Annual Report of the National Center for Meningococci 2009

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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 Hospital 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 2009 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 was therefore added to our systematic antibiotic susceptibility testing as a sensitive phenotypic marker for such resistance detection.

These last 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 2009, the CNM has received 58 strains of *Neisseria meningitidis* isolated from normally sterile specimens like blood (n=39), CSF (n=13), blood and CSF (n=1), lymph node (n=1), joint fluid (n=3), and a respiratory strain isolated from a septicemic patient (n=1). These strains correspond to 73% of the invasive meningococcal cases notified to the SFOPH (Swiss Federal Office for Public Health) (n=80).

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 assessed 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 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. Nalidixic acid resistance, a necessary first step towards quinolone resistance development, 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* encoding gene (6). In 2008, an analysis of *penA* gene sequences 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 been previously described by Taha *et al*. (2007) (7). Sequences were then analysed against 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, United Kingdom (8). The DNA typing method chosen in all countries of Europe is now MultiLocus Sequence Typing (MLST) thanks to its reproducibility, reliability, and affordable cost. MLST determination reflects the variation present in the nucleotide sequence of 400-500 bp 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 CNM has developed a rapid and sensitive nucleic acid amplification method to detect a meningococcal infection and to identify the serogroup at the gene 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 this 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

In 2009, Swiss laboratories have sent proportionally more strains than during previous years. Fifty eight strains representing 73% of all the 80 cases of invasive meningococcal diseases (IMD) notified to the Swiss Federal Office of Public Health were received in our laboratory (Figure 1). The incidence of IMD remains below 1 case per 100’000 inhabitants and is always very low in Switzerland. All strains except one isolated from a sterile origin had a serogroup that could be defined. Similarly to the other European countries, the serogroup B appears always predominant (52%) and, when compared to the previous year, the proportion of serogroup C (29%)

remained constant (Figure 2). It is the first time that the serogroup Y appears significantly present in Switzerland with a proportion of 14% (Figure 3, Table 1).
In 2009, we have performed 24 direct PCR assays on samples from 20 different patients. These samples were constituted of CSF, blood or joint fluids. Of them, 14 were positive for the detection of *Neisseria meningitidis* and 10 were negative. All specimens were tested for the presence of inhibitors and none was inhibited. The direct PCR detection allowed diagnosing 7 invasive cases which were PCR positive for *Neisseria meningitidis* but remained culture-negative. Of these 7 additional patients, all were infected with serogroup B strains.
As observed during the previous years, MLST type 11 constituted the most predominant genotype: it represented 26% of all MLST types. All strains of MLST type 11 belong to serogroup C except one strain of serogroup B that has probably undergone a capsular switch. For the first time, the second MLST most frequently identified was the MLST type 23 (14%), with all isolates corresponding to serogroup Y. MLST type 41 (strains of serogroup B) was relatively rare (5%), as opposed to what was recorded during the previous years. Various other MLST types were also present in one or two instances (Table 2).
In 2007, new breakpoints for different antibiotics were defined by CLSI (4) and new recommendations were published by the European Monitoring Group on Meningococci (13). With these new breakpoints, the susceptibility of strains to penicillin remained high in 2009 (64% of strains susceptible) in accordance with the European data. Thirty six percent of strains were intermediate for this antibiotic MIC between 0.094 and 0.38mg/L). Other tested antimicrobial agents (Table 3) were highly active against meningococci with the exception of erythromycin. As in previous years, no resistance against ciprofloxacin was detected in the isolates: all MICs for nalidixic acid were very low (0.75mg/L; break point for sensitivity=8mg/L) which is reassuring in that it reduces the risk of observing fluroquinolone resistance in Switzerland. Resistance to rifampicin which is occasionally observed following chemoprophylaxis was not detected in the strains tested in 2009.
The analysis of the *penA* gene sequence of each strain received in 2009 was 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 for 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 exists between the MIC values and the presence of mutations in the *penA* gene. All strains with a MIC <0.094mg/L (n=48) showed no mutations and the nine isolates with a MIC >0.125mg/L displayed 5 known amino acid sequence changes in the trans peptidase-encoding region of the *penA* gene. The exception to this rule was only for a strain that showed a MIC of 0.19 but no mutation in the penA gene.

