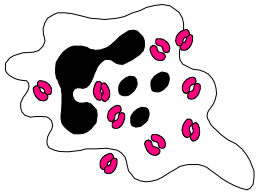
Typing 

Epidemiology
and
Diagnostics

Population genetics of the pathogenic *Neisseria* and its relevance to molecular epidemiology and typing methods

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The pathogenic *Neisseria* species are naturally transformable, and there is increasing evidence that this mechanism of genetic exchange is important for their evolutionary and population biology. There are several reports of both intra- and inter-species recombinational events in gonococcal and meningococcal genes but the main evidence for frequent recombination comes from population genetics.

In most bacterial species, recombination appears to be less important than mutation in effecting evolutionary change, resulting in populations consisting of largely independent clonal lineages. Clonal populations arise because mechanisms for the exchange of chromosomal genes are rarely used in nature and/or because different lineages rarely meet each other. Clonal populations are usually recognized (often uncritically) by the presence of linkage disequilibrium (non-random association) between alleles in the population, using data derived from multilocus enzyme electrophoresis (MLEE), and by the recovery of indistinguishable isolates that are temporally and geographically unassociated (1). Bacterial populations become increasingly less clonal as the extent of recombination increases, and it has been estimated that they will appear to be non-clonal when a change in an electrophoretic allele is about ten times more likely to occur by recombination rather than mutation (2).

Population structure of meningococci. Isolates of serogroup A meningococci, which cause pandemic disease with very high attack rates, are highly clonal (3,4). The population structure of meningococci of other serogroups (referred to as serogroup B and C meningococci for convenience) is more complex. MLEE studies of serogroup B and C meningococci isolated from invasive disease appear to suggest that these populations are clonal as significant linkage disequilibrium is found (5). However, in most developed countries, for every 100,000 individuals, there will be about 5,000 carriers of serogroup B and C meningococci, but probably only about 5 cases of invasive disease. Sampling of the isolates from invasive disease therefore only samples about 0.1% of the total meningococcal population and can lead to sampling problems that can distort the underlying population structure. Recent studies have suggested that meningococcal populations are basically non-clonal, but that hyper-endemic strains arise at intervals, and become over-represented in populations obtained from invasive isolates (6). The presence of many isolates of the same, or similar, electrophoretic type introduces linkage disequilibrium into these populations, which makes them appear more clonal than they are. Removal of this sampling bias uncovers the non-clonal nature of the population (6). As is expected for a non-clonal population, there is little relationship between serological markers and electrophoretic type in serogroup B and C meningococci, whereas there is a clear relationship in the clonal serogroup A isolates (5).

It may be thought that focusing on the large fraction of meningococci that are living as harmless "commensals" is inappropriate, and that it is the minority of isolates from invasive disease which should attract all of our attention. However, it is very possible that recombination between isolates from carriers, or between these strains and closely-related commensal *Neisseria* species, produces the particularly favorable combinations of alleles that lead to the unusually virulent or transmissible strains which occasionally arise to cause hyper-endemic disease. It is also very likely that the relatively rapid diversification of the hyper-endemic strains of serogroup B and C meningococci is a consequence of the accumulation of recombinational replacements with chromosomal DNA from carried isolates.

Population structure of gonococci. Two MLEE studies of populations of gonococci have failed to detect linkage disequilibrium, suggesting that the alleles in the population are broken up by frequent recombination (6-8). In support of this view there is little association between gonococcal auxotype, serotype and electrophoretic type (9). Linkage equilibrium also implies that different lineages must meet each other. The presence of mixed infection in a disease like gonorrhea would not be surprising, particularly in the highly sexually active subpopulations (core groups or super-spreaders) that are believe to maintain gonorrhea in the population (10). Even in gonococci the population structure may be complex, since there are many reports in the literature of isolates with similar phenotypes that have loosely been described as clones. In many cases this may be due to the use of typing systems that are not sufficiently discriminatory. However, there are some cases where there is strong support for the existence of gonococcal clones. The best case is that of the arginine-, hypoxanthine- and uracil-requiring isolates (AHU^-) which share a number of unusual phenotypic characters (11). We have recently shown that 101 AHU^- gonococci isolated over a 39 year period were of a single electrophoretic type (92%) or differed from this ET at a single locus (12). AHU^- gonococci are clearly a relatively long-lived clone within a basically non-clonal population. How this clone avoids rapid diversification by recombinational exchanges with other gonococci is at present unclear, although it does not appear to be due to a defect in their ability to be transformed. In meningococci, it is possible to argue that serogroup A meningococcal lineages avoid being broken up by recombination because they rarely meet other lineages, as a consequence of their epidemic lifestyle. This type of argument is less easy to apply to gonococci, but possible reasons for the increased stability of AHU^- isolates can be put forward.

Population structure and its relationship to epidemiology. The population biology of meningococci and gonococci is complex, with basically non-clonal populations (excepting serogroup A meningococci and AHU^- gonococci). It might be expected that clones cannot exist within non-clonal populations but, as as described above, this is not strictly true. However, in contrast to highly clonal species such as *Salmonella enterica*, where clones persist virtually unchanged over many decades (e.g. the typhi serovar, or clone), the clones in non-clonal populations should be ephemeral, since new successful lineages that rise in frequency in the population will be rapidly diversified by recombinational exchanges with other lineages. The rates at which meningococci diversify appears to vary very considerably. In serogroup A strains the rate is sufficiently low to lead to a clearly clonal population structure. In serogroup B and C meningococci this rate appears to be sufficiently high to lead to a basically non-clonal population. However, certain strains of serogroup B and C appear to diversify more slowly, and

give rise to the hyper-endemic transient clones that are a feature of the epidemiology of meningococcal disease (13). The rate of diversification is presumably a function of the rate at which recombinational exchanges with other lineages occur. This could reflect differences in "transformability", but this is not necessarily so, as differences in the frequency and duration of carriage, and the rate of transmission between individuals, will also markedly effect the rate of diversification by reducing contact between lineages (4). Fortunately for epidemiological purposes, even in the non-clonal serogroup B and C meningococci, lineages may still be recognizable over time periods that are significant in human terms (months or years), although extremely short on evolutionary time scales (transient clones).

A knowledge of the population biology of meningococci and gonococci is useful for guiding studies of their molecular epidemiology. Clearly the information that can be obtained, and the typing techniques that have to be used, will be very different for the highly clonal serogroup A meningococci compared to the non-clonal gonococci. In serogroup A meningococci the stability of clones has allowed the well known studies of Achtman and colleagues which have traced the pandemic spread of the major lineages (clones) that cause epidemic meningococcal disease (3). In this case, the stability of the clones, combined with the detection of minor variants, provides the ideal situation for charting the spread of disease from country to country over a period of several decades. In the less clonal serogroup B and C meningococci this type of long term study becomes more difficult since variation accumulates too rapidly, and in gonococci it should be impossible, since the rate of diversification is probably so high that the gonococci isolated in 1996 will be completely different from those in 1986. The rapid diversification of gonococci does not mean that molecular epidemiology is impossible, but just that the questions that can be asked are limited to the spread of isolates within a short time frame. It is very unlikely that gonococci diversify so rapidly that the spread of gonococcal strains over periods of weeks, or months, cannot be followed. Fortunately it is this type of time frame that is of importance in the epidemiology of gonorrhea, e.g. for identifying new strains introduced into a community, or the identification of isolates from sexual contacts.

Population structure and typing methods for meningococci. Is a knowledge of the population structure of the pathogenic *Neisseria* of any help in deciding which types of genes, or gene products, are most suitable for typing isolates for epidemiological studies? As stressed in the previous paragraph, the choice of typing scheme, and the epidemiological questions that can be answered, will be very different if bacterial clones are stable over many decades, or become unrecognizable over a matter of weeks or months, as a consequence of diversification by frequent recombinational exchanges. In highly clonal bacteria (e.g. the serogroup A meningococci) there is good congruence between trees constructed from MLEE data and from the sequences of house-keeping genes. In some highly clonal species the horizontal transfer of genes specifying the variable cell surface structures used for serological typing is rare, and even serology can mark clones (e.g. in *Salmonella enterica*). In these cases ribotyping, pulsed field gel electrophoresis, PCR with arbitrary primers, or MLEE, should each provide a consistent measure of the relationships between strains, and the choice of characters to use for typing is to some degree arbitrary. The clear advantage of MLEE over the other methods is the ease with which the relationships between isolates can be quantitated.

In the less clonal serogroup B and C meningococci the choice of typing method depends on the question being addressed. Most of the questions relate to the nature of the strains causing meningococcal disease, and their relationship to similar disease-causing isolates recovered within a community, a country, or other countries. As serogroup B and C meningococci should diversify relatively rapidly by the accumulation of recombinational exchanges, the ease with which this can be done should depend on the amount of time since the strains had a common ancestor. Thus, isolates recovered from a localized outbreak within a school, caused by the same strain, will have a very recent common ancestor and can easily be shown to be identical, or very similar, by a number of methods (e.g. pulsed field gel electrophoresis or MLEE, and even serology). However, it becomes more difficult to decide whether these isolates are members of one of the transient clones of serogroup B or C meningococci currently causing hyper-endemic disease by comparing them to reference isolates from other countries, as substantial variation in pulsed field gel patterns, MLEE profile, and serology, may have occurred since the strains had a common ancestor. In this case a more thorough evaluation of the relationship of these isolates to the members of the hyper-endemic clonal complexes, and the overall meningococcal population, using quantitative methods (e.g. MLEE) may be needed. This problem becomes more acute as the time from the origin of the hyper-endemic clone increases, as the clonal complexes currently causing hyper-endemic diseases should continue to diversify by recombinational exchanges (probably at different rates) (13) until the members of these complexes can no longer be distinguished from the background population of meningococci. Unfortunately, new hyper-endemic transient clones will inevitable arise, which will diversify and disappear, to be replaced by further transient clones.

Population structure and typing methods for gonococci. A knowledge of the population structure of gonococci can be used to develop suitable typing systems. If recombination is common in gonococci there should be a vast array of different genotypes within the population. This should mean that almost all randomly selected gonococci will be distinguishable, provided sufficiently high resolution typing systems are used. The commonly used typing systems lack the high degree of resolution required to distinguish the vast array of genotypes expected in a non-clonal population. High resolution typing schemes such as MLEE should be ideal for distinguishing gonococci. Unfortunately, the level of sequence variation in gonococcal house-keeping genes is low, and a large number of enzymes have to be assayed to provide a highly discriminatory method. As much of the interest in gonococcal typing is to recognize isolates from sexual contacts, or strains newly introduced into a community, it is allowable to use genes for typing in which variation accumulates very rapidly. We have chosen to use a highly variable gene family (the opa genes), that evolves very rapidly (probably by recombination), as the basis of a typing method for the short term epidemiology of gonorrhea. Using these genes it has been possible to devise a typing scheme that appears to distinguish all gonococcal isolates, unless they are from sexual contacts or a short chain of disease transmission (14). This typing method (opa-typing) appears to provide a much higher resolution than other methods and appears to allow us to predict sexual contacts simply by the fact that they share gonococci with identical opa-types.

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Epidemiology, Typing and Diagnostics

Microevolution during epidemic spread of subgroup III serogroup A meningococci

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Subgroup III meningococci caused a pandemic meningitis wave in the 1960s-1970s, which affected China, Scandinavian countries and Brazil (1-3). A second pandemic wave began in China and Nepal in the early 1980's and spread via an epidemic in Mecca during the Haj pilgrimage of 1987 to East, Central and West Africa (4,5). Bacteria were also exported from Mecca to Europe and the USA without causing epidemics. 300 isolates from these diverse sources were analyzed for genetic variation of the *iga* and *opa* sequences and of pulsed-field gel electrophoresis (PFGE) patterns.

iga and some *opa* genes have been transmitted via clonal descent over long periods of time: variable regions of the *iga* gene and the *opaA*, *opaF* and *opaH* alleles were identical in subgroup III and IV-1 bacteria isolated in the 1960's, indicating that they had been inherited from one strain which was the ancestor of both subgroups at a much earlier time. However, during the subgroup III Mecca outbreak, the *iga* gene was replaced through horizontal genetic exchange involving at least 2 kb of DNA by an allele from an unrelated organism. Similarly, *opaH* was replaced through horizontal genetic exchange by the *opaI* allele and concurrently a DNA stretch 4.5 kb upstream of the *opaH* allele was replaced by a homologous DNA stretch containing an *Nhe*I site. The proximity of this DNA stretch to *opaH* suggests that these exchanges reflect recombination with a DNA fragment of >5 kb size. Finally, *opaF* suffered a 1 bp mutation during the Mecca epidemic. The 3 loci, *iga*, *opaF* and *opaH* are widely separated on the genome and must represent 3 independent genetic changes. Indeed, analysis of subgroup III meningococci isolated during and shortly after the Mecca outbreak revealed 4 strains representing the parental genotype, numerous strains representing the new genotype and 2 intermediate recombinants which had acquired the *opaF* mutation and the novel *iga* allele but still retained the region encompassing *opaH*. The ancestral and intermediate genotypes did not spread and were never recovered again among later isolates.

Analysis of bacteria isolated from different countries during both pandemic waves revealed extensive geographically localized genetic variation. A third *iga* allele was found in the early 1960's in China which differed by 1 nucleotide from the 'pre-Mecca' pattern. Numerous strains were found where *opa* alleles had translocated by gene conversion to yield recombinant *opa* loci and a few strains where *opa* alleles had been imported by horizontal genetic exchange or had suffered point mutations. Two additional strains were found among isolates from China in the early 1980's where a sequence variant of the DNA stretch containing the *Nhe*I site had been imported by horizontal genetic exchange. All the variants were localized to individual countries or geographical regions and did not spread extensively. Thus both pandemics were associated with continued genetic variation in individual countries while the basic pattern of these alleles did not change during extensive spread, except for the Mecca outbreak. The results are interpreted as reflecting repeated sequential bottlenecks which purify epidemic bacteria and occasionally allow clonal replacement (6,7).

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Identification and typing of *Neisseria meningitidis por*B from cerebrospinal fluid using nested PCR and biotin labeled probe hybridization

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The epidemiology of the antigenic characteristics of the class 3 outer membrane protein of *Neisseria meningitidis* has been important both in identification of outbreak strains and for the development of OMP vaccines. Information regarding the genetic basis for antigenic diversity between strains has increased our understanding of traditional serotypes distinctions. We and others have previously described the variable regions of the *por*B gene encoding the class 3 and class 2 proteins and an identification technique using hybridization of oligonucleotide probes to the variable regions of several important serotypes.(1,2,3) We have expanded our panel of probes to include all the major sequence types at each of the variable regions based on the prototype stains expressing class 3 OMP. Several of these probes have identified variable region homology between non-serotypeable strains and known serotype strains. Of eight Brazilian non-typeable stains tested, five expressed class 3 OMP and 4 of five had at least one VR in common with known serotype strains.

To examine the epidemiology of specific *por*B genotypes among disease causing strains from a population where group B disease is endemic, we are analyzing a series of 100 CSF samples from Brazil using PCR and subsequent VR hybridization. Polymerase chain reaction amplification of *por*A from low copy numbers in cerebrospinal fluid has been described.(4) We have used a similar PCR strategy using outer and nested primers which are external to the VRs to amplify the *por*B gene directly from CSF. Eight CSF samples with known culture results (2 group B and 2 group C *N. meningitidis*, 3 culture negative and 1 *Streptococcus pneumoniae*) were examined in a preliminary study. PCR accurately identified the *N. meningitidis* CSF samples. PCR products from the two group B samples hybridized the VR1-4 probe corresponding to serotype 4 and PCR products from the two group C samples hybridized the class 2 probe.

We have developed a nested PCR amplification of *por*B from cerebrospinal fluid with subsequent hybridization of the PCR products allowing both diagnosis and typing information directly from CSF samples. The epidemiology of individual variable region genotypes shown by this method may aid in the selection of strains for vaccine development. This technique may also be of importance in vaccine trials to analyze culture negative suspected cases (between 1/3 and 1/2 of cases in Brazil) and to asses the ability to protect against some non-serotypeable stains based on individual variable region type.

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Epidemiology, Typing and Diagnostics

Assessment of molecular typing methods for differentiation of *Neisseria gonorrhoeae*

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Over the past forty years, various typing schemes have been devised to characterise isolates of *Neisseria gonorrhoeae.* Traditionally, auxotyping and serotyping have been accepted as the two major phenotypic systems for the typing of gonococci. However, these methods lack the discriminatory power to differentiate many gonococcal isolates (1) and have not examined variation of the entire gonococcal genome. With new developments in genetics, molecular techniques are being increasingly used to differentiate gonococcal isolates (2, 3). Sixty four clinical samples of *N. gonorrhoeae* were isolated from consecutive male patients presenting with acute urethritis at a STD clinic in Durban, South Africa. Isolates were typed using serotyping, macrorestriction and pulsed-field gel electrophoresis (PFGE) and the polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs) of the *por* gene. Isolates were serotyped, using the Genetic Systems panel (Syva, Palo Alto), on the basis of their reaction with antibodies to IA or IB Por, the principal protein of the outer membrane of *N. gonorrhoeae* (4). Testing was done in duplicate for each isolate. Isolates were differentiated into 5 IA and 12 IB serovars. There were four major serovars, viz., IA-6, IB-3, IB-1 and IA-3. The IA-6 serovar predominated, constituting 29% of the population. This is a trend peculiar to strains from the African continent (5). The two major PIB strains, IB-3 and IB-1, comprised 19% and 13% of the population, respectively. Overall, 53% of the isolates belonged to PIB serovars. Genomic DNA was prepared by enzymatic and detergent lysis of gonococcal cells embedded in low melting point agarose plugs (2). Plugs were restricted overnight in 150 ml restriction buffer containing 20 U of *Nhe*I, a low-frequency cleavage enzyme that recognises 6 bp sequences containing the tetranucleotide CTAG, an infrequent sequence in most bacterial genomes. Isolates were subjected to PFGE in a contour-clamped homogeneous electric field (CHEF) apparatus - CHEF Mapper System (Biorad) for 26 h with pulse times of 1-25s. Fingerprints consisting of 10-18 fragments (1-485 kb) were obtained and allowed for the differentiation of the entire gonococcal genome. Restriction patterns of isolates with identical serovars showed various degrees of genetic variation ranging from being clonal, to clonally related, or being independent strains which were unrelated. However, the majority of isolates showed multiple similarities between 1-145 kb, suggesting a common ancestry. It has been suggested that serotyping does not adequately discriminate the IB-3 and IB-1 serovars (1). PFGE allows for intra- and inter-serotypic discrimination, especially with regards to the IA-6, IB-3 and IB-1 serovars. Thus, PFGE appears to enhance the discrimination of each isolate, when used in conjunction with serotyping. The *por* gene of each isolate was amplified by the polymerase chain reaction using specific primers targeted to the *por* gene, viz., ^{84}ATGAAAAAATCCCTGATTGCCC^{105} and ^{1064}TTAGAATTTGTGGCGCAGA^{1046} (6). Amplification of the *por* gene consisted of 35 cycles of 1 min at 94°C, 2 min at 45°C and 3 min at 70°C. An initial and final step of 5 min at 94°C and 10 min at 72°C were included. With each of the PIA-expressing isolates, PCR amplimers of approximately 0.9-1.0 kb were obtained, compared to the 1.1 kb fragment obtained from the

PIB-expressing isolates. The resulting amplimers were restricted with *Cfo*I, *MspA*1I and *Hpa*II (3,7) and run on 6% non-denaturing polyacrylamide gels to generate fingerprints with 2-3, 3-4 and 2-4 fragments, respectively. Amplification of a specific segment of DNA, the *por* gene, limits the number of restriction fragments obtained, allowing easy and accurate interpretation of fragment patterns. Both PFGE of the entire genome and PCR-RFLP of the *por* gene allow good discrimination of the non-clonal gonococcal isolates and augment the traditionally accepted phenotypic serotyping system.

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Molecular typing of *Neisseria gonorrhoeae* to identify sexual contacts.

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Objective. A molecular typing technique has been developed for use in studying the transmission of gonorrhea in sexual networks. A highly discriminative method that gives unique patterns for unlinked isolates but indistinguishable profiles for isolates from sexual contacts is required. To achieve this degree of discrimination we have examined diversity in the opa gene family.

Method. The opa genes were amplified by PCR, digested by Taq1 to produce multiple fragments and end-labeled with ^{32}P-dCTP. The resultant fragments were separated on a non denaturing polyacrylamide gel and exposed to X-ray film1. RFLPs were compared using GelCompar and the degree of similarity determined by Pearson's correlation coefficient. This method was evaluated using selected strains from a collection of gonococcal isolates which were tested retrospectively and included isolates from known sexual contacts and from consecutive patients not known to be contacts. Isolates are also being tested from two prospective studies of consecutive patients attending St.Mary's Hospital, London and the Royal Hallamshire Hospital, Sheffield. Phenotypic analysis using auxotype/serovar (A/S) classes is also being performed. Detailed epidemiological data is being independently collected.

Results. The method has proved to be highly discriminatory with strains isolated worldwide. Indistinguishable patterns were obtained within all the pairs/groups of isolates from known sexual contacts but were distinct between the clusters.

Analysis of isolates from patients from the prospective studies has shown two different populations. A total of 220 isolates have been analyzed from patients attending the clinic in London between April and September 1995 and appear as a heterogeneous population as identified by both the phenotypic and genotypic parameters tested. Phenotypic analysis showed a total of 20 serovars, 8 auxotypes and 46 auxotype/serovar (A/S) classes with 25% of isolates belonging to A/S class, NR/IB-1. Genotypic analysis found considerable variation in the profiles obtained by opa typing. Data collected from the patients identified only 21 pairs and one triplet of known sexual contacts. Of these, 21 of the 22 had concordant A/S classes and the opa profiles showed a correlation coefficient of >85% between paired isolates which is indicative of a high degree of similarity. The one discordant pair were isolates from two male patients known to be contacts but both of whom were known to have multiple partners. The A/S classes were different (NR/1B-8 AND NR/IB-7) and the opa profiles were dissimilar. This could suggest the presence of a mixed infection or infection from another contact.

Analysis of the 140 isolates collected from patients in Sheffield between April 1995 and January 1996 has shown a more homogenous population. Phenotypic analysis showed 11 serotypes, 6 auxotypes and 24 A/S classes with 62/140 (44%) belonging to a single A/S class, Arg/IB-3. Genotypic analysis showed that the opa profiles of the Arg/IB-3 isolates were similar (correlation coefficient of >80%) suggesting the presence of a large cluster in this population. The opa profiles of the remaining isolates were largely heterogeneous with a number of pairs and two other smaller clusters.

Conclusion. We have shown opa-typing to be a highly discriminative technique. In a retrospective study it was able to identify isolates from known sexual contacts. Prospective studies have identified diverse gonococcal populations in different cities. The implications for the transmission of gonorrhea in these sites will be discussed.

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Epidemiology, Typing and Diagnostics

The molecular epidemiology of the *por*B gene of *Neisseria meningitidis* isolated in England and Wales

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Surface antigens of the meningococcus, such as PorA and PorB, are important as epidemiological markers and potential vaccine components (1,2,3). Monoclonal antibodies that identified meningococcal serotypes and subtypes, introduced in the 1980's greatly improved their characterisation compared with polyclonal reagents (4) but many organisms remained not or only partially characterised for these proteins (5). Nucleotide sequence analyses of *por*A and *por*B have since been used to identify the reasons for this: in addition to completely novel variants there are minor variants of these antigens that do not react predictably with a given monoclonal antibody (6).

