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Antibiotic Susceptibility Pattern of *Neisseria meningitides* Isolates from Asymptomatic Carriers in Gurage zone, Southern Ethiopia.

By Fikerte Mulatu (BSc)

A thesis Submitted to Department of Medical Laboratory Sciences,College of Health Science AddisAbaba University In Partial Fulfillment Of The Requirements For The Degree Of Masters Of Sciences In Clinical Laboratory Sciences (Diagnostic And Public Health Microbiology Specialty Track).

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Abbreviations

AAERC	AHRI/ALERT Ethical Review Committee
AAU	Addis Ababa University
AHRI	Armauer Hansen Research Institute
ALERT	All Africa Leprosy, Tuberculosis and Rehabilitation Training
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BSC	Biological safety cabinet
CDC	Center for Disease Control and Prevention
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standard Institute
COHS	College of Health Science
CSF	Cerebrospinal Fluid
DERC	Department Ethics and Research Committee
DMLS	Department of Medical Laboratory Sciences
DSS	Demographic surveillance site
GGT	γ-glutamyl- transferase
MD	Meningococcal disease
MHA	Muller Hinton Agar
MIC	Minimum inhibitory concentration
ND	Non determinant
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
PCR	Polymerase chine reaction
QC	Quality control
SOAHS	School of Allied Health Sciences

Abstract

Background: *Neisseria meningitides* represents a pathogen of great public health importance in both developed and developing countries. Resistance to some antimicrobial agents used either for therapy of invasive infections or for prophylaxis of case contacts has long been recognized. However, there is a limited data in relation with the antimicrobial resistance pattern of *Neisseria meningitides* in Ethiopia. Therefore; the aim of this study was to assess drug susceptibility pattern of *Neisseria meningitides* isolates from asymptomatic carrierers of all age groups at Meskan and Mareko Districts, Gurage Zone, Southern Nations, Nationalities and Peoples Regional State Ethiopia.

Methods: Cross-sectional study was conducted on 187 *Neisseria meningitides* isolates obtained from MenAfricar project.A total of 4110 study participants from Meskan and Mareko Districts, Gurage Zone, Southern Nations, Nationalities and Peoples Regional State, Ethiopia were screened for *Neisseria meningitides* by the project. Antimicrobial susceptibility test (AST) was done on stored *Neisseria meningitides* isolates.The activity of ten antimicrobial agents used for treatment and prophylaxis of meningococcal disease was investigated. The AST was performed for *Neisseria meningitides* isolates according to the criteria of the CLSI guide line by disk diffusion method. Data was analysed by using SPSS version 20.0 software.

Results: From 187 *Neisseria meningitides* isolates 8(4.28%) were serogroup X, 24(12.83%) were serogroup Y, 1(0.53%) was serogroup W135 and 154(82.35%) were non determinant (ND). Resistance for Cotrimoxazol was high accounting 116(62%) followed by Ciprofloxacine 112(60%), Cefotaxime 26(14%), Ceftriaxone 24(13%) , Meropenenem 21(11%) , Minocycline 15(8%), Rifampine 14(7.5%), Azithromycine 10(5%), Chloramphenicol 7(4%),Levofloxacin 6(3%) . 102(54.5%) isolates were resistance for more than one drug.

Conclusions: An antimicrobial resistance for *Neisseria meningitides* isolates from asymptomatic carriers for the present study is high. Continued surveillance of meningococci for antimicrobial resistance is necessary to monitor early detection of changes in susceptibility patterns that might affect recommendations for chemoprophylaxis and treatment.

Key words: *Neisseria meningitides,* serogroups, sensitivity pattern, drug resistance

1. Introduction

1.1. Background

Neisseria meningitides, the meningococcus, is a Gram-negative bacterium with a coccoid shape, and a pathogenic member of the Neisseriae family. Anton Weichselbaum first isolated the bacterium from the cerebrospinal fluid (CSF) of a patient and identified it as the cause of meningitis in 1887; he called the organism *Diplococcus intracellularis* (Weichselbaum,1887) (1).

The sole ecological niche of *Neisseria meningitides* is the mucosa of the oropharynx of humans. Meningococcal colonization of the respiratory tract, a phenomenon commonly referred to as carriage, represents a successful commensal relationship between the host and the bacterium, with the host experiencing no detectable pathology. On the other hand disease represents a failed or dysfunctional relationship with the host. Acquisition of *Neisseria meningitides* demands person-to-person transmission via direct contact or through dispersion of respiratory droplets from an infected to a susceptible individual. Although often protected by a polysaccharide capsule, meningococci are particularly sensitive to desiccation; thus, spread from one individual to another requires close contact. In closed or semi-closed settings, such as residential schools and military recruit camps, transmission increases dramatically and carriage prevalence may approach 100%.While carriage rates are very variable among human populations, point-prevalence carriage rates in Europe and the United States have been estimated to range from 10 to 35% in young adults and it is likely that, at one time or another during life, most individuals are colonized with meningococci (2).

Neisseria meningitides is a leading cause of bacterial meningitis and septicemia worldwide. Strains isolated from patients with these invasive infections are mainly of serogroups (capsular antigens) A, B, C, Y, and W135, which comprise various serotypes and serosubtypes (defined in terms of the antigenicity of the major outer membrane proteins PorB and PorA, respectively)(3).

Infections by *Neisseria meningitides* are significant causes of mortality and morbidity in young children and adolescents. The epidemiology of serious meningococcal disease is an area of considerable interest, and many unanswered questions surround this organism and the types of diseases it causes. Group A and group C meningococci are frequently the cause of major epidemic disease, particularly in underdeveloped countries and among the poorer segments of

society, perhaps reflecting certain risk factors associated with transmission, such as crowding and poor sanitation. The organisms may be asymptomatically carried in the oropharynx and nasopharynx of a variable percentage of individuals, and the rate of carriage is related to several factors such as age, socioeconomic class, and the presence of actual disease in a community (4).

Neisseria meningitides is not only a common bacterial commensal of the human upper respiratory tract (nasopharynx) but also an important and devastating human pathogen. Meningococci are gram-negative diplococci, can be encapsulated or un encapsulated, have a genome of about 2·1–2·2 m bases with roughly 2000 genes, and are the worldwide cause of epidemic meningitis and rapidly progressing fatal shock. The persistence of large serogroup A outbreaks in Africa, the emergence in different regions of serogroups Y, X, and W-135 in the past decade, and the persistence of serogroups B and C disease in many industrialized countries (5).

Antimicrobial treatment and chemoprophylaxis for patients with meningococcal disease and their close contacts is critical to reduce morbidity and mortality and to prevent secondary cases. Although an extended-spectrum cephalosporin such as ceftriaxone is recommended for empirical treatment of meningitis, some treatment guidelines recommend switching to penicillin G when *Neisseria meningitides* is confirmed. Ceftriaxone, ciprofloxacin, and rifampin are the currently recommended chemo prophylactic antimicrobials. Azithromycin was recommended as an alternative chemo prophylactic antimicrobial in eastern North Dakota and western Minnesota when ciprofloxacin resistance was first reported in North America (serogroup B) (6).

The value of monitoring antimicrobial resistance is important for *Neisseria meningitidis* .Although there is no global alert for the spread of resistant meningococcal strains, the emergence of resistance is correlated to the outcome of treatment and the successful prophylaxis of close contacts.The global rise of antimicrobial resistance in bacteria combined with the decreasing number of innovative antibacterial agents has led to warnings that we may soon lose our ability to treat bacterial infections (7).

Early antibiotic treatment of meningococcal disease is crucial for keeping the case fatality rate and risk of sequelae as low as possible. Comprehensive data regarding the antibiotic susceptibility of *Neisseria meningitides* in many African countries are limited, and no up-to-date extensive study of the antibiotic susceptibility of *Neisseria meningitides* is at hand (8).

1.2. Statement of the problem

Meningococci can be transmitted from human to human through direct contact with large droplet respiratory secretions (9).Meningococcal disease remains a major cause of child morbidity and mortality worldwide, despite improved diagnosis and treatment. The usual sources of meningococcal infection are asymptomatic human nasopharyngeal carriers. The meningococcal carrier state is an immunizing process, and production of serogroup-specific antibodies to meningococci can usually be identified within 2 weeks of colonization (10).

