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Prevalence of bacterial isolates from cerebrospinal fluid, their antimicrobial susceptibility pattern and associated risk factors with special emphasis on *streptococcus pneomoniae* among pediatrics suspected meningitis patients at Tikur Anbessa and Yekatit 12 specialized hospitals, Addis Ababa, Ethiopia.

By: Mulu Hassen (BSc)

A thesis submitted to the School of graduate studies of Addis Ababa University in partial fulfillment to the requirements of the degree of Master of Sciences in Clinical Laboratory Sciences (Diagnostic and public health microbiology)

Advisors: Gebru Mulugeta (BSc, MSc)

Kassu Desta (BSc, MSc, Assistant professor)

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SCHOOL OF GRADUATE STUDIES

"Prevalence of bacterial isolates from cerebrospinal fluid, their antimicrobial susceptibility pattern and associated risk factors with special emphasis on *streptococcus pneomoniae* among pediatrics suspected meningitis patients at Tikur Anbessa and Yekatit 12 specialized hospitals, Addis Ababa, Ethiopia."

By: Mulu Hassen (BSc)

School of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University

Approved by the Examining Board

______________________________	_______________	_______________
Chairman, Dep. Graduate Committee	Signature	Date

Advisors

Gebru Mulugeta (BSc, MSc, Lecturer)	_______________	_______________
	Signature	Date
Kassu Desta (BSc, MSc, Ass. Professor)	_______________	_______________
	Signature	Date
Dr.Adane Bitew	_______________	_______________
Internal Examiner	Signature	Date
Dr. Solomon	_______________	_______________
External Examiner	Signature	Date

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Table of contents

Contents **Pages**

List of tables

List of figures

List of acronyms and abbreviations

ABM	Acute bacterial meningitis
ATCC	American type culture collection
BAP	Blood agar plate
BSC	Bachelor of Sciences
CDC	Center for disease control and prevention
CSF	Cerebrospinal fluid
CLSI	Clinical and Laboratory standards Institute
CNS	Central nervous system
DRERC	Departmental research and ethics review committee
Hib	*Haemophilus influenza type b*
ICT	Immunochromatographic test
IRB	Institutional review board
ID	Identification number
MRSA	Methicillin resistant *staphylococcus aureus*
PI	Principal Investigator
PCR	Polymerase chain reaction
RPM	Revolution per minute
SD	Standard deviation
SMLS	School of medical laboratory sciences
SPSS	Statically package for social sciences
SOP	Standard operating procedure
TSI	Triple sugar iron
WBC	White blood cells
WHO	World health organization

Abstract

Background: Bacterial meningitis remains a major cause of mortality and morbidity in neonatology and pediatrics patients in many countries of the world including Ethiopia. Information on prevalence of bacterial meningitis, susceptibility of the causative microorganism to rationalize treatment and associated risk factors is scare among pediatrics groups.

Objective: To isolate bacteria's from CSF specimen, determine antimicrobial susceptibility pattern and to assess associated risk factors with special emphasis on *Streptococcus pneumoniae* among pediatrics suspected meningitis patients attending pediatrics clinic at Tikur Anbessa and Yekatit 12 specialized hospitals, Addis Ababa, Ethiopia.

Methodology: A hospital based cross sectional study was conducted at Tikur Anbessa and Yekatit 12 specialized hospitals, from September, 2013 to January, 2014. A consecutive sampling technique was used. Three hundred eighty five pediatrics patients attending the hospital at pediatrics ward that were gave CSF samples for diagnostic purpose were enrolled in the study. Samples were taken from them and analyzed according to standard microbiological (culture) procedures, antimicrobial susceptibility pattern were determined using disc diffusion technique and serological (BinaxNOW *streptococcus pneumoniae* antigen test) procedure was also done. Datas were double entered with EPI INFO version 3.5.3 and analyzed using SPSS version 21 software. Binary logistic regression was used to identify the association between variables. Sensitivity, specificity, PPV and NPV were used to see the performance of the ICT.

Result: Bacterial pathogens were isolated from 17 patients showing an isolation rate of 4.4%.Among these, 58.8% and 41.2% were gram negative and gram positive organisms respectively. The most commonly isolated bacteria were *S.pneumoniae* (35.3%), followed by *Neisseria meningitidis* (11.8%). Among all risk factors assessed, none of them were statistically significant with suspected meningitis cases ($p>0.05$). The antimicrobial sensitivity remained high for third generation cephalosporins for most of the isolates. The ICT increased the detection of pneumococcus over culture.

Conclusion The prevalence of bacterial isolates in this study was 4.4%. Antigen detection (BinaxNOW *S.pneumoniae* test in our study) is a better adjuvant to culture. Frequency of single as well as multiple drug resistance was very high among the bacterial isolates. Area specific periodic evaluation of antimicrobial susceptibility test will be important.

1. Introduction

1.1.Background

Meningitis also termed arachnoiditis or leptomeningitis, is an inflammation of the membranes that surround the brain and spinal cord, thereby involving the arachnoid, the piamater, and the interposed cerebrospinal fluid (CSF). The inflammatory process extends throughout the subarachnoid space around the brain, the spinal cord, and the ventricles [1].

It has been divided into bacterial meningitis and aseptic meningitis. Bacterial or pyogenic meningitis is an acute meningeal inflammation secondary to a bacterial infection that generally evokes a polymorphonuclear response in the CSF. Aseptic meningitis refers to a meningeal inflammation without evidence of pyogenic bacterial infection on Gram's stain or culture, usually accompanied by a mononuclear pleocytosis. Aseptic meningitis is subdivided into two categories: nonbacterial meningeal infections (typically viral or fungal meningitis), and noninfectious meningeal inflammation from systemic diseases (such as sarcoidosis), neoplastic disease (leptomeningeal carcinomatosis or neoplastic meningitis), or drugs [2].

Bacterial meningitis is an acute infection in which the meninges, the subarachnoid space, and the brain parenchyma are all frequently involved in the inflammatory reaction. This disease is characterized by severe headache, fever, intolerance to light and sound and rigidity of muscles, especially those of the neck. The central nervous system (CNS) inflammatory reaction from bacterial meningitis may result in decreased consciousness, seizures, raised intracranial pressure, and stroke [3].

The organisms most commonly responsible for bacterial meningitis are *Streptococcus pneumoniae (S.pneumoniae), Neisseria meningitidis (N.meningitidis), Streptococci group B, Listeria monocytogenes, and Haemophilus influenzae.* In children, meningococcal, *Haemophilus influenzae type b (Hib*), and pneumococcal infections are the most common causes [4].

Streptococcus pneumoniae bacteria are small (0.5-1.0µm) lanceolate, gram-positive, facultative anaerobic organisms. They are typically observed in pairs (diplococcic) but may also occur singularly or in short chains. Pneumococci are encapsulated, their surfaces composed of complex polysaccharides. Encapsulated organisms are pathogenic for humans and experimental animals, whereas organisms without capsular polysaccharides are not. Capsular polysaccharides are the primary basis for the pathogenicity of the organism. They are antigenic and form the basis for classifying pneumococci to different serotypes [5].

Meningitis caused by bacteria is a medical emergency. The therapeutic goal is to initiate antibiotic therapy within 60 minutes of patient arrival in the emergency room. In patients suspected of having bacterial meningitis, CSF should be obtained for cultures and empirically antimicrobial therapy initiated without delay. For many years β-lactams have comprised the corner stones of therapy and parenteral third generation cephalosporins such as ceftriaxone or cefotaxime are most commonly used [6, 7].

For the diagnosis of bacterial meningitis, CSF examination is mandatory. CSF culture is the "gold standard" for diagnosis, and it is obligatory to obtain the *in vitro* susceptibility of the causative microorganism and to rationalize treatment. CSF Gram staining, antigen tests, latex agglutination testing and PCR are additional diagnostic tools that might aid in etiological diagnoses, especially for patients with negative CSF cultures. Culture-based methods lack sensitivity, take long period of time to diagnose the patient, the BinaxNOW *S.pneumoniae* antigen test, an immunochromatographic membrane assay that detects the presence of the C polysaccharide cell wall antigen common to all pneumococcal serotypes, is useful for the rapid diagnosis from CSF samples of invasive pneumococcal disease [6].

Meningitis can occur at any age and in previously healthy individuals. There are some risk factors that predispose the individual to meningitis. Host risk factors can be grouped into four categories: age, demographic/socioeconomic factors, exposure to pathogens, and immunosuppression. Patients at the extremes of age: the elderly (age over 60 years) and pediatric patients (young children age younger than 5 years, especially infants/neonates) have an increased susceptibility to meningitis [2].

1.2. Statement of the problem

Bacterial meningitis is one of the most severe infectious diseases, causing neurologic sequelae and accounting for an estimated 200,000 deaths worldwide per year. Although most diseases occur in infants, the societal impact is also important because of the continued high incidence in healthy older children and adolescents. Despite many new antibacterial agents, bacterial meningitis fatality rates remain high, with reported rates between 2% and 30%. Furthermore, permanent sequelae, such as epilepsy, mental retardation, or sensor neural deafness are observed in 10%–20% of those who survive [8, 9].

It is a public health problem demanding early diagnosis, effective treatment, prevention and control. There are three main organisms that account for over 90% of the world's cases of meningitis. These are *N. meningitidis, S. pneumoniae* and *H. influenzae type b* (Hib). In Africa, meningitis prevails in the semiarid sub-Saharan area between 10º and 12º North latitude from West to East Africa, dubbed the meningitis belt. A region in sub-Saharan Africa, extending from Ethiopia in the east to the Gambia in the west and containing 15 countries with >260 million people, is known as the "meningitis belt" because of its high prevalence of endemic disease with periodic epidemics caused by *N.meningitidis* [9,10].

Endemic meningitis among children takes the form of sporadic cases or small clusters with an endemicity rate of 1.5/100,000 and 20/100,000 population in the developed and developing countries, respectively. At least 890,000 cases [500,000 in Africa; 210,000 in pacific countries; 100,000 in Europe and 80,000 in America] are estimated to occur annually. Of these cases, 160,000 and 135,000 of them are disabling and fatal, respectively [11].

Despite great advances in antimicrobial therapy, neonatal and pediatric life support measures, bacterial sepsis, and meningitis continue to be a major cause of morbidity and mortality in newborns, particularly in infants. A wide variety of spectrum of organisms has been described for cases of septic meningitis and this spectrum is subjected to geographical alterations. The organisms isolated are more often resistant to multiple antimicrobials, making treatment more difficult and leading to grave sequelae [10].

This calls for the need of bacteriological monitoring in pediatric wards and their antibiotic sensitivity pattern. Neonates are particularly vulnerable to infections, so any delay in the initiation of empirical therapy or wrong choice of antibiotic could be fatal. Antibiotics are usually administered before the laboratory results of CSF culture and sensitivity are available. To ensure appropriate therapy, current knowledge of the organisms that cause bacterial meningitis and their antibiotic susceptibility pattern in a particular setting or region is of utmost importance [6].

The gold standard diagnostic method continues to be culture for diagnosis of bacterial meningitis. Even though there are limitations of this method in the detection of meningitis cases. The limited diagnostic technique for this infection in the country remains a challenge for improving case detection rate. Therefore antigen detection of cerebrospinal fluid specimen provides a useful adjunct to culture-based diagnosis [5].

Over two-thirds of all cases of bacterial meningitis occur in children less than five years old. The disease can occur at any age and in previously healthy individuals, although some patients have an increased risk of meningitis including pediatrics However, there is limited information available on factors associated with an increased risk for bacterial meningitis in pediatrics [2].

Therefore, the aim of this study was to isolate *streptococcus pneumoniae* and other bacteria's from cerebrospinal fluid, their antimicrobial susceptibility pattern and associated risk factors on pediatrics suspected meningitis patients at Tikur Anbessa and Yekatit 12 specialized hospitals, Addis Ababa, Ethiopia, September, 2013 to January, 2014.

1.3. Significance of the study

To be able to diagnose and manage properly, pediatricians' have to be aware of the types of micro-organisms prevalent in the local community and their susceptibility against different antibiotics. What is true in the western may not be similar in other part of the world. There is paucity of objective data on the causative agents and their susceptibility in these hospital populations. The current study was undertaken to fill this gap.

All over the world, indiscriminate and irrational use of antimicrobials has led to the development of antimicrobial resistance owing to significant changes in microbial genetic ecology. Therefore studying types of micro-organisms prevalent and antimicrobial susceptibility pattern of them will have useful advantage for clinicians by aiding in the selection of proper drugs for pediatric meningitis diseases; it will also strengthens the struggle towards the achievement of the millennium development goal 4 of reducing child death by two-thirds by 2015.

In addition to generating data on the distribution of bacterial isolates from CSF specimen and their antimicrobial susceptibility pattern related risk factors will give appropriate information for policy makers. The data that can be obtained from this study will be part of the solution for current program or will be components for future studies.

This study was also carried out for detection of *S. pneumoniae* antigen in CSF samples which will be an alternative for the diagnosis of pneumococcal meningitis and knowing risk factors will be important for designing preventive strategies.

The purpose of this study was, therefore, to identify the prevalence of bacterial pathogens that cause bacterial meningitis, to determine their antimicrobial susceptibility pattern and associated risk factors among pediatrics suspected meningitis patients at Tikur Anbessa and Yekatit 12 specialized hospitals.

2. Literature Review

A comprehensive retrospective study presented with data on causes of bacterial meningitis and their susceptibility pattern among children at Children's Medical Center, a referral tertiary care center in Iran from 1998 to 2008. Of 11269 CSF cultures examined, 329 (2.9%) were positive for bacterial growth. *Staphylococci species* and gram-negative enteric organisms were the most common pathogens isolated. In addition, high rates of oxacillin and vancomycin resistance were found among *staphylococci*. In this study more than 80% of gram-negative enteric bacteria were resistant to ampicillin; they also found high rates of cephalosporin resistance among these organisms. Over 55% of *S. pneumoniae* were resistant to penicillin [12].

A study conducted in Nepal in 2011 to determine bacteriological agents, clinical profile and immediate outcome in patients admitted to children's ward of Patan Hospital with meningitis. The result showed that out of 7,751 children, 296 (3.8%) had meningitis. This was a group ranging from neonates to adolescents aged 18 years. Only 13 (4.4%) of CSF samples taken from them yielded positive culture reports. The organisms were pneumococcus, *Hib,* β-hemolytic *Streptococcus,* α-hemolytic *Streptococcus*), *N. meningitidis* and *Pseudomonaes*. Whereas *Hib was* isolated from young infants, pneumococci were found in the young as well as the old [13].

A prospective study was conducted to assess the etiology of childhood acute bacterial meningitis, in Turkey, in July 2008. Bacterial etiology was determined in 243 cases; *N. meningitidis* was detected in 56.5%, *S. pneumoniae* in 22.5%, and *Hib* in 20.5% of the PCR positive samples. Among *N. meningitidis* positive CSF samples, 42.7%, 31.1%, 2.2%, and 0.7% belonged to sero-groups W-135, B, Y, and A, respectively. This study highlights the emergence of sero-group W-135 disease in Turkey [9].

