Annual Report of the National Center for Meningococci 2008

Béatrice Ninet and Jacques Schrenzel
Hôpitaux Universitaires de Genève
Laboratoire Central de Bactériologie
Rue Gabrielle-Perret-Gentil 4
1211 Genève 14

Phone : 022 372 92 52 Fax 022 372 73 04

http://www.meningo.ch

Introduction

The incidence of meningococcal disease varies geographically in Europe but it remains one of the most severe childhood infections, with incidence rates of up to 50/100,000 for children aged 0-4 and mortality rates approaching 20%. Appreciable numbers of cases in other age groups, notably in young adults, are also detected in all countries with often a rapid onset of the disease, and a high rate of fatality or severe complications. The epidemiology and pathogenesis of *N. meningitidis* is defined by, 1) the virulence characteristics of different strains or clonal groups, 2) the human reservoir and the dynamics of meningococcal exposure (e.g. human transmission, acquisition and carriage), 3) the variable human susceptibility to the disease (1). There is currently no comprehensive childhood vaccine against this disease, the severity of which, combined with its rapid progression and non-specific symptoms, results in an unacceptable burden of childhood morbidity and mortality. In addition, the emergence of meningococci with reduced susceptibility to penicillin is a major concern. The public health management policies and the development of

effective vaccines are confounded by the epidemiology of meningococcal disease, which is itself governed by the complex population biology of the causative organism. Immediate management of invasive meningococcal infections requires prompt measures to confirm the diagnosis, to administer appropriate antimicrobial therapy and to prevent secondary cases in close contacts (vaccination and/or chemoprophylaxis). The National Center of Meningococci (= CNM) located at the University Hospitals of Geneva (Laboratory of Bacteriology) enhances the capability to detect outbreaks by a centralized characterization of all Swiss strains. Typing of meningococcal strains is based on variable outer membrane-expressed structures such as the capsule (serogroups), major outer membrane proteins (serotypes) and other outer membrane proteins (sero-subtypes). Another molecular method, considered as the method of choice by the European meningococcal network, the MultiLocus Sequence Typing (MLST) is also performed for providing a better reproducibility of the results, as well as for international comparisons.

The CNM is also in charge of the surveillance of the evolving antibiotic resistance profiles. The development of resistance of *N. meningitidis* to various antimicrobial agents is not particularly efficient but the emergence of intermediate resistance to penicillin has been well documented. It is related to the expression of altered forms of penicillin-binding proteins (PBP2) as a result of differences in the sequence of the *penA* gene. For this reason, all strains isolated in 2008 were analyzed by sequencing the *penA* gene and all different alleles observed were compared against a European database with concomitant analysis of their Minimal Inhibitory Concentration to penicillin Recently, the emergence of ciprofloxacin-resistant *Neisseria meningitidis* was described in North America and in France (2, 3). Nalidixic acid antibiotic was therefore added to our systematic antibiotic susceptibility testing as a first marker of this resistance detection.

During the last four years, we have developed molecular methods for non-culture diagnosis which do not require viable bacteria for accurate detection. In many cases, these molecular assays decrease the time for identifying a meningococcal infection and enable the detection of pathogens from culture-negative clinical samples. These PCR methods permit the identification of bacterial DNA from CSF and whole blood as well as the genogrouping of strains by segregating them into serogroups A, B, C, Y or W135.

Materials and Methods

During the year 2008, the NCM has received 41 strains of *Neisseria meningitidis* isolated from normally sterile specimens like blood (n=25), CSF (n=10), blood and CSF (n=4), pleural fluid (n=1) and other unknown origin (n=1). These strains correspond to 58% of the invasive meningococcal cases notified to the SFOPH (Swiss Federal Office for Public Health) (n=70).

