UNIVERSITI PUTRA MALAYSIA

MULTILOCUS SEQUENCE TYPING (MLST) AND PHYLOGENETIC ANALYSIS OF GROUP B STREPTOCOCCUS ISOLATES FROM DIFFERENT MALAYSIAN HOSPITAL

NUR AIMI LIYANA BINTI ABD AZIZ

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ABSTRACT

Multilocus Sequence Typing (MLST) and Phylogenetic Analysis of Group B Streptococcus Isolates from Different Malaysian Hospitals

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Introduction: Group B streptococcus (GBS) or also known as *Streptococcus agalactiae* is a normal flora that can be found in gastrointestinal and genitourinary tracts of healthy adults. However, invasive disease attributable to infection with GBS results in increasing incidence with a large variety of clinical diseases among newborn children, pregnant women and healthy adults. In Malaysia, literature research on GBS infections in human identified a low number of articles with regard to its serotypes distribution, sequence types (STs) and phylogenetic analysis. **Objective:** Therefore, this study aims to determine the serotypes distribution, sequence types (STs) and phylogenetic tree of GBS isolated from different hospitals origin in Malaysia in relation to the infected subjects. **Methodology:** A total of 40 GBS isolates were collected from 5 hospitals in Malaysia. The isolates were characterized on its strains by capsular serotyping using multiplex PCR, while the locus of seven housekeeping genes are characterized by using monoplex PCR. In addition, GBS isolates were analysed by multilocus sequence typing (MLST) to find the sequence type (ST) prior constructing the phylogenetic tree using Mega software to analyse the genetic relationship. **Results:** The most common serotype was III (n = 11), followed by VI (n = 7), Ia, Ib, II, V (n = 4 each), VII (n = 3), IV (n = 2), and IX (n = 1). The seven housekeeping genes were effectively amplified in 38 GBS isolates, while only six were identified in the other two isolates. MLST analysis revealed 15 different allelic profiles and STs. On the list of 15 STs, ST1 was the most common (n = 13), followed by ST17 (n = 5) and ST283 (n = 2). The sequences of seven housekeeping genes of each isolates were aligned and phylogenetically compared to 15 MLST reference sequences. There are two big branches with Clade I and II that mainly composed of the most isolates which are 25 and 6 respectively in the first branch. On the second branch that consisting of Clade III and IV, each of the clade has 2 and 5 isolates respectively. They were classified based on their resemblance to the isolates studied. **Discussion:** There is a variation in the serotypes distribution and sequence types (STs) by looking at the patterns in constructed phylogenetic tree of GBS strains that isolates from different Malaysian hospitals. The genotypic distribution patterns and MLST analysis on GBS in this research were mostly consistent with earlier findings from other countries. **Conclusion**: This implies that some specific genotypes tend to predominate in human infection settings. Therefore, for epidemiological research and vaccine development in Malaysia, further monitoring of GBS from different geographical regions is necessary.

Keywords: Group B streptococcus, *Streptococcus agalactiae,* serotyping, multilocus sequence typing, phylogenetic tree

ABSTRAK

Menaip Urutan Multilokus (MLST) dan Analisis Filogenetik Kumpulan B Streptokokus dari Pelbagai Hospital di Malaysia

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Pengenalan: Kumpulan B Streptokokus (GBS) atau dikenali sebagai *Streptococcus agalactiae* adalah flora normal yang dapat dijumpai di saluran gastrointestinal dan genitouriner orang dewasa yang sihat. Walaubagaimanapun, penyakit invasif yang disebabkan oleh jangkitan GBS memperlihatkan peningkatan kejadian dengan pelbagai penyakit klinikal dalam kalangan kanak-kanak yang baru lahir, wanita hamil dan orang dewasa yang sihat. Di Malaysia, penyelidikan literatur mengenai jangkitan GBS pada manusia mengenal pasti jumlah artikel yang rendah berkaitan dengan sebaran serotip, jenis urutan (ST) dan analisis filogenetik. **Objektif**: Oleh itu, kajian ini bertujuan untuk menentukan taburan serotaip, jenis urutan (ST) dan pokok filogenetik GBS dari pelbagai hospital di Malaysia berkaitan subjek yang dijangkiti. **Metodologi:** Sebanyak 40 isolat GBS dikumpulkan dari 5 hospital di Malaysia. Isolat dicirikan pada strainnya dengan serotaip kapsul menggunakan PCR multiplex, sementara lokus tujuh gen pengemasan dicirikan dengan menggunakan PCR monoplex. Di samping itu, isolat GBS dianalisis dengan menaip urutan multilokus (MLST) untuk mencari jenis urutan (ST) sebelum membina pokok filogenetik menggunakan perisian Mega untuk menganalisis hubungan genetik. **Keputusan:** Serotip yang paling banyak adalah III (n = 11), diikuti oleh VI (n = 7), Ia, Ib, II, V (n = 4 masing-masing), VII (n = 3), IV (n = 2), dan IX (n = 1). Tujuh gen pengemasan berkesan diperkuat dalam 38 isolat GBS, sementara hanya enam yang dikenal pasti dalam dua isolat yang lain. Analisis MLST mendedahkan 15 profil alel dan ST yang berbeza. Dalam senarai 15 ST, ST1 adalah yang paling banyak (n = 13), diikuti oleh ST17 (n = 5) dan ST283 (n = 2). Urutan tujuh gen pengemasan setiap isolat diselaraskan dan dibandingkan secara filogenetik dengan 15 urutan rujukan MLST. Terdapat dua cabang besar dengan Klad I dan II yang kebanyakannya terdiri daripada isolat paling banyak masing-masing adalah 25 dan 6 di cabang pertama. Pada cabang kedua yang terdiri daripada Klad III dan IV, masing-masing mempunyai 2 dan 5 isolat. Mereka dikelaskan berdasarkan kemiripannya dengan isolat yang dikaji. **Perbincangan:** Terdapat variasi dalam sebaran dan jenis urutan serotaip (ST) dengan melihat corak pada pohon filogenetik yang dibina dari strain GBS dari pelbagai hospital di Malaysia. Corak taburan genotipik dan analisis MLST pada GBS dalam penyelidikan ini selaras dengan penemuan sebelumnya dari negara lain. **Kesimpulan:** Ini menunjukkan bahawa beberapa genotip tertentu cenderung mendominasi dalam persekitaran jangkitan manusia. Oleh itu, untuk penyelidikan epidemiologi dan

pengembangan vaksin di Malaysia, perlu dilakukan pemantauan lebih lanjut terhadap GBS dari kawasan geografi yang berbeza.

Kata kunci: Streptokokus Kumpulan B, *Streptococcus agalactiae*, serotip, menaip urutan multilokus, pohon filogenetik

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LIST OF ABBREVIATIONS

adhp	alcohol dehydrogenase
ATCC	American Type Culture Collection
atr	amino acid transporter
bp	Base pair
CAMP	Christie–Atkins–Munch–Peterson
cfb	Complement factor b
CO^2	Carbon dioxide
CPS	Capsular polysaccharides
CSF	Cerebrospinal fluid
DFU	Diabetic foot ulcer
DNA	Deoxyribonucleic acid
EOD	Early-onset disease
GAS	Group A Streptococcus
GBS	Group B Streptococcus
glcK	glucose kinase
glnA	glutamine synthetase
H^20^2	Hydrogen peroxide
IgG	Immunoglobulin G
JKEUPM	Ethic Committee for Research Involving Human Subject
LOD	Late-onset disease
MEGA	Molecular Evolutionary Genetics Analysis
MLST	Multilocus sequence typing
NCTC	National Collection of Type Culture
NMRR	National Medical Research Register
PCR	Polymerase chain reaction
PDR	People's Democratic Republic
pheS	phenylalanyl tRNA synthase
RBC	Red blood cell
rpm	Revolution per minute
sdhA	Serine dehydratase
ST	Sequence type
SPSS	Statistical Package for the Social Sciences
S. aureus	*Staphylococcus aureus*
TB	Tuberculosis
TBE	Tris-borate-EDTA
tkt	Transketolase
UKM	Universiti Kebangsaan Malaysia
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

1.1 Background

Group B Streptococcus (GBS) which is also known as *Streptococcus agalactiae* is a type of gram-positive streptococcal bacteria. It has become a significant source of disease in newborn children, pregnant women and non-pregnant adults with underlying medical conditions. According to Nicola et al. (2003), GBS is a leading source of neonatal sepsis. In the elderly, it is considered as an emerging pathogen (Henning et al., 2001) and is a common cause of maternal sepsis. Though GBS is usually a commensal organism, it can be separated by up to 35% of healthy adults from genitourinary and gastrointestinal tracts (Bliss et al., 2002).

Capsular serotyping is an important epidemiological instrument in researching GBS. There are ten capsular serotypes that have been described. The ten distinct capsular polysaccharide antigens dependent GBS serotypes are namely Ia, Ib, II, III, IV, V, VI, VII, VIII and IX, respectively (Jannati E et al., 2012). Different GBS serotypes are correlated with strains immune to antibiotics, neonatal diseases or the presence of specific organs (Ferrieri et al., 2013; Alhhazmi et al., 2016). That answers the question why specific antibodies needed to protect the organism from being infected with GBS. Dele et al., (2004) also mentioned in their study, the antibodies to the GBS serum type-specific capsular polysaccharides (CPSs) of animals used in experiments and human neonates correlate with GBS disease protection. Then, the serotypes will be further grouped into distinct sequence types (STs) using multilocus sequence typing (MLST).

In molecular biology, multilocus sequence typing (MLST) is a procedure for the typing of multiple loci. It is a genotypic analysis that sequences up to 500-bp fragments of the seven housekeeping genes which are *adhP* (alcohol dehydrogenase), *pheS* (phenylalanyl tRNA synthase), *atr* (amino acid transporter), *glnA* (glutamine synthetase), *sdhA* (serine dehydratase), *glcK* (glucose kinase) and *tkt* (transketolase) to provide the genetic history information of a bacterial population (Ezhumalai et al., 2020; Tsai et al., 2019). The MLST database will provide the information on allelic profile and sequence types (STs). Therefore, the phylogenetic tree of the GBS isolates can be formed.

A phylogenetic tree of GBS isolates is a diagram that reflects the GBS evolutionary relationship and the diversity of each ST can be compared. According to Mogens, Christian and Hans (2012), a phylogenetic analysis of concatenated complete sequences of housekeeping genes with bp total number revealed a tree composed of different clades of many strains. One of the software in constructing the phylogenetic tree is using MEGA software. The existence of such clusters is also indicated by the trees built for each gene, a finding confirmed further by MEGA research (Nurul Huda et al., 2008).

1.2 Problem Statement

The pathogenicity of ten distinct Group B Streptococcus (GBS) serotypes depends on the capsular polysaccharide antigen. It also varies according to certain aspects. The global spread of serotyping of a given GBS serotype varies by geographic location, ethnic variation and research conductance period (Jannati et al., 2012). The concern in this area is in determining the serotype distribution among the GBS isolates from different Malaysian hospitals. According to Raabe and Shane (2019), invasive disease caused by GBS results in increasing incidence and a large variety of clinical diseases among newborn children, pregnant women and healthy adults. For instance, infections such as meningitis and septicemia in neonates and pregnant women.

