THE DEVELOPMENT OF A NOVEL ALGORITHM FOR DETECTION OF COMMON BACTERIAL, VIRAL AND FUNGAL AETIOLOGIC AGENTS OF MENINGOENCEPHALITIS

Edouard Ntagwabira

H80/91821/2013

A Thesis submitted in fulfilment for award of the degree of Doctor of Philosophy (Ph.D.) in Medical Microbiology of the University of Nairobi

University of Nairobi

2019

DECLARATION

This thesis is my original work and has not been submitted for a degree in any other University

Edouard Ntagwabira

Signed:

Date: 19/07/19

This thesis has been submitted for award of PhD degree with our approval as Supervisors:

Prof. Omu Anzala, MBChB, PhD

Director, KAVI- Institute of Clinical Research &

Professor, Department of Medical Microbiology,

University of Nairobi

Signed:

Date: 19/07/19

Jean Baptiste Mazarati, BSc, MSc, PhD

Head of Department of Biomedical Services

Rwanda Biomedical Center

Signed:

Date: 19/07/2019

Marianne Mureithi, BSc, PhD

Lecturer,

Department of Medical Microbiology,

University of Nairobi

Signed

Date: 19/7/19

DEDICATION

To my nuclear family members for their unconditional love and care that has always given me strength and courage to carry on. Thank you for your tremendous patience, encouragement, support and prayers.

I also dedicate it to my parents and other family members who were killed in the Rwanda Genocide against the Tutsi in 1994, who endeavoured their level best to make me who I am today. Thank you for your fore-sightedness, which encourages our family acquire high-level quality education.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

AIDS: Acquired Immunodeficiency Syndrome

API: Analytical profile index

CDC: Center for Disease Control

CHUB: Butare University Teaching Hospital

CHUK: Kigali University Teaching Hospital

CLSI: Clinical and Laboratory Standards Institute

CMV: Cytomegalovirus

CNS: Central Nervous System

CSF: Cerebral Spinal Fluid

Ct: cycle threshold

DNA: Deoxyribonucleic acid

EAPHLNP: East Africa Public Health Laboratory Network Project

EBV: Epstein–Barr Virus

Ep-TB: Extra Pulmonary Tuberculosis

EQC: External Quality Control

GCLP: Good Clinical Laboratory Practice

GCS: Glasgow Coma Scale

HSCT: Hematopoietic Stem Cell Transplant

HIV: Human Immunodeficiency Virus

HSV-1: Herpes Simplex Virus type I

HSV-E: Herpes Simplex Virus Encephalitis

HSV-M: Herpes Simplex Virus Meningitis

KAVI-ICR: Kenya AIDS Vaccine Initiative - Institute of Clinical Research

IC: Internal Control

KEMRI: Kenya Medical Research Institute

KFH: King Faisal Hospital

LAT: Latex agglutination test

MTA: Material Transfer Agreement

NHLS: National Health Laboratory Service

NRL: National Reference Laboratory

PCR: Polymerase Chain Reaction

RBC: Rwanda Biomedical Center

RMH: Rwanda Military Hospital

RNA: Ribonucleic acid

SOP: Standard Operating Procedure

SPIU: Single Project Implementation Unit

TB: Tuberculosis

TBM: Tuberculous Meningitis

T-I: Trans-Isolate

UoN: University of Nairobi

VZV: Varicella-Zoster Virus

WHO: World Health Organization

YPD: Yeast Extract Peptone Dextrose

ABSTRACT

Background:

The incidence of meningitis and encephalitis in developing countries is underestimated due to inadequate diagnosis as it is mostly based on conventional techniques with a limited detection capacity for viral, bacterial and fungal aetiological agents. This results in prescription of a combination of anti-viral, antifungal or antibiotics, as the aetiologic agent is unidentified. Accurate diagnostic results are required for appropriate treatment options.

Aim: This research was designed to make a contribution in improving quality of diagnostic and accuracy of meningoencephalitis in Rwanda.

Objectives:

1.To develop a novel algorithm for the detection of the most common bacterial, viral and fungal etiological agents of meningoencephalitis using both culture and Real-time Multiplex PCR methods.

2.To determine the drug susceptibility pattern of the detected bacterial agents of meningoencephalitis.

3.To evaluate and demonstrate the utility of molecular diagnostic method compared with conventional diagnostic methods in detection of etiologic agents of central nervous system infections in Rwanda.

Materials and Methods:

Using cross sectional laboratory based study design, 845 hospitalized patients with meningitis and encephalitis from four referral and four satellite hospitals were enrolled into the study. From each patient four sterile tubes were used to collect Cerebral Spinal Fluid (CSF) specimens for analysis using conventional and RT-Multiplex PCR methods. Two tubes were analysed on site for routine diagnosis: One for bacteriology and fungal detection and the other for cytology and biochemistry. The remaining two tubes were sent to the National Reference Laboratory (NRL) for conventional and RT- Multiplex PCR.

Results:

Objective 1: From the study, 152 viral, 49 fungal and 105 bacterial etiological agents of meningoencephalitis were detected. This represents 18%, 6% and 22% diagnostic capacities for viral, fungal and bacterial aetiologic agents respectively.

Objective 2: *Klebsiella pneumonia* and *Streptococcus agalactiae* were 100% sensitive to augmentin and 100% sensitive to cefotaxime respectively. *Staphylococcus aureus* was 90% sensitive to penicillin, oxacillin and methicillin.

Objective 3: Using conventional techniques**,** no viruses were detected. However, with Real- time Multiplex PCR a range of viruses, 152/845 (18%) were detected. In addition, different types of bacteria 185/845(22%) were detected using Real-time Multiplex PCR compared with the 59/845(7%), detected using conventional techniques.

Conclusion: The results from this study showed rapid and accurate diagnosis of etiological agents of Central Nervous System (CNS) infection. There was an improvement on diagnostic capacity from 9% using conventional methods, to 40% using a combination of conventional and Real time -Multiplex PCR methods. The use of RT- Multiplex PCR in detection of etiological agents of CNS infections will contribute to accurate diagnosis and early, appropriate treatment of patients with meningoencephalitis.

Recommendation: The Ministry of Health in Rwanda should consider developing a policy that includes the use of this algorithm by Healthcare Professionals and laboratory networks in order to improve quality of diagnosis for better treatment of patients with meningoencephalitis at all levels in the country.

CHAPTER ONE: INTRODUCTION

1.1. Background

Meningitis is a severe disease resulting from an acute inflammation of meninges. Encephalitis is the presence of an inflammatory process in the brain parenchyma associated with clinical evidence of brain dysfunction. Meningoencephalitis is mostly caused by viruses, bacteria, fungi and protozoa. The disease is known to cause significant morbidity and mortality (Baskin and Hedlund, 2007a).There are no reliable clinical symptoms at the initial stage of meningoencephalitis to differentiate between viral, bacterial, fungal or protozoal infections. All suspected cases of meningoencephalitis are therefore hospitalized. Early diagnosis is very important in order to prescribe appropriate drugs (Baskin and Hedlund, 2007a).

The etiology of central nervous system infections varies depending on age group and region. According to previously conducted research, approximately 30 to 40% of CNS infections are caused by bacteria, while 60 to 70% are caused by *Cryptococcus Species*, viruses and brain abscess (Mengistu et al., 2013).

Bacterial meningoencephalitis is often fatal in around 50% of untreated cases and causes permanent pathological changes (Campagne et al., 1999). Bacterial meningitis diagnosis is confirmed by CSF culture. Antibiotic susceptibility tests should be done to determine appropriate drugs for treatment in the first critical hours (Mengistu et al., 2013).

Streptococcus pneumoniae, *Neisseria meningitidis, Haemophilus influenzae, Listeria monocytogenes* and group B *Streptococci* are the most common causes of meningitis (Bartt, 2012), while *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* mostly cause meningococcal infection in children younger than five years (Taylor et al., 2012).

Isolation of bacteria from CSF using conventional techniques is unreliable due to 7 to 10 days incubation period and prior use of antibiotics before sample collection (Ntagwabira et al., 2017).

The neurological complications and mortality by viral meningitis is mostly caused by *Enteroviruses* (35 to 83%),and mumps (1 to 40%) (Minjolle et al., 2002). *Herpes viruses, Toga viruses, Bunya viruses,* lymphocytic choriomeningitis virus, measles and *Rubella viruses* also cause CNS infections.

Congenital *Cytomegalovirus (CMV)* infections mostly occur in patients with AIDS with significant morbidity and mortality (Bartt, 2012). In immunocompromised persons adenovirus is a common cause of viral infection (Cho and Mckendall, 2014). Acyclovir is the drug of choice but requires early treatment to reduce morbidity caused by *CMV* (Bartt, 2012).

Diagnosis of viral meningoencephalitis is known to be challenging, but with newer molecular techniques it has improved (Logan and MacMahon, 2008). Improved specific diagnosis enhances early treatment with favourable outcomes of viral encephalitis (Baskin and Hedlund, 2007a).

Cryptococcus neoformans, Candida albicans and *Aspergillus species* are the most common causes of fungal meningoencephalitis in immunocompromised patients (Baskin and Hedlund, 2007a). *Coccidioides immitis* or *Coccidioides posadasii* also causes fungal diseases. In endemic regions, this fungus accounts for about 20% of community-acquired pneumonia cases. It is a respiratory tract infection with symptoms resembling those of bronchitis or pneumonia which can persist for few weeks followed by spontaneous recovery in immunocompetent people. In most cases it is asymptomatic (Malo et al., 2014).

1.2. Problem Statement

Acute bacterial, viral and fungal meningoencephalitis is a serious disease that needs rapid and appropriate diagnosis for accurate treatment. These pathogenic and opportunistic infections are not properly detected using conventional methods in the Sub-Saharan region and particularly in Rwanda. As results the incidence of CNS infections is underestimated resulting to inappropriate treatment of patients.

The Direct microscopic examination, culture methods, antibody and antigen detection assays as conventional techniques in clinical microbiology laboratories for CSF analysis have several limitations on it sensitivity and specificity.

Based on previous data from a study conducted by National Reference Laboratory from four referral hospital laboratories in Rwanda for a period of four years (2009 to 2012), 91% of CSF) specimens collected from hospitalized patients having clinical suspicion symptoms of meningoencephalitis were found to be negative and only 9% were positive for different microorganisms (Ntagwabira et al., 2017).

1.3. Justification

In a retrospective cross-sectional study conducted on hospitalized patients with clinical suspicion of meningoencephalitis at four referral hospitals in Rwanda 2009 – 2012 (Ntagwabira et al., 2017). ,the 91% negative results from hospitalized patients suggest the conducted laboratory tests were not specific for suspected causative microorganisms or the likelihood that the laboratory diagnostic processes were incorrectly done. This could be due to lack of an appropriate algorithm for analysis of CSF samples or prior use of antibiotics before CSF collection thus interfering with isolation of pathogens cultured.

Despite numerous studies carried out, there are no clear, appropriate algorithms for diagnosis of CNS infections in East African countries thereby limiting timely clinical interventions.

Conventional diagnostic methods used were not adequately guiding physicians to effectively treat suspected meningoencephalitis patients. As a result, the high level of misdiagnosis not only leads to inadequate or inappropriate patient treatment but also to delayed decision making by the physicians in view of the 7 to 10 days waiting period it takes to obtain results from specimen culture. If this is the case, then lack of accurate diagnostic tools for isolation of meningoencephalitis causative agents may not only be leading to delays in starting appropriate treatment and therefore to increased prevalence of serious morbidity and mortality, but also to an underestimation of the burden of meningoencephalitis hence hampering formulation of effective control strategies.

This study aimed at making a contribution to improved quality and accurate results in laboratory diagnosis, appropriate therapy, early treatment and better management of patients with meningoencephalitis in Rwanda by developing, establishing and implementing conventional and molecular diagnostic techniques novel algorithms to detect the most common causative agents of meningoencephalitis in CSF.

It was envisioned that the new diagnostic algorithm would assist the Ministry of Health of Rwanda to achieve its mission by improving the quality of laboratory services within the laboratory network, promote health and boost quality of life in Rwanda. An achievement of this study will be to contribute to the attainment of the wider health sector aspirations for the country as enshrined in the health sector strategic plan.

Furthermore, this study envisioned that the developed algorithm would help the Ministry of Health in Rwanda to come up with policies for quick appropriate diagnosis and early treatment of meningoencephalitis and also implement the use of the developed novel algorithm within the medical laboratory network as it would greatly assist in patient care at all levels in the country.

The results obtained from the study will benefit health care facilities and the Ministry of Health by providing information on leading causes of meningitis and encephalitis in Rwanda, and the effectiveness of treatment using appropriate antibiotics from minimum inhibitory concentration and disc susceptibility testing.

In addition, it is also expected that the outcome from the research will help other medical laboratories in the African countries improve the quality of their services, such as in analysis of CSF samples by detecting and identifying the causative agents for meningoencephalitis using molecular techniques in their medical laboratories.

1.4. Objectives

1.4.1. Broad Objective
To compare detection capacities of conventional and Real-time Multiplex PCR methods of most common bacterial, viral and fungal etiological agents of meningoencephalitis in Rwanda.

1.4.2. Specific Objectives:

1. To develop a novel algorithm for the detection of the most common bacterial, viral and fungal etiological agents of meningoencephalitis using both culture and Real-time-Multiplex PCR methods.

2. To determine the drug susceptibility pattern of the detected bacterial agents of meningoencephalitis.

3. To evaluate and demonstrate the utility of the   Real-time-Multiplex PCR over conventional methods for detection of pathogens causing meningoencephalitis.

CHAPTER TWO: LITERATURE REVIEW

2.1. Bacterial, Viral and Fungal Meningoencephalitis

Meningitis is a serious CNS infection that causes acute inflammation of meninges, it is mostly caused by viruses, bacteria, fungi and protozoa. It causes substantial morbidity and mortality. Encephalitis is an inflammation in the brain parenchyma with symptoms of brain dysfunction. (Baskin and Hedlund, 2007a).

Central Nervous System is protected by the blood-brain barrier which creates an obstacle for pathogens which circulate in the blood without limiting vital nutrients reaching the brain (Coureuil et al., 2017). This CNS barrier is made of meninges i.e. dura mater, arachnoid mater and pia matter. Systemic bacteria can only cross this barrier through three sites. The first is the chroid plexuses whereby it interacts with loose capillaries in the endothelium and cross the monolayer of ependymal cells to reach CNS. The second is the capillaries of CNS barriers through interaction with capillaries of leptominange followed by interstitial fluid drainage towards meninges through lymphatic circulation. The last pathway is the non-hematogenous route, like crossing through arachnoid villi or olfactory nerve axonal transport for respiratory tract colonizing bacteria.

This mechanism is also used by intracellular organisms able to multiply in macrophages and dendritic cells facilitating them to cross through local immune cells of the host (Coureuil et al., 2017).

2.1.1. Bacterial Meningitis

Bacterial meningitis is usually caused by extracellular pathogens, such as *Neisseria meningitidis* *Haemophilus influenzae*, and *Streptococcus pneumoniae.* This bacteria are the most common causes of meningitis in infants and adults globally (Thigpen et al., 2011).

Laboratory diagnosis plays a critical role in meningitis surveillance by efficiently detecting the causative agents and guiding the appropriate treatment options for patients according to the antimicrobial susceptibility results (Aguilera et al., 2002).

Haemophilus influenzae is a small Gram negative bacilli or coccobacilli. The most frequent infection is caused by type b strains especially in unvaccinated infants in developing countries (Pandit et al., 2005).

Streptococcus pneumoniae is a Gram-positive lanceolate diplococcus bacterium, but it can also be seen in short chains of cocci which may be found in intracellular or extracellular spaces. It is alpha-haemolytic on blood agar, sensitive to optochin and is bile soluble (Watt et al., 2009). Pneumococcal meningitis is a rare and severe disease that can develop into a life-threatening medical emergency. Early diagnosis and timely appropriate treatment is highly recommended to minimize the risk of adverse outcomes (Johnson, 1996).

Streptococcus agalactiae is the most common cause of neonatal meningitis in industrialized countries which occurs during or just after birth (Kim, 2016).

Group B *Streptococcus* is a Gram positive cocci in chain. They are beta-haemolytic, catalase-negative and facultative anaerobes. They are surrounded by a polysaccharides capsule helping in sub-classification into 10 serotypes. In general, they are harmless commensal bacteria colonizing

gastrointestinal and genitourinary tracts, but can cause opportunistic infections including meningitis in immunocompromised persons (Berardi et al., 2010).

In pregnant women, hemorrhaging at the time of delivery can allow bacteria to invade the blood stream and cause post-partum bacteremia, septicemia or even meningitis. It can also be transmitted to the baby while passing the colonized birth canal and causing neonatal sepsis which can lead to neonatal meningitis resulting in death or long-term disabilities (Leclercq et al., 2016).

Laboratory diagnosis relies on isolating beta-haemolytic bacteria on blood agar plate under 5-10% CO_2, followed by biochemical and serological identification. Group B *Streptococci* is catalase negative and reactive to group B antiserum of Lancefield grouping kit. On Gram stain they are Gram positive cocci in chains (Steer and Plumb, 2011).

Escherichia coli, usually normal flora of gastro-intestinal tract, is Gram negative rod-shaped bacteria. It causes neonatal meningitis and in the nosocomial setting, after penetration of brain barrier due to cranio-cerebral injury or after neurosurgical procedures (Grundmann et al., 2011). Community-acquired cases are mostly exclusively associated with immunosuppression , old age, diabetes mellitus or chronic alcoholism (Elaldi et al., 2013). Infection with *Escherichia coli* is associated with higher mortality rate compared with other bacterial meningitis (Gaschignard et al., 2011).

