Characterization of invasive serogroup Y meningococci in Italy: prevalence of ST-23 complex/Cluster A3

Cecilia Fazio, Arianna Neri, Stefania Starnino, Tonino Sofia, Paola Mastrantonio, Paola Stefanelli

Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

SUMMARY

The percentage of *Neisseria meningitidis* serogroup Y isolated from patients with invasive meningococcal disease (IMD) in Italy has increased from 1998 to 2006. In this study, phenotypic features and genetic relatedness have been investigated in all serogroup Y meningococci isolated during that period. Multilocus sequence typing (MLST) identified the ST-23 complex/Cluster A3 as the major clonal complex in 88.8% of the strains. That complex included all strains belonging to the sequence type (ST) 23 isolated from 1998 to 2004, whereas the ST-3171 was prevalent among strains in the years 2005 and 2006. The STs 23 and 3171 differ for only one nucleotide in the phosphoglucomutase (*pgm*) housekeeping gene. Over 80% of serogroup Y ST-23 complex/Cluster A3 strains showed phenotype Y:14:NST and 85% of the latter resulted indistinguishable by pulsed-field gel electrophoresis analysis. In 2005, serogroup Y meningococci with decreased susceptibility to penicillin were isolated for the first time in Italy. In the following year, three of the seven strains showed this phenotype. The results of this study allow us to draw a profile of the molecular characteristics of invasive serogroup Y in Italy and will be helpful to monitor the spread of this serogroup in the next years.

KEY WORDS: Serogroup Y, *Neisseria meningitidis*, ST-23

Received March 07, 2008 *Accepted* March 27, 2008

INTRODUCTION

In Europe, two main meningococcal serogroups, B and C, are responsible for the majority of all invasive meningococcal disease (IMD) (Trotter *et al.*, 2007). Differently, serogroup Y *Neisseria meningitidis* causes generally a small percentage of cases (Connolly *et al.*, 1999), but it is more frequently found in healthy carriers (Claus *et al.*, 2005; Yazdankhah *et al.*, 2004). In the US serogroup Y represents one of the major causes of meningococcal disease with an increase from 2% in 1989-1991 to 19-28% in 2000-2005 (McEllistrem *et al.*, 2004, Pace *et al.*, 2007).

Data from the EU-IBIS (European Union Invasive Bacterial Infections Surveillance Network, www.euibis.org/reports.htm) showed that in 2006 the average percentage of *N. meningitidis* serogroup Y strains was about 3% in Europe, with a maximum rate of 10-11.8% in Sweden and Norway, respectively.

In Italy, serogroup B and C meningococci represent 62% and 28% respectively of all strains sent to the National Reference Laboratory (NRL) at the Istituto Superiore di Sanità in 2006, whereas *N. meningitidis* serogroup Y accounted for 7%.

We investigated the molecular features and genetic relatedness of sporadic serogroup Y meningococci isolated from invasive diseases over a nine-year period, from 1998 to 2006.

Phenotypic and genotypic methods, such as whole-cell ELISA, susceptibility to penicillin G,

Corresponding author
Dr. Paola Mastrantonio
Department of Infectious, Parasitic
and Immune-mediated Diseases
Istituto Superiore di Sanità,
Viale Regina Elena, 299 - 00161 Rome, Italy
E-mail: paola.mastrantonio@iss.it

pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and PorA VR typing were used in the analysis.

METHODS

Bacterial strains and serotyping

Within the National Surveillance of bacterial meningitis in Italy, established in 1994, each year the NRL receives an average 80% of strains isolated by local hospital laboratories throughout the country. During the period 1998-2006, the NRL received 885 *N. meningitidis* strains. The serogroup of the strains was confirmed by sero-agglutination and/or by a multiplex PCR of the *siaD* locus (Taha, 2000). Sero/subtypes were determined by standard whole-cell ELISA (Abdillahi *et al.*, 1987), using monoclonal antibodies (NIB SC, Herts, UK).

Susceptibility to penicillin G and *pen*A gene analysis

Susceptibility to penicillin G was determined by E-test method (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar (Oxoid) supplemented with 5% of sheep blood and incubated with 5% CO_2 at 35°C for 24h, according to the manufacturer's instructions. The breakpoints were MIC≥1 mg/L for resistant strains, MIC>0.06 mg/L to MIC<1 mg/L for intermediate, and MIC≤0.06 mg/L for susceptible strains, as recommended by the European Monitoring Group for Meningococci (EMGM) (Vázquez, 2007).

