Chapter One

Introduction

Bacterial infections are significant threat to the health of neonates and adults. The throat and skin of the human host are the principal reservoirs for the bacterial pathogen.

The genus Streptococcus, a heterogeneous group of Gram-positive bacteria, has broad significance in medicine and industry. Streptococci are Gram-positive, non-motile, non-spore forming, catalase-negative that occur in pairs or chains, and produce grey mucoid 2 mm diameter colonies. Older cultures may lose their Gram-positive character. Most Streptococci are facultative anaerobes, and some are obligate (strict) anaerobes. Most require enriched media (blood agar). Streptococci are essential in industrial and dairy processes and as indicators of pollution (Patterson, 1996).

Streptococci are classified on the basis of colony morphology, haemolysis, biochemical reactions, and (most definitively) serologic specificity. They are divided into three groups by the type of haemolysis on blood agar: β-haemolytic (clear, complete lysis of red cells), α haemolytic (incomplete, green haemolysis), and γ haemolytic (no haemolysis).

Serologic grouping is based on antigenic differences in cell wall carbohydrates (groups A to V, except I and J), in cell wall pili-associated protein, and in the polysaccharide capsule in group B Streptococci. Diagnosis is based on cultures from clinical specimens. Serologic methods can detect group A or B antigen; definitive antigen identification is by the precipitin test. Bacitracin sensitivity presumptively differentiates group A from other β-haemolytic Streptococci (B, C, and G); group B Streptococci typically show hippurate hydrolysis; group D is differentiated from other viridans Streptococci by bile solubility and optochin sensitivity (Patterson, 1996).

Various species of Streptococci are important ecologically as part of the normal microbial flora of animals and humans; some can also cause diseases that range from acute to sub-acute or even chronic (Patterson, 1996).

Group A Streptococci (GAS) or *Streptococcus pyogenes* has been recognized as an important human pathogen, since early days of modern microbiology, and it remains among the top ten causes of mortality from an infectious disease (Ralph and Carapetis, 2013).

Group B Streptococci (GBS) or *Streptococcus agalactiae* is a major cause of sepsis and meningitis in neonates and infants and of invasive

disease in pregnant and non-pregnant women, presumably immunocompromised adults, and the elderly. Nine GBS serotypes have been described (Borchardt, *et al.*, 2004).

The roles of group C and F Streptococci in causing endemic pharyngitis are still controversial, although group C Streptococci are implicated in the outbreaks of pharyngitis and associated disorders. Group C beta hemolytic Streptococcal infections are uncommon in humans, and group C beta hemolytic Streptococcal pneumonia is exceedingly rare (Al-charrakh, *et al.*, 2011).

The Viridans Group Streptococci (VGS) are human commensal, colonizing the gastrointestinal and genitourinary tracts in addition to the oral mucosa. VGS are generally considered to be of low pathogenic potential individuals. However, in certain patient populations, VGS can cause invasive disease, such as endocarditis, intra-abdominal infection, and shock (Christopher and Carey, 2010).

Enterococci are commensal of the human and animal gastrointestinal tract and opportunistic pathogens that cause urinary tract infections (UTIs), endocarditis and sepsis (Louise, *et al.*, 2012).

Koppe, *et al.*, (2012) found that *Streptococcus pneumoniae* is both a frequent colonizer of the upper respiratory tract and a leading cause of life-threatening infections such as pneumonia, meningitis and sepsis.

Group G Streptococci (GGS) are usually, but not exclusively, beta-hemolytic. *S. canis* is an example of a GGS which is typically found on animals, but can cause infection in humans.

Among methods that allow quicker bacterial identification from growing colonies, the study of Jonathan, *et al.*, (2013) who found that, Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry was demonstrated to accurately identify bacteria routinely isolated from blood culture in clinical biology laboratories, in order to speed up the identification process.

Objectives

General objective

To determine the phenotypic and molecular characterization of Streptococcal infection in Sudanese patients

Specific objective

1/ To isolate and identify Streptococcal groups in patients suffering from different disease conditions.

2/ To study the phenotypic and genotypic characters of the isolated organisms by biochemical and serological methods.

3/ To improve the laboratory diagnosis of Streptococcal infection in Sudan through introduction of Polymerase Chain Reaction (PCR) technique.

4/ To study the sensitivity of the isolated organisms to antibiotics.

Chapter Two

Literature Review

2-1 Identification of Pathogenic Bacteria

The rapid identification of pathogenic bacteria is important for earlier effective patient management and antimicrobial therapy, especially for the infant patient, whose immunological system is not fully developed. However conventional microbiogical techniques of bacterial identification, culture and isolation of pathogenic bacteria, identification by biochemical and serological assay, are time-consuming and require intensive labour. On the basis of special gene sequence, Polymerase Chain Reaction (PCR) provides a simple and rapid way to identify bacteria. But it is difficult to identify all of bacteria species which are suspicious of pathogenic agents (Shen and Feng, 2004).

Identifying different types of organisms within a species is called typing. Traditional typing systems based on phenotype, such as serotype, biotype, and phage-type or anti bio-gram, have been used for many years. However, the methods that examine the relatedness of isolates at a molecular level have revolutionized the ability to differentiate among

bacterial types (or subtypes). The choice of an appropriate molecular typing method (or methods) depends significantly on the problem to solve and the epidemiological context in which the method is going to be used, as well as the time and geographical scale of its use. Therefore, typing techniques should have excellent typeability to be able to type all the isolates studied (Sabat, *et al.*, 2013).

2-2 Taxonomy of Streptococci

Since the division of the Streptococcus genus into Enterococci, Lactococci and Streptococci in 1984, many changes in the nomenclature and taxonomy of the Streptococcus genus have taken place. The application of genetic comparisons has improved the proper classification of the different species. The Lancefield system of serogrouping the Streptococci by the expression of beta-haemolysis on blood agar plates are still very useful for the identification of Streptococci for patient management. The Lancefield grouping system cannot be used in itself for accurate identification of specific beta-hemolytic species, but it can be a useful part of the identification procedure (Ritchard, 2002).

Riapis and Briko (2008) performed a comparative analysis of main trends of taxonomy, classification, and nomenclature of prokaryotic

organisms and Streptococci. Phylogenetic classification of Streptococci was thoroughly considered. Original classification of Streptococci based on pathogenicity and ecological-epidemiological criteria was proposed for medical purposes. It was shown that according to ecological-epidemiological criterion Streptococci can be referred to as agents of anthroponoses, zoonoses, and sapronoses.

2-3 Sore Throat

The sore throat decision rule can identify both patients who are so likely to have Group A Beta Haemolytic Streptococci (GABHS) that a confirmatory test is not needed and patients who are so unlikely to have GABHS that further testing is unrewarding. Using the rule will successfully identify most patients who need treatment for GABHS infection, while decreasing antibiotic use for sore throat by about 80%. The 4 most useful features to look for in diagnosing GABHS are enlarged sub-mandibular glands, throat exudates, fever, and absence of cough and runny nose (Graham, 2007).

A study carried by Ayse (2014) resulted in that *Streptococcus pyogenes* (Lancefield group A Streptococci (GAS) is a common human pathogen which causes a variety of diseases with a great difference in severity. Person-to-person spread of *S. pyogenes* occurs from an infected

person by respiratory droplets or by direct contact with secretions of the nose or throat.

Management of pharyngitis is commonly based on features which are thought to be associated with Lancefield Group A Beta-Haemolytic Streptococci (GABHS) but it is debatable which features best predict GABHS. Non-group A strains share major virulence factors with group A, but it is unclear how commonly they present and whether their presentation differs (Little, *et al.*, 2012).

A study performed by Giordano, *et al.*, (2001) for assessing genetic diversity of Streptococci. It resulted in that, 114 Group A Streptococcus (GAS) isolates were recovered from pediatric pharyngitis patients in Rome, Italy. These isolates comprised 22 different M protein gene (*emm*) sequence types, 14 of which were associated with a distinct Serum Opacity Factor (*SOF*) /fibronectin binding protein gene sequence type.

The throat and skin of the human host are the principal reservoirs for the bacterial pathogen *Streptococcus pyogenes*. The peak incidence for Streptococcal pharyngitis and impetigo varies with season and locale, leading to wide spatial and temporal distances between throat and skin strains. *Streptococcus pyogenes* strains are among the most prevalent of pathogenic bacteria which afflict humans. They have a worldwide

distribution, and humans are the only known biological host. *S. pyogenes* can cause a life-threatening illness, such as toxic shock syndrome, or trigger autoimmune disorders, such as rheumatic fever. However, most often *S. pyogenes* causes only mild infection at either the throat or skin, leading to pharyngitis or impetigo respectively (Awdhesh, *et al.*, 2002).

Studies on the carriage rate of beta-hemolytic Streptococci among children form an important component of public health practice to prevent disease complications. Throat swabs collected from asymptomatic school children were inoculated into appropriate media for isolation of beta-hemolytic Streptococci. They were identified by standard biochemical methods and sero-grouped. Antibiotic sensitivity was evaluated using the Kirby-Bauer disk diffusion method. The study resulted in that Beta-hemolytic Streptococci were isolated in a percentage of 7.7% throat swabs and Group F was the predominant sero-group isolated. Moreover the highest resistance observed among all the beta-hemolytic Streptococci was to trimethoprim-sulfamethoxazole (Devi, *et al.*, 2011).

Adult group C beta-hemolytic Streptococcal pharyngitis has a prevalence of approximately 5% and can present with a broad spectrum of severity. Negative rapid test was observed in a woman with severe

pharyngitis. This presentation illustrates the importance of a systematic approach for evaluating patients with negative rapid strep tests and worsening pharyngitis. Current guidelines assume that group A beta-hemolytic Streptococcus is the only important treatable cause of episodic pharyngitis. These guidelines dissuade physicians from using antibiotics, unless a firm diagnosis of group A beta-hemolytic Streptococcus can be made. The study presents a woman who presented with worsening pharyngitis and negative rapid antigen tests for group A pharyngitis. This presentation raises important diagnostic considerations for the General Internist (Mobin, *et al.*, 2007).

The role of large colony Streptococci groups C or G as pathogenic agents in sore throat have been questioned. A study by Lindbaek, *et al.*, (2005), aimed to analyze clinical features of patients with large colony Streptococci groups C or G compared with patients with group A Streptococci (GAS) and with negative cultures. The results showed that out of 306 patients with a sore throat, 244 were adults and 62 were children under 10 years old; 40% were men. One hundred and twenty-seven had GAS, 33 had Streptococci groups C or G, and 146 had negative throat cultures. It was concluded that patients with tonsillitis caused by Streptococcus groups C or G have, to a large extent, the same clinical

picture as patients with GAS. Large colony Streptococci groups C and G should be considered as throat pathogens in line with GAS.

A study was conducted by Al-charrakh, *et al.,* (2011). She concluded that groups C and F Streptococci were implicated as a cause of acute pharyngitis in 6.2% of the specimens among other groups of Streptococci. Most of these isolates have the ability to produce more than one virulence factor. There was a high rate of resistance among isolates for β-lactam antibiotics; however, they were highly susceptible to vancomycin, Ofloxacin, and clindamycin.

In a study of the prevalence *Streptococcus pyogenes* group A (GAS) strains colonizing the upper respiratory tracts of children, Riza, *et al.,* (2003), showed that the organism plays an important role in the spread of this bacterial infection, especially among children at school, day-care centers, orphanages, and home. The molecular epidemiology of the isolates may provide useful information about the origin and spread of this infectious agent, allowing for more effective control measures. The prevalence of nasopharyngeal *Streptococcus pyogenes* was 14.3%. Isolates were found to be susceptible to all antibiotics tested.

Detailed epidemiologic studies of impetigo, particularly among indigenous communities in the Pacific among whom rheumatic fever is endemic, indicated that the disease was remarkably prevalent. In contrast, group A Streptococcal pharyngitis occurs no more frequently than in regions wherein rheumatic fever is rare. Studies of molecular epidemiology reveal that overall there is a greater diversity of group A Streptococcal strains in tropical regions, and skin-associated strains appear predominant. These skin strains may move between skin and throat, and there is increasing evidence of skin-associated strains being linked to cases of rheumatic fever (Parks, *et al.*, 2012).

Bacterial sore throats respond well to antibiotics, whereas viral ones do not. However, Streptococcal throat infection remains a leading cause for physician visits, and researchers have long struggled to determine how best to treat it. The best clinical management of patients with sore throat depends on both the clinical probability of group A Streptococcal infection and clinical judgments that incorporate the importance ratings of the individual patients as well as practice circumstances (Dalalah and Magableh, 2008).

Diagnosis and correct treatment of group A Streptococcal sore throat is important particularly to prevent non-suppurative sequelae. Clinical

findings continue to be used to differentiate Streptococcal infection from viral sore throat. The American Academy of Pediatrics recommends that Streptococcal sore throat diagnosis should always be performed by microbiological identification methods. A study was aimed to evaluate the accuracy of clinical diagnosis in comparison with culture and rapid test. It concluded that the microbiologic method is necessary for the correct prescription of antibiotics in children with Streptococcal sore throat (Santos and Berezin, 2005).

A study was designed by Steer, *et al.*, (2009) to estimate the prevalence of carriage of Beta-Hemolytic Streptococci (BHS) and the incidence of BHS culture-positive sore throat in school aged children in Fiji. The study concluded that GAS culture-positive sore throat was more common than expected. Group C and group G Streptococci were frequently isolated in throat cultures, although their contribution to pharyngeal infection was not clear. The molecular epidemiology of pharyngeal GAS in the study differed greatly from that in industrialized nations and this has implications for GAS vaccine clinical research in Fiji and other tropical developing countries.

Streptococcus pyogenes or group A Streptococcus (GAS) is a common cause of vulvo-vaginitis in pre-pubertal females but is

uncommonly isolated from the vaginal swabs of adult females. A study aimed to describe the clinical and laboratory findings of adult females with GAS isolated from vaginal swabs in a community and hospital laboratory. It concluded that isolation of GAS from the vaginal swabs of adult females is uncommon. In the community setting it may represent infection with vulvo-vaginitis or asymptomatic colonization. In the hospital setting, its isolation is frequently associated with pregnancy-related infectious complications (Upton and Taylor, 2013).

Streptococcus pyogenes (group A) is a major pathogen capable of causing wide range of diseases in different age group of people. In a study 100 patients were presented with the complaint of sore throat. All the patients were divided in four age groups. *Streptococcus pyogenes* colonies were confirmed on the basis of beta-haemolysis, bacitracin sensitivity test, and latex agglutination test for group A. Forty two out of a total of 100 samples, were confirmed as group A Streptococcus. From the study, it has been observed that all age groups, with a maximum occurrence in 5-15 years of age, were suffering from group A Streptococcal pharyngitis. Therefore every case of sore throat especially affecting children should be investigated to detect the causative agent for initiation of proper therapy (Ray, *et al.*, 2010).

The European Society for Clinical Microbiology and Infectious Diseases established the Sore Throat Guideline Group to write an updated guideline to diagnose and treat patients with acute sore throat. In diagnosis, Center clinical scoring system or rapid antigen test can be helpful in targeting antibiotic use. The Center scoring system can help to identify those patients who have higher likelihood of group A Streptococcal infection. In patients with high likelihood of Streptococcal infections, physicians can consider the use of Rapid Antigen Test (RAT). If RAT is performed, throat culture is not necessary after a negative RAT for the diagnosis of group A Streptococci (Pelucchi, *et al.,* 2012).

A study by Alexandre, *et al.*, (2014), at Ponta Grossa, a midsize city of southern Brazil; aimed to determinate the prevalence of oropharyngeal colonization by GABHS in pediatric population and to estimate the effectiveness of Anti Streptolysin-O (ASO), compared to culture, in presence of infection; and design an unpublished investigative algorithm of rheumatic fever's suspicion, based on needs identified in worldwide consensus. It was an epidemiologic, observational and transversal study, involving children younger than 12 years. Secretion of posterior oropharynx was collected for culture; and peripheral blood for determination of ASO. It resulted in that; the prevalence encountered was

3.9%, and 25.5% of the children showed reagent ASO. This serological test demonstrated quantitatively and qualitatively significant associations to the GABHS presence. The results emphasize the need for similar studies in other populations, to provide better targeting of the diagnosis and treatment of oropharyngitis by GABHS.

2.4 Vaginal Bacteria

Group B Streptococcus (GBS) is one of the most important bacteria in the majority of maternal and neonatal infections, such as chorioamnionitis, endometritis, bacteremia, sepsis and meningitis. During pregnancy, GBS screening is one of the recommended strategies that are recommended by the Center of Disease Control (CDC). A study by Hamedi, *et al.*, (2012) in Iran, aimed to determine the rectovaginal colonization prevalence among pregnant women, and also the rate of transmission to their offspring. Between June 2008 and April 2009, two hundred pregnant women admitted in the department of Obstetrics and Gynecology (Ghaem Hospital, Mashhad) were enrolled in the study. Samples from maternal rectum and vagina as well as neonate ear and umbilical cord were taken for culture. It resulted in that; the colonization rate for GBS in pregnant women and their neonates was around 6% and 5%, respectively. All the carrier mothers were cases with premature rupture

of membranes (at least 18 hours before delivery). In terms of colonization, there was a significant correlation between mothers and newborns, and more than 80% of neonates from GBS carrier mothers were colonized by GBS.

Streptococcus agalactiae is a major pathogen of neonates and immunocompromised adults. Prior studies have demonstrated that, beyond the neonatal period, *S. agalactiae* rarely causes invasive infections in children. However, during 2004-2005, *S. agalactiae* was the causative agent of 60 meningitis episodes in children aged 3 months to 12 years from Angola (Florindo, *et al.*, 2011).

A retrospective study carried by Wloch, *et al.*, (2013) to analyze the frequency of group B Streptococci carriage in pregnant women from the region of Krakow Poland, was carried out between 2008- 2012. The study included the pregnant women between 35 and 37 weeks of gestation, studied in accordance with the guidelines of the Polish Gynecological Society (2008). A high percentage of pregnant women who are carriers of group B Streptococci were demonstrated. During the study period, it was in the range of 25-30%, with an average value equal to 28%. The results confirm the need for taking swabs from both the vagina and anus, since 15% of GBS-positive patients showed only rectal carriage

In a prospective observational study in Egypt to detect the magnitude of group B Streptococcal (GBS) colonization and disease among a sample of pregnant women, 95 pregnant females, 35-37 weeks of gestational age, attending the antenatal outpatient clinic at AlFayom University Hospital between September 2006 and June (2007), all participants were screened with vaginorectal swabs by a conventional GBS PCR assay. Participants were grouped into group A (GBS present, 17 patients) and group B (GBS absent, 78 patients). Ninety-five infant data were also recorded. All neonates of group A (17 out of 95 with known positive maternal GBS) underwent collection of simultaneous specimens from surface sites for PCR before their first bath and within four hours of birth. The study resulted in that GBS carriage rate in the study sample was 17.89%. It was concluded that maternal GBS carriage is associated with a significant increase in neonatal infection rate but is not associated with an increase in neonatal intensive care admission (Elbaradie, *et al.,* 2009).

Group B Streptococcus colonization in pregnant women usually has no symptoms, but it is one of the major factors of newborn infection in developed countries. In Iran, there was a little information about the prevalence of maternal colonization and newborns infected with group B Streptococcus. A study was conducted in Iran to find out its prevalence

among Iranian pregnant women and its vertical transmission to their newborns. It resulted in that 50% symptomatic neonates were born from the mothers with positive vaginal culture for group B Streptococcus. About 63% had absolute neutrophil count more than normal, and 9.1 % newborns were omitted from the study. Therefore, 50% of neonates showed clinical feature, whereas Para-clinical test was required to detect the infection for the rest of neonates who showed no signs or symptoms (Shirazi, *et al.*, 2014).

Genital colonization by Group B Streptococcus (GBS) in pregnant women in their third trimester, which is a known risk factor of morbidity and mortality among newborns, was studied. Dechen, *et al.,* (2010), studied the prevalence and the correlates of vaginal colonization by GBS among pregnant women. Three high vaginal swabs were obtained from all the pregnant women admitted at term and in preterm labor. The first set of swabs was cultured on 5% Sheep blood agar plates. The second set of swabs were inoculated into Todd-Hewitt broth and then subcultured in 5% sheep blood agar plates and the third one for Gram staining. It resulted in that, the main outcome measures were the presence of GBS infection in comparison to the age group, gravida, and gestational age, Premature Rupture Of Membrane (PROM), preterm labor and association with febrile

spells of the present pregnancy. It resulted in that the culture positivity rate of GBS was 4.77% and coexistent organisms isolated were Candida species (36%), *Staphylococcus aureus* (8%) and Enterococcus species (8%). It concluded that GBS infection among pregnant women was significantly correlated with the gestational age, PROM and preterm labor.

Streptococcus agalactiae (GBS) vaginal pathogenicity is not uniformly acknowledged throughout the literature. Accordingly, genital itching and burning, along with leukorrhea in women, were commonly and almost exclusively referred to bacterial vaginosis, candidiasis and trichomoniasis. Conversely, GBS virulence for vagina was recognized in the past, as the organism has been observed to potentially cause local inflammation and discharge. A study depiced a case where a non-hemolytic (γ-hemolytic) GBS strain was found to be the etiological agent of vaginal infection, such uncommon *S. agalactiae* phenotypes are hard to be recognized and may be therefore responsible for misdiagnosing and underestimation of GBS vaginitis prevalence (Vincenzo, *et al.,* 2013).

Group B Streptococci (GBS) are the most frequent cause of severe, life-threatening neonatal infections. Significant infections are also seen in adult women as a complication of pregnancy and in the elderly and immunocompromised hosts. Jannati *et al.*, (2012) found that, commonly,

10–40 % of pregnant mothers carry GBS in their genitourinary and gastrointestinal tracts and maternal colonization with GBS is the predominant risk factor for the development of invasive neonatal GBS disease. They concluded that, GBS carriage screening at weeks 35-37 of gestation and subsequent Intrapartum Antibiotic Prophylaxis (IAP) for culture-positive women is recommended in many countries.