Klebsiella pneumoniae is an aerobic, rod shaped, Gram-negative, lactose-fermenting, non-motile bacterium. It is not a common pathogen, but it affects immunocompromised persons and causes nosocomial infections (Pomar et al., 2013) with capsule polysaccharide as it virulence factors, it can affect immunocompetent persons and cause meningitis (Lin et al., 2016).

Although *Klebsiella pneumoniae* meningitis is associated with high mortality rate, the third-generation cephalosporin (cefotaxime, ceftriaxone, or moxalactam) provide excellent antimicrobial activity both in vitro and in vivo (Teckie and Karstaedt, 2015). Appropriate therapeutic drugs are an important factor for patient survival and neurological outcome. Therefore, diagnosis should be done as soon as possible and empirical therapeutic drugs should be readily available (Bouadma et al., 2006).

Staphylococcus aureus is Gram positive cocci, grouped in clusters with plasma coagulation capacity due to its coagulase enzyme. It causes rare meningitis but can be fatal. (Aguilar et al., 2010). Staphylococcal meningitis is diagnosed by CSF culture and biochemical identification.

 The *Staphylococcus aureus* is catalase positive and coagulase positive. Linezolid is regarded as a good treatment option due its capacity to penetrate the CNS easily. In severe cases and community-acquired methicillin resistant *Staphylococcus aureus* infections, trimethoprim/sulfamethoxazole or rifampin in addition to vancomycin is recommended (Aguilar et al., 2010). Individuals with underlying chronic conditions have a higher risk of mortality (Van De Beek, Drake and Tunkel, 2010).

2.1.2. Bacterial Meningoencephalitis Diagnosis

Adequate CSF culture is normally used to identify causative bacteria of meningitis and also to determiner antimicrobial susceptibility of the isolated bacteria in order to provide appropriate antibiotics for treatment (Mengistu et al., 2013).

However, the lumbar puncture should be done before antibiotic treatment is prescribed so that the positivity of CSF culture increases, since antibiotics taken before CSF collection may cause negative CSF cultures (Mengistu et al., 2013). The PCR technique for diagnosis of

meningoencephalitis infection has been proven as useful meningitis detection method even after antibiotics have been prescribed (Issa et al., 2003).

2.1.3. Antimicrobial drugs for treatment of bacterial meningoencephalitis

Results obtained from antimicrobial susceptibility are reported as either sensitive, resistant or intermediate. Bacteria are said to be sensitive to an antimicrobial agent if the agent shows ability to inhibit the growth of bacteria on in-vitro culture. This means that the satisfactory results are expected when it is used to treat infection caused by that sensitive bacteria.

 Resistance is defined as failure to kill or inhibit growth of bacteria on in-vitro culture. This means that an infection caused by resistant microorganism cannot be treated using the resistant antimicrobial agent drug (Pal Chugh et al., 2012).

Intermediate sensitivity is the moderate activity of an antimicrobial agent to the tested microorganism (it is in between sensitivity and resistance). In case of lack of sensitive drugs, higher doses of intermediate drugs   may be used. In this case the toxicity of the antimicrobial agents has to be taken into consideration (Pal Chugh et al., 2012).

Inappropriate use of antimicrobial agents may cause high resistance to antimicrobial agents. Bacteria resistance to some antibiotics can be overcome by altering the dosage regimens or inhibiting the resistance mechanism (e.g., beta-lactamase inhibitors), whereas other resistance mechanisms can only be overcome by using an antibiotic from a different class. (Chugh et al., 2011).

 A study by Y. Chugh et al.2011, found   that Gram positive bacteria   causing meningitis   are highly sensitive (100%) to linezolid and vancomycin followed by piperacillin-tazobactam (95.45%), amikacin (90.91%), cefoperazone-sulbactam (86.36%), meropenem (86.36%) and pristinamycin (83.33%) (Chugh et al., 2011).

Gram-positive isolates have been reported to be sensitive to vancomycin and ceftriaxone while a large number of gram-positive isolates have a multi-drug-resistance pattern to cefotaxime, erythromycin, chloramphenicol and penicillin. A systematic microbial susceptibility pattern is therefore highly recommended in clinical settings before patient treatment (Al Khorasani and Banajeh, 2006).

A study of susceptibility pattern of gram negative bacteria isolated from CSF samples has demonstrated that Gram-negative bacteria are 100% sensitive to meropenem and to pristinamycin recommended for *Neisseria* followed by piperacillin tazobactam (94%) and observed 100% susceptibility of *E. coli* to meropenem, piperacillin-tazobactam and cefoperazone-sulbactam (Pal Chugh et al., 2012).

2.2. Viral Meningoencephalitis

Viral meningitis is frequently found in young children but it is also observed in patients of all ages. Due to lack of diagnosis capacity, most viral meningitis cases worldwide are not reported. Specific viral detection is very important because appropriate treatment will be offered to patients and unnecessary antibiotics and hospital stay will be reduced. They mainly cause encephalitis and aseptic meningitis. Encephalitis is also caused by *Herpes simplex (HSV)* and *Varicella-zoster (VZV)* viruses, *Cytomegalovirus, Epstein-Barr virus (EBV),* and human *Herpesvirus* 6 (*HHV*-6) in immunosuppressed patients (McGill et al., 2018).

2.2.1. Laboratory Diagnosis of Viral meningoencephalitis

The Diagnosis of viral meningitis is done by CSF sample analysis. In addition, CSF cytology assists in the exclusion of neoplastic meningitis, viral meningitis is often characterized by a lymphocyte pleocytosis particularly in enteroviral meningitis. The polymerase chain reaction is a new rapid, sensitive and specific method for detection of RNA and DNA viruses in CSF samples, which improves routine virological diagnosis and reduce costs of drugs and inpatient stays (Koskiniemi et al., 2001).

2.2.2. Antiviral drugs for treatment of viral meningoencephalitis

Suspected or confirmed CNS viral infection should be treated using anti-viral drugs like acyclovir for *Herpes, Varicella-zoster* encephalitis, ganciclovir and foscarnet for *Cytomegalovirus* encephalitis and pleconaril for *Enterovirus* encephalitis.

There is no specific therapy for *Adenovirus* infection other than supportive and symptomatic treatment, since most infections are self-limiting in the setting of a normal immune response and do not require specific therapy (Lion, 2014).

2.3. Fungal Meningoencephalitis

2.3.1. Cryptococcus neoformans

Cryptococcus neoformans and Cryptococcus gattii are the main causes of fungal meningitis. They are opportunistic human pathogens which cause life-threatening meningitis associated with high morbidity and mortality (Boulware et al., 2010).

Cryptococcal infection is transmitted by inhalation of infectious propagules, that initially colonize the lung and then enter central nervous system, the yeast can persist in human body by expressing virulence capsule protein, melanin. Despite the strong association with HIV globally, some cases are found in non-HIV individuals (Butler et al., 2012).

2.3.2. Diagnosis of Cryptococcal meningitis

Cryptococcus neoformans detection in CSF specimen using conventional methods is carried out by Crag latex agglutination and microscopic Indian ink tests to identify capsulated yeast, and culture on Sabouraud Dextrose Agar (Scriven et al., 2017).

Polymerase chain reaction based techniques for detection of *C. neoformans*, have been developed for early and rapid detection for DNA from CSF specimen which is very reproducible and stable over time (Chang et al., 2013).

2.3.3. Coccidioidomycosis

Coccidioidomycosis is infection caused by *Coccidioides immitis* or *Coccidioides posadasii*, both dimorphic saprophytic fungi normally found in soil and cause infection to human by inhalation of spores after soil disruption. These fungi are common causes of pneumonia which may lead to systemic infection and meningitis (Taylor et al., 2012).

The infection is mostly asymptomatic. However, some cases are associated with clinical symptoms like respiratory distress and skin lesions. The primary infection site is the respiratory tract. In immunocompetent people, symptoms resemble bronchitis or pneumonia and can persist for a few weeks followed by spontaneous recovery. In endemic regions, this fungus accounts for about 20% of community-acquired pneumonia cases (Malo et al., 2014).

Diagnosis of Coccidioidomycosis is conducted through a combination of different methods, such as clinical symptoms, radiographic imaging, and laboratory results. Laboratory diagnosis is conducted through microscopic identification of diagnostic cells in body fluids and tissue biopsy stained using the Papanicolaou method or Grocott's methenamine silver staining (Pappagianis and Coccidioidomycosis Serology Laboratory, 2007). The two causative agents of coccidioidomycosis (*C. immitis* and *C. posadasii*) are very similar, and can only be distinguished by molecular techniques with specific nucleotide primers which have been developed for *C. immitis* DNA amplification in PCR assay (Malo et al., 2014).

Treatment of coccidioidomycosis requires long term anti-fungal therapy, lasting approximately three to six months. In immunocompromised individuals the recommended drugs for disseminated infection are oral fluconazole and intravenous amphotericin B. Other alternatives include itraconazole and ketoconazole for milder disease. For coccidioidal meningitis, the medication of choice is fluconazole due to its penetration into CSF (Botero Aguirre and Restrepo Hamid, 2015).

2.3.4. Histoplasmosis

This is an infection caused by *Histoplasma capsulatum,* normally found in soil from bird droppings. Humans are infected through inhalation of infectious elements released by disruption of soil during excavation or construction. It is primarily a lung infection but can disseminate to

other organs, and become fatal if not treated adequately. Histoplasmosis is observed more frequently among immunocompromised people especially HIV positive patients. In immunocompetent individuals, the primary infection leads to development of immune memory resulting in partial protection against this fungus in case of reinfection (Kauffman, 2007). The infection incubation period is 3 to 17 days after exposure. It is mostly an infection without clinical manifestations, since intact immune system provides protection.

Among immunocompromised individuals chronic histoplasmosis can develop tuberculosis-like lung disease. Disseminated histoplasmosis affects different organs including the brain causing histoplasmal meningitis which is always fatal if not adequately treated.

Diagnosis of histoplasmosis is based on detection of fungus in samples from infected organs. Antigens or antibodies are detected from blood, urine or CSF samples using ELISA. It often requires confirmation by culturing on Sabouraud or on YPD agar (Nielsen et al., 2012).

In the majority of immunocompetent individuals, there is no treatment required because it gets cured spontaneously. Antifungal medications used to treat severe cases of acute histoplasmosis and all cases of chronic and disseminated histoplasmosis are amphotericin B, followed by oral itraconazole (Nielsen et al., 2012).

CHAPTER THREE: MATERIALS AND METHODS

3.1. Study Design

This was a laboratory based cross-sectional study.

3.2. Study Area

The study was conducted at the National Reference Laboratory using samples collected from eight selected sites. These were four referral hospitals consisting of King Faisal Hospital, Rwanda Military Hospital, Butare University Teaching Hospital, Kigali University Teaching Hospital; and four satellites hospitals namely, Gisenyi Hospital, Kibungo Hospital, Nyagatare Hospital and Byumba Hospital (See appendix 1).

These hospitals were selected in order to cover all the provinces in Rwanda. A key selection criteria was that the hospitals had to have a laboratory bacteriology section that had the capability of examining CSF specimens using conventional diagnostic methods. The CSF samples were collected in 3 departments namely Emergency, Internal Medicine and Paediatrics. All collected adequate specimens were taken to the study site laboratory for diagnosis, packaging and shipment to National Reference Laboratory. The study samples were collected between February 2017 and February 2018.

3.3. Study Population

Eight hundred and forty-five (845) hospitalized patients with clinical signs and symptoms of meningoencephalitis, both males and females of all ages. CSF samples were obtained from each of these patients and examined for causative pathogens using macroscopy, microscopy, staining culture and RT- multiplex PCR. The collected 845 samples were distributed as follows; King Faysal Hospital (37), Rwanda Military Hospital (84) Butare University Teaching Hospital (154),

Kigali University Teaching Hospital (391), Gisenyi Satellite Hospital (27), Kibungo Satellite Hospital (55) Nyagatare Satellite Hospital (29) and Byumba Satellite Hospital (68). Only adequate specimens collected from patients with signs and symptoms indicated in the case report form were used for this study.

3.4. Sample size calculation

The sample size calculation was carried using the Fishers formula (Fisher et.al., 1998):

$$n= [Z^2_{\alpha/2} (p) (1-p)] /d^2$$

Z= is the   corresponding value to the 95% confidence interval

Based on National Laboratory Network surveys on CSF specimen analysis in Rwanda at four referral hospitals, the diagnostic capacity of identifying causative agents of meningoencephalitis using conventional method was only 8.6%. Using the application of a novel algorithm for diagnosis of specific suspected causative aetiologic agents, an improvement in the diagnostic capacity by at least 40% was desirable.

Thus the minimum sample size required was calculated using the following parameters:

P=0.086 δ=0.40

$$n = (Z_{\alpha/2}+ Z_\beta)^2 [P (1-P)/(\delta)^2]$$

$$n= (2.82)^2[0.078604]/ (\delta)^2 = 0.6250904496/\delta^2$$

$Z_{\alpha/2}$: Is the   corresponding value to the 95% confidence interval = 1.96

Z_β : Value corresponding to power of the test taken to be 80% and corresponds to a value 0.86

P: Prevalence of the diagnostic capacity for causative agents of meningoencephalitis in different referral hospitals in Rwanda from CSF specimens.

δ= is the error of margin usually taken around 5% but for the current this varied in accordance to the proposed simulation method.

N= is estimated samples size

Thus the sample size required for the study was as given below:

δ=40% of 8.6%= 0.0344

$n = (2.82)^2[0.078604] / (0.0344)^2 = 845$

3.5. Selection Criteria

3.5.1. Inclusion criteria

All consenting and assenting inpatients and coma patients' proxies of all ages hospitalized with clinical signs and symptoms of meningoencephalitis indicated in the case report form in the selected sites during the study period in Rwanda, who signed consent and assent forms.

3.5.2. Exclusion criteria

All non-consenting, non-assenting patients and coma patients' proxies of all ages hospitalized with signs and symptoms of meningoencephalitis indicated in the case report form in selected study sites during the study period in Rwanda. Patients with the following were excluded from the study: INR > 1.5, platelet counts < 50,000, intracranial mass, partial/complete spinal block or acute spinal trauma.

3.6. Ethical considerations

This study was reviewed and approved by both Rwanda National Ethical Committee and Kenyatta National Hospital/University of Nairobi Ethics and Research Committee prior to its commencement. The approval numbers are No 572/RNEC/2041, KNH/UON ERC-P561/09/2014 respectively). The approvals were renewed annually, see appendices 3A-F, 4A&B, 5 A&B). Privacy and confidentiality of the patients was upheld at all stages of the study.

3.7. Recruitment of Study Teams

The Principal Investigator and study team trained from National Reference Laboratory met with the management of the study sites and explained the purpose of the research and its importance. Thereafter, the management of the hospitals nominated study teams who included medical doctors, nurses and laboratory personnel based on their capacity. The identified study teams were trained on the study materials, sample collection, storage and transportation and all required materials were distributed from National Reference Laboratory to the sites. A research monitor visited all the selected sites to evaluate the readiness of the sites to carry out the study and once he gave a directive or positive report, the recruitment of participants and sample collection began. Regular monitoring visits were conducted to ensure that staff on sites follow procedures correctly for quality and accurate study results.

3.8. Recruitment of Study Subjects

All consecutive, eligible patients were enrolled into the study by consulting doctors in emergency, internal medicine and paediatric departments until the required sample size was reached.

The clinicians examined the patients for symptoms and abstracted signs like photophobia using light, tested altered mental status to determine confusion using Glasgow Coma Scale (GCS) and meningeal signs using Kerning and Brunziski signs.

In addition, they were responsible for selection of meningitis cases to be enrolled and filling of study case report form. Every patient enrolled was assigned a serial registration number. A pre-designed case report form was used to obtain patient history and socio-demographic information (See appendix 2).

3.9. Quality Assurance at sites

Continuous monitoring of all procedures was regularly conducted to ensure quality of all study steps. Orientation training of research sites medical and laboratory personnel was done before starting recruitment of study participants and regular monitoring visits were done to ensure that staff follow procedures correctly and understood their role. Before sample collection, the Standard Operating Procedures were developed and distributed to all study sites, for use during sample collection, transportation and analysis.

3.10. Sample collection and transport

All lumbar puncture procedures were done by physicians under aseptic conditions using a CSF collection kit. Before specimen collection, the patients were positioned as illustrated in Figure1, and the skin along a line drawn between the two iliac crests was disinfected with 70% alcohol and povidone iodine to clear the surface and remove debris, oils. The skin was then allowed to dry completely before sample collection. The spinal needle was then positioned between the 2 vertebral spines at the fourth and fifth lumbar vertebrae and introduced into the skin with the bevel

of the needle facing up as illustrated in Figure 3. Accurate placement of the needle helped to collect the fluid, which normally is clear and colorless without blood.

Once the needle was in position, the CSF pressure was measured and a sample of 1 to 3 ml of CSF was collected into each of the 4 sterile screw up tubes. Four sterile separate tubes each with at least 1 ml each were needed. The tubes were labelled with the patient's name, date and time of specimen's collection and unique identification number that was matched on both request forms and report forms. After collection of CSF, 2 tubes were analysed on site, one for biochemistry and cytology and the other for pathogen detection using routine/conventional diagnostic methods. The two remaining tubes were transported to NRL, one for pathogen detection by routine/conventional methods and the other one by molecular diagnostic method.

CSF samples were transported to the site laboratories within 15 minutes after collection and processed immediately on site in microbiology and clinical chemistry laboratories. Where it was not possible to be analysed within 1 hour, samples were inoculated into Trans-Isolate (T-I) medium for culture (see Figure 2).

Samples for molecular diagnosis were transported in cold triple packaging and stored in - 20°C or - 70°C, waiting for 96 samples for analysis. Before inoculation in T-I medium the aluminium cover was removed using sterile forceps and the stopper was disinfected with 70% alcohol and allowed to dry. For inoculation of CSF into T-I medium, sterile syringes and needles were required 0.5 to 1 ml of CSF was inoculated into T-I medium and after inoculation the T-I medium bottle were mixed several times. If transportation for T-I medium to National Reference Laboratory was delayed to the next day or longer, averting needle (sterile cotton plugged hypodermic needle) was inserted through the rubber stopper of the T-I medium bottle, which ensured growth and survival of the bacteria. The used needles were discarded in a puncture resistant, autoclavable container.

