Population biology and epidemiology

The population biology of the pathogenic *Neisseria*

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In a highly clonal species the population consists of independent non-recombining lineages, that may be lost by stochastic extinction, or increase in frequency under selection or random drift. In such species (*e.g. Salmonella enterica*), the very low rate of recombination between genes of different lineages results in strong linkage disequilibrium between alleles in the population. The extent of recombination within bacterial species can therefore be inferred from measurements of linkage disequilibrium (1). Significant linkage disequilibrium has been detected in several species and it has been suggested that recombination is rare in bacteria. However, recombination may be much more common in some naturally-transformable species, including *N. gonorrhoeae* and *N. meningitidis*. Linkage disequilibrium was not detected in two gonococcal populations, implying that the extent of recombination (and the frequency of mixed infections) must be high in nature (1-3). Gonococci therefore have a non-clonal population structure in which alleles in the population are constantly being shuffled, and in which clones cannot emerge, or have only a transitory existence. As a consequence, epidemiologically-unrelated gonococcal isolates recovered in different regions, or at different times, should be distinct, provided suitable methods that give sufficient powers of discrimination are used (4).

In serogroup B and C meningococci there appears to be less recombination than in gonococci and the population appears superficially to be clonal (1,6). However, we have argued that there is considerable recombination in this population and that, in the longer term, the population structure is non-clonal, but this is obscured by the linkage disequilibrium introduced by the emergence and spread of transitory hyper-endemic isolates (*e.g.* the ET-5 or ET-37 complexes) which arise at intervals, flourish and are subsequent broken up by recombination (1). If the distorting effect of these successful clones is eliminated, the alleles in serogroup B and C populations are close to linkage equilibrium. In contrast to gonococci, and serogroup B and C meningococci, analysis of the extent of linkage disequilibrium in serogroup A meningococci suggests a clearly clonal population structure (6, unpublished results).

Recombination within *Neisseria* populations can also be detected at the molecular level, either by the non-congruence of trees derived from different genes, or of gene

trees compared to trees derived from electrophoretic data. Furthermore, recombination can be detected by the presence of mosaic gene structure in which, for example, the sequence of a gene from strain A may be identical to that from strain B in the first half of the gene, but may differ significantly in the second half, which may be the same as the sequence of the gene from strain C. Mosaic structure is commonly found in genes encoding gonococcal or meningococcal cell surface structures (7), but this provides little information on the extent of recombination, as recombinational events within these genes may be rare, but they arise to prominence as a result of positive selection for genetic variation (*e.g.* from the human immune response). Indeed, recombination within cell surface genes has been found even in bacterial species which have strongly clonal structures and in which recombination in nature is thought to be rare. Molecular evidence for recombination must therefore be sought in house-keeping genes in which these events should be selectively neutral. In highly clonal species there is good congruence between different gene trees and trees derived by isoenzyme analysis (8). Unfortunately, house-keeping genes of gonococci are extremely uniform (*e.g.* no differences between the *recA* genes of 10 isolates (3), and a maximum of 1/668 differences in the *adk* gene of 10 other isolates; E. F. and B.G.S., unpublished) and there is far too little variation to build robust trees, or to find significant evidence of recombination. Thus, extensive recombination may occur within gonococcal populations (as predicted by the absence of linkage disequilibrium), but it is invisible because of lack of sufficient variation.

House-keeping genes of meningococci are more variable but in most cases there is still insufficient variation to build robust trees (9). However, visual inspection of the sequences from serogroup B and C isolates shows little sign of congruence between different gene trees. However, the *glnA* gene of *N. meningitidis* is sufficiently variable to look for recombination, and examination of the sequences of this gene from different meningococcal isolates shows clear evidence of intra-genic recombination (J.Z. and B.G.S., unpublished). In contrast, there is good congruence between the gene trees of serogroup A meningococci, confirming the restricted recombination between isolates of the major group A lineages (E.F. and B.G.S., unpublished).

Recombination between the pathogenic *Neisseria* and the closely-related commensal *Neisseria* species also occurs, both in genes under positive selection (*e.g.* those encoding penicillin-binding proteins), and in house-keeping genes (9,10). Surprisingly, however, there is little evidence of recombination in nature between the two closely-related pathogenic species (3). The relative ecological separation between meningococci and gonococci probably provides a major component in their genetic isolation in nature. The extreme uniformity of gonococcal house-keeping genes suggests that the species has been through a recent severe bottleneck which we have suggested may have been speciation - *i.e.* gonococci may have arisen very recently (3).

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Analysis of chromosomes from *Neisseria meningitidis* strains

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The strain *Neisseria meningitidis* 44/76 has been used for the production of the serogroup B meningococcal vaccine in Norway (2). The chromosome of 44/76 was analysed by pulsed field gel electrophoresis after restriction enzyme digestion of the DNA. A physical map of the chromosome has been constructed by aligning the fragments. The restriction enzyme *SfiI* cut the genome in 9 fragments ranging from 980 to 20 kb designated by letters A through I. Several *NheI* sites have been localized on the physical map, and 21 gene probes have been assigned to the map. The *pilE*, -G, -T, and -Q genes are more than 80 kb apart.

44/76 belongs to the ET-5 complex (1). The ET-5 strains studied contained 8 - 9 *SfiI* fragments ranging in size from 20 to 980 kb. The largest fragment, A, was between 820 - 980 kb in size, and fragment B varied from 460 to 610 kb. Fragment G of 40 kb was not detected in some of the strains resulting in hybridization of a gene probe from fragment G to fragment A. The *asd* gene probe, usually localized on fragment A, was sometimes found on fragment B, which is localized more than 200 kb away from fragment A on the 44/76 chromosome. Additional clone complexes were studied. All cluster D strains had a similar *Sfi*I pattern; this pattern was distinct from that of ET-5.

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Molecular methods in the determination of the relationships between meningococcal population biology and epidemiology

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Establishing the relationships between the population genetics and epidemiology of bacterial pathogens enhances our understanding of disease and consequently improves our ability to design novel means of prophylaxis and treatment. This is particularly true in the development and testing in field trials of new vaccines, where information on the mechanism and rates of change of the targeted pathogen can be crucial to the long-term success of the vaccine. Recent studies have shown that *Neisseria meningitidis* has a complex population structure, greatly influenced by horizontal genetical exchange (3). Further, within the species, bacteria associated with epidemiologically different forms of the disease have different population structures (5). Horizontal genetical exchange generates much of the antigenic diversity of this species (2,8,9), which is a major factor contributing to the difficulties encountered in the development of new vaccines against serogroup B meningococci (10). This diversity extends to other genes with the result that no single genetic or antigenic target is necessarily a reliable in the determination of the relationships among meningococcal isolates. This hampers the collection of accurate epidemiological data.

To overcome these difficulties, it is important to develop new procedures for the characterisation of meningococcal isolates and to understand the strengths and weaknesses of current approaches. Our studies have concentrated on the acquisition of data by several DNA-based techniques that provide different and complementary information. These approaches have included: pulsed field gel electrophoresis (PFGE) fingerprinting, for the investigation of the relationships between chromosomes (1); DNA hybridization techniques, for identifying the genes encoding antigenic variants (4); and direct nucleotide sequence analysis, for investigating the relationships between genes (6,9). These technique have been applied to (i) strains previously defined by conventional serological typing and multilocus enzyme electrophoresis and (ii) otherwise untyped isolates, providing new insights into the population genetics and epidemiology of the meningococcus.

Pulsed field gel electrophoresis fingerprinting. The meningococcus has a genome in the range of 2Mb, which when digested with appropriate restriction endonucleases, can be resolved into fingerprints of 12-25 bands by PFGE. The advantage of this

technique is that the whole chromosome is subject to analysis. In addition, if a number of different endonucleases are used it is possible sample up to 50 separate sites on the chromosome rapidly. It is a reasonable expectation that changes in restriction endonuclease sites are selectively neutral, making them ideal for measuring the divergence among isolates.

A remarkable feature of the meningococcus is the heterogeneity of the chromosome structures, and hence the PFGE fingerprint patterns, observed in the meningococcal isolates from endemic strains. This is consistent with most carried meningococci belonging to a panmictic population (3,7). By contrast, strains from particular epidemic or hyper-endemic outbreaks have similar PFGE fingerprints, which are therefore useful in the identification and characterisation of disease outbreaks. The stability of the fingerprints is also instructive, with minor changes indicating the rates of genetical change in the meningococcal population. Serogroup A meningococci are genetically the most stable of the epidemic/hyper-endemic strains, with fingerprints persisting unchanged over longer periods of time than those of serogroup B or C meningococci.

Direct nucleotide sequence analysis. The most precise information on the movement and rates of change of particular genes or parts of genes in the meningococcal population comes from direct nucleotide sequence analysis and related techniques such as 'T-tracking'.The nucleotide sequence analysis of major subcapsular antigens from meningococci associated with a variety of epidemiologies has established that different selection pressures and different mechanisms of variation are important in each epidemiologically distinct group of strains. Of the major disease-causing groups, Group A meningococci are again are the most stable, although there is evidence of horizontal genetical exchange generating their antigen genes in the distant past (9). In serogroup C isolates of the ET-37 complex, antigenic variation has occurred by the accumulation of point mutations, whereas in the predominantly serogroup B ET-5 complex many recently arising mosaic genes can be seen, implying that horizontal genetical exchange is the most significant mechanism for antigenic variation in these bacteria.

DNA-based analyses have demonstrated that the strains associated with each of the major epidemiologies of meningococcal disease exhibit characteristic population structures. These structures arise as a consequence of the relative importance of point mutation and horizontal genetical exchange. Whether diverse epidemiological behaviour is the consequence or cause of differences in population genetics is an intriguing, but as yet unanswered, question.

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Analysis of *Neisseria meningitidis* class 3 outer membrane protein gene variable regions and type identification using genetic techniques

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Antibodies to the class 2/3 OMPs are bactericidal and antigenic diversity between these proteins forms the basis of the current serotyping classification. The class 3 protein genes of several serotypes have been sequenced and areas of variability described (1, 3) which correspond to the surface exposed loops of the of the mature porin protein (2). It is expected that these loops contribute to the serotype specific antigenic epitopes. We have examined the variable regions of 13 class 3 OMPs as they relate to serotype and have developed oligonucleotide probes for identification and classification using genetic techniques.

Complete class 3 gene sequences were obtained from prototype strains for serotypes 3, 14, 17, 18, and 19 representing serotypes not previously reported in the literature. A Brazilian outbreak strain, BB1350 (serotype 4), and M978 (serotype 8), for which VR1 and VR2 were previously described (3), were also sequenced and analyzed. The *porB* gene sequences for strains of serotypes 1, 4, 12, 15, and 21 were obtained from GenBank. Multiple sequence alignment was performed using translated amino acid sequences and revealed large areas of highly conserved sequence and several well defined areas of sequence variability. Amino acid differences between serotypes were noted in 46 different positions, 43 of which were located in outer exposed loops. All non-conserved amino acid changes were localized to four regions of major variability (VR1, VR2, VR3, VR4) corresponding to loops I, V, VI, and VII of the folded protein.

Multiple sequence alignment on individual variable regions was performed using nucleic acid sequences. Analysis of VR-1 shows four distinct subgroups: serotypes 15 and 17 each have unique sequences, serotypes 4 and 21 share a common sequence and serotypes 1, 3, 8, 14, 18, and 19 are identical. In VR-2, unique sequences for serotypes 4a (M981), and 17 were found. For VR-3, serotypes 1 and 12 are identical, serotypes 4 and 17 are identical, serotypes 21 and 14 differ by one base, and serotypes 3, 8, and 19 are identical and differ from 18 by one base. In VR-4, serotypes 1, 12, 15, 17, and 21 each have distinct sequences while serotypes 3, 4, 8, 18 and 19 are identical.

Hybridization assays using several biotin 5' end-labelled class 3 oligonucleotide probes, and a Class 2 probe were performed using dot blots of genomic DNA from each prototype serotype strain. Additionally, nine serotype 15 strains were hybridized with the VR1-15 probe, and fourteen serotype 4 strains and eight serotype 21 strains were hybridized with probes VR1-4, VR2-4a, VR4-4 and VR4-21. The probes for Class-2, VR1-15, VR1-17, VR4-12, and VR4-21 were specific for DNA from strains of the corresponding serotypes. VR1-15 identified the series of serotype 15 strains, and the probes VR1-4, and VR4-21 when used in tandem, correctly identified the series of serotype 4 and serotype 21 strains.

We have thus expanded the sequence data of *N. meningitidis* class 3 proteins to include 6 additional serotypes, and have described significant similarities and differences between VR sequences of distinct serotypes. A subgroup of serotype 4 strains was demonstrated. Hybridization assays demonstrated the ability to identify VR genotypes and distinguish serotypes using biotin labelled oligonucleotide probes. This information may be useful in strain selection for vaccine development, epidemiologic studies to determine the prevalence of individual VR genotype especially among non-serotypeable strains, and combined with PCR, in identification of culture negative suspected meningococcal cases.

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Molecular analyses of meningococcal serosutyping antibodies

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The quality of epidemiological data is pivotal in the design of improved meningococcal vaccines and is critically dependent upon the availability of appropriate reagents for the characterisation of isolates. Meningococcal isolates are characterized serologically on the basis of capsular polysaccharide into serogroups which are further subdivided, according to their major outer membrane protein (OMP) antigens, into serotypes (determined by the mutually exclusive class 2 and 3 OMPs) and serosubtypes (determined by the class 1 OMP), and according to their lipooligosaccharide into immunotypes (1). The class 1, 2 and 3 OMPs are related porins that have loops, of variable length and amino acid sequence, extending from the cell surface (4,9). The class 1 OMP has comparatively large cell surface loops that include the epitopes recognized by the murine monoclonal antibodies (MAbs) used for serosubtyping. In many cases the epitopes that determine meningococcal serosubtypes have been mapped using linear peptides (6). However, the range of MAbs that recognize class 1 OMP epitopes is limited and even when an antibody that reacts with a particular epitope exists, nucleotide sequence analyses have revealed epitope variants that react unpredictably (3,10). As a consequence, a considerable proportion of isolates continue to be reported as non-subtypeable (3).

Numerous variants of particular serosubtype epitopes have been identified, many of which fail to react with the corresponding MAbs. Analysis of the nucleotide sequences of class 1 OMP genes in endemic strains from the UK suggests that these variants have arisen by the repeated selection of point mutations, insertions and deletions. Several families of serosubtype variants have been studied in detail: i) eight amino acid sequences similar to the P1.10 epitope have been identified of which only two, P1.10 itself and P1.10e, react with the existing anti-P1.10 reagent; ii) meningococcal isolates from a local epidemic in the Gloucester area of the UK in the mid-1980s (5) and from a recent cluster of cases in Norway express distinct variants of the P1.16 epitope (7); and iii) variants of the P1.7 epitope have been described that are only recognised by the anti-P1.7 MAb when the class 1 OMP is denatured (10). The P1.2 family was chosen for further studies on the molecular interaction between meningococcal class 1 OMP epitopes and their corresponding MAb for a number of reasons: four variants of P1.2 have been identified that only differ from one another by one or two amino acids; there are three murine hybridomas producing

monoclonal antibodies that differentiate between P1.2 variants; meningococci expressing P1.2 variants are frequently associated with disease (eg bacteria of the ET37 complex); and the P1.2 epitope has been included in a recombinant OMV vaccine that is under consideration for clinical trials.

The genes encoding the V_H and V_L domains of the monoclonal antibodies that recognize P1.2 variants have been cloned and their nucleotide sequences have been determined. A comparison of their deduced amino acid sequences showed that the V_H domains of all three antibodies are similar, whereas there was greater variation between V_L domains. Further comparison with the previously published amino acid sequence of an anti-P1.15 monoclonal antibody (2) revealed greater differences between the antibodies which recognise structurally distinct epitopes. These data indicate that the ability of the MAbs to differentiate between P1.2 variants is determined by their light chains, whilst the ability to differentiate between the P1.2 and P1.15 epitopes is probably determined by both the V_H and V_L domains. Given that the murine hybridomas used to produce the anti-P1.2 reagents were isolated independently, the high degree of similarity amongst these MAbs probably results from the selection methods employed in isolating the hybridomas from which they are produced. It is, therefore, unlikely that the structure of these antibodies is typical of the human immune response to the P1.2 epitope, where a greater diversity of antibody structure would be expected.

The cloning and expression in bacteria of genetically modified V_H and V_L domains as single chain Fv fragments, Fab' and $F(ab')_2$ fragments (8), together with the elucidation of the structures of these antibodies bound to their respective epitopes, will permit future typing regents to be engineered with specificities more relevant to meningococcal epidemiology and reduce the number isolates that are currently described as non-typeable.

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Use of PCR for detection and typing of *tetM* determinant in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae exhibiting high-level plasmid-mediated resistance to tetracycline (TRNG) were identified in 1984, firstly in the USA (1) and subsequently in the Netherlands (2). We have evaluated PCR, using sequences derived from *Ureaplasma urealyticum tetM*, for the detection of TRNG. *tetM* was detected in 90 strains known to be TRNG but not in 19 strains known to exhibit chromosomal resistance to tetracycline (3). *tetM* was also detected in 22 of 266 isolates tested from consecutive patients attending St. Mary's Hospital between January-August 1993.

Genetic diversity within the *tetM* determinant was determined using both a *HpaII* digest of the product from the PCR used for detection and by the use of an additional three sets of primers. The analysis of 64 TRNG showed the presence of two patterns. The first pattern produced three fragments of approx. 350, 250 and 150bp on digestion of the PCRP with *HpaII* and the *tetM* amplified with all primer pairs. The second pattern produced two fragments of 660 and 150bp and the *tetM* amplified only with the primers used for detection. The *tetM* determinant from 170 strains isolated in different parts of the world has subsequently been studied. The first type of *tetM* was found in the majority of strains and is consistent with the *tetM* found among TRNG initially isolated in the USA (4). The second type of *tetM* was found in only 29 (17%) TRNG and is consistent with the *tetM* found initially amongst TRNG from the Netherlands (4).

N. gonorrhoeae carrying the *tetM* determinant have now spread to many parts of the world. The PCR we have evaluated detects *tetM* in all TRNG tested and has shown genetic diversity within the *tetM* determinant found in *N. gonorrhoeae*.

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Patterns of PCR-RFLP of *Neisseria meningitidis* group B and their epidemiologic significance

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The cause of epidemic cerebrospinal meningitis (ECM) was mainly *Neisseria meningitidis* group A in China. We have established a method for typing *Neisseria meningitidis* by analysis of polymerase chain reaction (PCR) - restriction fragment length polymorphisms (RFLP) in the *porA* gene encoding the Class 1 outer membrane protein (OMP). The results obtained by the above method were consistent with those of serosubtyping 103 strains of *Neisseria meningitidis* group A by means of monoclonal antibodies (McAbs) against the class 1 OMP in whole cell ELISA. However, the strains of *Neisseria meningitidis* group B which were isolated from the patients of ECM seemed to have a tendency to increase in recent years in China. The currently available vaccine could not provide an effective protection against the infection of *Neisseria meningitidis* group B in the prophylactic practice. In order to survey the change of serogroups of *Neisseria meningitidis* in China and to select the representative strains to produce vaccine against infection of *Neisseria meningitidis* group B, the PCR-RFLP typing method was used for further typing the strains of *Neisseria meningitidis* group B and analysing the epidemiological significance of their RFLP patterns. In the amplification of porA gene with primers described by Maiden, the 1116bp fragment which could be split into 827bp and 289bp fragments by restriction endonuclease EcoRI was obtained from all 100 group B strains which included 63 case strains and 35 carrier strains as well as 2 reference strains. It could be explained that all group B strains possessed of the porA gene encoding class 1 OMP. In the above strains examined, 40 strains yielded an additional 900bp product which couldn't be split by EcoRI. After all DNA of *por*A gene of group B strains was amplified by PCR and then was digested with restriction endonuclease MspI, 6-10 fragments were found. Among others, the largest fragment occurred in the range of about 400bp, but the patterns of fragments were diverse in the range under 300bp. According to the number of fragments and their difference of molecular weights, 100 strains group B could be divided into 33 distinct RFLP patterns (b1-b33). As compared with the group A strains, RFLP patterns of group B strains seemed to be more complex, but a predominant pattern (b20) was distinct in group B strains. The characteristics of the predominant pattern (b20) were as follows: 1. it accounted for 31.63% of total isolates examined in the research; 2. b20, the important RFLP pattern, was mainly isolated from patients (29/31); 3. the proportion of b20 was

increasing from 12.0% to 56.3% in recent years; 4. all 31 strains with pattern b20 were subtype P1.2; 5. b20 possessed 7 bands in which 6 larger bands were similar to those of b21, b22, b23 and these RFLP patterns accounted for 50% of total strains tested; 6. the pattern 620 was characterized by widely regional distribution, which occurred in 9 of 15 provinces investigated. These data demonstrate that PCR-RFLP technique is a convenient, quick and highly discriminative method that can provide a new tool of molecular epidemiology for *Neisseria meningitidis*; b20 and closely relative RFLP patterns have important value for tracing the spread of *Neisseria meningitidis* in epidemiologic surveillance, and b20 may be considered as a candidate in developing new vaccine against group B meningococcal meningitis.

