ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Department of Microbial, Cellular and Molecular Biology

Etiology, Disease Severity and Diagnostic Challenges of Bacterial Meningitis during Non-epidemic Seasons in Ethiopia

By Wude Mihret Woldemedhin

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Supervisor: Professor Beyene Petros

A DISSERTATION SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN BIOMEDICAL SCIENCE

June, 2017

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Ethiopia

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Etiology, Disease Severity and Diagnostic challenges of Bacterial meningitis during Non-epidemic Seasons in Ethiopia

By

Wude Mihret Woldemedhin

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the PhD Degree of Philosophy in Biology (Biomedical Science)

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DEDICATION

This thesis work is dedicated to my Lord Jesus Christ, my husband Dr Moges Alemu, my kind and thoughtful mother W/ro Bizunesh Kebede, my father Ato Betemariam Demisse, and my children Kalkidan Moges, Dagmawi Moges, Wongelawit Moges and Kaleb Dawit who have been the source of inspiration and joy empowering all my achivments.

ACKNOWLEDGEMENT

My first and foremost acknowledgment goes to my Lord and my Savior Jesus Christ, who is always with me and has been opening my ways of success since childhood. Next, I would like to appreciate my advisor Professor Beyene Petros for his knowledgeable guidance and quality critics, which took me from my undergraduate study to this end. My deep thanks also go to Dr Abraham Aseffa former Director of Armauer Hansen Research Institute (AHRI), who has assisted my study in many ways. My gratitude also goes to my advisor Dr Gunnstein Norheim, from Norwegian Institute of Public Health (NIPH), who arranged for my two visits to Norway for carrying out laboratory training, experiment, course attendance and thesis write up at NIPH/Oslo. My gratitude also goes to his blessed family (his wife Marianne, cheerful daughter, Johana and son, Tsigur) who all together gave color to my stay at Oslo/Norway. Lots of my admiration goes to Professor Dominique Caugant, the lead chief Scientist in bacterial meningitis, for allowing the Norwegian Research Council Grant to carry out this study and inviting me to her lab at NIPH/Oslo. I highly admire and thank Dr. Einar Rosenqvist, who gave his kind permission to pay for my expenses for Oslo visits. I would also like to praise Mr Jan Oksnes, the lab engineer at NIPH/Oslo lab who trained me on Real Time PCR technology. I am deeply indebted to Mrs Inger Marie for training me on Qiagen DNA extraction and multi locus sequence typing (MLST) lab protocols respectively. My thanks also goes to WHO collaborating center and NIPH/Oslo for donation of trans-isolate media (TIM) and devices for sample collection. Moreover for their donation of freezers, fridges, incubators, centrifuges, as well as meningitis culture detection reagents for both the clinical and research lab. Professor Petter Brandtzaeg, Mrs Berit Brusletto, Dr Reidun Øvstebø, Mrs Anne-Marie Siebke Trøseid, Oslo University team, Dr Lisbeth Naess, Mrs Kalpana and Dr Hanna NIPH/Oslo team are all acknowledged for their contribution in the immunological part of the study. The entertainment I was provided, kind and friendly atmosphere

created by NIPH/Oslo team especially Dr Gunnstein Norheim, Dr Solomon Yimer, his wife Mrs Engidu and his sons, Dr Carolyn, Mrs Karen, Mrs Diana, Dr Benthe and Dr Hanna who left in my mind unforgettable memories. Ethiopian Public Health Institute (EPHI), Federal Ministry of Health (FMOH), World Health Organization (WHO) offices, Mr Abiyot, Mrs Netsanet and Ato Solomon are deeply appreciated for their collaboration in sharing reports of meningitis. My respect and many thanks also go to the kind entertainment provided by Dr Shewit and his family during my stays in Oslo. The meningitis consortium research project between Tikur Anbessa Hawassa and Gondar study site University hospitals (MenTAHG) study coordinators, Mr Yared Merid (Hawassa University Hospital), Professor Afework Kassu (formerly at Gondar University Hospital), Dr Workeabeba Abebe (Tikur Anbessa University Hosipital), health care workers and participants are all appreciated. Lots of my admiration goes to Dr Lawrence Yamuah, Head of AHRI Data management Unit, for his input on data management, Mr Samuel Ayele for training and assisting me in SPSS data analysis. All AHRI data section is also appreciated for entering the raw data of the MenTAHG study into computer data base. I would also like to forward my gratitude to Mr Biruk Yeshitela who was always by my side to assist me in my activities as bacteriology team leader. I am grateful for the close team spirit shown to me by Melaku Yidnekachew, Mr Wondale Mekonen, Mrs Tsehaynesh Lema, Dr Demissew Beyene, Miss Marchign and all AHRI staff. My appreciation also goes to Mr Adugna Aberra (AHRI), Dr Abebe Getahun, Dr Adane Mihret, Dr Dagim Jirata, Dr Martha Zewde and Dr Rawleigh Hawei who kindly assisted me in editing my thesis. I would also like to thank Mrs Elfnesh Benti, Mrs Meseret Mengiste and the other Christian sisters who were praying about my study. Last but not least, my dearest husband Dr Moges Alemu, my daughters Kalkidan and Wongelawit, my sons Dagmawi and Kaleb my nieces Belen and Kidist and my nephew Eyuel are all appreciated for the love and support they provided me during my study period.

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LIST OF ACRONYMS

AAU: Addis Ababa University

ADP: Adenosine Di Phosphate

AHRI: Armauer Hansen Research Institute

AIF: Apoptosis inducing factor

BAP: Blood agar plate

BBB: Blood brain barrier

Bp: Base pair

BSA: Bovine serum albumin

CBC: Complete blood count

CD: Cluster of differentiation

CDC: Centers for Disease Control

CFR: Case fatality rate

CFU: Colony forming unit

CNS: Central nervous system

CRF: Case record form

CSF: Cerebrospinal Fluid

CT: Cycle threshold

DNA: Deoxyribo-neucleic acid

Dx: Diagnosis

ECM: Extra cellular matrix

EDTA: Ethylene-diamine tetra acetic Acid

Fet: Ferric enterobactin transport

G-CSF: granulocyte colony-stimulating factor

GGT: Gamma-glutamine-transpeptidase

Hinf: *Haemophilus influenzae*

Hsf: Haemophilus surface fibril

ICAM: Intracellular adhesion molecule

ICCS: Intra cytoplasmic cytokine staining

IDSR: Integrated Disease Surveillance and Response

IFN-γ: Interferon-gamma

Ig: Immuno-globulin

IL: Interleukin

IL-1β: Interleukin one beta

IL-1RA: Interleukin one receptor antagonist

IP: Interferon gamma Induced protein

IUPAC: International Union of Pure and Applied Chemistry

IPC: Internal Positive Control

IRAK: Interleukin-1 receptor-associated kinase

GLOBVAC: Global health and vaccination research

GNDC: Gram negative diplococcus

GPDC: Gram positive diplococcus

GPSR: Gram positive short rod

GPR: Gram positive rod

LAL: Limulus Amebocyte Lysate

LOS: Lipooligosaccharide

LPS: Lipopolysaccharide

LPT: Lipopolysaccharide transport

LTA: Lipoteichoic acid

MCP: Monocyte chemo-attractant protein

MCMBD: Microbial Cellular Molecular Biology Department

Men: Meningitis

MenTAHG: Meningitis study between Tikur Anbessa, Hawassa, Armauer Hansen Research Institute and Gondar University hospitals

MM: Meningococcal meningitis

MIP-1α: Macrophage inflammatory protein 1 alpha

MIP-1β: Macrophage inflammatory protein 1 beta

MLST: Multi-Locus Sequence Typing

MMP: Matrix metalloproteinases

MOPS: N-Morpholinopropane sulfonic acid

mRNA: Messenger ribo-nucleic acid

MTA: Material transfer agreement

MyD88: Myeloid differentiation-88

MAPK: Mytogen activated protein kinsase

OMP: Outer membrane protein

NAD: Nicotinamide adenine dinucleotide

NERC: National Ethical Review Committee

NF-$_k$B: Nuclear factor kappa B

NIPH: Norwegian Institute of Public Health

Nm: *Neisseria meningitidis*

NP: Nasopharengial

NPV: Negative predictive value

NTC: Non-template Control

NG: Non-groupable

PARP: Poly ADP- ribose polymerase

PBS: Phosphate buffer saline

PCR: Polymerase Chain Reaction

PI: Principal investigator

Ply: Pneumolysin

PM: Pneumococcal Meningitis

PPV: Positive predictive value

PsA-TT: Serogroup A meningococcal polysaccharide-tetanus toxoid conjugate vaccine (MenAfriVac)

RANTES: Regulated-on-activation normal T-cell expressed and secreted

RDT: Rapid diagnostic test

RT PCR: Real Time Polymerase Chain Reaction

SA-PE: streptavidin-phycoerythrin

SASG: sero-agglutination sero-grouping

SBA : Serum bactericidal activitiy

SDS: Sequence detection software

SIRPα: Signal inhibitory regulatory protein alpha

SOP: Standard Operative Procedure

Spn: *Streptococcus pneumoniae*

SPSS: Statistical Package for Social Sciences

TIM: Trans Isolate Medium

TLR: Toll like receptor

TNF-α: Tumor necrosis factor alpha

TRAIL: TNF-related apoptosis-inducing ligand

VITOX: Isovitalex

VR: Variable region

WBC: White blood cells

WHO: World Health Organization

ABSTRACT

Bacterial meningitis (BM) is a severe infectious disease of the nervous system that needs urgent medical attention. Ethiopia, a country located at the eastern end of the "meningitis belt", is frequently affected by meningitis epidemics. Studies have rarely focused on non-epidemic season strains of BM and use of less sensitive diagnostic tools have impeded characterization of its causative organisms. A prospective case-based study was launched from 2012-2013 on 139 patients clinically diagnosed with BM. The objective of the study was to obtain recent data on the frequent etiologies of BM, diagnostic challenges and disease severity in Ethiopia during non-epidemic seasons. Cerebrospinal fluid (CSF) samples taken from the study participants were subjected to bacterial culture, molecular and immunological lab analyses while sera were evaluated by immunological assays. The patient's ages were varying from 2 days to 78 years old and > 50% of the total study population were in ≤ 12 years old age category. Younger age groups than olders, males than females faced higher level of BM induced disease severity (i.e. death or sequelae). Bacterial culture detection test performed at the research lab recovered 14 (10.1%) live isolates out of 139 CSFs. These comprises of *N. meningitidis* (n = 5, serogroups A (n = 4) + W-135 (n = 1)), *S. pneumoniae* (n= 8), and *H. influenzae* (n =1) from the CSFs of the patients. Real time PCR (RT-PCR) detected a total of 46 (33.1%) etiologic agents of BM composing *N. meningitidis* in 27/46 (58.7%), genogroups A (11/27, 40.7%), W-135 (7/27, 26%), C (1/27, 3.7%), X (1/27, 3.7%) and non-groupable (7/27, 26%), *S.pneumoniae* in 18/46 (39.1%) and *H. influenzae* in 1/46 (2.2%). Hospital lab versus research lab results for 50 patients whose clinical lab data were found were compared and

the hospital lab results showed less specificity for *N. meningitidis* (91% versus 100%) and *S. pneumoniae* (95% versus 100%), decreased PPV for *N. meningitidis* (50% versus 100%) and *S. pneumoniae* (33% versus 100%), decreased NPV for *N. meningitidis* (66% versus 68%) and *S. pneumoniae* (85% versus 93%) respectively. Levels of IL-4, IL-8, IL-12/p70, INF-γ, MCP-1, MIP-1α, MIP-1β, RANTES, TRAIL and MMP9 in the CSF were significantly elevated in BM caused by *S. pneumoniae* compared to that of *N.meningitidis*, consistent with a worsened outcome by the former. Significantly different ($P < 0.05$) levles of median endotoxin activities (measured by Limulus Amoebocyte Lysate/LAL) were detected among *N. meningitidis* positive patients with different clinical conditions. *Streptococcus pneumoniae* positive patients who died (n = 3) had significantly high ($P < 0.05$) levels of MMP9 and IL-1β compared to the survived ones. Fifty patients out of 139 were reported to develop sequelae in age groups ≤4 (epilepsy, n = 1), ≤ 4 (raised intracranial pressure, n = 1), ≥ 40 (quadriparesis, n = 1), 20-29 (confusion in age group, n = 20-29), while seizure was reported in age groups ≤4 (n = 22), 5-12 (n = 7), 13-19 (n = 4), 20-29 (n = 7) and ≥ 40 (n = 4). Seventeen patients out of the 48 with specifically reported sequelae were RT-PCR confirmed positive for BM etiologic agents. Types of sequelae were not specifically reported for two patients. Three *N. meningitidis* and 6 *S. pneumoniae* positive patients who experienced sequelae showed increased trends of IL-1β, IL-6 and IL-1rα compared to those with no sequelae and negative controls. Affordable, multivalent meningitis vaccines composed of serogroups A, C, W-135 and X are urgently needed for use in Ethiopia and possibly in all countries within the African meningitis belt.

1. INTRODUCTION

Meningitis is a medical emergency caused by inflammation of the meningeal layer of the central nervous system (CNS), in response to infectious agents and their products or non-infectious agents (Doran *et al.*, 2016). The inflammation and swelling extends across the different layers of the meninges (the pia matter, arachnoid or subarachnoid) (Teach me anatomy, 2015-2016) (Fig. 1).

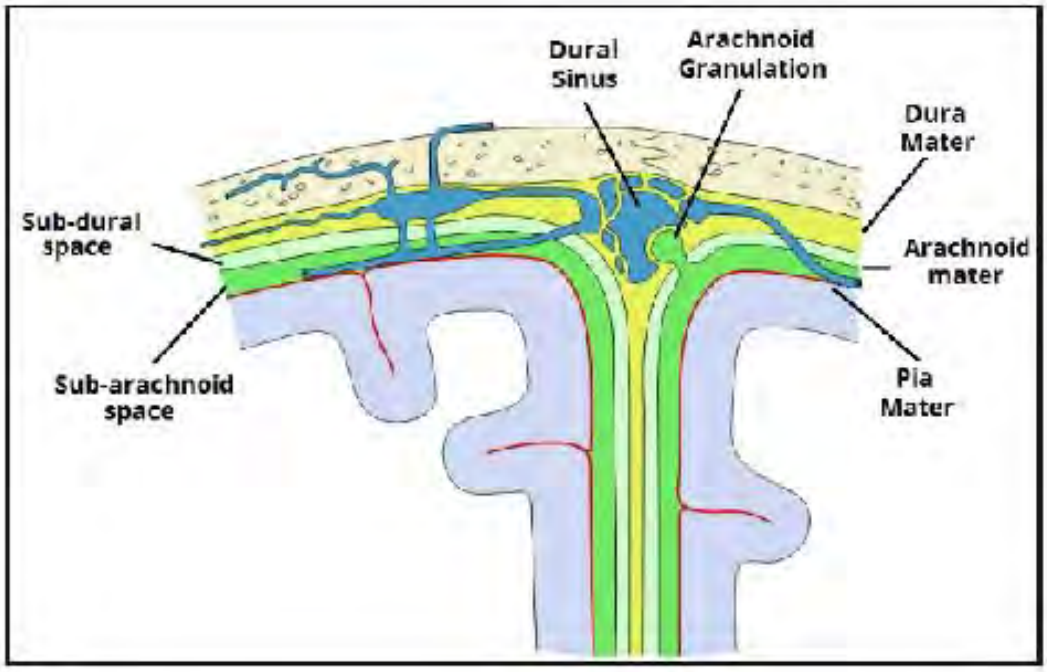


Fig. 1: The human brain and parts of the meninges
Adapted from Teach me anatomy, (2015-2016).
http://teachmeanatomy.info/neuro/structures/meninges/

In spite of the advances in medical care with introduction and widespread use of antibiotics, meningitis has still remained a cause of high morbidity and mortality. The age related incidence of BM differs between regions, with the very youngest (<5 years) demonstrating highest incidence (Thompson *et al.*, 2006).

Signs and symptoms with which the disease is characterized are sudden onset of headache, fever, and stiffness of the neck, at times accompanied by nausea, vomiting, photophobia, altered mental status with lowered consciousness and bulging of the fontanelle in infant cases (Thompson *et al.,* 2006).

Meningococcal meningitis was first described by Vieusseux in 1805 in Geneva, Switzerland. Occurrence of meningitis was also found documented in ancient texts in written documents of Hippocrates (Pappas *et al.,* 2008). Though meningitis was first recorded as an outbreak in Geneva in 1805 (Greenwood *et al.,* 2006), reports show that Gaspard Vieusseux (1746-1814), Andre Matthey (1778-1842) in Geneva, and Elisa North (1771-1843) in Massachusetts have also described epidemics of meningococcal meningitis (Tyler, 2009). Occurrences of several other epidemics in Europe and the United States were also described around these time periods. In Africa, the first outbreak of the disease was described in 1840 while epidemics became much more common in the 20th century (Codjoe, S.N., Nabie, 2014). The first major epidemics in Africa were reported in Nigeria as well as Ghana in 1905-1908 and in Ethiopia in 1901 and 1935 (Castellani, 1939).

Initial identification of the meningococcus bacteria as a causative agent of BM was reported by Anton Weichselbaum, an Austrian bacteriologist in 1887 (Christodoulides, 2013; Buchillet and de Lamballerie, 2010). Later in the 19th century diverse types of bacterial agents were identified as etiologic agents of meningitis. This includes Group B Streptococcus, *Escherichia coli, Listeria monocytogenes, Mycobacterium tuberculosis* and others. Pathogens including viral, fungal, protozoal and helminthic agents as well as

several non-infectious agents (chemicals) were also understood to cause meningitis (Forleo-Neto *et al.,* 1999). The chemical agents include non-steroidal anti-inflammatory drugs and antibiotics. Overall, compared to other agents contributing to the cause of meningitis, bacterial etiologic agents take the upper hand with regard to disease severity as well as causation of death (Matthews *et al.,* 2007).

Bacterial meningitis has a rapid disease progression, which may lead to permanent disability or death in a matter of hours. Accurate identification of etiologic agents is needed in order to initiate the best treatment rapidly to reduce risk of death and sequeale in survivors (Matthews *et al.,* 2007).

The disease has remained a cause of substantial morbidity and mortality both in developing and developed countries (Chanteau *et al.,* 2006). Amongst all bacterial etiologic agents of meningitis, *Neisseria meningitidis, Streptococcus pneumoniae,* and *Haemophilus influenzae* were identified as frequent etiologic agents of BM (Tyler, 2009).

Bacteria causing the disease have very high potential of genetic polymorphism and emergence of new strains is likely to occur. Due to this diverse situation, studying etiologic agents and circulating strains of BM requires routine epidemiological surveillance (Laval *et al.,* 2006).

Carriage of bacterial strains that may cause meningitis is one of the risk factors for disease occurence. Meningitis causing bacteria adhere and colonize nasopharyngial tissue and can migrate across basal layer of cells to enter the blood circulation (Laval *et al.,* 2006).

After crossing the blood brain barrier the organism reaches the CSF and causes BM. The pathogens can also use other routes of entry to progress towards the CSF (Drevets *et al.,* 2004).

Individuals who have been non-carriers and later acquire the bacteria through transmission from others have the chance of progression from carriage to disease (Laval *et al.,* 2006). Social habits such as gathering, kissing and sharing food or utensils can induce spread of the bacterial agent from a carrier person to other person (Mate, 2013). Exposure to respiratory droplets through coughing and sneezing can also spread the bacterial agent (Manchanda *et al.,* 2006).

The risk of disease occurrence can increase on exposure to a crowd where there are diseased individuals or carrier droplets. Susceptible people in settings with an elevated degree of crowding such as schools, sites of religious pilgrimage, military camps, universities and day-care centers or preschool attendants are at greater risk of contracting bacterial meningitis. Transmission rate of virulent clones is higher, and invasive disease often occurs within the first week after acquisition of the pathogen (Manchanda *et al.,* 2006).

People of any age group can be affected by the disease (Kim, 2010). Early age groups are highly affected by negative outcome of BM as compared to older age groups (Gessner *et al.,* 2010). Meningitis may have nonspecific symptoms among infants and children aged less than 2 years. Neck stiffness, usually seen in adult people with meningitis may be absent in this age group (Thompson *et al.,* 2006).

The knowledge about transition from carrier to disease state by some hosts while others remain carriers has been obscure for a long period of time. Studies report the presence of diverse factors contributing to the transition from carriage to disease (Caugant and Maiden, 2009).

Pathogen structural components (e.g., pili, Fig. 2), genetic compositions of pathogen as well as host-pathogen interaction are the major factors contributing to adhesion, colonization, crossing basal cell layer of the host nasopharengeal tissue, crossing blood brain barrier (BBB) and finally disease causation (Ribet and Cossart, 2015).

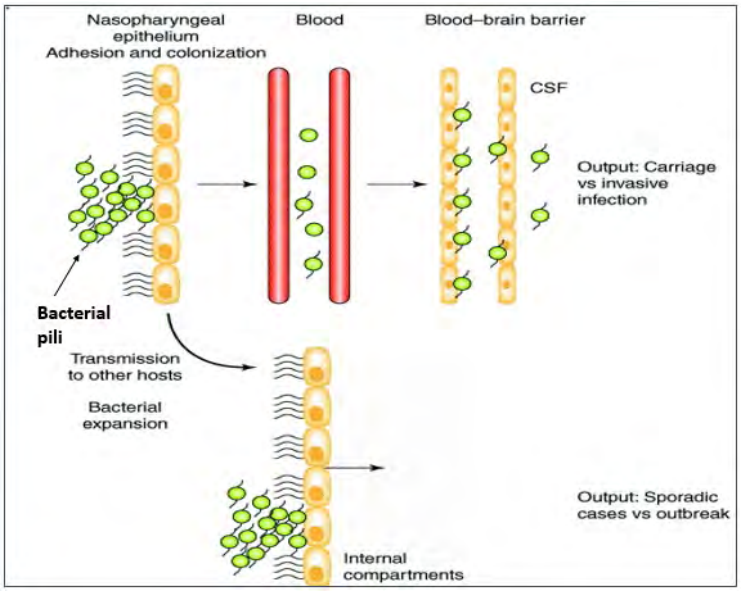


Fig. 2: Adherence, colonization and migration of bacterial pathogen on and across nasopharyngeal tissue and across blood and blood brain barrier
Adapted from Taha *et al.* (2002).

Among the Neisseria family there are species (e.g., *N. lactamica*) that lack all or part of the pathogenic capsule loci such as α 2→8, α 2→9, 6DGlcα 2→4, 6DGal α 2→4, α 1→

6 N-acetyl D-mannosamine, α 1→4 N-acetyl D-glucosamine regions (Fig. 3) possessed by serogroups B, C, Y, W135, A and X (Dolan-Livengood *et al.*, 2003).

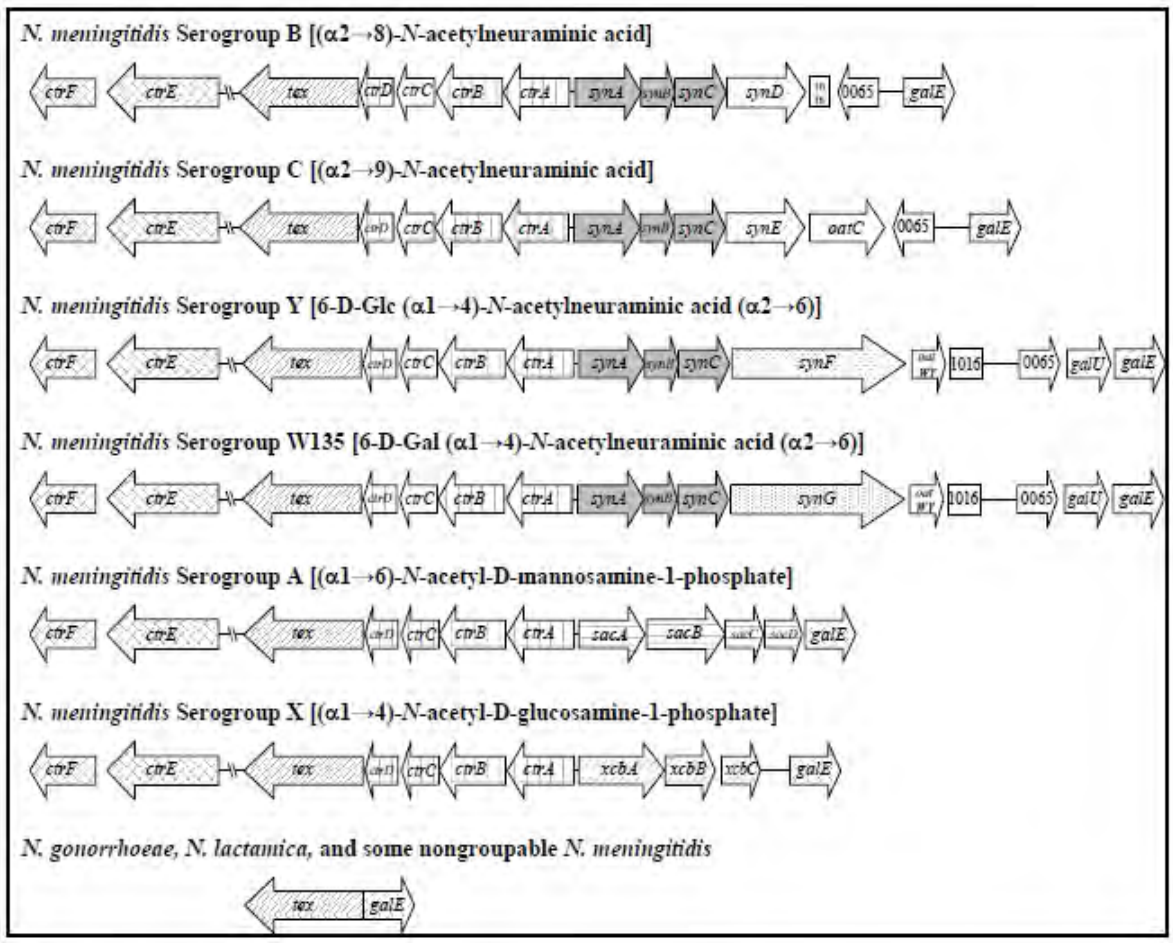


Fig. 3: Genetic maps of the capsule gene complex of *N. meningitidis*
Adapted from Dolan-Livengood *et al.* (2003)

Genes encoding proteins synthesizing the specific pathogenic capsule are forming the referred serogroups of *N. meningitidis* inducing pathogenesis (Dolan-Livengoog *et al.*, 2003). The galE regions of the ctr gene of *N. meningitidis* are the capsule transport operon, which are highly conserved among all the 5 serogroups. Capsule biosynthesis and

transport loci can have genetic organization indicated at the loci next to the galE regions (Dolan-Livengood *et al.* 2003).

1.1 Global epidemiology of bacterial meningitis

Bacterial meningitis has undergone dramatic changes in global epidemiology during the past decade (Scheld *et al.,* 2002). Globally 500,000 cases and between 50,000 and 135,000 deaths are reported annually due to meningococcal meningitis and sepsis (Chang and Stephens, 2012; Cox, 2012; Vestergård and David, 2008).

A surveillance report for global epidemics of meningococcal disease in all age groups from 1970-1996 showed that *N. meningitidis* serogroups A, B and C were the main etiologic agents of BM (WHO, 1998) (Fig. 4). Additional serogroups like W-135, X and Y which were not reported in the 1970-1996 period are currently (Hedari *et al.,* 2014) emerging as important causes of BM (Fig. 4). Serogroup Y meningococcus was the commonest disease-causing serogroup in 2006 in Colombia and Venezuela. Countries like Argentina, Canada, Chile, Columbia, Mexico, the United States and Venezuela experienced low levels of BM during this period (Sáfadi and Cintra, 2010).

In general, serogroups that cause invasive disease differ in different regions of the world, and change over time (Leimkugel *et al.,* 2009). An observational study on bacterial meningitis etiology in England and Wales during years 2004-2011 (Okike *et al.,* 2013) showed that *N. meningitidis* followed by *S. pneumoniae* were causing the highest incidence of BM in all age groups. The main etiologic agents of BM in Africa in infants

from 0-5 years were reported to be *H. influenzae* followed by *S. pneumoniae* and *N.meningitidis* (Lussiana *et al.,* 2011).

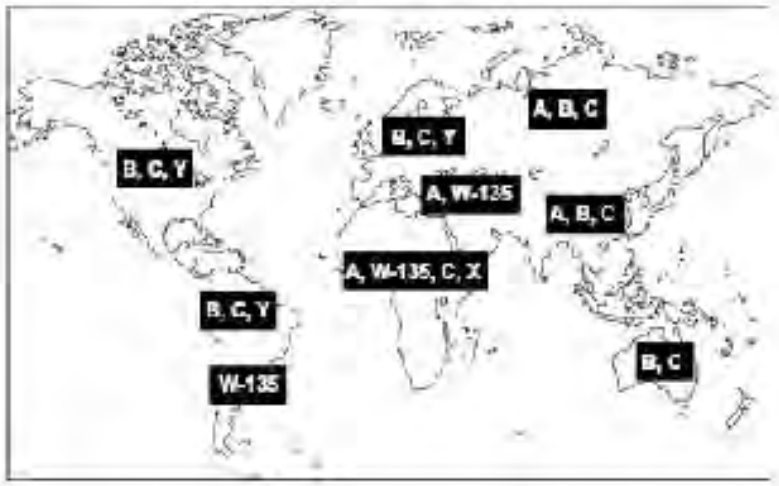


Fig. 4: Global serogroup distribution of invasive meningococcal disease
Letters are designating lists of *N. meningitidis* serogroups found at the site where they are labeled
Adapted from Hedari *et al.* (2014)

Relatively recent outbreaks caused by serogroup X have occurred between 2006 and 2010 in Kenya, Niger, Togo, Uganda and Burkina Faso. In reference with the global distribution of meningococcal serogroups, this serogroup was previously a rare etiologic agent of BM (Lingappa *et al.,* 2003), whereas serogroup W-135 was reported to cause disease in countries such as the Sudan and Saudi Arabia located at the eastern Mediterranean region. These countries have high endemic rates of serogroup A disease as well. The outbreaks they experienced during the hajj season occurred with these same serogroups (Lingappa *et al.,* 2003).

Uninterrupted surveillance and intervention on endemic and sporadic (non-epidemic) as well as rapid identification of organisms causing outbreaks are critical actions for the design and mobilization of preventative interventions to reduce the incidence of BM (Makwana and Riordan, 2007).

1.2 Epidemiology of bacterial meningitis in Africa

Disease occurrence could be higher among the low income countries populations, due to increased crowding. This converges with African meningitis belt countries stretching from Senegal in the west to Ethiopia in the east. The disease has remained to be a common cause of death in Africa (Rajasingham *et al.,* 2015; Rosenstein *et al.,* 2001).

More than 400 million people living in the 26 countries of African meningitis belt are affected with BM every year. In this region 900, 000 cases were reported in the last 20 years (1995–2014) and 10% of the cases resulted in deaths, while another 10–20% developed neurological sequelae (WHO, 2015).

This area suffers cyclic epidemics of meningitis that affect persons of all ages, with attack rates ranging from 100 to 800 per 100,000 populations (LaForce and Okwo-Bele, 2011; Memish *et al.,* 2010). The largest epidemic outbreak that has occurred in this part of the world was in 1996, when over 250,000 cases occurred and 25,000 people died as a consequence of the disease (LaForce and Okwo-Bele, 2011).

Neisseria meningitidis (37–52%), *Streptococcus pneumoniae* (27–43%), and *Haemophilus influenzae* (5–31%) were estimated to be the most common etiologic agents of BM during non-epidemic situations among African meningitis belt countries such as

Burkina Faso, Ghana, Niger, and Mali (Rajasingham *et al.,* 2015). In a study carried out in Burkina Faso among persons aged 1 month to 67 years, the incidences of meningococcal meningitis, pneumococcal meningitis, and *H. influenzae* typ b meningitis occurred among 19 cases of 179 (10.6%) patients, 17 cases out of 162 patients (10.5%), and 7 cases out of 68 (10.3%) patients per 100,000 persons per year, respectively (du Châtelet *et al.,* 2005).

A report from a study in Niger in 2006 found *N. meningitidis* to be the cause of BM in 95% of cases in the epidemic and 59% in the non-epidemic seasons (Rajasingham *et al.,* 2015). In general, more than 50% of all meningococcal meningitis cases are reported from the countries located within the "meningitis belt" (Guo *et al.,* 2015). Intermittent devastating outbreaks of BM occur with attack rates as high as 1% of the population in the African meningitis belt during major epidemics (Lingani *et al.,* 2015).

Reports from this region show that most of the previous epidemics have been due to serogroup A *N. meningitidis* while some have been due to serogroups C, W-135, and X (Lingani *et al.,* 2015). Determination of carriage prevalence is important to map the potential clones that in the future may lead to disease acquisition by susceptible people (WHO, 2015; Trotter and Greenwood, 2007).

Studies show that higher carriage rate of meningococci occur in lower socioeconomic classes (Yazdankhah and Caugant, 2004). Another major risk factor is the frequent exposure to desert sand dust, causing disruption of the mucosal barrier (Jusot *et al.,* 2017). Carriage studies of *N. meningitidis* in Mali in 1974, Nigeria in 1976, 1982, 1999

and Ghana in 2007 (Trotter and Greenwood, 2007) have shown a high level of fluctuation in percentage carriage prevalence across different age groups (Fig. 5). Two large studies have recently been performed to assess the carriage of meningococci in the African meningitis belt. First, a study led by the Norwegian Institute of Public Health in Burkina Faso, found that the overall meningococcal carriage prevalence prior to vaccination in the general population was 3.98%; and highest among the 15 to 19 year-olds for males and among the 10 to 14 year-olds for females. Serogroup Y was highest (2.28%), followed by serogroups X (0.44%), A (0.39%), and W-135 (0.34%) (Kristiansen *et al.*, 2011).

Secondly, a meningococcal bacterial carriage consortium study was done from 2009-2012 and termed MenAfricar. The study was run among seven African meningitis belt countries extending across the meningitis belt from east to west (Ethiopia, Chad, Nigeria, Niger, Ghana Mali, and Senegal) prior and post serogroup A meningococcal polysaccharide-tetanus toxoid conjugate vaccine (PsA-TT/MenAfriVac) implementation (MenAfricar Consrtium, 2015; Kristiansen *et al.*, 2015; Novak *et al.*, 2012). Based on the report from MenAfricar Consrtium study, with the exception of northern Cameroon, substantial transmission of serogroup A meningococci was detected in 2011 and 2012 only in Chad (MenAfricar Consrtium, 2015). A carriage rate approaching 1% was found in rural areas of Chad. The reason forwarded for this low level (less than the estimated 10% carriage on the basis of limited available information) finding may be that the timing of the survey was during a naturally low level of transmission of serogroup A meningococcus in the African meningitis belt countries (MenAfricar consortium, 2013).