When a strain is received by the CNM, its serogroup is immediately determined with two types of latex agglutination kits. The serogroups A, C or Y-W135 are performed with the Pastorex^{TM} meningitis kit (Bio-Rad, Pasteur, Paris, France) and the serogroup B with the Welcogen *N. meningitidis* B/*E. coli* K1 reagent (Remel Europe Ltd, Dartford, UK). As soon as a fresh culture is available (usually two days after the reception of the strain), the isolate is tested for its antimicrobial susceptibility profile (Minimal Inhibitory Concentration = MIC) to the following nine antimicrobial agents: penicillin, cefuroxime, ceftriaxone, minocycline, rifampicin, erythromycin, azythromycin, ciprofloxacin, and chloramphenicol. Values of the E-test (AB Biodisk, Sweden, distributed in Switzerland by Bio-Rad) on Mueller-Hinton 5% sheep blood agar are interpreted according to the American CLSI recommendations (4). But for cefuroxime and erythromycin, no value is provided by the CLSI and therefore the criteria proposed by the British Society for Antimicrobial Chemotherapy (5) are applied. For the first time this year, nalidixic acid resistance which is the first step to detect an emergence of quinolone resistance was also tested by MIC.

The only well-known mechanism involved in the development of intermediate resistance to penicillin is the expression of altered forms of PBP2 as a result of differences in the sequence of the *penA* codifying gene (6). In 2008, an analysis of these sequences of the *penA* gene of all Swiss strains was established in our laboratory. A 512 bp region of the *penA* gene was chosen for sequencing; the conditions for amplification and sequence determination have previously been described by Taha and al. (2007) (7). Sequences were then analysed in a European database accessible at the following URL: http://neisseria.org/nm/typing/penABlast.

Serogroups, serotypes and sero-subtypes are determined with a dot-ELISA technique based on a set of 27 monoclonal antibodies purchased from the National

Institute for Biological Standards and Controls, Herdfordshire, Great Britain (8). The DNA typing method chosen in all countries of Europe is now MultiLocus Sequence Typing (MLST) due to its reproducibility, portability, reliability, affordable cost and adequate throughput. MLST determination reflects the variation present in the nucleotide sequence of 400-500bp internal fragments from seven housekeeping-genes (9). The following loci are examined by sequencing: *abcZ* (putative ABC transporter), *adk* (adenylate kinase), *aroE* (shikimate deshydrogenase), *fumC* (fumarate), *gdh* (glucose-6-phosphate deshydrogenase), *pdhC* (pyruvate deshydrogenase subunit) and *pgm* gene (phophoglucomutase).The MLST profile corresponding to a MLST number is then accessible in the MLST database via the following URL: http://pubmlst.org/ neisseria/ (10).

The NCM has developed a rapid and sensitive nucleic acid amplification method to detect a meningococcal infection and to identify the serogroup at the genetic level. Nucleic acids of CSF or blood-EDTA are automatically extracted with the MagNAPure Compact system (Roche Diagnostic Ltd.). DNA is amplified with a real-time PCR to detect the *ctrA* gene (capsular transport gene specific of *Neisseria meningitidis*) (11) and, whenever the first PCR assay is positive, we perform a second amplification to detect the genes encoding the specific polysialyltransferase (*siaD* gene) for B, C, Y and W135 serogroups and *mynB* gene for A serogroup, respectively (12). This laboratory assay is offered to all Swiss laboratories or hospitals with a rapid response, especially for paediatric patients.

Results

The 41 strains received in 2008 represent only 59% of the 70 cases of invasive meningococcal diseases (IMD) notified to the Swiss Federal Office of Public Health (Figure 1). With an incidence below 1 case per 100’000 inhabitants, the IMD are very rare in Switzerland in comparison with other European countries where the overall incidence ranges between < 1 per 100’000 up to 5 per 100’000 inhabitants. All Swiss 2007 epidemiological data were published in October 2008 by the SFOPH (13) and are available on their web site: http://www.bag.admin.ch. All strains isolated from sterile origin had a serogroup that could be defined. Similarly to the other European countries, the serogroup B appears always largely predominant (63%) and, when

compared to the previous year, the proportion of serogroup C (32%) has slightly decreased (Figure 2). Only two strains belonged to other serogroups: one strain was a serogroup Y and one a serogroup W135. As the number of strains is relatively low, it is difficult to derive a trend based on these data, but again the two serogroups B and C encompass almost all serogroups detected in Switzerland in 2008 (Figure 3, Table 1).