With the increasing convenience and availability of automated nucleotide sequence technology it is now becoming feasible to obtain the gene sequences for antigens from a large number of meningococcal case and carrier isolates. In addition to characterising strains, the availability of such comprehensive data for *por*A and *por*B can provide the basis for detailed epidemiological and population genetic analyses which will improve our understanding of the population structure and antigenic diversity of the meningococcus. Nucleotide sequence data has also enabled the development of DNA-based approaches for rapid and accurate *por*B characterisation of meningococcal isolates, providing enhanced disease surveillance by the identification of antigenic variation within dominant serotypes such as serotype 4. The DNA-based approach also allows the rapid identification and characterisation of case isolates with a novel serotype such as serotype 22.

The identification of the meningococcal *por*B gene by a PCR ELISA assay has been modified to enable non-culture confirmation of meningococcal disease in clinical specimens. Non- culture diagnosis has improved case ascertainment and the usefulness of the assay for the diagnosis and confirmation of several institutional outbreaks of meningococcal disease in the UK has been demonstrated.

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Clonal analysis of *Neisseria meningitidis*: Sequence studies on the 16S ribosomal RNA

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Neisseria meningitidis is a major human pathogen and is the commonest cause of bacterial meningitis in children in the UK. Two thirds of all meningitis cases are due to infection with group B meningococci, the remainder being due predominantly to serogroup C (1). Studies on the population genetics of *N. meningitidis* have shown it to have essentially a clonal structure. Serogroup C meningococci are responsible for both sporadic and endemic disease being intermediate between genetically diverse serogroup B and the relatively homogeneous serogroup A. We have applied genotypic typing methods to determine the clonal relationship of recent UK isolates of serogroup C meningococci (2,3). Eleven clones were identified by StuI REA with two of the clones accounting for 64% of strains. In order to further assess the genetic relatedness of the UK serogroup C isolates we have sequenced part of the 16S ribosomal RNA gene and compared the results with those obtained from StuI REA.

Chromosomal DNA from serogroup C isolates was purified and the 5' half of the 16S rRNA gene amplified using primers Po and Pc3mod. The amplicon was purified from excess primers and dNTPs using Centricon C-100 columns. The products were sequenced using Applied Biosystems dye dideoxyterminator cycle sequencing kits on an ABI 373A automated sequencer fitted with a stretch upgrade. Internal primers as well as Po and Pc3mod were used to sequence the fragments on both strands (4). Data was analysed using the Seqed sequence analysis program. Twelve isolates were sequenced and analyzed representing seven of the eleven StuI REA clones plus one serogroup A isolate.

The complete sequence of the 5' 750bp from each isolate was obtained. Comparison of the twelve sequences identified 10 polymorphic sites located throughout the entire 750bp fragment. All sequences from the same StuI REA clone were identical. Some of the StuI REA clones also had identical sequences. StuI REA clones 1,2,3 and 9 had identical 16S rRNA sequences although their StuI REA Dice coefficients varied from 59-74%. Comparison of the sequences from StuI REA clones 1,7,8 and 11 showed them to differ at 4 of the 10 polymorphic sites except clones 7 and 11 which differed at 8 of the 10 sites.

The StuI REA Dice coefficients for clones 1,7,8 and 11 varied between 49-95% but generally less than those having the same 16S rRNA sequence. Isolate A7 differed at 3-7 of the 10 sites when compared to the REA serogroup C clones.

Sequencing of the 16S rRNA from different species has provided an invaluable tool for the simple and rapid discrimination both among and within genera. It is used to study the evolution of bacteria and to determine accurately most phylogenetic relationships up to about the species level (5). During the course of our studies we observed a number of differences within the 16S

rRNA genes of *N. meningitidis*. Comparison of the data with our results from StuI REA showed that the differences occurred between clones and not within clones. This suggests that the mutations within the 16S rRNA gene have occurred as these organisms have evolved. The structural constraints on the sequence of the small ribosomal RNA ensures that mutations within the gene are relatively infrequent hence their utility in phylogenetic analysis. Our observation of identical 16S sequences within clones further supports the clonal structure of *N. meningitidis* populations.

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Measurement of antibodies against meningococcal capsular polysaccharides B and C in ELISA: An approach towards an improved surveillance of meningococcal disease.

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Objective. To establish ELISAs specific for meningococcal capsular polysaccharide B and C antibodies and thereby improving the surveillance of serogroup B and C meningococcal disease.

Material. Paired sera from 162 patients with and 101 patients without laboratory evidence of meningococcal disease were used in the evaluation of the tests.

Methods. Microtitre plates were precoated with poly-l-lysine and subsequently coated with purified serogroup B or C polysaccharide (1) or with buffer without polysaccharide as control for non-specific binding (2). Test serum was diluted 1:100 and for each assay an in-house standard reference, a positive and negative quality control serum were employed as controls. The test was performed using peroxidase labelled rabbit anti human IgG and/or IgM antibody and ortho-phenylenediamine as substrate. Absorbance values were read at a wavelength of 490 nm. Each test result was determined as the difference between the optical density (OD) in the antigen well and that of the corresponding control well. The OD test result of each serum sample was transformed to au/ml by the linear portion of the standard reference curve based on a log/log scale. Competitive inhibition by purified B and C polysaccharide was assessed using the same assay principle.

Results. Three ELISAs, anti-B IgM, anti-C IgM and anti-C IgG were established. On the basis of four antibody determinations for each of the three positive quality control sera per week during 10 weeks, the interassay coefficients of variation were estimated to range between 1-19%. The binding of anti-capsular antibodies of the standard reference sera was inhibited 96-99% by preincubation with the homologous polysaccharide.

Sixty out of 76 patients (79%) with culture-confirmed serogroup B disease had high anti-B IgM titres or a significant change in titre; anti-B IgM antibodies waned rapidly in children < 4 years of age; 26 out of 27 patients (96%) with culture-confirmed serogroup C disease had high anti-C IgM and/or IgG antibody titres or a significant change in titre (IgM: 93%; IgG:70%). In patients without meningococcal disease, 19% of children < 4 years of age and 72% of those > 4 years of age had low levels of anti-B IgM antibodies. In contrast, < 10% of these had anti-C IgM and/or IgG antibodies. In 50 out of 59 patients (85%) with culture-negative, but with clinical and serological evidence of meningococcal disease, a definitive serogroup specific diagnosis was established by examination of paired sera in ELISA.

Conclusions. Though the B and C polysaccharides are homopolymers of the same poly-sialic acid and a cross-reactive epitope exists (3), the methods developed were shown to be powerful tools in discriminating between serogroup B and C disease. A serogroup specific diagnosis may be established in > 80% of culture-negative cases of meningococcal disease. As serogroups B

and C meningococci account for practically all cases of meningococcal disease in industrialized countries, the availability of these tests may have implications by improving the surveillance and prevention of the disease.

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Neisseria meningitidis strains that were not serosubtypable in whole-cell ELISA

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Neisseria meningitidis is world-wide a major cause of bacterial meningitis and septicemia. Elevated incidence of meningococcal disease was documented in the province of Québec, Canada, since the beginning of 1991 (1, 2). In an effort to combat this high incidence and its accompanying fatalities, health officials initiated mass immunization programs in the province (3).

Outer membrane proteins (OMPs) of meningococci are of particular interest because they form the basis of serotyping and serosubtyping (4). The use of monoclonal antibodies raised against the class 1 OMP has identified differences in the class 1 OMP and this forms the basis of serosubtyping. Outbreaks of meningococcal disease have been linked with the emergence of specific meningococcal subtypes in Brazil, Chile and England. Proper identification of subtypes is thus essential in epidemiological analyses of meningococcal disease. The currently used protocol for routine serosubtyping relies exclusively on ELISAs using whole-cells and mAbs against the class 1 OMP (5). However, this procedure fails to yield serosubtype information in some strains. Reasons suggested for this failure include masking of epitopes on the cell surface (6) and the presence of novel variable regions for which no mAbs are currently available (7).

Isolated from all patients hospitalized with invasive meningococcal disease in Québec (1993-1994) were 174 meningococcal strains. Of these, 48 (28%) were non-serosubtypable in ELISAs using whole-cells (M. Lorange, Laboratoire de Santé Publique du Québec). These 48 strains were characterized by OMP profiles and ELISAs using outer membrane vesicles. Forty of the 48 strains expressed class 1 OMP indicating that the inability to assign a serosubtype was not due to the absence of the class 1 OMP. 17% (8/48) of the strains did not make the class 1 OMP. In these strains, inability to assign a serosubtype is due to absence of the class 1 OMP on the cell surface. The frequency of isolation of class 1 OMP-deficient strains was thus 5% (8/174). This percentage is significant when considering the design of vaccines that are based on the class 1 OMP: 5% of meningococcal strains would not be targets for such vaccines. Fifteen of the 40 strains that expressed the class 1 OMP were serosubtypable in ELISAs using OMVs. Thus, ELISA using OMVs improves the serosubtyping information that is obtained for epidemiological analyses. However, not all strains will be subtyped using these two procedures. For complete subtyping information, molecular techniques have been proposed (7).

To determine whether the eight strains that do not express the class 1 OMP contained the *porA* gene, we used *porA*-specific primers in PCR. The predicted 1.1 kbp fragment was obtained in 6 of the 8 strains. Thus, in these 6 strains, lack of expression was not due to deletion of the gene. From the 2 class 1 OMP non-producing strains for which no amplification products were obtained in PCRs, restricted genomic DNA was subjected to Southern hybridization using *porA*-specific probes in order to assess whether the *porA* gene was present. Fragments from restricted

genomic DNA of both strains hybridized to the *porA*-specific probe indicating that at least part of the gene was present in these strains. Inability to amplify *porA* sequences from these two strains may be attributed to deletion or to mutations in the primer regions in these sequences. Following the report (8) that variable expression of the class 1 OMP is related to the spacing between the -10 and -35 regions of the promoter and the number of G residues between them , we amplified and sequenced the promoter regions of the 8 strains that did not express the class 1 OMP. Two control strains that produced the class 1 OMP had a stretch of 10 and 12 G residues, respectively, between the -10 and -35 regions. The strains that did not express the class 1 OMP had stretches of 9, 10, 11 or 12 residues but in 7 out of the 8 strains, one of the G residues in the stretch was changed to an A. Thus, variations of sequences between the -10 and -35 regions may determine gene expression in meningococci.

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Appearance of *N. meningitidis* serogroup B:ET15 in Canada.

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Serogroup C meningococcal disease has increased in Canada during the past several years due to the emergence of a specific bacterial genotype, ET15 (1), which is a member of the ET37 complex (2,3). This increased incidence has been accompanied by a number of outbreaks (4-6) which have led to mass-vaccination programs to interrupt the spread of disease (5,6).

Prior to August, 1993, nearly 700 group C:ET15 disease isolates were obtained from patients while no group B:ET15 isolate was found. From August 1993, onwards, nine B:ET15 disease (blood/CSF) isolates and one urethral isolate were obtained from patients ranging in age from 4 months to 48 years. These B:ET15 strains were serotype 2a, subtype P1.2, P1.2,5 or P1.- and expressed opa protein epitopes which are also prevalent on C:ET15 isolates. Analysis of these isolates by pulsed-field gel electrophoresis using restriction enzymes *Bgl*II, *Spe*I and *Not*I (7) revealed that all B:ET15 strains had chromosomal genotypes that differed from each other, yet were similar to PFGE genotypes previously obtained for C:ET15 strains in Canada. This suggests that the B:ET15 strains examined so far are derived from multiple ancestral genotypes, all of which are very closely related to the more commonly recovered C:ET15 disease-causing strains.

Sharing of ETs between strains of different serogroups has been observed previously by Caugant et al. (2). Genes encoding enzymes required for synthesis of chemically different serogroup B, C, Y and W135 polysaccharides may be partly non-homologous (8). The B:ET15 phenomenon may, therefore, prove to be attributable to qualitative differences in the capsular-polysaccharide genes arising on an ET15 genotypic background, perhaps as a consequence of localized recombination. It is of special interest to note that, with the exception of one isolate, the occurrence of B:2a:ET15 among Canadian disease isolates did not begin until about 7 months after mass-vaccination campaigns were initiated. Therefore, the human immune response to group C polysaccharide may have contributed to the appearance of B:ET15 strains by conferring a selective advantage to rare genetic variants in the bacterial population. Regardless of the reasons for appearance of B:2a:ET15 strains in Canada, it is clear that continuous surveillance is needed to monitor the potential emergence of this genotype, particularly in view of the fact that there is still no satisfactory group B vaccine.

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Antimicrobial susceptibility test for evaluation of *Neisseria meningitidis* C isolated during an urban epidemic, Rio de Janeiro 1993-1995

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Geographical information about antimicrobial susceptibility of *Neisseria meningitidis* became a significant factor in the investigation. Emergence of *N. meningitidis* with relative resistance (penRR, MIC≥0.1 to 1μg/ml) and rarely resistant to penicillin (MIC>1μg/ml) has been reported during the last decade (1). Most of the reports come from Europe (1). Nevertheless, a few reports from North America of penRR *N. meningitidis*, have appeared in medical literature recently (2,3). This phenomenon does not seem to be related to a particular serogroup (4). Another significant problem for Public Health, is the development of resistance to rifampicin (MIC≥0.25μg/ml), currently used for chemoprophylaxis of meningococcal disease (MD). Sulphonamide has not been used in clinical practice in most countries, because the presence of high prevalence of resistant strains. Although discrimination between resistant (MIC≥10μg/ml) and susceptible *N. meningitidis* to sulphonamide has been shown as an useful epidemiological tool (5).

Determination of the antimicrobial susceptibility pattern of *N. meningitidis* to penicillin (PN), rifampicin (RA) and sulphonamide (SD) is our aim. We have decided to work with these three drugs because of their clinical and epidemiological importance.

Minimum inhibitory concentrations (MIC) of 73 clinical isolates of *N. meningitidis* C to PN, RA and SD, were determined by the agar dilution procedure, according to NCCLS guidelines (6). *N. meningitidis* were obtained from the Instituto Estadual de Infectologia São Sebastião (IEISS), the Reference Centre for MD in Rio de Janeiro, Brazil. The strains were isolated between 1988-1995. 80% of the bacteria belongs to the epidemic wave registered from 1993 -1995, in the city of Rio de Janeiro (UVE/IEISS/SESRJ).

All *N. meningitidis* were fully susceptible to PN (MIC≤0.06μg/ml). Of the total, 45% were resistant to SD. Seven strains (9%) were resistant to RA. Three had a MIC value for RA of 0.25μg/ml and four of 0.5μg/ml.

Determination of regional MIC values of *N. meningitidis* is the first step for an efficient surveillance program. The high resistance level demonstrated for sulphonamide is a significant observation and may serve for epidemiological surveillance. Group C strains characterised in different epidemics registered in Brazil in the 90's were sulphonamide-susceptible (7). The epidemic registered in Rio de Janeiro is associated with a shift in the age-distribution of the disease towards older children and teenagers (UVE/IEISS/SESRJ). Certain *N. meningitidis* are more likely to be associated with disease in older age-groups, like a particular sulphonamide-resistant phenotype of the ET-5 complex described in England (5). Resistance to RA among

clinical isolates poses a serious problem for the control of MD secondary cases. A significant proportion of MD secondary cases (6%) have been registered in Rio de Janeiro, where chemoprophylaxis with RA is current recommended (8). The presence of RA-resistance detected in this study may in part explain the failures of chemoprophylaxis in our region. Nevertheless, we must take into account that some secondary cases are the result of the failure to detect all close contacts of an index case (8).

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Epidemiology and molecular analysis of epidemic meningococcal disease related to group C *Neisseria meningitidis* in a Brazilian metropolis: Rio de Janeiro, 1993-1995

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Since the first report of epidemic meningococcal disease (MD) in Rio de Janeiro in 1921, three other epidemics have been reported: two in 1974 (groups C and A) and one in 1989 (group B) (1). Since 1992, disease incidence has steadily risen in Rio de Janeiro, a city in the south-east of Brazil, with a population close to 6 million. In 1995, the epidemic peaked, with a recorded incidence of 9.5/100,000. The increased incidence was related to the presence of *Neisseria meningitidis* C. Epidemics of MD serogroup C have affected some southern Brazilian cities in the 90's and more than 70% of strains have been characterised as C:2b:P1.3 sulphonamide-sensitive (ET-40; ET-11 complex) (2).

The general purpose of the study is to provide clinical and epidemiological information about MD during an epidemic wave and describe the causative strain. The investigation took place in the Instituto Estadual de Infectologia São Sebastião (IEISS), the local Reference Centre for MD in Rio de Janeiro. The IEISS has notified in the media, 72% of all known cases in the city of Rio de Janeiro. Cases study were carried out involving the investigation of all patients admitted to the hospital between 1989-1995. Data for 1995 is not yet definitive. Either clinical case definition (fever and a purpuric rash), or bacteriologic criteria (*N. meningitidis* isolated from blood or cerobrospinal fluid or a positive CSF latex test) eligible cases will be included in the study. The Bacteriology Laboratory of the IEISS characterises more than 90% of *N. meningitidis* isolates obtained in the metropolitan area and stores the bacteria at -70°C. Sero/subtype are currently determined in the Instituto Adolfo Lutz (IAL) using monoclonal antibodies (2a,2b,4,8,15,17,19,23; P1.2,P1.3,P1.4,P1.7,P1.9,P1.14,P1.15,P1.16) and whole-cell suspensions. The electrophoretic types (Ets) were determined by analysis of allelic variations in 13 enzymes (electromorph profile) as previously described (3). Minimum inhibitory concentrations (MIC) of sulphonamide (SD, sulphonamide-resistant MIC≥10μg/ml) were determined following a standard guideline (4).

4161 MD patients have been identified between 1989-1995, 51% of which are residents in the city of Rio de Janeiro ('89-'92: 1115 cases; '93-'95: 1017 cases). Patients were classified as meningitis (32%), septicaemia and meningitis (60%) and septicaemia (8%). Lethality was 10% and clinical presentation was a significant determinant for a fatal outcome. Of the total, 60% presented a bacteriological criteria. Between '89-'92, 12% were group C and between '93-'95, 41% ('93: 20%, '94: 35%, '95: 59%). A shift in the age-distribution of the disease was observed during the epidemic period. The proportion of cases in those 10-19 years was 19% (15-19 years: 6%) before 1993 and 24% (15-19 years: 12%) from 1993-1995. For group C disease, media age

was 12 years and median was 10 years and for group B disease media was 11 years and median was 6 years.

Analysis of the epidemic period included 342 clinical isolates of *N. meningitidis*, 203 group B and 139 group C. 81% of group B strains were of serotype 4. Among group C strains, 73 were of serotype 2b (‘93: 8; ‘94: 16; ‘95: 49) and 44 of 2a (‘93: 10; ‘94: 14; ‘95: 20). Among group C: 2b strains in 1995, 90% were C:2b:nt. 84 group C strains were analysed by starch-gel electrophoresis. 78% were isolated during the epidemic wave (‘93-’95). 60 (95%) group C strains of the serotypes 2a or 2b were related to a unique clone of the ET-40. The ET-40 was first described in the city of São Paulo (2). 14 new Ets were found among serotypes W135:23, C:4 and C:NT. 33% of the ET-40 strains were sulphonamide-resistant.

Multilocus enzyme electrophoresis was an important tool in defining the epidemic strain. The increase of serogroup C disease, associated to two different serotypes, has been investigated in Spain recently (5). The epidemic strain described in Rio de Janeiro shows striking differences when compared with reports of recent epidemics in our country, particularly the trend to affect older age groups (2,6). A new subtype may be associated with the C:2b:nt mentioned above. Although we have not included all monoclonal antibodies for the subtype screen that were previously described.

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Antigenic variation of the class 1 outer membrane protein in an emerging *Neisseria meningitidis* clone in the Netherlands

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The class 1 outer membrane protein P1 of *Neisseria meningitidis* is a vaccine candidate against meningococcal infection. Antigenic variation of the class 1 outer membrane protein P1, was studied. The prevalence of serogroup B P1.4 subtype among Dutch isolates increased 200 fold since 1980, and is associated with the five-fold increase in serogroup B meningococcal disease in the last 15 years. Screening of the strain collection of the Netherlands Reference Laboratory for Bacterial Meningitis for this subtype, revealed that P1.4 has been present in the Dutch population since 1965. Sequence analysis showed that *porA* genes of genotypically distinct P1.4 strains have different VR1 regions, but contain identical VR2 regions. This indicates that the VR2 region encoding the P1.4 epitope may have spread through the meningococcal population via horizontal gene transfer. Genotyping of P1.4 strains showed that one cluster of strains, the ET24/25 cluster, is responsible for the increase since 1980. ET24/25 strains not expressing the P1.4 subtype, seem to have lost the P1.4 epitope encoding region by replacement via horizontal gene transfer or by partial deletion via recombination between direct repeats.

Non-culture diagnosis and serogroup determination of meningococcal B and C infection by a sialyltransferase (*siaD*) PCR ELISA

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In the UK each year, an increased proportion of suspected meningococcal infections remain unconfirmed by culture, due in part to the increasing practice of pre-admission parenteral antibiotic treatment and reluctance to perform lumbar punctures(1). This is evident by the differential between the number of culture proven case isolates referred to the Public Health Laboratory Service (PHLS) Meningococcal Reference Unit (MRU) and the number of notified cases of meningococcal disease recorded by the Office of Population Censuses and Surveys (OPCS)(2). The rapid non-culture confirmation of meningococcal disease is of growing importance for case ascertainment and epidemiological surveillance.

Recently, there have been several publications describing the use of PCR for the detection of meningococcal DNA in clinical specimens(3,4,5,6), however, none of these PCRs provide epidemiologically useful information about the causative organism. The identification of the serogroup of the organism is essential for effective outbreak control and contact management. During recent years, the majority of meningococcal infections in the UK were due to either serogroup B (70%) or C (30%)(2). To combine the non-culture diagnosis of meningococcal infection from CSF, whole blood and serum with serogroup (B and C) identification, a polymerase chain reaction assay (PCR), based on a restriction fragment length polymorphism (RFLP) in the meningococcal serogroup B and C *siaD* gene, was developed.

The similarity between the biochemical nature of meningococcal polysialic acid B (α2-8 linked sialic acid) and C (α2-9 linked sialic acid) capsules(7) has so far hampered the identification of target sequences to differentiate meningococci expressing serogroup B and C capsules. Hybridization occurs between serogroup B, C, W135 and Y capsular genes involved in directing sialic acid synthesis, but not the serogroup-specific sialyltranferases involved in the polymerization of the sialic acid to the polysialic acid chain (8). Nucleotide sequencing of the sialyltransferase (*siaD*) genes of serogroup B and C meningococci (9) has enabled identification of sequence differences allowing the specific identification of serogroup B or C *siaD* genes.

The PCR assay was adapted to an ELISA format incorporating hybridization with serogroup-specific B and C oligonucleotide probes. The specificity for CSFs was 100% and sensitivities were respectively 81, 63 and 30% for CSFs, whole blood and sera.

