Chapter 8
Streptococci

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8.1 Introduction

Streptococci are a heterogeneous group of bacteria consisting of more than 50 species. The genus is comprised of a wide variety of both pathogenic and commensal Gram-positive bacteria which are found to inhabit a wide range of hosts, including, but not limited to, humans, horses, pigs, dogs, fish, horses, and cows. Within the host, streptococci often colonize the mucosal surfaces of the mouth, upper respiratory tract, alimentary tract, and genitourinary tract. In certain circumstances, they may also inhabit the skin, heart or muscle tissue. Many streptococci are known to cause human disease, some species being highly virulent and responsible for major diseases. *Streptococcus pyogenes*, *S. pneumoniae*, and *S. agalactiae* are particularly notable as causes of serious infections in man. In recent years, increasing attention has been given to epidemiologic significance of streptococcal species other than the "big three." For example, recently it has been observed in a population-based study that the invasive disease burden attributable to beta-hemolytic *S. dysgalactiae* subsp. *equisimilis* approximated that of *S. pyogenes* [1]. Also, the importance of the viridans streptococcal species is increasingly highlighted in various disease manifestations (dental caries, bacteremia, meningitis, periodontal disease, suppurative infections, pneumonia [2]). *S. anginosus* is also increasingly associated with suppurative infections in children and adults [2]. *S. suis* commonly found as a pathogen in pigs, has become increasingly noted as a cause of severe systemic infections (meningitis and sepsis) in humans [3]. *Streptococcus salivarius* is among the most common normal flora of the mouth and has been increasingly associated with iatrogenic meningitis associated with lumbar puncture [4].

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8.2 Classification of Streptococci

The system for classifying streptococci into major categories was introduced at the beginning of the twentieth century and is based on a series of characteristics of the organisms: (a) colony morphology and hemolytic reactions on blood agar, (b) serologic specificity of the cell wall group-specific substance and other cell wall or capsular antigens, (c) biochemical reactions and resistance to physical and chemical factors, and (d) ecologic features. More recently, molecular genetics have also been used to study the streptococci. Combinations of the above methods have permitted the classification of streptococci for purposes of clinical and epidemiologic convenience, but as knowledge has evolved, new methods have been introduced with the result that several classification systems have been described. In some cases, different species names have been used to describe the same organisms; in other instances, some members of the same species have been included in another species or classified separately. The genera *Enterococcus* and *Lactococcus*, for example, now include some species previously classified as group D and group N streptococci, respectively [5]. In spite of these exceptions to the traditional rules of streptococcal taxonomy, hemolysis and serologic tests can still be used to divide the streptococci into broad categories as a first step in identification and typing of clinical isolates.

8.2.1 Hemolysis on Blood

The type of hemolytic reaction displayed on blood agar has long been used to classify the streptococci. Streptococci are designated as exhibiting either alpha, beta or gamma hemolytic activity. β-hemolysis is associated with complete lysis of red cells surrounding the colony, whereas α-hemolysis is a partial or "green" hemolysis associated with reduction of red cell hemoglobin. Nonhemolytic colonies have been termed γ-hemolytic. Hemolysis is affected by the species and age of red cells, as well as by other properties of the base medium. Group A streptococci (almost always *S. pyogenes*) are nearly always beta-hemolytic; Group B streptococci (*S. agalactiae*) are normally beta-hemolytic but can also manifest alpha or gamma hemolysis. *S. pneumoniae* are normally alpha-hemolytic but can cause β-hemolysis during anaerobic incubation. Most of the oral streptococci are either alpha-hemolytic or nonhemolytic. While the property of hemolysis is not entirely reliable as a streptococcal species marker, it is nonetheless essential for routine rapid screens used for the identification of *S. pyogenes*, *S. pneumoniae* and *S. agalactiae.*

