

Genetic characterisation of the emerging invasive *Neisseria meningitidis* serogroup Y in Sweden, 2000 to 2010

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Neisseria meningitidis serogroups B and C have been responsible for the majority of invasive meningococcal disease in Europe. Recently, an increase of *N. meningitidis* disease due to serogroup Y has been noted in Sweden (in 2010, the proportion was 39%, with an incidence of 0.23 per 100,000 population), as well as in other northern European countries. We aimed to investigate the clonal pattern of the emerging serogroup Y in Sweden during 2000 to 2010. The serogroup Y isolates identified during this time (n=85) were characterised by multilocus sequence typing and sequencing of the *fetA*, *fHbp*, *penA*, *porA* and *porB* genes. The most frequent clone (comprising 28 isolates) with identical allele combinations of the investigated genes, was partly responsible for the observed increased number of *N. meningitidis* serogroup Y isolates. It was sulfadiazine resistant, with genosubtype P1.5-2,10-1,36-2, sequence type 23, clonal complex 23, *porB* allele 3-36, *fetA* allele F4-1, *fHbp* allele 25 and *penA* allele 22. The first case with disease due to this clone was identified in 2002: there was a further case in 2004, six during 2006 to 2007, eight during 2008 to 2009, with a peak of 12 cases in 2010. An unusual increase of invasive disease in young adults (aged 20–29 years) caused by this clone was shown, but no increase in mortality rate was observed.

Introduction

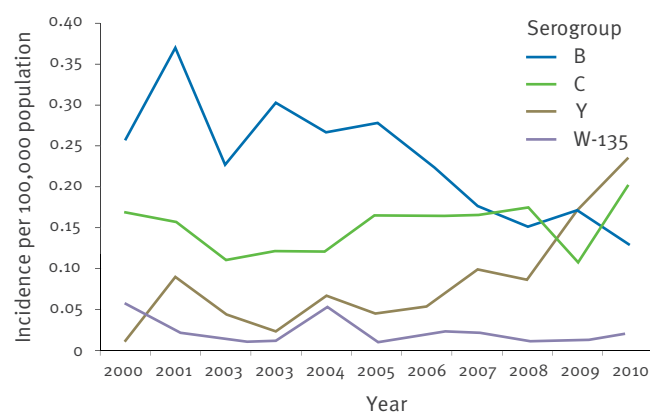
Neisseria meningitidis is a Gram-negative bacterium that is an obligate commensal of humans: it is carried without symptoms in the pharynx by about 10% of the general population. It can occasionally cause severe infection, mainly septicaemia and meningitis, when it crosses the epithelial barrier to invade the bloodstream and the meninges [1]. Meningococcal disease is most common in infants but the carriage rate is highest in young adults. Despite treatment and modern intensive care, the disease is still fatal in about 10% of cases [2].

N. meningitidis is divided into different serogroups, depending on the biochemical composition of the capsule surrounding the bacterium, but only isolates belonging to the A, B, C, W-135, X and Y serogroups

have a major role in causing disease [1]. Different hyperinvasive lineages of *N. meningitidis* cause disease with a unique epidemiology and the distribution of the serogroups is highly regional [2]. In Africa, especially in the so-called meningitis belt, the disease is mostly caused by serogroup A, but serogroups C, W-135 and X may also be involved. In Asia, serogroup A is also the most common serogroup. In Europe, North and South America and Australia, serogroups B and C have for decades been the dominating serogroups [3]. In the mid-1990s, however, the incidence of disease due to serogroup Y increased substantially in the United States (US) and today one third of the *N. meningitidis* infections in that country are caused by this serogroup [4,5]. During the last decade, there has also been an increase of meningococcal disease caused by serogroup Y in Canada and Colombia [6,7]. In addition, some northern European countries, displayed higher proportions of disease caused by this serogroup: for example, in Norway in 2009 and 2010, the proportion was 31%; in Finland, it was 38% in 2010 [8].