The scientific literature contains a wealth of susceptibility data for clinical meningococci isolates associated with a variety of medical conditions. There is, however, a lack of data that describe the antimicrobial susceptibilities of *Neisseria meningitides* strains that colonize the nasopharynx. Knowledge of susceptibility patterns and of trends in the resistance of colonizing strains may be of great value in establishing a policy for empirical antimicrobial treatment of meningococcal disease (MD) and in developing appropriate prophylactic regimens for eradication of the carrier state in persons at high risk of developing serious infection (11).

Neisseria meningitides with reduced susceptibility to peniciline is common in many areas of the world, though the clinical significance of this resistance has not yet been established. In addition, resistance to rifampicine has been reported and such strains have resulted in prophylaxis and treatment failures. Recently, sporadic resistance to ciprofloxacine, an antibiotic commonly used for chemoprophylaxis of non-pregnant adults in many countries, has been reported throughout the world, including Europe, south America, Australia, Asia,and North America(12).

Reduced susceptibility to ciprofloxacin and resistance to rifampicin have also been reported from many countries.Resistance to ceftriaxone is claimed to have been identified and was reported from India (13).

The continuously increasing antibiotic resistance in many bacterial pathogens is a serious public health threat worldwide and there have been numerous reports of emerging resistance in meningococci during the past decades (13).

There are limited data on the AST pattern of *Neisseria meningitides* in Ethiopia in general and in the study settings in particular.

1.3. Significance of the study

So far, to our knowledge, there is only one study that investigates drug susceptibility test of *Neisseria meningitides* in Ethiopia (38). We noted *Neisseria meningitides* is developing resistance to several antibiotics. Therefore, generating information about the AST of *Neisseria meningitides* is highly required.

It is crucial to identify resistance in the meningococcus in an early state of the disease in order to prevent treatment failure because of the administration of inappropriate antibiotics. Surveillance is necessary to monitor trends in their susceptibilities to antimicrobial drugs to advise clinicians on appropriate empirical therapy and chemoprophylaxis and also it is crucial to continuously monitor the antibiotic susceptibility of meningococci to avoid future treatment failures. Hence this study gave us a good indication to know AST pattern of *Neisseria meningitides* in our country and it is a crucial and must know the problem to improve the drug administration.

2. Literature review

2.1. Epidemiology of *Neisseria meningitides* and serogroup distribution

Invasive meningococcal disease is a significant health problem worldwide. In Africa, particularly in the sub-Saharan, so-called meningitis belt, epidemics of acute meningitis can reach incidence rates of 1,000 cases per 100,000 inhabitants, and in individual communities, attack rates as high as 1:10 for the population have been reported. During these epidemics, a mortality rate of about 10% is usually reported, which most probably is an underestimation. These epidemics in the African meningitis belt have historically been caused mostly by a limited number of *Neisseria meningitides* serogroup A clones. During recent years, strains of other serogroups, such as serogroups C, W-135, and X, have also been involved (8).

The five common serogroups A, B, C, Y and W135 are responsible for about 90% of infections caused by *Neisseria meningitides*, serogroups A, B and C account for most cases of meningococcal disease throughout the world, with serogroups A and C predominating throughout Asia and Africa and serogroups B and C responsible for the majority of cases in Europe and the Americas. In recent years, the number of cases involving serogroup Y has increased; from 1996 to 1998, one third of cases in United States were due to serogroup Y. Israel and Sweden are the only countries other than the United States that have reported an increase in serogroup Y disease. Serogroup W-135, currently accounting for only 4% of cases in the United States, was reported in 15 to 20% of isolates received by the Centers for Disease Control and Prevention between 1978 and 1980 (14).

Epidemic rates of meningococcal disease varies from <1- 3/100,000 in many developed nations to 10-25/100,000 in some developing countries. This difference in attack rates reflects the difference in pathogenic properties of *Neisseria meningitides* strains prevalent and differences in socioeconomic and environmental conditions. The proportion of cases caused by each serogroup varies by age group; more than half of cases among infants aged <1 year are caused by serogroup B, for which no vaccine is available (14).

In the United States, most cases of invasive meningococcal disease are caused by isolates belonging to serogroup B or C and only very rarely to group A. Recently, disease due to serogroup Y, which is commonly associated with pneumonia and was previously associated with outbreaks in military populations, has increased to approximately one-third of all cases. Serogroup W135 has been associated with military population outbreaks and was responsible for

a large outbreak during the Muslim pilgrimage in the Middle East, hajj, in 2000. While most cases in Europe and the Americas are due to serogroups B and C, serogroups A and C are most common in Africa and Asia. Outbreaks that occur in the "meningitis belt" of Africa in some years have had an attack rate of 500 to 1,000 cases per 100,000 populations and a mortality rate of approximately 10% (15).

An outbreak of pyogenic meningitis occurred in western part of India (Surat, Gujrat) during 1985-87.A total of 197 cases of meningitis with 34 deaths were reported during a period of one-and-a-half years. *Neisseria meningitides* was the predominant pathogen isolated from 66 out of 138 CSF samples. Recently migrated males of productive age groups drawn from the states of Uttar Pradesh and Orissa were predominantly affected. Male to female ratio was found to be 7.2:1. Pregnancy and childbirth appeared to be important predisposing factors in females. Nine cases were reported from the family contacts of cases (14).

Since 1960, the incidence of meningococcal meningitis in the United States has been stable, at approximately 0.9-1.5 cases per 100,000 people per year. Most cases occur during winter and early spring. Serogroups B and C have caused most cases of meningococcal meningitis in the United States since World War II. An increased frequency of serogroup B and Y meningococci has been noted since 1990.The frequency of localized out breaks has increased since 1991 (16).

In Africa Within individual countries in the meningitis belt (a region of savanna that extends from Ethiopia in the east to Senegal in the west), major epidemics occur with a periodicity of 5 – 10 years. Between the epidemics, the rate of disease falls markedly, but is still considerably higher than in the western world. The West and Central African epidemics of meningitis are often enormous. For example, in 1996 more than 150,000 cases were reported, with nearly 16,000 deaths as a result. Attack rates as high as 1:10 have been observed in individual communities. Furthermore, the estimated figures are most likely an underestimation, which is due to both a breakdown of the normal reporting systems and the fact that many patients with septicaemia die before they reach a hospital. The epidemics usually start in the beginning of the dry season and end quickly when the rains start, only to break out again when the subsequent dry season starts. The reason for this pattern is not fully understood, but environmental factors, such as absolute humidity and dust concentrations, have been confirmed as important factors (13).

Meningococcal carriage is highly age dependent. The great majority of studies have been conducted in industrialized societies, and in such settings meningococcal carriage is usually very rare in infancy and increases with age, carriage prevalence reaching a peak during the teenage years (16).18 Studies in Africa have shown a less consistent age distribution, carriage prevalence being generally highest in younger children aged 5 to 14 years (18).

2.2. Transmission and carriage

The dynamics of meningococcal transmission, acquisition and carriage in humans are a second major influence on the incidence and likelihood of meningococcal disease. The natural habitat and reservoir of the meningococcus are the upper respiratory nasopharyngeal mucosal membranes. As noted, *Neisseria meningitides* is carried by ~8–20% of the normal population, but the prevalence of carriage varies widely and does not directly predict disease. However, without meningococcal carriage there is no meningococcal disease and the rates of meningococcal disease are influenced by factors that enhance exposure and transmission, carriage rates of strains with different virulence potential, and host factors. Transmission is by direct contact with or inhalation of meningococcus in large droplet nuclei that are acquired through very close contact with respiratory secretions and saliva. Acquisition of meningococci may be transient, result in invasive disease or lead to colonization (carriage). Meningococcal disease usually occurs 1–14 days after acquisition. The inoculum size required for infection is unknown but, based on infection of *N. gonorrhoeae* in a human urethral challenge model, may be 10^3–10^4 organisms mL^{-1}. Transmission is facilitated by close contact, coughing and spreading of secretions. Once meningococci reach human epithelial cells, a series of interactions with host epithelial cells occurs, leading to effacement of the epithelial surface, microcolony formation and/or epithelial cell invasion (19).

