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MOLECULAR EPIDEMIOLOGY OF NEISSERIA MENINGITIDIS

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1. ABSTRACT

Neisseria meningitidis poses a major disease burden on human beings. Meningococcal typing has a longstanding tradition for epidemiological surveillance of the disease. Genetic and antigenetic variability resulting from horizontal genetic exchange has been exploited for this purpose. *Neisseria meningitidis* served as the bacterial prototype organism for the development of multi locus sequence typing, which has replaced multi locus enzyme electrophoresis as the gold standard for meningococcal typing. Due to the rapid emergence of new porin variants serotyping by monoclonal antibodies is currently being replaced by DNA sequencing of variable regions of the *porA* gene. Sequence data were used to characterize the population structure of meningococci in carriage and disease. The advances in molecular epidemiology of meningococcal disease, and the rapid exchange of DNA sequence data via curated internet websites have resulted in an interactive international network, which is capable of identifying newly emerging clones within weeks.

2. INTRODUCTION: MENINGOCOCCAL DISEASE

The gram negative β-proteobacterial species *Neisseria meningitidis* is restricted to the human host and does not survive in the environment. Spread of meningococci between humans is supported by close contact and exchange of nasopharyngeal secretions, e.g. in households or institutions (1). The nasopharynx is the primary site of meningococcal colonization. The frequency of colonized individuals within a population depends on the epidemiological situation and a variety of individual risk factors, e.g. age and smoking. Figures of 10% carriage rate represent the lower range, and it is likely that nasopharygeal swabbing results in underestimates (2). Colonization of the nasopharynx is mediated by pili and outer membrane proteins such as Opc and Opa which interact with epithelial cells (3-7). Carriage can last for a couple of months and may induce a strain specific immunity directed against a variety of targets such as capsular polysaccharides, lipopolysaccharide, and porins (8). Frequent encounter of a human population with a circulating pathogenic strain has been reported to induce herd immunity (9). Protective immunity may also be the result of colonization with apathogenic *Neisseria* species such as *N. lactamica*, which might explain the low prevalence of meningococcal carriage in young children (10-16). As the result of the induction of bactericidal antibodies during the circulation of a strain in a population escape variants are selected (9,17-22). By genetic transformation, escape variants change surface exposed antigens, which are targets to bactericidal antibodies.

Despite of high carriage rates, the incidence of meningococcal disease in Europe and North America is low with approximately 1-3 cases in 100.000 inhabitants per year (23). Secondary cases are rarely observed (reviewed in 24). Nevertheless, there have been several examples of notable raises of the incidence of meningococcal disease in several industrialized countries throughout the past century, which forced public health systems to implement several vaccination campaigns (25-29). Furthermore, massive outbreaks of meningococcal disease in the meningitis belt of sub-Saharan Africa occur regularly during the dry season and are accompanied by considerable number of deaths (24,30). A world-wide mobility of pathogenic clones of meningococci has been demonstrated. Serogroup A meningococci of subgroup III-1 spread from Asia to Africa within few years (31). A highly pathogenic variant of serogroup C meningococci (the ET-15 clone) emerged in Canada and migrated to Europe and Australia (32). Within weeks, serogroup W-135 meningococci causing disease among Hajjis participating in the pilgrimage to Saudi Arabia were also found in Asia, Europe and North America (33). Global monitoring of the migration of pathogenic variants is needed and demands the use of internationally available and portable typing systems.

Despite its low incidence in industrialized countries, meningococcal disease attracts public attention. This is due to the rapid course of the disease, its considerable mortality, the high frequency of sequelae, and the young age of the victims (34,35). Most cases of meningococcal disease present with septicemia or meningitis. Respiratory infections and conjunctivitis are rare. Genetic predisposition has been shown to increase the susceptibility to meningococcal disease or its severity (36-44).

Effective antimicrobial agents are available against meningococci. Nevertheless, the mortality and the frequency of sequelae remain unacceptably high. Therefore, much research is performed to push forward the development of an effective vaccine against meningococci. The fact that the sequences of two meningococcal genomes have now been published illustrates these efforts (45,46). Vaccine development is hampered by the genetic and antigenetic variability of meningococci. Porin vaccines always need adaptation to the current epidemic clone causing disease and their application must be lead by typing data. Vaccine development is furthermore hampered by the poor immunogenicity of surface components like the serogroup B capsule. Therefore, in contrast to the elimination of meningitis causing *Haemophilus influenzae* serotype b, meningococcal disease will probably never be eliminated by a single polysaccharide conjugate vaccine. The extensive and successful use of a polysaccharide vaccine directed against only one serogroup (serogroup C) during the UK vaccination campaign starting in 1999 (47) raised concerns about the induction of serogroup switching by pathogenic isolates (48,49). There are no data supporting these concerns, but molecular epidemiology of carrier and disease isolates is currently performed in the UK to study changes in the population structure following the alteration of herd immunity. A meningococcal vaccine target should be highly immunogenic and should not vary upon immune selection. The target should be expressed in all pathogenic strains. Ideally, the target should not be present in apathogenic isolates, so that the bacterial ecology of the nasopharynx is only minimally disturbed by vaccination. A handful of experimental targets fulfilling several of these criteria were identified with the help of genome sequence data (50). The induction by vaccination of bactericidal activity against all pathogenic clonal lineages and sequence types will be a crucial point. The evaluation of a broad bactericidal activity can be accomplished by use of comprehensive strain collections, which have been characterized using molecular epidemiology.

