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2017

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Molecular characterization of non-groupable *Neisseria meningitidis* causing invasive disease in South Africa

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Submitted by

Karistha Ganesh

(1153542)

To



The University of the Witwatersrand,

Faculty of Health Sciences

In fulfilment of the requirements for the degree of

Master of Science in Medicine

Date of submission: 27 October 2017

Supervisor: Dr M. du Plessis

Co-supervisors: Dr M. Allam and Dr A. von Gottberg

Place: Johannesburg

DECLARATION

I, Karistha Ganesh, declare that the dissertation (by research publication) entitled 'Molecular characterization of non-groupable *Neisseria meningitidis* causing invasive disease in South Africa', which is being submitted to the University of the Witwatersrand for the degree of Master of Science in Medicine, is my own work. My contribution to this study included the following:

- Background research and writing a literature review
- Writing the funding proposal for a National Health Laboratory Services Research Trust award specifically for this project, which was awarded to Dr Mignon du Plessis (supervisor)
- Writing the study proposal containing the initial aims, objectives and design
- Application to the Human Research Ethics Committee (University of the Witwatersrand) for clearance of research
- Sample processing which included re-testing the isolates obtained through the national laboratory-based surveillance for the purposes of mastering techniques to obtain this degree:
    - Characterization using phenotypic methods (culture, Gram strain, oxidase, API-NH, serogrouping using capsule specific antibodies)
    - Antimicrobial susceptibility testing
    - Preparation of isolates for long-term storage
    - DNA extraction for PCR (boiling method)
    - Performing real-time PCR (*ctrA*, *sodC* and genogrouping)
    - DNA extraction (manual kit extraction) and quantification for genome sequencing
    - Quality analysis of sequenced genomes and assembly; and submission of assembled sequences and demographic data for uploading onto the *Neisseria* PubMLST database for annotation and analysis
    - Validation of an electron microscopy method for visualization of bacterial capsules
        - Performing a literature search for electron microscopy protocols

- Preparation of isolates for staining
- Troubleshooting
- Data analysis and critical review of the book chapter in which this work was published
- I have also trained in Dr Monica Birkhead's laboratory (observation of the transmission electron microscopy protocols being performed)

➢ Data analysis

- Analysis of results from phenotypic assays, real-time PCR, antimicrobial susceptibility testing and whole genome data
- Molecular characterization of the South African non-groupable meningococci as well as 89 other capsule null isolates on *Neisseria* PubMLST, using genome data (serogroup, capsule null allele, *rplF* typing, PorA, FetA, sequence type, clonal complex)
- Analysis of the capsule polysaccharide synthesis locus to determine mechanisms for capsule loss; and flanking regions to determine acquisition/ loss of capsule genes
- Phylogenetic comparison of the South African capsule null isolates and publically available capsule null genomes on *Neisseria* PubMLST using seven-locus MLST, ribosomal MLST and core genome MLST
  - Construction of phylogenetic trees
  - Quantifying the degree of relatedness between isolates
  - Performing statistical analysis
  - Examining the nucleotide sequences of variable loci between isolates and obtaining annotations from the *Neisseria* PubMLST database
- Identification of genetic markers for differentiation of carriage and invasive capsule null meningococci (virulence analysis)

- Obtaining PubMLST annotations ('NEIS') for 117 previously published putative virulence loci
- Performing Genome Comparator analysis for the South African capsule null isolates and publically available capsule null isolates on *Neisseria* PubMLST, and interpretation of output

- Analysis of vaccine antigens (fHbp, NHBA, NadA) in the South African and 89 publically available capsule null genomes on *Neisseria* PubMLST
- Troubleshooting
- Sourcing patient and isolate information which was accessible (submitted to the Centre for Respiratory Diseases and Meningitis as part of the national laboratory-based surveillance)

➢ Data interpretation

- Interpretation of phenotypic, PCR and genome data in conjunction with clinical information and previously published data
- Writing of the manuscript and dissertation (including preparation of all tables and figures for both documents)

➢ Preparation of poster and power point presentations; and presentation at local and international forums

All of the above were conducted under the supervision of Dr's Mignon du Plessis, Mushal Allam and Anne von Gottberg at the Centre for Respiratory Diseases and Meningitis, National Institute for Communicable Diseases, National Health Laboratory Services, Johannesburg. This work has not been previously submitted for any other degree or examination by the University of the Witwatersrand or any other institute.



Karistha Ganesh

27 October 2017
Date



For my parents,

Naresh and Anitha Ganesh

CONFERENCE PROCEEDINGS

Ganesh K, Allam M, Maiden MCJ, Jolley KA, Wolter N, von Gottberg A and du Plessis M. Molecular characterisation of two capsule null locus meningococci causing invasive disease in South Africa. Poster presentation: *19th International Pathogenic Neisseria Conference*, Asheville, United States of America, 12-17 October 2014.

Birkhead M and Ganesh K. Phenotypic characterisation of bacterial pathogens using transmission electron microscopy and cationic dyes. Poster presentation: *52nd Conference of the Microscopy Society of Southern Africa*, Stellenbosch, South Africa, 2-5 December 2014.

Ganesh K, Allam M, Maiden MCJ, Jolley KA, Wolter N, von Gottberg A and du Plessis M. Molecular characterization of invasive capsule null *Neisseria meningitidis* in South Africa. Poster presentation: *Faculty of Health Sciences Biennial Research Day and Post Graduate Expo*, Johannesburg, South Africa, 1 September 2016.

Ganesh K, Allam M, Wolter N, Bratcher HB, Harrison OB, Lucidarme J, Borrow R, de Gouveia L, Meiring S, Birkhead M, Maiden MCJ, von Gottberg A and du Plessis M. Molecular characterization of invasive capsule null *Neisseria meningitidis* in South Africa. Oral presentation: *National Institute for Communicable Diseases Research Forum*, Johannesburg, South Africa, 26 April 2017.

LIST OF PUBLICATIONS

Manuscript

Ganesh K, Allam M, Wolter N, Bratcher HB, Harrison OB, Lucidarme J, Borrow R, de Gouveia L, Meiring S, Birkhead M, Maiden MCJ, von Gottberg A and du Plessis M. Molecular characterization of invasive capsule null *Neisseria meningitidis* in South Africa. BMC Microbiol 2017 Feb 21;17(1):40.

Book chapter

Birkhead M, Ganesh K, Nlangisa K, Koornhof HJ. Transmission electron microscopy protocols for capsule visualisation in pathogenic respiratory and meningeal bacteria. In: Microscopy and imaging science: practical approaches to applied research and education, ed. by Méndez-Vilas A. Spain: Formatex Research Center, 2017.

ABSTRACT

Background

The meningococcal capsule is an important virulence determinant. Unencapsulated meningococci lacking capsule biosynthesis genes and containing the capsule null locus (*cnl*) are predominantly non-pathogenic. Rare cases of invasive meningococcal disease caused by *cnl* isolates belonging to sequence types (ST) and clonal complexes (cc) ST-845 (cc845), ST-198 (cc198), ST-192 (cc192) and ST-53 (cc53) have been documented. The clinical significance of these isolates however remains unclear. We identified four invasive *cnl* meningococci through laboratory-based surveillance in South Africa from 2003 through 2013, which we aimed to characterize using whole genome data.

Results

One isolate [NG: P1.7-2,30: F1-2: ST-53 (cc53)] contained *cnl* allele 12, and caused empyema in an adult male with bronchiectasis from tuberculosis, diabetes mellitus and a smoking history. Three isolates were NG: P1.18-11,42-2: FΔ: ST-192 (cc192) and contained *cnl* allele 2. One patient was an adolescent male with meningitis. The remaining two isolates were from recurrent disease episodes (eight months apart) in a male child with deficiency of the sixth complement component, and with the exception of two single nucleotide polymorphisms, contained identical core genomes. The ST-53 (cc53) isolate possessed alleles for NHBA peptide 191 and fHbp variant 2; whilst the ST-192 (cc192) isolates contained NHBA peptide 704 and fHbp variant 3. All four isolates lacked *nadA.* Comparison of the South African genomes to 65 additional *cnl* genomes on the PubMLST *Neisseria* database (http://pubmlst.org/neisseria/), determined that most putative virulence genes could be found in both invasive and carriage phenotypes.

Conclusions

Although rare, invasive disease by *cnl* meningococci may be associated with host immunodeficiency and such patients may benefit from protein-based meningococcal vaccines.

ACKNOWLEDGMENTS

I would like to express my gratitude to the Almighty, for blessing me with the opportunity to pursue the degree of Master of Science (Medicine), and for the people who have contributed towards this research.

SCIENTIFIC:

To my supervisors, Dr's Mignon du Plessis, Mushal Allam and Anne von Gottberg from the Centre for Respiratory Diseases and Meningitis, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg, South Africa; for contributing towards the design of this study, sharing your expertise to guide the data analyses and constructive criticism of the manuscript. Thank you for your constant support and for believing in my capabilities.

To the members of the GERMS-SA surveillance network, as well as the staff of the Centre for Respiratory Diseases and Meningitis, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg, South Africa; this research was made possible through your efforts.

To Dr Nicole Wolter from the Centre for Respiratory Diseases and Meningitis, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg, South Africa; for contributing towards the study design and constructive criticism of the manuscript. Thank you for your constant encouragement and support.

To Dr Monica Birkhead from the Centre for Emerging and Zoonotic Diseases, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg, South Africa; for your assistance in validating an electron microscopy method for visualization of polysaccharide capsules from Gram-positive and Gram-negative bacteria (1).

This research made use of the *Neisseria* Multilocus Sequence Typing website (http://pubmlst.org/*neisseria*/) developed by Dr Keith A. Jolley and Dr Martin C. J. Maiden, Department of Zoology, University of Oxford, Oxford, United Kingdom. I would like to thank Dr Martin C. J. Maiden for contributing towards the conception of this study and critical review of the manuscript. Additionally, I would like to acknowledge Dr Odile B. Harrison and Dr Holly B. Bratcher for curation of the PubMLST *Neisseria* database which enabled automatic annotation of the assembled genomes for all 94 isolates analysed in this study (using BIGSdb), and for review of the manuscript.

To Dr Ray Borrow and Dr Jay Lucidarme from the Meningococcal Reference Unit, Public Health England, Manchester Medical Microbiology Partnership, Manchester Royal Infirmary, United Kingdom; for co-ordinating the genome sequencing of four additional sequence type (ST)-192, capsule null meningococci from Burkina Faso [PubMLST identification no.: 35416, 35417, 35418 and 35419] (2-4), and review of the manuscript.

To Professor Sanjay Ram from the University of Massachusetts Medical School, Division of Infectious Diseases and Immunology, United States of America; for providing DNA for the invasive capsule null meningococcal isolate A4BZ577 (ST-198) [PubMLST id: 37603], which was sequenced during this study (3;5).

To Mrs Linda de Gouveia and Dr Susan Meiring from the Centre for Respiratory Diseases and Meningitis, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg, South Africa; for assisting me with obtaining additional demographic information for the South African patients presenting with invasive meningococcal disease by capsule null isolates, from the respective hospitals in which these patients were admitted (this information was not submitted through the national laboratory-based surveillance); and review of the manuscript.

To the staff of the Core Sequencing Facility, National Institute for Communicable Diseases of the National Health Laboratory Service and The Oxford Genomics Centre, University of Oxford, for their services i.e., sequencing the South African non-groupable *N. meningitidis* isolates.

FUNDING

This work was supported by a National Health Laboratory Service Research Trust development grant (grant number 94442) to Dr Mignon du Plessis. Bill and Melinda Gates Foundation funding was awarded to Miss Karistha Ganesh to attend and present this work, in part, at the *19th* International Pathogenic *Neisseria* Conference, Asheville, North Carolina, USA, 12-17th October 2014 (award reference number: 307494-01-00-64512). Dr Odile B. Harrison, Dr Holly B. Bratcher and Dr Martin C.J. Maiden were supported by the Wellcome Trust (grant 087622). The maintenance of the *Neisseria* Multilocus Sequence Typing website is funded by the Wellcome Trust (grant 104992).

PERSONAL

To my guiding lights, my parents Naresh and Anitha Ganesh, and my brother Nivesh Ganesh; for your constant encouragement throughout my research.

LIST OF ABBREVIATIONS AND SYMBOLS

~: Approximately

°C: Degrees Celsius

Δ: Gene deletion

µl: Microliter

ATCC: American Type Culture Collection

BIGSdb: Bacterial Isolate Genome Sequence Database platform

bp: Base pair

C6: Sixth complement component

cc: Clonal complex

CFU: Colony forming units

cgMLST: Core genome MLST

cnl: Capsule null locus

CO_2: Carbon dioxide

Contig: Contiguous sequence

COPD: Chronic obstructive pulmonary disease

cps: Capsular polysaccharide synthesis locus

CRDM: Centre for Respiratory Diseases and Meningitis

CSF: Cerebrospinal fluid

DNA: Deoxyribonuclease acid

DMP: Diagnostic Media Products

ENA: European Nucleotide Archive

FetA: Ferric enterochelin receptor

fHbp: Factor H-binding protein

GERMS-SA: Group for Enteric, Respiratory and Meningeal Disease Surveillance

HIV: Human Immunodeficiency Virus

hr: Hour

IMD: Invasive meningococcal disease

kDa: Kilodalton

LPS: Lipooligosaccharide

MCC: Meningococcal serogroup C/diphtheria toxoid vaccine

MIC: Minimum inhibitory concentration

min: Minute

ml: Millilitre

MLEE: Multilocus enzyme electrophoresis

MLST: Multilocus sequence typing

N. meningitidis: *Neisseria meningitidis*

NadA: Neisserial adhesin A

ND: Not defined

NG: Non-groupable

NHBA: Neisserial heparin-binding antigen

NHLS: National Health Laboratory Services

NICD: National Institute for Communicable Diseases

OMP: Outer membrane protein

PCR: Polymerase chain reaction

PorA: Porin A

PorB: Porin B

R: Resistant

rMLST: Ribosomal MLST

RT-PCR- Real-time PCR

S: Susceptible

SA: South Africa

SBA: Serum bactericidal assay

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

sec: Second

SNP: Single nucleotide polymorphism

ST: Sequence type

TEM: Transmission electron microscopy

UK: United Kingdom

USA: United States of America

VR: Variable region

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CHAPTER ONE: LITERATURE REVIEW

1.1. Background

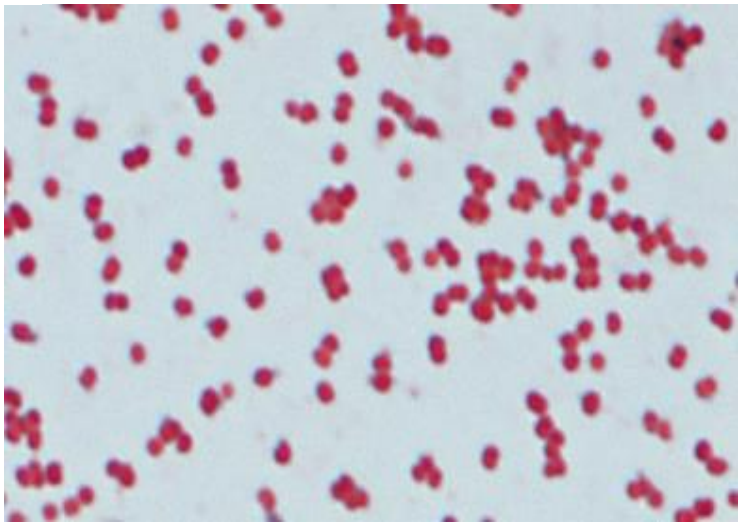
Neisseria meningitidis (meningococcus) is a commensal bacterium of the human upper respiratory tract but is also an important cause of septicemia and meningitis, particularly in children less than five-years-old and young adults (6-13). Approximately 1.2 million cases of invasive meningococcal disease (IMD) are reported globally, per annum; with ~10% of IMD cases being fatal and up to 20% of survivors experiencing long-term sequelae such as deafness, limb amputation and mental impairment (14;15). Further, the meningococcus is transmitted via aerosolized respiratory secretions and may cause large scale outbreaks (16;17). The rapid disease manifestation, devastating long-term complications and significant impact on public health makes precise identification and characterization of *N. meningitidis* an important aspect for the monitoring, control and prevention of IMD.

The first well described outbreak of meningococcal disease was by Vieusseux and occurred in 1805 in Geneva, Switzerland (18). It was only in 1887, that Weichselbaum successfully isolated the etiological agent from the cerebrospinal fluid (CSF) of six fatal cases of IMD, and named it '*Diplococcus intracellularis meningitidis*'. The asymptomatic carriage state of the meningococcus precedes IMD and was later established in 1896, whereby Kiefer isolated the bacterium from throat swabs taken from healthy carriers.

N. meningitidis is a Gram-negative diplococcus that is oxidase and catalase positive, and may be differentiated from other *Neisseria* spp. by fermentation of glucose and maltose (Figure 1 A) (19). Meningococci are fastidious and grow optimally on blood-enriched media, incubated in a moist environment between 35-37°C and containing 5% CO_2. The bacterium is non-haemolytic and

appears as smooth, translucent/ grey colonies which are convex in shape (Figure 1 B). In addition, meningococci may be encapsulated or unencapsulated.

