Epidemiology of *Streptococcus agalactiae* (Group B Streptococci) in Dublin Maternity Hospitals

A thesis presented for the degree of Doctor of Philosophy

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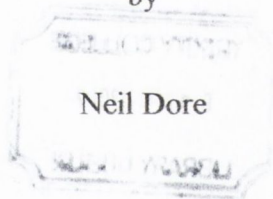
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Table of Contents

Chapter 1 Introduction

Chapter 3 Serotype and Sepsis

iv

Index of figures

Index of tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Declaration

This thesis is submitted by the undersigned to the University of Dublin for examination for the degree of Doctor of Philosophy. The work herein is entirely my own and has not been previously submitted for a degree to this or any other university.

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Neil Dore

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To my parents, Sheila and David

and to Laura

Summary

Streptococcus agalactiae (Group B Streptococci) are Gram-positive encapsulated cocci distinguished from other streptococci by their narrower zones of β-haemolysis. Group B Streptococci (GBS) was first described as an important pathogen in the 1970's and since that time has remained the primary cause of bacterial infection in new-born babies, resulting in disease at birth and up to 3 months of age.

GBS often colonises the vagina, although carriage here may be intermittent. At any one time, the vaginae of approximately a quarter of all women of childbearing age in Ireland are colonised with GBS. This organism becomes particularly important in childbirth especially if there are signs of infection. GBS causes two types of infection in new-born babies depending on time of infection onset: termed early and late-onset. A number of predisposing factors, in addition to vaginal carriage during pregnancy indicate an increased risk of infant GBS sepsis and suggest prompt antimicrobial treatment. These factors include obstetric complications, low birth weight, foetal hypoxia, the presence of GBS in the maternal urine and the birth of a previous sibling with GBS disease. This research project sought to generate essential epidemiological and virulence-related information about GBS isolates associated with invasive disease in Ireland.

Group B streptococcal isolates (159) obtained from three maternity hospitals in Dublin were serotyped. The serotype distribution of the isolates was: Ia, 19.5%; Ib, 18.9%; II, 10.7%; III, 29.5%; IV, 1.9%; V, 15.1%; non-typeable, 4.4%. There was a statistically significant association between the serotype and invasive status (carriage or infection) of isolates ($P < 0.005$), but no significant association between serotype and degree of invasiveness was demonstrated.

The discriminatory capacity of Random amplified polymorphic DNA (RAPD) fingerprinting and Pulsed-field gel electrophoresis (PFGE) typing were determined in order to evaluate their suitability for epidemiological analysis of GBS. The Simpson's index of diversity, D, for the RAPDnm primer with Irish isolates was 0.989, while an index of 0.918 was obtained for the RAPD1 primer. For PFGE using the restriction endonuclease *SmaI* the Simpson's index of diversity of Irish GBS isolates was 0.956. Results showed genetic heterogeneity not only between different serotypes but also among

isolates belonging to same serotype, suggesting that molecular genotyping may be more advantageous than capsular serotype in differentiating GBS isolates. Nine RAPDnm type groups, 7 RAPD1-type groups, and 8 PFGE groups were identified with isolates having a similarity averages of 57, 67.7 and 73%, respectively. The data show that the RAPDnm primer demonstrated a greater discriminatory power than the RAPD1 primer.

All 159 isolates were analysed for the *bac*, *bca*, *hylB*, *pepB*, and *rib* genes. The presence or absence of each gene was not associated with the invasive status of isolates. Statistically significant associations were revealed between *bca* and *hylB*(IS*1548*) ($P =$ 0.0004) and between *bac* and *bca* ($P = 0.014$). The *bac*, *bca*, *hylB*(IS*1548*) and *rib* genes and the numbers of tandem repeats in the *bca* gene showed significant associations with serotype. Almost 50% of serotype III isolates possessed either the *bac* gene or *bca* gene or both and 55–65% of strains of serotypes Ia, Ib and II possessed the *rib* gene. Most serotype III isolates had IS*1548* in their *hylB* genes. Serotype Ib was the only serotype in which more than half of the strains contained more tandem repeats in the *bca* gene than the overall mean for the GBS population studied of 7.4 repeats.

Publication arising from thesis

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Abbreviations

bp:	basepairs
BSA:	bovine serum albumin
c.f.u:	colony forming units
c.p.m.:	counts per minute
CDC:	Centres for Disease Control
δ:	standard deviation
dATP:	2' - deoxyadenosine triphosphate
dCTP:	2' - deoxycytidine triphosphate
dGTP:	2' - deoxyguanosine triphosphate
DNA:	deoxyribonucleic acid
dTTP:	2' - deoxythymidine triphosphate
EDTA:	ethylenenediaminetetra - acetic acid, tetrasodium salt
EtBr:	ethidium bromide
FSB:	final sample buffer
h:	hour
ICUD:	intra-uterine contraceptive device
Ig:	immunoglobulin
kb:	kilobase pairs or 1000 bp
L agar:	Luria agar
L broth:	Luria broth
M:	molar
µg:	microgram
mg:	milligram
min:	minute
µl:	microlitre
ml:	millilitre
µM:	micromolar
mM:	millimolar

NJ:	neighbour joining
PCR:	polymerase chain reaction
r.p.m.:	revolutions per minute
RAPD:	random amplified polymorphic DNA
RFLP:	restriction fragment length polymorphism
RNA:	ribonucleic acid
s:	second
S_D:	similarity coefficient
SDS:	sodium dodecyl sulphate
SSC:	salt, sodium citrate
TBE:	tris-borate EDTA
Tris:	tris (hydroxymethyl) aminoethane
UPGMA:	unweighted-pair group method average linkage
UV:	ultraviolet
v/v:	volume per volume
w/v:	weight per volume

CHAPTER 1

Introduction

1.1 Historical Background

Group B streptococci (GBS) or *Streptococcus agalactiae* was first named *Streptococcus agalactiae contagiosae* by Kitt (1893) when the bacterium was first discovered in milk and infected udders of cows, leading to economic problems in the dairy industry (Bergey, 1957). However, the story of GBS and GBS human disease really began with Lancefield (1933). In Lancefield's initial serological studies, GBS were of bovine origin, primarily from acute infections such as bovine mastitis, as they had not yet been detected among streptococcal strains of human origin. However, when streptococci from a wider variety of clinical conditions and from healthy carriers were examined, GBS were detected quite often (Brown, 1939). Through her collaboration with Dr. Colebrook and Dr. Hare at Queen's Charlotte's Hospital, London Lancefield first described human strains from women whose puerperium was afebrile and also from urinary tract infections (UTI's) (Hare and Colebrook, 1934).

Lancefield (1934) described three type-specific polysaccharide antigens of GBS (i.e., types I, II and III). She believed that some mucoid strains of streptococci depended on capsular polysaccharide for virulence and showed that elicited antibodies to the type-specific polysaccharides of GBS were protective in mice. The type I antigen was resolved later into two types, namely, Ia and Ib (Lancefield, 1938).

Between the years 1934–1941, the first reports of GBS sepsis and fatality were documented. GBS were at one time only seen as unusual cases of postpartum infection (Fry, 1938) but since the 1960's these facultative anaerobes have been recognised as an important pathogen and have emerged the primary cause of bacterial infection in new-born babies in hospitals around the world (Hood *et al.*, 1961). The increased incidence and importance of GBS was further highlighted by Eickhoff *et al.* (1964), who described an overall neonatal sepsis rate of 5.2% in the Boston City Hospital over a nineteen month period. GBS were found to be the most common pathogen and were the primary pathogen in ten cases of septic abortion. Serological typing using Lancefield's sera revealed that type Ia was the predominant serotype in these early-onset neonatal infections. Butter and de Moor (1967) published a paper on GBS describing 22 cases of GBS neonatal

1

meningitis and four cases of sepsis over a 6-year period. Other papers from around the world soon followed indicating clearly that GBS were a leading cause of neonatal infection (e.g., Cayeux, 1972; McCracken, 1973; Quirante *et al.*, 1974). This trend is seen to this date with the epidemiology of GBS receiving considerable attention. In 1996 the Centres for Disease and Control and Prevention (CDC), the American College of Obstetricians and Gynaecologists (ACOG), and the American Academy of Paediatrics (AAP) issued consensus guidelines on the prevention of perinatal GBS disease (Schuchat, 1996).

1.2 GBS Bacteriology

Even prior to the development of Lancefield's serological classification of streptococci, GBS were recognised as a distinct species, called *Streptococcus agalactiae*, by their characteristic colony morphology and haemolytic activity (Ayers *et al.*, 1922). GBS are non-motile, Gram-positive encapsulated cocci, which grow as grayish mucoid colonies on blood agar plates. They are distinguished from other streptococci by their zone of β-haemolysis on blood agar plates, which is much narrower and less definite than those of other streptococci of human origin (Fig. 1.1).

1.3 GBS Carriage

Although babies can acquire GBS from hospital sources or from other members of the family, the maternal genital tract is the main reservoir leading to infection of neonates. Around a third of all adults carry GBS as a normal commensal in their intestines that, once present, rarely can be eradicated (Hall *et al.*, 1976). In addition GBS often colonise the vagina, although carriage here may be intermittent. At any one time, the vagina in approximately one in four women of child-bearing age in Ireland is colonised with GBS (Kieran *et al.*, 1998; Cafferkey M, unpublished data). Between 50–70% of infants born to vaginal carriers have been shown to acquire GBS from their mothers (Dillon *et al.*, 1987; Stoll and Schuchat, 1998). The relative density of GBS in the birth canal influences the occurrence of neonatal disease (Anthony *et al.*, 1979; Hoogkamp-Korstanje *et al.*, 1982).

Table 1.1 summarises the factors that have been shown to increase the

2

Figure 1.1. Colony morphology of Group B Streptococci (GBS) on blood agar and Gram stain appearance.

(A) GBS streaked on a blood agar plate with zones of β-haemolysis shown in insert.
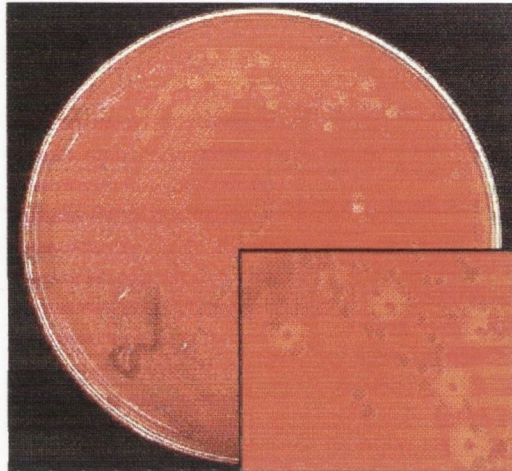
(B) Gram stain showing Gram-positive cocci in chains.

⊢⊣
1 μm

likelihood of GBS vaginal carriage. Vaginal carriage has been shown to be more common in youngerwomen, both pregnant and non-pregnant, especially in teenagers. GBS carriage occurs significantly more often among African Americans, suggesting that ethnic background may be a factor associated with increased carriage. In non-pregnant women, GBS is more often demonstrable in the first half of the menstrual cycle; this is possibly related to increased GBS adherence to the vaginal epithelium at this time (Tamura *et al.*, 1994). Finally, higher sexual activity among women has been shown to be associated with higher carriage of GBS. Supportive evidence of this is that male consorts of vaginal carriers have been shown to harbour the same strains of GBS (Gardner *et al.*, 1979; Yamamoto *et al.*, 1999).

Apart from the host factors that increase the likelihood of GBS carriage, the organism itself plays a role. GBS have the ability to adhere to surface receptors such as fibronectin and laminin, which are present on human epithelial cells (Tamura and Nittayajarn, 2000). Recently, GBS have also been found to adhere to cytokeratin (Tamura and Nittayajarn, 2000). It is hypothesised that the adherence to cytokeratin, as well as other factors (Section 1.4), is important for colonisation by GBS at sites of keratinised epithelium such as the vagina or of damaged epithelial cells at other sites.

Table 1.1. Factors which increase the prevalence of carriage.

Factors	Reference
Maternal age (<20 years)	Ancona *et al.*,1980
Ethnic background	Zangwill *et al.*,1992; Goldenberg *et al.*, 1995; Cambell *et al.*, 2000
Phase of menstrual cycle (1st half)	Baker *et al.*,1977
Sexual transmission	Baker *et al.*,1977; Yamamoto *et al.*,1999

1.4 GBS Infections

GBS, an aetiological agent of bovine mastitis, were considered for many years to be an occasional and opportunistic agent of human disease but were subsequently recognised as a major cause of morbidity and mortality in the neonatal period, frequently causing bacteraemia and meningitis. In adults, GBS occur most often in certain groups, such as diabetics, pregnant and post-partum women, and patients who are immunocompromised, in whom they cause serious local infections and disseminated disease (Wessels *et al.*, 1993; Hussain *et al.*, 1995). Over the last 27 years GBS have been isolated with increasing frequency as the primary aetiological agent of a wide variety of human pathological conditions, including neonatal sepsis and meningitis, omphalitis, septicaemia, puerperal infections including septic abortion, UTI, endocarditis, pneumonia empyema, abscesses, wound and skin infections, peritonitis, osteomyelitis, arthritis, and otitis media (Wilkinson *et al.*, 1973; Jackson *et al.*, 1995)

Meningitis is described as acute inflammation of the membranes covering the brain or spinal cord, or both (Huang *et al.*, 2000). It can be caused by bacteria, viruses, protozoa, yeasts, and fungi, usually introduced from elsewhere in the body (Shanson, 1988). The characteristic clinical features of acute meningitis include headache, irritability, fever, and neck stiffness. Bacterial meningitis can be primary or secondary. Primary meningitis develops through the spread of bacteria from the bloodstream to the meninges, while the less commonly seen secondary meningitis arises from the direct spread of infection from infected ears, trauma or surgery. However, neonates, the elderly and the immunocompromised patients may develop meningitis without such characteristic features, e.g., nausea and vomiting, to suggest a central nervous system (CNS) infection. Examination of the cerebrospinal fluid by means of a lumbar puncture permits a specific diagnosis. Antibiotic therapy has reduced mortality and decreased the incidence of such complications as brain damage and paralysis.

Puerperal infection is suspected when the mother's temperature rises to $\geq 38°C$ on any two successive days after the first 24 h post partum and other causes are not apparent. Even in the first 12 h post partum, a significant fever must be evaluated by examining the lungs and uterus and obtaining cultures of the urinary tract. The most common cause of

fever in early puerperium is dehydration, but after 2 or 3 days of low-grade fever, an abrupt rise in temperature indicates infection. Infections directly related to delivery commonly affect the genital tract, occurring in the uterus or parametria. Bladder and kidney infections also commonly occur soon after delivery. Other causes of fever, such as pelvic thrombophlebitis and breast infection, tend to occur after the 3rd day postpartum. Febrile chorioamnionitis during labour may be followed by secondary endometritis, myometritis, parametritis, or puerperal pyrexia. Certain predisposing conditions are associated with an increased risk of normal vaginal bacteria, such as anaerobic streptococci and staphylococci, to migrate to the uterus and to cause disease during the puerperium; these include anaemia, prolonged rupture of the membranes, prolonged labour, operative or traumatic delivery, repeated examination, retention of placental fragments within the uterus, and post-partum haemorrhage. The same predisposing conditions enable other bacteria to multiply in the uterus and vagina, the most commonly found organisms being *E. coli*, coagulase-negative staphylococci, enterococci and anaerobic cocci. Although vaginal delivery cannot be made sterile, post-partum infections are uncommon today because of better aseptic techniques.

Puerperal infection typically begins with uterine tenderness as a sign of uterine infection. Uncommonly, peritonitis or pelvic thrombophlebitis (with risk of pulmonary embolism) or both may complicate the illness. Endotoxaemia, endotoxic shock, and renal tubular or cortical necrosis may follow particularly more serious forms of puerperal sepsis and may be fatal.

GBS can also cause pneumonia that has characteristic clinical featues of coughing, pleuritic chest pains and fever. The pneumonia, which is more acute and severe than atypical pneumonia due to *Mycoplasma* or non-bacterial organisms, can cause production of mucopurulent sputum. Empyema, which is defined as a collection of pus within the pleural cavity, may be the result of a primary infection or result from infection of a pleural effusion or haemothorax.

Osteomyelitis is an acute or chronic infection of bone and bone marrow and is characterized by pain, high fever, and an abscess at the site of infection. The infection

which may be caused by a variety of microorganisms gaining access to the bone through an open wound or fracture or through the bloodstream.

Septic arthritis is described as a clinical manifestation of late-onset disease in neonates and requires prolonged antibiotic treatment to ensure an uncomplicated outcome. In adults, septic arthritis due to GBS has also been documented and is often associated with age and risk factors such as diabetes mellitus, cancer, cardiovascular disease, chronic renal insufficiency, alcoholism, intravenous drug abuse, human immunodeficiecy viral infection, neurological disease and cirrhosis.

Otitis media is an infection or inflammation of the middle ear more commonly caused by *Streptococcus pneumonia* and *Haemophilus influenzae* but also GBS. This inflammation often begins when infections that cause sore throats, colds, or other respiratory or breathing problems spread to the middle ear. Seventy-five percent of children experience at least one episode of otitis media by their third birthday. Almost half of these children will have three or more ear infections during their first three years. It is estimated that medical costs and lost wages because of otitis media amount to $3.5 billion dollars a year in the United States (Swarts *et al.*, 2000). Although otitis media is primarily a disease of infants and young children, it can also affect adults.

1.5 Risk Factors for GBS Infection of Neonates

A number of predisposing factors (Table 1.2), in addition to vaginal carriage during pregnancy, indicate an increased risk of infant GBS sepsis and suggest the need for antimicrobial prophylaxis. These factors include the obstetric complications detailed previously (Section 1.4), low birth weight, foetal hypoxia, the presence of GBS in the maternal urine and the birth of a previous sibling with GBS disease. Colonisation plus one or more obstetric predisposing factors increases the risk of infant GBS disease by approximately 12-fold. At least 75% of early-onset GBS infection and 90% of resultant deaths follow deliveries with one or more of these risk factors. Although GBS are sensitive to common antibiotics like penicillin, successful treatment of infections is often hampered by the rapidity with which the disease develops.

7

Table 1.2. Predisposing factors for increased risk of GBS disease (Schuchat, 1998).

Risk Factors

Premature delivery

Low birth weight

Increased interval between membrane rupture and delivery

Rupture of membranes before labour onset

Amnionitis, Intrapartum fever

Maternal vaginorectal colonisation with GBS

African American race

Maternal age, <20 years

GBS bacteriuria during current pregnancy

Low level of antibody to type-specific capsular polysaccharide

Previous stillbirth or spontaneous abortion

Multiple gestation/sibling of affected twin

Previous delivery of infant with GBS disease

Caesarean infection

Urinary tract infection in pregnancy

Prolonged duration of intrauterine monitoring

Degree of colonisation

Prolonged rupture of membranes

1.6 Group B Streptococcal Disease in Infants

This organism becomes particularly important in childbirth especially when there are signs of infection. GBS cause two types of infection in newborn babies, depending on the time of onset of infection, termed early- and late-onset (Table 1.3). GBS has been recognised as a cause of premature delivery, stillbirths, late miscarriage and, in some studies, preterm rupture of membranes (Regan *et al.*, 1981). Pregnancy-associated GBS disease is most often manifest during labour or within the first few days of an infant's life and is termed early-onset disease. Newborn babies can acquire GBS by aspiration of infected amniotic fluid or during passage through the birth canal. Since the 1970s, GBS have been the principal cause of sepsis and meningitis during the first week of life (i.e., early-onset disease). GBS also cause late-onset infections (at >7 days of age but rarely after the third month) (Gardner *et al.*, 1980).

1.6.1 Early-onset disease

Approximately 80% of all GBS infections occur in newborn babies within the first few hours of birth or as late as the 6th day of life and are termed early-onset. Signs of infection are usually apparent at birth. Newborns with early-onset GBS disease acquire the organism intrapartum from their mothers, who are colonised with GBS in the genital tract. Most early-onset disease results from the spread of the organism in the amniotic fluid and leads to invasive disease in some infants, with perinatal transmission often occurring across intact membranes. Early-onset GBS infection is normally characterised by the rapid development of respiratory distress, associated with septicaemia (CDC, 1997). Infection in babies is commonly associated with a number of maternal obstetric complications, such as premature or prolonged rupture of membranes at delivery, preterm delivery, septic or traumatic delivery and maternal peripartum fever (Hillier *et al.*, 1995; Schuchat *et al.*, 1996).

The reported attack rate worldwide for this type of disease is 0.7–3.7/1000 live births with mortality rates ranging from 50–75% (Boyer, 1995; Baker and Edwards, 1995; Schuchat and Schrag, 2000). With premature babies this mortality rate can approach 90–

Table 1.3. Main symptoms of GBS in neonates (Ferrieri, 1985).

Early-onset GBS disease [frequently seen at birth or within a few hours of birth]

A.   Pneumonia with bacteraemia

Respiratory distress syndrome with bacteraemia

Transient respiratory distress ('wet lung') with bacteraemia

B.   Bacteraemia with meningitis

C.   Bacteraemia without meningitis

D.   Pneumonia without bacteraemia (heavily colonised with GBS at mucosal sites)

Late-onset GBS disease

A. Meningitis (with or without detectable bacteraemia)

B. Osteomyelitis (with or without detectable bacteraemia)

Table 1.4. Incidence of neonatal GBS disease from 1986–1996 in the United States (Schuchat, 1998).

Setting	Date of Study	No. of Births	Incidence EOND*	LOND**
Single hospital	1986–1994	119,931	1.95	0.24
Nine hospitals	1987– 1989	61,809	3.21	
Multistate population based	1990	180,000	1.4	0.3
Multistate population based	1991–1992	124,464	1.8	
Twelve hospitals	1991–1993	7,606[a]	5.9[a]	7.7[a]
Multistate population based	1993–1995	190,000	1.7	

[*] Early Onset Neonatal Disease (cases per 1,000 births)

[**] Late Onset Neonatal Disease (cases per 1,000 births)

[a] Very low birth weight (<1,500 g) only; number of births for late-onset rate was 6,911

Table 1.5. Incidence of early-onset group B streptococcal disease in Active Bacterial Core Surveillance Areas, United States, 1998–1999 (Schuchat, 1999).

State	1998		1999	
	No.	Incidence[†]	No.	Incidence
California	16	0.39	14	0.34
Connecticut	21	0.49	10	0.23
Georgia	55	0.92	44	0.74
Maryland	33	0.47	27	0.38
Minnesota	28	0.43	25	0.39
New York[¥]	7	0.49	1	0.07
Oregon	11	0.56	10	0.51
Tennessee	19	0.58	18	0.55
Total	190	0.55	153	0.39

[†] Calculated as cases per 1,000 live births.
[¥] Cases from seven Rochester counties.

100%. The attack rates for early-onset disease can vary from hospital to hospital (Table 1.4 and Table 1.5). When meninigitis is seen, which is rare in early-onset disease, serotype III isolates account for four out of five of the cases (Anthony *et al.*, 1979). The majority of survivors of early-onset disease experience no long-term problems.

1.6.2 Late-onset disease

Late-onset infection, the second category of GBS disease in infants, usually presents itself as meningitis or osteomyelitis or both that may be with or without detectable bacteraemia. Late-onset disease occurs in babies more than 7 days old and up to 8–12 weeks of age (Kenny and Zedd, 1977; Paredes *et al.*, 1977; Bingen *et al.*, 1992). In late-onset disease it is rare to see any unusual obstetric problems, like those seen in early-onset, during delivery or in the post-partum period. Late-onset infection has an attack rate worldwide of 0.5–1.8/1,000 (Chatellier *et al.*, 1997) with an eventual mortality rate of 14–18%, that is considerably lower than that of early-onset (Table 1.4). However, of the survivors of GBS meningitis, up to a third suffer long-term mental or physical disability or both, from mild learning disabilities to severe mental retardation, impaired sight, impaired hearing and lung damage. In approximately one in eight survivors, the disabilities may be severe.

1.7 GBS-Related Morbidity in Women

The recognition of the medical significance of GBS increased immensely after the publication by Eickhoff *et al.* (1964). The importance of GBS disease is based on the fact that the organism is often isolated from patients with urinary tract infections, wound infections and a host of other infections in an adult. GBS often colonise the urogenital tract of women and a correlation has been seen between urogenital infections and carriage of these organisms. Ascending spread leads to amniotic infection that can result in maternal sepsis. GBS are also a leading cause of chorioamnionitis and are one of several bacteria now thought to enhance the risk of preterm rupture of membranes. GBS can also cause endometritis and wound infection in women who have recently given birth.

1.8 GBS Disease in the Elderly and Those with Underlying Medical Conditions

Cases of invasive GBS disease, defined as isolation of GBS from a normally sterile site, occur among non-pregnant adults (Trivalle *et al.*, 1998). Among adults, the incidence of GBS disease increases with age. Non-pregnant adults manifest sepsis, pneumonia, soft-tissue infections such as cellulitis and arthritis, and urinary tract infections (UTI's) complicated by bacteraemia. Case-fatality rates for invasive GBS disease are now higher among adults than in the newborn and are significantly higher among those aged over 65 than in younger adults. Independent risk factors associated with GBS disease in non-pregnant adults include diabetes mellitus, cirrhosis, renal failure, stroke, and breast cancer (Schuchat, 1999). The risk of GBS infection was increased 11- to 30-fold in persons with diabetes mellitus who were 20 to 64 years old but was only 3.7- to 5.7-fold higher in people older than 65 years of age (Farley *et al.*, 1993). Farley *et al.* (1993) estimated that the risk was 28-fold higher in patients with breast cancer between the ages of 20 and 49 but only 4-fold higher in patients between 50 and 69 years of age. Older age, independent of any underlying medical condition, increases the risk of invasive GBS disease.

1.9 Antibiotic Resistance Patterns of GBS

The intravenous or intramuscular injection of antimicrobial agents after the onset of labour or rupture of the membranes is highly effective in reducing neonatal colonisation and infection with GBS. GBS isolates are susceptible to penicillin and this remains the antibiotic of choice for GBS prophylaxis and therapy (Table 1.6). Several reports have described penicillin-tolerant GBS isolates (Severin *et al.*, 1976; Cunningham *et al.*, 1992; Betriu *et al.*, 1994). The tolerance of an organism has been described as an MBC/MIC ratio of 1/32 or greater after 24 h of incubation. The tolerance is a rate of killing lower than that in non-tolerant organisms, and can be calculated using time-kill curves (Handwerger and Tomasz, 1985). Betriu *et al.* (1994) found two strains out of one hundred examined with intermediate suceptibility to penicillin (MIC 0.25–2.0 $\Box$g/ml) and seventeen isolates that were tolerant to penicillin. Betriu *et al.* (1994) also described isolates with intermediate suceptibility to ampicillin (MIC 4.0 $\Box$g/ml). A similar study described GBS remaining sensitive to penicillin-G and ampicillin, with 90% and 86%

Table 1.6. Antimicrobial susceptibility of GBS strains (Betriu *et al.*, 1994).

Antimicrobial agent	MIC (µg/ml)		
	Range	50% [a]	90% [a]
Penicillin	≤0.03–0.5	0.03	0.06
Ampicillin	≤0.06–1.0	≤0.06	0.12
Cephalothin	≤0.12–16	0.5	0.5
Cefotaxime	≤0.12–16	≤0.12	≤0.12
Imipenem	≤0.12–2	≤0.12	≤0.12
Erythromycin	≤0.25–>4	≤0.25	≤0.25
Clindamycin	≤0.12–>2	≤0.12	≤0.12
Tetracycline	≤1–>8	>8	>8
Ciprofloxacin	≤0.5–>2	1	2
Rifampicin	≤0.5–>2	≤0.5	≤0.5
Sulphamethoxazole-trimethroprim	≤0.5–>2	≤0.5	≤0.5
Gentamycin	≤4–>8	>8	>8
Vancomycin	0.5–>4	1	2
Chloroamphenicol	≤0.25–>16	4	4

[a] MIC is for 50% and 90% of isolates, respectively

sensitive to clindamycin and cefoxitin, respectively (Berkowitz *et al.*, 1990). The frequency of GBS resistance to erythromycin and clindomycin has been reported as 12% (out of 200 tested) and 8% (out of 49 tested), respectively (Goodrum *et al.*, 2000). Although GBS strains continue to be susceptible to penicillin, erythromycin and clindamycin resistance have been reported in 7.4% and 3.4% of invasive GBS isolates, respectively, and in 16% and 15% of genitourinary isolates, respectively (Schuchat, 1999). Alternatives, such as a cephalosporin, may be more appropriate than these two drugs for prophylaxis in penicillin-allergic women.

No widespread increase in the incidence of neonatal sepsis due to organisms that are penicillin resistant has been identified in the context of either intrapartum or postnatal prophylaxis programmes. However, the concern is whether increased use of antimicrobial prophylaxis in obstetric care will lead to the emergence of antimicrobial resistance among other perinatal pathogens.

1.10 Prematurity and GBS

There are some reports suggesting a relationship between GBS and prematurity. The incidence of invasive GBS disease (i.e., sepsis and meningitis) is higher among preterm infants than among those born at term, although 74% of early-onset GBS and 56% of late-onset cases occur in full-term infants. Antibody transport across the placenta is reduced early in gestation. Preterm infants born to colonised mothers may have a higher risk of disease because lower amounts of protective maternal antibodies have ben transported across the placenta. Premature rupture of membranes (i.e., rupture before spontaneous onset of labour) is strongly associated with early-onset GBS disease. Premature rupture of membranes earlier than 37 weeks occurs in 30–40% of preterm deliveries. GBS, and several other bacteria, may cause premature rupture through a variety of mechanisms, including secretion of proteases that degrade collagen and weaken foetal membranes.

Regan *et al.* (1981) reported an investigation involving 13,646 women in several centres in the USA. Heavy colonisation, defined as isolation of the organism from direct plating, was associated with a 1.5-fold higher risk of preterm low birthweight, after adjustment for other factors, and was present in 9.1% of the women. The same

16

multicentre study group reported that bacterial vaginosis was associated with a 1.4-fold increased risk of preterm low birthweight and was present in 16% of the cohort. Antibiotic treatment trials for bacterial vaginosis have reduced preterm low birthweight in some populations but an erythromycin trial among women with GBS colonisation failed to reduce premature delivery, although the relative risks in these two groups were similar (Schuchat, 1998 and 2001). The erythromycin trial had limited power to identify an impact among heavily colonised women, and the agent used did not significantly reduce GBS carriage. Larger studies that focus on heavily colonised women and use other antimicrobial agents (or vaccines, if these prove to reduce genital colonisation) are needed to determine whether interventions aimed at GBS colonisation can affect preterm or low-birthweight deliveries or both.

1.11 Clinical Laboratory Practices

Generally, genital tract specimens require selective measures to be taken if GBS are to be detected. The prevention guidelines from the CDC (Fig. 1.2) recommend that swabs be taken from both the vagina and rectum and transferred into selective broth medium, e.g., Todd-Hewitt broth supplemented with either colistin (10 µg/ml) and nalidixic acid (15 µg/ml) (Lim broth) or with gentamycin (8 µg/ml) and nalidixic acid (15 µg/ml) (SBM broth). After incubation for 18 to 24 h the selective broth is then subcultured onto a sheep blood agar plate for another 18 to 24 h. Organisms suggestive of GBS (β-haemolytic or non-haemolytic, Gram-positive, and catalase-negative cocci) are inspected and identified. Various slide agglutination tests, such as the Streptex test (Murex Biotech), are used for specific Lancefield group identification. If any risk factors are identified (Table 1.1) or if there is a positive screen for GBS, then intrapartum penicillin is given.

1.12 Epidemiology

The epidemiology of GBS human infections has received considerable attention since this pathogen emerged. Several genotypic typing methods have been applied to epidemiological studies of GBS (Table 1.7). Investigation of chromosomal genetic diversity and serotype diversity of national collections of strains could help us to

Figure 1.2. Algorithm for prevention of early-onset group B streptococcal (GBS) disease in neonates, using prenatal screening at 35–37 weeks' gestation.

Risk factors:
Previous infant who had invasive GBS disease
GBS bacteriuria during this pregnancy
Delivery at <37 weeks' gestation*

— Yes → Give intrapartum penicillin

— No ↓

Collect rectal and vaginal swabs for GBS
Screening at 35–37 weeks' gestation

— **GBS positive** → Give intrapartum penicillin

Not done, incomplete, or results unknown ↓

Risk factors:
Intrapartum temperature (>38.0°C)
Membrane rupture >18 h

— Yes → Give intrapartum penicillin**

— No ↓

GBS negative

No intrapartum prophylaxis

* If membranes rupture at <37 weeks' gestation, and the mother has not begun labour, collect specimen for group B streptococcal culture and either (a) administer antibiotics until cultures are completed and the results are negative or (b) begin antibiotics only when positive cultures are available. No prophylaxis is needed if culture obtained at 35–37 weeks' gestation was negative.

**Broader spectrum antibiotics may be considered at the physician's discretion, based on clinical indications (Schuchat et al., 1996).

Table 1.7. Phenotypic and genotypic typing methods applied to GBS.

Typing Methods	References
Phenotypic Methods	
Serotyping	Kvam *et al.*,1995; Lin *et al.*,1997;Harrison *et al.*,1998; Paoletti *et al.*, 1999
Genotypic Methods	
Multilocus enzyme electrophoresis (MLEE)	Helmig *et al.*, 1993; Quentin *et al.*,1995
Pulsed-field gel electrophoresis (PFGE)	Fasola *et al.*,1993; Gordillo *et al.*,1993; Dmitriev *et al.*, 1997; Rolland *et al.*,1999
Random amplified polymorphic DNA (RAPD) analysis	Bert *et al.*,1996; Chatellier *et al.*, 1997
Restriction endonuclease analysis(REA)	Denning *et al.*, 1989; Blumber *et al.*, 1992; Fasola *et al.*,1993
Ribotyping	Blumberg *et al.*, 1992; Huet *et al.*, 1993; Chatellier *et al.*, 1996

understand the transition from a commensal organism to a disease-causing organism and/or help us to recognise particularly virulent clones. Unfortunately, no modern genotypic or extensive phenotypic typing methods have become universally accepted in the clinical environment for GBS and therefore identification still depends on older clinical laboratory practices.

1.12.1 Serotyping

The earliest and most common methods for the characterisation of microorganisms relied primarily on structural features associated with a particular microorganism. The "gold-standard" phenotypic typing method used today in the study of GBS is serotyping. GBS associated with human disease are generally encapsulated by serologically distinct capsular polysaccharides and are classified into one of nine types on this basis, i.e., Ia, Ib, II, III, IV, V, VI, VII and VIII. With minor exceptions, the type-specific polysaccharides are all composed of the same four component sugars, namely, glucose, galactose, *N*-acetylglucosamine and *N*-acetylneuramic acid or sialic acid (Section 3.1.3).

1.12.2 Genotypic characterisation

Epidemiological studies of microorganisms rely on precise and reproducible methods for typing organisms. With modern molecular biological techniques it has become possible to directly examine and compare the genomes of invasive and non-invasive organisms (Hall *et al.*, 1998). This could enable recognition of putative invasive types. Over the last several years, some of the more frequently used genotypic approaches for characterising bacterial isolates have been applied to GBS and include restriction endonuclease analysis of chromosomal DNA (REA), random amplified polymorphic DNA analysis (RAPD), ribotyping, and pulsed-field gel electrophoresis (PFGE) (Table 1.7). Conventional polymerase chain reaction (PCR) methods that employ high stringency conditions and primers for specific sequences have also been used for genotyping for example isolates with antibiotic resistance genes. Another common and efficient typing method used for other bacterial species is plasmid fingerprinting. However, although GBS have been shown to carry plasmids, they appear to do so uncommonly. Therefore, plasmid fingerprinting is

unlikely to prove an adequate system of typing GBS for epidemiological purposes, unless an outbreak strain happens to carry an unusual plasmid or set of plasmids. Before a typing method can be adopted for routine use, it needs to be assessed in terms of typeability, reproducibility, discriminatory power, ease of performance and ease of interpretation.

1.12.2.1 Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis is a variation of the PCR technique (Section 1.13) employing an oligonucleotide primer that is not targeted to amplify any specific bacterial DNA sequence. Rather, at low annealing temperatures, the primer will hybridise at multiple random chromosomal locations and initiate DNA synthesis. RAPD analysis is a fast and simple typing method which gives a characteristic and reproducible pattern of amplified DNA fragments that may be sufficiently discriminatory to investigate epidemic or nosocomial GBS invasive infections. This method has been used successfully for typing several medically important organisms, including groups A, C and G streptococci (Bert *et al.*, 1996). There is also a report in the literature documenting the suitability of RAPD analysis for the differentiating isolates of GBS (Chatellier *et al.*, 1997).

1.12.2.2 Ribotyping

In this technique, chromosomal DNA restriction digests produced by frequent cutting endonucleases are separated by conventional agarose gel electrophoresis and then the DNA fragments are transferred onto a nylon membrane. The DNA on the membrane is then hybridised with a chemically or radioactively labelled ribosomal RNA gene probe. The patterns of restriction fragments of chromosomal DNA hybridising with the ribosomal gene probe have been applied successfully to the characterisation of strains from many different species, e.g., *Streptococcus pneumoniae*, *Streptococcus suis* and *Salmonella Salmonella enterica* serovar Typhi (Altwegg *et al.*, 1989a and 1989b; Staats *et al.*, 1998; Song *et al.*, 2000). Restriction fragment length polymorphism (RFLP) analysis of restriction endonuclease digests of DNA from GBS is a relatively easy technique to perform and the system appears to be very reproducible with good discriminatory power (Blumberg *et al.*, 1992; Huet *et al.*, 1993; Chatellier *et al.*, 1996).

21

1.12.2.3 Pulsed-field gel electroporesis (PFGE)

Another method that is frequently used in typing bacterial isolates is pulsed-field gel electrophoresis (PFGE). This typing procedure involves the use of restriction enzymes that digest the bacterial chromosome at infrequent sites. However, such large fragments are not readily separated by conventional gel electrophoresis. Instead this technique involves the use of a pulsed-field gel developed by Schwartz and Cantor (1984). PFGE requires the DNA molecules to change their direction of migration periodically during electrophoresis as the electric field alternates to get clear separation of DNA fragments. The pattern of restriction fragments obtained is characteristic for each strain and provides an estimate of the genomic relationship between strains (Bert *et al.*, 1997).

1.12.3 Criteria for a discrimnatory epidemiological typing method

Epidemiological typing can be carried out by a variety of techniques as described above. An assessment of which typing method is the most efficient and provides the most valuable results depends on a number of factors, namely, typeability, reproducibility, and discrimination (Hunter and Gaston, 1988). Typeability and reproducibility are the characteristics which are the easiest to quantify. The typeability using a particular method is the percentage of distinct bacterial strains that can be assigned a positive typing marker while the reproducibility is the percentage of strains that give the same result on repeat testing.

After estimation of typeability and reproducibility it is the discriminatory power of a typing method that provides the most information on the value of a particular typing method. The discriminatory power of a typing method is its ability to distinguish between unrelated strains by determining the types defined by the typing procedure and the relative frequencies of these types. Hunter and Gaston (1988) suggested the use of a single numerical index of discrimination (D), based on the probability that two unrelated strains sampled from a test population will be placed into two separate groups. This probability can be calculated by Simpson's index of diversity (Simpson, 1949). In developing new typing methods a large discriminatory index is desirable if the typing results are to be

interpreted with confidence. Two or more typing methods can be combined in order to get an acceptable discriminatory index of greater than 0.90.