8.2.2 Antigenic Types

The β-hemolytic streptococci are further classified on the basis of a scheme developed by Rebecca Lancefield (1895–1981) that tests the serologic reactivity of carbohydrate antigens (C substance) derived from acid extraction of the bacterial

cell walls [6]. Recognized serogroups are given letter designations from A–H to K–V. Some group antigens are shared by multiple species; however, in general, only a single pathogenic species each comprises groups A (*S. pyogenes*) and B (*S. agalactiae*). Other streptococci with pathogenic potential are found within the β-hemolytic strains of groups C, F and G (*S. equisimilis or S. anginosus*), but only rarely is disease associated with group D (*S. bovis*, *S. durans*, *or S. avium*) or other Lancefield groups. *S. pneumoniae* lacks a group specific antigen and likewise, no group antigen is present in the various viridans streptococcal species (e.g., *S. mutans*, *S. sanguis*, *S. salivarius*, and *S. milleri*). A reliable summary of group antigen associations, biochemical associations, and nomenclature changes within the genus has been published [7].

8.2.3 Molecular Tools

The introduction of DNA-based approaches during the 1960s heralded a new era when genotypic studies combined with chemotaxonomic data allowed major developments to be made in the classification of bacteria in general, and of Gram-positive cocci in particular. One of the most useful tools applied to the revision of the classification system for the *Streptococcus* genus is the application of 16S rRNA gene sequencing [8].

8.2.4 Shared Features in Typing Schemes for the Three Major Streptococcal Pathogens

For the 3 major streptococcal pathogens discussed below, there is a common theme in that for each there is a major surface virulence factor that is expressed as one of many different antigenic types. This is particularly true for pneumococci and GAS that express more than 90 antigenically distinct polysaccharide capsules and more than 100 different M virulence proteins, respectively. In comparison, GBS is known to express a relatively modest array of 9 or 10 different capsular polysaccharides. The pneumococcal capsular polysaccharides have long been utilized in successful multivalent vaccines, while these GAS and GBS surface molecules have been long assessed as vaccine candidates. For each of these three pathogens there has been a long-standing serologic typing scheme of the antigenic types of these major surface virulence factors, and for each there have been DNA-based serotype-deduction methods introduced that suitably substitute for serologic typing. Additionally, for each of these organisms a multilocus sequence typing (MLST) scheme has been developed that determines clonal type. The two approaches (deduction of serologic types and MLST) have revealed that there are strong associations of serological types with given MLST types (STs), and that all three of these species undergo a great deal of intraspecies genetic exchange. For the purposes of epidemiologic typing of large sets of isolates, we have found that deduction of the combination of primary surface antigen serotype and ST serves us best.

MLST is highly discriminating and well suited towards identifying clusters of isolates with identical or closely related genotypes within the three different species. MLST, which employs seven genomically unlinked housekeeping loci [9–11], is highly suitable for the epidemiological analysis of bacterial isolates since it provides results that are portable and available through Internet databases (see www.mlst.net). These databases currently contain more than 6,700 pneumococcal STs, 586 GAS STs, and 551 GBS STs (accessed May 2011). A closely related MLST scheme has been developed for *S. dysgalactiae* subsp. *equisimilis* and *S. canis* which has revealed a significant degree of horizontal exchange of housekeeping loci between *S. pyogenes* and *S. dysgalactiae* subsp *equisimilis* [12, 13].

For all three species, DNA banding pattern based methods have been applied with great success, especially pulsed field gel electrophoresis (PFGE). PFGE and other similar genomic banding pattern assessment methods have proven utility in localized outbreak settings; however, the genotyping information is not readily cross-comparable within global databases, and for this reason is not well suited for large strain distribution studies. In comparison, STs are simple, digital identifiers that can be readily related to component allelic sequence files on the Internet (see www.mlst.net).

8.3 Group A Streptococci (*S. pyogenes*)

Among the pathogenic hemolytic streptococci, *S. pyogenes*, or group A streptococci (GAS), has the most diverse spectrum of acute disease and post-infectious sequelae within the species, with manifestations including acute pharyngitis (“strep throat”), impetigo, rheumatic fever, scarlet fever, glomerulonephritis, streptococcal toxic shock syndrome, and necrotizing fasciitis [14]. GAS express a variety of both cell surface and extracellular virulence factors, with the M-protein being the single most studied and appreciated virulence feature of GAS. The M-typing process can be made more strain-specific by inclusion of two other GAS-characterization methods: T-protein antigen agglutination profiles and sequence typing of the streptococcal serum opacity factor (SOF) [15, 21].

