Clonal Waves of Meningococcal and Pneumococcal Meningitis in a Region of the Meningitis Belt of Sub-Saharan Africa

INAUGURALDISSERTATION

zur

Erlangung der Würde einer Doktorin der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät der

Universität Basel

von

Julia Leimkugel

aus

Mülheim an der Ruhr (Deutschland)

Basel 2006

Genehmigt von der Philosophisch- Naturwissenschaftlichen Fakultät
Der Universität Basel auf Antrag von

Prof. Dr. Marcel Tanner, Prof. Dr. Dieter Ebert und Prof. Dr. Gerd Pluschke,

Basel, den 19. September 2006

Prof. Dr. Hans-Jakob Wirz
Dekan

dedicated to my mother

Table of Contents

Acknowledgements

This thesis was part of a collaboration between the Swiss Tropical Institute, Basel and the Navrongo Health Research Institute, Ministry of Health, Ghana.

My sincerest thanks go to Prof. Gerd Pluschke for his advise and support in the lab and in the field in any situation. I appreciate very much his open mind for any idea and question and his uncomplicated way of handling complex issues. His door was always open, not only for scientific questions. I am especially grateful for his understanding in difficult periods last year.

Furthermore, I would like to thank the members of my PhD committee, Prof. Marcel Tanner, for his continuous interest in the study, his support and encouragement and Prof. Dieter Ebert for acting as my coreferee. I am very grateful for any discussion and contributions from Tom Smith, Sébastien Gagneux and Thomas Junghanss. Prof. Gasser in Basel and Dr Ingrid Ehrhard in Würzburg I would like to thank for technical support.

In the STI, I appreciated very much the unique working atmosphere, with its ecological niche for everyone. I did truly enjoy working in this institute. Here my first thanks go to Valentin Pflüger and Jean Pierre Dangy for working closely together with me, for their support and friendship, for bearing my chaotic organisation and for a good time in Ghana. Special thanks to all current and former members of the Molecular Immunology Group Charlotte Huber, Christine Bannholzer, Claudia Daubenberger, Daniela Schütte, Diana Diaz, Eliane Arnold, Elisabetta Peduzzi, Denise Vogel, Dorothy Yeboah-Manou, Franziska Schwager, Marija Curcic, Markus Müller, Martin Naegeli, Marco Tamborrini, Max Bastian, Michael Käser, Niels Pfeiffer, Rolf Spirig, Shinji Okitsu, Sibylle Siegrist, Simona Rondini, Tatjana Zalac, Theresa Ruf, Tobias Jäggi, and Verena Christen. Furthermore, I would like to thank Christine Walliser, Agnes Dorée, Eliane Ghilardi and Margret Slaoui for their support in the preparation of my various journeys to Ghana, and Heidi Immler, Madeleine Buchholzer, Beatrice Wäckerli, Rolf Dürr, Yvette Endriss and Paul Haas for every day help.

In Navrongo, I am grateful for the good cooperation with my ghanaian collegues. Many individuals have helped to organize and perform this study in spite of various obstacles.

Dr. Abraham Hodgson, and my ghanaian counterpart Dr. Abudulai Forgor, I would like to thank for the good and close collaboration and many fruitful discussions. I appreciate very much the work of Akalifa Bugri and Abdul Wahab in the bacteriology lab of NHRC. The logistic support of Titus Tei and Margret Bugase in Accra, is greatly acknowledged. Cletus Tindana, Christie Amalba, Stanley Welaga, Raymond Allou, John Krugu, Joe Asampana, Oscar Bangre, Elizabeth Awine and the computer unit in Navrongo assisted during the colonization surveys and performed very important work in the field. Emefa, Mark, and all the kitchen team in Navrongo made my stay in the center enjoyable, especially when providing me with breakfast at any timepoint of the day when returning from the field. Ernest, Danzumah, Maxwell, Ken have been exceptional drivers, not only in the field but also during the long and exhausting trips to Accra. I would like to thank Cindy at the Lufthansa office in Accra, for uncomplicated handling of the dry shipper with all the samples.

In particular, I would like to thank all participants of the study for their willing participation throughout eight years of study period. The field visits for the colonization study have been a unique experience. Furthermore, I would like to appreciate the support of the local chiefs and health authorities of the Kassena Nankana District, the Upper East Region, Brong Ahafo Region and Ghana Health Services.

Deepest thanks go to my parents and to Philip, Pia and Jona for their support and their trust, in loving memory to my grandparents for all their guidance and their faith in me and to Gero for his love and patience especially in the last weeks and months.

This work was financially supported by the Meningitis Research Foundation, UK and the Meningitis Vaccine Project.

Summary

Bacterial meningitis remains one of the major health problems in Sub-Saharan Africa and contributes significantly to childhood morbidity and mortality. The three most important agents are *Neisseria meningitidis, Streptococcus pneumoniae*, and *Haemophilus influenzae*. All three pathogens are common colonizers of the human nasopharynx, invasive disease is usually a rare event. While meningitis caused by *Haemophilus influenzae* (Hib) and *Streptococcus pneumoniae* (pneumococcus) is mostly endemic and affects certain risk groups, *Neisseria meningitidis* (meningococcus) is known for its potential to cause meningitis epidemics especially in Sub-Saharan Africa. In the so called African Meningitis Belt, epidemics of meningococcal meningitis reccur every 8-12 years with incidence rates of up to 1% in the affected population, typically caused by serogroup A. Recently, outbreaks of serogroup W135 have raised general concern. The dynamics of these epidemic cycles is uncompletely understood. As meningitis cases in the early phase of epidemics usually fulminantly shoot up, outbreaks are often only detected when the epidemic is already on course. Also a lack of functional infrastructure in the respective countries, contributes to the delayed initiation of intervention measures, to prevent the majority of cases such as emergency immunization of the affected population with meningococcal polysaccharide vaccine .

After a major meningitis epidemic in Northern Ghana in 1998 a long-term colonisation and disease study was initiated in the Kassena Nankana District (KND), by a joined collaboration of the Navrongo Health Research Center and the Swiss Tropical Institute. The study aims to enhance the understanding of the dynamics of meningococcal meningitis epidemics in the African Meningitis Belt for early outbreak detection and improved intervention. This thesis as part of the long term study, concentrates on epidemiological characteristics of colonisation and disease and the association of the population of meningococcal carriage- and patient-isolates. Furthermore, the impact of emerging clones (e.g. serogroup W135) and other bacterial species (in particular *S. pneumoniae*) causing acute bacterial meningitis in Northern Ghana was investigated.

During eight years study period completed so far, we observed sequential waves of colonisation with pathogenic and apathogenic meningococcal genoclouds that typically lasted for three to four years. Epidemiological trends were profoundly different from those observed in industrialized countries. The carried populations of meningococci were i) less

stable in genotype composition, ii) less diverse during the peaks of colonization waves, iii) non-groupable (NG) strains were comparatively rare and iv) the hyperinvasive genoclouds responsible for all culture-reconfirmed meningococcal meningitis cases were not a minority, but dominated, representing 71% of the colonisation isolates.

Serogroup A meningococci caused two outbreaks during the study period. Sequence Type (ST) 5 bacteria were detected during a post-epidemic outbreak in 1998 and colonisation persisted until 1999. While A ST5 meningococci have never been isolated again in the KND, closely related A ST7 meningococci emerged in 2001, causing substantial outbreaks between 2002 to 2004 before disappearing from the district in late 2005. In between, an outbreak of serogroup X bacteria occured in 2000 and 2001, with colonisation rates of up to 20%, but only a limited number of cases. Even though the meningococcal population observed in the KND over eight years exhibited a low genetic diversity, constant microevolution was observed in the different genoclouds.

W135 meningococci, exhibiting epidemic potential in neighbouring Burkina Faso only caused sporadic meningitis cases in Ghana and no major wave of colonisation and disease. However, W135 strains isolated from single meningitis patients were indistinguishable from Burkinian epidemic isolates by PFGE analysis and in certain patient communities efficient clonal colonisation with carriage rates of up to 20% was observed.

Hib meningitis was recorded only infrequently in the KND since routine vaccination with the Hib polysaccharide-protein conjugate vaccine had been implemented in Ghana in 2000. In contrast, *S. pneumoniae* caused an outbreak of pneumococcal meningitis in the KND between 2000 and 2003, that revealed features characteristic for meningococcoal meningitis such as seasonality, clonality and a broad age spectrum of the patients. Serotyping and MLST analysis showed the dominance of a serotype 1 clonal complex, which has repeatedly been isolated in various African countries. PFGE analysis and pspA-sequencing of the outbreak strains in comparison with reference strains representing three serotype 1 lineages, confirmed the clonal relationship of the Ghanaian isolates and indicated a phylogenetic association of the three serotype 1 lineages.

The observed lack of a temporally stable and genetically diverse resident pharyngeal flora of meningococci might contribute to the susceptibility of the population in the African meningitis belt to meningococcal disease epidemics. Because capsular conjugate vaccines are known to impact meningococcal carriage, effects on herd immunity and potential serogroup replacement should be monitored following the introduction of such vaccines. Furthermore the emergence of new genoclouds of non-vaccine serogroups with

epidemic potential or other species such as *S. pneumoniae* needs to be carefully examined to evaluate the need for other vaccines or a change of the intervention strategies.

Zusammenfassung

Bakterielle Meningitis stellt eines der grossen Gesundheitsprobleme in Sub-Sahara Afrika dar und trägt dort nach wie vor erheblich zu Kindermorbidität und -mortalität bei. Die drei wichtigsten Erreger sind *Neisseria meningitidis, Streptococcus pneumoniae*, und *Haemophilus influenzae*. Alle drei Pathogene sind Bestandteil der normalen bakteriellen Flora des menschlichen Nasen-Rachenraums, invasive Krankheiten bilden die Ausnahme. Während durch *Haemophilus influenzae* (Hib) und *Streptococcus pneumoniae* (Pneumokokkus) verursachte Meningitis üblicherweise endemisch auftritt und zumeist bestimmte Risikogruppen betrifft, ist *Neisseria meningitidis* (Meningokokkus) bekannt für sein Potential, Meningitis-Epidemien in Afrika südlich der Sahara auszulösen. Mit Inzidenzraten von bis zu 1% der betroffenen Bevölkerung treten Meningokokken-Epidemien im sogenannten Afrikanischen Meningitis Gürtel alle 8-12 Jahre auf. Epidemien werden primär durch Erreger der Serogruppe A verursacht. Seit einigen Jahren erregen jedoch Ausbrüche der bislang als relativ apathogen angesehenen Serogruppe W135 Besorgnis. Die Dynamik der epidemischen Zyklen ist noch nicht aufgeklärt. Da die Zahl der Meningitisfälle zu Beginn eines Ausbruchs explosionsartig ansteigen, werden Epidemien oft erst in fortgeschrittenem Stadium erkannt. Mangelhafte Infrastruktur in den Ländern des Meningitis Gürtels trägt dazu bei, dass nötige Interventionen, insbesondere Massenimpfungen der betroffenen Bevölkerung zu spät initiiert werden, um die Mehrzahl der Fälle zu verhindern.

Nach einer Meningitis Epidemie im Norden Ghanas wurde 1998 mit einer Langzeit-Studie der Meningokokkenkolonisation und Erkrankungen im Kassena Nankana Distrikt (KND) begonnen- ein gemeinsames Projekt des Navrongo Health Research Center und des Schweizerischen Tropeninstituts. Die Studie hat sich zum Ziel gesetzt, zu einem tieferen Verständnis der Dynamik der Meningokokken-Epidemien im Afrikanischen Meningitis Gürtel beizutragen, um Früherkennung und Interventionen zu verbessern. Als ein Teil dieser Langzeitstudie befasst sich die vorliegende Arbeit mit der Epidemiologie von Kolonisation und Krankheit und dem Verhältnis der von Trägern und Patienten isolierten Meningokokken-Populationen. Ausserdem wurde die Bedeutung von aufkommenden neuen Klonen, z.B. der Serogruppe W135, und anderer bakteriellen Meningitiserregern (insb. *S. pneumoniae*) untersucht.

Während der acht Studienjahre wurden aufeinanderfolgende Wellen der Koloniation mit pathogenen und apathogenen Meningokokken-„Genoclouds“ beobachtet, die etwa drei bis vier Jahre lang andauerten. Hierbei zeigten sich deutliche Unterschiede zur Epidemiologie der Kolonisation mit Meningokokken in Industrieländern. Die Population der Trägerisolate war i) weniger konstant in ihrer Zusammensetzung, ii) weniger divers während der Kolonisationswellen, iii) nicht-serogruppisierbare Stämme wurden vergleichsweise selten isoliert und iv) die hyperinvasiven „Genoclouds“, die für alle kultur-rückbestätigten Meningokokken-Meningitis Fälle verantwortlich waren, dominierten mit 71% unter den Trägerisolaten.

Meningokokken der Serogruppe A verursachten im Verlauf der Studie zwei Meningitis Ausbrüche im KND. Erreger des Sequenz Typ (ST) 5 wurden während des Ausbruchs von 1998 isoliert, die Kolonisation mit diesen Erregern dauerte bis 1999 an. Danach jedoch wurden sie nie wieder im KND nachgewiesen. Meningokokken der Serogruppe A, ST7, kamen 2001 auf und verursachten Ausbrüche von 2002 bis 2004, bevor sie Ende 2005 wieder aus dem Distrikt verschwanden. In der Zwischenzeit sorgte eine Welle von Serogruppe X Meningokokken für Kolonisationraten bis zu 20%, aber nur für eine begrenzte Zahl von Fällen. Obwohl die Meningokokken Populationen im KND über acht Jahre hinweg nur eine begrenzte genetische Diversität aufwiesen, wurde mittels Pulsed Field Gel Electrophorese (PFGE) eine fortwährende Mikroevolution der „Genoclouds“ beobachtet.

W135 Meningokokken, die in Burkina Faso ein Potential zu Meningokokken Ausbrüchen gezeigt hatten, verursachten in Ghana nur vereinzelte Meningitis Fälle. Es kam weder zu einer Epidemie noch zu einer grösseren Kolonisationswelle. Die vereinzelten W135-Isolate von ghanaischen Meningitis Patienten waren mittels PFGE von den epidemischen Isolaten aus Burkina Faso nicht zu unterscheiden. Im Heimatdorf eines Patienten beobachteten wir klonale W135 Kolonisation mit Trägerraten von bis zu 20% beobachtet.

Haemophilus-Meningitis wurde im KND nur sehr selten festgestellt, da seit dem Jahre 2000 in Ghana der Hib Konjugat-Impfstoff in das Standard-Impfschema für Kleinkinder eingegliedert ist. Hingegen haben wir im KND zwischen 2000 und 2003 einen Ausbruch an Pneumokokken-Meningitis beobachtet. Dieser Ausbruch wies typische Charakteristika der Meningokokken-Meningitis, wie Saisonalität, Klonalität und ein breites Altersspektrum der Patienten, auf. Serotyp-Bestimmung und Multi Locus Sequence Typing (MLST) zeigten die Dominanz eines Serotyp 1 klonalen Komplexes, der schon wiederholt in verschiedenen afrikanischen Ländern isoliert wurde. PFGE und Sequenzierung des Pneumokokken Oberflächen Protein A Gens (pspA) im Vergleich zu weiteren Serotyp 1 Linien bestätigte

die klonale Verwandtschaft der ghanaischen Isolate und wiesen weiterhin auf eine enge phylogenetische Beziehung der drei Serotyp 1 Linien hin.

Das Fehlen einer stabilen und mannigfaltigen Meningokokken-Flora könnte zur erhöhten Empfänglichkeit für Meningokokken-Epidemien im Meningitis Gürtel beitragen. Da Imfpungen mit Kapsel-Konjugat Impfstoffen sich auf die Kolonisation mit Meningkokokken auswirken können, sollten mögliche Effekte auf Herd-Immunität und ein potentieller Austausch der Serogruppe bei einer Einführung dieser Impfstoffe im Meningitis Gürtel genauestens überwacht werden. Weiterhin sollte das Aufkommen neuer „Genoclouds“ von Serogruppen, die nicht in den aktuellen Imfpungen enthalten sind oder anderer Bakterienspezies wie *S. pneumoniae* sorgfältig untersucht werden, um so den Bedarf für neue Impfstoffe oder neue Interventionen zu evaluieren.

List of Tables

List of Figures

Abbreviations

Burst	Based Upon Related Sequence Types
CSF	Cerebrospinal Fluid
CSM	Cerebrospinal Meningitis
DLV	Dilocus variant
DNA	Desoxyribonucleic acid
ET	Electrophoretic Type
Hib	*Haemophilus influenzae* type B
KND	Kassena-Nankana District
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
NDSS	Navrongo Demographic Surveillance System
NHRC	Navrongo Health Research Center
OMP	Outer Membrane Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
PS	polysaccharide
pspA	pneumococcal surface protein A
SLV	Single Locus Variant
ST	Sequence Type
STI	Swiss Tropical Institute
VNTR	Variable Number Tandem Repeats
WHO	World Health Organization

Chapter 1

Introduction

Bacterial meningitis is a medical emergency and remains a major cause of mortality and morbidity throughout sub-Saharan Africa (Hart C.A. & Cuevas L.E. 2003;Greenwood 2004). From post-mortem interviews it has been estimated that about 2 percent of deaths in children under 5 years of age are due to meningitis, thus an overall 200'000 cases of bacterial meningitis cases have been extrapolated to occur in African children each year, 70'000 of these with fatal outcome (Greenwood 2004). The most important causative agents of bacterial menigitis are *Neisseria meningitidis* (the meningococcus), *Streptococcus pneumoniae* (the pneumococcus) and *Haemophilus influenzae* type b (Hib) (Cadoz *et al.* 1981;Campagne *et al.* 1999;Palmer *et al.* 1999;Peltola 2001). *Haemophilus influenzae* is well known for causing childhood meningitis, and *S. pneumoniae* mostly affects elderly, infants, and immunocompromised patients. Epidemics of bacterial meningitis are usually associated with *Neisseria meningitidis* (Mar *et al.* 1979;Moore 1992;Makela *et al.* 1992). All three species are common colonizers of the human nasopharynx; colonisation only occasionally causes disease. These bacteria spread from person to person predominantly via aerosol droplets. In most instances, meningitis results from colonisation and subsequent invasion of the bloodstream; it may also develop due to a bacterial spread from a focus of infection such as otitis media (Hart C.A. & Cuevas L.E. 2003;Greenwood 2004).

Symptoms may set off suddenly with fever, the disease ususally progresses fast. Typical signs for the involvment of the meninges include headache, irritability, nausea, vomiting, and neck stiffness. Convulsions may occur especially in children. Clinical diagnosis of acute bacterial meningitis might be difficult when characteristic symptoms are absent, e.g. in the old and the very young patients. Cerebral malaria is an important differential diagnosis in areas with high malaria endemicity (Hart C.A. & Cuevas L.E. 2003). Fatality rates are highly dependant on timely treatment and therefore varies substantially in different reports. In a meta-analysis of various African studies, fatalitiy rates of 8% for meningococcal meningitis, 29% for Hib meningitis and 45% for pneumococcal meningitis have been cumulatively calculated (Peltola 2001).

Neisseria meningitidis

Neisseria meningitidis, a gram negative diplococcus initially called *Diplococcus intracellularis*, was isolated for the first time in 1887 (Weichselbaum A. 1887). Currently, meningococci are classified into 13 serogroups according to the chemical structure of their polysaccharide capsule (Yazdankhah & Caugant 2004). Being an obligat human pathogen, it usually colonizes the nasopharynx and only occasionally causes invasive disease. Only a few serogroups are associated with invasive disease, namely the serogroups B,C,Y and W135, with sialic acid containing polysaccharide capsules, and the serogroup A, with a capsule composed of mannosaminephosphate (Poolman *et al.* 1995). The polysaccharide capsule can confer resistance to host complement mediated attack mechanisms leading to the survival and spread of the bacteria in the bloodstream.

The annual incidence of meningococcal disease in industrialized countries is about 1-5 cases per 100'000. Highest incidence rates are usually recorded during early childhood and in teenagers and young adults. Limited outbreaks of disease are often associated with overcrowding and resulting high transmission of meningococci, such as in schools, student dormitories or military campls. Apart from contact with a case and overcrowding, exposure to smoke has been identified as a possible risk factor. In Europe and Northern America a seasonal increase of meningococcal cases is usually recorded during winter months (Cartwright 1995;Tikhomirov *et al.* 1997).

Serogroup specific epidemiology

Historically, serogroup A meningococci have caused most large epidemics of meningococcal meningitis (Greenwood 1999). Before the second world war, large meningitis epidemics were also recorded from Europe and North America, but since then serogroup A meningococcal meningitis have only rarely been reported from these regions. Exceptions are an outbreak in Finland in the late 1960s (Achtman 1995b) and sporadic cases as a result of serogroup A pandemics (Zhu *et al.* 2001). In 1998/99, only 0.7% of the meningococcal meningitis cases registered in Europe were attributed to serogroup A (Cartwright *et al.* 2001).

However, serogroup A bacteria have been responsible for three pandemic waves ov meningococcal meningitis outbreaks since the mid 1960's. The first wave detected in China in the mid 1960's, spread to northern Europe (Russia and the Scandinavian countries; 1969-mid 1970's), and Brazil (mid 1970's). A second pandemic wave began in

the early 1980s in China and Nepal. It caused a meningitis outbreak in Mecca during the annual hajj in 1987, and was distributed with returning pilgrims to most countries of the globe, triggering a series of severe epidemics throughout Africa. In 1996, these bacteria were predominantly responsible for more than 150'000 cases of meningococcal disease reported from Africa (Tikhomirov, Santamaria, & Esteves 1997). A third pandemic began in China in 1993. Since 1994 these subgroup III bacteria have caused outbreaks in Mongolia, Moscow and Africa. Details of these pandemic waves will be discussed later.

Serogroup B, only sporadically isolated in Africa, is currently the most common cause of meningococcal meningitis in Europe, with approximately two third of the reported cases (Cartwright, Noah, & Peltola 2001). Serogroup C is increasingly causing localized outbreaks in Europe and North America, which promoted the efforts towards the development of a serogroup C polysaccharide-conjugate vaccine (see below). Some of the larger outbreaks in Africa have also been attributed to serogroup C (Broome *et al.* 1983;Whittle *et al.* 1975). Serogroup X meningococcal meningitis cases have been reported occasionally from North-America (Ryan & Hogan 1980), Europe (Grahlow *et al.* 1986;Pastor *et al.* 1985), and Australia (Hansman 1983). Outbreaks of serogroup X have been reported increasingly from Niger since the early 1990 (Etienne *et al.* 1990;Djibo *et al.* 2003;Boisier *et al.* 2005) and from Northern Ghana in 2000/01 (Gagneux *et al.* 2002b). Only 1.5% of the meningococcal cases reported in Europe have been attributed to serogroup Y (Cartwright, Noah, & Peltola 2001). However, in the US the proportion of meningococcal cases caused by serogroup Y increased from 2% during 1989-1991 to 37% during 1997-2002 (Bilukha & Rosenstein 2005). W135 meningococci have been associated with ~1-8% of all cases of sporadic meningococcal disease worldwide (Mayer *et al.* 2002) and in Africa only isolated cases have been described initially (Denis *et al.* 1982;Kwara *et al.* 1998). In 2000, serogroup W135 meningococci caused an outbreak with several hundred cases during the annual Hajj in Mecca. Thereafter isolated W135 cases were seen in several countries due to bacterial spread with returning pilgrims (Taha *et al.* 2000;Aguilera *et al.* 2002;Hahne *et al.* 2002b). Between 2002 and 2004, these W135 bacteria were partly responsible for large outbreaks in Burkina Faso and Niger and have been isolated in several countries all over the *Meningitis Belt*.

The African Meningitis Belt

The first documented outbreak of bacterial meningitis in sub-saharan Africa has been recorded in Northern Nigeria in 1905 and was confirmed by the detection of diplococci in the cerebrospinal fluid (CSF) (McGahey K 1905). The first epidemic in Ghana (Gold Cost) between 1906-08 started only one year later. Evidence that epidemic cerebrospinal meningitis (CSM) was not prevalent before that time comes from early African literature, reports from the first European explorers to West Africa and accounts obtained from the local populations at the time of the first major epidemics. It has been suggested that the bacteria causing the 1906/08 outbreak may have been introduced by pilgrims returning from Mecca. At that time, they travelled with caravans through the Sahara via Sudan, where an epidemic occurred a few years previously (Greenwood 1999).

Lapeysonnie, in his comprehensive report “La méningite cérébro-spinale en Afrique” published in 1963, defined the *African Meningitis Belt*, south of the Sahara and north of the tropical rain forest, within the 300-1100mm mean annual rainfall isohytes, stretching from Upper Volta (today Burkina Faso) to Sudan, with overall 28 Mio inhabitants. Based on numerous records collected from the first half of the 20th century, he described the typical epidemiology of bacterial meningitis in this region. He characterized the periodicity of outbreaks, disease causing agents, clinical aspects, general morbidity, treatment, carriage and climatic factors, in detail for five of the countries and in general for the entire region (Lapeyssonnie 1963). Still today, most of the features described by Lapeysonnie are valid, when analysing the pattern of meningococcal meningitis in Africa.

Both, endemic and epidemic meningococcal meningitis is seasonal, peaking during the dry and hot season of the year (December - May) and rapidly declining with the onset of the rains (Moore 1992). Large epidemics occur in 8-14 year-cycles, with incidence rates of up to 1% in the general population. While in endemic settings highest rates of meningococcal meningitis are recorded in young children, a shift in the age distribution has been observed during major epidemics, where often teenagers and young adults experience the highest attack rates (Moore 1992;Lapeyssonnie 1963).

The determinants of meningococcal meningitis epidemics have not been completely understood. Yet, a combination of factors related to the host, the pathogen and environmental conditions might contribute to this phenomenon. The loss of herd immunity in the interepidemic period or the emergence of new meningococcal clones that may escape the immune system of the host might increase the susceptibility of a population.

But the appearence of a new clone does not always cause an epidemic outbreak. Certain communities in close vicinity to outbreak villages have escaped the epidemic and it has been hypothized, that exposure and transmission of the clone during the rainy season might induce sufficient levels of immunity to prevent invasive disease in most individuals in the following dry season. The "2nd hit" theory suggests, that invasive disease might be favored in combination with a predisposing factor, such as levels of circulating monomeric IgA elicited by cross reactive bacteria colonizing the gut (Moore 1992;Greenwood 1999;Tikhomirov, Santamaria, & Esteves 1997;Griffiss 1982;Greenwood *et al.* 1987). High titers of IgA may block the triggering of the complement mediated serom bactericidal activity by IgG and IgM.

In the last 50 years, the affected region of the *Meningitis Belt* has extended further from Ethiopia in the East to as far as Senegal in the West, probably due to climate changes (Moore 1992). Over 300 Mio people live in this region, and outbreaks happen more often and irregularly than initially described by Lapeysonnie. Furthermore, in Eastern and Southern Africa meningococcal meningitis outbreaks are increasingly reported (Figure 1.1). These observations are in a high level of agreement with the current updated maps of the 300-1100mm mean annual rainfall isohytes (Savory *et al.* 2006;Molesworth *et al.* 2002).

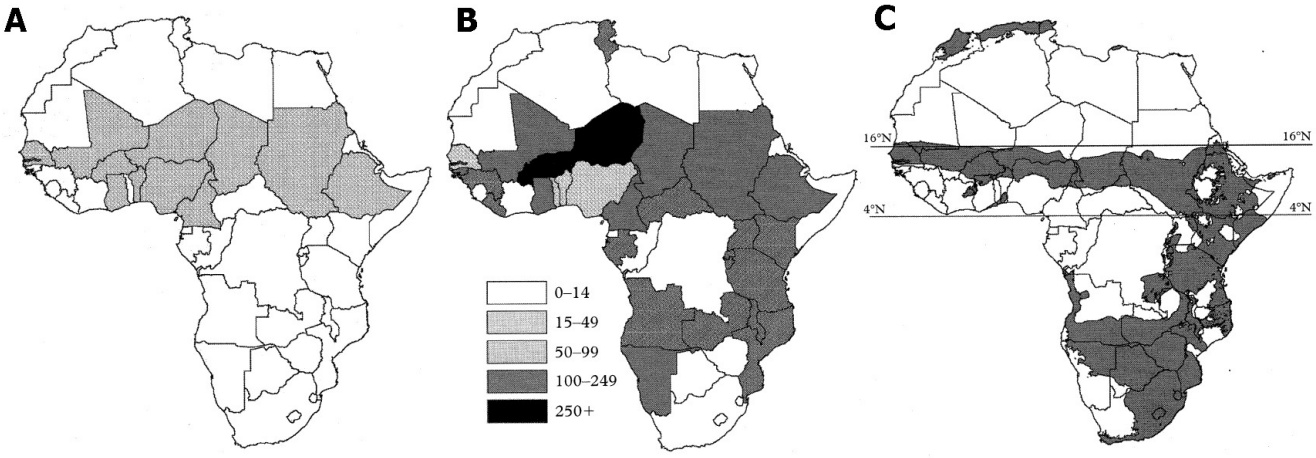


Figure 1.1 The Extension of the African Meningitis Belt A Countries included in the epidemic meningitis Belt (WHO); B Maximum rates (cases per 100'000 population reported in routine WHO surveillance data between 1981 and 1999; C Area of 300-1100 mm annual rainfall (based on 1920-1980 climatology). (source: Molesworth et al. 2002)

The largest recent outbreak of meningococcal meningitis occurred in 1996, with more than 150'000 cases all over Africa. Almost 80'000 of these occurred in Nigeria, and more than 40'000 in Burkina Faso (Tikhomirov, Santamaria, & Esteves 1997). After frequent outbreaks between 1994 and 2003, with altogether more than 700'000 cases throughout the Meningitis Belt, fewer outbreak have been recorded since then in the countries belonging to the WHO surveillance system (WHO 2005).

Management of epidemics

In the pre-antibiotic era, the mortality of meningococcal meningitis was around 80%. The introduction of sulphonamids in 1938 reduced the mortality to 10% and less. However, emerging resistance against sulphonamids in several African countries led to the replacement of these drugs in the early 1970s (Greenwood 1999). Currently, a single intramuscular injection of oily chloramphenicol is the recommended standard treatment in countries with meningococcal meningitis outbreaks. The recommendations of WHO also suggest Penicillin G (intravenous) and third generation cephalosporines (WHO 1998). As these intravenously administered drugs need to be applied several times over a period of several days, the one-shot-therapy with chloramphenicol is usually preferred during meninigits epidemics. A recent non-inferiority study proved the effivacy of a single dose treatment of cetriaxone during an outbreak in Niger (Nathan *et al.* 2005). However, third generation cephalosporines have so far not been considered as standard regimens because of cost considerations.

To date, the most important intervention strategy to control meningococcal meningitis epidemics are mass vaccinations with antimeningococcal vaccines based on the polysaccharide capsule as soon as the epidemic is detected. The available vaccines include a quadrivalent formulation, comprising serogroups A, C, Y, W135, and bivalent- and trivalent serogroup formulations primarily used in the African Meningits Belt. Due to the explosive increase in cases at the beginning of major outbreaks, epidemics have often been detected too late to prevent most of the cases by emergency vaccination (Greenwood 1999). To standardise response to emerging epidemics at the district level, WHO has developed emergency response recommendations using a threshold principle. The alert threshold with 5 meningitis cases per 100'000 population per week is used to sound an early warning and to check for epidemic preparedness. The epidemic threshold

is used to confirm the emergence of an epidemic and launch mass vaccinations. The epidemic threshold in high risk situations is 10 cases, in non-high risk situations 15 cases per 100'000 per week (WHO 2000).

Streptococcus pneumoniae

As surveillance on bacterial meningitis in Africa is dominated by the interest in meningococcal disease, comprehensive information about other pathogens is rare. A meta-analysis of 50 studies from different african regions between 1960 and the 1990s reviewed incidence rates of bacterial meningitis and the causative agents. The pneumococcus was the leading causative agent of non-epidemic meningitis and other bacterial diseases, followed by *H. influenzae* (Peltola 2001). Between 2003 and 2005, WHO reported that 25% of positive CSF samples contained *S. pneumoniae*.

Streptococcus pneumoniae belongs to the large heterogeneous group of the streptococci. The pneumococcus is an alpha-hemolytic, gram positive and facultative anaerobe oval diplococcus, often found in short chains. It is surrounded by a thick polysaccharide capsule and can be divided into 90 serotypes. Serotypes that exhibit close serological cross-reactivity are grouped into serogroups. Thus, of the 90 serotypes, 58 belong to 20 serogroups containing 2 to 4 serotypes (Dowson 2004)

Due to their narrow host specificity, humans are virtually the only natural reservoir for pneumococci. As part of the normal commensal flora, they usually colonize their host without causing severe pathology. Transmission of *S. pneumoniae* occurs via respiratory droplets from person to person. Although disease occurs in only a small proportion of individuals colonized by pneumococci, the ubiquity of the organism in human populations results in a large burden of disease and yearly 700.000 to 1 Million deaths are currently attributed to pneumococcal disease (www.who.int) The most commonly identified pneumococcal infections are community acquired pneumonia, bacterial meningitis, acute otitis media and acute bacterial sinusitis (Butler 2004;Bogaert *et al.* 2004a). In temperate regions, cases of pneumococcal disease are most common during the cold months. Even though this phenomenon is not yet completely understood, low humidity, crowding of the susceptible hosts and associated viral infections have been attributed to it (Dowell *et al.* 2003;Talbot *et al.* 2005). Most pneumococcal infections occur sporadically, but outbreaks

of pneumonia, meningitis, and conjunctivitis are occasionally reported, usually from closed or semi-closed communities (Butler 2004).

The incidence rates of pneumococcal disease are highest in the very young and the elderly individuals. The increased risk of infants is likely related to an immature immunological response, to the polysaccharide capsule, and a high prevalence of colonisation. Factors contributing to the higher rates of pneumococcal disease and case fatality among the elderly include higher rates of underlying medical conditions and age-related immune dysfunction (Castle 2000). Certain ethnic groups appear to be at higher risk for pneumococcal infections. In the US, rates of invasive pneumococcal disease are higher for blacks and native Americans than for whites and persons of other ethnic background (Cortese *et al.* 1992;Pastor *et al.* 1998;Robinson *et al.* 2001). Similarly, rates of invasive pneumococcal disease among Aborigines in central Australia are some 20 to 30 times higher than those reported for non-Aborigines living in Australia (Torzillo *et al.* 1995;Trotman *et al.* 1995). Factors contributing to these disparities in disease rates are poorly understood and may include differences in living conditions and higher prevalence of immunocompromising conditions (Dworkin *et al.* 2001). Finally, the risk and severity of pneumococal infection is increased for persons with certain chronic medical conditions, including functional or anatomic asplenia, HIV infection, chronic obstructive pulmonary disease, asthma, cirrhosis, diabetes, and conditions with impaired immune system (Butler 2004).

The pneumococcal polysaccharide capsule is essential for virulence as it acts as protection against phagocytosis. Anticapsular antibodies are the major defence mechanism against infection. Non-encapsulated variants are avirulent in experimental infections and the amount of polysaccharide expressed correlates with the degree of virulence (Kim & Weiser 1998) Out of the known 90 serotypes a relatively small number causes most invasive infections. The number of serotypes responsible for a given percentage of invasive disease is remarkably constant within each age group and geographical region. Only 2-3 serotypes and 4-5 serotypes account for ~50% of the cases in infants and children/adults, respectively. In contrast, the relative importance of a specific serotype to cause invasive disease varies in the different age groups and by geographic locations (Table 1.1) (Hausdorff *et al.* 2000). However, a meta-analysis comparing carriage- and disease isolate collections from seven studies in distinct geographical settings indicated differences in invasiveness between pneumococcal serotypes.

Commonly carried serotypes were less likely isolated from invasive disease and “invasive” serotypes (such as 1, 5, 7), were only seldomly obtained from healthy carriers (Brueggemann *et al.* 2004). From these “invasive” serotypes, small outbreaks with a limited genetic diversity of disease isolates have been reported (Porat *et al.* 2001;Gratten *et al.* 1993;Henriques *et al.* 2001). However, it is not clear, whether the polysaccharide capsule alone determines this virulence or whether other genotype-associated properties also determine the nature of the host-pathogen relationship.

Table 1.1 Contribution of individual serogroups to invasive disease, by region and age group (relative ranking is in decreasing order of importance) source: Haussdorff et al. 2000 (part I)

Age group, region	1st	2d	3d	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	Patients with meningitis, %
Young children																	
United States and Canada	14 (27.8)	6 (17)	19 (14.3)	18 (8.6)	23 (7.4)	9 (7.3)	4 (6.3)	7 (1.4)	3 (1.2)	1 (1.1)	15 (1.1)	12 (0.6)	22 (0.6)	11 (0.5)	10 (0.5)	33 (0.5)	10.4
Asia	1 (11.7)	19 (10.8)	6 (9.9)	5 (9.1)	14 (8)	7 (6.4)	23 (5.3)	12 (4.1)	9 (3.9)	2 (3.8)	15 (3.7)	18 (3.5)	3 (2.7)	31 (1.8)	16 (1.8)	4 (1.7)	29.5
Africa	6 (23.8)	14 (18.9)	1 (12.7)	19 (11.7)	23 (4.2)	5 (4)	15 (3.8)	18 (3.6)	4 (3)	7 (2.6)	9 (2.1)	8 (1.6)	20 (1.6)	12 (1.4)	2 (1.3)	29 (1.2)	40.1
Europe	14 (18.7)	6 (15.4)	19 (12.7)	18 (9.6)	23 (8.1)	9 (6.3)	1 (6.1)	7 (4.7)	4 (3.6)	5 (2.1)	3 (1.8)	24 (1.3)	15 (1.3)	33 (0.8)	10 (0.7)	38 (0.6)	25.8
Latin America	14 (22)	6 (13.9)	5 (9.2)	1 (8.2)	19 (7.9)	23 (7.9)	18 (5.5)	9 (4.4)	7 (3.8)	3 (2.1)	4 (1.8)	15 (1.6)	10 (1.2)	16 (0.8)	8 (0.8)	12 (0.8)	45.1
Oceania	14 (24)	6 (15.9)	19 (14.2)	18 (6.6)	23 (6.4)	9 (6.3)	4 (4.2)	7 (4.1)	1 (2.9)	33 (1.9)	8 (1.5)	5 (0.9)	22 (0.9)	45 (0.8)	3 (0.6)	12 (0.6)	19.8
Older children and adults																	
United States and Canada	4 (12.4)	14 (12.1)	9 (10.2)	6 (8.1)	12 (7.1)	19 (6.7)	23 (6.5)	1 (4.8)	3 (4.5)	18 (3.7)	8 (3.2)	7 (3.1)	22 (2.5)	11 (1.4)	15 (1.3)	20 (1)	6.7
Asia	1 (19.2)	3 (7.8)	5 (7.1)	6 (5.6)	19 (6.7)	7 (6.1)	14 (5.3)	23 (5.3)	12 (3.8)	4 (3.4)	10 (2.9)	9 (2.8)	16 (2.3)	34 (2.2)	15 (2.1)	18 (1.8)	13.4
Africa	1 (28.6)	19 (8.9)	14 (8.2)	6 (6.3)	3 (5.4)	12 (4.8)	7 (4.7)	5 (4.4)	4 (3.7)	25 (3.6)	9 (3.2)	18 (3.1)	15 (2.6)	29 (2.6)	8 (2.2)	10 (2)	15.1
Europe	14 (10.3)	3 (8.9)	9 (8.6)	19 (8)	1 (7.6)	6 (7.5)	23 (6.6)	7 (6.6)	4 (6.3)	8 (4.5)	18 (2.9)	12 (2.5)	5 (2.5)	22 (1.9)	15 (1.9)	11 (1.9)	10.4
Oceania	14 (12.6)	4 (9.4)	19 (9.4)	9 (9)	1 (8)	6 (7.1)	3 (6.5)	7 (6.5)	23 (6)	22 (3.7)	18 (3.5)	8 (3.1)	33 (1.5)	12 (1.3)	11 (0.5)	20 (0.5)	6.1

NOTE. Data are serogroup no. (% of all serotypes isolated).

Dynamics of nasopharyngeal colonisation

The upper respiratory tract represents the natural habitat for many bacterial species. In children, the nasopharyngeal flora becomes established soon after birth (Sulikowska *et al.* 2004). A broad variety of microorganisms, including *S. pneumoniae*, *H. influenzae* and *N. meningitidis,* can colonise the nasopharynx. Attendance to day care centers, schools, military camps often promotes transmission, resulting in higher carriage rates. Bacterial colonisation of healthy individuals and person to person transmission are therefore important mechamisms for dissemination of the pathogen within the community (Bogaert *et al.* 2004c).

Nasopharyngeal colonisation is a dynamic process both in terms of turnover of colonising species and genotypes. Interspecies competition is thought to affect the composition of the nasopharyngeal flora. The resident flora, including alpha-hemolytic streptococci, may inhibit colonisation by transient invading species (Faden *et al.* 1990). Furthermore, the different pathogenic species may influence each other (Bogaert, van Belkum, Sluijter, Luijendijk, De Groot, Rumke, Verbrugh, & Hermans 2004c). During in

vitro experiments, the growth of *H. influenzae*, *M. catarrhalis* and *N. meningitidis* was inhibited in the presence of and during coculture with *S. pneumoniae* which has been attributed to its production of hydrogene peroxide (Pericone *et al.* 2000). In contrast, in vivo mice experiments have recently shown, that components of *H. influenzae* stimulate complement dependant killing of *S. pneumoniae* by recruitment of neutrophils, thus indicating a role of the innate immune response in the interspecies competition for colonisation of mucosal surfaces (Lysenko *et al.* 2005).