3. TYPING OF N. MENINGITIDIS

Meningococcal typing for a long time relied on serological methods for typing (51,52). These methods utilize a standardized set of monoclonal antibodies supplied by the NIBSC (UK; http://www.nibsc.ac.uk/). The serogroup determines the capsular polysaccharide, the serotype the porin (Por) B, the serosubtype the PorA. The advantage of serological typing is that it provides information about the antigens which are vaccine targets, i.e. the capsule and PorA. However, there are several disadvantages of serological typing: a considerable proportion of carriage isolates temporarily switch off capsule synthesis (53); escape variants of the porins demand constant generation of new specific antibodies. Therefore, serosubtyping by monoclonal antibodies is currently being replaced by DNA sequencing of the variable regions (VR) 1 and VR 2 of *porA* (54-57).

Serological typing data may give hints about the clonal lineage of meningococci. For example, the antigenetic formula C:2a:P1.2,5 is frequently seen in meningococci of the electrophoretic type (ET)-37 complex (new designation: sequence type (ST)-11 complex). However, it should be taken into consideration that serological typing in contrast to multi locus enzyme electrophoresis (MLEE) (58-61) or multi locus sequence typing (MLST) (62,63) relies on genes under diversifying selection and therefore does not provide information about the population structure of meningococci.

For surveillance of outbreaks methods with a higher resolution than that of serological typing and MLST are sometimes needed, e.g. pulsed field gel electrophoresis (53,64,65), genotyping by randomly amplified polymorphic DNA (RAPD) (66), or fluorescent amplified-fragment length polymorphism genotyping (67,68). Furthermore, a combination of MLST and *porA* sequencing increases the resolution of MLST (56). Some clonal lineages require specialized approaches: the highly pathogenic ET-15 clone can only be discriminated from other members of the ST-11 complex by revealing a non-synonymous point mutation in the 5´-region of the *fumC* gene, which is not included in the regular MLST scheme (69).

In the following, we will discuss the most important DNA sequence based methods which are widely used nowadays, i.e. MLST, *porA* sequence typing, capsular genotyping, and molecular monitoring of antimicrobial resistance.

3.1 Multi locus sequence typing

N. meningitidis served as the prototype organism to establish MLST (63). The concept and philosophy of MLEE were adopted. Seven genes were chosen for sequence typing, which code for housekeeping enzymes and which were considered not to be subject to immune selection. In contrast to MLEE, not only non-synonymous changes of the DNA sequence affecting the electrophoretic charge of the protein are detected, but also other non-synonymous and synonymous changes. Therefore less genes are required for typing by MLST when compared to MLEE. The seven loci are not linked physically. Of the 2.2 Mbp genome of meningococci, 3,284 bp are sequenced accounting for 0.15% of the whole genome. Each new sequence found at a locus resembles a new allele which is assigned a consecutive number. The combination of 7 numbers provides the ST. Related STs, i.e. those sharing at least 4 or 5 alleles, are regarded to belong to one genetic lineage (70). Lineage assignments can be accomplished using the computer program BURST which was developed by E. Feil and M.-S. Chan. BURST is a "web-implemented clustering algorithm