8.3.1 M (emm) Typing

Classic M protein serotyping proved to be invaluable for more than 60 years for resolving more than 100 antigenic types of GAS [16]. A key feature to the M serotyping scheme is that the type-specific, N-terminal region of the protein correspondingly serves as the major type-specific protective antigen. During the past 20 years, unavailability of typing reagents and difficulties in their preparation and maintenance have seen the development of an alternative PCR/DNA-sequencing method called *emm* typing to deduce M serotypes. *emm* typing has greatly extended the utility of the M typing scheme, primarily due to the wide availability of PCR and DNA sequencing technology. In addition to the obvious technical advantages, the

technique offers much less subjectivity than M serotyping in interpretation. For example, GAS strains recovered from relatively remote tropical regions are often nontypeable using M-serotyping; however, strains are always typeable when using M-protein gene (*emm*) sequencing [17]. Classical T agglutination is a useful and relatively simple procedure that is used to augment the strain identification potential of *emm* typing [15], allowing the division of approximately 30 different T protein profiles identifiable using a slide agglutination test [18]. Discovery of the T antigen genes [19] has lead to a straightforward PCR-based scheme proposed to be a reliable replacement [20]. The combination of M (*emm*) type and T agglutination types allows for the quick identification of many global GAS clones [15, 21]. For example, the T1, *emm1* strain is by far the most recognized global GAS strain [22], almost invariably corresponding to MLST type 28 (ST28) [MLST is discussed below]. The same observation holds true for other predominant types such as T3/*emm3* (ST15), T12/*emm12* (ST36), and T28/*emm28* (ST52). Although *emm* type associations with specific GAS clones are strong, there are many known exceptions of *emm* types associated with unrelated clonal types [17, 21, 23].

M protein gene (*emm*) sequence typing is the most widely used method for resolving GAS strains, having replaced the more technically challenging M serologic typing scheme. When examined at a global level, *emm* typing has revealed broad geographic differences in the epidemiology and strain distribution of this species [24]. This genotypic typing scheme is based upon the region of the M protein gene (*emm*) that encodes the type-specific region of the M protein and has become a useful and reliable epidemiologic tool [3, 25, 26]. *emm* typing is independent of *emm* gene expression and can discriminate between biologically distinct isolates that may be only weakly antigenic or nontypeable, allowing for deduction of known M-serotypes and classification of isolates that have new *emm* genes and/or M protein serotypes. This system relies upon the use of two highly conserved primers to amplify a large portion of the *emm* gene [25]. The hypervariable sequence encoding M serospecificity lies adjacent to one of the amplifying primer sequences, allowing for direct sequencing of 150 nucleotides of the 5′ end which displays the highest level of sequence polymorphism; >180 different *emm* types have been described to date [27]. The Centers for Disease Control and Prevention (CDC) maintains a database (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm) that allows for accurate identification of *emm* types using established parameters for identification. The database contains sequences for all identified *emm* types as well as any allelic variations within the defined type-specific region of these types. In addition, this database contains an extensive database of *emm* genes found in *S. dysgalactiae* subsp. *equisimilis* [12, 28] and less commonly in *S. canis* [12].

8.3.2 emm Subtypes

Isolates with small alterations in the *emm* 5′ terminus relative to the major *emm* type subtype can potentially have altered susceptibility to type-specific opsonic antibodies elicited against the M protein [21]; however, such variants are generally effectively

