Introduction

Meningococcal infection is a major public health problem in European countries with a wide spectrum of disease from meningococcemia and meningococcal meningitis to rapidly fatal septicemia. They remain a leading cause of bacterial meningitis and sepsis in infants and adolescents with a great impact in the population. A good characterization of all isolated strains in Switzerland is important to set off preventive measures in certain populations at risk like a recommendation of vaccination (1). The epidemiological surveillance by the National Center for Meningococci (NCM) contributes to implement these measures. The NCM is a key for this epidemiological surveillance by an efficient biological characterization of all strains and a surveillance of their antimicrobial resistance patterns. The bacteriology laboratory of the University Hospitals of Geneva has performed this task since 1990 in collaboration with the Swiss Federal Office of Public Health (SFOPH). Classification and characterization of clinical isolates have been based on phenotypic characteristics of variable outer membrane-expressed structures such as the capsule (serogroups), major outer membrane proteins (serotypes) and other outer membrane proteins (sero-subtypes).
For many studies however, serological methods suffer a number of limitations, including an incomplete coverage of the antibody panels employed and inconsistent correspondence with genetic relationships (2). Many methods based on DNA technology have been proposed including ribotyping, random amplified polymorphic DNA (RAPD), fluorescent amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) but the comparison of these techniques among laboratories remains difficult. Other sequencing methods like the Multi Locus Sequence Typing (MLST), the sequencing of two regions of the porA gene (porA-VR1 and porA-VR2 corresponding to sero-subtypes) and the sequencing of the fet-A gene (encoding for an iron repressible outer-membrane protein) are reproducible methods that are now considered as the methods of choice to characterize meningococci within the European Meningococcal Network (European Meningococcal Disease Society, EMGM). The CNM performs also the surveillance of antibiotic resistance profiles. The spread of antibiotic resistance is also a predominant problem and it must be analyzed to monitor best practices in therapy. The development of resistances of N. meningitidis to antimicrobial agents is not particularly efficient but the decrease in penicillin susceptibility observed over the last 10 years due to alterations in penicillin-binding proteins (PBPs) has underscored the importance of such analyses. To manage accurately and rapidly invasive meningococcal infections, the confirmation of N. meningitidis by culture remains essential. As early antibiotic treatment is recommended when invasive meningococcal infection is suspected, isolation of viable organism is however often compromised. Molecular methods for non-culture diagnosis and laboratory confirmation of such bacterial infections have also been developed and are now currently used to detect infections.

Materials and Methods

During the year 2010, the CNM has received 39 strains of Neisseria meningitidis isolated from normally sterile specimens like blood (n=24), CSF (n=12), blood and
CSF (n=1), and joint fluid samples (n=2). These strains correspond to 70% of the invasive meningococcal cases notified to the SFOPH (n=56).

When a strain is received at the CNM, its serogroup is immediately determined by latex agglutination kits. The serogroups A, B, C Y and W135 are assessed with the Pastorex™ meningitis kit (Bio-Rad, Pasteur, Paris, France). As soon as a fresh culture is available (usually the day 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, azithromycin, ciprofloxacin, and chloramphenicol. Values of the E-test (AB Biodisk, Sweden, distributed in Switzerland by bioMérieux) on Mueller-Hinton 5% sheep blood agar are interpreted according to the CLSI recommendations (3). But for cefuroxime and erythromycin, no value is provided by the CLSI and therefore the criteria proposed by the British Society for Antimicrobial Chemotherapy (4) are applied. Nalidixic acid resistance, a necessary first step towards quinolone resistance development, was also tested by MIC.

Since 2008, an analysis of penA gene sequences of all Swiss strains was established in our laboratory to detect the expression of altered forms of PBP2. The PCR and sequencing method have been previously described by Taha and al. (2007) (6). Sequences were analysed against a European database accessible at the following URL: http://neisseria.org/nm/typing/penABlast. Until 2010, serogroups, serotypes and sero-subtypes were determined with a dot-ELISA technique based on monoclonal antibodies. This method has been substituted by new DNA typing sequencing methods as recommended by the EMGM:

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

Multi Locus Sequence Typing (MLST sequence type and clonal complex) reflects the variation present in the nucleotide sequence of 400-500 bp internal fragments from seven housekeeping-genes (7). 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 (phosphoglucomutase). The
PorA VRs and FetA VR sequencing methods have been established according to the published data in the web site www.neisseria.org. All allele determinations are then accessible in the different databases through the following URL: http://pubmlst.org/neisseria/.

