



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

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► To cite this version:

Aziza Razki, Eva Hong, Khalid Zerouali, Houria Belabbes, Khadija Aitmouss, et al.. Molecular Characterization of Invasive Isolates of *Neisseria meningitidis* in Casablanca, Morocco.. *Journal of Clinical Microbiology*, 2018, 56 (7), pp.e00445-18. 10.1128/JCM.00445-18 . pasteur-02069133

HAL Id: pasteur-02069133

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Submitted on 30 Mar 2019

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3 Molecular characterization of invasive isolates of *Neisseria meningitidis* in
4 Casablanca-Morocco.

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17 Running Title: Meningococcal disease in Morocco

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25 **Keywords: Meningococcal disease; Serogroup B disease; Typing; MLST;**

26 **Whole genome sequencing**

Abstract

Meningococcal epidemiology may change unpredictably and typing of isolates of *Neisseria meningitidis* is crucial for the surveillance of invasive meningococcal disease (IMD). Few data are available of the meningococcal epidemiology in countries of North Africa. We aimed to explore the invasive meningococcal isolates from the Casablanca region in Morocco. We used whole genome sequencing (WGS) 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 genotyping of most of these isolates (n=62) belong to the sequence type (ST-33) of 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 to an expansion of local isolates in the Casablanca region as also reported with similar 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.

Introduction

Neisseria meningitidis (the meningococcus), a Gram negative capsulated bacterium, is the causative agent of invasive meningococcal disease (IMD). IMD is characterized mainly by meningitis and/or septicemia but other clinical forms are also described (1).

N. meningitidis causes globally an estimated 500000 cases of invasive meningococcal disease (IMD) per year with 10% fatality rate as well as permanent sequelae among 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 responsible for almost all the disease worldwide (3). Capsular polysaccharide-based conjugate vaccines are available against serogroups A, C, W and Y while proteins-based vaccines are available against isolates of serogroup B (3).

The incidence of IMD varies worldwide with low incidence in industrialized 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, IMD occurs either as sporadic cases with occasional outbreaks in the industrialized countries such as in Europe. However, major epidemics occur periodically in the African meningitis belt, a region that spans countries between Senegal in the west and 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 epidemics of meningococcal disease occurred in the meningitis belt that have been caused by serogroup A but serogroup A decreased in the meningitis belt since the introduction of the conjugate vaccine against isolates of serogroup A but isolates belonging to serogroups C, W and X emerged (4, 7-9). Moreover, serogroup W seems to undergo a worldwide increase since 2013 (10). Few data are available from North Africa countries (6, 11) and IMD is mainly considered as travel disease linked to the

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

95 **Materials and Methods**

96 **Ethical Statement**

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

104 **Characterization of the isolates**

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

Results

Description of cases of cases of IMD

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

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% of 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 days 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 ($0.12 \text{ mg/L} \leq \text{MIC} \leq 0.25 \text{ mg/L}$).

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

There were several fypes 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

(cc174). The two serogroup W isolates were of ST-11 and ST-13257 that both belonged to the cc11 as ST-13257 was single locus variant of the ST-11.

Genomic analysis of isolates

To describe the relationships of the isolates, all the 105 cultured isolates (for the period 2010-2016) were subjected to cgMLST analysis using the BIGSdb tools in the PubMLST database (see Materials and Methods). The isolates grouped into several lineages. The major cluster corresponded to the cc32 isolates that were quite separated 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 relationships of these isolates from Morocco to all other isolates sharing the same combination and that were available on the PubMLST (accessed on 26/12/2017). In addition to the 58 isolates from Morocco, there were 69 other isolates between 1976 and 2017 and were from the UK, France, Brazil, Cuba, Italy, Slovenia, South Africa, Canada, Denmark, Greece, Ivory Coast and Switzerland. The phylogenetic trees showed clear clustering of the Moroccan isolates distinctly from the isolates from other countries. Only few isolates from France and the UK were clustered with the Moroccan isolates (Fig. 3B). Isolates from Casablanca region harbored the genes encoding the antigens of the vaccines against serogroup B (Supplementary Table).

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).

Discussion

This report used WGS analysis as a tool for complete characterization of invasive 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). Recent data from Tunisia also suggested that serogroup B predominates in the country between 1998 and 2013 (23). Serogroup B isolates were also reported in 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 with little molecular typing data.

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

236 representativeness, but few exhaustive data are available due to limitations in
237 reporting, diagnosis and typing (6).

