

Molecular Characterization of Invasive Isolates of Neisseria meningitidis in Casablanca, Morocco.

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4	Casablanca-Morocco.
3	Molecular characterization of invasive isolates of <i>Neisseria meningitidis</i> in
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27 Abstract

28 Meningococcal epidemiology may change unpredictably and typing of isolates of Neisseria meningitidis is crucial for the surveillance of invasive meningococcal 29 disease (IMD). Few data are available of the meningococcal epidemiology in 30 countries of North Africa. We aimed to explore the invasive meningococcal isolates 31 32 from the Casablanca region in Morocco. We used whole genome sequencing (WGS) 33 to characterize 105 isolates from this region during the period 2011–2016. Our data showed that the majority (n=100) of the isolates belonged to serogroup B. The 34 genotyping of most of these isolates (n=62) belong to the sequence type (ST-33) of 35 36 the clonal complex cc32. They also showed the same PorA and FetA markers and clustered together on the basis of WGS phylogenic analysis and seemed to correspond 37 to an expansion of local isolates in the Casablanca region as also reported with similar 38 39 isolates in several other countries in the world.

These data suggest that serogroup B isolates may predominate in Morocco. They also
have important impact on designating vaccination strategies.

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49 Introduction

50 Neisseria meningitidis (the meningococcus), a Gram negative capsulated bacterium, is the causative agent of invasive meningococcal disease (IMD). IMD is characterized 51 mainly by meningitis and/or septicemia but other clinical forms are also described (1). 52 *N. meningitidis* causes globally an estimated 500000 cases of invasive meningococcal 53 disease (IMD) per year with 10% fatality rate as well as permanent sequelae among 54 55 survivors(2). Meningococci are classified into 12 serogroups based on biochemical properties of their capsular polysaccharides but 6 of them (A, B, C, W, Y and X) are 56 responsible for almost all the disease worldwide (3). Capsular polysaccharide-based 57 58 conjugate vaccines are available against serogroups A, C, W and Y while proteins-based vaccines are available against isolates of serogroup B (3). 59

The incidence of IMD varies worldwide with low incidence in industrialized 60 61 countries between 0.3–4 cases per 100,000 population but the incidence can approach 1000 per 100,000 during epidemics in countries of the sub-Saharan Africa(4). Indeed, 62 IMD occurs either as sporadic cases with occasional outbreaks in the industrialized 63 countries such as in Europe. However, major epidemics occur periodically in the 64 African meningitis belt, a region that spans countries between Senegal in the west and 65 66 Ethiopia in the east (5). The distribution of serogroups also varies in space and time. Serogroup B predominates in Europe, North America and Australasia (6). Periodic 67 epidemics of meningococcal disease occurred in the meningitis belt that have been 68 caused by serogroup A but serogroup A decreased in the meningitis belt since the 69 introduction of the conjugate vaccine against isolates of serogroup A but isolates 70 belonging to serogroups C, W and X emerged (4, 7-9). Moreover, serogroup W seems 71 to undergo a worldwide increase since 2013 (10). Few data are available form North 72 Africa countries (6, 11) and IMD is mainly considered as travel disease linked to the 73

74	Hajj and Umrah pilgrimages (12). Previous data from the Casablanca region in
75	Morocco obtained between January 1992 and September 2000 showed predominance
76	(75.5%) of serogroup B isolates with strains of the phenotype
77	(serogroup:serotype:serosubtype) B:4:P1.15 representing 74.8% of all serogroup B
78	isolates (13). We aimed in this work to follow the recent evolution of IMD in
79	Casablanca region that represents 20% of the population of Morocco using whole
80	genome sequencing (WGS) approach in order to provide more genetic resolution on
81	the isolates and their relationship to other isolates that are currently circulating
82	worldwide.
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95 Materials and Methods

96 Ethical Statement

Isolates were obtained as part of routine activity of the Bacteriological laboratory at Hospital Ibn Rochd in Casablanca, and were analyzed anonymously for the epidemiological surveillance and control of communicable disease in the Moroccan Community. Ethical approval and informed consent were thus not required. A case of IMD was defined as the recovery of an isolate of *N. meningitidis* or meningococcal DNA from a normally sterile body site, such as blood, cerebrospinal fluid (CSF), joint aspirates.

104 Characterization of the isolates

PCR was used to confirm suspected cases with negative culture (N=45) as previously 105 described (14). The 105 cultured invasive isolates available from cases of IMD at the 106 Ibn Rochd hospital for the period 2011-2016. All isolates were cultured on GCB 107 medium plated supplemented with Kellogg supplements (15). Isolates were identified 108 on the basis of typical morphology of colonies, Gram stain, oxidase test and API NH 109 test (bioMerieux, Marcy l'Etoile, France), according to the manufacturer's 110 instructions. Antibiotic susceptibility testing was performed using Etest Minimal 111 inhibitory concentrations (MICs) for Penicillin, amoxicillin, ciprofloxacin and 112 were determined by the E-test (Oxoid) methods according to 113 Ceftriaxone, manufacturers' instructions [15] and interpreted according to the latest EUCAST 114 115 guidelines, as previously recommended and described for *N. meningitidis* (16).

