MOLECULAR DETECTION OF VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES OF *STREPTOCOCCUS PYOGENES* ISOLATED FROM THE OROPHARYNX OF FEBRILE PATIENTS IN ADAMA AND WONJI, ETHIOPIA.

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A THESIS SUBMITTED TO GRADUATE PROGRAM OF ADDIS ABABA UNIVERSITY COLLEGE OF HEALTH SCIENCES SCHOOL OF MEDICINE DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY IN PARTIAL FULFILLMENT FOR THE DEGREE OF MASTER IN MEDICAL MICROBIOLOGY.

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ABBREVIATIONS AND ACRONYMS

AAU	Addis Ababa University
ACSHMC	Adama Comprehensive Specialized Hospital Medical College
AGN	Acute Glomerulonephritis
AHRI	Armauer Hansen Research Institute
ALB	Alkaline Lysis Buffer
ALERT	All Africa Leprosy, Tuberculosis and Rehabilitation Training Center
AOR	Adjusted Odd Ratio
APC	Antigen Presenting Cell
APHRRLC	Adama Public Health Research and Referral Laboratory Center
ARF	Acute Rheumatic Fever
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
bp	Base Pair
BSC	Biosafety Cabinet
CDC	Centers for Disease Control and Prevention
CLSI	Clinical Laboratory Standard Institute
$cMLS_B$	Constitutive Macrolide Lincosamide Streptogramin B
CO_2	Carbon Dioxide
COR	Crude Odd Ratio
DNA	Deoxyribo - Nucliec Acid
ER	Erythromycin Resistant
ERC	Ethical Review Committee
ERGAS	Erythromycin Resistant Group A Streptococci
erm	Erythromycin Ribosomal Methylase
GAS	Group A Streptococcus
GPC	Gram Positive Cocci
ICR	Inducible Clindamycin Resistant
ID	Identification number
iMLSB	Induced Macrolide Lincosamide Streptogramin B

MDR	Multidrug Resistant
MHA	Mueller Hinton Agar
MHC	Major Histocompatibility Complex
MIC	Minimum Inhibitory Concentration
MLSB	Macrolide Lincosamide Streptogramin B
MRGAS	Macrolide Resistant Group A streptococci
NF	Necrotizing Fasciitis
Pbp	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PI	Principal Investigator
PYR	Pyrrolidonyl Arylamidase
RHD	Rheumatic Heart Disease
rRNA	Ribosomal Ribonucleic Acid
SAg	Superantigen
SBA	Sheep Blood Agar
SETA	Severe Typhoid Fever Surveillance in Africa
SOPs	Standard Operating Procedures
Spe	Streptococcal Pyrogenic Exotoxins
SPSS	Statistical Package for the Social Sciences
ssa	Streptococcal Superantigen
STSS	Streptococcal Toxic Shock Syndrome
TCR	T-cell Receptor
TR	Tetracycline Resistant
UK	United Kingdom
URTI	Upper Respiratory Tract Infection
USA	United States of America
UV	Ultra violet
VF	Virulence Factor
WHO	World Health Organization

ABSTRACT

Background: *Streptococcus pyogenes* also called Group A Streptococcus (GAS) is a significant human pathogen that causes several diseases including pharyngitis, skin infections, rheumatic fever and rheumatic heart disease. GAS produces a variety of virulence factors such as toxins/superantigens, proteases, DNases. Detection of virulence genes can be used to determine the pathogenic potential of a strain. Although GAS is generally thought to be susceptible to β-lactams, an increasing trend of treatment failures and resistance to macrolides and tetracyclines have been observed. There is a paucity of data on the molecular epidemiology of virulence factors and antibiotic-resistance of *S. pyogenes* in Ethiopia.

Objective: To assess virulence factors, antimicrobial susceptibility profile and drug resistance genes of oropharyngeal *S. pyogenes* isolated from febrile patients.

Method: A cross-sectional study was conducted on 201 *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Oromiya, Ethiopia. Standard microbiological procedures were used for bacterial growth and identification. Bacterial DNA was extracted using an alkaline lysis buffer. Multiplex PCR was conducted to identify virulence factors for the production of superantigens (SpeA, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, SmeZ and ssa); proteases (SpeB, spyCEP, scpA, Mac and sic); and DNases (sdaB, sdaD, sdc and spd3), erythromycin resistance genes [mef(A), erm(B) and erm(A)-TR] and tetracycline resistance genes [tet(M), tet(O), tet(K)and tet(L)]. SPSS version 27 was used for statistical analysis. Majority of the results were summarized by frequency and percentage distributions.

Result: This study was performed on 201 *S. pyogenes* isolates. We investigated the presence of 20 different GAS virulence factors and identified 18 of them with variable proportions. Relatively higher frequencies of Mac (24.9%), SmeZ (16.9%), scpA (15.4%), sic (12.9%), SpeK (12.4%) and SpeI (10.0%) genes were identified. Our antibiotic susceptibility testing showed that 10% of the GAS isolates were non susceptible to penicillin. High resistance rate to tetracycline (54.2%), quinupristin-dalfopristin (43.3%) and erythromycin (19.9%) were also observed. The overall multidrug resistant (MDR) rate was 14.9%. However, all GAS isolates were 100% sensitive to linezolid. M phenotype was observed in 85% of erythromycin resistant GAS isolates and mef (A) gene was identified in 96.9% of the erythromycin non susceptible GAS isolates. Among tetracycline resistant genes tet(M) was identified in 98.2% of the tetracycline non susceptible GAS isolates.

Conclusion: In this study, we have identified 121 (60.2%) of the GAS isolates had at least one or more types of virulence factors and again more than half of the isolates,156 (77.6%) were resistant to one or more types of antibiotic classes. We also found that, an emerging penicillin non susceptible GAS isolates contrary to the previous reports and this might be an alarming condition. The most common erythromycin resistance mechanism in our study was efflux pumping which was encoded by the mef(A) gene where as that of tetracycline resistance mechanism was target site modification by ribosomal protection protein which was encoded by the tet(M) gene. Linezolid may be one of the promising drugs for the treatment of drug resistant GAS strains. The identification of different virulence factors and the increasing trends of drug non susceptibility especially to penicillin among GAS isolates requires a concern for continuous surveillance of the pathogen.

Key Words: *Streptococcus pyogenes*, Group A Streptococci, antibiotic sensitivity testing, drug resistance genes, virulence factor, toxin, superantigen, Protease, DNase, Ethiopia

1 INTRODUCTION

1.1 Background Information

Streptococcus pyogenes, also called Group A streptococcus (GAS) is a medically important gram-positive bacteria of the genus Streptococci with genome size of about 1.85 – 1.9 Mb (1–3). It is one of the major human pathogenic bacteria that cause a wide range of diseases ranging from mild pharyngitis (strep-throat) and impetigo (skin infection) to severe and life-threatening infections such as necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) (2–7). If untreated, the mild infections of pharyngitis and impetigo can lead to immune-mediated post infectious sequelae such as acute rheumatic fever (ARF), rheumatic heart disease (RHD) and acute glomerulonephritis (AGN), which are a major problem worldwide, especially in developing countries (7–9).

GAS is responsible for about 750 million infections annually, of which approximately 18 million cases and 517,000 deaths per year are associated with severe GAS disease making it one of the top bacterial causes of global morbidity and mortality (3,7,9–11). Much of the GAS-associated mortality occurs in low-income regions and populations. Of the GAS infections, RHD and serious streptococcal invasive diseases are associated with the greatest global mortality rate up to 25% (9,12). The case fatality rate of NF and STSS are much higher and could reach to 50% in some cases (13). Hence, GAS is ranked among the top 10 human pathogens causing infection-related deaths in the world (3).

GAS disease has been described in all human populations, but school-aged children are the most affected group worldwide (1,5,9,10,14). Streptococcal pharyngitis which is characterized by fever and sore throat is the most common type of disease caused by GAS and again it is highly prevalent in school age children (15–17).

S. pyogenes harbors many different virulence factors that contribute to its complex pathogenicity. These include, extracellular colonization factors, toxins or superantigens, tissue-degrading enzymatic factors and antibiotic resistance genes (1,3).

According to Severe Typhoid Surveillance in Africa (SETA) project, there was a high report of febrile cases in Adama and Wonji, Ethiopia and large amount of oropharyngeal S. *pyogenes* were identified from febrile patients living in this area (18). S. *pyogenes* can exist as an asymptomatic carriage or acute pharyngitis in the human oropharynx and hence, it is important to know the profile of oropharyngeal GAS isolates as an important input for infection prevention and control strategies.

However, in Ethiopia there is limited data about GAS virulence factors and antibiotic susceptibility profile in general and among febrile patients in particular. Therefore, such a study is useful in generating valuable evidence for epidemiological investigation of GAS infections.

1.2 Statement of the Problem

Recently, GAS is a major cause of global infection-related morbidity and mortality (19). GAS primarily causes upper respiratory tract infections and most frequently causes pharyngitis among children and adults (6,15,20–23). The annual case of pharyngitis is estimated to be about 600 million cases worldwide (7,9). Etiologically, GAS is responsible for about 5% to 30% of pharyngitis cases (20).

Among microbial causative agents of pharyngitis, GAS is the only agent that requires an etiologic diagnosis and specific treatment because it can trigger post-streptococcal systemic complications such as acute rheumatic fever (ARF) and acute glomerulonephritis (AGN) which occur 1-3 weeks after the pharynx infection with GAS (20,21,24). ARF is the result of an autoimmune response to GAS pharyngitis in which antibodies created in response to GAS infection cross-react to one's own tissues (15)**.** The long-term damage to cardiac valves caused by ARF, which can result from a single severe episode or from multiple recurrent episodes of the illness, is known as rheumatic heart disease (RHD) and is a notable cause of morbidity and mortality in resource-poor settings around the world (9,25). Severe cases of ARF and RHD are estimated to kill half a million people each year globally (9,26).

ARF is a preventable, leading cause of pediatric heart disease (27). Developing countries have the highest documented rates of rheumatic fever worldwide (28). Globally, more than 282,000 new

cases of RHD are seen per year with the highest prevalence rate of about 2 per 100 inhabitants in developing countries (15). In Ethiopia, RHD has an overall prevalence rate ranging from 4.6 to 7.1 per 1000 children, which is among the highest rates in the world (28).

The pharynx serves as a reservoir for GAS that causes pharyngitis or sore throat, as well as invasive infections with STSS and post-streptococcal sequelae like RHD (2). These infections impose significant loss of health and wealth on society and the problem is common in developing countries like Ethiopia where the prevalence of RHD is the highest in the world (13,28).

The pathogenic properties of GAS strains are often linked to the production of multiple virulence factors such as toxins/superantigens, enzymes (DNases, proteases) and antibiotic resistance genes. The presence of these virulence factors is an indicator of its invasiveness. Profile of the virulence factors encoded by the particular strain is part of strain characteristics and their presence/absence can be used as a diagnostic method and simple tool in clinical diagnosis (17). However, in Ethiopia, there was no data about GAS virulence factors. This study has described the exotoxins/superantigens, proteases and DNases of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia.

Until today, *S. pyogenes* remains universally sensitive to penicillin which has been the drug of choice for streptococcal infections for more than 80 years (22). For patients with penicillin or beta-lactam antibiotics allergies, macrolide drugs are recommended as an alternative therapy. However, high macrolide-resistant GAS strains are on the rise worldwide (6). The prevalence of erythromycin-resistant (ER) *S. pyogenes* strains has been reported from an increasing number of countries in recent years (22,29,30). Meanwhile, ineffective or delayed treatment, and infection with drug-resistant GAS leads chronic immune-mediated disorders such as ARF and AGN (31–34). But the antibiotic susceptibility profile and antibiotic resistance genes of GAS is not well studied in our country, particularly in Adama and Wonji, Ethiopia.

1.3 Rationale and Significance of the Study

Since the 1980s, *S. pyogenes* continues to have devastating effects on public health and the national economy as they mainly affect children and young adults across the world (35,36). As the epidemiology of GAS infections evolves, a rapid and reliable characterization of the isolates remains essential for epidemiological analysis and infection control (3).

Virulence factors such as toxins, proteases or DNases present in the particular GAS strain can be the predictor of its invasiveness (17,37,38). Streptococcal pyrogenic exotoxins (Spe) act as a superantigen which abnormally activates enormous T-lymphocytes which in turn induces fever and massive release of cytokines. High release of cytokines resulted in multiorgan failure, shock, and even death (31,39). The distribution of virulence factors varies in different areas/regions, climatic conditions and countries of the world (17). Epidemiological studies, which provide the distributions of the type of *S. pyogenes* strains in communities, are of basic importance for the diagnosis, identification, pathogenesis, prompt treatment and control of GAS infections (3,40).

Penicillin has been used routinely for treating GAS infections for the last eight decades and yet most studies indicated the susceptibility of GAS to penicillin treatment. However, an increasing trend of erythromycin and tetracycline resistance *S. pyogenes* have been observed during the last few years in different parts of the world (31,41–43). The main factors attributed to drug resistance was over-prescription and random use of antibiotics (8,44). Hence, understanding the antibiotic susceptibly profile and identification of the drug resistance genes of *S. pyogenes* play vital role in intervening GAS infections.

Therefore, characterization of the different virulence factors of GAS as well as understanding its antibiotic resistance profile and mechanism of resistance is important for the study of epidemiology, pathogenesis, clinical diagnosis and for taking appropriate interventions.

2 LITERATURE REVIEW

2.1 Virulence Factors of *S. Pyogenes* (GAS)

S. pyogenes contains a large number of virulence factors (VFs) which are either secreted or cell bound and contribute to its pathogenicity (1,42,45). These VFs impair the host immune system by mediating adhesion to host epithelial cells and resisting to opsonophagocytosis through degradation of chemokines and chemotactic factors like IL-8, activation of massive non-specific T cell subpopulation, massive cytokine release, cleavage of IgG, and degradation of neutrophil extracellular traps (NETs) (46). The surface expressed VFs are vital for colonization, establishment of infection and the development of disease whereas the secreted VFs are the major mediators of tissue damage and toxicity seen during active infection (47). M protein is one of the potent surface expressed VFs whereas streptococcal pyrogenic exotoxins or streptococcal superantigens, proteases and DNases are secreted VFs (45).

M protein is a fibrillar cell wall protein that facilitates adhesion of GAS to human cells and prevents opsonophagocytosis of the GAS by the immune cells, meanwhile contributing to the overall burden of GAS infections (3,12). M protein is probably the most notorious virulence factor that these bacteria possess (42). To date, according to CDC and other studies, more than 250 emm types have been identified and reported (3,25,46,48,49). But emm typing is a genome sequencing-based method which is technically and financially costly and not feasible in this study.

GAS superantigens (SAgs), commonly referred to as the streptococcal pyrogenic exotoxins (Spe) are also considered to be chief contributors of GAS pathogenesis (1,3,40,50–53). Their importance in disease pathogenesis resides in their ability to bypass the regular antigen presentation process and overstimulate immune activation. Conventional antigen presentation by antigen presenting cells (APCs) to T cells is mediated by the interaction of the major histocompatibility complex (MHC) molecules and the T-cell receptor (TCR). This process stimulates approximately 0.01% of the T-cell repertoire. Conversely, unprocessed SAgs bind directly to the MHC II molecules on the surface of APCs and to the Vβ variable region of the TCR, resulting in the stimulation of about 25% of the T cell population followed by the massive release of inflammatory cytokines (53). These inflammatory cytokines induce aggressive immunological reactions leading to tissue

damage, multiorgan failure, toxic shock syndrome, and even death of the patient (2,31,36,54,55). Since the major biological niche for S. pyogenes is the upper respiratory tract, SAgs have likely evolved to function in the context of asymptomatic nasopharyngeal colonization and/or pharyngitis (52).

GAS strains that cause invasive infections usually produce one or more of the following superantigens (SAgs): SpeA, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, SmeZ, and ssa. These are responsible for super-antigenic activation of T cells (2). Some of these SAgs are chromosomally encoded; others are related to the presence of mobile genetic elements, lysogenic bacteriophages, or putative bacteriophages (17,52,53). Most super antigenic toxins, such as SpeA and SpeC, are often harbored and disseminated by genetic mobile elements, such as lysogenic bacteriophage (2,45,56). SAgs are well recognized in the pathogenesis of scarlet fever and the streptococcal toxic shock syndrome (TSS) (52). SpeA and SpeC toxins have been recognized as the most likely causative agents of STSS in most cases (3,47). On the other hand, cases of scarlet fever have been correlated with the carriage or acquisition of speA, speC and ssa (57). Furthermore, studies also show that the expression of speL, speM and speK genes have been associated with acute rheumatic fever (53).

GAS also releases a wide array of proteases (peptidases) that catalyze the hydrolysis of host cell peptide bonds facilitating GAS entry deep into the tissue. The most proteases identified in GAS are cysteine protease B, previously known as streptococcal pyrogenic exotoxins B (SpeB), Mac, C5a peptidase (ScpA) and SpyCEP protease as well as streptococcal inhibitor of complement (sic) protein (53).

SpeB is not a toxin but cysteine proteinase that degrades a wide variety of host and bacterial cellular and extracellular components including matrix glycoproteins and complement components. Therefore, SpeB is involved in bacterial evasion from the host defense system and in systemic dissemination. In general, SpeB plays an important role in the internalization and penetration of GAS into human cells and tissues (2).

The other pathogenicity of GAS relies on GAS DNases. DNases contribute to the pathogenesis of *S. pyogenes* through cleavage of host cell neutrophil activity. Most common GAS, DNases are

SdaB, SdaD, Sdc and Spd3. Many DNases are prophage associated, and therefore they are only found in certain *S. pyogenes* strains (47).

Recently, the simultaneous detection of 20 GAS virulence factors (spd3, sdc, sdaB, sdaD, speB, spyCEP, scpA, mac, sic, speL, K, M, C, I, A, H, G, J, smeZ and ssa) are developed in a four low volume multiplex PCR reaction method (6,17).