Summary of key observations:

- No increase in number of invasive isolates
- Serogroup Y now appears third after serogroup B and serogroup C
- Penicillin remains highly active against *Neisseria meningitidis*
- No quinolone resistant strain was detected

Discussion

The repartition of strains (serogroups, serotypes and MLST types) has slightly changed in 2009, as compared to 2008. In Switzerland, serogroup B strains of *Neisseria meningitidis* remain the first pathogens responsible of invasive meningococcemia, corresponding to half of all analysed strains. The monitoring of the different serogroups 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 for reliable comparisons of results between countries. These subtypes results are in accordance with European subtype data with a majority of P1 subtypes like P1.7, P1.5 and P1.14. This subtype's determination can be important in case of a local epidemic from serogroup B strains in order to compare with other neighbour's countries and possibly to develop a specific vaccine.

For the first time, we have participated to an external quality control distributed by QCMD (London, England) in order to compare the performances of our center with that of other European countries. Every determination was correct except for two MLST results due to an inversion of different tubes during alleles sequencing. When the two strains were reanalysed, the true MLST were defined. The inversion of tubes was due to a very large number of analyses handled in the same time. We have therefore decided to analyze only a few isolates simultaneously to minimize such risk.

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 MLST observed, with the majority of isolates belonging to serogroup C. In 2009, we have detected a unique isolate with a capsular switch that corresponds to a change of polysaccharidic capsule due to pressure of immunization (14). This prevalence of capsular switch requires surveillance to detect changes in the meningococcal population structure that may affect the effectiveness of meningococcal vaccines.

In 2009, four points should be particularly discussed:

1- An increase of serogroup Y strains was detected in 2009 with a specific MLST (MLST23). These isolates were described in the entire American continent, i.e. in the United States but also in South America region (15). In Europe, Italy has published the same trend of infections with serogroup Y strains between 1988 to 2006 (16). This trend of increase with serogroup Y, MLST 23 strains is interesting; however nobody has a good explanation for this emerging serogroup. The potential invasive of these strains and their spread are still unclear.

2- In June 2009, the EMGM group has proposed a new consensus for the laboratory methods to be used for improved discrimination of circulating meningococcal strains. Laboratory surveillance should rely only on molecular and sequence-based typing data. The previously recommended serotyping scheme including serogroup (capsule), serotype (porB antigen) and serosbtype (PorA antigen) suffers from increasing lack of serotype and serosubtype specific antibodies. Currently, about one third of circulating meningococcal strains cannot be fully typed by serological methods. Based on these published recommendations, the labnet consortium fully agreed on the molecular typing scheme:

Serogroup: PorA(vr1): PorA(vr2): FetA (vr1): clonal complex (MLST).

In Switzerland, this high resolution scheme will probably help to characterise the new serogroup Y, MLST 23 strains. This new approach will allow us to better understand if these swiss infections are due to a unique or different clones. In Geneva, we have implemented and validated these methods to retrospectively analyse all invasive strains (isolates of 2009) as well as for our prospective surveillance (all isolates received in our CNM since January 2010).

3- For the antibiotic susceptibility testing, the results are very similar to the previous years. The proportion of strains with reduced sensitivity to penicillin is similar as in 2009 (36%). But only 9 isolates had a MIC of >0.125mg/L that perfectly correlates with the proven polymorphism in the *penA* gene encoding penicillin-binding protein. This sequencing assay has been implemented in our laboratory to compare our data with those from other European countries. We have found exactly the same five mutations in the Swiss strains as those described by the European surveillance network (6). The EUCAST gives the value of = or< 0.06 for susceptible isolates and 0.12 to 0.25 for intermediate isolates. Isolates with MIC of 0.094 (determined by E test) are problematic according to the breakpoint of the EUCAST and are not correlated with molecular mutations in the *penA* gene. For these reasons, a modification of interpretation of penicillin sensitivity will be performed in 2010. All isolates with a MIC of 0.094 will be interpreted as susceptible. This decision has been also proposed by the EMGM. The development of quinolone resistance has been also particularly controlled because it is crucial for specifying the post-exposition prophylaxis after 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 important surveillance analysis.

4.- PCR is 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 due to early onset of antibiotic treatment. Molecular tools also offer the invaluable advantage for culture-independent typing of meningococci.

Current and Further developments:

1. The immunological typing schemes exhibit a number of limitations including incomplete coverage and difficulties in production and provision of reagents. For these reasons, we have developed these last few month new molecular typing methods: amplification and sequencing of the two variables regions of the *porA* gene (VR1, VR2) and the *fetA* gene, an iron-regulated meningococcal OMP gene. We plan to evaluate all our results and to formally

compare them with previous methods, and particularly to serological typing. Ultimately, these methods will replace the other, once our validation will be completed.