Besides, in Malaysia, literature searches on GBS infections in humans have identified a low number of articles since the 1980s. An old previous study in Malaysia reported that the genital carriage rate of GBS in parturient woman was 9.7% and the average annual incidence of neonatal GBS septicemia in babies born in hospital was 0.4/1000 live births (Eskandarian et al., 2013). Current status is not known due to the lack of studies.

1.3 Justification of Study

Since GBS is known to be the major source of invasive diseases, it would be beneficial to look at genotypic background of the GBS isolates by conducting Multilocus Sequence Typing (MLST) and phylogenetic analysis to understand the dissemination pattern of the isolates in relation to the diseased subjects.

1.4 Objectives

1.4.1 General Objective

In general, this study is to determine the serotype distribution, sequence types and phylogenetic tree of invasive Group B Streptococcus (GBS) isolated from different hospitals originating in Malaysia in relation to the infected subjects.

1.4.2 Specific Objective

This study is specifically aimed:

1. To determine the serotype distribution of GBS isolates.
2. To determine the sequence types (STs) of GBS isolates using multilocus sequence typing (MLST).
3. To determine the MLST phylogenetic tree of GBS isolates.

1.5 Hypothesis

There is a variation in the serotype distribution and sequence types (STs) by looking at the patterns in constructed phylogenetic trees of invasive GBS strains that isolates from different Malaysian hospitals.

CHAPTER 2

LITERATURE REVIEW

2.1 *Streptococcus agalactiae*

2.1.1 Characteristics

Group B Streptococcus GBS, also known as *Streptococcus agalactiae*, is a gram-positive streptococcal bacterium in pairs with a proclivity for forming short chains (Nuccitelli, Rinaudo & Maione, 2015) on gram stain. Moreover, it is characterized as a facultative anaerobe that is beta-haemolytic and catalase-negative.

Being a facultative anaerobe, GBS is nearly impossible to grow in the presence of oxygen, but can alter the mechanism. According to Hentges (1996), the most adaptable creatures are facultative organisms for they prefer to take oxygen as a terminal electron acceptor, but they may also metabolise without it by reducing other chemicals. This adaptation of GBS can be toxic and pathogenic. As emphasised by Johri et al. (2003), only short intervals of low oxygen levels were required to reverse the comparatively poor invasiveness detected when GBS was grown in the absence of oxygen. This indicates that GBS will respond quickly to accessible oxygen.

In addition, GBS is known to be a beta hemolytic streptococci. According to Whidbey et al. (2013), the ornithine rhamnolipid pigment in GBS is responsible for its hemolytic and cytolytic action. GBS colonies appeared grey to whitish-grey in colour and surrounded by a faint zone of beta hemolysis in the Columbia blood agar that contained sheep blood 5%v/v. This is because GBS produces a toxin that induces full haemoglobin lysis in RBCs (Six et al., 2016; Hanna & Noor, 2021). Apart from that, GBS is characterized by not producing catalase. This can be observed by doing a catalase test in which no bubbles form. Catalase is an enzyme that converts hydrogen peroxide, a toxic chemical, into water and oxygen bubbles (Nandi et al., 2019).

2.1.2 Infection

Due to the characteristics exhibited by GBS, it has become a major cause of infection in non-pregnant individuals, pregnant women and newborns with underlying medical conditions. As stated by Henning et al. (2001), GBS has become an emerging pathogen in the elderly. Moreover, in pregnant women, GBS is considered as a common cause of maternal sepsis (Kalin et al., 2015) which then developed to be the leading source of neonatal sepsis in newborns (Shah & Padbury, 2014). Although GBS is actually a normal flora that lives in vagina as well as gastrointestinal and genitourinary tract in healthy adults as a commensal organism which commonly exhibit asymptomatic colonization. However, it may transform into an invasive pathogen. This will cause several clinical manifestations in adult humans. As mentioned by Rajagopal (2009), adult GBS infection may cause a variety of symptoms, including skin, soft tissue, and urinary tract infections, as well as bacteremia, pneumonia, arthritis, and endocarditis.

GBS is believed to be the primary source of maternal colonisation since it is the typical microbiota in the vagina of healthy women. This suggests that GBS is capable of quickly adapting to different host conditions and exhibit some symptoms. Bloodstream infections, meningitis, osteomyelitis, and endocarditis are among the invasive maternal illnesses caused by GBS in pregnant and postpartum women (Shabayek & Spellerberg, 2018). In addition, which also reported by Shabayek and Spellerberg (2018), bacteriuria, amnionitis, fasciitis, cellulitis, endometritis, and wound infections are non-invasive maternal illnesses that are linked with episiotomies or caesarean births.

Neonatal colonisation and infection are strongly linked to maternal colonisation at the time of birth. As stated by Morgan and Cooper (2018), with or without membrane breach, the foetus is vulnerable to ascending infection into the amniotic fluid. It is classified as early-onset, late-onset and late-late-onset based on the age of the neonatal presentation. Early-onset disease (EOD) of GBS is defined as a disease that occurs during less than seven days of life (Hanna & Noor, 2021), while late-onset disease (LOD) appears between the ages of 7 and 90 days; GBS infection in babies three months or older is characterised as late-late onset which is also known as later-onset or very late-onset illness (Hon et al., 2017). The clinical presentations vary according to the age presentation.

As reported by Heath and Jardine (2014), sepsis (69%) is the most common symptom of early-onset group B streptococcal infection, followed by pneumonia (26%), respiratory distress (13%), and, in rare instances, meningitis (11%). In another study, sepsis was detected in the blood samples of 8 patients (26.7%), followed by pneumonia (n = 4, 13.3%), tissue abscess, meningitis (n = 3, 10% each), and TB (n = 1, 3.3%) among the 30 isolates from sterile locations (Suhaimi et al., 2017). In EOD of GBS, the majority of the neonates get sick during the first 24 hours. This is due to the GBS maternal infection. Maternal vaginal colonisation as well as maternal gut colonisation could be the genesis of GBS in the early onset GBS illness (Hon et al., 2017). Early-onset GBS can lead to death if untreated.

In contrast, it is less likely to be lethal for late-onset GBS with fever or meningitis as their common symptoms. This is because of the difference in their way of infection although horizontal transmission is still feasible after delivery. According to Sorge et al. (2009), GBS may infiltrate, survive and cross the blood-brain barrier and contribute to blood-brain barrier penetration and pathogenesis of meningitis. Instead of GBS colonisation of the birth canal, preterm birth is the most major maternal risk factor for late onset infections (Hon et al., 2017). The decreased time for maternal IgG to reach the newborn might explain this.

2.2 Capsular Serotyping

For GBS research, serotyping is one of the useful epidemiological techniques with capsular polysaccharide (cps) that acts as a significant contributor to GBS pathogenesis. As reported by Jannati E et al. (2012), based on capsular polysaccharide antigen, there are 10 different GBS serotypes which are Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX. However, the antigenicity and immunogenicity of capsular antigens varies by serotype and is linked to the invasiveness of GBS infections. For instance, according to a epidemiological research specifically in Malaysia conducted by Suhaimi et al. (2017), serotype Ia was the most prevalent serotype (n = 27, 45%), followed by serotype III (n = 10, 16.7%), V (n = 9, 15%), VI (n = 8, 13.3%), VIII (n = 2, 3.3%), and VII (n = 1, 1.7%), and the three upmost common serotypes which are Ia, III, and V often linked with invasive illness in pregnant women, neonates, and non-pregnant people, respectively.

This was consistent with other local study from a teaching hospital which revealed by Karunakaran et al. (2009), Ia was the most prevalent serotype, accounting for 22.2% of 45 isolates followed by serotype VI (17.8%), III and V (13.3% each). Additionally, in another GBS Malaysian study, serotype Ia was the prevalent capsular polysaccharide (38.9%) among the 18 GBS isolates in adults and neonates, followed by VI, V and III (Eskandarian et al., 2013). In contrast, another study was done in the following year at Malaysian hospital which serotype VI (22.3%) was found to be significantly overrepresented from the total of 103 GBS isolates compared to an earlier research among adults and newborns, followed by VII (21.4 %), III (20.4%), Ia (17.5%), V (9.7%), II (7.7 %) and IV (1 %) (Eskandarian, 2014).

While in other epidemiological studies in North America, serotypes Ia, Ib, II, III, and V are the most commonly associated with invasive illness (Raabe & Shane, 2019). The most frequent serotype causing invasive illness in non-pregnant people in the United States (US) is group V (29% in 2005–2006), followed by serotypes Ia, II, and III (Skoff et al., 2009). From 2003 to 2013, a research of serotypes linked to invasive GBS illness in Alberta, Canada, found that serotype III (20%) was the most common, followed by serotypes V (19%), Ia (19%), Ib (13%), and II (11%) (Alhhazmi, Hurteau & Tyrrell, 2016). It shows that the disease-causing serotypes vary by location and between invasive and colonising isolates.

2.3 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is an unambiguous method for identifying bacterial isolates based on the sequences of internal segments of usually seven housekeeping genes. As mentioned by Li et al. (2019), for GBS, the internal segments of seven housekeeping genes (*adhP, pheS, atr, glnA, sdhA, glcK,* and *tkt*) were sequenced to accomplish the typing. Internal segments of each gene of around 450-500 bp are chosen because they can be read correctly on both strands using an automated DNA sequencer. The various sequences found within a bacterial species are allocated as unique alleles for each house-keeping gene, and the alleles at each of the seven loci form the allelic profile or sequence type for each isolate (ST). Each species isolate is therefore clearly identified by a set of seven numbers that correspond to alleles at the seven house-keeping loci. Because it offers data that can be readily compared across labs via the Internet, MLST is especially well suited for epidemiological research.

According to Ezhumalai et al. (2020), from a Malaysian hospital setting, ST1 being the most common (42%) among the 51 viable GBS isolates, followed by ST28, ST23 (n = 2 for each); and ST19, ST24, ST144, ST103, ST485 (n = 1 for each). ST1 shown to be linked to the majority of invasive isolates. As emphasized by Flores et al. (2015), phylogenetic study in United States showed a time dependent pattern of genetic diversification, which corresponded to the development of ST1 GBS as significant human disease agents in the 1990s. The sequence type also may be varied according to the ages and the underlying health condition. As identified by Tien et al. (2011), based on Taiwan epidemiological study, ST1 strains were the most invasive in adults, whereas ST17 strains were the most invasive in newborns; while ST19 was found in both adults and newborns. In contrast, at the same setting, ST17, a hypervirulent clone, was responsible for 68% of invasive GBS illnesses and 71% of culture-proven meningitis (Lee et al., 2019). Whereas, ST1 contributes as the most frequent (32%) sequence types colonizing GBS strains in pregnant women followed by ST12 (22%), and ST23 (15%) (Lee et al., 2019).

Interestingly, in other epidemiological study, another sequence type became the most hypervirulent clone that accounted for a significant percentage of invasive GBS. For instance, ST283. According to Barkham (2019), from 1995 to 2017, ST283 was identified in all invasive Asian samples studied which in Lao PDR, it was responsible for 29/38 (76%) human isolates, 102/139 (73%) in Thailand, 4/13 (31%) in Vietnam, and 167/739 (23%) in Singapore. It is believed to have a strong connection with foodborne transmission. As stated by Delannoy et al. (2013), ST283 has been linked to invasive illness in non-pregnant people, and it was identified from fish in Thailand.