However, in Ethiopia the most circulating superantigens, proteases and DNases are not well-known. Thus, this study will try to generate important data on the distribution of oropharyngeal GAS virulence factors in Adama and Wonji, Ethiopia.

2.2 Antibiotic Susceptibility Profile of *S. pyogenes* (GAS)

GAS is an important public health problem causing significant morbidity and mortality worldwide (58). In the last 40 years, serious GAS infections have shown dramatic increases throughout the world because of antigenic change and antibiotic resistance (35). Currently, GAS is one among the top life-threatening infectious pathogens. Among the numerous diseases it causes, pharyngeal infection is the commonest type and the infection rate is higher among children (59). Throat culture is the gold standard for diagnosing pharyngitis caused by streptococcus and it has a sensitivity ranging between 90% and 95% (20). However, the absence of early diagnoses, in appropriate treatment and or infection with drug-resistant strain of pharyngeal and skin infection by GAS leads to the complex systemic and fatal type of diseases like RHD and STSS (28,31–34).

Antimicrobial resistance is considered a serious drawback in the management of patients with infectious diseases; but still, penicillin is the antibiotic of choice for treating GAS infections in those patients not allergic to it (22,31,41,42). However, resistance to other antimicrobial agents was reported worldwide (60). A sudden worldwide increase in antimicrobial resistance in *S. pyogenes* has been observed during the last few years (31,43). In patients who are allergic to penicillin or beta-lactam antibiotics, macrolides and lincosamides are alternative treatment choices (20,22). However, an increased prevalence of macrolide-resistant GAS (MRGAS) has been reported in many countries of the world (2,6,25). Recent reports on tetracycline resistance (TR) identified the ineffectiveness of this drug against infections with GAS (31,61–65). A fluctuating

trend has been observed in the antibiotic resistance pattern from different parts of the world (8). Resistance to macrolide and tetracycline among GAS has been possibly linked to the spread of a particular clone by selection pressure due to over-prescription and random usage of these antibiotics (8,31). The increased use of macrolide antibiotics is correlated with an increased rate of resistance to erythromycin among isolates of GAS (56).

Macrolide resistance in GAS was first reported in 1968, and since then, the resistance has been progressively increasing worldwide (66). An increasing erythromycin resistance (ER) GAS isolates was noted in the 1990s, and in some countries, this resistance peaked in the early 2000s (66,67). The rates vary between 5% and 40%, with the highest prevalence in Asia and the lowest prevalence in Europe and the USA, suggestive of a geographic variation in the resistance rates and the prevalence of resistance mechanisms. The earlier studies from India also reported about the sharp increase of 2%–38% macrolide resistance among GAS isolates (8). Recent macrolide resistance proportions have been reached 32.8% in Spain (reaching 60% in some regions of the country), 30% - 40% in Japan, 40% in Belgium and 22.8% in Greece; and the proportion of macrolide resistance GAS is reportedly high in China which is 98.4% (6,24,54,68–71).

Performing double disk diffusion test (D -test) is also important for the identification of phenotypic macrolide resistance. D-test for GAS is primarily performed to determine inducible clindamycin resistance (ICR) by macrolide resistance strains. Initially macrolide or erythromycin resistance and clindamycin sensitive or intermediate *S. pyogenes* isolates were used for D-testing. While performing D-test, we may identify three categories of macrolide resistance phenotyping also called Macrolide Lincosamide Streptogramin B (MLS_B) resistance phenotyping. These are: M phenotype, constitutive MLS_B ($cMLS_B$) and induced MLS_B (iMLSB) resistance phenotype (5,22,60,67).

M phenotypes are those resistant to macrolide antibiotic (erythromycin) only but susceptible to lincosamide (clindamycin) and streptogramin B. cMLSB resistant phenotype is when *S. pyogenes* is resistant to both erythromycin and clindamycin at the same time. If *S. pyogenes* is initially resistant to erythromycin and sensitive to clindamycin by disk diffusion method when done separately or placed far apart, but becomes resistant to clindamycin when placed nearby with

erythromycin 12mm apart and the resistant is characterized by blunting shape of resistance around clindamycin on the side adjacent to erythromycin, we call it iMLSB resistant phenotype or ICR, i.e., clindamycin resistance is induced from erythromycin resistant strain. Studies showed that, mef(A) gene which encodes macrolide efflux pumping is associated with M phenotype whereas erm genes [erm(B) and erm(A)-TR] which encodes target site modification by erythromycin methylase enzyme are associated with cMLSB or iMLSB phenotypes (5,22,67,72).

Knowing the macrolide resistance phenotypes are helpful in understanding the drug resistance genes and mechanism of resistance, especially in resource poor settings where there are limited advanced molecular tests like polymerase chain reaction (PCR). This provides important information for appropriate treatment and to prevent the invasive spread and also to prevent infection with drug resistance strains.

However, the drug resistance profile and macrolide resistance phenotyping of GAS among febrile patients in Ethiopia, particularly in Adama and Wonji were not known. Hence, this study generated data and provided some important information for the existing gaps.

2.3 Erythromycin and Tetracycline Resistance Genes of *S. pyogenes* (GAS)

2.3.1 Erythromycin Resistance Genes

Major mechanisms of macrolide resistance in GAS are target site modification and macrolide efflux pumping (8). Several resistance genes, mef(A), erm(B), and erm(A) subtype erm (TR), are associated with ER in GAS (12,49). mef(A) encodes an efflux pump which selectively removes macrolides from the bacterial cell and is commonly associated with the M phenotype (5,24). erm(B) and erm(A) -TR encode 23S rRNA methylases which mediate target site modification and result in cMLSB and or iMLSB resistant phenotyping. Some studies indicate, erm(B) is commonly associated with the cMLSB phenotype, while erm(A) -TR is associated with the iMLSB phenotype (5,67,72).

2.3.2 Tetracycline Resistance Genes

The tetracycline resistance (TR) genes in GAS are; tet(O), tet(M), tet(K) and the tet(L) genes (67,73). The tetracycline resistance mechanisms in GAS are ribosomal protection protein and drug efflux pump. tet(O) and tet(M) encodes proteins that interfere with the binding of tetracycline to its target 30S ribosome in bacteria which often co-occur with ER genes, due to carriage of the resistance determinants on the same mobile genetic element (12,67). These genes code for tetracycline resistance ribosomal protection proteins. The tet(K) and the tet(L) genes, codes for efflux-mediated tetracycline resistance (3,73).

Hence, surveillance of drug resistance genes and the newly variant strains play pivotal roles in infection prevention and control of GAS diseases in developing nations like Ethiopia. But in Ethiopia, particularly in Adama and Wonji, the distributions of drug resistance GAS stains are not characterized at all particularly among febrile patients. Probably, this study will be the first of its kind which will try to answer such limitations.

3 OBJECTIVES

3.1 General Objective

To determine the distribution of virulence factors and drug resistance genes of *Streptococcus pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia.

3.2 Specific objectives

1. To determine the distribution of virulence factors of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia.

2. To determine the antibiotic susceptibility profile of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia.

3. To identify erythromycin and tetracycline resistant genes of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia.

4 MATERIALS AND METHODS

4.1 Study Area

The study was conducted on *S. pyogenes* isolated from the oropharyngeal swab samples of febrile patients living in Adama city and Wonji Town during the Severe Typhoid Fever Surveillance (SETA) project. The SETA project was a 3-year multicounty surveillance study conducted in six African Countries including Ethiopia. In Ethiopia, the SETA project was conducted between October 2017 and September 2019, primarily to address the burden of invasive salmonellosis among children and adults (18). Different clinical samples including oropharyngeal swab samples were collected for the investigation of potential pathogens from febrile patients during the project.

Adama City is one of the conference centers located in Oromia Regional State, East Shoa Zone, in the Great Rift Valley, about 100 KM South East of the capital, Addis Ababa, and has a total population of about 456,868 (223,560 Male and 233,308 Female). Wonji Town is also a town in Oromia Region, East Shoa Zone,16 KM South of Adama City with an estimated total population of 29,886 and the area is commonly known by irrigation farms particularly sugarcane irrigation which is an input for Wonji sugar factory (74).

4.2 Study Design and Period

A cross-sectional study design was conducted from October 2022 to August 2023 for the detection of virulence factors, antibiotic resistance genes and for the determination of antibiotic susceptibility profile of 201 *S. pyogenes* isolates. Patient clinical information and *S. pyogenes* isolates were obtained retrospectively from SETA project, AHRI, Ethiopia.

4.3 Study population

This study was performed on 201 *S. pyogenes* isolated from the oropharynx of febrile patients visiting Adama Comprehensive specialized Hospital Medical College, Wonji Shoa Alem Tena Health Center, Wonji Gefersa Health Center and Kuriftu Health Center during the study period.

4.4 Variables of the Study

4.4.1 Dependent Variables

GAS virulence factors, antibiotic susceptibility profile, phenotypic macrolide resistance, erythromycin and tetracycline resistance genes.

4.4.2 Independent variables

Socio- demographic factors: age, sex, residence, family size; health-related factors: recent antibiotic treatment, duration of fever, family fever illness within the last 28 days; clinical conditions: headache, vomiting and rash which were obtained from SETA project clinical data recorded during patient recruitment and sample collection.

4.5 Operational Definitions

- Febrile patients: patients with an objective fever of greater than 37°C (>37°C) axillary or fever reported for three or more consecutive days (≥3 days) within the last 7 days (18).
- *Streptococcus pyogenes* is also called Group A streptococcus (GAS) and hence could be used interchangeably.
- Multidrug resistant *S. pyogenes*: is *S. pyogenes* resistant to three or more antibiotic groups at the same time (75).
- Non-susceptibility or reduced susceptibility: any inhibition zone below or less than the susceptibility or sensitivity breakpoint set by the Clinical Laboratory Standard Institute (CLSI) guideline.
- Penicillin non-susceptible: any inhibition zone of below or less than 24mm for penicillin.
- Ceftriaxone non-susceptible: any inhibition zone of below or less than 24mm for ceftriaxone.

4.6 Clinical Data and *S. pyogenes* Isolates

Patient clinical information and S. *pyogenes* isolates were obtained from AHRI up on permission and ethical approval by AHRI/ALERT Ethical Review Committee.

4.7 Laboratory Works

4.7.1 Culture and Identification

In order to avoid extreme temperature fluctuation, *S. pyogenes* isolates were initially transferred from -80°C to -20°C for 4 hours and then to room temperature for 30 minutes. Then, using sterile plastic loop and aseptic safety procedures in BSC level 2, the isolates were inoculated on blood agar base (Oxoid, UK) containing 7% Sheep Blood and incubated at 37°C for 18 to 20 hours (76). AnaeroGen sachet (Thermo Scientific Oxoid, UK) system which generates 5 - 10% CO_2 atmosphere was also included in the incubation process since *S. pyogenes* is a facultative anaerobe bacterium. A colony characteristic of β- hemolytic, catalase (3% H_2O_2) test negative, gram positive cocci in chain and bacitracin (Oxoid, UK) sensitive or pyrrolidonyl arylamidase (PYR) (Hardy Diagnostics, USA) test positive is a bacteriological characteristic of *S. pyogenes*. GAS with such characteristics was used for DNA extraction and antibiotic susceptibility testing (AST).

4.7.2 DNA Extraction

As a template for PCR reaction, GAS DNA was extracted by using alkaline lysis buffer (ALB) as described earlier (77). A pure colony of *S. pyogenes* isolates were picked by sterile 1µl plastic inoculating loop and inserted into a sterile eppendorf tube containing 20µl ALB labeled with isolate identification number (ID). The suspension was mixed well and eppendorf tube were closed and placed into a preheated heat block (Peqlab, Germany) at 95°C. After 15 minutes the tubes were taken out from the heater and centrifuged at high speed (15,000g) for 5 seconds by eppendrof centrifuge. Then 180µl of nuclease free water (Ambion, USA) were added and recentrifuged at 13,000g for 5 minutes to pellet the debris under the bottom whereas the supernatant is a crude DNA extract. Aseptically, 150µl of the supernatant (crude DNA-extract) was transferred to a new sterile eppendorf tube labeled with the same isolate ID. The concentration and purity of the DNA were determined by using Nanodrop 2000 spectrophotometer (Thermofisher, USA) at 260/280 nm UV absorbance. 1 µl of crude DNA were applied to the nanodrop 2000. A DNA concentration of ≥10ng/µl at 260 nm UV absorbance and a DNA purity of 1.7 to 2.0 at an absorbance ratio of 260/280 nm were used for PCR reactions (35). The extracted DNA was stored at -80 ℃ for later use.

4.7.3 Detection of Virulence Factors

The detection of 20 virulence factors (eleven toxins/superantigens, five Proteases and four DNases) were performed by 4 sets of low volume conventional multiplex PCR technique described earlier with minor modifications (17). Primers were purchased from Eurogenetec, Belgium. In mix-1 a multiplex PCR consisting of six superantigen genes, namely speL, speK, speM, speC, smeZ and speI were involved; in mix 2 five superantigens (ssa, speA, speH, speG and speJ); in mix 3 four DNases (spd3, sdc, sdaB and sdaD) and in mix 4 five proteases (speB, spyCEP, scpA, mac and sic) genes were added. Within each reaction, all the target genes have a size difference of more than 50 bp. Each mix was different by the volume of molecular grade water and template DNA used. What was common with in each mix were; the final reaction volume per tube was 15µL, the volume of 2X HotStarTaq PCR Master Mix used was 7.5µL and the volume of forward as well as reverse primers used was 0.2µL of 20nM from each primer were commonly applied for all mixes. In mix-1, the volume of molecular grade water and template DNA was 2.3µL and 2.8 µL; in mix-2 & mix-4, 2.6µL and 2.9 µL; in mix-3, 2.9µL and 3 µL respectively. Within each run and in all mixes, positive and negative controls were included. All the reaction mixtures are conducted on a cold PCR plate. Same template DNA was used within each mix. In a majority of cases a 96 well plate was used and for some reactions, PCR tubes were also used for amplification. Amplification was performed by Bio-Rad T100 Thermal cycler. For mix-1, 2 and 3; a PCR program was set as initial denaturation 95°C for 3 minutes, followed by 40x of denaturation (95°C for 15 seconds), annealing (60 °C for 2 minutes) and elongation (72 °C for 2 minutes) with a final extension of 72 °C for 7 minutes were employed. For mix-4, only annealing temperature was different from the 3 mixes which was 52.5°C for 45 minutes. Gel electrophoresis were employed to detect the target amplicon by using a 1.5% agarose gel (1.5g agarose in 100 ml 1X TAE buffer) stained with 3 µL ethidium bromide and electrophoresed at 120V for 80 minutes. 10µL PCR amplicons were mixed with 2µL of 6% loading dye to settle down the amplicon when applied to the gel well. 5µL 100 bp DNA ladders (Promega, USA) were used as a ruler to indicate the size of DNA in the gel. Visualization of the gel result was done by using a gel imaging system, GelPRo gel documentation system (Cleaver Scientific, UK). The image was saved. (Table1,2 and 3 below).

4.7.4 Antibiotic Sensitivity Testing

Antimicrobial susceptibility testing was performed by modified Kirby-Bauer disk diffusion technique against ten different classes of antibiotics on Mueller Hinton Agar (MHA) (Oxoid, UK) plate supplemented with 5% defibrinated sheep blood according CLSI guidelines (76). Pure and fresh 18 - 20 hours, colony growth of *S. pyogenes* from 7% SBA was picked by sterile plastic inoculating loop and inserted into a tube (16 mm diameter glass tube) containing 2ml sterile normal saline. This bacterial suspension was adjusted to a turbidity of 0.5 McFarland standard by using DEN-1 McFarland Densitometer (Biosan, USA). The standard suspension was inoculated on to the susceptibility test agar, MHA with 5% sheep Blood by using sterile cotton tipped swab (76). Excess or overflowing inoculum from the swab was removed by pressing and rotating the swab firmly against the side of the tube above the level of the suspension. The swab was streaked all over the surface of the medium three times, rotating the plate through an angle of 60 °C after each application. Finally, the swab was rotated around the edge of the agar before discarding into 1% bleach solution. The inoculum or streaked plate was left for 5 minutes for drying at room temperature with lid closed before applying the antibiotic disks. The antibiotic disks were applied on the plate manually by using sterile needle-tip at a distance of 24 mm between each disk and 15 mm from the border. A maximum of 9 disks on 150 mm and 4 disks on 100 mm plates were applied. The recommended antibacterial agents (with their concentration in bracket) for β-hemolytic streptococcus species according to CLSI guideline, were Penicillin G (10 units), Erythromycin (15µg), Clindamycin (2µg), Tetracycline (30µg), Chloramphenicol (30µg), Vancomycin (30µg), Quinupristin-dalfopristin (15µg), Ceftriaxone (30µg), Ofloxacin (5µg) and Linezolid (30µg) (76). Chloramphenicol (30µg) and Vancomycin (30µg) disks were purchased from Abtek Biologicals, UK and all the other disks were purchased from Oxoid, UK. The susceptibility plate containing antibiotic disks were incubated at 37^0C for 20-24 hours with AnaeroGen sachet (Thermo Scientific Oxoid, UK) which releases about 5-10% CO_2 concentration. After 20-24 hours of incubation, the zone of inhibition around the disks were measured by vernier caliper (Mitutoyo, Japan) and the results were interpreted as sensitive, intermediate and resistant based on CLSI guideline (76). As a control strain, S. *pneumonia* (ATCC 49619) were used for quality control of disk diffusion tests (76). The resistant isolates were stored at – 80°C for later use.