An analytical study was conducted in Pakistan in 2012 to isolate and identify the causative pathogen, antibiotic sensitivity testing and success rate of empirical antibiotic therapy in pyogenic meningitis. Seventeen different bacteria were isolated. The most commonly occurring bacteria were coagulase negative *Staphylococci* (25%), *E. coli* (12.5%), *K. pneumoniae* (8.3%), *S.pneumoniae* (8.3%) and *P.aeruginosa* (8.3%). All the bacteria were sensitive to vancomycin (96.7%), meropenem (76.7%), amikacin (75%), ciprofloxacin (65.3%), chloramphenicol (46.5%), ceftazidime (44.2%), cefepime (41.9%), co-amoxiclav (38.0%), oxacillin (34.8%), cefotaxime (21.4%), penicillin (20.7%), ceftriaxone (18.6%), cefuroxime (14%) and ampicillin (6.9%). The combination of sulbactam and cefoperazone showed antimicrobial sensitivity of 81.4%. The success rate of empirical antibiotic therapy was 91.7 % [14].

A study conducted in India from 2007-08 to determine antimicrobial susceptibility of Gram positive CSF isolates in septic meningitis in a tertiary care hospital. CSF was collected from 638 admitted children clinically suspected of septic meningitis. Of the samples tested 102 (15.99%) were culture positive of which 45 (44.12%) were found in children aged 1-12 years. The isolates in 66 (64.71%) cases were Gram positive of which 36 (54.55%) were *Streptococcus spp*., 24 (36.36%) *Staphylococcus aureus* and 6 (9.09%) cases coagulase negative *Staphylococcus*. Both *Streptococci* and coagulase negative *Staphylococci* were highly sensitive (100%) to Linezolid, Vancomycin and Piperacillin-Tazobactam. However, *Staphylococcus aureus* were 100% sensitive to Linezolid and Vancomycin but it was only 87.5% sensitive to Piperacillin-Tazobactam combination. The *Streptococcus species* showed a high degree of resistance to Tetracyclin 91.67%, Co-trimoxazole 88.89% and Penicillin 63.89%. *Staphylococcus aureus* showed resistance to the tune of 83.33% each to Tetracycline and Co-trimoxazxole and 79.17% with Penicillin. In case of coagulase negative *Staphylococcus,* Co-trimoxazole showed resistance in 83.33%, Penicillin in 66.67% and Tetracycline in 50% cases [10].

Another study conducted in Ahmedabad from 2010-2011, bacterial pathogens were isolated from 205 samples showing an isolation rate of 13.94%. Gram's stain positivity was 61.95%. Among the isolated organisms, 69.26% were gram negative bacilli and 30.74% were gram positive cocci. The most commonly isolated bacteria were *K. pneumoniae* (22.92%) & *S. aureus* in 19.02%. Pyogenic meningitis was more common in pediatric patients than adults. *K. pneumoniae* and Enterococci spp. were most common isolated in neonatal age group. Most common organisms isolated in neurosurgical patients were *P.aeruginosa* and *S.aureus.* 09.10% gram negative organisms were extended spectrum β-lactamase (ESBLs). Only 2 Gram positive isolates were methicillin resistant *S.aureus* (MRSA) [15].

A prospective study in Morocco was conducted in 2012 to investigate the causes of invasive bacterial diseases in children in order to inform antibiotic therapy and vaccine choices. Of 238 children aged ≤ 5 years admitted to the Children's Hospital of Casablanca for invasive diseases over a 12-month period, 185 were diagnosed with bacterial infection: seventy six had chest-X-ray-confirmed pneumonia, 59 had meningitis and 50 had sepsis. *S.pneumoniae* was the most common pathogen identified, followed by *N.meningitidis* (all group B) and *H. influenzae.* The rate of penicillin non-susceptibility was 62.5% among *S. pneumonia* isolates and 11.1% among *N. meningitidis.* All the isolates were ceftriaxone-susceptible. Of the 11 *H. influenzae* isolates, only 1 produced a beta-lactamase. The five predominant *S.pneumoniae* serotypes were 19F, 14, 23F, 6B and 19A and the theoretical coverage of the 7, 10 and 13-valent pneumococcal conjugate vaccines was 60%, 78% and 91% respectively [16].

A cross-sectional descriptive study of routinely collected antibiotic susceptibility data from the Namibia Institute of Pathology database which were results of CSF culture and sensitivity from 2009-2012 at Namibia were analyzed. The most common pathogens isolated were *Streptococcus species, Meningitidis, Influenzae, Staphylococcus, and Escherichia coli.* The common isolates from CSF showed high resistance to penicillin. Over one third (34.3%) of *Streptococcus* were resistance to penicillin. The sensitivity to cephalosporin remained high for *Streptococcus, Neisseria, E. coli and Haemophilus* [17].

A study carried out in Libya in 1998 with a 14 month period, 77 children with a presumptive diagnosis of acute bacterial meningitides were investigated with a rate of isolation 0.8%. Children <1 year of age were more affected (64.9%).A total of 48 isolates identified; *H. influenzae* (33.8%) was predominant followed by *S.pneumoniae* (26.0%), *klebsiella spp* (6.5%) and *N.meningitidis* (2.6%). Many of the bacterial isolates were sensitive to gentamycin, cefotaxime, and ceftriaxone and least sensitive to tetracycline and ampicillin [18].

A study in Nigeria from October 2005 to December 2006, to see the etiology of invasive bacteria isolates from children with severe infections in a Nigerian hospital. Fourteen out of 67 (20.9%) of the CSF samples yielded bacterial isolates: *S. pneumoniae*, 3 (4.5%); *H. influenzae*, 8 (11.9%); *Haemophilus* spp, 1 (1.5%); *E. coli*, 1 (1.5%); and atypical coliform, 1 (1.5%). Gram-negative coliform isolates were predominantly resistant to penicillin based antibiotics and co-trimoxazole but sensitive to third-generation cephalosporin's and quinolones [19].

A hospital-based surveillance of ABM among children admitted to Manhic, a District Hospital (Maputo, Mozambique) in 2006. CSF samples were collected from 642 children <15 years of age with suspected meningitis. ABM was confirmed in 43 (7%) of the 642 cases. Hib (14 cases), pneumococcus (9 cases), and meningococcal (7 cases) represented ~70% of confirmed cases. All 9 pneumococci isolates were susceptible to chloramphenicol, and 8 were susceptible to penicillin (the additional 1 had intermediate resistance). For the 10 Hib isolates tested, only 1 was susceptible to chloramphenicol, and 5 were susceptible to ampicillin [20].

A similar study conducted in Egypt to determine bacterial isolates from CSF specimen from hospital patients in Cairo, 1977-78.The result showed that from 1627 samples, there were 77 bacterial isolates (22%). *Pneumococci* were the most common and the serotype distribution was similar to that reported from other parts of Africa; second were *meningococci*; thirdly *Haemophilus influenzae* type b were isolated. There were many clear CSF specimens that were found to contain *pneumococci*, *meningococci* or *H. influenzae* type b, confirming the need for more comprehensive laboratory facilities for accurate diagnosis of the etiology of bacterial meningitis [21].

A comparable study conducted in Alexandria, Egypt three hundred and ten patients (195 males and 115 females) were included in the study. About 65.2% of them were infected with acute bacterial meningitis and 34.8% were infected with aseptic meningitis. In this study, ABM was caused by *H.influenzae* (21%), *S.pneumoniae* (13.9%), *N.meningitidis* (14.2%) and other undetermined bacteria (16.1%). ABM showed significant association with age group 1-9 years (66.3%), low socio-economic class (96%), working mother (83.2%), more than two smokers in the family (62.9%) and cold seasons(fall 35.1% and winter 48.5%) [11].

A study conducted in 2011 in Hawassa Referral Hospital, South Ethiopia to assess drug resistance pattern of *S.pneumoniae* among clinically diagnosed cases of pneumonia, meningitis and otitis media. The result showed that 61.2% were males. Of cultured 152 patients' samples 21.4% growth *S.pneumoniae*. The highest resistance rate was seen for ampicillin and penicillin but lowest for chloramphenicol. Sixty four point two percent (64.2%) of the isolates were resistant to two or more antimicrobial agents [22].

Another study conducted in 2005 Gondar University Teaching Hospital, Northwest Ethiopia to identify bacterial pathogens that cause meningitis and to assess the antibiotic susceptibility patterns of the isolates from the CSF of acute bacterial meningitis samples. The result showed that bacterial pathogens were isolated from 22 patients showing an isolation rate of 5.6%. The most commonly isolated bacteria were *N.meningitidis* 10(45.5%) and *S.pneumoniae* 7(31.8%). Among gram positive organisms *S. pneumoniae* showed a high level of drug resistance against chloramphenicol 4(57%), tetracycline 3 (43%), co-trimoxazole 3(43%), ampicillin 3(43%), and gentamicin 1(14%). Among gram negative bacteria, *N.meningitidis* was found to be resistant to co-trimoxazole 5(50%), chloramphenicol 3(30%), gentamicin 3(30%) and ampicillin 2(20%). The single isolate from *Proteus species* was found to be resistant to co-trimoxazole and tetracycline. *E. coli* was found to be resistant to all antibiotics except for gentamicin and ciprofloxacin. Multiple drug resistance was observed in 50% of the isolates. No organism was found to be resistant to ciprofloxacin [23].

A similar study in Gondar was conducted from 2002-2012 to determine bacterial profile and antimicrobial susceptibility pattern of bacterial meningitis among neonates and children at Gonder university hospital, Northwest Ethiopia. From 2170 CSFs bacterial pathogens were isolated from 97 patients with an isolation rate of 4.5%.*S.pneumoniae* 35(36.1%), followed by *N.meningitidis* 28(28.9%) and *Hemophilus species* 9(9.3%).The gram positive bacteria were constituted 52(54.7%).Among gram positive level of drug resistance against Co-trimoxazole 24(68.6%),gentamycin 23(65.7%),tetracycline 14(40%),penicillin 12(34.3%) and chloroamphinicol 9(25.8%).of the gram negative bacteria *N. meningitidis* was found resistance to Co-trimoxazole 24(85.8%) , tetracycline 19(67.8%) ,ampicilin 9(52.2%), gentamycin 7(25%) and penicillin 6(21.55%).Multidrug resistance was observed in 61.9% of the isolated bacteria [24].

Another similar study in Gondar was conducted to assess the common bacterial isolates and their antimicrobial resistance pattern. CSF samples delivered for culture from September 2009 to march 2011 was retrospectively collected from microbiology registration book. Bacteria were isolated from 75 patients which makes the isolation rate 4.3%, 3.7% among males and 5.1% among females the common bacterial isolates were *S.pneumoniae, N.meningitidis* and *E.coli*. *S.pneumoniae* showed a high level of drug resistance against cotrimoxazole, gentamycin and tetracycline. The single isolate of *viridian streptococci* was 100% resistance to ciprofloxacin, erythromycin, gentamycin and cotrimoxazole. *N.meningitidis* was 95.3% and 57% resistant to cotrimoxazole and gentamycin respectively while they were sensitive to penicillin (100%) and ceftriaxone (90.9%). *E.coli* showed 100% resistance to cotrimoxazole, tetracycline, ampicilin and naldixic acid but the isolates were sensitive to ceftriaxone [25].

3. Objectives

3.1.General objective:

- To determine prevalence of bacterial isolates from cerebrospinal fluid, their antimicrobial susceptibility pattern and associated risk factors with special emphasis on *S.pneumoniae* among pediatrics suspected meningitis patients at Tikur Anbessa and Yekatit 12 specialized hospitals, Addis Ababa, Ethiopia, Sept, 2013 to Jan, 2014.

3.2.Specific objectives:

- To determine the prevalence of bacterial isolates from CSF specimen among pediatrics suspected meningitis patients.
- To determine the antimicrobial susceptibility pattern of bacterial isolates.
- To identify associated risk factors with bacterial meningitis.
- To compare the prevalence of *S.pneumoniae* isolates based on culture and BinaxNOW *Streptococcus pneumoniae* antigen test results.

4. Materials and methods

4.1. Study Area

The study was conducted at pediatric departments of Yekatit 12 and Tikur Anbessa specialized hospitals, Addis Ababa, Ethiopia. Based on 2007 census results Addis Ababa has a total population of 2,738,248, consisting of 1,304,518 men and 1,433,730 women, and from which 195,932 are children under five years of age. With an estimated area of 530.14 square kilometers (204.69 square mile), this capital city has an estimated density of 5,165.1 inhabitants per square kilometer (13,378 / m^2).

Tikur Anbessa specialized Hospital is the largest teaching hospital found in the college of health science, Addis Ababa University, Ethiopia, with about 800 beds. It is staffed by 130 specialists and 50 non-teaching doctors. The emergency department sees around 80,000 patients per year.

Yekatit 12 hospital is a tertiary care center that has been on renovation for the last few Years. It is one of the hospitals under Addis Ababa city administration health bureau having pediatrics, surgery, gynecology, psychiatry, HIV care and an outpatient clinic that has been giving routine health services for the city community and other referral cases from different regional states of Ethiopia.

Study sites were selected according to pediatric facilities (senior pediatrician) as well as laboratory test available (microbiology culture test availability or vicinity to the microbiology laboratories).

4.2. Study design and period

A hospital based cross sectional study was conducted from September, 2013 to January, 2014 at Yekatit 12 and Tikur Anbessa specialized hospitals Addis Ababa, Ethiopia.

4.3. Population

4.3.1. Source population

All pediatrics patients attending at Yekatit 12 and Tikur Anbessa specialized hospital pediatric clinics during the study period.

4.3.2. Study population

Pediatrics patients who were suspected to have bacterial meningitis willing to give CSF sample for their diagnostic purpose.

4.4. Eligibility

4.4.1. Inclusion criteria

All patients ≤15 year with suspected meningitis and willing to give CSF sample for their diagnostic purpose and their mothers/guardians signed a consent were included in the study.

4.4.2. Exclusion criteria

Those with pneumococcal vaccine in the past one week, who started antibiotics before fourteen days and who were not willing to participate during the study period were excluded.

4.5. Study variable

4.5.1. Dependent variables

- Bacterial isolates from CSF specimen
- Comparison of BinaxNOW *S.pneumoniae* antigen test with culture

4.5.2. Independent variables

- Socio demographic characteristics.
- Associated risk factors

4.6. Sample size and sampling technique

Since the study is based on a single population proportion the sample is calculated as follows;

$$n = \frac{(Z\alpha/_{2})^{2}P(1-P)}{d^{2}}$$

where; n is the sample size to be determined, $Z\alpha/_2$ is 1.96, p is the prevalence (4.5 %) (Mulat D et al,2012),1-p is (95.5%) and d is margin of allowable error. Since p<10%, d=p/2, **d=0.0225** according to Naing *et al* [28].Using the above formula and considering 95% confidence interval;

$$n = \frac{(1.96)^2(0.045)(0.955)}{(0.0225)^2}$$

$$n = 326$$

- Therefore consecutive sampling technique was used until the sample size determined was met.