In fact, there was a major outbreak of GBS among human involving fish consumption occurred in the year of 2015. As remarked by Chau et al. (2017), in Singapore, a large epidemic of group B Streptococcus (GBS) infection was linked to the eating of yusheng, a Chinese-style raw fish dish. This virulent ST infects the fish and become invasive isolates that can be harmful to human. ST283 has been found to colonise and infect a variety of farmed freshwater fish, as well as produce a particularly severe invasive illness in humans (Wang et al., 2017, Kalimuddin et al., 2017). Therefore, the use of freshwater fish in all ready-to-eat raw fish meals was prohibited in Singapore, and surprisingly it showed a lower GBS infection cases frequency. As mentioned by Rajendram et al. (2016), although rare invasive ST283 infections have been detected, the number of reported invasive GBS infections in Singapore has declined.

CHAPTER 3

METHODOLOGY

3.1 Sample Collection

The approval for conducting the sample collection was granted by the Ethics Committee which are National Medical Research Register (NMRR), Ethic Committee for Research Involving Human Subject (JKEUPM) and Universiti Kebangsaan Malaysia (UKM). A total of 40 *Streptococcus agalactiae* samples were available in the glycerol stock in -20°C freezer. These GBS isolates were collected from sterile sites of newborns, pregnant adults and non-pregnant adults such as cerebrospinal fluid, blood, pus, tissue and peritoneal fluid. There are five different Malaysian hospitals involved in this study which are Hospital Sultanah Aminah, Hospital Serdang, Hospital Universiti Kebangsaan Malaysia, Hospital Melaka and Hospital Tuanku Jaafar.

3.2 Bacterial Subculture

5% columbia sheep blood agar is an agar that can be used to subculture bacteria including *Streptococcus pyogenes* ATCC™ 19615. Sheep blood enables hemolytic responses to be detected and provides the X factor (heme) required for the development of a wide variety of pathogenic organisms. It has been the preferred source of blood in blood agar because sheep's red blood cells are particularly susceptible to the hemolytic toxins generated by bacteria, resulting in hemolytic zones surrounding the colonies over time. Colonies are often bigger and growth is more luxuriant on this medium than on other blood agar bases.

The samples in glycerol stocks were retrieved from the -20°C freezer and thawed in the ice for a few minutes. The samples were then streaked on the columbia sheep blood agar by using a wire loop and incubated in a CO^2 incubator at 37°C overnight. On the next day, small colonies that appear white to grayish colour were expected to grow on the blood agar due to haemolytic properties of the GBS.

3.3 Identification of GBS by phenotypic test

3.3.1 Gram stain

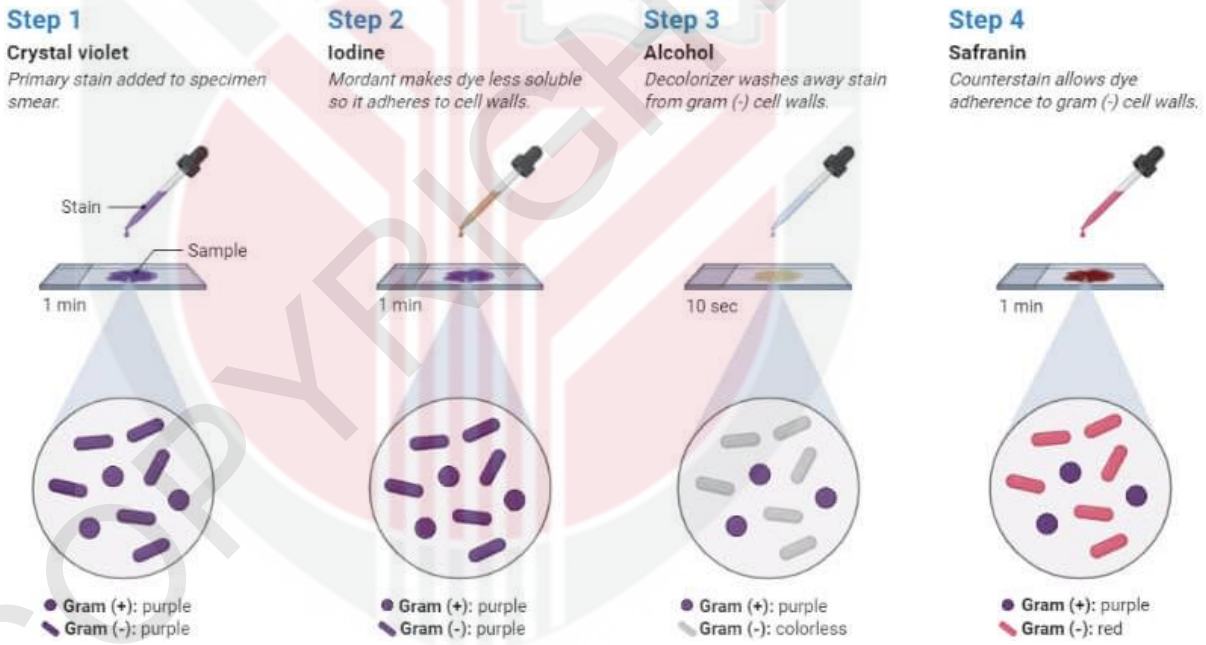


Figure 3.1: Gram staining procedure

The basic technique which is gram staining, was used to identify gram properties of the bacteria. This technique always can differentiate the bacterial species into two groups which are gram-positive bacteria and gram-negative bacteria. By identifying peptidoglycan, the staining distinguishes bacteria based on the physical and chemical characteristics of their cell walls. When examined under a microscope, *Streptococcus* species were gram-positive and appeared as short and round blue pairs or chain cocci. For the gram staining method, the specimen was prepared from 24 hours inoculated 5% columbia sheep blood agar (Isolab Sdn Bhd, Malaysia) A thin smear of bacterial specimens is made on a glass slide using a combination of isolated colonies and a few drops of water. After allowing the slide to air dry, it was heat fixed. The staining process begins with a one-minute soak of the specimen in crystal violet. After that, the dye was washed away with distilled water. Then, a drop of alcohol is placed on the violet stain to decolorize it. This procedure was completed for no more than 10 seconds before being washed with distilled water to remove any remaining alcohol. Next, the specimen was counter-stained for a minute with safranin. The slide was blotted dry with a paper towel after the colour was washed off. The specimen was examined using the oil immersion method under a light microscope (100X magnification). If the observation revealed gram-positive or violet-coloured cocci in chains, the bacterial isolates were presumed to be streptococci.

3.3.2 Catalase Test

The catalase test is very useful for determining if gram-positive cocci are either staphylococci or streptococci. The bacterial colonies were suspended in hydrogen peroxide for this test. As *Streptococcus agalactiae* is a streptococcus species, it does not generate catalase (catalase-negative), which will not create water and oxygen when combined with hydrogen peroxide. The oxygen was evident through the suspension's bubbling for staphylococci. The procedure began with a 3% H^2O^2 solution put on a clean slide. A small quantity of bacterial colony was transferred from an agar plate and mixed with hydrogen peroxide using a sterile loop. The fast evolution of oxygen within 5-10 seconds as shown by bubbling indicates positive results, while the lack of bubbles is considered as a negative result for catalase test which is expected in this study.

3.3.3 Latex Agglutination

The identification of streptococcal groups A, B, C, D, F, and G using a latex agglutination test Lancefield (Oxoid™, UK) discovered that the majority of pathogenic streptococci had unique carbohydrate antigens that allow them to be classified into categories. These streptococcal group antigens may be isolated from cells and shown to be present using latex particles coated with group-specific antibodies. In the presence of homologous antigen, these latex particles will agglutinate, but in the absence of such antigen, they will stay in smooth suspension. Streptococcal Grouping Kit is a latex agglutination test for determining the streptococcal group, with reagents for groups A, B, C, D, F and G included.

Bacterial cultures must be performed initially in this test. A bacteriological loop was used to select two to five test colonies equal to 2-3 mm of growth, which were subsequently emulsified in the enzyme preparation. It will then be incubated in a water bath for 10 minutes at 37°C. After 5 minutes of incubation, remove each tube and shake vigorously for 2-3 seconds before continuing the incubation. Before being used, it will be removed and let to cool at room temperature. One drop of latex was dispensed into each of the reaction card's circular circles. Using a pasteur pipette, one drop of produced extract was applied to each of the 6 rings. The mixture is then spread over the whole surface of the ring using a different mixing stick supplied for each ring. Because all of the samples are Group B streptococcus, agglutination is anticipated to occur in the group B ring.

3.3.4 CAMP Test

The CAMP test (Christie–Atkins–Munch–Peterson) is a method for identifying group B β-hemolytic streptococci (*Streptococcus agalactiae*) based on the production of a substance (CAMP factor) that enlarges the region of hemolysis caused by a β-hemolysin derived from *S. aureus (Staphylococcus aureus).* Certain bacteria (particularly group B streptococci) generate a diffusible extracellular hemolytic heat-stable protein (CAMP factor) that works in tandem with *S. aureus* beta-lysin to induce increased red blood cell lysis. On sheep blood agar, group B streptococci are streaked perpendicular to a streak of S. aureus. A positive reaction manifests itself as an arrowhead zone of hemolysis adjacent to the intersection of the two streak lines. Bacteriophage NCTC 8-17 *Streptococcus agalactiae* that was obtained from National Collection of Type Culture (MCTC; Colindale, London, United Kingdom) was used as positive control for the assay.

3.4 Genomic DNA Extraction

Genomic DNAs were extracted from GeneAll Exgene Mini genomic extraction kits (GeneAll, South Korea) according to manufacturer's instructions. In this procedure, lysozyme enzymes need to be prepared first by multiplying 0.003g/100µL with the GP buffer. The pellet cells were then centrifuged for 1 minute at maximum speed in a 1.5 mL microcentrifuge tube, and the supernatant was discarded. After that, the pellet was completely resuspended in 180 µL of the prepared enzyme mixture and incubated for 30 minutes at 37°C. After adding 20 µL of Proteinase K solution and 200 µL of Buffer BL, the mixture was incubated at 56°C for 30 minutes followed by 95°C for 15 minutes. After that, the tube was spinned down for a few seconds to remove any remaining droplets from the inside of the lid. 200 µL of absolute ethanol was added to the sample, which was pulse-vortexed and quickly spun down. The whole mixture was transferred to the SV column and centrifuged for 1 minute at over 8000 rpm before being replaced with fresh collecting tubes. The same procedures were performed with 600 µL of buffer BW and 700 µL of buffer TW, but the pass-through for TW buffer was disregarded and the SV column was reinstated back into the collecting tube. To remove any remaining wash buffer, the tubes were centrifuged at high speed for 1 minute. In a new 1.5 mL microcentrifuge tube, the SV column was inserted. Buffer AE was added in a 200 µL volume and incubated for 1 minute at room temperature. Finally, centrifuge at full speed for 1 minute before using Nanodrop to quantify the DNA.

