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ABSTRACT Neisseria meningitidis, a leading cause of bacterial meningitis and other serious infections, is responsible for approximately one-third of cases of bacterial meningitis in the Children's Hospital of Tunis. The serogroup distribution, antibiotic susceptibility and antigenic and molecular characteristics of N. meningitidis isolates were determined in patients aged 3 days–13 years between February 1998 and June 2013. In all 107 invasive strains of N. meningitidis were isolated. Reduced susceptibility to penicillin G was seen in 55.7% of isolates, with a low level of resistance in all cases; 28.4% showed a low level of resistance to amoxicillin. Serogroup B isolates were the most frequent (80.4%), followed by serogroups C (12.2%) and A (5.6%). Isolates of serogroup A had the same antigenic formula (A:4:P1.9), the same variable regions VR1, VR2 and VR3, and belonged to the same clonal complex (CC5). Isolates of serogroups B and C were more heterogeneous with several antigenic formulae. The most frequent clonal complex in these isolates was CC35. Serogroup B accounted for a large

percentage of our isolates with marked diversity.

Caractérisation de souches invasives de Neisseria meningitidis isolées à l'Hôpital d'enfants de Tunis, en Tunisie

RÉSUMÉ La bactérie Neisseria meningitidis, l'une des causes principales de méningite bactérienne et d'autres infections graves, est responsable d'environ un tiers des cas de méningite bactérienne à l'Hôpital d'enfants de Tunis. La distribution par sérogroupe, la sensibilité aux antibiotiques et les propriétés antigéniques et moléculaires des isolats de N. meningitidis ont été déterminées chez des patients âgés de 3 jours à 13 ans entre février 1998 et juin 2013. En tout, 107 souches invasives de N. meningitidis ont été isolées. Une sensibilité réduite à la pénicilline G a été observée dans 55,7 % des isolats, avec un bas niveau de résistance pour tous les cas ; et 28,4 % ont montré un bas niveau de résistance à l'amoxicilline. Les isolats du sérogroupe B étaient les plus fréquents (80,4 %), suivis par les sérogroupes C (12,2 %) et A (5,6 %). Les isolats du sérogroupe A avaient la même formule antigénique (A:4:P1.9), les mêmes régions variables VR1, VR2 et VR3, et appartenaient au même complexe clonal (CC5). Les isolats des sérogroupes B et C étaient davantage hétérogènes, avec plusieurs formules antigéniques. Le complexe clonal le plus fréquent de ces isolats était CC35. Le sérogroupe B comptait pour un pourcentage élevé des isolats avec une diversité marquée.

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Received: 16/05/15; accepted: 17/03/16

Introduction

Neisseria meningitidis is a major public health concern, particularly in children (1). Invasive meningococcal infections can occur in different clinical forms. Meningococcal meningitis is the most common entity and the outcome is usually good if patients are rapidly and well treated. However, the less common septic shock with the classical purpura fulminans is rapidly fatal (1,2). The case-fatality rate of invasive disease is ~10% in treated cases but varies widely depending on the clinical presentation and the infecting isolate. The highest case-fatality rates are observed in meningococcaemia without meningitis (2,3).

The natural reservoir of N. meningitidis is the human nasopharyngeal mucosa. The carriage rate of N. meningitidis is

Unencapsulated isolates are commonly found in the nasopharynx in asymptomatic carriers but rarely cause invasive disease. Carriage induces a systemic humoral response, which is strain specific. Usually, invasive meningococcal isolates are encapsulated (5). The capsular polysaccharide is the major virulence factor for this bacterium. The composition of the capsule defines 12 different serogroups.

Meningococcal infections can be avoided using capsule-based vaccines. However, such vaccines are serogroup specific. Serogroups included in meningococcal vaccines are A, C, W and Y. Two types of capsule-based vaccines are currently available (6,7). The first is the meningococcal polysaccharide vaccine that has been available since 1970 (8). More recently, meningococcal conjugate vaccines have been developed (3). In addition, protein-based vaccines targeting serogroup B isolates have been licensed in Europe, Canada, US and Australia (9).