Some meningococcal strains are highly transmissible but rarely cause long-term carriage (eg, ST-11, serogroup C). Other meningococci can be less virulent but are frequently transmitted (eg, ST-23, serogroup Y strains). The pattern of meningococcal carriage in sub-Saharan Africa remains unclear, although more studies are being done before the introduction of conjugate vaccines. In sub-Saharan Africa, the serogroup A carriage rate might be as high as 10% during outbreaks but this rate is usually low between epidemics. In the few studies that have been undertaken in Africa, serogroup A carriage has been highest in older children and young adults.

Meningococcal carriage is affected by age, intimate personal contact, crowding (eg, bars, dormitories), and smoking (5).

Damage to the upper respiratory tract by co-infections (eg, mycoplasma, influenza, and other respiratory viral infections), smoking, very low humidity, drying of mucosal surface, and trauma induced by dust, predisposes to carriage and meningococcal disease. Carriage has been linked to secretor status of glycoprotein ABO blood group antigens that are water soluble and to ethnic background. In a large UK study, social behavior (eg, attendance at pubs or clubs, intimate kissing, and cigarette smoke or exposure to passive smoke) was most highly associated with the risk of meningococcal carriage (5).

The majority of individuals will, at one time or another throughout life, harbor the bacterium asymptomatically in the throat. Thus, invasive disease is a very rare outcome of infection, usually occurring shortly after acquisition of the bacterium by a susceptible host. Causing disease is of no importance for the spread of meningococci and the population biology of N. meningitides (Maiden, 2004); most patients with meningococcal disease have not been in contact with another case. The bacterium is transmitted between individuals when respiratory droplets from an infected – but symptom-less – person are spread to other individuals through close contact (20).

The relation between carriage rate and invasive disease is not clearly understood. Carriage studies can provide valuable information on epidemiology, pathogenesis, serogroup distribution and possible transmission patterns, information which helps understanding the potential effects of control programs, such as vaccination (21).

As meningococcal are transmitted by healthy carriers, conjugated vaccines that prevent colonization, in addition to protecting vaccinated individuals against invasive disease, have shown to be very efficient to control disease (22).

As the capsule is immunogenic and probably the most important virulence factor, capsular polysaccharide of serogroups A, C, W and Y has been used as vaccine antigens. Until very recently, A/C and A/C/W polysaccharide vaccines have been the only vaccines available for use in sub-Saharan Africa (23).

A monovalent serogroup A tetanus toxoid-conjugated vaccine, MenAfriVac, has been developed with the goal of eliminating the devastating NmA epidemics in Africa. The vaccine was shown

to be safe and immunogenic and its low price made it appropriate for routine vaccination and mass vaccination campaigns in Africa. MenAfriVac was prequalified by the World Health Organization (WHO) in June 2010 to be given as a single dose in the age group 1–29 years (23).

In a study performed in Norway, the carriage rate among a troop of military recruits was higher than 70% (24). A surveillance study performed in the spring and autumn of 1998 in Poland showed that meningococcal carriage among military recruits was dynamic, with overall carrier rates ranging between 36 and 61% within 2-month periods (25).

Meningococcal carriage increases rapidly among university students in the first month of the academic year and much of this increase probably occurred during the first week (26).Carriage prevalence of *Neisseria meningitides* is generally higher among household contacts of meningococcal patients (27,28,29) than in the general population. The overall carriage rate of *Neisseria meningitides* among household contacts in New Zealand was 20.5% (30). The study demonstrated that age-specific carriage in children under 5 years of age was low at 5.8% but increased to 35.1% in 15– 19-year-olds. Using phenotypic characteristics, 50% of household carriers harboured the same strain as the patient (1).

A recent study showed that the carriage rate might be underestimated when using conventional nasopharyngeal swabbing. By using immunohistochemistry for detection of *Neisseria meningitides* in patients undergoing tonsillectomy, it was found that meningococci were present in 45% of the samples, while only 10% were positive by culture of nasopharyngeal swabs (31). Comparison of culture of throat swabs and PCR for specific detection of *Neisseria meningitides* in carriers demonstrated that the sensitivity of throat swab culture was higher than a PCR assay based on ctrA, a gene involved in export of the meningococcal capsule. PCR was shown to be a useful adjunct to culture for detecting nasopharyngeal carriage, but it failed to detect some non capsulated strains (32).

2.3. Colonization of *Neisseria meningitides*

Meningococci that elaborate a capsule can lead to invasive disease. The capsule protects them from desiccation and from host immune mechanisms. Smoking and concurrent viral infections of the upper respiratory tract diminish the integrity of the respiratory mucosa and increase the likelihood of invasive disease. Crowding living conditions also facilitate disease spread, since individuals from different areas have different strains of meningococci (16).

Risk factors for meningococcal disease include organism, host, and environmental factors. Persons with persistent complement component deficiencies (e.g., C5—C9, properidin, factor H, or factor D) or functional or anatomic asplenia are at increased risk for invasive meningococcal disease (33).

Crowded living conditions can facilitate respiratory droplet transmission of meningococci. College freshman residing in dormitories are at greater risk of acquiring meningococcal disease than are college students not living in dormitories. Active or passive smoking and recent upper respiratory tract infections also increase risk of disease. Historically, blacks and persons of low socioeconomic status have been found to be at higher risk for meningococcal disease than whites and persons of high socioeconomic status, however in recent years these differences have diminished. Race and socioeconomic status are likely markers for differences in risk factors such as household crowding, exposure to tobacco smoke, and urban residence (9).

Meningococcal disease rates in children younger than 1 year peak at 0-6 months. More than 50% of meningococcal disease in children 0-6 months is caused by serogroup B; serogroup Y is also more prevalent in this age group. In time, children gradually become exposed to meningococci and develop bactericidal antibodies. By the time they reach adulthood, 65%–85% of persons possess bactericidal antibody against meningococcal disease (9).

Those who have close contact with case-patients, such as household members, are at a substantially increased risk for acquiring carriage and disease. Rates of secondary disease are also elevated among daycare workers and attendees as well as among schoolchildren (9).

2.4. Antibiotic Susceptibility Pattern of *N. meningitides*

Neisseria meningitides represents a pathogen of great public health importance in both developed and developing countries. Resistance to some antimicrobial agents used either for therapy of invasive infections or for prophylaxis of case contacts has long been recognized, although specific guidelines for susceptibility testing have not been fully developed (15).

Neisseria meningitides does not commonly show resistance for many antimicrobial agents. Low-level resistance to penicillin is common in some areas of the world, though the clinical significance of this resistance has not yet been established. Meningococcal resistance to sulfonamides, rifampicin (also referred to as rifampin), and chloramphenicol has also been described. Chloramphenicol tends to be the empiric drug of choice for treating patients with

meningitis caused by *Neisseria meningitides*; rifampicin and sulfonamides are often used for prophylaxis (34).

Increasing antimicrobial resistance among bacterial pathogens is a serious public health threat worldwide and today resistance can be found in almost every bacterial species for which antibiotic therapy exists. Bacteria can be naturally resistant to an antibiotic (intrinsic resistance), which is often due to lack of the target molecule. Bacteria may also be genetically altered and thereby gain resistance to an antibiotic (acquired resistance). Depending on the drug and the bacterium different mechanisms can confer antibiotic resistance .The main mechanisms are: decreased uptake of antibiotic, increased export of antibiotic, Inactivation or modification of target, Alternative target, Inactivation or modification of antibiotic (13).

Reduced susceptibility to ciprofloxacin and resistance to rifampicin have also been reported from many countries. Furthermore, there have been rare reports of chloramphenicol resistant meningococcal isolates from Australia, France and Vietnam Resistance to ceftriaxone is claimed to have been identified and was reported from India. These strains, however, have not been further examined, comprehensively phenotypically and genetically characterized, and/or confirmed by an independent laboratory (13).

