Identification of Potential Pathogens of Pneumonia in Sputum Samples from Adult Patients at the University Teaching Hospital in Lusaka

A Dissertation Submitted to the University of Zambia in Partial Fulfilment of the Requirements for the Degree of Master of Science in Medical Microbiology

By

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Declaration

I, John Mulemena, declare that this is my own work. It is being submitted for the Degree of Master of Science in Medical Microbiology at the University of Zambia, Lusaka. It has not been submitted for any degree at this or any other university.

_____ 31st August, 2016

John Mulemena

Certificate of Approval

Dissertation Title: Identification of Potential Pathogens of Pneumonia in Sputum Samples from Adult Patients at the University Teaching Hospital in Lusaka

This dissertation of **John Mulemena** has been approved in partial fulfilment of the requirements for the degree of Master of Science in Medical Microbiology at the University of Zambia.

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Abstract

Introduction: Pneumonia is a leading cause of morbidity and a significant cause of mortality worldwide. Although information is available on pneumonia in children in Zambia, the incidence in adults in many parts of Africa including Zambia is unknown. Knowledge of the local aetiological agents of pneumonia is critical for making rational decisions about treatment as differences in aetiology may result in poor response to therapy chosen to cover pathogens common in studies done in high income countries.

Objective: The objective of this study was to identify aetiological agents of pneumonia in adult patients and determine their antibiotic susceptibility patterns at the University Teaching Hospital in Lusaka.

Materials and Methods: This was a cross-sectional study carried out from March 2014 to August 2014. A total of 312 sputum samples from adults suspected of pneumonia were cultured and of which 146 samples were also analysed by PCR. Antibiotic susceptibility testing was done on bacterial isolates according to Clinical Laboratory Standards International guidelines.

Results: Of the 312 samples cultured, 56.1% (175/312) yielded probable pathogens with the most common being *Moraxella catarrhalis* 20.8% (47/226), *Pseudomonas aeruginosa* 19.9% (45/226), and *Klebsiella pneumoniae* 14.2% (32/226). Almost all (10/11) *K. pneumoniae* isolates were multidrug resistant (7 of 9 drugs) and were ESBL positive. About 71% of the 146 samples tested using PCR yielded human cytomegalovirus 24.3% (44/181), *K. pneumoniae* 17.7% (32/181), *H. influenzae* non-type b 16.0% (29/181), *S. pneumoniae* and *S. aureus* 9.4% (17/181) each. Other agents, which included Rhinovirus (5.5%, 10/181), *M. catarrhalis* (4.4%, 8/181), *Pneumocystis jirovecii* (3.9%, 7/181), Respiratory Syncytial Virus (RSV) A/B (3.9%, 7/181), Adenovirus (1.7%, 3/181), Human bocavirus (1.1%, 2/181), Human metapneumoviruses A/B (1.1%, 2/181), Parainfluenzae type 1 (1.1%, 2/181), Parainfluenzae type 2 (1.1%, 2/181), Parainfluenzae type 4 (1.1%, 2/181), *Salmonella species* (0.6%, 1/181), *Mycoplasma pneumoniae* (0.6%, 1/181), Influenza virus type B (0.6%, 1/181), Human coronavirus 63 (0.6%, 1/181), and Parainfluenzae type 2 (0.6%, 1/181) were also detected. Multiple agents were detected in 42% of samples analysed by PCR. About 29.5% (13/44) of these specimens

harboured *K. pneumoniae* and *H. influenzae* non-type B. The culture and PCR methods detected 30.1% and 69% community acquired agents, respectively.

Conclusion: The study showed a wide variety of potential pathogens that included bacteria, viruses and fungi. PCR detected more organisms than the culture method, which included viruses and fungal agents. Some of the samples yielded multiple organisms which would makes it difficult to determine the causative agent of pneumonia in a patient. Most of the bacterial agents isolated displayed the multidrug resistant phenotype. These data shows the importance of employing better diagnostic methods, such as molecular tools, for identifying potential pathogens associated with pneumonia. The high drug resistance patterns observed with bacterial isolates presents physicians with very limited treatment options for the affected patients, which may require resorting to more expensive drugs. This calls for an urgent review of treatment practices at the University Teaching Hospital to avoid complications in the patients

Dedication

I dedicate this work to my late father, Mr. Amos John Mulemena, for his encouragement, emotional and spiritual support during the time of my study.

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List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
AMR	Antimicrobial Resistance
API-20E	Analytical Profile Index-20 Enterobacteria
ATCC	American Type Culture Collection
CAP	Community Acquire Pneumonia
CLSI	Clinical and Laboratory Standards Institute
CMV	Cytomegalovirus
DNA	Deoxyribonucleic Acid
ESBLs	Extended-Spectrum-β-Lactamases
FTD	Fast Track Diagnostics
GNC	Gram Negative Cocci
GNCB	Gram Negative Coccobacilli
GNR	Gram Negative Rods
GPC	Gram Positive Cocci
GPDC	Gram Positive Diplococci
GPR	Gram Positive Rods
HAP	Healthcare Associated Pneumonia
HIV	Human Immunodeficiency Virus
HPF	High Power Field
HTM	Haemophilus Test Medium

LPF	Low Power Field
MDR	Multidrug Resistance
MGIT	Mycobacteria Indicator Growth Tube
MHB	Müeller Hinton agar with 5% sheep blood
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear Cells
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
SECs	Squamous Epithelial Cells
UN	United Nations
UNZABREC	University of Zambia Biomedical Research Ethics Committee
UTH	University Teaching Hospital
WHO	World Health Organisation

Chapter 1

Introduction

1.0 Introduction

1.1 Background

Pneumonia is a disease of the lung parenchyma distal to the terminal bronchioles (Black, 2008), and is mainly caused by infection with bacteria, viruses, fungi and less commonly by parasites (Peto *et al*., 2014). Occasionally, inhaled chemicals can cause lung inflammation and lead to pneumonia (Ezzati *et al*., 2001). The disease is associated with clinical and radiological evidence of consolidation of part or parts of one or both lungs (Cao *et al*., 2013). It may also be complicated by presence of effusion, empyema, abscess and sepsis which may lead to lung failure, and eventually death (Mandell, 2007). The most recent estimate of the Global Burden of Disease Study revealed that lower respiratory tract infections, including pneumonia, are the fourth most common causes of death globally, exceeded only by ischaemic heart disease, stroke and chronic obstructive pulmonary disease (Lozano *et al*., 2013; Acharya *et al*., 2014).

Globally, pneumonia is the leading cause of morbidity and mortality in all age groups, with up to 30% of patients requiring hospitalisation (Zar *et al*., 2013; Ho, 2014; Micek *et al*., 2014). Most deaths occur in low income countries (Lozano *et al*., 2013). According to the World Health Organisation (WHO), 1.6 million deaths per annum in adults are attributed to pneumonia and will be amongst the leading four causes of death by 2030 (WHO, 2015). About 5% of patients hospitalised for pneumonia require treatment in an intensive care unit, and these severely ill patients have a mortality rate of about 35% (Torres *et al*., 2013). The disease places a

considerable burden on healthcare systems and society, with an annual estimated cost of approximately €10 billion, which is mainly attributed to hospitalization and lost working days (Torres *et al*., 2013).

It is estimated that community acquired pneumonia (CAP) cause almost one million adult deaths per year in Asia (Peto *et al*., 2014). According to the WHO, a good number of these deaths occur in the elderly, but a large number occur in those with good life expectancy, including 160 000 among those aged 15–59 years (WHO, 2008). In Sub-Saharan Africa, the annual mortality attributed to pneumonia is estimated at about 4 million per year (Zar *et al*., 2013).

The pattern of microbiological organisms causing pneumonia in high income countries has been well characterized (Gutiérrez *et al*., 2005; Charles *et al*., 2008). However, in most low-income countries, such as Zambia, there is a paucity of data concerning the burden of pneumonia in adults despite an increase in the number of cases (Hartung *et al*., 2011; Micek *et al*., 2014). Increased risk of pneumonia is common among immune compromised, alcoholics, smokers, and patients with chronic kidney disease, chronic liver disease, chronic lung disease, and the very young and old (Torres *et al*., 2013; Chakwe, 2014).

Treatment of pneumonia using antibiotics is usually empirical, given that an aetiological diagnosis is achieved in only half the cases and is usually determined late or retrospectively (Huijskens *et al*., 2013; Peto *et al*., 2014). There is considerable concern about the emerging resistance among the usual pathogens of pneumonia to most commonly used antimicrobial

agents (Fuller *et al*., 2005; File *et al*., 2010; Welte, 2012). Hence, proper selection of empirical treatment depends on the common pathogens identified in previous aetiological studies.

1.2 Statement of the Problem

Pneumonia is a life threatening disease requiring prompt pathogen identification and therapeutic intervention in order to control severity of the infection (Zar *et al*., 2013). At the University Teaching Hospital (UTH), it is among the top five leading causes for hospital admission (Chakwe, 2014). However, a large proportion of pneumonia cases have unknown aetiology, and for those that are known, there is little or no data on their antibiotic resistance patterns. Thirdly, the quality of sputum is not assessed during microbiological analysis, and this may be making the cost of analysing the samples very high subjecting the laboratory to nasopharyngeal organisms that may not be associated with pneumonia. There are also differences in geographical distribution of agents of pneumonia and this poses a challenge in establishment of empirical treatment. This has led to increased morbidity and mortality, and has further contributed to the mismanagement of pneumonia patients (Welte, 2012). While in high income countries a number of new pathogens have been identified and newer antimicrobial agents are available, in Zambia there are no standardised reports on the incidence of aetiological agents of pneumonia and their drug resistance patterns.

1.3 Justification of the Study

Determining the microbial aetiology of pneumonia is challenging, particularly due to difficulties in obtaining appropriate lower respiratory specimens for diagnostic testing. Sputum is the lower respiratory specimen most commonly collected from adults with pneumonia. However, sputum quality has a large bearing on the interpretation of culture results (Murdock *et al,* 2009).

Oropharyngeal flora of the upper respiratory tract usually contaminate the sputum sample, and a large number of different species overgrow putative pathogens, thereby precluding their isolation and identification (Ziyade and Yagci, 2010). This can lead to the incorrect conclusion that an organism colonizing the upper airways is causing pneumonia. Consequently, it has become standard practice for diagnostic laboratories to assess the quality of an expectorated sputum specimen to confirm that it has been obtained from the lower respiratory tract. This study would help in providing information on the importance of proper collection of sputum specimens by clinical and nursing staff in order to facilitate the isolation of lower respiratory pathogens by the microbiology laboratory.

Several studies have revealed that there are differences in distribution of causative agents of pneumonia (Felmigham *et al*., 2002; Lynch *et al*., 2009; Fieldman *et al*., 2012; Micek *et al*., 2014). Therefore, knowledge of the local aetiological agents of pneumonia is critical for making rational decisions about treatment as differences in aetiology may result in poor response to therapy chosen to cover pathogens common in studies done in high income countries (Holter *et al*., 2015). With this has come the recognition not only that resistance patterns change over time but also that there are significant geographical and regional differences in the patterns of pathogen and resistance (Ho *et al*., 2014). As a result of these differences, the empirical antimicrobial therapy and vaccination programmes recommended in high income countries may not be directly applicable to countries such as Zambia. This study will contribute to the identity, as well as the understanding of antimicrobial susceptibility patterns of bacterial pathogens commonly associated with pneumonia in adults at the UTH. Thus, appropriate treatment can be provided to the affected patients through the understanding of these aetiological agents. This is

the first study in Zambia to simultaneously investigate the quality of sputum, microbiological aetiology and antimicrobial susceptibility patterns of bacteria from adult patients with pneumonia.

1.4 Research Question

What are the common pathogens that are associated with pneumonia in adults at the University Teaching Hospital in Lusaka?

1.5 Objective

1.5.1 General Objective

To identify potential pathogens in sputum specimens from adults suspected of having pneumonia at the University Teaching Hospital in Lusaka.

1.5.2 Specific Objectives

1.5.2.1 To determine the quality of sputum samples from adults suspected of having pneumonia using the Bartlett score.

1.5.2.2 To identify the potential pathogens present in sputum specimens from adults suspected of having pneumonia.

1.5.2.3 To determine patterns of antimicrobial resistance among bacterial pathogens isolated from sputum samples of adults suspected of having pneumonia.

Chapter 2

Literature Review

2.0 Literature Review

2.1 Aetiology of Pneumonia

Pneumonia is often categorised as "typical" or "atypical" (Bedi, 2006). Typical pneumonias are mainly caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (Huijskens *et al*., 2013). With atypical pneumonia, the infection is caused by *Mycoplasma pneumoniae*, *Legionella pneumophilia*, *Chlamydophila pneumoniae*, *Chlamydia psittaci* and *Coxiella burnetli*, and all these organisms are collectively referred to as atypical organisms (Cunha, 2010). Community acquired pneumonia (CAP) include both typical and atypical pneumonias and but is most commonly caused by bacterial infection (Ho, 2014). Infections by fungi such as *Pneumocystis jirovecii* are responsible for a small number of CAP cases and are more likely to occur in immunocompromised patients because of weakened immunity (Chakwe, 2014).

Early onset of healthcare-associated pneumonia (HAP) is often associated with *M. catarrhalis*, *H. influenzae*, and *S. pneumoniae*, while the late onset of HAP is frequently associated *K. pneumoniae*, *P. aeruginosa* or *S. aureus*, including methicillin-resistant *Staphylococcus aureus* (MRSA) (Edwards *et al*., 2009). Viruses, such as Influenza A and B or Respiratory Syncytial Virus, can cause early and late onset healthcare-associated pneumonia, whereas yeasts, fungi, such as *Candida albicans* and *Pneumocystis jirovecii*, *Legionella* are usually pathogens of late onset pneumonia (Chakwe, 2014).