The *siaD* PCR has been used in several outbreak investigations, whereupon non-culture identification of the serogroup from serum or whole blood DNA confirmed the serogroup to be the same as that of the outbreak strain in the absence of positive culture confirmation. In one instance this was substantiated by a subsequent throat swab isolate from the *siaD* PCR diagnosed

case. Following an outbreak of meningococcal disease amongst a student community caused by a B:15:P1.7,16 sulphonamide resistant (R) strain, both serogroupable and non-groupable isolates were cultured from nasopharyngeal swabs from students investigated. The non-groupable (NG) and B:15:P1.7,16 R isolates examined were all identified as serogroup B by *siaD* PCR ELISA. During another outbreak investigation caused by a C:2a:P1.2 R strain amongst secondary school children, 3 NG and 3 C:2a:P1.2R were confirmed as serogroup C.

The serogroup-specific *siaD* PCR ELISA is a significant addition to currently available tests for non-culture diagnosis of meningococcal infection and outbreak investigation.

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Epidemiology of meningococcal meningitis in Niamey, Niger: 1989-1995

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Introduction: Although epidemics of meningococcal meningitis (MM) within the "meningitis belt" of SubSaharan Africa are well-described, few reports with population-based data describe MM between epidemics in the context of bacterial meningitis in general. Our objective was to describe the epidemiology of bacterial meningitis in Niamey, Niger (1995 population est.: 547,739) during 1989 to 1995, a period which included an epidemic (1994/5).

Methods. The bacteriology laboratory at CERMES evaluates all cerebrospinal fluid (CSF) specimens obtained at the National Hospital of Niamey, where all Niamey residents with suspected meningitis are hospitalized. We reviewed laboratory and hospital records associated with CSF specimens submitted between June 1989 and May 1995. A case of bacterial meningitis was defined as occurring when at least one of the following criteria were met: direct exam with $\geq$100 WBC/ml of CSF, detection of antigen from CSF by latex agglutination, or isolation of bacteria from CSF culture. To permit evaluation of information related to each meningitis season, we analyzed data for the 12 months from June to May, rather than for calendar years.

Results. Of a total of 3,706 bacterial meningitis cases, 2505 (67.6%) were due to *N. meningitidis*. Among those, 1808 occurred during the 1994-95 epidemic. The annual incidence (cases per 100,000) of meningitis due to each pathogen was:

Year	***N. meningitidis***	***S. pneumoniae***	***H. influenzae***
1989-90	38.7	16.3	15.6
1990-91	33.4	15.1	13.9
1991-92	52.2	18.8	9.1
1992-93	29.3	17.3	11.0
1993-94	3.8	11.9	12.3
1994-95	346.3	7.3	5.2

N. meningitidis was the principal pathogen associated with bacterial meningitis in each year except 1993-4, when the incidence of meningococcal meningitis fell substantially. During 1993-4, the incidence of meningitis due to *S. pneumoniae* and *H. influenzae* was similar to previous years. Seasonal variation was evident during both epidemic and nonepidemic years. The majority of cases occurred during the dry season, from December to May, with peak disease during March and April of each year. Incidence was consistently higher in males compared with females (male to female ratio: 1.5:1). The case fatality ratio for meningococcal meningitis was 7.8%, which was substantially lower than case fatality of meningitis due to *S. pneumoniae* (45%) or *H. influenzae* (36%). The vast majority (82.5%) of meningococcal meningitis cases occurred among persons less than 20 years of age. In the nonepidemic years, the highest incidence of

disease occurred among children 10-14 years of age (62/100,000). During the epidemic, high rates of disease occurred in all age groups <30 years old.

Age (years)	Annual incidence 1989-94	Annual incidence 1994-95
< 1 year	32	421
1-4	37	516
5-9	49	489
10-14	62	499
15-19	40	484
20-29	12	129
30-39	8	66
40-49	4	30

Among meningococcal cases, serogroup A predominated in all years except 1991-92. During 1991-92, serogroup C caused more than 50% of meningococcal meningitis. The incidence of serogroup C meningococcal disease peaked at 29 cases per 100,000 during 1991-2. The epidemic of 1994-95 was caused by serogroup A, subtype 4:1.9, clone III-1 (1).

Conclusions. These data suggest that *N. meningitidis* is the leading cause of meningitis in Niamey in endemic as well as epidemic years. Most disease is due to serogroups A and C, suggesting that serogroup A/C meningococcal conjugate vaccines currently under development offer the potential to prevent substantial endemic disease as well as preventing periodic epidemics. Meningococcal disease incidence fell markedly during 1993-4, the year preceding a major serogroup A epidemic. Since the incidence of meningitis due to *H. influenzae* and *S. pneumoniae* were stable during 1993-4, the meningococcal disease trough was unlikely to reflect changes in diagnostic practices or laboratory methods. The low rate of disease in 1993-4 may reflect the absence of circulating strains during that year. Subsequent introduction of the virulent III-1 clone into a highly susceptible population probably accounted for the 1994-5 epidemic. We are not aware of other meningococcal epidemics preceded by marked meningococcal disease troughs. Evaluation of longitudinal surveillance data in other meningitis belt countries would help determine whether meningococcal epidemics might be predicted by troughs in meningococcal disease in previous years, which might then provide an early alert to the risk of subsequent epidemics.

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Improved surveillance of meningococcal disease in Norway by continual connection of the epidemiological and bacteriological data

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At the end of 1994, the Department of Bacteriology, NIPH, Oslo, became National Reference Laboratory (NRL) for meningococcal disease. At the same time, the Norwegian Infectious Diseases Notification System was integrated into the Department. This new structure permitted a continual surveillance of the epidemiological situation and allowed early intervention on population groups at high risk of meningococcal disease.

In the 18-month period, from September 1, 1994 to February 29, 1996, a total of 220 cases were notified in Norway and the disease causing isolate was received at NRL for 203 (92%) of the cases. All isolates were analysed by serogrouping, serotyping, and multilocus enzyme electrophoresis (1). Of the 203 patient strains, 73% were serogroup B, 21% were serogroup C, and 4% were serogroup Y. The 9 cases caused by serogroup Y were significantly older: 7/9 patients were between 39 and 91 years old. While serogroup B disease was at 66% in the first 6-month period, it increased again to over 75% in the last year.

The proportion of ET-5 complex strains increased again from just over 40% in the first to 54% in the last six-month period, while the proportion of ET-37 complex strains remained low (under 10%). The main epidemiological change observed is that 50% of the serogroup C strains were serotype 15:P1.7,16, and belonged to the ET-5 complex. These strains were responsible for a large part of the new increase in disease due to ET-5 strains. Twelve cases (6%) were caused by clones of lineage III, that has been associated with the recent hyperendemic wave of disease in The Netherlands (2).

Fourteen cases (2 of them fatal) were caused by a clone-complex that only newly was associated with disease in Norway. While the first three cases occurred in the same region within a month, the additional cases were spread through the country. Most of these isolates were serologically characterized as B:4:NST. DNA sequence analysis of the *por*A gene of these strains revealed an identical sequence (P1.18,25) coding for a porin against which no monoclonal antibodies have been developed so far.

In the described 18-month period there were six pairs of associated cases and one group of three cases occurring within the same communities in the course of one day and up to ten weeks, and caused by identical isolates. In addition, a one-year old girl underwent two episodes of meningococcal disease 5 months apart, caused by the same strain (B:15:P1.7,16; ET-5).

Two outbreaks were identified. The first one occurred in the Sør-Trøndelag county from April to December 1995 and was caused by a clone variant of the ET-5 complex, with serological characteristics B:15:P1.7,16. It involved 10 cases (8 culture-verified cases and 2 cases likely due

to the same clone), one of them being fatal. A carrier study around the cases showed a very low prevalence of the outbreak clone. Carriers of the virulent strain were treated prophylactically with ciprofloxacin. The second outbreak was in the Hordaland county from September 1995 to February 1996 and was caused by an unusual ET-5 strain in that it belonged to serogroup C. Following our recommendation, serogroup C vaccination of all children between the age of 2 to 5 and young people from 13 to 21 years old (3 950 individuals) was performed in February 1996 in the part of Hordaland county afflicted by the outbreak.

The new organisation of meningococcal surveillance in Norway has permitted the early recognition of two outbreaks, allowing intervention on the population at risk. It has also shown that the number of associated cases, outside the close contacts, may be higher than earlier estimated (3).

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Detection of *Neisseria gonorrhoeae* in clinical samples by the polymerase chain reaction

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The gold standard for diagnosis of gonorrhoea has always been the culture and the presence of gram-negative diplococci in smears of genital discharge (1). However, these methods have several disadvantages including false-negative results and several days may elapse before cultural diagnosis can be established (1). Non-cultural methods offer several advantages over conventional procedures because diagnosis is not dependent upon the presence of viable micro-organisms for microbial isolation and turnaround times can be significantly reduced (1). The development of the polymerase chain reaction (PCR) has been shown to offer a sensitive and specific alternative approach for the diagnosis of a variety of bacterial pathogens. In the present study, a PCR assay (2) was used for direct detection of *Neisseria gonorrhoeae* in clinical specimens of 200 consecutive adult males presenting with acute urethritis at a STD clinic in Durban, South Africa. Ho *et al*. (2) developed this assay by targeting the *cppB* gene found on the 2.6 MDa cryptic plasmid or integrated into the gonococcal chromosome. Swabs, containing urethral exudate, were collected and suspended in phosphate-buffered saline, pH 7.3. Samples were centrifuged and resulting pellets suspended in 100 ml 1 x PCR buffer with 0.45% non-ionic detergent Tween 20 and 200 mg/ml proteinase K. Cell suspensions were incubated at 50-60°C for 1 h, heated to 95°C for 10 min (2) and suspensions stored at -20°C for future use. Crude clinical lysates and 30 pure cultures of *N. gonorrhoeae* were amplified using two 20-mer oligonucleotide primers HO1 (5' GCTACGCATACCCGCGTTGC 3') and HO3 (5' CGAAGACCTTCGAGCAGACA 3') (2). 40 cycles of amplification were performed, each of which consisted of a 30s denaturation step at 94°C, a 1 min annealing step at 55°C and a 30s template elongation step at 74°C. Amplified product was analysed by electrophoresis in a 2% agarose gel, which was examined for the presence of a 390 bp fragment. The 390 bp amplimer was obtained for the pure cultures as expected, the specificity of which was confirmed by restricting the amplified product with *Msp*I, to give two fragments of 250 and 140 bp. A sensitivity of 100% by PCR was obtained for the pure culture. Initial PCR assays of clinical samples using pure culture parameters did not yield the expected 390 bp amplimer. Instead smears that stopped exactly at the 390 bp amplimer position or smears without a distinct stop were obtained. In order to optimise the PCR assay for clinical specimens, the amount of crude lysate used as the template DNA was decreased and the annealing temperature increased from 55°C to 60°C. The required 390 bp product was, however, not obtained for all samples. Decreasing the dNTP concentration, increasing the $MgCl_2$ concentration, chelating excess phosphate ions with sodium citrate, decreasing the number of reaction cycles and phenol-chloroform extraction of the crude lysate, have been attempted in an effort to further optimise the assay. Compared to Ho *et al*. (2), who obtained a sensitivity of 100% and a specificity of 88.9%, preliminary evaluation of 50 clinical specimens showed that of 42 specimens that were culture positive, only 37 were PCR positive. Among the 8 culture negative specimens, four were found to be PCR positive. The large number of false-negative PCR results seen could be as a

result of (i) the inability of primers to anneal with appropriate sites and prime amplification in all strains; (ii) inhibition of the *Taq* polymerase by a substance present in the extracts of clinical specimens; and (iii) low numbers of gonococci present in exudate as a function of length of infection. The use of the PCR assay as a diagnostic tool is promising but requires optimisation and further evaluation.

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Prevalence of *Neisseria meningitidis* serotype 22 in Germany

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The serotypes/serosubtypes are important epidemiological markers of *Neisseria meningitidis* isolates. Antigenic variations of the outer membrane proteins (OMPs) of classes 2 and 3 define the serotype, those of class 1 the serosubtype of a meningococcal strain. Six hundred and forty-four (45,3%) out of 1421 *N. meningitidis* strains isolated between January 1989 and March 1996 from patients and carriers in Germany, were non-typable (NT) with the currently available monoclonal typing reagents. The National Reference Laboratory in Prague has produced a new serotype 22-specific monoclonal antibody (McAb) which reacts with the epitope of serotype-specific class 2 OMP (1). Its suitability for extending the panel of McAbs currently used for strain discrimination was tested. The isolates examined comprised all subculturable non-typable (NT) as well as non-subtypable (NST) strains of the years 1989-1993 and all NT strains from 1994 to March 1996. Forty-five (11,3%) of 397 meningococcal NT isolates of Germany tested reacted with this new McAb (serotype 22) in whole-cell ELISA (2). The highest prevalence of serotype 22 was seen in 1993 (25%, 7 out of 28 NT/NST strains). Thirty-three (73,3%) of meningococcal serotype 22 isolates derived from patients with invasive disease and 11 (24,4%) from carriers, the origin of one serotype 22 strain remained unknown. With the exception of one isolate, which was serogroup C, all serotype 22 strains belonged to serogroup B (44 strains) although 280 serogroup B and 49 serogroup C strains were examined. The prevalent antigenic formula of serotype 22 strains from 1994 to 1996 (27 isolates) was B:22:NST (10 isolates) and the second most common B:22:P1.2,5 (5 isolates). The geographic distribution of serotype 22 demonstrates that it is found almost exclusively in the region of the former FRG and shows a cluster in Berlin.
Conclusion: Employment of McAb 22 in meningococcal typing provides additional valuable epidemiological data.

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Random Amplified Polymorphic DNA (RAPD) genotyping of serogroup A meningococci yields results comparable to those of Multilocus Enzyme Electrophoresis (MEE).

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The RAPD genotyping method was applied to 1 representative each of the 84 Neisseria meningitidis serogroup A electrophoretic types (ETs) previously described (1). With few exceptions, the subgroup structure was similar by both methods but one strain (ET28 in subgroup I) was assigned to a different subgroup by RAPD and MEE. Further tests showed that six other isolates of ET28 were assigned to subgroup I by RAPD and MEE.

These results were extended by comparing additional strains from individual ETs of serogroup A. Again, the results of RAPD and MEE were fully consistent. Finally, an independent sample of 18 serogroup A strains isolated in The Netherlands was also analyzed by RAPD and MEE. These bacteria again yielded comparable results between the two methods and showed that isolates from the Netherlands included bacteria from subgroups which had not been formerly described. One such strain was assigned to the ET5 complex, which is more typical of serogroup B strains (2).

RAPD analysis was faster and involved less work than MEE. Strains can be linked to previously characterized genotypes in a computerized database, and dendrograms based on cluster analyses can be generated easily. These properties of RAPD make it suitable for quickly assigning new isolates of serogroup A bacteria to known subgroups, for
defining new subgroups and possibly for similar purposes with epidemic outbreaks caused by serogroups B and C.

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Epidemic meningococcal disease and tobacco smoke: A risk factor study in the Pacific Northwest

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Background. *Neisseria meningitidis* causes an estimated 2,600 cases of bacterial meningitis and sepsis annually in the United States, with an incidence of one case per 100,000 population and a case-fatality rate of 10 to 15 percent. Since 1992, Oregon and southwest Washington have experienced a substantial increase in the incidence of meningococcal disease, with rates reaching four to eight times the national average. Most of these infections were caused by a group of closely related serogroup B meningococcal strains belonging to the enzyme type 5 (ET-5) complex. The only meningococcal vaccine currently licensed in the United States does not protect against serogroup B disease. Thus, identifying modifiable risk factors for meningococcal disease may be the only means of altering the course of this epidemic.

Antecedent viral infection, household crowding, tobacco smoke exposure, and bar patronage have all been implicated as risk factors for meningococcal disease. However, many of these studies were performed with small numbers of cases, and none assessed the independent contribution of each factor or evaluated the magnitude of risk associated with specific exposures. In this report, we describe the results of a large population-based study to define independent risk factors for meningococcal disease. Using these adjusted estimates of risk, we calculated the proportion of disease associated with specific exposures to develop public health recommendations.

Methods. We performed a case-control study of risk factors for invasive meningococcal disease, comparing 129 cases (67% serogroup B) identified through population-based surveillance in Oregon and southwest Washington (3.5 million aggregate population) with 274 age- and area-matched controls. A case-patient was defined as any resident of the study area from whom *N. meningitidis* was cultured from a normally sterile site between January 1 and December 31, 1994. Control households were identified through a process of random digit dialing, using the modified Mitofsky-Waksberg method. Study subjects or their guardians were interviewed via telephone by trained personnel using a standardized questionnaire. Matched odds ratios were calculated and assessed using maximum likelihood estimates. Dose-effect relationships were investigated for variables with more than two ordered levels. Conditional logistic regression analysis was performed to determine independent risk factors for disease. Adjusted population attributable risks were computed using methods appropriate for multivariate analysis with pair-matched data. Similar results were observed when the analyses were stratified by causative serogroup.

Results. Among children less than 18 years of age, having a mother who smokes was the strongest independent risk factor for invasive meningococcal infection (OR, 3.8; CI, 1.6 to 8.9).

Low maternal education, lack of a primary physician and living in a household with other children, and attending school with 30 or more students per classroom were also independent risk factors for meningococcal disease in children. Using a humidifier in the month of interest was protective, and despite its strong correlation with both the smoking and education variables, church attendance was also associated with a decreased risk of disease.

Among adults, having a chronic underlying illness (OR, 10.8; CI, 2.7 to 43.3) and passive tobacco smoke exposure (OR, 2.6; CI, 1.0 to 6.9) were independently associated with invasive meningococcal infection. In addition, active smoking conferred an elevated risk of disease, although this association did not remain statistically significant after adjusting for the other factors (OR, 2.4; CI, 0.9 to 6.6). Risk of meningococcal disease increased with increasing passive smoke exposure in all age groups, and 37 percent (CI, 15 to 65) of meningococcal disease in children could be attributed to maternal smoking.

Conclusions. We found that having a mother who smokes was the strongest independent risk factor for meningococcal disease in children. Results were most dramatic for children less than 5 years old. Adults who were exposed to passive or active tobacco smoke were also more likely to develop disease. Previous studies have implicated tobacco smoke as a risk factor for invasive meningococcal infection (1,2). However, this is the first study to assess the magnitude of risk conferred by tobacco exposure when adjusting for all of the other factors identified, including socioeconomic status and household crowding.

Several studies have addressed the biologic plausibility of tobacco smoke facilitating invasive bacterial disease. Through either its mechanical effects on the respiratory mucosa or its functional effects on the immune response (3), tobacco smoke may directly promote the adherence and/or invasion of meningococci. The association between smoking and meningococcal disease may also be due in part to its indirect predisposition to viral respiratory illness (4). Finally, tobacco smoke, both active and passive, has repeatedly been identified as a risk factor for meningococcal carriage (5,6).

This study provides further evidence that tobacco smoke is a potentially modifiable risk factor for a significant proportion of meningococcal disease. Causality is supported by the strength of its independent association with disease, the demonstration of a dose-response relationship, and biologic plausibility. Reductions in tobacco smoking, especially among mothers with young children, may substantially decrease the incidence of meningococcal disease.

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Epidemiology of meningococcal disease in the Republic of Ireland

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Previous laboratory record review has documented *Neisseria meningitidis* as a cause of significant morbidity in Irish children (1). Establishing current meningococcal disease (MD) incidence rates and serogroup distribution is required to update epidemiological patterns of this disease. A laboratory based surveillance system for culture confirmed MD was established in November 1994 in the Republic of Ireland (ROI) (population 3.5 million). Monthly return forms provide details of cases (name, age, gender, site of isolate, clinical diagnosis, serogroup and survival status). Data are presented here for cases with *N. meningitidis* cultured from deep sites (blood, cerebro-spinal fluid (CSF), and joint fluid) for 1995.

The occurrence of 209 cases throughout 1995 constitutes an annual MD incidence of 5.9 per 100,000 population for the ROI with the rate varying between 8.5 per 100,000 population in the health board with the highest incidence and 0.5 per 100,000 population in that with the lowest incidence. Females comprised 100 (52.6%) cases and 104 (50%) cases occurred in the 4 month period December through March. Nineteen (9.1%) cases died. The highest age-specific annual incidence occurred in children under 1 year (88 cases per 100,000). Serogroup data were available for 195 (93.3%) cases, of which group B comprised 105 (53.8%) cases and group C 87 (44.8%) cases. While the national incidence of serogroup C disease was 2.5 per 100,000 population one health board region had a group C incidence rate of 4.3 per 100,000 population. The mean age of cases with serogroup B disease (7.5 years, range 0.08-68.90) was significantly lower than that for serogroup C disease (9.6 years, 0.17-88.00) ($p=0.02$). Of the two health board regions with the greatest overall MD incidence rates the Eastern Health Board had significantly more cases due to serogroup B than serogroup C (66 versus 34 cases) compared to the Southern Health Board (16 versus 23 cases) (Odds ratio=2.79, 95% confidence interval 1.21-6.46, $p<0.01$).

This surveillance system is the first to document the epidemiology of MD nationally in the ROI. The incidence rate of 5.9 per 100,000 population is one of the highest rates in western Europe and about 4.5 times that reported in the United States (US) (2). The fatality rate of 9.1% is a minimum rate as this data is provided by a laboratory-based system rather than by clinicians. The annual age-specific incidence for meningococcal disease in children under 5 years (43 cases per 100,000) is 72% higher than that for Haemophilus influenzae type b (Hib) disease prior to the introduction of Hib conjugate vaccine in the ROI (3). MD is now the most serious infectious disease which is currently non-vaccine preventable by primary immunisation in the ROI. The variation in MD incidence rates between different regions in the ROI is wide with that in the highest incident health board being 17 times higher than the rate in lowest incident health board. The current lack of sufficiently effective vaccines for primary prevention of MD is a major drawback in controlling this disease. If the currently available polysaccharide meningococcal A

& C vaccine was to be 100% effective and provided to all children at the earliest effective age of 2 years only 29% of all MD in the ROI could be prevented. However, when a conjugate meningococcal vaccine for group C disease becomes available the proportion prevented could be increased to 42% of all disease nationally (assuming vaccine efficacy from 2 months of age). Whatever impact a meningococcal group C conjugate vaccine can make in reducing the burden of this disease, an effective group B vaccine will be necessary to achieve the ultimate - meningococcal disease eradication.

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Production of monoclonal antibodies against *Neisseria meningitidis* that recognize specific and cross reactive antigens

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Meningococcal meningitis and septicemia caused by *Neisseria meningitidis* continue to be worldwide public health problems. The mortality rate is high, and the disease occurs in epidemics.

The monoclonal antibody directed against *N. meningitidis* was established by hybridoma technology (1). Clone 8C7Br1 was obtained from a fusion of mouse spleen cells with the murine myeloma line X63-Ag8.653. Mice were immunized with two doses of live meningococci given both intraperitoneally and intravenously three days before fusion (2). The meningococcal strain used in this study was B:4:P1.9 recovered from patients with meningococcal disease in Brazil. The selection of monoclonal antibodies was initially based on their binding with the homologous strain by ELISA. Immunoblotting of SDS-PAGE resolved, *Neisseria gonorrhoeae*, *Haemophilus influenzae b*, *Escherichia coli*, *Bordetella pertussis*, *Salmonella typhimurium*, *Shigella flexneri* and *Bacillus subtilis* were used to demonstrate antibody cross reactivity. The MAb 8C7BR1 recognized their target antigen, a 50 kDa protein, in different serotypes and subtypes of *N. meningitidis*, *Neisseria lactamica*, *N. gonorrhoeae*, *B. pertussis*, *S. typhimurium* and *S. flexneri.* In *E. coli*, *H. influenzae b* and *B. subtilis* the monoclonal recognized a peptide of 65, 60 and 70 kDa, respectively. The interesting finding that different electrophoretic mobilities were obtained when reacting MAb 8C7Br1 with Brazilian case and reference strains of *N. meningitidis* are under investigation. We have generated the hybridoma cell line which produces IgM monoclonal antibodies reactive against specific antigens of different serogroups, serotypes and subtypes of *N. meningitidis* . These monoclonal antibody provide new tools for antigenic analysis of *N. meningitidis*.