Simpson's index of diversity:

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{S} n_j (n_j - 1)$$

N= total number of strains in sample population
S= total number of types described
n_j= number of strains belonging to j^{th} type

1.13 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) for amplification of a specific nucleic acid sequence was introduced by Saiki *et al.* (1985). This technique allows production of multiple copies of specific nucleic acid target sequences quickly and exponentially through the use of thermostable *Taq* polymerase. PCR employs two oligonucleotide primers, oriented in the 5′ to 3′ direction, which are complementary to opposing strands of the target sequence. When the primers anneal to the single-stranded, heat-denatured target sequence, they form a double-stranded duplex which mimics the double-stranded priming region of natural DNA replication and can, therefore, allow DNA synthesis by DNA polymerase in the 5′ to 3′ direction. This three-step procedure – denaturation, annealing and extension of DNA – constitutes the basis of PCR and is allowed to continue until a sufficient degree of amplification is achieved. Each cycle of amplification essentially doubles the yield of target sequence, resulting in exponential accumulation which allows better manipulation of the desired sequence.

1.14 GBS Virulence Factors

Apart from their capsular polysaccharide many other cell-associated and extracellular proteins are thought to be associated with virulence of GBS (Table 1.8 and Section 5.2 and 5.3). These putative virulence determinants of GBS include the major surface-associated C proteins (α, β, γ and δ), the Rib proteins the extracellular enzyme hyaluronidase and the cell-associated oligopeptidase.

1.14.1 Alpha (α) C-protein

The α C-protein (Section 5.2.1) is a surface-associated antigen encoded by the *bca* gene and antibodies to this protein are protective when elicited in animal models (Madoff *et al.*, 1991). Biochemical and immunological characterisation of this protective C-protein has been reported (Michel *et al.*, 1994). It has been suggested that the repeating units of the α C-antigen represent a mechanism for phenotypic and genotypic variability, providing natural sites for genetic rearrangements that generate antigenic diversity (Madoff *et al.*, 1996).

1.14.2 Beta (β) C-Protein

The β C-protein (Section 5.2.2) is a surface-expressed antigen encoded by the *bac* gene. Although earlier believed to be different forms of the C-protein, the α and β antigens are now known to be phenotypically distinct and genetically unrelated proteins (Bevanger, 1983; Johnson and Ferrieri, 1984). The β antigen does not possess tandem repeats as seen in the α C-protein. Moreover, it has an ability to bind the Fc region of human IgA (Russel-Jones *et al.*, 1984; Jerlström *et al.*, 1996). Since IgA is the predominant defence immunoglobulin against microbial infection at mucosal surfaces, non-productive sequestering of IgA may be a mechanism for avoiding this line of host defence (Tomasi and Plaut, 1985).

24

Table 1.8. The putative virulence factors that are postulated to be involved in GBS disease.

Protein	Gene	Protein type	Reference
Alpha C-protein	*bca*	Surface protein	Michel *et al.*, 1992; Kling *et al.*, 1997
Beta C-protein	*bac*	Surface protein	Russell-Jones *et al.*, 1984
Rib	*rib*	Surface protein	Stålhammar-Carlemalm *et al.*, 1993
Sip	?	Surface protein	Brodeur *et al.*, 2000
R1–R4 proteins	?	Surface protein	Kvam *et al.*, 1999
γ and δ C-protein	?	Surface protein	Brady *et al.*, 1988
Alp2 and Alp3	*alp2 and alp3*	Surface protein	Lachenauer *et al.*, 2000
Laminin-binding protein	*lmb*	Surface proteins	Granlund *et al.*, 2000
β-Haemolysin	*cyl*	Cytolytic toxin, cell-associateed	Gibson *et al.*, 1999; Pritzlaff *et al.*, 2001
Pep	*pepB*	Oligopeptidase, cell-associated	Lin *et al.*, 1996
Superoxide dismutase	*sodA*	Metalloenzyme, call-associated	Gaillot *et al.*,1997; Poyart *et al.*, 1995, 2001
Hyaluronidase	*hylB*	Extracellular enzyme	Lin *et al.*, 1994
CAMP factor	*cfb*	Extracellular enzyme	Hassan *et al.*, 2000
C5a peptidase	*scpB*	Enzyme degrading complement factor 5a	Adderson *et al.*, 2000

1.14.3 Rib Protein

Studies of the Rib protein (Section 5.2.3) encoded by the *rib* gene have shown that it shares several properties with the α C-protein, e.g., it is a surface protein with variable numbers of tandem repeats (Ståhlhammer-Carlemalm *et al.*, 1993). Similar to the α C-protein, the repetitive structure of the Rib protein may play a role in evasion of type-specific host immunity in GBS infection by giving rise antigenic variation (Ståhlhammer-Carlemalm *et al.*, 1993).

1.14.4 Pep Protein

GBS produces a cell-associated oligopeptidase (Pep protein) that is encoded by the *pepB* gene (Section 5.3.1). The GBS oligopeptidase has the ability to hydrolyse the synthetic collagen-like substrate *N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA) (Lin *et al.*, 1996) and also to degrade a variety of small bioactive peptides. It has been postulated that this enzyme's activity might contribute to the premature rupture of membranes at childbirth (Lin *et al.*, 1996).

1.14.5 Hyaluronidase

Hyaluronidase, encoded by the *hylB* gene, is one of several proteins secreted by GBS that are believed to contribute to strain virulence (Section 5.3.2). Hyaluronidase cleaves hyaluronic acid (a component of the extracellular matrix of many tissues) to yield the disaccharide *N*-acetylglucosamine–glucuronic acid. It is believed that hyaluronidase may facilitate tissue invasion by bacteria (Milligan *et al.*, 1978; Lin *et al.*, 1994).

1.14.6 Other putative virulence factors

Several other proteins are believed to participate in the pathogenicity of GBS (Table 1.8). However, the other putative virulence factors described herein were not examined in the present study.

Superoxide dismutase, encoded by the *sodA* gene, is found in a variety of Gram-positive bacteria including GBS (Poyart *et al.*, 1995, 2001). This enzyme has the ability to convert superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) which, in turn, is

metabolised by catalase either alone or together with peroxidase. These enzymes constitute one of the major defense mechanisms of cells against oxidative stress and, hence, play a role in the pathogenesis of bacteria by blocking the oxygen-dependent mechanism of microbial killing after phagocytosis by macrophages or neutrophils (Pesci *et al.*, 1994).

Lachenauer *et al.* (2000) reported alpha-like proteins, Alp2 and Alp3, members of the C protein family. Like the α C-protein and Rib protein genes, the genes that encode these proteins contain a tandem repeat region, but they also contain an additional non-tandem repeat region similar to the IgA-binding region of the β C-protein. Alp3 was present on most serotype V and serotype VIII strains but not on strains of other serotypes.

The β-haemolysin produced by GBS is also speculated to be an important virulence factor. GBS β-haemolysis is thought to enhance the pathogenicity of the organism through direct tissue injury or activation of the host inflammatory response (Pritzlaff *et al.*, 2001). GBS have the ability to cross polarised meningeal endothelial cells, a step in the pathogenesis of GBS meningitis, through cell injury due to β-haemolysin production (Nizet *et al.*, 1998; Huang *et al.*, 2000).

Brodeur *et al.* (2000) identified Sip, surface immunogenic protein, which unlike other surface proteins (e.g., Rib and α C-protein) does not vary in size and is present in all nine serotypes.

1.15 Vaccine Development

Although antibiotics are effective in preventing and treating GBS infections (Boyer and Gotoff, 1986), the best long-term solution for elimination of GBS disease would be the use of an effective vaccine. Also, even though the CDC prevention guidelines have been successful, this frequent use of antibiotics might lead to the emergence of resistant infections by GBS or other bacteria (Schuchat, 1998).

The capsular polysaccharides are important virulence factors and are targets of protective immunity (see Chapter 3). Because GBS vaccines composed of capsular polysaccharide antigens were sub-optimally immunogenic in adults (Baker *et al.*, 1988, 1990), conjugate vaccines were developed by coupling the capsular polysaccharide to

27

tetanus toxoid, a procedure that gives a greater immune response to the capsular polysaccharide (Kasper *et al.*, 1996). The first conjugate vaccines used oligosaccharides of defined size. Studies with these vaccines revealed a capsular polysaccharide chain length that was optimal for eliciting high levels of functional antibody in animals. More recent conjugate vaccines used the entire native capsular polysaccharide structure linked to several sites on the protein. These, like all conjugate vaccines, displayed vastly improved immunogenicity in animals compared to uncoupled capsular polysaccharide.

An effective vaccine would consist of a multivalent GBS conjugate for use as a maternal vaccine for the prevention of neonatal GBS disease. This vaccine may also be implemented for use among non-pregnant adults.

1.16 Aims of project

This research project sought to generate essential epidemiological and virulence-related information about GBS isolates associated with invasive disease in Ireland.

- To characterise by molecular methods carriage and disease-causing GBS isolates recovered from various anatomical sites from gynaecological and maternity patients and infants attending Dublin's three main maternity hospitals (The Rotunda Hospital; The National Maternity Hospital, Holles Street, and The Coombe Womens' Hospital).

- To attempt to associate or correlate genotypic characteristics (the presence or absence of genes encoding putative virulence factors) with the carriage or invasive status of isolates.

- To assess whether molecular typing methods (RAPD typing, PFGE) can be used as an alternative to the current "gold standard" method of serotyping and also whether they can be used to identify carriage or invasive isolates of GBS.

- To investigate (i) the occurrence and number of repeats in the *bca* gene and the presence or absence of IS sequences (IS*1548*) in the *hylB* gene to assess whether these traits are markers for increased or decreased virulence of GBS and (ii) their association with serotype designation of isolates.

28

- To assess whether GBS represent a spectrum of pathogenic strains whose properties may differ between serotype or molecular fingerprint type.

The information gained may be used to:

- determine the phylogenetic relationships between invasive and carriage isolates
- assess evolving patterns and disease trends in relation to the isolates of GBS nationwide
- assess the likely effectiveness of serotype-specific GBS vaccines currently under development.

CHAPTER 2

Materials and Methods

2.1 General Microbiological Procedures

2.1.1 Culture media and growth conditions

S. agalactiae isolates were routinely cultured on blood agar plates, supplemented with 5% defibrinated horse blood (Columbia base; Oxoid, Hampshire, UK). All isolates were incubated at 37°C for 18–20 h. Before genomic DNA extractions were performed, a single colony from each isolate was inoculated into 10 ml Todd-Hewitt Broth (Oxoid) and incubated at 37°C in an orbital incubator set at 180 r.p.m. (Stuart Scientific). *Escherichia coli* strain DH5α was routinely cultured on L-agar, pH 7.4 (Lennox, 1955) at 37°C, and for liquid culture in L-broth, pH 7.4 (Lennox, 1955) at 37°C for 16 h with shaking in an orbital incubator.

2.1.2 Chemicals and enzymes

All chemicals were purchased from the Sigma Chemical Company Ltd. (Poole, Dorset, U.K.) or from Boehringer Mannheim (Dublin, Ireland) and were of Analar grade or equivalent. The Streptex latex streptococcal grouping kit was purchased from Murex Biotech Ltd. (Dartford, U.K.) while the Group B streptococcal typing sera were purchased from Mast Diagnostics (Mast Group Ltd., Merseyside U.K.). Both the grouping and typing procedures were performed according to manufacturer's instructions. Restriction endonucleases and *Taq* DNA polymerase (5000 units/ml) were purchased from Promega (Madison, WI., USA) and were used according to manufacturers' instructions. DNA probe labelling was performed using the AlkPhos Direct DNA labeling kit (Amersham, U.K.) according to manufacturers' instructions.

2.2 Subjects and *S. agalactiae* Isolates

2.2.1 Sources of isolates

During 1997–1999, one hundred and fifty nine GBS isolates were recovered from infants and women with invasive disease and from asymptomatic adult carriers attending Dublin's three main maternity hospitals. Ninety-five of the *Streptococcus* strains were received from Dr. Mary Cafferkey and the Micobiology Laboratory Staff at The Rotunda Hospital, Dublin. Forty-four of the strains were received from Dr. Phillip Troy at the National

Maternity Hospital, Holles street, Dublin. The remaining twenty strains were received from Stephen Dempsey at The Coombe Hospital, Dublin. The three hospitals, combined collectively, participate in approximately 40% of all births in Ireland each year. Isolates were recovered from pregnant women and non-pregnant adults from a variety of sites such as blood specimens, high vaginal swabs, caesarian section swabs, umbilical cord swabs and placental tissue swabs. A reference GBS isolate, *Streptococcus agalactiae* American Type Culture Collections (ATCC) 13813 (synonyms: NCTC 8181; G19, A. Stableforth), was obtained from the culture collection of the Department of Microbiology, Trinity College Dublin.

2.2.2 Characterisation of isolates according to invasive or carriage status

Each GBS isolate was classified into one of five categories based on its isolation site and whether there was microbiological or histological evidence of invasion or infection or both. Isolates from vaginal swabs with no eventual morbidity to mother or child were classified as carriage isolates with "0" grade invasion. Organisms were termed invasive if they were recovered from normally sterile sites and were graded between "1+" and "4+" according to the signs of infection at their recovery site. Isolates recovered from the uterine cervix or placenta (chorial plate) with no histological signs of inflammation of the placenta and no pus formation prominent were termed "1+". Isolates recovered from wound infections, an infected intra-uterine contraceptive device (IUCD) or from the placenta with histological signs of inflammation were termed "2+". An isolate was termed "3+" when it was recovered from a patient with mastitis. Isolates in groups "2+" and "3+" were considered to be of the same epidemiological status. Isolates recovered by blood culture were indicative of severe invasive disease and were termed "4+". These were designated as the primary cause of sepsis and in some cases were responsible for foetal death (Cafferkey, Personal communication).

2.3 Identification of *S. agalactiae*

2.3.1 Verification as a Group B streptococcus

All β-haemolytic isolates were identified as GBS using Streptex reagents according to the manufacturer's instructions (Murex Biotech Ltd.) and stored in cryobeads (Protect, Technical Services Consultants, Heywood, Lancashire, UK) at –20°C. Prior to specimen analysis thawed cultures were routinely streaked out on sheep blood agar plates and grown at 37°C. For liquid culture, organisms were subcultured from the blood agar plates into Todd-Hewitt broth and incubated at 37°C for 16 h in an orbital incubator.

2.3.2 Serotyping

A total of 159 GBS carriage and invasive isolates from neonates, pregnant women and non-pregnant adults were serotyped by the Lancefield method with commercially available antisera (Mast Diagnostic) to the capsular polysaccharide antigens Ia, Ib, II, III, IV and V according to manufacturer's instructions. All reagents were supplied in the kit. Briefly, cells were harvested by centrifugation at 3,000 X *g* from 10 ml Todd-Hewitt broth cultures that had been incubated for 16 h at 37°C. The cell pellet was resuspended in 0.5 ml of fresh Todd-Hewitt broth containing four drops of swine pancreatic extract and one drop of phenol red as a pH indicator. The pH of the mixture was adjusted to 8.0–8.5 by the addition of 0.2 M sodium hydroxide and was then incubated for 1 h at 37°C. Cells were harvested by centrifugation for 20 min at 3,000 X *g*. The pellet was resuspended in 0.5 ml phosphate buffered saline (pH 7.2) and 50 μl of the resulting suspension was mixed directly with 15 μl of each individual antiserum on a glass slide. Agglutinations were recorded as strongly positive or negative when compared with a control reaction that used sterile physiological saline instead of antiserum. Isolates of known serotype were used as positive controls. Agglutination in a particular antiserum indicated the presence of that particular polysaccharide capsule. Cells that caused spontaneous agglutination or yielded weak agglutination reactions were retested. On retesting, cells that showed spontaneous agglutination were termed non-typeable.

2.4 DNA Extractions

2.4.1 Preparation of chromosomal DNA

Purified chromosomal DNA for use in all experiments was prepared using the Wizard genomic DNA extraction kit (Promega) with the following modifications. Cells from an overnight 10 ml Todd-Hewitt broth culture were harvested by centrifugation at 3,000 X g and resuspended in 480 µl of 50 mM EDTA. Then, 60 µl of lysozyme (10 mg/ml) and 60 µl of mutanolysin (10 mg/ml) were added. The solution was mixed briefly and then incubated at 37°C for 60 min. Following incubation, the samples were centrifuged at 16,000 X g for 2 min before discarding the supernatant fluid. To complete cell lysis the pellet was gently resuspended in 600 µl Nuclei Lysis Solution (Wizard kit) and incubated at 80°C for 10 min. The mixture was incubated for a further 30 min at 37°C with 3 µl RNase solution (Wizard kit). Cellular protein was then precipitated by the addition of 200 µl of the Protein Precipitation Solution (Wizard kit). The sample was allowed to sit on ice for 5 min prior to centrifugation at 16,000 X g for 3 min. The supernatant fraction was transferred to a 1.5 ml tube containing 600 µl of room temperature isopropanol and mixed by inversion. The DNA precipitate was then recovered by centrifugation at 16,000 X g for 2 min and washed with 70% ethanol, centrifuged, dried and resuspended in 100 µl Rehydration Solution (Wizard kit). The concentration of DNA was read by measuring the absorbence of a sample at A260 as well as A280 on a spectrophotometer. For good quality DNA without a lot of protein this ratio was between 1.8 and 2. The A260 of DNA with a concentration of 40 mg/ml was 1 OD unit.

2.4.2 Preparation of *S. agalactiae* chromosomes for pulsed-field gel electrophoresis

A single GBS colony was inoculated into 10 ml of Todd-Hewitt broth and placed at 37°C overnight (16 h). The cells were then harvested by centrifugation at 3,000 X g and washed in 500 µl of wash buffer (1 M NaCl, 10 mM Tris-HCl pH 7.6). Cells were resuspended in 50 µl of wash buffer and warmed at 55°C for 2–3 min. Bacterial suspensions were mixed with an equal volume of 1% chromosomal grade agarose (Seakem), transferred into plug moulds (1.5 mm x 10 mm x 20 mm, BioRad) and allowed to solidify at 4°C for 10–15 min. Once solid, the plugs were transferred into sterile vials and stored at 4°C. When

required, each plug was placed in 3 ml of lysis buffer (0.1 M EDTA pH 8.0, 10 mM Tris, 0.5% Brij, 0.2% deoxycholate, 0.5% Sarkosyl, 60 µg/ml RNase and 100 µg/ml each of lysozyme and mutanolysin) and incubated at 37°C to lyse the bacteria. The bacteria were lysed in the plugs for 20 h at 37°C. The lysis buffer was decanted and the plugs were incubated overnight (16 h) in 3 ml of ESP buffer (0.2 M EDTA, pH 8.0, 1% Sarkosyl and 1mg/ml of proteinase K) at 56°C.

The ESP buffer was decanted and the plugs were rinsed in 3 ml of sterile distilled water, with gentle shaking for 15 min. The water was decanted and the plugs were washed twice in 3 ml of TE buffer (1M Tris-HCl, pH 8.0, 0.25 M EDTA) and 30 µl of 100 mM phenylmethylsuphonylfluoride (PMSF) for 30 min with gentle shaking to inactivate the proteinase K and nucleases. The plugs were then washed once in 3 ml of sterile distilled water and twice in 3 ml of TE buffer for 15 and 30 min, respectively. The plugs were stored in 1 ml of TE buffer at 4°C until needed for restriction endonuclease cleavage.

Restriction endonuclease cleavage was performed by cutting off a slice, approximately ¼, of an agarose plug and incubating it for 30 min in 250 µl of the relevant restriction buffer and then for 16–20 h with 20 U of restriction enzyme and fresh buffer at the relevant temperature for the enzyme.

2.5 General DNA Procedures

2.5.1 Restriction endonuclease digestion of chromosomal DNA

Chromosomal DNA (5 µg) from a GBS reference strain ATCC 13813 was digested with 10 units each of 20 restriction endonucleases separately (*Alu*I, *Asp*700, *Bam*HI, *Bsm*I, *Cla*I, *Dde*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*II, *Mae*III, *Mnl*I, *Msp*I, *Mva*I, *Pst*I, *Pvu*II, *Rsa*I, *Sau*96I, *Taq*I and *Xba*I) according to manufacturer's recommendations (Promega).

2.5.2 Electrophoresis of DNA

2.5.2.1 Agarose gel electrophoresis

Horizontal 1% (w/v) agarose (Sigma) gels were cast in 0.5 X TBE buffer [10 X TBE: 10.8% (w/v) Trizma base, 5.5% (w/v) boric acid, 9.8% (w/v) EDTA), containing 0.5

μg/ml of ethidium bromide (EtBr)] into horizontal gel trays. Restriction endonuclease-generated DNA fragments in 1 X final sample buffer [30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 3.8% (w/v) EDTA] were applied to the gel wells and electrophoresis was performed at 30 V for 16 h or 100 V for 5 h. A 1-kb DNA ladder (Gibco, BRL, Bethseda, U.S.A.) was used as a molecular size standard.

Following electrophoresis, DNA bands were visualised on a UV transilluminator (wavelength 312 nm; TFX-20M). Gels were photographed using Polaroid black and white print film, type 667. Gel image acquisition, enhancement and analysis (Section 2.9) was also performed using Alpha Imager Software (Alpha Innotech Corporation, USA).

2.5.2.2 Pulsed-field gel electrophoresis (PFGE)

For PFGE, the DNA fragments were subjected to electrophoresis on 1.0% SeaKem (FMC Bioproducts, Rockland, ME, USA.) agarose gels in 0.5 X TBE buffer using a modified contour-clamped homogeneous electric field (CHEF) system. The running conditions were 5.6 V/cm at 14°C for 20 h. The pulse times were 1–15 sec with a linear ramp. A 50-kb DNA lambda ladder (BioRad) was used as the molecular weight size marker. After electrophoresis, gels were stained in ethidium bromide (EtBr, 0.5 μg/ml) for 15 min and destained in distilled water for 15 min and photographed as described (Section 2.5.3).

2.5.3 Gel visualisation

All agarose gels were either photographed under UV transillumination onto Polaroid film as described (Section 2.5.2.1) or were visualised using an Alpha Imager™ 1220 Documentation system (Alpha Innotech Corporation, USA). This system incorporates the AlphaEase software which allows image capture and storage. Once stored, profiles can be analysed and reproduced onto Sony Polaroid film. For subsequent analysis of the gels, computer images were generated, and molecular weights of bands were obtained using Phoretix Software (Phoretix International, UK; Section 2.9 and Figure 2.1).

2.6 Polymerase Chain Reaction (PCR)

2.6.1 Oligonucleotides

The oligonucleotides used in this study are listed in Tables 2.1 and 2.2. All oligonucleotides were obtained as dried DNA pellets from Genosys Research Products Ltd. (Cambridge, UK). On arrival, the pellets were reconstituted in an appropriate volume of sterile distilled water to give a final concentration of 200 pmol/ml. Once reconstituted, aliquots of the oligonucleotides were made and stored at –20°C. Prior to use, the oligonucleotides were diluted 10-fold to a concentration of 20 pmol in sterile distilled water and stored at 4°C for a maximum period of 3–4 weeks ready for use.

2.6.2 Random amplified polymorphic DNA (RAPD) fingerprinting

Oligonucleotide primers were synthesised by Genosys Biotechnologies Ltd., UK. The sequences of the oligonucleotide primers used are shown in Table 2.1. To determine the primers that yielded the most discriminatory profiles of amplified products, polymerase chain reactions were performed with separate individual primers on extracted DNA from a number of GBS isolates. Reactions were performed in 0.2 ml thin-walled PCR tubes in a final reaction mixture (50 µl) containing 40 pmoles of oligonucleotide primer, 8 mM $MgCl_2$, 250 µM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim), 2.5 U of *Taq* DNA polymerase (Promega) and the appropriately diluted 10 X reaction buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton X-100) supplied with the DNA polymerase and ~20 ng of DNA template. The reaction mixtures were mixed gently and placed in a Perkin-Elmer GeneAmp PCR 2400 DNA thermal cycler. The cycling programme was 10 cycles of [10 s at 94°C, 30 s at 36°C, and 2 min at 68°C] followed by 20 cycles of [10 s at 94°C, 30 s at 36°C and 2 min at 68°C (with an increment of 20 s per cycle)]. A final extension step was performed for 7 min at 72°C. Following amplification, the reaction mixtures were electrophoresed on 2% (w/v) agarose gels containing EtBr (0.5 µg/ml). A 100-bp DNA ladder (Gibco) was used as a molecular size standard.

Table 2.1. Sequence of oligonucleotide primers used for random amplified polymorphic DNA (RAPD) fingerprinting.

Name	Primer Sequence	Size	Reference
RAPD2	5'-TATCGTATTGCGCCCGGG-3'	18 nt	Seppala *et al.*,1994
RAPD3	5'-CCTATCGCTACCAGTCGG-3'	18 nt	This study
RAPDnm	5' -TGTTGGGCAACCTGATTG-3'	18 nt	Olcen *et al.*, 1995
RAPD1	5'-AAGTAAGTGACTGGGGTGAGCG-3'	22 nt	Versalovic *et al.*,1991
OPS16	5'-AGGGGGTTCC-3'	10 nt	Chatellier *et al.*, 1996
OPS11	5'-AGTCGGGTGG-3'	10 nt	Chatellier *et al.*, 1996
AP42	5'-AACGCGCAAC-3'	10 nt	Chatellier *et al.*, 1996
A4	5'-GCATCAATCT-3'	10 nt	Chatellier *et al.*, 1996

Table 2.2. Primers designed for use in amplification of virulence factor gene DNA Sequences.

Gene	Name	Primer sequence (5'-3')	Nucleotide numbers	T_A (°C)	Product size (bp)	Ref. (Genbank Accession nos.)
bac	BacF	GCAGTTCATATTGGAAGG	74 – 91	45	830	Michel et al., 1991
	BacR	AATTTCTTGATCCAGACCAGC	904 – 924			(X58470.1)
bca	BcarptF	GTCGAATTCAGTACCGGATAAAGATAAAT	755 – 774	60	246	Michel et al., 1991
	BcarptR	GTCGAATTCGTTTTGGTGTCACATGAAGG	982 – 1000			(M97256.1)
	BcaF (repeat region)	CCATCGATATAGTTGCTGCATCTACA				
	BcaR (repeat region)	CGGGATCCATCCTCTTTTTTCTTAGAAAC				
hylB	HylIS2F	CCGTTATCAGTTACAGGTC	771 – 789	45	714/2031	Granlund et al., 1998
±IS*1548*	Hyl3R	GTCGATGTAAGAACCGTCAGC	1464 – 1484			(U15050.1and
						Y14270.1)
pepB	PepBF	ATCTAGCGATAGGACGGG	816 – 833	45	417	Lin et al., 1996
	PepBR	CGTTCAGTAAAAGCACGA	1218 – 1235			(U49821.1)
rib	RibF	CAGATGCCGATAAGA	755 - 769	39	237	Stålhammar-
	RibR	TACGCGGATCGACAA	1214 – 1228			Carlemalm et al.,
						1998 (U58333.1)

2.6.3 Amplification of putative virulence genes

PCR was used to amplify a specific target sequence of DNA within the genes encoding five putative virulence factors. Primer sequences for each of the virulence genes were designed based on the gene sequences that were retrieved from GenBank database (Table 2.2). Amplification was carried out in 0.5 ml thick-walled PCR tubes in 50 μl reaction mixtures containing 20 pmoles of each primer, 2.5 mM MgCl , 250 μM of each dATP, dCTP, dGTP, and dTTP (Boehringer Manheim), 2.5 U of *Taq* DNA polymerase, 10 X buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton X-100) and ~10 ng of template DNA. Amplification involved 35 cycles consisting of [1 min at 94°C, 1 min at the appropriate annealing temperature (see Table 2.2) followed by 2 min at 72°C] on a MJ research PCR thermal cycler. The optimum annealing temperature was determined using the formula:

$$T_A(°C) = [2(A+T)]+[4(G+C)]-5$$

Following amplification, the reaction mixtures were electrophoresed on 2% (w/v) agarose gels containing EtBr (0.5 μg/ml). The DNA products were visualised and photographed under UV light and then scanned into the Alpha Imager Documentation System as described later.

2.6.4 Estimation of number of tandem repeats in the *bca* gene

Isolates identified as possessing the *bca* gene were analysed further to estimate the number of tandem repeats in the *bca* gene. The forward primer was chosen within the region of the gene encoding the N-terminus of the protein and the reverse primer from the 3'-end of the gene. Amplification was carried out as before in a final volume of 50 μl with the following cycling conditions – 25 cycles [10 s at 94°C, 30 s at 50°C and 2 min at 72°C (with an increment of 10 s after each cycle)] with a terminal extension at 72°C for 7 min. Following amplification, products were electrophoresed on 1% agarose gels containing EtBr (0.5 μg/ml). A 1-kb ladder was used as a molecular size standard.

2.7 Dot-blot Hybridisation

2.7.1 Preparation of labelled probe

A PCR-amplified product of the putative virulence genes of GBS was labelled using the AlkPhos Direct labelling system (Amersham) and used according to manufacturer's instructions. The AlkPhos Direct labelling system involves the direct labelling of probe DNA with a specially developed thermostable alkaline phosphatase enzyme. This is achieved by completely denaturing the probe and adding a cross-linker that covalently couples the enzyme to the nucleic acid probe. Labelling reactions were carried out 37°C in 1.5-ml microfuge tubes and the reaction components were added in the following order:

DNA (100 ng)	10 µl
Reaction buffer	10 µl
Labelling reagent [containing 0.1% (w/v) sodium azide]	2 µl
Cross-linker solution [containing 4.7% (v/v) formaldehyde]	10 µl

The reaction mixtures (32 µl) were incubated at 37°C for 30 min, after which the probe was used immediately or stored in 50% glycerol at -20°C.

2.7.2 Preparation of blots

Chromosomal DNA was purified as described above and 200 ng of DNA was blotted onto positively charged nylon membranes filters (Hybond™ N+, Amersham).

2.7.3 Hybridisation and detection

Blots prepared as described above were pre-wetted in 2 X SSC buffer (3.0 M NaCl and 0.3 M sodium citrate). Each blot was rolled separately into a cylindrical shape with a piece of nylon mesh and put into a hybridisation bottles. The bottles were rolled horizontally until the meshes and filters had unwound to cover the walls of the bottles. Each filter was allowed to equilibrate in a solution of 2 X SSC buffer to 37°C for about 20 min in the rotary hybridisation oven. Once the temperature of the 2 X SSC solution had reached 55°C, it was poured away and the blots were pre-hybridised in pre-hybridisation buffer

41

[AlkPhos Direct hybridisation buffer containing 0.5 M NaCl and 4% (w/v) blocking reagent] for at least 15 min at 55°C in the rotary hybridisation oven (Hybaid). The labelled probe was then added to the buffer to give a concentration of 5–10 ng/ml. Hybridisation was carried out at 55°C overnight.

Post-hybridisation stringency washes consisted of two primary washes and two secondary washes. Primary washes were performed at 55°C for 10 min each in 50 ml of a solution containing 2 M urea, 0.1% (w/v) SDS, 50 mM sodium phosphate, 150 mM NaCl, 10 mM $MgCl_2$ and 0.2% (w/v) blocking reagent (Amersham). Two secondary washes were performed at room temperature for 5 min each in secondary wash buffer. Fresh secondary wash buffer was made by diluting the secondary stock solution (1 M Tris base, 2 M NaCl, pH 10.0) 1:20 in sterile distilled water and adding $MgCl_2$ to give a final concentration of 2 mM.

Blots were drained of excess secondary wash buffer before 30–40 ml/cm^2 of detection reagent was added. The blots were left at room temperature for 5 min and then wrapped in Saran Wrap and exposed to a sheet of autoradiography film (Biomax ML; Kodak) in an X-ray film cassette with an intensifying screen (Hypercassette™, Amersham). Film exposures times varied depending on the blot, ranging from one hour to overnight, prior to development by an X-ray developing machine (Radiology Department, The Children's Hospital, Temple Street).

Nylon membranes were stripped of bound probe by incubating blots in 0.5% (w/v) SDS solution at 60°C for 60 min. The blots were then rinsed in 100 mM Tris (pH 8.0) for 5 min, wrapped in Saran Wrap and stored at -20°C between reprobings.

2.8 Automated Fluorescent DNA Sequencing

Fluorescence-based sequence analysis of double-stranded PCR fragments (Rao, 1994) was carried out using an ABI Prism BigDye terminator cycle sequencing ready reaction kit according to manufacturer's instructions on a Perkin Elmer Applied Biosystems model 310 fluorescent DNA fragment analyser.

2.8.1 Purification of PCR products for sequencing

Each PCR product was amplified using the primers listed in Table 2.2. Following amplification, the PCR products were purified (Qiagen column kit) to remove any contaminating primers or excess reaction components that might interfere with the sequencing reaction and 2 µl was checked by electrophoresis on an agarose gel for concentration and purity. The product was only used for subsequent sequencing reactions if a concentration of 5 µg/µl was obtained.

2.8.2 Dye terminator cycle sequencing of PCR products

The primers used in the sequencing reactions were the same ones as used in the primary PCR reactions (Table 2.2). All products were sequenced in forward and reverse directions. Sequencing buffer (200 mM Tris, 5 mM $MgCl_2$, pH 9.0) was prepared and used to dilute the BigDye terminator mix. Cycle sequencing reactions (5 µl) were set up in 0.2-ml thin-walled PCR tubes. Each reaction contained the following:

Big Dye terminator mix	2 µl
Sequencing primer (1 pmol/µl)	2 µl
PCR product	1 µl

The reactions were mixed and centrifuged briefly. They were then placed on a MJ research PCR thermal cycler and subjected to 25 cycles consisting of denaturation at 90°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. Following amplification 15 µl of sterile distilled water was added to each sample and they were then stored at 4°C for subsequent purification.

2.8.3 Precipitation of extension products

After cycle sequencing the reaction products were purified by ethanol precipitation to remove any excess reaction components. The 20-µl reactions were transferred into a sterile 1.5-ml Eppendorf tube containing 2 µl of 3 M sodium acetate, pH 4.6 and 50 µl of 95% ethanol. The contents of the tubes were mixed and left to precipitate for 15 min at

room temperature. They were then centrifuged at 14,000 X *g* for a further 15–30 min. Immediately after centrifugation, the supernatant fraction was removed and discarded. The samples were recentrifuged and any remaining supernatant fraction was removed. The pellets were then washed with 500 μl of 70% ethanol. After mixing, the samples were centrifuged for a further 10 min and the supernatant fractions removed. The pellets were then dried for 2 min at 90°C.

2.8.4 Preparation of samples for electrophoresis on ABI 310 Genetic Analyser

Each pellet was reconstituted in 20 μl of Template Suppression Reagent (Applied Biosciences) or deionised formamide and heated to 94°C for 2 min to denature the DNA. The samples were then chilled on ice, vortexed thoroughly and centrifuged briefly. The samples were stored at 4°C until ready to load onto the capillary. Prior to loading, the samples were transferred to 0.5-ml sample tubes and covered with a tube septum to avoid evaporation on the autosampler.

2.8.5 Preparation of the capillary for electrophoresis

The ABI 310 Genetic Analyser was set up according to manufacturer's instructions (Perkin Elmer, Applied Biosciences). Performance optimised polymer-6% (Pop 6), the polymer matrix used in the capillary for sequencing, was equilibrated to room temperature before it was loaded into the syringe. The syringe was then screwed into the pump block and the pump block was filled with polymer. The capillary used for short-read sequencing (47 cm x 50 μm internal diameter) was then installed by connecting one end to the pump block and one end near the electrode. The anode buffer reservoir was filled with Genetic Analyser buffer and installed on the pump block. The thermoplate was preheated to 50°C and the samples loaded onto the autosampler. Details of the run were entered into both the sample and the injection sheets in the computer's software and the run was started. Samples were injected into the capillary at 5 kV for 30 s. The end of the capillary near the cathode was then placed in buffer and electrophoresis was continued at 15 kV for 36 min.

2.8.6 Data analysis and storage

The raw data were collected by the data collection software on the ABI Prism 310. The data were then transferred into the sample manager where they were analysed by the sequencing analysis software and converted to DNA sequence data ready for visual analysis.

2.9 Computer-Assisted Image Analysis

Once the image was stored using the Alpha Imager™ 1220 Documentation system (Alpha Innotech Corporation, USA), profiles were analysed and compared using the 1D Professional Advanced Analysis v4.01 and database v1.12 software package (Phoretix International, UK) (Fig. 2.1).

Background subtraction was carried out on each gel using the rolling disc method set at 70. All lanes were optimised for band detection by adjusting the noise reduction and the percentage maximum peak value. Each gel contained two or three lanes with standard molecular weight markers. These lanes were used to normalise the gels using particular bands as Rf (Retardation factor) markers. Rf lines identify sets of bands that should match. To facilitate comparisons between gels suitable high and low bands were used as Rf marker bands. In this way Rf values were used to compare bands across gels run to different lengths. The molecular weights for each of the fragments were then computed using these Rf values. A similarity matrix was created using the band-based Dice similarity coefficient , S_D, equal to the ratio of twice the number of common bands to each pair of strains to the sum of all bands in two strains (Dice *et al.*, 1945). A variation tolerance allowance of 0.9% was allowed for experiment-to-experiment variation. Using this parameter, molecular standards derived from different gels grouped together at a mean linkage level of 95–100%. From the similarity data generated, a Neighbour joining group method (Saitou and Nei, 1987) was used to cluster the patterns and create an evolutionary tree. This method involves the successive clustering of the most closely related pairs of organisms.

2.10 Statistical Analysis

GBS attack rates were estimated with binomial confidence intervals and compared by Fisher's Exact test. Data for serotype and infection status in relation to the presence or absence of genes encoding putative virulence factors were analysed using frequency tables, chi-squared (χ^2) tests of association and goodness of fit, Fisher's Exact tests, and hierarchical log-linear analysis. Analysis of the *bca* gene tandem-repeat data was done by the Kolmogorov-Smirnov Goodness of Fit test, the Mann-Whitney U-test and Kruskal-Wallis non-parametric tests. $P < 0.05$ was considered statistically significant. The analyses were carried out with the SPSS statistical analysis package for Windows Version 10.0. These studies were done in conjunction with Dr. M. Kaliszer (Department of Community Health, TCD).

Figure 2.1. The major steps involved in analysing RAPD and PFGE profiles to generate a dendogram.