The aetiological agents of pneumonia may vary with age, co-morbidities, use of immunosuppressive or antimicrobial drugs and severity of disease (Reynolds *et al*, 2010). Since there is weakened immunity in immunocompromised, patients are defined by susceptibility to infection with organisms of little native virulence in normal individuals. Each group of hosts such as AIDS, solid organ transplant recipients, or hematopoietic cell transplant recipients, has enhanced susceptibility to a subset of pathogens depending upon the nature of the underlying immune defects (Fishman and Rubin, 1998). In patients with chronic obstructive pulmonary disease, *H. influenzae* and *M. catarrhalis* are commonly encountered (Feldman *et al*., 2007). Alcoholic patients are at increased risk of infection with *S. pneumoniae, Legionella pneumophilia* and mixed infections (Zar *et al*., 2013). The acute intoxicating effects of alcohol and the attendant risk of secretions or foreign material entering into the trachea and lungs are components in the development of alcohol-associated pneumonia. This is necessitated by chronic damaging chemical imbalance in the cell and consequent cellular dysfunction within the layer of epithelium lining the airway as well as macrophages in the airway (Bechara *et al*. 2005; Kamat *et al*. 2005). In the last two decades, other pathogens such as influenza B, parainfluenza, adenovirus, Hantavirus, and human metapneumovirus have been implicated, and detected, as a result of the rapid development of molecular diagnostic techniques which have enhanced specificity and sensitivity (Huijskens *et al*., 2013; Liu *et al*., 2013; Ho 2014).

Fungal pathogens such as *Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Sporothrix schenckii, Cryptococcus neoformans* may cause sporadic cases of pneumonia, but more likely in endemic areas such as the United States of

America (Orin, 2005). *Histoplasma capsulatum* is common in the Mississippi and Ohio River valleys and south-east parts of the country, while *Coccidioides immitis and Blastomyces dermatitides* are mainly associated with the southwestern United States of America (Orin, 2005). In Africa, particularly sub-Sahara, *Pneumocystis jirovecii* has been increasingly reported in areas where it had previously been thought to be a rare pathogen especially in persons with HIV infection (Fisk *et al*., 2003).

Rapid and accurate aetiological diagnosis of pneumonias in patients suspected with pneumonia is essential to establishing the local prevalence patterns of disease. However, in low-income countries, such as Zambia, the identification of aetiological agents for pneumonia has been a big challenge due to limited diagnostic capacity. Therefore health centres with capabilities to perform advanced diagnostic techniques should monitor disease trends and obtain data on potential pathogens of pneumonia in order to improve treatment algorithms.

2.2 Epidemiology of Pneumonia

Aetiological epidemiology and diagnosis are important issues in adult pneumonia, with particular challenges in identifying the causative pathogens based on patient clinical features (Bartlett, 2011; Liu *et al*., 2013). The aetiological agent profile of pneumonia is not the same across various countries but varies within the same country with time due to differences in the frequency of use of antibiotics, environmental pollution, awareness of the disease and life expectancy (Micek *et al*., 2014).

In recent years, there has been a change in the relative frequency of the different pathogens, a change attributable to the increase in the number of patients who are older or who have underlying disease and the development of more sensitive diagnostic techniques (Huijskens *et al*., 2013). However, current evidence suggests that pneumonia in adults is most commonly caused by bacterial infection (Bartlett, 2011; Patterson *et al*., 2012). *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* account for at least 50% of pneumonia cases, with *S. pneumoniae* being the most commonly identified pathogen (Khalil *et al*., 2013; Regasa, 2014). *Mycoplasma pneumoniae, Chlamydia pneumoniae, Chlamydia psittaci, Coxiella burnetii* and *Legionella pneumophilia* are responsible for up to 15% of cases and may be co-pathogens in other cases (Shariatzadeh *et al*., 2005).

In elderly patients, *S. pneumoniae* remains the most common pathogen with *M. pneumonia* and *L. pneumophila* being less frequent (Huijskens *et al.*, 2013; Takahashi *et al*., 2013). However, other studies have shown conflicting results. A 12-month prospective multicentre study conducted in India whose main focus was to identify new and emerging aetiological agents of pneumonia, showed that the most common causative agents of pneumonia were *S. pneumoniae* (15.3%), *H. influenzae* (10.9%), *Legionella spp*. (6.7%) and *C. pneumoniae* (6.1%) (Fang *et al*., 1990). The highest mortality cases were associated with *S. aureus* (50%), whilst *C. pneumoniae* (4.5%) was associated with the lowest mortality cases (Fang *et al*., 1990).

In a Chinese study conducted on 590 adults with community-acquired pneumonia, the most common agent isolated was *M. pneumoniae* (20.7%), followed by *S. pneumoniae* (10.3%), *H. influenzae* (9.2%), *C. pneumoniae* (6.6%), *K. pneumoniae* (6.1%), *L. pneumophila* (5.1%), *S.*

aureus (3.8%), *E. coli* (1.6%), *M. catarrhalis* (1.3%), and *P. aeruginosa* (1.0%). Among the viruses detected, adenovirus was the most common (8.7%), followed by influenza virus B (6.5%), respiratory syncytial virus (4.3%) and influenza virus A (2.7%) (Liu *et al*., 2009). Another study conducted in Beijing, China, between November 2010 to October 2011 using multiplex and quantitative real-time PCR, viruses accounted for 36.4% of the pathogens detected, while *M. pneumoniae* and bacteria accounted for 18.0% and 14.4%, respectively, from 500 samples tested. About 10.6% of the cases had mixed viral infections, and influenza A virus represented the greatest proportion of infections (Liu *et al.*, 2013). In a study conducted in Central Vietnam on 286 sputum samples tested for bacterial infections, 28% were positive for *H. influenzae*, while 23% were positive for *S. pneumoniae.* Further testing of 357 samples for viral infection using PCR, revealed that 21% were positive for respiratory viruses which included respiratory syncytial virus (RSV), rhinovirus, human metapneumovirus, human bocavirus, and parainfluenza and 9% were positive for influenza A (Takahashi *et al*., 2013).

A prospective observational study conducted in northern Israel, in which 127 subjects were enrolled, showed that at least one pathogen was found in 66.7% of the subjects with the following pathogen distribution: *S. pneumoniae* (18.3%), atypical pathogens (52.4%) and viruses (45.2%). The most frequent isolates were *C. pneumoniae* (20.6%) and influenza virus A or B (19.0%) (Shibli *et al.*, 2010).

A cross sectional study conducted in southern Ethiopia in 2013 in which a total of 170 adult patients with typical symptoms of pneumonia were enrolled, showed that the most frequent isolated bacteria was *S. pneumoniae* (11.8%), followed by *S. aureus* (8.8%), *P. aeruginosa*

(5.8%), *K. pneumoniae* (4.7%), *E. coli* (2.4%), *H. influenzae* (2.4), *P. mirabilis* (0.6%) and *P. vulgaris* (0.6%) (Regasa, 2014). A similar study conducted in Egypt in five major military hospitals on 239 adult patients, identified *S. pneumonia* (36.4%) as the major causative agent for CAP, followed by *S. aureus* (7%), *K. pneumoniae* (4.8%), *P. aeruginosa* (2.1%), *E. coli* (1.6%), *Staphylococcus spp*. (1.6%), *Streptococcus hominis* (1.6%), and *S. pyogenes* (1.6%). On the other hand in cases of HAP the most common microorganism identified as being *K. pneumoniae* (23.1%) followed by *P. aeruginosa* (17.3%), *E. coli* (11.5%), *Acinetobacter species* (7.7%), *S. haemolyticus* (7.7%), MRSA (5.8%), *Candida species* (5.8%) (Khalil *et al*., 2013).

In a retrospective laboratory-based study conducted in Benin City in Nigeria on 1539 patients with symptoms of pneumonia, *K. pneumoniae* was the predominant isolate (30.16%) recovered, followed by *H. influenzae* (17.05%), *S. aureus* (15.41%), and *Acinetobacter species* (0.66%) (Egbe *et al*. in 2011). In contrast, a study conducted on 3,406 patients with acute respiratory infection in rural western Kenya, *S. pneumoniae* (16%) emerge as most frequently isolated bacteria, followed by non-Typhi *Salmonella* (3%) and *M. pneumonia* (0.7%), while influenza A virus was the most frequently (38%) virus (Feikin *et al*., 2012).

However, aetiological agents of pneumonia are poorly characterized in many low income countries, especially sub-Saharan Africa, because of limited availability of resources. In Zambia little is known about the epidemiology of pneumonia.

2.2.1 Pneumonia in HIV Infection

The lower respiratory tract is an important target of Human Immunodeficiency Virus (HIV)-associated complications. Patients with HIV are at increased risk of having pneumonia and other pulmonary conditions (Huang and Crothers, 2009). Pneumonia is a common problem for many Human Immunodeficiency syndrome virus (HIV) positive people, even for those who have high CD4 cell counts or are responding well to HIV treatment (Afessa and Green, 2000). Opportunistic pneumonias are major causes of morbidity and mortality among HIV-associated pulmonary complications and are frequent reasons for referral to respiratory specialists for diagnostic evaluation and treatment (Huang and Crothers, 2009). A number of opportunistic infections have been associated with HIV which includes bacterial, fungal, viral, and parasitic pneumonias. WHO/UNAID has revealed that there has been an increase in morbidity and mortality worldwide due HIV/AIDS infection (WHO, 2008). A significant proportion of these deaths are due to opportunistic pneumonias. In sub-Saharan Africa it has been estimated that there are about 22 million cases of HIV/AIDS, 5% of which accounts for are associated with adults aged between 15 and 49 (WHO, 2008). Studies have shown that the incidence of bacterial pneumonia among persons with HIV infection is greater than that among persons without HIV (Afessa B and Green, 2000; Hartung *et al*., 2011). *S. pneumoniae* is the most common, followed by *H. influenzae, P. aeruginosa,* and *S. aureus*. Rarely, bacterial pneumonia can be caused by *L pneumophila, M. pneumoniae,* and *C. pneumoniae*.

Pneumocystis jirovecii is another common cause of pneumonia in HIV-positive people with suppressed immune systems (Thomas and Limper, 2004). *P. jirovecii*-associated pneumonia has

been increasingly reported in sub-Saharan Africa where it had previously been thought to be a rare pathogen (Huang *et al*., 2011; Wasserman *et al*., 2013). Before the HIV/AIDS epidemic. Cytomegalovirus (CMV) is the most frequent viral pneumonia seen in persons with HIV infection (Ruuskanen *et al*., 2011). Most disease occurs in CMV-seropositive individuals and disease represents reactivation of latent infection, rather than new infection (Huang and Crothers, 2009). Cytomegalovirus has often been detected at the time of *P. jirovecii*-associated pneumonia diagnosis from HIV-infected adults and without evidence from lung biopsy, it can be difficult to discriminate asymptomatic viral shedding from active disease (Williams *et al*., 2001). In a Malawian study on pneumonia, 27% of an HIV-positive patients were infected with *P. jirovecii* pneumonia, followed by *Mycobacteria tuberculosis* (22%) and the rest had of the patients (29%) had multiple isolates of bacteria (Hartung *et al*., 2011).

2.3 Clinical Presentation of Pneumonia

In most cases pneumonia manifests following a lower respiratory tract infection, and the onset is usually sudden, with half of the cases presenting with a shaking chill (Bradley *et al*., 2011). Temperature rises during the first few hours to 39-40ºC (Peto *et al*., 2014). The pulse accelerates and a sharp pain in the involved hemi thorax is usually observed. The cough is initially dry with pinkish or blood-flecked sputum. Gastrointestinal symptoms such as, anorexia, nausea, vomiting, abdominal pain, diarrhea may be mistaken as acute abdominal inflammation (Peto *et al*., 2014).

2.4 Diagnosis of Pneumonia

2.4.1 Clinical Diagnosis of Pneumonia

Current diagnostic modalities focus on clinical assessments even while attempting to identify the aetiological agent (Feldman *et al*., 2007). Symptoms suggestive of pneumonia include rales, rhonchi, and a dull thud, an indication of presence of consolidation in which the lung becomes filled with fluid and pus, and pleural effusion (Ebell, 2010; Bradley *et al*., 2011). The chest radiograph is the cornerstone in the initial diagnostic evaluation of pneumonia and is recommended in both the inpatient and outpatient settings because it helps in a number of ways such as to confirm the diagnosis, delineate the extent of the consolidation, indicate the presence of underlying disorders and denote the presence of complications (Dalhoff *et al*., 2013). Chest radiographs reveal a lobar distribution and an air space pattern of disease. However, the chest radiographical features of pneumonia are not consistent or characteristic enough to suggest the most likely causative agent (Feldman *et al*., 2007; Black, 2008; Cao *et al*., 2013). Where an X-ray is not available no single clinical sign or symptom can predict the presence of pneumonia, but the most accurate diagnosis is achieved by combining a number of simple clinical features such as cough plus another lower tract symptom, e.g. (dyspnoea, pleuritic pain), new focal chest signs on examination e.g. (bronchial breathing) or one of the following signs sweating, fever, shivers, myalgia, or pyrexia (Dalhoff *et al*., 2013). Most studies which have been undertaken in developed settings lack standardized case definition, most studies undertaken have focused on radiologically confirmed pneumonia (Diederen *et al*., 2009; Johansson *et al*., 2010; Lieberman *et al*., 2010).

2.4.2 Laboratory Diagnosis of Pneumonia

2.4.2.1 Sputum Microscopy

Microscopic screening should be performed as a contingency for acceptability of culture to assess suitability of sputum specimen and to detect the usual pathogens. Sputum specimens are frequently contaminated with oropharyngeal flora and several organisms are capable of either carriage or pathogenicity (Bartlett, 2011). This poses a challenge in determining the real causative agent of pneumonia among the many organisms present in the specimen. The Gram staining technique is usually recommended to be performed on all sputum specimens prior to culturing to ensure that excessively contaminated sputum specimens are identified and to predict the result of culture and assist in the interpretation of the results (Bartlett 2011; Dalhoff *et al*., 2013). When there is no correlation between culture and smear, the culture report may not indicate the aetiology of lower respiratory tract infection (Mariraj *et al*., 2011). However, a positive Gram stain may predict a subsequent positive culture result (Mandell *et al*., 2007).