2. The EMGM agreed that the establishment of a meningococcal strain collection is necessary for licensing of serogroup B vaccines. The first step to implement a representative European Meningococcal Strain Collection is to bring in real time the data in the European database. The EMERT database is in place and used by different countries. The EMERT management team asked us to include the Swiss data. Once we will have the approval of the SFOPH, we plan to participate in this centralization of the European data.

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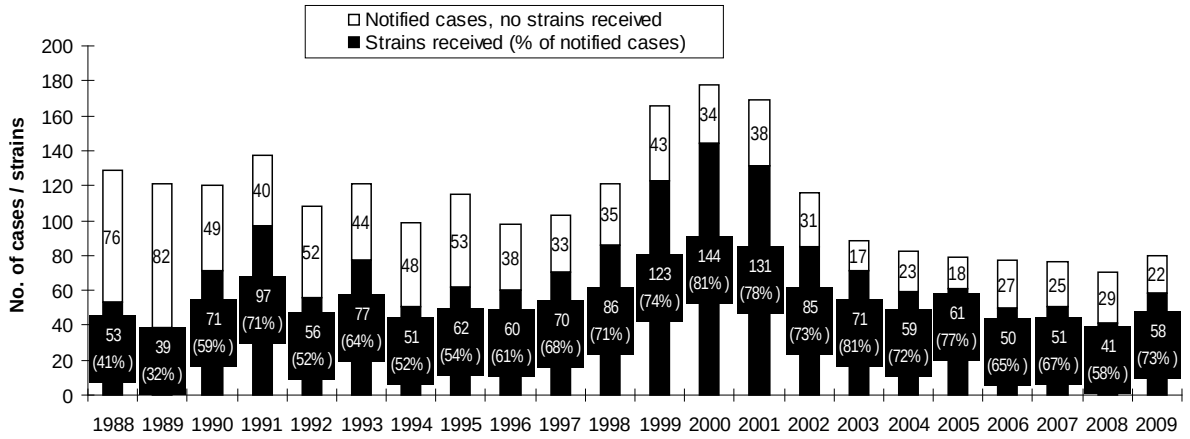

Figure 1: Comparison of the annual number of *N. meningitidis* strains received at the National Center for Meningococci in Geneva and the number of invasive meningococcal infections notified to the Swiss Federal Office of Public Health from 1988 to 2009

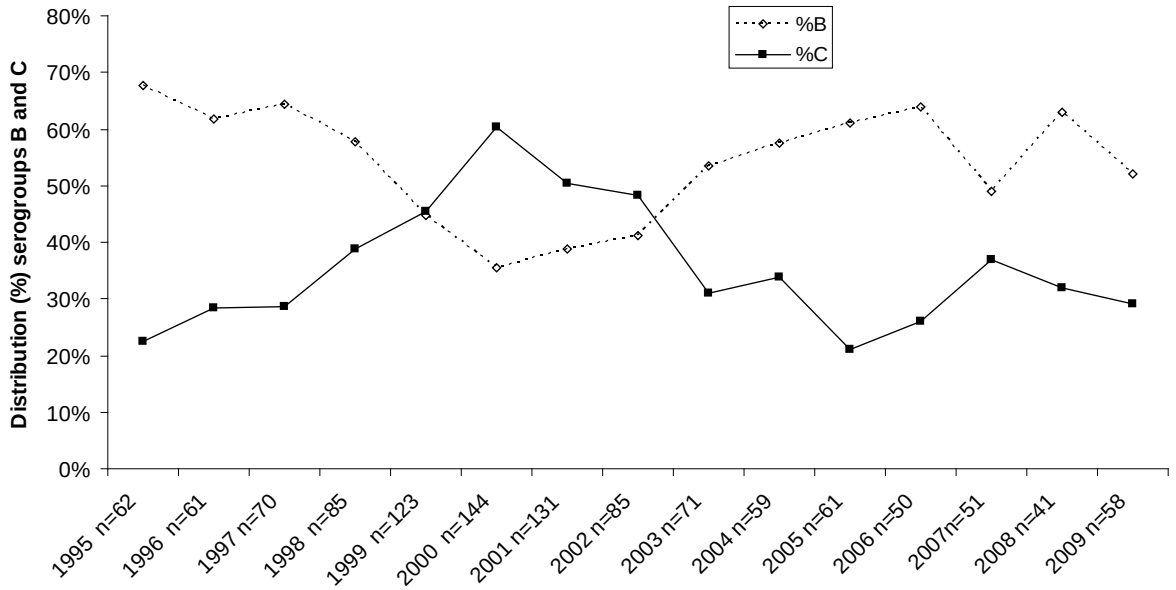

Figure 2: Distribution of serogroups B and C of *N. meningitidis* from 1995 to 2009