targeted by vaccines raised against the major subtype [29]. The CDC database includes subtypes assigned on the basis of any alterations within the coding region for the predicted 50 N-terminal residues of the processed M protein. A 60 codon region is employed for this subtyping scheme that includes 10 codons of signal sequence that allows precise identification of the mature M protein N-terminus (see http://www.cbs.dtu.dk/services/SignalP/ for reliable program for identifying signal cleavage sites). For example, *emm*68.1 contains a 7 codon deletion within the 5′ 150 bases encoding the mature M protein relative to *emm*68. Subtype information may increase the specificity of epidemiologic information and can be valuable in tracking specific strains. Common *emm* types are subdivided into stable subtypes on the basis of this 150 base type specific region. For example subtype *emm*3 0.1 accounts for the majority of type *emm*3 isolates in the United States (about 75–80%), while subtype *emm*3.4 accounts for about 20% of *emm*3 isolates [30]. A GAS strain with a novel subtype (*emm3.17*) of the very common type *emm*3 was recovered from blood and tissue of an allograft recipient and also from the autopsy of the deceased tissue donor, providing a definitive epidemiologic link [31]. The nomenclature of this subtyping scheme is simple. Any variation within the 180 bases encoding the predicted 50 N-terminal M protein residues plus 10 signal sequence residues relative to the reference strain (designated with a 0.0; e.g., *emm3.0*, *emm6.0*, *emm12.0*, etc.) is assigned a subtype (e.g., *emm*3.1, *emm*3.2, *emm*6.1, *emm*6.2, *emm*12.1, *emm*12.2, etc.). For any *emm* sequence not found to share sequence identity with one of the approximately 1,200 180 base entries in the CDC database (ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/), the associated raw data trace file is sent along with relevant strain information to the CDC *emm* database curator for inclusion within the database (ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/).

8.3.3 sof Genes from Group A Streptococci

The anti-opacity factor (AOF) type, conferred by the *sof* (serum opacity factor gene) has been widely used as a tool for strain characterization. SOF production occurs in approximately half of all known M/*emm* types, and this production correlates highly with specific M types [32] or *emm* types [21, 25, 33]. The identification of the gene encoding SOF, the *sof* gene, and the subsequent development of tests to detect this gene by PCR amplification and sequencing of a 450–650 bp fragment, have added another tool for GAS characterization [21, 34, 35]. The co-presence of identical *emm* and *sof* sequence types are highly predictive of clonal groups predicted by MLST and can allow rapid resolution in certain instances where different clones have been observed to share the same *emm* type [17]. There is a nearly complete correlation of *sof*-gene presence with SOF production, making the *sof* PCR test a practical alternative to classical phenotypic SOF determination.

8.3.4 *Other Approaches for Typing GAS*

8.3.4.1 *emm* Amplicon Restriction Analysis

To improve sequencing efficiency and economy, restriction digests of *emm* amplicons can be subjected to gel electrophoresis and the resulting enzyme restriction patterns compared. For large collections of isolates, strains with common T-types and opacity factor (OF) reactions, displaying identical *emm* amplicon restriction profiles with *Dde* I and with *Hinc* II + *Hae* III, can then be grouped together, and *emm* sequencing can be performed on a smaller sample (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). Almost invariably, this *emm* type is highly conserved among the entire group of isolates [36, 37]. *emm* amplicon restriction patterning is a quick method to detect isolate sets that share highly conserved *emm* genes and is particularly useful in outbreak settings. However, it should be noted that certain *emm* types (e.g., types *emm5* and *emm6*) display a large number of different restriction profiles due to the presence of unstable tandem sequence repeats, while other types (for example types *emm1* and *emm12*) display a single predominant restriction profile.

Numerous other useful genotypic methods have been developed for the typing of GAS isolates. These include additional restriction enzyme techniques such as Virtyping [38], ribotyping [39, 40], random amplification of polymorphic DNA (RAPD) [41, 42], and pulsed-field gel electrophoresis (PFGE) [22, 43].

8.4 Group B Streptococci (*S. agalactiae*)

S. agalactiae, or Lancefield's group B streptococcus (GBS), a facultative gram-positive diplococcus with an ultrastructure similar to other Gram-positive cocci, was originally known for causing bovine mastitis and was not demonstrated to be a human pathogen until 1938. Although GBS is generally carried asymptomatically, it can cause invasive disease in newborns, pregnant women, and immunocompromised or chronically ill (e.g., diabetic) adults. Invasive infections in neonates can result in pneumonia, sepsis, or meningitis. Early-onset disease (EOD) occurs within the first week. Late-onset disease (LOD) occurs after the first week and accounts for most meningitis cases and deaths. Because recommendations for intrapartum antibioprophylaxis (IAP) for mothers in labor at risk for GBS infection have been widely implemented in many countries and continue to evolve [44], the incidence of EOD has declined to <1/1,000 births, but the incidence of LOD has remained unchanged [45].

