Molecular Techniques for the Investigation of Meningococcal Disease Epidemiology

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Abstract

Meningococcal disease remains a major cause of childhood morbidity and mortality world wide and no comprehensive vaccine is available against the causative organism, *Neisseria meningitidis*. Molecular studies of the diversity of this bacterium have provided a number of key insights into its biology, which have implications for control of meningococcal disease. These have included the identification of hyperinvasive lineages and the correlation of genetic type with antigenic type and disease epidemiology. In practical terms, such studies have enabled the application of DNA-based technologies in the development of improved methods for diagnosis and epidemiological monitoring. These data are of especial importance with the current, and ongoing, development and introduction of new meningococcal vaccines.

Index Entries: *Neisseria meningitidis*; meningococcal disease; molecular diagnosis; molecular epidemiology; population biology.

1. Introduction

Neisseria meningitidis, the meningococcus, is primarily a harmless commensal bacterium that colonizes the naso- and oropharynx of adult humans. However, this gram-negative diplococcus has the potential to cause two devastating disease syndromes, meningitis and fulminant septicemia, that can occur either separately or in combination ***(1,2)***. Once they have successfully invaded in the bloodstream from their primary colonization site, meningococci have the capacity to grow rapidly ***(3)*** and also to cross the blood-brain barrier ***(4)***, both features being of central importance in pathogenesis. Disseminated meningococcal infection is frequently accompanied by the release of highly toxic lipooligosaccharides which cause extensive tissue damage and severe toxic shock ***(5)***. The progress of the disease is swift and death often occurs within hours of the onset of symptoms ***(6)***. Meningococcal disease is well known as a major cause of morbidity and mortality among small children, even in those countries where it is relatively rare in absolute terms, and remains a high priority for public health services world wide due to the high mortality rates of fulminant septicemic disease (which can be up to 40% even when intensive supportive therapy is available), the high proportion of sequela in patients who have recovered (including brain damage and digit or limb loss), and the age groups most susceptible (young children and to a lesser extent young adults) ***(7,8)***.

The severity of meningococcal disease is often exacerbated by the notorious difficulties in its diagnosis ***(9)***. The initial stages of both syndromes are nonspecific and, in the case of fulminant disease, the early signs of the purpuric rash can be small and easily overlooked or misinterpreted. The rapid progress of the infection makes these difficulties in diagnosis potentially fatal as it is vital for successful management that parenteral antibiotics are administered as early as possible in the course of the disease ***(9)***. The emphasis on early treatment, preferably before hospitalization ***(10)***, and the maintenance

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of antibiotic therapy on admission to hospital, reduces the likelihood of isolating a meningococcus from clinical samples from approx 50% to less than 5% ***(11)*** and has resulted in an increasing proportion of cases that cannot be confirmed by laboratory culture of the organism ***(12,13)***. This decrease in laboratory confirmed cases, and the accompanying loss of epidemiological information, is occurring at a time when such data are particularly important due to the testing and introduction of novel vaccines ***(14)***. It has been suggested that for certain patient groups an improved diagnosis system could increase the number of confirmed cases by as much as 60% ***(15)***.

Meningococcal disease is associated with four distinct patterns of epidemiological spread ***(16,17)***: endemic; hyperendemic; localized epidemic; and large-scale epidemic/pandemic. In Europe and the Americas, endemic disease with annual attack rates of 1–5 cases per 100,000 population is prevalent, with occasional hyperendemic outbreaks (10–15 cases per 100,000, over periods of months or years), or localized disease outbreaks (20–30 cases per 100,000, the outbreaks persisting for a number of weeks). By contrast, large scale epidemic/pandemic outbreaks of meningococcal disease (up to 1000 cases per 100,000) occur periodically in Africa and Asia: these represent the most serious manifestation of infection by *Neisseria meningitidis* and can cause tens of thousands of cases and thousands of deaths during the course of an outbreak ***(18,19)***.

Currently, there are no effective childhood vaccines that protect against all meningococci, although serogroup A and C polysaccharide-based vaccines that can interrupt outbreaks caused by organisms expressing these serogroups in older children and adults have been available since the late 1960s ***(20)***. New protein-conjugate A and C polysaccharide vaccines provide the prospect of acceptable infant vaccines against meningococci that express capsules of these serogroups ***(21)***. Unfortunately, despite intensive research interest in this area ***(22)***, there is little prospect of an effective vaccine against serogroup B meningococci in the immediate future.

