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Phenotypic and genotypic characterization of meningococcal isolates in Tunis, Tunisia: High diversity and impact on vaccination strategies

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ABSTRACT

Objectives: The aim of this study was to characterize *Neisseria meningitidis* (Men) isolates in Tunisian paediatric patients with invasive meningococcal disease (IMD) in order to target therapeutic and preventive strategies.
Methods: Fifty-nine isolates of Men and four cerebrospinal fluid samples that were culture-negative but Men-positive by PCR (NC-MenPPCR) (2009–2016) were collected from IMD patients. Isolates were analysed for their antimicrobial susceptibility. Whole-genome sequencing (WGS) was used to characterize isolates and multilocus sequence typing for NC-MenPPCR. Coverage of Men serogroup B (MenB) was determined by Genetic Meningococcal Antigen Typing System (gMATS) and fHbp expression by ELISA.
Results: MenB was the predominant type (88.9%). The majority of isolates (81%) had reduced susceptibility to penicillin G with altered *penA* alleles. The clonal complex CC461 (27.1%) was the most frequent. Among the MenB vaccine targets neisserial heparin binding antigen (NHBA) and fHbp, the predominant variants were NHBA118 (30.8%) and fHbp peptide 47 (25%), respectively. The *nadA* gene was present in 17.3% of isolates. Using gMATS, 36.5% of MenB were predicted to be covered by the 4CMenB vaccine. ELISA showed that 92.4% of the MenB were expected to be killed by anti-fHbp antibodies.
Conclusions: MenB was the leading serogroup in IMD, and more than 90% had a sufficient level of fHbp expression for vaccine coverage. The study results will be useful for the Tunisian vaccination programme.

Introduction

Neisseria meningitidis (meningococcus, Men) is a gram-negative bacterium that causes meningitis and/or septicaemia. The virulence of Men is linked to many bacterial factors, but the major one is the capsule. Meningococcal strains are classified into 12 serogroups based on the structure and the expression of the capsule, but invasive meningococcal disease (IMD) is generally associated with only six of them: A, B, C, W, Y, and X; these cause sporadic cases, small clusters, and epidemics worldwide. However, the distribution of meningococcal serogroups varies over time and from one country to another (Acevedo et al., 2019).

Several polysaccharide or conjugate vaccines are available against Men serogroups A, C, Y, and W. However, for Men serogroup B (MenB), which is predominant in many parts of the world including Tunisia (Saguer et al., 2016), capsular polysaccharide is not a suitable vaccine component due to potential auto-immunity concerns. In the genomic era, reverse vaccinology and high throughput screening have allowed the development of two protein-based vaccines that target serogroup B meningococci. One vaccine, 4CMenB (Bexsero) contains three proteins – factor H-binding protein (fHbp), neisserial heparin binding antigen (NHBA), and *Neisseria* adhesin A (NadA) – in addition to the porin A (PorA) from the outer membrane

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vesicles (OMV) expressing the PorA P1.4. (Serruto et al., 2012). The second vaccine, the bivalent rLP2086 MenB-FHbp (Trumenba), is composed of two lipidated variants of fHbp (Zlotnick et al., 2015).

In addition to the severity and changing epidemiology of IMD, there have been increasing reports of antibiotic resistance in recent years, particularly to beta-lactams, which are used as the first-line treatment (Deghman et al., 2017). Thorough characterization of meningococcal isolates is therefore crucial for the management of cases and for the implementation of preventive strategies. Molecular typing of meningococcal isolates by multilocus sequence typing (MLST) is based on the allelic variation in seven housekeeping genes; MLST classifies the isolates into sequence types (ST) and clonal complexes (CC) (Maiden et al., 1998). Molecular typing of Men also involves the investigation of polymorphisms and the level of expression of vaccine antigens (Vogel et al., 2013; McNeil et al., 2018).

Data on the characterization of virulence factors in Men strains circulating in North African countries are limited, with little information published in this area (Tali-Maamar and Rahal, 2003; Saguer et al., 2016; Razki et al., 2018).

The aim of this study was to better characterize Men strains isolated from Tunisian paediatric IMD patients, in order to better target therapeutic and preventive strategies.

Materials and methods

Bacterial isolates

This study included 59 isolates of Men isolated in the Laboratory of Microbiology at Bechir Hamza Children's Hospital in Tunis (Tunisia) during the period 2009–2016. In addition, four cerebrospinal fluid (CSF) samples collected in the same laboratory that were culture-negative but positive for Men by PCR (NC-MenPPCR) were also included.