4.7. Data collection and processing

4.7.1. Data and specimen collection

A structured questionnaire was used to collect socio demographic and risk factors data on suspected cases of meningitis, medical history and the required information of each patient by asking care takers or the responsible clinician.

The CSF specimen taken by the physician using lumbar puncture was used for white blood cell count, gram stain, differential, protein, culture and BinaxNOW *S.pneumoniae* antigen test. The volume and gross appearance of CSF i.e. clear, bloody/traumatic, cloudy and xanthochromic were recorded. For cloudy specimen gram stain was done before centrifugation, if not the CSF specimen was centrifuged for Gram stain. The CSF was also centrifuged if the volume of fluid was greater than 1 ml, centrifuge for 15 min at 2500-3000 rpm.

4.7.2. Transport of specimens

After collection, the CSF samples were immediately transported to the clinical microbiology laboratory of Yekatit 12 and Tikur Anbessa specialized hospitals, where the laboratory investigations (BinaxNOW *S.pneumoniae* antigen test (Binax, Inc., Scarborough, Maine 04074 USA), culturing (inoculation), species identification and susceptibility test) were performed.

4.7.3. Specimen processing , culture and identification

4.7.3.1.Microbiological investigation

All the specimens were inoculated onto Blood agar, chocolate agar and Macconckey agar. The blood and chocolate agar plates were incubated at 35-37°C in a candle jar with capnophilic(5-10% co_2) environment; Macconckey agar was incubated aerobically, which was used for the isolation of gram negative aerobic bacteria that need oxygen for their growth and examined for the presence of any bacterial growth after 24 hours. Those plates showing no growth were incubated for another 24 hours.

The plates were examined macroscopically for morphological and presumptive identification. The suspected isolates were identified by colonial morphology such as Hemolytic reaction (on blood agar plate), pigment production or color changes surrounding carbohydrate fermenting colonies (on MacConkey agar plate).Other identification methods were Colony characteristics (white, golden yellow, pink, grey in color or mucoid in appearance) and Gram's stain under microscopic examination. Conventional biochemical tests (such as catalase, coagulase, oxidase, hydrogen sulphide test, urease, indole, citrate, sugar utilization tests and bile solubility test), susceptibility to Optochin were also used to identify the bacteria. Cultures of these samples were performed according to standard microbiological methods (Annex VIII) [29].

4.7.3.2.Antimicrobial susceptibility test

Antimicrobial susceptibility pattern of the isolates were studied by using the criteria of Clinical and Laboratory Standards Institute (CLSI) and Kirby-Bauer technique by disc diffusion method on Muller-Hinton Agar (Oxoid, Ltd, England) for gram negative isolates. Muller-Hinton Agar supplemented with 5% Sheep blood was also used for fastidious bacteria such as *S.pneumoniae* and *N.meningitidis*. From a pure culture 3-5 pure colonies of bacteria were taken and transferred in to a tube containing 5 ml sterile nutrient broth (Oxoid) and mixed gently until the turbidity of the suspension become adjusted to a McFarland 0.5 standard.

Using sterile cotton swab, the bacteria were seeded evenly over the entire surface of Mueller-Hinton agar (Oxoid) for gram negative bacteria's and fastidious bacteria like *S.pneumoniae* , *N.meningitidis* etc. were seeded on Mueller-Hinton agar supplemented with 5% of sheep blood agar by rotating at 60^{o}C for at least three times[36].

The plates were left at room temperature to dry for 3 to 5 minutes and a set of antibiotic discs (Oxoid) with the recommended concentrations were placed on the surface of a Muller Hinton agar. The following antibiotics discs were used (Ampicillin (AMP,30µg) ,Gentamycin (GN,10µg),Sulfomethoxazole/Trimethoprim(SXT,1.25µg),Chloroamphenicol(C,30µg,Ciprofloxacin(CIP,5µg),Tetracycline(TTC,30µg),Clindamycin(Cln,2µg),Penicillin(P,10unts),Norfloxacin(NOR,10µg),Vancomycin(Van,30µg),Ceftazidime(CAZ,30),Cefuroxime/sodium(CXM,30µg),Cephazolin (KZ,30µg),Cefotaxime (CTX,30µg) and cefoxitin(FOX,30µg).

Finally, fastidious organisms seeded were incubated at 37°C in a candle jar of capnophilic environment and the other plates incubated at 37^{o}C for 24 hours aerobically. Diameters of growth inhibition around the discs were measured by caliper and interpreted as sensitive, intermediate or resistant as per the standard protocol (Annex VIII) [36].

4.7.3.3.BinaxNOW *S. pneumoniae* antigen test

The BinaxNOW *S. pneumoniae antigen* test (Binax, Inc., Scarborough, Maine 04074 USA) is an immunochromatographic assay that uses a rabbit anti-*S.pneumoniae* antibody, conjugated to visualizing particles, to bind any soluble pneumococcal antigen (C polysaccharide) present in the urine and CSF samples [31].

A negative sample gave a single pink-to-purple colored control line in the top half of the window, indicating a presumptive negative result. A positive sample also gave two pink-to-purple colored lines. Specimens with low levels of antigen may give a faint patient line. Any visible line is positive. If no lines were seen, or if just only the sample line was seen, the assay was invalid .Invalid tests should be repeated [31].

CSF samples were immediately tested using BinaxNOW or otherwise the CSF was stored at room temperature until the next day to perform the tests.

4.8. Data quality assurance

The pre-analytical, analytical and post-analytical phases were controlled throughout the process of the study. Moreover site assessment and pre-test were conducted prior to the actual data collection. Data collection was done after the data collectors received on-site training. The data were cheeked for completeness and representativeness prior to entry.

4.8.1. Pre-analytical considerations

SOPs for specimen collection was applied. The specimen (CSF) had been collected and handled properly. CSF was collected in a sterile tube. Each specimen was clearly labeled. As much as possible these specimens were received in the laboratory and processed soon after collection.

4.8.2. Analytical considerations

The sample (CSF) was spread on a solid culture medium which was first checked for sterility and performance of culture media then the remaining tested on BinaxNOW *S.pneumoniae* antigen test. The sterility of culture media were checked by incubating 3-5 % of the batch at 35 – 37^{o}C overnight and observed for bacterial growth. Those media which showed growth were discarded.

The performance of culture media were also controlled by using various quality control strains such as *E. coli* ATCC 25922 and *S.aureus* ATCC 25923. The organism identified based on colony characteristics and identification tests. Quality assurances for the BinaxNOW *S.pneumoniae* antigen tests were conducted according to the manufacturer's direction.

i) Daily quality control: The BinaxNOW *S. pneumoniae* test contains built-in positive and negative procedural controls. The manufacturer's minimum recommendations for daily quality control was to document these procedural controls for the first sample tested each day.

ii) External positive and negative controls: BinaxNOW test kits contain positive and negative control swabs. These swabs were monitoring the entire rapid test assay. Good laboratory practice suggests the use of positive and negative controls to ensure that: test reagents were working and the test was correctly performed.

4.8.3. Post analytical considerations

Verification and interpretation of the test results were done properly. The data entry was done by two individuals and checked for agreement. Cross checking of the data with the result paper was also conducted.

4.9. Data processing and analysis

Data was entered into EPI info version 3.5.1 and transferred to SPSS version 21 for analysis. Binary logistic regression were performed for identifying risk factors which had significant association with suspected bacterial meningitis cases and multiple logistic regression for identifying factors which had significant association with this meningitides disease. Odds ratio with 95% confidence intervals (CIs) that do not include 1.00 and *P* values <0.05 were considered statistically significant. Tables and graphs were used for data presentation.

4.10. Dissemination of results

After conducting the research, results was presented to the department of medical laboratory sciences, college of health sciences, Addis Ababa University and other concerned bodies. The manuscript will be submitted to peer reviewed journals for publication.

4.11. Ethical considerations

The proposal was ethically approved by the Departmental research and ethics review committee (DRERC) of the department of Medical Laboratory Science, School of Allied Health Science, College of Health Science, Addis Ababa University. Addis Ababa regional health bureau was reviewed again, approved and gave permission letter. Permission was also obtained from Tikur Anbessa and Yekatit 12 specialized hospitals. Informed consent was gained from every participant's mother/guardian. Patient identities was coded and any information was kept confidential. Patients positive with serological and culture method was consulted with their physician and ensured proper treatment.

4.12. Project management

Cross-checking and data cleaning was done and missing information obtained by going back to the questionnaire. All laboratory and clinical data were recorded on appropriate record during the study period and the data was stored in a CD, flash, floppy disk, attached on Email address.

4.13. Operational definition

Suspected bacterial meningitis: fever (temperature ≥38°C) and at least one of the following meningeal signs: convulsions, bulging fontanelle in children or stiff neck; poor sucking or irritability; prostration or lethargy; or petechial or purpural rash. [26].

Probable bacterial meningitis: A suspected meningitis case (as defined above) with CSF examination showing at least one of the following: turbid appearance, leukocytosis (> 100 cells/mm^3), leukocytosis (10-100 cells/ mm^3) and either an elevated protein (>100 mg/dl) or decreased glucose (< 40 mg/dl).

Confirmed meningitis: A suspected meningitis case that is laboratory-confirmed by growing (i.e. culturing) or identifying (i.e. by Gram stain, antigen detection, immunochromotography, PCR or other methods) a bacterial pathogen (Hib, pneumococcus or meningococcus) in the CSF or from the blood in a child with a clinical syndrome consistent with bacterial meningitis.

Qualitative tests: those candidate methods that provide only two categorical responses (i.e. positive/negative or yes or no results).

Gold standard: a non-specific term that indicates a processes or material(s) is the best available approximation of the truth.

Suspect: A clinically compatible case that is not yet laboratory confirmed and is not epidemiologically linked to a confirmed case [27].

Pediatrics: Age less than or equal to fifteen year.

5. Results

5.1.Socio-demographic characteristics

A total of 385 pediatric patients participated in this study. Fifteen subjects were excluded from the study because of reasons like guardians/mothers refused to consent to take part in the study.

From pediatric participants included in the study 234(60.8%) of them were males and 151 (39.2%) females with age range of 1 day to 15 years with mean age (standard deviation) of 2.58(±4.25) years. One hundred fifty four (40.0%) of them were neonates (birth to 1month), 94 (24.4%) of them were infants and others were one year to fifteen year (137(35.6%)) (Figure 5.1). Study subjects mothers/guardians educational status were, 128(33.2%) unable to read and write and the others (257(66.8%)) were at primary and above schools. Majority of them were urban dwellers, 277 (71.9%) while 108 (28.1%) were lived in rural areas (Table 5.2).

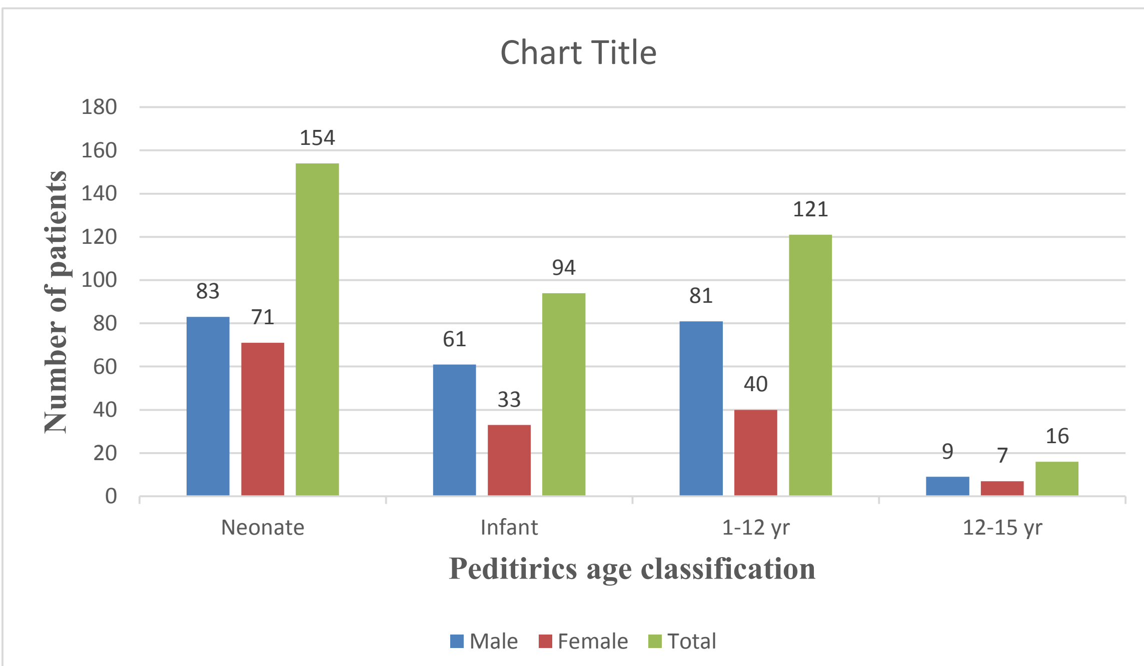

Figure 5.1: Age and sex distribution of patients (n=385) investigated for suspected cases of meningitis at Yekatit 12 and Tikur Anbessa specialized Hospitals from Sept, 2013 to Jan, 2014.

5.2.Prevalence of Bacterial Isolates

The CSF appearance was clear in 278 (72.2%), slightly cloudy 82(21.3%) and turbid in 25 (6.5%) of the cases. The turbid appearance of CSF was an indication of presence of bacterial meningitis. In most of the cases WBC count remained normal (n=329, 85.5%, WBC<05 cells). However, it was increased in some of meningitis patients (n = 56, 14.5%), among these most of them had a high percentage (>70%) of polymorphonuclear cells (n = 36 (64.3%), and in others increased lymphocyte (n=20, 35.7 %) count was observed.

Among a total of 385 suspected meningitis cases bacterial pathogens were isolated from 17 pediatric patients showing an isolation rate of 4.4%.The gram stain detected 20 suspected cases of meningitis than culture which detected 17 suspected cases of meningitis.

The number of pathogens isolated were greater in females than males. Pathogen isolation rate was higher in neonates 9(6.5%) than in the other age groups [infants 4(2.9%), children 4(4.6%) and none from adolescents]. Out of 9 species, 2 species were gram positive and 7 species were gram negative bacteria's. Gram negative bacteria's 10(58.8%) were more than gram positive bacteria's 7(41.2%). *S. pneumoniae* 6(35.3%) was found to be the most frequent isolate followed by *N.meningitidis* 2(11.8%), *Proteus species* 2(11.8%), *E.coli* 2(11.8%), *Klebsiella pneumoniae*, *Acenitobacter species, Citrobacter species, Pseudomonas aeruginosa* and *Streptococcus pyogenes* each 1 (5.9%). The majority of *S. pneumoniae* 4(66.7%) were isolated from neonates. Multiple infections were not observed. The type and frequency of pathogens isolated from CSF is shown in Table 5.1.