3.5 PCR for Genotyping Confirmation

The optimization was done by targeting the *cfb* gene. A 25 µl reaction mixture, which consisted of 12.5 µl of mastermix (Bioline, London, United Kingdom)), 0.1 µl of Forward primer (TTTTCACCAGCTGTATTAGAGTA), 0.1 µl of reverse primer (GTTCCCTGAACATTATCTTTGAT) 7.5 µl of nucleus water and 3 µl of DNA template was prepared in the tubes. The reaction tubes were then placed in thermocycler and amplified according to the program as the following: initial denaturation at 95 °C for 15 min followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 1 min, a final extension at 72 °C for 10 min and holding at 4°C.

3.6 Capsular Typing of GBS using Multiplex PCR

The Multiplex polymerase chain reaction (Multiplex PCR) is a common technique for making numerous copies of a DNA segment using DNA templates that contain the specific segment. Forward and reverse primers, DNA polymerase, DNA template, deoxyribonucleotide triphosphates (dNTPs), and reaction buffer are all components of PCR.

In a thermocycler machine, the whole reaction occurs. Due to the high temperature in the thermocycler, DNA templates are first denatured and split into two strands. When the temperature lowers to a particular degree, known as the annealing temperature, specialised primers for the targeted gene bind to a specific location in the DNA templates. The primers are created in accordance with the golden principles of primer design. By linking together, the dNTPs present in the reaction mixture, DNA polymerase starts to synthesize new strands of DNA beginning from each primer linked to the DNA template. The freshly produced DNA strands will serve as the DNA template for the next cycles. The cycle continues until the desired number of cycles has been reached.

In this study, Multiplex PCR was used to determine the ten distinct GBS serotypes which are Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX. The Multiplex PCR reaction mixtures were prepared in a total volume of 25 µL per 0.2 mL microcentrifuge tube for 40 samples as shown in Table 3.1. Forward and reverse primers used were according to the studied serotypes. For instance, forward and reverse primers for capsular polysaccharide synthesis gene *(cps) including cpsL, cpsG, cps J-Ib, cps J-2, cps G 2-6, cpsJ-4, cps N-5, cps I-6, cps I-7, cps J-8* and *cps I-9* were used in determining GBS serotype according to Imperi et al. (2010). Table 3.2 shows the name and sequence of the nineteen primers used in the multiplex PCR assay. The reaction mixture was then subjected to PCR in a thermocycler according to the setup as shown in Table 3.3. The PCR products were then stored in -20°C freezer and proceeded with gel electrophoresis.

Table 3.1: Concentration and volume of respective polymerase chain reaction reagents for determining a serotype in a single preparation.

Reagents	Concentration	Volume (µL)
		1X
MyTaq™ HS Red Mix	-	12.50
Forward primer	1.0µM	1
Reverse primer	1.0µM	1
Nuclease free water	-	7.5
DNA	-	3

Table 3.2: Name and sequence of the primers used in the multiplex PCR assay

Gene	Sequences (5'to3')		Amplicon size(bp)
cpsL	F	CAATCCTAAGTATTTTCGGTTCATT	688
	R	TAGGAACATGTTCATTAACATAGC	
cpsG	F	ACATGAACAGCAGTTCAACCGT	272
	R	ATGCTCTCCAAACTGTTCTTGT	
cpsJ-Ib	F	GCAATTCTTAACAGAATATTCAGTTG	621
	R	GCGTTTCTTTATCACATACTCTTG	
cpsJ-2	F	CATTTATTGATTCAGACGATTACATTGA	465
	R	CCTCTTTCTCTAAAATATTCCAACC	
cpsG-3-6	F	ACATGAACAGCAGTTCAACCGT	352
	R	TCCATCTACATCTTCAATCCAAGC	
cpsJ-4	F	CATTTATTGATTCAGACGATTACATTGA	538
	R	CCTCAGGATATTTACGAATTCTGTA	
cpsN-5	F	ATGCAACCAAGTGATTATCATGTA	582
	R	CTCTTCACTCTTTAGTGTAGGTAT	
cpsI-6	F	GAATTGATAACTTTTGTGGATTGCGATGA	470
	R	CAATTCTGTCGGACTATCCTGATG	
cpsI-7	F	GAATTGATAACTTTTGTGGATTGCGATGA	179
	R	TGTCGCTTCCACACTGAGTGTTGA	
cpsJ-8	F	TATTTGGGAGGTAATCAAGAGACA	438
	R	GTTTGGAGCATTCAAGATAACTCT	
cpsI-9	F	CTGTAATTGGAGGAATGTGGATCG	229
	R	AATCATCTTCATAATTTATCTCCCATT	

Table 3.3: Thermocycling conditions of Multiplex PCR for GBS isolates

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	1 minute	1
Denaturation	95	15 minutes	30
Annealing	55	15 minutes	
Extension	72	10 minutes	
Final extension	72	5 minutes	1
Hold	4	Pause	1

3.7 Gel Electrophoresis

Using an electric field, gel electrophoresis was utilised to analyze and segregate the PCR products according to their sizes and charges. An electronic weighing scale was used to weigh 1.36g of agarose powder, and 1X Tris-borate-EDTA (TBE) buffer (Vivantis Sdn Bhd, Malaysia) was made by diluting 100 mL of 10X TBE buffer with 900 mL of sterile distilled water. Then, in a 250 mL Scott container, 1.36g agarose powder (Vivantis Sdn Bhd, Malaysia) was mixed with 80 mL 1X TBE buffer to make a 1.7% agarose gel. The agarose powder was fully dissolved after swirling the liquid in a circular motion. After that, the mixture was heated in the oven for 2 minutes to ensure full disintegration.

5µL of EtB“Out” nucleic acid staining solution was added by using a 1-10µL pipette to the mixture after it had been heated. After that, the gel solution was poured into the gel cast and allowed to cool at room temperature. The agarose gel was moved to a gel tank when it hardened, and a 1X running TBE buffer was added until it covered the surface of the gel. Using a 1-10µL pipette, 5µL of 1kb DNA ladder was put into the first well of the agarose gel, followed by 5µL of PCR products in the following wells. After that, the agarose gel with loaded DNA ladder and PCR products was run in an electric field for 60 minutes at 80 V. The agarose gel was then examined under the Gel Doc UV trans-illuminator once the separation was completed.

3.8 DNA Sequencing

To extract the whole sequence of the amplified products and compare with nucleotide sequence databases, DNA sequencing was conducted on representative PCR results. These will validate whether or not the amplified products are of interest to our research. 20L of PCR product was transferred to a 1.5 mL Eppendorf tube and labelled for DNA sequencing. A 0.5 mL microcentrifuge tube was filled with 10L of each primer and labelled. The PCR products and primers were put in microcentrifuge tubes on a tube holder and submitted to Apical Scientific Sdn. Bhd. for sequencing. Multiple sequence alignment was then used to examine the DNA sequences.

3.9 Multiple Sequence Alignment

The DNA sequences retrieved from Apical Scientific Sdn. Bhd. were trimmed by using MEGA 7 according to the MLST references of the seven housekeeping genes. The preserved DNA sequences were then blasted in PubMLST to search for the allelic profile from the databases. Multiple sequence alignment was performed for all representative DNA sequences of the 40 samples to get the sequence types (STs) in order to build a phylogenetic tree.

3.10 Phylogenetic Analysis

The trimmed DNA sequences from representative PCR products and databases for GBS isolates were analyzed in MEGA 7 software. The DNA sequences were aligned and subjected to phylogenetic analysis. A phylogenetic tree was constructed to study the genetic relationship between the sequence type of the 40 GBS isolates.

3.11 Statistical Analysis

Data obtained from analysis of the samples such as genes distribution on its source of isolation, serotype and STs of the GBS isolates were summarised and analyzed by using Statistical Package for the Social Sciences (SPSS) Version 25. Chi-square test is done in order to determine the association between variables, such as sequence types (STs) with serotypes, serotypes with source of isolates and source of isolates with sequence types (STs). It shows significant associations between the categorical variables if the *p* value is less than 0.05.

CHAPTER 4

RESULTS

4.1 Characteristics of GBS Isolates

Data and sample collection were done with the involvement of five different Malaysian hospitals which are 16 samples from Hospital Sultanah Aminah, 8 from both Hospital Serdang and Hospital Universiti Kebangsaan Malaysia and 4 samples from Hospital Melaka as well as Hospital Tuanku Jaafar which give a total of 40 samples. All of them were collected starting from May 2019 throughout more than a year until almost the end of December 2020. The research population varied in age from newborns to 81 years, with a mean of 40.3 (±27.1) years. Out of 40 patients involved, 17 (42.5%) of them are male and 23 (57.5%) are female. The majority of the study subjects were Malay with the number of 24 (60%) followed by 10 (25%) Chinese and 6 (15%) of Indians.

The samples were collected from different sources. Most of the samples were taken from blood (26; 65%) followed by tissue (6; 15%), pus (3; 7.5%), cerebrospinal fluid (2; 5%), as well as bone, peritoneal fluid and skin biopsy with 1 (2.5%) for each source.

Sepsis was the most common consequence of GBS infections (14, 35%), accounting for the majority of cases in this study. Apart from that, serious consequences such as septic arthritis, abscess, and cellulitis were also found in patients with positive GBS infections in this research, accounting for 7.5% of these three sequelae. Additionally, other diseases such as diabetic foot ulcer (DFU), congenital pneumonia, meningitis, and pneumonia account for instances of GBS infection in study participants. Furthermore, wet gangrene, necrotizing fasciitis, tenosynovitis, peritonitis, mitral valve stenosis, deep mycosis infection, acute gastroenteritis, cardiogenic shock syndrome and meningoencephalitis contribute for only minority cases with 2.5% for each clinical syndrome.

The most often encountered underlying medical condition in patients with positive GBS infections was immunocompromised individuals with type 1 diabetes mellitus, which accounted for the majority of GBS infections (16, 40%). It is followed by neonatal jaundice, which affects 7 (17.5%) of all study participants. Additionally, other underlying medical conditions such as pregnancy, renal disease, neonatal hypoxic-ischemic encephalopathy, forensic case, respiratory disease, autoimmune disease or immunosuppressive condition, maternal pyrexia, cardiovascular disease, skin and soft tissue disease, and altered mental status were minor indicators of the infections which approximately accounting less than 10% cases in this study.