1. RAPD-PCR

2. Electrophoresis

3. Data Capture

4. Preliminary Visual analysis

5. Band detection

6. Normalisation of gel by Rf lines

7. Mol.wt. calibration using Rf

Phoretix 1D Advanced

8. Database

9. Matching

10. Dendogram construction

Phoretix 1D Database
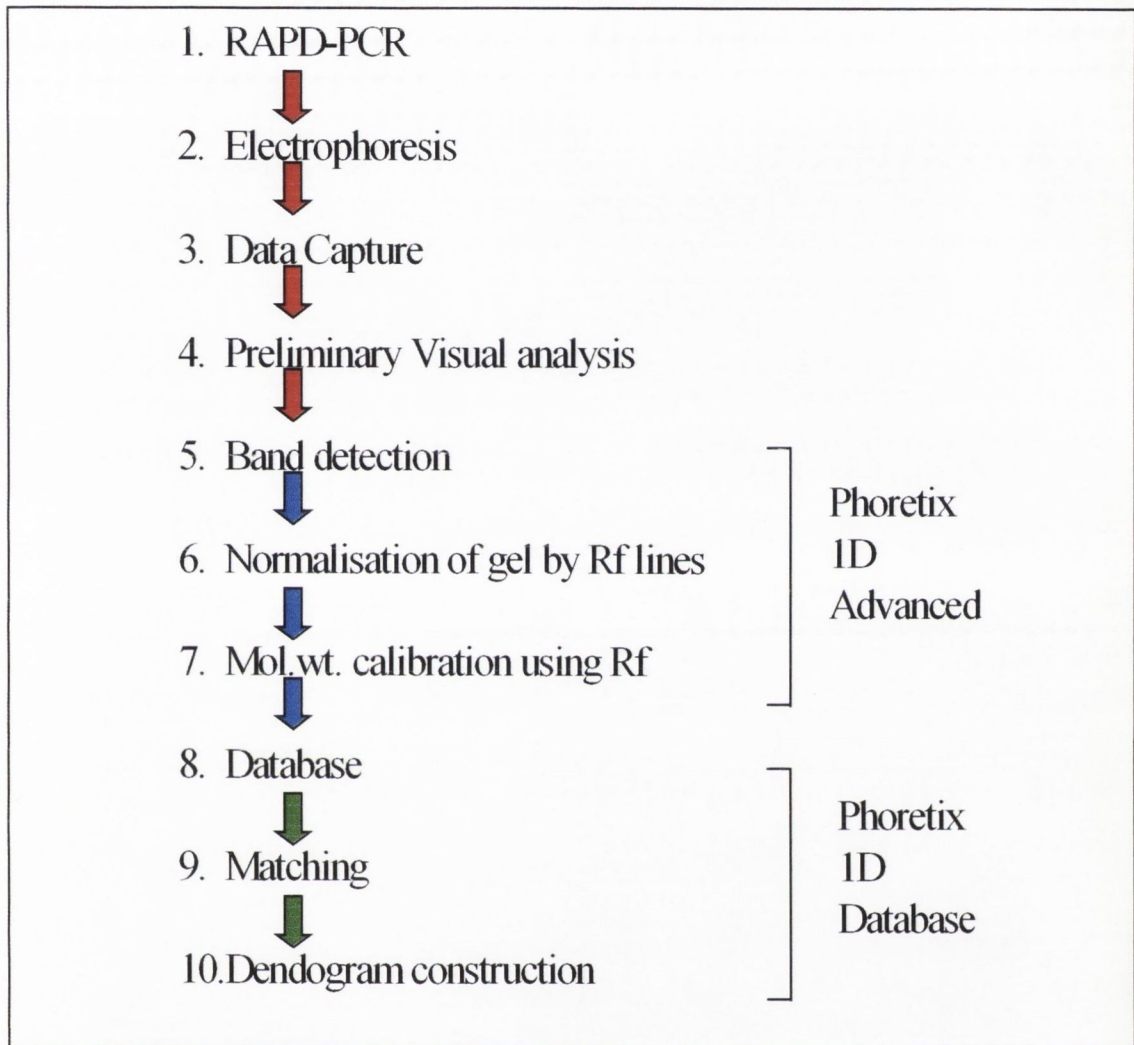
CHAPTER 3

Serotype and Sepsis of GBS

3.1 Introduction

Group B streptococci remain the most frequent cause of systemic infection in neonates. GBS may also be carried in the vagina of pregnant women without apparent clincal consequences for the newborn. Why some infants and mothers develop infection and others remain asymptomatic has not yet been fully answered. GBS capsular polysaccharides play crucial roles as major virulence factors for the organism. In particular, the presence of sialic acid residues as side-chain termini of the polysaccharides is a key determinant of GBS virulence (Tissi *et al.*, 1998).

3.1.1. Serotype distributions and epidemiology

Early studies of US populations indicated that GBS strains isolated from pregnant women and healthy neonates were evenly divided among serotypes I, II and III. However, new serotypes have been described in the past decade (type IV in Europe, type V in the United States, types VI and VIII in Japan) (Kvam *et al.*, 1995; Blumberg *et al.*, 1996; Lachenauer *et al.*, 2000). Although each of the nine known serotypes has been recovered from infants with serious infections, strains producing the type III antigen cause more than two-thirds of all neonatal disease in the United States (Baker *et al.*, 1995, Bhusan *et al.*, 1998). In contrast to the United States and other developed countries where type III is a predominant serotype, the most frequently identified serotype in Mexico is Ia, with serotype III found in only low frequency. Serotype Ia has also been mentioned as an important invasive serotype elsewhere (Hood *et al.*, 1961). Butter and de Moor (1967) reported a predominance of type Ia in the overall population (45%) and in infections (70–77%). In Mexico a higher proportion of nontypeable GBS has been reported, with approximately 12% of isolates being termed non-typeable (Palacios *et al.*, 1997). This figure contrasts with less than 1% of isolates in the majority of developed countries (Baker *et al.*, 1995) Reports have described serotype Ib as being significant in the Indian population (Jelinkova *et al.*, 1977) while serotypes VI and VIII are important serotypes in Japan (Lachenauer *et al.*, 2000).

Distribution studies have revealed population shifts of known GBS serotypes and the emergence of new types (Harrison *et al.*, 1998). For instance, although serotype III

isolates have been the most prevalent serotype in invasive disease in the United States in the past, strains of serotype V are now emerging as a major clinical problem (Blumberg *et al.*, 1996, Hickman *et al.*, 1999). Indeed, in Zimbabwe serotype V colonization is as prevalent in pregnancy as serotype III. No similar study has been carried out for the Irish population, apart from a report published by Kieran *et al.* (1998). These authors identified GBS carriage in 25% of women of child-bearing age, the predominant serotype being type III. The population shifts and emergence of new serotypes will have implications for the formulation of a multivalent vaccine. The purpose of this part of the study was to evaluate by means of population and statistical analysis, whether or not GBS serotypes correlated to the sepsis status of a mother or child in the Irish population.

3.1.2 Bacterial identification and typing

Early identification methods for microorganisms relied on tests that were based on the phenotypic properties of an organism. Strains were assigned to groups based on phenotypic characteristics that could be easily examined, such as staining properties, pigmentation, morphology, motility, nutritional requirements, acid formation and spore formation. However, one of the disadvantages of such methods is that tests derived for one particular organism are not always useful for other organisms (Busse *et al.*, 1996). Since the introduction of modern taxonomic methods, it has now been recognised that over-dependence on a small number of properties for classification purposes can lead to errors (Towner and Cockayne, 1994).

The main biological typing techniques of biotyping, phage typing, bacteriocin typing and serotyping are well established and have been applied to a wide variety of microorganisms (Murray *et al.*, 2000). Biotyping identifies organisms through examination of cultural and biochemical characteristics of an organism while phage typing is based on the sensitivity of bacterial strains to defined collections of bacteriophages which have been selected to provide maximum differentiation within a particular species. In general the biotype of a species is only useful when it is combined with other typing techniques, while phage typing is a technically complex procedure.

Serotyping is based on the variation in the antigenic components of the bacterial cell. Structural components such as capsules, cell envelopes, flagella and intracellular and extracellular molecules have been used in serological studies. In general, the serotype of a bacterial strain is a relatively stable and reli0idemiological tool. The main disadvantages seem to be associated with problems in antisera production and standardization of methodology.

3.1.3 Serotyping of GBS

GBS possess a distinct polysaccharide capsule. With minor exceptions, the type-specific polysaccharides are all composed of the same four component sugars, namely, glucose, galactose, N-acetylglucosamine and N-acetylneuramic acid, or sialic acid. Serotypes Ia and Ib have the same structure and components except for the way the core antigen is attached to the native antigen (Fig. 3.1). Based on reactions with specific antigens GBS are classified into one of nine types according to the antigenicity of their capsular polysaccharides. Serotyping has become the "gold-standard" phenotypic typing method used today in the study of GBS. The strains isolated from clinical cases usually belong to one of the major capsular types (Ia, Ib, II and III), but five new serotypes have been described in the last 15 years: IV, V, VI, VII, and VIII (Motlova *et al.*, 1986; Kogan *et al.*, 1995; Lachenauer *et al.*, 2000). Although some possible changes in the structures of antigens, related particularly to lysogenic conversion, have been noted (Meitert and Meitert, 1978), in general it seems that the serotype of a bacterium is a relatively stable and reliable typing marker. Occasionally, nontypeable strains, i.e, strains bearing capsular type antigens serologically different from the aforementioned classical serotypes, are isolated from either healthy subjects or patients.

All GBS capsular polysaccharides are immunologically distinct (Tissi *et al.*, 1998). The immune response against GBS is largely against the capsular antigens through their recognition by specific antibodies elicited in a host. Futhermore, the sialic acid residues present in capsular polysaccharide side chain have been shown to inhibit complement activation by the alternate pathway in the absence of any type-specific antibody, allowing the GBS to impede phagocytosis (Tissi *et al.*, 1998). Poorly capsulated strains or strains

Figure 3.1. Structures and structural modifications of the repeating units of the GBS polysaccharide antigens types Ia, Ib, II, III, IV, V and VIII. 1= native antigen; 2=core antigen, sialic acid removed; 3=degalactosylated antigen, side chain removed; 4= backbone antigen, complete side chain removal.

Ia

$$[4) \ \beta-D-glcp(1 \longrightarrow 4) \ \beta-D-galp(\ 1 \longrightarrow \ ]_n$$
--------------------------------- 4

↑ (1-3)

β–D-glcpNAc
--------------------------------- 3

↑ **(1-4)**

β–D-galp
--------------------------------- 2

↑ (2-3)

α–D-NeupNAc   1

Ib

$$[4) \ \beta-D-glcp(1 \longrightarrow 4) \ \beta-D-galp(\ 1 \longrightarrow \ ]_n$$
--------------------------------- 4

↑ (1-3)

β–D-glcpNAc
--------------------------------- 3

↑ **(1-3)**

β–D-galp
--------------------------------- 2

↑ (2-3)

α–D-NeupNAc   1

II

$$[4) \ \beta-D-glcpNAc \ (1 \longrightarrow 3) \ \beta-D-galp(1 \longrightarrow 4) \beta-D-glcp(1 \longrightarrow 3) \ \beta-D-glcp(1 \longrightarrow 2) \ \beta-D-galp(1 \longrightarrow]_n$$
2

↑(1-6)   ↑(2-6)

β–D-galp   α–D-NeupNAc

1

III

[4) β–D-glc*p*(1⟶6) β–D-glc*p*NAc*p*(1⟶3)β–D-gal*p*(1⟶]ₙ   4

        ↑ (1-4)

     β–D-gal*p*   2

        ↑ (2-6)

    α–D-Neu*p*NAc   1

IV

[4) α–D-glc*p*(1⟶4) β–D-gal*p*(1⟶4]β–D-glc*p* ⟶]ₙ   4

           ↑

     β–D-glc*p*NAc   3

           ↑

     β–D-gal*p*   2

           ↑

    α–D-Neu*p*NAc   1

V

[4) α-D-glc*p*-(1⟶4)-β-D-gal*p*(1⟶4]-β-D-glc*p*-(1⟶]ₙ   4

    ↑ (1-6)   ↑ (1-3)

   β–D-glc*p*NAc β–D-glc*p*
--- 3
    ↑ (1-4)

   α–D-Neu*p*NAc   2

    ↑ (2-3)

  β–D-Neu*p*NAc   1

VIII

⟶4)[β–D-glc*p*NAc (1⟶4) β–D-gal*p*(1⟶4)β–L-rha*p*(1 ⟶]ₙ

             ↑(3-2)

      α–D-Neu*p*NAc

        1

whereas highly capsulated strains resist phagocytic killing. The absence of *N*-acetylglucosamine in serotype VI isolates does not reduce the virulence of strains in comparison to other encapsulated GBS. Rubens *et al.* (1987) demonstrated that a specific, single mutation that completely interupted capsule expression resulted in a GBS isogenic mutant that was avirulent compared to the wild-type strain (Wessels *et al.*, 1992; Rubens, 1993 and 1995). The biosynthesis of such complex capsular polysaccharides requires several genes involved in monosaccharide synthesis and transport, oligosaccharide polymerisation, and cell wall attachment, as is seen in the studies of *E.coli* K1 capsule biosynthesis (Silver *et al.*, 1982).

The GBS serotype distribution of both carriage and invasive isolates is unknown in Ireland. Knowledge of the serotype distribution of virulent isolates may be valuable for obstetricians in making early decisions with respect to deliveries with a high risk of infection.

3.2 Results

3.2.1 Epidemiology of Group B streptococcal sepsis in the Rotunda Hospital (1991-1999).

During the decade following recognition of the importance of GBS disease in mothers and newborns, tremendous advances in treating infections and improving survival were achieved (Lannering *et al.*, 1983). During the 1990's several approaches had been put forward for the prevention of infection with GBS.

Although there has been a demographic increase in GBS disease, a nine-year audit of GBS disease in the Rotunda Hospital, Dublin did not demonstrate this trend. As can be seen in Table 3.1 there was an increase in the number of mothers attending the Rotunda Hospital and hence an increase in the number of live births over the last five years. Concomitant with the increase in the number of patients, there was an increase in documented morbidity in mothers due to GBS sepsis (0.12/1,000 mothers between 1991-94 to 0.55/1,000 mothers between 1995-99). This increase was statistically significant (P=0.007, Fisher's Exact test, 1 DF). However, the criteria for taking a blood culture changed in 1995. At that time a policy of septic screening was introduced for mothers with pyrexia of >38°C in labour or post partum. In contrast, the number of babies with confirmed GBS sepsis did not increase (P=0.42). Routine antenatal screening for GBS carriage is not performed at the hospital. However, since 1995 a policy has been implemented whereby intrapartum prophylaxis is administered to mothers with documented GBS carriage who have recognised risk factors and also to mothers with a previous history of invasive disease in a previous infant or stillbirth due to GBS. Similar findings in neonatal sepsis rates have been reported from other centres through the adoption of preventive consensus guidelines (Schrag *et. al*, 2000; Schuchat *et al*, 2000, 2001).

Over the last nine years the incidence of early-onset neonatal sepsis in this hospital has peaked at 0.44/1,000 live births (95% CI: 0.21–0.80/1,000). This compares with a reported demographic attack rate for this type of disease of 0.7–3.7/1000 live births (Boyer, 1995; Baker and Edwards, 1995; Schuchat and Schrag, 2000). Late-onset GBS infection with an attack rate worldwide of 0.5–1.8/1000 is also well above that seen in this

Table 3.1. Epidemiology of Group B streptococcal sepsis in the Rotunda Hospital (1991-1999).

Parameter	1991-1994 (GBS attack rate)	1995-1999 (GBS attack rate)
No. of Mothers attending for antenatal care	25,306 [31,633][a]	32,974
Confirmed GBS sepsis (Mothers)	3 (0.12/1,000)	18 (0.55/1,000)
No. of live births	22,960[28,700][a]	30,562
Confirmed GBS sepsis (live births)	13 (0.57/1,000)	12 (0.39/1,000)
Early-onset Disease	10 (0.44/1,000)	11(0.36/1,000)
Late-onset Disease	3 (0.13/1,000)	1(0.03/1,000)

[a] Nos. in square brackets are those for five-year periods based on the yearly averages

Irish hospital (0.03–0.13/1,000; 95% CI for the peak rate of 0.13–0.38/1,000). An audit of invasive GBS infection in the neonate is currently underway at all Irish maternity units in collaboration with the British Paediatric Surveillance Unit (M. Cafferkey, personal communication).

3.2.2 Sepsis status of the 159 GBS isolates

All of the GBS isolates were divided into a sepsis status category between "0" and "4+" depending on the site of isolation and eventual morbidity or mortality to the mother or child (Table 3.2, Section 2.2.2). Of the 159 isolates sepsis status, 87 (52.8%) were carriage isolates ("0" sepsis status), representing those obtained from higher/lower vaginal and rectal swabs from pregnant and non-pregnant women and causing no morbidity to mother or child (Table 3.3).

The remaining 72 strains were termed invasive isolates. Any of the isolates obtained from specimens retrieved from normally sterile sites were designated "1+". No histological signs of inflammation and no pus were prominent. These isolates represented 18 (11.3%) of the 159 isolates. This "1+" category is not in the strictest of terms exclusively invasive isolates, as the positive swabs were retrieved from sites that potentially might have been contaminated while specimens were collected. In the case of the "2+" isolates, which also represented 11.3% of the 159 isolates, definite signs of invasion were apparent with either wound infections, histological inflammation or pus formation. Only 3 (1.9%) of the isolates were obtained from patients with mastitis and therefore were grouped in a category alone designated "3+". The final and most significant category, "4+", represented 20.8% of all cases. These 33 invasive isolates (23 from mothers and 10 from infants) were recovered from sites either after foetal death or from blood cultures or both. As a percentage of invasive cases, including the "1+" category studied in this project, this "4+" category represented 45.8% of cases.

3.2.3 Serotype distribution of the 159 GBS isolates

All 159 GBS carriage and invasive isolates were serotyped according to the Lancefield method with commercially available antisera (Mast Diagnostic) to the capsular polysaccharide antigens Ia, Ib, II, III, IV and V (Section 2.3.2). Only 4 of the isolates

Table 3.2. The sepsis status and isolate numbers of the 159 isolates.

Isolate Reference No.*	Isolate Name	Sepsis Status	Serotype	Isolate Reference No.*	Isolate Name	Sepsis Status	Serotype
1	203	0	NT	41	H1997	0	II
2	237	0	II	42	173	0	III
3	247	0	Ib	43	183	0	III
4	250	0	III	44	8076	0	III
5	251	0	Ia	45	2524	0	III
6	255	0	III	46	2011	0	III
7	278	0	III	47	2008	0	III
8	7368	0	V	48	7105	0	III
9	C2	0	V	49	7106	0	III
10	C3	0	III	50	H524	0	III
11	C5	0	Ia	51	H887	0	III
12	C6	0	Ia	52	H1059	0	III
13	C7	0	III	53	H1870	0	III
14	C10	0	II	54	H1712	0	III
15	C11	0	III	55	H1969	0	III
16	C12	0	NT	56	222	0	IV
17	C13	0	Ia	57	184	0	V
18	C14	0	III	58	187	0	V
19	C16	0	III	59	246	0	V
20	C19	0	III	60	7450	0	V
21	C22	0	III	61	7470	0	V
22	C23	0	NT	62	H701	0	V
23	C24	0	NT	63	H708	0	V
24	C27	0	Ib	64	H1516	0	V
25	200	0	Ia	65	H2234	0	V
26	2186	0	Ia	66	271	0	NT
27	H703	0	Ia	67	204	1+	III
28	H777	0	Ia	68	273	4+	Ia
29	H1240	0	Ia	69	219	4+	Ib
30	H1356	0	Ia	70	H1068	3+	Ia
31	H1999	0	Ia	71	H896	4+	Ia
32	202	0	Ib	72	McCann	4+	Ia
33	235	0	Ib	73	188	4+	NT
34	8161	0	Ib	74	220	4+	Ib
35	167	0	II	75	234	4+	III
36	189	0	II	76	242	1+	Ia
37	243	0	II	77	248	1+	V
38	254	0	II	78	279	2+	Ia
39	H1766	0	II	79	C8	1+	V
40	H1963	0	II	80	C26	2+	Ia

Isolate Reference No.*	Isolate Name	Sepsis Status	Serotype	Isolate Reference No.*	Isolate Name	Sepsis Status	Serotype
81	C28	1+	II	121	208	4+	III
82	C29	4+	NT	122	B497	4+	III
83	7763	0	III	123	W2444	4+	III
84	205	1+	Ib	124	H2003	4+	III
85	206	1+	Ib	125	H1842	4+	III
86	227	1+	Ib	126	186	4+	V
87	258	1+	Ib	127	H1741	4+	V
88	259	1+	Ib	128	H1066	2+	Ib
89	H1646	1+	Ib	129	H1692	0	III
90	H1645	1+	Ib	130	1741	4+	V
91	175	1+	III	131	H4341	4+	III
92	194	1+	III	132	H4183	2+	Ib
93	H823	1+	III	133	270	0	II
94	7552	1+	IV	134	272	0	IV
95	1744	1+	V	135	7543	0	Ia
96	H1635	1+	V	136	8429	0	II
97	181	2+	Ia	137	8600	0	V
98	H1502	2+	Ia	138	8857	0	II
99	H2000	2+	II	139	820	0	Ia
100	H578	2+	III	140	8718	2+	Ib
101	2292	2+	V	141	8099	2+	III
102	H852	2+	V	142	299	0	III
103	H1711	2+	V	143	320	0	V
104	H1338	3+	Ia	144	1135	0	Ib
105	H1666	3+	Ia	145	1630	0	II
106	170	4+	Ia	146	1588	0	III
107	185	4+	Ia	147	1845	0	Ia
108	245	4+	Ia	148	1929	0	Ia
109	H1234	4+	Ia	149	1813	2+	Ia
110	35	4+	Ib	150	3042	4+	III
111	215	4+	Ib	151	1795	0	III
112	226	4+	Ib	152	5057	0	Ib
113	229	4+	Ib	153	5026	0	Ib
114	230	4+	Ib	154	5036	2+	III
115	H901	4+	Ib	155	5002	2+	Ib
116	H970	4+	Ib	156	5452	0	III
117	H913	4+	Ib	157	4532	2+	Ib
118	233	4+	II	158	3656	2+	III
119	H964	4+	II	159	8132	0	V
120	207	4+	III				

* Reference nos. were assigned to this collection for storage purposes.

Table 3.3. The frequency and percentage of carriage isolates "0" and invasive isolates which caused infection graded between "1+" and "4+".

Status	Frequency	Percent	
		All cases	Invasive
0	87	52.8	—
1+	18	11.3	25.0
2+	18	11.3	25.0
3+	3	1.9	4.2
4+	33	20.8	45.8
Total cases	159	100.0	
Total invasive cases	72		100.0

were classified as truely non-typeable as they caused spontaneous agglutination in all antisera. Three of the isolates caused agglutination with two of the antisera. Each of these isolates had a strong reaction with antiserum IV along with either antiserum type Ib, type II or type V and were designated non-typeable. Together the non-typeable isolates represented 4.4% of all the isolates studied.

The overall serotype distribution, shown in Figure 3.2, was Ia (19.5%), Ib (18.9%), II (10.7%), III (29.5%), IV (1.9%), V (15.1%), and non-typeable (4.4%).

The serotype distribution varied somewhat from hospital to hospital. The most frequently recovered GBS serotype in each hospital was type III. Type Ib isolates were more frequently isolated in The Rotunda and Holles Street Hospitals compared to The Coombe Hospital (Table 3.4).

3.2.4 Simpson's Index of diversity

An assessment of serotyping as an efficient typing method is based on a number of factors: typeability, reproducibility, and discrimination. Serotyping fulfils the characteristics of typeability and reproducibility. The discriminatory power was calculated by Simpson's index of diversity. This single numerical index of discrimination (D) is based on the probability that two unrelated isolates from the test population will be placed into different typing groups. In using a successful typing system the index of diversity should be as close to 1.0 as possible.

The index is calculated by taking the sum of n_j $(n_j -1)$, i.e. the total number of isolates for each type, j, in the typing system. This sum is then divided by $N(N-1)$ where N is the total number of isolates examined in the study. After the division this figure is then subtracted from 1.0. The closer the discriminatory index is to 1.0 determines the usefulness of a typing system for discriminating isolates. In this study the Simpson's index of diversity for serotyping of isolates in Ireland was 0.81 (Fig. 3.3).

Figure 3.2. GBS serotype distribution among patients in all three hospitals.

GBS Serotype Distribution
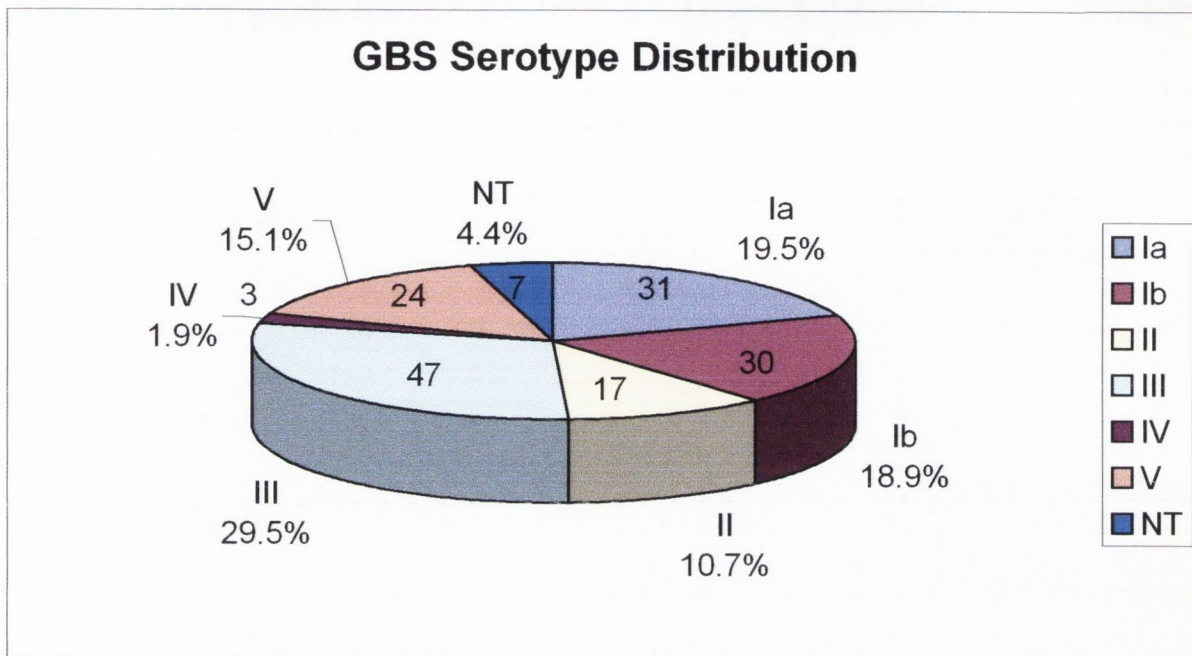
Table 3.4. Serotype distribution of GBS in three Dublin Maternity Hospitals.

Serotype	Rotunda Maternity Hospital	National Maternity Hospital, Holles St.	Coombe Maternity Hospital	Totals for Three Dublin Maternity Hospitals
Ia	15 (16.0%)	12 (26.7%)	4 (20.0%)	31 (19.5%)
Ib	22 (23.4%)	7 (15.5%)	1 (5.0%)	30 (18.9%)
II	10 (10.6%)	5 (11.1%)	2 (10.0%)	17 (10.7%)
III	28 (29.8%)	12 (26.7%)	7 (35.0%)	47 (29.5%)
IV	3 (3.2%)	0	0	3 (1.9%)
V	13 (13.8%)	9 (20.0%)	2 (10%)	24 (15.1%)
NT	3 (3.2%)	0	4 (20%)	7 (4.4%)
Carriage	52 (55.3%)	19 (42.2%)	16 (80.0%)	87 (54.7%)
Invasive	42 (44.7%)	26 (57.8%)	4 (20.0%)	72 (45.3%)
Totals	94	45	20	159

Figure 3.3. Simpson's index of diversity for serotyping of isolates in Ireland

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{s} n_j (n_j - 1)$$

No. of strains (N)= 159

No. of types = n_1 (31 , Ia); n_2 (30, Ib); n_3 (17, II); n_4 (47 , III); n_5 (3, IV); n_6 (24 ,V); n_7 (7, NT).

D = 1- [(31 x 30 + 30 x 29 + 17 x 16 + 47 x 46 + 3 x 2 + 24 x 23 + 7 x 6) / (159 x 158)]

   = 1-[(930 + 870 + 272 + 2162 + 6 + 552 + 42) / 25122]

   = 1- [4834/25122]

   = 0.81

3.2.5 Comparison of sepsis and serotype

Each serotype was assessed to see if there was any association of the serotypes with a specific sepsis category (Tables 3.5). Within each serotype, except non-typeable strains, isolates displayed a spectrum of invasive capacity. However, the numbers of isolates were too small to measure an association between grade of invasiveness and serotype, even excluding the 10 serotype IV and nontypeable strains.

Accordingly, sepsis categories "1+" and "2+" were grouped together as were categories "3+" and "4+". However, even when the categories of invasiveness were variously combined, no statistically significant association of degree of invasiveness with serotype was demonstrated (Table 3.6). The highest adjusted residuals (i.e., deviation from expected values) were observed with serotypes Ib, II and III (i.e., -3.3, 2.0 and 1.7, respectively). The -3.3 adjusted residual was associated with the carriage isolates of serotype Ib. This figure indicates that there was a negative association of serotype Ib with the carriage category of isolates. The adjusted residuals 2.0 and 1.7 for serotypes II and III, respectively, indicated that a large percentage of each serotype was classified as carriage isolates.

Finally, the "1+", "2+", "3+" and "4+" categories were combined together as invasive GBS (Table 3.7). Capsular polysaccharide type Ib GBS was found to be the most frequent invasive type with 22 (73.3%) of the 30 isolates termed as invasive. Of the serotype Ia isolates 51.6% were classified as invasive. In contrast, 76.5% of the serotype II GBS isolates were classified as carriage. Although serotype III isolates represented 29.6% of the total population, only 36.2% of this serotype contributed to invasive disease. Excluding the non-typeable and serotype IV strains, overall the serotypes of the GBS isolates have a statistically significant association with the carriage or invasive status of isolates ($\chi^2 = 14.9$, DF= 4, $P = 0.005$). Serotypes Ib and II have statistically significant adjusted residuals and serotype III marginally so. Serotype Ib has more invasive isolates than expected, while serotypes II and III have less than expected. Serotype Ia isolates were observed to be evenly distributed between the carriage and invasive categories while serotype V had a slightly higher percentage in the carriage category (58.3%).

Table 3.5. Serotype distribution of carriage and invasive isolates of 159 GBS isolates.

Serotype	No. of carriage isolates "0"	Nos. of invasive isolates*				Total
		"1+"	"2+"	"3+"	" 4+"	
Ia	15	1	5	3	7	31
Ib	8	7	5	-	10	30
II	13	1	1	-	2	17
III	30	4	4	-	9	47
IV	2	1	-	-	-	3
V	14	4	3	-	3	24
NT	5	-	-	-	2	7
Totals	87	18	18	3	33	159

* For designation of codes see Materials and methods

Table 3.6. Frequencies, percentage, and adjusted residuals of the important serotypes within the different sepsis categories.

Serotype		Sepsis			Total
		"0"	"1+" and "2+"***	"3+ and "4+"***	
Ia	Count	15	6	10	31
	%	18.8%	17.1%	29.4%	20.8%
	Adjusted Residual	-0.7	-0.6	1.4	
Ib	Count	8	12	10	30
	%	10.0%	34.3%	29.4%	20.1%
	Adjusted Residual	-3.3	2.4	1.5	
II	Count	13	2	2	17
	%	16.3%	5.7%	5.9%	11.4%
	Adjusted Residual	2.0	-1.2	-1.2	
III	Count	30	8	9	47
	%	37.5%	22.9%	26.5%	31.5%
	Adjusted Residual	1.7	-1.3	-0.7	
V	Count	14	7	3	24
	%	17.5%	20.0%	8.8%	16.1%
	Adjusted Residual	0.5	0.7	-1.3	
Total	Count	80	35	34	149
	%	100.0%	100.0%	100.0%	100.0%

*** Categories "1+" and "2+" were combined together as were categories "3+" and "4+".

Table 3.7. The association of serotype with sepsis with the adjusted residuals given.

Serotype		Status		Total
		Carriage	Invasive**	
1a	Count	15	16	31
	% within SEROTYPE	48.4	51.6	100
	Adjusted Residual	-0.7	0.7	
1b	Count	8	22	30
	% within SEROTYPE	26.7	73.3	100
	Adjusted Residual	-3.3	3.3	
II	Count	13	4	17
	% within SEROTYPE	76.5	23.5	100
	Adjusted Residual	2.0	-2.0	
III	Count	30	17	47
	% within SEROTYPE	63.8	36.2	100
	Adjusted Residual	1.7	-1.7	
V	Count	14	10	24
	% within SEROTYPE	58.3	41.7	100
	Adjusted Residual	0.5	-0.5	
Total	Count	80	69	149
	% within SEROTYPE	53.7	46.3	100

** Sepsis status categories "1+", "2+", "3+", and "4+" combined.

3.2.6 Distribution of carriage and invasive isolates and their serotypes in Dublin maternity hospitals

Each of the 159 isolates were assessed by serotype to see if there was an association with carriage and invasive status in the three Dublin maternity hospitals (Table 3.8). The numbers of isolates were too small to measure an association between invasive and carriage status between the Coombe hospital and the other two hospitals.

For the most part the Rotunda and Holles Street Hospitals display similar trends to the overall observed invasive and carriage status. However, some differences in the invasive status between the two hospitals are noteworthy. Capsular polysaccharide type Ib GBS was found to be the most frequent invasive type in the Rotunda Hospital with 15 out of 22 (68%) designated invasive. All serotype Ib isolates obtained from Holles Street Hospital were invasive. A higher frequency of carriage isolates within serotype II was noted in the Rotunda Hospital (i.e. 9 out of 10 isolates) than in Holles Street Hospital (3 out of 5 isolates).

Similar frequencies of invasive status for serotype III was observed in the Rotunda and Holles Street Hospital while, allowing for small numbers, all serotype III isolates obtained from the Coombe were designated carriage.

Table 3.8. Distribution of carriage and invasive GBS isolates by serotype in the three Dublin maternity hospitals

Serotype	Rotunda Maternity Hospital		National Maternity Hospital, Holles St.		Coombe Maternity Hospital		Totals for Three Dublin Maternity Hospitals	
	Carriage	Invasive	Carriage	Invasive	Carriage	Invasive	Carriage	Invasive
Ia	7	8	5	7	3	1	15	16
Ib	7	15	0	7	1	0	8	22
II	9	1	3	2	1	1	13	4
III	16	12	7	5	7	0	30	17
IV	2	1	0	0	0	0	2	1
V	9	4	4	5	1	1	14	10
NT	2	1	0	0	3	1	5	2
Totals	52	42	19	26	16	4	87	72

3.3 Discussion

Serotyping is one of the classic strain typing techniques. It is still an important tool for routine identification and typing of many bacterial species, such as meningococci and pneumococci, particularly when used in conjunction with other typing methods. Polysaccharide capsules of several organisms are thought to play important roles as virulence factors in human infections. The primary function of bacterial capsules in pathogenesis is to help the pathogen to evade phagocytic host-defense mechanisms. The type-specific capsular polysaccharide antigen of GBS is secreted as a soluble polysaccharide but is also covalently attached to the peptidoglycan layer. Infection of the neonate usually occurs via the mother, although cross-infection has also been suggested in some instances (Christensen *et al.*, 1985). To determine serotype distribution changes in Ireland the serotype needs to be monitored for both carriage isolates and those that may lead to eventual morbidity to mother or child.

GBS isolates are classified into nine serotypes on the basis of the arrangement of component monosaccharides into a unique repeating unit in their capsular polysaccharide (Fig. 3.1). GBS capsules have been shown to be antigenically distinct and highly immunogenic and are known to be essential virulence factors. A study demonstrated that a specific, single mutation that completely interrupted capsule expression in a GBS serotype III isogenic mutant resulted in diminished virulence in animal models compared to the wild-type strain (Rubens *et al.*, 1987).

Group B streptococci occur normally in the vagina, throat and faeces. The overall carriage rate has been reported as 25% in Irish women (Kieran *et al.*, 1998). This is typical of carriage rates that are seen around the world, with some exceptions such as Mexico. Only 10% of pregnant Mexican women are colonised with GBS (Palacios *et al.*, 1997). The incidence of GBS disease in the Rotunda Hospital over a nine-year period was 0.47/1,000 live births. This incidence is considered relatively low and contrasts to incidence of GBS disease observed in other countries. Indeed, during a 10-year study period in Finland an incidence of GBS disease was 0.76/1,000 live births (Kalliola *et al.*, 1999) while an incidence of 0.77/1,000 livebirths was observed over a 5-year period in the United States (Schuchat *et al.*, 2001).

Type III together with serotypes Ia, Ib, and II remain the most prevalent polysaccharide capsular types (Table 3.9). However, there has been dramatic change in prevalence of serotype during the last decade such as the increase in serotype V in the United States and serotypes VI and VIII in Japan. The remarkable increase in the incidence of serotype V has occurred in the 1990's, when the percentage of serotype V climbed from 2.6% in 1992 to 14% in 1993 and then 20% in 1994 (Blumberg et al. 1996). Serotype Ib in Irish GBS was found to be the most frequent invasive type with 22 of the 30 serotype Ib isolates (73.3%) designated as invasive. However, non-typeable GBS are frequently reported and represent 4.4% of Irish isolates.

Of 159 isolates tested in this present study six serotypes were represented and only 4.4% of isolates were termed non-typeable. This is a higher percentage than that seen in the United States (Table 3.9) but lower than that seen in Mexico where 12% of isolates were termed non-typeable (Palacios et al., 1997). Sellin et al. (1992) described reversible non-typeable GBS phase where enrichment of low-density subpopulations with hypotonic Percoll gradient centrifugation before typing has been successful. This suggests that the Irish non-typeable isolates may be due to the occurrence of newer serotypes VI, VII and VIII, an inactivating insertion sequence or mutation in genes essential for capsule expression (e.g. cpsD) or that the isolate is a reversible non-encapsulated phase variant. However, there are no commercially available antisera to identify these serotypes VI–VIII.

The predominance of serotype III among Irish isolates is similar to the findings reported by Christensen et al. (1985) on American isolates. However, unlike the findings of Christensen et al. (1985), serotype III is not responsible for the majority of invasive cases in the present study. Of all the serotype III isolates only 36.2% of this serotype contributed to invasive disease. In the present study, isolates termed as invasive comprise 77.3% of all isolates belonging to serotype Ib which accounted for 17% of the strain population.

Table 3.9. World-wide distribution of main GBS serotypes.