For GBS, serotyping is the most commonly used phenotypic assessment but does not reveal information regarding genetic identity, so many investigators now use genotypic techniques in conjunction with certain phenotypic techniques.

8.4.1 Serotyping

A crucial factor in GBS virulence is the production of an antigenically variable polysaccharide capsule, also used for strain typing. The classification of GBS has evolved over time and currently ten serotypes have been described (Ia, Ib, II-IX) [46]. The distributions of these serotypes, however, vary by geographic location and study population. In the United States serotypes Ia, II, and III, and since the early 1990s serotype V are more commonly associated with invasive disease [47].

Several phenotypic methods have been devised for serotyping GBS and include the Lancefield capillary precipitin method (regarded as the "gold standard") [48], double immunodiffusion [49], coagglutination [50], enzyme immunoassay [51], and latex agglutination [52] methods. Serotyping does, however, have limitations as these tests often have a complicated interpretation, require specific reagents that may not be readily available for routine use in many laboratories and may not be sensitive enough to detect important differences among epidemiologically unrelated strains. In addition, an increasing number of isolates are being classified as nontypeable (~4–9%) by classical serotyping due to mutations in their capsular genes, the presence of reversible nonencapsulated phase variant, or more rarely due to expression of a new capsular serotype [46].

In recent years, various molecular serotyping approaches based on the detection of serotype-specific genes of the capsular region have been developed and have included either PCR in conjunction with sequencing [53], hybridization [54] or enzymatic restriction cleavage pattern analysis [47, 55] and more recently multiplex-PCR approaches [56, 57]. These molecular approaches have made it possible to assign a molecular serotype to many nontypeable isolates and thereby reduce their numbers. Since GBS capsular polysaccharides are candidate components of multivalent vaccines, it is important to deduce capsular serotypes of GBS isolates that have lost the ability to produce capsule subsequent to the infection process. These techniques are also attractive because they are reproducible, specific, and easy to perform and are particularly well adapted for GBS capsular polysaccharide typing in large-scale epidemiological studies [53, 54, 56, 58, 59]. Others studies have additionally used the presence of surface proteins and/or the genes encoding them to characterize the isolates [60]. Although these proteins generally correlate with a capsular serotype, this is not always so.

8.4.2 Protein and DNA Based Fingerprinting of GBS

Population genetic methods have been applied to GBS strains, as well as GAS and pneumococci, to investigate genotypes associated with disease, assess genetic variation within genotypes, and examine the role of recombination in the generation of new genotypes. These include RFLP, PFGE, multilocus enzyme electrophoresis typing (MLEE), and MLST [10, 61–63]. MLST is the more sensitive molecular version of MLEE, a method that plots electrophoretic mobility of housekeeping

enzymes. Based upon housekeeping gene alleles, MLST has subdivided GBS strains into numerous STs [10], with over 500 STs being described to date (http://pubmlst.org/sagalactiae/). Some STs group together into clusters following phylogenetic analyses using eBURST and four major clonal complexes (CCs) (ST1, ST17, ST19, and ST23) have been identified among clinical GBS strains [10]. The distribution of CCs has been shown to differ between colonizing and invasive strains [10, 62, 64]. The ST17 serotype III strains have been associated with neonatal disease in several populations and may have an enhanced ability to cause disease [10, 62, 64, 65].

8.5 *Streptococcus Pneumoniae*

S. pneumoniae (the pneumococcus) is a common colonizer of the respiratory tract and is a prevalent opportunistic pathogen. This organism is a global scourge as a leading cause of bacterial pneumonia, meningitis, otitis media, and sinusitis.

Accurate molecular epidemiologic resolution of pneumococcal isolates is crucial for understanding changes in their population and evolutionary biology as trends in pneumococcal disease are influenced by selective factors in the environment. Serotyping has been the primary method to understand the epidemiology of specific strains of *S.pneumoniae* for decades since vaccines are targeted against the capsular polysaccharides. More recently, additional subtyping techniques and methods for genetic characterization have provided powerful tools for elucidating the epidemiology of pneumococcal disease outbreaks, biologic responses to selection exerted by antimicrobials and vaccines, and the global spread of specific pathogenic clones.