The application of molecular techniques to study the epidemiology and population biology of *N. meningitidis* has provided important insights that have begun to elucidate some of the reasons for the difficulties experienced in the development of antimeningococcal vaccines. Further, there have been many attempts to improve the techniques and reagents available for diagnosis and epidemiological monitoring by the exploitation of molecular approaches. Finally, in addition to its intrinsic importance, the study of meningococci has provided a number of paradigms and techniques which have more general application. Here we shall review the principal molecular techniques used to study meningococcal epidemiology and discuss the insights obtained from their application.

2. Analysis of Meningococcal Diversity

The study of meningococcal diversity has proved to be of the utmost importance in understanding the spread of meningococcal disease and has recently relied on molecular techniques, especially the application of high-throughput nucleotide sequence determination for isolate characterization. Some of the aspects of meningococcal diversity which have been well studied from the point of view of epidemiology and population biology are discussed here.

2.1. The Meningococcal Serological Typing Scheme

In common with other bacterial pathogens, the characterization for *N. meningitidis* relied for many years on the serological reactivity of cell surface components ***(23)***. The primary level of serological characterization for this organism is the serogroup, which is based on the differential immunological reactivity of the various polysaccharides which meningococci can express to form a capsule. There are 13 meningococcal serogroups ***(24)***, but of these only organisms expressing the capsules that define serogroups A, B, and C commonly cause disease, with serogroup Y and W135 organisms causing most of the remaining cases ***(2)***. This feature makes the meningococcal capsule a principal, and arguably the only clearly defined, meningococcal virulence factor. Expres-

sion of a capsule is essential for survival in the host blood stream although, apparently paradoxically, expression of the capsule must be switched off before invasion of host tissue can occur ***(25)***.

Further immunological characterization of meningococcal isolates is based on "subcapsular" antigens. Serotypes are antigenic variants of the outer membrane porin, PorB, and serosubtypes variants of the related PorA protein ***(26,27)***. Variation in lipooligosaccharide (LOS), the major glycolipid of the outer leaflet of the meningococcal outer membrane, is rather less frequently employed in routine isolate characterization and defines "immunotypes" ***(28)***. The whole scheme is conventionally written in the order serogroup serotype serosubtype immunotype, with each character separated by colons, thus:

B:15:P1.7,16:L3,7,9

Serotype, serosubtype (prefix P1.) and immunotype (prefix L) may have several characters for one isolate, which are separated by commas. Originally polyclonal sera were employed for immunological isolate characterization but monoclonal antibodies are now available for many serotypes, serosubtypes, and immunotypes. There are a number of problems with the serological typing scheme ***(29)*** including a lack of comprehensive reagents and poor correlation of serological characteristics with the genetic relationships of isolates ***(30,31)***. Molecular techniques have made a number of contributions to resolving these problems by improving our understanding of antigen synthesis and expression and providing more direct means of identifying protein variants.

2.2. Capsular Antigens

The capsules of serogroup B and C meningococci are composed of homopolymeric sialic acids. In the case of serogroup B polysaccharides the sialic acid residues are "α2,8-linked, whereas the serogroup C capsule is composed of "α2,9-linked polysialic acids. Sialic acids are also constituents of the serogroup W135 and Y capsules with additional galacatose and glucose residues, respectively ***(32–35)***. The polysaccharide capsule of serogroup A meningococci is rather different, being a polymeric "α1,6-linked N-acetyl-D-mannosamine-1-phosphate ***(33)***. Polyclonal antisera and monoclonal antibodies are commonly available for capsular typing ***(36–40)*** and are most useful for serogrouping of disease isolates. However, carriage isolates frequently remain not serogrouped by this set of antibodies, because capsular types other than A, B, C, W135, and Y are expressed or, alternatively, because capsule expression undergoes phase variation resulting in a capsular negative phenotype ***(41–43)***. To overcome this problem, molecular serogrouping methods based on the identification of serogroup specific capsular genes have been developed.