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Detection of bacterial DNA in cerebrospinal fluid with a simultaneous assay for *Neisseria meningitidis, Haemophilus influenzae* and Streptococci by a seminested PCR strategy

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Primers specific to conserved and variable regions in the 16S rRNA gene were selected for the simultaneous detection of *Neisseria meningitidis, Haemophilus influenzae*, and streptococci in cerebrospinal fluid (CSF) by a seminested PCR assay. The nucleotide sequences of two third of the 16S rRNA gene in N. *meningitidis, H. influenzae, and S. pneumoniae, S. agalactiae and Staphylococcus epidermidis* were determined. Sequence comparisons of the 16S rRNA genes indicated species and genus specific domains suitable for primer design.

The assay was divided in two DNA amplifications. The first contained a universal primer set resulting in a 1.0 kb DNA product, independent of which bacterial species was present in the PCR mixture. The second amplification, using four primers, was based on a 1:500 dilution from the first PCR incubation. The length of the amplified PCR fragment in the second reaction was correlated to the bacterial species.

The high specificity of the primers was documented after testing 133 bacterial strains of 28 different species. A total of 304 stored clinical CSF samples , including 125 samples from different patients with bacterial meningitidis, were assayed to explore the diagnostic sensitivity and specificity for bacterial meningitis.

In conclusion the assay showed high sensitivity (0.94) and specificity (0.96) with the clinical samples though some false results were obtained, the reasons for which will be discussed.

Continued spread of meningococcal disease caused by serogroup A *Neisseria meningitidis* strains of subgroup III in Africa (1989-1994). Origin of serogroup A strains isolated in France (1987-1994)

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Since 1989, we have studied strains of *Neisseria meningitidis* isolated in twelve African countries. We have also examined 62 serogroup A strains isolated in France 1987-94 (5). Strains were collected from the following African countries: Chad (1993, 11 strains; 1994, five strains); Morocco (1989, 90 strains including 25 from cerebrospinal fluid and 65 from the rhinopharynx)(4); Niger (1991, eight strains); Central African Republic (1992, 24 strains)(3); Algeria (1992, five strains); Burundi (1992 five strains); Cameroon (1993, seven strains, 1994 six strains); Guinea Conakry (1993, 10 strains); Zambia (1993 nine strains); Rwanda (1993, two strains); Sudan (1993 one strain); and Mali (1993 one strain, 1994 11 strains). A number of these strains were isolated in Norway from Trans-Isolate medium (1) which had been inoculated with CSF from patients in Africa. Characterization involved determination of the antigenic formula (serogroup:serotype:subtype), multilocus enzyme electrophoresis (2,6), and assessment of sensitivity to sulfonamides.

Most of the African isolates belonged to the second world pandemic caused by strains A:4:P1.9/clone III-1. However, some other clones of subgroup III were also found, in particular, in Morocco and Guinea. A clone IV-1 strain was isolated in the Central African Republic in 1992 (3), and strains belonging to ET-37 complex were detected in Niger, Guinea and Mali.

In a number of countries, the geographic distribution of systemic meningococcal disease was found to have extended beyond the traditional zones, which in the north and south had been described by the 300 and 1100 mm isohyetal lines, respectively (The Meningitis Belt). This e.g. the case of Morocco, the Central African Republic (the region around the city of Bozoum), Algeria, Burundi, southern Cameroon, Guinea Conakry, and Zambia.

The epidemiological characteristics of the patients were also found to have changed : the patients were older and the illness was more severe.

In certain regions, correlations were evident between extension of the geographic distribution of meningitis and climatic changes. At present, this phenomenon is being studied in the field. In addition to the effects that these new conditions were having, there is also a possibility that the subgroup III-1 strains were more virulent than previously.

Of the 62 strains of *N. meningitidis* studied in France during this period, 50 serogroup A strains (up to 5% of all isolates) were isolated from 1987 to 1991; these mostly belonged to clone III-1. Since 1992, the number of strains of *N. meningitidis* A isolated has fallen to levels experienced prior to 1987. In addition, with the exception of strains of African origin (one in Somali), they no longer uniformly belong to clone III-1. In Africa, the extension of clone III-1 can be clearly traced: it spread from the east in 1988/1989 toward the west and south, and was isolated again during the first quarter of 1994. In France, as in other industrialized countries, the epidemic ended abruptly at the end of 1987/beginning of 1988.

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Epidemiology of *Neisseria meningitidis* - impact of new techniques

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There is a long history of laboratory effort to distinguish between meningococci, driven by the realisation that certain strains have differences that affect the epidemiology of human infection. The early subdivision into serogroups with polyclonal sera directed against the polysaccharides has been useful and successful but quality assurance surveys demonstrate some shortcomings in serogroup determination. The precise structure of the polysaccharides has been elucidated and monoclonal antibodies are now available. These will undoubtedly improve the specificity of whatever technique is used. Study of related strains isolated from a community outbreak has shown just how variable capsule expression can be, not only in nasopharyngeal isolates but also from case isolates, and occasional strains have capsules in the limit of detectability by latex agglutination. This does not necessarily affect the overall epidemiological picture but is a reminder about the phenotypic variability of meningococci can affect capsules just as in the occurrence of C15 P1.16 strains in the course of a prolonged B15 P1.16 outbreak (1). Moreover transformation, of which this presumably is an example, can and does affect other surface expressed antigens. For many epidemiological purposes, not the least for close observation of particular disease-producing strains and how they move locally, nationally, and internationally, much finer discrimination is required. It has become clear that the application of typing and serotyping dependent on class 2/3 and class I outer membrane proteins although very helpful is not entirely sufficient for this purpose. Not least is that phenotype determination gives little information about the genotype or genetic group to which the meningococci in question belongs. Multilocus enzyme electrophoresis (MLEE) is a highly successful technique for clonal analysis and has been instrumental in our understanding of international and national epidemiology. Newer DNA-based techniques of pulsed field gel electrophoresis (PFGE) and restriction length polymorphisms (RFLP) can provide genotypic data that can enhance the discrimination of MLEE (2).

Although the introduction of monoclonal antibodies directed against epitopes on the class 2/3 (typing) and class I (subtyping) outer membrane proteins improved specificity, problems remain. These arise from the organism e.g. variation in expression of the antigens and the inherent variability of the epitopes due to genetic transfer by transformation. There are also reagent problems in that there are typing and subtyping epitopes that are not recognised by the existing monoclonals, either

because the epitopes are yet to be recognised or they are minor variants of known subtypes that do not react with the appropriate reagent. A sub-typing scheme based on the polymerase chain reaction PCR has been developed (3) and application of this technique produces a significant improvement in sub-typeability.

In 1993 in England and Wales 17% of Group B isolates and 35% of Group C strains were not subtypeable. Using the DNA-based subtyping system for the Group B strains both variable regions (VR1 & VR2) were identified in 43%, VR1 only in 34%, VR2 in 8% of the not subtypables and only 14% remained not subtypeable. For Group C isolates both VR1 & VR2 were identified in most strains and only 1.8% remained not subtypeable. Clearly with this method it is now possible to define the scope of variation in class 1 OMP isotopes. What remains is to estimate the frequency of change so that evaluation of the epidemiological importance of the recognition of these variations can be made. Results obtained with DNA-based methods should also facilitate the generation of new and useful sub-typing monoclonal antibodies. With regard to variability and rate of change there is some data already available. Direct sequencing of the gene encoding for the P1.16 epitope has so far demonstrated three variants, and for these there is also some epidemiological data. B15 Pea and B15 P1.16b were found to be present in many separate locations in England and Wales (4) and historical data showed that each variant tended to be restricted to a locality and to have persisted for up to a decade without evidence of change. B15 Pea and B15 P1.16c have been found in Norway (5) and the new variant P1.16c was present over the 1987-91 period at about the 5% level. These variants, generated by mutations in the sequence encoding for the VR2, appear to be relatively stable and long-lasting in the population of B15 P1.16 strains in European countries.

Further application of the newer DNA-based techniques will define both the distribution and rate of change of more outer membrane proteins associated with epidemiological typing and in a much more comprehensive way than previously. Such data will not only improve our appreciation of the epidemiology, but will be relevant in the quest for satisfactory group B meningococcal vaccines.

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Enhanced epidemiological surveillance for meningococcal disease by DNA-based sub-typing of meningococci

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Existing methods for strain identification of meningococci are based upon the detection of antigenic differences in surface components of the organism. However, although of proven epidemiological value, many meningococcal isolates do not react or can only be partially typed with the existing reagents. Problems associated with the serotyping and subtyping of meningococci arise from the ability of the organism to undergo antigenic variation of the serotype and subtype determinants. This variation occurs amongst genetically closely related organisms, for example in isolates belonging to the ET-5 complex, which has been responsible for a number of recent outbreaks in North West Europe and elsewhere. Antigenic variants arise at unpredictable intervals and are stable insofar as they persist for some years. The capacity for antigenic modulation combined with the phenomenon of clonal replacement results in a constant requirement for the expansion of the typing reagent panel.

Recently, with the development of DNA amplification techniques, it has become possible rapidly to determine the DNA sequence of a particular gene or DNA fragment. The DNA sequences of the meningococcal class 1 (1) outer membrane protein (OMP) from a number of different subtypes are known and the epitopes recognised by the monoclonal antibodies used for serosubtyping have been identified. The determination of the sequences of the various class 1 OMP genes from isolates expressing each of the different subtype epitopes has enabled the development DNA-based methods for subtyping meningococci.

The DNA-based subtyping of meningococci uses a novel approach that combines the polymerase chain reaction (PCR) with DNA hybridization (2). With the development of a system for determining the presence of DNA sequences relevant to the variable regions of the class I outer membrane proteins, the next step was to evaluate the method for usefulness with clinical isolates. Most of the group B and C isolates sent to the Meningococcal Reference Unit in 1993, if they were not subtypeable with the monoclonal antibody, were examined by this method.

In 1993, from a total of 929 group B isolates examined, 570 (61%) were not typeable and 162 (17%) were not sub-typeable. In the same period 319 group C strains were received 55 (17%) of which were not typeable and 111 (35%) were not sub-typable. Of the 162 strains group B strains that were not subtypeable with monoclonal antibodies, 130 were examined by the DNA-based system. Both variable regions (VR1 and VR2) were identified in 56 strains (43%), VR1 only in 45 strains (34%), VR2 only in 10 strains (8%), and only 19 (14%) remained non subtypeable.

The 130 strains examined came from 171 Health Districts, an average of less than 1 strain per district so that the epidemiological impact of these results was only apparent in larger districts or cities. Nevertheless, in 21 Health Districts the additional subtyping information improved information on relationships of strains. For example, in Leeds, 2 out of 23 not subtypeable strains were shown to be similar to 10 others that subtyped with monoclonal antibody, making a difference to the size and appreciation of this cluster of strains.

With the group C strains, 111 (55%) were not sub-typable with monoclonal antibodies and all but 6 of these were sub-typed by the DNA-based system. In the majority of isolates both VR1 and VR2 were identified except that 11 strains gave a result with VR1 only and 2 strains with VR2 only. The overall result of only 6 (1.8%) of group C strains remaining not sub-typable meant a significant increase in epidemiological information was available. The group C strains came from 132 Health Districts and in 33 (25%) of these Districts a clearer view was obtained of the relatedness or otherwise of the nonsub-typeable strains. For example 3 out of 6 strains in one District were not sub-typeable; two were shown to be similar to other strains and one was different; in another District all three isolates were not sub-typable but were shown to be similar to each other and to other sub-typed strains in other Districts helping to get a better picture of a city-wide cluster of strains. Similar results were seen in another city. We therefore concluded that, particularly for the group C strains, a very definite gain in epidemiological interpretation was afforded by use of the new system. The reduction from 35% to 1.8% nonsub-typable was greater and perhaps more useful than the group B reduction of 17% down to 2%.

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Identification of epidemiologically important meningococcal genotypes by Restriction Fragment Length Polymorphism analysis

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Meningococcal strains are classified based upon variation in serologically defined determinants. The application of techniques such as Multilocus Enzyme Electrophoresis has enabled a better understanding of the population genetics of meningococci (1). Knowledge of meningococcal population genetics has provided information regarding the epidemiological behaviour of meningococci. Many pathogenic bacteria characteristically have a clonal population structure and much of the disease which occurs at a particular time is caused by a small number of pathogenic clones. Similarly meningococcal epidemiology is characterised by periodic hyperendemic activity or epidemics, due to the emergence of a pathogenic clone which then goes onto cause epidemics in different localities over a period of time. Recent advances in molecular biology, particularly polymerase chain reaction-based nucleotide sequence analysis have provided information important to our understanding of the epidemiology of meningococcal disease. From the accumulated sequence information of a number of genes for group B and C meningococci it has become apparent that there is considerable horizontal exchange of genetic material between strains resulting in panmictic populations (2). This is manifest in the epidemiology of the organism by the emergence of a pathogenic clone with a dominant phenotype which subsequently becomes diverse. The promiscuous behaviour of group B and C meningococci means that epidemiological characterisation using phenotypic markers based upon serologically defined determinants often obscures the genetic relationship between strains and can confound the epidemiology.

Recent advances in molecular biology have been applied to develop techniques that will provide information regarding the genotype of strains referred to national reference centres in order to rapidly identify strains belonging to pathogenic clones without the resource requirements of MEE. We have analysed restriction fragment length polymorphisms, amongst random and outbreak associated strains, using a genomic probe and restriction enzyme digestion of meningococcal chromosomal DNA to identify genotypes associated with identified pathogenic clones. Amongst 245 strains identified as RFLP type 1 associated with strains related to the ET-type

5 complex which have caused outbreaks of group B disease in several parts of the United Kingdom and elsewhere during the 1970's and 80's. Amongst this collection of strains there a 10 different phenotypes. Amongst the isolates examined were a collection of strains expressing the phenotype B4:P1.10 which were isolated over a period from 1984 and largely confined to a single Health district.

Unlike group B strains, group C meningococci appear phenotypically homogeneous. However RFLP analysis demonstrated considerable genotypic heterogeneity. Of 229 group C strains examined 7 phenotypes and 74 genotypes were identified. Amongst 29 C2a:NT strains and 67 C2a:P1.2 strains 9 and 26 RFLP types were identified respectively. Similarly for each of the two groups of C2b strains examined (67 C2b:NT and 21 C2b:P1.2) 8 genotypes were identified. The genotypic differentiation of meningococcal strains using RFLP analysis is supported by data obtained by 16S rRNA restriction fragment length polymorphisms and pulse field gel electrophoresis.

The development of an RFLP-based system has enabled the rapid identification of pathogenic genotypes amongst regional clusters of strains and determined their association with known pathogenic genotypes responsible for outbreaks of disease elsewhere thus providing enhanced epidemiological surveillance for meningococcal disease.

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DNA-based methods for assessing the diversity of serotype determinants of *Neisseria meningitidis*

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The development of novel means for controlling meningococcal disease requires extensive epidemiological information. Such data is particularly important in the assessment of new vaccines and is also necessary for improving our understanding of the evolutionary mechanisms and the genetic diversity of *Neisseria meningitidis* populations. To date, strain characterisation of meningococci has been based on the identification of capsular and subcapsular antigens with immunological reagents (4). The serotype of a meningococcal isolate is commonly used in epidemiological studies and is determined by the antigenically variable class 2 or 3 outer membrane proteins (OMP). At present these are identified with a panel of monoclonal antibodies (Mab's)(1).

Over the past few years, the number of Group B strains isolated in England and Wales that remain non-typeable with the current reagent panel has increased from around 30% to over 60% in 1993. This has resulted in less complete epidemiological data. The rise in non-typeable strains may be due to the introduction of novel genes encoding non-typeable class 2 or 3 OMP's, mutations arising in existing genes that prevent the interaction of the monoclonal antibodies, or the failure of antibodies to detect their target antigens because of poor expression or masking by another antigen. Unlike the class 1 OMP (the subtyping antigen), which has relatively long linear epitopes in surface loops I and IV that can be mimicked by linear peptides (6,7), the class 2 and 3 OMP's epitopes are smaller, occur in different surface loops, and are difficult to mimic with linear peptides (3,9). The surface loops of the class 2 and 3 OMP's may form conformations that are not stable in small linear peptides or more than one surface loop may be involved in the epitopes recognised by the Mab's. In either case, it is possible that class 2 or 3 proteins that differ by a small number of amino acid residues may present immunologically distinct epitopes, despite being closely related genetically. To investigate this problem, the present work has exploited DNA-based techniques (i) to define the non-typeable genes at the genetic level and (ii) to develop more comprehensive typing schemes. Analysis of the antigens at the level of their genes provides more detailed information on the relationships between strains. Two techniques have been used to assess and identify the genetical diversity

of the class 2 and 3 OMP genes: 'T-track' analysis (8); and direct nucleotide sequence analysis of polymerase chain reaction (PCR) products (2).

Seven Group B non-typeable (NT) strains, collected in 1993 by the UK Meningococcal Reference Laboratory (MRL), were chosen to be a representative sample of the non-typeable strains isolated that year (NT strains accounted for 61% of isolated Group B strains). From 'T-track' analyses, 6 of the 7 strains had class 3 genes identical to the Type 4 class 3 OMP. The remaining strain was confirmed as non-typeable, possessing a previously unseen 'T-track' pattern. These results imply that the majority of the NT strains isolated in 1993 were serotype 4 and that the inability to type these strains using serological techniques was not due to the introduction of a novel gene but due to failure of the antibody to detect the Type 4 antigen or a very closely related variant of this antigen. The predominant subtypes of the Group B NT stains were P1.15 (32%) and P1.4 (24%), subtypes commonly associated with type 4 in UK isolates.

The DNA-based methods were used to characterise 30 strains used in the European Monitoring Group on Meningococci (EMGM) External Quality Assurance Scheme (EQAS). These strains have been chosen using a number of criteria to be representative of the range of meningococci isolated. Each laboratory participating in the scheme grouped, typed, and subtyped the strains. By 'T-tracking' the amplified class 2 or 3 OMP genes, 24 of the 30 strains were successfully typed (four of the 24 EQAS strains were non-typeable with the current panel of Mab's). The remaining 6 strains, 5 of which were non-typeable with the Mab's, had novel 'T-track patterns'. The genes from these strains are currently being sequenced.

Using DNA-based typing techniques, such as 'T-tracking' the amplified class 2 and 3 OMP genes, it is possible to positively type the large number of strains that remain untyped using methods based on the antigenic properties of *Neisseria meningitidis*. By looking directly at the DNA sequence encoding the class 2 or 3 OMP, the genetical relationships between strains can be more clearly identified than when comparing proteins, whose conformation may be markedly altered by minor changes in amino acid sequence. To complement the 'T-tracking' typing method, a DNA dot-blot system, similar in principle to that used for subtyping meningococcal strains (5), is under development. Used in tandem, these techniques could provide a rapid and reliable way of typing and subtyping the large number of strains which are otherwise untypeable, so allowing more complete epidemiological studies to be undertaken.

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Population biology and epidemiology, poster 113.

Monoclonal antibody to serotype 17 of *Neisseria meningitidis* and their prevalence in Brazilian states

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Neisseria meningitidis are Gram-negative diplococci responsible for cases of meningococcal disease all over the world. The epidemic potential of *N. meningitidis* serogroup B and C is clearly a function of their serotype antigens more than of their capsular polysaccharides. Until recently, hyperimmune sera were used to detect typing antigens on the bacteria. The advent of monoclonal antibodies (Mab's) offered the opportunity to eliminate many of the cross-reactions and have improved the accuracy and reproducibility of meningococcal serotyping. We have produced a MAb to the outer membrane protein of the already existent serotype 17 that have been detected by the use of hyperimmune rabbit sera. The prevalence of this serotype epitope is low in the Brazilian strains. By using the MAb 17 we could not decrease the percentage of nontypeable serogroup C strains. However, there was a decrease in nontypeable strains to 13% in serogroup B strains and to 25% in the other serogroups.

Population biology and epidemiology, poster 114.

Ribotyping as an additional molecular marker of *Neisseria meningitidis* Serogroup C epidemic strains

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The ribotyping molecular method was used as an additional epidemiological marker for detection epidemic strains. *Neisseria meningitidis* serogroup C, referred as ET-11 complex strains, responsible for the increasing number of meningococcal disease cases during 1990 to 1993 in the south Brazil were analyzed. The analysis by ribotyping of these strains showed only a single rRNA gene restriction pattern (Rb2), obtained with *Cla*I restriction enzyme. In addition to multilocus enzyme electrophoresis and serotyping, this method provided useful information about the clonal characteristics of the *Neisseria meningitidis* serogroup C:2b:P1.3 strains isolated in Brazil in this period.

A PCR assay for differentiating two groups of 25.2 Mda TetM *Neisseria* plasmids

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High-level tetracycline resistance (MICs of ≥ 16 mg/ml) in *N. gonorrhoeae* (TRNG) is due to the presence of a 25.2 Mda plasmid carrying the Tet M structural gene. This gene codes for a ribosomal protection mechanisms and has been inserted into the gonococcal conjugative 24.5 Mda plasmid (4,6). The first TRNG was isolated in the United States in 1983, but not recognized until later (6). Subsequently, TRNG was isolated in Canada, Europe, England, Africa and Asia, while the 25.2 Mda Tet M plasmid has been found in *N. meningitidis*, *Kingella denitrificans* and *Eikenella corrodens* (1,2,5). Restriction endonuclease analysis mapping suggested that two different restriction patterns were found for different 25.2 Mda Tet M plasmid carried by *N. gonorrhoeae* (1,2). Gascoyne *et al* (1) named the two patterns; American type with patterns identical to plasmids from TRNG original isolated in the United States and described by us (6) and; Dutch type because the first isolate was from a TRNG isolated in The Netherlands. However, these two patterns are not limited to these geographical locations (1,2).