The Bartlett sputum grading system is the commonly used method employed in the assessment of the quality of sputum. This system assesses the number of epithelial cells and neutrophils per low power field and quantifies the relative number of each type of bacteria seen per high power field (Bartlett, 1974). A good quality sample of sputum sample is defined as one with more than 25 polymorphonuclear cells and less than 10 squamous epithelial cells per Low Power Field (LPF) (Lim *et al*., 2009; Noltes, 2011).

2.4.3.2 Culture of Sputum

Contemporary standards for high-quality microbiological analysis for pneumonia include various components: type of specimen, pathogenic potential of various organisms, concentrations of organisms recovered, and the influence of prior antibiotics (Dalhoff *et al*., 2013). Therefore, it is recommended that cultures be performed on deeply cough–produced sputum specimens obtained before antibiotic treatment and plated within 2 hours of collection or stored at 4ºC to prevent overgrowth of contaminants (Bartlett, 2011). Culture is performed on blood, chocolate, MacConkey and Sabouraud agar plates 37ºC for 18 to 24 hours. Some of the commonly isolated potential pathogens include *S. pneumoniae, S. aureus, S. pyogenes, H. influenzae, Enterobacteriaceae, M. catarrhalis, Pseudomonas species* and fungi (Huijskens *et al*., 2013).

2.4.3.3 Molecular Diagnosis

Nucleic acid amplification techniques such as Polymerase Chain Reaction (PCR) are currently available and are highly sensitive for the detection of nucleic acid sequences from viruses, fungi and bacteria in clinical specimens (Saiki, 1990; Pinar *et al*., 2004; Mustafa *et* al., 2011; Zar *et al*., 2013). Another advantage of PCR over culture method is its ability to identify respiratory pathogens after initiation of antibiotic therapy (Johansson *et al*., 2008). These amplification techniques are particularly advantageous for the detection of fastidious or difficult-to-culture organisms and they have reduced the percentage of unknown cause of pneumonia to under 50% (Wiersinga *et al*., 2012; Huijskens *et al*. 2013; Ho, 2014).

Most of the previous studies with PCR have focused on the detection of single bacteria. PCR was employed in study conducted in Spain in which *Streptococcus pneumoniae* was targeted. *S.*

pneumoniae was detected in in 22 out 40 patients suspected of pneumonia, yielding a sensitivity of 55% (Lorente *et al.,* 2000). In a prospective study conducted in the United States of America PCR revealed *S. pneumoniae* in 44% of patients (Michelow *et al*., 2002). In a study conducted in England in adult patients to detect *S. pneumoniae*, PCR was positive in 31 out of 60 patients, giving a sensitivity of 52% (Smith *et al*., 2009). However, the diagnostic utility of PCR for a single infectious agent is limited, especially for pneumonia patients. Multiplex real-time PCR methods have provided an answer to this since they are rapid and are able to simultaneously detect multiple pathogens in a single clinical specimen. These methods have contributed to proper treatment, avoidance of inappropriate antibiotic administration and reduced the expense of public health concerns (Benson *et al.,* 2008; Mustafa *et al*., 2011; Nomanpour *et al*., 2012).

2.5 Treatment of Pneumonia

Treatment of pneumonia is usually empirical and different options are offered. Individual choice of therapy is best guided by thorough knowledge of commonly encountered pathogens in the region or practice environment and a full appreciation of their usual susceptibility patterns which has a major impact on the prognosis of the patient (Micek *et al*., 2014; Peto *et al*., 2014). In high income settings where incidence of aetiological agents of pneumonia have been well characterized and empirical treatment determined, guidelines for the management of CAP have been developed by different organisations . In the United States the most widely referenced guidelines have been those published by the Infectious Diseases Society of America and the American Thoracic Society (Jackson *et al*., 2004). While the guidelines are mostly applicable to other parts of the world, local antibiotic resistance patterns, drug availability, and variations in

health care systems suggest that modification of these guidelines is prudent for local use (Mandell *et al*., 2007).

Inclusion of antibiotics targeted against atypical pathogens has been recommended in outpatient treatment of CAP but differs between guidelines, mandatory in some and optional in others (Gupta, 2012; Wiersinga *et al*., 2012). Recent recommendations emphasize that in selecting an antimicrobial regimen, history of antibiotic usage in the 3 months prior to initiation of treatment and national/local resistance rates should be taken into consideration (Nair *et al*., 2011). With in-patient treatment, most of the guidelines for treating CAP in hospitalized patients recommend coverage for both typical and atypical pathogens (Wiersinga *et al*., 2012).

According to guidelines for treatment of CAP in the United State, all patients receive treatment for *S. pneumoniae* and atypical organisms because CAP is more often caused by these pathogens (Watkins *et al*. 2011). A macrolide or doxycycline is recommended for previously healthy out-patients with no antibiotic use in past three months. For out-patients with comorbidities or antibiotic use in past three months a respiratory fluoroquinolone e.g. levofloxacin, gemifloxacin, or moxifloxacin, or a β-lactam antibiotic such as high-dose amoxicillin, amoxicillin/clavulanate, or cefpodoxime plus a macrolide are recommended. A respiratory fluoroquinolone, or a β-lactam antibiotic plus a macrolide are recommended for inpatients and β-lactam antibiotic e.g. ceftriaxone, cefotaxime, or ampicillin/sulbactam, plus azithromycin or a respiratory fluoroquinolone for inpatients, in the intensive care unit (Zar *et al*., 2013).

In South Africa, a middle income country, no data is available from high quality randomised controlled trials to provide optimal antimicrobial treatment guidelines. However, observational studies conducted recommended the use of combined antimicrobial therapy a β-lactam and a macrolide (Zar *et al*., 2013). Patients below the age of 65 years without comorbid illness are treated with a high-dose of oral amoxicillin, while those aged 65 years and above and adults with comorbidities, such as those with HIV infection, are usually treated with amoxycillin-clavulanate or selected oral cephalosporins such as cefuroxime or cefpodoxime (Zar *et al*., 2013). In order to limit the development of resistance, fluoroquinolones are not used as routine first-line therapy. However, fluoroquinolones with extended Gram-positive coverage, i.e. moxifloxacin and gemifloxacin, are the preferred agents because of their superior microbiological efficacy against *S. pneumoniae*. For macrolide/azalide-resistant *S. pneumoniae* or patients with severe allergy to standard β-lactam agents, telithromycin is recommended (Feldman *et al*., 2007).

It is important to note that much of the available data on treatment of pneumonia are from high income countries. Therefore, there is an urgent need to expand research and newer treatment regimens in low-income countries such as Zambia. Currently recommended drugs for empirical therapy of patients suspected with pneumonia in Zambia include benzyl penicillin administered intravenously for 5 days in adults and in children it is administered intramuscularly in 4 divided doses for 7 days as soon as symptoms subside and respiratory rates are controlled change to oral medication. Recommended oral medication include, amoxycillin, ceftriaxone (for those allergic to penicillin) and erythromycin as outlined in the Standard Treatment Guidelines, 2013.

2.6 Antimicrobial Drug Resistance

It is clearly evident that antibiotic resistance is highly prevalent and increasing among pathogens causing pneumonia and that antibiotic-prescribing habits have changed as a consequence of perceived problems with resistance (Feldman 2004; Mandell *et al*. 2007; Lynch *et al*., 2009; Zar *et al*., 2013). *S. pneumoniae* has developed resistance to a myriad of antibiotics, including the penicillins and cephalosporins, macrolides, and fluoroquinolones, leading to the risk of emergence of multidrug resistant strains (Feldman, 2004; Lynch *et al*., 2009; Song *et al*., 2010). The mechanisms of β-lactam resistance in *S. pneumoniae* infection are principally related to alterations in the penicillin binding proteins (Lynch *et al*., 2009). Macrolide resistant strains employs two different mechanisms specifically, low-level resistance which is due to an active efflux pump mechanism (*mef(A)* gene), and high-level resistance due to a ribosomal methylation mechanism affecting the macrolide binding site (*erm(B)* gene) (Livermore 2002; Lynch *et al*., 2009; Song *et al*., 2010). Some strains of *S. pneumoniae* with this type of resistance mechanisms have been observed in different parts of the world, and in some areas, such as South Africa, in certain cases these mechanisms do coexist, translating overall into high-level resistance (Zar *et al*., 2013).

Fluoroquinolones inhibit bacterial DNA synthesis by interacting with the intracellular drug target DNA gyrase and the enzymes topoisomerase IV which are involved in DNA replication (Lynch *et al*., 2009). Resistance develops by mutations occurring in the quinolone resistance gene determining region, involving *gyrA* and *parC*. However, two different levels of resistance have been observed with Fluoroquinolones thus, low and high level resistances. In the case of low-level resistance, mutations most commonly take place in *parC*, and isolates are susceptible to the

newer fluoroquinolones, whereas in high-level resistance are seen in dual mutations affecting both *parC* and *gyrA* (Lynch *et al*., 2009).

In the presence of high-level resistance with dual mutations, treatment failures with fluoroquinolones have been reported (Lynch *et al*., 2009; Fuller *et al*., 2005). Resistance of the pneumococcus to fluoroquinolones is currently very uncommon but is increasing worldwide (Lynch *et al*., 2009; Song *et al*., 2010).

Resistance of *H. influenzae* and *M. catarrhalis* is commonly due to emergence of β-lactamase production. Two types of β-lactamase resistant genes have been observed with *H. influenzae* either TEM-1 or ROB-1. ROB-1 is novel β-lactamase type (Tristram *et al*., 2007; Feldman *et al*., 2012). In case of *M. catarrhalis* the β-lactamases are most commonly those termed BRO-1, BRO-2, or BRO-3 (Hoban *et al*., 2002; Livermore, 2002).

Methicillin Resistant *Staphylococcus aureus* (MRSA) are uniformly resistant to all available penicillins and other β-lactam agents such as oxacillin, and amoxicillin. MRSA infections have become common in United States of America, accounting for greater than 50% of staphylococcal infections in the outpatient setting (Cataldo *et al*., 2010). In the community, most MRSA infections are skin and pneumonia infections. More severe or potentially life-threatening MRSA infections occur most frequently among patients in healthcare settings (Dalhoff *et al*., 2013). While 25% to 30% of people are colonized in the nose with *Staphylococcus*, less than 2% are colonized with MRSA (Gorwitz *et al*., 2008).

Klebsiella pneumoniae and *E. coli*, over several years have shown a progressive increase in antimicrobial resistance, including the presence of extended spectrum β-lactamase (ESBL) production (Livermore *et al*., 2001; Paterson *et al*., 2004; Dalhoff *et al*., 2013). ESBL are enzymes that mediate resistance to extended-spectrum third generation cephalosporins such as ceftazidime, cefotaxime, and ceftriaxone, and monobactams e.g., aztreonam but do not affect cephamycins e.g., cefoxitin and cefotetan or carbapenems e.g., meropenem or imipenem (Bonnet, 2004; Dalhoff *et al*., 2013). The presence of an ESBL-producing organism in a clinical infection can result in treatment failure if one of the above classes of drugs is used. ESBLs can be difficult to detect because they have different levels of activity against various cephalosporins (Livermore *et al*., 2001). Pneumonia due to *K. pneumoniae* and *E. coli* have been noted in patients with HAP, as well as those with community-acquired infections, particularly in certain areas of the world (Marques *et al*., 2010; Song *et al*. 2011).

In a study conducted in Beijing, China, a total of 63 isolates of *S. pneumoniae* were submitted for antimicrobial susceptibility testing and the proportions of intermediately or fully resistant isolates to penicillin were 19.0% and 3.2%, respectively. The prevalence of isolates considered non-susceptible to azithromycin and fluroquinolones were 79.4% and 6.3%, respectively. Of the 56 isolates of *H. influenzae* tested, 8.4% produced β-lactamase and 11.9% were non-susceptible to ampicillin (Liu *et al*., 2009). In a Hungarian study, remarkably low (<2%) penicillin resistance rates were observed in an epidemiological survey on *S. pneumoniae* isolates (Dobay *et al*, 2008). The other β-lactam antibiotics showed very good efficacy of 97–99% sensitivity. On the contrary, the macrolide resistance of the isolates was high (40%) and clindamycin resistance

recorded at 30%. No resistance was observed with quinupristin-dalfopristin and fluoroquinolones.

In an Indian study where a total of 126 clinical isolates of *S. pneumoniae* were tested, 5%, 20% and 23% of the isolates exhibited resistance to penicillin, erythromycin and ciprofloxacin, respectively (Shariff *et al*., 2013). A further study conducted to determine the serotype distribution and susceptibility patterns of *S. pneumoniae* in eastern India, revealed that 77.1% were resistant to at least one antibiotic and those strains that were resistant to penicillin belonged to type 8 (25%) (Devi *et al*., 2012). None of the *S. pneumoniae* tested were resistant to vancomycin, while 94.2% and 96.2% were resistant to cefotaxime and ceftriaxone, respectively. Resistance to erythromycin was 33.3%, followed by cotrimoxazole (44.0%), tetracycline (38.7%), clindamycin (18.18%), and levofloxacin (4.7%) (Yasin *et al*, 2011).

A study to provide an overview on the prevalence of antibiotic resistance among *H. influenzae* isolates obtained from pulmonary outpatient departments of 13 hospitals in the Netherlands between 2005 and 2010 showed that resistance to amoxicillin fluctuated between 22% and 33% during the study period (Geelen *et al*., 2013). Amoxillin-cluvanate resistance varied between 2.3% in 2007 and 8.6% in 2009. There were also no significant changes observed in the prevalence of resistance to amoxillin-cluvanate. Approximately 11% of the *H. influenzae* isolates were β-lactamase-positive and also amoxicillin-resistant and amoxillin-cluvanate susceptible. Overall approximately 5% of the isolates were β-lactamase-negative, amoxicillin-resistant, and amoxillin-cluvanate resistant. Doxycycline resistance fluctuated markedly during the study period between 46% in 2008 and 10% in 2006 (Geelen *et al*, 2013).