A



B

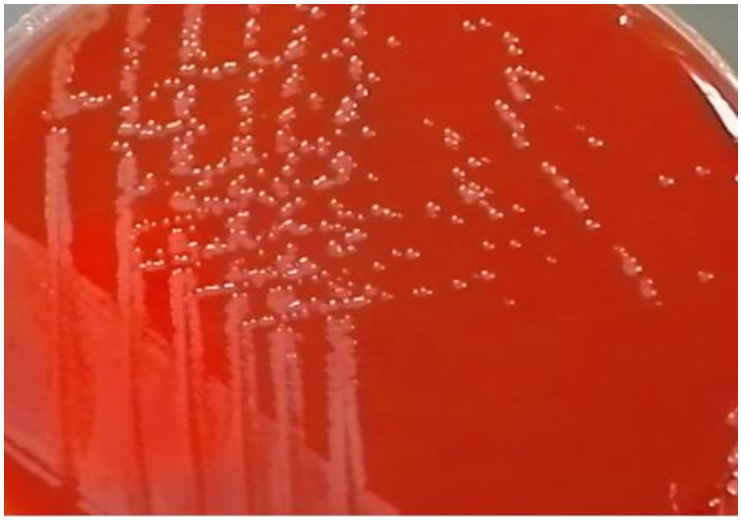


Figure 1. Microbiological characteristics of *Neisseria meningitidis*. A) Gram's stain of *N. meningitidis* (American Type Culture Collection (ATCC) 13077, serogroup A), showing Gram-negative diplococci (X1000). B) A 24 hour (hr) culture of *N. meningitidis* (ATCC 13077, serogroup A) on 5% horse blood agar showing convex, smooth, translucent/ grey colonies which are non-haemolytic.

1.2. Phenotypic characterization of *N. meningitidis* using the polysaccharide capsule

The polysaccharide capsule is regarded as an important virulence determinant, and promotes survival of the meningococcus in the bloodstream by preventing complement-mediated lysis and opsonophagcytosis (20-23). Further, most cases of IMD are caused by encapsulated strains (6-13). Twelve antigenically different polysaccharide capsules have been described, and classify the bacterium into serogroups (A, B, C, E, H, I, K, L, W, X, Y and Z) (24). In addition, the meningococcal capsule is an important component of polysaccharide-based vaccines which target serogroups A, C, W and Y.

The first meningococcal serological typing scheme was described in 1915 by Gordon and Murray (25), which classified *N. meningitidis* into types I-IV. The typing scheme was revised in 1950 by the Subcommittee on the Family *Neisseriaceae* of the International Committee on Bacteriological Nomenclature (International Association of Microbiologists), and classified *N. meningitidis* into serogroups A, B, C and D (26). Serogroups X, Y and Z were subsequently described in 1961; E (previously 29E or Z') and W (previously 135 or W135) in 1968; H, I and K in 1981 and L in 1983 (27-31).

Enzymes for capsular synthesis and transport are encoded by a single cluster of genes, termed the capsular polysaccharide synthesis (*cps*) locus (Figure 2) (24). The *cps* locus is divided into six regions which are arranged in order of D-A-C-E-D'-B. Enzymes for polysaccharide synthesis are encoded by genes in region A, which vary according to serogroup and are therefore used to design genogrouping assays (24;32). Genes in regions B (*ctrE-ctrF*) and C (*ctrA-ctrD*) encode enzymes for transport of capsular material (33). Region D comprises non-functional rhamnose biosynthetic pathway genes (*rfbA- rfbC*), and *galE* which is involved in lipopolysaccharide (LPS) biosynthesis; whilst region D' is a truncated, non-functional version

of region D (34). Region E is not involved in polysaccharide synthesis nor transport, but is hypothesized to be involved in the regulation of these processes (35). Meningococci belonging to serogroup D were determined to be unencapsulated based on the fact that they contained serogroup C genes and internal stop codons in *ctrA* and *ctrE*, and did not agglutinate with antisera (24).

Non-groupable refers to a meningococcal isolate which cannot be classified into one of the 12 defined serogroups, using phenotypic and genotypic methods. Slide agglutination serogrouping assays are based on antigen-antibody binding, and a positive result is indicated by agglutination with one serogroup-specific antiserum and a negative saline control (36). Serological assays are however subjective as the intensity of the reaction is reliant on the density of the cell suspension and quality of antiserum used. On occasion, some meningococci may agglutinate with more than one serogroup-specific antiserum and are termed as 'polyagglutinating', e.g. isolates expressing more than one capsule phenotype (37-39). In addition, some meningococci do not agglutinate and are regarded as 'non-reactive', or agglutinate in the saline control and are classified as 'autoagglutinating'; both of which may occur as a result of capsule loss due to horizontal genetic exchange, slipped-strand mispairing, point mutations or gene deletion in the *cps* locus (40-42). According to Castillo *et al.* (36), polyagglutinating, non-reactive and autoagglutinating meningococci are reported as 'non-groupable'. Although genogrouping assays such as real-time polymerase chain reaction (RT-PCR) directly infer serogroup information from nucleotide sequences, these assays do not indicate capsule expression (32). Genome data may be used to provide additional resolution for determining mechanisms for non-groupability (39;43).

The presence of a polysaccharide capsule may be confirmed using transmission electron microscopy (TEM). However, polysaccharide capsules are hydrated structures and are difficult to preserve during sample preparation. Previous protocols to address this problem included the use of cationic dyes such as ruthenium red or alcian blue, together with fixatives including formaldehyde and glutaraldehyde with the addition of L-lysine acetate (primary fixation), and osmium tetroxide (post-fixation) (1;44;45). These methods were however not universal as capsular staining may depend on the cell wall composition (Gram-positive or Gram-negative) and the cationic dye used (1). Results from both phenotypic and genotypic assays should therefore be considered to confirm the presence and expression of *cps* genes.

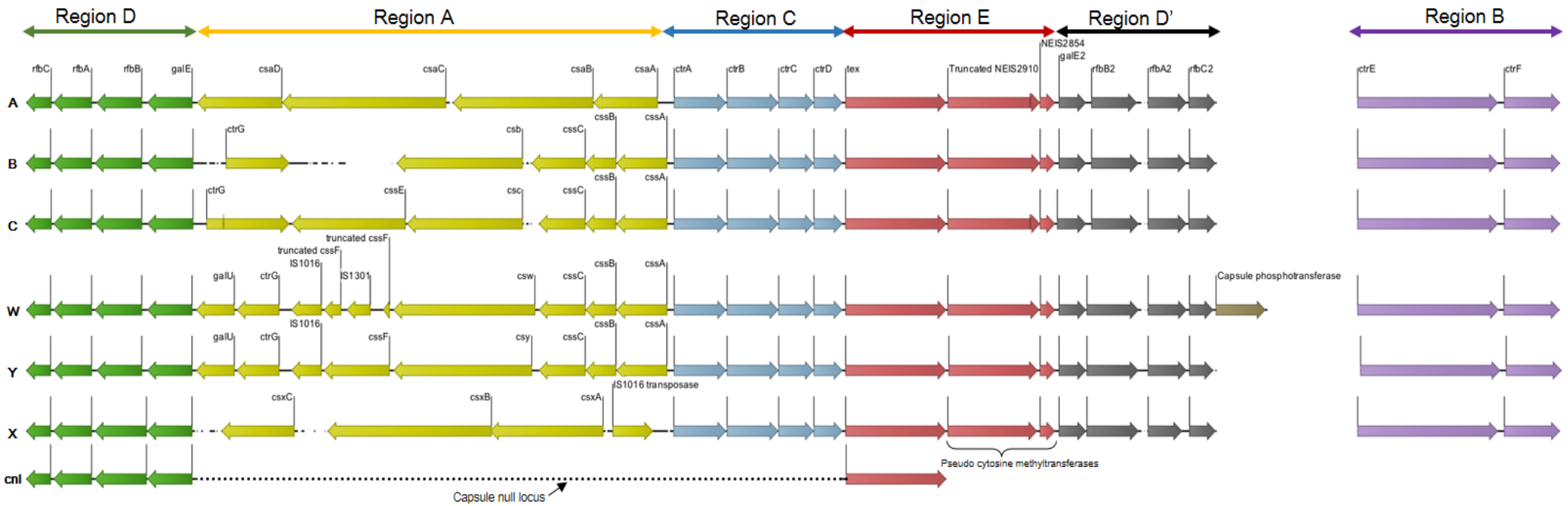


Figure 2. Arrangement and nomenclature of genes in the *Neisseria meningitidis* capsular polysaccharide synthesis (*cps*) locus for the six most common invasive serogroups A (isolate Z2491, GenBank accession no. AL157959), B (H44/76, CP002420), C (FAM18, AM421808), W (WUE171, HF562992), X (α388, HF562988) and Y (α162, HF562989), adapted from Harrison *et al.* (24).The *cps* is divided into six regions arranged in order of D-A-C-E-D'-B, and genes in region A vary according to serogroup. Meningococci containing the capsule null locus (*cnl*) (5957, PubMLST accession no. 29312) lack the genes required for polysaccharide synthesis (region A) and transport (region B and C).

1.3. Molecular characterization

1.3.1. Outer membrane proteins

Meningococci of the same capsular type (serogroup) are genetically and antigenically diverse, and capsular typing alone does not provide sufficient data for IMD surveillance. Typing of meningococcal outer membrane proteins (OMP) provides additional strain information such as serotype and serosubtype. Meningococcal OMP's are categorized into five classes based on molecular weight (46). Serosubtyping is based on porin A (PorA), which is a class 1 (46 kDa) OMP and denoted as P1. (47). The PorA protein structure contains eight surface-exposed loops with the highest amino acid diversity observed in loops I and IV, which bind to monoclonal antibodies in serological assays (48). Loops I and IV are designated as major variable regions (VR) and are referred to as VR1 and VR2, respectively. Serotyping is based on the porin B (PorB) antigen which possesses class 2 (41 kDa) or 3 (38 kDa) homology (49).

Due to the high variability of OMP's, monoclonal antibody panels do not encompass all known antigen variants (50). In addition, serological assays rely on culture positive specimens and expression of the relevant target, and results are poorly reproducible (51;52). To address these problems, molecular typing schemes for meningococcal characterization were developed and are hosted on the PubMLST *Neisseria* database (http://pubmlst.org/neisseria/) (53). The PubMLST *Neisseria* database is publically available and regularly updated, and provides a standardized platform for comparison of data between different laboratories. The PubMLST *Neisseria* database hosts antigen typing schemes for PorA VR1 and VR2 (*porA*) (54), PorB (*porB*) (55), the ferric enterochelin receptor (*fetA*) (56) and antigens which are included in OMP vaccine formulations including factor H-binding

protein **(***fHbp*) (57), neisserial adhesin A (*nadA*) (58;59) and neisserial heparin-binding antigen (*nhba*) (60).

The FetA antigen is expressed under conditions of iron limitation and may be classified into one of five families, based on loop VII (61;62). The meningococcal fHbp antigen recruits factor H to the bacterial surface to avoid complement-mediated lysis (63). The fHbp antigen may be classified into three variants (variants 1-3) (64) or two subfamilies (A and B) (65). Subfamily A corresponds to variants 2 and 3, and subfamily B corresponds to variant 1. The NadA antigen is involved in adhesion and invasion of human epithelial cells, and is predominantly associated with invasive meningococci as opposed to carriage strains (58;59;66). The NHBA antigen binds heparin and increases resistance to killing by human sera, as well as contributes to adhesion to human epithelial cells (60;67).

1.3.2. Multilocus sequence typing

Loci which encode OMP's are highly variable and do not provide information on meningococcal population structure. The multilocus enzyme electrophoresis (MLEE) approach was previously used to detect microevolution in a bacterial populations, based on the electrophoretic movement of metabolic enzymes which are encoded by conserved housekeeping loci (68). However, MLEE data are poorly reproducible and difficult to compare amongst laboratories. Alternatively, meningococcal population structure may be determined by directly indexing variation in the nucleotide sequences of housekeeping genes (fragment size ~500 bp), using multilocus sequence typing (MLST) (69). Multilocus sequence typing data are therefore unambiguous, reproducible, standardized and may be readily compared between laboratories using the PubMLST *Neisseria* database.

The nucleotide sequences of seven housekeeping genes i.e., *abcZ* (putative ABC transporter), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *fumC* (fumaratehydratase), *gdh* (glucose-6-phosphate dehydrogenase), *pdhC* (pyruvate dehydrogenase subunit) and *pgm* (phosphoglucomutase) are queried on the PubMLST *Neisseria* database, and every unique sequence for each locus is assigned a numeric identifier or allele number (69). The PubMLST *Neisseria* database is regularly updated, with each new sequence being assigned a new allele number. The unique combination of seven allele numbers (allelic profile) is defined as a sequence type (ST) e.g. *abcZ* [allele no. 35]-*adk* [24]-*aroE* [15]-*fumC* [48]-*gdh* [6]-*pdhc* [48]-*pgm* [41], is the allelic profile for *N. meningitidis* isolates belonging to ST-192. A group of related ST's which contain ≥ 4/7 allele matches is defined as a clonal complex (cc), for *Neisseria* spp. (70).

Multilocus sequence typing however provides limited resolution as this method is based on nucleotide variation in only seven genes which are not under selective pressure and remain conserved. Meningococci belonging to the same sequence type may exhibit considerable variation in other parts of the genome (71), hence MLST is unable to distinguish between closely related isolates. Multilocus sequence typing data can therefore be combined with sequence data from more rapidly evolving genes to provide more resolution. The combined data for serogroup, PorA, FetA, sequence type and clonal complex, form the meningococcal strain designation as follows (51):

- Serogroup: P1. VR1 family-subfamily, VR2 family-subfamily: F family-subfamily: ST (cc)
- Example:
  - Stain designation: NG: P1.7-2,30: F1-2: ST-53 (cc53)
    - NG: non-groupable

- P1.7-2,30: PorA VR1 family 7 and subfamily 2, VR2 family 30
- F1-2: FetA VR family 1 and subfamily 2
- ST-53: sequence type 53
- cc: clonal complex 53

1.3.3. The Bacterial Isolate Genome Sequence Database (BIGSdb) platform

The use of next-generation sequencing enables cost-efficient characterization of *N. meningitidis* and in addition, provides higher resolution than MLST (72;73). The genome of *N. meningitidis* is ~2 million bp, and encodes ~2000 open reading frames (74-76). Since 2012, the PubMLST *Neisseria* typing databases have been hosted by the Bacterial Isolate Genome Sequence Database (BIGSdb) platform, which is a database system that was developed for the flexible storage and analysis of sequencing data of any size, including single or multiple genes and whole genome data (77). Following DNA extraction, genome sequencing and *de novo* assembly of short paired-end reads into contiguous sequences (contigs) to form draft genomes, the assembled sequences can be uploaded to the respective isolate record on the PubMLST *Neisseria* database and annotated using BIGSdb (Supplementary Figure S1) (77). Like the MLST approach which is based on allelic diversity, the BIGSdb platform also utilizes the gene-by-gene concept (53;77;78). The prefix 'NEIS' is allocated to all genes identified in *Neisseria* spp., followed by a unique numeric gene identifier and respective allele number, e.g. NEIS1364 is the unique PubMLST *Neisseria* database identifier for the *porA* locus, with 201 sequence variants/ alleles being described at the time of this study (accessed 11/1/2017).

The BIGSdb platform permits analysis of single or multiple loci which are incorporated into functional typing schemes, including metabolism, capsule, genetic information processing,

antigen typing, iron acquisition, etc (77). In addition, genome data may be analysed using schemes which are extensions of the seven-locus MLST approach, including the ribosomal multilocus sequence typing (rMLST) scheme which comprises 53 universal loci encoding ribosome protein subunits; and the core genome MLST (cgMLST) scheme comprising 1605 conserved *N. meningitidis* loci (72;73). Another typing method implemented by BIGSdb, enables *Neisseria* spp. identification by using a single rMLST locus, more specifically a 413 bp fragment of the 50S ribosomal protein L6 (*rplF*) gene (79). The genetic relatedness amongst isolates using single or multiple loci may be evaluated using the Genome Comparator tool in BIGSdb which constructs phylogenetic trees based on the number of differing loci, using the NeighbourNet Algorithm (77).

1.4. Meningococcal pathogenesis

N. meningitidis is first acquired through respiratory secretions and attaches to the non-ciliated mucosal epithelial cells of the human oropharynx, which is the bacterium's sole ecological niche (80;81). The meningococcus then multiplies locally, leading to asymptomatic carriage. Parasite-directed endocytosis is initiated by the meningococcus and the phagocytic vacuoles pass through the epithelial cell so that the bacterium may enter the extracellular matrix, or may migrate back to the cell surface for transmission to another host.

