

Molecular Epidemiology of Meningococcal Disease in Northern Ghana

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Prof. Dr. A. Zuberbühler
Dekan

dedicated to my family,
my friends, and the
marvellous people of Ghana

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SUMMARY

Meningococcal disease remains a major public health concern, especially in the African Meningitis Belt where large meningitis epidemics with attack rates of up to 500/100,000 recur every 8-12 years. The factors precipitating epidemics are largely unknown. Epidemics are therefore unpredictable which often leads to control measures being initiated too late to be effective. Following a major meningitis epidemic that occurred in northern Ghana in 1997, a collaborative research project was developed between the Swiss Tropical Institute and the Navrongo Health Research Center, in order to address several research questions relevant to the epidemiology of meningococcal disease in Ghana. This research partnership built the framework of the present thesis, which concentrated on the molecular epidemiological aspects of the project.

During the dry season of 1998, there was a second meningitis outbreak in the Kassena-Nankana district (KND) of northern Ghana. All suspected meningitis patients were recruited at the local health facilities, lumbar punctures carried out before treatment and the cerebrospinal fluid (CSF) specimen sent to the field laboratory for analysis. In 50 of 92 CSF samples analyzed, serogroup A *Neisseria meningitidis* were detected. All serogroup A *N. meningitidis* isolates recovered were of the A:4:P1.9,20 phenotype. Analysis of representative isolates by multilocus sequence typing (MLST) and by restriction fragment length polymorphism (RFLP) of *opa*, *iga* and *ingA* genes showed that they belonged to subgroup III (sequence type 5) of *N. meningitidis* and had RFLP patterns characteristic of serogroup A subgroup III bacteria isolated in Africa after the 1987 Mecca epidemic. RFLP analysis of six polymorphic loci in a global collection of 502 isolates of subgroup III, serogroup A *N. meningitidis* identified nine 'genoclouds', consisting of genotypes that were isolated repeatedly, plus 48 less frequent descendent genotypes.

Starting during the second outbreak, a series of five 6-monthly carriage surveys of 37 randomly selected households were carried out in KND. As serogroup A *N. meningitidis* carriage decreased, that of X meningococci increased dramatically to reach 18% (53/298) of the people sampled during the dry season of 2000. This coincided with a further outbreak of disease, this time caused by serogroup X. The Ghanaian serogroup X strains were analyzed by MLST and pulsed-field gel electrophoresis (PFGE) along with other serogroup X isolates from different countries

of Africa, Europe and North America. The European and American isolates were highly diverse. However, one clonal grouping was identified among sporadic disease and carrier strains isolated over the last two decades in the UK, The Netherlands, Germany and the USA. In contrast to the diversity among the European and American isolates, most carrier and disease isolates recovered in Ghana and other countries of the African Meningitis Belt over the last thirty years belong to a second clonal grouping. Based on the PFGE results, two genoclouds were identified within the second clonal grouping, one of which caused an outbreak in Niger in 1997 and the other of which caused the outbreak in KND in 2000.

Patterns of carriage of *N. lactamica* in KND were unrelated to those of *N. meningitidis*. Non-serogroupable (NG) strains of *N. meningitidis* were infrequent. This contrasts with industrialized countries where asymptomatic nasopharyngeal carriage of *N. meningitidis* is common and up to 50% of the strains carried are NG.

The nine genoclouds of subgroup III meningococci have caused three pandemic waves of disease since the mid-1960's, with the 1997-8 outbreaks in KND forming part of the second wave. The third wave was imported from East Asia to Europe and Africa in the mid-1990s, and may well lead to renewed epidemic serogroup A disease in Europe and the Americas. The finding that a serogroup X meningococcal clonal grouping has caused outbreaks in Africa, supports concerns that polysaccharide vaccines, which have been in use for more than a decade might be selecting for non-vaccine serogroups and argues for the development of a comprehensive conjugate vaccine including serogroup X polysaccharide. The dynamics of meningococcal carriage that were observed in KND suggest that in the African meningitis belt, the populations become colonized in waves of different meningococcal strains, and the occurrence of epidemics of disease depends on the virulence of these strains. Carriage of NG meningococci may protect against meningococcal disease by eliciting cross-reactive immunity against pathogenic strains and the low levels of carriage of such organisms in the African meningitis belt may thus increase susceptibility to epidemics.