Country (State)	Serotype (%)							References
	Ia	Ib	II	III	IV	V	Other*	
Ireland	19.5	18.9	10.7	29.5	1.9	15.1	4.4	This study
Canada	17	10	9	19	–	31	14	Tyrrell *et al.*, 2000
Finland	23	11	6	47	8	1	7	Kalliola *et al.*, 1999
France	15.4	nd	19.5	24	nd	13.6	nd	Adam *et al.*, 1994
India	23	11	12	nd	nd	nd	24	Radhakrishnan *et al.*, 1995
Japan	8.8	8	nd	11.2	nd	nd	52.4	Mikamo *et al.*, 2000
Korea	nd	35.8	nd	23.9	nd	nd	nd	Chong *et al.*, 1993
Morocco	32.2	nd	nd	39	nd	10.2	nd	Aitmhand *et al.*, 2000
Sweden	18	nd	nd	62	nd	9	nd	Berg *et al.*, 2000
United States (Maryland)	40	9	6	27	–	15	3	Lin *et al.*, 1998
United States (Ohio)	19	12	10	16	4	27	1	Goodrum *et al.*, 2000
United States (Texas)	31.6	7.9	25	22.4	–	11.8	1.3	Hickman *et al.*, 1999
Zimbabwe	11	–	1	41.8	3.3	37.4	2	Moyo *et al.*, 2000

* Includes serotypes VI–VIII as well as non-typeable; nd = not determined

The present study indicates that serotyping of GBS is both useful and reliable, if care is taken in the interpretation of test results. Serotyping of Irish GBS isolates fulfils the characteristics of typeability and reproducibility. The discriminatory power of serotyping in the Irish population, calculated by Simpson's index of diversity, gave a result of 0.81. This index indicates that if two strains were sampled randomly from the population, then on 81% of occasions they would fall into different types. This index is high in comparison to serotyping in other organisms. For example serotyping provides less discrimination for organisms such as *Serratia marcescens* or *Candida albicans* which have indexes of 0.57 and 0.44, respectively.

Knowledge of the serotype distribution of both carriage and invasive Irish isolates will permit assessment of the likely effectiveness of the future capsule-based vaccines. A multivalent GBS vaccine for use in Ireland would currently include types Ia, Ib, II, III and V. This formulation would provide coverage against 93.7% of the isolates in this study. However, the serotype distribution of GBS throughout Ireland needs to be monitored continually especially with respect to the emergence of new non-typeable organisms.

CHAPTER 4

Genotypic Characterisation

4.1 Introduction

Electrophoresis is increasingly being used as a tool to study bacterial populations and to investigate the epidemiology of bacterial infection. Traditional methods of strain typing, such as bacteriophage typing and serotyping, have been supplemented or replaced in some laboratories with newer molecular methods, such as plasmid fingerprinting, ribotyping, PCR-based methods and chromosomal DNA analysis, e.g., restriction endonuclease analysis (REA) and pulsed-field gel electrophoresis (PFGE) (Section 1.12.2).

In the study described here random amplified polymorphic DNA analysis (RAPD) and pulsed-field gel electrophoresis (PFGE) of chromosomal DNA were performed on the collection of 159 isolates to assess the potential utility of these techniques as tools for investigating the epidemiology of GBS invasive disease.

4.2 Pulsed-field gel electrophoresis (PFGE)

Schwartz and Cantor (1984) first described pulsed-field gel electrophoresis (PFGE). The development of PFGE, which allows the separation of DNA fragments as large as 10 Mb, has enabled the complete genome of most bacteria studied to be viewed as a single electrophoretic profile. Previously, cloning was required for mapping of genomic DNA, which was time-consuming and laborious. The PFGE method has facilitated the construction of physical maps of bacterial chromosomes and is a more efficient method than conventional gel electrophoresis which can only separate DNA fragments up to ~50 kb.

This technique was first utilised for the separation of yeast chromosomes through the use of infrequent-cutting restriction endonucleases and an electrophoresis system in which the electric field alternates in direction. Large genomic fragments are not readily separated by conventional gel electrophesis. In contrast, a PFGE system enables DNA molecules to change their direction of migration periodically, separation being based on the fact that larger DNA molecules change direction more slowly than smaller molecules resulting in the separation of the fragments through retardation rather than sieving which occurs in conventional gel electrophoresis. The mobility of different molecules is a linear function of their molecular size and resolution can be achieved by adjusting the pulse time

of the electric field. Other physical factors are also important. It is known that changes in temperature, voltage, agarose concentration and buffer strength will also affect the mobility of the DNA molecules. The pattern of restriction fragments obtained is characteristic for each strain and provides an estimate of the genomic relationship between strains (Towner and Cockayne, 1994).

Several different PFGE systems are available but the most popular systems are those that rely on the contour-clamped homogeneous electric fields (CHEF). These systems rely on a hexagonal electrode array to produce a highly uniform electric field, which produces straight lanes and good resolution of DNA fragments up to and exceeding 5 Mb in size. The discriminatory value and information generated by PFGE can be increased by using more than one infrequent-cutting restriction endonuclease. A number of investigators have found that typing of strains by PFGE offers greater differentiation of strains than methods such as ribotyping and that it is a reliable and reproducible fingerprinting technique (Gordillo *et al.*, 1992).

4.3 Random amplified polymorphic DNA (RAPD) fingerprinting

Since prior knowledge of the DNA sequence is often required before PCR-based tests can be applied (Caetona-Anollés *et al.*, 1991b), PCR-based strategies to amplify short arbitrary stretches of DNA from a target genome were developed (Welsh and McCelland 1990, Williams *et al.*, 1990; Caetona-Anollés *et al.*, 1991a). Welsh and McCelland (1990) designed arbitrary oligodeoxynucleotide primers of 20–34 nucleotides in length and used the method of PCR to amplify bacterial DNA fingerprints in a method called arbitrarily primed PCR (AP-PCR). Williams *et al.* (1990) used shorter arbitrary primers (9–10 nucleotides in length) to amplify DNA polymorphisms from a variety of plant, human and bacterial templates. They called this method random amplified polymorphic DNA (RAPD) analysis. Caetona-Anollés *et al.* (1991a) described a similar technique called DNA amplification fingerprinting (DAF) which uses primers as short as five nucleotides in length.

The methods described by Welsh and McCelland (1990) and Caetona-Anollés *et al.* (1991a) require radioactive labelling and silver staining, respectively, and therefore are

cumbersome and unsuitable for general laboratory use. Therefore, the RAPD method of Williams *et al.* (1990) was deemed the most practical method for use in the present studies. The RAPD patterns can be used for genetic mapping as well for analysis of genetic distance and phylogeny reconstruction (Williams *et al.*, 1993). Caetona-Anollés *et al.* (1991a) noted that the bands were of two types, namely, those that were phylogenically conserved and those that were strain specific.

The advantages of RAPD-PCR are that the amount of DNA needed for RAPD analysis is much smaller than that needed for REA or PFGE, and digestion by a restriction endonuclease is unnecessary. Also, prior knowledge of the target DNA sequence is not required, the technique does not require the labelling of the nucleotide primer, and the procedure is fast and relatively simple. RAPD has been demonstrated to be useful for the analysis of a collection of closely related strains with similar phenotypic characteristics, but with different pathogenicity properties which are difficult to analyse (Bainbridge and Heale, 1996). The technique is an excellent epidemiological tool for grouping species, identifying pathogenic strains and detecting atypical strains or contaminants.

There have been a number of reports describing the ineffectiveness of this technique because of non-reproducibility or the lack of inter-laboratory reproducibility of the amplification protocol (Penner *et al.*, 1993). The final PCR products of a RAPD-PCR depend on a number of variables, such as the sequence and primer length as well as the PCR reaction conditions which can also influence standard PCR reactions. Magnesium concentrations, genomic DNA extraction procedures, PCR cycling conditions, the ratio of template to primer concentration, the type of thermocycler and the concentration and type of *Taq* polymerase are all important factors in the reproducibility of results. For all these reasons before RAPD-PCR can be considered to be a useful epidemiological tool for a particular application it is essential, that amplification conditions are optimised and standardised for the species being examined. This method has been used successfully for typing several medically important organisms, including groups A, C and G streptococci (Seppälä *et al.*, 1994 and Bert *et al.*, 1996). There is also a report in the literature documenting the suitability of RAPD analysis for the differentiating isolates of GBS (Chattelier *et al.*, 1997).

4.4 Molecular phylogeny using DNA fingerprinting

Molecular phylogeny is an attempt to reconstruct evolutionary history using molecular characteristics such as variations in the genome of an organism. All DNA fingerprinting procedures require band and lane matching in one form or another whether the band patterns be generated by PCR-based techniques, such as random amplified polymorphic DNA analysis (RAPD), or by restriction fragment length polymorphisms. Evolutionary relationships can be constructed using DNA data by a method known as genetic distances. The genetic distances method makes use of overall similarities and differences at the DNA level.

In this thesis all relationship studies involved the use of the Phoretix 1D Advanced and Phoretix 1D Database software. Phoretix 1D Advanced is a flexible gel-analysis package that includes tools for molecular size calculation, overcoming gel distortions and normalisation between gels using Rf (Retardation factor). The Phoretix 1D Database then allows comparison of lanes from many gels to produce lane-relationship dendograms using the UPGMA method (unweighted pair group method using arithmetic averages) or the Neighbour Joining Method and tables of relationship data. These methods take the input data and derive from them some measure of similarity or difference between strains and from this construct a tree that tries to match these data.

The simplest of the distance methods is a type of cluster algorithm that is known as UPGMA (Sokal and Sneath, 1963). This method has gained popularity primarily because of its simplicity and speed (although many other distance methods are as fast). In this method, the distance between two clusters is calculated as the average distance between all pairs of objects in the two different clusters. This method obviously assumes that the species are all still existing and that all rates of change are equal. Although this is a clear assumption of the method, there are many examples where rates of evolution vary between species (Sokal and Sneath, 1963).

Another very popular distance method is the Neighbour Joining Method (Saitou and Nei, 1987). This method attempts to correct the UPGMA method for its strong assumption that the same rate of evolution applies to each branch. Hence this method yields an unrooted tree. A modified distance matrix is constructed to adjust for differences

in the rates of evolution of each branch. Similar to the UPGMA method, the least distant pairs of nodes are linked and their common ancestral node is added to the tree, while their terminal nodes are cut from the tree. This continues until only two nodes remain. There are many other methods to reconstruct trees via distance measures (e.g. Fitch and Margoliash, 1967) almost all of which are very rapid (Hillis and Bull, 1993).

4.5 Results

4.5.1 Optimisation of RAPD-PCR parameters

To identify primers that generate informative genetic polymorphisms by PCR, five unrelated GBS strains (251, Ia; 35, Ib; II; 255, III; 186, and 237, V) were selected from the collection of 67 isolates available at the time these studies were initiated. The strains were isolated from various different anatomical sites and belonged to different serotypes. The five strains were obtained from a blood culture, the vaginas of asymptomatic women and from a neonate suffering from meningitis.

Eight oligonucleotides (Table 2.1) were appraised individually for their possible usefulness in detecting genetic polymorphisms according to the following constraints, namely, that they possessed a suitable G + C composition and contained no palindromic sequence. Four of the primers (RAPD2, OPS11, A4 and OPS16), assessed individually, resulted in no amplification products or gave only a small number of amplimers. The remaining four primers (RAPD1, RAPD3, AP42 and RAPDnm), also assessed individually, gave reproducible patterns comprising of a suitable number of amplimers (5–12 amplimers) over a broad size range (0.1–2.0 kb), especially primers RAPD1 and RAPDnm.

The latter two primers were used in further analysis. The RAPD1 oligonucleotide primer was first described by Versalovic *et al.* (1991). The RAPDnm primer had previously never been used in RAPD analysis. These oligonucleotide primers RAPDnm and RAPD1 were 18 bp in length and 22 bp in length, respectively. Caetona-Anollés *et al.* (1991b) reported that a higher primer G + C content results in more RAPD-PCR products. The results seen with primers RAPD1 and RAPDnm, each of which had a G + C content of 50%, were consistent with this.

Using the RAPD conditions described in Section 2.7.2, the two primers, when used individually, reproducibly produced amplimers that could be scored for similarity between the GBS strains. To ensure reproducible amplification products were obtained from the GBS isolates in this study, a modified cycling programme was chosen. This cycling programme was compared with those of Bert *et al.* (1996) and Seppälä *et al.*

(1994). The cycling programme that was chosen was a little longer than the others tested as it resulted in clearer and sharper banding patterns.

For reproducibility, the Mg^{2+} concentration plays an important role in the RAPD-PCR reaction. As the concentration of Mg^{2+} increases, some DNA segments are amplified more efficiently than others and therefore the magnesium ion concentration must be optimised for each bacterial species. To determine the optimal Mg^{2+} concentration for GBS, a concentration titration was performed from 1–10 mM $MgCl_2$ using the PCR reaction and cycling conditions described in Section 2.7.2. The optimal concentration of $MgCl_2$ appeared to be between 7.5 mM and 8.5 mM (Figure 4.1). The PCR amplification was repeated with the optimised $MgCl_2$ concentration (8 mM) to ensure that this yielded reproducible patterns. Since RAPD studies have reported that the model and/or type of the thermal cycler (Penner *et al.*, 1992) and the source of the *Taq* polymerase (Fani *et al.*, 1993) can play a role in the reproducibility of results, these parameters remained unaltered throughout this study, namely, Perkin-Elmer GeneAmp PCR 2400 DNA thermal cycler and Promega, respectively.

4.5.2 RAPD-PCR analysis of GBS strains using primer RAPDnm

The RAPD-PCR amplification products obtained using the RAPDnm primer were fractionated on 2% agarose gels. The GBS reference strain (ATCC 13813) was used as a positive control in each gel along with a negative control (sterile distilled water). A 100-bp molecular weight marker was used in the first and last lanes to calculate the Rf values of amplimers and hence the molecular weights of individual bands in every profile. The RAPDnm primer generated patterns of between 2–16 bands of between 0.05–2 kb in size.

The molecular weights and numbers of DNA amplimers generated by the RAPDnm primer were put into the database and resolved into a numerical matrix of similarity coefficients. All clinical GBS isolates were assessed for their genetic relatedness using the RAPDnm primer. Similarity coefficient (S_D) values were determined for all possible isolate pair combinations from the RAPD-PCR profiles.

RAPD results with this primer showed that, taken altogether, all isolates shared a 57% similarity average. Among the 159 strains analysed, 128 RAPDnm patterns were

Figure 4.1. Gel electrophoresis analysis of the RAPD–PCR products obtained in a MgCl$_2$ concentration titration (7.0 mM to 9.0mM) with the RAPDnm and RAPD1 primers.

identified. Of the comparisons, 41 pairs resulted in a $S_D \geq 0.95$ (Table 4.1). From these data it is noticeable that 32 out of the 41 strain pairs had similar invasive or carriage status. Eighteen of the strain pairs had both the same serotype and invasive or carriage status. Also, isolate pairs were intermingled between all three hospitals.

The similarity data generated were then used to estimate genetic distances and subsequently construct a dendogram describing genetic similarity. The dendogram was generated by the Neighbour Joining Method. Using this method GBS were divided into nine distinct RAPD type groups which were designated I to IX as indicated by the dendogram (Figure 4.2). Figure 4.3 illustrates the type of RAPD-PCR profiles obtained for GBS strains within the nine clusters using the RAPDnm primer. Several groups of isolates that were either invasive or carriage isolates could be distinguished by their RAPD clusters at the genomic level. Three of the groups (VI, VII and VIII) comprised mainly carriage isolates. Of RAPDnm-type group VI, 73.6% (14 out of 19) were carriage isolates, of RAPDnm-type group VII, 78.9% (15 out of 19) were carriage isolates and of RAPDnm-type group VIII, 100% (10 out of 10) were carriage isolates. Two groups consisted mainly of invasive isolates. Of RAPDnm-type group II, 81.8% (9 out of 11) were invasive isolates and of RAPDnm-type group V, 70.6% % (12 out of 17) were invasive isolates. RAPDnm-type groups I, IV, and IX, contained a mixture of invasive and carriage isolates (Table 4.2). Any isolates that did not fall into a group of three or more isolates were termed non-clusterable (NC). Using the RAPDnm primer 10 isolates did not cluster into groups (Figure 4.4)

The clustering analysis was not able to differentiate isolates by serotype. However, half of the invasive serotype Ib isolates (11 out of 22) grouped into RAPDnm-type group V. This cluster group also provided most of the isolate-pair combinations with an $S_D \geq$ 0.95 (10 out of 41, seven of which had the same serotype and sepsis status). Clustering was not as distinct for any of the other serotypes, although some grouping is noticed for serotype III in RAPDnm-type group IV, i.e., 19 of 47 serotype III isolates.

The discriminatory capacity of the RAPD typing was determined in order to evaluate the suitability of the RAPDnm primer for epidemiological analysis of GBS. Using the RAPDnm primer the Simpson's index of diversity for Irish GBS isolates was 0.989.

Table 4.1. The sepsis status, serotype and isolate nos. of the isolate pairs that share a similarity coefficient, $S_D \geq 0.95$ using the RAPDnm primer.

Cluster	Isolates with $S_D \geq 0.95$					
	Isolate	Serotype	Sepsis Status	Isolate Pair No.	Serotype	Sepsis Status
I	H4532*	Ib	2+	H1066	Ib	2+
II	1744*	V	1+	H1635	V	1+
	7470	V	0	H852	V	2+
IV	250*	III	0	255	III	0
	250*	III	0	8076	III	0
	208	III	4+	7763	III	0
	5036*	III	2+	H578	III	2+
V	H913	Ib	4+	202	Ib	0
	H913*	Ib	4+	226	Ib	4+
	H913*	Ib	4+	229	Ib	4+
	226*	Ib	4+	229	Ib	4+
	202	Ib	0	226	Ib	4+
	202	Ib	0	229	Ib	4+
	229*	Ib	4+	230	Ib	4+
	258*	Ib	1+	259	Ib	1+
	219*	Ib	4+	H901	Ib	4+
	230*	Ib	4+	H901	Ib	4+
VI	184*	V	0	187	V	0
	187*	V	0	H701	V	0
	C12*	NT	0	C24	NT	0
	C7*	III	0	C11	III	0

Cluster	Isolates with $S_D \geq 0.95$					
	Isolate	Serotype	Sepsis Status	Isolate	Serotype	Sepsis Status
VI	C5	Ia	0	C27	Ib	0
	C5	Ia	0	C24	NT	0
	C6	Ia	0	C12	NT	0
	C6	Ia	0	C24	NT	0
	3656	III	2+	H1240	Ia	0
VII	8099	III	2+	8718	Ib	2+
	299	III	0	8429	II	0
	299	III	0	8099	III	2+
	299	III	0	320	V	0
	320	V	0	1588	III	0
	C7*	III	0	C11	III	0
VIII	C16	III	0	C23	NT	0
	247	Ib	0	251	Ia	0
	247*	Ib	0	5026	Ib	0
	251	Ia	0	5026	Ib	0
IX	7106	III	0	7552	IV	1+
	8161	Ib	0	H2234	V	0
	C28	II	1+	C29	NT	4+
	234	III	4+	H823	III	1+
	234	III	4+	H1645	Ib	1+

* Pairs of the same serotype and invasive or carriage status

Figure 4.2. Genetic relationships of the 159 GBS as estimated by clustering analysis of RAPD types with the RAPDnm primer. The dendogram was generated using the Dice coefficient with Neighbour Joining algorithm. Branch lengths are shown proportional to the amount of evolutionary change.
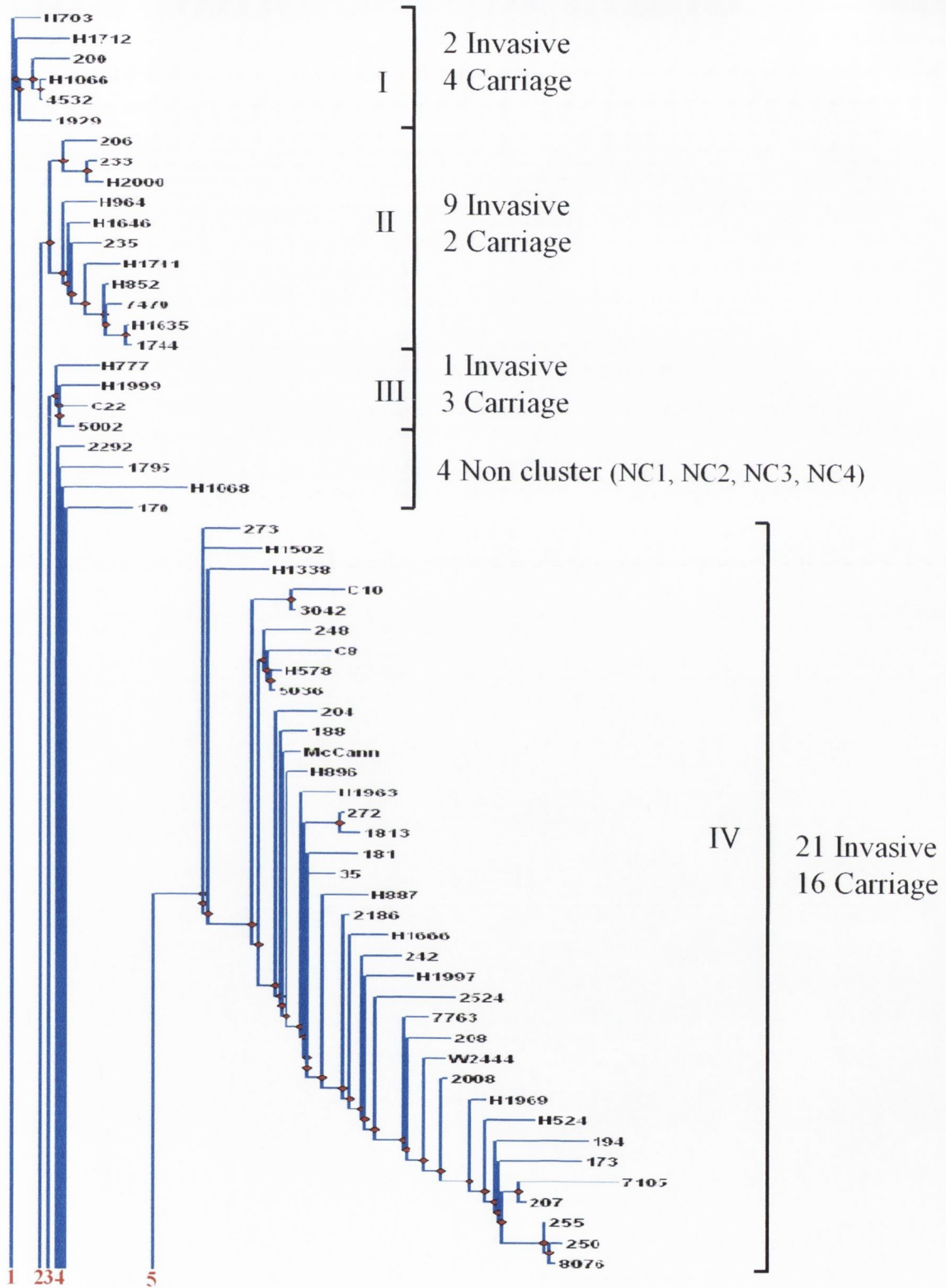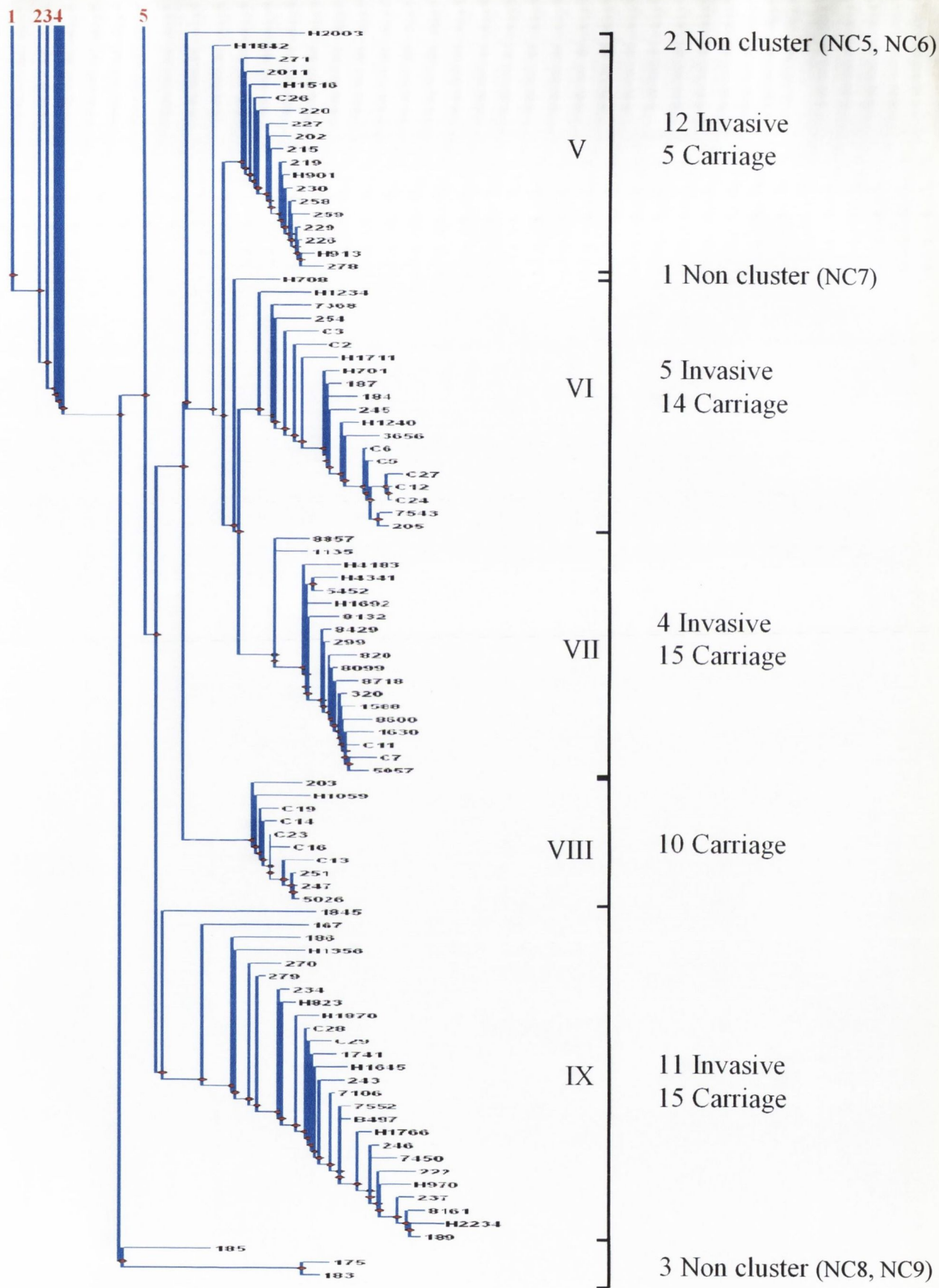
Figure 4.3 Representative GBS profiles obtained for clusters I–IX using the RAPDnm primer. A 100-bp molecular weight ladder (L) is used as a marker.
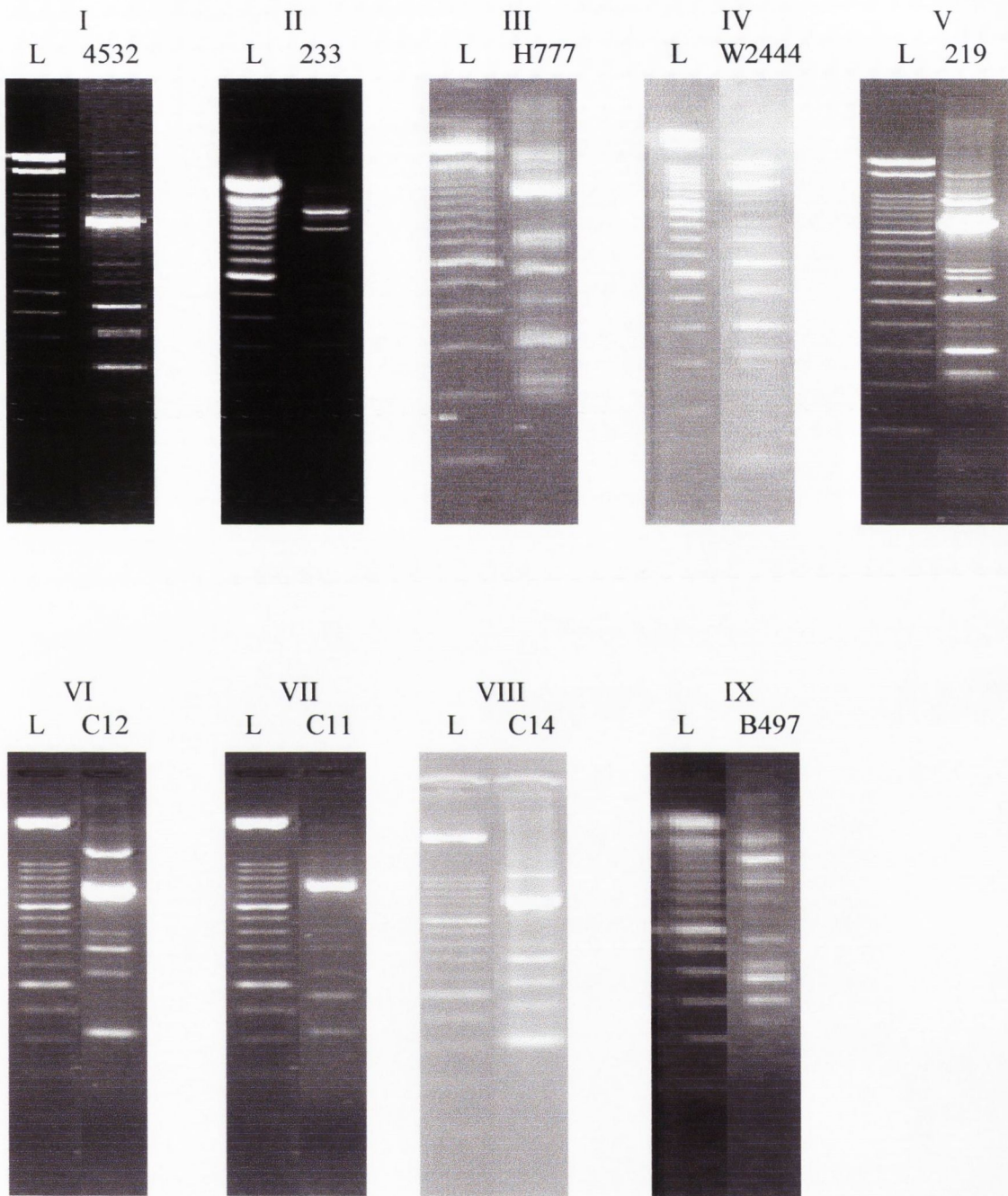
Table 4.2. The sepsis status and serotype distribution of the clusters found using the RAPDnm primer.

RAPDnm Cluster	No. of isolates by serotype							No. of isolates by status	
	Ia	Ib	II	III	IV	V	NT	Invasive	Carriage
I	3	2	–	1	–	–	–	2	4
II	–	3	3	–	–	5	–	9	2
III	2	1	–	1	–	–	–	1	3
IV	10	1	3	19	1	2	1	21	16
V	1	12	–	2	–	1	1	12	5
VI	6	2	1	2	–	6	2	5	14
VII	1	4	3	8	–	3	–	4	15
VIII	2	2	–	4	–		2	0	10
IX	3	3	7	5	2	5	1	11	15
Non–Cluster	3	–	–	5	–	2	–	7	3

Figure 4.4. GBS profiles obtained for non-clustering (NC) isolates using the RAPDnm primer. A 100-bp molecular weight ladder (L) is used as a marker.
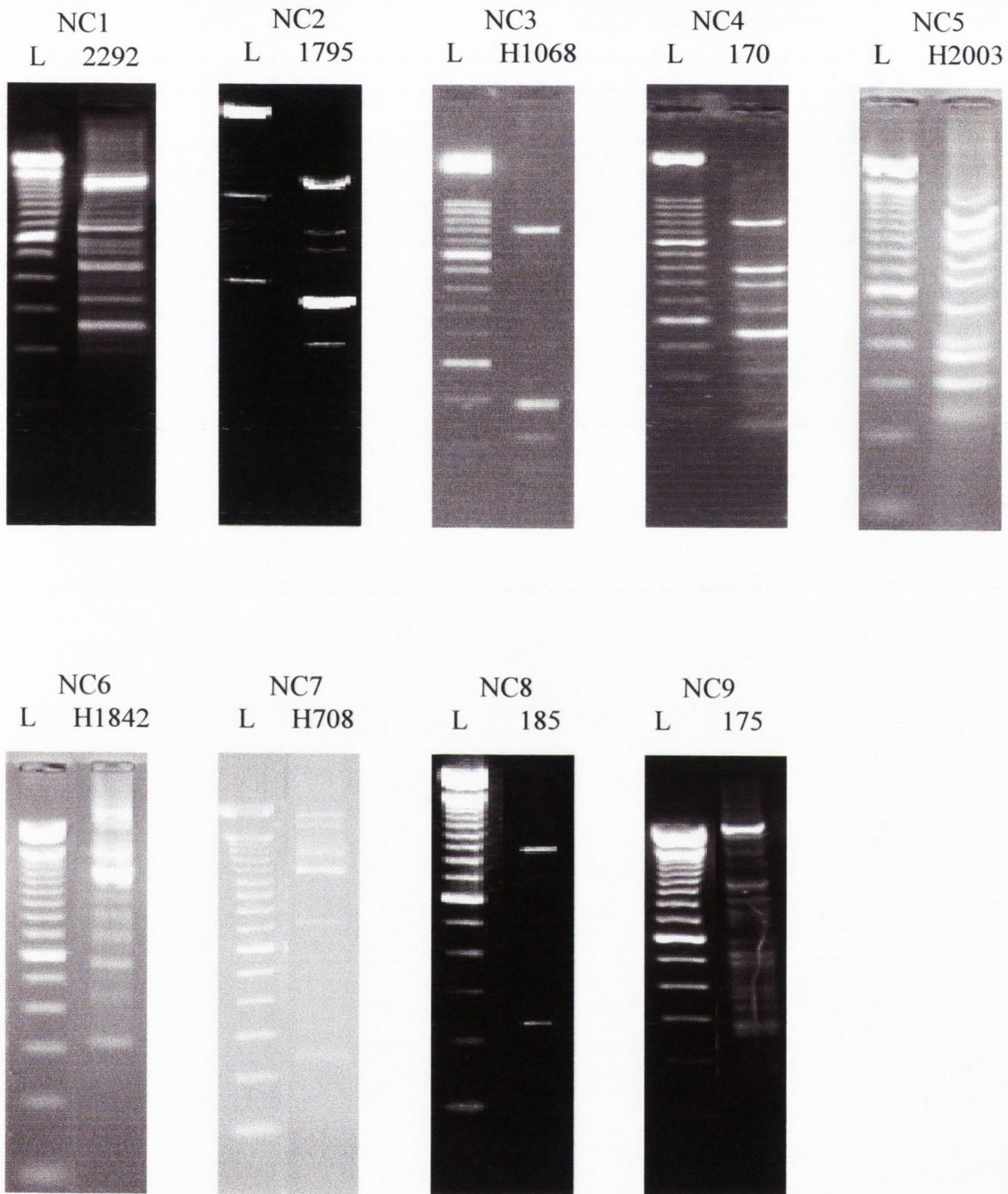
4.5.3 RAPD-PCR analysis of GBS strains using primer RAPD1

The PCR products obtained using the RAPD1 primer were fractionated on 1% agarose gels. As described previously (Section 4.5.2) the GBS reference strain (ATCC 13813) was used as a positive control in each gel along with a negative control. A 1-kb molecular weight marker was used in the first and last lanes to calculate the Rf values and hence molecular weights of individual bands in every profile. The RAPD1 primer generated patterns of between 6–15 bands of between 0.1–3 kb in size. The molecular weights and numbers of amplimers generated by this primer were put into the database, as described previously. Similarity coefficient (S_D) values were determined for all possible isolate-pair combinations with the RAPD1 profiles. One hundred and forty-six different profiles were obtained from the 159 isolates. Of the comparisons, 15 pairs resulted in a $S_D \geq 0.95$ (Table 4.3). Eleven of the strains had both the same serotype and invasive or carriage status and isolate pairs were intermingled between all three hospitals. RAPD results with this primer showed that, taken altogether, all isolates shared a 73% similarity average.

The similarity data generated were then used to estimate genetic distances and to subsequently construct a dendogram describing genetic similarity. The dendogram was generated using the Neighbour Joining algorithm. GBS were divided into seven distinct RAPD1-type groups which were designated i to vii, with 10 of the isolates not differentiated into a distinct cluster (nc) as indicated by the dendogram (Figure 4.5). Figure 4.6 shows representative RAPD1 profiles obtained in each of the seven clusters and Figure 4.7 shows the RAPD1 profiles for the non-cluster (nc) isolates. The clustering analysis was not able to differentiate isolates as invasive or carriage isolates. Only RAPD1-type group vi represented invasive or carriage traits with 16 out of 18 (88.09%) being carriage isolates. The clustering analysis did, however, seem to have the ability to differentiate a large number of the isolates by serotype (Table 4.4). Two of the RAPD1-type groups, iii and vi, comprised of 67.7% (21 of the 31) of the serotype Ia isolates. A large number of the serotype Ib isolates, 46.7% (14 of the 30), were observed in RAPD1-type group iii also. Clustering was not as distinct for any of the other serotypes, although some grouping is notable for serotype Ib in RAPD1-type group iii and for serotype V in RAPD1-type groups iii and vi (Table 4.4).

Table 4.3. The sepsis status, serotype and isolate nos. of the isolate pairs that share a similarity coeffiecient, $S_D \geq 0.95$ using the RAPD1 primer.