Invasive disease is not part of the normal meningococcal life-cycle, as the bacterium cannot be easily transmitted to another host from the bloodstream or cerebrospinal fluid (CSF). Reasons for causing IMD are poorly understood, and the meningococcus is thus regarded as an 'accidental pathogen' (82). Entry into the bloodstream may occur between or through the capillary endothelial cells (81). Once in the bloodstream, the meningococcus may multiply rapidly resulting in high bacterial loads and meningococcal endotoxin (LPS). Symptoms of

meningococcemia include sudden onset of fever, petechial rash leading to purpura fulminans, hypotension and shock (83;84). The meningococcus may then cross the brain vascular endothelium and invade the meninges and CSF causing symptoms of meningitis, including sudden onset of fever, nausea, vomiting, stiff neck, photophobia, headache and disorientation (81;83;84). Meningococcemia and meningitis are the most common manifestations of IMD, however *N. meningitidis* may also cause focal infection such as arthritis, pneumonia, conjunctivitis, urethritis and pericarditis (83;84).

1.5. Meningococcal carriage

Meningococcal carriage is an important aspect in understanding disease transmission and determining measures for control. This includes planning of immunization programs for meningococcal conjugate vaccines which reduce carriage and provide indirect immunity, as well as treatment for close contacts (85;86). Meningococcal carriage prevalence in healthy individuals was reported to be ~10% in high-income countries (87;88). However, carriage rates vary according to age group and were shown to be low during early childhood (under five years of age) (86;89). This observation may be due to children less than five-years-old having the highest carriage prevalence of *Neisseria lactamica*, which was shown to inhibit meningococcal carriage during nasal-inoculation experiments in young adults (90;91). Carriage prevalence peaked during late adolescence (18-26 years) in Europe and the United Kingdom (UK), and was associated with social behaviour such as kissing, crowding (attendance at night clubs) and smoking (including passive smoke) (87;88;92).

Meningococcal carriage in Africa has been described in the ‘African meningitis belt’ (comprising 26 Sub-Saharan countries from Ethiopia to Senegal), which experiences the highest annual incidence of IMD in the world. However, results from carriage studies

conducted in this region prior to 2009 were not consistent, with carriage prevalences reported to be between 2-35%, and peaks observed in variable age groups (93;94). Reasons for the observed heterogeneity include sampling different age groups at different times of the year and using different swabbing techniques and testing methods (86;94). From 2010 through 2012, the MenAfriCar consortium conducted cross-sectional surveys and longitudinal household surveys to monitor meningococcal carriage and transmission in seven African meningitis belt countries; following the implementation of mass vaccination campaigns to reduce serogroup A cases, which was most prevalent at the time (86;95). These surveys were conducted using standardized methodology and included both the rainy and dry seasons, as well as a broad range of age groups. The meningococcal carriage prevalence estimated from 48490 participants who were sampled across the African meningitis belt during the cross-sectional surveys, was lower than high-income countries at 3% (86). Risk factors for carriage included age (5-14 years), gender (male), season (dry), crowding, smoking within a household and indoor kitchen facilities. In contrast to findings from the UK, social behaviour was not a risk factor for meningococcal carriage however, the MenAfriCar surveys were limited to information regarding the frequency of attendance at social gatherings. The carriage prevalence estimated from 980 members of 133 households who were sampled as part of the longitudinal household survey was 25%; and the average carriage duration of an individual was estimated to be 3.4 months, though persistent carriage of up to 5.8 months was observed in 20 individuals (95).

Non-groupable meningococci are frequently associated with meningococcal carriage (40;41;86;96) and non-invasive disease (97;98). Approximately 16-46% of meningococci from asymptomatic carriers were determined to be unencapsulated (40;41;86). One group of unencapsulated meningococci which harboured the capsule null locus (*cnl*), constituted 46%

of *N. meningitidis* isolates obtained from healthy carriers in the African meningitis belt (86). Capsule null meningococci lack the genes required for capsule-specific polysaccharide synthesis (region A) and capsular transport (region B and C) within the *cps* locus, and contain the characteristic *cnl* allele (Figure 2) (40). The *cnl* is a non-coding DNA fragment of ~113-368 bp. At the time of this study, 26 unique alleles were defined in the PubMLST *Neisseria* database, with some alleles also identified in *N. lactamica* and *N. gonorrhoeae* (Accessed 11/1/2017) (40).

The capsule null meningococci obtained from asymptomatic carriers belonged to a limited number of genetic lineages, including cc53, cc1117, cc198, cc845, cc1136, cc41/44 and cc192 (4;40;99). Previous genome data suggested that the *cps* was acquired through horizontal gene transfer into a non-pathogenic ancestor resembling *cnl* meningococci (75;100). Further, *cnl* meningococci belonging to cc198 or cc1136 were most closely related to *N. gonorrhoeae* and *N. lactamica*, and showed no signs of recombination in the conserved loci which flank the *cnl* (*galE* and *tex*); suggesting that these isolates may resemble unencapsulated ancestors of *N. meningitidis* (101). Alternatively, capsule null meningococci may be derived from encapsulated isolates. Genome analysis of a *cnl* isolate belonging to cc53 determined that this isolate was more closely related to encapsulated meningococci, as opposed to other *cnl* isolates belonging to cc198 and cc1136. In addition, the cc53 *cnl* isolate showed evidence of recombination within the *galE* and *tex* loci. Further, cc41/44 predominantly comprises isolates belonging to serogroups B and C, and *cnl* isolates from this clonal complex additionally contained genes in region B of the *cps*, which are usually absent (40).

1.6. Disease epidemiology-encapsulated meningococci

The epidemiology of IMD varies globally and is mostly attributable to encapsulated isolates belonging to serogroups A, B, C, W, X and Y (6-13;15). Increased risk of IMD was associated with the dry season and age(12;102), previous respiratory disease (103;104), crowding (17;105) and host immunosuppression (106-108). In industrialized countries such as England and Wales, Europe and the United States of America (USA), the annual IMD incidence was reported to be ≤1 case/100 000 population, with most cases being children younger than five years of age with disease caused by serogroup B (7;9;13). Invasive disease due to serogroups C, W and Y were also observed in industrialized countries, and predominantly occurred in adults older than 25 years of age. Serogroup W cases were however reported to be increasing in England and Wales (since 2009) and Europe (since 2011) (9;13;109). From 2000-2002, an increase in serogroup W cases was observed in England and Wales amongst Hajj pilgrims (109), a group which has historically been associated with increased risk of IMD, predominantly due to serogroups A and W (17). Increasing numbers of invasive serogroup Y cases in England and Wales was also reported since 2007 (13;110).

The African meningitis belt is characterized by its unique high incidence of IMD, which ranges between 10->100 cases/100 000 population (102;111). Reasons for the high disease incidence are largely unknown and hypothesized to be associated with extreme environmental conditions during the dry season when most outbreaks occur (94;102). The high IMD incidence in this region may be related to dust wind from the Sahara which damages local mucosal defences, thereby increasing chances of developing IMD upon infection. In addition, irritant coughing may facilitate transmission. Prior to the introduction of a serogroup A conjugate polysaccharide vaccine in 2010, most cases of IMD were

associated with serogroup A (6;11). Disease due to serogroups C (16), W (112) and X (6), have since become more prevalent in the belt (11).

Historically, peaks of IMD in South Africa were observed during the 1970's, 1990 and 1996 in the Gauteng province, in adult black mine workers; and were due to serogroup A (113). During the 1970's however, serogroup B became prevalent in mixed race children in the Western Cape province, and has remained as one of the leading causes of IMD in South Africa. In 2005, a peak in IMD incidence due to serogroup W was observed, with most cases being reported in the Gauteng province, in children younger than one-year-old (114). In 2015, IMD incidence in South Africa was reported to be 0.28 cases/100 000 population (12).

1.7. Disease epidemiology-unencapsulated meningococci

Despite the high prevalence of unencapsulated meningococci in carriage, cases of IMD by this group are uncommon. Fourteen cases of IMD by *cnl* isolates have been published (2;5;115-117), or described in the PubMLST *Neisseria* database (Table 1). These cases occurred in seven countries including Germany (116), Canada (5;115), Burkina Faso (2), China (117), Ireland, UK and Austria. Patient demographic information was unknown for most cases but where available, *cnl* meningococci caused disease in both immunocompetent and immunocompromised patients; and most patients recovered after treatment with antibiotics (2;116;117). One case of IMD which occurred in Canada, in an immunocompetent patient was fatal (5). Overall, the 14 invasive *cnl* isolates belonged to one of five clonal complexes including cc198 (n=5), cc1136 (n=4), cc192 (n=3), cc845 (n=1) and cc53 (n=1).

Cases of invasive disease by unencapsulated meningococci were also reported in the USA, in immunocompromised twin infants (118). Further, one of the twins developed a second

episode of IMD due to the same strain (six months apart), based on identical OMP profiles using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The genetic mechanism for capsule loss in these isolates was unknown. One case of IMD by an unencapsulated isolate belonging to ST-7962 was reported in China, in an immunocompetent infant male (119). The loss of capsule expression was due to slipped-strand mispairing in the *csb* gene, in region A of the *cps* locus.

Table 1. Patient demographic data and strain characterization of previously described cases of invasive disease by unencapsulated *Neisseria meningitdis* isolates.

Patient no.	Gender	Age (years)	Underlying disease	Outcome	Sequelae	Year	Country	Specimen	Strain designation	Mechanism for capsule loss	Reference
1	Unknown	42	Leukemia	Recovered	Unknown	2001	Germany	Blood	NG: P1. 18,25: F5-2: ST-845 (cc845)	*cnl*	(116)
2	Male	12	Unknown	Recovered	None	2003	Burkina Faso	CSF	NG: P1.18-11,42: FΔ: ST-192 (cc192)	*cnl*	(2)
3	Male	13	Unknown	Recovered	None	2003	Burkina Faso	CSF	NG: P1.18-11,42:FΔ: ST-192 (cc192)	*cnl*	(2)
4	Female	11	Unknown	Recovered	None	2004	Burkina Faso	CSF	NG: P1.18-11,42-1: FΔ: ST-192 (cc192)	*cnl*	(2)
5	Female	13	Immunocompetent	Fatal	N/A	2004	Canada	CSF	NG: P1.19,13-9: F5-5: ST-198 (cc198)	*cnl*	(5)
6	Female	13	Unknown	Unknown	Unknown	2006	Canada	Blood	NG: P1.18,25-1: F5-5: ST-198 (cc198)	*cnl*	(115)
7	Female	7	Immunocompetent	Recovered	Unknown	2015	China	CSF	NG: P1. 18,25: F5-70: ST-2146 (cc198)	*cnl*	(117)
8	Unknown	Unknown	Unknown	Unknown	Unknown	2013	UK	Unknown	NG: P1.18,25-14: F-ND: ST-823 (cc198)	*cnl*	PubMLST
9	Unknown	Unknown	Unknown	Unknown	Unknown	2012	Austria	Unknown	NG: P1.18,25-15: F5-5: ST-823 (cc198)	*cnl*	PubMLST
10	Unknown	Unknown	Unknown	Unknown	Unknown	2015	Ireland	Unknown	ND: P1.18-4,25-31: F3-9: ST-1136 (cc1136)	*cnl*	PubMLST
11	Male	10	Unknown	Unknown	Unknown	2009	Canada	Blood	NG: P1.18-4,25: F4-1: ST-1136 (cc1136)	*cnl*	PubMLST
12	Unknown	Unknown	Unknown	Unknown	Unknown	2015	Ireland	Unknown	NG: P1.18-4,25-31: F3-9: ST-1136 (cc1136)	*cnl*	PubMLST
13	Unknown	Unknown	Unknown	Unknown	Unknown	2010	UK	Unknown	NG: P1.12-17,4-1: F3-6: ST-1136 (cc1136)	*cnl*	PubMLST
14	Unknown	<1	Unknown	Unknown	Unknown	2012	Ireland	Unknown	NG: P1.7,30: F1-6: ST-53 (cc53)	*cnl*	PubMLST
15	Male	<1	C6 deficiency	Recovered	None	1986	USA	CSF	NG: P1.ND: F-ND: ND (-)	Unknown	(118)
15	Male	<1	C6 deficiency	Recovered	None	1986	USA	Blood	NG: P1.ND: F-ND: ND (-)	Unknown	(118)
16	Male	<1	C6 deficiency	Recovered	None	1986	USA	CSF	NG: P1.ND: F-ND: ND (-)	Unknown	(118)
17	Male	<1	Immunocompetent	Recovered	Unknown	2009	China	CSF	NG: P1.7-2,14: F4-21: ST-7962 (-)	Slipped-strand mispairing	(119)

Abbreviations: C6, sixth complement component; N/A, not applicable; NG, nongroupable; P1, PorA; F, FetA; ST, sequence type; cc, clonal complex; Δ, gene deletion; ND, not defined; *cnl*, capsule null locus; PubMLST, PubMLST *Neisseria* database (http://pubmlst.org/neisseria/)

1.8. Vaccines

The first polysaccharide vaccines were developed during the 1960's, and targeted *N. meningitidis* serogroups A or C (120). Polysaccharide vaccines were developed in bivalent (A and C), trivalent (A, C and W) and quadrivalent (A, C, W and Y) formulations (121). However, meningococcal capsular polysaccharide molecules are T-cell-independent antigens which elicit poor immunologic memory and provide limited protection; and repeated doses lead to immunologic hyporesponsiveness (122). In addition, polysaccharide vaccines are poorly immunogenic in children younger than five years of age (123;124). Polysaccharide vaccines were also unable to eradicate meningococcal carriage (125). These drawbacks prompted the development of monovalent (A or C) and quadrivalent (A, C, W and Y) formulations of conjugate polysaccharide vaccines.

Meningococcal conjugate vaccines elicit a T-cell dependent immune response, and therefore provide lasting protection (126). In addition, conjugate vaccines induce immunity in children younger than five-years-old and adults (127;128); and provide indirect immunity by reducing meningococcal carriage (86;129). The meningococcal serogroup C-diphtheria toxoid vaccine (MCC) was the first meningococcal conjugate vaccine to be developed and licensed, and was introduced into the routine infant immunization schedule for individuals younger than 19 years in the UK, in 1999 (129). The MCC vaccine was implemented in response to increasing numbers of serogroup C IMD cases in the UK (130). According to recent data from England and Wales (2015/2016), the number of IMD cases due to serogroup C was reduced by 96% since vaccine implementation (13). Conjugate serogroup C vaccines have since been implemented in routine immunization programmes in European countries, Canada, New Zealand and Australia (121).

The success of the MCC vaccine prompted the development of a low-cost monovalent serogroup A polysaccharide-tetanus toxoid vaccine (MenAfriVac™, Serum Institute of India, Pune, India), as part of the Meningitis Vaccine Project (131). Mass vaccination campaigns were initiated in 2010, and targeted 217 million people living in 15 African meningitis belt countries. Although the incidence of IMD due to serogroup A was significantly reduced, cases of disease due to serogroups C, W and X were also reported in African meningitis belt countries, emphasizing the need to develop a low-cost multivalent vaccine (11).

In 2015, the quadrivalent conjugate meningococcal vaccine (A, C, W and Y) was introduced into the national immunization programme for adolescents in England and Wales, due to the increase in serogroup W cases (132). Quadrivalent conjugate meningococcal vaccines were also licensed for use in adolescents in the USA (133). Quadrivalent polysaccharide vaccines, Mencevax ACW135Y® (GlaxoSmithKline, Belgium, Europe) and Menomune®–A/C/Y/W-135 (Sanofi Pasteur Inc., Pennsylvania, USA), have been available in South Africa since 2003 and 2007, respectively (107). The quadrivalent conjugate meningococcal vaccine, Menactra® (Sanofi Pasteur Inc., Pennsylvania, USA) was recently introduced in 2014 (134). Meningococcal vaccines are however not routinely used in South Africa as public health priorities are directed towards high burden diseases such as HIV and tuberculosis, but are available privately and are recommended for individuals at high risk of acquiring IMD, including patients with terminal complement deficiencies and anatomical or functional asplenia (107;134). Other high risk groups include travellers to areas at increased risk of epidemics, Hajj pilgrims, university students, infants and children.

Serogroup B is a major cause of IMD in industrialized countries. The serogroup B capsule is composed of α2→8 linked N-acetylneuraminic acid, which is similar to the polysialic

structures in human brain tissue, and may therefore act as an autoantigen (135;136). Meningococcal OMP's are immunogenic and therefore form the basis of serogroup B vaccines, which were initially developed to specifically control outbreak strains in Norway (137), Chile (138), Cuba (139) and New Zealand (140). The strain coverage of these OMP vaccines were however limited, considering that meningococcal OMP's are highly variable due to selection pressure.