8.5.1 Serotyping

The capsular polysaccharides of *S. pneumoniae* represent a remarkably diverse group of polymers that play an essential role in the virulence of the organism. Serotypes vary in the extent to which they are carried in the nasopharynx and the degree to which they are recovered from different disease states. The Quellung reaction, which uses commercially (or in-house) available factor (typing) sera, is able to divide pneumococci into serogroups and serotypes. These sera have been developed by a process of multiple cross-absorptions, which render them specific for the immunochemical differences between the pneumococcal capsular polysaccharides (CPSs) [66]. At present, 93 individual serotypes are recognized by their patterns of reactivity with the factor sera [67, 68].

Due in part to the large number of serotypes that are encountered in infection and carriage, the standard Quellung reaction test for serotyping pneumococci is labor-intensive and time-consuming, and requires a certain level of experience to be performed satisfactorily. An agglutination method with anti-rabbit IgG-coated latex particles sensitized to pooled and select individual serotype-specific antisera (PCV7

serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F) for serogrouping/serotyping *S. pneumoniae* has been developed and is commercially available [69]. The latex agglutination method is simpler and faster but is intended to narrow the identification down to a group or pool of serotypes and then Quellung can be done using specific antisera for each serotype in the group or pool.

Flow cytometric methods for the serotyping of pneumococci have recently been reported [70, 71]; however, these methods require monoclonal antibodies against the pneumococcal serotype-specific polysaccharide and other reagents that are not readily available to most laboratories.

The high cost of antisera, subjectivity in interpretation, and technical expertise requirements associated with these serologic-based methods have led to a renewed interest in alternative methods to identify the capsular polysaccharides of *S. pneumoniae* and many other bacteria. Central to this renaissance have been the molecular characterization and complete nucleotide sequence determination of the capsular loci for *S. pneumoniae* [72]. One widely used method for PCR serotype deduction involves a sequential multiplex PCR-based serotyping scheme easily adaptable to different serotype distributions [73–75] that presently includes the 40 serotype specificities that are most commonly encountered (http://www.cdc.gov/ncidod/biotech/strep/pcr.htm). This PCR approach has the potential to greatly reduce reliance upon conventional serotyping and provides serotype-determining potential to laboratories that lack type-specific antisera and other reagents needed for conventional serotyping, yet have the equipment necessary for DNA amplification and electrophoresis. This approach has been extended to deducing pneumococcal serotypes present in nasopharyngeal secretions [76, 77] and has great application for deducing serotypes from clinical specimens when causal pneumococcal strains cannot be recovered [78, 79]. It is important to realize that these methodologies and schemes will continue to be refined as additional serotypes are added and primer sets updated to improve specificity. More recently, alternative PCR-based approaches such as real-time PCR and reverse line blot hybridization have also been explored for serotyping pneumococcal isolates [80–82].

8.5.2 Methods for Fingerprinting Pneumococci

Motivated by the emergence of antimicrobial resistance in the 1990s and the spread of resistant organisms worldwide, various subtyping methods have been evaluated to differentiate strains of *S. pneumoniae*. Typing methods such as ribotyping, BOX fingerprinting, ERIC-PCR, pulsed-field gel electrophoresis and restriction fragment-end labeling of small DNA fragments have been widely used to subtype pneumococcal isolates [83] and have long been employed to identify "serotype switch" events where capsular biosynthetic loci have been transferred between distinct genetic lineages [84]. Alterations in penicillin-binding proteins (PBPs) are the major mechanism of resistance to penicillins and cephalosporins in *S. pneumoniae*. The organism possesses several high-molecular-mass PBPs, and most of the high-level penicillin resistance is due to alterations in PBP 1a, 2b, and 2x.