Epidemiological information regarding sex, age, and the region of residence were collected. The identification of isolates was based on conventional methods such as Gram-staining and oxidase test. Complete identification was performed using the API NH system (bioMérieux, France). After identification, isolates were stored at −80 °C in heart–brain broth supplemented with 15% glycerol for further use.

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MIC) were determined using E-test strips (bioMérieux, France) following the European Committee for Antimicrobial Susceptibility Testing guidelines (www.eucast.org). The antibiotics tested were penicillin G, amoxicillin, cefotaxime, rifampicin, and ciprofloxacin. The MIC interpretative breakpoints of the National Reference Centre of Meningococci (NRCM) at the Pasteur Institute in Paris were adopted for all isolates (Table 1). The control strain used was *Streptococcus pneumoniae* ATCC 49619. For all isolates, the production of beta-lactamase was tested by nitrocefin disc (bioMérieux, France).

Table 1
Minimum inhibitory concentration interpretative breakpoints used in this study.

Antibiotics	Susceptible (mg/l)	Resistant (mg/l)
Penicillin G	<0.125	>1
Amoxicillin	<0.25	>2
Cefotaxime	≤0.25	>0.25
Rifampicin	≤0.25	>0.25
Ciprofloxacin	<0.064	≥0.064

Molecular typing

DNA extraction and molecular identification

In the case of CSF samples, DNA extraction and purification were performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Identification and genogrouping were performed by real-time PCR (rt-PCR) using the MenSerogroup-20 kit (Diagenode, Belgium).

For bacterial culture, boiled extracts were prepared and stored at −20 °C prior to PCR. The identification of Men was confirmed by two multiplex PCRs with specific primers targeting the contact-regulated A gene (*crgA*) (Deghmane et al., 2019), which is implicated in the regulation of gene expression, the capsular transport A gene (*ctrA*), the putative exporter gene *NMB1732*, the Cu–Zn superoxide dismutase gene (*sodC*), and the gamma-glutamyl transpeptidase gene (ggt) (Takahashi et al., 2004; Dolan Thomas et al., 2011; Guilhen et al., 2013).

All isolates were genogrouped by multiplex PCR with the amplification of specific capsular genes: *csaB* for serogroup A (MenA), *csb* for serogroup B (MenB), *csc* for serogroup C (MenC), *csxA* for serogroup X (MenX), *csy* for serogroup Y (MenY), and *csw* for serogroup W (MenW) genes (Deghmane et al., 2019).

MLST, porA, fetA, *and* penA *sequencing*

The NC-MenPPCR CSF samples were genotyped by MLST, *porA* and *fetA* typing, as described previously (Maiden et al., 1998; Thompson et al., 2003). The *penA* gene was amplified, purified, and then sequenced (Taha et al., 2007). The sequence analyses were performed using CLC Main Workbench 5.0.2 (Qiagen). The alleles, STs, and CCs were identified using the *Neisseria* MLST database (http://pubmlst.org/neisseria).

Whole-genome sequencing (WGS)

The genomic DNA was extracted from Men isolates using the Roche MagNa Pure 96 system (Roche Molecular Systems, Pleasanton, IL, USA), and the preparation of libraries was performed with the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). WGS was realized using the Illumina NextSeq 500 technology, as described previously (Hong et al., 2018), at the NRCM, Pasteur Institute, Paris. All sequences were assembled de novo to generate contigs with SPAdes (CAB, St Petersburg State University, St Petersburg, Russia). The BIGSdb genome comparator tools available in PubMLST were used to compare the Men isolates using the core genome MLST (which used 1605 loci) (Jolley and Maiden, 2010). Genomes from reference strains and isolates from Morocco available in PubMLST were included. The characteristics of these strains have been described in previous reports (Lucidarme et al., 2015; Taha et al., 2016; Guiddir et al., 2018; Razki et al., 2018).

Coverage of MenB isolates by the 4CMenB vaccine was predicted using the Genetic Meningococcal Antigen Typing System (gMATS). Isolates were predicted to be covered or not covered if they possessed identical alleles to those reported by Muzzi et al. to be covered or not covered, respectively. If a different allele was present, the isolate was indicated to be unpredictable and half of these unpredictable isolates were considered as covered (Muzzi et al., 2019).