4.7.5 Determination of Macrolide Resistance Phenotypes

Erythromycin resistant *S. pyogenes* isolates were a candidate for Macrolide Resistance Phenotyping which is also called Macrolide–lincosamide– streptogramin B (MLS_B) resistance phenotyping. This was determined by the double disk diffusion test (D-test) in which initially erythromycin resistant and clindamycin sensitive or intermediate phenotypes were placed side by side with a close proximity of 12mm apart according to CLSI guideline (76). The suspension turbidity, the susceptibility media, mode of inoculum, incubation conditions and inhibition zone measurement were similarly treated as of antibiotic sensitivity testing procedure. MHA plates supplemented with 5% sheep blood were inoculated with a suspension of group A streptococci that met a 0.5 McFarland turbidity standard. An erythromycin disk (concentration, 15µg per milliliter) and a clindamycin disk (concentration, 2µg per milliliter) were placed 12 mm apart on the plate after 5 minutes of inoculation. Zones of inhibition were examined after incubating at 37°C for 20 - 24 hours with 5-10 % CO_2 generating AnaeroGen sachet. After 20-24 hours incubation, any blunting shape of the inhibition zone around the clindamycin disk (D-test positive) indicates inducible clindamycin resistant (ICR) or inducible MLSB phenotype (resistance to most macrolide, lincosamide, and streptogramin B antibiotics); resistance to both erythromycin and clindamycin indicates a constitutive MLSB phenotype; and those isolates resistant to erythromycin (ER) only but susceptibility to clindamycin were categorized as M phenotype (resistance to macrolide antibiotics only (22,67).The isolates were stored at – 80°C for later use.

4.7.6 Detection of Erythromycin and Tetracycline Resistance Genes

4.7.6.1 Detection of Erythromycin Resistance Genes

All erythromycin-resistant and intermediate isolates were screened for erythromycin-resistance genes [mef(A), erm(B), erm(A) subtype TR] by conventional multiplex polymerase chain reaction (PCR) described previously with slight modifications (5,22,67,78). Primers were purchased from Eurogenetec, Belgium. A total volume of 15µL per tube was prepared by mixing 7.5µL 2 x HotStarTaq PCR Master Mix, 3.3µL of molecular grade water, 0.2µL of 20nM forward primers from each of the 3 genes as well as 0.2µL of 20nM reverse primers from each of the 3 genes and 3µL template DNA. Reagent mixing was done on a cold PCR plate. The PCR amplification was done by Bio-Rad T100 Thermal cycler with a cycling condition of initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation (95°C for 15 seconds), annealing (51 °C for 2 minutes) and elongation (and 72 °C for 2 minutes) with a final elongation temperature of 72 °C for 7 minutes. Positive and negative controls were included through the processes. *S. pyogenes* susceptible to erythromycin were used as negative controls. *S. pneumoniae* (P6 and P8) for the detection of erm(B) and mef(A) genes respectively, and *S. aureus* (S7) for the detection of erm(A) genes were used as a positive control strain. Gel electrophoresis were employed to detect the target amplicon by using a 1.5% TAE agarose gel stained with 3µL ethidium bromide at 120V for 80 minutes. 10µL PCR amplicons were mixed with 2µL of 6% loading dye to settle down the amplicon when applied to the gel well. 5µL 100 bp DNA ladders (Promega, USA) were used as a ruler to indicate the size of DNA in the gel. Visualization of the gel result was done by using a gel imaging system, GelPRo gel documentation system (Cleaver Scientific, UK). (Table1,2 and 3 below).

4.7.6.2 Detection of Tetracycline Resistance Genes

For tetracycline resistant (TR) and intermediate *S. pyogenes*, the presence of the tet(M) and tet(O), tet(k) and tet(L) genes were screened by conventional multiplex PCR as described previously with slight modifications (12,67). Primers were purchased from Eurogenetec, Belgium. A final volume of 15µL per tube was prepared by mixing 7.5µL 2 x HotStarTaq PCR Master Mix, 2.9µL of molecular grade water, 0.2µL of 20nM forward primers from each of the 4 genes as well as 0.2µL of 20nM reverse primers from each of the 4 genes and 3µL template DNA. Reagent mixing was done on a cold PCR plate. The PCR amplification was done by Bio-Rad T100 Thermal cycler with a cycling condition of initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 2 minutes and elongation at 72°C for 2 minutes with a final elongation/extension temperature of 72°C for 7 minutes. Positive and negative controls were included during the amplification. Gel electrophoresis was employed to detect the target amplicon by using a 1.5% TAE agarose gel stained with 3µL ethidium bromide at 120V for 80 minutes. 10µL PCR amplicons were mixed with 2µL of 6% loading dye to settle down the amplicon when applied to the gel well. 5µL 100 bp DNA ladders (Promega, USA) were used as a ruler to indicate the size of DNA in the gel. Visualization of the gel result was done by using a gel imaging system, GelPRo gel documentation system (Cleaver Scientific, UK). The image was labeled and saved. (Table1,2 and 3 below).

Table 1: List of primers used for the detection of GAS VFs, ER genes and TR genes

Gene	Primer sequence (Forward = F)	Primer sequence (Reverse = R)	Size (bp)	Gene type	Reference
Virulence Factors: Toxins/ superantigens					
SpeL	CCTGAGCCGTGAAATTCCCA	ACACCAGAATTGTCGTTTGGT	657	VF/Toxin	(17)
SpeK	CCTTGTGTGTGTATCGCTTGC	TTGCTGTCCCCCATCAAACT	568	VF/Toxin	(17)
SpeM	ATCGCTCATCAAACTTTTCCT	CCTTGTGTGTGTATCGCTTGC	496	VF/Toxin	(17)
SpeC	GCCAATTTCGATTCTGCCGC	TGCAGGGTAAATTTTTCAACGACA	405	VF/Toxin	(17)
SmeZ	TTTCTCGTCCTGTGTTTGGA	TTCCAATCAAATGGGACGGAGAACA	246	VF/Toxin	(17)
SpeI	TTCATAGACGGCGTTCAACAA	TGAAATCTAGAGGAGCGGCCA	176	VF/Toxin	(17)
ssa	AAGAATACTCGTTGTAGCATGTGT	AATATTGCTCCAGGTGCGGG	678	VF/Toxin	(17)
SpeA	AGGTAGACTTCAATTTGGCTTGTGT	GGGTGACCCTGTTACTCACG	576	VF/Toxin	(17)
SpeH	TGAGATATAATTGTCGCTACTCACAT	CCTGAGCGGTTACTTTCGGT	480	VF/Toxin	(17)
SpeG	TGGAAGTCAATTAGCTTATGCAG	GCGAACAACCTCAGAGGGCAAA	384	VF/Toxin	(17)
SpeJ	TCCTTGTACTAGATGAGGTTGCAT	GGTGGGGTTACACCATCAGT	286	VF/Toxin	(17)
Virulence Factors: DNases					
spd3	ATCGTCGTACTTGGCAAGGTT	GCCGCTTCTTCAAACTCTTCG	784	VF/DNases	(17)
sdc	AAGCTTAGAAACTCTCTCGCCA	AGTTCCAGTAATAGCGTTTTTCCGT	600	VF/DNases	(17)
sdaB	TATAGCGCATGCCGCCTTTT	TGATGGCGCAAGCAAGTACC	440	VF/DNases	(17)
sdaD	TTTACGCTGAATCGGGCACT	GGCTCTGGTTTGCTTTCCCA 295	295	VF/DNases	(17)
Virulence Factors: Proteases					
speB	AGACGGAAGAAGCCGTCAGA	TCAAAGCAGGTGCACGAAGC	952	VF/ Proteases	(17)
spyCEP	GATCCGGCCCATCAAAGCAT	AGCTGCCACTGATGTTGGTG	786	VF/ Proteases	(17)
scpA	GCTCGGTTACCTCACTTGTCC	CAATAGCAGCAAACAAGTCACC	622	VF/ Proteases	(17)
Mac	TCTTGCCCTGTTGAAAGTGT	CGAGGTGGTATTTTTGACGCC	389	VF/ Proteases	(17)
sic	TTACGTTGCTGATGGTGTATATGGT	TTTGATAGAGGGTTTTCAGCTGGC	150	VF/Proteases	(17)
Erythromycin Resistance (ER) Genes					
erm(A) -TR	ATAGAAATTGGGTCAGGAAAAGG	TTGATTTTTAGTAAAAAG	530	ER Genes	(5,73)
erm(B)	GAAAAGGTACTCAACCAAATA	AGTAACGGTACTTAAATTGTTTAC	639	ER Genes	(5,73)
mef(A)	AGTATCATTAATCACTAGTGC	TTCTTCTGGTACTAAAAGTGG	348	ER Genes	(5,73)
Tetracycline Resistance (TR) Genes					
tet(K)	TATTTTGGCTTTGTATTCTTTCAT	GCTATACCTGTTCCCTCTGATAA	1159	TR Genes	(5,73)
tet(L)	ATAAATTGTTTCGGGTCGGTAAT	AACCAGCCAACTAATGACAATGAT	1077	TR Genes	(5,73)
tet(M̈)	GAACTCGAACAAGAGGAAAGC	ATGGAAGCCCAGAAAGGAT	740	TR Genes	(5,73)
tet(O)	AACTTAGGCATTCTGGCTCAC	TCCCACTGTTCCATATCGTCA	519	TR Genes	(5,73)

Key: ER: Erythromycin resistance; TR: Tetracycline resistance; VF: Virulence factor

Table 2: Summary of PCR reaction set up per tube for ER genes, TR genes and VFs of GAS.

Reagent	Resistant Gene Reaction Mixes		Virulence Factor Reaction Mixes			
	ER Gene Reaction Mixes	TR Gene Reaction Mixes	Mix-1	Mix-2	Mix-3	Mix-4
2X HotStarTaq PCR Master Mix	7.5µL	7.5µL	7.5µL	7.5µL	7.5µL	7.5µL
Molecular Grade Water	3.3µL	2.9µL	2.3µL	2.6µL	2.9µL	2.6µL
20nM Forward Primer (FP)*	0.2µL*3	0.2µL*4	0.2µL*6	0.2µL*5	0.2µL*4	0.2µL*5
20nM Reverse primer (RP)*	0.2µL*3	0.2µL*4	0.2µL*6	0.2µL*5	0.2µL*4	0.2µL*5
Template DNA	3µL	3µL	2.8µL	2.9µL	3µL	2.9µL

*Indicates the number of forward and reverse primers included in a single multiplex PCR reaction mix. In ER gene reaction mixes, 3 types of forward and 3 types of reverse primers were added in a single reaction. Similarly, in TR gene reaction mixes 4FP, 4RP; in Mix1 6FP, 6RP; in Mix2 5FP, 5RP; in Mix 3 4FP, 4RP and in Mix 4 5FP,5RP were added in a single PCR reaction. ER: Erythromycin resistance; TR: Tetracycline resistance; PCR: Polymerase Chain Reaction; FP: Forward Primer; RP: Reverse Primer VFs: Virulence factors.

Table 3: Summary of PCR program set up for ER genes, TR genes and VF detection of GAS.

PCR Cycling Condition	Number of Cycles	ER Reaction Mixes		TR Reaction Mixes		Virulence Factor Reaction Mixes				Gel Electrophoresis
						Mix-1, 2 and 3		Mix-4		
		Temp.	Time	Temp.	Time	Temp.	Time	Temp.	Time	
Initial Denaturation	1X	95°C	3 min	95°C	3 min	95°C	3 min	95°C	3 min	1.5%gel
Denaturation	40X	95°C	15 sec	95°C	15 sec	95°C	15 sec	95°C	15 sec	
Annealing		51 °C	2 min	60°C	20 sec	60°C	20 sec	52.5°C	45 sec	1.5g agarose in 100 ml of 1x TAE buffer
Elongation		72°C	2 min	72°C	2 min	72°C	2 min	72°C	2 min	
Final Elongation	1X	72°C	7 min	72°C	7 min	72°C	7 min	72°C	7 min	Run at 120V for 80 minutes

Key: ER: Erythromycin resistance; TR: Tetracycline resistance; VF: Virulence Factors.

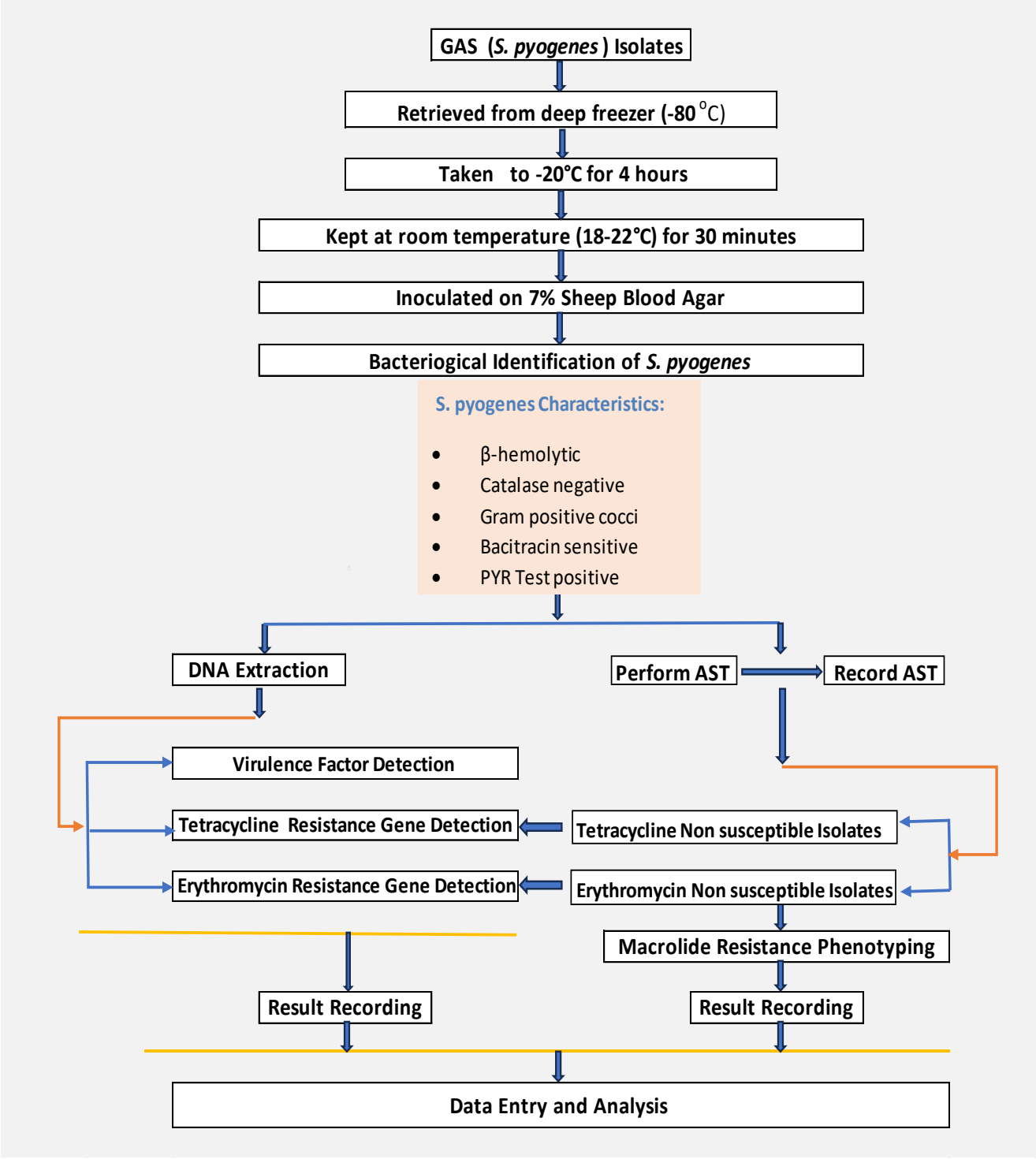


Figure 1: General Laboratory Work Flow: Identification, AST and Molecular Detection of Virulence and Antibiotic Resistance Genes of GAS Isolates.

4.8 Quality Control

Universal safety precautions and standard operating procedures (SOPs) were followed during specimen handling, culture preparation, inoculation, isolation, extraction, storage, master mix preparation/ reagent mix up and gel electrophoresis. Culture and master mix procedures were processed in Biosafety Cabinet (BSC) Level 2. Culture media were prepared according to the manufacturer's instructions by sterilization at 121 °C for 15 minutes. After preparing culture media, 5% of the batch was incubated for sterility check. Similarly, growth support and β-hemolysis test was done by inoculating *S. pyogenes* (ATCC 19615) and *S. pneumoniae* (ATCC 49619). The incubation condition for both sterility and growth support test was at 37 °C for 24 - 48 hours with 5 -10% CO_2 generating AnaeroGen sachet. To exclude contaminants and check the sterility of diluents (normal saline, molecular grade water and nuclease free water), each of them were inoculated on two 7% Sheep Blood Agar at 37 °C for 24 - 48 hours, one plate aerobically (without CO_2) and the other with 5-10 % CO_2 generating AnaeroGen sachet. American Type Culture Collection (ATCC) standard reference strains were used to check the quality of medium, antibiotic susceptibility testing (AST) and PCR products. The quality of PYR test and Bacitracin test were done by using *S. pyogenes* (ATCC 19615) as a positive control and *S. agalactiae* (ATCC 13813) as a negative control. Gram staining reagents were also checked weekly with known gram positive and gram negative ATCC organisms. These ATCC organisms were; *S. pyogenes* (ATCC 19615), *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922). *E. coli* was grown aerobically on 7% SBA at 37 °C for 16 - 18 hours.

4.9 Data Management and Analysis

Data was entered into excel spreadsheet, cleaned and exported to SPSS software version 20 for analysis. Frequency distribution of the variables were done to check the completeness and consistency of the data. The characteristics of study participants and majority of the findings were summarized by proportions (frequency and percentages), tables and graphs. Bivariate logistic regression was used to demonstrate the relationship between an independent variable and the outcome variable. Variables with a p-value of ≤ 0.25 in the bivariate analysis were included in the multivariate logistic regression model to identify variables that were independently associated with the outcome variable. The presence and strength of association between independent and outcome

variables were calculated using an adjusted odds ratio with a 95% confidence interval (CI). A P-value < 0.05 in the multivariate logistic regression indicates statistically significant association.

4.10 Ethical Consideration

The actual work of this project started after obtaining ethical approval from two Ethical Review Committees namely; Department Research Ethical and Review Committee (DRERC) of Department of Microbiology, Immunology and Parasitology School of Medicine College of Health Sciences Addis Ababa University, Meeting Number: DRERC/003/2022 and AHRI/ALERT Ethics Review Committee (AAERC), Protocol Number PO-54-22.