The CNM provides to all Swiss laboratories or hospitals a rapid determination of meningococcal infection by PCR amplification. The CNM has used a rapid and sensitive nucleic acid amplification method to detect meningococcal infection and to identify the serogroup at the gene level. Nucleic acids of various clinical samples are automatically extracted with the MagNAPure Compact system (Roche Diagnostic Ltd.). DNA is amplified with a real-time PCR to detect the ctrlA gene (capsular transport gene specific of Neisseria meningitidis) (8) 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/W135 serogroups and mynB gene for A serogroup, respectively (9). In 2010, a new PCR method was also developed to characterize specifically the serogroup Y (synF gene) or W135 (synG gene) which can be useful for culture negative samples (10).

Results

In 2010, Swiss laboratories have sent the same proportion of strains as during previous years. Thirty nine strains representing 70% of all 56 cases of invasive meningococcal diseases (IMD) notified to the Swiss Federal Office of Public Health were received by our laboratory (Figure 1). The incidence of IMD remains always very low in Switzerland. All strains isolated from a sterile origin could be defined to the serogroup level. Similarly to the other European countries, the serogroup B appears predominant (38%) and, when compared to the previous year, the proportion of serogroup C (26%) has decreased slightly (Figure 2). It is the first time that the serogroup Y appears significantly present in Switzerland with a proportion of 33% (Figures 2, 3, 4).

In 2010, we performed 37 direct PCR assays on samples from 35 different patients. These samples were constituted of 27 CSF and 10 from other various specimens (blood, respiratory specimens, biopsies). Of them, 13 revealed positive for the detection of Neisseria meningitidis and 24 were negative. All specimens were tested
for the presence of inhibitors and none was inhibited. The direct PCR detection allowed diagnosing 10 additional invasive cases which were PCR positive for *Neisseria meningitidis* but remained negative by culture. Of these 10 additional patients, 4 were infected with a serogroup B strain, 5 with serogroup C and one with serogroup Y/W135.

For the first time and contrary to the previous years, the MLST type 23 constituted the most predominant genotype: representing 26% of all MLST types. All strains of MLST type 23 belong to serogroup Y. The second most frequently identified MLST was the type 11 (20%), with all isolates corresponding to serogroup C.

Various and new other MLST types were also detected in one or two instances (Table 1) specifically among serogroup B strains.

In 2007, new breakpoints for different antibiotics were defined by CLSI (3) and new recommendations were also published by the European Monitoring Group on Meningococci (11). These recommendations were updated in 2010 for penicillin but a divergence was observed in the breakpoints for penicillin between CLSI and the European group. The European group has chosen a value of 0.094 corresponding to several mutations in the *penA* gene and the CLSI a more conservative value of 0.06.

We applied the European recommendations and with this new breakpoint, a small number of strains were characterized as showing decreased susceptibility to penicillin. No strain was defined as a resistant isolate (CMI ≥ 0.5). Resistance to rifampicin which is occasionally observed following chemoprophylaxis was not detected in strains tested in 2010. The classical susceptibility of meningococci to quinolones appears to evolve over the last few years (12) and has been carefully monitored. In Switzerland, no intermediate or resistant strain has been detected so far. Other tested antimicrobial agents were active against meningococci (Table 2).

The analysis of the *penA* gene sequence of each strain received in 2010 revealed very informative. As described in the European data project (EU.MenNet project), five polymorphic sites appear to 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 confirmed between the MIC values and the presence of mutations in the *penA* gene. Three strains with a MIC >0.094 mg/L showed five mutations in the trans-peptidase-encoding region of the *penA* gene. These three strains had a MIC
value of 0.25, 0.19 and 0.125, respectively. All strains with a MIC<0.094 showed no mutation in the penA gene.