Streptococcus agalactiae (Group B Streptococcus, GBS) are the most common cause of serious bacterial infections in newborn and adults with underlying medical disorders. At the time of birth, GBS is vertically transmitted to the neonate, and it is estimated that 50% or more of newborns exposed to GBS will become colonized if the mother is a carrier (Georgia, *et al.,* 2013).

During a national surveillance program on Group B Streptococci (GBS) maternal carriage and neonatal infections, a GBS strain isolated from a pregnant woman's vagino-rectal swab was non typable by either serological or molecular methods. Further molecular characterization demonstrated that the strain lacked the entire capsular locus, possibly by a recombination event that excised. The natural loss of the capsular locus by GBS isolated from a human has never been described so far. Such an event, while possibly a dead-end from the evolutionary point of view, leaves a

still able-to-colonize organism unrecognizable by the vaccines currently under development (Creti, *et al.*, 2012).

Streptococcus agalactiae regarded mainly as a pathogen of neonates and pregnant women, is increasingly affecting non pregnant adults. In adults, *S. agalactiae* causes skin and soft tissue infections, bacteremia, urinary tract infections, pneumonia, osteomyelitis, meningitis, endocarditis, and Streptococcal toxic shock syndrome. Risk factors associated with invasive GBS in adults are old age, diabetes mellitus, neurologic diseases, cirrhosis or other liver diseases, stroke, breast cancer, and renal failure. *S. agalactiae* colonizes the lower gastrointestinal and genitourinary tracts of 30 to 50% of healthy adults, and an estimated 20 to 30% of all pregnant women are carriers. *S. agalactiae* can be isolated from vaginal or rectal swabs, and prenatal screening for colonization of pregnant women is recommended (Charlotte, *et al.*, 2011).

During the last few decades, group B Streptococcus (GBS) has emerged as an important pathogen. The major reservoirs for GBS are the vagina and the peri-anal regions/rectum, and the colonization of these regions is a risk factor for subsequent infection in pregnant women and newborns. A study was performed in India to determine the prevalence of GBS colonization in the vagina and rectum of pregnant women and the

antibiotic susceptibility pattern of the isolates. It aimed also to identify risk factors associated with GBS colonization. It resulted in that GBS colonization rate was 2.3%. It concluded that the GBS colonization rate was low. No resistance to penicillin or clindamycin was seen, while the majority of the isolates were resistant to tetracycline (Vijayan, *et al.,* 2011).

Group B Streptococci are leading cause of infections in newborns, pregnant women, and older persons with chronic medical illness. Kimberly, *et al.,* (2013), found that In addition to maternal cervicovaginal colonization and neonatal infection that results from the vertical transmission of bacteria from mothers to their infants, GBS can also cause urinary tract infection (UTI). The spectrum of GBS UTI includes asymptomatic bacteriuria (ABU), cystitis, pyelonephritis, urethritis, and urosepsis. GBS ABU is particularly common among pregnant women, although those most at risk for cystitis due to GBS appear to be the elderly and immunocompromised individuals.

A study by Horvath, *et al.,* (2013), assessed the benefits of a chemoprophylaxis program based on screening women for GBS infection between 30 and 32 weeks of pregnancy in a population with a high rate of premature births. It resulted in that, there were 0.2% infected newborns in the study cohort, and 1 of 8 with sepsis died. Also 0.7% infected newborns

in the historical cohort, and 29 of 31 with sepsis died. It was concluded that screening women early in a population with a high rate of premature births may simplify preterm labor management.

Group B Streptococcus is the leading cause of neonatal infections. Oviedo, *et al.*, (2013), characterized GBS colonization in pregnant women, current serotypes, resistance phenotypes and genes associated with virulence. Vaginal-rectal swabs from pregnant women were studied. GBS strains were identified by conventional and serological methods. Serotypes were detected using Strep-B Latex. Resistance phenotypes were determined by the double-disk test. Genes were studied by PCR. Maternal colonization was 9.38%.

The capsular polysaccharide (CPS) is an important virulence factor and a vaccine target of the major neonatal pathogenic GBS. Population studies revealed no strong correlation between CPS type and multilocus sequence typing (MLST) cluster, with the remarkable exception of the worldwide spread of hyper virulent GBS CC17, which were all until recently CPS type III. The study showed that GBS CC17 hyper virulent strains have switched one of their main vaccine targets. Thus, continued

surveillance of GBS population remains of the utmost importance during clinical trials of conjugate GBS vaccines (Bellais, *et al.*, 2012).

A group B Streptococcus vaccine for pregnant women would add to the currently available vaccines given during pregnancy to protect mothers and their infants against serious and potentially lethal diseases, including tetanus, influenza, pertussis and meningococcal infection. Implementation of the administration of these high priority vaccines during routine prenatal care would result in a maternal immunization program with the potential to have a positive impact in public health globally, by reducing maternal and neonatal morbidity and mortality (Munoz and Ferrieri, 2013).

2.5 Urinary Tract Infection

Urinary tract infections (UTI) can lead to poor maternal and prenatal outcomes. Investigating epidemiology of UTI and antibiotics sensitivity among pregnant women is fundamental for care-givers and health planners. A cross sectional study was conducted at Khartoum North Teaching Hospital Antenatal Care Clinic between February-June 2010 by Hamdan, *et al.*, (2011) to investigate epidemiology of UTI and antibiotics resistance among pregnant women. UTI was diagnosed using mid stream urine culture on standard culture media. The results showed that 28.0% were

symptomatic and 71.9% asymptomatic. The prevalence of bacteriuria among symptomatic and asymptomatic pregnant women was 12.1%, and 14.7% respectively, and the overall prevalence of UTI was 14.0%. In multivariate analyses, age, gestational age, parity, and history of UTI in index pregnancy were not associated with bacteriuria.

UTI is a common bacterial infection known to affect the different parts of the urinary tract and the occurrence is found in both males and females. Despite the fact, that both the genders are susceptible to the infection, women are mostly vulnerable due to their anatomy and reproductive physiology. The infection is usually caused as a consequence of bacterial invasion of the urinary tract including the lower and the upper urinary tract. Among the bacterial species *Escherichia coli* account to 80% to 85% of the infection followed by Staphylococcus species that constitutes 10% to 15%. In addition, bacterial species such as Klebsiella, Pseudomonas, Proteus and Enterococcus species play a minor role in conferring the infection. A variety of parameters are related to UTI including age, parity, gravidity, pregnancy and association of diseases augment the condition of the infection (Ranganathan, 2014).

A study was undertaken to prospectively evaluate a *Streptococcus pneumoniae* urinary antigen test for diagnosis of pneumococcal pneumonia

among patient and control groups between 2004 and 2006. Non-concentrated urine samples were tested using an immunochromatographic assay, the NOW *S. pneumoniae* antigen test. The urinary antigen test was positive in 9 (15.3%) of 59 patients enrolled in the study and in 8 (73%) of 11 patients with pneumococcal pneumonia confirmed by conventional methods. The study concluded that the urinary antigen test can supplement conventional microbiological tests in the diagnosis of pneumococcal pneumonia (Ercis, *et al.,* 2006).

Recurrent urinary tract infections (RUTI) are common in pregnant women and may cause serious adverse pregnancy outcomes for both mother and child including preterm birth and small-for-gestational-age babies. Interventions used to prevent RUTI in pregnant women can be pharmacological (antibiotics) or non-pharmacological. So far little was known about the best way to prevent RUTI in pregnant women. The study of Schneeberger, *et al.,* (2012) assessed the effects of interventions for preventing recurrent urinary tract infections in pregnant women showed that primary maternal outcomes were RUTI before birth and preterm birth (before 37 weeks). The primary infant outcomes were small-for-gestational age and total mortality. It resulted in that, no significant differences were

found for the primary outcomes: recurrent urinary tract infection before birth and preterm birth.

Bacteriuria is defined as the presence of bacteria in urine, regardless of the number of colony-forming units per mL (CFU/mL). A study carried by Allen, *et al.*, (2012) to provide information regarding the management of GBS bacteriuria to midwives, nurses, and physicians who are providing obstetrical care. Results were restricted to systematic reviews, randomized controlled trials, and relevant observational studies. Its recommendations were quantified using the evaluation of evidence guidelines developed by the Canadian Task Force on Preventive Health Care. The recommendations in this guideline were designed to help clinicians identify pregnancies in which it is appropriate to treat GBS bacteriuria to optimize maternal and prenatal outcomes, to reduce the occurrences of antibiotic anaphylaxis, and to prevent increases in antibiotic resistance to GBS and non-GBS pathogens.

Urinary tract infection in pregnancy is associated with significant morbidity for both the mother and the baby. A study aimed to determine the bacterial profile and antibiotic resistance pattern of the urinary pathogens isolated from pregnant women at Felege Hiwot Referral

Hospital Bahirdar, Ethiopia showed that a total of 367 pregnant women with and without symptoms of urinary tract infection were enrolled as a study subject. Organisms were identified from mid-stream clean catch urine samples and antibiotic susceptibility was performed using bacteriological standard tests. Bacteriological screening of urine samples revealed growth of bacteria in 8.5% and 18.9% for symptomatic and asymptomatic pregnant women respectively, with overall prevalence of 9.5%. The most common isolates detected were *E. coli* (45.7%) followed by coagulase negative Staphylococcus (17.1%) and *S. aureus* (8.6%). Gram-negative bacteria showed resistance rates in the range of 56.5% – 82.6 % against trimethoprim/sulfamethoxazole, tetracycline, amoxicillin and ampicillin. Gram positive isolates showed resistant rate ranging from 50–100% against tetracycline, trimethoprim-sulphamethoxazole, amoxicillin and penicillin-G. It was concluded that, the isolation of bacterial pathogens both from symptomatic and asymptomatic pregnant women that are resistance to the commonly prescribed drug calls for an early screening of all pregnant women to urinary tract infection (Tazebew, *et al.,* 2012).

Streptococcus agalactiae is one of the uropathogens responsible for urinary tract infections (UTI) in children, pregnant women, and elderly

people with chronic underlying diseases. A study was performed by Shayanfar, *et al.*, (2012) to determine the prevalence of urinary tract isolates of group B Streptococci (GBS) in a group of females referred to a referral University Hospital in Iran. Urine analysis and urine culture results were reviewed. Bacteriuria, colony count, pyuria and demographic data of patients were also evaluated, (20.1%) of them had positive urine cultures. GBS was the isolated microorganism in 8.92% cases, yielding a prevalence of 1.79% in total study population.

Urinary tract infections (UTI) are one of the most common infections among women worldwide. *E. coli* often causes more than 75% of acute uncomplicated UTI, however, little is known about how recurrent UTIs and indiscriminate use of antimicrobials affect the aetiology of UTIs. A study was aimed to establish the aetiology of UTI in a population of recurrent and self-medicated patients referred from pharmacies to a hospital in Hanoi, Vietnam and to describe genotypes and antimicrobial susceptibility of the associated bacterial pathogens. The aetiology of bacterial pathogens associated with UTI was established by phenotypic and molecular methods. *Enterococcus faecalis* isolates were typed by Multi Locus Sequence Typing (MLST), Pulsed-Field Gel Electrophoresis (PFGE) and antimicrobial susceptibility testing (Louise, *et al.,* 2012).

2.6 Blood Culture

Blood culture has been the gold standard for the detection of sepsis pathogens. Continuously monitored blood culture systems have reduced the detection time of positive blood cultures, but there are few rapid and accurate methods for identifying pathogens directly from these samples. Nucleic-acid-based methods (DNA probe method, fluorescent in situ hybridization, PCR) may offer one solution, the accuracy and feasibility of one of them was studied. The Genotype blood culture assays (Hain Life science, Nehren, Germany). The two Genotype BC panels identify 41 bacterial species. Additionally, the methicillin resistance-gene and the vancomycin resistance-genes can be detected. The genotype procedure includes three steps: DNA isolation, multiplex amplification with biotinylated primers, and strip-based reverse hybridization and detection (Santra, *et al.,* 2012).

Rapid and reliable identification of bacteria directly from blood culture is an important criterion in clinical practice to guide appropriate antibiotic therapy. A study of the performance of the AccuProbe (Gen-Probe, Inc., San Diego, Calif.) in direct identification of *Staphylococcus aureus*, *Streptococcus pneumoniae*, Enterococci, and group A and B

Streptococci from positive blood culture bottles was evaluated by using 6-year routine clinical laboratory blood culture material from Paijat-Hame Central Hospital, Lahti, Finland. With the Enterococcal and group A and B Streptococcal probes, the diagnostic performance of the test was excellent at a cut off value of 50,000 relative light units (RLU) as recommended by the manufacturer (Lindholm and Sarkkinen, 2004).

Rapid identification of pathogens directly from positive blood cultures can play a major role in reducing patient mortality rates. A study evaluated the performance of the Verigene Gram-Positive Blood Culture (BC-GP) for detection of commonly isolated Gram-positive organisms as well as associated resistance markers from positive blood cultures. Positive blood cultures were analyzed using the BC-GP assay within 12 h for the detection of 12 different organisms, including Staphylococci, Streptococci, and Enterococci, as well as for the presence of 3 resistance markers (*mecA*, *vanA*, and *vanB*). Results were compared to those of routine laboratory methods for identification and susceptibility testing. For identification of organisms and detection of resistance markers in 178 mono microbial positive blood cultures, the BC-GP assay showed 94% and 97% concordance, respectively, with routine methods. After 25 poly microbial cultures were included, the results showed 92% and 96% agreement for

identification and resistance markers, respectively, for a total of 203 positive cultures. The BC-GP assay is capable of providing rapid identification of Gram-positive cocci as well as detection of resistance markers directly from positive blood cultures at least 24 to 48 h earlier than conventional methods (Linoj, *et al.*, 2013).

Bloodstream infection remains a major cause of morbidity and prolonged hospital stay in children. Samuel, *et al.*, (2013) found that, in neonates and infants, it is a life-threatening emergency, and therefore identifying the common bacteria and their susceptibility patterns will provide information necessary for timely intervention.

Streptococcus urinalis was isolated from a blood culture of a 60-year-old man with a history of urethral stricture. This species has been recently described as a new member of the pyogenic subgroup of Streptococci that cause urinary tract infections (Peltroche, *et al.*, 2012).

The viridans groups Streptococci (VGS) are a heterogeneous group of organisms that can be human commensals, colonizing the gastrointestinal and genitourinary tracts in addition to the oral mucosa. VGS are generally considered to be of low pathogenic potential in immunocompetent individuals. However, in certain patient populations,

VGS can cause invasive disease, such as endocarditis, intra-abdominal infection, and shock. Within the VGS, the rates and patterns of antimicrobial resistance vary greatly depending upon the species identification and the patient population. In general, *Streptococcus mitis* group organisms are resistant to more antimicrobial agents than the other VGS species. A review of recent pediatric-specific data regarding the clinical manifestations of VGS revealed that the *Streptococcus anginosus* group (SAG) organisms may be important pathogens in pediatric patients and that the VGS may contribute to disease in patients with cystic fibrosis. It also appears that rates of antimicrobial resistance in VGS in pediatric patients are surpassing those of the adult population (Christopher and Carey, 2010).

An unidentified strain of the viridans group of Streptococci was isolated from a human blood sample. It was distinguished from all other recognized species of the *Streptococcus sanguinis* group by several biochemical characteristics. Phylogenetic analysis based on 16S rRNA gene sequence comparisons clustered this strain with *Streptococcus ferus* (mutans group) but phylogenetic analysis based on rpoB and sodA gene sequence comparisons included it in the *S. sanguinis* group. The isolates showed 95.4 and 95.2 % 16S rRNA gene sequence similarity to *S. ferus*

and *S. sanguinis*, respectively, confirming it as belonging to a novel taxon, for which the name *Streptococcus massiliensis* sp. nov. was proposed (Glazunova, *et al.*, 2006).

A study suggested that underlying valvular heart disease and persistent bacteremia are independently associated with Viridans Streptococcal Infective Endocarditis (VSIE). The study recommends routine echocardiography in patients who have these conditions. Previous steroid use and immunosuppressive therapy have both been shown to be associated with poor prognosis in patients with Viridans Streptococcal Bacteremia (VSB). Many studies have shown that viridans Streptococci cause various and severe infections and play an important role in the etiology of primary bacteremia following immunosuppressive therapy. In light of these findings, there was growing interest among clinicians about viridans Streptococci and their role in infection. The study reported that previous steroid use and immunosuppressive therapy are independently associated with mortality due to VSB (Soo-Kyeong, *et al.*, 2013).

2.7 Resistant to Antibiotics

Resistance to antibiotics is a major public-health problem, and studies that link antibiotic use and resistance have shown an association but not a causal effect. Macrolide azithromycin and clarithromycin were used

to investigate the direct effect of antibiotic exposure on resistance in the oral Streptococcal flora of healthy volunteers. The study showed that, notwithstanding the different outcomes of resistance selection, Macrolide use is the single most important driver of the emergence of Macrolide resistance in vivo. Physicians prescribing antibiotics should take into account the striking ecological side-effects of such antibiotics (Malhotra-Kumar, *et al.,* 2007).

2.8 Molecular detection of Streptococci

Developments in molecular based microbial detection methods, such as polymerase chain reaction (PCR), have significantly contributed to rapid identification and quantification of unknown biological agents. Public health and clinical laboratories are often tasked with developing PCR-based assays for detecting unknown pathogens in complex matrices **(**Nathan and Jayne, 2012).

Extraction of DNA, RNA, and protein is the basic method used in molecular biology. Currently, there are many specialized methods that can be used to extract pure biomolecules, such as solution-based and column-based protocols. Manual method has certainly come a long way over time with various commercial offerings which included complete kits containing most of the components needed to isolate nucleic acid. Automated systems

designed for medium-to-large laboratories have grown in demand over recent years. It is an alternative to labor-intensive manual methods. The technology should allow a high throughput of samples; the yield, purity, reproducibility, and scalability of the biomolecules as well as the speed, accuracy, and reliability of the assay should be maximal, while minimizing the risk of cross-contamination (Siun and Beow, 2009).

2.9 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) using universal bacterial primers and subsequent DNA sequence analysis of the PCR product was used to detect beta-haemolytic Streptococci in clinical samples. *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Streptococcus dysglactiae* were identified in 19 primary culture-negative cases of infections from 203 patient samples with detectable bacterial DNA (Grarup, *et al*., 2008).

A study described the construction and use of a multiple-locus variant-repeat assay (MLVA) on 126 well-characterized human GBS strains, consisting mostly of invasive Norwegian strains and international reference strains, based on in silico whole-genomic analysis of the genomes of strains A909, NEM316, and 2603V/R, 18 candidate loci were selected and investigated by PCR (Radtke, *et al.*, 2010).

Accurate identification of isolates belonging to genus Enterococcus, Streptococcus, coagulase-negative Staphylococcus, and Lactococcus at the species level is necessary to provide a better understanding of their pathogenic potential, to aid in making clinical decisions, and to conduct epidemiologic investigations, especially when large blind samples must be analyzed. It is useful to simultaneously identify species in different genera using a single primer pair (Xuerui, *et al.*, 2012).

A 761-bp portion of the *tuf* gene from 28 clinically relevant Streptococcal species was obtained by sequencing amplicons generated using broad-range PCR primers. The specificity of the PCR assay was verified using 102 different bacterial species, including the 28 Streptococcal species. Genomic DNA purified from all Streptococcal species was efficiently detected. The Streptococcus-specific assay was highly sensitive for all 28 Streptococcal species tested. The *tuf* sequence data was also used to perform extensive phylogenetic analysis, which was generally in agreement with phylogeny determined on the basis of 16S rRNA gene data. However, the *tuf* gene provided a better discrimination at the Streptococcal species level that particularly useful for the identification of very closely related species (Francois *et al.*, 2004).

A comparative study evaluated three different PCR-based capsular gene typing methods applied to 312 human and bovine *Streptococcus agalactiae* (group B Streptococcus [GBS]) isolates and compared the results to serotyping results obtained by latex agglutination. Among 281 human isolates, 27% could not be typed by latex agglutination. All 312 isolates except 5 could be typed by the three PCR methods combined. Two of these methods were multiplex assays. Among the isolates that were typeable by both latex agglutination and capsular gene typing, 94% showed agreement between the two methods. However, each of the PCR methods showed limitations. One of the methods did not include all 10 recognized serotypes, one misidentified eight isolates of serotypes Ib and IV as serotype Ia, and one did not distinguish between serotypes VII and IX. For five isolates that showed aberrant patterns in the capsular gene typing, long-range PCR targeting the cps operon disclosed large insertions or deletions affecting the cps gene cluster. In the same study a sensitive flow cytometric assay based on serotype-specific antibodies was attempted applied to 76 selected isolates that were nontypeable by latex agglutination revealed that approximately one-half of these did express capsular polysaccharide (Yao, *et al.*, 2013).

A study aimed to evaluate diagnostic tests in order to introduce a diagnostic strategy to identify the most common Gram-positive bacteria (Pneumococci, Enterococci, β-haemolytic Streptococci and *S. aureus*) were found in blood cultures within 6 hours after signalling growth. It resulted in that, during the validation period, 150 (91%) of a total of 166 Gram-positive cocci (119 in clusters, 45 in chains or pairs and 2 undefined morphology) were correctly identified as *S. aureus*, CoNS, Pneumococci, Enterococci or group A Streptococci (GAS), group B Streptococci (GBS), group G Streptococci (GGS) within 6 hours with a minimal increase in work-load and costs. The remaining samples (9%) were correctly identified during the next day. No samples were incorrectly grouped with this diagnostic strategy and no patient came to risk by early reporting. It was concluded that a simple strategy gives reliable and cost-effective reporting of more than 90% of the most common Gram-positive cocci within 6 hours after a blood cultures become positive. The high specificity of the tests used makes preliminary reports reliable. The reports can be used to indicate the focus of infection and not the least, support faster administration of proper antimicrobial treatment for patients with serious bacterial infections (Larsson, *et al.*, 2014).