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Prevalence and variation in class 5 expression by serogroup A,C and B during epidemics in Brazil

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The incidence of meningococcal Disease (MD) in Brazil has been monitored since the serogroup A and C epidemics that occurred between 1971 and 1974[1]. In 1988, the incidence of B MD in the greater São Paulo, area state of São Paulo , was over 4.06 per 100,000 inhabitants ,suggesting a new epidemic wave in this region.

The major proteins of the outer membrane of *Neisseria meningitidis* are designated class 1 through class 5. Each strain of *N. meningitidis* also variably expresses up to four distinct class 5 proteins. These heat -modifiable proteins are 25-30 kDa strain variants that differ only in their expression pattern.

Until now, the frequency of most of the class 5 proteins expressed by the Brazilian meningococcal strains had not been determined. Here we present the results of such tests. The production of two novel monoclonal antibodies specific for epitopes present in the class 5 protein derived from a Brazilian *N .meningitidis* epidemic strains (5.8 and 5.9) will contribute greatly to the determination of class 5 proteins to be included as antigens in the future vaccine for meningitis.

We analyzed 63 strains of serogroup A and 60 strains of serogroup C (from the 1972-1974 epidemics), and 136 strains of serogroup B(from the 1988-1994 epidemics). The serotypes and subtypes of *N. meningitidis* B studied were, respectively B:4: P1.15, B:4:P1.9 ,B:4:P1.7, B:4:P1.3, B:4:P1.14, B:4:P1.16, B:4:NT and B:NT:NT. The strains were recovered from blood or cerebrospinal fluid of patients with systemic disease. The anti-class 5 MAbs selected for this study were : 3E6(5.1), 3B4-C7 (5.3) ,1B61C7(5.4), 3DH9F5G8(5.5), 5F1F4-T3 (5.3) and the two new monoclonal antibodies C14F10Br2 (5.8) ,7F11B5Br3 (5.9). To prepare *N. meningitidis* samples (whole cells) for Dot-blot we used the method described in (2)·

Our results demonstrated that the expression of class 5 proteins in the *N. meningitidis* B Brazilian strains studied is highly heterogeneous. A vaccine for *N. meningitidis* B to be prepared in Brazil should contain not only the (5.5) antigen but also (5.4) and (5.c), because the expression of the latter one was found to be highly significant by us. The new monoclonal antibodies C14F10Br2 (5.8) reacted with (3.6%) of the serogroup B investigated, yet MAb 7F11B5Br3 (5.9) reacted with (8.8%) of serogroup A, (5%) of serogroup B and (5%) C strains of *N. meningitidis* .

We would like to emphasize the importance of establishing a broad set of antigenic characteristics of the prevalent meningococcal species found in each epidemic region, in order that truly effective vaccines and immunoprophylactic programs can be produced.

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A monoclonal antibody as a probable immunological differential marker to discriminate bacterial from non-bacterial meningitis

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In public health laboratory the diagnosis of *Neisseria meningitidis* infection is usually accomplished by bacteria culture, counter immunoeletrophoresis and latex agglutination (1).

A correct diagnosis of bacterial meningitis at an early stage of infection is of fundamental importance to the clinician to select the appropriate treatment promptly to avoid serious sequelae.

The partially treated bacterial meningitis cases, as well as those in which neither laboratory tests nor clinical symptoms show a clear distinction between bacterial and lymphocytic meningitis, and even cases of viral meningitis which present, at the on set of infection, a CSF (cerebrospinal fluid) pattern suggestive of a bacterial cause, continue to pose problems for correct diagnosis and, consequently, for early appropriate treatment.

Additional parameters indicating the presence or absence of a bacterial or viral agent would be helpful, especially in countries where meningitis is still frequent.

The new monoclonal 8C7Br1 directed against *N. meningitidis* was established by hybridoma technology (2). The monoclonal antibody was initially chosen based on their binding capacity with *N. meningitidis* strain by ELISA . The monoclonal antibody presented cross reactivity with *Neisseria gonorrhoeae, Haemophilus influenzae b, Escherichia coli, Bordetella pertussis, Salmonella typhimurium, Shigella flexneri and Bacillus subtilis.*

We used a new monoclonal antibody for testing by Dot-ELISA technique. A total of 168 CSF selected in diagnostic routine at Immunology Division of Adolfo Lutz Institute by counter immunoelectrophoresis and bacterial culture. The selected samples were obtained from patients with positive diagnosis of meningitis by *N.meningitidis B, N.meningitidis C, H.influenzae b, Streptococcus pneumoniae* and *E.coli.* Other CSF samples were from patients with some others neurological disorders. In order to see cross reactivity we used antigenic preparations of adenovirus, enterovirus and influenzae virus.

The new monoclonal antibody presented an agreement of reaction of 95% by Dot -ELISA reaction with the CSF of bacterial selected, meanwhile the MAb did not show reactivity with the selected viral antigen preparations or with CSF samples from patients with other neurological disorders.

The potential use of the new monoclonal antibody in Dot-ELISA reaction as a possible differential immunological marker in discriminating bacterial from lymphocytic meningitis justifies future research.

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Neisseria gonorrhoeae is clonal - sometimes

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The incidence of Gonococcal infection in Grampian region was the highest in Scotland in 1992, 1993 and 1994. Despite a considerable decrease in 1994, it remained more than twice that of the whole country. Grampian also differed from other regions in that 1A isolates were commoner, accounting for around 78%, 73% and 75% of isolates; the 1A-2 serovar was particularly common. The proportion of 1B isolates in Grampian (22%, 27% and 25%) was low compared with the other centres where 1B accounted for around 70% of all isolates (Young & Moyes, 1996). Seventy-six 1992,1993 and 1994 clinical isolates of various serotypes were obtained from the Scottish *Neisseria gonorrhoeae* Reference Laboratory including 40 strains from Grampian and 36 strains from Lothian. Genomic DNA was subjected to pulsed-field gel electrophoresis (PFGE) after digestion with low-frequency cleavage (LFC) endonuclease (Nhe1). The restriction patterns generated were reproducible, stable and easy to read. PFGE was compared to restriction endonuclease analysis (REA) with high-frequency cleavage (HFC) endonuclease (Hind III). To analyse the results of the Nhe1 and Hind III patterns Dice analysis as well as "Gel Compar" were carried out to facilitate further subdivision of the serogroups. Strains with values of >90% were considered to be clonally related. 37 patterns by PFGE and 36 patterns by REA were identified. A large number of the patterns, particularly those from 1A-2 serovars, were similar, many being identical. These findings clearly demonstrate the potential of PFGE as a highly discriminatory tool, for the epidemiological investigation of gonorrhoea. The temporal and geographical distribution of strains with these patterns indicated that a clone of *N. gonorrhoeae* was circulating in Scotland during the study period.

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Macrorestriction profiles of *Neisseria meningitidis* using pulsed-field gel electrophoresis: Novel epidemiological examples.

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The epidemiological characterization of *Neisseria meningitidis* is currently based upon variation in surface components using serological based methods. However, the epidemiology of meningococci is confounded by the underlying genetic diversity. One technique demonstrated to be useful for the genotyping meningococci is pulsed-field gel electrophoresis (PFGE) in which the genetic relatedness over the whole genome is considered (1,2,3) and is an important method for outbreak identification (4) and cluster analysis (2).

A practicable PFGE method for the characterization of meningococci received at the MRU was investigated, using the restriction enzymes *Sfi*I, *Spe*I and *Nhe*I. The standardized electrophoresis conditions used were; 200v, forward to reverse ratio = 1, switch times 20 to 25 seconds over a 22 hour time period, using the CHEF-DRII apparatus (Biorad). Four novel epidemiological examples are presented to illustrate the application of PFGE for genotyping *N. meningitidis.*

1. *In vivo* mouse model: The stability of the PFGE macrorestriction profiles were shown by the typing of isolates recovered from an infant mouse model of meningococcal infection (5). The original inoculum (B:15:P1.7,16R) and the isolates obtained from the nose, lungs and blood demonstrated identical PFGE DNA types using *Sfi*I and *Spe*I.

2. Stonehouse Meningococcal Survey (SMS): Epidemiologically related isolates obtained from volunteers in the SMS (6) were investigated using PFGE. Seven individuals with two or more isolates of phenotypes (B:15:P1.16R, B:15:NTR or NG:15:P1.16R) were PFGE typed. All the isolates had previously been considered genotypically similar based upon RFLPs using a random DNA-probe (7). PFGE typing demonstrated three main *Sfi*I and six main *Spe*I PFGE types, (where the type definition was based upon three or more fragment differences) (8). There was a single predominant PFGE type with either enzyme (with closely associated subtypes), but repeated isolates from some individuals demonstrated a diversity of profiles.

3. Military recruit study: The methodology was used to track the transmission and carriage of meningococci of a particular phenotype (X:4) amongst military recruits during a longitudinal study of *N. meningitidis* acquisition and carriage. The majority of the 59 X:4 isolates examined by PFGE were categorised into five *Sfi*I and four *Spe*I PFGE types. Two PFGE types predominated, corresponding to the X:4:P1.2 isolates and a single X:4:P1.16 isolate. The identifying of PFGE types has allowed a more accurate study of the acquisition and carriage of meningococci in the troops. The X:4:P1.2 and X:4:NT isolates were shown to be acquired by several recruits of a particular troop half-way through training. The X:4:P1.16 PFGE type was more frequently isolated from a particular troop, in which carriage was limited.

4. School outbreak: Following a C:2a:P1.2R associated school outbreak investigation 44 of 48 isolates examined were indistinguishable by *Spe*I and *Sfi*I PFGE typing. All three NG:2a:P1.2R isolates were similar to the outbreak PFGE type whereas single C:2a:P1.2R and the C:2a:NT:R isolates demonstrated distinct genotypes.

Genetic stability during meningococcal disease was demonstrated *in vivo* using PFGE on isolates from an infection mouse model. This has not been previously described. Genetic stability following transmission was demonstrated for a large number of isolates during a school outbreak of serogroup C infection. PFGE genotyping of isolates obtained from military recruits facilitated studies of the acquisition, carriage and transmission of meningococci in a closed community. The SMS volunteers showed that some individuals acquired and carried phenotypically similar meningococci but of diverse PFGE genotype over the two year time period. This may suggest frequent genetic events or acquisition of new strains amongst individuals in an open epidemiological context.

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Molecular analysis of *Neisseria meningitidis* class 3 outer membrane protein in the strains recognized by the Monoclonal antibody CB-Nm.2

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The bactericidal monoclonal antibody (Mab) CB-Nm.2 (1,2), specific for class 3 outer membrane protein of *N. meningitidis*, was assayed in Enzyme linked immuno-sorbent assay (ELISA) with a panel of 86 *N. meningitidis* strains. Fifty six strains belonging to seven serogroups: A, B, 29E, L, X, Y, Z and five serotypes: 1, 4, 5, 12, and 13, reacted with CB-Nm.2. The *por*B gene, coding for four different class 3 proteins, was cloned and sequenced. The translated amino acid sequences were compared with five previously published sequences. Sequence alignment revealed the five amino acid region (S/T)VETG located into the major variable region (VR) VR1, which was present in all *N. meningitidis* strains recognized by CB-Nm.2 and was not present in the strains negative in ELISA. Synthetic peptides, containing the predicted antigenic determinant from strains B385 and H355, were designed. Mouse antiserum obtained against the synthetic peptides recognized *Neisseria* strains in whole cell Dot-blot, but synthetic peptides failed to react with the Mab.

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Meningococci causing disease in South Australia 1971 through 1995: A 25 year study

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For the 25 year period 1971 through 1995, isolates of meningococci from children, adolescents and adults with meningococcal disease in South Australia (SA) were examined by serogrouping, serotyping and serosubtyping. Serogrouping was by slide agglutination using Wellcome (now Murex Diagnostics) sera1. For the period 1971 through 1989 serotyping/subtyping was done by FA in Ottawa by ELISA with whole cell meningococci as coating antigen and monoclonal antibodies2. For the period 1990 through 1994 typing/subtyping was done by AL in Adelaide with ELISA using the RIVM meningococcal serosubtyping kit, which now (1996) includes a total of 19 monoclonal antibodies; the method used was as described in the kit product insert except that a commercial substrate was used (Behring). In 1995 a dot blot technique also with RIVM monoclonal antibodies was introduced; this technique uses less antibody than does ELISA and tests in parallel with ELISA showed equal specificity; the dot blot method was easier to read.

For the period of the study the incidence of meningococcal disease varied considerably from year to year. During the period there were no identified outbreaks of meningococcal disease. However, a small epidemic of meningococcal disease occurred in Central Australia in the late 1980's; this may have spread to involve aborigines in SA accounting for the increased number of group A meningococcal infections in SA in 1988 and 1989.

Serogroup B predominated in 22 of the 25 years studies and constituted 126 (55%) of isolates from 230 cases of meningococcal bacteremia and/or meningitis. Group C was next in prevalence, predominating in three years (1984, 1989, 1994) and constituted 55 (24%) of isolates. Of the other groups, Y (20 isolates or 8%) and A (17, 7%) were most common. There were 9 isolates of Group W135 and single isolates identified as groups X or Z; one isolate was non-groupable.

For the 19 year period 1971 through 1989, the main types were 4, 2a, 15 and 14 in that order, and the main subtypes were P1.2, P1.1 and P1.10, in that order3,4.

For the six year period 1990 through 1995 the commonest types were 4, 14 2b and 15; subtypes were diverse, the least uncommon were P1.2 and P1.4.

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PFGE-RFLP analysis of meningococci of the phenotype C:2b:P1.2 causing geographically diverse outbreaks of disease in Australia

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Background. Meningococci of the phenotype C:2b:P1.2 were associated with a number of outbreaks of disease in geographically diverse areas of Australia in 1990 - 1991. Historically, this phenotype was also the commonest sporadic isolate in some areas, but has declined in incidence in 1994 - 1995.

Aim. To compare PFGE-RFLP patterns of the C:2b:P1.2 outbreak isolates with the patterns obtained from randomly selected sporadic isolates 1990 - 1994.

Organisms tested. A total of 35 strains were examined. Twenty strains of serogroup C *Neisseria meningitidis* isolated from epidemiologically confirmed outbreaks, 12 sporadic invasive isolates from 1990-1991 and three strains from 1993 - 1994 were examined by PFGE-RFLP. These last three strains were selected to represent other serogroups and a non-invasive strain which shared the C:2b:P1.2 phenotype of outbreak strains. These organisms were: an invasive serogroup Y:NT:NT isolate, a serogroup B:2b:P1.2 strain and a non-invasive (throat) isolate of a C:2b:P1.2. Both outbreak and sporadic strains were from geographically diverse regions of Australia several thousands of kilometers apart..

Methods. Serotyping and serosubtyping. Serotyping and serosubtyping was performed using a modified immuno dot-blot method and monoclonal antibodies from RIVM, Netherlands.

PFGE-RFLP Whole genomic DNA from the 35 isolates were prepared and restricted with either SpeI or NheI. The restriction digestion fragments for each enzyme was examined by PFGE using the CHEF DRII system (BioRad), stained with ethidium bromide and photographed under ultra-violet transillumination using Polaroid 667 film. Banding patterns were visually compared with molecular weight markers.

Results. Serotyping and serosubtyping:- Nineteen outbreak strains were of the phenotype C:2b:P1.2 (and P1.5[1])and one was C:2b:NT. The 12 sporadic isolates belonged to a variety of phenotypes:

- six were C:2b:P1.2 (and P1.5[1]),
- one was C:2b:NT
- one was C:NT:P1.2
- two C:2a:P1.5,2.
- one B:NT:P1.12,16
- one B:NT:NT.

PFGE-RFLP - Visual examination of the fingerprints for both enzymes showed that 17 of the outbreak strains and seven sporadic strains all with the phenotype C:2b:P1.2 had identical fingerprints. A further two strains (one outbreak and one sporadic isolate) with the phenotype C:2b:NT also had fingerprints identical to the above 24 strains. The two remaining C:2b:P1.2 outbreak strains had two banding differences from the common fingerprint suggesting they were very closely related but based on the epidemiological links would be classified as the same strain.

The banding patterns obtained for the other seven strains, none of which had the phenotype C:2b:P1.2, differed from that obtained for the twenty outbreak and eight sporadic strains.

Discussion. The PFGE-RFLP results suggest that one clone with the phenotype C:2b:P1.2 was involved in invasive disease and associated with geographically diverse outbreaks in Australia during 1990 - 1991. Sporadic isolates of the phenotype C:2b:P1.2 from the same period appeared to belong to the same clone. Furthermore, the same fingerprint has been obtained for recent sporadic isolates of this phenotype. Ongoing PFGE of larger numbers of sporadic isolates belonging to this phenotype will indicate whether there is more than one clone of C:2b:P1.2 associated with invasive disease in Australia.

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Public Health Microbiology, Centre for Public Health Sciences, Cooper's Plain, Queensland.
Microbiology Diagnostic Unit, University of Melbourne, Parkville, Victoria
Department of Microbiology, Princess Margaret Hospital for Children, Subiaco, Western Australia.

[1]Not all strains had the P1.5 epitope detected.

Examination of the usefulness of three genotype methods to characterize epidemiologically related meningococci

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Background. In October 1993, three children from one family presented to the Accident and Emergency Department of a local hospital suffering from meningococcal meningitis. Throat swabs collected from forty close household contacts yielded a further four strains of *Neisseria meningitidis*. One patient had a repeat isolate of *N. meningitidis* after rifampicin prophylaxis. One week after the three co-primary cases had occurred, an elderly person resident in the same local government area presented with meningococcal disease. No epidemiological link between these cases could be established. These ten isolates form the basis of this study.

Aim. To examine three co-primary cases and contact isolates and assesses the ability of three genotypic techniques to distinguish amongst these strains and the potential use of these techniques for future meningococcal strain differentiation.

Methods. All strains were serogrouped against serogroups A, B, C, W135, Y, Z (Murex Diagnostics). MICs were performed by a standardized agar plate dilution technique. All strains were serotyped and serosubtyped with monoclonal antibodies from RIVM, Netherlands, using a modified immuno dot-blot technique(1,2). Genotypic analysis was undertaking by the following methods: Random amplified polymorphic DNA fingerprinting (RAPD-PCR)(3,4), *por A* gene PCR amplicon restriction enzyme amplification (*por A* PCR-AREA)(5) and restriction fragment length polymorphism detection using pulsed field gel electrophoresis (PFGE-RFLP)(6,7,8). The reproducibility/stability of banding patterns obtained with these techniques was evaluated.

Results. All invasive isolates and two of the five contact strains were found to belong to phenotype C:2b:P1.2. The strain isolated after rifampicin prophylaxis was also C:2b:P1.2. One contact was NG:2b:P1.2 and the other NG:NT:P1.15. All three genotypic methods found the three co-primary invasive cases and four of the five contacts strains to be identical. The other invasive case, the elderly patient, gave identical banding patterns to the three co-primary cases using RAPD-PCR and *por A* gene PCR-AREA. This same strain, when analysed using PFGE-RFLP, yielded results suggestive of being closely related to the three co-primary cases. The fifth contact strain, NG:NT:P1.15 had banding patterns distinctive from the other strains using all three methods.

RAPD-PCR banding patterns suffered from a lack of reproducibility when different batches of Taq polymerase was used.

Discussion. Phenotyping may not distinguish amongst isolates and in particular, nasopharyngeal isolates may not be typeable by the available phenotypic methods. However, all

strains examined were typeable by the three genotypic techniques and all three techniques correctly identified organisms which were strongly epidemiologically related.
1) PFGE-RFLP is a widely used technique, can be used to establish clonal relationships(6,8) and has the added advantage that both the equipment and reagents can be applied to many other organisms. However, it is expensive and may take up to a week to obtain results.
2) The technique of *por A* gene PCR-AREA was found to be reliable and provide rapid results. However, this technique examines only a small segment of the genome. *por* A PCR-AREA may be used as an intermediary method for non phenotypeable strains and PFGE-RFLP performed as a confirmatory genotypic tool.
3) RAPD-PCR, whilst initially promising, suffered from reproducibility problems when different Taq polymerase batches and/or suppliers were used.
The need for further strain differentiation and the final analysis of the results thus obtained should always be made in conjunction with the epidemiological data to hand.

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Meningococcal disease isolate surveillance, New South Wales, Australia, 1994 - 1995

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Introduction. The Australian National Neisseria Network was established in 1994 as a means of laboratory based meningococcal disease surveillance and provides serogroup, MIC and outcome data on invasive disease. The NNN is affiliated with the European Monitoring Group on Meningococci. In New South Wales additional data on serotypes and serosubtypes of meningococci are available for 1994 - 1995. The results of laboratory and epidemiological data for 118 meningococcal isolates are presented. The data presented here relates to invasive isolates only and strains from either throat carriage or other sites were not included in this analysis.

Results. Age distribution and seasonality. In 1995, the seasonal variation in incidence of invasive disease differed from the pattern observed in 1994. More than 25% of cases of invasive disease occurred between January and March 1995 (summer) as opposed to 12% of cases in the corresponding period in 1994. However, the peak incidence for both 1994 and 1995 still occurred during the winter period, July - September. The age distribution for both years was bi - modal with the majority of cases being reported in children under 4 years of age with a second peak in 15-19 years age group.

Serogroup and serotype/subtype data. In 1995, serogroup B accounted for 69% and serogroup C for 27% of invasive strains. This pattern was markedly different from 1994 where serogroup B accounted for only 40% of strains and serogroup C accounted for 57% of invasive strains. The commonest phenotypes of the 1995 serogroup B strains were B:2b:P1.10 (20%) and B:NT:NT (17%). Sixteen phenotypes were found amongst the remaining 25 serogroup B strains. In 1994, the commonest phenotypes of serogroup B were 2b:P1.10 (38%) and NT:NT (17%). Forty-one per cent of the 1995 serogroup C strains were C:2b:P1.5,2 and 31% were C:2a:P1.5,2. In 1994, the commonest phenotypes of serogroup C were 2b:P1.5,2 (44%) and 2a:P1.5,2 (38%). There were two serogroup Y isolates and two non-groupable isolates included over this period. No serogroup A strains were received/reported.

Mortality Outcome data for 1995 was provided for 45 of 58 cases with two deaths being reported. Both fatal cases had serogroup B meningococci isolated from cerebrospinal fluid and /or blood cultures. One fatal case was a 19 year old male and the other was a male of unknown age. In 1994 there were 6 deaths noted in 44 of 60 cases where outcome was notified. One death had serogroup B meningococcus isolated from CSF. The other five deaths were due to septicemia. One patient had serogroup B isolated and four had serogroup C meningococci isolated.