Novel conserved surface antigens to develop a broad range serogroup B vaccine were later identified from the genome of a virulent serogroup B strain (MC58), in a process is called 'reverse vaccinology' (141). The antigens were recombinantly expressed in *Escherichia coli*, purified and used to immunize mice, which were monitored for an immunological response. Antigens for vaccine development were selected based on their potential to induce broad protection against a variety of serogroup B strains (142). The Bexsero® or 4CMenB vaccine (Novartis Vaccines, Sienna, Italy), contains fHbp (variant 1.1), NadA (peptide 8, variant 2/3) and NHBA (peptide 2) (143;144). The Bexsero® vaccine also contains outer membrane vesicles prepared from *N. meningitidis* strain NZ98/254 which expresses the PorA antigen P1.4 (144). The Bexsero® vaccine was approved for use in 37 countries including Australia, USA, EU/EEA countries and Canada (145). Additionally, the bivalent fHbp vaccine Trumenba® (Pfizer, New York, USA) which contains both subfamily A and B variants (peptides A05 and B01) was licensed for use in the USA, in 2014 (146;147). Bexsero® and Trumenba® were also recommended for patients at risk of serogroup B disease including those with persistent complement deficiency, in the USA (148). Serogroup B vaccines are however not yet available in South Africa.

1.9. Virulence

According to MLST data, meningococci are genetically diverse, with 12897 ST's belonging to 52 clonal complexes being defined on the PubMLST *Neisseria* database (Accessed 14/04/2017). However, most cases of IMD are caused by only a few clonal complexes or 'hypervirulent lineages' (149;150). Conversely, meningococci isolated from asymptomatic carriers are more diverse, and belong to clonal complexes which are infrequently associated with invasive disease (151;152). Despite the lack of the polysaccharide capsule, unencapsulated meningococci which are predominantly associated with carriage, were reported in cases of IMD in apparently healthy patients (5;117;119). Further, invasive *cnl* meningococci belonging to cc192 were shown to resist serum killing, and were comparable to an encapsulated serogroup B strain (2). These findings therefore imply that non-capsular virulence mechanisms may also play a role in the invasive potential of *N. meningitidis.* Comparative genome hybridization and whole genome sequencing which have been used to differentiate between commensal and pathogenic *Neisseria* spp. (153-155), and more specifically commensal and pathogenic meningococci (71;75;156-159); have failed to define a pathogenome. Although mobile genetic elements have been significantly associated with invasive meningococci, their contribution to meningococcal virulence is unclear (71;156).

1.10. Diagnosis

In South Africa, a 'possible case' of IMD is defined as a clinical diagnosis of meningitis or septicemia which may be due to *N. meningitidis,* however other diagnoses are also considered (107). A 'probable case' is defined as the clinical diagnosis of meningitis and/or septicemia with *N. meningitidis* being the most likely cause of disease. These definitions differ to those used in other parts of the world for instance, in Europe, a probable case is defined as any individual meeting the clinical criteria for IMD with an epidemiological link (160); whilst

laboratory detection of *N. meningitidis* using latex agglutination is required to classify a probable case of IMD in the USA (161). The definition of a 'confirmed case' of IMD in South Africa is similar to those used in the USA and Europe, and includes the identification of *N. meningitidis* from a normally sterile site specimen (blood, CSF, pleural fluid) by culture, Gram's stain and/or antigen detection-latex agglutination result, or a positive PCR result (107;160;161). The use of genotypic assays are particularly important in cases where antibiotic therapy was administered prior to specimen collection, which may result in culture-negative specimens. Real-time PCR assays which are sensitive, specific and allow for rapid diagnosis, were developed for the identification of *N. meningitidis* by detection of the superoxide dismutase (*sodC*) gene, which is able to detect both encapsulated and unencapsulated meningococci (162). Meningococci which are potentially encapsulated may be indicated by detection of the capsule transport gene (*ctrA*), and further characterized using serogroup-specific targets (32).

1.11. Treatment and public health response in South Africa

In South Africa, all suspected cases of IMD are immediately notified to a local health authority. Probable and confirmed cases prompt a public health response which includes the identification of close contacts and administering post-exposure chemoprophylaxis, and identifying other cases of IMD in the same area which may indicate a cluster of disease (107). Intravenous benzyl penicillin is the first drug of choice for proven meningococcal septicemia and meningitis cases for 5-7 days, followed by a single-dose of ciprofloxacin or two-day course of rifampicin to eradicate carriage (107;163). Ceftriaxone or cefotaxime were however recommended as the empiric therapy for suspected bacterial meningitis. Ciprofloxacin, ceftriaxone or rifampicin are administered to close contacts of confirmed and probable cases within 24 hrs of identification of the index case, but still may be effective 10

days after exposure. A ‘close contact’ is regarded as an individual who has had prolonged contact with respiratory secretions within seven days illness onset of the case e.g. persons within a household (107). In addition, prophylactic treatment is administered to individuals who have had transient close contact with the case and were directly exposed to large respiratory droplets within 10 days of illness onset e.g. in health-care settings whereby procedures such as mouth-to-mouth resuscitation were performed. Although not routinely practiced, life-long treatment with penicillin is recommended for patients with complement deficiency to prevent recurrent episodes of IMD (108).

1.12. Aim

To characterize invasive non-groupable *N.meningitidis* isolates obtained through the South African national laboratory-based surveillance, from January 2003 through December 2013.

1.13. Objectives

- To describe cases of invasive disease caused by non-groupable *N. meningitidis* in South Africa
- To phenotypically characterize the invasive non-groupable meningococci using standard microbiological methods and serological serogrouping
- To phenotypically confirm the presence or absence of a capsule using TEM
- To determine the minimum inhibitory concentrations (MIC) of each non-groupable isolate to penicillin, chloramphenicol, rifampicin, ciprofloxacin, trimethoprim-sulfamethoxazole and ceftriaxone
- To determine the genetic mechanism for capsule loss by analysis of the *cps* locus using real-time PCR and whole genome data

- To describe the molecular characteristics of each non-groupable isolate using real-time PCR, *rplF* typing and examining the translated protein products encoded by the *porA* and *fetA* loci from genome data. Sequence type and clonal complex were also determined
- To determine the phylogenetic relatedness of the South African *cnl* meningococci to other *cnl* meningococcal genomes which were publically available on the PubMLST *Neisseria* database, using seven-locus MLST, rMLST and cgMLST
- To identify non-capsular mechanisms for meningococcal virulence by comparing previously described putative virulence genes (n=117) in invasive and carriage *cnl N. meningitidis* genomes available on the PubMLST *Neisseria* database
- To determine the effect of protein-based serogroup B vaccines (Bexsero® and Trumenba®) on *cnl* meningococci *in silico*, by examining the translated protein products of *fHbp, nadA* and *nhba*

CHAPTER TWO: MATERIALS AND METHODS

2.1. Meningococcal surveillance, 2003-2013

National laboratory-based surveillance for IMD in South Africa was established in 1999 (164) and was enhanced in 2003 through the Group for Enteric, Respiratory and Meningeal Disease Surveillance (GERMS-SA) (114). Approximately 200 microbiology laboratories from the private and public sector submitted meningococcal isolates and/or clinical specimens together with patient demographic information to the National Institute for Communicable Diseases (NICD) for confirmation and characterization. Additional information such as underlying disease, HIV status, antiretroviral use and patient outcome were also sought from hospital records. A case of IMD was defined as the identification of *N. meningitidis* from a normally sterile site specimen by culture, Gram's stain and/or antigen detection-latex agglutination result, or a positive PCR result. If a case of IMD was reported ≥21 days after the first episode, it was regarded as a new case.

2.2. Bacterial culture and characterization

At the NICD, *N. meningitidis* identification was confirmed using standard microbiological methods including, culture on 5% horse blood agar (Diagnostic Media Products (DMP), Johannesburg, South Africa) (24 hr, 5% CO_2, 37°C), oxidase test and Gram's stain (19). Pure meningococcal cultures (24 hr, 5% horse blood agar) were stored in 1 ml of 10% skim milk (DMP, Johannesburg, South Africa) at -70°C.

Phenotypic serogrouping was performed by preparing a milky suspension of single colonies (24 hr, 5% horse blood agar) in 0.5 ml 10% formal saline, and mixing 10 µl of the cell suspension with 10 µl of individual capsule-specific antibodies (Remel Biotech Ltd, Dartford, UK) to detect serogroups A, B, C, W, X or Y, on a glass slide. The antigen-

antibody suspensions and 10 µl of the formal saline-cell suspension with no antibody (control), were shaken for 2 minutes (min) and observed for agglutination. A positive reaction was characterized as a negative saline control and agglutination with one capsule-specific antibody. Isolates were reported as non-groupable if agglutination was observed in the saline control (autoagglutination), or if agglutination occurred with more than one monoclonal antibody (polyagglutination), or if no agglutination was observed in the saline control and antisera (non-reactive/ negative) (36).

Real-time PCR was used to confirm phenotypic identification and slide agglutination serogrouping results. DNA was extracted from a single colony (24 hr, 5% horse blood agar) which was added to 200 µl of nuclease free water (Anatech Analytical Technology, Michigan, USA), and boiled at 95°C for 10 min. The boiled suspensions were briefly centrifuged and tested using real-time PCR to detect *ctrA* and serogroups A (*csaB*), B (*csb*), C (*csc*), W (*csw*), X (*csxB*), and Y (*csy*) (Supplementary Table S1) (32). From 2003 through 2013, we identified five IMD isolates which were negative for *ctrA* and six serogroups. *N. meningitidis* identity was reconfirmed using API-NH (bioMérieux, Marcy-l'Étoile, France), and real-time PCR to detect the superoxide dismutase (*sodC*) gene (Supplementary Table S1) (162). All real-time PCR assays incorporated the following cycling conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 seconds (sec) and 60°C for 1 min; using the Applied Biosystems 7500 Fast real-time PCR platform (Applied Biosystems, California, USA). The five non-groupable isolates were further characterized by whole genome sequencing.

2.3. Antimicrobial susceptibility testing

Minimum inhibitory concentrations for penicillin, chloramphenicol, rifampicin, ciprofloxacin, trimethoprim-sulfamethoxazole and ceftriaxone were determined using the E-test® (bioMérieux, Marcy-l'Étoile, France) method. Briefly, 3 ml saline suspensions of 1-2 x 10^8 CFU/ml were prepared from 18-24 hr colonies on 5% horse blood agar, and inoculated onto Mueller-Hinton agar supplemented with 5% defibrinated sheep blood (DMP, Johannesburg, South Africa) to form an even lawn of growth. The inoculated plates were dried for 15 min before placing a maximum of two E-test® strips per plate, followed by incubation for 24 hrs at 37°C and 5% CO_2. After incubation, MIC's were interpreted using Clinical and Laboratory Standards Institute guidelines (165).

2.4. DNA extraction and quantification for genome sequencing

The Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) was used to extract DNA from the invasive South African non-groupable isolates for genome sequencing. In summary, a sweep of overnight culture (3-5 µl) was suspended in 600 µl of Nuclei Lysis Solution and incubated at 80°C for 5 min. After cooling to room temperature, 3 µl of RNase Solution was added to the cell lysate, mixed by inversion and incubated at 37°C for 30 min. The lysate was cooled to room temperature and 200 µl of Protein Precipitation Solution was added. The suspension was vortexed for 20 sec, incubated on ice for 5 min and centrifuged at 13,000 x *g* for 3 min. The supernatant was transferred to a sterile 1.5 ml microtube, to which 600 µl of room temperature isopropanol (Sigma Aldrich, France, Europe) was added, and gently mixed. The suspension was centrifuged at 13,000 x *g* for 2 min and the supernatant was discarded. The pellet was washed with 600 µl of 70% ethanol (Sigma Aldrich, France, Europe), and centrifuged at 13,000 x *g* for 2 min. The ethanol was carefully aspirated and the pellet was allowed to dry for 10-15 min, before being re-suspended in 100 µl of DNA

Rehydration Solution. The DNA extracts were incubated overnight at 4°C prior to quantification, and thereafter stored at -20°C.

The DNA extracts were quantified using the Qubit® 2.0 fluorometer (Invitrogen, Oregon, USA) and Qubit® dsDNA BR assay kit. A mastermix was prepared from 199 µl BR buffer and 1 µl BR reagent per sample, and 195 µl of the mixture was added to 5 µl of extracted DNA. The DNA-mastermix suspension was incubated at room temperature for 2 min before quantification.

2.5. Genome sequencing, assembly and annotation

Library preparation was performed using the Nextera XT DNA Library Prep Kit (Illumina, California, USA) according to manufacturer's instructions, and sequenced using the Illumina platform. The reads were *de novo* assembled using Velvet (version 1.2.08) combined with the VelvetOptimiser script (version 2.2.4) to a draft level (166;167). The minimum output contiguous assembly size was set to 100 bp with scaffolding turned off and all other parameters were set as default. No read trimming was performed. The sequence assemblies were uploaded into PubMLST.org/ *Neisseria*. Annotation of the genomes was performed using the PubMLST *Neisseria* database, which implements the BIGSdb platform and are publically available [PubMLST: 29306, 29312, 37616, 41860 and 41961] (77). Additionally, Illumina sequencing was performed for four non-groupable cc192 isolates from Burkina Faso (two carriage and two invasive) at Public Health England, Colindale [PubMLST: 35416, 35417, 35418 and 35419] (Supplementary Table S2) (2;4). Sequence reads were also deposited in the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena), for the South African [accession: ERR519863, ERR519789, ERR519785, ERR1805704 and

ERR1805705] and Burkina Faso isolates [accession: ERR903637, ERR903631, ERR903647 and ERR903634].

2.6. Identification of capsule null isolates

Genome Comparator, a BIGSdb tool, was used to verify PCR negative results for serogroups A, B, C, X, W and Y, and to determine if isolates were serogroups E, H, I, K, L or Z; or harboured a *cnl* allele (PubMLST *Neisseria* database locus identifier: NEIS2743) (24;40;77). Capsule regions A and C were further investigated to confirm the presence of the *cnl* allele using CLC Genomics Workbench version 7.5.1 (CLC bio, Aarhus, Denmark). Additional non-groupable *N. meningitidis* genomes harbouring a *cnl* allele were identified in the PubMLST *Neisseria* database at the time of this analysis, for phylogenomic comparison with the South African isolates (Supplementary Table S2) (Accessed: 01 July 2016). In addition, genes flanking the *cnl* allele, namely, *galE* (NEIS0048) and *tex* (NEIS0059), were compared in all isolates in the PubMLST *Neisseria* database.

2.7. Strain typing of capsule null isolates

Species identity was confirmed *in silico* by the presence of *sodC* (NEIS1339) and analysis of a 413 bp fragment of the 50S ribosomal protein L6 (*rplF,* NEIS0147) (79). Multilocus sequence type (ST) and peptide typing fragments for porin A (PorA) variable regions (VR) 1 and 2, ferric enterochelin receptor (FetA) VR, factor H-binding protein (fHbp), neisserial adhesin A (NadA) and neisserial heparin-binding antigen (NHBA), were identified from the whole genome data (51;57;58;60).

2.8. Phylogenomic comparison of capsule null meningococci

The Genome Comparator tool was used to construct phylogenetic networks to assess the relationships between the South African *cnl* isolates and additional *cnl* genomes. Isolates were compared using seven MLST genes, 53 rMLST genes and 1605 core genes [cgMLST scheme v1.0] (69;72;73). The distance matrices were visualized as Neighbor-net phylogenies and annotated using SplitsTree version 4.13.1 (168). The degree of relatedness between isolates was quantified by calculating the mean number of differing core loci between isolates and statistical significance was determined using the Fisher's exact test ($p<0.05$). Loci which were absent in at least one isolate or incomplete as a result of being situated at the end of a contig, were excluded from analysis. Functional annotations for variable core loci were determined using the PubMLST *Neisseria* database.

2.9. Identification of genetic markers for potential differentiation of carriage and invasive capsule null meningococci

The Genome Comparator tool was used to examine 117 previously defined putative virulence loci, in 51 carriage and 14 invasive capsule null isolates with known epidemiology (Supplementary Table S3) (75). The ST-192 (cc192) isolates from Malawi were not included in the analysis as genome data were not available on the PubMLST *Neisseria* database at the time of analysis (22 December 2016). A mutually exclusive gene or allele was defined as being present in all isolates in one group (carriage or invasive) and absent in the other.

2.10. Transmission electron microscopy

As part of a validation to visualize encapsulated Gram-positive and Gram-negative bacteria, encapsulated *N. meningitidis* control isolates belonging to serogroups A (ATCC®-13077™), B (ATCC®-13090™), W (ATCC®-35559™) and Y (ATCC®-35561™), and an

unencapsulated *N. lactamica* strain (ATCC-23970™); were visualized using previously described TEM methods which were modified as stipulated in Birkhead *et al.* (1). These methods included negative staining (1), simultaneous glutaraldehyde-osmium tetroxide fixation adapted from Frank *et al.* (169), pre-incubation with a cationic dye such as ruthenium red or alcian blue pyridine variant and *en bloc* staining, adapted from Hayat (170); L-lysine acetate fixation with a cationic dye such as ruthenium red or alcian blue (pyridine or 8XG variant) adapted from Jacques and Graham (171), Erlandsen *et al.* (172) and Hammerschmidt *et al.* (44). The L-lysine acetate fixation with alcian blue (pyridine variant) stained capsular material from all encapsulated *N. meningitidis* controls and did not indicate capsule around *N. lactamica* (although staining of extracelluar material between cells was observed), and was therefore used to visualize the South African non-groupable isolates.

