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# Hyperinvasive genotypes of *Neisseria meningitidis* in France

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## ABSTRACT

Clinical isolates of *Neisseria meningitidis* from cases of meningococcal disease, collected between January 2000 and December 2004, were identified and typed at the French National Reference Centre. A representative subset of 546 isolates from among 2882 isolates was further genotyped by multilocus sequence typing to determine their genetic lineages (clonal complexes) and the degree of diversification among different clonal complexes. Representative isolates of the main clonal complexes were tested for their virulence in mice and for proapoptotic effects on human epithelial cells. High genetic diversity in some genetic lineages (ST-32 and ST-41/44) was correlated with heterogeneity in virulence in mice and proapoptotic effects on human epithelial cells. In contrast, the homogeneous genetic structure of isolates of the ST-11 clonal complex, regardless of their serogroup, correlated positively with a fatal outcome of the infection, increased virulence in mice and increased proapoptotic effects on human epithelial cells.

**Keywords** Apoptosis, epidemiology, genetic lineages, invasion, *Neisseria meningitidis*, virulence

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## INTRODUCTION

*Neisseria meningitidis* is found most frequently as a commensal bacterium of the human nasopharynx, but can occasionally cause a life-threatening invasive infection [1]. It is estimated that 10% of adults are asymptomatic carriers, although carriage rates can be affected by temporal, geographical and seasonal factors [2]. The annual incidence of invasive meningococcal infections in different countries of Europe varied between <1/100 000 population and up to 14.3 and 4.9/100 000 in 1999 and 2004, respectively [3].

Meningococcal isolates can be grouped, according to polymorphisms in seven housekeeping genes, into clonal complexes that comprise closely related isolates varying by no more than two loci [4]. Hyperinvasive isolates are found in a limited number of clonal complexes, although carriage isolates are very diverse [5–7]. New variants in the meningococcal population appear continuously following frequent horizontal exchanges of DNA [8]. Enhanced transmission and the acquisition of variants may increase the genetic heterogeneity of carriage isolates [9].

Meningococcal invasion depends on the ability of the bacterium to colonise and multiply in its host, and also on its ability to invade sterile sites, e.g., the blood and cerebrospinal fluid, leading to meningococcaemia and meningitis, respectively [10,11]. Induction of apoptosis of cells of the epithelial barriers may be an important feature involved in bacterial virulence and invasiveness, and it has been suggested that this feature correlates with the severity of the disease [12,13]. However, bacterial modifications that enhance virulence may not influence transmissibility/acquisition (ecological fitness). The small number of genetic lineages associated with invasive meningococcal infections may reflect the fact that the disease is not part of the transmission cycle, but is an epidemiological dead-end, as bacteria that have invaded the blood or the cerebrospinal fluid are no longer transmitted [10,11]. Indeed, isolates that express the phenotypes of invasive isolates are rare in the overall meningococcal population.

It is not clear why invasive isolates are clustered in a few clonal complexes and what limits

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their diversification. Therefore, isolates of major clonal complexes are often considered to be hyper-virulent, mainly on the basis of their involvement in epidemics with high fatality rates, but without any evidence of a specific genetic profile of virulence and pathogenicity, despite the availability of fully sequenced genomes [14]. Therefore, the aims of the present study were to analyse the genetic structure and diversification of a large collection of clinical isolates from invasive meningococcal infections in France, spanning a 5-year period, and to analyse the functional impact of the genetic structure and diversification of isolates of each main clonal complex on their virulence in mice and on the induction of apoptosis of human epithelial cells.

## MATERIALS AND METHODS

### Characterisation of bacterial isolates

Isolates from cases of invasive meningococcal infection in France are sent systematically to the National Reference Centre for Meningococci for full characterisation and typing. Epidemiological and clinical data, including whether there was an immediate fatal outcome (<24 h after admission), are also recorded. Bacteria were grown on plates of GCB medium with Kellogg supplements [15]. Serogroups were determined by agglutination using specific in-house rabbit antibodies to *N. meningitidis* serogroups A, B, C, Y and W135. Phenotypes (serogroup:serotype:serosubtype) were determined as described previously [16].

Isolates were genotyped by multilocus sequence typing (MLST) as described previously [4,17]. Alleles, sequence types (STs) and clonal complexes were assigned using the *Neisseria* MLST database (http://pubmlst.org/neisseria). Cluster analysis was performed using the BURST algorithm as described previously [18] (http://www.mlst.net). The BURST algorithm clusters isolates if they differ by no more than two loci, and then defines an ancestral ST (founder ST) in each cluster; it also defines STs varying by a single locus (single-locus variants; SLVs), STs varying by two loci (double-locus variants; DLVs) and satellite STs that vary by more than two loci, but which are still linked indirectly because they are no more than two loci different from the other SLVs or DLVs.