CSF were handled as potentially infectious clinical specimens in order to maintain a safe working environment for health care workers and laboratories. During manipulation of CSF specimens, proper biosafety guidelines were adhered to and CSF was analysed in biosafety cabinets. Cerebrospinal fluid is normally sterile but contaminants can easily be introduced by non-application of aseptic techniques, both in the wards and in the laboratory.

Examination of CSF is an emergency and specimens should be examined without delay. The preliminary results of tests i.e. Gram stain, latex agglutination and white cell count, were immediately reported to the attending physician or a senior nursing staff in the ward immediately. Before a lumbar puncture was done the laboratory staff were informed, to enable the laboratory to be ready to receive and examine the specimen immediately.
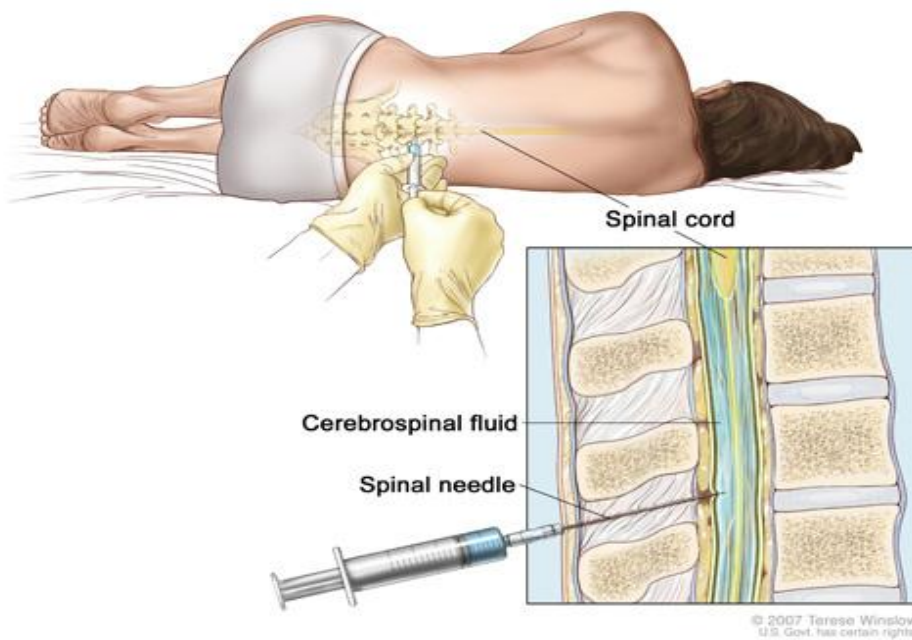
Figure 1: Lumbar puncture to collect sample of cerebrospinal fluid (Ajello et al., 1984)

Figure 2: Picture of bottle of Trans- Isolate medium used in the study

3.11. Onsite sample processing

The volume of collected CSF samples was recorded   and its gross appearances noted as clear, bloody, cloudy, or xanthochromic. Clear CSF samples were tested using cryptococcal latex agglutination and India ink microscopy for detection of *C. neoformans* antigen.

 Adequate turbid samples were tested using latex agglutination for detection of specific polysaccharide surface antigens, for most common bacteria as causative agents of meningitis.

 The specimen was then centrifuged for 20 min at 1,500 to 3,000 ×*g* if the volume recorded was >1 ml. The sediment was vortexed vigorously for at least 30 s yeast cells surrounded by a characteristic polysaccharide capsule, respectively to re-suspend the pellet.

Using a sterile pipette, media was inoculated by placing 1 or 2 drops of sediment on an alcohol-rinsed slide, allowing drop to form a large heap. The slide was then air dried on a slide warmer before being gram stained as described by Fouad et al., 2014.

The CSF gram stained smears were examined and interpreted immediately and all positive smears were immediately reported to the physician and nursing unit by telephone. The telephone notification was documented.

The glucose concentration in CSF was determined using enzymatic oxidation method (peroxidase) and quantified using spectrophotometer COBAS C311.

The CSF protein concentration is considered as one of the most important indicators of meningoencephalitis. The protein concentration in CSF sample was determined using spectrophotometer Cobas C311 automated machine. Normal protein ranges to be considered was 15 to 60 milligrams per mg/dl or 0.15 to 0.6 mg/ml.

The second tube was used Indian ink staining for detection of *Cryptococcus neoformans*. Indian ink test was done using one drop of Indian ink reagent mixed with one drop of fresh CSF sample on slide and covered by cover slide then examined under microscopy in order to identify capsulated yeast. The capsule of Cryptococcus in Indian ink wet mount preparation appears as clear zone around the yeast in a black field.

Twenty-five µl of cryptococcus antigen positive control, negative control and was added to CSF specimen onto the ring slide, then 25 µl of cryptococcal latex to each ring was added. Using applicator sticks, each ring was thoroughly mixed, and rotated by hand or ring slide placed on a rotator set to 100 rpm (+/- 25) for 5 minutes at room temperature. The results were read immediately by looking for cryptococcus antibodies.

3.11.1. Culture examination

All collected CSF samples in four referral hospitals were transported within 1 h and centrifuged at 1000 $\times g$ for 10 to 15 min with supernatant, used for rapid diagnostic test. Sediment was used for gram stain and primary plating on chocolate, blood agar, MacConkey agar and sabouraud dextrose agar. All plated and thioglycolate broth media were examined for macroscopic evidence of growth. With no visible growth on the culture media, broth was re-incubated and negative plates were examined daily for 72 h before discarding. Broth media was also examined daily for 5 to 7 days before discarding.

3.11.2 Culture with growth and organism identification

From colony appearance, colony was picked to prepare gram stain broth, if positive. Semi quantitative growth was put on plated media and gram stain prepared for each morphotype. The microorganisms were identified based on the morphology of colonies, and by biochemical reaction and serotyping.

Antimicrobial sensitivity was also done according to the isolated microorganisms. The physician was notified of culture findings and antibiotic sensitivity patterns.

After analysis the results were immediately recorded in the log book and sent to the attending physician for either initiation or continuation of treatment. The CSF T-I inoculated bottles were incubated at 37°C in candle jar and untreated CSF tube ,fourth tube was kept at -20°C until shipment to NRL for DNA/RNA extraction and analysis using molecular method.
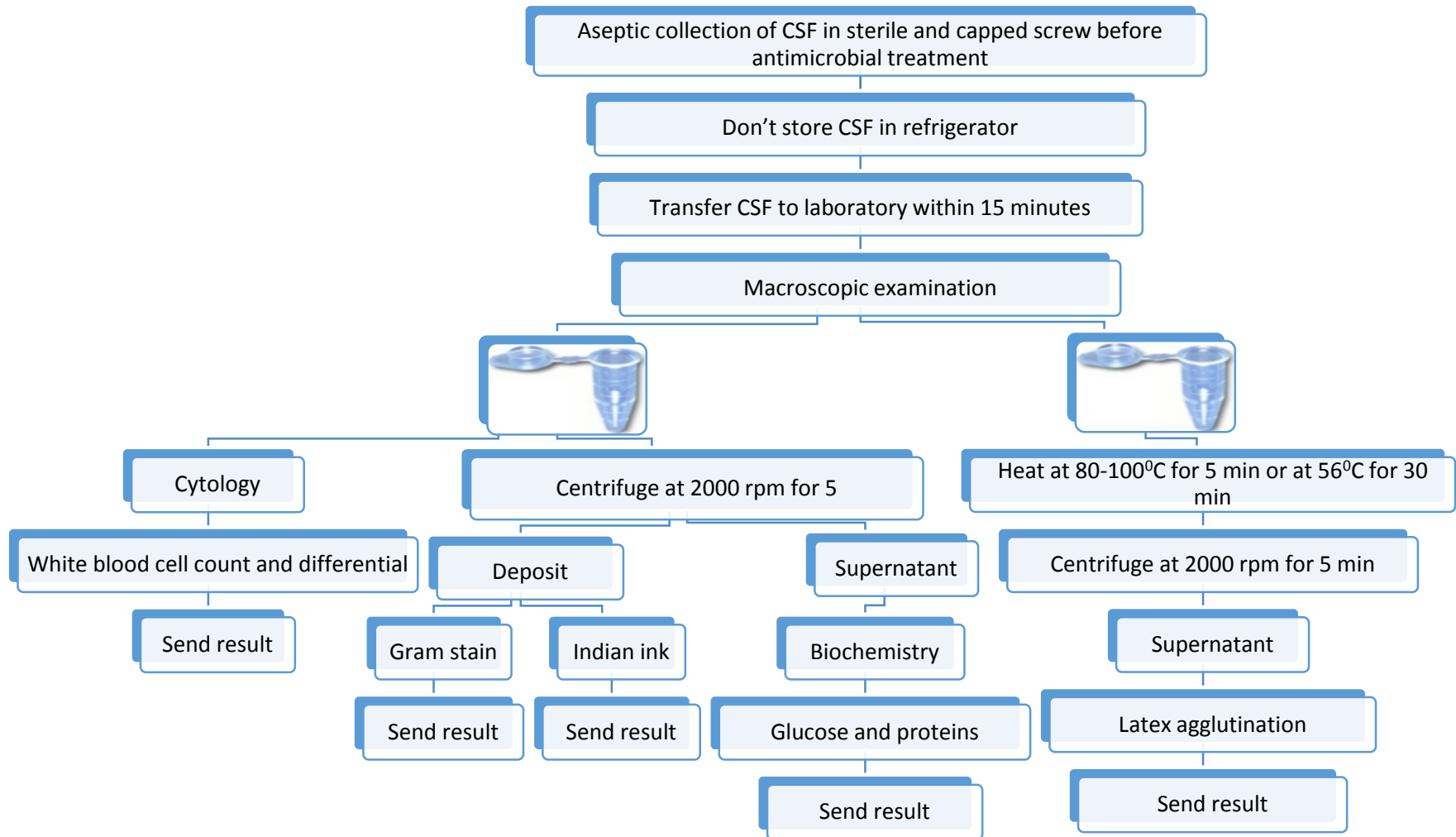
Figure 3: Existing algorithm for diagnosis of CNS infections using conventional method in DHS and PHs in Rwanda 2018

3.11. 3. Sample shipment and transportation from sites to NRL

Among four collected tubes the third and fourth CSF sample tubes were transported to NRL for analysis. The third was inoculated in Trans-Isolate as soon as possible without delay after collection and transported at room temperature. The fourth tube was transported in a cool box with ice in triple packaging. All specimens were sent with their signed consent/assent and case report forms in 4 hours after specimen collection to NRL.

3.12. Quality Assurance on conventional method

To ensure quality and accuracy of study results, the method was validated using the known positive samples selected from the NRL archived strains of *Neisseria meningitis, Streptococcus pneumoniae*, and *Haemophilus influenzae* B, from the NHLS, South Africa *Cryptococcus neoformans* from NRL archive as well as ATCC bacterial species (*Neisseria meningitis, Streptococcus pneumoniae, Escherichia coli* and *Haemophilus influenzae* B) were incorporated in each culture processing lot. All samples were processed according to the Standard Operating Procedures.

3.13. Sample processing at NRL using conventional method

The specimen in T-I was aseptically inoculated on chocolate agar, sheep blood agar, and MacConkey agar. The remaining specimen in T-I bottles was incubated at 35-37°C in ~5% CO_2 then checked daily for 7 days for turbidity, to enhance growth in case of failure of the initial culture The venting needle was inserted in the TI bottle through the bottle rubber stopper plugged with sterile cotton wool, in order to promote growth and survival of bacteria.

When T-I turned turbid, culture on blood and chocolate agar was done then incubated at 35-37°C in a ~5% CO_2 for 24 to 48 hours before reporting negative culture and also on MacConkey agar and incubated at 35-37°C for 18 to 24 hours. In absence of turbidity after 7 days, sterility of sample was tested by systemic inoculating specimen on sheep blood, chocolate and Mac Conkey agar, before discarding the T-I media.

In presence of growth young colonies were used for Gram staining then biochemical testing (API 20E, API NH, catalase, coagulase, oxidase, CAMP test) and serotyping. Antimicrobial susceptibility test was done on isolated bacteria using recommended antibiotic discs on Mueller-Hinton agar, Kirby-Bauer method and Clinical and Laboratory Standards Institute (CLSI) catalogue for result interpretation.

One drop of CSF specimen from molecular samples was inoculated on Yeast Extract Peptone Dextrose (YPD) and incubated at 35- 37°C, then checked after 48 hours for growth, if no growth, the plate was re-incubated and checked daily up to 7 days. The specimen for molecular testing was tightly closed and stored at -20°C. The colonies growing on YPD were identified using Indian ink and Cryptococci antigen latex agglutination (CSF analysis steps followed at NRL are summarized in Figure 7).

The results were recorded into Laboratory Information System and Epi-Info data base. All positive results were reported to attending physician by phone call and all printed results were sent by sample transportation system once in a week to each study site.

3.14. Real- time multiplex Polymerase Chain Reaction Assay Validation

3.14.1 Quality control

Known positive control strains used during culturing above were also used as the positive controls for the bacteria detection and specificity of the PCR assays. Accuracy and reliability of the study was validated and verified using known positive samples selected from the following NRL archived strains: *Neisseria meningitis, Streptococcus pneumoniae, Haemophilus influenzae B,* and *Cryptococcus neoformans.*

ATCC bacterial species (*Neisseria meningitis, Streptococcus pneumoniae, Escherichia coli and Haemophilus influenzae* B, and also strains for positive control from the NHLS, South Africa and from NRL archive were used. 10% of each isolated microorganism and 5% of negative samples were sent to Kenya AIDS Vaccine Initiative - Institute of Clinical Research for external quality control which confirmed the study accuracy.

3.14.2. Nucleic acid extraction

Nucleic acid was extracted from the above known positive controls archived from NRL using an automated nucleic acid extraction platform (QIAsymphony SP-Qiagen, Germany). The QIAsymphony DSP virus /pathogen mini kit was used according to manufacturer's instruction with a starting volume of 250μl and an elution volume of 60μl.

The nucleic acid was   then purified by washing in a series of wash buffer on rod covers before finally being released in the elution buffer. Sterile nuclease free water was used as a negative control of extraction.

The extracted nucleic acid was collected in elution racks, labelled and quantified using QiAxpert nucleic acid quantifier system (Qiagen, Germany) to establish the quality and quantity of the

eluates. The quality of extracted nucleic acid was validated on wavelengths 230/260 and 260/280 ratios to check for sample purity. The eluted nucleic acid was used directly in Real time-PCR and the excess stored at -80^{0}C for long storage or 4 to 8°C for short storage.

3.14.3. Master mix preparation

Pre-optimized Commercial RT-multiplex PCR kits for in-vitro diagnostics were used. The kits comprising of PCR buffer, primer and probe mix(PPmix) and Taq polymerase enzyme were provided in three separate tubes and required constitution of the mastermix before adding the templates. Both automated and manual mastermix preparations were evaluated for their efficiency in processes and also on optimal reagent usage. QIAgility (Qiagen, Germany) liquid handler was used for the mastermix. The system was programmed to pipette 12.5 µl of PCR buffer, 1.5µl of PPmix, 1.5µl of the enzyme and 2 µl of internal control into a 1.5ml tube and later aliquoted 15µl into individual 0.1ml PCR ml tubes that were compatible with the thermalcycler (Rotor Gene Q, QIAGEN-Germany). 10µl of the extracted sample nucleic acid was added to the mastermix into each tube including the extracted negative control sample. Positive and negative controls from the manufacturer were included in each run. In total, we used four RT-multiplex PCR kits namely: FTD bacterial meningitis   FTD Neonatal meningitis, and FTD Neuro9 all from Fast Track Diagnostics, Luxembourg, Germany and *Cryptococcus neoformans* Real-TM kit from Sacace Biotechnologies, Italy.

The PCR setup for the *Cryptococcus neoformans* entailed 10 µl of PCR mix -1, 1.5 µl of PCR mix-2, 0.5 µl of DNA Polymerase, 2 µl of internal control and sterile water into each PCR reaction tube followed by 10 µl of the extracted nucleic acid. Specificity of the assays was tested by amplifying known strains in the kits without their targets. The tubes were sealed, labelled and

transferred to the real time thermalcycler (Rotor Gene Q -Qiagen, Germany) for amplification. (See   protocol on appendix 7).

3.14.4. Amplification

The DNA was amplified simultaneously in the same tube by polymerase chain reaction. In multiplex PCR, a cocktail of primers and probes targeted multiple pathogens. The presence of specific pathogen sequences in the reaction was detected by an increase in fluorescence observed from the relevant dual-labeled probe, and reported as a cycle threshold value (Ct) by the Real-time thermocycler. The assays used murine *Cytomegalovirus* (m *CMV*) and *Brome mosaic virus (BMV)* as the internal controls which were introduced into each PCR reaction tube during the PCR setup to check for PCR efficiency.

 In this study we used the three pre-optimized kits from Fast Track Diagnostics (FTD bacterial meningitis, FTD Neonatal meningitis kit, FTD Neuro9 kit). The kits used) similar amplification programme involving an initial enzyme activation step at 95^0C for 15' minutes and 5 cycles comprising of denaturation at 95^0C for 5 seconds, annealing step at 60^0C for 20 seconds and extension step at 72^0C for 15 seconds without fluorescence detection. This was followed by 40 cycles comprising of denaturation at 95^0C for 5 seconds, annealing step at, 60^0C for 15 seconds and extension step at 72^0C for 15 seconds with fluorescence detection on four detection channels of the thermocycler as indicated on table 1.