For the *pen*A gene analysis, chromosomal DNA was extracted by using the QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The *pen*A gene was amplified and sequenced using primers and conditions described by Zhang *et al.* (1990); the amplicon of 402 bp, corresponding to the amino acid residues 441 to 574 of the transpeptidase domain of the PBP2 protein, was purified and sequenced to identify the corresponding *pen*A allele on the http://neisseria.org web site (Taha *et al.*, 2007).

MLST and PorA VR typing

MLST of seven genes (*abc*Z, *adk*, *aro*E, *fum*C, *gdh*, *pdh*C, *pgm*) was performed as described by Maiden *et al.* (Maiden *et al.*, 1998). Primers, determination of sequence alleles and designation of sequence types are those described on the MLST website (http://neisseria.org/typing/mlst).

For PorA VR typing, the Variable Regions (VR) VR1, VR2 and VR3, were sequenced following procedures already described (Mölling *et al.*, 2000). The deduced amino acid sequences of VR1 and VR2 were submitted to the *N. meningitidis* PorA variable regions database (http://neisseria.org/nm/typing/pora), whereas the VR3 corresponded to those indicated by Mölling *et al.* (2000).

PFGE

PFGE was performed as already described (Hartstein *et al.*, 1995). Briefly, the bacterial DNAs were digested with 30 U of the restriction endonuclease *Nhe*I (NewEngland, Biolabs) overnight at 37°C. Identical profiles were confirmed by using the restriction endonuclease *Spe*I (30U). The CHEF-MAPPER II apparatus (Bio-Rad) was used with the following parameters: voltage of 4.5V/cm, pulse time of 1s to 30s, run time of 24 h. The gel was stained with 0.5 mg/L of ethidium bromide, exposed to ultraviolet light, and photographed. Tenover's criteria (Tenover *et al.*, 1995) were followed to determine relationship among strains. The molecular sizes of the PFGE fragments were determined by the Diversity Database software (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Statistical analysis was performed using Student's *t* test. A P value of less than 0.01 was considered statistically significant.

RESULTS

From 1998 to 2006, out of 885 *N. meningitidis* isolates from invasive disease sent to the NRL, 18 were typed as serogroup Y. Although the NRL did not receive meningococcal isolates from every case of IMD in Italy, those received during this study period were very likely the majority of the strains isolated from IMD and thus very representative of the population.

Before 2005 the proportion of serogroup Y isolates ranged between 0 and 4% of the total number of strains sent each year to the NRL, but an

increase up to 7% was observed during 2006. Patients infected by serogroup Y meningococci were older than patients infected by other serogroups, with a median age of 35 years, compared to 19 years for serogroup B and C (P<0.01). 72% of infections due to serogroup Y cases occurred in females. Eleven serogroup Y strains were isolated from cerebrospinal fluid, six from blood and one from the eye of a meningitis case. About 33% of the patients had septicaemia alone as clinical picture (Table 1).
Six phenotypes were identified. In particular, thirteen strains (72%) were Y:14:NST, the remaining five showed different sero/subtypes: Y:14:P1.5, Y:NT:P1.5, Y:NT:P1.5,2, Y:15:P1.5, Y:15:P1.16 (Table 1).

Susceptibility to penicillin G and penA gene analysis

Overall, five serogroup Y isolates (29%) showed a decreased susceptibility to penicillin (PenI), with a MIC range between 0,094 and 0,19 mg/L (Table 1). PenI strains belonging to serogroup Y appeared in Italy for the first time in 2005, when the two serogroup Y strains isolated that year showed a decreased susceptibility to penicillin. During 2006 the percentage of serogroup Y meningococci with decreased susceptibility to penicillin was 42.8%.
Sequence analysis of the 402 bp region of the *pen*A gene of each strain showed the presence of 3 different alleles: 20, 9 and 22. Both of the alleles 9 and 20 were characterized by a mosaic nucleotide gene structure in the *pen*A transpeptidase domain with identical amino acid sequences. On the contrary, the allele 22 differed for 14 amino acids and for the lack of mosaicism in the gene. No association was detected between *pen*A alleles and MIC values and/or serosubtypes among meningococci Y strains with decreased susceptibility to penicillin.