Colonisation with meningococci is low in infancy, and has its peak in the 5-15 year old children. Rates of transmission and carriage increase in closed and semi-closed communities, such as military recruits, university students, and in the household contacts of patients with meningococcal disease (Yazdankhah & Caugant 2004). In non-epidemic settings, in industrialized countries approximately 10% of healthy individuals carry *N. meningitdis* at any time (Cartwright *et al.* 1987;Caugant *et al.* 1994). About 50% of carrier isolates lack the capsule and are therefore serologically not serogroupable (Claus *et al.* 2002;Caugant *et al.* 1988;Jolley *et al.* 2000). There is evidence that loss of capsule enhances the capability of meningococci to colonize the human nasopharynx (Hammerschmidt *et al.* 1996). The population of carried meningococci is usually highly diverse. Carriage of the disease causing lineages is infrequent (Caugant, Kristiansen, Froholm, Bovre, & Selander 1988;Yazdankhah *et al.* 2004). During African meningitis epidemics colonisation rates up to 33 % with the epidemic strain have been reported (Greenwood et al. 1987). It has been observed that after the end of epidemics carriage declines quickly and that endemic carriage rates are low (Hassan-King *et al.* 1988). While disease is highly seasonal in the *African Meningitis Belt,* transmission does not appear to be associated with season (Greenwood *et al.* 1984;Blakebrough 1979)

Pneumococcal colonisation is highest in young children, reaching up to 100% in the under five year olds, before it decreases again in older children and adults. One of the strongest risk factors for pneumococcal carriage in infants is the presence of colonized siblings. This has been observed in both high-income (Gray *et al.* 1980;Leino *et al.* 2001) and low-income countries (Coles *et al.* 2002). Children at their peak age of pneumococcal carriage (i.e. 2-5 years) seem to be a main source of pneumococcal transmission (Givon-Lavi et al 2002, Leino et al 2001). Overall, pneumococcal carriage is markedly greater in low-income countries than in high-incomce countries (Feikin *et al.* 2003;Lloyd-Evans *et al.*

1996;Montgomery *et al.* 1990). However, no robust comparative investigations have analysed the risk factors associated with this difference.

Serological and Molecular Typing of *N. meningitidis* and *S. pneumoniae*

Agglutination of bacteria with antisera targeting the polysaccharide capsules specifically differentiates meningococci into 13 serogroups and pneumococci (using the “Quellung reaction”) into over 90 serotypes. For meningococci, an additional classification into serotypes based on differences in the class 2 and 3 outer membrane proteins (OMP, PorB) and sero-subtypes depending on variation in the class 1 OMP (PorA) using monoclonal antibodies has been commonly applied (Poolman, van der Ley P.A., & Tommassen J. 1995). However, these classical serological methods have their limitation because adequate typing reagents are not available for all serotypes or serogroups. Furthermore, many strains isolated from the nasopharynx do not react serologically due to down-regulation or modification of capsule expression or loss of genes involved in capsule production (Claus *et al.* 2005).

Several molecular typing methods have been developed for a more comprehensive differentiation of isolates for epidemiological surveillance and analysis of bacterial evolution. Herein, problems with different demands on the typing method may be adressed. Short-term epidemiological questions, for example aiming to understand the microevolution of a pathogen during an outbreak, require a typing method based on rapidly accumulating genetic variation using for example genes that are under immune selection. Typing methods based on conservative genes, that accumulate (nearly) neutral variation (e.g. housekeeping genes) exhibit phylogenetically more reliable information on the global spread and evolution of the organism. As these genes are usually very conserved several loci are usually included in a multilocus typing approach for as sufficient resolution.

Multi Locus Enzyme Electrophoresis, MLEE, uses the natural variation in electrophoretic mobility of proteins such as cytoplasmatic allozymes (enzyme variants) or outer membrane proteins as markers for different genetic alleles on the bacterial chromosome. Bacterial strains with indistinguishable electrophoretic patterns are assigned a common Electrophoretic Type, ET. Depending on the different laboratories, by cluster analysis of meningococcal strain collections, groups of related ETs have been designated subgroups

(for serogroup A), clusters or complexes (for serogroup B and C). Because of the difficulty in interlaboratory comparability, a DNA sequence based approach, Multi Locus Sequence Typing (MLST), was developed in 1998 (Maiden *et al.* 1998) to replace MLEE. 400 to 500 bp long fragments of seven housekeeping genes are sequenced and the sequences compared. Each unique sequence is assigned a unique allele number, the set of alleles results in the sequence type (ST). Strain collections are compared on the basis of shared alleles. Clusters of closely related isolates, where each strains shares the majority of alleles (usually six of seven alleles) with at least one other isolate of the cluster are designated clonal complexes (Feil *et al.* 2004). Using the e-BURST software, recent evolutionary developments are modelled using a simple algorithm. BURST (based upon related sequence types), focuses on the identification of clonal complexes, which are assumed to share a recent common ancestor, within a bacterial population. The assumed founding genotype has increased in frequency and diversified by the accumulation of mutations and/or recombinational replacements resulting in the emergence of closely related variants. Furthermore, the founding genotype should be identifiable according to its highest number of single locus variants (SLV) (Figure 1.2).

A different approach, pulsed field gel electrophoresis (PFGE) is based on restriction digestion of genomic DNA. Genomic DNA is carfully prepared by lysing bacteria embedded in agarose blocs and subsequent digest of DNA with rare cutting restriction enzymes. The separation of the DNA fragments on the gel is performed by electric pulses coming from the different angels of a hexagonal electrode. The large DNA pieces are separated according to the velocity of reorientation within the electric field towards the anode. The result is a fingerprint of the DNA pieces generated by the restriction digest. Closely related strains will share all or at least most of the band pattern (Tenover *et al.* 1995). This method is suitable to compare strain collection within one lab. It usually has a high resolution and short term evolutionary changes can be detected. However, results are difficult to digitalize and inter-laboratory comparison is problematic.

Population structure and bacterial evolution

During asexual reproduction in bacterial populations, chromosomal variation occurs by *de novo* mutations, which can spread only by being passed on to the descendants of the cells in which they arose ("vertical transmission"), and new lineages emerge by the accumulation of such mutations over successive generation. However, bacteria also possess a number of parasexual mechanisms for the horizontal genetic exchange of chromosomal DNA. The three most important mechanisms, conjugation, transduction, and transformation do not involve the exchange of complete chromosomal genetic material, but rather small genome segments (Smith *et al.* 1991;Spratt & Maiden 1999).

The first studies on bacterial population dynamics using MLEE, indicated that in the populations examined, most isolates belong to a limited number of closely related genotype clusters Thus, a clonal structure of bacterial populations was concluded suggesting that uniform groups of pathogens in diverse species arose from a common ancestor (Musser *et al.* 1988;Selander *et al.* 1985;Musser *et al.* 1987;Achtman 2004). Consequently, the distribution of chromosomal polymorphisms within an asexual (clonal) bacterial population would be non-random, or in linkage disequilibrium (Spratt & Maiden 1999).

The clonal paradigm of bacteria was challenged in 1993 by MLEE studies of *Neisseria gonorrhoeae* and *Bacillus subtilis* populations (O'Rourke & Stevens 1993). Maynard Smith and collegues then postulated that recombination is so frequent in some species, that clonal complexes might exist only temporarily and epidemic spread might therefore result in transient apparent clonality (Smith *et al.* 1993a). Furthermore, they stated that in case of an emerging lineage with increased invasive capacity, the analysis of isolates obtained exclusively from disease can result in an overestimate of the significance of the epidemic clone as a consequence of sampling bias. As recombination seemed so frequent, it was found impossible or difficult to reconstruct the phylogenetic framework of evolution of many pathogenic species (Feil *et al.* 2001).

The large databases collected since the introduction of MLST in 1998 indicate that a spectrum of population structures exists. It reaches from the extremes of strictly clonal, e.g. *Salmonella enterica* (Boyd *et al.* 1996), where apparently no recombination has occurred in the evolutionary history of the species, to 'panmictic', e.g. *Helicobacter pylori*

(Go *et al.* 1996), where recombinational exchanges are sufficiently frequent to randomize the alleles in the population and to prevent the emergence of stable clones. The term ‘epidemic clonal’ has been used to describe a situation, where a particularly effective lineage within a basically non-clonal bacterial population arises and rapidly spreads, so that, in the short term, a large number of related organisms come to predominate the population (Smith et al. 1993a;Smith *et al.* 2000). All three types of structure, clonal, panmictic and epidemic clonal, can be present in a single bacterial species (Spratt & Maiden 1999).

However, it is now more and more accepted, that the distinction between clonality and frequent recombination is too simplistic to reflect the variety of population structures that are found in diverse bacterial species (Achtman 2004), and new tools and algorithms are needed to classify and understand the diversity (Feil & Enright 2004). It has been recently recognized that a number of species are of recent decent and possess little sequence diversity. *Yersinia pestis*, according to MLST results, is a clone of *Y. pseudotuberculosis* that has evolved in the last few millenia (Achtman *et al.* 1999). Also *Mycobacterium tuberculosis*, *M. ulcerans*, *Bacillus anthracis* and some other pathogens are highly uniform and may be of recent decent. These various groups of pathogens define a novel population structure, possibly best designated as “Young”, which does not fall into the clonal versus recombination debate (Achtman 2004).

The population structure of N. meningitidis and S. pneumoniae

Both, the meningococcus and the pneumococcus are antigenically and genetically highly diverse. MLEE- and MLST data have shown a high level of recombination in the house keeping genes of both species. Most recent analysis of these data has quantified the relative contribution of recombination vs. mutation and shown that the generation of novel alleles of these housekeeping loci in pneumocooci is driven predominantly by recombination rather than point mutation. The number of alleles that arise by recombination in contrast to mutation is estimated to 10:1 for pneumococci and 4:1 for meningococci (Feil *et al.* 2000).

Pneumococci as well as meningococci colonizing the nasopharynx live alongside closely related bacterial species, such as viridans Streptococci and other Neisseria species

respectively. For both pathogens, interspecies recombination with closely related bacteria occupying the same ecological niche has been described, further enhancing the diversity of the species. For example penicillin-resistant pneumococci have acquired altered penicillin-binding proteins by interspecies recombination (Coffey et al 1995). Acquisition of foreign genes or gene-segments during epidemic spread of Neisseria meningitidis has been reported for the *iga* gene, the *opc* gene, the *porA* gene (Maiden *et al.* 1996), and the *tbp*-gene (Linz *et al.* 2000).

For both pathogens the bacterial capsule is essential for causing invasive disease and is considered as one of the major virulence factors. However, comparisons of the disease-associated and carried populations have shown that most invasive disease is caused by a minority of serological grouping and genotypes.

The major invasive meningococcal serogroups are B and C for Europe and Northern America and serogroup A (and C) for developing countries. The MLST database of Neisseria species comprises up to date more than 5000 STs and over 7000 bacterial isolates (http://pubmlst.org/neisseria/) isolated from diseased patients and healthy carriers since 1917. Analysing isolates from meningococcal carriage vs. disease has revealed, that certain STs, especially those belonging to the ST-1, ST-4, ST-5, ST-8, ST-11, ST-32, and ST-41/44 clonal complexes are overrepresented in collections of disease-associated isolates relative to their frequency in collections of asymptomatically carried isolates. However, the analysis of three meningococcal isolate collections (I. carriage population in the Czech Republic, II. a temporally and geographically equivalent set of disease isolates, III. the global diversity of meningococcal isolates in the second part of the 20th century) indicated that overall patterns of diversity, as measured by absolute levels of polymorphism, allele frequency spectra, and linkage disequilibrium, were similar in collections of disease-associated and carried meningococci, despite the dominance of hyperinvasive lineages among the disease associated isolates (Jolley *et al.* 2005).

The existence of invasive clones in the presence of frequent recombination seems to be a contradiction. Clonal expansion is usually associated with epidemic spread, where a large number of similar strains emerge in a very short time. Meningococcal meningitis epidemics in any one country only last for about 2-3 years and not only disease, but also

carriage returns to endemic levels very quickly (Hassan-King, Wall, & Greenwood 1988). It has been suggested that towards the end of an epidemic the frequency of both genetic escape variants and and serological variants increases dramatically, presumably due to immune pressure. But most of these variants seem to be less fit than their parents and are lost by bottlenecks during the spread from country to country (clonal reduction) (Achtman 2004). As a result, isolates from a pandemic spanning multiple countries and continents can be indistinguishable, even by very sensitive methods, whereas multiple unique variants can be isolated in each country. The resulting population type, describing a genotype plus its closely related descendants, was designated “genocloud” (Zhu *et al*. 2001). The three pandemic waves of serogroup A, subgroup III meningococci since the mid 1960’s were by analysis of six polymorphic loci associated with the sucsessive replacement of individual dominant genoclouds by others. These genoclouds exhibited very close relationship with each other, the strains causing the second and third pandemic wave by MLST were identified as ST5 and ST7 bacteria respectively, differing only in one of seven alleles. However, this genetic evolution is non-reversible; genoclouds only exist transiently before they are continously eliminated during spread from country to country. In addition they disappear from individual countries and areas after several years, even in the absence of vaccination (Zhu *et al.* 2001). Capsule switch of pathogenic meningococci as a mechanism to escape serogroup specific immunity has been reported (Swartley *et al.* 1997), but it is not known, to which extent this occurs in natural populations.

Also the epidemiology of *S. pneumoniae* shows, that usually at any time and place only a small group of serotypes is responsible for the majority of invasive disease (in contrast to a highly diverse carriage population) (Enright & Spratt 1998). The MLST database of *S. pneumoniae* comprises to date almost 3600 isolates that constitute 2180 ST, and 184 clonal complexes. The extend of clustering among all genotypes within the current pneumococcal MLST database can be displayed by a “population snapshot”, an e-Burst diagram, comprising the complete MLST database (Figure 1.3). A high diversity within serotypes has been described. For many serotypes only one or two clonal complexes are recovered from invasive disease in different countries. For serotype 14, two unrelated clones (ST9 and ST124) have been found in various studies (Enright & Spratt 1998;Brueggemann *et al.* 2003). In other cases, such as serotypes 6A and 19A, the population seems to be more heterogeneous and no single ST has been identified as the predominant cause of invasive disease in different countries (Spratt *et al.* 2004). In many

cases, global spread of pneumococcal clones has been associated with emergence and manifistation of drug resistance in commonly carried serotypes (Klugman 2002). These clones, which have appeared within the last 30 to 50 years have been well monitored and they have been shown to quickly diversify. Good evidence has been found for frequent recombinational exchange of the pneumococcal capsular locus, reported repeatedly from major antibiotic resistant clones, that have been extensively studied (Figure 1.2) (Coffey *et al.* 1998;Ramirez & Tomasz 1999;Jefferies *et al.* 2004).

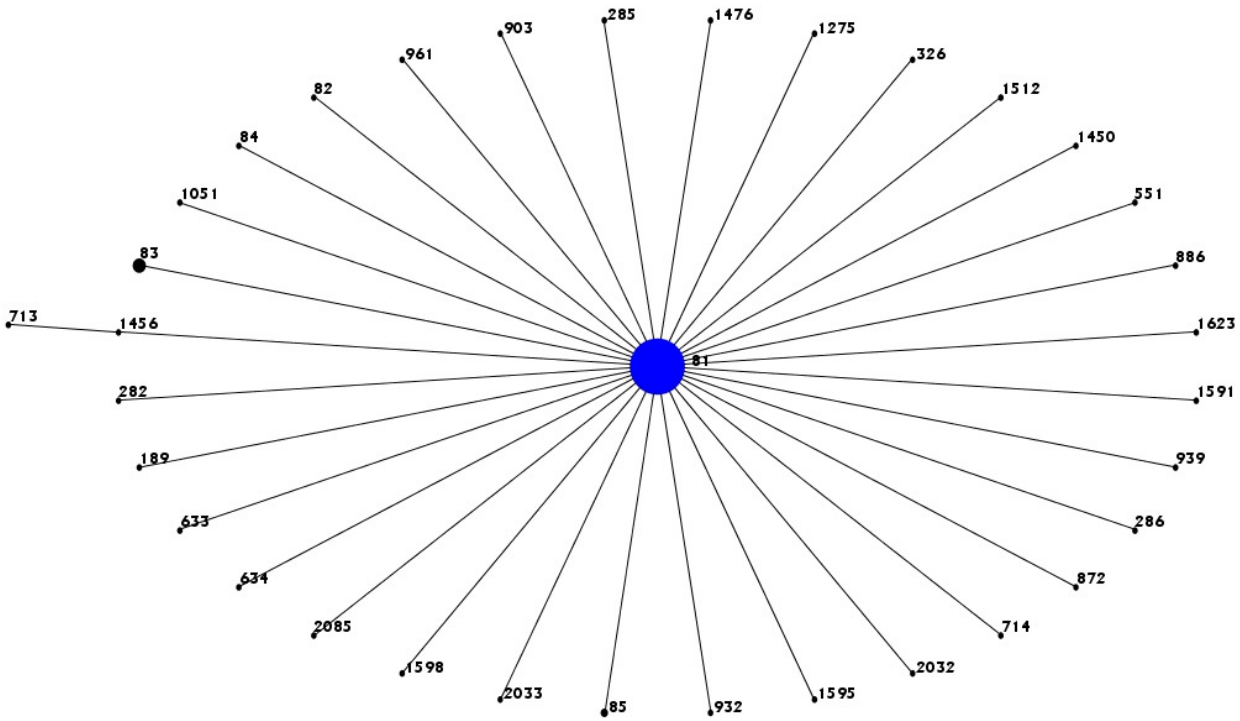


Figure 1.2 E-Burst diagram of the ST81 clonal complex. The MLST database comprises a large number of isolates sharing the ST81 and a number of closely related isolated, most of them are Single Locus Variants (SLV) of ST81. The E-Burst diagram strongly predicts that ST81 is the founding genotype (blue). All isolates of the clonal complex are all multiple antibiotic resistant. The clone is known as Spain^{23F}-1. Isolates showing ST81 are of different seotypes: 23F, 19F, 19A, 14, 9V indicating a history of serotype switching.

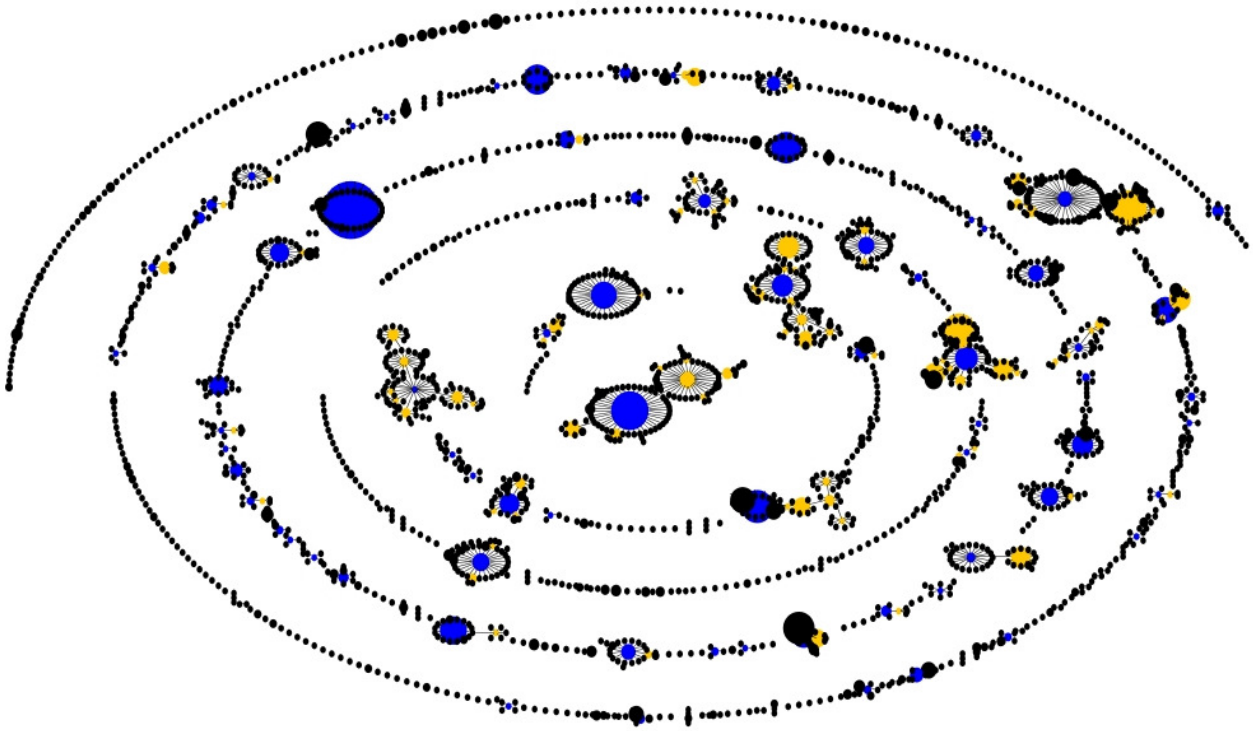


Figure 1.3 Population snapshot by e-Burst of the *S. pneumoniae* MLST database according to its status in June 2006. Each ST is represented by one dot. Single locus variants are connected by lines, blue dots represent predicted founders of a clonal comples, yellow dots show predicted subgroup founders.

While the population structure of meningococci and pneumcocci is well studied and genomes of several isolates have been sequenced, it is still unclear, which factors make the individual genoclouds more virulent than others. Also the mechanisms responsible for the emegence and dissapearence of patricular clones are not understood. Systembiological approaches will be required to give deeper insight into the underlying host-pathogen interaction at the population level. Molecular epidemiological studies will help to identify groups of isolates that should be selected for high-throughput genomic and proteomic analysis with the aim to identify genetic constellations responsible for successful competition at the colonisation level and for higher virulence in particular host populations.

Rationale and research frame work

The work presented here is part of a long-term collaboration between the Swiss Tropical Institute, Basel, and the Navrongo Health Research Centre in Ghana that attempts to better understand the dynamics of bacterial meningitis in the *African Meningitis Belt*. Even though the meningitis epidemics of the last decades have been studied in detail in the various countries, the factors precipitating these epidemics are still largely unknown, and outbreaks remain unpredictable. The collaboration started after a major epidemic that occurred in the dry season of 1997 in Northern Ghana with the establishment of a meningitis surveillance system in the Kassena Nankana District and a longitudinal colonisation survey. In the first phase of this collaboration (reported in the PhD theses of A. Hodgson and S. Gagneux), the epidemiological features of the outbreak of 1997 were closely analysed, and the risk factors and sequelae were studied. Furthermore, the molecular epidemiological characteristics of a serogroup A post-epidemic outbreak in 1998 and of a serogroup X colonisation wave were analysed in detail.

This thesis presents part of the second phase of this collaboration, focussing on long-term analysis of the relationship between colonisation and disease during a 8-year period, and determining the role of newly emerging pathogenic clonal complexes of *N. meningitidis* and *S. pneumoniae*.

Chapter 2

Goal and Objectives

Goal and Objectives

Goal

The goal of the long-term colonisation and case study in Northern Ghana is to contribute to a better understanding of the epidemiology of bacterial meningitis in the Meningitis Belt of Sub-Saharan Africa. In particular we were interested in the association of meningococcal colonisation and disease. Furthermore we aimed to examine the significance of other emerging meningococcal clones such as W135 meningococci, and other bacterial pathogens causing cerebrospinal meningitis in particular *S. pneumoniae* and *H. influenzae* B in the Kassena Nankana District of Northern Ghana.

Objectives

1. To analyse continuously collected CSF samples from the KND and health facilities from neighbouring districts in Northern Ghana, to isolate the respective causative agents and to characterize meningococcal and pneumococcal strains by serological and molecular methods.

2. To monitor the colonisation with *N. meningitidis* and *N. lactamica* in the KND by twice yearly carriage surveys. To analyse the population structure of the colonizing meningococcal population, to relate this to the disease causing strains and to examine long term trends of colonisation and disease.

3. To investigate the threat of W135 meningococcal meningitis epidemics in Ghana by molecular epidemiological analysis of ghanaian W135 case isolates and investigation of their spread in the healthy population by located colonisation surveys.

4. To analyse the epidemiological and microbiological characteristics of pneumococcal meningitis in Northern Ghana

5. To examine the population structure of *S. pneumoniae* isolates dominating in Northern Ghana in comparison with reprtesantative strain of a global collection of isolates.

Chapter 3

An outbreak of serotype 1 *Streptococcus pneumoniae* meningitis in Northern Ghana with features characteristic of epidemic meningococcal meningitis

Julia Leimkugel[1], Abudulai Adams Forgor[2], Sébastien Gagneux[1*], Valentin Pflüger[1], Christian Flierl[1], Elizabeth Awine[2], Martin Naegeli[1], Jean-Pierre Dangy[1], Tom Smith[1], Abraham Hodgson[2] and Gerd Pluschke[1]

[1] Swiss Tropical Institute, Basel, Switzerland

[2] Navrongo Health Research Centre, Ministry of Health, Navrongo, Ghana

*present address: Division of Infectious Diseases and Geographic Medicine, Stanford University Medical Centre, Stanford, USA

This article has been published in:

Journal of Infectious Diseases (2005),**195**: 192-199

Abstract

Background The Kassena-Nankana District (KND) of northern Ghana lies in the African meningitis belt where epidemics of bacterial meningitis have been re-occurring every 8-12 years. These epidemics are generally caused by *Neisseria meningitidis*, an organism considered uniquely capable of causing meningitis epidemics.

Methods We recruited all suspected meningitis cases in the KND between 1998 and 2003. Cerebrospinal fluid samples were collected and analysed by standard microbiological techniques. Bacterial isolates were subjected to serotyping, multi-locus sequence typing (MLST) and antibiotic resistance testing.

Results A continual increase in the incidence of pneumococcal meningitis was observed from 2000 to 2003. This outbreak exhibited strong seasonality, a broad host age spectrum, and clonal dominance, all of which are characteristic of meningococcal meningitis epidemics in the African meningitis belt. The case fatality rate for pneumococcal meningitis was 44.4%, the majority of pneumococcal isolates were antibiotic sensitive and expressed the serotype 1 capsule. MLST revealed that these isolates belonged to a clonal complex dominated by sequence type (ST) 217 and its two single-locus variants ST303 and ST612.

Conclusions The ST217 clonal complex of *S. pneumoniae* represents a hypervirulent lineage with a high propensity to cause meningitis. In addition, our results suggest that this lineage might have epidemic potential. Serotype 1 is not included in the currently licensed paediatric heptavalent pneumococcal vaccine. Mass vaccination targeting hypervirulent serotypes with a less complex conjugate vaccine should therefore be considered.

Introduction

Neisseria meningitidis, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) are the most common causes of acute bacterial meningitis (Hart C.A. & Cuevas L.E. 2003). Meningitis caused by *N. meningitidis* has been considered unique with respect to its epidemic occurrence. A region of sub-Saharan Africa extending from Ethiopia to Senegal, designated the 'meningitis belt', has been particularly vulnerable to meningococcal disease epidemics. In addition to sporadic disease, which occurs mainly during the annual dry season, epidemics have occurred in the meningitis belt every 8-12 years over the past 100 years (Greenwood 1999;Achtman 1995a).

Information about the epidemiology of pneumococcal meningitis in the African meningitis belt is fragmentary, but some studies have found *S. pneumoniae* to be the most important causative agent of bacterial meningitis in certain areas (Mar, Denis, & Cadoz 1979). The incidence in these areas is 10-20 cases per 100,000 and year, which is about ten times higher than in Western Europe and the United States (Greenwood 1987;Hausdorff, Bryant, Paradiso, & Siber 2000). Cases of *S. pneumoniae* meningitis occur throughout the year, and most studies report the youngest (<2) and the oldest (> 60) age groups to be at greatest risk (Mar, Denis, & Cadoz 1979;Greenwood 1987). For unknown reasons, the case fatality rate for pneumococcal meningitis (about 50%) is five to ten times higher than for meningococcal meningitis.

Although there are about 90 pneumococcal serotypes known, only a limited number account for most of the invasive infections. The serotype distribution varies with time, location and age group (Hausdorff, Bryant, Paradiso, & Siber 2000). Clonal dominance and global spread has been described for a small number of highly successful, (often multi-) drug resistant pneumococcal clones (Klugman 2002). Serotype 1 is one of the most common serotypes causing invasive disease worldwide, particularly in Africa (Greenwood *et al.* 1980;Hausdorff, Bryant, Paradiso, & Siber 2000;Brueggemann & Spratt 2003). It has a high attack rate but is rarely isolated from healthy carriers or mild occult bacteraemia. Outbreaks of invasive serotype 1 pneumococcal disease have occurred in several communities (Dagan *et al.* 2000;Gratten, Morey, Dixon, Manning, Torzillo, Matters, Erlich, Hanna, Asche, & Riley 1993;Hausdorff, Bryant, Paradiso, & Siber 2000;Mar, Denis, &

Cadoz 1979;Porat, Trefler, & Dagan 2001;Henriques, Kalin, Ortqvist, Akerlund, Liljequist, Hedlund, Svenson, Zhou, Spratt, Normark, & Kallenius 2001;Tugwell *et al.* 1976).

The present study was conducted between 1998 and 2003 in the Kassena-Nankana District (KND) in northern Ghana. Following a large meningococcal meningitis epidemic in the dry season of 1997, all suspected meningitis patients were recruited prospectively. Cerebrospinal fluid (CSF) samples were taken and analysed by standard microbiological techniques. Between 2000 and 2003, a continuous increase in incidence of pneumococcal meningitis was observed. We demonstrate that the epidemiological and bacteriological features of this outbreak closely resemble the ones usually associated with meningococcal disease epidemics. The implications of these observations for the control of bacterial meningitis in the African meningitis belt are discussed.

Methods

Study area

The KND has a population of 140,000 and lies within the Guinea Savannah woodland area of northern Ghana. Two major seasons exist, a short wet season from May to October and a long dry season for the rest of the year. The general population is rural except for those living in the town of Navrongo, which has a population of 20,000. People live in compounds with an average of 10 inhabitants.

Patients

CSF samples were collected from January 1998 to December 2003 from suspected meningitis patients reporting to the War Memorial Hospital, Navrongo, or to one of four Health Centres in the KND. In line with the standard diagnostic procedures in Ghana, samples were analysed at the laboratory of the War Memorial Hospital for confirmation of the clinical diagnosis. Additional samples were obtained from the Regional Hospital of the Upper East Region in Bolgatanga, and from health facilities in the Bongo and Bulsa Districts. In 1998 and 1999, only samples collected during the dry season were analysed. Thereafter, samples obtained from the few suspected meningitis cases presenting during the wet season were also included. Ethical clearance for the study was obtained from the responsible institutional review boards and the Ghanaian Ministry of Health. Clinical and demographic information was recorded from all patients. Personal data were linked with the database of the Navrongo Demographic Surveillance System (NDSS). The

denominators used for calculation of incidence rates represent the average annual district population between 1995 and 1999 (Nyarko *et al.* 2002).

Analysis of CSF

CSF samples were analysed by direct Gram staining. Boiled CSF-supernatants were tested serologically for capsular polysaccharide antigens of *N. meningitidis* (serogroups A, B, C and W135), *S. pneumoniae* and Hib (Slidex meningite-Kit, Bio Merieux, Pasteurex-Kit, BIO RAD #61718). CSF specimens were inoculated on blood-, chocolate-, and Thayer Martin Agar and incubated in candle jars for 24 hours at 37˚C. *S. pneumoniae* colonies were identified based on colony morphology, Gram staining behaviour and resistance to Optochin (Taxo P discs, BD #231046). All pneumococcal isolates were serotyped with the Quellung Reaction using antisera from the Statens Serum Institute, Copenhagen.

Antibiotic resistance testing

All isolates from the KND were tested for resistance to penicillin G, chloramphenicol (the two antibiotics commonly used for standard therapy of bacterial meningitis in Ghana), cefotaxime, and ciprofloxacin using E-test strips (Isenberg Henry D.(ed.) 1998). Breakpoints of the NCCLS protocol have been applied. For ciprofloxacin 4μg/ml has been taken as breakpoint for resistance (Brueggemann et al. 2002). The ATCC 49619 strain was included as control.

Multi-Locus Sequence Typing (MLST)

Bacteria were grown overnight in Todd Hewitt medium. DNA extraction (Vela Coral *et al.* 2001), MLST (Enright & Spratt 1998) and direct sequencing of PCR products with an ABI Prism 310 Genetic Analysis System was performed according to standard protocols. Allelic profiles were analysed using applications available on the MLST homepage *(www.mlst.net).* For the analysis of the relationships between closely related isolates the eBurst software (http://eburst.mlst.net) was used with the most stringent group definition (6/7 alleles identical). All allelic profiles obtained were compared to the complete listing of STs available in the database.

Results

Meningitis cases

Between 1998 and 2003, a total of 140 meningococcal, 117 pneumococcal and 14 Hib meningitis cases were confirmed by culture and/or Latex agglutination assay in the KND. The number of pneumococcal cases remained low during the first two years of the study, but increased continuously during the following years (Figure 3.1). Two subsequent outbreaks of serogroup A meningococci were reported during the study period. After the large meningococcal meningitis epidemic in Ghana 1997, 50 confirmed serogroup A cases occurred in 1998 (Gagneux *et al.* 2000). After two years of absence, from 2001 onwards serogroup A meningococcal cases re-emerged causing yearly outbreaks until 2004 (Chapter 5). The number of Hib meningitis cases remained low throughout the study period and included mainly children below 7 years of age (Figure 3.1).

The vast majority of meningococcal and of pneumococcal meningitis cases occurred during the dry season (Figure 3.2). The pneumococcal meningitis cases peaked one to two months earlier than the meningococcal cases. During the rest of the year only sporadic meningitis cases, mostly caused by *S. pneumoniae*, were observed.

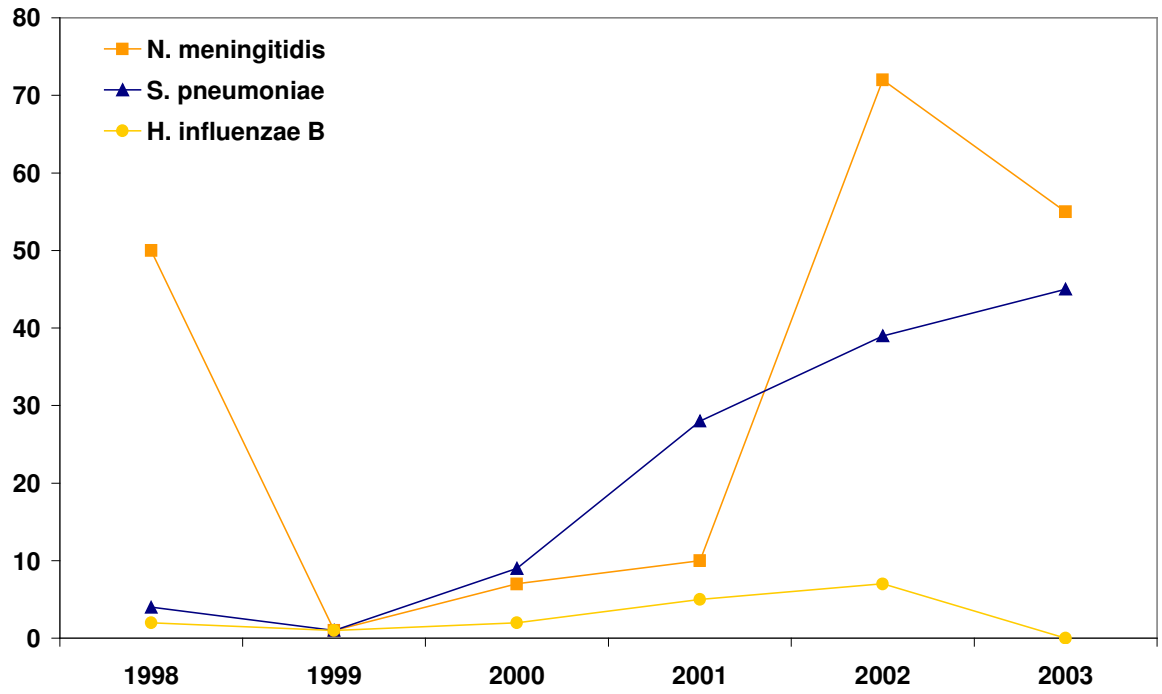


Figure 3.3.1 Number of laboratory-confirmed (cultivation and/or latex agglutination) **meningitis cases in the Kassena-Nankana District of northern Ghana between 1998 and 2003.**

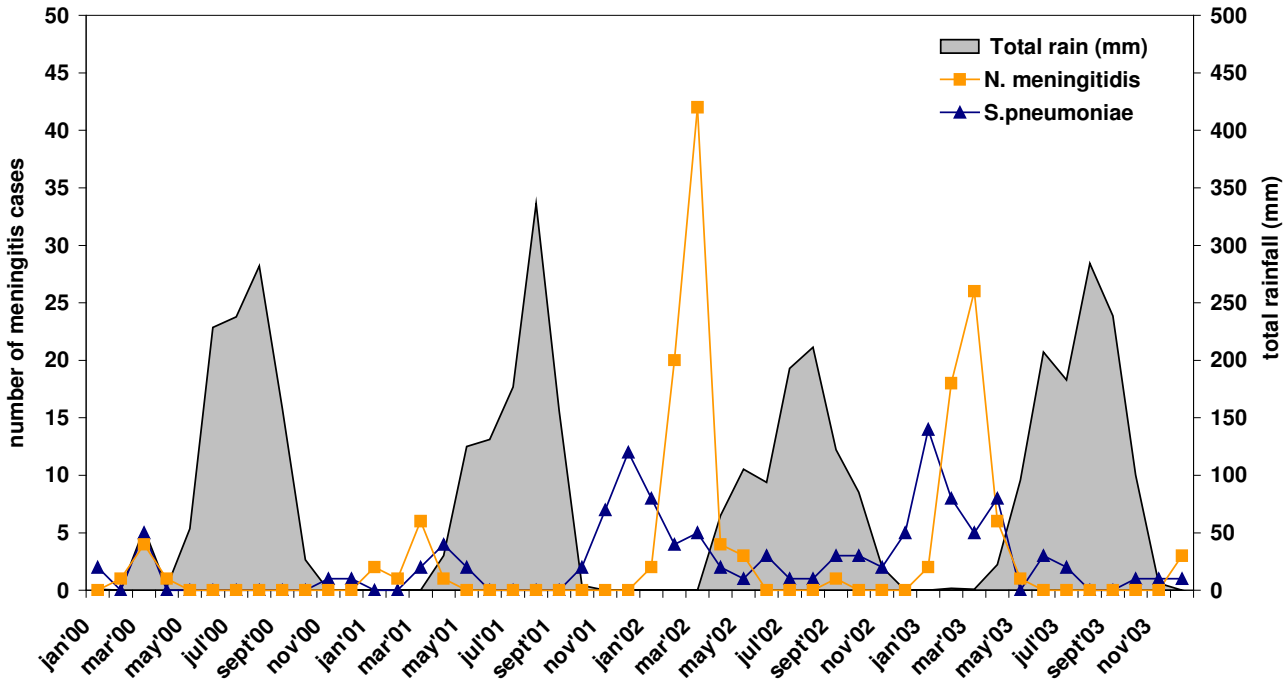


Figure 3.3.2 Seasonal patterns of rainfall and number of pneumococcal and meningococcal meningitis in the KND. (monthly rainfall data from the Meteorological Station of the KND)

The populations of both meningococcal and pneumococcal meningitis patients exhibited a broad age range (Figure 3.3). Infants <1year had the highest incidence for both pneumococcal and meningococcal meningitis (43 cases/100,000 per year). For pneumococcal meningitis, the incidence in all other age groups was 15 - 26/100,000. For meningococcal meningitis the incidence was comparable for children of all age groups, and decreased steadily for the older age groups. As a result, the incidence of pneumococcal meningitis in the >60 year age group was significantly higher than for meningococcal meningitis (2.6/100'000 versus 23.4/100'000). The overall case fatality rate was 44.4% (51/117) and 4.3% (6/140) for pneumococcal and meningococcal meningitis, respectively.

The geographic location of the homes of 74 pneumococcal and 102 meningococcal meningitis patients was mapped using the NDSS, but neither pneumococcal nor meningococcal cases were geographically clustered (data not shown). Furthermore no significant family clustering was observed.

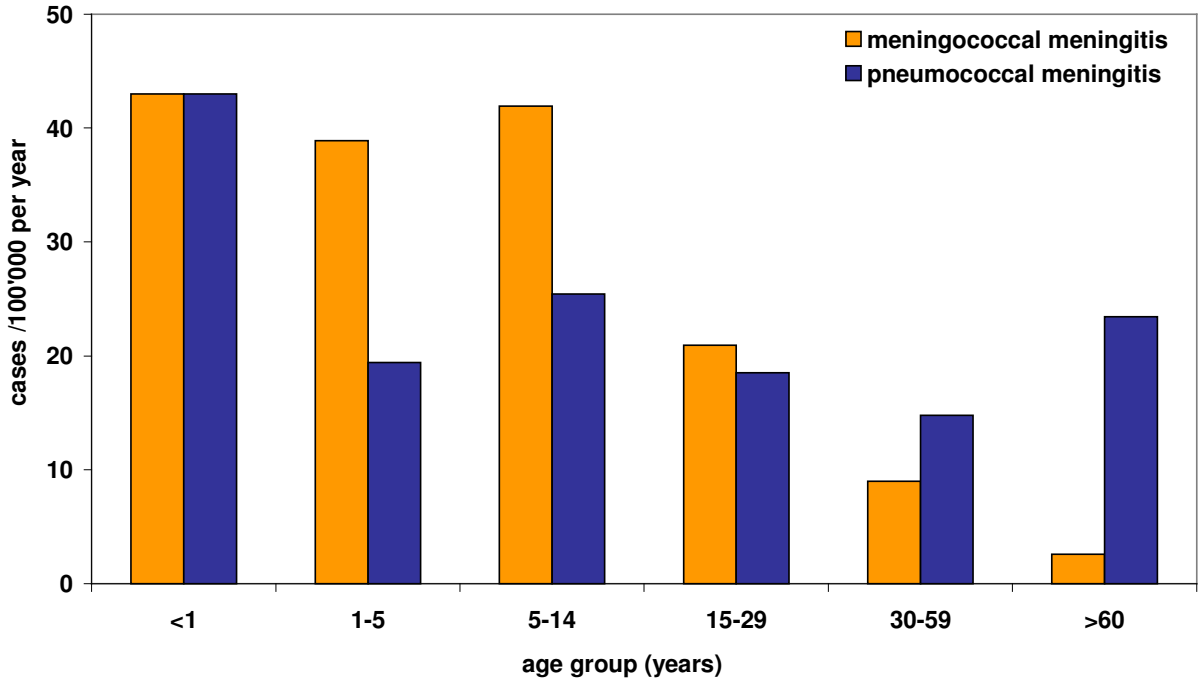


Figure 3.3.3 Incidence (laboratory confirmed cases by latex agglutination or culture) **of meningococcal (orange bars) and pneumococcal (blue bars) meningitis in the KND.**

Characterisation of pneumococcal isolates

Between 1998 and 2003, 76 pneumococcal disease isolates were obtained from meningitis patients in the KND. Fifty-eight of these (76.3%) belonged to serotype 1, which represented the dominating serotype throughout the study (Table 3.1). The 18 non-serotype 1 isolates from the KND belonged to nine other serotypes. Only a third (2/6) of the paediatric disease isolates (<5 year old) were serotype 1, the remaining belonged to serotype 3 and 14. In contrast, in older children (5-14 years), young adults (15-29 years) and grown-ups (30-59 years) the serotype 1 ratio was >80 % (24/29, 11/12 and 11/14, respectively). In patients >60 years the percentage of serotype 1 isolates was 56 % (5/9).