ZUSAMMENFASSUNG

Meningokokken-Meningitis ist ein Gesundheitsproblem von weltweiter Bedeutung. Grosse Epidemien mit Inzidenzen von über 500/100'000 treten regelmässig alle 8-12 Jahre im Afrikanischen Meningitisgürtel auf. Die Faktoren, die zu diesen Epidemien führen sind grösstenteils unbekannt und Kontrollmassnahmen werden dadurch oft zu spät eingeleitet. Im Jahre 1997 wurde der Norden Ghanas von einer schweren Epidemie heimgesucht. Eine wissenschaftliche Zusammenarbeit zwischen dem Schweizerischen Tropeninstitut und dem Navrongo Health Resarch Center wurde daraufhin ins Leben gerufen, mit dem Ziel, verschiedene Fragestellungen zur Epidemiologie der Meningokokken-Meningitis in Ghana anzugehen. Diese wissenschaftliche Zusammenarbeit bildete den Rahmen für diese Dissertation, die sich mit den molekular-epidemiologischen Aspekten befasst hat.

Während der Trockenzeit des Jahres 1998, traf eine zweite Meningitis Epidemie den Kassena-Nankana Distrikt (KND) im Norden Ghanas. Meningitis Patienten wurden im Distrikthospital and an den Gesundheitszentren rekrutiert. Eine Lumbarpunktion wurde vor der Behandlung durchgeführt und die Liquorproben zum Feldlabor transportiert. Serogruppe A *Neisseria meningitidis* konnte in 50 von 92 analysierten Liquorproben identifiziert werden. Alle kultivierten Serogruppe A *N. meningitidis* Stämme hatten den Phänotyp A:4:P1.9,29. Eine Anzahl repräsentativer Stämme wurde mit „Multilocus Sequence Typing“ (MLST) und „Restriction Fragment Length Polymorphism“ (RFLP) von *opa*, *iga* und *ingA* Genen analysiert. Diese Analysen zeigten, dass diese Bakterien zur Subgruppe III (Sequenztyp 5) von *N. meningitidis* gehören. Ihre RFLP Muster waren charakteristisch für Serogruppe A, Subgruppe III Meningokokken, die nach der 1987er Epidemie in Mekka in Afrika isoliert wurden. Die genetische Variabilität einer globalen Sammlung von 502 Subgruppe III Isolate wurde mit RFLP von 6 polymorphen Loci analysiert. Neun „Genoclouds“ wurden dabei identifiziert, die aus Genotypen bestehen, die öfters isoliert wurden. Daneben wurden weitere 48 verwandte, aber seltene Genotypen gefunden.

Während der zweiten Meningitis-Epidemie im KND, wurde eine Serie von fünf Trägerstudien gestartet, in der 37 zufällig ausgewählte Haushalte halbjährlich besucht wurden. Während die Serogruppe A *N. meningitidis* Trägerrate abnahm, stieg diejenige von Serogruppe X Meningokokken dramatisch an und erreichte 18% (53

von 298 getesteten Personen) in der Trockenzeit des Jahres 2000. Eine weitere Meningitis-Epidemie, diesmal durch Serogruppe X verursacht, begleitete diesen Anstieg der Trägerrate.

Die Ghanaischen Serogruppe X Stämme wurden zusammen mit einer Sammlung von anderen Serogruppe X Isolaten aus verschiedenen europäischen, amerikanischen und afrikanischen Ländern mit MLST und Pulsfeld Gel Elektrophorese (PFGE) analysiert. Die europäischen und amerikanischen Stämme waren sehr unterschiedlich. Eine klonale Gruppe wurde aber identifiziert, bestehend aus Isolaten von gesunden Trägern und von sporadischen Krankheitsfällen, die während den letzten beiden Jahrzehnten in Grossbritannien, Holland, Deutschland und USA isoliert worden waren. Im Gegensatz zu der Heterogenität der europäischen und amerikanischen Isolate gehört die Mehrheit der Stämme, die in den letzten 30 Jahren in Ghana und in anderen Ländern des Afrikanischen Meningitisgürtels isoliert wurden, zu einer klonalen Gruppe. PFGE Resultate zeigten, dass diese klonale Gruppe sich in zwei „Genoclouds“ aufteilen lässt. Bakterien der einen „Genocloud“ haben eine Epidemie in 1997 in Niger verursacht und Bakterien der anderen die 2000er Epidemie im KND.

Die Kolonisation des Nasopharynx mit *N. lactamica* hatte keinen Einfluss auf diejenige mit *N. meningitidis*. Nicht-serogruppierbare (NG) Stämme waren selten, was im Gegensatz zu den Industriestaaten steht, wo Trägertum häufig ist und bis zu 50% der Trägerisolate NG sind.