This approach exploits the knowledge of the molecular mechanisms of capsule expression. The genes required for the expression of a capsular polysaccharide in meningococci are clustered at a single chromosomal location, the *cps* locus ***(44)***. Within this region, 16 genes have been assigned to 5 gene loci in accordance with their biochemical function. The genetic organization of these loci is almost identical in meningococci expressing all serogroups analyzed so far and is summarized in **Fig. 1**. Region A contains the genes required for the biosynthesis of the capsular polysaccharide ***(45)***; in loci encoding the synthesis serogroups B, C, W135, and Y this region comprises the *siaA*, *siaB*, *siaC,* and *siaD* genes which direct the synthesis of the sialic acid containing capsules. The *siaA*, *siaB,* and *siaC* genes are highly conserved ***(46)*** as they perform the same function for meningococci expressing each of these serogroups, namely the synthesis of monomeric sialic acid and its activation to form CMP-sialic acid ***(45)***. The activated form of sialic acid is the substrate for the polysialyltransferases, which direct the polymerization of the activated sialic acid monomers. The *siaD* genes are the only capsule biosynthesis genes with functional and nucleotide sequence specificity for these four serogroups. The *siaD* genes of serogroup B and C meningococci share a 64.4% identity ***(45,46)*** and are unrelated to the *siaD* genes of serogroup W135 and Y meningococci, which are more than 98% identical to each other ***(43)***. There is one polymorphic region between nucleotides 885 and 1029 in the 3,114-

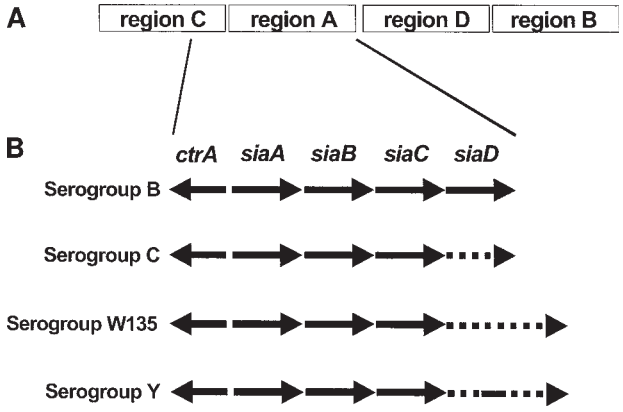

Fig. 1. Meningococcal capsular operons. **(A)** Schematic depiction of the organization of the genes of the capsule gene cluster (cps) indicating the functional regions A (biosynthesis genes), B (phospholpid substitution of polysaccharide chains), C (cell suface transport of capsular polysaccharide), D (LOS bioynthesis). **(B)** Organization of the sialic acid biosynthesis genes. *siaA-C* are highly conserved among serogroup B, C, W135 and Y meningococci. *siaD* exhibits functional and nucleotide sequence heterogeneity.

bp *siaD* genes of serogroup W135 and Y, which confers serogroup specificity, and that can be used to distinguish both serogroups (H. Claus and M. Frosch, unpublished observations). The sequence divergence among the *siaD* genes of all serogroups provides the basis for the design of serogroup specific oligonucleotides for PCR-based grouping system. This scheme can be extended by the amplification of the *myn* genes that direct capsule biosynthesis in serogroup A meningococci ***(47)***.

The biosynthesis region A of the capsule operon is flanked by regions directing phosholipid substitution of the polysaccharide genes (region B) ***(48)***, LOS biosynthesis (region D) ***(49)***, and capsular polysaccharide transport to the cell surface (region C) ***(50)***. This latter region comprises four genes, *ctrA*, *ctrB*, *ctrC,* and *ctrD*, which all show a very high degree of sequence identity in all serogroups analyzed so far ***(51)***. Consequently, one of these genes, *ctrA*, is an attractive target sequence for nonculture detection of meningococci.

2.3. Porin Antigens

The *porB* and *porA* genes, which encode the serotype (PorB) and the serosubtype (PorA) antigens of the meningococcus respectively, are among the best studied of all meningococcal genes and a very large number of alleles for each of these loci have been characterized by nucleotide sequence determination ***(52–58)***. Comparisons of the deduced amino acid sequences of many variants of both PorA and PorB have demonstrated they are related members of a family of *Neisseria* porins ***(54,59–62)***. For both PorA and PorB, specific serological reactivities reside in variable surface loops of the porin structure. In the case of PorA, the serosubtyping antigen, most sequence variability resides in the first and forth surface-exposed loops of the putative porin structure, described as Variable Region 1 (VR1) and VR2: there is a third, less variable, region corresponding to the fifth putative loop (VR3 or sVR) ***(53,54)***.