The isolates were sent to the Institut Pasteur, Paris, France for WGS. Serogrouping
and genogrouping were performed as previously described (14). Genomic DNA was
extracted using the Roche MagNA Pure 96 system (Roche, Pleasanton CA 9, USA).

Sequencing was performed by Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA, USA) and assembled as previously described (17). Sequences are available through the PubMLST database which runs on the Bacterial Isolate Genome Sequence Database (BIGSdb) platform (18). WGS data were analyzed on the PubMLST to extract sequence type (ST) and clonal complexes according to the multilocus sequence typing analysis. Other alleles corresponding to genes of interest for antibiotic susceptibility were also extracted. WGS data were also analyzed using a "gene-by-gene" approach available through the PubMLST Genome Comparator tool using N. meningitidis core genome MLST (cgMLST) v1.0 that includes 1605 core loci (18). SplitsTree4 (version 4.13.1) was used to visualize the resulting distance matrices as Neighbor-net networks (19). The IDs of all these isolates are given in the supplementary Table to allow retrieving of WGS sequence in FASTA formats.

138 **Results**

139 Description of cases of IMD

In Morocco, notification of clinically suspected cases of IMD should be reported to 140 the regional health authority and Case notification forms are sent to the Department of 141 Epidemiology, Ministry of Health. The annual incidence is 2 to 3.6 per 100,000 142 inhabitants with 700-850 suspected cases per year fro the entier country. The Ibn 143 144 Rochd University Hospital usually receives cases from the Casablanca region (about 20% of the entire country population). The surveillance for IMD is performed under 145 146 the national recommendations mentioned above. Cultured bacterial isolates and samples can be sent to the Institut Pasteur of Morocco in Casablanca for identification 147 and typing. 148

One hundred and forty five suspected meningococcal cases were reported and confirmed by cultured and/or PCR in Casablanca between 2011 and 2016. These cases were estimated to correspond to 25% to 40% des suspected reported cases in Casablanca. Of these 145 biologically confirmed cases, 40 (27.6%) were primary clinical materials (CSF) with no positive culture and were confirmed by PCR and 105 (72.4%) of viable cultured isolates. Most of the isolates (71%) were from CSF and 29% from blood.

We analyzed 105 isolates from Casablanca between 2011 and 2016. The age of patients ranged from 3 day to 61 years the median age is 3 years. The majority of isolates belonged to serogroup B (100 isolates; 95% for the entire period). One isolate was of serogroup C and two isolates of serogroup W and two isolates of serogroup Y. The annual distribution of isolates during this period according to serogroups is depicted in Fig.1. We detected 19% of the isolates with reduced susceptibility to penicillin G (0.12 mg/L \leq MIC \leq 0.25 mg/L).

164 Analysis of the genetic markers of the isolates

Data on the MLST were extracted from WGS and isolates were grouped into clonal complexes (Fig. 2A). Twenty-five different sequence types (STs) were identified among the isolates that were grouped into 10 clonal complexes (cc). However, 78 isolates (74% of all tested isolates) belonged to one clonal complex, the cc32. ST-33, that belonged to the cc32, was the most frequent ST (n=62 isolates) in the cc32 in in the collection tested in Casablanca. Few isolates differed from ST-33 by one locus (single locus variants) or by two loci (double locus variants) (Fig. 2B).

The isolates harbored several alleles of *penA* encoding the penicillin binding protein2 172 (PBP2). Alterations of PBP2 were directly linked to the reduction of susceptibility to 173 penicillin G in meningococci (20). Four highly related alleles (penA1, penA2, penA3 174 and *penA22*) were found in 85 isolates that were previously described to correspond 175 to the penicillin G-susceptible isolates (20). Twenty isolates harbored diverse and 176 altered alleles (penA9, penA19, penA33, penA35, penA217, penA295, penA709 and 177 penA710) were previously described to correspond to isolates with reduced 178 susceptibility to penicillin G. Indeed, the isolates harboring modified penA alleles 179 correlated with MIC values of penicillin G. 180

There were several fines types (the combination of variable regions VR1 and VR2 of PorA and the variable region of FetA) but the finetype P1.19,15:F5-1 was the most frequent and was represented by 58 isolates that were all of serogroup B and cc32. These isolates were present throughout the study period (2011-2016).

The unique serogroup C isolate belonged to the ST-3327 of the cc865. The two serogroup Y isolates were different and belonged to ST-1627 (cc167) and ST-1466 187 (cc174). The two serogroup W isolates were of ST-11 and ST-13257 that both
188 belonged to the cc11 as ST-13257 was single locus variant of the ST-11.