4.11 Declaration

The investigators in this project declare no conflict of interest.

5 RESULTS

5.1 Socio Demographic and Clinical Characteristics of GAS Positive Patients

In our study 201 *S. pyogenes* isolated from febrile patients were included. The age range of *S. pyogenes* positive patients were 1-78 years with a mean of 24.58 and standard deviation of 17.6%. 108 (53.7%) of *S. pyogenes* were isolated from male patients. Majority of the *S. pyogenes* isolates were obtained from patients with age of ≥15 years old [132 (65.7%)] whereas 69 (34.3%) were isolated from < 15 years old patients. Forty-one (20.4%) of the GAS isolates were isolated from school age children, 5 -14 years old. Concerning the household size, 126 (62.7%) of the GAS isolates were obtained from patients with a family size ranging from 4-6, while 57 (28.4%) and 18 (9.0%) of the GAS isolates were obtained from patients with a family size of 1-3 and > 6 respectively. Moreover, 174 (86.6%) of the GAS positive patients live in urban areas. Most of the GAS positive patients, 161 (80.1%) had a fever of ≥3 days; and 151 (75.1%) were symptomatic for headache. Fourteen (7.0%) of the GAS isolates were identified from febrile patients who had taken antibiotic treatment with in the last one month whereas, 10 (5.0%) were from those who had family member fever illness with in the last one month, 4 (2.0%) were from those who had throat infection, 42 (20.9%) were from those who had vomiting and 2 (1.0%) were obtained from those febrile patients who had rash (Table 4 below).

Table 4: Sociodemographic and clinical characteristics of oropharyngeal *S. pyogenes* positive febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Variables	Category	Frequency	
		No	%
Gender	Male	108	53.7
	Female	93	46.3
Age	< 5 years	28	13.9
	5-14 years	41	20.4
	15-44 years	104	51.7
	≥45 years	28	13.9
Residence	Urban	174	86.7
	Rural	27	13.4
Household size	1-3	57	28.4
	4-6	126	62.7
	>6	18	9.0
Fever Duration	< 3 Days	40	19.9
	≥3 Days	161	80.1
Family member fever illness (≤ 1 month)	Yes	10	5.0
	No	191	95.0
Recent antibiotic treatment (≤ 1 month)	Yes	14	7.0
	No	187	93.0
Headache	Yes	151	75.1
	No	50	24.9
Throat Infection	Yes	4	2.0
	No	197	98.0
Vomiting	Yes	42	20.9
	No	159	79.1
Rash	Yes	2	1.0
	No	199	99.0

5.2 Virulence Factors (Toxins/Superantigens, Proteases and DNases)

In this study, we have screened 20 different GAS virulence factors; eleven toxins/superantigens, five proteases and four DNases in all 201 *S. pyogenes* isolates. Eighteen different VFs were identified. All types of toxins and proteases were identified with variable frequencies. The most commonly identified toxins/superantigens were, SmeZ 34 (16.9%), SpeK 25 (12.4%), SpeI 20 (10.0%), SpeJ 10 (5.0%) and SpeM 9 (4.5%) whereas low frequencies of SpeL 5 (2.5%), SpeA 5 (2.5%), SpeH 4 (2.0%), SpeG 3 (1.5%), SpeC 2 (1.0%), and ssa 2 (1.0%) were identified. The frequency of proteases was, Mac 50 (24.9%), scpA 31 (15.4%), sic 26 (12.9%), spyCEP 15 (7.5%) and speB 10 (5.0%). We have identified very low DNases with a frequency of 4 (2.0%) for sdaB, 3 (1.5%) for sdaD whereas sdc and spd3 genes were not detected at all. Multiple co-existence of VFs up to 8 different genes per an isolate was also observed.

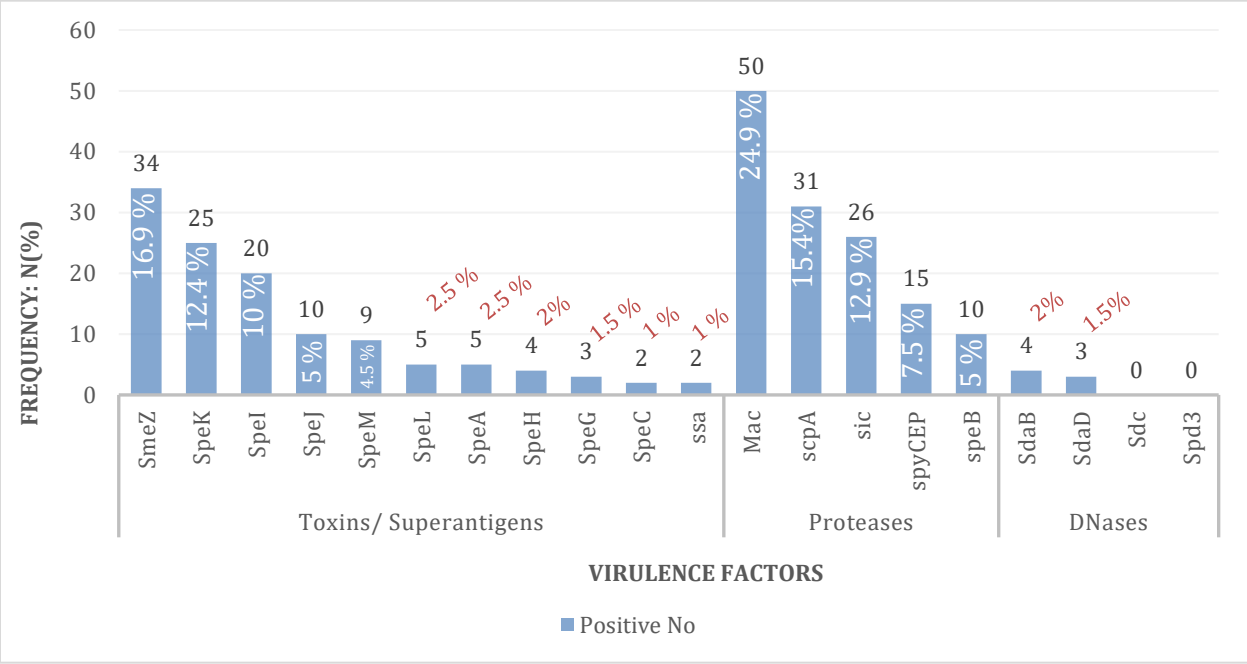


Figure 2: Detection of virulence factors of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

5.2.1 Co-occurrence of Virulence Factors (Superantigens, Proteases and or DNases)

In this study, 121 (60.2%) of the isolates had at least one or more virulence factors and 70 (34.8%) were positive for two or more virulence factors whereas 80 (39.8%) of the isolates had none of the virulence factors investigated. The maximum co-existence of virulence factors in this study was 8 which was found in 1 (0.5%) of the isolates. Isolates with a multiple virulence factor of 3, 4, 5, 6 and 7 were 14 (7.0%), 3 (1.5%), 6 (3.0%), 2 (1.0%) and 3 (1.5%) respectively. The proportion of GAS isolates harboring only one type of virulence factor were 51 (25.4%).

Table 5: Co-occurrence status of virulence factors of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Multiple VF	Frequency	
	No	%
VF0	80	39.8
VF1	51	25.4
VF2	41	20.4
VF3	14	7.0
VF4	3	1.5
VF5	6	3.0
VF6	2	1.0
VF7	3	1.5
VF8	1	0.5
≥VF2	70	34.8
≥VF1	121	60.2

Key: VF: Virulence Factor, VF0: Negative for all the investigated VFs, VF1 Positive for only one VF, VF 2: Positive for two VFs, VF 3: Positive for three VFs, VF 4: Positive for four VFs, VF 5: Positive for five VFs, VF 6: Positive for six VFs, VF 7: Positive for seven VFs, VF8: Positive for eight VFs; ≥ VF2: Positive for two or more VFs: ≥ VF1: Positive for at least one or more VFs, N: Number, %: Percent.

5.2.1.1 Co-occurrence of Toxins/Superantigens

Seventy (34.8%) isolates were at least positive to one or more toxins/superantigens, 29 (14.4%) isolates were positive to two or more toxins/superantigens whereas 131 (65.2%) isolates had none of the investigated toxins. The maximum multiple toxins detected were 7 which was detected in 1 (0.5%) isolate. Isolates with a multiple toxin of 3, 4 and 5 were 6 (3%), 3 (1.5%) and 1 (0.5%) respectively. Forty-one (20.4%) of the isolates had only one type of toxin.

Table 6: Co-occurrence status of toxins/superantigens of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Toxins/ Superantigens	**Frequency**	
	No	%
T0	131	65.2
T1	41	20.4
T2	18	9.0
T3	6	3.0
T4	3	1.5
T5	1	0.5
T6	0	0.0
T7	1	0.5
≥T2	29	14.4
≥T1	70	34.8

Key: T0: Negative for all toxins, T1: Positive for only one type of toxin, T2: Positive for two toxins, T3: Positive for three toxins, T4: Positive for four toxins, T5: Positive for five toxins, T6: Positive for six toxins, T7: Positive for seven toxins, ≥T2: Positive for two or more toxins: ≥T1: Positive for at least one or more toxins, N: Number, %: Percent.

5.2.1.2 Co-occurrence of Proteases

From a total of 201 oropharyngeal *S. pyogenes* isolates, 83 (41.3%) were positive to at least one or more proteases and 36 (17.9%) were positive to two or more proteases whereas 118 (58.7%) were negative for the investigated proteases. Those isolates positive to 2,3 and 4 proteases were 25 (12.4%), 9 (4.5%) and 2 (1.0%) respectively. Forty-seven (23.4%) of the isolates had only one type of protease.

Table 7: Co-occurrence status of proteases of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Proteases	Frequency	
	No	%
P0	118	58.7
P1	47	23.4
P2	25	12.4
P3	9	4.5
P4	2	1.0
≥P2	36	17.9
≥P1	83	41.3

Key: P0: Negative for all proteases, P1: Positive for only one protease, P2: Positive for two proteases, P3: Positive for three proteases, P4: Positive for four proteases, ≥P2: Positive for two or more proteases: ≥P1: Positive for at least one or more protease, N: Number, %: Percent.

5.2.1.3 Co-occurrence of DNases

Only 7 (3.5%) of the isolates were positive to one type of DNases and no co-occurrence of DNases were observed. The remaining 194 (96.5%) isolates were negative for the investigated DNases.

Table 8: Distribution of virulence factors, toxins/superantigens, proteases, DNases and selected top four occurring virulence genes of oropharyngeal *S. pyogenes* with in different sociodemographic and clinical characteristics of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Variables	Category	All Virulence Factors (n=121)	Toxins/SAgs (n=70)	Proteases (n=83)	DNases (n=7)	Mac (n=50)	SmeZ (n=34)	scpA (n=31)	Sic (n=26)	SpeK (n=25)
		N (%)	N (%)	N (%)	N (%)		N (%)	N (%)	N (%)	N (%)
Gender	Male	64 (52.9)	39 (55.7)	41 (49.4)	2 (28.6)	25 (50.0)	21 (61.8)	15 (48.4)	13 (50.0)	15 (60.0)
	Female	57 (47.1)	31 (44.3)	42 (50.6)	5 (71.4)	25 (50.0)	13 (38.2)	16 (51.6)	13 (50.0)	10 (40.0)
Age	< 5 years	21 (17.4)	11 (15.7)	14 (16.9)	0 (0.0)	9 (18.0)	6 (17.6)	6 (19.4)	3 (11.5)	2 (8.0)
	5-14 years	22 (18.2)	12 (17.1)	18 (21.7)	1 (14.3)	9 (18.0)	4 (11.8)	6 (19.4)	4 (15.4)	5 (20.0)
	15-44 years	61 (50.4)	37 (52.9)	39 (47.0)	3 (42.9)	2 1(42.0)	18 (52.9)	16 (51.6)	14 (53.8)	16 (64.0)
	≥45 years	17 (14.0)	10 (14.3)	12 (14.5)	3 (42.9)	11 (22.0)	6 (17.6)	3 (9.7)	5 (19.2)	2 (8.0)
Residence	Urban	103 (85.1)	59 (84.3)	70 (84.3)	6 (85.7)	43 (86.0)	27 (79.4)	24 (77.4)	22 (84.6)	21 (84.0)
	Rural	18 (14.9)	11 (15.7)	13 (15.7)	1 (14.3)	7 (14.0)	7 (20.6)	7 (22.6)	4 (15.4)	4 (16.0)
Household size	1-3	28 (23.1)	15 (21.4)	19 (22.9)	3 (42.9)	12 (24.0)	5 (14.7)	7 (22.6)	6 (23.1)	6 (24.0)
	4-6	80 (66.1)	48 (68.6)	51 (61.4)	3 (42.9)	26 (52.0)	23 (67.6)	20 (64.5)	16 (61.5)	17 (68.0)
	>6	13 (10.7)	7 (10.0)	13 (15.7)	1 (14.3)	12 (24.0)	6 (17.6)	4 (12.9)	4 (15.4)	2 (8.0)
Fever Duration	< 3 Days	27 (22.3)	16 (22.9)	21 (25.3)	2 (28.6)	12 (24.0)	9 (26.5)	9 (29.0)	7 (26.9)	6 (24.0)
	≥3 Days	94 (77.7)	54 (77.1)	62 (74.7)	5 (71.4)	38 (76.0)	25 (73.5)	22 (71.0)	19 (73.1)	19 (76.0)
Family member fever illness (≤ 1 month)	Yes	8 (6.6)	3 (4.3)	5 (6.0)	0 (0.0)	4 (8.0)	3 (8.8)	1 (3.2)	1 (3.8)	1 (4.0)
	No	113 (93.4)	67(95.7)	78 (94.0)	7 (100.0)	46 (92.0)	31 (91.2)	30 (96.8)	25 (96.2)	24 (96.0)
Recent antibiotic treatment (≤ 1 month)	Yes	6 (5.0)	3 (4.3)	5 (6.0)	1 (14.3)	4 (8.0)	1 (2.9)	2 (6.5)	2 (7.7)	1 (4.0)
	No	115 (95.0)	67 (95.7)	78 (94.0)	6 (85.7)	46 (92.0)	33 (97.1)	29 (93.5)	24 (92.3)	24 (96.0)
Headache	Yes	88 (72.7)	53 (75.7)	61 (73.5)	6 (85.7)	35 (70.0)	28 (82.4)	23 (74.2)	21 (80.8)	17 (68.0)
	No	33 (27.3)	17 (24.3)	22 (26.5)	1 (14.3)	15 (30.0)	6 (17.6)	8 (25.8)	5 (19.2)	8 (32.0)
Throat Infection	Yes	1 (0.8)	0 (0.0)	1 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	No	120 (99.2)	70 (100.0)	82 (98.8)	7 (100.0)	50 (100.0)	34 (100.0)	31 (100.0)	26 (100.0)	25 (100.0)
Vomiting	Yes	1 (0.8)	18 (25.7)	24 (28.9)	2 (28.6)	13 (26.0)	9 (26.5)	9 (29.0)	8 (30.8)	7 (28.0)
	No	120 (99.2)	52 (74.3)	59 (71.1)	5 (71.4)	37 (74.0)	25 (73.5)	22 (71.0)	18 (69.2)	18 (72.0)
Rash	Yes	1 (0.8)	0 (0.0)	1 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)
	No	120 (99.2)	70 (100.0)	82 (98.8)	7 (100.0)	50 (100.0)	34 (100.0)	30 (96.8)	26 (100.0)	25 (100.0)

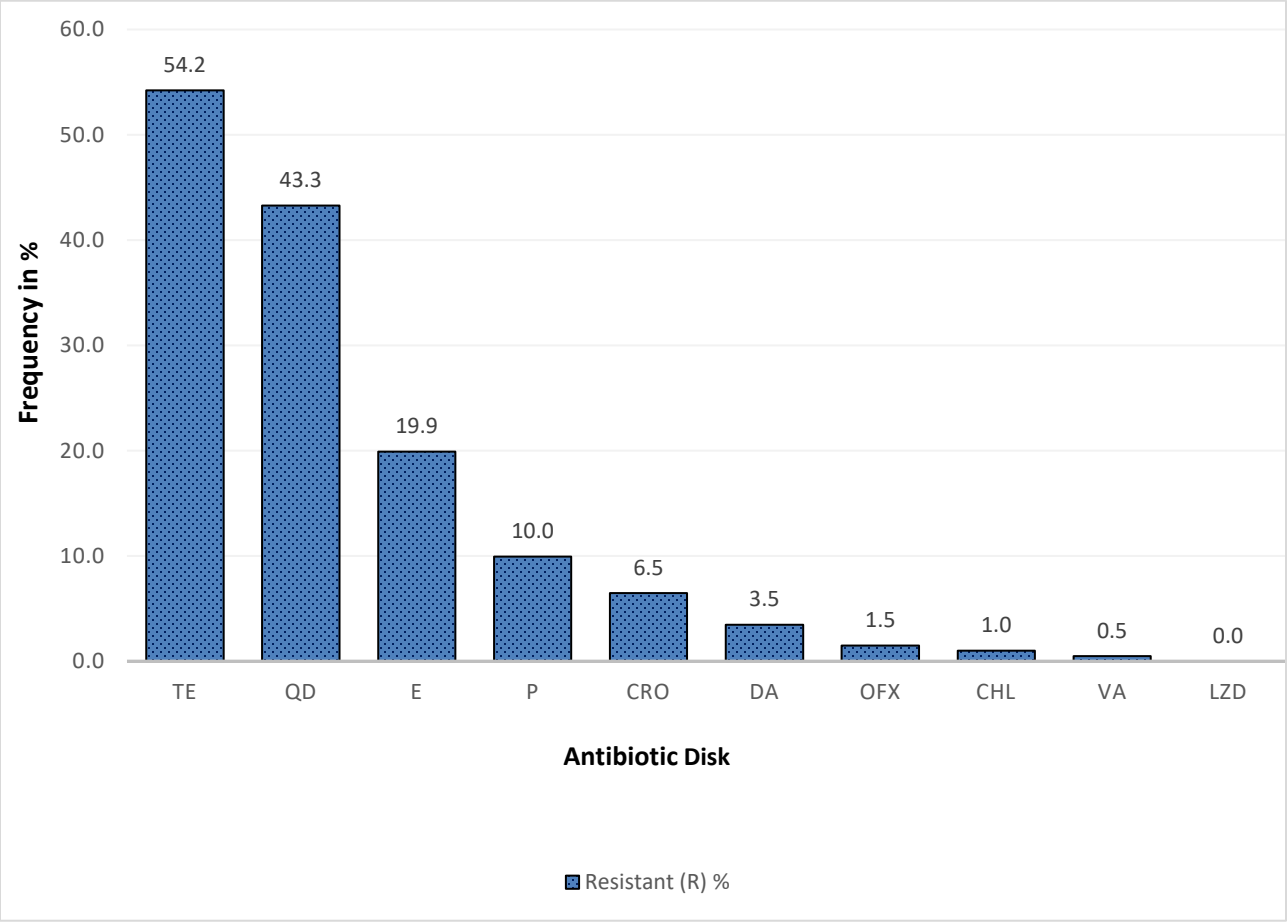
5.3 Antibiotic Sensitivity Profile of Oropharyngeal GAS Isolates

Antibiotic sensitivity testing (AST) was done for all 201 *S. pyogenes* isolates against ten different classes of antibiotics by using modified Kirby-Bauer disk diffusion technique. Accordingly, the top three resistant antibiotics were: tetracycline 109 (54.2%), quinupristin-dalfopristin 87 (43.3%) and erythromycin 40 (19.9%). Moreover, a substantial proportion of the isolates 20 (10.0%) were non susceptible to penicillin G. All the other antibiotics tested had a resistance rate of less than 10.0%. Resistance to ceftriaxone, clindamycin, ofloxacin, chloramphenicol, and vancomycin were 13 (6.5%), 7 (3.5%), 3 (1.5%), 2 (1.0%) and 1 (0.5%), respectively. All 201 (100%) *S. pyogenes* isolates were sensitive to linezolid. Intermediate results were relatively high against quinupristin-dalfopristin 54 (26.9%) and erythromycin 25 (12.4%) whereas intermediate results against clindamycin and ofloxacin were very low which was 2 (1.0%) for each of them.