designed for use on multilocus sequence typing (MLST) data sets" (http://www.mlst.net/BURST/burst.htm). BURST clusters sequence types, which share a defined number of loci. It furthermore defines the most likely founder genotype from which variants decended. The use of seven loci per strain usually is sufficient to obtain a robust typing result. More loci may be needed for theoretical analysis of the population structure of the organism. In meningococci, most polymorphisms are the result of recombination, not of point mutations (71). Data from carrier isolates show that much new variation comes from new mixtures of alleles, whereas novel polymorphisms are rarely found (62). Since meningococci served as the model organism for establishing MLST, the database accessible via the website http://neisseria.org/nm/typing/mlst/ is now very large (in April 2002, there were 2,747 Neisserial isolates, and 1,811 sequence types). Isolates from more than 50 countries have been deposited, the oldest isolate was collected in 1917. A large number of isolates has been obtained from healthy carriers, so that the bias of strain collections dedicated to pathogenic meningococci has been overcome. Approximately 300 isolates are *N. lactamica*, *N. gonorrhoeae*, and *N. cinerea* isolates, respectively, indicating that the *Neisseria* MLST protocol with some minor modifications can now be considered a whole genus MLST. Although the data on *Neisseria* other than meningococci have not yet been published by Martin Maiden and colleagues, this approach will allow to compare population structures of the species and it will allow to test the current taxonomic concept of *Neisseria* species. The well organized structure of the MLST database is reflected by the implementation of an international management team responsible for the structure of the database and for the designation of lineages. Funding of the database is currently provided by the Wellcome Trust and by the European Union via the EU-MenNet (http://neisseria.org/nm/emgm/eumennet/). Taken together, the *Neisseria* research community is in the lucky position to have access to a virtual strain collection providing essential information for everyone interested in molecular epidemiology. It should be mentioned finally that MLST is usually performed from bacterial cultures, but non-culture identification of the ST has been published allowing MLST also in culture negative cases (72).

3.2. The PorA variable region database

As stated above, serological typing of meningococci included the determination of porin types. Meningococci express two porins, PorA and PorB. PorA variants determine the serosubtype, PorB variants determine the serotype (52). Although internationally available monoclonal antibodies were used for many years, the approach was hampered by an increasing number of isolates which could not be typed by the set of antibodies. Furthermore, serosubtyping by monoclonal antibodies significantly underestimates the variability of *porA* (73). Determination of the amino acid sequences of the variable regions (VR) 1 and VR2 of PorA by DNA sequencing allowed to substitute for serosubtyping (54-57,74). Thanks to the initiative of Martin Maiden, Ian Feavers, and colleagues, an invaluable database was created allowing sequence comparisons (http://neisseria.org/nm/typing/pora/). The website contains lists of VR1 and VR2 types and illustrates that many variants do not bind an established monoclonal antibody. VR1 and VR2 amino acid sequences are ordered into families of related sequences, thus maintaining the traditional nomenclature. It should be noted that the nomenclature is still a matter of debate (73,75). Furthermore, some authors use VR3 in addition to VR1 and VR2 (76). A website for determination of the meningococcal *porB* sequence type has recently been created (http://neisseria.org/nm/typing/porb/). With this tool available, the serotype can now also be identified by PCR and sequence analysis.

3.3. The capsular serogroup

The most important pathogenicity factor of meningococci is the capsular polysaccharide (reviewed in 77). This antigen is used for serogroup determination. Pathogenic isolates express the serogroups A, B, C, W-135, and Y (reviewed in 78). Serogroup determination is important for vaccination campaigns, because different polysaccharide vaccines containing (i) pure A and C polysaccharide, (ii) combinations of the A, C, W-135, and Y polysaccharides, or (iii) serogroup C polysaccharide conjugated to protein carriers are available. The serogroup B polysaccharide is only poorly immunogenic because the human neural cell adhesion molecule is glycosylated with an identical polymeric sugar (79,80). Meningococci can switch off the capsule production during colonization of the nasopharynx (53). Molecular mechanisms for phase variation have been described (81,82). We recently typed 822 meningococcal carrier isolates from Bavaria, Germany, by MLST and determined the capsular serogroup. Furthermore, we determined the capsular genotype for all isolates. For all strains harboring polysialyltransferase genes specific to the serogroups B, C, W-135, and Y (n=541) we found that approximately 69% expressed the polysaccharide (Claus, Maiden, Frosch, and Vogel, manuscript in preparation). Thus, molecular techniques are needed for serogrouping of carriage isolates. Furthermore, the rapid administration of antimicrobial agents in patients who are suspected to suffer from meningococcal disease has increased the number of cases of meningococcal disease without positive culture from blood or cerebrospinal fluid. For this reason, DNA amplification by PCR is now widely used to diagnose meningococci (83). For culture negative patients with meningococcal disease the capsular genotype is then determined by molecular tools (84,85). The basis for this molecular approach was laid by the work of Matthias Frosch, who identified the capsule synthesis operon composed of the *sia* genes (86). The serogroups B, C, W-135, and Y can now be determined by PCR primers identifying serogroup specific *siaD* genes, which encode the polysialyltransferases (21,87,88). Alternative protocols have been developed for serogroup A meningococci (89).