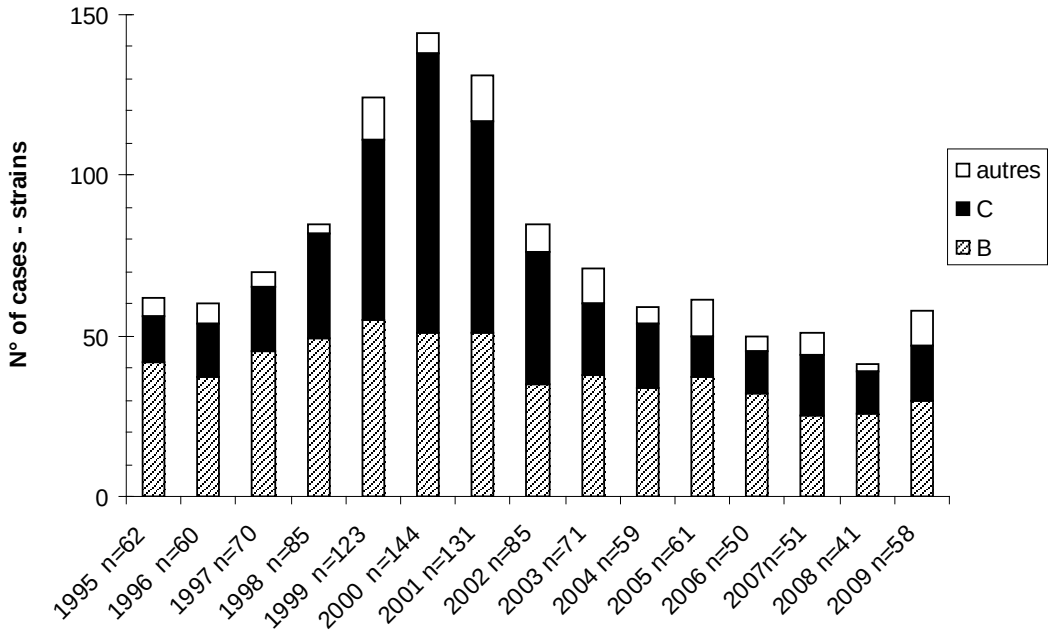

Figure 3: Serogroup distribution of invasive meningococcal isolates 1995-2009

Table 1: Distribution of serogroups and serotypes of *N. meningitidis* strains isolated in Switzerland 2009

	Serotype	1	4	15	2a	Unknown	Total	
Serogroup								
B		4	9	5	1	11	30	52%
C					12	5	17	29%
Y						8	8	14%
W135						2	2	3%
Unknown						1	1	2%
Total		4	9	5	13	27	58	
		7%	15%	9%	22 %	47%		100%

Table 2: Distribution in 2009 of the most frequent MLST types for meningococci in Switzerland according their serogroups

MLST	11	23	32	41	NDM*	Other MLST**	Total
Serogroup B	1		3	3	3	20	30
Serogroup C	14				1	2	17
Serogroup Y		8					8
Serogroup W135						2	2
NDS**					1		1
Total (%)	26%	14%	5%	5%	9%	41%	100%

NDM*: Not defined MLST

NDS**: Not defined Serogroup

**: Other MLST: 21 different MLST types (number of strains ≤ 2)

Table 3: Inhibitory activity of 9 antimicrobial agents on 41 meningococci isolated in Switzerland during 2009

Agent	Minimal Inhibitory Concentration (µg / ml) range	50%	90%	Breakpoint sensitive ≤ µg/ml	% sensitive
Penicillin	0.023-0.38	0.064	0.19	0.06*	64%
Cefuroxime	0.047-2	0.25	0.75	1**	97%
Ceftriaxone	<0.01	<0.01	<0.01	0.12*	100%
Minocycline	0.064-0.5	0.19	0.58	2***	100%
Rifampicin	<0.01-0.064	0.016	0.047	0.5***	100%
Erythromycin	0.19-1	0.5	1	0.5**	55%
Azithromycin	0.125-1.5	0.75	1	2***	100%
Ciprofloxacin	<0.01-0.016	<0.01	0.012	<0.03*	100%
Chloramphenicol	0.38-1.5	1	1	2*	100%

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