Cluster	Isolates with $S_D \geq 0.95$					
	Isolate No.	Serotype	Sepsis Status	Isolate Pair No.	Serotype	Sepsis Status
II	H1646*	Ib	1+	258	Ib	1+
III	H1066*	Ib	2+	H4183	Ib	2+
	200	Ia	0	243	II	0
IV	270	III	0	272	IV	0
	227*	Ib	1+	259	Ib	1+
	227*	Ib	1+	H1645	Ib	1+
V	8099	III	2+	299	III	0
VI	820*	Ia	0	1845	Ia	0
	820*	Ia	0	1929	Ia	0
	1929*	Ia	0	1845	Ia	0
	247	Ib	0	251	Ia	0
VII	H887*	III	0	H1059	III	0
	H1870*	III	0	H1712	III	0
Non–Cluster	215*	Ib	4+	219	Ib	4+
	H708*	V	0	H1516	V	0

* Pairs of the same serotype and invasive or carriage status

Figure 4.5. Genetic relationships of the 159 GBS as estimated by clustering analysis of RAPD types with the RAPD1 primer. The dendogram was generated using the Dice coefficient with Neighbour Joining algorithm. Branch lengths are shown proportional to the amount of evolutionary change.
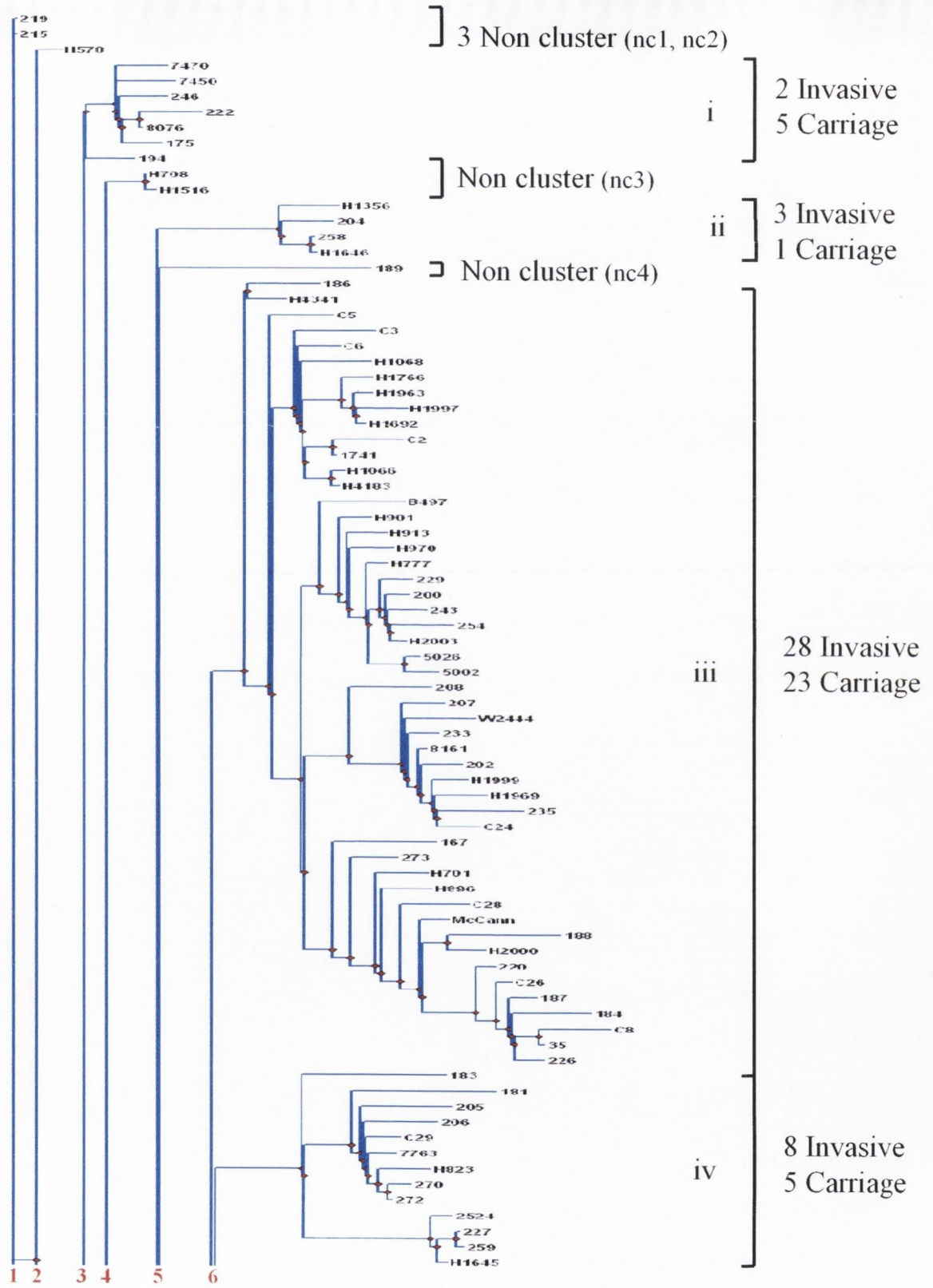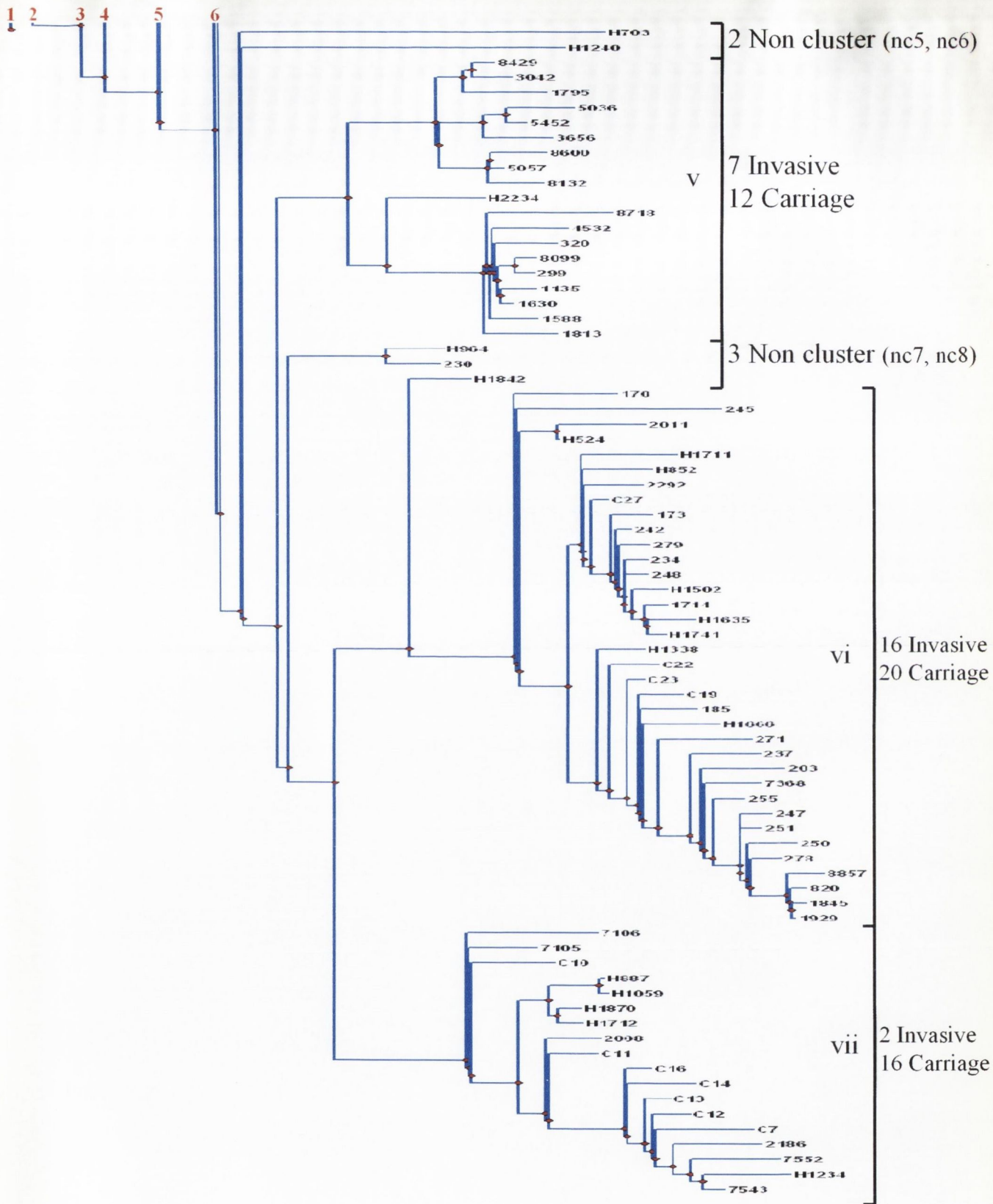
Figure 4.6. Representative GBS profiles obtained for clusters i–vii using the RAPD1 primer. A 1-kb molecular weight ladder (L) is used as a marker.
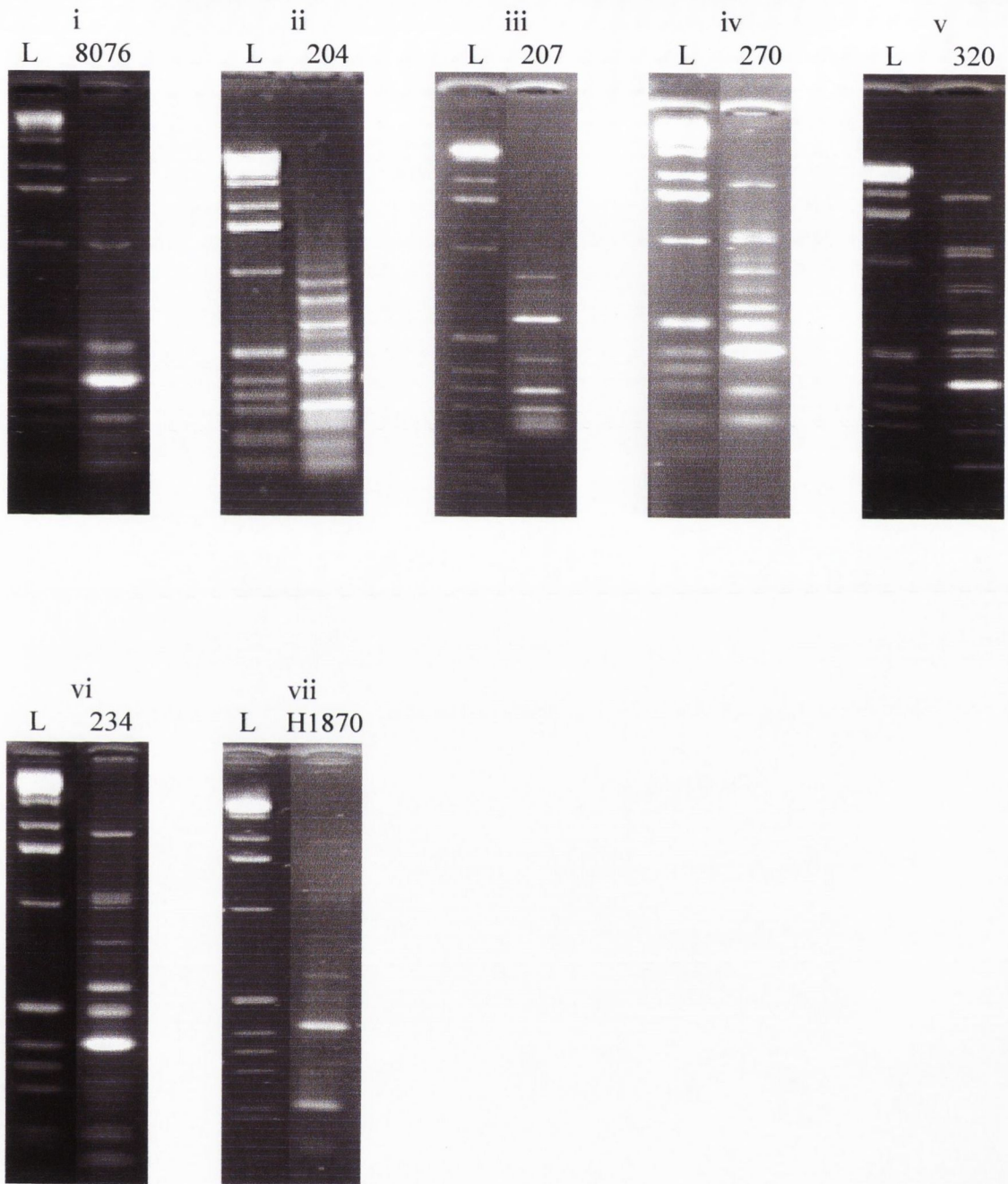
Figure 4.7. GBS profiles obtained for non-clustering (nc) isolates using the RAPD1 primer. A 1-kb molecular weight ladder (L) is used as a marker.
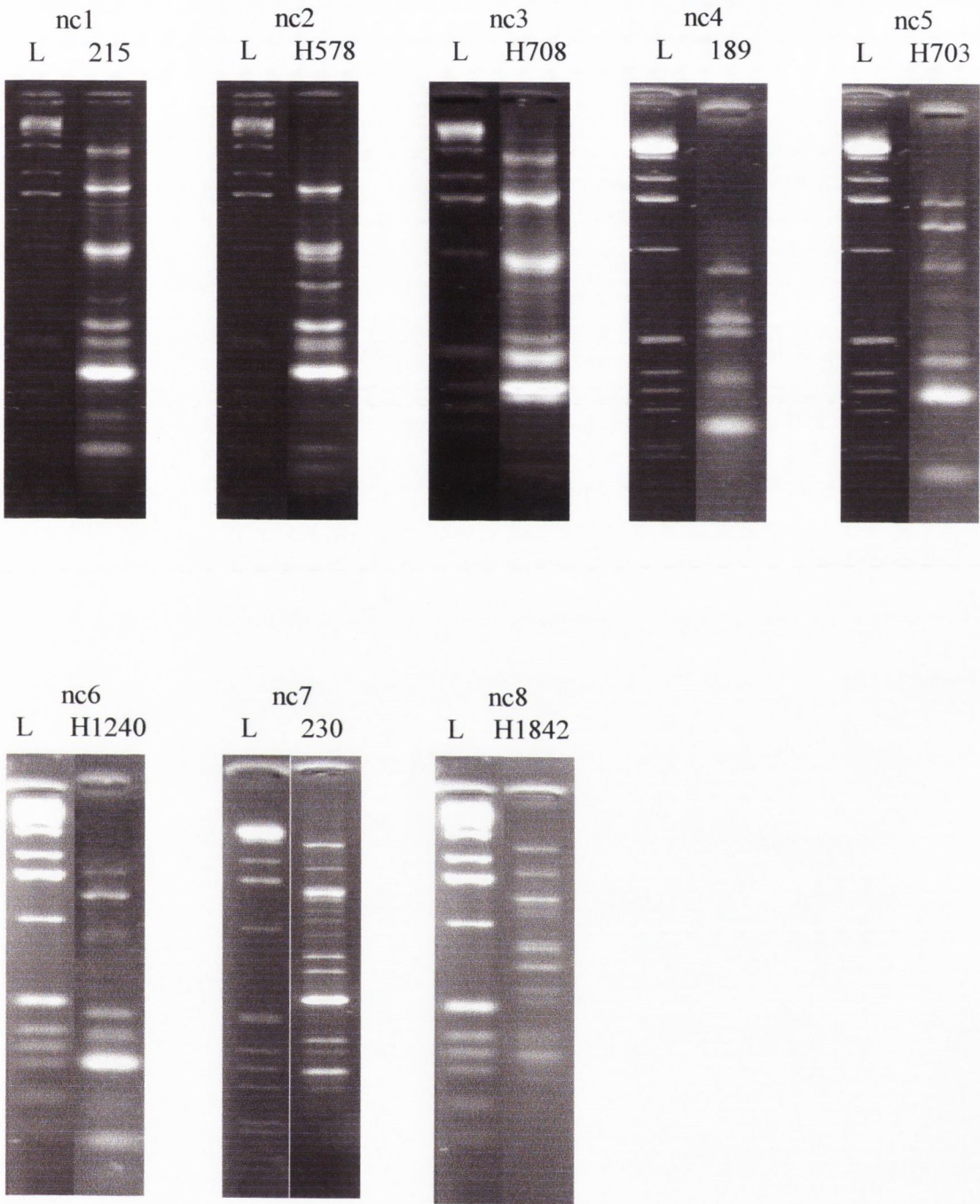
Table 4.4. The sepsis status and serotype distribution of the clusters found using the RAPD1 primer.

RAPD1 Cluster	No. of isolates by serotype							No. of isolates by status	
	Ia	Ib	II	III	IV	V	NT	Invasive	Carriage
i	–	–	–	3	1	3	–	2	5
ii	1	2	–	1	–	–	–	3	1
iii	10	14	9	9	–	7	2	28	23
iv	1	5	1	4	1	–	1	8	5
v	1	4	2	8	–	4	–	7	12
vi	12	2	2	9	–	8	3	16	20
vii	4	–	1	11	1	–	1	2	16
Non–Cluster	2	3	2	2	–	2	–	6	5

The discriminatory capacity of the RAPD typing was determined in order to evaluate the suitability of the RAPD1 primer for epidemiological analysis of GBS. Using the RAPD1 primer the Simpson's index of diversity for Irish GBS isolates was 0.918.

4.5.4 Pulsed-field gel electrophoresis analysis

Four of the collection of 159 isolates, namely, strains 7450, 271, C23 and 2524, were unrecoverable from cryobeads and therefore had to be omitted from this study. Chromosomal DNA embedded in agarose plugs of the remaining 155 GBS strains were digested with restriction endonuclease *SmaI* and fractionated on 1% agarose gels using the CHEF system. A lambda (λ) DNA ladder was used in the first and last lanes to calculate the Rf values and molecular weights of the banding profiles. Restriction with *SmaI* yielded between 6 and 17 fragments ranging from 50 to 500 kb in size. Chromosome restriction profiles were visualised and analysed using the Alpha Imager and Phoretix software as previously described. The molecular weights and number of restriction fragments were put into the database and the similarity coefficients were determined for all possible pair combinations.

PFGE profiles demonstrated that isolates within the population were genetically diverse. Among the 155 strains analysed, 116 PFGE patterns were identified. The deduced genetic relationships between the 155 strains of GBS are shown in the dendogram in Figure 4.8. Eight PFGE groups, A–H, were identified with a 67.7% similarity average between isolates. Figure 4.9 illustrates representative PFGE profiles obtained for GBS strains within the eight clusters, while Figure 4.10 demonstrates the profiles obtained for the four isolates that did not cluster into any group.

The clustering analysis was not able to differentiate between invasive or carriage isolates. PFGE patterns within the serotypes differed greatly, with almost a different PFGE pattern obtained per isolate. Serotypes III and V were the most homogeneous of the serotypes and clustered in particular groups. Fourteen of the 23 strains (60.9%) of serotype V belonged to PFGE groups A and F and, 19 of the 46 strains (41.3%) of serotype III belonged to PFGE group F (Table 4.5).

Figure 4.8. Genetic relationships of the 159 GBS as estimated by clustering analysis of PFGE types The dendogram was generated using the Dice coefficient with Neighbour Joining algorithm. Branch lengths are shown proportional to the amount of evolutionary change.

1	2	3	4

H1692
272
H913
H703
H777
H970
H901
H2000
5002
5057
5036
5026
C3

E

6 Invasive
7 Carriage

3656
4532
8132
C29
C28
C14
8099
320
C16
C22
H1059
C24
H2234
H1516
H708
1630
H1741
H4341
H4183
270
186
H1066
H1842
1741
7540
C7
7105
C11
8076
H887
C19
H1870
H1712
H1069
299
1588
205
206
237

F

14 Invasive
25 Carriage

35
1744
H1711
H578
C10
185
170
1845
1929

G

6 Invasive
3 Carriage

246
H1997
C26
8718
H1766
234
220
247
251
248
2008
7106
245
242
H1068
188
H1646
7763
243
H1963
254
227
259
McCann
H896
258
250
255

H

15 Invasive
13 Carriage

7470
H701

Non cluster (*NC'3*)

Figure 4.9. Representative GBS profiles obtained for clusters A–H using PFGE. A λ ladder (L) is used as a molecular weight marker.
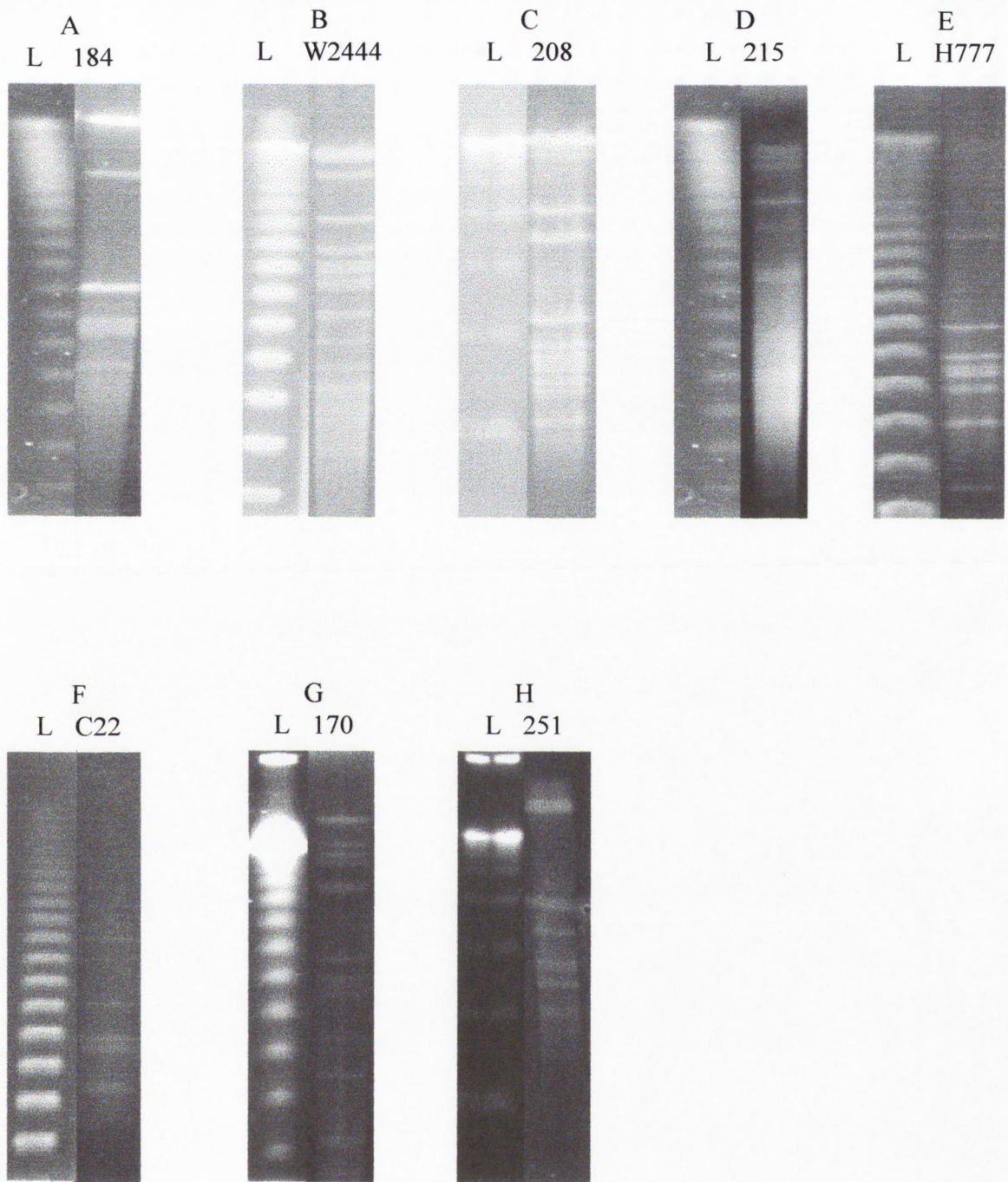
Figure 4.10. Representative GBS profiles obtained for non-clustering (*NC*) isolates using PFGE. A λ ladder (L) is used as a molecular weight marker.
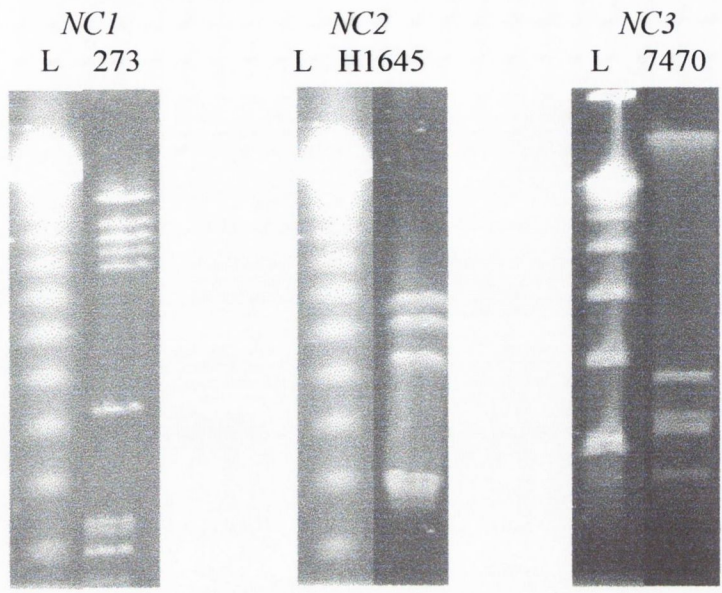
Table 4.5. The sepsis status and serotype distribution of the clusters found using PFGE.

PFGE Cluster	No. of isolates by serotype							No. of isolates by status	
	Ia	Ib	II	III	IV	V	NT	Invasive	Carriage
A	1	3	–	4	–	6	1	8	7
B	4	–	3	5	–	1	–	5	8
C	1	–	1	1	–	–	–	2	1
D	10	7	2	7	2	2	1	14	17
E	2	6	1	3	1	–	–	6	7
F	1	5	4	19	–	8	2	14	25
G	4	1	1	1	–	2	–	6	3
H	7	7	5	6	–	2	1	15	13
Non–Cluster	1	1	–	–	–	2	–	2	2
NR*	–	–	–	1	–	1	2	–	4

* Not recoverable from cryobeads for PFGE analysis.

The discriminatory capacity of the PFGE typing was determined in order to evaluate its suitability for epidemiological analysis of GBS. For PFGE with *SmaI* the Simpson's index of diversity of Irish GBS isolates was 0.956.

4.6 Discussion

For several years serotyping of GBS capsular polysaccharides has been the standard method used for typing. One limitation of serotyping of GBS is that isolates may display different invasive traits within a serotype. Also, the frequency of non-typeable strains is increasing being as high as 12% in some countries (Palacios *et al.*, 1997). During the last 15 years, DNA polymorphism assays, such as Restriction Endonuclease Analysis (REA) of genomic DNA and analysis of rRNA genes (Ribotyping) have been applied to the typing of GBS (Blumberg *et al.*, 1992). These methods have been shown to differentiate clones among GBS isolates of the same serotype. The most recently introduced DNA polymorphism assays for typing GBS are RAPD and PFGE (Chatellier *et al.*, 1997; Dmitriev *et al.*, 1997). The RAPD method is faster, technically easier, and more economical than the older genomic typing methods such as ribotyping and REA, while PFGE has become the typing method of choice for many organisms, e.g., *Staphylococcus aureus* (Bannerman *et al.*, 1995).

Evaluation of a typing method according to the criteria of typeability, reproducibility, and discriminatory power is required before it can be used for epidemiological purposes. For RAPD to be used as an epidemiological typing method, selection of an appropriate primer and optimisation of PCR conditions are of great importance for maximisation of the discriminatory power. In the present study the typeability and reproducibility of the RAPDnm and RAPD1 primers were excellent, with both being near 100%. As with all typing methods, the discriminatory power of RAPD depends on the genetic diversity of the population of isolates being examined. The discriminatory power can be calculated using the Simpson's index of diversity. The Simpson's index of diversity, D, for the RAPDnm primer with Irish isolates was 0.989, while an index of 0.918 was seen for the RAPD1 primer. The closer the discriminatory index is to 1.0 determines the usefulness of a typing system for differentiating isolates. It has been shown to be possible to increase the discrimination power of RAPD analysis by using two primers together (Micheli *et al.*, 1993). Three previous studies have reported genetic charaterisation of GBS isolates of human origin by RAPD (Chatellier *et al.*, 1997, Limansky *et al.*, 1998 and Martinez *et al.*, 2000). Chatellier *et al.* (1997) used a

combination of four primers to obtain a D value of 0.90, while Limansky *et al.* (1998) used a partially degenerate oligonucleotide to obtain a D value of 0.98. In the most recent report, Martinez *et al.* (2000) described the use of a combination of three oligonucleotide primers to achieve a D value of 0.99. However, in the present study equally good discrimination was achieved by using only one primer. This was also seen in the studies of *Streptococcus pyogenes* and *Streptococcus uberis* isolates by Seppälä *et al.* (1994) and Jayarao *et al.* (1992) respectively.

The current study aimed to identify the clones of GBS that displayed invasive ability. Results showed genetic heterogeneity not only between different serotypes but also among isolates belonging to same serotype. This suggests that RAPD typing may be more advantageous than capsular serotype in differentiating GBS isolates. The data clearly show that the RAPDnm primer demonstrated a greater discriminatory power than the RAPD1 primer in identifying invasive status.

Our understanding of GBS disease may also be improved by analysing the genetic structure of GBS through the use of PFGE. PFGE is a powerful tool for resolving large DNA molecules. Due to its high discriminatory power and reproducibility it has been used successfully in typing bacterial isolates, examination of evolutionary divergence between strains, and investigation of common source outbreaks of infection (Arbeit, 1999). One major drawback with the use of PFGE as a typing method is the duration of the procedure. The standard protocol for a bacterial sample can take up to 8 working days before a result is achieved. Since completion of this study a rapid PFGE protocol for GBS has been developed which has increased reproducibility, achieved higher image quality, and a reduced completion time by as many as 5 days (Benson and Ferrieri, 2001).

Some correspondence to serotype can be seen in cluster analysis following PFGE of GBS genomic DNA. All isolates shared a 67.7% similarity average with serotypes III and V clustered into particular PFGE groups. The similarity average is consistent with a study of a French GBS population where Rolland *et al.* (1999) described a 62% similarity average. In the report, Rolland *et al.* (1999) found that serotypes Ia and III clustered into particular PFGE groups. Another report by Elliot *et al.* (1998) emphasises the heterogeneity of GBS isolates by the finding of 17 different PFGE patterns among the 45

107

serotype V isolates examined. As no similarity average was obtained or relationship studies performed on the 57 GBS isolates in the report by Benson and Ferrieri (2001), no comparison can be made to the present study.

PFGE of chromosomal DNA provides an efficient method for determining DNA sequence variations. In contrast to ribotyping which is a painstaking and laborious method that involves the use of three restriction enzymes to discriminate strains. PFGE requires only one restriction enzyme digestion (*SmaI*). In addition the recently proposed modifications to the PFGE method may reduce the time required to less than 3 days. PCR-based methods of analysing bacterial genomic DNA have also proven to be fast, sensitive and reliable for determining genetic relationships among invasive and carriage isolates (Pooler *et al.*, 1996). RAPD and PFGE randomly explore most of the bacterial genome and probably identify more minor parts of the genome not essential for bacterial survival and therefore identifies more genetic heterogeneity between strains. Phylogenetic markers may be used to identify strains within the GBS population that are able to invade the central nervous system of neonates. It would be necessary to test clinical isolates of other national collections to ensure that virulent strains present particular patterns or characteristics.

In conclusion, the results argue for the existence of particular Irish GBS strains that are invasive. While PFGE analysis differentiates Irish GBS isolates within different serotypes, RAPD-PCR using primer RAPDnm used in parallel with serotyping would appear to be highly suitable for epidemiological typing of Irish GBS isolates.

CHAPTER 5

GBS Virulence Factors

5.1 Introduction

Molecular typing methods can be divided into groups based on the type of macromolecule targeted for the typing, e.g., lipolysaccharide (LPS) and fatty acid based methods, protein based methods and nucleic acid techniques. Although, the first two methods have been used successfully to type a variety of different bacterial species, they only examine the phenotype of the particular bacterial species. Genotypic methods are directed specifically at the DNA or RNA molecules of the bacterial species and are not dependent on the growth environment of the species.

The analysis of the DNA of a species is becoming increasingly important in the studies of evolutionary ecology, population studies and systematics. Studying the DNA of an organism has several advantages over conventional typing methods, namely, (1) the genotype rather than the phenotype is assayed, (2) the methods are general to any type of DNA, and (3) DNA can be prepared from small amounts of sample and is relatively stable. While nucleic acid techniques for the identification and typing of microorganisms have been developed, the target nucleic acid may be available only in limited quantities, e.g., tissue, food, water and environmental samples or clinical specimens, unless the organism has been cultivated. This problem can be overcome by artificially amplifying the amount of target nucleic acid available for analysis, allowing a million-fold amplification of the nucleic acid sequence of interest.

The polymerase chain reaction (PCR) for amplification of specific nucleotide sequences was introduced by Saiki *et al.* (1988) and since then has proved to be a very important scientific technique in understanding the genetic makeup of all organisms. Following the introduction of PCR it was recognised that the method provided a sensitive approach for the detection and identification of different properties of organisms, such as the genes that encode particular virulence proteins.

The mechanisms of GBS pathogenicity as well as the determinants involved have received notable attention in recent years. It appears that a variety of bacterial products are involved in the virulence of this streptococcal species. Based on *in vitro* and animal model studies of GBS, several virulence factors have been described, a major one being

the capsular polysaccharides [Chapter 3]. Other factors are also thought to be associated with virulence of GBS (Table 1.8).

Although assays for virulence proteins have been used successfully to type a variety of different organisms, these techniques suffer from the limitation that they only analyse the phenotype of the organism and not the genotype. Lately, the focus of studies has begun to switch towards a more molecular genetic approach in order to define the mechanisms of GBS-induced disease. The results of studies from gene cloning and nucleotide sequencing have led to a better understanding of the organism itself and its virulence mechanisms. It is suggested preferable to analyse the genotype of an organism since this type of analysis does not rely on the expression of particular genes encoding virulence factors, and is therefore not subject to phenotypic variation (Maeland et al., 2000).

A variety of studies have demonstrated diversity in the distribution of recognised and putative virulence factors among GBS (e.g., Johnson and Ferrieri, 1984; Chun et al., 1991; Jackson et al., 1994; Ferrieri 1988 and 1997). Associations have been suggested between the presence or absence of some of these factors and the virulence of GBS (e.g., Chun et al., 1991; Berner et al., 1999; Gibson et al., 1999). By and large such studies have examined the distribution of each virulence factor or its gene separately without accounting for possible associations between virulence factors or without considering the genomic background of the isolates, for example, using serotype as a surrogate marker.

In the present study, the relationships of genes encoding (putative) virulence factors (Section 1.14 and Section 5.2), either alone or together, with serotype, carriage or invasive status and with each other were also investigated using multivariate and hierarchical log-linear statistical analysis.

5.2 Surface Proteins

Lancefield (1975) showed that antibodies raised to the C-proteins in rabbits protected mice that were challenged with GBS expressing the C-proteins. The C-proteins and the Rib protein are surface-associated antigens that are now thought to play a major role in both virulence and immunity. Two of the main C-proteins, α (trypsin-resistant) and β

(trypsin-sensitive), have been characterised biochemically and immunologically (Michel *et al.*, 1991, 1994). Two additional antigenic components, designated γ and δ, have also been shown to be associated with the C-protein (Brady *et al.*, 1988).

5.2.1 Alpha (α) C-protein

The α C-protein of GBS is a surface-expressed antigenic determinant present on many clinical isolates that is capable of eliciting protective antibody-mediated immunity in experimental animals (Madoff *et al.*, 1991). This protein, like the capsular polysaccharides previously described (Section 3.1.3) and the M proteins of Group A streptococi, may confer resistance to opsonophagocytic killing in the absence of type-specific antibody (Madoff *et al.*, 1996).

The protein comprises several domains (Fig. 5.1) including a C-terminal membrane anchor domain that is typical of those found in other surface proteins of Gram-positive cocci (Madoff *et al.*, 1991). The antigen also possesses an N-terminal region of 185 amino acids which is followed by a series of identical tandem repeats, each 82 amino acids in length.

The α C-protein is present in approximately 50% of all clinical isolates and in approximately 70% of non-type III GBS isolates (Gravecamp *et al.*, 1996). The molecular size of the protein varies with the number of tandem repeats it contains. Strains of GBS have been reported that express α C-proteins ranging in size from 62.5 to 167 kDa (Madoff *et al.*, 1996).

The α C-protein is encoded by the *bca* gene. The complete gene sequence consists of 3063 nucleotides (nt), based on 9 tandem repeats. The signal sequence is encoded by the first 123 nt, followed by a N-terminal region of 555 nt. The tandem repeating units begin at nucleotide 679 of the DNA sequence and each repeating unit consists of 246 nt followed by a single partial repeat of 33 nt. Following the repeating units is a small C-terminal region compounding 148 nt (Michel *et al.*, 1992).

The repeats found in the α C-protein are unique for several reasons. Firstly they are larger than those found in other Gram-positive surface proteins. They are identical at

Figure 5.1. Schematic representation of the structure of the α C-protein gene indicating the relative positions of the signal region, N-terminal region, repeat (R) regions and the anchor region. The repeat region is emphasised. The positions of the primers used in amplifying the bca gene and the bca repeat region are indicated.
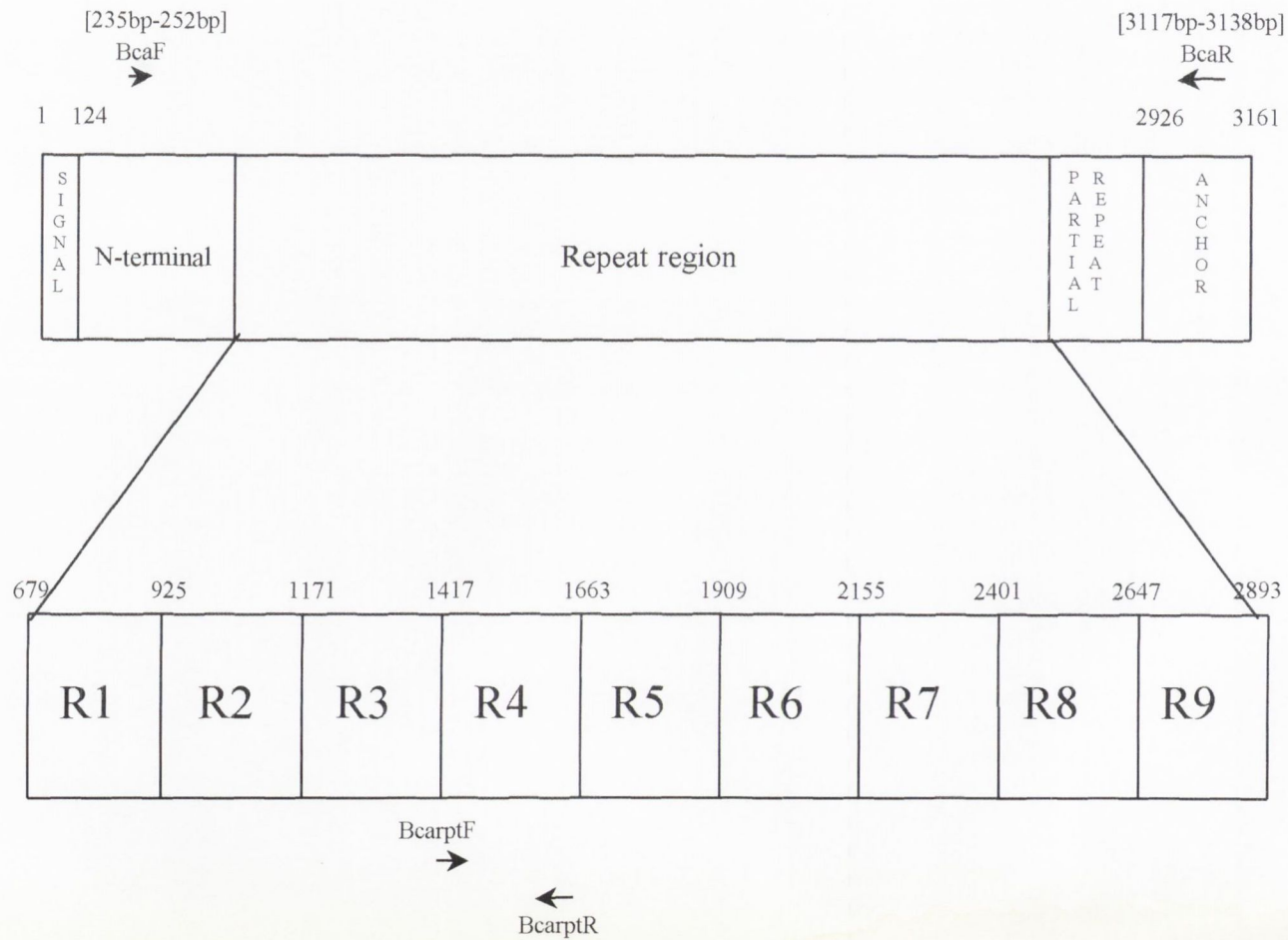
the nucleic acid level and the size of the α C-protein expressed correlates to the number of the repeat units. Analysis of the nucleotide sequence also failed to show codons within the repeat region that could cause early termination of translation (Michel *et al.*, 1992). This suggests that size variability in the α C-protein results from recombination of intragenic repeats. The tandem repeats could provide convenient fixed recombination sites for deletion or duplication of a repeat unit. Deletion would reduce the size of the gene and might occur during DNA replication by unequal crossover or mispaired template slippage, which would occur in frame (Michel *et al.*, 1992). Duplication of DNA could be a mechanism to create antigenic diversity. Madoff *et al.* (1996) demonstrated that neonatal isolates with altered α C-proteins arose from maternal isolates by intragenic deletion in the *bca* gene. They further showed that such deletions occurred frequently in a mouse model of GBS infection in the presence of immune serum raised to the α C-protein. It is thought, therefore, that loss of repeat units from the α C-protein could result in:

- loss of binding affinity because of lower valency of the protein (i.e., fewer antibody-binding sites)
- loss of possible conformational epitope or epitopes
- poorer presentation of an epitope or epitopes at the cell surface when a smaller protein is expressed.