Table 4.1: Characteristics of patients with GBS infections

Variables	Frequency	%
Age Mean (SD)	40.3 (27.1)	
Gender		
Male	17	42.5
Female	23	57.5
Ethnicity		
Malay	24	60.0
Chinese	10	25.0
Indian	6	15.0
Hospital		
Sultanah Aminah	16	40.0
Serdang	8	20.0
Universiti Kebangsaan Malaysia	8	20.0
Melaka	4	10.0
Tuanku Jaafar	4	10.0
Source		
Blood	26	65.0
Tissue	6	15.0
Cerebrospinal fluid	2	5.0
Pus	3	7.5
Bone	1	2.5
Peritoneal dialysis fluid	1	2.5
Skin biopsy	1	2.5

Table 4.2: The clinical syndromes found in patients with GBS infections

Clinical Syndromes	Frequency	%
Diabetic foot ulcer DFU	2	5.0
Wet gangrene	1	2.5
Necrotizing fasciitis	1	2.5
Tenosynovitis	1	2.5
Sepsis	14	35.0
Congenital pneumonia	2	5.0
Peritonitis	1	2.5
Meningitis	2	5.0
Septic arthritis	3	7.5
Abscess	3	7.5
Mitral valve stenosis	1	2.5
Cellulitis	3	7.5
Deep mycosis infection	1	2.5
Acute gastroenteritis	1	2.5
Pneumonia	2	5.0
Cardiogenic shock syndrome	1	2.5
Meningoencephalitis	1	2.5

Table 4.3: The underlying medication conditions of patients with GBS infections

Underlying Medical Condition	Frequency	%
Neonatal jaundice	7	17.5
Pregnant women	1	2.5
Diabetes Mellitus	16	40.0
Renal Disease	2	5.0
Neonatal Hypoxic-Ischemic encephalopathy	1	2.5
Forensic case	2	5.0
Respiratory disease	4	10.0
Autoimmune disease or immunosuppressive condition	2	5.0
Maternal pyrexia	1	2.5
Cardiovascular disease	2	5.0
Skin and soft tissue disease	1	2.5
Altered mental status	1	2.5
Death	3	7.5

4.2 GBS Isolates Identification

Columbia agar with 5% sheep blood is a suitable and differential agar for the growth of *Streptococcus agalactiae*. Columbia blood agar with 5% sheep blood was originally red in colour. Due to the bacterium's haemolytic capabilities, small bacteria colonies with a whitish grey appearance formed on blood agar after an overnight incubation at 37°C. As shown in Figure 4.1, the blood agar bacterial growth shifted from red to whitish grey due to the full lysis of red cells in the medium around and underneath the colonies. As a result, all 40 isolates from the glycerol stock in the -20°C freezer that grew on the 5% Columbia sheep blood agar were verified as beta haemolysis streptococcus bacteria because they were able to alter the colour of the blood agar from red to whitish grey following overnight incubation at 37°C. However, further identifications are necessary since Group A Streptococcus (GAS) and Group B Streptococcus (GBS) exhibit the same properties.

Additional phenotypic tests for GBS identification, including gram stain, catalase test, latex agglutination, and CAMP test, were performed, and it was verified that all 40 bacterial isolates were *Streptococcus agalactiae*. To begin, the whitish grey colonies on blood agar were the primary indicator of streptococcus presence. Throughout the isolation process, only colonies with a whitish grey colour were chosen to make pure colonies. (Figure 4.1). Following that, gram staining was performed on all presumed streptococci and it was confirmed that the isolates are streptococcus due to their round, purple-stained cocci (gram positive), and in pairs or short chains. (Figure 4.2).

The phenotypic identification test proceeded with a catalase test. The catalase test of *Streptococcus agalactiae* showed a negative result with no presence of bubbles as all other streptococci when tested with 3% hydrogen peroxide. In contrast, there are bubbles present when tested with staphylococcus bacteria even though both of the bacteria are gram positive. (Figure 4.3 (a) and (b)). In addition, confirmatory test using latex agglutination was performed in order to determine in which group the streptococcus will be. Based on Figure 4.4, the latex agglutination test showed that all the 40 isolates were confirmed to be in group B as there was agglutination formed when the bacteria mixed with the B grouping reagent, while absent in other five groups.

If the laboratory is unable to identify group B streptococci (GBS) using the Lancefield grouping process, there are various microbiological tests that can be performed to determine whether or not the organism is GBS. One of the tests is the CAMP test. The CAMP test takes use of GBS's ability to generate CAMP factor, while the majority of other hemolytic streptococci do not. As shown in Figure 4.5, all 40 isolates exhibit an arrowhead shape zone when streaked perpendicular to a streak of *Staphylococcus aureu*s (vertical streak). Hence, it is confirmed that the samples collected were all *Streptococcus agalactiae* or GBS.

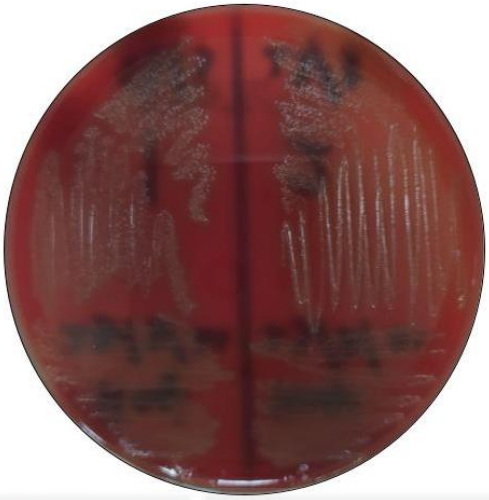


Figure 4.1: GBS colonies grew on Columbia agar with 5% sheep blood after being incubated at 37°C for an overnight period

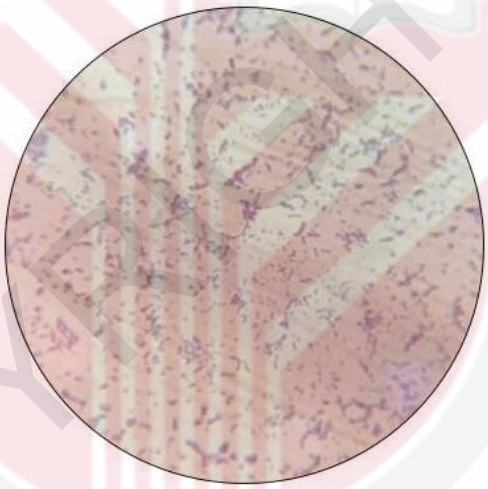


Figure 4.2: Short, round, gram positive cocci and in pairs or chains of GBS isolates under 100X magnification

Figure 4.3: (a) Negative catalase test of representative *Streptococcus agalactiae* (b) Positive catalase test of representative staphylococcus bacteria

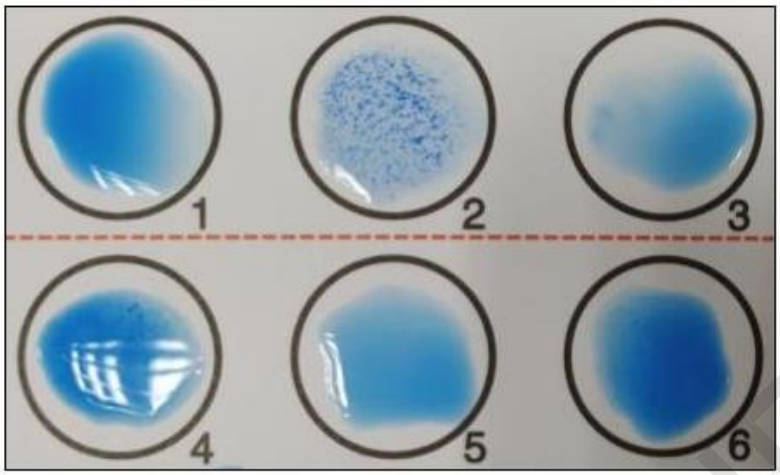


Figure 4.4: Positive agglutination for latex grouping reagent group B (2) and no agglutination formed on latex grouping reagent groups A, C, D, F and G (1, 3, 4, 5 and 6 respectively)

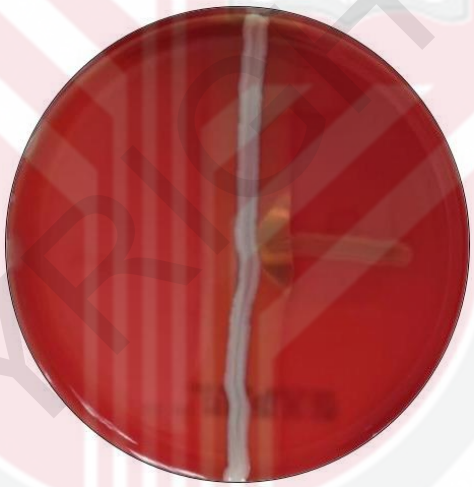


Figure 4.5: At the intersection of the *Staphylococcus aureus* and test organism streaks, there is presence of a distinct arrowhead shape zone.

4.3 Genotyping Confirmation

PCR-based assays for GBS were performed for genotypic confirmation which targeted the *cfb* gene that encodes for the CAMP factor of *Streptococcus agalactiae.* The isolates are confirmed to be GBS if there is the presence of *cfb* gene in the strains of *Streptococcus agalactiae.* All the 40 GBS isolates from symptomatic individuals were positive as a result of using a conventional PCR technique targeting the *cfb* gene. The samples showed single bands of the expected size of the *cfb* gene (153bp), as shown in Figure 4.6.

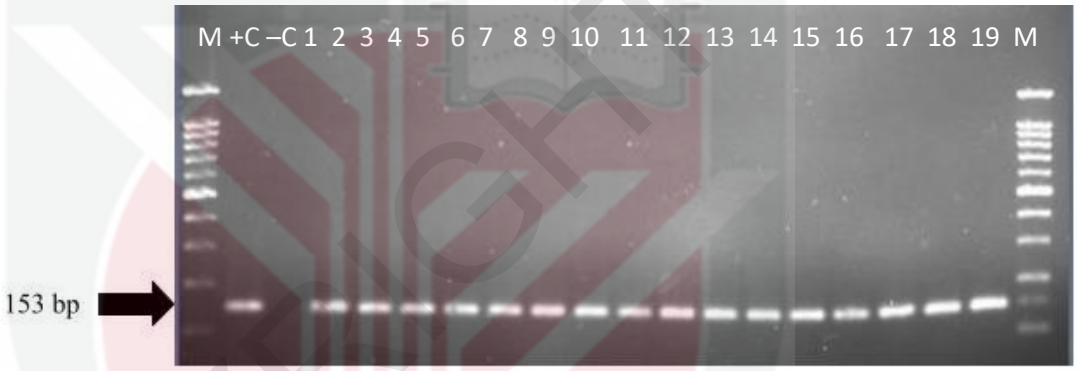


Figure 4.6: Electrophoresis of the GBS *cfb* gene PCR product on an agarose gel. Lanes 1 and 20 are DNA ladders (100 bp); lane 2 represents a positive control; lane 3 represents negative control; and the remaining lanes represent positively amplified genes with the expected band size of approximately 153 bp

4.4 Distribution of Serotypes

Multiplex PCR was used to determine the ten distinct GBS serotypes which are Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX. The most prevalent was serotype III (n=11), followed by serotype VI (n=7), serotype Ia, Ib, II, V (n=4 each), serotype VII (n=3), serotype IV (n=2), and serotype IX (n=1). Serotype VIII was not found in this study (Table 4.4).

Table 4.4: Serotypes distribution of *Streptococcus agalactiae*

Serotypes	Frequency	Percent (%)
Ia	4	10.0
Ib	4	10.0
II	4	10.0
III	11	27.5
IV	2	5.0
V	4	10.0
VI	7	17.5
VII	3	7.5
IX	1	2.5

4.5 Multilocus Sequence Typing analysis

The sequences of the seven housekeeping genes were successfully amplified in 38 GBS isolates, whereas only six of the seven housekeeping genes were found in the other two isolates. The MLST analysis confirmed the presence of 15 distinct allelic profiles and STs. Among the 15 STs, ST1 was the most prevalent ST (n = 13) followed by ST17 (n = 5); ST3, ST12, ST26 (n = 3 for each); ST283 (n =2); ST19, ST23, ST24, ST28, ST130, ST196, ST335, ST459 and ST861 (n = 1 for each). (Table 4.5).