Drug sensitivity testing showed that all but two of the 58 serotype 1 strains from the KND were completely susceptible to penicillin G, cefotaxime, chloramphenicol and ciprofloxacin. Minimal inhibitory concentrations (MIC) determined for the two strains (both isolated in 2002) showing antibiotic resistances were: strain P1036**:** penicillin G 0.5 μg/ml (intermediate), cefotaxime 2μg/ml (resistant), chloramphenicol: 5 μg/ml (intermediate); strain P1037: penicillin G 0.5 μg/ml (intermediate), cefotaxim 1 μg/ml (intermediate), chloramphenicol 8 μg/ml (resistant).

All isolates from the KND and 15 isolates from neighbouring districts were analysed by MLST. The results showed that all serotype 1 isolates were clonally related (Table 3.2). Ten distinct STs were identified; but all shared at least six of seven alleles with one other ST. ST217 and its two single locus variants ST612 and ST303 dominated. In addition, single locus variants of the three dominating STs were sporadically found. All isolates obtained in 1998 and 2000 had ST217. ST303 isolates dominated from 2001 onwards (6/15 in 2001, 9/18 in 2002 and 14/20 in 2003).

An eBurst analysis was done including the STs of the Ghanaian stains and all strains available in the MLST database (Figure 3.4). Three of the 10 STs found in the Ghanaian isolates (ST217, ST303 and ST612) have been previously described in altogether 34 serotype 1 lineage B isolates (Brueggemann & Spratt 2003). 16 of these isolates came from Africa, the others from Israel, Europe or the United States. In addition, Brueggemann et al. (Brueggemann & Spratt 2003) defined three lineage B associated STs (ST613, ST614 and ST 618) represented by four African and one European isolate. The eBurst diagram (Figure 3.4) demonstrates, that all Ghanaian serotype 1 strains found in this study and all the lineage B isolates described by Brueggemann et al. are part of a single clonal complex in which all isolates share 100% genetic identity at six or seven MLST housekeeping loci with at least one other member of the group.

Of the non-serotype 1 isolates, only the serotype 14 strains exhibited allelic profiles closely related to those of the serotype 1 complex (Table 2). One of the serotype 14 strains (ST1324) was a single locus variant of ST1323 (shown in Figure 3.4), two (ST1314 and ST1315) were double locus variants of ST1323 and the remaining isolate (ST1313) shared five alleles with ST1314 and four alleles with ST1323.

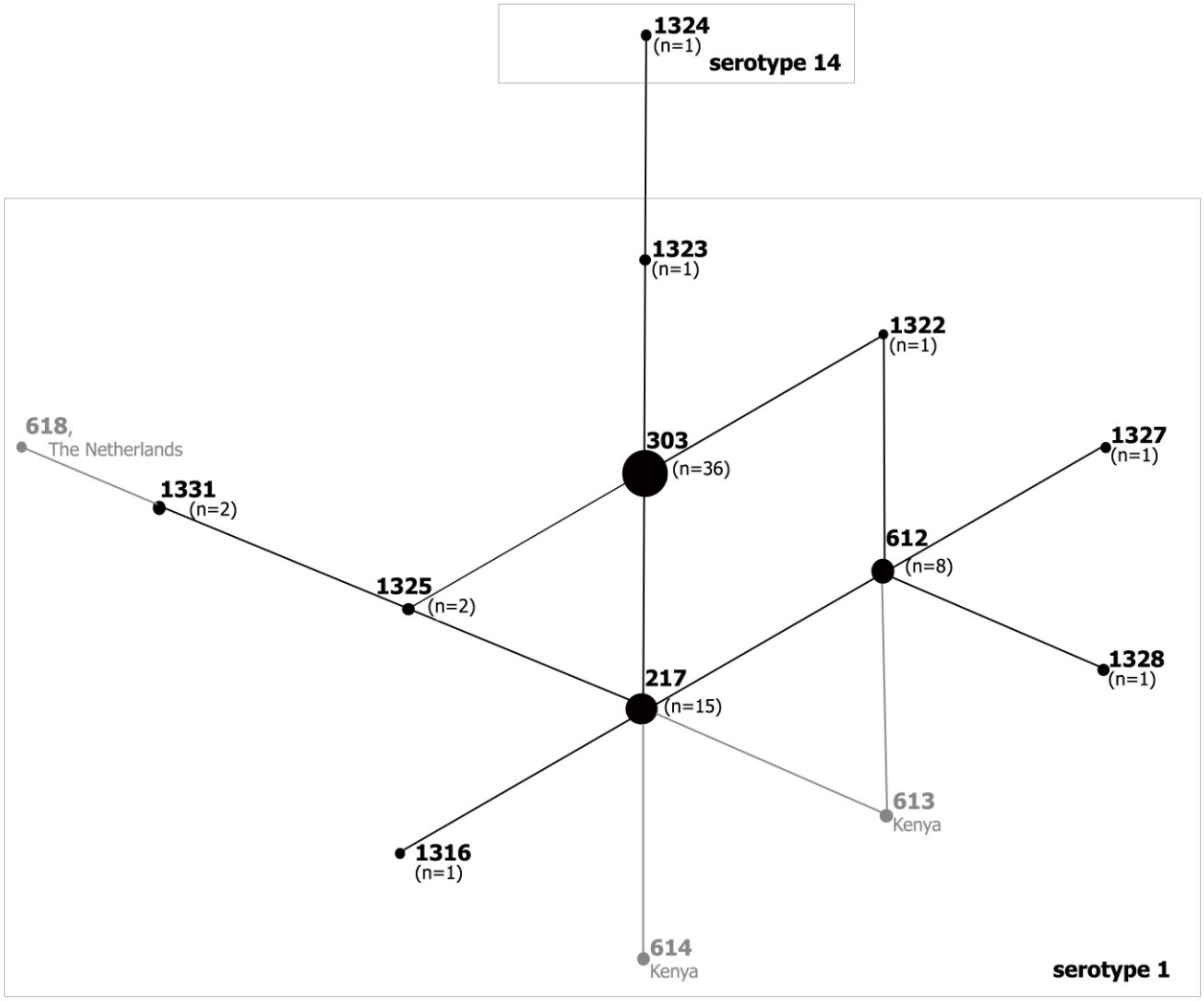


Figure 3.4 e-BURST diagram of the sequence type (ST) 217 clonal complex.

Included are all Ghanaian serotype 1 *Streptococcus pneumoniae* isolates and 1 Ghanaian serotype 14 *S. pneumoniae* isolate from the present study as well as all serotype 1 lineage B *S. pneumoniae* isolates described byBrueggemann et al. (Brueggemann & Spratt 2003). Lines connect all single-locus variants with each other. Black circles indicate STs found in northern Ghana in the presentstudy, and the no. of isolates found are given. Gray circles indicate serotype 1 lineage B–associated STs not found in northern Ghana [9], and the countries of origin of the isolates are given. Shown is a modification of the original diagram produced by e-BURST, which was edited for the no. of isolates, the origin of non-Ghanaian isolates, and multiple single-locus variant connections.

Table 3.1 Host age distribution of serotype 1 and non–serotype 1 *Streptococcus pneumoniae* isolates from the Kassena-Nankana District of northern Ghana, found between 2000 and 2003.

Age group (years)	**<1**	**1-4**	**5-14**	**15-29**	**30-59**	**>60**	**n.s.***	**Total**	***age range***	***median***
No. of isolates serotyped	2	4	29	12	14	9	6	76	*$^{4}/_{12}$ to 85 y*	*14y*
Serotype 1 isolates	0	2	24	11	11	5	5	58	*$1^{9}/_{12}$ to 72 y*	*15y*
Non-serotype 1 isolates	2	2	5	1	3	4	1	18	*$^{4}/_{12}$ to 85 y*	*13y*
Serotypes of non-serotype 1 strains	14^{+}	3^{+}	3^{+}, 7F, 8, 12F	8	6A, 8, 10F	8, 12F, 14, 38	2			

Table 3-2 Serotype distribution and Sequence Types (STs) of *S. pneumoniae* strains isolates in northern Ghana, found between1998 and 2003.

Serotype	ST	No. of isolates	Yr. of isolation	Allelic Profile							origin (district)
				aroE	gdh	gki	recP	spi	xpt	ddl	
Serotype 1	217	15	1998-2003	10	18	4	1	7	19	9	KND (13), Bongo (1), Builsa (1)
	612	8	2001-2003	10	18	4	1	7	19	31	KND (7), Bolgatanga (1)
	303	36	2001-2003	10	5	4	1	7	19	9	KND (29), Bolgatanga (7)
	1322	1	2001	10	5	4	1	7	19	31	KND
	1316	1	2002	2	18	4	1	7	19	9	KND
	1325	2	2002	10	8	4	1	7	19	9	KND
	1331	2	2002	13	8	4	1	7	19	9	KND
	1327	1	2003	10	18	4	1	13	19	31	KND
	1328	1	2003	10	18	4	1	7	21	31	KND
	1323	1	2003	10	5	4	1	7	21	9	KND
Serotype 2	74	1	1998	2	13	4	1	6	6	14	KND
Serotype 3	458	7	2001	2	32	9	47	6	21	17	KND (3), Bolgatanga (4)
Serotype 4	1321	1	2002	8	8	47	18	46	122	31	Bolgatanga
Serotype 6°	1320	1	2002	7	13	8	6	6	8	8	KND
Serotype 7F	1326	1	2002	10	16	4	1	6	21	9	KND
Serotype 8	1317	1	2003	7	5	15	11	83	58	70	KND
	1318	1	2000	7	9	15	11	83	58	70	KND
	1335	1	2003	7	9	4	60	83	28	70	KND
	1319	1	2003	7	9	15	11	83	25	70	KND
Serotype 10F	909	1	2003	2	42	2	1	6	19	20	KND
Serotype 12F	989	1	2003	12	5	89	8	6	112	14	KND
	1330	1	2003	12	5	89	8	13	112	14	KND
Serotype 14	1324	1	2002	10	5	4	17	7	21	9	KND
	1313	1	2003	2	5	4	12	7	21	14	KND
	1315	1	2003	2	5	9	1	7	21	9	KND
	1314	1	2003	2	5	4	1	7	21	14	Builsa
Serotype 38	1329	1	2003	12	5	4	10	42	49	9	KND

Discussion

N. meningitidis is regarded as uniquely capable of causing bacterial meningitis epidemics. Our observation of a meningitis outbreak caused by *S. pneumoniae* in the KND of northern Ghana is therefore intriguing. The outbreak exhibited epidemiological features characteristic of African meningococcal epidemics (Greenwood 1999), including strong seasonality, a broad host age range and clonal dominance. The increase in pneumococcal meningitis was accompanied by two successive outbreaks of meningococcal meningitis. In the KND the burden of disease for pneumococcal meningitis has met criteria for the alert status of the WHO definition of epidemic meningococcal outbreaks (threshold of 5 cases per 100,000 per week) and in the neighbouring Bolgatanga District even criteria for the epidemic status with a threshold of 10 cases were fulfilled in March 2001. Cases of both meningococcal and pneumococcal meningitis were concentrated in the dry season, suggesting that similar factors might have triggered both types of outbreak. Such factors may include damaged mucosal defences due to the extreme environmental conditions and/or co-infections of the nasopharynx (Greenwood 1999). Care was taken to avoid a bias associated with the well-known seasonality of meningococcal meningitis in the study area. Standardized guidelines for lumbar puncture were applied to avoid that lumbar punctures were less likely to be performed during the wet season.

Interestingly, the pneumococcal meningitis cases peaked one to two months earlier than meningococcal meningitis. This may reflect either the very high invasive capacity of the causative clonal complex of serotype 1 pneumococci or indicate that the factors which trigger pneumococcal and meningococcal meningitis are not entirely the same. In this context, differences in climatic conditions during the early dry season (including the Harmattan period with cold nights and extremely dusty air) and the late dry season (intensive heat), may be relevant. The broad age range in both meningococcal and pneumococcal meningitis cases shows that age related differences in the capacity of natural and adaptive immune effector functions are less important for susceptibility to invasive disease than in other epidemiological situations. Lack of spatial clustering suggests that colonisation with the serotype 1 pneumococci is not focal.

Clonally related bacteria from a common epidemiological source often show limited genotypic variation (Feil 2004). Groups of frequent genotypes plus their epidemiologically associated descendents have been designated *clonal complexes* (Feil 2004) or *genoclouds* (Zhu, van der, Falush, Brieske, Morelli, Linz, Popovic, Schuurman, Adegbola, Zurth, Gagneux, Platonov, Riou, Caugant, Nicolas, & Achtman 2001) on the basis of a

threshold level of MLST allelic identity. The pneumococcal outbreak in the KND was caused by a clonal complex of serotype 1 pneumococci. The three most frequently found STs (ST217 and its two single-locus variants ST303 and ST612) have been described before (Brueggemann & Spratt 2003), indicating that these genetic variants evolved prior to the outbreak in the KND. However, some of the infrequently isolated locus variants, such as ST1316, ST1322, ST1327 and ST1328 may have emerged locally. It is interesting to note, that ST1331 and ST1325, which were found each twice in the Ghanaian isolates link a ST618 isolate from The Netherlands to the clonal complex.

Serotype 1 pneumococci are a common cause of invasive disease in many parts of the world, but are only rarely found among healthy carriers (Brueggemann & Spratt 2003;Hausdorff, Bryant, Paradiso, & Siber 2000;Sandgren *et al.* 2004). Studies comparing the prevalence of *S. pneumoniae* subgroups from invasive disease and from carriage showed that individual serotypes may differ more than a 100 fold in their potential to cause invasive disease (Brueggemann, Griffiths, Meats, Peto, Crook, & Spratt 2003;Sandgren, Sjostrom, Olsson-Liljequist, Christensson, Samuelsson, Kronvall, & Henriques 2004). Individual clonal complexes belonging to the same serotype have different abilities to cause invasive disease (Sandgren, Sjostrom, Olsson-Liljequist, Christensson, Samuelsson, Kronvall, & Henriques 2004), suggesting that complex-specific virulence determinants might be important as well. It is not clear whether the virulence of the three major subgroups of serotype 1 pneumococci with distinct geographic distribution (Brueggemann & Spratt 2003;Gonzalez *et al.* 2004) is primarily determined by the capsular serotype and therefore uniform, or whether lineage-specific genetic differences modulate the potential to cause particular types of invasive disease. Our results suggest that the ST217 associated clonal complex might have a particular propensity to cause meningitis. However, further studies are needed in order to verify whether this observation reflects a true bacterial phenotype or merely the influence of host and/or environmental factors.

We do not know whether the ST217 clonal complex has recently been imported into northern Ghana or whether it has been present for a longer time without causing more than sporadic disease. Clonal dissemination of *S. pneumoniae* is usually associated with antibiotic resistance (Klugman 2002), but we observed no significant resistance in the Ghanaian isolates. Other factors must therefore have led to the increased incidence of pneumococcal meningitis in the KND. Vaccination against *S. pneumoniae* is uncommon in

Ghana. However, the massive immunization campaigns with a meningococcal A+C carbohydrate vaccine that have been repeatedly carried out throughout the study period might have played a role. *S. pneumoniae* and *N. meningitidis* both colonize the human nasopharynx, and effective interventions against one of these bacteria is likely to promote competing micro-organisms. Vaccinations with conjugate vaccines have been shown to reduce nasopharyngeal carriage of the vaccine type bacteria and to lead to replacement by bacteria not included on the vaccine (Bogaert *et al.* 2004b;Lipsitch 1999). Even though polysaccharide vaccines, such as the unconjugated *N. meningitidis* A + C vaccine used in the KND, are generally thought to have no effect on the prevalence of nasopharyngeal carriage (Greenwood 1999), repeated immunization against *N. meningitis* might still modify the bacterial flora of the nasopharynx (Fernandez *et al.* 2003). Thus, it is conceivable that the increase in pneumococcal meningitis in the KND, as well as the recently observed outbreaks of non-A, non-C meningococcal meningitis (Gagneux, Hodgson, Smith, Wirth, Ehrhard, Morelli, Genton, Binka, Achtman, & Pluschke 2002b;Djibo, Nicolas, Alonso, Djibo, Couret, Riou, & Chippaux 2003;Chonghaile 2002) may have been promoted by mass vaccination against *N. meningitis*. It will be important to investigate more closely the interactions between these bacteria, especially in the context of vaccination (Bogaert, De Groot, & Hermans 2004a).

Serotype 1 is not included in the currently licensed paediatric heptavalent pneumococcal vaccine. This vaccine contains polysaccharides from the seven serotypes (4, 6B, 9V, 14, 18C 19F and 23F) that cause over 85% of severe pneumococcal infections in infants and young children in the USA and Canada (Bogaert, Hermans, Adrian, Rumke, & De Groot 2004b;Hausdorff, Bryant, Paradiso, & Siber 2000). The vaccine covers 70% of paediatric disease isolates from Europe, but only 67% and 43% of those from Africa and Asia, respectively (Hausdorff, Bryant, Paradiso, & Siber 2000). In the KND serotypes 3, 7F, 8, 12 and 14 accounted for the non- serotype 1 cases in patients below 15 years of age. The 'paedriatric' serotypes (e.g. 6, 14, 9, 1, 5) (O'Dempsey *et al.* 1996) were rarely found. Here, the heptavalent conjugate vaccine would have covered 5.7% (2/35) of all cases and 22% of the non-serotype 1 cases in this age group. A nonavalent conjugate vaccine including serotype 1 is currently being developed, but such a complex conjugate vaccine may be too expensive for mass immunization in the African meningitis belt. However, mass vaccination targeting hypervirulent serotypes with a less complex conjugate vaccine should be considered, since increasing trends in pneumococcal meningitis have also been observed in other districts of Ghana (data not shown). Predominance of serotype 1 and a

broad age spectrum also seem to be features of the current pneumococcal meningitis situation in Burkina Faso (Parent, I *et al.* 2005;Robbins *et al.* 2005). In view of the high case fatality rate of *S. pneumoniae* meningitis, there is also an urgent need for improved treatment options suitable for countries with limited resources.

Acknowledgements

This work was supported in part by a grant of the Meningitis Research Foundation. We acknowledge the use of the pneumococcal MLST database which is located at Imperial College London and is funded by the Wellcome Trust. We thank Mr Alhassan and his team from the Bolgatanga hospital for access to the data and the provision of samples, the district health authorities of the KND for their support and the health facilities of Bongo and Sandema for their kind collaboration. Furthermore, we would like to acknowledge A. Bugri and A. Wahab for their indispensable contribution in the laboratory in Navrongo, the fieldworkers of NHRC for their efforts, and Prof. Gasser (Basel) for his support and practical advise.

Chapter 4
Emergence of W135 meningococcal meningitis in Ghana

Abudulai Adams Forgor[1], Julia Leimkugel[2], Abraham Hodgson[1], Akalifa Bugri[1], Jean-Pierre Dangy[2], Sébastien Gagneux[2*], Tom Smith[2] and Gerd Pluschke[2]

[1]Navrongo Health Research Centre, Ministry of Health, Navrongo, Ghana
[2]Swiss Tropical Institute, Basel, Switzerland

*present address: Division of Infectious Diseases and Geographic Medicine, Stanford University Medical Centre, Stanford, USA

This article has been published in
Tropical Medicine and International Health (2005), **10**:1229-1234

Summary

Neisseria meningitidis serogroup W135, well known for a long time as a cause of isolated cases of meningococcal meningitis, has recently increasingly been associated with disease outbreaks of considerable magnitude. Burkina Faso was hit by W135 epidemics in the dry seasons of 2002-2004, but only four W135 meningitis cases were recorded between February 2003 and March 2004 in adjoining Ghana. This reconfirms previous findings that bottlenecks exist in the spreading of new epidemic *N. meningitidis* clones within the meningitis belt of sub-Saharan Africa. Of the four Ghanaian W135 meningitis patients one died and three survived, of which one had profound neurosensory hearing loss and speech impairment. All four disease isolates were sensitive to penicillin G, chloramphenicol, ciprofloxacin and cefotaxime and had the multi-locus sequence type (ST) 11, which is the major ST of the ET-37 clonal complex. Pulsed-field gel electrophoresis (PFGE) profiles of the Ghanaian disease isolates and recent epidemic isolates from Burkina Faso were largely identical. We conducted meningococcal colonisation surveys in the home communities of three of the patients and in the Kassena Nankana District located at the border to Burkina Faso. W135 carriage rates ranged between 0 and 17.5%. When three consecutive surveys were conducted in the patient community with the highest carrier rate, persistence of W135 colonisation over a period of one year was observed. Differences in PFGE profiles of carrier isolates taken at different times in the same patient community were indicative of rapid microevolution of the W135 bacteria, emphasising the need for innovative fine typing methods to reveal the relationship between W135 isolates.

Introduction

Epidemic meningococcal disease has occurred in the meningitis belt of sub-Saharan Africa for approximately 100 years (Greenwood 1999). Historically the epidemics have been primarily caused by *Neisseria meningitidis* serogroup A. Serogroup W135 meningococci identified in 1968 (Evans *et al.* 1968) and first described in Africa in 1982 (Denis, Rey, Amadou, Saliou, Prince-David, M'boup, Cadox, Mar, & Etienne 1982) were initially considered to be a rare cause of invasive disease. However, two W135 meningitis outbreaks coinciding with pilgrimage seasons for Hajj in 2000 and 2001 (Taha, Achtman, Alonso, Greenwood, Ramsay, Fox, Gray, & Kaczmarski 2000;Lingappa *et al.* 2003) were followed by a first large scale epidemic in Burkina Faso in 2002 (Taha *et al.* 2002;Decosas & Koama 2002). Since then, each year Burkina Faso has been hit by mixed meningitis epidemics caused by W135 and A meningococci. In Saudi Arabia W135 meningococci

were responsible for 13% of all meningococcal disease between 1995 and 1999 and have been present to a notable degree at least since 1990 (Lingappa, Al Rabeah, Hajjeh, Mustafa, Fatani, Al Bassam, Badukhan, Turkistani, Al Hamdan, Al Jeffri, Al Mazrou, Perkins, Popovic, Mayer, & Rosenstein 2003). From 2002 onwards vaccination with the quadrivalent meningococcal polysaccharide vaccine (A/C/Y/W135) therefore became a visa requirement to participate in the Hajj (Wilder-Smith *et al.* 2003a). Already before the outbreaks in 2000 the danger of W135 meningitis epidemics in Africa was recognized (Kwara, Adegbola, Corrah, Weber, Achtman, Morelli, Caugant, & Greenwood 1998).

The Hajj outbreaks probably led to the expansion of a particular W135 clone within the electrophoretic type-37 (ET-37) complex (Mayer, Reeves, Al Hamdan, Sacchi, Taha, Ajello, Schmink, Noble, Tondella, Whitney, Al Mazrou, Al Jefri, Mishkhis, Sabban, Caugant, Lingappa, Rosenstein, & Popovic 2002;Popovic *et al.* 2000). A high acquisition rate of W135 meningococci (15-17%) in pilgrims has been reported (Wilder-Smith *et al.* 2003b). Throughout the world these carriers have transmitted Hajj-related W135 bacteria after returning home (Aguilera, Perrocheau, Meffre, & Hahne 2002;Hahne *et al.* 2002a;Wilder-Smith, Barkham, Ravindran, Earnest, & Paton 2003b). Related W135 strains also belonging to the ET-37 complex have been circulating worldwide since at least 1970 (Mayer, Reeves, Al Hamdan, Sacchi, Taha, Ajello, Schmink, Noble, Tondella, Whitney, Al Mazrou, Al Jefri, Mishkhis, Sabban, Caugant, Lingappa, Rosenstein, & Popovic 2002) and currently both the Hajj-related epidemic strain and Hajj-unrelated local W135 strains seem to be responsible for sporadic W135 cases worldwide (Hahne, Handford, & Ramsay 2002a;Taha *et al.* 2004). Genetic drift of the Hajj-related strain (Hahne, Handford, & Ramsay 2002a) complicates the analysis of the relationship between W135 isolates by standard typing techniques, such as pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) considerably.

In spite of its border with Burkina Faso, no outbreak of W135 meningococcal meningitis has so far occurred in Ghana. Here, we describe properties of four W135 strains isolated between February 2003 and March 2004 from the cerebrospinal fluid (CSF) of Ghanaian meningitis patients and provide evidence for spreading and rapid microevolution of the causative W135 meningococci.

Materials and Methods

Disease isolates

In the respective hospitals, CSF samples were taken for diagnostic purpose, latex agglutination was performed and the causative agents were isolated by culture using standard microbiological techniques. Bacterial isolates of all four Ghanaian W135 cases were transferred for further analysis to the Navrongo Health Research Centre, where serological grouping was reconfirmed by PCR. Reference isolates of W135 meningococci were obtained from M. Achtman, Berlin (isolated in Mecca, 2000, strains Z9230 and Z9232), and D. Caugant, Oslo (isolated during the outbreaks in Burkina Faso of 2001 (BF01/01, BF24/01), 2002 (BF06/02, BF67/02) and 2003 (BF01/03)).

Carrier isolates

In three of the affected communities and two control communities throat swabs have been taken and analysed for colonisation with *N. meningitidis* and *N. lactamica*. Community K1 is a small village located in a rural setting directly on the main truck road between the south and the north of Ghana (Fig. 1). Nearly the whole population of the village participated in the study. In December 2003 a control community located 2 km away from K1 was included. Communities B1 and B2 are located in Bolgatanga, the Upper East Region's capital. Here throat swabs were taken from the affected and the closest neighbouring compounds (including the majority of the about 30 inhabitants per compound).

After obtaining informed consent, throat swabs were taken and directly plated onto Thayer Martin Agar. The plates were incubated at 37ºC within eight hours after sampling for 24-48 hours. Two colonies with neisserial morphology were sub-cultured from each plate. *N. meningitidis* and *N. lactamica* colonies were identified as previously described (Gagneux, Hodgson, Smith, Wirth, Ehrhard, Morelli, Genton, Binka, Achtman, & Pluschke 2002b) by standard bacteriological methods. Ethical clearance was obtained from the responsible institutional and national ethical approval committees.

Characterisation of bacterial isolates

Meningococcal isolates were serogrouped with serogroup-specific antisera (Difco). Results were reconfirmed by PCR (Taha 2000;Bennett *et al.* 2004;Orvelid *et al.* 1999). All W135 isolates were analysed by pulsed field gel electrophoresis (PFGE) after digestion

with *NheI* as previously described (Morelli *et al.* 1997). All disease isolates were tested for resistance to penicillin G, chloramphenicol, cefotaxime, and ciprofloxacin with E-test strips (Isenberg Henry D.(ed.) 1998) using the NCCLS breakpoints. Selected strains were analysed by multi-locus sequence typing (MLST). DNA extraction (Vela Coral, Fonseca, Castaneda, Di Fabio, Hollingshead, & Briles 2001), PCR (Maiden, Bygraves, Feil, Morelli, Russell, Urwin, Zhang, Zhou, Zurth, Caugant, Feavers, Achtman, & Spratt 1998) and sequencing of PCR products with an ABI Prism 310 Genetic Analysis System were performed according to standard protocols on the MLST homepage (http://pubmlst.org/neisseria/). Allelic profiles were analysed using applications available on the MLST homepage.

Results

Characterization of W135 disease isolates from Ghana

Between February 2003 and March 2004, four cases of W135 meningococcal meningitis were reported by the regional hospitals in Tamale, Bolgatanga and the Korle Bu Teaching hospital (Accra), respectively. Patients came from the centre, the south or the north of the country (Figure 4.1) and were between 3 and 17 years of age (Table 4.1). One patient died, and of the three survivors one had profound sequelae.

All four disease isolates were sensitive to penicillin G, chloramphenicol, ciprofloxacin and cefotaxime. PFGE profiles of all four Ghanaian disease isolates were compared with disease isolates from the Hajj outbreak in Mecca 2000 and from Burkina Faso between 2001 and 2003. The Burkinian strains isolated in 2001 and 2002 showed identical profiles (shown for strain BF67/02, Figure 4.2, lane 5, profile C), whereas the 2003 isolate appeared to be very closely related (Figure 4.2, lane 6, BF01/03, profile D). Profiles of the Ghanaian disease isolates were largely identical (Figure 4.2, lanes 7-10, profile D) and indistinguishable of that of the 2003 strain from Burkina Faso (Figure 4.2, lane 6). The reference disease isolates from the Hajj outbreak in 2000 had a distinct, but related PFGE profile (shown for strain N11421, Fig. 2, lane 4, profile B; both strains had an identical profile). All four Ghanaian disease isolates had the multi-locus sequence type (ST) 11, which is the major ST of the ET-37 clonal complex.

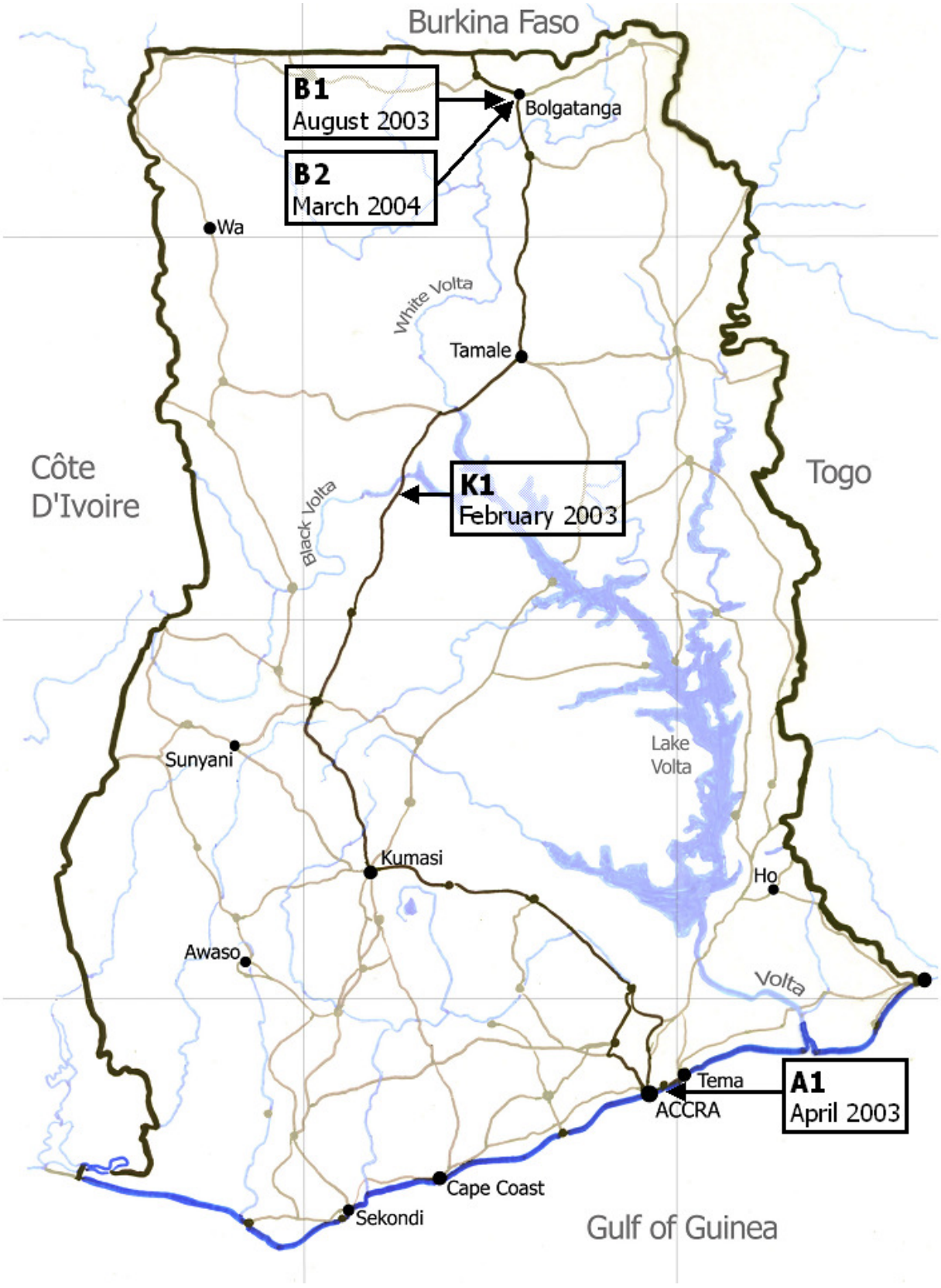


Figure 4.1 Map of Ghana showing the location of home communities of W135 meningitis patients. The sample time points were as follows: Community K1: I) April 2003, II) December 2003 (including control community), III) April 2004. Community B1: December 2003, Community B2: March 2004. The home community of patient A1 could not be identified and has not been sampled.

Table 4.1 W135 cases reported to the Ghanaian Disease Control Authorities in 2003 and 2004

Patient ID	Time of disease onset	Village/ City	Region	Age (yrs)	Sex	Outcome	Sequelae
K1	February 2003	Kpalkpalgbeni	Brong Ahafo	3	male	survived	*multiple
A1	April 2003	Accra	Greater Accra	4	male	died	unknown
B1	August 2003	Bolgatanga	Upper East	17	female	survived	none
B2	March 2004	Bolgatanga	Upper East	3	male	survived	+multiple

* Profound neurosensory hearing loss, speech impairment, transient ataxia hyperactive left knee and challis reflexes

+ Arthritis of the knee joints and occasional episodes of brief startling attacks during the first week after discharge but stopped thereafter

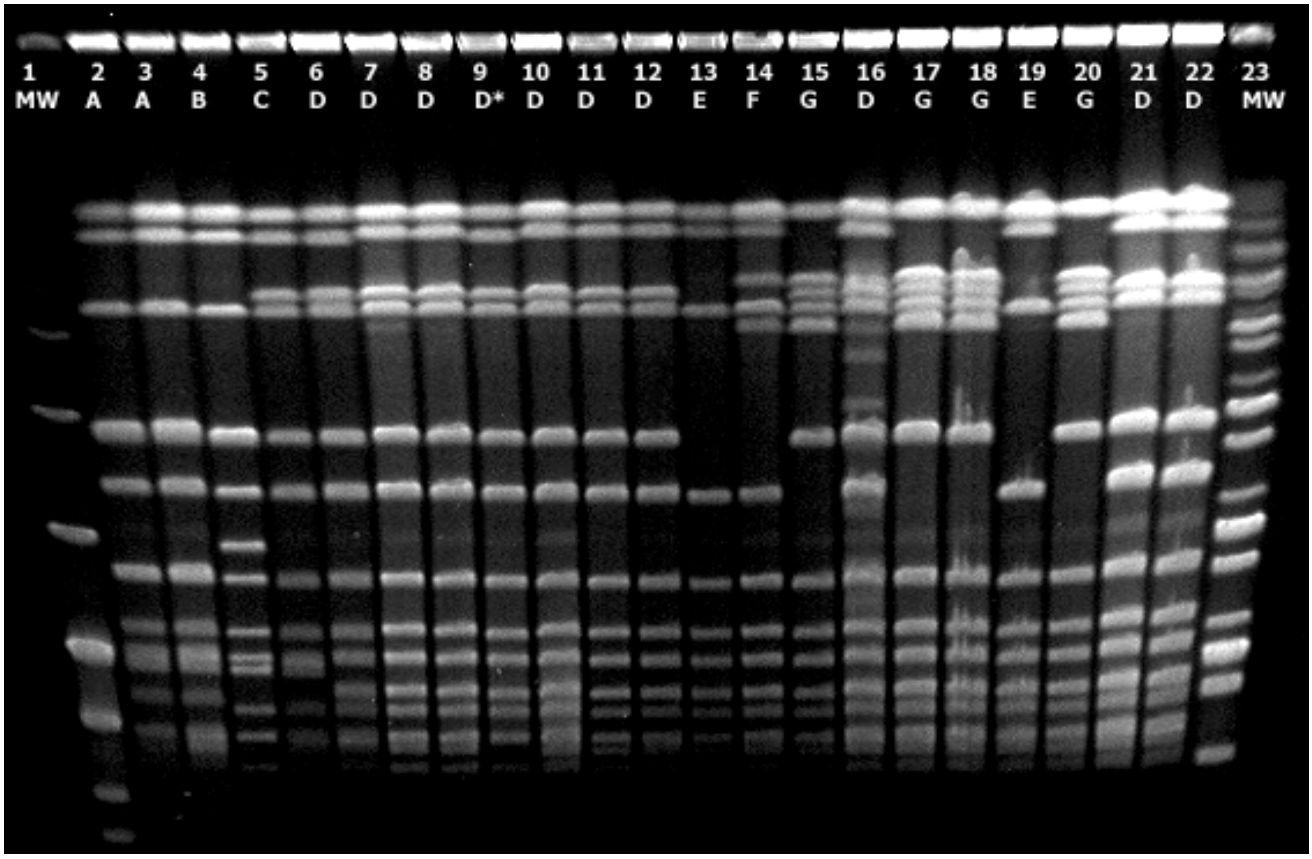


Figure 4.2 PFGE profile of W135 carrier and disease isolates (lane: strain No.; origin)

Indicated on the gel are the different band profiles of the W135 strains (A-G). 1: MW marker; 2, 3: N1621, N1622, KND 1998, carriage; 4: N1421 (Z9230), Mecca 2000, reference strains; 5: N1627 (BF67/02), Burkina Faso 2002, case; 6: N1628 (BF01/03), Burkina Faso 2003, case; 7: N1681 Ghana 2003, patient K1; 8: N1682, Ghana, 2003, patient A1 9: N1683, Ghana 2003, patient B1; 10: N1846, Ghana 2004, patient B2; 11, 12: N1485, N1487, community K1, April 03, carriage; 13, 14, 15: N1633, N1640, N1636, community K1, Dec 03, carriage; 16, 17: N1848, N1857, community B2, March 04, carriage; 18, 19, 20: N1951, N1953, N1959, community K1, April 03, carriage; 21, 22: N1888, N1903, KND, April 04, carriage; 23: MW marker

Table 4.2 Carriage of different serogroups of *N. meningitidis* and of *N. lactamica* in home communities of three W135 meningococcal meningitis patient and in a neighbouring control community.

Time of survey	Community (patient ID)	Volunteers swabbed (n)	Colonisation rate % (n)						
			N. lactamica	*N. meningitidis*	W135	A	X	Y	NG*
April '03	home (K1)	103	7.8 (8)	24.3 (25)	17.5 (18)	2 (2)	0	1 (1)	3 (3)
Dec. '03	home (K1)	100	3 (3)	15 (15)	13 (13)	0	0	1 (1)	1(1)
April '04	home (K1)	96	5.2 (5)	8.3 (8)	3.1 (3)	1 (1)	0	1 (1)	3 (3)
Dec. '03	control (K1)	100	1 (1)	9 (9)	0	2 (2)	2 (2)	2 (2)	3 (3)
Dec. '03	home (B1)	110	8.2 (9)	3.6 (4)	0	0	0	1 (1)	3 (3)
April '04	home (B2)	100	4 (4)	7 (7)	2 (2)	0	2 (2)	0	2 (2)

* Non groupable (NG) strains were negative both in serological tests and in serogroup A and W135 specific PCR analyses

Table 4.3 Age distribution of colonisation with *N. lactamica* and W135 and non-W135 *N. meningitidis* in the patient home community K1 (cumulated data from all three surveys).

Age group (yrs)	Frequency of colonisation						
	<1	**1-4**	**5-9**	**10-14**	**15-19**	**20-39**	**>40**
N. meningitidis serogroup W135	1/6 *(16.7%)*	5/78 *(6.4%)*	7/44 *(15.9%)*	7/37 *(18.9%)*	6/24 *(25.0%)*	8/93 *(8.1%)*	0/17 *(0%)*
non-W135 N. meningitidis	0/6 *(0%)*	1/78 *(1.3%)*	0/44 *(0%)*	4/37 *(10.8%)*	3/24 *(12.5%)*	5/93 *(5.3%)*	1/17 *(5.9%)*
N. lactamica	2/6 *(33.3%)*	9/78 *(11.5%)*	1/44 *(2.3%)*	1/37 *(2.7%)*	0/24 *(0%)*	0/93 *(0%)*	1/17 *(5.9%)*

W135 colonisation in patient communities and clonal diversity of bacteria

Three consecutive *N. meningitidis* colonisation surveys were performed in community K1, the first six weeks after the emergence of the case, in February 2003. In April 2003, 17.5% of 103 inhabitants (about 90% of the total population of the village) were colonized with W135 meningococci. Thereafter, the W135 colonisation rate declined to 13% in December 2003 and to 3 % in April 2004. In addition, a few carriers of other meningococci were found. *N. lactamica* colonisation rates were between 3 and 8%. *N. meningitidis* A, X and Y, but no W135 carriers were found in a neighbouring control community included in the December 2003 survey (Table 4.2).

Cummulated data from all three surveys conducted in community K1 were used to analyse the age distribution of colonisation with W135 meningococci in comparison to other serogroups found and to *N. lactamica* (Table 4.3). Logistic regression, including random effects to allow for repeated assessment of the same individuals, indicated that the ratio of carriage prevalence of *N. meningitidis* to that of *N. lactamica* increased with age (Chi-square=7.6, 1 degree of freedom, p=0.006), however there was no significant age trend in the ratio of W135 to other *N. meningitidis* (Chi-square 0.8, 1 d.f. p=0.4).