Die 9 „Genoclouds“ der Subgruppe III Meningokokken haben seit den 1960er Jahren drei pandemische Krankheitswellen verursacht. Die 1997-8er Epidemie im KND war Teil der zweiten dieser Wellen. Die dritte Welle wurde in den 1990er Jahren aus Ostasien nach Europa und Afrika importiert und könnte zu einem neuen Aufkommen von epidemischer Serogruppe A Krankheit in Europe führen. Die Tatsache, dass eine Serogruppe X klonale Gruppe mehrere Epidemien in Afrika verursacht hat, unterstützt den Gedanken, dass die Polysaccharid Impfstoffe, die in Afrika seit mehr als 10 Jahren intensiv verwendet werden, Serogruppen selektionieren, die nicht im Impfstoff enthalten sind. Diese Resultate sprechen dafür, einen umfassenden Konjugatimpfstoff zu entwickeln, der auch Serogruppe X Polysaccharid enthält.

Die Dynamik des Meningokokken Trägertums im KND deutet darauf hin, dass die Populationen im Afrikanischen Meningitisgürtel durch verschiedene Meningokokkenstämme wellenartig kolonisiert werden, und dass das Aufkommen von Epidemien von der Virulenz dieser Stämme abhängt. Im Hinblick darauf, dass das Trägertum von NG Stämmen vor Meningokokken Erkrankung schützen könnte, indem es kreuz-reagierende Immunität fördert, könnte eine geringe Trägerrate solcher Organismen im Afrikanischen Meningitisgürtel zu einer erhöhten Empfindlichkeit für Epidemien führen.

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ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
CSF	Cerebrospinal Fluid
CI	Confidence Interval
CSM	Cerebrospinal Meningitis
ELISA	Enzyme-linked Immunosorbent Assay
ET	Electrophoretic Type
KND	Kassena-Nankana District
LRT	Likelihood Ratio Test
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
NHRC	Navrongo Health Research Center
NJ	Neighbor Joining
OMP	Outer Membrane Protein
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
ST	Sequence Type
STI	Swiss Tropical Institute
WHO	World Health Organization

Chapter 1

INTRODUCTION

1.1 The history of meningococcal meningitis in Africa¹

The first clear account of an outbreak of meningococcal meningitis, sometimes called cerebrospinal meningitis (CSM), is given by Vieusseux (1806), who described a typical epidemic that occurred in 1805 in Geneva, Switzerland. Cases may have occurred previously, lost among reports of ‘spotted fevers’, but large epidemics of CSM are so dramatic that it seems unlikely that these would have passed unreported by the observant physicians who practiced in Europe in the 17th and 18th centuries (Greenwood 1999). In 1806, another typical outbreak was described in Medfield, Massachusetts, USA, the first report of the disease in the New World (Danielson & Mann 1806). Throughout the 19th and early part of the 20th centuries, outbreaks of CSM occurred on many occasions across the USA and throughout Europe.

The causative agent of CSM, a Gram-negative diplococcus initially called *Diplococcus intracellularis* but now known as *Neisseria meningitidis* (the meningococcus), was described for the first time in 1848 (Marchiafava 1848), and was first cultured from patients with CSM by Weichselbaum in Vienna (1887).

When epidemic CSM first reached West Africa and how it got there will probably never be known definitely but it is likely that the first major epidemics occurred around 100 years ago. Evidence that epidemic CSM was not prevalent before that time comes from 3 main sources – early African literature, reports from the first European explorers to West Africa and accounts obtained from the local population at the time that the first major epidemics were reported (Greenwood 1999). The first proven outbreak of CSM in West Africa, established by the detection of diplococci in cerebrospinal fluid, occurred in Northern Nigeria in 1905 (McGahey 1905). Several recent epidemics of CSM in Africa have been caused by meningococci introduced into West Africa by pilgrims on their return from the Hajj (Morelli *et al.* 1997) and it has been hypothesized that the meningococcus responsible for the 1905 epidemic in Nigeria was brought in by the same process (Greenwood 1999).

In the Gold Coast (Ghana), an epidemic of CSM was reported for the first time in 1906; it seems probable that this was caused by the same epidemic strain that caused the Nigerian outbreak. The epidemic started in the north-west of the Gold Coast, spread widely throughout the area during the following dry season (Horn 1908).

¹ This section is mainly taken from the Manson Lecture by Greenwood (1999).

It spread rapidly westwards into the territories under French colonial rule and outbreaks of CSM have occurred across West Africa every few years ever since. In Ghana, epidemics were subsequently reported in 1919/20, in 1939/40, in 1945, in 1949/50 (Waddy 1957), in 1961, in 1972/73 (Belcher *et al.* 1977), in 1984 (A. Amankwa, personal communication) and in 1997/98 (Tikhomirov *et al.* 1997).



Figure 1.1. The African Meningitis Belt (Source: Moore 1992).