**Summary of key observations:**
- A decrease in the number of invasive isolates in 2010
- Serogroup Y strains now appears second after serogroup B strains
- Penicillin remains highly active against *Neisseria meningitidis*

**Discussion**

In Switzerland the number of invasive meningococcal disease (IMD) is very small but the repartition of strains has changed in 2010 as compared to the previous years. Serogroup B strains of *Neisseria meningitidis* remain the first pathogens responsible for invasive meningococcemia but a clone has emerged belonging to serogroup Y. This clone can be characterized by the following data:

Serogroup Y:PorA(vr1) 5-2:PorA(vr2) 10-1 : FetA (vr1) F4-1 : clonal complex ST-23.

The Y isolates are known to be commonly distributed across all continents and correlate with invasiveness, particularly in the North America where one third of IMD cases are now caused by this serogroup. For example in the United States, between 1999 and 2002, fifty seven percent of *Neisseria meningitidis* Y isolates belonged to the single common clone ST23/ P1.5-2, 10-1. These isolates were described in the entire American continent, i.e. across all United States but also in the South American region (13). In Europe, Italy has published the same trend of invasive infections caused by serogroup Y strains between 1988 to 2006 (14). In the rest of Europe, the proportion of IMD cases caused by serogroup Y strains remains low (overall 5,8%). In the contrary, this percentage is clearly increasing in Switzerland as compared to the other neighboring countries like in France (3% in 2009). During 2009 and 2010, 13 patients were older than 40 years which is very rare for IMD caused by other serogroups. The monitoring of the emerging endemic strains is helpful to the epidemiology and pathology of IMD and these new results have encouraged us to submit a poster to the ECCMID 2011 (Milano, Italy, May 2011). It is a way to alert other European countries of our specific Swiss situation.
As the strains of *Neisseria meningitidis* serogroup Y are now important in Switzerland, a new PCR method was implemented in our laboratory to confirm serogroup agglutination but also to perform a direct determination in clinical specimens. It is difficult to find a specific target to differentiate serogroup Y and serogroup W135 strains but we identified a successful method published in 2009 (10).

In June 2009, the EMGM group achieved consensus for the laboratory methods and variables to be used for high discrimination of circulating meningococcal strains. It was confirmed that laboratory surveillance should rely only on molecular and sequence-based typing data. The proposed scheme is therefore as follow:


In Geneva, we have implemented and validated these methods to retrospectively analyze all invasive strains (isolates of 2009) as well as for our prospective surveillance (all isolates received since January 2010). For this reason we communicated the results at the SFOPH with a new database encompassing this molecular typing scheme.

A European collection of data was established in 2010 (EMERT = European Meningococcal Epidemiology in Real Time). EMERT collects data about strains causing meningococcal disease throughout Europe in real time. Participating reference laboratories are requested to submit details of all isolates causing disease, thus enabling data to be shared for comparison. As soon as we received approval from SFOPH, we started introducing our data by batches, several times a year, in order to compare our epidemiologic situation with other European countries. This contributes to centralize data and helps to increase our visibility to other countries.

For the antibiotic susceptibility testing, the results are very similar to the previous years. The proportion of strains with reduced sensitivity to penicillin is very low (only three strains) and only these strains display mutations in the *penA* gene (values of MIC of 0.125, 0.19, 0.25). We have found exactly the same five mutations in the Swiss strains as those described by the European surveillance network (5). The correlation between the detected mutations and the sensitivity to penicillin is perfect and the decision of the EUCAST to interpret isolates with a MIC of 0.094 as susceptible seems to be correct.
PCR is now a very efficient method to directly detect the presence of *Neisseria meningitidis* from clinical specimens particularly in case of a culture-negative meningococcal infection. 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. A specific target (*ctrA* gene) is widely used in most laboratories since ten years (8). We have published in December 2010 that isolates with polymorphism in this gene may rarely generate false-negative results (15). The false negative case with this PCR had a positive culture with a serogroup B strain with the type B:-::P1.2,5, ST-269. To assess the frequency of such polymorphism, we tested by the real-time PCR our collection of *N. meningitidis* clinical isolates ST-269. These strains are very rare in Switzerland. The four serogroup B, ST-269 isolates, collected from 2004 to 2009, were tested by the *ctrA* real-time PCR and the results were negative for two of them. These two strains had the same sero-subtyping. In addition to our experience, it has also been reported that some non-invasive strains of *N. meningitidis* do not have the *ctrA* gene.