Table 5.1 Bacterial profile isolated from culture of CSF among suspected meningitis pediatrics patients at Yekatit 12 and Tikur Anbessa specialized Hospitals from Sept, 2013 to Jan, 2014.

Organisms	**Neonate(1-30 day)**			**Infant(1mon-1yr)**			**Children(1yr-12yr)**			**Number**
	M	**F**	**T**	**M**	**F**	**T**	**M**	**F**	**T**	**Total (%)**
S.pneumoniae	1	3	4	0	1	1	0	1	1	6(35.3)
N.meningitidis	0	1	1	0	0	0	1	0	1	2(11.8)
E.coli	0	0	0	2	0	2	0	0	0	2(11.8)
Citrobacter species	1	0	1	0	0	0	0	0	0	1(5.9)
Proteus species	1	0	1	0	1	1	0	0	0	2(11.8)
S.pyogenes	0	0	0	1	0	1	0	0	0	1(5.9)
Acenitobacter species	1	0	1	0	0	0	0	0	0	1(5.9)
Klebsiella pneumoniae	0	0	0	0	0	0	0	1	1	1(5.9)
Pseudomonas aeruginosa	0	1	1	0	0	0	0	0	0	1(5.9)
Total	4	5	9	3	2	5	1	2	3	17(100)

M=Male F=Female T=Total

5.3.Possible risk factors for Bacterial meningitis

Table 5.2: Association of selected risk factors for bacterial meningitis among suspected cases of meningitis with prevalence of bacterial meningitis (N=385) among pediatrics patients at Tikur Anbessa and Yekatit 12 specialized Hospitals, Sept 2013 to Jan, 2014.

Variables		**Bacterial growth**		**COR** 95%CI	**P-value**
		Pos Nº (%)	Neg Nº (%)		
Sex	Female	9(6)	142(94)	0.5(0.2-1.5)	0.242
	Male	8(3.4)	226(96.6)	1	
Address	Urban	12(4.3)	265(95.7)	1	
	Rural	5(4.6)	103(95.4)	0.9(0.3-2.7)	0.898
Educational status	Illiterate	6(4.7)	121(95.3)	1.43(0.5-3.8)	0.48
	Formal edu & Above	10(3.9)	247(96.1)	1	
Occupation of mother/guardian	House wife	13(4.7)	263(95.3)	0.77(0.2-2.4)	0.655
	Others	4(3.7)	105(96.3)	1	
Way of cooking food	Non-Electric	14(5.5)	239(94.5)	2.6(0.7-9.2)	0.137
	Electric	3(2.3)	129(97.7)	1	
No of house partition	≤4	17(4.7)	343(95.3)	80(0)	0.998
	≥4	0(0)	25(100)	1	
No of people sharing the same room with the child	≥4	6(5.1)	111(94.9)	1.2(0.4-3.4)	0.671
	2-3	11(4.1	256(95.9)	1	
Day care attendance	Yes	3(4.8)	60(95.2)	1.1(0.3-3.9)	0.884
	No	14(4.3)	308(95.7)	1	
Industry left surplus product	Yes	1(5.0)	19(95.2)	1.1(0.1-9.0)	0.898
	No	16(4.4)	346(95.6)	1	
Has your child been sick in the previous 10 days &hospitalize	Yes	1(2.3)	42(97.7	0.486(0.06-3.7)	0.486
	No	16(4.7)	324(95.3)	1	
Exposure to smoker in the house	Yes	3(8.3)	33(91.7)	2.12(0.6-7.7)	0.255
	No	14(4.1)	327(95.9)	1	

From all the risk factors assessed, 71.9% of caretakers were urban dwellers'. The majority of them (128, 33.2%) involved in the study were Illiterate followed by mothers taken formal education and above (257, 66.7%) (Figure 5.2).Two hundred seventy six (71.7%) of the mothers involved in this study were housewives while 35(9.1%), 46(11.9%), 11(2.9%) of mothers were working in governmental or non-governmental institutions, engaged in private business and farmers respectively.

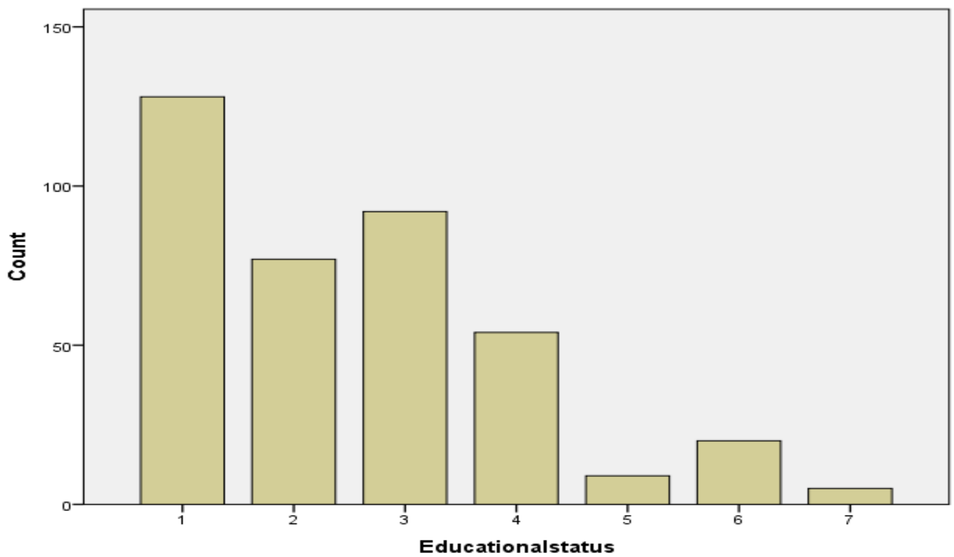

Figure 5.2. The educational levels of mothers whose children were involved in the study at Yekatit 12 and Tikur Anbessa specialized Hospitals from September, 2013 to January, 2014. **(Key for educational levels; 1=Illiterate, 2= below 4th grade, 3=below 8th grade 4=High school, 5=certificate, 6=diploma, 7=1st degree and above)**

The majority of pediatric subjects 198(51.4%) were solely feeding on breast milk while 62(16.1%), 22(5.7%), 99(25.7%) of subjects were feeding on formula milk only, combination of formula milk and breast milk; and additional foods respectively. Based on number of house partition the pediatrics participants (360, 93.5%) were lived in house having <4 partitions and the others (25, 6.5%) were lived in house partitions ≥4. Two hundred sixty seven (69.5%) of them were sharing with 2-3 peoples, while 117 (30.5%) were sharing ≥4 peoples the same room. Among the total, 322 (83.6%) participants were not used day care center. Industries that left surplus products were 20 (5.2%) in participants' environment. Habit of smoking of caretakers were recorded as 36(9.4%).

Forty one percent used wood, 34.2% used electricity and 24.4% used kerosene for cooking food. Near to fifteen percent (14.5%) of participants were taken BCG vaccine and 95.5% were vaccine free before seven days of sample collection. History of hospitalization in the previous month were seen in 11.2% of participants. Among all these variables none of them were showed significant association with suspected meningitis cases ($p>0.05$). (Table 5.2)

5.4.Antibiotics susceptibility pattern of bacterial isolates

Among the gram positive organisms *S.pneumoniae* showed a high level of drug resistance against Tetracycline 3 (50%), clindamycin 3(50%), penicillin 2(33.3%), vancomycin 2(33.3%), Trimethoprim/sulfomethoxazole 2(33.3%) and chloramphenicol 1(16.7%).However all the isolates were sensitive to ceftriaxone and cefotaxime. One *S.pyogenes* was found resistant to tetracycline but was sensitive to chloramphenicol, clindamycin and cefoxitin. The pneumococcal isolates was proved to be more sensitive to third generation cephalosporin in this study while least resistance was shown by these organisms. There was increasing resistance among major pathogen which cause meningitis to most of the traditional antimicrobial agents used as initial therapy prior to the availability of bacteriological agents. The *S.pneumoniae* isolates have shown full susceptibility for the drugs chloramphenicol, ceftriaxone and cefotaxime. (Table 5.5)

All *E.coli* showed resistance to ampicillin and tetracycline, 50% resistance to cefazolin, gentamycin, norfloxacin, cefotaxime, ceftriaxone, ceftazidime and nalidixic acid. However, all were sensitive to chloramphenicol and kanamycin.

All *Proteus species* were resistant to ampicillin, tetracycline, cefazolin, gentamycin, chloramphenicol, cefotaxime, kanamycin and ceftriaxone while they were sensitive to norfloxacin and nalidixic acid. *Pseudomonas aeroginosa* was resistance to Ampicillin, cefazolin and chloramphenicol, but sensitive to the rest of the drugs tested. (Table 5.3)

The single isolate of *Klebissella pneumoniae* was sensitive to norfloxacin, cefotaxime, ceftazidime, and nalidixic acid while resistant to the rest of the drugs tested. *Acenitobacter species* was resistant to most of the drugs though sensitive to tetracycline, norfloxacin, and nalidixic acid. *Citrobacter species* was also resistant to ampicillin, cefazolin but sensitive to the rest of the drugs tested.

Table 5.3: Antimicrobial sensitivity pattern of gram negative bacterial isolates from CSF (N=10), Yekatit 12 and Tikur Anbessa specialized Hospitals, Sept, 2013-Jan, 2014.

Organisms		Antimicrobial agent											
		AMP No	TE No	KZ No	GN No	NOR No	C No	CTX No	K No	CRO No	CAZ No	NA No	CIP No
Ecoli	S	0	0	1	1	1	2	1	2	1	1	1	-
	R	2	2	1	1	1	0	1	0	1	1	1	-
Proteus species	S	0	0	0	0	2	0	0	0	0	0	2	2
	R	2	2	2	2	0	2	2	2	2	2	0	0
K. pneumoniae	S	0	0	0	0	1	0	1	0	0	1	1	-
	R	1	1	1	1	0	1	0	1	1	0	0	-
P.aeroginosa	S	0	1	0	1	1	0	1	1	1	1	1	-
	R	1	0	1	0	0	1	0	0	0	0	0	-
Acenitobacter species	S	0	1	0	0	1	0	0	0	0	0	1	-
	R	1	0	1	1	0	1	1	1	1	1	0	-
Citrobacter species	S	0	1	0	1	1	1	1	1	1	1	1	-
	R	1	0	1	0	0	0	0	0	0	0	0	-

S=Sensitive, **R=**Resistant, **AMP**=Ampicillin**, TE**=Tetracycllin, **NOR**=Norfloxacin, **CIP**=Ciprofloxacin, **CTX**=Cefotaxime, **CAZ**=Cephtazidime, **KZ**=Cephazoline, **CXM**=Cefuroxime, **C**=Chloramphenicol, **GN**=Gentamicin, **KZ**=Cefazoline, **K**=kanamycin, **CRO**=Ceftriaxone, **NA**=Nalidixic acid.

From gram negative bacterial isolates all *N.meningitidis* were resistant to chloramphenicol and trimethoprim/sulfomethoxazole while all were sensitive to ceftriaxone, ciprofloxacin, Cefuroxime and azithromycin.

Table 5.4: Antibiotic sensitivity pattern of *N.meningitidis* from CSF (N=2), Yekatit 12 and Tikur Anbessa specialized Hospitals, Sept, 2013 to Jan, 2014.

Organism		Antimicrobial agent					
		C No	CRO No	CIP No	SXT No	CXM No	AZ No
N.meningitidis	S	0	2	2	0	2	2
	R	2	0	0	2	0	0

S=sensitive, **R=**resistant **C=**Chloramphenicol, **CRO=**Ceftriaxone, **CIP**=Ciprofloxacin,

SXT=Sulfomethoxazol/Trimethoprim, **CXM**=Cefuroxime, **AZ=**Azithromycin,

Table 5.5 Antibiotic sensitivity pattern of gram positive bacterial isolates from CSF (N=7), Yekatit 12 and Tikur Anbessa specialized Hospitals, Sept, 2013 to Jan, 2014.

Antimicrobial agent	**Bacterial isolates**					
	S.pneumoniae			***S.pyogenes***		
No (%)	**S**	**I**	**R**	**S**	**I**	**R**
P	4(66.7)	0(0)	2(33.3)	-	-	-
VA	4(66.7)	0(0)	2(33.3)	-	-	-
TE	3(50)	0(0)	3(50)	0(0)	0(0)	1(100)
SXT	4(66.7)	0(0)	2(33.3)	-	-	-
C	5(83.3)	0(0)	1(16.7)	1(100)	0(0)	0(0)
CLN	3(50)	0(0)	3(50)	1(100)	0(0)	0(0)
CTX	6(100)	0(0)	0(0)	0(0)	0(0)	0(0)
CRO	6(100)	0(0)	0(0)	0(0)	0(0)	0(0)
FOX	-	-	-	1(100)	0(0)	0(0)

P=penicillin, **VA**=vancomycin, **TE**=Tetracycline, **SXT**=Sulfomethoxazol/Trimethoprim, **C=**chloroamphenicol, **CLN**=Clidamycin **CTX=**Cefotaxime, **CRO**=Ceftriaxone, **FOX**=Cefoxitin.

5.5. Multiple drug resistance pattern of the isolates (N=17)

Among gram positive organisms *S.pneumoniae* showed a high level of multi-drug resistance 2(33%) while from gram negatives *Proteus* species 2(100%) followed by *Klebsiella pneumoniae* 1(100%) and *Acenitobacter* 1(100%) showed high level of multi-drug resistance.(Table 5.6)

Table 5.6 Lists of multi-drug resistance pattern for each bacterial isolates (N=17) of pediatrics suspected meningitis patients at Yekatit 12 and Tikur Anbessa Hospitals, Sept, 2013-Jan, 2014.

Bacterial isolates	Total No	Anti-microbial sensitivity pattern					
		R0	**R1**	**R2**	**R3**	**R4**	**≥R5**
N.meningitidis	2	0(0)	2(100)	0(0)	0(0)	0(0)	0(0)
E.coli	2	0(0)	1(50)	0(0)	0(0)	0(0)	1(50)
Proteus	2	0(0)	0(0)	0(0)	0(0)	0(0)	2(100)
Klebissella pneumoniae	1	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)
Pseudomonas aeroginosa	1	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)
Acinetobacter	1	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)
Citrobacter	1	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)
S.pneumoniae	6	1(16.6)	3(50%)	0(0)	2(33.3)	0(0)	0(0)
S.pyogenes	1	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)

R0: no resistance, **R1**: resistance to one drug, **R2**: resistance to two drug, **R3**: resistance to three drugs, **R4**: resistance to four drugs, **≥R5**: resistance to five and above drugs.

5.6. Prevalence of *S.pneumoniae* based on BinaxNOW *streptococcus pneumoniae* antigen test versus culture.

Among 385 samples tested 8 samples were positive for the antigen test, from these six participants were culture positive for *S.pneumoniae* and the other 2 were culture negative. Using CSF culture as a gold standard BinaxNOW *streptococcus pneumoniae* antigen test result was positive in 100% of patients with culture positive pneumococcus and was negative in 99.4 % of patients with a culture positive for another pathogen and culture negative cases.

The performance of qualitative test is most commonly described by sensitivity and specificity. In children with suspected cases of bacterial meningitis BinaxNOW *S.pneumoniae* antigen test sensitivity was 100% (95% CI, 40.9-92.9), while specificity was 99.4 %(95% CI, 98-99.9). The disease prevalence of pneumococcal meningitis was 1.6 %.The predictive value of a test combines target condition prevalence with sensitivity and specificity. The positive predictive value (PPV) and negative predictive value (NPV) were 75% and 99.5 % respectively.

Table 5.7.2×2 contingency table for BinaxNOW *S.pneumoniae* antigen test and culture of suspected meningitis pediatrics suspected meningitis patients at Yekatit 12 and Tikur Anbessa specialized Hospitals, Sept, 2013-Jan, 2014.

		Gold standard (culture)						
		Positive	Negative	Total	Sensitivity	Specificity	PPV	NPV
BinaxNOW *S.pneumoniae* antigen test	Positive	6	2	8	100%	99.4%	75%	99.5 %
	Negative	0	377	377				
	Total	6	379	385				

6. Discussion

Now a days despite availability of potent antimicrobial and sophisticated intensive care units, bacterial meningitis continues to be a significant cause of morbidity and mortality among children [15].This is reflected by the fact that the global burden of the disease in childhood is at least 1.2 million cases of bacterial meningitis every year with 135,000 deaths [24].

An accurate laboratory confirmation of the etiology in acute bacterial meningitis is essential to provide optimal patient therapy, appropriate case contact management, and reasoned public health actions. Prospectively, it also provides information upon which to base decisions regarding immunization programs, especially for countries without routine vaccination against the main acute bacterial meningitis pathogens [9]. Thus, the data presented in this study could provide information of immediate public health importance to clinicians in the study area on the selection of antimicrobial agents for the treatment of patients suffering from bacterial meningitis.

Laboratory investigations of CSF specimens in suspected cases of bacterial meningitis are extremely important for prompt diagnosis and management of patients. In our study, Gram stain on CSF provided an evidence of the causative bacteria in 20 suspected meningitis pediatric patients and by culture detection in 17 cases. In line with our study several studies have reported Gram's staining as the most useful test for identifying bacterial meningitis, as it revealed more positive cases than cultures [10].This may be due to gram stain is positive in 60 to 80 percent of untreated cases of bacterial meningitis and in 40 to 60 percent of partially treated cases [37].

The isolation rate of bacteria found in the present study (4.4%) is comparable to previous study conducted in Nepal by Ansari *et al.* (3.8%), Gondar by Zelalem *et al*. 4.3%, lower than a study in Namibia by Mengistu *et al.* (6.9%) ,Gondar by Andargachew *et al.*(5.6%),Gondar by Mulat *et al.*(4.5%), [13,25,17,23,24].The low number of isolation rate in the present study is due to (1) Some of the cases were not bacterial meningitis in the first place; they were probably viral meningitis but because of the borderline CSF picture, got labeled and treated as bacterial meningitis, or (2) the increase accessibility of vaccination in our country and (3) over clinical diagnosis of meningitis of all febrile neonates, infants and children has done unnecessary lumbar puncture.

On the opposite the rate of isolation in our study is higher than studies conducted at Lokmanya Tilak municipal medical college, Mumbai where isolation rate of 1.7% [32].This may be due to the epidemiological nature of the organisms.

The majority of etiological agents for meningitis in our study were gram negative organisms. This is similar to study in Turkey by Ceyhan *et al*, Pakistan by Tajdin *et al.* and Gondar by Andargachew et *al*. [9, 14, 23]. To the contrary to our study, gram positive bacteria were predominant isolate in CSF culture of the study in, Namibia by Mengistu *et al.,* Gondar by Mulat *et al*. [17, 24]. This may be due to slight change of shift of pathogens from gram positives to gram negatives.

S.pneumoniae and *N.meningitidis* were the most common isolated pathogens causing meningitis which are in agreement with previous studies in Morrocco by El Mdaghri et *al.,* Mozambique by Roca *et al.,* Gondar by Mulat *et al.* and Gondar by Zelalem *et al.* [16, 20, 24, 25]. Recently, *Hemophilus influenzae* type b (Hib) once a common cause of meningitis, has disappeared in developed nations due to the effectiveness of the extended immunization program of this countries. Even though *Hib* remains a significant public health problem in countries like Ethiopia having shortage of medical supplies.

In this study, *S.pneumoniae* was the predominant bacteria in agreement with study in Nepal by Ansari *et al.*, Gondar by Mulat *et al.* and unlike studies in Pakistan *coagulase negative staphylococci* by Tajdin *et al.*, Nigeria *Haemophilus influenzae type b* by Anthony *et al.*, in Mozambique *Haemophilus influenzae type b* by Roca *et al.*, Egypt *Haemophilus species* by Farag *et al.*, Gondar *N.meningitidis* by Andargachew *et al*. [11,13,14,19,20,23,24]. However no *Haemophilus species* found in our study. The reason for this is not clear but may be due to the new vaccine being given as part of routine child immunization activities, which might have reduced the occurrence of invasive diseases due to *H.influenzae*.

There was no association between risk factors suggested for bacterial meningitis by using binary logistic regression for each risk factor. These were occupation of the mother/guardian, educational status of the mother/guardian, way of cooking food, Nº of house partition, use of day care center, Industry that left surplus products improperly, way of feeding, habit of smoking, taken vaccine within 7 days, history of hospitalization in the previous ten days had no significant association with bacterial growth based on meningitis cases. This contrasts a study conducted in Vietnam by Talarico et *al*. and in North America by Orin et *al*.: Nº of people sharing the same room with the child, day care attendance and house hold size have significant association with pneumococcal meningitis disease in multivariate statistical model of this studies [33, 39]. This may be due to the low number of culture positive bacterial meningitis cases in our study.

All over the world, indiscriminate and irrational use of antimicrobials has led to the development of antimicrobial resistance owing to significant changes in microbial genetic ecology [10]. There was increasing resistance among major pathogens which cause meningitis to most of the traditionally antimicrobial agents used as initial therapy prior to the availability of bacteriological agents [14]. Antimicrobial resistance of *S.pneumoniae, N.meningitidis* and *H. Influenzae* to commonly prescribed antibiotics has become common now a days. Several gram negatives isolated from CSF also developed antimicrobial resistance to conventional antibiotics regimens and emerging multidrug resistance strains [25].

We found that 33.3% of *S.pneumoniae* isolates were resistant to penicillin in agreement with a study conducted in Pakistan by Tajdin *et al*., Nigeria by Anthony *et al.,* and Gondar by Mulat *et al*. [14, 19, 24]. In contrary to a study in Gondar by Adargachew et *al*. no *S.pneumoniae* was resistant to penicillin [23]. This may be due to miss use of penicillin in the study area and the bacteria develops resistance through time. These findings support the use of a third generation cephalosporin for pneumococcal meningitis in areas where penicillin resistance is prevalent, at least until penicillin susceptibility is known. Since *S.pneumoniae* have shown full susceptibility to the drugs: chloramphenicol, ceftriaxone and cefotaxime. The only isolate *S.pyogene* was found resistant particularly to tetracycline but was sensitive to chloramphenicol, clindamycin and cefoxitin. (Table 5.5).

From gram negatives which are among the most causative agents of meningitis, including *N.meningitidis* were resistant to most commonly prescribed drugs like chloramphenicol .However both of these strains are sensitive to ciprofloxacin in the present study contrary to a study conducted in Gondar by Mulat *et al*. [24]. The susceptibility of the organism isolated to fluoroquinoloes was generally good in our study.

Even though there are geographical differences in the drug resistance pattern of bacteria, the problem is at an alarming rate indicating growing emergence of multidrug resistance strains.53% of the isolates showed multiple antibiotic resistance (Table 5.6) in consistent with a study conducted in India by Modi *et al*., Gondar by Mulat *et al.* [15,24]. The highest resistance rate was seen for Penicillin, Ampicillin, Vancomycin, and Tetracycline. This may be due to the absence of guidelines regarding the selection of drugs and social trend of inappropriate use of a commonly prescribed drugs. The highest multiple drug resistance was also observed among gram negative bacteria's from which *Proteus species* showed highest multiple antibiotic resistance similar with previous studies in Iran by Rezaeizadeh *et al*. and Gondar by Mulat *et al.* [12, 24].

Mostly clinicians' initiate antimicrobial therapy prior to the identification of the infecting agent and resistance study will play a crucial role in helping to understand trends in predominant pathogens and the impact of resistance on empiric choice.

Streptococcus pneumoniae (or *pneumococcus*) is a major cause of bacterial meningitis in the developing world. When compared to culture, the BinaxNOW *S.pneumoniae* immuno chromatographic test was >99% sensitive for the diagnosis of pneumococcal meningitis and yielded two false positive results. Even though the positive cases were much lower than the negatives. It identified additional pneumococcal cases among patients with a negative CSF culture further supported by negative test results with culture proven non pneumococcal cases. Antigen detection of CSF specimens provides a useful adjunct to culture-based diagnosis. Our study is in agreement with a multisite study conducted by Moisi *et al.,* a study conducted by Saha *et al.* in Bangladeshi with a result of ICT for pneumococcal antigen in CSF was 100% sensitive and specific in diagnosing pyogenic pneumococcal meningitis and could detect ≈30% more pneumococcal meningitis cases than with culture alone. [26, 40].

7. Limitation of the study

- Our study has limitation which is in relation to isolation of bacteria’s’ were merely by conventional biochemical tests and colony characteristics and does not use molecular tests like PCR.
- The study identifies only bacterial meningitis on suspected meningitides cases.
- The number of positive isolates of *S .pneumoniae* for the kit (BinaxNOW *S.pneumoniae* antigen test) comparison with culture was low.

8. Conclusion

In conclusion the prevalence of bacterial isolates in this study was 4.4%, most of the isolates were found CSF drawn from neonates. The predominant causes of meningitis were *S.pneumoniae*, *N.meningitidis*, *E.coli* and *proteus species*. The isolation rate of bacterial pathogens from CSF was found to be low in this study. Hence, antigen detection (BinaxNOW *S.pneumoniae* antigen test in our study) is a better adjuvant to culture, though a gold standard requires 48 hours, trained medical personnel and an equipped laboratory. This antigen test would help in making an early diagnosis and initiation of therapy, as providing a timely intervention was lifesaving and as it could help in reducing morbidity. Among all risk factors assessed, none of them were statistically significant with suspected meningitis cases ($p>0.05$). The frequency of single as well as multiple drug resistance was very high among the bacterial isolates. In line with this efforts are still needed to control the increase in antibiotic resistance by adopting a policy/guidelines for proper antibiotic use.

9. Recommendation

According to our findings we recommend the following points:

- We did only the bacteriological profile of the CSF but doing the full microbiology of the sample: the fungal, viral and bacterial profile or large scale studies with many factors will have important implication for clinicians' for proper patient treatment.
- We recommend that the ICT will be used as an adjunct to culture in pneumococcal meningitis cases if it studied including large number of positive samples.
- Area specific periodic evaluation of antimicrobial susceptibility pattern of micro-organisms on suspected cases of bacterial meningitides should be performed. Stress should also be given on the restrained and rationale use of antimicrobials.

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Annexes

Annex I: Information sheet (for mothers or guardians English version)

Name of the Organization: Addis Ababa University, College of Health Sciences, School of Allied Health Sciences, Department of Clinical Laboratory Sciences.

Title of the Research Project: 'Prevalence of bacterial isolates from cerebrospinal fluid, their antimicrobial susceptibility pattern and associated risk factors with special emphasis on *streptococcus pneomoniae* among pediatrics suspected meningitis patients at Tikur Anbessa and Yekatit 12 specialized Hospitals, Addis Ababa, Ethiopia.'

Name of Investigator: Mulu Hassen (BSc, MSc candidate)

Introduction

You are invited to participate in a study to be conducted by MSc student at Addis Ababa University, college of health sciences, School of allied health science, Department of medical laboratory science, It is aimed at determining the spectrum of bacterial isolates, their drug susceptibility pattern and associated risk factors with special emphasis on *streptococcus pneumoniae* among pediatrics suspected meningitis patients at Black Lion and Yekatit 12 specialized hospitals which is important to know the distribution of the bacteria to control the disease , knowing risk factors will be important for designing preventive strategies and also useful for drug choice, please read the following statements and ask any unclear points before you agree to participate.

Participation in this study is exclusively voluntarily. If you are not interested to participate to your child or if you once decide to participate and with draw your children at any time, there will be no consequences and your child will get all the services provided in the hospital with no problems. If you decide to participate your child, you have to sign on the consent form and you may obtain a copy of this information sheet.

What is expected from you and your child as a participant of the study?

As a participant of this study your children are expected to agree that after CSF specimen collected for his diagnostic purpose by responsible physician using lumbar puncture is done will be collected from your child specimen. In addition you are expected to give answers for some questions about yours and your child health and socio demographic conditions. You need to know that the results might be discussed with appropriate individuals out of this hospital. But the name of you or your child, address and phone number will not be disclosed and rather than identification code will be used in such conditions.

How much time will I and my child spent to participate in this study?

You will spend 20-25 minutes until the specimen is collected, the questionnaire is filled and the consent is signed.

What are the risks of participating in this study?

There are no anticipated risks to your participation. Because I took CSF specimen drawn for your children diagnostic test purpose. So there is no risk.

How our information is to be kept in secret?

All information that you give and the results from your child specimen will be used for this study only. Only limited number of professionals will have access to the information. All the information will be encoded in a computer and will be password protected.

What are the benefits from participation?

Since this study is MSc student research, there will be no payment for participants. But your child participation is important for studying the prevalence of *streptococcus pneumoniae* bacteria, other bacteria's isolated from CSF specimen and their drug susceptibility to know the distribution of this bacteria and associated risk factors.

What are your rights as a participant of this study?

You have the right to withdraw your child from the study at any time and all the services provided in the hospital will not be discontinued. You have also welcomed if you have any question for further explanations about the study. You can get the results of the analysis.

What can I do if I have a problem or question?

Please direct any questions or problems you may encounter during this study to

Mulu Hassen
Department of medical laboratory science, School of Allied health science, College of health sciences, Addis Ababa University, Addis Ababa Ethiopia.

Mobile: +251-92-0-47-05-29

Email: muluh2233@gmail.com

For additional information, please contact Addis Ababa University, Medical faculty institutional review board (IRB) office at:

Tell. +251-11-5-53-87-34

Fax +251-11-5-51-1-51-30-99

P.O.Box 9086, Addis Ababa, Ethiopia

Email:aaumfirb@yahoo.com

Agree to participate?