Originally the designation of serosubtypes was on the basis of antibody reactivity ***(63–65)*** and most of the serosubtype-specific monoclonal antibodies have been shown to react with contiguous peptide epitopes that are located within VR1 or VR2. However, as soon as multiple sequences of *porA* genes became available it was apparent that much diversity was missed by the antibodies and that the sequences present in the loops could be divided into VR "families" which contained related but distinct sequences. These studies also showed that there were a number of reasons for the lack of comprehensive coverage of the serosubtype monoclonal antibodies. In some cases isolates expressed VR families in either VR1, VR2, or both, for which no monoclonal antibody was available, whereas other nonsubtypable isolates expressed variants of VRs families that did not react or reacted poorly with the relevant monoclonal antibody ***(57,66,67)***. For example, it was shown that a single polymorphism between two family members could completely abolish antibody reactivity ***(68)*** while other changes did not affect antibody binding ***(66)***. The particular assay conditions employed could also change antibody reactivity to particular PorA proteins ***(69)***. Phase variation by changes in the promoter region of the *porA* gene ***(70)*** and interruption and inactivation of the gene by an insertion element ***(71)*** can also result in serosubtyping failure.

These observations led to the proposal that the peptide sequence of the VRs deduced from nucleotide sequences of the *porA* gene should be used as the basis for subtype family definition, with variants indicated by the addition of letter to the subtype family name thus: P1.16a ***(57,67,68)***. The increasing diversity of PorA proteins, however, resulted in a number of problems with this system: first, there were so many variants that the addition of letters did not provide sufficient family member designations; and second, some of the families which were originally defined by monoclonal antibody reactivity were difficult to distinguish on the basis of their amino acid sequences. A new system has been devised which maintains as much consistency as possible with the previous system and which utilizes numbers for the family member definitions providing for unlimited expansion of the number of family members. This nomenclature is available on the world wide web at http://outbreak.ceid.ox.ac.uk/porA-vr.

Although the serotype antigen, the meningococcal PorB protein, is related to the PorA protein, and conforms to a similar structural model ***(62)***, the antigenic variability of this protein is rather different ***(55,56,72)***. First, a given meningococcal isolate has one of two mutually exclusive genes at the *porB* locus encoding either a class 2 or a class 3 PorB protein; there are numerous variants of both class 2 and class 3 PorB proteins. Second, although antigenic diversity among PorB proteins also resides in the putative surface loops of the proposed porin structure, the surface loops of PorB variants are smaller and less diverse. Third, in both class 2 and class 3 PorB proteins different surface loops vary compared with PorA. Finally, the serotype monoclonal antibodies do not in general react with contiguous peptides in epitope scanning experiments although it is possible to associate particular loops with particular serotypes in some cases ***(73–75)***. DNA-based methods for the identification of serotypes have been developed, which use oligodeoxyribonucleotide probes, defined by nucleotide sequence analyses of PorB proteins, to identify differences in surface loops by hybridization ***(76,77)***, but ultimately nucleotide sequence determination of most of the *porB* gene is required to unambiguously identify a given allele ***(75)***.

2.4. Diversity in Housekeeping Genes

Selection pressures imposed by the host immune system on antigen genes can distort the inter-isolate relationships inferred from characterization of such genes, making closely related isolates appear to be very different, while grouping otherwise unrelated isolates that happen to share a particular antigen gene allele at a particular locus. These problems can be avoided by indexing the variation present at multiple housekeeping loci, which are distributed around the bacterial chromosome and are under stabilizing selection for conservation of function, so that the genetic changes which accumulate are selectively neutral or nearly so. The first method to exploit this approach was multi locus enzyme electrophoresis (MLEE) ***(78)***.

Although MLEE was successful in establishing the population structure of the meningococcus and in identifying the major disease-associated lineages ***(30,79)***, there were a number of problems with this approach. The allelic variants were inferred by the differential mobility of the proteins which they encoded during starch gel electrophoresis. This had the disadvantage that candidate housekeeping loci for multi locus sequence typing (MLST) had to encode a protein (usually an enzyme) which could be identified by staining after electrophoresis, thereby limiting the loci which could be exploited by this method. Further, the differential mobility of the variant proteins relied on charge differences in the allelic variants, so truly neutral mutations, those which generated synonymous changes, were not detected. The identification of allelic variants also required an extensive collection of reference isolates and the technique was relatively complex and time consuming, although very high throughput could be achieved in well set up laboratories. Consequently, few laboratories implemented the approach routinely.

MLST was designed to overcome the problems associated with MLEE. It was first developed for the meningococcus ***(80)*** and has since been extended to a number of other bacterial species ***(81–84)***. The approach was similar to that employed by MLEE, in that the variation present at multiple loci around the chromosome that encoded proteins

performing essential metabolic functions ("housekeeping" genes) were identified and employed to establish allelic profiles (electrophoretic types, or ETs for MLEE and sequence types, or STs for MLST). Relationships among allelic profiles reflected, and were therefore used to establish, genetic relationships among isolates.