Although various diagnostic approaches for pathogen detection have been proposed, most were too expensive, lengthy or limited in specificity for clinical use. Hyun, *et al.,* (2013), studied a new assay diagnostic platform for the rapid detection and phenotyping of common clinical pathogens. The assay makes use of magnetic nanoparticles (MNPs) and oligonucleotide probes to specifically detect target nucleic acids from the pathogen. It concluded that; nucleic acid-based techniques allow the specific typing of species with high enough sensitivity to circumvent lengthy culturing processes. The 16S rRNA sequence information has been used for bacterial classification and taxonomy, so typing methods based on these databases have emerged as the preferred technique for microbial identification over traditional culture and biochemical assays.

Comet assay provides a rapid and reliable way to screen genotoxic effects of a wide variety of nanoparticles. Single cell gel electrophoresis assay (SCGE) or comet assay gained in popularity as a standard technique and is widely used for testing novel particles or chemicals for genotoxicity, monitoring environmental contamination with genotoxins, human biomonitoring and molecular epidemiology, and fundamental research in DNA damage and repair (Bowman, *et al.,* 2012).

2- 10 Sequencing

Recently, analysis of microbial epidemics has been revolutionized by whole-genome sequencing. Nahuel, *et al.*, (2013) recently sequenced the genomes of 601 type *emm59* Group A Streptococcus (GAS) organisms responsible for an ongoing epidemic of invasive infections in Canada and some of the United States. The epidemic has been caused by the emergence of a genetically distinct, hyper virulent clone that has genetically diversified. The ease of obtaining genomic data contrasts with the relatively difficult task of translating them into insightful epidemiological information. Authors sequenced the genomes of 90 additional invasive Canadian *emm59* GAS organisms. They used an improved bioinformatics pipeline designed to rapidly process and analyze whole-genome data and integrate strain metadata. They discovered that *emm59* GAS organisms are undergoing continued multiclonal evolutionary expansion.

The molecular mechanisms underlying pathogen emergence in humans is a critical but poorly understood area of microbiologic investigation. Serotype V group B Streptococcus (GBS) was first isolated from humans in 1975, and rates of invasive serotype V GBS disease significantly increased starting in the early 1990s. Flores, *et al.*, (2015)

found that 92% of the serotype V GBS strains isolates were multilocus sequence type (ST) 1. Elucidation of the complete genome of a 1992 ST-1 strain revealed that this strain had the highest homology with a GBS strain causing cow mastitis and that the 1992 ST-1 strain differed from serotype V strains isolated in the late 1970s by acquisition of cell surface proteins and antimicrobial resistance determinants. Whole-genome comparison of 202 invasive ST-1 strains detected significant recombination in only eight strains. The remaining 194 strains differed by an average of 97 SNPs. Phylogenetic analysis revealed a temporally dependent mode of genetic diversification consistent with the emergence in the 1990s of ST-1 GBS as major agents of human disease. Thirty-one loci were identified as being under positive selective pressure, and mutations at loci encoding polysaccharide capsule production proteins, regulators of pilus expression, and two-component gene regulatory systems were shown to affect the bacterial phenotype. These data revealed that phenotypic diversity among ST-1 GBS is mainly driven by small genetic changes rather than extensive recombination, thereby extending knowledge into how pathogens adapt to humans.

Enterococcus faecalis ATCC 29212, a vancomycin-sensitive strain, has been extensively used as a representative control strain for clinical and

laboratory experiments. A study reported the draft genome and annotation of this strain, containing 3,027,060 bp, with a G+C content of 37.2% in 126 consigns (≥500 bp). It was increasingly recognized as a nosocomial pathogen. It was isolated from human urine, and was used as a control strain to assess the quality of commercially prepared microbiological culture media, susceptibility testing of pathogens to antibiotics, commercialized biochemical identification of bacterial species and microbiological quality testing of food and animal feeding stuffs. The need for genome-level information is indicated by the fact that *E. faecalis* strains are now regarded as opportunistic pathogens and those hospital-associated strains typically share a similar genetic composition and resistance to clinically relevant antibiotics. The study sequenced and annotated the genome of ATCC 29212 to enable broader applications of this organism for comparison of genotypic differences among members of this species (Eun, *et al.*, 2012).

Chapter Three

Materials and Methods

3-1 Study Design

3-1 -1 Type of Study

The type of this study was a Cross-sectional hospital- based study carried from September 2013 to November 2016.

3-1-2 Study Area

1- Khartoum Ear Nose and Throat Teaching Hospital (Khartoum E. N. T. Teaching Hospital)
2- Saudi Maternal Hospital
3- Omdurman Teaching Hospital
4- Khartoum North Teaching Hospital

3-1- 3 Samples

Five hundred samples were collected in this study including 150 (30%) throat swabs, 125 (25 %) vaginal swabs, 125 (25 %) urine samples and 100 (20 %) blood samples.

The number of each sample was calculated according to the type

of infection which was obtained from the Ministry of Health records (Tonsillitis, UTI, Urine and blood during 2012) {Appendix 1}, according to the number of each infection recorded.

3-1- 4 Study Population

Patients included in this study were those who attended the study hospitals suspected with Streptococcal infection.

3-1-5 Ethical Consideration

The study took into account the regulations and the instructions of the University Ethical Committee. The administrations of the hospitals were informed about the type of the samples that were collected. Nature and the goal of the study were explained to all candidates. The confidentiality of the patients was established by coding of the questionnaires. All investigations were carried out for patients free of charge and they were not claimed for analysis expenses.

3-1-6 Inclusion Criteria

Untreated patients suspected with Streptococcal infection.

3-1-7 Exclusion Criteria

Patients who were under treatment with antibiotic or treated seven days before collection were excluded.

3-2 Methodology

3-2-1 Data Collection

All information required from patients was collected with the questionnaire {Appendix 2}.

3-3 Sterilization

3-3-1 Sterilization of Swabs and Urine Containers

Ready sterilized swabs and urine containers were purchased.

3-3-2 Sterilization of Petri Dishes

Sterilized plastic Petri dishes were purchased. Glass Petri dishes were sterilized in a hot air oven at 160 ºC for one hour.

3-3-3 Sterilization of Equipment

Test tubes, flasks, graduated pipettes and other glass wares used were sterilized in a hot air oven at 160 ºC for one hour.

Bottles and plastic tips were sterilized by autoclaving at 15 lb/sq inch for 15 minutes at 121 ºC.

3-3-4 Sterilization of Culture Media and Solutions

Media and solutions were sterilized by autoclaving at 15 lb/sq inch for15 minutes at 115 ºC.

3-4-1 Sample Collection

Throat swabs were collected from the specific patients who attended Khartoum E. N. T. Teaching Hospital suffering from sore throat.

Vaginal swabs were collected from pregnant women who attended the Saudi Maternal Hospital at the third trimester (35 – 37 weeks of gestation).

Urine and blood samples were collected from suspected patients who attend Omdurman and Khartoum North Teaching Hospitals. Some of the urine samples were collected also from same pregnant women in addition to the vaginal swabs.

Blood was collected under aseptic technique (Sodium Polyanethol Sulphonate (SPS) was used as anticoagulant) and the samples were transferred directly into Tryptone soya broth diphasic media (which was purchased ready for use).

3-4-2 Samples Preservation

After collection, all samples were kept at refrigerator temperature for laboratory investigation.

3-5 Reagents

All reagents were prepared as described by Barrow and Felltham

2003 {Appendix 3}.

3-5-1 Hydrogen Peroxide

This solution was prepared in a concentration of 3% and was used for the catalase test.

3-5-2 Bile Salts Solution

This solution was prepared in a concentration of 10% and was used for the bile solubility test.

3-5-3 Normal Saline Solution

This solution was prepared in a concentration of 0.85% and was distributed into test tubes of 5 ml each and the tubes were then sterilized by autoclaving at 15 lb/sq inch for 15 minutes at 121 ºC. Then 4 ml were used as a suspension solution to keep the bacterial culture for the extraction of the DNA and one ml was used for the bile solubility test.

3-6 Blood

Fresh defibrinated sheep blood was used in the preparation of blood agar medium. It was collected from sheep's jugular veins in sterile 10 ml syringes containing sodium citrate as an anticoagulant, it was mixed gently and it was stored in a refrigerator.

3-7 Types of Media

A: Solid Media

3-7-1 Blood Agar

The medium was prepared and sterilized by autoclaving at 15 lb/sq inch for 15 minutes at 121 ºC. Then it was cooled to 55 ºC and 50 ml of fresh sheep blood were added to 950 ml nutrient agar (5%), mixed and distributed into sterile Petri dishes 10 ml and 20 ml in each dish and stored in refrigerator after solidification. It was used for culturing throat swabs, vaginal swabs and urine samples. It was also used for identification of the type of haemolysis, Bacitracin and Optochin sensitivity and the CAMP reaction. (**Christie Alkins and Munch-Petersen = CAMP**).

3-7-2 Bile Salt Agar

Bile salt agar was prepared, sterilized and distributed into sterile Peri dishes and stored in a refrigerator after solidification, it was used for detection of the growth of the organisms isolated in 40% bile salt agar.

3-7-3 Muller Hinton Agar

Muller Hinton was prepared, sterilized and distributed into sterile Petri dishes and stored in refrigerator after solidification. It was used for sensitivity to antibiotics.

B: Liquid Media

3-8-1 Nutrient Broth

This medium was prepared; the pH was adjusted to 7.4. The medium was then distributed into test tubes (5 ml each) and sterilized by autoclaving at 15 lb/sq inch for 15 minutes at 121 ºC. After cooling; 0.25 ml cow serum was added into each tube (5% serum) and it was then stored at refrigerator temperature. It was used for the catalase test.

3-8-2 Aesculin Broth

Aesculin broth medium was prepared and the pH was adjusted to 7.4. The medium was then distributed into test tubes (5 ml each) and sterilized by autoclaving at 15 lb/sq inch for 15 minutes at 121 ºC and stored at refrigerator temperature. It was used for Aesculin test.

3-8-3 Arginine Broth

Arginine broth medium was prepared and the pH was adjusted to 7.4. The medium was then distributed into test tubes (5 ml each) and sterilized by autoclaving at 15 lb/sq inch for 15 minutes at 121 ºC and it was then stored at refrigerator temperature. It was used for Arginine test.

3-8-4 Nessler's Reagent

Nessler's reagent was prepared as describe by Barrow and Felltham (2003). It was used in Arginine test.

3-8-5 Hippurate Broth

Hippurate broth medium was prepared and it was then distributed into test tubes (5 ml each), it was sterilized by autoclaving at 15 lb/sq inch for 20 minutes at 121 ºC. It was used for detection of Hippurate hydrolysis.

All types of media described in this study were prepared as described by Barrow and Felltham (2003) {Appendix 3}.

3-8-6 Lancefield Grouping Kit

Lancefield grouping kit was provided ready for use from Saudia Arabia and it was used as described by the manufactures (Remel Europe Ltd. Clipper Boulevard Kent, D A2 6 PT UK) {Appendix 4}.

3-9-1 Culturing and Identification of Organism

Swabs and urine samples were streaked on the surface of blood agar plates and the plates were incubated under 10% Co_2 for overnight at 37 ºC. When no growth was observed, plates were reincubated for further 48 hrs before they were discarded as negative.

The type of haemolytic reaction displayed on blood agar was

observed and used to classify the Streptococci to β- Haemolylic Streptococci, which was associated with complete lyses of red cells surrounding the colony, whereas α-hemolytic Streptococci appeared partial or "greening" haemolysis associated with reduction of red cell hemoglobin or γ- non-haemolytic Streptococci. Haemolysis was not used as a stringent identification criterion.

3-9-2 Gram Stain

The Gram stain was done from the overnight growth. A colony of the tested organism was emulsified in a drop of sterile normal saline placed on a clean slide and allowed to dry then fixed by passing the slide three times over a flame then stained for one minute by crystal violet, washed with tap water and iodine solution was then added for one minute as a mordant, then washed with tap water, acetone was then added for 10 seconds as a decolourizing agent, washed with tap water, dilute carbol fuchsin was then added as a counter stain for half a minute , washed with tap water and the slide was then allowed to air dry and examined microscopically.

Organisms that showed Gram-positive reaction, were subjected to further examination and organisms that showed Gram-negative reaction, were excluded.

3-9-3 Diagnosis of Gram-Positive Organisms

All Gram-positive samples were subjected to further examinations including different biochemical tests.

3-10 Biochemical Tests

All biochemical tests were done as described by Barrow and Fellthamm (2003).

3-10-1 Catalase Test

Few drops of 3% hydrogen peroxide were added to the tested organism which was previously cultured for overnight at 37 ºC in 5 ml nutrient broth to which 5% serum was added. Positive result was detected by immediate production of air bubbles. Streptococci are considered as catalase-negative. Catalase positive samples were excluded.

3-10-2 CAMP Reaction

The CAMP reaction was done on a 5% sheep blood agar plates by streaking a strain of *Staphylococcus aureus* that produces ß –haemolysins, then the tested organisms were streaked at right angles to it. The tested organisms were not allowed to touch the *Staphylococcus aureus* inoculum. The tested organism was diagnosed to be *S. agalactiae* if after overnight

incubation at 37 ºC there was an arrow- head shaped area of haemolysis were the Staphylococcus organism met the tested organism.

3-10-3 Aesculin Test

Hydrolysis of Aesculin was done by inoculating the tested organism in Aesculin broth and incubated aerobically at 37 ºC and was examined daily up to five days for blackening; which indicates hydrolysis of Aesculin.

3-10-4 Arginine Test

Arginine hydrolysis was done by inoculating the tested organism in 5 ml of Arginine broth and after 24 hrs incubation, 0.5 ml of the culture was added to 4.5 ml distilled water and after shaking, 0.25 ml of Nessler's reagent was added, appearance of brown colour indicates positive result.

3-10-5 Growth on Bile

Growth on 40% bile was performed by streaking the tested organism on 10% and 40% bile agar plates and was incubated at 37 ºC for 24 – 48 hrs. Growth indicates resistance to bile.

3-10-6 Bile Solubility

A heavy inoculum of the tested organism was emulsified in one ml of normal saline and 0.25 ml of 10% bile salt sodium deoxycholate was

added and the tube was incubated at 37 ºC for 15 minutes. This dissolves positive organism by clearing the turbidity within 10-15 minutes. Negative organisms were not dissolved and therefore there was no clearing of the turbidity.

3-10-7 Hippurate Hydrolysis

Hippurate hydrolysis for Streptococci was done by inoculating the tested organism in Hippurate broth for 2- 4 days at 37 ºC, then a centrifugation of the culture was made and 0.5 ml of 50% sulphuric acid was added to 2 ml of the supernatant, appearance of white precipitate indicates hydrolysis of the Hippurate. (*Streptococcus agalactiae* positive others are negative).

3-10-8 Bacitracin Sensitivity

Bacitracin discs were placed on the surface of blood agar plate which was previously streaked with the tested organism. The plate was then incubated for 18 – 24 hrs at 37 ºC. Sensitivity to Bacitracin was shown by inhibition of the bacterial growth around the disc; resistant organism grew up to the disc.

3-10-9 Optochin Sensitivity

Optochin disc was placed on the surface of blood agar plate which was previously streaked with the tested organism and the plate was then incubated for 18 – 24 hrs at 37 ºC. Sensitivity to Optochin was shown by inhibition of bacterial growth around the disc for a distance of at least 5 mm; resistant organism grew up to the disc or may show a small inhibition zone not more than 1-2 mm.

3-10-10 Serological Method

Serologic methods can detect group A or B antigen. Bacitracin sensitivity differentiates group A from other β-haemolytic Streptococci (B, C, G); group B Streptococci typically shows Hippurate hydrolysis; group D is differentiated from other viridans Streptococci by bile solubility and Optochin sensitivity.

3-10-11 Lancefield Grouping

Serological identification of the organism was made by using the commercial Lancefield-grouping antisera. Few colonies of the tested organism were placed in a test tube containing 0.4 ml of the reacting enzyme solution and it was then incubated at 37 ºC for 10 minutes or up to one hour, then 0.04 ml of this culture was placed on a separate well of the

examination card provided (containing 6 wells), and a drop of each antisera (specific for group A, B, C, D, F and G) was added to the drop of tested organism culture in a separate well and was mixed, and finally, it was observed for appearance of agglutination up to one minute. A positive control was also provided and it was used to compare the agglutination of the tested organism as described by the manufacturing company.

3-11 Blood Culture

Blood samples were collected from patients who were suspected with Streptococcal infection (white blood cell count > 13000/ comm.), and inoculated directly into tryptone soya broth media bottles and were incubated at 37 ºC and were examined daily up to seven days for appearance of turbidity which indicates bacterial growth; samples that showed turbidity were subjected to further examination after streaking them on blood agar plates. The plates were incubated under 10% Co_2 overnight at 37 ºC and then their examination was carried out as for other samples bearing that Gram's stain was done first on a smear which was made from the Buffy coat after centrifugation of a blood sample collected in EDTA anticoagulant containing bottle.

3-12 Sensitivity to Antibiotics

Sensitivity to antibiotics was done by the disc diffusion method by adding few colonies from a growth of the tested organism into a nutrient broth medium to which 5% serum was added and then incubated for 2-3 hours at 37 ºC. Then about 1 ml of this culture was spread on the surface of Muller Hinton agar plates; by using commercial sensitivity disc containing Penicillin (10 units), Augmenting (30 µgm), Vancomycin (30 µgm), Erythromycin (10 µgm), Fluidic acid (10 µgm), Chloramphenicol (30 µgm), Amikacin (30 µgm), ampicillin (10 µgm), Cotrimoxozole (25 µgm), Cephalexin (30 µgm), Tetracycline (30 µgm), Cefotaxme (30 µgm), Gentamycin (10 µgm), Ciprofloxacin (05 µgm), and Ofloxacin (05 µgm). The plates were then incubated at 37 ºC for overnight; the sensitivity was indicted by the inhibition zone around each disc.

3-13 Polymerase Chain Reaction (PCR)

3-13-1 DNA Extraction

The DNA extraction was done using saturated bacterial culture which was previously kept in a normal saline solution at freezing temperature. The protocol used is a modified protocol; was often referred to as plasmid “mini-prep,” yield fairly clean DNA quickly and easy. Samples were centrifuged to pellet the bacterial cells then 0.2 ml of ice-

cold solution one (50 mM Glucose, 25 mM Tris HCl and 10 mM EDTA) was added to each pellet and vortexed. Solution two (0.2 N NaOH and 1%SDS) was then added, mixed by inversion and incubated at room temperature for 5 minutes. Ice-cold solution three (5 M acetic acid and 3M potassium acetate) was then added. Sample was vigorously shacked to release DNA and kept at room temperature for 10 min. Centrifugation and transformation of supernatant into fresh micro centrifuge tube using clean disposable transfer pipette was done. This fraction step separates the DNA from the cellular debris. The tube was then filled with Ice-cold Isopropanol which effectively precipitates nucleic acids. A centrifugation was then made to precipitate the milky pallet of the DNA at the bottom of the tube and the supernatant was poured off without dumping out the pellet. Then ice-cold 70% ethanol was added before another centrifugation was done and the supernatant was poured off. Finally samples were dried then dissolved in double distilled water and kept at – 20 ºC until used.

3-13-2 DNA Amplification (PCR)

Polymerase Chain Reaction (PCR) amplification was done using a genus- specific primer for Streptococci: (ST R1 5'- GTA CAG TTG CTT CAG GAC GTA TC-3' and STR2 5'- ACG TTC GAT TTC ATC ACG TTG-3') (Vivantis Technologies) {Appendix 5}. PCR reaction was

performed using a touchdown program profile: 94 °C for 2 min as an initial denaturation followed by 14 cycles during which annealing temperature was 63°C, decreasing per cycle for 14 cycles until the annealing temperature 55 °C was reached. The program was then continued using final temperature as annealing step for additional 35 cycles of: 45 s at 94 °C, 45 s of annealing and 1 min of extension at 72 °C. Finally the reaction was ended with 5 min at 72 °C. The mixes contained 1 µl (10 mM) of each forward and reverse primer, 1 µl of 25 mM MgCl2, 1µl of 10 mM dNTPs, 1 unit of Taq DNA polymerase, 2.5µl of 10X PCR buffer (10 mM tris-HCL pH 8.3, 50 mM KCL), ~150 ng of DNA and the volume was completed to 25 µl by double distilled water. PCR products were subjected to gel electrophoreses using 2% agarose in Tris Boric EDTA (TBE).

3-14- Quality Control

The isolates were subjected to sequencing using two other pairs of primers including the 16 S rRNA Primer: F (5'-AGA GTT TGA TCC TGG CTC AG-3' and 16 S rRNA R 5'-AAG GAG GTG ACT CAG CCG CA-3') {Appendix 6} and the TUF primer: (TseqF 5'-AAY ATG ATI ACI GGI GCI CAR ATG GA -3' and TseqR 5'- CCI ACI GTI CKI CCR CCY TCR CG -3') {Appendix 6}.

3-15 Electrophoresis (Agarose Gel)

The products were analyzed in 2% agarose gel and stained with 0.05% ethidium bromide in 1x Tris Boric EDTA (TBE) {Appendix 7} running buffer. The agarose gel was prepared by adding 2 gm of agarose to 100 ml of 1XTBE buffer and melting the mixture in a microwave. The mixture was cooled to reach 60°C before 2 µl of ethidium bromide solution were added. The gel was poured into electrophoresis apparatus, the comb was placed until solidification of gel. Then the comb was removed and the samples were loaded. Three micro liters of PCR products were mixed with 5 µl of the loading dye (Bromophenol blue dye) and loaded into gel wells. A hundred bp DNA ladder was used for the determination of band size and negative controls with no template DNA were included in all reactions.