A Nigerian study designed to reveal susceptibility patterns of bacterial isolates showed that fluoroquinolones (ofloxacin and ciprofloxacin), β-lactams (amoxicillin-clavulanate, cefuroxime, cetazidime, ceftriaxone), and gentamicin had moderate to high activity, while all the bacteria were resistant to cotrimoxazole, tetracycline, erythromycin, and cloxacillin (Egbe *et al*., 2011).

In Zambia, there is very little data on drug resistance patterns of bacterial pathogens, excluding *Mycobacterium tuberculosis*, isolated from sputum in adult patients suspected of pneumonia despite laboratory records suggesting existence of multi-drug resistant organisms.

Chapter 3

Materials and Methods

3.0 Materials and Methods

3.1 Study Design

This was a laboratory-based cross-sectional study using sputum samples collected from adult patients suspected of pneumonia.

3.2 Study Site

This study was carried out at the University Teaching Hospital (UTH). The UTH is located in Lusaka, the Capital City of Zambia and has a bed capacity of approximately 2000 and comprises several clinics and admission wards. The Bacteriology Laboratory at the hospital is the reference centre for all microbiology work in Zambia and is actively involved in research, diagnosis and disease outbreak surveillance of bacterial pathogens of national importance.

3.3 Sampling Frame

Using a convenient sampling approach, all sputum samples from adult patients suspected of having pneumonia were screened for inclusion by checking for demographic and clinical details on laboratory request forms. Three hundred and twelve sputum samples from adult patients at UTH submitted to the Bacteriology Laboratory for routine bacteriological examination were analysed prospectively. These samples were collected from March 2014 to August 2014.

3.3.1 Inclusion Criteria

Inclusion criteria were 18 years and above. Only one sample of expectorated sputum from each patient was examined with a clinical diagnosis of pneumonia. In this study pneumonia was defined as least one symptom of cough, sputum production, breathlessness, chest pain, and haemoptysis, and an abnormality on a chest radiograph consistent with pneumonia. The assessment of pneumonia was based on clinical suspicion by attending physicians.

3.3.2 Exclusion Criteria

Sputum specimens collected from patients younger than 18 years of age or those samples without clinical and demographic data on laboratory request forms were excluded from the study. Patients with co-morbidities such as lung cancer were also excluded.

3.4 Sample Size

Approximately 22% of pneumonia cases are reported at the University Teaching Hospital (Kasali, 2006). In order to estimate the prevalence within 5% (or 0.05) and considering a 95% confidence level, a minimum sample size of 264 will be used as obtained from the formula above.

$n = \frac{z^2 p(1-p)}{e^2}$, where z= 95% confidence interval level (or 1.96), $p = 22\%$ (or 0.22) is the sample proportion, and e = standard error of the proportion, $e^2 = (0.05)^2 \approx 0.0025$.

Therefore, n = ~264.

3.5 Sampling Technique

Convenience sampling was applied in the study because it is relatively easy and inexpensive to conduct. Only sputum samples from adult patients suspected of having pneumonia with clear clinical and demographic data submitted to the Bacteriology Laboratory during the study period were included in the analysis.

3.6 Determination of the Quality of Sputum Specimens using the Bartlett Score

Gram-stained smears were made from most visually purulent portions of each sputum specimen. The quality of sputum was assessed by determining the numbers of squamous epithelial cells (SECs) and polymorphomonuclear cells (PMN) in Gram-stained smear of the specimen (Bartlett 1974; Murray and Washington, 1975). The quality of specimen was assessed by determining the number of SECs and PMNs within the following categories: <10, 10-25, or >25 cells per representative (100x) low power fields (LPF). The presence of PMN was graded as +1 and +2, whereas SECs were graded as -1 and -2 after observing a minimum of 20 LPF. The scores were added and the specimens with zero or less scores were classified as being of poor quality (Baron, 2015). The smears were interpreted as indicated below (Table 3.1).

Table 3.1: Bartlett Grading System

Number of neutrophils per LPF (x10 objective)	**Grade**
< 10	0
10 – 25	+1
>25	+2
Presence of mucus	+1
Number of epithelial cells per LPF (x10 objective)	**Grade**
< 10	0
10 – 25	-1
>25	-2
Bartlett score (Total Score)	

LPF, Low Power Field. Adapted from Bartlett (1974)

An attempt was also made to predict the morphology of bacteria in sputum by assessing their morphology and quantity. This helps in selecting the appropriate identification methods and assessing the severity of infection. This was achieved by microscopy under high power field (HPF) after staining with Gram stain. Microorganisms seen in smear under HPF (1000x) were described according to the classic Gram stain morphotypes: Gram negative rods (GNR), Gram positive cocci in clusters (GPC clusters), Gram negative coccobacilli (GNCB), Gram positive in chains (GPC in chains), Gram negative diplococci (GNDC), Gram positive cocci single cells (GPC singles), Gram negative cocci (GNC), Gram positive rods (GPR), Gram positive diplococci (GPDC) and yeast cells. The quantification of the bacteria on smears was achieved as follows: 1, scanty; 2-9, 1+; 10-99, 2+; and ≥100, 3+.

3.7 Identification of Potential Pathogens in Sputum

3.7.1 Phenotypic Identification of Potential Pathogens

Demonstration of the presence of potential pathogenic bacteria in sputum is vital in helping to confirm cases of suspected pneumonia. The isolation and identification of bacteria were carried out by standard cultural and biochemical methods for the suspected bacteria (Barrow and Feltham, 2003). The most purulent portion of each specimens was inoculated on to sheep blood, MacConkey, Sabouraud's and chocolate agar plates, streaked out using a standard four quandrant streaking method, and incubated at 37ºC for 24-48 hours. Cultures were examined at 24 hours and 48 hours interval, and predominant organisms were identified according to the furthest quadrant with visible colonies (First quadrant = scanty; second quadrant = 1+; third quadrant = 2+; fourth quadrant = 3+) (Figure 2.1).

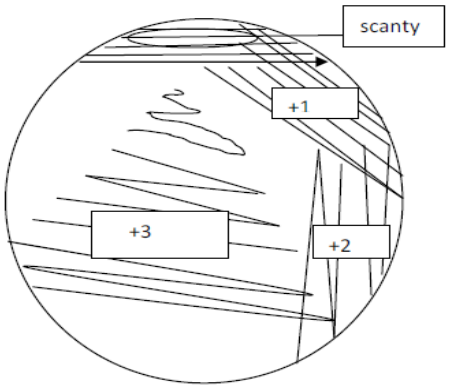

Figure 3.1: Assessment of the relative quantity of bacterial growth on an agar plate using the four quadrant streaking method. Scanty, growth in the first quadrant; 1^+, growth in the second quadrant; 2^+, growth in the third quadrant; and 3^+, growth in the fourth quadrant.

Background mixed oropharyngeal flora (including viridans streptococci, commensal *Neisseria*, coagulase-negative staphylococci, yeasts (except *Cryptococcus*), diphtheroids and *Capnocytophaga* were quantified as a group, but not identified further.

For the isolation of *Mycobacterium tuberculosis*, sputum samples were processed by the standard N-acetyl- L-cysteine (NALC)-NaOH method and concentrated at $3000 \times g$ for 15 min. The sediment was reconstituted to 2.5 ml with phosphate buffer (pH 6.8) to make suspensions for the smears and cultures. The samples were then cultured on the BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson Microbiology systems, Sparks, MD, USA) according to the manufacturers' recommendations. The cultures were monitored for 6 weeks until they were positive for growth. The isolates from MGIT 960 tubes were confirmed to be *M. tuberculosis* by microscopic observation for serpentine cord formation after Ziehl-Neelsen staining, and the Capilia TB assay (TAUNS, Japan) according to the manufacturer's instructions. Contamination of cultures was ruled out by inoculating samples from the MGIT machine onto blood agar plates.

3.7.2 Molecular Detection of Pathogens in Sputum

3.7.2.1 Nucleic Acid Extraction

Before DNA extraction, sputum samples were first subjected to digestion with freshly prepared 0.1% Sputasol (Oxoid Ltd, Cambridge, UK) in a 1:1 ratio at room temperature until completely dissolved. DNA was extracted on the easyMag instrument (bioMérieux, Marcy I'Etoile, France) according to the manufacturer's instructions using the "on-board lysis" protocol. DNA was eluted in a final volume of 110µl. The concentration of DNA was estimated by ultraviolet spectroscopy at 260nm. A DNA sample with an optical density (OD) of 1 at 260nm corresponds to a DNA concentration of 50µg/ml of double-stranded DNA. The purity of the DNA was determined by a DNA/protein absorbance ratio of 260nm/280nm. The DNA was then stored at -20^{o}C until required.

3.7.2.2 Detection of Respiratory Pathogens by Multiplex PCR

To detect the respiratory pathogens, a commercial multiplex PCR kit, FTD Respiratory Pathogens 33 Kit (Fast Track Diagnostics, Junglinster, Luxembourg) was used. This kit utilises an 8-tube multiplex PCR technique for the simultaneous detection and identification of the following respiratory organisms: influenza A, H1N1, influenza B, rhinovirus, coronavirus NL63, 229E, OC43, HKU1, parainfluenza 1, 2, 3, 4, human metapneumovirus A/B, bocavirus, *Mycoplasma pneumoniae*, respiratory syncytial virus A/B, adenovirus, enterovirus, parechovirus, *Chlamydia pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, cytomegalovirus, influenza C, *Pneumocystis jirovecii*, *Haemophilus influenzae* type B, *Bordetella* species (except *Bordetella parapertussis*), *Moraxella catarrhalis*, *Klebsiella pneumoniae*, *Legionella* species and *Salmonella* species. An AgPath-ID One-Step enzyme (Invitrogen, Karlsruhe, Germany) was used in the PCR reaction on an Applied Biosystems 7500 Real-Time PCR cycler (Life Technologies, California, USA). Thermocycling conditions were as follows: 50°C for 15 min and 95°C for 10 min, followed by 40 cycles of 95°C for 8s and 60°C for 35s.

3.8 Determination of the Antibiotic Susceptibility Patterns of Bacterial Isolates

There is an increasing demand on the clinical laboratory to determine the antibiotic susceptibility or resistance of various pathogenic bacteria. This helps in guiding physicians to determine antimicrobial agents that are effective in treating the infections caused by the bacteria being tested. The antimicrobial susceptibility testing was carried using the Kirby-Bauer disc diffusion method using the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). Briefly, one to two isolated colonies from freshly streaked plates were suspended in 1ml saline,

adjusted to a 0.5 McFarland standard and spread evenly with a sterile cotton swab onto a Müeller-Hinton agar (Oxoid Ltd, Cambridge, UK) plate surface. After the surface of the agar plate had dried for about 5 minutes, antibiotic disks were applied onto the inoculated plate and incubated for 16 to 24 hours at 37°C. For testing *H. influenzae isolates, Haemophilus* Test Medium (HTM) (Oxoid Ltd, Cambridge, UK) was used, while Müeller Hinton agar (Oxoid Ltd, Cambridge, UK), supplemented with 5% sheep blood, was used for testing *S. pneumoniae*. The zones of inhibition were measured using a Vernier calliper and end points determined based on the areas showing no bacterial growth visible. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as controls. Results were interpreted according to the CLSI guidelines (CLSI, 2012). The 16 antimicrobials tested included amoxicillin/clavulanic Acid, ampicillin, ciprofloxacillin, chloramphenicol, tetracycline, cefotaxime, co-trimoxazole, penicillin, gentamicin, ceftazidime, imipenem, erythromycin, clindamycin, vancomycin, oxacillin, and amoxycillin-cluvanic acid.

Bacterial isolates suspected to be ESBL producers were subjected to a confirmatory test using the combination disc method. This test was carried out by spreading a standardised inoculum on Mueller-Hinton agar (Mast diagnostics, Mast group Ltd, Merseyside, UK) and placing an indicator cephalosporin and a cephalosporin/clavulanic acid combination disc on the same plate (e.g. cefotaxime and cefotaxime/clavulanic acid). After incubation for 18-24 hours at 37^{o}C, the zones of inhibition of the indicator cephalosporin and cephalosporin/clavulanic acid were measured using vernier callipers and compared. Confirmation of ESBL production was indicated by the zone size of the cephalosporin/clavulanic acid being greater than the indicator cephalosporin (i.e., ≥5mm).

3.9 Data Analysis

Raw data were entered and cleaned in Microsoft excel and exported to SPSS version 16.0 Software (IBM, Armonk, New York, USA) for final coding and analysis. Frequency and percentages distribution were generated to describe the relative proportions of relevant variables. The outcome variable was defined as sputum positive for pneumonia associated pathogen (s) by PCR and/ or culture and stratified according to demographic data. Data were presented in tables and graphs. Chi square and odds ratios were calculated to ascertain association between patients' demographic characteristics and the outcome. A *p*-value at a level of 0.05 considered statistically significant.

3.10 Ethics Considerations

This study was a laboratory-based study, with no direct contact with patients. Permission to use patients' data and sputum samples and stored bacterial isolates was obtained from the UTH Management. To maintain patient confidentiality, all specimens were assigned study numbers. Ethics clearance for the study was sought from the University of Zambia Biomedical Research and Ethics Committee (UNZABREC). The ethics clearance certificate reference number was 008-05-14 (Appendix 1).