Table 4.5: Sequence Types (STs) of the GBS isolates

No of Isolates	%	Allelic profile							Sequence Types (STs)
		adhp	***pheS***	***atr***	***glnA***	***sdhA***	***glck***	***tkt***	
13	32.5	1	1	2	1	1	2	2	1
3	7.5	1	1	4	1	1	3	2	3
3	7.5	10	1	4	1	3	3	2	12
5	12.5	2	1	1	2	1	1	1	17
1	2.5	1	1	3	2	2	2	2	19
1	2.5	5	4	6	3	2	1	3	23
1	2.5	5	4	4	3	2	3	3	24
3	7.5	1	1	5	4	1	4	6	26
1	2.5	1	1	3	5	2	2	2	28
1	2.5	9	8	4	1	5	3	4	130
1	2.5	1	1	3	1	1	12	2	196
2	5.0	9	5	7	1	3	3	2	283
1	2.5	1	1	43	2	2	2	2	335
1	2.5	1	1	3	1	41	12	2	459
1	2.5	1	1	89	2	2	2	2	861

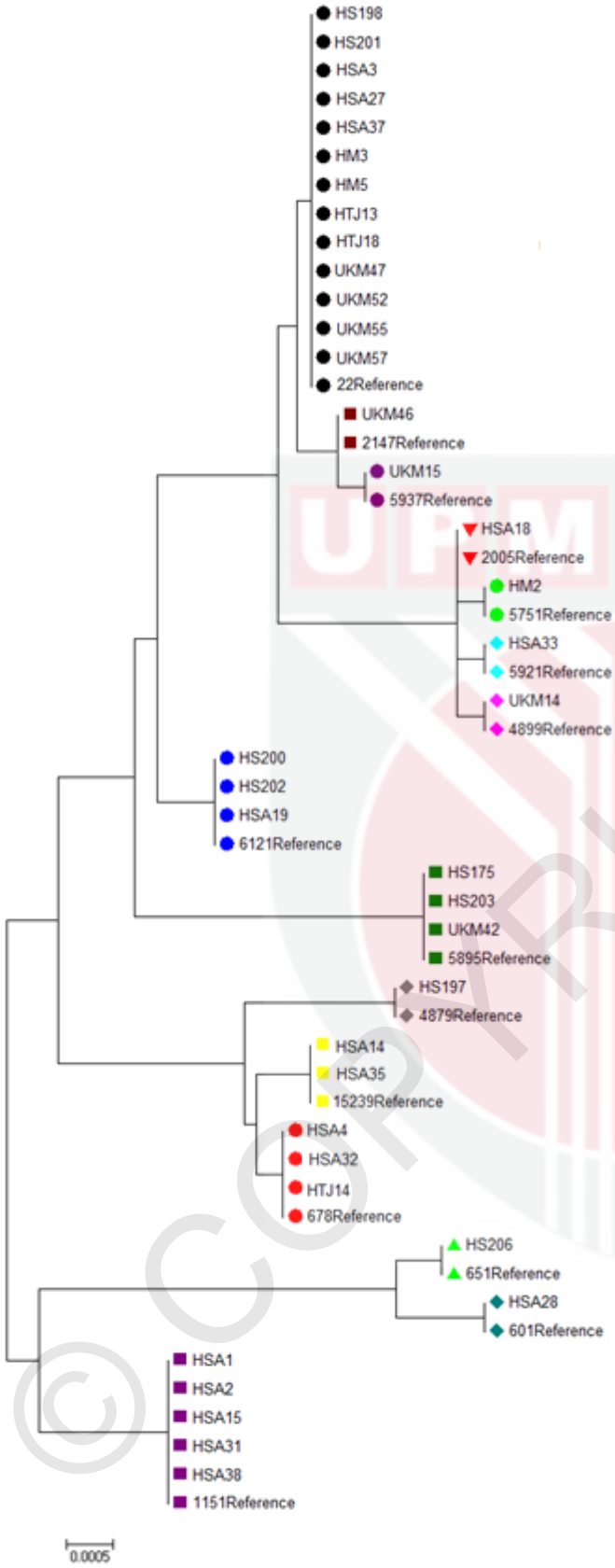
Table 4.6: Serotypes distribution among the sequence types

		Serotypes									Total
		Ia	Ib	II	III	IV	V	VI	VII	IX	
ST	**1**	1	1	1			1	6	3		13
	3			1	2						3
	12		3								3
	17				5						5
	19	1									1
	23	1									1
	24	1									1
	26						3				3
	28			1							1
	130									1	1
	196					1					1
	283										2
	335										1
	459					1					1
	861										1
Total		4	4	3	11	2	4	6	3	1	38

4.6 Multilocus Sequence Typing Phylogenetic tree

All nucleotide sequences of the seven housekeeping genes for the respective isolates were aligned in the specified order and subjected to phylogenetic analysis among all the 38 isolates (labelled with hospital's acronym followed by numbers) and 15 reference sequences from the MLST database (labelled with identity number followed by Reference).

The tree was branched out into two big branches which consisted of Clade I and Clade II in the first branch; while Clade III and Clade IV in the second branch. Among the four clades, there was one major group consisting of 25 isolates from this current study (Clade I), followed by six isolates in Clade II. Additionally, Clade III consists of two isolates, while there are five isolates in the fourth clade. The clades also showed clear segregation among the STs, where clade I represented predominantly ST1, followed by ST196, ST459, ST19, ST335, ST861, ST28, ST3 and ST26. All reference sequences of the different STs were clustered according to their respective similar ST of the isolates in this study as shown in Figure 4.7.



Source	ST	Serotype
PD	ST1	VI
Tissue	ST1	VI
Blood	ST1	VI
Blood	ST1	Ib
Blood	ST1	Ia
Blood	ST1	II
Tissue	ST1	V
Tissue	ST1	VII
Blood	ST1	VI
Blood	ST1	VII
Bone	ST1	VII
Blood	ST1	VI
Blood	ST1	VI
Rectovaginal swab	ST1	V
Blood	ST196	IV
Rectovaginal swab	ST196	IV
Pus aspirate	ST459	IV
Vaginal swab	ST459	IV
Blood	ST19	Ia
Rectovaginal swab	ST19	V
Blood	ST335	III
Rectovaginal swab	ST335	III
Blood	ST861	III
Rectovaginal swab	ST861	III
Blood	ST28	II
Rectovaginal swab	ST28	II
Tissue	ST3	II
Blood	ST3	III
Blood	ST3	III
Blood	ST3	II
Blood	ST26	V
Blood	ST26	V
Blood	ST26	V
Rectovaginal swab	ST26	V
Blood	ST130	IX
Vaginal swab	ST130	IX
Blood	ST283	III
Pus aspirate	ST283	III
Blood	ST283	III
Blood	ST12	Ib
Tissue	ST12	Ib
CSF	ST12	Ib
Blood	ST12	Ib
Blood	ST24	Ia
Blood	ST24	Ia
Pus	ST23	Ia
Blood	ST23	Ia
Blood	ST17	III
Blood	ST17	III
Blood	ST17	III
Blood	ST17	III
CSF	ST17	III
Blood	ST17	III

Figure 4.7: MLST phylogenetic tree

4.7 Statistical Analysis

Statistical analysis using chi-square test was performed in IBM SPSS version 25 to identify the significant associations between the serotypes and sequence types of GBS, as well as the sources of isolation, together with the predominant clinical syndrome and underlying medical condition which are sepsis and diabetes mellitus respectively. The significant relationship was indicated by p-value which is less than 0.05. As a result, there is no significant relationship between all serotypes of GBS and the sources as shown in Table 4.6. This same goes to the association between 15 sequence types and the sources that shows no potential significant between them as shown in Table 4.7. Besides, all the serotypes of GBS in this study did not differ by the major clinical syndrome which is sepsis, $p > 0.05$ as shown in Table 4.8. Additionally, as can be seen by the frequencies cross tabulated in Table 4.9, there is no significant relationship between all GBS serotypes and diabetes mellitus in this research. However, there is potential significant association between few serotypes of GBS and the most prevalent sequence type which is ST1 with $p < 0.05$ observed in serotype III, VI and VII (Table 4.10). This same goes to the association between few ST and the most prevalent serotype which is serotype III. As shown in table 4.11, there are potential significant association between

Table 4.7: The association between serotypes of GBS and the sources

Serotypes		Sources			X^2	P-Value
		Blood	CSF	Others		
Ia	**Yes**	3 (75.0%)	0 (0.0%)	1 (25.0%)	0.328	0.731
	No	23 (63.9%)	2 (5.6%)	11 (30.6%)		
Ib	**Yes**	2 (50.0%)	1 (25.0%)	1 (25.0%)	3.746	0.819
	No	24 (66.7%)	1 (2.8%)	11 (30.6%)		
II	**Yes**	2 (50.0%)	0 (0.0%)	2 (50.0%)	0.969	0.423
	No	24 (66.7%)	2 (5.6%)	10 (27.8%)		
III	**Yes**	9 (81.8%)	1 (9.1%)	1 (9.1%)	3.379	0.111
	No	17 (58.6%)	1 (3.4%)	11 (37.9%)		
IV	**Yes**	1 (50.0%)	0 (0.0%)	1 (50.0%)	0.459	0.581
	No	25 (65.8%)	2 (5.3%)	11 (28.9%)		
V	**Yes**	3 (75.0%)	0 (0.0%)	1 (25.0%)	0.328	0.731
	No	23 (63.9%)	2 (5.6%)	11 (30.6%)		
VI	**Yes**	4 (57.1%)	0 (0.0%)	3 (42.9%)	0.972	0.512
	No	22 (66.7%)	2 (6.1%)	9 (27.3%)		
VII	**Yes**	1 (33.3%)	0 (0.0%)	2 (66.7%)	2.116	0.182
	No	25 (67.6%)	2 (5.4%)	10 (27.0%)		
VIII	**Yes**	0 (0.0%)	0 (0.0%)	0 (0.0%)	-	-
	No	26 (65.0%)	2 (5.0%)	12 (30.0%)		
IX	**Yes**	1 (100.0%)	0 (0.0%)	0 (0.0%)	0.552	0.475
	No	25 (64.1%)	2 (5.0%)	12 (30.0%)		