In 2008, we have performed 49 direct PCR assays on various samples. Of these, 14 were positive for the detection of *Neisseria meningitidis* and 35 were negative. All specimens were tested for the presence of inhibitors. The direct PCR detection from blood, CSF or skin biopsy has allowed diagnosing 7 invasive cases which were PCR positive for *Neisseria meningitidis* but have remained culture negative. Of these 7 additional patients, 3 were infected with a serogroup C strain, 3 with a serogroup B and one with a strain with no defined serogroup

As observed during previous years, MLST type 11 constituted the most predominant genotype: it represented 27% of all MLST types. All strains of MLST type 11 belong to serogroup C. Specifically, 9 strains were of complete serotype C:2a:P1.5 and two of complete serotype C:2a:P1.-. The second MLST most frequently identified was the MLST type 41 (10%), with all isolates corresponding to serogroup B. MLST type 2816 (also strains of serogroup B) was the third type most predominant (7%). Various other MLST types were also present in one or two instances (Table 2). No epidemic clone has been detected according to this typing method.

In 2007, new breakpoints for different antibiotics were defined by CLSI (4) and new recommendations were published by the European Monitoring Group on Meningococci (14). With these new breakpoints, the susceptibility of strains to penicillin remained high in 2008 (66% of strains susceptible). Contrary to the last years (before 2007) and due to this better standardisation, our results are now in accordance with the European data: Switzerland doesn’t show an unusual level of penicillin resistance in European countries, Thirty one percent of strains were intermediate for this antibiotic (CMI between 0.094 and 0.25mg/L) and one isolate was reported as resistant (CMI = 0.75mg/L). The same strain was also exceptionally intermediate to cefuroxime with a CMI of 1.5mg/L. Other tested antimicrobial agents (Table 3) were active against meningococci with the exception of erythromycin (only 29% of susceptible strains). As in previous years, no resistance against ciprofloxacin was detected in the isolates. All CMI for nalidixic acid were very low (0.5mg/L) which

is reassuring in terms of risk to observe fluroquinolone resistance in Switzerland. Resistance to rifampicin which is occasionally observed following chemoprophylaxis was not detected in strains tested in 2008.

For the first time, we have also analysed the *penA* gene sequence of each strain received in 2008 and the results revealed very informative. As described in the European data project (EU.MenNet project), five polymorphic sites might differentiate penicillin susceptible from intermediate strains and those five specific positions are the main keys to the definition of intermediate resistance to penicillin. In our experiments, the same mutations responsible for antimicrobial resistance were also observed and a very good correlation existed between the CMI values and the presence of mutations in the *penA* gene. All strains with a CMI <0.125mg/L (n=33) showed no mutations and the eight isolates with a CMI >0.125mg/L displayed 4 or 5 known amino acid sequence changes in the trans peptidase-encoding region of the *penA* gene.

Discussion

The repartition of strains (serogroups, serotypes and MLST types) has not changed significantly in 2008, as compared to 2007. Serogroup B strains remain at the same level as the year before, corresponding to about two thirds of all strains analysed. The monitoring of the level of serogroup C, Y or W135 strains is essential for vaccine recommendations from a public health standpoint as well as for the secondary prophylaxis of contacts. The more precise determination of subtypes remains valuable allowing a reliable comparison of inter-country results. These subtypes results are in accordance with European subtype data. The MLST method is used as a powerful tool to rapidly detect clusters of strains and to identify a clone that is particularly associated with the disease. The MLST type 11 was the predominant one observed, with all isolates belonging to serogroup C. This year, and in contrary to other years, we have not detected isolates with capsular switching that corresponds to a change of polysaccharidic capsule due to pressure of immunization (15). No epidemic clone was detected in 2008 but some MLST were particularly controlled; for