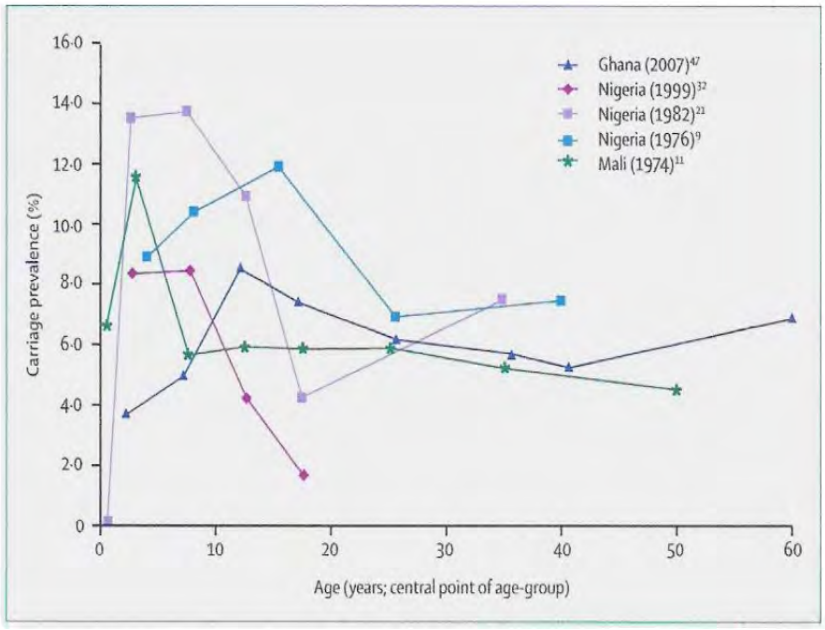


Fig. 5: Carriage prevalence of *N. meningitidis* in Ghana (2007), Nigeria (1976, 1982, and 1999) and Mali (1974) across different age groups
Adapted from Trotter and Greenwood (2007)

A study in introduction of the conjugate PsA-TT vaccine and its impact in Burkina Faso showed a dramatic decrease in the incidence of serogroup A meningitis and carriage (Kristiansen *et al.*, 2015; Novak *et al.*, 2012). This was again verified in a separate study in Chad (Daugla *et al.*, 2014). With regard to the need in more focused supervision of the most affected countries, studies are showing that meningitis belt countries (Fig. 6) such as Benin, Burkina Faso, Cameroon, the Central African Republic, Chad, Côte d'Ivoire, the Democratic Republic of the Congo, Ethiopia, the Gambia, Ghana, Guinea, Mali, Mauritania, Senegal, South Sudan, Sudan and Togo need to have continued surveillance for etiologies of meningitis to enable a rational assessment of the need for vaccines and their effect after implementation (Chang *et al.*, 2012; Kaninda *et al.*, 2000; Pandya *et al.*, 2015).

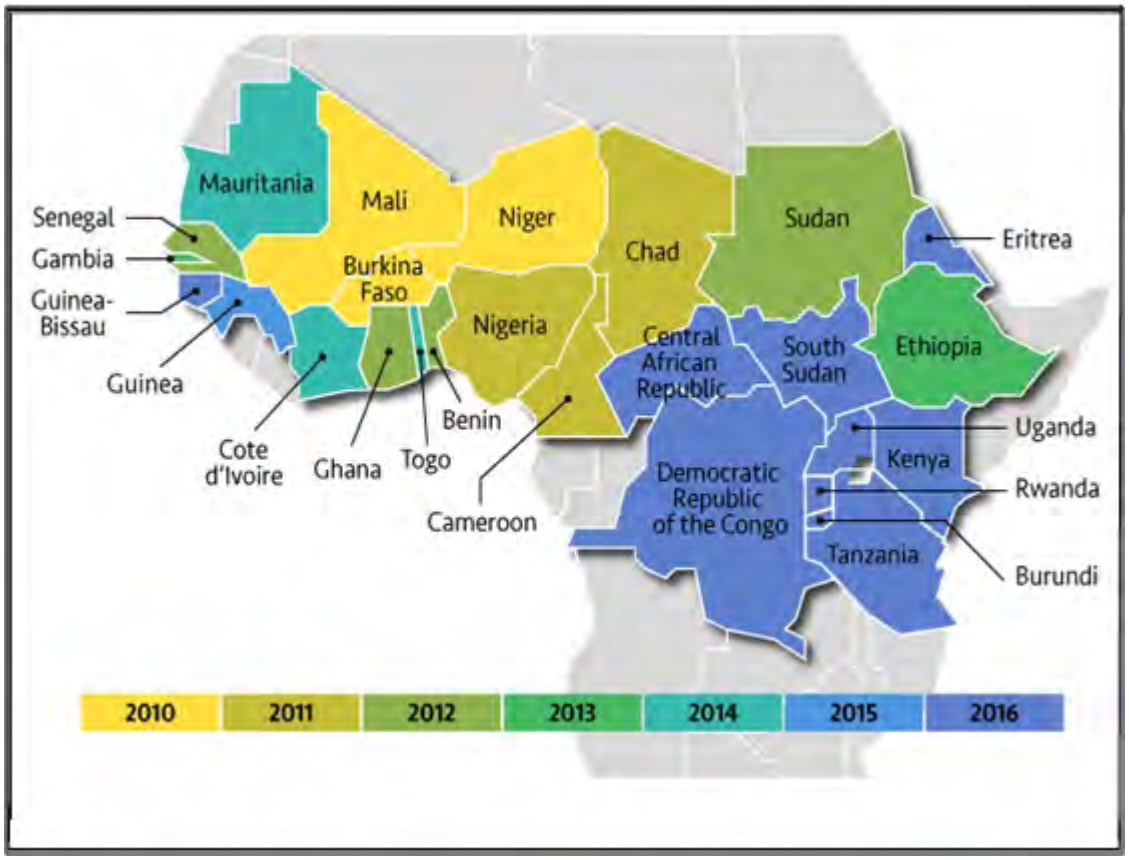


Fig. 6: MenAfriVac vaccine roll out across the 26 countries of Africa's meningitis belt over the course of seven years
Adapted from *Program for Appropriate Technology in Health (*PATH*)*/David Simpson. Corresponding coloures of the years and countries are indicating the duration that vaccine was initiated in the specified country

The most common cause of BM among patients diagnosed in the meningitis belt during the period 2008 to 2009 was *N. meningitidis* (81.6%) with mainly serogroups A (75%) and W135 causing outbreaks (Maïnassara *et al.*, 2014). However, this pattern has changed in the last decade, mainly due to the implementation of the serogroup A meningococcal conjugate vaccine in meningitis belt countries (Fig. 6). Natural

fluctuations occur alongside, with emergence of other serogroups of meningococcal meningitis such as serogroups W-135 as well as X (Novak *et al.,* 2012).

In part this may be explained by worldwide mobility of people which increased both the type and magnitude of disease burden of BM (Alberer *et al.,* 2015; Gushulak and MacPherson, 2004). However, there is a high natural diversity in serogroups among isolates detected among asymptomatic carriers, including serogroup X and W-135 (Bårnes *et al.,* 2016). Population movements may impact in transmission of new serogroups to neighboring meningitis belt countries where no previous reports of such serogroups are detected (Bårnes *et al.,* 2016).

It was reported that serogroup W-135 was responsible for the meningitis cases between 1988 and 2001 in 13 African countries of the meningitis belt (Nicolas *et al.,* 2005). Whereas, the epidemic which occurred in the meningitis belt countries in the 1996 was caused by *N. meningitidis* serogroup A (Mohammed *et al.,* 2016). With regard to strain distribution, meningococcal serogroup A strain ST-7 emerged in the mid-1990s and was totally replaced by ST-5 since 2002. These two STs of meningococci had been responsible for causing hundreds of thousands of cases (Caugant and Nicolas, 2007).

Identification of W-135 ST-11 is a great concern for meningitis belt countries, in particular since this serogroup is not included in the MenAfriVac vaccine (Nicolas *et al.,* 2005). Other than these, clones such as ST-2881, ST-181, ST-751 and two major clonal complexes, and a few other clones were detected to occur sporadically (Nicolas *et al.,* 2005). Studies carried out on the meningitis belt countries especially in the western part

of Africa have shown that other bacteria, in particular pneumococcal bacteria, also cause significant proportion of the meningitis cases (Leimkugel *et al.,* 2005; Traore *et al.,* 2009).

1.3 Epidemiology of bacterial meningitis in Ethiopia

Ethiopia, a sub-Saharan African country, located at the eastern end of meningitis belt of Africa is endemic for BM. The country, has the second largest population (≈ 100 million in 2013) among the countries located in the meningitis belt (Molesworth *et al.,* 2002).

Meningococcal epidemics have been documented in Ethiopia since 1901 and 1935 and prior to these years as well (Castellani, 1939). After the meningitis epidemic was reported in 1935 in Ethiopia, subsequent outbreaks have occurred later in 1940, the 1950s, 1964, 1976, 1977, 1981 to 1983, and 1988 to 1989 (Habte-Gabr *et al.,* 1984; Haimanot *et al.,* 1990).

Neisseria meningitidis serogroup A, W and C were causing meningococcal meningitis in the years from 2001-2005 in the meningitis belt of Africa (Mohammed *et al.,* 2016, Djibo *et al.,* 2003). Outbreaks of meningitis between 2006 and 2010 in Kenya, Niger, Togo, Uganda, and Burkina Faso have occurred due to serogroup X, a previously rare cause of sporadic meningitis. This serogroup has been as well responsible for the latter 1,300 cases of meningitis among the 6,732 reported annual cases (Mohammed *et al.,* 2016).

The outbreaks have caused panic in the community even to the extent of causing an epidemic hysteria especially in north-west part of the country triggered by the local epidemic of meningococcal meningitis (Maru, 1982). Hence, the disease has remained a

serious health threat for Ethiopia. The major cause of the epidemics was serogroup A *N.meningitidis*, while serogroup C was also identified in 1981 and during outbreaks in 1983-84, 2000 and 2003 (Norheim *et al.,* 2006). Recently, conjugate vaccines against *H. influenaze* serotype B, *S. pneumoniae* (PCV10), and *N. meningitidis* serogroup A (MenAfriVac) were introduced in Ethiopia in the subsequent years of 2007, 2011 and 2013-2015, respectively (Kristiansen *et al.,* 2015; Rueda *et al.,* 2015).

Introduction of the MenAfriVac has led to considerable level of reductions in the incidence of suspected meningitis cases and epidemics. Hence this has proven that the vaccine has promoted a substantial effect in the reduction of serogroup A meningococcal meningitis (Trotter *et al.,* 2017). However, epidemic of meningitis was reported to occur in Nigeria in 2017 of March. A total of 1,407 suspected cases and 211 deaths have been reported from 40 local government areas of five states of Nigeria since December 2016 (WHO, 2017).

Bacterial meningitis occurs in Ethiopia particularly in the dry season from December to June and there have been frequent meningococcal epidemics. It was reported that the 1989 outbreak in Ethiopia was due to the spread of the disease from pilgrims of other African countries returning from Mecca (Moore, 1992). Frequent outbreaks were also reported to occur from neighboring countries. The northern, north-western and south-western parts of Ethiopia, traditionally included in the meningitis belt region, were mainly the geographical locations where the outbreaks have occurred prior to 1988. Reports have shown that the outbreaks have further included the capital city of the country, Addis Ababa (Haimanot *et al.,* 1990; Norheim *et al.,* 2006; Agzew, 1985). The

occurrence of the outbreak of meningitis in the southern part of Ethiopia from 1988-1989 was described in a recent cross-sectional study on 325 neonates admitted at Gondar University Hospital (Kokeb and Desta, 2016). The study reported that meningitis is the common cause of neonatal mortality at the neonatal unit of the hospital (Kokeb and Desta, 2016). Although global mortality rate ranges from 10-25%, reports from some studies have shown that neonatal morality may even exceed from this (Aletayeb *et al.,* 2010).

Diverse serotypes of meningococcal and Pneumococcal meningitis are causing disease to a wide age range of people. Occurrence of frequent epidemics and scarcity of surveillance, has made measurement of incidence of meningitis complicated especially in low-income countries (PetterBrandtzaeg *et al.,* 2012).

Studies show the nearby elimination of meningitis caused by *H. influenzae* resulting from vaccine introduction while, all-age pneumococcal meningitis has decreased by around 25%, with little data from low-income settings. The effect of a new serogroup A meningococcal conjugate vaccine in the reduction of this serotype is reported from African meningitis belt countries. Whereas, reports from several high-income countries, show the near elimination of serogroup C meningococcal meningitis (PetterBrandtzaeg *et al.,* 2012).

Other studies showed that Addis Ababa has also suffered from an outbreak leading to over 850 cases and 33 deaths between June and August 2000. The cause of BM, which

resulted in high morbidity and mortality were identified to be *N.meningitidis*, *H.influenzae* and *S. pneumoniae* (Von Gottberg *et al.,* 2008; Muhe and Klugman, 1999).

Absence of reliable data regarding circulating etiologic agents of BM, in resource poor African countries like Ethiopia where the magnitude of the disease is high makes the issue of building a case for vaccine prevention even worse (Berkley *et al.,* 2001).

Despite frequent outbreaks of meningococcal disease and a high likely burden of BM in Ethiopia, utilization of sensitive diagnostic methods, timely inoculation of CSF into culture media is poorly managed even in the reference hospital laboratories located at the capital city of the country (Gebremariam, 1998). Absence of patient sample transportation media such as trans-isolate media (TIM) for routine patient sample diagnosis is also one of the challenges especially in areas where CSF is needed to be transported to referral labs (Norheim *et al.,* 2006). Use of less sensitive methods as Gram stain will still give some information if used and reported consistently, complemented by culture. Lack of a consistent system and referral laboratories performing culture based confirmation may lead to false negative results. Apart from culture, where available, and Gram stain, clinical diagnosis and treatment is dependent on CSF pleocytosis (Gebremariam, 1998).

Reference studies using highly sensitive diagnostic methods to identify circulating etiologic agents of BM and strains are needed to update physicians and inform vaccine development strategies. This is particularly true for neonatal BM where a diversity of pathogens could play a role. For instance, a report compiled from a 10-year population based retrospective study in Addis Ababa using biochemical methods showed that only

55 cases of neonatal meningitis were identified. For instance, a report compiled from a 10-year population based retrospective study in Addis Ababa using biochemical methods showd that only 55 cases of neonatal meningitis were identified. The report showd that twenty-two (40%) of the infants died and BM etiologic agents such as *Klebsiella pneumoniae, Escherichia coli* and Enterobacter spp were isolated from their CSF using routine biochemical lab diagnosis. These together accounted for 67% of all the bacterial isolates (Gebremariam, 1998). Seven out of the 33 (21%) surviving newborns developed neurological complications. The short-term sequeale reported in the study were hydrocephalus, spastic paresis and seizures (Gebremariam, 1998).

The disease burden and frequency of identified pathogens is likely underestimated with these less sensitive biochemical diagnostic methods. Major epidemics usually occur in the country every 8 to 12 years and the last official report with a significant number was in 2001 when 6,964 cases were reported with 330 deaths. The largest number of cases occurred during the outbreaks in Amhara, Southern Nations and Nationalities of People's Region (SNNPR) and Oromia regions (Norheim *et al.,* 2006). The years 2000 to 2003 were also the periods in which Ethiopia encountered epidemics with 855, 6266, and 2329 cases in the first three years, respectively and 3540 cases in the last year. The case fatality rates (CFRs) in these last same years were 2.2, 5.0, 5.0 and 4.7, respectively (Norheim *et al.*, 2006).

Assessing the national as well as district level burden of the disease, trend of cases and deaths, and seasonal variation on the incidence of BM is important. Using descriptive data on clinical laboratory diagnosed BM etiologic agents is of high importance for

patient management purposes. Confirmation of the diagnosis with highly sensitive diagnostic assays is valuable to estimating the actual burden of the disease and etiologic agents in order to tailor or select the appropriate vaccine fitting the actual serogroups (WHO, 2015). For instance, the officially reported number of suspected meningitis cases (WHO, 2015), covering the period of 21 years from 1994 to 2014 showed decreasing trends in the belt stretching from Senegal in the west to Ethiopia in the east (Fig. 7).

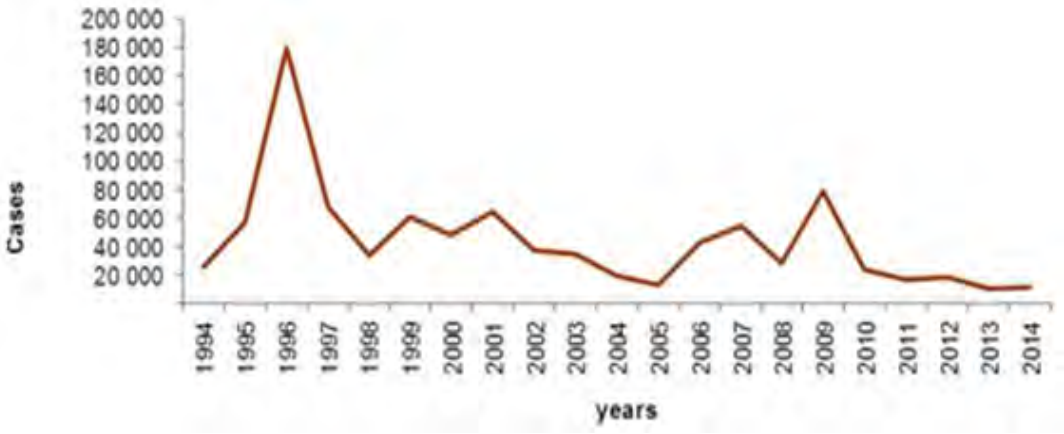


Fig. 7: Meningitis cases in the African belt countries stretching from Senegal in the west to Ethiopiain the east, 1994-2014

Adapted from WHO (2015).

In this report, every year BM cases impact the 26 meningitis belt countries in which more than 400 million people live. Of all the cases reported in the last 20 years (1995–2014), 10% resulted in deaths, while another 10–20% developed neurological sequelae (WHO, 2015). An earlier report by WHO (2014) presented the continued burden of the disease with regard to suspected meningitis cases (with a median of 1,056) during 2001–2010. The major cause of these epidemics has been serogroup A meningococci though

serogroup C was also reported during the periods of outbreaks occurring in 1981, 1983–84, 2000 and 2003 (Norheim *et al.*, 2006).

Although the fluctuation in occurrence of BM occurs globally and renders the disease to be highly unpredictable, the challenge with the less sensitive diagnostic assays aggravates the problem especially in countries of the meningitis belt. A recent unpublished retrospective study based on patient card records of Gondar and Hawassa (Ethiopia) University hospitals showed the presence of varied levels of fluctuations in the occurrence of BM cases especially among the former (Fig. 8A) ones. The Gondar University Hospital and Hawassa University Hospital patients visited the hospitals during a five year (2007-2011, Fig 8A) and a two year periods (2010-2011, Fig 8B), respectively (Ahmed, 2012).

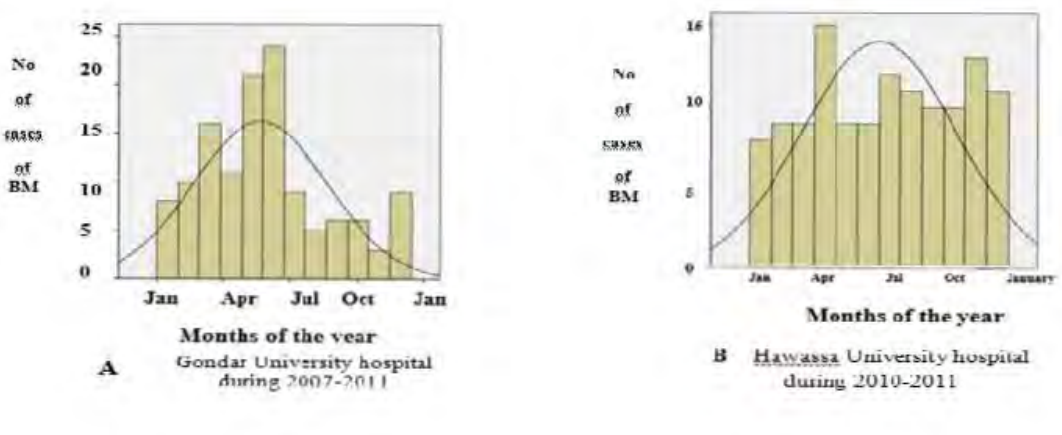


Fig. 8: A. Seasonal variation in the cases of bacterial meningitis at Gondar University hospital during 2007-2011, B. Seasonal variation in the cases of bacterial meningitis in Hawassa University hospital during January 2010-December 2011

Adapted from Ahmed (2012)

The data from Gondar showed that the number of BM patients was the highest in June as compared to April in Hawassa (Ahmed, 2012). The report showed that *N. meningitidis* was the major etiologic agent of BM in the month of June while this dropped to nil during October and November. Cases of BM in males were higher in frequency than females (Ahmed, 2012).

Highest number of cases from Gondar was found at the age range of 0-12 months (29/109, 26.6%) followed by 1-4 years (25/109, 22.9%). The third most highly affected age group was 25-39 years of age (18/109, 16.5%). Etiologic agents of BM in the Gondar study were *S. pneumoniae* in 35.4%, *N. meningitidis* in 27.7%, *H. influenzae* in 9.1% and others in 28.9% of the cases (Ahmed, 2012). The highest number of cases from Hawassa University Hospital were young adults in the 15-24 year of age (38/143, 26.6%) followed by adults between the ages of 25-39 years (29/143 cases, 20.3 %). Children from 0-12 months and 1- 4 years showed identical occurrences of the disease (16, 11.2%). *Escherichia coli*, *S. aureus*, Salmonella species, Acinetobacter, *S. pyogens*, other Streptococci species, Pseudomonas, Providencia species, *Morganella morganii*, *K.pneumoniae*, Enterobacter were among the other least identified bacterial agents from the CSF.

Since this report was based on routine hospital diagnosis using Gram stain and latex agglutination, there may also be occurrence of under-reporting as seen in other studies (Brouwer *et al.,* 2010). In the Netherlands, a study found under-reporting of meningococcal disease when comparing laboratory records, hospital clinical records and national surveillance records (De Greeff *et al.,* 2006).

Baseline incidence of meningococcal disease around the world is typically 0.8-1 case per 100,000 population. Assuming full recording in Ethiopia, one may estimate a minimum of around 950 cases per year. Moreover, in spite of the serious health challenges due to BM, epidemics of meningococcal disease in African countries are commonly detected too late and admisinstration of antibiotic as well as steroid may not impact on the survival of many cases (Lewis *et al.,* 2001). Hence, the reports obtained from the less sensitive routine clinical diagnostic methods underestimate the burden of the disease. This means that even more samples could have turned out positive than the one reported so far if sensitive diagnostic tools have been applied for reporting purpose. For instance, a recent study by Diawara *et al.* (2016) showed that sensitivity of culture detection method compared to real time-PCR detection from CSF for *S. pneumoniae* was 55.56 % (CI 40.00-70.35). Whereas, sensitivity of culture detection method compared to real time-PCR detection for *N. meningitidis* was 34.62 % (CI 21.97- 49.09). The specificity, PPV and NPV of both were ranging from 81 to 100 %.

Reports from reference studies based on PCR assist physicians to estimate strains of etiologic agents for patient management purposes as well as for public health authorities for selection of appropriate vaccine (Chaudhuri *et al.*, 2008). A weekly based meningitis epidemic registry from clinical data was initiated in the year 2009 (personal communication with weekly WHO meningitis registry) in Ethiopia composing 11 regions of the country. This program would have been benefited if more sensitive diagnostic methods were employed especially for such countrywide data reporting purposes that could be used by central referral lab and decision making in selection of vaccine.

1.4 Microbiology of frequent etiologic agents of bacterial meningitis

Neisseria meningitidis is an aerobic, nonmotile, diplococcic Gram negative bacterium belonging to the genus Neisseria (Fig. 9B). It is a human-specific bacterium that can commensally colonize the extracellular part of the nasopharyngeal mucosa (Auburtin *et al.,* 2002). Meningococci produce catalase, harbor the enzyme oxidase and are best isolated on chocolate agar or blood agar. It shows grayish, non-hemolytic, round, convex, smooth, moist and glistening colonies with a clearly defined edge (Manchanda *et al.,* 2006).

Growth is stimulated by CO_2, humidity and acid they produced from carbohydrates oxidatively. Like other gram-negative bacteria, the organism is surrounded by an outer membrane composed of lipids, outer membrane proteins (OMPs) and lipopolysaccharides. Attached to this outer membrane is a polysaccharide capsule in the pathogenic meningococci (Manchanda *et al.,* 2006).

Complex genetic diversity is possessed by meningococci than most other pathogenic human bacteria. This is partly due to horizontal intra-species gene recombination and incorporation from closely related *Neisseria* species. Serotyping (i.e. determining the capsular group) is essential for the development of vaccination strategies. Exchange of the genetic material is also responsible for capsule switching from one serogroup to other.

For instance, capsular biosynthetic and transport operons of the major meningococcal serogroups may switch from the B to C capsule in the outbreak strain and this may be the result of allelic exchange of the polysialyltransferase (Swartley *et al.,* 1997). This in turn

is supposed to be the result of transformation and horizontal DNA exchange *in vivo* of a serogroup C capsule biosynthetic operon. Hence closely related virulent meningococcal clones may not be recognized by traditional serogroup-based surveillance (Swartley *et al.*, 1997).

Capsule switching may be an important mechanism of bacterial virulence and a challenge to human health with regard to use of vaccines that merely provide serogroup-specific protection (Manchanda *et al.*, 2006). The bacteria may be encapsulated with polysaccharide capsule or may remain unencapsulated. In rare instances, meningococcal isolates without capsule can cause disease (Ganesh *et al.*, 2017). Severe disease like meningitis is caused by the encapsulated strains and the bacteria occurs intracellular or extracellular in the polymorphonuclear leukocytes (Leimkugel *et al.*, 2005).

The specific polysaccharides are designated as A, B, C, H, I, K, L, M, X, Y, Z, 29E and W-135 (serogroup D is no longer recognized). Of the 13 serogroups, only 6 (A, B, C, W-135, X and Y) are known to cause severe diseases such as BM (Leimkugel *et al.*, 2005). The bacteria is further classified into 20 serotypes (on the basis of class 2 or 3 OMP antigens), 10 subtypes (identifying class 1 OMP antigens) and 13 immunotypes on the basis of lipooligosaccharide antigenic properties of immunoglobulin A1 (IgA1) proteases and pili are used for additional typing. For instance serological typing B: 4: P1.4 can indicate serogroup (B), serotype (4) and subtype (P1.4) (Manchanda *et al.*, 2006).

Streptococcus pneumoniae is a bacterium with characteristic lancet shaped Gram positive diplococci. It sometimes occurs in short chains. Its lipoteicohic acid layer plays a major role in inducing pathogenesis in host tissue. *Streptococcus pneumoniae* is the classic example of a highly invasive, extracellular bacterial pathogen. Microbiological characteristics of *S. pneumoniae* (Fig. 9) show that the organism is a Gram-positive (e.g., Fig 9A), alpha-hemolytic, aero-tolerant, encapsulated, diplococcus bacteria belonging to the genus Streptococcus (Tonnaer *et al.,* 2006).

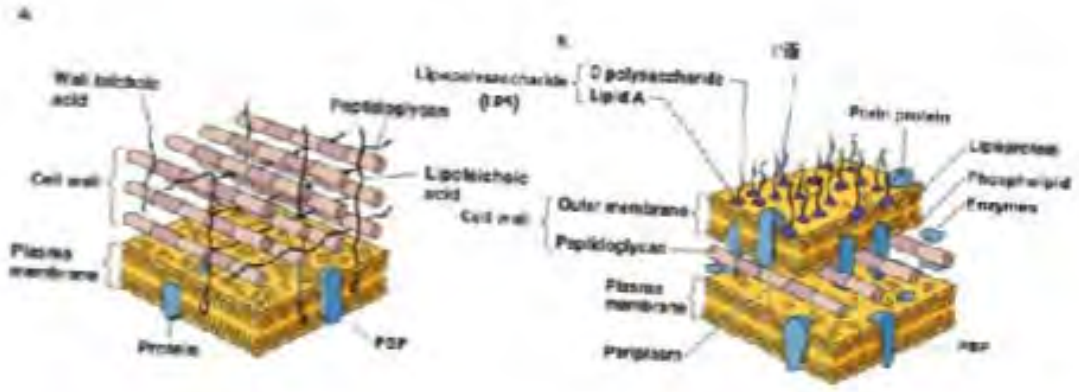


Fig. 9: Graphical illustration of Gram-positive (A) and Gram-negative (B) cell wall structures Adapted from Tortora *et al.* (2010)

Streptococcus pneumoniae can be differentiated from *Streptococcus viridians* by using Optochin (P) antibiotic (Ethyl-hydro-cupreinean) discs, too toxic for therapeutic use, since the former organism is susceptible to this test (Perilla *et al.,* 2003). Large green zone surrounding growing *S. pneumoniae* colonies can be observed during inoculation onto blood medium, due to lysis of hemoglobin. The colonies on culture media vary in appearance depending on the degree of encapsulation of the organism. Heavily encapsulated strains can have large colonies, several millimeters in diameter, appearing

gray and mucoid, while less heavily encapsulated organisms usually have smaller colonies (Xayarath and Yother, 2007).

Haemophilus influenzae are bacteria possessing the characteristic feature of Gram negative (e.g., Fig. 9B) short rods. Studies from microbiological characteristics of *H.influenzae* show that the organism has non hemolytic, opaque cream-to-gray colonies with no specific arrangement. The bacteria has smooth, pale, grey or transparent colony bearing a convex shape. As *H. influenzae* is a highly fastidious organism, usage of antibiotics prior to CSF collection as well as slight procedural modification for culture based isolation reduces its isolation rate (Welinder‐Olsson *et al.*, 2007).

Approximately 5 % of healthy individuals are colonized with strains of serotypes a-f (Manchanda *et al.*, 2006) and the rate increases for children compared to adults (Gilsdorf, 1998). Studies show that most of the strains of *H. influenzae* are opportunistic pathogens which usually dwell in their host without causing disease. These bacteria are not found in the environment and do not colonize or infect other animal species. Apart from asymptomatic colonization, the bacterial strains that possess type b capsule *H. influenzae* type b (Hib), can also cause significant human disease such as bacteremia and meningitis in nonimmune infants and children (Gilsdorf, 1998). A study by Su *et al.* (2014) has showed that encapsulated *H. influenzae* type f (Hif) have increased incidence of invasive disease even after vaccination with *H. influenzae* type b (Hib). The study was focusing towards investigating for possible genomic characteristics that may shed light on the pathogenesis of *H. influnzae*. However presence of antigenic property that may provide cross-protection against Hib was not elucidated (Su *et al.*, 2014).

Life-threatening, invasive infections in otherwise healthy children or adults are rarely caused by the non-encapsulated (non-typeable) *H. influenzae*. However, if infection occurs, it induces localized respiratory diseases, such as otitis media, sinusitis, pneumonia, meningitis and bronchitis (Gilsdorf, 1998). The cascade of reactions they induce starting from the mechanism of attachment to host cells to disease causation is still vague (Dando *et al.,* 2014).

1.5 Immunopathogenesis of bacterial meningitis

Different bacterial pathogens utilize varying types of immunopathogenesis mechanisms. Meningococcal bacterial interaction with the host cell surface is effected by binding with the host cell (Fig. 9B) CD46 molecule through its pilli (Capecchi *et al.,* 2005; Sanders *et al.,* 2011).

Experiments investigating roles played by bacterial capsular protein show the contribution of adhesion factors of *N. meningitidis* Type 4 pilli (T4p) in transition from carriage to disease state (Hallström *et al.,* 2008; Sanders *et al.,* 2011; Coureuil *et al.,* 2017). Bacterial surface-associated filamentous structures such as T4p of most encapsulated bacteria (Sutherland *et al.,* 2010) are important for adhesion, colonization as well as disease causation (Proft and Baker, 2009). Crossing of nasopharyngeal epithelial cells occurs through continued extension and retraction of Tfp prior to crossing epithelial cells. This has been discovered using cell model systems such as monolayers of polarized Calu-3 cell lines (columnar epithelial cells), derived from bronchial epithelial cells (Sutherland *et al.,* 2010). Other than Tfp, endotoxin or lipopolysaccharide (LPS) or lipo-

oligosacharides (LOS) (Fig. 9B) are the major component of outer membrane of the bacteria (Mogensen *et al.*, 2006).

Lipopolysacharides (LPS) and peptidoglycans are the major inflammatory substances in Gram-negative bacteria (Sanders *et al.,* 2011). Immunopathogenesis of Gram negative bacteria like *H. influenzae* is induced through TLR2 upregulation which is promoted through synergistic activity of Glucocorticoids by negative cross talk with inhibitory molecules (Wang *et al.*, 2016).

Conversely, lipoteichoic acid (LTA), teichoic acid as well as peptidoglycan (PGN) are factors that induce pathogenesis (Fig. 9A) in Gram positive bacterial agents such as *S.pneumoniae* (Xu *et al.,* 2015). In general, the sequential steps in the pathogenesis of BM involve colonization on the mucosal cells, transition to blood either transcellularly or paracellularly across the mucosal cells of the host and entry to the CNS by breaching the BBB. This induces three other major steps (bacterial invasion, Nf-kB activation and leukocyte migration) responsible for the pathogenesis of BM so as to promote production of proinflammatory molecules (Wang *et al.*, 2016; Sanders *et al.,* 2011) (Fig. 10 and 11). The host pathogen interactions on the cell surface induce inflammatory stimuli that activate NF-$_k$B pathways.

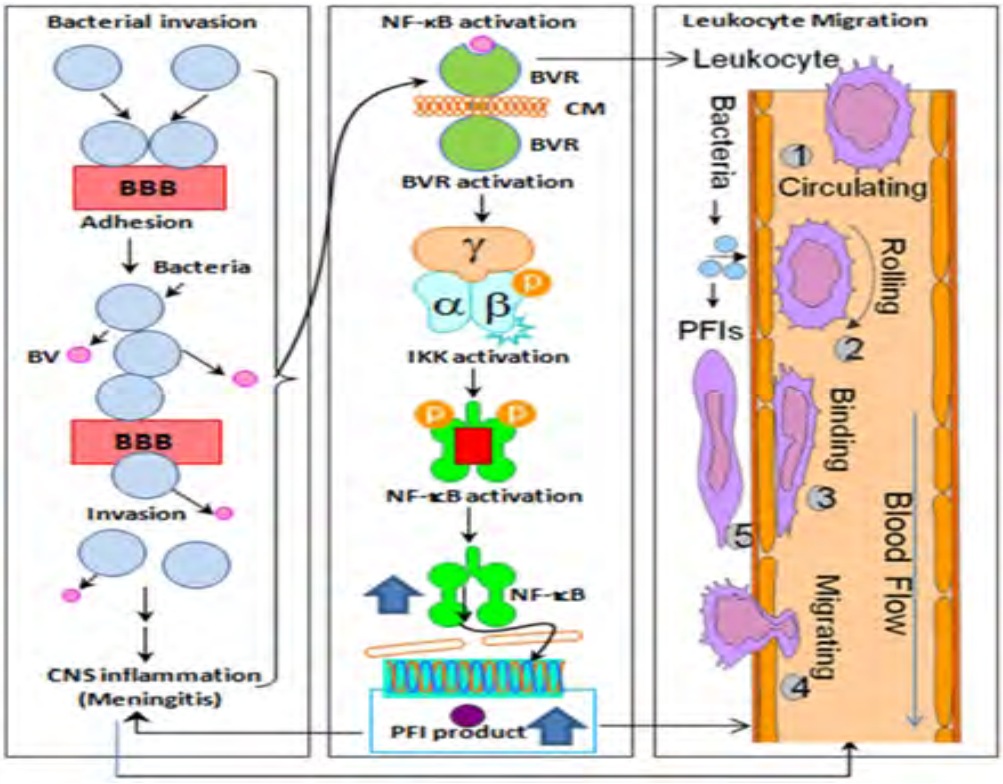


Fig. 10: Flow chart of the pathogenic triad in bacterial meningitis
Adapted from Wang *et al.* (2016)

The initiated signal relays through myeloid differentiation-88 (MyD88) protein pathways in which mytogen activated protein kinsase (MAPK) is also functioning (Sanders *et al.*, 2011). Bacterial cell wall components such as LTA as well as LPS are crucial in initiating inflammatory signaling of the innate immune system via Toll-like receptors 2 and 4 (TLR2 and TLR4) (Fig. 11 and 12).

Activation of NF-κB induced by *S. pneumoniae* depends on TLR2 TLR4, and their ligands lipoteichoic acid (LTA) and lipopolysaccharide transport (LPT) (Mogensen *et al.*, 2006). Variety of transcription factors including NF_kB coordinate the induction of many

genes encoding different inflammatory mediators (Mogensen *et al.*, 2006; Wang *et al.* 2016; Gaikwad *et al.*, 2016).