All isolates from the 18 W135 carriers in community K1 in April 2003, revealed identical PFGE profiles (shown for strains N1485 and N1487, Figure 4.2., lanes 11 and 12, profile D), indistinguishable from those of the Ghanaian disease isolates (Figure 4.2 lanes 7-10). However, some genetic diversification became apparent in the December 2003 colonisation survey. While the isolates of nine (of thirteen) W135- carriers revealed the original band profile (data not shown), the isolates from the other four exhibited three new variant profiles (Figure 4.2, lanes 13-15, profile E, F, G). Two of the three variant PFGE profiles, but not the original profile, were found again in the last colonisation survey in April 04 (Figure 2, lanes 18-20, profile E and G).

In community B1 and B2 only one colonisation survey was performed, three months and three weeks, respectively, after the emergence of the case. While no W135 meningococci were found in community B1 in community B2 W135 isolates of two carriers were obtained with the same PFGE profile as the Ghanaian disease isolates (Figure 4.2, lane 16, profile D, Table 4.2). In addition, from one of them a variant strain was isolated with a PFGE profile (Figure 4.2, lane 17, profile F) identical to a variant profile found in colonisation isolates from community K1.

W135 colonisation in a long-term colonisation survey in northern Ghana

Within the framework of a longitudinal *N. meningitidis* colonisation and disease study in the Kassena Nankana District (KND) of northern Ghana (Gagneux, Hodgson, Ehrhard, Morelli, Genton, Smith, Tanner, Binka, Achtman, & Pluschke 2000;Gagneux, Hodgson, Smith, Wirth, Ehrhard, Morelli, Genton, Binka, Achtman, & Pluschke 2002b), no W135 meningococcal meningitis case was recorded between 1998 and 2004. During these seven years of twice yearly colonisation surveys only single carriers of W135 meningococci have been identified in 1998 (1/300 in April and 1/299 in November 1998) (Gagneux, Hodgson, Ehrhard, Morelli, Genton, Smith, Tanner, Binka, Achtman, & Pluschke 2000). However, in April 2004 a W135 colonisation rate of 0.9% (3/350) was found.with isolates showing the same PFGE profile (Figure 4.2, lanes 21 and 22, profile D) as the Ghanaian disease isolates (Figure 4.2, lanes 7-10). Profiles of the two 1998 carrier isolates (Figure 4.2, lanes 2 and 3, profile A), were identical among each other but distinct from all other profiles observed in this study.

Discussion

In spite of the consecutive W135 epidemics in Burkina Faso in 2002 - 2004, no major outbreak of W135 disease has been observed so far in Ghana, demonstrating that bottlenecks exist for the spreading of epidemic strains within the meningitis belt, as already described for serogroup A meningococci (Achtman 1995a). The four isolated Ghanaian cases described in this paper have probably only been reported because of intensive national surveillance and awareness. W135 strains belonging to the ET-37 complex have been present in Ghana before the Mecca outbreak (Gagneux, Hodgson, Ehrhard, Morelli, Genton, Smith, Tanner, Binka, Achtman, & Pluschke 2000) and sporadic W135 cases may have easily remained undetected before the year 2000.

PFGE analysis demonstrate that the four Ghanaian W135 meningitis isolates were closely related to recent disease isolates from Burkina Faso, indicating, that these meningitis cases were caused by epidemic-related strains and not by local strains of the ET-37 complex. At least in the north of Ghana colonisation with the Burkina Faso epidemic-related strain is detectable. While visitors from Burkina Faso are frequently met in the border communities B1 and B2, it is not possible to guess the origin of the disease causing W135 strain of patient A1 living close to Accra. In the case of community K1, located in the middle of Ghana, contact to nomads may have been the source of the W135

bacteria, as a part of a neighbouring community frequently moves to Burkina Faso and back.

W135 carriage rates of healthy contacts in the three home communities of W135 meningitis patients, were very different. W135 carriers were found in the home communities (K1 and B2) of the index cases aged 3 years but not in B1, the home community of the 17 year old patient. Age of the patients may play a role, as suggested by findings of a study carried out during an serogroup C outbreak in Brazil, where contact carriage rates were highest in households, where the index case was an infant (Cartwright 1995). Carriage rates of outbreak strains tend to be higher in closed or partially-closed communities than in an open communities (Cartwright 1995). The rural community K1 has the features of a semi-closed community, where inhabitants lived very closely together and shared all living activities, while the urban communities B1 and B2 were much more open and loose. This may explain, why the highest (18%) carriage rate was observed in community K1. The age distribution of W135 colonisation, was not unusual, as the pattern observed in community K1 was characteristic for meningococci in general (Cartwright 1995).

Changes of the PFGE profile of colonisation isolates with time demonstrate that microevolution of W135 may be rapid. N. meningitidis is a naturally transformable species and there is evidence that microevolution is driven more frequently by recombination than by mutation. The observed genetic drift can make it very difficult to distinguish between epidemic-related and local W135 strains belonging to the same ET-37 complex and to prove epidemic spread of a particular clone. While available techniques are suitable to analyse the global population structure of other meningococcal serogroups (Lingappa, Al Rabeah, Hajjeh, Mustafa, Fatani, Al Bassam, Badukhan, Turkistani, Al Hamdan, Al Jeffri, Al Mazrou, Perkins, Popovic, Mayer, & Rosenstein 2003), new approaches are required for studying the molecular epidemiology of *N. meningitidis* W135.

An affordable vaccine against W135 meningococci (e.g., a trivalent groups A, C, and W135 polysaccharide vaccine) is now available and has been successfully used to contain outbreaks of W135 meningitis in Burkina Faso (Ahmad 2004). As Burkina Faso epidemic-related W135 meningococci now seem to spread into Ghana, intense surveillance efforts at national and regional levels for timely detection of a potential W135 epidemic is an important issue in future years.

Acknowledgements

This study was supported by grant GAT.0779-01476-GRT of the Meningitis Vaccine Project. We would like to acknowledge the National Disease control unit of the Ghana Health Service and the National Public Health Reference Laboratory for releasing the CSF samples of the index case and of the case from Accra. Furthermore we are grateful to the laboratory personnel of the Bolgatanga Regional hospital for making available the other two CSF samples. We thank the CSM team of the NHRC for assistance during the study and the communities, health authorities (Upper East Regional Health Directorate, Bolgatanga Municipal Health Directorate, Kintampo District Health Directorate, Kintampo Health Research Centre, Navrongo Health Research Centre) as well as all study participants for the cooperation accorded us. Finally we would like to acknowledge D. Caugant and M. Achtman for helpful comments on the manuscript and the supply with W135 Reference isolates.

Chapter 5

Clonal Waves of Colonisation and Disease of *Neisseria meningitidis* in the African Meningitis Belt

An Eight Years Longitudinal Study in Northern Ghana

[1] Julia Leimkugel, [2] Abraham Hodgson, [2] Abudulai Adams Forgor, [1] Valentin Pflüger, [1] Jean-Pierre Dangy, [1] Tom Smith, [3] Mark Achtman, [1*] Sébastien Gagneux and [1] Gerd Pluschke

[1] Swiss Tropical Institute, Basel, Switzerland

[2] Navrongo Health Research Centre, Ministry of Health, Navrongo, Ghana

[3] Max Planck Institute for Infection Biology, Berlin, Germany

*present address: The Institute for Systems Biology, Seattle, USA

This article has been submitted to
Public Library of Science, Medicine

Abstract

Background The Kassena-Nankana District (KND) of northern Ghana lies in the African meningitis belt where epidemics of meningococcal meningitis have been re-occurring every 8-12 years for the last 100 years. The dynamics of meningococcal colonisation and disease are incompletely understood.

Methodology/Principle Findings Between February 1998 and November 2005, pharyngeal carriage of *N. meningitidis* in the KND was studied by twice yearly colonisation surveys. Meningococcal disease was monitored throughout the 8-year study period, and patient isolates were compared to the colonisation isolates. The overall meningococcal colonisation rate of the study population was 6.0%. All culture-confirmed patient isolates and the majority of carriage isolates were associated with three sequential waves of colonisation with encapsulated (A ST5, X ST751, A ST7) meningococci. Compared to industrialised countries, the colonising meningococcal population was less constant in genotype composition over time, genetically less diverse during the peaks of the colonization waves, and a smaller proportion of the isolates was non-serogroupable. We observed a broad age range in the healthy carriers, resembling that of meningitis patients during large disease epidemics.

Conclusions The observed lack of a temporally stable and genetically diverse resident pharyngeal flora of meningococci might contribute to the susceptibility to meningococcal disease epidemics in the African meningitis belt. Because capsular conjugate vaccines are known to impact meningococcal carriage, effects on herd immunity and potential serogroup replacement should be monitored following the introduction of such vaccines.

Introduction

The highest burden of meningococcal meningitis occurs in the 'meningitis belt' of sub-Saharan Africa; a region stretching from Senegal to Ethiopia with an estimated population of 300 million (Greenwood 1999;Lapeyssonnie 1963). Within individual areas of the meningitis belt, major disease epidemics occur in irregular cycles every 8–12 years, with attack rates ranging from 100 to 1000 per 100,000 population (WHO 1998). Epidemics start in the early dry season, stop abruptly at the onset of the rains, but may break out again in the following dry season. Low humidity and high temperatures may favour the occurrence of meningococcal disease by damaging mucosal surfaces and the immune defence. In any one country, epidemics only last for two to three years (Greenwood 1999). The periodicity of these epidemics is not well understood, nor is it possible to predict them accurately. The current approach for control of meningococcal disease epidemics is based on early detection of the disease by the epidemic threshold of 10-15 cases per 100,000 inhabitants per week (WHO 2000) followed by mass immunisations with polysaccharide vaccines (WHO 1998). However, in settings with limited resources, effective surveillance and timely interventions are difficult to implement. Therefore vaccination campaigns are often delayed (Greenwood 1999).

N. meningitidis can be classified into thirteen serogroups based on the chemical composition of its polysaccharide capsule (Yazdankhah & Caugant 2004). Serogroup A accounts for most epidemics in the African meningitis belt, but C and W135 epidemics have also been reported (Greenwood 1999;WHO 2005). Meningococci that cause epidemics are genetically closely related; specific genotypes plus their epidemiologically associated genetic descendants constitute genoclouds (Zhu *et al.* 2001). The two most recent meningococcal disease pandemics originated in Asia and were caused by serogroup A meningococci belonging to two related genoclouds (Zhu *et al.* 2001). These two genoclouds have been assigned the sequence types 5 (ST5) and ST7, respectively, based on Multi-Locus Sequence Typing (MLST) (Zhu *et al*. 2001;Maiden *et al.* 1998). Serogroup W135 meningococci used to be a rare cause of invasive disease. However, two recent W135 meningitis outbreaks in Mecca were followed by major epidemics in Burkina Faso (WHO 2005;Taha et al. 2000).

N. meningitidis is a commensal of the human nasopharyngeal mucosa. It is transmitted by aerosol droplets or through contact with respiratory secretions. Because meningococcal transmission is independent of disease, characterisation of the carrier state is crucial for

understanding the epidemiology of meningococcal disease. Multiple colonisation studies have been performed in industrialized countries, but little is known about the meningococcal colonisation dynamics in Africa. Here, we report the findings of the (to our knowlede) first long-term colonisation study carried out in the African meningitis belt.

Materials and Methods

Study area

The study was conducted in the Kassena-Nankana District (KND) of the Upper-East Region of Ghana. It lies within the guinea Savannah woodland and has two major seasons; a short wet season from June to October and a long dry season for the rest of the year. The district-population is about 140,000, most of them rural, except for the 20,000 inhabitants of Navrongo town. In the KND, people live in compounds with an average of 10 inhabitants (http://www.indepth-network.net/dss_site_profiles/navrongo.pdf). Between 1997 and 2002, yearly vaccination campaigns with meningococcal serogroup A/C polysaccharide vaccine targeted the whole district population. Between 2003 and 2005, smaller campaigns were carried out. In 2003, 80% of the study participants reported to have been vaccinated within the previous three years. Ethical clearance for this study was obtained from the responsible institutional review boards.

Ethical Approval

Ethical clearance for this study was obtained from the relevant institutional review boards and the Ghana Ministry of Health. Informed consent was obtained from all study participants.

Colonisation isolates

Thirty-seven residential compounds were randomly selected from a complete listing of the district population using the Navrongo Demographic Surveillance System (NDSS) (Binka F 1999). The sample size was chosen to include a total population of about 300 individuals per survey, which means a carriage rate of 5% at any one survey (corresponding to a relatively frequent infection) can be distinguished at a significance level of 5% from those of infrequent infections carried by only 2.5% of the population.

Throat swabs were taken twice per year from all inhabitants of the 37 compounds present at the time of the visit who agreed to participate. A total of 16 surveys have been performed since March 1998, in each of them between 292 and 350 study participants have been swabbed (Table 5.1). The number of participants swabbed in single compounds ranged from 0 to 30 (mean 8.57, median 6), fluctuations occurred due to travelling activities. However, only three individuals refused to participate in the study, as a result one of the compounds was discontinued after the 1st visit. Another compound was replaced in April 2002 after being deserted by its inhabitants. The age distribution of the study participants was comparable with the overall age distribution in the KND (Table 5.2).

At the compound visit, the throat swab was taken and directly inoculated on Thayer-Martin agar plates (Gagneux et al. 2000). Two colonies with neisserial morphology were sub-cultured from each positive plate. N. meningitidis and N. lactamica colonies were identified by standard bacteriological methods as previously described (Gagneux et al. 2000).

Disease isolates

Suspected meningitis patients presenting at the War Memorial Hospital (WMH), Navrongo, or one of the four Health Centres of the KND were recruited throughout the study period. A suspected meningitis patient was defined by sudden onset of fever and stiff neck, or fever and stiff neck and altered mental status, in accordance with WHO-guidelines (WHO 1998). A lumbar puncture was performed before treatment from all suspected meningitis patients, and the cerebrospinal fluid specimen was analyzed in the laboratory of the WMH as described previously (Gagneux et al. 2000). In 1998–1999, only samples collected during the dry season were analyzed. Thereafter, samples collected from the few patients with suspected meningitis who presented during the wet season were also included.

Characterisation of bacterial isolates

Meningococci were serogrouped with serogroup-specific antisera (Difco) according to the manufacturer's instruction. In a subset of isolates, serological typing was confirmed by PCR (Taha 2000;Bennett, Mulhall, & Cafferkey 2004). All isolates were analysed by pulsed-field gel electrophoresis (PFGE) after digestion of genomic DNA with NheI (Morelli *et al.* 1997). MLST was performed as described (Maiden *et al*. 1998).

Statistical Methods

As a measure of the invasiveness of the different genoclouds we computed the ratio of cases to the approximate number of carriers in the district (Table 5.3). To estimate the latter we assumed that each survey was representative of the six month period in which it was carried out, and that average duration of carriage is six months, corresponding approximately to the half life of Neisseria meningitidis carriage determined in a study in University students in the United Kingdom (Ala'Aldeen *et al.* 2000). The diversity (D) (Table 5.1) of the meningococcal carriage population was determined with respect to the serogroup distribution for each of the 16 surveys and for the overall study period using the Simpson Index (Simpson E.H 1949;Grundmann *et al.* 2001).

The unadjusted proportion of samples carrying each bacterium provides a consistent and unbiased estimate of carriage prevalence, however because of the random sampling of the population the clustering in the sampling procedure introduced extra-binomial variation, which was allowed for in the estimates of standard errors (Table 5.1) by calculating robust (sandwich) estimates (Williams 2000) using Stata version 8.0 (Stata Corporation, TX, USA).

Risk ratios and confidence intervals were calculated using EpiInfo v3.3.2 but these analyses treated the different samples as statistically independent and consequently some of the tests carried out are anticonservative because of the effects of repeated sampling of the same individuals. We draw attention to the implications of this where it applies.

Results

Clonal waves of meningococcal colonisation and disease

We monitored the dynamics of pharyngeal carriage of *N. meningitidis* and bacterial meningitis in the KND of northern Ghana from February 1998 to November 2005; three major waves of clonal colonisation and disease with encapsulated meningococci were observed. A meningitis epidemic occured in the dry season of 1996/97 with 1396 suspected meningitis cases in the KND, but because of lack of infrastructure no laboratory analysis was performed. (Hodgson A. *et al.* 2002) This epidemic was followed by a smaller outbreak with 50 laboratory-confirmed serogroup A meningitis cases in the following dry season. A total of 36 isolates were culture confirmed and identified as subgroup III, ST5 bacteria (Gagneux et al. 2000), that spread throughout the meningitis belt after an epidemic in Mecca in 1987 (Nicolas *et al.* 2001). Carriage of the serogroup A ST5 meningococci decreased steadily from 2.7% (8/301) in April 1998 to 0.3% (1/308) in November 1999 (Figure 5.1a). Thereafter, none of the clinical or colonisation isolates from the KND belonged to the serogroup A ST5 genocloud. In 2000, no serogroup A meningococci were isolated from either patients or carriers. However, in 2001, a new wave of serogroup A meningococcal colonisation and disease started. All serogroup A carrier and disease strains isolated since then belonged to a new genocloud of serogroup A meningococci associated with ST7 that was observed for the first time in Africa in 1995 (*Zhu et al*. 2001). Although colonisation was still low in April 2001 (i.e. was <0.3%), seven serogroup A ST7 meningitis cases were identified between February and March 2001. In the following three years, serogroup A ST7 colonisation rates of 1.2% to 4.3% were observed. In spite of yearly serogroup A/C polysaccharide mass-immunisations, this low level of colonisation was associated with repeated serogroup A ST7 meningitis outbreaks in the KND (Figure 5.1a). Laboratory-confirmed cases numbered 70 between January and May 2002, 56 were identified between January and May 2003, and 114 were identified between December 2003 and April 2004. Thereafter, the serogroup A ST7 colonisation rate dropped below 1% and only two serogroup A ST7 meningitis cases were recorded in February 2005.

Between the two waves of serogroup A colonisation and disease, we documented a wave of colonisation with a serogroup X ST751 genocloud (Figure 5.1b) (Gagneux *et al.* 2002a;Gagneux *et al.* 2002b). The extensive spread of this low-virulent serogroup was associated with a total of 15 meningitis cases between 1998 and 2003. Serogroup X

carriage and disease peaked in the dry seasons of 1999/2000 and 2000/01 with colonisation rates of 17.3 and 15.1%, respectively. While in the peak of the serogroup X wave the ratio of serogroup X cases to carriers was found to be between 0.1 and 0.3:1,000, the observed ratio of serogroup A cases versus carriers during the A ST7 outbreak was between 16.8 and 42.3:1,000 in the respective dry seasons (Table 5.3).

Since November 2003, 23 non(sero)groupable (NG) ST192 carriage isolates with closely related PFGE-patterns were collected (Figure 5.1b). With 3.8% (12/313) their colonisation rate peaked in November 2004. NG ST192 strains isolates have been previously reported from The Gambia and Niger. (http://pubmlst.org/perl/mlstdbnet/mlstdbnet.pl?page=st-query&file=pub-nm_isolates.xml).

Investigation of Bacterial isolates

Throughout the study period, 1145 suspected meningitis patients were recruited and their CSF samples were analyzed in the laboratory. Overall, 311 meningococcal meningitis cases were confirmed by latex agglutination. Furthermore, a pneumococcal meningitis outbreak with dominance of serotype 1 occurred between 2000 and 2003 (Chapter 3), while overall only 17 *H. influenzae* meningitis cases were identified. We obtained meningococcal isolates in 197/311 (63%) of the confirmed cases. Latex aggluntination confirmed the serogroup A capsule for all 114 cerebrospinal fluid samples that were negative in culture.

All recovered *N. meningitidis* isolates belonged to the three dominating genoclouds of encapsulated meningococci (36 serogroup A ST5, 148 serogroup A ST7 and 15 serogroup X strains). With respect to colonization, 289 (5.8%) of the pharyngeal samples contained *N. lactamica* and 302 (6.0%) *N. meningitidis.* Point prevalence of the different serogroups at the 16 individual surveys is given in Table 5.1. All serogroup A (n=55) and serogroup X (n=161) carriage isolates belonged to the three genoclouds causing the major sequential colonisation waves. In addition, 16 NG isolates shared ST and PFGE-patterns with the serogroup A ST5 (2 isolates), serogroup A ST7 (2 isolates) or serogroup X (12 isolates) isolates, respectively (Figure 5.1a). These colonisation isolates thus represented unencapsulated variants of the respective genoclouds. There was no evidence for an accumulation of the non-encapsulated variants towards the end of the colonisation waves

(Figure 5.1). In some cases, encapsulated and NG variants of the same genocloud were found simultaneously in the same compound.

Bacterial carriage showed only moderate clustering by compound. Of the 39 compounds sampled at least once, 31 (79%) provided at least one *N. lactamica* and 36 (92%) at least one *N. meningitidis* positive sample. Of the 583 individual compound visits, 158 (27%) yielded *N. lactamica* and 155 (27%) *N. meningitidis*. To compare these numbers with those expected if the bacteria were randomly distributed between compounds, we selected 289 of the samples in the database at random and found that 208 different visits including 38 compounds were represented, while 302 samples chosen at random corresponded to 213 visits to 37 of the compounds.

The diversity index D (Table 5.1) varied strongly between the single-sampling time points. Highly homogenous carriage populations were found in the course of colonisation waves with peak values of D=0.855 in April 2001 for the X wave, and D=0.583 in April 2003 for the A ST7 wave. In contrast, carriage isolates were much more divers in the transition phase between two clonal waves with the lowest values of D=0.194 in November 1998 during the transition from A ST5 to X and D=0.278 in April 2002 during the transition from X to A ST7. Overall, the serogroup diversity in the KND (D=0.349, 95% CI 0.306-9.393) was slightly lower than in a carriage study in Europe (D=0.282, 95% CI 0.251-0.313) (Yazdankhah et al. 2004).

Low background of meningococci unrelated to the clonal waves

Only from 16.6% (50/302) of the meningococcal carriers, colonisation isolates were unrelated to the dominating serogroup A, X and NG ST192 genoclouds (Figure 5.1c). Although neighbouring Burkina Faso was hit by repeated W135 ST11 epidemics in the dry seasons of 2002-2004, in the KND, carriers of the epidemic strain were only found in April 2004 (3/350; 0.9%) and November 2004 (2/313; 0.6%), and not a single W135 meningitis case was recorded between 1998 and 2005 (Chapter 4). Single carriers of W135 ST11 meningococci were also identified in April (1/300) and in November 1998 (1/299) (Gagneux et al. 2002b), two years prior to a first documented W135 meningitis outbreak in Mecca (Taha et al. 2000). While serogroup Y meningococci (21 isolates) and serogroup Y ST168 related NG strains (7 isolates) were isolated in 10 out of the 16 individual surveys, carriage of serogroup B and serogroup 29E meningococci was rare (Table 5.1). Carriage of serogroup Y meningococci was strongly associated with one particular compound,

where during eight of the 16 surveys, 67% (14/21) of the serogroup Y strains were isolated. Altogether, only eight NG isolates had PFGE-patterns and STs unrelated to the dominating serogroup A, X, Y and NG ST192 genoclouds (Table 1). While the *N. lactamica* carriage rate remained relatively constant (4.7%– 9.3%) for six years, it declined after April 2004 to 0.3% in April 2005 (Figure 5.1d). We observed no significant correlation between the A/C meningococcal polysaccharide vaccine immunisation status and meningococcal carriage of all serogroups (RR=1.11; p=0.81), of serogroup A (RR=0.9; p=0.92), or of *N. lactamica* (in the >2year old RR=0.7, p=0.3). These P-values do not allow for the effect of clustering in the sampling. An analysis that allowed for clustering would give even higher P-values, confirming the absence of effect

Age distribution of carriers and patients

Colonization with meningococci in the KND exhibited a broad age range (Figure 5.2a). It peaked in teenagers and young adults (median age 17.9 years; range 5 months to 84 years). In contrast, the carriage rate of *N. lactamica* was highest in the <5 age group (Figure 5.2b). During the 1996/97 epidemic the age pattern of clinically diagnosed meningitis patients (median age 17.8 years; range 3 months- 80 years) resembled that of meningococcal carriers (Figure 5.2c), the incidence rates of males (n=628, IR=0.95%) and females (n=713, IR=0.98%) were comparable (RR=0.97, p=0.59). In contrast, during the post-epidemic A meningococcal disease outbreaks between 1998 and 2005, the incidence of meningitis was highest in children <10 years of age and decreased steadily with age (Figure 5.2c). The median age of A ST5 cases in 1998 and of A ST7 cases in 2001-2005 was comparable (8.0 years; range 4 months- 64 years versus 10.0 years; range 2 months- 75 years, respectively). However, between 2001 and 2005 the incidence rate of males (n=159, IR=0.049%) was significantly higher (RR=2.0, p<0.0001) than of females (n=89, IR=0.024%). The case fatality rate of A meningococcal meningitis was much higher during the A ST5 post-epidemic outbreak in 1998 (20%; 10/50) than during the epidemic in 1996/97 (4.7%; 65/1396) or during the A ST7 outbreaks in 2001-2005 (4.8%; 11/238).

Table 5.1 Carriage rates in % during 16 carriage surveys in the Kassena Nankana District.

	Survey	samples	*N. lactamica*	*N. meningitidis*	serogroup A	serogroup X	serogroup Y	serogroup W135	serogroup 29E	serogroup B	non groupable	PFGE pattern of NG strains	Simpson's Index of Diversity *
			% (s.e.)	% (s.e.)	% (s.e.)	% (s.e.)	% (s.e.)	% (s.e.)	% (s.e.)	% (s.e.)	% (s.e.)		D (CL)
1	Apr 98	300	9.3 (2.7)	4.7 (1.6)	2.7 (1.1)		1.3(1.3)	0.3 (0.3)			0.3 (0.3)	A	0.374 (0.173; 0.575)
2	Nov 98	299	8.7 (2.2)	3.0 (1.3)	1.0 (0.6)		0.7(0.7)	0.3 (0.3)			1.0 (0.5)	A, X, NT	0.194 (0.092; 0.296)
3	Apr 99	292	8.2 (1.8)	5.1 (1.5)	0.7 (0.5)	3.4 (1.4)	0.7(0.7)				0.3 (0.3)	NT	0.448 (0.181; 0.715)
4	Nov 99	308	9.7 (2.0)	4.2 (1.7)	0.3 (0.3)	1.9 (1.1)	0.6(0.4)				1.3 (0.7)	X, NT (3)	0.282 (0.134; 0.43)
5	Apr 00	298	8.4 (2.4)	19.8 (3.7)		17.4 (4.0)	1.3(0.9)				1.0 (0.7)	X, Y, NT	0.78 (0.642; 0.918)
6	Nov 00	301	6.0 (2.4)	13.6 (6.0)		11.0 (4.3)					2.7 (1.9)	X(7), Y	0.678 (0.527; 0.829)
7	Apr 01	310	8.4 (2.2)	17.1 (2.9)		15.8 (2.6)					1.3 (0.7)	X, Y(3)	0.855 (0.729; 0.981)
8	Nov 01	306	6.5 (2.2)	2.0 (0.8)		1.3 (0.6)			0.3 (0.3)		0.3 (0.3)	X	0.4 (0.015; 0.785)
9	Apr 02	339	4.7 (1.4)	2.7 (1.0)	1.2 (0.7)	0.6 (0.4)	0.9(0.7)						0.278 (0.162; 0.394)
10	Nov 02	319	5.6 (1.5)	2.8 (1.3)	1.9 (1.2)	0.6 (0.4)	0.3(0.3)						0.444 (0.139; 0.749)
11	Apr 03	312	5.4 (1.6)	2.9 (1.1)	2.2 (1.1)		0.3(0.3)		0.3(0.3)				0.583 (0.243; 0.953)
12	Nov 03	297	6.4 (2.1)	3.4 (1.2)	1.3 (0.8)	1.0 (0.6)	0.3(0.3)				0.7 (0.5)	192, X	0.222 (0.096; 0.349)
13	Apr 04	350	3.7 (1.3)	8.0 (2.2)	4.3 (1.5)		0.3(0.3)	0.9 (0.8)	0.3 (0.3)	0.3(0.3)	2.0 (0.7)	192(5), A(2)	0.341 (0.194; 0.488)
14	Nov 04	313	1.9 (0.8)	5.8 (1.9)	0.6 (0.5)			0.6 (0.6)			4.2 (1.9)	192(12), Y, NT	0.608 (0.347; 0.869)
15	Apr 05	321	0.3 (0.3)	3.1 (1.0)	0.9 (0.5)						2.2 (0.7)	192(5), NT(2)	0.533 (0.301; 0.765)
16	Nov 05	334	0.6 (0.4)	0.6 (0.4)							0.6 (0.4)	NT(2)	1.0 (0)

Table 5.1. cont'd

s.e. indicates the standard error of the % calculated with allowance for the cluster sampling. For NG strains the PFGE patterns are given, if more than one NG strain was isolated, the number of carriers are added in brackets;

A, X, Y is the typical pattern of the respective serogroups, NT= Non-typable, PFGE-pattern is not known. 192 is the NG ST192-pattern.

* D and its confidence limits were determined according to Grundmann et a.[17] on the basis of serogroup diversity.

Table 5.2 Age distribution of the overall study population during eight years colonization surveys in comparison with the overall population in the KND according to NDSS surveys between 1995-1999

	Study Population Swabbed				Total population of the KND+			
	Male	%*	Female	%	male	%	female	%
<5	319	6.43	340	6.85	9780	7.07	9721	7.03
5-10	322	6.49	501	10.10	10329	7.47	9932	7.18
10-15	375	7.56	344	6.93	10007	7.23	9077	6.56
15-20	266	5.36	294	5.93	7585	5.48	6520	4.71
20-30	253	5.10	346	6.97	7587	5.48	9325	6.74
30-40	164	3.31	328	6.61	5935	4.29	9114	6.59
40-50	246	4.96	211	4.25	4928	3.56	7443	5.38
>50	302	6.09	351	7.07	9092	6.57	11960	8.65
total	2247	45.28	2715	54.72	65243	47.16	73092	52.84

* % of the total population

+ Mean denominator of the population between 1995 and 1999

Table 5.3 Ratio of meningitis cases versus 1000 carriers for serogroup A and X at the different timepoints

Period	Serogroup A			Serogroup X		
	carrier (n)	cases(n)	ratio* (95% CI)	carrier (n)	cases (n)	ratio (95% CI)
Jan-Jun98	8	43	11.7(5.4, 28.7)		1	∞
Jul-Dec98	3					
Jan-Jun99	2			10	1	0.2(0, 1.5)
Jul-Dec99	1			6		
Jan-Jun00				52	7	0.3(0.1, 0.6)
Jul-Dec00				33		
Jan-Jun01				49	3	0.1(0, 0.4)
Jul-Dec01		7	∞	4		
Jan-Jun02	4			2	1	1.2(0, 23.5)
Jul-Dec02	6	69	42.3(15.8, 159.5)	2		
Jan-Jun03	7	1	0.4(0, 3.2)			
Jul-Dec03	4	52	16.8(7.6, 43.7)	3	1	0.7(0, 8.9)
Jan-Jun04	15	2	1.1(0.1, 7.5)			
Jul-Dec04	2	111	18.7(10.9, 34.6)			
Jan-Jun05	3	2	1.5(0.1, 13.5)			
Jul-Dec05						

* The total population of the KND was estimated at 138’335 (INDEPTH

next page: Figure 5.1

Waves of colonisation and disease in the KND from April 1998 until November 2005.

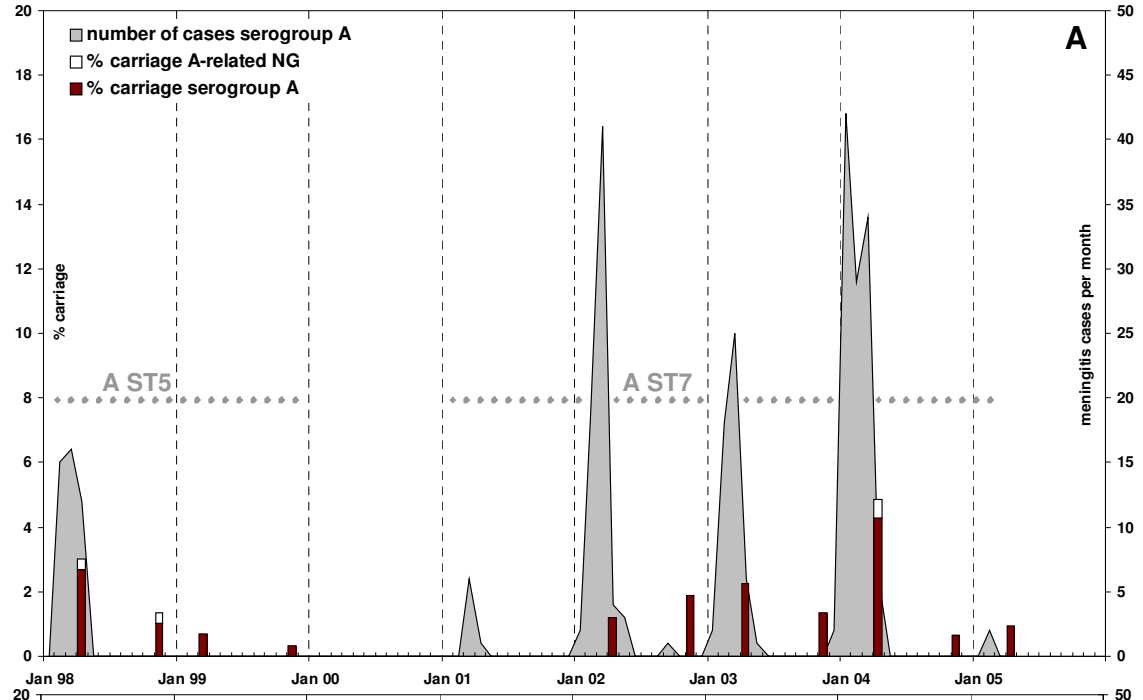
Carriage rates recorded during 16 colonisation surveys (April and November each year) and monthly numbers of confirmed meningitis cases of *N. meningitidis.*

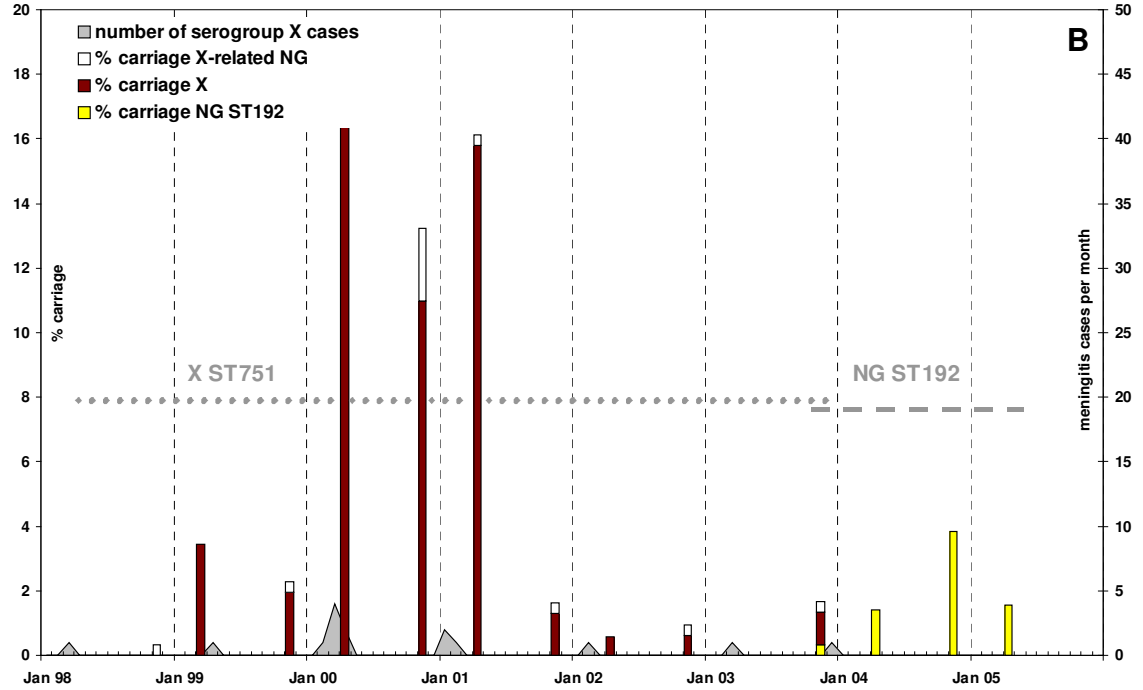
a) Genoclouds of serogroup A ST5 and ST7 meningococci

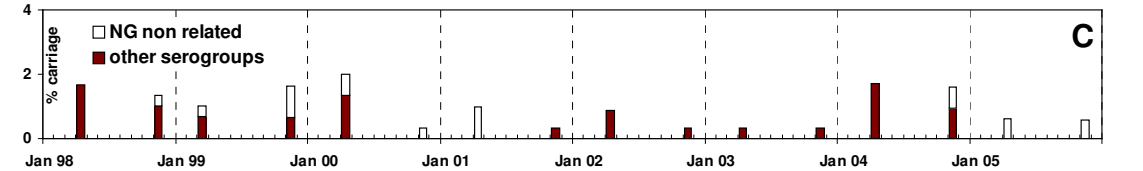
b) genoclouds of serogoup X ST851 and NG ST192 meningococci.

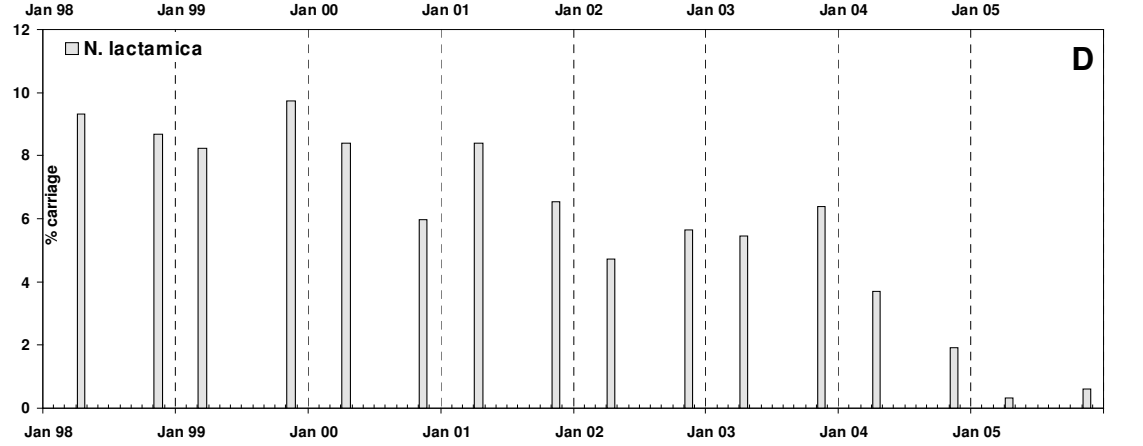
c) Carriage rates of other serogroups and meningococci non related to the A, X, or NG ST192 genoclouds.

d) Carriage rates of *N. lactamica*


number of cases serogroup A
% carriage A-related NG
% carriage serogroup A
A
% carriage
meningitis cases per month
A ST5
A ST7
Jan 98
Jan 99
Jan 00
Jan 01
Jan 02
Jan 03
Jan 04
Jan 05


number of serogroup X cases
% carriage X-related NG
% carriage X
% carriage NG ST192
B
% carriage
meningitis cases per month
X ST751
NG ST192
Jan 98
Jan 99
Jan 00
Jan 01
Jan 02
Jan 03
Jan 04
Jan 05


NG non related
other serogroups
C
% carriage
Jan 98
Jan 99
Jan 00
Jan 01
Jan 02
Jan 03
Jan 04
Jan 05


N. lactamica
D
% carriage
Jan 98
Jan 99
Jan 00
Jan 01
Jan 02
Jan 03
Jan 04
Jan 05

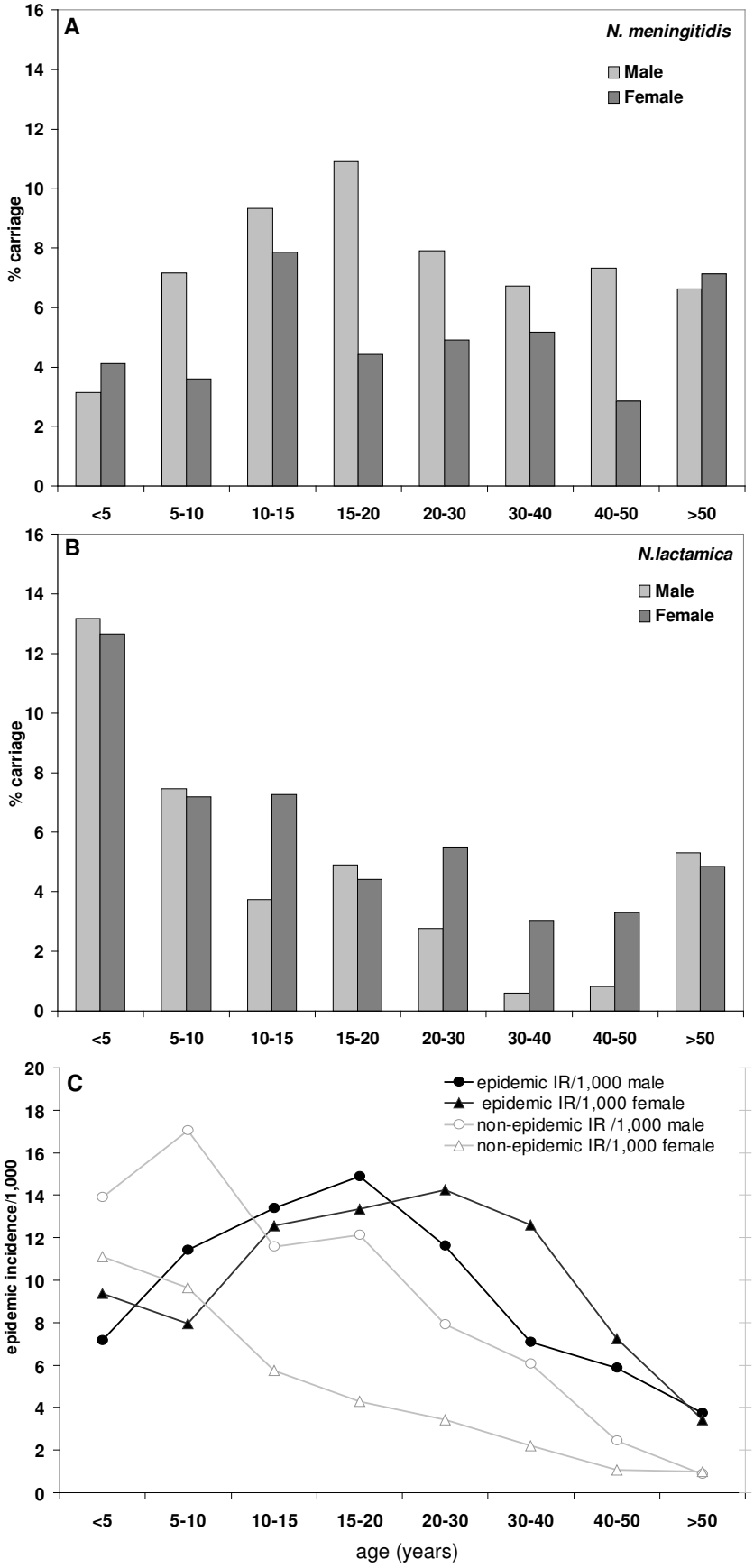


Figure 5.2 Age and Sex Pattern of Colonisation and Disease

A) Carriage of meningococci in the different age groups (all serogroups and NG, cumulation of all surveys) of the male (light grey bars) and female (dark grey bars) population.