An extensive survey of published and unpublished records, many obtained by personal visits to hospitals and ministries of health across West Africa, enabled Lapeyssonnie (1963) to produce the definitive report on CSM in West Africa during the first half of the 20th century: *La méningite cérébrospinale en Afrique* (Lapeyssonnie 1963). He documented in detail the epidemiological features of CSM in Africa and drew attention to the fact that it is only in a restricted area of Africa that the

infection behaves in such a characteristic and peculiar way. This led him to define the 'African meningitis belt', bounded to the north by the Sahara and to the south by areas of tropical rain forest. In the 36 years since it was first published the concept of the African meningitis belt has held up well. However, it is now known that the belt extends further west than originally envisaged, reaching as far as Senegal, Guinea and the eastern half of The Gambia (Moore 1992) (Figure 1.1).

1.2 The epidemiology of meningococcal meningitis in Africa today

The current epidemiology of meningococcal meningitis in Africa differs little from that described by Lapeyssonnie in 1963. It has been suggested that epidemics have become more frequent and that they have lost some of their periodicity but this is difficult to document as epidemics have always occurred in an unpredictable way (Greenwood 1999). The characteristic epidemiological features of epidemic meningococcal meningitis in Africa are summarized below.

Periodicity

Within individual countries of the meningitis belt, major epidemics of meningococcal meningitis occur with a periodicity of 8-12 years (Moore 1992); the pattern of epidemics in Burkina Faso shown in Figure 1.2 is characteristic. Although the incidence of meningococcal infection falls markedly between epidemics, it nevertheless remains several times higher than that found in industrialized countries (Tikhomirov *et al.* 1997). In the African meningitis belt, major epidemics usually last for 2 or 3 dry seasons, dying out during the intervening rainy season (Moore 1992).

Size

African epidemics of meningococcal meningitis are often enormous with attack rates that may exceed 500 per 100,000 population. In 1921, an epidemic in Nigeria caused 45,000 deaths in Sokoto Province (population 1.36 million) alone (Blair 1921). In 1996, 80,000 cases were reported in Nigeria and 40,000 in Burkina Faso (Tikhomirov *et al.* 1997). One year later, 20,000 cases occurred again in Burkina Faso, 20,000 in Ghana, and 10,000 in Mali. In 1999, more than 30,000 cases occurred in

Sudan (WHO 1999), and more than 10,000 occurred in Niger in 2000 (WHO 2000a). Figures such as these, massive though they are, are nearly always substantial underestimates because, during the stress of a major epidemic, routine reporting systems frequently break down. In addition, many patients with the septicaemic form of meningococcal disease die before they reach a hospital or health center so that they are never recorded in official statistics.

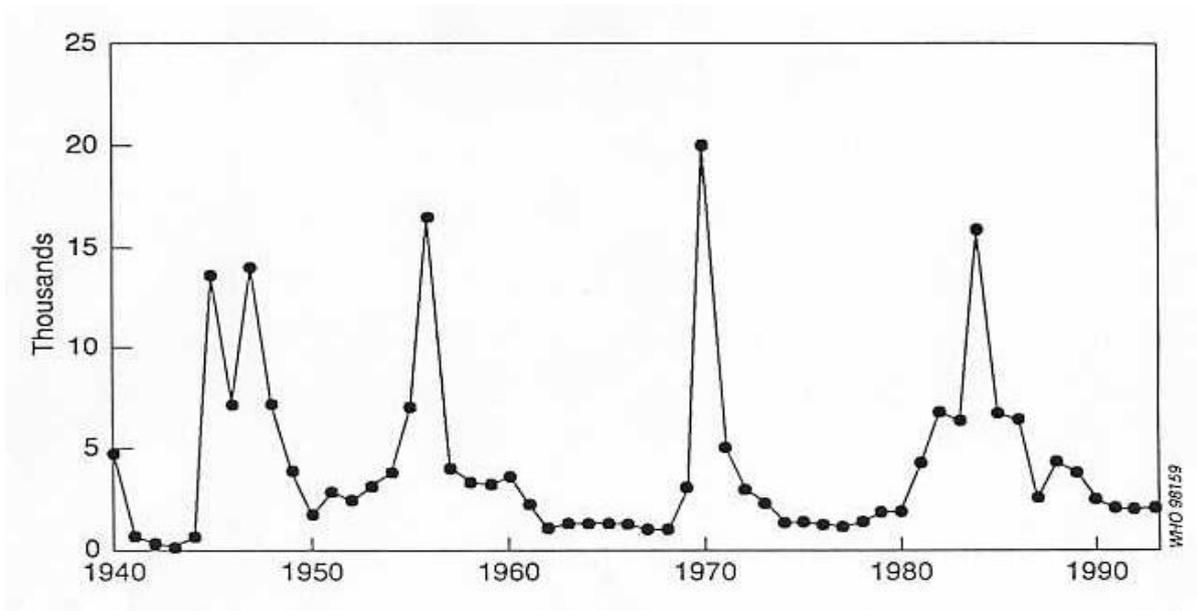


Figure 1.2. Annual number of meningitis cases, Burkina Faso, 1940-1993 (Source: WHO 1998).