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

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Acknowledgments

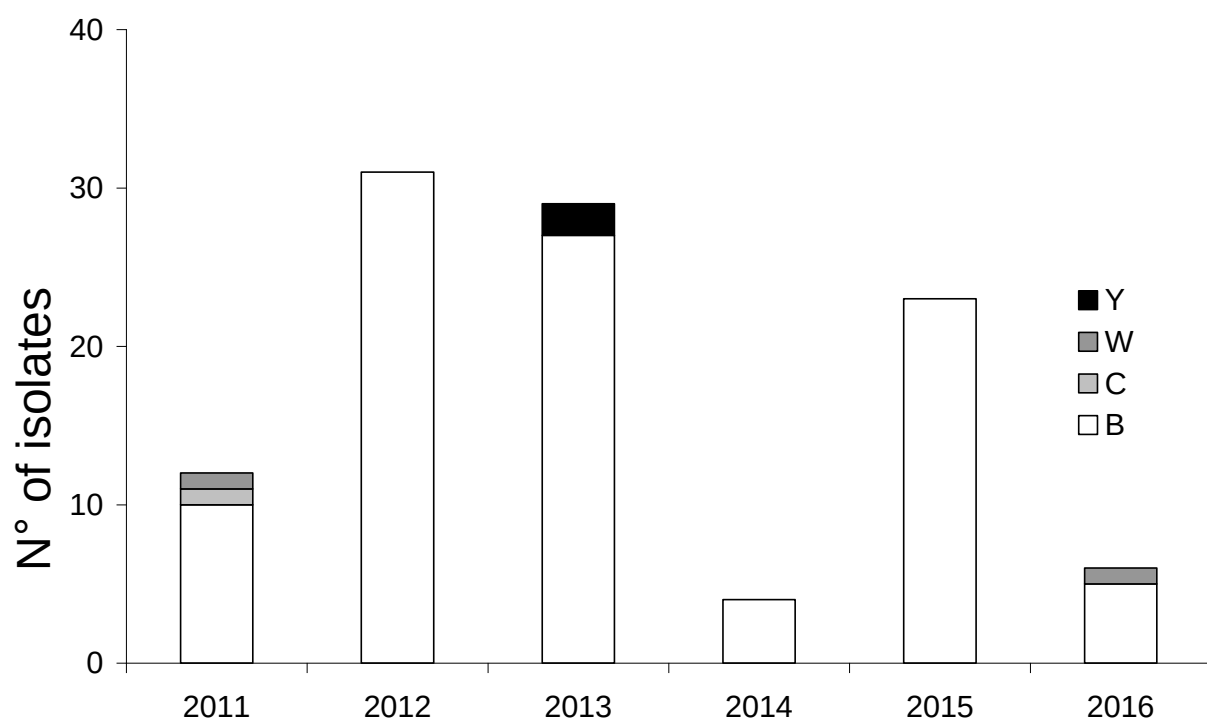
This work was supported by the Institut Pasteur, ACIP grant N° A03-&4. This publication made use of the Neisseria Multi Locus Sequence Typing website (<https://pubmlst.org/neisseria/>) developed by Keith Jolley and sited at the University of Oxford (18). The development of this site has been funded by the Wellcome Trust and European Union. We also acknowledge the PIBNET-P2M platform at the Institut Pasteur.