B) Carriage of *N. lactamica* in the different age groups (cumulation of all surveys) of the male (light grey bars) and the female population (dakr grey bars).

C) Age spectrum of incidence rates of meningococcal meningitis in the male (circles) and female (triangle) population of the KND in the epidemic of 1996/97 (dark grey) versus the interepidemic period 2001 to 2005. Denominator is the district population 1995-99. On the primary Y-axis the epidemic incidence rates and on the secondary Y-axis the interepidemic incidence rates are indicated.

Discussion

Here, we present the results of the first longitudinal study (to our knowleded) on meningococcal colonisation and disease in the meningitis belt of sub-Saharan Africa. The study revealed features which are in many aspects remarkably different from findings of colonisation studies conducted in Europe and North America (Caugant et al. 1988;Maiden et al. 2004;Jolley et al. 2000;Yazdankhah & Caugant 2004;Claus et al. 2005). The population of meningococci carried in the KND was i) less constant in the genotype composition, ii) less genetically diverse during the peaks of the colonization waves , iii) it included fewer NG strains and iv) virulent encapsulated strains were dominant. Indeed, the A ST5, A ST7 and X ST751 meningococci responsible for all 197 culture-reconfirmed meningitis cases represented 71% (216/302) of the colonisation isolates.

These results allow us to describe the major developments in meningococcal carriage and disease in the KND over an eight year study period that included both meningococcal outbreaks and the interepidemic period. We recorded very good compliance of the study participants. However financial and organisational constraints did not allow sampling intervals shorter than 6 months, and this may have been too long for a robust analysis of acquisition rates and duration of carriage. The relatively small number of 300 study members also led to a rather low sensitivity, and thus to the late detection of the serogroup A ST7 colonisation in the district. Meningococcal colonisation with minor clones might also have been undetected.

In industrialised countries, approximately 10% of individuals from the general population are carrying meningococci at any one time (Cartwright et al. 1987). In children younger than four years, carriage rates are <3%. They increase to 20–40% in teenagers and young adults (Blackwell *et al.* 1990;Cartwright et al. 1987;Caugant et al. 1988;Caugant et al. 1994) and decrease again to <10% in older age-groups. In contrast, invasive meningococcal disease is most common in young children and in teenagers. Current endemic rates of meningococcal disease in most industrialized countries range from less than one to five cases per 100,000 population. In industrialised settings, meningococcal strains collected from patients and carriers differ genetically and serologically (Caugant et al. 1988). Typically, the carried populations of meningococci are highly diverse, with a low representation of the invasive serogroups A, B, C, Y and W135 (Maiden 2004;Jolley et al. 2000;Yazdankhah & Caugant 2004;Claus et al. 2005). The genetic composition of the strains carried is relatively constant over time, and up to 50% are serologically non-

groupable (Yazdankhah & Caugant 2004;Cartwright, Stuart, Jones, & Noah 1987). Encapsulation is thought to reduce adherence to pharyngeal epithelial cells, and loss of expression of capsular polysaccharide may be an adaptation to long-term carriage (Cartwright 1995). Colonisation with NG strains may be beneficial to the host by eliciting cross-reactive immune responses to non-capsular meningococcal surface antigens (Cartwright 1995).

The observed lack of a stable and genetically diverse resident pharyngeal flora of meningococci in the KND may explain why incoming new clones may spread so successfully in populations of the African Meningitis Belt. The instability in the resident flora evidently leads to clonal waves of colonisation typically lasting for about four years and, in the case of hypervirulent lineages, disease outbreaks or epidemics. We found that the case to carrier ratio was generally much higher for serogroup A than for serogroup X meningococci, reflecting the marked difference in virulence between these two serogroups. Only in the dry season of 2001 at the beginning of the A ST7 colonisation and disease wave did we find patient isolates that were unrepresented during the corresponding colonisation survey. The highest A ST7 colonisation rate (4.3% in April 2004) was associated with the largest meningococcal meningitis outbreak observed during the entire study period. These data give no strong indication for a change in the case to carrier ratio in the course of the serogroup A ST7 outbreak.

New contact of the population with genoclouds that have epidemic potential does not always lead to high colonisation rates. For example, we recovered isolates resembling those responsible for the 2002-2004 epidemics in Burkina Faso from a few carriers in KND in 2004, but we did not observe any wave of W135 colonisation. Importantly, fluctuations of the pharyngeal microflora of the population are not confined to the meningococci. For example, the *N. lactamica* colonisation rate also changed in the course of the study. In addition, an outbreak of pneumococcal meningitis occurred during the study period with features (seasonality, clonality and a broad age spectrum) characteristic of meningococcal epidemics (Chapter 3). Increasing herd immunity may be responsible for the disappearance of dominating genoclouds. However, changes in herd immunity do, not fully explain the complete disappearance of the A ST5 genocloud two years after the 1996/97 epidemic nor the emergence of the closely related A ST7 genocloud after only a short time interval.

The age distribution of healthy carriers in the KND with peak carriage rates in teenagers and young adults was similar to many European colonisation studies (Caugant et al. 1994;Cartwright, Stuart, Jones, & Noah 1987;Yazdankhah & Caugant 2004). The incidence of meningitis during the disease outbreaks in the years 1998-2005 was highest in children <10 years, comparable to endemic disease in industrialised countries. It is thought, that immune responses elicited by colonisation with meningococci and other antigenically cross-reactive microorganisms are responsible for the decreased disease susceptibility in the older age groups. This may imply that natural serum antibody-mediated immunity against invasive disease is developing much more efficiently than secretory IgA-mediated protection against colonisation.

However, during the epidemic in 1996/97, the age-distribution of meningitis patients resembled that of meningococcal colonisation, consistent with reports of most large meningococcal epidemics(Greenwood *et al.* 1979;Greenwood 1999;Lapeyssonnie 1963;Moore 1992). During the epidemic the disease susceptibility of the whole population was increased and an overall incidence rate of 970/100'000 was recorded. The fact that also in children <10 years the epidemic incidence of meningitis was exceeding endemic attack rates dramatically, argues against the '2 hit' hypothesis, associating susceptibility to disease with blocking serum IgA elicited by colonisation of the gut with cross-reactive microorganisms (Griffiss 1982).

The factors that initiate epidemics in the meningitis belt are incompletely understood. Contact of a population with a hyperinvasive new genocloud that is antigenically distinct enough to escape natural immunity may lead to an epidemic. Loss of natural immunity in exposed individuals over time and new birth cohorts may make a population increasingly susceptible. However, epidemics are not always associated with the appearance of a new clone (Greenwood 1999). This suggests a role of environmental triggers, such as periods of hot, dry, and dusty weather, co-pathogens or social factors. In spite of intense annual A/C polysaccharide vaccination campaigns carried out in the KND since 1998, outbreaks with IR of up to 80 per 100,000 occurred between 2002 and 2004. Even though, this incidence was less than a tenth of the disease burden during the 1996/97 epidemic in the KND, it was still several times higher than in industrialized countries (Tikhomirov, Santamaria, & Esteves 1997). It is not clear, whether herd immunity elicited by the serogroup A ST5 epidemic, lack of environmental triggers or the vaccination campaigns have prevented a large A ST7 epidemic.

Meningococcal vaccines protect individuals from disease by eliciting bactericidal serum antibodies (Borrow *et al.* 2001). Recent studies following the introduction of conjugate C vaccines in the United Kingdom have demonstrated that capsule conjugate vaccines also affect carriage and transmission by inducing mucosal immune responses (Snape & Pollard 2005;Maiden & Stuart 2002). Herd immunity may play a key role in the control of meningococcal infection using meningococcal conjugate vaccines (Ramsay *et al.* 2003). An affordable serogroup A conjugate vaccine may soon become available and introduced in the African meningitis belt (http://www.meningvax.org). Serogroup replacement and the emergence of escape variants (Maiden & Spratt 1999) are potential consequences of limited-spectrum vaccines, but these factors cannot be well understood without carriage studies. Our data suggest that successive waves of meningococci replace each other in the meningitis belt, some leaving little trace in disease surveillance statistics because of their low invasiveness. Once conjugate vaccines are introduced it will be critical to ensure that there is no replacement of vaccine serogroups with invasive alternatives. Carriage studies will play an important role in monitoring bacterial dynamics in order to anticipate any such problems and to prepare responses, such as mass vaccination with a supplementary carbohydrate vaccine.

Acknowledgements

The study was funded by the Stanley Thomas Johnson Foundation, the Meningitis Research Foundation and the Meningitis Vaccine Project. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing or the report. We are grateful for support and contributions of E. Arnold, F. Binka, S. Droz, I. Ehrhard, B. Genton and M. Tanner. In the Navrongo Health Research Center, we thankfully appreciate the assistance of A. Bugri, S. Abudulai and A. Wahab in the laboratory, all nurses and health workers in the War Memorial Hospital, Navrongo and the Health Centers of the KND, C. Tindana with all fieldworkers and drivers for excellent work in the field, and T. Tei and M. Bugase for logistic support. We acknowledge the use of the NDSS database and we thank all study participants for their trust and contribution.

This publication made use of the Neisseria Multi Locus Sequence Typing website (http://pubmlst.org/neisseria/) developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford. The development of this site has been funded by the Wellcome Trust and European Union.

Chapter 6

Genetic diversification of *Neisseria meningitidis* during waves of colonisation and disease in the meningitis belt of sub-Saharan Africa

[1] Julia Leimkugel, [2] Abudulai Forgor, [1] Jean-Pierre Dangy, [1] Valentin Pflüger, [1*] Sebastien Gagneux, [2] Abraham Hodgson, [1] Gerd Pluschke

[1] Swiss Tropical Institute, Basel, Switzerland

[2] Navrongo Health Research Centre, Ministry of Health, Navrongo, Ghana

*present address: Division of Infectious Diseases and Geographic Medicine, Stanford University Medical Centre, Stanford, USA

This article has been submitted to

Vaccine

Abstract

Although *Neisseria meningitidis* is a highly variable organism, most invasive disease is caused by a minority of genotypes. Hypervirulent lineages have been identified and their pandemic spread has been traced. During a longitudinal meningococcal colonisation study in a district of northern Ghana clonal waves of carriage and disease were observed. Genetic diversification of genoclouds was analysed by Pulsed Field Gel Electrophoretic (PFGE) analysis of isolates from healthy carriers and from meningitis patients. Even during the limited time of persistence in the district, microevolution of the dominating genoclouds took place. Population genomic analyses are required to understand the genetic basis for the emergence of new lineages with epidemic potential, which is of crucial importance for the development of long-term global vaccination strategies against meningococcal disease.

Introduction

Neisseria meningitidis is a common commensal of the upper respiratory tract of humans. Most invasive disease is caused by a minority of genotypes. Although genetic analyses have provided evidence for frequent recombination in *N. meningitidis*, it is possible to trace the pandemic spreading of certain hypervirulent genotypes (Morelli *et al*. 1997). However, constant microevolution leads to their genetic diversification. Dominating genotypes plus their clonally related and epidemiologically associated descendents have been designated genoclouds or clonal complexes. Such genoclouds persist only transiently and change their composition constantly through microevolution (Zhu et al. 2001).

Since disease is not necessary for meningococcal transmission, characterization of the carrier state is crucial for understanding the epidemiology of meningococcal disease. Our results from the first long term meningococcal colonisation and disease study in the African Meningitis Belt revealed features, which were profoundly different from those observed in industrialized countries. All isolates from meningitis patients and the majority of strains from healthy carriers were associated with three sequential waves of colonisation with encapsulated (A ST5, X ST751 and A ST7) meningococci. Compared to industrialised countries, the colonising meningococcal population was less constant in genotype compsition over time and genetically less diverse during the peaks of the colonisation waves. Only smaller proportions of the isolates belonged to other serogroups such as W135 and Y or were non-serogroupable. The sequential waves of colonisation with pathogenic and apathogenic meningococcal genoclouds typically lasted for four years (Chapter 5).

From this long term study, several groups of epidemiologically related isolates became available for genomic fingerprinting analyses: 1) sequence type 5 (ST5) isolates were collected from healthy carriers and meningitis patientes in 1998 and 1999. They had been introduced to Africa after an epidemic in Mecca during the Hajj 1987 and subsequently spread throughout the Meningits Belt.; 2) ST7 A meningococci emerged in the KND from April 2001 until April 2005. This genocloud has been recorded in Africa since 1995 (Nicolas et al. 2001); 3) in between the two serogroup A waves, serogroup X meningococci caused a fulminant wave of colonisation and sporadic meningitis cases. Outbreaks of serogroup X meningococcal meningitis have been reported increasingly from Niger since the early 1990 (Etienne et al. 1990;Djibo et al. 2003;Boisier et al. 2005); 4) serogroup Y meningococci were isolated sporadically from carriers throughout the entire study period. While in most settings, serogroup Y is only infrequently attributed to invasive

disease, in the US recently a third of all meningococcal meningitis cases were caused by serogroup (Bilukha & Rosenstein 2005); 5) serogroup A135 isolates becoming available for genetic fingerprinting analysis included colonization strains isolated in the years 1998 and 2004 from the KND, disease isolates from CSF of patientes from Burkina Faso and from other areas of Ghana, and colonisation isolates from residential compounds of W135 index cases from different regions of Ghana (Chapter 4). Also serogroup W135 only occasionally caused invasive disease, until recently outbreaks during the Hajj in Mecca and in the African Meningitis Belt has enhanced the awareness to this serogroup and its epidemic potential (Taha *et al*. 2000;Decosas & Koama 2002;Boisier *et al*. 2005); 6) isolates from a wave of NG ST192 meningococci, that was recorded between November 2003 and April 2005.

Comparative genetic fingerprinting analysis of carriage and disease isolates by Pulsed Field Gel Electrophoresis (PFGE) and Multi-locus sequence typing (MLST) revealed genetic diversification processes during clonal expansion and allowed to study the spatio-temporal spreading of new variants. Common and distinctive features will be presented in this report.

Materials and Methods

Study area

The study was conducted in the Kassena-Nankana District (KND) of the Upper-East Region of Ghana. It lies within the guinea Savannah woodland and has two major seasons; a short wet season from June to October and a long dry season for the rest of the year. The district-population is about 140,000, most of them rural. People live in compounds with an average of 10 inhabitants.

Bacterial isolates

Carriage isolates were obtained from twice yearly colonisation surveys performed in the KND since April 1998 in thirty-seven residential compounds that were randomly selected from a complete listing of the district population (Gagneux *et al.* 2002b). A total of 16 surveys have been performed since March 1998. A throat swab was taken from all consenting compound members present at the time of the visit and directly inoculated on

Thayer-Martin agar plates (Gagneux *et al*. 2000). Two colonies with neisserial morphology were sub-cultured from each positive plate. *N. meningitidis* colonies were identified by standard bacteriological methods as previously described (Gagneux *et al.* 2000).

Disease isolates were obtained from all suspected meningitis patients in the KND through an intensive surveillance system. In the case of W135 meningococcal meningitis samples were received from hospitals in Tamale and Accra. A suspected meningitis patient was defined by sudden onset of fever and stiff neck, or fever and stiff neck and altered mental status, in accordance with WHO-guidelines (WHO 1998). A lumbar puncture was performed before treatment, and the cerebrospinal fluid specimen was analysed as described previously (Gagneux *et al.* 2000).

Characterisation of bacterial isolates

Meningococci were serogrouped with serogroup-specific antisera (Difco) according to the manufacturer's instruction. In a subset of isolates, serological typing was confirmed by PCR (Taha 2000;Bennett, Mulhall, & Cafferkey 2004). All isolates were analysed by pulsed-field gel electrophoresis (PFGE) after digestion of genomic DNA with *NheI* (Morelli et al. 1997) a subset of bacteria has furthermore been typed with *SpeI*. MLST was performed as described (Maiden *et al.* 1998).

Spatial distribution

The spatial distribution of Serogroup A ST7 carrier- and patient compounds were mapped with MapInfo, using data from the Navrongo Demographic Surveillance System (NDSS) (Binka F 1999).

Results:

Serogroup A

Following a large epidemic in the dry season of 1996/97, serogroup A sequence type 5 (ST5) isolates were isolated from meningitis patients during a post-epidemic outbreak in 1998. Carriage of the ST5 meningococci decreased steadily from 2.7% in April 1998 to 0.3% in November 1999 before their disappearance from the KND since the year 2000 (Gagneux *et al.* 2000). The 36 disease isolates obtained in 1998 revealed three distinct PFGE patterns after *NheI* digest (I, II, and III, track 1-3, Figure 6.1), a spatial clustering of the distinct types could not be proven (Gagneux *et al.* 2000). The eight carrier strains isolated during the outbreak in early 1998 revealed pattern I and II (Figure 6.1., tracks 4, 9, 11, 12, 15-17, 19), identical to disease strains. Six ST5 strains were isolated from carriers between Nov. 1998 and Nov. 1999. While three isolates showed three new types (IV-VI, Figure 6.1), pattern I persisted in the remaining three strains (Figure 6.1, tracks 7, 10, 13). One carrier was found positive in four subsequent surveys, showing three different PFGE patterns (Figure 6.1, Carrier 1, tracks 4-7). Two Non-serogroupable meningococcal strains from carriers revealed ST5 by MLST and pattern I by PFGE analysis (Figure 6.1, tracks 11 and 18).

The emergence of the A ST7 genocloud in the KND was confirmed by culture in three serogroup A cases in early 2001 before carriage of A ST7 meningococci was first detected one year later. Between 2002 and 2004, A ST7 colonisation rates of 1.2 to 4.3% in the KND were associated with repeated A ST7 meningitis outbreaks; the highest carriage rate (4.3% in April 2004) coincided with the largest outbreak with 114 confirmed ST7 cases in the dry season of 2003/04. Thereafter A ST7 carriage dropped to below 1% and only two more A ST7 meningitis cases were recorded in February 2005. The A ST7 PFGE pattern was closely related to the A ST5 pattern (Figure 6.2, tracks 1 and 2). Between 2001 and 2005, altogether six A ST7-PFGE patterns were obtained after *NheI* digest of all 41 ST7-carrier and 147 ST7-disease isolates from the KND (Table 6.1 and Figure 6.2). In 2001, all three case isolates revealed PFGE type 7.1 (Table 6.1). Throughout 2002 and 2005, PFGE type 7.0 clearly dominated, with 85% (123/144) of the disease and 68% (28/41) of the carrier isolates. While the initially found pattern 7.1 persisted from 2001 to 2004, the remaining types (7.2-7.5) have only been isolated anecdotally from patients (Table 6.1).

Throughout the outbreaks of 2002 until 2004, serogroup A carriage and disease was concentrated in the densely populated east of the district. Here, local carriage rates of up

to 10% have been recorded (Figure 6.3). Strains of pattern 7.0 and 7.1 have been isolated repeatedly from the same compound at the same visit.

Serogroup X

In between the two serogroup A outbreaks, the relatively apathogenic serogroup X meningococci caused a fulminating wave of colonisation in the KND, associated with altogether 15 meningitis cases between 1998 and 2003 (Gagneux, Hodgson, Smith, Wirth, Ehrhard, Morelli, Genton, Binka, Achtman, & Pluschke 2002b). According to PFGE analysis after *NheI* and *SpeI* digestion, virtually all strains from carriers and patients isolated until November 2000 belonged to three phylogenetic clusters. All three clusters appeared in the KND when colonisation was first detected in April 1999. Differences in the spatial distribution were observed, but the case to carrier ratio was comparable for all three clusters (Gagneux, Hodgson, Smith, Wirth, Ehrhard, Morelli, Genton, Binka, Achtman, & Pluschke 2002b). Carriage with X-meningococci decreased after 2001 but was detectable on a low level in the KND until the end of 2003. Carrier and case-isolates obtained since 2001 in the KND had similar PFGE patterns as the isolates from 1999 and 2000 (Figure 6.4, track 1-10). However, sporadically new variants emerged in cases and carriers (Figure 6.4, track 11-20).

Serogroup Y

In the KND, serogroup Y meningococci were isolated on a low level with colonisation rates of up to 1.3% continuously between April 1998 and April 2000 and again between April 2002 and April 2004. During the entire study period no serogroup Y case was recorded in the KND. It is remarkable, that Y carriage was strongly associated with one particular residential compound, where altogether 14 (67%) of the 21 Y isolates came from. It was the only Y-positive compound between April 1998 and November 1999 and colonisation rates of up to 28% (4/14) were recorded. Initially, all strains isolated in the compound were identical according to *SpeI* and *NheI* digestion (Y1), the first variant was seen in April 1999 (Y2). Serogroup Y isolates that were obtained between 2002-2004 showed distinct but closely related PFGE patterns after *NheI* digest (Figure 6.5, pattern Y3-Y7). After each period of serogroup Y carriage, sporadic NG strains have been isolated from healthy carriers that showed closely related PFGE patterns, in Nov. 2000/April 2001 (Figure 6.5, track 5-7) and No. 2004 (Figure 6.5, track 14).

Serogroup W135

Four W135 meningococcal meningitis cases were recorded in Ghana between February 2003 and March 2004 (Chapter 4). The ghanaian W135 disease isolates, all ST11 by MLST, revealed identical PFGE patterns after *NheI* digest and were closely related to burkinian outbreak strains from 2001 and 2002, and undistinguishable from a case isolate of 2003 (Chapter 4). Carriage rates in different ghanaian patient communities were found to be highly variable. Whereas in one of the communities not a single W135 carrier was found, in another patient communities 18% of the inhabitants were colonized with W135 meningococci. Here, all carrier isolates revealed identical *NheI* patterns at the first visit. Two additional colonisation surveys showed diversification of the carriage isolates and finally the replacement of the original variant within one year (Chapter 4). Sporadic colonisation with W135 bacteria in the KND in 1998 and 2004 did not result in a major wave of carriage and disease. However, the 2004 carriage isolates had identical PFGE pattern to the 2003 case isolate from Burkina Faso (Chapter 4).

Non-groupable

Only 18.1% (55/304) of the meningococcal isolates during eight years of colonisation study were non serogroupable (NG). Most of the strains were related to the dominating encapsulated genoclouds by PFGE analysis. Between November 2003 and April 2005 we detected a wave of clonally related NG meningococci in the carriage population, of which representative isolates showed ST192 by MLST. While no case of NG 192 meningococci was detected throughout this period, the colonisation rate peaked in November 2004 (3.8%). From altogether 24 strains, we saw one dominating PFGE type in 18 isolates (75%) throughout the whole period (Figure 6.5, pattern1). Four closely related patterns (see Figure 6.5, pattern 2-4, pattern 5 not shown) were found between April 2004 and April 2005, one of them was isolated more than once (pattern 2).

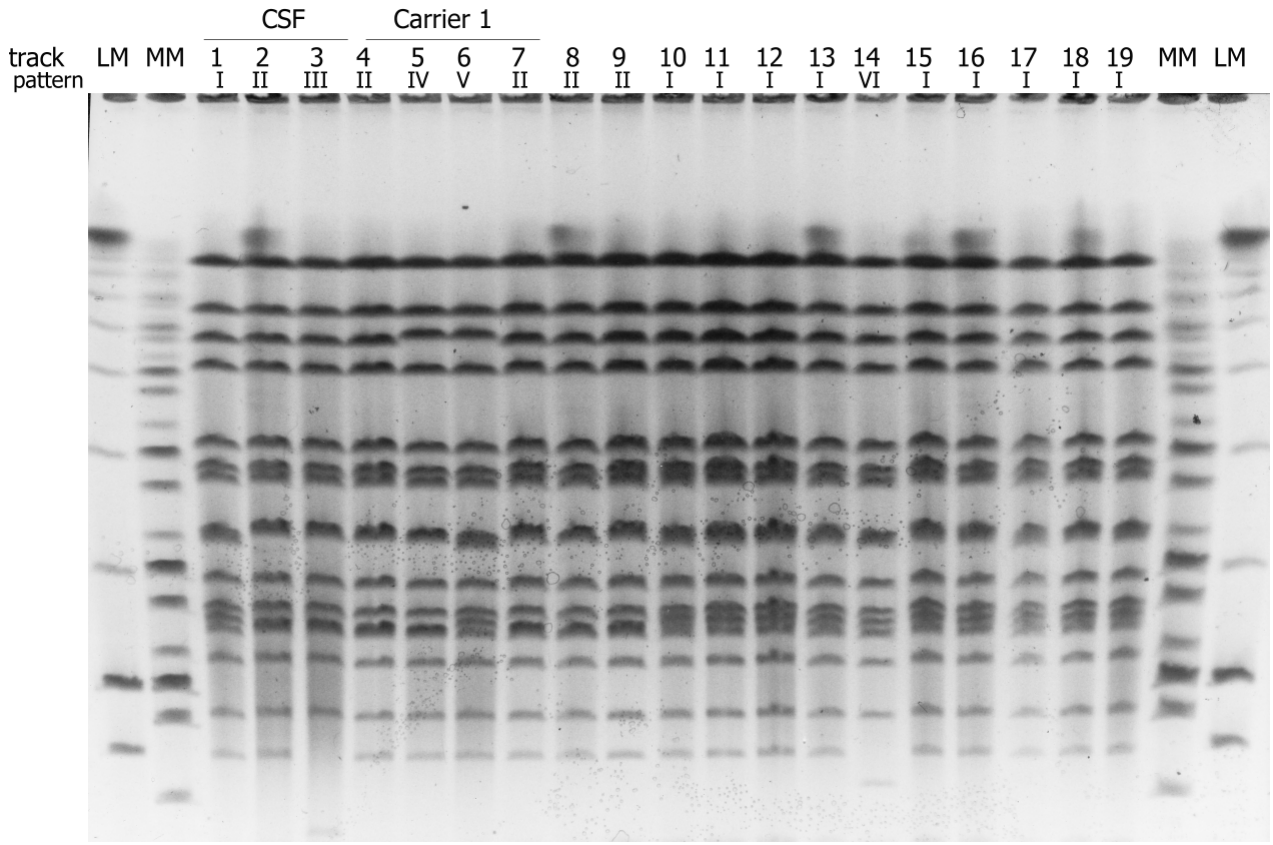


Figure 6.1 PFGE patterns of *NheI* digested chromosomal DNA of serogroup A ST5 meningococci isolated in the KND 1998-1999. Strains were loaded in tracks 1-19 in the following order (track: strain): 1:Z7057; 2: Z7060; 3: Z7067; 4: Z8319; 5:Z8324; 6: Z8326; 7: Z9567; 8: Z8349 (NG); 9: Z8314; 10: Z8323; 11: Z8315; 12: Z8316;13: Z8322; 14: Z8325; 15: Z8318; 16: Z8320; 17: Z8321; 18: Z8342; 19: Z8317. Molecular weight markers were loaded in the flanking tracks as indicated (LM: lowrange marker; MM: midrange marker). CSF indicate isolates recovered from meningitis patients during the 1998 outbreak in the KND. All other strains were isolated from healthy carriers. A ST 5 bacteria were isolated from the same person (Carrier 1) during four subsequent surveys

Table 6.1 Distribution of PFGE pattern among the meningococcal disease and carriage isolates from the Neisseria meningitidis serogroup A ST7 wave in the KND

year	2001	2002	2003	2004	2005
PFGE type			**Disease isolates (n)**		
7.0		38	27	56	2
7.1	3	1	1	14	
7.2		1			
7.3			2		
7.4			1		
7.5				1	
			Carrier isolates (n)		
7.0		8	10	10	3
7.1		2	1	7	

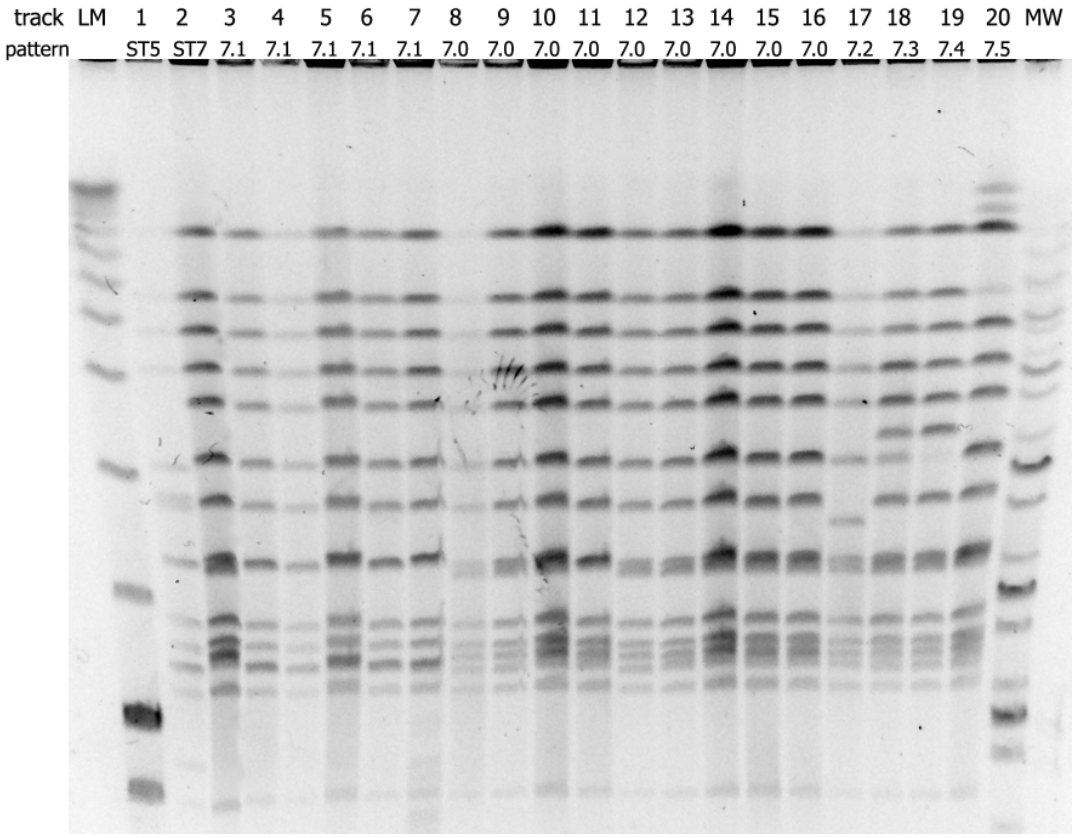


Figure 6.2 PFGE patterns of *NheI* digested chromosomal DNA of serogroup A ST7 meningococci isolated in the KND 2001-2005. Strains were loaded in the following order (Track:strain number): 1: N1267; 2: N1325; 3: N1264; 4: N1479, 5: N1841; 6: N1472; 7: N1606; 8: N1362; 9: N1552; 10: N1812; 11: N2009, 12: N1465; 13: N1604, 14: N1890; 15: N1990; 16: N2018; 17: N1359; 18: N1555; 19: N 1573; 20:1839 Molecular weight markers were loaded in the flanking tracks as indicated (LM: lowrange marker; MM: midrange marker).

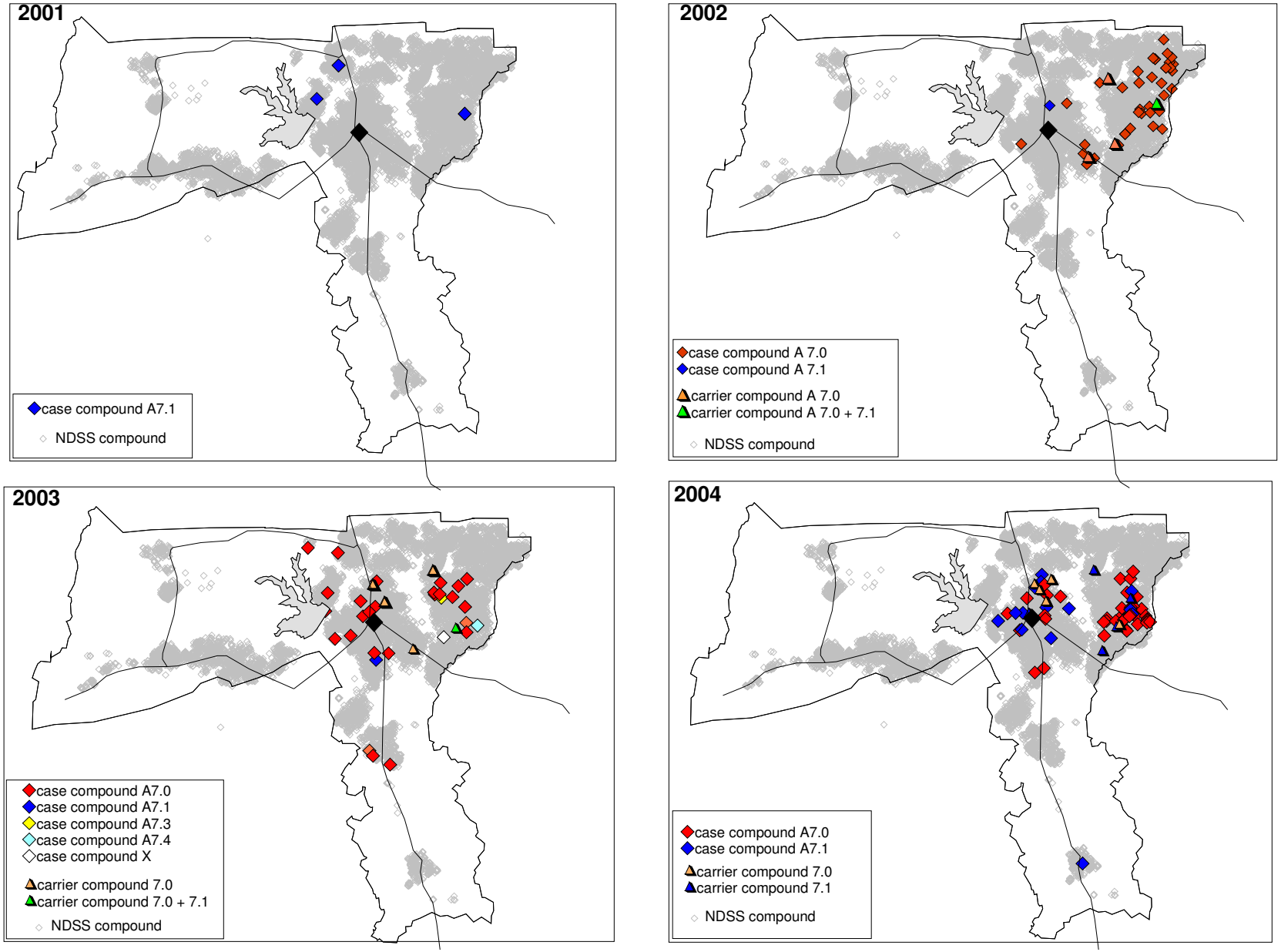


Figure 6.3 Spatial distribution of serogroup A ST7 carrier- and case- compounds in the KND between 2001 and 2004.

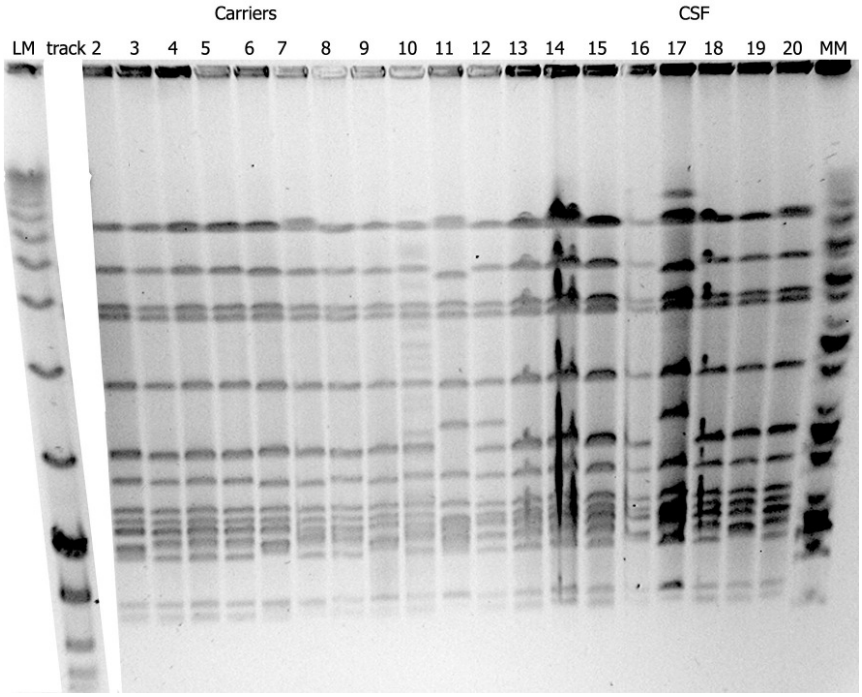


Figure 6.4 PFGE patterns of *NheI* digested chromosomal DNA of serogroup X meningococci isolated in the KND between 2001 and 2003. Strains were loaded in the following order (Track:strain number) 2: N1117; 3: N1242 ; 4: N1243 ; 5: N1287; 6:N1289; 7:N1299; 8: N1311; 9:N1382; 10: N1409; 11: N1447; 12: N1475; 13: N1760; 14: N1764; 15: N1768; 16: N1258; 17: N1260; 18: N1261; 19: N1326; 20: N1568. Molecular weight markers were loaded in the flanking tracks as indicated (LM: lowrange marker; MM: midrange marker).

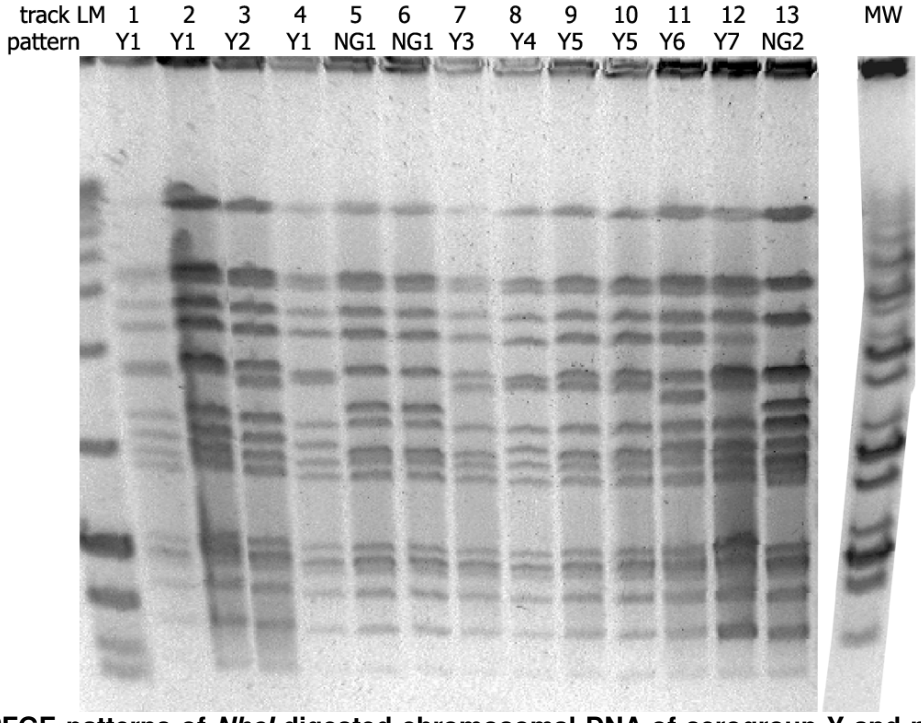


Figure 6.5 PFGE patterns of *NheI* digested chromosomal DNA of serogroup Y and noun-groupable meningococci isolated in the KND between 1998 and 2004. Strains were loaded in the following order (Track:strain number) 1:N1274; 2: N2087; 3: N2089 ; 4: N1001 (NG) ; 5: N1226 (NG); 6:N1247(NG); 7:N1383; 8: N1407; 9:N1443; 10: N1591; 11: N1756; 12: N1899; 13: N1980 (NG) Molecular weight markers was loaded in the flanking track as indicated (LM: lowrange marker)

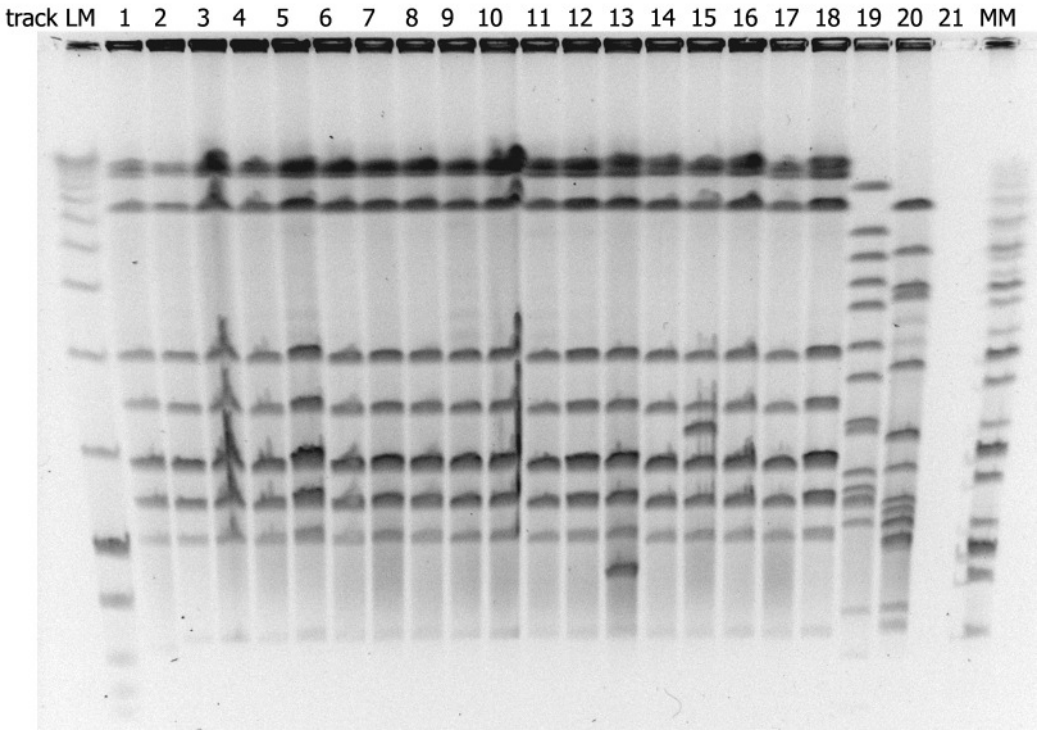


Figure 6.6 PFGE pattern of *NheI* digested chromosomal DNA of non-groupable ST192 meningococci. Strains were loaded in the following order (Track:strain number): 1: N1766, 2: N1907; 3: N1914, 4: N1917; 5: N1934, 6: N1970; 7: N1974; 8: N1978; 9: N1985; 10: N1992, 11: N1998, 12: N2004; 13: N2006; 14: N2012; 15: N2014; 16: N2015, 17:N2017; 18:N2022; 19: A ST7 reference; 20: X reference

Discussion

It has been suggested, that meningococcal epidemics are initiated by contact of a population with a particularly fit genotype which is transmitted efficiently (Zhu et al. 2001). After a few years the descendants of such fit strains seem to disappear from individual areas (Morelli *et al*. 1997;Achtman 1997). They may, though, cause further epidemics or pandemics by successful competition with other lineages for transmission to other geographical areas. Clonal expansion is accompanied by genetic microevolution. Sequential evolutionary bottlenecks have been made responsible for the apparent paradox between the occurrence of extensive recombination in meningococcal populations and the clonal uniformity observed in epidemic lineages (Achtman 2004).