Chapter 4

Results

4.0 Results

4.1 Determination of the Quality of Sputum Samples using the Bartlett Score

4.1 Demographic Information of Patients

A total of 312 sputum samples from adult patients clinically suspected to have pneumonia at UTH were selected and included in this study. Of these, 51.9% (162/312) of the samples were obtained from male patients, while 48.1% (150/312) were obtained from female patients. The median age of the patients was 38 years (interquartile range: 18 years) (Table 4.1).

Table 4.1. Demographic characteristics of study patients (n=312)

Variables	Frequency (Percentage)	Variables	Frequency (Percentage)
Gender			
Male	162 (51.9%)		
Female	150 (48.1%)		
Age			
18-34yrs	134 (42.9%)		
35-44yrs	83 (26.6%)		
45-54yrs	43 (13.8%)		
>54yrs	52 (16.7%)		

4.2 Determination of the Quality of Sputum Specimens

Three hundred and twelve sputum samples were analysed, and out of these 52.6% (164/312) were of good quality, and while 47.4% (148/312) were of poor quality. Overall, these high quality samples had about 49.4% >25 PMNs per LPF, indicating the presence of infection (Table 4.2).

Table 4.2: Comparison of neutrophil and epithelial cell quantity in sputum samples from adult patients with pneumonia

	Number of neutrophils per LPF							
	<10		**10-25**		**>25**		**All**	
	N	%	N	%	N	%	N	%
Number epithelial cells per LPF								
<10	59	18.9	8	2.6	97	31.1	164	52.6
10-25	13	4.2	1	0.3	20	6.4	34	10.9
>25	66	21.2	11	3.5	37	11.9	114	36.5
All	138	44.2	20	6.4	154	49.4	312	100

Percentages represent specimens among cases in whom sputum was collected and had available culture results (N=312). Abbreviations: LPF, low power field

Bacteria of various morphotypes were detected on primary Gram stain done directly on the sputum samples. Six different bacterial morphotypes were identified, including yeasts cells, in both good and poor sputum samples (Table 4.3). Gram negative rods (GNR) and Gram positive cocci (GPC) were the commonly seen morphotypes.

Table 4.3: Bacterial morphotypes detected in sputum samples.

	Morphotype						
Quality of Sputum	GNR	GPC	GNCB	GPDC	GPR	GNC	Y
Good	49 (41.5%)	96 (42.7%)	3(75%)	4(80%)	1(50%)	16(45.7%)	12(41.4%)
Poor	69 (58.5%)	129 (57.3%)	1(25%)	1(20%)	1(50%)	19(54.3%)	17(58.6%)
Total	118(100%)	225 (100%)	4(100%)	5(100%)	2(100%)	35(100%)	29(100%)

GNR, Gram negative rods; GNCB, Gram negative coccobacilli; GPC, Gram positive Cocci; GNC; Gram negative cocci; GPR, Gram positive rods; GPDC; Gram positive diplococcic; Y, Yeast cells.

Comparison of the Gram stain with culture results revealed concordance in 26.8% (44/164) of the good quality specimens and 24.3% (36/148) of the poor quality specimens (Table 4.4).

Table 4.4: Concordance between sputum Gram stain and sputum culture in 312 samples

		Sputum Gram Stain concordant with culture	
Gram stain indicative of infection		Yes	No
Good quality (164)			
Yes	52.6% (164/312)	26.8% (44/164)	73.2% (120/164)
Poor quality (148)			
Yes	47.4% (148/312)	24.3% (112/148)	75.7% (112/148)
Total	312		

There was no association between culture positivity and quality of sputum (*p*-value 0.491).

4.3 Identification Potential Pathogens Present in Sputum Specimens

4.3.1 Phenotypic Identification of Potential Pathogens by Culture Methods

Out of the 312 sputum samples analysed, only 56.1% (175/312) yielded bacteria or yeast cells while 43.9% (137/312) had no significant growth. A total of 226 individual isolates, comprising 11 different bacterial species and 1 fungal species, were identified. These included the following microorganisms: *Moraxella catarrhalis* (20.8%, 47/226), *Pseudomonas aeruginosa* (19.9%, 45/226), *Mycobacterium tuberculosis* (16.8%, 38/226), *Candida albicans* (16.4%, 37/226), *Klebsiella pneumoniae* (14.2%, 32/226), *Staphylococcus aureus* (4.4%, 10/226), *Enterobacter cloacae* (1.8%, 4/226), *Klebsiella oxytoca* (1.8%, 4/226), *Haemophilus influenzae* (1.3%, 3/226), *Escherichia coli* (0.9%, 2/226), *Citrobacter diversus* (0.9%, 2/226) and *Streptococcus*

pneumoniae (0.9%, 2/226) (Figure 3.1). There was no statistical significance difference in culture positivity with respect to sex (p=0.181).

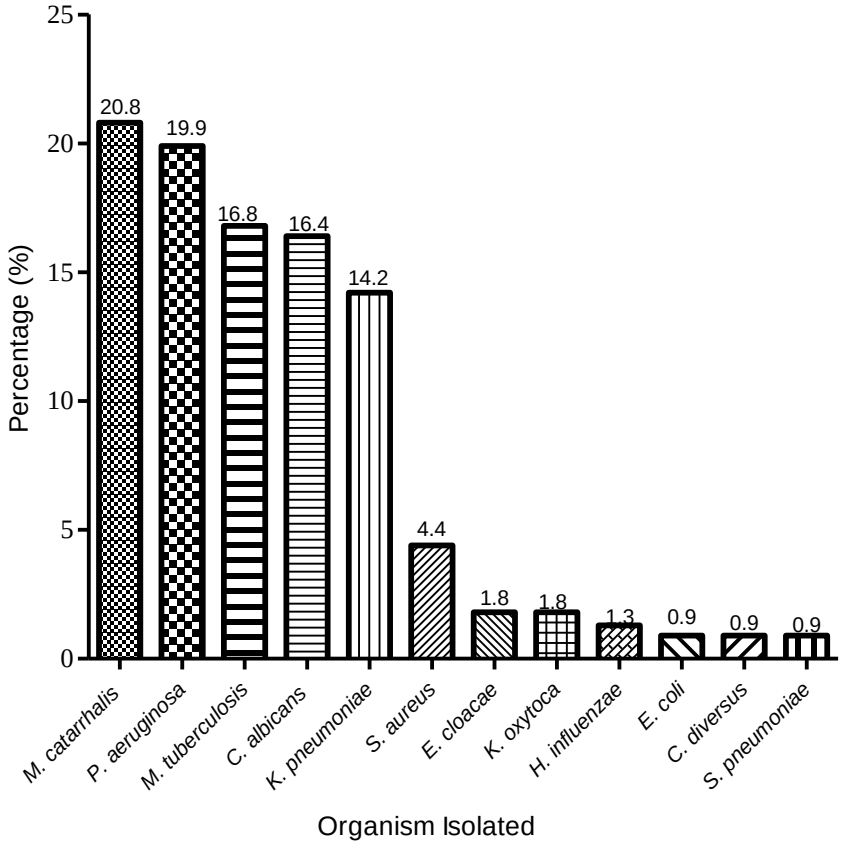

Figure 4.1 Range of microorganisms isolated from sputum.

4.3.2 Detection of Respiratory Pathogens by Multiplex PCR

Of the 312 sputum samples only 146 samples were available for PCR analysis, and the following group of organisms were detected: 79.5% (116/146) bacterial agents, 52.7% (77/146) viral agents and 4.8% (7/146) fungal agents.

There were 181 total single organisms detected from the specimens. The distribution for the organisms was as follows: Cytomegalovirus (24.3%, 44/181), *K. pneumoniae* (17.7%, 32/181),

H. influenzae non-type B (16.0%, 29/181), *S. aureus* (9.4%, 17/181), *S. pneumoniae* (9.4%, 17/181), *H. influenzae* type B (6.1% ,11/181), Rhinovirus (5.5%, 10/181), *M. catarrhalis* (4.4%, 8/181), *Pneumocystis jirovecii* (3.9%, 7/181), Respiratory Syncytial Virus (RSV) A/B (3.9%, 7/181), Adenovirus (1.7%, 3/181), Human bocavirus (1.1%, 2/181), Human metapneumoviruses A/B (1.1%, 2/181), Parainfluenzae type 1 (1.1%, 2/181), Parainfluenzae type 2 (1.1%, 2/181), Parainfluenzae type 4 (1.1%, 2/181), *Salmonella species* (0.6%, 1/181), *Mycoplasma pneumoniae* (0.6%, 1/181), Influenza virus type B (0.6%, 1/181), Human coronavirus 63 (0.6%, 1/181), and Parainfluenzae type 2 (0.6%, 1/181) (Figure 4.2).

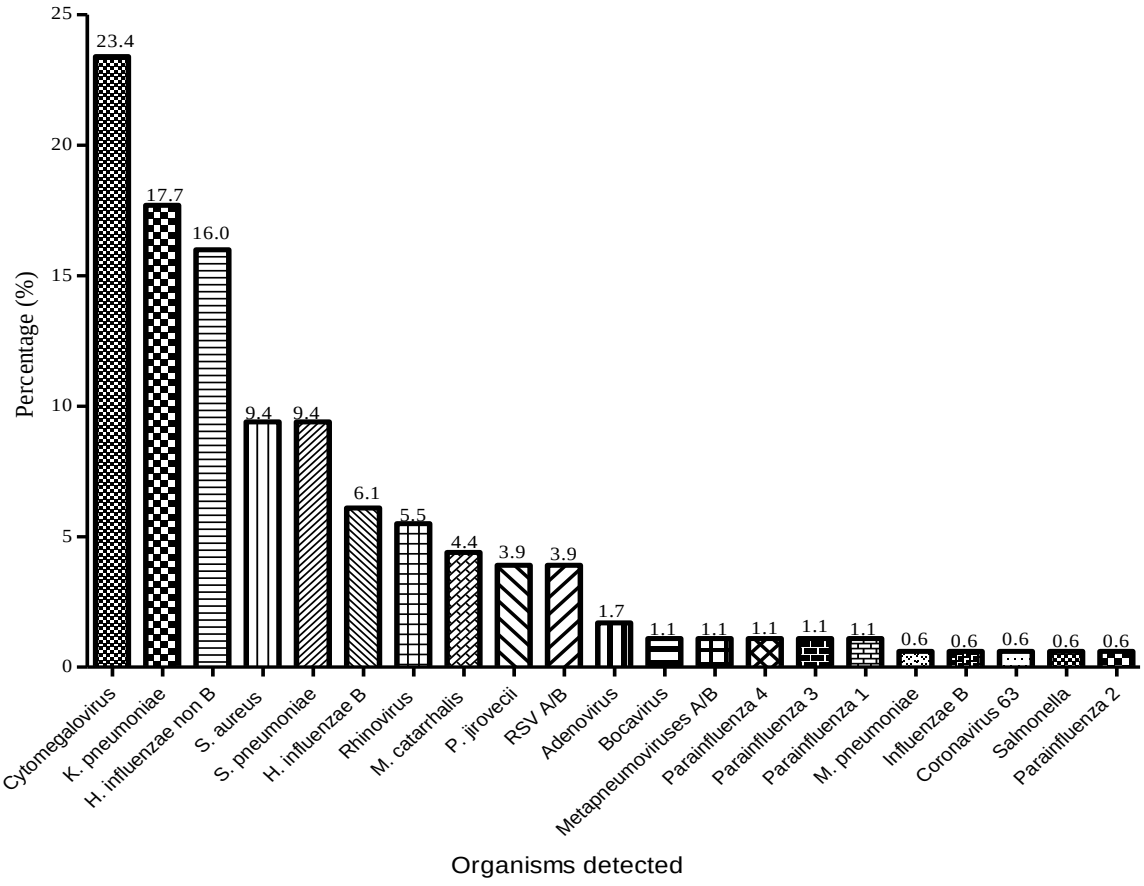

Figure 4.2: Distribution of organisms detected by PCR from sputum.

Forty-four of the 116 PCR positive specimens (79.5%) harboured more than one type of organism (coinfections) (Table 3.4). About 29.5% (13/44) of these specimens harboured *K. pneumoniae* and *H. influenzae* non-type B. The majority of the specimens had three to five different organisms (Table 3.4). More than half of the specimens (56.8%, 25/44) harboured both bacteria and viruses, followed by those with bacteria only (27.3%, 12/44), bacteria and fungi only (9.1%, 4/44) and viruses only (4.5%, 2/44).

