Addis Ababa University

College of Health Sciences

School of Medicine

Department of Microbiology, Immunology, and Parasitology

Microbial Profile, Antimicrobial Resistance, and Outcome of Presumptive Meningitis Patients at the University of Gondar Comprehensive Specialized Hospital, Gondar, Northwest Ethiopia

By: Derso Wale (BSc, MSc Candidate)

A Thesis Submitted to the Department of Microbiology, Immunology, and Parasitology, College of Health Sciences, Addis Ababa University for the Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology

January, 2025

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ABBREVIATIONS AND ACRONYMS

AHRI	Armauer Hansen Research Institute
AMR	Antimicrobial resistance
AOR	Adjusted odds ratio
AST	Antimicrobial sensitivity test
BBB	Blood-brain barrier
B-CSFB	Blood cerebrospinal fluid barrier
BM	Bacterial meningitis
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
CSF	Cerebrospinal fluid
DEPC	Diethyl pyro carbonate
DNA	Deoxyribose nucleic acid
DNTP	Deoxynucleotide triphosphate
DPT-HI Hep	Diphtheriae pertussis, tetanus, *H. influenzae* type B, hepatitis B
DTT	Dithiothreitol
EBV	Epstein-Barr virus
EV	Enterovirus
GCS	Glasgow Coma Scale
GOS	Glasgow Outcome Scale
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
LP	Lumbar puncture
MenAfrivac	*Neisseria meningitis serogroup A* vaccine
$MgCl_2$	Magnesium chloride
MHA	Mueller-Hinton agar
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PMNs	Polymorphonuclear neutrophils
RNA	Ribonucleic acid

RPM	Revolution per minute
RT-PCR	Reverse transcriptase polymerase chine reaction
SPSS	Statistical package for social science
TAE	Tris-acetate EDTA
UoGCSH	University of Gondar Comprehensive Specialized Hospital
VM	Viral meningitis
VZV	Varicella zoster virus
WBC	White blood cell

ABSTRACT

Background: Meningitis is a life-threatening disease characterized by inflammation of the meninges. It remains a significant public health challenge, particularly in resource-limited settings where delayed presentation to healthcare facilities and comorbidities are more common. In Ethiopia, the etiology of meningitis is rarely identified, and treatment is often empirical, primarily targeting bacterial pathogens. However, this approach poses challenges due to the dynamic nature of pathogen prevalence and the increasing threat of antimicrobial resistance.

Objectives: The study was conducted to assess the microbial profile, antimicrobial resistance, and outcome of presumptive meningitis patients at the University of Gondar Comprehensive Specialized Hospital, Gondar, Northwest Ethiopia, 2023.

Methods: A prospective cross-sectional study was conducted from August 01 to December 31, 2023, on 195 presumptive meningitis patients at the University of Gondar Comprehensive Specialized Hospital. A cerebrospinal fluid sample was collected from each study participant and analyzed using culture and PCR techniques for the detection of bacterial and viral etiologies. The antimicrobial susceptibility of isolates was determined using the disc diffusion method. Patient outcomes were assessed using the Glasgow Outcome Scale. Descriptive statistics were employed. Bivariate and multivariable analyses were also done to identify factors associated with unfavorable patient outcomes. In all the cases, a *p*-value <0.05 at 95% confidence level was considered statistically significant.

Results: The overall prevalence of meningitis among the 195 patients with presumptive meningitis was 114 (58.5%). Bacterial pathogens were detected in 55 (28.2%) of the study participants, which constituted 55/114 (48.2%) of the PCR-confirmed cases. *E. coli* was the most commonly detected pathogen 25 (21.9%), followed by *H. influenzae* 9 (7.9%), *S. pneumoniae* 6 (5.3%), *N. meningitidis* 4 (3.5%), *S. aureus* 3 (2.6%), *K. pneumoniae* 1 (0.9%), *L. monocytogene* 1 (0.9) and mixed infections involving both *E. coli* and *K. pneumoniae* 3 (2.6%). Viral pathogens were detected in 59 (30.3%) of the study participants, which constitute 59/114 (51.8%) of the PCR-identified etiologies. The most frequently detected virus was enterovirus 53 (46.5%), followed by herpes simplex virus-1 5 (4.4%), and Epstein-Barr virus 1 (0.9%). A total of 14 (7.2%) CSF samples were found positive for bacterial growth in culture. The most frequently isolated pathogens were *S. pneumoniae* and *K. pneumoniae* for 4 each, followed by *E. coli* and *S. aureus* 2 each, and *H. influenzae* and *N. meningitidis* 1 each. With regard to drug resistance, 3/4

(75%) of *K. pneumoniae* exhibited resistance to both trimethoprim-sulfamethoxazole and ampicillin and 2/4 (50%) to meropenem, cefotaxime, and ciprofloxacin. *E. coli* isolates showed 1/2 (50%) resistance to ceftazidime, tetracycline, ciprofloxacin, chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin. 1/4 (25%) of *S. pneumoniae* isolates were resistant to erythromycin, trimethoprim-sulfamethoxazole, ciprofloxacin, and rifampin. 2/2 (100%) of the *S. aureus* isolates were resistant to both chloramphenicol and rifampin. Unfavorable outcomes on leaving the hospital were documented in 27/195 (13.8%) of the study participants. Glasgow Coma Scale (GCS) (AOR = 0.658, 95% CI = 0.524–0.827), the presence of comorbidity (AOR = 9.221, 95% CI = 1.580–53.815), and duration of disease onset to hospital visit (AOR = 1.604, 95% CI = 1.146–2.242) were factors independently associated with the unfavorable outcome; however, dexamethasone use does not confer benefit or harm in multivariate analysis.

Conclusion: A considerably high prevalence of meningitis was detected. Viral meningitis was more prevalent than bacterial, but most presumptive meningitis patients were empirically treated for bacterial infections. High rates of antibiotic resistance among gram-negative bacterial isolates were observed. Most of the study participants experienced favorable outcomes; however, unfavorable outcomes were associated with low GCS, the presence of comorbidity, and delayed presentation to healthcare facilities. The use of dexamethasone as adjunctive treatment in presumptive meningitis patients does not confer benefit or harm.

Keywords: Meningitis, PCR, Antimicrobial resistance, Patient outcome

1 INTRODUCTION

1.1 Background

Meningitis is a life-threatening disease characterized by the inflammation of the meninges, which is a membrane that surrounds the brain and spinal cord (Marcus and Walter, 2022, Assegu Fenta et al., 2020). The disease can be caused by infectious and noninfectious etiologies. Infectious agents such as bacteria and viruses are the most common cause of meningitis (G. B. D. Meningitis Collaborators, 2018). Bacterial meningitis (BM) is the most severe form, posing life-threatening risks and serious complications, particularly with delayed diagnosis (Gudina et al., 2018). While viral meningitis (VM) is usually less severe, it still presents significant public health challenges (Wami et al., 2021).

Potentially all pathogenic microbes can cause meningitis. Previously, *Neisseria meningitidis (N. meningitidis), Streptococcus pneumoniae (S. pneumoniae), and Haemophilus influenzae (H. influenzae)* accounted for about 75–80% of bacterial meningitis cases worldwide (C. Wang et al., 2023). However, *Escherichia coli (E. coli) and others* increased in importance after the introduction of vaccines and caused significant morbidity in specific age groups and underlying conditions (Carnalla-Barajas et al., 2022, Dagnew et al., 2013).

Viral agents of meningitis are the most commonly detected microorganisms in the cerebrospinal fluid (CSF) (Barnes et al., 2018, Geteneh et al., 2021). Several viruses were implicated in the causation of VM. Enteroviruses (EVs) are the most common viruses that cause meningitis (Geteneh et al., 2021, Tarai and Das, 2019, Wami et al., 2021). Due to limited infrastructure and skilled human capital, the etiology of VMs is rarely identified in developing countries like Ethiopia. Many of the patients with VM were misdiagnosed, mistreated with antibiotics, and hospitalized, even though those cases were self-limiting and most affected required supportive management only except for the herpes family viruses (Bartt, 2012, Mount and Boyle, 2017, Wami et al., 2021).

Although the etiology of meningitis varies, there were no significant distinctions in the clinical signs and symptoms (Al-Qahtani et al., 2022, Mcgill et al., 2016). Fever, neck stiffness, impaired mental state, and headache are the common symptoms. Less frequent vomiting, sensitivity to light (photophobia), sensitivity to sound (phonophobia), Kernig's sign, and Brudzinski's sign are observed among cases (Adjei et al., 2018, Al-Qahtani et al., 2022).

Rapid and accurate diagnosis of the etiologic agents of meningitis is mandatory for successful management (Ahmed et al., 2023, Geteneh et al., 2021, Tarai and Das, 2019, Wami et al., 2021). Etiological diagnosis of meningitis could be achieved with different methods. CSF analysis is the main contributor to the diagnosis (Ahmed et al., 2023). CSF microscopy, biochemistry, and culture remain the mainstay of diagnosis; however, nowadays molecular techniques are increasingly useful (Ellis et al., 2019). It improves the diagnosis as it is more sensitive, rapid, and not affected by the viability (Zeighami et al., 2021).

In recent years, managing BM has been increasingly difficult due to the rise of antimicrobial resistance (AMR), a global issue that is especially severe in developing countries like Ethiopia (Assegu Fenta et al., 2020, Hibstu et al., 2022, Tigabu et al., 2021). This is due to the lack of well-equipped bacteriological laboratories and antimicrobial resistance surveillance systems. The rise of AMR limits treatment options and contributes to poor patient outcomes, especially in resource-constrained settings where alternatives to first-line antibiotics may not be readily available (Gudina et al., 2016a).

Adjunctive corticosteroid therapy in combination with antibiotics is more effective than antibiotic therapy alone in reducing the risk of unfavorable patient outcomes (Alamarat and Hasbun, 2020, Rayanakorn et al., 2020). However, this is still controversial, especially in resource-limited settings (Gudina et al., 2016b, Gudina et al., 2018).

1.2 Statement of the problem

Meningitis is a devastating disease with substantial mortality and significant sequelae in survivors; it remains a major global public health challenge (Carter and Mcgill, 2022, Venkatesan, 2021). Cases and outbreaks are a threat in all countries of the world but are more pronounced in developing countries (Assegu Fenta et al., 2020). Despite breakthroughs in diagnosis, treatment, and vaccinations, in 2017 there were 5 million reported cases of meningitis worldwide, with 390,000 subsequent deaths (The, 2020). One-fourth of meningitis survivors in Africa develop neurological sequelae (Aku et al., 2017).

Annually, BM affects 1.2 million people and causes more than 30,000 deaths worldwide (Marcus and Walter, 2022). In Africa, the highest burden of BM occurs in an area of sub-Saharan Africa commonly known as the “meningitis belt,” stretching from Senegal to Ethiopia. Mortality from BM in this region exceeds 50%; around 1 in 10 people die, and 1 in 5 develop

severe complications (Wall et al., 2017). This is also true in Ethiopia (Awulachew et al., 2020, Hibstu et al., 2022). VM is common, with an annual incidence of 7.6 per 100,000 adults (Mount and Boyle, 2017). Despite this high morbidity and mortality of meningitis in Ethiopia, studies reporting patient outcomes are limited. Further, to the best of our knowledge, no study has been conducted regarding VM in the study area.

Meningitis demands prompt treatment; however, the rising antibiotic resistance of the pathogens poses significant challenges in managing patients effectively (Ali et al., 2021). In 2021, there were an estimated 4.71 million bacterial AMR-associated deaths (G. B. D. Antimicrobial Resistance Collaborators, 2024).

1.3 Rationale and significance of the study

Meningitis requires instant treatment, but antimicrobial resistance changes over time and varies from place to place due to different reasons. In Ethiopia, the treatment of meningitis is mostly empirical. Therefore, to manage meningitis patients rapidly and properly, data on the types of microorganisms prevalent in the local community and their up-to-date antimicrobial resistance profile against different antibiotics is an urgent need. However, the microbial profile and antibiotic resistance of the pathogens causing meningitis in Ethiopia have not been well characterized, especially in this study area. To our knowledge, there is no data regarding viral meningitis in the study area. Furthermore, whether adjunctive anti-inflammatory therapies like dexamethasone improve patient outcomes or not remains controversial, particularly in resource-limited settings where meningitis is more pronounced. Therefore, to the best of our knowledge, this study is the first to explore the microbial profile (BM and VM), bacterial antibiotic resistance pattern, and outcome of presumptive meningitis patients in Ethiopia at the University of Gondar Comprehensive Specialized Hospital (UoGCSH). As the hospital has the role of a referral center, data generated from this research will provide valuable insights into the current BM and VM causes of meningitis. Particularly, the data on antimicrobial resistance will help clinicians choose appropriate treatments. In general, this study will aid in the improvement of care for patients in the study site and other similar settings.

2 LITERATURE REVIEW

2.1 Prevalence of meningitis

Studies show varying prevalence of meningitis. A multicenter observational cohort study conducted in England revealed that the prevalence of meningitis in presumptive meningitis patients was 638 (57%) (Mcgill et al., 2018). A study carried out in Colombia reported an overall positivity rate of 97 (15.2%) (Penata et al., 2020). A prospective study conducted in Ethiopia showed 21 (10%) PCR positivity (Barnes et al., 2018).

Several studies compare VM and BM, showing that VM generally outnumbers BM. A multicenter observational cohort study conducted in England showed viral etiologies were positive in 231 (36%) cases, and 99 (16%) cases were BM (Mcgill et al., 2018). Another cross-section study conducted in Egypt showed that VM is more common at 87.1% than BM at 12.8% (Dawod et al., 2019). Studies from different countries, including Ethiopia, also showed a similar trend (Barnes et al., 2018, Penata et al., 2020).

2.1.1 Bacterial meningitis

BM is an inflammation of the meninges that occurs in response to bacteria and bacterial products. Potentially all pathogenic bacteria possibly cause meningitis, however. Previously, *S. pneumoniae, N. meningitidis, and H. influenzae* accounted for about 75–80% of the BM cases (G. B. D. Meningitis Collaborators, 2018). However, *E. coli, S. aureus, K. pneumoniae,* and others increased in importance after the introduction of vaccines and caused significant morbidity in specific age groups and underlying conditions (Carnalla-Barajas et al., 2022, Dagnew et al., 2013). Its prevalence varies by region and study population.

A retrospective study conducted in Finland showed that from a total of 148 CSF samples cultured, causative bacteria were detected in 50 (33.8%). In this study, the most common pathogens in CSF culture were *S. pneumoniae* 11 (7.4%) (Niemela et al., 2023).

Another retrospective study conducted in China found that out of 1,610 cultured CSF samples, causative bacteria were identified in 515 (32.0%). *E. coli* were the commonest pathogens detected in 195 (24.7%) (C. Wang et al., 2023).

Another prospective observational study conducted in Pakistan also showed that *E. coli* was the most common with 12.4% (Bhatti et al., 2024). A similar multicenter retrospective study in China also revealed similar findings (Peng et al., 2021).

A cross-sectional study carried out in Pakistan revealed that there were 321 (35.9%) confirmed cases of bacterial meningitis from 894 CSF samples. The most common etiology was *S. pneumoniae* 14.7% (130/894), followed by N. meningitidis accounted for 5.7% (51/894), H. influenzae for 2.5% (22/894), S. aureus for 6.9% (61/894), E. coli for 4.5% (40/894), and K. pneumoniae for 1.9% (17/894) (Ali et al., 2021).

Another study carried out in Iraq revealed that among 432 presumptive meningitis patients, culture was done, and bacteria were isolated in 33 (7.6%) CSF samples. Among 33 positive cases, 18 (54.5%) were *S. pneumoniae*, 2 (6%) were *E. coli*, 2 (6%) were *K. pneumoniae*, and 1 (3.1%) were *S. aureus,* and 1 (3.1%) were *H. influenzae (Saadi et al., 2017).*

Another cross-sectional study conducted in Kenya showed that using PCR bacterial agents, Out of 196 samples, 22 (11.2%) were positive for bacterial infections, including S. pneumoniae in 12 cases (54.5%), N. meningitidis in 7 cases (31.8%), and H. influenzae in 3 cases (13.6%) *(Gituro et al., 2017).*

Another hospital-based study conducted in southwest Ethiopia at Jimma University Hospital showed that causative bacteria were isolated in 26 (28.9%) patients (Gudina et al., 2018).

A hospital-based cross-sectional study conducted in Dilla University Referral Hospital showed that from a total of 287 CSF samples cultured, causative bacteria were detected in 38 (13.2%). The most commonly identified isolate was S. pneumoniae, found in 13 cases (34.2%), followed by S. aureus in 7 cases (18.4%), N. meningitidis, and Escherichia coli, each in 6 cases (16%). H. influenzae was isolated in 4 children (10.5%) with meningitis, while S. agalactiae was responsible for another 10.5% of cases (Awulachew et al., 2020).

A cross-sectional study was conducted in Debere Markos Comprehensive Specialized Hospital among 152 meningitis-suspected patients' bacteria isolated in 17 (11.2%) patients. The leading bacterial isolates were S. aureus and K. pneumoniae, each representing 29.4% (5/17) of the cases (Hibstu et al., 2022).

A hospital-based cross-sectional study conducted at Felege Hiwot Specialized Hospital showed that out of 176 CSF samples, 8 (4.5%) were found to be bacteriological culture-positive (Tesera et al., 2020).

A multisite cross-sectional study conducted in Ethiopia showed that among 139 BM, suspected patients *N. meningitidis* was isolated in 19.4% and *S. pneumoniae* was isolated in 12.9% of the patients (Mihret et al., 2016).

A retrospective cross-sectional study conducted at UoGCSH revealed a bacterial meningitis prevalence of 1.28% among adult patients. The most frequently identified isolate was S. pneumoniae (32%), followed by S. aureus (12.80%), E. coli (12.80%), and N. meningitidis (10.60%) (Tigabu et al., 2021).