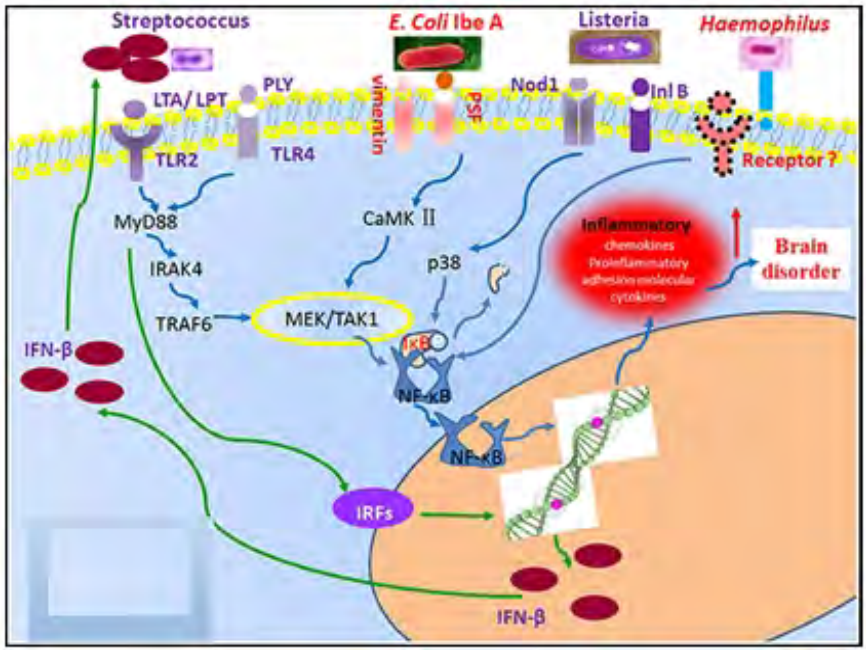


Fig. 11: Schematic representation of signal cascade in pathogenesis of *S. pneumoniae* and other bacterial pathogens including *H. influenzae*
Adapted from Wang *et al.* (2016)

Subsequent brain damage occurs as a result of proinflammatory molecules produced through the signal cascades initiated during host pathogen interaction (Scheld *et al.* 2002; Gaikwad *et al.* 2016). This is promoted through the activity of proinflammatory cytokines (IL-1β and TNF-α) which are potent activators of NF-kB (Fig. 12).

Variation in CD46 expression, alteration in the type and route of signal cascade, and type of pathogen inducing the signal cascade in the host cell may determine the type and level of cytokine production, which in turn determines degree of disease severity (Grandgirard *et al.*, 2013).

As the pathogen intrudes into the CNS by breaching the BBB, an uncontrolled expression of proinflammatory mediators and elevated expression of adhesion molecules both on the endothelium (e.g. intercellular adhesion molecule, (ICAM) as well as on neutrophils leads to subsequent massive influx of leukocytes into the subarachnoid space (Scheld *et al.,* 2002). Granulocyte colony stimulating factor (G-CSF), chemokines such as IL-8 and MIP-1 function to activate leukocytes and their infiltration into the CNS after introduction of a pathogen such as *N. meningitidis* into this compartment (Crawford *et al.,* 2008; Croxen and Finlay, 2010; Koedel *et al.,* 2002; Sadikot *et al.,* 2005). This initiates an increase in the level of CD14 expression in association with a polymorphism in the CD14 gene which was shown in meningococcal meningitis patients with severe disease progression (Biebl *et al.,* 2009).

Genetic variants in the complement factor H region are associated with susceptibility to meningococcal disease. Tumor-necrosis factor-α (TNF-α) gene polymorphism is also associated with invasive meningococcal infection (Warrel *et al.,* 2003). Increased susceptibility and outcome can also be induced by types of gene coding for mannose-binding lectin, Fc-γ receptors (CD16, CD32), and TLR-4 (Warrel *et al.,* 2003). Studies from Gambia and United Kingdom suggest that the most common polymorphism in TLR-4 gene (Asp299Gly) was not highly represented among cases. Increased disease severity was reported to occur in association with polymorphism in genes coding for interleukin-1 receptor antagonist (IL-1RA) and interleukin-1 (IL-1) (Warrel *et al.,* 2003).

Damaged function of the BBB which occurs during meningitis is induced as a result of generation of reactive oxygen metabolites and proteolytic enzymes (Biebl *et al.*, 2009). Elevated production and influx of the proinflammatory cytokines such as IL-6, IL-12, TNF-α, IL-2, IL-1 and chemokines into the CNS are events following the damage to the BBB. The elevation in the level of these cytokines may induce disease severity in BM patients (Barichello *et al.*, 2013; Echchannaoui *et al.*,2002).

Magnitude of disease severity of BM caused by *S. pneumoniae* can be reflected by increased level of pro-inflammatory cytokines in response to production of pneumolysin (Ply) that circulates in the CNS (Yushchenko *et al.*, 2000). Studies show that significantly higher CSF concentrations of IFN- , MCP-1, and matrix-metalloproteinase-9 (MMP-9) are associated with pneumococcal meningitis associated disease severity (Yushchenko *et al.*, 2000; Gray *et al.*, 2014). The disease severity and resulting death and sequelae in survivors occurs due to the activity of proinflammatory cytokines which activate proteolytic enzymes (MMP-9) that degrades the extracellular matrix (ECM) (Scheld *et al.*, 2002)..

Oxidants produced due to activity of the proinflammatory cytokines and leukocytes promote lipid peroxidation and loss of membrane integrity. This, as well as DNA damage will result in cell injury resulting in death or sequeale in survivors. Levels of different proinflammatory cytokines and chemokines may be higher during CNS infection caused by *S. pneumoniae* as compared to its infection with *N. meningitidis* and *H. influenzae* (Scheld *et al.*, 2002).

In general, production of proinflamatory cytokines such as IL-1, IL-6, interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and chemokines are rapidly increasing in the CSF during inflammation induced by bacterial agents. Studies show that the pneumolysin (Ply) peptide is a potent inducer of pneumococcal induced immunopathogenesis including BM (Mogensen *et al.,* 2006; Serón *et al.,* 2015; Zwijnenburg *et al.,* 2006). The common intra-cellular adapter protein, myeloid differentiation factor 88 (MyD88) is used by the majority of TLRs on host cells exposed to bacterial peptides including Ply (Barichello *et al.,* 2013; Gray *et al.,* 2014).

Influx of activated neutrophils and progressing inflammatory activity may continue as a result of increased level of bacterial cell wall components e.g., LPS of Gram negative bacteria as well as domain 4 Ply of Gram positive bacteria such as *S. pneumoniae* (Gray *et al.,* 2014). This induces production of proinflammatory cytokines and chemokines by microgelial cells and astrocytes (Fig. 12).

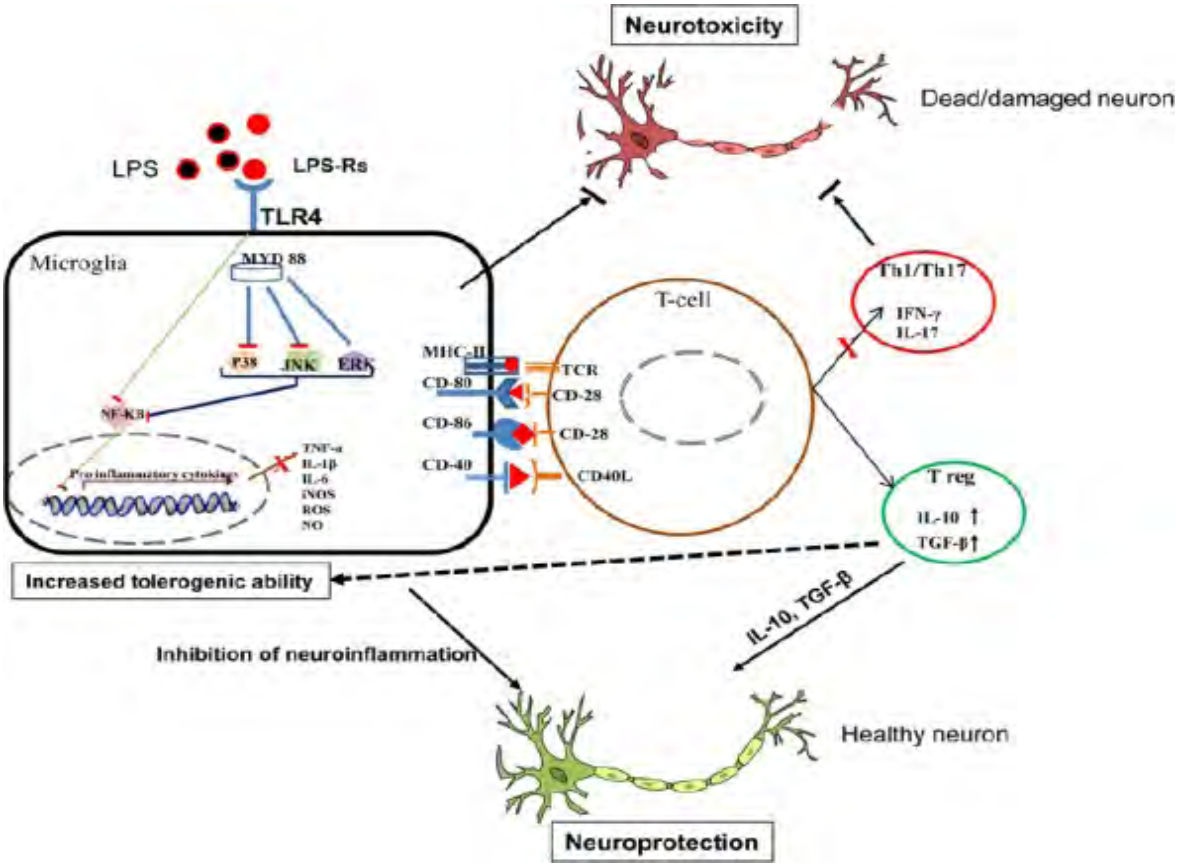


Fig. 12: Signal cascade showing production of pro-inflammatory and anti-inflammatory cytokines induced by bacterial LPS; their outcomes in neuronal death/damage or neuroprotection Adapted from Gaikwad *et al.* (2016).

Tissue damage, death, and sequela in survivors of BM patients may be induced due to decreased level of TRAIL (Steinwede *et al.*, 2012), less degree of apoptosis and subsequent failure in limiting inflammatory function of activated neutrophils (Koedel *et al.*, 2002). Studies have elucidated the role of TNF-related apoptosis inducing ligand (TRAIL) through regulating inflammatory neutrophil induced apoptosis in order to promote resolution of inflammation and minimize level of BM associated disease severity (McGrath *et al.*, 2011).

Recent studies show the presence of significant risk of severe infections caused by bacteremia which could at times trigger cerebral injury, even without penetration of viable bacteria into the CNS and cause adverse outcomes including death during childhood (Strunk *et al.,* 2014). Bacterial meningitis mediated brain damage occur through release of proinflammatory and anti-inflammatory cytokines/chemokines which in due time results in production of oxidants such as reactive oxygen species and reactive nitrogen intermediates. These terminal mediators of brain damage in BM cause deleterious outcomes including death or damage (Fig. 12) of neurons (Koedel and Pfister, 1999). The pathways of neuronal cell death may be induced through necrotic, caspase-independent apoptotic, and caspase-dependent apoptotic pathways (Van der Flier *et al.,* 2003).

On the other hand, necrotic cell death is induced after damage to cell membranes through progression of lipid peroxidation leading to loss of membrane integrity and depolarization. All these as well as DNA damage occur due to bacterial and host oxidants resulting in energy-consuming activation of poly ADP-ribose polymerase (PARP). When DNA repair is ineffective because of the magnitude of the damage, massive energy depletion will cause necrotic cell death (Koedel and Pfister, 1999). Elevated levels of calcium oxidants, excitatory amino acids, and bacterial exotoxins such as the Ply of *S.pneumoniae* contribute to these events. The resulting PARP activation due to activity of bacterial toxins contributes to necrotic death. It also primarily causes damage to the mitochondrial outer membranes with PARP-dependent release of apoptosis-inducing factor (AIF) (Braun *et al.,* 2001).

The pili, fimbriae and outer membrane proteins (OMPs) of *H. influenzae* are important virulent factors for causation of BM. The pili's major involvement in causation of BM is associated with initial colonization as with most other bacterial agents possessing this structure (Hallström *et al.,* 2008; Sanders *et al.,* 2011). *H. influenzae* type b (Hib) porin may contribute to elevation in production of cytokines and increase in blood brain barrier (BBB) permeability as a result of signal cascades it induces after attaching to cell surface molecules of the host. Both pilus and nonpilus adhesions of *H. influenzae* contribute to adherence to extra cellular matrix (ECM) proteins. Haemophilus surface fibrils (Hsf) are the major nonpilus adhesion factors in Hib. Meningitis, septicemia and epiglottitis are the life threatening diseases caused by Hib among the encapsulated *H. influenzae* while non-typable *H. influenzae* accounts for the majority of local disease and upper and lower respiratory tract infections such as acute otitis media, bronchitis and sinusitis (Hallstrom *et al.,* 2006). However, little is understood about immunopathogenesis induced by *H. influenzae* especially with regard to causation of BM (Dando *et al.,* 2014).

1.6 Disease severity in bacterial meningitis

Severe neurological sequelae can be encountered by patients who contract BM. Cognitive impairment can occur after facing the disease even in those patients who have a relatively good clinical outcome (Van de Beek *et al.,* 2002; Merkelbach *et al.,* 2000). Manifestation of the disease severity is as a result of non-progressive disorder of motor function such as cerebral palsy, (CP) caused by irreversible damage to the brain. The disorder may result in sequelae such as seizures, mental retardation, visual and hearing defects (Okike *et al.,* 2013). This occurs mainly due to peptides (e.g., pneumolysin in

case of pathogenesis induced by *S. pneumuoniae*) which translocate to mitochondria and induce pore formation in mitochondrial membranes (Braun *et al.*, 2007; Bermpohl *et al.*, 2005).

Large scale fragmentation of host cell DNA and apoptosis associated cell death may promote release of apoptosis inducing factor (AIF) from damaged mitochondria. This type of cell death may occur in a caspase independent manner and the host cells exposed to live pneumococci or pneumolysin cannot be rescued by caspase inhibitors. Other studies on related matters also show that disease severity is mainly dependent on host-pathogen interaction (Caws *et al.*, 2008; Bermpohl *et al.*, 2005).

In a study done in Brazil, twelve patients with pneumococcal meningitis had significantly higher levels of interferon-γ in CSF as compared to other 12 patients with meningococcal meningitis (Coutinho *et al.*, 2013). Subsequently, CSF samples from 14 BM patients from Burkina Faso with *S. pneumoniae* and 22 patients with *N. meningitidis* meningitis were analyzed in the same lab where samples from Brazilian patients were analyzed (Grandgirard *et al.*, 2013; Coutinho *et al.*, 2013). Interferon-γ, MCP-1 and MMP-9 were significantly higher in patients with pneumococcal meningitis suggesting a different cytokine profile with more intense production and possible association with disease severity (Grandgirard *et al.*, 2013). As in the study by Coutinho *et al.* (2013), TNF- , IL-1 , IL-1ra, and IL-6 circulating in the CNS were significantly higher in BM patients who died as compared to survivors (Coutinho *et al.*, 2013). Moreover, elevated production of pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin-1

(IL-1), interleukin-6 (IL-6), interferon gamma (IFN-γ), chemokines like interleukin-8 (IL-8) were suggested to be contributory factors to pathogenesis and disease severity of BM (Coutinho *et al.,* 2013). The study by Coutinho *et al.* (2013) reported that the ability to release high levels of IFN-γ and other proinflammatory cytokines with low number of cells in the CNS are important factors for pathogenesis of BM especially when *S.pneumoniae* is the causative agent of BM.

Other studies also showed that leukocytes start to appear in the CSF and their number elevates after initial production of cytokines such as TNF-α, IL-1, IL-6, and IL-8 in the CSF of patients with BM (Balding *et al.,* 2003). Regarding site of production of the cytokines, Ramesh *et al.* (2013) reported that (Fig. 13) cells normally present in the CNS (endothelial cells, microglial cells, and astrocytes) produce increased levels of cytokines after entry of bacterial agents into this compartment. Monocytes introduced from inflammatory exudates of the circulation are the other major origin of cytokines in the CSF. Anti-inflammatory cytokines such as IL-10 and TGF-β can lessen the inflammatory process during BM through inhibition of production of pro-inflammatory cytokines such as TNF-α. In general, cytokines are formed by a wide variety of cells including monocytes, macrophages, microglial cells, astrocytes, and endothelial cells. The respective pathological conditions are associated with levels and types of these inflammatory proteins circulating in the CNS (Ramesh *et al.,* 2013) (Fig. 13). Signatures of pro-inflammatory molecules and increased intensity of activated inflammatory processes in the CSF usually is associated with severe disease leading to death or sequelae in survivors (Bociaga-Jasik *et al.,* 2003; Grandgirard *et al.,* 2013).

With regard to antibody mediated immune responses, dissecting the specificities of human antibody responses following disease caused by serogroup A meningococci may be important for the development of improved vaccines. A previous study of sera obtained from 71 Ethiopian patients and 113 controls during meningococcal meningitis outbreaks by bacteria of sequence type 7, in 2002 and 2003, showed that meningitis A (MenA) disease induced significant increases in immunoglobulin G (IgG) against LOS L11 strain and Neisserial adhesinA (NadA) (Norheim *et al.,* 2008).

The report from the study has proved that the IgG levels against LOS remained elevated following disease and anti-NadA IgG levels were significantly higher in acute-phase sera than in controls. Moreover, the IgG antibody levels against LOS and NadA correlated significantly with serum bactericidal activity against MenA strains. Recommendations from the study by Norheim *et al.* (2008) highlighted that future studies on immune responses during MenA disease should consider the high levels of anti-MenA polysaccharide IgG commonly found in the population so as to clarify the role of antibodies against subcapsular antigens in protection against MenA disease (Norheim *et al.,* 2008).

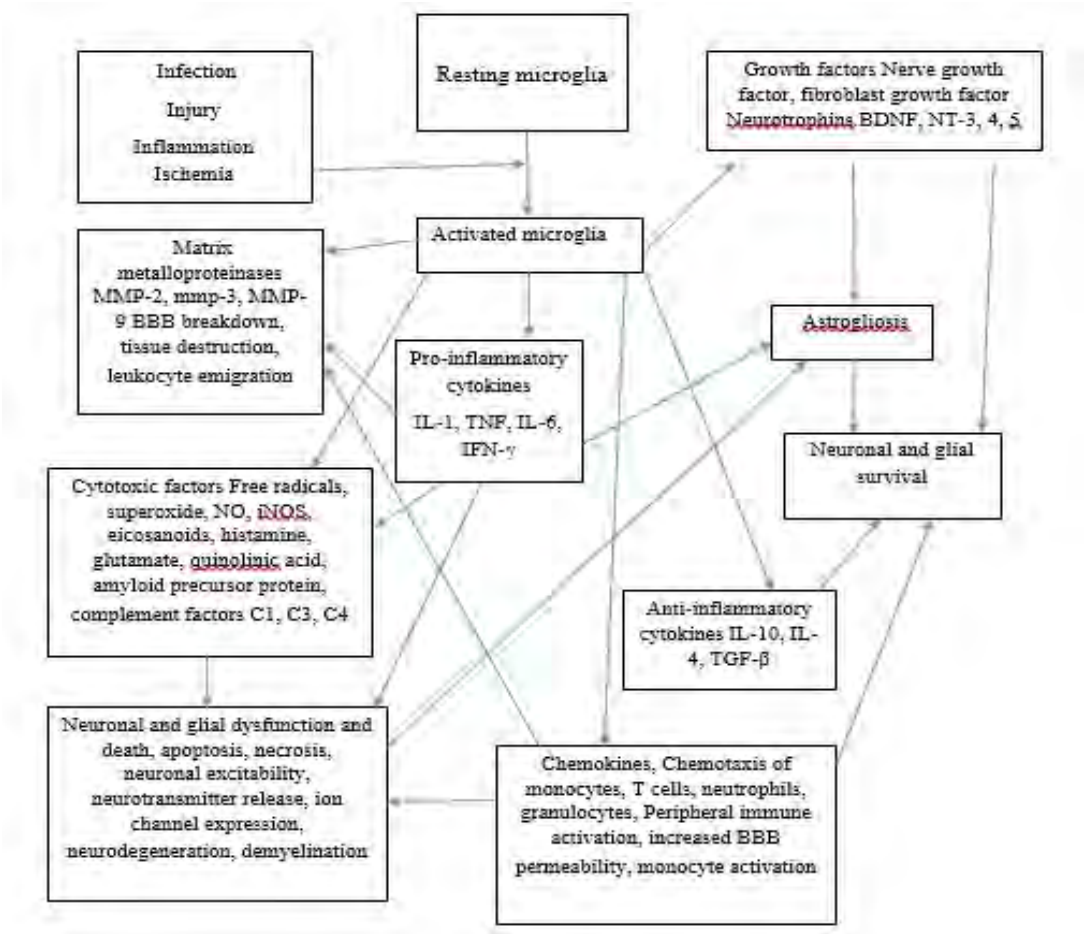


Fig. 13: Central role of microglia in neuroinflammation
Adapted from Ramesh *et al.* (2013)

Studies showing correlation between level of IgG and serum bactericidal activity (SBA) in individuals who have anti-meningococcal vaccine may be used in determining level of protection of the antibody and lowering disease severity (Guerin *et al.,* 2008). Genetic factors or burden of multiple infections may also aggravate disease severity resulting in death or long term sequelae (Naranbhai *et al.,* 2014; Chandran *et al.,* 2011). Diversity of cell surface molecules of the host and genetic variation are factors determining levels of disease severity. The concentration of bacterial capsular polysaccharide triggers host

immune responses which correlate with clinical disease severity manifested by death or sequeale in survivors (Coutinho *et al.*, 2013; Grandgirard *et al.*, 2013).

Antibodies against *N. meningitidis* serogroups A, C, W-135, and Y capsular polysaccharides are bactericidal and confer protection at concentrations of 1 to 2 μg/ml of serum. Evaluation of efficacy of vaccines such as Bexasero and Trumenba may prove potency of antibodies for conferring protection, as previous efforts showed that the capsular polysaccharide of *N. meningitidis* serogroup B bacteria have antigenic similarity with neuronal cell membranes (Feavers and Maiden , 2017).

. Bactericidal and opsonophagocytic antibodies recognizing surface-exposed epitopes of the outer membrane protein, especially PorA, are playing a big role for protection. Antibodies which are anti-lipopolysaccharides (anti-LPS) recognize commonly shared epitopes among virulent and non-pathogenic Neisseria (Warrel *et al.*, 2003).

1.7 Diagnosis of bacterial meningitis

Clinical diagnosis of BM is based on signs and symptoms of meningitis which include headache and neck stiffness associated with fever, petecchiae, seizure, ecchymosis, confusion or impaired consciousness, vomiting, and inability to tolerate light (photophobia) or loud noises (phonophobia) (Best and Hughes, 2008). Rather than neck rigidity, bulging of fontanel may be present in children as a clinical picture of BM (Berkley *et al.*, 2004; Best and Hughes, 2008). Children often exhibit only nonspecific symptoms, such as irritability and drowsiness, while presence of rash may also indicate meningococcal infection. Most children with meningococcal infection have purpuric rash

(mainly in Caucasians), fever and delayed capillary refill (Cascio and Heath, 1996; Marc *et al.,* 2002; Pfeiffer *et al.,* 2003). Clinical diagnosis through using signs and symptoms are essential steps to empirically and urgently initiate patient management as BM is a medical emergency (Attarpour-yazdi *et al.,* 2014).

Gram staining, available at almost all clinical laboratories is used to support the empirical clinical diagnostic arm for patient management purpose. Culture diagnostic tests (through biochemical detection) such as seroagglutination test, antibiotic sensitivity test, and growth response to nutritional factors are among the routine clinical laboratory diagnostic methods (Pittman *et al.,* 2014; WHO, 2011). These culture diagnostic tests in combination with other tests or alone are used in referral level hospital laboratories for diagnosing *N. meningitidis, S. pneumoniae* and *H. influenzae,* respectively (Mohammadi *et al.,* 2013; Pittman *et al.,* 2014). Although culture diagnostic tests are used in referral level hospital laboratories (where the facility is available) for patient management diagnostic support purpose, none of the culture diagnostic tests are entirely satisfactory because of limited sensitivity and specificity (Lusiana *et al.,* 2011; Amsalu *et al.,* 2005; Perilla *et al.,* 2003).

Due to challenges in the diagnosis of acute BM in resource poor countries, fatal consequences are usually faced by the patients (Scarborough and Thwaites, 2008). For instance, sensitivity of Gram stain test, in developing countries is only 25-40% when compared to 80-85% in developed countries (Nagarathna *et al.,* 2012). Delays in CSF sample processing and not using sample transporting media such as trans-isolate media

(TIM) if the sample could not be diagnosed immediately after collection due to some logistic reasons are challenges for detection of etiologic agents of BM (Caugnant *et al.,* 1999).

Although they do not help to determine the specific type of bacteria, the WBC count in BM is higher than 1,000 per mm^3 among eighty-seven percent of patients. The number of white blood cells (WBCs) per mm^3 of CSF would be up to 5 in healthy adults and may rise to 20 WBCs per mm^3 in healthy newborns. Patients with viral meningitis more commonly have less than 100 WBCs per mm^3 (Seehusen *et al.,* 2003).

Higher sensitivities and specificities (< 100 %) are consistently shown by molecular diagnostic assays in comparison with routine biochemical or clinical diagnostic assays (Richardson *et al.,* 2003; Saravolatz *et al.,* 2003). In addition to higher sensitivity and ease of laboratory protocol, molecular diagnostic tests such as real time PCR is a highly convenient method to be selected for amplification of gene of interest (Wu *et al.,* 2013; Saravolatz *et al.,* 2003; Corless *et al.,* 2001). In general WHO recommends use of either culture or PCR detection or both as gold standard methods for diagnosis of BM for reporting country wide data (WHO, 2014).

Studies show that countries like Ethiopia, need to use improved laboratory diagnostic systems and update laboratory infra-structure, undertake monitoring and evaluation of systems as well as procurement and maintenance of equipment to combat major infectious diseases (Nkengasong *et al.,* 2009).

1.8 Management of patients diagnosed with bacterial meningitis

Since BM is a medical emergency, admission of the suspected patient to a hospital or health center is necessary. Initiating antibiotic therapy and close monitoring with frequent repeated prognostic evaluations are necessary steps to be taken for patient management. Bacterial meningitis is an emergency CNS infection that needs to be treated with antibiotics urgently. Other than administration of antibiotics anti-inflammatory drugs like Dexamethasone can attenuate inflammation. The patient may not be rescued (i.e., may die or remain with permanent sequelae if surviving) even with provision of antibiotics (Brouwer *et al.*, 2010).

Mean opening cerebrospinal pressure and cerebral perfusion pressure improve in patients administered with anti-inflammatory drugs while patients who are administered with antibiotics alone will have worse health outcomes (Van de Beek *et al.*, 2004, Molynexu *et al.*, 2002). Whereas, delay in initiating antibiotic therapeutic intervention may aggravate the disease (Proulx *et al.*, 2005).

Apart from immune responses, patient recovery from the disease of BM depends on pharmacodynamics properties of antibiotics. The nature or capacity of the drugs to diffuse across or access the BBB is very important since they must penetrate the CNS to optionally act (Andes and Craig, 1999). Lipid solubility, molecular weight, ionization, and protein binding capacity are determinant factors in the ability of chemotherapeutic agents to treat BM effectively. Lipophilic agents, such as fluoroquinolones,

chloramphenicol, rifampin and sulfonamides are capable of entering the CSF by diffusion (Nau *et al.,* 2010).

Single dose regimens of ceftriaxone or chloramphenicol have been recommended to ensure rapid and effective treatment during epidemics of meningococcal meningitis. Effective treatment is simple, cheap and can be made available even at the most peripheral level. Single dose regimens are not effective against pathogens such as *S.pneumoniae* or *H. influenzae.* Treating patients with BM caused by these pathogens require longer courses as per WHO guide lines (WHO, 2014).

1.9 Immunization against bacterial meningitis

With regard to protective immune response researches have focused more on blood rather than mucosal protection. However not much is known to date about the mucosal arm of the immune response against the bacterial agents which mainly cause BM. Hence more studies are needed to research nasopharyngeal mucosa immune responses against the bacterial agents prior to their entry into the circulation during initiation of disease (Johswich *et al.*, 2015).

Vaccination against bacterial meningitis was introduced in the early 20th century (Tyler, 2009). The progress in development of the vaccines especially against *N. meningitidis* and *H. influenzae* was improved in later years (Tyler, 2009). Studies that implement vaccine intervention against BM in different geographical regions recommend the need for continued surveillance to monitor effectiveness of vaccine protection (Ali *et al.,* 2014). Quadrivalent anti-meningococcal vaccine comprised of serogroups A, C, W-135

and Y (Menactra or Menveo) and anti-meningococcal monovalent (MenAfrivac or MenA-TT) and *H. influenzae* bivalent (MenHiebrix) vaccines (Table 1) are conjugate vaccines. These and several others conjugate vaccines are manufactured based on the requirement of different age groups and circulating serogroups for a given population at risk (Crum-Cianflone *et al.* 2016) (Table 1).

Effective routine immunization against bacterial pathogens such as *N. meningitidis, S.pneumoniae* and *H. influenzae* is necessary to have a significant impact in preventing invasive diseases like BM. Studies done after provision of vaccine have shown that the levels of protection obtained can be measured. The vaccine may also reduce carriage of the bacterial agents (especially the encapsulated ones) within nasopharyngeal mucosal tissue (Bårnes *et al.,* 2016; Segal and Pollard, 2004).

Efficacy of the specific vaccine can be determined for surveillance purpose by measuring level of serum bactericidal antibody (SBA) pre and post vaccine administration. Serum bactericidal activity level is used as an immunological correlate of protection for approval and license of vaccine for specified age groups and populations (Snape *et al.,* 2005). Reports have shown that vaccination campaigns have significantly reduced the incidence of meningococcal disease. Vaccination with MenAfriVac (Table 1) vaccination has reduced carriage of serogroup A *N. meningitidis* and has induced herd protection. Clinical trials have also demonstrated that the conjugate MenAfriVac is safe, and has induced a superior immune response compared to polysaccharide vaccines and provides immunological memory (Kristiansen *et al.,* 2015). The MenAfriVac serogroup A vaccine has been administrated to Ethiopians aged 1-29, in three phases. Phase 1, phase 2 and

phase 3 vaccinations were implemented in 2013, 2014 and 2015, respectively. The first phase was administered to the population living in the west and north west parts of the country, while the second phase was administered to the population dwelling in the central and southern parts of the country (Kristiansen *et al.,* 2015; Muhammad, 2013). The third phase was administered in 2015 for the population living in the east and north east parts of the country. Implementation of a routine long-term anti-meningococcal vaccination strategy addressing population of all high risk regions is still a gap not yet addressed (Kristiansen *et al.,* 2015).

Table 1: Meningococcal polysaccharide, conjugate and protein vaccines

Type of vaccine	**Serogroups included**	**Trade Name**	**Approved Ages**
Polysaccharide	A, C, W-135, Y	Menomune (MPSV-4)	2 years and older
Conjugate	A, C, W-135, Y	Menactra (MCV4)	9 months-55 years
Conjugate	A, C, W-135, Y	Menveo (MCV-4)	2 months-55 years
Conjugate	C, Y and Hib	MenHibrix	6 weeks-18 months
Protein	B	Trumenba	10-25 years+
Protein	B	Bexsero	10-25 years+
Conjugate	C	Meningitec (MenC-CRM)	≥12 months
Conjugate	C	Menjugate (MenC-CRM)	≥12 months
Conjugate	C	NeisVac-C (MenC-TT)	≥12 months
Conjugate	A	MenAfrivac (MenA-TT)	1-29 years
Conjugate	A, C, W-135,Y	Nimenrix (MenACWY-TT)	≥12 months
Conjugate	Hib, Men C	Menitorix (Hib-MenC-TT)	Primary series 1.5-12 months. Booster: 12 months to 2 years

Adapted from Crum-Cianflone *et al.* (2016). Notes: Manufacturer details: Abbreviations: MenA: meningococcus serogroup A; MenC: meningococcus serogroup C; TT: tetanus toxoid; Hib*: Haemophilus influenzae* serotype b; CRM: cross reacting material for cornyebacterium diphtheria toxin.

Polysaccharide serogroup A tetanus toxoid conjugate (PsA-TT) vaccine implementation among nine hyperendemic countries (Chad, Cameroon, Nigeria, Benin, Ghana, Senegal, Sudan, Gambia, and Ethiopia), representing a target population of 150 million vaccinated people, showed significant decrease of disease incidence (Diomandé *et al.,* 2015). Significant reduction of oropharyngeal carriage of group A meningococci was shown not only in vaccinated individuals but also among unvaccinated individuals, demonstrating the vaccine's ability to generate herd protection and prevent group A epidemics (Kristiansen *et al.,* 2013). Conjugate vaccines against *H. influenzae* serotype b (Table 1), *N. meningitidis* serogroup A (MenAfriVac) (Table 1) and *S. pneumoniae* (PCV-10) (Table 2) are also generating such herd protection (Kristiansen *et al.,* 2015).

The composition of the pneumococcal vaccines depends on the number of *S. pneumoniae* serotype polysaccharide antigens they contain and whether or not these antigens are conjugated to a protein carrier (WHO, 2012).

The older unconjugated pneumococcal vaccines (Table 2) are pneumococcal polysaccharide vaccines (PPSV) while the newer vaccines are pneumococcal conjugated vaccines (PCV). Currently available PPSV contain 23 purified capsular polysaccharide antigens of *S. pneumoniae* serotypes. Whereas PCV consists of protein-polysaccharide combinations which contain variable number of (7, 10 or 13) capsular polysaccharides antigens bound to a protein carrier (non-toxic diphtheria toxin CRM197, or D-protein of *H. influenzae*). The PCVs induce immunological memory in children less than 2 years of age while PCV13 is also approved for use in adults who are 50 and older (WHO, 2012).

Table 2: Pneumococcal unconjugated polysaccharide and conjugate vaccines

Vaccine	Vaccine antigens Excipients	Vaccine antigens Excipients
Unconjugated (PPSV)	Pneumococcal 23-valent vaccine consists of 25 µg of each capsular polysaccharide antigens from the serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.	Dissolved in isotonic saline solution with phenol (0.25%) or thimerosal (0.01%) added as preservative.
Conjugated (PCV)	Pneumococcal conjugate 7-valent vaccine consists of 2-4 µg of capsular polysaccharide antigens from the serotypes 4, 6B, 9V, 14, 18C, 19F, 20, and 23F conjugated to non-toxic diphtheria toxin (CRM197). Pneumococcal conjugate 13-valent vaccine consists of 2 - 4 µg polysaccharides of the capsular antigens of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F and 23F, individually conjugated to a nontoxic diphtheria CRM197). Pneumococcal conjugate 10-valent vaccine consists of 1 microgram of polysaccharide for serotypes 1, 5, 6B, 7F, 9V, 14 and 23F, and 3 micrograms of serotypes 4, 18C and 19F.	Aluminum phosphate No preservatives PCV13 also contains polysorbate 80, and succinate buffer. Adapted from WHO, (2012).

Adapted from WHO, (2012)

2. THE RATIONALE OF THE STUDY

In spite of the decline in morbidity and mortality caused by BM, the disease is still considered to be a serious health threat especially in the regions known as the ‘’meningitis belt’’ of Africa. The focus of many studies on BM has been on epidemic seasons, while less attention has been given to non-epidemic seasons, which are sporadic and characterized by endemic manifestations of the disease (Ramakrishnan *et al.,* 2009). Data on non-epidemic season BM in Ethiopia are rare. As part of the “Meningitis belt” countries, Ethiopia also suffers from recurrence of meningitis epidemics for which *N.meningitidis* serogroup A was reported to be largely responsible. Occasional outbreaks of other serogroups (such as W-135 and X) have also been reported from studies done in other meningitis belt countries (Chippaux *et al.,* 1999).