At the end of electrophoreses, the gel tray was carefully transferred into a gel documentation system and examined under UV light.

3-16 DNA Sequencing

The selected PCR products were sent to Macrogen Company (Korea) for sequencing using the amplified primer. Sequencing reactions were performed for both directions (Forward and reverse) for each sample.

The obtained sequences were analyzed using different software packages. Firstly, sequences were aligned using BIO-EDIT and MEGA 6 software. Sequences were cleaned by removing the regions at the beginning and at the tail of the traces.

All sequences were blasted at NCBI to confirm and identify Streptococcus sp. at species level. For each species comparable sequences were downloaded and used for alignments and phylogenetic analysis. Accession numbers of GeneBank sequences used are displayed in the alignments and trees.

Multiple sequence alignments were performed using MEGA5. The evolutionary history was inferred using the Neighbor-Joining method. The optimal trees with the sum of branches lengths were shown (Fig 5 & 6). The percentage of replicate trees in which the associated taxi clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 727 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 to evaluate the best technique for identification of Streptococci.

Chapter Four

RESULTS

Out of 500 samples that were collected from patients suspected with Streptococcal infection, 115 samples of Streptococci were isolated from patients suffering from sore throat (76.67%) using a total number of 150 throat swabs. From 125 vaginal swabs, four isolates of Streptococci were isolated (3.2%), while 8 Streptococci were isolated from 125 samples of urine (6.4%) and five Streptococci were isolated from hundred samples of blood (5%). The percent of each type from the total number of samples was as follows: throat samples 23%, vaginal samples 0.8%, urine samples 1.6% and the blood samples 1%. Hence Streptococci isolated from the whole samples constituted 132 (26.4%) as shown in Table (1).

Table (1): **Number of Streptococci isolated from each type of sample and their percentages**

Type of samples	No of samples	Percent from total samples (500)	No of positive Strep	Percent of positive Strep	Percent from total No
Throat	150	30%	115	76.67%	23%
Vaginal	125	25%	4	3.2%	0.8%
Urine	125	25%	8	6.4%	1.6
Blood	100	20%	5	5%	1%
Total	500	100%	132	26.4	26.4

The primary identification of the collected samples included the growth on blood agar media, Gram's stain reaction (Figure 1), catalase test (Figure 2) and the type of haemolysis that appears on the surface of the blood agar plate.

All of 150 throat swab samples showed growth on blood agar except one sample, for the Gram stain reaction, 146 samples were found to be Gram-positive while three samples were Gram-negative. For catalase reaction, 115 samples showed catalase-negative reaction, while the other samples (31) showed catalase-positive reaction. For the type of haemolysis there were 93 samples appeared as β haemolysis and 22 samples appeared as α. Non-haemolytic (γ) was not found.

All the samples of the vaginal swabs (125) showed growth on the blood agar medium and Gram-positive staining reaction but only four samples were recognized as Streptococci as they showed catalase-negative reaction, while the remaining (121) were found as catalase-positive. These samples also appeared only as β haemolytic organisms.

All the urine samples (125) showed growth on blood agar but only 34 samples reacted as Gram-positive organisms, while the others (91) were found to be Gram-negative organisms. For the catalase reaction, only 8 organisms were recognized as Streptococci (catalase-negative) as the

remaining samples (26) showed catalase-positive reaction. Out of these 8 samples, three samples appeared as β haemolytic organisms, five samples appeared as α haemolytic and γ (non-haemolytic) was not found.

For the blood samples (100 samples), 55 showed growth on blood agar whereas 45 samples did not grow. Out of these 55 samples, 21 samples reacted as Gram-positive organisms but the remaining samples (34) were Gram-negative organisms. Using the catalase reaction, only five samples were negative, while the 16 remaining samples were catalase-positive. The type of haemolytic reaction was β haemolytic in four samples, one sample appeared as α haemolytic reaction and γ (non-haemolytic) was not found as shown in Table (2).

Table (2): **Primary identification of samples**

Type of sample	**Growth on blood agar**	**No growth**	**Gram stain**		**Catalase**		**Haemoslysis**		
			Pos.	**Neg.**	**Pos**	**Neg**	**β**	**α**	**γ (No)**
Throat	149	1	146	3	31	115	93	22	-
Vaginal	125	-	125	-	121	4	4	-	-
Urine	125	-	34	91	26	8	3	5	-
Blood	55	45	21	34	16	5	4	1	-
Total	454	46	326	128	195	132	104	27	-

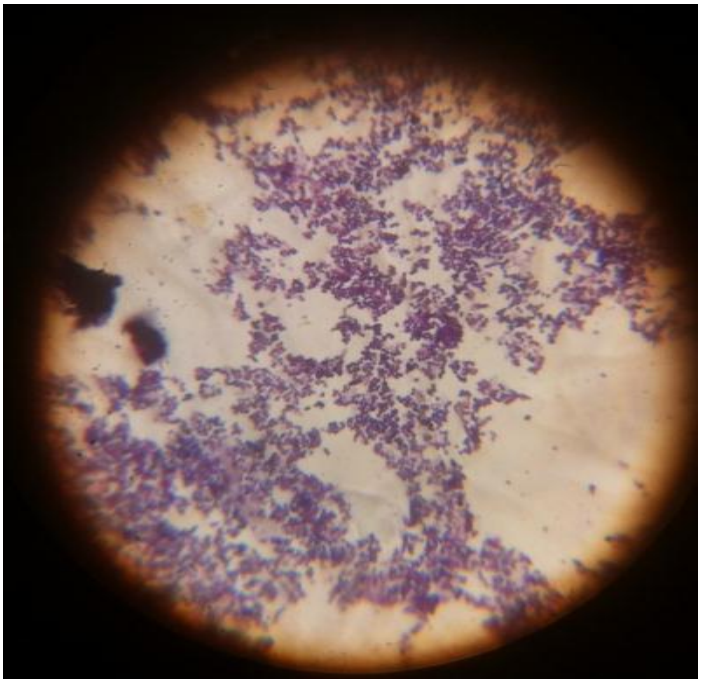

Figure (1): Gram stain

Figure (2): Catalase test
Left: negative reaction
Right: positive reaction

Samples that were primarily identified as Streptococci were subjected to secondary identification using different biochemical reactions including aesculin and arginine hydrolysis, growth on 10% and 40% bile agar media, bile solubility, hippurate hydrolysis, bacitracin and optochin sensitivity in addition to CAMP reaction.

Out of 115 throat samples eighty three samples were found positive to aesculin and 32 were negative, only one sample grew on 10% bile, the others are negative. However all the samples did not grow on 40% bile as all of them also were not soluble in the bile and did not hydrolyze the hippurate. One hundred and eleven samples were sensitive to bacitracin and the other 4 samples were resistant. For optochin sensitivity and CAMP reaction, all the throat samples were negative.

Two of the 4 samples of vaginal swabs hydrolyzed aesculin, while the other two did not. The 4 samples hydrolyzed arginine and they did not grow in 10% and 40% bile agar. Also they did not solublized bile and did not sensitive to bacitracin and optochin but they hydrolyzed the hippurate and showed positive CAMP reaction.

Two of the urine samples hydrolyzed aesculin, while the other 6 did not, 7 samples hydrolyzed arginine and one sample did not. Only one sample grew in 10% bile and all samples did not grow in 40% bile, and did

not solublized bile, not hydrolyzed hippurate and not sensitive to bacitracin and optochin, while two of them reacted positively to CAMP but the other six reacted negatively.

One of the blood samples hydrolyzed aesculin; the other four did not. The 5 samples hydrolyzed arginine; did not grow on 10% and 40% bile; one of them solublized bile, while the others did not. Two samples hydrolyzed hippurate, the others did not. The five samples were found resistant to bacitracin; one sample was sensitive to optochin, the other four resisted. Two samples reacted as CAMP positive and the other three were negative, as shown in Table (3).

Table (3): Biochemical reactions of catalase-negative organisms

Type of reaction	Throat swab		Vaginal swab		Urine		Blood	
	pos.	neg.	pos.	neg.	pos.	neg.	pos.	neg.
Aesculin	83	32	2	2	2	6	1	4
Arginine	113	2	4	-	7	1	5	-
Growth on 10% bile	1	114	-	4	1	7	-	5
Growth on 40% bile	-	115	-	4	-	8	-	5
Bile solubility	-	115	-	4	-	8	1	4
Hippurate	-	115	4	-	2	6	2	3
Bacitracin	111	4	-	4	-	8	-	5
Optochin	-	115	-	4	-	8	1	4
CAMP reaction	-	115	4	-	2	6	2	3

Key: According to Barrow and Feltham (2003)

Bacitracin sensitive = group A

CAMP pos. + Hippurate hydrolysis = group B

Optochin pos. + bile solubility = *S. pneumoniae*

Grouping of Streptococci, according to their biochemical reaction, showed that 107 samples were group A from the throat samples, four samples group F and four samples were not typable. According to Lancefield grouping 111 samples were found to belong to group A and four samples to group F.

For the vaginal swab, the four samples were grouped as group B Streptococci by the two methods.

From the eight urine samples, two samples were grouped as group B and the other six as group D by the two methods.

Out of the five blood sample, two were grouped as group B Streptococci, two group D Streptococci and one as *S.pneumoniae* according to the biochemical results; but according to Lancefield grouping, two samples were grouped as group B Streptococci, two as group D Streptococci and the last sample was not typable as shown in Table (4).

Table (4): Grouping of isolated Streptococci

Group / Sample	Biochemical grouping					Lancefield grouping					Non typable	
	A	B	C	D	F	A	B	C	D	F	Bio.	Lan. kit
Throat	107	-	-	-	4	111	-	-	-	4	4	-
Vaginal	-	4	-	-	-	-	4	-	-	-	-	-
Urine	-	2	-	6	-	-	2	-	6	-	-	-
Blood	-	2	-	2	-	-	2	-	2	-	-	1

Table (5) shows sensitivity to antimicrobial drugs and as the table shows all isolates were found highly sensitive to Penicillin, (except those isolated from blood culture which were moderately sensitive to Penicillin and highly sensitive to Vancomycin), Augamentin, Vancomycin and Erythromycin, all of the samples were mildly sensitive to Fusid acid and Cloxacillin while they were all resistant to Chloramphenicol Tetracycline Gentamycin and Ciprofloxacin except the urine samples which were seen with mild sensitivity to Ciprofloxacin.

Table (5): Sensitivity of isolated Streptococci to antimicrobial drugs

sample	No tested	Pen	Aug	Van	Ery	Fus	Cox	Chl	Tet	Cip	Gen
Throat	115	xxxx	xxx	xxx	xx	xx	x	-	-	-	-
Vaginal	4	xxxx	xxx	xxx	xx	x	x	-	-	-	-
Urine	8	xxx	xxx	xx	x	x	x	-	-	x	-
Blood	5	xx	xx	xxxx	x	x	x	-	-	-	-

Pen=Penicillin, Aug=Augamentin, Van=Vancomycin, Ery= Erythromycin, Fus=Flusidic acid, Clox= Cloxacillin, Chl= Chloramphenicol, Tet= Tetracycline,

Cip= Ciprofloxacin, Gen =Gentamycin.

Key

XXXX = highly sensitive

XXX = sensitive

XX = moderately sensitive

X = weak sensitive

- = resistant

Table (6) shows the number of samples for which the PCR was done or not according to the type of primer used.

Out of 115 throat samples, the PCR results were positive for 112 samples by the use of two primers; the genus- specific *tuf* primer and the other pair of *tuf* primers used to amplify a larger part of the gene. Three samples were negative by both primers. Both primers produced the expected band size (200 bp and 700 bp, respectively) (Figure 3 and 4).

All the fifty five samples (middle column) subjected to PCR by the use of the 16 s rRNA primer (which was used as another confirmatory result), produced the expected size band.

For the four samples that were isolated from the vaginal swabs the PCR was successful to all of them by the use of the two *tuf* primers. However, only two samples were successfully subjected to 16 s rRNA primer. For the other two samples DNA was not available for PCR.

For the eight urine samples, all samples were positive for the PCR using the two *tuf* Primers, while only seven samples were used successfully to amplify the 16s rRNA gene primer. DNA was not available for the one left sample of that group.

For the blood samples the PCR was also positive for all samples (N=

5) using genus specific *tuf* primer and the second *tuf* primer. PCR was also positive for the available three DNA samples using the 16s rRNA primer.

Table (6): PCR results by the use of the three types of primers

Type of sample	Genus specific	16 s rRNA primer	*Tuf.* Primer	PCR failed
Throat	112	43	112	3
Vaginal	4	4	4	-
Urine	7	5	7	1
Blood	5	3	5	2

Table (7) shows the results of samples that were sent for sequencing by each type of primer. Each selected sample (total= 35) was subjected to two sequencing reactions; the first sequencing analysis was done by the forward primer and the second by the reverse primer. For the *tuf* gene specific primer, five selected PCR products (throat = 2, urine 2, blood 1) were used to confirm identification process using that primer. All samples resulted in typical Streptococcus sequence.

By the use of the second *tuf* gene primer, 23 samples were sent for sequencing (Throat = 13, vaginal = 2, urine = 5, blood = 3). The sequencing results were readable for 18 samples, while the quality of the sequence was bad in the five samples.

Seven PCR products were selected for sequencing using the 16 s rRNA primer (Throat =3, urine = 2, blood = 2). Sequencing results were successful in four samples, while three samples were not good for subsequent analysis.

Table (7): Sequencing results for the selected

Positive Streptococci samples

Type of primer	Throat	Vaginal	Urine	Blood	Seq. succeed	Seq. failed
Genus specific	2	-	2	1	5	-
16s (r RNA)	3	-	2	2	4	3
***Tuf* gene**	13	2	5	3	17	6
Total	18	2	9	6	26	9

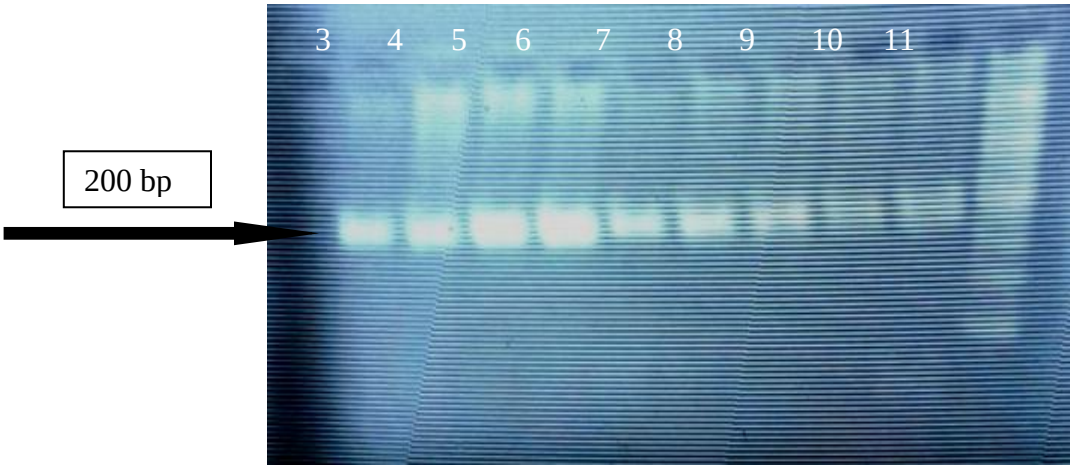

Figure (3): Gel electrophoresis result for the PCR amplification Genus - specific primer of streptococci.

Lane 1 = negative control. Lanes 2 -10 amplified DNA samples. Lane 11 100 bp DNA molecular Marker

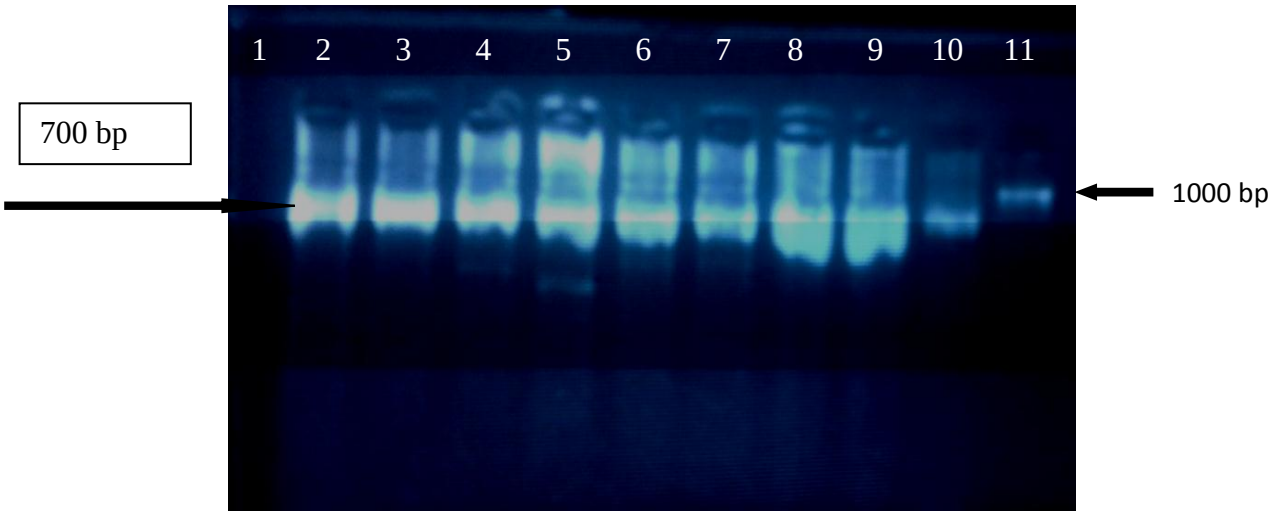

Figure (4): Gel electrophoresis result for the PCR amplification *tuf* primer of streptococci.

Lane 1 = negative control. Lanes 2 -10 amplified DNA samples. Lane 11 1000 bp DNA molecular Marker

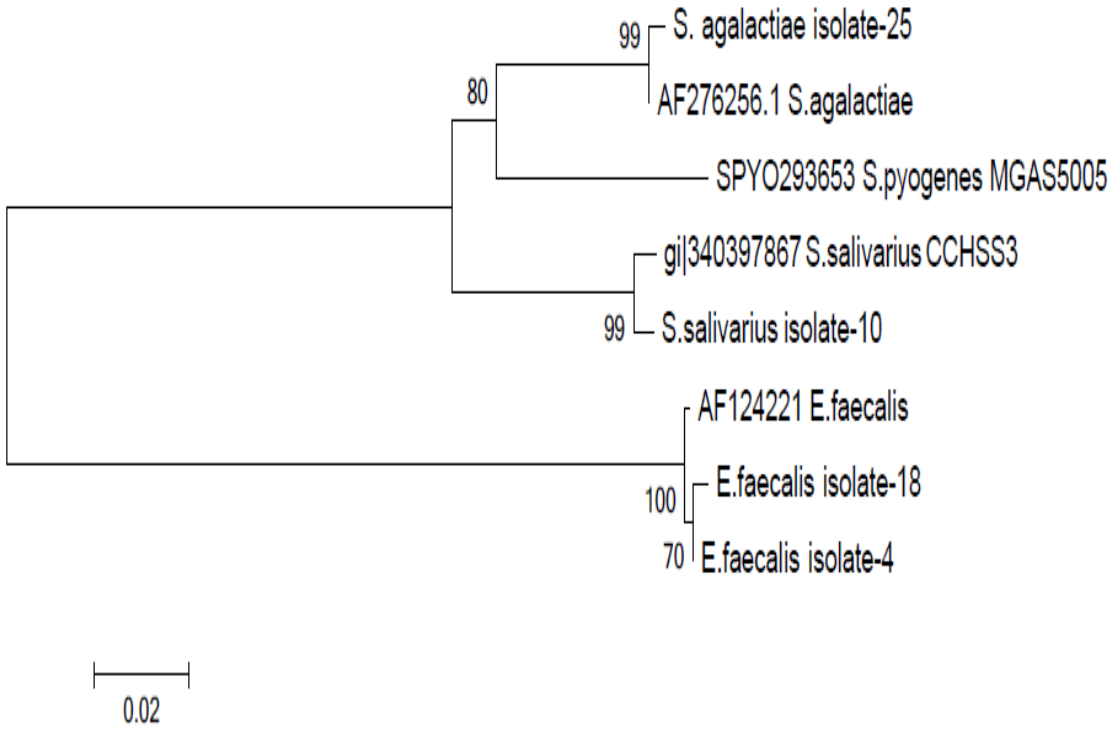

Figure: (5) A Neighbor-Joining Phylogenetic tree of Streptococcus sp. (including group D) based on a 691 bp portion of elongation factor (*Tuf*) generated using the MEGA5 software. The value on each branch represents the percentage of bootstrap supporting replications. A total of 1,000 bootstrap replications were calculated. All sequences used were obtained either from this study or from the following sources: GenBank (http://www.ncbi.nlm.nih.gov). Gen Bank accession numbers are given in the case of Gen Bank sequences

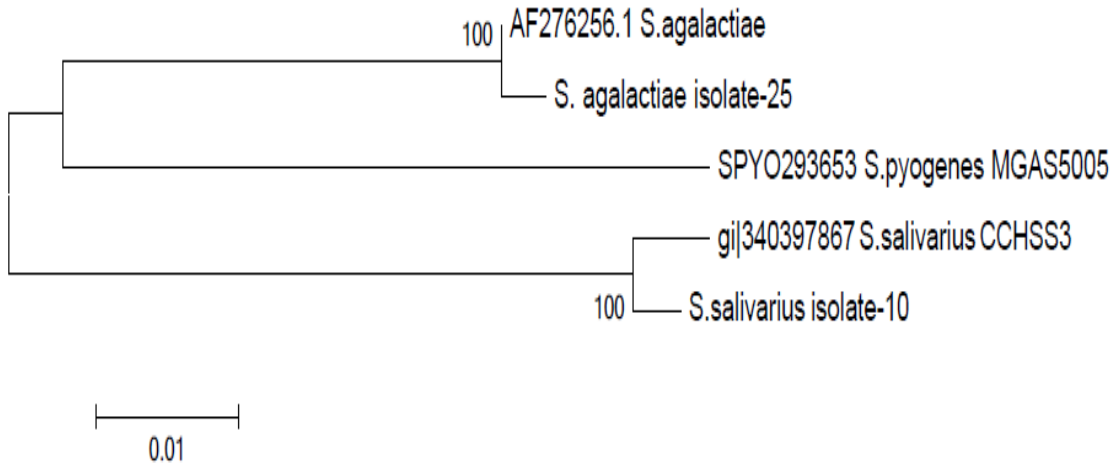

Figure (6): A Neighbor-Joining Phylogenic tree for Streptococcus sp. (excluding group D) based on a 691-bp portion of elongation factor (*Tuf*) generated using the MEGA5. The value on each branch represents the percentage of bootstrap supporting replications. A total of 1,000 bootstrap replications were calculated. All sequences used were obtained either from this study or from the following source: GenBank (http;//www.ncbi.nlm.nih.gov) GenBank accession numbers are given in the case of GenBank sequences.