Seasonality

Epidemics nearly always start in the early part of the dry season when it is hot, dry and dusty, build up to a peak at the end of the dry season, and then stop abruptly at the onset of the rains, only to break out again during the following dry season (Moore 1992). As an example, Figure 1.3 shows the number of hospital admissions for meningococcal disease in Zaria, Nigeria from 1977 to 79, in relation to the absolute humidity, the mean maximum temperature, and the presence of the Harmattan, a dusty wind blowing from the Sahara.

The mechanisms underlying this seasonal association have never been clearly defined. It is possible that droplet transmission is more efficient under conditions of

low rather than high absolute humidity. This has never been properly investigated, although one study found higher bacterial numbers in the air during the dry season than during the rainy season (Ghipponi *et al.* 1971). However, longitudinal carriage studies conducted in the African meningitis belt showed that, in contrast to meningococcal disease, asymptomatic carriage of meningococci was not seasonal (Greenwood *et al.* 1984) (Chapter 5).

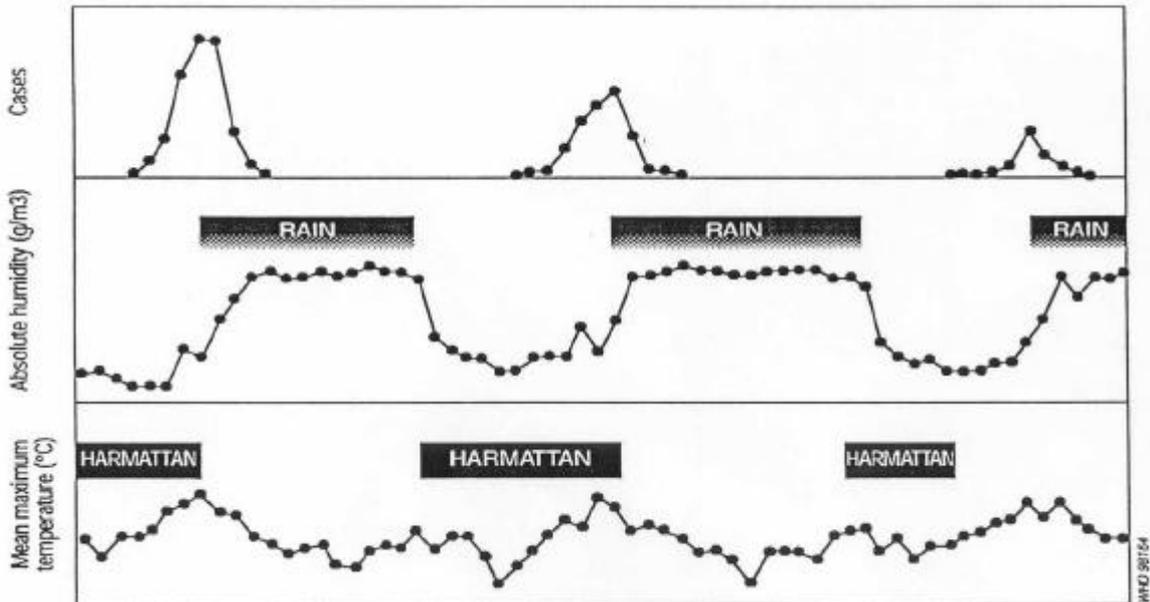


Figure 1.3. Relation of seasonal climatic factors to hospital admissions for meningococcal disease in Zaria, Nigeria, 1977-79 (Source: WHO 1998).

An alternative explanation for the seasonality of meningococcal disease in the African meningitis belt has therefore been proposed (Greenwood 1999), in that infections with the epidemic strain continue throughout the rainy season but that the ratio of cases to asymptomatic carriers declines, thus resulting in an apparent disappearance of the epidemic (Figure 1.4b).