- ☐ Yes
- ☐ No

Annex II- Subject information sheet (for mothers /guardians, Amharic version)

አዲስ አበባ ዩኒቨርሲቲ,የጤና ሣይንስ ኮሌጅ,የአላይድ ጤና ሣይንስ ት/ቤት,የሕክምና ላቦራቶሪ ሣይንስ ክፍል

<u>እድሜያቸው ከአስራ አምስት አመት በታች በሆኑ ልጆች ላይ የህብለ ሰረሰር ፈሳሽ ተወስዶ ለሚሰራው ስትሬፕቶኮከስ ኒሞንያ የተባለዉ ተህዋስያን/ባክቴሬያ እና ሌሎች ባክቴርያወች ምን ያህል የተሰራጩ እንደሆነ ለማወቅ ጥናት ለሚሳተፉ ልጆች እናቶች/አሳዳጊ-ች የተዘጋጀ መረጃ</u>

በአዲስ አበባ ዩኒቨርሲቲ፣ጤና ሳይንስ ኮሌጅ የሕክምና ላቦራቶሪ ሳይንስ ት/ክፍል የማስተርስ ድግሪ ተማሪ የመመረቂያ ጥናት ላይ እዲሳተፉ ተጋብዘዋል፡፡እባክዎ በዚህ ጥናት ለመሳተፍ ከመስማማትዎ በፊት ከዚህ ቀጥሎ የሚገኘዉን ምንባብ በጥሞና ያንብቡና ግልጽ ያልሆነዉን/ትን ማንኛዉም ሃሳብ ይጠይቁ፡፡

<u>መግቢያ</u>

የጥናቱ ርዕስ « እድሜያቸው ከ15 አመት በታች በሆኑ ልጆች ላይ የህብለ ሰረሰር ፈሳሽ ተወስዶ ስትሬፕቶኮከስ ኒሞንያ እና ሌሎች ተህዋስያን/ባክቴሬያ ምን ያህል የተስፋፉ እንደሆነ ለማወቅና ለህክምና ስኬታማነት የሚያሳድረው ተፅእኖ/ችግር/ ለማጥናት ነው፡፡ አላማዉም ይህ ባክቴሬያ ምን ያህል የተስፋፉ እንደሆነና ለህክምና ስኬታማነት የሚያሳድረው ተፅእኖ/ችግር/ ለማግኘት አማራጭ መንገድ ይሆናል፡፡

እርስዎ ና ልጅዎ በዚህ ጥናት ላይ የሚኖራችሁ ተሳትፎ ሙሉ በሙሉ በበጎ ፈቃደኝነት ላይ የተመሰረተ ነዉ፡፡በዚህ ጥናት ዉስጥ ላለመሳተፍ ወይም ለመሳተፍ ከወሰኑ በኀላ ለማቋረጥ የሚወስኑ ቢሆንም እንኩዋ በዚህ ሆስፒታል የሚሰጠዉ ማንኛዉም አገልግሎት አይቋረጥም፡፡በጥናቱ ለመሳተፍ የሚስማሙ ከሆነ የስምምነት ቅጹ ላይ በጹሁፍ ወይም በጣት ፊርማ ማስቀመጥ ይጠበቅዎታል፡፡ከፈለጉ ይህንን መረጃ አንድ ቅጅ ለራስዎ ሊያስቀሩ ይችላሉ፡፡

<u>ልጄ የጥናቱ ተሳታፊ በመሆኑ የሚጠበቅበት ምንድን ነው?</u>

በዚህ ጥናት ለመሳተፍ የሚስማሙ ከሆነ ለልጅወ ለራሱ ምርምራ የህብለ ሰረሰር ፈሳሽ ናሙና ከልጄወ ላይ ከተወሰደ የታዘዘለትን የላቦራቶሪ ምርመራ ተሰርቶ ሲያልቅ የተረፈዉ ናሙና እንደሚወሰድና ለጥናቱ እንዲዉል መስማማት ይጠበቅብወታል፡፡ከተወሰደዉ ናሙና ላይ የሚገኙ መረጃዎች ከዚህ ሆስፒታል ዉጭ ለሚገኙና ለስራዉ አግባብነት ላላቸዉ ሰዎች ቢነገር የማይቃወሙ መሆኑን መስማማት ይጠበቅብዎታል፡፡ይሁን እንጅ ይህ አይነቱ መረጃ የርስዎን እንዲሁም የልጅዎን ማንነት የሚገልጡ መረጃዎችን ማለትም ስም፤አድራሻና የስልክ ቁጥር የመሳሰሉትን መረጃዎቸን አይጨምርም፡፡ ይልቁንም ለዚህ አገልግሎት ብቻ የሚዉል ልጅዎን ለማወቅ የሚያስችል መለያ ቁጥር ጥቅም ላይ እንዲዉል ይደረጋል፡፡በተጨማሪም ስለርስዎና ስለልጅዎ አጠቃላይ የጤና ሁኔታ ለሚቀርቡ አንዳንድ ተጨማሪ ጥያቄዎች መልስ መስጠት ይጠበቅብዎታል፡፡

<u>በዚህ ጥናት መሳተፍ ምን ያህል ጊዜ ይፈጃል?</u>

የተዘጋጀዉን መጠይቅ ለመሙላት፤የስምምነት ቅጹ ላይ ለመፈረምና ናሙና ለመስጠት ከ20-25 ደቂቃ ያስፈልጋል፡፡

<u>በዚህ ጥናት መሳተፍ የሚያስከትላቸዉ ችግሮች ምንድን ናቸዉ?</u>

ናሙና በሚሰበሰብበት ወቅት ምንም አይነት የከፋ ችግር አያጋጥምዎትም፡፡ናሙና የሚሰበሰበዉ ከልጅወ ከተቀዳዉ የህብለ ሰረሰር ናሙና ሃኪሙ ያዘዘለት ቤተ-ሙከራ ተሰርቶ ሲያልቅ የተረፈዉ ናሙና ነዉ::

<u>የልጄ የህክምና መረጃ በሚስጥር ተጠብቆ መቆየት የሚችለዉ እንዴት ነዉ?</u>

ስለራስና ስለልጅ የሰጡት ማንኛዉም መረጃና ከተወሰደዉ ናሙና ላይ የተገኘዉ የላቦራቶሪ ዉጤት የሚዉለዉ ለጥናቱ አላማ ብቻ ነዉ፡፡ ይህን ማህደር ሊያገኙ የሚችሉት የተወሰኑ የጥናቱ ተባባሪ ሰራተኞች ብቻ ናቸው፡፡ከዚያም በላይ ስለ እርስዎና ስለ ልጅዎ ያለውን ማንኛውንም መረጃ የተለየ የይለፍ ቃል ባለው የኮምፒውተር የመረጃ ማህደር ውስጥ እንዲቀመጥ ይደረጋል ፡፡

<u>በዚህ ጥናት መሳተፍ የሚያስገኛቸው ጥቅሞች ምንድን ናቸው ?</u>

ይህ ጥናት የማስተርስ ዲግሪ መመረቂያ እንደመሆኑ መጠን ለተሳታፊዎች ገንዘብ አይሰጥም፡፡ ነገር ግን የእርስዎ ተሳትፎ ህጻናትን ለመርዳትና ስትሬፕቶኮከስ ኒሞንያ እና ሌሎች ተህዋስያን/ባክቴሬያ ምን ያህል የተስፋፉ እንደሆነና ለህክምና ስኬታማነት ይረዳል፡፡

<u>የልጄ በዚህ ጥናት ተሳታፊ መሆኑ መብቱ ምንድን ናቸው ?</u>

በጥናቱ ውስጥ ያላችሁን ተሳትፎ በማንኛውም ጊዜ የማቋረጥ ሙሉ መብት የተጠበቀ ከመሆኑም በላይ ልጅን ከጥናቱ በማግለል ምክንያት የሚቀርበት ምንም አይነት የሆስፒታል አገልግሎት አይኖርም ፡፡ከዚህም በተጨማሪ ጥናቱ በተመለከተ ማንኛውንም አይነት ጥያቄ የመጠየቅና ገለጻ የማግኘት መብት አለበት፡፡የላብራቶሪ ምርመራ ውጤቱንም በነጻ ማግኘት ይችላሉ፡፡

<u>ጥያቄ ካለኝ ወይም ችግር ቢያጋጥመኝ ምን ማድረግ ይገባል?</u>

ይህንን ጥናት በተመለከተ ወይም ከዚህ ጥናት ጋር በተዛመደ መልኩ ስለሚያጋጥሙ ድንገተኛ አደጋዎች ወይም ጥያቄ ካለዎት በሚመለከተው አድራሻ ይጠቀሙ፡፡

ሙሉ ሀሰን

የህክምና ላባራቶሪ ሳይንስ ት/ክፍል: የአላይድ ጤና ሳይንስ ት/ቤት: የጤና ሳይንስ ኮሌጅ : አዲስ አበባ ዩኒቨርሲቲ

ሞባይል: +251-92-0-47-05-29

ኢሜይል: muluh2233@gmail.com

ለመሳተፍ ይስማማሉ?

- □ እስማማለሁ
- □ አልስማማም

ለተጨማሪ መረጃዎች የአዲስ አበባ ዩኒቨርሲቲ ህክምና ፋኩልቲ ኢንስቲትዩሽናል ሪቬው ቦርድ ይጠይቁ፡፡

ስ.ቁ +251-11-5-53-87-34

ፋክስ +251-11-5-51-1-51-30-99

ኢሜይል aaumfirb@yahoo.com

Annex-III- Consent Form (for mothers/guardians, English version)

I have been informed about the study which is aimed at determining the prevalence of bacterial isolates from CSF of suspected meningitis patients, and their drug susceptibility pattern and associated risk factors among pediatrics patients. For this study CSF sample is required from my children. The aims of the study were explained to me.

I am also informed that all the information contained within the questionnaire is to be kept confidential. Moreover I have been well informed of my right to keep hold of information, decline to cooperate and make my child withdraw from the study.

It is therefore with full understanding of the situation that I gave the informed consent voluntarily to the researcher to use the CSF taken from my child for the investigation. In addition, I have had the opportunity to ask questions about it and received clarification to my satisfaction. I have also been informed that the benefit of participation is to get the results of analysis from my child sample analyzed for free.

Participant code: ___________________ Signature: _____________

Date: ______________

For those who can't read the information

Advisor nurse name _________________ Signature: _____________

Date _______________

Annex-IV - Consent form (for mothers/guardians, Amharic Version)

<u>**የተሳታፊወች ስምምነት ማረጋገጫ ቅጽ**</u>

እኔ ከላይ የተጠቀሰኩት የተሳታፊዉ እናት/ተንከባካቢ ‹‹**እድሜያቸው ከአስራ ስምንት አመት በታች በሆኑ ልጆቸ ላይ የህብለሰረሰር ፈሳሽ ተወስዶ ለሚሰራው ስትሬፕቶኮከስኒሞንያ የተባለዉና ሌሎች ተህዋስያን/ባክቴሬያ ምን ያህል የተሰራጩ እንደሆነ እና እነዚህ ታህዋስን/ባክቴሪያ/ ለምን ያህል ለተለያዩ መድሃኒቶች የተለማመዱ/የተቋቋሙ መሆናቸዉን ለማሳየት የሚጠና**›› ጥናት በቂ ገለጻ ተደርጎልኛል፡፡ለጥናቱም ከልጆች የተወሰደ የህብለሰረሰር ፈሳሽ ናሙና እንደሚያስፈልግ ተገልጾልኛል፡፡የጥናቱንም አላማዎችም ተረድቻለሁ፡፡

በመጠይቁ ላይ የገለጽኳቸው መረጃዎች በሙሉ በሚስጥር የተጠበቁ እንደሚሆኑ ተነግሮኛል፡፡በጥናቱ ላይ ያለመሳተፍና ማንኛውንም መረጃ ያለመስጠት እንዲሁም በማንኛውም ጊዜ ከጥናቱ ራሴን የማግለል መብቴ የተጠበቀ እንደሆነ ተገልጾልኛል፡፡

ስለዚህ ለዚህ ጥናት መረጃና የስምምነት ቃሌን የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳትና በፍጹም ፍቃደኝነት ነው፡፡ከሰረሰርጌ አንጎል ላይ የሚወሰደው ለምርምር እንደሚውልም ተረድቻለው፡፡በተጨማሪም ጥያቄ ለመጠየቅ ተፈቅዶልኝ ለማወቅ የፈለኩትን ያህል ማብራሪያ አግኝቻለሁ፡፡የዚህ ጥናት ተሳታፊ በመሆኔ የማገኘው ጥቅም የሁሉንም ምርመራ ውጤት በነጻ ማግኘት እንደሆነ ተረድቻለሁ፡፡

የሚስጥር ቁጥር ----------------------------- የተሳታፊዉ ወላጅ ወይም አሳዳጊ ፊርማ /የጣት አሻራ -----------------------

ቀን---------------------------------------

(የስምምነት ቅጹን ማንበብ ለማይችሉ ተሳታፊዎች)

የአማካሪ ነርስ ስም --- ፊርማ --

ቀን----------------------

Annex-V **Questionnaire (English version)**

Questionnaire for the study on '**Prevalence of bacterial isolates from cerebrospinal fluid, their antimicrobial susceptibility pattern and associated risk factors with special emphasis on *streptococcus pneomoniae* among pediatrics suspected meningitis patients at Tikur Anbessa and Yekatit 12 specialized hospitals, Addis Ababa, Ethiopia.'**

Instruction: fill the information, either thick in the appropriate boxes by using "right" or a word or phrases where required. Code number ____

I. **Socio-demographic factors and Patient Identification**

1. Age of participant pediatric________
2. Sex of participant pediatric ________
3. Address □ urban □ Rural__________ Card number_________
4. Educational level of the mother

   □Unable to write and read □Certificate

   □Below fourth grade □ Diploma

   □5th to 8th grade □ First degree and above
5. Occupation of the mother

   □Student □House wife

   □Employed □ Jobless

   □Private work □ others

   □Farmer
6. How do you cook your food? You can put marks on more than one option

   □Use of wood for heat □ Use of electric for heat

   □Use of kerosene for heat □ other source of energy
7. No of house partition

   □ Greater than or equal to four □ less than four
8. No of people sharing the same room with the child

   □<2 □ 2-3 □>4
9. Is your child attending to day care center?

   □ Yes □ No

10. Is there any industry in your village that left its surplus products inappropriately?

 Yes No

II. Health related information

11. Has your child been sick and hospitalize for any reason during the previous month?

Yes No

12. If yes, when---------------------------describe the illness------------------------
13. Is he/she taking any prescribed medication during the previous week and now?