WGS analysis was used to determine the MLST alleles, the ST, the CC, and the outer membrane proteins (PorA, PorB, FetA, fHbp, NadA, and NHBA). Moreover, we determined the allele sequences for the *penA*, *gyrA*, and *rpoB* genes encoding the targets of beta-lactams, ciprofloxacin, and rifampicin, respectively. Alleles that were previously reported to be responsible for resistance or reduced susceptibility were indicated as altered alleles (Taha et al., 2007).

Analysis of the level of expression of fHbp *by ELISA*

All MenB were tested for the level of expression of fHbp using monoclonal antibodies anti-fHbp rabbit IgG at 1:1000 for 1 h at 37 °C and anti-rabbit IgG-HRP conjugate (Jackson ImmunoResearch) at 1:5000 for 1 h at 37 °C for chemiluminescent detection. The optical densities (ODs) were recorded at 492 nm. Two positive controls were used: LNP18468 and LNP18465 (for variant 1/subfamily B and variant 2 or 3/subfamily A, respectively). The results were then expressed as a ratio to the reference strain and described as covered or not covered by vaccination according to the coverage threshold (9%), as described previously (Hong et al., 2012).

Statistical analysis

The median levels of C-reactive protein (CRP) in the blood, and glucose and protein levels in the CSF were calculated using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA).

Results

Epidemiological and clinical characterization of the study population

From January 2009 to December 2016, 73 cases of IMD were confirmed in the laboratory at Bechir Hamza Children's Hospital in Tunis, giving an estimated annual incidence rate of IMD of 1.5 per 100 000 children in 2014 in Tunis and the surrounding area (http://census.ins.tn/fr/resultats). This hospital is a public paediatric hospital that provides tertiary care for Tunis and the surrounding area. Among confirmed cases, 64 had a positive culture, six had a positive PCR, and three had both a positive direct examination and positive soluble antigen.

For this study, 59 viable meningococcal isolates among the 64 positive culture cases were recovered in the laboratory and included. These were from three sites: CSF ($n = 51$), blood ($n = 7$), and respiratory specimen ($n = 1$). The remaining five strains did not grow after storage at −80 °C. The distribution of Men isolates varied according to age group. The most represented age group was children under 1 year of age (44.6%), followed by the 1–4 years group (30.4%), the 5–14 years group (17.9%), and the 15–17 years group (7.1%). The male to female sex ratio was 1.1.

The cases were associated with important inflammatory responses as estimated by the levels of CRP in the blood, with a median value of 196.66 mg/l (range 7.7–16 990 mg/l). The glucose levels in the CSF ranged between 0 and 6.9 mmol/l (median 1.71 mmol/l), while the protein levels varied between 0.15 and 5.01 g/l (median 1.3 g/l).

Strain typing

Serogroup distribution

All Men strains harboured the *crgA*, *sodC*, *NMB1732*, and *ggt* genes. Only one isolate lacked the *ctrA* gene and was therefore predicted to be non-capsulated. This isolate was indeed non-groupable and was isolated from the CSF of a 2-year-old child with no underlying pathology.

Among the four NC-MenPPCR CSF samples, three were serogroup B and one was serogroup W.

For all IMD cases, MenB was the most frequently identified (56 cases; 88.8%), followed by MenY (three cases; 4.7%), MenA, MenC, and MenW (one case each; 1.6% each).

Antibiotic susceptibility testing

With regard to antibiotic susceptibility, all isolates were beta-lactamase-negative and were susceptible to cefotaxime (MIC ≤ 0.25 mg/l). For penicillin G and amoxicillin, 48 strains (81.3%) were intermediately resistant, but no isolate was fully resistant. MIC_{50} and MIC_{90} were 0.25 mg/l and 0.38 mg/l, respectively, for penicillin G and 0.75 mg/l and 1 mg/l, respectively, for amoxicillin. All isolates were susceptible to rifampicin (MIC ≤ 0.25 mg/l). For ciprofloxacin, only one strain was resistant (MIC ≥ 0.064 mg/l).