Studies have shown that isolates of GBS vary in their susceptibility to opsonophagocytic killing in the presence of monoclonal antibody to the α C-protein. The extent of killing was proportional to the size of the α C-protein expressed and to the number of tandem repeats within the gene (Madoff *et al.*, 1996).

5.2.2 Beta (β) C-Protein

The α and β C-protein antigens were originally thought to be different forms of the same surface protein, but are now known to be phenotypically and genetically unrelated. The β C-protein, encoded by the *bac* gene, can be a surface-expressed or secreted antigen. The deduced amino acid sequence of the *bac* protein (1134 residues) includes a signal

114

sequence of 37 amino acids and a putative membrane anchor region at the C-terminal end. The processed form of the protein, 1097 residues, has a calculated molecular weight of 123.8 kDa. There are no cysteines in the Bac protein, suggesting a fibrillar structure (Hedén *et al.*, 1991).

The β C-protein does not possess tandem repeats as seen in the α C-protein but it has an ability to bind the Fc region of human IgA (Russel-Jones *et al.*, 1984). The IgA-binding region has been identified as a 14-kDa domain at the N-terminal end of the protein. This protein was found to be immunogenic in rabbits (Ustinovitch *et al.*, 1999). Since IgA is the predominant immunoglobulin defence against bacterial infection at mucosal surfaces, non-productive sequestering of IgA may be a mechanism for avoiding the first line of host defence. In recent reports the frequency of occurrence of the β C-protein in GBS strains was found to be approximately 27% and almost confined to type Ib strains (Madoff *et al.*, 1991). The presence of truncated, non-IgA binding forms of the antigen has also been reported (Brady *et al.*, 1989). Kreikemeyer and Jerlström (1999) developed a strategy for the construction of a heterologous expression mutant using the Bac protein as a model antigen. The *bac* gene, including its own promoter, was cloned into a *Escherichia coli-Enterococcus faecalis* shuttle vector and was maintained extrachromosomally in a *bac*-deficient GBS strain. The resulting mutant expresses the β-antigen on its surface at higher levels than the parent strain which can be used in animal models to assess its importance in GBS pathogenesis. This genetic tool could prove to be beneficial in proving the involvement of other bacterial factors in the pathogenesis of GBS.

5.2.3 Rib Protein

Studies of the Rib protein encoded by the *rib* gene have shown that it shares many properties of the α C-protein, in that it is a surface protein with variable numbers of tandem repeats (Ståhlhammer-Carlemalm *et al.*, 1993). Sequence analysis of the *rib* gene and the deduced amino acid sequence from a type III strain demonstrated an exceptionally long signal peptide and 12 repeats that account for 80% of the sequence of the mature protein (Wäsfeldt *et al.*, 1996). Although the Rib protein and α C-protein show 61% residue identity in their N-terminal regions and 47% identity in their repeat regions, they

do not cross-react immunologically (Ståhlhammer-Carlemalm *et al.*, 1993). Similar to the α C-protein, the repetitive structure of the Rib protein may play a role in evasion of type-specific host immunity in GBS giving rise to antigenic variation. In contrast to the α and β C-proteins, the Rib protein was found to be expressed in most isolates of GBS thought to be associated with invasive disease, including almost all strains of serotype III (Ståhlhammer-Carlemalm *et al.*, 1993).

5.2.4 Alpha (α)-like proteins Alp2 and Alp3

Lachenauer *et al.* (2000) reported newly discovered mosaic proteins of the C-protein family in GBS serotypes V and VIII. Interestingly these proteins, designated Alp2 and Alp3, possess similarities to the Rib protein and α C-protein. Alp2 contains the same N-terminal region and tandem repeat region as the Rib protein. The Alp3 protein on the other hand shows similarities to the C-terminal region of Rib. It is suggested that, since the α C-protein and Rib protein generally do not co-exist as independent proteins on serotypes, these mosaic proteins Alp2 and Alp3 have possibly been created through intragenic recombination or horizontal gene transfer. The Alp2 and Alp3 proteins could possibly confer virulence attributed to both the α C- and Rib proteins onto organisms harbouring these proteins (Lachenauer *et al.*, 2000). These proteins contain a region of tandem repeats similar to that of the α C-protein and Rib proteins, but they also contain a non-tandem repeat region which has IgA-binding ability like that of the β C-protein. Also, apart from the repeats, the Alp3 protein is almost identical to the sequence of the R28 protein from *Streptococcus pyogenes* (Ståhlhammer *et al.*, 1999). Gene transfer among streptococcal species has been reported previously with the M12 protein (Simpson *et al.*, 1992). The *emm12* gene has been demonstrated in group A streptococci and group G streptococci. However, the Alp3 protein has so far only been found in serotype V and serotype VIII strains of GBS and therefore is unlikely to be a factor in the pathogenicity mechanisms of other GBS serotypes.

5.3 Enzymes

5.3.1 Oligopeptidase

GBS produces a cell-associated oligopeptidase (originally thought to be a collagenase) that is encoded by the *pepB* gene. The *pepB* gene encompasses an opening reading frame (1800 nt) for a 69.6-kDa protein, whose amino acid sequence shows 66.4% identity to the oligopeptidase of *Lactococcus lactis*. The GBS oligopeptidase has the ability to hydrolyse the synthetic collagen-like substrate *N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA) (Lin *et. al*, 1996). The *pepB* gene product has also demonstrated the ability to degrade a variety of small bioactive peptides (Table 5.1). It has been postulated that the oligopeptidase might contribute to the premature rupture of membranes through damage to the amniotic collagen fibrils at childbirth and possibly to infection of the neonate (Lin *et. al*, 1996). There are no available data on the frequency of occurrence of the *pepB* gene or of expression of the oligopeptidase enzyme in GBS isolates.

5.3.2 Hyaluronidase

Hyaluronidase has been described as a "spreading factor" from the studies of McClean (1941) and is one of several proteins secreted by GBS that are believed to contribute to strain virulence. It cleaves hyaluronic acid (a component of the extracellular matrix of many tissues) to yield a disaccharide of *N*-acetylglucosamine and glucuronic acid. When acting on hyaluronic acid the enzyme makes an initial random cleavage and then rapidly and progressively degrades the resulting polysaccharide chains to yield the disaccharide product. It is believed that degradation of hyaluronic acid may facilitate tissue invasion (Pritchard *et al.*, 1994) and possibly promote persistant colonisation of the vagina by GBS (Baker and Pritchard, 2000).

All GBS serotypes have been shown to contain the hyaluronidase gene (*hylB*), although varying levels of enzyme production have been noted. Strains of GBS type Ib were described as being low producers of hyaluronidase (Lin *et al.*, 1994). Milligan *et al.* (1978) reported that disease-causing strains of serotype III GBS were much more likely to produce high levels of the enzyme than carriage strains.

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117

Table 5.1. Characterisation of the substrate specificity of the oligopeptidase PepB from GBS (Lin *et al.*, 1996).

Substrate	Cleavage site(s)
FALGPA	Furanacryloyl-Leu-Gly-Pro-Ala
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Ile-Ser-bradykinin	Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Neurotensin	Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Adrenocorticotropin Fragment 1-10	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly
Substance P fragment 2-11 (small bioactive peptide)	Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$

↓   Primary cleavage sites

↓   Minor cleavage sites

5.4. GBS Insertion Sequences and IS*1548* in the *hylB* gene

Insertion elements (IS) are mobile genetic elements that facilitate horizontal gene transfer and genetic rearrangements. In GBS, five different insertion elements have been identified and sequenced (Rubens *et al.*, 1989; Granlund *et al.*, 1998; Spellerberg *et al.*, 2000; Granlund *et al.*, 2001). IS*861* is a 1,442 bp insertion sequence identified in the type III GBS. Multiple copies of IS*861* were observed throughout the chromosome of this GBS strain. One of the copies is located near genes involved in GBS capsular polysaccharide synthesis. IS*861* shares greater than 30% homology with IS*3* and IS*150* of *Escherichia coli*, primarily in the region of their putative transposases.

IS*1381*, an insertion sequence previously described in *Streptococcus pneumoniae*, was cloned from GBS strain A909. The presence of multiple copies of IS*1381* in strain A909 prompted further study of the insertion element to assess its effectiveness as a subtyping tool. IS*1381* was found by Southern blot analysis to be present in 18 (72%) of 25 unrelated GBS isolates tested. For two mother-infant pairs of isolates, a single additional insertion was seen in the isolate obtained from the infant in each case, suggesting that an additional insertion occurred between maternal colonization and infection of the infant.

Spellerberg *et al.* (2000) reported a novel insertion sequence, designated ISSa*4*, which exhibits close resemblance to the IS*892* family first identified in lactococci (Yu *et al.*, 1995). Interestingly, copies of ISSa*4* could only be detected in GBS isolated in 1997 or the following years. It is suggested that one previously identified non-haemolytic strain is due to the presence of the ISSa*4* insertion sequence in the *cylB* gene, which encodes the membrane-spanning domain of the putative haemolysin transporter of GBS (Spellerberg *et al.*, 2000).

Recent studies report conflicting data to those of Milligan *et al.* (1978) in that the most invasive organisms did not produce hyaluronidase due to the presence of the insertion sequence IS*1548* in the *hylB* gene (Fig. 5.2) (Granlund *et al.*, 1998). The insertion sequence is 1317 bp long and the insertion point of this IS element maps to nucleotide 1050 of the *hylB* sequence. Of all GBS serotypes examined by Granlund *et al.* (1998), 86% of colonising isolates produced hyaluronidase, whereas only 38%(5/13) and

119

Figure 5.2. Relative positions of the primers used in amplifying the hyaluronidase gene with the insertion element IS*1548*.
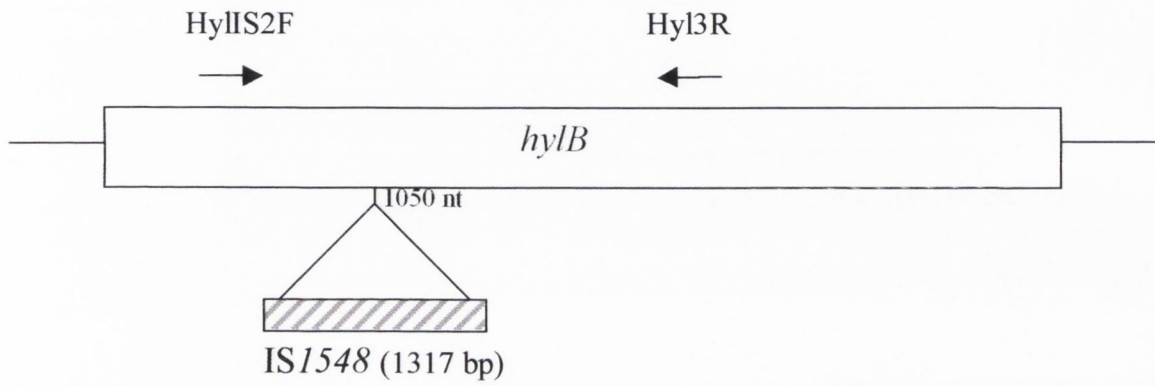
73% (11/15) of GBS isolates associated with endocarditis and other invasive diseases, respectively, produced hyaluronidase. This low proportion of hyaluronidase-producing isolates associated with invasive disease was due primarily to the fact that 11 of the 28 isolates contained the IS*1548* element inserted into the *hylB* gene. Between 3–8 copies of the IS*1548* element were present in the genomes of serotype III GBS and in all Group A streptococci examined. The exact reason for the disruption of hyaluronidase production by the IS element and its role in virulence are unknown, but it is thought that the lack of hyaluronidase would be beneficial for a propensity to adhere to and survive on host tissues.

Granlund *et al.* (2001) recently described an active, self-splicing group II intron, GBSi1. Sequences highly similar to GBSi1 exist also in *Streptococcus pneumoniae*. GBSi1 is located downstream of the C5a-peptidase gene (*scpB*) in some groups of GBS that lack the insertion element IS*1548*.

5.5 Results

5.5.1 PCR detection of the genes encoding five putative virulence factors

Figure 5.3 shows that PCR products of the expected sizes were obtained using the primers described in Table 2.1. When GBS DNA was used as a template in PCR, the *bca* gene primers BcarptF and BcarptR amplified a 246-bp product while the *bac* gene primers BacF and BacR amplified an 830-bp product. The *pepB* gene primers PepBF and PepBR amplified a 417-bp PCR product while the *rib* gene primers RibrptF and RibrptR amplified the smallest product of 237 bp. The identities of the *bac*, *bca*, *rib* and *pepB* PCR products were verified by DNA sequencing and alignment with the gene sequences available from the Genbank database, demonstrating that the primers used were specific for the individual genes and that no cross-contamination or non-specific binding of other genes occurred. Figure 5.4 shows partial gene sequences obtained using the primers mentioned above. BLAST analysis of the sequences obtained verified that PCR products were gene-specific. The primers HylIS2f and Hyl3R flanking the *hylB* gene amplified either a 714-bp or a 2031-bp PCR product, the difference in the *hylB* product sizes being identified by sequencing and BLAST analysis as the IS*1548* element. The occurrences of the *bac*, *bca* and *rib* genes in strains were confirmed by dot-blot hybridisation tests using specific, sequence-verified gene probes comprising alkaline phosphatase-labelled PCR products (Fig. 5.5).

5.5.2 Association of virulence genes with carriage or invasive status

All 159 GBS isolates were examined by PCR for the presence of five genes encoding putative virulence factors. Figure 5.6 shows a schematic diagram representing the genotypic patterns seen for all 159 isolates. All except one of the 159 GBS isolates (isolate H1969 of serotype III) possessed the *hylB* gene. The larger *hylB*(IS*1548*) PCR product was detected almost exclusively from serotype III isolates with two exceptions, isolate C10 of serotype II and H1711 of serotype V. Forty-two of the isolates (26.6%) had a *hylB* gene with the IS*1548* insertion sequence. The 2031-bp product was amplified from 40 of the 47 (85.1%) serotype III isolates. All but one of the invasive serotype III isolates possessed the insertion sequence (16 out of 17 invasive isolates versus 24 out of

Figure 5.3. PCR amplification of the *bac*, *bca*, *hylB*, *hylB*(IS*1548*), *pepB* and *rib* genes of GBS.
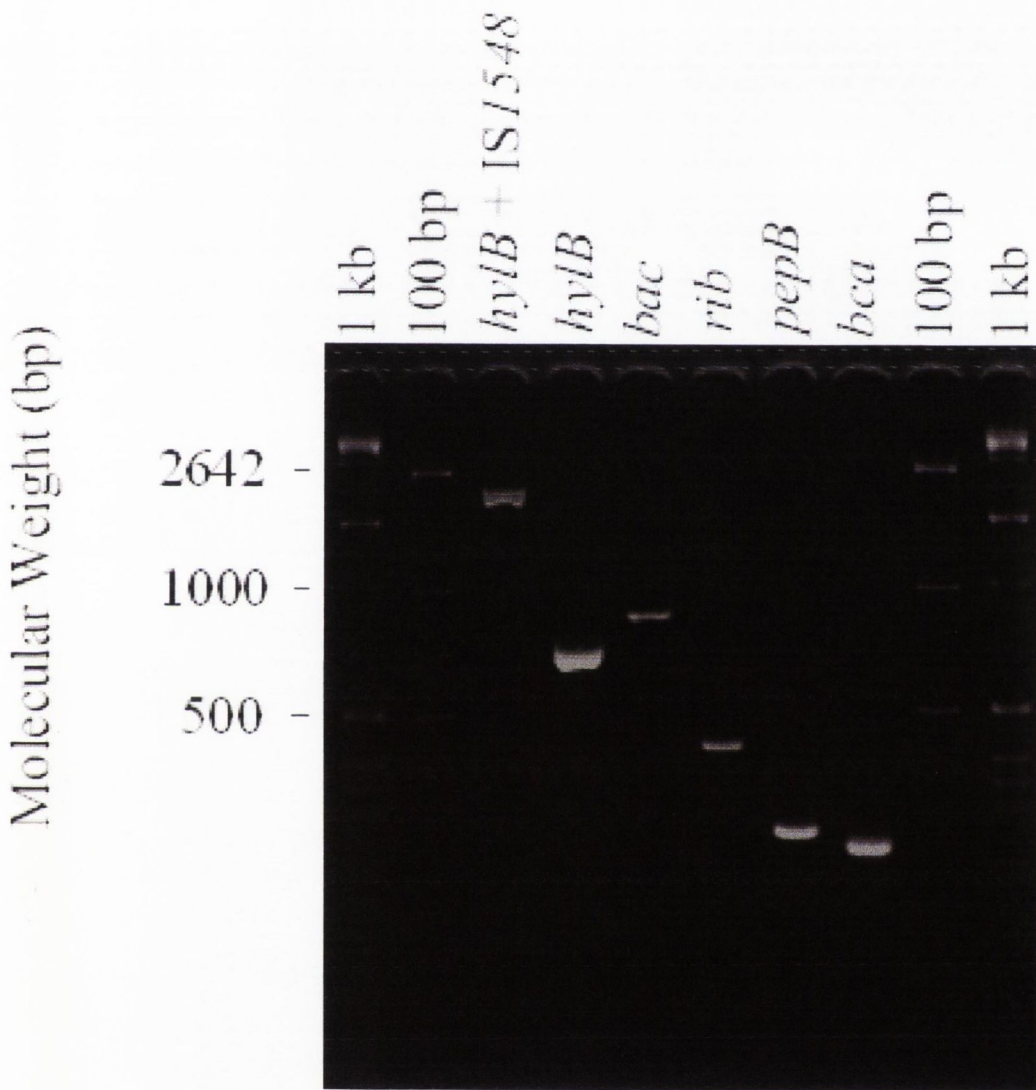
Figure 5.4. Partial sequences alignments of PCR products (Query) with Genbank gene sequences (Sbjct). Genbankgene sequence accession numbers are in italics.

(a) *X58470.1 – Streptococcus agalactiae bac* gene for Fc receptor

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Query:    1  cgaacctttacttcggcatcttcttttttatctgcttgttgagcttgtttacgaatatct  59
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  869  cgaacctttacttcggcatcttcttttttatctgcttgttgagcttgtttacgaatatct  810

Query:   60  tcgattttttcagcatagaatctttatcttcatttgtgattcccttttgctctgccatt  119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  809  tcgattttttcagcatagaatctttatcttcatttgtgattcccttttgctctgccatt  750

Query:  120  ttttcaacttgacttttttgatgattaagctctttgtcaatattgcttagatctactttc  179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  749  ttttcaacttgacttttttgatgattaagctctttgtcaatattgcttagatctactttc  690

Query:  180  gtatctgactgtttcagagtt  200
             |||||||||||||||||||||
Sbjct:  689  gtatctgactgtttcagagtt  669
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(b) *M97256.1 – Streptococcus agalactiae* group B C-protein alpha-antigen gene

```
Query:    1  aatatctgtcttaattgtatctaatcctgttaaaacctcagtacgactttcttccgtcca  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  675  aatatctgtcttaattgtatctaatcctgttaaaacctcagtacgactttcttccgtcca  616

Query:   61  cttaggatcgtttgcattcgttctaacttcttcaatcttatccctcaaggttgttgaatc  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  615  cttaggatcgtttgcattcgttctaacttcttcaatcttatccctcaaggttgttgaatc  556

Query:  121  ataaatagttaaagcaacactaactatatcaatttgtccatcagtctttacatgtggtag  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  555  ataaatagttaaagcaacactaactatatcaatttgtccatcagtctttacatgtggtag  496

Query:  181  tccatcttcacc  192
             ||||||||||||
Sbjct:  495  tccatcttcacc  484
```

(c) *U49821.1 – Streptococcus agalactiae* group B oligopeptidase PepB (pepB) gene

```
Query:    1    cagcatacttacgctaaaacattacaaacaaatgtgaaatctcaaaattttaaggctcgt  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  880    cagcatacttacgctaaaacattacaaacaaatgtgaaatctcaaaattttaaggctcgt  939
```

Query: 61   gtgcatcattatcaatcagcacgccaatcagctctatctgccaattttattccagaagag 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 940 gtgcatcattatcaatcagcacgccaatcagctctatctgccaattttattccagaagag 999

Query: 121 gtctacgaaactctaattaagacagttaatcatcatttacc 161
            |||||||||||||||||||||||||||||||||||||||||
Sbjct: 1000 gtctacgaaactctaattaagacagttaatcatcatttacc 1040

(d) *U58333.1 – Streptococcus agalactiae* group B surface protein Rib (*rib*) gene

Query: 1   agacaccgaaggcagaagattctattggtaacttaccagatcttccgaaaggtacaacag 59
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3416 agacaccgaaggcagaagattctattggtaacttaccagatcttccgaaaggtacaacag 3475

Query: 60   tagcctttgaaactccagttgatacggcaacaccgggagacaaaccagcaaaagttgttg 119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3476 tagcctttgaaactccagttgatacggcaacaccgggagacaaaccagcaaaagttgttg 3535

Query: 120 tgacttacccagatggttcaaaagatactgtagatgtgactgttaaggttgtcgat 176
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3536 tgacttacccagatggttcaaaagatactgtagatgtgactgttaaggttgtcgat 3591

(e) *U15050.1 – Streptococcus agalactiae* hyaluronate lyase gene

Query: 1   atgaaactaacggcaaaaacatcaaaactatcaaactggattctaatcgcactttccttt 59
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 901 atgaaactaacggcaaaaacatcgaagctatcaaactggattctaatcgcactttccttt 960

Query: 60   ggaaagatttagataatctcaataatt 86
            |||||||||||||||||||||||||||
Sbjct: 961 ggaaagatttagataatctcaataatt 987

(f) *Y14270.1 – Streptococcus agalactiae* insertion sequence IS*1548*

Query: 1   cacgccaaacgcatgaagaaacttttgttttcagaaaactagattcataatgacaaaatc 59
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 29   cacgccaaacgcatgaagaaacttttgttttcagaaaactagattcataatgacaaaatc 88

Query: 60   aatcaaagtagtacactatagatgaggtgactacgatgattgattttattatttctattg 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 89   aatcaaagtagtacactatagatgaggtgactacgatgattgattttattatttctattg 148

Query: 121 atgattgcgcagttgaattggatagtcgtcaatcttggaaaattcgctaccc 176
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 149 atgattgcgcagttgaattggatagtcgtcaatcttggaaaattcgctaccc 200

Figure 5.5. Dotblot hybridization of the 159 GBS isolates for the presence of the (a) *bac*, (b) *bca* and (c) *rib* genes. Isolate numbers correspond to those of Table 3.5.

(a) *bac*
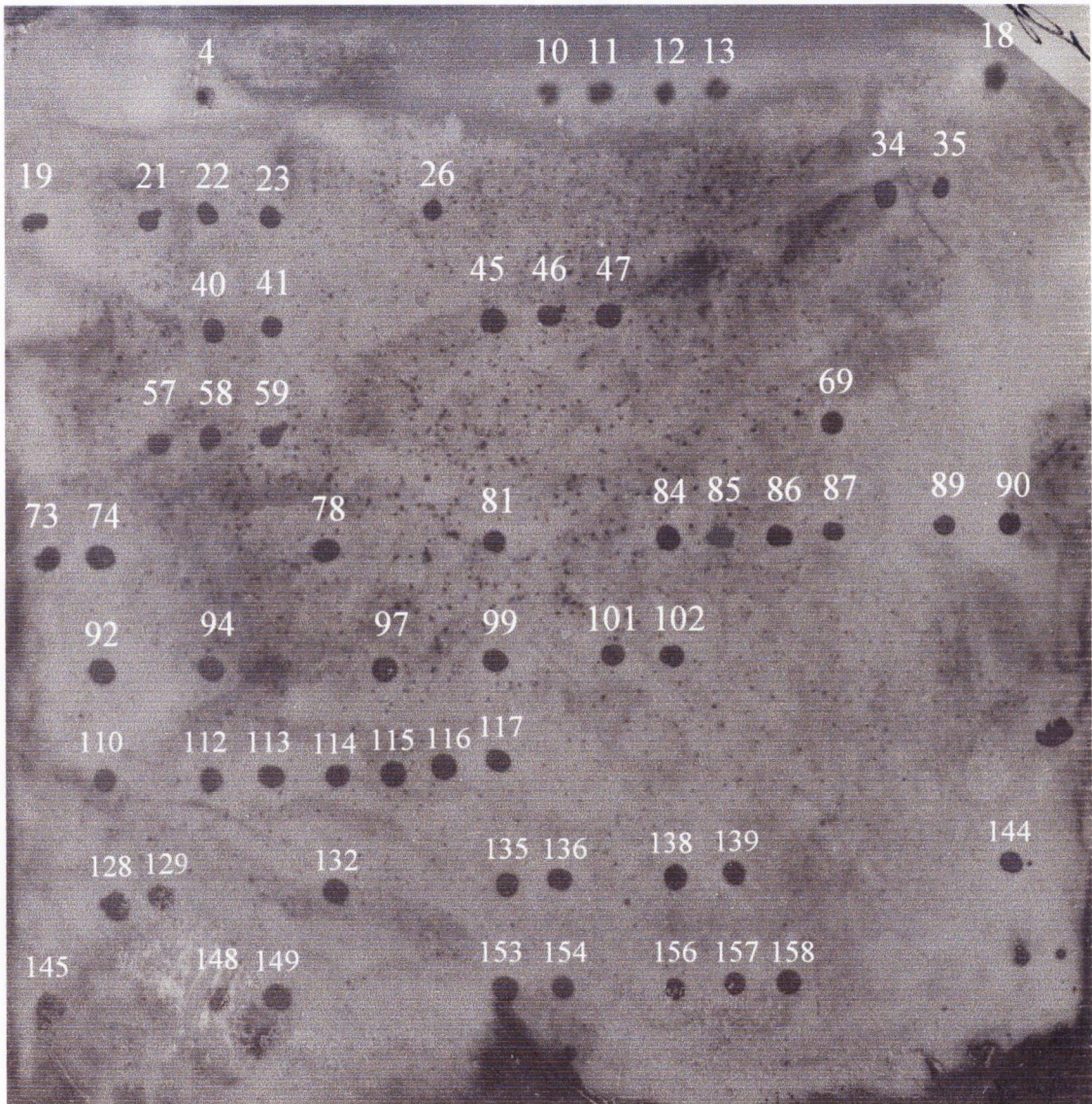
(b) *bca*
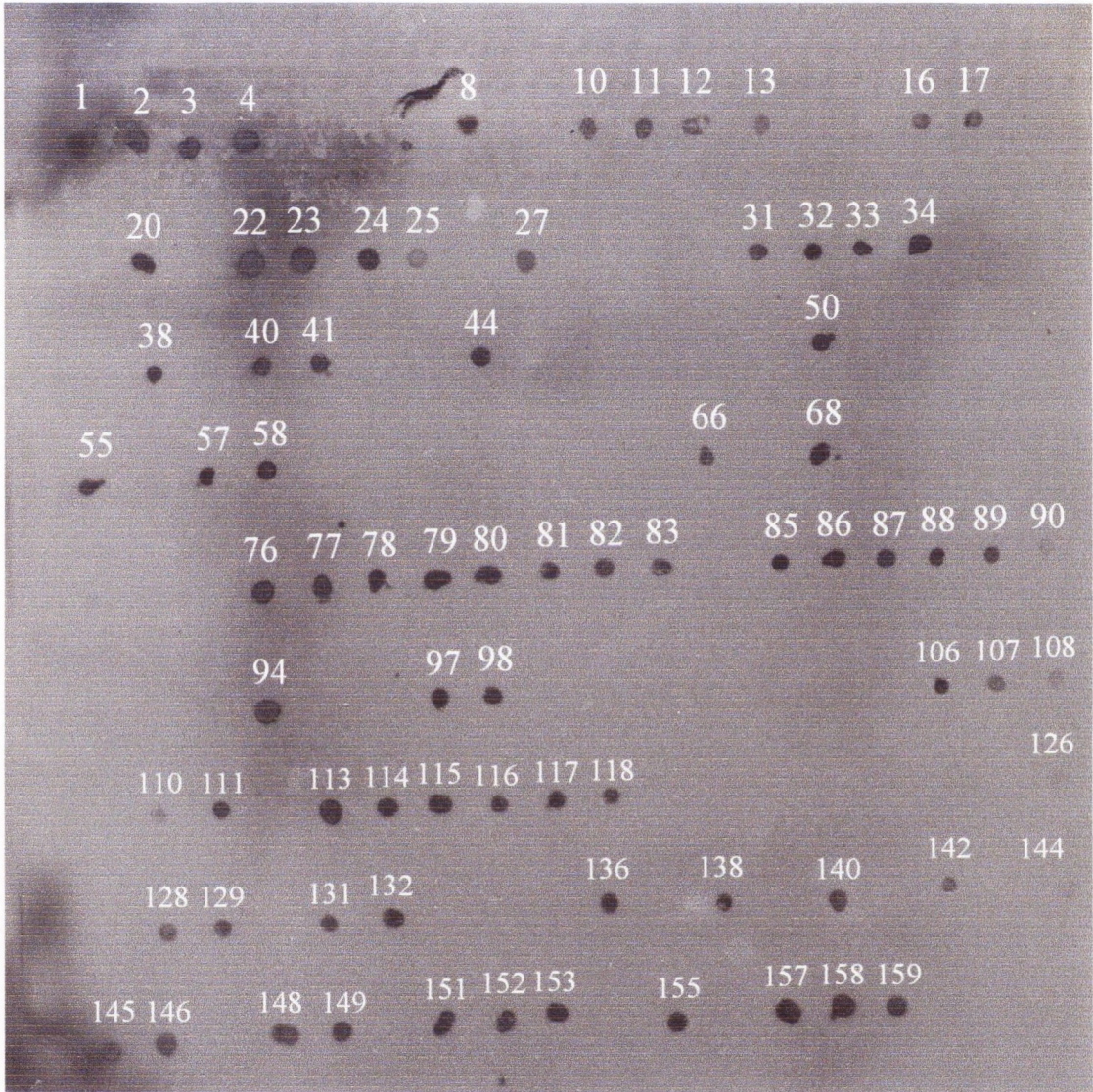
(C)   *rib*
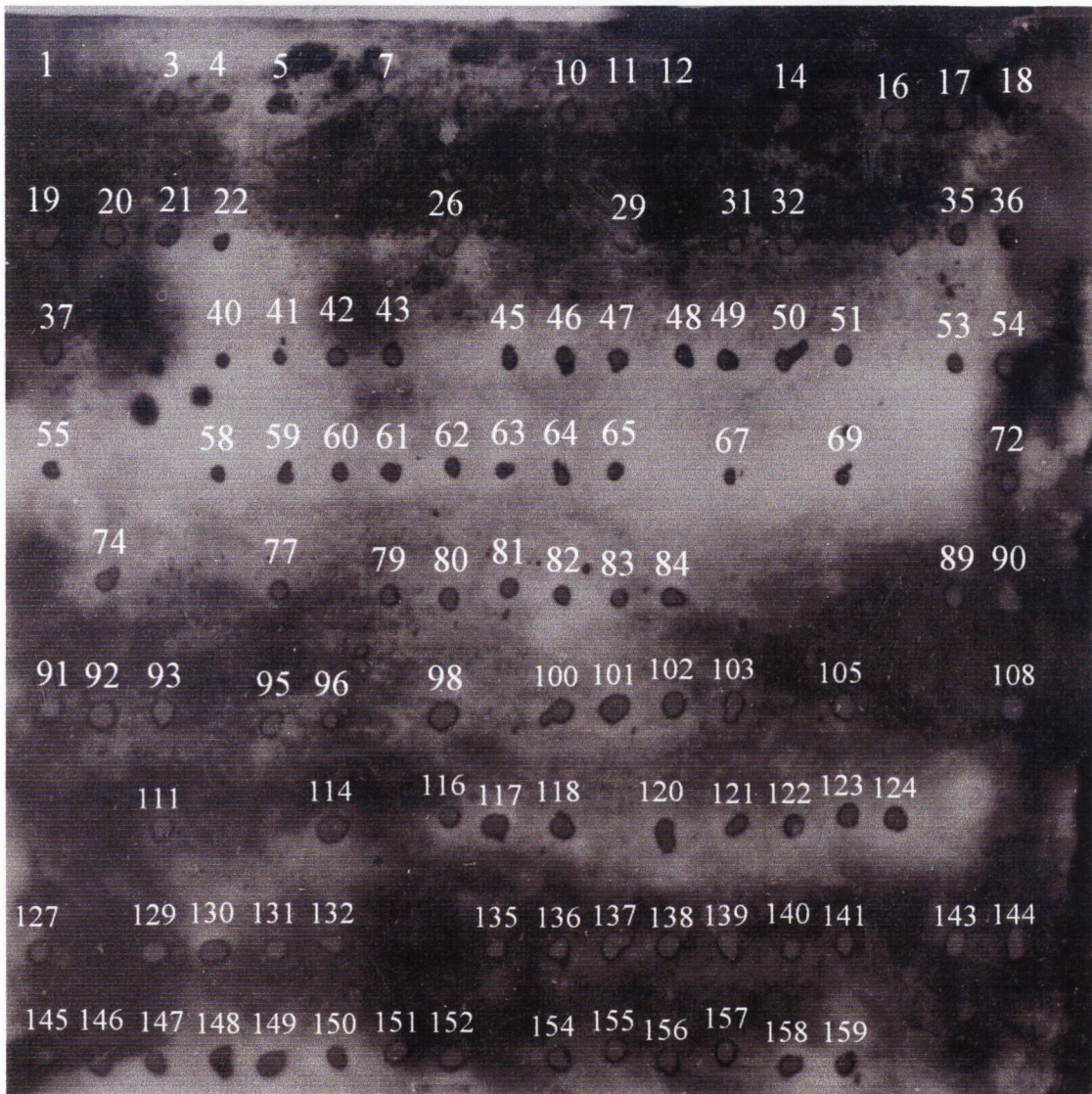
Figure 5.6. PCR banding patterns of the *bca*, *bac*, *rib*, *pepB*, *hylB* and *hylB*(IS*1548*) genes in the 159 GBS isolates.
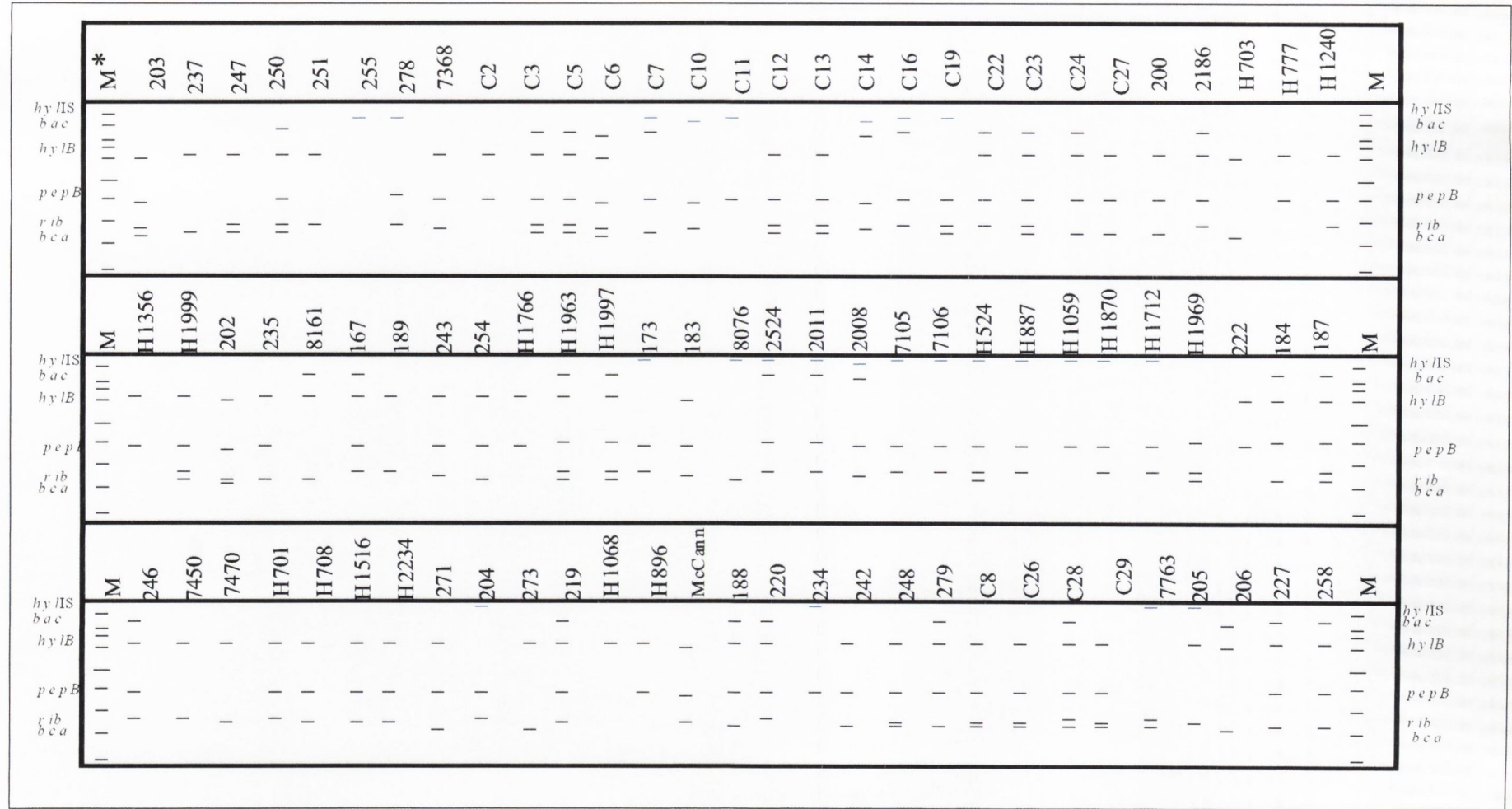
* 100bp ladder was used as a molecular weight marker, M.

