ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES
SCHOOL OF BIOMEDICINE AND LABORATORY SCIENCES

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY

Prevalence and Immune Correlates of Risk in Community-Acquired Meningitis: A Study at ALERT and Hawassa University Comprehensive Specialized Hospitals, Ethiopia.

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JUN, 2025

ADDIS ABABA, ETHIOPIA

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A research thesis submitted to Addis Ababa University, School of Biomedicine and laboratory sciences, Department of Microbiology, Immunology and Parasitology in the partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology

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JUN, 2025

Addis Ababa, Ethiopia

Acknowledgement

I would like to thank everybody who contributed to this work in any way. I sincerely thank my advisor, Dr. Tamrat Abebe (PhD), and co-advisor, Mr. Shemse Sebre (PhD fellow), for their guidance and support. My gratitude also extends to Dr. Getachew Tesfaye (PhD) for giving me the opportunity to participate in his research project, "Enhancing diagnosis of acute meningitis in meningitis belt resource-limited setting, Ethiopia" at AHRI.

I deeply appreciate the financial support from the Armauer Hansen Research Institute (AHRI) and the African Academy of Sciences through the ARISE-PP project.

Finally, I thank the data collectors (physicians and nurses), study participants, and laboratory technicians at ALERT and Hawassa University Hospitals for their essential contributions to participant recruitment and laboratory procedures.

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List of Abbreviations and Acronyms

AHRI: Armauer Hansen Research Institute

AIDS: Acquired immunodeficiency syndrome

ALERT: All Africa Leprosy Rehabilitation and Training

ATCC: American Type Culture Collection

CAM: Community-acquired meningitis

CoP: Correlate of protection

CoR: Correlate of risk

CRP: C-reactive protein

CSF: Cerebrospinal fluid

DMIP: Department of Microbiology, Immunology, and Parasitology

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme linked immunosorbent assay

GBD: Global Burden of Disease

HIV: Human immunodeficiency virus

MAC: Membrane attack complex

NSAIDs: Non-steroidal anti-inflammatory drugs

PCR: Polymerase chain reaction

PCT: procalcitonin

SPSS: Statistical Package for Social Science [Later named as "Statistical Product and Service Solutions"]

TBM: Tuberculosis Meningitis

TCC: Terminal Complement Complex

WHO: World Health Organization

Abstract

Background: Meningitis poses a major public health challenge in sub-Saharan Africa, often triggering devastating epidemics. Although it can affect individuals of all ages, infants, children, and immunocompromised persons are at the greatest risk. In Ethiopia, meningitis continues to be a significant public health concern due to its potential for outbreaks, high mortality rates, and the considerable burden it places on the healthcare system.

Objective: This study was aimed to assess the prevalence of etiologic agents causing meningitis and to evaluate immune correlates of risk among clinically suspected meningitis cases in Ethiopia.

Methods: An institutional-based cross-sectional study was conducted from August 2023 to June 2024 at ALERT Comprehensive Specialized Hospital and Hawassa University Comprehensive Specialized Hospitals. A total of 201 clinically suspected meningitis cases of all age groups (except neonates) were enrolled. Cerebrospinal fluid (CSF) samples were analyzed using conventional culture, microscopy, and Conventional multiplex PCR to identify bacterial and viral pathogens. Immune correlates of risk-including CD4+ T-cell counts and plasma complement levels (C5, C5a, C9, Factor D, MBL, Factor I, Properdin)- were compared among cases with bacterial meningitis, viral meningitis cases and healthy individuals. Univariate and multivariate logistic regression analyses were performed to identify significant associations.

Results: The overall prevalence of bacterial meningitis detected by classical CSF culture was 5.5% (n = 11), with *Klebsiella pneumoniae* 3/11(1.5%) being the most frequently isolated organism. In contrast, multiplex PCR identified a substantially higher prevalence of bacterial meningitis at 36.8% (n = 74), with *Escherichia coli* 30/74(14.9%), *Streptococcus pneumoniae* 15/74(7.5%), and *Neisseria meningitidis* 11/74(5.5%) as the predominant pathogens. *Klebsiella pneumoniae 9/74(4.5%), Streptococcus agalactiae 4/74(2%), and Listeria monocytogenes 2/74(1%)* were also rarely detected. Antimicrobial resistance was alarmingly high, with 72.7% of bacterial isolates exhibiting resistance to three or more antibiotics. The overall prevalence of viral meningitis was 34.3% (n = 69), with Human Enteroviruses (28.9%) representing the most common viral etiology. Overall 65% [13/20] of patients with viral meningitis have CD4 deficiency [<200cells/mm3]. 54% [27/50] of patient with bacterial meningitis have Properdin deficiency followed by 52% [26/50] C5- Deficiency and 46% [23/50] C9- deficiency. Analysis of immune correlates revealed that deficiencies in complement components C5 (AOR = 2.50, 95% CI: 1.60–3.90, p < 0.001), C9 (AOR = 3.09, 95% CI: 1.31 7.30, p = 0.007), and Properdin (AOR = 1.80, 95% CI: 1.20–2.70, p = 0.003) were significantly associated with an increased risk of bacterial meningitis. For viral meningitis, low CD4+ T-cell levels were significantly associated with increased risk (AOR = 2.59, 95% CI: 1.60–3.90, p = 0.035).

Conclusion and recommendation: In conclusion, our study confirms a significant burden of bacterial and viral meningitis in our study settings, disproportionately affecting young children. Our findings highlight specific immune correlates of risk for meningitis: deficiencies in terminal complement components (C5, C9, and Properdin) significantly increase susceptibility to bacterial meningitis, whereas low CD4+ T-cell levels are associated with a higher risk of viral meningitis. These findings highlight the urgent need for enhanced diagnostic capabilities, expanded vaccination programs, stringent antibiotic stewardship, and the implementation of immune profiling for better patient management in Ethiopia. Future research should track long-term outcomes and evaluate intervention effectiveness.

Keywords: Meningitis, bacterial meningitis, viral meningitis, immune correlates, complement components, CD4 counts, Ethiopia.

CHAPTER ONE: INTRODUCTION

1.1. BACKGROUND

Meningitis, a severe infection of the brain and spinal cord membranes, is a devastating global health challenge due to its high fatality rate and propensity for long-term complications. While epidemics occur worldwide, they are particularly prevalent in sub-Saharan Africa's "Meningitis Belt." Though meningitis can stem from non-infectious causes like autoimmune disorders or cancer, infectious agents—including bacteria, viruses (aseptic meningitis), fungi, and rarely parasites—are the primary drivers, with bacterial meningitis accounting for the highest global burden *(Hersi et al., 2017; Thakur and Wilson, 2018; WHO, 2025*).

Bacterial meningitis can be caused by various Gram-negative bacteria, including *N. meningitidis*, *P. aeruginosa*, *E. coli*, and *H. influenzae*, as well as Gram-positive bacteria such as *S. aureus*, *S. pneumoniae*, Group B Streptococcus, and *L. monocytogenes*. Additionally, *M. tuberculosis* and *T. pallidum* are known to cause meningitis in immunocompromised individuals *(Hersi et al., 2021, 2017). N. meningitidis*, which causes meningococcal meningitis, has the potential to produce large epidemics (WHO, 2025). There are 12 serogroups of *N. meningitidis* that have been identified, 6 of which (A, B, C, W, X and Y) can cause epidemics *(WHO, 2025).*

Bacterial meningitis accounts for a notable percentage (6-8%) of hospital admissions in Ethiopia, with case fatality rates reported as high as 7.5% to 28% in some studies (*Amare et al., 2018).* Studies in Ethiopia have reported varying prevalence of bacterial meningitis based on age group studied, methodologies used and geographical locations. The prevalence ranges from 1.28% in Gondar *(Tigabu et al., 2021),* 8.5% in Hawassa (Ali, 2024), and 11.2% in Debre Markos *(Hibstu et al., 2022).*

Non-polio enteroviruses, including group B coxsackievirus and echovirus, are the most frequent viruses that cause aseptic meningitis. Other viral causes include: mumps, parechovirus, measles, influenza, arboviruses (West Nile, La Crosse), and herpesviruses (such as Epstein Barr virus, Herpes simplex virus 1 and 2, and Varicella-zoster virus) *(Cassady And Whitley, 2014; Hersi et al., 2021; Vasilopoulou et al., 2011).* Depending on the study population and methodology, prevalence rates of viral meningitis in Ethiopia have varied, ranging from 5.6% to 26.7%.

Human Enteroviruses (HEVs) have been found to be the primary causative agents *(Geteneh et al., 2025).*

Fungal meningitis is caused by different types of fungi such as *Cryptococcus neoformans*, Candida species, Aspergillus species, *Blastomyces dermatitidis, Histoplasma capsulatum,* and *Coccidioides immitis* (Hersi et al., 2021; Klinger et al., 2000). Studies have shown varying prevalence rates of fungal meningitis in patients diagnosed with meningitis in Ethiopia. One study found that 8.5% of patients clinically diagnosed with meningitis were positive for cryptococcal meningitis. Other studies show even higher rates of cryptococcal meningitis among HIV-infected patients, ranging from 6.2% to 11.7% depending on the specific patient group and CD4 count *(Tufa and Denning, 2019).*

Globally, parasitic meningitis is generally considered rare compared to bacterial and viral meningitis, but its incidence varies greatly depending on geographic location, environmental factors, and host immune status. The most common parasitic causes of meningitis/menengoencephlitis worldwide include: *Strongyloides stercoralis*, *Angiostrongylus cantonensis*, *Baylisascaris procyonis*, and *Naegleria fowleri*; *Acanthamoeba* (Mallewa and Wilmshurst, 2014). While parasitic infections are highly prevalent in *Ethiopia (Assemie et al., 2021)* and some can lead to meningitis, specific prevalence data for parasitic meningitis itself is scarce due to diagnostic limitations.

Meningitis can occur in people of all ages, but babies, children, and those with a weakened immune system (iatrogenic, transplant recipients, congenital immunodeficiencies, AIDS) are at the highest risk. Higher risk also occurs in people with chronic medical disorders (renal failure, diabetes, adrenal insufficiency, and cystic fibrosis), extremes of age, undervaccination, and those who live in crowded conditions (overcrowded households, student or military housing, or refugee camps) *(Hersi et al., 2022; WHO, 2025).* Smoking can also raise the risk of certain types of meningitis *(Pilat et al., 2021).*

1.2. STATEMENT OF PROBLEM

Meningitis remains a much-feared disease worldwide with a high case fatality rate and a propensity to cause epidemics that present a major challenge for health systems, economies and society. In 2019 alone, there were an estimated 2.51 million new cases and 236,000 deaths worldwide, with children under five disproportionately affected (1.28 million cases and 112,000 deaths) (Wunrow et al., 2023). As per the 2016 Global Burden of Disease (GBD) Study, the incident cases of meningitis have increased globally from 2.50 million (2.19–2.91) in 1990 to 2.82 million (2.46–3.31) in 2016 *(Zunt et al., 2018).*

Globally, over 1.2 million people are affected by bacterial meningitis each year (*Ghia and Rambhad, 2021)*. Burden of bacterial meningitis is unevenly distributed across the globe. The most common bacterial etiologies of meningitis vary by age. Group B Streptococcus is common in infants less than 2 months of age while Streptococcus pneumoniae is the most common in all other age groups, with the exception of 11 - 17 year old, where Neisseria meningitidis is still the most common cause. Listeria monocytogenes and gram-negative bacteria such as Escherichia coli, Klebsiella, Enterobacter, Pseudomonas aeruginosa are other less common causes *(Hersi et al., 2021).*

Viruses are the most common cause of meningitis around the world. Viral meningitis has an estimated incidence ranging from 0.26 to 17 cases per 100,000 people. Enteroviruses cause more than 90% of all viral meningitis cases *(Cantu, 2019; Tattevin et al., 2019).*

Meningitis can be acquired in the community setting or hospital setting. The latter group has often been classified as nosocomial meningitis (Hospital-acquired meningitis) because a different spectrum of microorganisms (i.e, resistant gram-negative bacilli and staphylococci) are the more likely the etiologic agents, and different pathogenic mechanisms are associated with the development of this disease (T*unkel et al., 2017*). Hospital-acquired meningitis is typically caused by gram-positive bacteria like Coagulase-negative Staphylococci (CoNS), especially *Staphylococcus epidermidis,* and *Staphylococcus aureus* (including MRSA) as well as gram-negative bacteria: Enterobacteriaceae (e.g., *Klebsiella* species, *Enterobacter* species, *Escherichia coli, and Serratia marcescens*), *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Panic et al., 2022). Common etiologic agents of CAM vary greatly depending on the patient's age and vaccination status, but broadly include: *Streptococcus agalactiae* (Group B Streptococcus -

GBS), *Streptococcus pneumoniae* (Pneumococcus), *Neisseria meningitidis* (Meningococcus), *Haemophilus influenzae type b* (Hib), *Listeria monocytogenes,* human enteroviruses, HSV1& 2 and mumps viruses *(Boisson et al., 1999; Diederik van de Beek et al., 2016*).

Epidemics of meningitis are seen across the world, particularly in meningitis belt of sub-Saharan Africa (WHO, 2021). The meningitis belt of sub-Saharan Africa runs across the continent from Senegal to Ethiopia. This region is prone to major epidemics of meningococcal meningitis, with a high case fatality and serious sequelae that place a heavy strain on national and local health services *(Mazamay et al., 2021; WHO, 2019).* The reason for the susceptibility of this region of Africa to major epidemics of meningococcal disease is at least in part related to its climatic features, with outbreaks occurring mainly in the hot, dry season (Sultan et al., 2005). According to WHO, an estimated 22 414 new cases of meningitis and 1261 deaths were reported in the African meningitis belt in 2019 *(WHO, 2023).*

Ethiopia, being in the meningitis belt of sub-Saharan Africa, is one of the countries most affected with bacterial meningitis (Bedetti et al., 2019). Bacterial meningitis accounts for a notable percentage (6-8%) of hospital admissions in Ethiopia, with case fatality rates reported as high as 28% in some studies (Amare et al., 2018). Viral etiologies of meningitis (aseptic meningitis) are rarely identified in Ethiopia owing to a lack of advanced laboratory settings (Wami et al., 2021). Depending on the study population and methodology, prevalence rates of viral meningitis in Ethiopia have varied, ranging from 5.6% to 26.7%. Human Enteroviruses (HEVs) have been found to be the primary causative agents (*Geteneh et al., 2025*).

Ethiopia is among the countries most affected by meningitis, a severe public health challenge with significant morbidity and mortality (WHO, 2021). Despite this critical burden, the routine management of meningitis in Ethiopia remains predominantly based on syndromic surveillance and clinical presentations rather than confirmed etiological diagnoses *(Gudina et al., 2016; Hibstu et al., 2022).* This diagnostic gap has profound implications for targeted treatment, antimicrobial stewardship, and effective public health interventions. Furthermore, limited data exists on the post-vaccine epidemiology of meningitis etiologies in our country.

Thus, this study is designed to assess the magnitude of meningitis, its etiologic agents and immune correlates of risk among clinically suspected meningitis cases in selected hospitals in

Ethiopia. By doing so, this research will contribute to improving diagnostic capabilities and risk assessment strategies in a setting where meningitis management is largely based on clinical presentations due to limited resources.

1.3. SIGNIFICANCE OF THE STUDY

In countries with a high burden of meningitis, Ethiopia being in meningitis belt, enhancing diagnostics through developing resources, uninterrupted surveillance and checking the patterns of etiologic agents of meningitis is crucial for accurate diagnosis, improve patient management and controlling infectious meningitis or taking effective public health measures in general.

This study is significant because it provides comprehensive epidemiological data on the prevalence of bacterial and viral meningitis pathogens in a high-burden setting, using both classical and molecular diagnostic methods to improve detection accuracy. Furthermore, assessing the immune correlate of risk helps to determine the level of associated risk factors predisposing to infectious meningitis in the community. These insights are crucial for developing targeted interventions, improving risk stratification, and guiding vaccine and therapeutic development tailored to the local epidemiological context.

Additionally, the findings can inform public health policies by highlighting gaps in current diagnostic and treatment approaches, emphasizing the need for enhanced surveillance and immunological assessments. Ultimately, this study contributes to reducing the burden of meningitis through improved diagnosis, prevention, and personalized management strategies in Ethiopia and comparable regions with high meningitis incidence. Finally, the resulting data will serve as a baseline for further future similar but larger studies.

CHAPTER TWO: LITERATURE REVIEW

2.1. ETIOLOGIC AGENTS OF MENINGITIS

Meningitis can stem from infectious (bacteria, viruses, fungi, parasites) or non-infectious processes (autoimmune disorders, cancer, drug reactions) (Table1) *(Ginsberg, 2004; Hersi et al., 2017; WHO, 2021).*

Common bacterial meningitis *include N. meningitidis, S. aureus, E. coli, H. influenzae, S. pneumoniae, Group B Streptococcus (GBS), P. aeruginosa, and L. monocytogenes. In immunocompromised individuals, M. tuberculosis and T. pallidum can also cause meningitis (Hersi et al., 2017, 2017*).

Viral (Aseptic) Meningitis are more common but often underreported due to milder symptoms (Kohil et al., 2021), and most frequently caused by non-polio enteroviruses (especially group B coxsackievirus and echovirus). Other viral agents include Herpesviruses (Epstein-Barr, Herpes simplex 1 and 2, Varicella-zoster), measles, influenza, mumps, Parechovirus, and arboviruses (West Nile, La Crosse) *(Biaukula et al., 2012; Hersi et al., 2021).*

Fungal Meningitis is Primarily associated with immunodeficiency, fungal meningitis can be caused by Coccidioides, Cryptococcus, Histoplasma, Blastomyces, and Candida species *(Hersi et al., 2022; Klinger et al., 2000).*

Parasitic meningitis is less common, parasitic causes include *Strongyloides stercoralis, Angiostrongylus cantonensis, Baylisascaris procyonis, Naegleria fowleri, and Acanthamoeba* (*Klinger et al., 2000; Singhi and Saini, 2019)* (see Table 1).

Table 1: Common Etiologic Agents of Meningitis

Category	Pathogen	General Characteristics	References
Bacterial	*Streptococcus pneumoniae*	Leading cause in adults; declining due to PCV13/PPSV23 vaccines.	*(D# van de Beek et al., 2016)*
	Neisseria meningitidis	Cause invasive meningococcal disease (IMD), including meningitis and septicemia	*(Nguyen and Ashong, 2024)*
	Haemophilus influenzae type b (Hib)	Rare where the Hib vaccine is used.	*(Mitchell et al., 2019)*
	Group B Streptococcus (GBS)	Leading neonatal cause; vertical transmission.	*(Nanduri et al., 2019)*
	Listeria monocytogenes	High mortality in elderly/pregnant.	*(Charlier et al., 2017)*
	E. coli	Most common causes of bacterial meningitis in newborns, particularly premature infants and those with low birth weight	*(Murray, 2024)*
	Klebsiella pneumoniae	Healthcare-Associated Infections (Nosocomial); High incidence of carbapenem resistance	*(Yang et al., 2024)*
Viral	Enteroviruses (Coxsackie, Echo)	Enteroviruses (Coxsackie, Echo)	*(Kohil et al., 2021)*
	Herpesviruses (HSV-1/2, VZV)	HSV-1: Encephalitis; VZV: Post-shingles.	*(Kohil et al., 2021)*
	Arboviruses (West Nile)	Mosquito-borne; neuro-invasive forms.	*(McGill et al., 2018)*
Fungal	Cryptococcus neoformans	AIDS-defining; subacute presentation.	*(Rajasingham et al., 2022)*
	Coccidioides spp.	Southwest USA/Latin America.	*(Galgiani et al., 2016)*
Parasitic	*Naegleria fowleri*	Rare freshwater amoeba; >97% fatal.	*(Sazzad et al., 2020)*

The bacteria causing meningitis differ by age (See Table-2): *Streptococcus pneumoniae* and *Neisseria meningitidis* commonly affect children and adults, while *Streptococcus agalactiae*,

Escherichia coli, and *Listeria monocytogenes* are primary causes in newborns *(Brouwer et al., 2010). Haemophilus influenzae* can cause meningitis across all age groups but is most prevalent in children under five *(Hall et al., 2021*). Common etiologic agents in different age groups are described below (See Table-2).

Table 2: Most Common Meningitis Pathogens by Age Group

Age Group	Bacterial	Viral	Fungal	References
Newborns (0–1 mo.)	GBS, *E. coli, Listeria monocytogenes*	Enteroviruses, HSV-2	*Candida*	*(Heath et al., 2017)*
Infants (1–12 mo.)	S. *pneumoniae, Hib*	Enteroviruses	Rare	*(O'brien et al., 2000)*
Children (1–18 yrs.)	*N. meningitidis Hib*	Enteroviruses, Mumps	Cryptococcus (HIV+)	*(Tugume et al., 2023)*
Adults (18–50 yrs.)	*S. pneumoniae*	Enteroviruses, HSV-1	Cryptococcus (HIV)	*(McGill et al., 2018)*
Elderly (>50 yrs.)	*S. pneumoniae, Listeria*	VZV	*Coccidioides*	*(D#̂ van de Beek et al., 2016)*

Prior to widespread immunization, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b (Hib) were the main causes of bacterial meningitis *(Oordt-Speets* et al., 2018). Vaccination efforts have significantly altered this epidemiology, notably leading to a drastic reduction in Hib meningitis among infants and children due to routine Hib vaccines (*Short and Tunkel, 2000; Weisfelt et al., 2006)*. However, in high-income countries, pneumococcal meningitis remains a serious concern in adults, associated with high fatality and neurological complications, despite medical advancements *(Van de Beek et al., 2004).*

2.2 BURDEN OF MENINGITIS

Meningitis epidemics are a global concern, especially within the sub-Saharan African "meningitis belt" (Figure 1). This region, which stretches across 26 countries from Senegal to Ethiopia, faces the highest incidence of meningococcal meningitis, with rates soaring to 1,000 cases per 100,000 people during dry season epidemics (WHO, 2025).

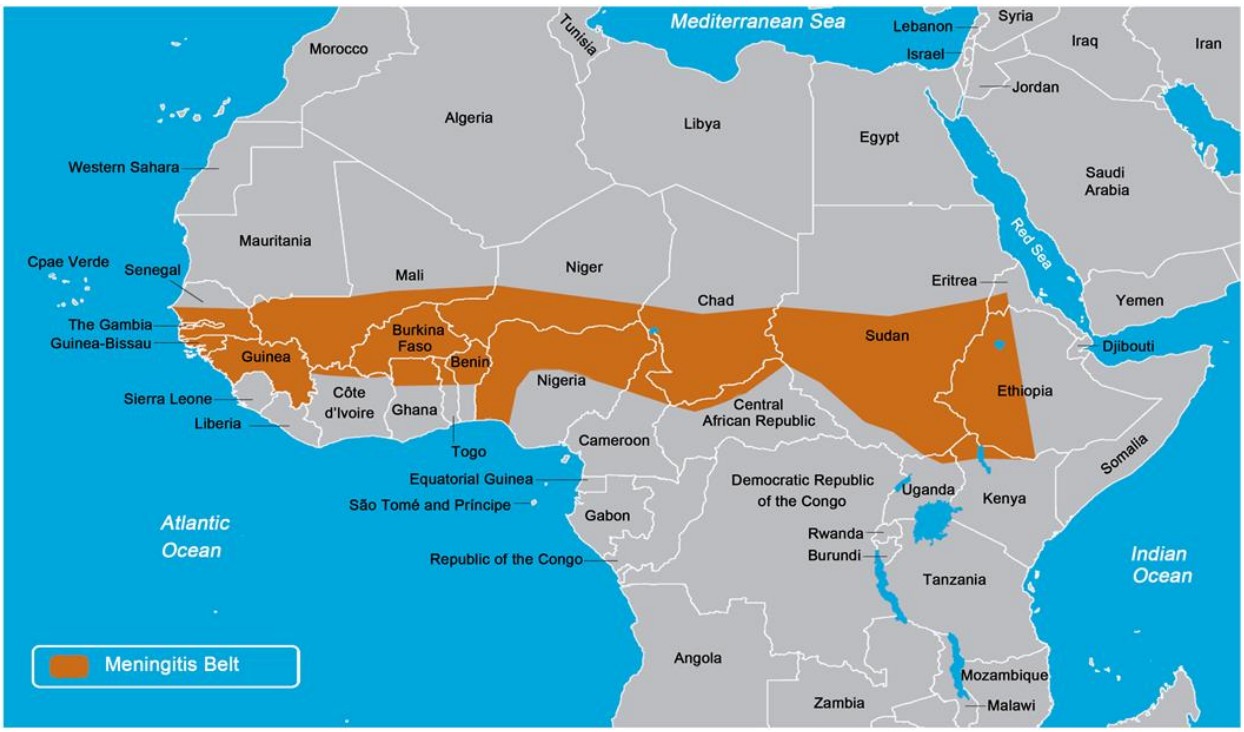

Figure 1: Map of Africa highlighting the African meningitis belt *(Asiedu-Bekoe, 2016).*

Meningitis outbreaks in Ethiopia have been documented in written accounts since 1901 (Habte-Gabrel et al., 1984) Haimanot et al., 1990). 1981 and 1989 outbreaks were the largest ever recorded in Ethiopia with 50,000 and 45,806 cases, and 990 and 1686 deaths respectively*(Haimanot et al., 1990*)..

A study conducted at a pediatric hospital in Addis Ababa from 1993 to 1995 revealed that bacterial meningitis accounted for approximately 5.5% of all hospital admissions *(Mohammed et al., 2000; Muhe and Klugman, 1999).*

A five-year study (1998-2003) at Gondar University Hospital found that *N. meningitidis* was the most common cause (28%) of bacterial meningitis in children under 14, followed by *S. pneumoniae***.** (7%) and *H. influenzae* (6.5%) (Tegene et al., 2015). Separately, a study in Butajira, Ethiopia, reported that meningitis caused almost 1% of 10,700 deaths between 1987 and 2008 *(Byass et al., 2010).*

A cross-sectional study by Wami AA, et al. (2021) in five Addis Ababa public hospitals found that out of 146 suspected meningitis cases, HEVs were detected in 39 (26.7%). Most HEV cases (28, or 71.9%) occurred in infants under one year old *(Wami et al., 2021).*

Before the AIDS epidemic, Cryptococcosis had a lower global prevalence. However, in Africa, meningitis occurs in up to 30% of AIDS patients, with about 90% of AIDS patients infected with *C. neoformans* developing meningitis(*Hoang et al., 2004*). Studies have shown varying prevalence rates of cryptococcal meningitis in HIV-positive patients, ranging from 49% to 100% in India *(Satpute et al., 2006*) and 7% in Ethiopia (Woldemanuel and Haile, 2001).

2.3 TRANSMISSION

Meningitis-causing organisms have varied transmission routes. Many bacteria (e.g., meningococcal, pneumococcus, *Haemophilus influenzae*) and some viruses are carried in the nose and throat, spreading via respiratory droplets or throat secretions. Some viruses are also vector-borne. Close, prolonged contact, like kissing, sneezing/coughing on someone, or cohabiting with an infected person, facilitates disease spread (WHO, 2021; Yazdankhah and Caugant, 2004). These bacteria can occasionally enter the bloodstream, reaching the spinal fluid to rapidly cause meningitis and/or septicemia *(Brandtzaeg and van Deuren, 2012).*

2.4 IMMUNE CORRELATES OF RISK IN COMMUNITY-ACQUIRED MENINGITIS (CAM)

Understanding the factors that predispose individuals to developing community-acquired meningitis is paramount for improving diagnostic strategies, guiding preventive measures, and developing effective treatment interventions. In the context of infectious diseases, an immune correlate of risk (CoR) is defined as an immunologic biomarker that is associated with the subsequent occurrence of a clinical endpoint, in this case, the development of meningitis (Gilbert et al., 2023). These biomarkers can provide insights into the likelihood of an individual developing the disease based on their immune status prior to or at the early stages of infection. It is important to distinguish CoR from an immune correlate of protection (CoP), which is a biomarker that reliably predicts the efficacy of a vaccine against a specific clinical endpoint *(Gilbert et al., 2023).* While CoPs are valuable for vaccine development and licensure, CoRs can help elucidate the mechanisms of disease susceptibility in the broader population, including those who may not have been vaccinated or for whom vaccines are unavailable. The probability of an endpoint occurring can be estimated by analyzing biomarker levels, even in the absence of a placebo arm in vaccine trials, highlighting the broader applicability of the CoR concept *(Plotkin and Gilbert, 2012).*

2.4.1 Role of Innate Immunity in Susceptibility

The innate immune system provides the body's first line of defense against invading pathogens, and its components play a critical role in determining an individual's susceptibility to community-acquired meningitis.

A. **Physical and chemical Barriers**

Blood-Brain Barrier (BBB), composed of endothelial cells with tight junctions, normally restricts the entry of pathogens and immune cells into the brain and spinal cord. However, in meningitis, this barrier can be compromised, allowing bacteria to invade *(Ransohoff and Brown, 2012*). The meninges, the protective membranes surrounding the brain and spinal cord, also play a role in preventing infection. Inflammation of the meninges (meningitis) disrupts this protection (Patel et al., 2025). Antimicrobial Peptides (AMPs), secreted by various cells, have also a direct antimicrobial activity against bacteria (*Mook-Kanamori et al., 2011*).

B. **Complement System Deficiencies**

Deficiencies in certain complement components, notably those in the terminal pathway (C5-C9) and Properdin (X-linked) have been strongly linked to a significantly increased risk of invasive and often recurrent meningococcal infections(Lewis and Ram, 2014). Individuals with these deficiencies are less able to form the membrane attack complex (MAC), which is essential for directly lysing meningococcal bacteria (Sadarangani, 2018). People with terminal complement deficiencies (C5–C9) have 10,000x higher risk of *Neisseria* meningitis. Low MBL levels correlate with severe pneumococcal meningitis *(Brouwer et al., 2010)*. Alternative Pathway Regulatory Protein Deficiencies (Factor D, Properdin, Factor H, Factor I) can lead to its over activation and increased susceptibility to specific bacterial infections *(Lewis and Ram, 2014).*

C. Phagocytic cell dysfunction (including macrophages and neutrophils)

Phagocytic cells, including macrophages and neutrophils, are another vital component of the innate immune system, playing a crucial role in defense against meningitis-causing pathogens. Impaired phagocytic function, which can occur in individuals with conditions such as diabetes or chronic alcoholism, has been associated with an increased risk of bacterial meningitis, suggesting that effective clearance of pathogens by these cells is crucial for preventing disease *(Camargo and Husain, 2014*). During bacterial meningitis, the majority of recruited leucocytes are

neutrophils-as well as a smaller number of monocytes- and they eradicate bacteria *(Gerber and Nau, 2010).*

D. Pattern recognition receptors (PRRs)

Sensing of microbial components is mainly achieved through highly conserved germline encoded family of proteins known as pattern recognition receptors (PRRs) of which the family of Toll-like receptors (TLRs) has been studied most extensively. PRRs recognize microbial (viral, bacterial and fungal) components, known as pathogen associated molecular patterns (PAMPs) that are essential for their survival and are therefore difficult for them to alter (Mogensen, 2009). Toll-like receptors (TLRs) play a crucial role in detecting bacterial PAMPs. For instance, TLR2, TLR4 and TLR9 are capable of recognizing structures of *N*. meningitidis *(Johswich, 2017).* TLR3 recognizes genomic dsRNA of dsRNA viruses or dsRNA that are produced during replication of ssRNA viruses or DNA viruses. TLR3 deficiency causes an increase in infection rates of HSV-1 *(Kawasaki et al., 2011).* In fungal infection, Toll-like (TLR), NOD-like (NLR), RIG-I-like (RLR) and C-type lectin-like receptors (CLR) are four receptor families that contribute to the recognition *(Plato et al., 2015).*

2.4.2 Adaptive Immunity and Meningitis Risk

The adaptive immune system has been considered to play a minimal role in the early host response during bacterial meningitis. Humoral immunity, mediated by antibodies, is critical in protecting against encapsulated bacteria. These antibodies, often specific to the polysaccharide capsule of the bacteria, can facilitate the clearance of pathogens through mechanisms such as opsonophagocytosis and complement activation (Gilbert et al., 2023). There is sound clinical evidence that patients with combined B- and T-cell deficiency, such as severe combined immunodeficiency, are more susceptible to recurrent bacterial infections than immunocompetent individuals (Ribes et al., 2016),

A. **Anti-Polysaccharide Antibody Deficiency** (**IgG2/IgG4 subclass deficiencies)**

A lack of adequate acquired protective antibodies against specific bacterial pathogens is a well-established major risk factor for developing bacterial meningitis, especially in vulnerable populations like young children and the elderly (Goldstein and Overturf, 2003). Several specific antibody deficiencies have been linked to an increased risk of meningitis. For instance,

individuals with IgG subclass deficiency, particularly IgG2 deficiency, may experience recurrent infections with encapsulated bacteria, although invasive infections like bacterial meningitis are extraordinarily rare in this context. Specific antibody deficiency (SPAD), characterized by an impaired ability to produce high-quality antibodies against certain bacteria despite normal overall immunoglobulin levels, can also increase susceptibility to respiratory infections, including pneumonia and potentially meningitis caused by encapsulated organisms (Lear et al., 2006).

B. **CD4+ T-Cell Depletion**

Cellular immunity, primarily involving T cells, is essential for controlling intracellular pathogens and plays a role in defense against viruses and fungi that can cause meningitis (Rajasingham et al., 2022). CD4+ T cells, particularly those mediating Th1 responses characterized by the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), are critical for controlling intracellular bacteria like *Listeria monocytogenes* and fungal pathogens such as *Cryptococcus neoformans* (Tugume et al., 2023). Impaired cell-mediated immunity, often observed in individuals with HIV infection, transplant recipients receiving immunosuppressive drugs, and those with certain hematological malignancies significantly increases the risk of fungal meningitis, especially cryptococcal meningitis (Onyishi and May, 2022). T-cell responses are also important in controlling viral infections that can lead to meningitis. Notably, in enterovirus infections, the status of cellular immunity appears to be a stronger correlate of clinical outcome than humoral immunity *(Specht et al., 2024).*

2.4.3 Influence of Host Factors on Immune Risk

Several host factors can significantly influence an individual's immune status and consequently their risk of developing community-acquired meningitis.

A. Age.

Age is a critical factor, with infants and young children, as well as older adults, exhibiting a higher susceptibility. Infants and young children have immature immune systems, and the protection afforded by maternal antibodies wanes over the first few months of life, leaving them more vulnerable to various pathogens. Adolescents and young adults face an increased risk of meningococcal meningitis, potentially due to close living conditions in settings like dormitories.

Older adults are also at higher risk due to the age-related decline in immune function, a process known as immunosenescence, and the presence of underlying health conditions (comorbidities) *(Adriani et al., 2015).*

B. **Asplenia**

The absence or dysfunction of the spleen (asplenia), whether due to surgical removal or functional impairment, significantly elevates the risk of severe bacterial infections, including meningitis. This is particularly true for encapsulated organisms such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae*, as the spleen plays a crucial role in clearing these bacteria from the bloodstream. Asplenia increases the risk of infections and, in some cases, can lead to a case of severe sepsis known as overwhelming post-splenectomy infection (OPSI), which has a very high mortality rate *(Adriani et al., 2015).*

C. Autoimmune diseases and meningitis

Autoimmune disorders, like rheumatoid arthritis, Systemic Lupus Erythematosus, and inflammatory Bowel Diseases syndrome, can increase the odds of developing meningitis *(Hersi et al., 2021).*

D. Other Disease conditions

Several diseases that damage the immune system can increase the risk of community-acquired meningitis (See Table 3). HIV/AIDS is a common such condition and can be a risk factor for bacterial, viral, and fungal meningitis. Diabetes mellitus has also been reported as a risk factor for both bacterial and fungal meningitis. Similarly, alcohol use disorder is also linked with an elevated risk of meningitis (*Hersi et al., 2022)* (See Table 3).

Table 3: Summary of immune correlates of risk in community-acquired meningitis, categorized by major pathogen types

Pathogen	**Immune Correlates of Risk**	**Implications**
Neisseria meningitidis	-Complement deficiency (C5–C9) -Properdin deficiency - Low bactericidal antibody titers	High risk in individuals with terminal complement defects; vaccine responsiveness may be impaired
Streptococcus pneumoniae	- Defective opsonization	Common in asplenic and elderly

	- IgG2 subclass deficiency - TLR2/TLR4 polymorphisms	individuals; poor outcomes linked to excessive inflammation
Haemophilus influenzae	-IgG2 deficiency -Complement defects - Lack of prior immunization	Pediatric risk; Vaccines highly effective at reducing disease burden
Listeria monocytogenes	- Impaired cell-mediated immunity - T-cell lymphopenia (e.g., HIV, cancer)	Often affects neonates, elderly, and immunosuppressed; intracellular pathogen
Mycobacterium tuberculosis	- Defective Th1 response - Low IFN-γ or IL-12 - TNF-α blockade	TB meningitis common in HIV+ or biologic-treated patients
Cryptococcus neoformans	- CD4+ T-cell deficiency -Impaired macrophage activation - High CSF IL-10	Most common cause of fungal meningitis in AIDS; high IL-10 may predict poor outcome
Enteroviruses	-Generally mild in immunocompetent - Risk in neonates and humoral immune deficiencies	Self-limiting in most; severe in agammaglobulinemia or very young
Herpes Simplex Virus (HSV)	-TLR3 pathway defects (in HSV-1 encephalitis) -Poor innate CNS immune sensing	Can cause severe encephalitis; early antiviral therapy critical

2.5 OTHER RISK FACTORS FOR MENINGITIS

Risk factors for meningitis are diverse and include chronic medical disorders like renal failure, diabetes, adrenal insufficiency, and cystic fibrosis, as well as extremes of age and undervaccination. Immunosuppressed states, whether iatrogenic, due to organ transplantation, congenital immunodeficiencies, or AIDS, significantly increase susceptibility. Living in crowded conditions also poses a risk. Other specific risk factors involve the presence of a ventriculoperitoneal (VP) shunt or an external ventricular drain, alcohol use disorder, penetrating head injury, lumbar puncture, and cranial or spinal surgeries that open the dura. Additionally, bacterial endocarditis, malignancy, Dural defects, IV drug use, sickle cell anemia, and splenectomy are recognized as contributing factors (Hersi et al., 2022)

2.5 PATHOGENESIS OF MENINGITIS

Bacterial meningitis develops when bacterial virulence factors overwhelm the host's defenses in the subarachnoid space. The process begins with the colonization of the nasopharynx. From there, the bacteria can invade local tissues and enter the bloodstream. Common meningitis-causing pathogens like *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* possess an outer polysaccharide capsule, which is a key virulence factor that prevents phagocytosis and complement pathway activation, thus aiding their survival and spread (Livorsi et al., 2011; Serrats et al., 2010; Virji, 2009) (Figure.2).

Following survival and replication in the bloodstream, bacteria can breach the blood-brain barrier and enter the subarachnoid space. The cerebrospinal fluid (CSF) offers limited protection against infection due to low levels of local antibodies and complement activity. Bacterial proliferation and white blood cell accumulation in the CSF intensify the local inflammatory response within the subarachnoid space, leading to the production and release of inflammatory mediators such as cytokines (IL-1, IL-6, TNF), prostaglandins (especially prostaglandin E2), and leukotrienes (especially leukotriene B4)(Quagliarello and Scheld, 1992). This inflammation of the meninges and ventricles results in a polymorphonuclear response, elevated CSF protein, and glucose consumption. Inflammatory damage and tissue destruction, often forming abscesses, are more severe in cases of gram-negative meningitis (Yazdankhah and Caugant, 2004).

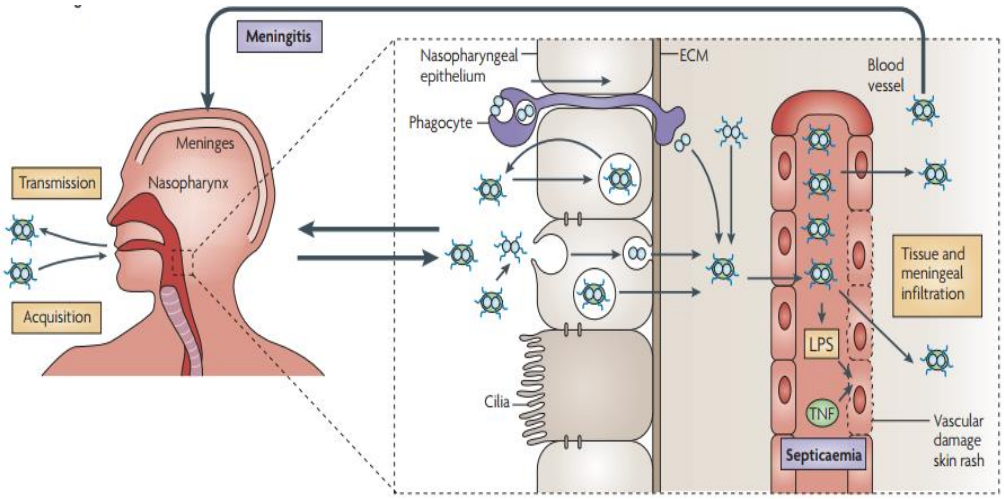

Figure 2: Stages in the pathogenesis of *N. meningitidis* (*Virji, 2009)*

2.6 CLINICAL PRESENTATION OF MENINGITIS

Although the "classic triad" of fever, headache, and neck stiffness is indicative of meningitis, it's present in under 50% of cases (*Heckenberg et al., 2008*). Early symptoms are often non-specific (fever, headache, malaise), with meningeal irritation signs (neck stiffness, photophobia, altered consciousness) appearing later (*Van de Beek et al., 2004*). These specific signs may be absent in unconscious, very young, or immunocompromised patients, complicating diagnosis (Cabellos et al., 2008). Other potential symptoms include nausea, vomiting, back rigidity, shock, seizures, unconsciousness, and skin bleeding; a petechial rash is characteristic of *N. meningitidis* infection *(Ramakrishnan et al., 2009).*

2.7 COMPLICATIONS OF MENINGITIS

Untreated meningitis, particularly bacterial meningitis, can lead to severe complications such as hearing defects, speech abnormalities, intellectual impairment, learning difficulties, and seizures *(Koomen et al., 2003; Ramakrishnan et al., 2009)*. The rate of these complications can be as high as 50% (Grimwood et al., 2000). The likelihood and severity of these issues depend on the causative organism, patient age, disease severity, and quality of treatment. Complications are more frequent and often long-lasting in young children, potentially causing focal neurological deficits like limb paralysis, developmental disabilities, cerebral abscesses, and hydrocephalus (Aronin et al., 1998; Pfister et al., 1993). While most childhood complications resolve within 2-3 years, about 10% of affected children may experience lifelong impairments *(Namani et al., 2011).*

2.8 DIAGNOSIS OF MENINGITIS

Meningitis diagnosis primarily relies on clinical examination followed by a lumbar puncture (LP) and analysis of cerebrospinal fluid (CSF) for appearance, cell counts, protein, and glucose. Rapid tests like Gram stain and latex agglutination offer presumptive identification, while definitive diagnosis often requires bacterial isolation from CSF or blood cultures, or detecting the target DNA by polymerase chain reaction (PCR) (WHO, 2011). When LP is contraindicated, supportive evidence can come from serum inflammatory markers, blood cultures, skin biopsies, and urine antigen testing. CSF analysis is critical because clinical signs alone are insufficient to differentiate meningitis types; it should be performed promptly unless specific contraindications like anticoagulation or local infection are present *(Brouwer et al., 2010).*.

Detailed CSF analysis involves observing its appearance (turbidity indicates high leukocytes), measuring opening pressure (typically elevated in bacterial meningitis), and performing a Gram stain for immediate presumptive identification of common bacterial culprits like *S. pneumoniae*, *N. meningitidis*, and *L. monocytogenes*. Cell counts and chemistry are crucial: a CSF leukocyte count exceeding 5cells/μL confirms meningitis, with WBC≥1000 cells/μL and neutrophilic predominance strongly suggesting bacterial infection. Hypoglycorrhachia (low CSF glucose) and elevated CSF protein also indicate bacterial meningitis, although values can overlap with other forms, particularly after antibiotic pretreatment or in specific infections *(Hase et al., 2014) (Pappas et al., 2001)* (see Table 4).

Table 4: Typical CSF findings in healthy adults and adult patients with meningitis *(Pappas et al., 2001)*

	Opening pressure (cm H_2O)	CSF leukocytes (cells/μL)	Predominant cell type	CSF protein (g/L)	CSF-to-blood glucose ratio
Normal CSF	≤20	0–5	Lymphocytes	0.15–0.45	0.6
Bacterial meningitis†	>20	≥1000	Neutrophils	≥1.0	≤0.5
Viral meningitis‡	≤20	<1000	Lymphocytes (may be neutrophilic in 1st 48 h)	<1.0	0.6
Tuberculous meningitis	Normal or elevated	100–500	Lymphocytes	≥1.0	≤0.5
Eosinophilic meningitis	Normal or elevated	<1000	≥10% eosinophils	<1.0	0.6
Cryptococcal meningitis	>20	<200	Lymphocytes	>0.45	≤0.5

† Excluding *Listeria* meningitis, where CSF leukocytes are often <1000 cells/μL and CSF may be lymphocytic.

‡ Excluding HSV and VZV meningitis, where protein may be ≥1.0 g/L.

CSF: cerebrospinal fluid; HSV: herpes simplex virus; VZV: varicella-zoster virus.

Other than CSF analysis, Latex agglutination is a diagnostic test that has been utilized for the etiological diagnosis of bacterial meningitis, providing results in less than 15 min (Tunkel et al., 2004). Serum Inflammatory Markers (Lactate, procalcitonin and C-reactive protein) are helpful in distinguishing bacterial from non-bacterial meningitis (D van de Beek et al., 2016; Vikse et al., 2015). CSF culture remains the gold standard for the diagnosis of bacterial meningitis; aerobic culturing techniques are obligatory for community-acquired bacterial meningitis. Anaerobic culture may be important for the investigation of CSF shunt meningitis Blood cultures are also valuable to detect the causative organism and establish susceptibility patterns if CSF cultures are negative or unavailable. Finally, skin biopsies can be of additional diagnostic value for patients with suspected meningococcal meningitis *(Cruz et al., 2018; Stephens et al., 2007).*

2.8.1. Diagnostic challenges in Resource limited Settings

Diagnosis of CNSI is challenging, and a large proportion of patients never receive a confirmed microbiological diagnosis, particularly in resource-limited settings where access to modern diagnostic platforms is limited. Overcoming diagnostic challenges and the early institution of empirical antibiotic therapy and, when feasible, adjunctive steroid therapy constitutes the pillars of reducing the disease burden of bacterial meningitis in resource-limited settings like Ethiopia *(Villalpando-Carrión et al., 2024; WHO, 2011; Wondimu et al., 2023).*

2.9. MANAGEMENT AND PREVENTION OF MENINGITIS

2.9.1. Management

Immediate medical attention is essential in cases of bacterial meningitis. Initial treatment with parenteral benzylpenicillin or ceftriaxone has been recommended for patients who presented to primary care with probable meningococcal cases (McGill et al., 2016). Once arrived at hospital, the patient should be placed in droplet precautions to prevent potential nosocomial transmission of meningococcal infection and resuscitated as required. CSF cultures should be quickly obtained, and dexamethasone and antibiotics should be given within 1hr of admission. The importance of timely therapy has been demonstrated in multiple studies, with one study showing an increase in mortality of 13% and another showing an increase in mortality and disability of 30% (Aronin et al., 1998) for every hour that treatment is delayed *(Aronin et al., 1998).*

As empirical therapy, most guidelines recommend dexamethasone plus ceftriaxone in the initial treatment of community-acquired bacterial meningitis in adults. While some guidelines

recommend the addition of vancomycin *(Aronin et al., 1998; Tunkel et al., 2004).* For viral meningitis, dexamethasone and antibiotics can be discontinued if CSF parameters are consistent with viral meningitis, provided that the clinical suspicion for bacterial meningitis is low. Enteroviral meningitis is self-limiting and requires only supportive care. HSV meningitis is also self-limiting in immunocompetent patients; symptomatic treatment alone is reasonable, although 7–10 days of antiviral treatment *(Noska et al., 2015).*

2.9.2. Prevention

Preventing bacterial meningitis relies heavily on immunization and chemoprophylaxis. Vaccines, including the widely used Hib protein polysaccharide conjugate vaccine and the pneumococcal conjugate vaccine (first introduced as a 7-valent vaccine in the US in 2000), are cornerstones of prevention. In sub-Saharan Africa, where meningococcal serogroup A is prevalent and causes epidemics, a conjugate vaccine is available through the Meningitis Vaccine Project (MVP), a WHO and PATH initiative aiming to provide low-cost vaccine to 250 million people across 25 African countries *(Lexau et al., 2005).* Chemoprophylaxis protects close contacts; the CDC recommends starting meningococcal prophylaxis within 24 hours of contact or pathogen identification, and up to 14 days after disease onset if reporting is delayed (Bilukha and Rosenstein, 2005)... Recommended antibiotics for eliminating N. meningitidis carriage in chemoprophylaxis include rifampicin, ciprofloxacin, and ceftriaxone *(Trestioreanu et al., 2013).*

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CHAPTER THREE: OBJECTIVES

3.1. GENERAL OBJECTIVE

To determine the prevalence of etiologic agents and assess immune correlates of risk in Community-acquired meningitis at All Africa Leprosy Rehabilitation and Training (ALERT) Comprehensive specialised Hospital and Hawassa University Comprehensive Specialised Hospitals, Ethiopia.

3.2. SPECIFIC OBJECTIVES

- To determine the prevalence of bacterial pathogens causing community-acquired meningitis among patients at ALERT Comprehensive Specialized Hospital and Hawassa University Comprehensive Specialized Hospital.
- To determine the prevalence of viral pathogens causing community-acquired meningitis among patients at the study sites.
- To assess immune correlates of risk associated with community-acquired meningitis, including evaluation of immune parameters that may influence susceptibility to bacterial and viral meningitis.

CHAPTER FOUR: MATERIALS AND METHODS

4.1. STUDY AREA

The study was conducted at ALERT Comprehensive Specialized Hospital and Hawassa University Comprehensive Specialised Hospitals, Ethiopia. ALERT Comprehensive specialised Hospital is a medical facility in Addis Ababa, specializing in Hansen's disease, also known as "leprosy". Currently, ALERT Comprehensive Specialized Hospital has evolved into a multidisciplinary healthcare institution, offering over 29 specialized medical services and ranking as Ethiopia's third-largest hospital in terms of patient flow. With a dedicated team of 2000 staffs, ALERT serves over 1,500 patients daily and more than 450,000 patients annually. There is currently a 240-bed in the hospital, which includes dermatology, ophthalmology, and surgery departments, also an orthopedic workshop, and a rehabilitation program.

The Hawassa research University Teaching Hospital (Hawassa university compressive specialized hospital) is a public hospital in Hawassa, Sidama State, Ethiopia. HUCSH serves as a referral center for much of southern part of Ethiopia, including nearby Oromia and other southern states. The hospital was established in 1998 and it is the biggest hospital in the southern Ethiopia. The hospital has a capacity of 250 beds and has one open system general ICU with a capacity of 8 beds for comprehensive care of all critically ill patients from all specialties.

4.2. STUDY DESIGN AND PERIOD

An institution-based cross-sectional study was conducted from August 2023 to June 2024.

4.3. STUDY POPULATION

All individuals visiting ALERT Comprehensive specialised Hospital and Hawassa University Comprehensive Specialised Hospitals during the study period who are clinically diagnosed with meningitis and fulfill the inclusion criteria were enrolled in the study.

4.4. INCLUSION AND EXCLUSION CRITERIA

4.4. 1 Inclusion criteria

All patients of all age-group who were clinically suspected of meningitis and showed willingness to participate in the study (through consent) were included in the study.

4.4. 2. Exclusion criteria

- Patients for whom lumbar puncture is contraindicated were excluded from the study primarily due to patient safety concerns and the potential for severe, irreversible adverse events.
- Neonates were also excluded from the study since it is technically more challenging to obtain sufficient specimen in neonates due to their fragile anatomy.
- Presumptive meningitis patients who refused to give consent were excluded.
- Patients who are critically ill and unable to give blood samples were excluded.

4.5. VARIABLES OF THE STUDY

4.5. 1. Dependent variables

- Prevalence of Meningitis (Bacterial, viral)
- Antimicrobial susceptibility profiles of isolated pathogens
- Status of immune-correlates

4.5.2. Independent variables

- Socio-demographic factors
  - ✓Age
  - ✓ Sex
  - ✓ Residence
  - ✓ Occupation
- Vaccination status
- Level of Immunity

4.6. SAMPLE SIZE DETERMINATION AND SAMPLING TECHNIQUE

Sample Size Determination.

The sample size was determined using a single-population proportion formula based on a prevalence rate of 17% reported in a previous study conducted in Ethiopia (Yeshidinber, 2020).

$$n = \frac{\left[Z_{a}/_{2}\right]^{2} p[1-p]}{d^{2}}$$

$$n = \frac{[1.96]^{2} * 0.17[1-0.17]}{(0.05)^{2}}$$

$$n = 217$$

Adding a nonresponse rate (10%), the final sample size becomes =239

Where n = sample to be drawn

z= the standard normal deviation corresponding to specified CI (95%) = 1.96

p = proportion among the study population = 17% (0.17) from the previous study (Yeshidinber, 2020).

d = marginal error = 5%

Sampling Technique

A non-probability, consecutive sampling technique was employed to recruit participants until the target sample size was achieved within the study period. This approach was selected because meningitis is a relatively rare condition, making random sampling methods impractical for obtaining a sufficient sample size within the limited timeframe of an MSc study. By systematically enrolling all eligible cases that met the study criteria during the data collection period, this method ensured efficient recruitment while maintaining methodological rigor.

Proportional Allocation

Sample allocation was proportionally distributed across study sites based on the patient flow recorded in the preceding three months. During this period, ALERT Hospital had evaluated 53 suspected meningitis cases, while HUCSH had recorded 36. Accordingly, we initially assigned 142 participants to ALERT and 97 to HUCSH. After the screening, 201 eligible patients were enrolled—142 from ALERT and 59 from HUCSH—refuting adjustments for exclusion of unqualified specimen (insufficient, contaminated or mishandled).

4.7. OPERATIONAL DEFINITIONs

Presumptive meningitis patients: patients with sudden onset of fever (>38.5°C rectal or >38.0°C axillary) and one or more of the following such as neck stiffness, altered consciousness, another meningeal sign or petechial or purpural rash.

Community-acquired meningitis (CAM): Community-acquired meningitis (CAM) is meningitis contracted outside of a healthcare environment. It is acquired in the general community, rather than

in a hospital or other medical facility as a complication of procedures or hospitalization (Diederik van de Beek et al., 2016; Van de Beek et al., 2006).

Hospital-acquired meningitis (HAM): Hospital-acquired (nosocomial) meningitis refers to an infection of the meninges (membranes surrounding the brain and spinal cord) that develops during a hospital stay, typically more than 48 hours after admission. It is often linked to invasive procedures, like neurosurgery or the insertion of catheters, or to complications from head trauma (Sahiledengle et al., 2020; Valdoleiros et al., 2022).

Aseptic meningitis: A syndrome characterized by acute onset of meningeal symptoms (stiff neck, fever, and headache), and cerebrospinal fluid pleocytosis (excessive lymphocytes), with no laboratory evidence of bacterial or fungal organisms. Aseptic meningitis is a syndrome of multiple etiologies, but many cases are caused by a viral agent.

Confirmed case: A clinically compatible illness diagnosed by a physician as aseptic meningitis, with no laboratory evidence of bacterial or fungal meningitis; or a viral isolate from cerebrospinal fluid; or a viral isolate from blood with a clinically compatible illness diagnosed by a physician.

Bacterial meningitis:

Suspected meningitis case: Any person with sudden onset of fever (>38.5 °C rectal or 38.0 °C axillary) and neck stiffness or other meningeal signs, including bulging fontanelle in infants.

Confirmed meningitis case: Any suspected case that is laboratory confirmed by culture or identification of (i.e., by polymerase chain reaction) a bacterial pathogen (N. *meningitidis, S. pneumoniae or H. influenzae type b*) in the CSF or blood.

Healthy Controls: defined as age- and sex-matched individuals without a history of meningitis or other acute infections, recruited from the same geographic area and community as the cases to ensure comparable environmental exposure and rigorously screened to be free of all target pathogens via culture and PCR.

4.8. DATA COLLECTION PROCEDURES

4.8.1. Demographic characteristics and risk factors

Sociodemographic and clinical data were collected by assigned physicians and Nurses using a pre-structured questionnaire. The purpose of the study was explained to the study participants or their

guardians, and those who provided their consent or assent were recruited to participate in the study.

4.8.2. Specimen collection and transportation.

CSF Sample collection

After obtaining consent, 3 mL of CSF was aseptically collected by the attending physician following standard procedures. This sample was divided into three sterile tubes: Tube 1 for chemistry and immunology, Tube 2 for bacteriology, and Tube 3 for cell count and differential. Each tube was labeled with patient information, collection time, and tube number. CSF Samples in tube-2 were transported to the microbiology laboratory within ten minutes at ambient temperature, and analysis began within 30 minutes. Before conventional lab analysis, a minimum of 1 mL of CSF from Tube 2 was transferred to a cryotube in a safety cabinet, coded, documented, and stored at -20 °C for later PCR. These PCR samples were then transported in a cold box to AHRI and stored at -80 °C until ready for PCR analysis.

Blood Sample collection

For patients undergoing lumbar puncture, 4 mL of blood was collected in EDTA tubes by nurses immediately after CSF collection. These blood samples were labeled with patient information and promptly transported to the laboratory along with the CSF samples. Upon arrival, the blood samples were centrifuged at 6000 rpm for 10 minutes. The separated plasma was then aseptically transferred via a sterile Pasteur pipette to a cryotube within a safety cabinet, coded, documented in a logbook, and stored at -20 °C. The plasma was subsequently transported in a cold box to AHRI and stored at -80 °C until thawed for complement assay analysis

4.8.3. Specimen Processing

I. CSF analysis (appearance, cell count, and chemistry)

Once arrived at the laboratory, the CSF was examined for its appearance; clear, cloudy, turbid, or bloody or if there is any clots or xanthochromia. Cloudiness often indicates infection. Unclear or turbid fluid denotes significant leukocyte content in the sample. Differential cell count was performed using a hematology analyzer. Clinical chemistry analyzer was used to measure glucose and total protein.

II. Microscopic Examination

Smears were made from the sediment of centrifuged CSF sample (centrifuged for 20 minutes at 2000rpm) and stained with Gram's stain. Examined under the microscope for the presence of bacteria (Gram-positive or Gram-negative), based on morphology and gram reaction

III. Cerebrospinal Fluid (CSF) Culture and Bacterial Identification

Upon arrival at the laboratory, the CSF sample was inoculated onto Blood Agar (BAP), Chocolate Agar (CAP), and MacConkey Agar plates for primary culture and incubated at 37°C. The Chocolate and Blood Agar plates were placed in a candle jar to create a microaerophilic environment (5–10% CO_2), facilitating the growth of fastidious bacteria, while the MacConkey Agar plates were incubated aerobically.

Following this, our strategy to ensure comprehensive pathogen identification, particularly for organisms that might be present in very low numbers or are difficult to culture, included direct molecular detection via PCR. This approach allowed us to identify pathogens that might otherwise be missed by conventional culture methods alone.

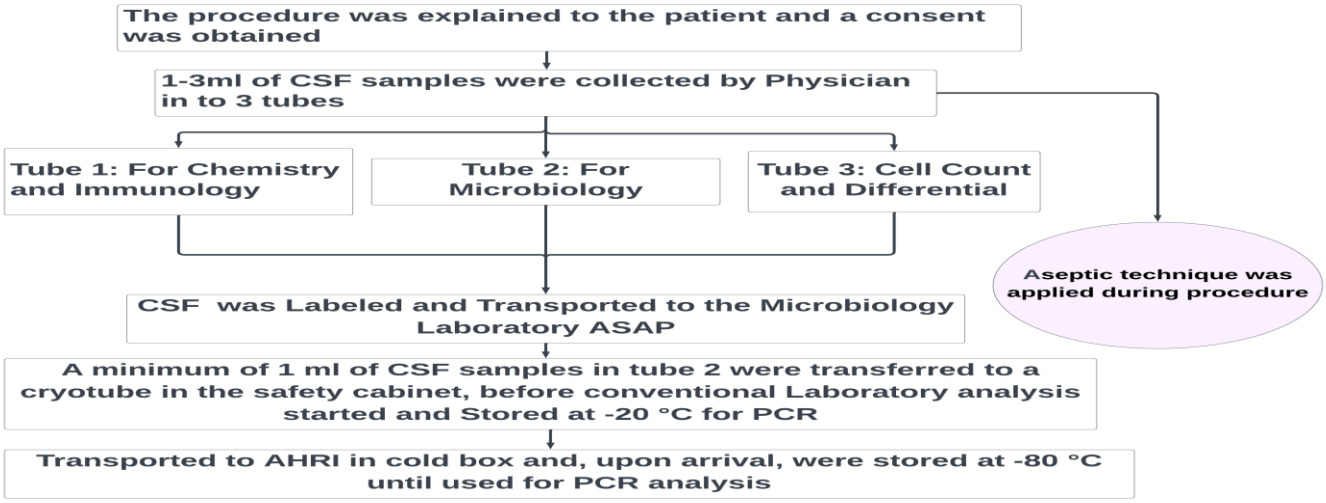
The culture plates were initially examined for bacterial growth after 18 to 24 hours of incubation. If no growth was visible, incubation continued for a maximum of 72 hours before a negative result was finalized. To identify positive CSF cultures, a combination of colony morphology, Gram-stain characteristics, and biochemical testing was employed, following standard microbiological practices (Cheesbrough, 2004). For Gram-negative organisms, including both Enterobacteriaceae and non-Enterobacteriaceae, identification involved a series of tests such as indole production, H_2S production, citrate utilization, motility, urease activity, oxidase reaction, and carbohydrate fermentation, with the aid of API 20E identification kits from BioMérieux, France. Gram-positive cocci were differentiated using catalase, coagulase, bacitracin susceptibility, and optochin sensitivity assays.

IV. Antibiotic Susceptibility Testing

Antibiotic susceptibility testing (AST) was performed on all bacterial isolates using the Kirby-Bauer disk diffusion method, adhering to Clinical and Laboratory Standards Institute (CLSI, 2022) guidelines. The following antibiotics were tested (abbreviation, concentration):

Ampicillin (AMP, 10 µg), Ceftriaxone (CRO, 30 µg), Ciprofloxacin (CIP, 5 µg), Chloramphenicol (C, 30 µg), Trimethoprim-Sulfamethoxazole (SXT, 1.25/23.75 µg), Gentamicin (GM, 10 µg), Meropenem (MEM, 10 µg), Imipenem (IMP, 10 µg), Vancomycin (VA, 30 µg), Cefepime (FEP, 30 µg) and Cefotaxime (CTX, 30 µg)

Inoculated plates were incubated at 37±2∘C for 24–48 hours, with conditions (ambient air or 5% CO_2) adjusted based on the bacterial species. After incubation, the diameters of inhibition zones around each disk were measured to the nearest millimeter using a calibrated ruler. Results were categorized as sensitive, intermediate, or resistant according to CLSI (2022) interpretive criteria.



- **Diagrammatic workflow of CSF sample Collection, Transportation and Processing.**

V. Polymerase chain reaction (Conventional-PCR)

Nucleic acids extraction

Nucleic acids were extracted from CSF samples using a Bioer NPA-32P automated nucleic acid purification machine (HANGZHOU BIOER TECHNOLOGY CO., LTD., China). 300µL of CSF volume was used for each extraction procedure. For quality control, DNA was extracted from corresponding ATCC strains for all targeted bacteria using the same procedure.

PCR protocol Optimization

Our multiplex conventional PCR was optimized in the following six (6) steps to ensure efficient and specific amplification of multiple target DNA sequences in a single reaction. Our goal was to achieve strong, specific bands for all targets without primer dimers or non-specific amplification.

Step-1: Selection of primers combination

Primer pairs that have similar melting temperatures (Tm) [typically within a 5^0Crange] and minimal self-complementarity or cross-complementarity were selected to avoid primer-dimers and non-specific amplification. Primer pairs that have distinct amplicon sizes were selected to facilitate separation and visualization on an Agarose gel. Each primer pair's specificity and efficiency was verified by performing individual singleplex PCR reactions with each primer pair before combining them in a multiplex reaction.

Step-2: Annealing Temperature Optimization: Gradient PCR test was performed to determine the optimal annealing temperature.

Step-3: Primer Concentration Optimization: Concentration of each primer was adjusted to obtain uniform bands of each amplified targets.

Step-4: $MgCl_2$ Concentration Optimization: $MgCl_2$ concentration was optimized by testing a range of concentration multiplex reaction.

Step-5: Template DNA Concentration and Quality: The optimal concentration of template DNA was selected by testing ranges of concentration. DNA purity was assessed using A260/280 and A260/230 ratios.

Step-6: Cycling Conditions Adjustment: PCR cycling parameters were adjusted until the optimum condition was obtained.

Master Mix Preparation

The Go-Taq® Green Master Mix: (Promega, Cat-No. M7122), which contains Taq DNA polymerase, dNTPs (10mM), MgCl2 (25mM) and reaction buffers (5X Green buffer), was used, following the manufacturer's recommended cycling conditions with adjustments for our specific target and primer set, which are consistent with common practices for this master mix. The final volume of each PCR tube contained 25 µL reaction mixture composed of 5 µL of 5X buffer, 2.5 µL of MgCl2 (25mM), 0.5 µL dNTPs (10mM) of the master mix, 0.5 µL of each forward and reverse primer, 0.1 µL Taq polymerase, 5 µL of extracted DNA and 10.9 µL nuclease-free water to complete the final volume.

Nucleic acid amplification and Detection

Nucleic acid amplification was performed in a T100 LASEC thermocycler (BIO-RAD) using the following 35-cycle program: **Initial denaturation:** 94∘C for 3 minutes, **Denaturation:** 94∘C for 10 seconds, **Annealing:** 60∘C for 30 seconds, **Extension:** 72∘C for 30 seconds, **Final extension:** 72∘C for 5 minutes and **Hold:** 12°C indefinitely

The thermal cycler's lid temperature was set to 105∘C, and the entire program ran for approximately 1 hour and 20 minutes. Subsequently, PCR products were separated by gel electrophoresis at 400 amps and 120 volts for 45 minutes. The products were then visualized under UV light using a CS-DARKROOM (Cleaver Scientific), with identification based on their amplicon sizes. Specific primers for the target organisms used in this study can be found in (see Table 5 and 6).

Conventional multiplex PCR

Conventional multiplex PCR was utilized for the detection of bacterial DNA. Bacterial targets were multiplexed into two groups for amplifying primer specific genes. The first group **[Group-1]** comprises Eco (*E. coli*), Kpn *(K. pneumonae*), Sau (*S. aureus*), and Lm (*L. monocytogenes*). The second group **[Group-2]** consisted of CtrA (*N. meningitides*), LytA (*S. pneumoniae*), Hpd (*H. influenzae*), and Sag (*S. agalactiae*). And viral targets were detected in singleplex PCR. Gel images showing the amplified bands of each target can be found below [see figure 3].

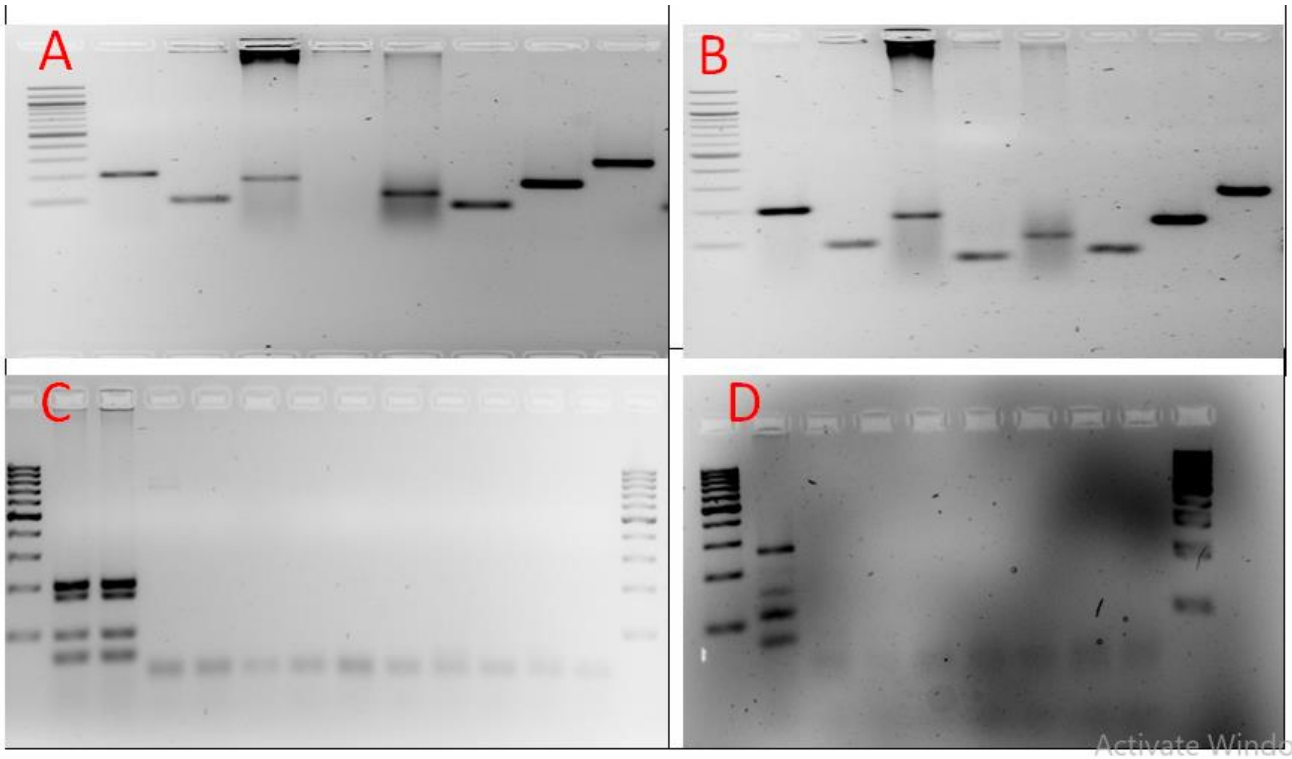


Figure 3: Gel images showing amplified bands of each target in singleplex [A and B] and Multiplex [C and D] reactions against 100bp ladder. **Image** A&B: amplified bands of each target in singleplex from left to right: - E. coli [204bp], S. aureus [97bp], K, Pneumoniae [176bp], L. monocytogenes [64bp], N .meningitidis [111bp], S. Pneumoniae [75bp], H. influenzae [151bp] and S. agalactiae [260bp]. **Image-C**: Group-1 multiplex from top to bottom: E. coli [204bp], K, Pneumoniae [176bp], S. aureus [97bp], and L. monocytogenes [64bp]. **Image-D:** Group-2multiplex from top to bottom: S. agalactiae [260bp], H. influenzae [151bp], N. meningitidis [111bp], and S. Pneumoniae [75bp].

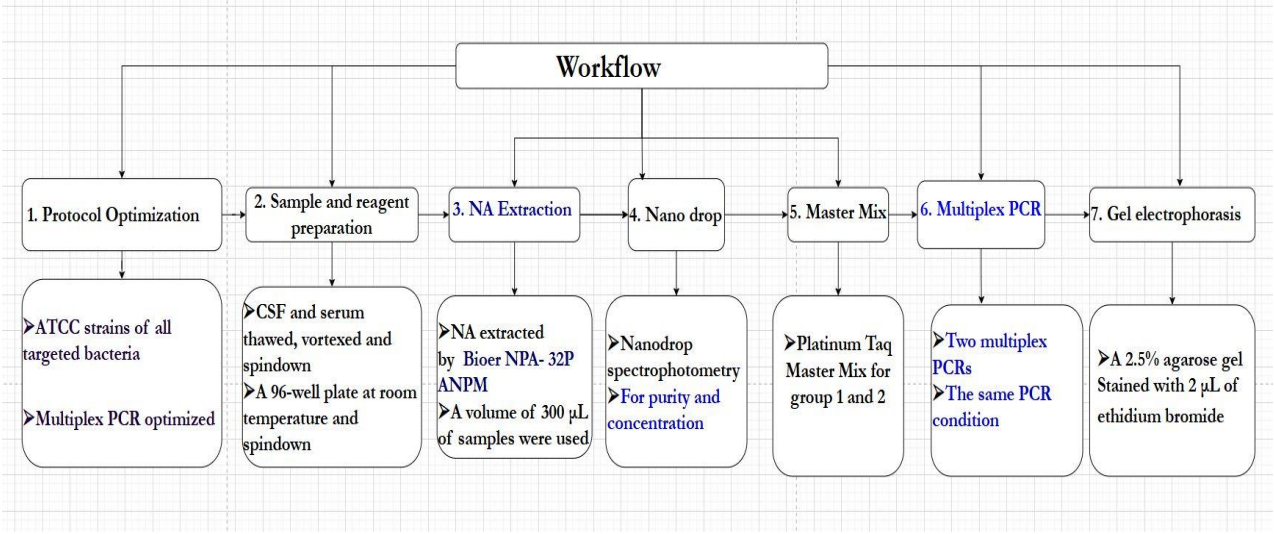


Figure 4: Diagrammatic Work-flow of Molecular-Analysis Procedures

Table 5: Specific primers used for the bacterial targets in this study

,Organism	Target gene	Oligo-name	Sequence (5' to 3')	Amplicon size (bp)	TM	GC content	*References*
N. meningitidis	*ctr A*	*ctrA*-F	GCTGCGGTAGGTGGTTCAA	111	60.3	57.8	*(Karimi et al., 2023)*
		ctrA-R	TTGTCGCGGATTTGCAACTA		58.8	45.0	
S. pneumoniae	*LytA*	*lytA*-F	ACGCAATCTAGCAGATGAAGCA	75	60.4	45.4	*(Wang et al., 2014)*
		lytA-R	TCGTGCGTTTTAATTCCAGCT		45.0	42.8	
H. influenzae	*Hpd*	*Hpd*-F	GGTTAAATATGCCGATGGTGTTG	151	58.3	43.4	*(De Gier et al., 2016)*
		Hpd-R	TGCATCTTTACGCACGGTGTA		60.3	47.6	
E. coli	*Rrna*	*Eco-*F*rRNA*	GGGAGTAAAGTTAATACCTTTGC	204	55.3	39.1	*(Wang et al., 2014)*
		Eco-Rr*RNA*	CTCAAGCTTGCCAGTATCAG		56.2	50.0	
L. monocytogen	*Hly*	Lm-F*hly*	CAT GGCACCACCAGC ATCT	64	60.4	57.8	*(Wang et al., 2014)*
		Lm-R*hly*	ATC CGCGTGTTTCTTTTCGA		58.5	45.0	
S. aureus	*Fem A*	Sau-F*femA*	TGCTGGTGGTACATCAAA	97	53.5	44.4	*(Wang et al., 2014)*
		Sau-R*femA*	ACGGTCAATGCCATGATTTAA		56.2	38.1	
S. agalactiae	*Cfb*	Sag-F*cfb*	ATGATGTATCTATCTGGAACTCTAGTG	260	57.7	37.0	*(Wang et al., 2014)*
		Sag-R*cfb*	CGCAATGAAGTCTTTAATTTTTC		53.5	30.4	
K. kpneumoniae	*RcsA*	Kpn-F*rcsA*	GGATATCTGACCAGTCGG	176	53.5	55.6	*(Dong et al., 2015)*
		Kpn-R*rcsA*	GGGTTTTGCGTAATGATCTG		55.0	45.0	

Table 6: Specific primers used for the viral targets in this study

Name of viruses	Oligo-name	Sequence (5' to 3')	Amplicon size (bp)	TM	GC content	*References*
HSV-1	HSV1-gpB-F	CCACCGTCAGCACCTTCAT	127	60	57.9	*(Pormohammad et al., 2020)*
	HSV1-gpB-R	CGCTGGACCTCCGTGTAGTC		62.27		
HSV-2	HSV2-gpB-F	CCACCGTCAGCACCTTCAT	127	60	57.9	*(Jerome et al., 2002; Pormohammad et al., 2020)*
	HSV2-gpB-R	CGCTGGACCTCCGTGTAGTC		62.27		
EBV	EBV3-9F	CTGACACTTTAGAGCTCTGGAG	228	57.89	50	*(Liu et al., 2022)*
	EBV3-9R	GGCCCTGACCTTTGGTGAAGTCA		64.79		
VZV	VZV-DNApolF	GCGCTCTAACGTTCGAGAAAGT	60	61.23	47.6	*(Wong et al., 2016)*
	VZV-DNApolR	CGCATAGCCAACCAGTCTTTT		59.19		
Enterovirus	PanEV2-5′NTRF	CAT GGT GCG AAG AGT CGA TTG A	138	57.27	47.6	*(Pabbaraju et al., 2015)*
	PanEV2-5′NTRR	5CACCCAAAGTAGTC GGTTCCGC		59.93		
Mumps virus	MumV-F	TTCAGGGAACCAACTCGTTGA	171	59.51	47.6	*(Liu et al., 2022)*
	MumV-R	CTTCGGAGGATGAGACCATGAT		59.37		

VI. Assessment of Immune correlates of risk

CD4+ T-cell counts and percentages were measured from whole blood samples collected in EDTA tubes using BD FACSPresto CD4 Counter [Multicolor Fluorescence Imaging Cytometry]. The system uses single-use, disposable cartridges. Each cartridge contains a disc of dried fluorescently labeled antibodies specific to various cell surface markers. Crucially for CD4 counting, these include antibodies for: CD4 (To identify CD4+ T cells), CD3 (To identify all T lymphocytes (as CD4+ cells are a subset of T lymphocytes), CD45 (A pan-leukocyte marker used for gating and identifying white blood cells). It can directly enumerate the absolute CD4 count (cells/µL) and calculate the CD4 percentage (the proportion of CD4+ cells among lymphocytes). For the assessment of plasma complement component deficiencies, including terminal components C5, C5a, C9, as well as Factor D (Adipsin), MBL, Factor I, and Properdin, stored plasma samples were utilized. These measurements were performed using Luminex® xMAP® technology with the MILLIPLEX® Human Complement Expanded Magnetic Bead Panel 1 kit. A detailed description of the specific assay procedures and the criteria used to define 'deficiency' for each component can be found in Annex III.

4.9 QUALITY ASSURANCE.

Data quality was ensured through use of standardized data collection materials, pretesting of the questionnaires, proper training before the start of data collection and intensive supervision during data collection by the principal investigator.

Sample processing, culture, microscopy, and PCR assays were performed by qualified laboratory personnel. Standard operating procedures (SOPs) were used for CSF handling, inoculation techniques, media preparation, incubation, Gram staining, biochemical identification, and PCR execution.

Each PCR run included validated positive controls (known positive nucleic acid extracts from target pathogens) and negative controls (molecular grade water, and extraction blank) to monitor for amplification success and contamination, respectively.

4.10. STATISTICAL ANALYSIS AND INTERPRETATION

Data was analyzed by using Statistical Product and Service Solutions (SPSS) version 26.0 software. Frequency and percentage were calculated to summarize the results and presented in Tables and Graphs.

For statistical analysis of the immune correlates of risk for bacterial and viral meningitis we obtained demographic data (age, sex), CD4+ T-cell counts, and plasma complement component concentrations (C5, C5a, C9, Factor D (Adipsin), MBL, Factor I, Properdin) from patients comprising 50 cases with bacterial meningitis, 20 cases with viral meningitis, and 20 healthy controls [rigorously screened to be free of all target pathogens via culture and PCR.]

Then all immune component measurements (CD4+ T-cells, C5, C5a, C9, Factor D, MBL, Factor I, Properdin) were categorized into binary variables indicating 'deficiency' (Yes/No) based on their predefined counts and/or plasma concentrations. Univariate and multivariate logistic regression models were employed to evaluate the effect of these independent variables, along with age and sex, on the dependent variable (diagnosis of bacterial or viral meningitis). A two-sided p-value of less than 0.05, calculated at a 95% confidence interval, was considered statistically significant.

4.11. ETHICAL CONSIDERATION

Ethical approval for the study was secured from the Department of Microbiology-Immunology and Parasitology Research Ethics Review Committee (DRERC/004/2024) at Addis Ababa University, and the AHRI/ALERT Ethics Review Committee (AAERC) (protocol number PO-55-22). Before enrollment, all eligible participants received clear explanations of the study's purpose, benefits, and confidentiality measures. Adult volunteers provided written informed consent. Minors under 18 gave assent after their family or guardian consented. Participant confidentiality was maintained by using codes instead of personal identifiers.

4.12. DISSEMINATION OF RESEARCH FINDINGS.

The finding of the study will be submitted to Addis Ababa University, DMIP. The data will be made known to the scientific community through conference presentation and publication in reputable national and international journals.

CHAPTER FIVE: RESULTS

5.1. Socio-Demographic Characteristics of Study Subjects

A total of 201 patients of all age groups (except neonates) with suspected meningitis were included in the study, of whom 56.2% were males. The majority of stud participants (83.1%, n=167) were children under 5 years of age, with 59.2% being (<1years) Infants [see**Table-7].**

Table 7: The distribution of Socio-demographic characteristics of patients suspected with Meningitis at ALERT and HUCSH, 2023/24

Variables		Frequency	Percentage
Sex	Male	113	56.2%
	Female	88	43.8%
Age	(<1years) Infants	119	59.2%
	(1 to <5 years) Young children	48	23.9%
	(5–17years) Children/Adolescents	24	11.9%
	(18-64 years) Adults	9	4.5%
	(≥65 years)elderly	1	0.5%
Sites	ALERT-Hospital	142	70.6%
	HUCSH	59	29.4%

5.2. Clinical Presentation of Study Participants before Admission

The median duration of illness before hospital presentation was varied by age group. Young infants (29-90 days) typically presented after 24 hours of symptom onset (IQR: 7.0-72.0) whereas older infants and children presented later, with a median of 72 hours (IQR: 24.0-96.0). Both groups exhibited febrile temperatures upon presentation with mean body temperatures of 38.1°C (SD=0.8) in young infants and 38.3°C (SD=0.9) for older infants and children. The most frequently observed clinical features at admission included irritability or lowered consciousness (73%), fever (59.2%), vomiting (48%), decreased breastfeeding (43.3%), and seizure (37%) (See Figure: 5).

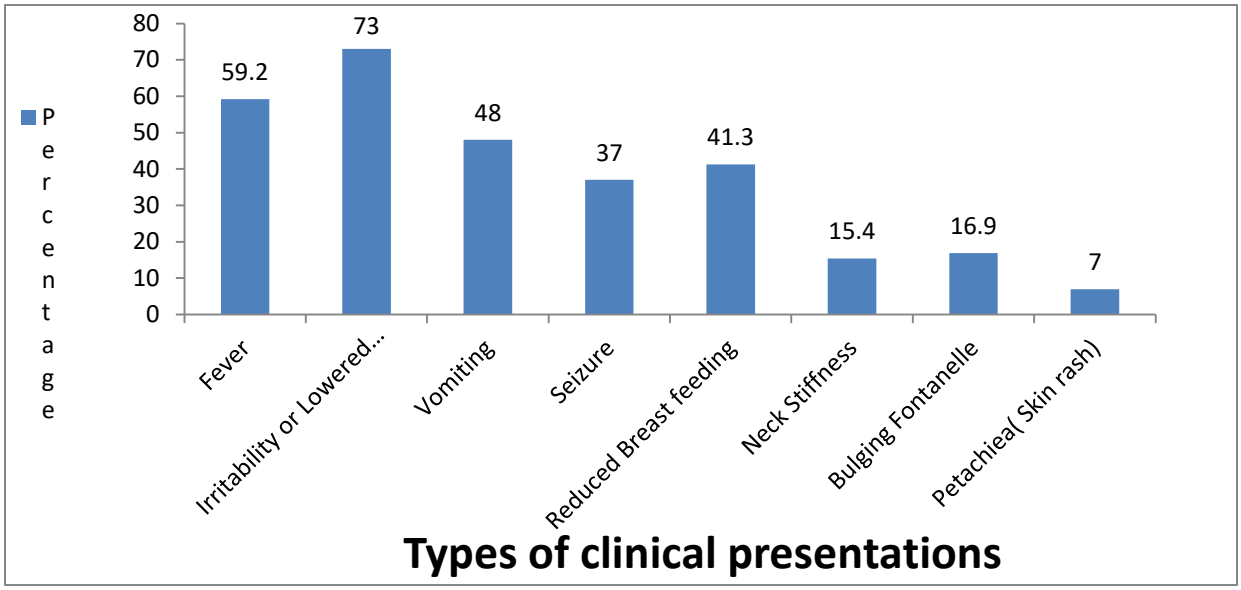


Figure 5: Clinical presentations of patients suspected with Meningitis at ALERT and HUCSH, 2023/24

5.3. Prevalence of Bacterial Pathogens of Meningitis

5.3.1. CSF Culture and Microscopy

The overall prevalence of culture-positive bacterial meningitis cases was 5.5% (n=11) of which 7 cases were also positive by microscopy. Among the 11 culture-positive cases, two patients had multiple infections. The bacterial pathogens isolated includes *Klebsiella pneumoniae* (3 cases 1.5%), *S. pneumoniae* (2 cases 1%), *E. coli* (2 cases 1%), Acinetobacter(2 cases 1%), and one each case of *N. Meningitidis*, *S. aureus*, Coagulase negative staphylococci (CoNS) and Enterobacter (Figure-6).

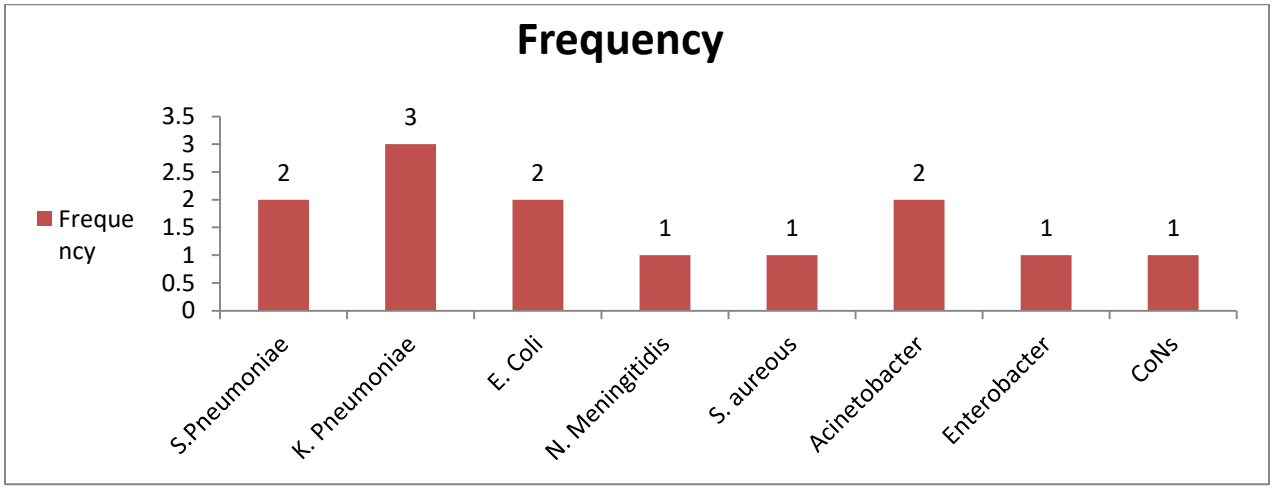


Figure 6: Bacterial species identified by CSF culture at ALERT and HUCSH, 2023/24

5.3.2. Antimicrobial Resistance Patterns of Bacterial Isolates

Key resistance patterns among Gram-positive isolates included *S. pneumoniae* resistance (one out of two isolates) to erythromycin, trimethoprim-sulfamethoxazole, ciprofloxacin, rifampin and *S. aureus* one isolate (100% resistance to chloramphenicol, rifampin), but remained susceptible to tetracycline.

For Gram-negative isolates, notable resistance was seen in *K. pneumoniae* resistance (two out of three isolates) (trimethoprim-sulfamethoxazole, ampicillin), *E. coli* (multiple drugs), and Acinetobacter two isolates (100% resistance to tetracycline, ciprofloxacin, meropenem, imipenem). *N. meningitidis* displayed intermediate resistance to ciprofloxacin and chloramphenicol but was otherwise susceptible.

5.3.3. CSF Conventional PCR

The overall prevalence of bacterial etiologies identified by conventional multiplex PCR among patients suspected of meningitis was 36.8% (n=74) including cases of multiple infections. *Escherichia coli* was the most frequently isolated pathogen accounting for 40.5% of all PCR-positive cases and representing 14.9 % (n=30) of all suspected meningitis patients. This was followed by *Streptococcus pneumoniae* detected in 7.5% (n=15) and N. meningitidis in 5.5% (n=11) of cases (see Figure-7).

A total of 43 (21.4%) cases of bacterial meningitis occurred in males while 31 (15.4%) in females. *E. coli* and *S. agalactiae* were predominantly observed in young infants and preschool children accounting for 63.3% (19 of 30) *E. coli* meningitis cases 75% [3 of 4] *S. agalactiae cases* respectively.

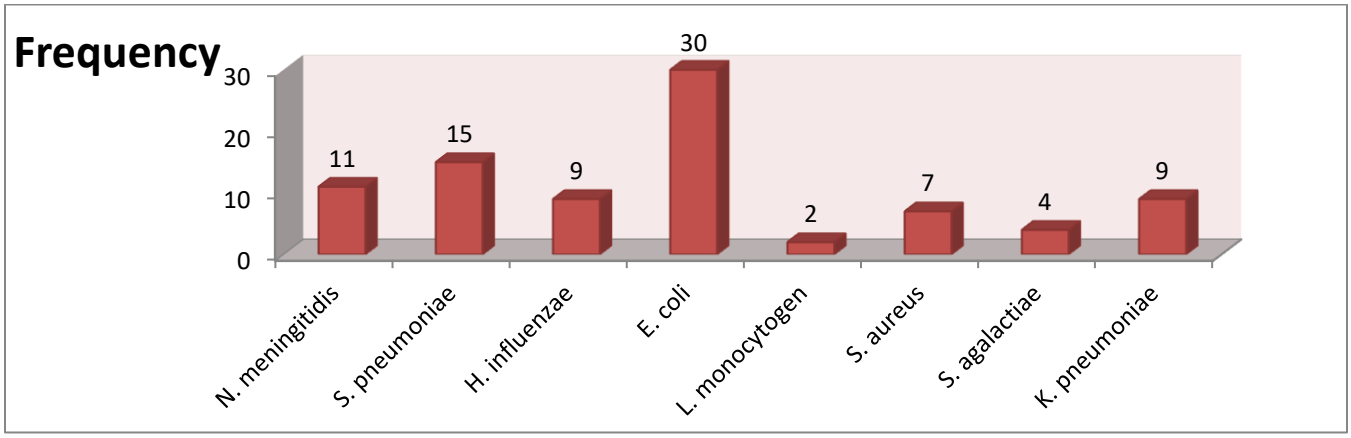


Figure 7: Frequency of bacterial etiologies identified from patients suspected of meningitis by Conventional multiplex PCR at ALERT and HUCSH, 2023/24

5.4. Prevalence of viral pathogens of meningitis [aseptic meningitis]

Polymerase chain reaction (PCR) test was performed targeting six viral pathogens known to cause meningitis: four DNA- viruses (Herpes Simplex virus-1, Herpes Simplex virus-2, Epstein-Barr virus, and Varicella-Zoster) and two RNA- viruses (Mumps virus and Enteroviruses). The overall prevalence of viral meningitis was 34.3% [n=69]. Human enteroviruses were the most commonly identified causes of aseptic meningitis 28.9% [n=58] of cases, followed by HSV-1 at 2.5% [n=5], HSV-2 1.5% [n=3] and EBV 1% [n=2] [see Figure 8].

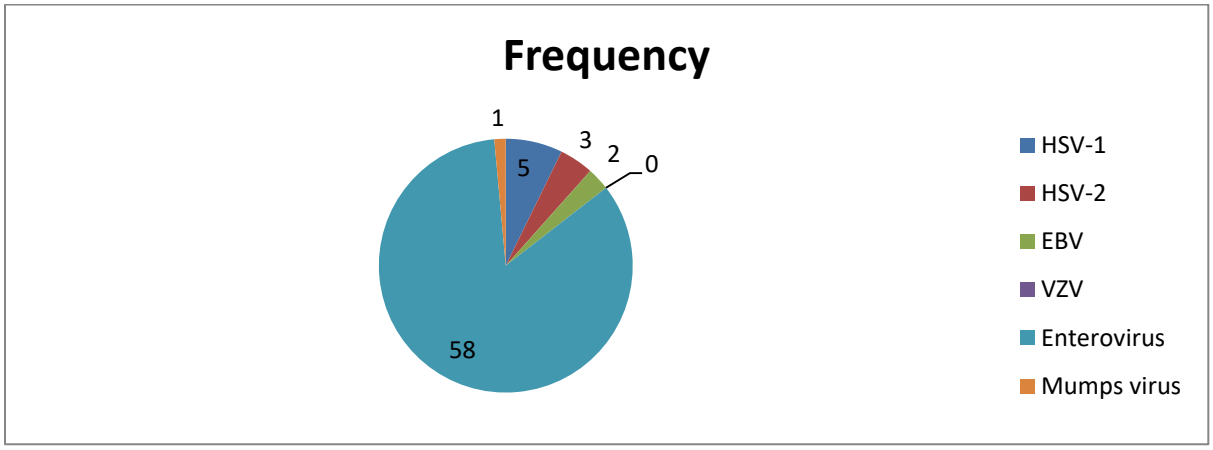


Figure 8: Frequency of etiologies of viral meningitis identified by PCR from patients suspected with Meningitis at ALERT and HUCSH, 2023/24

5.5. Immune correlates of risk for community-acquired meningitis

5.5.1. Baseline characteristics of patients and controls

The mean age of participants was 8.96±7.62 years for bacterial meningitis cases, 7.68±7.21 years for viral meningitis cases, and 8.23±7.42 years for healthy controls. Males constituted (51.1%) of the cohort and 60% of participants resided in Addis Ababa. Demographic characteristics were comparable between cases and controls, with no significant differences observed [see Table-8].

Table 8: Base-line Characteristics of study participants: Two-tailed P values were calculated by Fisher's exact test between infection states as follows: P1 = Bacterial meningitis vs Viral meningitis; P2 = Bacterial meningitis vs Healthy controls; P3 = Viral meningitis vs Healthy controls.

characteristics	**Bacterial Meningitis [n=50]**	**Viral Meningitis [n=20**	**Healthy Controls [n=20]**	**p-Values**		
				p-1	p-2	P-3
Mean age (yrs.) **(± SD)**	8.96 (7.62)	7.68(7.21)	8.23(7.42)	0.065	0.541	0.601

Gender male	26(52%)	9(45%)	11(55%)	0.57	0.647	0.875
Residence						
Addis Ababa	31(62%)	11(55%)	12(60%)	0.324	0.335	0.524
Oromia	11(22%)	6(30%)	4(20%)	0.412	0.365	0.468
Sidama	8(16%)	3(15%)	4(20%)	0.078	0.485	0.587

SD= standard deviation

5.5.2. Key Immune Correlates of Bacterial Meningitis Risk

This study identified distinct immune profiles associated with viral and bacterial meningitis. Individuals with viral meningitis exhibited low CD4 levels, whereas those with bacterial meningitis showed significantly reduced levels of terminal complement complex (TCC) components—C5, C5a, and C9—as well as Properdin, compared to controls. 54% [27/50] of patient with bacterial meningitis have Properdin deficiency followed by 52% [26/50] C5-Deficiency and 46% [23/50] C9- Deficiency. In contrast, mannose-binding lectin (MBL), Factor D (Adipsin), and Factor I levels showed no significant differences across case and control groups [see Table-9].

Univariate and multivariate logistic regression analyses were performed for all variables. The multivariate analysis showed no significant association between low CD4 levels and the risk of developing bacterial meningitis. However, deficiencies in several plasma complement components were significantly associated with increased odds of bacterial meningitis after adjusting for age, sex, and other immune correlates.

Specifically, C5 deficiency was associated with a 2.50-fold increase in the odds of bacterial meningitis (adjusted odds ratio [AOR] = 2.50; 95% confidence interval [CI]: 1.60–3.90; $p < 0.001$). Similarly, C9 deficiency increased the odds by 3.09 times (AOR = 3.09; 95% CI: 1.31–7.30; $p = 0.007$). Properdin deficiency was also significantly associated with bacterial meningitis, increasing the odds by 1.80 times (AOR = 1.80; 95% CI: 1.20–2.70; $p = 0.003$). While C5a deficiency showed a statistically significant association with increased odds of bacterial meningitis (AOR = 6.67; 95% CI: 0.54–82.0; $p = 0.038$), the wide confidence interval indicates considerable uncertainty regarding the precise effect size.

Collectively, these findings suggest that deficiencies in these complement components may predispose individuals to bacterial meningitis by compromising critical innate immune mechanisms.

Table 9: Univariate and multivariate logistic regression analysis of immune- correlates of risk factors for BM

Variables	**Bacterial Meningitis Cases 50**	**Controls 20**	**Unadjusted OR (95% CI)**	**Adjusted OR (95% CI)**	**p-value**
Deficiency (No [Ref.])	**Yes: N[%]**	**Yes: N[%]**			
CD4 Deficiency<200cells/mm3	6[12%]	5[25%]	7.09(0.92-54.49)	4.04(0.44-37.12	0.217
C5- Deficiency	26[52%]	5[25%]	3.20 (2.10–4.90)	2.50 (1.60–3.90)	**<0.001**
C5a- Deficiency	23[46%]	5[25%]	2.53(0.28-22.12)	6.67(0.54-82.0)	**0.038**
C9- Deficiency	23[46%]	8[40%]	1.23(0.08-0.68)	3.09 (1.31-7.30)	**0.007**
Factor D- Deficiency	17[34%]	10[50%]	0.51(0.24-1.07)	0.55(0.24-1.25)	0.157
MBL- Deficiency	19[38%]	10[50%]	0.62(0.29-1.33)	11.63 (0.78-1.72)	0.075
Factor I- Deficiency	15[30%]	6[30%]	1.04(0.34-3.16)	0.57(0.16-2.06)	0.398
Properdin- Deficiency	27[54%]	7[35%]	2.10 (1.50–2.95)	1.80 (1.20–2.70)	**0.003**

AOR adjusted odds ratio, **CI** confidence interval, **UOR** Unadjusted/crude odds ratio, **MBL** Mannose Binding lectin

5.5.4. Key Immune Correlates of Bacterial and Viral Meningitis Risk

This study identified distinct immune profiles associated with viral meningitis. Overall 65% [13/20] patients with viral meningitis have low CD4 levels [<200cells/mm3] counts. Multivariate analysis was performed to evaluate the association between various immune components and the risk of developing viral meningitis. The analysis revealed a statistically significant association between low CD4 levels [<200cells/mm3] and an increased risk of viral meningitis (adjusted odds ratio [AOR] = 2.59; 95% confidence interval [CI]: 1.60–3.90; $p = 0.035$). This 2.59-fold increase in odds underscores the critical and irreplaceable role of CD4+ T-helper cells in orchestrating effective antiviral immunity. The findings suggest that even moderate reductions in CD4+ T-cell counts may substantially impair viral clearance and elevate susceptibility to viral meningitis.

Notably, none of the other immune components (complement) assessed demonstrated a statistically significant association with viral meningitis risk in this study [see Table-10].

Table 10: Univariate and multivariate logistic regression analysis of immune- correlates of risk factors for VM

Variables	**Viral Meningitis Cases [20]**	**Controls [20]**	**Unadjusted OR (95% CI)**	**Adjusted OR (95% CI)**	**p-value**
Deficiency (No [Ref.])	**Yes: N[%]**	**Yes: N[%]**			
CD4 Deficiency <200cells/mm3	13[65%]	5[25%]	3.24 (2.10–4.90)	2.59 (1.60–3.90)	**0.035**
C5- Deficiency	6[30%]	6[30%]	1.62(0.80-3.29)	0.75(0.25-2.20)	0.605
C5a- Deficiency	4[20%}	5[25%]	2.78(0.33-23.41)	1.20(0.12-11.8)	0.874
C9- Deficiency	7[35%]	8[40%]	0.476(0.19-1.17)	0.69(0.22-2.16)	0.534
Factor D-Deficiency	7[35%]	10[50%]	0.51(0.24-1.07)	0.55(0.24-1.25)	0.157
MBL- Deficiency	8[40%]	10[50%]	7.09 (0.92-54.49)	4.04(0.44-37.12	0.217
Factor I-Deficiency	9[45%]	6[30%]	1.83(0.73-4.54)	1.63(0.59-4.5)	0.338
Properdin-Deficiency	8[40%]	7[35%]	1.90(0.71-5.06)	1.46(0.44-4.84)	0.528
AOR adjusted odds ratio, **CI** confidence interval, **UOR** Unadjusted/crude odds ratio, **MBL** Mannose Binding lectin					

CHAPTER SIX: DISCUSSION

6.1. General Findings

Meningitis remains to be a complex and significant public health challenge, characterized by a diversified etiologic agents and varied prevalence across different regions and populations, both in Ethiopia and around the globe. This study, conducted at two major hospitals in Ethiopia—ALERT Comprehensive Specialized Hospital and Hawassa University Comprehensive Specialized Hospital—involved 201 clinically suspected meningitis cases. This study provides results about the magnitude of bacterial meningitis both by classical culture and PCR, 5.5% and 36.8%, respectively. It also identified the prevalence viral meningitis-by PCR- to be 34.3%, with human enteroviruses predominance (28.9%). Additionally, our study found significant association between deficiency in terminal complement complex (TCC) components (C5, C5a, and C9)—as well as-Properdin and risk of bacterial meningitis. Low CD4+ T cell levels were found to be linked with increased risk of viral meningitis.

6.2. Clinical Presentation and Demographics

Our study found that the majority of stud participants (83.1%) were children under 5 years of age, with 59.2% being (<1years) Infants. Similar data also reported from different parts of Ethiopia; Dilla 72.5% were under five *(Sileshi Elias et al., 2021),* Gonder 71% (Wondimu et al., 2023) and Hawassa 72.3% (Ali, 2024). The finding also agrees with global data indicating that children under five years of age are at the highest risk for meningitis *(WHO, 2021; Wondimu et al., 2023).*

In this study, Common clinical presentations observed in patients with suspected meningitis were lowered consciousness (73%), followed by fever (59.2%), vomiting (48%), and decreased breastfeeding (43.3%). Comparable patterns of clinical presentations of meningitis, were reported from other parts of Ethiopia with fever, irritability, and altered consciousness being the most common symptoms *(Hibstu et al., 2022; Østergaard et al., 2005; Tigabu et al., 2021; Yeshidinber, 2020).*

6.3. Prevalence of Bacterial Meningitis

The current study, documented an overall 5.5% prevalence of culture-positive bacterial meningitis. Comparable results were reported by previous studies in Ethiopia; for instance (4.7%) in Addis Ababa at Tikur Anbessa Specialized Hospital *(Reta and Zeleke, 2016)* and

(8.5%) Hawassa University Comprehensive Specialized Hospital (HUCSH*) (Ali, 2024).* Significantly lower rates were reported from Gondar 1.73% *(Tigabu et al., 2021)* and higher prevalence was reported from Debre Markos 11.2% *(Hibstu et al., 2022).* These varying prevalence rates might be attributed to difference in geographical locations and age -group studied.

Our conventional multiplex PCR identified an overall 36.8% prevalence of bacterial meningitis in the study setting. This finding is in agreement with previous studies have also reported similar rates: 33.1% in Ethiopia *(Mihret et al., 2016),* 32% in China *(Wang et al., 2023),* and 33.8% in Finland (Niemelä et al., 2023). Our finding shows a lower prevalence than studies from Lithuania 66.3% *(Rynkevič et al., 2024)* and Pakistan 88.9%; *(Bhatti et al., 2024).* This difference might be due to the presence of predisposing factors (such as immunocompromising conditions like pediatric age), which can increase susceptibility to infection.

However, our PCR finding shows a higher prevalence than several studies reported from different parts of Ethiopia and few other countries; where the reported prevalence ranging from 1.28% in Gondar to 13.2% in Hawassa in Ethiopia (Ali, 2024; Hibstu et al., 2022; Tigabu et al., 2021), and from Iraq 7.6% (Saadi et al., 2017), and Kenya 11.2% (Gituro et al., 2017). The difference might come from the difference in methodology (some studies only used culture-based methods), which may underestimate the actual prevalence of meningitis due to its lower sensitivity.

In our study, *E. coli* is the most common pathogen detected (14.9% of cases) by PC, accounting for 40.5% of all PCR- positive cases. Our finding is in agreement with previous studies that have identified *E. coli* as a significant cause of neonatal and infant meningitis, particularly in low-resource settings (Ghia and Rambhad, 2021). Similar findings were also reported from China (28.5%) (Jiang et al., 2017) and Korea (Rhie et al., 2018), where *E. coli* was reported as the most common cause of bacterial meningitis. This *E. coli* predominance might be attributed to the fact that the majority of our study participants were young children and infants and *E. coli* is known to be a common cause of bacterial meningitis in these age groups. In contrast, some studies from Ethiopia and other countries have reported *S. pneumoniae* as the predominant bacterial pathogen of meningitis *(Awulachew et al., 2020; Block et al., 2022; Tegene et al., 2015).* This could be

attributed to certain factors like difference in geographical locations and age group studied since etiologies of meningitis greatly vary by age group.

Interestingly, the prevalence of bacterial meningitis identified by CSF culture was much lower (5.5%) compared to PCR (36.8%). This discrepancy indicates the limitations of classical-culture methods in detecting etiologies of bacterial meningitis. Our study proved PCR to be a more sensitive diagnostic tool for identifying bacterial and viral pathogens, especially in cases of prior antibiotic treatment that might have reduced the viability of bacteria in culture.

6.4. Antimicrobial Resistance

Major challenge faced by current healthcare system is continuous rise of antibiotics resistant bacteria (Bhatti et al., 2024; Hibstu et al., 2022). We evaluated AMR and antimicrobial susceptibility (AMS) in all bacterial isolates. We found among gram-negative isolates that, *E. coli* exhibited 50% resistance to first generation (tetracycline and ampicillin), second generation (ciprofloxacin), third generation (Ceftazidime), chloramphenicol, and trimethoprim-sulfamethoxazole. Similar results were also reported in several studies conducted in Ethiopia, which have reported 40% resistance to ciprofloxacin and 50% to chloramphenicol (Tigabu et al., 2021), 40% resistance to tetracycline (Tegene et al., 2015), 60% resistance to ampicillin (Awulachew et al., 2020), and 50% resistance to ciprofloxacin *(Assegu Fenta et al., 2020).*

In contrast to this study, the antibiotic resistant *E. coli* was reported for penicillin (12%), Ceftriaxone (44%), Co-trimoxazole (52%), chloramphenicol (52%) (Edmond et al., 2010) and ceftriaxone (86.7%), Ciprofloxacin (40%), and gentamycin (26.7%) from India (Raj and Reddy, 2013). In our study, *K. pneumoniae* showed 66.6% resistance (trimethoprim-sulfamethoxazole, ampicillin). The emerging resistance in *K. pneumoniae* was due to its ability to produces an enzyme that degrades the carbapenem and beta-lactam ring structures. This resistance is an emerging problem due to misuse and overuse of extended-spectrum cephalosporin drugs *(Nayeem et al., 2024).*

Among gram-positive organisms, *S. pneumoniae* isolates were found to be 25% resistant to erythromycin, trimethoprim-sulphamethoxazole, ciprofloxacin, and rifampin, and 100% sensitive to vancomycin. Comparable findings also reported from different parts of the country, which reported resistance to chloramphenicol 22.2%, erythromycin 20%, and 87% sensitivity to

vancomycin (Tigabu et al., 2021). In our study, *S. aureus* showed 100% resistance of towards chloramphenicol. Comparable results were from UoGCSH *(Tigabu et al., 2021)* and Debre Markos, Ethiopia *(Hibstu et al., 2022).*

On the other hand, there is a significantly high rate of resistance of microorganisms, 72.7% of bacterial isolates, to two or more antibiotic resistance (Multidrug resistance) including commonly prescribed third-generation cephalosporin (ceftriaxone of Cefotaxime) in the current study. Roughly similar findings were reported from Gondar *(Wondimu et al., 2023)* and Hawassa (Assegu Fenta et al., 2020). No bacterial isolates were resistant to Vancomycin in this study, being this drug is indicated as a therapeutic option in the case of multidrug-resistant bacteria, together with the cephalosporin drugs. Comparable results were reported from Gondar (Tegene et al., 2015) Hawassa (Daka et al., 2011) and India *(De Anuradha et al., 2016).*

6.5. Prevalence of Viral Meningitis (Aseptic Meningitis)

The current study also identified the prevalence viral meningitis-by PCR- to be 34.3%, with human enteroviruses predominance (28.9%). Comparable findings were from previous studies in Ethiopia; 26.7% prevalence was reported from cross- sectional study at 5 hospitals in Addis Ababa *(Wami et al., 2021*) and systematic review reported prevalence ranging from 5.6% to 26.7% *(Geteneh et al., 2025).*

In contrast, one study from Uganda reported a prevalence of 5.9% *(Page et al., 2017).* This apparent variability in reported prevalence across African countries might be due to several of factors, including differences in the intensity and scope of surveillance activities, the availability and utilization of advanced diagnostic technologies.

In our study, prevalence of Herpes simplex virus (HSV) types 1 and 2 were (2.5% and 1.5%, respectively). These findings align with Meta-Analysis study on Worldwide Prevalence of Herpes Simplex Virus Encephalitis and Meningitis, which reported prevalence of Herpes simplex virus (HSV) types 1 and 2 as 1% and 4% respectively. And prevalence of Epstein-Barr virus (EBV) was 1% and prevalence of Varicella-Zoster virus (VZV) was 0%. These low frequencies may reflect the limited role of these viruses in causing meningitis in the study population.

6.6. Immune Correlates of Risk

In current study, we identified specific immune markers that are strongly associated with the risk of developing bacterial and viral meningitis. Our multivariate logistic regression analysis showed that certain immune markers are associated with the risk of developing bacterial or viral meningitis.

According to our multivariate logistic regression analysis, deficiencies in terminal complement components [C5 and C9], and Properdin, were significantly associated with increased odds of developing bacterial meningitis. The complement system is a crucial arm of the innate immune system, fundamental for host defense against bacterial infections, particularly through opsonization, direct lysis via the Membrane Attack Complex (MAC), and inflammation (Dunkelberger and Song, 2010). In our finding, we observed strong association of C5 deficiency (AOR = 2.50, 95% CI: 1.60–3.90, $p < 0.001$) and C9 deficiency (AOR = 3.09, 95% CI: 1.31–7.30, $p = 0.007$) with the increased the odds of bacterial meningitis. This finding is consistent with the known roles of these components in forming the MAC, which is critical for lysing bacterial pathogens, especially Gram-negative bacteria (Yekani and Memar, 2023). Similarly, the observed association between Properdin deficiency and increased odds of bacterial meningitis (AOR = 1.80, 95% CI: 1.20–2.70, $p = 0.003$) aligns well with existing literature (Ellison III et al., 1983; Goldstein and Overturf, 2003; Lundbo and Benfield, 2017; Yekani and Memar, 2023). Properdin is the sole positive regulator of the alternative complement pathway, and its deficiency is well-established to predispose individuals to invasive bacterial infections, including meningitis, particularly from *Neisseria meningitidis* and other encapsulated bacteria *(Goldstein and Overturf, 2003) (Specht et al., 2024).*

While C5a deficiency also showed a statistically significant association with increased odds of bacterial meningitis (AOR = 6.67, 95% CI: 0.54–82.0, $p = 0.038$), the broad confidence interval suggests that this estimate may be imprecise, warranting further investigation in larger sample size. In general, these results strongly suggest that a compromised complement system significantly predisposes individuals to bacterial meningitis.

For viral meningitis, our investigation of immune correlates of risk revealed a statistically significant association between low CD4+ T cell levels and an increased risk of developing the condition (AOR = 2.59, 95% CI: 1.60–3.90, $p = 0.035$). This finding agrees with the general

understanding of the role of CD4+ T cells in antiviral immunity. CD4+ T cells help antiviral CD8+ T cells in two main ways: they maximize CD8+ T cell population expansion during a primary immune response and also facilitate the generation of virus-specific memory CD8+ T cell populations. In addition to their helper functions, CD4+ T cells contribute directly to viral clearance. They secrete cytokines with antiviral activities and, in some circumstances, can eliminate infected cells through cytotoxic killing (Boonnak and Subbarao, 2012; Brien et al., 2008; Swain et al., 2012). Therefore, our findings perfectly match with existing immunological principles and prior research describing the essential role of CD4+ T cells in effective antiviral immunity.

CHAPTER SEVEN: LIMITATION OF THE STUDY

Though our study provides significant input to the current understanding of meningitis in Ethiopia, we acknowledge the following limitations:-

1. The findings of this study may not represent the whole country, since the study was conducted at only two major hospitals
2. The cross-sectional nature of the study limits the establishment of direct cause-and-effect relationships between immune correlates and risk of developing meningitis.
3. The study did not assess the long-term consequences of meningitis. Thus lack important information about the sequelae and overall burden of the disease.
4. Small sample size, though adequate, may not be enough to assess immune correlates of risk at species level.
5. Since neonates were excluded from our study, our findings may not be generalizable for newborns.

CHAPTER EIGHT: CONCLUSION AND RECOMMENDATION

8.1. Conclusion

To sum-up, our study provides compelling evidence of the significant burden posed by both bacterial and viral meningitis in Ethiopia, disproportionately affecting young infants and preschool children. Using PCR a significantly higher number of bacterial and viral pathogens of meningitis were detected compared to the classical culture method. We have identified specific immune correlates of risk: deficiencies in terminal complement components (C5, C9, and Properdin) significantly increase susceptibility to bacterial meningitis, while low CD4+ T-cell levels are associated with an elevated risk of viral meningitis. These insights are crucial for public health, strongly advocating for the enhancement of diagnostic infrastructure, strategic expansion of vaccination efforts, rigorous antibiotic stewardship, and the adoption of immune profiling as a tool for risk stratification and improved patient management in Ethiopia.

8.2. Recommendation

Based on the findings of our study we forward the following recommendations to concerned bodies:

1. The high prevalence of both bacterial and viral meningitis indicates the need for enhanced diagnostic capabilities, by adopting advanced techniques like PCR, offer rapid and accurate pathogen identification
2. The high prevalence of bacterial meningitis and predominance of *E. coli*, *S. pneumoniae*, and *N. meningitidis* as causative agents, indicates the for expansion vaccination program to include the pathogens that were previously not targeted.
3. Implementing and enforcing antimicrobial stewardship programs helps to address the high levels of antimicrobial resistance observed in both Gram-positive and Gram-negative isolates.
4. Routine monitoring of CD4 counts in immunocompromised individuals could help to identify those at higher risk of developing viral meningitis, allowing for timely preventative measures.
5. Health education Campaigns promoting improved Hygiene and Sanitation may help in reducing the incidence of viral meningitis, especially Enteroviral meningitis.

6. Continued Surveillance might be important for monitoring changes in the epidemiology of the disease, especially in the context of evolving vaccine introduction and coverage.
7. Future research should prioritize longitudinal studies to evaluate the long-term effects of meningitis and the efficacy of interventions in reducing the toll of this devastating disease.

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Annex- I: Information sheet, Assent and Consent form

Information Sheet for Adults (≥18 years) (English Versions)

Name of Organization: - Department of Microbiology, Immunology and Parasitology, College of Health Sciences, Addis Ababa University

Principal Investigator: Jemal Aman

Title: Assessment of the prevalence of meningitis and immune correlates of risk in community-acquired meningitis at ALERT and Hawassa University Comprehensive Specialised Hospitals, Ethiopia.

Introduction

I am asking you to take part in a research to be conducted by Master of Science candidate in Medical Microbiology, from Addis Ababa University. The team of this research includes principal investigator and advisors from Addis Ababa University Microbiology, Immunology and Parasitology department. I want to be sure that you understand the purpose and your responsibilities in the research before you decide if you want to be part of the study. Please ask us to explain any words or information that you may not understand.

Purpose of the study: The purpose of the study is to determine prevalence of meningitis and assess the immune correlates of risk in community-acquired meningitis at ALERT and Hawassa University Comprehensive Specialised Hospitals, Ethiopia.

Possible Benefits: Participation in this research may not benefit you directly. But results from this study will be used to inform decisions in implementation and strengthening of programs aimed at controlling, rapid diagnosis and detection of meningitis, its management and earlier treatment initiation in Ethiopia.

Possible Risks: The study has no major risks for the participants. However, there may be pain during CSF and Venous blood collection. Generally, it is very unlikely that participation in this research will expose you to any physical, social or psychological risks. Participation in the Research:-You are free to decide if you want to be part of this research or not.

Confidentiality: - I will protect information about you taking part in this research to the best of

our ability. I will neither use your name in any reports nor discuss your participation with anyone outside the research team.

Payment: - No payments will be made for participation.

Leaving the Research: You may end your participation in this study at any time without any negative consequence to you

Your rights as a Participant

This research has been reviewed and approved by the Addis Ababa University ethical review board. If you have any questions about how you are being treated by the study or your rights as a participant you may contact through email: jamaaluna@gmail.com.

Information Sheet for Adults (≥18 years) (Amharic Versions)

ለአዋቂዎች የመረጃ ወረቀት (የአማርኛ ትርጉም)

የድርጅቱ ስም: - በአዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ፣ የማይክሮ ባዮሎጂ ኢሚውኖሎጂ እና ፓራሲቶሎጂ ትምህርት ክፍል

ዋና መርማሪ፡ ጀማል አማን።

ርዕስ፡- **Assessment of the prevalence of meningitis and immune correlates of risk in community-acquired meningitis at ALERT and Hawassa University Comprehensive Specialised Hospitals, Ethiopia**

መግቢያ

በአዲስ አበባ ዩኒቨርሲቲ የሜዲካል ማይክሮባዮሎጂ ትምህርት ክፍል በሳይንስ ማስተር እጩ በሚካሄደው ምርምር ላይ እንድትሳተፉ እጠይቃለሁ። የዚህ ጥናት ቡድን ከአዲስ አበባ ዩኒቨርሲቲ የማይክሮ ባዮሎጂ፣ ኢሚውኖሎጂ እና ፓራሲቶሎጂ ትምህርት ክፍል, ዋና ተመራማሪ እና አማካሪዎችን ያካትታል። ዓላማውን እና የጥናቱ አካል መሆን መፈለግዎን ከመወሰንዎ በፊት በምርምር ውስጥ ያሉ ሀላፊነቶች መረዳቱን እርግጠኛ መሆን እፈልጋለሁ።እባክዎ እርስዎ የማይረዱትን ማንኛውንም ቃል ወይም መረጃ ለማስረዳት ይጠይቁ።

የጥናቱ ዓላማ

የጥናቱ ዓላማ የማጅራት ገትር በሽታ ስርጭትን ለመለየት እና በማህበረሰብ አቀፍ የማጅራት ገትር በሽታ የመከላከል ተዛማችነት በአለርት እና በደብረ ብርሃን ዩኒቨርሲቲ ሆስፒታሎች ግምገማ ለማድረግ ነው።

ሊሆኑ የሚችሉ ጥቅሞች:- በዚህ ጥናት ውስጥ መሳተፍ በቀጥታ አይጠቅምዎትም። ነገር ግን የዚህ ጥናት ውጤቶች የማጅራት ገትር በሽታን በፍጥነት ለመመርመር እና ለመለየት ፣ በሽታን ለመቆጣጠር እና የታቀዱ ፕሮግራሞችን አፈፃፀም ማጠናከር እና ውሳኔዎችን ለማሳወቅ ጥቅም ላይ ይውላል።

ሊሆኑ የሚችሉ አደጋዎች፡ ጥናቱ ለተሳታፊዎች ትልቅ ስጋት የለውም። ሆኖም ግን, CSF እና ደም በሚሰበሰብበት ጊዜ ህመም ሊኖር ይችላል። በአጠቃላይ በዚህ ምርምር ውስጥ መሳተፍ ለማንኛውም አካላዊ፣ ማህበራዊ ወይም ስነልቦናዊ አደጋዎች አያጋልጥዎትም።

በምርምር ውስጥ ተሳትፎ:- የዚህ ጥናት አካል መሆን አለመፈለግዎን ለመወሰን ነፃ ነዎት።

ሚስጥራዊነት:- በተቻለን መጠን በዚህ ጥናት ላይ ስለመሳተፍዎ መረጃን እጠብቃለሁ። ስለ እርስዎ ተሳትፎ ከተመራማሪው ቡድን ውጪ ከማንም ጋር አልወያይም። በየትኛውም ዘገባ ላይ ስማችሁን አልጠቅስም።

ክፍያ፡- ለተሳትፎ ምንም ክፍያ አይከፈልም.

ጥናቱን ለቅቆ መውጣት፡- በማንኛውም ጊዜ ምንም አይነት አሉታዊ ውጤት ሳይኖር ተሳትፎዎን ማቆም ይችላሉ።

የእርስዎ መብቶች እንደ ተሳታፊ

ይህ ጥናት በአዲስ አበባ ዩኒቨርሲቲ የሥነ ምግባር ገምጋሚ ቦርድ ታይቶ ጸድቋል። በጥናቱ እንዴት እየተስተናገድህ እንዳለህ ወይም እንደ ተሳታፊ መብት ማንኛውም አይነት ጥያቄ ካሎት በኢሜል፡ jamaaluna@gmail.com መጠየቅ ይችላሉ።

Consent Form for Adult Participants (≥18 years) (English version).

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. I voluntarily consent that I would participate in this study to give the specimen required for laboratory analysis and I understand that I have the right to withdraw from the study at any time.

Patient's Name _____signature _____Date_____

Investigator's name _____signature _____Date_____

Consent Form for Adult Participants (≥18 years) (Amharic version).

ለአዋቂዎች ተሳታፊዎች የስምምነት ቅጽ (የአማርኛ ትርጉም)

ከላይ ያለውን መረጃ አንብቤዋለሁ። ጥያቄዎችን እንድጠይቅ እድል ተሰጥቶኛል እና ጥያቄዎቼ አጥጋቢ ምላሽ አግኝተዋል። በዚህ ጥናት ውስጥ ለላቦራቶሪ ትንታኔ የሚያስፈልገውን ናሙና ለመስጠት እሳተፋለሁ እና በማንኛውም ጊዜ ከጥናቱ የመውጣት መብት እንዳለኝ ተረድቻለሁ። ያንን በፈቃዴ እስማማለሁ።

የታካሚ ስም:- _____ ፊርማ:-_____ ቀን:-_____

የመርማሪው ስም፡-_____ፊርማ:-_____ ቀን፡-_____

Information sheet for parents/guardians (English version)

Name of Organization: - Department of Microbiology, Immunology and Parasitology, College of Health Sciences, Addis Ababa University.

Principal Investigator: Jemal Aman
Title: Assessment of the prevalence of meningitis and immune correlates of risk in community-acquired meningitis at ALERT and Hawassa University Comprehensive Specialised Hospitals, Ethiopia.

Introduction

I would like to conduct a research entitled "Assessment of the prevalence of meningitis and immune correlates of risk in community-acquired meningitis at ALERT and Hawassa University Comprehensive Specialised Hospitals, Ethiopia". I am requesting you to allow your child to voluntarily participate in this study, and inform you about the purpose, responsibility of investigators or data collectors to keep confidentiality and how I am going to use the data. Please take as much time as you need to read or listen to the information provided here.

Purpose of the study: The purpose of the study is to determine prevalence of meningitis and assess the immune correlates of risk in community-acquired meningitis at ALERT and Hawassa University Comprehensive Specialised Hospitals, Ethiopia.

Possible Benefits: Participation in this research may not benefit your child directly. But results from this study will be used to inform decisions in implementation and strengthening of programs aimed at controlling, rapid diagnosis and detection of meningitis, its management and earlier treatment initiation in Ethiopia.

Possible Risks (during sample collection): The study poses no major risks to your child. However, there may be pain during CSF and Venous blood collection. Generally, it is very unlikely that participation in this research will expose your child to any physical, social or psychological risks.

Participation in the Research: - You are free to decide if your child wants to be part of this research or not

Confidentiality: - I will protect information about your child taking part in this research to the best of our ability. I will neither use your child name in any reports nor discuss your child participation with anyone outside the research team.

Payment: - No payments will be made for participation.

Leaving the Research:- You may end the participation of your child in this study at any time without any negative consequence to your child.

Your child's right as a Participant

This research has been reviewed and approved by the Addis Ababa University ethical review board. If you have any questions about how you are being treated by the study or your rights as a participant you may contact through email: jamaaluna@gmail.com.

Information sheet for parents/guardians (Amharic version)

ለወላጆች/አሳዳጊዎች የመረጃ ወረቀት (የአማርኛ ትርጉም)

የድርጅቱ ስም: - በአዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ፣ የማይክሮ ባዮሎጂ ኢሚውኖሎጂ እና ፓራሲቶሎጂ ትምህርት ክፍል

ዋና መርማሪ፡ ጀማል አማን።

ርዕስ፡- Assessment of the prevalence of meningitis and immune correlates of risk in community-acquired meningitis at ALERT and Debre Birhan University Hospitals, Ethiopia.

መግቢያ

“የማጅራት ገትር በሽታ ስርጭትን ለመለየት እና በማህበረሰብ አቀፍ የማጅራት ገትር በሽታ የመከላከል ተዛማችነት በአለርት እና በደብረ ብርሃን ዩኒቨርሲቲ ሆስፒታሎች ግምገማ ለማድረግ” በሚል ርዕስ ጥናት ማድረግ እፈልጋለሁ።በዚህ ጥናት ላይ ልጅዎ በፈቃደኝነት እንዲሳተፍ እንድትፈቅዱ እጠይቃለሁ። ስለ መርማሪዎች ወይም መረጃ ሰብሳቢዎች ምስጢራዊነትን የመጠበቅን ዓላማ፣ ኃላፊነት እና መረጃውን እንዴት እንደምጠቀም ያሳውቅዎታል። እባክዎን ለማንበብ ወይም እዚህ የቀረበውን መረጃ ለማዳመጥ የሚፈልጉትን ያህል ጊዜ ይውሰዱ።

የጥናቱ ዓላማ

የጥናቱ ዓላማ የማጅራት ገትር በሽታ ስርጭትን ለመለየት እና በማህበረሰብ አቀፍ የማጅራት ገትር በሽታ የመከላከል ተዛማችነት በአለርት እና በደብረ ብርሃን ዩኒቨርሲቲ ሆስፒታሎች ግምገማ ለማድረግ ነው።

ሊሆኑ የሚችሉ ጥቅሞች

በዚህ ጥናት ውስጥ መሳተፍ በቀጥታ ልጅዎን ሊጠቅም አይችልም።ነገር ግን የዚህ ጥናት ውጤቶች የማጅራት ገትር በሽታን በፍጥነት ለመመርመር እና ለመለየት ፣ በሽታን ለመቆጣጠር እና የታቀዱ ፕሮግራሞችን አፈፃፀም ማጠናከር እና ውሳኔዎችን ለማሳወቅ ጥቅም ላይ ይውላል።

ሊሆኑ የሚችሉ ስጋቶች (ናሙና በሚሰበስቡበት ወቅት)

ጥናቱ በልጅዎ ላይ ትልቅ ስጋት አያስከትልም። ሆኖም ግን, በ CSF እና ደም በሚሰበሰብበት ጊዜ ህመም ሊኖር ይችላል። ባጠቃላይ በዚህ ጥናት ውስጥ መሳተፍ ልጅዎን ለማንኛውም አካላዊ፣ ማህበራዊ ወይም ስነ-ልቦናዊ አደጋዎች ሊያጋልጥ ይችላል ተብሎ የማይታሰብ ነው።

በምርምርው ውስጥ መሳተፍ

ልጅዎ የዚህ ጥናት አካል መሆን አለመፈለጉን ለመወሰን ነጻ ነዎት።

ምስጢራዊነት

ልጅዎ በዚህ ጥናት ላይ የሚሳተፍበትን መረጃ በተቻለን መጠን እጠብቃለሁ። በማንኛውም ሪፖርቶች የልጅዎን ስም አልጠቀምም ወይም የልጅዎን ተሳትፎ ከጥናት ቡድኑ ውጭ ከማንም ጋር አልወያይም።

ክፍያ: - ለተሳትፎ ምንም ክፍያ አይከፈልም።

ጥናቱን መልቀቅ፡- በማንኛውም ጊዜ በልጅዎ ላይ ምንም አይነት አሉታዊ ውጤት ሳይኖር የልጅዎን ተሳትፎ በዚህ ጥናት ማቆም ይችላሉ።

የልጅዎ የተሳታፊነት መብት

ይህ ጥናት በአዲስ አበባ ዩኒቨርሲቲ የሥነ ምግባር ገምጋሚ ቦርድ ታይቶ ጸድቋል። በጥናቱ እንዴት እየተስተናገድህ እንዳለህ ወይም እንደ ተሳታፊ መብት ማንኛውም አይነት ጥያቄ ካሎት በኢሜል፡ jamaaluna@gmail.com መጠየቅ ይችላሉ።

Consent form for parents/guardians (English Version)

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. I voluntarily consent that my child participates in this study provided he/she gives assent to give specimen for laboratory investigation, be a participant in this study and understand that I have the right to withdraw my child from the study at any time .

Parent's/guardian's name _____Signature _____Date_____

Investigator name _____Signature _____Date_____

Consent form for parents/guardians (Amharic Version)

ለወላጆች/አሳዳጊዎች የስምምነት ቅጽ (የአማርኛ ትርጉም)

ከላይ ያለውን መረጃ አንብቤዋለሁ። ጥያቄዎችን እንድጠይቅ እድል ተሰጥቶኛል እና ጥያቄዎቼ አጥጋቢ ምላሽ አግኝተዋል። ልጄ በዚህ ጥናት ውስጥ እንዲሳተፍ በፈቃደኝነት እስማማለሁ።ልጄን በማንኛውም ጊዜ ከጥናቱ የማውጣት መብት እንዳለኝ ተረድቻለሁ።

የወላጅ/አሳዳጊ ስም_____ፊርማ:-_____ ቀን፡-_____

የመርማሪው ስም፡-_____ፊርማ:-_____ ቀን፡-_____

Assent form for children (English version).

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. I voluntarily assent that I would participate in this study provided that my parents/guardians give their consent and understand that I have the right to withdraw from the study at any time.

Participant name _____Signature _____Date_____

Investigator name _____Signature _____Date_____

Assent form for children (Amharic version).

የፍቃድ ቅጽ ለህፃናት (የአማርኛ ቅጂ)።

ከላይ ያለውን መረጃ አንብቤዋለሁ። ጥያቄዎችን እንድጠይቅ እድል ተሰጥቶኛል እና ጥያቄዎቼ አጥጋቢ ምላሽ አግኝተዋል። ወላጆቼ/አሳዳጊዎቼ ፈቃዳቸውን ከሰጡ እና በማንኛውም ጊዜ ከጥናቱ የመውጣት መብት እንዳለኝ እስከተረዱ ድረስ በዚህ ጥናት ላይ እንድሳተፍ በፈቃዴ ወስኛለሁ።

የተሳታፊው ስም _____ፊርማ:-_____ ቀን፡-_____

የመርማሪው ስም፡-_____ፊርማ:-_____ ቀን፡-_____

Annex II: Questionnaire

Protocol Title: Assessment of the prevalence of meningitis and immune correlates of risk in community-acquired meningitis at ALERT and Hawassa University Comprehensive Specialised Hospitals, Ethiopia.

Principal Investigator: Jemal Aman

Patient's Card No-_____Date of Interview_____

Introduction

Thanks so much for your permission. I am **Jemal Aman**, a student pursuing a Master of Medical microbiology Program at Addis Ababa University School of medicine, Addis Ababa, Ethiopia. This interview is being conducted as part of a research on assessment of the prevalence of meningitis and immune correlates of risk in community-acquired meningitis. I would be very much grateful if you would kindly find some time to answer these questions. Your views, opinions and contributions are very valuable and important and would go a long way to help me to assess the prevalence of meningitis and immune correlates of risk in community-acquired meningitis. This study is strictly for academic purpose and I can assure you of the confidentiality on any information that you would provide.

Thanks for your cooperation.

Fill in the space provided or tick in box provided to represents the answer.

Part I: Patient Identifications

- AHRI Code _____ Hospital Card Number: _____
- Age in years: _____ or Age in months (if <12 months) _____
- Sex: □ F □ M

Part II: Socio- demographic characteristics

- **Patient's residence:**
- ✓ Region/City/zone/district of residence: _____ S/city/Town/Village: _____
- ✓ Father/mother /guardian name: _____Phone number _____
- ✓ Date seen (D/M/Y): _____ / ____ / _____ Date of onset: ____ / ____ / _____

ONLY FOR ADULT PARTICIPANTS

- **Marital status:** □ Single □ Married □ Divorced/Widowed
- **Education:** □ No education □.primary □.Secondary □college/university and above
- **Occupation:** □Teacher □Soldier □Merchant □Other
- **Income per month: _____birr**

Part III: Patient medical history

1	Did the patient experience any of the following symptoms and signs?	Yes	No
I	Fever (≥38.5°C)		
Ii	Impaired consciousness or irritability		
Iii	Vomiting or reduced ability to suck for breastfeeding		
Iv	Stiffness of the neck or the back		
V	Bulging fontanelle of infant patient		
Vi	Petechiae: ≤ 5 mm?		
Vii	Seizures?		
Viii	Lowered consciousness?		

2. Has the patient vaccinated for meningitis? ❑ YES NO ☐ . If yes, what is the name of vaccine?_____

3. **NB!** This Question is to be answered by the health care worker: Is meningitis outbreak announced currently at the region where the patient comes from? ❑ YES ❑ NO

4. Antibiotic treatment given before admission? ❑ YES ☐ NO ☐ Don't know. If yes, what is the name of antibiotics he/she took? _____

5. **Comorbidities**: **Yes/No**

i) HIV: _____

ii) Diabetis:_____
iii) Hypertention: _____ iv)Other(specify): ____

Part IV: Clinical status on admission to hospital

- Temp. (in ºC): _____
- Date of specimen collection: _____
- Time of specimen collection: _____ Hrs. _____ Min
- Specimen collected: ☐ Blood ☐ CSF ☐ Throat swab
- Appearance of CSF: ☐ Clear ☐ Turbid ☐Hematic (blood stained) ☐ Xanthochromic (**yellowish)**
- Date and time of inoculation in the T-I: _____ and _____HH _____Min
- Type of tests performed: ☐ Cytology ☐ Gram stain

CBC of circulation:

- ✓ Total WBC: _____
- ✓ Neutrophil count: _____%Neutrophil: _____
- ✓ Lymphocyte count: _____% Lymphocyte count: _____

CSF WBC Count

- ✓ Total WBC: _____
- ✓ Neutrophil count: _____%Neutrophil: _____
- ✓ Lymphocyte count: _____% Lymphocyte count: _____

- ✓ CSF protein: _____
- ✓ CSF glucose: _____

Part V: Bacteriology laboratory results

1. **Gram-stain:** ☐ GPD ☐ GND ☐GPB ☐ GNB ☐ Negative ☐ other pathogen

2. **PCR:** date of PCR: _____ Type of PCR: ☐ Real-time ☐ Conventional

3.**Organism Identified:**

☐ NmA ☐ NmC ☐ NmW ☐ NmY ☐ NmB ☐ NmX ☐ *S. pneumoniae* ☐ Hib ☐ *H. influenzae* Indeterminate ☐ StrepB ☐ Other pathogens (specify): _____

☐ Negative

Part VI: - Antibiotic Susceptibility Test result:

1.	Ceftriaxone:	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
2.	Penicillin G:	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
3.	Oxacillin:	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
4.	Vancomycin	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
5.	Moxifloxacin	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
6.	Ampicillin	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
7.	Cefepime	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
8.	Meropenem	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
9.	Ceftazidime	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done
10.	Cotrimoxazole	☐ Sensitive	☐ Resistant	☐ Intermediate	☐ Not done

Annex III: Sample collection, transportation, and processing.

1. Sample collection and transportation

I. Cerebrospinal fluid (CSF) collection and transportation

The collection of CSF is an invasive procedure and should only be performed by experienced personnel under aseptic conditions.

A. Lumbar Puncture procedure

1. Gather all materials from the CSF collection kit and a puncture-resistant autoclavable container for used needles.

2. Wear surgical mask and sterile latex or nitrile gloves that are impermeable to liquids and

Change gloves between every patient.

3. Label the collection tubes with appropriate information: patient's name, date and time of specimen collection and Unique Identification Number. Be sure this number matches the number on both the request and report forms.

4. Ensure that the patient is kept motionless during the lumbar puncture procedure, either sitting up or lying on the side, with his or her back arched forward so that the head almost touches the knees in order to separate the lumbar vertebrae during the procedure.

5. Disinfect the skin along a line drawn between the crests of the two ilia with 70% alcohol and povidone-iodine to clean the surface and remove debris and oils. Allow to dry completely.

6. Position the spinal needle between the 2 vertebral spines at the L4-L5 level and introduce into the skin with the bevel of the needle facing up.

* Accurate placement of the needle is rewarded by a flow of fluid, which normally is clear and colorless.

7. Remove CSF (1 ml minimum, 3-4 ml if possible) and collect into sterile screw-cap tubes. If

3-4 ml CSF is available, use 3 separate tubes and place approximately ml into each tube.

8. Withdraw the needle and cover the insertion site with an adhesive bandage. Discard the needle in a puncture-resistant, autoclavable discard container.

9. Remove mask and gloves and discard in an autoclavable container.

10. Wash hands with antibacterial soap and water immediately after removing gloves.

11. Transport the CSF to a microbiology laboratory within 1 hour for culture and analysis.

« If that is not possible, inoculate CSF into T-I medium.

12. In the event of a needle-stick injury or other skin puncture or wound, wash the wound liberally with soap and water. Encourage bleeding.

13. Report a needle-stick injury, any other skin puncture, or any contamination of the hands or body with CSF to the supervisor and appropriate health officials immediately as prophylactic treatment of the personnel performing the procedure may be indicated.

B. Inoculating and transporting T-I medium

T-I is a biphasic medium that is useful for the primary culture of meningo-cocci and other etiological agents of bacterial meningitis (S. pneumoniae and H. influenzae) from CSF. It can be used as a growth medium as well as a holding and transport medium. T-I media should be stored at 4°C and warmed to room temperature (25°C) before use.

1. Label the T-I bottle with appropriate information: patient name, date and time of CSF inoculation, and Unique Identification Number. Be sure this number matches the number on both the request and report forms.

2. Use sterile forceps to pull the aluminum cover of a T-I bottle away from the rubber stopper and disinfect the stopper with 70% alcohol. Allow to dry.

- Do not use povidone-iodine as it may be carried into the medium by the passing needle and would inhibit growth of bacteria.
- Do not completely remove the aluminum cover.

3. Use a sterile syringe and needle to inoculate 0.5ml of CSF into the T-I medium. The remaining CSF should be kept in the collection tube. It should not be refrigerated, but should be maintained at room temperature (20-25°C) before Gram staining and other tests. Discard the needle in a puncture-resistant, autoclavable discard container.

4. After inoculations invert the T-I bottle several times to mix

5. If transport to a reference laboratory is delayed (next day or longer), insert a venting needle (sterile cotton-plugged hypodermic needle) through the rubber stopper of the T-I bottle, which will encourage growth and survival of the bacteria.

- Be sure that the venting needle does not touch the broth.

6. Incubate inoculated T-I medium at 35-37°C with ~5% CO (or in a candle-jar) overnight or until transport is possible. If transportation is delayed more the 4 days, remove the vented T-I bottle from the incubator or candle jar and place at room temperature until shipment.

7. Remove the venting needle and wipe the rubber stopper with 70% alcohol before shipping. It is essential to avoid contamination when sampling the bottles to obtain specimens aseptically.

8. If the T-I bottle can be transported for a reference laboratory the same day, do not vent the bottle until it arrives in the receiving laboratory.

- For culture: The inoculated T-I media should be sent from the health facility to the reference lab within 24 hours.
- PCR: Tube 2 (cryotube 2) should be sent to AHRI, along with T-I media for PCR testing. Unlike inoculated T-I media, cryotubes should be refrigerated or frozen during storage and transported to AHRI under reverse cold-chain system.

9. Upon arrival, vent the T-I bottle, incubate at 35-37% with ~5% CO (or in a candle-jar), and observe daily for turbidity in the liquid phase for up to 7 days.

- If turbidity is observed, culture onto a blood agar plate (BAP) and a chocolate agar plate (CAP) immediately.
- If no turbidity is observed, culture onto a BAP and a CAP on day 4 and day 7.
- If T-I medium appears to be contaminated, selective media such as Modified Thayer- Martin and chocolate agar with bacitracin may be used.

If T-I vials cannot reach the laboratory of reference within 24 hours

1. Ventilate the T-I vial with a sterile cotton plugged needle. The Needle should not dip into the culture media (broth).

2. Store the ventilated T-I vial in an upright position at room temperature. Make sure it is away from excessive heat, direct sunlight, and dust.

3. Before transporting the vial, remove the ventilating needle from the top of the T-I vial. (This will prevent leakage and contamination during shipment). Disinfect the top of the T-I vial with 70% alcohol and replace the metallic cover.

4. Transport the T-I vial at room temperature in a sealed plastic bag to minimize the risks of contamination. And attach the case investigation form.

II. Procedure for venous blood collection

1. Select a sterile, dry, preferably plastic syringe of the capacity required, 10 ml.

2. Apply a soft tubing tourniquet arm band to the upper arm of the patient.

Do not apply the tourniquet too tightly or for longer than 2 minutes. Ask the patient to make a tight fist which will make the veins more prominent.

3. Using the index finger, feel for a suitable vein, selecting a sufficiently large straight vein that does not roll and with a direction that can be felt*.

*If a vein cannot be felt, apply a pressure cuff above the elbow and raise the pressure to 80 mm (deflate the cuff once the needle is in the vein).

4. Cleanse the puncture site with 70% ethanol and allow drying. Do not re-touch the cleansed area.

5. With the thumb of the left hand holding down the skin below the puncture site, make the venipuncture with the bevel of the needle directed upwards in the line of the vein. Steadily withdraw the plunger of the syringe at the speed it is taking the vein to fill*. Avoid moving the needle in the vein. If the plunger is withdrawn too quickly this can cause hemolysis of the blood and the collapse of a small vein.

6. When sufficient blood has been collected, release the tourniquet and instruct the patient to open his or her fist. Remove the needle and immediately press on the puncture site with a piece of dry cotton wool. Remove the tourniquet completely. Instruct the patient to continue pressing on the puncture site until the bleeding has stopped.

7. Remove the needle from the syringe and carefully fill the container(s) with the required volume of blood. Discard the needle safely. Do not attempt to re-sheath it because this can result in needle-stick injury.

Important: Do not fill a container with the needle attached to the syringe. Forcing the blood through the needle can cause haemolysis.

8 Mix immediately the blood in an EDTA or citrate anticoagulated container. When required, immediately label carefully all the blood samples.

9 Check that bleeding from the venipuncture site has stopped. Cover the area with a small dressing. Using the index finger, feel for a suitable vein, selecting a sufficiently large straight vein that does not roll and with a direction that can be felt*.

*If a vein cannot be felt, apply a pressure cuff above the elbow and raise the pressure to 80 mm (deflate the cuff once the needle is in the vein)

2. Specimen Processing

I. CSF processing.

A. Gram stain procedure for CSF

1. Centrifuge the CSF for 10-15 minutes at 1000 x g, if >1 ml is available.

2. Divide a glass slide into two sections using a marker. Use one section for the unknown CSF and the other section for a known organism for QC.

3. Prepare a smear by placing 1-2 drops of the well-mixed CSF sediment on the slide, allowing the drop(s) to form one large slightly turbid, uniform suspension.

« To prepare a smear using an isolate, add a small drop of sterile water or physiological saline to the slide and create a slightly turbid, uniform suspension of cells from an overnight culture.

4. Let the suspension air dry. The suspension MUST be completely dry before proceeding.

5. Fix the smear by the flooding the slide with 95% methanol for a minimum of 2 minutes

Rinse with distilled water. Shake off excess water.

- If methanol is not available, heat-fix the smears by quickly passing the slide through a flame three times. Do not over-heat the slide as over-heating will cause significant distortion or destruction of the cells.
- It is possible to use simple water if distilled water is not available for the entire Gram stain procedure.

6. Flood the slide with crystal violet ammonium oxalate for 1 minute to stain. Rinse with distilled water. Shake off excess water.

- Avoid touching the slide with the tip of the reagent bottle or applying liquid directly onto the smear.

7. Flood the slide with Gram's iodine for 1 minute. The iodine acts as a mordant as it binds the alkaline crystal violet dye to the cell wall. Rinse with distilled water. Shake off excess water.

8. Decolorize with 95% ethanol until no more stain washes off (5-10 seconds may be enough). Rinse with distilled water. Shake off excess water.

- It is essential to view decolorization closely: gram-positive bacteria can be made to appear gram-negative by over-decolorization and gram-negative bacteria can be made to appear gram-positive by under decolorization.

9. Counterstain with safranin for 30 seconds or with carbol-fuchsin for 10-15 seconds. Rinse with distilled water. Shake off excess water.

10. Gently blot the slide using bibulous paper or a clean paper towel. Let air dry.

11. When dry, examine the stained smear under a microscope with 100X oil immersion objective.

Reading the Gram stain results (under microscopic examination):

• Gram-positive organisms will appear dark violet or purple.

• Gram-negative organisms will appear red or pink (from the counterstain).

B. Procedure for primary culture directly from CSF

1. For culture and analysis, inoculate 1-5 drops of CSF (depending on volume received in laboratory) directly onto both a BAP and CAP within 1 hour after collection.

- If the CSF was centrifuged, use 1 drop of the well-mixed sediment for primary culture.

2. Using a sterile bacteriological loop, cross-streak the inoculum to obtain single, isolated colonics.

- Disposable loops are preferred, but if using a wire loop, it must be sterilized prior to each step of the plate-streaking process.
- BAP and CAP that have been properly streaked.

3. A back-up broth (e.g., brain-heart infusion broth with proper supplements) should be inoculated with some of the sediment pellet.

4. Agar plates and broth inoculated with the CSF sediment should be incubated for 18-24 hours at 35-37°C with ~5% CO; (or in a candle-jar).

C. Molecular Method

A. Samples and reagent preparation

a. Material and reagents

- CSF and Blood samples
- 96-well plate containing magnetic beads and extraction reagent
- 96-well plates
- Pipettes and tips
- Personal protective equipment (PPE)

b. Procedure

1. Thaw CSF and blood samples at room temperature.
2. Shortly Centrifuge at 6000 rev/min for a few seconds
3. Bring all centrifuged samples to room temperature.
4. Equilibrate a 96-well plate containing magnetic beads and extraction reagent to room temperature.
5. Centrifuge the equilibrated plate according to the manufacturer's instructions.

B. DNA extraction

a. Automation purification

1. Reagent Preparation

Add 500µL Lysis Buffer to columns 1 and 7 of the 2.2 mL. 96-deep-well plate, 500µL Wash Buffer I to the columns 2 and 8, 500µL Wash Buffer II to the columns 3 and 9, 70µL Elution Buffer to the columns 5 and 11, 175µL pure water, and 25µL MagaBio Reagent to the columns 6 and 12 (the magnetic beads should be mixed thoroughly before use). Put the 96 well-prepacked reagents at room temperature. Shake the 96-well plate upside down three times, and tear off the plastic bag. Centrifuge the pre-packed reagent for a few seconds (or swing by hand a few times) to avoid reagent adhering to the wall of the tubes. Tear off the aluminum foil film of the 96-well plate and identify the direction of the plate (magnetic beads in columns #6 and #12).Add 300µL sample to the 96 well plate columns #1 and #7.

2. Place a 96-deep well plate on the instrument, and install the 8-strip tips on the instrument.

3. Run the program according to the following procedures:

Step	Well	Name	Waiting time (min:ss)	Mixing time (min:ss)	Magnetic time (min:ss)	Adsorption	Speed	Volume (microliter)
1	6	Beads	0:0	0:0	0:15		M	200
2	1	Binding	0:0	3:00	0:35	√	F	700
3	2	Wash 1	0:0	0:30	0:20	√	F	500
4	3	Wash 2	0:0	0:30	0:20	√	F	500
5	5	Elution	1:0	2:00	0:25	√	F	70
6	6	Discard	0:0	0:0	0:0		M	200

Temperature settings: elution temperature: 80 °C. Heating during elution begins at Step 5.

4. After the automatic purification is over, elution buffer in columns 5 and 11 is transferred to a clean nuclease-free 0.5 mL centrifuge tube; store at -20° if not using.

C. Master Mix preparation

a. Material and reagents

- Materials Required
- PCR Buffer (10x): 2.5 µL
- $MgCl_2$: 1.2 µL
- dNTPs: 1.0 µL
- Primers: 1.0 µL of each primer (2 primers total = 2.0 µL)
- Molecular Grade Water: 13.1 µL
- Platinum Taq Polymerase: 0.2 µL
- Template DNA: 5.0 µL (added later)
- PCR Tubes
- Pipettes and Tips

b. Procedure

1. Calculate the total volume for the master mix (without template DNA) is 20 µL.
2. Combine all reagents in single PCR tube according volume requered (2.5 µL of 10x PCR Buffer, 1.2 µL of $MgCl_2$, 1.0 µL of dNTPs, 2.0 µL of primers (1.0 µL of each primer), 13.1 µL of molecular grade water, 0.2 µL of Platinum Taq Polymerase) for one PCR reactions.
3. Gently pipette vortex to mix the components without introducing bubbles.
4. Briefly centrifuge the tube to ensure all components are at the bottom.
5. To PCR tube containing the master mix, add 5.0 µL of the DNA template.
6. Mix gently by vortex to ensure the template is well incorporated into the master mix.
7. Ensure the PCR tube is properly sealed to prevent evaporation during the PCR process.

D. PCR Procedure

a. Protocol

1. Set the Program of thermal cycler (T100 LASEC BIO-RAD) with the following cycling conditions

- Initial denaturation: 94°C for 3 minutes
- Cycling (35 cycles):
- Denaturation: 94°C for 10 seconds
- Annealing: 60°C for 30 seconds
- Extension: 72°C for 5 minutes
- Final extension: 72°C for 10 seconds
- Lid temperature: 105°C

2. Place the PCR tubes in the thermal cycler and start the program.
3. Run the PCR program (approximately 1 hour and 20 minutes).
4. Store the amplified products at 2-8°C until gel electrophoresis is performed.

E. Reagent preparation procedures

I. 50x TAE Buffer Preparation

a. Materials and reagents

- Tris Base: 484 g
- Glacial Acetic Acid: 114.2 mL
- EDTA (0.5 M): 200 mL
- Deionized Water: Approximately 2L
- pH Meter or pH Indicator Strips: For pH adjustment
- Beaker or Mixing Container
- Magnetic Stirrer: For mixing
- Volumetric Flask or Graduated Cylinder

b. Procedure

1. Weigh 484 g. Tris base and dissolve it in distilled water.
2. Add 114.2 ml of glacial acetic acid (100% stock) and 200 ml of 0.5 M EDTA (pH 8).
3. Adjust the final volume to 2 L with distilled water.
4. Stir the solution overnight for complete dissolution.
5. Measure the pH on the second day using a universal indicator.

6. Label the container and store the 50x TAE buffer.

II. 1x TAE buffer from 50x stock

1. Dilute 20 ml of 50x TAE stock solution with 980 ml of distilled water.
2. Mix the solution thoroughly.
3. Label the container and use the 1x TAE buffer for the experiment.

III. 100-bp DNA Ladder Preparation

a. Materials and reagents

- Thermo Scientific GeneRuler 100 bp DNA Ladder
- Nuclease-free water
- 6X DNA loading buffer (containing blue tracking dye)
- Microcentrifuge tubes
- Pipettes and pipette tips
- Vortex mixer
- 20°C freezer for storage

b. Procedure

1. ALIQUOT 1µL of the Thermo Scientific GeneRuler 100 bp DNA Ladder.
2. 4µL of nuclease-free water is added to the 1 µL DNA ladder. The ease of adding nuclease-free water makes the DNA ladder reduce to a working concentration best suited for the gel electrophoresis process.
3. Add 1µL of 6X DNA loading buffer with blue tracking dye. This loading buffer is used for viewing the DNA during electrophoresis and for proper loading into the gel wells.
4. Vigorously mix the solution using a vortex for a short period of time. The solution is supposed to be uniform now; there should not be any extra bubbles present in the solution.
5. The DNA ladder tube is marked with an identifying label. The date of preparation as well as the contents are indicated.
6. The mixture of the DNA ladder solution was stored at -20 °C. The tube should be tightly sealed to protect against contamination and degradation.

7. When ready to use, thaw the DNA ladder solution on ice. Mix gently before loading onto the agarose gel for electrophoresis.

IV. 2.5% Agarose Gel Preparation

a. Materials and reagents

- Agarose powder
- Analytical balance
- TAE (Tris-Acetate-EDTA) buffer
- Heating device (hot plate)
- Flask
- Graduated cylinder
- Ethidium bromide solution
- Casting tray with well-forming comb
- Nitrile Gloves

b. Procedure

1. Weigh 2.5 grams of agarose powder with an analytical balance.
2. Add measured agarose powder to 100 milliliters of TAE buffer in a flask.
3. Gently mix the mixture to make sure the agarose powder is uniformly distributed in the buffer.
4. Use a hot plate to gently heat the agarose-buffer mixture until the agarose powder is fully dissolved.
5. Monitor closely the content of the solution in order to prevent over-boiling.
6. Keep heating until the solution gets clear and all the agarose particles have dissolved.
7. Let the hot agarose solution cool down.
8. While the agarose solution is still hot, add about 2 microliters of Ethidium bromide solution to it. Ethidium bromide is a fluorescent dye that intercalates with DNA, thus permitting DNA bands to be visualized under UV light. Besides, gloves must be worn and working must be done in a hood when handling Ethidium bromide due to the mutagenic properties of it.
9. Carefully pour the warm agarose solution into a casting tray.
10. Place the comb in the tray to form wells for loading samples.
11. Leave the gel to solidify for 20 min at RT.
12. It will solidify and should be firm but opaque when chilled correctly.

13. Gently remove the comb from the solidified gel, taking care not to tear the wells.

Safety First: Handling Ethidium Bromide

The primary safety concern highlighted is **Ethidium bromide (EtBr)**, a chemical commonly used to visualize DNA. It's explicitly labeled as a **mutagen**, meaning it can cause genetic mutations that may lead to cancer. To protect yourself, always wear gloves and safety goggles throughout the entire procedure. Handle EtBr with extreme care to avoid contact. Proper disposal is also crucial; make sure to follow your institution's specific guidelines for waste containing Ethidium bromide.

F. Gel Electrophoresis and Visualization

a. Materials and reagent

- PCR product
- Loading dye
- 100-bp DNA ladder
- Agarose gel
- UV machine (Vilber)
- Gel electrophoresis equipment (gel tray, comb, power supply)
- Pipettes and tips
- Micro centrifuge tube

b. Procedure

1. To mix 25 µL of the PCR product in a micro centrifuge tube and then add 5 µL of loading dye. Pipette it gently up and down to mix evenly
2. Centrifuge briefly for a few seconds to collect this mixture at the bottom
3. Load 10 µl of your PCR product carefully into the wells of submerged agarose gel prepared for each well, taking care not to introduce air bubbles
4. In another well load 5 µL of 100 bp DNA ladder to compare the size
5. Connect the power supply after closing the gel electrophoresis chamber
6. Set the voltage at 120 V, 400 amp, and run it for an appropriate time of around 45 minutes
7. For observing loading dye migration, monitor the progress of electrophoresis.
8. Remove the gel from the chamber when the electrophoresis is complete.

9. Observe DNA bands under a Vilber machine.

10. Compare the size of the PCR product with those of the DNA ladder

11. Photograph the gel, recording the outcome.

II. Procedures for Blood specimen processing

A. Procedures for Complement assay using Luminex® xMAP® technology

I) Principle

Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.

II) Reagents and Material

Reagents

Reagents	Volume	Quantity	Cat. No
Human Complement Expanded Panel 1 Standard	Lyophilized	1 vial	HCMPEX1-8019
Human Complement Expanded Panel 1 Quality Controls 1 and 2	Lyophilized	2 vials	HCMPEX1-6019
Set of one 96-Well Plate with 2 Sealers	--	1 plate, 2 plate sealers	-
Assay Buffer	30 mL	1 Bottle	L-AB
10X Wash Buffer	60 mL	1 Bottle	L-WB
Bead Diluent	3.5 Ml	1 Bottle	LBD
Human Complement Expanded Panel 1 Detection Antibodies	5.5 mL	1 Bottle	HCMPEX1-1019
Streptavidin-	5.5 mL	1 Bottle	MC-SAPE7

Phycoerythrin			

Bead/Analyte Name	Luminex® Bead Region	Customizable 9 Analytes (20X concentration, 200 µL) Available	Cat. No.
Anti-Human C2	19	✓	HCC2-MAG
Anti-Human C4b	43	✓	HCC4B-MAG
Anti-Human C5	44	✓	HCC5-MAG
Anti-Human C5a	45	✓	HCEXC5A-MAG
Anti-Human C9	46	✓	HCEXC9-MAG
Anti-Human Factor D (Adipsin)	73	✓	HCEXCFD-MAG
Anti-Human MBL	74	✓	HMBL-MAG
Anti-Human Factor I	76	✓	HCFI-MAG
Anti-Human Properdin	77	✓	HCEXCFP-MAG

Preparation of Plasma Samples

• Plasma collection using EDTA as an anti-coagulant is recommended.

1. Centrifuge for 10 minutes at 1000 x g within 30 minutes of blood collection.Remove plasma and assay immediately or aliquot and store samples at -20 °C.
2. Avoid multiple (> 2) freeze/thaw cycles.
3. When using frozen samples, thaw samples as rapidly as possible, put on ice and assay immediately. This prevents complement activation in samples, which will affect results.
4. Mix samples well by vortexing and centrifuge prior to use in the assay to remove particulates.
5. Plasma samples should be diluted 1:1,000 in the Assay Buffer provided in the kit. For example, in a tube, 5 µL of plasma may be combined with 120 µL of Assay Buffer (1:25), then add 5 µL of the 1:25 diluted plasma to 195 µL Assay Buffer. When further dilution beyond 1:1,000 is required, use Assay Buffer as the diluent.

Immunoassay Procedure

• Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.

• Allow all reagents to warm to room temperature (20-25°C) before use in the assay.

• Diagram the placement of Standards 0 (Background), Standard 1 through 7, Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate verticallyby default.) It is recommended to run the assay in duplicate.

• If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps, so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).

2. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.

3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 ng/mL standard (Background).

4. Add 25 µL of Assay Buffer to the background, standards, control, and sample

wells.

5. Add 25 µL of Sample (diluted1:1000) into the appropriate wells.

6. Vortex Mixing Bottle and add 25 µL of the Mixed or Premixed Beads to each well.

(Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)

7. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hours) at 2-8°C. Alternatively, incubate for 2 hours at room temperature (20-25°C).

8. Gently remove well contents and wash plate 3 times following instructions listed

in the PLATE WASHING section.

9. Add 50 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)

10. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). DO NOT ASPIRATE AFTER INCUBATION.

11. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Detection Antibodies.

12. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).

13. Gently remove well contents and wash plate 3 times following instructions listed in the PLATE WASHING section.

14. Add 150 µL of Sheath Fluid PLUS (or Drive Fluid PLUS if using MAGPIX®) to all wells. Re-suspend the beads on a plate shaker for 5 minutes.

15. Run plate on Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® with xPONENT® software or xMAP® INTELLIFLEX with INTELLIFLEX Software.

16. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (Note: For diluted samples, final sample concentrations should be multiplied by the dilution factor. For samples diluted as per protocol instructions, multiply by 1,000. If using another dilution factor, multiple by the appropriate dilution factor.

DECLARATION SHEET

I, the undersigned, Medical Microbiology student declare that this thesis is my original work in partial fulfillment of the requirement for the degree of Master of Medical Microbiology.

Name of principal investigator: Jemal Aman (MSc Candidate) **Signature: _____**

Place of submission: Addis Ababa University, College of Health Science, School of Medicine, Department of Microbiology, Immunology and Parasitology

Date of submission: **_____**

Advisors:-

This thesis paper has been submitted for examination with our approval as advisors.

Advisor	**Co-Advisor**
Dr. Tamrat Abebe (PhD, Ass. Professor)	Mr. Shemse Sebre (MSc, PhD Candidate)
Signature: _____	Signature: _____
Date: _____	Date**:** _____