example the MLST type 269 is associated with serogroup B disease and it has recently emerged in Québec, Canada (16) or the MLST 2816 which had increased in Switzerland in 2007, particularly in the area of Luzern.
For the antibiotic susceptibility testing, the results are very similar to the previous year. The proportion of strains with a reduced sensitivity to penicillin has slightly decreased. But one strain was defined as resistant to this antibiotic and 20% of all isolates had a CMI of >0.125mg/L that perfectly correlated with a proven polymorphism in the *penA* gene encoding penicillin-binding protein. This sequencing assay has been developed in our laboratory to compare our data with those of other European countries. We have found exactly the same five mutations in the Swiss strains as those described by the European surveillance network (6). Overall, all other antibiotics currently used against meningococci remain very active but we are aware of resistance appearing in other continents (2) as well as in neighbour countries like France (3). This new problem, namely the development of fluoroquinolone resistance, is crucial for specifying the prophylaxis of meningococcal contacts. We have therefore followed the guidelines provided by the CLSI, suggesting relying on nalidixic acid for surveillance purpose. In Switzerland, we have seen that it is not a current problem but we will of course continue this surveillance analysis.
PCR is a now a very efficient method to directly detect the presence of *Neisseria meningitidis* from clinical specimens. We have received samples (blood or CSF) from different places (directly from hospitals or other laboratories) with a good feedback of applicants. Molecular assays decrease the time of identification of *N. meningitidis* but also improve case ascertainment by confirming infections in culture-negative clinical samples. In 2009, it will be important to analyse these results with the SFOPH in order to better define the real incidence in our country. The method and the application of this PCR to chronic meningococcemia has been published with our clinical collaborators in the University Hospitals of Geneva (17).

Further developments: The immunological typing schemes exhibit a number of limitations including incomplete coverage and difficulties in production and provision of reagents. For these reasons, we plan to develop next years other molecular typing methods. Amplification and sequencing of the three variables regions of the *porA* gene (VR1, VR2 and VR3) which code for the major constituent of the outer

membrane will be performed according to new global recommendations. Secondly, the nucleotide sequencing of the *fetA* gene, an iron-regulated meningococcal OMP gene, is now employed in reference laboratories throughout the world for genotyping purposes. We plan to evaluate and implement, if applicable, the published protocols.

Publications: The method and the application of the PCR method to chronic meningococcemia has been published with our clinical collaborators in the University Hospitals of Geneva (17). In collaboration with SFOPH, the NCM has also published all 2007 epidemiological data on severe infections occurring in Switzerland (12) and we participated to an oral presentation in the 16th international Pathogenic Neisseria Conference in September 2008 at Rotterdam (18).

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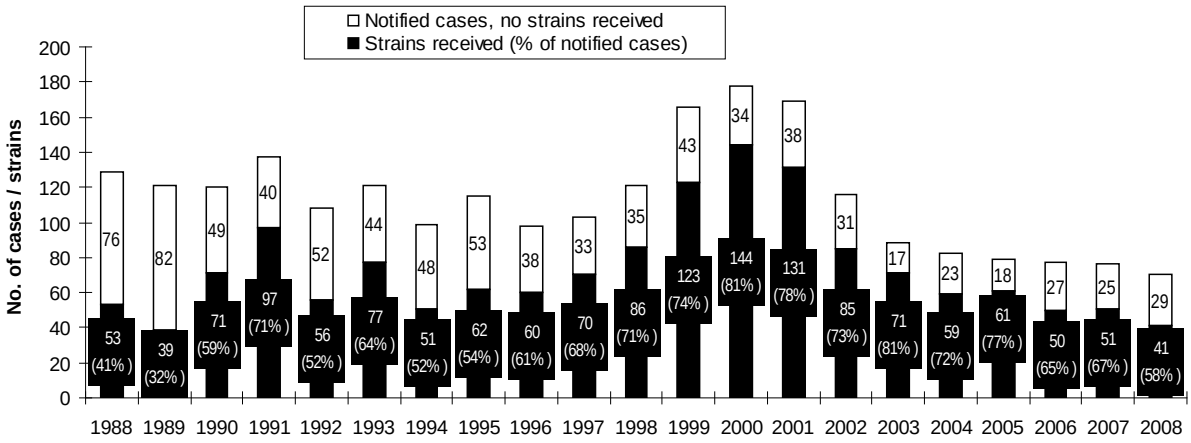