Few data are available from North African countries about serogroup distribution and molecular characterization of N. meningitidis, particularly in children (10,11). North African countries are located between sub-Saharan Africa, where isolates of serogroups A and W are responsible for periodic epidemics of meningococcal meningitis (12) and Europe, where isolates of serogroup B are predominant and responsible for sporadic cases of meningococcal invasive diseases (13). In North African countries, serogroup A and B isolates are predominant. Serogroup A strains are homogeneous and have the same antigenic formula, A:4:P1.9 (2,10,11,14), as the pandemic clone that caused large epidemics in many African countries within the meningitis belt between 1990 and 1997 (14,15). In Tunisia, previous studies have involved a small number of isolates (n = 23), and have demonstrated that serogroup B is predominant among invasive N. meningitidis isolates (2). The multi- component meningococcal B vaccine (4CMenB) is not yet licenced in Tunisia, so a study of a larger collection of isolates allows us to better guide the prophylaxis and specify possible large-scale vaccination. N meningitis is one of the commonest organisms responsible for meningitis in our country. However, few data on the isolation rate and antibiotic susceptibility pattern are available. Such data are valuable for the empirical treatment of patients.

The aim of this study was to explore the serogroup distribution, antibiotic susceptibility and antigenic and molecular diversity of meningococcal isolates in a paediatric population in Tunisia in order to guide the empirical treatment and prophylaxis of invasive meningococcal diseases.

Methods

Bacterial isolates

We studied 107 N. meningitidis isolates obtained from clinical specimens at the Children's Hospital of Tunis from February 1998 to June 2013. After storage at –80 °C, bacterial isolates were grown at 37 °C in 5% CO_2 in GC medium base (Difco, Bordeaux, France) with Kellogg supplements. Culturing, identification and typing of the isolates are all part of the routine management and surveillance of meningococcal disease according to the guidelines of the Tunisian Department of Health.

Antibiotic susceptibility

Antibiotic susceptibility was performed by the disc diffusion method and included penicillin G, amoxicillin, cefotaxime, chloramphenicol and rifampin. Decreased susceptibility to beta-lactam antibiotics was detected using a 5-µg oxacillin disc. The minimum inhibitory concentrations (MICs) of these antibiotics were defined by E-test methods (bio-Mérieux, Marcy l'Etoile, France) on Mueller–Hinton agar (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 5% sheep blood and incubated at 37 °C in 5% CO_2 for 18–24 h. Results were interpreted according to the Antibiogram Committee of the French Society for Microbiology (CA-SFM) guidelines (www.sfm-microbiologie.org). Beta-lactamase was detected using a nitrocefin disc (bio-Mérieux). Internal quality control for antimicrobial susceptibility testing was performed using Pseudomonas aeruginosa ATCC 27553, Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923 strains according to CA-SFM guidelines.

Serological typing

Serogroups were determined for all isolates by slide agglutination using specific antibodies of N. meningitidis serogroups A, B, C, W and Y (Pastorex Meningitis; BioRad, France). Isolates were sent to the Institut Pasteur, Paris, France for phenotyping (serotypes and serosubtypes), which was determined for 56 isolates that were successfully recovered before February 2007 (41 serogroup B, 8 serogroup C, 6 serogroup A and 1 serogroup Y). Phenotyping was performed using whole-cell ELISA, as previously described (16,17). Sera for 6 serotypes (1, 2a, 2b, 4, 14, 15) and 13 serosubtypes (P1.1, 2, 4–7, 9, 10 and 12–16) were tested. Antigenic formula was determined by the combination of serogroup: serotype: serosubtype.

Molecular typing

Genomic DNA extraction of meningococcal isolates was performed using the thermal shock method. The DNA extracted was stored at –20 °C. All isolates were genogrouped using a capsule multiplex polymerase chain reaction (PCR) (18). Only isolates before February 2007 (n = 56) were further genotyped by porA sequencing and multilocus sequence typing (MLST). For MLST and porA sequencing, gene sequences were amplified, purified and sequenced as

previously described (19,20). Alleles, sequence types (STs) and clonal complexes (CCs) were assigned using the Neisseria MLST database (http://pubmlst.org/neisseria). The deduced amino acid sequences of variable regions VR1 and VR2 were assigned geno-subtype numbers according to the N. meningitidis PorA variable regions database (http://neisseria.org/nm/typing/pora). The sequences of VR3 variants were classified according to the sequences described by Clarke et al. (21).

Results

Between February 1998 and June 2013, 107 invasive isolates of N. meningitidis were isolated from cerebrospinal fluid (n = 92; 86%) and blood (n = 14; 13.1%). One isolate (0.9%) was isolated from articular puncture. The patients' age ranged from 3 days to 13 years (mean = 3.2 years; median = 2.3 years) and gender ratio (male/female) was 1.26.