A study conducted in army recruits in a training camp at Malaysia by Rohani et al. showed that the serogroups of randomly selected 210(17.9%) of the 1181 *Neisseria meningitides* isolates,One hundred isolates were subjected to antibiotic susceptibility testing based on almost (100%) susceptibility of *Neisseria meningitides* to commonly used antibiotics where the minimum significant number for testing was 60-80 isolates. The antibiotic susceptibility patterns of 100 *Neisseria meningitides* strains to cotromixazole, chloramphenicol, rifampin, levofloxacin, cefotaxime and penicillin were determined by a disc diffusion method using Mueller Hinton II agar with 5% sheep blood. All strains subjected to antibiotic sensitivity testing were susceptible to chloramphenicol, rifampin, levofloxacin, cefotaxime and penicilin. Ten *meningococcal* strains (12.5%) were less susceptible to penicillin by disc diffusion but all fell within the susceptible category based on the MIC of ≤ 0.064µg/ml measured by E-Test. The MICs ranged between 0.023µg/ml and 0.064µg/ml. The rate of resistance to cotrimoxazole was 84%.Various serogroups were detected including A (3.3%) and W135 (4.7%) and 171 (81%) were either serogroup X, Y or Z. About 11% of the isolates were non-serogroupable due to the limited number of antisera available. Our finding agrees with that of others that serogroups X, Y and Z

are common serogroups carried by the normal population. Resistance to trimethoprime-sulphamethoxazole was high among these isolates and the rate was almost similar to other reports. The widespread resistance to this antibiotic is possibly due to the early introduction of sulphonamides (35).

Study conducted Greek children in 2004 showed all isolates were sensitive to ceftriaxone, rifampicin and chloramphenicol. Only one (4.5%) isolate was resistant to co-trimoxazole, while five (22.7%) showed intermediate resistance to penicillin.Five serogroups were represented: serogroups A (one isolate, 4.5%), B (five isolates, 22.7%), C (seven isolates, 31.8%), D (one isolate, 4.5%), and W135 (three isolates, 13.7%). Five (22.7%) isolates were non-serogroupable (10).

Another study conducted on Cankaya municipality schools of Ankara province in 2005 the antibiotic susceptibilities of *Neisseria meningitides* isolates were determined by agar dilution method for penicillin, sulfadiazine, rifampicin, and azithromycin. *Neisseria meningitides* carriage prevalence was found as 10.4% with serogroup B being the most predominant (47.5%). five strains (4.2%) had decreased sensitivity to penicillin. The resistance against sulfadiazine was 54.4%, while it was 26.9% against azithromycin. No rifampicin-resistant strains were detected. Rifampicin should be the first drug of choice both for the treatment of meningococcal carriers and for the prophylaxis of the subjects who had been in contact with patients with meningococcal infection (36).

Another study from Punjab (Ludhiana) has studied 170 cases of meningococcal meningitis. Among these cases 96 were found culture positive. The study reported all their isolates to be sensitive to most of the common antibiotics including penicillin, chloramphenicol, ampicillin and sulphadiazine.84 Isolates obtained from the recent episode (early 2005) of spurt of cases in and around Delhi were subjected to drug susceptibility testing by MIC method (Etest). Most of the isolates were susceptible to commonly used drugs. All the isolates were sensitive to penicillin, ampicillin, rifampicin and ceftriaoxone. As regards to ciprofloxacin, about two third of the isolates tested were found to be ‘non susceptible’. All the isolates were found resistant to cotrimoxazole (14).

A study conducted by L. arreaza *et al.* in 2000 the activities of seven antimicrobial agents used for treatment and prophylaxis of meningococcal disease was investigated against 901 *Neisseria meningitides* isolates, 112 of which were recovered from patients and 789 of which were

recovered from asymptomatic carriers. The proportions of isolates with decreased susceptibility to penicillin were 55.3 and 39.0%, respectively. Penicillin- and ampicillin-intermediate strains were more common among serogroup C meningococci than among non-serogroup C meningococci from both patients and carriers (11).

One study in South Africa in 2008 showed that data from an active national laboratory-based surveillance program from January 2001 through December 2005 were analyzed. A total of 1,897 cases of invasive meningococcal disease were reported, with an average annual incidence of 0.83/100,000 population. Of these cases, 1,381 (73%) had viable isolates available for further testing; 87 (6%) of these isolates tested intermediately resistant to penicillin (Peni). Peni meningococcal isolates were distributed throughout all provinces and age groups, and there was no association with outcome or human immunodeficiency virus infection. The prevalence of Peni was lower in serogroup A (7/295; 2%) than in serogroup B (24/314; 8%), serogroup C (9/117; 8%), serogroup Y (22/248; 9%), or serogroup W135 (25/396; 6%) ($P = 0.02$) (36).38

The prevalence of isolates with decreased susceptibility to penicillin is lower in South Africa than in Europe but similar to the prevalence reported for the United Kingdom and the United States. Although there were fluctuations, the prevalence exceeded 10% only in 1 of 5 years studied, and no increase in prevalence was observed over the study period. Some studies have shown a steady increase in the prevalence of Peni isolates since they were first detected, whereas others have shown a stable prevalence over time. We found that serogroup A was associated with the lowest prevalence of decreased penicillin susceptibility. Other studies have shown the prevalence of Peni isolates to be higher among serogroup C and W135 isolates (37).

Other Study showed that Susceptibilities of *Neisseria meningitides* isolates (n =137) recovered during the period 2000–2006 in 18 different African countries, mainly those within the meningitis belt, to 11 different antibiotics all isolates were β lactamase negative and susceptible to ceftriaxone (MIC = 0.002 µg/ml), chloramphenicol (MIC, 0.38 to 1.5 µg/ ml), and ciprofloxacin (MIC, 0.002 to 0.012 µg/ml). Three of the isolates (2%) displayed reduced susceptibility to penicillin G; two of these isolates were in serogroup A (isolated in Ethiopia and Somalia), and one was in serogroup Y (isolated in Senegal). The two isolates with the highest penicillin G MICs (MIC, 0.25 µg/ml and 0.38 µg/ml for isolates in serogroups A and Y, respectively) were the sole isolates displaying reduced susceptibility to ampicillin and cefuroxime. The serogroup Y isolate was also resistant to penicillin V (MIC = 1.5µ g/ml).

Overall, 1 of 82 serogroup A isolates (<1%) displayed reduced susceptibility to rifampin (MIC = 0.38 µg/ml). Fifty two percent of the isolates were resistant to tetracycline, 74% were resistant to erythromycin, and 94% were resistant to sulfadiazine. For rifampin, two susceptible populations could be observed, one comprising mainly serogroup W-135 and Y isolates, displaying very low MICs, and one with higher MICs, comprising mainly serogroup A and X isolates. For tetracycline, a similar pattern was observed, with a resistant population (MIC, 2 to 6 µg/ml) of mainly serogroup A isolates, belonging to ST-7 or ST-2859, and a susceptible population (MIC, 0.064 to 0.38 µg/ml) consisting of isolates representing other serogroups and STs (8).

A study in Ethiopia by Assefa et al., 2013 indicates, all isolated *Neisseria meningitides* were susceptible to chloramphenicol, erythromycin and tetracycline. The results demonstrated that the highest resistance rates of *Neisseria meningitides* against cotrimoxazole 14 (100%), ceftriaxone 7 (50.0%) and ciprofloxacin 3 (21.4%), Contrary to this study, a study of meningococcal carriage in Greece showed that no strains were resistant to ceftriaxone or ciprofloxacin. Therefore, this result noted that *Neisseria meningitides* is developing a resistance to the antibiotics, ceftriaxone and ciprofloxacin, appropriate for management of meningococcal meningitis epidemic response. However, the overall finding of the result showed relatively chloramphenicol, ceftriaxone and ciprofloxacin, as most effective antibiotics in the currently included community. *Neisseria meningitides* nasopharyngeal carriage was considerably higher among older children. These high numbers of carrier children could serve as reservoirs for the transmission of *Neisseria meningitides* to the community, which could lead to serious meningococcal disease .Although the number of isolated *Neisseria meningitides* may be too small to draw meaningful conclusions, there is indication of higher cotrimoxazole and ciprofloxacin resistances too (38).

3. Objectives of the study

3.1. General Objective

- To determine the antimicrobial Susceptibility pattern of *Neisseria meningitides* isolates obtained from asymptomatic carriers.