Table 4.5: Mixed aetiological agents of pneumonia detected in sputum samples using PCR

Mixed isolates	**Frequency (%)**
K .pneumoniae + H. influenzae non type B	13(8.9)
Cytomegalovirus + *K. pneumoniae*	7(4.8)
S. pneumoniae + K .pneumoniae	7(4.8)
Cytomegalovirus + *H. influenza non type B*	6(4.1)
S. aureus+ H. influenzae non type B	6(4.1)
S. pneumoniae + H. influenza non type B	5(3.4)
H. influenza non type B+ H. influenza type B	4(2.7)
Cytomegalovirus+ *S. aureus*	4(2.7)
S. pneumoniae + K. pneumoniae+ H. influenza non type B	4(2.7)
S. aureus+ P. jiroveci	3(2.1)
Cytomegalovirus+ Respiratory syncytial virus A/B	3(2.1)
Cytomegalovirus + *M. catarrhalis*	3(2.1)
Rhinovirus + *S. aureus*	2(1.4)
S .pneumoniae + H. influenza type B	2(1.4)
Cytomegalovirus + *S. pneumoniae*	2(1.4)
Cytomegalovirus+ Rhinovirus+ *K .pneumoniae*	2(1.4)
S. pneumoniae+ S. aureus	2(1.4)
Cytomegalovirus + Adenoviruses + *H. influenza non type B*	2(1.4)
S. pneumoniae +S. aureus+ P. jiroveci	1(0.7)
Cytomegalovirus+ Coronavirus *63+K.pneumoniae*	1(0.7)
Cytomegalovirus+ Rhinovirus + Parainfluenza 3	1(0.7)
Respiratory syncytial virus A/B + *K. pneumoniae+ H. influenza non type B*	1(0.7)
Metapneumoviruses A/B+ *H. influenza type B*+ Parainfluenza 1	1(0.7)
Influenza type B+ K. pneumoniae	1(0.7)
K .pneumoniae+ P. jiroveci	1(0.7)
K .pneumoniae + Parainfluenza 1	1(0.7)
S. pneumoniae + K. pneumoniae + S. aureus	1(0.7)
K. pneumoniae + Parainfluenza 3	1(0.7)
Cytomegalovirus + *K. pneumoniae + H. influenza non type B*	1(0.7)
Cytomegalovirus+ Parainfluenza 2	1(0.7)
K. pneumoniae+ H. influenza non type B+ H. influenza type B	1(0.7)
Cytomegalovirus + Bocavirus + *K .pneumoniae+ S. aureus*	1(0.7)
Cytomegalovirus+ Rhinovirus+ *S. pneumoniae*	1(0.7)
Cytomegalovirus+ Adenoviruses+ *S. aureus + H. influenzae non type B+ M. catarrhalis*	1(0.7)
H. influenzae non type B + H. influenzae type B + M. catarrhalis	1(0.7)
K. pneumoniae + Salmonella	1(0.7)
Adenoviruses + *M. pneumoniae+ S. aureus+ H. influenza non type B+ H. influenza type B*	1(0.7)
Cytomegalovirus+ Bocavirus+ *M. catarrhalis*	1(0.7)
K .pneumoniae + S. aureus + H. influenza non type B	1(0.7)
S. pneumoniae + M. catarrhalis	1(0.7)
Cytomegalovirus + *S. pneumoniae + H. influenza non type B*+ Parainfluenzae 4	1(0.7)
Respiratory syncytial virus A/B + *S. aureus + M. catarrhalis*	1(0.7)
Cytomegalovirus+ Rhinovirus+ *S. aureus+ H. influenza non type B+ P. jiroveci*	1(0.7)
K. pneumoniae + H. influenza non type B+ P. jiroveci	1(0.7)

4.3.3 Comparison Between PCR and Culture Methods

An attempt was made to determine the congruence between PCR and culture methods in the identification of bacteria in the 146 specimens subjected to PCR. As observed in Table 3.6 below, the PCR technique detected more bacteria than the culture method.

Table 4.6: Comparison between PCR and Culture methods

Organism	**PCR positive n (%)**	**Culture positive n (%)**
K. pneumoniae	32(21.9)	17(11.6)
H. influenzae	29(19.9)	2(1.4)
S. aureus	17(11.6)	6(4.1)
S. pneumoniae	17(11.6)	1(0.7)
H. influenzae B	11(7.5)	0(0.0)
M. catarrhalis	8(5.5)	5(3.4)
Salmonella species	1(0.7)	0(0.0)

4.4 Determination of the Antibiotic Susceptibility Patterns of Bacterial Isolates

The antibiotics used in the antibiotic susceptibility assay were selected based on the antibiotic list used at the UTH. Out of the bacterial isolates, only *S. pneumoniae* was sensitive to almost all the antibiotics used. It was only resistant to tetracycline (50%) but 100% sensitive to chloramphenicol, tetracycline, cefotaxime, co-trimoxazole, penicillin and erythromycin. For the other bacteria, the following were their antibiotic resistance patterns: *P. aeruginosa* exhibited resistance patterns to gentamicin (51%), ceftazidime (33%), ciprofloxacin (27%), and imipenem (18%). All *K. pneumoniae* isolates exhibited 100% resistant to ampicillin, followed by co-trimoxazole (66%), cefotaxime (53%), chloramphenicol (50%), ciprofloxacin (47%), ceftazidime (44%) and imipenem (6%); *S. aureus* was resistant to ciprofloxacin (90%), penicillin (90%), tetracycline (80%), co-trimoxazole (80%), gentamicin (70%), erythromycin (70%), and

vancomycin (20%); *H. influenzae* showed resistance to co-trimoxazole (100%), tetracycline (100%), cefotaxime (67%), chloramphenicol (33%) and ciprofloxacin (33%); *K. oxytoca* was resistant to co-trimoxazole (100%), gentamicin (75%), ciprofloxacin (50%), chloramphenicol (50%), cefotaxime (50%) and ceftazidime (50%). The isolates were, however, sensitive to imipenem (100%); *E. cloacae* was 50% resistant to ciprofloxacin, chloramphenicol, cefotaxime, co-trimoxazole and ceftazidime, but showed low resistance to imipenem (25%). *E. coli* was 100% resistant to ampicillin, gentamycin, cefotaxime and ceftazidime and 50% resistant to ciprofloxacin. The isolates were, however, sensitive to imipenem (100%). All these data are summarised in Table 3.7 below.

Table 4.7: Antimicrobial resistance profile of bacterial isolates from sputum cultures collected from adult patients at UTH

Bacterial Isolates	**Total No.**	**Resistance profile of antimicrobial agents (R %)**														
		AML	**CIP**	**CH**	**TET**	**CTX**	**SXT**	**P**	**CN**	**CAZ**	**IPM**	**ERY**	**DA**	**VA**	**OX**	**AMC**
P. aeruginosa	45	ND	12(27)	ND	ND	ND	ND	ND	23(51)	15(33)	8(18)	ND	ND	ND	ND	ND
Klebsiella pneumoniae	32	32(100)	14(44)	16(50)	ND	17(53)	21(66)	ND	15(47)	15(47)	2(6)	ND	ND	ND	ND	1/11(9)
S. pneumoniae	2	ND	ND	0(0)	0(0)	0(0)	1(50)	0(0)	ND	ND	ND	0(0)	ND	ND	ND	ND
Staphylococcus aureus	10	ND	9(90)	6(60)	8(80)	ND	8(80)	9(90)	7(70)	ND	ND	7(70)	7(70)	2(20)	8(80)	ND
Citrobacter diversus	2	2(100)	0(0)	1(50)	ND	1(50)	1(50)	ND	1(50)	0(0)	0(0)	ND	ND	ND	ND	ND
Haemophilus influenza	3	3(100)	0(0)	1(33)	1(33)	2(67)	3(100)	ND	ND	ND	ND	ND	ND	ND	ND	0/2(0)
Enterobacter cloacae	4	4(100)	2(50)	2(50)	ND	2(50)	2(50)	ND	2(50)	2(50)	1(25)	ND	ND	ND	ND	ND
Escherichia coli	2	2(100)	1(50)	2(100)	ND	2(100)	2(100)	ND	2(100)	2(100)	0(0)	ND	ND	ND	ND	0/2(0)
Klebsiella oxytoca	4	4(100)	2(50)	2(50)	ND	2(50)	4(100)	ND	3(75)	2(50)	0(0)	ND	ND	ND	ND	0/2(0)

AML- Ampicillin, CIP- Ciprofloxacillin, CH- Chloramphenicol, TET- Tetracycline, CTX- Cefotaxime, SXT- Co-trimoxazole, P- Penicillin, CN- Gentamicin, CAZ- Ceftazidime, IPM- Imipenem, ERY- Erythromycin, DA- Clindamycin, VA- Vancomycin, OX- Oxacillin, AMC-Amoxycillin-cluvanic acid, ND- Not done.

After testing all the 32 *K. pneumoniae* isolates for ESBL production, 34.4% (11/32) were found to be resistant to cefotaxime, ceftazidime and cefpodoxime, suggesting that they were potential producers of ESBL. Further analysis of the isolates with a confirmatory test (combination discs: cefotaxime-clavulanic acid, ceftazidime clavulanic acid and cefpodoxime clavulanic acid) showed that 91% (10/11) were ESBL-producers.

An attempt was also made to profile multiple antimicrobial resistance patterns of the bacterial isolates. The majority of the *K. pneumoniae* isolates exhibited multidrug resistance (MDR) (68.8%, 22/32) with 13 different patterns. The commonest pattern was ampicillin-chloramphenicol-cefotaxime-cotrimoxazole-gentamicin-ceftazidime (15.6%, 5/32), followed by ampicillin-ciprofloxacin-chloramphenicol-cefotaxime-cotrimoxazole-gentamicin-ceftazidime (15%, 4/32), and ampicillin-chloramphenicol-cotrimoxazole (9.4%, 3/32) (Table 3.4). MDR was defined as resistance to three or more drugs.

Pseudomonas aeruginosa isolates had three different MDR patterns, the commonest being ciprofloxacin-gentamicin-ceftazidime (13.3%, 6/45), followed by ciprofloxacin-gentamicin-ceftazidime-imipenem (8.9%, 4/45) and gentamicin-ceftazidime-imipenem (2.2%, 1/45). *S. aureus* had eight MDR patterns: ciprofloxacin-chloramphenicol-tetracycline-cotrimoxazole-penicillin-gentamicin-erythromycin-clindamycin (10%, 1/10), ciprofloxacin-chloramphenicol-tetracycline-cotrimoxazole-penicillin-gentamicin-erythromycin-clindamycin (10%, 1/10), ciprofloxacin-chloramphenicol-tetracycline-cotrimoxazole-penicillin-gentamicin-erythromycin (10%, 1/10), ciprofloxacin-tetracycline-cotrimoxazole-penicillin-gentamicin-erythromycin-clindamycin (10%, 1/10), chloramphenicol-tetracycline-cotrimoxazole-penicillin-gentamicin-

clindamycin (10%, 1/10) and tetracycline-cotrimoxazole-penicillin (10%, 1/10). *K. oxytoca* group had three different patterns each exhibiting 25% (1/4). *E. cloacae* displayed the following MDR patterns: ampicillin-ciprofloxacin-chloramphenicol-cefotaxime-cotrimoxazole-gentamicin–ceftazidime-imipenem (25%, 1/4) and ampicillin-ciprofloxacin-chloramphenicol-cefotaxime-cotrimoxazole-gentamicin-ceftazidime (25%, 1/4). *C. diversus*, had only one pattern, ampicillin- chloramphenicol -tetracycline- cefotaxime -gentamicin (25%, 1/4) and *E. coli*, had two patterns, ampicillin-ciprofloxacin-chloramphenicol-cefotaxime-cotrimoxazole-ceftazidime (50%, 1/2) and ampicillin-chloramphenicol-cefotaxime-cotrimoxazole-gentamicin-ceftazidime (50%, 1/2) (Table 4.8).

Table 4.8: Antimicrobial resistance patterns of bacteria isolated from sputum in adults at the UTH

Antimicrobial Resistance Patterns	**No. of Isolates (%)**
***Klebsiella pneumoniae* (32)**	
AML-CIP-CTX-SXT-CN-CAZ-IPM	1(3.1)
AML-CIP-CH-CTX-SXT-CN-CAZ	4(12.5)
AML-CH-CTX-SXT-CN-CAZ	5(15.6)
AML-CIP-CTX-SXT-CN-CAZ	1(3.1)
AML-CIP-CH-CTX-SXT-CN	1(3.1)
AML-CH-CTX-CAZ-IPM	1(3.1)
AML-CIP-CTX-SXT-CAZ	1(3.1)
AML-CH-CTX-SXT-CN	1(3.1)
AML-CIP-CTX-SXT	1(3.1)
AML-CH-CN	1(3.1)
AML-CH-SXT	1(3.1)
AML-SXT-CN	1(3.1)
AML-CH-SXT	3(9.4)
Total MDRs	**22(68.8)**
***Pseudomonas aeruginosa* (45)**	
CIP-CN-CAZ-IMP	4(4.4)
CIP-CN-CAZ	6(13.3)
CN-CAZ-IMP	1(2.2)
Total MDRs	**11(24.4)**
***S. aureus* (10)**	
CIP-CH-TET-SXT-P-CN-E-DA	1(10)
CIP-CH-TET-SXT-P-CN-E-VA	1(10)
CIP-CH-TET-SXT-P-CN-E	1(10)

CIP-TET-SXT-P-CN-E-DA	1(10)
CIP-CH-TET-P-CN-E-DA	1(10)
CH-TET-SXT-P-CN-DA	1(10)
TET-SXT-P	1(10)
Total MDRs	**7(70)**
***K. oxytoca* (4)**	
AMP-CIP-CH-CTX-SXT-CN-CAZ	1(25)
AMP-CTX-SXT-CN-CAZ	1(25)
AMP-CIP-CH-SXT	1(25)
Total MDRs	**3(75)**
***E. cloacae* (4)**	
AMP-CIP-CH-CTX-SXT-CN-CAZ-IPM	1(25)
AMP-CIP-CH-CTX-SXT-CN-CAZ	1(25)
Total MDRs	**2(50)**
***H. influenzae* (3)**	
AMP-CH-TET-CTX-SXT	1(33.3)
AMP-CTX-SXT	1(33.3)
AMP-SXT	1(33.3)
Total MDRs	**3(100)**
***C. diversus* (1)**	
AMP-CH-TET-CTX-CN	1(25)
Total MDR	**1(25)**
***E. coli* (2)**	
AMP-CIP-CH-CTX-SXT-CN-CAZ	1(50)
AMP-CH-CTX-SXT-CN-CAZ	1(50)
Total MDR	**2(100)**

AML- Ampicillin, CIP- Ciprofloxacin, CH- Chloramphenicol, TET- Tetracycline, CTX- Cefotaxime, SXT- Co-trimoxazole, P- Penicillin, CN- Gentamicin, CAZ- Ceftazidime, IPM- Imipenem, ERY- Erythromycin, DA- Clindamycin, VA- Vancomycin, OX- Oxacillin, AMC-Amoxycillin-cluvanic acid.