FIGURE 1

Incidence of invasive *Neisseria meningitidis* caused by serogroups B, C, Y and W-135, Sweden, 2000–2010^a



^a In 2010, the number of *N. meningitidis* isolates were: 12 (serogroup B), 19 (serogroup C), 22 (serogroup Y) and 2 (serogroup W-135).

Until recently, meningococcal disease in Sweden has followed the European serogroup distribution pattern, with the disease being caused mainly by serogroups B and C. However, the incidence of invasive *N. meningitidis* caused by serogroup Y started to increase in Sweden in the mid-2000s, rising from 0.04 per 100,000 population in 2005 to 0.23 per 100,000 population in 2010 (Figure 1). The reasons for the dramatic shift in serogroup distribution are unknown. One explanation could be that a new serogroup Y clone has been introduced in Sweden. To investigate this, extensive genetic characterisation must be conducted.

The European Meningococcal Disease Society has published a designation scheme for genetic characterisation of *N. meningitidis* based on serogroup, genotype based on analysis of the variable regions of the *porA* gene (encoding porin A, an outer membrane protein), *fetA* (encoding FetA, an iron-regulated outer membrane protein), sequence type (ST) and clonal

complex (cc), the last two being determined by multi-locus sequence typing (MLST) [9]. In addition, other gene targets such as *porB* (encoding porin B, another outer membrane protein), *penA* (encoding penicillin-binding protein 2, involved in penicillin susceptibility) and *fHbp* (encoding factor H-binding protein, which is a promising novel vaccine antigen) can be used for more discriminatory characterisation.

The aim of this study was to investigate by genetic characterisation the clonal pattern of the emerging invasive *N. meningitidis* serogroup Y isolates in Sweden between 2000 and 2010.

Methods

Bacterial isolates

The Swedish Institute for Infectious Disease Control (SMI) is mandatorily notified of all invasive cases of meningococcal disease by clinicians, using the European Union

TABLE 1

Primers for all target genes used in real-time PCR to characterise invasive *Neisseria meningitidis* serogroup Y isolates, Sweden, 2000–2010 (n=85)

Target gene	Primer	Primer sequence (5'→3')	Length of amplicon ^a (base pairs)	Reference
<i>abcZ</i>	P ₂ C	TCCCCGTCGTAAAAACAATC	856	[14]
	P ₁ C	TGTTCCGCTTCGACTGCCAAC		
<i>adk</i>	P ₁ B	CCAAGCCGTGTAGAATCGTAAACC	697	
	S ₂	CAATACTTCGGCTTTCACGG		
<i>aroE</i>	P ₁ B	TTTGAAACAGGCGGTTGCGG	835	
	P ₂ B	CAGCGGTAATCCAGTGCGAC		
<i>fumC</i>	S ₁	TCCGGCTTGCCGTTTGTCAG	530	
	S ₂	TTGTAGGCGGTTTGGCGAC		
<i>gdh</i>	P ₁ B	CTGCCCCGGGGTTTTCATCT	677	
	P ₂ B	TGTTGCGCGTTATTTCAAAGAAGG		
<i>pdhC</i>	P ₁ B	CCGGCCGTACGACGCTGAAC	818	
	P ₂ B	GATGTCGGAATGGGGCAAACA		
<i>pgm</i>	P ₁	CTTCAAAGCCTACGACATCCG	1,186	
	S ₂ A	GGTGATGATTTCGGTYGCRCC		
<i>penA</i>	1F	ATCGAACAGGCGACGATGTcb	697	[15]
	ModGcDown3-R	CGGGGATATAACTGCGGCCGTC ^c		[16]
	1R ^d	GATTAAGACGGTGTGTTGACGG ^c	512	[15]
<i>fetA</i>	s12	TTCAACTTCGACAGCCGCCTT ^b	429	[17]
	s15	TTGCAGCGCGTCRTACAGGCG ^c		
<i>fHbp</i>	F	TGACCTGCCTCATTGATGC	950	[18]
	R	CGGTAAATTATCGTGTTGCGACGGC		
	5CE2086 mod ^d	TATGACTAGGAGYAAACCTG	882	Modified from [19]
<i>porB</i>	S ₁	GCAGCCCTTCCTGTTGCAGC	973	[17]
	S ₂	TTGCAGATTAGAATTTGTG		

^a Amplicon lengths in MC58 [13].