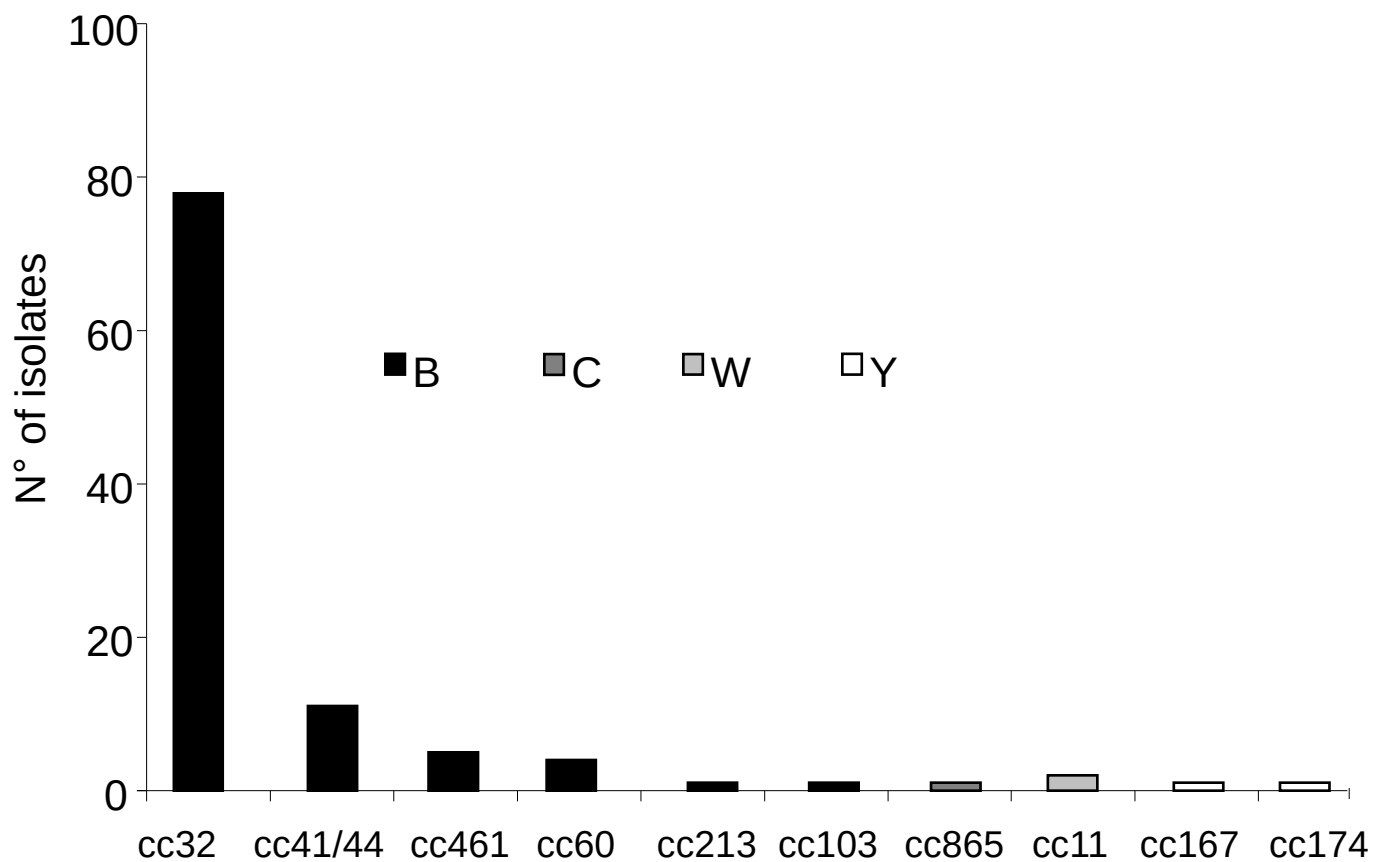
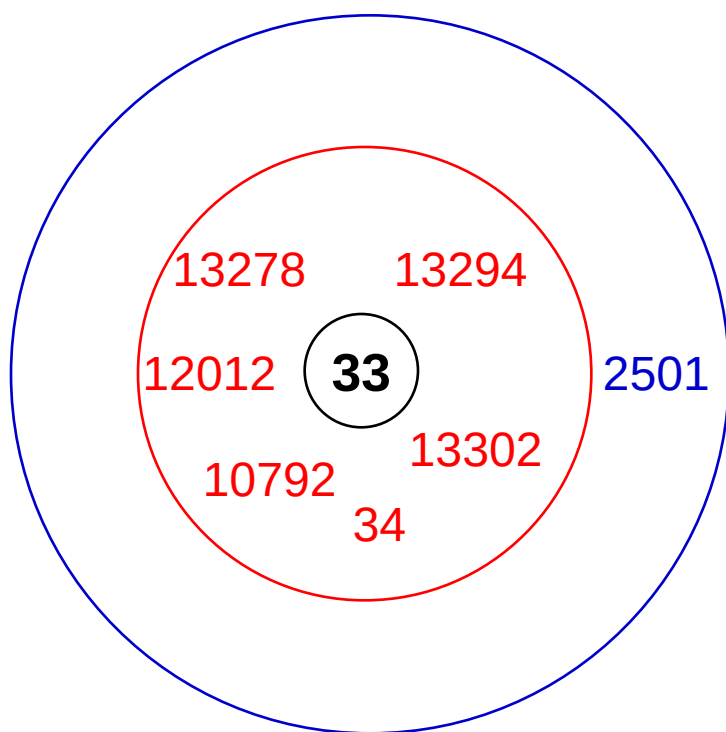
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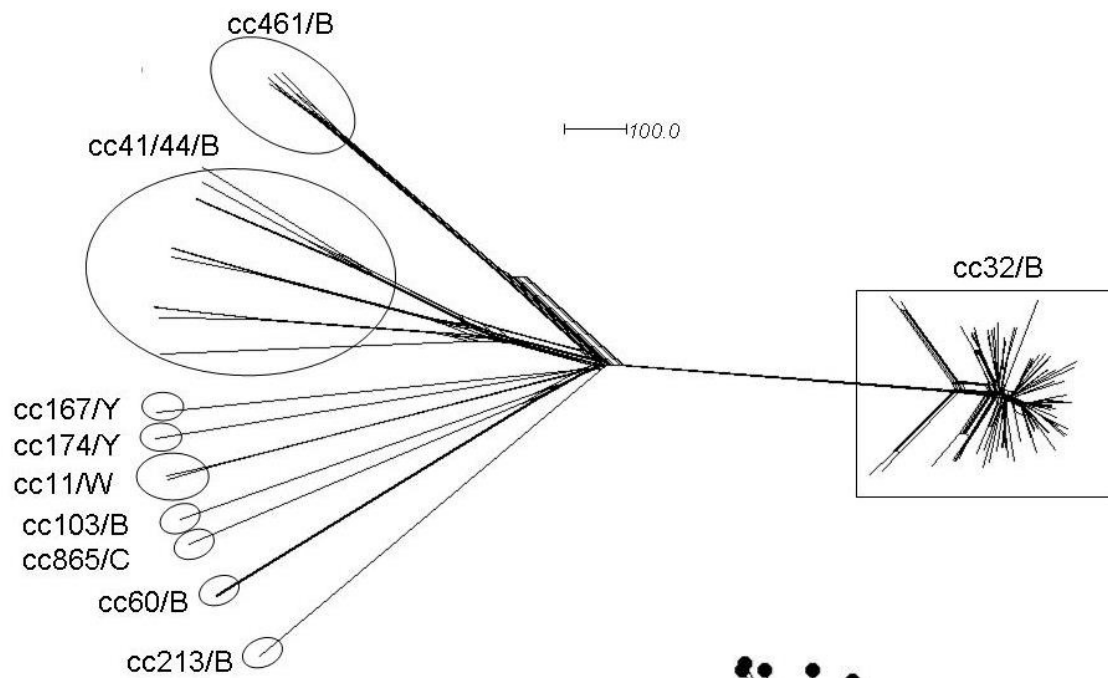
Figure1. Distribution of the isolates of the study according to their serogroups and year of isolation.