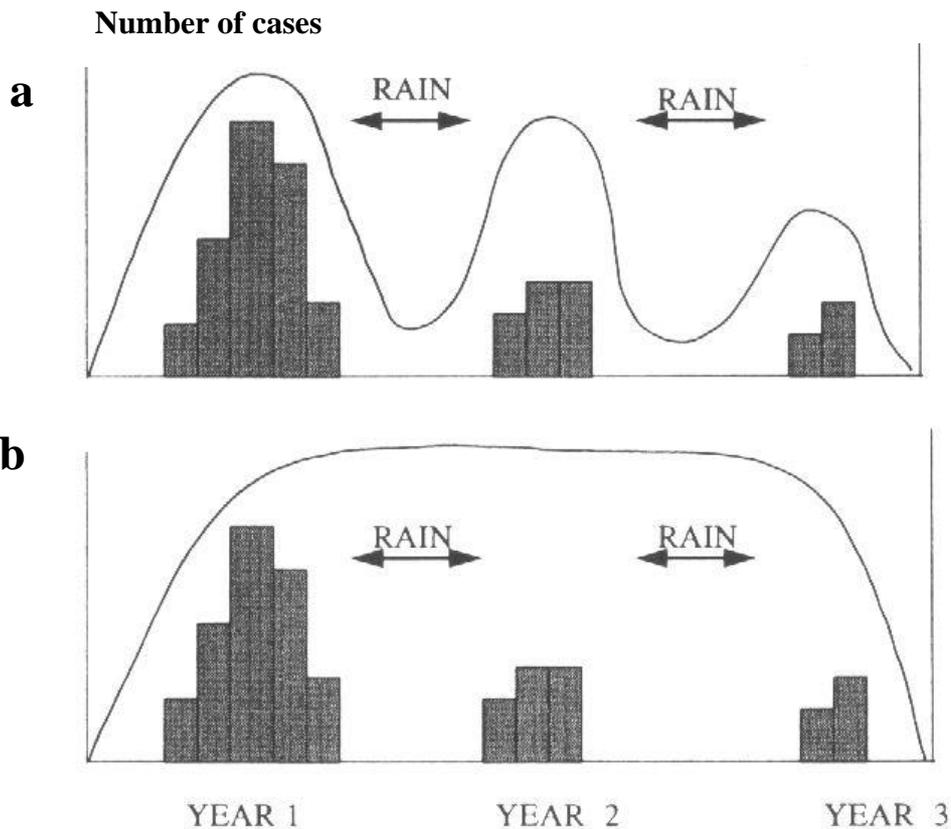


Figure 1.4. Two alternative possible explanation for the seasonal pattern of meningococcal meningitis in Africa. The line above the bars indicates the level of transmission (Source: Greenwood 1999).

Two pieces of evidence support this hypothesis. By means of repeated nasopharyngeal swabbing, Blakebrough documented the spread of a serogroup A meningococcus through a Nigerian village during the rainy season in the absence of any cases of meningitis in this or in surrounding villages, although many cases occurred in the neighbourhood during the preceding and the following dry seasons (Blakebrough 1979). The second piece of evidence comes from a study in The Gambia in which a small number of sera were collected from children during a malaria survey in the year preceding a major outbreak of serogroup A meningococcal disease in the area (Greenwood *et al.* 1985). A rise in group A meningococcal antibody titre was demonstrated in paired samples during the rainy season that preceded the outbreak,

suggesting that the epidemic strain had already begun to circulate in the area without causing clinical disease.

How might the ratio of asymptomatic carriers to clinical cases, usually at least 100:1, be changed by climate-associated factors? The simplest explanation is that the extreme environmental conditions present at the end of the dry season – high temperature, low absolute humidity and the Harmattan – damage the local mucosal defenses so that the risk that these will be breached on exposure to a potentially virulent meningococcus is enhanced (Greenwood 1999, Moore 1992). Other potential factors that might be important for epidemics to develop are discussed below.

Serogroup

The major conventional classification of meningococci is based upon the chemical structure of their capsular polysaccharide (Poolman *et al.* 1995). Thirteen serogroups based on the antigenicity of these capsular polysaccharides are currently recognized (Tikhomirov *et al.* 1997). Most meningococcal epidemics in Africa have been caused by bacteria belonging to serogroup A. Although this serogroup used to be the main cause of meningococcal disease in Europe and the USA, it has become very rare since World War II (Cartwright 1995a). Serogroup B bacteria which are currently the most frequent cause of meningococcal meningitis in Europe and the USA has been isolated only very rarely in Africa. In contrast, meningococci belonging serogroup C, which are increasingly causing disease in Europe and the USA, have made an important contribution to some epidemics in Africa (Broome *et al.* 1983, Whittle *et al.* 1975). Meningococci belonging to the rarer serogroup W135 have caused isolated cases of meningitis in Senegal, Mali and The Gambia (Denis *et al.* 1982, Kwara *et al.* 1998). In 2000, serogroup W135 bacteria caused a major outbreak during the annual Hajj pilgrimage in Mecca. Serogroup W135 meningitis was subsequently reported among a series of pilgrims returning from Saudi Arabia and their contacts (Popovic *et al.* 2000, Taha *et al.* 2000). Serogroup W135 disease has again been reported this year (2001), among pilgrims attending the Hajj pilgrimage as well as among their contacts (WHO 2001a). Serogroup X meningococci are even rarer than serogroup W135 bacteria and have caused only a limited number of sporadic meningitis cases (Chapter 3, Hansman 1983, Pastor *et al.* 1985, Ryan & Hogan 1980, Grahlow *et al.* 1986, Riou