^b Universal forward sequence adaptor (5'-GTTTTCCAGTCACGACGTTGTA-3') added to the 5'-end of the primer [15].

^c Universal reverse sequence adaptor (5'-TTGTGAGCGGATAACAATTTC-3') added to the 5'-end of the primer [15].

^d The penA1R and fHbp 5CE2086mod primers were used to complement the penA ModGcDown3-R and fHbp F primers, respectively, due to polymorphism in the annealing sites.

case definition [10] and the corresponding isolates are sent to the Swedish National Reference Laboratory for Pathogenic Neisseria. Basic epidemiological data (age, sex, area of residence and clinical site of isolation) are gathered routinely for all isolates from cases. We analysed all invasive *N. meningitidis* serogroup Y isolates in Sweden between 2000 and 2010 (n=85) from a total of 637 invasive isolates collected during this time period. The isolates were from clinical specimens of cerebrospinal fluid (n=11), blood (n=73) and joint fluid (n=1) and were cultured on chocolate agar.

Serogrouping and genosubtyping

The isolates were routinely serogrouped by co-agglutination [11] and subsequently genosubtyped (analysis of *porA* variable regions), as previously described [12]. Antibiotic susceptibility was determined using Etest (AB Biodisk, Sweden). As a reference, a serogroup B strain MC58 [13] was included.

Real-time PCR

The DNA used for amplification and sequencing was prepared from bacterial colonies by boiling or using the Bullet BUGS'n BEADS kit (NorDiag ASA, Norway). The genes targeted in MLST (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC* and *pgm*), together with *fetA*, *fHbp*, *penA* and *porB*, were amplified by real-time PCR using the PCR primers shown in Table 1. In each PCR run, MC58 was used as positive control.

DNA sequencing and sequence alignment

The PCR products were purified by vacuum filtration and subsequently cycle sequenced. The primers used for sequencing of the genes used for MLST were those recommended [14], except for *pdhC*, for which the PCR primer P2B used to amplify *pdhC* was used for sequencing. The *fHbp* gene was sequenced with the respective PCR primers as well as gna1870 s2 and gna1870 s3 [18]. The *fetA* and *penA* genes were sequenced with the oligonucleotide sequences of the adaptors (Table 1) attached to the respective PCR primer. When *penA*1R was used, it was used for both PCR and sequencing. The *porB* gene was sequenced with the PCR primers as well as 8U, 8L [17] and PB7f2 (5'-TYGGCAACGTAACGC-3'), where Y is C or T.

The sequence alignments were assembled using ChromasPro software version 1.33 (Technelysium Pty Ltd, Australia). The different sequences were assigned allele numbers using the *N. meningitidis* sequence query database [20]. A clone was defined by all isolates sharing the same genosubtype, ST, sulfadiazine susceptibility and combination of *penA*, *fetA*, *fHbp* and *porB* alleles.

Data analysis

We evaluated whether each sequenced gene could be used to discriminate between strains, on the basis of Simpson's index of diversity [21]. The discrimination (D)-index determines the probability that two randomly picked strains will be separated into different typing groups. A high D-index (close to 1) divides the isolates into many small groups (high discriminatory capacity),

whereas a low D-index (close to 0) indicates that the typing target only divides the isolates into a few large groups (low discriminatory capacity). Confidence intervals of 95% were calculated [22].

Minimum-spanning trees of the MLST profile data were created to investigate the ST clusters. The links were determined with Prim's algorithm and the clustering was created with the BURST (based on related STs) algorithm [23]. The *porA* genosubtypes and *porB*, *penA*, *fetA* and *fHbp* alleles were subsequently distributed over the different STs in the minimum-spanning trees, to describe the variation of different genes within each ST.