3.4. Molecular monitoring of antimicrobial resistance in meningococci

In contrast to gonococci, resistance to antimicrobial agents is of minor importance for meningococcal disease (reviewed in 24). However, the experience with penicillin resistance of pneumococci is a warning sign to keep

Table 1. Meningococcal lineages, examples

Genetic lineage (MLST)	Previous designation (MLEE)	Predominant serogroup	Genome sequence available	Characteristics
ST-1 complex	Subgroup I/II	A	No	Associated with epidemic disease
ST-4 complex	Subgroup IV	A	Yes	Associated with epidemic disease
ST-5 complex	Subgroup III	A	No	Associated with epidemic disease
ST-8 complex	Cluster A4	C	No	Associated with endemic disease
ST-11 complex	ET-37 complex	C	Yes [1]	Associated with endemic disease
ST-32 complex	ET-5 complex	B	Yes	Associated with endemic disease
ST-44 complex	Lineage 3	B	No	The ST-44 complex comprises both virulent and avirulent STs
ST-22 complex	-	W-135	No	Frequently isolated from carriers, infrequently isolated from invasive disease
ST-23 complex	Cluster A3	Y	No	Frequently isolated from carriers, infrequently isolated from invasive disease
ST-60 complex	-	29E [2]	No	Frequently isolated from carriers, infrequently isolated from invasive disease
ST-53 complex	-	capsule null locus (*cnl*) [3]	No	Frequently isolated from carriers

NOTE: [1] in progress at the Sanger Center; [2] own unpublished data (Claus *et al.*, manuscript in preparation); [3] reference 95

on monitoring the development of antimicrobial resistance in meningococci. Furthermore, the acquisition of a chloramphenicol acetyltransferase gene by meningococci isolated in France and Vietnam has been reported (90). Much more work has been done on penicillin resistance. The group of Brian Spratt demonstrated that *penA* mosaic genes of penicillin resistant isolates were generated by DNA uptake from commensal *Neisseria* (91,92). This work was an early indication of the importance of horizontal gene transfer for the population structure of meningococci. It has then been shown by Taha *et al.* that *penA* genes of penicillin sensitive isolates were uniform (93). In contrast, resistant isolates showed highly diverse *penA* genes due to horizontal gene transfer. These data were used to set up molecular tools for non-culture prediction of penicillin sensitivity (94). The current knowledge of the molecular epidemiology of antimicrobial resistance in meningococci will be invaluable if resistance rates should start to rise.

4. POPULATION STRUCTURE OF MENINGOCOCCI

N. meningitidis is a naturally competent species which takes up DNA from the environment. This feature is the basis for the observation of horizontal gene transfer in meningococci. Despite of horizontal gene transfer, clonal lineages of *N. meningitidis* have been described. Clonal lineages are under diversifying selection, and may have the tendency to spread globally (reviewed in 70). Work in the eighties by D.A. Caugant *et al.*, who used MLEE, already indicated that clonality is much more evident among disease isolates than among carriage isolates (58). Several virulent clonal lineages are listed in Table 1. In contrast to disease isolates, the high degree of genetic diversity of meningococci isolated from healthy carriers is impressive as has been shown by sequence typing of Czech carriage isolates (62). In collaboration with Martin Maiden, we recently identified 322 STs among 822 isolates, which were collected from healthy carriers within five months in Bavaria, Germany (Claus *et al.*, manuscript in preparation). 219 STs of the 322 STs were singletons, which means that they were found only once in the strain collection. The high frequency of singletons demonstrated that it is a difficult task to cover all of the genetic diversity of the meningococcal population. It should nevertheless be mentioned that some apathogenic lineages can be isolated at high frequencies suggesting a high level of fitness and clonal expansion. For example, we could isolate from Bavarian carriers several abundant apathogenic lineages which lacked the capsule locus *cps* and thus were unable to produce the major pathogenicity factor of meningococci, the polysaccharide capsule (35).

5. FUTURE PERSPECTIVES

In many reference laboratories, MLST is still regarded a labor intensive, time consuming, and expensive method. We are convinced that further technical improvements will overcome these shortcomings. Costs could possibly be reduced by the establishment of MLST centers. For Europe, an integral part of the agenda of the EU-MenNet (coordinator: Matthias Frosch) is to set up a pan-European infrastructure for the surveillance of meningococcal disease. This approach includes the creation of a European Meningococcal MLST Center (EMMC) which is located in Oxford, UK, and which is directed by Martin Maiden. The EU-MenNet is linked to the European Monitoring Group on Meningococci (EMGM), a network of the European reference laboratories on meningococcal disease.

Despite of such advances and the benefits of a uniform technique to study the molecular epidemiology of meningococci, new techniques are constantly emerging and will be evaluated. We are currently comparing DNA/DNA hybridization on microarrays with MLST. Furthermore, the genocloud concept based on a combination of MLST data with *tbp*, *opa*, *iga*, and IS*1106* typing represents a new development based on MLST (96). Because of the extreme speed of progress of DNA sequencing technology, we are convinced that future MLST protocols will demand sequencing of much more than the current 3 kb per isolate. The data obtained from a single organism will then be highly discriminatory, they will allow precise theoretical consideration about the population structure, and they will give detailed information on antimicrobial susceptibility and the availability of vaccine targets.

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