3.2. Specific Objectives

- To determine drug susceptibility pattern of *Neisseria meningitides.*
- To determine drug susceptibility patterns of each serogroups of *Neisseria meningitides.*

4. Hypothesis

The antimicrobial susceptibility pattern of *Neisseria meningitides* is different from the previous study conducted in Ethiopia.

5. Materials and Methods

5.1. Study Area

The study was conducted at AHRI Addis Ababa, Ethiopia. Isolates were collected by the MenAfricar project from Meskan district in Gurage Zone, Southern Nations, Nationalities and Peoples Regional State, Ethiopia.

5.2. Study Design and period

Cross sectional study was conducted from July to November 2016 on *Neisseria meningitides* isolates obtained from the MenAfricar project.some sociodemographic factors including age, sex, residence were taken from the data base of the project retrospectively while for AST is a prospective cross sectional study.

5.3. Source Population

Neisseria meningitides Isolates collected during MenAfricar project from Meskan and Mareko Districts, Gurage Zone, Southern Nations, Nationalities and Peoples Regional State, Ethiopia.A total of 4110 study participants were screened.

5.4. Study population

One hundred eighty seven *Neisseria meningitides* isolates collected from MenAfricar project at AHRI.

5.5. Inclusion and Exclusion criteria

5.5.1.Inclusion criteria:

Neisseria meningitides confirmed isolates stored at AHRI.

5.5.2 Exclusion criteria:

Isolated samples those auto and poly agglutinate for serotype.

5.6. Variables of the study

5.6.1. Dependent variable

- Antimicrobial Susceptibility pattern of *Neisseria meningitides*

5.6.2. Independent variables

- Socio demographic characteristics
- Serogroups of *Neisseria meningitides*

5.7. Sample size and sampling methods

All stored isolates which were confirmed as *Neisseria meningitides* were included except poly and auto agglutinate isolates by using convenient sampling method.

5.8.Data collection

5.8.1. Information collection for positive *Neisseria meningitides* isolate

The following data was collected from AHRI bacteriology laboratory log book: AHRI identification code, gram staining, serogroup reaction, bio chemical test result (oxidase positive, Gram negative diplococci ,ONPG negative, GGT positive and Tributyrin negative bacteria) to make sure they were typically *Neisseria meningitides* isolates.

5.8.2. Socio demographic data

Check list was used to retrieve some socio demographic data including age, sex, and residence from AHRI data unit. This information was collected during the main project time.

5.8.3. Sub culturing of *Neisseria meningitides*

Since the isolate was biochemically identified as *N.meningitides* and all isolates were stored at-80ºc.and preserved using brain heart infusion (BHI) with beads. We took one bead and sub cultured on blood agar (BA) media and incubated in 5% CO2 at 37ºC for 18-24 hours. Only pure colonies with the characteristics of *N. meningitides* were further processed for antimicrobial susceptibility testing.

5.8.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out on isolates *of Neisseria meningitides by* using disc diffusion technique on Mueller-Hinton agar supplemented with 5% sheep blood. A suspension of the test organism was prepared by taking 3-5 colonies from blood agar plate by emulsifying in 1 ml of sterile physiological saline and incubated at 37ºC until the turbidity of the suspension become matched with turbidity standard equivalent to 0.5 McFarland. Using a sterile swab, the surface of Mueller-Hinton with 5% sheep blood agar was completely covered by

pressing and rotating the swab against the side of the tube above the level of suspension. After the plate has dried (3-5 minutes), the discs were evenly distributed on the inoculated plate using sterile forceps and incubated in 5% CO2 at 37ºc for 24 hours according to the guideline recommendations of CLSI-2014. Antibacterial susceptibility test was done for a numbers of antibiotics including Ceftriaxone (30µg), Cefotaxime (30µg), Meropenem(10µg), Azithromycin (15µg), Minocycline(30µg), Ciprofloxacin(5µg), Levofloxacin 5 µg, Trimethoprimsulfam ethoxazole (1.25/ 23.75 µg) , Chloramphenicol (30µg), and Rifampin (5µg). Diameters of the zone of inhibition around the disc are measured to the nearest millimeter using a graduated caliper in millimeters, and results were classified as sensitive, intermediate, and resistant according to CLSI (CLSI, 2014) (39).

5.9. Data Management

The data obtained kept on a secured, password protected computer and hard copies of data collection worksheets was kept securely locked for the duration of the study period.

5.10. Data Analysis

Data analysis and cleaning was done by using SPSS version 20.0 software. Data was double entered to check consistency. Frequency count and percentage used to clean and check the accuracy of data entry. Prevalence figures calculated for the total study population and separately by age groups. Chi-square test was used compare results between participants with different age groups and with the previous findings from the literature. *P-value* less than 0.05 were considered statistically significant.

5.11. Quality Assessment

The quality of our work assessed by sterility of culture media checked by incubating 3-5 % of the batch at 35^{0}c – 37^{o}c overnight to see contamination by any organisms. Those media which showed growth was discarded. *S. pneumoniae* ATCC 49619 were used as the quality control strains for each run as recommended by CLSI- guide lines.

5.12. Ethical Consideration

The proposal was approved and ethically cleared by Department Ethics and Research Committee (DERC) of Addis Ababa University (AAU), College of Health Science (COHS), School of Allied Health Sciences (SOAHS), Department of Medical Laboratory Science (DMLS) and

AHRI/ ALERT Ethical Review Committee (AAERC). As this was a study on stored samples, we obtained the AAERC for waiver of consent.

5.13. Dissemination of Results

Study results were submitted to Addis Ababa University, College of Health Science, School of Allied Health Sciences, Department of Medical Laboratory Sciences (DMLS), and AHRI/ALERT. Then the findings of the study will be presented to Department of Medical Laboratory Sciences, to the scientific community through scientific presentation and finally we will sent for publication on peer reviewed scientific Journals.

6. Results

6.1. Socio demographic characteristics

The *Neisseria meningitides* were isolated from all age groups. Among 187 *Neisseria meningitides* positive individuals 99(53%) were males and 88 (47%) were females. The highest carriage rate was observed among the age groups of 1-10 (33%) and 11-20 years (33%).The lowest rate was observed among age groups of < 1years old (3%). The highest carriage rate was observed in rural area 128(68%) than urban area 59(32%) (Table 6-1).

Table 6.1. Socio-demographic characteristics for nasopharyngeal carriage rate of *N. meningitides.*

Characteristics of the study population (n=187)		**N**	**%**
Sex	Male	99	53
	Female	88	47
Residence	Urban	59	32
	Rural	128	68
Age	<1	6	3
	1-10	61	33
	11-20	61	33
	21-30	25	13
	31-40	17	9
	>41	17	9

6.2. Serogroup distribution of *Neisseria meningitides* isolates

In this study, serotype Y accounted 12.8% (24) and majority of them 82.3 % (154) were non determinant (Table 6.2)

Table 6. 2- Serogroup Distribution of *Neisseria meningitides*.

Serogroup	Freq.	Percent
∗ND	154	82.35
W-135	1	0.53
X	8	4.28
Y	24	12.83
Total	187	100.00

∗ND - Non determinant

6.3. Antimicrobial susceptibility pattern of *Neisseria meningitides* isolates

The Antimicrobial susceptibility pattern of *Neisseria meningitides* isolates is shown table 6.3.About 62 % (116/187) of *Neisseria meningitides isolates were* resistance to cotrimoxazol and the least resistance 6(3%) was observed for Levofloxacin. Moreover, *Neisseria meningitides* isolates showed resistance to any of one drug was 49(26.2%) and 102 (54.5%) isolates were multidrug resistance (resistance to more than one antibiotic) and 36 (19.3%) isolates were susceptible to all antibiotics.

Cefotaxone, Ceftriaxone, Meropenem, Azitheromycin and Minocycline drugs have only susceptible cut of point according to CLSI guide line. So far, our finding showed resistance because they have lower from the susceptible cut of point.

For Levofloxacin there is no cut of point on CLSI guide line. Fortunately six isolates were having no zone of inhibition. So we have taken this result as resistance.

Table 6.3. Drug susceptibility profile of *Neisseria meningitides* (No=187).