Similarly, the pre-optimized commercial real-time PCR kits was used for detection of *Cryptococcus neoformans* and entailed an initial enzyme activation step at 95^0C for 15' minutes and 5 cycles comprising of denaturation at 95^0C for 5 seconds, annealing step at 60^0C for 20 seconds and extension step at 72^0C for 15 seconds without fluorescence detection, followed by 40 cycles comprising of denaturation at 95^0C for 5 seconds, annealing step at, 60^0C for 20 seconds

and extension step at 72^0C for 15 seconds with fluorescence detection on green and yellow at the annealing step (60^0C).

3.14.5. Interpretation of the PCR assays

Specific probes were detected on four different channels on Rotor Gene Q, matched up with specific pathogens as shown on table 1. The detection involved analysing the PCR assay upon completion using Rotor Gene Q software to check for contamination by analysing the negative controls and establishing PCR amplification efficiency.

The threshold was set at a point above negative control and the Ct value for the positive and internal controls was below 33 while the amplification exhibits positive (i.e. exponential trace). Any specimen displaying an exponential trace with a Ct value below 33 is considered positive. A detection guide outlined in table 1 was provided by the manufacturer for establishing the pathogens detected in the positive samples. Appearance of any curve in the negative control was considered as potential contamination, and therefore the results obtained were not interpretable. The whole run including extraction had to be repeated. The run was also repeated where the positive and internal controls did show amplification curves.

The validation of the RT multiplex PCR Assays was considered successful upon detection of the known NRL strains used as positive controls of nucleic acid purification. The assays also amplified the positive and internal controls provided in the kits while both the negative control of purification and the one provided in the kit did not amplify.

Table1: Detection guide for the targets in the four RT- Multiplex PCR kits used for analysis of CSF specimens from patients with CNS infections.

Kit Name	Tube	Green Dye(Wavelenth-520nm)	Yellow Dye(Wavelenth-550nm)	Orange Dye(Wavelenth-610nm)	Red Dye(Wavelenth-670nm)
FTD Neuro9	1	*EBV*	*CMV*	IC	*NC*
	2	*HSV*1	*HSV*2	IC	*VZV*
	3	*EV*	*PV*	IC	-
	4	*HHV*6	*B*19	*HHV*7	IC
FTD Bacterial Meningitis kit	1	*H. influenzae*	*N. meningitis*	IC	*S. Pneumonia*
FTD Neonatal meningitis kit	1	*Group B Streptococci*	IC		*Listeria monocytogenes*
	2			*E.coli*	
Cryptococcus neoformans Real-TM kit	1	IC	*Cryptococcus neoformans*		

3.14.6. Processing of Patient samples using Real-time Multiplex PCR

Upon validation of the PCR assays, all the 845 patient samples were processed using the above validated assays. The quality of the assays was continuously monitored by running positive, negative ad internal controls in each run. The automated extraction system extracted 96 samples including a positive and negative controls. The Rotor gene was able to run up to 72 reaction tubes per run. Analysis was done manually by visual inspection of the amplification profiles for every sample on each wavelength channel.

3.15. Data entry and analysis

Epi Info7 software was used for data entry and data analysis was done using standard statistical method like univariate, bivariate and multivariate analysis, the data was analysed using Epi Info 7, MS Excel, SPSS and Stata 16.

CHAPTER FOUR: RESULTS

Eight hundred and forty-five CSF specimens collected from meningoencephalitis suspected patients were analysed using conventional and RT Multiplex PCR diagnostic techniques. Female were 434 (51%) while male was 411(49%) of the total study population.

4.1.   Characteristics of study population

The demographic representation of the age groups is shown in figure 4, x axis represents age group while y axis represents frequency as a percentage. The most frequently affected population was the age group 25 to 34 years' old which constituted 21% of the patients seen, while the least frequently affected age group was that aged 5 to 14 years old constituting 9% of the patient population.
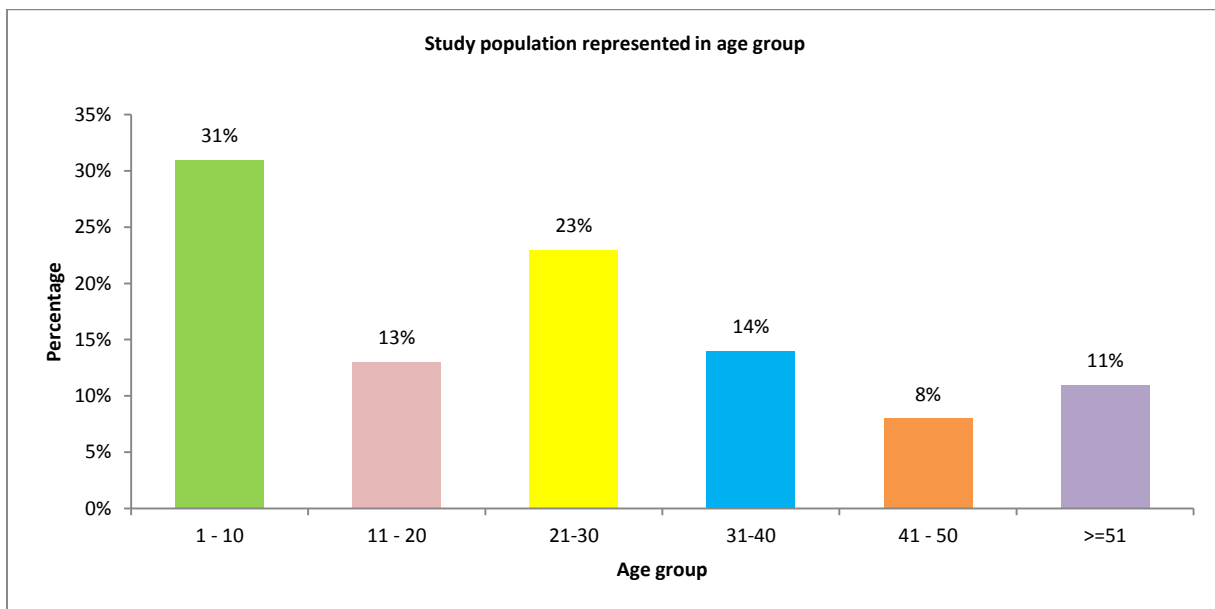
Figure 4: Demographic representation of study population by age group (n=845)

4.2. Clinical Characteristics of study population

Table 2: Frequency of CNS infections signs and symptoms by age group

Signs and symptoms	0 – 4 years	5 – 14 years	15 – 24 years	25 – 34 years	35 – 44 years	45 – 54 years	>= 55 yrs	Total
Chills	0	2	2	1	5	1	2	13
Coma	5	7	15	12	8	7	11	65
Confusion GCS	19	20	40	67	56	30	32	264
Convulsions	75	31	28	21	15	10	10	190
Cranial nerve abnormalities	1	0	1	2	0	0	0	4
Fever	124	65	130	136	95	57	56	663
Headache	32	49	151	160	122	69	75	658
Lethargy	28	18	21	19	15	7	14	122
Neck stiffness	19	20	74	58	53	32	31	287
Neurological deficit	1	3	4	6	3	2	6	25
Irritability	43	28	62	69	57	37	37	333
Photophobia	21	19	52	66	52	27	31	268
Kernig/ Brunziski sign	3	2	10	7	8	1	5	36
Nausea	43	35	70	64	68	28	33	341
Vomiting	43	34	57	43	46	21	17	261

The most frequently observed sign was fever, at 663 (76%) while headache was the most frequently reported symptom by 658 (75%) of the patients. Only 4(0.5%) and 13(1.5%) had cranial nerve abnormalities or chills, respectively as shown in table 2.

4.3. **Conventional methods results analysed on study sites.**

Gram staining results in the figure 5 below shows bacteria identified in CSF of the patients with meningoencephalitis. X axis represents age groups while Y axis represents number of isolates. Only a very small number of the CSF samples 37(4%) showed bacteria on Gram staining. The most frequently seen bacteria were gram negative bacilli *Escherichia coli* and *Klebsiella pneumoniae*) with positivity rate of 23(62%), followed by gram positive cocci (*Staphylococcus aureus* and *Streptococcus agalactiae*) 10 (27%) and gram negative diplococci (*Neisseria meningitidis*) 3(8%). The least seen was gram positive diplococci (*Streptococcus pneumoniae*) 1(3%). Gram negative bacilli were most frequently identified in the CSF of 0 to 4-year-old children, whereas gram positive cocci were most frequently identified in the CSF of those aged 55 years and above. No bacteria were seen on Gram staining in CSF obtained from the 34 to 44 years' age group. The Gram staining results from study sites shows that, the most affected are age group 0-4 with 15 (65%) out of 23, followed by age group >= 55 with 4(40%) out of 10. The less affected age was age group 34 - 44 with 0(0 %) out of 37.
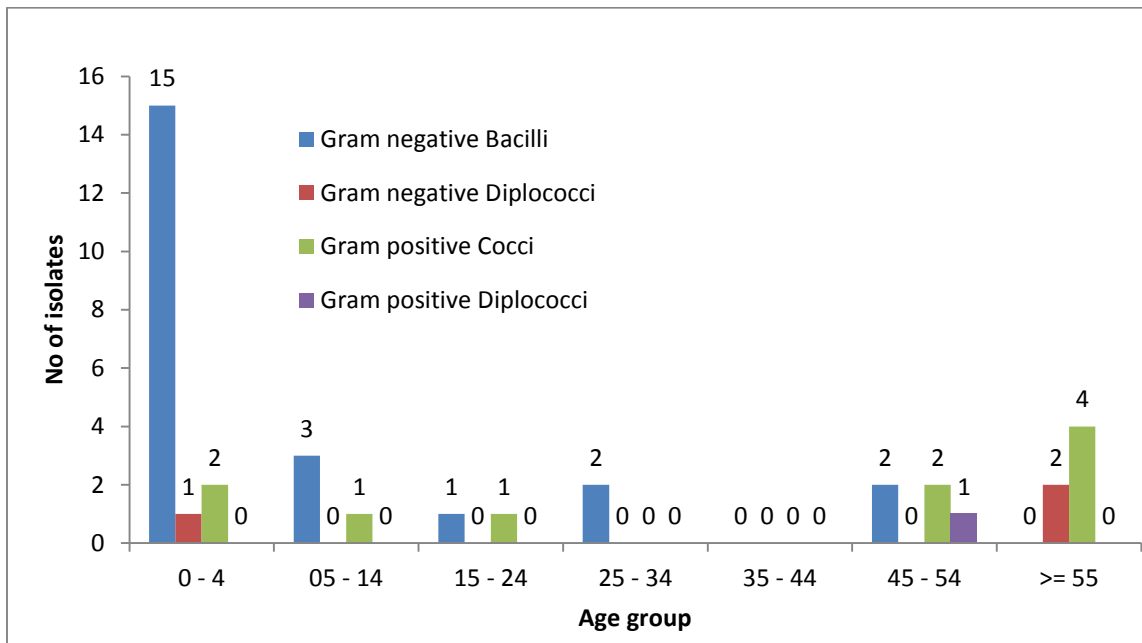
Figure 5: Gram staining results by age group

Using Indian ink, *Cryptococcus neoformans* were identified at each study site as shown in figure 6 below: X axis represents study sites while Y axis represents number of isolates. CHUB 14 out of 154 samples (9 %), followed by KFH 3out 37(8%), Gisenyi 2 out of 27 (7%), Nyagatare 2 out of 29 (7%), CHUK 20 out of 391 (5%), RMH 3out of 84(4%), Kibungo 2 out of 55(4%) and Byumba 2 out of 68(3%).The prevalence of *Cryptococcus neoformans* was slightly higher in men 25/411 (6%) than in women 23/434 (5%).
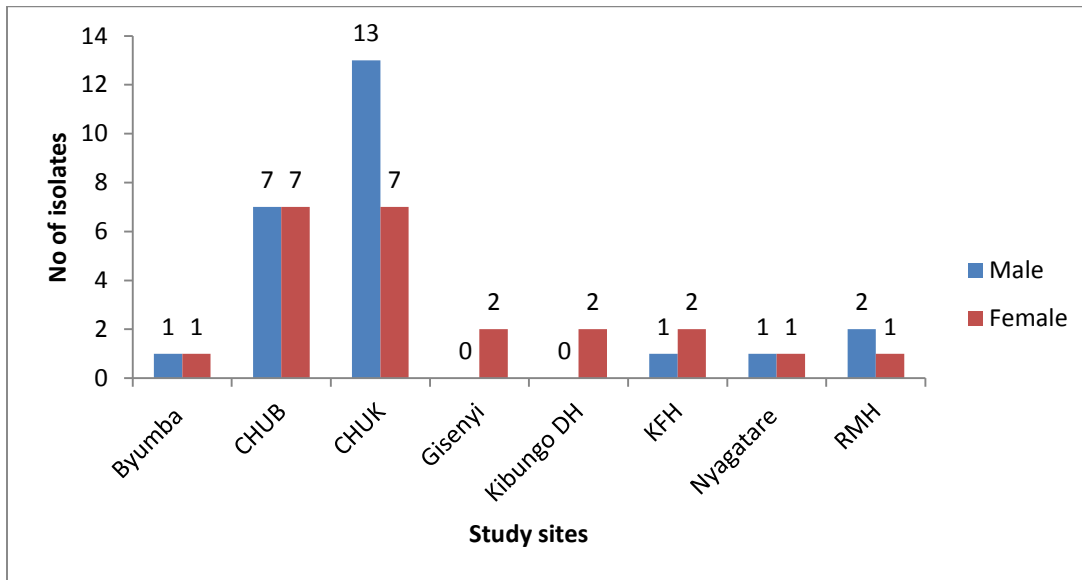
Figure 6: *Cryptococcus neoformans* identified using Indian ink by study sites and gender.

Table 3: Frequency of pathogen detected by sites using culture at National Reference Laboratory.

Pathogen	Byumba	CHUB	CHUK	Gisenyi	Kibungo	KFH	Nyagatare	RMH	Total
Streptococcus agalactiae	0	0	3	0	1	1	0	0	5
Cryptococcus. neoformans	1	14	22	2	2	3	2	3	49
Escherichia coli	0	8	21	0	0	0	0	1	30
Klebsiella pneumoniae	0	3	1	0	0	0	0	0	4
Staphylococcus coagulase neg.	0	1	3	0	0	0	3	2	9
Staphylococcus aureus	0	2	1	0	2	2	0	3	10
Streptococcus pneumoniae	0	0	2	0	0	0	1	2	5
Total	1	28	53	2	5	6	6	11	112

The table 3 above shows Culture results by study sites at NRL. Only 112 (13%) of the specimens had microorganisms isolated. *Cryptococcus neoformans* was the most common microorganism isolated with positivity rate of 49(44%), followed by *Escherichia coli* accounting for 30 (27%), *Staphylococcus auerus* with 10(9%), *Staphylococcus* coagulase negative 9(8%), *Klebsiella. pneumoniae* 4 (4%). *Streptococcus agalatiae* and *Staphylococcus pneumoniae* each accounted for 5 (5%) of the isolated microorganisms.

Table 4: The relationship between pathogens isolated and HIV status

Isolated pathogen	HIV Positive N= 148	HIV Negative N= 697	Total N=845
Cryptococcus neoformans	26 (18%)	23 (3%)	49 (6%)
Escherichia coli	1 (1%)	29 (4%)	30 (4%)
Klebsiella. pneumoniae	0 (0%)	4 (1%)	4 (0%)
Staphylococcus coagulase negative	4 (3%)	5 (1%)	9 (1%)
Staphylococcus aureus	2 (1%)	8 (1%)	10 (1%)
Streptococcus group B	1 (1%)	4 (1%)	5 (1%)
Streptococcus pneumoniae	1 (1%)	4 (1%)	4 (0%)

In the table 4 above, *Cryptococcus neoformans* was more detected in HIV patients with prevalence of 18% while it was 3% in HIV negative among hospitalized patients. The second most prevalent pathogen in HIV positive patients was *Staphylococcus* coagulase negative with a prevalence of 3% against 1% found in HIV negative patients. No *Klebsiella pneumoniae* was found in HIV positive patients.

Table 5: The relationship between patient outcome with HIV status

HIV status	Patient outcome		Total
	Death	Recovered	
Negative	38 (6%)	659 (94%)	697
Positive	12 (8%)	136 (92%)	148
Total	50 (6%)	795 (94 %)	845

Pearson Chi-Square = 1.547, df = 1 and a p-value = 0.214 (not significant > 0.05)

In patients with CNS infections, the mortality rate was higher in HIV patients at 18%, than in HIV negative at 6% as shown in table 5 above.

Table 6: The pathogen isolates compared with CSF protein

Pathogen	< 45 mg/dl	≥ 45 mg/dl	Total
Streptococcus agalactiae	2	3	5
Cryptococcus neoformans	3	46	49
Escherichia coli	19	11	30
Klebsiella pneumoniae	1	3	4
Staphylococcus coagulase negative	0	9	9
Staphylococcus aureus	5	5	10
Streptococcus pneumoniae	4	1	5
Total	34	78	112

Pearson Chi-Square = 40.8, df = 60 and a p-value = 0.9 (not significant > 0.05)

Table 6 above shows that infection with *Staphylococcus* coagulase negative had higher CSF protein concentration than other isolated microorganisms, 9/9 100%, followed by *Cryptococcus neoformans* 46 / 49 CSF protein concentration (93.9 %). The normal protein concentration in CSF is below 45mg/dl.