Whereas, meningococcal meningitis is prevalent primarily during the epidemic seasons, *S. pneumoniae* and *H. influenzae* endemically occur as BM etiologic agents during the non-epidemic seasons (Xie *et al.,* 2013). Therefore, determining the etiologic agents of BM during the non-epidemic seasons, the challenges with diagnostic approaches and levels of disease severity will be important steps for designing interventions. Strains from non-epidemic seasons are also contributing to incidence of BM during epidemic seasons. Studies conducted by Nicolas *et al.* (2005) and Norheim *et al.* (2006) regarding strain distribution of *N. meningitidis* serogroup A in the meningitis belt of Africa, showed that ST5, the previously common strain identified during epidemic seasons, has been replaced by ST7 between 1995 and 2000. Studies showed similarity of strains of *N. meningitidis* serogroup A isolated during the epidemic season with those reported during the non-

epidemic season (Bevanger *et al.,* 1998; Salih *et al.,* 1990), suggesting that strains from the later season are responsible for a vast proportion of cases of BM occurring during the former seasons (Ramakrishnan *et al.,* 2009; Laforce *et al.,* 2009).

Although studies have reported relative success with vaccine-induced control of serogroup A *N. meningitidis,* determining the strain distribution of serogroup A *N.meningitidis* during the non-epidemic season will be essential for the purpose of detection of surveying the introduction of newly coming strain (Ramakrishnan *et al.,* 2009).

Disease severity and subsequent outcome of death and sequelae in survivors can be mediated through activated microglial cells which induce neuronal and glial cell injury through elevated production and dysregulation of cytokines and chemokines (Ramesh *et al.,* 2013). Given the impact meningococcal infections have had in sub-Saharan Africa, Ethiopia in the years 1981 and 1989, it is of interest to study the dynamic interaction between levels of *N. meningitidis* in the circulation and subarachnoid space and clinical presentation. This interaction has been extensively studied in Europe but little in Africa (Brandtzaeg *et al.,* 1992; Van Deuren *et al.,* 1994). Furthermore, studies show that BM caused by *S. pneumoniae* induce a higher case fatality rate and more sequelae than *N.meningitidis* meningitis in Africa and industrialized countries (Ramakrishnan *et al.,* 2009; Gessner *et al.,* 2010). The biological basis explaining this difference has not been fully elucidated. One hypothesis is that the inflammatory responses to the Gram negative *N. meningitidis* and the Gram positive *S. pneumoniae* in the subarachnoid space are quantitatively different and this may impact outcome. Few studies have compared the

cytokine profiles in the cerebrospinal fluid (CSF) of the two pathogens, analyzed in the same laboratory (Coutinho *et al.*, 2013; Grandgirard *et al.*, 2013). The findings from the current study will provide a better understanding about the actual diversity of BM etiologic agents and describe the level of disease severity and associated phenomenon with them especially during the non-epidemic season. Since Ethiopia has the second largest population among African meningitis belt countries, recent data on etiologies of BM and disease severity would be of significance. The need for such studies is also recommended by other parallel studies (Grandigrad *et al.,* 2013; Coutinho *et al.,* 2013). Such studies are of invaluable significance for identifying newly prevalent strains and those which need regular monitoring. They are also potential resources for informing vaccine composition based on the circulating strains. Health care professionals and vaccine protection units will benefit by data generated from such studies. The data generated will aid the improvement of patient care service, through appropriate therapeutic intervention and vaccine selection. Hawassa University and Gondar University Hospitals are tertiary level referral Hospitals to which patients are referred to from the Southern and Northern regions of the country, respectively (Fig. 14). Tikur Anbessa (located in Addis Ababa) University hospital (Fig. 14), is also a tertiary level, specialized, referral hospital where patients are referred to from all over the country (Norheim *et al.,* 2006; Mulu, 2014). The Gondar (Amhara region) and Hawassa (Southern Nations, Nationalities and peoples region) University hospitals were recommended as focal meningitis study sites since people living at these geographical

locations have been facing frequent outbreaks of the disease (Ahmed, 2012; Mengistu *et al.*, 2003; Habte-Gabr *et al.*, 1984; Enaro *et al.*, 1999).

Fig. 14: **Map of Ethiopia showing locations of the study sites (Gondar, Hawassa and Tikur Anbessa/Addis Ababa University hospitals). Air distances from Addis Ababa to Gondar and Hawassa are ≈ 420 km and 220 km, respectively. Adapted from Embassy of France in Ethiopia, Addis Ababa.**

3. HYPOTHESIS

Bacterial meningitis during the non-epidemic season is of multiple etiologies, dominated by *N. meningitidis, S. pneumoniae and H. influenzae* in Ethiopia. Disease severity caused by these major etiologic agents is related to the levels of pro-inflammatory cytokines and chemokines in the cerebrospinal fluid.

4. OBJECTIVES

4.1 General Objective

To identify the most common bacterial causes of meningitis (*N. meningitidis, S.pneumoniae, H. influenzae*) in patients clinically diagnosed with BM and admitted to three referral hospitals in Ethiopia during the non-epidemic seasons and determine the levels of disease severity

4.2 Specific Objectives

To determine:

1. The frequency of *N. meningitidis, S. pneumoniae* and *H. influenzae* in BM patients diagnosed during the non-epidemic season
2. The different strains of frequent BM etiologic agents and genogroups of *N.meningitidis* by using real time-PCR and Multi-locus sequence typing (MLST)

3. The difference in performance (sensitivity and specificity) of culture diagnostics (routine biochemical assays) of BM between clinical bacteriology laboratories in Hawassa, Gondar and Tikur Anbessa University hospitals and research bacteriology laboratory (Armauer Hansen Research Institute (AHRI))

4. The level of disease severity in bacterial meningitis patients infected with *N.meningitidis*, *S. pneumoniae* and *H. influenzae*

5. The effect of pre-hospital admission antibiotic usage in routine clinical bacteriology diagnosis of BM

6. The levels of bacterial DNA copy numbers in serum and CSF of patients and correlate with disease severity

5. MATERIALS AND METHODS

5.1 Study sites

Three tertiary level referral teaching University hospitals (Hawassa University Hospital (HUH) located 270 km south of Addis Ababa, Gondar University Hospital (GUH) located 750 km northwest of Addis Ababa and Tikur Anbessa Specialized University Hospital (TASUH) located in Addis Ababa were sites where patients were recruited for this study. The study sites were visited by the study team prior to initiation of the meningitis consortium study (MenTAHG). The study was coordinated by Armauer Hansen Research Institute.

5.2 Study design

This study is a cross-sectional study in which patients clinically suspected with pyogenic meningitis were enrolled based on their sequential flow of hospital visit.

The visit to the study sites had a capacity building component so as to upgrade the clinical bacteriology lab through assessing the gaps and replenishing them with consumables, reagents and equipment in short supply especially as needed for diagnosis of meningitis. Discussions were held during the visit with the health care workers in order to upgrade the hospitals' bacteriology labs instrumental capacity with regard to diagnosis of bacterial meningitis. The capacity building was subsequently accomplished by supply of lab reagents, instruments, lab facilities (appliances/devices) such as refrigerators, freezers, centrifuges, incubators, lumbar puncture (LP) needles and CSF collection tubes

and trans-isolate media (TIM), and blood agar base so as to upgrade the referred hospitals' clinical laboratory bacteriology labs with regard to diagnosis of BM.

Manpower training on routine clinical lab biochemical methods was given with the objective of maintaining uniformity to the routine culture (biochemical) detection tests. Standard operative procedures (SOPs) of the routine clinical lab diagnostic protocols were prepared and submitted to each clinical bacteriology lab and AHRI bacteriology lab prior to initiation of sample processing. Uniform guidance in terms of specific team training was given for the health care workers who run sample collection, complete case record form and sample handling in the clinical lab. Further discussions were held with the study site coordinators and health care workers as per requirement.

5.3 Study population

Patients of all age groups and both gender, who have attended Gondar, Hawassa and Tikur Anbessa University hospitals from 2012-2013, clinically diagnosed to have BM with regard to signs and symptoms were enrolled into this study. The enrollment was based on patient agreement through signed informed consent (for adult patients, i.e. those 18 and above) and assent (for those less than 18 years old) after having detailed explanation about the research project through using preplanned patient information sheet. The information sheet's detailed content was explained by interpreating it into the local language of the study participant.

Sample size estimation

Since bacterial meningitis is a disease having unpredictable occurrence (Schaffner *et al.,* 2014) and the study focuses the non-epidemic season with anticipated sporadic recruitment (Agier *et al.,* 2013), the desired number of sample size for this study was estimated through convenient sampling so as to include sufficient number of patients to be able:

- To provide a reliable estimate of BM etiologic agents in the selected areas
- To estimate challenges in diagnosis of BM
- To assess level of disease severity among the proportion of patients that turn positive for the frequent etiologic agents of BM

In a non-epidemic situation the frequent etiologic agents of BM may cause 2-20% of the meningitis cases. With an aim of including at least 20 patients with confirmed BM disease, and assuming that 10% of the cases are caused by the frequent etiologic agents of BM, around 200 patients with bacterial meningitis was the target sample recruitment size (Table 3).

Table 3: Number of bacterial meningitis patients required to be included to identify frequent etiologic agents of BM disease cases and disease severity

Proportion of patients clinically diagnosed with bacterial meningitis					
Recruitment aim	**2%**	**10%**	**20%**	**50%**	**100% (outbreak)**
20 BM patients	1000	200	100	40	20
40 BM patients	2000	400	200	80	40

This may be possible if e.g. approx. 70 bacterial meningitis patients can be recruited from each of the three selected hospitals to participate in the study from 2012-2013.

Assuming that bacterial meningitis is diagnosed in 5% of admissions, a hospital admitting 70 bacterial meningitis cases will therefore require a total admission of 1400 outpatients (all conditions) over a non-epidemic period.

5.4 Inclusion and Exclusion Criteria

Inclusion Criteria

Patients of all age groups visiting the three University hospitals and clinically diagnosed to have acute pyogenic meningitis (bacterial meningitis)

Exclusion Criteria

Patients in a moribund clinical condition, suffering from conditions such as a bleeding disorder as well as severe malnutrition or severe anemia

5.5 Sample collection and handling

I. Cerebrospinal fluid (CSF) sample collection and handling

Depending on the age of the patient lumbar puncture CSF was collected aseptically by the attending physician using needle measuring 1.5 inches (3.8 cm) in infants, 2.5 inches (6.3 cm) in children, and 3.5 inches (8.9 cm) in adults. Cerebrospinal fluid was collected from a total of 165 recruited patients clinically diagnosed to have BM. The CSF was handled as shown in Fig. 15. The clinician who attended the patient collected a total of 1-1.5 ml of CSF with the standard CSF collection procedure for routine patient care diagnostic purpose.

Fig. 15: Flow chart showing CSF sample handling and transportation
Cerebrospinal fluid collection and transporatation were done based on standard procedures (WHO, 2011; Ajello *et al.* 1984)

II. Blood sample collection

A total volume of 2 to 5 ml venous blood was drawn from peripheral veins for the purpose of routine patient care diagnostic support (i.e., for complete blood count, CBC) in hospital laboratory. Blood was drawn aseptically by a laboratory technician and handled as shown in Fig. 16.

Fig. 16: Flow chart showing blood sample handling and transportation

III. Case record form filling and assignment of study IDs for the study participants

A predefined case record form (CRF) addressing clinical investigation details defining BM was developed prior to recruitment of the study participants. Patient personal data comprising clinical information on admission and the immediate outcome, i.e., death or survival and immediate severe sequelae such as deafness or neurological impairment were recorded. The attending physician or assisting health care worker completed the CRF based on information obtained from patient clinical investigation. Assignment of study ID for patient who gave informed consent or assent (parent/guardian consent) was as per sequence of enrolment into the study.

5.6 Laboratory Methods

5.6.1 Bacteriology

Blood and CSF samples were handled according to Fig. 16 and 17, respectively, for patient routine clinical diagnostic care purposes. The serum (aliquoted into endotoxin free Eppendorf tubes) and CSF (aliquoted into plain tubes) transported to AHRI lab at -20 °C car freezer were again preserved in a -20°C freezer at AHRI lab till they were shipped on dry ice to NIPH/Oslo for subsequent use of meningococcal DNA (from serum and CSF) and cytokines (from CSF) analysis. The CSF in TIM transported to AHRI lab at ambient temperature was immediately inoculated on blood, chocolate and Thayer-Martin (T-M) agar media plates (Fig. 17) through sterile sample inoculation procedures. It was then incubated at 37°C (Thermo-scientific incubators, USA) supplied with 5% CO_2. The remaining CSF in TIM was preserved at -20°C for future use of DNA extraction and PCR

amplification. Possible growth of organisms was checked from the inoculated culture media within alternative duration of 24, 48 or 72 hours. Oxidase (BD USA) test was run based on methods on the standard microbiology lab guideline (WHO, 2011) on a single colony culture media growth briefly by adding 2-3 drops of the test reagent *N*,*N*,*N′*,*N′*-tetramethyl-*p*-phenylenediamine (TMPD), which is a redox indicator showing a dark blue color when oxidized, and colorless when reduced. A dark blue color change shows that the organism is oxidase positive i.e., indicating that the bacterium contains cytochrome c oxidase and can therefore utilize oxygen for energy production with an electron transfer chain. Typically, Neisseria is oxidase positive organism. Gram staining (BD, USA) test was done based on methods on the standard microbiology lab guideline (WHO, 2011) for both oxidase positive and negative organisms. Subsequent culture detection tests were carried out as per referred biochemical test algorithms (Fig. 17). Oxidase positive Gram negative diplococci (GNDC) bacteria were tested with gamma-glutamyl-trans-peptidase (GGT) reagent (Rosco Diagnostica, Denmark) based on methods described on the standard microbiology lab guideline (WHO, 2011; MenAfriCar Consortium, 2013) to distinguish *N. meningitidis* from other oxidase positive Neisseria species. Briefly a loopful of 18 to 24 hr. culture growth of oxidase positive GNDC organisms were suspended in 250 µl of normal saline and the suspension was incubated with GGT enzyme substrate at 37 °C for 4 hrs before observation for the indicative orange color.

Fig. 17: Flow chart showing CSF sample inoculation into culture media plates and further biochemical tests as in WHO (2011)

Further biochemical tests such as tributrin and Ortho-Nitrophenyl-Galactopyranoside (ONPG) (Fig. 18) were run for ruling out *N. meningitidis* from *Moraxicella catarallis and N. lactamica* based on methods for identifying *N. meningitidis* as per general microbiology test guideline (Basta *et al.,* 2013). Rapid Tributyrin (Remel, USA) test was run on the oxidase positive, Gram-negative diplococci organisms. Each tube for the test was inoculated aerobically with a 3 mm loopful of the test isolate from a pure, 18-24 hour culture, and mixed thoroughly.

The isolate inoculated into the culture tube containing the test reagent was incubated at 33-37°C in an incubator for 2-4 hours. Further incubation was done up to 24 hours when tests were found non-reactive. After the incubation period the contents were visually inspected and yellow color development was interpreted as a positive test while absence of colour change was interpreted as negative test for *Moraxella catarrhalis. Moraxella catarrhalis* (ATCC 25238) was used for positive control and *Neisseria lactamica* (ATCC 23970) was used for negative control. The Ortho-Nitrophenyl-β-galactopyranoside (ONPG) (Thermo scientific, UK) biochemical test (based on hydrolysis of ONPG molecule into galactose and ortho-nitrophenol in the presence of β-galactosidase) was run for the oxidase positive Gram negative diplococci organisms to differentiate *N. meningitidis* (which is negative for ONPG) from other Neisseria species such as *N. lactamica* (which is positive for ONPG). Ortho-Nitrophenyl-β-(beta)-galactopyranoside (ONPG) is normally colorless but in the presence of β-galactosidase it hydrolyzes the ONPG molecule into a yellow color that can be used to check for enzymatic activity by means of colorimetric assay (at 420 nm wavelength). β-galactosidase is required for lactose utilization, so the intensity of the color produced can be used as a measure of the enzymatic rate. Briefly, one ONPG test disc was placed into a sterile tube and 0.1 ml of sterile 0.85% sodium chloride was added. The colony under test was picked with a sterile loop and emulsified in the tube containing the disc and 0.85% sodium chloride. The content in the tube was incubated at 35°C. After 6-24 hours of incubation visual inspection was done and yellow colouration was interpreted as a

positive ONPG test while colourless response was interpreted as negative ONPG test.*Neisseria lactamica* was used for positive control and *N. meningitidis* was used for negative control. Bacteria found to be GGT positive, tributrine and ONPG negative were classified as *N. meningitidis.*

Serogrouping of *N. meningitidis* was carried out with seroagglutination test based on methods on the standard microbiology lab guideline (WHO, 2011). Oxidase negative, GPDC organisms were tested with optochin (Oxoid UK) antibiotic susceptibility test based on methods described in the standard microbiology lab guideline (WHO, 2011) to determine whether or not the organism under investigation was *S. pneumoniae.* Optochin (Ethyl hydrocupreine hydrochloride) is a quinine derivative that can be used to differentiate *S. pneumococci* from *Streptococci viridians* (WHO, 2011) with sensitivity greater than 95%.

To run optochin antibiotic susceptibility test (Fig. 18), suspected colonies were inoculated in blood agar media after preparing 0.85% diluted culture lysate by mixing the loop full of the culture lysate with 0.9% normal saline to the level of McFaraland standard. Optochin antibiotic discs were mounted on the diluted culture lysate inoculated into blood agar medium.

The media was incubated at 37°C, 5% CO_2 supplied incubator alternatively at 12 or 24 or 72 growth hours. When growth inhibition of the inoculated organism was observed (in > 14 mm around 6 mm disk) at the zone of the antibiotic disc, the organism was identified as *S. pneumoniae*.

Fig. 18: Flow chart showing biochemical test algorithms run in the study as in WHO lab guideline, (2011) and MenAfricar consortium, (2013)

On the other hand, oxidase negative, Gram negative short rods were tested with XV (X + V, Fig. 18) (Oxoid UK) nutritional factors based on methods on the standard microbiology lab guideline (WHO, 2011) for testing growth response of *H. influenzae* (indicative of presence of *H. influenzae*) at the XV zone of nutritional factor (satellite colonies) (WHO, 2011).

5.6.2 Molecular Methods

I. Genomic DNA extraction

Genomic DNA was extracted after thawing the frozen CSF in TIM preserved at -20°C using Qiagen spin column (Qiagen Hilden, Germany) DNA extraction kit based on the company's instruction. Briefly, 200 µl of the CSF in TIM was added into 1.5 ml micro-centrifuge tube in which 20 µl proteinase K (Qiagen Biotechnology, Germany) was added for general digestion of protein. After thorough mixing, 200 µl of buffer AL (Lysis buffer, composing Tris-HCl/EDTA salts and Sodium Dodecyl Sulphate) was added, vortexed for 15 seconds, incubated consecutively at 56 °C for 10 minutes, 95° C for 5 minutes and briefly centrifuged at 3000 rpm for 1 minute. Twenty µl of 96% ethanol was added to the mixture, pulse vortexed for 15 seconds and briefly centrifuged at 3000 rpm for 1 minute. The mixture (a total of 440 µl) was carefully transferred to Qiamp spin column, placed within 2 ml collection tube and centrifuged twice at 8000 rpm for 1 minute. The mini spin column was transferred to a fresh 2 ml collection tube after adding 500 µl buffer AW1 (Wash Buffer) used to denature the proteins so that they can pass through the filter. It was then centrifuged twice at 8000 rpm for 1 minute. The filtrate was discarded after every centrifugation. Five hundred µl of buffer AW2 (324 ml wash buffer

composed of 70% ethanol) was added into the mini-spin column to wash the salts out. The content was centrifuged at 14,000 rpm twice for 3 minutes. Finally fifty µl of buffer AE (elution buffer/10 mM Tris-Cl. 0.5 mM EDTA at pH 9.0) was added to the mini spin column to allow the DNA removed/eluted successfully from the silica membrane. The content was centrifuged at 8000 rpm for 1 minute and incubated at room temperature (15-25°C). The DNA was preserved at -20° C until needed for PCR amplification.

II. Conventional Multiplex PCR

Conventional multiplex PCR was run for all the 139 DNA samples using PorA, LytA and BexA gene primers (Qiagen Biotechnology, Germany) of *N. meningitidis, S. pneumoniae* and *H. influenzae* respectively. The PCR master mix was prepared so as to have a final total volume of 20 µl of the mix per PCR tube. To prepare the master-mix, each of the PCR mix constituent for individual PCR tube was multiplied with the number of samples, positive and negative controls with an additional 2 µl of extra volume of each component to adjust pipetting errors. Each PCR tube contained 12.5 µl of 100 Pm/µl Taq DNA polymerase enzyme (hot start), 0.1 µl of 100 Pm/µl of PorA forward and reverse primers each, 0.1 µl of 100 Pm/µl LytA forward and reverse primers each, 0.1 µl of 100 Pm/µl of BexA forward and reverse primers (Appendix I) each and 6.9 µl of PCR grade water. The PCR master mix was pulse vortexed gently 5 to 6 times. Twenty µl of the PCR master mix was aliquoted into each labeled PCR tube in the PCR mix room within biosafety cabinet level 2. Five µl of each genomic DNA sample (CSF in TIM), positive controls from the DNAs of *N. meningitidis, S. pneumoniae* and *H. influenzae* extracted from American Type Culture Collection (ATCC) laboratory strain number 35562, 49619,

35056 of the respective organisms and negative control (PCR grade water processed together with samples during DNA extraction) were added into the corresponding PCR tubes in a separate room. The conventional PCR multiplex reaction for amplifying Por A, LytA and Bex A genes was initiated by activation of hot start Taq polymerase at 95°C for 5 min. This was followed by 94°C of denaturation for 3 min in 35 cycles. Denaturation was continued at 94°C for 40 seconds followed by 58°C of hybridization for 30 seconds, and extension at 72°C for 30 seconds. Final extension was carried out at 72°C for 10 minutes, followed by a 4°C hold temperature. Another conventional multiplex PCR amplification was carried out for capsule genogrouping to identify *N. meningitidis* genogroups from samples whose Por A genes were amplified and visualized on agarose gels. Genogroups A, W-135 and Y were the focus of interest for genogrouping with conventional PCR by using the respective primer pairs of SacB, MynB and SiaD genes of *N. meningitidis* genogroup A, W-135 and Y (Appendix II). Genogroup determination of amplifying W-135 and A was carried out in conventional multiplex PCR by preparing PCR master mix calculated to have a volume of 27 µl PCR mix per individual PCR tube. The master mix was prepared by mixing 0.5 µl of 10 pmol/µl of each forward and reverse primer, 6 µl of fire Pol Solis Biodyne mix and 18µl of PCR grade water per PCR reaction tube. After aliquoting 27 µl of the mix into each PCR reaction tube in the PCR mix room, three µl of patient sample DNA, positive controls DNA and negative control (PCR grade water processed together with samples DNA extraction) each was added into the respective labeled PCR reaction tube in a separate room. The PCR program used for conventional multiplex PCR genogroup determination was 95°C denaturation of 3

minutes continued with 35 cycles of annealing temperature having individual heating step of 95° C for 30 minutes, 55° C for 30 minutes and 72° C for 1 minute. A 72°C extension was continued for 10 minutes followed by a final hold temperature of 4°C. Amplification of SiaD gene of genogroup Y was run in a separate PCR since the molecular Wt. of the PCR product of SaiD gene is similar (Appendix II) with mynB gene of genogroup W-135. Specified primer pairs for genogroup Y amplification were added into 27 µl of PCR mix prepared with similar volume of constituents with the other genogroups to amplify *N.meningitidi*s genogroup Y with similar PCR program used for amplifying the other genogroups.

III. Agarose gel electrophoresis

Agarose gel electrophoresis was run for PCR amplified products to visualize the respective bands and determine their molecular wt. size using 100 bp reference molecular weight markers. To prepare the 1.5% agarose gel, briefly 0.75 gram of agarose was added to 50 ml of 1x TAE (Tris acetate Ethylene-diamine-tetra acetic-acid) buffer. The agarose solution was melted using microwave oven for 3 min at 900 watts. When the agarose solution was cooled to about 50°C, 4 µl of ethidium bromide (10mg/ml) was added into the solution. The agarose solution was directly poured into gel casting tray with well forming comb and allowed to polymerize within 20 to 30 minutes. The polymerized gel in the casting tray was submerged into 250 ml of 1X TAE buffer, covered with the buffer. The comb was removed carefully by lifting it gently at one end. Six to eight µl of 100 bp DNA ladder, PCR products of sample DNAs, positive and negative controls each mixed

with 2 µl of loading dye (Bromophenole blue) were loaded separately into individual wells. The tank was connected with cover attached to electrodes. Power supply was turned on to initiate the gel electrophoresis by adjusting the power to 150 volts. Gel electrophoresis was allowed to run for 45 minutes from negative to positive polar charges. Presence of bands on the gel was checked through visualizing the gel with gel doc system (BioRAD, USA) supplied with a UV light, from which gel picture was taken for documentation of results.

IV. Real time PCR

Real time Multiplex PCR was run based on real-time-PCR application guideline (RT-PCR application guideline, 2006) for all the 139 DNA samples using ctrA, lytA and omp genes of *N. meningitidis, S. pneumoniae* and *H. influenzae,* respectively. Master mix was prepared in a dedicated PCR mix room by mixing 10 µl of 2x Taqman fast Universal PCR master mix with 0.6 µl of 10 µM ctrA-F and ctrA-R primers of *N. meningitidis,* 0.8 µl of 5 µM Ned/Atto550 probe omp, 0.4 µL of 10 µM F373 and R424 primers (Appendix III) of *S. pneumoniae,* 0.8 µl of 5 µM Fame probe ctrA-P, 0.4 µl of 10 µM omp P2-F and omp P2-R primers of *H. influenzae,* 0.8 µl of 5 µM Cy5 probe pb400, 2 µl of Vic 10 x Exeo-internal positive control (IPC) mix, 0.4 µl of 50x Exeo IPC DNA and 0.4 µl nuclease free water. Eighteen µl of the prepared master mix was aliquoted into wells of 96 well plates, based on pre-planned plate layout. Dedicated wells of samples (as per sample ID), positive and negative controls were labelled on the 7500 fast system computer software provided plate layout. The PCR plate was sealed with a sterile one

time use plate sealer and moved from PCR mix room to DNA mixing room where 2 µl of 1:25 diluted genomic DNA of each patient sample, positive and negative controls were added into dedicated wells in duplicates. Sterile, PCR grade water processed together with patient samples DNA extraction was used for negative control. The plate was then sealed again with fresh plate sealer and centrifuged at 1000 x g for 1 minute and placed into ABI 7500 Fast real-time PCR (Applied Biosystems, Foster City, CA, USA) system pre-warmed 30 minutes prior to loading the PCR plate. The PCR program of 45 cycles was run through 95°C denaturation for 20 minutes, 60°C annealing for 30 minutes and 60 °C extension for 30 minutes, followed by cooling at 4°C. Samples were interpreted as positive when Real-time PCR reactions showed a cycle threshold (Ct) value of < 40. If Ct was >35, the sample was retested. Samples positive for ctrA gene of *N. meningitidis* were subjected to capsule genogrouping in single real-time PCR genogroup identification of *N.meningitidis* genogroup A, B, C, Y, W-135 and X after preparing PCR master mixes independently for each genogroup by using their specific primer pairs as well as probes (Appendix IV). Master-mix to amplify genogroup A was prepared by mixing 10 µl of 2x Taqman fast Universal PCR master mix, 1.8 µl of 10 µM F2531 and R2624 primers, 0.8 µl of 5 µM PB839i Fam probe and 3.6 µl of nuclease free water. Master-mix to amplify *N. meningitidis* genogroup B was prepared by mixing 10 µl of 2x Taqman fast Universal PCR master mix with 0.6 µl of 10 µM F737 and R882 primers, 0.4 µl of 5 µM Pb839i Fam probe and 6.4 µl of nuclease free water. Master-mix to amplify *N. meningitidis* genogroup C was prepared by mixing 10 µl of 2x Taqman fast universal PCR master-mix with 1.8 µl of 10 µM F478, 0.6 µl of 10 µM R551 primers, 0.4 µl of 5 µM Pb495 Fam

probe and 5.2 µl of nuclease free water. Master-mix to amplify *N. meningitidis* genogroup W135 was prepared by mixing 10 µl of 2x TaqMan fast Universal PCR master mix with 0.2 µl of 10 µM F857 primer, 1.8 µl of 10 µM R964 primer, 0.8 µl of 5 µM Pb907i Fam probe and 5.2 µl nuclease free water. Master-mix to amplify *N. meningitidis* genogroup X was prepared by mixing 10 µl of 2x Taqman fast Universal PCR master mix with 1.8 µl of 10 µM F173 primer, 1.8 µl of 10 µM R237 primer, 0.4 µl of 5µM Pb196 Fam probe and 4.0 µl of nuclease free water. Master-mix to amplify *N. meningitidis* genogroup Y was prepared by mixing 10 µl of 2 x Taqman fast Universal PCR master-mix with 1.8 µl of 10 µM F787 primer, 1.2 µl of 10 µM R929 primer, 0.4 µl of 5 µM Pb1099i Fam probe and 4.6 µl of nuclease free water. Eighteen µl of each master mix prepared for the required volume of specimen for amplifying *N. meningitidis* genogroups A, B, C, W-135, and Y and X were aliquoted into dedicated wells based on pre-planned plate layout each time. Plate for testing each genogroup was sealed with sterile onetime use plate sealer and moved to DNA mixing room to add 2 µl of 1:25 diluted genomic DNA of each patient sample, positive and negative controls. The plates were centrifuged each time at 1000 x g for 1 minute and placed into 7500 Fast system SDS software RT-PCR machine (Applied Biosystems, guideline Norway, 2004) pre-warmed 30 minutes prior to loading each PCR plate at each time to amplify each genogroup. The PCR was programmed as 95°C denaturation for 20 minutes, 60°C annealing for 30 minutes and 60 °C extension for 30 minutes. The three stages were processed for 45 cycles followed by cooling at 4°C of final temperature. Interpretations of the results of real-time PCR were processed as follows. Non-template controls (NTCs) and negative controls should

produce amplification curves that are straight lines near zero. The amplification plots of the positive controls and any unknown specimens that generate curves should all be sigmoidal. The commercial master mix used contain ROX, a reference dye, BEX an internal positive control (IPC) dye and HEX, sample dye. Proper functionality of the real-time PCR test was confirmed through assuring that the amplification plot and plate sample value for ROX in each reaction resembled those of the none template and negative controls. Interpretations of results were carried out if these criteria were met. Determining positive or negative results for the presence or absence of the etiologic agents of interest were done by following cutoff values used at Meningitis Laboratory at Centers for Disease Control (CDC). Positive samples are those with cycle threshold (Ct) $\leq$ 36, samples with Ct > 36 to 40 were considered to be equivocal and were retested after diluting the template DNA in PCR-grade water to reduce any inhibitors that may be interfering with the reaction. Samples that remain to have Ct $\geq$ 40 were interpreted as negative samples. The amplification plots were analyzed when they were smooth and sigmoidal in shape. If plots were lacking these characteristics, the samples were considered for retesting. Genes of *N. meningitidis* genogroup A, B, C, W-135, X and Y namely sacB, synD, synE, synG, xcbB and synF, respectively, were targeted from α-N acetyl regions of the capsular polysaccharide of the organism.

V. Multi-Locus-Sequence Typing (MLST)

Multi-locus sequence typing (MLST), a test using a set of housekeeping genes was run to characterize strains of organisms. Tests were performed at Norwegian institute of Public Health (NIPH/Oslo/Norway) lab after isolated strains were sent through using signed material transfer agreement MTA. Genomic DNA extracted from pure *N. meningitidis* and *S. pneumoniae* isolates were used to perform multi locus sequence typing (MLST) as previously described (Enright and Spratt, 1998; Maiden *et al.,* 1998). *Haemophilus influenzae* was not considered for this procedure as it was merely one isolate which was detected during RT-PCR. Real Time PCR confirmed positive live bacterial strains of *N.meningitidis* and *S. pneumoniae* were seeded into culture media plates, and DNA was extracted from the growing bacteria. Multilocus sequence typing was done to check variation between strains at variable number of genes depending on desired degree of discrimination. Extracts of DNA were used for PCR amplifications and MLST with seven housekeeping gene primers of *N. meningitidis* (Appendix V and VI) as well as *S.pneumoniae*. Each of the seven genes abcZ, adk, aroE, gdh, pdhC, pgm, and fumC were amplified by PCR. Identification of the 2 variable region peptide loops of the porA protein was carried out by sequencing the porA gene. Multilocus sequence typing and ferric enterobactin transport genotyping were also performed. The MLST PCR amplification reaction set-up for *N. meningitidis* had a total volume of 49 µl of the mix and 1 µl of the template DNA. To prepare a total volume of 49 µl master mix, 41.5 µl of PCR grade water was mixed with 5.0 µl of 10 x buffer, 1 µl of 10 mM dNTPs (with a final concentration of 200 µM), 1µL of 20 µM forward primer as well as reverse primer

each with a final conc. of 0.4 μM and 0.5 μl of DNA polymerase. One μl of template DNA was then added to the individual reaction mix. The PCR cyclic conditions for *N.meningitidis* MLST amplification and sequencing using the referred forward and reverse primer pairs (Appendix VII) (except for pgm) were as follows: A 1 x initial 94°C denaturation of 4 minutes followed by 94°C denaturation of 1 minute, 55°C annealing of 1 minute and 72°C extension of 1 minute which were repeated in 35 cycles. A 1 x 5 minutes 72°C extension was then followed by 4°C holding temperature. The PCR cycling conditions for pgm gene of *N. meningitidis* MLST amplification was initiated with a 1 x 4 minutes denaturation with 95°C. This was followed by 94°C denaturation for 1 minute, 65°C annealing for 1 minute and 72°C extension for 1 minute which were repeated for 35 cycles. A 1 x 72°C extension for 5 minutes was then run and followed by 4°C holding. The last two steps were repeated once more by altering the 5 minutes to 2 minutes for the last step. The MLST PCR amplification reaction set-up using the forward and reverse primers for *S. pneumoniae* (Appendix VIII and IX) was as follows. To prepare a total volume of 25 μl master mix, 18.5 μl of PCR grade water was mixed with 2.5 μl of 10 x buffer, 0.5 μl of 10 mM dNTPs with a final conc. of 200 μM, 0.5 μL of 20 μM forward primer as well as reverse primer each with a final conc. of 0.2 μM, 0.5 μl of DNA polymerase. Template DNA was finally added to the individual reaction mix at a volume of 2.5 μl. Once the PCR reaction mixes were set-up, the PCR tubes were immediately placed in the PCR machine and the PCR program was run. The PCR cycling conditions for *S. pneumoniae* MLST amplifications was initiated with a 1 x 94°C denaturation of 5

minutes. The 94°C denaturation was continued for 15 seconds followed by a 54°C annealing for 30 seconds, 72°C extension for 45 seconds which was all repeated for 10 cycles through adding 10 seconds to the extension. This step was repeated again for 20 cycles. Another step with a 1 x 72°C extension was then run for 10 minutes followed by 4°C hold temperature. Prior to running reaction clean-up and sequencing, which was done through gel filtration columns (a solid phase reversible immobilization magnetic bead based system), the success of the PCR amplification was checked through running agarose gel electrophoresis. Sequencing of the PCR products was performed on both strands using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (ABI Prism 377 instrument Applied Biosystems, Foster City, CA) according to the manufacturer's instructions using 5% Long Ranger gels (FMC Bioproducts, Rockland, ME). The set of sequencing primers used for *N. meningitidis* were different from those used for PCR amplification of the seven housekeeping genes, while the same first set of seven primers which were used for PCR amplification of the house keeping genes of *S.pneumoniae* were used for sequencing. Alleles and STs were assigned, as described previously by querying the Neisseria MLST database as shown in Norheim *et al.* (2006). The sequences of internal fragments used were of a length to provide sufficient variation for identification of different alleles. The DNA sequence variations were directly measured by the method in the set of house-keeping genes. The characterized strains were then measured by their unique allelic profiles. Ferric enterobactin transport (FetA) genotyping was performed by sequencing the variable region (VR) in FetA. The respective sequence data was searched against the PubMLST database at

http://pubmlst.org Bio-informatics techniques to arrange, manage, analyze and merge all the huge sets of biological data produced during sequencing and identification processes of MLST. Multi-locus sequences were analyzed and allele numbers were given after collection of the data generated. Sequences differing even with a single nucleotide level were assigned as different alleles and determined using the seven house-keeping genes of different sequences. The data composing alleles at a loci was analyzed, numbered and combined into an allelic profile to whom, sequence type (ST) was assigned. In the final section of processing MLST relatedness between isolates, the strains were structured through comparing allelic profiles.