Molecular analyses

MLST analysis identified a major clonal complex. Sixteen strains (88,8%) belonged to the ST-23 complex/Cluster A3 and in particular, within this clonal complex, seven strains, isolated from 1998 to 2004, were identified as ST-23, one strain isolated in 2005 as ST-3974, and eight strains, isolated from 2005 to 2006 as ST-3171. ST-23 is frequently recognised in serogroup Y meningococci. On the contrary, ST-3171 and ST-3974 are very rare. According to information in the Neisseria PubMLST database (http://pubmlst.org), ST-3974

TABLE 1 - *Clinical features of patients and phenotypic characteristics of serogroup Y meningococci from invasive meningococcal disease (IMD).*

Strain	*Year of isolation*	*Age of patients (yrs)*	*Clinical presentation*	*Site of isolation*	*Phenotype*	*MIC (mg/L) for penicillin G*
1	1998	18	meningitis	*CSF	Y:14:nst	0.023
2	2000	46	septicaemia	Blood	Y:14:nst	0.064
3	2002	29	meningitis	CSF	Y:14:P1.5	0.047
4	2004	31	meningitis	CSF	Y:14:nst	0.047
5	2004	12	septicaemia	Blood	Y:15:P1.5	0.064
6	2004	3	meningitis	CSF	Y:14:nst	§n.d.
7	2004	79	septicaemia	Blood	Y:14:nst	0.032
8	2004	18	meningitis	CSF	Y:14:nst	0.023
9	2004	74	meningitis	CSF	Y:15:P1.16	0.064
10	2005	52	meningitis	CSF	Y:14:nst	0.19
11	2005	8	meningitis	CSF	Y:14:nst	0.125
12	2006	70	meningitis	CSF	Y:14:nst	0.125
13	2006	3	septicaemia	Blood	Y:14:nst	0.094
14	2006	59	septicaemia	Blood	Y:14:nst	0.023
15	2006	55	septicaemia	Blood	Y:14:nst	0.064
16	2006	46	meningitis and septicaemia	Eye	Y:nt:P1.5	0.047
17	2006	7	meningitis and septicaemia	CSF	Y:14:nst	0.047
18	2006	18	meningitis	CSF	Y:nt:P1.5,2	0.125

*CSF: Cerebrospinal fluid; §n.d.: not done.

was detected in a non groupable strain (NG), isolated in 1999 from a carrier in the United Kingdom. ST-3171 was detected in two meningococci, one of them (serogroup Y) isolated from a carrier in 1999 in the United Kingdom;the second (NG) was isolated in Malawi, but other information is missing. The ST-3171 differs from ST-23 only for one nucleotide, the sixth in the allelic sequence of the *pgm* gene, whereas the ST-3974 shows thirty different nucleotides in the pyruvate dehydrogenase subunit C (*pdh*C) gene.
Three variable regions, named VR1, VR2 and VR3, contained in OMP1 extramembranous surface-exposed loops, were studied. DNA sequencing of the *por*A gene revealed a low degree of variability (Table 2) among the 13 strains with phenotypes Y:14:NST characterized by PorA Variable Region types 5-2 for VR1, 10-1 or 10-2 for VR2 and 36b for VR3. Three strains, serotyped as Y:14:P1.5, Y:NT:P1.5, Y:NT:P1.5,2, respectively showed the same VR combination 5-2, 2-2, 36b. The *por*A gene sequences for the two isolates with serotype 15 were also analyzed and showed the VR combination 5-1, 10-4, 36b (strain subtype P1.5), 21, 16, 37-1 (subtype P1.16) (Table 2).
In addition to the ST-23 complex, two other clonal complexes were present among meningococci Y circulating in Italy in the year 2004: the ST-167 complex (ST-168) and ST-174 complex (ST-1466), differing from ST-23 for all the 7 housekeeping genes or for five, respectively (Table 2).
All those belonging to ST-23 complex, except strains 1, 2, 16 and 18 (Table 1), showed the same pulse type in PFGE analysis (data not shown).