We have observed waves of colonization and disease by meningococcal genoclouds in the KND of northern Ghana (Chapter 5). While constant microevolution was detected in all dominating lineages, patterns of diversification were not uniform. For example, initial cases of the serogroup A ST7 meningococci in the district were caused by a particular

PFGE variant, but a closely related type soon spread more successfully and dominated during the subsequent four years among case and carrier isolates. Further variants appeared anecdotally. In contrast, serogroup X meningococci emerged in the district in three phylogenetic clusters which in the following showed different patterns of dissemination and diversification (Gagneux *et al*. 2002b). Both, the X ST751 and the A ST7 genoclouds completely disappeared from the district after 3-4 years of extensive spread (Chapter 5).

Contact of a population with a new virulent genotype is not always followed by extensive transmission and outbreaks of invasive disease. Although we detected in the KND a low level of colonization with W135 bacteria with PFGE patterns identical to that of the Burkinian epidemic clone and found a high W135 colonisation rate in a Ghanaian W135 meningitis patient community, Ghana was not hit by a major wave of W135 colonization and disease. This indicates that not only bacterial factors, but also host and environmental factors contribute to transmission and epidemic potential of individual genoclouds. The same is suggested by our findings with Y meningococci. Over the years these have been isolated in the KND at a low frequency in 10 of the altogether 16 carriage surveys. While the Y genocloud was successful and persistent in one particular compound, it exhibited only a low transmission efficacy in the general population.

It has been suggested that towards the end of epidemics the frequency of variants increases and that these strains represent escape variants from herd immunity (Achtman 2004). Diversification found in our study in the PFGE patterns of A ST5, X ST751 and NG ST192 isolates towards the end of their spread support this assumption. Also during W135 spread in a patient community we observed clonal diversification and replacement of the original variant within one year (Chapter 4). Changes in PFGE patterns are indicators of genomic diversification, but cannot directly be associated with structural and functional variation. However, in the near future high throughput DNA sequencing methods will allow comparing the genomes of large numbers of bacterial isolates. This will make it possible to assess whether herd immunity is in fact leading to the selection of escape variants that carry mutations in crucial antigens, such as outer membrane proteins. Identification of the proteins that are diversifying and thus are likely to represent targets for protective immune responses will have major implications for the selection of candidate antigens for multivalent protein-based vaccines.

New genotypes are often lost during spread of genoclouds to other areas and may thus have a lower transmission efficacy in a naive host population than their parental genotypes. On the other hand, occasionally variants arise, which are efficiently transmitted and develop into the dominating genotype of new genoclouds. Differences in the dominating variants between countries imply that bottle necks exist in the dissemination, even during epidemic periods with high carriage rates. It is not understood, why serogroups, such as X and W135 which were for a long time considered as having a low pathogenicity start to cause epidemics (Taha et al. 2000;Decosas & Koama 2002;Boisier *et al.* 2007). It is expected that population genomic analyses will reveal in future how mutations, genetic exchange and selection shape the evolution of new *N. meningitidis* lineages. This may help to understand the genetic basis for the emergence of new lineages with epidemic potential (Shao *et al.* 2006), which is crucially important for the development of a successful long-term global vaccination strategy against meningococcal disease.

Acknowledgments

We would like to thank all the study participants for their trust and cooperation during the study. Furthermore, we acknowledge the use of the Navrongo DSS database, the support of A. Bugri and A. Wahab in the lab of the NHRC as well as C. Tindana and all field workers for their contribution in the field and in data management. The study was sequentially funded by the Stanley Thomas Johnson Foundation, the Meningitis Research Foundation and the Meningitis Vaccine Project

Chapter 7

Conservation of the Pneumococcal surface protein A (PspA) sequence in a hypervirulent lineage of serotype 1 *Streptococcus pneumoniae*

Julia Leimkugel[1], Valentin Pflüger[1], Abudulai Adams Forgor[2], Martin Nägeli[1], Christian Flierl[1], Sébastien Gagneux[1*], Gerd Pluschke[1]

[1]Swiss Tropical Institute, Basel

[2]Navrongo Health Research Centre, Ghana

*present address: The Institute for Systems Biology, Seattle, USA

This article will be submitted to
Journal of Clinical Microbiology

Abstract

Serotype 1 pnenumococci are only rarely isolated from healthy carriers, but have been implicated in meningitis outbreaks. Based on Multi-Locus Sequence Typing (MLST) data they have been subdivided into three lineages, with each strain sharing not less than four of seven alleles with at least one other isolate of the same lineage. The three lineages (designated A, B and C) have distinct geographic distribution patterns with the majority of lineage B isolates originating from Africa. In our Pulsed Field Gel Electrophoresis (PFGE) analysis of serotype 1 isolates, strains belonging to the same lineage had closely related, but not identical PFGE patterns. These data reconfirmed MLST-based assignment of isolates to the three lineages and provided further evidence for recombinational diversification. Sequence analysis of the gene encoding the pneumococcal surface protein A revealed a surprisingly high intra-lineage conservation of this hypervariable vaccine candidate antigen. All 15 isolates belonging to the hypervirulent lineage B and the majority of lineage C isolates analysed shared a unique *psp*A allele, which has not been described in other serotypes. These findings have major implications for protein-based vaccine development against hypervirulent groups of pneumococci, such as the lineage B of serotype 1.

Introduction

Streptococcus pneumoniae is one of the most important opportunistic pathogens worldwide. Ranging from mild respiratory tract infections to invasive disease such as sepsis and meningitis it is responsible for estimated 700.000 to 1 million deaths per year (www.who.int). While invasive disease is usually a rare event, symptomless colonization with *S. pneumoniae* is common especially in infants with colonization rates of nearly 100% at 1 year of age (Crook *et al.* 2004). DNA-recombination within the pneumococcal population is frequent and the strains isolated from patients and carriers usually exhibit a broad genetic variability (Feil, Smith, Enright, & Spratt 2000;Jefferies, Smith, Clarke, Dowson, & Mitchell 2004). Over 90 different serotypes determined by the chemical structure of the polysaccharide capsule are known, but at any place and time only a limited number of serotypes are responsible for the majority of invasive infections. The composition of the dominating invasive serotypes varies from place to place and with time (Hausdorff, Bryant, Paradiso, & Siber 2000). The relevance of the capsular type in comparison to non-capsular factors for the virulence of pneumococcal genotypes is currently incompletely understood. While certain serotypes are genetically highly diverse and not a single genotype can be assigned responsible for the majority of invasive

infections (Spratt, Hanage, & Brueggemann 2004), in other serotypes only one or two lineages are frequently isolated from patients in different countries (Brueggemann, Griffiths, Meats, Peto, Crook, & Spratt 2003).

Pneumococcal surface protein A (PspA) is regarded as one of the major virulence factors of *S. pneumoniae*, that is present in virtually all pneumococcal isolates (Crain *et al.* 1990). The pspA gene is highly variable (Hollingshead *et al.* 2000) and has a mosaic structure consisting of five domains, a signal peptide, an alpha-helical domain, a proline-rich region, a choline binding domain and a C-terminal tail. The most variable part is the N-terminal alpha-helical domain, which is exposed to the host immune system. Hollingshead et al. proposed a classification for PspA based on the sequence divergence of a stretch located just before the proline rich region, designated the clade-defining region (CDR). The classification divides PspA into three families, which can be subdivided into 6 clades (Hollingshead, Becker, & Briles 2000). In spite of its high sequence variability, considerable cross-reactivity among PspA molecules has been demonstrated. PspA is therefore evaluated as potential vaccine candidate for a protein based anti-pneumococcal vaccine (Virolainen *et al.* 2000;Nabors *et al.* 2000;Briles *et al.* 2000).

In addition to the classical typing of bacteria with serological and biochemical techniques, a range of molecular typing methods have been developed to examine the population structure and evolution of microbial pathogens in detail. Multi-Locus Sequence Typing (MLST), developed in 1998 for *Neisseria meningitidis* has since then been extended to a broad variety of pathogens (http://mlst.net). It is based on the sequence variability in house-keeping genes that are thought to accumulate changes slowly and selectively neutral. Analysing the variability of seven alleles, this method gives a good resolution for global molecular epidemiology (Enright & Spratt 1998;Feil, Smith, Enright, & Spratt 2000). Pulsed Field Gel Electrophoresis (PFGE) is based on electrophoretic separation of fragments of genomic bacterial DNA generated by digestion with rare cutting restriction enzymes, yielding strain specific band patterns. PFGE usually exhibits a high discriminatory power and can be used for short-term epidemiological questions such as the investigation of outbreaks (Porat, Trefler, & Dagan 2001;Decousser *et al.* 2004;Givon-Lavi *et al.* 2002) .

Pneumococcal serotypes vary substantially in the ratio of the frequency of isolation from invasive disease and from nasopharyngeal carriage (Smith *et al.* 1993b;Brueggemann,

Peto, Crook, Butler, Kristinsson, & Spratt 2004). Serotype 1 lineages have been found responsible for outbreaks in several communities (Porat, Trefler, & Dagan 2001;Henriques, Kalin, Ortqvist, Akerlund, Liljequist, Hedlund, Svenson, Zhou, Spratt, Normark, & Kallenius 2001). In contrast, it is usually only rarely isolated from healthy carriers, suggesting a high virulence (Smith, Lehmann, Montgomery, Gratten, Riley, & Alpers 1993b;Lloyd-Evans, O'Dempsey, Baldeh, Secka, Demba, Todd, McArdle, Banya, & Greenwood 1996;Syrogiannopoulos *et al.* 2002;Brueggemann, Peto, Crook, Butler, Kristinsson, & Spratt 2004). However, it is not clear, whether all serotype 1 lineages exhibit the same degree of virulence (Musher 2006), since some reports indicate that serotype 1 strains cause only low-level bacteraemia and are associated with a lower mortality than other serotypes (Sandgren *et al.* 2005). A global MLST analysis identified a limited number of serotype 1 associated sequence types (STs) and defined three genetic lineages. Within these, each strain shared at least four of seven MLST alleles with at least one other member of the lineage. Lineage B and C isolates were to a certain degree related, as most of them had three alleles (aroE 10, gki 4, recP 1) in common. In contrast, most lineage A strains did not share a single allele with lineage B strains and only one allele with lineage C strains, respectively (Brueggemann & Spratt 2003). The three lineages exhibited a distinct geographic distribution pattern. Lineage A was only isolated in Europe and Northern America, whereas the majority of the lineage B isolates originated from Africa (Brueggemann & Spratt 2003).

Between 1998 and 2003 we observed a pneumococcal meningitis outbreak in Northern Ghana, where the majority of the cases were caused by serotype 1 pneumococci. MLST analysis revealed a clonal relationship of these strains, all of them belonging to the ST217 clonal complex belonging to lineage B (Chapter 3). Here we describe the clonal relationship between various serotype 1 strains based on MLST, PFGE and pspA gene sequence analysis. The outbreak strains of Northern Ghana were compared to representatives of all three serotype 1 pneumococcal lineages (Brueggemann & Spratt 2003).

Materials and Methods

Bacterial Isolates

63 serotype 1 pneumococcal strains were isolated from CSF of meningitis patients in the context of a long-term study on bacterial meningitis in the Kassena Nankana District (KND) in Northern Ghana between 1998 and 2005. Additional strains from CSF were obtained from the Bolgatanga Regional Hospital in the Upper East Region (n=10) and from health facilities in the Bongo (n=1) and Builsa Districts (n=1). Furthermore four serotype 1 pneumococcal isolates isolated in 2005 during a pilot study on bacterial meningitis in the Nouna Health District in Burkina Faso were examined. The bacteria have been identified and isolated from CSF of suspected meningitis patients using standard microbiological methods (Chapter 3). Serotyping has been performed with the Quellung Reaction using serotype specific antisera from the Statens Serum Institute in Kopenhagen. According to the lineage definition of serotype 1 pneumococcal isolates by Brueggemann et al., all Ghanaian and Burkinian serotype 1 isolates belonged to lineage B (Brueggemann & Spratt 2003). Reference isolates from the other lineages A (n= 6) and C (n= 4) were obtained from A. Brueggemann, Oxford.

Multilocus Sequence Typing (MLST)

Bacteria were grown overnight in Todd-Hewitt medium. DNA extraction, MLST, and direct sequencing of polymerase chain reaction products by use of an ABI Prism 310 genetic analysis system was performed in accordance with standard protocols. Allelic profiles were analyzed by use of applications on the MLST home page (available at: http://spneumoniae.mlst.net/). For analysis of the relationships between closely related isolates, eBURST software (available at: http://eburst.mlst.net/) with the most stringent group definition (6/7 alleles identical) was used. All allelic profiles obtained were compared with the complete listing of the sequence types (STs) available in the database. The diversity of STs within the lineages was assessed using the E-Burst Software with respect to the current state of the database.

Pulsed Field Gel Electrophoresis (PFGE)

All serotype 1 isolates have been analysed by PFGE, after modifying a protocol which was previously described (McEllistrem *et al.* 2000). Briefly, bacteria were grown overnight on Colombia Agar containing 5% sheep blood, in the log phase, they were harvested and inoculated into 5ml sterile PBS and adjusted to a density of ~5McF.The cells were

centrifuged for 10min, washed and centrifuged again. The pellet was suspended in 1.5% agarose blocs (1ml) and poured into the moulds for solidification. DNA lysis was performed first in 2.5ml Lysisbuffer (0.1M EDTA pH=8; 1M Nacl; 6mM Tris HCl pH8; 0.5% Brij 58, 0.5% N-lauryl-sarcosine, 0.5% Sodiumdeoxycholate; supplemented with 50ug/ml RNAase and 5mg/ml Lysozyme) overnight at 37°C, after this in 2.5ml ESP Buffer (0.5M EDTA, pH=8, 1% N-lauryl-sarcosine, 0.1% Proteinase K) overnight at 50°C. The blocs were stored at 4-8°C in TE Buffer (10mM Tris HCl;1mM EDTA). The digest was performed with 15U *SmaI* at 25°C overnight.

PspA sequencing

Selected strains from the Ghanaian serotype 1 pneumococcal meningitis outbreak and the pilot study in Burkina Faso were compared with the lineage A and C reference strains isolated worldwide (Brueggemann & Spratt 2003).

A 1000bp long stretch of the variable region of the PspA gene was sequenced following a modified protocol of Hollingshead et al. (Hollingshead, Becker, & Briles 2000). Briefly, a 1200 bp long product was obtained by PCR with the primers SKH2 5' CCACATACCGTTTTCTTGTTTCCAGCC3' and a modified LSM12 primer LSM12* 5' CCAGCGTCGCTATCTTAGGGGCTGGTT3' using standard conditions with the annealing temperature of 62.5°C. PCR products were purified by PEG precipitation. The 1000bp piece was sequenced by use of the ABI Prism 310 genetic analysis system and alignment of DNA and protein sequence was performed using the online available ClustalW software (http://www.ebi.ac.uk/clustalw/).

Results:

PFGE analysis reconfirms the MLST-based distinction between three serotype 1 lineages

Currently serotype 1 strain data submitted to the MLST database comprise 49 STs which belong to 6 clonal complexes within the three lineages (Table 1). Three singletons can not be assigned to a lineage. Lineage B now consists of the ST217 clonal complex with 17 STs and one singleton (ST302). Lineage C comprises three clonal complexes (ST615, ST1031, ST300) with each 2-3 STs. Overall within each Lineage B and C all STs share at least 5/7 alleles with one other ST of the respective lineage. The average distances of the STs within lineage B calculated by E-Burst analysis range from 1.64 to 4.0, for lineage C from 1.83 to 2.66. Lineage A reveals higher diversity by E-Burst analysis consisting of two clonal complexes (ST306 and ST305 clonal complex) and one singleton (ST1247) whith

only four common alleles. The average distance of STs within lineage C ranges from 2.05-4.3

Serotype 1 strains of the same lineage exhibited closely related PFGE patterns of *SmaI* digested DNA (Figure 7.1). As expected from the sharing of multiple MLST alleles, PFGE patterns of lineage B (Figure 7.1, track 12-20) and lineage C (Fig. 1, track 8-11) isolates had more (up to 8/12) bands in common, than lineage A (Figure 7.1, track 1-7) with B or C isolates. Furthermore, it could be confirmed, that lineage A isolates exhibited a higher degree of intra-lineage diversity of PFGE patterns than lineages B and C. In accordance with the E-Burst results, the six lineage A strains analysed here by PFGE could be assigned to the two clonal complexes. Representatives of the ST305 clonal complex (a ST304 and a ST305 isolate) differed by only two bands in PFGE (Figure 7.1, track 2, 3). Four representatives of the ST306 clonal complex had closely related PFGE patterns, which differed markedly (in up to seven bands) from those of the ST305 clonal complex.

Table 7.1 MLST diversity of the serotype 1 lineages. Current available serotype 1 strain data submitted to the MLST database has been analysed by E-Burst, clonal complexes have been assigned and the average genetic distances of STs within each lineage was assesed

	MLST and E-burst Data			**PFGE Analysis**	
Lineage	**Clonal complex /Singletons**	**No. of ST**	**average distances of STs** (range within lineage)	**No of strains**	**No of STs**
A	ST306	15		4	4
	ST305	5	2.05-4.3	2	2
	ST1247	singleton			
B	ST217	17	1.6-4.0	73	11
	ST302	singleton			
C	ST615	3		3	3
	ST300	2	1.83-2.66	1	1
	ST301	2			
not assigned	ST870	singleton			
	ST2210	singleton			
	ST150	singleton			

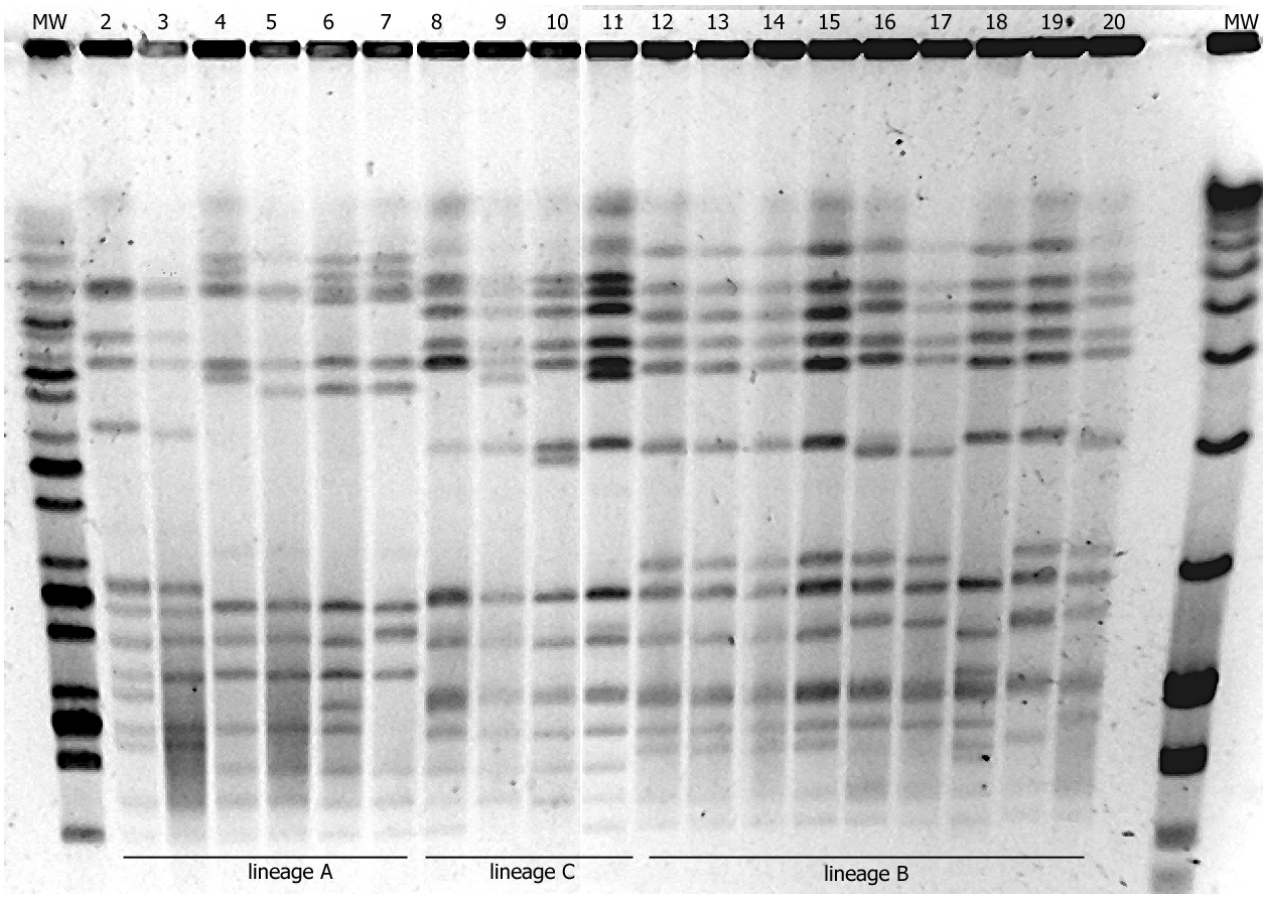


Figure 7.1 PFGE profiles of serotype 1 isolates from all three different lineages. DNA was digested with *SmaI*. Indicated on top of the lanes are the lineages of the respective strains. (Lane: strain-No., ST,)

1: MW Marker, 2: sl_4, ST304, 3:sl_30, ST305, 4: sl_2, ST227, 5: sl_11, ST228, 6: sl_3, ST306, 7: sl_38, ST617, 8: sl_71, ST611, 9:sl_102, ST616, 10: INV1871, ST300, 11: NCTC7465, ST615, 12: P1010, ST303, 13: P1012, ST303, 14: P1004, ST217, 15:P1031, ST217, 16: P1026, ST612, 17: P1052, ST612, 18: P1100, ST618, 19: P1032, ST303, 20: P1007, ST217

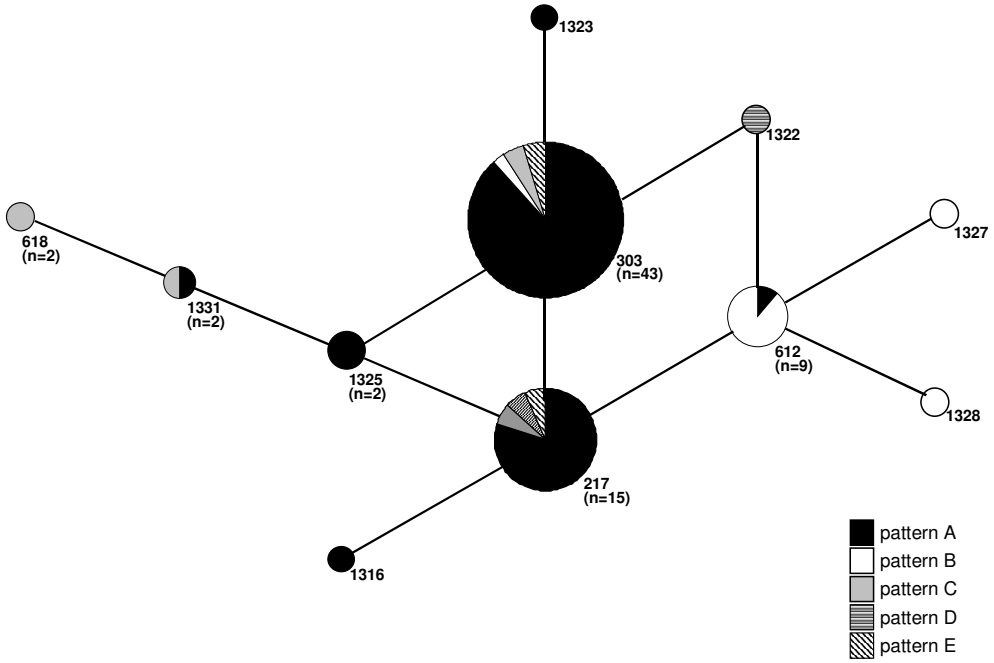


Figure 7.2 Modified E-Burst diagram and Pulsed Field-Gel Electrophoresis types of the ghanaian serotype 1 strains belonging to lineage B. The Pulsed Field Patterns are given by A-E, indicated in parenthesis is the number of strains found with the respective patterns, if more than one strain was isolated.

Genetic diversity of serotype 1 isolates belonging to lineage B

Based on MLST analysis, all 73 Ghanaian and Burkinian isolates analysed could be assigned to one clonal complex (Figure 7.2) with three dominating STs (ST217, ST303 and ST612). All remaining STs were only sporadically isolated (chapter 3). To date, the global collection of serotype 1, lineage B strains comprises in total 18 different allelic profiles (Table 7.1); eleven of these were represented in the strain collection from Northern Ghana and Burkina Faso. The close relationship between all lineage B isolates was also reflected by similarity of their PFGE patterns. Five closely related major patterns (designated 1A-1E) were identified (Figure 7.1). Pattern 1A (Figure 7.1, lanes 12-15) dominated and was found in association with seven of the 11 STs (ST217, ST303, ST612, ST1316, ST1323, ST1325, ST1331) in 56 of the 78 isolates included in the analysis (Figure 7.2). 11 strains (associated with four different STs; Figure 7.2) showed pattern 1B (Figure 7.1, lanes 16, 17). While no strong general association between PFGE patterns and STs was observed, eight of the nine ST612 strains and two strains that were single-locus variants (ST303 and ST1327) of ST612 showed pattern B. On the other hand, the six isolates with PFGE pattern 1C were associated with four different STs and the groups of ST303 and ST217 isolates were associated each with four different PFGE patterns.

Intra-lineage conservation of the pspA gene sequence

Sequence analysis of the *psp*A gene revealed a high degree of intra-lineage sequence identity. All 15 lineage B isolates analysed had an identical pspA gene sequence, representing an allele (designated BC) of this hypervariable protein, that has not been described previously (Figure2). While three of the four lineage C isolates shared this allele with the lineage B strains, the fourth lineage C strain, isolated in 1948, had a different allele (designated A), also found in five of the six lineage A isolates analysed. One lineage A strain had a third allele (designated A*) (Table 2). Following the clade definition of Hollingeshead

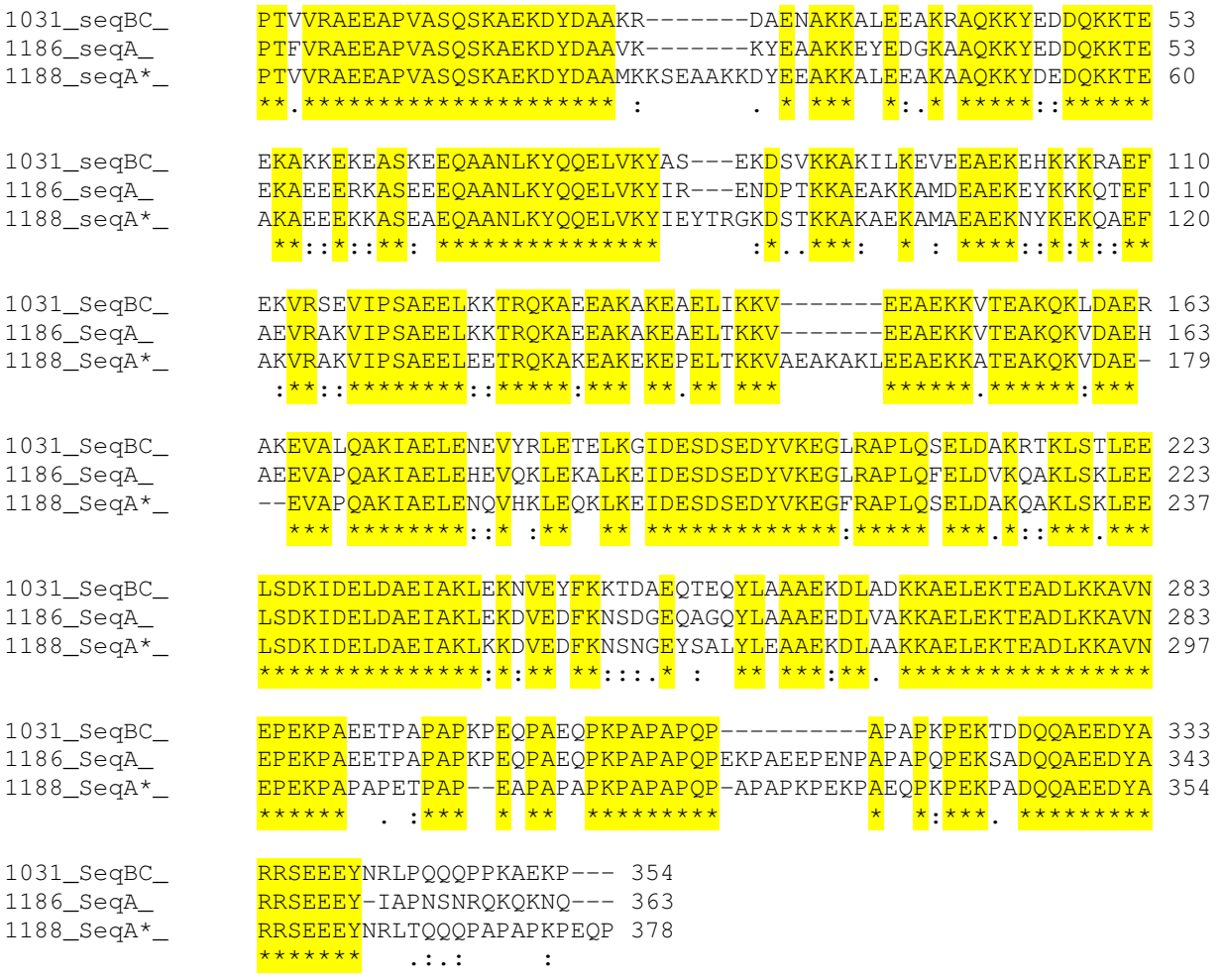


Figure 7.3 Clustal alignment of the three representative protein sequences of PspA. A summary of the sequencing results in comparison with the other typing methods is presented in table 2. SeqBC and SeqA share 78% sequence identity, SeqBC and SeqA* as well as SeqA and SeqA* share 74% identity.

Table 7.2 Geographic origin and characteristics of strains representing three serotype 1 lineages.

ST	No of strains	Lineage	PFGE-type	PspA allele	origin
306	1	A		SeqA	USA 2001
617	1	A		SeqA	Norway 2001
228	1	A		SeqA	Spain 2001
227	1	A		SeqA	USA 2000
304	1	A		SeqA	USA 2001
305	1	A		SeqA*	Denmark 2000
303	6	B	1A ,1E	SeqBC	Ghana
217	2	B	1A	SeqBC	Ghana
612	4	B	1A ,1B	SeqBC	Ghana
618	1	B	1C	SeqBC	Ghana
1322	1	B	1D	SeqBC	Ghana
1325	1	B	1A	SeqBC	Ghana
1331	1	B	1C	SeqBC	Ghana
615	1	C		SeqA	USA 1948
611	1	C		SeqBC	South Africa 2001
616	1	C		SeqBC	Poland 1995
300	1	C		SeqBC	UK 2001

Discussion

Using three different genetic features, the sequence diversity of seven house-keeping genes, sequence analysis of the hyper-variable surface protein PspA and a fingerprint of genomic DNA digested by a rare cutting restriction enzyme, we compared the intra- and inter-lineage diversity of serotype 1 pneumococcal isolates. While MLST is commonly used for global and long-term epidemiological analysis (Maiden, Bygraves, Feil, Morelli, Russell, Urwin, Zhang, Zhou, Zurth, Caugant, Feavers, Achtman, & Spratt 1998;Enright & Spratt 1998), PFGE is often more useful for local or short-term epidemiological investigations, such as the analysis of the stability of emerging clones in the course of an outbreak (Porat, Trefler, & Dagan 2001). Our PFGE analysis supported MLST data-based sub-division of serotype 1 isolates into three major lineages and distinction of two subgroups of lineage A isolates. All three types of genetic analysis indicated that serotype 1 lineages B and C are more closely related to each other than to lineage A. However, the finding, that a lineage C strain isolated in 1948 (Table 7.2) had the PspA allele dominating in the lineage A isolates, suggests a common ancestor of all three serotype 1 lineages.

In an analysis of a large collection of recent lineage B isolates from Ghana and Burkina Faso both MLST and PFGE demonstrated substantial diversification. According to MLST, all isolates, covering overall 11 STs, belonged to the same clonal complex, where each member was sharing six of seven alleles with at least one other member of the complex. Five major PFGE patterns within this complex could be distinguished by PFGE analysis with one restriction enzyme. Individual STs were associated with a range of PFGE patterns and vice versa. Only PFGE pattern B was associated with a particular ST (ST612). Interestingly also in other African settings such as in Burkina Faso and the Gambia (unpublished results), closely related serotype 1 lineage B strains have been isolated, and the latest STs, which have been added to the ST217 clonal were found in Niger, Burkina Faso and Egypt (http://mlst.net), indicating that this hypervirulent clonal complex is widely distributed. It is not known, since when pneumococci belonging to this lineage circulate in Africa. Meningococcal genoclouds responsible for meningitis outbreaks and epidemics are known to persist only transiently and disappear completely from respective geographical regions after three to four years (Achtman 2004). However, it is not known whether the same applies for pneumococcal genoclouds with epidemic potential. Even though incidence rates of pneumococcal meningitis have declined after three years of outbreak in the KND, cases caused by serotype 1 lineage B pneumococci continued to occur. In this light, it will be important to further examine the virulence

potential and immunological properties of this pneumococcal lineage, to evaluate its capability to cause epidemic meningitis in African countries especially in view of the introduction of meningococcal conjugate vaccines and potential strain replacements.

The cell wall bound protein PspA is exposed to the immune system of the host and has been shown to be highly immunogenic. It exhibits high sequence variability and is thought to be under much stronger selection pressure (Hollingshead, Becker, & Briles 2000) than the presumably neutral allelic variation in the house keeping genes analysed by MLST. Surprisingly, intra-lineage variation of PspA was much lower than allelic diversity of the housekeeping genes. It is assumed that a single nucleotide site in a meningococcal housekeeping gene is about 50-fold more likely to change as a result of recombination than as a result of mutation. It has been suggested that due to their very low carriage rate, serotype 1 pneumococci are less likely to exchange genetic material with other pneumococcal strains than other pneumococci (Porat, Trefler, & Dagan 2001). However, both MLST and PFGE results are indicative for substantial recombinational diversification of serotype 1 lineage B pneumococci. Therefore other factors are likely to be responsible for the high conservation of the PspA gene. The unexpected lack of diversification of the PspA protein in the clonal complex may have major implications for the design of a protein-based vaccine against emerging hypervirulent lineages.

Chapter 8
General Discussion and Conclusions

General Discussion and Conclusions

This thesis focussed on the dynamics of bacterial meningitis during a long-term study in the KND of Nothern Ghana. Characteristics of colonisation and disease were adressed and the population of the carrier- and patient-isolates were put in relationship to each other. Furthermore we examined the impact of emerging clones (e.g. W135) and other bacterial species causing acute bacterial meningitis in Northern Ghana. In addition to the detailed discussion in the seperate chapters of this thesis, the results of the study are discussed here in a broader context with respect to immunological and evolutionary aspects and in view of strategies for future research and an improved management of bacterial meningtis epidemics.

Recent epidemiological trends in the African Meningitis Belt

During the last 15-20 years a series of major epidemics and smaller outbreaks of meningococcal meningitis have occured within various regions of Sub-Saharan Africa. Numerous reports documented the emergence and spread of three major pathogenic meningococcal genoclouds in the African Meningitis Belt (Fekade & Zawde 1992;Salih *et al.* 1990;Haimanot *et al.* 1990;Guibourdenche *et al.* 1996;Niel *et al.* 1997;Riou *et al.* 1996;Lengeler *et al.* 1995;Mohammed *et al.* 2000;Aplogan *et al.* 1997;Hodgson et al. 2002;Gagneux *et al.* 2000;Nicolas *et al.* 2000;Jacobsson *et al.* 2003;Sow *et al.* 2000;Mengistu *et al.* 2003;Nicolas *et al*. 2001;Nicolas *et al.* 2005;Fonkoua *et al.* 2002;Decosas & Koama 2002;Boisier *et al.* 2005). The first two genoclouds were serogroup A subgroup III bacteria, associated with ST5 and ST7. These originated in Asia, invading Africa as result of pandemic spread (Zhu *et al* 2001). ST5 bacteria were recorded in Africa from 1988 until 2001, causing fulminant epidemics, peaking with more than 150'000 cases counted throughout the Meningits Belt in 1996 (Tikhomirov *et al.* 1997). From 1995 onwards, ST7 meningococci started causing disease outbreaks, whereby in some countries an overlap of the ST5 and ST7 waves was recorded. (Nicolas et al. 2001;Nicolas et al 2005). Finally, since 2001 the ST5 genocloud had completely disappeared, while ST5 meningococci continue to cause outbreaks. In Northern Ghana a major epidemic in 1996/97 caused overall more than 18'000 meningitis cases and 1'352 cases in the KND. During a post-epidemic outbreak in the KND in 1998, 50 serogroup A cases were recorded, ST5 was reconfirmed by culture in all 36 isolates. No serogroup A ST5 cases were recorded after 1998. The ST7 wave reached Northern Ghana in 2001 and

caused subsequent outbreaks between 2002 and 2004 with incidence rates of up to 80/100'000 in the KND.

With W135 ST-11 meningococci, belonging to the ET-37 complex, a third clone within a short period of time, caused various outbreaks in Africa. Even though isolated sporadically in Africa since decades, only after a first epidemic in Mecca 2000 W135 ST11 bacteria had a major impact on meningococcal meningitis incidence in the African Meningitis Belt. Between 2001 and 2004 they contributed substantially to major epidemics in Burkina Faso and Niger (Boisier *et al*. 2005;Parent *et al*. 2005;Taha *et al*. 2002), usually in mixed outbreaks with serogroup A ST7. Furthermore, limited outbreaks or case clusters were reported from Chad, Senegal, Cameroon, Benin (Nicolas *et al.* 2005), Sudan and Kenya, (http://www.who.int/csr/don/archive/year/en/). In spite of its vicinity to Burkina Faso, no major W135 outbreak occured in Ghana. Between March 2003 and March 2004, four W135 ST11 cases were reported throughout the country probably only identified as a result of intensive surveillance activities. The bacterial isolates were undistinguishable from epidemic strains from Burkina Faso by Pulsed Field Gel Electrophoresis.

Serogroup X meningococci were responsible for a clonal wave of carriage up to 20% and altogether 15 cases of meningitis in the KND between 1998 and 2003. They have been responsible for substantial meningitis outbreaks in Niger between 1995 and 2000 (Djibo *et al.* 2003). Reports on further outbreaks in 2006 (M.Laforce, personal communication) may indicate that virulence and epidemic potential of this genocloud is also increasing.

Finally, since 2003 the WHO meningococcal reference centre in Ouagadougou recorded that meningococcal outbreaks declined throughout the African Meningitis Belt (WHO 2005). However, a major outbreak in Burkina Faso in 2006 is currently being investigated. Even though, the outbreaks of the last two decades have been analysed in detail and epidemiological and immunological features have been characterized, there is still a lack of understanding on the spread and dissappearance and on the distinct patterns of dispersal of the various meningococcal genoclouds within various regions of the Meningitis Belt.

Recently, an elevated importance of pneumococcal meningitis has been recorded in non-epidemic periods (Parent *et al.* 2005;Gordon *et al.* 2000). Little data is available on the serotype distribution from many regions of the Meningitis Belt. However, in selected areas

serotype 1 (Parent *et al.* 2005) or serotype 5 (Campbell *et al.* 2004) have been associated with the majority of invasive pneumococcal cases. Between 2000 and 2003 also in Northern Ghana an outbreak of pneumococcal meningitis occured. More than 70% of the isolates from KND and representative isolates from neighbouring districts belonged to serotype 1. The outbreak showed features characteristic of meningococcal meningitis such as clonality, seasonality and a broad age spectrum. It has therefore raised some concern about the epidemic potential of *S. pneumoniae*. Since 2004, the incidence of pneumococcal meningitis has declined in the KND.