VI. DNA quantification for *N. meningitidis*

Meningococcal DNA detection and quantification was performed on CSF and serum samples using the Taqman PCR (Perkins-Elmer Applied Biosystems 7700 automated PCR platform, Norwalk, Connecticut) as in Hackett *et al.* (2002). The Taqman assay is able to detect and quantify meningococcal DNA from fluid samples of patients RT-PCR diagnosed positive for the bacteria. Sufficient serum volumes were available from 23 meningococcal patients to enable quantification of the copy number of meningococcal DNA. Five sera from patients with pneumococcal meningitis were used as negative controls. Briefly capsular gene (ctrA) common to all *N. meningitidis* serogroups was amplified using the respective primers and probes (Appendix III). The Real time PCR detection of the ctrA gene (4-5 hours) provides quantification of bacterial load residing per ml of sample. The quantification of DNA for all patient samples confirmed to be positive for *N. meningitidis* was performed on a single plate using the same standards.

Intra-assay variability was detected to be minimal. The bacterial load is expressed as genome copy number per milliliter of CSF or serum. As each bacterium contains only a single capsular gene, DNA load equates to bacterial load. The copy number of *N.meningitidis* DNA was determined through using quantitative PCR. Sequence specific hybridization probes (0.3 μM per reaction) and Light Cycler Fast Start DNA master hybridization probe mix (Roche, Germany) were used in the detection system with a detection limit of 2.5 $x10^2$ per Ml.

5.6.3 Immunodiagnostic techniques

I. Determining levels of inflammatory mediators in cerebrospinal fluid

Cerebrospinal fluid preserved at -20° C was thawed on ice and measurements of inflammatory mediators concentration (in pg/ml) levels were performed for 43 patients (27 with meningococcal meningitis and 16 out of 18 (having sufficient samples) with pneumococcal meningitis. The levels were quantified by Bio-Plex Pro Cytokine, Chemokine, and Growth Factor 27-plex assay (Bio-Rad, Hercules, CA) for the following 18 cytokines and chemokines. Interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-18, granulocyte colony stimulating factor (G-CSF), interferon-γ (INF-γ), INF-γ-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory proteins 1 alpha (MIP-1α), macrophage inflammatory proteins 1β (MIP-1β), regulated on activation normal T cells expressed and secreted (RANTES), tumor necrosis factor (TNF) and TNF related apoptosis inducing ligand (TRAIL). All thawed samples were vortexed and spun down at 10000 x g for 10 min at 4°C. All CSF samples were diluted in sample diluent prior to analysis in 1/4 and

1/50 ratio. Further dilution to 1/100 and 1/1000 were needed for some samples for measuring levels of IL-6, IL-8, IP-10 and MIP-1α. Bio-Plex Pro cytokine array system is a novel technology designed to run an assay of multiplex magnetic bead-based array system to quantitate multiple cytokines from fluid samples e.g., serum, CSF. The assay composes fluorescently dyed microspheres (beads) to which bio-molecules are tightly attached with. The color-coded beads permit simultaneous detection of 27 cytokines or more in a single well of a 96-well plate (Bio-Rad, Hercules, and CA). The color-coded beads are 5.6 μm polystyrenes to which antibody specifically directed against the cytokine of interest is covalently coupled. In addition to the colour-coded magnetic beads the immunoassay makes use of detection antibodies, standards, assay buffer, wash buffer, sample diluents, standard diluents, detection antibody diluents, streptavidin-PE, a flat bottom plate and sealing tape. The advantage of using this assay over other immune-assays in detection of the inflammatory mediators is that it enables quantification of multiple protein biomarkers in a single well of a 96-well plate in 3-4 hours. The total volume of diluted CSF used for each patient for this assay was 50 μl. The assays were performed based on manufacturer's instructions making use of StatLIA immunoassay technology software package. The Bio-Plex Pro cytokine analysis software incorporates a weighted five-parameter logistic curve-fitting method used to calculate sample cytokine and chemokine concentrations (ver. 3.2; Brendan Scientific, Inc.). A flow cytometer apparatus equipped with two lasers and associated optics are also used in the system to measure biochemical reactions that occur on the surface of the microsphere beads.

Fluorescent outputs discriminately express the read out of analytes through coloured peaks by a high-speed digital signal processor.

Matrix metallopeptidase-9 (MMP-9) in CSF was quantified by Bio-Plex Pro Human MMP Assay according to the manufacturer's instruction (Bio-Rad, Hercules, CA). Briefly, 50 µl of 1x diluted, vortexed beads were added to pre-wet (with 100 µl Bio-Plex assay buffer) filter plate. The plate was washed twice with 100 µl Bio-Plex wash buffer. Patient samples, standards, diluents (for blank well) and control were vortexed and 50 µl of each specimen was aliquoted to the respective well according to the pre-planned plate layout. The plate was covered with sealing tape and protected from light with aluminum foil and incubated on a shaker at 1000 g at RT for 1 hr. Detection antibodies were vortexed for 15 seconds and quick-spinned while the plate was kept in incubation for 10 minutes. Detection antibodies were used at a onetime dilution. The plate was washed thrice with 100 µl wash buffer. One time diluted and vortexed twenty five µl detection antibody was then added to each well. It was again covered and incubated on a shaker at 1000 g, as described above, in the dark for 30 minutes at room temperature. Bio-Plex manager software protocol was used through the standard values and units provided in the assay kit. One hundred times streptavidin-phycoerythrin (SA-PE) was then vortexed for 5 seconds and quick-spun 10 minutes before incubation period. After plate was washed thrice with 100 µl wash buffer, 50 µl of one time diluted and vortexed SA-PE was added to each well. The plate was again sealed and incubated on a shaker at 1000 g, as described above, in the dark for 30 minutes at RT. The beads were resuspended in 125 µl assay buffer after the plate was washed three times with 100 µl wash buffer. The plate was then

sealed with a tape and placed on a shaker at 1000 g for 30 seconds. The fluorescent signal in the sample was determined using a BioRad analyzer at excitation/emission wavelengths of 340 nm/465 nm.

II. Limulus amebocyte lysate (LAL) assay

Lymulus ameobocyte lysate is endotoxin activity measuring assay that can be used for quantification of levels of endotoxin i.e. lipopolysaccharide (LPS) for meningococci circulating in the CSF or plasma. Studies show that levels of LPS in the CSF and plasma are quantitatively closely associated with inflammatory mediators, clinical symptoms, and outcome (Bjerre *et al.,* 2000). The levels of LPS were determined with Chromo-LAL® (Associates of Cape Cod, Inc, East Falmouth, MA) according to the manufacturer's instruction through Limulus Amebocyte Lysate (LAL) gelatin test resulting with complete clotting of the lysate which is indicative of a positive reaction for presence of bacterial LPS (Young *et al.,* 1972). The lipid-A part of endotoxin molecule can activate the gelation of the limulus lysate extracted from blood of horse shoe crab (Young *et al.,* 1972). Fifty microliter of the standard sample was dispensed into the appropriate endotoxin-free reaction tube in a 37°C ± 1°C block or water bath. A blank tube and four endotoxin standards were run in duplicate for each assay to determine patient sample LPS level. The blank tubes contain 50 µl of LAL reagent and distilled water instead of sample. At time T = 0, fifty µl of LAL was added into each reaction tube. Limulus amoebocyte lysate timing is begun as it is added to the first reaction vessel. The mixture was thoroughly mixed without vortexing. At time T=10 minutes, hundred microliter of substrate solution (pre-warmed to 37°C ± 1°C) was then added into each reaction tube

and mixed throughly. Hundred microliter of stopping reagent was added at T = 16 minutes and thoroughly mixed. Absorbance of each reaction tube was read at 405–410 nm using distilled water to adjust the photometer to zero absorbance. The endotoxin activity was visualized using Bromothymol blue. Unspecified activity of endotoxin was compared to the *E.coli* standard endotoxin (Bjerre *et al.*, 2000). The test was run not only for samples tested positive for *N. meningitidis* with biochemical test but also for those samples which tested positive for the referred bacteria through Real Time PCR detection method.

5.7 Data Analysis

Data on clinical diagnosis based on patient symptoms, patient socio-demographic profiles and outcome from the disease were recorded on pre-planned and tested case record form (CRF). The data from CRF were entered into SPSS statistical software program. Data from bacteriological/biochemical/culture detection, molecular and immunological laboratory diagnosis of patient samples were recorded in laboratory log book and computer software were entered into a separate SPSS (SPSS Inc., Chicago, IL) statistical data base. The data were then merged and comparison between groups of patients and different results were carried out based on clinical parameters per site, species or serogroup by the Pearson's chi square test (asymptotic) or the Fisher's exact test (if the number of cases in each of the cross-tabulation table are less than 5) using SPSS. Real time PCR detected *S. pneumoniae* and *N. meningitidis* positive samples were used for statistical analysis in association with the rest of the data generated in this study. Spearman non-parametric Mann-Whitney U-test was used for comparison of medians of

numerically reported clinical parameters between patients with meningococcal or pneumococcal disease as confirmed by RT-PCR. Since the cytokine and chemokine levels did not have normal distribution, the data was analyzed using non-parametric Kruskal Walis test. Difference in the median level of cytokines and chemokines were compared with RT-PCR positive result of *S. pneumoniae* and *N. meningitis* patient samples in reference with negative controls. Correlation was constructed by r language gg plot 2 packages. This statistical software program is preferred since it is highly recommended for analyzing non-parametric data. Presence or absence of statistical significances between the parameters compared were checked. Analyzed data were represented by box and scatter plots.

5.8 Ethical Consideration

This study was reviewed by both institutional and national ethical review committees and ethical clearance was obtained from Ethiopian National Ethics Review Committee (NERC) as well as all the national and International institutions involved in this research project. The three University hospitals/study sites (Gondar, Hawassa and Tikur Anbessa) were communicated to recruit consented/assented patients who were clinically diagnosed with BM. Patients requested to participate in this study have given written and signed informed consent or assent prior to enrollment into the study. Prior to the request for enrollment detailed explanation was given to each patient or patient parent or guardian (when the patient is under 18 or unconscious) by using pre-planned patient information sheet. Further explanation was also given to patient or parent/guardian using patient's ethnic language. In case of participant disinterest to continue his/her enrollment in the

study, possibility of withdrawal from the study including discarding of biological samples as well as any patient clinical data were explained clearly. Clear explanation was also given assuring that being willing or unwilling to participate in the study or withdrawal from the study after enrollment does not affect the routine clinical diagnosis and patient care service. All the pros and cons that the patient may face during enrollment into the study were explained to the patient or patient guardian. Possible presence of risk during CSF collection through the routine LP procedure was explained to the patient or guardian. The fact about CSF collection procedure is done merely for the sake of patient clinical diagnosis purpose was explained clearly and understood by the participants/guardians. The risk during LP for CSF collection for routine patient care diagnostic purpose is some discomfort that the patient practices through introducing infection by the needle going through the skin and not using sterile needle, or headache after the procedure. All routine aseptic measures were however taken prior to introducing needle to the patient and each time sterile needle was used. The risks however have nothing to do with participation in this study as CSF uptake is a routine procedure practiced for patient diagnosis and care during suspicion for meningitis. Hence, the patient was not subjected to extra sample collection for the sake of this study since it was only the residual leftover sample (from the volume collected for routine clinical diagnosis), which was used for the study. If anyhow the patient came across LP procedure associated risk, proper patient care would soon be given by the attending physician. This study is supervised by advisors from Addis Ababa University (AAU), Armauer Hansen Research Institute/AHRI (host institute), and external partners (Norwegian Institute of Public Health/NIPH/Oslo).

6. RESULTS

6.1 Socio-demographic and clinical characteristics

Patient enrolment into this study was based on consecutive patient flow into the site University hospitals. The enrolled study participants were 165 patients and 26 patients were excluded from the study due to absence of completed CRF. Hence a total of 139 residual CSF and sera left over from routine patient care clinical lab diagnosis were used for this study. The enrolled 139 study participants were composing 92 (66.2%) from Gondar University Hospital (GUH), 27 (19.4%) from Hawassa University hospital (HUH), and 20 (14.4%) from Tikur Anbessa University hospital (TAUH). Frequency of these clinically diagnosed BM patients who visited the site hospitals during non-epidemic seasons, based on signs and symptoms are showed in Fig 19. The highest number (n = 27) of patients were diagnosed in April, 2013 followed by March, 2013 (n = 15), while the lowest frequency of patients (n = 2) occurred in January 2013. The bars in Fig. 19 are not based on laboratory diagnostic test results. The youngest enrolled patient was 2 days old while the oldest was 78 years old.

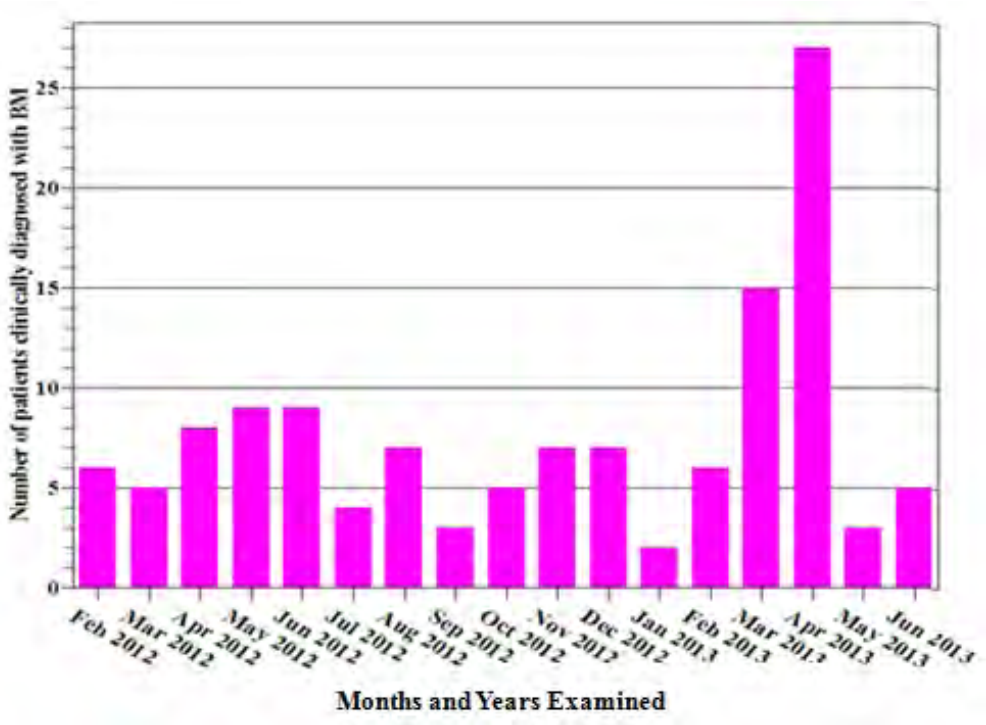


Fig. 19: Number of cases clinically diagnosed as bacterial meningitis from Hawassa, Gondar and Tikur Anbessa University hospitals during non-epidemic seasons of 2012-2013

Forty eight out of the 139 (34.5%) patients were in the age category of ≤ 4 while 26/139 (18.7%) were in the age category of 5-12 altogether composing more than 50% of the total study population (Table 4). Age groups were categorized in such a way that frequency of etiologies and levels of disease severity could be clearly observed in the most affected age groups i.e. the ≤ 4 and 5-12. Among all the clinical manifestations and general clinical conditions (Fig. 20-23), age group ≤ 4 years of age, followed by 5-12 were those who appeared with high frequencies as compared with the remaining age categories. Impaired consciousness was more frequent in younger children ($P < 0.05$). Statistical analysis of patients' general clinical condition was carried out only for 117

patients since data for the remaining 22 patients were left unrecorded during CRF filling at the study site hospitals.

Table 4: Age category of study participants clinically diagnosed with bacterial meningitis at the three University hospitals, 2012-2013

Age Category in yrs.	TAUH	GUH	HUH	Total	Percentage
≤ 4	14	27	7	48	34.5
5-12	5	15	6	26	18.7
13-19	1	6	6	13	9.4
20-29	0	16	4	20	14.4
30-39	0	11	1	12	8.6
≥ 40	0	17	3	20	14.4
Total	20	92	27	139	100

Other clinical conditions such as seizure, stiff neck and back rigidity have also been reported (data not shown in figures). Forty four out of the 138 (31.9%) patients experienced seizure of which 22 (50%) were in the ≤ 4 yrs age categories. Stiff neck and back rigidity were reported by 72/136 (52.9%) patients. Eighteen out of the 72 (25%) were in the age range of 5-12 yrs. Fever and impaired consciousness are statistically significant across the different age groups of patients ($P < 0.05$, Chi-square). Such overall statistical significance was, however, not apparent among the different age groups of patients, who experience vomiting ($P > 0.05$, Fig. 20) and the range of general clinical conditions (Fig. 23, $P > 0.05$). Of all the general clinical conditions observed from the patients diagnosed with BM, “acutely sick looking, irritable and lethargic” was prominent

among all the age groups of patients.

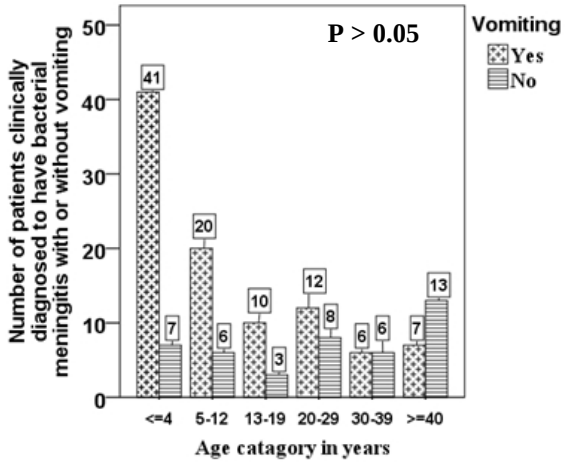


Fig. 20: Frequency of different age groups of patients at Hawassa, Gondar and Tikur Anbessa University Hospitals, clinically diagnosed as bacterial meningitis, with and without vomiting; 2012-2013

P < 0.05

Fig. 21: Frequency of different age groups of patients at Hawassa, Gondar and Tikur Anbessa University Hospitals clinically diagnosed as bacterial meningitis with and without fever, 2012-2013

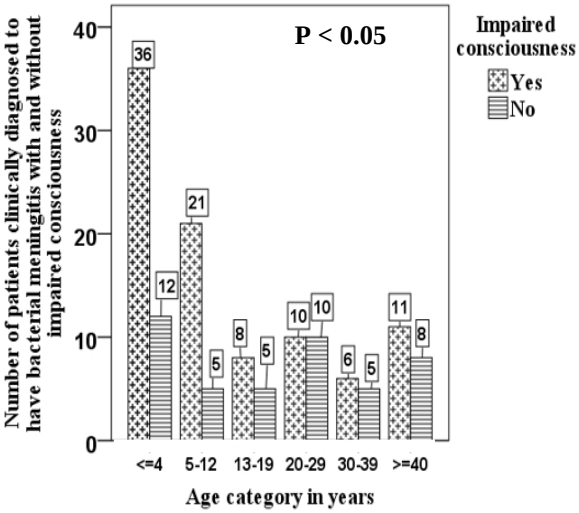


Fig. 22: Frequency of different age groups of patients clinically diagnosed as BM with and without impaired consciousness at Hawassa, Gondar and Tikur Anbessa University Hospitals, 2012-2013

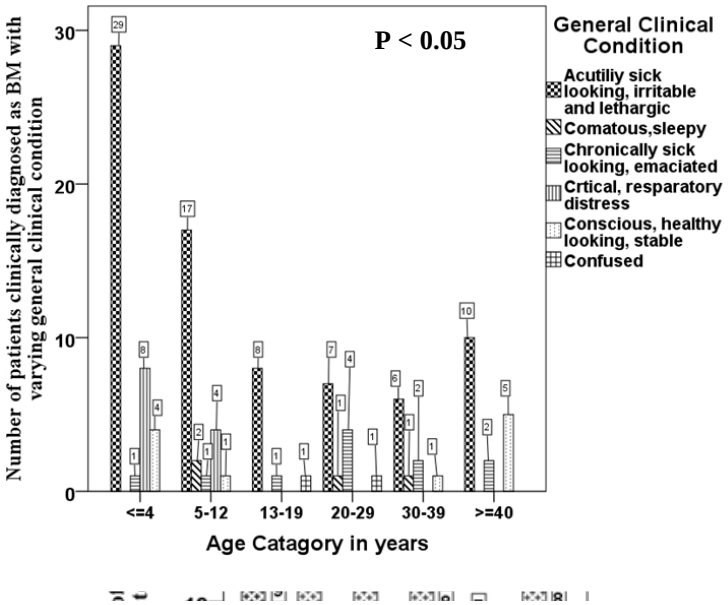


Fig. 23: Frequency of different age groups of patients clinically diagnosed as BM at Hawassa, Gondar and Tikur Anbessa University Hospitals; their general clinical conditions, 2012-2013

It is clear that the signs and symptoms (Figs. 20, 21, 22) as well as general clinical conditions (Fig. 23) overlap in some patients.

Antibiotic uptake prior to hospital admission was reported by 45/138 (32.6%) patients. Twenty patients out of the 139 (14.4%) patients clinically diagnosed with BM have died. Of the 20 patients who died, general clinical condition was retrieved only for 18. Seven out of the 18 (38.9 %) died patients have reported to have antibiotic treatment before hospital admission. General clinical conditions of acutely sick looking irritable and lethargic (3 patients); chronically sick looking and emaciated (1 patient); critical with respiratory distress (1 patient); conscious and healthy looking (1patient); and confused general clinical condition (1 patient) were observed in these seven patients. Out of the 20 patients who died, 5 (25%) were in the age group ≤ 4 yr, while age groups 20-29 yr and ≥ 40 yr were each composed of 4 (20%) patients. Age groups 5-12 yr and 13-19 yr were each comprised of 2 (10%) patients and within the age range of 30-39 yr were 3 (15%) patients.

Immediately occurred sequelae were recorded to occur in 50/139 (36%) of patients. The recorded types of immediately occurred sequelae were specified only for 4 patients stated as confusion (n = 1), epilepsy (n = 1), quadriparesis (n =1) and increased intracranial pressure (n=1), while the types of sequelae which the remaining 46 patients faced were not showed in the record. Fifty percent of the sequelae were faced by patients at younger age group especially by those at ≤ 4 yrs old compared to the patients at older age groups.

Chi-square statistical analysis showed the presence of significant (P = 0.01) association (Fig. 24) between age groups and sequelae.

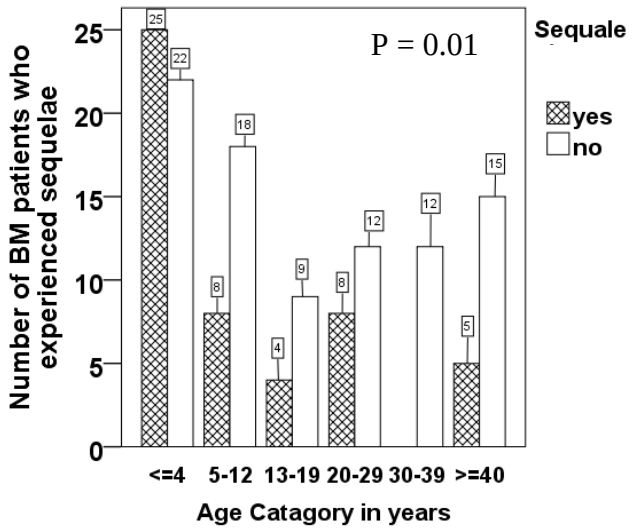


Fig. 24: Bacterial meningitis patients who experienced sequelae

Seven out of the 20 died patients also showed sequelae prior to death and these were among those whose sequelae were not specified.

6.2 Culture detection (biochemical) test results

Of the total 139 CSF in TIM inoculated into culture media plates only 71 (51.1%) showed colony growth while no colony growth was detected on the remaining 68 (48.9%). Culture detection (through biochemical tests) test results revealed a total of 14 organisms to be positive for BM etiologic agents targeted by the current study involving *N.meningitidis* (5/14, 35.7%), *S. pneumoniae* (8/14, 57.1%) and *H. influenzae* (1/14,

7.1%). No organisms of interest of the current study was found with culture detection from colonies isolated from two patient samples, one with Gram negative diplococci mixed with Gram positive diplococci (GNDC + GPDC) and the other with GPDC mixed with Gram positive streptococci (GPDC + GPSC) after repeated sub-culturing of single colonies. Both samples were oxidase negative (Table 5). Among the patients who reported to have antibiotic up take prior to hospital admission 45/138 (32.6%), were having 20 positive and 25 negative culture detection test results were found.

Table 5: Gram stain and culture detection test results

139 CSF inoculated onto culture media. (71 culture growth positive samples)										
Gram staining and biochemical tests run depending on applicability on culture positive samples										
Oxidase positive organisms and their Gram stain status		**Gammaglutamil transpeptidase test (GGT)**	**Tributrin**	**Ortho-Nitrophenyl-β-galactoside (ONPG)**	**Sero-agg. test results for Nmen serogroups A,C, W, X, Y**	**Final impression on culture detection**	**Oxidase negative organisms and their Gram stain status**		**Optochin antibiotic sucseptibility test**	**Growth response towards XV (X+V) nutritional factors**	**Final impression on culture detection**
Coco Bacilli (Gram variable)	**1**	**NA**	**NA**	**NA**	**NA**	**NA**	**Coco Bacilli (Gram variable)**	**-**	**NA**	**negative**	**Unknown**
GNC/short rod	**1**	**NA**	**NA**	**NA**	**NA**	**NA**	**GNC/short rod**	**5**	**NA**	**NA**	**Unknown**
GNDC	**8**	**5 pos. 3 neg**	**5 neg. + 3 others**	**5 neg + 3 others**	**5 pos. (4A + 1W)**	**→ *Nmen* = 5**	**GNDC**	**6**	**NA**	**NA**	**Unknown**
GNDC + GPDC	**-**	**NA**	**NA**	**NA**	**NA**	**NA**	**GNDC + GPDC**	**1**	**NA**	**NA**	***Unknown***
GNR	**7**	**NA**	**NA**	**NA**	**NA**	**NA**	**GNR**	**12**	**NA**	**1**	**→ *Hinf* =1**
GPC	**9**	**NA**	**NA**	**NA**	**NA**	**NA**	**GPC**	**2**	**1**	**NA**	**→ *Spn* =1**
GPDC	**5**	**NA**	**NA**	**NA**	**NA**	**NA**	**GPDC**	**10**	**6**	**NA**	**→ *Spn* =6**
GPDC + GPSC	**-**	**NA**	**NA**	**NA**	**NA**	**NA**	**GPDC + GPSC**	**1**	**1**	**neg**	**negative**
GPR	**2**	**NA**	**NA**	**NA**	**NA**	**NA**	**GPR**	**-**	**NA**	**NA**	**Unknown**
***GPSC**	**1**	**NA**	**NA**	**NA**	**NA**	**NA**	**GPSC**	**-**	***GPSC**	**NA**	**→ *Spn* =1**
Total	**34**							**37**			

Coccobacilli (CB), Gram negative cocci (GNC), Gram negative diplococcic (GNDC), Gram positive diplococcic (GPDC), Gram negative rod (GNR), Gram positive Cocci (GPC), Gram positive streptococci (GPS), Streptococcus (Strept), Gram positive rod (GPR), Gram positive cocci (GPC), * Oxidase variable, → indicate culture positive results, shaded columns indicate oxidase positive samples test results, while unshaded columns indicate oxidase negative samples test results

6.3 Molecular diagnostic test findings

I. Conventional multiplex PCR amplification results

Genomic DNA was extracted from all culture positive and negative CSF samples and subjected to PCR amplification. Of 139 genomic DNA samples subjected to conventional multiplex PCR amplification, a total of 18 (12.9%) genes were amplified. Seven out of the 18 (38.9%) were PorA genes of *N. meningitidis*, 10 (55.6%) were LytA genes of *S. pneumoniae* and 1 (5.6%) was BexA gene of *H.influenzae.* The amplified conventional PCR products were subjected to gel electrophoresis. Amplified positive control DNA extracted from American type culture collection (ATCC) reference bacteria were loaded in lanes 9, 10 and 11 of the agarose gel (Fig. 25). Patient sample DNA amplicons loaded in lanes 3 and 4 were Lyt A genes of *S. pneumoniae* (390 bp bands) while lane 7 comprised the PorA gene of *N. meningitidis* (1100 bp bands) (Fig. 25).

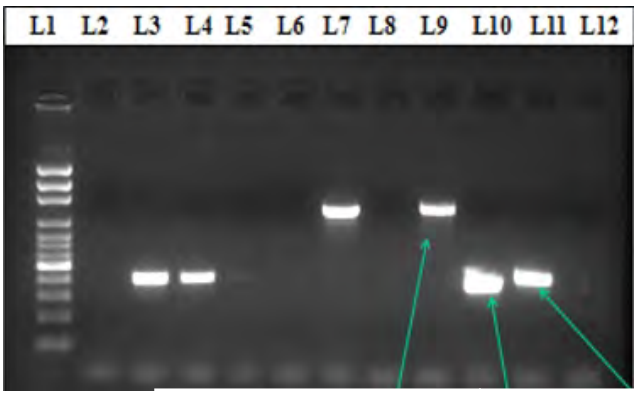
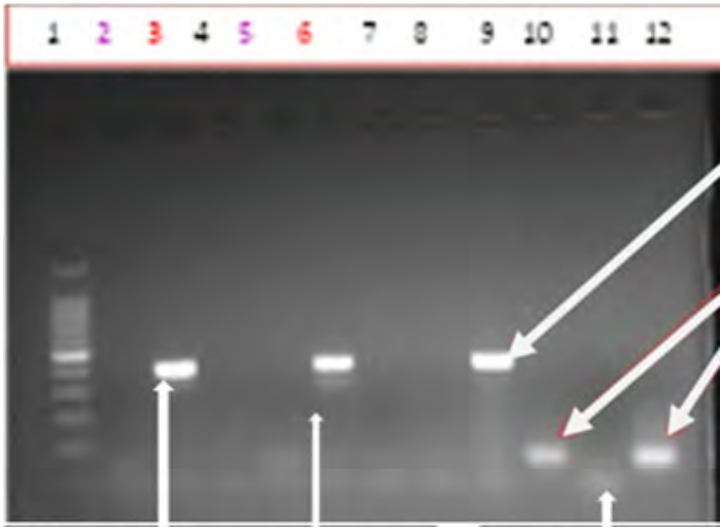


Fig. 25: **Agarose Gel electrophoresis showing conventional multiplex PCR amplification product bands of Por A, Lyt A and Bex A genes of *N. meningitidis, S. pneumoniae* and *H. influenzae***

Lanes 2, 5, 6 and 8 are also patient sample PCR products, which turned negative for the bacterial genes of interest. One out of the 139 patient samples was PCR positive for BexA gene (343bp) of *H. influenzae* (not shown in the figure). Lane 10 is a positive control for the Bex A gene (Fig. 25) of *H. influenzae* (343 bp). Samples positive for Por A gene of *N. meningitidis* were run with a second conventional PCR for genogrouping purpose. Six out of the seven samples (85.7%) were positive for SacB gene of *N. meningitidis* serogroup A while 1 (14.3%) was positive for MynB gene of *N. meningitidis* serogroup W-135. No Siad gene of genogroup Y were amplified from patient samples using conventional PCR amplification. Gel electrophoresis of the second conventional multiplex PCR amplicons of SacB (genogroup A, lane 3 and 6) and MynB (genogroup W-135, lane 10) genes of *N.meningitidis* genogroups are shown in Fig. 26.



Lane 9: 400 bp Sac B gene of *N. meningitidis* genogroup A (positive control)

Lane 10 and 12, patient samples and positive control of 120 bp mynB gene of *N. meningitidis* genogroup W_{135} Rrespectively

Lane 3 and 6: Patient samples with Sac B gene of *N. meningitidis* genogroup A PCR amplfication

Lane 11: negative control (dH_2O)

Fig. 26. Agarose gel electrophoresis picture showing conventional multiplex PCR amplicons of *N. meningitidis* genogroup A and W -135

Lane 2, 4, 5, 7 and 8 were loaded with patient samples conventional PCR products, which have remained negative for any of the genes of interest of *N. meningitidis* (Fig. 26).

II. Real Time Multiplex PCR amplification results

Real Time multiplex PCR amplification was run for all the 139 study participants DNA extracted from CSF in TIM using the Qiagen DNA extraction kit. The PCR was run in duplicates and with internal positive controls (IPCs). Out of the total of 139 DNA samples, RT-PCR amplified 46 (33.1%) of the genes of interest from the three BM etiologic agents. This consisted of ctrA genes of *N. meningitidis* in 27/46 (58.7%), LytA gene of *S. pneumoniae* in 18/46 (39.1%) and omp gene of *H.influenzae* in 1/46 (2.2%).

Ninety three out of the 139 patient samples were negative with the RT-PCR test. Out of the 93 negatives 42 had (non-*Nm/Spn/Hinf*) growths on culture media while the remaining 51 had no growth. Amplification of the internal positive control (IPC), has confirmed that the RT-PCR experiments have worked and the results can be accepted as shown (Figs. 27, 28 and 29). The sigmoidal amplification reaction curves (Fig. 27, 28 and 29) as well indicate that the results from the samples in the reaction mixes are acceptable as in the guide line of the test protocol.

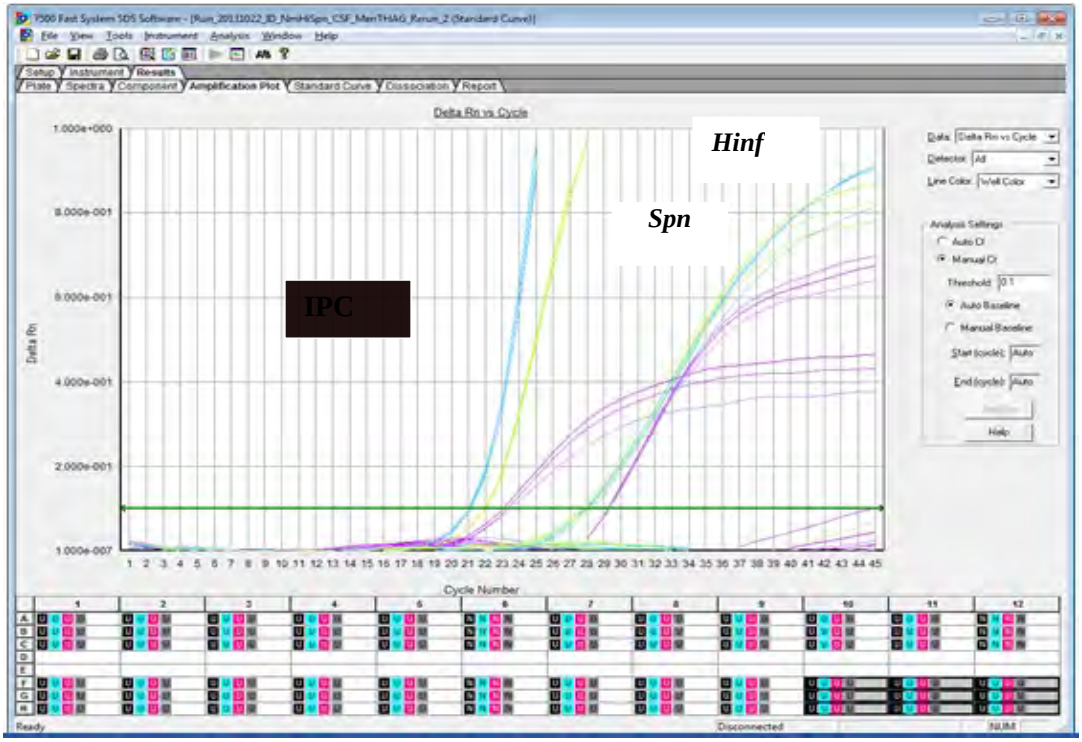


Fig. 27: Real time PCR amplification fluorescence sigmoidal curves for ctrA, lytA and omp genes of *S. pneumoniae* and *H. influenzae* respectively using 7500 Fast System SDS Software

The curves are showing that each test was run in triplicate. Average of the three cycle thresh hold (CT) values from the triplicate test of a single sample were taken for interpreting the result. In cases when only two out of the three curves were amplified the test was repeated and average of the two CT values were used for interpretation of the result if the same result was obtained.