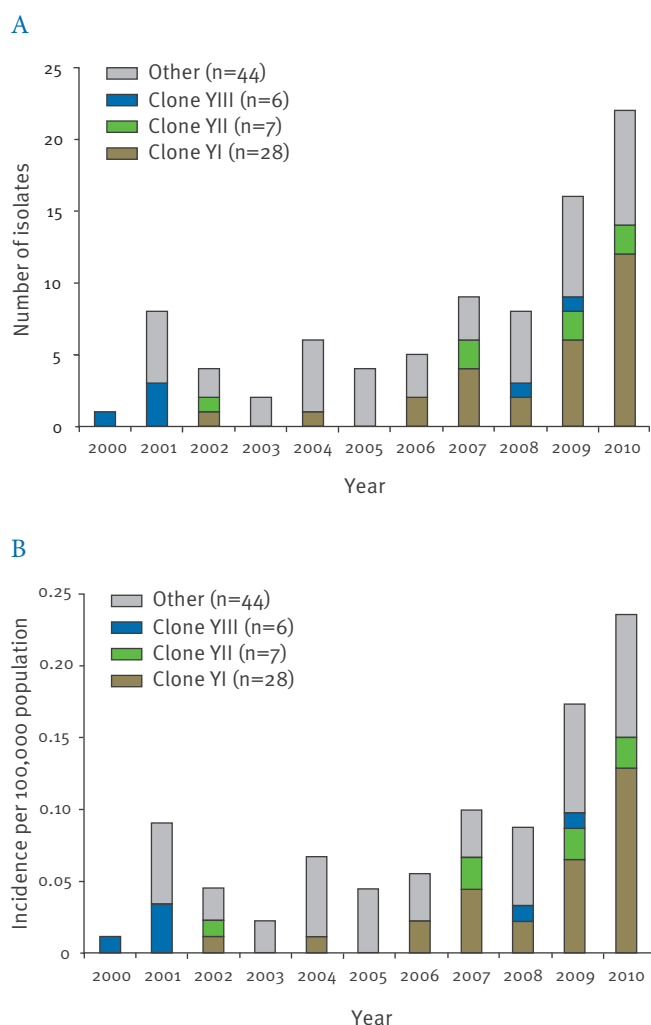
Results

Genetic characterisation

Using genosubtyping, MLST, and *porB*, *fetA*, *penA* and *fHbp* analysis, we found three distinctive clones comprising more than five isolates each. The most frequent clone (n=28), referred to as Clone YI, was sulfadiazine

FIGURE 2

Distribution over time of the three most common invasive *Neisseria meningitidis* serogroup Y clones (YI, YII and YIII) and all other invasive serogroup Y isolates, shown by (A) number of isolates and (B) incidence, Sweden, 2000–2010 (n=85)



resistant, with genosubtype P1.5-2,10-1,36-2, ST 23 (cc23), *porB* allele 3-36, *fetA* allele F4-1, *fHbp* allele 25 and *penA* allele 22. The second most frequent (Clone YII; n=7) was sulfadiazine susceptible, with genosubtype P1.5-1,2-2,36-2, ST 23 (cc 23), *porB* allele 2-55, *fetA* allele F5-8, *fHbp* allele 25 and *penA* allele 22. The third most frequent (Clone YIII; n=6) was sulfadiazine susceptible, with genosubtype P1.5-1,2-2,36-2, ST23 (cc23), *porB* allele 3-36, *fetA* allele F5-8, *fHbp* allele 25 and *penA* allele 1. The remaining 44 isolates included clones of two to five identical isolates (n=21) and 23 isolates with individual genetic profiles.

Distribution of clones and other isolates

Compilation of the epidemiological data on the isolates and genetic characterisation data generated a pattern of the distribution of the clones over time (Figure 2). There was no indication of an epidemiological link between the notified cases. The age distribution of patients with invasive disease caused by the three most common clones and all other serogroup Y isolates is shown in Figure 3. An increase of invasive disease caused by Clone YI was seen in young adults (aged 20–29 years). Overall, this clone was significantly more prone ($p<0.05$ Mann–Whitney U test) to cause disease in a younger age group (~median age: 47 years; interquartile range: 20–70), compared with the isolates with other genetic profiles. These other clones and isolates had the usual pattern of serogroup Y regarding age groups affected, i.e. they affected mainly elderly people (median age: 72 years; interquartile range: 36–84).