The major difference was that in MLST, nucleotide sequence determination was used to identify the alleles which were employed to generate the allelic profile. This meant that any gene could be chosen from the genome, a process now simplified by the availability of complete genome sequences, amplified by the polymerase chain reaction (PCR) and its nucleotide sequence determined by rapid cycle sequencing. In this way, all of the genetic variation present at a given locus was identified including, importantly, synonymous nucleotide sequence changes. The greater resolution of alleles, compared to that available from starch gel electrophoresis, made it possible to achieve similar resolution of isolates with a seven locus MLST system to that achieved by a 14 locus or more MLEE system ***(80)***. As nucleotide sequence determination is a generic technique, it is possible for most molecular biology laboratories to undertake MLST studies with no additional equipment. In addition, an increasing number of commercial services offer nucleotide sequence determination services. Nucleotide sequence data are definitive and readily transferred electronically via the Internet, and it was possible to establish virtual isolate collections accessible from the world wide web which could be queried remotely (http://neisseria.mlst.net). The STs are unambiguous and independent of the laboratory in which they are determined, so it is possible for direct comparisons to be made with data from other laboratories which is stored on the website. An important feature of the MLST website is that it is curated and for a novel allele designation to be assigned it is necessary to deposit the raw sequence data to ensure that sequence errors are not introduced into the databases.

In practice 400–500 bp fragments of housekeeping genes, rather than complete genes were employed for MLST. This enabled the alleles (or, more correctly, allele fragments) to be determined on both strands with only two nucleotide sequencing primers, considerably reducing costs and increasing the speed of the approach. In all bacterial species so far analyzed, allelic fragment of this size were sufficient to establish genetic relationships among isolates ***(81–84)***. For MLST, each distinct allelic fragment is identified by a unique allele number, which is arbitrary and assigned in order of description. The STs therefore comprise seven numbers, one for each locus, as illustrated in **Table 1** which shows the typical STs for some of the more important meningococcal hyperinvasive lineages.

2.5. Genes Conferring Antibiotic Resistance

Multiple nucleotide sequence analyses have been performed on three chromosomal genes encoding proteins that can confer antibiotic resistance on meningococci: *rpoB* which encodes the β subunit of DNA-directed RNA polymerase, mutations in which confer resistance to rifampicin ***(85)***; *dhps* which encodes dihydropterate synthase, mutations in which confer resistance to sulphonamide drugs ***(86)***; and *penA* which encodes penicillin binding protein 2, mutations in which confer resistance to β-lactam antibiotices ***(87)***. These studies have shown that it is relatively easy for rifampicin resistance to occur spontaneously by mutation ***(85)***. For both *dhps* ***(86,88,89)*** and *penA* ***(90,91)***, antibiotic resistance spreads by horizontal genetic exchange rather than arising *de novo* by mutation. In the of penicillin resistance and perhaps sufonamide resistance, interspecies gene transfer has been involved ***(92)***.

2.6. Genome Wide Diversity

Until very recently, molecular approaches to the comparisons of whole genomes have been limited to fingerprint analyses, producing patterns from chromosomal DNA, usually after the DNA has been digested with one or more restriction endonuclease. In some cases the fingerprints are resolved by pulsed field gel electrophoresis (PFGE) ***(93–96)***, in others conventional electrophoresis ***(96,97)***, southern hybridization with DNA probes has been used to generate such fingerprint patterns ***(98)***. Random amplified polymorphic DNAs (or RAPDs),

Table 1
Characteristic Sequence Types Associated with Particular Meningococcal Lineages

MLEE Lineage designation	MLST Lineage designation	Locus *abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
Subgroup I	ST-1 complex	1	3	1	1	1	1	3
Subgroup IV	ST-4 complex	1	3	3	1	4	2	3
Subgroup III	ST-5 complex	1	1	2	1	3	2	3
Cluster A4	ST-8 complex	2	3	7	2	8	5	2
ET-37 complex	ST-11 complex	2	3	4	3	8	4	6
ET-5 complex	ST-32 complex	4	10	5	4	6	3	8
Lineage 3	ST-41 complex	3	6	9	5	9	6	9

where chromosomal fingerprints are generated by PCR amplification with "random" oligodeoxyribonucleotide primers ***(99)*** is an alternative approach. Whilst these methods are useful in establishing identity of isolates none is effective in establishing genetical relationships among strains, and care has to be taken in applying phylogenetic analyses to fingerprint data. The advent of complete genome sequences allows more detailed comparisons, but on a very small number of isolates. For example, the two isolates sequenced so far appear to have different gene orders, but more data are required to establish how widespread this is ***(100,101)***.