More recently, a polymerase chain reaction (PCR) assay which amplifies a portion of the sequence within the structural *tetM* gene has been used on TRNG isolates (3). When the PCR products were cut with *Hpa*II, two restriction patterns were observed. We have now developed a PCR assay which looks at the downstream regions of the Tet M determinant and have found two different size PCR products. Nineteen TRNG isolates with known and unknown restriction endonuclease patterns, for their 25.2 Mda Tet M plasmid, were used. The bacteria were isolated from different geographical areas between 1985-1990.

For comparisons, nine *N. meningitidis*, from different geographical areas and isolated between 1985-1991, carrying the Tet M plasmid were also examined. Small scale plasmid preparations, which contained both plasmids and chromosomal DNA were used as the template in the PCR assay. Two different sized PCR amplified products were generated when the TRNG isolates were used as the template. Six isolates yielded the smaller PCR product and of these, five had previously been characterized and known to carry the 25.2 Mda Tet M plasmid with the Dutch type restriction endonuclease pattern. The remaining 13 TRNG isolates yield the larger PCR product

and of these seven were known to carry the 25.2 Mda Tet M plasmid with the American type restriction endonuclease pattern. In contrast, the nine *N. meningitidis* had only one PCR product which was indistinguishable in size from the smaller PCR product produced by the "Dutch" TRNG isolates.

These results are compatible with the data presented by Swartley *et al* (8). As with our other PCR assays, proteinase K treated bacteria could replace purified DNA as the template for the PCR assay. This would allow for more rapid determination and reduce the supplies needed. More isolates will need to be examined, but the preliminary data suggests that the two different sized PCR products correlate with the two previously described restriction endonuclease patterns in the TRNG isolates and could be a useful addition in the epidemiologic study of these bacteria.

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PCR AREA Differentiates between strains of *Neisseria meningitidis*

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We successfully applied PCR for the early detection of meningococcal meningitis (1) using primers which amplify a DNA sequence of the meningococcal *dhps* gene. Genetic alterations within this sequence have been shown by us to confer sulfonamide resistance (2). We further studied the ability of restriction endonuclease analysis of the PCR amplicons (PCR AREA) to differentiate between individual strains of *N. meningitidis*. Eight isolates of the isoenzyme ET-5 complex had identical PCR AREA band patterns whereas 8 isolates of other ET-complexes all had different band patterns. Using PCR AREA we were also able to identify the disease-causing strain of *N. meningitidis* in the throat of a healthy contact of a patient with meningococcal septicemia. By statistical analysis of the band patterns by a computer program (GelCompar®), we were able to construct a dendrogram showing the genetic distance between individual strains. We conclude that PCR AREA can be used to type meningococci.

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An approach to study the molecular genetics of *Neisseria meningitidis*: Partial sequence of *rpoB* gene and genetic variability

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Using the technique of polymerase chain reaction (PCR), a 624 bp DNA fragment, which belongs to the ß-subunit of DNA-directed RNA polymerase (*rpoB*, EC 2.7.7.6.) of *Neisseria meningitidis* serogroup B was amplified. The fragment was cloned and sequenced. In contrast to the known sequences of gram negative rods, *N.meningitidis* shows an insertion of about 36 bp. A phylogenetic tree, based on the amino acid translation of this sequence, was constructed (1), including some other eubacterial as well as archaebacterial and chloroplast sequences, too. In the resulting tree *N. meningitidis* forms a distinct branch within a cluster of gram-negative rods. This was separated from another cluster formed by *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Bacillus subtilis.*

In addition, two oligonucleotides were chosen from the sequence. These highly specific oligonucleotides were labelled to perform a hybridization study with a variety of amplified fragments belonging to *Neisseria lactamica*, *Neisseria flava, Neisseria subflava, Neisseria sicca* and seven *N.meningitidis* serogroups. Results indicate genetic differences not only within the genus *Neisseria* but also within the species *N. meningitidis* at this specific part of an essential gene.

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Characterization of *Neisseria meningitidis* by polymerase chain reaction and restriction endonuclease digestion of the M-6 gene

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Neisseria meningitidis is a pathogen responsible for serious invasive disease through the world. The lack of an effective vaccine against all serogroups constitutes a problem in the control of meningococcal disease. Several outer membrane proteins (OMP's) have been examined as vaccine candidates, with only the class 1 protein inducing high titre bactericidal antibodies, which are protective in an experimental model for meningococcal infection (3).

Besides the major OMP's, other surface proteins are under investigation in several laboratories (1, 2), particularly highly conserved OMP's that will potentially confer protection against meningococcal disease in humans. Our group has previously isolated, cloned and expressed in *Escherichia coli* the *M-6* gene, coding for a high molecular weight protein (P64k) which is common to many meningococcal strains (4). Using chromatographic methods the protein has been purified to homogeneity.

To characterize this meningococcal antigen further at the molecular level, we developed a rapid method to evaluate *N. meningitidis* isolates on the basis of genetic variabilities within their *M-6* gene. Strains were differentiated on the basis of restriction fragment length polymorphism (FRLP) patterns obtained after polymerase chain reaction (PCR) of the *M-6* gene and restriction endonuclease digestion with *Hpa*II, which has 13 sites within the gene. We applied this method to 14 serogroup B and 1 serogroup I strains, epidemiologically representative of distinct geographic areas. This allowed differentiation of all 15 strains into 4 distinct RFLP patterns compared with 13 different serotypes and 7 subtypes. Finally, the PCR-RFLP technique, together with the nucleotide sequence of the *M-6* gene from one of each of the 4 RLFP patterns of these strains, revealed a high degree of homology either at the DNA levels; which implies that this gene is conserved among different meningococci.

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PCR for diagnosis of meningococcal meningitis: its application to the cerebrospinal fluids collected during the Norwegian serogroup B vaccine protection trials

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Because of the often rapid and dramatic development of systemic meningococcal disease (SMD), it is recommended that antibiotic treatment starts as early as possible, even before admittance to hospital. The consequence of this is a significant lowering of the proportion of suspected SMD cases confirmed by culture.

Alternative diagnostic tests, such as direct detection of antigens in cerebrospinal fluid (CSF), are not very sensitive. An ELISA serological test based on capsular polysaccharide antigens was developed during the Norwegian serogroup B vaccine protection trial (1). We now employed a nested PCR test to detect meningococcal DNA in CSF, adapted from the procedure described by Saunders *et al*. (2). This test, based on the gene coding for the PorA outer membrane protein, also has the advantage that the subtype of the infecting strain can be determined through DNA sequencing of the PCR product.

Two sets of primers that specifically amplified the *porA* gene from the different subtypes of *Neisseria meningitidis* were selected. The method was tested on CSF samples from 87 patients with various disease etiology, including 37 patients with SMD. When testing 21 CSF's from well-characterized clinical categories of meningococcal disease (3), only 15 were found positive after the nested PCR. Of the 6 negative CSF's, 5 were from non-meningitis patients, while the last one was culture negative, but contained a huge amount of LPS, corroborating a meningitis diagnosis. However, a positive reaction was obtained for this sample after dilution, suggesting the presence of inhibitory substances. All 50 CSFs from patients with non-meningococcal etiology proved negative.

The method was applied to all 63 CSF's from culture negative patients collected between 1987-1993 in the course of the Norwegian serogroup B vaccination trials (1). Seven of the 63 CSF's were positive after nested PCR. Further, none of the negative CSF's were found to have inhibitors. Only two CSF's were positive with the antigen test, and both were PCR positive. Serum pairs of 42 patients had been analysed for serological serogroup response in ELISA (1). The 7 PCR positive

CSF's belonged to patients with a serological response judged as either typical for or compatible with SMD.

Sequence analysis of the PCR products revealed that 4 of the 7 positive CSF's were infected with a strain of subtype P1.7,16; as for the epidemic ET-5 strain (4). One was P1.12,13a, which is a newly identified subtype for some strains of the ET-5 complex recovered mainly from patients from the West coast of Norway (5); this patient was from Bergen. One strain was of a rare subtype, as far as we know, not associated with the ET-5 complex. The last strain was P1.5,2, a subtype often associated with serogroup C strains in Norway, which was in agreement with the serological response for that patient.

The described PCR technique proved more sensitive than antigen testing and was in agreement with the serological results for meningitis patients. It gave the possibility to determine the subtype of the disease-causing strains in patients involved in the protection trials, and also when no isolate was available.

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DNA sequence analysis of antigenic diversity in *Neisseria meningitidis* class 3 outer membrane proteins

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Strains of *Neisseria meningitidis* express their *porB* gene to produce either a class 2 or class 3 outer membrane protein which are differentiated by their molecular weight. Based on antigenic variation in the PorB, strains are classified into about 15 serotypes. Serogroup B meningococci carrying class 3 protein have been associated with epidemic disease in recent years in several parts of the world. Anti-PorB monoclonal antibodies have been demonstrated to be bactericidal in vitro and to be protective in animal models (3, 5).

Until now, a limited number of porB genes have been sequenced to try to define the serotype determining epitopes (1, 2, 6), and four variable regions have been identified. Due to variation in different parts of the molecule, it has been difficult to relate special serotype determinants to the sequences.

To obtain additional knowledge about serotype determinants, we sequenced 14 porB genes of strains expressing 8 different class 3 serotypes. The study was designed to take advantage of the presence of single and combined serotypes to determine the epitopes responsible for binding the respective serotyping monoclonal antibodies. The entire reading frames of the genes were generated by PCR, and sequenced directly using the thermal cycling procedure. The strains were also characterized by multilocus enzyme electrophoresis and SDS-PAGE.
The sequence data were analysed using the neighbour-joining method (4). Published DNA sequences from 8 additional strains were also included in our analysis for comparison (1, 2, 6). The resulting phylogenetic tree showed that the sequences fell into two distinct groups: one group consisting only of the serotype 15 sequences, and the other, more heterogeneous, containing the remaining serotypes.

Analysis of mutations outside the variable regions revealed no correlation to serotype specificity. However, the point mutations in the 22 strains were generally located in identical positions, indicating either mutational hot-spots or a common ancestry.

Due to the rather homogenous sequences within each of the two groups, and the lack of common variable regions between the groups, it was impossible to locate the

epitopes to single, linear sequences. On the contrary, all serotype monoclonal antibodies seemed to recognize mainly conformational, discontinuous epitopes in the polypeptide sequences, as suggested by immunoblotting (Wedege, unpublished results). An exception may be the serotype 4 monoclonal, which is totally dependent on the presence of a glutamate (E) in the second position in the loop 1 sequence VEHNGGQVVSVETGT. Based on the variation in the three other variable regions, it seems likely that this monoclonal has its epitope restricted to loop 1.

Nearly identical PorB sequences were demonstrated in genetically distant strains showing that the *porB* gene can be transferred horizontally as an entity. SDS-PAGE analyses revealed a surprising lack of correlation between the deduced molecular weights of the different PorB proteins and their relative migration. This may reflect strain specific differences in processing signal peptides from the full length PorB proteins, or may be caused on strong interactions between PorB and other strain-specific molecules.

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Rapid diagnosis of meningococcal meningitis by the polymerase chain reaction

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Polymerase chain reaction (PCR) was used for diagnosis of meningococcal meningitis by amplification of IS1106 insertion sequence of *Neisseria meningitidis*. All 21 strains of *Neisseria meningitidis* yielded a specific 596bp fragment but 14 non-meningococcal strains did not. The sensitivity of amplification was about 12.5fg. PCR amplified fingerprints were generated from the repetitious sequence of IS1106. The band patterns of strains were quite diverse. With the aid of the IS1106-PCR, 20 of 21 cerebrospinal fluids (CSF) and 12 of 14 acute sera of suspected patients were PCR-positive. In the above samples tested, 8 CSFs and 4 sera which were collected from patients with positive culture of meningococcal meningitis were also PCR-positive. In order to confirm the specificity of the IS1106-PCR, 12 recovery sera of cases, 3 contacts' sera and 20 normal sera were examined and they were PCR-negative. The results of the present work suggest that IS1106-PCR is a sensitive and specific method for rapid diagnosis of meningococcal meningitis.

Key words: Meningococcal meningitis; Polymerase chain reaction (PCR); Diagnosis.

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Comparative evaluation of suspicious meningococcal cases with nPCR and ELISA

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A meningococcal outer membrane protein vaccine was tested in Iquique, Chile, where an epidemic of *Neisseria meningitidis*, B:15:P1.(7),3 was occurring. Surveillance from October, 1987 through March, 1990 (2) was done in evaluating the efficacy of this vaccine. Within this time period, 112 patients presented with symptoms classified by physicians as suspicious, moderately suspicious or highly suspicious for meningococcal disease. Gram stains and cultures were negative from all these cases, and none of them could be confirmed as meningococcal disease for the purposes of vaccine evaluation.

Further testing of patient samples has been performed to evaluate their status. Of the samples collected from these 112 patients, 79 sets of CSF and acute and convalescent sera were available for further analysis. The samples of CSF were tested for the presence of meningococcal DNA using a highly sensitive and specific nested polymerase chain reaction (nPCR) previously described (1). Fourteen of fifteen confirmed cases were positive by this technique; 18 samples of CSF collected from patients with other etiology were negative.

Acute and convalescent sera were analyzed for antibodies directed to outer membrane complex (OMC) of strain 8529(B:15:P1.(7),3), a typical epidemic strain. IgG levels in acute and convalescent sera were quantitated by ELISA to assess patient response. In analysis of 36 confirmed cases from this study, an increase in IgG greater than or equal to 2 fold was detected in 31 of these cases, or 86%. None of the 19 serum pairs from patients determined to be free of meningococcal disease exhibited a 2 fold rise in IgG against 8529 OMC.

In analysis of specimens from 74 of the 79 patients, 54 samples were negative by both methods. Twenty were positive by at least one of the two methods, eight were positive by both methods, five were only positive by nPCR, and seven were only positive by ELISA. One suspicious case had a high antibody level in the acute serum and did not show an increase, but was positive on nPCR. Further examination of these samples is ongoing. Discrepancies between the two methods may be due to lack of antibody response in true positive cases; an increase in antibody levels due to carriage, but without disease or experimental error. ELISA and nPCR are valuable

adjuncts to conventional clinical diagnosis of meningococcal disease, particularly where it is important to obtain accurate identification of all cases.

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Importance of the use of subtype P1.9 monoclonal antibody for the serotyping of *Neisseria meningitidis* Serogroup B strains in Brazil

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The monoclonal antibody 5F81A4 directed at outer membrane proteins of *N. meningitidis* was obtained in the U.S. in 1992. We basically used the technique described by Kohler & Milstein (1975). The clones were selected by ELISA. Antibody specificity was determined by Western blotting using outer membrane antigens of a homologous *N. meningitidis* strain. The monoclonal antibody obtained was specific for *N. meningitidis* B class 1. Dot-ELISA using standard subtype strains only reacted with M982 (P1.9 standard). We used *N. meningitidis* strains of different serotypes obtained in Brazil in 1992. Subtype P1.9 was predominantly related to serogroup B, serotype 4, representing 6.8% of the subtyping of serogroup B strains in Brazil in 1992. No significant differences were observed in the prevalence of subtype P1.9 among strains isolated in different Brazilian states. These results are important because Brazil has a broad heterogeneity of *N. meningitidis* B strains. An effective vaccine should probably contain outer membrane proteins of the different prevalent subtypes, and bacteria belonging to subtype P1.9, which were previously considered to be untypeable, should probably be part of the preparation of a vaccine against this microorganism.

The physical map of a Group A strain of *Neisseria meningitidis* shows a region of complex DNA rearrangement relative to the chromosomes of *Neisseria gonorrhoeae* strains FA1090 and MS11

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Macrorestriction maps of *Neisseria gonorrhoeae* strains FA1090 (2, 3) and MS11 (1) have previously been constructed. Within the limits of resolution of these two macrorestriction maps, these two strains were shown to be nearly identical in the organization of genetic markers located on the chromosome.

Neisseria gonorrhoeae and *Neisseria meningitidis* have been shown to be closely related by a number of criteria, including DNA homology (4), although very different clinical spectra of disease are associated with these two species. The key virulence attributes that are responsible for the differences in infections caused by the two pathogens have not been identified. We have constructed a macrorestriction map of *Neisseria meningitidis* in order to compare the genetic organization of these two organisms in hopes of identifying major structural rearrangements that may account for differences in clinical disease.

Neisseria meningitidis strain Z2491 is a Group A strain (subgroup IV-1) isolated in the Gambia in 1983. To construct the macrorestriction map, cloned gonococcal and meningococcal genes were used to probe Southern blots of DNA digested with each of six restriction enzymes and separated on pulsed-field gels. In this manner we determined how fragments from different digests overlapped, and thus the order of the fragments on the map. A total of 116 markers have been located on the Z2491 map. The sizes of the chromosomes of the two pathogenic *Neisseria* species are the same (2.2 Mb) and the genetic organization is quite similar over approximately 60 percent of the chromosome. The remaining 40 percent of the chromosome contains large regions of DNA that have been rearranged or inverted in Z2491 when compared to the gonococcal maps. These differences are complex and must have occurred by multiple independent events.

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Pulsed field gel electrophoresis analysis of the ET-37 complex of *Neisseria meningitidis*

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Introduction: Meningococcal disease occurs with four distinct epidemiologies: large-scale, rapidly spreading epidemics, occasionally constituting global pandemics; localised epidemic outbreaks, often associated with a particular population or area; hyper-endemic disease, which may occur from time-to-time in a country that normally experiences endemic disease; and sporadic, endemic disease (2). Each of these epidemiological types of disease represents a different problem in terms of public health management and control. Interestingly, although a number of socioeconomic, geographical, and climactic conditions influence the epidemiology of this disease, genetically different meningococci are responsible for each epidemiology. A knowledge of the genetical relationships among isolates of meningococci is therefore central to understanding the dynamics of disease in a given country at a particular point in time. This information is also necessary if the evolution of new meningococcal strains is to be understood and anticipated.

The ET-37 complex of bacteria is a group of meningococcal isolates defined by multilocus enzyme electrophoresis (MLEE) (4). These strains have been isolated from many countries over a relatively long period of time and have been responsible for a number of known localised epidemic outbreaks. The ET-37 strains are typically, but not necessarily, C:2a:P1.5,2. Unlike the subgroups of serogroup A, which can be considered to be clones (1,3) and are antigenically homogenous, MLEE analyses reveal that ET-37 is a complex of less closely related organisms that exhibit a degree of antigenic variation. In the present analysis this complex of bacteria was characterised by pulsed field gel electrophoresis (PFGE) fingerprinting.

Methods and strains: Three groups of strains were included in the analysis: a collection of ET-37 strains supplied by Dr. M. Achtman of the Max-Planck-Institut für molekulare Genetik, Berlin; a collection of isolates from the UK Meningococcal Reference Laboratory, supplied by Drs D.M. Jones and A. Fox; and a collection of strains from a recent outbreak of disease in the Czech Republic, supplied by Dr. Paula Kris, National Institute of Public Health, Prague, Czech Republic. PFGE fingerprinting was carried out as previously described (1).

Results: Forty-four strains were examined by digestion of chromosomal DNA preparations with the restriction endonuclease *Sfi*I. The resultant fragments were separated by PFGE to generate a fingerprints of 20 bands with sizes ranging from 20 - 550 kbp. On a single gel, accurate measurements were possible for 17 of these bands in the size range 20 - 300 kbp. These bands represented about half of the 2Mbp chromosome and were used in the subsequent analyses. The *Sfi*I fingerprints from all the strains were similar, with a pattern that was characteristic for ET-37 strains, but were not all identical. However, the strains from a particular outbreak, for example those from the recent outbreak in the Czech Republic, shared the same fingerprint pattern. One NG:NT:P1.2 Czech strain had a very different fingerprint indicating this suspect strain not to be ET-37, which was consistent with MLEE data (Dr. P. Kris, personal communication). Another NG:NT:P1.5,2b (P1.2b=P1.y) strain had the ET-37 associated fingerprint, which was also consistent with MLEE data (4). Some strains, suspected of being members of the ET-37 complex on the basis of their serogroup, serotype, and serosubtype, were shown to have different fingerprint patterns, while others that had serological properties not normally associated with ET-37 possessed the characteristic fingerprint.

Discussion: Fingerprint patterns generated by the endonuclease *Sfi*I can be used to identify members of the ET-37 complex, and to distinguish strains from different localised outbreaks independently of serological or MLEE analyses. The *Sfi*I fingerprints of this complex were less stable than those observed for the subgroups of serogroup A meningococci (1). Nucleotide sequence and serological data indicates that horizontal genetical exchange is less frequent in ET-37 complex than in the predominantly serogroup B ET-5 complex. These data suggest that the accumulation of point mutations is the major mechanism for the generation of variation in the ET-37 complex.

The ET-37 complex is a group of genetically related meningococci that have a slightly elevated potential to cause disease. These bacteria are globally distributed and have persisted over several decades at least (4). Over this time, although they have changed significantly at the chromosomal level and in some of their subcapsular antigens, they have not lost their potential to cause localised, limited disease outbreaks. They do not appear to exchange DNA with other meningococci as frequently as some other complexes of bacteria, for example ET-5, but on the other hand, ET-37 is not as antigenically and genetically as stable as the Serogroup A meningococci.

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Clonal analysis of *Neisseria meningitidis* serogroup B serotype 15 strains of Germany using pulsed-field gel electrophoresis

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The molecular epidemiology of *Neisseria meningitidis* group B serotype 15 strains belonging to various serosubtypes was examined. After digestion with the rare cutting restriction endonuclease *Nhe* I the DNA of 58 strains was separated by pulsed-field gel electrophoresis (PFGE). The strains were isolated both in the old and new countries of Germany from 1983 through 1994 and were serosubtyped as follows: B:15:P1.7,16 (25 isolates), B:15:P1.16 (22 isolates), B:15:P1.1,7 (4 isolates), B:15:P1.7 (3 isolates) and B:15:NST (3 isolates). The B:15:P1.7,16 strains mainly derived from the territory of the former GDR (region of Leipzig, 80%), whereas the B:15:P1.16 strains mainly (68%) came from the territory of the former FRG. The 58 strains yielded 17 different fingerprints. The most common pattern consisted of 9 restriction fragments (364 kb, 315 kb, 242 kb, 170 kb, 110 kb, 95 kb, 65 kb, 55 kb, 30 kb). Fifty-six % (14 isolates) of the B:15:P1.7,16 strains, 41% (9 isolates) of the B:15:P1.16 strains and one isolate each of B:15:P1.7 and of B:15:NST showed this pattern. The restriction profile second most common also consisted of 9 fragments (394 kb, 315 kb, 280 kb, 242 kb, 170 kb, 110 kb, 65 kb, 55 kb, 30 kb). Five isolates of B:15:P1.7,16 and one isolate of B:15:P1.16, which produced this pattern, originated from the region of the former GDR (isolated 1988-1992). Four B:15:P1.1,7 strains with the identical fingerprint were isolated in a kindergarten in North West Germany.

Taken together, strains of the antigenic composition B:15:P1.7,16 had 10 different restriction patterns, whereas strains of the antigen formula B:15:P1.16 yielded 13 distinct profiles. Six of the 17 different fingerprints were found within various serosubtypes. Eleven patterns belonged to single isolates. The variation among most of the 17 different restriction profiles was minimal. Only three of the patterns showed significant differences to the other fingerprints. Identical fingerprints were found within various serosubtypes of serotype 15 strains. So the same group as distinguished by PFGE included strains of distinct subtypes. On the other hand, isolates of identical sero/serosubtypes could be differentiated by PFGE restriction patterns. One group of *N. meningitidis* with the same restriction pattern was prevalent in the territories of the former GDR and FRG.

Analysis of meningococci from a New Zealand hyperendemic using serotyping, subtyping and restriction fragment length polymorphism typing

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Introduction: New Zealand is currently experiencing a hyperendemic of *Neisseria meningitidis* invasive disease which began in the second half of 1991. This study seeks to analyse the relationships between isolates received from cases of invasive meningococcal disease during and prior to this period, using serotyping and subtyping and also using Restriction Fragment Length Polymorphism (RFLP) typing.

Materials and Methods: Since 1989, isolates from all cases of meningococcal invasive disease have been solicited for surveillance from all over New Zealand by ESR Health: Communicable Disease Centre . For this study, one isolate from each case was characterised by serotyping and subtyping and by RFLP. Serotyping and subtyping was based on the method of Abdillahi and Poolman (1) using whole cell ELISA. The method for RFLP used the rare cutting restriction enzyme *Sfi* 1 and Pulsed Field Gel Electrophoresis and was derived from that of Single and Martin (6) and that of Bygraves and Maiden (2).

Results: For the years 1989-1990 the average annual incidence rate of invasive meningococcal disease was 1.5 per 100,000 population. From the middle of 1991 there has been an increased number of cases peaking at 200 in 1993, giving an annual rate of 5.9 per 100,000. These rates were based on laboratory confirmed and notified cases and represented an increase across all age-groups.

The relative proportion of serogroup B isolates rose from 41% in 1989 to a peak of 74% in the first half of 1994. The proportion of serogroup C isolates also rose from 17.6% in 1989 to 34% in 1993 and has fallen to 24% in the half year to June 1994. Serogroup A has represented less than 2% of isolates annually since 1990.

Serotyping and subtyping has identified a number of combinations with one particular strain predominating. This strain, B:4:P1.4, emerged as the dominant strain in mid 1991, coincident with the start of the hyperendemic. Prior to 1991 this strain had been identified only 3 times. In 1991 it was found 14 times, 13 of them in the second half of the year, giving a rate of 62% of sero/sub typeable serogroup B isolates in those 6 months. The numbers of this strain have continued to rise and currently represent 82% (18/22) of sero/sub typeable serogroup B isolates for the first 23 weeks of

1994. This strain has been found throughout New Zealand with no obvious geographical focus. Initially, it appeared to have an unequal distribution across age-groups, in 1992 accounting for 33% of sero/sub typeable serogroup B isolates from under 5 year olds compared with 64.5% for the 15 to 24 age-group. Recent data suggests a more even distribution of the strain across age-groups. RFLP analysis, while incomplete, indicates that the majority of isolates of B:4:P1.4 belong to the same RFLP type, Ba, suggesting a clonal relationship.

While strain B:4:P1.4 has dominated over the last 3 years there has also been an overall increase in other meningococci belonging to a wide range of genotypes. A number of clusters of related cases of meningococcal disease have occurred since 1991. In addition to the dominant strain, B:4:P1.4, these have involved other meningococci including a sulphonamide resistant strain B:15:P1.7,16, RFLP type Bi.

Discussion: The background incidence of meningococcal disease in New Zealand has been similar to that reported in other countries, showing a higher rate of infection in pre-school children and a dominance of serogroup B over other serogroups. From the middle of 1991 rates increased significantly in all age-groups to peak at 5.9 per 100,000 population in 1993. Coincident with this rise was the emergence of a dominant strain, B:4:P1.4, RFLP type Ba.

Serogroup B epidemics have previously been linked with the introduction of new strains, for example, in Norway in 1974 with strain B:15:P1.16 (3), and in Brazil with strain B:4:P1.15 (4). Although our strain, B:4:P1.4, was not found with any frequency before 1991 it subsequently became the most prevalent type. However, it still only represented 32.5% (39/120) of all isolates causing invasive disease in 1992, for example. Therefore, the increase cannot be attributed to this strain alone. Results of serosubtyping and genetic fingerprinting indicate considerable heterogeneity among other meningococcal isolates, suggesting that other factors may also have contributed to the increase observed. A similar pattern of events occurred in the Netherlands during the 1980s, including the emergence of a meningococcus expressing the same sero/sub type, B:4:P1.4. (5). The relationship of our strain to that found in the Netherlands needs investigation and has implications for vaccine development.

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Genomic fingerprinting of meningococcal group C ET-15 disease isolates by pulsed field gel electrophoresis

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A single enzyme electrophoretic type (ET-15) of *Neisseria meningitidis* has been associated with an increase in meningococcal group C disease in Canada during the past several years (1). The emergence of ET-15 has been accompanied by a series of clusters or focal outbreaks which have occurred in different geographical areas (1,5). Genomic fingerprinting, through pulsed-field gel electrophoresis (PFGE) of chromosomal DNA, has been used to further delineate the genetic relationships among ET-15 isolates from one such outbreak in the National Capital Region of Canada (5). PFGE has now been used to characterize meningococcal ET-15 isolates from six additional clusters or focal outbreaks which occurred in diverse geographical areas across Canada.

Based on previous studies (5), three restriction endonucleases, *Bgl*II *Not*I, and *Spe*I were used to generate genomic fingerprints from DNA obtained from meningococci grown on GC medium at 36°C in 5% CO_2.

Cluster 1: Eight cases of group C disease occurred in a metropolitan area during a period of 11 weeks. Five of the eight strains had the same PFGE profile (PFP). Four of the five identical isolates came from patients during a nine-day period. The same PFP was associated with a case of group C disease in the area 20 months earlier. Of the three remaining isolates, two were of a second and identical PFP and one exhibited a third distinct PFP which was also associated with a case of group C disease 10 months earlier.

Cluster 2: During a period of 8 days there were 4 cases of group C disease involving three schools in the same city subdivision. All 4 disease isolates exhibited the same PFP. The identical PFP was exhibited by a group C disease isolate, which was recovered 1 week later in another part of the province from a person who had recently visited the same city.

Cluster 3: Two cases of group C disease and a case of polyarthritis occurred in a town during a 2 week period. The index case occurred in a high school student. A few days later polyarthritis developed in a young woman whose brother attended the

same high school. The PFP of the throat isolate from the woman was identical to the blood isolate of the index case. Twenty days after the index case the mother of the young woman with polyarthritis developed group C disease with the blood isolate having an identical PFP as the other two isolates.

Cluster 4: During a 17-day period, 4 cases of group C disease occurred in a Canadian city. The four isolates exhibited reduced susceptibility to penicillin and identical PFPs. Seven additional cases, from which isolates had the same PFP and reduced susceptibility to penicillin, occurred during the next 14 months in that city and immediate area. Seven other ET-15 isolates from group C disease occurring in the province during this period were sensitive to penicillin and were of different PFPs.

Cluster 5: Nine cases of group C disease occurred during a period of 6 days in Victoria County in Ontario. Seven isolates were available and all were of the same PFP.

Cluster 6: Four cases of group C disease occurred in a metropolitan region in Ontario during a period of 8 days. All 4 isolates were of the same PFP. The isolate from a fifth case, which occurred just outside the region during that time, had a different PFP.

Isolates from clusters 1 to 5 all exhibited different PFP's. However, isolates from clusters 5 and 6, which occurred 4 years apart, exhibited similar PFP's. There was no correlation between PFP and outcome of disease in patients. Seventeen PFP's were found among 25 non-disease-associated ET-15 carrier isolates.

Genetic transformation, which is a common process in *Neisseria meningitidis* (4), results in genomic variation due to minor differences in nucleotide base sequences. Thus, certain genotypic methods appear to be useful for more detailed characterization of outbreak stains. For example, a study employing PFGE has resolved differences between various subgroup III strains within serogroup A (2) and investigation of restriction fragment length polymorphisms employing cloned probes has detected genomic variation within a single enzyme electrophoretic type of group B meningococci (3). Our studies indicate that genomic variation exists for ET-15 meningococcal isolates. The use of PFGE to detect such genomic subtypes should help to delineate future outbreaks of group C disease caused by ET-15 meningococci.

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Emergence of a new virulent clone within the ET-5 complex of serogroup B meningococci in Norway

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In the late 1980s, B:15 meningococci with subtype P1.12 emerged among isolates from patients with systemic meningococcal disease in Norway (4). Between 1987 and 1992, 24 B:15:P1.12 strains were detected, and P1.12 was the third most common subtype among B:15 strains. All B:15:P1.12 strains belonged to the ET-5 complex (1), but 17/24 isolates (71%) were a new clone (ET-5c) which has not been seen elsewhere in the world. Except for one strain, all ET-5c isolates were from patients in two neighbouring counties in Western Norway.

A monoclonal antibody (MAb) against the unknown variable region 2 (VR2) in the P1.12 subtype protein was developed. This MAb (202, G-12) and the reference subtype P1.13 MAb (MN24H10.75 from J.T. Poolman, RIVM, Bilthoven, The Netherlands) reacted with 19 and 4 strains, respectively, of the B:15:P1.12 isolates, whilst one strain showed no binding. Judged from immunoblots, MAbs 202, G-12 and P1.13 reacted with a linear and a conformational epitope, respectively. Both antibodies showed subtype-specific bactericidal activity. Sequencing of *porA* genes demonstrated a series of four threonines in the deduced VR2s of strains reacting with MAb 202, G-12, whereas five threonines were found in the P1.13 positive strains. The former sequence is similar to one recently designated P1.13a (3). Therefore, MAb 202, G-12 is a P1.13a specific antibody. Its specificity is also supported by the reaction with serogroup A strains of clone IV-1 whose VR2s contain the P1.13a subtype (3). The one P1.12 isolate without reaction with the P1.13a and P1.13 Mab's had a sequence of nine threonines in VR2 and expressed a class 1 porin of higher molecular weight in SDS-gels.

IgG antibodies against the P1.7,16 protein, induced after vaccination with Norwegian meningococcal vaccine, did not cross-react on immunoblots with the P1.12,13a strains, showed antibody activity against the P1.12,13a protein. These antibodies did not cross-react with the P1.7,16 protein. The dominant antibody activity in these two

patient sera was directed against the P1.12 epitope in VR1. One of the sera showed an additional weak binding to the P1.13a subtype region. Epitope mapping with synthetic peptides demonstrated that this binding was directed to the same sequence of 11 amino acids which also reacted with MAb 202, G-12. The lack of cross-reactivity of vaccine-induced class 1 protein IgG antibodies may imply reduced effect of the Norwegian meningococcal vaccine against strains in the new clone.

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Meningococcal disease in Europe: Concerted action on all aspects of disease management

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The demand for regular meetings to promote European collaboration for the management of meningococcal disease is greater than at any other time in recent history. This need arises from the lack of rapid exchange of comparable data on meningococci between European countries. The ongoing geopolitical dynamics in Europe, the epidemic potency of different strains and progress towards group B vaccine development call for a comprehensive collaboration and surveillance. Furthermore although increasing numbers of pheno- and genotyping methods have been developed and are in use, they are of limited accessibility and reagent availability.

Hitherto, meetings of representatives from European National Reference Laboratories for Meningococcal Disease were organised by the World Health Organisation but ceased some 8-10 years ago. Informal discussions between European National Reference Laboratory representatives confirmed the continuing need for a European forum. It was therefore decided to create a European Monitoring Group for Meningococci (EMGM) to bring together, by regular meetings, representatives from as many National Reference Centres throughout Europe as possible and to identify areas for concerted action.

To expedite this initiative financial support was obtained from the Austrian Ministry of Health and a number of commercial organisations who provided sufficient funds to hold an inaugural meeting in Graz, Austria in May 1993 (1). The meeting was attended by delegates from 23 European States including many of the new Republics in Eastern Europe. The first meeting of the EMGM was structured to provide an update for all the delegates in key areas of importance to the management of meningococcal disease. Speakers addressed the following subjects: diagnosis of meningococcal infection, standardisation of methods for strain characterisation and anti-polysaccharide antibody determination, vaccines past, present and future, and the role of the reference laboratory. The meeting concluded with open discussions of issues arising from the formal programme and a business agenda for the EMGM initiative. The EMGM was greeted with enthusiastic support from participating

delegates. A consensus regarding the aims of the EMGM to progress European collaboration for the management of meningococcal disease was achieved. A work programme was formulated to be completed before the next meeting in May 1994. This programme was directed at three main areas:

1) pan- European surveillance
2) establishment of an External Quality Assurance Scheme for strain characterisation
3) audit of reference laboratory performance.

The 2nd meeting was held in Austria during May 1994, the meeting was attended by representatives form additional European states. The second meeting was organised to encourage greater involvement by the participants. An update of European Surveillance based on data provided by all those participating in the EMGM followed by a series of presentations by participants on topics of interest including changes in surveillance and or epidemiology, outbreaks, laboratory acquired infections and strain characterisation. These were followed by presentation of the results obtained from the various concerted actions completed by the group during 1994. The concerted actions were pan-European surveillance, the institution of an External Quality Assurance scheme for strain characterisation and an Audit of reference laboratory function. The final part of the meeting was dedicated to round table discussions on the following subjects, surveillance, diagnosis, strain characterisation and audit. Progress towards consensus regarding the basic data set to be collected for surveillance, European criteria for diagnosis of meningococcal disease, the standardisation of reagents for strain characterisation and the next round of audit proposals for the group was achieved.

The EMGM provides an important forum to progress the control of meningococcal disease throughout Europe, illustrated by the surveillance data for approximately 7,000 cases which occurred throughout Europe during 1993-94. The creation of a group to facilitate pan-European collaboration on the management of meningococcal disease will accelerate progress towards the control and prevention of meningococcal disease throughout Europe.

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Factors affecting meningococcal carriage and acquisition in military recruits

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We are performing a longitudinal study of meningococcal carriage and acquisition in military recruits in training and examined some of the factors associated with this, including the mucosal and systemic immune response. Recruits enter the base in troops of 30-45 every 4-6 weeks. They range in age from 16-26. After an initial period of 2 weeks in a barrack room accommodating the whole troop, they are housed in dormitories with 4 men to a room.

During the first week of training, the nature of the study was explained to recruits who were asked to take part. Those who agreed gave written consent. Posterior pharyngeal swabs were obtained at approximately 4 weekly intervals throughout the 30 week training period. Swabs were plated directly onto a selective medium and the plates incubated within 1 hour. Meningococci were identified, serogrouped and serotyped using standard methods. Data on smoking habits and exposure were collected using a standard questionnaire at the beginning and end of the survey. Each time swabs were obtained, the recruits were questioned about respiratory illness in the preceding month. A total of 10 troops are included in the survey and 324 recruits have taken part. The initial rate of carriage on entry was surprisingly high, with 35% recruits being positive. Acquisition of meningococci occurred throughout the training period. Among the recruits who completed the survey, less than 20% remained negative throughout the study period.

The predictive value of a single negative swab was determined by examining results from carrier recruits who were found to be positive with the same strain on at least 2 swabs 2 or more months apart. It was assumed that carriage would have been continuous and therefore that intervening swabs should also have been positive. In practice 75% of these specimens were positive. Extrapolating from these results, the sensitivity in detecting carriage of a single swab was 83%, 92% for 2 swabs and 100% for 3 or more swabs. On this basis, acquisition was defined as isolation of a meningococcus from a recruit after 2 previous negative samples. Using this definition, acquisition has been studied in the recruits. Data on the effects of season, respiratory infection and smoking on carriage and acquisition will be presented, and the dynamics of transmission of meningococcal strains both within and between will be described.

Dynamic characteristics of the meningococcal carrier state

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Objective: To investigate the rates and fluctuations of the meningococcal carrier state within a three months period in a semi-closed community. Three hundred and thirty-one Danish male military recruits from the camp Hoevelte, 25 kilometres north of Copenhagen were used as the study population. During the week the recruits lived in 4-5 or 8-10 beds dormitories, while during most weekends they returned to their home regions.

Methods: At the entry to military on November 1st, 1992 and 14, 30, 60 and 90 days later a swab for culture of meningococci was taken from both tonsils. All swabs were taken by one person (JA) and inoculated immediately on selective chocolate agar medium. Culture and identification of *Neisseria meningitidis* were performed by conventional methods. Isolates of *N. meningitidis* were characterized by serogrouping, -typing and -subtyping (1, 6) and by the susceptibility to sulphonamide, penicillin, rifampicin and ciprofloxacin.

Results: Out of the 331 recruits enrolled in the study, 327 were investigated at the entry to military; on the subsequent four occasions the number of participants varied between 203 and 301; growth of *N. meningitidis* was obtained from 557 (41,5%) out of a total of 1343 swabs. Regarding the carrier state the following definitions were used:

Constant Carrier: Positive culture for meningococci from all samples taken, irrespective of the number of occasions at which the recruit was examined.
Intermittent Carrier: At least one positive and one negative culture for meningococci from a recruit examined on at least two occasions.
Non-Carrier: Negative culture for meningococci from all samples taken, irrespective of the number of occasions at which the recruit was examined.

The mean carrier rate at the five dates of sampling was 41.7% (range 40.1-44.8%). At the entry to military the carrier rate was 40.1% and at the end of the three-months period it was 44.8%. Although the mean carrier rate was rather constant, the individual meningococcal carrier state in between sampling dates varied for 13-18% of the recruits; the frequencies of loss and acquisition of meningococci were equal within the first two months. The slight increase in the overall carrier rate during the last

month can be explained by the fact that the frequency of acquisition then was higher than that of loss of pharyngeal carriage of meningococci. The acquisition rate was higher among the recruits in the larger dormitories than among those in the smaller ones. Intermittent carriage of identical isolates occurred and this observation may indicate that the pharyngeal colonisation at intervals may be below detectable levels.

Out of the 331 recruits, 140 were examined on all five possible occasions; 32 (23%) were constant carriers, 51 (36%) were intermittent carriers, and 57 (41%) were non-carriers, i.e. 85 (61%) were carriers on at least one occasion. The acquisition rate was highest within the first month. Overall 54% (178/331) were carriers on at least one occasion and 46% were non-carriers; at the end of the study period 32% were categorized as intermittent carriers. In total 557 *N. meningitidis* isolates were identified and characterized; 74% belonged to serogroup B, 6% to serogroup C; 15% to other serogroups (X, Y, Z, W-135, 29E) and 5% were non-groupable; 34% were non-typeable and 23% were non-subtypeable. The most prevalent strain was serogroup B:4:P1.1,7 (12/178 carriers = 6.7%), while B:15:P1.7,16, the most prevalent strain isolated from patients with meningococcal disease within the same period in Denmark, represented 5.1% (9/178) of the carrier strains.

Conclusions: Compared with the findings in other studies the overall carrier rate among Danish military recruits was rather constant (40-45%) during the three months period of study (2, 4, 5). However, a marked fluctuation in the individual carrier state was observed. Although weekend contact with families and friends was common the majority of *N. meningitidis* strains found throughout the three months were phenotypically identical to the strains present among the recruits at the entry to military. The acquisition rate was highest within the first month, while colonisation of intermittent carriers was a predominant phenomenon during the last two months. Crowding may influence the acquisition rate (3, 7).

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Phenotypic and genotypic characterization of *Neisseria meningitidis* isolates from carriers in households with infants

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Objective: To assess the discriminatory capacity of phenotypic versus genotypic characterization of *Neisseria meningitidis* serogroup B carrier strains isolated from households in which infants aged 0-4 years and the mothers were carriers.

Background: In a study from the Faroe Islands (2) it was previously shown that healthy children aged 0-4 and 5-14 years had substantially higher risk of being carriers of *N.meningitidis* if the mothers were so, especially if the mother was carrier of *N.meningitidis* B:15.

Material: In March-April 1985 serogroup B *N.meningitidis* strains were isolated from 35 carriers in 14 households with 15 children aged 0-4 year (2). The total number of household members was 54. Six strains isolated from Faroese patients with serogroup B meningococcal disease during 1985 were included in the study.

Methods: All strains were phenotypically characterized by serotyping (panel: 1, 2a, 2b, 4, 14, 15, 16), serosubtyping (panel P1.1, P1.2, P1.6, P1.7, P1.9, P1.15, P1.16)(1) and susceptibility to sulphonamides (resistance: MIC $8 mg sulphamethoxazole/l). The genotypes were determined by the multilocus enzyme electrophoresis (MEE) profile (ET) and by the DNA-fingerprinting pattern (DFP)(3).

Results: The overall carrier rate for the 14 households studied was 69% (37/54). Of the 37 meningococcal isolates, one was serogroup Y, one was serogroup 29E and the remaining 35 were serogroup B. Out of the 35 serogroup B carrier strains nine (26%) were both typeable and subtypeable, whereas 14 (40%) were non-typeable (NT) and another 14 (40%) were non-subtypeable (NST); two strains were NT:NST; nine strains (26%) were sulphonamide-resistant. Five out of six serogroup B strains from patients with meningococcal disease were B:15:P1.16 and one was B:4:P1.7; four out of five (80%) of B:15:P1.16 strains were sulphonamide-resistant. By MEE 17 distinctive ETs were identified. At a genetic distance of 0.15 there were 13 lineages; of these four were represented by a clone of two closely related ETs.

The discriminator's capacity of DFP when applied to carrier strains within a household and to the epidemic strains versus carrier strains of the same phenotypes was

equivalent to MEE. The majority of group B carrier strains (94%) were typeable and/or subtypeable. Among these strains two phenotypes (15:P1.16 and 2a:NST) included each two closely related ET's and one phenotype (NT:P1.9) included two ET's with a genetic distance of 0.35. Two ET's included each two phenotypes: ET7 strains were either 1:NST or 2a:NST, and ET4 strains were either NT:NST or NT:P1.6. The discriminatory capacity using resistance to sulphonamide as a marker was limited since the majority of carrier strains (74%) were susceptible. However for the two ET's belonging to the same serotype (2a:NST) a phenotypic distinction between the two types could be obtained by serotyping combined with results of susceptibility testing.

Within 8 households all members carried identical strains, within 4 households two different strains and within 2 households three different strains were isolated. Seven out of 35 carrier strains (20%) were identical to or closely related to the epidemic B:15:P1.16 strain. The seven strains were isolated from two members (mother and child) in each of three households and from a 9-year old child in a household where the two other members carried sulphonamide-susceptible B:2a:N-ST strains. The three households with B:15:P1.16 carriers were all living in a hyperendemic area (2).

Comments: By the methods used the overall discriminatory capacity of phenotypic and genotypic characterization by MEE was equivalent. DNA-fingerprinting is only considered suitable for comparison of a limited number of strains. In ten households the mothers and the infant carried identical strains, in three households the mother and the infant carried different strains and in one household with two infants one carried the same strain as the mother and the second one carried another strain. In all cases the similarities and differences were identified whether using phenotypic or genotypic characteristics.

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The epidemiological cycle of meningococcal infection in Russia (1969-1993)

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The aim of this investigation is to present a pattern of epidemiological cycle of meningococcal infection (MI) in Russia in 1969-1993. During that period two peaks of epidemic rise of morbidity were registered (1). Between these peaks incidence of MI decreased but did not fall to the pre-epidemic level, and this fact makes it possible to consider the whole 25-years period as one inseparable cycle. Major factors influencing on the origin of the two peaks of morbidity within this cycle were determined. The role of social, age, etiological, territorial and immunological factors in the formation of each peak was evaluated.

The first peak of morbidity began since 1969 and continued on the average over 5 years with a maximum in 1972-1973 (incidence rate 7.8 and 8.7 per 100,000).The intensity of this increase varied in the different regions of the country. Maximum incidence rates were registered in: Tuva Republic (242.2 per 100,000), Tatarstan (62.3 per 100,000), Udmurtia (30.7 per 100,000), city of Moscow (26.5 per 100,000). On the peak of morbidity a proportion of the patients among urban and rural population was 4.0:1.0 and 4.5:1.0, and during decrease of the first peak in 1977 and 1975 2.5:1.0 and 1.6:1.0 respectively. Among patients children up to 14 years old accounted for 70% of cases and there was 5-6 times increase in the number of patients in adolescent and young adult groups. Serogroup A (Men-A) meningococci were isolated from patients in 80% of cases. In 1976 in Yaroslavl (Central region of Russia) immunological structure of the healthy population against meningococci was studied (2). Results of this study confirmed the predominating role of the Men-A in the morbidity due to MI. Depending on the age of investigated persons the number of seropositive samples with the titres 1:20 and above in passive haemagglutination test was up to 23-57%. Antibody levels against other serogroups of meningococci was significantly lower.

Generally in Russia, during the second peak the increase of morbidity was gradual, rather intensive, with a maximum in 1984 (9.0 per 100,000). The highest incidence rate was registered in the Far East (13.4 per 100,000), in Western Siberia (12.3 per 100,000) and in Mordovia (19.8 per 100,000). In 1984 proportion of the patients among urban and rural population was 1.4:1.0, and in 1993 - 1.1:1.0. Children up to

14 years old accounted for 83% of cases. In 1984 children incidence rate was 33.8 per 100,000 against 20.3-25.3 per 100,000 at the maximum of the first peak, and among adults it was 2.1 and 3.4-3.3 per 100,000 respectively. During the rise of the second peak a change of a leading meningococcal serogroup occurred. In 1987-1988 Men-A was identified in 13-10%, Men-B in 42-68% respectively. By 1992 Men-B became predominating in etiology of MI and accounted for 73-95.4% of the number of classified strains. In 1987 in Yaroslavl population immunological structure against meningococci was repeatedly studied (3). Antibodies against Men-B were found out in 95.0% of cases, among adults in 54.0% of cases the titres were 1:40 and above. The similar antibody levels against Men-A were only in 15% of cases, and this proved the change of the leading pathogen serogroup in the etiology of generalized forms of MI. The pattern of the second peak of epidemics was similar to the increase of morbidity in the North-Western countries of Europe.

Analysis of morbidity with consideration of territorial factor and intensity of epidemic peaks reveals two patterns of epidemic cycle: "European" and "Asiatic". At the same time in a number of regions an increase of morbidity was not accompanied with clear-cut peaks, i.e. it was of mixed pattern.

This investigation establishes that in Russia the first and the second peaks of MI differed in: intensity of the increase, decrease period and their duration; unequal involvement in epidemic process of age groups and different social strata; predominating meningococci serogroups in etiology of generalized forms of MI during first and second peaks;- population immunological status against meningococci. Results of the investigation made it possible to identify two-peak pattern of epidemiological cycle of MI in Russia and to assess the role and significance of certain factors in the origin of the two-peak pattern of epidemiological cycle.

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Neisseria meningitidis and bacterial meningitis in Moscow

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Etiological deciphering of purulent meningitis plays a significant role for performing adequate aetiotropy therapy in time. Bearing in mind the reduction of the incidence of meningococcal infection in Moscow, the role of other etiological organisms is increasing. To compare bacteriological diagnostic state and to define their structural changes there were taken two periods of control, 1984 and 1993, with the ten-year interval between them.

It was recorded before that the ten-year period of control is the most significant for comparison and makes it possible to reveal the reliable differences between the parameters considered. The control was carried out at Moscow clinical infectious hospital N 2 and the conditions of work during the two periods were alike. For the 1st control period the laboratory got information from 665 patients; 507 cases were classified as meningococcal infection and 158 as others purulent meningitis.

The classifying analysis of patients with meningococcal infection and purulent meningitis showed that children under 14 were more often exposed to meningococcal infection. The meningococcal infection was diagnosed in 321 cases among children against 186 among adults. The correlation of children and adults with diagnosis of purulent meningitis were alike; 82 cases were registered among children and 76 among adults.

Clinical diagnosis was confirmed in 26% cases with meningococcal infection and in 40.5% with purulent meningitis. In all these cases only positive culture and exposure of antigen by express methods were taken into account.

In all there were 136 cultures of *Neisseria meningitidis*, that made 68% of the total number of strains. Only strains got out of spinal fluid and patient blood were taken into consideration. Among all *N. meningitidis* strains group A made up 41.9%, group B - 47.7% and group C - 5.8%. The number of strains of *Streptococcus pneumoniae* was 32 (16.5%) and *Haemophilus influenzae* type b 18 (9%). During the 2nd control period the laboratory got data from 237 patients: 121 cases were classified as meningococcal infection and 116 as other types of purulent meningitis. That means the correlation of these clinical forms were like (1:1) while during the 1st period the

ratio was 3.2:1. The meningococcal infection was diagnosed in 79 cases among children against 42 among adults. Ages of patients with diagnosis of purulent meningitis was quite different. Children accounted for 40 cases (34.5%),adults for 76 cases (65.5%).

Clinical diagnosis was confirmed in 32% of cases among the patients with meningococcal infection and in 39% among patients with other forms of purulent meningitis. That didn't differ from the percentage of bacteriological confirmation of clinical diagnosis in 1984. In total we got 27 cultures of *N. meningitidis,* which made up 44.2% of the whole amount; that's considerably less than in 1984. *N. meningitidis* of group B predominated and the percentage was 62.9%. The number of strains of *Streptococcus pneumoniae* accounted for 21 (34.4%), *H. influenzae* for 4 (6.5%). The information above shows a considerable increase in *N. meningitidis* group B in comparison with 1984, and a reduction in *N. meningitidis* group A from 41.9% to 11.1%. Particular attention should be paid to the increase of cases with *Streptococcus pneumoniae* meningitis; the percentage increased from 16.6% in 1984 to 34.4% in 1993. This fact reflects the peculiarity of the 2nd control period.

Clinical course of meningococcal disease in one district of the Czech Republic

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An unusually high incidence of meningococcal disease in Northern Moravia (Czech Republic) is described. The highest incidence was recorded in two regions (Olomouc and Bruntal), the age-group of 15-19 years being the most affected. Twenty-three meningococcal diseases were reported from the beginning of February to the end of June 1993, and twelve cases occurred in the second part of 1993 only. Circulation of a new strain of *Neisseria meningitidis* C:2a:P1.2 (detected in the Czech Republic for the first time) had the greatest influence on the high incidence and serious course with a high fatality rate of these diseases.

Meningococcal diseases manifested themselves as meningitis, sepsis or combination of both. About 50% of patients showed complications, e.g. severe disturbances of consciousness, neurological disorders, arthritis, myocarditis, extensive necrosis of soft tissues. Four patients died. Some patients exhibited an unusual beginning of the disease, e.g. two patients were operated on because of acute abdominal pain, some patients had diarrhoea and others suffered from joint pains. Niclasson criteria have been used to assess the prognosis of meningococcal invasive diseases.

Characterization of nontypeable and nonsubtypeable *Neisseria meningitidis* strains isolated in the Czech Republic

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Introduction: The mean annual percentage of *N.meningitidis* strains, isolated from invasive meningococcal diseases in the Czech Republic in 1980-1993 and characterized as nontypeable (NT) and/or nonsubtypeable (NST) in our NRL, reaches almost 80% of all patient strains. Such level is significantly higher than those reported in other countries (1,2,3) and can be explained by the presence of specific meningococcal clone(s) in the area, the population of which was rather isolated for the past decades from the other ones. High proportion of NT/NST (i.e. NT:NST, NT:subtypeable, typeable:NST) strains causes difficulties in epidemiological data evaluation.

We have screened the collection of meningococcal NT/NST patient strains, which were found by Whole-Cell ELISA not to interact with the currently available panel of typing/subtyping monoclonal antibodies (McAb's), for the expression of the outer membrane proteins (OMP) using SDS-PAGE. The strains showing positive expression of Class 1 and Class 2/3 OMP were clustered to allow to select significant clone for novel type/subtype-specific McAb preparation.

Materials and Methods: Strains; meningococcal strains were isolated from CSF and blood samples obtained from patients with invasive meningococcal disease. After biochemical identification, bacteria were serogrouped with slide agglutination kit (Diagnostics Pasteur, Bio Merieux). The strains were stored either lyophilized, either at -70°C in Brain Heart Infusion medium, grown on Mueller-Hinton agar with 5% of sheep blood at 37/C and harvested after 18h growth. Whole-cell ELISA; the standard procedure (4) was used for strain serotype/subtype determination and for hybridoma McAb reactivity estimation. The currently available panel of McAbs specific towards following type or subtype antigens (kindly provided) was used: 1, 2a, 2b, 2c, 4 (J.T.Poolman), 4 (W.D.Zollinger), 11, 14, 15, 16, 21 or P1.1, P1.2, P1.3, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.13, P1.14, P1.15, P1.16, P1.x, P1.y, respectively. OMP isolation; the procedure of Barenkamp (5) was modified. Single-plate cell suspension was sonicated 5 min at 4/C in 0.01M Tris-HCl pH=8.0, intact cells were removed by low-speed centrifugation and membrane fraction was obtained by repeated ultracentrifugation of supernatants at 100,000 g (Beckman L8-

70, SW60Ti) followed by extraction with 1% N-lauroylsarcosine. SDS-PAGE; Laemmli discontinuous alkaline conditions (11%T running gel, pH 8.8, Bio-Rad Mini-Protean II) was used for screening of OMP isolate patterns. Hybridoma preparation; performed using Sp2 myeloma cell line and PEG-induced fusion after whole-cell immunization of BALB/c mice with the suspension of heat-inactivated meningococcal culture in PBS in 4 doses (0.1 ml each, 10^9 CFU/ml). Western immunoblotting; in semi-dry Towbin arrangement (pH 8.3, 0.1% SDS, Bio-Rad Trans-Blot SD) was used following SDS-PAGE for checking of hybridoma McAb reactivity towards NT/NST antigens.

Results and Discussion: A collection of 315 *N .meningitidis* invasive patient strains was screened by whole-cell ELISA using the currently available panel of noncapsular antigen-specific McAb's. The mean annual proportion of NT/NST strains in the total of isolated patient strains was 78.1%, the percentage of serogroups among NT/NST strains in the 1980-1993 period was 66.3% (B), 26.0% (C), 6.9% (A), 0.8% (W135, X, Y). These data are in correlation with the distribution of serogroups among patient strains in the Czech Republic.

All NT/NST OMP isolates studied showed expression of Class 2/3 proteins, expression of Class 1 protein was negative in 2 strains and reduced in 6 strains, as observed previously (6). In our collection the Class 1 OMP pattern relative mobilities (RM) showed a high homogeneity (a great majority of strains were identical in this feature, only 4 strains belonged to other RM group with uniformed lower RM). This observation is consistent with the data for serogroup B strains by Kayhty (3), but differs from the situation among serogroup A strains, where seven RM variants of Class 1 OMP were found (6,7).

The situation in Class 2/3 OMP RM screening was completely different, NT/NST strains could be clustered into seven distinct RM groups, which results corresponds with RM variant heterogeneity in serogroup B and C strains (2,8) and the homogeneity in group A strains (2,9). The most frequent Class 2/3 OMP patterns were those of isolates with lower RM, i.e. those of RM groups 1 (29.5%) and 2 (35.8%). The remaining RM groups were much less frequent - group 3 (11.6%), 4 (6.3%), 5 (5.3%), 6 (7.3%), 7 (4.2%).

Strain 483.93, belonging to the RM group 2 and showing strong expression of either Class 2/3 or Class 1 OMP, was chosen for murine immunization and hybridoma preparation. The results of whole-cell ELISA and Western immunoblot show specific activity of some of hybridoma McAb's towards RM group 2 strains without any cross reactivity with other NT/NST or reference strains in the first testing.

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New epidemiological situation in the Czech Republic due to *Neisseria meningitidis* C:2a:P1.2 (P1.5)

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Introduction: Meningococcal strains isolated from patients, contacts and healthy carriers have been investigated since 1970 and collected since 1973. As new methods of investigation of *Neisseria meningitidis* are being introduced in our laboratory the possibilities of identifying its epidemiological markers are improving. Thanks to the collection of lyophilized strains it is possible to investigate the strains isolated in the past using the new methods and to improve our epidemiological knowledge. *N. meningitidis* strains isolated from patients with invasive meningococcal disease in the Czech Republic showed a prevalence of serogroup B and a different distribution of serotypes and subtypes with a high proportion of nontypeable and/or nonsubtypeable strains in comparison with the strains isolated in other Western European countries for the whole period 1973-1992. For the same period ET-types of meningococci isolated in the Czech Republic were heterogeneous and different from the ET-5 or ET-37 clones causing outbreaks of invasive meningococcal disease in the world (1, 2).

Epidemiological data (routine notification and NRL surveillance data) are being analyzed as well. It is possible to follow mortality and morbidity from meningococcal meningitis in the Czech Republic back to 1921 and 1945 respectively. In the Czech Republic, the last epidemic of meningococcal meningitis occurred in the 1950's, when the morbidity rate reached the peak, i.e. 14.8/100 000 (4, 5). After that a gradual decline in notified cases was observed until the middle 1980's when the incidence of meningococcal meningitis became only sporadic. Highest age-specific morbidity was found in the lowest age groups, with a relative shift into the older age groups during the epidemic period. The increase of the incidence was reported in the middle 1980's and age and antigenic shifts were recognized (3,4,5). More reliable and early indicators of an expected epidemic were identified (4) and their monitoring has been conducted since. A new epidemiological situation for the invasive meningococcal disease appeared in the Czech Republic in 1993 and was recognized quite early thanks to the long term monitoring and the analysing of epidemiological and microbiological data. We present a changes recognized in this new emergency situation.

Results and Discussion: The new epidemiological situation for meningococcal invasive disease began in the Czech Republic in spring 1993. The investigation of noncapsular antigens was very useful in the recognition of two local outbreaks in one region of the Czech Republic. A new strain *N. meningitidis* C:2a:P1.2 (P1.5) was identified as causative agent. Further investigation of P1.2 and P1.5 relationships suggests that growth conditions may influence the P1.5 positivity. Class 5 and pili antigens show a uniformity in *N. meningitidis* C:2a:P1.2 (P1.5) (strains from patients and their contacts), while *N. meningitidis* C "non 2a:P1.2 (P1.5)" and *N. meningitidis* B have heterogeneous Class 5 and pili properties. ET-typing using multilocus electrophoresis revealed that this *N. meningitidis* C:2a:P1.2 (P1.5) belongs to the ET-37 clone. This strain had never been found in the Czech Republic before, at least since 1973.

This new clone is causing the highest morbidity and fatality rates in the age group of 15-19 years (20% fatality). Vaccination, using A+C polysaccharide vaccine (Merieux), which was focused on the most affected localities and age groups probably prevented the meningococcal invasive disease caused by *N. meningitidis* C for further spreading in the "vaccinated" localities (as showed in our other presentation). Nevertheless, meningococcus C:2a:P1.2 (P1.5) spread to all regions of the Czech Republic during the next winter/spring season (1993/1994), keeping its high morbidity in the age group of 15-19 years, a high fatality rate and severe and atypical clinical manifestations. The spread of this new clone is very quick as documented by a rapid increase in the percentage of *N. meningitidis* C isolated from patients. For the whole year 1993 this figure was 48%, in June 1994 reached 73% (the percentage of *N. meningitidis* C was maximally 30% in the past). The percentage of *N. meningitidis* C:2a:P1.2 (P1.5) increased from 1992 to 1994 as well (from 0% to 88%). *N. meningitidis* B keeps its heterogeneity of serotypes and subtypes, with a high proportion of NT and NST strains, as identified in the past (see our other presentation). This spread of the new *Neisseria meningitidis* C:2a:P1.2 (P1.5) strain seems to be in good correlation with the low level of herd immunity against C:2a:P1.2, detected in the Czech population in the past (as showed in our other presentation).

In the new emergency situation there was a need for renewing the guidelines for the measures to be taken in the focus of the invasive disease and establishing the criteria for epidemiological indication of vaccination. Renewed guidelines for clinicians were prepared as well, because of the frequent incidence of an atypical course of meningococcal disease.

The incidence of invasive meningococcal disease has an increasing trend in the Czech Republic which is in correlation with the increase of the age index, calculated by Peltola's formula (6). Considering it as a predictor of an epidemic, we may expect an accelerated increase of the incidence of meningococcal invasive disease in the Czech

Republic, because it reached high values during a relatively short period (1.6 in October 1993 and 4.5 in June 1994).

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Significance of bactericidal antibodies directed against capsular and noncapsular antigens of *Neisseria meningitidis*

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Introduction: The bactericidal antibody assay was recommended by WHO for detection of protective meningococcal antibodies in 1976 (1). Our laboratory started to use a micromodification of this test (2) in 1982. Immunological surveys of meningococcal antibodies in healthy population of the Czech Republic have been conducted since 1984. The reference *Neisseria meningitidis* strains, selected for production of polysaccharide A and C vaccines, have been used as antigen sources. Encouraged by the advances in research on serotype and serosubtype antigens of *Neisseria meningitidis* we used for the survey in 1989 a selected collection of six *Neisseria meningitidis* strains, showing different serogroup, serotype and serosubtype antigenic properties. Their selection reflected the collection of strains used previously for the testing and the second criteria were the antigenic properties the most commonly found in the Czech Republic. The aim of our study was to assess statistical correlation between the antibodies directed against serogroup, serotype and serosubtype antigens of *Neisseria meningitidis.*

Materials and Methods: The sera collected in the Czech Republic by the National Serum Bank at the Center of Epidemiology and Microbiology in NIPH were investigated (3). From one district, 750 samples of sera of all age groups of population (males and females shared equally), without any acute febrile disease and ATB treatment were obtained. The results of blind testing of the code numbered sera were submitted to the Department of Statistics of the NIPH for analysis. Percentage of sera positivity and geometric means of different antibody levels in correlation to the age and sex were determined. The correlation between positive sera against different antigens of *Neisseria meningitidis* strains was analyzed by the use of the x^2-test and t-test.

Bactericidal antibody microassay was used (2) in our laboratory modification (4). Reference and field *Neisseria meningitidis* strains used as serogroup, serotype and serosubtype antigens for tests in l989; A l027 - A : 4, 2l : (P1.10), B l6B6 - B : 2a : Pl.2, P1.5, B 98/88 - B : NT : Pl.2, P1.5 (P1.10), B 24/89 - B : 4 : Pl.l5, C 7606 - C : 2a : Pl.2, P1.5 and C l/8l - C : 4, 2l : P1.5 (P1.10). The selection of serotype and serosubtype patterns was based on the serotypes and subtypes prevalence in the Czech Republic that time and on the serotypes and

subtypes of previously used reference strains. Nevertheless, we have not used sero/subtype identical strains in all cases because of presence of non-serotypeable or non-serosubtypeable strains.

Results and Discussion: There were found differences in levels of bactericidal meningococcal antibodies against different antigens of *Neisseria meningitidis* strains in healthy population of the Czech Republic. In the immunological survey carried out in l989, the highest percentage of positive sera was found for antibodies anti A l027 and anti B 24/89, medium values for antibodies anti B 98/88 and anti C l/8l and the lowest ones for antibodies anti C 7606 and anti B l6B6. Similar three value levels were found in both males and females population. As in previous years, males population showed higher levels of antibodies.

The correlation of positive sera was evaluated for different antibodies by the x^2 - test and t - test. Correlation significant on 0.l% level, in both tests, was found between following antibodies; anti A l027 and anti C l/8l, anti A l027 and anti B 24/89, anti C l/8l and anti B 24/89, anti C l/8l and anti B 98/88. The strains showing this antibody correlation share identical serotypes (except the last couple, where B98/88 is non-serotypeable) and in 50% identical subtypes, but have different serogroups.

This high correlation which was found between antibodies against identical *Neisseria meningitidis* serotypes and in 50% of subtypes in contrast to the antibodies against identical serogroups, suggests that the antibodies directed against noncapsular antigens are more significant for the defense mechanisms of organism. This hypothesis is in correlation with a rapid spread of the new clone of *Neisseria meningitidis*, causing serious invasive diseases in the Czech Republic recently (as showed in our other presentation). This *Neisseria meningitidis* C is carrying the new noncapsular antigens (2a : P1.2, P1.5) and the herd immunity against them is very low in the Czech population of all age groups.

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Serotypes and subtypes of *Neisseria meningitidis* strains isolated in Moscow during the decrease of morbidity (1989-1991)

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The outer membrane protein antigens (OMPA) of meningococcal isolates attract the attention of investigators in different countries (2-5, 8-10). In Russia, studies on the strains of *Neisseria meningitidis* group B isolated from patients in 1980's were also undertaken (6). No predominating serological type/subtype was revealed among the isolates. The aim of the present study was the epidemiological evaluation of serological characteristics of a relatively large collection of meningococcal strains isolated from healthy persons (carriers) in Moscow and suburbs in 1989-1991.

We studied 260 strains isolated from 218 healthy adult carriers of 11 closed communities. In four communities one-two cases were observed during the study. For serotype/subtype determination whole-cell ELISA method (1) was used with MCAb to 17 OMPA; type antigens (TA) - 1, 2a, 2b, 4, 14 and 15; subtype antigens (SA) - P1.1, P1.2, P1.4, P1.6, P1.7, P1.9, P1.10, P1.12, P1.14, P1.15 and P1.16. The MCAb were kindly provided by Dr. J.T.Poolman. Nine serogroup A strains were sent to Prof. M.Achtman for a more detail study.

Serotype/subtype appeared to be a stable property of the strain. Thus, among 29 carriers studied sequentially for two or more times, only in five persons the serotype/subtype changed. At the same time, the change of the serogroup of these isolates was observed. We considered these cases as infection with a new strain. A definite OMPA composition of the strains was characteristic of each community. These observations allowed us to confirm that serotype/subtype characteristic of the meningococcal strain is a valid epidemiological marker.

Among the collection of strains studied no predominating serological variant was revealed. Among 218 isolated originally strains, 13 belonged to serogroup A; 28 strains to serogroup B; 31, to serogroup C; 11, to serogroup X; 44, to serogroup Y;

10, to serogroup 29E-Z; 33, to serogroup W-135; 48 strains (22.0%) were nontypeable.

In 139 (63.3%) strains, serotype and/or subtype were determined: in 54 (24.6%), serotype and subtype; in 21 (9.6%), only serotype; in 64 (29.3%), only subtype. Among 75 typeable strains, the TA 15 predominated (48.0%); TA 4 was encountered in 24.0%; TA 1, in 17.3%; TA 14, in 9.3%; TA 2b, in 1.3% (1 strain). Among 118 subtypeable strains, the most common SA were: P1.12 (22.0%); P1.16 (20.3%); P1.7 (17.8%); P1.4 (16.9%). 37.3% out of 118 subtyped strains had two SA, mostly P1.4 and P1.12. SA P1.4 alone was never encountered. The composition of TA and SA 15:P1.16 was most common, in 19 out 54 strains with determined serotype and subtype. Eleven out of 19 strains with formulation 15:P1.16 belonged to the serogroup Y; only 3, to the serogroup B and one, to the serogroup C; others were nongroupable. All strains with this formulation were isolated from the communities where no cases were registered. Among 28 serogroup B strains, only 10 belonged to "ET5-complex" (4, 5) all were isolated from the communities free of cases. The serogroup C strains were also very heterogenous. The serogroup A strains were less heterogenous than B and C. Eleven possessed TA 4; two were nontypeable. According to Prof. Achtman laboratory data, 8 strains isolated from the case contacts belonged to a formerly unknown subgroup VI-I based on TA:SA formulation (4,21:P1.5,10) as well as pilin protein (I and IIa) and OMPA of Class 5. Epidemiological significance of this subgroup is unclear so far.

In general, the serotype/subtype pattern of meningococcal strains spread in Moscow reflected their wide antigenic heterogeneity characteristic of the period of sporadic morbidity (7). Indeed, since 1986 (6.9 per 100,000) - a stable decrease of morbidity in Moscow was observed (3.4 and 2.4 per 100,000 in 1991 and 1993, respectively). This decrease occurred after a 18-year period of elevated morbidity with a peak in 1970 (26.5 per 100,000). Thus, prevalence of heterogenous OMPA variants of *N. meningitidis* strains spread in population represents positive epidemiological sign. No correlation between the OMPA composition of meningococcal strains and their antilysozyme and hyaluronidase activities was observed.

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Serum bactericidal activity in a secondary school population following an outbreak of meningococcal disease

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Introduction: Immunity to *Neisseria meningitidis* correlates with the presence of bactericidal activity in normal sera, and there is evidence that carriage plays a role in induction and maintenance of natural protection to meningococci (3). In a survey of secondary school children carried out following an outbreak of meningococcal disease, we found that carriage of meningococci, particularly the outbreak strain (B:4:P1.15) was associated with the non-secretor phenotype (1).

The objectives of this study were to use a bactericidal assay: to determine if carriage of meningococci or secretor status was associated with serum bactericidal activity; and to assess levels of isotypes, either total or specific for *N. lactamica* or *N. meningitidis*, associated with serum bactericidal activity related to carriage or secretor status.

Subjects and methods: Sera from 67 secretors and 34 non-secretors were randomly selected from the samples obtained from school children (12-18 years of age) two and a half weeks after an outbreak of meningitis (1). *N. lactamica* was from the departmental collection and the following isolates of meningococci were obtained from Dr. R.J. Fallon, Meningococcal Reference Laboratory, Glasgow: B:4:P1.15; B;4:-; C:4:-; B:15:P1.7,16; NG:4:- . The strains were grown on modified New York City medium (MNYC) for 24 hr in a humidified atmosphere with 10% CO_2. The methods for testing bactericidal activity and absorption of the human complement source were reported previously (2). The reciprocal of the highest serum dilution at which there was at least an 80% decrease in viable count compared with the controls was recorded as the bactericidal titre.

Sera were assayed by quantitative ELISA for determination of total IgA, IgG or IgM and isotypes specific for the strains (6). Immunoglobulin levels of sera with bactericidal activity were compared with those for sera without bactericidal activity by the Mann-Whitney U test. Comparison of the bactericidal titre data was made after conversion of the titres to $\log_2$.

Results: Most sera had high titres of antibodies to *N. lactamica* (82.4%) and the non- groupable strain of meningococci (88.9%), but not to capsulate isolates: B:4:P1.15 (%); B:15:P1.7,16 (29.6%); B:4:- (31.5%); C:4:- (27.8%). Bactericidal activity was higher for both carriers and secretors, but the differences were not significant. Compared with sera in which there was no killing, in bactericidal sera there was no marked difference in levels of total or specific IgA or IgM. IgG levels were significantly higher in sera bactericidal for all four capsulate strains.

Carriers had significantly higher levels of IgG to *N. lactamica* in sera with bactericidal activity for each of the four capsulate strains compared with sera in which there was no killing: B:4:P1.15 (P = 0.02); B:15:P1.7,16 (P = 0.05); B:4:- (P = 0.004); C:4:- (P = 0.003). While there were higher levels of IgG *to N. lactamica* in sera of non-carriers with bactericidal activity, this was significantly associated only with killing of B:4:P1.15 (P = 0.02) and B:4:- (P = 0.003)

Sera with bactericidal activity obtained from secretors had significantly higher levels of IgG to *N. lactamica*: B:4:P1.15 (P = 0.01); B: 15:P1.7,16 (P = 0.005); B:4:- (P = 0.0001); C:4:- (P = 0.05). There was no significant association between IgG levels and bactericidal activities among non-secretors.

Discussion: Rosenqvist and colleagues (5) found IgG antibodies were significantly higher for carriers than for non-carriers before and after vaccination. In the non-vaccinated population in this study, among non-carriers there was no significant association between serum killing and the presence of IgG to these strains; but among carriers, killing of the capsulate isolates was associated with higher levels of IgG to *N. lactamica*. Although carriage was significantly associated with the non-secretor phenotype, there was no significant difference in bactericidal activity associated with secretor status.

While it has been suggested that carriage of bacteria with cross-reactive antigens might induce IgA antibodies that compete with IgG and IgM (4), we found no evidence for this. IgM levels of bactericidal and non-bactericidal sera were comparable. The higher levels of IgG to *N. lactamica* found in the sera with bactericidal activity to all the capsulate strains used suggest these antibodies are directed against common antigens.

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Meningococcal disease in Romania 1971-1992

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Meningitis due to *Neisseria meningitidis* is a cyclic endemo-epidemic disease in Romania. After the epidemic of 1970 in which there were 1051 cases with a morbidity of 5.2% (5), the incidence decreased dramatically ranging from 191 to 534 cases per year with a corresponding morbidity of 0.9-2.3%. In 1985, the numbers of cases began to rise sharply reaching a peak of 2,632 and a morbidity rate of 11.4% in 1987. This has decreased to 250-270 cases per year in 1990 and 1991 with morbidity rates of 1.1 and 1.2% respectively. Mortality closely followed the morbidity curve with a maximum of 0.4% in 1984. Fatality was higher during endemic periods with highest levels of 7.9% (1972), 8.9% (1975) and 6.3% (1990-1991) and lowest (1.4%) in 1980.

In contrast to countries in Europe and the Americas where serogroups B or C have been the predominant cause of meningococcal disease (1,3,5,6,7,10), both the Romanian epidemics were due to serogroup A. In the interepidemic years, the incidence of cases due to group A decreased to 5.6% (1974) and disease due to group A virtually disappeared in 1991. Group A was replaced by group B, 66.7% in 1975 and 90.9% in 1992 among patient isolates and 24.5% among carriers in 1992. While serogroup B was not detected in 1983-1984 or 1986-1990 among either patients or carriers, serogroup C was permanently present. In post epidemic years 1974 and 1990, they were responsible for 38.9% and 23.1% of cases respectively. Non-groupable strains were frequently isolated in interepidemic years, especially from carriers. No differences in the distribution of serogroups were observed among isolates from the 24 counties in Romania compared with Bucharest.

During the epidemic years, clinical disease due to serogroup A was relatively mild, reflected in the lower fatality rates (3.5% in 1987) compared with the endemic periods when group B was most prevalent (8.9% in 1975 and 7.9% in 1992). Males (56%) were more often affected than females (44%). A higher seasonal incidence in April-May and October-December was usually noted. The incidence of meningococcal disease was greatest among the 0-1 year age group during both the epidemic (morbidity of 100% in 1987) and endemic periods, followed by a continuous decline with age. There were no significant differences between urban and rural areas except Bucharest and another large town where the age group most affected was 1-4 years and a new small peak in the 15-24 age range in endemic years and in the 25-44 age range in epidemic periods.

The majority of 20 strains examined for serotype and subtype in reference laboratories in Greece and Scotland did not react with the serotype or subtype monoclonal reagents used to differentiate strains in northwest Europe. Among isolates from patients, 54% were non-typeable as were 89% from carriers. The most prevalent serotype was 14 which is common among strains isolated in Greece (8). Subtype P1.15 was found exclusively among patient strains and P? among isolates from carriers. *N. meningitidis* strains (649) isolated between 1980-1992 were sensitive to penicillin and chloramphenicol. There were strains resistant to tetracycline (0.1%), ampicillin (0.5%), rifampicin (0.7%), erythromycin (1.4%), kanamycin (7.7%), streptomycin (14.2%), gentamicin (18.3%), sulphonamides (24.7%). As in Greece (10), there have been recent reports of penicillin insensitive isolates (12%) (2).

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Characteristics of meningococcal isolates from patients and carriers in the Balkans

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Introduction: Our previous study of *Neisseria meningitidis* isolates from patients and carriers in Greece found that the majority were not typeable by the monoclonal reagents used successfully in northwest Europe and the Americas (3). As has been observed for meningococcal strains in Spain, nearly half the isolates from patients and carriers in Greece were insensitive to penicillin (4,5). In Romania, there are periodic epidemics due to serogroup A meningococci. The mortality associated with these epidemics is lower than that associated with disease due to serogroups B and C during inter-epidemic periods (1). In this study we wished to determine if the isolates from Romania would resemble those from Greece or those from western European countries.

Materials and methods: Isolates from Romanian patients and carriers (33) were examined by the reference laboratories in both Athens and Glasgow for serogroup, serotype and subtype. They and 43 Greek isolates from patients and 34 from children who were carriers were examined for multilocus enzyme electrophoresis (MLEE) by Dr. D.A. Caugant.

Results: MLEE analysis of 14 enzyme loci were carried out on isolates from Greek children with meningococcal disease. The strains were classified into 18 different enzyme types. None belonged to the ET-5 complex associated with disease in northern Europe. Two strains represented clones of the ET-37 complex. Nearly half the isolates belonged to a group of clones previously designated as cluster A4 which was associated with disease in several European countries in the 1970's but has not been a major cause of disease recently in other parts of Europe. Only one ET found among the patient isolates was identified among 34 isolates obtained during a survey of school children in Athens for meningococcal carriage. The dominant ET-37 complex was not found among the carrier isolates.

Our recent studies of 33 isolates from patients and carriers in Romania indicate that, as with the Greek strains, the majority of serogroup B isolates are not serotypeable with the available monoclonal reagents. As with the Greek strains, none of the Romanian isolates belonged to the ET-5 complex.

Discussion: Analysis of isolates from patients and carriers in two Balkan countries found that the monoclonal reagents used for typing meningococci in north and west Europe and the Americas are not useful for strains from eastern Europe. Similar results have been reported for isolates from the Czech Republic; 50-80% of the Czech strains are non-typeable by the reagents currently available (2). While the Czech Republic and Romania have been relatively closed populations, Greece is a major holiday destination for tourists from north west Europe; however, the Greek isolates appear to be more like those from other eastern European countries.

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Group C meningococcal disease in São Paulo, Brazil: A re-emerging pathogen

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Introduction: Meningococcal disease (MD) was described in São Paulo in the beginning of this century (2). An outbreak from 1945 to 1951 has occurred, with its peak in 1947. A new outbreak due to group C began in 1971; overlapped by group A in 1974, causing the largest epidemic ever seen in São Paulo. A vaccination campaign were directed at the control of group A/C MD in 1975. Since 1976, group B has been the most frequently isolated. In 1988, another epidemic started, caused by group B strain B:4:P1.15. In 1989 initiated a change in group C strain distribution, from C:2a to C:2b (ET-11 complex), never isolated before (3). Since 1990 the proportion of group C has increased. The purpose of this study is describe the behaviour of group C MD in Greater São Paulo in period of 1990 to 1993.

Materials and methods: Greater São Paulo includes the City of São Paulo and 36 other nearby municipalities. All MD cases that occurred between January of 1990 and December of 1993 have been analyzed. Meningococcal infection was diagnosed on the basis of (*a*) typical clinical symptoms with skin manifestations; (*b*) microscopy of cerebrospinal fluid (CSF); (*c*) detection of *N. meningitidis* antigens by counterimmunoelectrophoresis (CIE) or latex agglutination in CSF or blood; and (*d*) cultures of CSF and blood. The *N. meningitidis* strains were serotyped and subtyped by using monoclonal antibodies and whole-cell suspensions prepared as described by Abdillahi and Poolman by DOT Immunoblot technique (1). Monoclonal antibodies 2b, 4, 17, P1.7, P1.9, P1.14, and P1.15 were produced at Adolfo Lutz Institute. The others, 2a, 8, 15, P1.2, P1.3, and P1.16 were kindly provided by W. D. Zollinger and C. E. Frash of Bethesda, USA.

Results: In the period of study, group C accounted for 111 cases in 1990 (7.36 cases/1,000,000), 149 in 1991 (9.69 cases/1,000,000), 108 in 1992 (7.03 cases/1,000,000), and 81 in 1993 (4.68 cases/1,000,000). In distribution by groups of *N. meningitidis*, group C was responsible for 32% of all MD cases in 1990, 45% in 1991, 32% in 1992, and 34% in 1993. Among children under 5 years of age,

group C accounted for 30.63% in 1990, 55.71% in 1991, 74.39% in 1992, and 70.88% in 1993 of MD cases.

Two vaccination campaigns took place in the period, first in 1990 directed at the control of group B/C, and second in 1991 at the control of group C, with 90% population coverage under 20 years old.

Case fatality rate for group C MD were 9.52% in years 1990-1993. When stratified for clinical presentation form, fatality rates highly differ, with 41.18% for septicemia, 12.86% for meningitis with septicemia, and 2.19% for meningitis.

Among group C *N. meningitidis* isolates collected, the C:2b strain represented average of 80% in years 1990-1993, without significant variations. Subtype C:2b:P1.3 increased from 62.3% in 1990 to 77.1% in 1993. The majority of C:2b strain were from ET-11 complex, ribotype Rb2.

Discussion: Since 1988, an outbreak of MD has been in progress in Great São Paulo. Group B *N. meningitidis* seems to be the responsible for the vast majority of cases. However, since 1990, the proportion of group C is increasing. The highest incidence rate of group C occurred in 1991. The proportion of cases due to group C was 45%, quite similar to group B, with 51% in the same year (considering only the grouped samples).

The proportion of MD cases in children under 5 years old in years increased from 31% in 1990 to 74% in 1992. It suggests that a non epidemic pattern took place. Overall period case fatality rate average was 9.52%. However, significant difference was found related to clinical presentation form. Despite efforts to control MD in Greater São Paulo, the present epidemic is still on its course. The vaccination campaigns impact has been lower than desirable to prevent group C MD.

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Group B meningococcal disease in São Paulo, Brazil: An epidemiological and microbiological study

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Introduction: Half of meningococcal disease (MD) cases in the State of São Paulo occurs in Greater São Paulo. Group B *Neisseria meningitidis* has predominated over other groups since the end of 70s epidemic. Since 1979 the epidemic strain from ET-5 complex has been detected. The same strain is associated with outbreaks in Norway, Chile, and Cuba (A, B). In 1988 a new epidemic took place, with annual incidence rate of 4,06 cases/100.000. Strain B:4:P1.15 was the most important strain in this new epidemic (A). The purpose of this study is to describe the Group B MD in the period of 1990 to 1993 in Greater São Paulo.