Column headers (vertical, repeated for each group): *rib bca*, *pepB*, *hy IB*, *hy IIS bac*

Group 1	Group 2	Group 3
M	M	M*
1588	H913	259
1845	233	H1646
1929	H964	H1645
1813	297	175
3042	208	194
1795	B497	H823
5057	W2444	7552
5026	H2003	1744
5036	H1842	H1635
5002	186	181
5452	H1741	H1502
4532	H1066	H2000
3656	H1692	H578
8132	1741	2292
M	H4341	H852
	H4183	H1711
	270	H1338
	272	H1666
	7543	170
	8429	185
	8600	245
	8857	H1234
	820	35
	8718	215
	8099	226
	299	229
	320	230
	1135	H901
	1630	H970
	M	M

30 carriage isolates). Analysis of the results for the other four genes showed that the population of GBS was genetically diverse. Fisher's Exact tests of association between the presence of each of these genes and the carriage or invasive status of isolates demonstrated that none of these associations was statistically significant (the smallest P value was 0.39 for the *rib* gene).

5.5.3 Association between the occurrences of the *bac, bca, pepB, hyl*(IS*1548*) and *rib*

Unadjusted pairwise associations (Fisher's Exact test) between the *bac, bca, hylB* (IS*1548*), *pepB* and *rib* genes revealed that most of these genes were associated to some extent. Of the 10 associations tested, four were statistically significant and two were borderline significant.

The presence of so many pairwise associations pointed to more complex associations. Accordingly, partial associations were examined by a hierarchial log-linear model. This analysis gave two statistcally significant associations, viz., *bca* versus *hylB*(IS*1548*) (P = 0.0004) and *bac* versus *bca* (P = 0.014) and also indicated a potential three-way interaction between *bac* versus *bca* versus *rib* (P = 0.043).

The combinations (Table 5.2) primarily responsible for the three-way interaction are (i) where the *bac* and *bca* genes are both absent or present and the *rib* gene is, respectively, present or absent (patterns B and G) and (ii) where the *bca* and *rib* genes are both present or absent and the *bac* gene is, respectively, present or absent (patterns D and E). Patterns B and G are more frequent than expected, whereas patterns D and E occur less frequently than expected.

The associations among the genes may be modified after adjusting for their interactions with the serotypes. However, a larger data set would be required to perform this analysis.

Table 5.2. Interactions between the presence and absence of the *rib*, *bca* and *bac* genes.

Pattern	bac	bca	rib	Observed Frequency	Expected Frequency*	Residual	Adjusted Residual†	Nos. of isolates by serotype							No of isolates by status		Total
								Ia	Ib	II	III	IV	V	NT	Invasive	Carriage	
A	-	-	-	17	15.26	-1.74	0.61	6	0	3	5	2	1	0	7	10	17
B	-	-	+	39	32.32	6.68	1.96	5	0	3	19	0	13	0	18	22	40
C	-	+	-	16	15.85	0.15	0.05	6	3	2	2	0	2	1	6	10	16
D	-	+	+	25	33.56	-8.56	-2.50	5	6	1	7	0	3	3	11	14	25
E	+	-	-	2	9.76	-7.76	-3.08	0	1	1	0	0	0	0	2	0	2
F	+	-	+	20	20.66	-0.66	-1.21	3	3	1	9	0	3	0	7	12	19
G	+	+	-	16	10.13	5.87	2.30	2	9	0	1	1	1	2	11	5	16
H	+	+	+	24	21.45	2.55	0.81	4	8	6	4	0	1	1	10	14	24

* Expected frequencies are based on a model of complete mutual independence

† An adjusted residual approximately >2 is statistically significant at $P = 0.05$

5.5.4 Association of genotypic traits with serotype

Table 5.3 shows the numbers and percentages of positives for each of the five genotypic traits – *bac, bca, pepB, rib, and hylB* (IS*1548*) – examined within each serotype. Because the *hylB* gene was detected in all the GBS isolates but one, the *hylB* gene itself was excluded. Serotype IV and the non-typeable strains were excluded because of the small numbers (total of 10 isolates accounting for 6.3% of strains). The *rib, bca, bac* and *hylB* (IS*1548*) genes show significant associations with serotype (Table 5.3).

As the table shows, the percentages of isolates positive for the *pepB* gene within each serotype was relatively constant (range 75.0%–83.9%). Hence, there was no association between this gene and serotype. The distribution of *rib* gene by serotype was the next least variable (range 54.4–83.09%). This gene was most prominent in serotypes III and V. The distributions of bac^+ (range 25.0–70.0%) and bca^+ (range 29.2–86.7%) genes were similar in their ranges and in their distributions across the serotypes except for serotype Ia. The most variable was the *hylB*(IS*1548*) gene which was present in 85% of serotype III isolates and virtually absent in all others. In fact 40 of the 42 isolates positive for this gene were of serotype III. Serotype Ib has a higher proportion than expected of bac^+ and bca^+ isolates, the converse applying to serotype III, which had a very similar pattern of occurrence of bac^+, bca^+ and rib^+ isolates to serotype V.

5.5.5 Association of *bca* repeats with serotype and invasive and carriage status

Madoff *et al.* (1996) and Gravekamp *et al.* (1998) reported that deletions in the number of repeats in *bca* genes occurred under selective pressure *in vivo* and that a smaller number of repeats helped the survival of an organism against the host's immune system. Accordingly, a stratified random sample of 59 of the 73 bca^+ GBS excluding serotype IV and non-typeable isolates (Ia = 15, Ib = 23, II = 6, III = 11, V = 4) was characterised to determine the numbers of tandem repeats within their *bca* genes using primers that flanked the repeat regions.

The PCR primers BcaF and BcaR gave rise to differently sized amplimers depending on the number of tandem repeats within the *bca* gene (Fig. 5.7). In Table 5.4 the numbers of tandem repeats are documented according to the serotype and invasive or

133

Table 5.3. Frequencies of occurrence of genotypic traits within serotypes of 149 GBS isolates.

Serotype	bac^+	bca^+	$HylB$ (IS1548)$^+$	$pepB^+$	rib^+	Total
			No. of isolates of given genotype (%)			
Ia	9 (29.0)	17 (54.8)	0	26 (83.9)	17 (54.8)	31
Ib	21 (70.0)	26 (86.7)	0	23 (76.7)	17 (56.7)	30
II	8 (47.1)	9 (52.9)	1 (5.9)	13 (76.5)	11 (64.7)	17
III	14 (29.8)	14 (29.8)	40 (85.1)	39 (83.0)	39 (83.0)	47
V	6 (25.0)	7 (29.2)	1 (4.2)	18 (75.0)	20 (83.3)	24
Total	58 (38.9)	73 (49.0)	42 (28.2)	119 (79.9)	104 (69.8)	149
P	0.002	<0.001	<0.001	0.868	0.018	

Figure 5.7. Agarose gel showing GBS isolates with varying sizes of the repeat region in the *bca* gene.
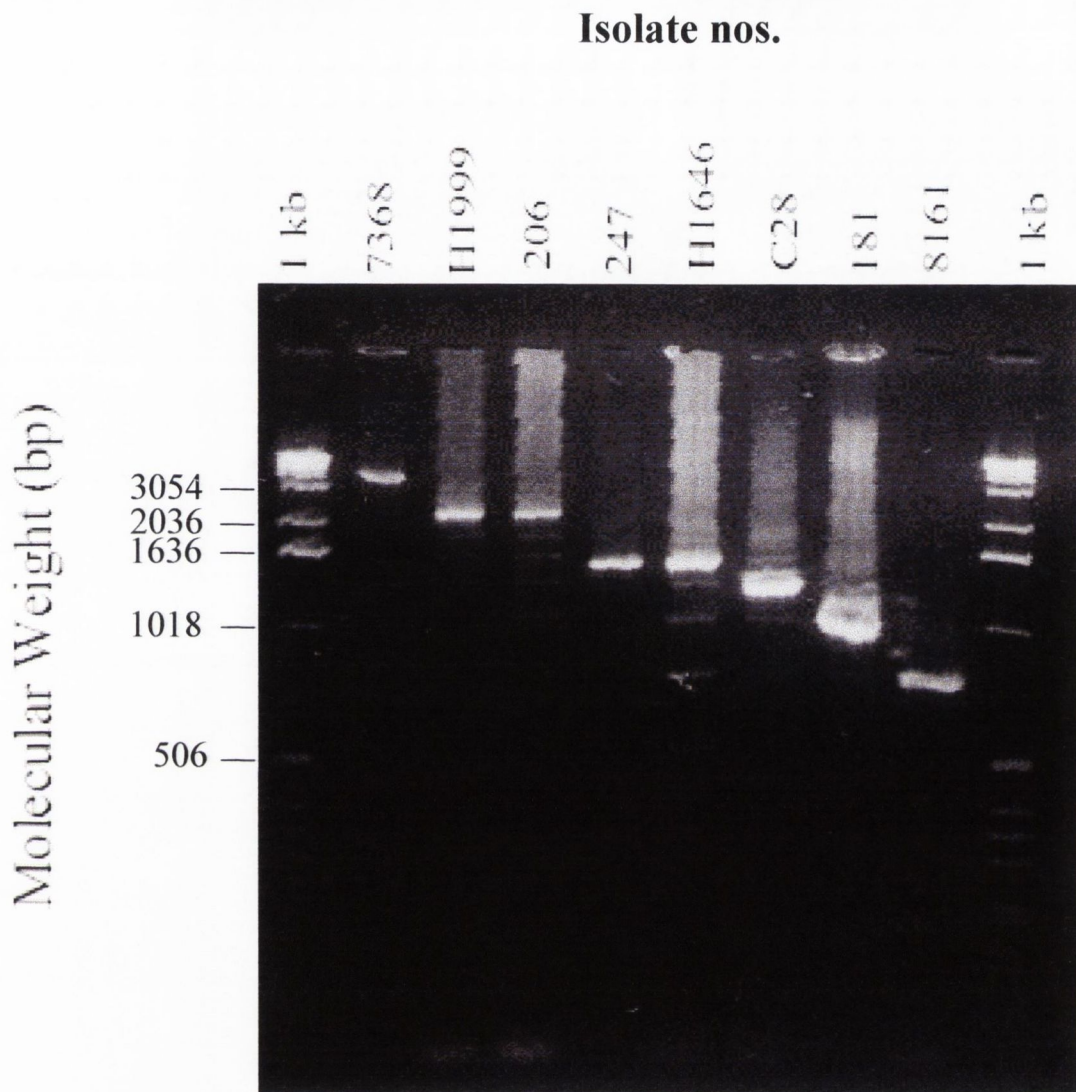
Table 5.4. Determination of the number of *bca* tandem repeats in 59 *bca*[+] GBS isolates.

Number of *bca* repeats[a]	Size of PCR products (bp)	No. of isolates by serotype					No. of isolates by status		Total
		Ia	Ib	II	III	V	Invasive	Carriage	
1	918	1	-	1	-	-	1	1	2
2	1164	3	2	-	2	2	3	6	9
3	1410	-	4	1	-	-	4	1	5
4	1656	-	1	2	2	1	2	4	6
5	1902	2	-	-	1	-	1	2	3
6	2148	3	1	-	1	-	3	2	5
7	2394	1	1	-	-	-	-	2	2
9	2886	1	2	1	3	-	4	3	7
10	3132	-	-	-	1	-	-	1	1
11	3378	2	3	-	-	-	3	2	5
12	3624	1	2	-	-	1	2	2	4
14	4116	1	5	1	-	-	5	2	7
15	4362	-	1	-	-	-	-	1	1
16	4608	-	1	-	1	-	1	1	2
Mean repeats		6.6	9.1	5.8	6.9	5.0	7.9	7.0	7.4
Median repeats		6.0	11.0	4.0	6.0	3.0	9.0	6.0	6.0

[a] The molecular sizes of the products were determined relative to a 1-kb marker ladder and the number of repeats calcuated on the basis of a repeat size of 246nt

carriage status of isolates. The repeat number ranged from 1–16 repeats within these isolates. The data obtained suggest that the occurrence of any numbers from 1–16 is equally likely. When the distribution of the numbers of tandem repeats for the whole sample was compared with a uniform distribution, the difference was not statistically significant (Kolmogorov-Smirnov Goodness of Fit test, $P = 0.1$)

Potential associations between the number of tandem repeats and the serotype or invasive status of GBS were analysed. No difference was found in the distributions of tandem repeats between invasive and carriage isolates (Mann-Whitney U-test, $P = 0.7$), or among the distributions of tandem repeats in serotypes Ia, Ib, II and III (Kruskal-Wallis test, $P = 0.23$). Serotype V was excluded since it had only 4 strains in the sample. On visual inspection of Table 5.4, however, serotype Ib GBS appear to possess higher numbers of *bca* tandem repeats than found in other serotypes, as indicated by the cluster of isolates with 11 to 16 repeats and its substantially higher median. Serotype Ib is the only serotype in which more than half of the strains contained more tandem repeats than the overall mean of 7.4 repeats. There is a statistically significant difference between its tandem-repeat distribution and that of the other four serotypes combined (Mann-Whitney U-test, $P = 0.025$)

5.5.6 Serotype III

As serotype III dominated in the present study, a more detailed analysis of the characteristics of this group was undertaken. The high incidence of serotype III isolates possessing the *hylB* (IS*1548*) gene, as already indicated, was noteworthy. The rates of the occurrence of serotype III strains bearing the *hylB* (IS*1548*) gene were similar for carriage isolates in the three maternity hospitals, namely, 5 out of 7, 6 out of 7 and 13 out of 16, respectively. In the two hospitals that contributed 87.4% of all the GBS studied, the incidences of the *hylB* (IS*1548*) among invasive isolates were 5 out of 5 and 11 out of 12, respectively. Thus, serotype III isolates bearing the *hylB* (IS*1548*) gene occur widely across the greater Dublin area and are not a feature of strains from a particular suburban catchment area. Thus, the presence of the *hylB* (IS*1548*) gene may be a useful genotypic

marker for a potential risk of serotype III invasive disease, since 16 out of 17 invasive serotype III isolates were identified with this characteristic.

Isolates of gene pattern E (Table 5.5) accounted for 46.8% (22 of 47) of serotype III isolates and 55% of those possessing the *hylB* (IS*1548*) gene (gene patterns E–H, 22 out of 40), 10 of which were carriage isolates and 12 invasive. Of the remaining 18 isolates possessing the *hylB* (IS*1548*) gene ± either the *bac* or *bca* gene or possessing both genes (gene patterns F–H), 14 were carriage and 4 invasive isolates (χ^2= 4.31, DF = 1, *P* <0.05). Of the 22 pattern-E isolates, 17 possesssed the *rib* gene (7 carriage, 10 invasive), 17 possessed the *pepB* gene (9 carriage, 8 invasive), and 14 possessed both genes (7 carriage, 7 invasive). Of the gene pattern F–H isolates, 15 strains had the *rib* gene (11 carriage, 4 invasive), 16 had the *pepB* gene (12 carriage, 4 invasive) and 14 possessed both genes (10 carriage, 4 invasive).

5.5.7 Association of RAPD and PFGE clusters with genotypic traits, serotype and status

All molecular fingerprinting results (Chapter 4) were combined with genotypic trait, serotype and sepsis results (Appendix, this chapter). However, a larger data set would be required to perform association analysis between genotypic traits and RAPD or PFGE types.

Table 5.5. Characteristics of *Streptococcus agalactiae* serotype III isolates.

Pattern	No. of Isolates	Source		*hylB**	*hylB*(IS*1548*)	*bac*	*bca*
		Carriage	Invasive				
A	2	1	1	+	–	–	–
B	2	2	0	+ / –	–	–	+
C	1	1	0	+	–	+	–
D	2	2	0	+	–	+	+
E	22	10	12	+	+	–	–
F	7	6	1	+	+	–	+
G	8	6	2	+	+	+	–
H	3	2	1	+	+	+	+

* For the purposes of this table *hylB* and *hylB*(IS*1548*) are regarded as mutually exclusive variants of the hyaluronidase gene

5.6 Discussion

Serotyping remains the classical epidemiological tool in the investigations of invasive GBS disease to date. However, in many cases it provides insufficient information to definitively distinguish epidemiologically related or unrelated GBS isolates. In addition, serotyping does not allow sufficient assessment of genetic relatedness or diversity among isolates and a more discriminatory typing system for GBS is needed.

Attempts to correlate the presence of specific recognised or putative virulence factors with GBS disease or its severity have been made previously (e.g., Chun *et al.*, 1991; Granlund *et al.*, 1998; Gibson *et al.*, 1999). In such studies the roles of particular putative virulence factors have been analysed separately without taking possible linkages between putative virulenc factors into account. This approach may bias conclusions on the role of putative virulence factors in disease by not accounting for the confounding effect of other putative virulence factors that have not been tested for and by ignoring potential synergistic effects between putative virulence factors.

Based on serological tests GBS strains of different polysaccharide type have been shown to express particular cell-surface proteins. For example, the Bca protein has been found largely on strains of serotypes Ia, Ib and II, the Rib protein on strains of serotype III and the Alp3 protein on most isolates of serotypes V and VIII (Johnson and Ferrieri, 1984; Lachenauer *et al.*, 1996 and 2000). The genotypic data obtained in the current study are somewhat at variance with these general phenotypic trends. The data in Tables 5.2 and 5.3 indicate that approx. 50% of serotype III strains possess the *bac* or *bca* genes or both, while 55–65% of the strains of serotypes Ia, Ib and II possess the *rib* gene. In contrast, the prevalence of the *bac* and *bca* genes in the same population of serotypes Ia, Ib and II is in concordance with the PCR, hybridization and serological data in several studies over the past decade (Suvorov *et al.*, 1997, Berner *et al.*, 1999; Rolland *et al.*, 1999; Brodeur *et al.*, 2000). The same is true of the prevalence of the *rib* gene and the Rib protein in serotype III strains. The sizes of the PCR products, the PCR product sequencing data and alignments, and the dot-blot hybridization results all confirm the identity of these genes. Thus, the genotypic data either (i) represent novel observations that have been missed because of previous emphasis on phenotypic testing or (ii) indicate

the presence of silent *bac*, *bca* and *rib* genes in these serotypes or (iii) indicate the presence of genes encoding as yet unrevealed proteins with sequence similarities to the *bca*, *bac* and *rib* genes. Indeed, the recent description of a previously unknown surface protein of group B streptococci designated Sip (Brodeur *et al.*, 2000) and of a laminin-binding protein (Rocha *et al.*, 1999) and the unpublished sequence data on an epsilon surface antigen (Accession No. U33554) and an R5 surface protein (Accession No. AJ133114) in the GenBank database emphasize that there may be further unrevealed wall-associated proteins in this bacterium.

In the present study, to assess if there was any association between the presence or absence of genes encoding putative virulence factors and serotype or invasiveness, isolates were screened for five genes using PCR amplification. Correlations between the distributions of the *bac*, *bca*, *hylB*(IS*1548*) and *rib* genes and serotype revealed significant associations. Moreover, the joint occurrence of the *bac* and *bca* genes was associated with serotype Ib and their joint absence with serotypes III and V, confirming published epidemiological data (Johnson *et al.*, 1984; Kvam *et al.*, 1995; Maeland *et al.*, 2000). The *bac*$^+$ *bca*$^+$ genotype may therefore be a feature of serotype Ib. No phenotypic characterization of isolates for gene products was performed herein. Thus, it might be argued that to analyse associations with invasiveness based solely on genotypic characteristics could be misleading. However, Maeland *et al.* (2000) have shown, at least in the case of the β C-protein antigen, that PCR-based *bca* gene detection and antibody-based Bca protein detection demonstrated good concordance. In contrast, 7 of 14 reference and prototype strains that were serologically negative for the α C-protein possessed the *bca* gene by PCR (Maeland *et al.*, 1999) and 16 of 52 (30.8%) clinical isolates were similarly Bca$^-$ but *bca*$^+$ (Maeland *et al.*, 2000). Given also that Bca proteins with different numbers of tandem repeats are antigenically different (Madoff *et al.*, 1996), our approach underscores the need for molecular genetic characterization. Indeed, as pointed out by Maeland *et al.* (2000), lack of expression of a cell-surface protein *in vitro* does not mean absence of expression *in vivo*, since the environmental stimuli and the nature of the regulatory cascades controlling GBS gene expression *in vivo* are unknown.

The present study also investigated the mutual interactions of these virulence genes

in a hierarchial log-linear model. This analysis indicated a three-way interaction between the presence or absence of the *bac*, *bca* and *rib* genes. These three genes were found to be present or absent in specific patterns in some serotypes. Suvorov *et al.* (1997) found that the size of the *Eco*RI chromosomal fragments hybridizing with a *bca* gene probe correlated with the genomic presence or absence of the *bac* gene. These data suggest linkage between these genes, although PFGE hybridization data suggest that the *bac* and *bca* genes are not adjacent. No statistically significant associations were demonstrated between invasiveness and serotype or between the presence of each of the five genes tested for and invasive status. Interestingly, the majority of the Irish serotype III isolates, both invasive and carriage, possessed a *hylB* gene bearing the insertion sequence IS*1548*. The inactivation of the *hylB* gene by this insertion element (Granlund *et al.*, 1998) and the frequency of occurrence of the *hylB* (IS*1548*) gene in both carriage and invasive isolates suggest that hyaluronidase does not play an important role in invasion of the serotype III isolates. Recently Sellin *et al.* (2000) were able to split serotype III isolates into two distinct clusters, designated III$_1$ and III$_2$, by *cps* gene cluster RFLP analysis. A covariation with insertion sequence IS*1548* in the *hylB* gene was suggested since allelic type III$_1$ harboured IS*1548* in the *hylB* gene. Thus, 85% of the Irish serotype III isolates would appear to be of this allelic type on this basis.

Stemming from the GBS population studies of Musser *et al.* (1989), who identified two lineages of serotype III isolates based on MLEE analysis of 11 genetic loci, many studies have attempted to identify virulence-associated properties or alleles of genes associated with these two clusters of strains (e.g., Quentin *et al.*, 1995; Hauge *et al.*, 1996; Sellin *et al.*, 2000). While hyaluronidase production was associated with the so-called high virulence ET1 clone of Musser *et al.* (1989), more recent studies have associated an IS-inactivated *hylB* gene and a hyaluronidase-negative phenotype with serotype III strains from invasive neonatal infections and endocarditis (Hauge *et al.*, 1996; Granlund *et al.*, 1998; Rolland *et al.*, 1999; Sellin *et al.*, 2000). While it is difficult to compare frequencies of occurrence of the *hylB*(IS*1548*) gene between studies because of the differing populations of strains selected or collected, a surprising feature of the Dublin serotype III isolates was the high incidence of this gene among both carriage and invasive

isolates. The pattern-E isolates (Table 5.5) very closely resemble the division-I Danish strains of Hauge *et al.* (1996) by being additionally *bac⁻ bca⁻*. Several studies have reported low or quite differing incidences of the α and β C-proteins or of their genes in serotype III strains (Chun *et al.*,1991; Quentin *et al.*, 1995; Maeland *et al.*, 1997; Suvorov *et al.*,1997). The finding of *bac⁺ bca⁺* strains of serotype III suggest that these are probably of a different clonal lineage.

Three of the so-called "low virulence strains" of Musser *et al.* (1989) possessed the *hylB*(IS*1548*) and clustered with the Danish isolates in being of ribotype 4, *hylB* RFLP type 2 and C5a peptidase RFLP type 2 (Hauge *et al.*, 1996). From the present studies the majority of this clonal lineage appear to possess the *rib* and *pepB* genes. Thus, the *hylB*(IS*1548*) gene may be a useful marker for the pathogenic potential of this serotype III clone for which risk of invasive infection appears to be high, but definition of clear virulence determinants remain elusive.

It is clear from the present and other studies (Musser *et al.*, 1989; Quentin *et al.*, 1995; Hauge *et al.*, 1996; Rolland *et al.*, 1999) that the pathogenicity of GBS strains is either multifactorial or that some as yet undiscovered virulence factor, or both, may be important. Indeed, over the last few years novel putative virulence factors have been found in group A streptococci and in enterococci (Hanski and Caparon, 1992; Rich *et al.*, 1999; Rocha and Fischetti, 1999) as well as GBS (Brodeur *et al.*, 2000; Lachenauer *et al.*, 2000; Rocha *et al.*, 1999). However, the nature of the predisposing factors to infection in the neonate and mother may be more crucial for GBS infection (Christensen *et al.*, 1985).

Since this study was completed Lachenauer *et al.* (2000) have described newly discovered α-like proteins namely, Alp2 and Alp3. These mosaic proteins share several characteristic features with the α C- and Rib proteins. Alp2 contains the same N-terminal, tandem repeat region possibly derived from the Rib protein and an additional repeat derived possibly from the α C-protein. The Alp3 N-terminal half are highly similar to the α and β C-proteins while the C-terminal end is almost identical to the Rib protein. Neither the *bca* primers used herein nor the *bca* PCR product showed sequence identity with the repeat sequences in the *rib* or *alp3* genes. Although the BcarptF and BcarptR primers show sequence identity with the *alp2* gene, these sequences are on the same DNA strand

143

covering about 120 nt. The RibF primer shows identity to the *rib* gene, but not to the *alp2* and *alp3* sequences. Thus, only a *rib* PCR product would be amplified. Accordingly, the PCR products detected are undoubtedly indicative of the presence of the *bca* and *rib* genes.

In conclusion, in contrast to most previous studies using univariate analysis, the present findings using multivariate analysis suggest that some previously established associations between virulence factors and GBS disease require further clarification and that individual gene markers are not necessarily good predictors of invasive capacity, as previously shown for PCR detection of the *bac* gene (Chun *et al.*, 1991). PCR detection of several putative virulence factor genes, along with serotyping, may eventually prove to be a more appropriate method for identifying potentially invasive GBS isolates in a clinical laboratory. The identification of virulence factors produced by the most common serotypes may aid in the development of an effective vaccine for GBS disease.

APPENDIX

Table A1. RAPDnm types of all isolates with associated hospital, genotype, serotype, and sepsis statu

HOSPITAL	Isolate Name	SEROTYPE	RAPDnm TYPE	STATUS	SEPSIS	Genotype					
						bac	bca	rib	pepB	hylB	hylIS
Rotunda	1929	1a	I	Carriage	0	+	+	+	+	+	−
Rotunda	200	1a	I	Carriage	0	−	+	−	+	+	−
Holles	H703	1a	I	Carriage	0	−	+	−	−	+	−
Holles	H1712	III	I	Carriage	0	−	−	+	+	+	+
Rotunda	4532	1b	I	Invasive	2+	+	+	+	+	+	−
Holles	H1066	1b	I	Invasive	2+	+	+	−	+	+	−
Rotunda	235	1b	II	Carriage	0	−	+	−	+	+	−
Rotunda	7470	V	II	Carriage	0	−	−	+	−	+	−
Rotunda	206	1b	II	Invasive	1+	+	+	−	−	+	−
Holles	H1646	1b	II	Invasive	1+	+	+	+	+	+	−
Rotunda	1744	V	II	Invasive	1+	−	−	+	−	+	−
Holles	H1635	V	II	Invasive	1+	−	−	+	+	+	−
Holles	H2000	II	II	Invasive	2+	+	−	−	+	+	−
Holles	H852	V	II	Invasive	2+	+	−	+	+	+	−
Rotunda	233	II	II	Invasive	4+	−	+	+	−	+	−
Holles	H964	II	II	Invasive	4+	−	−	−	+	+	−
Holles	H1741	V	II	Invasive	4+	−	−	+	+	+	−
Holles	H1999	1a	III	Carriage	0	−	+	+	+	+	−
Holles	H777	1a	III	Carriage	0	−	−	−	+	+	−
Coombe	C22	III	III	Carriage	0	+	−	+	+	+	−
Rotunda	5002	1b	III	Invasive	2+	−	+	+	+	+	−
Rotunda	272	IV	IV	Carriage	0	−	−	−	+	+	−
Rotunda	2186	1a	IV	Carriage	0	+	−	+	+	+	−
Coombe	C10	II	IV	Carriage	0	−	−	+	+	+	+
Holles	H1963	II	IV	Carriage	0	+	+	+	+	+	−
Holles	H1997	II	IV	Carriage	0	+	+	+	+	+	−
Rotunda	173	III	IV	Carriage	0	−	−	+	+	+	+
Rotunda	2008	III	IV	Carriage	0	+	−	+	+	+	+
Rotunda	250	III	IV	Carriage	0	+	+	+	+	+	−
Rotunda	2524	III	IV	Carriage	0	+	−	+	+	+	+
Rotunda	255	III	IV	Carriage	0	−	−	−	−	+	+
Rotunda	7105	III	IV	Carriage	0	−	−	+	+	+	+
Rotunda	7763	III	IV	Carriage	0	−	+	+	−	+	+
Rotunda	8076	III	IV	Carriage	0	−	+	−	−	+	+
Holles	H1969	III	IV	Carriage	0	−	+	+	+	−	−
Holles	H524	III	IV	Carriage	0	−	+	+	+	+	+
Holles	H887	III	IV	Carriage	0	−	−	+	+	+	+
Rotunda	242	1a	IV	Invasive	1+	−	+	−	+	+	−
Rotunda	194	III	IV	Invasive	1+	+	−	+	+	+	+
Rotunda	204	III	IV	Invasive	1+	−	−	+	+	+	+
Rotunda	248	V	IV	Invasive	1+	−	+	+	+	+	−
Coombe	C8	V	IV	Invasive	1+	−	+	+	+	+	−
Rotunda	1813	1a	IV	Invasive	2+	+	+	+	+	+	−
Rotunda	181	1a	IV	Invasive	2+	+	+	−	+	+	−
Holles	H1502	1a	IV	Invasive	2+	−	+	+	+	+	−
Rotunda	5036	III	IV	Invasive	2+	+	−	+	+	+	+
Holles	H578	III	IV	Invasive	2+	−	−	+	+	+	+
Holles	H1338	1a	IV	Invasive	3+	−	−	−	+	+	−
Holles	H1666	1a	IV	Invasive	3+	−	−	+	+	+	−
Rotunda	273	1a	IV	Invasive	4+	−	+	−	−	+	−
Holles	H896	1a	IV	Invasive	4+	−	−	−	+	+	−
Holles	McCann	1a	IV	Invasive	4+	−	−	+	+	+	−
Rotunda	35	1b	IV	Invasive	4+	+	+	−	+	+	−
Rotunda	3042	III	IV	Invasive	4+	−	−	+	+	+	+
Rotunda	207	III	IV	Invasive	4+	−	−	+	+	+	−

HOSPITAL	Isolate Name	SEROTYPE	RAPDnm TYPE	STATUS	SEPSIS	Genotype					
						bac	bca	rib	pepB	hylB	hylIS
Rotunda	208	III	IV	Invasive	4+	−	−	+	−	+	+
Rotunda	W2444	III	IV	Invasive	4+	−	−	+	+	+	+
Rotunda	188	NT	IV	Invasive	4+	+	+	−	+	+	−
Rotunda	202	1b	V	Carriage	0	−	+	+	+	+	−
Rotunda	2011	III	V	Carriage	0	+	−	+	+	+	+
Rotunda	278	III	V	Carriage	0	−	−	+	+	+	+
Rotunda	271	NT	V	Carriage	0	−	+	−	−	+	−
Holles	H1516	V	V	Carriage	0	−	−	+	+	+	−
Rotunda	227	1b	V	Invasive	1+	+	+	−	+	+	−
Rotunda	258	1b	V	Invasive	1+	+	+	−	+	+	−
Rotunda	259	1b	V	Invasive	1+	−	+	−	+	+	−
Coombe	C26	1a	V	Invasive	2+	−	+	+	+	+	−
Rotunda	215	1b	V	Invasive	4+	−	+	+	+	+	−
Rotunda	219	1b	V	Invasive	4+	+	−	+	+	+	−
Rotunda	220	1b	V	Invasive	4+	+	−	+	+	+	−
Rotunda	226	1b	V	Invasive	4+	+	−	−	−	+	−
Rotunda	229	1b	V	Invasive	4+	+	+	−	+	+	−
Rotunda	230	1b	V	Invasive	4+	+	+	+	−	+	−
Holles	H901	1b	V	Invasive	4+	+	+	−	+	+	−
Holles	H913	1b	V	Invasive	4+	+	+	+	+	+	−
Rotunda	7543	1a	VI	Carriage	0	+	−	+	+	+	−
Coombe	C5	1a	VI	Carriage	0	+	+	+	+	+	−
Coombe	C6	1a	VI	Carriage	0	+	+	+	+	+	−
Holles	H1240	1a	VI	Carriage	0	−	−	+	+	+	−
Coombe	C27	1b	VI	Carriage	0	−	+	−	+	+	−
Rotunda	254	II	VI	Carriage	0	−	+	−	+	+	−
Coombe	C3	III	VI	Carriage	0	+	+	+	+	+	−
Coombe	C12	NT	VI	Carriage	0	−	+	+	+	+	−
Coombe	C24	NT	VI	Carriage	0	+	+	−	+	+	−
Rotunda	184	V	VI	Carriage	0	+	+	−	+	+	−
Rotunda	187	V	VI	Carriage	0	+	+	+	+	+	−
Rotunda	7368	V	VI	Carriage	0	−	+	−	+	+	−
Coombe	C2	V	VI	Carriage	0	−	−	−	+	+	−
Holles	H701	V	VI	Carriage	0	−	−	+	+	+	−
Rotunda	205	1b	VI	Invasive	1+	+	−	+	−	+	−
Rotunda	3656	III	VI	Invasive	2+	+	+	+	+	+	+
Holles	H1711	V	VI	Invasive	2+	−	−	+	+	+	+
Rotunda	245	1a	VI	Invasive	4+	−	+	+	+	+	−
Holles	H1234	1a	VI	Invasive	4+	−	−	−	+	+	−
Rotunda	1135	1b	VII	Carriage	0	+	+	+	+	+	−
Rotunda	5057	1b	VII	Carriage	0	−	+	+	+	+	−
Rotunda	1630	II	VII	Carriage	0	+	+	+	+	+	−
Rotunda	8429	II	VII	Carriage	0	+	+	+	−	+	−
Rotunda	8857	II	VII	Carriage	0	+	+	+	+	+	−
Rotunda	299	III	VII	Carriage	0	−	+	−	+	+	+
Rotunda	1588	III	VII	Carriage	0	−	+	+	+	+	+
Rotunda	5452	III	VII	Carriage	0	+	−	+	+	+	+
Holles	H1692	III	VII	Carriage	0	+	+	+	+	+	+
Rotunda	320	V	VII	Carriage	0	−	−	+	+	+	−
Rotunda	8132	V	VII	Carriage	0	−	+	+	+	+	−
Rotunda	8600	V	VII	Carriage	0	−	−	+	+	+	−
Rotunda	820	1a	VII	Carriage	0	+	−	+	+	+	−
Coombe	C11	III	VII	Carriage	0	−	−	−	+	+	+

HOSPITAL	Isolate Name	SEROTYPE	RAPDnm TYPE	STATUS	SEPSIS	Genotype					
						bac	*bca*	*rib*	*pepB*	*hylB*	*hylIS*
Coombe	C7	III	VII	Carriage	0	+	+	−	+	+	+
Rotunda	8718	1b	VII	Invasive	2+	−	+	+	−	+	−
Holles	H4183	1b	VII	Invasive	2+	+	+	+	+	+	−
Rotunda	8099	III	VII	Invasive	2+	−	−	+	−	+	+
Holles	H4341	III	VII	Invasive	4+	−	+	+	+	+	+
Rotunda	5026	1b	VIII	Carriage	0	+	+	−	+	+	−
Rotunda	251	1a	VIII	Carriage	0	−	−	+	−	+	−
Coombe	C13	1a	VIII	Carriage	0	−	+	+	+	+	−
Rotunda	247	1b	VIII	Carriage	0	−	+	+	−	+	−
Coombe	C14	III	VIII	Carriage	0	+	−	+	+	+	+
Coombe	C16	III	VIII	Carriage	0	+	−	+	+	+	+
Coombe	C19	III	VIII	Carriage	0	−	+	+	+	+	+
Holles	H1059	III	VIII	Carriage	0	−	−	−	+	+	+
Rotunda	203	NT	VIII	Carriage	0	−	+	rib	+	+	−
Coombe	C23	NT	VIII	Carriage	0	+	+	+	+	+	−
Rotunda	270	II	IX	Carriage	0	−	−	−	+	+	−
Rotunda	1845	1a	IX	Carriage	0	−	−	+	+	+	−
Holles	H1356	1a	IX	Carriage	0	−	−	−	+	+	−
Rotunda	8161	1b	IX	Carriage	0	+	+	−	−	+	−
Rotunda	167	II	IX	Carriage	0	+	−	+	+	+	−
Rotunda	189	II	IX	Carriage	0	−	−	+	−	+	−
Rotunda	237	II	IX	Carriage	0	−	+	−	−	+	−
Rotunda	243	II	IX	Carriage	0	−	−	+	+	+	−
Holles	H1766	II	IX	Carriage	0	−	−	+	+	+	−
Rotunda	7106	III	IX	Carriage	0	−	−	+	+	+	+
Holles	H1870	III	IX	Carriage	0	−	−	+	+	+	+
Rotunda	222	IV	IX	Carriage	0	−	−	−	+	+	−
Rotunda	246	V	IX	Carriage	0	+	−	+	+	+	−
Rotunda	7450	V	IX	Carriage	0	−	−	+	−	+	−
Holles	H2234	V	IX	Carriage	0	−	−	+	+	+	−
Holles	H1645	1b	IX	Invasive	1+	+	+	+	+	+	−
Coombe	C28	II	IX	Invasive	1+	+	+	+	+	+	−
Holles	H823	III	IX	Invasive	1+	−	−	+	−	+	+
Rotunda	7552	IV	IX	Invasive	1+	+	+	−	−	+	−
Rotunda	279	1a	IX	Invasive	2+	+	+	−	+	+	−
Holles	H970	1b	IX	Invasive	4+	+	+	+	+	+	−
Rotunda	234	III	IX	Invasive	4+	−	−	−	+	+	+
Rotunda	B497	III	IX	Invasive	4+	−	−	+	+	+	+
Coombe	C29	NT	IX	Invasive	4+	−	+	+	+	+	−
Holles	1741	V	IX	Invasive	4+	−	−	rib	+	+	−
Rotunda	186	V	IX	Invasive	4+	−	+	−	−	+	−
Rotunda	2292	V	NC1	Invasive	2+	+	−	+	+	+	−
Rotunda	1795	III	NC2	Carriage	0	−	+	+	+	+	−
Holles	H1068	1a	NC3	Invasive	3+	−	−	−	−	+	−
Rotunda	170	1a	NC4	Invasive	4+	−	+	−	+	+	−
Holles	H2003	III	NC5	Invasive	4+	−	−	+	+	+	+
Holles	H1842	III	NC6	Invasive	4+	−	−	−	−	+	+
Holles	H708	V	NC7	Carriage	0	−	−	+	+	+	−
Rotunda	185	1a	NC8	Invasive	4+	−	+	−	−	+	−
Rotunda	183	III	NC9	Carriage	0	−	−	+	+	+	−
Rotunda	175	III	NC9	Invasive	1+	−	−	+	+	+	+

Table A2. RAPD1 types of all isolates with associated hospital, genotype, serotype, and sepsis status