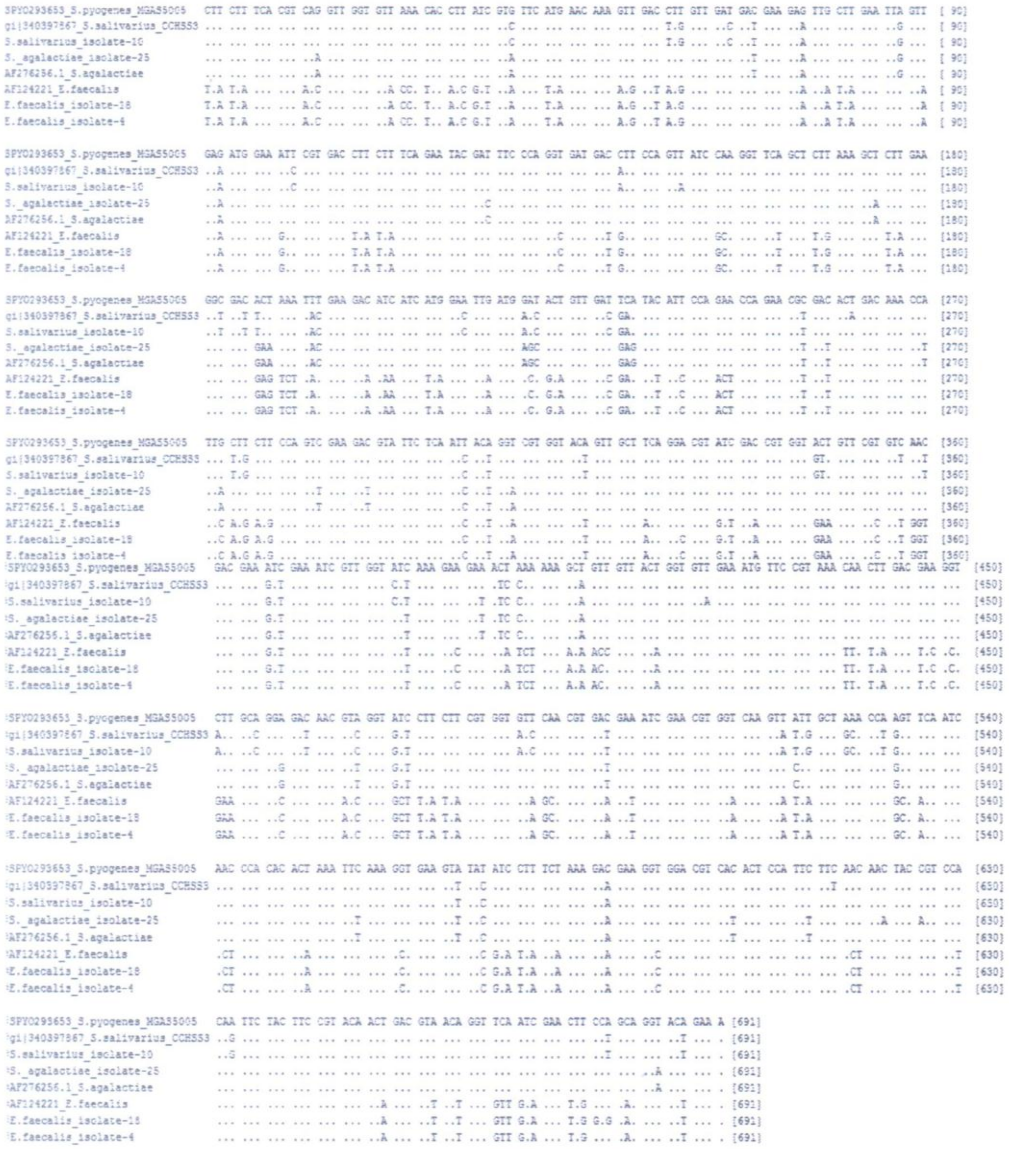

Figure (7): Multiple sequence alignments of all Streptococcus sp. (including group D) sequences using *tuf* elongation factor gene sequences performed using MEGA5. Four isolates of the current study and four NCBI partial sequences were used in the alignment representing Streptococcus sp. Accession numbers precedes GenBank sequences.

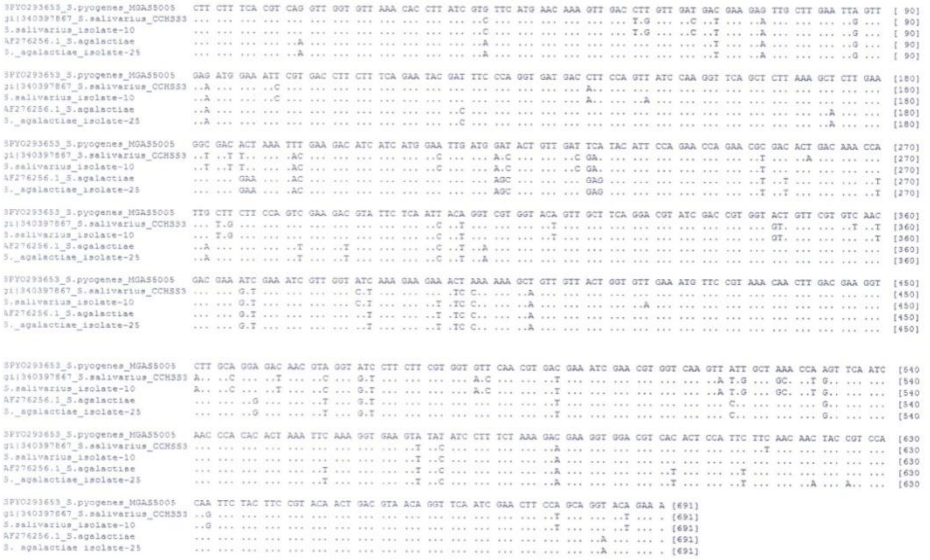

Figure (8): Multiple sequence alignments of identified Streptococcus sp. using *tuf* elongation factor gene sequences and performing using MEGA5. Four isolates of the current study and four NCBI partial sequences were used in the alignment representing Streptococcal sp. Accession numbers proceeds GenBank sequences.

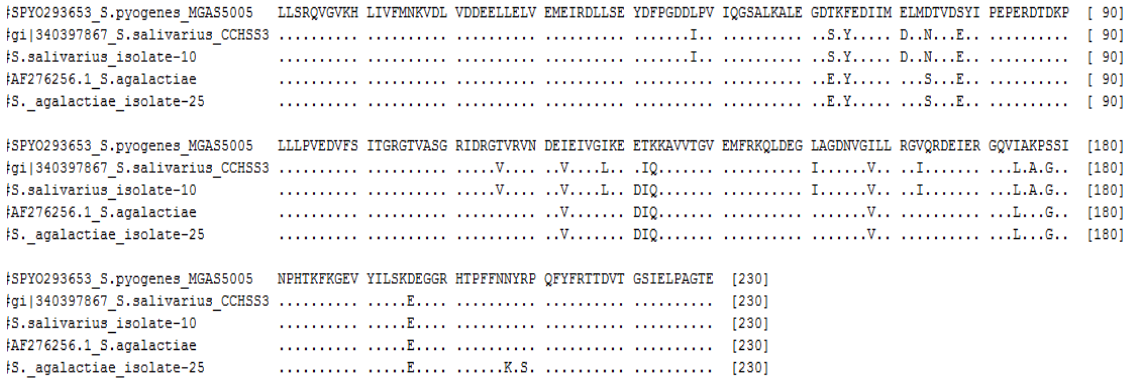

Figure (9): Multiple sequence alignment of all Streptococcus sp. Sequences (excluding group D) using tuf elongation factor gene translated sequences generated using MEGA5 software. Two isolates of the current study and thee NCBI partial sequences were used in the alignment representing Streptococcus sp. Accession numbers proceeds GenBank sequence

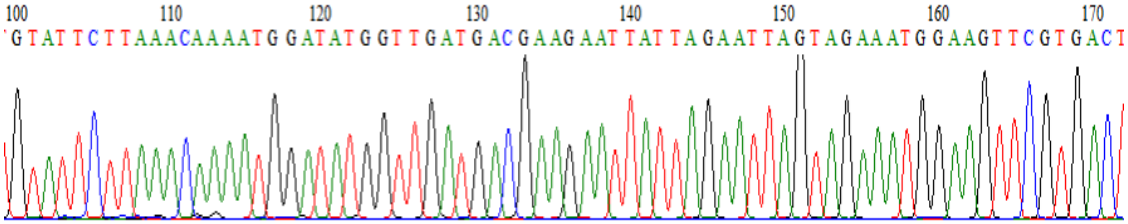

Figure (10): The quality of analyzed sequences. All sequences of bad quality were not included in subsequent analysis. Enterococcus sp. isolated from urine sample.

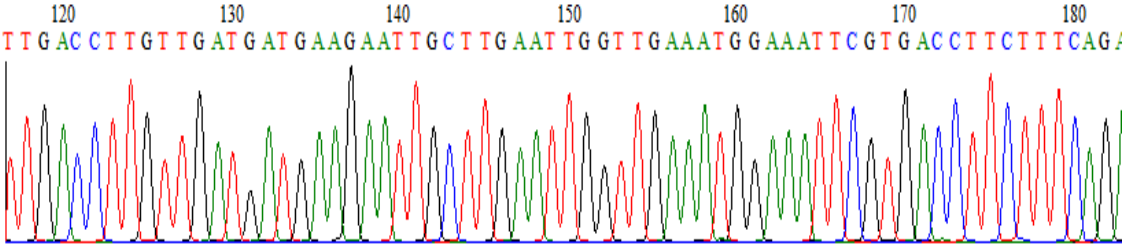

Figure (11): The quality of analyzed sequences. All sequences of bad quality were not included in subsequent analysis. Streptococcus sp. isolated from blood sample.

Table (8) shows comparison between the biochemical grouping, serological grouping (Lancefield grouping) and molecular grouping (sequencing).

All samples of throat that were diagnosed as group A Streptococci (*S. pyogenes*) and those which could not be typable by biochemical reaction and those were diagnosed as group A Streptococci (*S. pyogenes*) by the Lancefield grouping were differentiated by the molecular grouping as there were 3 samples diagnosed as *S. salvarius*, 3 samples Staphylococcus sp., while 2 samples were found as Enterococci species and there were 5 samples that their sequences could not be clearly read by the use of the *tuf* primer. By the use of the 16s rRNA one sample was diagnosed as *S. salvarius* and one was diagnosed as Staphylococcus species, while the last one could not be clearly read. By the use of the genus specific primer one sample was diagnosed as *S. salvarius* and the second as viridans group.

For the two vaginal samples, one sample was diagnosed as Enterococci; the second sample could not be clearly read by the use of the *Tuf* gene. The molecular diagnosis by the other two primers was not done. The biochemical and serological diagnosis resulted in group B Streptococci by the two methods for all samples.

Urine samples resulted in that 3 samples were diagnosed as Enterococci (group D) by the use of the *tuf* primer but one was diagnosed as Staphylococcus species and one could not be clearly read by the use of the same primer. By the use of the 16s r RNA primer two samples could not be clearly read, while one was diagnosed as Enterococci and one as Staphylococcus species. For the genus specific primer the two selected samples resulted in viridans group (group D). Using the biochemical and Lancefield grouping, 4 samples were diagnosed as Enterococci (group D) and one as group B in both methods.

For the blood samples, one was diagnosed as *S. agalactiae* (group B) and the two other samples could not be read by the use of the *tuf* primer, while no samples were diagnosed by the 16s r RAN primer, one sample was diagnosed by the genus specific primer as viridans group (group D). The biochemical grouping resulted in one sample as *S. pneumoniae* which was not provided with the Lancefield kit but the result was the same for other 4 samples as two were diagnosed as viridans group (group D) and the other two as group B Streptococci.

Table (8): Comparison between biochemical, serological and molecular grouping of streptococci

Sample No	Site of collection	*Tuf* primer	16 s rRNA	Genus specific	Biochemical grouping	Lancefield grouping
2	throat	could not be read	*S. salvarius*	*S. salvarius*	group F	group F
9	throat	Staph	-	-	group A	group A
18	throat	Staph.	Staph.	-	group A	group A
35	throat	Enterococci	-	-	group A	group A
40	throat	*S. salvarius*	could not be read	viridans	group A	group A
43	throat	Enterococci	-	-	group A	group A
16	throat	could not be read	-	-	group A	group A
91	throat	could not be read	-	-	group A	group A
92	throat	*S. salvarius*	-	-	group A	group A
93	throat	could not be read	-	-	group A	group A
1	throat	*S. salvarius*	-	-	group F	group F
124	throat	could not be read	-	-	group A	group A
5	throat	Staph	-	-	group A	group A
1	vaginal	could not be read	-	-	group B	group B
2	vaginal	Enterococci	-	-	group B	group B
1	urine	*E. faecalis*	-	-	Enterococci	group D
2	urine	Staph	Staph	viridans	Enterococci	group D
124	urine	*E. faecalis*	could not be read	viridans	Enterococci	group D

Sample No	Site of collection	*Tuf* primer	16 s rRNA	Genus specific	Biochemical Grouping	Lancefield grouping
48	urine	could not be read	could not be read	-	group B	group B
81	urine	*E. faecalis*	Enterococci	-	Enterococci	group D
96	blood	could not be read	-	-	*S. pneum*.	-
47	blood	group B	-	-	group B	group B
24	blood	could not be read	-	viridans	viridans	group D

Statistical analysis

Cross tables

Table (9): Cross table of case processing summary (sex)

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Sex * total result	500	100.0%	0	.0%	500	100.0%

Crosstabs

Table (10): Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Age group * total result	500	100.0%	0	.0%	500	100.0%

Table (11): Cross table of sex total result

	Tal result		Total
	+ve	-ve	
Count	59	91	150
% within sex	39.3%	60.7%	100.0%
% within total result	44.7%	24.7%	30.0%
% of Total	11.8%	18.2%	30.0%
Count	73	277	350
% within sex	20.9%	79.1%	100.0%
% within total result	55.3%	75.3%	70.0%
% of Total	14.6%	55.4%	70.0%
Count	132	368	500
% within sex	26.4%	73.6%	100.0%
% within total result	100.0%	100.0%	100.0%
% of Total	26.4%	73.6%	100.0%

Table (12): Age group * total result Cross tabulation

			Total result		Total
			+ve	-Ve	
age group	1 – 17	count	83	91	174
		% within age group	47.7%	52.3%	100.0%
		% within total result	62.9%	24.7%	34.8%
		% of Total	16.6%	18.2%	34.8%
	18 – 50	count	42	240	282
		% within age group	14.9%	85.1%	100.0%
		% within total result	31.8%	65.2%	56.4%
		% of Total	8.4%	48.0%	56.4%
50 and more		count	7	37	44
		% within age group	15.9%	84.1%	100.0%
		% within total result	5.3%	10.1%	8.8%
		% of Total	1.4%	7.4%	8.8%
Total		count	132	368	500
		% within age group	26.4%	73.6%	100.0%
		% within total result	100.0%	100.0%	100.0%
		% of Total	26.4%	73.6%	100.0%

Table (13 a): Chi-Squire Tests

	Value	df	Asymp. Sig. (2- sided)
Person Chi-Squire	62.340 [a]	2	.000
Likelihood Ratio	60.429	2	.000
Linear-by-linear association	48.441	1	.000
N of Valid Cases	500		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.62.

Table (13 b): Chi-Squire Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Person Chi-Squire	18.447 [a]	1	.000		
Continuity Correction [b]	17.509	1			
Likelihood Ratio					
Fisher's Exact Test	18.410	.000		.000	.000
Linear-by-linear Association	500				
N of Valid Cases [b]					

a. Cells (.0%) have expected count less than 5. The minimum expected count is 39.60.
b. Computed only for a 2x2 table.

Chapter Five

Discussion

Routine bacterial identification is based on phenotypic tests, including Gram staining, culture and growth characteristics in addition to biochemical patterns. Complete identification is routinely achieved within three days, but may be longer for fastidious or a typical organisms.

As expected *S. pyogenes* was the major isolated pathogen in the current study (93%). *S. pyogenes* is a major pathogen capable of causing a wide range of diseases in different age groups of people. The prevalence of the carriage of GAS was previously found to be more common than expected by Steer, *et al.*, (2009) who also isolated group G and group C from throat cultures. In the present study, in addition to group A Streptococci group F was also isolated (3.4%).

There were four organisms which could not be clearly grouped by biochemical reactions into group A or Group C because they showed bacitracin sensitivity, which is related to group A, but the type of haemolysis is similar to group C which is bacitracin negative. The current throat results are similar to those of Al-Charrkh, *et al.* (2011) in isolating group F Streptococci as a cause of sore throat together with group C;

however none of the current tested samples was identified as group C. The present findings seem to differ from the results of Mobin, *et al.* (2007) who isolated only group C Streptococci as a cause of pharyngitis in a recurrent case and Lindbaek, *et al.* (2005) who isolated group C and group G as a cause of sore throat. These results highlight the importance of other possible groups like group F which might complicate the consequent antibiotic treatment. These findings are also in agreement with Graham (2007) and Dalalah *et al*. (2008) in proving that the proper identification of the groups is necessary for the correct use of antibiotics. Ignoring these groups in the diagnosis will lead to misuse of antibiotics and recurrence of infections after a short period of antibiotic treatment resulting in resistant bacteria.

Collected data in the current study indicated that sore throat infection was mostly incriminated to *S. pyogenes* (GAS) which was found to be the main cause of tonsillitis especially in children but, the diagnosis depended only on symptoms and physical examination of the tonsils and pharynx. About 50% of cases of pharyngitis were caused by other groups and they were mainly isolated from adults. Laboratory investigations were rarely requested and include only ASO titer, ESR and CBC. Bacteriological

investigations, if requested, include bacitracin sensitivity only which is a stringent criterion for GAS.

These presentations illustrate the importance of a systematic approach for evaluating patients with negative rapid Streptococci GAS tests and to avoid worsening sore throat infections. The current study also showed that the recommended diagnostic approach to the adult sore throat patient focuses on the possibility of group A beta-hemolytic Streptococci. Subsequently, current guidelines consider group A beta-hemolytic Streptococcus as the sole group for antibiotic treatment. These guidelines ignores none -group A beta–hemolytic Streptococcal pharyngitis (especially group C or group G beta–hemolytic Streptococcal pharyngitis and peritonsillar abscess) infections leading to negative rapid strep tests and worsening during the first week of symptoms. Although Beta-hemolytic group C Streptococcus is a less common cause of acute pharyngitis, it has both similar microbiology and presentation to group A beta-hemolytic Streptococci. The diagnosis of group C Streptococcus should be considered in a patient with negative rapid antigen detection test and a worsening clinical course because it can cause severe pharyngitis. We would suggest that worsening pharyngitis patients should have a throat

culture to test for both group A and non-group A beta-hemolytic Streptococci.

Group B Streptococcus (GBS), when present during pregnancy, it is either colonizer or pathogenic, as its presence in the intestinal tract is normal. In the last few decades *Streptococcus agalactiae* or Group B Streptococcus (GBS) has gained importance due to its ability to cause serious neonatal infections. In developed countries GBS is a leading cause of sepsis and meningitis in neonates (Shirazi, *et al.,* 2014).

This study isolated GBS bacteria from 4 pregnant women out of the 125 studied cases (3.2%). Detection rate reached 4% when Gram negative samples were excluded. Maternal colonization by GBS was observed to range from 4% to 40% in several studies conducted worldwide (Jannati, 2012). Although the frequency of GBS colonized female was less than the rates of nearby countries like Egypt (17%) (Elbaradie, 2009); we consent that the screening test for this group of bacteria is recommended to be done during pregnancy. The lower rate of GBS colonization in this study might be due to the fact that only vaginal swabs (no rectovaginal swabs) were collected. Rectovaginal swabs could have increased the detection rates. We also found that GBS should be screened among pregnant women at the age of 35 - 37 weeks of gestation of pregnancy as stated before by

Elbaradie (2009).

In agreement with the recommendation of the Center of Disease Control (CDC) and Shirazi *et al.*, (2014), these results indicated that screening test for this group of bacteria during pregnancy is recommended. The results of the current study signify a strong need for further biochemical reactions to be done in addition to antimicrobial sensitivity. Detection rates although slightly less but are in agreement with Vijayan (2011) who isolated (GBS) in a ratio of 2.3%., Shirazi, *et al.*, (2014) isolated the same type of bacteria with a ratio of 4.8%, and with Hamedi, *et al.*, (2012) who isolated GBS with a slightly higher ratio (6%).