Chapter 5

Discussion

5.1 Discussion

Examination of sputum is the primary approach to determining the aetiology of pneumonia in patients. However, the determination of the microbial aetiology of pneumonia is challenging because lower respiratory secretions are always contaminated with oropharyngeal flora present in saliva (Hammitt *et al*., 2012). This has a large impact on the interpretation of sputum culture results as some of the poorly collected specimens may be entirely composed of upper respiratory secretions. This situation can lead to incorrect conclusion that an organism colonising the upper airway is causing pneumonia (Anevlavis *et al*., 2009). Consequently, it has become standard practice for diagnostic laboratories to assess the quality of expectorated sputum specimens to confirm that it has been obtained from the lower respiratory tract.

Data obtained in this study showed that about half of the specimens were of good quality. This was contrary to the results obtained in other studies conducted in India and United States of America in which the good quality sputum samples were 65% and 79% respectively (Mariraj *et al*., 2011; Mokkapati *et al*., 2013). The difference may be attributed to the fact that clinical staff and patients involved in specimen collection are not taught the value of collecting good specimen. This may lead to incorrect generation of results by the diagnostic laboratory. Therefore, good quality sputum samples depend on proper education of healthcare workers and patients. When age and gender were considered it was observed that they did not play a role in determining the quality of the sputum samples.

Gram stain results further revealed that there were a total of six morphotypes which included Gram negative rods (GNR), Gram Negative Coccobacilli (GNCB), Gram Positive Cocci (GPC), Gram Negative Cocci (GNC), GPR-Gram Positive Rods (GPR), Gram Positive Diplococcic (GPDC) and yeasts. GNR and GPC were the commonly observed morphotypes and were predominately found in poor quality samples. Other studies have reported similar findings. Studies conducted in both India and Thailand have showed high GPC and GNR in sputum samples (Mokkapati *et al*., 2013). This could possibly be due contamination with oropharyngeal flora which over grew due to delay in transporting samples to the laboratories from clinic and wards. Therefore, strict measures should be put in place by UTH management to ensure that sputum specimens are transported to Bacteriology laboratory without delay.

Data presented in this study, showed that morphotytes and culture results had a concordance of 51.1% positivity. This finding was in conformity with a similar study conducted in the United States which showed a concordance of 57% (Shariatzadeh and Marrie, 2009). To the contrary, a study conducted in Thailand showed a higher concordance, while lower concordance was observed in Iraq study (Mubarak *et al*., 2012). This may in part be due to pre-hospitalisation treatment with antibiotics prescribed by general practitioners prior to collection of sputum sample. Antibiotics may inhibit or kill potential pathogens if treatment with antibiotics is initiated before sample collection. A more systematic approach of obtaining clinical information such as prior administration of antibiotic before sample collection should be included in future studies.

Pneumonia can be caused by a wide range of organisms, and published reports have revealed the isolation of different organisms, and this has been attributed to differences in patient groups, presence of epidemic organisms and scope of investigation (Reynolds *et al*., 2010). Culture methods allow phenotypic identification of the causative agents and provides an opportunity for performing antimicrobial susceptibility tests, which in turn, allows modification of empirical treatment (Holter *et al*., 2015). In this study, 57.1% of sputum samples were found to be positive for potential pathogens associated with pneumonia using culture methods. This yield was higher than what was obtained in other studies conducted in Malaysia, Egypt, Nigeria and Ethiopia (Egbe *et al*., 2011; Khalil *et al*., 2013; Mustafa *et al*., 2011; Regasa, 2014). The difference was attributed in part to variations in sensitivity of culture methods employed in different studies. The data also showed that there was no significance difference between men and women, but this was not consistent with data from countries such as Nigeria where most of the positive samples were obtained from women (Egbagbe *et al*., 2006; Okesola *et al*., 2007). This difference may be ascribed to differences in methods used in selection of patients included in the study.

Culture results showed that *Moraxella catarrhalis* was the most isolated organism, followed by *P. aeruginosa, C. albicans*, *K. pneumoniae, K. oxytoca* and *E. coli*. These observations are contrary to findings from other studies conducted in Nigeria and Ethiopia which reported *K. pneumoniae* and *S. pneumoniae* as the most predominate organisms respectively (Egbe *et al*., 2011; Regasa, 2014). Some studies have indicated that *M. catarrhalis* is an important cause of exacerbations of chronic obstructive pulmonary disease in adults (Larsen *et al*., 2009). However, in our study it cannot be inferred that this organism was a cause of pneumonia because of limited access to clinical information and it was predominately isolated in poor quality sputum samples.

The study also revealed that *P. aeruginosa* was the second most isolated potential pathogen. This finding is higher than other studies conducted in Iran, Nigeria and Ethiopia (Hasheni *et al*., 2010; Fiberesima *et al*., 2008; Regasa, 2014). Generally most studies conducted in different parts of the world showed that Gram negative bacilli accounts only 3-10% of total prevalence (Bartlett and Mundy, 1995). However, a lot of Gram negative bacilli which included *P. aeruginosa* were isolated in this study. The higher incidence seen in our study may truly represent the pattern of local flora, as some of the Gram-negative bacilli (such as *P. aeruginosa* and *K. pneumoniae*) were endemic in other parts of Africa and would be important implications in the choice of antibiotics therapy. This brings an attention in the area to conduct further investigation and in fact change in trend of infection may bring to new approach to diagnosis of pneumonia in adults patients. Furthermore, a change toward Gram negative bacteria and opportunistic organism may occur with increasing age and the severity of the concomitant medical illness.

Improved detection of many bacterial and viral pathogens associated with pneumonia has been observed with the use of PCR (Templeton *et al*., 2005). The advantage of PCR technique is its ability to detect microorganisms also after initiation of antibiotic treatment (Johansson *et al*., 2008). Recent studies have shown that application of both PCR and culture methods have high and wider microbial yield (Holter *et al*., 2015). This study utilised PCR as a tool for identifying organisms from the sputum samples and significant number of potential pathogens were detected. Potential pathogens were detected in 71% of sputum samples which is in line with results from other studies, although results vary considerably from 39% to 76% (Jennings *et al*., 2008; Charles *et al*., 2008; Lieberman *et al*., 2010; Johansson *et al*., 2010; Holter *et al*., 2015).

This variation is attributed to variation in distribution of aetiology of pneumonia in adults in different geographic regions (Micek *et al*., 2014).

On the other hand, Human cytomegalovirus was detected as the most common single organism using PCR. The most affected age group was between 18 and 44. The high incidence of CMV may be attributed to rise in HIV/AIDS infections within this age group in Zambia. However, in this study, it was not possible to link our findings to HIV infections due to limited access to clinical information of patients. *Klebsiella pneumoniae* was found as the second most frequent potential pathogen in all age groups. Other potential pathogens detected included *H. influenzae* non-type B, *S. aureus*, *S. pneumoniae*, *H. influenzae* type B, rhinovirus, *M. catarrhalis*, *Pneumocystis jirovecii*, Respiratory Syncytial Virus (RSV) A/B, parainfluenza virus, adenovirus, Human metapneumoviruses A/B, Human bocavirus, Human coronavirus 63, influenza type B, parainfluenza type, parainfluenza type, parainfluenza type 4, *Salmonella species*, *Mycoplasma pneumoniae*, influenza virus type B, Human coronavirus 63 and parainfluenza type 2. This is an important finding, suggesting that novel empirical antimicrobial treatment be considered. On the contrary in Norway *S. pneumoniae* was most frequently detected followed by influenza, rhinovirus, respiratory syncytial virus, parainfluenza virus, enterovirus, metapneumovirus and adenoviruses (Holter *et al*., 2015). Another similar study which was done in Netherlands identified *S. pneumoniae* as the most common organism, followed by coxiella burnetii and influenza A virus (Huijskens *et al*., 2012). Other reports have shown that viral infection in patients with pneumonia varies from 4% to 39% (Liu *et al*., 2009). In the present study, respiratory viruses accounted for 52.9% of tested sputum samples. The higher rates of viral

pathogens that were found as a single or combined agent can be attributed to the novel laboratory tests that were used.

In the present study, PCR detected multiple isolates in the 30% of the samples analysed. This is consistent with the fact that the incidence of mixed infections does not usually exceed 30% as has been observed in another study (de Roux *et al*., 2006). Mixed isolates have also been reported in various studies conducted elsewhere (Johansson *et al*., 2010; Gencay *et al*., 2013; Liu *et al*., 2013). *K. pneumoniae* and *H. influenzae* non type B were the most frequently found coinfections in the multiple infected patients. Similar finding were observed in other studies conducted in Nigeria and Ethiopia (Egbagbe *et al*., 2006; Regasa, 2014). The identification of mixed infection is very important for treatment strategies and to avoid a false impression of clinically resistant strains. It has further been observed that establishing a microbiological diagnosis for pneumonia from samples with mixed isolates is challenging as no single pathogen can be said to cause the infection. Interactions between different pathogens *in vivo* are yet to be elucidated (Liu *et al*., 2013). Multiple concurrent infections might interfere with the pulmonary cleansing function, and thus helping to establish a setting for pneumonia. It is not well understood whether a viral infection alone causes pneumonia or acts in conjunction with other respiratory pathogens, and a number of investigators postulate that a viral infection is followed by a secondary bacterial infection (Gencay *et al*., 2010; Small *et al*., 2010; Ruuskanen *et al*., 2011). Several studies have shown that viral infection is an important activator of secondary bacterial infection. Prior viral infection might impair mucosal barriers in the respiratory system and make the host susceptible to bacterial infection (Van der Sluijs *et al*., 2010; Wunderink, 2010).

Comparison of bacterial PCR and culture results revealed differences, with PCR yielding more positives than culture, which confirmed the increased sensitivity of PCR reported by others (Templeton *et al*., 2005; Oosterheert *et al*., 2005; Holter *et al*., 2015). A high percentage of negative sputum cultures may be due to fastidious bacteria, viral agents, and previous antibiotic therapy. A number of studies have revealed that during antibiotic treatment sputum samples become rapidly negative for bacteria in contrast to the PCR technique that remain positive (Stralin *et al.*, 2005; Kee *et al.*, 2010; Liu *et al*., 2009) besides conventional culture methods cannot detect viral infection. In this study, sputum culture detected only one case of *S. pneumoniae* and *H. influenzae*, confirming that it is an insensitive test (Loens *et al*., 2009), as was revealed by PCR results in which *S. pneumoniae* and *H. influenzae* cases were much higher. The high diagnostic yield of aetiological agents of pneumonia that was revealed with the application of PCR technique demonstrates that aetiology, nowadays, can be established in the majority of pneumonia patients if multiple techniques are employed in routine practice.

The increase in antimicrobial resistance (AMR) among bacterial agents of pneumonia, especially from MDR strains, is challenging global prospects for fighting pneumonia in adults. In this study nearly all the organisms isolated exhibited multi drug resistance patterns. *P. aeruginosa*, showed 51% resistance to gentamycin, which is comparable with the study conducted in Ethiopia (Regasa *et al*., 2015). However, it showed low resistance to ciprofloxacin, ceftazidime and imipenem. Similarly study conducted in Nigeria showed low resistance for ciprofloxacin and no resistance for ceftriaxone. However, ceftriaxone was not tested in this study. *Klebsiella pneumoniae* isolates were all resistant to ampicillin and high resistance was also seen with

cotrimoxazole, which was parallel with studies conducted in Nigeria where all isolates were resistant to cotrimoxazole (Okesola *et al*., 2008). Some of the *Klebsiella pneumoniae* isolates were MDR strains, with common patterns being ampicillin-chloramphenicol-cefotaxime-cotrimoxazole-gentamicin-ceftazime and ampicillin-ciprofloxacin-cefotaxime-cotrimoxazole-gentamicin-ceftazidime. *K. pneumoniae* also exhibited high level of multi-drug resistance and this was similar to studies conducted in Asia and South Africa where *K pneumoniae* had substantial rates of multi-drug resistance (Feldman *et al*., 1995; Rammaert *et al*., 2012). This study also demonstrated significant occurrence of β-lactamase-producing *K. pneumoniae. S. aureus* was resistant to all traditional first line drugs and 70% of the isolates exhibited multi drug resistance. This was similar to results obtained in a study conducted in USA, China and Nigeria (Cataldo *et al*., 2010; (Okesola et al., 2008; Okeke *et al*., 2005). All *H. influenzae* isolates showed 100% resistance to ampicillin and cotrimoxazole which is comparable with study conducted in USA (Doern *et al*., 1999). However, moderate resistance was observed against chloramphenicol, tetracycline and cefotaxime and this was in line with, a study done in Nigeria where isolates were resistant to the same drugs (Okesola *et al*., 2008). These differences could be due to variations in antibiotic prescribing habits among different countries. Although *E. coli* was almost resistant to all antibiotics tested only two isolates were tested. In the present study, different bacterial species had high level of resistance pattern to different antibiotics, unfortunately, the number of positive cultures for *K. oxytoca*, *E. cloacae*, *Haemophilus influenzae, C. diversus*, *S pneumoniae*, and *E. coli* were too small for a valid statistical analysis to decide the empirical treatment. According to Clinical Laboratory Institute, empirical treatment can only been established if >30 isolates are subjected to susceptibility testing. Ongoing surveillance is needed for bacterial pathogens in order to determine the empirical treatment.

However, based on our finding all isolates exhibited MDR to these commonly used antimicrobial agents at UTH. Only few drugs like Imipenem was found effective. If used more and more without control, these isolates may also develop resistance to these drugs too. The emergence of resistant microorganisms has a significant impact on treatment outcomes and poses a challenge to healthcare. Prescription of antibiotics without laboratory guidance and over sales of antibiotics without proper drug prescription may be some of the different factors that can contribute for this high level drug resistant pattern. The other reason, sale of antibiotics are a rife in Zambia as laws and regulations are not strict and not followed on the sale and purchase of antimicrobial drugs (Pandey and Sharma, 1994; Hang'ombe, 1999). Therefore, drug prescription for patients should be laboratory evidence based. Alternative management modalities should be sought, especially in developing countries where introduction of new brand drugs is economically challenging and the disease is also prevalent and severe. Provided that empiric therapy is unavoidable in management of pneumonia the efficacy of antimicrobials at hand is important. However, in developing countries like Zambia, the available drugs are limited enforcing use of similar antimicrobials frequently. These, in turn, in the absence of sufficient and appropriate guiding studies may elaborate the problem. As a result, the problem might have been under scored. We believe the antibiotic resistance observed is higher than has been demonstrated in this study.