Table 9: Antibiotic susceptibility profile of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Antibiotics	Resistant (R)		Intermediate (I)		Sensitive (S)		Non susceptible ("R" + "I")	
	No	%	No	%	No	%	No	%
Tetracycline (TE)	109	54.2	4	2.0	88	43.8	113	56.2
Quinupristin-dalfopristin (QD)	87	43.3	54	26.9	60	29.9	141	70.1
Erythromycin (E)	40	19.9	25	12.4	136	67.7	65	32.3
Penicillin G (P)	20*	10.0*	0	0.0	181	90.0	20	10.0
Ceftriaxone (CRO)	13*	6.5*	0	0.0	188	93.5	13	6.5
Clindamycin (DA)	7	3.5	2	1.0	192	95.5	9	4.5
Ofloxacin (OFX)	3	1.5	2	1.0	196	97.5	5	2.5
Chloramphenicol (CHL)	2	1.0	0	0.0	199	99.0	2	1.0
Vancomycin (VA)	1	0.5	0	0.0	200	99.5	1	0.5
Linezolid (LZD)	0	0.0	0	0.0	201	100.0	0	0.0

Key: R: Resistant, I: Intermediate, S: Sensitive, *: Non susceptible for P and CRO in place of resistant



Key: TE: Tetracycline, QD: Quinupristin-dalfopristin, E: Erythromycin, P: Penicillin G, CRO: Ceftriaxone, DA: Clindamycin, OFX: Ofloxacilin, CHL: Chloramphenicol, VA: Vancomycin, LZD: Linezolid, N: Number, %: Percent.

Figure 3: Antibiotic resistance status of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Table 10: Bivariate and multivariate logistic regression analysis of factors associated with tetracycline resistant *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Variables	Category	Frequency N (%)	Tetracycline (R + I) GAS Isolates N (%)	Bivariant		Multivariant	
				P-value	COR (95% CI)	P-value	AOR (95% CI)
Gender	Male	108(53.7%)	60(53.1%)	1			
	Female	93(46.3%)	53(46.9%0	0.838	1.06(0.606,1.854)		
Age	< 5 years	28(13.9%)	18(15.9%)	1.000	1.000 (0.335,2.984)		
	5-14 years	41(20.4%)	22(19.5%)	0.381	0.643 (0.24,1.726)		
	15-44 years	104(51.7%)	55(48.7%)	0.284	0.624(0.263,1.479)		
	≥45 years	28(13.9%)	18(15.9%)	1			
Residence	urban	174(86.6%)	101(89.4%)	0.189	1.729(0.764,3.913)	0.159	1.853(0.785,4.374)
	Rural	26(13.4%)	12(10.6%)	1		1	
Household size	1-3	69(34.3%)	29(25.7%)	1		1	
	4-6	123(65.7%)	72(63.7%)	0.43	1.287(0.687,2.412)	0.135	0.414(0.13,1.317)
	>6	18(9.0%)	12(10.6%)	0.245	1.931(0.637,5.854)	0.365	0.605(0.204,1.794)
Family member fever illness (≤ 1 month)	Yes	10(5.0%)	5(4.4%)	1			
	No	191(95.0%)	108(95.6%)	0.685	1.301(0.365,4.643)		
Recent antibiotic treatment (≤ 1 month)	Yes	14(7.0%)	12(10.6%)	0.036	5.109(1.113,23.46)	0.045*	4.851(1.04,22.641)
	No	187(93.0%)	101(89.4%)	1		1	

Key: R: Resistant, I: Intermediate, 1: Reference, COR: Crude odd ratio, AOR: Adjusted odd ratio, CI: Confidence Interval, *: statistically significant at 95%, N: Number, %: Percent

From this bivariate and multivariate analysis, we observed that those patients who had recent antibiotic treatment with in the last one month develop tetracycline resistance 4.85 times higher than those who did not take antibiotic treatment [AOR = 4.851, 95% CI (1.04 - 22.641)]. Therefore, previous antibiotic treatment was significantly associated with reduced susceptibility or resistance to tetracycline (only those variables with P-value of ≤0.25 on their Bivariate analysis are incorporated in the table).

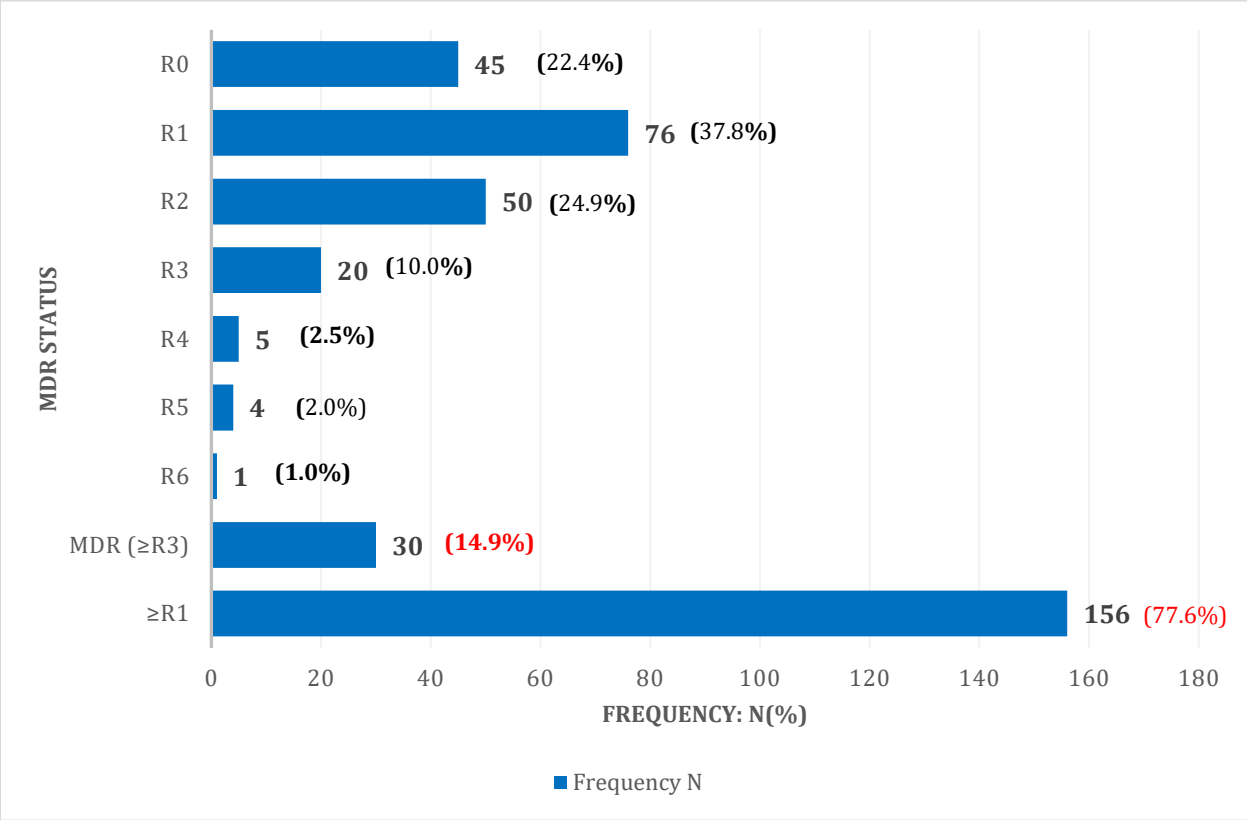
Table 11: Distribution of the antibiotic resistant oropharyngeal *S. pyogenes* isolates within different Sociodemographic and clinical characteristics of febrile Patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Variables	Category	TE (RI=109)	QD (RI=87)	E(RI=40)	P (NS=20)	CRO(RI=13)	DA(RI=7)	OFX(RI=3)	CHL(RI=2)	VA(RI=1)
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Gender	Male	57 (52.3)	43(49.4)	27(67.5)	12(60.0)	10(76.9)	4(57.1)	1(33.3)	1 (50.0)	0(0.0)
	Female	52(47.7)	44(50.6)	13(32.5)	8(40.0)	3(23.1)	3(42.9)	2(66.7)	1(50.0)	1(100.0)
Age	< 5 years	17(15.6)	12(13.8)	10(25.0)	11(55.0)	3(23.1)	2(28.6)	0(0.0)	1 (50.0)	0(0.0)
	5-14 years	22(20.2)	20(23.0)	8(20.0)	5(25.0)	2(15.4)	3(42.9)	1(33.3)	0(0.0)	0(0.0)
	15-44 years	52(47.7)	42(48.3)	17(42.5)	2(10.0)	5(38.5)	2(28.6)	1(33.3)	1 (50.0)	0(0.0)
	≥45 years	18(16.5)	13(14.9)	5(12.5)	2(10.0)	3(23.1)	0(0.0)	1(33.3)	0(0.0)	1(100.0)
Residence	Urban	98(89.9)	77(88.5)	34(85.0)	18(90.0)	11(84.6)	6(85.7)	3(100.0)	2(100.0)	1(100.0)
	Rural	11(10.1)	10(11.5)	6(15.0)	2(10.0)	2(15.4)	1(14.3)	0(0.0)	0(0.0)	0(0.0)
Household size	1-3	26(23.9)	26(29.9)	13(32.5)	5(25.0)	3(23.1)	2(28.6)	2(66.7)	1 (50.0)	0(0.0)
	4-6	71(65.1)	55(63.2)	25(62.5)	13(65.0)	7(53.8)	5(71.4)	0(0.0)	1 (50.0)	1(100.0)
	>6	12(11.0)	6(6.9)	2(5.0)	2(10.0)	3(23.1)	0(0.0)	1(33.3)	0(0.0)	0(0.0)
Fever Duration	< 3 Days	17(15.6)	18(20.7)	8 (20.0)	5(25.0)	3(23.1)	2(28.6)	0(0.0)	0(0.0)	0(0.0)
	≥3 Days	92(84.4)	69(79.3)	32(80.0)	15(75.0)	10(76.9)	5(71.4)	3(100.0)	2(100.0)	1(100.0)
Family member fever illness (≤ 1 month)	Yes	5(4.6)	5(5.7)	2(5.0)	1(5.0)	0(0.0)	0(0.0)	1(33.3)	0(0.0)	0(0.0)
	No	104(95.4)	82(94.3)	38(95.0)	19(95.0)	13(100.0)	7(100.0)	2(66.7)	2(100.0)	1(100.0)
Recent antibiotic treatment (≤ 1 month)	Yes	11(10.1)	8(9.2)	3(7.5)	3(15.0)	2(15.4)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	No	98(89.9)	79(90.8)	37(92.5)	17(85.0)	11(84.6)	7(100.0)	3(100.0)	2(100.0)	1(100.0)
Headache	Yes	79(72.5)	61(70.1)	23(57.5)	7(35.0)	8(61.5)	3(42.9)	2(66.7)	2(100.0)	1(100.0)
	No	30(27.5)	26(29.9)	17(42.5)	13(65.0)	5(38.5)	4(57.1)	1(33.3)	0(0.0)	0(0.0)
Throat Infection	Yes	2(1.8)	2(2.3)	0(0.0)	2(10.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	No	107(98.2)	85(97.7)	40(100.0)	18(90.0)	13(100.0)	7(100.0)	3(100.0)	2(100.0)	1(100.0)
Vomiting	Yes	23(21.1)	18(20.7)	10(25.0)	4(20.0)	5(38.5)	4(57.1)	0(0.0)	2(100.0)	0(0.0)
	No	86(78.9)	69(79.3)	30(75.0)	16(80.0)	8(61.5)	3(42.9)	3(100.0)	0(0.0)	1(100.0)
Rash	Yes	1(0.9)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	No	108(99.1)	87(100)	40(100.0)	20(100.0)	13(100.0)	7(100.0)	3(100.0)	2(100.0)	1(100.0)

Key: TE: Tetracycline, QD: Quinupristin-dalfopristin, E: Erythromycin, P: Penicillin G, CRO: Ceftriaxone, DA: Clindamycin, OFX: Ofloxacin, CHL: Chloramphenicol, VA: Vancomycin, RI: Resistant Isolates, NS: Non susceptible, N: Number, %: Percent

5.4 Multidrug Resistant (MDR) Status of Oropharyngeal GAS Isolates

In our study, the magnitude of multidrug resistant (MDR) oropharyngeal *S. pyogenes* isolates were 30 (14.9). Those isolates resistant to four, five and six groups of antibiotics were 5 (2.5%), 4 (2.0) and 1 (0.5) respectively. Majority of the isolates, 156 (77.6%) were at least resistant to one or more types of antibiotics whereas 45 (22.4%) were sensitive to all types of antibiotics.



Key: R0: Sensitive to all antimicrobial groups, R1: resistance to only one antimicrobial groups, R2: resistance to two antimicrobial groups, R3: resistance to three antimicrobial groups, R4: resistance to four antimicrobial groups, R5: resistance to five antimicrobial groups, R6: resistance to six antimicrobial groups, MDR(≥R3): Multidrug resistant (resistance to three or more antimicrobial groups at the same time), ≥R1: resistant to at least one or more groups of antibiotics, N: Number, %: percent.

Figure 4: Magnitude of multidrug resistant (MDR) status of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