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

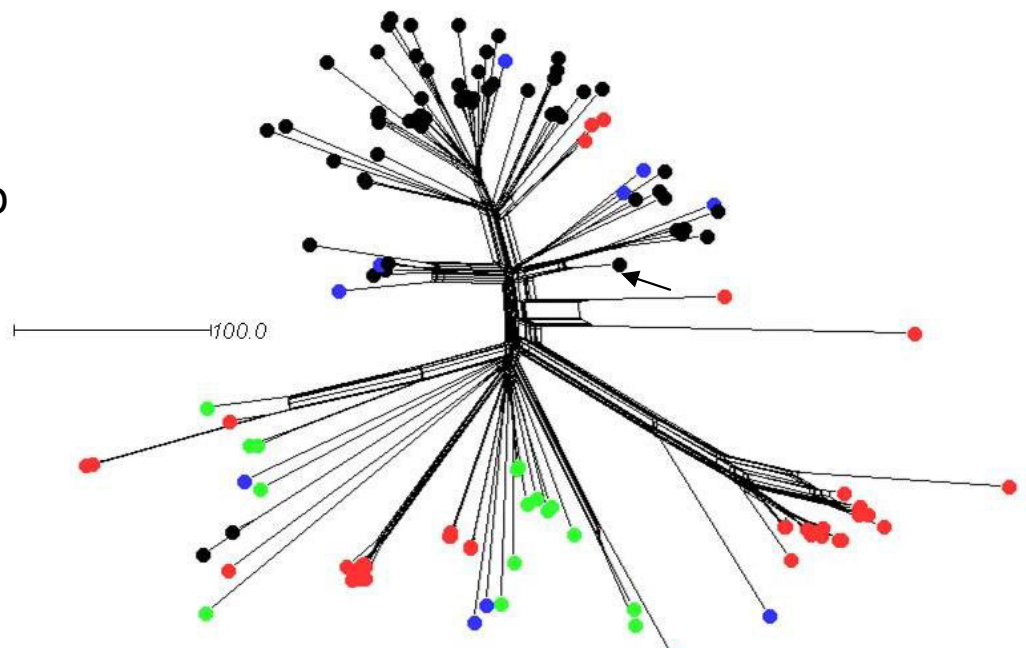
Figure 3. (A) Neighbor-net phylogenetic network of all invasive cultured isolates received of the study from the region of Casablanca, Morocco (2011-2016). The tree was drawn on the basis of cgMLST. The 10 clonal complexes and the corresponding serogroup of the isolates are indicated. (B) Neighbor-net phylogenetic network of all serogroup B isolates showing the finetype P.19,15:F5-1 in the PubMLST database: 58 isolates from Morocco in addition to 69 other isolates between 1976-2017 and were from the UK, France, Brazil, Cuba, Italy, Slovenia, South Africa, Canada, Denmark, 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 isolates from our collection (black circle) B depicted within the tree of all invasive 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 Anglo-French-Hajj.



A**B**

A**B**

- Morocco
- UK
- France
- Others

**C**