Also, *Haemophilus influenzae* remains an important causative agent for meningitis in children in countries, where the Hib conjugate vaccine has not been implemented yet. In Mali, yearly incidence rates of over 150/100'000, in Burkina Faso of 97/100'000 in children under 1 years of age have been reported recently (Sow *et al.* 2005;Yaro *et al.* 2006). Overall it has been estimated to be 40-60/100'000 cases in the under 5 year old children (WHOWHO 2003). Ghana is one of the very few African countries where the Hib conjugate vaccine has been implemented in the routine immunization scheme since the year 2000 (personal communication). As a results, in the KND Hib meningitis has virtually dissappeared in the past years and we have only recorded isolated cases.

Natural Immunity and the Dynamics of Colonisation and Disease

Protective immunity to pneumococcal (Snapper C M *et al.* 2004), meningococcal (Goldschneider *et al.* 1969) or Hib meningitis (Kelly *et al.* 2004) depends primarily on bactericidal and/or opzonizing antibodies. These may be elicited by nasopharyngeal colonisation with these bacteria (Goldblatt *et al.* 2005) or due to contact with species sharing cross reacting antigens. In the development of natural immunity to meningococci related colonizers of the human nasopharynx such as *N. lactamica* and *M. catarrhalis* may be involved (Braun *et al.* 2004;Troncoso *et al.* 2000). Cross-reactions have also been observed between pneumococcal polysaccharides and polysaccharides of *Klebsiella* species and non-groupable streptococci (Lee & Koizumi 1981;Lee *et al.* 1984).

Production of anti-pneumococcal antibodies has been shown to begin as early as 6 months of age and increased markedly in the age between 12 and 18 months, coinciding with the age of highest pneumococcal carriage rates and acute otitis media (Soininen *et al.* 2001). Even though meningococcal colonisation usually peaks in older children and

adolescents, young children are frequently colonized by *N. lactamica* that could lead to an early development of anti-meningococcal antibodies (Gold *et al.* 1978). Immunity is usually capsular group specific, but also antibodies against protein antigens may be relevant. Cell surface exposed proteins are currently investigated as candidate antigens for non-serogroup/serotype specific vaccines for *N. meningitidis* and *S. pneumoniae*.

Responses of the mucosal immune system to colonisation leading to the formulation and secretion of IgA may have relevance in the development of natural immunity (Simell *et al.* 2002). Whereas local anticapsular IgA secretion has been reported in infants in response to pneumococcal colonisation and vaccination, salivary IgG has only infrequently been detected in this age group in contrast to older age groups (Simell, Kilpi, & Kayhty 2002;Nieminen *et al.* 1999). Salivary IgG is thought mainly to be derived from serum, but it may also be produced locally (Zhang *et al.* 2002). The contribution of secretory IgA versus IgG in the prevention of colonisation or disease is unclear. Studies with toddlers that have been vaccinated with 9-valent pneumococcal conjugate vaccine showed an inverse correlation between acquisition of new *S. pneumoniae* strains and serotype -specific anti-PS serum IgG (Dagan & Lipsitch 2004). Meningococcal PS vaccines induce mucosal IgA and IgG antibodies in adolescents. Whereas the titers of salivary IgA induced by pure group A/C- PS-vaccine and group C conjugate vaccine were comparable, salivary IgG titers were significantly higher after the conjugate vaccine (Zhang *et al.* 2000). This indicates, that IgA might not be the primary determinant in the protection against colonisation as it is seen after immunization with conjugate vaccines.

It has been repeatedly shown, that the immunogenicity of pneumococcal serotypes varies. This has been seen in response to colonisation (Soininen, Pursiainen, Kilpi, & Kayhty 2001;Goldblatt, Hussain, Andrews, Ashton, Virta, Melegaro, Pebody, George, Soininen, Edmunds, Gay, Kayhty, & Miller 2005) as well as to vaccination (Korkeila *et al.* 2000). Recently, this has also been reported for different meningococcal serogroups. Whereas low carriage prevalence of serogroup A and C have been associated with high anti-A and anti-C antibody titers in a non-vaccinated Nigerian population (Blakebrough *et al.* 1982), 15% serogroup W135 colonisation failed to induce putative protective immune response in the majority of the population in Burkina Faso (Mueller *et al.* 2006).

In Northern Ghana, we have observed a low diversity of the carried meningococcal population. Clonal waves of encapsulated meningococci followed each other and dominated for three or for years carrier- and patient-isolates but dissappeared thereafter. Background colonisation with non-groupable meningococci and isolates of other

serogroups was very low. The resulting low exposure to meningococcal antigens may be one determinant of insufficient antimeningococcal herd immunity and enhanced susceptibility of the population towards newly emerging meningococcal clones every few years. Whereas for unkown reasons there is no stable and diverse meningococcal colonisation in the African study population, around 10% colonisation with a diverse meningococcal population is typically established in human populations in Europe and the US (Yazdankhah & Caugant 2004;Cartwright *et al.* 1987). Meningococcal meningitis epidemics have occured until the second world war also in Europe and Northern America, equally dominated by serogroup A bacteria. The discontinuation and the failure of pandemic clones to cause significant outbreaks in these regions may indicate a change in the host-pathogen relationship since this time. Serogroup A meningococci of ST5 disappeared from the KND in 1999 possibly due to sufficient herd- immunity against colonisation as a result of its epidemic spread. However, it is remarkable, that closely related bacteria of the same serogroup could establish themselves in the district only 1.5 years later. This indicates, that also the development of non-anticapsular immune responses might play a role in the spread and disappearance of meningococcal genoclouds in the Menigitis Belt.

Also the epidemiology of pneumococcal colonisation and disease reveals several differences between developing countries and Europe and Northern America. These are for example reflected in the serotype distribution and in the overall prevalence of colonisation and disease. Whereas in Europe and the US commonly carried serotypes such as 6B, 19F, 23 also dominate in disease, in Africa the highly pathogenic serotypes 1 and 5 are highly prevalent. Interestingly, this shift in pneumococcal serotype distribution seems to be a result of the last 50 to 70 years. Before, the “invasive” serotypes that are common in Africa also caused a higher proportion of invasive disease in the US. A meta-analysis of 15 studies published between 1928 and 1998 in the US, reports a significant increase of the serogroups included in the 7-valent pneumococcal conjugate vaccine, while the fraction of so called “epidemic” serotypes 1,3, and 5 significantly decreased (Feikin & Klugman 2002). The authors suggest that the introduction of antibiotics might have lead to this change in serotype-distribution. Widespread use of antibiotics changed the selective pressure in the human nasopharynx and as a results antibiotic resistance might have emerged more often in the serotypes that were commonly carried than in the “invasive” serotypes (such as 1, 3, and 5) which are carried infrequently. The reduction of their proportion in the population over the last decades might thus be a consequence.

Differences in bacterial colonisation and disease between different human populations, particularly between high and low-income countries, have been attributed to differences in living standards and hygienic conditions. Also unkown genetic differences in human populations have been suggested to impact different pneumococcal colonisation rates or serotype distribution as seen in indigenous populations of Northern America and Australia (Bogaert et al. 2004a;Crook et al. 2004). The ecosystem of the human nasopharynx is very complex and it is almost impossible to attribute a single factor for the observed epidemiological differences. However, I think, that the introduction of vaccines and antibiotics in the second half of the 20th century has markedly influenced the immune system of the host and the selective forces in the bacterial ecosystem of the nasopharynx. Differences in vaccine-coverage and use of antibiotics might therefore also play a role in the regulation of the complex system of colonisation and disease of the various respiratory pathogens.

In Northern Ghana, we have seen that not only meningococcal and *N. lactamica* colonisation but the whole system of bacterial meningitis is highly dynamic. Meningococcal colonisation and disease was dominated by successive clones which persisted for three to four years and dissappeared afterwards. *N. lactamica* colonisation, even though stable over the first few years has decreased continuously since April 2004. A pneumococcal serotype 1 clone caused an outbreak, with a pattern typical of *N. meningitidis*. And an epidemic clone of W135 meningococci, that has caused major epidemics in the neighboring country fails to induce a wave of colonisation and disease due to unknown reasons.

The more we study the dynamics of meningococcal colonisation and disease, the more we appreciate its complexity, but the more new questions arise: What is the virulence potential of newly emerging clones such as the recent serogroup W135, but also serogroup X genocloud? Which factors do promote epidemic spread in certain regions and not in others? What is the significance of potential cofactors?

It will be important to investigate the potential environmental and microbiological factors more indepth for a better understanding of when and how an emerging clone will cause an epidemic or not. The dynamics of nasopharyngeal colonisation is a very important determinant for the dynamics of bacterial menigitis. Implementing prospective case vs colonisation studies like the one presented here in a larger scale covering more regions of the African Meningitis Belt and investigating some of the questions raised above such as the continuing analysis of possible environmental and host- factors as well as genomic and

proteomic analysis of the differences in the pathogen will contribute to the understanding of epidemiological factors associated with epidemic spread of colonisation and disease.

Intervention strategies against bacterial meningitis in Africa

A) Treatment

For the treatment of meningitis patients, apart from pharmakodynamic considerations, in epidemic situations the affordability of the drug is a major concern. Parenteral penicillin or ampicillin, given every 4-6 hours for at least 4 days, are recommended by the WHO guidelines as the drug of choice in the treatment of meningococcal meningitis (WHO 1998). However, during epidemics, the application of single dose oily chloramphenicol, injected intramuscularly, is generally preferred and the standard treatment in most countries of the African Meningitis Belt. The third-generation cephalosporins, ceftriaxone and cefotaxime, are excellent alternatives but up to now have been considerably more expensive. A short course treatment with a single injection of ceftriaxone has recently been evaluated in a non-inferiority trial during an epidemic in Niger. The treatment failure as well as neurological sequelae were comparable in the patients treated with ceftriaxone in comparison to the ones that received the conventional chloramphenicol-treatment (Nathan *et al*. 2005). As the patent protection of ceftriaxone has recently ended, a significant reduction in the cost of treatment can be expected and it can be a powerful alternative in the managment of patients during epidemics.

Chloramphenicol exhibits lower antibacterial activity against *S. pneumoniae* than against *N. meningitidis.* High mortality rates 50% in setting of low resources in contrast to about 20% in industrialized countries are a major threat associated with pneumococcal meningitis. These have been attributed to a delayed CSF sterilisation in pneumococcal meningitis (Koedel *et al.* 2002). Also in the KND, where Chlorampenicol is the standard treatment for suspected meningitis patients, we have observed mortality rates of 44% of pneumococcal meningitis in contrast to 4.3% of meningococcal meningitis between 2000 and 2003. It has been shown, that Ceftriaxone has a better better clinical efficacy as compared to oily chloramphenicol in bacterial meningitis due to *S. pneumoniae* (WHO 1997). Rising incidence rates of pneumococcal meningitis in sub-saharan Africa are a further rationale for a careful re-evaluation of the standard treatment regimen against bacterial meningitis. Yet there is no indication of rising antibiotic resistance against Ceftriaxone in Sub-Saharan Africa. The pneumococcal isolates from Northern Ghana

showed good in-vitro sensitivity against Chloramphenicol, Penicillin G and Cefotaxim. Only isolated strains have shown reduced activity of Chloramphenicol and Cefotaxim respectively.

B) Vaccination

Until recently, only a bivalent A/C PS- vaccine and a tetravalent PS-vaccine against A, C, Y, and W135 were available wordwide against meningococcal disease. In various trials these vaccines were proved safe and immunogenic in older children and adults, with an overall calculated vaccine efficacy of 95% for the prevention of serogroup A disease in the first year after immunization (Patel & Lee 2005). Purified polysaccharides act as T-cell independent antigens due to their highly repetitive chemical structure. Thus, they do not induce long lasting immunological memory, the antibody titers elicited usually only last for a few years. Furthermore, mostly poor immune responses are measured in infants with immature B cell system (Jodar *et al.* 2002). Only serogroup A polysaccharide has been shown immunogenic in infants <2years but protective immunity after one dose only lasts a short time and even after repeated injections booster responses are poor (MacLennan *et al.* 1999;Reingold *et al.* 1985). Immune hyporesponsivness has been reported after repeated vaccination with serogroup C (Jokhdar *et al.* 2004).

In the light of the vast meningococcal meningitis epidemics in the second half of the 1990s, before the introduction of conjugate vaccines, heavy debates have been carried out on the best vaccination strategies in the African Meningitis Belt. It was repeatedly suggested to implement routine vaccination schemes against meningococcal disease, but only in a few countries, this has been adopted. In Egypt a measurable reduction in the serogroup A meningococcal disease after the introduction of a school based immunzation scheme has been reported (Nakhla *et al.* 2005). Also in Benin, routine immunization in two departments with coverage rates of 50-60% of the population, lead to the prevention of large meningococcal meningitis outbreaks between 1994 and 1997 (Hassan *et al.* 1998).

In the KND, yearly vaccination campaigns between 1997 and 2002 targeted the complete district population. Later on smaller campaigns were carried out, especially in schools, creating at least for some age groups a system of routine immunization. Since 2002, up to 80% of our carriage-study members reported to have been vaccinated within the last three years reflecting a high coverage rate in the district. Nevertheless, substantial serogroup A

ST7 meningitis outbreaks occured in the district between 2002 and 2004. Even though some of the patients reported to have been vaccinated, the proportion of vaccine failure is difficult to quantify, as vaccination is not reliably documented with vaccination cards. Furthermore, other unkown environmental or host-pathogen related factors might influence the occurence of outbreaks in the respective regions.Therefore, the data obtained so far is too little and incomplete to assess the population wide effect of routine immunization campaigns for the prevention of meningococcal outbreaks. However, for the majority of the sub-saharan countries of the Meningitis Belt, routine vaccination with multiple dosage in young children, will not be realizable due to financial and organizational contraints.

In 2000, WHO recommendations where published that call for intense meningitis surveillance and stocking of vaccines at the beginning of the dry season. Alert and epidemic thresholds were defined with a specific plan of action to ensure early detection and response to meningococcal meningitis epidemics. Even though these thresholds have been found effective for the prediction of epidemics (Kaninda *et al.* 2000), lack of stocking and infrastructure may still lead to delayed responses by national health authorities in some regions of the Meningitis Belt.

Against pneumococcal disease the so far available 23-valent pneumococcal polysaccharide vaccine covers about 90% of the serotypes associated with invasive disease anywhere. Being also a pure polysaccharide vaccine, it has the same limitations as the meningococcal PS vaccine. Currently, pneumococcal vaccination is recommended by WHO for individuals at increased risk of invasive pneumococcal disease, such as elderly, particularly those living in institutions, patients suffering from chronic organ failure or immunodeficiencies. For the African Meningitis Belt, so far pneumococcal vaccines have not been considered to be included in a standard vaccination scheme due to its failure in infants and the lack of immunological memory. However the large burden of pneumococcal disease in sub-saharan Africa due to pneumonia and meningitis emphasises the urgent need for intervention strategies against *S. pneumoniae* in this part of the world.

Vaccine development

Capsular polysaccharides have been found to induce anti-capsular antibodies mediating complement bactericidal activity and/or opsonophagocytosis (Snapper C M, Colino J, Khan A Q, & Zheng Q W 2004;Goldschneider, Gotschlich, & Artenstein 1969). Thus, multi-

serotype/-serogroup polysaccharide vaccines are in use for both *S. pneumoniae* and *N. meningitidis* (Lesinski & Westerink 2001). To circumvent the limitations associated with pure PS antigens a new generation of vaccines has been developed. The purified capsular polysaccharide or oligosaccharide antigens have been covalently linked to immunogenic carrier proteins, converting the antigen to a thymus dependant antigen and increasing its immunogenicity. The first conjugate vaccine for use in humans was against *H. influenzae* type B (Granoff *et al.* 1993). A dramatic decline of Hib disease occurred where the Hib conjugate vaccine was introduced. Also in Northern Ghana, since the introduction of the conjugate vaccine in 2000, Hib meningitis has lost its importance. Only 15 Hib meningitis cases have been recorded between 2000 and 2002 in the KND, and none since 2003. The ability of Hib conjugate vaccines to reduce nasopharyngeal colonisation by the induction of mucosal immunity is thought to contribute substantially to the >97% reduction in the incidence of HiB infections observed in Finland, the UK and the USA after the incorporation of the vaccine into infant vaccination schemes (Peltola *et al.* 1992;Booy *et al.* 1997;Adams *et al.* 1993). Following the overwhelming success of Hib conjugate vaccines, conjugate vaccines for meningococcal and pneumococcal disease have been developed and in part already introduced.

The first meningococcal conjugate vaccine against serogroup C was introduced into routine immunization in the UK in Novemver 1999 (Jodar et al. 2002). The immediate consequence was an overall decrease in serogroup C invasive disease by over 85% between 1999 and 2001 in the targeted age groups (Balmer *et al.* 2002). Later on it was shown, that in contrast to pure polysaccharide vaccines, this capsule conjugate vaccines also affect carriage and transmission by inducing mucosal immunity (Maiden & Stuart 2002;Ennes *et al.* 1992) and might therefore contribute to the development of herd immunity in the non-vaccinated population. On the other hand, strain replacement, the emergence of escape variants or capsule switching are major concerns associated with the introduction of conjugate vaccines (Maiden & Spratt 1999). Even though no direct indication for capsule switch has been obtained a significant increase in genetic diversity of meningococcal isolates as been recorded in the first years after the introduction (Diggle & Clarke 2005). Also in the KND we do not have indications for frequent capsule switch in the colonisation isolates analysed.

Another concern connected with the introduction of conjugate vaccines is the duration of protection induced. Data collected since the introduction of the conjugated HiB vaccine in

the early 199s indicate that vaccine effectiveness and duration of protection depend on the schedule of immunisation and significant waning of antibody titers occurs in children that have been vaccinated during infancy (Kelly, Moxon, & Pollard 2004). Comparable results have been obtained after the introduction of meningococcal serogroup C conjugate vaccine. Even though good long-term vaccine efficacy was observed in older children and adults, waning antibody titers and reduced vaccine efficacy was measured after one year following infant immunization (Trotter *et al.* 2004;Borrow *et al.* 2002). However immunological memory was demonstrated in children aged 4 years by rapid increase of SBA titers after the administration of a booster dose (Borrow *et al.* 2002). Therefore modified immunization schedules with late booster doses have been suggested. A quadrivalent conjugate vaccine (A,C,Y, W135) has been licensed in the US in January 2005 (Vu *et al.* 2006), but this vaccine is too expensive for routine use in the Meningitis Belt.

To date, no meningococcal conjugate vaccine has been licensed for the the African Meningitis Belt. A serogroup A conjugate vaccine for the use in all age groups and a serogroup A/C formulation for the implementation into the current EPI vaccine using a heptavalent formulation for infants in Africa, are currently tested in clincal trials (Soriano-Gabarro *et al.* 2004). Effective immunization schedules need to be developed carefully, as waning antibody titers in older children and adolescents might have large implications in the prevention of meningococcal disease, as peak incidence rates are reported in these age groups. School based immunzation of older children might therefore have a greater long-term impact than infant immunization with several booster doses where lack of compliance might reduce the herd effect of this vaccine. During the last 20 years we have seen a strong dominance of serogroup A meningococcal meningitis in the Meningitis Belt. On the other hand we have recorded a potential of other serogroups for epidemic spread. Yet, we cannot predict the upcoming epidemiological trends and we can not be sure of the consequences of the introduction of a serogroup A conjugate vaccine and whether it will be as successful as the introduction of the Hib vaccine. Therefore, continuous surveillance needs to monitor the emergence of new clones, possibly of non-vaccine serogroups of Neisseria meningitidis and other species with an epidemic potential such as *S. pneumoniae*.

Pneumococcal conjugate vaccines with up to 11 serotypes linked to a protein carriers have been designed, all of them showed to be safe and immunogenic in infants and

toddlers (Obaro *et al.* 2000;Ahman *et al.* 1996;Shinefield *et al.* 1999;Esposito *et al.* 2005). As too much carrier antigen coupled to protein carrier may impair the antibody response to the polysaccharides (Bogaert *et al.* 2004b), conjugate vaccines are limited in their maximum number of serotypes included. A heptavalent conjugate vaccine has been licensed in the US in 2000 and shortly after in Europe. Formulated according to the most prevalent serotypes in the US and Canada, it has a potential coverage of over 85% of pneumococcal isolates in the US, but only 60-70% for Europe and around 55% for Asia. In Africa this rate might be even lower, between 33% of all strains from adults and up to 67% of pneumococcal isolates from young children could be covered by the 7V-conjugate vaccine (Hausdorff *et al* 2000). During the pneumococcal outbreak in Northern Ghana, this vaccine would have covered less than 10% of all serotyped pneumococcal cases. The nona-valent formulation which is currently undergoing clinical trials reveals a better coverage rate as it comprises in addition to the 7V-vaccine formulation the serotypes 1 and 5. These serotypes are prominent in Africa and Asia causing localized outbreaks such as in Northern Ghana. A randomised vaccine trial of the 9V conjugate vaccine in children in the Gambia has shown a high efficacy against radiological pneumonia, a substantial reduction of all-cause hospital admission and improved child survival (Cutts *et al.* 2005).

The consequences of the introduction of pneumococcal polysaccharide conjugate vaccine has been closely monitored in various studies. It has been described that carriage of vaccine serotypes declined significantly, whereas non-vaccine-serotypes increased and the total carriage rate stayed constant. Furthermore a shift of drug resistance towards the non-vaccine serotypes was observed (Frazao *et al.* 2005). The pathogenic potential of non-vaccine serotypes is not known, however an increase in episodes of acute otitis media has been observed in vaccinated children (Eskola *et al.* 2001). Capsule switch has been reported in several multi drug resistant clones and seems to be a frequent phenomenon in pneumococcal populations. Also during the pneumococcal outbreak in Ghana we have seen indications for a capsule switch from serotype 1 to 14, as the serotype 14 isolates were closely related to the ST217 clonal complex. The introduction of conjugate vaccines comprises a great potential to fight the disease burden caused by this pathogen. On the other hand, it will be unavoidable to closely monitor the changing pneumococcal epidemiology in response to this intervention.

Table 8.1 Serotypes included in current (un-)conjugated pneumococcal PS vaccine formulations

Vaccine-formulation	Serotypes included	Status
PS-Protein : 7-valent	4, 6B, 9V, 14, 18C, 19F, 23F	licensed
PS-Protein :9-valent	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5	in development
PS-Protein :11-valent	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 3, 7F	in development
PS : 23-valent	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 3, 7F, 2, 8, 9N, 10A, 12F, 15B, 17F,19A, 20, 22F, 33F	licensed

Certain proteins of the pneumococcal cell wall may also be accessible for antibodies and represent target structure for protein-based vaccines. Animal studies have also shown a protective effect of immunoglobulins directed against selected pneumococcal cell surface-associated proteins, including pneumococcal surface protein A (pspA), pneumococcal surface adhesin A (psaA), and pneumolysin (Paton *et al.* 1993). These proteins are currently leading candidates for protein based pneumococcal vaccines (Briles *at al.* 2000). Although PspA shows considerable heterogenicity in clinical isolates, Nabors and colleagues found broadly cross-reactive antibodies to heterologues PspA molecules after immunization with a single recombinant PspA preparation (Briles *et al.* 1998;Nabors *et al.* 2000). Surprisingly the pspA gene in ghanaian serogroup1 isolates turned out to be highly conserved, suggesting further investigation of its potential as a vaccine candidate.

The impact and afforability of anti-pneumococcal vaccination with conjugate vaccines in infants and high-risk groups in developing countries with the greatest burden of pneumococcal disease, especially in infants is still under evaluation. Considering, that a full vaccination regimen with conjugate vaccines might be unaffordable in many countries, as an additional cost-effective measure for the protection of newborns during the first three months of life single immunization of pregnant women with the 23valent-polysaccharide vaccine has been suggested after the transfer of maternal antibodies has been shown via the placenta and the breast milk of immunized mothers (Shahid *et al.* 1995;Obaro *et al.* 2004). The development of protein based antipneumococcal vaccines or less complex conjugate vaccines focussed on the serotypes prevalent in Africa, might be an alternative in fighting the high burden of pneumococcal disease in this region.

Outlook

During the course of the long term meningococcal colonisation and disease study performed so far, we have observed the close association between colonisation and disease and found that bacterial meningitis in the African Meningitis Belt is highly dynamic. Even though, through improvement of surveillance activities, outbreaks can now be detected earlier, still emergency vaccination campaigns are often initiated too late to have a major impact on the course of epidemics. This implies the need for further improvement in the implementation of already existing intervention strategies and efficient support of countries suffering from epidemics.

Recently newly emerging meningococcal clones have been detected, in Africa and Asia. Serogroup W135 ST2881 meningococci have been isolated since 2003 from meningitis patients in Niger, Benin and Nigeria. ST2881only shares one MLST locus with ST11, is therefore not closely related (Nicolas *et al*. 2005) and its pathogenic potential not well characterized. In the Anhui province in China, a new serogroup C clone has been responsible for several outbreaks between 2003 and 2005. By MLST and PFGE analysis it was shown, that these bacteria did not belong to the previously known major clonal complexes responsible for the major disease burden in the 20th century, but constituted a new clonal complex, ST4821 (Shao *et al*. 2006). Further spread of these bacteria within China and to other countries needs to be monitored carefully to evaluate the potential risk of causing a future wave of meningococcal meningitis in the African Meningitis Belt and to ensure the preparation of health authorities to answer possible epidemics timely.

Reference List

Achtman M (1995a) Epidemic spread and antigenic variability of Neisseria meningitidis.*Trends Microbiol.* 3, 186-192.

Achtman M (1995b) Global Epidemiology of Meningococcal Disease: In Meningococcal Disease ed. Cartwright Ked, John Wiley & Sons Ltd., West Sussex, pp.159-176.

Achtman M (1997) Microevolution and epidemic spread of serogroup A Neisseria meningitidis--a review.*Gene* 192, 135-140.

Achtman M (2004) Population structure of pathogenic bacteria revisited.*Int J.Med.Microbiol.* 294, 67-73.

Achtman M, Zurth K, Morelli G *et al.* (1999) Yersinia pestis, the cause of plague, is a recently emerged clone of Yersinia pseudotuberculosis.*Proc.Natl.Acad.Sci.U.S.A* 96, 14043-14048.

Adams WG, Deaver KA, Cochi SL *et al.* (1993) Decline of childhood Haemophilus influenzae type b (Hib) disease in the Hib vaccine era.*JAMA* 269, 221-226.

Aguilera JF, Perrocheau A, Meffre C, & Hahne S (2002) Outbreak of serogroup W135 meningococcal disease after the Hajj pilgrimage, Europe, 2000.*Emerg.Infect.Dis.* 8, 761-767.

Ahmad K (2004) Vaccination halts meningitis outbreak in Burkina Faso.*Lancet* 363, 1290.

Ahman H, Kayhty H, Tamminen P *et al.* (1996) Pentavalent pneumococcal oligosaccharide conjugate vaccine PncCRM is well-tolerated and able to induce an antibody response in infants.*Pediatr.Infect.Dis.J.* 15, 134-139.

Ala'Aldeen DA, Neal KR, Ait-Tahar K *et al.* (2000) Dynamics of meningococcal long-term carriage among university students and their implications for mass vaccination.*J.Clin.Microbiol.* 38, 2311-2316.

Aplogan A, Batchassi E, Yakoua Y *et al.* (1997) [An epidemic of meningococcal meningitis in the region of Savanes in Togo in 1997: research and control strategies].*Sante* 7, 384-390.

Balmer P, Borrow R, & Miller E (2002) Impact of meningococcal C conjugate vaccine in the UK.*J.Med.Microbiol.* 51, 717-722.

Bennett DE, Mulhall RM, & Cafferkey MT (2004) PCR-based assay for detection of Neisseria meningitidis capsular serogroups 29E, X, and Z.*J.Clin.Microbiol.* 42, 1764-1765.

Bilukha OO & Rosenstein N (2005) Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP).*MMWR Recomm.Rep.* 54, 1-21.

Binka F NPPJAKMB (1999) Assessing population dynamics in a rural African society; Findings from the Navrongo demographic surveillance system.*Journal of Biosocial Science* 31, 375-391.

Blackwell CC, Weir DM, James VS *et al.* (1990) Secretor status, smoking and carriage of Neisseria meningitidis.*Epidemiol.Infect.* 104, 203-209.

Blakebrough, I. S. Epidemiological and laboratory studies of meningococcal infections in Nigeria. 1979. University of Liverpool, UK.
Ref Type: Thesis/Dissertation

Blakebrough IS, Greenwood BM, Whittle HC, Bradley AK, & Gilles HM (1982) The epidemiology of infections due to Neisseria meningitidis and Neisseria lactamica in a northern Nigerian community.*J.Infect.Dis.* 146, 626-637.

Bogaert D, De Groot R, & Hermans PW (2004a) Streptococcus pneumoniae colonisation: the key to pneumococcal disease.*Lancet Infect.Dis.* 4, 144-154.

Bogaert D, Hermans PW, Adrian PV, Rumke HC, & De Groot R (2004b) Pneumococcal vaccines: an update on current strategies.*Vaccine* 22, 2209-2220.

Bogaert D, van Belkum A, Sluijter M *et al.* (2004c) Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children.*Lancet* 363, 1871-1872.

Boisier P, Djibo S, Sidikou F *et al.* (2005) Epidemiological patterns of meningococcal meningitis in Niger in 2003 and 2004: under the threat of N. meningitidis serogroup W135.*Trop.Med.Int Health* 10, 435-443.

Boisier P, Nicolas P, Djibo S *et al.* (2007) Meningococcal meningitis: unprecedented incidence of serogroup x-related cases in 2006 in niger.*Clin.Infect.Dis.* 44, 657-663.

Booy R, Heath PT, Slack MP, Begg N, & Moxon ER (1997) Vaccine failures after primary immunisation with Haemophilus influenzae type-b conjugate vaccine without booster.*Lancet* 349, 1197-1202.

Borrow R, Goldblatt D, Andrews N *et al.* (2001) Influence of prior meningococcal C polysaccharide vaccination on the response and generation of memory after meningococcal C conjugate vaccination in young children.*J.Infect.Dis.* 184, 377-380.

Borrow R, Goldblatt D, Andrews N *et al.* (2002) Antibody persistence and immunological memory at age 4 years after meningococcal group C conjugate vaccination in children in the United kingdom.*J.Infect.Dis.* 186, 1353-1357.

Boyd EF, Wang FS, Whittam TS, & Selander RK (1996) Molecular genetic relationships of the salmonellae.*Appl.Environ.Microbiol.* 62, 804-808.

Braun JM, Beuth J, Blackwell CC *et al.* (2004) Neisseria meningitidis, Neisseria lactamica and Moraxella catarrhalis share cross-reactive carbohydrate antigens.*Vaccine* 22, 898-908.

Briles DE, Hollingshead S, Brooks-Walter A *et al.* (2000) The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection.*Vaccine* 18, 1707-1711.

Briles DE, Tart RC, Swiatlo E *et al.* (1998) Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA).*Clin.Microbiol.Rev.* 11, 645-657.

Broome CV, Rugh MA, Yada AA *et al.* (1983) Epidemic group C meningococcal meningitis in Upper Volta, 1979.*Bull.World Health Organ* 61, 325-330.

Brueggemann AB, Coffman SL, Rhomberg P *et al.* (2002) Fluoroquinolone resistance in Streptococcus pneumoniae in United States since 1994-1995.*Antimicrob.Agents Chemother.* 46, 680-688.

Brueggemann AB, Griffiths DT, Meats E *et al.* (2003) Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype- and clone-specific differences in invasive disease potential.*J.Infect.Dis.* 187, 1424-1432.

Brueggemann AB, Peto TE, Crook DW *et al.* (2004) Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children.*J.Infect.Dis.* 190, 1203-1211.

Brueggemann AB & Spratt BG (2003) Geographic distribution and clonal diversity of Streptococcus pneumoniae serotype 1 isolates.*J.Clin.Microbiol.* 41, 4966-4970.

Butler JC (2004) Epidemiology of pneumococcal disease: In The pneumococcus eds. Tuomanen E, Mitchell T, Morrison D, & Spratt BG, ASM Press, Washington, pp.148-168.

Cadoz M, Denis F, & Mar ID (1981) [An epidemiological study of purulent meningitis cases admitted to hospital in Dakar, 1970-1979].*Bull.World Health Organ* 59, 575-584.

Campagne G, Schuchat A, Djibo S *et al.* (1999) Epidemiology of bacterial meningitis in Niamey, Niger, 1981-96.*Bull.World Health Organ* 77, 499-508.

Campbell JD, Kotloff KL, Sow SO *et al.* (2004) Invasive pneumococcal infections among hospitalized children in Bamako, Mali.*Pediatr.Infect.Dis.J.* 23, 642-649.

Cartwright K (1995) Meningococcal Carriage and Disease: In Meningococcal Disease ed. Cartwright Ked, John Wiley & Sons, West Sussex, pp.115-147.

Cartwright K, Noah N, & Peltola H (2001) Meningococcal disease in Europe: epidemiology, mortality, and prevention with conjugate vaccines. Report of a European advisory board meeting Vienna, Austria, 6-8 October, 2000.*Vaccine* 19, 4347-4356.

Cartwright KA, Stuart JM, Jones DM, & Noah ND (1987) The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica.*Epidemiol.Infect.* 99, 591-601.

Castle SC (2000) Clinical relevance of age-related immune dysfunction.*Clin.Infect.Dis.* 31, 578-585.

Caugant DA, Hoiby EA, Magnus P *et al.* (1994) Asymptomatic carriage of Neisseria meningitidis in a randomly sampled population.*J.Clin.Microbiol.* 32, 323-330.

Caugant DA, Kristiansen BE, Froholm LO, Bovre K, & Selander RK (1988) Clonal diversity of Neisseria meningitidis from a population of asymptomatic carriers.*Infect.Immun.* 56, 2060-2068.

Chonghaile CN (2002) Meningitis in Africa--tackling W135.*Lancet* 360, 2054-2055.

Claus H, Maiden MC, Maag R, Frosch M, & Vogel U (2002) Many carried meningococci lack the genes required for capsule synthesis and transport.*Microbiology* 148, 1813-1819.

Claus H, Maiden MC, Wilson DJ *et al.* (2005) Genetic analysis of meningococci carried by children and young adults.*J.Infect.Dis.* 191, 1263-1271.

Coffey TJ, Enright MC, Daniels M *et al.* (1998) Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of Streptococcus pneumoniae.*Mol.Microbiol.* 27, 73-83.

Coles CL, Rahmathullah L, Kanungo R *et al.* (2002) Nasopharyngeal carriage of resistant pneumococci in young South Indian infants.*Epidemiol.Infect.* 129, 491-497.

Cortese MM, Wolff M, Almeido-Hill J *et al.* (1992) High incidence rates of invasive pneumococcal disease in the White Mountain Apache population.*Arch.Intern.Med.* 152, 2277-2282.

Crain MJ, Waltman WD, Turner JS *et al.* (1990) Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of Streptococcus pneumoniae.*Infect.Immun.* 58, 3293-3299.

Crook DW, Brueggemann AB, Sleeman KL, & Peto TE (2004) Pneumococcal Carriage: In The pneumococcus 1 edn. eds. Tuomanen E, Mitchell TJ, Morrison D, & Spratt BG, ASM Press, Washington, pp.136-147.

Cutts FT, Zaman SM, Enwere G *et al.* (2005) Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial.*Lancet* 365, 1139-1146.

Dagan R, Gradstein S, Belmaker I *et al.* (2000) An outbreak of Streptococcus pneumoniae serotype 1 in a closed community in southern Israel.*Clin.Infect.Dis.* 30, 319-321.

Dagan R & Lipsitch M (2004) Changing the Ecology of Pneumococci with Antibiotics and Vaccines: In The Pneumococcus 1 edn. eds. Tuomanen E, Mitchell T, Morrison D, & Spratt BG, ASM Press, Washington, pp.283-313.

Decosas J & Koama JB (2002) Chronicle of an outbreak foretold: meningococcal meningitis W135 in Burkina Faso.*Lancet Infect.Dis.* 2, 763-765.

Decousser JW, Pina P, Viguier F *et al.* (2004) Invasive Streptococcus pneumoniae in France: antimicrobial resistance, serotype, and molecular epidemiology findings from a monthly national study in 2000 to 2002.*Antimicrob.Agents Chemother.* 48, 3636-3639.

Denis F, Rey JL, Amadou A *et al.* (1982) Emergence of meningococcal meningitis caused by W 135 subgroup in Africa.*Lancet* 2, 1335-1336.

Diggle MA & Clarke SC (2005) Increased genetic diversity of Neisseria meningitidis isolates after the introduction of meningococcal serogroup C polysaccharide conjugate vaccines.*J.Clin.Microbiol.* 43, 4649-4653.

Djibo S, Nicolas P, Alonso JM *et al.* (2003) Outbreaks of serogroup X meningococcal meningitis in Niger 1995-2000.*Trop.Med.Int.Health* 8, 1118-1123.

Dowell SF, Whitney CG, Wright C, Rose CE, Jr., & Schuchat A (2003) Seasonal patterns of invasive pneumococcal disease.*Emerg.Infect.Dis.* 9, 573-579.

Dowson CG (2004) What is a Pneumococcus?: In The pneumococcus 1 edn. eds. Tuomanen E, Mitchell T, Morrison D, & Spratt BG, ASM Press, Washington, pp.3-14.

Dworkin MS, Ward JW, Hanson DL, Jones JL, & Kaplan JE (2001) Pneumococcal disease among human immunodeficiency virus-infected persons: incidence, risk factors, and impact of vaccination.*Clin.Infect.Dis.* 32, 794-800.

Ennes HS, McRoberts JA, Hyman PE, & Snape WJ, Jr. (1992) Characterization of colonic circular smooth muscle cells in culture.*Am.J.Physiol* 263, G365-G370.

Enright MC & Spratt BG (1998) A multilocus sequence typing scheme for Streptococcus pneumoniae: identification of clones associated with serious invasive disease.*Microbiology* 144 (Pt 11), 3049-3060.

Eskola J, Kilpi T, Palmu A *et al.* (2001) Efficacy of a pneumococcal conjugate vaccine against acute otitis media.*N.Engl.J.Med.* 344, 403-409.

Esposito S, Pugni L, Bosis S *et al.* (2005) Immunogenicity, safety and tolerability of heptavalent pneumococcal conjugate vaccine administered at 3, 5 and 11 months post-natally to pre- and full-term infants.*Vaccine* 23, 1703-1708.

Etienne J, Sperber G, Adamou A, & Picq JJ (1990) [Epidemiological notes: meningococcal meningitis of serogroup X in Niamey (Niger)].*Med.Trop.(Mars.)* 50, 227-229.

Evans JR, Artenstein MS, & Hunter DH (1968) Prevalence of meningococcal serogroups and description of three new groups.*Am.J.Epidemiol.* 87, 643-646.

Faden H, Stanievich J, Brodsky L, Bernstein J, & Ogra PL (1990) Changes in nasopharyngeal flora during otitis media of childhood.*Pediatr.Infect.Dis.J.* 9, 623-626.

Feikin DR, Davis M, Nwanyanwu OC *et al.* (2003) Antibiotic resistance and serotype distribution of Streptococcus pneumoniae colonizing rural Malawian children.*Pediatr.Infect.Dis.J.* 22, 564-567.

Feikin DR & Klugman KP (2002) Historical changes in pneumococcal serogroup distribution: implications for the era of pneumococcal conjugate vaccines.*Clin.Infect.Dis.* 35, 547-555.

Feil EJ (2004) Small change: keeping pace with microevolution.*Nat.Rev.Microbiol.* 2, 483-495.

Feil EJ & Enright MC (2004) Analyses of clonality and the evolution of bacterial pathogens.*Curr.Opin.Microbiol.* 7, 308-313.

Feil EJ, Holmes EC, Bessen DE *et al.* (2001) Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences.*Proc.Natl.Acad.Sci.U.S.A* 98, 182-187.

Feil EJ, Li BC, Aanensen DM, Hanage WP, & Spratt BG (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data.*J.Bacteriol.* 186, 1518-1530.

Feil EJ, Smith JM, Enright MC, & Spratt BG (2000) Estimating recombinational parameters in Streptococcus pneumoniae from multilocus sequence typing data.*Genetics* 154, 1439-1450.

Fekade D & Zawde D (1992) Epidemic meningococcal meningitis in adult Ethiopians in Addis Abeba, Ethiopia, 1988.*Ethiop.Med.J.* 30, 135-142.

Fernandez S, Arreaza L, Santiago I *et al.* (2003) Impact of meningococcal vaccination with combined serogroups A and C polysaccharide vaccine on carriage of Neisseria meningitidis C.*J.Med.Microbiol.* 52, 75-77.

Fonkoua MC, Taha MK, Nicolas P *et al.* (2002) Recent increase in meningitis Caused by Neisseria meningitidis serogroups A and W135, Yaounde, Cameroon.*Emerg.Infect.Dis.* 8, 327-329.

Frazao N, Brito-Avo A, Simas C *et al.* (2005) Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of Streptococcus pneumoniae in healthy children attending day-care centers in Lisbon.*Pediatr.Infect.Dis.J.* 24, 243-252.

Gagneux S, Hodgson A, Ehrhard I *et al.* (2000) Microheterogeneity of serogroup A (subgroup III) Neisseria meningitidis during an outbreak in northern Ghana.*Trop.Med.Int.Health* 5, 280-287.

Gagneux S, Wirth T, Hodgson A *et al.* (2002a) Clonal groupings in serogroup X Neisseria meningitidis.*Emerg.Infect.Dis.* 8, 462-466.

Gagneux SP, Hodgson A, Smith TA *et al.* (2002b) Prospective study of a serogroup X Neisseria meningitidis outbreak in northern Ghana.*J.Infect.Dis.* 185, 618-626.

Givon-Lavi N, Fraser D, Porat N, & Dagan R (2002) Spread of Streptococcus pneumoniae and antibiotic-resistant S. pneumoniae from day-care center attendees to their younger siblings.*J.Infect.Dis.* 186, 1608-1614.

Go MF, Kapur V, Graham DY, & Musser JM (1996) Population genetic analysis of Helicobacter pylori by multilocus enzyme electrophoresis: extensive allelic diversity and recombinational population structure.*J.Bacteriol.* 178, 3934-3938.