Table 4.8: The association between sequence types of GBS and the sources

Sequence Types		Sources			X^2	P-Value
		Blood	CSF	Others		
1	**Yes**	8 (61.5%)	0 (0.0%)	5 (38.5%)	2.284	0.342
	No	18 (72.0%)	2 (8.0%)	5 (20.0%)		
3	**Yes**	2 (66.7%)	0 (0.0%)	1 (33.3%)	0.234	0.859
	No	24 (68.6%)	2 (5.7%)	9 (25.7%)		
12	**Yes**	1 (33.3%)	1 (33.3%)	1 (33.3%)	5.523	0.393
	No	25 (71.4%)	1 (2.9%)	9 (25.7%)		
17	**Yes**	4 (80.0%)	1 (20.0%)	0 (0.0%)	4.004	0.307
	No	22 (66.7%)	1 (3.0%)	10 (30.3%)		
19	**Yes**	1 (100.0%)	0 (0.0%)	0 (0.0%)	0.474	0.509
	No	25 (67.6%)	2 (5.4%)	10 (27.0%)		
23	**Yes**	0 (0.0%)	0 (0.0%)	1 (100.0%)	2.876	0.105
	No	26 (70.3%)	2 (5.4%)	9 (24.3%)		
24	**Yes**	1 (100.0%)	0 (0.0%)	0 (0.0%)	0.474	0.509
	No	25 (67.6%)	2 (5.4%)	10 (27.0%)		
26	**Yes**	3 (100.0%)	0 (0.0%)	0 (0.0%)	1.503	0.240
	No	23 (65.7%)	2 (5.7%)	10 (28.6%)		
28	**Yes**	1 (100.0%)	0 (0.0%)	0 (0.0%)	0.474	0.509
	No	25 (67.6%)	2 (5.4%)	10 (27.0%)		
130	**Yes**	1 (100.0%)	0 (0.0%)	0 (0.0%)	0.474	0.509
	No	25 (67.6%)	2 (5.4%)	10 (27.0%)		
196	**Yes**	1 (100.0%)	0 (0.0%)	0 (0.0%)	0.474	0.509
	No	25 (67.6%)	2 (5.4%)	10 (27.0%)		
283	**Yes**	1 (50.0%)	0 (0.0%)	1 (50.0%)	0.666	0.492
	No	25 (69.4%)	2 (5.6%)	9 (25.0%)		
335	**Yes**	1 (100.0%)	0 (0.0%)	0 (0.0%)	0.474	0.509
	No	25 (67.6%)	2 (5.4%)	10 (27.0%)		
459	**Yes**	0 (0.0%)	0 (0.0%)	1 (100.0%)	2.876	0.105
	No	26 (70.3%)	2 (5.4%)	9 (24.3%)		
861	**Yes**	1 (100.0%)	0 (0.0%)	0 (0.0%)	0.474	0.509
	No	25 (67.6%)	2 (5.4%)	10 (27.0%)		

Table 4.9: The association between serotypes of GBS and sepsis

Serotypes		Sepsis		X^2	P-Value
		Yes	No		
Ia	Yes	1 (25.0%)	3 (75.0%)	0.195	0.663
	No	13 (36.1%)	23 (63.9%)		
Ib	Yes	1 (25.0%)	3 (75.0%)	0.195	0.663
	No	13 (36.1%)	23 (63.9%)		
II	Yes	1 (25.0%)	3 (75.0%)	0.195	0.663
	No	13 (36.1%)	23 (63.9%)		
III	Yes	4 (36.4%)	7 (63.6%)	0.012	0.912
	No	10 (34.5%)	19 (65.5%)		
IV	Yes	1 (50.0%)	1 (50.0%)	0.208	0.652
	No	13 (34.2%)	25 (65.8%)		
V	Yes	2 (50.0%)	2 (50.0%)	0.440	0.513
	No	12 (33.3%)	24 (66.7%)		
VI	Yes	4 (57.1%)	3 (42.9%)	1.829	0.182
	No	10 (30.3%)	23 (69.7%)		
VII	Yes	0 (0.0%)	3 (100.0%)	1.746	0.192
	No	14 (37.8%)	23 (62.2%)		
VIII	Yes	0 (0.0%)	(0.0%)	-	-
	No	14 (35.0%)	26 (65.0%)		
IX	Yes	0 (0.0%)	1 (100.0%)	0.552	0.463
	No	14 (35.9%)	25 (64.1%)		

Table 4.10: The association between serotypes of GBS and diabetes mellitus

Serotypes		Diabetes mellitus		X^2	P-Value
		Yes	No		
Ia	Yes	3 (75.0%)	1 (25.0%)	2.269	0.137
	No	13 (36.1%)	23 (63.9%)		
Ib	Yes	2 (50.0%)	2 (50.0%)	0.185	0.671
	No	14 (38.9%)	22 (61.1%)		
II	Yes	2 (50.0%)	2 (50.0%)	0.185	0.671
	No	14 (38.9%)	22 (61.1%)		
III	Yes	2 (18.2%)	9 (81.8%)	3.009	0.087
	No	14 (48.3%)	15 (51.7%)		
IV	Yes	2 (100.0%)	0 (0.0%)	3.158	0.079
	No	14 (36.8%)	24 (63.2%)		
V	Yes	1 (25.0%)	3 (75.0%)	0.417	0.524
	No	15 (41.7%)	21 (58.3%)		
VI	Yes	2 (28.6%)	5 (71.4%)	0.462	0.502
	No	14 (42.4%)	19 (57.6%)		
VII	Yes	2 (66.7%)	1 (33.3%)	0.961	0.333
	No	14 (37.8%)	23 (62.2%)		
VIII	Yes	0 (0.0%)	0 (0.0%)	-	-
	No	16 (40.0%)	24 (60.0%)		
IX	Yes	0 (0.0%)	1 (100.0%)	0.684	0.414
	No	16 (41.0%)	23 (59.0%)		

Table 4.11: The association between serotypes and sequence type 1

Serotypes		Sequence Type 1		X^2	P-Value
		Yes	No		
Ia	Yes	1 (25.0%)	3 (75.0%)	0.169	0.685
	No	12 (35.3%)	22 (64.7%)		
Ib	Yes	1 (25.0%)	3 (75.0%)	0.169	0.685
	No	12 (35.3%)	22 (64.7%)		
II	Yes	1 (33.3%)	2 (66.7%)	0.001	0.974
	No	12 (34.3%)	23 (65.7%)		
III	Yes	0 (0.0%)	11 (100.0%)	8.050	0.005
	No	13 (48.1%)	14 (51.9%)		
IV	Yes	0 (0.0%)	2 (100.0%)	1.098	0.301
	No	13 (36.1%)	23 (63.9%)		
V	Yes	1 (25.0%)	3 (75.0%)	0.169	0.685
	No	12 (35.3%)	22 (64.7%)		
VI	Yes	6 (100.0%)	0 (0.0%)	13.702	0.001
	No	7 (21.9%)	25 (78.1%)		
VII	Yes	3 (100.0%)	0 (0.0%)	6.264	0.014
	No	10 (28.6%)	25 (71.4%)		
VIII	Yes	0 (0.0%)	0 (0.0%)	-	-
	No	13 (34.2%)	25 (65.8%)		
IX	Yes	0 (0.0%)	1 (100.0%)	0.534	0.471
	No	13 (35.1%)	24 (64.9%)		

Table 4.12: The association between sequence type and serotype III

Sequence Types		Serotype III		X^2	P-Value
		Yes	No		
1	Yes	0 (0.0%)	13 (100.0%)	8.050	0.005
	No	11 (44.0%)	14 (56.0%)		
3	Yes	2 (66.7%)	1 (33.3%)	2.253	0.139
	No	9 (25.7%)	26 (74.3%)		
12	Yes	0 (0.0%)	3 (100.0%)	1.327	0.256
	No	11 (31.4%)	24 (68.6%)		
17	Yes	5 (100.0%)	0 (0.0%)	14.132	0.001
	No	6 (18.2%)	27 (81.8%)		
19	Yes	0 (0.0%)	1 (100.0%)	0.418	0.523
	No	11 (29.7%)	26 (70.3%)		
23	Yes	0 (0.0%)	1 (100.0%)	0.418	0.523
	No	11 (29.7%)	26 (70.3%)		
24	Yes	0 (0.0%)	1 (100.0%)	0.418	0.523
	No	11 (29.7%)	26 (70.3%)		
26	Yes	0 (0.0%)	3 (100.0%)	1.327	0.256
	No	11 (31.4%)	24 (68.6%)		
28	Yes	0 (0.0%)	1 (100.0%)	0.418	0.523
	No	11 (29.7%)	26 (70.3%)		
130	Yes	0 (0.0%)	1 (100.0%)	0.418	0.523
	No	11 (29.7%)	26 (70.3%)		
196	Yes	0 (0.0%)	1 (100.0%)	0.418	0.523
	No	11 (29.7%)	26 (70.3%)		
283	Yes	2 (100.0%)	0 (0.0%)	5.182	0.025
	No	9 (25.0%)	27 (75.0%)		
335	Yes	1 (100.0%)	0 (0.0%)	2.521	0.117
	No	10 (27.0%)	27 (73.0%)		
459	Yes	0 (0.0%)	1 (100.0%)	0.418	0.523
	No	11 (29.7%)	26 (70.3%)		
861	Yes	1 (100.0%)	0 (0.0%)	2.521	0.117
	No	10 (27.0%)	27 (73.0%)		

CHAPTER 5

DISCUSSION

In our study, multiplex PCR revealed the distribution of the serotypes which serotype III (27.5%) was the most prevalent, followed by serotype VI (17.5%), serotype Ia, Ib, II, V (10.0% each), serotype VII (7.5%), serotype IV (5.0%), and serotype IX (2.5%). This result was consistent with a study in Alberta, Canada from 2003 to 2013 which found that serotype III (20%) was the most common, followed by serotypes V (19%), Ia (19%), Ib (13%), and II (11%) in a research of serotypes linked to invasive GBS illness (Alhhazmi, Hurteau & Tyrrell, 2016). On the other hand, there was an epidemiological research specifically in Malaysia conducted by Suhaimi et al. (2017), found that serotype Ia was the most prevalent serotype (n = 27, 45%), followed by serotype III (n = 10, 16.7%), V (n = 9, 15%), VI (n = 8, 13.3%), VIII (n = 2, 3.3%), and VII (n = 1, 1.7%). Also, other local study from a teaching hospital which revealed by Karunakaran et al. (2009), Ia was the most prevalent serotype, accounting for 22.2% of 45 isolates followed by serotype VI (17.8%), III and V (13.3% each). Additionally, in another GBS Malaysian study, serotype Ia was the prevalent capsular polysaccharide (38.9%) among the 18 GBS isolates in adults and neonates, followed by VI, V and III (Eskandarian et al., 2013). In contrast, another study was done in the following year at Malaysian hospital which serotype VI (22.3%) was found to be significantly overrepresented from the total of 103 GBS isolates compared to an earlier research among adults and newborns, followed by VII (21.4 %), III (20.4%), Ia (17.5%), V (9.7%), II (7.7 %) and IV (1 %) (Eskandarian, 2014).

For the ST, MLST studies revealed 15 STs among 38 GBS isolates in this research, with ST1 being the most common which contribute approximately 32.5% of the strain collection. A previous study conducted by Ezhumalai et al. (2020), from a Malaysian hospital setting, ST1 being the most common which accounted for 42.0% among the 51 viable GBS isolates. In another study, ST1, ST17, ST19, and ST23 comprised up two-thirds of a strain collection from the United Kingdom, the United States, Japan, New Zealand, Israel, Singapore, and Thailand, according to Jones et al (2003).