Beta-lactam susceptibility testing showed that 55.7% of N. meningitidis isolates had decreased susceptibility to penicillin G (called hereafter MDSP) (MIC90 = 0.5 mg/L) (Table 1). Among the MDSP, 30 isolates (28.4%) had reduced susceptibility to amoxicillin. However, no isolate was beta-lactamase-producing and all were susceptible to cefotaxime (Table 1). Three (2.8%) isolates had reduced susceptibility to chloramphenicol (Table 1).

Serogroup B was the most frequent (n = 86; 80.4%), followed by serogroup C (n = 13; 12.2%) and serogroup A (n = 6; 5.6%). Two isolates (1.8%) belonged to serogroup Y.

ELISA showed that all 6 serogroup A isolates had the same antigenic formula (A:4:P1.9). The porA sequencing revealed the same variable regions VR1 (20), VR2 (9) and VR3 (35-1). By MLST, serogroup A isolates belonged to CC5 but were of 2 sequence types (4 isolates were ST-7 and 2 were ST-5832).

Two serogroup C isolates were of serotype 2a, 4 of serotype 4 and 2 were nontypeable (NT). Several serosubtypes were detected among these isolates and 7 different antigenic formulae were obtained (Table 2). The porA sequencing allowed us to predict the variable regions VR1, VR2 and VR3 that define the subtypes for all those isolates. Genotyping by MLST showed that serogroup C isolates belonged to 5 different STs that were clustered into 3 CCs, among which CC35 was the most frequent (n = 4, 50%) (Table 2). Three strains belonged to CC11 and had the same antigenic formula, P1.5,2 (Table 2). The serogroup Y isolate was NT and non-serosubtypeable (NST) and belonged to ST-167 (CC167). porA sequencing revealed variable regions VR1 (5-1), VR2 (10-1) and VR3 (36-2).

Serogroup B isolates were predominant (41 of 56 isolates; 73%) and were heterogeneous at the phenotypic level (Table 3). Six serotypes and 8 serosubtypes were detected among these isolates. However, most of them were NT (n = 20) or NST (n = 11). Isolates belonging to serogroup B showed highly heterogeneous structure with 16 different combinations of serotype: serosubtype. The antigenic formula B:NT:P1.14 was the most frequent (n = 7). The porA sequencing revealed sequences of VR1, VR2 and VR3 in all serogroup B isolates except 2 that failed to show PCR amplification for porA and may correspond to a PorA-negative isolate. VR1, VR2 and VR3 regions showed even more diversity than that revealed by serological typing with 8, 10 and 7 different sequences respectively (Table 3). MLST confirmed this diversity at the genetic level. Twenty-three different STs that were clustered into 12 distinct CCs were obtained. In 2 isolates, new sequences not belonging to any known CC were identified (designated as types ST-3452 and ST-3453 and saved at the pubMLST database in the Tunisian collection) (Table 3). The most frequent CC among serogroup B isolates was CC35 (10 isolates; 24.4%), which were grouped into 3 STs (ST-35, ST-3451 and ST-5806). However, the hyperinvasive CC41/44, CC32 and CC269 were only represented by 2 (4.9%), 4 (9.7%) and 1 (2.4%) of the Tunisian isolates, respectively.

In total, the most frequent CC among the 56 genotyped isolates was CC35, which accounted for 25% (14 isolates of serogroups B and C respectively) (Table 4). The hyperinvasive lineages (CC5, CC11, CC32, CC41/44 and CC269) were represented by 18 isolates (32%), regardless of the serogroups. There were 12 isolates (24%) in serogroups B and C.

Discussion

N. meningitidis is a frequent cause of invasive bacterial disease in children, especially meningitis. The annual incidence of meningococcal disease varies from 0.5 to 6/100 000 inhabitants for developing countries (22). In sub-Saharan Africa the incidence can be much higher. The highest incidence is seen in countries of the meningitis belt, where it can be as high as 10 to 1000/100 000 inhabitants (22). Some studies have been conducted in Algeria, Morocco and Tunisia with regard to phenotyping and genotyping, but none was large enough to define the annual incidence in these countries or to allow good typing of the strains involved (1,2,10,11).