Yes No

14. If yes, what type?
15. How do you feed your child? You can put marks on more than one options

Only breast milk some additional foods

Only formula milk breast milk and formula milk

16. Have you ever been smoke cigarette/ is there anyone who smokes cigarettes in the house hold or neighbors?

Yes No

17. If yes for (Q-15), how frequently do you smoke?

Always occasionally

18. Have you ever been taken any vaccine during the last 7 days?

 Yes No

19. If yes for (Q-17) what type____

Annex-VI **Questionnaire (Amharic version)**

አዲስ አበባ ዩኒቨርሲቲ፡ የጤና ሳይንስ ኮሌጅ፡የአላይድ ጤና ሳይንስ ት/ቤት፡የሕክምና ላቦራቶሪ ሳይንስ ት/ክፍል

እድሜያቸው ከ15 አመት በታች በሆኑ ልጆች ላይ የህብለ ሰረሰር ፈሳሽ ተወስዶ ለሚሰራው ስትሬፕቶኮከስ ኒሞንያ የተባለዉና ሌሎች ተህዋስያን/ባክቴሬያ ምን ያህል የተሰራጩ እንደሆነ እና እነዚህ ታህዋስን/ባክቴሪያ/ ለምን ያህል ለተለያዩ መድሃኒቶች የተለማመዱ/የተቋቋሙ መሆናቸዉን ለማሳየት የሚጠና ጥናት ለሚሳተፉ ልጆች እናቶች የተዘጋጁ መጠይቆች

የሚስጥር ቁጥር --------

<u>ክፍል ፩፡</u> አካባቢያዊና ማህበረሰባዊ ሁኔታዎች

1. ልጁን የተመለከተ መረጃ

   እድሜ -----------------አመት

2. ፆታ፡ □ ወንድ □ ሴት
3. አሁን የሚኖሩበት አካባቢ?

   □ ከተማ □ ገጠር

4. የእናት ወይም የአሳዳጊ የትምህርት ደረጃ

- □ ማንበብ መጻፍ የማትችል
- □ ከአራተኛ ክፍል በታች
- □ ከአምስተኛ እስከ ስምንተኛ ክፍል
- □ ሁለተኛ ደረጃ
- □ ሰርትፊኬት
- □ ዲፕሎማ
- □ የመጀመሪያ ዲግሪ እና ከዛ በላይ

5. የእናት የስራ ሁኔታ

- □ ተማሪ
- □ የቤት እመቤት
- □ ተቀጣሪ(የመንግስት /የግል)
- □ ስራ የሌላት
- □ የግል ስራ
- □ ሌላ
- □ ግብርና

6. ምግብዎን እንዴት ያበስላሉ?ከአንድ በላይ ምርጫ መምረጥ ይችላሉ፡፡

□ የእንጨት እሳት በመጠቀም

□ ጋዝ በመጠቀም

□ ኤሌክትሪክ በመጠቀም

□ ሌላ ነገር በመጠቀም

7. የቤታችሁ ክፍል ቁጥር ስንት ነዉ?

□ ከአራት ያነሰ

□ ከአራት በላይ

8. ከልጅዎ ጋር አንድ ክፍል የሚጋሩ ሰዎች ቁጥር ስንት ነዉ?

□ <2

□ 2-3

□ >4

9. ልጅዎ በልጆች ማቆያ/ትምህርት ቤት ዉስጥ ?

□ ይዉላል

□ አይዉልም

10. በአካባቢያችሁ ተረፈ-ምርቱን ያለአግባብ የሚያስወግድ ኢንዱስትሪ አለ?

□ አለ

□ የለም

<u>ክፍል ፪- የስነ-ጤና መረጃ</u>

11. በአለፉት ወራቶች ልጅዎ ታሞ/ታማ ያዉቃል/ታዉቃለች?

□ አዎ

□ የለም

12. መልሱ አዎ ከሆነ መች?-----------ህመሙ ምን እንደነበር ይግለጹ------------

13. ልጅዎ መድሃኒት በመዉሰድ ላይ ነዉ/ነች?

□ አወ

□ አይደለም

14. መልሱ አወ ከሆነ ምን አይነት መድሃኒት?--------------------------

15. ልጅዎ ከዛሬ ሰባት ቀን በፊት ክትባት ወስዶ ነበር?

- አወ
- አይደለም

16. መልሱ አወ ከሆነ የምን ክትባት------------------

17. ልጅዎን ምን ይመግባሉ?ከአንድ በላይ ምርጫ መምረጥ ይችላሉ፡፡

- የእናት ጡት ብቻ
- የእናት ጡትና የቆርቆሮ (የታሸገ)ወተት
- የቆርቆሮ (የታሸገ)ወተት
- ሌሎች ተጨማሪ ምግቦች

18. በቤት ዉስጥ ወይም በአካባቢያችሁ ሲጋራ የሚያጨስ ሰዉ አለ?

- አለ
- የለም

19. ለቁጥር 17 መልስዎ አዎ ከሆነ ለምን ያህል ጊዜ ያጨሳሉ?

ሀ) ሁልጊዜ ለ) አልፎ-አልፎ

መጠይቁን ጨርሰዋል፡፡

አመሰግናለሁ፡፡

Annex VII– **Laboratory data collection format**

1. Patient ID. -----and CSF sample ID ------
2. Date of sample collection -----/-----/------
3. Type of specimen: CSF
4. Appearance of specimen-------------------
5. WBC count---------------------------------
6. Protein result--------------------------------
7. Gram stain result--------------------------
8. *Streptococcus pneumoniae* antigen test result--
9. Culture growth: Yes-----------No---------
10. Name of bacteria, if isolated---------------
11. Biochemical identification test results---
12. . Antimicrobial susceptibility testing

Antimicrobial	S (mm)	I (mm)	R (mm)
o ERY- Erythromycin (15ug)	------------	-------------	----------
o GE- Gentamycin (10 ug)	------------	-------------	----------
o CIP- Ciprofloxacin (5ug)	------------	--------------	----------
o TE- Tetracycline (30ug)	------------	--------------	----------
o PE-Penicillin (5ug)	------------	--------------	----------
o AMP- Ampicillin (30 ug)	------------	--------------	----------
o CRO- Ceftriaxone (30ug)	------------	--------------	----------
o K-Kanamycin (5ug)	------------	--------------	----------
o NA-Nalidixic acid (30ug)	------------	--------------	----------
o CAF-Chloramphenicol (30ug)	------------	--------------	----------
o DOX-Doxycycline (30ug)	------------	--------------	----------
o VA-Vancomycin (30ug)	------------	--------------	----------
o Norfloxacin (NOR,10µg)	------------	--------------	----------
o Trimethoprim- Sulfamethoxazole(SXT,1.25µg)	------------	--------------	----------
o Cefuroxime-Sodium (CXM,30µg)	------------	--------------	----------
o Cefotaxime (CTX,30µg)	------------	--------------	----------
o Cephazoline (KZ,30µg)	------------	--------------	----------
o Ceftazidime (CAZ,30µg)	------------	--------------	----------
o Cefoxitin (FOX,30µg)	------------	--------------	----------

Annex VIII-SOP for preparation of culture media, collection and processing of specimens, Culturing and Identification.

1. Specimen collection and preparation (cerebrospinal fluid)

1. CSF samples should be collected in a sterile tube by responsible physician using lumbar puncture and should be labelled properly.
2. Record volume and gross appearance of CSF i.e. clear, bloody, cloudy, xanthochromic. If the specimen is cloudy, a gram stain should be done before the centrifugation.
3. Centrifuge the CSF specimen for Gram stain.
4. Centrifuge the specimen: If the volume of fluid is greater than 1 ml, centrifuge for 15 min at 2500-3000 rpm .Remove supernatant and vortex the sediment vigorously for at least 30 seconds. If less than 1 ml is received, do not centrifuge but vortex the specimen.
5. Using a sterile pipette, inoculate the media and streak for isolated colonies. In addition, add a single drop of fluid to the 4th quadrant of the plate and allow to air dry. Do not streak out this drop of fluid.
6. Interpretation of Cultures: Examine the MacConkey, blood and chocolate agar plates daily for 72 hours.

Laboratory procedures

2. Culturing

2.1.Materials and Equipment needed (some of them)

- Culture media,
- Petri dish,
- Culture tube,
- Inoculating loop, Straight wire,
- Bunson burner,
- Sample of bacteria (Positive and Negative control organisms),
- Bio-safety cabinet,
- Incubator,
- Refrigerator,
- Autoclave,
- PH meter,
- Flask,
- Measuring cylinder,
- Aluminum foil,
- Boiler and
- Balance

2.2.Preparation of culture media

The general procedure includes;

1. Weighing and dissolving: - weighing is based on the manufacturer's direction and then multiply or reduced based on the amount of media needed. Dissolving is mediated by mixing very well by rotating 360^0 until all the powders mixed and finally boiling to get homogenous solution.
2. Sterilization: - majority of culture media are sterilized by being autoclaved. It is performed by placing in autoclave of 121 ^oC for 15 minute. This ensures the destruction of bacterial endospores as well as vegetative cells. It is important to sterilize a medium at the correct temperature and for the correct length of time as instructed in the method of preparation.
3. Add heat susceptible chemicals or substances such as blood in case of blood agar, and certain antibiotic supplements.
4. PH testing: - the PH of most culture media is near neutral. An exception is alkaline peptone water. The simplest way of testing the PH of a culture medium is to use narrow range pH paper. It can be tested by dipping a narrow range PH paper in to a sample of the

medium when it is at room temperature before dispensing and comparing the color of the paper against the PH color chart.

5. Dispensing: - before this we have to cool the media based on the manufacturer's direction but in case of agar media we have to cool a temperature of between 45-50^{o}C because agar in nature is solidifying agent so below this temperature will solidify the media before dispensing. Dispensing should be until it covers the surface of the dish on flat and sterile surface to get uniform depth at room temperature. Wait 10-15 minute until it solidify and invert the media and wait hours (6hr most of the time).
6. Quality control test: - sterility and performance test. Performance test is used to check quality of culture media prepared (whether it can be used to perform the intended test) using control organisms. We have to inoculate the control organism that grows and not grow on the prepared media and check whether it performs its actual activity. Incubate 4-5% of prepared media for sterility testing overnight on incubator, if there is growth we have to discard the batch.
7. Labeling and Storage: - All culture media must be clearly labeled with its name, preparation and expire date. Plates of culture media should be stored at 2-8^{o}C, preferably in sealed plastic bags to prevent loss of moisture. When in use, the media must be protected from direct light, especially sun light.

2.3. Inoculation

Inoculation is the next step in culturing of samples where placing the specimen on appropriate culture media takes place. The most common culture media used for inoculation of CSF sample during the study includes; MacConkey, chocolate and Blood agar.

2.4. Aseptic techniques

- Decontaminate the work bench before starting the day work and after finishing.
- Use a safety cabinet when working with hazardous pathogens and wear protective clothing, gloves and face mask.
- Flame sterilize wire loops, straight wires and metal forceps before and after use.
- Flame the necks of specimen bottles, culture bottles and tubes after removing and before replacing caps or plugs.
- When inoculating, do not let the tops or caps of bottles and tubes touch non-sterile surface. This can be avoided by holding the top cap between fingers in hands.

2.5.Incubation

Following to inoculation, incubation was performed because after placing the sample on the appropriate media we have to create suitable environment for the growth of pathogenic organisms. The most common suitable environment for pathogenic bacteria is the environment that resembles human body so we have to incubate in the incubator of 37^{o}C for overnight in aerobic or micro aerophilic environment according to the bacterial species expected.

3. Tests for bacterial identification.

3.1.Gram Stain for the CSF specimen

Purpose

- Used to classify bacteria as gram positive and gram negative based on their gram reaction.

Principle

Gram's stain is that cells are first fixed to slide by heat or alcohol and stained with a basic dye (e.g. crystal violate), which is taken up in similar amounts by all bacteria. The slides are then treated with a Gram's iodine (iodine KI mixture) to fix (mordant) the crystal violet stain on Gram positive bacteria, decolorized with acetone or alcohol, and finally counter stained with Safranin.

Required

- Crystal violet (initial stain), Lugol's iodine (mordant or binding agent), Acetone–alcohol (decolorizer) and Safranin (counter stain) were the reagents commonly used for gram staining technique,
- Immersion oil,
- Slides, Forceps, Washer, Staining Bottles,
- Flame (bunson burner), Cotton, Match and Microscope.

Procedure

1. Place the fixed slide on the staining rack.
2. Cover the heat-fixed smear with crystal violet; leave for 10 seconds.

Wash off with water and drain well.

3. Cover the smear with Gram's iodine; leave for 10 seconds.

Wash off promptly with water and drain.

4. Holding the slide over the sink at a 45° angle, RUN the decolorizing agent (acetone/alcohol) over the slide and watch the excess run off the bottom edge of the slide. Continue decolorizing until the purple dye no longer runs off the slide with the decolorizer, (i.e., the excess dripping off the bottom edge of the slide is colorless).Immediately wash with water and drain.
5. Cover the smear with basic fuchsin or safranin (red counterstain); leave for 10 seconds.

Wash with water and drain; air-dry in an upright position.

6. Leave until dry and look for a microscope (100 xs)

7. Report as gram positive (blue coccii or rods) and gram negative (red rods or coccii) and others accordingly.

3.2.BinaxNOW *streptococcus pneumoniae* antigen test

Principle

The BinaxNOW *S. pneumoniae* urinary antigen test is an immunochromatographic assay that uses a rabbit anti-*S.pneumoniae* antibody, conjugated to visualizing particles, to bind any soluble pneumococcal antigen (C polysaccharide) present in the urine or CSF sample. The resulting complex is immobilized by a band of rabbit anti-*S.pneumoniae* antibodies adsorbed onto a nitrocellulose membrane (sample line). A second band of goat anti-rabbit immunoglobulin G (control line) captures excess visualizing complex. A swab is dipped into the urine or CSF and inserted into the test device; a buffer solution is added, and the device is closed. The result is read by eye, after 15 min. A pink to purple color on both the sample and control lines indicates a positive antigen test. Color on the control line alone indicates a negative test. Absence of color on the control line indicates an invalid test.

Procedure

1. Bring patient sample(s) and /or liquid to room temperature (15-30ºc), then swirl gently to mix. Remove device from its pouch **just before use** and lay flat.
2. Dip a Binax swab into the sample to be tested, completely covering the swab head. If the swab drips, Touch swab to side of collection container to remove excess liquid.
3. There are two holes on the inner right panel of the device. Insert swab into the **BOTTOM** hole (swab well).Firmly push upwards so that the swab tip is fully visible in the top hole. **DO NOT REMOVE SWAB.**
4. Hold Reagent A vial vertically,1/2 to 1 inch above the device .slowly add **three(3)** free falling drop of reagent A to the **BOTTOM** hole.
5. Immediately peel off adhesive liner from the right edge of the test device. Close and securely seal the device. Read result in window 15 minutes may be inaccurate. However, some positive patients may produce a visible sample line in less than 15 minutes.