Antimicrobials with Disk content		**No**	**%**
Azithromycine/AZM- 15µg	I	-	-
	R	10	5
	S	177	95
Cefotaxime/ CTX- 30µg	I	-	-
	R	26	14
	S	161	86
Ceftriaxone /CRO -30µg	I	-	-
	R	24	13
	S	163	87
Ciprofloxacin in/CPR/CIP- 5µg	I	11	6
	R	112	60
	S	64	34
Chloramphenicol/C -30µg	I	53	28
	R	7	4
	S	127	68
Meropenem/M EM -10µg	I	-	-
	R	21	11
	S	166	89
Levofloxacin 5 µg	I	-	-
	R	6	3.2
	S	181	96.8
Rifampine/RD -5µg	I	42	22.5
	R	14	7.5
	S	131	70
Trimethoprim sulfamethoxazole1/SXT 1.25/23.75µg	I	8	4
	R	116	62
	S	63	34
Minocycline -30µg	I	-	-
	R	15	8
	S	172	92

The AST patterns of *Neisseria meningitides* was assessed with gender and there is no significant difference between AST pattern and gender of the study participants. (Table 6.4)

Table 6.4. Association between Gender and Antibiotic Susceptibility Pattern.

	Males(n=99)	**Female(n=88)**	**P-value**
Azithromycine			
Sensitive	95(96%	82(93%)	0.399
Resistant	4(4%)	6(7%)	
Cefotaxim			
Sensitive	86(87%)	75(85%)	0.746
Resistant	13(13%)	13(15%)	
Ceftriaxone			
Sensitive	86(87%)	77(87.5%)	0.897
Resistant	13(13%)	11(12.5%)	
Ciprofloxacin			
Sensitive	39(39%)	36(41%)	0.833
Resistant	60(61%)	52(59%)	
Chloramphenicol			
Sensitive	95(96%)	85(97%)	0.820
Resistant	4(4%)	3(3%)	
Meropenem			
Sensitive	88(89%)	78(89%)	0.956
Resistant	11(11%)	10(11%)	
Levofloxacin			
Sensitive	97(98%)	84(95%)	0.328
Resistant	2(2%)	4(5%)	
Rifampine			
Sensitive	94(95%)	79(90%)	0.179
Resistant	5(5%)	9(10%)	

Trimethoprim sulfamethoxazole			
Sensitive	38(38%)	33((37.5%)	0.901
Resistant	61(62%)	55(62.5%)	
Miocycline			
Sensitive	92(93%)	80(91%)	0.612
Resistant	7(7%)	8(9%)	

Similarly the antibiotic resistance for specific drugs are different for all age groups but highest percentile of resistance seen at age group 11≤20 years for Trimethoprim sulfamethoxazole and the lowest resistance was seen for age group <1years old however ,there is no significant difference between age group and AST patterns (table 6.5) .

Table 6.5. Association between age and Antibiotic Susceptibility Pattern.

Drugs	Age group in year												P-value
	<1		1 ≤10		11≤ 20		21 ≤30		31≤ 40		>40		
	R	S	R	S	R	S	R	S	R	S	R	S	
Azithromycine	0	6	2	59	5	56	0	25	2	15	1	16	0.462
Cefotaxim	2	4	11	50	7	54	4	21	1	16	1	16	0.418
Ceftriaxone	1	5	12	49	6	55	3	22	0	17	2	15	0.341
Ciprofloxacin	3	3	38	23	41	20	14	11	5	12	11	6	0.124
Chloramphenicol	0	6	1	60	4	57	0	25	1	16	1	16	0.589
Meropenem	3	3	5	56	8	53	3	22	1	16	1	16	0.055
Levofloxacin	0	6	3	58	1	60	1	24	0	17	1	16	0.812
Rifampine	0	6	7	54	5	56	1	24	0	17	1	16	0.577
Trimethoprim sulfamethoxazole	4	2	37	24	41	20	15	10	8	9	11	6	0.775
Miocycline	0	6	5	56	5	56	1	24	2	15	2	15	0.886

6.4. Antimicrobial Susceptibility pattern of *N. meningitides* with different serogroup

We have also assessed the AST pattern with different serogroup and 21 of the 24 serotype Y isolates were resistant to at least one antibiotic and only one isolates for serogroup W135 resistance for CTX and CIP. However, the difference is not significant, though the number of serotype was small to do comparison (Table 6.6).

Table 6.6. Antibiotics resistance for individual serogroup

Serogroup and No of isolates	**Antibiotics**										
		AZM	**CTX**	**CRO**	**CIP**	**C**	**MEM**	**LEVO**	**RD**	**SXT**	**MINO**
X(8)	S	8	7	7	4	8	7	8	8	3	6
	R	0	1	1	4	0	1	0	0	5	2
W-135(1)	S	1	0	1	0	1	1	1	1	1	1
	R	0	1	0	1	0	0	0	0	0	0
Y(24)	S	22	22	21	11	24	23	23	24	7	22
	R	2	2	3	13	0	1	1	0	17	2
ND(154)	S	146	132	134	60	147	135	149	140	60	143
	R	8	22	20	94	7	19	5	14	94	11

Azithromycine /AZM, Cefotaxime /CTX, Ceftriaxone / CRO, Ciprofloxacin/CPR/CIP, Chloramphenicol /C, Meropenem /MEM, Levofloxacin/LVF, Rifampine/RD, Cotrimoxazol/SXT, Minocycline.

6.5. Multiple drug resistance patterns

Our findings showed 55(29.4%) of isolates were resistance to two drugs, 9(4.8%) were resistance to four drugs. From the total number of isolate, serogroup ND 13/154 isolates were resistance for more than five drugs (Table 6.7).

Table 6.7. Multi-drug resistance pattern for *Neisseria meningitides* isolates.

Serogroup	**Anti-microbial sensitivity pattern**						
	R0	R1	R2	R3	R4	≥R5	Total
ND	31	38	45	19	8	13	154
X	2	2	2	1	-	1	8
Y	3	9	7	3	1	1	24
W-135	-	-	1	-	-	-	1
Total	36	49	55	23	9	15	187
Percentile	19.3%	26.2%	29.4%	12.3%	4.8%	8%	100%

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

7. Discussion

Neisseria meningitides represents a pathogen of great public health importance in both developed and developing countries. Resistance to some antimicrobial agents used either for therapy of invasive infections or for prophylaxis of case contacts has long been recognized (15).

Increasing antimicrobial resistance among bacterial pathogens is a serious public health threat worldwide and today resistance can be found in almost every bacterial species for which antibiotic therapy exists (13).

The prevalence of *Neisseria meningitides* in our study was 320 (7.8%) from the total population we used it is comparable to the study conducted in Ethiopia by *Bårnes GK, et al*. (22) in Arba Minch, southern Ethiopia 6.6 % , in Burkina Faso 7.86% (23),in Turkey by *Gazi H. et.al.* 6.2% (4) and study conducted in Greece (7.2%) (17).In contrary higher prevalence of carriage rate reported in India (10.4%) (36) And lower nasopharyngeal carriage of *Neisseria meningitides* was reported in healthy Dutch children 46 (1.5%) (17) And in Greece children (4.0%) (10). our results showed higher nasopharyngeal carriage rate of *Neisseria meningitides* among older children less than twenty years old. The high prevalence of *Neisseria meningitides* could be due to the fact that many children may be immune debilitated due to the diseases, which were the reason for children to visit the hospital. This may, therefore, suggest that large numbers of meningococcal carriers are at high risk of developing invasive meningococcal diseases and the family members, their peers with whom they interact in the community are at risk of acquiring the pathogens.

Our finding showed only 36/187(19.3%) isolates were not having resistance for drugs what we used, the others 151(80.7%) had drug resistance which indicates more than eighty percent of our isolates were resistance for standard antibiotic for this bacteria. Moreover, *Neisseria meningitides* isolates showed resistance to one drug was 49(26.2%), multidrug resistance (resistance to more than one antibiotic) was 102 (54.5%).

Neisseria meningitides isolates resistant were 116(62%) to Cotrimoxazol, 112(60%) to Ciprofloxacine, 26(14%) to Cefotaxime, 24(13%) to Ceftriaxone, 21(11%) to Meropenenem, 15(8%) to Minocycline, 14(7%) to Rifampine, 10(5%) to Azithromycine, 7(4%) to Chloramphenicol and 6(3%) to Levofloxacin.