Antimicrobial susceptibilities 1994 - 1995. The penicillin MICs of 117 strains were determined using standardized agar dilution methods. Forty six (39%) strains were “sensitive” ($\leq$ 0.03 mg/L)

and 71 (61%) strains were “less sensitive” (0.06 - 0.5 mg/L). The MICs ranged from between 0.016 and 0.25mg/L. All 117 strains tested (one strain was non-viable) were sensitive to ceftriaxone, rifampicin and ciprofloxacin. One isolate had a raised MIC to chloramphenicol.

Discussion. The cases reported during 1995 were all sporadic with no reports of clusters or outbreaks of cases. However, in 1994 there were several interesting cases including two children from the same school who were both infected with a C:2a:P1.5 strain. Staff and children were offered vaccination. .
The increase in serogroup B invasive disease in 1995 was not associated with a corresponding increase in one particular phenotype. No significant change in serogroup C phenotypes was found between 1994 and 1995 although the frequency of serogroup C strains markedly decreased. The four common phenotypes were the same in both years.
The penicillin MIC data for 1994 - 95 as summarized, indicates that penicillin based treatment regimens remains suitable for use in the State of New South Wales, Australia.

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Stability of the meningococcal *porA* gene in serial isolates from military recruits

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The meningococcal *porA* gene encodes the class 1 outer membrane protein, which determines sero-subtype specificity and is an important component of candidate vaccines against meningococcal disease. Sequence variations in two specific regions of the *porA* gene (VR1 and VR2) encode differences in the predicted amino acid sequence of two surface-exposed loops of the proposed porin structure (1). Only a limited number of subtypes are recognized by currently available sero-subtyping monoclonal antibodies and sequence variations can occur within a subtype which are not detected by these monoclonal antibodies.

Sequence variation of VR1 and VR2 has been used to examine serologically identical isolates from cases of meningococcal disease and household contacts. Whilst cases and contacts showed a high degree of sequence homology, significant differences were detected in some family groups (2). Such differences suggest that it is possible to use sequence information to differentiate between potential sources of infection, which appear identical using serological methods. An alternative explanation might be the rapid emergence of variants during meningococcal carriage, due to direct mutation within the *porA* gene, or recombination through horizontal exchange between strains. Sequence data obtained from isolates taken at a single time point, or from different individuals cannot differentiate these possibilities. Therefore, we have used DNA sequence analysis to assess the stability of the *porA* gene, studying serial isolates of meningococci cultured from the same individuals over a 30 week period.

Isolates were obtained as part of a longitudinal study of meningococcal throat carriage in cohorts of military recruits undergoing a 30 week training program (3). Many isolates were not readily identified by serotyping, most being either non-groupable, non-typable or non-subtypable. Serologically indistinguishable isolates were selected from individuals who exhibited serial colonization by meningococci and sequence analysis of VR1 and VR2 performed to determine the stability of the *porA* gene over the period of study. Similarly indistinguishable isolates from additional subjects within the cohort and from subjects from other cohorts within the study were also studied. Twenty-five isolates from eight subjects were sequenced, with up to five serial isolates per subject. Single isolates from a further five subjects were also sequenced. Sequence data for VR1 and VR2 from these isolates show the dynamics of transmission and the stability of *porA*.

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Optimizing ascertainment of meningococcal infection in England and Wales - impact of novel diagnostic techniques and reconciliation of available datasets between the national reference laboratory and other surveillance schemes

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Meningococcal disease remains one of the most feared bacterial infections with a predilection for children and young adults and a mortality of 10-12% in England and Wales. Attempts to lower mortality and serious morbidity have been made by United Kingdom health departments recommending that primary care physicians give parenteral penicillin when arranging hospital admission of suspected cases.

The stepwise fall in laboratory ascertained meningococcal infection in England and Wales from a high of 1,500 cases in 1990 to 1,129 in 1994 (1) may be part of the normal cyclical pattern seen in this country, however, the decrease in culture proven cases has not been mirrored by clinical case notifications and the discrepancy between these totals has increased over time. A possible explanation is that preadmission penicillin has adversely affected laboratory diagnosis while media publicity and heightened doctor awareness has increased notifications. This impression is supported by analysis of the mortality and notification data which show a falling mortality rate while notifications of predominantly septicemic meningococcal infection have risen.

Conjugate group C meningococcal vaccines are undergoing immunogenicity trials and early results show promise. The proportion of meningococcal disease burden represented by group C infections in England and Wales fell from 39% in 1988 to 26% in 1994. This, along with the fact that group C infections in England and Wales are more uniformly distributed across age bands than group B disease with about 25% cases occurring in patients aged over 25 years, means that any assessment of vaccine efficacy will need optimized case ascertainment and characterization of infecting organisms from a wide population base. Non-culture diagnostic methods have been developed to this end.

Serodiagnosis based on finding IgM and IgG antibody to outer membrane proteins (OMPs) and group B and C polysaccharide has been available for some years. Cases identified by this means rose from 84 in 1992 to 174 in 1994, the increase in numbers being due to greater use of the investigation by clinicians.

In autumn 1995, an evaluation of PCR for diagnosis of meningococcal infection was commenced. PCRs for the *IS1106* (2), class 2/3 OMPs (*porB*) and sialyltransferase (*siaD*) genes were developed, the last two providing epidemiological as well as diagnostic information. Guidelines for optimal laboratory investigation of suspected cases which included specimens to obtain for non-culture diagnosis were published and widely distributed (3).

The start of the PCR evaluation period (October - December 1995) coincided with a sharp upsurge in meningococcal disease activity in parts of England. In addition to an increase in culture proven cases to the highest recorded total for this quarter, specimens from large numbers of cases from whom no isolates could be obtained were submitted for investigation by non culture methods - both PCR and serology. Initial assessment of the PCR tests was carried out on 476 evaluable specimens sent from 311 patients. These gave positive reactions on material from 138 of the patients and an isolate was subsequently obtained from 47 of them. These in all but one case, confirmed the PCR group and/or typing when this was available. The 91 cases identified by PCR alone represented an 20% increase in laboratory ascertained infection and serodiagnosis further added to this total. Additional details along with data from the rest of the evaluation phase will be presented.

An attempt was also been made to identify overlap between cases investigated by laboratories and those statutorily notified (where no laboratory confirmation is necessary) and assess the extent to which clinical judgment alone was used to designate cases as meningococcal infection and also to see what proportion of laboratory proven cases were never formally notified.

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Clonal distribution of disease-causing *Neisseria meningitidis* in the county of Telemark, Norway 1987-95; studied by PCR Amplicon restriction endonuclease analysis (PCAREA)

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Sequence analysis of the dhps gene of *Neisseria meningitidis* shows the presence of both conserved and variable regions (1). This allows construction of PCR primers that are homologous with conserved regions, but whose amplicons contain variable regions. We have shown that such primers can be used both for detection (2) and typing (3) of meningococci.. Detection is based on visualization of an amplicon of expected size, while typing is done by HhaI amplicon restriction endonuclease analysis (PCRAREA).

In the present study, we used PCR AREA to determine the clonal distribution of all meningococci from culture-confirmed meningococcal disease in the county of Telemark, Norway (165.000 inhabitants) during the period from November 1987 to March 1995. There were 42 isolates from 42 patients. For typing, we used the NM7/NM6 set of primers which amplify a sequence of 634 bp. All isolates were additionally serogrouped, serotyped, their sulfonamide susceptibility determined. Chromosomal DNA fingerprinting cleaving DNA with HindIII was performed on all isolates. We also collected information on the patients age, sex, clinical presentation and outcome, and place of residence.

We were able to identify 11 different meningococcal PCRAREA classes among the 42 isolates. The predominant classes were class I, II and III containing 15, 8 and 6 isolates, respectively. The apparent intraclass identity and interclass variability of these three classes were confirmed by chromosomal DNA fingerprinting. Class I isolates were all sulfonamide resistant; were of serogroup B, and 12 of 14 which were typed, contained the serotype 15 protein and ten isolates were of subtype P1.7,16. All class II isolates were sulfonamide resistant, were of serogroup C and of serotype 2a:P1.2,5. The class III isolates were all sulfonamide sensitive, of serogroup B and contained the serotype 16 protein. The remaining classes (IV-XI) were more heterogeneous.

Epidemiologically, class I isolates were predominant in 1987 and 1992, but were absent in 1989, 1990 and 1994. Class II isolates were present each year until 1992, but have not been seen since. Class III appeared in 1991 when it was the predominant class, was present in 1992, but has since disappeared.

The Grenland area, which is the most densely populated area of Telemark (120.000 inhabitants), had an incidence over the study period of 20 cases/100.000 inhabitants. The corresponding figure for the 45.000 inhabitants of remaining Telemark, was 38. Ten out of 15 class I isolates were from outside Grenland; an incidence during the study period of 22/100,000 inhabitants. In Grenland the incidence of class I was 4/100,000 inhabitants. Class II and class III isolates, however, were almost exclusively seen in the Grenland area.

24 of the patients were below 6 years of age, 12 were between 11 and 20 years, and 6 were between 39 and 77 years of age. There was no patients between 20 and 39 years of age. The mean age of the patients was 14.3 years, but we observed that the mean age varied from year to year; in 1987 (5 patients) the mean age was 6.5 years, in 1988 (8 patients) 19.5 years, in 1989 (2 patients) 38.5 years, 1991 (7 patients) 15.2 years, 1992 (8 patients) 6.1 years and in 1993 (2 patients) 0.8 years. The most predominant classes among children from 0-5 years of age were classes other than class I,II, and III (39%), class I occurred in 35%, class II in 13% and class III in 17% of these patients.
Twenty-nine (69%) patients were male and 13 (31%) female. The different classes of meningococci were equally distributed in the two sexes.

A total of 17 patients were diagnosed as having septicemia alone, 12 had septicemia and meningitis, eleven had meningitis and 2 had benign meningococcemia. We observed no significant association between any class of meningococci and clinical presentation. Four patients (9.5%) died from their disease. Those who died had disease caused by class I or II meningococci.

There is a need for a rapid and reliable method to differentiate between clones of meningococci. The PCRAREA method based on primers from the dhps gene, has the advantage that it can be used both for diagnosis of meningococcal disease and for typing of the bacterium directly in CSF. PCRAREA can also be used for detecting and typing meningococci in pure culture, in mixed culture (primary isolation) and may also be used on throat specimens and blood. PCRAREA may therefore, be applied for the rapid identification of the disease-causing strain in healthy contacts of patients with meningococcal disease, for screening selected populations for pathogenic meningococci (military recruits), and for the surveillance of variation in clone distribution in a population giving valuable information for vaccine recommendations. The PCRAREA band patterns can be digitized and stored in digital databases. There exist PC programs which compare band patterns, automatically recognize and identify patterns, and allow the construction of dendrograms. Our work progresses along these lines.

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Molecular epidemiology of meningococcal disease in Iceland 1977-1995.

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Iceland is an island in the North-Atlantic ocean with approximately 270,000 inhabitants. There are >100,000 annual arrivals and departures of travellers from the island.

There is one central microbiology laboratory serving the whole country where most meningococcal isolates have been identified and data on patients has been collected by one physician. These facts make Iceland a unique place to study the molecular epidemiolgy of meningococcus.

An epidemic of meningococcal disease started in Iceland in 1975 reaching a peak incidence of $37.7/10^5$ cases in 1976. The incidence declined during 1977-78, but remained hyperendemic until 1988. In 1989 the incidence rose again reaching $11.3/ 10^5$ in 1994, but declined to 5.4 in 1995. Since 1977 isolates of meningococci from patients have been stored frozen and by the end of 1995 the collection consisted of 231 viable isolates. In addition, 150 isolates were obtained during an investigation of a cluster of cases in 1991, and carrier rate of college students in 1993.

A recent study compared different molecular methods to differentiate among meningococcal isolates and found that pulsed field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MEE) and ribotyping were comparable, both with respect to typeability and discrimination of strains (1).

The available patient strains were analysed by serotyping (ST), sero-subtyping (SST) and multilocus enzyme electrophoresis (MEE) (141 isolates). Pulsed field gel electrophoresis (PFGE) on *Sfi*I digested whole chromosomal DNA (381 isolates) (2) but modified according to Maslow et al. (3). Susceptibility to penicillin (P), sulfadiazine (S) and rifampin (R) (231 isolates) was determined using the E-test™.

From 1976 through 1984 serogroup B (predominantly B 2b:P1,2)
was most prevalent with serogroup A seen sporadically in 1976 and in 1980 through 1982. None of the group A strains were analysed by ST or SST. However, by PFGE seven of eight isolates were identical while one was unrelated. Serogroup C was initially isolated in 1978. It remained sporadic until 1982 but has subsequently been hyperendemic. Analysis of the serogroup C isolates by ST and STT showed three contiguous “clones” since 1982. These data correlate well with the genotyping data as determined by both PFGE and MEE. The incidence of serogroup B was low between 1984 and 1990 when a surge in the number of serogroup B cases occurred. Initially ST and STT analysis of the serogroup B isolates showed mostly group 21:P1.16 (seen in serogroup C isolates also) with occasional 15:P1.7,16. From 1992 the latter has, however,

predominated and was in fact responsible for an outbreak between 1992 and 1995 (ET-5 complex).

By PFGE, the isolates (n = 231) were allocated to 35 genotypes (clones) and further into 11 subgroups according to previously published criteria (4). One hundred forty one isolates were analysed by MEE and they were assigned to 42 electrophoretic types. The results were well correlated between both methods (PFGE and MEE). By PFGE fourteen genotypes included ≥ 3 isolates and five genotypes > 10 isolates. With the exception of two years (1988-89) three of the five genotypes caused disease yearly. The three most common genotypes have been in our community since 1980, 1981 and 1989. Two of these (ETs 19-22 and ET-37 complex) are of serogroup C; one of them (ETs 19-22) so far has only been identified in Iceland and one of group B (ET-5 complex). Twenty one of 35 PFGE genotypes (60%) occurred as sporadic cases representing 10% of patient isolates.

All patient isolates were susceptible to R and P. One genotype showed an intermediate susceptibility (MIC = 0,125μg/ml) to P. Resistance to S (MIC > 125 μg/ml)was noted in several genotypes (ET-5 complex).

The study demonstrates that more than one strain was responsible for meningococcal disease at any given time. Usually there was a dominant epidemic strain occurring with one or more sporadic strains. A particular clone may cause sporadic disease for several years (up to 10 years) before causing an epidemic. Further, among sporadic cases, at least 5 years may lapse between occurrences of cases infected with the same strain.

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Antigenic variations within the genetic clone ET-15/37 of *Neisseria meningitidis* occurring in the Czech Republic

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A new genetic clone of *Neisseria meningitidis* appeared in the Czech Republic in 1993 and caused an unusual epidemiological and clinical situation in invasive meningococcal disease characterized by increased morbidity and fatality rates, a changed age distribution, local outbreaks, and changed clinical courses. The clone was recognized using multilocus electrophoresis as ET-15 belonging to ET-37 complex and was practically identical to the clone identified in Canada at the beginning of the 1990's (1) This clone, which was quite new in the Czech Republic, occurred in 1993 in two districts of the country. It showed the following phenotypic characteristics: C:2a:P1.2(P1.5). The variability of the P1.5 serosubtype seems to be attributable to the conditions of *Neisseria meningitidis* culture. Class 5 proteins were uniform (P5.II, P5.III) and pili as well (II b). The agent was responsible for a high fatality rate (20%) and the highest morbidity in the age group 15-19 years (2). In the district with the highest incidence of invasive meningococcal disease, a targeted vaccination with A+C polysaccharide meningococcal vaccine in the most affected age group (with the age specific morbidity 57 per 100,000) prevented the spread of the disease caused by *Neisseria meningitidis* C (3,4)

The new clone spread all over the country later (1994-1996) and changes were recognized in the age distribution of invasive meningococcal disease: a shift to the age groups 1-4 years, 0-11 months and adults (5). Two antigenic variants in which either serotype or serosubtype had not been determined by WCE were found in 1994 and in the first part of 1995: *Neisseria meningitidis* C:**NT**:P1.2(P1.5) and *Neisseria meningitidis* C:2a:**NST**, respectively. In the second part of 1995 a new very important antigenic change was revealed: serogroup B appeared in combination with serotype 2a and serosubtype P1.2(P1.5). These strains **B**:2a:P1.2(P1.5) belong to the genetic clone ET-15/37 like the previous antigenic serogroup C variant. Alarming is the high fatality rate due to serogroup B variants (20%), while that due to serogroup C variants has a decreasing trend: 20% in 1993, 13.8% in 1994 and 8.4% in 1995.

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The new serotype of *Neisseria meningitidis*

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A high percentage (50-80%) of the *Neisseria meningitidis* strains isolated in the Czech Republic since 1973 remained non-typable (NT) and/or non-subtypable (NST) in spite of a large collection of monoclonal antibodies (MAbs) used for the whole-cell ELISA (WCE) (1). A hypothesis arose that the isolated *Neisseria meningitidis* strains might be different from those used in the world for MAbs production and therefore new meningococcal serotypes and/or subtypes might be present among meningococci isolated in the Czech Republic. A project focused on the problem of NT/NST *Neisseria meningitidis* was started and a new serotype candidate has been identified (2, 3).

Construction of producing hybridomas was made by the classical method of fusing a murine myeloma line SP2/0-Ag14 cells and activated lymphocytes obtained from BALB/c mice immunized by the whole-cell antigen. Cell fusion was initiated using PEG 1550 in the conditioned RPMI 1640 medium. The highly positive clones were selected for production of murine ascites. The Ig class and sub-class of MAb was determined by means of Isotype AB-STAT^{TM}-I test (SangStat Medical Corporation). The hybridomas, clones and ascites were tested by WCE and immunoblotting and showed good sensitivity and specificity. The new MAb does not react with any of the serotype/subtype reference strains but does react with the strain used for its production at a 1:1,000 dilution. The immunoblotting has allowed to recognize that the new MAb reacts with the epitope of the serotype-specific Class 2 OMP. Therefore this new MAb was designated serotype 22-specific, as the last number of serotype-specific MAb available is 21 of Dr. Zollinger.

A collection of 97 *Neisseria meningitidis* B:NT strains isolated from cerebrospinal fluid or blood of patients with invasive meningococcal disease in the Czech Republic between 1973 and 1994 was serotyped with the new MAb and 37% of these strains gave positive WCE result. The period of 22 years was divided into two sub-periods: 1973-1992 and 1993-1994, to allow to see whether there was a difference in the strains incidence after the appearance of the new clone *Neisseria meningitidis* C:2a:P1.2(P1.5), ET-15/37 in the Czech Republic in 1993. A lower percentage of the new serotype 22 has been found in the later period but the difference is not statistically significant (40.4% versus 35.6%).

In 1995, the new serotype 22-specific MAb has been included into our MAbs collection used for routine WCE serotyping. Fifty-nine *Neisseria meningitidis* B:NT strains isolated from individuals in different clinical situations (i.e. invasive meningococcal disease, respiratory disease, contact, carrier) in the Czech Republic from January 1995 to May 1996 were serotyped and 26 of them (44.1%) appeared positive with the new serotype 22-specific MAb. Seven of the *Neisseria meningitidis* B:22 strains were isolated from patients with invasive meningococcal

disease mostly aged 0-4 years. The significance of the new serotype 22 candidate was underlined recently: it was recognized in the *Neisseria meningitidis* B strains isolated from three died patients. The geographical distribution of the new serotype meningococci is general all over the Czech Republic.

These results indicate the epidemiological and clinical significance of the new serotype candidate 22. The new MAb was offered to seven European laboratories (in Athens, Bilthoven, Glasgow, Graz, Heidelberg, Manchester and Oslo) and their first results are shown in other presentations. An international discussion of the importance of this new meningococcal serotype is desirable, including a suggestion of its final numbering, because the number 22 has been already used for the serotype-specific monoclonal.

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The molecular characterization of a new meningococcal serotype: serotype 22

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It is now recognized that *Neisseria meningitidis* particularly serogroup B organisms are genetically diverse due in part to frequent recombination between strains. This results in constant emergence of new serotypes often heralded by increasing numbers of nontypable strains. Large numbers of Group B meningococci isolated in the Czech Republic were non-serotypable (NT) with the available panel of monoclonal antibodies (MAbs) (1). Recently, a significant new meningococcal serotype designated serotype 22 was identified amongst nontypable isolates from the Czech Republic which is found in 37% of the Czech B:NT strains isolated from invasive meningococcal disease within a 22 year period (1973-1994) (2). This demand necessitated the production of a serotype 22 specific monoclonal antibody for inclusion in the serotype reagent panel, illustrating the need for constant expansion of the reagent panel to include new serotypes. Following the introduction of the serotype 22 MAb, 44% of the Czech B:NT isolates from 1995 were identified as serotype 22. Since then serotype 22 strains have been identified outside the Czech Republic including England and Wales where both serogroup B and C serotype 22 isolates have been identified.

DNA-based typing methods for *Neisseria meningitidis* may overcome many of the problems inherent to the serological typing system. The characterization of the serotype 22 *porB* gene of Czech and UK isolates by molecular fingerprinting techniques, namely T-tracking and direct nucleotide sequence analysis identified two class 2 *porB* sequence types amongst the Czech isolates. These were designated type 22a and type 22b. Amongst the UK isolates analyzed only the serotype 22b type was identified. The two serotype 22 types have common sequences in variable loops I and VI of the gene. Sequence data was used to design a serotype 22 specific oligonucleotide probe for incorporation in a *porB* serotype specific DNA probe panel for PCR-based *porB* DNA typing. Furthermore, the molecular characterization of the serotype 22 including the design of the serotype specific probe can be performed in a reasonably short time allowing the rapid expansion of the serotype probe panel in response the emergence of new serotypes.

This presentation illustrates the potential of DNA-based methods for the rapid characterization of *porB* variation and the identification of new epidemiologically important serotypes. Furthermore, the increased discrimination achieved using DNA-based typing as shown here by the identification of serotypes 22a and 22b provides enhanced surveillance for the better understanding of the epidemiology of meningococcal disease.

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Antigenic phenotype of meningococcal strains isolated from patients and carriers in Greece using the new monoclonal antibody designated 22

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The National Meningococcal Reference Laboratory in Greece started serotyping and subtyping of *Neisseria meningitidis* strains in 1989 using the whole-cell ELISA (WCE) (1-3). In spite of a large collection of monoclonal antibodies (MAbs) used, the majority of the Greek *Neisseria meningitidis* isolates do not react with the serotype- and subtype-specific MAbs available at present. The percentage of the non-typable (NT) and/or non-subtypable (NST) isolates ranges from 40 to 60% indicating, that the antigenic phenotypes associated with the outbreaks in Britain and Scandinavia are not common in Greece (4).

A new serotype preliminarily designated 22 resulted from the research of the National Reference Laboratory for Meningococcal Infections in the Czech Republic and a new MAb has been produced recently. We are presenting the attempt to serotype the Greek meningococcal non-typable isolates with the new serotype 22-specific MAb.

A collection of 430 non-typable meningococcal strains from both patients (52) and carriers (378) was tested against the type 22-specific MAb. Approximately 10% and 9.5% of the meningococcal strains isolated from patients and carriers, respectively, were serotypable with the new serotype-specific MAb. The most frequent phenotype combination found was B:22:P1.13 among the patients' isolates and B:22:P1.7 and A:22:P1.5 among the carriers' isolates.

It was shown that the significant portion of Greek meningococcal strains remained still non-serotypable with the enlarged serotype and subtype reagents panel. On the contrary, a high percentage (29.7%) of the carrier strains isolated from immigrant schoolchildren originated from Russia, which were previously non-typable, reacted with the new MAb.