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190 Genomic analysis of isolates

To describe the relationships of the isolates, all the 105 cultured isolates (for the 191 period 2010-2016) were subjected to cgMLST analysis using the BIGSdb tools in the 192 193 PubMLST database (see Materials and Methods). The isolates grouped into several lineages. The major cluster corresponded to the cc32 isolates that were quite separated 194 195 from the other serogroup B isolates (Fig. 3A). The isolates of serogroup B:P1.19,15:F5-1:cc32 were highly related, we therefore compared by cgMLST the 196 relationships of these isolates from Morocco to all other isolates sharing the same 197 combination and that were available on the PubMLST (accessed on 26/12/2017). In 198 addition to the 58 isolates from Morocco, there were 69 other isolates between 1976 199 and 2017 and were from the UK, France, Brazil, Cuba, Italy, Slovenia, South Africa, 200 Canada, Denmark, Greece, Ivory Coast and Switzerland. The phylogenetic trees 201 showed clear clustering of the Moroccan isolates distinctly from the isolates from 202 other countries. Only few isolates from France and the UK were clustered with the 203 Moroccan isolates (Fig. 3B). Isolates from Casablanca region harbored the genes 204 encoding the antigens of the vaccines against serogroup B (Supplementary Table). 205 206 The serogroup W/cc11 isolates were compared to other W/cc11 isolates that we have

recently published in France and that belonged to the two major sublineages: the "Anglo-French-Hajj" sub-lineage and "South American-UK" sub-lineage with its two variants the "original UK strain" and the "UK 2013-strain (21, 22). This analysis clustered the two Moroccan isolates within the "Anglo-French-Hajj" (Fig. 3B).

212 Discussion

This report used WGS analysis as a tool for complete characterization of invasive 213 214 meningococcal isolates from the Casablanca region of Morocco. Our data showed the majority of the isolates were of serogroup B in agreement with a previous report (13). 215 Recent data from Tunisia also suggested that serogroup B predominates in the 216 country between 1998 and 2013 (23). Serogroup B isolates were also reported in 217 218 Algeria between 1992-2001 (24). A shift from a serogroup A to serogroup B was noted in Egypt since 1991(25). However, the data remained scarce from North Africa 219 220 with little molecular typing data.

The previous work on the isolate in Casablanca region reported the phenotype P1.15 221 for the isolate of serogroup B (13) that is in agreement with our findings on the typing 222 of PorA (P1.19,15). Moreover, the WGS data were also available on for one group 223 B/cc32 isolate from Morocco from 1994 on the PubMLST database (ID 26047) and 224 clustered together with the recent isolates (26) (Figure 2). Our Data suggest the 225 persistence of these isolates in the region and may further suggest a recent clonal 226 expansion of these isolates with most of the isolates belonging to the major sequence 227 type (ST-33) and few single locus and double locus variants. Of interest, the cgMLST 228 analysis showed that these isolates seem to differ from related isolates reported in 229 other countries suggesting an expansion of a local strain in the Casablanca region. 230 231 These related isolates were responsible for large expansion as was reported in Cuba and Brazil and lead to vaccination campaigns (27). The availability of protein-based 232 vaccines may provide a possible strategy to control the expansion of those isolates. 233 234 These isolates harbor genes encoding the vaccine antigens. However, expression assays are required to confirm coverage (28). One limitation of our data is their 235

representativeness, but few exhaustive data are available due to limitations inreporting, diagnosis and typing (6).

The use of the WGS approach provide a thorough understanding of the circulating meningococcal isolates that provide powerful tools for high resolution in epidemiological surveillance and should help decision making to tailor vaccination strategies.

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362 Acknowledgments

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382 Legends of figures

Figure1. Distribution of the isolates of the study according to their serogroups andyear of isolation.

Figure2. (A) Distribution of the isolates of the study according to their serogroups and clonal complexes. (B) BURST analysis of the isolates of the clonal complex cc32 on the basis of the seven loci of the MLST. The most frequent sequence type (ST-33) is in the central circle (black). The middle circle (red) represents the single locus variants for the ST-33 and the outer circle (bleu) represents the double locus variant from the ST-33

391 Figure 3. (A) Neighbor-net phylogenetic network of all invasive cultured isolates received of the study from the region of Casablanca, Morocco (2011-2016). The tree 392 393 was drawn on the basis of cgMLST. The 10 clonal complexes and the corresponding serogroup of the isolates are indicated. (B) Neighbor-net phylogenetic network of all 394 serogroup B isolates showing the finetype P.19,15:F5-1 in the PubMLST database: 58 395 isolates from Morocco in addition to 69 other isolates between 1976-2017 and were 396 from the UK, France, Brazil, Cuba, Italy, Slovenia, South Africa, Canada, Denmark, 397 398 Greece, Ivory Coast and Switzerland. The isolates from Morocco 1994 is indicated by an arrow (ID 26047) (C) Neighbor-net phylogenetic network of the two serogroup W 399 400 isolates from our collection (black circle) B depicted within the tree of all invasive 401 serogroup W isolates from France (period 2010-2016). Cases are classified in three groups using WGS data: "original UK strain", "UK 2013-strain" and the 402 Anglo-French-Hajj. 403