Figure 1: Comparison of the annual number of *N. meningitidis* strains received at the National Center for Meningococci in Geneva and the number of invasive meningococcal infections notified to the Swiss Federal Office of Public Health from 1988 to 2008

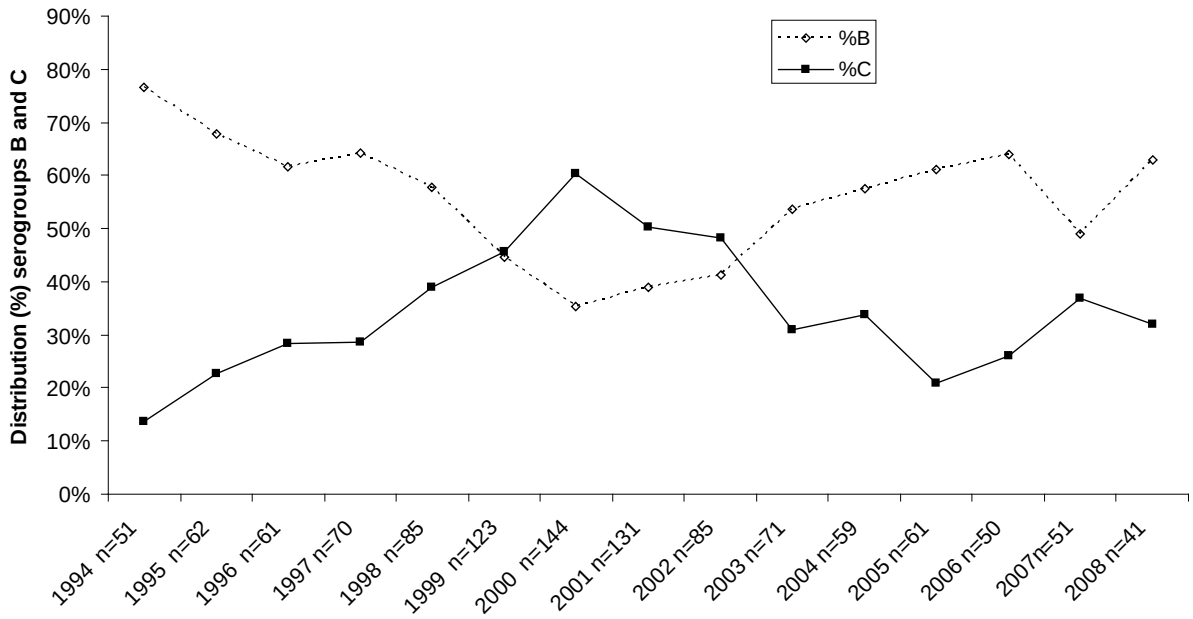

Figure 2: Distribution of serogroups B and C of *N. meningitidis* from 1991 to 2008