We also studied the geographical distribution of the three most common serogroup Y clones and all other serogroup Y isolates, which showed a higher incidence of Clone YI in young adults (aged 15–25 years) in the central parts of Sweden (data not shown). No such

geographical pattern was seen for elderly cases with invasive isolates of Clone YI or cases with Clone YII, Clone YIII or any of the other isolates.

The mortality rate among patients with disease caused by serogroup Y was 13% (11 of 85). Two of the 11 deceased patients were infected with Clone YI.

The genes that displayed the highest variability in the minimum-spanning trees (*porA*, *porB* and *fetA*) are shown in Figure 4; the *penA* and *fHbp* genes were found to be fairly conserved (data not shown). These findings were further confirmed by the D-indices calculated for the genes used in MLST, and *porA*, *porB*, *fetA*, *fHbp* and *penA* genes (Table 2).

Discussion and conclusion

Our results show that the most common invasive *N. meningitidis* serogroup Y isolate, clone YI, was partly responsible for the increase of meningococcal disease caused by this serogroup in Sweden in recent years. This clone was prevalent at the beginning of the 2000s, with one isolate being identified in both 2002 and 2004 (incidence of 0.01 per 100,000 population in each year). The number of isolates gradually increased, from two isolates in 2006 to a peak of 12 in 2010 (incidence of 0.02 and 0.13, respectively, per 1000,000 population). No outbreaks or clusters of cases due to serogroup Y infection were noted in Sweden during 2000 to 2010.

The reasons for the increased incidence of invasive disease caused by Clone YI, compared with the other clones and isolates, among younger people are unclear. However, a similar pattern for disease due to serogroup Y has been observed in Canada, where the proportion of cases with this serogroup in the age group 10–19 years increased from 11.8% in 1999 to 26.3% in 2003, with a peak of 41.4% in 2001 [6]. In the US, however, no such increase has been observed among persons aged 15–24 years [25].

FIGURE 3

Age distribution of patients with invasive meningococcal disease caused by the three most common *Neisseria meningitidis* serogroup Y clones (YI, YII and YIII) and all other invasive serogroup Y isolates, Sweden, 2000–2010 (n=85)