**Conclusions:**

In 2010 we have performed many changes to standardize our techniques in accordance with all our neighbours. In 2011, we plan to update the written references for all these new methods and then certify them.

For the PCR detection we would like to implement additional target(s) to exclude false negative PCR results due to absent or variant *ctrA* gene target.

For the antibiotic, we plan to test only those molecules recommended by the scientific organizations, after a discussion with the SFOPH.
References


- 3. CLSI/NCCLS antimicrobial susceptibility testing standards: performance standards for antimicrobial susceptibility testing. Vol 27., N°1, January 2007


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 1995 to 2010.
Figure 2: Distribution of serogroups B, C and Y of *N. meningitidis* from 1995 to 2010
Figure 3: Serogroup distribution of invasive meningococcal isolates 1995-2010
Figure 4: Typing of *N. meningitidis* strains isolated in Switzerland 2010

**Typing of serogroup B strains**
- porAVR1:5; porA VR2:2; fetA VR1:F5-1
- porAVR1:5; porA VR2:16-59; fetA VR1:F3-3
- porAVR1:21; porA VR2:4; fetA VR1:F5-8
- porAVR1:22; porA VR2:14; fetA VR1:F1-28
- porAVR1:22; porA VR2:14; fetA VR1:F5-5
- porAVR1:18-1; porA VR2:3; fetA VR1:F1-5

**Typing of serogroup Y strains**
- porAVR1:5-1; porA VR2:2-2; fetA VR1:F2-16
- porAVR1:5-1; porA VR2:10-4; fetA VR1:F3-6
- porAVR1:5-2; porA VR2:10-1; fetA VR1:F4-1
- porAVR1:5-2; porA VR2:10-1; fetA VR1:F5-12
- porAVR1:5-2; porA VR2:10-12; fetA VR1:F4-1
- porAVR1:5-2; porA VR2:10-28; fetA VR1:F4-1

**Typing of serogroup C strains**
- porAVR1:5; porA VR2:2; fetA VR1:F3-3
- porAVR1:22; porA VR2:14; fetA VR1:F5-5
- porAVR1:5-1; porA VR2:10-8; fetA VR1:F3-6
- porAVR1:5-1; porA VR2:10-8; fetA VR1:F5-8
Table 1: Distribution in 2010 of the most frequent MLST types for meningococci in Switzerland according their serogroups

<table>
<thead>
<tr>
<th>MLST</th>
<th>11</th>
<th>23</th>
<th>41</th>
<th>213</th>
<th>2816</th>
<th>NDM*</th>
<th>Other MLST**</th>
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<tbody>
<tr>
<td>Serogroup B</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td></td>
<td></td>
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<tr>
<td>Serogroup C</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serogroup Y</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serogroup W135</td>
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<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

NDM*: No defined ST-Type

NDS**: No defined serogroup

**: Other MLST: 10 different ST-types (number of strains ≤ 2)
Table 2: Inhibitory activity of 9 antimicrobial agents on 39 meningococci isolated in Switzerland during 2010

<table>
<thead>
<tr>
<th>Agent</th>
<th>Minimal Inhibitory Concentration (µg / ml)</th>
<th>Breakpoint sensitive ≤µg/ml</th>
<th>% sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>range</td>
<td>50%</td>
<td>90%</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.032-0.25</td>
<td>0.064</td>
<td>0.094</td>
</tr>
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<td>Cefuroxime</td>
<td>0.064-1</td>
<td>0.19</td>
<td>0.38</td>
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<td>Ceftriaxone</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.094-0.75</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>&lt;0.01-0.064</td>
<td>0.012</td>
<td>0.023</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.064-1</td>
<td>0.38</td>
<td>0.75</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.125-1</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&lt;0.01-0.016</td>
<td>&lt;0.01</td>
<td>0.012</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.38-1.5</td>
<td>0.75</td>
<td>1</td>
</tr>
</tbody>
</table>

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