5.2 Conclusion

Quality specimens were only obtainable with only 50% of the specimens, and the most common morphotypes detected were GPC and GNR. By combining conventional diagnostic methods with real-time PCR techniques for both common bacteria and a number of respiratory viral agents, a

higher microbial yield was detected. Cytomegalovirus and *Moraxella catarrhalis* were the leading causative agent using PCR and culture method respectively. Mixed infections were frequent, with *K. pneumoniae* and *H. influenzae* non-type B being the most common organisms. All the isolates tested against ampicillin were 100% resistant. *K. pneumoniae, S. aureus* and *P. aeruginosa* exhibited multi drug resistance to more than two antibiotics. The levels of antibiotic resistance among *K. pneumoniae* and *S. aureus* causing pneumonia are high in our settings. Our results indicate that potential viral infections should be given more attention in adult pneumonia cases. This is the first study in Zambia to simultaneously investigate the quality of sputum, microbiological aetiology and antimicrobial susceptibility patterns of bacteria from adult patients with pneumonia.

5.3 Limitations of the Study

- Not all sputum samples were subjected to PCR analysis owing to limited resources allocated to this study. All the samples were supposed to have been processed using both culture and PCR in order to give a true picture of the potential pathogens in the samples.
- The data obtained in this study were restricted to the patients from Lusaka, and may not be extrapolated to representative of the entire Zambian population.
- No clinical information was obtainable to determine the prior use of antibiotics because of poor record keeping at the hospital. Some patients are allowed to carry their clinical records home, making it difficult for the investigator to access the data.

5.4 Future Directions

- It would of interest to extend this study to other parts of Zambia in order to give an accurate picture of the respiratory pathogens circulating in the country.
- Future studies should include the collection of clinical and epidemiological information so as to give an insight of who the most vulnerable groups are, as well as common risk factors in Zambia.
- National-wide studies should be conducted to ascertain existence of MDR strains of *K. pneumoniae*, *S. aureus* and *E. coli*.
- An effective national surveillance system for pneumonia pathogens should be set up for their monitoring and control as well as control of antimicrobial resistance by the Ministry of Health (MOH).
- MOH should endeavour to improve laboratory capacity to improve diagnostic capacity of the country for pneumonia diagnosis.
- Clinicians should also ensure that antibiotic susceptibility testing is done before administration of antibiotics.
- Unsuitable samples should be rejected in order to reduce the costs of processing sputum samples.

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9.0 Appendices

Appendix I: Ethics Approval Letter

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

8th May, 2014.

Your Ref: 008-05-14.

Mr. John Mulemena,
Ndola College of Biomedical Sciences,
Postal Agency,
Ndola.

Dear Mr. Mulemena,

RE: SUBMITTED RESEARCH PROPOSAL: **"IDENTIFICATION OF POTENTIAL PATHOGENS IN SPUTA FROM ADULTS SUSPECTED OF HAVING PNEUMONIA AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA" (REF. No. 008-05-14)**

Your application for a waiver of ethics review for the aforementioned protocol was reviewed. The waiver is hereby granted. The approval was conducted in line with the University of Zambia Biomedical Research Ethics Committee guidelines on granting waiver of Ethics review.

CONDITIONS:

- The waiver is based strictly on your submitted proposal. Should there be need for you to modify or make changes to the proposal; you will need to seek clearance from the Biomedical Research Ethics Committee.
- This waiver does not release you from the obligation of ensuring confidentiality.
- If you need any clarifications please consult this office.
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. J.C. Munthali
CHAIRPERSON

Date of approval: 8th May, 2014. **Date of expiry:** 7th May, 2015.

Appendix II: Targeted Gene Sequences of the Pathogens Within The FTD Resp 33.

Pathogen	Target
adenovirus	Hexon gene
bocavirus	NP1 gene
Bordetella pertussis	IS481 gene
Chlamydia pneumoniae	RNA polymerase beta chain gene
cytomegalovirus	US7 +US8 genes
coronavirus 43	nucleocapsid protein (N) gene
coronavirus 63	nucleocapsid protein (N) gene
coronavirus 229	nucleocapsid protein (N) gene
coronavirus HKU1	nucleocapsid protein (N) gene
enterovirus	parts of domain IV and V
Haemophilus influenzae	ompP2 gene
Haemophilus influenzae type B	BexA gene
human metapneumovirus A	fusion glycoprotein (F) gene
human metapneumovirus B	fusion glycoprotein (F) gene
influenza A	matrix gene (pos1)
influenza B	segment 8 NS1/NEP
influenza C	matrix gene
Klebsiella pneumoniae	khe hemolysin gene
Legionella species	16S rRNA
Moraxella catarrhalis	copB gene
Mycoplasma pneumoniae	adhesin P1
parainfluenza 1	hemagglutinin-neuraminidase (HN) mRNA
parainfluenza 2	hemagglutinin-neuraminidase (HN) mRNA
parainfluenza 3	hemagglutinin-neuraminidase (HN) mRNA
parainfluenza 4	fusion protein gene
parechovirus	5`untranslated Region (UTR)
Pneumocystis jirovecii	(mtLSU)rRNA gene
respiratory syncytial virus A	nucleocapsid protein gene

respiratory syncytial virus B	nucleoprotein mRNA
rhinovirus	5`untranslated Region (UTR)
Salmonella species	tetrathionate subunit B (ttrB)
Streptococcus pneumoniae	LytA gene
Staphylococcus aureus	sensor histidin kinase vick gene

Appendix III: Nucleic Acids Extraction and Amplification Machines

A. NucliSENS EasyMAG Nucleic Acids Extraction Machine (Biomeriuex)

Item	**Catalog #**
Nuclisens Magnetic Silica	280133
Nuclisens Extraction Buffer 3 (4x1L)	280132
Nuclisens Extraction Buffer 1 (4x 1L)	280130
Nuclisens Extraction Buffer 2(4X1L)	280131
Nuclisens lysis buffer 4x1l	280134
Nuclisens easymag disposable	280135

B. 7500 Real Time PCR Systems (Applied Biosystems)

Item	**Catalog #**
Optical Adhesive Cover +96 plate	4314320
Optical 96-well reaction plates (MicroAmp)	4306737

Appendix IV: Antibiotic Susceptibility Panels (OXOID, UK)

The following antibiotics were tested on isolates recovered from sputum samples.

Acinetobacter

Drugs	**Disc Concentration**	**S ≥**	**I**	**R ≤**
Gentamicin	10 µg	15	13-14	12
Ciprofloxacin	5 µg	21	16-20	15
Imipenem	10 µg	16	14-15	13

Alpha-Haemolytic Streptococci

Drugs	**Disc Concentration**	**S ≥**	**I**	**R ≤**
Penicillin		MIC ≤ 0.12	MIC 0.25-27	MIC ≥ 4
Cefotaxime	30 µg	28	26-27	25
Imipenem	30 µg	17		

Beta-Haemolytic Streptococci

Drugs	**Disc Concentration**	**S ≥**	**I**	**R ≤**
Penicillin	10 Units	24		
Erythromycin	15 µg	21	16-20	15
Clindamycin	2 µg	19	16-18	15
Tetracycline	30 µg	23	19-22	18

Enterobacteriaceae

Drugs	**Disc Concentration**	**S ≥**	**I**	**R ≤**
Ampicillin	10 µg	17		16
Amoxicillin/clavulanic acid	20/10 µg	18	14-17	13
Gentamicin	10 µg	15	13-14	12
Contrimoxazole	1.25/23.75 µg	16	11-15	10
Cefotaxime	30 µg	23	15-22	14
Chloramphenicol	18	13-17	12	
Ciprofloxacin	5 µg	21	16-20	15
Ceftazidime	30 µg	18	15-17	14
Ceftriaxone	30 µg	21	14-20	13
Imipenem	10 µg	23	20-22	19
Cefoxitin	30 µg	18	15-17	14

***Haemophilus influenzae*:**

Drugs	**Disc Concentration**	**S ≥**	**I**	**R ≤**
Ampicillin	10 µg	22	19-21	18
Amoxicillin/Clavulanic acid	20/10 µg	20		19
Ciprofloxacin	5 µg	21		
Chloramphenicol	30 µg	29	26-28	25
Tetracycline	30 µg	29	26-28	25
Cefotaxime	30 µg	26		
Cotrimoxazole	1.25/23.75 µg	16	11-15	10

Pseudomonas aeroginosa

Drugs	**Disc Concentration**	**S ≥**	**I**	**R ≤**
Gentamicin	10 µg	15	13-14	12
Ciprofloxacin	5 µg	21	16-20	15
Ceftazidime	30 µg	18	15-17	14
Imipenem	10 µg	16	14-15	13

Staphylococcus aureus

Drugs	**Disc Concentration**	**S ≥**	**I**	**R ≤**
Gentamicin	10 µg	15	13-14	12
Chloramphenicol	30 µg	18	13-17	12
Ciprofloxacin	5 µg	21	16-20	15
Erythromycin	15 µg	23	14-22	13
Penicillin	10 Units	29		28
Cotrimoxazole	1.25/23.75 µg	16	11-15	10
Cefoxitin	30 µg	22		21
Oxacillin	1 µg	13	11-12	10
Vancomycin				
Tetracycline	30 µg	19	15-18	14
Clindamycin	2 µg	21	15-20	14

Streptococcus pneumoniae

Drugs	**Disc Concentration**	**S ≥**	**I**	**R ≤**
Chloramphenicol	30 µg	21		20
Cotrimoxazole	1.25/23.75 µg	16	11-15	10
Tetracycline	30 µg	23	19-22	18
Oxacillin	1 µg	20		
Erythromycin	15 µg	21	16-20	15

Appendix V: Media and Reagents

A. Media

MacConkey Agar (Oxoid Ltd, Basingstone, Hampshire, England)

50g of MacConkey agar base in 1litre distilled water

Autoclave at 121^0C for 15 minutes.

Blood Agar (Oxoid Ltd, Basingstone, Hampshire, England)

37.5g in 1000ml of distilled water.

Autoclave at 121^0C for 15 minutes.

Cooled to 50-55^0C and added 5-7% sterile sheep blood.

Mixed well before pouring.

Chocolate Blood Agar (Oxoid Ltd, Basingstone, Hampshire, England)

37.5g in 1000ml of distilled water.

Autoclave at 121˚C for 15 minutes.

Added 5-7% sterile blood sterile blood the temperature is till 75-80^{0}C.

Mueller Hinton Agar (Mast Diagnostics, Mast Group Ltd, Merseyside, UK)

38.0g in 1000ml of distilled water

Autoclave at 121˚C for 15 minutes.

Haemophilus Test Medium (REMEL, UK)

21.5g in 500ml of distilled water.

Autoclave at 121˚C for 15 minutes.

Cooled to 50-55^{0}C and added HTM supplement SR 0158 as directed.

Mixed well before pouring.

Mueller Hinton Agar with 5% sheep blood (REMEL, UK)

38.0g in 1000ml of distilled water

Autoclave at 121˚C for 15 minutes.

Cooled to 50-55^{0}C and added 5-7% sterile sheep blood.

Sabouraud Dextrose Agar (REMEL, UK)

65g in1000ml of distilled water

Autoclave at 121˚C for 15 minutes.

Triple Iron Sugar (TSI) Agar (REMEL, UK)

59.5g in 1000ml of distilled water

Autoclave at 121˚C for 15 minutes.

Simmons Citrate Agar (HiMedia Laboratories Pvt. Ltd. India)

24.2g in 1000ml distilled water

Autoclave at 121˚C for 15 minutes.

Urease Agar (HiMedia Laboratories Pvt. Ltd. India)

24.01g of urea base in 950ml distilled water

Autoclave at 115˚C for 20 minutes.

Cool to 50˚C and add 50ml of sterile 40% urea

Lysine Iron Agar (HiMedia Laboratories Pvt. Ltd. India)

34.56g in 1000ml distilled water

Autoclave at 121˚C for 15 minutes.

Sulfide Indole Motility (REMEL, UK)

30g in 1000ml distilled water.

Autoclave at 121˚C for 15 minutes

B. Reagents

Normal Saline

0.89% Sodium Chloride

0.89g Sodium chloride in 100ml distilled water

Autoclave at 115˚C for 15 minutes.

McFarland standard 0.5

Barium chloride (1.175%) 0.05ml

Sulphuric acid (1%) 9.95ml

2% Sodium Deoxycholate (bile salt) Solution (REMEL, UK)

2 g of sodium deoxycholate

100 ml sterile distilled water.

1% Oxidase Reagent from oxidase powder

0.1 g of tetramethyl-p-phenylenediamine dihydrochloride

10 ml of sterile distilled water.

Mixed well and then let stand for 15 minutes.

Dithiothreitol (Oxoid #SR0233)

0.5mg dithiothreitol.

Dissolved in 50 ml of molecular grade water.

Vortex for 1 minute.

BactiCard Neisseria (REMEL, UK)

BactiCard Neisseria Test cards 25.

5-Bromo-4-chloro-3-indolyl butyrate (IB).

5-Bromo-4-chloro-3-indolyl-β-D-galactoside (BGAL).

Gamma-glutamyl- β-naphthylamide (GLUT).

L-Proline- β-naphthylamide (PRO).

Rehydrating Fluid 8 ml.

Colour Developer (0.02% para-Dimethylaminoainnamaldehyde).

Expected values

Organism	**IB**	**BGAL**	**GLUT**	**PRO**
Moraxella catarrhalis	+	+	-	+
Neisseria lactamica	-	+	-	+
Neisseria gonorrhoeae	-	-	-	+
Neisseria meningitidis	-	-	+	Variable