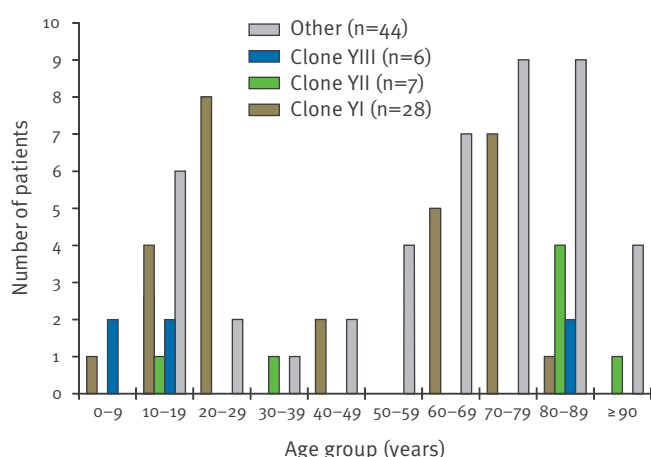


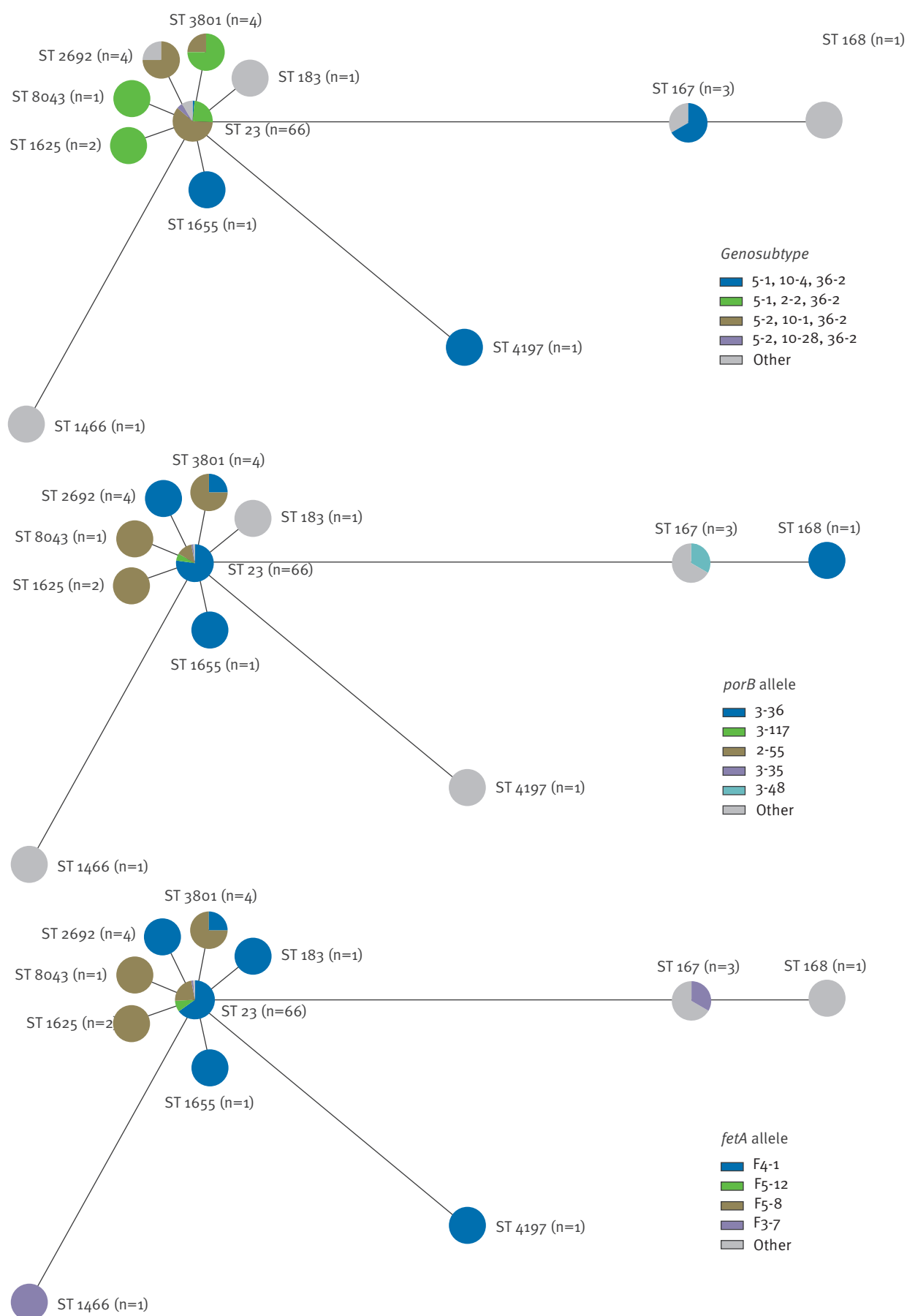
TABLE 2

Discrimination indices for sequence types and *porA*, *porB*, *fetA*, *fHbp*, *penA* genes, invasive *Neisseria meningitidis* serogroup Y isolates, Sweden, 2000–2010 (n=85)

Target	Number of types	Number (%) of isolates with the most common type	Discrimination index (95% CI)
<i>porA</i>	15	45 (53)	0.65 (0.57–0.74)
<i>fetA</i>	7	53 (62)	0.55 (0.45–0.65)
<i>porB</i>	9	58 (68)	0.51 (0.39–0.62)
Sequence types	12	66 (78)	0.39 (0.26–0.53)
<i>penA</i>	4	72 (85)	0.27 (0.15–0.39)
<i>fHbp</i>	6	78 (92)	0.16 (0.5–0.26)

FIGURE 4

Minimum-spanning trees based on multilocus sequence typing of all invasive *Neisseria meningitidis* serogroup Y isolates, Sweden, 2000–2010 (n=85)



The circles denote different sequence types. The genosubtype (*porA*) and *porB* and *fetA* alleles are distributed over the different sequence types.