3. Molecular Diagnosis of Meningococcal Disease

For confirmation of a clinical diagnosis it is necessary to detect meningococci in samples of cerebrospinal fluid (CSF), serum, or whole blood. When the organism cannot be cultured, this has traditionally been achieved by techniques such as microscopy, coagglutination, or latex agglutination, but these methods are often of low sensitivity and specificity ***(102)***. The amplification of bacterial genes from clinical samples by the PCR provides alternative methods for detecting bacteria in clinical samples which are rapid and potentially highly sensitive and specific ***(14)***. Most success has been achieved with CSF samples, but the reluctance of many physicians to take CSF samples ***(103)*** has lead to an increasing interest in using serum or whole blood samples, which are more readily obtained but which are more difficult specimens for PCR-based diagnostic techniques.

Diagnostic techniques that use the PCR require small quantities of clinical material, are as rapid or faster than culture, and the test-specific reagents, the oligodeoxyribonucleotide primers, are inexpensive. Modification of conventional protocols, such as the use of seminested primer sets, can achieve very high sensitivities, equivalent to 1 colony forming unit per amplification reaction ***(104)***. Sensitivity and specificity can be further improved by including hybridization assays with membrane filters (Southern blots) or in micro titer wells (PCR-ELISA) ***(105,106)***. In addition, the amplified genes obtained can be further analyzed, e.g., by digestion with restriction endonucleases, hybridization with specific probes, or nucleotide sequence determination, to provide not only diagnostic but also epidemiological data from the same sample ***(14)***.

As it is in principle possible to amplify any bacterial gene by the PCR, careful choice of the target genes for these procedures is important. This choice should be made on the grounds, not only of test specificity and sensitivity, but also of the epidemiological value of the information that can be obtained by analysis of the amplified gene. Once a gene target has been chosen, extensive evaluation of clinical samples, including double-blinded trials is necessary before reliance can be placed on a given method for diagnosis. Although double-blinded trials of some methods have been carried out, no extensive comparative analyses of different PCR diagnostic techniques for meningococcal disease have been published to date. The data from a number of separate studies based on different target genes are summarized here.

Because bacteria other than the meningococcus, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, group B streptococci, and *Listeria monocytogenes* cause meningitis, a primary requirement of nonculture diagnosis is species identification. All eubacteria have *rrn* genes in multiple copies in their chromosomes which comprise conserved and species-specific variable regions of gene sequence ***(107,108)***. Several methods exploit this by amplification of *rrn* genes from clinical samples derived from suspected cases of meningitis ***(102,109–112)***. A primary PCR can be used to amplify a product from all eubacterial *rrn* genes which can be followed by one or two secondary amplifications with species-specific primer sets that produce differentially sized amplicons which are characteristic for each species. The species specific products are readily distinguished by the separation of the amplicons using agarose gel electrophoresis. Such methods require only small samples of CSF of ~10 µL ***(102,109)***.

Further confirmation of infection with a meningococcus can be achieved with a PCR test based on the gene encoding the conserved capsular transport protein, *ctrA* ***(113)***. After confirmation of a meningococcal infection, the most important information for the clinician and public health physician is the serogroup of the causative organism. This information enables appropriate public health measures such as vaccination, administration of prophylactic antibiotics, and counseling of contacts to be undertaken ***(114)***. A PCR test that distinguishes alleles of *siaD*, the gene encoding a sialyltransferase involved in the synthesis of both serogroup B, C, Y, and W135 capsules, has been developed ***(115,116)***. This procedure is based on specific sequences of the *siaD* gene that distinguish operons encoding serogroup B, C, W135, and Y isolates. Both the *ctrA* and *siaD* based technique has been adapted to a micro-titer well PCR-ELISA hybridization and ABI-Taqman™ automated format ***(117)***.

The *porA* and *porB* genes have not been extensively used in diagnosis, but a number of groups have successfully amplified these genes from clinical specimens ***(77,104,118)***. Using appropriate primers it is possible to amplify both the *porA* and *porB* genes in the same experiment ***(55)*** and, as the amplified genes have characteristic sizes when examined by agarose gel electrophoresis, this is a potentially useful characteristic for confirming the presence of meningococci. The dihydropterate synthase *(dhps)* gene has been amplified from at least one clinical specimen ***(119)***. Extensive studies in clinical specimens have not been carried out, but in principle this technique allows the identification of sulphonamide resistance, a commonly determined epidemiological characteristic, even when the organism is not culturable. A similar approach is also, in principle, applicable to the *penA* gene.