DISCUSSION

This study indicates that serogroup Y meningococci are still an uncommon cause of invasive disease in Italy, however the proportion of IMD isolates belonging to this serogroup increased in 2006 up to 7% of all isolates typed at the NRL, in agreement with the trend already described in other countries (CDC. 2007, EU-IBIS 2006). Moreover, all the serogroup Y meningococci received in 2006 belonged to ST-23 complex/Cluster A3, already described as the most frequent ST/Clonal Complex associated with invasive serogroup Y meningococci (http://neisseria.org/nm/typing/mlstdb/).
Interestingly, all these serogroup Y meningococci were ST-3171, of which to our knowledge there is no mention in the literature on invasive

TABLE 2 - *Molecular characteristics of serogroup Y strains isolated in Italy from 1998 to 2006.*

	MLST		*Alleles*							*porA*
Strain	*ST*	*Clonal complex*	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	*VR1, VR2, VR3*
1	23	ST-23/A3	10	5	18	9	11	9	17	5-2, 10-2, 36b
2	23	ST-23/A3	10	5	18	9	11	9	17	5-2, 10-2, 36b
3	23	ST-23/A3	10	5	18	9	11	9	17	5-2, 2-2, 36b
4	23	ST-23/A3	10	5	18	9	11	9	17	5-2, 10-1, 36b
5	168	ST-167	**2**	**16**	**6**	**17**	**9**	**18**	**8**	5-1, 10-4, 36b
6	23	ST-23/A3	10	5	18	9	11	9	17	5-2, 10-1, 36b
7	23	ST-23/A3	10	5	18	9	11	9	17	5-2, 10-1, 36b
8	23	ST-23/A3	10	5	18	9	11	9	17	5-2, 10-1, 36b
9	1466	ST-174	6	5	**173**	**13**	5	**24**	17	21, 16, 37-1
10	3974	ST-23/A3	10	5	18	9	11	**226**	17	5-2, 10-2, 36b
11	3171	ST-23/A3	10	5	18	9	11	9	**120**	5-2, 10-2, 36b
12	3171	ST-23/A3	10	5	18	9	11	9	**120**	5-2, 10-2, 36b
13	3171	ST-23/A3	10	5	18	9	11	9	**120**	5-2, 10-2, 36b
14	3171	ST-23/A3	10	5	18	9	11	9	**120**	5-2, 10-1, 36b
15	3171	ST-23/A3	10	5	18	9	11	9	**120**	5-2, 10-1, 36b
16	3171	ST-23/A3	10	5	18	9	11	9	**120**	5-2, 2-2, 36b
17	3171	ST-23/A3	10	5	18	9	11	9	**120**	5-2, 10-1, 36b
18	3171	ST-23/A3	10	5	18	9	11	9	**120**	5-2, 2-2, 36b

Alleles different from those of the main ST, the ST-23 of ST-23 complex/ClusterA3, are indicated in bold.

serogroup Y meningococci. This sequence type seems to have replaced ST-23 predominant in previous years.

The appearance in 2005 and the increase during 2006 of serogroup Y meningococci showing a decreased susceptibility to penicillin (PenI) is noteworthy. This phenotype is widespread all over Europe, especially among serogroup C strains, and extensively reported in the literature (Stefanelli *et al.*, 2003; Thulin *et al.*, 2006; Vázquez *et al.*, 2007). Fifty percent of meningococci serogroup Y belonging to ST-3171 of the ST-23 complex/Cluster A3 showed a decreased susceptibility to penicillin and were genetically related as shown by PFGE analysis.

This is the first report on the characterization of sporadic cases of *N. meningitidis* serogroup Y in Italy. Despite the low number of isolates, the data here reported suggest there is an increase in the presence of serogroup Y menincococci very likely due to the circulation, since 2005, of strains belonging to sequence type 3171.

ACKNOWLEDGMENTS

We are indebted to the microbiologists of the hospital laboratories participating in the Italian National Surveillance of Bacterial Meningitis.

This work was partially supported by the Ministry of Health-CCM grant n° N17 " Surveillance of Vaccine Preventable Diseases".

REFERENCES

Abdillahi H., Poolman J.T. (1987). Whole-cell ELISA for typing *Neisseria meningitidis* with monoclonal antibodies. *FEMS Microbiol. Lett.* **48**, 367-371.

Claus H., Maiden M.C.J., Wilson, D.J., McCatrhy N.D., Jolley K.A., Urwin R., Hessler F., Frosch M., Vogel U. (2005). Genetic analysis of meningococci carried by children and young adults. *J. Infect. Dis.* **191**, 1263-1271.

Connolly M., Noah N., on behalf of the European Meningitis Surveillance Group. (1999). Is serogroup C meningococcal disease increasing in Europe? A report of surveillance of meningococcal infection in Europe 1993-6. *Epidemiol. Infect.* **122**, 41-49.