In this research, all the 38 isolates from the five hospital settings were invasive with ST1 being the most prevalent (32.5%) followed by ST17 (12.5%); ST3, ST12, ST26 (7.5%); ST283 (5.0%); ST19, ST23, ST24, ST28, ST130, ST196, ST335, ST459 and ST861 (2.5%). These findings are in line with those of a research performed in local setting which found that ST1 was the most frequent invasive ST (60.9%), followed by ST28, ST23 (8.7% for each); ST19, ST24, ST144, ST103, ST485 (4.3% for each) (Ezhumalai et al., 2020). This is similar to Taiwan epidemiological study conducted by Tien et al. (2011), which found that ST1 was the most frequent invasive ST (29.4%), followed by ST12 (20.6%), ST17 (17.6%), and ST23 (14.7%). Specifically, ST1 strains were the most invasive in adults, whereas ST17 strains were the most invasive in newborns; while ST19 was found in both adults and newborns. A Swedish research, on the other hand, found that the invasive STs were ST17, ST19, ST23, and ST1 (Luan et al., 2005). In another study in Taiwan, ST17 a hypervirulent clone, was responsible for 68% of invasive GBS illnesses (Lee et al., 2019).

GBS serotypes Ia, Ib, II, III, V, VI and VII were the most common in ST1 in our research. Jones et al. (2003) identified GBS serotypes Ib, III, V, VI, VIII, and the non-typeable serotype in ST1, which is mostly consistent with our findings. ST17 was found to exclusively contain serotype III, which was linked to invasive neonatal illness in many investigations (Ezhumalai et al., 2020; Jones et al., 2003; Tien et al., 2011; Luan et al., 2005). Similar to our research which discovered that ST17 contained serotype III, but this serotype was linked to invasive illness in both in neonates and adults. In a similar research, Wang et al. (2015), discovered that ST17 predominated, accounting for 80% of serotype III invasive GBS isolates. ST19 was also shown to have a GBS serotype III that was linked to colonising infection, according to Martins et al. (2012) which contradict to our research that found serotype Ia in ST19.

In another investigation conducted by Tien (2011) revealed that serotype V was mainly found in ST1. According to Luan et al. (2005), serotype V has been documented among non-pregnant individuals in the United States and Sweden since the late 1990s. After serotypes Ia, Ib, II, and III, Yoon et al. (2015) found that serotype V was the sixth most frequent serotype, causing infection in both newborns and adults. All these studies were contrasted with but our research as there is only 2.6% (n = 1) among the 38 GBS isolates accounted for serotype V in ST1. Serotype VI was the most common serotype found in ST1 (n = 6) in this research. Serotype VI was shown to be the most common in ST1 according to a Taiwanese research conducted by Lin et al. (2016).

Last but not least, by looking at the result of phylogenetic analysis in this study, the clades demonstrated a distinct segregation of the STs. For instance, certain GBS strains or particularly ST1 have been identified from all hospitals involved in this study. This suggest that the genetic lineages have been dispersed. As mentioned by Dzaraly et al. (2021), according to the phylogenetic tree, many isolates have a similar genetic ancestry. Besides, as a consequence of their grouping, the majority of isolates had a significant degree of genetic similarity, with some having comparable serotypes and STs. Therefore, it is reasonable to assume that they started from a single source and subsequently spread across the population.

CHAPTER 6

CONCLUSION

6.1 Conclusion

In conclusion, there is a variation in the serotypes distribution and sequence types (STs) by looking at the patterns in constructed phylogenetic tree of invasive GBS strains that isolates from these five different Malaysian hospitals. The genotypic distribution patterns and MLST analysis on GBS in this research were mostly consistent with earlier findings from other countries.

6.2 Recommendation

This implies that some specific genotypes tend to predominate in human infection settings. Therefore, for epidemiological research and vaccine development in Malaysia, further monitoring of GBS from different geographical regions is necessary

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APPENDICES

Appendix A

Gantt Chart

	2020				2021			
	January	**February**	**March**	**April**	**May**	**June**	**July**	**August**
Proposal writing								
Ethical approval								
Isolation and Identification								
DNA extraction and PCR								
MLST								
Data analysis								
Thesis writing and submission								

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(*Medical Research & Ethics Committee*)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Kompleks Institut Kesihatan Negara
Blok A, No 1, Jalan Setia Murni U13/52,
Seksyen U13, Bandar Setia Alam,
40170 Shah Alam, Selangor.
Tel: 03-3362 8888/8205

Ref : KKM/NIHSEC/ P19-962 (9)
Date: 04-February-2020

ABDULRAHMAN MANSOOR MOHAMMED MUTHANNA
UNIVERSITY PUTRA MALAYSIA (UPM)

Dear Sir/ Mdm,

AMENDMENTS FOR STUDY: NMRR-19-876-46665 (IIR)
Protocol No :
Identification and molecular characterization of Streptococcus agalactiae isolated from Malaysian Hospitals

Your amendment submission dated 22-January-2020 is referred.

2. Amendments of the following have been received and reviewed with reference to the above study:

Documents received and reviewed with reference to the above study:

1. Declaration of Conflict of Interest (COI), version No 2 and version date 31-12-2019.
2. Study Proposal, version No 3 and version date 31-12-2019.
3. Recording form(Data collection form), version No 3 and version date 31-12-2019.
4. Patient information sheet (English) & Informed Consent Form (English) version No 2 and version date 31-12-2019.
5. Patient information sheet (BM) & Informed Consent Form (BM) version No 2 and version date 31-12-2019.
6. Addition of study sites:
   a. Hospital Melaka
   b. Hospital Sungai Buloh
7. Addition of new investigators:

Investigators	Study Sites	Investigators' role	Investigator's document
Dr. Lim Bee Bee	Hospital Melaka	Co / Sub Investigator at the site	CV, GCP Certificate
Dr. Chua Hui Shan	Hospital Melaka	Co / Sub Investigator at the site	CV
Dr. Nur Hanani Binti Ahmad	Hospital Sungai Buloh	Co / Sub Investigator at the site	CV, GCP Certificate

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(*Medical Research & Ethics Committee*)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Kompleks Institut Kesihatan Negara
Blok A, No 1, Jalan Setia Murni U13/52,
Seksyen U13, Bandar Setia Alam,
40170 Shah Alam, Selangor.
Tel: 03-3362 8888/8205

Ruj.Kami: KKM/NIHSEC/ P19-962 (6)
Tarikh : 03 -Jun-2019

ABDULRAHMAN MANSOOR MOHAMMED MUTHANNA
UNIVERSITY PUTRA MALAYSIA (UPM)

Dato'/ Tuan/ Puan,

SURAT KELULUSAN ETIKA: NMRR-19-876-46665 (IIR)
Identification and molecular characterization of Streptococcus agalactiae isolated from Malaysian Hospitals

Dengan hormatnya perkara di atas adalah dirujuk.

2. Bersama dengan surat ini dilampirkan surat kelulusan saintifik dan etika bagi projek ini. Segala rekod dan data subjek adalah SULIT dan hanya digunakan untuk tujuan kajian dan semua isu serta prosedur mengenai *data confidentiality* mesti dipatuhi. Kebenaran daripada Pengarah Hospital / Institusi di mana kajian akan dijalankan mesti diperolehi terlebih dahulu sebelum kajian dijalankan. Dato' / Tuan / Puan perlu akur dan mematuhi keputusan tersebut dan undang-undang lain yang berkaitan, termasuklah Akta Akses Kepada Sumber Biologi dan Perkongsian Faedah 2017.

3. Penyelidik- penyelidik dan lokasi kajian yang terlibat ialah:

HOSPITAL SERDANG
Dr Lailatul Akmar binti Mat Nor

HOSPITAL SULTANAH AMINAH
Dr Anis Roziana binti Mohmad

HOSPITAL TUANKU JAAFAR, SEREMBAN
Dr Marlindawati Mohd.Ali

PUSAT PERUBATAN UNIVERSITI KEBANGSAAN MALAYSIA (PPUKM)
Dr Zalina binti Ismail

UNIVERSITY PUTRA MALAYSIA (UPM)
Dr Mohd Nasir Mohd Desa
Dr Syafinaz Binti Amin Nordin
Abdulrahman Mansoor Mohammed Muthanna (Penyelidik Utama)

4. Adalah dimaklumkan bahawa kelulusan ini adalah sah sehingga **02-Jun-2020.** Tuan/Puan perlu menghantar dokumen-dokumen seperti berikut selepas mendapat kelulusan etika. Borang-borang berkaitan boleh dimuat turun daripada laman web Jawatakuasa Etika & Penyelidikan Perubatan (JEPP) (http://www.nih.gov.my/mrec).

i. ***Continuing Review Form*** selewat-lewatnya dalam tempoh 2 bulan (60 hari) sebelum tamat tempoh kelulusan ini bagi memperbaharui kelulusan etika.
ii. ***Study Final Report*** pada penghujung kajian.
iii. Mendapat kelulusan etika sekiranya terdapat pindaan ke atas sebarang dokumen kajian / lokasi kajian / penyelidik. Pihak JEPP mempunyai hak untuk menarik balik kelulusan etika sekiranya terdapat perubahan dokumen kajian yang tidak diisytiharkan.

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Kompleks Institut Kesihatan Negara
Blok A, No 1, Jalan Setia Murni U13/52,
Seksyen U13, Bandar Setia Alam,
40170 Shah Alam, Selangor.
Tel: 03-3362 8888/8205

Ref : KKM/NIHSEC/ P19-962 (7)
Date: 03 -June -2019

ABDULRAHMAN MANSOOR MOHAMMED MUTHANNA
UNIVERSITY PUTRA MALAYSIA (UPM)

Dear Sir / Mdm,

ETHICS INITIAL APPROVAL: NMRR-19-876-46665 (IIR)
Identification and molecular characterization of Streptococcus agalactiae isolated from Malaysian Hospitals

This letter is made in reference to the above matter.

2. The Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (MOH) has provided ethical approval for this study. Please take note that all records and data are to be kept strictly **CONFIDENTIAL** and can only be used for the purpose of this study. All precautions are to be taken to maintain data confidentiality. Permission from the District Health Officer / Hospital Administrator / Hospital Director and all relevant heads of departments / units where the study will be carried out must be obtained prior to the study. You are required to follow and comply with their decision and all other relevant regulations, including the Access to Biological and Benefit Sharing Act 2017.

3. The investigators and study sites involved in this study are:

   HOSPITAL SERDANG
   Dr Lailatul Akmar binti Mat Nor

   HOSPITAL SULTANAH AMINAH
   Dr Anis Roziana binti Mohmad

   HOSPITAL TUANKU JAAFAR, SEREMBAN
   Dr Marlindawati Mohd.Ali

   PUSAT PERUBATAN UNIVERSITI KEBANGSAAN MALAYSIA (PPUKM)
   Dr Zalina binti Ismail

   UNIVERSITY PUTRA MALAYSIA (UPM)
   Dr Mohd Nasir Mohd Desa
   Dr Syafinaz Binti Amin Nordin
   Abdulrahman Mansoor Mohammed Muthanna (Penyelidik Utama)

4. The following study documents have been received and reviewed with reference to the above study:

Documents received and reviewed with reference to the above study:

1. Study Protocol_Version 2, dated 27-May-2019
2. Patient Information Sheet & Informed Consent Form_English_Version 1, dated 27-May-2019
3. Patient Information Sheet & Informed Consent Form_Malay_Version 1, dated 27-May-2019
4. Data Collection Form_Version 1, dated 22-Apr-2019
5. Investigator's documents : Declaration of Conflict of Interest (COI), IA-HOD-IA, and CV: