

## **EU-MenNet**

# **Impact of meningococcal epidemiology and population biology on public health in Europe**

**Final report  
2001-2005**

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## Objectives and achievements

The EU-MenNet was built to improve our understanding of the spread of meningococcal disease, which is one of the most severe childhood infections in Europe. This goal of the consortium has been achieved by integrated epidemiological and population genetic studies that employed the latest molecular isolate characterisation techniques and electronic data transfer via the Internet. The consortium has enhanced European expertise in this area by implementing integrated data collection, management, and interpretation and by establishing a number of specific research and development programmes. The insights thus gained and the European infrastructure that has been built up for research and surveillance will be of future value for the further improvement of public health and clinical management of meningococcal disease within Europe.

The EU-MenNet consortium has adopted a multi-disciplinary and collaborative approach to the study of meningococcal population biology. The network has put in place the collaborative structures and technological expertise required to obtain data and samples. Sequence based typing, specifically multilocus sequence typing (MLST), has been made available throughout Europe for research and surveillance purposes and by the creation of a European Meningococcal MLST Centre (EMMC WP 1 and 4). Improved PCR and microarray based techniques have been established to improve diagnosis and strain characterisation directly from clinical specimens (WP2 and WP3). The microarray technology also has been proven to be a valuable tool for characterisation of virulence attributes of hyperinvasive and hypervirulent lineages (WP3).

Hypothesis driven studies addressed the areas of carriage and vaccination; antibiotic resistance; and clinical severity of meningococcal disease. The data from the carriage studies were used to investigate the effect of large scale vaccination on the spread of meningococcal variants expressing non vaccine antigens (WP4) and to understand the relation between carrier and disease causing strains (WP5). After standardization of test systems it became possible to determine the spread of antibiotic resistant meningococci through Europe and to correlate antibiotic susceptibility with currently circulating meningococcal lineages (WP6). In WP7 particular hyperinvasive and hypervirulent lineages have been defined by characterising by MLST the isolates obtained from large numbers of patients with known severity of meningococcal disease as assessed by a variety of clinical scoring paradigms.

Thus, a network of competence including all European Reference Laboratories on Meningococci has been set up and a European infrastructure has been put in place for the epidemiological surveillance of meningococcal disease, that will provide all prerequisites for appropriate preventive measures for the control of meningococcal disease. The leading scientists in the field of meningococcal epidemiology and population biology, who established the consortium and built up the existing infrastructure are looking forward to an intense and close collaboration with national and European health authorities in order to combat one of the most severe and fearsome infections and to reduce the meningococcal disease burden in Europe.

## List of Working packages

<b>WP no.</b>	<b>Title</b>	<b>Responsible Participants</b>
1	Establishment of a European Meningococcal MLST Centre (EMMC)	Martin Maiden
2	Non-culture detection and typing of meningococci	Andrew Fox, Muhamed-Keir Taha, Ulrich Vogel
3	Implementation of the microarray technology for whole genome comparisons and isolate characterisation	Ulrich Vogel, Matthias Frosch
4	Establishment of a European Meningococcal Epidemiology Centre (EMEC)	Mary Ramsay
5	Studies on the carriage of meningococci in Europe	Dominique Caugant, Paula Kriz, Georgina Tzanakaki
6	Spread of antibiotic-resistant meningococci in Europe	Julio Vasquez
7	Studies on hypervirulent meningococci	Simon Kroll, Martin Maiden
8	Project co-ordination	Matthias Frosch, Dominique Caugant, Sigrid Heuberger, Martin Maiden, Mary Ramsay

## Workpackage 1

### Establishment of a European Meningococcal MLST Centre (EMMC)

**Participants :** Martin C.J. Maiden

#### Collaborations with partners in other workpackages

Collaboration with partners in work package 4 has established linked databases containing epidemiological and molecular typing data.

Collaboration with partners in work package 5 on the analysis of MLST data from carried meningococci, established quantitative assessments of hyper invasiveness for meningococcal clonal complexes.

Collaboration with partners in work package 7 has generated MLST profiles for 230 isolates from patients with clinical scores.

#### Objectives

- To obtain a comprehensive overview of the lineages of *N. meningitidis* responsible for meningococcal disease throughout Europe which is linked to epidemiological surveillance;
- To establish systems for submission of isolates to the central MLST facility and distribution of low-cost reagents from the MLST facility;
- To disseminate techniques throughout Europe to ensure ongoing collection of these data and to promote best practice Europe-wide.

#### Results

##### Submission of isolates and distribution of reagents

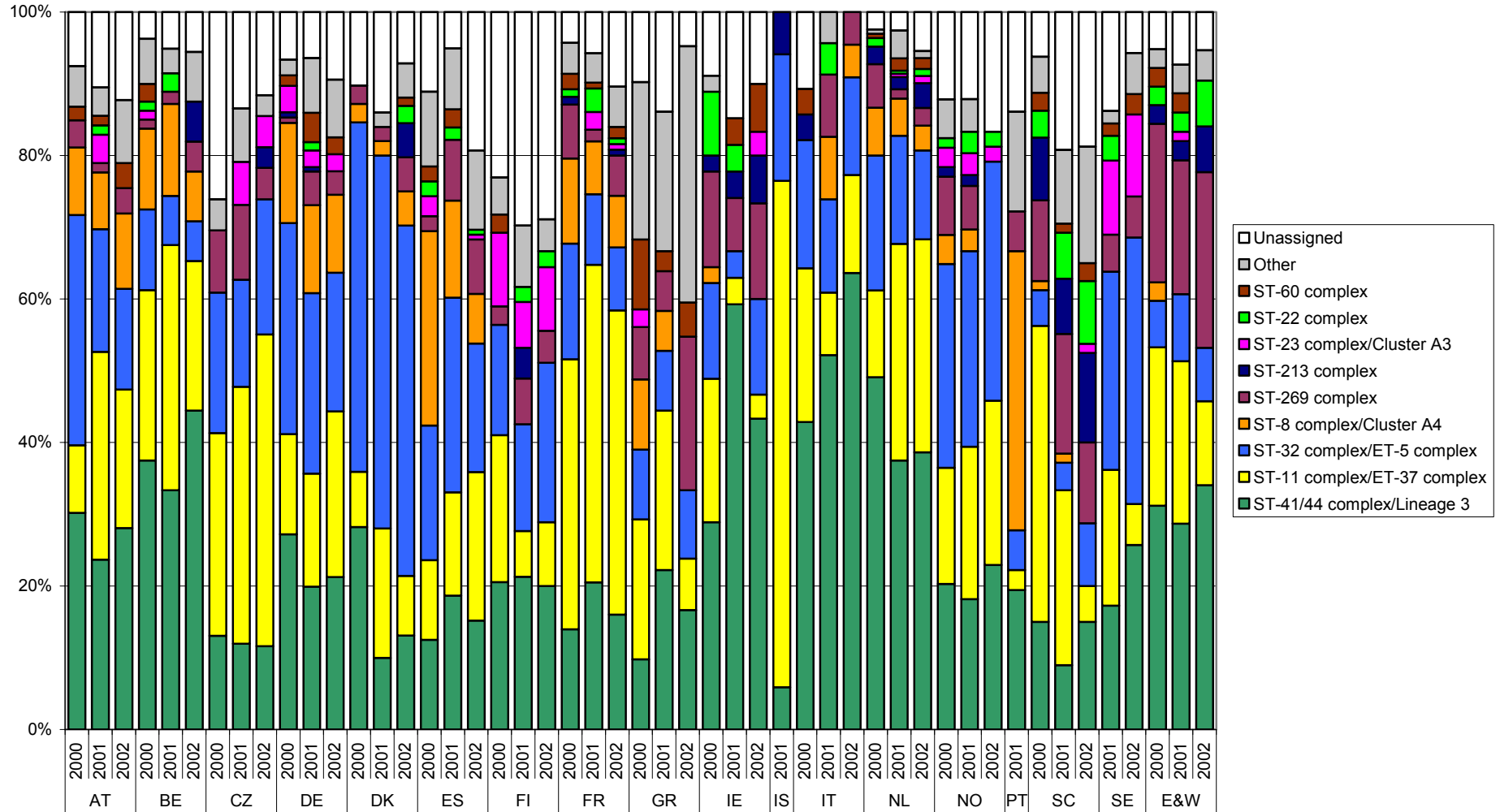
Infrastructure for the receipt of samples and distribution of reagents from Oxford was successfully established. Results from the first batches of submitted samples indicated that the initial protocol employed for the collection of samples was less robust than anticipated. Samples were originally boiled in water and shipped on dry ice to the EMMC, but it became apparent that if the samples were not boiled immediately after the addition of water, the cells would lyse and release nucleases, which would damage the DNA. The results were not consistent between centres, with some samples producing excellent results while others failed completely to PCR amplify. The substitution of PBS buffer for the water in the protocol improved the stability of the samples and yielded good results from the majority of those submitted. Additionally, the samples could be sent at room temperature in the normal post without suffering any noticeable degradation.

The bulk-buying power of the sequencing facility enables it to offer sequencing reagents to participating centres at a cheaper price than they could source them locally.

##### Typing of meningococcal disease isolates

The EMMC has complete allelic profiles for 3993 isolates from the target years of 2000-2002, significantly more than the 3000 isolates committed to at the outset of the project. The majority of these were sequenced within the EMMC, although some participating centres also submitted data. Additionally, PorA variable region sequencing has been performed on approximately 2600 isolates. Data from 18 European countries is represented. A total of 998

### Clonal complex distribution seen in participating countries



different sequence types (STs) have been identified. Despite this diversity, a small number of STs accounted for most of the isolates, ST-11 (20%), ST-32 (8%) and ST-41 (7%). Further, the isolates were resolved into 25 different clonal complexes, the main ones being ST-41/44 complex (Lineage 3) (25%), ST-11 (ET-37) complex (22%), ST-32 (ET-5) complex (18%), ST-8 complex (Cluster A4) (7%) and ST-269 complex (6%). These top five clonal complexes make up 78.0% of all the isolates. The distributions of clonal complexes found were broadly similar, although the prevalence of each was slightly different among countries (see figure). Differences included the absence of ST-8/Cluster A4 isolates and the higher prevalence of ST-269 complex isolates in the UK in comparison to other countries. Over the three years there were no major changes in the prevalence of clonal complexes across Europe and the main ones predominated each year.

### Database development

The requirements of this project necessitated the need to develop database software to hold the MLST sequencing results and to make these available to participating centres in a timely manner. The open-source software that is used to run many of the publicly available MLST databases, mlstdbNet, was improved to allow the following:

1. Improved data upload interfaces, so that results from the sequence assembly pipeline could be added to the online database as soon as it was generated with minimal manual data entry.
2. Restricted access of data depending on log-on credentials. This enabled participating centres to log on to the web database and view only their sequencing results. Coupled with the immediate upload of results, each centre was able to follow the progress of typing of their isolates.
3. A distributed structure so that isolate databases could be separate from the main profiles database yet still benefit from centralised assignment of sequence types and allele sequences. By linking to the main profiles database (hosted on <http://pubmlst.org>), sequence type and clonal complex assignments could be made automatically on completion of an allelic profile. Further, newly recognised assignments, for example of a new clonal complex, were automatically defined in the isolate database as soon as they were made.
4. Enhanced analysis tools so that complex breakdowns of multiple fields could be performed.

The improvements made to the software have been added to its code-base so that the new functionality is freely available, such as for the *Neisseria* PubMLST database (<http://pubmlst.org/neisseria/>).

### Linking of EMMC and EU-IBIS surveillance databases

The EMMC database contains only the information that is required by the MLST Centre to type the samples submitted. In order for detailed epidemiological analyses to be performed, the typing information needed to be integrated with the EU-IBIS surveillance data held at the CDSC in Colindale. Since both datasets were updated frequently, such links had to be between the live data, rather than simply importing one set of data in to the other database.

To facilitate such a link, a database mirroring system was established that allowed the EU-IBIS database server to make a secure connection to the EMMC database each evening, and synchronise a local copy of the EMMC database that resided on the EU-IBIS server. Links between the two datasets were made for queries by means of a lookup table that contained details of the id numbers as submitted to EU-IBIS and to the EMMC. The technical aspects were achieved relatively easily, but it was found that reference laboratories submitting different id numbers for their samples to the two centres without providing

reconciliation between the two hampered the linking. Cleaning of the data and cross-referencing the different id numbers of particular samples was performed at the CDSC and required the most effort.

## Outlook

This work package has successfully established a working infrastructure for the molecular characterisation of isolates and the integration of molecular typing with and epidemiological data. Together with the training provided to personnel from a wide range of European laboratories, this has generated a framework for the continued pan-European surveillance of meningococcal disease to a high level of precision yet at very modest cost.

The data generated provide uniquely valuable information on the meningococcus of great value in the design and implementation of public health measures, especially vaccination. The structure put in place for meningococcal disease in this programme of work is a paradigm applicable to other bacterial pathogens.

## Publications

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## Workpackage 2

### Non-culture detection and typing of meningococci

**Participants:** Andrew Fox, Muhamed-Kheir Taha, Ulrich Vogel

#### Collaborations with partners in other workpackages

Together with Julio Vazquez (WP6) methods for non-culture prediction of sensitivity to antibiotics were developed.

#### Objectives

- to provide accurate non-culture identification and typing of meningococcal strains using molecular approaches. Methods to be developed may then be applicable in all National Reference Laboratories (NRL). This would permit a unified and homogenous approach and the implementation of quality assessment schemes among NRLs;
- to improve surveillance for meningococcal disease by accurate identification of serogroups, serotypes and subtypes;
- to provide non-culture prediction of meningococcal clones associated with altered sensitivity to antibiotics (penicillin).

#### Results

##### Standardization of non-culture identification and genogrouping of *N. meningitidis* :

A Quality Assurance Study for nonculture confirmation of meningococcal infection using nucleic acid amplification was completed during 2003. A total of twenty samples of which eighteen were cerebrospinal fluid samples were distributed to twelve reference centres in eleven countries. The samples tested in this study were collected, boiled and centrifuged before being sent to the participating laboratories. For future studies it may be better to send frozen samples, although the bacterial DNA in boiled samples seemed to withstand transport at room temperature quite well. It was not essential to purify DNA from CSF unless a PCR inhibitory effect is observed.

The participating laboratories were requested to perform their standard non-culture approach. All participating laboratories first carried out a meningococcal 'screening' PCR. Each laboratory performed its in-house polymerase chain reaction assays covering a range of meningococcal gene targets (*crgA*, *ctrA*, 16S rDNA and *porA*). Genogrouping was subsequently performed using oligonucleotides designed to amplify the *siaD* and *mynB/sacC* genes. The participating laboratories also performed genogrouping assays on the sample (*siaD* and *mynB/sacC*). The reference laboratories compared reasonably well for the nonculture detection of meningococcal infection by nucleic acid amplification versus conventional diagnostic methods with mean sensitivity and specificity of 90% and 93% respectively. However, the performance of genogrouping was more variable between laboratories with mean sensitivity and specificity of 75% and 95%, respectively.

This study highlights the reliability of molecular non-culture diagnosis of *N. meningitidis*. It became evident, that the various gene targets used for meningococcal non-culture detection were equivalent in sensitivity and specificity for the confirmation of meningococcal infection. Although genogrouping for serogroup B gave the best correlation between participants the performance of genogrouping was variable between laboratories and in particular for serogroups A and W135. Further efforts are needed to harmonize protocols in Europe.

### Development of protocols for non-culture typing by MLST of *N. meningitidis* in clinical samples

Protocols for non-culture strain characterisation of meningococci direct from clinical specimens were developed. Initially, alternative primer sets were developed for each of the MLST loci to increase the sensitivity of the locus specific PCRs for direct amplification from cerebrospinal fluid (CSF), whole blood (EDTA) and serum. Alternative primer sets and amplification strategies including Touch Down PCR were unable to achieve the required sensitivity for many specimens from non-culture confirmed meningococcal infections. A nested approach was therefore adopted using nested primer sets for each housekeeping gene locus. The nested protocol was evaluated on clinical specimens including whole blood and CSF from culture proven cases for which corresponding isolates were available and which contained varying numbers of meningococcal genome copies (as determined by semi-quantitative real time PCR assays). The nested MLST protocol was found to provide accurate MLST sequence typing direct from clinical specimens with a sensitivity of 10-100 genome copies per PCR reaction. This protocol was used to obtain MLST data in a number of case cluster/outbreak investigations and has been presented as a European non-culture MLST protocol. A nested PCR-protocol was also developed for the meningococcal variable regions VR1 and VR2 of the *porA* gene to provide sequence typing data direct from clinical specimens for this important antigen gene encoding meningococcal subtype variations.

Novel methods were developed based upon an analysis of the MLST data and development of single nucleotide based polymorphism (SNP) assays for the detection of the ST-11 clonal complex using real time PCR assays. These novel assays involved the development of primer and probe combinations which detect clonal complex specific alleles. The use of a combination of allelic discrimination probe primer sets provides high specificity for the ST-11 clonal complex. Real time PCR on both the Roche lightcycler and ABI TaqMan systems provides exquisite sensitivity and rapid identification of both isolates and non-culture confirmed cases due to organisms belonging to the hypervirulent ST-11 clonal complex. The availability of the assays on multiple real time PCR platforms provides high flexibility. The assays have also been applied for cost effective large scale screening of clinical samples from non-culture confirmed cases to provide essential information for enhanced surveillance for infections due to ST-11 complex following the introduction of the Men C conjugate vaccine into the United Kingdom.

### Non-culture prediction of meningococcal clones associated with altered sensitivity to antibiotics (penicillin).

A study for non-culture detection of susceptibility of *N. meningitidis* to penicillin G was completed in three member laboratories of the European Monitoring Group on Meningococci (EMGM). A panel of thirteen clinical samples (cerebrospinal fluids) corresponding to 13 culture-confirmed cases of invasive meningococcal infection was distributed to the three laboratories as well as the corresponding bacterial isolates. Minimal inhibitory concentrations (MIC) of penicillin G were determined for the isolates. Each laboratory used an "in house" PCR-based method to determine alterations of *penA* gene, known to be associated with the reduced susceptibility to penicillin G. Nucleotide sequences of the 3' end of *penA* gene were also determined. A good correlation was also observed between genotyping of *penA* and the phenotypic determination (MIC) of susceptibility to penicillin G. The results obtained on *penA* in the samples, using the three methods, correlated very well with results for the bacterial isolates as well as with sequence data. These data suggest that genotyping may be used to predict susceptibility of *N. meningitidis* to penicillin G. These data strongly argue for the use of genotyping of *penA* as a tool to determine meningococcal susceptibility to penicillin G in culture negative cases. While nucleotide sequencing of *penA* may be the gold standard in genotyping of *penA*, the less expensive PCR-based approach reported in this study may be more rapid to test a large number of isolates and clinical samples.

### Monoclonal antibodies against *NmeDI* specific to ST-11 and ST-8 meningococci

We have developed a monoclonal antibody against the endonuclease *NmeDI* specific to ST-8 and ST-11 meningococci. The antibody reacted with the antigen in Western blots and dot blots. The antibody was evaluated using a large variety of meningococcal isolates from invasive meningococcal disease as well as from healthy carriers. Furthermore, the specificity was assessed by using strains from a variety of bacterial species. The antibody provides a useful tool for the rapid identification of meningococci of the ST-11 and ST-8 complexes. There will be a potential for direct culture-independent detection of the *NmeDI* antigen in clinical samples (e.g. in carriage studies). However, further technical issues will have to be solved before this feature is becoming available. The antibody has been published and is available upon request (Claus et al. 2003).

### Improvement of QA (quality assurance) schemes

We developed a model for integrated QA schemes that is based on the use of a collection of characterized clinical samples.

- Several “in house” methods (first-time validated) can be compared using this collection of samples and a gold standard which is the modal (consensus) results.
- Diagnostic accuracy/correlation can be analyzed by Kappa coefficient.
- Finally, standardization of protocols can be performed (finalise primers and, reaction conditions).

### **Outlook**

The EMGM (European Monitoring Group on Meningococci) may host a collection of well-characterized samples that can be distributed and used by laboratories for first-time validation and internal QA of non-culture diagnosis and typing. As guidelines for primers, gene targets, protocols for PCR-based diagnosis and typing are now available, the organization of regular (once a year) EQA study with laboratories of the EMGM becomes available in the future and national EQA studies may be organized by the local members of the EMGM in each country.

### **Publications**

Antignac, A., I.G. Boneca, J.C. Rousselle, A. Namane, J.P. Carlier, J.A. Vazquez, A. Fox, J.M. Alonso, M.K. Taha. 2003. Correlation between alterations of the penicillin-binding protein 2 and modifications of the peptidoglycan structure in *Neisseria meningitidis* with reduced susceptibility to penicillin G. *J. Biol. Chem.* 278: 31529-35.

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Taha, M.K., J.M. Alonso, M. Cafferkey, D.A. Caugant, S.C. Clarke, M.A. Diggle, A. Fox, M. Frosch *et al.* 2005. Interlaboratory comparison of PCR-based identification and genogrouping of *Neisseria meningitidis*. *J. Clin. Microbiol.* 43: 144-9.

## Workpackage 3

### Implementation of the microarray technology for whole genome comparisons and isolate characterisation

**Participants:** Ulrich Vogel, Matthias Frosch

**Collaborations with partners in other workpackages:** Dominique Caugant, Martin Maiden

#### Objectives

Implementation the microarray technology

- to study differentially expressed genes;
- to perform genome-wide comparison of genes present in hypervirulent, hyperinvasive and apathogenic meningococci;
- for the typing of meningococci.

#### Results

##### Use of microarray for multilocus sequence typing of meningococci

Multilocus sequence typing (MLST) has become the gold standard for typing of a variety of bacterial and fungal micro-organisms. At the beginning of the activities of the consortium, however, there was a great deal of discussions about the costs and workload implied by the use of MLST. The advent of microarray technology combined with its successful use especially for transcriptome analysis raised the question whether this technology might be useful for multilocus sequence typing.

Microarray technology has been adapted to single nucleotide polymorphism analysis. However, the so-called tiling technology requires an excessive number of oligonucleotides. The database at the EMMC provides a comprehensive compilation of MLST sequences. Furthermore, there it is a consensus that the database covers almost all polymorphisms present in nature. Therefore, we developed microarrays based on polymorphism-directed oligonucleotide design for typing of *Neisseria meningitidis*. The rationale behind this approach was to minimize the number of microarray probes by exploiting the comprehensive knowledge of polymorphisms combined in the *Neisseria* MLST website. As a first step, we performed several experiments to define the technical features of the microarrays to be developed. These experiments using model oligonucleotides of 28-32 base-pairs in length lead to hybridisation protocols which were highly specific. Single point mutations could be elucidated unless they were located at the termini of the oligonucleotide probes. The oligonucleotide length seemed to be the most important parameter, but also the GC content affected the hybridisation intensities. 50 to 140 oligonucleotides were designed for each of three loci selected for further validation of the protocols. However, despite of several optimisation steps in oligonucleotide design, the rate of misidentification of oligonucleotides remained >1.8% even in consecutive validation experiments using arrays representing the genetic diversity at three MLST loci. We assumed that the high density of polymorphic sites and the extensive GC-content variations at *N. meningitidis* MLST loci hindered the successful implementation of MLST microarrays based on polymorphism-directed oligonucleotide design.

The question is whether the tiling technology might have been more successful than our approach. The tiling technology was shown to work for MLST of *S. aureus*, as was published during the course of our work. However, *S. aureus* clearly exhibits less GC content variation

and a smaller number of polymorphic sites. Therefore, we would not be optimistic with regard to the application of tiling technology to the meningococcal MLST. Furthermore, the question of target amplification from meningococcal strains should be addressed. Multiplex PCR is possible but might result in a DNA yield of different concentrations with regard to the different loci. The DNA concentration has to be adjusted prior to the labelling process. Finally, our own experiences and calculations suggest that standard capillary sequencing is cheaper and quicker than the microarray approach. Since capillary sequencing is highly accurate, highly standardized, portable, increasingly available to many laboratories, and becoming even cheaper with time, it is our appraisal that microarray technology does not represent an alternative to capillary sequencing with regard to meningococcal MLST.

#### Use of microarray for comparison of meningococcal strains

A large number of cases due to serogroup C meningococci are caused by a clonal lineage designated the sequence type (ST)-11 complex. A novel derivative of the ST-11 complex emerged in the nineteen-eighties and was defined as the electrophoretic type (ET)-15 clone. The clone attracted public health interest in many countries because its appearance was frequently associated with rises in the incidence of invasive meningococcal disease. A single nucleotide polymorphism for the identification of ET-15 meningococci has previously been described by us and allows for a presumptive identification of ET-15 meningococci without multilocus enzyme electrophoresis. However, single nucleotide polymorphisms might not be stable, but rather temporary traits blurred by horizontal gene transfer during ongoing microevolution. Therefore, we screened a collection of ST-11 complex strains composed of ET-15 and non ET-15 strains for ET-15 specific DNA-fragments by DNA/DNA-hybridisation using microarrays purchased from Eurogentec. A highly effective and simple algorithm for binary assignment of the presence or absence of genes was developed which was applicable to this specific subset of strains. The algorithm relied on our knowledge of genes present in serogroup B and serogroup A meningococci, respectively, but absent from ST-11 meningococci. Highly reproducible results were obtained, which were confirmed by PCR analysis for selected cases.

The hybridisation analysis confirmed the clonal nature of the ET-15 clone which exhibited a smaller degree of heterogeneity than other strains of the ST-11 complex. Four chromosomal loci were significantly associated with the ET-15 clone, but were lacking in other ST-11 complex strains. Those four loci now enrich our repertoire of specific traits of the ET-15 clone, which can be utilized for unambiguous strain identification (Claus et al., manuscript in preparation). Furthermore, in a related project we have developed fingerprinting assays using probes derived from one of the specific traits, which can be utilized for fine typing of ET-15 meningococci, e.g. in cluster analysis (Elias et al., manuscript in preparation).

The data suggest that a variety of genetic changes were required before the successful emergence of a new distinct meningococcal clone became evident. The alterations had been fixed during worldwide epidemic spread. The selective benefit of the genetic changes is unknown. None of the changes identified until now had an obvious bearing on the expression or function of well-known virulence factors. Also transcriptome analysis of the same set of strains did not provide us with testable hypotheses. Therefore, it remains obscure whether there is a molecular basis for the assumed hypervirulence of ET-15 meningococci.

#### **Outlook**

MLST will remain the method of choice for unambiguous typing of meningococci. The strategy followed by the EU-MenNet to establish the EMMC proved to be highly successful. The use of microarray analysis should not be considered as an alternative approach. It might rather compromise the now achieved harmonisation of Europe-wide data. ET-15 specific traits will be introduced for identification procedures by reference laboratories in order to achieve reliable and comparable results in epidemiological investigations. Comprehensive

microarrays are now available for transcriptome analysis of genetically diverse meningococcal isolates.

### **Publications**

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## Workpackage 4

### Establishment of a European Meningococcal Epidemiology Centre

**Participants:** Mary Ramsay

This project built on an existing infrastructure funded by DG-SANCO – the European Union Invasive Bacterial Infection Surveillance (EU-IBIS; HT 2003 790 917 and 2001 CVG4-015). Participants include epidemiologists from the national surveillance centres and microbiologists from the national reference laboratories in all EU countries ([http://www.euibis.org/meningo/euibis\\_partners\\_meningo.htm](http://www.euibis.org/meningo/euibis_partners_meningo.htm)). The project aimed to establish a focus for further epidemiological work in the area of meningococcal disease.

#### Collaborations with partners in other workpackages

In collaboration with WP1, the European MLST centre, a web-enabled database has been created including linked epidemiological and MLST information for cases from several EU countries. This database is available to participants for running simple web-based queries.

#### Objectives

- To create a European Meningococcal Epidemiology Centre;
- To analyse surveillance data and other epidemiological information;
- To validate models of the transmission dynamics of *Neisseria meningitidis* in the UK and adapt for use in other European countries;
- To predict the impact of different vaccination strategies on meningococcal carriage and disease in different European countries.

#### Results

##### Development of a website and electronic submission capacity

Rather than creating a new 'European Meningococcal Epidemiology Centre' website, a decision was made to upgrade the existing EU-IBIS website ([www.euibis.org](http://www.euibis.org)) for three reasons. First, we wanted to ensure consistency and felt that upgrading a site that was already known to and used by collaborating partners was preferable to launching a new site. Second, the funds were not provided to purchase a dedicated server to host the website, as set out in the original grant proposal. Third, since the time of the EU-MenNet grant proposal EU-IBIS has secured additional funding (until October 2006) which will help to ensure that the website is sustained and continues to be serviced and upgraded. We have described the role of EU-MenNet on the website (<http://www.euibis.org/eumennet.htm>).

We enlisted the expertise of the Bioinformatics Unit at the Health Protection Agency, who have the appropriate knowledge and skills to develop the website. The content of the website has been revised to provide more information and the website is now more 'user-friendly' with drop down menus and clear mapping. (<http://www.euibis.org/index.htm>)

Users are able to view the meningococcal disease database online and generate tables and charts ([http://www.euibis.org/meningo/meningo\\_statistics.htm](http://www.euibis.org/meningo/meningo_statistics.htm)). Access to the online database is username and password restricted via a log-in system (<http://www.euibis.org/members/login.htm>). It was agreed at an EU-MenNet / EU-IBIS management meeting that each participating country could have access to all data that had previously been published, but that each participant would only be allowed to view and download unpublished data from their own country. On the participants only site a rapid

reporting system for B2a and B2b strains has been established. Participants are able to submit this data on-line and to view charts of this data. A bulletin board for the rapid dissemination of information on meningococcal infection has been established.

### Epidemiological Analysis

We have conducted three major epidemiological analyses under the auspices of EU-MenNet. First, we reviewed the ascertainment of meningococcal disease in Europe, through a review of published and unpublished reports. Second, we analysed the associations between death from meningococcal disease and host and strain characteristics, including clonal complex (as ascertained in work package 1). Third, we compared the effectiveness of the routine infant MCC vaccine schedules in England and Spain.

### Ascertainment of meningococcal disease

Some of the variation in the incidence of meningococcal disease across Europe is likely to be due to differences in case ascertainment. It is important to try to quantify the extent of under-ascertainment particularly if international comparisons are to be valid. A review of the published and unpublished literature on the reporting and ascertainment of meningococcal disease in Europe was conducted in collaboration with European colleagues. In addition, we also reviewed the methods that may be used to conduct investigations into the degree of under-reporting or under-ascertainment in Europe. We intend to submit this manuscript to a peer-reviewed journal for publication.

### Factors associated with death from meningococcal disease in Europe, 1999-2002

Previous studies in Europe have shown that the outcome of meningococcal disease is associated with the phenotype of the infecting organism, in addition to clinical presentation (septicaemia having a higher fatality ratio than meningitis) and age (increasing mortality with increasing age). In recent years, several countries have experienced increases in serogroup C disease, with serotype 2a in particular being associated with a higher risk of death. DNA sequencing techniques, particularly multi-locus sequence typing (MLST) are increasingly used to characterise *N. meningitidis* isolates. The sequence type also appears to be an indicator of virulence, and the hypervirulent C2a strains are associated with sequence type 11 (ST-11). The sequence types can be further grouped phylogenetically into clonal complexes. There are considerable geographical and temporal variations in the epidemiology of meningococcal disease in Europe, only some of which is likely to be explained by differential reporting and case ascertainment. A Europe-wide analysis of case fatality enables us to examine whether the associations between phenotype or genotype (in collaboration with colleagues in work package 1) and fatal outcome are geographically consistent, which would be expected if these are truly markers of hypervirulence.

### Comparison of MCC vaccine effectiveness in Spain and England

In 1999 the UK became the first country to introduce the serogroup C meningococcal conjugate (MCC) vaccines in response to the increasing incidence of serogroup C disease from the mid-1990s onwards. The vaccine programme has successfully reduced the number of cases and deaths caused by serogroup C. MCC vaccines have subsequently been introduced in Spain, The Netherlands, Belgium, and Iceland. ([http://www.euibis.org/meningo/vacc\\_sched\\_meningo.htm](http://www.euibis.org/meningo/vacc_sched_meningo.htm)) The most common cause of disease in Europe is serogroup B, but to date there are no broadly effective serogroup B vaccines available.

The UK incorporated the MCC vaccines into the routine infant immunisation schedule and initiated a catch-up campaign targeting all children under the age of 18 years old at the end

of 1999. The success of the UK campaign was important in persuading other countries to introduce the MCC vaccines. Since the UK was the first country to introduce the vaccines, it has the longest time period available for follow-up and because of the large number of children and young adults vaccinated there is sufficient power to objectively evaluate the vaccines. Spain introduced the MCC vaccines in 2000, offering children under one year three doses of vaccine at 2, 4 and 6 months of age, in contrast to the UK's accelerated schedule at 2, 3 and 4 months of age. They also instigated a catch-up campaign for all children aged less than 6 years old.

Vaccine evaluation studies in England from mid-2003 onwards suggested that although the short-term effectiveness of the routine infant immunisation programme was high, it appeared to decline quickly. This information was shared with European colleagues informally through the surveillance network and was subsequently published in July 2004, using cases up to March 2004, by which time enough data had been collected to ensure that this finding was not a chance effect. The fall in efficacy to low levels in infants after only one year, despite evidence from clinical trials of immunological memory, raised questions regarding the appropriateness of the accelerated schedule, the importance of age at vaccination and the role of immunological memory in providing long-term protection.

Spanish epidemiologists contacted the HPA in May 2004 to compare their estimates of vaccine effectiveness in infants. The comparison was important, as Spain had used a slightly different schedule, with more time between each dose (2 months *versus* 1 month) and with infants receiving the last dose at an older age (6 months *versus* 4 months). We standardised the methods for assessing vaccine effectiveness, the results of which are presented in the table below.

#### MCC vaccine effectiveness following routine infant immunisation in England and in Spain

Cohort	Age at vaccination	Doses scheduled <sup>1</sup>	Period of observation	Cases (vaccinated)	Vaccine effectiveness (95% confidence intervals)		
					Overall	Within 1 year of scheduled	More than 1 year after scheduled
Spain	2, 4, 6 months	3	Q1 2001 - Q2 2004	36 (15)	95 (89,97)%	98 (94, 100)%	83 (39, 95)%
UK	2, 3, 4 months	3	Q1 2000 - Q1 2004	28 (21)	65% (4,86)%	93% (66,99)%	-84% (-75,62,71)%

<sup>1</sup>Vaccine effectiveness compares children eligible for complete vaccination who had received all scheduled doses versus 0 doses. Partially vaccinated children were excluded

The short-term effectiveness (within one year) is high (above 90%) in both countries. After one year the effectiveness declines significantly in both countries, to -84% in England in years 1-4 after vaccination, and to 83% in Spain in years 1-3.5 after vaccination. Although vaccine effectiveness is slightly higher in Spain (p=0.02), the decline in effectiveness over 1 year does not significantly differ from the decline seen in England (p=0.37). The reason for the higher effectiveness observed for more than 1 year after scheduled vaccination is because the effectiveness starts from a higher level. We can't assume the decline will be the

same as for us but as yet there is no evidence it differs. This work has been presented to the medicines regulatory authorities in the UK and Spain.

### Mathematical modelling studies

Mathematical models have been used to describe the transmission dynamics of a range of infectious organisms and diseases, where they can be applied to help understand the observed patterns of disease and to predict the potential impact of control programmes, such as vaccination. A range of different vaccine schedules and policy options can be explored, through model simulations. These models can be very useful tools, although their limitations - that they are by definition simplifications of the real world and depend upon the quality of the model assumptions and inputs - must also be recognised.

A realistic age-structured transmission dynamic model of meningococcal carriage and disease was developed in order to understand and predict the impact of serogroup C vaccination. The main model was developed and validated using data from England and Wales because, as outlined above, the follow-up time is longest, and more parameters (including, crucially, the impact of the MCC campaign on carriage prevalence) have been estimated here.

In order to validate the model, we compared the predicted number of cases to the observed number of cases in the UK each year from 1998/99 (pre-vaccine) to 2003/04. The model predicted the number and pattern of cases well, and in addition predicted that the prevalence of carriage would decline by 71% in the year following vaccination, which is close to that found by Maiden et al (66%, 95% CI = 28, 85%).

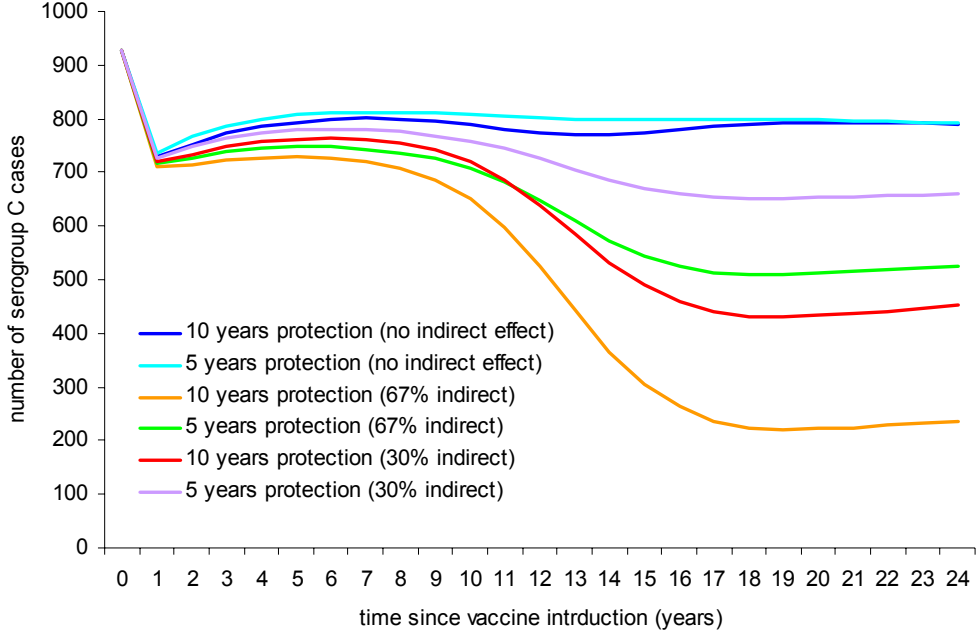
In the next stage we used the model to predict the impact of several different vaccination strategies, including routine infant vaccination without a catch-up, routine infant and routine teenage vaccination, and routine vaccination with a catch-up campaign. We assume that vaccinated individuals are protected (to a degree, in this case 67%) against acquiring serogroup C carriage. Vaccinating a large proportion of teenagers (in whom carriage prevalence is highest) in a one-off catch-up campaign therefore results in substantial herd immunity because there are much fewer susceptibles to maintain transmission. The model predicts that after such a large 'shock' the prevalence of serogroup C carriage will fall to very low levels and that the herd effects will persist for many years.

### *Modelling MCC vaccination in other countries*

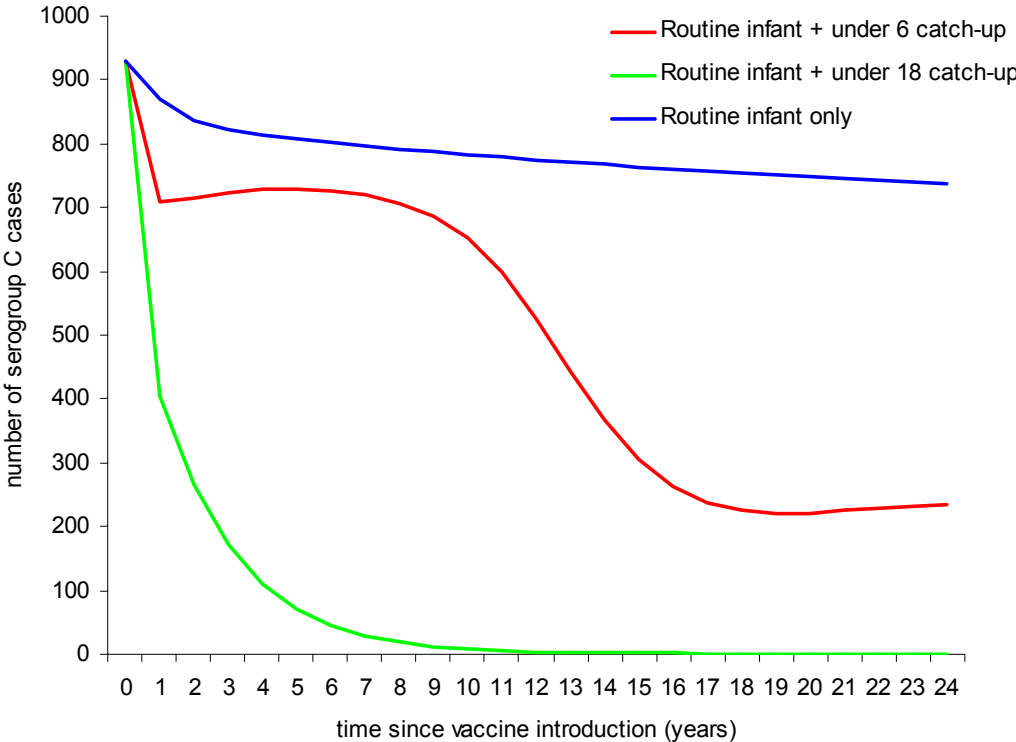
The insights from the UK model are qualitatively generalisable and can be used to investigate the impact of a range of vaccine schedules, which may be considered in other countries. The UK had experienced large herd immunity effects from the MCC campaign, but the herd immunity measured in Spain was much less. The mathematical models (described in the preceding section) were used to predict the herd immunity expected from an under 18 catch-up campaign (UK strategy) and a under 6 catchup campaign (Spain). The results of this comparison are shown in figure 1 below. Using the UK model to simulate the effect of the 'Spanish strategy' (i.e. routine infant vaccination at 2, 4 and 6 months of age with a catch-up campaign for all children under 6 years old) reveals that there will be little additional indirect protection as there will be little reduction in carriage prevalence (since 0-5 year olds rarely carry meningococci). It is clear that the UK campaign, which target a larger number of children, is more effective in reducing disease incidence. Furthermore, the models also show that by targeting teenagers, who are the most common carriers of meningococci, herd immunity is much greater than if only younger age groups are targeted.

From this model (base case scenario), we estimated that the Spanish strategy would lead to a 14% reduction in the number of cases in 1-4 year olds through herd immunity 3 years after the start of the vaccine campaign. This is slightly lower than the reduction of 35.14% (95% CI 15.4 to 86.6) found in unvaccinated children aged 1-5. As shown in figure 2 below, the model

**Figure 1:** Sensitivity of the model to assumptions regarding duration of protection conferred by the vaccines, and the degree of protection against carriage (Spanish strategy)



**Figure 2:** Comparison of UK (routine +under 18 catch-up) and Spanish vaccine strategies (routine + under 6 catch-up) using a mathematical model parameterised to UK data.



predictions are sensitive to assumptions regarding the duration of protection conferred by the vaccines, and the degree of protection against carriage. Our estimates for Spain are likely to be improved if we re-parameterise and re-calibrate the model using Spanish carriage and disease data.

Three ways of re-calibrating the model to make quantitative predictions for other countries have been investigated, and may be applied as appropriate and according to the available data. In all cases, the model inputs need adjusting, but the same model structure can be used, so little re-programming is needed. First (and ideally) the models can be re-calibrated using carriage prevalence and disease incidence data from country X. The forces of infection for serogroup C and 'other' infections can be re-estimated as can the risk of disease given infection. However, carriage studies are expensive and difficult to conduct, and as a result are conducted rarely. Very few European countries (Spain and the Czech Republic being the possible exceptions) have sufficient carriage data across a broad age range to allow the models to be individually parameterised for each country. Methods 2 and 3 therefore attempt to re-calibrate the models using just the age-specific disease data. One can assume either that the transmission of *N. meningitidis* is the same as the UK and adjust the risk of disease given infection, or that the risk of disease given infection is the same as the UK and transmission changes. Method 3 is probably more realistic, although the limitations of both must be recognised.

The models are available to be used at the request of any collaborating European centre.

## Outlook

The resource established under Eu-MenNet will continue to be of value to the EU-IBIS project, which is funded until October 2006. Further development of the Hib component of the EU-IBIS project is planned, building on the work established under Eu-MenNet. Outputs from the other work packages will be shared with EU-IBIS participants through the new website. As the use of MLST, and other sequence typing, in national reference laboratories becomes more common, EU-IBIS is hoping to modify the reportable data to incorporate this. Further analyses will then be enabled. Long term monitoring of the effectiveness of MCC vaccine in Europe will be continued to identify differences between strategies chosen in different EU countries.

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Trotter, C.L., Gay, N.J., Edmunds, W.J. Dynamic models of meningococcal carriage, disease and the impact of serogroup C conjugate vaccination. Submitted for publication.

## Workpackage 5

### Studies on the carriage of meningococci in Europe

**Participants:** Dominique A. Caugant, Paula Kriz, Georgina Tzanakaki

#### Collaborations with partners in other workpackages

Invasive meningococcal strains from Greece and Norway for 2000-2002 were analysed by MLST in Oslo. Strains isolated from patients in the Czech Republic in 2000-2002 were investigated by MLST partly in Oxford and partly in Prague. Data were forwarded to the European Meningococcal MLST Centre (WP 1).

Strains were provided to WP 3 for the whole genome comparisons.

Data were provided data to the European Meningococcal Epidemiology Centre (WP 4).

Penicillin-insensitive strains from the Czech Republic, Greece and Norway were sent to the partner in WP 6 for further characterisation.

#### Objectives

- Identification of meningococcal lineages circulating in the carrier state throughout Europe;
- Measurement of carriage of multiple lineages by individuals;
- Establishment of the duration of carriage for given lineages;
- Creation of a carrier strain collection centre.

#### Results

##### Harmonisation of isolate characterization from previous studies

To better understand the relation between carrier and disease causing strains, we performed a comparative study of the phenotypes and genotypes of patients and carriers in three European countries, the Czech Republic, Greece, and Norway, representing the Northern, Southern, and Eastern parts of Europe, using 667 strains collected in a 10-year period. All strains were analysed by phenotypic methods and MLST. A total of 314 patient isolates and 353 isolates from asymptomatic carriers were characterized. The frequency distribution of serogroups and clone complexes differed among countries and between disease and carrier isolate collections. While all but one patient strains were serogroupable, between 30 and 45% of the carrier isolates were non-serogroupable. Serogroup B strains predominated in both patients and carriers: about 30% in each country among carriers, and from 30 to 75% among patients. The serogroup distribution among carrier isolates was similar in the 3 countries, but marked differences were observed in the serogroup distribution among patient isolates.

While 20% of the disease isolates had sequence types not assigned to a known clonal complex, over one third of the carrier isolates fell into that not assigned category. Overall, the same clonal complexes were represented both among the carrier and the patient strains, but their distribution was not uniform among the 3 countries. A new hypervirulent clonal complex (ST-162 complex) was identified. A marked positive association of serogroup C with disease was evidenced. The association was 14 times stronger for serogroup C than for serogroup B. The ST-11 complex was strongly positively associated with disease, while the ST-23 complex was positively associated with carriage; associations for other clonal complexes were weaker. The genetic diversity of the clonal complexes differed. A single ST dominated

the ST-11 complex, while the ST-41/44 complex exhibited greater levels of diversity. These data robustly demonstrated differences in the distribution of meningococcal genotypes in disease and carrier isolates and among countries. Furthermore, they provided further evidence that differences in genotype diversity and pathogenicity exist between meningococcal clone complexes.

#### Development of a new PCR-based method for rapid discrimination of meningococcal clones

Repetitive DNA motifs with potential variable-number tandem repeats (VNTR) were identified in the genome of *N. meningitidis* and used to develop a new typing method. A total of 146 meningococcal isolates recovered from carriers and patients were studied. These included 82 of the 107 *N. meningitidis* isolates previously used in the development of MLST, 45 isolates recovered from different counties in Norway in connection with local outbreaks, and 19 serogroup W135 isolates of sequence type 11 (ST-11), which were recovered in several parts of the world. The latter group comprised isolates related to the Hajj outbreak of the year 2000 and isolates recovered from outbreaks in Burkina Faso in 2001 and 2002. All isolates had been characterized previously by MLST or multilocus enzyme electrophoresis (MLEE).

VNTR analysis showed that meningococcal isolates with similar MLST or MLEE types recovered from epidemiologically linked cases in a defined geographical area often presented similar VNTR patterns while isolates of the same MLST or MLEE types without an obvious epidemiological link showed variable VNTR patterns. Thus, VNTR analysis was found to be useful for fine typing of meningococcal isolates after MLST or MLEE typing. The method might be especially valuable for differentiating among ST-11 strains, as shown by the VNTR analyses of serogroup W135 ST-11 meningococcal isolates recovered since the mid-1990s. In particular, an intact IS1016 was found in the *pglE* gene of the Hajj 2000-related W135 strains which may have inactivated pilin glycosylation. This allowed to distinguish them from the W135 ST-11 isolates recovered during the epidemics in Burkina Faso in 2001 and 2002.

The method was then applied to the analysis of 31 strains of the ST-162 complex (identified in our first study) recovered from patients and healthy carriers in Greece in 1999 and 2002. VNTR-analysis demonstrated the heterogeneity of this newly identified clonal group. The method was also applied for characterization of a local outbreak occurring in a day-care center in the Athens area and to determine the spread of the outbreak strain among carriers. Although all strains isolated from patients and carriers exhibited the same phenotypic (B:4:P1.14) and genotypic (ST-162) characteristics, VNTR permitted to distinguish the particular variant causing the local outbreak. A simplified VNTR method was further developed for rapid identification of multiple carriage in individual carriers (see below).

#### New carriage studies

##### *Measurement of multiple carriage in individuals/Establishment of the duration of carriage for defined meningococcal lineages*

The high degree of genetic diversity of meningococcal populations has been attributed to its capacity to readily generate new genotypes through recombination. Horizontal genetic exchange between meningococcal strains must then occur during the carriage stage in the throat of healthy individuals colonized by multiple clones. Although numerous carrier studies have been performed, usually a single meningococcal colony from each sample is analysed and it is unknown how frequent carriage of multiple strains is. In an attempt to address that question, multiple colonies were isolated and characterized from consecutive throat samples of healthy carriers in the Czech Republic, Greece, and Norway.

Nasopharyngeal swabs were obtained monthly for 5-6 months from the same individuals. Cultivation of the sample was performed on selective media and usually up to 20 meningococcal colonies per throat sample were picked for storage and further investigation.

The genetic homogeneity of the colonies in each sample was screened by randomly amplified polymorphic DNA (RAPD) or by the VNTR method. Individual clones from each carrier were also characterized by serogrouping, serotyping and antibiotic susceptibility testing.

In Norway a carriage study in military recruits (n=126) was performed between February and July 2003. A total of 78 carriers (carriage rate = 61.9%) were identified. A total of 5902 individual colonies were isolated and preserved; 1897 colonies from 104 samples (19 carriers) were tested by VNTR. More than one clone was detected only in three samples (2 carriers) and confirmed by MLST. Overall 69% of the participants were stable carriers throughout the study period. The ST-23 complex predominated among the carried strains.

In the Czech Republic a carriage study was performed in high school students (n=206) between October 2002 and March 2003. All 6 samples were obtained from 190 (92%) of the volunteers. Thirty-three (16.0%) carriers were identified. A total of 1242 colonies were isolated and tested by RAPD. A total of 22 RAPD patterns were distinguished. RAPD variation within individual samples was detected in two samples from two different individuals and the presence of distinct clones was confirmed by MLST. The ST-22 complex and the ST-41/44 complex predominated among the carried strains.

In Greece two carriage studies were performed in university students (n=202) aged 18-25 years old, between February and June 2002 (n=102) and between February and June 2003 (n=100), respectively. Overall 37 (18%) carriers were identified and 43% of the carriers were stable carrier during the study period. About 2000 colonies were isolated and tested by RAPD and phenotypic methods (serogroup, serotype/ subtype). Minor changes with one of the 2 primers used were detected within individual samples of three carriers. None of these samples harboured more than one clone as evidenced by MLST. The ST-162 complex predominated among the carried strains.

These studies demonstrated that the commensal association of particular clones with a host is a long term relationship, with 45 to 70% of the carriers being persistent carriers and 90% of the carriers keeping the same clone for at least 5-6 months. Multiple carriage was rarely detected: under our experimental conditions, the large majority of the samples contained only a single meningococcal strain. However, a few cases of multiple carriage was detected, indicating that colonisation by multiple clones may result in the generation of novel genotypes that can be selected within the human host.

#### Definition of hyperinvasive and avirulent lineages

These carriage studies have improved our understanding of the population structure of the meningococci, providing evidence that meningococcal populations from healthy carriers are composed of a number of successful carrier clones that can be geographically widespread.

Furthermore, it was confirmed that hypervirulent ST-complexes are rare among strains from carriers. Variation in carriage rate of individual hypervirulent clone-complexes does not appear to be clearly related to the variation in incidence of disease caused by these clonal complexes. Thus, carriage studies using clonal analyses might not be useful to predict changes in the epidemiology of meningococcal disease.

Hypervirulent clones vary in their capability to establish a commensal relationship with their host, with especially the ST-11 complex being a poor colonizer.

#### Assembly of defined meningococcal collection

Strains from the new carriage studies (including multiple and repeated isolates) are stored in the laboratories of their respective origin and will be available upon request.

## Outlook

Further investigations of the following aspects are warranted:

- Because our studies showed that most carriers were stable carriers for a period of 5-6 months, it is clear that to determine the duration of carriage and how it varies for specific clones it will be necessary to follow individuals for much longer periods.
- There is a need to better assess the relation between carried and disease-causing clones and accurately determine the relative virulence potential of “hypervirulent” sequence types and clonal complexes.
- The geographical component of the diversity of carriage strains should be further ascertained. This might be especially important in relation to the introduction of meningococcal vaccines and estimation of the herd effect.
- Additional carriage studies, including extensive molecular strain characterization, should be performed before and after vaccination in countries where mass vaccination is introduced.
- The effect of vaccination in individual carriers should be studied and coupled to serological analyses.

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## Doctoral/diploma thesis

*PhD thesis by Konstantinos Kesanopoulos entitled: “Epidemiological investigation of the multiple carriage of Neisseria meningitidis strains isolated from students by molecular and conventional methods”. University of Ioannina, Medical School. To be defended.*

## Workpackage 6

### Spread of antibiotic resistant meningococci in Europe

**Participants:** Julio A Vázquez

#### Collaborations with partners in other workpackages

As part of the Quality Control assays we collaborated with WP 2 to compare different PCR based tools for defining the susceptibility levels to penicillin G.

#### Objectives

- To define the real extent of the resistance across Europe;
- To define the break-points to be used;
- To standardize methods and protocols for the MIC determination;
- To design a PCR based tool for detection of the susceptibility level to penicillin.

#### Results

##### Standardization of protocols for susceptibility testing

Previous studies carried out by the European Monitoring Group on Meningococci (EMGM) showed enormous differences in the methods used to determine the minimal inhibitory concentrations (MICs) in laboratories across Europe. Therefore, as a first step we standardized the methodology including the definition of break points. For this purpose a study was implemented together with 14 different laboratories, which used partially different methods for susceptibility testing. The design of the study included a collection of 17 well characterized meningococcal strains. The MICs to penicillin G, rifampin, cefotaxime, ceftriaxone, ciprofloxacin, and ofloxacin were determined by all the laboratories not only by their own protocol, but also by Etest and agar dilution according to a given unified protocol. Eleven strains were previously defined as having intermediate resistance to penicillin (Pen(I)) by sequencing and restriction fragment length polymorphism analysis of the *penA* gene. The MIC was determined by agar dilution and Etest with Mueller-Hinton agar (MH), MH supplemented with sheep blood (MH+B), and MH supplemented with heated (chocolated) blood. Several laboratories encountered problems obtaining confluent growth with unsupplemented MH. MH+B was considered to give the most congruent and reproducible results among the study laboratories. The modal MIC for MH+B for each antibiotic and method was calculated to define the MIC consensus, allowing assessment of each individual laboratory's data in relation to the others. The agreement in each antibiotic/method/medium combination was defined as the percentage of laboratories with a result within one dilution of the consensus result. For the whole study, an agreement of 90.6% was observed between agar dilution and Etest methods. The agreement in each laboratory/antibiotic/method combination ranged from 98.2% to 69.7%, with six laboratories demonstrating agreement higher than 90% and 11 more than 80%. The ability of the laboratories to detect the Pen(I) isolates ranged from 18.2% to 100%. Based on these studies a panel of serologically well defined meningococci with known penicillin resistance determinants has been defined, which now can be used to define limits and MIC breakpoints for meningococci and for quality control. We propose the use of four isolates with a high agreement between laboratories (EMGM 1, 2, 10, and 13) as reference strains for standardization of susceptibility testing in reference laboratories. These isolates will be made commonly available on request by the Spanish Reference Laboratory for Meningococci (National Institute of Health Carlos III).

After implementation of standardized protocols for antibiotic resistance testing we defined the breakpoints to be used in the definition of the real extent of resistance to penicillin in European meningococcal isolates. For this purpose, comparative susceptibility testing by E-test and sequencing of an internal fragment of the *penA* gene was performed for 43 meningococcal strains. All the strains for which the MIC was  $\leq 0.047$   $\mu\text{g/ml}$  as determined by E-test possessed *penA* alleles related to Pen<sup>s</sup> strains. Mosaic *penA* alleles were identified in all the strains for which the MIC was  $\geq 0.094$   $\mu\text{g/ml}$ . Among those strains for which the MIC was 0.064  $\mu\text{g/ml}$  ( $n = 9$ ), two groups were defined according to the *penA* gene sequence: five isolates showed *penA* alleles related with Pen<sup>s</sup> strains and four isolates possessed mosaic *penA* alleles. A similar correlation can be found between agar diffusion testing and *penA* polymorphism. All the strains for which the MIC was  $< 0.06$   $\mu\text{g/ml}$  showed *penA* alleles of Pen<sup>s</sup> meningococci, while mosaic alleles were identified in those for which the MIC was  $> 0.06$ . Once again there was a less clear correlation with the isolates for which the MIC was 0.06  $\mu\text{g/ml}$ , with three of eight strains showing mosaic *penA* alleles. It is important to note, that from 23 strains for which the MIC was 0.12  $\mu\text{g/ml}$  as determined by agar diffusion, 13 exhibited a MIC of 0.094  $\mu\text{g/ml}$  by E-test. Thus, meningococcal strains with MIC of 0.094  $\mu\text{g/ml}$  might occur frequently.

For those strains with penicillin G MIC was 0.064  $\mu\text{g/ml}$ , the MICs of other  $\beta$ -lactams (ampicillin, cefotaxime, and ceftriaxone) were tested. The most relevant finding was, that for all the strains showing mosaic structures at the *penA* gene, ampicillin MICs determined by E-test were  $\geq 0.125$   $\mu\text{g/ml}$ . However, for those strains possessing *penA* alleles related to susceptible strains, ampicillin MICs were lower ( $\leq 0.094$   $\mu\text{g/ml}$ ). According to these results, 0.094  $\mu\text{g/ml}$  should be used as the Pen<sup>i</sup> breakpoint when E-test is used as the susceptibility testing method. The heterogeneous situation found among strains for which the MIC was 0.064  $\mu\text{g/ml}$  as determined by E-test determines that isolates for which the MIC is at this level should be defined as susceptible in order to avoid an overestimation of the Pen<sup>i</sup> meningococcal population. The evaluation of different inocula and/or media in the E-test susceptibility testing method could be important for clarifying the confusing situation found for those isolates for which the MIC was 0.064  $\mu\text{g/ml}$ . However, the level of susceptibility to ampicillin might be used for the decision if those isolates for which the penicillin G MIC determined by E-test was 0.064  $\mu\text{g/ml}$  should be included in the Pen<sup>s</sup> or Pen<sup>i</sup> group.

#### Clonal distribution of penicillin resistance

The prevalence of meningococcal strains with intermediate penicillin resistance might be related to the occurrence of particular clonal lineages. In fact, previous studies have shown a possible relationship between the C:2b phenotype and intermediate resistance to penicillin. This correlation could explain the high percentage of Pen<sup>i</sup> meningococcal isolates in certain countries with a high prevalence of strains typed as C:2b (C:2b:NST and C:2b:P1,2,5).

In this study forty-four *Neisseria meningitidis* strains isolated from cases of meningococcal disease were included: forty-one C:2b strains and three B:2b isolates showing a possible capsular switching event. The analysis of the *penA* gene in Pen<sup>s</sup> strains showed a high identity among the *penA* gene alleles identified (alleles 1, 2, and 3). All of them encoded for the same protein sequence (PBP2-1). On the other hand, only 2 *penA* gene alleles (alleles 4 and 5), which encoded for 2 variants of PBP2 (PBP2-2 and PBP2-3), were found among the Pen<sup>i</sup> strains. These observations obtained for two clonal lineages (ST11 and ST8) are in contrast to previous results, which indicated high diversity of *penA* genes in Pen<sup>i</sup> strains in a wide range of clonal lineages. Therefore, we assume that, although Pen<sup>i</sup> strains have arisen on many occasions in Spain, resulting in a diversity of altered *penA* genes, resistance in those isolates causing most of the C:2b disease in Spain is caused by single genetic event that created the altered *penA* allele 4.

Although the PBP2 protein of the Pen<sup>i</sup> strains (PBP2-2 and PBP2-3) showed different polymorphisms, the level of penicillin susceptibility was the same, and the MIC ranged from 0.12 to 0.25  $\mu\text{g/mL}$ . This fact could indicate that only a small number of amino acid changes

might be necessary for the appearance of strains showing reduced susceptibility. However, it was difficult to assess which amino acid changes determine this level of reduced susceptibility. The analysis of the PBP2 sequence of C:2a Pen<sup>i</sup> meningococcal strains revealed an extra codon. This insertion codon does not exist in the *penA* gene sequences from Pen<sup>s</sup> strains. The role of the extra codon in the Pen<sup>i</sup> phenotype in *N. meningitidis* has been considered elsewhere. However, the present study confirms that the reduction in affinity of PBP2 cannot be entirely caused by that insertion, because strains that have a *penA* sequence without the extra codon (e.g., allele 4) show the same level of reduced susceptibility.

The C:2b strains, which belonged to 2 different genetic lineages, had the same *penA* gene sequence (allele 4). We suggest that interspecies recombination events between *N. meningitidis* and commensal *Neisseria* species determined the appearance of that allele (allele 4) in one of these genetic lineages, and then an intraspecies recombinational event probably resulted in the spread of this allele to the other lineage. Thus, recombination between the *penA* gene of commensal *Neisseria* species and that gene in a C:2b Pen<sup>s</sup> ST11 strain could have generated the C:2b Pen<sup>i</sup> ST11 strains (allele 4). After that, a recombination event between a C:2b Pen<sup>i</sup> ST11 (allele 4) and a C:2b Pen<sup>s</sup> ST8 isolate could explain the appearance of the C:2b Pen<sup>i</sup> ST8 (allele 4) isolates. Alternatively, the first recombination event could have occurred between the *penA* gene of commensal *Neisseria* species and a C:2b Pen<sup>s</sup> ST8 strain with subsequent transfer of allele 4 into a C:2b Pen<sup>s</sup> ST11 isolate.

#### Non-culture identification of altered *penA* genes

Taken advantage of the definition of 5 specific changes on the sequence of the strains with intermediate resistance, we designed a PCR method for molecular determination of intermediate penicillin susceptibility. By the establishment of a real time PCR assay in cooperation with WP 2 we were able to detect altered *penA* alleles directly in clinical samples.

#### **Outlook**

Having a standardized protocol for susceptibility testing of meningococci available now, it will be possible to determine the real extent of penicillin resistance across Europe. As penicillin is still the antibiotic of the first choice, it is pivotal to analyse the distribution of isolates with intermediate penicillin resistance and the possible emergence of fully resistant strains in the future.

#### **Publications**

Vázquez, J.A., L. Arreaza, C. Block, I. Ehrhard, S.J. Gray, S. Heuberger, S. Hoffmann, P. Kriz, P. Nicolas, P. Olcen, A. Skoczynska, L. Spanjaard, P. Stefanelli, M.K. Taha, G. Tzanakaki. 2003. An interlaboratory comparison of agar dilution and etest methods to determine the antimicrobial minimum inhibitory concentration of antibiotics used in management of *Neisseria meningitidis* infections. *Antimicrob. Agents Chemother.* 47: 3430-34.

Arreaza, L., C. Salcedo, B. Alcalá, M.J. Uría, R. Abad, R. Enríquez, J.A. Vázquez. 2004. *penA* gene sequencing in *Neisseria meningitidis*: the key to success in the definition of penicillin G breakpoints. *Antimicrob. Agents Chemother.* 48: 348-9.

Arreaza, L., B. Alcalá, C. Salcedo, L. de la Fuente, J.A. Vázquez. 2003. Dynamics of the *penA* gene in serogroup C meningococcal strains. *J. Infect. Dis.* 187: 1010-4.

## Workpackage 7

### Studies on hypervirulent meningococci

**Participants:** J. Simon Kroll ; Simon Nadel, Linda Bailey, Sena Plevnik

#### Collaborations with partners in other work packages

Multi-locus sequence typing of meningococci was done in cooperation with Martin Maiden (WP 1). Together with Mary Ramsay and Andrew Fox (WP 2, 4) meningococcal strains were identified in the Meningococcal Reference Unit in Manchester.

#### Objectives

- Sequence typing of isolates from >100 cases of meningococcal disease of known severity;
- Numerical definition of hyperinvasive meningococci on the basis of clinical scores and MLST data;
- Promulgation of treatment and scoring algorithms throughout Europe;
- Assembly of reference collection of hypervirulent meningococci submitted to the European Meningococcal Strain Collection Centre (EMSC).

#### Results

##### Identification of Patient-Strain Pairs

Five hundred and fifty five cases of meningococcal disease were identified from the records of admission of children to St Mary's Hospital, London, between 1996 and 2001. Most of these cases were referred for paediatric intensive care (PICU). Linkage of these cases to records at the HPA Meningococcal Reference Unit, Manchester UK, identified 127 cases with stored strains available for further study. In 28/127 the clinical records were missing or inadequate for objective evaluation of the severity of the case using GMSPS or PRISM (see below), leaving 99 Patient-Strain Pairs (PSPs) (92 PICU cases, 7 of lesser severity admitted to the infectious diseases ward). As most of the St Mary's cohort of patients, referred for intensive care, had very severe meningococcal disease, a complementary collection of PSPs was compiled, representing cases of meningococcal disease of lesser severity. Between 1997-1999 one of us (LB) participated in a study of avoidable causes of death in meningococcal sepsis. In the course of this UK study, two non-fatal cases of meningococcal disease were identified admitted to the same hospital close in time to each fatal case. These so-called control cases included many not requiring admission to PICU. As before, the records of these non-fatal cases were linked to records at the Manchester Meningococcal Reference Unit, to identify 108 with stored strains available for further study. In 22/108 the records were inadequate for objective clinical scoring, leaving 86 PSPs (25 PICU cases, 61 of lesser severity admitted to general paediatric wards).

We have thus amassed a collection of 185 PSPs (117 PICU cases), well in excess of the 100 planned.

##### Clinical Severity Scoring

Severity of disease may be assessed objectively by assigning scores to relevant, discriminant clinical and laboratory parameters. In the extremity of life-threatening illness, for

children admitted to an intensive care unit, one of the most widely used and best validated critical illness scores is the PRISM (Paediatric RiSk of Mortality) score (Pollack MM *et al.* Pediatric risk of mortality (PRISM) score. Crit Care Med 1988; 16: 1110–1116.). This is calculated from 14 (patho)physiological parameters evaluated over the first 24 hours of PICU admission, inputted into a computer programme to generate a percentage risk of mortality (ROM%). Provided the PRISM score is calculated by an experienced individual carefully following strict guidelines, it provides a reasonably reliable measure of illness severity. PRISM ROM%  $\geq 20\%$  is generally regarded as signifying very severe disease, assisting in reaching the decision for example to deploy scarce resources or relatively high-risk therapeutic modalities. To objectify the severity of meningococcal sepsis in particular, the Glasgow Meningococcal Septicaemia Prognostic Score (GMSPS) has been developed (Sinclair JF, Skeoch CH, Hallworth D. Prognosis of meningococcal septicaemia. Lancet. 1987 ii 38). This is a simpler scoring system than PRISM, and can readily be used by non-experts. It is widely used in Europe (see e.g. <http://www.sfar.org/scores2/gmsps2.html>). It is based on 7 readily observed clinical characteristics of a case, converted using a simple rule to a score taking a value between 0 and 15. It is calculated during the first 8 hours of an admission, does not rely on laboratory data beyond the measurement of base deficit (from a blood gas machine, widely available in hospital emergency departments), and a score of  $\geq 8$  is generally regarded as signifying disease sufficiently severe to warrant admission to PICU.

We have used PRISM and GMSPS in the evaluation of the severity of our cases of meningococcal disease, but from these descriptions it will be appreciated that while both are potentially useful in assessing the severity of a case, they have somewhat different relevance, and the results of applying the different scoring methods may be discordant. Most importantly, relatively milder cases of meningococcal disease, not rating admission to PICU, cannot be objectively assessed at all by PRISM. The GMSPS is particularly sensitive to manifestations of shock, and cases admitted to PICU that respond well and quickly to early fluid resuscitation may have a high GMSPS and yet a low PRISM. Both scoring systems may be applied retrospectively, but this must be done with caution. Absent information for particular scoring criteria lead by convention to scores of zero for that element, which can plainly mislead. GMSPS in particular relies on rather subjective assessments of the clinical features of a case, which may not be well recorded in the clinical notes.

In figure 1, the plot is divided into 4 quadrants. Points in the lower left and upper right quadrants correspond to cases with concordant severity scores: either both “low” or both “high”. Clinical notes for cases from which points with discordant scores were derived were re-examined. In each of the 7 cases with low GMSPS but high PRISM ROM%, the scores were confirmed. In one case the low GMSPS could be explained by there being a forced zero entry for one missing datum. For the rest, each case followed a similar pattern: a child admitted with meningococcal disease, not apparently severely ill, who deteriorated nearly 24 hours later, while in hospital, and was admitted to PICU where after a further 24 hours a high PRISM score could be calculated. There are not enough of such cases in our data set to be able to ascertain whether there are particular strains of meningococci responsible for this pattern of illness. The case notes of five patients with high GMSPS but low PRISM ROM% were selected at random for detailed review. In all cases the scores were confirmed, and in no case was the low PRISM score the result of one or more forced zero entries. In each case the presenting illness was dominated by haemodynamic shock which responded quickly to fluids and inotropes, so that the child was stabilised and greatly improved within a few hours of admission.

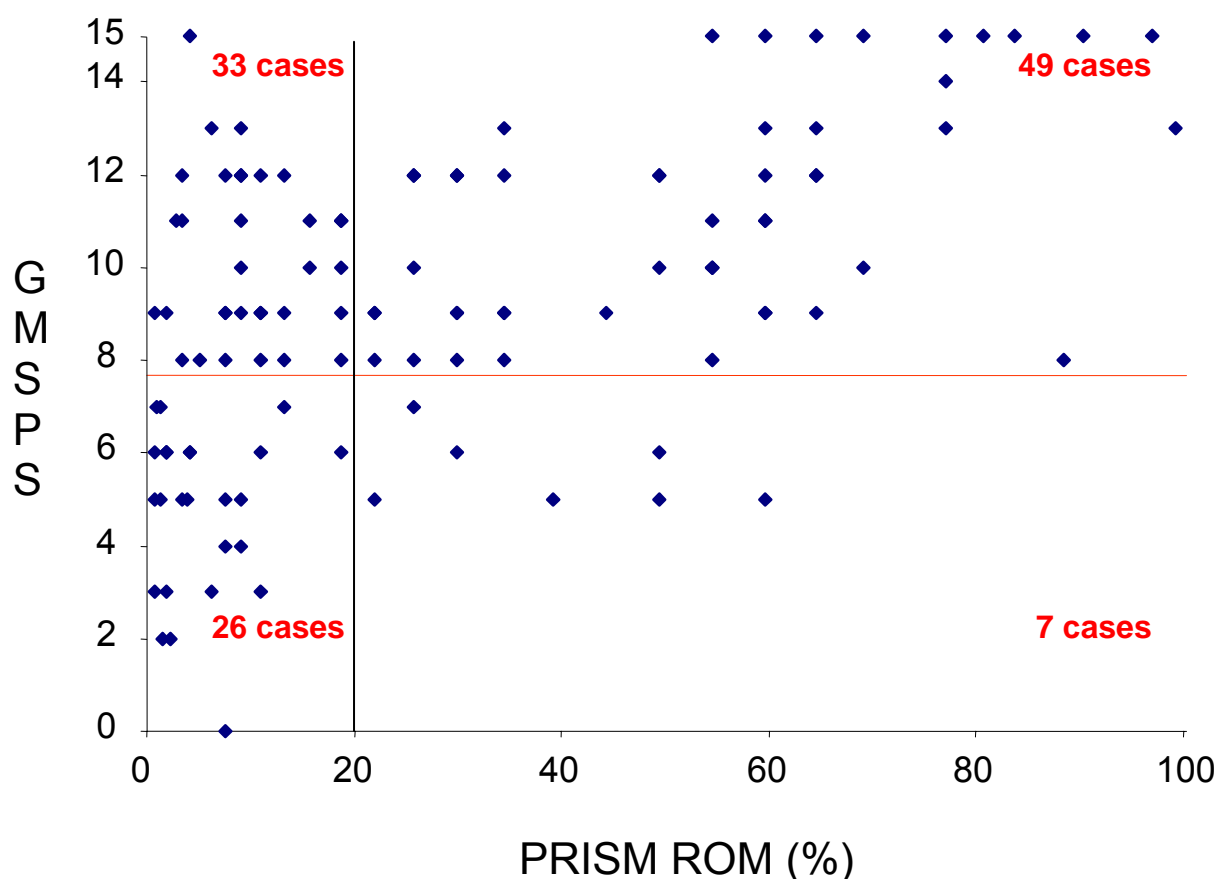


Fig 1  
Correlation of PRISM ROM% and GMSPS for 115 PICU cases where both scores could be determined. Each point corresponds to a single PSP, and horizontal and vertical lines divide the data points into conventional groups with very severe and less severe GMSPS and PRISM ROM% respectively.

#### Meningococcal strains and multi-locus sequence typing (MLST)

Meningococcal strains were recovered from the HPA collection in Manchester, and bacterial DNA prepared for MLST. Typing was carried out in Oxford, and the strains have been incorporated into the Oxford collection (WP 1). The results are shown in Fig 2. The data were examined for systematic changes in the frequency of recovery of different MLST complexes from cases over the period of the study. No such change occurred.