The sequences of the *penA* gene (encoding penicillin-binding protein 2, PBP-2), corresponding to 14 different alleles, were extracted from the WGS data of the 59 strains. The majority of isolates ($n = 48$) showed altered *penA* alleles. The allele *penA9* was the most frequent ($n = 17$), followed by *penA33* ($n = 16$), presenting MIC values between 0.125 mg/l and 0.5 mg/l, respectively. The altered alleles (295, 11, 14, 240, 572, 540, and 661) were also found among 15 other isolates. The five amino acid mutations in the transpeptidase domain of PBP2 (A510 V, F504 L, H541 N, I515 V, and I566 V) responsible for reduced penicillin susceptibility were all found in the isolates with altered *penA* alleles (MIC ≥ 0.125 mg/l for penicillin G). Only five wild-type *penA* alleles were found among 11 isolates. Among the NC-MenPPCR specimens, only three *penA* sequences were determined with *penA9* ($n = 2$) and *penA295* ($n = 1$) alleles.

Overall, 51 cases (81%) had altered *penA* alleles. The two predominant ones were *penA9* and *penA33* and these were found over the entire study period (Supplementary Material File 1).

Allele *gyrA2* was the most frequent ($n = 30$), followed by allele 3 ($n = 14$), allele 4 ($n = 14$), and allele 10 ($n = 1$). The last allele had altered amino acid sequences at residue D95 N within the quinolone resistance-determining region (QRDR) and it was from a MenC ciprofloxacin-resistant strain (MIC = 0.064 mg/l).

Concerning rifampicin resistance, which is linked to mutations in the region of the *rpoB* gene, we obtained 13 different alleles in 58 strains with no mutation conferring resistance to rifampicin, and the *rpoB* gene of one isolate did not assemble from the WGS (Taha et al., 2010). The alleles *rpoB5* ($n = 15$) and *rpoB18* ($n = 11$) were the most frequent alleles, followed by the allele *rpoB4* ($n = 7$). Two strains had new *rpoB* alleles, which were identified for the first time in this study.

Genomic analysis

The analysis of MLST profiles extracted from the WGS data showed that 59 meningococcal isolates belonged to 17 different STs distributed in 13 different CCs. The most frequent CC (CC461) belonged to serogroup B ($n = 16$), followed by CC41/44 ($n = 10$) and CC60 ($n = 8$). The most frequent ST was ST1946, belonging to CC461 (27.1%). Two new STs belonging to CC35 and CC41/44 were identified (Table 2).

The hyperinvasive clonal complexes CC41/44, CC32, and CC11 accounted for only 20.3% of strains in this collection. One isolate of serogroup A was detected and belonged to ST7 (CC5). For the CN-MenPPCR, MLST analyses identified three clonal complexes, CC865 ($n = 2$), CC11, and CC60. The distribution of CCs according to altered *penA* genes and study period is shown in Supplementary Material File 2.

We next performed a core genome MLST (cgMLST analysis) on the WGS data. As shown in Fig. 1, the 59 genomes of the meningococcal isolates were compared to other reference genomes from different populations. Isolates were grouped into several clusters, with few isolates that were separated from the clusters (unrelated isolates). The major cluster corresponded to isolates of CC461 and another cluster corresponded to CC41/44. These isolates circulated from 2009 to 2016 representing 27.1% and 17%, respectively, of all isolated strains. Two other clusters corresponded to CC865 and CC60, which circulated from 2009 to 2015; isolates of CC35 also clustered together and circulated from 2009 to 2010. Three small clusters corresponded to CC213, CC174, and CC162, which circulated between 2010 and 2015. Of interest, the only CC32 isolate from this study clustered closely to

Table 2
Distribution of strains by serogroup, sequence type, and clonal complex.

Serogroup	Clonal complex	Sequence type
B	CC461	ST1946 (*n* = 16)
	CC41/44 complex	ST41 (*n* = 1)
		ST6349 (*n* = 4)
		New (*n* = 1)
		ND (*n* = 4)
	CC60	ST60 (*n* = 3)
		ST2209 (*n* = 5)
		ND[a] (*n* = 1)
	CC865	ST3327 (*n* = 4)
		ND[a] (*n* = 2)
	CC35	ST35 (*n* = 2)
		New (*n* = 1)
		ND (*n* = 1)
	CC162	ST162 (*n* = 3)
	CC213	ST213 (*n* = 3)
	CC32 complex	ST33 (*n* = 1)
	CC103	ST4983 (*n* = 1)
	NA	ST3453 (*n* = 1)
		ST1111(*n* = 1)
		ST2888 (*n* = 1)
C	CC11 complex	ST11 (*n* = 1)
A	CC5 complex	ST7 (*n* = 1)
Y	CC174	ST1466 (*n* = 2)
	CC23 complex	ND (*n* = 1)
NG	CC103	ST4983 (*n* = 1)
W[a]	CC11[a]	ND[a] (*n* = 1)

NA, not assigned; ND, not determined; NG, non-groupable strain.
[a] Clonal complex obtained from cerebrospinal fluid negative culture but a *Neisseria meningitidis* positive PCR.