Materials and methods: Greater São Paulo includes the City of São Paulo and 36 other nearby municipalities. All MD cases that occurred between January of 1990 and December of 1993 have been analyzed. Meningococcal infection was diagnosed on the basis of (*a*) typical clinical symptoms with skin manifestations; (*b*) microscopy of cerebrospinal fluid (CSF); (*c*) detection of *N. meningitidis* antigens by counterimmunoelectrophoresis (CIE) or latex agglutination in CSF or blood; and (*d*) cultures of CSF and blood. The *N. meningitidis* strains were serotyped and subtyped by using monoclonal antibodies and whole-cell suspensions prepared as described by Abdillahi and Poolman by DOT Immunoblot technique (C). Monoclonal antibodies 2b, 4, 17, P1.7, P1.9, P1.14, and P1.15 were produced at Adolfo Lutz Institute. The others, 2a, 8, 15, P1.2, P1.3, and P1.16 were kindly provided by W. D. Zollinger and C. E. Frash of Bethesda, USA.

Results: The highest incidence rate of MD was in 1990, with 6.24 cases/100,000. Thereafter, it declined, reaching 4.63 cases/100,000 in 1993. The incidence rate maintained above endemic level in this period. From 1990 to 1992, 38% of all cases had the *N meningitidis* group detected; in around 25% the CSF culture, CIE, and/or latex agglutination were negative. By contrast, in 1993 32% of all cases had *N meningitidis* group detected. Group B accounted for 215 cases in 1990 (14.25 cases/1,000,000), 167 in 1991 (10.87 cases/1,000,000), 179 in 1992 (13.99

cases/1,000,000), and 147 in 1993 (9.56 cases/1,000,000), performing a total of 744 cases.

Group B proportion of strains analyzed was 63% in 1990, 51% in 1991, and 63% in 1992 and 1993. The strain B:4:P1.15, from epidemic clone ET-5, ribotype Rb1, has accounted for 70% of cases. In years 1990-1993, group C *N. meningitidis* proportionately increased, with highest proportional incidence of 45% in 1991. Three to 4% of cases were due to other groups.

Case fatality rate of MD in the period of study ranged from 18 to 20%, with different distribution according to clinical presentation. Highest fatality rate occurred in septicemia with average of 69.08%, 16.03% for meningitis with septicemia, and 7.73% for meningitis. By contrast, group B fatality rate was 12.47%, also with different distribution according to clinical presentation, with 65.52% for septicemia, 15.04% for meningitis with septicemia, and 3.45% for meningitis.

Discussion: Since 1988, there has been an MD epidemic in the Greater São Paulo, with a peak of infection in 1990. Group B *N. meningitidis* was the major cause of the increased disease incidence, but in recent years group C is becoming more frequent. In 1990, the population was submitted to a massive vaccination against group B and C MD, using a vaccine produced in Cuba, with coverage of 92% of children between 3 months to 6 years of age. However, epidemiological data evaluation show that this did not have an impact in MD incidence rate.

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Mathematical modelling of age-specific incidence of meningococcal disease

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Age-specific distribution of meningococcal disease reported in different countries has three general features: 1) rapid decrease of initially high incidence by age observed at ages of patients from one to seven years; 2) low and approximately constant incidence observed at ages greater than seven years; 3) second small peak on the age-specific distribution observed in some countries for the ages of patients of 15-20 years. This distribution could be easily explained using the reasonable assumption that initially susceptible, not immunized children, obtain resistance as a result of contact with pathogenic meningococci, whereas a small part of them receives the systemic infection. Later this resistance/immunization may be lost by time or updated as a result of new contacts with pathogen. In our analytical mathematical model the left part of age-specific distribution (the decline) provides the information on the rate of acquisition of resistance, the right part (the constant level) provides the additional information on the duration of immunization, and the "second peak" gives the opportunity to estimate the effects of behavioral and social factors.

1278 episodes of systemic meningococcal infection in Moscow region during last 10 years was analyzed. Our model approximates satisfactorily the age distribution of infection (correlation coefficient of model and observed functions greater than 0.99) and predicts the time constants of "susceptible" and "resistant" states. The same approach was used for analysis of age-specific distributions reported for several European countries and for the USA. The quality of approximation was also satisfactorily and the different values of time constants correspond to the different intensity of epidemic process. In addition, the age-specific distribution of meningococcal infection in the group of patients with late complement component deficiency was analyzed. In accordance with our previous data the age-specific incidence in this group was approximately constant, that is these patients are unable to obtain the sufficient resistance after contact with meningococci.

Probabilistic spatial-temporal model as a simulator for the epidemiology of directly transmitted infections: Updated version for meningococcal infection

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This computerized model simulates the epidemic process as the probabilistic process in real space. Each point of the free-form array ("town") represents an individual (up to 60000 points in array for our version that can be easily expanded for more powerful computer). Each individual may be in one of four states: healthy (H) that is susceptible to infection, carrier of meningococci (C), patient with systemic meningococcal disease (D), or immunized (I) after the experienced disease or carriage. The state of this individual at the next cycle of calculation (e.g. at next day) depends on the probabilities of transition during the time unit (cycle) from one state to another one: H6H, H6C, H6D, C6C, C6D, C6I, C6H, D6D, D6I, I6I, I6H, I6C, these probabilities are designated conventionally as P_{HH}, P_{HC}, P_{HD}, P_{CC}, P_{CD}, P_{CH}, P_{CI} etc. Some of the probabilities, namely P_{HC}, P_{HD}, P_{HC}, depend on *i*, the number of carriers among the neighbours of the individual (up to eight neighbours at any moment) and are marked as P^i_{HC}, P^i_{HD} and P^i_{HC}.

Any kind of dependence on the number of surrounding carriers may be determined, e.g. the infectiousness of carriers may be independent or has the cumulative effect. In order to simulate the output from the states more accurately, the delay time for each state may be given (an individual should spend certain delay time in this state and after that obtains the probabilities to leave it). All probabilities, delay times, and the initial numbers of individuals in each state are defined before the first cycle of calculation, the different individuals are distributed randomly in the above-mentioned array-"town" or any desirable part of "town". These probabilities could be changed also at any moment during calculation that may simulate temporal changes of susceptibility or infectiousness, e.g. seasonal effects. Additionally, the number of "running" or relocating individuals, who change their spatial positions in the array randomly after each cycle could be given before or during calculation. As the useful option, several subpopulations with different sets of parameters could be studied simultaneously in one model. Thus one can simulate different susceptibility or mobility of different biological or social groups (e.g. "children" and "adults"). Another option provides the opportunity to add the individuals to any group during the calculation, that may simulate, for example, an immunization program, if the "immunized" individuals are added. If necessary, the flow-chart of process, that is the scheme of states and

permitted transitions between states, can be easily modified or reduced. For example, we compared two flow- charts: for first one only transitions I6I, I6H were possible, that is contacts with carriers did not change the status of immunized individual. For second one the additional transition I6C was permitted, that is the immunized individual was able to become a carrier and thus to prolong his immunized state. The results of calculation are the temporal and spatial changes of the number of "healthy", "infected", "diseased", and "immunized" individuals.

The model simulates satisfactorily the known general features of meningococcal epidemic process and provides the opportunity to study the influence of parameters and initial conditions on the rate of spreading and stationary state of meningococcal infection. A range of different aspects of circulation of infection was simulated; several results will be presented including: the simulation of long-term epidemic waives, the problem of seasonal variation of morbidity, the effects of different mobility and communication of population, age-dependent effects and the influence of immunocompromised hosts, the comparison of different programs of immunization, etc. This model may be used both for investigatory and educational purposes. Its main preference in comparison to the recent analytical mathematical models is that model-simulator provides the opportunity to observe the effects of changing epidemic conditions without solving of sophisticated sets of equations.

Meningococcal disease as a problem of the special intensive care unit for patients with infectious diseases

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The systematic clinical investigation of meningococcal disease (MD) in Moscow was started in early seventies when the high incidence of disease was registered. From that time all patients with MD from Moscow city and Moscow region admitted to the Second Hospital of Infectious Diseases, where the ICU have been established in 1975.

We have studied clinical records of 3391 patients with MD admitted to the Hospital from 1984 to 1993 and especially the records of 1278 patients admitted to the ICU. Although the bacteriological studies of blood and CSF of all patients were made, the antibiotic treatment of patients before admission to the Hospital have led to the clinical diagnosis of MD was confirmed by positive culture only in 27% of cases.

Patients with MD admitted to the ICU were grouped to four clinical categories: 110 patients with meningitis (MG) alone, 313 patients with meningococcemia (MC) alone, 825 patients with combined meningitis and meningococcemia (MG+MC), and 31 patients with meningoencephalitis (ME). MD was complicated by brain swelling in 764 (60%) cases and by septic shock in 352 (28%) cases. 83% of patients were younger than 30 years; 62% (796) of patients were children (an age less than 14 years) and 80% of children were younger than 4 years.

The total mortality of patients with MD in the ICU was 17.6 % (225/1278 cases) or 6.6% (225/3391) for all patients with MD. The lethal outcome was caused by primarily central nervous system complications in 51 patients and by septic shock in 174 patients. In 167 cases septic shock was complicated by multiple organ failure (MOF).

Thus septic shock was the main cause of fatal outcome in patients with MD. 125 (75%) of deceased patients were children. Male:female ratio was 83:84. The age distribution for deceased patients did not differ from that for all patients with MD. The fatal outcome of septic shock was observed in 62 (37%) cases during first four hours, in 109 (65%) cases during twelve hours and in 128 (77%) cases during 24 hours after admission.

During the study period (1984-93) the yearly incidence of MD had decreased significantly ($p < 0.05$), but the yearly number of fatal cases was approximately the same. This phenomenon could be explained by that, although the number of patients with MG+MC had decreased, the number of patients with MC was constant, whereas MC was more fulminant clinical variant complicated often by septic shock.

The endotoxin levels have been measured in serum samples collected at admission to the ICU from 79 patients with MD confirmed by positive culture: 42 patients with MG+MC, 33 patients with MC and 4 patients with MG. The fatal outcome was observed in 14 (38%) of 37 patients with endotoxin level > 700 ng/l, in 16 (84%) of 19 patients with level > 3000 ng/l and in all 12 patients with level > 8000 ng/l. The average level (5660±1290 ng/l) was greater in samples from patients with MC than the level in samples from patients with MG+MC (2660±850 ng/l) or MG alone (160±120 ng/l). The significant positive correlation of serum endotoxin level and outcome of disease was observed in groups of patients with MC (correlation coefficient - 0.74), MG+MC (0.58) and in all patients (0.73). The median endotoxin level (12200 ng/ml) in serum of patients with MOF was approximately ten times greater than that (1300 ng/l) in patients without MOF. We had never observed the increase of endotoxin level in patient's sera after start of antibiotic treatment. Moreover, the endotoxin level had dropped to the undetectable level during several hours irrespective of the clinical variant of MD, patient's condition or complications.

The treatment of MD is well-known. However the complex of intensive therapy including antibiotic treatment, blood volume restoration, dopamine, corticosteroids, ventilation support, extracorporeal detoxication (plasmapheresis, blood exchange) was not effective in 17.3% of all patients admitted to the ICU. The possible reason is that the distinctive feature of meningococcal septic shock is the development of MOF at early stage of shock. The fatal outcome during first four hours after admission observed in 37% of dead patients with shock could be an evidence of irreversible MOF development in prehospital period. Correspondingly, the observation, that 77% of fatal outcomes take place during 24 hours after admission, indicates that the real control of MOF development is absent. The recent research of the role of meningococcal endotoxin, cytokines, complement and coagulation systems, etc. in pathogenesis of meningococcal disease demonstrates the principle mechanism of inflammation process and MOF development. But the clinical successes in the treatment of patients with MOF are not so prominent.

Single-dose ofloxacin to eradicate tonsillopharyngeal carriage of *Neisseria meningitidis*

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After an outbreak of 3 cases of serogroup B meningococcal disease at a Norwegian college, 84 of 392 (21%) subjects were tonsillopharyngeal carriers of *Neisseria meningitidis*. All strains were highly susceptible to ofloxacin. To eradicate meningococcal carriage, 80 volunteers received a single dose of 400 mg ofloxacin. Three days after treatment, all 75 evaluable volunteers were culture negative for *N. meningitidis*, and after 7 days none carried the strain that they harboured initially, as judged by DNA-fingerprinting. On day 33, 6 carriers were detected and 2 of these harboured strains with the same genomic pattern as before treatment. If we consider reisolation of the same strain after treatment as eradication failure, ofloxacin was 97.2% effective in eradicating meningococcal carriage for a period of 33 days. After ofloxacin treatment, no case of meningococcal disease occurred for 12 months. Six other carriers of *N. meningitidis* were treated with a single dose of ofloxacin, and all of them became culture-negative 1-2 hours after dosing. A single dose of ofloxacin was found effective in eradicating tonsillopharyngeal carriage of *N. meningitidis*, and may thus prevent outbreak and spread of meningococcal disease.

Antibiotic prescribing and meningococcal disease

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A protracted outbreak of meningococcal disease in Gloucestershire, England, has been associated mainly with a sulphonamide resistant meningococcus (1). An investigation was carried out to see if sulphonamide or cotrimoxazole prescribing by family doctors in high incidence Gloucestershire towns might be selecting meningococci and thereby precipitating disease.

Family doctor antibiotic prescribing patterns in four Gloucestershire communities with high meningococcal disease incidence were compared with four low incidence communities in the same county on two occasions separated by six months. On each occasion there was no difference in the prescribing pattern of any antibiotic (including sulphonamide and cotrimoxazole prescribing) with the single exception of a consistent and highly significant excess of erythromycin prescribing by family doctors in high incidence communities. This finding was unexpected. Possible mechanisms which were considered include:

1. selection of erythromycin resistant meningococci;
2. excess erythromycin prescribing in response to an intercurrent infection, eg. respiratory tract infection, which itself predisposes to meningococcal disease; 3. erythromycin prescribing causes meningococcal disease.

Further work showed that a representative selection of Gloucestershire meningococcal strains were all erythromycin sensitive. Patients acutely ill with meningococcal disease were not more likely than controls to have received recent erythromycin treatment.

Mycoplasma hominis, the only erythromycin resistant *Mycoplasma* species (5), has been incriminated as a cofactor in meningococcal disease in Africa (4) but was not isolated from any of 26 cases or 26 controls in a multi-centre UK follow-up study.

Meningococci are inhibited by some Gram-positive oral organisms, particularly streptococci (2), which are usually erythromycin sensitive. A possible mechanism by which high erythromycin prescribing might increase the incidence of meningococcal

disease could be through a reduction in oropharyngeal inhibitory flora facilitating increased meningococcal transmission and/or colonisation within communities.

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Identification of amino acid residues crucial for sulfonamide resistance in the chromosomal *dhps* gene in *Neisseria meningitidis* by site-directed mutagenesis

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Sulfonamide resistance in *Neisseria meningitidis* is encoded by an altered form of the chromosomal *dhps* gene. Most of the clinically isolated sulfonamide resistant meningococci have been found to contain a *dhps* gene (type 1) that differ about 10% from the corresponding gene in sulfonamide sensitive isolates. It was therefore concluded that the resistance had been introduced by horizontal gene transfer followed by homologous recombination rather than by accumulation of point mutations (1).

Recently, another type of resistance mediating *dhps* gene (type 2) was found in two strains of *Neisseria meningitidis*. Their genes were identical, although the isolates were of different serogroups, and they differed from the type 1 resistance gene by being more similar to the *dhps* genes in susceptible meningococci. Both types of resistance genes have been used for site-directed mutagenesis in order to detect the influence of individual amino acid changes on the resistance phenotype.

The proposed peptide sequence of the type 2 DHPS differed at 18 amino acid positions compared to the corresponding sequence in susceptible meningococci. Three of the alterations were in amino acid residues conserved in all known bacterial DHPS polypeptides including the enzymes encoded by the plasmid borne genes *sul*I and *sul*II. Two of these amino acids turned out to be crucial for resistance. These residues were both located in conserved areas of the enzyme, one in the N-terminal part and the other in the central part of the DHPS. These changes were not present in the DHPS polypeptide of the type 1 enzyme. The proposed peptide sequence of the type 1 DHPS had two extra amino acid residues located to the conserved central part of the enzyme. Deletion of these two codons gave an enzyme that still had DHPS activity but was sensitive to sulfonamides.

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Changes in the resistance to ampicillin and tetracycline of *Neisseria gonorrhoeae* isolated in Stockholm 1982-1993

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Resistance to ampicillin and tetracycline can be encoded by the chromosome or by resistance plasmids. Chromosomally encoded resistance is mediated through several loci with additive effects (1). Resistance plasmids usually mediate high level resistance. This study was conducted to investigate changes in the prevalence of *N. gonorrhoeae* strains resistant to ampicillin and tetracycline during 1982-1993 in Stockholm.

Samples from out-patient clinics in the southern parts of the Stockholm area were included. All strains in the study were either consecutive or randomly selected from consecutively collected strains. A total of 404 strains from 5 different time periods were included. The minimum inhibitory concentrations (MIC) were determined by the agar dilution method. Strains with a MIC value of $\geq$4.0 mg/L to ampicillin or $\geq$2.0 mg/L to tetracycline were regarded as resistant. Plasmid DNA was obtained using an alkaline lysis procedure (3). The preparations were analysed by agarose gel electrophoresis. Isolates with bands around 38-42 Kb were digested with the restriction endonuclease *Bgl*I to discriminate between the conjugative plasmid and the *tet*(M) carrying plasmids.

All *N. gonorrhoeae* strains isolated in the laboratory from 1988 to 1991 were analysed retrospectively for MIC values of tetracycline. Strains with a recorded MIC of $\geq$2.0 mg/L were screened with E-test. Three strains isolated in 1991 had MIC values of >16 mg/L. They were further analysed for presence of plasmids. The strains were classified into serovars with monoclonal antibodies of the Pharmacia panel. Fisher's exact test was used for the statistical analysis.

Ampicillin; the proportion of strains with chromosomally encoded resistance with a MIC of $\geq$4.0 mg/L to ampicillin was 0-1% during the study. The proportion of penicillinase (ß-lactamase) producing *N. gonorrhoeae* (PPNG) rose during the period from 2% (2/106) in 1982 to 13% (5/39) in 1993 ($p<0.02$). Eighty-five percent (28/33) of the PPNG strains were also resistant to tetracycline. ß-lactamase encoding plasmids of Asian and African types were found.

Tetracycline; the proportion of strains with chromosomally encoded resistance with a MIC$\geq$2.0 mg/L to tetracycline was 17% (18/106) in 1982 compared with 56%

(20/36) in 1993 ($p<0.001$). A total of 7 *tet*(M) carrying strains were demonstrated. The first three were isolated in 1991 and carried a *tet*(M) plasmid of American type. The remaining 4 were of Dutch type. Six out of 7 *tet*(M) strains also produced ß-lactamase. To our knowledge this is the first report of *tet*(M) carrying strains isolated in Sweden.

Serological classification: thirty-four strains carried resistance plasmids, they belonged to 18 serovars. Four serovars were represented by 3 isolates, 8 serovars by 2 isolates, and 6 serovars by 1 isolate.

Conclusions: The incidence of gonorrhoea has declined sharply in Sweden during the study period but the total number of PPNG strains has not changed. The proportion of PPNG strains, strains with chromosomally encoded resistance to tetracycline, and *tet*(M) carrying strains rose during the period. This is most likely due to the increasing proportion of imported *N. gonorrhoeae* strains in Stockholm (2). The serovars of the strains suggest that there is only a limited spread of strains with resistance plasmids in Stockholm. The enhanced proportion of resistance to both ampicillin and tetracycline emphasizes the necessity of culturing *N. gonorrhoeae* and to susceptibility test all gonococcal isolates.

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Variable sequences between the *carA* and *carB* genes of pathogenic and commensal *Neisseria* species

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The *carA* and *carB* genes of *Neisseria gonorrhoeae* are separated by a large, variable intervening sequence ranging in size from approximately 2.2 to 3.7 kb in 30 isolates examined. This variation occurs between gonococcal isolates of different auxotype/serotype/plasmid class, and within some classes, a property which may be useful in a typing scheme. The intervening sequence between the *carA* and *carB* genes of *N. gonorrhoeae* strain CH811 has been sequenced, revealing the presence of two novel 120 and 150 bp repeats named RS6 and RS7 which are thought to play a role in the variation observed. We now demonstrate that a large, variable, intervening sequence between *carA* and *carB* is also present in *Neisseria meningitidis* and 8 commensal *Neisseria* species. These intervening sequences were amplified by the polymerase chain reaction using primers homologous to the 3' end of *carA* and the 5' end of *carB* of *N. gonorrhoeae* CH811. The resulting amplicons varied in size from approximately 2.7 to 3.1 kb in 12 *N. meningitidis* isolates (comprising 5 serovars), and from approximately 1.4 to 4.1 kb in 17 isolates of 8 commensal *Neisseria* species. This suggests that the novel gene organization found in *N. gonorrhoeae* is present throughout species of the genus *Neisseria*. This variable intervening sequence may be useful for typing meningococcal and commensal *Neisseria* species. Restriction enzyme analysis (REA) indicates that the intervening sequence between *carA* and *carB* differs significantly in different *Neisseria* species; related REA patterns are seen within a species, though no obvious similarities in REA patterns are seen between species. However, the intervening sequence in *N. meningitidis* isolates hybridizes at high stringency to a probe consisting of the intervening sequence from *N. gonorrhoeae* CH811, suggesting some similarities exist between the intervening sequences of these pathogenic *Neisseria* species. The intervening sequences from the commensal *Neisseria* species either do not hybridize, or weakly hybridize, at high stringency to this probe, possibly due to lower similarity between these sequences. The novel repetitive sequences, RS6 and RS7, found in *N. gonorrhoeae* strain CH811, were used to probe Southern blots of all the intervening sequences examined, to determine the specificity of the RS6 and RS7 sequences. RS6 and RS7 did not hybridize at high stringency with the intervening sequences from any of the *Neisseria* species except for *N. gonorrhoeae*, suggesting RS6 and RS7 may be specific for *N. gonorrhoeae* in this region of the genome. Using primers homologous to conserved regions surrounding RS6 and RS7 sequences in strain CH811, we have developed a PCR protocol which can amplify a variable region in all gonococcal isolates tested. The possibility of a species-specific variable sequence which can be selectively amplified from gonococcal isolates merits further study.