The IS*1106* insertion sequence (IS), which is present in several copies in the meningococcal chromosome ***(120)***, has been used by several laboratories as a diagnostic PCR target. A number of double-blinded trials of methods based on IS*1106* sequence have been done on clinical specimens and several modifications in the technique have been published ***(11,15,121)***. Unfortunately, an insertion sequence is a poor choice as a target for a diagnostic approach, as these elements may cross species or genus boundaries. This behavior provides a possible explanation of the false positive results that have been obtained with the IS*1106*-based diagnostic techniques ***(122)***. The use of this target is further complicated by nucleotide sequence rearrangements within the IS ***(15)*** and amplification of IS*1106* provides no epidemiological data. For these reasons, this target should not be used in preference to the other amplification targets discussed above; however, the work on IS*1106* was the first to establish the utility of PCR techniques for meningococcal diagnosis and the system could be used in addition to one or more of the above methods for further confirmation of diagnosis.

4. Interpretation of Diversity: Meningococcal Population Biology and Epidemiology

Accurate isolate characterization is recognized as a cornerstone of successful epidemiological studies, but it is perhaps less widely appreciated that it is also necessary to interpret these data with appropriate theoretical frameworks if the maximum

public health benefit is to be gained ***(17,123)***. The advent of affordable and rapid nucleotide sequence determination techniques permits the generation of data that are both epidemiologically informative and directly applicable to studies of the epidemiology and population genetics of the organism ***(124)***. In particular MLST data has been exploited in a number of studies to investigate the population structure of the meningococcus ***(125,126)***.

The genetic and antigenic diversity of meningococcal populations, although considerable, is not random and is structured. Although a comprehensive understanding of this structuring is yet to be achieved, and it is unlikely that any one model will provide a complete description of meningococcal populations, a number of models are currently available which both provide testable predictions and represent an appropriate basis for the development of more sophisticated models with improved predictive power.

The essentially asexual nature of bacterial reproduction implies a clonal population structure ***(127)***, which is a consequence of each daughter cell being an exact replica, or clone, of its mother cell. Mutations occur during evolutionary time, but new alleles which arise in this way will be confined in the lineage in which they arose. Consequently clonal populations are characterized by linkage disequilibrium (nonrandom combination of alleles) and relatively low diversity, especially if the population undergoes diversity reduction events such as periodic selection or bottle necking which purge the population of diversity ***(128)***. Preliminary analysis of MLEE data, which identified clusters of organisms with related ETs and linkage disequilibrium, suggested that meningococcal populations were clonal ***(30)***; however, a number of lines of evidence have shown that this is not an appropriate model ***(128)***.

The calculation of the index of association, or I_A, provides one means of assessing whether linkage disequilibrium is due to clonality or an artifact generated by the frequency distribution of alleles in the population ***(129)***. For meningococcal data sets I_A analysis indicated that although the clonal descent model could explain relationships within complexes it was not an appropriate model of the relationships among clonal complexes ***(129)***. More support for this view was provided by the observation that identical alleles were found in genetically otherwise unrelated complexes for loci encoding both antigens and housekeeping proteins. Further, meningococcal data sets were not congruent, that is to say the phylogenetic relationships identified among members of different complexes depended on the loci examined, which is in contradiction to the clonal model which predicts that the phylogeny obtained for a given population will be independent of the gene chosen to reconstruct the phylogeny ***(126)***.

A likely explanation for this breakdown of clonality in the long term evolution of meningococcal populations is the fact that the meningococcus is transformable (naturally competent for DNA uptake), which permits meningococci to partake in a quasi-sexual process, horizontal genetic exchange—also referred to as "localized sex" ***(130)***. This process involves the exchange of small segments of genetic material among organisms which do not necessarily share a common mother cell, and in the meningococcus it is probably a consequence of autolysis of meningococcal cells, which releases DNA that can be taken up by other cells and incorporated into their genomes by homologous recombination ***(131)***. Nucleotide sequence determination of multiple alleles of various genes have demonstrated widespread occurrence of mosaic genes which is consistent with horizontal genetical exchange occurring regularly throughout the meningococcal genome ***(91,94,132)***. Indeed it is not uncommon to observe sequences that appear to have been imported into the meningococcal population from other species ***(133)***. This inter-species horizontal genetic exchange appears to have had an important role in the emergence of resistance to a number of antibiotics, including penicillin which is a first line drug for the treatment of meningococcal disease. Horizontal genetic exchange has important implications for epidemiological analyses as it is necessary to distinguish the clonal and horizontal spread of genetic or serological markers. Where horizontal spread occurs at high frequency, the epidemiologist