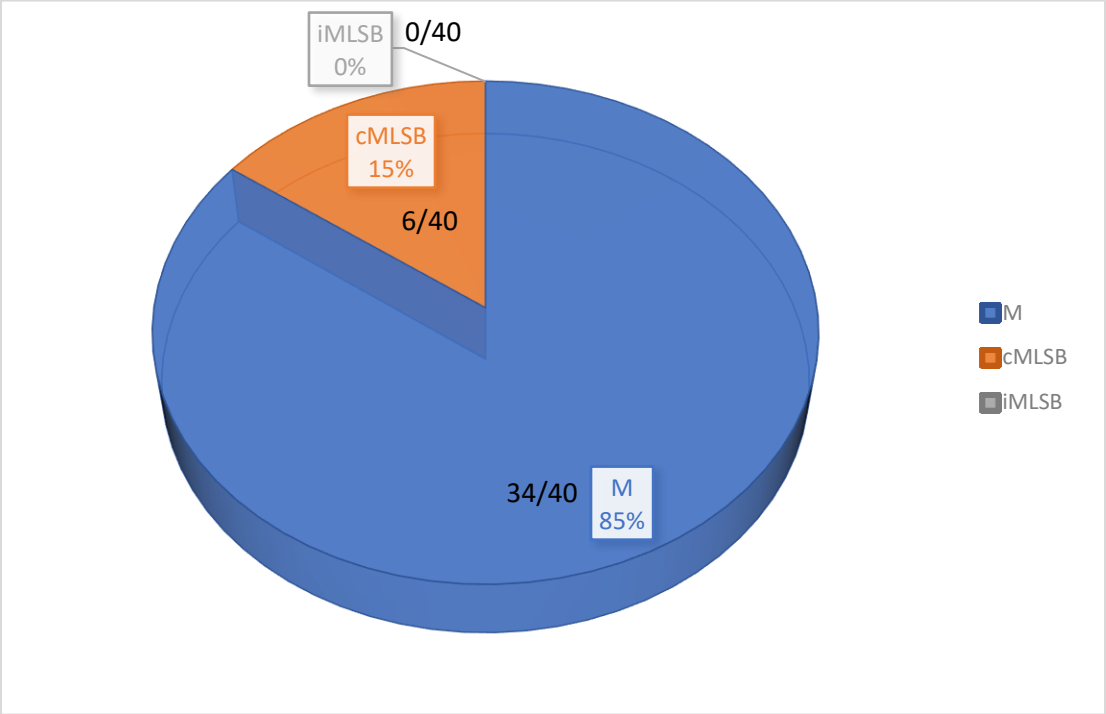
Table 12: Distribution of MDR, susceptible and resistant oropharyngeal *S. pyogenes* isolates within different Sociodemographic and clinical characteristics of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Variables	Category	MDR (RI = 30)		R0 (RI=45)	R1 (RI=76)	R2 (RI =50)	R3 (RI =20)	R4 (RI =5)	R5 (RI =4)	R6 (RI =1)
		No	%	%	%	%	%	%	%	%
Gender	Male	20	66.7	66.7	43.4	50.0	65.0	60.0	75.0	100.0
	Female	10	33.3	33.3	56.6	50.0	35.0	40.0	25.0	0.0
Age	< 5 years	7	23.3	4.4	11.8	20.0	15.0	40.0	50.0	0.0
	5-14 years	7	23.3	20.0	22.4	16.0	15.0	40.0	25.0	100.0
	15-44 years	12	40.0	66.7	51.3	46.0	55.0	20.0	0.0	0.0
	≥45 years	4	13.3	8.9	14.5	18.0	15.0	0.0	25.0	0.0
Residence	Urban	27	90.0	82.2	86.8	88.0	90.0	80.0	100.0	100.0
	Rural	3	10.0	17.8	13.2	12.0	10.0	20.0	0.0	0.0
Household size	1-3	10	33.3	26.7	32.9	20.0	40.0	20.0	25.0	0.0
	4-6	17	56.7	64.4	56.6	74.0	55.0	60.0	50.0	100.0
	>6	3	10.0	8.9	10.5	6.0	5.0	20.0	25.0	0.0
Fever Duration	< 3 Days	4	13.3	24.4	18.4	22.0	0.0	60.0	25.0	0.0
	≥3 Days	26	86.7	75.6	81.6	78.0	100.0	40.0	75.0	100.0
Family member fever illness (≤ 1 month)	Yes	1	3.3	2.2	7.9	4.0	0.0	20.0	0.0	0.0
	No	29	96.7	97.8	92.1	96.0	100.0	80.0	100.0	100.0
Recent antibiotic treatment (≤ 1 month)	Yes	3	10.0	0.0	7.9	10.0	10.0	0.0	25.0	0.0
	No	27	90.0	100.0	92.1	90.0	90.0	100.0	75.0	100.0
Headache	Yes	18	60.0	82.2	82.9	66.0	80.0	20.0	25.0	0.0
	No	12	40.0	17.8	17.1	34.0	20.0	80.0	75.0	100.0
Throat Infection	Yes	1	3.3	2.2	1.3	2.0	5.0	0.0	0.0	0.0
	No	29	96.7	97.8	98.7	98.0	95.0	100.0	100.0	100.0
Vomiting	Yes	8	26.7	20.0	19.7	20.0	20.0	40.0	25.0	100.0
	No	22	73.3	80.0	80.3	80.0	80.0	60.0	75.0	0.0
Rash	Yes	0	0.0	2.2	1.3	0.0	0.0	0.0	0.0	0.0
	No	30	100.0	97.8	98.7	100.0	100.0	100.0	100.0	100.0

Key: MDR: Multidrug resistant, RI: Resistant Isolate, R0: Sensitive to all antimicrobial groups, R1: resistance to only one antimicrobial groups, R2: resistance to two antimicrobial groups, R3: resistance to three antimicrobial groups, R4: resistance to four antimicrobial groups, R5: resistance to five antimicrobial groups, R6: resistance to six antimicrobial groups, No: Number, %: Percent.

5.5 Macrolide Resistant Phenotyping of Oropharyngeal GAS Isolates

Double disk diffusion test (D-test) was performed for 40 (19.9%) *S. pyogenes* isolates resistant to erythromycin with those isolates sensitive or intermediate to clindamycin disk placed edge to edge 12mm apart for phenotypic detection of inducible clindamycin resistant (ICR) as well for macrolide resistant phenotyping which is also called Macrolide Lincosamide Streptogramin B (MLS_B) resistant phenotyping. Accordingly, among 40 erythromycin resistant isolates, the M phenotype (resistant to erythromycin drug only) were 34 (85%) while constitutive MLS_B ($cMLS_B$) were 6(15%). Inducible ICR or MLS_B ($iMLS_B$) phenotype was not detected. All the intermediate isolates (n = 25) showed M phenotypic characteristics.



Key: M: Macrolide Resistant only, MLS_B: Macrolide Lincosamide Streptogramin B, $cMLS_B$: Constitutive MLS_B, $iMLS_B$: Inducible MLS_B

Figure 5: Magnitude of phenotypic macrolide resistance of *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

5.6 Erythromycin Resistant Genes

In our study, among 65 erythromycin non susceptible GAS isolates (40 resistant and 25 intermediate) screened for erythromycin resistant genes, the predominant gene identified was mef(A) with a frequency of 63 (96.9%) while the frequency of erm(B) and erm(A)-TR were 7 (10.8%) and 5 (7.7%) respectively. All the 25 erythromycin intermediate results were positive for the mef(A) gene and negative to erm(B) and erm(A)-TR genes. Fifty-eight isolates (89.2%) harbored only mef(A) gene whereas the remaining 7 (10.8%) isolates owned two or three erythromycin resistant genes in combinations. The frequency of mef(A) + erm(B) + erm(A)-TR was 3(4.6%), mef(A) + erm(B) was 2(3.1%), erm(A)-TR + erm(B) was 2(3.1%). Six (85.7%) clindamycin resistant isolates were also resistant to erythromycin, but one (14.3%) clindamycin resistant isolate was sensitive to erythromycin.

Table 13: Distribution of erythromycin resistant genes among erythromycin non susceptible *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Erythromycin Resistant Gene	Erythromycin non-susceptible isolates: "R" (40) + "I" (25) = 65			
	Positive		Negative	
	N	%	N	%
mef(A)	63*	96.9	2	3.1
erm(B)	7	10.8	58	89.2
erm(A)-TR	5	7.7	60	92.3

Key: *All the 25 erythromycin intermediate isolates were positive for mef(A) gene only and negative for erm(B) and erm(A)-TR genes, R: resistant, I: intermediate

Table 14: Co-occurrence of erythromycin resistant genes among erythromycin non susceptible *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, Oct. 2022 to Aug. 2023.

Erythromycin Resistant Gene	Erythromycin non-susceptible isolates: "R" (40) + "I" (25) = 65	
	No	%
mef(A) gene only	58*	89.2
mef(A) + erm(B)	2	3.1
erm(A)-TR + erm(B)	2	3.1
mef(A) + erm(B) + erm(A)-TR	3	4.6
mef(A) + erm(A)-TR	0	0.0
Total	65	100.0

Key: *All the 25 erythromycin intermediate isolates were positive for only mef(A) gene, R: resistant, I: intermediate.

5.7 Tetracycline Resistant Genes

Among 113 tetracycline non-susceptible *S. pyogenes* (109 resistant and 4 intermediate) isolates screened for tetracycline resistant genes, tet(M) gene was predominantly identified in 111 (98.2%) followed by tet(K), 29 (25.7%) and tet(O), 12 (10.6%). In this study tet(L) was not totally detected. Among the four tetracycline intermediate isolates, three harbored tet(M) and one harbored tet(M) and tet(K) genes. Seventy-three (64.6%) isolates harbored only tet(M) gene while 28 (24.8%) harbored tet(M) + tet(K), 9 (8%) harbored tet(M) + tet(O) and 1 (0.9%) harbored tet(M) + tet(k) + tet(O) genes. Two isolates (1.8%) harbored only tet(O) genes.

Table 15: Distribution of tetracycline resistant genes among tetracycline non-susceptible *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Tetracycline Resistant Gene	Tetracycline non-susceptible isolates: "R" (109) + "I" (4) = 113			
	Positive		Negative	
	N	%	N	%
tet(M)	111	98.2	2	1.8
tet(K)	29	25.7	84	74.3
tet(O)	12	10.6	101	89.4
tet(L)	0	0.0	113	100.0

Table 16: Co-occurrence of tetracycline resistant genes among tetracycline non-susceptible *S. pyogenes* isolated from the oropharynx of febrile patients in Adama and Wonji, Ethiopia, October 2022 to August 2023.

Tetracycline Resistant Gene	Tetracycline non-susceptible isolates: "R" (109) + "I" (4) = 113	
	N	%
tet(M) only	73[a]	64.6
tet(M) +tet(K)	28[b]	24.8
tet(M) + tet(O)	9	8.0
tet(O) only	2	1.8
tet(M) + tet(k) + tet(O)	1	0.9
tet(K) + tet(O)	0	0.0
Total	113	100.0

Key: [a]Three tetracycline intermediate isolates possessed tet(M) gene, [b]one tetracycline intermediate isolates possessed tet(M) +tet(K) gene, R: Resistant, I: Intermediate.

6 DISCUSSION

6.1 Virulence Factors: Toxins/Superantigens, Proteases and DNases

GAS produces an impressive arsenal of surface expressed and or secreted virulence factors (VFs) that facilitates its pathogenicity. The upper respiratory tract is a major biological niche for *S. pyogenes* (GAS) and serves as the main source of mild to severe invasive GAS diseases (52)**.**

In our study, we have screened all 201 oropharyngeal *S. pyogenes* isolates against 20 different VFs; eleven toxins/superantigens, five proteases and four DNases. Eighteen VFs were identified. All types of toxins and proteases were identified with variable proportions. More than half of the isolates, 60.2% (121/201) had at least one type of the VFs. Multiple co-existence of VFs up to 8 different genes in a single isolate was also identified. The proportion of isolates harboring virulence factors of 2, 3, 4, 5, 6, 7 and 8 were 70 (34.8%), 14 (7.0%), 3 (1.5%), 6 (3.0%), 2 (1.0%), 3 (1.5%) and 1 (0.5%) respectively. Co-existence of VFS were also common between and within toxins and proteases but not seen between DNases.

From eleven GAS toxins/superantigens screened, the predominantly identified genes were SmeZ 34 (16.9%), SpeK 25 (12.4%), SpeI 20 (10.0%), SpeJ 10 (5.0%) and SpeM 9 (4.5%) whereas SpeL 5 (2.5%), SpeA 5 (2.5%), SpeH 4 (2.0%), SpeG 3 (1.5%), SpeC 2 (1.0%) and ssa 2 (1.0%) were identified with very low frequencies. Even though, no sufficient data on GAS toxins/superantigens and other virulence factors were found, some studies conducted on a few superantigens/toxins of SpeA, SpeC, SpeG and ssa isolated from clinical samples showed higher than our findings with variable proportion. In Tunisia, speA (13.4%), speC (53.4%,) and ssa (13.4%) in Portugal, speA (13%), speC (23%) and ssa (38%); in Lebanon, ssa (36 %) and speG (30 %), in India, speC (85%). The findings of speI (1%) in Lebanon was lower than our findings and speA (4.2%) in India was somewhat comparable to our findings (3,22,31). The variable distribution of VFs might be related to geographical variation, type of clinical sample and the variable distribution of the genetic carrier/bacteriophages of the VFs.

The co-existence of toxins up to seven per isolate was also observed. Seventy (34.8%) isolates were at least positive to one or more toxin/superantigen and 29 (14.4%) isolates were positive to two or more toxins/superantigens. Isolates with a multiple toxin of 3, 4, 5 and 7 were 6 (3%), 3

(1.5%), 1 (0.5%) and 1 (0.5%) respectively. Forty-one (20.4%) of the isolates had only one type of toxin. The co-existence of these virulence factors may indicate the capacity of GAS to cause invasive and different clinical manifestations. We did not find similar studies conducted on the co-existence of toxins to compare with our results.

GAS toxins/superantigens are also called streptococcal pyrogenic exotoxins (Spe) (1). Clinical manifestations of disease have been linked to the presence of superantigens (SAgs) in streptococcal pathogens. Studies show that, cases of scarlet fever have been correlated with the carriage or acquisition of ssa, speA and speC. Additionally, speA and ssa, together with speK, and smeZ have been associated with invasive disease and Streptococcal Toxic Shock Syndrome (STSS). In most cases, SpeA and SpeC, toxins have been recognized as the likely causative agents of STSS. Furthermore, the expression of speL, speM and speK genes have been associated with Acute Rheumatic Fever (ARF) (47,53). Therefore, even if their proportions were low in our study, toxins/superantigens that play a role in the pathogenesis of ARF, STSS and scarlet fever have been identified.

GAS SAgs, play role in disease pathogenesis by directly binding to the MHC II molecules and TCR, resulting in the unusual stimulation of about 25% of the T cell population which in turn secretes a vast amount of proinflammatory cytokines such as IL-2, interferon-γ and tumour necrosis factor-α. This uncontrollable cytokine release is then thought to be responsible for the characteristic manifestations of STSS, such as systemic vasodilation, hypotension and multi-organ failure (53).

GAS proteases (peptidases) also play their own role in the pathogenesis of GAS. They catalyze the hydrolysis of host cell peptide bonds and facilitate GAS invasion deep into the host cell (53). In this study, we screened 5 types of proteases and identified all of them in our isolates with variable frequencies; Mac 50 (24.9%), scpA 31 (15.4%) and sic 26 (12.9%), spyCEP 15 (7.5%) and speB 10 (5.0%). Like GAS superantigens, no sufficient data on proteases were found. A study conducted in Lebanon and India identified SpeB as 87% and 58% respectively (3,31) which was much higher than our findings. Probably, mutation of SpeB regulatory gene may down regulates SpeB gene expression (53). Co-existence up to 4 proteases were also observed. Eighty-three (41.3%) were positive to at least one or more proteases and 36 (17.9%) were positive to two or

more proteases. whereas 118 (58.7%) were negative for the investigated proteases. Those isolates positive for 1, 2, 3 and 4 proteases were 47 (23.4%), 25 (12.4%), 9 (4.5%) and 2 (1.0%) respectively. However, we did not get studies conducted on the prevalence of protease as well as the co-existence of proteases to compare with our results.

SpeB is a broad-spectrum cysteine protease that degrades numerous host proteins including immunoglobulins, complement components, chemokines, cytokines, extracellular matrix proteins and numerous other host proteins facilitating GAS pathogenesis. During superficial GAS infections, SpeB helps GAS to degrade the host immune components and replicate in the cytosol of infected epithelial cells allowing establishment and dissemination into deeper tissues. Although SpeB is highly conserved across most *S. pyogenes* strains, its expression may vary depending on the type of infection. For example, 40% of isolates from patients with acute rheumatic fever produced SpeB, compared to only 5.5% of isolates from impetigo patients. Therefore, the variability in SpeB expression suggests that SpeB is more important for certain forms of disease progression. Loss of SpeB expression may happen through the mutations in the regulatory genes (53).

Mac protease specifically cleaves IgG antibodies allowing GAS to resist Ig-mediated phagocytosis and cytotoxicity (47,53). C5a peptidase (ScpA) specifically cleaves and inactivates the C5a complement factor and has been implicated to play a role in inhibiting the recruitment of phagocytes to the infectious site (53). SpyCEP protease cleaves human CXC chemokines, and usually up-regulated in human invasive GAS isolates (53). The streptococcal inhibitor of the complement (sic) gene inhibits the membrane attack complex of the complement system (47).

Another important GAS virulence factor are the DNases. DNases contribute to the pathogenesis of *S. pyogenes* by facilitating innate immune evasion of the pathogen. During infection, neutrophils release antibacterial granule proteins and chromatin to create neutrophil extracellular traps (NETs). NETs bind and trap bacteria and degrade bacterial virulence factors, ultimately resulting in bacterial death (47). GAS overcomes the activity of neutrophils by means of DNases. However; in our study, relatively low DNases were identified with a frequency of 4 (2.0%) for sdaB and 3 (1.5%) for sdaD whereas sdc and spd3 genes were not detected at all. No co-occurrence of DNases was also detected. DNase expression may be associated with active or invasive infections or they

are not common in the oropharyngeal environment. We did not get data of DNases from previous studies to compare with our results. Many DNases are prophage associated, and therefore they are only found in certain *S. pyogenes* strains resulting in variable distributions (47). Mutation, type of infection and variable distribution of bacteriophages may affect the distribution of GAS virulence factors including DNases (47,53).

6.2 Antibiotic Sensitivity Profile

Effective treatment of GAS infection is needed to prevent serious complications and the consecutive immune mediated diseases (79). Usually, penicillin and β-lactams are considered the primary treatment choice for uncomplicated GAS infections (80). For severe infections, a combination of high-dose penicillin and clindamycin is recommended (5). In case of allergic patients, macrolide drugs are recommended as the first alternative therapy (6,81,82). Other recommended alternatives drugs for GAS includes; streptogramin B, vancomycin, chloramphenicol, linezolid and fluoroquinolones. Linezolid, fluoroquinolones and chloramphenicol are used especially for invasive and clindamycin resistant streptococcal infections (80,81,83). However, a high proportion of drug resistance including macrolide- resistant GAS (MRGAS) has been reported in many countries of the world with variable geographical distribution (5,84). Antimicrobial resistance is considered a serious drawback in the management of patients with infectious diseases (3). One big issue of drug resistance worldwide is, drug overuse or an increased drug prescription for both human and animal consumption as well as inappropriate diagnosis and medications which is commonly seen in low- and middle-income countries (79).

In almost all studies, GAS was reported as sensitive to penicillin (5,79,80,85,86). However, in our study, we have identified 20 (10.0%) penicillin non susceptible *S. pyogenes* isolates. Similarly, few recent studies also reported the presence of penicillin non susceptible GAS isolates. In Eastern India intermediate susceptibility to penicillin was 23.57% (31), in Iran non susceptibility to penicillin was 4.2% and to amoxicillin was 38.3% (87) as well as non-susceptibility to penicillin have been reported from China, Japan, Iceland, Mexico and United States (88). Studies indicated that target site modification by penicillin-binding protein 2x (pbp2x) results in penicillin and other β-lactam resistance. Extensive or repeated exposure to β-lactam antibiotics is a factor for pbp2x point mutation (85).