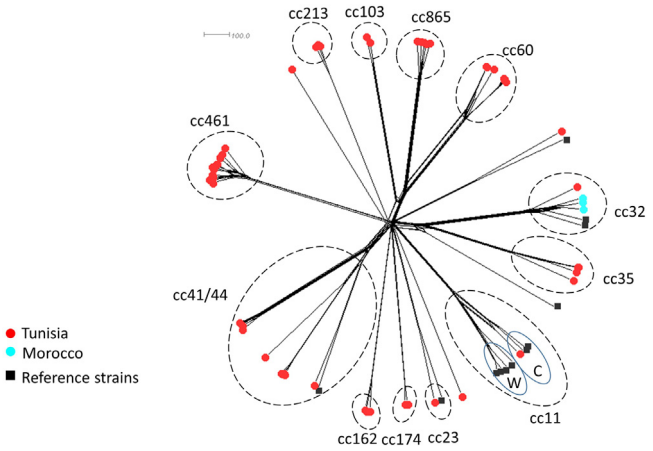

Fig. 1. A neighbour-network based on allelic profiles all the 59 isolates from Tunisia (red circles) using cgMLST analysis compared to reference strains (black squares) corresponding to different genotypes (IDs on the PUBMLST.org 2290(W/cc11) ; 240 (B/cc32) ; 613 (A/cc4) ; 698 (C/cc11) ; 40235(Y/cc23) ; 40389(C/cc11) ; 42037 (C/cc11); 42758(W/cc11) ; 27088(B/cc32) ; 39973(B/cc269) ; 51551(B/cc41/44) and 41857 W/cc11)). Three isolates from Morocco (cyan circles IDs 42116; 42117 and 42118 that are all ST-33 (cc32)) were also added for this comparison. The major clonal complexes for the Tunisian isolates are indicated. Groups (C and W) are also indicted for cc11 isolates.

expanding isolates from Morocco (Razki et al., 2018). *penA* altered alleles were variably distributed among these clusters. The most frequent altered *penA9* allele was detected in isolates from CC41/44, CC865, and CC60, while all CC461 harboured the same altered *penA* allele (*penA33*) (Supplementary Material File 2). Together, these data showed the high heterogeneity of the Tunisian meningococcal isolates (Fig. 1).

MenB strain characterization

Further focus was placed on serogroup B isolates, which were the most frequent in this study. The *porB* sequences were obtained for 43 strains of MenB that showed 14 different variable regions; the variant 3-14 (*n* = 17) was the most frequent. The *fetA* gene region encoding the variable region was obtained for all isolates, with 16 different FetA variant regions. The most frequent one was F3-9 (*n* = 15), followed by F5-8 (*n* = 8). The PorA sequences were obtained for 52 isolates and showed high diversity in the variable regions (VR1 and VR2). Twenty different PorA types (P1.VR1,VR2) were identified including 11 VR1 and 14 VR2, with the most frequent genetic formulae being P1.5,2 and P1.18-1,3 (*n* = 8 for each). A total of 23 finetypes (the combination of variable regions VR1 and VR2 of PorA and VR of FetA) were found and the finetype P1.18-1,3:F3-9 was the most frequent.

For NC-MenPPCR, we determined four VR of FetA (F1-1, F5-9, F5-8, and F1-5), while PorA VR2 typing was successful in only two cases (P1.14 and P1.2).

Vaccine antigens

Using WGS, the genes encoding the vaccine targeting serogroup B invasive isolates were also extracted.

The *nadA* gene was present in only nine strains (17.3%). Two strains had both NadA peptide 1 and 21, and seven had new NadA peptide (http://pubmlst.org/neisseria/) that belonged to CC60 (*n* = 4) and CC213 (*n* = 3).