et al. 1996). However, sergroup X bacteria have the potential to cause outbreaks, as was seen in Niger (Etienne *et al.* 1990, Campagne *et al.* 1999) and in Ghana (Chapter 5).

Meningococci belonging to an individual capsular polysaccharide serogroup can be sub-classified on the basis of the antigenic characteristics of their outer membrane proteins and lipopolysaccharides (Poolman *et al.* 1995), the electrophoretic mobility of housekeeping enzymes (Wang *et al.* 1992) or by direct analysis of their DNA (see below).

Causes of an epidemic

The factors that initiate African epidemics of meningococcal meningitis are not understood. Epidemics are sometimes associated with the appearance of a new clone but this is not always the case and, during epidemics, there may be an increase in cases of meningitis caused by meningococci belonging to non-epidemic strains and even in the incidence of cases of meningitis caused by the pneumococcus (Greenwood 1999). This phenomenon suggests the importance of environmental factors. It is possible that a new bacterial clone could be sufficiently antigenetically different from resident meningococci to allow it to escape the background immunity induced by previous asymptomatic nasopharyngeal infections. However, this has not been substantiated clearly and the relative contribution of antibodies to the serogroup A capsular polysaccharide, which is non-polymorphic, and of antibodies to the polymorphic outer membrane protein antigens to naturally acquired protective immunity are not known (Greenwood 1999). Accumulation of a population of non-exposed and hence non-immune individuals through births and in-migration since the previous outbreak, and loss of immunity in previously exposed individuals, are likely to be important contributors to an epidemic (Moore 1992).

A striking feature of epidemic meningitis in Africa is that some communities escape an outbreak, despite the fact that neighboring communities are affected severely (Greenwood *et al.* 1987). One possible explanation for this phenomenon is that protected communities are exposed to the epidemic strain during the rainy season, as discussed above. An alternative explanation is the '2 hit' theory which hypothesizes that invasive disease is most likely to occur when exposure to a meningococcus occurs

after infection with enteric bacteria that share antigenic-cross-reactivity with the meningococcus (Griffiss 1982). IgA may block binding of IgG and IgM in this situation and thus prevent complement activation. A third possible explanation is that some kind of ‘first hit’ is needed to precipitate an African epidemic. In industrialized countries, epidemics of influenza A seem to partially fill this role (Cartwright 1995b).

Predicting an epidemic

In Africa, epidemics of meningococcal disease are frequently not recognized until they are well under way. Thus, control measures are often initiated too late to be very effective. On the basis of data collected in Burkina Faso, Moore *et al.* (1992) found that a weekly attack rate of more than 15 cases per 100,000 population collected over 2 weeks was a sensitive and specific predictor of major epidemics of meningococcal disease. This model was integrated into the WHO emergency-response plan, which describes a strategy of using district-level surveillance to predict epidemics and begin mass vaccination (WHO 1998). While retrospective analysis of a data set collected in Ghana confirmed the usefulness of this threshold (Woods *et al.* 2000), a data analysis from Niger supported an alternative threshold of 5 cases per 100,000 over 3 weeks (de Chabaliier *et al.* 2000). This threshold-based approach to epidemic prediction requires a good system of surveillance for cases of meningitis; this is difficult to maintain during inter-epidemic periods when cases are few and when control of other infections is a priority.

1.3 Treatment of meningococcal meningitis in Africa

Before 1938, there was no effective treatment for meningococcal disease in Africa and the case fatality ratio was around 80% (Greenwood 1999). Serum therapy, employed in Europe and the USA with modest success (Cartwright 1995a), was tried in Africa but this was not a practical proposition in a tropical environment and epidemic situations. The introduction of sulphonamids in 1938 reduced mortality from meningococcal meningitis to 10% or less (Greenwood 1999). However, sulphonamid-resistant serogroup A meningococci began soon to be detected in several African countries and by the early 1970s sulphonamids could no longer be used to treat epidemic meningococcal disease. Today, a single injection of oily chloramphenicol is

the standard treatment in countries where epidemic meningococcal meningitis occurs (Greenwood 1999). However, appearance of meningococci resistant to chloramphenicol has recently been reported in Vietnam (Galimand *et al.* 1998).