Comparison between results of conventional and real time multiplex PCRs showed that real time PCR detected significantly higher (Fishers exact test: P `= 0.0001) number of samples to be positive for the three etiologic agents of BM

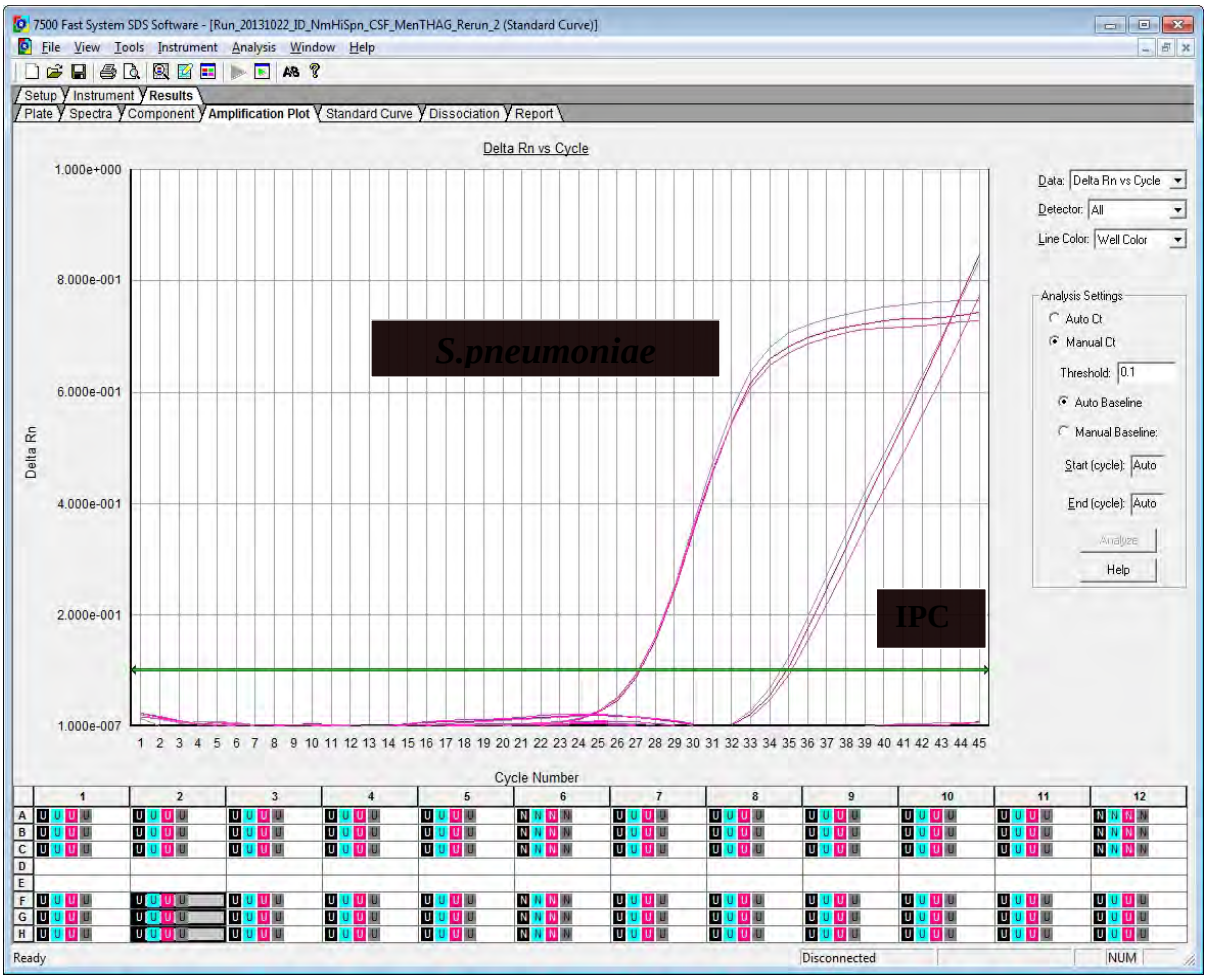


Fig. 28: Real Time PCR Sigmoid curves of internal positive control (IPC) and *S. pneumoniae*

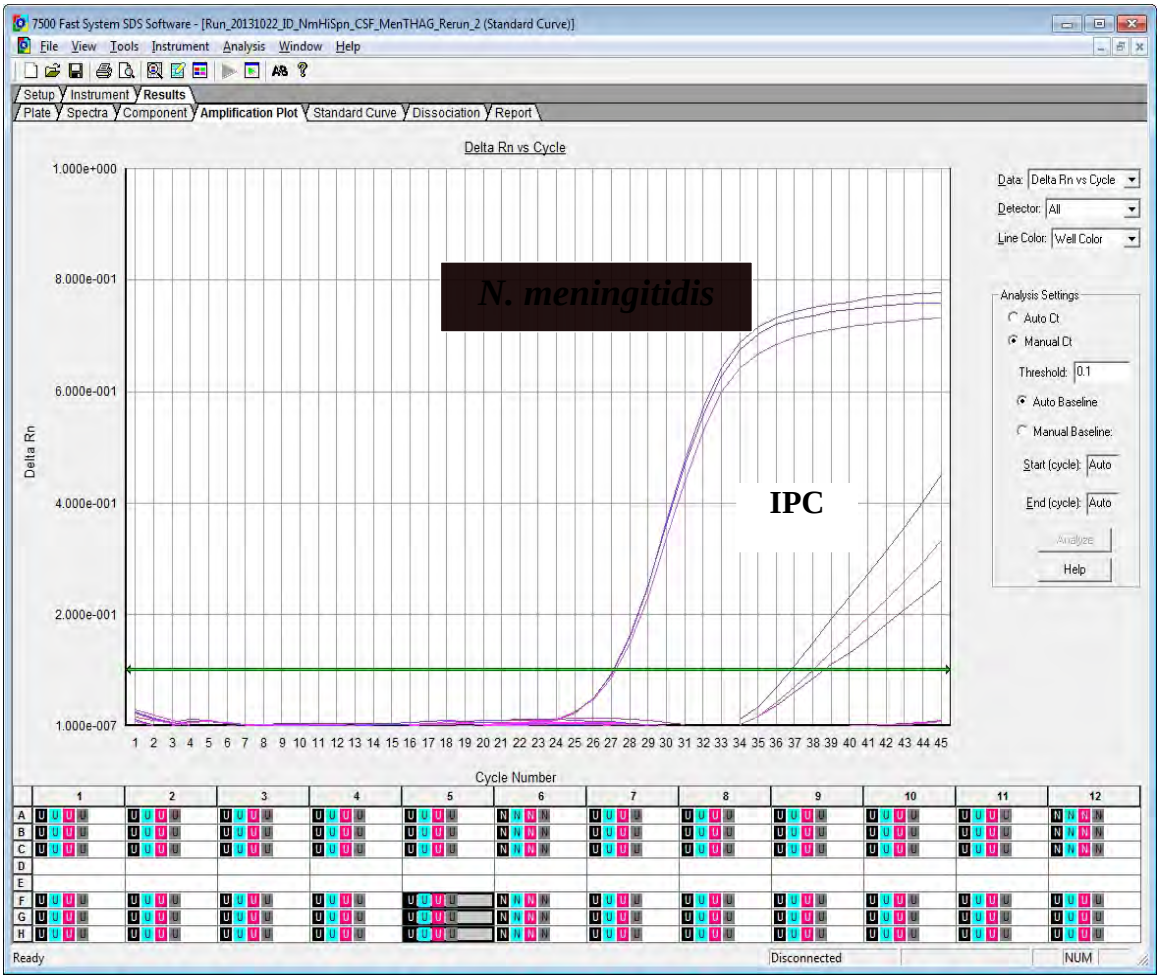


Fig. 29: Real Time PCR Sigmoid curves of internal positive control (IPC) and *N. meningitidis*

Genogrouping of the 27 DNAs positive for ctrA gene of *N. meningitidis* showed amplification of all the tested genogroups (Table 6) except genogroup B. Nonidentified *N. meningitidis* are those samples which didn't belong to any of the tested genogroups

Table 6: Frequency and percentage of *N. meningitidis* genogroups A, B, C, W-135, X and Y

Serogroup of *N. meningitidis* with ctrA gene positive results in Real Time PCR	**Target genes**	**Frequency**	**Percentage**
A	*SacB*	11	40.7
B	*SynD*	-	-
C	*SynE*	1	3.7
W-135	*SynG*	7	25.9
X	*XcbB*	1	3.7
Y	*SynF*	-	-
Non-groupable	When none of the target genes can be amplified	7	25.9

III. Multilocus sequence typing (MLST) Results

PorA gene sub-typing by PCR and gene sequencing was possible for 23 out of the total 27 CSF samples confirmed positive for *N. meningitidis* DNA since copy numbers of DNA were insufficient for the remaining 4 samples (Table 7). Two of the PorA variable regions (VRs) were determined in most of the cases of the

sequenced *N. meningitidis* DNA, while in some cases only one of the two PorA VRs was determined.

Multi-locus sequence typing (MLST) and PorA/FetA genotyping of 4 meningococcal strains showed that one was genogroup W, ST-11:P1.5, 2:F1-1, whereas 3 were genogroup A, ST-7:P1.20, 9:F3-1. ST-7: P1.20, 9 was predominant in Hawassa while ST-11 P: 1.5, 2 was the most common in Gondar (Table 7). Sequence

Table 7: PorA gene sub-typing of *N. meningitidis* through sequence identification of 23 samples from Gondar, Tikur Anbessa and Hawassa University Hospitals, 2012-2013

Site sites (University hospitals)	**P1.20,9**	**P1.5,2**	**P1.5-11,1-1**	**P1.,4**	**P1.,2**	**P1.5**	**P1.,9**	**Total**
Gondar	1	7	0	1	1	0	0	10
Tikur Anbessa (Addis Ababa)	0	0	0	0	0	0	1	1
Hawassa	9	0	1	0	0	1	1	12
Total	10	7	1	1	1	1	2	23

The 3 pneumococcal isolates that were recovered for MLST typing were ST-8875 (n=2) and ST-289 (n=1); all from Gondar.

6.4 Real time PCR test results in reference with clinical, socio-demographic and seasonal conditions

Among RT-PCR positive samples, the highest total frequency of BM etiologic agents were detected in patients of age group ≤ 4 (16/46, 34.8%) followed by patients of age group 5-12 (15/46, 32.6%) while the least frequent distribution was at age group 30-39 (1/46, 2.2%).

General clinical condition was retrieved for 39 of the total of 46 RT-PCR positive BM patients. “Acutely sick looking, irritable and lethargic” clinical condition was exhibited in 26/39 (66.7%) patients of whom 16/26 (61.5%, P > 0.05) were *N.meningitidis* positive and 10/26 (38.5%, P > 0.05) were *S. pneumoniae* positive. Among the remaining 13 of 39 patients, 10 (77%) who were positive for the ctrA gene of *N. meningitidis* were grouped by their general clinical condition as follows: Seven (53.9 %) were having general clinical condition of “respiratory distress”; 1 (7.7%) “comatose”; 1 (7.7%) “confused” and 1 (7.7%) “conscious, healthy looking and stable”. Three of the 13 were confirmed positive for *S. pneumoniae* of whom 2 were “comatose and sleepy” while one was “conscious, healthy looking and stable”.

Occurrence of sequelae was reported among 50/139 (36%) of the study participants. Specific type of seuelae was showed among 48 of the study participants while no record of the specific sequelae was found for the two study participants. Seizure was the sequelae faced by 44/48 (91.7%) while intracranial pressure, epilepsy, confusion and quadriparesis were reported to occur independently in 4/48 (8.3%) patients. Disease severity has occurred mainly in children of age group ≤ 4 and 5-12. In the ≤

4 age groups, death occurred in 5/20 (25%); while sequelae has occurred as seizure in 22/48 (46%), as epilepsy in 1/48 (2.1%) as increased intracranial pressure in 1/48 (2.1%)). In children of age group 5-12 death occurred in 2/20 (10%) and sequelae as seizure in 7/48 (14.6%). Whereas sequelae from the remaining older ages (i.e. ≥ 13 - 78) has occurred in a total of 20/48 (41%) of the patients. In these same older age groups of patients, death was reported to occur in 11/20 (55%). Seizure, the sequelae which occurred in 44 of the patients has occurred in a total of 15 (34%) BM patients confirmed positive for *N. meningitidis* (8/15) and *S. pneumoniae* (7/15) while only one (6.7%) patient with *S. pneumoniae* has faced epilepsy. The etiologic agent of BM in the patients who faced intracranial pressure, confusion and quadriparesis was not among the three BM etiologic agents in focus of this study. This is meant that the patients who faced the refereed clinical conditions were not diagnosed with any one of the bacterial meningitis etiologic agents under the focus of the current study.

Of the total 46 RT-PCR confirmed *N. meningitidis, S. pneumoniae* and *H. influenzae* positive patients enrolled into this study, relatively higher numbers of patients was observed (Fig. 30) in May 2012 (4 patients), June 2012 (3 patients), August 2012 (3 patients) and February 2013 (3 patients) Fig. 30. The impact of antibiotic therapy prior to hospital admission was shown in reduced level of positivity with culture detection method for the three organisms under the focus of the current study, which showed positive results only for 4 out of the 20 culture grown samples. Whereas, real time PCR has detected 10/20 organisms with culture growths and 5/25 organisms with no culture growths.

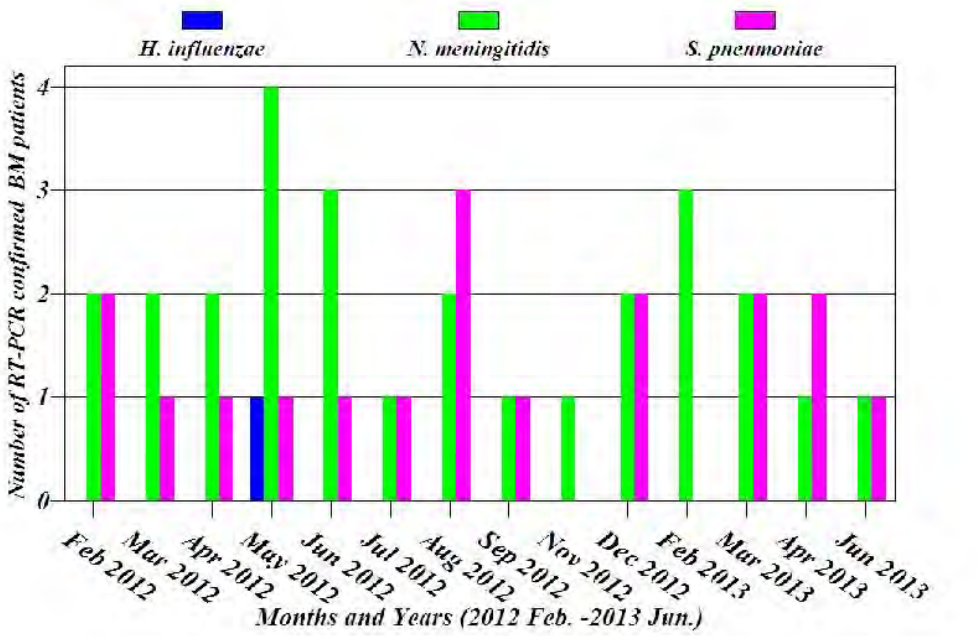


Fig. 30: **Number of RT-PCR confirmed *N. meningitidis, S. pneumoniae* and *H. influenzae* cases among clinically diagnosed bacterial meningitis patients from three University hospitals during non-epidemic seasons, Feb. 2012- June 2013**

Antibiotic treatment prior to hospital admission was reported by 45/138 (32.6%) patients (Table 8). Fifteen out of the 45 patients who had history of antibiotic uptake prior to hospital admission were diagnosed positive for the three BM etiologic agents composing *S. pneumoniae* (7/15, 46.7%), *N. meningitidis* (7/15, 46.7 %) and *H.influenzae* (1/15, 6.7%). Twenty five out of the 45 patient samples were culture negative while 20 were culture positive. Real time PCR detected BM etiologic agents among the 5 out of the 25 (20%) culture negatives (composing 1 *Nm*W-135, 2 *Nm*NG and 2 *Spn*) and 10 out of the 20 (50%) culture positives (composing 1 *Nm*A, 1 *Nm*W-135, 2 *Nm*NG, 1 *Hinf* and 5 *Spn*).

Neck rigidity was present frequently (though not with statistical significance) in meningococcal than in pneumococcal patients (70.4 % versus 55.6%; P= 0.354) respectively. Impaired consciousness (Table 8: 77.8% versus 72.2%, P = 0.04) were showed more often in meningococcal than pneumococcal meningitis patients. Whereas, back rigidity (data not shown on Table, 12.5% versus 21.4%, P= 0.022) and ecchymosis (0% versus 5.9%, P= 0.025) were present less often in meningococcal meningitis patients as compared to pneumococcal meningitis patients respectively.

Patients with impaired consciousness and > 37.5 °C body temperature were more commonly associated with *N. meningitidis* positivity than *S. pneumoniae* and associations of both findings were statistically significant with P value = 0.041 (Table 8). Age variation was significantly associated with BM etiologic agents (meningococcal meningitis was more common among younger and pneumococcal meningitis more common among older BM patients, P value < 0.05). Although more males were diagnosed with meningitis than females, there was no gender differences in the distribution of etiologies (P = 0.62).

The proportion of BM etiologic agents detected by RT-PCR among the total sample collected at each site was highest in Hawassa (19 of 27, 70.4%), followed by Addis Ababa, (7 of 20, 35.0%) and finally in Gondar (20 of 92, 21.7%). Among RT-PCR confirmed positives, 27 were *N. meningitidis* positive with median age of 7 years (range 5 days to 35 years), and *S. pneumoniae* confirmed positive patients were having median age of 10 years with a range of 2 months to 78 years.

Table 8: Distribution of *N. meningitidis* and *S. pneumoniae* (detected by RT- PCR) positivity in bacterial meningitis diagnosed patients among different socio-demographic and clinical parameters, 2012-2013.

Characteristics	**Cases**	***N. meningitidis***	***S. pneumoniae***	**P value†**
Age in years	**139 (%)**	**27 (%)**	**18 (%)**	
≤4	48 (34.5)	9 (33.3)	6 (33.3)	0.018
5-12	26 (18.7)	11 (40.7)	4 (22.2)	
13-19	13 (9.4)	4 (14.8)	1 (5.6)	
20-29	20 (14.4)	2 (7.4)	4 (22.2)	
30-39	12 (8.6)	1 (3.7)	0	
≥40	20 (14.4)	0	3 (16.7)	
Gender				
Male	83/139 (59.7)	17 (63.0)	10 (55.6)	0.619
Female	56/139 (40.3)	10 (37.0)	8 (44.4)	
Clinical findings				
Impaired consciousness	92/137 (67.2)	21 (77.8)	13 (72.2)	0.041
Neck stiffness	92/137 (67.2)	19 (70.4)	10 (55.6)	0.354
Vomiting	96/139 (69.1)	22 (81.5)	14 (77.8)	0.354
Fever > 37.5°C	40/138 (29.0)	25 (92.6)	15 (88.2)	0.041
Antibiotic treatment	45/138 (32.6)	7 (25.9)	7 (38.9)	0.480
Petecchiae > 5mm	4/138 (2.9)	1 (3.7)	2 (11.1)	0.555
Sequelae	50/138 (36.2)	8 (29.6)	8 (44.4)	0.198
Deaths	20/139 (14.4)	2 (7.4)	3 (16.7)	0.670

***Immediate severe sequelae; (sequelae faced by patients diagnosed with *Spn* were seizure, n = 7, epilepsy, n = 1) and patients diagnosed with *Nm* (seizure, n = 8)**

Epilepsy was faced by one *S. pneumoniae* diagnosed positive patient with age group ≤4 while raised intracranial pressure, quadriparesis and confusion were observed in three patients at age groups ≤ 4, ≥ 40 and 20-29 respectively each one with one of the sequelae but not diagnosed with any one of the three BM etiologic agents.

Whereas, 44 out of the 48 (91.7%) patients who experienced seizure from hospital admission to discharge were diagnosed to develop sequelae. They were in age groups ≤4 (n = 22), 5-12 (n = 7), 13-19 (n = 4), 20-29 (n = 7) and ≥ 40 (n = 4). Out of the 44 patients who experienced seizure, 7 with age groups (≤ 4, n = 4; 20-29, n = 2; ≥ 40, n = 1) were diagnosed positive with *S. pneumoniae* and 8 with age groups (≤ 4, n = 3; 5-12, n = 3; 13-19, n = 1; 20-29, n = 1) were diagnosed positive with *N.meningitidis* while the one patient with *H. influenzae* (with age group ≤ 4, n = 1) has also faced seizure. Percentage distribution of the target BM etiologic agents among the study sites showed the highest number in Hawassa with 19 (70.4%) of 27 samples, followed by Addis Ababa with 7 (35.0%) of 20 samples and Gondar with 20 (21.7%) of 92 samples. Most of the patients with confirmed *S. pneumoniae* were diagnosed at GUH (10 of 18, 55.6%) followed by HUH (5 of 18, 27.8%) with the least number of pneumococcal meningitis cases diagnosed from TAUH (3 of 18, 16.7%). Of the 11 *N. meningitidis* genogroup A diagnosed cases 9 were from HUH and 2 from TAUH. Of the 7 genogroup W-135 meningococcal cases 6 were from GUH and 1 from HUH (Fig. 31). Among the seven out of the 27 (25.9%)

N.meningitidis identified as non-groupable in this study, 3 (42.9%) belonged to GUH, 2 (28.6%) to HUH and 2 (28.6%) to TAUH (Fig. 31).

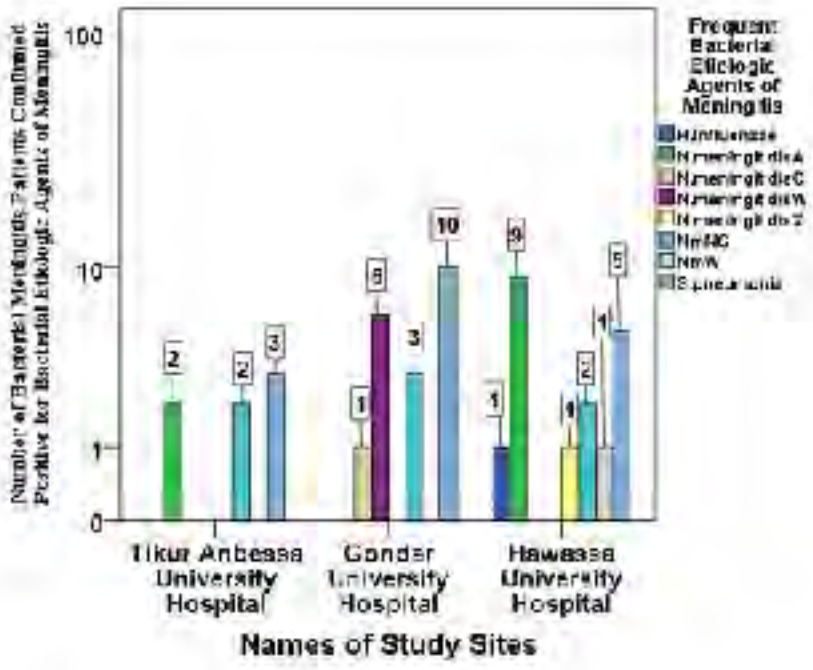


Fig. 31: Distribution of BM etiologic agents among 139 patients with clinical signs and symptoms of BM in Ethiopia, confirmed to be *N. meningitidis* (*Nm*A/C/W/X/NG), *S. pneumoniae* (*Spn*) and *H. influenzae* (*Hinf*) by RT-PCR among patients who visited Gondar Hawassa and Tikur Anbessa University hospitals, 2012-2013

Only one case of serogroup C (1/27, 3.7%) and one case of serogroup X (1/27, 3.7%) were detected from GUH and HUH patients, respectively. The rare serogroup of BM (serogroup X) was detected in patients of age group ≤ 4 (Table 9). Sixteen out of the 27 (59.3%) *N. meningitidis* detected in this study were sero-groups other than A (Table 9).

Table 9: Distribution of RT-PCR detected etiologic agents of bacterial meningitis by age category, 2012-2013

Organism	Age Category in years						Total
	≤ 4	5-12	13-19	20-29	30-39	≥ 40	
H. influenzae	1	-	-	-	-	-	1
N. meningitidis A	2	5	2	1	1	-	11
N. meningitidis C	-	1	-	-	-	-	1
N. meningitidis W-135	2	3	1	1	-	-	7
N. meningitidis X	1	-	-	-	-	-	1
N. meningitidis NG	4	2	1	-	-	-	7
S. pneumoniae	6	4	1	4	-	3	18
Total	16	15	5	6	1	3	46

NG = Non groupable

Thirty nine out of the 119 survivors were confirmed to be positive for the investigated etiologic agents with RT-PCR from which 15 (38.5 %) were *S.pneumoniae* and 24/39 (61.5 %) were *N. meningitidis* composing genogroups A (n = 10), W-135 (n = 6), X (n = 1), C (n = 1) and NG (n = 6) (Table 10). Out of the 20 died patients 7 were RT-PCR confirmed positive for BM etiologic agents. Thirty nine RT-PCR confirmed BM etiologic agents were found from survived patients. The ratio of patients who died to live among confirmed positives is 1: 7 (Table 10). Twelve out of the 20 patients who died had culture media growth of their inoculated CSFs. Six out of the 12 (50%) with culture media growth and one out of the 8 (12.5%) with no culture media growth samples from the died BM patients were confirmed positive with RT PCR for the etiologic agents of BM. This comprises 3

*S. pneumonia*e, 3 *N. meningitidis* (1 genogroup A, 1W-135, 1 NGNm) and 1 *H.influenza*e. Thirteen out of the 20 died belonged to the ninety three patients who were diagnosed with BM on clinical grounds without positive culture detection (biochemical) test results or RT-PCR findings.

Table 10: Real time PCR diagnosed bacterial etiologic agents of meningitis from CSF of died and survived patients clinically diagnosed as bacterial meningitis, 2012-2013

RT-PCR results	Died	Survived	Total
H.influenzae	1	0	1
N. meningitidis A	1	10	11
N. meningitidis C	0	1	1
N. meningitidis W-135	1	6	7
N. meningitidis X	0	1	1
Negative for *Nm, Spn* and *Hinf*	13	80	93
N. meningitidis NG	1	6	7
S. pneumoniae	3	15	18
Total	20	119	139

Among the 119 survivors of BM diagnosed either by clinical or microbiological criteria sequelae occurred in 50 (42%) patients. Types of sequelae were recorded only for 4 of 50 (8%) patients, and the sequelae were stated as “confused” (n = 1), “epilepsy” (n = 1), “quadriparesis” (n = 1) and “raised intracranial pressure” (n = 1).

6.5 Comparison of RT-PCR diagnostic test results with conventional PCR, culture detection (biochemical diagnostic) and white blood cell count findings

Of the total 46 bacterial agents identified by RT-PCR, 14 were also positive with culture detection while the remaining 32 (15 with culture media growth and 17 with no growth) showed negative results for culture detection. *Heamophilus influenzae* was detected equally with RT-PCR, conventional and culture detection tests. Conventional PCR, which amplified 18 organisms out of the 139 (Table 11) patient samples has also detected the 14 culture detection test positive samples.

The 4 additional samples detected by conventional PCR were among the culture detection test negative samples. With regard to the focus of interest of the current project, RT PCR detected 46 BM etiologic agents (Table 11) composing the three bacterial agents of interest.

Table 11: Comparison of frequency and percentages of conventional and RT-PCR

Organisms	Type of PCR	Genes amplified	Frequency and % with both	Total result with RT-PCR
N. meningitidis	Conventional PCR	Por A	7 (5%)	
	RT. PCR	CtrA	7 + 20 (19.4%)	27
S. pneumoniae	Conventional PCR	Lyt A	10 (7.2 %)	
	RT-PCR	Lyt A	10 + 8 (13%)	18
H. influenzae	Conventional PCR	Bex A	1 (0.7%)	
	RT-PCR	Omp	1 (0.7%)	1
	Conventional PCR	Negative	121 (87.1%)	
	RT-PCR	Negative	93 (66.9)	93
Total				139

This composes those diagnosed with culture detection (n =14, not shown on table), conventional PCR (n = 4 more in addition to those diagnosed with culture detection) and 28 detected merely with RT-PCR (Fig. 32).

Fig. 32: Flow chart showing number of samples detected to be positive or negative for *N.meningitidis, S. pneumoniae* and *H. influenzae* by biochemical, conventional and RT-PCR tests, 2012-2013

Twenty eight more samples which were not identified with conventional PCR and biochemical tests were detected by RT-PCR. In general, Real time PCR has detected a total of 46 organisms composing 18 *S. pneumoniae* (8 culture positive and 10 culture negative), 27 *N. meningitidis* (5 culture positive and 22 culture negative) (Table 12 and 13) and 1 *H. influenzae* (culture positive). Moreover, real time PCR detected a total of 5 (20%) organisms: 1 *N. meningitidis* W-135, 2 *N. meningitidis* non-typable and 2 *S. pneumonia* from twenty five culture negative samples taken from the 45 patients who reported to have antibiotic therapy prior to hospital admission. Whereas, 10 out of the 20 (50%) culture positive samples from the patients with antibiotic therapy prior to hospital admission were RT-PCR confirmed as *S. pneumoniae* (n= 5), *N. meningitidis* W-135 (n = 1), *N. meningitidis* non-groupable (n = 2), *N. meningitidis* A (n = 1) and *H. influenza*e (n = 1). No organism of interest under the focus of the current study was detected with RT-PCR among the two mixed colonies having Gram stain status of GNDC + GPDC and GPDC + GPSC. Further investigation is needed to examine the 93 samples which remained negative with Real time PCR. Culture detection test results with regard to detection of *S. pneumoniae* and *N. meningitidis,* of the research lab were analyzed in reference with RT-PCR results as PCR is recommended to be used as reference gold standard (WHO, 2014). Culture detection capacity of *N. meningitidis* and *S. pneumoniae* in the research lab in reference with RT-PCR detection showed low level of sensitivity (19%, CI: 0.06-0.38) and (44%, CI: 0.22-0.69), respectively (Table 12 and 13).

Table 12: Statistical significance of research lab culture detection test results of *N. meningitidis* in reference with RT-PCR detection among 139 study participants, 2012-2013

Culture detection Status for *N. meningitidis*	RT-PCR detection status for *N. meningitidis*, No (%) +	-	Sensitivity (%) (CI)	Specificity (%) (CI)	PPV (%) (CI)	NPV (%) (CI)	**Total**
Culture +	**5 (18.5%)**	**0**	19% (CI: 0.06-0.38)	100% (0.95-1.00)	100% (0.36-1.00)	84% (0.76-0.89)	5
Culture -	**22 (81.5%)**	112					134
Total	**27**	**112**					**139**

Table 13: Statistical significance of research lab culture detection test results of *S. pneumoniae* in reference with RT-PCR detection among 139 study participants, 2012-2013

Culture detection Status for ***Spn***	RT-PCR detection status for *S. pneumoniae,* No (%) +	-	Sensitivity (%) (CI)	Specificity (%) (CI)	PPV (%) (CI)	NPV (%) (CI)	**Total**
Culture +	**8 (44.4%)**	**0**	44% (0.22-0.69)	100% 0.96-1.00)	100% (0.52-1.00)	92 (0.86-0.96)	8
Culture -	**10 (55.6%)**	121					131
Total	**18**	**121**					**139**

More than four times high number of *N. meningitidis* and more than two times high number of *S. penumoniae* were detected with RT-RCR compared to that of culture detection tests (Table 12 and 13). The levels of sensitivities of the culture detection tests in reference with RT-PCR detection were highly reduced for both organisms.

6.6 Comparison of culture detection test results of hospital and research labs in reference with RT-PCR diagnostic test results

Clinical laboratory data record from the three site University hospitals were retrieved only for 50/139 (35.9%) study participants. Sub-analysis was done for comparison of clinical and research bacteriology lab biochemical test results of the 50 study participants after sorting and entering the corresponding data into the data base using their subject identifier codes. Culture detection test results of the hospital and research bacteriology labs were compared in reference with their RT-PCR test results. Out of the 50 study participants' CSF samples, 26 had growths on culture media with colony morphology of the organisms of interest (18 *N. meningitidis* and 8 *S. pneumoniae*) in both research and hospital labs. However, culture media growth status of the samples was not similar between the research and hospital labs. The culture detection test results of *N.meningitidis* among the research and hospital labs were having similar sensitivity (Table 14) while reduced specificity, PPV and NPV were found in the hospital lab results where fresh samples are tested. Culture detection test results of *S. pneumoniae* among the research lab were having higher sensitivity, specificity, PPV and NPV compared to that of hospital lab results (Table 15).

Table 14: Comparison of statistical significance in sub analysis for culture detection between research and clinical lab test results of *N. meningitidis* in reference with real time-PCR detection among 50 study participants, 2012-2013

Culture detection Status for ***Nm***	RT-PCR detection status for *N. meningitidis*, No (%) +	-	Sensitivity (%) (CI)	Specificity (%) (CI)	PPV (%) (CI)	NPV (%) (CI)	**Total**
Research lab +	**3 (16.7%)**	**0**	17% (0.04-0.41)	100% 0.84-1.00)	100% (0.19-1.00)	93% (0.53-0.81)	3
Research lab -	**15 (83.3%)**	32					47
Total	**18 (100%)**	**32**					**50**
Clinical lab +	**3 (16.7%)**	**3**	17% (0.04-0.41)	91% (0.75-0.98)	50% (0.12-0.88)	66% (0.50-0.80)	6
Clinical lab -	**15 (83.3%)**	29					44
Total	**18 (100%)**	**32**					**50**

Table 15: Comparison of statistical significance in sub analysis for culture detection between research and clinical lab test results of *S. pneumoniae* in reference with real time-PCR detection among 50 study participants, 2012-2013

Culture detection Status for ***Spn***	RT-PCR detection status for *S. pneumoniae,* No (%) +	-	Sensitivity (%) (CI)	Specificity (%) (CI)	PPV (%) (CI)	NPV (%) (CI)	**Total**
Research lab +	**5 (62.5%)**	**0**	62% (0.24-0.91)	100% 0.87-1.00)	100% (0.36-1.00)	93% (0.81-0.99)	5
Research lab -	**3 (37.5%)**	41					44
Total	**8 (100%)**	**41**					**49**
Clinical lab +	**1 (12.5%)**	**2**	12% (0.00-0.53)	95% 0.84-0.99)	33% (0.01-0.91)	85% (0.72-0.94)	3
Clinical lab -	**7 (87.5%)**	40					47
Total	**8 (100%)**	**42**					**50**

6.7 Blood cell count results in cerebrospinal fluid and the circulation

Patient clinical data including complete blood count (CBC), neutrophil count (differential) from whole blood and CSF, white blood cell (WBC) count as well as CSF neutrophil count (differential) were assessed for 52 (Gondar University Hospital) out of 139 patients whose data were found from hospital record. The median CSF WBC counts (Table 16) in both *S. pneumoniae* (450 cells/mm^3) and *N. meningitidis* (7200 cells/mm^3) positive BM patients were very high compared to the normal range (0-5). With regard to CSF WBC count, no statistically significant elevation was detected between *S.pneumoniae* positive BM patients and *N. meningitidis* positive BM patients.

Table 16: Median White Blood Cells Count in CSF and the circulation of Bacterial Meningitis Patients: 2012-2013

Etiologic agents of Bacterial Meningitis	CSF WBC Count Normal range (0-5 cells)/mm^3 If BM 100-20,000 (mean = 800)	CSF Neutrophil Count (n) Normal (<2) If BM (Neutrophil predominance > 80% PMN)	CBC of circulation (n) Normal range (5000-10000)	Circulation Neutrophil Count (70-75%)
N. meningitidis	7200	97.6	11050	80
S. pneumoniae	450	85	17850	80.1

CBC = Complete blood count, WBC = White blood cells, normal ranges are adapted from Tunkel *et al.* 2004 Statistical significance analysis in differences between *Nm* and *Spn* with regard to CSF WBC count showed (P value = 0.08), for CSF neutrophil count (P = 0.04), for CBC of circulation (P = 0.05) and for CSF neutrophil count (P = 0.733).