Briefly, polytop vials were used to punch out agar disks with single colonies (24hr, 5% horse blood), which were fixed using a primary fixative consisting of 2% methanol-free formaldehyde, 2.5% glutaraldehyde, 1.55% L-lysine acetate and 0.15% alcian blue pyridine variant for 20 min, on ice. The agar disks were then washed with chilled dyed buffer prepared from 0.1 M sodium cacodylate buffer (deionised distilled water, 0.09 M sucrose, 0.01 M $CaCl_2.2H_2O$ and 0.01 M $MgCl_2.6H_2O$) and 0.15% alcian blue to a final pH of 7.15; fixed on ice for 3 hrs using the primary fixative without 1.55% L-lysine acetate, and washed with chilled dyed buffer three times (20 min per rinse). Post-fixation was carried out by incubating the samples in 1% osmium tetroxide in dyed buffer for 1 hr on ice. After five rinses with chilled dyed buffer (20 min per rinse), the samples were dehydrated using a graded chilled ethanol series (10%, 20%, 35%, 50% and 70%) at 30 min intervals. After the 10% ethanol dehydration step, the colonies were scraped off the agar and placed into a 1.5 ml Eppendorf tube, which was centrifuged at 500 x *g*, 4°C for 15 min between subsequent dehydration

steps. The samples were incubated overnight on ice in 70% ethanol, followed by ethanol rinses at 90% (x 1) and 100% (x 4). The cells were then infiltrated on ice with 1:1 ethanol: London Resin White (medium, Agar Scientific, Stansted, UK) for 4 hrs, 1:2 ethanol: resin overnight, three changes of pure resin at 12 hr intervals; with centrifugation steps not exceeding 3,000 x *g* for 40 min between changes. The cells were transferred to gelatine capsules together with pure resin and polymerized for 48 hrs at 62°C. The capsules were sectioned into 70 nm sections using a Leica EM UC6 ultramicrotome (Leica-microsystems**,** Wetzlar, Germany) and stained with 4% uranyl acetate for 5 min. The ultrathin sections on 0.25% formvar-coated copper slot-grids were viewed at 80 kV on a BioTwin Spirit transmission electron microscope (FEI Company, Oregon, USA) with a Quemesa CCD camera (Olympus, Germany, Europe). *N. meningitidis* serogroup W strain (ATCC-35559™) was used as an encapsulated control, whilst *N. lactamica* strain (ATCC-23970™) was used as an unencapsulated control.

2.11. Ethics and consent to participate

GERMS-SA surveillance (M140159) and project-specific (M150349) ethics were approved by the Human Research Ethics Committee of the University of the Witwatersrand. Details of informed consent were included in the national-laboratory based surveillance program (protocol number: M140159). Briefly, written informed consent was obtained from parents or legal guardians if participants/ patients were minors; and assent was obtained from minors who were old enough to understand.

CHAPTER THREE: RESULTS

3.1. Identification and characterization of invasive capsule null meningococci

From 2003 through 2013, 4770 cases of IMD were reported, with viable isolates available for 2988 (63%) cases. We identified five *N. meningitidis* isolates that were phenotypically and genotypically negative for serogroups A, B, C, W, X and Y. The isolates were also *ctrA* PCR negative but were *sodC* PCR positive. Transmission electron microscopy confirmed the absence of a polysaccharide capsule (Figure 3).

One of the five isolates, 37616, did not contain a *cnl* allele, belonged to ST-11147 (cc41/44) and contained capsule transport genes in region B (*ctrE* and *ctrF*) and lipopolysaccharide synthesis genes (*rfbA-C* and *galE*) in region D of the *cps* locus. All region A genes except for *ctrG* were absent, as well as *ctrA* from region C. The remaining four *ctrA* negative isolates lacked genes in regions A and C (*ctrA-D*) and contained a 114 bp *cnl* allele. Further, these isolates contained all region D lipopolysaccharide synthesis genes and lacked region B genes. The locus was identified as *cnl* allele 12 in isolate 29312, and *cnl* allele 2 in isolates 29306, 41860 and 41961 (Figure S2) (40). Isolates 29306, 41860 and 41961 lacked the *fetA* locus. The finetypes for the four *cnl* isolates were as follows: isolate 29312 was NG: P1.7-2,30: F1-2: ST-53 (cc53), and isolates 29306, 41860 and 41961 were NG: P1.18-11,42-2: FΔ: ST-192 (cc192).

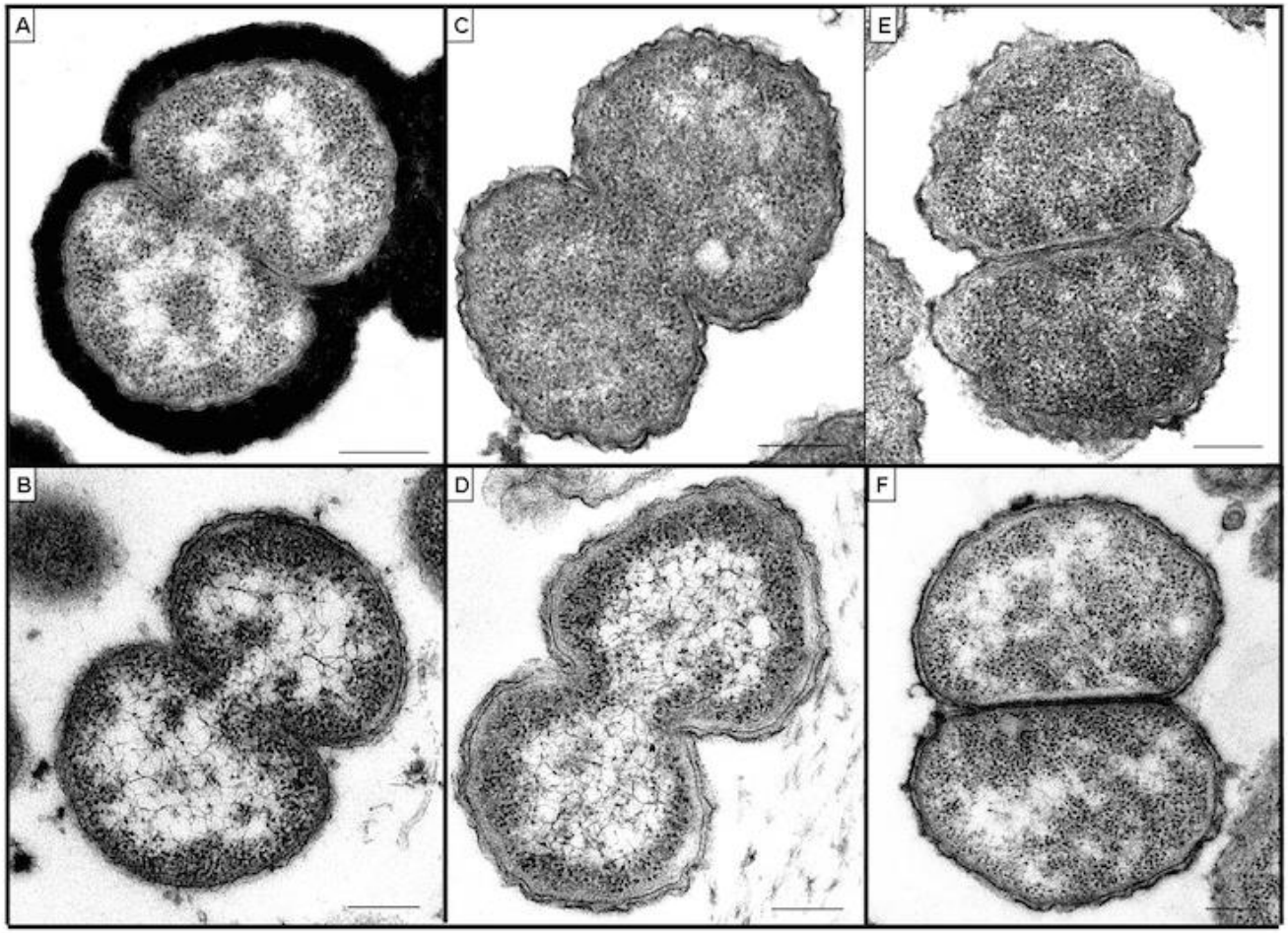


Figure 3. Transmission electron micrographs showing the presence of surface capsular polysaccharide for A) *Neisseria meningitidis* serogroup W (ATCC 35559) and B) absence of capsule for *Neisseria lactamica* (ATCC 23970). Clinical isolates from South Africa are depicted in C) 29312 D) 29306 E) 41961 and F) 41860. The scale bars represent 200 nm.

3.2. Clinical case descriptions

The four invasive *cnl* meningococci were isolated from three patients, including one patient with recurrent meningococcal disease (Table 2). All three patients responded well to antibiotic therapy and subsequently recovered from their IMD episodes. The four *cnl* isolates were susceptible to all antimicrobials except for trimethoprim-sulfamethoxazole. Additional information regarding vaccination status and long-term complications for all three patients was sought, but unfortunately these data were not available.

The first patient, an adult male, was previously diagnosed with multiple chronic illnesses including diabetes mellitus, hypertension, osteoarthritis and chronic obstructive pulmonary disease. He was a smoker, morbidly obese and had a right lower lobe lobectomy in 2003 due to damage from a previous tuberculosis infection. In 2006, he was diagnosed with empyema and *N. meningitidis* was cultured from the pleural fluid (isolate 29312). The second patient, an adolescent male, was diagnosed with meningococcal meningitis in 2010 (isolate 29306). Unfortunately, information regarding underlying disease conditions and the severity of disease could not be obtained. The third patient was a male child with deficiency of the sixth complement component (C6). In 2011, he was diagnosed with meningitis (isolate 41961), however, eight months later, in 2012, he presented with fever and disorientation and *N. meningitidis* was isolated from the blood (isolate 41860). He was prescribed life-long treatment with penicillin. His mother received a dose of the quadrivalent conjugate vaccine (Menactra®) in 2015.

Table 2. Patient demographic information and phenotypic and genotypic characteristics of four invasive capsule null (*cnl*) *Neisseria meningitidis* isolates identified through national laboratory-based surveillance in South Africa, 2003-2013.

Characteristic				
Patient	**1**	**2**	**3 (Episode 1)[a]**	**3 (Episode 2)[a]**
Gender	Male	Male	Male	Male
Age category (years)	45-64	15-24	5-9	5-9
HIV status	Negative	Unknown	Negative	Negative
Antiretroviral use	Not applicable	Unknown	Not applicable	Not applicable
Underlying disease	Diabetes mellitus, COPD	Unknown	C6 deficiency	C6 deficiency
Year of disease presentation	2006	2010	2011	2012
Province	Western Cape	Gauteng	Free State	Free State
Patient outcome	Recovered	Recovered	Recovered	Recovered
Specimen type	Pleural aspirate	CSF	CSF	Blood
Isolate				

Minimum Inhibitory				
Concentrations (µg/ml)				
Penicillin G	0.032 (S)	0.064 (S)	0.064 (S)	0.047 (S)
Ceftriaxone	≤ 0.002 (S)	≤ 0.002 (S)	≤ 0.002 (S)	≤ 0.002 (S)
Trimethoprim-sulfamethoxazole	8 (R)	12 (R)	3 (R)	3.8 (R)
Chloramphenicol	0.75 (S)	1 (S)	1 (S)	0.38 (S)
Rifampicin	0.008 (S)	0.032 (S)	0.064 (S)	0.032 (S)
Ciprofloxacin	0.008 (S)	0.008 (S)	0.006 (S)	0.006 (S)
Molecular characterization				
cnl allele (NEIS2743)	12	2	2	2
Strain designation	NG: P1.7-2,30: F1-2: ST-53 (cc53)	NG: P1.18-11, 42-2: FΔ: ST-192 (cc192)	NG: P1.18-11, 42-2: FΔ: ST-192 (cc192)	NG: P1.18-11, 42-2: FΔ: ST-192 (cc192)
Genome information				
Approx genome size (bp)	2,104,685	2,040,849	1,995,940	2,003,633
No. contigs	119	111	447	489

Neisseria PubMLST.org identification number	29312	29306	41961	41860

[a] Patient three presented with two episodes of invasive meningococcal disease in 2011 (episode 1) and 2012 (episode 2, 8 months later), respectively. He was diagnosed with deficiency of the sixth complement component (C6).

Abbreviations: COPD, chronic obstructive pulmonary disease; C6, sixth complement component; CSF, cerebrospinal fluid; S, susceptible; R, resistant; NG, non-groupable; P1, PorA; F, FetA; ST, sequence type; cc, clonal complex; Δ, gene deletion.

3.3. Comparison of South African and other capsule null meningococcal genomes

The four South African isolates were compared to 89 *cnl* meningococcal genomes available on the PubMLST *Neisseria* database, including four cc192 Burkina Faso isolates that were sequenced as part of this study (Supplementary Table S2). The remaining 85 isolates belonged to seven clonal complexes including cc53 (n=48), cc198 (n=13), cc41/44 (n=8), cc1136 (n=6), cc192 (n=6), cc1117 (n=3) and cc213 (n=1). All *cnl* meningococci (n=93) harboured *fHbp* and *nhba*, but lacked *nadA*. Phylogenetic analysis of 53 ribosomal MLST (rMLST) loci clustered the 93 *cnl* isolates by clonal complex (data not shown). Data for 646 of 1605 core genome MLST (cgMLST) loci were incomplete and were excluded from further analysis. Phylogenetic analysis of the remaining 959 of 1605 cgMLST loci in all *cnl* isolates (n=93), demonstrated clustering by clonal complex (Figure 4). Isolates within each respective clonal complex contained identical *cnl* alleles, except for cc41/44 which contained alleles 2 or 12; and cc192 which contained alleles 2 or 3. Additional analysis of 117 putative virulence loci in 51 carriage and 14 invasive *cnl* isolates including those from South Africa, determined no mutually exclusive loci or alleles (data not shown). Most putative virulence loci were identified in both carriage and invasive isolates (97/117), and the remaining 20 loci were absent in all 65 isolates.

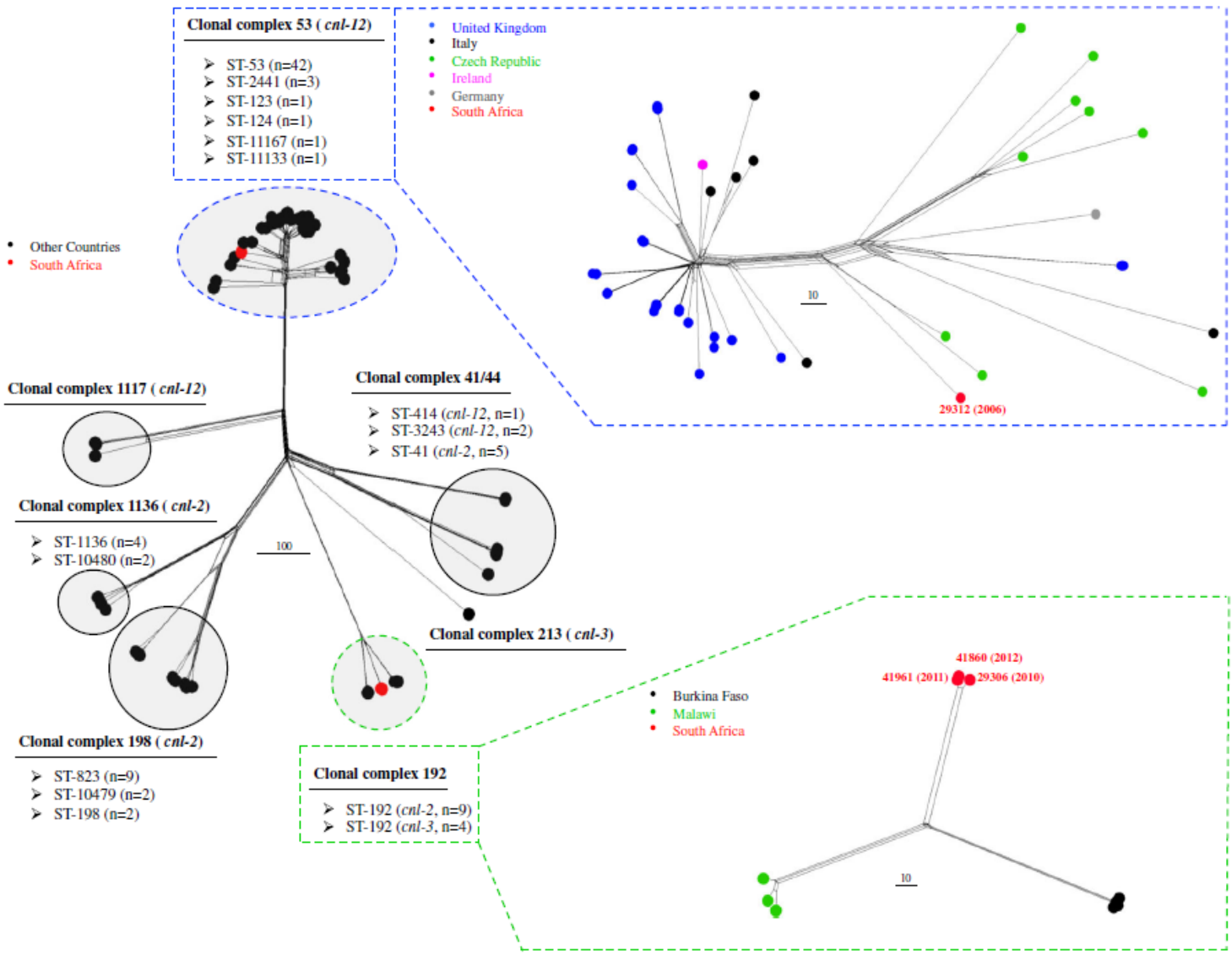


Figure 4. Phylogenetic analysis of 959 of 1605 core genes (cgMLST) genes in capsule null *Neisseria meningitidis* isolates (n=93) belonging to clonal complexes (cc) 53 (n=49), cc198 (n=13), cc192 (n=13), cc1136 (n=6), cc41/44 (n=8), cc1117 (n=3) and cc213 (n=1). Individual cgMLST phylogenies are also illustrated for cc53 and cc192. Clusters are highlighted in grey and the invasive South African isolates are represented by red nodes. Isolates 41860 and 41961 were from recurrent invasive disease episodes in the same patient. The scale bars represent the number of variant loci. All genomes are available on the pubmlst.org/neisseria website.