The second and third most common clones in Sweden, YII and YIII, consisted of few isolates per clone, which made the age distribution for cases with these clones difficult to interpret. It is interesting to note that in Maryland, US, two clones with different antigenic profiles seemed to be responsible for the increased number of invasive *N. meningitidis* serogroup Y isolates in the 1990s and that the clones are differently distributed over time [25]. The first, which shares parts of the genetic profile of clones YII and YIII in our study (cc23; P1.5-1,2-2; F5-8), was mainly responsible for early cases (before 1998), while the other clone, with the same genetic profile as the dominant Clone YI in our study (cc23; P1.5-2,10-1; F4-1), has dominated since 1998. In the Czech Republic, the most common clone (cc23; P1.5-2,10-1,36-2; F4-1) and second most common clone (cc23; P1.5-1,2-2,36-2; F5-8) present indistinguishable *porA* and *fetA* profiles and ST as the most common clones in Sweden, in the same order of frequency [8].

Clone YI does not seem to be related to increased mortality, although a difference would be difficult to detect because of the low incidence of meningococcal disease in general. The mortality rate of all the studied invasive serogroup Y isolates (13%) is within the range of the overall global mortality rate for meningococcal disease caused by all serogroups (about 10%) [2], but is considerably lower than the mortality rate for disease due to serogroup Y in Sweden in 1995 to 2005 (17%) [26]. The decreased mortality rate may be the result of the increased incidence of Clone YI in a younger age group (median age: 47 years).

The emergence of serogroup Y Clone YI is presumably due to multiple underlying factors, but the most probable is that it is an epidemiologically competent clone with increased pathogenicity. It is less likely that host adaptive immunity has decreased in different geographical areas concurrently. It is also possible that Clone YI possesses characteristics that lead to increased transmission efficiency. This possibility is difficult to investigate and no dynamic carriage studies have been performed in Sweden. However, in some carriage studies from elsewhere in Europe and the US, it was found that serogroup Y was one of the most common serogroups among carrier isolates [27-30].

The results from the minimum-spanning tree and D-indices indicate that in the case of serogroup Y, targeting the *penA* and *fHbp* genes yields a less discriminatory pattern than MLST or targeting the *porA*, *porB* and *fetA* genes. Although *penA* and *fHbp* seem to be too conserved to be of use for further differentiation of clones, these genes are still of interest for following penicillin resistance and the effect of promising novel vaccines that include the factor H-binding protein. Although none of the individual genes in our study reached the recommended discrimination level of 90% [21], a combination of target genes and inclusion of other serogroups probably could. However, using only typing tools with high D-indices may result in missing

isolates belonging to the same clone. Similarly, the apparent frequency of the clone would increase if only stable genes are targeted, as in MLST. Therefore, Clone YI is defined by what we think is an informative and appropriate number of target genes, which is also somewhat supported by the minimum-spanning trees and D-indices.

Further research could be carried out using characterisation tools that would increase the discrimination, such as variable number tandem repeat (VNTR) analysis, to provide more information about the clone. However, it will also be a challenge to customise the characterisation tools, to achieve an appropriate balance between the information required for the particular investigation and the time and cost.

In conclusion, the clone responsible for the recent emergence of *N. meningitidis* serogroup Y isolates was identified and described in this study. To achieve a complete epidemiological profile of the clone, it would be useful to examine invasive serogroup Y isolates from before 2000 and carry out additional typing. The general increase of meningococcal disease due to *N. meningitidis* serogroup Y has an important public health implication because this is one of the serogroups that is covered by available vaccines for meningococcal disease.

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