Table 7: The pathogen isolated compared with glucose concentration in CSF

Pathogen	≤40 mg/dl	>40 mg/dl	Total
Streptococcus agalactiae	3	2	5
Cryptococcus neoformans	36	13	49
Escherichia coli	9	21	30
Klebsiella pneumoniae	3	1	4
Staphylococcus coagulase negative	6	3	9
Staphylococcus aureus	4	6	10
Streptococcus pneumoniae	2	3	5
Total	63	49	112

Pearson Chi-Square = 43.9, df = 60 and a p-value = 0.9 (not significant > 0.05)

In the table 7 above, *Cryptococcus neoformans* was mostly isolated from samples with lower glucose concentration 36 / 49 (73.4%) followed by *Staphylococcus* coagulase negative 6/9 (66.6 %) compared to other isolated microorganisms. The normal glucose concentration in CSF is between 45 – 80 mg/dl.

4.4 Development of a novel algorithm for detection of most common bacterial, Viral and fungal etiological agents of meningoencephalitis.

In the existing CSF samples testing order, only gram staining, biochemistry and cytology were done on sites which have diagnostic limitations. With the new developed algorithms for CSF samples analysis on sites additional steps will be done as follows; culture, pathogen identification, susceptibility testing, submission of isolates and transfer all CSF samples to NRL for Real- time Multiplex PCR (see figure 10). At National reference laboratory, before new algorithm the diagnosis was limited to bacterial and fungal culture, isolation, susceptibility testing, biochemistry and cytology.

 The newly developed algorithm for NRL, will do all the above mentioned tests with addition of Real time Multiplex PCR to detect different viruses, bacteria and fungi in one day instead of 7 days and   confirm culture results from all isolated bacteria from the health facilities. Confirmed strains will be archived for research purposes (see figure 11).

The novel algorithm was developed for both conventional and molecular methods by combining diagnostic capacity in order to improve the overall diagnosis of CNS infections in Rwanda.

From the developed molecular diagnosis algorithm, 845 CSF samples were analysed. Ten types of viruses were detected in 152 samples and distributed as below*: Adenovirus* 38, *Epstein-Barr virus* 22, *Cytomegalovirus* 7*, Human Herpes virus* (6), *Human Herpes virus* (7) 3*, Herpes simplex virus* (1) 6, *Herpes simplex virus* (2) 3, *Enterovirus* 52 and *Human Parechovirus* 4*. Varicella-Zoster* 1 and *Parvovirus* (B19) 0. Similarly, 5 types of bacteria (*Streptococcus pneumoniae* 45, *Hemophilus influenzae* 25, *Neisseria meningitidis* 18, *Escherichia coli* 15, and *Listeria monocytogenes* 2) were detected in 105 samples of the 845 samples in which 88 were from patients over 5 years while 17 were from children under 5 years. *Escherichia coli* and *Listeria monocytogenes* were analysed and

detected in children under 5 years only. In addition, 49 samples of the 845 were positive for

Cryptococcus neoformans.
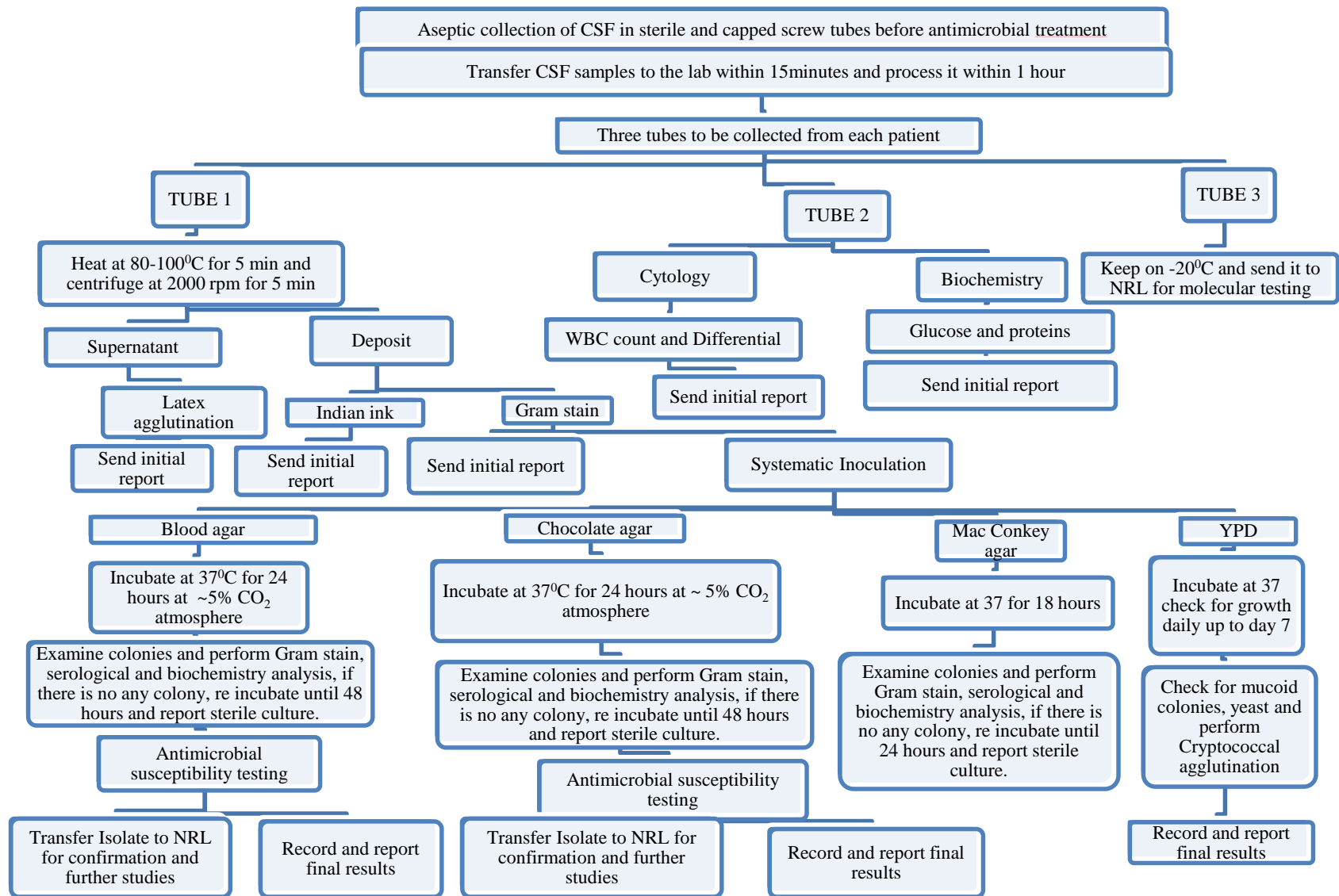
Figure 7: Developed novel algorithm for diagnosis of CNS infections using conventional method at District, Provincial and Referral Hospitals
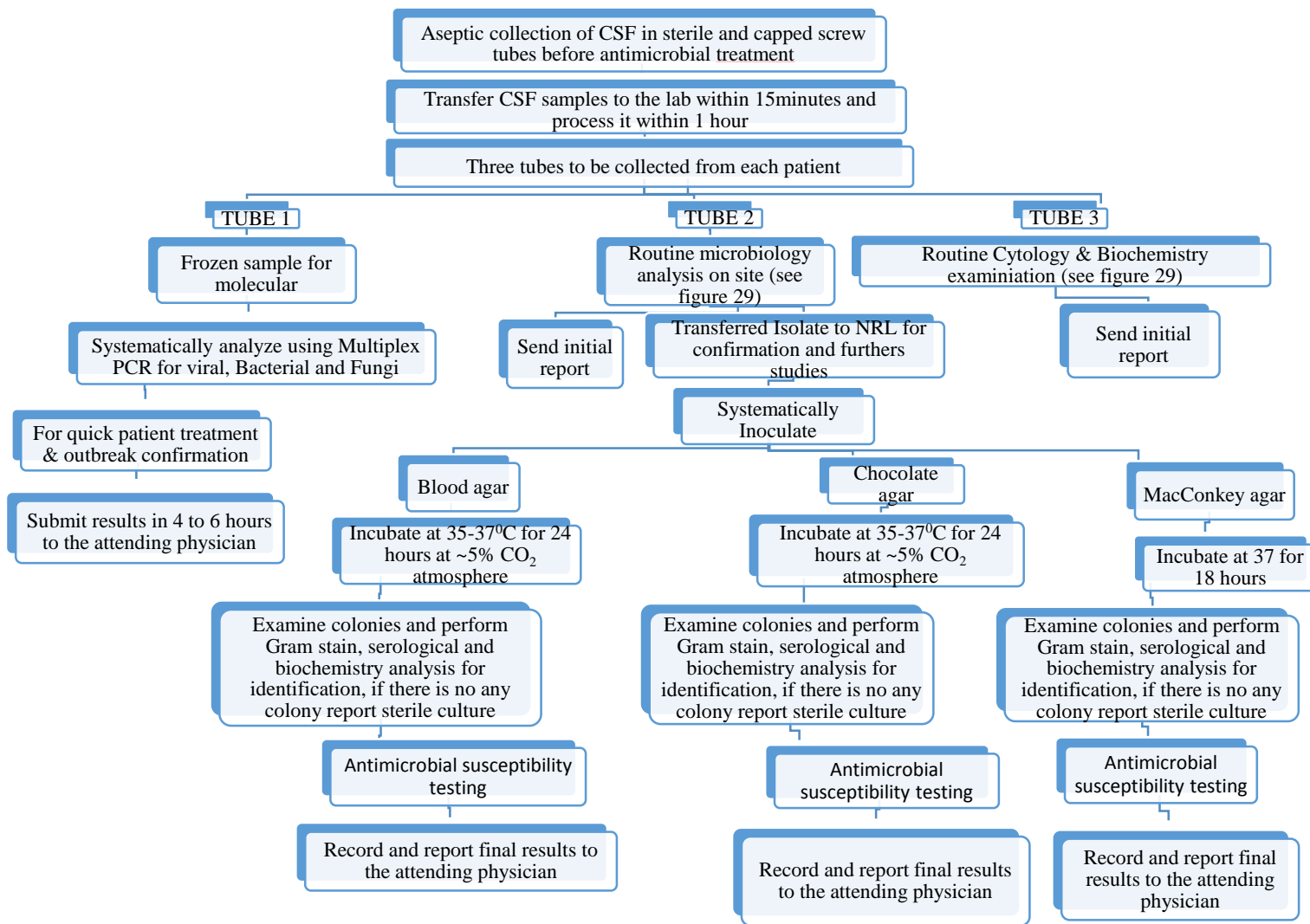
Figure 8: The developed novel algorithm for diagnosis of CNS infections using a combination of conventional and molecular methods at NRL

4.5 Determination of the drug susceptibility pattern of the detected bacteria agents of Meningoencephalitis

The drug susceptibility pattern was determined for all isolated bacteria using recommended antibiotic discs. The isolated bacteria were *Klebsiella pneumoniae, Escherichia coli, Streptococcus pneumoniae, Streptococcus agalatiae and Staphylococcus aureus*. The following antibiotics were used according to NRL standard operating procedures(SOPs); Ciprofloxacin, Tetracycline, Gentamycin, Sulfamethoxazole, Augmentin, Meropenem, Ampicillin, Azitreonam, Amikacin, Erythromycin, Penicillin, Cefotaxime, Linezolid, Piperacillin, Methicillin, Clindamycin and Oxacillin. The antibiotic was inoculated on Mueller-Hinton agar, Kirby-Bauer method and Clinical and Laboratory Standards Institute (CLSI) catalogue for result interpretation.

Representatives of susceptibility disc potency for isolated bacteria
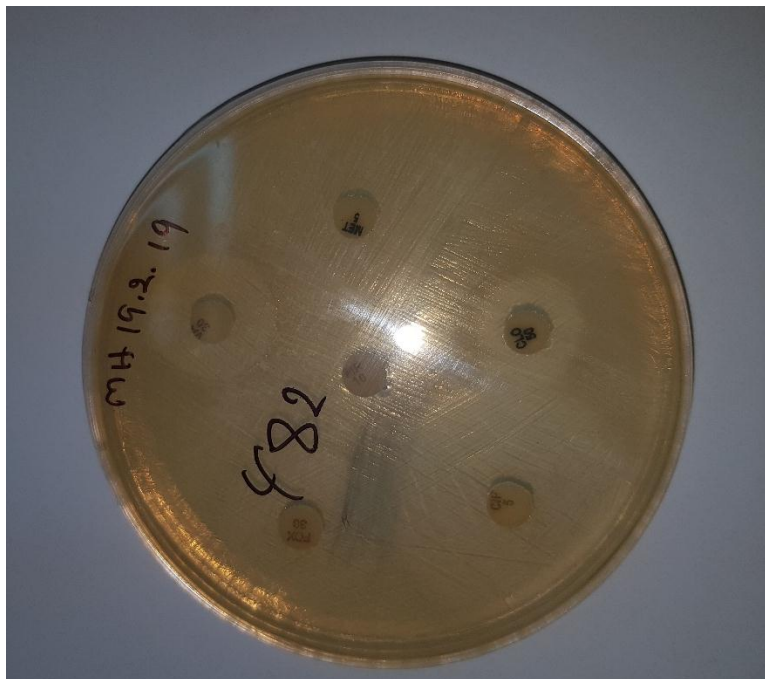
Figure 9 above shows a petri dish for sample 482 with *Escherichia coli* sensitive to amikacin, azitreonam, augmentin, sulfamethoxazole.

Figure 10 above shows a petri dish for sample No 44 with *Klebsiella pneumoniae* sensitive to augmentin, amikacin and meropenem.
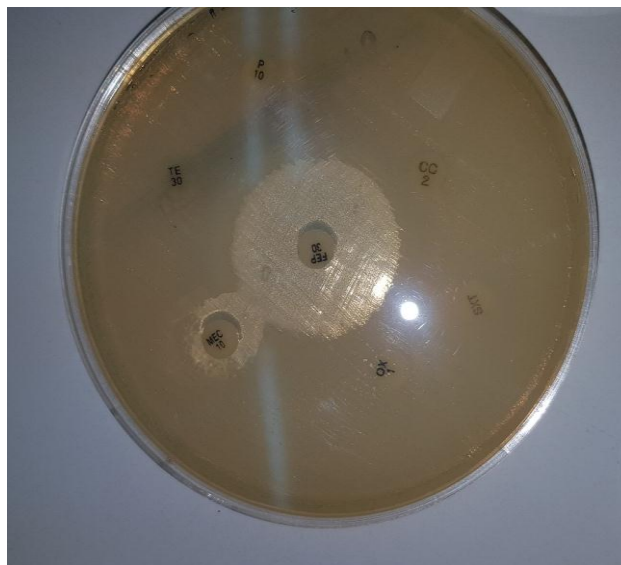
Figure 11 above shows a petri dish for sample No 132 with *Staphylococcus aureus* sensitive to oxacillin and methicillin and ciprofloxacin.

The results on interpreted susceptibility testing are indicated in the tables on the following pages.

Table 8: Sensitivity profile of isolated Escherichia coli and Klebsiella pneumoniae.

Pathogen	Meropenem	Sulfamethoxazole	Augmentin	Azitreonam	Amikacin
E.coli		74%	86%	83%	97%
K. pneumoni ae	75%	75%	100%	75%	75%

 Table 8 above shows that *Klebsiella pneumoniae* was 100% sensitive to augmentin and *Escherichia coli* was 97% sensitive to amikacin.

Table 9: Sensitivity profile of isolated Streptococcus agalactiae

Pathogen	Cefotaxime	Clindamycin	Linezolid	Piperacillin	Erythromycin
Streptococcus agalactiae	100 %	80%	80%	80%	80%

Streptococcus   agalactiae was 100% sensitive to cefotaxime and 80% sensitive to linezolid,

piperacillin, erythromycin and clindamycin as shown on table 9 above.

Table 10: Sensitivity profile of isolated Streptococcus pneumoniae

Pathogen	Piperacillin	Meropenem
Streptococcus pneumoniae	80 %	80%

Table 10 above shows that *Streptococcus pneumoniae* was 80% sensitive to meropenem and piperacillin.

Table11: Sensitivity profile of isolated Staphylococcus aureus

Pathogen	Oxacillin	Methicillin	Clindamycin	Penicillin	Tetracycline	Ciprofloxacin
S. aureus	90%	90%	80%	90%	70%	80%

Table 11 above shows *Staphylococcus aureus* was 90% sensitive to penicillin, oxacillin and methicillin. In addition, it was sensitive at 80% to ciprofloxacin and clindamycin.

4.6 Evaluation and demonstration of the utility of the molecular diagnostic technique over conventional techniques for detection of pathogens causing meningoencephalitis.

4.6.1. Outcome of culture at NRL

Table 12: Frequency of pathogen isolates by age group using culture at NRL

| Pathogen Isolated | Age group | | | | | | | |
	0 - 4	5 - 14	15 - 24	25 - 34	35 - 44	35 - 54	>55	Total
Streptococcus agalactiae	2	1	0	0	2	0	0	5
Cryptococcus. neoformans	1	3	7	6	10	12	10	49
Escherichia coli	23	4	1	0	1	1	0	30
Klebsiella pneumoniae	1	0	0	1	0	0	2	4
Staphylococcus coagulase negative	0	0	5	3	0	1	0	9
Staphylococcus aureus	2	1	0	3	1	2	1	10
Streptococcus pneumoniae	0	1	0	0	0	0	4	5
Sterile culture	104	63	156	166	118	59	67	732
Total	133	73	169	179	132	75	84	845

The positivity rates for isolated pathogen using culture at NRL was as follows: From 845 CSF samples cultured, 7% were positive for bacteria. The most isolated bacteria were *Escherichia coli*

30 (47%), followed by *Staphylococcus aureus* 10 (16%), *Staphylococcus* coagulase negative 9 (14%), *Streptococcus agalactiae* 5(8%), *Streptococcus pneumoniae* 5 (8%); and *Klebsiella pneumoniae*, 4(6%). Similarly, 49 samples out of 845 (6%) were positive for *Cryptococcus neoformans* as shown in table 12 above.