In the current study it was found that the commonest pathogens were Gram-positive organisms as they constituted 92 % of isolates. However, going further in the identification of these types of bacterial isolates was not in the scope of this study. Among the studies went into that direction the study by Hammoud, *et al.*, (2012) who isolated Staphylococcus, Enterococcus, Enterobacter and Candida but not GBS.

In this study only group B Streptococci was isolated from pregnant women as only colonizers (asymptomatic). The study showed that the diagnosis and correct treatment of group B Streptococci is important particularly to prevent its colonization in the urinogenital tracts of pregnant

women as a normal commensal or pathogen because it is one of the most important bacteria in the majority of maternal and neonatal infections (Hamedi, *et al.*, 2012).

The present data showed that, a substantial percentage of GBS carriers were otherwise asymptomatic. When a universal approach to screening is consistently applied, all of these asymptomatic GBS carriers should be screened and consequently treated with intrapartum prophylactic antibiotics. They should be overlooked, however, if a risk-based approach was used. The main findings of this study in this type of infection were: the prevalence of colonization of this group among pregnant women was higher than expected regardless to the women's age, parity and gestational age. Treating group B Streptococci carriers with benzathine penicillin G in the late third trimester eradicates or significantly reduces maternal group B Streptococcal colonization at delivery. This may provide an adjuvant therapy to those mothers at risk.

Urinary Tract Infection (UTI) is described as the microbial invasion of any tissues of the urinary tract and is the second most common clinical symptom for experimental antimicrobial treatment in primary and secondary care. Normally, the urinary tract is sterile, but urinary tract infections can be caused by a variety of conditions, asymptomatic or

symptomatic infections. Urinary tract infections (UTIs) are among the most common human infections and can occur at any time of life. Urinary tract infections are common during pregnancy. It is common among both genders with higher prevalence among women due to a variety of physiological changes during the course of pregnancy which enhances the occurrence of infection (Ranganathan, 2014).

Urine culture and sensitivity testing are the standard diagnostic investigations to detect the causative organism and to determine the type of antimicrobial therapy needed.

Enterococci are commensals of the human and animal gastrointestinal tract and opportunistic pathogens that cause urinary tract infections (UTIs), endocarditis, and sepsis. Nosocomial infections caused by enterococci have increased; these pathogens were considered recently as the third most common at hospitals after *Escherichia coli* and *Staphylococcus aureus* (Louise, *et al.*, 2012). Enterococcus sp. as pathogens have increased, but the sources of infection often remain unclear. Enterococci are frequently recorded as the cause of UTIs, wound infections, bacteremia, and endocarditis (Louise, *et al.*, 2012).

Again, the commonest pathogens in urine isolates were Gram-negative organisms as they constituted 72.8 % of isolates. Although the

current study was not intended to identify Gram negative organisms, Ranganthan (2014) isolated *Escherichia coli,* Staphylococcus species, Klebsiella, Pseudomonas and Proteus in addition to Enterococcus species.

According to the findings of this study, GBS can also cause urinary tract infections as it was diagnosed in two samples (1.6%). The present results are in agreement with those of Shayanfar, *et al.,* (2012) who isolated GBS from urine in a ratio of 1.79% and those of Louise, *et al.,* (2012) and Ranganathan (2014) who isolated Enterococci from urine.

Blood stream infection remains a major cause of morbidity and prolonged hospital stay in children. In neonates and infants, it is a life-threatening emergency (Samuel, *et al.,* 2013).

Blood culture is likely the most significant specimen type used for the diagnosis of bacterial infections, especially for blood stream infections. The blood culture represents a critical tool for the health care professional as a mean of detecting the dangerous presence of living organisms in the blood stream. A positive blood culture can suggest a definitive diagnosis and enable the targeting of therapy against the specific organism (Linoj, *et al.,* 2013) and (Jonathan, *et al.,* 2013).

Viridans Streptococci are groups of Streptococcus species that are nutritionally fastidious and mainly alpha-hemolytic on sheep blood agar. They are normally commensals, but when present in blood culture, they may be contaminants (0.6 – 6%) or invade the blood stream during the state of neutropenia. Studies have found that viridans Streptococci are among the most common organisms isolated from the cultures of bacteremia samples (Christopher and Carey 2010).

This study diagnosed five samples as Streptococci out of 100 samples. By the use of biochemical reactions the isolates were grouped into three groups: viridans Streptococci (Group D, two isolates), *S. agalactiae* (Group B, two isolates) and the last one was diagnosed as *S. penumoniae* (Bile solubility). While Lancefield grouping kit was not designated to detect the *S. penumoniae* other biochemical results confirmed the other four isolates grouping. These findings were in agreement with Lindoholm (2004) in isolating group B Streptococci and *S. pneumoniae* in addition to *S. aureus* and to those of Glazunova, *et al.*, (2006), Christopher and Carey, (2010) and Soo-Kyeong, *et al.*, (2013) in isolating viridans Streptococci.

In this study group B Streptococci was again isolated from blood culture, therefore the diagnosis and correct treatment of group B Streptococci is important.

In agreement with the recommendation of Linddholm and Sarkkinen, (2004) these results indicated that identification of bacterial groups isolated from blood culture is an important criterion. Thus further biochemical reactions and quick diagnostic tests are recommended in addition to antimicrobial sensitivity.

The results of the current study signify a strong need for further diagnostic methods to be done in addition to antimicrobial sensitivity. As GBS is a significant urinary pathogen in both pregnant and non-pregnant adults, its presence signals a need for screening for urinary tract abnormalities.

In our study, we found that most of the isolates respond well to antibiotics with high susceptibility to penicillin, augamentin and vancomycin as Dalalah (2008) found that sore throat bacteria respond to antibiotics.

Molecular biology based techniques allow fast and rapid identification of pathogens. Strategies based on polymerase chain reaction (PCR) and sequencing has shown particular promise as highly sensitive tools for microbiological identification. Conventional microbiogical techniques of bacterial identification, culture and isolation of pathogenic

bacteria, identification by biochemical and serological assay, are time-consuming and require intensive labor. On the basis of special gene sequence, polymerase chain reaction (PCR) was the basis of special gene sequence, polymerase chain reaction (PCR) was found to provide a simple and rapid way to identify bacteria (Shen and Feng, 2004).

The main goal of this part of the study was to introduce a rapid and sensitive method to detect and identify bacteria as the gene sequencing analysis remains the most reliable method by far, for the laboratory identification of Streptococci.

Firstly, PCR using universal Streptococci primers (*tuf* gene) was used in the study to confirm the microbial and serological diagnosis of selected Streptococci isolated.

Using the first set of *tuf* primer, PCR was able to identify Streptococcal genome in 100% of all vaginal, urine and blood. Moreover the primer was 97.3% sensitive in the detection of Streptococcus isolates from throat. Accordingly, the used *tuf* primer set was considered about 100% sensitive in the detection of Streptococcus samples. The lower detection rate in throat samples is negligible as it was due to bad DNA quality and quantity in the negative samples.

In agreement with Shen, (2004) who found that conventional microbiogical techniques of bacterial identification, culture and isolation of pathogenic bacteria, identification by biochemical and serological assay, are time-consuming and require intensive labour, on the basis of special gene sequence, Polymerase Chain Reaction (PCR) provides a simple and rapid way to identify bacteria.

Strategies based on polymerase chain reaction (PCR) and sequencing has shown particular promise as highly sensitive tools for microbiological identification.

The *tuf* sequences were used to select Streptococcus-specific PCR primers and to perform phylogenetic analysis and to perform extensive phylogenetic analysis, which was generally in agreement with phylogeny determined on the basis of 16S rRNA gene data which was also used in this study.

After PCR analysis the size of GBS amplification fragment was found to be 200 - 255 bp using the genus specific primer. As Xuerui, *et al.*, (2013) found, it will be more useful to identify species in different genera using a single primer pair.

In the present study the 5 samples that were selected for sequencing using the genus specific primer resulting in specific streptococci sequence, using the *tuf* gene sequencing 23 samples were selected for sequencing from the four types of samples as the sequencing was failed in three samples and when the 16s rRNA was used, 7 samples were selected for sequencing the sequencing was again failed in two samples resulting in 100%, 88% and 71.4% Streptococci sequence with a medium percent of 86.4% which was found to be a little pit lower than that found by Glazunova, *et al.*, (2006) who reached a percent of 95.2 - 95.4 % gene sequence similarity.

Comparing between biochemical, Lancefield and sequence grouping of Streptococci isolated in this study, the results of biochemical and Lancefield grouping were typical to each other except that there were two samples could not be clearly grouped by biochemical reaction, while one sample of *S. pneumoniae* which was isolated from one blood sample could not be grouped by the Lancefield kit because the anti-sera was not provided in the kit. Comparing these results with sequencing results, samples that were grouped as *S. pyogenes* (GAS) by the two methods were resulted in *S. salvarius* which is normally isolated from saliva and as it shares GAS in most of the biochemical reactions especially Bacitracin which may be

positive or negative in case of *S. salvarius* and known to be specific to GAS.

Sequencing of *tuf* gene revealed variable results. Of the 23 samples sent for sequencing only 16 (64%) samples were either readable or partially readable. Sequence analysis indicated that 62 % of these samples were related to Streptococcus species. Blast analysis revealed that of these were; three *S. salivarius* isolates from throat swabs and one *S. agalactiae* isolated from one blood sample. In addition to that, 6 samples were identified as group D Streptococcus species (now classified as Enterococcus species).

In comparison to the previously applied microbial techniques, all *S. salivarius* isolates were identified as *S. pyogenes*. It is tempting to speculate that the obtained discrepancy between classical microbial assays and sequencing technique might be due to mixed infection of more than one Streptococcus *sp* as both *S. salivarius* and *S. pyogenes* are inhabitant of human oral cavity. Also, in a research study, it was found that within the same Streptococcus genus, some species could actually inhibit the growth of other species (Upton, *et al.,* 2001). *Streptococcus salivarius* is the principal commensal bacterium of the oral cavity in humans. It is a normal inhabitant of the upper respiratory tract (Francesco, *et al.,* 2012). It can be transmitted by direct contamination of sterile body fluid, or it may enter the

blood stream by accident during dental work or when brushing the teeth. It therefore seems to be the pioneer in colonizing dental plaque; it creates favorable conditions so other species can begin to colonize (Rashmi, *et al.*, 2015). It is also a bacterium which plays the role of moderator, permitting the implantation of bacteria which are harmful to the health of the oral cavity (Rashmi, *et al.*, 2015).

Interestingly, the organism *Streptococcus salivarius* was identified to cause significant growth inhibition on *Streptococcus pyogenes* through a 22 amino acid lantibiotic (= antibiotic peptide) designated salivaricin A. (Upton *et al.*, 2001). Moreover, looking at the applied biochemical reactions it can be considered fairly possible to get confusing result if both organisms are present in the sample as the only differentiating reaction was arginine. Other biochemical reactions were either similar or uncertain.

The unexpected results of identifying 4 Staphylococcus spp. samples is most probably due to contamination during preparation of samples for molecular assay. Identification and isolation of Streptococcus colonies can be quite problematic but it was not possible in this case as the microbiology work was done correctly. There are a lot of similarities between Streptococcus (Strep) and Staphylococcus (Staph) that lead to confusion. Both are spherical, facultative anaerobic Gram-positive bacteria, common

members of the normal human microbiota, capable of resisting many forms of important antibiotics and some species are capable of being pathogenic, responsible for serious infections and even deaths directly or indirectly through virulence factors such as toxins. The key differentiating characteristics between these two groups of bacteria includes formation and cell division e.g. division occurs on a single axis growing in pairs or chains (Strep) as division in various directions on multiple axes (Staph) growing in grape-like clusters and their ability to produce catalase (enzyme to break down hydrogen peroxide) e.g. Strep is catalase negative as Staph is catalase positive.

In terms of site of collection, contamination was possible as often staph found on the skin or in the mucus membranes of healthy people and it can remain there without signs of infection.

Contamination may lead to misdiagnosis and thus would render false negative results. Several studies highlighted the effects of the possible contamination of biological sample using molecular assays. This study supports other asserts that contamination is an important complication of the molecular assays, which should be avoided through careful laboratory controls (Xu, *et al.*, 2005). Molecular finding are consequently should be carefully interpreted together with other laboratory findings, such as

culture, biochemical serological and the clinical scenario of the patient. Nevertheless, the molecular approach might be superior to culture during antimicrobial treatment of the patient and in the detection of bacteria with unusual growth requirements.

Moreover, the sensitivity and specificity of the culture techniques were found to be greatly affected by the presence of bacteria other than targeted organism on the culture media (McClean, *et al.*, 2011; Sarma, *et al.*, 2016). For this reason, isolated Streptococcus colonies might have been masked by the growth of other bacteria in the second round of culture for molecular assays. Contamination of the culture medium might also occur due to delay in processing, transport and culture of the swab samples or even delay in the process of DNA extraction.

To reconfirm the first run of PCR using the first set of *tuf* primers, 5 samples were selected for a second round of sequencing using the genus specific *tuf* primer. Results indicated that all five samples were Streptococci sequences supporting that the sequenced other organisms are due to contamination during DNA preparation. From the four types of samples as the sequencing was failed in three samples and when the 16s rRNA was used, 7 samples were selected for sequencing the sequencing was again failed in two samples resulting in 100%, 88% and 71.4%

Streptococci sequence with a medium percent of 86.4% which was found to be a little pit lower than that found by Glazunova, *et al.*, (2006) who reached a percent of 95.2 - 95.4 % gene sequence similarity.

Conclusion

In conclusion, the diagnostic methods in Sudan hospitals strongly need to be changed by introduction of modern diagnostic methods and equipments because isolation and identification of pathogenic bacteria by biochemical and serological assay are time-consuming and require intensive labour. On the basis of special gene sequence, Polymerase Chain Reaction (PCR) provides a simple and rapid way to identify bacteria and better results will be obtained if single species primers used.

It was concluded that, not only *S. pyogenes* is incriminated in sore throat infection and other groups can also cause sore throat and further examination should be made when Group A Streptococci (GAS) is not detected in patients suffering from sore throat.

There is also a strong need for further screening tests including biochemical reactions to identify the type of bacteria other than *Streptococcus agalactiae* that colonizes the pregnant women in maternal

Sudanese hospitals. Hence it is recommend that further identification methods should be done and a proper diagnosis of bacteria that colonizes the pregnant women to be reached. Detection methods should be adopted as routine work and follow-up programs to the neonates of infected women or colonizers might be needed as recommended by the center of disease control

Screening for streptococci in UTI infection and positive blood culture is recommended as an important test to be looked after in all hospitals to improve the correct diagnosis and to decrease the misuse of antibiotics.

In conclusion also, we have performed sequence analysis of streptococci showing that *tuf* generally offers a better discrimination power than 16S rDNA to distinguish streptococcal species. And we agree with Francois, *et al.,* (2004) in that, future developments will seek to combine this genus-specific assay with detection of species-specific *tuf* sequence polymorphisms to provide a molecular diagnostic tool for rapid and accurate diagnosis of streptococcal infections.

An accurate evaluation (using larger samples) to evaluate streptococcal invasive disease and to determine whether PCR is cost effective is required for the selection of appropriate preventive strategies.

Future work should focus on larger multicentre studies of streptococcal infections, evaluation of culture versus rapid detection methods, and randomized controlled trials of strategies for prevention of infection.

Recommendations

It was recommended that full bacteriological examination must be done as a routine practice for diagnosis in all hospitals in addition to PCR technique.

1- Sampling for throat infection should be carefully performed, especially in children to avoid miss-diagnosis
2- All pregnant women must be screened between 35 and 37 weeks to determine if they are carriers of GBS by taking vaginal and rectal swabs.
3- Introduction of a diagnostic strategy to identify the most common Gram-positive bacteria is recommended in the hospitals.
4- Combination between culture, biochemical, serology and molecular tools should be adopted to ensure the proper diagnosis
5- Application of molecular strategies in the detection of Gram-positive bacteria is recommended

6- Special laboratory set-up should be prepared to avoid contamination.

7- Antibiotic sensitivity screening test should be practiced before prescribing treatment.

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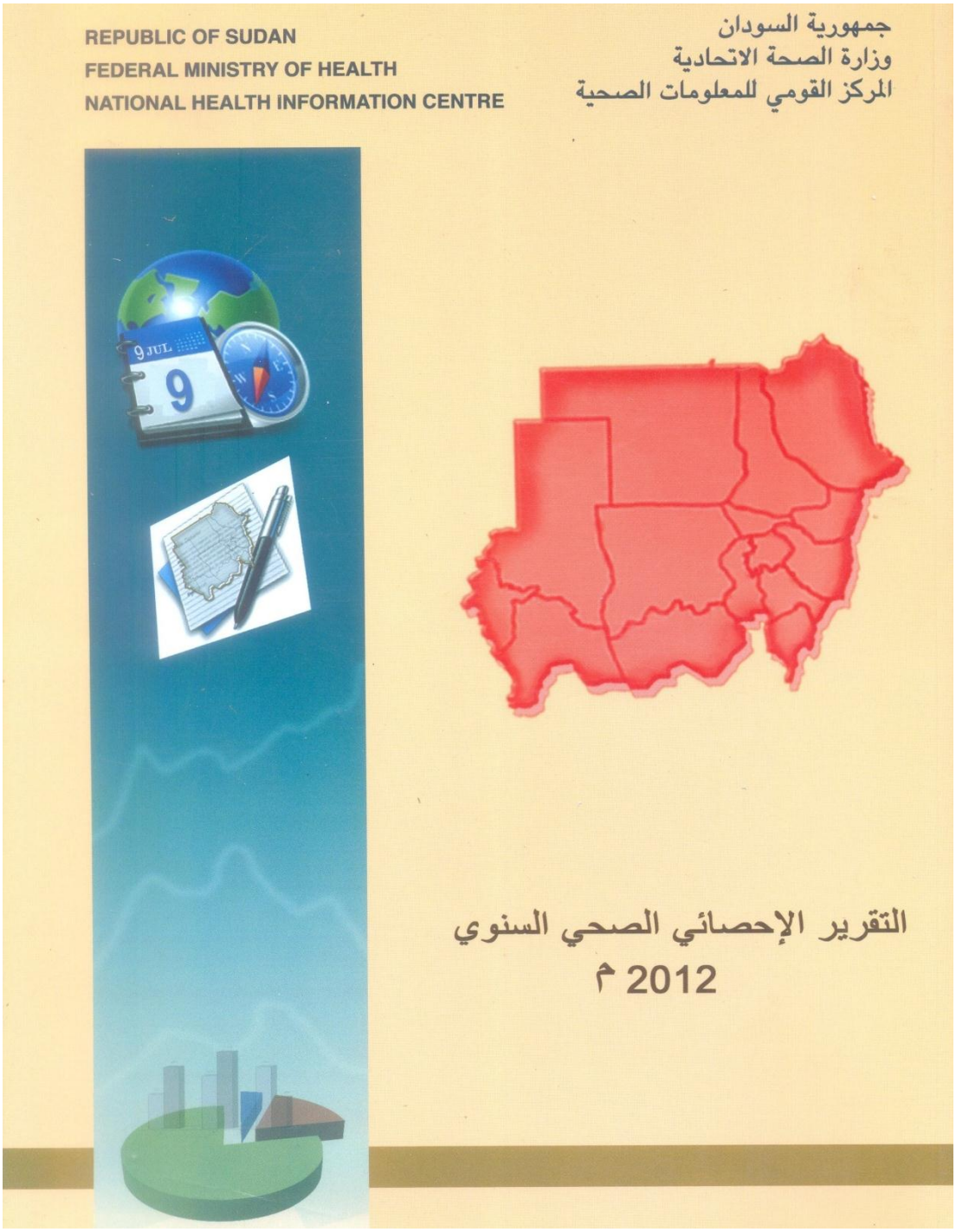
Appendixes

Appendix (1)

Annual statistical report (2012)



جدول رقم (2/3) أكثر عشرة أمراض ترددا على عيادات المؤسسات الصحية لعام 2012

Table (3/2) The (10) leading diseases treated in health units (outpatients) pre / 1000 Pop. 2012

المعدل لكل 1000 من السكان pre / 1000 Pop.	النسبة المئوية %	الاصابات cases	Diseases	رمز الدليل الدولى ICD-10	الأمراض
32	10	1105757	PNEUMONIA	J12	التهاب رئوى
28	9	964698	MALARIA	B54	الملاريا
20	6	710362	ACUTE TONSILLITIS	J03	التهاب حاد في اللوزتين
15	5	535927	DISORDERS OF URINARY TRACT	N39	اضطرابات بمجرى البول او المسالك البولية
14	4	490982	DIARRHOEA AND GASTROENTERITIS	A09	الاسهالات والنزلات المعوية
13	4	472427	ESSENTIAL HYPERTENSION	I10	ارتفاع ضغط الدم
13	4	444978	Other DISEASES OF RISPIRATORY SYSTEM	J95	أمراض بالجهاز التنفسى
12	4	411585	DIABETES MELLITUS	E13	السكرى
10	3	339142	INJURIES INVOLVING MULTIPLE BODY REGIONS	T00	اصابة سطحية بمواقع أخرى او متعددة او غير مضنية
9	3.	319780	Actue Bronchitis	J20	التهاب حاد في الشعب الهوائية
165	52	5795638	Total (10) Diseases		مجموع العشرة أمراض
151	48	5282797	Total of other diseases		مجموع بقية الأمراض
316	100	11078435	Grand Total		المجموع الكلى للأمراض

*نسبة لعدم وجود تصنيف جيد لأمراض المترددين يرد التصنيف في مجموعات

جدول رقم (3/3) أكثر عشرة أمراض ترددا على عيادات المؤسسات الصحية لعام 2012
بين الأطفال عمر (0 - 4)