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Factors relating to carriage of *Neisseria meningitidis* and the Lewis antigen phenotype

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There is a long history of investigations on carriage of *Neisseria meningitidis* in the Czech Republic. Carriage was about 10 % up to the late 1970s when it reached 80 % among the population in the 15-19 year age range; however, this was not associated with an increase in the incidence of invasive meningococcal disease. The *N. meningitidis* strains isolated from carriers were very heterogeneous for capsular and non-capsular antigens (1,2).

The new meningococcal clone ET-15/37 appeared in 1993 in the Czech Republic and caused a dramatic increase in the morbidity and fatality rates of this disease. A study of carriers of meningococci was initiated to analyze the factors influencing carriage and possible development of invasive meningococcal disease. It has been suggested that various meningococcal clones differ in their transmissibility and virulence among different populations.

The investigation began in February 1996 in the Czech district of Olomouc which had the highest incidence of invasive meningococcal disease. The new clone first appeared here in 1993 and a successful targeted vaccination program was carried out among the 15-19 year age group to control the outbreak (3). Two groups were investigated in this voluntary study: 116 children in the 1-4 year age range, 415 in the 15-19 year age range. The first sampling included a nasopharyngeal swab, blood and saliva specimens. The parents filled in a questionnaire on socioeconomic and health factors. Participants from whom *Neisseria* species were isolated were sampled again two weeks later for carriage and blood and saliva specimens were collected. A second questionnaire concerning the period between the two samples was administered.

The first sample found no meningococci in the younger age group and a carriage rate of 7.7 % for *Neisseria lactamica*. All these children were negative for both species when retested. *N. lactamica* was not isolated from any of the children in the older age group but the carriage rate was 6.2 % for *N. meningitidis*. All but 2 of the 33 carriers were positive for meningococci when retested (93.9%). At both samplings isolates from 38.7% of the carriers had the same capsular phenotype and 96.8% of the isolates had the same non-capsular antigens (serotype and subtype).

Analysis of ABO and Lewis blood groups has been completed for the older group. There was no difference in the distribution of ABO blood groups among the 34 carriers compared with the distribution of these groups among the population tested: A = 48%; B = 0%; O = 28%; AB = 3%. Assessment of Lewis blood group antigens found that there was an increase in the proportion of carriers among those individuals whose red cells were not agglutinated by either monoclonal anti-Lewisa or anti-Lewisb (Lewis-negative). There were 384 subjects whose red cells were agglutinated by the antibodies and 27 (7%) were carriers. There were 57 Lewis-negative subjects and 8 (14%) were carriers, but the differences were not statistically significant.

The sera are being investigated for bactericidal and opsonizing activities in relation to antibodies detected to bacterial components by ELISA. The data from the questionnaires are being analyzed and the preliminary results will be presented.

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Automated direct nucleotide sequence analysis in the study of meningococcal antigenic variation.

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The surface proteins of *N. meningitidis* are antigenically highly variable. Our previous studies of two meningococcal such proteins, PorA and PorB, which are important as epidemiological markers and potential vaccine components, indicated that, for a variety of reasons, serological analyses underestimate this variability (2,3,6,8,9). In addition, the monoclonal antibodies that identified meningococcal serotypes and subtypes provide information that is of limited use in understanding the mechanisms whereby antigenic variation arises. If multivalent vaccines which use cocktails of surface proteins are to be introduced, it is important that we have a comprehensive understanding of the diversity of these antigens in meningococcal populations, the mechanisms by which they change, and rate at which they change.

To overcome the problem of strains that could not be characterised by serological means, a number of groups developed DNA-based approaches for rapid characterization of isolates (e.g. Refs. 1 and 5). However, even these approaches do not necessarily guarantee comprehensive strain characterization (2). In the last few years automated nucleotide sequence technology has become increasingly available, rapid, and cost-effective. Determining the nucleotide sequences of potential antigens and typing targets has several advantages. It provides accurate, unambiguous and detailed data which, in addition to characterizing strains, can be used for detailed epidemiological and population genetic analyses. Further, the same methodology can be used for any gene target, requiring only oligonucleotide primers for that gene. Once complete a complete genome sequence is available for the meningococcus, any gene can be studied in multiple isolates by this approach.

We have instituted a program to assess the feasibility of using nucleotide sequence analysis for the routine characterization of meningococcal isolates and are sequencing the PorA protein from all strains isolated in the UK in the current year (November 1995 onwards; between 1000-2000 isolates). These data will be used in the following analyses:

(i) Comprehensive identification of PorA in the UK population of meningococci, providing a baseline of data for retrospective studies of antigenic change of these antigens over the last 20 years;
(ii) Comparisons these data with data obtained by serosubtyping;
(iii) Establish the feasibility of using nucleotide sequence analyses routinely;
(iv) Production of data suitable for testing of mathematical models of the evolution, persistence of antigenic variation, and strain structure (4,7).

In conclusion, direct automated nucleotide sequence analyses provide the prospect of comprehensive and accurate typing data that can be also applied to population studies and easily extended to the study of any genetic locus of the meningococcus.

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Confirmation of meningococcal sepsis using diagnostic nPCR of urine samples - a case report

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The life threatening nature of sepsis necessitates aggressive treatment upon patient presentation. *Neisseria meningitidis*, one causative agent of sepsis, is frequently difficult to culture from patient fluids as a result of empirical antibiotic treatment (1). Oftentimes, diagnosis of meningococcemia is presumptive, rather than confirmed. In an effort to confirm the etiology of such a case, a previously described nested polymerase chain reaction (nPCR) methodology (2) was modified for use with serum and urine. This technique confirmed the presence of meningococcal DNA, subtype P1.16, in the urine of a patient.

The patient was a young active duty male who was stationed in the DMZ in South Korea. He presented at an outlying clinic with systemic toxicity with fever, petechial rash, monoarticular arthropathy and conjunctivitis. He was treated empirically with a meningitic dose of IV ceftriaxone, fluids and hydrocortisone and was evacuated to the 121st General Hospital in Seoul where cultures were subsequently obtained.

He reported no recent insect bites, no recent sexual history nor any recollection of meningococcal vaccination. Laboratory evaluation and ancillary studies found coagulopathy/mild thrombocytopenia. Cerebrospinal fluid (CSF) examination was within normal limits with no antigens detected for *N. meningitidis* groups A and B, *S. pneumoniae* or *H. influenzae.* Skin lesions were biopsied, and rare Gram negative diplococci were observed. Urinalysis was normal. Cultures of blood, CSF, urine and skin biopsy tissue were negative. The patient was treated with intravenous penicillin G, showed immediate clinical response and was discharged after 10 days.

Concern remained that definitive diagnosis of meningococcal disease had not been made due to the remote possibility of gonococcal contacts. Frozen CSF, sera and urine samples were sent to WRAIR to attempt to confirm the diagnosis of meningococcemia by nPCR. Samples of CSF were treated as previously described (2). Controls included previously identified CSF samples as well as frozen urine samples from which gonococci had been cultured in a range from 0 CFU/ml to 8,000 CFU/ml (provided by Dr. K. Schmidt). Five hundred ml samples of serum and urine were centrifuged, and the pellet was resuspended in water. After heating, the urine and serum samples were processed like CSF samples. Nested PCR was performed as previously described, except that the concentration of meningococcal *porA* specific primers was increased to 250 pM/reaction. An ethidium bromide gel confirmed the amplification of DNA of the appropriate size from the urine as well as from the positive CSF controls, but not from the urine samples containing gonococci or from the serum samples. The DNA product from duplicate urine samples was gel purified and partially sequenced through the subtype specific variable region 2. Sequence analysis of this region determined that the DNA was meningococcal subtype P1.16.

Detection of meningococcal meningitis by nPCR has been demonstrated in retrospective analyses of CSF samples from vaccine trials (3,4). Suspected but unconfirmed cases of meningococcemia from these trials could not be assayed using CSF samples. Whole blood samples, rather than serum, might yield detectable amounts of DNA in association with white blood cells of the patient. However, whole blood is not usually collected and preserved. Urine samples are commonly collected and tested for meningococcal antigen by latex agglutination methods. Preservation of urine or urine sediment for nPCR analysis even with a normal urinalysis would be a simple addition to sample collection procedures. Further development of this nPCR with urine samples collected from meningococcemia patients will help to expand the effectiveness of PCR in confirming all cases of suspected meningococcal disease.

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Epidemic of serogroup B meningococcal disease in New Zealand has parallels with that observed in the Netherlands, 1980-1990 .

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New Zealand has been experiencing a meningococcal disease epidemic since mid-1991(1). Total numbers of cases have increased from an average of 51 per annum for the 1989-90 pre-epidemic period to 394 cases in 1995. This is an increase in the rate of disease over the total population from 1.5 per 100,000 for the years 1989-90 to 11.7 per 100,000 in 1995. Based on current numbers it is predicted that about 600 cases will occur in 1996 giving a rate of 17.5 per 100,000. These rates contrast with the rate of 2.2 per 100,000 recorded in Australia in 1994 (2). Disease rates are highest among our Maori and Pacific Islands children under the age of five with the highest rate of 278.8 cases per 100,000 occurring in Pacific Islands children under one year of age. In contrast a smaller peak in rates of disease, occurring in the 15-19 year age-group, has been experienced by Causasians. A total of 21 deaths occurred during 1995 giving a case-fatality rate of 5.3%. The case-fatality rate has been constant since 1992.

Most disease has been caused by serogroup B meningococci. In 1995 serogroup B accounted for 182 cases (76.8%) where a viable meningococcus was recovered. Serogroup C accounted for 49 (20.8%) of cases. Serogroup B with phenotype B:4:P1.4, emerged as dominant in mid-1991 coincident with the start of the epidemic (1). Since then isolates with this profile have increased as a proportion of all serogroup B meningococci tested. In 1995 B:4:P1.4 isolates represented 72% of all serogroup B meningococci. Although not included in this percentage, there have been some isolates of subtype P1.4 which are non-serotypable or are serotype 14. Numbers of such isolates appear to have increased in 1996. Macrorestriction fragment length polymorphism typing using *Sfi1* and pulsed field gel electrophoresis has shown that the majority of B:4:P1.4 isolates belong to a single pattern type although some have demonstrated distinct restriction fragment patterns.

A representative set of serogroup B and serogroup C isolates of varying serotypes and serosubtypes were sent to Dr. Dominique Caugant, Norway, for multilocus enzyme electrophoresis typing. Results show that all 13 isolates with phenotype B:4:P1.4 recovered since 1991, belong to clones of the same lineage III. Of note, two isolates recovered in 1989, one with phenotype B:4:P1.4 and the other with phenotype B:4:non-subtypable, did not belong to this lineage. The results are consistent with the concept that meningococci with phenotype B:4:P1.4 appear to have been introduced into New Zealand around 1991.

A similar increase in meningococci with phenotype B:4:P1.4 and belonging to lineage III was observed in the Netherlands in the 1980's (3). The insidious increase in incidence of meningococcal disease that has accompanied the recognition of meningococci of this phenotype in New Zealand since 1991 also has parallels with the pattern of meningococcal disease

observed in the Netherlands. The current development of outer membrane vesicle vaccines containing the Por A P1.7^{h},4 (4) offers some hope for the protraction of our epidemic.

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PCR-SSCP of clinical specimens for non-culture-based sub-typing of the meningococcus in clinical specimens

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Introduction. In the absence of a microbial culture, confirmation of the diagnosis can be made using the polymerase chain reaction (PCR) which can detect presence of meningococcal DNA in both CSF (1-4) and blood specimens, even after antibiotic treatment. However, present PCR tests do not give information about the sub-specific typing of meningococcal strain present in the disease. Sub-specific typing of strains from cases and carriers of the meningococcus is important for the following reasons: (i) a vaccine is currently available for serogroup A and C strains and it is therefore important to identify outbreaks associated with these serogroups; (ii) typing may be used to demonstrate epidemiological links between cases, and between cases and carriers in an outbreak situation; (iii) typing is important to monitor the changing epidemiology of disease; and (iv) multivalent serogroup B vaccines based on outer membrane proteins are presently being evaluated.

We have therefore developed a PCR-SSCP technique to obtain sub-specific typing information on meningococci present in blood and CSF. We amplified a segment of the variable region VR1 of the *por*A gene since VR1 is the target for many of the sero-subtyping antibodies and a great deal of information is available concerning sequence variation at this locus (5-8). PCR amplification was followed by single stranded conformational polymorphism (SSCP) analysis of the PCR product to detect differences in base sequence at the target locus. PCR-SSCP was used to demonstrate both identity and non-identity of meningococcal strains from clinical specimens and between clinical specimens and cultured strains.

Methodology. Clinical samples included blood and CSF cultures, boiled CSF, or DNA extracted from buffy coat or serum, as described (Newcombe et al) were obtained from Gloucester, Plymouth, Hereford and Cheltenham PHL. All meningococcal cultures had previously been typed by Meningococcal Reference Laboratory, Manchester PHL.
DNA was extracted from meningococcal cultures using standard DNA extraction protocols. The DNA was amplified using PCR WITH fluorescein labelled 12-dUTP incorporated into the PCR reaction mix. The products were analysed either by electrophoresis on the Applied Biosystems ABI 373A Automated DNA Sequencer.

Results. We investigated strains belonging to five different sero-subtypes. Each strain of the same sero-subtype tested was found to generate a distinct SSCP banding pattern. We next investigated eight strains of the same sero-subtype but differing in serogroup and/or serotype designations. Each of the eight strains gave identical SSCP banding patterns. We then demonstrated that clinical specimen produce the same banding patterns as DNA extracted from microbial cultures. We next examined four clinical specimens (2 serum 2 CSF) obtained from four different patients where subsequent *N. meningitidis* cultures were found to be of the same

p1.16,7 sero-subtype. Again, banding patterns that could be superimposed were obtained. Lastly, we examined DNA extracted from four clinical specimens (all CSF) obtained from four patients from whom *N. meningitidis* strains of differing sero-subtypes were isolated. Distinct banding patterns were obtained for each specimen.

Discussion. PCR-SSCP analysis may be used to demonstrate either identity or non-identity of strains present in clinical specimens taken from different patients. This technique may be used to establish the existence of an outbreak and to investigate where active transmission may be occurring within the community. Direct PCR-SSCP of clinical specimens may also be important for disease surveillance in situations where microbiological culture of patient specimens is negative.

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Meningoencephalitis. Notified cases between 1969 and 1995 in Argentina.

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An analysis of meningoencephalitis notified cases from 1969 to 1995 was made from the existing National Ministry of Health database. This study showed two outbreaks. The first occurred between 1974 and 1978 with the peak in 1976 and a rate of 15.2 cases per 100,000 inhabitants due to *Neisseria meningitidis* group C.

The second one began in 1992 with a peak in 1994 and a rate of 11.4 cases per 100,000 inhabitants. This last outbreak was due to a succession of outbreaks in restricted geographical areas of *Neisseria meningitidis* group B and group C with incidence rate between 0.2 and 18 cases per 100,000 inhabitants. *Neisseria meningitidis* group B was mostly observed in children under one year old. Meanwhile *N. meningitidis* group C was mostly detected in older children. During this second outbreak better microbiological detection agents were used and a better epidemiological study was performed. The mortality has not been variable during the last outbreak.

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Polymerase chain reaction of peripheral blood for the diagnosis of meningococcal disease.

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Confirmation of the diagnosis of meningococcal disease is becoming increasingly difficult. Confirmation of the diagnosis can be made from CSF in almost 90% of patients (1), but there is a a trend away from lumbar puncture especially in young children. Pre-admission antibiotic treatment reduces the chance of a positive blood culture to 5% or less (2). We demonstrated previously the value of a PCR designed to amplify a segment of the meningococcal insertion sequence IS*1106* (3), for specific and sensitive detection of *N. meningitidis* DNA in clinical CSF specimens (4). We here evaluated a DIG-PCR ELISA for the detection of meningococcal DNA in blood.

Patients and specimens. Peripheral venous blood samples (for standard haematological tests and anticoagulated with EDTA) were those taken on hospital admission from patients with suspected meningococcal disease and from controls. Samples were collected from patients in Gloucester, Hereford, Plymouth and Cheltenham. Almost always after overnight storage at 4°C, samples were centrifuged and divided into buffy coat, serum and red cell fractions. The samples were coded, stored at -20°C, and transported to the University of Surrey for DNA extraction and PCR analysis.

PCR sample preparation. DNA was purified from 50 ml blood buffy-coat or serum sample based on the method of Boom *et al* (5).

PCR amplification. Samples were handled in batches of 10 with 2 extraction blanks. 3 ml of diatom eluent was used in a 25 ml PCR reactions.

Detection of PCR products using DIG-PCR ELISA. This was available as a kit and performed essentially as described by the manufacturer (Boehringer manheim). An ELISA plate reader was used to record the result. Primers for the PCR and capture probe sequence were based on the meningococcal-specific insertion sequence IS*1106*(3).The PCR capture probe sequence was internal to the PCR product.

Coded clinical specimens from 80 patients with confirmed and suspected meningococcal disease and controls were examined by DIG-PCR ELISA. The sensitivity of the test in patients with confirmed meningococcal disease -either by culture or by the presence of gram-negative intracellular diplococci in CSF n these groups was 89% for serum specimens and 100% for blood buffy-coat. Using 95% confidence limits, the minimum value for the sensitivity of the test on blood buffy-coat was 86%. The specificity of the test was determined using specimens from control patient specimens and was found to be 100% for both serum and blood buffy-coat. Ten buffy-coat specimens were from patients with strong suspicion of meningococcal disease (fever with haemorrhagic rash) but from whom no organisms were isolated (Table 3 patient category

3). Using the PCR test on blood buffy-coat, confirmation of meningococcal infection was obtained in all of these patients. Four of the 25 patients with confirmed invasive meningococcal disease and 7 of the 10 patients with suspected meningococcal disease had been treated with parenteral benzylpenicillin before the specimen was collected. Blood cultures were uniformly negative on these patients but they all gave a positive PCR result. Buffy-coat specimens from a further eighteen patients with evidence of bacterial meningitis but without a haemorrhagic rash (Table 3 patient category 4) were examined and 5 of these were found to be positive for meningococcal DNA. None of these patients had been treated with antibiotics.

Conclusions. The PCR-DIG ELISA test is as rapid (result in less than 24 hours), as conventional culture (12-36 hours) but does not seem to be affected by prior antibiotic therapy. The PCR blood test is practical and convenient and could be readily applied in clinical laboratories. The test described here may therefore be of particular benefit for diagnosis of patients in whom lumbar puncture is contraindicated or in whom antibiotic treatment had rendered blood or CSF sterile, before specimens were taken.

Acknowledgements. This work was supported by the National Meningitis Trust.

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Comparison between DNA sequencing and fingerprinting by pulsed-field gel electrophoresis for strain typing : Preliminary results using 12 meningococcal strains and isolates of 4 further *Neisseria* species

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Neisseria meningitidis shows a high level of interspecific genetic diversity. Various typing methods have been employed for strain characterization including the examination of allozymes (MLEE) and the study of restriction fragment length polymorphisms RFLPs (either by cutting small PCR generated DNA fragments or by digesting the whole genome, which is known as pulsed-field gel electrophoresis PFGE). The finest resolution of strains is achieved by sequencing DNA but this method is expensive and not routinely used so far. Information on the genetic variability among strains may then be used to reconstruct the genetic relationships of the strains studied. Various studies concerning the genetic diversity of *Neisseriaceae* led to the conclusion that at least particular serogroups of meningococci are of more or less panmictic population structure (e.g. 1). This is a result of horizontal inter- and intraspecific genetic transfer (recombination). Interspecific horizontal transfer of DNA fragments could be detected for some gene loci (e.g. 2). By sequencing a fragment of the *rpoB* gene, coding for the second largest subunit of DNA directed RNA polymerase, no hints for recombination were found in 18 DNA fragments belonging to meningococci and four other *Neisseria* species. However, the meningococcal strains used showed a more or less clonal population structure, although belonging to various serogroups (3).

While the study of allozymes is on the protein level, both the analysis of RFLPs or DNA sequencing is on the genomic level. When evaluating genetic relatedness, point mutations which are recognized by both techniques to a different degree are used for the construction of clusters in a dendrogram. The aim of the present study was to clarify, whether there are differences in the genetic relationships between the strains, when dendrograms had been generated using the RFLP pattern or by using the sequence data.

A subgenic PCR fragment within the *rpoB* gene from 12 strains of meningococci and isolates of 4 other species of the genus *Neisseria* was sequenced. The meningococcal strains covered various serogroups which were studied previously (3, 4) together with *N. lactamica*, *N. gonorrhoeae*, *N. flava*, and *N. subflava*. Sequence analysis was performed on a 471 bp *rpoB* fragment. The sequences were aligned using the program CLUSTALW (5). Phylogenetic trees were constructed using various methods like UPGMA or neighbor joining (6, 7, 3).

The same strains were subjected to fingerprinting by PFGE. The neisserial genome was digested either with the restriction enzymes *Nhe*I or *Sfi*I. Restriction fragment patterns were analyzed using the program GelCompare. The program utilizes the UPGMA (6) or neighbor joining method (7) for reconstruction of the relationships among the strains by examining the patterns generated by the restriction enzyme. Observed RFLP patterns were analyzed separately for each

enzyme and afterwards for both enzymes in combination. The resulting genetic relationships between the strains, obtained by both methods, were compared.

Our preliminary results indicate that by applying different statistical methods for analyzing the results of each technique (PFGE or sequencing) the clustering of strains is comparable. When comparing the clustering between both techniques, however, certain differences are observed. These results are in accordance with the assumption of a more or less panmictic population structure.

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The high incidence of meningococcal disease in indigenous Australians

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Objectives. To describe the incidence and control of meningococcal disease in Aboriginal Australians.

Introduction. Aboriginal Australians constitute 1.6% of the country's 17.5 million people, and about 6% reside in the arid region of central Australia. Like many other indigenous population groups in developed countries, Aboriginal Australians suffer poor health. They have a very high incidence of infectious diseases and of the diseases of transition, such as diabetes. The annual incidence of invasive *Haemophilus influenzae* type b (Hib) disease in Aboriginal children under 5 years of age in central Australia in the pre-Hib vaccine era was 871/100,000, among the highest ever reported in the literature (1). Similarly, the mean annual incidence of invasive pneumococcal disease between 1985 and 1990 in this age group was 935/100,000 (2).

Methods. Data on notifications of meningococcal disease were obtained from the Communicable Diseases Network of Australia and New Zealand. Since 1991, the notifiable diseases dataset has included a field for identifying Aboriginality, but this is not obligatory. Three states with relatively high Aboriginal populations (Western Australia, Northern Territory and New South Wales), usually provided this information with some consistency; together they have a total population of 7.7 million, of which just over 151,000 are Aboriginal. Reports on outbreaks were obtained from peer reviewed journals and communicable disease bulletins. The definition of an outbreak was based on a publication reporting an outbreak in which at least 2 or more cases occurred in a defined Aboriginal community within 3 months.

Results. ***Incidence***: Aboriginality of the subjects notified with meningococcal disease was recorded as "unknown" in 35% of cases in the three states. To obtain minimal estimates of the incidence in the Aboriginal population, all cases with an "unknown" status were assumed to be non-Aboriginal. The mean annual incidence per 100,000, based on notifications in the three states between 1991 and 1994, was: 13 for the Aboriginal population (61 for children under 5 years), and 2.2 for the non-Aboriginal population (14 for children under 5 years). A study from northern Queensland reported a mean annual incidence per 100,000 of 20 for the Aboriginal population (61 for children under 5 years) and 1.6 for the non-Aboriginal population (15 for children under 5 years).