All MenB strains had the *nhba* and the *fhbp* genes (*n* = 52). Four isolates had new *nhba* genes (http://pubmlst.org/neisseria/) and different peptides were identified, with the most frequent ones being NHBA118 (*n* = 16) and NHBA24 (*n* = 14). An association was found between NHBA118 (30.8%) and CC461. The last vaccine antigen, fHbp, had high diversity in alleles encoding 16 different fHbp peptides, and fHbp peptide 47 (variant 3/subfamily A) was the most frequent (*n* = 13). Indeed, subfamily A (variants 2 or 3) was the most frequent (*n* = 34), followed by subfamily B (variant 1) (*n* = 18). The correlations between CCs and vaccine antigens (fHbp peptide and NHBA peptide) are shown in Supplementary Material Files 3 and 4. Genomic data on fHbp peptide, NHBA peptide, NadA peptide, and PorA (VR2) were available for 52 MenB isolates, including five isolates for which *fhbp* and *nhba* corresponded to new alleles. These data allowed coverage by the 4CMenB vaccine to be predicted on the basis of gMATS. Only eight isolates (17%) were predicted to be covered on the basis of combinations of fHbp (peptides 1, 4, and 14), NHBA (peptides 2 and 21), and PorA (P1.4), while 22 isolates were unpredictable and 22 isolates were predicted not to be covered. Muzzi et al. estimated that half of the unpredictable isolates would be covered; it was therefore estimated that the gMATS-based coverage of our MenB isolates was 36.5% (Muzzi et al., 2019).

Variation in the level of expression of fHbp by ELISA

As the gMATS method did not allow the vaccine coverage of all isolates to be predicted due to the heterogeneity of fHbp, we used our previously reported ELISA test to explore the expression of fHbp (Hong et al., 2012). This method measures the expression of fHbp regardless of the variant and allows the isolates to be classified into low, medium, and high levels of expression (Fig. 2). Only four isolates (7.6%), which all harboured fHbp peptide (subfamily A/variant 2), showed an ELISA expression level under the threshold that has been shown to be correlated with complement-dependent killing by anti-fHbp antibodies. This indicated that 92.4% of the MenB isolates in this study would be expected to be killed by anti-fHbp antibodies (Hong et al., 2012). Of interest, all variant 1 (subfamily B) showed higher levels than the threshold predicting complement-dependent killing by anti-fHbp antibodies (Fig. 2).

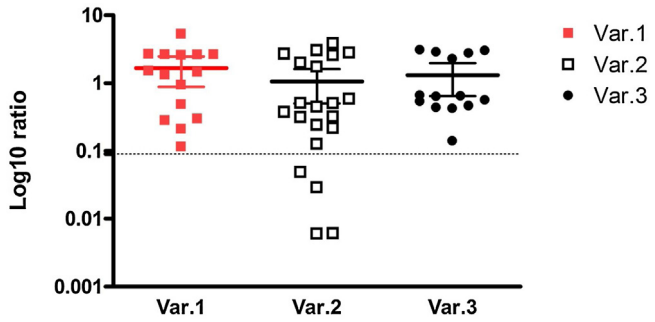

Fig. 2. The distribution of levels of expression of fHbp among *Neisseria meningitides*. Data are expressed by the ratio of expression of each isolate to the corresponding reference strain. Data are shown as mean with 95% confidence interval for isolates sharing varaints 1, 2 or 3.

Discussion

Limited data on the characterization of Tunisian Men isolates are available (Saguer et al., 2016). In Tunisia, there is no national surveillance network and no reference laboratory. In addition, no data on meningococcal disease are available in the Tunisian national surveillance network for antimicrobial resistance, due to the small number of isolates (https://www.infectiologie.org.tn/resistance.php).

In the region of Tunis, the incidence of IMD in 2014 was estimated at 1.5 per 100 000 children (http://census.ins.tn/fr/resultats). This rate is comparable to that of Morocco (2–3.6 per 100 000 inhabitants) (Razki et al., 2018) and Algeria (0.09 per 100 000 inhabitants in 2012) (Algérie Sante, 2012).

In this study, phenotypic and genotypic tools including WGS were used in order to better characterize Men strains isolated in a paediatric population, and the majority of IMD cases were found to belong to serogroup B (88.8%), in agreement with previous Tunisian studies (Saguer et al., 2016).

Also, the study findings are consistent with the serogroup distribution in North African countries (Algeria and Morocco) (Tali-Maamar and Rahal, 2003; Razki et al., 2018) and European countries (Whittaker et al., 2017). These data are quite distinct from the situation in the countries of the Sub-Saharan meningitis belt, where serogroups C, W, and X are predominant (WHO, 2019). Indeed, only one serogroup A isolate was detected in our study, and this belonged to CC5, which was prevalent in the meningitis belt prior to the implementation of the conjugate vaccine against serogroup A (MenAfriVac). In our study, the majority of IMD cases were observed in children under 5 years of age, with a peak among infants under 1 year.