3.3. Clonal complex 53

At the time of analysis, in addition to isolate 29312, complete genome data were available for 48 non-groupable cc53 isolates in the PubMLST *Neisseria* database. Forty-seven were carriage isolates from either the UK (n=31), Czech Republic (n=9), Italy (n=6) or Germany (n=1), and one was an invasive isolate from Ireland in 2012. The cc53 isolates (n=49) belonged to one of six STs, namely, ST-53 (n=42), ST-2441 (n=3), ST-123 (n=1), ST-124 (n=1), ST-11167 (n=1) or ST-11133 (n=1). All cc53 isolates harboured *cnl* allele 12 flanked by *galE* allele 16, and one of five *tex* alleles (5, 787, 222, 788 or 969). *GalE* allele 16 was present in one cc41/44 isolate, also harbouring *cnl* allele 12. All cc53 isolates possessed alleles 24 or 102 for the fHbp family 2/subfamily A antigen. The NHBA allele 65 (peptide 58) was present in all of the cc53 isolates, except the South African isolate which harboured allele 149 (peptide 191).

Using seven-locus MLST, the cc53 isolates were resolved into six clusters (data not shown). Ribosomal MLST further resolved these isolates into 21 clusters and the South African isolate had a unique rMLST profile (data not shown). Core genome MLST indicated that the South African isolate was more closely related to two carriage isolates circulating in the Czech Republic in 1993, than to carriage isolates from the UK, Italy and Germany, and the invasive isolate from Ireland (Figure 4). Overall, 221/959 (23%) cgMLST loci had identical nucleotide sequences amongst the cc53 isolates (n=49).

3.4. Clonal complex 192

Genome data were available in the PubMLST *Neisseria* database for four Burkina Faso isolates sequenced as part of this study, and six carriage isolates from Malawi. Analysis of the *cps* locus of the Burkina Faso isolates confirmed the absence of regions A, B and C and the

presence of *cnl* allele 3 flanked by *galE* allele 365 and *tex* allele 826 (2). In contrast, the South African and Malawi isolates harboured *cnl* allele 2 flanked by *galE* allele 161 and *tex* allele 235. All *galE* and *tex* alleles were unique to cc192 *cnl* isolates, and were not found in any meningococcal isolates in the PubMLST *Neisseria* database. The Burkina Faso isolates were finetype NG: P1.18-11,42: FΔ: ST-192 (cc192) whilst the South African and Malawi isolates were NG: P1.18-11,42-2: FΔ: ST-192 (cc192). All 13 isolates harboured allele 112 for the fHbp family 3/subfamily A antigen and lacked both the *fetA* and *nadA* loci. Eleven of 13 isolates had allele 621 for NHBA peptide 704.

Ribosomal MLST and cgMLST resolved the 13 cc192 isolates into three groups, which were congruent with country of origin. The South African and Malawi isolates were more closely related to each other (variable cgMLST loci: 183/959, 19%), compared to the Burkina Faso isolates, which differed from the South African meningococci by 201 (21%) loci and the Malawi meningococci by 217 (23%) loci, respectively (p=0.06) (Figure 4). Isolate 29306 from patient two, differed by 13 (1%) and 15 cgMLST loci (2%) from isolates 41961 and 41860 which were from recurrent IMD episodes in patient three, respectively. Isolates 41961 and 41860 differed from each other by two cgMLST metabolic loci (0.2%) (Supplementary Table 4). Further nucleotide analysis indicated that both genes differed by one nucleotide. Overall, 660/959 (69%) of cgMLST loci shared identical nucleotide sequences amongst the 13 cc192 isolates.

CHAPTER FOUR: DISCUSSION

Four invasive *cnl* meningococci were detected from three patients, including recurrent IMD in a C6 complement deficient patient. There was no epidemiological link between patients, and one isolate was NG: P1.7-2,30: F1-2: ST-53 (cc53) whilst the remaining three were NG: P1.18-11,42-2: FΔ: ST-192 (cc192). These genotypes were different to invasive *cnl* isolates reported in other countries (2;5;115-117). One invasive isolate did not contain a *cnl* allele, but lacked most genes from regions A and C of the *cps*. This was similar to that described previously in a non-groupable cc41/44 carriage isolate from the USA (41).

Meningococci of cc53 with *cnl* have previously been found in 7% of *N. meningitidis* isolates from healthy carriers in Germany (40;88). Meningococcal carriage data from the African meningitis belt suggest that cc53 is not common in this region (4;173;174) with searches in the PubMLST *Neisseria* database identifying only three other non-groupable cc53 carriage isolates from this region: two from Senegal and one in Ethiopia. In addition to an invasive Irish isolate which possessed the same finetype as the South African isolate, 11 other invasive non-groupable cc53 isolates were identified in Cuba, Cyprus, France and the UK, of which six isolates were NG: P1.7,30: F-ND: ST-53 (cc53). Carriage rates are currently unknown in South Africa and we have no knowledge of carried genotypes. Although we do not have genotypic data for all of our invasive isolates the earliest documentation of any cc53 strain in South Africa is the *cnl* isolate identified in 2006, and described in this study.

In contrast to cc53, cc192 has been reported among carriers in countries of the African meningitis belt including Burkina Faso (4), Ghana (174), The Gambia, Ethiopia, Mali, Uganda and Niger (PubMLST *Neisseria* database). According to the PubMLST *Neisseria* database, 95% (72/76) of cc192 isolates were isolated in Africa, and 4% (3/76) isolated in

Europe; as opposed to cc53 isolates which were predominantly isolated in Europe (74%, 312/419) and rarely observed in Africa (1%, 6/419). Furthermore, two cc192 carriage isolates were documented in Norway and Sweden, and one case of bacteremia was documented in France (PubMLST *Neisseria* database). The Swedish and French isolates shared the same strain designation as the South African and Malawi isolates, however genomic data were unavailable for these isolates. Three cases of invasive disease caused by *cnl* ST-192 isolates occurred in Burkina Faso in 2003 and 2004, two of which were included in our study along with two carriage isolates from 2003 (2;4). Core genome MLST analysis of 13 African isolates revealed three groups of cc192 isolates clustering according to their respective countries, however the dataset in this study was limited and more cc192 genomes would be required to validate this geographic clustering and fully describe the molecular epidemiology of this clone.

The lack of a polysaccharide capsule in disease-associated isolates implies that factors other than encapsulation may contribute to the ability of a strain to cause invasive disease, including underlying disease conditions of the host. Disease due to unencapsulated meningococci and recurrent IMD, have been described in immunocompromised patients who are deficient in terminal pathway complement proteins C5 through C9 (108;118;175-178). In our study, one patient with C6 deficiency had recurrent IMD with the same *cnl* strain. A similar case was described in the USA in a five-month-old male who was also diagnosed with C6 deficiency (118). He was first diagnosed with meningitis followed by bacteremia six months later. Both episodes were likely to have been caused by the same unencapsulated meningococcal strain, based on the fact that the isolates were non-groupable by phenotypic serogrouping and had identical outer membrane vesicle profiles on SDS-PAGE, however additional genotypic data were not available for confirmation. These cases of recurrent IMD

may suggest persistent carriage in close contacts which is further supported by Mueller *et al*. (4) who identified non-groupable cc192 meningococci at three consecutive monthly visits in six healthy carriers in Burkina Faso. In South Africa, chemoprophylaxis is recommended for close contacts of IMD patients to eradicate carriage, however this particular strain may have persisted within the family. This does not however exclude the possibility that the second isolate may have been re-acquired from an individual in the community. Philadelphia chromosome-positive (bcr-abl^{+}) common acute lymphatic leukemia was reported in a patient with IMD caused by a ST-845 (cc845) *cnl* isolate in Germany, in 2004 (116). In our study, the ST-53 (cc53) isolate was the only organism to be cultured from pleural fluid of a patient with empyema, indicating that this isolate was the most likely cause of invasive disease. Further, IMD by the ST-53 (cc53) isolate occurred in a patient who was immunocompromised due to diabetes mellitus and, in addition, presented with multiple chronic illnesses which may have contributed to his susceptibility to invasive disease with a *cnl* strain.

Molecular epidemiology and previous data from serum bactericidal assays (SBA) suggest that some groups of meningococci are more inclined to cause invasive disease than others, with encapsulated strains being more resistant to complement killing than their unencapsulated counterparts (2;115;116;150;179). Although the polysaccharide capsule has been shown to be an important virulence determinant, previous SBA data indicate that the ability of the invasive *cnl* ST-192 (cc192) isolates from Burkina Faso to resist complement killing in normal human sera, was comparable to an encapsulated serogroup B strain (2). The invasive ST-192 (cc192) isolates from Burkina Faso were also determined to be more resistant to complement killing than a carriage ST-53 (cc53) isolate and an invasive ST-845 (cc845) isolate, which had similar resistance profiles to each other and to an unencapsulated

serogroup B mutant. Exogenous lipooligosaccharide sialylation significantly increased resistance to complement killing in two invasive *cnl* isolates belonging to ST-198 (cc198), and was partially attributed to their ability to cause invasive disease in apparently healthy patients (5;115). However, this mechanism was not identified in the invasive ST-192 (cc192) isolates from Burkina Faso, the carriage ST-53 (cc53) isolate and the invasive ST-845 (cc845) isolate (2).

In agreement with previous genome studies, most putative virulence loci were present in both carriage and invasive *cnl* isolates (75;154;155). Although Joseph *et al.* (71) determined significant associations of mobile genetic elements with invasive meningococci, their contribution to meningococcal virulence is unknown. The ability of *cnl* isolates to cause invasive disease may likely be due to host risk factors, however differences in the virulence potential may also be explained by variation in gene expression. Predisposing factors for one patient with invasive disease that was caused by an ST-192 (cc192) isolate, were unknown. It is likely that this patient may have also presented with underlying disease, however SBA data for the invasive ST-192 (cc192) isolates from Burkina Faso, indicate that these isolates may cause invasive disease in healthy patients. In contrast, cc53 which was previously shown to be sensitive to normal human sera, may require an immunocompromised host to cause IMD, as demonstrated in our study. We did not perform SBA's to confirm these findings.

In South Africa, the quadrivalent polysaccharide vaccine and the quadrivalent conjugate vaccine which was recently introduced in 2015, are recommended for individuals with terminal complement deficiencies and may be offered to close contacts of IMD patients following post-exposure chemoprophylaxis. The vaccination status of all three patients as well as the contacts for the first two were unknown, however the mother of the patient with

C6 deficiency was administered a single dose of Menactra®, which is ineffective in preventing carriage of *cnl* meningococci which lack a capsule. All 94 *cnl* meningococci including those analyzed in this study lacked the *nadA* locus and the P1.4 antigen; and most isolates expressed fHbp variants which are not targeted by the Bexsero® vaccine (with the exception of isolates that belong to cc198 and cc41/44, which express variant 1 fHbp). The effectiveness of Bexsero® to target *cnl* meningococci in general would therefore be largely reliant on the expression and cross protective potential of NHBA (180). Although the bivalent fHbp vaccine Trumenba® potentially elicits broad spectrum bactericidal activity against serogroup B strains, its effect on fHbp variants and their level of expression in other serogroups and *cnl* meningococci is unknown (181).

Invasive meningococcal disease by *cnl* meningococci in South Africa is rare however such strains may have a heightened tendency to cause IMD in an immunocompromised host, potentially coupled with currently unknown non-capsular virulence mechanisms in the meningococcus.

CHAPTER FIVE: CONCLUSION

Through our robust and stable laboratory-based surveillance for IMD, we detected three cases of IMD caused by unencapsulated (*cnl*) *N. meningitidis*, including a repeat infection in one individual. One isolate belonged to lineage cc53 and, at the time of analysis, this clonal complex was most frequently identified in healthy carriers in Europe and the UK (PubMLST *Neisseria* database), however recent data from Southern Ethiopia reported ~10% of isolates from healthy carriers to be cc53 (99). The three remaining isolates were cc192 which was reported to be common amongst healthy carriers in the African meningitis belt (4;99;174).

The use of TEM in conjunction with phenotypic and genotypic data was used to confirm the absence of a capsule. Several previously described TEM assays were evaluated to visualize polysaccharide capsules from Gram-positive and Gram-negative bacteria (1). The use of formaldehyde-glutaraldehyde ruthenium red-osmium fixation was recommended for visualizing capsules expressed by Gram-positive bacteria, but was unable to stain capsules from *N. meningitidis* serogroup A. The use of alcian blue (pyridine variant) as a substitute for ruthenium red was recommended for Gram-negative bacteria, but occasionally demonstrated non-specific staining between unencapsulated meningococci. Considering that the TEM methods evaluated for the current study do not conclusively represent the presence of a capsule, the inclusion of positive and negative controls in addition to replicates are emphasized. Future validations should therefore aim to improve on or develop TEM assays which provide reliable results by for example, evaluating the performance additional cationic dyes on a broad collection of Gram-negative and Gram-positive isolates with variable capsule compositions.

The use of whole genome sequencing provided high resolution molecular characterization of these unusual *N. meningitidis* isolates and insight into the individual cases. Further, the PubMLST *Neisseria* database provided a standardized, portable, user-friendly platform for comparison of data with other publically available genomes from geographically distinct clinical isolates of the same lineage (77). Genomic data confirmed the phenotypic lack of capsule, and the presence/ absence of vaccine antigens and currently known virulence determinants. Although genome data may provide information on the presence of loci as well as their translated protein products, it does not indicate gene expression. This information is particularly important for determining vaccine effectiveness and virulence mechanisms, and should therefore be evaluated in future studies.

Consistent with previous genome studies, most putative virulence loci were identified in both carriage and invasive *cnl* meningococci analyzed in this study (71;75). In contrast, Schork *et al*. (182) identified a full length *opc* locus and a second *pilC* locus in a cc198 *cnl* isolate which displayed higher adhesion and invasion rates, compared to a cc53 carriage isolate. This comparison was however limited to two *cnl* genomes. Differences in pathogenic potential may be explained by variable expression of virulence loci in addition to those involved in processes such as metabolism and information processing (183;184). Considering that *N. meningitidis* is a strict human pathogen, the development of animal models to investigate virulence mechanisms of this pathogen *in vivo* is difficult. Alternatively, *ex vivo* models which mimic the human host together with systems biological approaches (including the use of microarrays) may be used to evaluate the expression of meningococcal virulence factors and determine potential differences between carried and invasive *cnl* meningococci (183).

The variable expression of virulence factors in *cnl* meningococci may also be highlighted by previous studies that demonstrate varying susceptibilities of *cnl* isolates to human sera, which may be linked to their ability to modify their cell surface by sialylation of the LPS, using exogenous sources of sialic acid (115). This mechanism was identified in invasive cc198 *cnl* isolates and increased their resistance to human sera, but was not used by *cnl* isolates belonging to cc845, cc53 and cc192 (2). In contrast to cc845 (invasive) and cc53 (carriage), the invasive Burkina Faso isolates belonging to cc192 were determined to be resistant to killing by human sera and may therefore cause disease in both immunocompentent and immunocompromised individuals. Considering that SBA's were not performed for the invasive South African *cnl* isolates, the virulence potential of these strains should also be further investigated. In addition, carriage data in South Africa will be useful to elucidate the circulation of these 'potentially' virulent strains. It is also recommended that the *sodC* target should be used to screen for IMD, which detects both unencapsulated and encapsulated meningococci, as opposed to *ctrA* which detects encapsulated isolates only. In our laboratory, both assays are used routinely.

Susceptibility to IMD by *cnl* meningococci may be influenced by host risk factors. One patient with C6 deficiency experienced two episodes of IMD, eight months apart. Whole genome analysis confirmed that the second IMD episode was caused by the identical strain. This observation suggests persistent carriage within the household or community and highlights the importance of administering chemoprophylaxis to close contacts upon identification of an IMD case, regardless of meningococcal encapsulation. Previous data suggest that protein-based vaccines such as Bexsero® and Trumenba® may have the potential to target non-serogroup B meningococci (185), including those harbouring a *cnl* (180). The

cnl meningococci analysed in this study (n=93) expressed fHbp (cc198 and cc41/44 specifically contained loci for variant 1 fHbp) and NHBA which was reported to be cross-reactive (142). Most *cnl* meningococci expressed fHbp variants 2 and 3 which were reported to be cross-reactive with each other, but not with variant 1 which is present in Bexsero® (64). Although the *nadA* locus was absent in all *cnl* isolates, NadA-1, NadA-2 and NadA-3 antigens were also shown to be cross-reactive (59). It was further established that immunogenicity to at least one target in Bexero® was sufficient to confer protection against IMD (186). The bivalent fHbp vaccine Trumenba® also conferred broad protection against variants expressed by serogroup B (146;181). Immunocompromised patients may therefore benefit from these vaccines for long-term protection. Serogroup B vaccines are however not available in South Africa. The mother of the patient with C6 deficiency received a single dose of the quadrivalent conjugate meningococcal vaccine which provides immunity and reduces carriage of serogroups A, C, W and Y; but does not confer protection against other serogroups and unencapsulated strains. The vaccination status of the C6 deficient patient was unknown, however he was put onto life-long treatment with penicillin. To provide long-term immunity, vaccination with the quadrivalent conjugate meningococcal vaccine is also recommended for individuals with complement deficiency in South Africa, although life-long treatment with penicillin may be considered to prevent recurrent IMD episodes.

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APPENDICES

Supplementary material

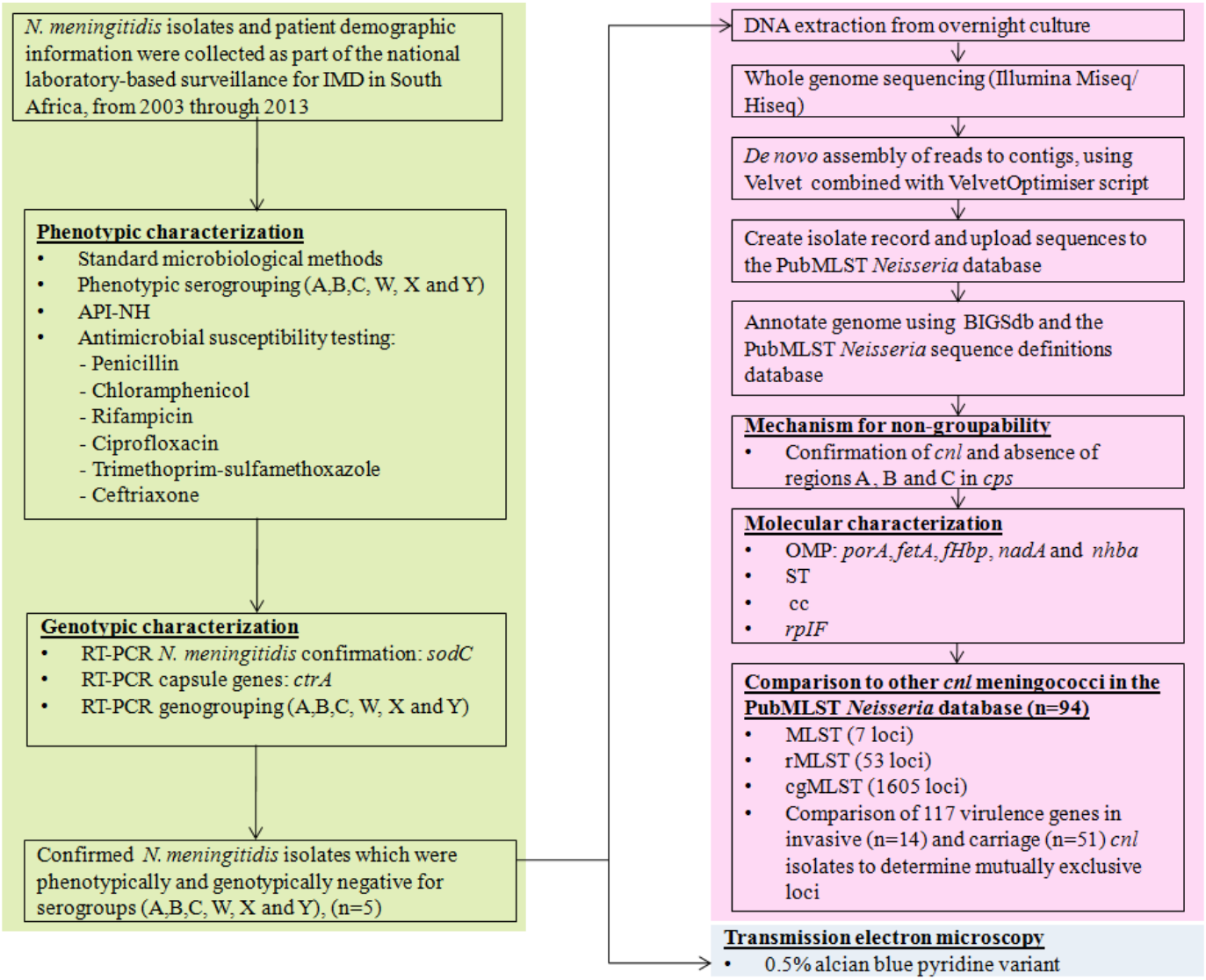


Figure S1. Workflow of phenotypic and genotypic methods used to characterize invasive capsule null *Neisseria meningitidis* isolates detected through the national laboratory-based surveillance in South Africa, from 2003 through 2013.

Table S1. Primers and probes used in real-time PCR assays for the detection of encapsulated and unencapsulated *Neisseria meningitidis* (32).

Organism	Target	Sequence 5'→3'	Concentration (nM)
N. meningitidis (encapsulated and unencapsulated)	*sodC* [a]	Forward GCACACTTAGGTGATTTACCTGCAT	300
		Reverse CCACCCGTGTGGATCATAATAGA	600
		Probe FAM-CATGATGGCACAGCAACAAATCCTGTTT-BHQ1	100
N. meningitidis (encapsulated)	*ctrA* [b]	Forward TGTGTTCCGCTATACGCCATT	300
		Reverse GCCATATTCACACGATATACC	900
		Probe FAM-AACCTTGAGCAA/BHQ1-dT/CCATTTATCCTGACGTTCT	100
N. meningitidis serogroup A	*csaB* [b]	Forward AAAATTCAATGGGTATATCACGAAGA	300
		Reverse ATATGGTGCAAGCTGGTTTCAATAG	900
		Probe HEX-CTAAAAG/BHQ1-dT/AGGAAGGGCACTTTGTGGCATAAT	100
N. meningitidis serogroup B	*csb* [b]	Forward GCTACCCCATTTCAGATGATTTGT	300
		Reverse ACCAGCCGAGGGTTTATTTCTAC	300
		Probe QUAS670 -AAGAGATGGGYAACAAC/BHQ2-dT/ATGTAATGTCTTTATTT	100
N. meningitidis serogroup C	*csc* [b]	Forward CCCTGAGTATGCGAAAAAAATT	900
		Reverse TGCTAATCCCGCCTGAATG	300
		Probe FAM-TTTCAATGC/BHQ1-dT/AATGAATACCACCGTTTTTTTGC	100
N. meningitidis serogroup W	*csw* [b]	Forward TATTTATGGAAGGCATGGTGTATG	100
		Reverse TTGCCATTCCAGAAATATCACC	900
		Probe FAM-AAATATGGAGCGAA/BHQ1-dT/GATTACAGTAACTATAA	200
N. meningitidis serogroup X	*csxB* [b]	Forward TGTCCCCAACCGTTTATTGG	900
		Reverse TGCTGCTATCATAGCCGCC	900
		Probe QUAS670 TGTTTGCCCACATGAATGGCGG-BHQ2	100
N. meningitidis serogroup Y	*csy* [b]	Forward TCCGAGCAGGAAATTTATGAGAATAC	900
		Reverse TTGCTAAAATCATTCGCTCCATAT	600
		Probe HEX-TATGGTG/BHQ1-dT/ACGATATCCCTATCCTTGCCTATAAT	100

BHQ, black hole quencher

[a]: The assay uses 12.5 µl of Taqman mastermix (Applied Biosystems, California, USA), per sample.

[b]: The assay uses 12.5 µl of Invitrogen mastermix (Applied Biosystems, California, USA) and 0.5 µl of 1:10 ROX, per sample.

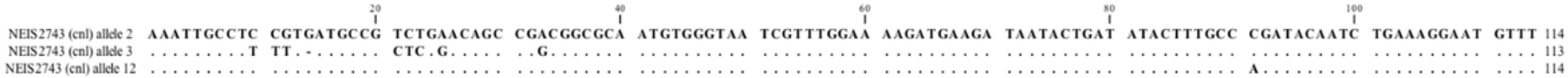


Figure S2. Nucleotide sequences of capsule null locus (*cnl*) alleles identified in invasive and carried *Neisseria meningitidis* isolates analyzed in this study (n=93), PubMLST *Neisseria* database locus identifier: NEIS2743.

Table S2. Epidemiological information, molecular characterization and genome characteristics of all *Neisseria meningitidis* isolates analyzed in this study (n=94) [a].

Clonal complex	PubMLST id	ENA accession	Country of Origin	Year of isolation	Infection status	Strain designation	Approx genome size (bp)	No. of contigs	N50 (bp)	fHbp family/ fHbp subfamily	NHBA peptide
ST-53	30	None	Germany	1999	Carriage	NG: P1.7,30-3: F5-5: ST-53 (cc53)	2145295	1	2145295	2/A	58
	972	ERR133719	Czech Republic	1993	Carriage	NG: P1.7,30: F1-2: ST-53 (cc53)	2086958	177	34059	2/A	58
	984	ERR133728	Czech Republic	1993	Carriage	NG: P1.7,30-2: F1-13: ST-53 (cc53)	2086353	155	40656	2/A	58
	1639	ERR137118	Czech Republic	1993	Carriage	NG: P1.7,30: F1-2: ST-53 (cc53)	2095123	198	34225	2/A	58
	1649	ERR137128	Czech Republic	1993	Carriage	NG: P1.7,30: F1-13: ST-53 (cc53)	2088196	150	41128	2/A	58
	1654	ERR137131	Czech Republic	1993	Carriage	NG: P1.7,30-2: F1-13: ST-123 (cc53)	2086742	169	34341	2/A	58
	2222	ERR137150	Czech Republic	1993	Carriage	NG: P1.7,30-4: F3-9: ST-124 (cc53)	2100494	175	39068	2/A	58
	8169	ERR137184	Czech Republic	1993	Carriage	NG: P1.7,30-5: F1-13: ST-53 (cc53)	2098030	152	37525	2/A	58
	8177	ERR133778	Czech Republic	1993	Carriage	NG: P1.12-4,16-8: F1-13: ST-53 (cc53)	2100968	163	33949	2/A	58
	8181	ERR133782	Czech Republic	1993	Carriage	NG: P1.7,30: F1-2: ST-53 (cc53)	2091984	194	33426	2/A	58
	26253	ERR406569	UK	2011	Carriage	NG: P1.7,30-7: F1-2: ST-53 (cc53)	2098335	184	42342	2/A	58
	26263	ERR406579	UK	2011	Carriage	NG: P1.7-2,30-5: F1-2: ST-53 (cc53)	2094245	171	45788	2/A	58
	26267	ERR406583	UK	2011	Carriage	NG: P1.7,30-2: F1-2: ST-53 (cc53)	2089516	127	62828	2/A	58
	26271	ERR406587	UK	2012	Carriage	NG: P1.7,30-3: F1-2: ST-53	2085831	181	53110	2/A	58

					(cc53)					
26274	ERR406590	UK	2012	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2099254	163	46913	2/A	58
26283	ERR406599	UK	2011	Carriage	NG: P1.7,30: F1-2: ST-2441 (cc53)	2093733	141	72612	2/A	58
26320	ERR406636	UK	2011	Carriage	NG: P1.7,30-5: F1-210: ST-53 (cc53)	2084589	152	49846	2/A	58
26325	ERR406641	UK	2012	Carriage	NG: P1.7,30-2: F1-2: ST-53 (cc53)	2099528	153	48389	2/A	58
26326	ERR406642	UK	2011	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2095080	173	40353	2/A	58
26336	ERR406652	UK	2012	Carriage	NG: P1.7,30-2: F1-2: ST-53 (cc53)	2095257	146	57623	2/A	58
26344	ERR406660	UK	2011	Carriage	NG: P1.7-2,30-1: F1-2: ST-53 (cc53)	2091793	154	57928	2/A	58
26350	ERR406665	UK	2011	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2074823	203	37591	2/A	58
26355	ERR406670	UK	2011	Carriage	NG: P1.7,30-5: F1-2: ST-53 (cc53)	2102044	188	42480	2/A	58
26365	ERR406680	UK	2012	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2093125	179	45837	2/A	58
26372	ERR406687	UK	2012	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2104019	159	42723	2/A	58
26382	ERR406697	UK	2011	Carriage	NG: P1.7,30-5: F1-210: ST-53 (cc53)	2084160	181	45756	2/A	58
26383	ERR406698	UK	2011	Carriage	NG: P1.7,30-2: F1-2: ST-53 (cc53)	2098926	192	43087	2/A	58
26394	ERR406709	UK	2011	Carriage	NG: P1.7,30: F1-2: ST-2441 (cc53)	2087485	217	38541	2/A	58
26399	ERR406714	UK	2011	Carriage	NG: P1.7,30: F1-2: ST-53 (cc53)	2115750	202	40895	2/A	58
26402	ERR406717	UK	2011	Carriage	NG: P1.7,30-2: F1-2: ST-53 (cc53)	2093395	199	40014	2/A	58
26415	ERR406730	UK	2011	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2091254	212	40601	2/A	58

26419	ERR406734	UK	2011	Carriage	NG: P1.7,30: F1-2: ST-53 (cc53)	2102519	171	45735	2/A	58
26444	ERR406759	UK	2011	Carriage	NG: P1.7,30-7: F1-2: ST-53 (cc53)	2099273	212	37220	2/A	58
26445	ERR406760	UK	2012	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2100574	163	37850	2/A	58
26451	ERR406766	UK	2011	Carriage	NG: P1.7,30: F1-2: ST-53 (cc53)	2081029	171	45568	2/A	58
26457	ERR406772	UK	2011	Carriage	NG: P1.7,30: F1-2: ST-53 (cc53)	2099175	184	39403	2/A	58
26466	ERR406781	UK	2012	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2095506	214	35648	2/A	58
26474	ERR406789	UK	2011	Carriage	NG: P1.7,30-2: F1-2: ST-53 (cc53)	2100594	148	45514	2/A	58
26500	ERR406815	UK	2012	Carriage	NG: P1.7,30-2: F1-2: ST-53 (cc53)	2097852	179	41400	2/A	58
26518	ERR406833	UK	2012	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2098884	155	42430	2/A	58
26522	ERR406837	UK	2012	Carriage	NG: P1.7,30: F1-2: ST-2441 (cc53)	2099850	135	80891	2/A	58
29312	ERR519789	South Africa	2006	Invasive	NG: P1.7-2,30: F1-2: ST-53 (cc53)	2104685	119	43300	2/A	191
36135	ERR976864	Ireland	2012	Invasive	NG: P1.7,30: F1-6: ST-53 (cc53)	2105077	119	63772	2/A	58
38889	None	Italy	2012	Carriage	NG: P1.7,30: F1-5: ST-53 (cc53)	2049750	189	18121	2/A	58
38943	None	Italy	2013	Carriage	NG: P1.7,30-3: F1-2: ST-53 (cc53)	2071425	159	22868	2/A	58
38946	None	Italy	2013	Carriage	NG: P1.18-1,3: F1-2: ST-53 (cc53)	2027967	250	13654	2/A	58
38953	None	Italy	2013	Carriage	NG: P1.7,30: F1-2: ST-53 (cc53)	2055930	176	19654	2/A	58
38954	None	Italy	2013	Carriage	NG: P1.7,30: F1-7: ST-11167 (cc53)	2066728	175	20704	2/A	58
38956	None	Italy	2013	Carriage	NG: P1.7,30-2: F1-5: ST-	2053748	195	18393	2/A	58

						11133 (cc53)					
ST-198	21389	ERR310995	Austria	2012	Invasive	NG: P1.18,25-15: F5-5: ST-823 (cc198)	2072066	138	51459	1/B	10
	26328	ERR406644	UK	2012	Unknown	NG: P1.18,25: F5-5: ST-823 (cc198)	2089889	113	74092	1/B	10
	26362	ERR406677	UK	2011	Unknown	NG: P1.18,25: F5-5: ST-823 (cc198)	2090590	157	53622	1/B	10
	26376	ERR406691	UK	2011	Unknown	NG: P1.18,25-1: F5-5: ST-10479 (cc198)	2098591	200	39537	3/A	10
	26454	ERR406769	UK	2011	Unknown	NG: P1.5-2,10-49: F5-5: ST-823 (cc198)	2084975	193	37200	1/B	10
	26458	ERR406773	UK	2012	Unknown	NG: P1.5-2,10-49: F5-5: ST-823 (cc198)	2080243	196	36697	1/B	10
	26481	ERR406796	UK	2011	Unknown	NG: P1.18,25-1: F5-5: ST-10479 (cc198)	2099838	168	51372	3/A	10
	26483	ERR406798	UK	2012	Unknown	NG: P1.5-2,10-49: F5-5: ST-823 (cc198)	2083366	131	48987	1/B	10
	26495	ERR406810	UK	2012	Unknown	NG: P1.18,25-37: F5-5: ST-823 (cc198)	2080149	166	54249	1/B	10
	26523	ERR406838	UK	2011	Unknown	NG: P1.18,25: F5-5: ST-823 (cc198)	2086643	113	55637	1/B	10
	28109	ERR473176	UK	2013	Invasive	NG: P1.18,25-14: F-ND: ST-823 (cc198)	2077201	143	37036	1/B	10
	37603	None	Canada	Unknown	Invasive	NG: P1.19,ND: F5-5: ST-198 (cc198)	2154992	25	7593	3/A	10
	34635	None	USA	1998	Carriage	NG: P1.18,25-1: F5-5: ST-198 (cc198)	1912936	360	188031	3/A	10
ST-1136	19999	ERR170970	UK	2010	Invasive	NG: P1.12-17,4-1: F3-6: ST-1136 (cc1136)	2061868	144	42306	3/A	554
	20568	None	Canada	2009	Invasive	NG: P1.18-4,25: F4-1: ST-1136 (cc1136)	2056076	152	27668	3/A	145
	26351	ERR406666	UK	2012	Unknown	NG: P1.18-4,25: F4-1: ST-10480 (cc1136)	2109512	196	34884	3/A	145

	26422	ERR406737	UK	2011	Unknown	NG: P1.18-4,25: F4-1: ST-10480 (cc1136)	2088468	233	33274	3/A	145
	36133	ERR976874	Ireland	2015	Invasive	NG: P1.18-4,25-31: F3-9: ST-1136 (cc1136)	2148812	108	55646	3/A	145
	36815	ERR957608	Ireland	2015	Invasive	NG: P1.18-4,25-31: F3-9: ST-1136 (cc1136)	2149725	116	56198	3/A	145
ST-41/44	26288	ERR406604	UK	2012	Unknown	NG: P1.17,16-3: F5-5: ST-3243 (cc41/44)	2163495	180	52379	1/B	710
	26293	ERR406609	UK	2012	Unknown	NG: P1.7-2,2: F1-5: ST-41 (cc41/44)	2098529	154	40808	1/B	2
	26378	ERR406693	UK	2012	Unknown	NG: P1.7-2,4: F1-5: ST-41 (cc41/44)	2102654	198	36139	1/B	2
	26463	ERR406778	UK	2011	Unknown	NG: P1.7-2,4: F1-5: ST-41 (cc41/44)	2097257	176	45840	1/B	2
	26484	ERR406799	UK	2011	Unknown	NG: P1.17,16-3: F5-5: ST-3243 (cc41/44)	2166337	228	36888	1/B	710
	26485	ERR406800	UK	2011	Unknown	NG: P1.7-2,4: F1-5: ST-41 (cc41/44)	2105468	190	43673	1/B	2
	26492	ERR406807	UK	2011	Unknown	NG: P1.7-2,4: F1-5: ST-41 (cc41/44)	2101154	137	45970	1/B	2
	38927	None	Italy	2013	Carriage	NG: P1.18-1,3: F1-84: ST-414 (cc41/44)	2090003	167	22192	2/A	2
	37616 [c]	ERR519785	South Africa	2013	Invasive	NG: P1.7-1,1: F3-20: ST-11147 (cc41/44)	2016190	181	19967	2/A	312
ST-1117	26341	ERR406657	UK	2012	Unknown	NG: P1.7-1,1: F3-7: ST-1117 (cc1117)	2114682	156	39419	2/A	239
	26998	ERR351662	UK	Unknown	Unknown	NG: P1.18-1,30: F3-7: ST-1117 (cc1117)	2108735	214	32834	2/A	ND[b]
	26999	ERR351663	UK	Unknown	Unknown	NG: P1.18-1,30: F3-7: ST-1117 (cc1117)	2108209	214	32838	2/A	239
ST-213	26467	ERR406782	UK	2012	Unknown	NG: P1.22,14: F5-5: ST-213 (cc213)	2224812	255	37780	2/A	248

ST-192	29306	ERR519863	South Africa	2010	Invasive	NG: P1.18-11,42-2: FΔ: ST-192 (cc192)	2040849	111	46259	3/A	704
	41961	ERR1805705	South Africa	2011	Invasive	NG: P1.18-11,42-2: FΔ: ST-192 (cc192)	1995940	447	7344	3/A	ND[b]
	41860	ERR1805704	South Africa	2012	Invasive	NG: P1.18-11,42-2: FΔ: ST-192 (cc192)	2003633	489	7130	3/A	ND[b]
	35416	ERR903637	Burkina Faso	2003	Invasive	NG: P1.18-11,42: FΔ: ST-192 (cc192)	2022469	252	19910	3/A	704
	35417	ERR903631	Burkina Faso	2003	Invasive	NG: P1.18-11,42: FΔ: ST-192 (cc192)	2025752	264	21188	3/A	704
	35418	ERR903647	Burkina Faso	2004	Carriage	NG: P1.18-11,42: FΔ: ST-192 (cc192)	2024513	227	24711	3/A	704
	35419	ERR903634	Burkina Faso	2004	Carriage	NG: P1.18-11,42: FΔ: ST-192 (cc192)	2020581	292	19063	3/A	704
	42224	None	Malawi	2005	Carriage	NG: P1.18-11, 42-2: FΔ: ST-192 (cc192)	2040471	94	56858	3/A	704
	42227	None	Malawi	2005	Carriage	NG: P1.18-11, 42-2: FΔ: ST-192 (cc192)	2042345	104	56856	3/A	704
	42237	None	Malawi	2005	Carriage	NG: P1.18-11, 42-2: FΔ: ST-192 (cc192)	2042666	107	56858	3/A	704
	42238	None	Malawi	2005	Carriage	NG: P1.18-11, 42-2: FΔ: ST-192 (cc192)	2041750	96	53482	3/A	704
	42251	None	Malawi	2006	Carriage	NG: P1.18-11, 42-2: FΔ: ST-192 (cc192)	2042182	100	54124	3/A	704
	42256	None	Malawi	2006	Carriage	NG: P1.18-11,42-2: FΔ: ST-192 (cc192)	2041768	98	54124	3/A	704

[a]: *nadA* was absent in all isolates analyzed; [b]: sequence data for *nhba* are incomplete; [c]: PubMLST ID 37616 is an invasive unencapsulated *N. meningitidis* isolate which lacks the genes required for polysaccharide synthesis and transport in regions A and C of the capsule polysaccharide synthesis locus, but does not possess the capsule null locus, and was therefore excluded from further analysis

Abbreviations: fHbp, factor-H binding protein; NHBA, neisserial heparin-binding antigen; NG, nongroupable; P1, PorA; F, FetA; ST, sequence type; cc, clonal complex; Δ, gene deletion; ND: not defined.

Table S3. Putative virulence loci (n=117) analyzed in 51 carriage and 14 invasive capsule null meningococci (75).

Gene	PubMLST*Neisseria* database locus identifier	Function
env/lpxC	NEIS0001	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosaminedeacetylase
kdtA	NEIS2152	3-deoxy-D-manno-octulosonic-acid transferase
lgtA	NEIS1902	Lacto-N-neotetraose biosynthesis glycosyltransferase
lgtB	NEIS1901	Lacto-N-neotetraose biosynthesis glycosyltransferase
lgtE	NEIS1900	Lacto-N-neotetraose biosynthesis glycosyltransferase
lgtF	NEIS1618	Beta-1,4 glucosyltransferase
lst	NEIS0899	Alpha-2,3-sialyltransferase
rfaC	NEIS2134	Heptosyltransferase I
lpxA	NEIS0168	UDP-N-acetylglucosamineacyltransferase
lpxB	NEIS0191	Lipid-A-disaccharide synthase
lpxD	NEIS0171	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase
pgm	NEIS0743	Phosphoglucomutase
rfaD	NEIS0773	ADP-D-beta-D heptoseepimerase
rfaE	NEIS0769	D-beta-D-heptose-7-phosphate kinase
rfaF	NEIS1456	Heptosyltransferase II
rfaK	NEIS1619	Alpha-1,2 N-acetylglucosaminetransferase
nadA	NEIS1969	Adhesin/invasin
omp85	NEIS0173	Outer membrane protein OMP85
ompH	NEIS0172	Putative outer membrane protein
opa	NEIS1719	Opa1800 OMP
opcA	NEIS2198	OpcA
opcB	NEIS1877	Hypothetical protein
pglA	NEIS0213	Pilin glycosyltransferase
pglB	NEIS0399	Protein glycosylation
pglC	NEIS0397	Pilin glycosylation protein
pglD	NEIS0396	Pilin glycosylation protein
pilC1	NEIS0371	Type IV pilus associated protein
pilC2	NEIS0033	Type IV pilus associated protein
pilE	NEIS0210	Pilus genes

pilF	NEIS1844	Pilus genes
pilG	NEIS1838	Pilus genes
pilT-1	NEIS0036	Type IV pilus retraction ATPase PilT
pilT-2	NEIS0721	pilT_like protein
pilV	NEIS0487	Minor pilin
porA	NEIS1364	PorA, porin, class 1 outer membrane protein
rmpM	NEIS1783	Outer membrane protein class 4
-	NEIS2438	Putative large exoprotein involved in heme utilization or adhesion of ShlA/HecA/FhaA family
-	NEIS0444	hemagglutinin
-	NEIS0443	TpsA activation/secretion protein TpsB
bcp	NEIS0704	Hypothetical protein
bfrA	NEIS1108	Bacterioferritin A
bfrB	NEIS1107	Bacterioferritin B
fbpA	NEIS0578	Major ferric iron binding protein
fbpB	NEIS0577	Putative iron-uptake permease inner membrane protein
fetB2	NEIS0340	Ferric enterobactin transporter binding protein
fur	NEIS0197	Ferric uptake regulation protein
-	NEIS1966	Putative inner membrane transport protein
-	NEIS1965	Putative inner membrane transport protein
-	NEIS1964	Putative membrane transport solute-binding protein
lbpA	NEIS1468	Lactoferrin binding protein A
lbpB	NEIS1469	Lactoferrin binding protein B
tbpA	NEIS1690	Transferrin binding protein A
tbpB	NEIS1691	Transferrin binding protein B
-	NEIS1887	TonB-dependent receptor protein, FhuA
-	NEIS0338	Ferric siderophore receptor protein
-	NEIS0387	TonB-dependent receptor
-	NEIS1282	Putative TonB-dependent receptor protein
nhba	NEIS2109	Putative periplasmic protein
tspA	NEIS1829	Neisseria-specific antigen protein TspA
tspB	NEIS1866	TspB protein
-	NEIS1806	putative zinc-binding protein
-	NEIS1805	hypothetical protein
frpC	NEIS1344	RTX iron-regulated frpc protein outer membrane

-	NEIS1560	Hemolysin
-	NEIS2434	RTX toxin activating lysine-acyltransferase
-	NEIS2502	putative FrpA/C-activating lysine-acyltransferase
dsbA	NEIS1760	Putative thiol:disulphide interchange protein
dsbA	NEIS1885	Thiol:disulfide interchange protein
dsbA	NEIS0273	Putative thiol:disulphide interchange protein
iga	NEIS0651	IgA1 protease
kat	NEIS0211	Catalase (KatA)
sodB	NEIS0825	Superoxide dismutase sodB
sodC	NEIS1339	Superoxide dismutase sodC
-	NEIS0035	PilT-like protein
-	NEIS2495	putative VapD-like protein
-	NEIS2103	putative protease
-	NEIS0528	putative periplasmic binding protein
-	NEIS0978	putative surface fibril protein
-	NEIS1658	periplasmic type I secretion system protein
-	NEIS1750	hypothetical protein
fabZ	NEIS0170	3R-hydroxymyristoyl ACP dehydrase
gna1870	NEIS0349	Factor H-binding protein
gna1946	NEIS1917	Putative lipoprotein
hap	NEIS1959	IgA-specific serine endopeptidase
hemH	NEIS0669	Ferrochelatase
hemH	NEIS0709	Phosphoribosylaminoimidazole-succinocarboxamide synthase
misR/phoP	NEIS0537	Putative two-component system regulator
misS/phoQ	NEIS0536	Putative two-component system sensor kinase
mtrC	NEIS1634	Membrane fusion protein
mtrD	NEIS1633	Drug efflux protein
mtrE	NEIS1632	Putative outer membrane lipoprotein
mtrR	NEIS1635	Transcriptional regulator mtR
narE	NEIS2492	ADP-ribosyltransferaseNarE
natC	NEIS1657	natC, periplasmic type I secretion system protein
nlpD	NEIS1418	Putative membrane peptidase
norZ	NEIS1548	Nitric oxide reductase
nspA	NEIS0612	Outer membrane protein

nth	NEIS0472	Endonuclease III
prc	NEIS1270	Putative carboxy-terminal processing protease
vacJ	NEIS1933	Periplasmic/outer membrane protein
vapA	NEIS1859	Autotransporter A VapA
virG	NEIS1454	Virulence associated protein
ctrG	NEIS0049	Putative transcriptional accessory protein
csc	NEIS0051	polysialyltransferase
ctrA	NEIS0055	capsule polysaccharide export outer membrane protein
ctrB	NEIS0056	capsule polysaccharide export inner membrane protein
ctrC	NEIS0057	capsule polysaccharide export inner membrane protein
ctrD	NEIS0058	capsule polysaccharide export ATP-binding protein
ctrE	NEIS0066	capsule translocation
ctrF	NEIS0067	capsule translocation
csaA	NEIS2157	csaA / UDP-N-acetyl-D-glucosamine 2-epimerase
csaB	NEIS2158	csaB / polymerase linking UDP-ManNAc monomers
csaC	NEIS2159	csaC / O-acetyltransferase
csaD	NEIS2160	csaD / capsule transport
csb	NEIS2161	polysialyltransferase
csw	NEIS2162	D-galactose and sialic acid
csy	NEIS2163	D-glucose and sialic acid

Table S4. Variable core loci (n=2) identified in clonal complex 192 capsule null *Neisseria meningitidis* isolates 41860 and 41961, obtained from a patient who experienced two episodes of invasive meningococcal disease in 2011 and 2012, respectively.

PubMLST locus ID	Alternate locus ID	PubMLST allele		Gene length (bp)	SNP position	SNP	Product	Function
		Isolate 41961	Isolate 41860					
NEIS0406	NMB1814	71	292	1080	954	G→A	3-dehydroquinate synthase	Amino acid metabolism
NEIS1155	NMB1254	12	241	594	513	C→T	GTP cyclohydrolase II	Metabolism of cofactors and vitamins

Abbreviations: SNP, Single nucleotide polymorphism

Ethics clearance certificate

R14/49 Miss Karistha Ganesh

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150349

NAME: (Principal Investigator)	Miss Karistha Ganesh
DEPARTMENT:	Clinical Microbiology and Infectious Diseases National Institute for Communicable Diseases
PROJECT TITLE:	Molecular Characterization of Non-Groupable Neisseria Meningitidis Causing Invasive Disease in South Africa
DATE CONSIDERED:	27/03/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Mignon du Plessis
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	24/03/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature

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27 October 2017

To whom it may concern

RE: Submission of MSc dissertation 2017 for Ms Karistha Ganesh, Wits student number 1153542

Ms Karistha Ganesh is currently a MSc student and employee within our department at the Centre for Respiratory Diseases and Meningitis (CRDM), National Institute for Communicable Diseases (NICD). The findings of her research for the project entitled 'Molecular characterization of non-groupable *Neisseria meningitidis* causing invasive disease in South Africa', was recently published in BMC Microbiology. A combined word document containing the final draft of the literature review and manuscript was evaluated for plagiarism using the 'turnitin' software. The highest similarity score (35%) corresponded to the published manuscript containing data from the current study, and permissions were obtained from all respective co-authors to use the article as part of her dissertation.
Manuscript title: Ganesh K, Allam M, Wolter N, Bratcher HB, Harrison OB, Lucidarme J, Borrow R, de Gouveia L, Meiring S, Birkhead M, Maiden MCJ, von Gottberg A and du Plessis M. Molecular characterization of invasive capsule null *Neisseria meningitidis* in South Africa. BMC Microbiol 2017 Feb 21;17(1):40.

Yours sincerely,

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FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Karisha Ganesh (Student number: 1153643) am a student registered for the degree of Master of Science (Medicine) in the academic year 2017.

I hereby declare the following:

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I, [Karistha Ganesh], student number [1153542], declare that this dissertation is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my dissertation .

Signature of Student ..... Date 22/2/2017

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Journal name, year, volume and page numbers: BMC Microbiology, 2017, 17, 1-10

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