HOSPITAL	Isolate Name	SEROTYPE	RAPD1 TYPE	STATUS	SEPSIS	Genotype bac	bca	rib	pepB	hylB	hylIS
Rotunda	8076	III	i	Carriage	0	−	+	−	−	+	+
Rotunda	222	IV	i	Carriage	0	−	−	−	+	+	−
Rotunda	246	V	i	Carriage	0	+	−	+	+	+	−
Rotunda	7450	V	i	Carriage	0	−	−	+	−	+	−
Rotunda	7470	V	i	Carriage	0	−	−	+	−	+	−
Rotunda	175	III	i	Invasive	1+	−	−	+	+	+	+
Rotunda	194	III	i	Invasive	1+	+	−	+	+	+	+
Holles	H1356	1a	ii	Carriage	0	−	−	−	+	+	−
Rotunda	258	1b	ii	Invasive	1+	+	+	−	+	+	−
Holles	H1646	1b	ii	Invasive	1+	+	+	+	+	+	−
Rotunda	204	III	ii	Invasive	1+	−	−	+	+	+	+
Rotunda	5026	1b	iii	Carriage	0	+	+	−	+	+	−
Holles	H1692	III	iii	Carriage	0	+	+	+	+	+	+
Rotunda	200	1a	iii	Carriage	0	−	+	−	+	+	−
Coombe	C5	1a	iii	Carriage	0	+	+	+	+	+	−
Coombe	C6	1a	iii	Carriage	0	+	+	+	+	+	−
Holles	H1999	1a	iii	Carriage	0	−	+	+	+	+	−
Holles	H777	1a	iii	Carriage	0	−	−	−	+	+	−
Rotunda	202	1b	iii	Carriage	0	−	+	+	+	+	−
Rotunda	235	1b	iii	Carriage	0	−	+	−	+	+	−
Rotunda	8161	1b	iii	Carriage	0	+	+	−	−	+	−
Rotunda	167	II	iii	Carriage	0	+	−	+	+	+	−
Rotunda	243	II	iii	Carriage	0	−	−	+	+	+	−
Rotunda	254	II	iii	Carriage	0	−	+	−	+	+	−
Holles	H1766	II	iii	Carriage	0	−	−	−	+	+	−
Holles	H1963	II	iii	Carriage	0	+	+	+	+	+	−
Holles	H1997	II	iii	Carriage	0	+	+	+	+	+	−
Coombe	C3	III	iii	Carriage	0	+	+	+	+	+	−
Holles	H1969	III	iii	Carriage	0	−	+	+	+	−	−
Coombe	C24	NT	iii	Carriage	0	+	+	−	+	+	−
Rotunda	184	V	iii	Carriage	0	+	+	−	+	+	−
Rotunda	187	V	iii	Carriage	0	+	+	+	+	+	−
Coombe	C2	V	iii	Carriage	0	−	−	−	+	+	−
Holles	H701	V	iii	Carriage	0	−	−	+	+	+	−
Coombe	C28	II	iii	Invasive	1+	+	+	+	+	+	−
Coombe	C8	V	iii	Invasive	1+	−	+	+	+	+	−
Coombe	C26	1a	iii	Invasive	2+	−	+	+	+	+	−
Rotunda	5002	1b	iii	Invasive	2+	−	+	+	+	+	−
Holles	H1066	1b	iii	Invasive	2+	+	+	−	+	+	−
Holles	H4183	1b	iii	Invasive	2+	+	+	+	+	+	−
Holles	H2000	II	iii	Invasive	2+	+	−	−	+	+	−
Holles	H1068	1a	iii	Invasive	3+	−	−	−	−	+	−
Rotunda	273	1a	iii	Invasive	4+	−	+	−	−	+	−
Holles	H896	1a	iii	Invasive	4+	−	−	−	−	+	−
Holles	McCann	1a	iii	Invasive	4+	−	−	+	+	+	−
Rotunda	220	1b	iii	Invasive	4+	+	−	+	+	+	−
Rotunda	226	1b	iii	Invasive	4+	+	−	−	−	+	−
Rotunda	229	1b	iii	Invasive	4+	+	+	−	+	+	−
Rotunda	35	1b	iii	Invasive	4+	+	+	−	+	+	−
Holles	H901	1b	iii	Invasive	4+	+	+	−	+	+	−
Holles	H913	1b	iii	Invasive	4+	+	+	+	+	+	−
Holles	H970	1b	iii	Invasive	4+	+	+	+	+	+	−
Rotunda	233	II	iii	Invasive	4+	−	+	+	−	+	−
Rotunda	207	III	iii	Invasive	4+	−	−	+	+	+	−
Rotunda	208	III	iii	Invasive	4+	−	−	+	−	+	+

HOSPITAL	Isolate Name	SEROTYPE	RAPD1 TYPE	STATUS	SEPSIS	Genotype					
						bac	*bca*	*rib*	*pepB*	*hylB*	*hylIS*
Rotunda	B497	III	iii	Invasive	4+	−	−	+	+	+	+
Holles	H2003	III	iii	Invasive	4+	−	−	+	+	+	+
Holles	H4341	III	iii	Invasive	4+	−	+	+	+	+	+
Rotunda	W2444	III	iii	Invasive	4+	−	−	+	+	+	+
Rotunda	188	NT	iii	Invasive	4+	+	+	−	+	+	−
Holles	1741	V	iii	Invasive	4+	−	−	+	+	+	−
Rotunda	186	V	iii	Invasive	4+	−	+	−	−	+	−
Rotunda	270	II	iv	Carriage	0	−	−	−	+	+	−
Rotunda	272	IV	iv	Carriage	0	−	−	−	+	+	−
Rotunda	183	III	iv	Carriage	0	−	−	+	+	+	−
Rotunda	2524	III	iv	Carriage	0	+	−	+	+	+	+
Rotunda	7763	III	iv	Carriage	0	−	+	+	−	+	+
Rotunda	205	1b	iv	Invasive	1+	+	−	+	−	+	−
Rotunda	206	1b	iv	Invasive	1+	+	+	−	−	+	−
Rotunda	227	1b	iv	Invasive	1+	+	+	−	+	+	−
Rotunda	259	1b	iv	Invasive	1+	−	+	−	+	+	−
Holles	H1645	1b	iv	Invasive	1+	+	+	+	+	+	−
Holles	H823	III	iv	Invasive	1+	−	−	+	−	+	+
Rotunda	181	1a	iv	Invasive	2+	+	+	−	+	+	−
Coombe	C29	NT	iv	Invasive	4+	−	+	+	+	+	−
Rotunda	1135	1b	v	Carriage	0	+	+	+	+	+	−
Rotunda	5057	1b	v	Carriage	0	−	+	+	+	+	−
Rotunda	1630	II	v	Carriage	0	+	+	+	+	+	−
Rotunda	8429	II	v	Carriage	0	+	+	+	+	+	−
Rotunda	1795	III	v	Carriage	0	−	+	+	+	+	−
Rotunda	299	III	v	Carriage	0	−	+	−	+	+	+
Rotunda	1588	III	v	Carriage	0	−	+	+	+	+	+
Rotunda	5452	III	v	Carriage	0	+	−	+	+	+	+
Rotunda	320	V	v	Carriage	0	−	−	+	+	+	−
Rotunda	8132	V	v	Carriage	0	−	+	+	+	+	−
Rotunda	8600	V	v	Carriage	0	−	−	+	+	+	−
Holles	H2234	V	v	Carriage	0	−	−	+	+	+	−
Rotunda	1813	1a	v	Invasive	2+	+	+	+	+	+	−
Rotunda	4532	1b	v	Invasive	2+	+	+	+	+	+	−
Rotunda	8718	1b	v	Invasive	2+	−	+	+	−	+	−
Rotunda	3656	III	v	Invasive	2+	+	+	+	+	+	+
Rotunda	5036	III	v	Invasive	2+	+	−	+	+	+	+
Rotunda	8099	III	v	Invasive	2+	−	−	+	−	+	+
Rotunda	3042	III	v	Invasive	4+	−	−	+	+	+	+
Rotunda	8857	II	vi	Carriage	0	+	+	*rib*	*pepB*	*hylB*	−
Rotunda	820	1a	vi	Carriage	0	+	−	+	+	+	−
Rotunda	1845	1a	vi	Carriage	0	−	−	+	+	+	−
Rotunda	1929	1a	vi	Carriage	0	+	+	+	+	+	−
Rotunda	251	1a	vi	Carriage	0	−	−	+	−	+	−
Rotunda	247	1b	vi	Carriage	0	−	+	+	−	+	−
Coombe	C27	1b	vi	Carriage	0	−	+	−	+	+	−
Rotunda	237	II	vi	Carriage	0	−	+	−	−	+	−
Rotunda	250	III	vi	Carriage	0	+	+	+	+	+	−
Coombe	C22	III	vi	Carriage	0	+	−	+	+	+	−
Rotunda	173	III	vi	Carriage	0	−	−	+	+	+	+
Rotunda	2011	III	vi	Carriage	0	+	−	+	+	+	+
Rotunda	255	III	vi	Carriage	0	−	−	−	−	+	+
Rotunda	278	III	vi	Carriage	0	−	−	+	+	+	+
Coombe	C19	III	vi	Carriage	0	−	+	+	+	+	+
Holles	H524	III	vi	Carriage	0	−	+	+	+	+	+
Rotunda	203	NT	vi	Carriage	0	−	+	+	+	+	−

Table A3. PFGE types of all isolates with associated hospital, genotype, serotype, and sepsis status.

HOSPITAL	Isolate Name	SEROTYPE	PFGE TYPE	STATUS	SEPSIS	bac	bca	rib	pepB	hylB	hylIS
Rotunda	8600	V	A	Carriage	0	−	−	+	+	+	−
Coombe	C27	1b	A	Carriage	0	−	+	−	+	+	−
Rotunda	183	III	A	Carriage	0	−	−	+	+	+	−
Rotunda	203	NT	A	Carriage	0	−	+	+	+	+	−
Rotunda	184	V	A	Carriage	0	+	+	−	+	+	−
Rotunda	7368	V	A	Carriage	0	−	+	−	+	+	−
Coombe	C2	V	A	Carriage	0	−	−	−	+	+	−
Rotunda	175	III	A	Invasive	1+	−	−	+	+	+	+
Rotunda	204	III	A	Invasive	1+	−	−	+	+	+	+
Coombe	C8	V	A	Invasive	1+	−	+	+	+	+	−
Holles	H1635	V	A	Invasive	1+	−	−	+	+	+	−
Holles	H1666	1a	A	Invasive	3+	−	−	+	+	+	−
Rotunda	226	1b	A	Invasive	4+	+	−	−	−	+	−
Rotunda	229	1b	A	Invasive	4+	+	+	−	−	+	−
Rotunda	3042	III	A	Invasive	4+	−	−	+	+	+	+
Rotunda	8429	II	B	Carriage	0	+	+	+	−	+	−
Rotunda	1795	III	B	Carriage	0	−	+	+	+	+	−
Rotunda	200	1a	B	Carriage	0	−	+	−	+	+	−
Rotunda	167	II	B	Carriage	0	+	−	+	+	+	−
Rotunda	189	II	B	Carriage	0	−	−	+	−	+	−
Rotunda	173	III	B	Carriage	0	−	−	+	+	+	+
Rotunda	278	III	B	Carriage	0	−	−	+	+	+	+
Rotunda	187	V	B	Carriage	0	+	+	+	+	+	−
Rotunda	181	1a	B	Invasive	2+	+	+	−	+	+	−
Holles	H1502	1a	B	Invasive	2+	−	+	+	+	+	−
Holles	H1338	1a	B	Invasive	3+	−	−	−	+	+	−
Holles	H2003	III	B	Invasive	4+	−	−	+	+	+	+
Rotunda	W2444	III	B	Invasive	4+	−	−	+	+	+	+
Rotunda	820	1a	C	Carriage	0	+	−	+	+	+	−
Holles	H964	II	C	Invasive	4+	−	−	−	+	+	−
Rotunda	208	III	C	Invasive	4+	−	−	+	−	+	+
Rotunda	1135	1b	D	Carriage	0	+	+	+	+	+	−
Rotunda	8857	II	D	Carriage	0	+	+	+	+	+	−
Rotunda	5452	III	D	Carriage	0	+	−	+	+	+	+
Rotunda	2186	1a	D	Carriage	0	+	−	+	+	+	−
Coombe	C13	1a	D	Carriage	0	−	+	+	+	+	−
Coombe	C5	1a	D	Carriage	0	+	+	+	+	+	−
Coombe	C6	1a	D	Carriage	0	+	+	+	+	+	−
Holles	H1240	1a	D	Carriage	0	−	−	+	+	+	−
Holles	H1356	1a	D	Carriage	0	−	−	−	+	+	−
Holles	H1999	1a	D	Carriage	0	−	+	+	+	+	−
Rotunda	202	1b	D	Carriage	0	−	+	+	+	+	−
Rotunda	235	1b	D	Carriage	0	−	+	−	+	+	−
Rotunda	8161	1b	D	Carriage	0	+	+	−	−	+	−
Rotunda	2011	III	D	Carriage	0	+	−	+	+	+	+
Holles	H524	III	D	Carriage	0	−	+	+	+	+	+
Rotunda	222	IV	D	Carriage	0	−	−	−	+	+	−
Coombe	C12	NT	D	Carriage	0	−	+	+	+	+	−
Rotunda	194	III	D	Invasive	1+	+	−	+	+	+	+
Holles	H823	III	D	Invasive	1+	−	−	+	−	+	+
Rotunda	7552	IV	D	Invasive	1+	+	+	−	−	+	−
Rotunda	1813	1a	D	Invasive	2+	+	+	+	+	+	−
Rotunda	279	1a	D	Invasive	2+	+	+	−	+	+	−
Rotunda	2292	V	D	Invasive	2+	+	−	+	+	+	−
Holles	H852	V	D	Invasive	2+	+	−	+	+	+	−

HOSPITAL	Isolate Name	SEROTYPE	PFGE TYPE	STATUS	SEPSIS	Genotype					
						bac	bca	rib	pepB	hylB	hylIS
Holles	H1234	1a	D	Invasive	4+	−	−	−	+	+	−
Rotunda	215	1b	D	Invasive	4+	−	+	+	+	+	−
Rotunda	219	1b	D	Invasive	4+	+	−	+	+	+	−
Rotunda	230	1b	D	Invasive	4+	+	+	+	−	+	−
Rotunda	233	II	D	Invasive	4+	−	+	+	−	+	−
Rotunda	207	III	D	Invasive	4+	−	−	+	+	+	−
Rotunda	B497	III	D	Invasive	4+	−	−	+	+	+	+
Rotunda	5026	1b	E	Carriage	0	+	+	−	+	+	−
Rotunda	5057	1b	E	Carriage	0	−	+	+	+	+	−
Holles	H1692	III	E	Carriage	0	+	+	+	+	+	+
Rotunda	272	IV	E	Carriage	0	−	−	−	+	+	−
Holles	H703	1a	E	Carriage	0	−	+	−	−	+	−
Holles	H777	1a	E	Carriage	0	−	−	−	+	+	−
Coombe	C3	III	E	Carriage	0	+	+	+	+	+	−
Rotunda	5002	1b	E	Invasive	2+	−	+	+	+	+	−
Holles	H2000	II	E	Invasive	2+	+	−	−	+	+	−
Rotunda	5036	III	E	Invasive	2+	+	−	+	+	+	+
Holles	H901	1b	E	Invasive	4+	+	+	−	+	+	−
Holles	H913	1b	E	Invasive	4+	+	+	+	+	+	−
Holles	H970	1b	E	Invasive	4+	+	+	+	+	+	−
Rotunda	270	II	F	Carriage	0	−	−	−	+	+	−
Rotunda	1630	II	F	Carriage	0	+	+	+	+	+	−
Rotunda	299	III	F	Carriage	0	−	+	−	+	+	+
Rotunda	1588	III	F	Carriage	0	−	+	−	+	+	+
Rotunda	320	V	F	Carriage	0	−	−	+	+	+	−
Rotunda	8132	V	F	Carriage	0	−	+	+	+	+	−
Rotunda	7543	1a	F	Carriage	0	+	−	+	+	+	−
Rotunda	237	II	F	Carriage	0	−	+	−	−	+	−
Rotunda	7105	III	F	Carriage	0	−	−	+	+	+	+
Rotunda	8076	III	F	Carriage	0	−	+	−	−	+	+
Coombe	C11	III	F	Carriage	0	−	−	−	+	+	+
Coombe	C14	III	F	Carriage	0	+	−	+	+	+	+
Coombe	C16	III	F	Carriage	0	+	−	+	+	+	+
Coombe	C19	III	F	Carriage	0	−	+	+	+	+	+
Coombe	C22	III	F	Carriage	0	+	−	+	+	+	−
Coombe	C7	III	F	Carriage	0	+	+	−	+	+	+
Holles	H1059	III	F	Carriage	0	−	−	−	+	+	+
Holles	H1712	III	F	Carriage	0	−	−	+	+	+	+
Holles	H1870	III	F	Carriage	0	−	−	+	+	+	+
Holles	H1969	III	F	Carriage	0	−	+	+	+	−	−
Holles	H887	III	F	Carriage	0	−	−	+	+	+	+
Coombe	C24	NT	F	Carriage	0	+	+	−	+	+	−
Holles	H1516	V	F	Carriage	0	−	−	+	+	+	−
Holles	H2234	V	F	Carriage	0	−	−	+	+	+	−
Holles	H708	V	F	Carriage	0	−	−	+	+	+	−
Rotunda	205	1b	F	Invasive	1+	+	−	+	−	+	−
Rotunda	206	1b	F	Invasive	1+	+	+	−	−	+	−
Coombe	C28	II	F	Invasive	1+	+	+	+	+	+	−
Rotunda	4532	1b	F	Invasive	2+	+	+	+	+	+	−
Holles	H1066	1b	F	Invasive	2+	+	+	−	+	+	−
Holles	H4183	1b	F	Invasive	2+	+	+	+	+	+	−
Rotunda	3656	III	F	Invasive	2+	+	+	+	+	+	+
Rotunda	8099	III	F	Invasive	2+	−	−	+	−	+	+
Holles	H1842	III	F	Invasive	4+	−	−	−	−	+	+
Holles	H4341	III	F	Invasive	4+	−	+	+	+	+	+
Coombe	C29	NT	F	Invasive	4+	−	+	+	+	+	−

HOSPITAL	Isolate Name	SEROTYPE	PFGE TYPE	STATUS	SEPSIS	Genotype					
						bac	*bca*	*rib*	*pepB*	*hylB*	*hylIS*
Holles	1741	V	F	Invasive	4+	−	−	+	+	+	−
Rotunda	186	V	F	Invasive	4+	−	+	−	−	+	−
Holles	H1741	V	F	Invasive	4+	−	−	+	+	+	−
Rotunda	1845	1a	G	Carriage	0	−	−	+	+	+	−
Rotunda	1929	1a	G	Carriage	0	+	+	+	+	+	−
Coombe	C10	II	G	Carriage	0	−	−	+	+	+	+
Rotunda	1744	V	G	Invasive	1+	−	−	+	−	+	−
Holles	H578	III	G	Invasive	2+	−	−	+	+	+	+
Holles	H1711	V	G	Invasive	2+	−	−	+	+	+	+
Rotunda	170	1a	G	Invasive	4+	−	+	−	+	+	−
Rotunda	185	1a	G	Invasive	4+	−	+	−	−	+	−
Rotunda	35	1b	G	Invasive	4+	+	+	−	+	+	−
Rotunda	251	1a	H	Carriage	0	−	−	+	−	+	−
Rotunda	247	1b	H	Carriage	0	−	+	+	−	+	−
Rotunda	243	II	H	Carriage	0	−	−	+	+	+	−
Rotunda	254	II	H	Carriage	0	−	+	−	+	+	−
Holles	H1766	II	H	Carriage	0	−	−	−	+	+	−
Holles	H1963	II	H	Carriage	0	+	+	+	+	+	−
Holles	H1997	II	H	Carriage	0	+	+	+	+	+	−
Rotunda	2008	III	H	Carriage	0	+	−	+	+	+	+
Rotunda	250	III	H	Carriage	0	+	+	+	+	+	−
Rotunda	255	III	H	Carriage	0	−	−	−	−	+	+
Rotunda	7106	III	H	Carriage	0	−	−	+	−	+	+
Rotunda	7763	III	H	Carriage	0	−	+	+	−	+	+
Rotunda	246	V	H	Carriage	0	+	−	+	+	+	−
Rotunda	242	1a	H	Invasive	1+	−	+	−	+	+	−
Rotunda	227	1b	H	Invasive	1+	+	+	−	+	+	−
Rotunda	258	1b	H	Invasive	1+	+	+	−	+	+	−
Rotunda	259	1b	H	Invasive	1+	−	+	−	+	+	−
Holles	H1646	1b	H	Invasive	1+	+	+	+	+	+	−
Rotunda	248	V	H	Invasive	1+	−	+	+	+	+	−
Coombe	C26	1a	H	Invasive	2+	−	+	+	+	+	−
Rotunda	8718	1b	H	Invasive	2+	−	+	+	−	+	−
Holles	H1068	1a	H	Invasive	3+	−	−	−	−	+	−
Rotunda	245	1a	H	Invasive	4+	−	+	+	+	+	−
Holles	H896	1a	H	Invasive	4+	−	−	−	+	+	−
Holles	McCann	1a	H	Invasive	4+	−	−	+	+	+	−
Rotunda	220	1b	H	Invasive	4+	+	−	+	+	+	−
Rotunda	234	III	H	Invasive	4+	−	−	−	+	+	+
Rotunda	188	NT	H	Invasive	4+	+	+	−	+	+	−
Rotunda	273	1a	NC1	Invasive	4+	−	+	−	−	+	−
Holles	H1645	1b	NC2	Invasive	1+	+	+	+	+	+	−
Rotunda	7470	V	NC3	Carriage	0	−	−	+	−	+	−
Holles	H701	V	NC3	Carriage	0	−	−	+	+	+	−
Rotunda	2524	III	ND	Carriage	0	+	−	+	+	+	+
Rotunda	271	NT	ND	Carriage	0	−	+	−	−	+	−
Coombe	C23	NT	ND	Carriage	0	+	+	+	+	+	−
Rotunda	7450	V	ND	Carriage	0	−	−	+	−	+	−

General Discussion

The bacterium *Streptococcus agalactiae*, better known as Group B streptococcus, has a worldwide geographical distribution and is the leading cause of meningitis, pneumonia and sepsis in neonates. GBS also causes serious infections in pregnant women and in immunocompromised adults. The considerable morbidity and mortality associated with GBS disease has prompted prevention strategies. Although the incidence of early onset GBS infection was already falling in the early 1990's, a further reduction in morbidity and mortality due to GBS is evident since the consensus prevention guidelines were published (Fig. 6.1). Between 1993 and 1998, the incidence of early-onset GBS disease declined by 65% in the United States. Factor *et al.* (2000) investigated the role of prevention policies in the decline of disease occurrence. Hospitals with the new prevention policy or a revised old one experienced a decline in cases of more than 50% between 1996 and 1997 ($P <$ 0.006) while those with no prevention policy had no significant decline ($P = 0.26$). Prevention protocols appear to have had no impact on late-onset disease (Fig. 6.1).

With the increasing pressures for reductions in the use of antimicrobial agents in animal and humans due to the increases of drug resistance, the necessity for improved understanding of the epidemiology of GBS has become apparent. For penicillin-allergic patients, susceptibility testing of GBS isolates is appropriate as resistance to erythromycin and clindamycin is common (Berkowitz *et al.*, 1990; Pearlman *et al.*, 1998). Further research is needed to determine convenient phenotypic or genotypic criteria for identifying virulent clone lineages among strains present in the genital tract. This would be valuable for obstetricians and could help them make early and accurate preventive decisions during deliveries with a high risk of infection. Also, for these reasons GBS remains an important target for vaccine development. This study sought to examine the epidemiology of GBS in Ireland by evaluating a range of genotypic typing methods and serotyping (Fig. 6.2).

Presently, development of the GBS vaccine is based on a few key types of capsular polysaccharides that are conjugated either to a tetanus toxoid or group B streptococcal proteins (Kasper *et al.*, 1996). A GBS vaccine for use in Ireland would currently include types Ia, Ib, II, III and V which would provide coverage against 93.7% of the isolates. However, the serotype distribution would need to be continually monitored to detect the

Figure 6.1. Invasive Group B Streptococcal disease in infants less than 1 week of age per 1,000 live births, Active Bacterial Core surveillance, 1993–1998 (Schuchat *et al.*, 2001).
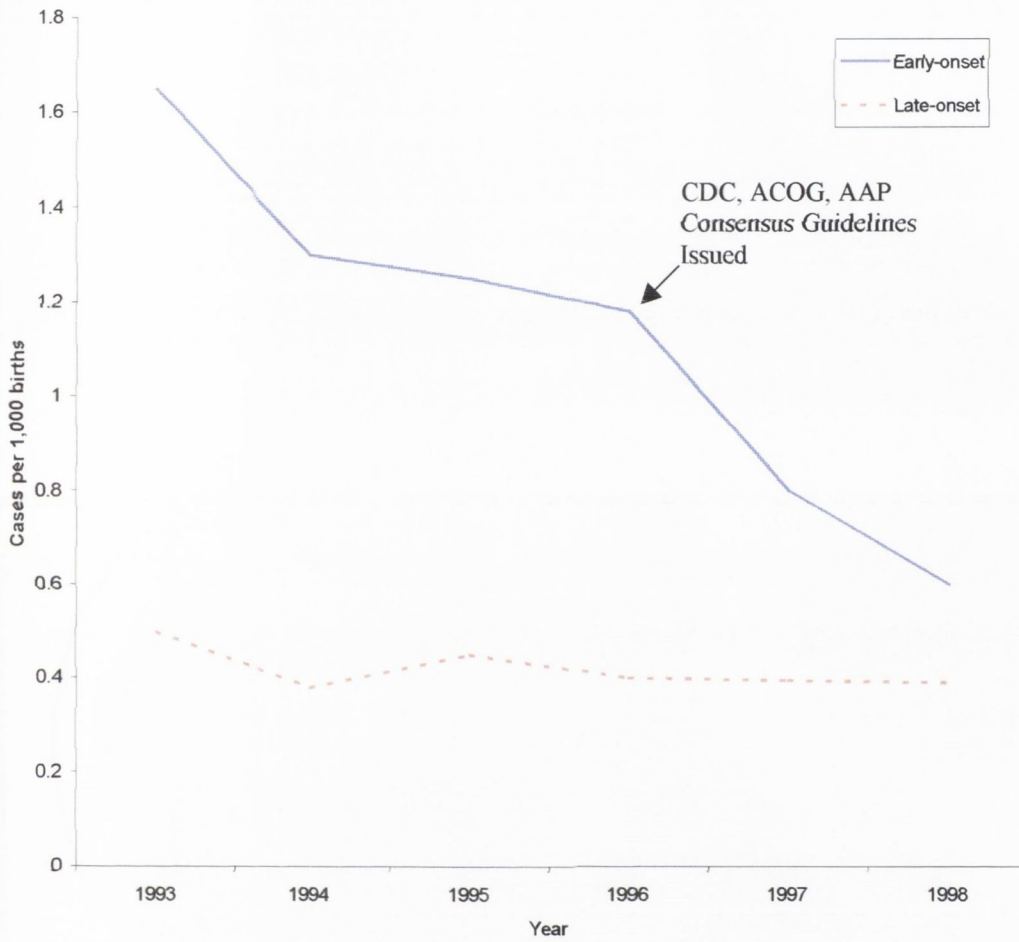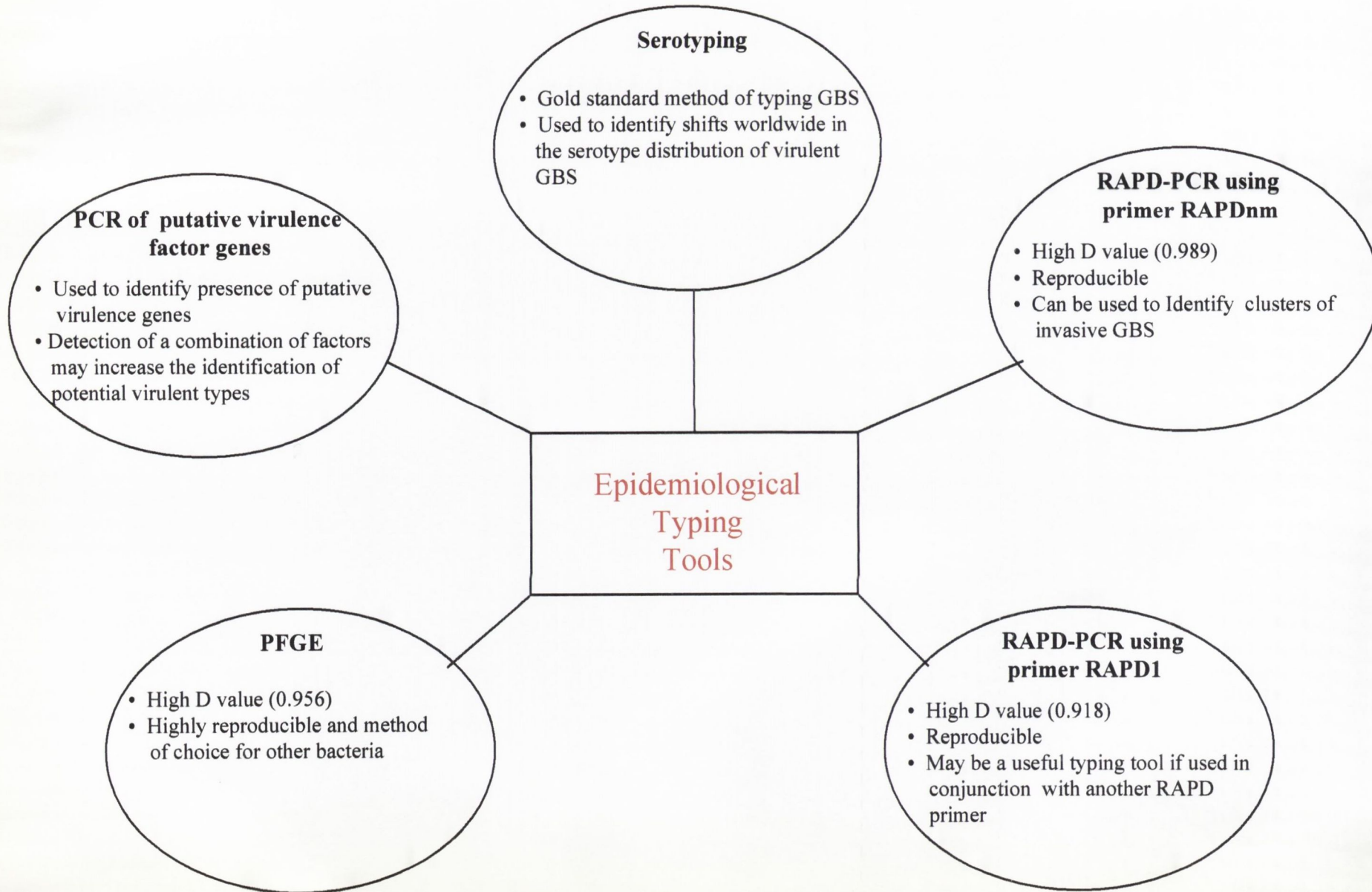
Figure 6.2. Schematic diagram of the epidemiological tools assessed in this study, with their main attributes listed in each case.

possible occurrence of the newer serotypes VI, VII and VIII and the effectiveness of future capsule-based vaccines.

Bacteriophage typing is an epidemiological method that has been used to help elucidate the epidemiology and pathogenisis of GBS, but it is cumbersome, requires specific reagents, and yields inconsistent results (Noya *et al.*, 1987). Moreover, ~30% of isolates are not typeable by the bacteriophage typing sets available (Colman, 1988). Plasmid analysis has been used successfully for this purpose in other organisms, but plasmids are uncommon in GBS.

Previous investigators examining GBS isolates of human origin have suggested that RAPD is superior to serotyping for epidemiological evaluations of this organsm. Results showed genetic heterogeneity not only between different serotypes but also among isolates belonging to same serotype (Chatellier *et al.*, 1997, Limansky *et al.*, 1998 and Martinez *et al.*, 2000). The present findings suggest that RAPD fingerprinting is more accurate than capsular serotype in differentiating invasive and carriage GBS isolates, especially using the RAPDnm primer which has a D value of 0.989 compared to 0.87 obtained with serotyping. PFGE, not unlike RAPD, had a high D value (0.956) but was not as beneficial in differentiating invasive and carriage Irish GBS isolates. The extensive genetic diversity among the 159 isolates has important implications for studies of the basic biology and epidemiology of this organism. Comparison of genetic patterns from isolates in other demographic regions could prove beneficial in determining a worldwide evolutionary genetic framework. Further analysis of RAPD profiles to elucidate whether particular fingerprint band patterns are common to invasive strains may allow the identification of novel virulence genes.
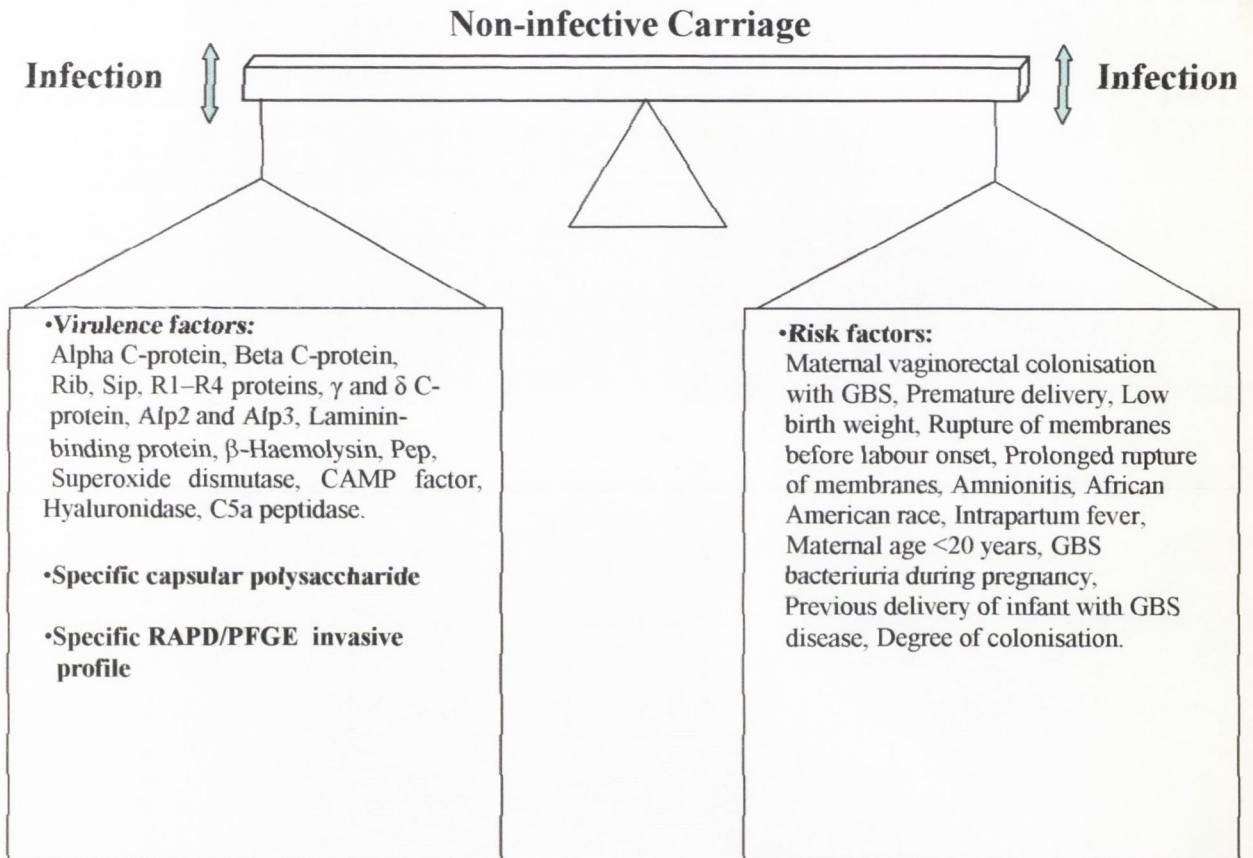
The main objectives of the virulence factor study were to analyse the potential relationships between genes encoding putative virulence factors, either alone or together, with serotype and to analyse potential relationships between genes encoding putative virulence factors and the invasive status of isolates. Genotypic findings from PCR analysis of virulence factors suggest that individual gene markers are not necessarily good predictors of invasive capacity (Chun et al., 1991) while detection of several factors increases the identification of potential virulent types. This present analysis indicated a

three-way interaction between the presence or absence of the *bac, bca* and *rib* genes. These three genes were found to be present or absent in specific patterns in some serotypes. Study of the hyaluronate lyase gene has led to speculation that alleles of this gene may be associated with virulence in GBS. An insertion element (IS*1548*) has been identified in the *hylB* gene and this *hylB* (IS*1548*) gene was associated with strains isolated from patients with endocarditis (Granlund *et al.*, 1998). Five different IS elements have been identified in GBS (Section 5.4) and multiple copies of IS*1548* elements have been identified throughout the GBS genome. These insertion sequences, which disrupt genes and may contribute to some phenotypic variation, may also introduce new restriction sites that may help in genotypic analysis of virulent GBS.

GBS colonises a substantial proportion of the Irish women population, many of which carry the organism without any causitive role in disease. It is clear from the present study that the pathogenicity of GBS strains is multifactorial or that some as yet undiscovered virulence factor may be important. However, defining factors that influence vertical transmission of and neonatal colonisation with GBS in neonates with predisposing risk factors may be as crucial for GBS infection (Fig. 6.3). One of the most prominent feature of GBS infections is the pronounced affect of age on incidence, i.e., neonates and adults >65 years old. The importance of GBS disease in adults is not as widely acknowledged as in neonates but further study could prove to be beneficial in identifying modes of GBS acquisition, transmission and pathogenesis. At present, little is known about the immunology of GBS disease among the elderly and whether GBS disease may be due to low levels of antibody production or a deficient cell-mediated response.

Data on associations of age and race with GBS carriage may be important but may only have local significance. Also it may be difficult to separate these from other socioeconomic factors. The incidence of GBS disease in African Americans is substantially greater than in other racial groups but this may be related to a higher prevalence of risk factors among this race such as increased GBS carriage, low birth weight and prematurity (Newton *et al.*, 1996).

Figure 6.3. Proposed maternal and bacterial factors for increased risk of GBS disease.

Non-infective Carriage

Infection

Infection

•*Virulence factors:*
Alpha C-protein, Beta C-protein, Rib, Sip, R1–R4 proteins, γ and δ C-protein, Alp2 and Alp3, Laminin-binding protein, β-Haemolysin, Pep, Superoxide dismutase, CAMP factor, Hyaluronidase, C5a peptidase.

•**Specific capsular polysaccharide**

•**Specific RAPD/PFGE invasive profile**

•**Risk factors:**
Maternal vaginorectal colonisation with GBS, Premature delivery, Low birth weight, Rupture of membranes before labour onset, Prolonged rupture of membranes, Amnionitis, African American race, Intrapartum fever, Maternal age <20 years, GBS bacteriuria during pregnancy, Previous delivery of infant with GBS disease, Degree of colonisation.

In conclusion, the characteristics of the Irish GBS population investigated herein argue for the existence of particular groups of strains responsible for invasive GBS and demonstrate that serotyping and RAPD-PCR can supply information about the genetic makeup of these strains. These data will be of considerable importance in assessing evolving patterns and disease trends in relation to the Irish GBS isolates and GBS isolated in other countries.

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180

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190

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