2.1.2 Viral meningitis

Viral meningitis, also usually termed aseptic meningitis, means meningitis caused by an unknown etiology, but the term is not recommended for use due to the presence of sophisticated molecular techniques that identify the etiologic agent (Al-Qahtani et al., 2022).

A prospective study conducted in China indicated that the etiology of viral meningitis was identified in 42.8% of suspected cases, and the leading pathogen was EV (37.7%) followed by herpes simplex virus-1 (HSV-1) in 13.9% and varicella zoster virus (VZV) 11.5 (Ai et al., 2017).

A prospective study conducted at a teaching hospital in Ethiopia found that the virus was identified in 57% of the PCR-positive samples. The study showed human herpes virus-6 was the most common isolated pathogen (Barnes et al., 2018).

A study conducted on repository samples of three teaching hospitals in Ethiopia in collaboration with AHRI showed that EVs were detected in 11 (12.8%) (Geteneh et al., 2021).

A cross-sectional study performed at selected hospitals in Addis Ababa, Ethiopia, showed that EVs were found in 39 (26.7%) of the 146 cases clinically suspected of meningitis (Wami et al., 2021).

2.2 Antimicrobial resistance profiles of bacterial isolates

Early antibiotic treatment improves patient outcomes, but the effectiveness of available antibiotics is threatened by the global emergence of AMR (Van De Beek et al., 2012). AMR is a phenomenon in which microorganisms become resistant to antimicrobial agents to which they were originally sensitive. It is among the most severe global public health threats, causing considerable morbidity and mortality in many developing countries, including Ethiopia (Abebe et al., 2019).

The primary cause of AMR arises from evolutionary pressure, leading organisms to develop defense strategies against antimicrobial or class of antimicrobials. This occurs via a range of resistance mechanisms, such as a modified antimicrobial target, enzymatic hydrolysis/degradation, efflux, and impermeability (Founou et al., 2017). This is generally due to either genetic mutations or the acquisition of new genetic material through horizontal gene transfer (Belay et al., 2024).

A study conducted in Iraq showed that *S. pneumonia* strains showed 100% sensitivity against vancomycin and 83% against erythromycin. Isolates of gram-negative bacilli (*E. coli* and *K. pneumoniae*) were 100% sensitive to imipenem but were 100% resistant (0% sensitivity to cefotaxime). All isolates of Staphylococci were sensitive to gentamicin but were resistant to cefotaxime (Saadi et al., 2017).

A hospital-based cross-sectional study conducted in Dilla University Referral Hospital showed that Among gram-negative bacteria: *E. coli* showed 42.9% resistance to tetracycline, 40% to ciprofloxacin, 60 % to chloramphenicol, 20% to Trimethoprim-Sulphamethoxazole, and 60 % to ampicillin. Among gram-positive: *S. pneumoniae* showed 23% resistance to rifampin and 15.4% to trimethoprim-sulphamethoxazole and erythromycin. *S. aureus* showed 42.9% resistance to erythromycin and tetracycline, 57.1% to chloramphenicol, and 71.4% to rifampin. While 71.4% sensitivity to trimethoprim-sulphamethoxazole and 71.4% ciprofloxacin (Awulachew et al., 2020).

A cross-sectional study from Hawassa University Hospital showed that gram-negative *E. coli* showed 50% resistance to ceftriaxone, cefotaxime, and ciprofloxacin. K. pneumoniae isolates exhibited complete resistance to ceftriaxone and cefotaxime. Gram-positive *S. pneumoniae* showed 60% resistance to chloramphenicol and were 100% sensitive to vancomycin. *S. aureus*

showed 100% resistance to cefotaxime, ciprofloxacin, and erythromycin (Assegu Fenta et al., 2020).

A cross-sectional study conducted in Debere Markos Comprehensive Specialized Hospital showed that gram-negative; *K. pneumoniae* showed 100% resistance to ceftriaxone, ampicillin, cefotaxime, chloramphenicol, and 80% to trimethoprim-Sulphamethoxazole while 100% sensitive to imipenem and meropenem. *N. meningitidis* and *H. influanzae* were 100% sensitive to cefotaxime and ceftriaxone, respectively. Among gram-positive *S. pneumoniae, it* was 100% sensitive to vancomycin and chloramphenicol (Hibstu et al., 2022).

A hospital-based cross-sectional study conducted at Felege Hiwot Specialized Hospital showed that among gram-negative; *E. coli* exhibited complete resistance to ampicillin and 66.7% resistance to Chloramphenicol and tetracycline. The isolated *K. pneumoniae* was resistant to ceftriaxone, ampicillin, trimethoprim-sulphamethoxazole, ciprofloxacin, and tetracycline (Tesera et al., 2020).

2.3 Epidemiology

The epidemiology of meningitis has been dynamic in the decades since the introduction of the vaccine, with the incidence and case fatality rates varying by region (Barichello et al., 2023). In 2019, meningitis caused 236,222 deaths and 15,649,865 years of life lost worldwide (Qu et al., 2023). Meningitis is an important cause of morbidity and mortality in Africa, with a high incidence in the meningitis belt (Qu et al., 2023, Barichello et al., 2023). Ethiopia is a country in sub-Saharan Africa, situated at the eastern end of the World Health Organization's (WHO) African meningitis belt. Nonetheless, the overall epidemiology of meningitis in Ethiopia is still not well understood because of insufficient data from well-conducted studies.

2.4 Pathogenesis

Pathogens causing meningitis cause meningitis in the following subsequent stages, which include mucosal colonization, microbial translocation of mucous membrane, and invasion into the intravascular space, followed by their survival and replication within the vascular system, leading to significant bacteremia, crossing the blood-brain barrier (BBB), and infecting the meninges (Doran et al., 2016).

A hallmark of many bacteria that infect the central nervous system (CNS) is their capacity to endure in the bloodstream by evading or resisting phagocytosis, either through the expression of a capsule or by infiltrating and surviving within polymorphonuclear neutrophils (PMNs) or macrophages. Meningitis may also result from direct invasion from adjacent infected tissues. However, all pathogens must overcome specific barriers, including the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (B-CSFB), to gain access to the brain(Doran et al., 2016).

Damage to the integrity and heightened permeability of the blood-brain barrier (BBB) facilitate central nervous system (CNS) infections, as these issues can enhance the infiltration of leukocytes and pathogens into the CNS, thereby hastening disease progression. Various factors have been identified as contributing to BBB disruption, including direct cellular damage caused by bacterial virulence factors and the activation of host-specific proteins or inflammatory pathways (Yang et al., 2023).

Crossing the protective barriers of the brain is necessary for the onset of meningitis. Bacteria utilize various strategies to penetrate these barriers and access the central nervous system (CNS). They rely on different virulence factors that allow them to attach to and pass through these barriers. These factors are involved in facilitating adhesion to and invasion of host cells, promoting intracellular survival, activating host cell signaling and inflammatory responses, and impacting barrier function. While some mechanisms differ among pathogens, others are shared across several species (Herold et al., 2019). Apart from these strategies, bacterial components induce an overshooting inflammatory reaction that eventually leads to brain damage (Liechti et al., 2015).

Viruses use two basic pathways to gain access to the CNS. Hematogenous and neuronal. Enterovirus is the prototype of hematogenous spread; on the other hand, the Herpes simplex virus has neural spread (Papa and Papadopoulou, 2018).

2.5 Clinical features

The most common symptoms of meningitis in adults are a headache, neck stiffness, high-grade fever, and altered mental status (Awulachew et al., 2020, Barnes et al., 2018, Wami et al., 2021). Less commonly sensitivity to light (photophobia) and sound (phonophobia), and vomiting are also seen, but in newborns and infants, these typical symptoms may not occur, or it may be

difficult to notice instead, babies may be slow or inactive, irritable, and feed poorly (Assegu Fenta et al., 2020, Barnes et al., 2018, Geteneh et al., 2021).

A prospective observational study conducted at Bedele General Hospital showed that the most frequently occurring clinical manifestation of meningitis was fever 164 (83.67%), neck rigidity 149 (76.02%), and irritability 122 (62.24%) (Bekele et al., 2021). This is also supported by another study in Ethiopia. Fever, headache, neck stiffness, and impaired consciousness were the most common clinical presentations of meningitis (Gudina et al., 2016a). A cross-sectional study conducted at selected Hospitals in Addis Ababa, Ethiopia showed that The most frequently noted clinical symptoms of viral meningitis included vomiting (75.5%), followed by fever (56.8%) and altered consciousness or irritability (50.7%) (Wami et al., 2021).

2.6 Laboratory diagnosis

Diagnosis of meningitis relies on the examination of CSF obtained from lumbar puncture (LP). It is the most ideal specimen, obtained under strict aseptic conditions (Khatib et al., 2017). For the patients who do not have another clinical contraindication to LP, CSF should be collected and analysis should be performed within 1 h of the presumptive diagnosis of meningitis (Dyckhoff-Shen et al., 2021).

Gram staining, cytochemical analysis, and culture of CSF are basic methods for the diagnosis of BM. Gram stain is more rapid, inexpensive, and has good specificity, but sensitivity is poor (10–93% depending on the organism and whether or not antibiotics were given before CSF collection) (Barnes et al., 2018). Bacterial load, and has a detection limit of approximately 10^4 colony-forming units/mL. A Gram staining can show Gram-negative coffee bean-shaped diplococci (*N. meningitidis*), Gram-positive diplococci (*S. pneumoniae*), Gram-negative coccobacilli (*H. influenzae*), chain-sapped Gram-positive cocci (*S. agalactiae*) or Gram-negative bacilli (*E. coli*).

CSF culture is considered the “gold standard” for diagnosing bacterial meningitis, yet it is only positive in 70–85% of those who have not received antimicrobial therapy before lumbar puncture. However, antimicrobial therapies are often started before clinical sample collection, suboptimal storage, and transportation conditions thereby decreasing the probability of confirming the bacterial pathogen by culture (Bahr and Boulware, 2014, Van De Beek et al.,

2016). In this case, CSF gram staining, cytochemical analysis, and PCR could provide additional information, especially when the CSF culture is negative.

The cytochemical composition of CSF varies in cases of different abnormalities. Classic abnormalities of CSF composition in bacterial meningitis are a pleocytosis of mainly polymorphic leukocytes, low glucose concentration, low CSF to blood glucose ratio, and elevated protein levels. In neonates, however, CSF leukocyte count, glucose, and total protein levels are frequently within the normal range or only slightly elevated (Van De Beek et al., 2016).

Viral meningitis is often associated with lymphocytic pleocytosis, normal glucose, and normal or slightly elevated protein or all the cytochemical analysis results may be within the normal range (Adjei et al., 2018). PCR-based assays of CSF have been suggested as a rapid, sensitive, direct, and specific diagnostic test for meningitis. This assay detects small amounts of deoxyribonucleic acid/ribonucleic acid (DNA/RNA) irrespective of pathogen viability (Zeighami et al., 2021). Studies showed that it is sensitivity up to 89% in samples collected on days 1–3 of antibiotic treatment, 70% on days 4–6, and 33% on days 7–10. In comparison, no CSF cultures were positive in samples collected more than 1 day after the initiation of antibiotics (Chala et al., 2022).

Table 2.1 Typical CSF parameters in meningitis

Parameters	**BM**	**VM**
Cell count	> 1000/µl	< 1000/µl
Cell type	PMN	Lymphocytic
CSF/serum glucose ratio	Decreased	Normal
Protein	> 100 mg/Dl	< 100 mg/dL

Source (Dyckhoff-Shen et al., 2021)

2.7 Management of meningitis

BM is a medical emergency; as a result, treatment should be started promptly. Empiric therapy is similar for most patients with presumptive meningitis with the considerations of local epidemiology, the patient's age, and the presence of specific underlying diseases or risk factors

(Van De Beek et al., 2012). Vancomycin plus a third-generation cephalosporin (either cefotaxime or ceftriaxone) is the initial empiric therapy due to the most common pathogen, *S. pneumoniae*, which is presumed to be resistant to penicillin (Van De Beek et al., 2012). In addition, for all patients beyond the neonatal age, dexamethasone should be administered before or at the time of initiation of antibiotics for all bacterial etiologies except *L. monocytogen* (Van De Beek et al., 2022). Once a bacterial pathogen has been identified, antibiotic therapy can be modified further for optimum treatment (Van De Beek et al., 2012). VM is generally self-limited with a good prognosis and requires only supportive care except for herpes simplex virus meningitis.

Generally, the duration of meningitis therapy is 10 to 14 days. However, it can be adjusted if the specific causative pathogen is identified. *N. meningitidis* for 7 days, *S. pneumoniae* for 10 to 14 days, *H. influenzae* for 7 to 10 days, *L. monocytogenes* at least for 21 days, Gram-negative bacilli at least for 21 days, and *S. aureus* at least for 14 days (Van De Beek et al., 2016).

L. monocytogenes is noteworthy because of its resistance to cephalosporins. Amoxicillin or ampicillin are effective against *L. monocytogenes* and should be given to immunosuppressed patients with meningitis who are at risk of this infection, including pregnant patients and those older than 50 years (Van De Beek et al., 2012).

Table 2.2 Empiric antibiotic treatment recommendation for bacterial meningitis

Age	**Standard treatment**
1 month to 50 years	Ceftriaxone + vancomycin
>50 years	Ceftriaxone + vancomycin + ampicillin
Immunocompromised	Vancomycin + ampicillin + either cefepime, meropenem, or ceftazidime

Table 2.3 Specific antimicrobial therapy for meningitis etiology

Microorganism		**Antimicrobial therapy**
N. meningitides	Penicillin sensitive	Penicillin G or amoxicillin
	Penicillin resistant	Ceftriaxone or cefotaxime
S. pneumoniae	Penicillin sensitive	Penicillin G
	Penicillin intermediate	Ceftriaxone or cefotaxime or cefepime
	Penicillin resistant	Ceftriaxone (or cefotaxime or cefepime) + vancomycin

H. influanzae		Ceftriaxone or cefotaxime or cefepime
Gram negative bacilli		Ceftriaxone or cefotaxime
S. aureus	Methicillin-sensitive	Nafcillin
	Methicillin-resistant	Vancomycin
S. agalactiae		Penicillin G or amoxicillin

Source (Van De Beek et al., 2016)

2.8 Meningitis vaccination

Vaccination against the most common pathogens that cause bacterial meningitis is recommended. *H. influenzae type b*, *S. pneumoniae*, and *N. meningitidis* infection are preventable with the use of currently available vaccines. In Ethiopia, vaccines against *H. influenzae* serotype b, *S. pneumoniae* (pneumococcal conjugate vaccine 10), and *N. meningitidis* serogroup A (MenAfriVac) were introduced in 2007, 2011, and 2013–2015, respectively (Mihret et al., 2016, Amare et al., 2018).

H influenza type b was delivered in combination with diphtheria, tetanus, pertussis, and hepatitis B as pentavalent (DTP-HibHep). DTP-HibHep and PCV are given in three doses to children at the age of six, ten, and fourteen weeks of birth. The MenAfriVac serogroup A vaccine has been given to Ethiopians aged 1 to 29 in three phases. Phase 1, Phase 2, and Phase 3 vaccinations took place in 2013, 2014, and 2015, respectively. The initial phase was targeted at the population residing in the western and northwestern regions of the country. These interventions with different coverage and uptake have disrupted the epidemiology of vaccine-preventable bacterial meningitis (Mihret et al., 2016).

Table 2.4 National EPI schedule

Vaccine	**Target group to be prevented**	**Age**
PCV	*S. pneumoniae*	At 6, 10, and 14 weeks
DPT-Hib-hep B	Meningitis associated with *H. influenzae* in combination with *C. diphtheria, B. pertussis, C. tetani,* and hepatitis B	At 6, 10, and 14 weeks
MenAfriVac	*N. meningtidis serogroup A*	1 to 29 years

2.9 Outcome of patients

A prospective observational study conducted at Bedele General Hospital showed that the magnitude of good treatment outcomes was 132 (67.35%), whereas 64 (32.6%) were poorly controlled. Corticosteroid use (AOR = 2.37, 95% CI: 1.17-4.81, *P* = 0.017) was a predictor of the meningitis treatment outcome (Bekele et al., 2021). Another cross-sectional study conducted at Felege Hiwot Referral Hospital found that about 15% of children with meningitis developed poor outcomes (Tewabe et al., 2018).

A retrospective study conducted in Ethiopia showed that patients with BM showed unfavorable outcomes in 36.7% (Gudina et al., 2016b). Another retrospective study conducted at Hiwot Fana Specialized University Hospital showed that 154 (77%) showed successful treatment outcomes, while 46 (23%) experienced poor treatment outcomes (Adem et al., 2020). Another retrospective study conducted at Dilla University Referral Hospital also showed that the overall proportion of favorable treatment outcomes was 92 (76.7%) while 28 (23.3%) had poor treatment outcomes (Sileshi Elias et al., 2021).

3 OBJECTIVES

3.1 General objective

- To assess the microbial profile, antimicrobial resistance, and outcome of presumptive meningitis patients at the University of Gondar Comprehensive Specialized Hospital, Gondar, Northwest Ethiopia, 2023

3.2 Specific objectives

- To determine the prevalence of meningitis-causing bacterial and viral pathogens from patients with presumptive meningitis
- To characterize the anti-microbial resistance profile of bacterial isolates
- To evaluate the outcome of patients with presumptive meningitis

4 METHODS AND MATERIALS

4.1 Study area

This study was conducted at the University of Gondar Comprehensive Specialized Hospital (UoGCSH). The hospital is located in Gondar city, 750 km northwest of Addis Ababa, the capital city of Ethiopia. It is one of the tertiary-level referral and teaching hospitals in the Amhara region, that serves an estimated seven million people residing in the Gondar city and surrounding zones. Data from the hospital's planning department showed that an average of 1,825 patients with presumptive meningitis get service annually. The number of admissions ranges from 180–200 patients per month, and an average of seven cases with suspected meningitis are admitted daily (source: UoGCSH administrative office).

4.2 Study period

The study was conducted from August 01 to December 31, 2023.

4.3 Study design

A prospective cross-sectional study design was used to assess the microbial profile, antimicrobial resistance, and outcome of patients with meningitis at UoGCSH, Northern West, Ethiopia.

4.4 Source population

All presumptive meningitis patients visiting UoGCSH.

4.5 Study population

All presumptive meningitis patients during the study period.

4.6 Sample size determination

The required sample size for this study was calculated based on the prevalence rate of bacterial meningitis of 13.2% as reported in a previous study (Awulachew et al., 2020), considering a confidence interval of 95% and a 5% marginal error. The sample size (n) was determined using the statistical formula for a single proportion as follows:

$$\mathbf{n} = \left[\frac{\left(\mathbf{z}\frac{\boldsymbol{\alpha}}{\mathbf{2}}\right)^{\mathbf{2}}\mathbf{p}(\mathbf{1}-\mathbf{p})}{\mathbf{d}^{\mathbf{2}}}\right], \text{ where}$$

d = the margin of error between the sample and the population

n = sample size

Z = 95% confident interval

P = prevalence of meningitis based on the previous study

$$\frac{(\mathbf{1.96})\mathbf{2} \times \mathbf{0.132}(\mathbf{1} - \mathbf{0.132})}{(\mathbf{0.05})\mathbf{2}} = \mathbf{177}$$

Considering a 10% nonresponse rate, the final sample size was 195.

4.7 Sampling technique

All presumptive meningitis patients based on clinical criteria who fulfilled the inclusion criteria during the study period were enrolled in the study conveniently until the desired sample size was attained.

4.8 Selection of study participants

4.8.1 Inclusion criteria

Presumptive meningitis patients greater than 28 days of age who visited the UoGCSH and were willing to participate in the study.

4.8.2 Exclusion criteria

- Patients who have known bleeding disorder
- Patients who have a known history of organ transplantation

4.9 Study variables

4.9.1 Dependent variables

Microbial profile, antimicrobial resistance, and patient outcome

4.9.2 Independent variables

Socio-demographic factors (such as age and sex), clinical manifestation (fever, neck stiffness, level of consciousness (GCS), seizure, kerning sign, brudzinski's sign, photophobia),

comorbidity, duration of disease onset to the hospital visit, history of antibiotics intake before the hospital visit.

4.10 Operational definitions

- **Presumptive meningitis** was defined according to clinical judgment, considering history and clinical signs and symptoms. Any person with a temperature ≥38ºC and/or neck stiffness and/or photophobia and/or altered mental state (Glasgow Coma Score ≤14).
- **Antimicrobial resistance** is a phenomenon in which microorganisms become resistant to antimicrobial agents to which they were originally sensitive.
- **Glasgow outcome scale** 1 = death, 2 = persistent vegetative state (coma or unresponsiveness). 3 = severe disability (hemiplegia, or paraparesis), 4 = moderate disability (improved with minor sequelae such as facial palsy), and 5 = good recovery (full recovery).
- **Unfavorable outcome**: defined as a GOS score of 1–4.
- **Favorable outcome:** defined as a GOS score of 5.
- **Consciousness** was assessed using the Glasgow Coma Scale (GCS) score for children >2 years old and adults and the Pediatric Glasgow Coma Scale (PGCS) for children <2 years old, and asses as follows (eye-opening: 4 = spontaneous, 3 = to sound, 2 = to pain, 1 = none: verbal response: 5 = age-appropriate vocalization, smile, or orientation to sound, interacts (coos, babbles), follows objects, 4 = cries, irritable, 3 = cries to pain, 2 = moans to pain, 1 = none: motor response; 6 = spontaneous movements command). 5 = withdraws verbally to (obeys touch (localizes pain), 4 = withdraws to pain, 3 = abnormal flexion to pain (decorticate posture), 2 = abnormal extension to pain (decerebrate posture), 1 = none), which has a score of 3 to 15. Full consciousness refers to a score of 15; a score of 9–14 was considered impaired consciousness, and patients with a score of ≤ 8 were classified as comatose (Tadesse et al., 2017).
- **Neck stiffness:** Passively flex the patient's neck. This test is positive if there is palpable resistance.
- **Kernig's sign:** Position the patient supine with their hips flexed to 90º. This test is positive when the knee cannot be extended beyond 180º (if "Kernig's angle'' <180º).
- **Brudzinski's sign:** Position the patient supine and passively flex their neck. This test is positive if this maneuver causes reflex flexion of the hip and knee.

- **Seizures** were considered only observed on admission as well as the history of seizures during 48 hours before hospitalization.

4.11 Data collection tools and test procedures

4.11.1 Data collection procedures

The data were collected using a pretested, structured questionnaire specifically prepared for this study after a thorough literature review. The questionnaires were developed in English and translated into the Amharic language, which is the local language in the study area. Slight modifications were made after the pre-test was performed on 5% of the study population at Alert Comprehensive Specialized Hospital. The questionnaire has four parts; the first part is for collecting data about the socio-demographic characteristics, and the second part is for collecting data concerning the clinical features of the study subjects on admission. The third part is for collecting data related to laboratory results, and the last one is to assess patient outcomes at the last date of hospital stay.

Before the actual data collection, brief explanations were given to the patients if the patient was 18 and above years old and conscious, to the guardian if the patient was unconscious or age under 11, or to both the patient and the guardian if the patient's age between 11 and 18, by the assigned physician about the purpose of the study and possible risks of participating in the study. Finally, written informed assents were obtained from the guardian for participants below 11 years old (both assent from the guardian and/or consent from the participant 11 to 18 years old), and consents were obtained from the study 18 or older conscious participants or assent from his/her guardian, and only those who signed participated in the study. Before actual data collection started, an orientation was given to data collectors about the aims and objectives of the project and how to collect and record data appropriately.

4.11.2 Demographic, clinical, and discharge outcome data collection

Socio-demographic variables, clinical variables at admission; and variables related to the patient outcome at the last date of hospital stay, were recorded through face-to-face interviewing of the participant or the guardian in case of severely ill and unable to communicate and physical examinations by the assigned physician, while microbiological analysis and results were documented by laboratory staff.

After collecting demographic and clinical data through interviews and physical examination the CSF samples were collected aseptically by the assigned physician as a part of the patient's routine clinical diagnostics.

Treatment the patient received, length of hospital stays, and death or discharge outcomes using the Glasgow Outcome Scale or left against medical advice were recorded by the assigned physician. For those who were regularly discharged, outcomes were categorized using the Glasgow Outcome Scale (1, death; 2, vegetative state; 3, severe disability; 4, moderate disability; 5, good recovery) at discharge. Later, the scores were dichotomized into favorable (5) and unfavorable (1 to 4) outcomes.

4.11.3 Lumbar puncture for CSF collection

The patient should be either sitting up or lying on the side, with his or her back arched forward. The skin was disinfected with 70% alcohol, 2% tincture of iodine, and 70% alcohol, respectively. Depending on the age of the patient, lumbar punctures were done using a needle measuring 1.5 inches (3.8 cm) for infants, 2.5 inches (6.3 cm) for children, and 3.5 inches (8.9 cm) for adults. The CSF sample was collected using sterile plastic tubes (Sartstedt, Germany) by following standard operating procedure (SOP) and transported to the laboratory within 30 minutes.

About 3 to 4 ml of CSF were collected in two sterile test tubes (about 2 ml each). The first test tube was sent to the hematology laboratory for white blood cell count and cell type, and subsequently to chemistry for glucose and protein determination. The second tube was transported to the bacteriology laboratory. Then, it was subjected to a naked-eye examination for macroscopic appearance, then approximately 0.5 ml of CSF was transferred to a Nuck tube inside safety cabinet level 2 and stored at -80 °C until transported to AHRI by car freezer and again preserved at -80^{o}C freezer at AHRI laboratory until PCR was done. The remaining CSF undergoes routine bacteriology analysis centrifuged at 30,000 revolutions per minute (RPM) for 15 minutes, the supernatant was discarded and the sediment was used for culture and gram stain.

4.11.4 Laboratory testing

4.11.4.1 Gross and microscopic examination

As soon as the CSF sample arrived at the bacteriology laboratory, it was subjected to macroscopic examination (clear, turbid, or xanthochromic). Then, the sample was centrifuged at 30,000 RPM for 15 minutes, smears were prepared from the sediments for Gram staining, and then examined for the presence of microbes.

4.11.4.2 CSF chemistry and cell count

CSF glucose and protein levels in the samples were estimated by using a standard chemistry analyzer (Mendery200), and CSF cell count was done manually by imprved-neubar chamber.

4.11.4.3 Culture and identification

Sediments of each centrifuged CSF sample were inoculated onto blood agar, chocolate agar, and MacConkey agar plates (Oxoid Ltd, Basingstoke, Hampshire, UK) following standard microbiological procedures. The chocolate and blood agar plates were incubated in a candle jar, which can provide 5-10% CO_2 concentration to create a microaerophilic condition for fastidious bacteria, while the inoculated MacConkey agar plates were incubated aerobically. After 18–24 hours of incubation, the plates were examined for bacterial growth. Plates that did not show any growth were further incubated for an additional 24 hours. All pure isolates were identified by their characteristic appearance on their respective media, and gram-reaction, and confirmed by the pattern of biochemical tests using standard microbiological methods. Gram-negative rods were identified by indole production, hydrogen sulfide (H_2S) production, citrate utilization, motility test, urease test, oxidase, and triple sugar iron (TSI) agar. For gram-positive cocci, catalase, coagulase, bacitracin, and optochin susceptibility tests were used.

4.11.4.4 Antimicrobial susceptibility testing

AST was performed for all pure bacterial isolates using the Kirby-Bauer disk diffusion method according to the criteria set by the Clinical and Laboratory Standards Institute (CLSI, 2022). 3 to 5 selected pure culture colonies from an overnight (18 to 24 hours) grown culture were taken and transferred to a tube containing 5 mL sterile normal saline and mixed gently until a homogenous suspension was formed and colony suspension, equivalent to a 0.5 McFarland standard was

reached. It was measured using a DEN-1B McFarland densitometer. A sterile cotton swab was used, and the excess suspension was removed by gentle pressing and rotation of the swab against the inside wall surface of the tube. The swab was evenly inoculated over the entire surface of unsupplemented Mueller Hinton agar (Oxoid Ltd.) or 5% Sheep blood-supplemented Mueller Hinton agar for isolates. The inoculated plates were left at room temperature to dry for 3-5 minutes, and a set of 5 to 10 antibiotic discs (Oxoid Ltd.) were then delivered to the surface of the 150-mm-diameter plate. The drugs used for disc diffusion testing were in the following concentrations: Ampicillin (AMP) (10 µg), Ceftriaxone (CRO) (30 µg), Ciprofloxacin (CIP) (5 µg), Chloramphenicol (C) (30 µg), Erythromycin (E) (15 µg), Trimethoprim-Sulphamethoxazole (SXT) (1.25/23.75 µg), Gentamycin (GM) (10 µg) Rifampicin (RA) (5 µg), Meropenem (MEM) (10 µg), Imipenem (IMP) (10 µg), Vancomycin (VA) (30 µg), Cefepime (FEP) (30 µg), Ceftazidime (CAZ) (30 µg), Cefotaxime (CTX) (30 µg), and Tetracycline (TE) (30 µg). The plates were then incubated in ambient air or 5% CO_2 as necessary at 37 ± 2ºC for 24-48 hours, depending on the organism tested. Diameters of the zone of inhibition around the disc were measured to the nearest millimeter using a graduated ruler in millimeters and interpreted as sensitive, intermediate, and resistant according to the CLSI 2022.

Reference strains

E. coli (ATCC-25922), *S. pneunoniae* ATCC-49619, *H. influenzae* ATCC-49247, *K. pneumoniae* ATCC-700603 and *S. aureus* (ATCC-25923) American Type Culture Collections were used as quality control throughout the study for culture, and antimicrobial susceptibility testing and for PCR. All the strains were obtained from the Ethiopian Public Health Institute. NATEVPOS-6MC for enterovirus, NATEBV-005 for Epstein-Barr Virus, and NATVZV-0005 for Vericella-Zoster virus were used as viral positive controls from NATtrol.

4.11.4.5 Molecular testing

Nucleic acid extraction

Extraction of nucleic acid from CSF samples was performed according to the protocol provided with the Bioer NPA-32P automated Nucleic Acid Purification method (Hangzhou Bioer Technology Co., Ltd, China) and High Pure viral nucleic acid Preparation Kit (Roche Life Sciences, Mannheim, Germany) for bacterial and viral nucleic acid extraction, respectively.

The concentration and purity of nucleic acid, DNA, or RNA of extract were determined using a Nanodrop Spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE) at 260, 260/280, and 260/230 nm.

Polymerase chain reaction (PCR) testing

Conventional singleplex and multiplex PCR for microbial etiology identification was performed with primers specific for amplifying the following genes: c*trA* (*N. meningitides*), *lytA* (*S. pneumoniae*), *hpd* (*H. influenzae*), hly (*L. monocytogenes*), 16S rRNA (*E. coli*), cfb (*S. agalactiae*), rcsA (*K. pneumonae*), femA (*S. aureus*), *gpB (HSV-1),* EBNA *(EBV),* DNAPol *(VZV),* 5′ NTR *(EnterV),* N gene *(MumV).*

Singleplex PCR was employed for the detection of all viral DNA and RNA. Multiplex PCR was utilized for the detection of bacterial DNA. Bacterial targets were multiplexed into two groups. The first group included *N. meningitidis*, *S. pneumoniae*, *H. influanzae*, and *S. agalactia*. The second group consisted of *E. coli*, *K. pneumoniae*, *S. aureus*, and *L. monocytogen*.

Platinum Taq PCR Master Mix (Thermo Fisher Scientific), which contains Taq polymerase, dNTPs, $MgCl_2$, and buffer was used for master mix preparation. Each PCR tube contained a 25 µL reaction mixture composed of 2.5 µL of 10× buffer, 1.2 µL of 50 Mm $MgCl_2$, 1 µL dNTP of the master mix, 1 µL of each forward and reverse primer, 0.2 µL Taq polymerase, 5 µL of DNA and 13.1 µL nuclease-free water to complete the final volume.

To prepare the master mix, each of the PCR mix constituents for individual PCR tubes was multiplied with the number of samples, positive and negative controls with an additional 2 µL of extra volume of each component to adjust pipetting errors. The PCR master mix was vortexed gently 5 to 6 times. 20 µL of the PCR master mix was aliquoted into each labeled PCR tube in the PCR mix room within biosafety cabinet level 2. Five µL of each genomic DNA or RNA sample, positive controls from the DNAs extracted from ATCC, and negative control (PCR grade water processed together with samples during DNA extraction) were added into the corresponding PCR tubes in a separate room.

PCR was performed using the Bio-Rad T100 thermal cycler. Amplification involved an initial denaturation at 94^{o}C for 3 min, followed by 35 cycles of denaturation (94^{o}C for 10 sec), annealing (60^{o}C for 30 sec), and extension (72^{o}C for 30 sec), with a final extension step (72^{o}C

for 5 min). The amplified DNA was separated by gel electrophoresis, stained with ethidium bromide, and visualized under UV transillumination. This protocol was adapted from the Platinum Taq polymerase protocol with minor modifications.

Table 4.1 Primers with their 5’ to 3’ sequence and respective nucleotide length used in the study

Organism	**Gene**	**Primer**	**Sequence (5' - 3')**	**Size (bp)**	**Reference**
N. meningitis	*ctrA*	ctrA-F	GCTGCGGTAGGTGGTTCAA	111	(Karimi et al., 2023)
		ctrA-R	TTGTCGCGGATTTGCAACTA		
S. pneumoniae	*lytA*	lytA-F373	ACGCAATCTAGCAGATGAAGCA	75	(Y. Wang et al., 2014)
		lytA-R424	TCGTGCGTTTTAATTCCAGCT		
H. influenzae	*hpd*	hpdF822	GGTTAAATATGCCGATGGTGTTG	151	(De Gier et al., 2016)
		hpdR952	TGCATCTTTACGCACGGTGTA		
E. coli	rRNA	Eco-FrRNA	GGGAGTAAAGTTAATACCTTTGC	204	(Y. Wang et al., 2014)
		Eco-RrRNA	CTCAAGCTTGCCAGTATCAG		
L. monocytogen	Hly	Lm-Fhly	CAT GGCACCACCAGC ATCT	64	(Y. Wang et al., 2014)
		Lm-Rhly	ATC CGCGTGTTTCTTTTCGA		
S. aureus	femA	Sau-FfemA	TGCTGGTGGTACATCAAA	97	(Y. Wang et al., 2014)
		Sau-RfemA	ACGGTCAATGCCATGATTTAA		
S. agalactiae	Cfb	Sag-Fcfb	ATGATGTATCTATCTGGAACTCT AGTG	260	(Y. Wang et al., 2014)
		Sag-Rcfb	CGCAATGAAGTCTTTAATTTTTC		
K. pneumoniae	rcsA	Kpn-FrcsA	GGATATCTGACCAGTCGG	176	(Dong et al., 2015)
		Kpn-RrcsA	GGGTTTTGCGTAATGATCTG		
Herpes simplex virus 1	*gpB*	HSV1-gpB-F	CCACCGTCAGCACCTTCAT	127	(Liu et al., 2022)

		HSV1-gpB-R	CGCTGGACCTCCGTGTAGTC		
Epstein-Barr virus	EBNA	EBV3-9F EBV3-9R	CTGACACTTTAGAGCTCTGGAG GGCCCTGACCTTTGGTGAAGTCA	228	(Liu et al., 2022)
Varicella-Zoster virus	DNAPol	VZV-DNApolF VZV-DNApolR	GCGCTCTAACGTTCGAGAAAGT CGCATAGCCAACCAGTCTTTT	60	(Pormohammad et al., 2020)
Enteroviruses	5′ NTR	PanEV2-5′NTR F PanEV2-5′NTR R	CATGGTGCGAAGAGTCGATTG A CACCCAAAGTAGTC GGTTCCGC	144	(Wami et al., 2021)
Mumps virus	N gene	MumV-F MumV-R	TTCAGGGAACCAACTCGTTGA CTTCGGAGGATGAGACCATGAT	171	(Liu et al., 2022)

For RNA virus two-step RT-PCR was conducted for the amplification of HEVs and mumps. EVs and mumps-specific primers were used to detect all EVs and mumps. Reverse transcription was performed in a 20 µL reaction composed of 0.2 µL of random hexamer, 1 µL of 10 mM dNTP, 1.8 µL DEPC water, and 10 µL template RNA 1 µL of 100 mM DTT, 1 µL of RNase inhibitor (40 units/µL), 0.8 µL of 10× RT buffer, 5 µL of superscript IV. The reaction mixture was then incubated at 65ºC for 5 minutes to synthesize cDNA, and the reaction was inactivated by incubating at 55ºC for 50 minutes, and 80ºC for 10 minutes. PCR amplification was done by adding 5 µL cDNA to 20 µL PCR master mix (constituting of 5 µL of 5 Green Go Taq Flexi Buffer, 2.5 µL 25 Mm $MgCl_2$, 0.5 µL 10 Mm dNTP, 0.3 µL of forward and reverse primer each (10 µM), 0.1 µL Go-Taq G2 Flexi DNA polymerase and 10.3 µL molecular grade water). The thermal cycling was adjusted at 94ºC for 2 minutes, and 35 cycles of denaturation at 94ºC for 30 seconds, followed by annealing at 58ºC for 30 seconds and extension at 72ºC for 30 seconds. The amplicon was visualized on 2.5% agarose gel. This protocol was obtained from the Go-Taq G_2 Flexi DNA polymerase protocol.

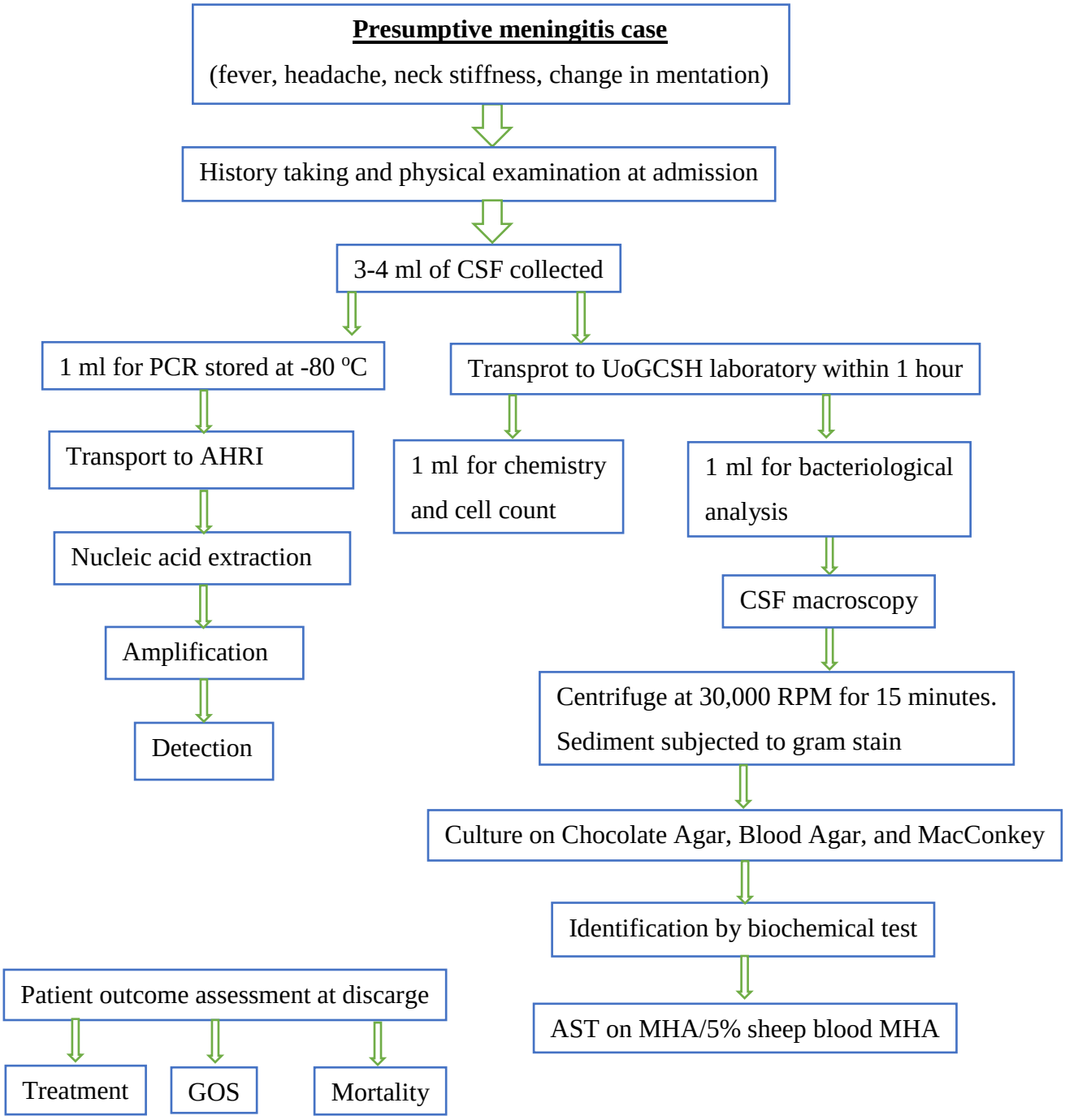

Figure 4.1 Flow chart of data collection, laboratory procedure, and patient outcome assessment of presumptive meningitis patients at the UoGCSH

4.12 Data quality control

Pre-tests were performed to assess the quality of the questionnaires before the actual data collection on 5% of the study population in the Alert Comprehensive Specialized Hospital, and minor amendments were made accordingly. All clinical evaluations were performed by

clinicians who were experienced in the care of patients. Orientation was given to all data collectors on how to collect and record data appropriately. The collected data were checked at different levels for completeness and consistency by the principal investigator at the end of each day. When an error was found at any level, the investigators with the assigned physicians traced it back and corrected it. Standard operating procedures (SOP) were followed during specimen collection, transportation, processing, interpretation, and reagent and media preparation following the manufacturer's instruction. The qualities of the culture media were checked by inoculating reference bacterial strains and 10% of the prepared media before inoculation were checked by incubating at 35–37 °C for 24 h and observing the growth of an organism. The performance of antibiotic discs was also checked by using the standard strains. Positive controls from NATrol (Helvetica Health Care, Geneva Switzerland) or ATCC strains and molecular-grade water were used as positive and negative quality controls respectively to ensure the quality of molecular test results. Double data entry was done by the principal investigator to keep the accuracy of the data.

4.13 Data analysis

The data were first coded, cleaned, and entered into Epi-Info v 3.1, then transformed and analyzed using Statistical Package for Social Science (SPSS) version 26 statistical software. The results of the analysis were presented in text, figures, or tables where appropriate. Frequency and percentage were used to summarize categorical variables. Skewed data were presented as a median with an interquartile range. The association between variables was assessed using binary logistic regression and multivariable regression analysis. All independent variables with $p<0.25$ in bivariate analysis were entered for multivariable analysis. Forward logistic regression analysis was done to identify the best-fit model. In all the cases, a *p*-value of $p<0.05$ was considered statistically significant.

4.14 Ethical consideration

The study was approved by the Research and Ethics Review Committee of the Department of Microbiology, Immunology and Parasitology reference DRERC/004/2023, and AHRI/ALERT Ethics Review Committee (AAERC) protocol number PO-55-22. Administrative permission was also obtained from UoGCSH. Detailed explanations were given about the objectives, and all the pros and cons that the patient may face during enrollment in the study were explained to the patient or patient guardian by using a pre-planned patient information sheet. A clear explanation

was also given: participation in the study or withdrawal from the study after enrollment does not affect the routine clinical diagnosis and patient care service. The fact that the CSF collection procedure is done for the sake of patient clinical diagnosis was explained clearly and understood by the participants/guardians. Strict confidentiality of responses is maintained. Data were collected after obtaining written informed consent from the study adult subjects or accent from parents/guardians for children, neonates, or the unconscious. Only subjects who signed the consent, or accent, participated in the study. Confidentiality was maintained by using a patient card number and research code instead of a patient name. The study was performed following the Declaration of Helsinki. This study was done under the supervision of advisors from Addis Ababa University (AAU) and Armauer Hansen Research Institute (AHRI).

4.15 Result dissemination

After completion of the study, the findings will be reported and defended at the AAU, School of Medicine, Department of Microbiology, Immunology, and Parasitology (DMIP). A copy will also be sent to the AAU Health Library and repository as baseline data for further research activities. Another copy will be sent to UoGCSH, and then the manuscript will be prepared and sent to a reputable international journal for publication.

Funding

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5 RESULTS

5.1 Study population

A total of 195 study participants with presumptive meningitis at UoGCSH were included in this study. Of these, 121 (62.1%) were males. The majority of the study participants (158, 81%) were under 14 years of age, and of these, children under five years constituted 60%. About half (96, 49.2%) of the study participants were rural residents (Table 5.1).

Table 5.1 Sociodemographic characteristics of presumptive meningitis patients at UoGCSH, Gondar, Ethiopia, 2023

Variable		Frequency	Percent
Age in years	<5	117	60
	5 - <14	41	21
	14 – 50	31	15.9
	>50	6	3.1
Sex	Male	121	62.1
	Female	74	39.1
Residence	Urban	99	50.8
	Rural	96	49.2

5.2 Clinical presentations of the study participants

In this study, the most common clinical presentation among patients with presumptive meningitis was fever, observed in 121 (62.1%), followed by neck stiffness in 114 (58.5%), and loss of consciousness in 80 (41%). About half of the patients 106 (54.4%) were vaccinated for vaccine-preventable meningitis etiologies. Moreover, 37 (19%) patients had taken some form of antibiotics prior to hospital admission (Table 5.2).

Table 5.2 Clinical condition and history of presumptive meningitis patients at UoGCSH, Gondar, Ethiopia, 2023

Clinical condition and history	Frequency	Percent
Fever	121	62.1
Neck stiffness	114	58.5
Loss of consciousness (GCS<14)	80	41
Seizures	75	38.5
Kernig's sign	29	14.9
Brudzinski sign	11	5.6
Rash	8	4.1
Photophobia	46	23.6
Vomiting	109	55.9
Comorbidity	8	4.1
Vaccination	106	54.4
History of antibiotics intake before hospital visit	38	19.5
Adjunctive dexamethasone therapy	79	40.5

5.3 Laboratory findings of cerebrospinal fluid examination

5.3.1 Routine laboratory findings

Out of the total 195 CSF samples collected, 152 (77.9%) appeared visually clear and 35 (17.9%) were turbid. Turbidity was observed in 25 (45.5%) samples from patients with confirmed bacterial meningitis and 3 (5.1%) from patients with confirmed viral meningitis.

The WBC counts of the CSF samples ranged from no cells to dense clusters of cells. The majority (137, 70.3%) of the samples had WBC counts of less than 5 cells/µL. Most of the CSF samples positive for viral meningitis (54/59, 91.7%) had a CSF WBC count of less than 5 cells/µL. In contrast, 16 (29.1%) out of the 55 confirmed bacterial meningitis patients had a WBC count of greater than 100 cells/µL. Additionally, 31 (56.6%) confirmed BM patients have a glucose level of less than 40 mg/dL, while 52 (88.1%) confirmed virus-infected patients have a glucose level of greater than 40 mg/dL. No virus-infected patient was found to have a protein level of >100 mg/dL (Table 5.3).

Gram staining of the CSF from presumptive meningitis was positive in 8 (4.1%) samples where 6 (75%) were gram-positive cocci, one (12.5%) gram-negative coccobacilli, and one (12.5%) gram-negative rods.

Table 5.3 CSF profile of patients with presumptive meningitis at UoGCSH, Gondar, Ethiopia, 2023

CSF analysis	**Presumptive meningitis**		**Microorganism detected**						**Microorganism not detected**	
	N	%	Bacteria		Virus		Total		N	%
			N	%	N	%	N	%		
Macroscopic appearance of CSF										
Crystal clear	152	77.9	25	45.5	54	91.5	79	69.3	73	90.1
Turbid	35	17.9	25	45.5	3	5.1	28	24.6	7	8.6
Blood-stained	8	4.2	5	9	2	3.4	7	6.1	1	1.3
CSF WBC										
<5 cells/µL	137	70.3	11	20	54	91.7	65	57	74	91.3
5-100 cells/µL	39	20	28	50.9	4	6.8	32	28.1	5	6.2
>100 cells/µL	19	9.7	16	29.1	1	1.7	17	14.9	2	2.5
CSF protein										
<46 mg/dL	141	72.3	18	32.7	49	83.1	67	58.8	74	91.4
46-100 mg/dL	38	19.5	21	38.3	10	16.9	31	27.2	7	8.6
>100 mg/dL	16	8.2	16	29.1	0	0	16	14	0	0
CSF glucose										
≤40 mg/dL	46	23.6	31	56.4	7	11.9	38	33.3	8	9.9
>40 mg/dL	149	76.4	24	43.6	52	88.1	76	66.7	73	90.1

5.3.2 Culture findings

A total of 14 (7.2%) CSF samples were found positive for bacterial growth. Gram staining detected half of the culture-positive CSF samples (7/14, 50%). One sample that demonstrated Gram-negative coccobacilli on Gram staining did not exhibit any growth in culture. The sample was confirmed positive for *H. influenzae* by PCR. The majority of the isolates were gram-negative bacteria (8/14, 57.1%). The most frequently isolated bacteria were *S. pneumoniae* and *K. pneumoniae* (each accounting for 4, 28.6%), followed by *E. coli* and *S. aureus* (each 2, 14.3%), and *H. influenzae* and *N. meningitidis* (each 1, 7.1%).

5.3.3 Anti-microbial resistance profile of bacterial isolates

Among the gram-negative bacterial isolates, *K. pneumoniae* showed 3/4 (75%) resistance to trimethoprim-sulfamethoxazole and ampicillin while 2/4 (50%) resistance was recorded to meropenem, cefotaxime, and ciprofloxacin. The *E. coli* isolates exhibited 1/2 (50%) resistance to ceftazidime, tetracycline, ciprofloxacin, chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin. Both the single isolates of *N. meningitidis* and *H. influenzae* showed intermediate resistance to ciprofloxacin and chloramphenicol respectively while no resistance was detected to the rest of the tested drugs (Table 5.4).

S. pneumoniae exhibited 1/4 (25%) resistance to erythromycin, trimethoprim-sulfamethoxazole, ciprofloxacin, and rifampin. The *S. aureus* isolates were 2/2 (100%) resistant to chloramphenicol and rifampin while 1/2 (50%) resistant to tetracycline (Table 5.5).

Table 5.4 Antibiotic susceptibility pattern of Gram-negative bacterial isolates from CSF of patients with bacterial meningitis at UoGCSH, Gondar, Ethiopia, 2023

	N. meningitidis **(N=1)**			***H. influenzae*** **(N=1)**			***E. coli*** **(N=2)**			***K. pneumoniae*** **(N=4)**		
Antibiotic Agent	S	I	R	S	I	R	S	I	R	S	I	R
CRO	1(100%)	-	-	1(100%)	-	-	2(100%)	0(0%)	0(0%)	2(50%)	1(25%)	1(25%)
MEM	1(100%)	-	-	1(100%)	-	-	1(50%)	1(50%)	0(0%)	2(50%)	0(0%)	2(50%)
SXT	1(100%)	0(0%)	0(0%)	ND	ND	ND	0(0%)	1(50%)	1(50%)	0(0%)	1(25%)	3(75%)
C	1(100%)	0(0%)	0(0%)	0(0%)	1(100%)	0(0%)	1(50%)	0(0%)	1(50%)	2(50%)	1(25%)	1(25%)
CAZ	ND	ND	ND	1(100%)	-	-	0(0%)	1(50%)	1(50%)	2(50%)	1(25%)	1(25)
CTX	1(100%)	-	-	1(100%)	-	-	2(100%)	0(0%)	0(0%)	2(50%)	0(0%)	2(50%)
FEP	ND	ND	ND	ND	ND	ND	2(100%)	-	0(0%)	3(75%)	-	1(25%)
IPM	ND	ND	ND	ND	ND	ND	2(100%)	0(0%)	0(0%)	4(100%)	0(0%)	0(0%)
AM	ND	ND	ND	1(100%)	0(0%)	0(0%)	0(0%)	1(50%)	1(50%)	0(0%)	1(25%)	3(75%)
CIP	0(0%)	1(100%)	0(0%)	ND	ND	ND	1(50%)	0(0%)	1(50%)	2(50%)	0(0%)	2(50%)
TE	ND	ND	ND	ND	ND	ND	1(50%)	0(0%)	1(50%)	2(50%)	1(25%)	1(25%)

CRO Ceftriaxone, MEM Meropenem, SXT Trimethoprim-Sulfamethoxazole, C Chloramphenicol, CAZ Ceftazidime, CTX Cefotaxime, FEP Cefepime, IPM Imipenem, AM Ampicillin, CIP Ciprofloxacin, TE Tetracycline S Sensitive, R Resistant, I Intermediate, *E. coli Escherichia Coli, N. meningitidis Neisseria meningitides, K. pneumoniae Klebsiella pneumoniae,* (ND) Not done, (−) Nonsucsuptible, N (%), observed value (percentage)

Table 5.5 Antimicrobial susceptibility pattern of Gram-positive bacterial isolates from CSF of patients with bacterial meningitis at UoGCSH, Gondar, Ethiopia, 2023

	***S. pneumoniae* (N=4)**			***S. aureus*(N=2)**		
Antimicrobial Agent	S	I	R	S	I	R
VA	4(100%)	-	-	ND	ND	ND
E	3(75%)	0(0%)	1(25%)	2(100%)	0(0%)	0(0%)
TE	2(50%)	2(50%)	0(0%)	1(50%)	0(0%)	1(50%)
SXT	1(25%)	2(50%)	1(25%)	2(100%)	0(0%)	0(0%)
C	3(75%)	-	1(25%)	0(0%)	0(0%)	2(100%)
GM	ND	ND	ND	2(100%)	0(0%)	0(0%)
RA	2(50%)	1(25%)	1(25%)	0(0%)	0(0%)	2(100%)
CIP	ND	ND	ND	1(50%)	1(50%)	0(0%)

VA Vancomycin, E Erythromycin, TE Tetracycline, SXT Trimethoprim-Sulfamethoxazole, C Chloramphenicol, GM: Gentamicin, RA Rifampin, CIP Ciprofloxacin S Sensitive, R Resistant, I intermediate, *S. pneumoniae Streptococcus pneumoniae*, *S. aureus staphylococcus aureus*, (ND) Not done, (−) Nonsucsuptible, N (%), observed value (percentage)

5.3.4 Molecular detection of pathogens from CSF

All 195 CSF samples from patients with presumptive meningitis were tested for the presence of pathogens using PCR. Of these, 114 samples were found positive, resulting in an overall detection rate of 58.5%. Overall, bacteria were detected in 55 CSF samples (28.2%), which constitute 48.2% of the 114 PCR-detected etiologies. All the culture-positive samples were also positive by PCR (Figure 5.2). Three of the four *K. pneumoniae* culture-positive CSF samples were found to be positive for both *K. pneumoniae* and *E. coli* using PCR.

E. coli was the most commonly detected etiology accounting for 25 (21.9%) followed by *H. influenziae* 9 (7.9%), *S. pneumoniae* 6 (5.3%), *N. meningitidis* 4 (3.5%), *S. aureus* 3 (2.6%), *K. pneumonae* 1 (0.9%), *L. monocytogene* 1 (0.9%) and mixed infections involving both *E. coli* and *K. pneumoniae* 3 (2.6%).

A total of 59 (30.3%) viral etiologies were detected in CSF samples from the patients with presumptive meningitis, which constitute 51.8% of the 114 PCR-identified etiologies.

Enterovirus was the most common, accounting for 53 (46.5%), followed by HSV 5 (4.4%) and EBV 1 (0.9%) (Figure 5.1).

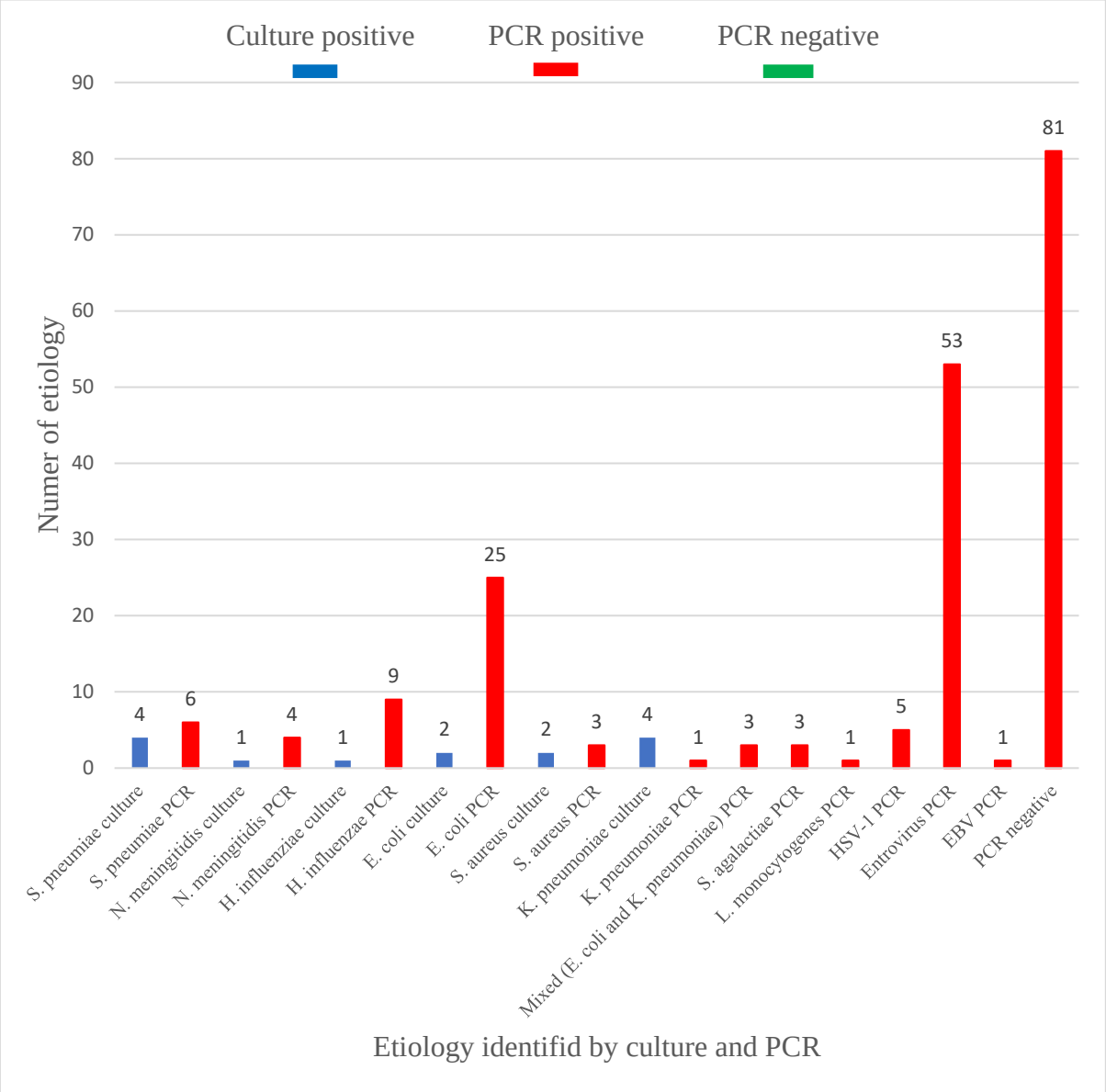

Figure 5.1 Comparison of the frequency of etiology of meningitis identified by culture and PCR at UoGCSH, Gondar, Ethiopia, 2023

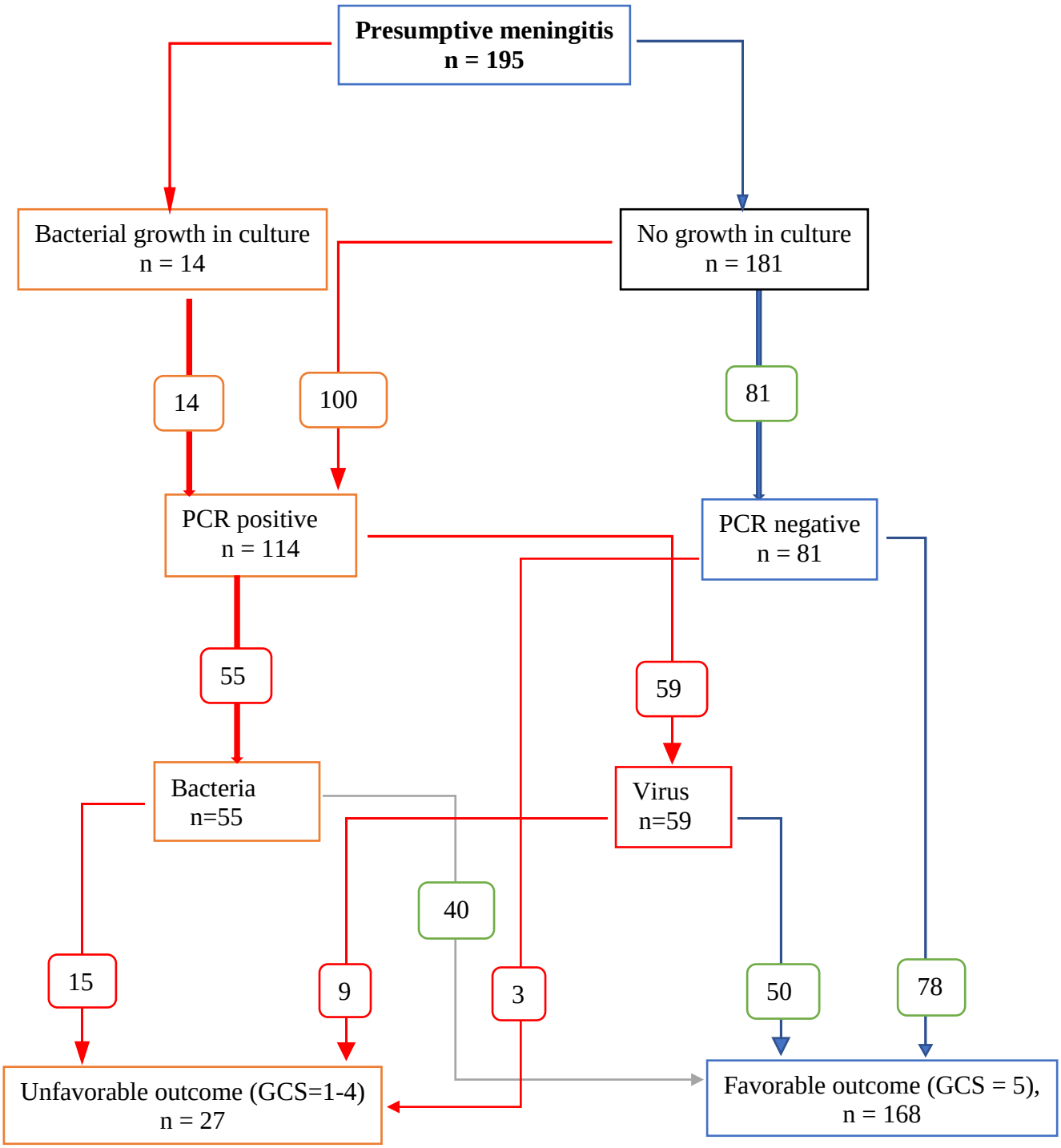

Figure 5.2 Culture and PCR test result and outcome category of presumptive meningitis at UoGCSH, Gondar, Ethiopia, 2023

5.4 Patient outcome and associated factors

The outcome of the 195 patients with presumptive meningitis was assessed using GOS. Accordingly, 27 (13.8%) had unfavorable outcomes (GOS 1-4) [Figure 5.2]. The mortality rate

of presumptive meningitis patients was 8 (4.1%). Three patients left the hospital against medical advice.

The majority of the patients with presumptive meningitis (177, 90.8%) received ceftriaxone therapy irrespective of culture or PCR findings. Most of the patients (175, 89.7%) received more than one antibiotic therapy. Of those 54 (27.7%) received a combination of ceftriaxone and vancomycin. Dexamethasone was given to 79 (40.5%) patients before the antibiotics or at the same time.

In bivariate logistic regression analysis, the following variables were found to be associated with unfavorable outcomes: neck stiffness, patient level of consciousness (GCS), duration of disease onset to hospital visit, comorbidity, history of antibiotics intake before hospital visit, CSF WBC, CSF protein, treatment with adjunctive dexamethasone, and *S. pneumoniae* meningitis (Table 5.6). However, on multivariate analysis, the effect of most of these variables disappeared except for low GCS (AOR = 0.658, 95% CI = 0.524–0.827), comorbidity (AOR = 9.221, 95% CI = 1.580–53.815), and duration of disease onset to hospital visit (AOR = 1.604, 95% CI = 1.146–2.242) that were independently associated with unfavorable outcome (Table 5.7).

Table 5.6 Bivariate analysis of factors associated with unfavorable outcome (GOS<5) at discharge in presumptive meningitis patients at UoGCSH, Gondar, Ethiopia, 2023

Variable		Outcome		Bivariate analysis	
		Favorable	**Unfavorable**	**COR (95% CI)**	***P*-value**
Age (Yrs.), median (IQR)		3.45(1.2-11)	3.50(0.8-11)	1.015(0.986-1.045)	0.326
Sex	Male	105	16	1	0.748
	Female	63	11	1.146(0.500-2.625)	
Neck Stiffness	No	75	6	1	0.034*
	Yes	93	21	2.823(1.084-7.349)	
GCS, mean [a]		14.1(1.6)	11.6(2.7)	0.609(0.506-0.733)	<0.001*
Seizure	No	108	12	1	0.053
	Yes	60	15	2.250(0.989-5.119)	
	Yes	5	3	4.075(0.915-18.16)	
Duration of disease onset to hospital visit in days, mean [b]		2.3(1.2)	3.73(1.9)	1.77(1.36-2.29)	<0.001*
Comorbidity	No	165	22	1	0.001*
	Yes	3	5	12.5(2.792-55.959)	
History of antibiotics intake	No	142	16	1	0.003*
before hospital visit	Yes	26	11	3.755(1.567-9.000)	
CSF WBC, cells/µL median (IQR)[b]		0(0-4)	17(0-1028)	1.003(1.002-1.004)	<0.001*
CSF glucose, mg/dL median (IQR)		52.5(44-66)	49(31-68)	0.981(0.955-1.007)	0.154
CSF protein, mg/dL median (IQR)[b]		32(22-44)	47(35-109)	1.024(1.012-1.035)	<0.001*
Adjunctive dexamethasone	No	105	11	1	0.036*
therapy	Yes	63	16	2.424(1.058-5.553)	
S. pneumoniae meningitis	Other	167	22	1	0.001*
	S. pneumoniae	1	5	37.955(4.24-340.0)	

OR adjusted odds ratio, **CI** confidence interval, **COR** crude odds ratio, **GCS** Glasgow coma scale, **mg/dL** milligram per deciliter, **IQR** interquartile range, **SD** standard deviation, **cells/µL** cells per microliter

* Statistically significant

[a] Odds ratio decreases with a unit increase in the predictor variable

[b] Odds ratio increase for a unit increase in the predictor variable

Table 5.7 Multivariable analysis of factors associated with unfavorable outcome (GOS<5) at discharge in presumptive meningitis patients at UoGCSH, Gondar, Ethiopia, 2023

Variable		Outcome		Multivariable analysis	
		Favorable	**Unfavorable**	**AOR (95% CI)**	***P*-value**
GCS, mean [a]		14.1(1.6)	11.6(2.7)	0.658(0.524-0.827)	<0.001*
Duration of disease onset to hospital visit in days, mean [b]		2.3(1.2)	3.73(1.9)	1.604(1.146-2.242)	0.002*
Comorbidity	No	165	22	1	0.014*
	Yes	3	5	9.221(1.58-53.82)	
CSF WBC (Cells/µL) median (IQR)		0(0-4)	17(0-1028)	1.002(0.988-1.003)	0.033

AOR adjusted odds ratio, **CI** confidence interval, **GCS** Glasgow coma scale, **IQR** interquartile range, **SD** standard deviation, **WBC** white blood cell, **cells/µL** cells per microliter

* Statistically significant

[a] Odds ratio decreases with a unit increase in the predictor variable

[b] Odds ratio increase for a unit increase in the predictor variable

6 DISCUSSIONS

This study aimed to identify the bacterial and viral etiologies of meningitis, characterize bacterial antimicrobial resistance patterns, and assess the outcomes of patients with presumptive meningitis at the UoGCSH, using both culture and PCR methods.

Meningitis is a medical emergency that requires early diagnosis and prompt therapy. To effectively manage the condition, rapid and accurate identification of the causative agent is crucial. However, In Ethiopia, therapy for meningitis is often empirical due to the limited availability of laboratory methods to differentiate bacterial from non-bacterial causes (Assegu Fenta et al., 2020, Barnes et al., 2018). As a result, virtually all patients with presumptive meningitis are treated for BM (Barnes et al., 2018, Wami et al., 2021). In this regard, PCR offers a strong alternative for detecting bacterial and viral meningitis (Zeighami et al., 2021, Barnes et al., 2018).

In this study, the overall prevalence of meningitis among the study participants was 58.5%. This is in agreement with a prior report of 57% from England (Mcgill et al., 2018), 59.6% from Croatia (Panic et al., 2022), and 52.3% from Mozambique (Nhantumbo et al., 2015). Whereas our finding is lower than the previous studies from Egypt, 93.8% (Alnomasy et al., 2021). This difference could be due to the difference in patient selection, as this study focused on presumptive meningitis cases whereas the study in Egypt involved hospitalized patients with abnormal CSF findings. On the other hand, our finding is higher than the previous studies from Germany (32.75%) (Pfefferle et al., 2020), Jimma, Ethiopia (10%) (Barnes et al., 2018), and Ethiopia 33.1% (Mihret et al., 2016). The higher prevalence of the current study may be due to the screening of CSF for many etiologic agents. However, previous studies have not identified more pathogens than the common ones. Additionally, about half of the study participants reside in rural areas with inadequate healthcare facilities, poor hygiene, poor literacy rate, and low socioeconomic status, which may contribute to the higher prevalence of the current study.

Findings from this study imply that the majority of cases had VM (51.8%) while BM constitutes 48.2% of PCR-positive samples. This aligns with previous studies from Colombia 45.5% VM vs. 43.5% BM (Penata et al., 2020) and Jimma, Ethiopia 57% VM vs. 33% BM (Barnes et al., 2018), showing that VM outnumbers BM. In contrast to our finding, a higher number of BM than VM was reported from Egypt: 72.9% BM vs. 27.1% VM (Ahmed et al., 2023) and 50.7% BM vs. 45.3% VM (Alnomasy et al., 2021), and from Germany: 53.7% BM vs. 48.2% VM

(Pfefferle et al., 2020). This could be due to differences in study population and seasonal variability, with viral meningitis, such as enterovirus, being more common in summer.