The majority of the isolates in this study had reduced susceptibility to penicillin G, with a correlation between penicillin resistance values and altered *penA* alleles. Five amino acid substitutions (F504 L, A510 V, I515 V, G541 N, and I566 V) were found in the transpeptidase region of PBP2 encoded by the *penA* gene (Thulin et al., 2006). The proportion of isolates with intermediate resistance to penicillin G increased from 55.7% in 2013 (Saguer et al., 2016) to 81% in 2016. This percentage was higher compared to other studies, such as in Morocco (19%) (Razki et al., 2018), Italy (45%) (Vacca et al., 2018), and the United States (16.7%) (Harcourt et al., 2015). Although reduced susceptibility to third-generation cephalosporins has been described recently (Deghmane et al., 2017), all of our isolates were susceptible to these antibiotics. The fact that all CC461 harboured an altered *penA* allele (*penA33*) may explain in part the high proportion of isolates with reduced susceptibility to penicillin G. However, this high proportion is also due to other altered alleles (such as *penA9*), which were detected in several clonal complexes.

The phylogenetic analysis of the 59 isolates of Men collected proved a high genomic variability and heterogeneity with distinct phylogenic clusters. The study data show that cluster CC461 was circulating in Tunisia over the entire study period, suggesting the expansion of a local isolate in the region of Tunis and surrounding area. In a previous study conducted in our hospital, cluster CC35 circulated from 1998 to 2007 (Saguer et al., 2016).

In Morocco, CC32 (and mainly ST33 isolates) is the predominant one (Razki et al., 2018). It is of interest that only one ST33 isolate was detected in the present study and this was related to the ST33 isolates from Morocco. This observation suggests that while serogroup B IMD seems to be prevalent in North Africa, the isolates may differ among the countries of that region. Among the European countries, CC41/44 is the most prevalent in France, Germany, and Italy (Vogel et al., 2013); however CC32, CC269, and CC213 are predominant in Spain (Abad et al., 2016). The present study also highlights the interest of using WGS to infer typing data, as well as antibiotic susceptibility data. Moreover, WGS allows the analysis of the genes encoding the proteins in vaccines targeting serogroup B (fHbp, NHBA, NadA, and PorA). Our data confirmed that NadA may not account for the coverage, as it was present in a low number of isolates in our study, in agreement with European data (Vogel et al., 2013). However, genomic data may not be enough to predict the coverage of MenB isolates by these vaccines, as only 36.5% were predicted to be covered by gMATS. This was due to the high number of fHbp variants for which no data were reported. Moreover, no MATS data are available for Tunisia to confidently evaluate the non-precitable isolates (Muzzi et al., 2019). The fact that all isolates harbouring fHbp variant 1 expressed enough proteins to be killed by antibodies further highlights the underestimation of coverage of our isolates by the 4CMenB vaccine. Indeed, the ELISA method used here allowed the prediction of coverage at 92.4%. This is in agreement with recent data, which have suggested that >91% of all meningococcal serogroup B isolates expressed sufficient levels of fHbp to be susceptible to bactericidal killing anti-fHbp antibodies induced by the bivalent rLP2086 MenB-FHbp (McNeil et al., 2018). Moreover, we successfully genotyped PCR-confirmed non-culturable CSF samples. This is essential for monitoring the epidemiology of IMD and vaccine coverage.

In conclusion, although the study results concern only one Tunisian hospital, they provide an insight into the epidemiology of paediatric IMD, given the lack of national data and in particular the lack of data concerning MATS typing. The study data on the serogroup distribution could be useful for informing vaccination programme policy. Indeed, the only available vaccine is the polysaccharide conjugated one (ACWY) and it is mandatory only for pilgrims. It is also recommended for those having extended stays in endemic regions (Sub-Saharan belt of Africa, some countries in Asia and South America) (Institut Pasteur de Tunis, 2019). Moreover, the meningococcal vaccines are not included in the routine childhood immunization programme in Tunisia.

Declarations

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Ethical approval: Ethical approval was not required. All samples collected and laboratory investigations performed in this work were part of the IMD management.

Conflict of interest: The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.11.013.

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