Table 13. Distribution of isolated pathogen by gender

		Gender	
Pathogen isolates	Total (n)	F (%)	M (%)
Cryptococcus neoformans	49	23(47%)	26(53%)
Escherichia coli	30	17(57%)	13(43%)
Klebsiella pneumoniae	4	3(75%)	1(25%)
Staphylococcus coagulase negative	9	4(44%)	5(56%)
Staphylococcus aureus	10	4(40%)	6(60%)
Streptococcus agalactiae	5	3(60%)	2(40%)
Streptococcus pneumoniae	5	3(60%)	2(40%)
Total	112	57	55

In table 13 above, pathogens isolated from culture are compared in relationship to gender. There is was significant difference in the isolated pathogens between men and women, since isolates from men were 55/411 (13%) and that from women were 57/434 (13%) Pearson Chi-Square=94, df 18 and a p-value=0.000 significant under 0.05.

.

4.6.2. Outcomes of RT-Multiplex PCR at NRL

Table 14: Frequency of viruses detected using RT multiplex PCR

Pathogen	Frequency	Positivity rate	Prevalence
Epstein-Barr virus	22	14 %	3 %
Cytomegalovirus	7	5%	1 %
Adenovirus	38	25 %	4 %
Herpes simplex virus 1	6	4 %	1 %
Herpes simplex virus 2	3	2%	0 %
Human Herpes virus 6	17	11 %	2 %
Human Herpes virus 7	3	2 %	0 %
Enterovirus	52	34 %	6 %
Human Parechovirus	4	3 %	0%
Total positive	152		18 %
Total sample	845		

The table 14 above shows that commercial validated RT-multiplex PCR kits for in-vitro diagnostics were used to detect up to 17 types of viruses, bacteria and fungus causing CNS infections.

Using the developed novel algorithm 845 CSF samples were collected and analysed. 152 (18%) were positive for the detected viruses.

In the figure 12 representing *Human adenovirus* below, the Ct value of the positive control is 25. The strongest Ct value was 22 seen in sample 2, which is stronger than positive control, followed by sample 145 and 195 both with Ct values of 24. The highest Ct value was 33 with lower positivity. The negative control did not show any Ct value.

Figure 12. Representation of *Human adenoviruses* (Ct values)

In figure 13 representing *Enteroviruses* below, the positive control Ct value was 21, strongest positive sample has Ct value of 33 which is sample 359 and 369. The lower positive has Ct value of 34 in which 18 samples were overlapping at the same Ct value.
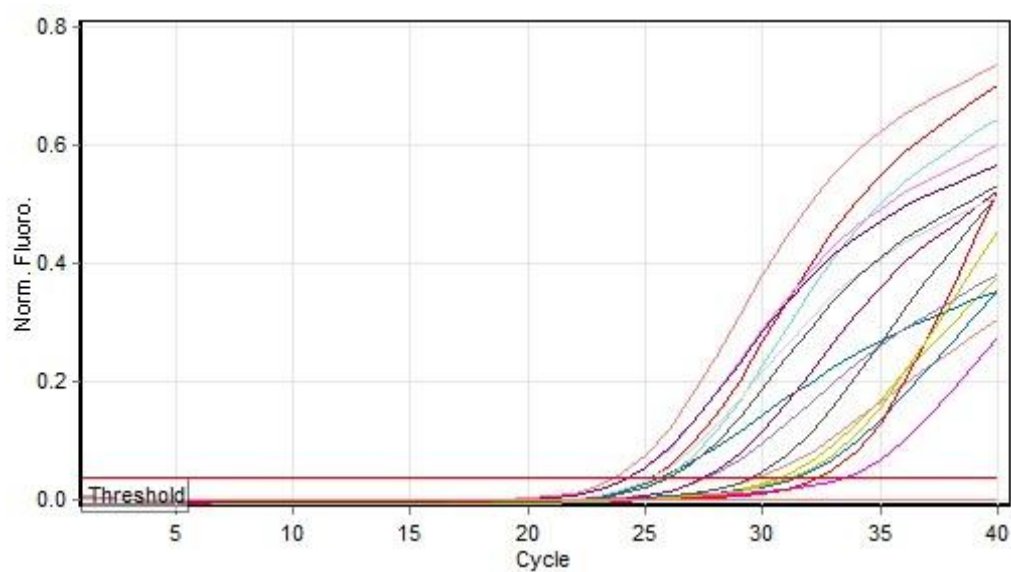The control negative does not show any Ct value.
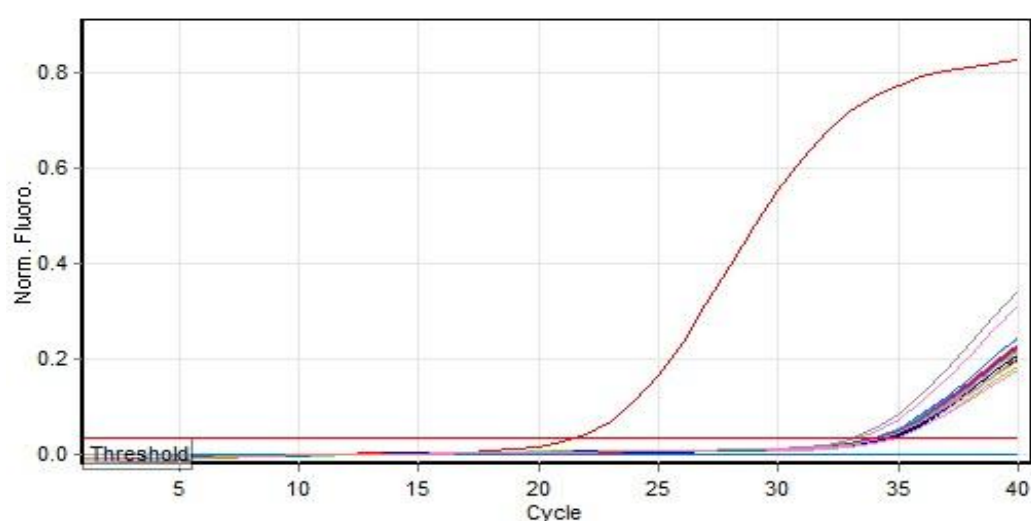
Figure13: Representation of *Enteroviruses* (Ct values)

In the figure 14 representing *Epstein Barr Virus* below, the positive control Ct value was 24, the strongest positive sample has Ct value of 22 which is sample 613, followed by Ct value of 30.

which is sample 581 followed by Ct value of 31 which is sample 592. The negative control does not show any Ct value.
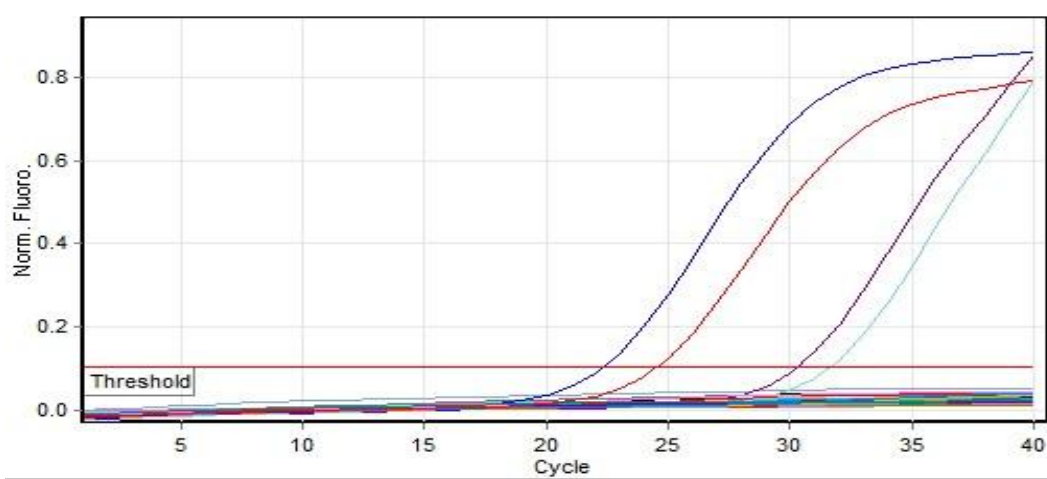
Figure14: Representation of *Epstein Barr Virus* (Ct values).

In the figure 15 representing *Human Herpes virus* 6, the positive control Ct value was 23 and the positive samples has Ct value of 25 which is sample 283 and Ct value of 31 which is sample 296. The negative control does not show any Ct value.
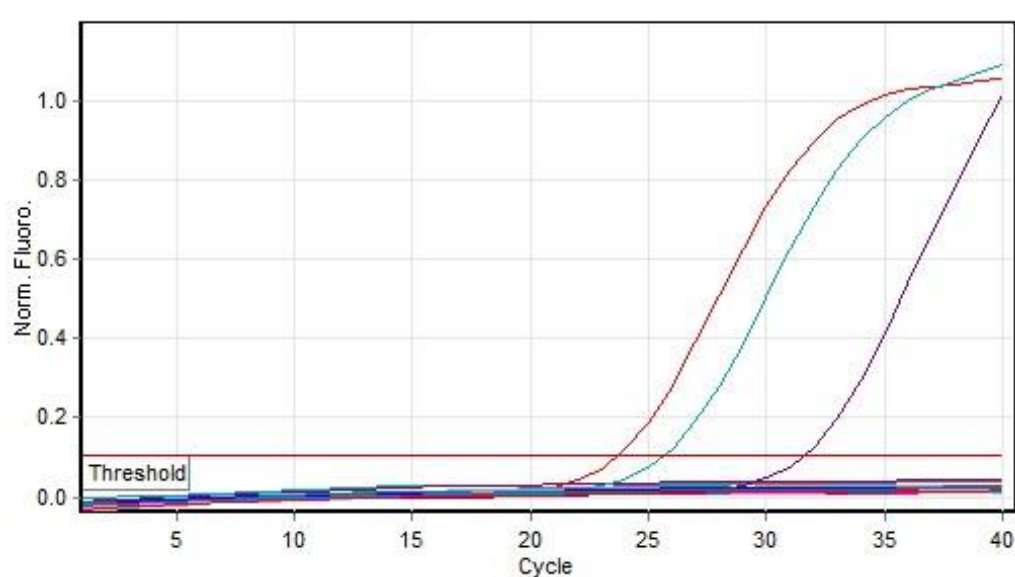
Figure15: Representation of *Human Herpes Virus* 6 (Ct values)

The figure 16 below represents *Herpes Simplex* 1 *virus*, the positive control Ct value was 21 and the positive sample has Ct value of 26 which is sample 682. The negative control does not show any Ct value.
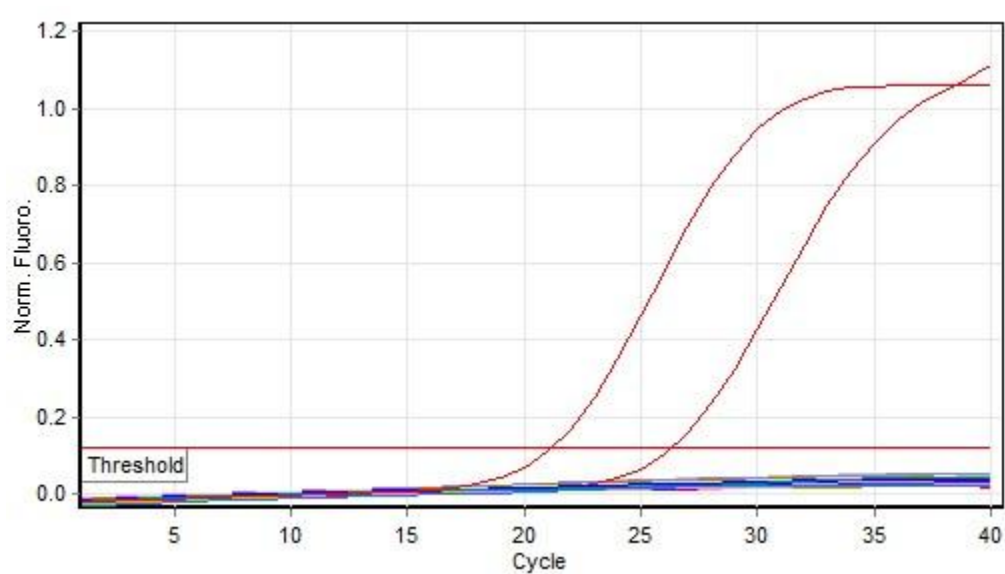
Figure 16: Representative of *Herpes Simplex 1 virus* (Ct values)

The figure 17 below represents *Cryptococcus neoformans,* the positive control Ct value was 21, strongest positive sample has Ct value of 33 which is sample 359 and 369. The lower positive has Ct value of 34 in which 18 samples were overlapping at the same Ct value. Negative control does not show any Ct value.
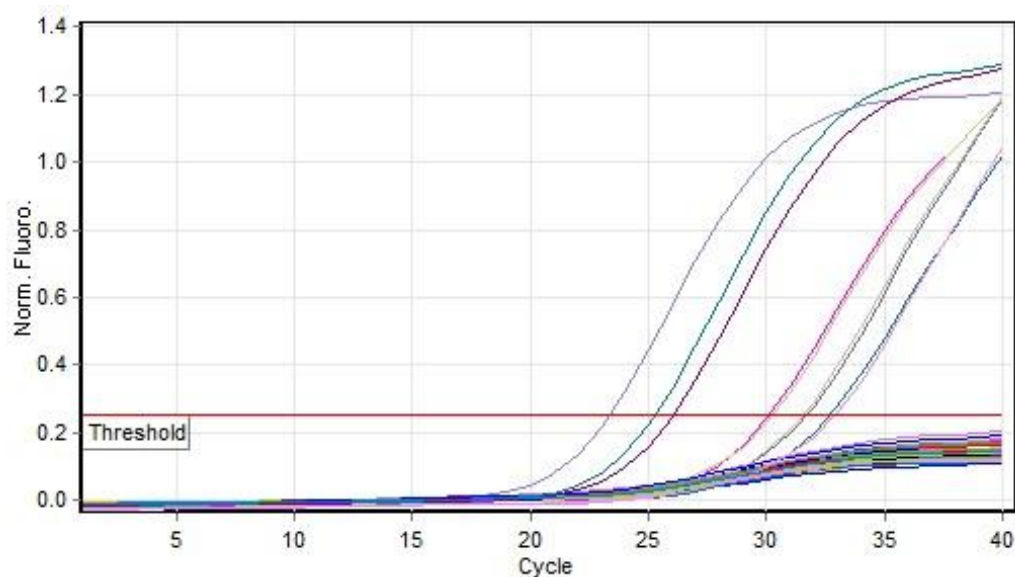

Figure 17: Representative of *Cryptococcus neoformans* (Ct values)

Table 15: Frequency of bacteria pathogens detected using molecular method

Pathogens	Frequency	Percentage
Hemophilus influenzae	25	2.90%
Neisseria meningitidis	18	2.10%
Streptococcus pneumoniae	45	5.3%
Total positive	88	4.2%
Total sample	845	10 %

 In the table 15 above, using molecular methods, 845 collected CSF samples were analysed for bacterial meningitis detection, 88 (10 %) samples were positive on the following microorganisms.

The most detected was *Streptococcus pneumoniae* 45 /88 isolated (51%) followed by and *Hemophilus influenzae* 25/ 88(28%) and *Neisseria meningitidis* with 18/ 88 (21%).

