agar contains the following in grams per litre according to Oxoid Ltd, Basingstoke, Hampshire, England:

Composition	Gram/litre
"Lab-Lemco" powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

pH 7.2-7.6

Preparation

To prepare the medium, 28grams of the powder was dissolved in 1 litre of distilled water. It was then boiled shortly to dissolve the medium completely. It was sterilized by autoclaving at 121^0C for 15 minutes.

Mode of Action

This is basic culture medium used to sub-culture organisms for maintenance purpose or to check the purity of subcultures from isolation plates prior to biochemical or serological tests.

Gram staining technique

The gram staining reaction is used to help identify pathogens in specimens and cultures by their gram reaction (gram-negative or gram-positive) and morphology. Pus cells can also be identified in gram smears.

Staining mechanism

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stain purple and Gram-negative bacteria have a thinner layer (10% of cell wall), which stain pink. Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space. There are four basic

steps of the Gram stain, which include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram's iodine), rapid decolorization with alcohol or acetone, and counterstaining with safranin or basic fuchsin.

Crystal violet (CV) dissociates in aqueous solutions into CV^+ and chloride (Cl^-) ions. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. The CV^+ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Iodine (I^- or I_3^-) interacts with CV^+ and forms large complexes of crystal violet and iodine (CV – I) within the inner and outer layers of the cell. When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the peptidoglycan layer is left exposed. The CV – I complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large CV – I complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan. The decolorization step is critical and must be timed correctly; the crystal violet stain will be removed from both Gram-positive and negative cells if the decolorizing agent is left on too long (a matter of seconds).

After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple color. Counterstain, which is usually positively-charged safranin or basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red color.

Some bacteria, after staining with the Gram stain, yield a *Gram-variable* pattern: a mix of pink and purple cells is seen. The genera *Actinomyces*, *Arthobacter*, *Corynebacterium*, *Mycobacterium*, and *Propionibacterium* have cell walls particularly sensitive to breakage during

cell division, resulting in Gram-negative staining of these Gram-positive cells. In cultures of *Bacillus, Butyrivibrio,* and *Clostridium* a decrease in peptidoglycan thickness during growth coincides with an increase in the number of cells that stain Gram-negative. In addition, in all bacteria stained using the Gram stain, the age of the culture may influence the results of the stain.

Gram staining protocol

1. Make a smear of the specimen that is to be stained on a slide. Heat the slide for few seconds until it becomes hot to the touch so that bacteria are firmly mounted to the slide.

2. Add the primary stain crystal violet and wait 1 minute. Rinse gently with water. This step colors all cells violet.

3. Add Gram's iodine, for 30 seconds. It is not a stain; it is a mordant. It doesn't give color directly to the bacteria but it fixes the crystal violet to the bacterial cell wall. All cells remain violet.

4. Wash with ethanol and acetone, the decolorizer. If the bacterium is Gram-positive it will retain the primary stain. If it is Gram-negative it will lose the primary stain and appear colorless.

5. Add the secondary stain, safranin, and wait 1 min, and then wash with water for a maximum of 5 seconds. If the bacterium is Gram-positive then the cell will retain the primary stain, will not take the secondary stain, and will appear black-violet. If the bacteria is Gram-negative then the cell will lose the primary stain, take secondary stain, and will appear red-pink.

6. Examine the smear microscopically, first with the x40 objective to check the staining and to see the distribution of material, and then with the oil immersion objective to report the bacteria and cells.