Hartstein A., Phelps C., Lemonte A. (1995). Typing of sequential bacterial isolates by pulsed-field gel electrophoresis. *Diagn. Microbiol. Infect. Dis.* **22**, 309-314.

Maiden M.C.J., Bygraves J.A., Feil E., morelli G., Russel J.E., Urwin R., Zhang Q., Zhou J., Zurth K., Caugant D.A., Feavers I.M., Achtman M., Spratt B.G. (1998). Multilocus sequence typing: a portable approach to the identification of clones within population of pathogenic microorganisms. *Pro. Natl. Acad. Sci. USA.* **95**, 3140-3145.

McEllistrem M.C., Kolano J.A., Pass M.A., Caugant D.A., Mendelsohn A.B., Pacheco A.G.F., Schutt K.A., Razeq J., Harrison L.H., Maryland Emerging Infections Program (2004). Correlating epidemiologic trends with the genotypes causing meningococcal disease, Maryland. *Emerg. Infect. Dis.* **10**, 451-456.

Mölling P., Unemo M., Bäckman A., Olcén P. (2000). Genosubtyping by sequencing group A, B and C meningococci; a tool for epidemiological studies of epidemics, clusters and sporadic cases. *APMIS.* **108**, 509-516.

Pace D., Pollard A.J. (2007). Meningococcal A, C, Y and W-135 polysaccharide-protein conjugate vaccines. A*rch. Dis. Child.* **92**, 909-915.

Stefanelli P., Carattoli A., Neri A., Fazio C., Mastrantonio P. (2003). Prediction of decreased susceptibility to penicillin of *Neisseria meningitidis* strains by real-time PCR. *J. Clin. Microbiol.* **41**, 4666-4670.

Taha M.K. (2000). Simultaneus approach for noncolture PCR-based identification and serogroup prediction of *Neisseria meningitidis*. *J. Clin. Microbiol.*, **38**, 855-857.

Taha M.K., Vázquez J.A., Hong E., Bennett D.E., Bertrand S., Bukovski S., Cafferkey M.T., Carion F., Christensen J.J., Diggle M., Edwards G., Enríquez R., Fazio C., Frosch M., Heuberger S., Hoffmann S., Jolley K.A., Kadlubowski M., Kechrid A., Kesanopoulos K., Kriz P., Lambertsen L., Levenet I., Musilek M., Paragi M., Saguer A., Skoczynska A., Stefanelli P., Thulin S., Tzanakaki G., Unemo M., Vogel U., Zarantonelli M.L. (2007). Target gene sequencing to characterize the penicillin G susceptibility of *Neisseria meningitidis*. *Antimicrob. Agents Chemother.* **51**, 2784-2792.

Tenover F.C., Arbeit R.D., Goering R.V., Persing D.H., Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.*, **33**, 2233-2239.

Thulin S., Olcén P., Fredlund H., Unemo M. (2006). Total variation in the *pen*A gene of *Neisseria meningitidis*: correlation between susceptility to beta-lactam antibiotics and *pen*A gene heterogeneity. *Antimicrob. Agents Chemother.* **50**, 3317-3324.

Trotter C.L., Chandra M., Cano R., Larrauri A., Ramsay, M.E., et al. (2007). A surveillance network for meningococcal disease in Europe. *Antimicrob. Agents Chemother.* **51**, 27-36.

Vázquez J.A., Enriquez R., Abad R., Alcalà B., Salcedo C., Arreaza L. (2007). Antibiotic resistant meningo-

cocci in Europe: Any need to act? *FEMS Microbiol. Rev.* **31**, 64-70.

VÁZQUEZ J.A. (2007). Resistance testing of meningococci: the recommendations of the European Monitoring Group on Meningococci. *FEMS Microbiol. Rev.* **31**, 97-100.

YAZDANKHAH, S. P., KRIZ, P., TZANAKAKI, G., KREMASTINOU, J., KALMUSOVA, J., ET AL. (2004). Distribution of serogroups and genotypes among disease-associated and carried isolates of *Neisseria meningitidis* from the Czech Republic, Greece and Norway. *J. Clin. Microbiol.* **42**, 5146-5153.

ZHANG QY., JANES D.M., SÀEZ NIETO J.A., PEREZ TRALLERO E., SPRATT B.G. (1990). Genetic diversity of penicillin-binding protein 2 genes of penicillin resistant of *Neisseria meningitidis* revealed by fingerprinting of amplified DNA. *Antimicrob. Agents Chemother.* **34**, 1523-1528.