The figure18 below represents *Streptococcus pneumoniae* detected. The positive control Ct value was 24.8, strongest positive sample has Ct value of 23 which is sample 57. The third positive was known *Streptococcus pneumoniae* sample one with Ct value of 26.6. The lower positive has Ct value of 31.8 which was known *Streptococcus pneumoniae* sample two.
The Control negative does not show any Ct value.
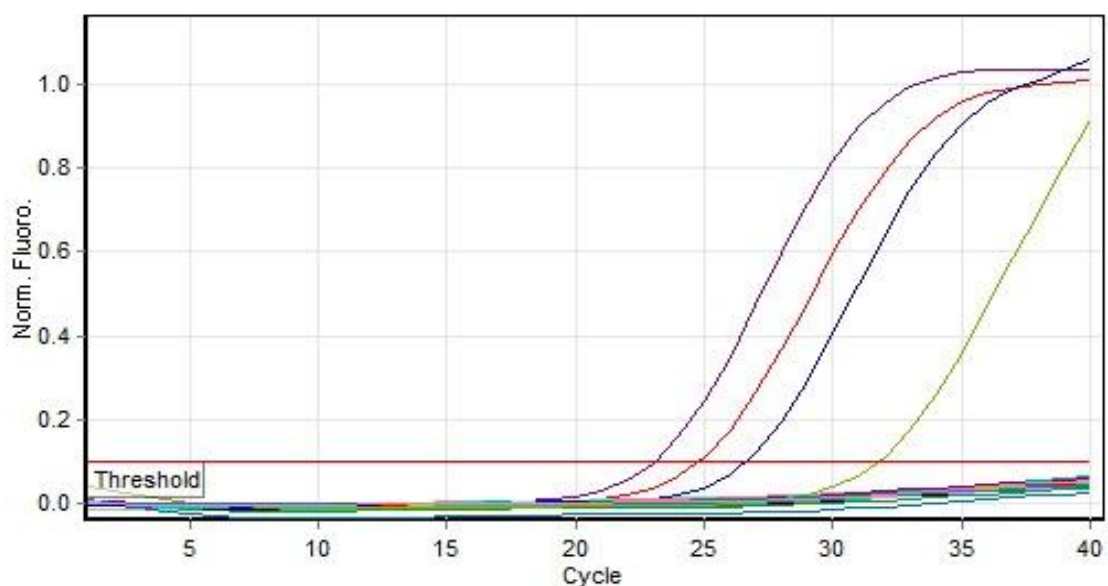
Figure 18: Representative of *Streptococcus pneumoniae* (Ct values)

The figure 19 below represents *Neisseria meningitidis* detected. The positive control one Ct value was 28.1. The positive control two Ct value of 28.9. The strongest Ct value was 17.9 which was sample 370 followed by known *Neisseria meningitidis* sample one with Ct value of 19.4 and known *Neisseria meningitidis* sample two with Ct value of 20. The lowest Ct value was 32 which was sample 371.The negative control does not show any Ct value.
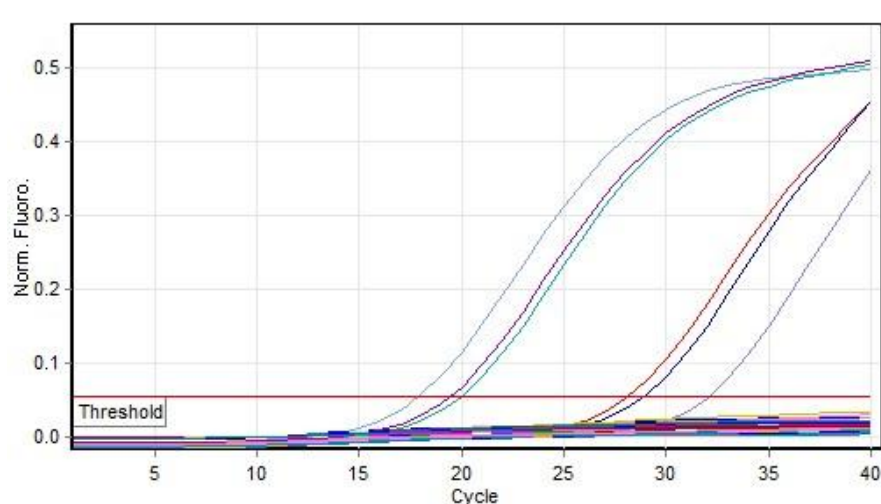
Figure 19: Representative of *Neisseria meningitidis* (Ct values)

The figure 20 below represents *Hemophilus influenzae* detected. The strongest Ct value was 18.8 which was sample 357. The positive control one has Ct value of 24.7. *Hemophilus influenzae* known sample has Ct value of 23.6. The positive control two has Ct value of 25. The lowest Ct value was 29.4 which was sample 371. The negative control does not show any Ct value.
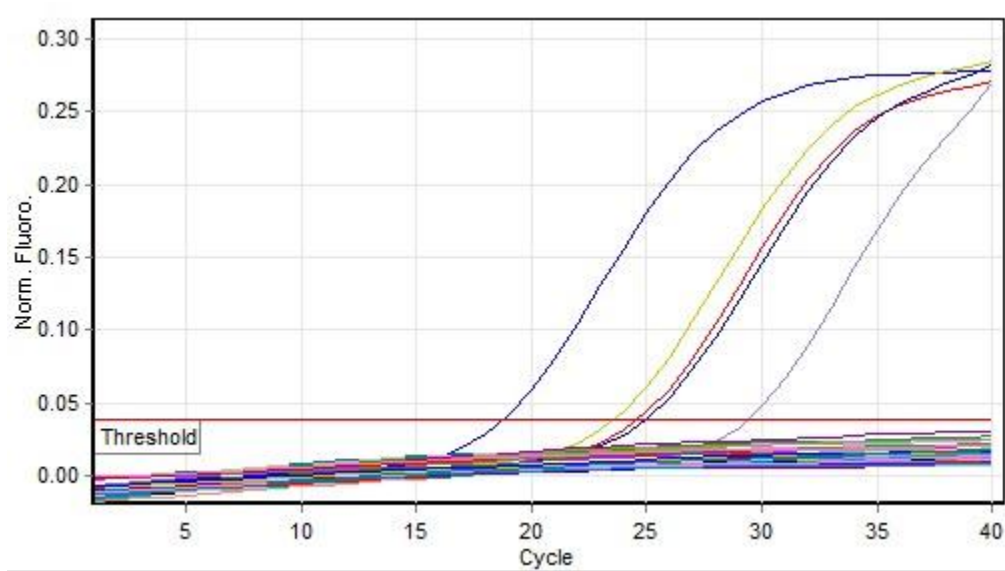
Figure 20: Representative of *Hemophilus influenzae* (Ct values)

Table16: Frequency of bacteria pathogens detected from neonatal CSF

Pathogen	Frequency	Prevalence
Listeria monocytogenes	2	1%
Escherichia coli	15	11%
Total Positive	17	12%
Total sample	138	

In the table 16 above, using a neonatal kit, 138 CSF samples were collected and analysed using molecular methods. 17/138 (12%) samples were positive on following microorganisms. The most detected organism was *Escherichia coli* 15/17 (88%) positive bacteria followed by *Listeria monocytogenes* 2/17 (12%).

The figure 21 below represents *Escherichia coli*. The strongest Ct value was 8 The positive control one has Ct value of 26 The known sample has Ct value of 26 The second strongest Ct value was 11 which was sample 559. The third Ct value was 12 which was sample 527. The fourth Ct value was 11.9 which was sample 513. The fifth Ct value was 12 which was sample 322. The sixth Ct value was 14.8 which was sample 452. The seventh Ct value was 26 which was sample 693. The lowest Ct value was 27 which was sample 426. The negative control does not show any Ct value.
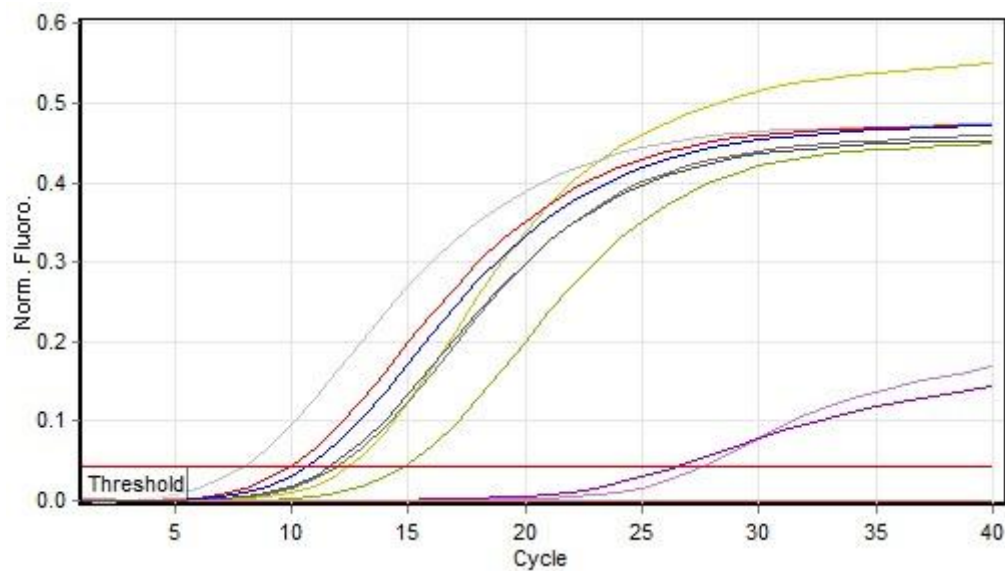
Figure 21: Representative of *Escherichia coli* (Ct values)

The figure 22 below represents *Listeria monocytogenes*. The strongest Ct value was 18.8 which was sample 543 followed by Ct value of 19.8 which was sample 570. The positive control has Ct value of 25. The negative control does not show any Ct value.
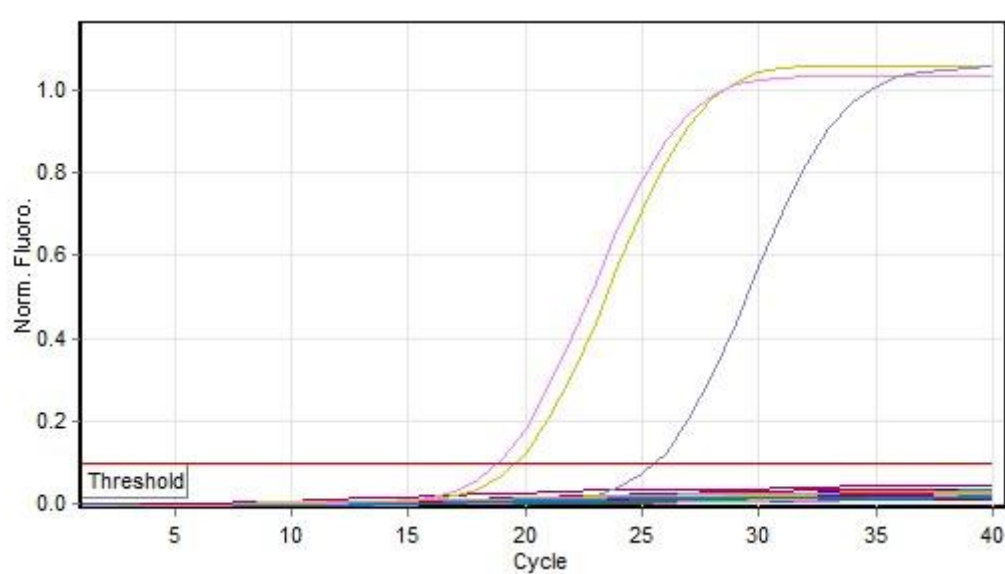
Figure 22: Representative of *Listeria monocytogenes* (Ct values)

The figure 23 below is a representative of internal control. The results shows that all Ct values were close together which indicates that the quality of results is quite accurate. This has been used in all samples including negative and positive controls. In some runs, the known sample from accredited external quality control laboratories were included in the runs to ensure the quality of results. The results are varied if the internal control (IC) have a Ct of less than 33.
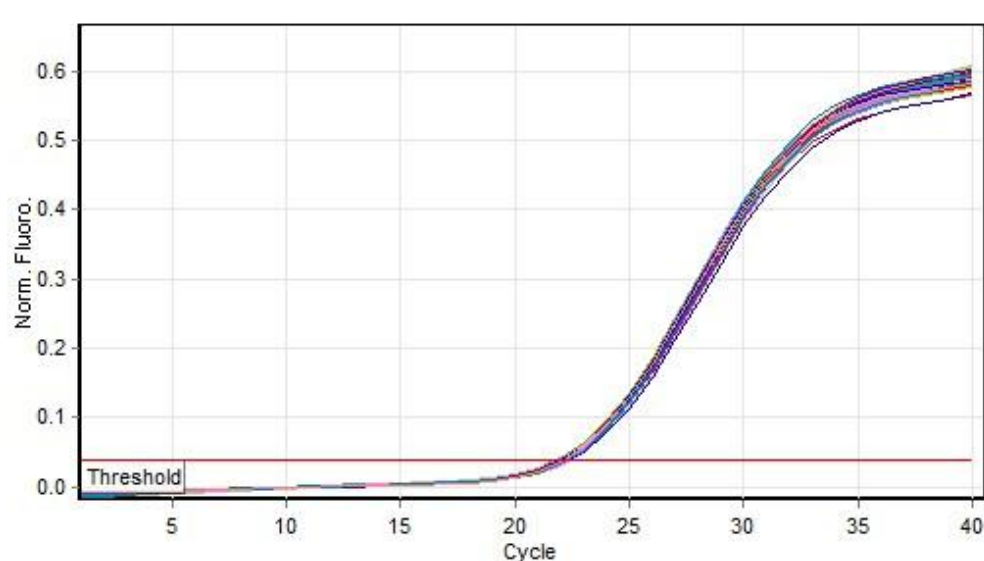
Figure 23: Representative of Internal control (Ct values)

CHAPTER FIVE: DISCUSSION

5. Discussion

The aim of this study was to develop a novel algorithm for detection of causative agents of meningoencephalitis using combination of conventional and molecular methods in Rwanda.

The study was encouraged by data collected from hospitalized patients in four referral hospitals namely King Faisal Hospital, Rwanda Military Hospital, Butare University Teaching Hospital, Kigali University Teaching Hospital for a period of 4 years from 2009 to 2012, which showed the diagnostic capacity of 9% positive and 91% negative. These results were inadequate for physicians to treat patients with appropriate drugs since, some bacteria, fungi and all viruses were not detected using conventional methods. Based on previous research, 75% of patient treatment is based on laboratory diagnosis. The 91% negative results from hospitalized patients with signs and symptoms of meningoencephalitis indicate limitation of appropriate treatment decisions by physicians. The developed novel algorithms combining both conventional and molecular techniques will help in improvement of diagnostic capacity for detection of causative agents of meningoencephalitis. The developed novel algorithm for molecular techniques has facilitated the establishment of new molecular laboratory at National Reference Laboratory, well equipped with high technology equipment from Germany and inaugurated by the Minister of Health. Laboratory staff have been recruited and trained on molecular techniques. The new technology established at NRL will be rolled out in the referral hospital laboratories, while the developed conventional algorithm will be established in referral, provincial and district hospitals laboratories.

The existing conventional methods before the development of the novel algorithm did not detect any viral infection, therefore treatment of patients suspected to have viral CNS infections was done based on signs and symptoms alone without differential diagnosis. The developed algorithm

improved diagnostic capacities by detecting 152 viral infections using Real-time Multiplex PCR. This represents 18% detection capacities of viral infections and is in agreement with similar study by (Koskiniemi et al., 2001) which reported Real-time Multiplex PCR as being specific and highly sensitive and rapid in detection of RNA and DNA viruses.

Real-time Multiplex PCR detected viruses as the most common cause of meningoencephalitis. *Enterovirus* had the highest positivity rate of 34%, followed by *Adenovirus* 25%, *Epstein-Barr virus* 15%, *Human Herpes virus* (6)11 %, and *Cytomegalovirus* 5%. The lowest positivity rate was *Varicella-Zoster* 1%. Moreover, based on our findings, molecular diagnostic method improved diagnostic capacity and therapeutic management of CNS infections by detecting multiple viral infections potentially responsible for mengoencephalitis , as reported similarly by (Quereda et al., 2000).

The detection capacity of bacterial infections improved from 7% using conventional to 22% using Real-time Multiplex PCR diagnostic methods. The usefulness of PCR diagnostic method is supported by previous studies (Issa et al., 2003) which emphasized the use of PCR diagnostic method in the detection of causative agents of CNS infection. However, the detection capacity of causative agents of fungal infections using conventional and Real-time PCR methods was the same at 6%.

Using Real- time Multiplex PCR different bacteria were detected using different Kits. Using bacterial meningitis Kit, 845 samples were analysed, 88 (10%) were positive for the following microorganisms; *Streptococcus pneumoniae* had a positivity rate of 45/88 (51%) followed by *Hemophilus influenzae* with positivity rate of 25/ 88(28%) and *Neisseria meningitidis* with positivity rate of 18/ 88 (21%).

Using Neonatal bacterial meningitis kit, 138 CSF specimens were analysed using Real time multiplex PCR.17/138 (12%) samples were positive for following microorganisms; *Escherichia coli* had a positivity rate 15/17 (88%) followed by *Listeria monocytogenes* 2/17 (12%). No Group B *Streptococcus* was detected.

Using both molecular and conventional techniques, 845 specimens were analysed. 49% were positive for *Cryptococcus neoformans. Cryptococcus neoformans* was more prevalent in HIV positive patients at 18% while it was 3% in HIV negative patients, as reported similarly by (Boulware et al., 2010).This indicates that *Cryptococcus neoformans* is mostly an opportunistic infection. The second most prevalent pathogen in HIV positive patients was *Staphylococcus* coagulase negative with a prevalence of 3% against 1% found in HIV negative patients.

The demographic representation of study population shows that about 65% of study population was between 24-34 years old. The highest prevalence of *Cryptococcus neoformans* was detected in CHUK study site. This could be due to HIV infection persons who had not actively participated in sensitized HIV screening in order to start ARV drugs treatment. The age group between 24 and 34 years old appears to be the most infected with HIV in this site since *C. neoformans* is primarily an opportunistic infection.

Escherichia coli was the most detected pathogen at CHUK. This could be due to referral nature of the hospital with paediatric speciality. CHUK study site had the most diverse number of pathogen isolates due to referral nature of the hospital.

 The study shows that the diagnostic capacity of etiological agents of CNS infection improved from baseline of 9 % using routine conventional methods (Ntagwabira et al., 2017), to 40% using developed novel algorithm combining both conventional and Real-time Multiplex PCR.

The antimicrobial susceptibility patterns of isolated bacteria causing meningitis, showed that *Klebsiella pneumoniae* was 100% sensitive to augmentin and 75% sensitive to meropenem, which is similar to previous research by (Pal Chugh et al., 2012) which reported 100% sensitivity of *Klebsiella pneumoniae* to meropenem.

Streptococcus pneumoniae was 100% sensitive to cefotaxime and 80% sensitive to piperacillin. This compares well with previous research which reported that *Streptococcus pneumoniae* had 94% susceptibility to piperacillin (Chugh et al., 2011)., and *Escherichia coli* was 97% sensitive to amikacin.

Streptococcus agalactiae was 100% sensitive to cefotaxime and 80% to linezolid, piperacillin erythromycin and clindamycin. *Staphylococcus aureus* was 90% sensitive to penicillin, oxacillin and methicillin and   80% sensitive to ciprofloxacin and clindamycin.

Due to the small number of isolated bacteria in this study (less 30% of the study population), these findings from the susceptibility profile cannot be used by the Ministry of Health to recommend as appropriate antibacterial treatment guideline but can be used as patient to patient treatment depending on isolated bacteria.

From the developed novel algorithm for diagnosis of central nervous system infections using conventional methods at district, provincial and referral hospitals, a recommendation that all isolates from these hospitals be transferred to the National reference laboratory for confirmation of bacterial isolates, antimicrobial susceptibility testing and further studies will guide the ministry of health to recommend every year appropriate antibiotics to be used for specific isolated bacteria in Rwanda health facilities .

The utility of molecular diagnostic method compared with conventional method was demonstrated. In the study, no viruses were detected using the conventional methods while a range of viruses, 152/845 (18%) were detected using Real time multiplex PCR. In addition, 185/845(22%) samples were detected as positive for different types of bacteria using the PCR compared with the 59/845(7%) that were positive using conventional methods. There was however no difference in the detection capacity of fungal agents between the two methods with a detection level of 6 %(49).