MLST complex	185 cases with $\geq 1$ severity score	
	Number (%)	
8	8 (4.3)	
11	61 (33.0)	
22	4 (2.2)	
32	14 (7.6)	
41/44	42 (22.7)	
269	36 (19.4)	
Other	20 (10.8)	

Fig 2. Meningococcal MLST complexes of strains recovered from PSPs (1996-2001)

The UK meningococcal carriage study, in which JSK was a participant, has yielded MLST data on carried strains in UK children in 1999. 731 carried strains (35.6% of the total) fell within the MLST complexes found in the invasive strains (Fig 2). This is in striking contrast to the situation with the invasive strains, where 89.2% of the total was in the designated complexes (Fig 2). The distribution of strains also differed strikingly between invasive and carried strains (Fig 3)

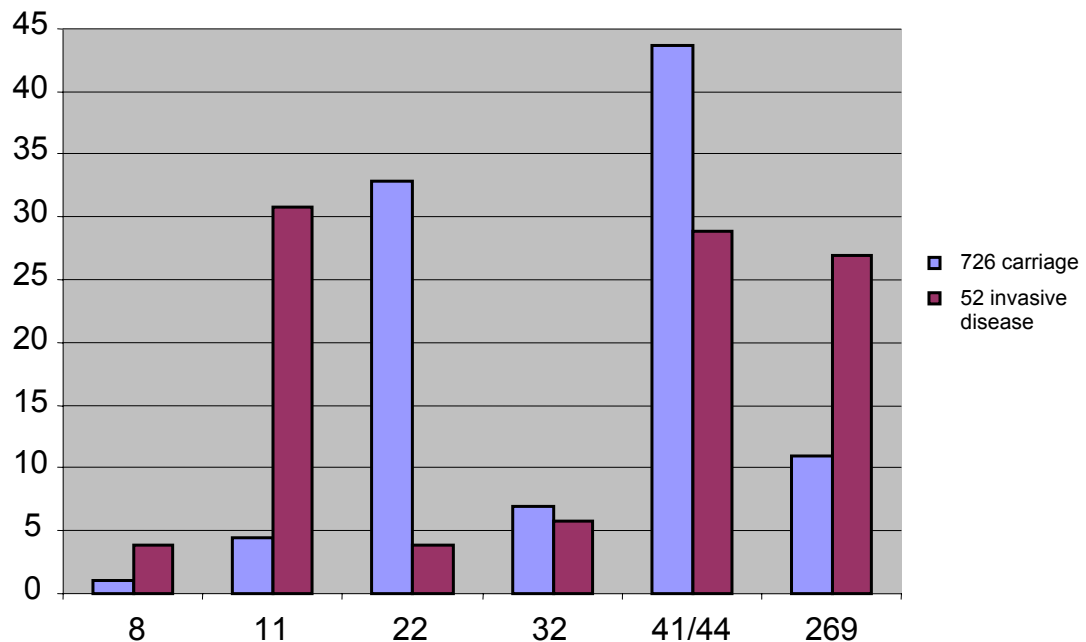


Fig 3. Carried and invasive meningococcal strains in 1999 (UK meningococcal carriage study)  
X-axis: MLST complex; Y-axis: percentage of total in each category.

Striking differences are seen between the carried and invasive populations. In particular, ST-11 complex strains are rarely isolated from healthy carriers, but frequently cause invasive disease. The same is true to a lesser extent of strains of the ST-269 complex. In contrast, ST-22 complex strains, for example, are frequently carried, but rarely cause invasive infection. And as pointed out already, there are many MLST complexes found in carriage strains which are apparently scarcely ever cause invasive disease. These observations accord with those of WP 5, and support the hypothesis that strains from a limited range of MLST complexes have hyperinvasive potential.

To explore the hypothesis that meningococcal strains of specific MLST complexes might be particularly prone to cause very severe disease, the proportion of strains falling within the different designated complexes was compared for:

- Non-PICU cases vs. PICU cases
- PICU: low vs high PRISM ROM%
- PICU: low vs high GMSPS

A closely comparable pattern was seen in each scenario, illustrated in Fig 4:

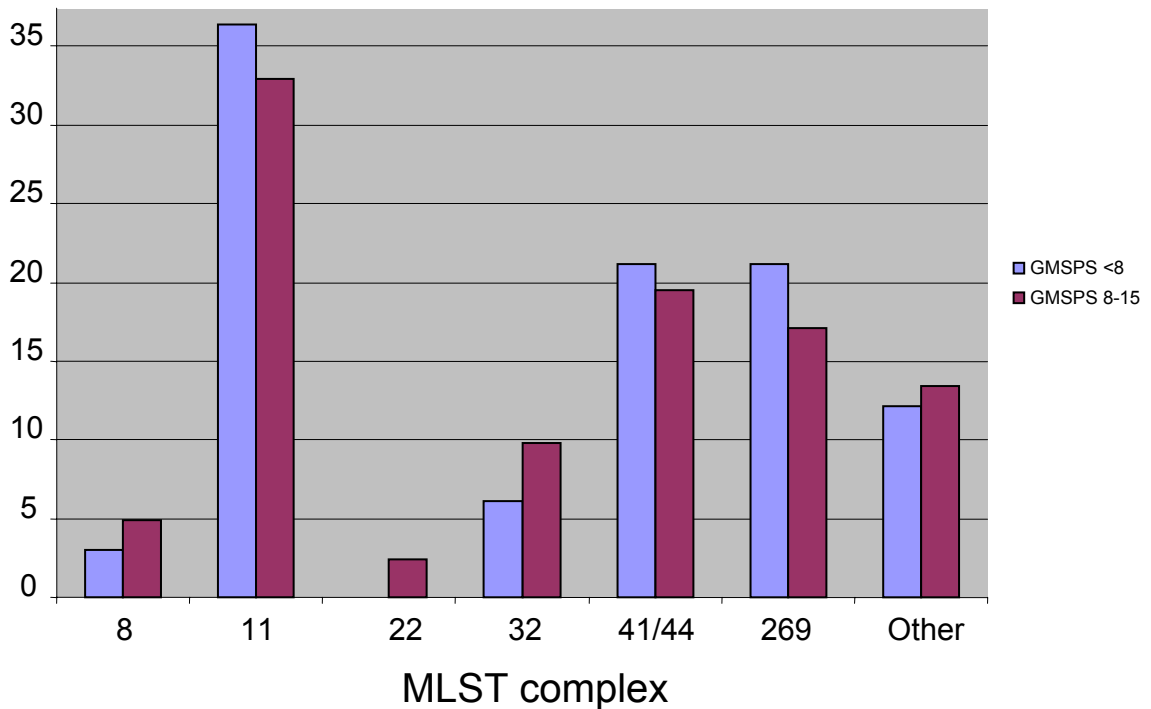


Fig 4: 115 PICU cases segregated by GMSPS  
Y-axis: percentage of clinical isolates in each category.

In contrast to the manifest difference between MLST complexes of strains found in carriers as opposed to cases of meningococcal disease, there was no difference found in strains responsible for more as opposed to less severe cases of disease.

Referring to Fig 1, the analysis presented in Fig 4 corresponds to a comparison of PSPs plotted in the upper two and lower two quadrants. Different analyses, for example incorporating all strains from PSPs with at least one high severity score and comparing them with strains from concordantly low-scoring PSPs, made no difference. Therefore, no MLST complex appears to represent strains of particularly virulent – as opposed to invasive – phenotype. As these results contradict to the findings of WP 4 further studies may be needed to clarify the correlation between outcome of disease and MLST genotypes.

### Conclusions and outlook

A highly restricted group of strains, not the same as carriage strains, cause most invasive meningococcal disease in the UK, indicating that some MLST complexes have a hyper-*invasive* clinical phenotype. However, in contrast to findings in WP 4 we found no evidence for an association of disease severity with MLST genotype. These contradictions need to be resolved in future studies and the clinical relevance of urgent strain characterisation (including MLST) beyond speciation and capsular typing needs to be defined.

In addition to strain characteristics disease severity may also reflect differences in host and situational factors which also should be addressed in future studies.

### Publications

Welch, S.B., S. Nadal. 2003. Treatment of meningococcal infection. Arch. Dis. Child. 88: 608-14.

Data presented in this report is being prepared for future publication.

## Workpackage 8

### Coordination

**Participants:** Matthias Frosch (coordinator), Dominique Caugant, Sigrid Heuberger, Martin Maiden, Mary Ramsay

#### Collaborations with partners in other workpackages

WP8 acted across all workpackages of the consortium.

#### Objectives

- Establishing a central management group comprising representatives of EMEC, EMMC, EMSC and public health monitoring institutions;
- Implementation of quality assurance schemes in public health epidemiological monitoring institutions;
- Organization of training and fellowship programmes;
- Providing guidelines for outbreak management and clinical management and giving recommendations on vaccination trials and vaccination programmes;
- Publication of manuscripts in refereed international journals.

#### Results

##### Management

The management group met regularly twice a year to discuss the progress made in the work packages and to coordinate the work between the Epidemiology, MLST and Strain Collection Resource Centres and the European Monitoring group on Meningococci. The annual and final reports have been compiled and regular meetings of the consortium and specific discussion groups have been organised. In close co-operation with the Epidemiology, MLST and Strain Collection Resource Centres and the European Monitoring Group on Meningococci the management group has also undertaken the choice of isolates from across Europe to be included in the MLST analysis and has ensured progress on the linking of the MLST data with the epidemiological records in co-operation with EU-IBIS headed by Mary Ramsay. This coordinated action now is the basis for a thorough exploitation of the infrastructure built up in the recent years by the EU-MenNet consortium for answering the central questions concerning the spread of meningococcal disease in Europe and in reducing meningococcal disease burden.

In all meetings the management group came to the conclusion that the EU-MenNet consortium was working perfectly well and there was always a very high degree of cooperation among the work packages. In this sense EU-MenNet was a true network in which individual partners collaborated strongly and regularly with other members of the network. The great success of the project becomes evident by the fact that all milestones were fulfilled at the end of the funding period and the envisaged deliverables are now available.

## Training

Training programmes have been established to provide assistance for the transfer of new technologies and quality assurance schemes to the National Reference Laboratories.

Three MLST training workshops have been held. The first was a three day residential course held in September 2002 at the University of Oxford and covered practical aspects of using sequencing techniques to type bacterial isolates, including high-throughput sample preparation, data assembly and analysis. The use of automated techniques for the setup and analysis of sequence data as well as developments in database software were also covered.

The second workshop was held in September 2003 in Lanzarote, during the EMGM meeting. This two day workshop concentrated on software used for the analysis of MLST data, and provided information concerning the integration of isolate databases and websites. Participants worked on their own laptops that were linked in an ad-hoc network to a Linux laptop that acted as a database server.

The final workshop was held in February 2005 at the University of Würzburg following the final meeting of the EU-MenNet consortium. This workshop introduced some of the advanced analysis tools that have been integrated in to the database software, and participants worked on a copy of the linked EMMC/EU-IBIS databases to answer specific epidemiological questions.

Feedback from all the workshops was positive and some participants who came to the first one, subsequently attended one or both of the following sessions.

## **Outlook**

A great need has been identified to continue the work of the EU-MenNet consortium for further achievements of the identified research priorities in the next couple of years. To further support sustaining of the established infrastructure, i.e. the MLST Centre, the Epidemiology Centre, and the Strain Collection Centre, the management group came to the decision to found a society covering the central objectives of the EU-MenNet. This society is called "EMGM – The European Meningococcal Disease Society". The coordinator of the EU-MenNet consortium became the first president of the society and other members of the consortium were elected to become board members of the society.

As this society together with the EU-MenNet consortium the newly established European CDC follow very similar aims it will be an important task in the future to establish a close collaboration and to offer the competence and knowledge for surveillance and controlling meningococci and meningococcal disease all over Europe. The management group also expects further support by the European Commission for the fight against one of the most important infectious diseases according to the regulations and standards on the control of communicable diseases in the Community, as stated in the DECISION No 2119/98/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 24 September 1998.

## **Publications**

A special volume of the FEMS Microbiological Reviews is scheduled for 2006, edited by Matthias Frosch and Martin Maiden, in which comprehensive reviews of the EU-MenNet consortium's work will be presented and guidelines for the laboratory diagnosis, clinical management and public health management will be given by experts of the consortium.