Outbreaks in Aboriginal communities: An outbreak between 1987 and 1991 in the central arid region of the country resulted in a mean annual incidence of 21/13228 in the Aboriginal population (3); it was caused by serogroup A, clonal subgroup I-1 (4), and none of the non-Aboriginal population of 25,000 people was affected. Between 1990 and 1994, outbreaks were reported in three communities in the northern part of the country, with attack rates of 12/1250 (5), 3/1000 and 7/6000. The former two were caused by *Neisseria meningitidis* C2b:P1.2 and the

latter by C2a:P1.2. All the outbreaks were controlled with community-wide vaccination programs.

The Aboriginal population in the arid centre was the only group in the country affected by the outbreak of serogroup A disease. Clonal subgroup I-1 also caused an epidemic in Auckland, New Zealand between 1985 and 1986, with high attack rates in the Polynesian population (6), and in the United States North West Pacific between 1975 and 1977, with high attack rates in native Americans (7). The same region in central Australia was affected by a larger outbreak of serogroup A disease between 1971 and 1974 (8) (clonal subgroup unknown), and outbreaks were not reported elsewhere in the country during this period.

The outbreak in the Aboriginal population in 1987 foreshadowed a steady rise in disease caused by serogroup B and C in the general population around the country. This incidence (based on notifications) increased from below 1, to a peak of 2.3 per 100,000 in 1994.

Conclusion

Aboriginal Australians have a high incidence of meningococcal disease. The causes of the high incidence of infectious diseases, and of overall poor health, are complex and probably related to adverse historical, cultural, social and economic factors. These, in turn, are reflected in measures of housing, education, employment and income.

The new meningococcal vaccines will have a role in controlling the disease in Aboriginal people. However, this strategy will have to be considered in the context of competing needs for resources to address the social and environmental determinants of ill health.

Several questions on the outbreak of disease caused by serogroup A remain unanswered: Why was the outbreak geographically restricted? Why were the non-Aboriginal residents in the epidemic area not affected? What is the significance of the temporal relationship between the outbreak and the subsequent rise in the incidence of disease caused by serogroup B and C around the country?

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Cochrane Reviews: The way ahead in the control of meningococcal disease

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Objective

To seek a commitment from individuals to work collaboratively in preparing and maintaining systematic, up-to-date reviews on the effect of interventions for controlling and preventing meningococcal disease.

The Problem

The effectiveness of interventions to control and prevent meningococcal disease has been reported by many original research studies.

As an example, studies on the serogroup A and C vaccines provide a spectrum of results and/or recommendations on: the age at which the vaccine is clinically protective; the duration of protection; the age at which a booster dose should be given; the need for vaccinating close contacts when a sporadic case occurs; the impact of vaccine on carriage of *Neisseria meningitidis*; and the population or setting the results are generalizable to.

Conclusions from a review of these studies will be determined by how the studies will be selected and scrutinized. They will vary in quality and generalizability; these, in turn, will depend on the study design and methods, including methods for selecting the study population, for measuring the outcome variables, and for conducting the analysis.

It is unreasonable and inefficient to expect all practitioners who want reliable information on the effectiveness of the interventions to unearth all the relevant evidence from original research. Most people rely on reviews of the primary research as a way of coping with the information overload confronting them. However, the quality of reviews often leaves much to be desired (1,2); this is because reviewers may not have approached their task with a respect of scientific principles, and in particular, a consideration of the control of biases and random errors. Furthermore, they may not have conducted exhaustive searches for all studies, including those published in the non-English language literature.

We need a systematic review of studies on the effectiveness of the interventions for controlling and preventing meningococcal disease, including specifically studies on the vaccines and the chemoprophylactic agents. The aims of the review would be:

- to identify all studies through an exhaustive search of the English and non-English language literature, including studies completed but not published;

- to select studies based on minimum quality criteria;

- to synthesize the information and provide estimates of effectiveness of the intervention measure, taking into account the expected heterogeneity of the results;

- to link the results of research to improved health outcomes; and

- to inform future research. For example, we could provide reliable estimates of the effectiveness of current vaccines against which the new vaccines can be compared, and identify important questions that researchers may have not have explored, or left unanswered in the earlier studies.

The Proposal

This conference provides a unique opportunity to identify individuals from many different countries, and with fluency in different languages, who will share in the vision of the Cochrane Collaboration (3).

The aims of the Collaboration are to prepare and maintain systematic reviews of randomized controlled trials (RCT) of the effects of health care, and to make this information readily available to decision-makers at all levels of the health care system. In the absence of RCT, the best available evidence is used. The Collaboration is guided by six principles: the shared will of contributors to collaborate with each other; building on people's commitment, enthusiasm and specific interests; minimizing unwarranted duplication of effort; avoiding bias by responding to criticism and disagreements through collegial resolution; keeping up-to-date with new evidence; and ensuring that the "*Cochrane Database of Systematic Reviews*" is available as widely as possible.

Working within this framework "...requires an ego that is satisfied by unselfish collaboration and group, rather than individual, recognition.....and the subservience of personal convictions to scientific evidence" (David Sackett 3).

The Cochrane Handbook provides a 'tool kit' of scientific strategies and tactics for preparing, updating, and disseminating the reviews, and for encouraging criticisms through a process of open peer review.

Conclusion

The Collaboration has identified the Acute Respiratory Infections Collaborative Review Group (ARI-CRG) as the initial site for locating individuals with an interest in meningococcal disease. The co-authors and the administrative centre of the Group, are based at the National Centre for Epidemiology and Population Health, at the Australian National University in Canberra, Australia.

Individuals interested in this initiative should contact Mahomed Patel at the conference. He invites colleagues to collaborate with him in developing further, a draft protocol for a systematic review of studies on the serogroup A vaccine. He is also keen, on behalf of the ARI-CRG, to talk

with individuals willing to review other specific interventions, and plans to convene an exploratory meeting of interested colleagues during the conference.

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Typing of *N. meningitidis* in Moscow: Prevalence of non-European strains.

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Seventy-five strains of *Neisseria meningitidis* were isolated from patients with systemic meningococcal disease in Moscow in 1993-1995. In contrast to most European countries 21% of the isolates were group A strains, 56% of strains group B, and 15% group C. In 1993-1995 the percentage of group A strains has a tendency to increase from 11% to 33% and group B to decrease correspondingly. In contrast, the European countries reported in 1994 less than 25 group A isolates from total number of 3594 isolates (<1%) (1).

Using the current panel of antibodies to class 2/3 outer membrane proteins, 81% of group B, 73% of group C and 31% of group A strains were non-serotypable. The same proportion of non typable strains was found among group B isolates obtained in Moscow in 1983-92 (2). Thus, either additional specific monoclonals for Russian strains or, preferably, genetic typing methods have to be elicited for class 2/3 proteins-based classification of such meningococci. According to their class 1 outer membrane protein, 24% of group B, 18% of group C, and 6% of group A strains were non-subtypable.

Twenty four different serotype/subtype combinations were found among 53 strains of group B and C. This heterogeneity was also in contrast to the situation in Europe; for example, in the Netherlands, having approximately the same territory and population as the Moscow region, 23 most frequent sero/subtype combinations were responsible for 75% of 475 Dutch group B and C cases in 1994 (3). Even for group A meningococci, five different subtype/serotype combinations were found among 16 Moscow strains, although usually only one group A clone predominates in certain region (4). No predominant serosubtype was found in Moscow; relatively most strains had the formulas B:NT:P1.2,5 and B:NT:P1.14 (24% and 14% of group B strains, correspondingly), C:4:P1.10 and C:NT:P1.2,5 (18% and 18%), A:4:P1.5,10 and A:NT:P1.10 (31% and 25%). Such strains are very rare in Western Europe whilst the common European strains, such as B:15:P1.7,16, B:NT:P1.4, B:4:P1.4, B:4:P1.15, B:2b:P1.10, C:2a:P1.2, C:2b:P1.10, C:2a:P1.5, C:2b:P1.2,5, C:2b:P1.2 (1, 3), were not found in Moscow. All strains were sensitive to penicillin (MIC < 0.16 mg/l), chloramphenicol (MIC < 2 mg/l) and rifampicin (MIC < 0.25 mg/l), whereas about 7% of European strains were reported to be penicillin-resistant in 1994 in total (1), although some countries reported no resistant strains.

In conclusion, meningococcal strains from Moscow showed considerable diversity of sero- and subtypes, probably evolved in post-epidemic situation in Russia. Differences in circulating strains and presumable immunity of population in Western Europe and Russia increases the probability of mutual exchange of strains and stresses the need of group B vaccine protecting both from West and East European variants of meningococci.

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Heteroduplex mobility analysis for identification of pathogens causing bacterial meningitis.

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Introduction. The classical bacteriological diagnosis of purulent meningitis by culturing takes at least 24 h and is strongly affected by antibiotic treatment before sampling (1). PCR techniques using a set of genus- or species-specific primers to diagnose meningitis are of limited value because many PCR runs should be performed to identify the possible pathogen. The use of universal PCR primers for amplification of the 16S rRNA gene of most pathogenic bacteria followed by hybridization with specific probes (2) is an alternative but cumbersome approach.

Objective. To evaluate heteroduplex mobility assay (HMA) for the identification of bacteria causing meningitides to the genus and/or species level.

Materials and methods. The bacterial strains used in this study were either clinical isolates from collection of the RIVM and the Reference Laboratory for Bacterial Meningitis, University of Amsterdam, the Netherlands or isolates stored at the Russian Collection of pathogenic microorganisms of the State Research Institute For Standardization and Control of Medical and Biological Preparations, Moscow, Russia. All strains were cultured on plates by standard methods. DNA samples for PCR were prepared according to Boom et al. (3) PCR and HMA were made as described elsewhere (4). Briefly, after 35 cycles of PCR with universal primers to bacterial 16S rRNA gene (1), amplicons produced from two different strains were mixed, denatured 2 min by heating at 95C, and cooled to form homo- and heteroduplexes. Then homo- and heteroduplexes were separated by 5% PAAG-electrophoresis at standard condition. Relative mobility of heteroduplexes was estimated using digitalized images of ethidium bromide stained gels.

Results. Thirty 16S rRNA gene sequences from representatives of 10 genera causing meningitis were picked up from Genbank and their 1 kb parts were aligned manually. Two primers in conserved regions were chosen. The length of primer-flanked fragment was 889 bp. DNA distances were calculated by counting mismatches after removal of small unpaired gaps using MEGA software. A theoretical possibility to differentiate all analyzed genera and even species within some genera by HMA was demonstrated. The estimated genetic distances between the analyzed 16S rRNA sequences of closest but different genera were always more than 12%. The distances between different species within a genus never exceeded 5%. The chosen primers were tested in PCR with DNAs from a panel of 40 bacterial species, isolated from CSF or blood of patients with bacterial meningitis.

Specific PCR products were observed for all the DNA tested. The collected PCR products were used as a panel of reference probes for HMA identification of any analyzed genus. A strong correlation between heteroduplex mobility and genetic distance was found for every pair of bacterial strains. Unspecific PCR amplification of the 16S rRNA gene in combination with HMA

distinguished ten following genera: *Neisseria*, *Streptococcus*, *Haemophilus*, *Staphylococcus*, *Escherichia*, *Listeria*, *Moraxella*, *Klebsiella*, *Pseudomonas*, *Campylobacter*. Moreover, as it was predicted by theoretical calculation of genetic distances, the discrimination of species within a genus by PCR-HMA was also possible for some genera. For example, *Haemophilus parainfluenzae* vs. *H. influenzae*, *Staphylococcus aureus* vs. *S. epidermidis* were simply discriminated. Almost all tested species within genus *Streptococcus* could be distinguished with exception of *S. pneumoniae* vs. *S. mitis* or *S. sanguis*. The total time for PCR-HMA test was only 5 h (2 h for PCR and 3 h for HMA).

Conclusions. PCR amplification of the 16S rRNA gene combined with HMA is a rapid, simple and relatively inexpensive method for identification of bacterial genera. For some genera the differentiation of species is also possible. This approach would be valuable for identification of bacteria found in the CSF, blood or other normally sterile body fluids. Such method is also promising as rapid screening method for phylogenetic classification of newly cultured unclassified microorganisms.

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Comparison of different methods to diagnose bacterial meningitis in Russia

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From 200 patients with clinical diagnosis of systemic meningococcal disease or bacterial meningitis of unknown etiology admitted to Moscow Hospital for Infectious Diseases in 1993-95, cerebrospinal fluid (CSF) and blood samples were obtained. AN additional 25 control samples were taken from patients with diseases other than bacterial meningitis. The laboratory diagnostic methods included bacterial culture, antigen detection (counter immune electrophoresis and latex agglutination) and DNA identification using polymerase chain reaction (PCR). PCR assays for the detection of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* were modifications of approaches elaborated by Ni et al. (1) (assay for detection of meningococcal insertion sequence IS1106) and Radstrom et al. (2) (assay for detection of bacterial 16S rRNA gene). The primers for IS element were changed that eliminated the occurrence of multi-band pattern, occasionally observed in (1). More specific primers for rRNA gene provided us the possibility to substitute seminested strategy (2) by one-run assay. Model experiments with 30 reference strains of different bacterial species and their extracted DNAs demonstrated 100% sensitivity and specificity of our PCR assays having detection limit of about 30 genome equivalents per sample. Control experiments included 30 CSF samples from Dutch patients having culture-confirmed bacterial meningitis (12, 10 and 8 cases caused by *N .meningitidis*, *S. pneumoniae*, and *H. influenzae* correspondingly). Sensitivity and specificity were equal 100% also.

Positive cultures were obtained from 66 of 200 Moscow patients (33%). The proportion of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* type b strains was 52%, 33%, and 10%, respectively. Low number of positive culture was caused by pre-clinical use of antibiotics (3). Comparing to microbiological methods, the antigen detection had the high specificity (100%), but a low sensitivity (about 67%).Using latex agglutination additionally 51 cases were diagnosed (meningococcal infection was found in 43 cases (84%) and pneumococcal meningitis in 8 cases).

PCR-based tests of our clinical specimens had the high specificity (97-98%) and sensitivity (more than 90%) in comparison to culturing as golden standard. Only two false-positive results was obtained. Both meningococcal PCR-tests (IS element and 16S RNA gene) were positive in the CSF samples of patient with cerebral insult and of patients with culture-positive pneumococcal meningitis. Both results were caused probably by contamination at the moment of sampling. Two false-negative PCR results were obtained in case of culture-positive pneumococcal meningitis. Six cases of meningococcemia without meningitis, confirmed by blood culture, were studied. From that, three CSF samples were positive in our PCR-assay, and 3 samples were negative. The latter results were considered also as false-negative, decreasing the sensitivity. The former results indicated that the traces of meningococcal DNA may be found even in the culture-negative CSF samples from patients without clinical meningitis.

As far as a few false-positive PCR results were observed, our PCR-based diagnosis might be considered as reliable, even if other tests were negative. Using PCR- tests, 95% of cases diagnosed microbiologically and/or immunologically were confirmed, and 56 cases was diagnosed additionally as meningococcal infection. Thus, in total the causative agent was identified in 173 cases (87%) of clinically suspected meningococcal infection or bacterial meningitis. The PCR-based tests were most informative. The application of PCR-methods for blood samples and a test to differentiate the sero(sub)types and serogroups of meningococci, would be the next stage of improvement of diagnosis.

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Outbreak of group A meningococcal disease in Moscow

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In January 1996 18 patients with systemic meningococcal disease were admitted to Moscow Hospital for Infectious Diseases, that was approximately the same as in corresponding period of previous years. 33% of cases were caused by group A, 50% by group B, and 17% by group C meningococci, that was also as in 1995 (1). The number of patients increased dramatically in February (51 patients), March (78) and April (41). The outbreak of meningococcal disease occurred in Vietnamese community in Moscow. Most patients worked in the same trading company and/or lived in the same hostels. First episode was registered at fifth week of 1996. The outbreak peaked at 10th week and practically stopped at 16th week. As a result, 26, 24, and 5 cases were registered among Vietnamese in February, March, and April correspondingly. Meningococci were isolated from 26 patients, all of strains were group A. The vaccination of the members of community and their contacts in public institutions was started at eighth week, using group A polysaccharide vaccine produced by Gabrychevsky Research Institute of Epidemiology and Microbiology. In total, about 3000 persons were vaccinated, that promoted the cessation of outbreak.

Five of 55 Vietnamese patients died (9%), male : female ratio was 62% to 38%. All non-survivors were males, four of them were younger than 2 years. Median age of patients was 11 years; 24 patients were younger than 6 years, nine belonged to 6-25 years group, 21 were from 26 to 50 years. The age distribution was affected probably by shifted age distribution among all Vietnamese migrants.

With a slight delay the incidence of meningococcal disease increased among other Muscovites. Some patients might be considered as the contacts of Vietnamese patients; patients25, 54, and 36 cases were registered in February, March, and April correspondingly (Vietnamese cases excluded). The increase started at eighth week of 1996 and reached a maximum at 12th week (17 cases per week). At end of April the situation was partly normalized (5 cases per week), that might reflect both the effect of vaccination and the seasonal effect. (Usually the meningococcal season in Moscow is February-March.) This increase was caused solely by group A meningococci, because the absolute number of group B and C episodes was stable from January to April. As a result, the percentage of group A cases among Muscovites increased up to 75% in March.

Among Muscovites with group A disease the case-fatality ratio was 12% and male : female ratio was 56% to 44%. The median age of patients was 19 years; 20% of patients were younger than 6 years, 39% belonged to 6-25 years group, 41% were from 26 to 70 years.

To date the genetic relationship between the strains, caused this group A outbreak, and the group A strains, circulating in Moscow in previous years (1), are not yet studied. Thus we could not

conclude either the new strain was introduced by Vietnamese migrants or the outbreak reflected their low resistance to common Moscow strains. Historically the highest epidemic of meningococcal disease in Russia during last fifty years started in late sixties, and the incidence of 27 cases/ 100000 individuals/ year was registered in 1970 in Moscow (2). Epidemiological (3) and genetic (4) data suggested that this epidemic might be caused by group A strain carried by Vietnamese migrants traveling in 1968 to Russia through China.

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Molecular typing of *Neisseria gonorrhoeae* by repetitive element sequence-based PCR in comparison with arbitrarily primed PCR analysis

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In this study, characterization of *Neisseria gonorrhoeae* isolates by serotyping using a panel of six monoclonal antibodies (1) raised against Protein IB encountered problems of non-reproducibility of co-agglutination reactions. Upon repeat testing, six of the IB-2 isolates were characterized as IB-3 and three have changed their serovar designations from IB2 to IB-6. Two PCR methods [arbitrarily primed PCR (AP-PCR) (2) and repetitive element sequence-based PCR (rep-PCR) (3)] were evaluated as rapid subtyping tools for *Neisseria gonorrhoeae* isolates. Rep-PCR identified eight distinct types while AP-PCR only identified three amongst the 19 isolates. Rep-PCR could clearly discriminate three strains present within cohort 1 from four epidemiologically unrelated strains isolated from a second cohort. With its higher discriminatory power and good day-to-day reproducibility, the rep-PCR is a rapid and sensitive subtyping tool which can complement the serotyping scheme. In laboratories where there is a lack of reagents or facilities to carry out the auxotype-serovar (A/S) classification scheme, rep-PCR typing may act as a substitute for immediate epidemiological tracking of *N. gonorrhoeae* infections in a community.

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Analysis of serogroup C *Neisseria meningitidis* causing sporadic meningococcal disease

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Serogroup C currently causes 64% of invasive *N. meningitidis* disease in metropolitan Atlanta and a similar proportion in much of the United States (1). An increase since 1991 in the number of outbreaks due to serogroup C meningococci in the United States has been noted (2). These outbreaks have been caused by strains with identical, or closely related enzyme types (ET), but little is known about the spread of endemic or epidemic strains within the United States. Understanding the epidemiology of endemic (sporadic) meningococcal disease may be critical to prevention of meningococcal epidemic outbreaks and case clusters. We used prospective population-based surveillance and molecular epidemiologic techniques to study sporadic serogroup C meningococcal disease in a metropolitan population of 2.34 million persons. During the five-year surveillance, in which no case clusters or outbreaks were noted, seventy-one cases of sporadic serogroup C meningococcal disease occurred (annual incidence 0.51/100,000). Eighty-four percent (52/62) of the serogroup C strains available for further study were members of the ET-37 complex by multilocus enzyme electrophoresis (MEE), including two enzyme types (ET's 17 and 24) responsible for 8 of 12 serogroup C outbreaks in the United States since 1991. Pulsed-field gel electrophoresis (PFGE) and serotyping confirmed the relatedness of these isolates. Sporadic cases caused by strains identical by all typing methods occurred over periods up to three years. Our study indicates that group C meningococcal strains which cause sporadic disease are the same as those causing epidemic outbreaks and suggests slow spread of these strains in a human population.

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A study of the sero/subtypes and antimicrobial resistance of *Neisseria meningitidis* isolated in Argentina during 1991-1996.

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The annual incidence of meningoencephalitis in Argentina remained within endemic levels until 1973. In 1974, a peak coinciding with the epidemic due to *N. meningitidis* in Brazil, with serogroup C predominance, was registered. Since 1978, a return to endemic levels and the emergence of serogroup B have occurred (1). The incidence of serogroups B and C remained within the expected levels until the `90s, when there was an increase in the number of cases due to serogroup B (in coincidence with other Latin American countries) (2). Epidemiological surveillance of the prevalent serotypes and subtypes is vital in order to orient vaccine production and to foresee their immunogenicity, as in the case of those for serogroup B meningococcus, which have been developed since 1985. From March, 1991 through May, 1996, 816 *N. meningitidis* isolates of patients from different regions of the country with a diagnosis of meningitis and/or meningococcemia, were studied. They were identified by biochemical technics and serogrouped by plate agglutination. The frequency of the different groups changed gradually. Up to 1994 inclusive, serogroup B predominated (79.1 %, n = 477) over the other ones circulating throughout the country (C, W135, X and Y). The growth in serogroup C was marked during the same year and it equalled serogroup B (50.2 %, n = 103 and 49.7 %, n = 102 respectively) in 1995-1996. Serotype and subtype determinations by ELISA were performed in 58.5 % of the isolates of serogroup B (n = 340) and in 41.3 % of those of serogroup C (n = 89) obtained during the 1991-96 period. The predominant combinations were: B:2b:P1.non-typable (NT), (25.0 %) and B:2b:P1.10 (24.1 %). followed by: B:15:P1.7,16, (7.3 %) and B:NT:P1.NT (6.4 %). The remaining 37.2 % belonged to other serotype and subtype combinations. As regards serogroup C, the prevalent combinations were: C:2b:P1.NT (51.7 % and C:2a:P1.2 (15.7 %). The remaining 32.6 % belonged to other combinations. Between 1991 and 1995, the susceptibility of 198 isolates to penicillin (PEN), ampicillin (AMP), ceftriaxone (CRO), rifampicin (RFA), trimethoprim-sulphamethoxazole (SXT), tetracycline (TET) and ciprofloxacin (CIP), was studied by the method of macrodilution in agar. 19 % revealed moderate susceptibility to PEN and AMP (MICs = 0.12-1 μg/ml) and no beta-lactamases were detected. None of the clinical isolates studied were resistant to CRO, RFA, TET or CIP. 51 % of the isolates had MICs for SXT > 0.5 μg/ml, while the MICs of the remaining population ranged between 0.004 and 0.064 μg/ml.