Macrolides have represented a major alternative to the use of penicillin and cephalosporins for the treatment of GAS infections. However, a high proportion of macrolide- resistant GAS (MRGAS) has been reported in many countries (89). The first macrolide-resistant S. *pyogenes* (MRGAS) was reported in 1968 and, since then the resistance rates have varied geographically and temporally, mainly associated with antibiotic use (66). In our study, 65 (32.3%) of the isolates had

reduced susceptibility to erythromycin; 40 (19.9%) were resistant and 25 (12.4%) were intermediate isolates. The proportion of erythromycin resistant GAS isolates in our finding was high and comparable with many other studies conducted in Greek (20.4%), in Lebanon (23%) and Northwest Ethiopia (21.4%) (3,79,84**;** higher than in Brazil (5.9%), Jigjiga, Ethiopia (6.1%), Iran (5.4%) and Tunisia (5.2%) (22,80,86,87); lower than in Gondar, Ethiopia (39.1%), Japan (41.3%) (5,83). The variability of these results may be related to macrolide drug exposure and sample size. It was noted that long-term exposure to azithromycin or erythromycin has been reported to increase the proportion of macrolide resistance in oropharyngeal streptococci (66). Application of antimicrobial stewardship including restriction of macrolide drugs in some countries have decreased the proportion of MRGAS (66).

The macrolide resistance mechanisms in GAS are efflux pumping and target site modification. The gene responsible for efflux pumping is mef(A) (macrolide efflux A) gene whereas that of target site modification is erythromycin ribosomal methylase (erm) genes which includes erm(B) and erm(A) subtype TR. mef(A) gene encodes the transmembrane- and ATP-binding domains of pump that efflux 14- and 15- carbon ring macrolides out of the cell (5,84). The C-14 and C-15 membered macrolides are erythromycin, clarithromycin and azithromycin. Hence, the mef(A) gene leads resistance to macrolide drugs (M phenotype). The erm(B) and erm (A)-TR encode 23S rRNA methylases which mediate target site modification and reduces the binding ability of erythromycin and clindamycin resulting in resistance to Macrolide, Lincosamide and Streptogramin B (MLS_B) drugs (MLS_B phenotype) (5,79,83,84). The macrolide resistance genes can also be encoded on mobile genetic elements, favoring lateral transfer of resistance (79,83).

In our study, among 65 erythromycin non susceptible GAS isolates (40 resistant and 25 intermediate), mef(A) gene was predominantly identified in 63 (96.9%) isolates whereas very low erm(B), 7 (10.8%) and erm(A)-TR, 5 (7.7%) were identified. All erythromycin intermediate (n = 25) results were positive for mef(A) gene only. Fifty-eight isolates (89.2%) harbored only mef(A) gene whereas the remaining 7 (10.8%) isolates harbored two or three erythromycin resistant genes in combination. The frequency of mef(A) + erm(B) + erm(A)-TR was 3 (4.6%), mef(A) + erm(B) was 2 (3.1%), erm(A)-TR + erm(B) was 2 (3.1%). The proportion of erythromycin resistant genes identified in our study was somewhat different from a study conducted in Spain which identified mef(A) in 58 (33.7%) isolates, erm(B) in 83 (48.3) and erm(A)-TR in 46 (26.7%) (23). The

variability may be due to the instability of GAS resistant strains and an inversion from erm to mef(A) genes (15). Six (85.7%) clindamycin resistant isolates were also resistant to erythromycin. Clindamycin inactivation by the lnuB (linB) gene results in resistance to clindamycin, and such isolate is phenotypically categorized as L phenotype (80).

In resource limited settings or in facilities with no molecular tests, double disk diffusion test (D - test) may be used to identify inducible clindamycin resistance (ICR) and also for macrolide resistance phenotyping. Initially, erythromycin resistant and clindamycin sensitive or intermediates isolates are placed side by side 12mm apart and incubated as of the standard microbiological technique. Accordingly, the isolate could be classified in to M phenotype (if resistant to macrolides drugs only), constitutive Macrolide Lincosamide Streptogramin B ($cMLS_B$) phenotype (if resistant to both Macrolide and Lincosamide) and inducible MLS_B ($iMLS_B$) phenotype which is also called inducible clindamycin resistant (ICR). In ICR ($iMLS_B$) phenotype, clindamycin resistance is seen when macrolide is present and it is characterized by blunting of the inhibition zone on the side adjacent to erythromycin disk (D shape) (76). Inducible resistance can result in treatment failure, as inducible clindamycin resistance is undetectable unless macrolides are also present (82). M phenotype is associated with mef(A) gene which encodes macrolide efflux pumping while MLS_B phenotype ($cMLS_B$/$iMLS_B$) is associated with erm(B), erm(A)-TR) genes which encodes target site modification (5,84).

In our study, among 40 erythromycin resistant isolates, M phenotype was identified with higher frequency of, 34 (85%) while constitutive MLS_B ($cMLS_B$) was 6 (15.0%). In this study, inducible MLS_B ($iMLS_B$) or CIR phenotype was not detected at all. All the 25 erythromycin intermediate isolates were M phenotype category. This indicates that efflux pumping which is encoded by the mef(A) gene is the major resistant mechanism that removes 14- and 15- carbon membered macrolide drugs out of the bacterial cell. However, there was a variability between our result and other studies conducted in other countries. A study conducted in Greek showed that, 1(10%) M phenotype, 3 (30%) cMLSB and 6 (60%) iMLSB (79); a study conducted in Brazil showed that 2 (10%) M phenotype, 7 (35%) cMLSB and 11 (55%) iMLSB (80); a study conducted in Spain showed that 49 (28.5%) M phenotype, 76 (44.1%) cMLSB and 46 (26.7%) iMLSB (90); a study conducted in Tunisia showed that 53.4% M phenotype, 46.6% cMLSB and no iMLSB phenotype (22); and a study conducted in Lebanon showed that 2% M phenotype, 3% cMLSB and 1%

iMLSB, phenotype (3). The difference between these findings indicated an inversion in the dominant phenotype of resistance to macrolides. In fact, many studies from multiple countries report significant temporal changes in the prevalence of macrolide resistance, suggesting the instability of the population of GAS strains, which has an impact on the prevalence of the macrolide resistance phenotypes and genotypes (22). The molecular identification of abundant mef(A) gene in erythromycin resistant isolates by molecular method supported the predominance of M phenotype in macrolide resistance phenotyping, since mef(A) gene which encodes efflux pumping is associated with M phenotype and both observed with the highest frequency.

Our laboratory investigation showed, high proportion of tetracycline resistance GAS isolates, 109 (54.2%). This finding was similar to a study conducted in Gondar, Ethiopia (56.5%), in Geek (40.8%), in Hawassa, Ethiopia (57.1%), in Jimma, Ethiopia (52.5%) and in Eastern India (53.57%) (31,44,79,83,91)**.** Many studies indicated high resistance to tetracycline was due to over and misuse of the drug. Our findings also showed the odds of developing tetracycline resistance because of previous antibiotic treatment was 4.85 times higher than those who were not treated [AOR = 4.851, 95% CI (1.04 - 22.641)]. Furthermore, tetracycline is one of the most relevant antimicrobials used in veterinary settings for the treatment of *S. agalactiae* in bovine mastitis (80).

Different genes like, tet(M), tet(O), tet(K) and tet(L) are responsible for tetracycline resistance. tet(M) and tet(O) encodes ribosomal protection proteins while tet(K) and tet(L) encodes efflux pumping. In this study, among tetracycline non susceptible GAS isolates (109 resistant and 4 intermediate), tet(M) gene was predominantly identified in 111 (98.2%) followed by tet(K), 29 (25.7%) and tet(O), 12 (10.6%). We did not identify tet(L) gene at all in our isolates. Three tetracycline intermediate isolates harbored tet(M) gene only while one harbored tet(M) and tet(K) genes in combination. Seventy-three (64.6%) isolates harbored only tet(M) gene while 28 (24.8%) harbored tet(M) + tet(K), 9 (8%) harbored tet(M) + tet(O) and 1 (0.9%) harbored tet(M) + tet(k) + tet(O) genes. Two isolates (1.8%) possessed only tet(O) genes. A study conducted in Spain indicated that the tet(M) gene was detected in 185 (78.1%) isolates, while tet (O) was detected in 81 (34.2%) (22). The findings of our study indicated that ribosomal protection encoded by tet(M) gene was the most common tetracycline resistant gene.

Our laboratory findings also showed that, GAS isolates were highly resistant to quinupristin-dalfopristin with a total reduced susceptibility of 70.1% (141/201) of which 43.3% (87/201) were resistant and 26.9% (54/201) were intermediate isolates. Even though no sufficient and recent data about the antibiotic sensitivity profile of GAS against quinupristin-dalfopristin was found, a study conducted in Lebanon long ago reported 100% sensitivity (3). The variation might be related to the study time period as exposure to drugs through time enhances resistance. Resistance to quinupristin-dalfopristin could happen through the production of ribosomal methylase enzyme that modifies the drug target site (5).

In this study, we have identified 13 (6.5%) ceftriaxone non susceptible isolates which was comparable with a study in Jigjiga, Ethiopia (4.1%) (86), Iran (8.1%) (87); lower than in Northwest Ethiopia (35.7%) (84) and Gondar (13%) (83). Studies from Greek and Lebanon reported 100% sensitivity to ceftriaxone (3,79). The variability might be related to sample size and study time period.

In case of severe invasive manifestations, clindamycin should be given with penicillin or other β-lactam antibiotics. The added benefit of clindamycin treatment is that it reduces the development of toxin-mediated symptoms like STSS. Since penicillin treatment can lead to lysis and toxin release, protein synthesis inhibitors like clindamycin that decrease toxin production can help mitigate excessive immune stimulation. Resistance to clindamycin is geographically variable across the world. In our study, low resistance to clindamycin,7(3.5%) was observed, probably because of low consumption. This result is more or less likely comparable with a study in, Brazil (5.5.%) (80), lower than in Gondar (9%) (83), Lebanon (9%) (3), Iran 12.4% (87), Japan (16.8%) (5) and much lower than Northwest Ethiopia (50.0%) (84), while a study in Eastern India reported 100% susceptibility (31).

Ofloxacilin, chloramphenicol, vancomycin and linezolid are alternative drugs used in invasive GAS infections with penicillin, in clindamycin resistant isolates or without penicillin, in penicillin allergic patients given their contraindication in concern. In our study, we have observed very low resistance to ofloxacin, chloramphenicol and vancomycin which were 3 (1.5%), 2 (1.0%) and 1 (0.5%) respectively while all GAS isolates were 100% sensitive to linezolid. Our finding was comparable with other studies; in Gondar, vancomycin and chloramphenicol were 100% sensitive

(83), in Greek susceptibility to linezolid and vancomycin were 100% (79). But in Iran resistance to vancomycin was 14.1% and to chloramphenicol was 8.4% (87); in Jigjiga resistance to chloramphenicol was (6.1%) and to vancomycin was (8.2%) (86); in North west Ethiopia resistance to vancomycin was 5 (35.7%) (84). This indicates the emergence of resistance strains against the prescription-controlled drugs even if there was sample size variability.

Currently, linezolid is one of the promising drugs in intensive care units. It has been approved for the treatment of complicated infections caused by drug resistant streptococcus species, enterococcus species, staphylococcus species, as well as drug resistant tuberculosis infections. The compound is a synthetic antibiotic that inhibits bacterial protein synthesis through binding to rRNA on both the 30S and 50S ribosomal subunits inhibiting the formation of initiation complex (92). In our finding, 100% sensitivity to linezolid also supports the effectiveness of this drug in treatment of GAS infection.

7 STRENGTHS AND LIMITATIONS

7.1 Strengths

- In our study, a large number of *S. pyogenes* (GAS) isolates were included for better explanation of the current GAS antibiotic sensitivity profile, macrolide resistance phenotyping, erythromycin and tetracycline resistance gene.
- We performed antibiotic sensitivity testing twice for each of penicillin non susceptible, ceftriaxone non susceptible and other drug resistance isolates, to be more confident on the resistance status of GAS isolates performed by the disk diffusion technique.
- To our knowledge this study is the first that combines GAS antibiotic sensitivity profiling, GAS macrolide resistance phenotyping and molecular characterization of GAS virulence factors, erythromycin and tetracycline resistance genes.

7.2 Limitations

Due to the unavailability of resources, minimum inhibitory concentration (MIC) or E test, which can evaluate AST better than disc diffusion test, characterization of penicillin binding protein 2x (PBP2x) for penicillin non susceptible isolates and drug resistant genes other than erythromycin and tetracycline as well as GAS emm typing was not performed in this study.

8 CONCLUSION AND RECOMMENDATIONS

8.1 Conclusion

In this study, we have investigated the presence of 20 virulence factors and the antibiotic susceptibility profile of 201 *S. pyogenes* isolates. We have observed that, more than half of the GAS isolates (60.2%) had at least one or more types of virulence factors and again 77.6% were at least resistant to one or more classes of antibiotics. Among the 20 different GAS virulence factors (eleven superantigens, five proteases and four DNases) screened, 18 of them were identified with variable proportions. Superantigens and proteases were more commonly identified than DNases. Probably, DNases were not common in the oropharyngeal environment. Co-existence of the virulence factors up to eight with in a single isolate were also observed which will indicate the virulence potential of the bacterium. More importantly and contrary to the previous studies, we have identified an emerging penicillin non susceptible GAS isolates (10%) and this might be an alarming condition. GAS remains highly resistant to tetracycline and erythromycin while the highest rates of non-susceptibility to quinupristin-dalfopristin (70.1%) among GAS isolates were observed in our study. However, all GAS isolates were 100% sensitive to linezolid, probably the promising drug for the treatment of drug resistant GAS strains. Majority of the erythromycin resistance GAS (ERGAS) isolates fall in the M phenotype category (resistance to macrolide drugs only). In our study, no inducible clindamycin resistance (ICR) was observed among ERGAS isolates. Efflux pumping which was encoded by mef(A) gene was the most erythromycin resistance mechanism whereas, target site modification which was encoded by tet(M) gene was the most common tetracycline resistance mechanism in our GAS isolates. Therefore, the presence and co-existence of different virulence factors and the increasing trend of drug non susceptibility especially to penicillin in GAS isolates will indicate, the probable occurrence and spread of severe, invasive and life-threatening GAS infections unless early control measures have been taken.

8.2 Recommendations

Based on our findings, we would like to recommend the following points.

- To reduce the increasing trends of drug resistance GAS infections, it is very important if the treatment protocols are supported with antibiotic sensitivity testing while empirical treatment of GAS infections should be minimized or evidence based.
- To prevent antimicrobial resistance, specific antibiotic utilization or antibiotic restriction has to be strengthened.
- Since non susceptibility to penicillin have been detected, active surveillance and characterization of penicillin resistance isolates should have to be of great clinical concern, as well as continuous surveillance of antibiotics resistance of *S. pyogenes* is important
- Expansion of diagnostic facilities with culture and AST capacity at least in hospital facilities must be encouraged to track antimicrobial resistance and for the right use of antibiotics.
- Regular characterization of the drug resistant and virulence factors of GAS isolates by reference, research and regional laboratories have to be encouraged
- A better communication among clinicians, regional laboratories, reference laboratory and research laboratories, ministry of health, regional health bureaus and stakeholders are very much important in order to generate robust data about antimicrobial resistance and virulence factors which will be useful input to guide or modify therapeutic protocols in the country for effective control and prevention strategies
- The Clinical Laboratory Standards Institute (CLSI), has to revise its guideline in setting intermediate and resistant breakpoints for penicillin, ceftriaxone and other β-lactam non susceptible GAS isolates since they have been reported and on the rise.

9 REFERENCES

1. Ahmad S. Molecular Characterization of *Streptococcus pyogenes* Isolated from Children with Pharyngitis. J Bacteriol Mycol Open Access. 2018;6(1):2007–9.

2. Amada BSH, Awabata SK, Akagawa IN. Review Molecular and genomic characterization of pathogenic traits of group A *Streptococcus pyogenes*. 2015;91(10):539–59.

3. Karaky NM, Araj GF, Tokajian ST. Molecular characterization of *Streptococcus pyogenes* group A isolates from a tertiary hospital in Lebanon. 2014;1197–204.

4. Strus M, Heczko PB, Goli E, Tomusiak A, Chmielarczyk A. The virulence factors of group A streptococcus strains isolated from invasive and non-invasive infections in Polish and German centres , 2009 – 2011. 2017;

5. Tanaka Y, Gotoh K, Teramachi M, Ishimoto K. Molecular epidemiology , antimicrobial susceptibility , and characterization of macrolide-resistant *Streptococcus pyogenes* in Japan. J Infect Chemother [Internet]. 2016;0–5. Available from: http://dx.doi.org/10.1016/j.jiac.2016.06.013

6. Tatara K, Gotoh K, Okumiya K, Teramachi M, Ishimoto K, Tanaka Y, et al. Molecular epidemiology , antimicrobial susceptibility , and characterization of fl uoroquinolone non-susceptible *Streptococcus pyogenes* in Japan *. J Infect Chemother [Internet]. 2019;(xxxx):8–12. Available from: https://doi.org/10.1016/j.jiac.2019.10.004

7. Brouwer S, Barnett TC, Rivera-hernandez T, Rohde M. *Streptococcus pyogenes*. 2016;

8. Abraham T, Sistla S. Trends in Antimicrobial Resistance Patterns of Group A Streptococci , Molecular Basis and Implications. Indian J Med Microbiol [Internet]. 2018;36(2):186–91. Available from: https://doi.org/10.4103/ijmm.IJMM_18_107

9. J.R. C, A.C. S, E.K. M, M. W. The global burden of group A streptococcal diseases. Lancet Infect Dis. 2005;

10. Cornick JE, Kiran AM, Vivancos R, Van Aartsen J, Clarke J, Bevan E, et al. Epidemiological and Molecular Characterization of an Invasive Group A Streptococcus emm 32.2 Outbreak. J Clin Microbiol. 2017;55(6):1837–46.