In this study, there was no sample in which the CSF culture showed bacterial growth and PCR was negative. Conventional methods (gram staining and bacterial culture) detected just 14 meningitis etiologies despite there being 55 PCR-positive CSF samples containing potentially culturable bacteria. These findings align with previous studies from Jimma, Ethiopia (Barnes et al., 2018), Ethiopia (Geteneh et al., 2020), and China (Molyneux et al., 2011). The low recovery rate of bacterial isolates in culture could be attributed to prior antibiotic treatment, or a low bacterial load due to some patients being uncooperative, and a small volume of CSF was collected.

In the present study, the prevalence of BM among presumptive meningitis patients using PCR was 55 (28.2%). This finding aligns with the previous studies from Ethiopia 33.1% (Mihret et al., 2016), China 32% (C. Wang et al., 2023), and Finland 33.8% (Niemela et al., 2023). Our analysis shows a lower prevalence than studies from Lithuania 66.3% (Rynkevic et al., 2024) and Pakistan 88.9%; (Bhatti et al., 2024). The possible explanation could be the presence of predisposing factors, as most of the patients were in the intensive care unit, and immunocompromising conditions, such as pediatric age, which may increase susceptibility to infection. However, our PCR analysis shows a higher prevalence than previous studies in Ethiopia, where the reported prevalence ranged from 1.28% to 13.2% (Assegu Fenta et al., 2020, Awulachew et al., 2020, Barnes et al., 2018, Hibstu et al., 2022, Birhanemeskel Tegene et al., 2017, Birehanemeskel Tegene et al., 2015, Tigabu et al., 2021) and from Iran 7.6% (Saadi et al., 2017) and Kenya 11.2% (Gituro et al., 2017). The difference may stem from the reliance on culture-based methods, which may underestimate the real prevalence, alongside the impact of vaccination reducing targeted pathogens and the subsequent emergence of non-vaccine-preventable pathogens.

The etiology of BM is constantly changing due to the introduction, expansion, and implementation of vaccines and the changing resistance patterns of pathogenic bacteria (Molyneux et al., 2011). In this study, our findings indicate that the common bacterial pathogen in presumptive patients was *E. coli,* which constitutes 25 (21.9%) of the identified pathogen. This was consistent with the study conducted in China (Jiang et al., 2017, Wu et al., 2023, C. Wang et al., 2023), and Korea (Rhie et al., 2018), which reported *E. coli* was the most common

cause of meningitis. However, our analysis contradicts findings from other studies in Ethiopia and other countries, which have reported *S. pneumoniae* as the predominant pathogen (Awulachew et al., 2020, Birhanemeskel Tegene et al., 2017, Block et al., 2022). The factors that influence *E. coli* to be more dominant than the others could be due to the reduction of *S. pneumoniae* meningitis due to vaccination for age, which is supported by about 60% of the participants aged less than five years.

Our analysis showed *H. influenzae* was the second most common causative agent of bacterial meningitis, with 9 (7.9%) of identified etiologies. This aligns with studies from UoGCSH 10% (Birehanemeskel Tegene et al., 2015) and 10.5% (Birhanemeskel Tegene et al., 2017), and Dilla, Ethiopia, 10.5% (Awulachew et al., 2020).

In this study, a total of 59 (30.3%) viral etiologies were detected in CSF samples from patients with presumptive meningitis. This is in agreement with the previous studies from Ethiopia, 26.7% (Wami et al., 2021), and China, 28.4% (L. P. Wang et al., 2022). However, the result of our analysis was lower than the previous studies from China 42.8% (Ai et al., 2017), Iran 46.2% (Hosseininasab et al., 2011), and Korea 63.3 (Song et al., 2024). The possible reason for this could be that this study focused on presumptive meningitis, whereas others targeted presumptive viral meningitis cases. On the other hand, our analysis shows a higher prevalence than studies from Ethiopia 12.8% (Geteneh et al., 2021), and Jimma, Ethiopia 5.5% (Barnes et al., 2018). This may be due to differences in the study population, study design, and the season of case occurrence with viral meningitis. The study from Ethiopia utilized repository samples, where the genetic material may have been compromised by storage conditions.

Enteroviruses were the most common viruses, accounting for 53 (46.5%) of the identified etiology. This finding aligns with the well-established understanding that EVs are the most detected pathogen in presumptive meningitis patients (Tarai and Das, 2019, Barnes et al., 2018, L. P. Wang et al., 2022, Ai et al., 2017).

The major challenge faced by the current healthcare system is the continuous rise of antibiotic-resistant bacteria (Assegu Fenta et al., 2020, Awulachew et al., 2020, Hibstu et al., 2022). Among gram-negative isolates, *E. coli* exhibited 50% resistance to ceftazidime, tetracycline, ciprofloxacin, chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin. These results are consistent with a study conducted in Ethiopia, which reported 40% resistance to ciprofloxacin and 50% to chloramphenicol (Tigabu et al., 2021), 40% resistance to tetracycline

(Birehanemeskel 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 our findings, lower levels of resistance were reported from Pakistan ceftazidime 17.5%, ciprofloxacin 30% (Ali et al., 2021), and UoGCSH chloramphenicol 10% (Birehanemeskel Tegene et al., 2015).

K. pneumoniae isolates were 50% resistant against meropenem, cefotaxime, and ciprofloxacin. In agreement with our findings, *K. pneumoniae* resistance was reported against meropenem 41.2%, and ciprofloxacin 41% from Pakistan (Ali et al., 2021) and from Debre Markos 100% to cefotaxime (Hibstu et al., 2022). The isolates were also 25% resistant to cefepime, ceftriaxone, and ceftazidime. In contrast to our finding, higher levels of resistance were reported from Debre Markos 100% to cefotaxime (Hibstu et al., 2022), and from Pakistan 64.7 to ceftazidime (Ali et al., 2021). The emerging resistance in *K. pneumoniae* was due to its ability to produce an enzyme that degrades the carbapenem and beta-lactam ring structures (Belay et al., 2024). This high resistance rate to B-lactam antibiotics among *K. pneumonia* and *E. coli* in this study is suggestive of the presence of extended spectrum-lactamases (ESBLs)-producing strains. Therefore, the use of carbapenems, imipenem in particular, for the treatment of meningitis caused by *K. pneumoniae* and *E. coli* may be helpful.

Among the gram-positive isolates, *S. pneumoniae* showed 25% resistance to erythromycin, trimethoprim-sulphamethoxazole, ciprofloxacin, and rifampin, and 100% sensitive to vancomycin. This is consistent with the previous studies in the study area with a resistance rate of chloramphenicol 22.2%, erythromycin 20%, and 87% sensitivity to vancomycin (Tigabu et al., 2021). Also, rifampicin resistance was 23% in Dilla University Referral Hospital, Ethiopia (Awulachew et al., 2020). The 100% resistance rates of *S. aureus* towards chloramphenicol in our study were comparable to the study from UoGCSH (Tigabu et al., 2021) and Debre Markos, Ethiopia (Hibstu et al., 2022), which reported 100% chloramphenicol resistance. Possible explanations for the high resistance could be due to exposure to a broad range of antibiotics, self-medication, and an incomplete dosage of antibiotics, leading *S. aureus* to develop new cellular processes to evade the effects of drugs (Belay et al., 2024). Even though AMR is a natural process, the overuse and misuse of antibiotics accelerate the emergence of drug resistance in pathogens (Belay et al., 2024).

The overall outcome of presumptive meningitis patients on leaving the hospital revealed that 13.8% had unfavorable outcomes (GOS 1-4). This is in agreement with the studies in Felege

Hiwot Referral Hospital, Ethiopia, 15% (Tewabe et al., 2018) and Dilla, Ethiopia, 23.3% (Sileshi Elias et al., 2021). In contrast to the present study, a higher number of unfavorable outcomes (36.7%) were reported from Jimma, Ethiopia (Gudina et al., 2016b, Gudina et al., 2018) and Bedele General Hospital, Ethiopia 32.6% (Bekele et al., 2021). This may be due to the study population and the etiology being isolated, as *S. pneumoniae* and *N. meningitidis* are the most common etiologies associated with unfavorable patient outcomes.

In the present study, low GCS (AOR = 0.658, 95% CI = 0.524–0.827) was one of the factors independently associated with unfavorable outcomes in multivariate logistic regression analysis after adjusting for potential confounders. This indicates that for each unit increase in the GCS score, the odds of the unfavorable outcome occurring decrease by approximately 34.2% (1-0.658). This is consistent with the study conducted in Jimma, Ethiopia, which showed low GCS (AOR = 0.766, 95% CI = 0.589–0.995) (Gudina et al., 2018) and the Netherlands (Van De Beek et al., 2004). This could be explained by the fact that prolonged altered consciousness increases the risk of neurological impairments, negatively impacting patient outcomes. Low GCS at admission may reflect complications from meningeal infection, possibly resulting from delayed hospital presentation.

Duration of disease onset to hospital visit (AOR = 1.604, 95% CI = 1.146–2.242) was also the factor independently associated with unfavorable outcomes. This means late presentation to the hospital by one day, 1.6 times more likely to develop unfavorable outcomes. Our analysis aligns with the study from Hiwot Fana Specialized University Hospital, Ethiopia (AOR 3.74, 95% CI 1.76–7.98) (Adem et al., 2020), Denmark (Koster-Rasmussen et al., 2008) and England (Mcgill et al., 2018). Reasons for hospital presentation delays may result from patient and healthcare-related factors, such as poor health-seeking behavior, limited access, misdiagnosis at primary care, and inadequate referral systems. This delay decreases pathogen yield and complicates case management, which may consequently contribute to unfavorable patient outcomes.

Comorbidity (AOR = 9.221, 95% CI = 1.580–53.815) was also another factor independently associated with unfavorable outcomes. This indicates that individuals with comorbidity are approximately 9.2 times more likely to experience unfavorable outcomes. The wide range of CI indicates variability, suggesting that while there is a strong association, there may be substantial uncertainty regarding the exact magnitude of the effect. This is consistent with the studies from Bedele General Hospital, Ethiopia (Bekele et al., 2021) and Denmark (Hovmand et al., 2023).

Patients with two or more diseases experienced worse outcomes. This can be attributed to the potential for drug-drug interactions resulting from the prescription of multiple medications to address their co-morbidities, which are likely to result in unfavorable outcomes.

In this study, 40.5% of patients were given dexamethasone by their attending physicians. Even though its administration was associated with unfavorable outcomes in bivariate analysis (AOR = 2.424, 95% CI = 1.058–5.553), the effects disappeared in multivariate analysis. This implies that the use of dexamethasone as adjunctive treatment in presumptive meningitis patients does not confer any benefit or potential harm. This is most likely due to the delayed presentation of patients, by which time the inflammatory process in the CNS has already started. However, the finding was inconsistent with Ethiopian studies associating dexamethasone use with unfavorable patient outcomes (Gudina et al., 2018, Gudina et al., 2016b, Tewabe et al., 2018) while others associated it with favorable outcomes (Bekele et al., 2021, Rahimi et al., 2022). This is because the corticosteroid dexamethasone is used as adjuvant treatment in meningitis to attenuate host inflammatory responses due to microbial invasion, which subsequently reduces neuronal death and brain damage.

Limitations of the study

This study was conducted at a single center, and serotypes were not identified. Furthermore, it did not include long-term follow-up, as outcomes were assessed only upon hospital discharge, which may limit the generalizability of real patient outcomes.

7 CONCLUSION AND RECOMMENDATIONS

7.1 Conclusions

In the present study, a considerably high prevalence of meningitis was detected. Relying solely on bacterial culture significantly underestimates the true prevalence, as molecular diagnostic methods demonstrated superior sensitivity in identifying microbial etiologies, including pathogens undetected by culture. Viral meningitis was more prevalent than bacterial meningitis, despite most presumptive meningitis patients receiving empirical treatment for bacterial infections. *E. coli* and enteroviruses were identified as the common bacterial and viral etiology of meningitis, respectively. High rates of antibiotic resistance among gram-negative bacterial isolates were observed.

Most study participants experienced favorable outcomes; however, unfavorable outcomes were associated with factors such as low GCS, the presence of comorbidity, and delayed presentation to healthcare facilities. The use of dexamethasone as adjunctive treatment in presumptive meningitis patients does not confer benefit or harm.

7.2 Recommendations

Based on the findings of our study we have made the following recommendations:

- Integration of PCR into routine laboratory procedures should be considered to enhance the detection of meningitis-causing pathogens.
- Patients with low GCS and comorbidities should receive special attention for better outcomes.
- Large-scale, multicenter studies are required to evaluate the association of adjunctive dexamethasone therapy and presumptive meningitis patient outcomes.

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ANNEXES

Annex I: Information sheet (English version)

My name is Derso Wale, and I am a second-year MSc student at Addis Ababa University Department of Medical Microbiology, Immunology, and Parasitology. I am here to research microbial profile, antibiotic resistance profile, and treatment and adjunctive therapy outcomes in meningitis-suspected patients at UoGCSH for the partial fulfillment of my MSc degree.

The objective of this study is to assess the prevalence, antimicrobial resistance profile, and evaluation of treatment and adjunctive therapy outcomes of meningitis in Gondar University Hospital, Gondar, North West Ethiopia. For this, we will take about 3 ml of CSF. There is no direct benefit you obtained from this study, but indirectly the result of the study will have importance to treating the patients and to use as a baseline for effective treatment in the absence of laboratory investigation. So, your participation is important for this research. The risk of this project is that you feel mild pain while samples are collected, except that there will be no severe risk to participating in the study.

The coding method will be applied in all the procedures; a code will be given to the questionnaire form and also to the CSF sample; the same code will be used for both the sample and laboratory result; and data will be analyzed accordingly so your results will be kept confidential and no one knows your result.

There is nothing you will lose if you are not willing to participate in the study or stop filling out the questionnaire in between, so it is your right to participate or not to participate in the study.

Would you be willing to participate?

Yes, I want to participate in the study. ☐ Please go to the next page.

No, I don't want to participate in the study. ☐ Thank you very much!

If you need further information or if you have related questions about this study, you can contact Addis Ababa University, School of Medicine, Department of Medical Microbiology, Immunology, and Parasitology, or the investigator using the following address:

Investigator: Derso Wale

Phone: 0918103887

E-mail: dersowale2799@gmail.com

Annex II: በምርምሩ ለመሳተፍ የስምምነት ማሳወቂያ (Amharic version)

ስሜ ደርሶ ዋለ ሲሆን በአዲስ አበባ ዩኒቨርስቲ የሁለተኛ አመት የማስተርስ ድግሪ የህክምና ማክሮባይሎጅይ ተማሪ ነኝ። ማጅራት ገትር በሽታ ስርጭት፤ መድሃኒቶችን መላመድ እና የህክምና ዉጤታቸው በሚል ርዕስ ላይ ለማስተርስ ዲግሪ የመመረቂያ ፅሑፍ እየሰራሁ እገኛለሁ።

የዚህ ጥናት አላማም የማጅራት ገትር በሽታ ስርጭት፤ መድሃኒቶችን መላመድ እና የህክምና ዉጤታቸውን ማወቅ ነው ።ለዚህም 3 ሚ.ሊ ህብረ ሰረሰር ናሙና ይወሰዳል። ከጥናቱ በቀጥታ የሚያገኙት ጥቅም የለም። ነገር ግን በተዘዋዋሪ መንገድ የጥናቱ ውጤት የህብረ ሰረሰር የቤተ ሙከራ ምርመራ ባልተደረገበት ጊዜ ውጤታማ ሕክምና ለማግኘት እንደ መሠረት ለመጠቀም ጥቅም ላይ የሚዉል ይሆናል። ስለዚህ የእርሶ ተሳትፎ ለዚህ ምርምር ጠቃሚ ነው። በዚህ ምርምር መሳተፍ ናሙናው ሲወሰድ ካለዉ መካከለኛ የህመም ስሜት ዉጭ እምብዛም የጎንዮሽ ጉዳት አይኖረውም። ለሁሉም የአሰራር ቅደም ተከተል የሚስጢር መለያ ቁጥር አሰራር ዘዴ እንጠቀማለን። ለመጠይቁም ሆነ ለህብረ ሰረሰር ናሙናው ሚስጢር ቁጥር እንጠቀማለን። የህብረ ሰረሰር ናሙናው እና የላብራቶሪ ውጤቱ ተመሳሳይ ሚስጢር ቁጥር ይኖራቸዋል። በመሆኑም የእርስዎ ውጤት ሚስጥራዊነቱ እንደተጠበቀ ነው። በጥናቱ በፍቃደኝነት አለመሳተፍ ምንም የሚያሳጣው ነገር አይኖርም። ስለዚህ በጥናቱ መሳተፍም ሆነ አለመሳተፍ የእርሶ መብት ነው። ከጥናቱ ጋር የተያያዘ ጥያቄ ካለዎት ወይም ተጨማሪ መረጃ ከፈለጉ በሚከተለው አድራሻ እኛን ማግኘት ይችላሉ።

የጥናት አድራጊው ስም፡- ደርሶ ዋለ

ስ.ቁ፡- 0918103887 ኢሜል፡- dersowale2799@gmail.com

Annex III: Consent form (English version)

Code no_____

Information about the study has been explained to me by the investigator. I have understood that the objective of this study is to assess the microbial profile, antimicrobial resistance, and evaluation of treatment and adjunctive therapy outcomes in meningitis, and the small amount of CSF that I will give will not hurt my health. It has also been explained to me that I have the right to stop participation at any time in between, and there is nothing I will lose if I refuse to participate. I agree to participate in the study and hereby approve my agreement with my signature.

Participant signature: _____Date_____

Investigator signature: _____Date_____

Wetness signature: _____Date_____

_____ Date _____

_____ Date _____

Annex IV: Accent form (English version)

Code no_____

Information about the study has been explained to me by the investigator. I understood that the objective of this study is to assess the microbial profile, antimicrobial resistance, and evaluation of treatment and adjunctive therapy outcomes in meningitis, and the small amount of CSF that my son/daughter will give will not hurt his/her health. The investigator has also explained to me that I have the right to refuse participation at any time in between, and there is nothing he/she will lose if I refuse to participate on my behalf. I agree on behalf of the son/daughter to participate in the study and hereby approve my agreement with my signature.