Table (3/3) The 10 leading diseases treated in health units for children age (0 – 4) 2012

النسبة المئوية %	جملة الاصابات Total cases	رمز الدليل الدولى ICD-10	الأمراض Diseases
22	513203	J12	التهاب رئوي Pneumonia
11	262061	B54	الملاريا Malaria
11	257994	A09	الاسهالات والنزلات المعوية Diarrhoea & E.G
8	191983	J03	التهاب حاد في اللوزتين Acute tonsillitis
5	117067	J20	التهاب حاد في الشعب الهوائية Actue Bronchitis
5	113240	J95	أمراض بالجهاز التنفسى DISEASES OF RISPIRATORY SYSTEM
3	63199	J00	التهاب الخيشوم (البلعوم الانفي) الزكام -نزلة البرد العادية ACTUE NASOPHARNGITIS
2	41453	K93	أمراض الجهاز الهضمى الأخرى Disorders Of Other Digestive Organs
2	39769	H11	اضطرابات أخرى بالعين Other Disorder Of Eye
2	39181	A06	الدوسنتاريا Dysentery
69	1639150		مجموع العشرة أمراض Total (10) diseases
31	729909		مجموع بقية الأمراض Total of other diseases
100	2369059		المجموع الكلى للأمراض Grand total

جدول رقم (1/4) الفحوصات المعملية للدم التي تمت بالمؤسسات الصحية المختلفة بالولايات 2012م
Table (4/1) Laboratory Investigations (Blood Sample) by states for2012

المجموع الكلى G.Total	Positive Case الحالات الايجابية									الولايات States
	أخرى Other	ترسيب E.S.R	سكرى Sugar	بولينا B.Urea	تايفويد Widal	ك البيضاء T.W.B.C	همقلوبين Hb	ملاريا Malaria	مجموع الفحوصات T. Sample	
201556	228	13189	2922	106	1205	62884	50759	70263	237612	الشمالية Northren
221926	976	11747	11789	6255	7212	92685	74152	17110	385801	نهر النيل R.Nile
68418	12348	2517	10388	1693	345	18605	10142	12380	139361	البحر الأحمر Red Sea
224500	9548	7835	10801	1425	5874	79313	56967	52837	437792	القضارف Gadareif
73241	776	426	29074	1651	10275	2801	1542	26696	157297	كسلا Kassala
2190982		213051	0	0	0	1037031	864118	76782	3516011	الخرطوم Khartoum
605624	16149	49548	61037	21852	20458	200040	177639	58910	991784	الجزيرة Gaziera
209382	4430	9308	13351	4018	21927	96868	59480	81853	425699	سنار Sinnar
133415	4459	5770	3420	133	5075	32779	27051	54728	173124	النيل الابيض W.Nile
183032	5034	5034	5547	104	13338	62105	16774	75096	319422	النيل الأزرق B.Nile
179891	4604	7527	5164	2924	2218	60850	31548	65056	462602	ش . كردفان N.kordofan
33101	377	1191	724	98	359	7594	3319	19439	39122	ج . كردفان S.Kordofan
73279	13276	1901	896	167	3275	15136	14302	24326	83244	ش. دارفور N.Darfour
35973	5912	3186	1327	3	1730	12422	2799	8594	85659	غ. دارفور w.Darfour
31239	338	331	493	13	978	18167	10347	572	55171	ج. دارفور S.darfour
4465559	78455	332561	156933	40442	94269	1799280	1400939	644642	7509701	السودان Sudan

جدول رقم (1/4) الفحوصات المعملية للدم التي تمت بالمؤسسات الصحية المختلفة بالولايات 2011م

Table (4/1) Laboratory Investigations (Blood Sample) by states for2011

المجموع الكلى G.Total	Positive Cases الحالات الايجابية								مجموع الفحوصات T. Sample	الولايات States
	اخرى Other	ترسيب E.S.R	سكرى Sugar	بولينا B.Urea	تايفويد Widal	ك البيضاء T.W.B.C	همقلوبين Hb	ملاريا Malaria		
136756	2577	7485	14120	4328	8994	48879	38291	12082	125700	الشمالية Northren
57452	632	3133	3660	37	3496	16884	9512	20098	90622	نهر النيل R.Nile
61572	3955	3092	10295	1595	129	18003	9042	15461	146918	البحر الاحمر Red Sea
15499	6536	8630	13371	1364	5196	73025	48001	59376	366971	القضارف Gadareif
10561	119	59	8	6	222	4203	581	5363	29971	كسلا Kassala
1333465	83802	464777	59157	51696	2374	108726	557053	5880	1530118	الخرطوم Khartoum
618883	16283	50524	73406	26796	26357	191310	175039	59168	1071496	الجزيرة Gaziera
292033	4830	10883	12868	4544	20446	94906	62218	81338	424267	سنار Sinnar
118996	4072	5687	3874	983	3932	26196	24140	50112	166728	النيل الابيض W.Nile
214990	1867	11615	1792	8770	14855	68294	34416	73381	354224	النيل الازرق B.Nile
303740	3564	7101	4614	2003	1734	133112	66623	84989	463941	ش . كردفان N.kordofan
24242	45	1400	913	257	563	4731	4923	11410	17682	ج . كردفان S.Kordofan
50786	10939	2621	874	126	2111	12140	14618	7357	85586	ش. دارفور N.Darfour
40748	1256	2370	438	395	421	11469	5063	19336	51839	غ. دارفور w.Darfour
23403	1500	2009	228	416	710	10370	6725	1445	6725	ج. دارفور S.darfour
3503126	141977	581386	199618	103316	91540	822248	1056245	506796	4932788	السودان Sudan

جدول رقم (11/4) المواليد ونوع الولادات المسجلة داخل وخارج المستشفيات
والمؤسسات الصحية الاخري خلال عام 2012م

Table (4/11) Types of registered Deliveries in and out side Health facilities 2012

المجموع الكلي	ولادة بالجفت Forceps		ولادة قيصرية Cesarean		ولادة طبيعية Normal Delivarly		المجموع	المواليد اموات Still Birth		المواليد أحياء Live Birth		الولايات States	
G. Total	توأم Twin	فرد Single	توأم Twin	فرد Single	توأم Twin	فرد Single	Total	أناث Females	ذكور Males	أناث Females	ذكور Males		
12037		7	94	3515	101	8320	12232	58	73	6057	6044	Northern	الشمالية
28867	3	157	198	5135	320	23054	29388	337	382	13888	14781	R. Nile	نهر النيل
15935		10	33	2051	220	13621	16188	84	107	7398	8599	Red Sea	البحر الأحمر
51420		1	91	2754	335	48239	51846	117	206	24036	27487	Gadarief	القضارف
40484		105		3097		37282	40484	156	174	19122	21032	Kassala	كسلا
154519	542	621	2375	5047	693	145241	158129	671	941	91279	65238	Khartoum	الخرطوم
77425	0	168	328	15792	703	60434	77425	340	506	38283	39327	Gezira	الجزيرة
31486	4	137	105	7069	169	24004	31764	165	281	14628	16690	N. Nile	النيل الابيض
12281	4	2	147	5	942	11181	13374	71	97	6283	6923	B. Nile	النيل الأزرق
24995	0	18	86	4889	224	19778	25305	104	119	11974	13108	Sinnar	سنار
25285	2	101	201	4896	343	19742	25831	237	519	11578	13497	N. Kordofan	ش كردفان
41220	2	111	234	6947	563	33363	42019	321	626	18976	22096	S. Kordofan	ج كردفان
19302	5	12	112	2204	452	16517	19871	277	395	8956	10243	N. Darfour	ش دارفور
1705	0	2	40	563	31	1069	1776	66	75	734	901	W. Darfour	غ دارفور
3524	17	135	41	1060	101	2170	3683	193	288	1558	1644	S.Darfour	ج دارفور
539454	579	1587	4085	65024	5197	462982	550346	3197	4789	274750	267610	SUDAN	السودان

Appendix 2

Questionnaire

Name ……

Sex: ……..

Age: ……..

Address: …….

Past infection: ……

Date: …….

Laboratory investigation made: ……

……..

Antibiotics used: ……..

Date: …….

Recurrent infection: ……..

Date: …….

Surgical treatment: ……

Date: …….

Type of sample:

O Throat swab

O Urine

O Blood

O Vaginal swab

Date of collection: ……..

Appendix 3

Statistical Analysis Tables

Table (14): Frequency of samples according to sex

Sex	Frequency	Percent
male	150	30.0
female	350	70.0
Total	500	100.0

Table (15): Age group of patients

	Age group	Frequency	Percent	Valid Percent	Cumulative Percent
Valid	**1-17**	174	34.8	34.8	34.8
	18 -50	282	56.4	56.4	91.2
	50 and more	44	8.8	8.8	100.0
	Total	500	100.0	100.0	

Table (16): Patients have known past infection before sample collection

	Frequency	Percent	Valid percent	Cumulative percent
Valid yes	242	48.4	48.4	48.4
no	258	51.6	51.6	100.0
Total	500	100.0	100.0	

Table (17): Patients for whom laboratory investigation made during their past infection

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid yes	81	16.2	16.2	16.2
no	419	83.8	83.8	100.0
Total	500	100.0	100.0	

Table (18): Use of antibiotic during past infection

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid yes	10	2.0	2.0	2.0
no	490	98.0	98.0	100.0
Total	500	100.0	100.0	

Table (19): Patients who had recurrent infection

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid yes	223	44.6	44.6	44.6
no	277	55.4	55.4	100.0
Total	500	100.0	100.0	

Table (20): Patients who had surgical treatment

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid yes	110	22.0	22.0	22.0
no	390	78.0	78.0	100.0
Total	500	100.0	100.0	

Table (21): Type of sample collected

	Frequency	Percent	Valid percent	Cumulative percent
Valid throat swab	150	30.0	30.0	30.0
urine	125	25.0	25.0	55.0
blood	100	20.0	20.0	75.0
vaginal swab	125	25	25.0	100.0
Total	500	100.0	100.0	

Table (22): Frequency of total positive result

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid **+ve**	132	26.4	26.4	26.4
-ve	368	73.6	73.6	100.0
Total	500	100.0	100.0	

Table (23): Number of positive results per samples

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	**throat swab**	115	87.1	87.1	87.1
	urine	8	6.1	6.1	93.2
	blood	5	3.8	3.8	97.0
	vaginal swab	4	3.0	3.0	100.0
	Total	132	100.0	100.0	

Table (24): Total of positive result according to sex

Sex		Frequency	Percent	Valid Percent	Cumulative Percent
male	**Valid +ve**	59	100.0	100.0	100.0
female	**Valid +ve**	73	100.0	100.0	100.0

Table (25): Total of positive result according to age

Age group		Frequency	Percent	Valid percent	Cumulative percent
1-17	**valid +ve**	83	100.0	100.0	100.0
18–50	**valid +ve**	42	100.0	100.0	100.0
50 and more	**valid +ve**	7	100.0	100.0	100.0

Appendix 4

Preparation of media and reagents

Hydrogen peroxide

This solution was prepared in a concentration of 3% by adding 3ml of hydrogen peroxide to 97 ml distilled water.

Bile salts solution

Bile salts	10 gm
Distilled water	100 ml

Normal saline solution

Sodium chloride	8.5 gm
Distilled water	1000 ml

Types of media

Solid media

Blood agar

Nutrient agar (nutrient broth + 2% agar)	950 ml
Defibrinated blood	50 ml

The pH was adjusted to 7.4. The medium was then sterilized and after cooling to 55 ºC, the blood was added. (5% blood agar).

Bile salt agar

a/ preparation of 10% bailed salt agar

Bile salts	10 gm
Distilled water	100 ml
Agar	2 gm

b/ preparation of 40% bailed salt agar

Bile salts	40 gm
Distilled water	100 ml
Agar	2 gm

Muller Hinton agar

Beef infusion	300 gm
Casein acid hydrolysate	17.5 gm
Starch	1.5 gm
Agar	17 gm
Distilled water	1000 ml

Dissolve by heating.

Liquid media

Nutrient broth

Nutrient broth powder	13 gm
Distilled water	1000 ml

Aesculin broth

Aesculin powder	1 gm
Ferric citrate gm	
Peptone water	15 gm
Distilled water	1000 ml

Arginine

Arginine powder	5 gm
Peptone	5 gm
Yeast extract	5 gm
Dipotassium hydrogen phosphate	2 gm

Glucose	0.5 gm
Distilled water	1000 ml

Nessler's reagent

Potassium iodide	5 gm
Freshly-prepared distilled water	5 ml
Cold saturated mercuric chloride solution	Added until a slight precipitate remains permanently after thorough shaking.
9-N sodium hydroxide	40 ml
Distilled water	complete to 100 ml

Allow to stand for 24 hrs.

Hippurate broth

Minced meat	540 gm
Water	1000 ml
Peptone	10 gm
Sodium chloride	05 gm
Sodium hippurate	10 gm

Allow the meat to infuse in the water overnight at 4 ºC. Skim the fat from the infused mixture, add the peptone and sodium chloride, boil for 30 minutes, filter, adjust the pH to 8.4 and boil for 20 minutes filter, adjust the pH to 7.4 and add 10 gm of sodium hippurate.

Gram stain solutions

Crystal violet

Crystal violet	20 gm
Ammonium oxalate	9 gm
Absolute ethanol	95 ml
Distilled water	to 1 liter

Filter before use

Lugal's Iodine

Iodine	5 gm
Potassium iodide	10 gm
Dist. Water	100 ml

Carbol fuchsin

Phenol	85 gm
Basic fuchsin	15 gm
Ethanol (95%)	250 ml
Distilled water	1250 ml

Mix the phenol and fuchsin, if necessary heat gently

Add the ethanol and distilled water and filter

Working solution

Dilute one volume of strong carbol fuchsin in 10 – 20 volumes of distilled water.

Appendix 5

Lancefield kit

Key Code TSMX7829
www.oxoid.com/ifu

Europe + 800 135 79 135 US 1 855 236 0910

CA 1 855 805 8539 ROW +31 20 794 7071

Streptex* EN

INTENDED USE

Streptex* is a rapid latex test system for use in the qualitative detection and identification of the Lancefield group of streptococci. Reagents are provided for groups A, B, C, D, F and G covering the majority of clinical isolates[6], group E streptococci are rarely isolated.

SUMMARY AND EXPLANATION OF THE TEST

The majority of species of Streptococcus possess group-specific antigens which are usually carbohydrate structural components of the cell wall. Lancefield showed that these antigens can be extracted in soluble form and identified by precipitation reactions with homologous antisera[12]. Several procedures for extracting the antigens have been described[3,4,10,13,17,18]. In the Streptex* system a simple enzyme extraction is employed and a more rapid acid extraction system is available (Streptex* Acid Extraction Kit, ZL59/R30951301).

The main use of the test is in identification of streptococci growing on agar plates, but satisfactory results have been reported with one hour extraction from pure broth cultures[8].

PRINCIPLE OF THE PROCEDURE

Group specific antigens are extracted from streptococci in a simple incubation step. Antigens are then identified using polystyrene latex particles which have been coated with group-specific antibodies. These latex particles agglutinate strongly in the presence of homologous antigen, and remain in smooth suspension in the absence of homologous antigen.

REAGENTS

KIT CONTENTS

Streptex*	50 tests (ZL50/R30950501)	200 tests (ZL61/R30164701)
1. Group A Latex (ZL51/R30950601)	1 dropper bottle (light blue cap)	4 dropper bottles (light blue caps)
2. Group B Latex (ZL52/R30950701)	1 dropper bottle (pink cap)	4 dropper bottles (pink caps)
3. Group C Latex (ZL53/R30950801)	1 dropper bottle (brown cap)	4 dropper bottles (brown caps)
4. Group D Latex (ZL54/R30950901)	1 dropper bottle (dark blue cap)	4 dropper bottles (dark blue caps)
5. Group F Latex (ZL56/R30951101)	1 dropper bottle (grey cap)	4 dropper bottles (grey caps)
6. Group G Latex (ZL57/R30951201)	1 dropper bottle (yellow cap)	4 dropper bottles (yellow caps)
7. Polyvalent Positive Control (ZL58/R30164601)	1 dropper bottle (red cap)	2 dropper bottles (red caps)
8. Extraction Enzyme (ZL55/R30951001)	2 bottles	8 bottles

should be stored at 2 to 8°C, when it will retain activity for at least three months after reconstitution, or until the date shown on the bottle label, whichever is the sooner. Alternatively the Enzyme may be stored in aliquots frozen at −15 to −25°C, when it will retain activity for at least six months, or until the date shown on the original bottle label, whichever is the sooner. DO NOT FREEZE AND THAW MORE THAN ONCE

CONTROL +

Polyvalent Positive Control†

One (ZL50/R30950501) or two (ZL61/R30164701) plastic dropper bottle(s) with a red cap containing a polyvalent extract of antigens from a representative strain of each streptococcal group A, B, C, D, F and G. The solution contains phosphate buffer pH 7.4 and 0.1% sodium azide as preservative.

The Polyvalent Positive Control should be stored at 2 to 8°C where it will retain activity at least until the date shown on the bottle label.

WARNINGS AND PRECAUTIONS

IVD

The reagents are for *in vitro* diagnostic use only.

For professional use only.

Please refer to the manufacturer's safety data sheet and the product labelling for information on potentially hazardous components.

HEALTH AND SAFETY INFORMATION

1. In accordance with the principles of Good Laboratory Practice it is strongly recommended that extracts at any stage of testing should be treated as potentially infectious and handled with all necessary precautions.
2. Non-disposable apparatus should be sterilised by any appropriate procedure after use, although the preferred method is to autoclave for 15 minutes at 121°C; disposables should be autoclaved or incinerated. Spillage of potentially infectious materials should be removed immediately with absorbent paper tissue and the contaminated area swabbed with a standard bacterial disinfectant or 70% alcohol. Do NOT use sodium hypochlorite. Materials used to clean spills, including gloves, should be disposed of as biohazardous waste.
3. Do not pipette by mouth. Wear disposable gloves and eye protection while handling specimens and performing the assay. Wash hands thoroughly when finished.
4. The freeze-dried Extraction Enzyme contains calcium chloride which is an irritant (Xi). Avoid contact with skin and eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
5. The Latex Suspensions and Polyvalent Positive Control contain 0.1% sodium azide which is classified per applicable European Economic Community (EEC) Directives

The reconstituted Extraction Enzyme should be stored at 2 to 8°C, when it will retain activity for at least three months after reconstitution, or until the date shown on the bottle label, whichever is the sooner. Alternatively the Enzyme may be stored in aliquots frozen at −15 to −25°C, when it will retain activity for at least six months, or until the date shown on the original bottle label, whichever is the sooner. DO NOT FREEZE AND THAW MORE THAN ONCE

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5. The Latex Suspensions and Polyvalent Positive Control contain 0.1% sodium azide which is classified per applicable European Economic Community (EEC) Directives as harmful (Xn). The following is the appropriate Risk (R) phrase.

Xn R22 Harmful if swallowed

Azides can react with copper and lead used in some plumbing systems to form explosive salts. The quantities used in this kit are small; nevertheless when disposing of azide-containing materials they should be flushed away with large volumes of water.

6. When used in accordance with the principles of Good Laboratory Practice, good standards of occupational hygiene and the instructions stated in these Instructions for Use, the reagents supplied are not considered to present a hazard to health.

ANALYTICAL PRECAUTIONS

1. Do not use the reagents beyond the stated expiry date.
2. Microbiological contamination of reagents must be avoided as this may reduce the life of the product and cause erroneous results.
3. Allow all reagents and samples to come to room temperature (18 to 30°C) before use. Immediately after use return reagents to the recommended storage temperature. Latex reagents which show signs of aggregation when dispensed for the first time may have been frozen and should not be used.
4. If the Extraction Enzyme solution becomes contaminated, as indicated by increasing turbidity during storage, it should be discarded.
5. It is important to hold the dropper bottles vertically and that the drop forms at the tip of the nozzle. If the nozzle becomes wet, a drop of incorrect volume will form around the end and not at the tip; if this occurs dry the nozzle before progressing.
6. Do not touch the reaction areas on the cards.

SPECIMEN COLLECTION AND PREPARATION OF CULTURES

For details of specimen collection and preparation of primary cultures a standard textbook should be consulted[6]. The media used normally include blood agar and in such case the haemolytic reaction of suspected streptococcal colonies must be noted prior to attempts at grouping. Streptococci growing in mixed culture on solid primary isolation media may be reliably grouped directly if they are not overgrown by organisms such as Klebsiella, Escherichia or Pseudomonas which may non-specifically agglutinate all the latex reagents. Streptex* grouping should not be attempted on primary cultures in liquid media. When grouping from primary cultures or impure subcultures which appear to contain streptococci (if a clear result is not obtained) it is recommended that pure subcultures of suspect colonies should be made for subsequent identification by Streptex*.

Organisms of groups A, B, C, F or G are normally beta-haemolytic. If an alpha- or non-haemolytic organism appears to belong to one of these groups the species identification should be confirmed by biochemical tests[7,16]. Since enterococci are relatively resistant to penicillin, differentiation of group D organisms into enterococcal *(Enterococcus spp.)* and non-enterococcal (group D streptococci) types should be carried out by a L-pyrrolidonyl-ß-naphthylamide (PYR) hydrolysis test (Remel, Code No. LP02/R30854301 and LP03/r30854401) or by culture in bile esculin and 6.5% NaCl broth[6] (Figure 3). Antigen production by group D streptococci is greatly improved by addition of 0.5 to 1% glucose to the medium[14], but with blood agar the haemolytic reaction will be obscured.

PROCEDURE

MATERIALS PROVIDED

Streptex* contains sufficient material for 50 tests (ZL50/R30950501) or 200 tests (ZL61/R30164701), see **Kit Contents**.