The rise in median CSF neutrophil count compared to the normal value (< 2) is excessively high (indicative of BM) in patients diagnosed positive with *N. meningitidis* as well as *S. pneumoniae* though slightly higher in BM patients positive for *N. meningitidis*

(Table 16). Moreover, the ratio of CSF to serum glucose level was three times much lower (20%) than the normal value (60%), in *N. meningitidis* confirmed positive patients, indicative of BM. Hence, CSF to serum glucose ratio of *N. meningitidis* positive BM patients was 0.2:1. No data was available with this regard in *S. pneumoniae* confirmed positive patients. Data regarding CSF protein level for patients diagnosed with either *S. pneumoniae* or *N. meningitidis* were also not found from hospital laboratories.

6.8 Status of culture and real time-PCR detection in patients with antibiotic therapy prior to hospital admission

Results about 138 patients regarding antibiotic therapy before hospital admission showing ¨yes" (for 45) ¨no¨ (for 68) and "do not know" (for 25) were recorded. With this regard, no data was available for one o the patients out of 139. Twenty out of the 45 (44.4%) were positive while 25/45 (55.8%) were negative with culture detection test. Ten out of the 20 (50%) culture detection test positives and 5 out of the 25 (20%) culture detection test negatives were detected positive for the bacterial agents in focus of the current study using real time -PCR. Out of the 20 samples with culture media growth, only 4 (1 *Hinf* and 3 *Spn*) samples were detected positive with culture detection method. These 4 were out of the 10 detected positive with real time PCR. Twenty five out of the 68 (i.e. 17 of 37 culture detection test positive and 8 of 31 negative) who responded ¨no¨ and 6 of 25 (2 of 14 culture positive and 4 of 11 culture negative) who responded ¨do not know¨ to antibiotic therapy prior to hospital visit have turned positive with real time-PCR. Eight (4 *Nm*A, 1*Nm*W and 3 *Spn*) out of 68 patients who responded no and 2 (*Spn*) out of the 25 patients who responded as ¨do not know¨ for antibiotic treatment prior to hospital

admission were detected with culture detection test. All these 10 including the 4 patients' samples who responded as ¨yes¨ for antibiotic treatment prior to hospital admission were detected positive with real time PCR.

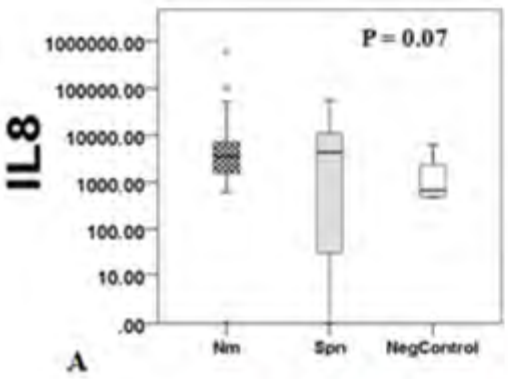
6.9 Comparison of levels of different inflammatory mediators between patients diagnosed positive for *S. pneumoniae* and *N. meningitidis*

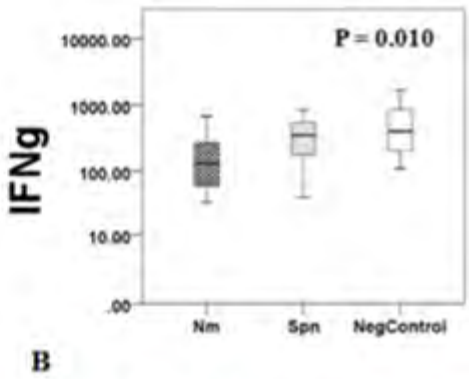
Levels of cytokines and chemokines were measured and compared between *S.pneumoniae* and *N. meningitidis* positive patients in comparison with samples from chronic meningitis cases negative for *S. pneumoniae* or *N. meningitidis* but diagnosed with TB meningitis, *Cryptococcus neoformans meningitis,* and others.

Out of the 27 *N. meningitidis* confirmed patients 2 (7%) died and 2 (7%) developed immediate severe sequelae.

Of the 16 confirmed, *S. pneumoniae* patients, 3 died and 3 developed immediate severe sequelae. Box plots were constructed to evaluate the differences between levels of cytokines and chemokines identified in CSF of *S. pneumoniae* positive, *N. meningitidis* positive BM patients and the negative control samples.

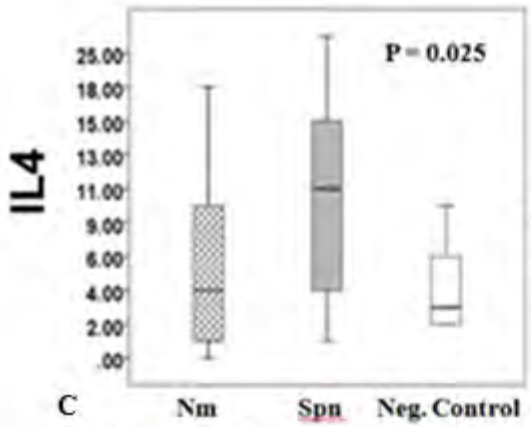
The levels of INF-γ (Fig. 33B), IL-4 (Fig. 33C), and IL-12/p70 (Fig. 33D) were significantly higher ($P < 0.05$) in *S. pneumoniae* positive BM patients compared to *N.meningitidis* positive BM patients in reference to the negative controls.

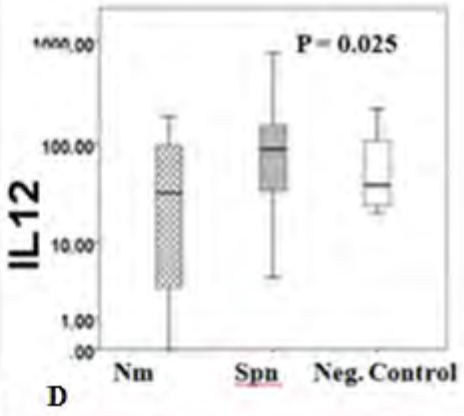




***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**





***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

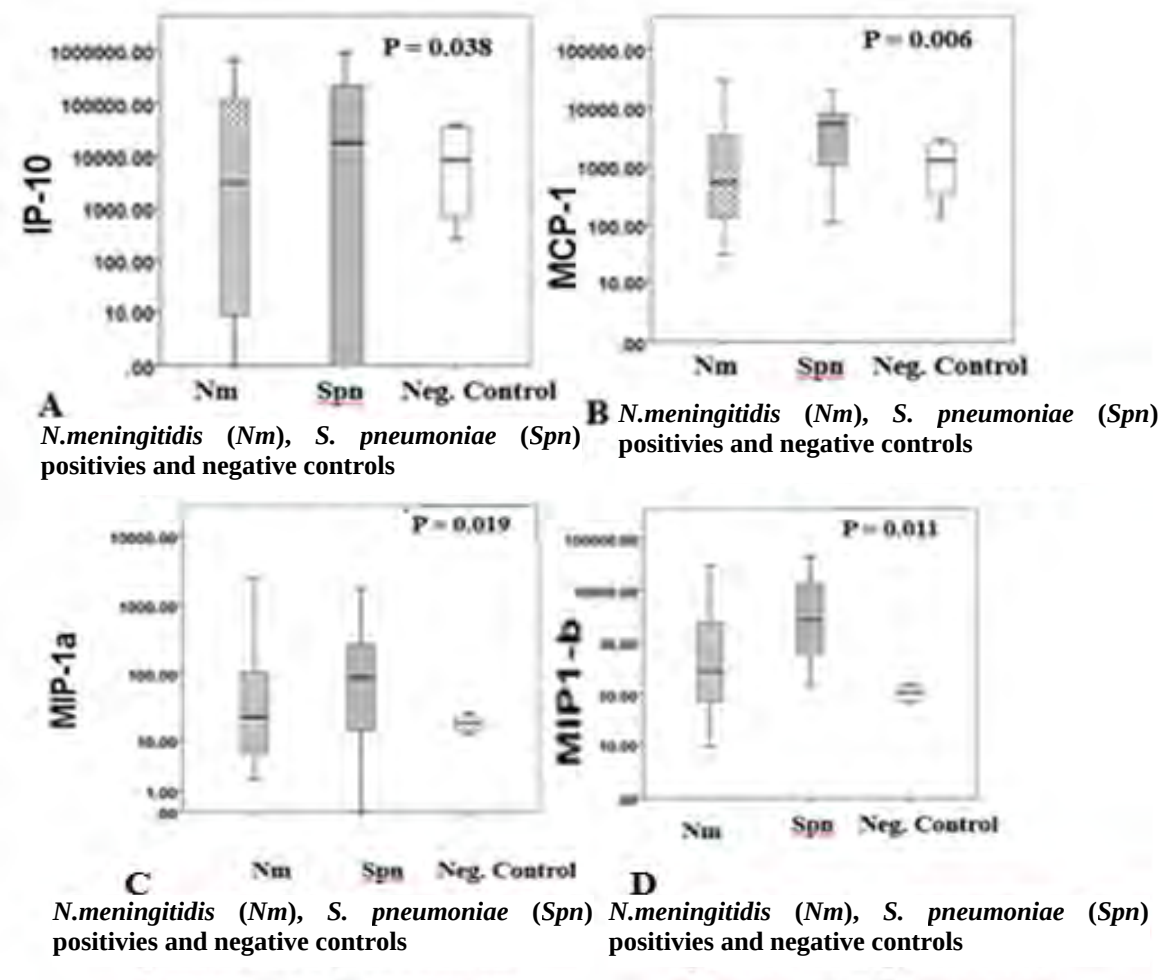
***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

Fig. 33: The levels of cytokines in *N. meningitidis, S. pneumoniae* positive BM patients and negative controls in Ethiopia, 2012-2013

33A: IL-8; 33 B: IFN-γ; 33 C: IL4; 33 D: IL-12/p70

No statistical significance (P > 0.05) was showed between *N. meningitidis* and *S.pneumoniae* positive BM patients with respect to IL-8 level though they both have higher levels compared to the negative controls (Fig. 33A). Levels of IP-10 (Fig. 34A), MCP-1 (Fig. 34B), MIP-1α (Fig. 34C) and MIP-1β (Fig. 34D) were significantly high (P

< 0.05) in BM patients positive for *S. pneumoniae* compared to those positive for *N.meningitidis* in reference to the negative controls.



***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

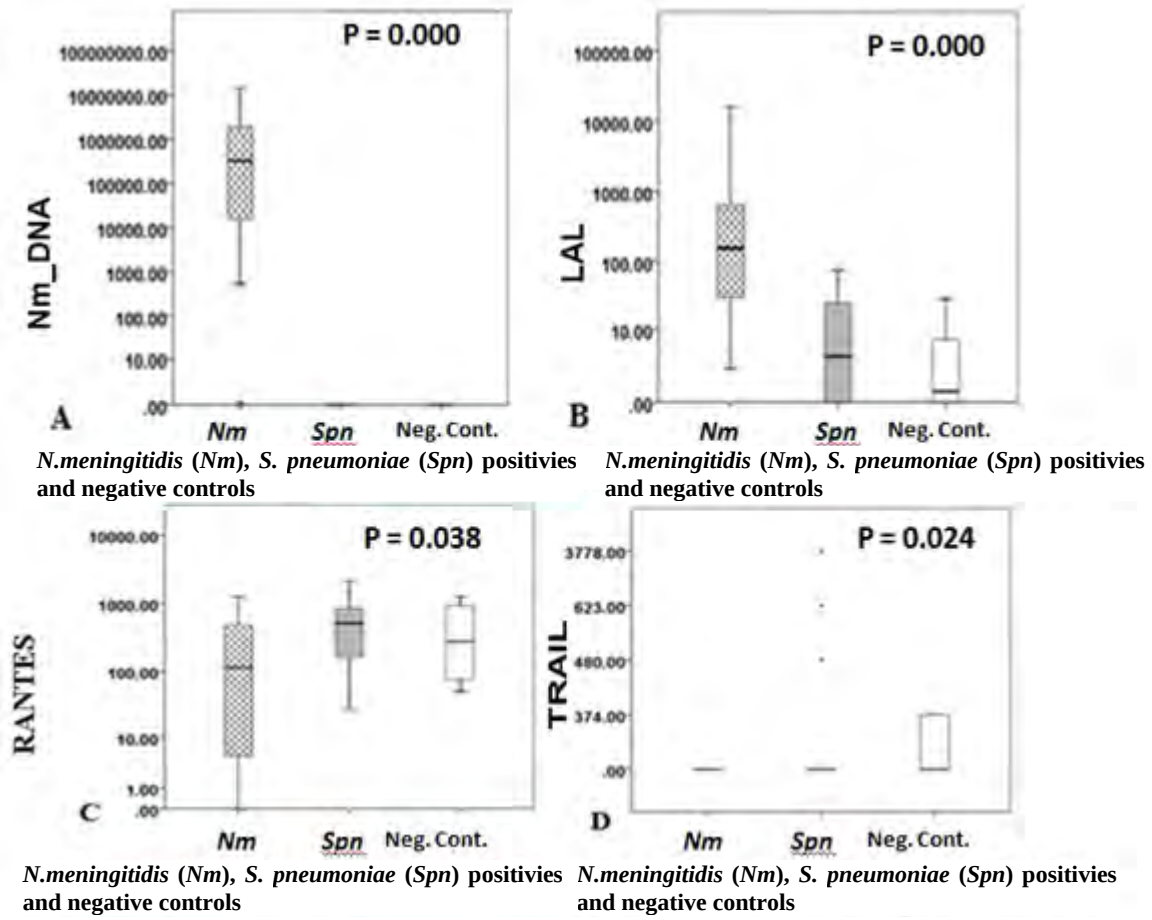
***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

Fig. 34: The levels of Chemokines in *N. meningitidis, S. pneumoniae* positive BM patients and negative controls in Ethiopia, 2012-2013

Figs. 34A: IP-10; 34B: MCP-1; 34C: MIP1α; and 34D: MIP1-β

Similarly, the level of RANTES (Fig. 35C) in *S. pneumoniae* positive BM patients was also significantly high (P < 0.05) compared to *N. meningitidis* positive BM patients in

reference to the negative controls. Whereas, three *S. pneumoniae* positive BM patients who are outliers were having significantly ($P < 0.05$) detectable level of TRAIL as compared to those patients with meningococcal meningitis (Fig. 35D) in reference to the negative controls.



***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

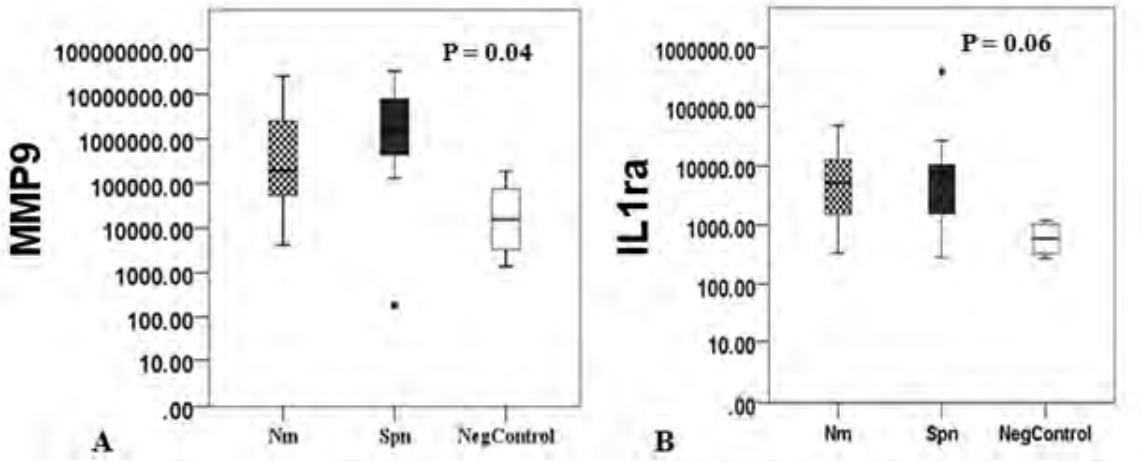
***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

Fig. 35: The levels of bacterial DNA, endotoxin, human Chemokines in *N. meningitidis, S. pneumoniae* positive BM patients and negative controls in Ethiopia, 2012-2013

Figs. 35A: Nm-DNA; 35B: LAL; 35C: RANTES; 35D: TRAIL

Obviously *N. meningitidis* DNA copy numbers (Fig. 35A) were significantly high (P < 0.05) among *N. meningitidis* positive BM patients compared to *S. pneumoniae* positive BM patients in reference to the negative controls. Meningococcal DNA was also found only in meningococcal meningitis patients as expected to be and also confirming the absence of contamination in the other groups. Levels of Limulus ameobocyte lysate (LAL) (Fig. 35B) or endotoxin activity were significantly higher (P < 0.05) in *N.meningitidis* positive BM patients compared to *S. pneumoniae* positive BM patients in reference to the negative controls. The levels of MMP-9 were also significantly high (P < 0.05) in *S. pneumoniae* positive BM patients compared to *N. meningitidis* positive BM patients in reference to the negative controls (Fig. 36A).



***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls** ***N.meningitidis* (*Nm*), *S. pneumoniae* (*Spn*) positivies and negative controls**

Fig. 36: The levels of MMP-9 (A) and IL1ra (B) in *N. meningitidis* and *S. pneumoniae* positive BM patients and negative controls in Ethiopia, 2012-2013

No statistically significant association (P > 0.05) was found in the levels of IL1ra between *S. pneumoniae* and *N. meningitidis* positive BM patients in reference to the negative controls (Fig. 36B). Correlations observed between levels of bacterial endotoxin activity (LAL) and IL-1ra (r = 0.464, P = 0.017) as well as LAL and MMP-9 (r = .495, P = 0.009) in *N. meningitidis* positive BM patients were statistically significant (Figs. 37A and 37B respectively).

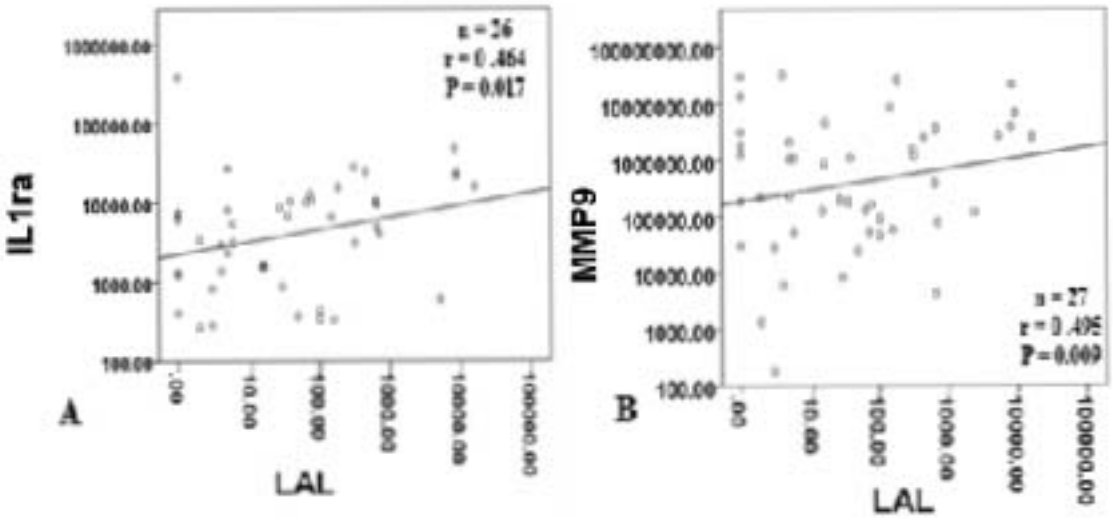


Fig. 37: Correlation of levels of LAL with IL1ra (A) and MMP9 (B) in *N. meningitidis* positive BM patients in Ethiopia, 2012-2013

6.10 The profiles of inflammatory mediators in the CSF of bacterial meningitis patients who either died or survived

Median levels of MMP-9 and IL-1b were significantly elevated in died *S. pneumoniae* positive BM patients as compared to *S. pneumoniae* positive BM patients who survived (P < 0.05, Table 17). The levels of Teichoic, Lipoteichoic acids, and MCP_1_MCAF also showed increases in trends among died BM patients confirmed positive for *S.pneumoniae*

as compared to the survived ones with similar pathogen (Data not showed in Table). Deceased pneumococcal meningitis patients had significantly higher median levels of IL-1β (P=0.04) and MMP-9 (P=0.02) than the survivors (Table 17). No statistical significance was found in levels of MMP-9 in *N. meningitidis* positive patients who were deceased compared to the survived ones though an increasing trend was shown in the former (Table 17) ones.

Table 17: Median MMP_9 and IL_1b levels in died versus survived *S. pneumoniae* and *N. meningitidis* positive BM patients in reference with negative controls, 2012-2013

Types of inflammatory mediators	**Died Median (Min. - Max.)**	**Survived Median (Min.- Max.)**	**Mann-Whitney**
MMP_9 (*Spn*)	**21.262x10^6** (13.348x10^6-30.267 x10^6)	**11.653x10^5** (183-32.842x10^6)	**0.021**
MMP_9 (*Nm*)	**226386** (47025-15.259x10^5)	**193913** (4142-25.547x10^6)	0.845
MMP_9 (Control)	**1368 (1368-1368)**	**18885 (22-188063)**	1.000
IL_1b_(*Spn*)	**3607** (5346-2665)	433.5 (7-6959)	**0.04**
IL_1b (*Nm*)	111 (8-1548)	702 (19-239179)	0.315
IL_1b_(Control)	3 (3-3)	**640.5** (.00-1194)	1:00

Since data for sequelae was recorded during admission at the hospital, whether certain percentage of the survived patients developed sequelae at a later stage is not known. A trend of elevated levels of median MMP_9 (Fig. 38A), MCP1 (Fig. 38B) and TNF-α (data not shown in graphs for TNFα) were observed in died BM patients with pooled pathogen compared to the survived ones.

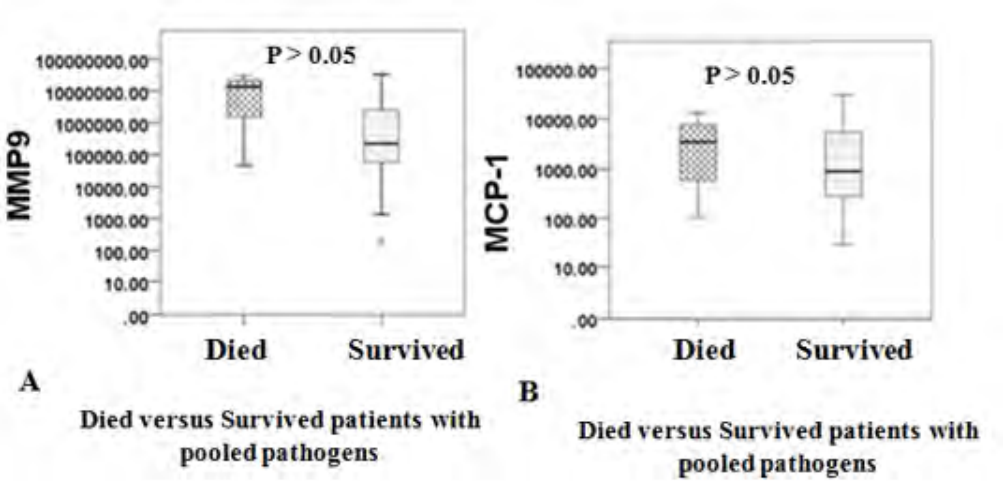


Fig. 38: The median levels of MMP-9 (A) and MCP1 (B) in fatal versus non-fatal cases of BM with pooled pathogen

Spearman non-parametric correlation analysis was carried out in endotoxin activity (LAL) in the CSF of *N. meningitidis* positive BM patients. The levels of endotoxin activity was significantly correlated to CSF *Nm*DNA (r = 0.45, P = 0.03, n=23), IL-1ra (r=0.46, P=0.02, n = 26) and MMP-9 (r = 0.50, P = 0.009, n=27) (Table 18).

Table 18: Correlation between IL1ra, MMP9 and LAL test results in *N. meningitidis* confirmed positive patients: 2012-2013

Factor 1	Factor 2	P value	r value	N	Conclusion
LAL	IL1ra	0.017	0.464	26	Significant direct correlation
LAL	MMP9	0.009	0.495	27	Significant direct correlation

The correlations between these variables (Table 18) are direct since all the figures shown are positive.

6.11 Meningococcal DNA, endotoxin (LPS) activity (LAL), clinical and immunological profiles in *N. meningitidis* confirmed positive bacterial meningitis patients

Only 23 patients had detectable CSF *Nm*DNA in the circulation and this group had a median of 8.2 x 10^5 (range < 2.5 x 10^2-1.4x10^7) copies of *Nm*DNA per ml. In 6 out of the 23 (26%) patients matched serum samples, detectable levels of *N. meningitidis* DNA was found with median concentration of 2.1 x10^4 (range 6.4 x10^3-1.8 x 10^5) copies per mL. The difference in copies of NmDNA between the CSF and serum was statistically significant (P < 0.05). The overall general clinical conditions of the patients and the number of copies of *N. meningitidis* DNA in their CSF also have statistically significant difference (P = 0.03, Fig 39A).

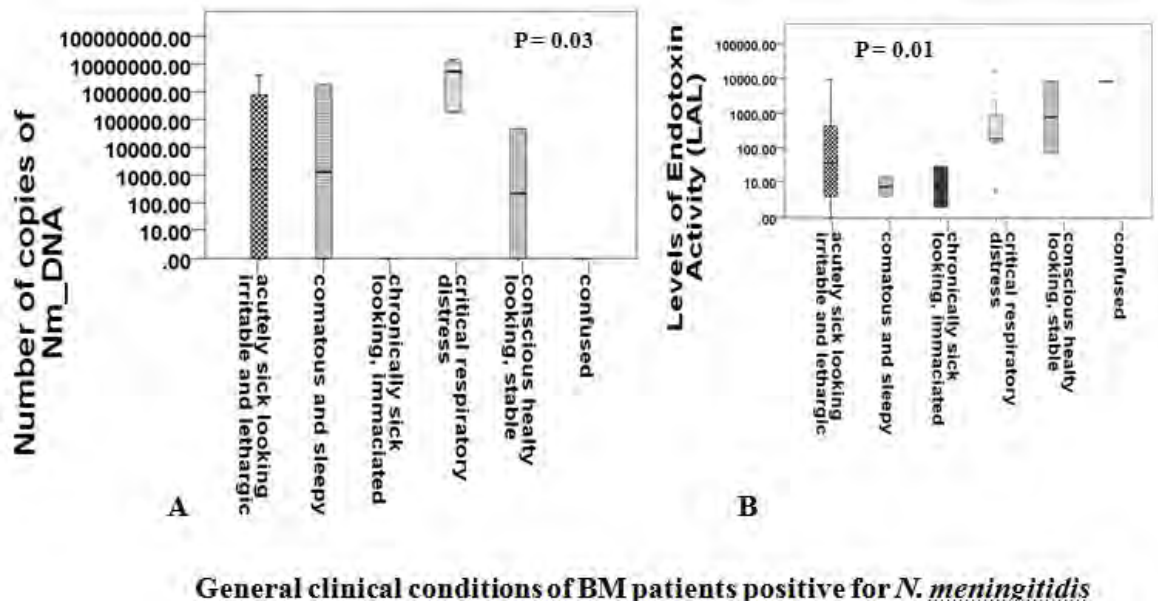


Fig. 39: General Clinical Conditions of BM patients confirmed positive for *N. meningitidis* and the association with the number of copies of NmDNA (A) and Endotoxin activity (LAL) (B)

Similarly the median endotoxin activities (LAL) among *N. meningitidis* positive BM patients was significantly different (P = 0.01) across multiple categories of general clinical conditions (Fig. 39 B). The median number of copies of *N. meningitidis* DNA was highest among BM patients with critical respiratory distress while the median levels of endotoxin activity was highest in confused BM patients compared to the other general clinical conditions (Fig. 39A and B). Patients with clinical conditions of "critical respiratory distress" have consistently maintained high levels of *N. meningitidis* DNA as well as endotoxin activity (Fig. 39 A and B). Significant differences were not observed in the levels of endotoxin activity, *Nm*DNA load and levels of cytokines, chemokines, or MMP-9 in the CSF across the different gender or age groups, or between deceased and surviving patients. No correlations were found between age and endotoxin activity as well as *Nm*DNA, whereas a negative correlation was apparent between age and levels of IL-18 (r=-0.45, P=0.02, n=26).

On the other hand, *Nm*DNA copy number was positively correlated with IL-1β (r=0.62, P=0.002, n=23) and MMP-9 (r=0.46, P=0.03, n=23) while negatively correlated with IL-6 (r=-0.45, P=0.03, n=23).

When the median copy number of *Nm*DNA in the CSF was compared to the copy number in the serum, the one in the CSF was 10-1000 folds higher. Correlations between meningococcal patients DNA concentration and cytokines/chemokines in the CSF were evaluated by Spearman's test (Table 19).

Table 19: Correlation between IL-6, IL-12, RANTES and *N. meningitidis* DNA concentration in bacterial meningitis patients in Ethiopia: 2012-2013

Factor	**Factor 2**	**P value**	**r value**	***N***	***Conclusion***
DNA	IL-6	0.006	-0.533	25	Significant inverse correlation
DNA	IL-12	0.05	-0.397	25	Significant inverse correlation
DNA	RANTES	0.038	-0.417	25	Significant inverse correlation

Significant inverse correlations were found between CSF meningococcal DNA and IL-6 (P =0.006), IL-12 (0.05) and RANTES (P = 0.04). The copy number of NmDNA in CSF was consistently higher than those observed in serum among 21 of the *N. meningitidis* positive patients with paired samples from serum and CSF. The highest level in CSF was $1.4x10^7$copies per mL with a corresponding serum level of $<1.0x10^5$ per mL. The highest serum level was $1.8x10^5$per mL with a corresponding CSF level of $3.0x10^6$ per mL.

7. DISCUSSION

The study has focused on patients who visited 3 University hospitals and clinically diagnosed with BM. The strength of the current study compared to previous ones carried out in the country is that the current study has determined etiologic agents of BM at multiple sites at the same time especially during non-epidemic seasons. Dominantly circulating meningococcal serogroups especially at the sites facing frequent epidemics (Gondar and Hawassa) were also addressed. Besides this, use of real time-PCR detection of BM etiologic agents, MLST typing and CSF transport medium to achieve better survival of strains have empowered the study to hit its targets. Measuring levels of cytokines, chemokines, endotoxin activities (LAL) and meningococcal DNA are the strong inputs that are used as landmarks to analyze levels of disease severity.

Age grouping used in the current study was constructed for the objective of observing detailed frequency of BM etiologic agents, clinical conditions, sequelae and death that were experienced in a higher frequency at the younger age groups of patients (≤ 4 and 5-12) compared to the olders. With regard to socio-demographic and clinical characteristics of the study participants, younger age (age range ≤ 4 seconded by 5-12) than older ones, more males than females, patients infected with *S. pneumoniae* than *N. meningitidis* were found out to be the population who faced the highest level of disease severity determined in terms of death or sequelae in survivors.

Patient generalized clinical conditions were also considered to be analyzed with regard to disease severity, though not in much focus as that of death and sequelae. The current

study is not population based or did not have a population denominator such as a demographic surveillance site from where all patients came. The report from this study refers only to patients who visited the specified study site hospitals.

During enrollment of the patients, highest peaks were reached in number of patients in April 2013 followed by March 2013. The months May and June followed by April were having enrollment of relatively higher number of suspected patients with BM. In all the 17 months (Feb. 2012- June 2013) of the current study, suspected patients for BM were enrolled into the study. Except the excluded three months (October 2012, January 2013 and May 2013, within which no BM etiologic agents were detected from patient samples) out of the 17, the three BM etiologic agents were detected from patient samples enrolled in the 14 months. A trend towards a seasonal pattern peaking in April was observed in both years in the number of suspected BM patients admitted to the hospitals. Though several climatic zones exist within the country, the climate in Ethiopia is characterized by a dry season during November/December and typically peaking in February-March-April (García-Pando *et al.*, 2014).

During the non-epidemic seasons, low air temperatures and frequent rains may lead to increased crowding and risk of transmission of bacterial etiologic agents of meningitis also increases as shown by other studies (Hodgson *et al.*, 2001).

Number of deaths encountered in the current non-epidemic season study based on documentation on CRF was 20/139 (14.4%). Results from culture detection tests (based

on biochemical tests) performed at the research laboratory have indicated isolation of 14 (10.8%) frequent etiologic agents of meningitis (5 = *N. meningitidis*, 8 = *S. pneumoniae* and 1 = *H. influenzae*) out of the total 139 patients.

The high number of median CBC in the circulation and WBC in the CSF in *S.pneumoniae* positive BM patients compared to *N. meningitidis* positive patients in the sub-group of the patients showed a similar pattern with the rest of the findings especially with regard to the inflammatory profiles of cytokines and chemokines.

This study has shown detection of BM in 33.1% (46/139) of the study participants using RT-PCR (sensitive method). Molecular typing showed that serogroup A meningococci isolated in Ethiopia in 2012–2013 were the same ST (ST7) as those causing the 2002–2003 outbreaks; both expressed PorA P1.20,9 (Haimanot *et al.,* 1990; Norheim *et al.,* 2006). These isolates also showed the same two outer membrane protein variants of PorA and FetA that are typical of ST-7. The serogroup W-135 isolates were ST11 with PorA P1.5,2, the same found among outbreak strains in other meningitis belt countries (Daugla *et al.,* 2014; Novak *et al.,* 2012; Caugant *et al.,* 2012).

The strains from the current study during non-epidemic seasons are thus the ones detected during earlier epidemic season confirming that newly introduced serogroup A strains have not been identified. However, earlier studies have indicated that clones from the epidemic season were identified among patients diagnosed during non-epidemic season (Salih *et al.,* 1990). Although new serogroup A strains are not found in the current study,

such a study should however be carried out regularly for an updated intervention purposes.

The second major finding of this study is generating real time-PCR based laboratory data for *N. meningitidis* genogroup W-135 and X to be the additional meningococcal etiologic agents causing BM among 139 patients visiting 3 hospitals in Ethiopia during the non-epidemic season. *Neisseria meningitidis* genogroup W-135 have been detected by PCR at AHRI lab from two sporadic cases in 2010 from patients who have been admitted to hospitals localized in Addis Ababa (personal communication). The relatively higher number of W-135 (7, 26%) in the current study is one of the prominent findings. The finding about the emergence of this serogroup in higher number unlike the past is in line with other studies in other African meningitis belt countries (Du Châtelet *et al.,* 2005).

Although not in higher number, occurrence of one case of serogroup X (1, 3.7%) in this study is the other unprecedented alarm and a new finding from BM patients in Ethiopia especially during non-epidemic season. Finding of this serogroup as a disease causing agent even during an epidemic season, in other meningitis belt countries was reported as an extraordinary outcome (Boiser *et al.,* 2007). The geographic variation in the meningococcal serogroup distribution with dominance of serogroup W-135 cases in Gondar and of serogroup A in Hawassa will likely affect the impact of the monovalent serogroup A vaccine.

The detection of one case of serogroup X meningococcal disease in Hawassa, southern Ethiopia, and serogroup C in Gondar, in this limited study shows the need for further studies to determine the magnitude of the problem in countrywide scope. Previous report from Ethiopia has also documented *N. meningitidis* serogroups A and C as a cause of outbreaks or sporadic disease (Xie *et al.,* 2013). The presence of serogroups W-135 and X cases, in line with the observed trends of their rise in the rest of the meningitis belt countries, shows that they need to be considered in vaccine selection or preparation strategy. Serogroup X cases have occurred in neighboring countries like Kenya and Uganda in 2006, and in several West-African countries in the last decades (Xie *et al.,* 2013). In parallel with our finding these studies indicate the advent of meningococcal serogroup X. This is because of the fact that this serogroup was previously a rare cause of sporadic meningitis, which was only reported as disease causing agent of BM outbreaks between 2006 and 2010 in meningitis belt countries such as Kenya, Niger, Togo, Uganda and Burkina Faso (Jafri *et al.,* 2013, Delrieu *et al.,* 2011). Appearance of serogroup X for which no vaccine is tailored to date and which showed antigenic variation from other serogroups sparks an interest with regard to the effort in the need for vaccine preparation that composes this serogroup (Hong *et al.,* 2013).