Contrary to the present study, a study done by Assefa *et al*.(38)all isolated *Neisseria meningitides* were susceptible to chloramphenicol, erythromycin and tetracycline but similar to our findings, even if they used small number of isolate, the highest resistance rates of *Neisseria meningitides* against cotrimoxazole were 14 (100%), ceftriaxone were 7 (50.0%) and ciprofloxacin were 3 (21.4%).

Reduced susceptibility to ciprofloxacin and resistance to rifampicin have also been reported from many countries. Resistance to ceftriaxone is claimed to have been identified and was reported from India (13).

Different to our findings study done by Rohani *et al*. showed that all strains subjected to antibiotic sensitivity testing were susceptible to chloramphenicol, rifampin, levofloxacin, cefotaxime and penicillin. The rate of resistance to cotrimoxazole was 84%.Resistance to trimethoprime- sulphamethoxazole was high among these isolates and the rate was almost similar to our reports. The widespread resistance to this antibiotic is possibly due to the early introduction of sulphonamides (35).

Contrary to our study in Greek children in 2004 showed all isolates were sensitive to ceftriaxone, rifampicin and chloramphenicol. Only one (4.5%) isolate was resistant to co-trimoxazole, while five (22.7%) showed intermediate resistance to penicillin (10).

Another study conducted on Cankaya municipality schools of Ankara province in 2005 the antibiotic susceptibilities of *Neisseria meningitides* isolates were determined for penicillin, sulfadiazine, rifampicin, and azithromycin. The resistance against sulfadiazine was 54.4%, while it was 26.9% against azithromycin **.**No rifampicin-resistant strains were detected. But ours were less resistance for azithromycin (5%) and 14(7%) of them were resistance for rifampicin.

Another study from Punjab (Ludhiana) has studied 84 Isolates obtained; all the isolates were sensitive to penicillin, ampicillin, rifampicin and ceftriaoxone. As regards to ciprofloxacin, about two third of the isolates tested were found to be 'nonsusceptible'. All the isolates were found resistant to cotrimoxazole (14).

Furthermore, it was observed that pathogenic strains were genetically related to isolates from carriers. Since asymptomatic carriers are presumably the major source of transmission of pathogenic strains, eradication of the carriage state may result in a significant reduction in invasive meningococcal disease (10).

Therefore, this result noted that *Neisseria meningitides* is developing a resistance to antibiotics, appropriate for management of meningococcal meningitis for epidemic response. However, the overall finding of our result showed relatively other than the two drugs (SXT and CIP) the others are effective antibiotics in the included community.

8. Strength and Limitation of the study

8.1. Strength of the study

Being this study used bacterial isolates from community based / household study it will represent the general population of the study site and the finding is more valid.

8.2. Limitation of the study

- We were unable to perform MIC test for Ampicillin and penicillin.

9. Conclusion

More than half of (65%) *Neisseria meningitides* isolates showed higher resistance rate for ciprofloxacin and Trimethoprim sulfamethoxazole. There was an indication of higher cotrimoxazole and ciprofloxacin resistances.

We have noted that carriage studies are important to improve our understanding of drug susceptibility patterns of *Neisseria meningitides* in the study settings.

10. Recommendation

The study results highlight the pressing need to consider large-scale antimicrobial resistance test for asymptomatic carriage. Some drugs are cheap and readily available to the local population, thus herald the emergence of resistant strains.

Antibiotic resistance surveillance should be done to monitor trends and to guide empirical treatment in the event of outbreaks.

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Annex I

Preparation of Mueller-Hinton agar plus 5% sheep (or horse) blood

To prepare Mueller-Hinton agar plus 5% sheep (or horse) blood

Follow manufacturer's instructions to prepare medium.

After autoclaving, cool medium to 50°C in a water bath.

Add 5% sterile, defibrinated sheep (or horse) blood, i.e., 50 ml of blood per liter of medium. (If a different volume of base medium is prepared, the amount of blood must be adjusted accordingly to 5%, e.g., 25 ml of blood would be added to 500 ml of base medium.)

a) Measure 60–70 ml of medium per plate into 15 x 150-mm plates, or measure 25–30 ml per plate into 15 x 100-mm plates. Agar should be poured into flat bottom glass or plastic Petri dishes on a level pouring surface to a uniform depth of 3–4 mm. using more or less agar will affect the susceptibility results. Agar deeper than 4 mm may cause false-resistance results, whereas agar less than 4 mm deep may be associated with a false-susceptibility report.
b) Freshly prepared plates may be used the same day or stored in a refrigerator (2°–8°C) for up to 2 weeks. If plates are not used within 7 days of preparation, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is on the surface, plates should be placed in an incubator (35°–37°C) until the moisture evaporates (usually 10–30 minutes). Do not leave lids ajar because the medium is easily contaminated.

Annex II

Standard Operating Procedure For Antimicrobial Sensitivity Testing (Disk Diffusion)

Aim

- To measure the susceptibilities of pathogenic bacteria to appropriate antimicrobials by a standardised disk diffusion method.

Principle

- Standard amounts of antibiotics impregnated onto absorbent paper are placed onto a specified agar plate seeded with a known concentration of bacteria. These plates are then incubated overnight in defined conditions (e.g. temperature and atmosphere). The antibiotic diffuses through the medium from the disk and if the bacteria are sensitive to the antibiotic then growth will be inhibited leaving a zone of clearing. The diameter of this zone is measured and compared with CLSI guidelines for zone sizes to determine whether the bacteria should be classified as sensitive, intermediately sensitive or resistant to the antibiotic (40).

- Various factors have been identified as influencing disk diffusion susceptibility tests. These include the type of medium, excess surface moisture on the medium, agar depth, disk potency, inoculum concentration, pH, cation content, incubation atmosphere, temperature and duration, and β-lactamase production by test organisms. It is therefore of the utmost importance to follow the CLSI guidelines on testing methods precisely in order for the CLSI interpretation tables to be valid. Regular quality control is also essential.

- CLSI methods are regularly reviewed and updated and so it is important that the most recent versions of CLSI standard documents are used.

- In some circumstances, accurate results cannot be achieved by simple disk diffusion testing and E-test MICs are required (described in SOP MIC-002).

Method

Reagents and equipment

- Agar plates (3-4 mm deep)
- Antibiotic impregnated disks
- Sterile saline (2-5ml)
- Sterile cotton tipped swabs
- Automatic disk dispenser or template with 5 or 6 disk spacing pattern
- Forceps
- Incubator with correct atmosphere at appropriate temperature
- Ruler

Inoculation:

- Subculture the organism to be tested onto a non-selective agar plate and incubate for 18-24 hours to obtain a pure growth.
- Remove the antibiotic disks from the fridge so they reach room temperature before the container is open (to avoid condensation and subsequent deterioration). Containers must contain active desiccant. Replace the disks and container in the refrigerator as soon as you have finished using them. Do not use disks past their expiry date.
- Using a straight wire or loop, touch at least six individual colonies from the pure culture and transfer them into sterile saline solution.
- Emulsify the colonies in sterile saline to give an equivalent turbidity of 0.5 McFarland Standard (equivalent to a growth of 1-2 X 10^8 CFU/mL for *E. coli* ATCC 25922).Check the turbidity by comparing with the standard by eye.

- Within 15 minutes after adjusting the turbidity of the inoculum, immerse a sterile cotton swab into the emulsion. Press the swab against the inner side of the tube, above the fluid level, to remove excess fluid.

- Use the appropriate plate and incubation conditions for *Neisseria meningitides:-media-* Mueller Hinton with 5% sheep blood and incubation condition -35-37°C in 5% CO_2 for 20-24h.

Application of disks

- Place disks of the appropriate antibiotics for the species on the plate using the automatic disk dispenser or manually using disk spacing template. Single disks may be handled using forceps.

- Avoid placing penicillin and cephalosporin disks next to each other.

- Disks need to be applied evenly on the agar surface; press gently on the disk after application.

- Because some antibiotics diffuse almost instantaneously, a disk should not be relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.

- Disks should be applied no later than 15 minutes after the plates have been inoculated. Similarly, once the disks are applied, they should be put in the incubator within a 15 minutes interval to prevent pre-diffusion of the antimicrobial at room temperature.