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Evolution of the second pandemic due to strains of *Neisseria meningitidis* A:4:P1.9/clone III-1. Survey in four African countries Niger, Burkina Faso, Cameroon and Chad October 1995-May 1996

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Since 1991(1), we have organized an epidemiological surveillance of the progression of a second pandemic due to strains A:4:P1.9/clone III-1 of *Neisseria meningitidis* based in four African countries of Niger, Burkina Faso, Cameroon and Chad. By the end of October 1995, we had analyzed 62 strains recovered from 76 registrations. The techniques used were serogrouping, sero and subtyping associated with multilocus enzyme electrophoresis (MLEE).

From Niger, 29 strains belonged to type A:4:P1.9/clone III-1, except three isolated in the same area which belonged to serogroup X. In general this year in Niger, the outbreak was weaker than in 1995 and was located in the south. The number of cases was less than 15 per 100,000 inhabitants.

In Burkina Faso, 32 strains were subcultured from 36 registrations. In 1995, only one strain belonged to the type A:4:P1.9/clone III-1. This year they are all of this type. In 1995, the endemic strains were Y:2a:P1.2,5/ET-37 complex.

From Cameroon, we received three strains this year, one W135:2a:P1.2,5 isolated in Garoua (North). From Yaoundé (South) one was B:4:P1.7,16 and one was A:4:P1.9/clone III-1.

Only one strain was studied in Chad and, it was identified as W135:NT:P1.2,5/ET-37 complex. From 1988(2) to 1996, the second pandemic constitued by serogroup A spread in Africa was responsible for severe outbreaks. The immunization with A+C vaccine is active to prevent the extension of the outbreaks, nevertheless we need to remain attentive to the detection and extension of the serogroups B,X,Y and W135.

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Evaluation of the cross reactivity of antisera raised to recombinant Transferrin binding protein 2 variants from *Neisseria meningitidis* against a genetically diverse collection of serogroup B strains.

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Transferrin binding protein 2 (Tbp2) is variable among strains of *Neisseria meningitidis* (1, 2) and is able to induce cross-reactive and bactericidal antibodies in the B16B6-like strains (3). To assess whether the same was true among M982-like strains in which Tbp2 is variable in size, specific antisera were produced against two recombinant Tbp2 variants from strain M982: one corresponding to the full length Tbp2 and one corresponding to the N-terminal half of the molecule, described as the human transferrin binding domain (5). A genetically diverse collection of serogroup B strains representing different genotypes, serotypes and subtypes and having different geographic origins was analyzed and the *tbp2* gene from each strain was amplified so as to determine the size of the gene. Strains with a 2.1 kb *tbp2* gene were selected and the reactivity of the antisera was tested on intact meningococcal cells in a dot blot assay. Ninety eight percent of the 58 strains reacted with the antiserum specific for the N-terminal half of Tbp2 while 74% of the strains reacted with the antiserum raised to full length Tbp2. In parallel, the bactericidal activity of the antisera was evaluated against M982-like strains. The results indicated that the N-terminal half of Tbp2 was sufficient to induce cross-reactive antibodies reacting with the protein on meningococcal cells but the presence of the C-terminal half of the protein seemed beneficial for the induction of cross-bactericidal antibodies.

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Serogroup Y meningococcal disease in the United States

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Background. Endemic disease is caused by heterogeneous meningococci belonging to a variety of serogroups while epidemics are usually caused by clonal *Neisseria meningiditis* strains belonging to a single serogroup. Among *N. meningitidis* strains causing endemic disease, the proportion belonging to a particular serogroup can vary dramatically over time. The reasons for this are not well understood but may be related to circulation of new clonal strains within particular serogroups or changes in population immunity.

From 1989 to 1991, serogroup Y meningococcal disease (SYMD) accounted for only 2% of endemic disease in U.S. surveillance (1), but by 1995, the proportion of SYMD increased to 31%. In the 1970s, SYMD was also recognized as a common cause of endemic disease in some U.S. populations (2,3) and was associated with several outbreaks in military personnel (4-6). In some military studies (4,5), but not others (6), serogroup Y was more likely than other serogroups to be associated with non-meningitic disease, especially pneumonia.

We used the recent increase in the proportion of endemic meningococcal disease caused by serogroup Y in the United States as an opportunity to: 1) better understand the variation in serogroup distribution of endemic meningococci and 2) more fully characterize the epidemiology and clinical illness associated with SYMD.

Methods. In 1992-1995, surveillance was conducted in three counties in the San Francisco metropolitan area, eight counties in the Atlanta metropolitan area, four counties in Tennessee, and the entire state of Maryland for an aggregate population of 12 million. A case was defined as a resident of the surveillance areas who had isolation of *N. meningitidis* from a normally sterile site.

Multilocus enzyme electrophoresis (MEE) (7) was used to characterize serogroup Y isolates from: 1992-1995 U.S. surveillance (n = 40), 1972 U.S. surveillance when SYMD accounted for 18% of the 324 isolates submitted to CDC (n = 7) (3), and 1970-1974 surveillance of U.S. military personnel (n = 12) (kindly provided by Dr. W. Zollinger, Walter Reed Army Institute of Research).

Results. In the active surveillance areas, the rate of SYMD increased from 0.12 per 100,000 persons in 1992 to 0.28 per 100,000 in 1995. The proportion of meningococcal disease caused by serogroup Y increased from 18.5 % in 1992 to 31.1% in 1995. The median age of the SYMD patients was 21.8 years, compared with 14.0 years for all other serogroups ($p = 0.005$). Fifty-one percent of SYMD patients were classified as black and 42% as white; only 25% of patients with other serotypes were classified as black and 67% white (odds ratio (OR) 2.5; 95% confidence interval (CI) 1.8-3.4). SYMD cases were more likely to be associated with pneumonia than were

the other serotypes (OR 4.2; CI 1.8-9.7). Serogroup Y did not differ from the other serogroups in case-fatality rate (10.3% vs. 9.6%).

Two closely related enzyme types (ET-508 and ET-501) represented 45% of the isolates tested from 1992 to 1995; however, these enzyme types were not present among the 1972 U.S. surveillance strains or the strains from the U.S. military personnel..

Conclusions. Serogroup Y meningococcal disease now represents a significant proportion of meningococcal disease in the United States. When patients with SYMD were compared to patients with meningococcal disease caused by other serogroups, they were older, more often black, and more likely to have pneumonia. At least part of the increase in SYMD may be due to the emergence of a distinct clone as characterized by MEE. Development of a serogroup Y conjugate component may be necessary to supplement meningococcal vaccines designed for control of endemic disease.

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Outbreaks of meningococcal disease in England and Wales in the winter of 1995/6

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Thirteen outbreaks of meningococcal disease in school settings were reported to CDSC between 1/10/95 and 31/12/95. Forty four cases were identified in these outbreaks, of whom five had died. Thirty were diagnosed as due to septicaemia, 11 as meningitis and three as both. Twenty six were confirmed by laboratory methods. Of the 13 clusters, eight had at least one serogroup C case, two had at least one serogroup B case and three consisted only of clinical cases.

The 13 outbreaks during a three month period represented a considerable increase when compared with the eight school outbreaks a year identified by CCDCs during the previous two years. A greater proportion of serogroup C cases were observed than would have been expected from the proportion of serogroup C cases identified at the Meningococcal Reference Unit during this period.

In two separate community outbreaks age specific attack rates of serogroup C disease reached levels of 40/100,000 and 160/100,000 within two month periods, compared with an attack rate of 4,3/100,000 in 1-19 year olds in England and Wales for the whole of 1995. A&C meningococcal vaccine and antibiotics were offered to 19,000 2-18 year olds in these outbreaks.

Considerable difficulties in managing these outbreaks, especially in relation to large scale immunization in outbreaks of serogroup C disease were encountered.

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Characterization of two porin genes present in *Neisseria flavescens*

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The genus *Neisseria* includes the pathogens, *N. meningitidis* and *N. gonorrhoeae* together with a variety of less disease-causing species which are considered to be 'commensal'. It has been postulated that carriage of commensal *Neisseria* species may stimulate the production, in humans, of cross-protective antibodies (1,2) but there is little evidence to confirm or refute this idea. Comparisons of predominant antigens in pathogenic and commensal *Neisseria* may therefore help to authenticate this theory and may assist in the development of new vaccines against pathogenic strains.

Extensive studies in *N. meningitidis* have identified porin proteins as being major surface antigens and this has led to their inclusion in novel vaccine approaches (3,4). The porins of different *Neisseria* species are related at the primary structural level and are thought to be similar in their tertiary structures, all having a β-barrel conformation with variable loop regions (5). Two porin alleles are found in *N. gonorrhoeae*, but individual gonococcal strains express only one of the alternative porin classes, PIA or PIB. However, *N. meningitidis* strains commonly express two major porin classes simultaneously, PorA and PorB, of which only PorB is essential for growth. It is thought that the commensal *Neisseria* usually contain only one porin gene which is characteristic for each species.

Nucleotide sequence analysis of porin genes in *N. meningitidis* has demonstrated that extensive variation exists in the loop regions of porins from different meningococcal strains. The same regions are responsible for serological differences recognized by monoclonal antibodies used in typing of clinical isolates. The greatest variation is seen in the loops of PorA and this has fueled speculation that PorA has an immunological role in helping the meningococcus to evade the human immune system. Horizontal genetic exchange has been described as one mechanism by which variation between *por*A genes from different meningococcal strains is perpetuated (6,7). As all the *Neisseria* are naturally competent for DNA uptake, horizontal genetic exchange also occurs between, as well as within, *Neisseria* species. An example of inter-species recombination is the development of penicillin resistance in *N. meningitidis*. An altered *pen*A gene in resistant meningococci was found to contain segments of DNA that had been acquired from *N. flavescens* (8). The common gene pool of *Neisseria* is hence a potential source of genetic variation.

Previous work showed that *N. flavescens* contained two porin genes (9). These genes were of almost identical size and so it was impossible to determine their nucleotide sequences by direct methods or to distinguish, by SDS-PAGE, whether there were one or two porins expressed. In the current work, the nucleotide sequences of the two genes were elucidated by cloning and subsequent PCR amplification of each gene separately. The presence of two porin genes in each of two further strains of *N. flavescens* was established by Southern blotting experiments. The results confirmed that the existence of two porin genes in *N. flavescens* was not a strain-specific

phenomenon. The two *N. flavescens* porin genes were compared with each other and with porin genes from other *Neisseria* species. One of the porin genes from *N. flavescens* shared more similarity (greater than 70% homology) with the porin gene from *N. sicca* than with the other porin gene from *N. flavescens*. The porin genes from both *N. sicca* and *N. flavescens* did not closely resemble any of the other porin genes examined. It is possible that inter-species transfer of a whole porin gene had occurred by horizontal genetic exchange as has been described previously for *N. meningitidis* and *N. gonorrhoeae* (10). Alternatively, *N. sicca* and *N. flavescens* may have previously existed as a single species and the second porin gene in *N. flavescens* was acquired upon species divergence. Further isolates need to be examined before these questions can be resolved.

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Molecular typing of *Neisseria meningitidis* strains using polymorphism of *pilA* gene.

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The identification and the characterization of meningococcal strains is quite important for the management of the infection and for the control of epidemics in the population.

A new molecular typing method for identification and characterization of *Neisseria meningitidis* is reported using the polymerase chain reaction (PCR)(1). We designed primers in the *pilA/pilB* locus and amplified the corresponding fragment which is subjected to restriction endonuclease analysis using three different enzymes. The restriction endonuclease patterns (REP) obtained were compared.

The choice of *pilA* locus for PCR-REP analysis is advantageous as *pilA* is universally present as a conserved gene in all meningococcal strains. Strains tested were from serogroups A, B, C, Y, W135, X and Z. Clonal isolates clustered together in distinct restriction endonuclease patterns (each corresponding to a particular *pilA* allele). PCR-REP-based classification coincided with electrotypes as determined by multi-locus enzyme electrophoresis (MLEE). Strains of serogroup A are less variable than those of serogroup B and C. Indeed, strains from serogroup A clustered in few PCR-REP types while strains from other serogroups (particularly B and C) showed extensive polymorphism in *pilA* with numerous PCR-REP types. Correlation serotype and serosubtype is incomplete particularly in strains belonging to serogroup B indicating the insufficience of serological classification alone in outbreaks and epidemics surveillance.

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The molecular epidemiology of *tet*M genes in *Neisseria gonorrhoeae*.

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Neisseria gonorrhoeae isolates with high-level (MIC>8mg/l) resistance to tetracycline (TRNG) were first detected in the USA in 1984 and first isolated in the UK in 1987. Tetracycline resistance in gonococci is mediated by a *tet*M determinant carried on a 25.2MDa conjugative plasmid (1). The restriction endonuclease map of the conjugative plasmid from a TRNG strain imported from the USA has been found to differ from a map derived from a strain isolated in Holland (2). These two types of *tet*M carrying conjugative plasmids were designated American and Dutch respectively. More recently the nucleotide sequences of the *tet*M genes from the +American and Dutch conjugative plasmids have been determined and found to differ significantly from each other (3).

We have designed three oligonucleotide primers that will hybridise with the *tet*M gene in a polymerase chain reaction (PCR). A universal forward primer that hybridizes with both variants is combined with reverse primers specific to each variant. This PCR assay amplifies the genes to produce products of 777 and 443 base pairs with the American and Dutch types of *tet*M respectively.

The specificity of the PCR assay was challenged using DNA preparations from 18 tetracycline sensitive isolates carrying the 24.5MDa conjugative plasmid and DNA preparations from three strains of *N. gonorrhoeae* with tetracycline MICs of 8mg/l. No product of any size was detected in the PCR assay. Twenty strains of *N. gonorrhoeae* carrying *tet*M conjugative plasmids of known restriction endonuclease pattern type (2) were also tested. Nineteen strains of these yielded the corresponding PCR product and one produced an American size PCR fragment from a Dutch type plasmid. This was reproducible and was confirmed as the correct result by another laboratory (4).

Four hundred and fourteen strains of TRNG isolated in the UK, but originating from infections caught in 48 countries worldwide as well as the UK, were investigated for *tet*M type. These strains had previously been typed using the auxotyping method of Copley and Egglestone (5), serotyped following the scheme of Knapp *et al* (6) and had the plasmid profile determined using a modification of the method of Birnboim and Doly (7).

Two hundred and ninety eight TRNG strains produced 777bp PCR products (American type *tet*M) and 116 produced 443bp PCR products (Dutch *tet*M). All TRNG isolated from strains originating in the Far East yielded the Dutch type and all strains originating in Africa, except one from S. Africa yielded the American type. Strains from other areas of the World yielded a mixture of types but with a predominance of the American type. b-lactamase-production was detected in 71% of strains carrying the American *tet*M and 66% of strains containing the Dutch *tet*M. The American type was found in 79% of strains carrying the 3.2MDa b-lactamase plasmid and the Dutch tetM type was found in 61% of strains carrying the 4.4MDa plasmid. Of the 15

strains originating from Indonesia 12 (80%) contained this latter combination of plasmids but these belonged to 9 different auxo/serotype (A/S) combinations. Of strains originating in the African continent, infections caught in Nigeria and Kenya contributed most strains, 18/25 (72%) contained the 3.2MDa b-lactamase plasmid. These comprised 6 A/S types, however 10/18 were a proline requiring IA6 serovar. Over the period of collection of TRNG strains (1988-1995) there was an increase in incidence of strains containing American *tet*M with either the 3.2MDa plasmid or no b-lactamase plasmid. Since 1991 there has been little overall change in other combinations of plasmids and *tet*M type.

These results are consistent with data previously collected on the global distribution of variants of *tet*M (4) and extend our knowledge of the molecular epidemiology of tetracycline resistance in the gonococcus.

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Assessment of serological response to meningococcal outer membrane proteins and capsular polysaccharide in the diagnosis of meningococcal infection

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Since 1991 the use of pre-admission antibiotic therapy of suspected cases of meningococcal infection in England and Wales, has resulted in a significant fall of culture confirmed cases when compared with those notified clinically. Attempts to try to confirm the diagnosis and additionally identify the serogroup of the infecting strain, where these are B or C by serodiagnosis have been made by the reference laboratories in England and Wales, and in Scotland.

Collections of acute and convalescent phase sera from patients with culture proven or clinically suspected meningococcal disease, with varying clinical presentations, were examined. Paired sera from patients with other proven infections served as controls.

Sera were initially screened using an enzyme-linked immunosorbent assay (ELISA) previously developed for the serodiagnosis of meningococcal infection. This ELISA uses outer membrane vesicles purified from three phenotypically diverse meningococci as antigen (1). Sera were then tested for antibodies to the serogroup A, B and C capsular polysaccharide by ELISA, using a modification of the CDC protocol (2, 3, 4).

Although most individuals responded to both the protein and the relevant polysaccharide antigen, some patients responded to one antigen only. However, the data indicate that a combination of antigens provides an extremely useful tool for the diagnosis of all forms of culture negative non-fatal meningococcal disease.

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P1.19 specificity of a previous P1.15 reference monoclonal antibody demonstrated by blotting methods, *porA* sequencing and peptide mapping

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A difference in specificity of the two P1.15 reference monoclonal antibodies (Mabs) produced by WD Zollinger (2-1-P1.15) and JT Poolman (MN3C5C) has been reported (1). MN3C5C recognizes a sequence of three amino acids in the variable region (VR) 2 of the Por A molecule (2), while the specificity of 2-1-P1.15 has not been reported.

Among meningococcal strains infecting patients in Norway in the period 1987-1995, we identified 37 strains reacting on dot-blots with 2-1-P1.15 of which 25 also bound MN3C5C. No strains that were only positive with MN3C5C, but not with 2-1-P1.15, were found. Furthermore, some strains which only bound 2-1-P1.15, also reacted with Mabs specific for other VR2 epitopes, thus identifying P1.1,15, P1.2,15 and P1.14,15 subtypes.

To understand the difference in specificity between the two P1.15 Mabs, sequence analyses of parts of the *PorA* encoding the two VR of PorA were undertaken. The *porA* of 8 strains, reacting with 2-1-P1.15 Mab, showed a sequence coding for a VR1 related to the P1.19 subtype (3), whether or not they reacted with MN3C5C (PPSKSQPQVKVTKA, P1.19, 6 strains; P**R**SKSQPQVKVTKA and P**L**SKSQPQVKVTKA, one strain each, designated P1.19b and P1.19c, respectively). These results suggested that 2-1-P1.15 could be a P1.19 subtype-specific Mab. Strains, that in addition reacted with P1.1, P1.2 or P1.14 specific Mabs, showed *porA* sequences compatible with these reactions, whereas strains binding MN3C5C had sequences encoding NNT or NNA in VR2. One isolate, reacting only with 2-1-P1.15, had the NNT sequence in VR2, but this was preceded by a proline instead of a glutamine, which seemed sufficient to annul the reaction with MN3C5C.

Partial peptide mapping of the epitope for 2-1-P1.15 was done by examining its binding in ELISA to 25 to 29-mer synthetic peptides which corresponded to loops 1, 4 and 5 of strain H355 porA. The results verified that 2-1-P1.15 did indeed react with a peptide corresponding to loop 1, thus confirming the P1.19 specificity of that Mab. On immmunoblots, 2-1-P1.15 bound weakly to the P1.19b variant, whereas no binding to P1.19c was observed, indicating that the epitope for 2-1-P1.15 must be located to the N-terminal end of VR1.

The Cuban B:4:P1.15 vaccine contains the P1.19,15 subtype protein (4). The demonstration of strains with subtype P1.19,15 and P1.19 strains without the P1.15 subtype made it possible to analyse theVR response in vaccinees receiving the Cuban vaccine. For this purpose, sera with class 1 protein antibody activity from five volunteers given the Cuban vaccine in the Icelandic trial (5), were immunoblotted against P1.19 strains with different VR2 subtypes and against

unrelated strains. The postvaccination sera responded mainly against the P1.19 region, and they showed the same specificity against the P1.19 variants as 2-1-P1.15, that is low or no binding.

In conclusion, the demonstration of a P1.19 specificity of the prior P1.15 reference Mab 2-1-P1.15 explains the difference in reaction between this and the other P1.15 reference Mab MN3C5C, and it enlarges the repertoire of VR1 specific Mabs. The use of different P1.19 strains showed that the class 1 protein response in vaccinees given the Cuban vaccine was directed against VR1 in contrast to the VR2 reaction found against protein P1.7,16 in volunteers receiving the Norwegian 15:P1.7,16 vaccine (6).

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School-based clusters of meningococcal disease in the United States: descriptive epidemiology and a case-control analysis

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Background. The occurrence of multiple cases of meningococcal disease (MD) in a school causes substantial concern in the local community. Routine postexposure chemoprophylaxis or vaccination is not recommended for school contacts. The approach to disease control in the setting of a school-based cluster of MD is problematic and limited by a lack of information on the epidemiology of and risk factors for such clusters.

Methods. We surveyed state health departments for school clusters of MD (defined as ≥2 infected students in grades K-12 with MD within a 30 day period) from January 1989 through June 1994. Each school with a MD cluster was geographically-matched to a control school with only 1 case of MD. We collected data on school characteristics, case-patients' school activities, and genetic relatedness of meningococcal isolates using multilocus enzyme electrophoresis (MEE) with 24 constitutive enzymes. Absenteeism data were reviewed to assess the role of antecedent illness.

Results. *Descriptive epidemiology*: We identified 22 clusters of MD in 15 states from all geographic areas of the United States, with a seasonal distribution similar to that seen with sporadic disease. The estimated incidence of secondary MD among schoolchildren 5-18 years of age was 2.4/100,000 population, a relative risk of 2.2. The median number of students/cluster was two (range 2-4). The number of school clusters with ≥2 students/cluster was significantly higher from that expected in a Poisson distribution of MD among children attending school (summary χ^2=284,282). Among cluster schools, 10 (36%) of 28 secondary cases occurred ≤2 days and 22 (79%) occurred ≤14 days after the index case. Among the 8 school with >2 cases, 50% of the additional cases occurred ≥2 days after the second case. *Case-control study*: Secondary schools (grades 7-12) accounted for 14 (74%) of 19 cluster schools compared with 9 (42%) of 19 control schools (p<0.05). In 16 (73%) of 22 clusters, interaction between case-patients was noted. The index patient in cluster schools was more likely than matched controls to have participated in a school-based activity ≤14 days before illness (OR=7.0, 95% confidence interval=0.9,57). Cluster and control schools did not differ with respect to school attendance prior to the onset of MD. *Laboratory*: Thirteen (59%) of 22 clusters were due to serogroup C, and 7 (32%) due to serogroup B, similar to the distribution among control schools. Isolates from among 10 (91%) of 11 individual school clusters were clonal by MEE.

Conclusions. Three fourths of school clusters occurred in a secondary school. Subsequent cases usually occurred within 2 weeks of the index case. After 2 cases of MD in a school, rapid initiation of a chemoprophylaxis program may prevent a substantial proportion of cases in this

setting. Continued surveillance may help determine if participation in group activities or circulating illnesses increase the risk of transmission in schools.