11. Vu T, Bessen DE, Guglielmini J, Steer AC, Carapetis JR, Melderen L Van. Updated model of group A Streptococcus M proteins based on a comprehensive worldwide study. 2012;

12. Li R, Liang C, Jiang L, Yuan C, Huang M. Biochemical and Biophysical Research Communications Structural determination of group A Streptococcal surface dehydrogenase and characterization of its interaction with urokinase-type plasminogen activator receptor. Biochem Biophys Res Commun [Internet]. 2019;(xxxx):1–6. Available from: https://doi.org/10.1016/j.bbrc.2019.01.102

13. Sánchez-Encinales V, Viviana G, Tamayo E, García-Arenzana JM, Muñoz-Almagro C MM. Causing Invasive Disease in Pediatric Population in Spain. Pediatr Infect Dis J. 2019;38(12):1168–72.

14. Cunningham MW. Pathogenesis of group a streptococcal infections. Clinical Microbiology Reviews. 2000.

15. Seidler KA, Seidler NW. ZŽůĞ ŽĨ ĞdžłđĂĐĞůůŽůĂđ ’ W , in *Streptococcus pyogenes* Virulence. 2013;(June):236–40.

16. Shaikh N, Leonard E, Martin JM. Prevalence of streptococcal pharyngitis and streptococcal carriage in children: A meta-analysis. Pediatrics. 2010;

17. Borek AL, Obsza K, Hryniewicz W, Sitkiewicz I. Detection of *Streptococcus pyogenes* virulence factors by multiplex PCR. 2012;943:529–33.

18. Park SE, Toy T, Maria L, Espinoza C, Panzner U, Mogeni OD, et al. The Severe Typhoid Fever in Africa Program : Study Design and Methodology to Assess Disease Severity , Host Immunity , and Carriage Associated With Invasive Salmonellosis. 2019;69(Suppl 6).

19. Osowicki J, Azzopardi KI, Mcintyre L, Rivera-hernandez T, Ong CY, Baker C. A Controlled Human Infection Model of Group A Streptococcus. 2019;4(1):1–13.

20. Martins L, Anjos M, Marcondes MB, Lima MF, Mondelli AL, Okoshi MP. Review Case Report Article Streptococcal acute pharyngitis. 2014;47(December 2013):409–13.

21. Edward A. Sykes, Vincent Wu, Michael M. Beyea, Matthew T.W. Simpson JAB. Approach to diagnosis and treatment. 2020;66:251–7.

22. Ksia S, Smaoui H, Hraoui M, Bouafsoun A. Molecular Characteristics of Erythromycin-Resistant *Streptococcus pyogenes* Strains Isolated. 2016;00(00):1–7.

23. Martin, Judith M, M.D. ERYTHROMYCIN-RESISTANT GROUP A STREPTOCOCCI IN SCHOOLCHILDREN IN PITTSBURGH. N Engl J Med. 2002;346(16).

24. Van Heirstraeten L, Coenen S, Lammens C, Hens N, Goossens H, Malhotra-Kumar S. Antimicrobial drug use and macrolide-resistant *Streptococcus pyogenes*, Belgium. Emerg Infect Dis. 2012;

25. Khosravi AD, Ebrahimifard N, Shamsizadeh A, Shoja S. ScienceDirect Isolation of *Streptococcus pyogenes* from children with pharyngitis and emm type analysis. J Chinese Med Assoc [Internet]. 2016;1–5. Available from: http://dx.doi.org/10.1016/j.jcma.2016.01.002

26. Mcmillan DJ, Vu T, Bramhachari P V, Kaul SY, Bouvet A, Shaila MS, et al. Molecular markers for discriminating *Streptococcus pyogenes* and S . dysgalactiae subspecies equisimilis. 2010;585–9.

27. Smoot JC, Korgenski EK, Daly JA, Veasy LG, Musser JM. Molecular Analysis of Group A Streptococcus Type emm18 Isolates Temporally Associated with Acute Rheumatic Fever Outbreaks in Salt Lake City , Utah. 2002;40(5):1805–10.

28. Abdissa A, Asrat D, Kronvall G, Shittu B, Achiko D, Zeidan M, et al. High diversity of group A streptococcal emm types among healthy schoolchildren in Ethiopia. Clin Infect Dis. 2006;

29. Wajima T, Murayama SY, Sunaoshi K, Nakayama E, Sunakawa K, Ubukata K. Distribution of emm type and antibiotic susceptibility of group A streptococci causing invasive and noninvasive disease. J Med Microbiol. 2008;

30. M. H, I. B-BB, A. D, E. S, S. BR, A. B. Epidemiological markers of *Streptococcus pyogenes* strains in Tunisia. Clin Microbiol Infect. 2011;

31. Ray D, Saha S, Sinha S, Pal NK, Bhattacharya B. Molecular characterization and evaluation of the

emerging antibiotic-resistant *Streptococcus pyogenes* from eastern India. BMC Infect Dis [Internet]. 2016;1–11. Available from: http://dx.doi.org/10.1186/s12879-016-2079-9

32. O'Loughlin RE, Roberson A, Cieslak PR, Lynfield R, Gershman K, Craig A, et al. The epidemiology of invasive group A streptococcal infection and potential vaccine implications: United States, 2000-2004. Clin Infect Dis. 2007;

33. Lamagni TL, Efstratiou A, Dennis J, Nair P, Kearney J, George R. Increase in invasive group A streptococcal infections in England, Wales and Northern Ireland, 2008-9. Euro Surveill. 2009;

34. S. N, R. K, P. R, H. V, N.K. G. Group A streptococcal sore throat in a periurban population of northern India: A one-year prospective study. Bull World Health Organ. 2001;

35. Liu D, Hollingshead S, Swiatlo E, Lawrence ML, Austin FW. Rapid identification of *Streptococcus pyogenes* with PCR primers from a putative transcriptional regulator gene. 2005;156:564–7.

36. Thenmozhi R, Balaji K, Kanagavel M, Pandian SK. *Streptococcus pyogenes*. 2010;306:110–6.

37. Proft T, Sriskandan S, Yang L, Fraser JD. Superantigens and streptococcal toxic shock syndrome. Emerg Infect Dis. 2003;

38. Lintges M, Van Der Linden M, Hilgers RD, Arit S, Al-Lahham A, René Reinert R, et al. Superantigen Genes Are More Important than the emm Type for the Invasiveness of Group A Streptococcus Infection. J Infect Dis. 2010;

39. Brouwer S, Barnett TC, Ly D, Kasper KJ, Oliveira DMP De, Rivera-hernandez T, et al. Prophage exotoxins enhance colonization fitness in epidemic scarlet fever-causing *Streptococcus pyogenes.* Nat Commun [Internet]. 2020; Available from: http://dx.doi.org/10.1038/s41467-020-18700-5

40. Agarwal S, Agarwal S, Pancholi P, Pancholi V. Role of Serine / Threonine Phosphatase (SP-STP) in *Streptococcus pyogenes* Physiology and Virulence * □. 2011;286(48):41368–80.

41. Detcheva A, Facklam RR, Beall B. Erythromycin-resistant group A streptococcal isolates recovered in Sofia, Bulgaria, from 1995 to 2001. J Clin Microbiol. 2002;

42. Bahnan W, Hashwa F, Araj G, Tokajian S. emm typing, antibiotic resistance and PFGE analysis of *Streptococcus pyogenes* in Lebanon. J Med Microbiol. 2011;

43. Arvand M, Hoeck M, Hahn H, Wagner J. Antimicrobial resistance in *Streptococcus pyogenes* isolates in Berlin. J Antimicrob Chemother. 2000;

44. Anja A, Beyene G, Mariam ZS, Daka D. Asymptomatic pharyngeal carriage rate of *Streptococcus pyogenes* , its associated factors and antibiotic susceptibility pattern among school children in Hawassa town , southern Ethiopia. BMC Res Notes [Internet]. 2019;1–6. Available from: https://doi.org/10.1186/s13104-019-4601-9

45. Cho KYUH, Port GC, Caparon M. Genetics of Group A Streptococci. 2019;

46. Cole JN, Barnett TC, Nizet V, Walker MJ. Molecular insight into invasive group A streptococcal disease. Nature Reviews Microbiology. 2011.

47. Shannon BA, Mccormick JK, Schlievert PM. Toxins and Superantigens of Group A Streptococci. 2019;1–15.

48. Id HK, Id LH, Id YK, Chowdhury S. Structural determination of *Streptococcus pyogenes* M1 protein interactions with human immunoglobulin G using integrative structural biology. 2021;1–19.

49. Ferretti JJ, Stevens DL, Fischetti V a. *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations. Streptococcus pyogenes Basic Biol to Clin Manifestations. 2016;

50. Rivera A, Rebollo M, Miró E, Mateo M, Navarro F, Gurguí M, et al. Superantigen gene profile, emm type and antibiotic resistance genes among group A streptococcal isolates from Barcelona, Spain. J Med Microbiol. 2006;

51. Mitchell DT, Levitt DG, Schlievert PM, Ohlendorf DH. Structural evidence for the evolution of pyrogenic toxin superantigens. J Mol Evol. 2000;

52. Kasper KJ, Zeppa JJ, Wakabayashi AT, Xu SX, Mazzuca DM, Welch I, et al. Bacterial Superantigens Promote Acute Nasopharyngeal Infection by *Streptococcus pyogenes* in a Human MHC Class II-Dependent Manner. 2014;10(5).

53. Barnett TC, Cole JN, Rivera-Hernandez T, Henningham A, Paton JC, Nizet V, et al. Streptococcal toxins: Role in pathogenesis and disease. Cell Microbiol. 2015;17(12):1721–41.

54. Domínguez MA, Aguilar L, Cercenado E, Gobernado M, García-perea A, Maran UG. Survey of emm Gene Sequences from Pharyngeal *Streptococcus pyogenes* Isolates Collected in Spain and Their Relationship with Erythromycin Susceptibility. 2003;41(6):2385–90.

55. Radcliff FJ, Clow F, Mahadevan M, Johnston J, Proft T, Douglas RG, et al. A potential role for staphylococcal and streptococcal superantigens in driving skewing of TCR V β subsets in tonsillar hyperplasia. Med Microbiol Immunol. 2017;

56. Čižman M, Pokorn M, Seme K, Oražem A, Paragi M. The relationship between trends in macrolide use and resistance to macrolides of common respiratory pathogens. J Antimicrob Chemother. 2001;

57. Friães A, Pinto FR, Silva-Costa C, Ramirez M, Melo-Cristino J. Superantigen gene complement of *Streptococcus pyogenes* - Relationship with other typing methods and short-term stability. Eur J Clin Microbiol Infect Dis. 2013;

58. Tamayo E, Montes M, Vicente D, Pérez-trallero E. *Streptococcus pyogenes* Pneumonia in Adults : Clinical Presentation and Molecular Characterization of Isolates 2006-2015. 2016;28:1–13.

59. Walker MJ, Cole JN. crossm Virulence Role of the GlcNAc Side Chain of the Lancefield Cell Wall Carbohydrate Antigen in Non-M1-Serotype Group A Streptococcus. 2018;9(1):1–12.

60. Malhotra-Kumar S, Lammens C, Piessens J, Goossens H. Multiplex PCR for simultaneous detection of macrolide and tetracycline resistance determinants in streptococci. Antimicrob Agents Chemother. 2005;

61. Gattringer R, Sauermann R, Lagler H, Stich K, Buxbaum A, Graninger W, et al. Antimicrobial susceptibility and macrolide resistance genes in *Streptococcus pyogenes* collected in Austria and Hungary. Int J Antimicrob Agents. 2004;

62. Ayer V, Tewodros W, Manoharan A, Skariah S, Luo F, Bessen DE. Tetracycline resistance in group A streptococci: Emergence on a global scale and influence on multiple-drug resistance. Antimicrob Agents Chemother. 2007;51(5):1865–8.

63. Brahmadathan KN, Anitha P, Gladstone P. Increasing erythromycin resistance among group A streptococci causing tonsillitis in a tertiary care hospital in southern India. Clin Microbiol Infect. 2005;

64. Jacob S, Lloyd C, Menon T. cMLS and M Phenotypes among *Streptococcus pyogenes* Isolates in Chennai. Indian J Med Microbiol. 2006;

65. Dipanwita R, Sukanta S, Somnath S, Sankar K, Dutta RN, Sujata B, et al. A preliminary sentinel surveillance report on antibiotics resistance trend of *Streptococcus pyogenes* in Kolkata region, India. Al Ameen J Med Sci. 2010;

66. Berbel D, Gonz A, Guillem L, Egea D, Jordi C, Ardanuy C. An Overview of Macrolide Resistance in Streptococci : Prevalence , Mobile Elements and Dynamics. 2022;1–23.

67. Huang C, Lai J, Huang I, Chen P, Wang H, Shiau Y, et al. Epidemiology and Molecular Characterization of Macrolide-Resistant *Streptococcus pyogenes* in Taiwan. 2014;52(2):508–16.

68. Rubio-Lápez V, Valdezate S, Álvarez D, Villalán P, Medina MJ, Salcedo C, et al. Molecular epidemiology, antimicrobial susceptibilities and resistance mechanisms of *Streptococcus pyogenes* isolates resistant to erythromycin and tetracycline in Spain (1994-2006). BMC Microbiol. 2012;

69. Zhou W, Jiang YM, Wang HJ, Kuang LH, Hu ZQ, Shi H, et al. Erythromycin-resistant genes in group A β-haemolytic Streptococci in Chengdu, Southwestern China. Indian J Med Microbiol. 2014;

70. Syrogiannopoulos GA, Grivea IN, Al-Lahham A, Panagiotou M, Tsantouli AG, Michoula AN, et al. Correction: Seven-Year Surveillance of emm Types of Pediatric Group A Streptococcal Pharyngitis Isolates in Western Greece. PLoS One. 2013;

71. Hirakata Y, Komatsu M, Muratani T, Kaku M. Drug susceptibility of bacteria isolated from pediatric respiratory infections at general practitioners' clinics to pediatric antibiotics. Jpn J Antibiot. 2009;

72. Sonia, K., Hanen, S., Amel, K., Anne B. Streptococcus pyogenes isolated in a Tunisian pediatric population: Emm types, T types, virulence factors and genes of resistance to macrolide and tetracycline. Malays J Microbiol. 2013;

73. Giovanetti E, Brenciani A, Lupidi R, Roberts MC, Varaldo PE. Presence of the tet (O) Gene in Erythromycin- and Tetracycline-Resistant Strains of *Streptococcus pyogenes* and Linkage with either the mef (A) or the erm (A) Gene. 2003;47(9):2844–9.

74. CSA. Ethiopian 2022 Project Population Census. 2022;(July):13.

75. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect [Internet]. 2012;18(3):268–81. Available from: http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x

76. Clinical and Laboratory Standards Institute (CLSI). M100 Performance Standards for Antimicrobial Susceptibility Testing. Clinical and Laboratory Standards Institute (CLSI). 2021.

77. Birnboim HC. A Rapid Alkaline Extraction Method for the Isolation of Plasmid DNA. Methods Enzymol. 1983;100(C):243–55.

78. Bingen E, Fitoussi F, Doit C, Cohen R, Tanna A, George R, et al. Resistance to macrolides in

Streptococcus pyogenes in France in pediatric patients. Antimicrob Agents Chemother. 2000;

79. Meletis G, Lazaros A, Ketikidis S, Floropoulou N, Tychala A, Kagkalou G, et al. Antimicrobial resistance rates of *Streptococcus pyogenes* in a Greek tertiary care hospital : 6-year data and literature review. 2023;37–42.

80. Barros RR. Antimicrobial Resistance among Beta-Hemolytic Streptococcus in Brazil : An Overview. 2021;

81. Mustafa Z, Ghaffari M. Diagnostic Methods , Clinical Guidelines , and Antibiotic Treatment for Group A Streptococcal Pharyngitis : A Narrative Review. 2020;10(October):1–10.

82. Johnson AF, Larock CN. Antibiotic Treatment , Mechanisms for Failure , and Adjunctive Therapies for Infections by Group A Streptococcus. 2021;12(November):1–9.

83. Tadesse M, Hailu Y. Prevalence , Antibiotic Susceptibility Profile and Associated Factors of Group A Streptococcal pharyngitis Among Pediatric Patients with Acute Pharyngitis in Gondar , Northwest Ethiopia. 2023;(March):1637–48.

84. Kebede D, Admas A, Mekonnen D. Prevalence and antibiotics susceptibility profiles of *Streptococcus pyogenes* among pediatric patients with acute pharyngitis at Felege Hiwot Comprehensive Specialized Hospital , Northwest Ethiopia. 2021;1–10.

85. Vannice KS, Ricaldi J, Nanduri S, Fang FC, Chochua S, Beneden CA Van, et al. *Streptococcus pyogenes* pbp2x Mutation Confers Reduced Susceptibility to β-Lactam Antibiotics. 2020;71(January 2018):2018–21.

86. Barsenga S, Mitiku H, Tesfa T, Shume T. Throat carriage rate , associated factors , and antimicrobial susceptibility pattern of group A Streptococcus among healthy school children in Jigjiga City , Eastern Ethiopia. BMC Pediatr [Internet]. 2022;1–8. Available from: https://doi.org/10.1186/s12887-022-03294-2

87. Khademi F, Vaez H, Sahebkar A, Taheri RA. Group A. 2021;36(1).

88. Yu D, Guo D, Zheng Y. A review of penicillin binding protein and group A Streptococcus with reduced- b - lactam susceptibility. 2023;(March):1–6.

89. Leclercq R. Mechanisms of Resistance to Macrolides and Lincosamides : Nature of the Resistance Elements and Their Clinical Implications. 2002;34.

90. Villal P, Marta B, Garrido N, Pino-rosa S. Clindamycin Resistance in Invasive *Streptococcus pyogenes* : A Retrospective Study of the Situation in Spain , 2007 – 2020. 2023;

91. Tesfaw G, Kibru G, Mekonnen D, Abdissa A. Prevalence of group A β-haemolytic Streptococcus among children with pharyngitis in Jimma town, Southwest Ethiopia. Egypt J Ear, Nose, Throat Allied Sci [Internet]. 2015;16(1):35–40. Available from: http://dx.doi.org/10.1016/j.ejenta.2015.02.001

92. Press D. Linezolid : a review of its properties , function , and use in critical care. 2018;1759–67.