Seven selected isolates from each of the main clonal complexes, representing the most frequent phenotypes, were studied in virulence and proapoptotic assays. The isolates were as follows: isolates of clonal complex ST-8 were LNP19935 (C:2a:P1.2,5), LNP21089 (C:2b:P1.2), LNP21317 (C:2b:P1.5,2), LNP21359 (C:2b:P1.2), LNP21661 (C:2b:P1.5,2), LNP21716 (C:2b:NST) and LNP21846 (B:2b:NST); isolates of clonal complex ST-11 were LNP17592 (W135:2a:P1.5,2), LNP19008 (C:2a:P1.5,2), LNP20342 (B:2a:P1.5), LNP20553 (C:2a:P1.5), LNP21515 (C:2a:P1.5), LNP21678 (C:2a:P1.5) and LNP21996 (B:2a:P1.5); isolates of clonal complex ST-32 were LNP19263 (B:15:P1.7), LNP19785 (B:14:P1.15), LNP19830 (B:4:P1.7,1), LNP20404 (B:14:P1.7,16), LNP21362 (B:14:P1.7,16), LNP21743 (B:4:NST) and LNP21866 (B:14:P1.7,16); and isolates of clonal complex ST-41/44 were LNP20927 (B:4:P1.4), LNP21521 (B:4:P1.4), LNP21680 (B:NT:NST), LNP21756 (C:NT:NST), LNP21784 (B:15:P1.4), LNP21806 (B:14:NST) and LNP21861 (B:4:P1.4).

### Virulence in mice

The virulence of the different isolates from the main clonal complexes was tested in a mouse model of sequential influenza A virus (IAV)–*N. meningitidis* infection [19]. In brief, female BALB/c mice aged 6 weeks (Janvier, Le Genet-Saint-Isle, France) were infected by intra-nasal administration of a 50-μL suspension containing 250 plaque-forming units of the IAV strain A/Scotland/H3N2/23/74, leading to transient pneumonia followed by spontaneous recovery and complete viral clearance within 10 days [19]. The mice were superinfected intra-nasally on day 7 with $1–5 \times 10^7$ CFU/mouse of each meningococcal isolate. Bacterial counts were determined in the lungs and blood of three mice/experiment at 24 h after meningococcal challenge by plating blood or lung homogenate samples on GCB medium. Data were analysed using the chi-square test, Student's *t*-test and analysis of variance (ANOVA), with $p \leq 0.05$ considered to be statistically significant.

### Induction of apoptosis of epithelial cells

Hec1-B epithelial cells were seeded in flat-bottomed 96-well tissue culture plates (Techno Plastic Products, Trasadingen, Switzerland) at a density of $5 \times 10^5$ cells/mL (100 μL/well) and incubated overnight at 37°C. The cells were then washed three times with phosphate-buffered saline and replenished with fresh medium. The cells were then infected with 100 μL of bacterial suspension at a multiplicity of infection of 10:1. Negative (non-infected cells) and positive (staurosporin-treated cells at a final concentration of 1 μM) controls were included in each experiment. Incubation was continued for 1, 4, 9 or 24 h at 37°C. The cells were then washed gently and stained using ApoPercentage (Biocolor Ltd, Newtownabbey, UK) according to the manufacturer's recommendations. Results were expressed as the mean $A_{550}$ values of assays performed in triplicate.

## RESULTS

### Meningococcal genotypes

In total, 2882 clinical isolates were received by the National Reference Centre for Meningococci between January 2000 and December 2004; of these, 546 (19%) were randomly selected to form the study set for analysing the genetic structure of the meningococcal population. The serogroup distribution among the total population (2882 isolates) of invasive isolates and the study set was equivalent, with 53% and 56% belonging to serogroup B, 33% and 30% to serogroup C, 10% and 9% to serogroup W135, and 3% and 3% to serogroup Y, respectively. It is worth noting that several isolates of serogroup W135, corresponding

to the Hajj-linked outbreak in 2000 [20], were included in the study set.

MLST identified 152 different STs among the 546 study set isolates. The STs were clustered into 15 clonal complexes, accounting for 506 (92.7%) isolates, with the remaining 40 (7.3%) isolates belonging to 26 STs that could not be assigned to any known clonal complex. The five most frequent clonal complexes were ST-11 ($n$ = 183, 33.5%), ST-41/44 ($n$ = 143, 26.2%), ST-32 ($n$ = 55, 10%), ST-8 ($n$ = 46, 8.4%) and ST-269 ($n$ = 31, 5.7%). The ten remaining clonal complexes each occurred at a frequency of <1.5%.