Thus, sequence differences in the genes encoding these PBPs have been exploited to assist in strain-typing *S. pneumoniae*. These methods include restriction fragment length polymorphism (RFLP) analysis of PCR products amplified from *pbp1a*, *pbp2b*, or *pbp2x*, or comparison of sequences of the amplified products [85]. An MLST scheme for *S. pneumoniae* was developed in 1998 [11] using sequence variation within internal fragments (about 500 bp) of seven housekeeping genes. Over 6,000 sequence types have been described at the pneumococcal MLST Web site (http://spneumoniae.mlst.net/). MLST combined with capsular serotyping is an effective means by which to detect serotype switch variants that occur through recombinational gene replacement at the capsular biosynthetic locus. Numerical MLST identifiers have allowed the research community to easily communicate and trace serotype switch events within the pneumococcal population genetic structure [86]; see http://www.sph.emory.edu/PMEN/pmen_criteria.html for nomenclature. MLST allows for insightful analysis of clonal structure and strain emergence within individual successful invasive serotypes. For example, MLST has revealed that invasive serotype 19A disease isolates within the United States are largely comprised of strains that appear likely to have originated within serotypes other than 19A [87], with the most rapidly emerging 19A strains appearing likely to have originated from serotype replacement events within strains formerly expressing 7-valent conjugate vaccine (PCV7) serotypes (4, 6B, 9V, 14, 18C, 19F, 23F).

The use of these typing methods combined with serotyping and antimicrobial-resistance patterns has allowed isolates from different epidemiological regions to be examined for potential relationships and the identification of persistent local and global clones [88]. Data from numerous surveillance projects performed from various countries over the past 30 years show that, although there is considerable diversity among resistant strains, a small number of highly successful clones have emerged within countries and in some cases have achieved massive geographical spread across both national and continental boundaries. A number of these international clones are described by the Pneumococcal Molecular Epidemiology Network that was established in 1997 to standardize laboratory methods and epidemiological definitions for identifying clones of pneumococci (http://www.sph.emory.edu/PMEN). Pneumococci belonging to some of these clones are not only widespread in the geographic sense but also represent a very large proportion of resistant strains in a given epidemiological setting. Isolates belonging to many of these clones have been isolated from pediatric as well as adult disease and as pneumococci colonizing the nasopharynx of healthy children.

8.6 Other Streptococci

Various phenotypic and genotypic approaches have also been developed for determining the molecular epidemiology and population structure of various streptococcal species other than GAS, GBS and *S. pneumoniae*. Recently, a MLST scheme for *S. oralis* [89], an important commensal of the oral microbiota, has been developed to analyze the species and further examine the population structure based on previous

genotypic studies [90]. Other MLST schemes have also been devised to type and speciate viridans and other streptococcal species (http://viridans.emlsa.net/) . Within our laboratory we have very recently found MLST of *S. salivarius* [91, 92] to be useful in tracing the oral carriage source of meningitis associated with lumbar puncture in two different outbreak investigations [93, 94].

S. suis has become an emerging pathogen causing severe systemic infections in humans and the occurrence of outbreaks has increased awareness and improved diagnostics in this species. Of the known 35 serotypes determined by agglutination with a panel of antiserum, only a limited number are responsible for the majority of disease [3]. Serotype 2 is considered to be the most pathogenic and genetic diversity has been studied using various typing techniques including random amplification of polymorphic DNA, PFGE and ribotyping [3, 95]. Analysis of the population structure using MLST [96] has identified 157 distinct STs (http://ssuis.mlst.net/) with ST-1, ST-27 and ST-29 as dominant clonal complexes within this species. In addition, heterogeneity within various zoonotic streptococci such as *S. uberis* and *S. zooepidemicus* has been recently studied using various typing approaches [97, 98].

8.7 eBURST Application to the Molecular Epidemiology of Streptococci

The elegant eBURST algorithm, where strains related to each other through sharing at least 6 of 7 MLST target sequences can be easily connected to create a clonal group, has proved to be a powerful tool for pneumococcal surveillance purposes, allowing for simple resolution of epidemiologically important clonal complexes [99]. In addition, eBURST provides the ability to display likely patterns of diversification of isolates from easily predicted founders within clonal complexes. The simplicity of this tool and its wide potential for streptococcal species in general is enabled by the fact that in streptococci studied to date, genomic changes occur primarily through recombination events between different strains rather than through mutation. Therefore, all MLST targets can be treated equally (regardless of divergence) through eBURST to effectively resolve genetic complexes in streptococcal species (see http://spneumoniae.mlst.net/eburst/, http://spyogenes.mlst.net/eburst/, and http://ssuis.mlst.net/eburst/ where eBURST can be run on entire species databases or selected MLST profiles).

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