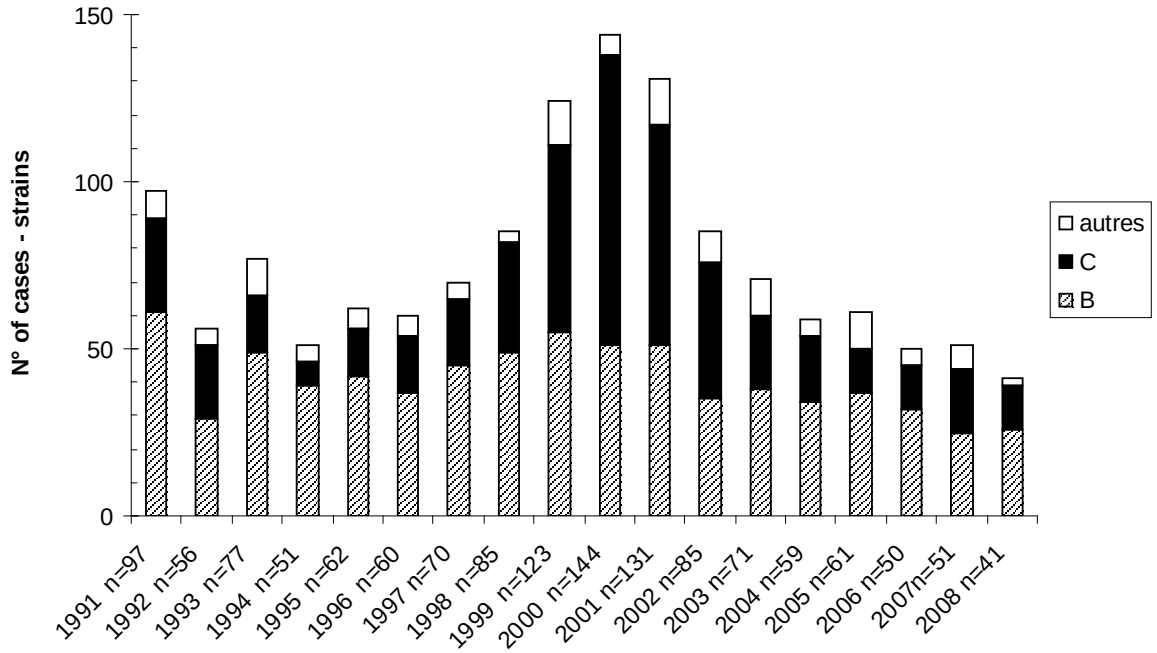

Figure 3: Serogroup distribution of invasive meningococcal isolates 1991-2008

Table 1: Distribution of serogroups and serotypes of *N. meningitidis* strains isolated in Switzerland 2008

Serotype	4	2a	14	15	1	unknown	Total	
Serogroup								
A								0%
B	9		1	6	4	6	26	63,5%
C	1	12					13	31,5%
W135						1	1	2,50
Y						1	1	2,5%
unknown								0%
Total	10	12	1	6	4	8	41	100%
	24%	29%	2.5%	15%	10%	19,5%	100%	

Table 2: Distribution in 2008 of the most frequent MLST types for meningococci in Switzerland according their serogroups

MLST	11	41	2816	22	23	ND*	Other MLST**	Total
Serogroup B		4	3			4	15	26
Serogroup C	11					2		13
Serogroup Y				1				1
Serogroup W135					1			1
Total (%)	27%	10%	7%	2,5%	2,5%	14,5%	36,5%	100%

*: Not defined MLST

**: Other MLST: 12 different MLST types (number of strains ≤ 2)

Table 3: Inhibitory activity of 9 antimicrobial agents on 41 meningococci isolated in Switzerland during 2008

Agent	Minimal Inhibitory Concentration (µg / ml) range	50%	90%	Breakpoint sensitive ≤ µg/ml	% sensitive
Penicillin	0.023-0.75	0.064	0.125	0.06*	66%
Cefuroxime	0.064-1.5	0.125	0.5	1**	98%
Ceftriaxone	<0.01	<0.01	<0.01	0.12*	100%
Minocycline	0.125-0.75	0.25	0.38	2***	100%
Rifampicin	<0.01-0.5	0.023	0.094	0.5***	100%
Erythromycin	0.25-2	0.75	1	0.5**	29%
Azithromycin	0.19-1.5	0.75	1	2***	100%
Ciprofloxacin	<0.01-0.01	<0.01	0.01	<0.03*	100%
Chloramphenicol	0.5-2	1	1	2*	100%

*CLSI/NCCLS 2007 (3) and EMGM working group
**British Society for Antimicrobial Chemotherapy (5).
*** CLSI/NCCLS 2007