The percentages of immediate fatal cases (i.e., death occurring in <24 h of admission) in the two sets were equivalent (8.8% and 9%, respectively, p 0.908). However, immediate fatality was significantly higher for cases involving clonal complex ST-11, which had a case fatality rate of 16%, compared to 7.1% for the entire population of isolates.

### Phenotypic and genotypic diversification within clonal complexes

The diversification of isolates within each of the four main clonal complexes was scored by calculating the percentage of SLVs, DLVs and satellite variants. Clonal complex ST-11 showed the least diversification, with only 10.4% of STs (mostly SLVs) differing from the founder ST. The proportion of diversified isolates (SLVs and DLVs) among the isolates of clonal complex ST-11 was significantly lower than that among the other isolates (p <0.0001). In contrast, isolates of clonal complexes ST-32 and ST-41/44 were more diversified than isolates of clonal complex ST-11 (p <0.001). The percentages of SLV, DLV and satellites were 39.5%, 19.7% and 5.7%, respectively, for ST-32, and were 23.6%, 10.8% and 15.3%, respectively, for ST-41/44. In clonal complex ST-8, 48% of isolates were variants, but these were mostly SLVs, with only 16% DLVs and no satellite variants.

Each of the four major clonal complexes (ST-11, ST-41/44, ST-32 and ST-8) contained a predominant serogroup (C, B, B and C, respectively). However, clonal complex ST-11, which was mostly serogroup C, also harboured a significantly high proportion of serogroups B and W135 (28.4%, p <0.00001). In contrast, the highly diversified clonal complex ST-32 comprised only serogroup B isolates. The other highly diversified clonal complex ST-41/44 comprised mainly serogroup B isolates, with only 4.2% of the isolates belonging to other serogroups.

### Impact of clonal complex diversification on virulence in mice

All of the meningococcal isolates tested induced pneumonia with subsequent bacteraemia in the mouse model of sequential IAV–*N. meningitidis* respiratory challenge. Isolates from the less diversified clonal complexes (ST-11 and, to a lesser extent, ST-8) generated higher bacterial loads in lungs at 24 h after infection (p <0.001 and p <0.05, respectively) (Table 1). Equivalent CFU counts were observed in the blood for all isolates. Bacterial counts in lungs for the ST-11 isolates, regardless of their serogroup (B, C or W135), were homogeneous, as indicated by the narrow 95% CI (Table 1). The W135 ST-11 isolate corresponded to the Hajj outbreak [20]. In contrast, isolates of ST-8, ST-32 and ST-41/44, belonging to serogroups C, B and B, respectively, showed variable CFU counts in lungs, depending on the challenge strain, with wider 95% CIs (Table 1).

### Induction of apoptosis

Isolates from each of the four major clonal complexes, ST-11 ($n$ = 5), ST-41/44 ($n$ = 5), ST-32 ($n$ = 4) and ST-8 ($n$ = 4), were analysed for their ability to induce apoptosis at 1, 4, 9 and 24 h after infection. All ST-11 isolates induced apoptosis, particularly at 24 h after infection. In contrast, only some isolates belonging to the other STs (ST-8, ST-32 and ST-41/44) were able to induce apoptosis (Fig. 1).

These findings correlated with the differences in immediate fatality associated with the isolates belonging to the different clonal complexes, with 25 of 49 immediately fatal cases being caused by

**Table 1.** Virulence in mice of isolates from the main clonal complexes

| Clonal complex | Blood[a] (95% CI) | Lungs[a] (95% CI) |
|---|---|---|
| ST-11 | 1.74 ± 0.21 (1.43–1.86) | 6.98 ± 0.71 (6.14–7.50) |
| ST-41/44 | 1.31 ± 0.45 (1.06–1.96) | 4.05 ± 0.95 (3.17–5.33) |
| ST-32 | 1.73 ± 0.45 (1.28–2.11) | 3.92 ± 0.95 (3.17–5.21) |
| ST-8 | 1.67 ± 0.61 (1.49–3.03) | 6.39 ± 0.0.98 (5.19–7.0) |

ST, sequence type.
[a]Geometric mean of $\log_{10}$ CFU/mL ± SE of samples collected at 24 h after infection from four mice/isolate, following challenge with seven different isolates of each clonal complex.