The third finding refers to dominating PorA serosubtypes of frequent bacterial etiologic agents causing BM. Previous studies in dominating serosubtypes among disease-causing meningococci in Ethiopia were limited only to show the 1989 epidemic with approximately 50,000 cases (Haimanot *et al.,* 1990; Norheim *et al.,* 2006). According to

the report from the 1989 epidemic, the etiologic agent which caused the epidemic was serogroup A meningococci of ST-5. Whereas, the 2002-03 outbreaks were caused by serogroup A meningococci of ST-7. Multilocus sequence typing data on the meningococci identified in this study revealed that serogroup A ST-7 are still causing disease in Ethiopia. The Ethiopian serogroup W-135 meningococci were of ST-11 with PorA serosubtype P1.5, 2. This serosubtype is the same as those observed among outbreak strains from other African meningitis belt countries (Caugant *et al.,* 2012). The result referring to identification of one of the two serogroup A isolates belonging to ST-7, is in line with a previous study in 2002-03 (Norheim *et al.,* 2006). This isolate also showed the same two outer membrane protein variants of PorA and FetA that are typical of ST-7.

In general, the meningococcal cases in the current study are composing serogroups A, C, W-135 and X. The PorA serosubtypes of MenA cases in the current study are the same as that of serogroup A which occurred during 1988-89 out-break while the sequence type of (ST-7) is new. Both serogroup A isolates have originated from Hawassa. The genogroup W-135 isolate belonging to ST-11, is the same clone as has caused several outbreaks and one epidemic in West Africa (Caugant *et al.,* 2012; Haimanot *et al.,* 1990). This isolate was isolated from a patient in Gondar. Both strains were expressing PorA serosubtype P1.20, 9 (Haimanot *et al.,* 1990; Norheim *et al.,* 2006). The STs found among the 3 isolates typed in this study were the same as observed in a recent pilot study in Ethiopia (Fentaw, 2013).

World health organization guideline (2014) based on studies by Boisier *et al.* (2009) and Borel *et al.* (2006), showed that PCR diagnostic assay or together with culture detection can be accepted as gold standard confirmatory diagnostic assay for detection of BM etiologic agents. Using this method of diagnosis is also recommended when antimicrobial therapy has been administered on patients and culture growth is inhibited (Sacchi *et al.*, 2011; Nagarathna *et al.*, 2012; Deutch *et al.*, 2006).

The guide line advises that the confirmatory diagnostic test result is strongly recommended to be carried out after rapid diagnostic tests (RDT) prior to initiation of vaccine (WHO, 2014). Other recent hospital-based retrospective study conducted at four teaching University hospitals in different regions of Ethiopia reported the continued occurrence of this challenge indicating reduced sensitivity of clinical diagnostic procedures especially in patients with severe neurological emergency disease such as BM (Gudina *et al.*, 2016).

Worsening outcome of BM may be due to the prevailing diagnostic dilemma and poor sensitivity of the standard diagnostic microbiology test (Brouwer *et al.*, 2012). If results from the current study's culture detection/biochemical diagnostic approach (10.1%) of BM etiologies were used for country wide report, it's obvious that the actual magnitude (32.1%) of BM etiologies according to real time-PCR detection would have been three times under-estimated. Hence, the study has confirmed the reality of the event of underestimation of reports due to diagnostic challenges. The problem would not merely remain a matter of under-estimation but also missing the circulating serotypes of the

etiologic agents which may be among the misdiagnosed agents. This has got a negative impact in vaccine serotype selection or tailoring since it with hold updating of vaccine based on the actual need of a target population. A recent study in Italy has reflected presence of similar challenge of under-estimation of actual magnitude of etiologic agents of BM (Azzari *et al.*, 2016), a situation which bias proper vaccine selection.

Diagnostic challenges are also loopholes for disruption of proper patient management. The recent study in Gondar (Ethiopia) by Gudina *et al.*, (2016), revealed that etiologic agents of BM were identified by clinical lab merely from 14/425 (3.3%) patients clinically diagnosed to have BM (Gudina *et al.*, 2016). Valuability of the biochemical test with respect to patient management purpose in developing countries like Ethiopia is not questionable even with its less sensitive diagnostic capacity.

The argument here is however about adapting the highly sensitive real time-PCR method for a relatively accurate reporting of country wide data, in which vaccine selection or tailoring depends on. Though usage of such highly sensitive molecular assay is not feasible for routine hospital lab diagnosis, it is anticipated that using the method would be essential for reporting of country wide data (WHO, 2011).

This situation has also been successfully applied in other meningitis belt countries and is being practiced as part of their national sentinel surveillance (WHO, 2011). Using this assay during analysis of samples from the weekly meningitis epidemic registry initiated in

Ethiopia could contribute and impact the evaluation of the MenAfriVac vaccine (Haimanot *et al.,* 1990; Norheim *et al.,* 2006).

In the current study, the hospital lab culture detection test rreal time RT-PCR test results have under-estimated the actual scenario. This is because of the finding in statistical sub-analysis which was carried out in comparing culture detection test results of the hospital and research labs on the 50 study participants. The results have showed that the research lab culture detection test outputs have achieved higher sensitivity, specificity, PPV and NPV compared to the hospital labs. This situation highlights presence of a gap in culture detection especially in the hospital lab showing an under-estimated picture as reported also from other resource poor countries in a study carried out from 2002-2010 (Maïnassara *et al.,* 2014; Gudina *et al.,* 2016). This occurs as a result of various limitations as reported by other studies among countries of the meningitis belt (Collard *et al.,* 2014). Retrospective study by Ahmed (2012) in Gondar and Hawassa has also reported that the challenge in BM hospital lab diagnosis may be due to limitation of resources and lack of properly trained staff.

This study, in line with other studies (Coutinho *et al.,* 2013; Grandgirard *et al.,* 2013), has confirmed that elevated disease severity with BM was induced by pneumococcus as compared to meningococcus. Moreover, in the current study the CSF was analyzed for lipopolysaccharide, a total of 18 different cytokines, chemokines and MMP9. The lipopolysaccharide levels in CSF were significantly correlated to the NmDNA copy number ($r = 0.45$, $P < 0.05$), interleukin-1 receptor antagonist ($r=0.46$, $P < 0.05$) and

MMP9 ($r = 0.50$, $P < 0.01$). Interleukin-4 (IL-4), IL-12p70, INF-γ, interferon gamma-induced protein 10, monocyte chemoattractant protein-1, macrophage inflammatory proteins 1α and 1β, RANTES and matrix metalloproteinase-9 were all significantly high ($P < 0.05$) in the pneumococcal than in the meningococcal group of BM patients suggesting that a specific pneumococcal cytokine profile may exist and possibly associated with a worse outcome.

The point in parallel finding of this study with the two previous studies (Coutinho *et al.,* 2013; Grandgirard *et al.,* 2013) is that infections with *S. pneumoniae* appear to have a worse outcome of disease severity than infections with *N. meningitidis.* Moreover, significant level of correlation ($P < 0.05$) was seen between levels of disease severity (with an outcome of death) and MMP-9 in *S. pneumoniae* positive BM patients as compared to *N. meningitidis* positive BM patients. A trend of high level of elevation of MMP-9 was also detected in *N. meningitidis* positive e died patients as compared to the survived ones, though no statistical significance was obtained. Similarly, levels of MMP-9 were also shown to have increasing trend in survived patients with sequelae as compared to those without sequelae. Reports from parallel studies showed that high concentration of MMP-9 is a risk factor for the development of post-meningitidal neurological sequelae (Leppert *et al.,* 2000). In the current study levels of LPS, endotoxin activity/LAL in the CSF of BM patients infected with *N. meningitidis* were significantly correlated with production of MMP-9 ($P < 0.01$) as well as IL1ra ($P < 0.02$).

In general, as has been observed in findings from other African and industrialized countries studies, patients with meningitis caused by *S. pneumoniae* has a higher case fatality rate and more sequelae than patients with *N. meningitidis* meningitis (Ramakrishnan *et al.,* 2009). The biological basis explaining this difference has not been yet fully delineated.

One hypothesis is that the inflammatory responses to the Gram negative *N. meningitidis* and the Gram positive *S. pneumoniae* in the CSF are different and may be reflected in the outcome induced on patients (Coutinho *et al.*, 2013; Grandgirard *et al.,* 2013). The studies on the Brazil's and Burkina Faso's BM patients have analyzed levels of inflammatory profiles in the CSF and correlate them with disease severity on patients. The study by the sixteen Brazilian patients with pneumococcal meningitis had significantly higher levels of interferon-γ in the CSF as compared with the twelve meningococcal meningitis patients (Coutinho *et al.*, 2013).

Similar with the findings of the current study, fourteen *S. pneumoniae* positive BM patients from Burkina had significantly higher levels of INF-γ, MCP-1 and MMP-9 compared to twenty two *N. meningitidis* positive BM patients. This finding suggests possible presence of different cytokine profile, "signature" (Grandgirard *et al.*, 2013).

With this regard, other studies suggest the reason for the increment in the levels of inflammatory cytokines and chemokines in BM patients is to promote the protective scenario of both innate and adaptive immune systems (Bailey *et al.,* 2006). However this

direction may at times divert to an invasive outcome with deleterious consequences resulting in death and sequelae in survivors (Nau and Brück, 2002). As reported in a study by Van de Beek *et al.* (2010), presence of reduced anti-inflammatory response was reported in patients with pneumococcal meningitis with median age of 9 months after adjunctive steroid administration (Arditi *et al.,* 1998; Van de Beek *et al.,* 2006).

The CSF in the current study was collected prior to steroid administration and the status of the anti-inflammatory cytokines e.g. IL-4 and IL-10 were still high in *S. pneumoniae* positive BM patients compared to the *N. meningitidis* positive BM patients unlike the report from Arditi and Van de Beek. On the other hand, in parallel with the finding of the current study, Kornelisse *et al.* (1997) has observed significantly higher level of elevation of IFN-γ in *S. pneumoniae* positive than *N. meningitidis* positive BM patients.

Hessle *et al.* (2000) has suggested that the increased production of IFN-γ in *S.pneumoniae* positive BM patients may be induced by IL-12, with TNF- α as a co-stimulator as infection with Gram-positive bacteria induce much more IL-12 than Gram-negative bacteria. In contrary to the findings of the current study, similar patterns of IL-4 were found in the brains of experimental animals infected with *S. pneumoniae* and *H. influenzae* (Diab *et al.,* 1997). Differences in levels of cytokine profiles induced by two bacterial strains may be interpreted with different immune-modulating approaches depending on the etiologic agent of the disease (Diab *et al.,* 1997).

The result from the current study indicates the regulatory effort of the immune system by increasing the production of anti-inflammatory cytokines during profuse production of

inflammatory cytokines so as to buffer the system in the absence of steroid administration, which would have been supporting the regulatory function when introduced to the patient.

The current study is the first detailed study of molecular inflammatory responses of meningococcal meningitis in East Africa. Further studies are needed in search for new inflammatory markers in patients with pneumococcal meningitis as compared with meningococcal meningitis. In the current study the median levels of proinflammatory cytokines such as IL-6, IL-12, INF-γ, IL-1b, IL-1 and chemokines such as IP-10 have shown elevation during a rise in MMP-9 indicating the presence of active infection. Where MMP-9 was elevated, the levels of MCP-1/MCAF, MIP-1α, RANTES and TNF-α were also increased especially in the died patients. In the current study the levels of RANTES produced in *S. pneumoniae* positive BM patients was significantly high compared to that of *N. meningitidis* positive patients though not to the negative controls which is a scenario that may need further explanation.

Studies show that the levels of production of RANTES are significantly higher in all forms of bacterial stimuli. Predominance in Gram-positive bacteria showed a higher level of RANTES compared to control group though the difference was not significant (Rammal *et al.,* 2017). Secretion of this chemokine by human cells of the CNS is dependent on the pathogen used to stimulate the cells (Fowler *et al.,* 2004).

As in the finding of the current study MMP9 was having significantly high levels of production in *S. pneumoniae* positive BM patients compared to that of *N. meningitidis*

positives and negative controls. This may be explained with regard to the findings of the study by Prager *et al.* (2017) showing that *S. pneumoniae* causes direct cytotoxic damage by employing H_2O_2, which reacts with the host's nitric oxide to form peroxynitrite. Peroxynitrite is a highly reactive oxidant that can be cytotoxic by a number of mechanisms that induces production of cytokines and MMPs as well as loss of membrane function and integrity Prager *et al.*, (2017).

The study by Grandgirard *et al.* (2013) has showed parallel findings of elevation of proinflammatory cytokines and chemokines in died pneumococcal meningitis patients as compared to the survived ones. In parallel to the findings by Grandgirard *et al.* (2013) the current study has also showed significant level of elevation ($P < 0.05$) of median MMP-9 in *S. pneumoniae* positive died patients compared to survived one's. The significant correlation found between LPS and IL-1b ($P < .005$, $r = .455$), as well as LPS and IL-1ra ($P < .005$, $r = .431$) in *N. meningitidis* positive patients of the current study seems indicative of sepsis. Although the levels of IL-1rα in this study as well as in other studies (Coutinho *et al.,* 2013; Grandgirard *et al.,* 2013) were found to be significantly higher in BM patients positive for *S. pneumoniae* than those patients positive for *N. meningitidis*, the pathogenic mechanism resulting with higher level of disease severity in the patients with the former pathogen is not yet elucidated.

Moreover, profiles of inflammatory mediators as well as MMP-9 in the CSF of patients who died as well as survived with sequelae were higher as compared to survived patients. Only six out of 23 (26%) of the *N. meningitidis* positive BM patients had detectable

NmDNA in the circulation. This group had the significantly higher levels of NmDNA in the CSF as compared to the remaining 17 patients without detectable NmDNA. A possible spillover of NmDNA is suggested to occur from the CSF to the circulation across ruptured BBB. This result from the DNA quantification has also confirmed the compartmentalized nature of meningococcal infection. The corresponding relationship between the levels of *N meningitidis* LPS and *N. meningitidis* DNA copy numbers in the CSF of patients participated in this study, the corresponding inflammatory response obtained by quantifying 18 different cytokines, chemokines and MMP-9 have showed an interesting result which can attract more attention to this line of research. The specific cytokine and chemokine profiles in pneumococcal meningitis in the current study as well as the two previous studies (Coutinho *et al.*, 2013; Grandgirard *et al.*, 2013) may call for future studies of specific mediators in an effort to better understand the underlying pathophysiology explaining the variation in status of disease severity.

Disease severity with regard to levels of cytokines and chemokines may also be associated with patient clinical condition (Ovstebo *et al.*, 2004). This study has showed that frequency of acutely sick looking, irritable and lethargic clinical condition was high at earlier age. The varied types of clinical conditions of patients enrolled into the current study were found to have statistically significant differences with number of copies of *N.meningitidis* DNA ($P = 0.03$) and endotoxin activity ($P = 0.01$). A study by Ovstebo *et al.* (2013) has also shown a parallel result indicating that the number of meningococci in

plasma and CSF appears to be the main determinant of the lipopolysaccharide levels, clinical presentation, and outcome.

The findings from the current study showing about disease severity in patients infected with *S. pneumoniae* as compared to patients infected with *N. meningitidis*, alarms for effective early pneumococcal vaccine administration. Such effort would also be of valuable importance with respect to establishment of herd immunity. Using improved chemotherapeutic intervention may also mitigate disease severity of BM (Van Furth *et al.,* 1996). In reference to the finding of this study, it is of interest to know whether the conjugate vaccines against *N. meningitidis* serogroup A and the ten serotypes of *S.pneumoniae* in Ethiopia will protect against the majority of BM cases caused by the different serotypes of the referred pathogens. For instance, vaccination of MenAfriVac was implemented in western areas (target population 29 million in 2013) of Ethiopia, southern and central part (target population of 27 million in 2014), and east and north eastern part (target population of 17 million in 2015) (WHO, 2014).

Assuming that this study is representative, a monovalent serogroup A vaccine which is administered even recently from 2013-2015 will only cover 40% (11/27) of at least the population (who came as cases) visiting referral hospitals (i.e., the cases in Southern areas of Ethiopia and in Addis Ababa). This is because about 60% of the remaining cases diagnosed with *N. meningitidis* serogroups W-135 (7/27), NG (7/27), C (1/27) and X (1/27) would have remained unprotected if the patients were included in the MenAfrivac mass vaccine campaign.

However, the high proportion of serogroup W-135 meningococci in northern Ethiopia is worrying, as is the serogroup X case due to the lack of vaccine that incorporate these serogroups. This calls for a fast-track approval process for affordable, multivalent polysaccharide vaccines such as ACW-135 polysaccharide vaccine to combat serogroup W-135 outbreaks, as done in Burkina Faso in 2012 (WHO, 2013) or quadrivalent conjugate vaccines against serogroups A, C, Y, W-135 and X for the meningitis belt countries as also suggested by some other studies (Xie *et al.,* 2013).

Though disease severity with regard to levels of cytokines and chemokines may not be always associated with patient clinical condition, this study has showed that frequency of patient clinical condition such as acutely sick looking, irritable and lethargic has increased in the extreme ages proving susceptibility to the disease in the youngest and oldest age groups.

On the other hand, as meningococcal meningitis is a compartmentalized infection (Brandtzaeg *et al.,* 1992), this study has also showed the presence of significantly higher levels of bacteria in the subarachnoid space than in the blood. This is showed by the copy numbers of NmDNA which was found in 10 to 1000-fold higher levels in CSF than in serum in the 21 Ethiopian meningococcal meningitis patients. The patients in the current study had marked clinical symptoms of meningitis but no septic shock. Other studies indicate that genetic constitution of certain patients might allow for more intense proliferation of the bacteria in both blood and CSF (Hellerud *et al.,* 2010). Such pattern

of bacterial load was shown in the CSF and serum in patients in Norway (Brandtzaeg *et al.*, 1992; Hellerud *et al.*, 2010).

Further investigation is needed to see the situation on this line in the population of African meningitis belt countries including Ethiopia. In the current study, proliferation of meningococcus in blood didn't reach levels associated with shock. This is because none of the patients with quantifiable copy number of meningococcal DNA reached 10^6 copies per mL. Previous studies suggest that patients passing this level of meningococcemia are predisposed to development of septic shock (Øvstebø *et al.*, 2004; Brandtzaeg *et al.*, 2012). Unlike patients with meningitis, many patients with meningococcal septicemia and persistent shock reveal levels of NmDNA copy numbers as high as 10^7 –10^8 per mL plasma or serum (Øvstebø *et al.*, 2004; Brandtzaeg *et al.*, 2012).

The outcome from this study can be a springboard for designing a large scale study for running assessment of the burden of BM and disease severity. This requires a national case-based surveillance system, combined with laboratory testing of CSFs using rapid and sensitive methods (Chanteau *et al.*, 2006).

8. CONCLUSIONS

1. *Streptococcus pneumoniae* and *N. meningitidis* were found to be the causative pathogens of BM detected from CSF samples collected from 139 patients suspected with BM during non-epidemic season (2012-2013) in Ethiopia. The finding of serogroup W-135 and X in addition to serogroup A is in contrast with previous reports and calls for continued alert for appropriate vaccine selection or development.

2. Infections with *S. pneumoniae* have more severe outcomes than infections with *N.meningitidis* as reported from South America and Burkina Faso.

3. Culture detection method has showed an under-estimated frequency of *S. pneumoniae* and *N. meningitidis*, the most frequently associated etiological agents of BM, as in the finding by use of real time-PCR detection in the current study. Hence, molecular detection particularly, real time-PCR has showed the highest sensitivity in detecting BM etiologic agents.

4. Multilocus sequence typing data on the meningococci identified in this study revealed that serogroup A ST-7 are still causing disease in Ethiopia.

5. Alerting health care workers, health policy makers and vaccine protection unit is necessary by notifying the emergence of new serogroups, which has not been reported before at the specified geographical location. The high proportion of serogroup W-135 meningococci in northern Ethiopia, which may be part of future outbreaks is a worrying finding (unlike bivalent so far). Moreover, almost 60% of the meningococcal meningitis

which was detected in the current study belongs to the serogroups other than A. A fast-track approval process for affordable, multivalent polysaccharide vaccines such as ACW-135 polysaccharide vaccine is suggested. Appearance of serogroup X, for which no vaccine is yet tailored, in patients of BM is also setting an alarm.

6. The reasons for underestimated magnitude of the disease in previous reports are due to delayed lab diagnosis and usage of results from less sensitive routine diagnostic methods for reporting purposes.

9. RECOMMENDATIONS

1. Employing sensitive diagnostic methods such as real time PCR, which increased the proportion of patients confirmed with bacterial etiological agents of meningitis is recommended during population based surveillance studies. The finding from this study promotes the idea of real-time-PCR positivity for diagnosing bacterial etiologic agent of meningitis as consensus gold standard in the presence or absence of culture diagnostic assay as recommended by WHO, 2014. Use of sample transportation media is also recommended for recovery of organisms if CSF could not be immediately inoculated into culture media, in order to facilitate analysis of the genotype. Health care workers need also to be alert about sample handling and urgency of laboratory diagnosis for such emergency diseases.Urgent and careful attention should be taken by health care workers in prioritizing bacteriological detection through timely inoculation of CSF into culture media not to miss the otherwise fastidious organisms.

2. Continuous studies of this type is essential especially in countries of the meningitis belt to design the strategy of preparing tailored vaccine for timely protection and prevention. The output from this study will be a good ground for planning wider scope study of similar objective to step towards selection of the right type of serogroup combination in future vaccine clinical trials. Quadrivalent conjugate vaccines composing serogroups A, C, Y, W-135 and are suggested for the meningitis belt countries. The idea of incorporation of serogroup X vaccine into the other combinations soon as it gets prepared is also recomeneded.

3. The laboratory verified analyses of CSF samples and case-based demographic data processed in this study could be baseline for further hospital based studies in the same areas where the prevalence of Men A would be expected to drop due to vaccination with MenAfriVac. The study has also showed the need to closely monitor the impact of the MenAfriVac monovalent serogroup A vaccine in Ethiopia with regard to coverage of the serogroups other than A.

4. Early childhood *S. pneumoniae* vaccine administration and evaluation of the efficacy of protection in the population is a matter which needs more attention. Similar type of vaccine given in previous years may not give effective protection. Selection or programming for tailoring of appropriate vaccine composing antigens against altered etiologic agent/s and strains is needed. Reinforcement of the population based surveillance of BM during non-epidemic seasons in Ethiopia is warranted, focusing on case-based reports with laboratory confirmed etiology using sensitive diagnostic methods as in this study and other meningitis belt countries.

5. An expanded network of hospitals and larger sample size is required to give more reliable estimates for the meningococcal serogroup distribution and pneumococcal isolates across Ethiopia. Wider scope of such a study with larger number of sample size is needed since the data generated will be useful to select tailored vaccine that encompasses the circulating serogroups both in epidemic and non-epidemic seasons. Proper data recording and handling need to be strengthened especially by hospital laboratory personnel since they are base line source data on which reports and subsequent outcomes

are relying. More focus should be given for elaborated task and work force in order to cover the population at risk with the timely tailored vaccine composing upcoming strains highlighted in this study. Upgraded therapeutic intervention composing down regulating elements of elevated levels of pro-inflammatory cytokines may mitigate disease severity in BM patients.

10. LIMITATIONS OF THE STUDY

The routine high turnover of health care workers has cost the study with repeated orientation for newly incoming batches of health care workers. This situation has resulted with some incomplete recording of CRFs. Magnitude of the challenges was varying among the different study sites. Direct microcopy, an investigation which needs centrifugation of CSF, was not done at the research lab due to sample volume insufficiency when used for the various molecular and immunological experiments. On the other hand antibiotic susceptibility testing was not done at the research lab due to failure of Greeves media into which certain portion of the culture lysate was preserved for future test. Moreover, this study reports only about the portion of the population of patients who have only got the chance to visit hospitals. Additional limitation is that long term sequelae that may be manifested among the survived patients could not be determined. The matter that all patients may have not been recruited may affect proportion of fatal vs. non-fatal cases and most fatal cases may have been missed at emergencies not recorded here.

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12. APPENDIXES

Appendix I: Sequences of oligonucleotide primer pairs used for conventional multiplex PCR amplification of *N. meningitidis, S. pneumoniae,* and *H. influenzae*

Organisms	**Target Gene**	**Sequences of oligonucleotide primers used**	**Molec.Wts of PCR products in bp**
N. meningitidis	*Por A*	F-730: 5'-AAA CTT ACC GCC CTC GTA3' , R-733:5'-TTA GAA TTT GTG GCG CAA ACC GAC-3'	1100
S. pneumoniae	*Lyt A*	F: 99-10: 5'-AAGAACAGATTTGCCTCAAGTCGGC-3' R: 99-11: 5'-TTGGTTATTCGTGCAATACTCGTGCG-3.	390
H. influenzae	*Bex A*	F: HI1: 5'-GTC GTT TGT ATG ATG TTG ATC CAG AC TAC-3' R: HI2: 5'-GCTT TGT CCA TGT CTT CAA AAT GAT G CAT-3'	343

Appendix II: Sequences of oligonucleotide primer pairs used for conventional multiplex PCR amplification of *N. meningitidis* genogroups

Organisms	Targeted gene	Sequences of oligonucleotide primers used	Product size in bp
N. meningitidis genogroup A	*SacB*	F 98-28 5'CGC AAT AGG TGT ATA TAT TCT TCC3' R 98-29 5'CGT AAT AGT TTC GTA TGC CTT CTT3'.	400
N. meningitidis genogroup W-135	*MynB*	F 98-32 5'CAG AAA GTG AGG GAT TTC CAT A A3' R- 98-33 5'CAC AAC CAT TTT CAT TAT AGT TAC3' TGT3'	120
Primer sequences for conventional PCR amplification of said *gene of N.meningitidis* genogroup Y			
N. meningitidis genogroup Y	*SiaD*	F: 5'CTCAAAGCGAAGGCTTTGGTTA3' R: 5'CTGAAGCGTTTTCATTATAATTGCTAA3'	120

Appendix III: Sequences of oligonucleotide primer pairs used for multiplex Real-time PCR (RT-PCR) amplification of ctrA, lytA and omp genes of *N. meningitidis*, *S.pneumoniae* and *H.influenzae* respectively

Target	Primer/ Probe name	RT- PCR Primers and Probes Nucleotide Sequence (5' to 3')
N. meningitidis ctrA primers	*ctrA-F*	5'TGTGTTCCGCTATACGCCATT3'
	ctrA-R	GCCATATTCACACGATATACC3'
Probe	*ctrA-P*	5'ACCTTGAGCAA¨T¨CCATTTATCCTGACGTTCT3'
S. pneumoniae lytA primers	*F373*	5'ACGCAATCTAGCAGATGAAGC3'
	R424	5'TCGTGCGTTTTAATTCCAGCT3'
Probe	*Pb400 Cy5*	5' FAMGCCGAAAACGC¨T¨TGATACAGGGAG BGQ3'
H.influenzae omp primers	*omp 2-F*	5'GGTTAAATATGCCGATGGTGTTG3'
	omp 2-R	5'TGCATCTTTACGCACGGTGTA3'
Probe	*omp P2-P*	5'TTGTGTACACTCCGT¨T¨GGTAAAAGAACTTGCAC3'
	omp P2-P	5'TTGTGTACACTCCGT¨T¨GGTAAAAGAACTTGCAC3'

Appendix IV: Sequences of oligonucleotide primer pairs and probes used for Real-Time singlex PCR amplifications of target genes of *N. meningitidis* genogroups

Target	Target genes	Primer/ Probe	RT- PCR Primers, Probes Nucleotide Sequence (5' to 3')
N. meningitidis A	*sacB*	F 2531	5’AAAATTCAATGGGTATATCACGAAGA3’
		R2624	5’ATATGGTGCAAGCTGGTTTCAATAG3’
Probe		PB839i Fam probe	5’AAGAGATGGGYAACAAC"T"ATGT3’
N. meningitidis B	*synD*	F737	5’GCTACCCCATTTCAGATGATTTGT3’
		R882	5’ACCAGCCGAGGGTTTATTTCTAC3’
Probe		Pb839iFam	5’AAGAGATGGGYAACAAC"T"ATGT AATGTCTTTATTT3’
N. meningitidis C	*synE*	F478	5’CCCTGAGTATGCGAAAAAAATT3’
		R551	5’TGCTAATCCCGCCTGAATG3’
Probe		Pb495Fam	5’TTTCAATGC"T"AATGAATACCACCGTTT TTTTGC3’
N. meningitidis	*synG*	F857	5’TATTTATGGAAGGCATGGTGTATG3’
W-135		R964	5’TTGCCATTCCAGAAATATCACC3’
Probe		Pb907i Fam	5’AAATATGGAGCGAATGATTACAGT AACTATAATGAA3’
N. meningitidis X	*xcbB*	F173	5’TGTCCCCAACCGTTTATTGG3’
		R237	5’TGCTGCTATCATAGCCGCC3’
Probe		Pb196Fam	5’TGTTTGCCCACATGAATGGCGG3’
N. meningitidis Y	*synF*	F787	5’TCCGAGCAGGAAATTTATGAGAATAC3’
		R929	5’TTGCTAAAATCATTCGCTCCATAT3’
Probe		Pb1099i Fam	5’TATGGTG"T"ACGATATCCCTATCCTTGC CTATAAT3’

Appendix V: *N. meningitidis* MLST scheme, including gene locus, amplicon length, and trimmed length of housekeeping gene sequence used for allelic determination

Housekeeping genes	Gene locus	Trimmed length
Putative ABC transporter	*abcZ*	433
Adenylate kinase	*Adk*	465
Shikimate dehydrogenase	*aroE*	490
Fumarate dehydrogenase	*fumC*	465
Glucose-6-phosphate dehydrogenase	*Gdh*	501
Pyruvate dehydrogenase subunit	*PdhC*	480
Phosphoglucomutase	*Pgm*	450

trimmed length of housekeeping gene sequence used for allelic determination

Appendix VI: MLST amplification primers for *N. meningitidis*

Gene locus	Primer name	Forward primer (5’-3’)	Primer name	Reverse primer (5’-3’)
abcZ	abcZ-PIC	TGTTCCGCTTCGACTGCCAAC	*abcZ-P2C*	TCCCCGTCGTAAAAAACAATC
adk	*adk-PIB*	CCAAGCCGTGTAGAATCGTAAACC	*adk-P2b*	TGCCCAATGCGCCCAATAC
aroE	*aroE-PIB*	TTTGAAACAGGCGGTTGCGG	*aroE-P2B*	CAGCGGTAATCCAGTGCGAC
fumC	*fumC-PIB*	TCCCCGCCGTAAAAGCCCTG	*fumC-P2B*	GCCCGTCAGCAAGCCCAAC
gdh	*gdh-PIB*	CTGCCCCCGGGGTTTTCATCT	*gdh-P2B*	TGTTGCGCGTTATTTCAAAGAAGG
PdhC	*pdhC-P2B*	CCGGCCGTACGACGCTGAAC	*pdhC-P2B*	GATGTCGGAATGGGGCAAACA
Pgm	*pgm-P1*	CTTCAAAGCCTACGACATCCG	*pgm-P2*	CGGATTGCTTTCGATGACGGC

Appendix VII: MLST sequencing primers for *N. meningitidis*

Gene locus	Primer name	Forward primer	Primer name	Reverse primer
abcZ	*abcZ-S1A*	AATCGTTTATGTACCGCAGR	*abcZ-S2*	GAGAACGAGCCGGGATAGGA
adk	*adk-SIA*	AGGCWGGCACGCCCTTGG	*adk-S2*	CAATACTTCGGCTTTCACGG
aroE	*aroE-S1A*	TCGGTCAAYACGCTGRTK	*aroE-S2*	ATGATGTTGCCGTACACATA
fumC	*fumC-S1*	TCCGGCTTGCCGTTTGTCAG	*fumC-S2*	TTGTAGGCGGTTTTGGCGAC
gdh	*gdh-S3*	CCTTGGCAAAGAAAGCCTGC	*Gdh-S4C*	RCGCACGGATTCATRYGG
pdhC	*pdhC-s1*	TCTACTACATCACCCTGATG	*pdhC-S2*	ATCGGCTTTGATGCCGTATTT
pgm	*pgm-S1*	CGGCGATGCCGACCGCTTGG	*pgm-S2A*	GGTGATGATTTCGGTYGCRCC

Appendix VIII: MLST amplification and sequencing primers for *S. pneumoniae*

Housekeeping genes	Gene locus	Trimmed length
Shikimate dehydrogenase	*aroE*	405
Glucose-6-phosphate dehydrogenase	*gdh*	460
Glucose kinase	*ski*	483
Transketolase	*recP*	450
Signal peptidase I	*Spi*	474
Xanthine phosphoribosyltransferase	*xpt*	486
D-alanine-D-alanine ligase	*ddl*	441

Appendix IX: *S. pneumoniae* MLST scheme, including gene locus, amplicon length, and trimmed length of housekeeping gene sequence used for allelic determination

Gene locus	Primer name	Forward primer (5'-3')	Primer name	Reverse primer (5'-3')
aroE3	*aroE-fwd*	TCCTATTAAGCATTCTATTTCTCCCTTC	*aroE-rev*	ACAGGAGAGGATTGGCCATCCATGCCCACACTG
Gdh4	*gdh-up*	ATGGACAAACCAGCNAGYTT	*gdh-dn*	GCTTGAGGTCCCATRCTNCC
Gki4	*ski-up*	GGCATTGGAATGGGATCACC	*ski-dn*	TCTCCCGCAGCTGACAC
recP3	*recP-fwd*	GAATGTGTGATTCAATAATCACCTCAAATAGAAGG	*recP-rev*	TGCTGTTTCGATAGCAGCATGGATGGCTTCC
Spi3	*spi-fwd*	CGCTTAGAAAGGTAAGTTATGAATTT	*spi-rev*	GAAGAGGCTGAGATTGGTGATTCTCGGCC
Xpt3	*xpt-fwd*	TTAACTTTTAGACTTTAGGAGGTCTTATG	*xpt-rev*	CGGCTGCTTGCGAGTGTTTTTCTTGAG
ddl3	*ddl-fwd*	TAAAATCACGACTAAGCGTGTTCTGG	*ddl-rev*	AAGTAGTGGGTACATAGACCACTGGG

Appendix X: Publication

Published

1. Mihret, W., Lema, T., Merid., Y., Kassu, A., Abebe, W., Moges B., Tenna, A., Woldegebriel, F., Yidnekachew, M., Mekonnen, W., Ahmed, A., Yamuah, L., Silamsaw, M., Petros B., Oksnes, J., Rosengivst, E., Ayele, S., Aseffa, A., Caugant D.A., Norheim G. (2016). Surveillance of Bacterial Meningitis, Ethiopia, 2012–2013. *Emerg Infect Dis.* **22** (1): 75-78.

   https://www.ncbi.nlm.nih.gov/pmc/?term=Surveillance+of+Bacterial+Meningitis%2C+Ethiopia%2C+2012%E2%80%932013

Submitted manuscript (To Emerging Infectious Diseases**)**

2. Inflammatory mediators in cerebrospinal fluids from meningococcal and pneumococcal meningitis patients in Ethiopia

   https://mc.manuscriptcentral.com/eid

DECLARATION

I, the undersigned, declare that this PhD Thesis is my original work, has not been presented by me or any other person for any degree in Addis Ababa or any other University. I also declare that all sources of materials used for the research project have been properly acknowledged.

Name: Wude Mihret Woldemedhin

Place: Addis Ababa University

Signature: _____

Date: _____

This Thesis has been submitted for examination with my approval as

Advisor: Beyene Petros, Prof.

Signature: _____

Date: _____