Guardians' signature: _____Date_____

Investigator signature: _____ Date_____

Wetness signature: _____ Date_____

_____ Date_____

Annex V: በምርምር ለመሳተፍ የፍቃደኝነት መዋዋያ ቅፅ (Amharic version)

መለያ ቁጥር _____

ጥናቱን በሚያካሂዱት ሰዎች ስለጥናቱ በቂ መረጃ ተሰጥቶኛል። የዚህ ጥናት አላማም _____ከኔ የሚወሰደዉ _____ በኔ ላይ ምንም አይነት የጤና ጉዳት የማያስከትል መሆኑን ተረድቻለሁ።እንዲሁም በጥናቱ ለመሳተፍ ፈቃደኛ ካለሆንኩ በጥናቱ ለመሳተፍ እንደማልገደድ ነገር ግን በዚህ ጥናት በመሳተፌ ለሳይንሳዊ እዉቀት ጠቃሚ መረጃ ማበርከትና ወደፊት በዚህ ዙሪያ ለሚሰሩ ስራዎች መሰረት የሚሆኑ መረጃዎችን መስጠት እንደምችል ተረድቻለሁ። በመሆኑም በዚህ ጥናት ላይ ለመሳተፍ የተስማማሁ መሆኔን በፊርማዬ አረጋግጣለሁ።

የተሳታፊ ፊርማ _____ ቀን _____

የጥናት አድራጊ ፊርማ _____ ቀን _____

የእማኝ ፊርማ_____ ቀን _____

_____ ቀን _____

_____ ቀን _____

Annex VI: Questionnaire (English version)

Identification No: _____ Hospital Card Number: _____

Date of Interview: ____/____/_____

Part 1: Socio-demographic characteristics			
No	Question	Response	
101	Age	_____year In months (if <12 months) _____	
102	Sex	☐ Male	☐ Female
103	Residence	☐ Urban	☐ Rural
Part 2: clinical characteristics			
201	Body T^{O} _____^{O}C		
202	Headache	☐ Yes	No ☐
203	Nacke stiffness	☐ Yes	No ☐
204	GCS _____		
205	Seizures	☐ Yes	No ☐
206	Kerning's sign	☐ Yes	No ☐
207	Brudzinski sign	☐ Yes	No ☐
208	Rash	☐ Yes	No ☐
209	Photophobia	☐ Yes	No ☐
210	Phonophobia	☐ Yes	No ☐
211	Vomiting	☐ Yes	No ☐
212	Reduced ability to suck for breastfeeding	☐ Yes	No ☐
213	Comorbidity	☐ Yes Specify_____	No ☐

214	Meningitis vaccination status	☐ vaccinated	☐ Not vaccinated
215	History of antibiotics intake before hospital visit	☐ Yes	No ☐
216	Pre-hospital illness duration _____ in days		

Part 3: Laboratory results

A: CSF sample

301	CSF Appearance	☐ Clear ☐ Turbid ☐ Hematic(blood-stained) ☐ Xanthochromic (yellowish)				
302	Gram-stain	☐ GPD ☐ GND ☐ GPB ☐ GNB ☐ Negative ☐ other specify_____				
303	Indian ink	☐Fungal elements are seen ☐No fungal element seen				
304	CSF cell Count	Total WBC_____				
		Neut _____				
		Lymp _____				
305	CSF Biochemistry	CSF protein _____				
		CSF glucose _____				
306	Culture Result	☐*N. meningitides* ☐*S. pneumonia* ☐*H. influenzae* ☐ GBS ☐*E. coli* ☐*S. aureus* ☐*K. pneumonia* ☐Other specify: _____ ☐No growth				
307	AST	Ceftriaxone	☐ S	☐ R	☐ I	☐Not done
		Penicillin G	☐ S	☐ R	☐ I	☐Not done
		Oxacillin	☐ S	☐ R	☐ I	☐Not done
		Vancomycin	☐ S	☐ R	☐ I	☐Not done

		Moxifloxacin	☐ S	☐ R	☐ I	☐Not done
		Ampicillin	☐ S	☐ R	☐ I	☐Not done
		Cefepime	☐ S	☐ R	☐ I	☐Not done
		Meropenem	☐ S	☐ R	☐ I	☐Not done
		Ceftazidime	☐ S	☐ R	☐ I	☐Not done
		Cotrimoxazole	☐ S	☐ R	☐ I	☐Not done
308	PCR result	☐Nm ☐*S. pneumonia* ☐*H. influenzae* ☐GBS ☐Enterovirus ☐Herpes simplex virus 1 ☐Herpes simplex virus 2 ☐Other pathogens (specify): _____ ☐Negative				
B: Blood sample						
309	WBC	TLC_____ Diff Neut_____ Lymp _____ RBC_____ Hemoglobin_____ Plateletes _____				
310	Blood chemistry	Blood glucose_____ Serum protein_____				
311	Blood culture result	☐*N. meningitides* ☐*S. pneumonia* ☐*H. influenzae* ☐ GBS ☐*E. coli* ☐*S. aureus* ☐*K. pneumonia* ☐Other specify: _____				

		☐No growth				
312	AST	Ceftriaxone	☐ S	☐ R	☐ I	☐Not done
		Penicillin G	☐ S	☐ R	☐ I	☐Not done
		Oxacillin	☐ S	☐ R	☐ I	☐Not done
		Vancomycin	☐ S	☐ R	☐ I	☐Not done
		Moxifloxacin	☐ S	☐ R	☐ I	☐Not done
		Ampicillin	☐ S	☐ R	☐ I	☐Not done
		Cefepime	☐ S	☐ R	☐ I	☐Not done
		Meropenem	☐ S	☐ R	☐ I	☐Not done
		Ceftazidime	☐ S	☐ R	☐ I	☐Not done
		Cotrimoxazole	☐ S	☐ R	☐ I	☐Not done
C: Throat swab sample						
313	PCR result	☐*N. meningitides* ☐*S. pneumonia* ☐*H. influenzae* ☐GBS ☐Enterovirus ☐Herpes simplex virus 1 ☐Other pathogens (specify): _____ ☐Negative				
Part 4: Treatment and Adjunctive Therapy Outcomes						
401	Treatment given	☐ Ceftriaxone ☐Vancomycin ☐ Meropenem ☐ Ceftazidime ☐ Ampicillin				

		☐ Cefepime ☐ Penicillin G ☐ Other specify_____
402	Adjunctive therapy	☐ Yes ☐ No If yes ☐ Dexamethasone ☐ Other specify_____
403	LHS	_____days
404	GOS at discharge	☐ 1. Death ☐ 2. Persistent vegetative state ☐ 3. Sever disability ☐ 4. Moderate Disability ☐ 5. Good recovery

Annex VII: የመረጃ መጠየቂያ ቅፅ (Amharic Version)

ክፍል1፡ ማህበራዊ ሁኔታ			
ተ.ቁ	ጥያቄ	መልስ	
101	እድሜ	_____ በአመት በወራት (ከ12 ወራት በታች ሲሆኑ) _____	
102	ፆታ	☐ ወንድ	☐ ሴት
103	መኖርያ	☐ ከተማ	☐ ገጠር
ኪፍል 2: ክሊንካል			
201	ሙቀት _____^{O}C		
202	ራስ ምታት አለወት	☐ አዎ	አይ ☐
203	የአንገት ህመም	☐ አዎ	አይ ☐
104	ራስን መቆጣጠር አለመቻል	☐ አዎ	አይ ☐
205	የሚጥል	☐ አዎ	አይ ☐
206	የከርኒግስ ሚልኪት	☐ አዎ	አይ ☐
207	የብሩድዘንሲኪ ምልክት	☐ አዎ	አይ ☐
208	የቆዳ መላላጥ	☐ አዎ	አይ ☐
209	ብርሃን መፍራት	☐ አዎ	አይ ☐
210	ድምጥ መፍራት	☐ አዎ	አይ ☐
211	ማስታወክ ለህጣናት	☐ አዎ	አይ ☐
212	ጡት መጥባት አለመቻል	☐ አዎ	አይ ☐
213	የማጅራት ገትር ክትባት	☐ ተከትቢያለሁ	☐ አልተከተብሁም
214	ምልኪቱ ከጀመርዎ ስንት ጊዜ ሂነው _____ በቀን		
ክፍል 3: የላቦራቶሪ ውጤት			

301	ህብረ ሰረሰር ናሙናዉ በአይን ሲታይ ሲታይ	☐ ንጥህ ☐ ድፍርስ ☐ደም የተቀለቀለበት ☐ዛንቶክሮሚክ (ቢጫ)				
302	በግራም ስቴይን ሲቀለም	☐GPD ☐GND ☐GPB ☐GNB ☐Negative ☐other				
303	በ ኤንዲያን ኢንክ ሲቀለም	☐የፈንገስ ዉጤቶች አሉ ☐የለም				
304	የህብረ ሰረሰር የህዋስ ቁጥር	የነጭ ደም ሴል ብዛት_____				
		የኒውትሮፊል ብዛት_____				
		የሊይምፕስይት ብዛት_____				
305	የህብረ ሰረሰር ንጥረ ነገሮች	ፕሮቲን _____				
		ግሉኮስ _____				
306	በማሳደጊያ እና በመለታ የተለዩ ተህዋስያን	☐N. meningitides ☐*S. pneumonia* ☐*H. influenzae* ☐ group B Streptococci ☐*E. coli* ☐*S.aureus* ☐*K. pneumonia* ☐ሌላ ካለ ጥቅስ: _____ ☐የተገኘ የለም				
307	መድሃኒት የመላመድ ሚርመራ	Ceftriaxone	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
		Penicillin G	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
		Oxacillin	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
		Vancomycin	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
		Moxifloxacin	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
		Ampicillin	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
		Cefepime	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
		Meropenem	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
		Ceftazidime	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም

		Cotrimoxazole	☐አልተላመደም	☐ ተላምድዋል	☐መካከለኛ	☐አልተሰራም
308	የፒሲር ሚርመራ ዉጤት	☐NmA ☐NmC ☐NmW ☐NmY ☐NmB ☐NmX ☐*S. pneumonia* ☐Hib ☐*H. influenzae* Indeterminate ☐Strep B ☐Entrovirus ☐Herpes simplex virus ☐C. neoforman ☐ሌላ ካለ ጥቀስ_____ ☐የለም				

ክፍል 4: ህክም እናየህክም ዉጤት ግምገማ		
401	የሚዎስደው መድሃኒት	☐ Ceftriaxone ☐Vancomycin ☐ Meropenem ☐ Ceftazidime ☐ Ampicillin ☐ Cefepime ☐ Penicillin G ☐ Other specify_____
402	አድጀንቲቭ ህክምና	☐ አወ ☐ አይ አዎ ከሆነ ☐ Dexamethasone ☐ ሌላ ከሆነ ጠቀስ_____
403	ለስንት ቀን ህክምናዉን ሲከታተል ቆየ	_____በቀናት
404	ከተቁዋሙ ሲወጣ የነበረበት ሁነታ	☐ 1. አርፉዋል ☐ 2. በከፍተኛ ህመም ሰመመን ዉስጥ ☐ 3. በከፍተኛ ህመም ☐ 4. በመካከለኛ ህመም ☐ 5. በጥሩ ሁኔታ ድንዋል/ለች

Annex VIII: Laboratory procedures

I. Lumbar puncture procedure

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

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

3. Label the collection tubes with the 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 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 it 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 of CSF is available, use 3 separate tubes and place approximately 1 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 the mask and gloves and discard them 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.

II. Gram staining procedure

1. Label the slides clearly with the date and ID number.

2. Making smears by spreading evenly and covering an area on a slide.

3. After drying the smears, the slide should be left in a safe place to air-dry, protected from flies and dust.

4. Fix the dried smear by using methanol alcohol.

5. Cover the fixed smear with the crystal violet stain for 1 minute.

6. Rapidly wash off the stain with clean water.

7. Cover the smear with lugol's iodine for 1 minute.

8. Wash off the iodine with clean water.

9. Decolorize rapidly (5 seconds) with 3% acetone alcohol. Wash immediately with clean water.

10. Cover the smear with safranin for 1 minute.

11. Wash off the stain with clean water.

12. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry.

13. Examine the smear microscopically, first with the 40 X objective to check the staining and to see the distribution of materials, and then with the oil immersion objective to look for bacteria and cells.

Result

- Gram-positive bacteria: dark purple
- Gram-negative bacteria: pale to dark red

III. Culture media preparation

Materials and Equipment used

- Bio-safety cabinet
- Culture tube, Inoculating loop, Straight wire
- Benson burner
- Culture media

- Incubator, Refrigerator
- Autoclave
- PH meter
- Flask
- Measuring cylinder, Aluminum foil
- Boiler, Balance, and Petri dish

1. Weighing and dissolving of culture media: weighing is based on the manufacturer's direction and then multiplies or reduces based on the amount of media that we need. Use completely clean glassware, plastic, or stainless-steel equipment that has been rinsed in pure water. Add the powdered ingredients to the distilled water. Dissolving is mediated by mixing very well by rotating until all the powders are mixed and finally boiling to get a homogenous solution.

2. Sterilization: most culture media are sterilized by being autoclaved. It is performed by placing it in an autoclave at 121 ºC for 15 minutes. This ensures the destruction of bacterial endospores as well as vegetative cells. It is important to sterilize a medium at the correct temperature and for the correct length of time as instructed in the method of preparation.

3. Add heat-susceptible chemicals or substances, such as blood in the case of blood agar, and certain antibiotic supplements.

4. PH testing of culture media: the PH of most culture media is near neutral. An exception is alkaline peptone water. The simplest way of testing the PH of a culture medium is to use narrow-range pH paper. It can be tested by dipping a narrow range of PH paper into a sample medium when it is at room temperature before dispensing and comparing the color of the paper against the PH color chart.

5. Dispensing of culture media: before this, we have to cool the media based on the manufacturer's direction, but in the case of agar media, we have to cool a temperature of between 45-50 ºC because agar in nature is a solidifying agent, so below this temperature will solidify the media before dispensing. Dispensing should be done until it covers the surface of the dish on a flat and sterile surface to get uniform depth at room temperature. Wait 10-15 minutes until it solidifies.

6. Quality control test of culture media: sterility and performance test. A performance test is used to check the quality of culture media prepared (whether it can be used to perform the intended test) using control organisms. We have to inoculate the control organism that grows and does not grow on the prepared media and check whether it performs its actual activity. Incubate 4-5% of prepared media for sterility testing overnight on the incubator; if there is growth, we have to discard the batch.

7. Labeling and Storage of Culture Media: All culture media must be clearly labeled with their name, preparation, and expiration date. Plates of culture media should be stored at 2-8 ºC, preferably in sealed plastic bags to prevent loss of moisture. When in use, the media must be protected from direct light, especially sunlight.

Inoculation

Immediately before inoculating a culture medium, check the medium for visual contamination or any change in its appearance that may indicate deterioration of the medium, e.g., darkening in color. When inoculating, or seeding, culture media, an aseptic (sterile) technique must be used. This will prevent contamination of cultures and specimens.

Prevent infection of the laboratory worker and the environment.

Inoculation is the next step in the culturing of samples, where placing the specimen on appropriate culture media takes place. The most common culture media used for the inoculation of CSF samples include MacConkey, chocolate, and blood agar.

After inoculation of the sample in the appropriate media

1. Decontaminate the workbench before starting the day's work and after finishing.

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

Incubation

Following inoculation, incubation will be performed, because after placing the sample on the appropriate media, we have to create a suitable environment for the growth of pathogenic organisms. The most common suitable environment for pathogenic bacteria is the environment that resembles the human body, so we have to incubate in an incubator at 37 ºC overnight in the aerobic or microaerophilic environment according to the bacterial species expected.

IV. Biochemical testing procedures

Identification of Gram-positive bacteria:

Gram-positive cocci will be identified based on their Gram reaction, catalase, Mannitol salt agar, and coagulase test results.

Catalase test: This test is used to differentiate those bacteria that produce the enzyme catalase, such as *staphylococci* (positive), from non-catalase-producing bacteria such as *streptococc*i. Catalase acts as a catalyst in the breakdown of hydrogen peroxide into oxygen and water.

Procedure

1. Add 2–3 ml of hydrogen peroxide solution into a slide.
2. Using a sterile wooden stick, pick colonies of the test organism.
3. Mix with hydrogen peroxide solution, then look for bubbling.
4. Interpretation active bubbling--positive test

No release of bubbles-negative test

Coagulase test: This test is used to differentiate *S. aureus,* which produces the enzyme coagulase, from another *Staphylococcus* spps. Coagulase, causes plasma to clot by converting fibrinogen to fibrin.

Procedure

1. Place a drop of physiological saline on two separate slides.
2. Emulsify the test organism in each of the drops to make a thick suspension.
3. Add one drop of plasma to one of the suspensions and mix gently. Look for clumping of the organism within 10 seconds.

4. Interpretation

Clumping within 10 seconds: *S. aureus*

No clumping within 10 seconds: other *staphylococcus* species

Mannitol salt agar test principle

Enzymatic Digest of Casein, Enzymatic Digest of Animal Tissue, and Beef Extract provide the nitrogen, vitamins, and carbon in Manitol Salt Agar. D-manitol is the carbohydrate source. In high concentrations, sodium chloride inhibits most bacteria other than *Staphylococcus*. Phenol red is the pH indicator. Agar is the solidifying agent. Bacteria that grow in the presence of a high salt concentration and ferment mannitol produce acid products, turning the phenol red pH indicator from red to yellow.

Interpretation; Yellow colonies with yellow zones: ferments mannitol

-Red colonies with no yellow zones: do not ferment mannitol.

Procedure Inoculate specimens on the medium as primary isolation or inoculate isolated colonies onto the medium for differentiation. Incubate at 37ºC for 24 hours. Look for colony morphology.

Result-Positive: yellow colony and may have a yellow halo around the colony.

Negative: no growth of bacteria (*E. coli*) or growth with colorless or pink colonies (CONs)

Identification of Gram-negative bacteria: based on their test result with a series of biochemical tests.