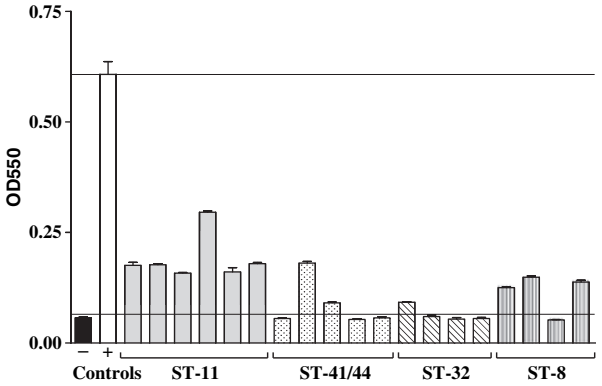


**Fig. 1.** Ability of representative isolates from the four most frequent clonal complexes to induce apoptosis of Hec-1-B cells at 24 h after infection. Negative controls (white bar) were uninfected cells and positive controls (black bar) were cells treated with staurosporin at a final concentration of 1 μM. Experiments were performed in triplicate and the results are expressed as geometric means of absorbance values at 550 nm ($OD_{550}$) ± standard deviation (bars).

isolates of clonal complex ST-11 (p 0.026). In contrast, no significant correlation was revealed between serogroup and an immediate fatal outcome.

## DISCUSSION

A few genotypes of *N. meningitidis* are known to be prominent in invasive infections [6,21]. Transmissibility and virulence are not linked, since invasive infection is a dead end in the natural evolutionary life-cycle of *N. meningitidis* [10]. The continuous genetic evolution caused by horizontal transfer of DNA and allelic recombination would lead to genotypic and phenotypic diversification of transmissible isolates [22,23]. This can be tested using functional and genomic analysis by sequencing the same regions in these isolates. Such an approach may be more informative than completely sequencing the genome of just a few isolates [24].

The present study set of 546 isolates, selected from among 2882 invasive isolates, was representative of the entire population of invasive *N. meningitidis* strains in France during the period 2000–2004. The most striking findings were the high number of isolates belonging to clonal complex ST-11, and the low level of diversification within this complex, with only 10.4% of isolates differing from the founder ST. However, this low level of diversification should be further confirmed by analysing polymorphism in a larger set of genes (i.e., more than the seven housekeeping gene fragments used in the MLST analysis). The low level of diversity observed might indicate the recent emergence of this clonal complex, unlike the other, probably older, clonal complexes (ST-8, ST-32, ST-41/44 and ST-269) that contain more SLVs, DLVs and satellite variants, as determined using the BURST algorithm. The epidemiological data are consistent with this hypothesis, since the meningococcal outbreaks observed during the study period were caused by isolates of clonal complex ST-11 belonging to serogroups C and W135 [25,26].

Virulence assays using isolates belonging to clonal complex ST-11 generated homogeneous results in mice in comparison with isolates from the other clonal complexes tested, irrespective of their serogroup. Several studies have suggested that apoptosis can be manipulated by infectious microorganisms [13]. Inducing apoptosis during sepsis has also been suggested to correlate with the severity of the disease [13]. All ST-11 isolates tested were able to induce apoptosis in Hec-1-B epithelial cells, but this induction was heterogeneous in isolates belonging to other genetic lineages.

These data emphasise the importance of molecular typing of *N. meningitidis* isolates in predicting meningococcal virulence, as opposed to simply determining the serogroup. A serogroup C to serogroup B capsule switch in clinical isolates belonging to clonal complex ST-11 has recently been reported, with conserved virulence [27]. Moreover, isolates belonging to this clonal complex were associated more significantly with an immediate fatal outcome than were isolates belonging to the other invasive clonal complexes in the present study set. Immediate fatality may be a clinical marker of hyper-virulence of meningococcal isolates. An association between fatal meningococcal disease and meningococcal clonal complex ST-11 has also been observed in *N. meningitidis* isolates from culture-confirmed cases of meningococcal disease in England and Wales between 1993 and 2000 [28]. Similar findings have been reported for *Streptococcus pneumoniae*, in which both genotype and capsular type determine pathogenic behaviour [29].

The present results obtained with a large collection of invasive isolates raise questions concerning the basis for the concept of 'hyper-virulence' in

*N. meningitidis*. Such a concept can hardly be applied to a bacterium that is normally found in healthy carriers and for which no structural basis of pathogenicity has been revealed by full genome sequencing [14]. Moreover, carriage isolates seem to differ genetically and phenotypically from invasive isolates [6]. Epidemic hyperinvasive isolates have been shown to be present only rarely among carriers [5] However, the present study suggests that some virulence-associated criteria can be assigned to the main invasive meningococcal genotypes. Isolates of the ST-11 clonal complex were genetically homogeneous, virulent in mice (Table 1) and induced apoptosis (Fig. 1), regardless of their serogroup, and can clearly be considered to be hyper-virulent, even in an exposed immunocompetent host, following acquisition. This was less obvious for ST-8, and not at all obvious for ST-32 and ST-41/44, which were represented by isolates that were heterogeneous in terms of virulence in mice and that were less proapoptotic. In such cases, expression of meningococcal virulence may depend to a greater degree on an increased susceptibility of the exposed host, either because of genetic defects [1] or because of associated co-factors of meningococcal infection, e.g., influenza [19].

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