Before the introduction of Haemophilus influenzae serotype b (Hib) vaccine in the national vaccine programme in Tunisia, N. meningitidis was the third most common cause of bacterial meningitis (23,24). After inclusion of Hib vaccine in the national immunization calendar, N. meningitidis has become the second most frequent cause of bacterial meningitis after Streptococcus pneumoniae (data presented at the Meningitis Research Foundation International Conference: Meningitis and septicaemia in children and adults, London, 2009).

There is little information regarding decreased antibiotic susceptibility of meningococcal isolates from African countries (25). The first isolate of MDSP was described in Spain in 1985 and their numbers have been increasing since then.. In this study, we found that 55.7% of the strains were MDSP, however, none of them exhibited beta-lactamase activity and all isolates were susceptible to cefotaxime. In a previous study in Tunisia, 34% of the strains were MDSP (26). This is a high percentage of MDSP compared to that in French (34%) and Canadian (21.7%) studies (27,28). However, in Spain, the percentage of MDSP is higher (67%) (29). In other countries, it was found to be lower and accounted 3.5% in Algeria (11), 4.3% in Morocco (30), and 24.6% in Portugal (31). As expected for the African countries, rifampicin remained consistently active. Studies from France (27) showed rare resistant isolates.

Meningococcal serogroup distribution differs between regions and countries (22). Isolates of serogroup B and to a lesser extent serogroup C are predominant in Europe and America (22,32). Serogroups A and W are predominant in the sub-Saharan meningitis belt. Additionally, serogroup X has been isolated in that region, while isolates of serogroup B are rare, if any (33). More recently, serogroup C isolates of a new sequence type (ST-10217) were responsible for outbreaks in Northern Nigeria (34). Our data suggest that, unlike the sub-Saharan countries, serogroup B is frequent in Tunisia. Indeed, serogroup B was the most frequent serogroup in our study followed by serogroups C and A. This distribution pattern is similar to the serogroup distribution in Morocco (10,11).

All serogroup A isolates belonged to CC5 with the same phenotype (A:4:P1.9). The CC5 isolates dominated (74.8%) among meningococcal isolates from countries of the African meningitis belt and were remarkably homogeneous in their PorA (33). As in Europe and America, in our study, serogroup B was the most frequent followed by isolates of serogroup C. However, the phenotypes and genotypes of the circulating isolates differed from those observed in Europe and America (27,35). Isolates of serogroup B display a remarkable heterogeneity. In fact, we found 16 different antigenic formulae (serotype: subtype), belonging to 23 STs and 12 CCs. The isolates belonging to CC35 were the most frequent in both serogroups B and C and accounted for 25% of all the genotyped isolates. This genotype has been rarely found among invasive isolates in Europe (32). A study on invasive strains of serogroups B and C has shown that CC35 strains lack the fetA gene (36). Further studies such as FetA VR sequencing are needed to explore this hypothesis for the Tunisian strains and to analyse their pathogenesis. One striking aspect is that, unlike Europe, where 77% of the isolates belong to the hyperinvasive CC8, CC11, CC32, CC41/44 and CC269 (37), our study showed that these CCs were only present in 24% of all genotyped isolates of serogroups B and C. The hyperinvasive isolates were predominant not only in Europe but also in New Zealand, Brazil and North America (3).

Despite the absence of molecular categorization of the entire collection studied, this is believed to be the largest epidemiological analysis of meningococcal invasive strains in Tunisia. Our study revealed great heterogeneity of N. meningitides isolates, particularly among serogroup B. This heterogeneity can be explained by the geographical position of Tunisia and the travel pattern of people leaving or visiting the country. We identified two new sequence types (ST-3452 and ST-3453) that were saved at the pubMLST database in the Tunisian collection. Epidemiology of meningococcal infections in North African countries is different from that in sub-Saharan countries. A surveillance network is needed to establish a reliable vaccination policy, especially for the use of conjugate vaccines or newly developed protein vaccines against serogroup B isolates (4).

Acknowledgements

We thank Dr Jean-Michel Alonso (Unité des Neisseria and Centre National de Référence des Méningocoques, Institut Pasteur, Paris) for discussion and support for this work.

Funding: This work was supported in part by WHO (EMRO/TDR Small Grants Scheme for Operational Research in Tropical and Other Communicable Diseases (SGS05/59), the French Ministry of Foreign Affairs (3C2-002/E) and the Pasteur Institute, Paris.

Competing interests: None declared.

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