Indole test: To detect the ability of an organism to degrade the amino acid tryptophan and produce indole. It is important in the identification of *Enterobacteriaceae*.

Principle:

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

Procedure:

The bacteria to be tested are inoculated in peptone water, which contains the amino acid tryptophan, and incubated overnight at 37 ºC. Following incubation, a few drops of Kovac's reagent are added. Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol, and concentrated HCl. Ehrlich's reagent is more sensitive in detecting indole production in anaerobes and non-fermenters. The formation of a red or pink colored ring at the top is taken as positive.

Triple sugar iron agar

Triple Sugar Iron Agar (TSI) is used to determine if bacteria can ferment glucose and/or lactose and if it can produce hydrogen sulfide or other gases. In addition, TSI detects the ability to ferment sucrose. These characteristics help distinguish various *Enterobacteriaceae*.

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

TSI contains three sugars: glucose, lactose, and sucrose. Lactose and sucrose occur within 10 times the concentration of glucose (1.0% versus 0.1%). Phenol red (a pH indicator that is yellow below pH 6.8 and red above it). A yellow slant on TSI indicates the organism ferments sucrose and/or lactose. A yellow butt shows that the organism fermented glucose. The black precipitate in the butt indicates hydrogen sulfide production. Production of gases other than hydrogen sulfide is indicated either by cracks or bubbles in the media or the media being pushed away from the bottom of the tube. Hydrogen sulfide (H2S) is produced by bacterial anaerobic degradation of the two sulfur-containing amino acids, cysteine and methionine. Hydrogen sulfide is released as a by-product when carbon and nitrogen atoms in the amino acids are consumed as nutrients by the cells. Under anaerobic conditions, the sulfhydryl (-SH) group on cysteine is reduced by cysteine desulfurase. The agar contains high levels of peptones (sources of cysteine and methionine) and ferrous sulfate as an indicator. When H2S is produced, the ferrous ion reacts with it to give ferrous sulfide, an insoluble black precipitate.

Procedure

1. The triple sugar iron agar slant was inoculated by stabbing the butt and drawing the stick over the surface of the slope.

2. Incubated at 35-37^{o}C for 18 to 24 hours.

3. Looked for black precipitate formed.

Result

- Acid deep (yellow)/alkaline slant (red): glucose fermented, lactose, and/or sucrose not fermented.
- Acid deep (yellow)/acid slant (yellow): lactose and/or sucrose fermented.
- Alkaline deep and slant (all red): glucose, sucrose, and lactose not fermented.
- Deep split or displaced: gas production.
- Deep blackened: H2S production.

Urease test

Some bacteria produce the enzyme urease, which catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. Organisms that do not produce this enzyme cannot metabolize

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

Methods

1. Obtain two urease broths from the refrigerator.

2. Inoculate one broth using an aseptic technique. Leave the other broth uninoculated (this will be used as a control).

3. Incubate at an appropriate temperature for 24 to 48 hours (do not exceed 48 hours for this test). Observe the color to indicate the result.

Results

Positive (Pink coloration within 18 to 24 hours)

Negative (Orange coloration after 18 to 24 hours)

Oxidase test (Filter Paper Method)

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

1. Soak a piece of filter paper in the reagent solution.

2. Scrape some fresh growth from the culture plate with a disposable loop or stick and rub it onto the filter paper or touch a colony with the edge of the paper.

3. Examine for blue color within 10 sec.

Citrate utilization test:

Principle:

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator, bromothymol blue. The medium also contains inorganic ammonium salts, which are utilized as a sole source of nitrogen. Utilization of citrate involves the enzyme citritase, which breaks down citrate into oxaloacetate and acetate. Oxaloacetate is further broken down into pyruvate and CO2. Production of Na2CO3 as well as NH3 from utilization of sodium citrate and ammonium salt, respectively, results in alkaline PH. This results in a change of the medium's color from green to blue.

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

Motility testing

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

Methods

1. Obtain a motility agar tube from the back shelf.

2. Use an inoculating pick. Straighten the pick as much as possible.

3. Make a stab inoculation (about 2/3 of the way into the agar) from your unknown stock culture. Try to make the stab (in and out) as straight as possible. Straightness is important because you will be evaluating the amount of growth away from the stab. A messy stab will be difficult to evaluate.

4. Incubate at an appropriate temperature for 24 to 48 hours (up to 72 hours).

5. Observe your culture by holding it up to a light source.

Lysine decarboxylase (LDC)

The acids produced by the bacteria from the fermentation of glucose will initially lower the pH of the medium and cause the pH indicator to change from purple to yellow. The acid pH activates the enzyme that causes the decarboxylation of lysine to amines and the subsequent neutralization of the medium. This results in another color change from yellow back to purple. Bacteria that decarboxylate lysine turn the medium purple. In addition, bacteria that produce H2S appear as black colonies.

V.Antimicrobial susceptibility testing

Disc diffusion susceptibility tests

A disc of blotting paper is impregnated with a known volume and appropriate concentration of an antimicrobial, and this is placed on a plate of susceptibility testing agar uniformly inoculated with the test organism. The antimicrobial diffuses from the disc into the medium, and the growth of the test organism is inhibited at a distance from the disc that is related to the susceptibility of the organism. Strains susceptible to the antimicrobial are inhibited at a distance from the disc, whereas resistant strains have smaller zones of inhibition or grow up to the edge of the disc.

Procedure

1. Prepare a suspension of the test organisms by emulsifying 3–5 pure colonies of the organism in 5 ml of normal saline.
2. Much of the turbidity of suspension is within the turbidity standard.
3. With a sterile swab, take the sample from the suspension (squeeze the swab against the side of the test tube to remove the excess fluid).
4. Spread the inoculums evenly over the Muller-Hinton agar plate with the swab.
5. Using a sterile forceps or needle, place the antimicrobial disc on the inoculated plate.
6. Incubate the plate aerobically at 35-37^{o}C for 18-24 hours.
7. Read the test after checking that the bacterial growth is neither heavy nor light. Measure the radius of the inhibition zone.

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

Extracted Clinical Laboratory Standard Institute (CLSI) M100, 2022, 32nd Edition

Antibiotic susceptibility pattern of Gram-negative bacteria from CSF

		N. meningitidis (T-21)			H. influenzae (T-2E)			E. coli/K. P (T-2A)		
Antimicrobial Agent	Disc content	S	I	R	S	I	R	S	I	R
Ceftriaxone (CRO)	30µg	≥34	-	-	≥26	-	-	≥23	20-22	≤19
Meropenem (MEM)	10µg	≥30	-	-	≥20	-	-	≥23	20-22	≤19
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75µg	≥30	26-29	≤25				≥16	11-15	≤10
Chloramphenicol (C)	30µg	≥26	20-25	≤19	≥29	26-28	≤25	≥18	13–17	≤12
Ceftazidime (CAZ)	30µg				≥26	-	-	≥21	18-20	≤17
Cefotaxime (CTX)	30µg	≥34	-	-	≥26	-	-	≥26	23-25	≤22
Cefepime (FEP)	30µg							≥25	-	≤18
Imipenem (Kim et al.)	10µg							≥23	20-22	≤19
Ampicillin (AM)	10µg				≥22	19-21	≤18	≥17	14-16	≤13
Ciprofloxacin (Principi and Esposito)	5µg	≥35	33-34	≤32				≥26	22-25	≤21
Tetracycline (TE)	30µg							≥15	12-14	≤11
Rifampin (RA)	5µg	≥25	20-24	≤19						
Doxycycline (DO)	30µg							≥14	11-13	≤10

T= Table

N. meningitidis

0.5 McFarland standard inoculum from Chocolate agar = 2X if from SBA, to MHA with 5% SBA, control *S. Pneumoniae*: ATCC 49619

5 Disc in 150 mm, 2 Disc in 100mm

H. influenzae

0.5 McFarland standard *H. influenzae* inoculum from chocolate to HTM

HTM control *H. influenzae* ATCC 49247

9 Disc in 150 mm, 4 Disc in 100mm

E. Coli/*K. Pneumoniae*

0.5 McFarland standard inoculum from SBA to MHA at ambient air

12 Disc in 150 mm, 6 Disc in 100mm

Hemophilus test medium (HTM) preparation

1. Prepare a fresh hematin stock solution by dissolving 50 mg of hematin powder in 100 mL of 0.01 mol/L NaOH with heat and stirring until the powder is thoroughly dissolved.
2. Add 30 mL of the hematin stock solution and 5 g of yeast extract to 1 L of MHA, and autoclave.
3. After autoclaving and cooling, add 3 mL of NAD stock solution (50 mg NAD dissolved in 10 mL distilled water, filter sterilized) aseptically.

Antibiotic susceptibility pattern of Gram-positive bacteria from CSF

		S. pneumoniae (T-2G)			*S. aureus* (T-2C)		
Antimicrobial Agent	Disc content	S	I	R	S	I	R
Oxacillin (OX)/cefoxitin (FOX)	FOX 30µg				≥22	-	≤21
Vancomycin (VA)	30µg	≥17	-	-			
Erythromycin (E)	15µg	≥21	16-20	≤15	≥23	14-22	≤13
Azithromycin (AMZ)	15µg	≥18	14-17	≤13	≥18	14-17	≤13

Tetracycline (TE)	30µg	≥28	25-27	≤24	≥19	15-18	≤14
Doxycycline (DO)	30µg	≥28	25-27	≤24	≥16	13-15	≤12
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75µg	≥19	16-18	≤15	≥16	11-15	≤10
Chloramphenicol (C)	30µg	≥21	-	≤20	≥18	13-17	≤12
Gentamycin (GM)	10µg				≥15	13-14	≤12
Rifampin (RA)	5µg	≥19	17-18	≤16	≥20	17-19	≤16
Ciprofloxacin (CIP)	5µg				≥21	16-20	≤15

T=Table

S. Pneumoniae

0.5 McFarland standard inoculum from SBA to MHA with 5% sheep blood & 5% CO2

Deterioration of OX disk content is best assessed with S. aureus ATCC 25923, with an acceptable range of 18–24 mm on unsupplemented MHA

9 Disc in 150 mm, 4 Disc in 100mm

S. aureus

0.5 McFarland standard inoculum from SBA to MHA, ambient air

12 Disc in 150 mm, 6 Disc in 100mm

VI. CSF WBC count procedure

1. Mix the CSF by inverting up and down 3 to 5 times.
2. Dilute the CSF 1 into 2 (1 drop of CSF with 1 drop of isotonic methylene blue diluting fluid).
3. Prepare a bright line improved Neubauer counting chamber and cover glass.
4. Using a fine-bore Pasteur pipette, carefully load the sample into the counting chamber.
5. Wait about 2 minutes for the cells to settle.
6. Count the cells microscopically.

VII. CSF biochemistry measurement procedure

A. Protein measurement procedure

1. Warm the reagents to room temperature.
2. Mix 1000 µL of biuret reagent R1 with 20 µL of CSF sample.
3. Incubate for 10–15 minutes at room temperature.
4. Measure the absorbance at 520 nm.

B. CSF glucose measurement procedure

1. Warm the reagents to room temperature.
2. Mix glucose oxidase reagent R1 with 20 µL of CSF sample.
3. Incubate for 10–15 minutes at room temperature.
4. Measure the absorbance at 520 nm.

VIII. Molecular Method

A. Samples and reagent preparation

a. Material and reagents

- CSF sample
- 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 of 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 (μL)
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, the elution buffer in columns 5 and 11 is transferred to a clean nuclease-free 0.5 mL centrifuge tube; stored at -20° if not used.

b. High Pure Viral Nucleic Acid Kit protocol

1. To a nuclease-free 1.5 mL microcentrifuge tube, add

- 200 μL CSF.
- 200 μL freshly prepared Working solution (carrier RNA-supplemented Binding Buffer) and mix well.
- 50 μL Proteinase K solution and mix immediately.
- Incubate for 15 minutes at +56°C.

2. Add 100 μL Binding Buffer and mix.
3. To transfer the sample to a High Pure Filter Tube:

- Insert one High Pure Filter Tube into one Collection Tube.
- Pipette entire sample into the upper reservoir of the Filter Tube (maximum 700 μL)

4. Insert the entire High Pure Filter Tube assembly into a standard tabletop centrifuge.

- Centrifuge 1 minute at 8,000 × g.

5. After centrifugation:

- Remove the Filter Tube from the Collection Tube; discard the flow through liquid and the Collection Tube.
- Insert the Filter Tube into a new Collection Tube.

6. After re-inserting the Filter Tube:

- Add 500 µL Inhibitor Removal Buffer to the upper reservoir of the Filter Tube.
- Centrifuge 1 minute at 8,000 × g.

7. After centrifugation:

- Remove the Filter Tube from the Collection Tube; discard the flow through liquid and the Collection Tube.
- Insert the Filter Tube into a new Collection Tube.

8. After removal of inhibitors:

- Add 450 µL Wash Buffer to the upper reservoir of the Filter Tube.
- Centrifuge 1 minute at 8,000 × g and discard the flow through.

9. After the first wash and centrifugation:

- Remove the Filter Tube from the Collection Tube; discard the flow through liquid and the Collection Tube.
- Insert the Filter Tube into a new Collection Tube.
- Add 450 µL Wash Buffer to the upper reservoir of the Filter Tube.
- Centrifuge 1 minute at 8,000 × g and discard the flow through.
- Leave the Filter Tube-Collection Tube assembly in the centrifuge and spin it for 10 seconds at maximum speed (approximately 13,000 × g) to remove any residual Wash Buffer.

10. Discard the Collection Tube and insert the Filter Tube into a nuclease-free, sterile 1.5 mL microcentrifuge tube.

11. To elute the viral nucleic acids:

- Add 50 µL Elution Buffer to the upper reservoir of the Filter Tube.
- Centrifuge the tube assembly for 1 minute at 8,000 × g.

12. The microcentrifuge tube now contains the eluted viral nucleic acids.

- Use the eluted nucleic acids directly in PCR (10 to 20 µL DNA eluate) or RT-PCR (3.5 µL viral RNA). Alternatively, store the eluted viral RNA at −80°C, or the viral DNA at +2 to +8°C or at −15 to −25°C for later analysis.

C. Nanodrop procedures

a. Materials and reagents

- Nanodrop spectrophotometer
- Nuclease-free water
- Extracted nucleic acid sample

b. Procedure

1. First, make sure the volume of the nucleic acid extracted sample falls within the range recommended by the NanoDrop device.

2. Blank the NanoDrop device using nuclease-free water following the manufacturer's directions.

3. Add the 1 µL of the extracted sample to the NanoDrop measuring surface.

4. Run the device and obtain the measure at both 260/230 and 260/280 nm.

5. Calculate the ratio:

- A ~1.8 ratio indicates a relatively pure DNA sample.
- A ~2.0 ratio indicates that it is pure RNA.

D. 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 a single PCR tube according volume required (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 the 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.

E. PCR Procedure

a. Protocol

1. Set the Program of the thermal cycler (T100 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.

F. Reagent preparation procedures

A. 50x TAE Buffer Preparation

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

a. 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.

B. 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.

C. 100-bp DNA Ladder Preparation

b. 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

c. 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. 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.

D. 2.5% Agarose Gel Preparation

- 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 a well-forming comb
- Nitrile Gloves

d. 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 to prevent overboiling.
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 work must be done in a hood when handling ethidium bromide due to its mutagenic properties.
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 Note**:** Ethidium bromide is a mutagen, so wear gloves and handle it with care. Dispose of waste containing ethidium bromide according to your institution's guidelines. Wear gloves and safety goggles throughout the procedure to protect yourself from potential hazards. The concentration of agarose used may vary depending on the size of the DNA fragments to be separated. Always use nuclease-free materials and work in a clean environment to prevent contamination.

G. 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
- Microcentrifuge tube

b. Procedure

1. Mix 25 µL of the PCR product in a microcentrifuge tube and then add 5 µL of loading dye. Pipette it gently up and down to homogenize 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 that of the DNA ladder
11. Photograph the gel, recording the outcome.

Annex IX: Declaration

Declaration of investigator

I, the undersigned student of Medical Microbiology, hereby declare that this thesis is my original work submitted as part of the requirements for the master's degree in Medical Microbiology.

Principal investigator: Derso Wale Mesele

Signature: _____

Date of submission: _____

Declaration of advisors

We, the undersigned Advisors, declare that this thesis is our original work in partial fulfillment of the requirement for the master's degree of Medical Microbiology for the stated student above to our best knowledge. We confirmed that this thesis is ready for defense with our approval as the university advisor(s).

Advisors	Signature	Date
1. Dr. Alem Abrha	_____	_____
2. Dr. Seifegebriel Teshome	_____	_____
3. Dr. Getachew Tesfaye	_____	_____

Department of Microbiology, Immunology andParasitology (DMIP)

Department Research Ethics Review Committee (DRERC)

Meeting No: DRERC/004/2023 Date: 26 June 2023

Protocol Title: Microbial profile, antimicrobial resistance and treatment and adjunctive therapy outcome of meningitis suspected patients at the University of Gondar Comprehensive Specialized Hospital				
Principal Investigator	Derso Wale			
Institute/Department	CHS-AAU/DMIP			
Type of review	[X] Initial Review:	[ ] Amendment	[ ] Other (specify):_____	
Elements Reviewed	[ ] Attached	[ ] Not attached		
Decision of the meeting	[X] Approved	[ ] Disapproved		
Action Required	[ ] Send to IRB	[X] Authorize Implementaion		

Obligations of the PI:

i. Should comply with the standard international and national scientific and ethical guidelines
ii. All amendments and changes made in protocol and consent form needs DREC approval
iii. The PI should report Serious Adverse Events (SAE) within 10 days of the event
iv. End of the study, including thesis work and manuscript should be reported to the DREC

Follow up report expected in:

3 Months_____ 6 Months __X__ 9 Months _____ one year _____

Asrat Hailu (Prof)

Chair, DRERC

Signature AH

Date: 26/06/2023

Department Stamp