The results from this study indicate that, Real time Multiplex PCR is the best method to use for detection of causative agents of meningoencephalitis, after considering its cost compared with conventional method and turn- around time of patients results of 1 day for Real time Multiplex PCR and 7 to 11 days for conventional methods.

The cost of patient's hospitalization for seven to ten days waiting for the results is higher than a difference of two to four dollars cost for PCR test for conventional and Real time Multiplex PCR respectively. Furthermore, the cost of treatment with non-specific drugs will be reduced and other costs like those of molecular laboratory reagents and consumables will be negotiated in order to lower the costs of tests. In addition, the diagnostic results turnaround time of one day will significantly reduce mortality.

The Real-time PCR method facilitates rapid and early detection of causative agents of Central Nervous System infections with turn-around time of one day compared to conventional methods that take seven to ten days which delays treatment of patients with meningoencephalitis.

The findings from this research will furthermore help in use of appropriate drugs for treatment of patients. This will in turn prevent development of resistance to various drugs.

The use of the new diagnostic methods will help in disease surveillance to determine prevalence of meningoencephalitis accurately especially in Rwanda and Sub-saharan Africa in general.

6. Limitations

The RT multiplex PCR kits used in the study had specific number of targeted pathogens to be detected. This means that some other non-targeted pathogens could have been missed. Additionally, the study was not able to detect tuberculous and parasitic meningitis due to lack of reagents and consumables due to budget constraints.

Despite use of combined conventional and molecular methods, the detection capacity of etiological agents of meningoencephalitis was 40%. There is still need to conduct additional studies using various kits with more probes to enhance etiological diagnosis of meningoencephalitis.

7. Conclusion

This study shows that the diagnostic capacity of etiological agents of CNS infection improved from baseline of 9% using routine conventional methods, to 40% using developed novel algorithm combining both conventional and Real-time Multiplex PCR methods.

The novel algorithm was useful in detection of 18.0% of specific viral pathogens from CSF that were not previously detected using routine conventional diagnostic methods.

The study showed that from all the isolated bacteria causing meningitis, *Klebsiella pneumoniae* and *Streptococcus pneumonia*e were 100% sensitive to augmentin and cefotaxime respectively, while *Streptococcus pneumoniae* was highly resistant at, 60% to cefotaxime.

8. Recommendations

1. In order to improve the detection of causative agents of meningoencephalitis, all CSF samples collected from patients with meningoencephalitis should be transported to the National Reference Laboratory for molecular diagnosis. This will help in prompt treatment using appropriate drugs.

2. Based on the small number of isolated bacteria in this study, the susceptibility profile can only be used for patient treatment, but cannot help the Ministry of Health to recommend appropriate antibacterial treatment guidelines. Further studies on systematic susceptibility patterns should be done in clinical settings with more isolates to help policy makers come up with treatment guidelines for CNS infections.

3. The Ministry of Health in Rwanda should consider developing a policy that includes the use of this algorithim by health care professionals and laboratory networks in order to improve diagnosis and treatment of patients with meningoencephalitis at all levels in the country.

4. Additional studies with larger samples size should be done to increase detection capacity of etiologic agents of meningoencephalitis including *Mycobacterium tuberculosis, Toxoplasma gondii,* and *Naegleria fowleri.*

REFERENCES

Aguilar, J. et al. (2010) 'Staphylococcus aureus Meningitis', Medicine, 89(2), pp. 117–125. doi: 10.1097/MD.0b013e3181d5453d.

Aguilera, J.-F. et al. (2002) 'Outbreak of serogroup W135 meningococcal disease after the Hajj pilgrimage, Europe, 2000.', Emerging infectious diseases, 8(8), pp. 761–7. doi: 10.3201/eid0805.010422.

Ajello, G. W. et al. (1984) 'Trans-isolate medium: a new medium for primary culturing and transport of Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae.', Journal of clinical microbiology, 20(1), pp. 55–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6430956 (Accessed: 22 August 2018).

Bartt, R. (2012) 'Acute Bacterial and Viral Meningitis', CONTINUUM: Lifelong Learning in Neurology, 18(6), pp. 1255–1270. doi: 10.1212/01.CON.0000423846.40147.4f.

Baskin, H. J. and Hedlund, G. (2007a) 'Neuroimaging of herpesvirus infections in children.', Pediatric radiology, 37(10), pp. 949–63. doi: 10.1007/s00247-007-0506-1.

Baskin, H. J. and Hedlund, G. (2007b) 'Neuroimaging of herpesvirus infections in children.', Pediatric radiology, 37(10), pp. 949–63. doi: 10.1007/s00247-007-0506-1.

Berardi, A. et al. (2010) 'Group B Streptococcus Early-Onset Disease in Emilia-Romagna: Review After Introduction of a Screening-Based Approach', The Pediatric Infectious Disease Journal, 29(2), pp. 115–121. doi: 10.1097/INF.0b013e3181b83cd9.

Botero Aguirre, J. P. and Restrepo Hamid, A. M. (2015) 'Amphotericin B deoxycholate versus liposomal amphotericin B: effects on kidney function', Cochrane Database of Systematic Reviews, (11), p. CD010481. doi: 10.1002/14651858.CD010481.pub2.

Bouadma, L. et al. (2006) 'Adults with spontaneous aerobic Gram-negative bacillary meningitis admitted to the intensive care unit', Clinical Microbiology and Infection, 12(3), pp. 287–290. doi: 10.1111/j.1469-0691.2005.01346.x.

Boulware, D. R. et al. (2010) 'Clinical features and serum biomarkers in HIV immune reconstitution inflammatory syndrome after cryptococcal meningitis: a prospective cohort study.', PLoS medicine. Edited by D. G. Lalloo, 7(12), p. e1000384. doi: 10.1371/journal.pmed.1000384.

Brouwer, M. C., Tunkel, A. R. and Beek, D. van de (2010) 'Epidemiology, Diagnosis, and Antimicrobial Treatment of Acute Bacterial Meningitis', Clinical Microbiology Reviews. American Society for Microbiology (ASM), 23(3), p. 467. doi: 10.1128/CMR.00070-09.

Butler, E. K. et al. (2012) 'Long term 5-year survival of persons with cryptococcal meningitis or asymptomatic subclinical antigenemia in Uganda.', PloS one. Edited by D. W. Cameron, 7(12), p. e51291. doi: 10.1371/journal.pone.0051291.

Campagne, G. et al. (1999) '[Epidemiology and control of bacterial meningitis in children less than 1 year in Niamey (Niger)].', Bulletin de la Societe de pathologie exotique (1990), 92(2), pp. 118–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10399603 (Accessed: 23 August 2018).

Chang, C. C. et al. (2013) 'Clinical and mycological predictors of cryptococcosis-associated immune reconstitution inflammatory syndrome.', AIDS (London, England), 27(13), pp. 2089–99. doi: 10.1097/QAD.0b013e3283614a8d.

Chaudhuri, A. et al. (2008) 'EFNS guideline on the management of community-acquired bacterial meningitis: report of an EFNS Task Force on acute bacterial meningitis in older children and adults', European Journal of Neurology, 15(7), pp. 649–659. doi: 10.1111/j.1468-1331.2008.02193.x.

Cho, T. A. and Mckendall, R. R. (2014) 'Clinical approach to the syndromes of viral encephalitis, myelitis, and meningitis', Handbook of Clinical Neurology. Elsevier, 123, pp. 89–121. doi: 10.1016/B978-0-444-53488-0.00004-3.

Chugh, Y. et al. (2011) Study of antimicrobial sensitivity pattern of Gram-positive CSF isolates among children suffering from septic meningitis in a tertiary care hospital. Available at: http://medind.nic.in/jac/t11/i4/jact11i4p274.pdf (Accessed: 19 September 2018).

Coureuil, M. et al. (2017) 'A journey into the brain: insight into how bacterial pathogens cross blood-brain barriers.', Nature reviews. Microbiology, 15(3), pp. 149–159. doi: 10.1038/nrmicro.2016.178.

Elaldi, N. et al. (2013) 'Community-acquired CTX-M-15-type ESBL-producing Escherichia coli meningitis: a case report and literature review.', Journal of infection in developing countries, 7(5), pp. 424–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23669434 (Accessed: 18 September 2018).

Gaschignard, J. et al. (2011) 'Neonatal Bacterial Meningitis: 444 Cases in 7 Years.', The Pediatric infectious disease journal, 30(3), pp. 212–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21416693 (Accessed: 18 September 2018).

Grundmann, H. et al. (2011) 'A framework for global surveillance of antibiotic resistance', Drug Resistance Updates, 14(2), pp. 79–87. doi: 10.1016/j.drup.2011.02.007.

Hodson, E. M. et al. (2005) 'Antiviral medications to prevent cytomegalovirus disease and early death in recipients of solid-organ transplants: a systematic review of randomised controlled trials', The Lancet, 365(9477), pp. 2105–2115. doi: 10.1016/S0140-6736(05)66553-1.

Issa, M. et al. (2003) 'PCR of cerebrospinal fluid for diagnosis of bacterial meningitis during meningococcal epidemics; an example from Sudan.', Scandinavian journal of infectious diseases, 35(10), pp. 719–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14606610 (Accessed: 18 September 2018).

Johnson, F. T. (1996) Acute Encephalitis. Available at: https://academic.oup.com/cid/article-abstract/23/2/219/366873 (Accessed: 18 September 2018).

Kauffman, C. A. (2007) 'Histoplasmosis: a clinical and laboratory update.', Clinical microbiology reviews, 20(1), pp. 115–32. doi: 10.1128/CMR.00027-06.

Al Khorasani, A. and Banajeh, S. (2006) 'Bacterial profile and clinical outcome of childhood meningitis in rural Yemen: A 2-year hospital-based study', Journal of Infection, 53(4), pp. 228–234. doi: 10.1016/j.jinf.2005.12.004.

Kim, K. S. (2016) 'Human Meningitis-Associated Escherichia coli.', EcoSal Plus, 7(1). doi: 10.1128/ecosalplus.ESP-0015-2015.

Koskiniemi, M. et al. (2001) 'Infections of the central nervous system of suspected viral origin: a collaborative study from Finland.', Journal of neurovirology, 7(5), pp. 400–8. doi: 10.1080/135502801753170255.

Kyaw, M. H. et al. (2006) 'Effect of Introduction of the Pneumococcal Conjugate Vaccine on Drug-Resistant Streptococcus pneumoniae', New England Journal of Medicine, 354(14), pp. 1455–1463. doi: 10.1056/NEJMoa051642.

Leclercq, S. Y. et al. (2016) 'Pathogenesis of Streptococcus urinary tract infection depends on bacterial strain and β-hemolysin/cytolysin that mediates cytotoxicity, cytokine synthesis, inflammation and virulence', Scientific Reports, 6(1), p. 29000. doi: 10.1038/srep29000.

Lin, H.-A. et al. (2016) 'Regulator of the mucoid phenotype A gene increases the virulent ability of extended-spectrum beta-lactamase-producing serotype non-K1/K2 Klebsiella pneumonia', Journal of Microbiology, Immunology and Infection, 49(4), pp. 494–501. doi: 10.1016/j.jmii.2014.08.023.

Lion, T. (2014) 'Adenovirus infections in immunocompetent and immunocompromised patients.', Clinical microbiology reviews. American Society for Microbiology (ASM), 27(3), pp. 441–62. doi: 10.1128/CMR.00116-13.

Logan, S. A. E. and MacMahon, E. (2008) 'Viral meningitis.', BMJ (Clinical research ed.), 336(7634), pp. 36–40. doi: 10.1136/bmj.39409.673657.AE.

Malo, J. et al. (2014) 'Update on the diagnosis of pulmonary coccidioidomycosis.', Annals of the American Thoracic Society, 11(2), pp. 243–53. doi: 10.1513/AnnalsATS.201308-286FR.

McGill, F. et al. (2018) 'Incidence, aetiology, and sequelae of viral meningitis in UK adults: a multicentre prospective observational cohort study', The Lancet Infectious Diseases, 18(9), pp. 992–1003. doi: 10.1016/S1473-3099(18)30245-7.

Mengistu, A. et al. (2013) 'Antimicrobial sensitivity patterns of cerebrospinal fluid (CSF) isolates in Namibia: implications for empirical antibiotic treatment of meningitis.', Journal of pharmaceutical policy and practice. BioMed Central, 6, p. 4. doi: 10.1186/2052-3211-6-4.

Minjolle, S. et al. (2002) Detection of herpesvirus genomes by polymerase chain reaction in cerebrospinal fluid and clinical findings, Journal of Clinical Virology. Available at: www.elsevier.com/locate/jcv (Accessed: 25 August 2018).

Nielsen, J. S. et al. (2012) 'Intravitreal anti-vascular endothelial growth factor therapy for choroidal neovascularization secondary to ocular histoplasmosis syndrome.', Retina (Philadelphia, Pa.), 32(3), pp. 468–72. doi: 10.1097/IAE.0b013e318229b220.

Ntagwabira, E. et al. (2017) 'African Journal of Microbiology Research Challenges in diagnosis of central nervous system infections using conventional method: Need for better approach in Rwanda', 11(35), pp. 1379–1385. doi: 10.5897/AJMR2017.8627.

Pal Chugh, Y. et al. (2012) Internet Journal of Medical Update Prevalence of Gram-negative Pathogens and their antimicrobial susceptibility in bacterial meningitis in pediatric cases, Internet Journal of Medical Update. Available at: http://www.akspublication.com/ijmu (Accessed: 19 September 2018).

Pandit, L. et al. (2005) 'Diagnosis of partially treated culture-negative bacterial meningitis using 16S rRNA universal primers and restriction endonuclease digestion', Journal of Medical Microbiology, 54(6), pp. 539–542. doi: 10.1099/jmm.0.45599-0.

Pappagianis, D. and Coccidioidomycosis Serology Laboratory (2007) 'Coccidioidomycosis in California state correctional institutions.', Annals of the New York Academy of Sciences, 1111(1), pp. 103–11. doi: 10.1196/annals.1406.011.

Pomar, V. et al. (2013) 'Spontaneous gram-negative bacillary meningitis in adult patients: characteristics and outcome', BMC Infectious Diseases. BioMed Central, 13(1), p. 451. doi: 10.1186/1471-2334-13-451.

Quereda, C. et al. (2000) 'Diagnostic utility of a multiplex herpesvirus PCR assay performed with cerebrospinal fluid from human immunodeficiency virus-infected patients with neurological disorders.', Journal of clinical microbiology, 38(8), pp. 3061–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10921978 (Accessed: 19 September 2018).

Richardson, A. R. et al. (2002) 'Mutator clones of Neisseria meningitidis in epidemic serogroup A disease.', Proceedings of the National Academy of Sciences of the United States of America, 99(9), pp. 6103–7. doi: 10.1073/pnas.092568699.

Saarinen, M. et al. (1995) 'Spectrum of 2,836 cases of invasive bacterial or fungal infections in children: results of prospective nationwide five-year surveillance in Finland. Finnish Pediatric Invasive Infection Study Group.', Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 21(5), pp. 1134–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8589133 (Accessed: 22 August 2018).

Scriven, J. E. et al. (2017) 'The CSF Immune Response in HIV-1-Associated Cryptococcal Meningitis: Macrophage Activation, Correlates of Disease Severity, and Effect of Antiretroviral Therapy.', Journal of acquired immune deficiency syndromes (1999), 75(3), pp. 299–307. doi: 10.1097/QAI.0000000000001382.

Steer, P. J. and Plumb, J. (2011) 'Myth: Group B streptococcal infection in pregnancy: Comprehended and conquered', Seminars in Fetal and Neonatal Medicine, 16(5), pp. 254–258. doi: 10.1016/j.siny.2011.03.005.

Stephens, D. S. (2007) 'Conquering the meningococcus.', FEMS microbiology reviews, 31(1), pp. 3–14. doi: 10.1111/j.1574-6976.2006.00051.x.

Taylor, W. R. et al. (2012) 'The Spectrum of Central Nervous System Infections in an Adult Referral Hospital in Hanoi, Vietnam', PLoS ONE. Edited by J. A. Stoute. Public Library of Science, 7(8), p. e42099. doi: 10.1371/journal.pone.0042099.

Teckie, G. and Karstaedt, A. (2015) 'Spontaneous adult Gram-negative bacillary meningitis in Soweto, South Africa', International Journal of Infectious Diseases, 30, pp. 38–40. doi: 10.1016/j.ijid.2014.10.006.

Thigpen, M. C. et al. (2011) 'Bacterial meningitis in the United States, 1998-2007.', The New England journal of medicine, 364(21), pp. 2016–25. doi: 10.1056/NEJMoa1005384.

Van De Beek, D., Drake, J. M. and Tunkel, A. R. (2010) Downloaded from www.nejm.org at CAUL on March 16, N Engl J Med. Available at: www.nejm.org (Accessed: 18 September 2018).

Watt, J. P. et al. (2009) 'Burden of disease caused by Haemophilus influenzae type b in children younger than 5 years: global estimates', The Lancet, 374(9693), pp. 903–911. doi: 10.1016/S0140-6736(09)61203-4.

Zaal, M. J. W., Völker-Dieben, H. J. and D'Amaro, J. (2003) 'Visual prognosis in immunocompetent patients with herpes zoster ophthalmicus.', Acta ophthalmologica Scandinavica, 81(3), pp. 216–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12780396 (Accessed: 25 August 2018).

APPENDICES

1. Appendix 1: Case report form
2. Appendix 2: Study sites map
3. Appendix 3: A and B- Consent forms
4. Appendix 4: A and B- Assent forms
5. Appendix 5: Ethics Approvals
6. Appendix 6: Molecular protocols
7. Appendix 7: Published paper 1 and 2 abstracts