Relationship of source of health care and antimicrobial susceptibility patterns of *Neisseria gonorrhoeae*

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In the United States, monitoring of gonococcal susceptibilities through the Gonococcal Isolate Surveillance Project (GISP) is based on samples collected from patients who receive care at public STD clinics. However, in many locations, including Seattle/King County, Washington, the majority of cases of gonorrhoea are reported by health care providers other than STD clinics. To assess the generalizability of antimicrobial susceptibility monitoring based on isolates recovered from STD Clinic patients, we analyzed susceptibility test results by source of the patient's health care for gonorrhoea cases diagnosed in Seattle/King County, Washington.

During 1992-1993, clinical laboratories provided 2746 *Neisseria gonorrhoeae* isolates, representing the equivalent of 77.7% (2746/3520) of reported cases of gonorrhoea. Isolates were available from all culture positive cases diagnosed by the STD clinic and from 65.9% (1506/2285) of cases seen by other health care providers. Minimum inhibitory concentrations (MICs) of ceftriaxone, cefixime, ofloxacin, penicillin and tetracycline were determined by agar dilution (1). Isolates were further characterized by auxotype and Protein I (*Por*) serovar (2). Phenotypes were defined by auxotype/serovar (A/S) class and R-factor plasmid content.

Regardless of source of care, the phenotype most frequently isolated was Proto/IB-3, susceptible to all the antimicrobials tested. Plasmid-mediated resistance (PPNG, TRNG, or PPNG/TRNG) was detected in 9.8% (270/2746) of isolates. Rates did not differ significantly between isolates from STD Clinic patients (9.4%) and other facilities (10.1%). The proportions of isolates with apparent chromosomal mediated resistance (CMRNG) to penicillin were similar for isolates from STD patients (12.0%) and isolates from patients at other sources of health care (11.6%). Likewise, the proportions of isolates resistant to tetracycline (CMRNG) were similar, 18.0% and 16.9% for STD and all other patients, respectively.

For all isolates, geometric mean MICs and MIC_{90} values to the broad spectrum cephalosporins, were 0.005 ：g/ml and 0.03 ：g/ml to ceftriaxone and 0.012 ：g/ml and 0.03 ：g/ml to cefixime. The same maximum values (0.125 ：g/ml) were observed for both groups. Geometric mean and MIC_{90} values for ofloxacin were 0.016 ：g/ml and 0.03 ：g/ml, regardless of source of health care. Decreased susceptibility to ofloxacin (MICs 0.125-0.5 ：g/ml) was detected in 13 (0.5%) isolates; 7 were recovered from STD Clinic patients and 6 were recovered from patients seeking care elsewhere. Isolates with decreased susceptibility to ofloxacin were more likely to be recovered in 1993 (0.8%) than in 1992 (0.2%) ($p<.05$). Six

different phenotypes were identified among these 13 isolates with decreased susceptibility to ofloxacin. Individual phenotypes were as likely to be first recovered from STD patients (3 phenotypes) as from non-STD patients (3 phenotypes).

In Seattle/King County, Washington, antimicrobial susceptibility surveillance based on specimens from the STD Clinic provides an adequate representation of all cases from which isolates are available.

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Epidemiologic analysis of *Neisseria gonorrhoeae* by pulsed-field gel electrophoresis

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Pulsed-field gel electrophoresis (PFGE) is a method allowing separation of relatively large DNA fragments with enzymes that cleave uncommon restriction sites (4). Analysis of PFGE patterns can differentiate gonococci within the same auxotype/serovar (A/S) class (3,6). PFGE was used to examine two different groups of gonococcal isolates: 1) AHU/IA-1,2 strains (n=49) recovered in Seattle from 1988-1993 and 2) Pro/IB-5,7 strains (n=9) with decreased susceptibility to fluoroquinolones isolated in four western United States cities during 1993-1994. PFGE patterns of two or more isolates were judged identical if all visible fragments were shared.

AHU/IA-1,2 strains have been associated with disseminated gonococcal infection, and asymptomatic urethral infection in men and subclinical cervical infection in women, and are highly susceptible to antimicrobial agents (2,5). This A/S class has caused gonorrhoea epidemics in the United States and Europe (2). A random sample of AHU/IA-1,2 isolates from each year was examined with the enzymes *Xba*I and *Nhe*I. PFGE analysis produced 9 *Xba*I and 6 *Nhe*I patterns among 49 isolates. Two *Xba*I patterns and two *Nhe*I patterns accounted for 63% (31/49) and 84% (42/49) of isolates from all years, respectively. Organisms with the same *Xba*I-- *Nhe*I patterns decreased in frequency from 47% (10/21) of all isolates in 1988-89 to 11% (1/9) of all isolates in 1993. A second combination of patterns was first detected among isolates from 1992 and in 1993 accounted for 55% (5/9) of isolates.

Gonococci with decreased susceptibility to fluoroquinolones (minimum inhibitory concentrations [MIC] $0.125 :g/ml of ciprofloxacin) have recently been described from the United States (1). Isolates of the same A/S class representing two different levels of decreased fluoroquinolone susceptibility were examined using the enzymes *Xba*I, *Nhe*I and *Spe*I: two $-lactamase positive strains with MIC's of 2 :g ciprofloxacin/ml recovered in Hawaii and seven $-lactamase negative strains with MICs of 0.25-0.5 :g ciprofloxacin/ml recovered in four Western United States cities (Honolulu, HI, Seattle and Tacoma, WA and Portland, OR). Isolates of the latter resistance phenotype were first recovered between August and October 1994 in these four cities. Isolates with MICs of 2.0 :g/ml differed from each other by PFGE patterns produced with all three enzymes and from the isolates with MICs of 0.25-0.5 :g/ml by PFGE patterns of two or more enzymes. Except for a single fragment difference in one isolate after *Spe*I digestion, PFGE patterns of six of the seven isolates with MICs of 0.25-0.5 :g/ml were indistinguishable from each other. The

seventh isolate (Honolulu, HI) differed from the other six isolates by patterns generated with all three enzymes.

PFGE patterns of AHU/IA-1,2 subtypes were less diverse than other previously studied types (3,6). Among organisms with decreased susceptibility to fluoroquinolones, common PFGE patterns of isolates recovered in Seattle and Tacoma, WA and Portland, OR strongly support the spread of a single gonococcal strain to populations in relatively diverse geographic settings over a short time period.

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Gonorrhoea surveillance among female commercial sex workers from Mexico City

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In Mexico little is known about the prevalence and risk factors of STDs besides HIV/AIDS, in groups of people prone to acquire and transmit these diseases (3). Regarding gonorrhoea, there are some studies conducted in recent years by our group that have addressed mostly the prevalence, antimicrobial resistance rates, treatment and some molecular epidemiology markers of gonococci isolated from both males and females belonging to high and low risk populations (1,2,4). However, a better epidemiological assessment was lacking in terms of obtaining reliable information on patients' behaviour, identifying factors related to the presence of gonorrhoea and characterizing *Neisseria gonorrhoeae* strains from female commercial sex workers (FCSW) located at different working sites.

Objective: To estimate and compare the prevalence of gonorrhoea in different groups of FCSW according to their characteristics, and describe risk factors associated to the disease. Also, to determine resistance to penicillin, plasmid types and serovars of strains isolated.

Methods: Two sampling approaches were followed, first between March 1992 and February 1993, 862 women who participate in a continuous AIDS detection program in a Mexico City STD clinic, were enrolled in this study. Second, a sampling frame was constructed in order to identify prostitution facilities in an area of the city known to have such activities and later a random selection of massage parlours, bars and "street points" was made to conduct our survey; 327 women interviewed and sampled at their working sites were studied in this way between April and November 1993. Informed consent was given by all participants and a properly designed and validated questionnaire was applied to FCSW by trained personnel. Afterwards, an endocervical swab was taken by a physician and streaked directly onto Thayer-Martin medium plates, that were put inside candle jars and transported immediately in a portable incubator from Mexico City to our laboratory in Cuernavaca.

Gonococci were isolated and identified by standard procedures (5), techniques for serotyping, beta-lactamase detection and plasmid extraction were those described elsewhere (4,6). Data analysis depending on sample size, was done with Pearson's and Kruskal-Wallis chi square tests. Gonorrhoea patients were given appropriate treatment (1,2).

Results: The frequency of gonococcal infection for the whole population was 2.8% (33/1189). The prevalence observed in each selected group showed no difference, 2.4% (21/862) vs 3.7% (12/327). However, once the FCSW were grouped according to their working site, we found that among women attending the STD clinic the highest prevalence belonged to those working on the streets (3%, 15/506), in comparison to girls from bars (0.8%, 3/356). In this case, we could not identify the source of 3 other strains isolated. Women randomly selected also proved to have a bigger prevalence if they worked in hotels ("street points"), in fact all cases diagnosed were found among them for a prevalence of 5.5% (12/216), while FCSW in massage parlours (53) and bars (58) were negative.

Furthermore, factors associated to gonococcal disease for this women in addition to working site, were age, time in prostitution, socioeconomic level of clients and school years. Thus, all cases were women younger than 27 and with 6 years or less in prostitution, 85% of cases had clients with a low socioeconomic level and 70% had elementary education as the highest. Other findings included that 73% of positive patients said to have been using condoms regularly, 90% were asymptomatic, 100% had not received any antibiotic treatment in six previous months and in the case of patients from the sampling frame, 11 out of 12 had no regular sex partners.

Penicillin resistance was 15% overall, but considering separately the groups studied, resistance was only detected in women attending the clinic (23.8%, 5/21); 4 IB-1 strains had the 4.4 MDa "Asia" plasmid and 1 IA-10 strain showed the same plasmid.

Discussion: Gonorrhoea seems to be a classical STD that is decreasing in industrialized countries due to several causes like appropriate diagnosis and treatment of cases and contacts, probably the use of condoms, changes in sexual habits because of AIDS awareness and efficacious surveillance programs of core groups. As far as we can tell from our limited experience assessing FCSW from Mexico city, we have observed during this study a decreased gonorrhoea prevalence in comparison to some years ago (4), penicillin resistance remains a problem but apparently in a localized fashion that needs further attention and molecular epidemiology markers may be the same through the last 7 years or so in this population group.

Interestingly, epidemiological characteristics of FCSW groups have been identified that will allow intervention measures to help control and prevent gonorrhoea, in combination with other STDs that we are also studying. There remains the need to maintain and improve these activities, extending them also to other high risk behaviour populations in Mexico City and other parts of the country to complete an epidemiological picture of gonorrhoea in Mexico.

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A comparison of three methods for the culture confirmation of *Neisseria gonorrhoeae* strains currently circulating in the UK

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During 1991 the Gonococcus Reference Unit (GRU) became aware, through enquiries and the receipt of cultures, that some strains of *Neisseria gonorrhoeae* were failing to give a positive result in the MicroTrak fluorescent antibody test (FAT) used for culture confirmation in the referring laboratory. The GRU had been aware for some time that the monoclonal antibodies used for serotyping, produced by Genetic Systems for Syva, were failing to react with some referred strains and some strains isolated from Bristol patients. As a result of this experience a prospective study was initiated to compare the MicroTrak *Neisseria gonorrhoeae* Culture Confirmation Test (Syva Co, Palo Alto, USA) with two other culture confirmation tests.

Two hundred *Neisseria* sp. isolated from male patients attending the Department of Genitourinary Medicine, Bristol Royal Infirmary, were identified using sugar utilisation in cysteine trypticase base agar (Becton Dickinson, Cockeysville, USA), the Phadebact Monoclonal GC Test (Karo Bio Diagnostics AB, Huddinge, Sweden) and MicroTrak. The same tests were also applied to 206 unselected *Neisseria gonorrhoeae* strains collected from male patients in Leeds undertaking a therapeutic trial, to 20 strains referred to the GRU because of difficulties with the Syva FAT and to 121 strains which had not reacted with the serotyping antibodies.

All strains were auxotyped using the defined medium of Copley and Egglestone (1) to test for nutritional requirement for proline, arginine, hypoxanthine and uracil. In addition the ability of ornithine to replace a requirement for arginine was tested. Monoclonal antibodies, raised against the major outer membrane protein I of *N. gonorrhoeae* and produced by Genetic Systems, were used to serotype strains. Six monoclonal antibodies were specific for protein IA strains and six were specific for protein IB strains. The co-agglutination reaction pattern with each panel was used in the nomenclature scheme of Knapp *et al*. (2) to determine the serovar.

The sugar utilisation test confirmed the identity of 99% of gonococci from Bristol and 97% of gonococci from Leeds. The Syva FAT confirmed 75% of Bristol isolates and 83% of Leeds isolates whereas the Phadebact test confirmed all isolates from both cities. Half of the referred 'FAT negative' isolates were confirmed to give a negative result in the Syva FAT negative, however, only 10% of the rest gave a strong reaction in our laboratory. All serotyping-antibody negative strains were negative in the FAT, although all these and all of the 20 strains that give a negative result in the FAT gave positive reactions in the other culture confirmation tests.

The typing tests revealed a greater diversity amongst the FAT negative strains from Leeds than those from Bristol. Five auxotypes were found in Leeds strains (74% prototrophic) compared to two auxotypes (98% prototrophic) in Bristol FAT negative strains. The FAT negative strains from Leeds were found to comprise five serovars (two IA6, three IA2, one each of IB1, IB2 and IB3) and 77% were non-typeable. Three serovars (one IA16, seven IB20 and one IB2) were found in Bristol FAT negative strains (81% non-typeable).

A previous evaluation of MicroTrak in Edinburgh, UK (3) reported 98.2% sensitivity and 100% specificity with 110 clinical isolates. The same group evaluated the Phadebact test using 1367 *Neisseria* sp. isolated in the same city from consecutive attending patients (4). This test yielded a sensitivity of 99.7% and a specificity of 100%. We have found considerable differences in the sensitivity of MicroTrak but not for the Phadebact test or sugar utilisation for the identification of isolates made in two geographically distinct areas of the UK.

Our results suggest that the Syva FAT would not be suitable, if used as a single test, for culture confirmation in Bristol or Leeds.

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Comparative typing of gonococci using pyrolysis mass spectrometry: Preliminary experiments

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Introduction: Pyrolysis mass spectrometry (PMS) is a rapid, simple, automated, high throughput method for fingerprint typing, that has been successfully applied in outbreak investigations of a broad range of organisms (5,6). Costs are low, < £1 per isolate revenue and < £2 per isolate for amortised cost of apparatus (£45,000). Colony samples are heated to 530/C, yielding a mixture of volatile products whose composition reflects the composition of the sample. Mass spectrometric analysis of the products yields spectra that can be compared by computerised statistical analysis. Gonococci were examined in the first published PMS typing study (2), but the work was not pursued. In the current study, blind coded cultures were analysed in PMS, and the results compared with those of standard typing methods.

Materials and Methods: *Cultures*; these were from the Gonococcus Reference Unit freeze-dried culture collection and comprised of penicillinase producing strains (PPNG) and isolates from a pair of sexual contacts. The identity of strains had been confirmed using standard methods. The geographical source of each strain was sought by the issue of a questionnaire to the referring laboratory.

Standard typing methods; the auxotype of each strain was determined by testing for nutritional requirement for proline (P^-), arginine (A^-), hypoxanthine (H^-) and uracil (U^-) on a defined medium (3). In addition the ability of ornithine (A^o) to replace a requirement for arginine was tested. Monoclonal antibodies (supplied by Syva, Palo Alto, USA) were used in a co-agglutination test to determine the serovar (4). Plasmid DNA was purified from PPNG strains and profiles determined using the methods of Bennett *et al* (1). Seven plasmid combinations were found amongst the strains used in the study: 3.0 + 24.5MDa (3C); 3.2MDa (AF); 3.2 + 24.5MDa (AFC); 3.2 + 25.2MDa (AFT); 4.4MDa (AS); 4.4 + 24.5MDa (ASC) and 4.4 + 25.2MDa (AST).

The strains selected for preliminary evaluation of PMS comprised: six replicate cultures of a single strain (serovar - IB1, auxotype - non-requiring (NR), plasmid type - AS from India); five isolates from the two contacts in a sexual abuse incident (6D9, $A^oH^-U^-$, (non-PPNG), UK); cultures of four pairs of isolates that were clearly geographically and temporally distinct, but indistinguishable in all routine typing methods (IA6, P^-, AST, from Africa and Malaysia; IB1, P^-, AFC, from Ethiopia and UK; IB1, P^-, AS, from Bulgaria and the Philippines; and IB4, P^-, AS, from Germany and Thailand); and cultures of 10 further unrelated isolates (IB3, NR, 3C, UK; IB3,

NR, AF, UK; IB3, NR, AFT, Tunisia; IB3, NR, ASC, Malaysia; IB1, P^-, ASC, Vietnam; IB4, P^-, AFC, UK; IB1, NR, 3C, UK; IB1, NR, AFT, UK; IA6, P^-, AF, Poland; and IB4, P^-, AST, Cambodia). Strains were maintained on beads in glycerol broth at -70°C, and subcultured onto blind-coded heated-blood agar slopes.

Pyrolysis mass spectrometry; organisms were subcultured on to lysed blood agar plates from a single batch and incubated at 37°C for 24 h in humidified air with 5% CO_2; duplicate cultures of ten organisms were prepared as standards to determine the cut-off similarity for indistinguishable cultures. A sweep of colonies (*ca.* 50 : g dry wt) was taken with a straight wire and spread on to each of four pyrolysis foil. Foils were heated at 80/C for 10 min within 10 min of sampling to dehydrate the specimen and destroy enzyme activity. The foils were placed in a PYMS 200X pyrolysis mass spectrometer (Horizon Instruments, Sussex, UK) and analysed sequentially, pyrolysing in vacuum at 530/C for 4 s and analysing products from mass 50 to 150 in the subsequent 60 s. Spectra were recorded and analysed by multivariate statistical methods described elsewhere (5).

Results and Discussion: Two groups comprising five and six cultures respectively were indistinguishable, and one group of two cultures was on the borderline of resolution. The remaining cultures were clearly distinct. On breaking the code, the group of six cultures represented the duplicate cultures of the same clinical isolate, and the group of five cultures represented the isolates from the sexual abuse case. The marginally related pair represented isolates from the UK and from Tunisia of distinct serotyping patterns (IB1 and IB3) but identical auxotype (NR) and plasmid profile (AFT). The failure to unequivocally resolve one unrelated pair of isolates indicates that some work on optimising culture conditions to obtain maximal differentiation may be necessary. However, the compositional similarity within the replicate culture and the sexual abuse isolates sets was readily detected, and the clear dissimilarity of other isolates, irrespective of similarities in conventional typing, indicate the high resolution of PMS.

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Genetic diversity of *por* for differentiating between isolates of *Neisseria gonorrhoeae*

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The *por* gene of *Neisseria gonorrhoeae* exists as a single gene with two alleles and encodes for the major porin, Por (1). Monoclonal antibodies to immunologically distinct epitopes on the two subclasses of Por, IA and IB, have been used for serological classification of *N. gonorrhoeae* (2). We have explored the use of diversity within the *por* gene as an alternative method for typing *N. gonorrhoeae*.

The *por* gene was amplified using primers that extend from the initiation codon to its stop codon (3) producing a fragment of approximately 1004 base pairs (bp) from IA strains and of 1071 bp from IB strains. The PCR products were digested using *Tha*I, *Rsa*I and *Hae*III and the fragments separated by agarose electrophoresis. The method was evaluated using a panel of isolates of *N. gonorrhoeae*. These isolates differed in their year and country of isolation, auxotype/serovar class, susceptibility to antibiotics and mechanism of resistance. The ability to distinguish between two different isolates was assessed using the Simpson's Index of diversity (4), where a value of D=1 indicates that all the population members differ from each other and a value of D=0 indicates they are all identical. The ability to identify isolates from a common source was tested using isolates from known contact pairs.

PCR products from the panel of 18 isolates produced 5 different patterns (RFLPs) with both *Tha*I and *Hae*III and 4 patterns with *Rsa*I. When each enzyme was used individually the discrimination index was approximately 0.7 but all three used in combination gave an index of 0.98. Isolates from pairs of known sexual contacts gave identical patterns with all enzymes.

Diversity within the *por* gene has proved to be a reproducible and discriminative method for differentiating between isolates of *N. gonorrhoea*e that gives identical results in isolates from a common source. Phenotypic typing using antibodies to specific epitopes on Por is simple and easy to perform but has a subjective endpoint which can reduce reproducibility. Genotypic typing of *por* is more complex but produces a more objective result.

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