Gold R, Goldschneider I, Lepow ML, Draper TF, & Randolph M (1978) Carriage of Neisseria meningitidis and Neisseria lactamica in infants and children.*J.Infect.Dis.* 137, 112-121.

Goldblatt D, Hussain M, Andrews N *et al.* (2005) Antibody responses to nasopharyngeal carriage of Streptococcus pneumoniae in adults: a longitudinal household study.*J.Infect.Dis.* 192, 387-393.

Goldschneider I, Gotschlich EC, & Artenstein MS (1969) Human immunity to the meningococcus. I. The role of humoral antibodies.*J.Exp.Med.* 129, 1307-1326.

Gonzalez BE, Hulten KG, Kaplan SL, & Mason EO, Jr. (2004) Clonality of Streptococcus pneumoniae serotype 1 isolates from pediatric patients in the United States.*J.Clin.Microbiol.* 42, 2810-2812.

Gordon SB, Walsh AL, Chaponda M *et al.* (2000) Bacterial meningitis in Malawian adults: pneumococcal disease is common, severe, and seasonal.*Clin.Infect.Dis.* 31, 53-57.

Grahlow WD, Ocklitz HW, & Mochmann H (1986) Meningococcal infections in the German Democratic Republic 1971-1984.*Infection* 14, 286-288.

Granoff DM, Holmes SJ, Osterholm MT *et al.* (1993) Induction of immunologic memory in infants primed with Haemophilus influenzae type b conjugate vaccines.*J.Infect.Dis.* 168, 663-671.

Gratten M, Morey F, Dixon J *et al.* (1993) An outbreak of serotype 1 Streptococcus pneumoniae infection in central Australia.*Med.J.Aust.* 158, 340-342.

Gray BM, Converse GM, III, & Dillon HC, Jr. (1980) Epidemiologic studies of Streptococcus pneumoniae in infants: acquisition, carriage, and infection during the first 24 months of life.*J.Infect.Dis.* 142, 923-933.

Greenwood B (1987) The Epidemiology of Acute Bacterial Meningitis in Tropical Africa: In Bacterial Meningitis, Academic Press Inc. (London) Ltd., pp.61-91.

Greenwood B (1999) Manson Lecture. Meningococcal meningitis in Africa.*Trans.R.Soc.Trop.Med.Hyg.* 93, 341-353.

Greenwood B (2004) Meningitis: In Principles of Medicine in Africa 3d edn. eds. Parry E, Godfry R, Mabey D, & Gill G. Campbridge, pp.305-315.

Greenwood BM, Blakebrough IS, Bradley AK, Wali S, & Whittle HC (1984) Meningococcal disease and season in sub-Saharan Africa.*Lancet* 1, 1339-1342.

Greenwood BM, Bradley AK, Cleland PG *et al.* (1979) An epidemic of meningococcal infection at Zaria, Northern Nigeria. 1. General epidemiological features.*Trans.R.Soc.Trop.Med.Hyg.* 73, 557-562.

Greenwood BM, Greenwood AM, Bradley AK *et al.* (1987) Factors influencing susceptibility to meningococcal disease during an epidemic in The Gambia, West Africa.*J.Infect.* 14, 167-184.

Greenwood BM, Hassan-King M, Onyemelukwe G *et al.* (1980) Pneumococcal serotypes in West Africa.*Lancet* 1, 360.

Griffiss JM (1982) Epidemic meningococcal disease: synthesis of a hypothetical immunoepidemiologic model.*Rev.Infect.Dis.* 4, 159-172.

Grundmann H, Hori S, & Tanner G (2001) Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms.*J.Clin.Microbiol.* 39, 4190-4192.

Guibourdenche M, Hoiby EA, Riou JY *et al.* (1996) Epidemics of serogroup A Neisseria meningitidis of subgroup III in Africa, 1989-94.*Epidemiol.Infect.* 116, 115-120.

Hahne S, Handford S, & Ramsay M (2002a) W135 meningococcal carriage in Hajj pilgrims.*Lancet* 360, 2089-2090.

Hahne SJ, Gray SJ, Jean F *et al.* (2002b) W135 meningococcal disease in England and Wales associated with Hajj 2000 and 2001.*Lancet* 359, 582-583.

Haimanot RT, Caugant DA, Fekadu D *et al.* (1990) Characteristics of serogroup A Neisseria meningitidis responsible for an epidemic in Ethiopia, 1988-89.*Scand.J.Infect.Dis.* 22, 171-174.

Hammerschmidt S, Muller A, Sillmann H *et al.* (1996) Capsule phase variation in Neisseria meningitidis serogroup B by slipped-strand mispairing in the polysialyltransferase gene

(siaD): correlation with bacterial invasion and the outbreak of meningococcal disease.*Mol.Microbiol.* 20, 1211-1220.

Hansman D (1983) Meningococcal disease in South Australia: incidence and serogroup distribution 1971-1980.*J.Hyg.(Lond)* 90, 49-54.

Hart C.A. & Cuevas L.E. (2003) Bacterial Meningitis: In Manson's Tropical Diseases 21 edn. eds. Cook G.C. & Zumla A., Elsevier Science Limited, pp.981-994.

Hassan J, Massougbodji A, Chippaux JP, Massit B, & Josse R (1998) Meningococcal immunisation and protection from epidemics.*Lancet* 352, 407-408.

Hassan-King MK, Wall RA, & Greenwood BM (1988) Meningococcal carriage, meningococcal disease and vaccination.*J.Infect.* 16, 55-59.

Hausdorff WP, Bryant J, Paradiso PR, & Siber GR (2000) Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I.*Clin.Infect.Dis.* 30, 100-121.

Henriques NB, Kalin M, Ortqvist A *et al.* (2001) Dynamics of penicillin-susceptible clones in invasive pneumococcal disease.*J.Infect.Dis.* 184, 861-869.

Hodgson A., Smith, T., Gagneux, S., Enos K.E., Adjuik, M., Pluschke, G., Binka, F., and Genton, B. Meningococcal meningitis in northern Ghana: epidemiological features of the 1997 outbreak in the Kassena-Nankana district. Meningococcal Meningitis in Northern Ghana: Epidemiological and Clinical Features, Risk Factors, Survival and Sequelae. 19-39. 2002. University of Basel. 2002.
Ref Type: Thesis/Dissertation

Hollingshead SK, Becker R, & Briles DE (2000) Diversity of PspA: mosaic genes and evidence for past recombination in Streptococcus pneumoniae.*Infect.Immun.* 68, 5889-5900.

Isenberg Henry D.(ed.) (1998) Essential Procedures for Clinical Microbiology, American Society for Microbiology .

Jacobsson S, Issa M, Unemo M *et al.* (2003) Molecular characterisation of group A Neisseria meningitidis isolated in Sudan 1985-2001.*APMIS* 111, 1060-1066.

Jefferies JM, Smith A, Clarke SC, Dowson C, & Mitchell TJ (2004) Genetic analysis of diverse disease-causing pneumococci indicates high levels of diversity within serotypes and capsule switching.*J.Clin.Microbiol.* 42, 5681-5688.

Jodar L, Feavers IM, Salisbury D, & Granoff DM (2002) Development of vaccines against meningococcal disease.*Lancet* 359, 1499-1508.

Jokhdar H, Borrow R, Sultan A *et al.* (2004) Immunologic hyporesponsiveness to serogroup C but not serogroup A following repeated meningococcal A/C polysaccharide vaccination in Saudi Arabia.*Clin.Diagn.Lab Immunol.* 11, 83-88.

Jolley KA, Kalmusova J, Feil EJ *et al.* (2000) Carried meningococci in the Czech Republic: a diverse recombining population.*J.Clin.Microbiol.* 38, 4492-4498.

Jolley KA, Wilson DJ, Kriz P, McVean G, & Maiden MC (2005) The influence of mutation, recombination, population history, and selection on patterns of genetic diversity in Neisseria meningitidis.*Mol.Biol.Evol.* 22, 562-569.

Kaninda AV, Belanger F, Lewis R *et al.* (2000) Effectiveness of incidence thresholds for detection and control of meningococcal meningitis epidemics in northern Togo.*Int J.Epidemiol.* 29, 933-940.

Kelly DF, Moxon ER, & Pollard AJ (2004) Haemophilus influenzae type b conjugate vaccines.*Immunology* 113, 163-174.

Kim JO & Weiser JN (1998) Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of Streptococcus pneumoniae.*J.Infect.Dis.* 177, 368-377.

Klugman KP (2002) The successful clone: the vector of dissemination of resistance in Streptococcus pneumoniae.*J.Antimicrob.Chemother.* 50 Suppl S2, 1-5.

Koedel U, Scheld WM, & Pfister HW (2002) Pathogenesis and pathophysiology of pneumococcal meningitis.*Lancet Infect.Dis.* 2, 721-736.

Korkeila M, Lehtonen H, Ahman H *et al.* (2000) Salivary anti-capsular antibodies in infants and children immunised with Streptococcus pneumoniae capsular polysaccharides conjugated to diphtheria or tetanus toxoid.*Vaccine* 18, 1218-1226.

Kwara A, Adegbola RA, Corrah PT *et al.* (1998) Meningitis caused by a serogroup W135 clone of the ET-37 complex of Neisseria meningitidis in West Africa.*Trop.Med.Int.Health* 3, 742-746.

Lapeyssonnie L (1963) La méningite cérébro-spinale en Afrique.*Bull.World Health Organ* 28, SUPPL-114.

Lee CJ & Koizumi K (1981) Immunochemical relations between pneumococcal group 19 and Klebsiella capsular polysaccharides.*J.Immunol.* 127, 1619-1623.

Lee CJ, Koizumi K, Henrichsen J *et al.* (1984) Capsular polysaccharides of nongroupable streptococci that cross-react with pneumococcal group 19.*J.Immunol.* 133, 2706-2711.

Leino T, Auranen K, Jokinen J *et al.* (2001) Pneumococcal carriage in children during their first two years: important role of family exposure.*Pediatr.Infect.Dis.J.* 20, 1022-1027.

Lengeler C, Kessler W, & Daugla D (1995) The 1990 meningococcal meningitis epidemic of Sarh (Chad): how useful was an earlier mass vaccination?*Acta Trop.* 59, 211-222.

Lesinski GB & Westerink MA (2001) Novel vaccine strategies to T-independent antigens.*J.Microbiol.Methods* 47, 135-149.

Lingappa JR, Al Rabeah AM, Hajjeh R *et al.* (2003) Serogroup W-135 Meningococcal Disease during the Hajj, 2000.*Emerg.Infect.Dis.* 9, 665-671.

Linz B, Schenker M, Zhu P, & Achtman M (2000) Frequent interspecific genetic exchange between commensal Neisseriae and Neisseria meningitidis.*Mol.Microbiol.* 36, 1049-1058.

Lipsitch M (1999) Bacterial vaccines and serotype replacement: lessons from Haemophilus influenzae and prospects for Streptococcus pneumoniae.*Emerg.Infect.Dis.* 5, 336-345.

Lloyd-Evans N, O'Dempsey TJ, Baldeh I *et al.* (1996) Nasopharyngeal carriage of pneumococci in Gambian children and in their families.*Pediatr.Infect.Dis.J.* 15, 866-871.

Lysenko ES, Ratner AJ, Nelson AL, & Weiser JN (2005) The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces.*PLoS.Pathog.* 1, e1.

MacLennan J, Obaro S, Deeks J *et al.* (1999) Immune response to revaccination with meningococcal A and C polysaccharides in Gambian children following repeated immunisation during early childhood.*Vaccine* 17, 3086-3093.

Maiden MC (2004) Dynamics of bacterial carriage and disease: lessons from the meningococcus.*Adv.Exp.Med.Biol.* 549, 23-29.

Maiden MC, Bygraves JA, Feil E *et al.* (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms.*Proc.Natl.Acad.Sci.U.S A* 95, 3140-3145.

Maiden MC, Malorny B, & Achtman M (1996) A global gene pool in the neisseriae.*Mol.Microbiol.* 21, 1297-1298.

Maiden MC & Spratt BG (1999) Meningococcal conjugate vaccines: new opportunities and new challenges.*Lancet* 354, 615-616.

Maiden MC & Stuart JM (2002) Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination.*Lancet* 359, 1829-1831.

Makela PH, Takala AK, Peltola H, & Eskola J (1992) Epidemiology of invasive Haemophilus influenzae type b disease.*J.Infect.Dis.* 165 Suppl 1, S2-S6.

Mar ID, Denis F, & Cadoz M (1979) [Epidemiologic features of pneumococcal meningitis in Africa. Clinical and serotypical aspects (author's transl)].*Pathol.Biol.(Paris)* 27, 543-548.

Mayer LW, Reeves MW, Al Hamdan N *et al.* (2002) Outbreak of W135 meningococcal disease in 2000: not emergence of a new W135 strain but clonal expansion within the electophoretic type-37 complex.*J.Infect.Dis.* 185, 1596-1605.

McEllistrem MC, Stout JE, & Harrison LH (2000) Simplified protocol for pulsed-field gel electrophoresis analysis of Streptococcus pneumoniae.*J.Clin.Microbiol.* 38, 351-353.

McGahey K (1905) Report on an outbreak of epidemic cerebro-spinal meningitis in Zungeru during February and March 1905.*Journal of Tropical Medicine* 8, 210-216.

Mengistu G, Mitiku K, & Teferi W (2003) Analysis and reporting of meningococcal meningitis epidemic in north Gondar 2001-2002.*Ethiop.Med.J.* 41, 319-331.

Mohammed I, Nasidi A, Alkali AS *et al.* (2000) A severe epidemic of meningococcal meningitis in Nigeria, 1996.*Trans.R.Soc.Trop.Med.Hyg.* 94, 265-270.

Molesworth AM, Thomson MC, Connor SJ *et al.* (2002) Where is the meningitis belt? Defining an area at risk of epidemic meningitis in Africa.*Trans.R.Soc.Trop.Med.Hyg.* 96, 242-249.

Montgomery JM, Lehmann D, Smith T *et al.* (1990) Bacterial colonization of the upper respiratory tract and its association with acute lower respiratory tract infections in Highland children of Papua New Guinea.*Rev.Infect.Dis.* 12 Suppl 8, S1006-S1016.

Moore PS (1992) Meningococcal meningitis in sub-Saharan Africa: a model for the epidemic process.*Clin.Infect.Dis.* 14, 515-525.

Morelli G, Malorny B, Muller K *et al.* (1997) Clonal descent and microevolution of Neisseria meningitidis during 30 years of epidemic spread.*Mol.Microbiol.* 25, 1047-1064.

Mueller JE, Yaro S, Traore Y *et al.* (2006) Neisseria meningitidis serogroups A and W-135: carriage and immunity in Burkina Faso, 2003.*J.Infect.Dis.* 193, 812-820.

Musher DM (2006) Pneumococcal serotypes and virulence.*J.Infect.Dis.* 193, 477-478.

Musser JM, Bemis DA, Ishikawa H, & Selander RK (1987) Clonal diversity and host distribution in Bordetella bronchiseptica.*J.Bacteriol.* 169, 2793-2803.

Musser JM, Kroll JS, Moxon ER, & Selander RK (1988) Clonal population structure of encapsulated Haemophilus influenzae.*Infect.Immun.* 56, 1837-1845.

Nabors GS, Braun PA, Herrmann DJ *et al.* (2000) Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules.*Vaccine* 18, 1743-1754.

Nakhla I, Frenck RW, Jr., Teleb NA *et al.* (2005) The changing epidemiology of meningococcal meningitis after introduction of bivalent A/C polysaccharide vaccine into school-based vaccination programs in Egypt.*Vaccine* 23, 3288-3293.

Nathan N, Borel T, Djibo A *et al.* (2005) Ceftriaxone as effective as long-acting chloramphenicol in short-course treatment of meningococcal meningitis during epidemics: a randomised non-inferiority study.*Lancet* 366, 308-313.

Nicolas P, Decousset L, Riglet V *et al.* (2001) Clonal expansion of sequence type (ST-)5 and emergence of ST-7 in serogroup A meningococci, Africa.*Emerg.Infect.Dis.* 7, 849-854.

Nicolas P, Norheim G, Garnotel E, Djibo S, & Caugant DA (2005) Molecular Epidemiology of Neisseria meningitidis Isolated in the African Meningitis Belt between 1988 and 2003 Shows Dominance of Sequence Type 5 (ST-5) and ST-11 Complexes.*J.Clin.Microbiol.* 43, 5129-5135.

Nicolas P, Raphenon G, Guibourdenche M *et al.* (2000) The 1998 Senegal epidemic of meningitis was due to the clonal expansion of A:4:P1.9, clone III-1, sequence type 5 Neisseria meningitidis strains.*J.Clin.Microbiol.* 38, 198-200.

Niel L, Lamarque D, Coue JC *et al.* (1997) [Chronical of a declared meningococcal meningitis epidemic (Goma, Zaire, August 1994)].*Bull.Soc.Pathol.Exot.* 90, 299-302.

Nieminen T, Kayhty H, Leroy O, & Eskola J (1999) Pneumococcal conjugate vaccination in toddlers: mucosal antibody response measured as circulating antibody-secreting cells and as salivary antibodies.*Pediatr.Infect.Dis.J.* 18, 764-772.

Nyarko, P., Wontuo, P., Nazzar, A., Phillips, J., Ngom, P., and Binka, F. Comparing Mortality Patterns at INDEPTH Sites. Navrongo DSS, Ghana. INDEPTH network. Population and Health in Developing Countries. (1), 247-256. 2002. Ottawa, ON, International Development Research Centre. 2002.
Ref Type: Serial (Book,Monograph)

O'Dempsey TJ, McArdle TF, Lloyd-Evans N *et al.* (1996) Pneumococcal disease among children in a rural area of west Africa.*Pediatr.Infect.Dis.J.* 15, 431-437.

O'Rourke M & Stevens E (1993) Genetic structure of Neisseria gonorrhoeae populations: a non-clonal pathogen.*J.Gen.Microbiol.* 139, 2603-2611.

Obaro SK, Adegbola RA, Chang I *et al.* (2000) Safety and immunogenicity of a nonavalent pneumococcal vaccine conjugated to CRM197 administered simultaneously but in a separate syringe with diphtheria, tetanus and pertussis vaccines in Gambian infants.*Pediatr.Infect.Dis.J.* 19, 463-469.

Obaro SK, Deubzer HE, Newman VO *et al.* (2004) Serotype-specific pneumococcal antibodies in breast milk of Gambian women immunized with a pneumococcal polysaccharide vaccine during pregnancy.*Pediatr.Infect.Dis.J.* 23, 1023-1029.

Orvelid P, Backman A, & Olcen P (1999) PCR identification of the group A Neisseria meningitidis gene in cerebrospinal fluid.*Scand.J.Infect.Dis.* 31, 481-483.

Palmer A, Weber M, Bojang K, McKay T, & Adegbola R (1999) Acute bacterial meningitis in The Gambia: a four-year review of paediatric hospital admissions.*J.Trop.Pediatr.* 45, 51-53.

Parent DC, I, Traore Y, Gessner BD *et al.* (2005) Bacterial meningitis in Burkina Faso: surveillance using field-based polymerase chain reaction testing.*Clin.Infect.Dis.* 40, 17-25.

Pastor JM, Fe A, Gomis M, & Gil D (1985) [Meningococcal meningitis caused by Neisseria meningitidis of the X serogroup].*Med.Clin.(Barc.)* 85, 208-209.

Pastor P, Medley F, & Murphy TV (1998) Invasive pneumococcal disease in Dallas County, Texas: results from population-based surveillance in 1995.*Clin.Infect.Dis.* 26, 590-595.

Patel M & Lee CK (2005) Polysaccharide vaccines for preventing serogroup A meningococcal meningitis.*Cochrane.Database.Syst.Rev.* CD001093.

Paton JC, Andrew PW, Boulnois GJ, & Mitchell TJ (1993) Molecular analysis of the pathogenicity of Streptococcus pneumoniae: the role of pneumococcal proteins.*Annu.Rev.Microbiol.* 47, 89-115.

Peltola H (2001) Burden of meningitis and other severe bacterial infections of children in africa: implications for prevention.*Clin.Infect.Dis.* 32, 64-75.

Peltola H, Kilpi T, & Anttila M (1992) Rapid disappearance of Haemophilus influenzae type b meningitis after routine childhood immunisation with conjugate vaccines.*Lancet* 340, 592-594.

Pericone CD, Overweg K, Hermans PW, & Weiser JN (2000) Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract.*Infect.Immun.* 68, 3990-3997.

Poolman JT, van der Ley P.A., & Tommassen J. (1995) Surface Structures and Secreted Products of Meningococci: In Meningococcal Disease ed. Cartwright Ked, John Wiley & Sons Ltd., West Sussex, pp.21-34.

Popovic T, Sacchi CT, Reeves MW *et al.* (2000) Neisseria meningitidis serogroup W135 isolates associated with the ET-37 complex.*Emerg.Infect.Dis.* 6, 428-429.

Porat N, Trefler R, & Dagan R (2001) Persistence of two invasive Streptococcus pneumoniae clones of serotypes 1 and 5 in comparison to that of multiple clones of serotypes 6B and 23F among children in southern Israel.*J.Clin.Microbiol.* 39, 1827-1832.

Ramirez M & Tomasz A (1999) Acquisition of new capsular genes among clinical isolates of antibiotic-resistant Streptococcus pneumoniae.*Microb.Drug Resist.* 5, 241-246.

Ramsay ME, Andrews NJ, Trotter CL, Kaczmarski EB, & Miller E (2003) Herd immunity from meningococcal serogroup C conjugate vaccination in England: database analysis.*BMJ* 326, 365-366.

Reingold AL, Broome CV, Hightower AW *et al.* (1985) Age-specific differences in duration of clinical protection after vaccination with meningococcal polysaccharide A vaccine.*Lancet* 2, 114-118.

Riou JY, Djibo S, Sangare L *et al.* (1996) A predictable comeback: the second pandemic of infections caused by Neisseria meningitidis serogroup A subgroup III in Africa, 1995.*Bull.World Health Organ* 74, 181-187.

Robbins JB, Schneerson R, & Gotschlich EC (2005) Surveillance for bacterial meningitis by means of polymerase chain reaction.*Clin.Infect.Dis.* 40, 26-27.

Robinson KA, Baughman W, Rothrock G *et al.* (2001) Epidemiology of invasive Streptococcus pneumoniae infections in the United States, 1995-1998: Opportunities for prevention in the conjugate vaccine era.*JAMA* 285, 1729-1735.

Ryan NJ & Hogan GR (1980) Severe meningococcal disease caused by serogroups X and Z.*Am.J.Dis.Child* 134, 1173.

Salih MA, Ahmed HS, Karrar ZA *et al.* (1990) Features of a large epidemic of group A meningococcal meningitis in Khartoum, Sudan in 1988.*Scand.J.Infect.Dis.* 22, 161-170.

Sandgren A, Albiger B, Orihuela CJ *et al.* (2005) Virulence in mice of pneumococcal clonal types with known invasive disease potential in humans.*J.Infect.Dis.* 192, 791-800.

Sandgren A, Sjostrom K, Olsson-Liljequist B *et al.* (2004) Effect of clonal and serotype-specific properties on the invasive capacity of Streptococcus pneumoniae.*J.Infect.Dis.* 189, 785-796.

Savory EC, Cuevas LE, Yassin MA *et al.* (2006) Evaluation of the meningitis epidemics risk model in Africa.*Epidemiol.Infect.* 1-13.

Selander RK, McKinney RM, Whittam TS *et al.* (1985) Genetic structure of populations of Legionella pneumophila.*J.Bacteriol.* 163, 1021-1037.

Shahid NS, Steinhoff MC, Hoque SS *et al.* (1995) Serum, breast milk, and infant antibody after maternal immunisation with pneumococcal vaccine.*Lancet* 346, 1252-1257.

Shao Z, Li W, Ren J *et al.* (2006) Identification of a new Neisseria meningitidis serogroup C clone from Anhui province, China.*Lancet* 367, 419-423.

Shinefield HR, Black S, Ray P *et al.* (1999) Safety and immunogenicity of heptavalent pneumococcal CRM197 conjugate vaccine in infants and toddlers.*Pediatr.Infect.Dis.J.* 18, 757-763.

Simell B, Kilpi TM, & Kayhty H (2002) Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal capsular polysaccharides in children.*J.Infect.Dis.* 186, 1106-1114.

Simpson E.H (1949) Measurement of Diversity.*Nature* 163, 688.

Smith JM, Dowson CG, & Spratt BG (1991) Localized sex in bacteria.*Nature* 349, 29-31.

Smith JM, Feil EJ, & Smith NH (2000) Population structure and evolutionary dynamics of pathogenic bacteria.*Bioessays* 22, 1115-1122.

Smith JM, Smith NH, O'Rourke M, & Spratt BG (1993a) How clonal are bacteria?*Proc.Natl.Acad.Sci.U.S.A* 90, 4384-4388.

Smith T, Lehmann D, Montgomery J *et al.* (1993b) Acquisition and invasiveness of different serotypes of Streptococcus pneumoniae in young children.*Epidemiol.Infect.* 111, 27-39.

Snape MD & Pollard AJ (2005) Meningococcal polysaccharide-protein conjugate vaccines.*Lancet Infect.Dis.* 5, 21-30.

Snapper C M, Colino J, Khan A Q, & Zheng Q W (2004) The Humoral Immune Response To Streptococcus Pneumoniae: In The Pneumococcus 1 edn. eds. Tuomanen E, Mitchell T, Morrison D, & Spratt BG, ASM Press, Washington, pp.367-381.

Soininen A, Pursiainen H, Kilpi T, & Kayhty H (2001) Natural development of antibodies to pneumococcal capsular polysaccharides depends on the serotype: association with pneumococcal carriage and acute otitis media in young children.*J.Infect.Dis.* 184, 569-576.

Soriano-Gabarro M, Rosenstein N, & LaForce FM (2004) Evaluation of serogroup A meningococcal vaccines in Africa: a demonstration project.*J.Health Popul.Nutr.* 22, 275-285.

Sow AI, Caugant DA, Cisse MF, Hoiby EA, & Samb A (2000) Molecular characteristics and susceptibility to antibiotics of serogroup A Neisseria meningitidis strains isolated in Senegal in 1999.*Scand.J.Infect.Dis.* 32, 185-187.

Sow SO, Diallo S, Campbell JD *et al.* (2005) Burden of invasive disease caused by Haemophilus influenzae type b in Bamako, Mali: impetus for routine infant immunization with conjugate vaccine.*Pediatr.Infect.Dis.J.* 24, 533-537.

Spratt BG, Hanage WP, & Brueggemann AB (2004) Evolutionary and Population Biology of Streptococcus Pneumoniae: In The Pneumococcus 1 edn. eds. Tuomanen E, Mitchell T, Morrison D, & Spratt BG, ASM Press, Washington, pp.119-135.

Spratt BG & Maiden MC (1999) Bacterial population genetics, evolution and epidemiology.*Philos.Trans.R.Soc.Lond B Biol.Sci.* 354, 701-710.

Sulikowska A, Grzesiowski P, Sadowy E, Fiett J, & Hryniewicz W (2004) Characteristics of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis isolated from the nasopharynges of asymptomatic children and molecular analysis of S. pneumoniae and H. influenzae strain replacement in the nasopharynx.*J.Clin.Microbiol.* 42, 3942-3949.

Swartley JS, Marfin AA, Edupuganti S *et al.* (1997) Capsule switching of Neisseria meningitidis.*Proc.Natl.Acad.Sci.U.S A* 94, 271-276.

Syrogiannopoulos GA, Katopodis GD, Grivea IN, & Beratis NG (2002) Antimicrobial use and serotype distribution of nasopharyngeal Streptococcus pneumoniae isolates recovered from Greek children younger than 2 years old.*Clin.Infect.Dis.* 35, 1174-1182.

Taha MK (2000) Simultaneous approach for nonculture PCR-based identification and serogroup prediction of Neisseria meningitidis.*J.Clin.Microbiol.* 38, 855-857.

Taha MK, Achtman M, Alonso JM *et al.* (2000) Serogroup W135 meningococcal disease in Hajj pilgrims.*Lancet* 356, 2159.

Taha MK, Giorgini D, Ducos-Galand M, & Alonso JM (2004) Continuing diversification of Neisseria meningitidis W135 as a primary cause of meningococcal disease after emergence of the serogroup in 2000.*J.Clin.Microbiol.* 42, 4158-4163.

Taha MK, Parent DC, I, Schlumberger M *et al.* (2002) Neisseria meningitidis serogroups W135 and A were equally prevalent among meningitis cases occurring at the end of the 2001 epidemics in Burkina Faso and Niger.*J.Clin.Microbiol.* 40, 1083-1084.

Talbot TR, Poehling KA, Hartert TV *et al.* (2005) Seasonality of invasive pneumococcal disease: temporal relation to documented influenza and respiratory syncytial viral circulation.*Am.J.Med.* 118, 285-291.

Tenover FC, Arbeit RD, Goering RV *et al.* (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing.*J.Clin.Microbiol.* 33, 2233-2239.

Tikhomirov E, Santamaria M, & Esteves K (1997) Meningococcal disease: public health burden and control.*World Health Stat.Q.* 50, 170-177.

Torzillo PJ, Hanna JN, Morey F *et al.* (1995) Invasive pneumococcal disease in central Australia.*Med.J.Aust.* 162, 182-186.

Troncoso G, Sanchez S, Moreda M, Criado MT, & Ferreiros CM (2000) Antigenic cross-reactivity between outer membrane proteins of Neisseria meningitidis and commensal Neisseria species.*FEMS Immunol Med.Microbiol.* 27, 103-109.

Trotman J, Hughes B, & Mollison L (1995) Invasive pneumococcal disease in central Australia.*Clin.Infect.Dis.* 20, 1553-1556.

Trotter CL, Andrews NJ, Kaczmarski EB, Miller E, & Ramsay ME (2004) Effectiveness of meningococcal serogroup C conjugate vaccine 4 years after introduction.*Lancet* 364, 365-367.

Tugwell P, Greenwood BM, & Warrell DA (1976) Pneumococcal meningitis: a clinical and laboratory study.*Q.J.Med.* 45, 583-601.

Vela Coral MC, Fonseca N, Castaneda E *et al.* (2001) Pneumococcal surface protein A of invasive Streptococcus pneumoniae isolates from Colombian children.*Emerg.Infect.Dis.* 7, 832-836.

Virolainen A, Russell W, Crain MJ *et al.* (2000) Human antibodies to pneumococcal surface protein A in health and disease.*Pediatr.Infect.Dis.J.* 19, 134-138.

Vu DM, Welsch JA, Zuno-Mitchell P, Dela Cruz JV, & Granoff DM (2006) Antibody persistence 3 years after immunization of adolescents with quadrivalent meningococcal conjugate vaccine.*J.Infect.Dis.* 193, 821-828.

Weichselbaum A. (1887) Ueber die Aetiologie der akuten Meningitis cerebrospinalis.*Fortschritte der Medizin* 5, 573-583.

Whittle HC, Evans-jones G, Onyewotu I *et al.* (1975) Letter: Group-C Meningococcal Meningitis in the Northern Savanna of Africa.*Lancet* 1, 1377.

WHO. Antimicrobial and support therapy for bacterial meningitis in children. Report of the meeting of 18-20 June 1997, Geneva, Switzerland. WHO/CHD/98.6, 1-29. 1997. Geneva, Switzerland, WHO.
Ref Type: Report

WHO. Control of epidemic meningococcal disease. WHO practical guidelines. http://www.who.int/csr/resources/publications/meningitis/WHO_EMC_BAC_98_3_EN/en/ (2nd), 1-82. 1998. WHO.
Ref Type: Electronic Citation

WHO (2000) Detecting meningococcal meningitis epidemics in highly-endemic African countries.*Wkly.Epidemiol.Rec.* 75, 306-309.

WHO. Update on the AFRO Paediatric Bacterial Meningitis Surveillance Network. (38), 1-6. 2003. WHO, Regional Office for Africa . Vaccine Preventable Disease Bulletin. WHO.

Ref Type: Serial (Book,Monograph)

WHO (2005) Enhanced surveillance of epidemic meningococcal meningitis in Africa:a three-year experience.*Wkly.Epidemiol.Rec.* 80, 313-320.

Wilder-Smith A, Barkham TM, Chew SK, & Paton NI (2003a) Absence of Neisseria meningitidis W-135 Electrophoretic Type 37 during the Hajj, 2002.*Emerg.Infect.Dis.* 9, 734-737.

Wilder-Smith A, Barkham TM, Ravindran S, Earnest A, & Paton NI (2003b) Persistence of W135 Neisseria meningitidis carriage in returning Hajj pilgrims: risk for early and late transmission to household contacts.*Emerg.Infect.Dis.* 9, 123-126.

Williams RL (2000) A note on robust variance estimation for cluster-correlated data.*Biometrics* 56, 645-646.

Yaro S, Lourd M, Naccro B *et al.* (2006) The epidemiology of Haemophilus influenzae type b meningitis in Burkina Faso.*Pediatr.Infect.Dis.J.* 25, 415-419.

Yazdankhah SP & Caugant DA (2004) Neisseria meningitidis: an overview of the carriage state.*J.Med.Microbiol.* 53, 821-832.

Yazdankhah SP, Kriz P, Tzanakaki G *et al.* (2004) Distribution of serogroups and genotypes among disease-associated and carried isolates of Neisseria meningitidis from the Czech Republic, Greece, and Norway.*J.Clin.Microbiol.* 42, 5146-5153.

Zhang Q, Choo S, Everard J, Jennings R, & Finn A (2000) Mucosal immune responses to meningococcal group C conjugate and group A and C polysaccharide vaccines in adolescents.*Infect.Immun.* 68, 2692-2697.

Zhang Q, Choo S, & Finn A (2002) Immune responses to novel pneumococcal proteins pneumolysin, PspA, PsaA, and CbpA in adenoidal B cells from children.*Infect.Immun.* 70, 5363-5369.

Zhu P, van der EA, Falush D *et al.* (2001) Fit genotypes and escape variants of subgroup III Neisseria meningitidis during three pandemics of epidemic meningitis.*Proc.Natl.Acad.Sci.U.S A* 98, 5234-5239.

Appendix

Meningitis caused by *Haemophilus influenzae* in the Kassena Nankana District of Northern Ghana

During the long-term study on bacterial meningitis in the Kassena Nankana District (KND) of Northern Ghana, meningitis patients were recruited in the War Memorial Hospital of Navrongo and four health centers between February 1998 and November 2005. CSF was taken from all suspected meningitis cases for diagnostic purposes and Hib diagnosis was based on gram stain and Latex Agglutination. Furthermore, laboratory-data was obtained from the Regional Hospital in Bolgatanga for the period of 2000 until 2002.

Both datasets indicate a low significance of Hib meningitis in Northern Ghana. In the KND, between 1998 and 2002, altother only 17 cases of Haemophilus meningitis have been diagnosed. After 2002 until 2005 no single Hib meningitis case was recorded in the KND. While from four patients the outcome of the disease is not known, five of these patients died. Of two patients the age was not known, however, 11/15 patients were up to five years old (median age: 5 years, age range: 6 months to 55 years).

These data are in accordance with laboratory results from the Bolgatanga Hospital between 2000 and 2002. While in total 580 suspected meningitis patients were recruited and 65 meningococcal meningitis and 191 pneumococcal meningitis cases were seen, only 9 Hib meningitis cases were recorded in this time period.

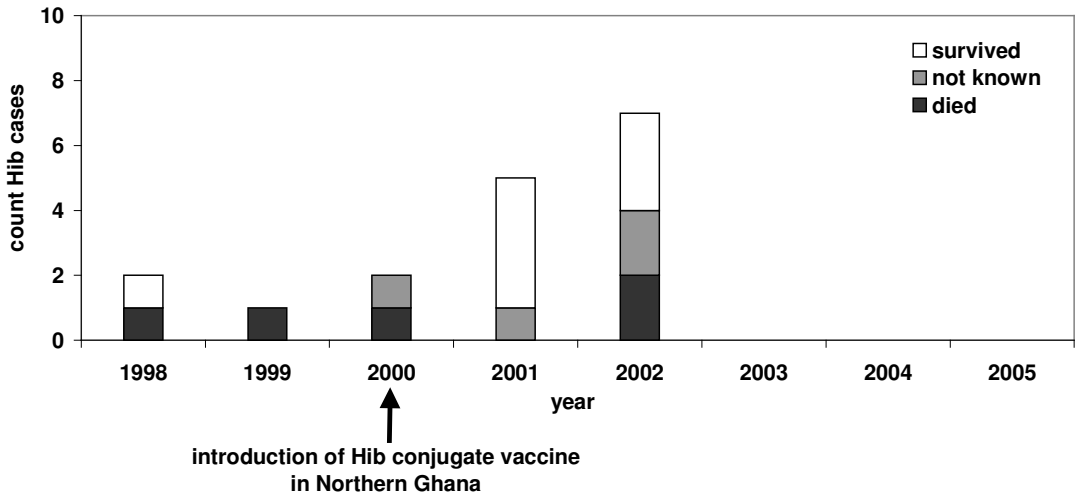


Figure 1 Hib meningitis cases in the KND between 1998 and 2005, reported in the laboratory of the War Memorial Hospital, Navrongo. Diagnosis was performed with Latex agglutination.

The incidence of Hib meningitis seen in these two settings of Northern Ghana is lower than in many other regions of sub-saharan Africa. This is most probably due to the introduction of the Hib conjugate vaccine into the routine immuninzation scheme of infants in the year 2000 in Ghana as one of the first African countries (personal communication).

Wherever the vaccine is not yet implemented, *Haemophilus influenzae* remains an important causative agent for meningitis in children. In Mali, yearly incidence rates of over 150/100'000, in Burkina Faso of 97/100'000 in children under 1 years of age have been reported recently (Sow et al. 2005;Yaro et al. 2006). Overall it has been estimated to be 40-60/100'000 cases in the under 5 year old children (WHO 2003). Even though we do not have solid pre-vaccination data from the district, the low significance of Hib meningitis between 1998 and 2002 and its dissapearence in 2003 until 2005 indicate the positive effect of Hib immunization.

Curriculum Vitae

Name: Susanne Julia Marianne Leimkugel

Date of Birth: 08th June 1976

Nationality: german

Languages studied: german, english, french, spanish, latin, ancient greek

Education:

1982- 1986	Catholic Primary School, Mülheim an der Ruhr
1986- 1995	Otto-Pankok Gymnasium, Mülheim an der Ruhr Abitur in July 1995
1995- 2000	Albert-Ludwigs University, Freiburg 1st state board examination August 1997 2nd state board examination March 2000
June 2001	Final Degree in Pharmacy (Approbation as pharmacist)
Since Jan. 2002	PhD studies in Microbiology at the Swiss Tropical Institute in Basel and the Navrongo Health Research Center, Navrongo, Ghana. Thesis entitled: "Clonal Waves of meningococcal and pneumococcal meningitis in a region of the meningitis belt of sub-saharan Africa"

Working Experience:

March 1998	Stage at the Sha'are Zedek Hospital, Jerusalem
Mai- Oct. 2000	Stage at the School of Pharmacy, London, Pharmacognosy Unit
Oct. '00- Mai '01	Stage in a public pharmacy (Oppspring Apotheke, Mülheim an der Ruhr)

Courses attended

Aug.-Sept. 2005 European Course of Tropical Epidemiology, Verona

During my PhD studies at the Swiss Tropical Institute, I attended courses taught by the following lecturers: Weiss N, Pluschke G, Daubenberger C, Felger I, Vounatsou P, Smith T, Tanner M.

Conferences and Workshops attended

Annual Meeting of the *Swiss Society of Tropical Medicine and Parasitology* and *Swiss Society of Microbiology*, Basel (Poster) March 2003

Annual Meeting of PhD students of Swiss Society of Tropcial Medicine and Parasitology, Münchenwiler, Switzerland, October 2003

Tropenkongress Würzburg, Germany. Annual meeting of the *Deutsche Gesellschaft für Tropenmedizin* and *Swiss Society for Tropcial Medicine and Parasitology*, September 2004

Annual Meeting of PhD students of Swiss Society of Tropical Medicine and Parasitology, Münchenwiler, Switzerland, October 2004

Publications

Gibbons S, **Leimkugel J**, Olawatuyi M, Heinrich M. Activity of Zanthoxylum clava-herculis extracts against multi-drug resistant methicillin-resistant Staphylococcus aureus (mdr-MRSA). Phytotherapy Research, 2003 Mar;17(3):274-5.

Leimkugel J, Forgor AA, Gagneux S, Pflüger V, Flierl C, Awine E, Naegeli M, Dangy JP, Smith TA, Hodgson A, Pluschke G, An outbreak of serotype 1 *Streptococcus pneumoniae* meningitis in Northern Ghana with features characteristic of epidemic meningococcal meningitis, Journal of Infectious Diseases (2005),195: 192-199

Forgor AA, **Leimkugel J**, Hodgson A, Bugri A, Dangy JP, Gagneux S, Smith TA, Pluschke G, Emergence of W135 meningococcal meningitis in Ghana, Tropical Medicine and International Health (2005), 10:1229-1234

Leimkugel J, Hodgson A, Forgor AA , Pflüger V, Dangy JP, Smith TA, Achtman M, Gagneux S, Pluschke G, Clonal Waves of Colonisation and Disease of *Neisseria meningitidis* in the African Meningitis Belt: An Eight Years Longitudinal Study in Northern Ghana, accepted by PLoS Medicine

Leimkugel J, Forgor AA, Dangy JP, Pflüger V, Gagneux S, Hodgson A, Pluschke G, Genetic diversification of *Neisseria meningitidis* during waves of colonisation and disease in the meningitis belt of sub-Saharan Africa, submitted to Vaccine

Leimkugel J, Pflüger V, Forgor AA, Nägeli M, Flierl C, Gagneux S, Pluschke G, Conservation of the Pneumococcal surface protein A (PspA) sequence in a hypervirulent lineage of serotype 1 *Streptococcus pneumoniae,* will be submitted to Journal of Clinical Microbiology

Forgor AA, Hodgson A, **Leimkugel J**, Adjuik M, Pflüger V, Bangre O, Dangy JP, Pluschke G and Smith TA, Survival and Sequelae of Pneumococcal Meningitis in Northern Ghana, submitted to Clinical Infectious Diseases