may well follow the spread of the marker genes rather than individual strains. To accommodate recombination in bacterial populations two further models of bacterial population structure have been proposed, nonclonal (sometimes referred to as panmictic), where recombination is sufficiently frequent to eliminate clonal structure in a population and so called "epidemic clonal," where the rapid spread of one particular lineage results in the over representation of that genotype in the population, giving an impression of clonality ***(129,134)***. The extremes of strictly clonal and completely nonclonal are probably very rare in natural populations of bacteria, with most bacterial population structures containing both clonal and nonclonal elements, the ratio of which will depend on the relative level of horizontal genetic exchange per generation. Meningococci are, over the long term, nonclonal but particular lineages (clonal complexes) do exist and can persist for periods of time of at least several decades.

A further complication of the population structure of meningococci is the relationship of carrier isolates to those obtained from patients with invasive disease ***(135)***. For understandable reasons, the majority of isolate collections comprise meningococci obtained from diseased patients. Individual meningococci, however, must survive and spread as efficient commensals as disease is not a route for transmission and in any case is very rare compared to colonization. Genetic analyses of collections of disease and carriage isolates have established that it is only a minority of genotypes, the twelve or so "hyperinvasive lineages," which cause the majority of disease, whereas carried meningococcal populations are much more diverse and contain numerous genotypes which are rarely if ever associated with disease ***(136)***. The use of genomic techniques to characterize and compare representatives of hyperinvasive and noninvasive lineages provides a tantalizing prospect of understanding meningococcal pathogenesis. Current models, therefore, envisage a meningococcal population which comprises a diverse collection of lineages, all of which persist solely as a virtue of their capacity to spread as commensal inhabitants of the human nasopharynx. The population is considered to be dynamic with its composition, in terms of lineages, changing with time. In addition these lineages diversify, mainly by the recruitment of new genetic material.

Further structure in meningococcal populations is presented by the antigen genes. The first population studies of meningococci established that the genes and operons that encode the surface structures of the organism also move by horizontal genetic exchange; however, while this occurs rapidly in evolutionary terms, presumably in response to the immune selection which meningococci experience during carriage, structures in the combination of antigen genes are preserved. This is consistent with theoretical frameworks that envisage the structuring of antigen combinations as a consequence of the costs imposed on bacteria by sharing of antigens. In other words, the theory predicts that antigenic variants will be more successful if they are as diverse as possible from other meningococci ***(137)***. As more data, especially from carried meningococci, becomes available some of the predications from such models can be more completely tested and the models developed appropriately.

5. Conclusions

Despite much research over the last one hundred years, and especially in the last 30, comprehensive and effective public health interventions against meningococcal disease remain elusive. The most attractive solution would be the development of vaccines against each of the five meningococcal capsular antigens which are associated with invasive disease, but although it seems likely that conjugate polysaccharide vaccines will soon be available against serogroup A, C, Y, and W-135 expressing meningococci, uncertainty remains for vaccines against serogroup B organisms, which causes the largest proportion of disease in many countries ***(138)***. Unfortunately the serogroup B polysaccharide capsule is especially poorly immunogenic ***(139)***, probably because of identity with host antigens ***(140)*** so that, in addition to the technical problem of inducing a good immune response, safety and regulatory issues will have to be resolved. These may prove to be

insurmountable. There are many alternative candidate antigens for meningococcal vaccines, including for example the PorA and PorB proteins, but none of these has yet provided a solution ***(138)***. At best, vaccines against such components may provide partial protection against particular disease causing lineages.

The recent introduction of protein-conjugate serogroup C polysaccharide vaccines in the United Kingdom ***(141)***, along with the use of outer membrane vesicle vaccines developed against specific meningococcal lineages in Norway ***(142)***, Cuba ***(143)***, and elsewhere ***(144)*** has established the precedent for the introduction of vaccines which protect against only a proportion of the meningococcal population. However, with our current understanding of the complicated population biology of the meningococcus it is impossible to assess the impact that these partial solutions might have in populations where carriage rates of meningococci are high: it is certainly not impossible that while providing short term benefit they could have marginal, or even paradoxical, effects on disease levels in the longer term ***(145)***. Molecular tools such as MLST provide us with the opportunity to follow the effects of such vaccine introductions on meningococcal populations and will hopefully lead to improved management of this terrible disease.

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