MATERIALS REQUIRED BUT NOT PROVIDED

- Pipette to measure and dispense 0.4 ml volumes.
- Bacteriological loop.
- Pipettes which deliver a drop volume of 40 µl.
- Water bath at 37°C.
- Glass or plastic test tubes 8 to 12 mm internal diameter, one per organism to be grouped.

TEST PROCEDURE

CAUTION: Precautions appropriate to the handling of live cultures should be taken while performing the tests.

A suggested outline scheme for grouping organisms from primary plates or subculture is shown in Figure 3.

Step 1 Dispense 400 µl Extraction Enzyme into an appropriately labelled test tube for each culture to be grouped.

Step 2 Using a bacteriological loop, make a light suspension of the culture in a tube of the enzyme solution. A single sweep of growth should be sufficient: it is frequently possible to obtain a result by picking as few as 5 large colonies to emulsify in the enzyme, if they adhere adequately to the loop. If the culture is not pure, it is recommended that streptococcal colonies should be picked from an area which contains as few contaminants as possible.

Step 3 Incubate the suspension at 37°C in a water bath (or in a beaker of water equilibrated to 37°C in an incubator) for a minimum of 10 minutes or any time up to 1 hour. Shake the tube after 5 minutes incubation.

Step 4 Resuspend each of the latex suspensions by shaking vigorously for a few seconds. Hold the dropper bottle vertically and dispense one drop (20 µl) of each latex suspension onto a separate circle on a Reaction Card.

NOTE: It is important when using dropper bottles that they are held vertically and that the drop forms at the tip of the nozzle. If the nozzle becomes wet an incorrect volume will form around the end and not at the tip; if this occurs dry the nozzle before progressing.

Step 5 Using a pipette, place one drop (40 µl) of extract in each of the six circles on the reaction card.

Step 6 Mix the contents in each circle in turn with a mixing stick, and spread to cover the complete area of the circle. Use a separate stick for each circle and discard it for safe disposal after use.

Step 7 Rock the card gently for a maximum of one minute. The card should be held at normal reading distance (25 to 35 cm) from the eyes. Do not use a magnifying lens. The patterns obtained are clear cut and can be recognised easily under all normal lighting conditions.

Step 8 Discard the used Reaction Card for safe disposal.

Step 9 Ensure that the reagents are returned to the refrigerator (2 to 8°C), using the storage rack provided.

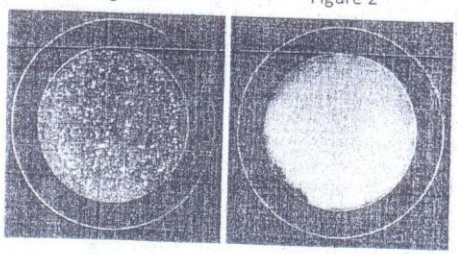
RESULTS

READING OF RESULTS

A positive result is indicated by the development of an agglutinated pattern showing clearly visible clumping of the latex particles (Figure 1).

The speed of appearance and quality of agglutination depends on the strength of the antigen extract; with a strong extract large clumps of latex particles will appear within a few second of mixing, but with a weak extract the reaction will take much longer to appear and the clumps of latex particles will be small.

Figure 1 Figure 2



In a negative result the latex does not agglutinate and the milky appearance remains substantially unchanged throughout the one-minute test (Figure 2). Note, however, that faint traces of granularity may be detected in negative patterns, depending on the visual acuity of the operator.

QUALITY CONTROL

Quality control testing should be run with each shipment and new kit lot number received. Each laboratory should follow their state and local requirements.

In normal use the performance of the test is assured by the presence of obvious agglutination in one latex suspension only, the other five suspensions showing no agglutination. This pattern of reaction may be regarded as sufficient on most occasions to demonstrate the specificity of the reagents and the efficiency of the enzymatic extraction procedure. When there is a different pattern of reaction, the following procedures are recommended:

a) **Test of the reactivity of the latex suspensions (Positive Control Procedure)**

Dispense one drop (40 µl) of Polyvalent Positive Control either in place of the test sample or in addition to it after no reaction has taken place in one minute. Mix the contents of each circle with a fresh mixing stick covering the area of the circle. After rocking the card gently for one minute, definite agglutination should occur with all the test latexes.

†Additional Polyvalent Positive Control is available (ZL58/R30164601).

b) **Test for specificity of agglutination (Negative Control Procedure)**

To ensure that agglutination of a latex suspension is specific, particularly in cases of very weak agglutination or where more than one suspension is agglutinated by a single extract, repeat the positive test (or tests) simultaneously with parallel test(s) using one drop of Extraction Enzyme instead of bacterial extract.

The latex suspension should not show significant agglutination in the presence of Extraction Enzyme alone and the result serves as a control for direct comparison with the pattern obtained in the presence of the bacterial extract.

c) **Test of enzyme extraction procedure**

Carry out the complete test procedure on a stock culture of known group. Occasional tests with a variety of known groups should be employed to evaluate the accuracy and efficiency of the complete test system, including the operator.

INTERPRETATION OF RESULTS

As a general rule only beta-haemolytic streptococci provide reliable results in grouping procedures[6,8]. There are exceptions to this rule since the majority of strains of group D streptococci are either alpha-haemolytic or non-haemolytic and some strains of group B are non-haemolytic. Organisms of group D should be further classified as Enterococcus or group D streptococcus by culture in bile-esculin and 6.5% NaCl broths or PYR test[6] (Remel, Code No. LP02/R30854301 and LP03/R30854401); those reacting with groups A, C, F or G may, if necessary, be identified by appropriate biochemical procedures[16].

Strong rapid agglutination in only one of the six latex suspensions indicates the identity of the strain under test and delayed, weak reactions with the same extract should be ignored. Similar strength of agglutination of more than one latex suspension (but not all) indicates that the extract may contain a mixture of streptococcal groups or other bacteria containing cross-reacting antigens and further isolation procedures and/or biochemical tests should be performed. Some strains of group D streptococci have been found which appear also to possess group G antigen[1,11]. These strains will react with both group D and group G latex reagents and may be confirmed as group D if desired by the bile-esculin test[6]. For epidemiological reasons and because some of these strains possess an unusually high level of antibiotic resistance[1], it is important that they should be identified correctly.

A delayed, weak reaction in a single latex suspension usually indicates the identity of the strain under test and if possible the test should be repeated using a heavier cell suspension. When agglutination is so weak as to give rise to doubt in interpretation the test for specificity described in **Quality Control Procedures** (b) should be carried out: comparison to the two patterns will indicate the correct result.

Agglutination of all the latex reagents, which characteristically has a stringy or thread-like appearance, indicates either (a) over-inoculation of the Extraction Enzyme, in which case extraction may be repeated using a lighter suspension, or (b) contamination with an interfering organism (see **Limitations of the Procedure**) which should be eliminated by further subculture. False agglutination due to either of these causes can usually be eliminated by heating the extract in boiling water for three minutes. If none of the latex suspensions show agglutination it is likely that the culture does not belong to any of the groups covered in the test. Negative results may also be due to the use of too few organisms for extraction, particularly with group D strains – some of which yield less antigen than other groups and group F strains which have minute colonies – some of which adhere strongly to the agar surface. If a culturally-identified streptococcus does not give definite agglutination with any of the latex suspensions, it may be desirable to repeat the extraction with a larger amount of culture.

LIMITATIONS OF THE PROCEDURE

False negative results can occur if an inadequate amount of culture is used for extraction (see section **Interpretation of Results**). Some strains of *Streptococcus bovis* and *Enterococcus faecium* (group D) may not be grouped easily.

Occasional false positive results may occur with organisms from unrelated genera, for example, Klebsiella, Escherichia or Pseudomonas which may non-specifically agglutinate all latex reagents. However by examination of cultural characteristics on growth media the operator can usually eliminate these from testing.

The existence of antigens common to organisms from heterologous species or genera has been demonstrated in some streptococci[2,5,15], and consequently the possibility of cross reactions of this type occurring in streptococcal grouping systems cannot be eliminated. The group D antigen is common to organisms of streptococcal groups Q, R and S[5,15].

Enterococci are relatively resistant to penicillin, but serological procedures do not differentiate between them and group D streptococci. Biochemical tests can be used for this purpose, such as PYR hydrolysis (Remel, Code No. LP02/R30854301 and LP03/R30854401) or growth in broth containing 6.5% NaCl. For details of the biochemical differentiation of streptococci a standard text book should be consulted[6].

EXPECTED RESULT

Extracts of streptococci belonging to serogroups A, B, C, D, F or G will give strong rapid agglutination with the corresponding latex suspension.

SPECIFIC PERFORMANCE CHARACTERISTICS[19]

Clinical studies were carried out in four centres in Great Britain and two in Canada on a total of 743 streptococcal cultures (663 beta-haemolytic and 80 alpha- or non-haemolytic). 290 primary cultures, 451 subcultures and 2 broth cultures were tested. The results obtained by Streptex* following both 10 minute and 60 minute extractions were compared with those found using an established reference method.

Results from 703 streptococcal cultures of groups A, B, C, D, F and G (638 beta- and 65 alpha- or non-haemolytic) are shown in Tables 1 and 2. Streptex* correctly identified 698 cultures (99%) after 10 minutes extraction and all 703 after 60 minutes. 4 beta-haemolytic cultures (1 group B, 1 group D, 1 group F and 1 group G) were missed by Streptex* after 10 minutes but correctly identified after 60 minutes extraction. One beta-haemolytic culture was grouped after 10 minutes as G but after 60 minutes extraction as B. The culture, heavily contaminated with corynebacterium spp., was not available for further study. The corynebacterium spp. from this culture did not react with Streptex*.

An additional 13 beta- and 3 alpha- or non-haemolytic cultures gave positive reactions with more than one streptococcal group with either the reference, Streptex* or both methods. These were presumed to be mixed cultures but were not available for confirmation.

Twenty four streptococcal cultures which were not grouped as A, B, C, D, F or G using the reference method did not react with Streptex*.

Table 1

Culture Identification (10 Minute Extraction)

	Streptex* Result						
Reaction	A	B	C	D	F	G	No
Established Method A	149						
B		214[a]				1	1
C			64				
D				120[b]			1
F					14		1
G						137	1

[a] = 197 beta- + 17 alpha- or non-haemolytic streptococcus
[b] = 72 beta- + 48 alpha- or non-haemolytic streptococcus

Table 2

Culture Identification (60 Minute Extraction)

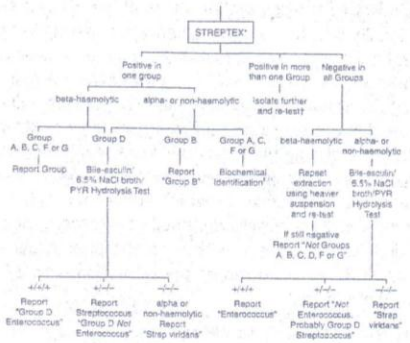
	Streptex* Result					
	A	B	C	D	F	G
Established Method A	149					
B		216[a]				
C			64			
D				121[b]		
F					15	
G						138

[a] = 199 beta- + 17 alpha- or non-haemolytic streptococcus
[b] = 73 beta- + 48 alpha- or non-haemolytic streptococcus

Figure 3

Suggested Scheme for Grouping Streptococci*[2,9]

Inspect streptococcal culture for type of haemolysis and cultural characteristics.
(If alpha-haemolytic, rule out *Streptococcus pneumoniae*).
Subculture if suspected organism is scanty or overgrown.



†Rare strains have been encountered which appear to possess more than one group of antigen. After confirming the proper operation of the reagents (see **Quality Control Procedures**), problem strains should be submitted to a Reference Laboratory for identification.

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[19] **Data on file**

PACKAGING

REF ZL50/R30950501.....50 tests
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IVD	*In vitro* diagnostic medical device
	Consult instruction for use (IFU)
	Temperature limitation (Storage Temp.)
LOT	Batch code (Lot Number)
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Appendix 6

Preparation of TBE (250 ml)

Tris base	2.695 gm
Boric acid	1.376 gm
EDTA	0.186 gm
pH	8.3

Running buffer

TBE	25 ml
Distilled water	225 ml

Appendix 7

Figures

Figure (12): Aesculin

Left = negative Right = positive

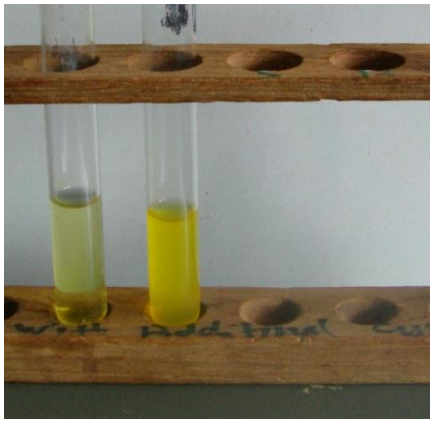

Figure (13): Arginine

Left = negative Right positive

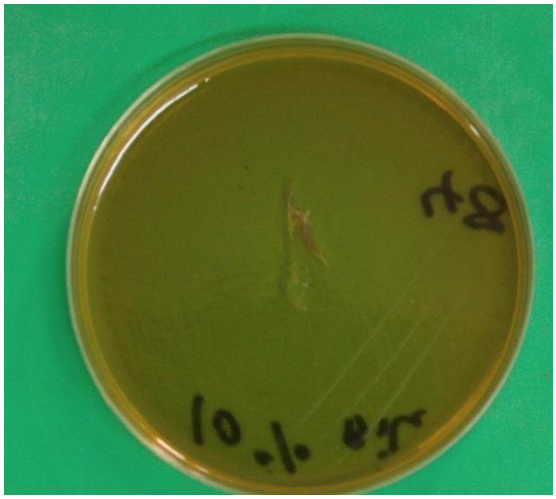

Figure (14 a): growth on 10% bile

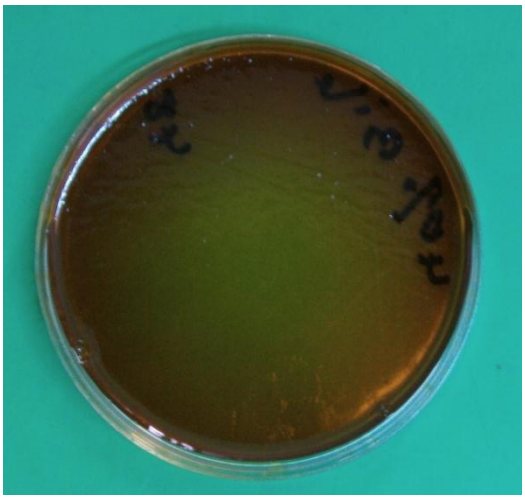

Figure (14 b): growth on 40% bile

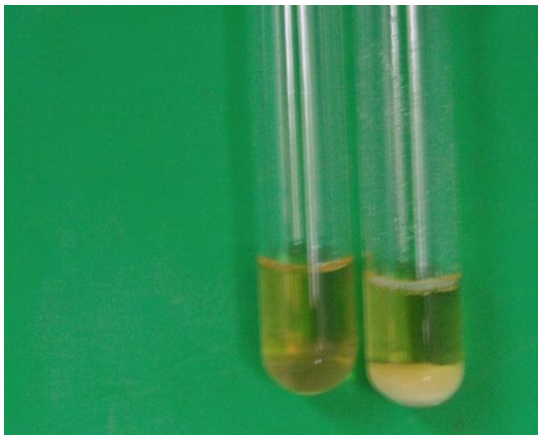

Figure (15): Hippurate hydrolysis
Left = negative Right = positive

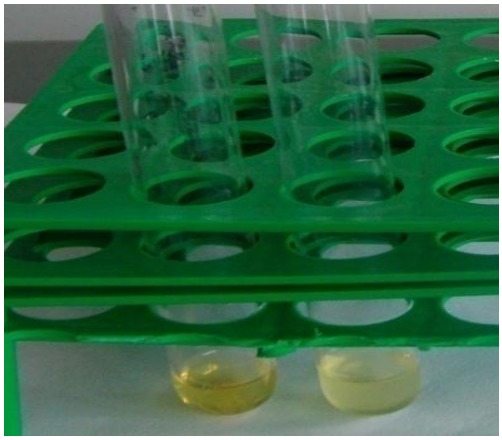

Figure (16): Bile solubility
Left = positive Right = negative

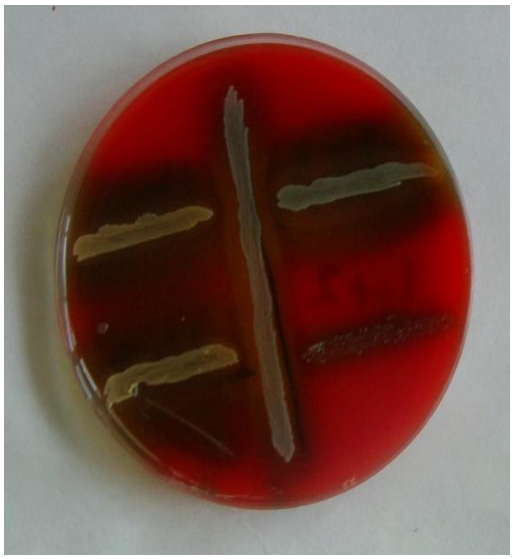

Figure (17 a) CAMP negative

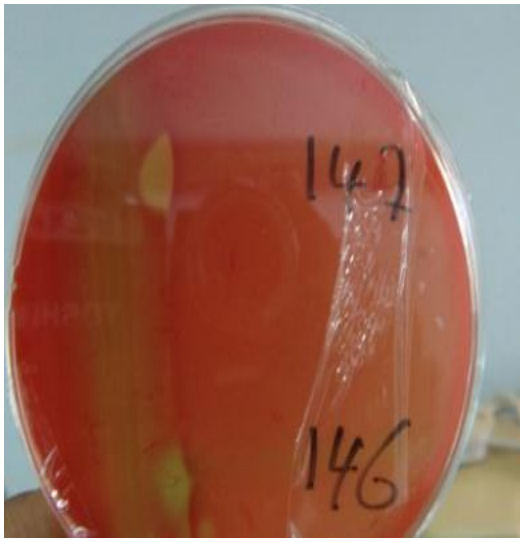

Figure (17 b): CAMP positive

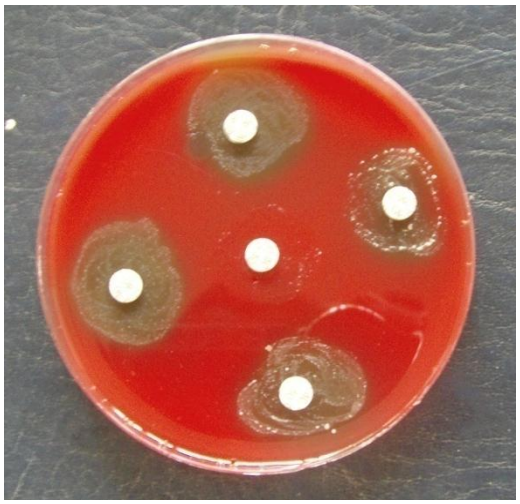

Figure (18): Bacitracin sensitivity

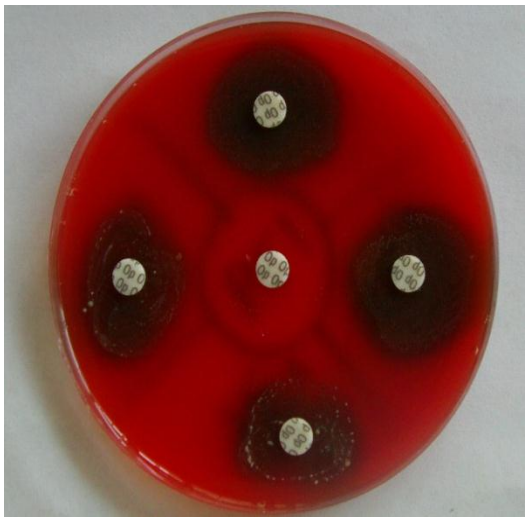

Figure (19): Optochin sensitivity

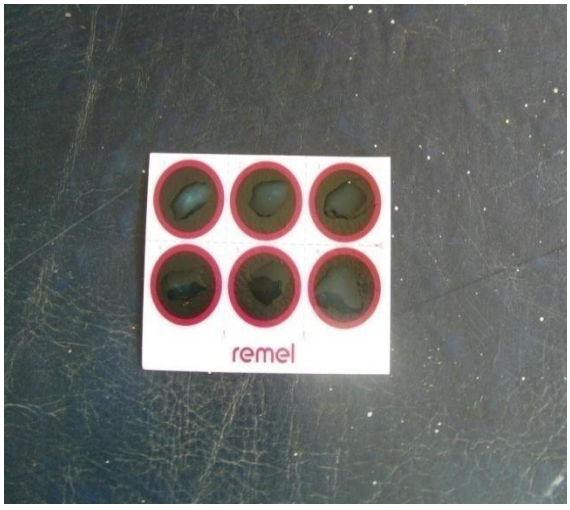

Figure (20): Lancefield agglutination

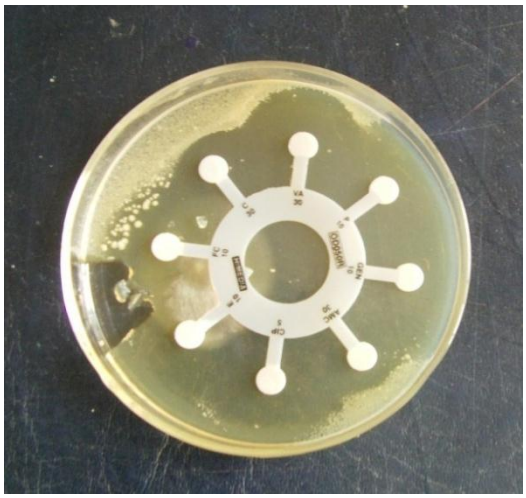

Figure (21): antibiotic sensitivity