Interpretation of the result

A negative sample will give a single pink-to-purple colored control line in the top half of the window, indicating a presumptive negative result. This control line means that the detection part of the test was done correctly, but no S.pneumonia antigen was detected.

A positive sample will give two pink-to-purple colored lines. This means that antigen was detected .Specimens with low levels of antigen may give a faint patient line. **Any visible line is positive.**

If no lines are seen, or if just the sample line is seen, the assay is invalid .Invalid tests should be repeated.

Reporting of the result

Positive CSF Positive for pneumococcal meningitis

Negative CSF Presumptive negative for Pneumococcal meningitis. Infection due to *S.pneumoniae* cannot be ruled out since the antigen present in the sample may be below the detection limit of the test [34].

3.3.Biochemical testing procedures

Identification of gram positive bacteria

Gram-positive cocci will be identified based on their gram reaction, catalase and coagulase tests results.

Catalase test

This test is used to differentiate those bacteria that produce the enzyme catalase such as *staphylococci* from non-catalase producing bacteria such as *streptococci*.

Principle:

Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more than 24 hours old.

Required

- 3% H_2O_2.

Method:

1. Pour 2-3 ml of the H_2O_2 solution into a test tube
2. Using sterile wooden sticks(swab)remove several colonies of test organism and immerse in the hydrogen peroxide solution
3. Look for immediate bubbling

Result: Active bubbling………………………..Positive result

No bubbling ……………………………Negative result

Control: Positive control ……………………………….*staphylococcus species*

Negative control………………………………*streptococcus species*

Bile solubility test

This helps to differentiate *S.pneumoniae*, which is soluble in bile and bile salts, from other alpha hemolytic streptococci (*viridian streptococci* which are insoluble).

Principle:

Heavy inoculums of a test organism are emulsified in saline and bile salt sodium deoxycholate is added. This dissolves *S.pneumoniae* by clearing of the turbidity within 10-15 minutes. Viridians and other *streptococci* are not dissolved and therefore there is no clearing of turbidity.

Method:

1. Emulsify several colonies on the test organism in a tube containing 2 ml saline, to give a turbid suspension.
2. Divide the organism suspension between two tubes.
3. To one tube add two drops of the sodium deoxycholate reagent and mix.
4. To the other tube (negative control), add two drops of sterile distilled water and mix.
5. Leave both tubes for 10-15 min at 35-37 0c.
6. Look for clearing of turbidity in the tube containing the sodium deoxycholate.

Results

Clearing of turbidity . . . . Probably *S. pneumoniae*

No clearing of turbidity . . . . Organism is probably not *S. pneumoniae*

Controls

Bile solubility positive control…………… *Streptococcus pneumoniae*

Bile solubility negative control………………. *Enterococcus faecalis*.

Coagulase test

The test is used to differentiate *staphylococcus aureus* (+ve) from other staphylococcus spp (-ve).

Principle: in the presence of the enzyme coagulase, the addition of commercial rabbit plasma produces a clumping reaction.

Required

Rabbit plasma, the plasma should be allowed to warm to room temperature before being used

Procedure

1. Place a drop of physiological saline on two separate slides.
2. Emulsify the test organism in each of the drop to make thick suspension.
3. Add one drop of plasma to one of the suspensions and mix gently.
Look for clumping of the organism within 10 seconds.
4. Interpretation:
Clumping within 10 seconds ------------------*S. aureus*
No clumping within 10 seconds --------------other *staphylococcus species*

Manitol fermentation test

Mannitol salt agar is a differential and selective media. It is selective because its high salt concentration (7.5 %) inhibits the growth of most bacteria. However, *Staphylococcus* is able to tolerate this high salinity.

Mannitol salt agar is differential because it contains the sugar mannitol and phenol red, a pH indicator. When mannitol is fermented, acid products are produced and the pH drops. Phenol red is yellow in color below pH 6.8. Thus, mannitol fermenters such as *Staphylococcus aureus* will have a yellow halo around them. Mannitol non fermenters such as *Staphylococcus epidermidis* will leave the MSA media unaltered (pink).

Identification of gram negative bacteria:-

Identification of gram negative bacteria will be based on their test result with a series of biochemical tests.

Procedure

1. Prepare a suspension of the test organism with nutrient broth by adding 3-4 colony of test organism in 5 ml nutrient broth.
2. A loop full of the bacterial suspension is inoculated in to indole, citrate agar, triple sugar iron agar, lysine decarboxylase agar, oxidase, urea agar and motility medium.
See in detail below one by one.
3. Incubate at 35-37^{O}C for 18-24 hours.
4. Look for color change (turbidity for motility) of the medium.
5. Identify the test organism by considering the result of biochemical tests.

Indole test

Principle:

Some bacteria can produce indole from amino acid tryptophan using the enzyme typtophanase. Production of indole is detected using Ehrlich's reagent or Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color. An alcoholic layer concentrates the red color as a ring at the top.

Required:-Kovac's reagent, Tubes, Inoculating loop and Incubator.

Procedure:

Bacterium to be tested is inoculated in peptone water, which contains amino acid tryptophan and incubated overnight at 37^{o}C. Following incubation few drops of Kovac's reagent are added. Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and concentrated HCl. Ehrlich's reagent is more sensitive in detecting indole production in anerobes and non-fermenters. Formation of a red or pink colored ring at the top is taken as positive.
Example: *Escherichia coli*: Positive; *Klebsiella pneumoniae*: Negative

Citrate utilization test:

Principle:

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen. Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO_2.Production of Na_2CO_3 as well as NH_3 from utilization of sodium citrate and ammonium salt respectively results in alkaline PH. This results in change of medium's color from green to blue.

Procedure: Bacterial colonies are picked up from a straight wire and inoculated into slope of Simmon's citrate agar and incubated overnight at 37^oC. If the organism has the ability to utilize citrate, the medium changes its color from green to blue.

Triple sugar iron agar

Triple Sugar Iron Agar (TSI) used to determine if bacteria can ferment glucose, and/or lactose and if it can produce hydrogen sulfide or other gases. In addition, TSI detects the ability to ferment sucrose. These characteristics help distinguish various *Enterobacteriacae*, including *Salmonella* and *Shigella*, which are intestinal pathogens.

Procedure: Bacterial colonies are picked up from a straight wire and the tube is inoculated by stabbing into the agar butt (bottom of the tube) with an inoculating wire and then streaking the slant in a wavy pattern. Results are read at 18 to 24 hours of incubation.

TSI contains three sugars: glucose, lactose and sucrose. Lactose and sucrose occur in 10 times the concentration of glucose (1.0% versus 0.1%). Ferrous sulfate, phenol red (a pH indicator that is yellow below pH 6.8 and red above it), and nutrient agar are also present. A yellow slant on TSI indicates the organism ferments sucrose and/or lactose. A yellow butt shows that the organism fermented glucose. Black precipitate in the butt indicates hydrogen sulfide production. Production of gases other than hydrogen sulfide is indicated either by cracks or bubbles in the media or the media being pushed away from the bottom of the tube.

Oxidase test (Filter Paper Method)

The oxidase test is used to determine if an organism possesses the cytochrome oxidase enzyme.

1. Soak a piece of filter paper in the reagent solution.
2. Scrape some fresh growth from the culture plate with a disposable loop or stick and rub onto the filter paper or touch a colony with edge of paper.
3. Examine for blue colour within 10 sec.

Lysine decarboxylase agar

A selective isolation medium for *Salmonellae* and *Shigella*. Low in nutrients this medium relies on a small amount of Sodium deoxycholate for selectivity. The indicator system is complex. Most enteric organisms except *shigella* will ferment xylose to produce acid however the *salmonellae* will also decarboxylate the lysine to keep the pH neutral. At near neutral pH the *salmonella* can produce H_2S from the reduction of thiosulphate producing black or black centered colonies. *Citrobacter spp*. can also decarboxylate lysine, however the acid produced by fermentation of both lactose and sucrose will keep the pH too acid for H_2S to be produced

Urease test

Some bacteria produce the enzyme Urease, which catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. Organisms that do not produce this enzyme cannot metabolize urea. Urea broth has a minimal amount of yeast extract along with urea. Organisms that cannot metabolize urea will have insufficient nutrients for growth. Urea hydrolysis will result in a pH increase because of the production of ammonia. The pH indicator phenol-red, will turn pink with this pH increase. However, the presence of strong buffers in the medium requires a large amount of ammonia production to cause a color change. Thus, only strong hydrolyzers of urea will turn the broth pink (indicating a positive result). This should happen within 24 hours.

Results

+ = Positive (Pink coloration within 24 hours to 48 hours)

- = Negative (Orange coloration after 24 to 48 hours)

V = Variable

Methods

1. Obtain two Urease Broths from the refrigerator.
2. Inoculate one broth using aseptic technique. Leave the other broth uninoculated (this will be used as a control).
3. Incubate at an appropriate temperature (whatever temperature your organism grows well at). Incubate for 24 to 48 hours (do not exceed 48 hours for this test).
4. Obtain your broths from the incubator and observe the color.

Motility testing

To test for motility, use a sterile straight wire to pick a well-isolated colony and stab the motility medium to within 1 cm of the bottom of the tube. Be sure to keep the straight wire in the same line it entered as it is removed from the medium. Incubate at 35°C for 24 hours or until growth is evident. A positive motility test is indicated by a red turbid area extending away from the line of inoculation. A negative test is indicated by red growth along the inoculation line but no further. See the methods below in detail.

Methods

1. Obtain a motility agar tube from the back shelf.
2. Use an inoculating pick. Straighten the pick as much as possible.
3. Make a stab inoculation (about 2/3 of the way into the agar) from your unknown stock culture. Try to make the stab (in and out) as straight as possible. Straightness is important because you will be evaluating the amount of growth away from the stab. A messy stab will be difficult to evaluate.
4. Incubate at an appropriate temperature for 24 to 48 hours (up to 72 hours).
5. Observe your culture by holding it up to a light source.

H_2S production

Principle

- Hydrogen sulfide (H_2S) is produced by bacterial anaerobic degradation of the two sulfur-containing amino acids, cysteine and methionine. Hydrogen sulfide is released as a by-product when carbon and nitrogen atoms in the amino acids are consumed as nutrients by the cells. Under anaerobic conditions the sulfhydryl (-SH) group on cysteine is reduced by cysteine desulfurase. The agar contains high levels of peptones (sources of cysteine and methionine) and ferrous sulfate as an indicator. When H_2S is produced, the ferrous ion reacts with it to give ferrous sulfide, an insoluble black precipitate.

Required

- TSI Agar Slant, Inoculating loop and Incubator.

Procedure

1. The triple sugar iron agar slant was inoculated by stabbing the butt and drawing the stick over the surface of the slope.
2. Incubated at 35-37^{o}C for 18 to 24 hours.
3. Looked for black precipitate formed.

Result

- Acid deep (yellow)/alkaline slant (red):- glucose fermented, lactose and/or sucrose not fermented.
- Acid deep (yellow)/acid slant (yellow):- lactose and/or sucrose fermented.
- Alkaline deep and slant (all red):- glucose, sucrose, and lactose not fermented.
- Deep split or displaced: - gas production.
- Deep blackened: - H_2S production.

Voges Proskauer (VP) test:

Principle:

While MR test is useful in detecting mixed acid producers, VP test detects butylene glycol producers. Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In these test two reagents, 40% KOH and alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alphanaphthol to produce red color. Role of alpha-naphthol is that of a catalyst and a color intensifier.

Procedure:

Bacterium to be tested is inoculated into glucose phosphate broth and incubated for at least 48 hours. 0.6 ml of alpha-naphthol is added to the test broth and shaken. 0.2 ml of 40% KOH is added to the broth and shaken. The tube is allowed to stand for 15 minutes. Appearance of red color is taken as a positive test. The negative tubes must be held for one hour, since maximum color development occurs within one hour after addition of reagents [35].

Examples: *Escherichia coli*: Negative;
Klebsiella pneumoniae: Positive

VI. Antimicrobial susceptibility testing

1. Optochin test

S.pneumoniae strains are sensitive to the chemical Optochin (ethyl hydrocupreine hydrochloride). Optochin sensitivity allows for the presumptive identification of alpha-hemolytic streptococci as S. pneumonia, although some *pneumococcal* strains are Optochin-resistant. Other alpha-hemolytic streptococcal species are Optochin-resistant.

A. Performing the Optochin test

1. Grow the strains to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO_2.

2. Use a disposable loop to remove an isolated colony from the overnight culture on the BAP and streak onto one half of a BAP.

Two different isolates can be tested on the same plate, but care must be taken to ensure that the cultures do not overlap.

3. Place a P disk within the streaked area of the plate and incubate the BAP overnight at 35-37°C with ~5% CO_2 (or in a candle-jar).

4. Observe the growth on the BAP near the P disk and measure the zone of inhibition, if applicable.

B. Reading the Optochin test results

- ✓ Using a 6 mm, 5 mg disk, a zone of inhibition of 14 mm or greater indicates sensitivity and allows for presumptive identification of *pneumococci*.
- ✓ Zones of inhibition should be measured from the top surface of the plate with the top removed.
- ✓ Use either calipers or a ruler with a handle attached for these measurements. Measure the diameter of the zone holding the ruler over the center of the surface of the disk when measuring the zone of inhibition. In the case of an isolate completely resistant to Optochin, the diameter of the disk (6 mm) should be recorded [35].

2. Antibiotic susceptibility testing

Procedure

1. Prepare a suspension of the test organism by emulsifying several colony of the organism in a small volume of nutrient broth.

2. Much the turbidity of suspension with turbidity standard.

3. With a sterile swab take sample from the suspension (squeeze the swab against the side of the test tube to remove the excess fluid).

4. Spread the inoculums evenly over the Muller-Hinton agar plate with the swab

5. Using a sterile forceps or needle, place the antimicrobial disc on the inoculated plate.

6. Incubate the plate aerobically at 35-37^{o}C for 18-24 hours.

7. Read the test after checking that the bacterial growth is neither heavy nor light. Measure the radius of the inhibition zone.

8. Interpret the reaction of the test organism to each antibiotics used as sensitive, intermediate, or resistance as per the standard.

Sensitive ____________________zone of radius is wider or equal to the control

Intermediate __________________zone of radius is > three mm smaller than the control

Resistance ___________________ no zone of inhibition [36].

Declaration

I the undersigned, declare that this is my original work and has not been presented for a degree in this or any other university and all sources of materials used for this thesis have been acknowledged.

Principal investigator: Mulu Hassen (Bsc,MSc. Student, CLS, AAU)

E-mail: muluh2233@gmail.com Phone: +251-920-47-05-29

Signature-----------------------------

Approval of advisors

1. Gebru Mulugeta (Msc,AAU)

   E-mail:gebrumulugeta@gmail.com Phone: +251-911-75-76-00

   Signature-------------------------Date-----------------------------

2. Kassu Desta

   E-mail-tulukassu@gmail.com Phone: +251-911-10-70-99

   Signature--------------------------Date------------------------------

   Place ---------------------------- Date of submission----------------------