- Invert the plates and incubate in the correct atmosphere for the appropriate time.

- Agar plates should not be placed in stacks of more than 10 because the middle plates will take longer to reach the incubator temperature. This delay could cause overlarge zones.

Reading and interpreting results

- After the incubation is complete, remove the plates from the incubator and measure the zone diameter, in mm, using a ruler. To measure zone diameter the ruler has to be held on the back of the inverted plate over a dark, non-reflecting background, and illuminated

from above (except oxacillin and vancomycin, which should be read with transmitted light i.e. plate held up to light source and any discernible growth within the zone of inhibition taken as indicative of resistance).

- The diameter of the zone of inhibition includes the diameter of the disk. The end of the zone should be taken as the area showing no obvious visible growth that can be detected with unaided eyes. Ignore faint growth of tiny colonies that can only be detected with a magnifying lens at the edge of the zone of inhibited growth

- When measuring zones on Mueller-Hinton plates with blood, the zone of growth inhibition should be measured NOT the zone of haemolysis inhibition. The zones should be measured from the upper surface of the agar, illuminated with reflected light, with the cover removed.

- The growth on the plates must be even and near confluent. If there are only isolated colonies, the test must be repeated.

- For staphylococci, the cefoxitin result should be reported as "oxacillin".Isolates that are resistant to cefoxitin should be reported as resistant to all beta-lactams (i.e. penicillin, oxacillin, co-amoxiclav, ceftriaxone)

- Compare the measured zone size with that for the species and antibiotic combination in Appendix 1.Record the zone diameter and the category taken from the table. If the organism and zone size is not included in the table then refer to the CLSI documents for further information. Results can usually be put into one of the categories below:

- Susceptible (S) includes isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

- Intermediate (I) includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels, and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g. quinolones and β-lactams in urine) or when a higher than normal dosage of a drug can be used (e.g. β-lactams). The

"intermediate" category also includes a "buffer zone" which should prevent small, uncontrolled technical factors from causing major discrepancies in interpretation, especially for drugs with narrow pharmacotoxicity margins.

- Resistant (R) includes isolates that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate MICs or zone diameters that fall in the range where specific microbial resistance mechanisms are likely (e.g. β-lactamases) and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

Quality assurance

- Regular testing of the correct quality control strains is essential to provide assurance that the media and disks being used most frequently are performing satisfactorily. Check the relevant CLSI documents and perform relevant quality control at the same time as doing the test.

Limitations

- Although the quality control carried out during manufacture of antibiotic disks is usually of a high standard, disk content may deteriorate during storage. This is one reason why regular quality control is essential. Correct storage and rotation of disk stocks is essential to maintain this quality. The main stock of disks should be stored at -20°C with a small quantity for current use being kept at 4°C. The disks required for the day's work should be brought to room temperature before opening the container. Desiccant should be changed regularly and kept in sealed containers. The oldest disks must be used first and always before their expiry date.

Annex III

Risk assessment

<table>
<tr><td colspan="2">COSHH risk assessment - University of Oxford COSHH Assessment Form</td></tr>
<tr><td>Description of procedure
Determination of bacterial antimicrobial susceptibilities by disk diffusion</td><td>Substances used
1. Cultured bacteria
2. Antimicrobial impregnated disks</td></tr>
<tr><td>Quantities of chemicals used
5ml of bacterial suspensions</td><td>Frequency of SOP use
Daily</td></tr>
<tr><td>Hazards identified
1. Autoclaved liquid
2. Potentially infectious material in sample
Potentially pathogenic bacteria</td><td>Could a less hazardous substance be used instead?
No</td></tr>
<tr><td colspan="2">What measures have you taken to control risk?
1. Training in good laboratory practices (GLP)
2. Appropriate PPE (lab coat, gloves, eye protection)
3. Use of biosafety cabinet for reading of plates /follow-up of BSL-3 organisms (e.g. B. pseudomallei)</td></tr>
<tr><td colspan="2">Checks on control measures
Observation and supervision by senior staff</td></tr>
<tr><td>Is health surveillance required?
No</td><td>Training requirements
1. GLP
2. Specific training in this SOP</td></tr>
<tr><td>Emergency procedures
1. Report all incidents to Safety Adviser
2. Use eyewash for splashes
3.Clean up spills using 1% Virkon or chemical spill kit</td><td>Waste disposal procedures
1.Sharps discarded into appropriate rigid containers for incineration
2.Infectious waste discarded into autoclave bags or 1% Virkon solution prior to autoclaving and subsequent incineration
3. Chemical waste disposed of according to manufacturer’s instructions</td></tr>
</table>

Annex IV

Antimicrobial disk testing panels and interpretations

Zone Diameter Interpretive Standards for *Neisseria meningitides* (CLSI-2014)

Test/report group	Antimicrobial agent	Disk content	Zone diameter interpretive criteria (nearest whole mm)		
			S	I	R
PENICILLINS	Penicillin		-	-	-
	Ampicillin		-	-	-
CEPHEMS	Cefotaxime or	30µg	≥34		
	Ceftriaxone	30µg	≥34		
CARBAPENEMS	Meropenem	10 µg	≥ 30		
MACROLIDES	Azithromycin	15 µg	≥ 20		
TETRACYCLINE	Minocycline	30 µg	≥ 26		
FLUOROQUINOLONES	Ciprofloxacin	5 µg	≥ 35	33-34	≤ 32
	Levofloxacin	-	-	-	-
FOLATE PATHWAY INHIBITORS	Sulfisoxazol Trimethoprim-sulfamethoxazole	1.25/23.75µg	≥ 30	26-29	≤ 25
PHENICOLS	Chloramphenicol	30 µg	≥ 26	20-25	≤ 19
ANSAMYCINS	Rifampin	5 µg	≥ 25	20-24	≤ 19

Annex V

Laboratory Check list

First I would see bacteriology log book and were selected positive *Neisseria meningitides* sample ID and register on my own book. All isolated samples were preserved by Beads almost all isolated samples were in a good condition.

1. Select *Neisseria meningitides* from their log book positive for GGT and negative for Tributrine and ONPG.

2. Select serogroups by their agglutination test.

3. Collect information from data manager, like socio-demographic data and will compare and contrast with laboratory information.

Socio demographic data collection from data room chick list for positive *Neisseria meningitides* isolates.

AHRI ID	**Age**	**sex**	**residence**				

Bacteriology laboratory data collection from log book for positive *Neisseria meningitides* isolates chick list.

AHRI ID	**Gram stain** Gram negative diplococci	**ONPG** negative	**GGT** positive	**Tributyrin** negative	**Serogroup**	**Oxidase** positive	

Then everything is ready-

Isolates to be tested should be cultured onto blood agar plate and incubated in a CO2 enhanced atmosphere (5% CO2 in a CO2-incubator or candle-extinction jar) at 35±2ºC for 20-24h.prior to testing.

As I mentioned above follow the procedure and control the result by using laboratory sheet. Subculture by blood agar media.

Subculture date:---/-------/----- Performed by:----------					
Sample ID	AHRI ID	Having growth	Insufficient growth	No growth	Remark

:-Insufficient growth (may have one or two inoculums will subculture again).

DST media after subculture MHA plus 5% sheep blood.

Date of testing for DST:-----/---------/---------- Performed by:---------- Interpretation of susceptibility: S=susceptible I=intermediate R=resistant								Remark
Sample ID	AHRI ID	Organism	Antibiotic#1	Antibiotic#2	Antibiotic#3	Antibiotic#4	Antibiotic#5	
01			mm S I R	mm S I R	mm S I R	mm S I R	mm S I R	

Sample sheet for recording data and quality control information: - After 20-24h.of incubation, will check the results for the QC strain(s) against the standard acceptable ranges; if they are within control limits, will continue reading results for the test isolate and record disk diffusion result in mm.

Declaration:

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

Fikerte Mulatu _____ _____

Principal investigator Signature Date

Approval of advisors:

Mr. Kassu Desta (PhD candidate) _____ _____

Signature Date

Mr.Biruk Yeshitila (MSc) _____ _____

Signature Date

Dr.Adane Mihret (PhD) _____ _____

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Place:

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Date of submission _____