1.4 Vaccines

Polysaccharide vaccines

In 1969, Gotschlich *et al.* (1969) described the development of highly immunogenic serogroup A and C meningococcal vaccines based on purified meningococcal capsular polysaccharides. The following year it was reported that a serogroup C meningococcal polysaccharide vaccine gave a high degree of protection against serogroup C meningococcal meningitis in American military recruits (Artenstein *et al.* 1970) and a serogroup A polysaccharide vaccine was soon shown to be equally effective in preventing serogroup A meningococcal disease in Egypt (Wahdan *et al.* 1973), the Sudan (Erwa *et al.* 1973), and Upper Volta (Ettori *et al.* 1977). Subsequently, serogroup A + C meningococcal polysaccharide vaccines have been used extensively in Africa where they have been shown to be very effective at bringing epidemics rapidly under control (Greenwood 1999). However, meningococcal polysaccharide vaccines are poorly immunogenic in young children and do not induce long-lasting, T cell-dependent immunological memory (Reingold *et al.* 1985). Furthermore, meningococcal polysaccharide vaccines do not seem to reduce the prevalence or incidence of nasopharyngeal carriage of serogroup A or C meningococci, as was found in studies conducted in Nigeria (Blakebrough *et al.* 1983) and The Gambia (Hassan-King *et al.* 1988).

Polysaccharide / protein conjugate vaccines

In contrast to conventional polysaccharide vaccines, polysaccharide/protein conjugate vaccines induce strong immunity also in young infants. Even more importantly for the prevention of epidemic meningococcal disease, they induce T cell-dependent immunological memory that is likely to be long lasting, especially if boosted by exposure to naturally circulating bacteria (Greenwood 1999). On the basis of experience with Hib and pneumococcal conjugate vaccines it is likely that

meningococcal conjugate vaccines will also have some effect on nasopharyngeal carriage.

An early trial of a meningococcal serogroup A + C conjugate vaccine (Sclavo) undertaken in The Gambia showed that the group C component of the vaccine was immunogenic in young infants and that it induced immunological memory (Leach *et al.* 1997, Twumasi *et al.* 1995). Unfortunately, the group A component of this vaccine was not effective in inducing immunological memory. However, another serogroup A + C conjugate vaccine in which the meningococcal polysaccharides are coupled to diphtheria toxin (Pasteur Mérieux) has given encouraging results during a pilot trial conducted in Niger and a larger immunogenicity study of this vaccine is now underway there (Greenwood 1999).

1.5 Bacterial population genetics and evolution

Whereas eukaryotic organisms have evolved mechanisms of sexual reproduction in which extensive genetic recombination occurs as an integral part of propagation, the bacteria reproduce asexually by binary fission, with each haploid mother cell giving rise to two genetically identical daughter cells. In the absence of sexual processes, 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, and new lineages emerge by the accumulation of such mutations over successive generations (Spratt & Maiden 1999). This transmission of genetic information can be regarded as ‘vertical’, as it passes exclusively from mother to daughter cell. However, bacterial populations are not entirely asexual since recombinational exchanges occur, mobilizing small genome segments among lineages and species, a process that has been termed ‘localized sex’ (Maynard Smith *et al.* 1991). Localized sex disrupts clonal population structures by providing a means of reassorting genetic variation, thereby enabling mutations to escape the lineage in which they arose. This type of transfer of genetic information can be regarded as ‘horizontal’, since genetic material is being moved between cells that do not necessarily share a recent common ancestor (Spratt & Maiden 1999). The three most important mechanism of horizontal genetic exchange are conjugation, transduction and transformation. Plasmids, prophages, transposons and insertion

sequences can also be transferred horizontally, providing mechanisms for mobilizing DNA among distantly related bacteria.

In the absence of the horizontal genetic exchange of chromosomal genes, a given mutation will be associated with the other mutations that have accumulated in the chromosome during the history of the lineage in which it arose. Consequently, the distribution of chromosomal polymorphisms within an asexual (clonal) bacterial population will be non-random, or in linkage disequilibrium (Spratt & Maiden 1999). This contrasts with populations of sexual organisms where mutations are continually reassorted, resulting in linkage equilibrium, i.e. mutations at different sites occur in more or less random combinations.

In asexual bacterial populations, differences in the frequencies of particular lineages will occur over time as a consequence of selection or stochastic events. When mutations that increase fitness arise, the lineages that contain them will increase in frequency, resulting in the loss of other lineages, and this process (periodic selection) reduces the genetic diversity within the population (Levin 1981). Similarly, bacterial populations are subject to rapid expansions and severe bottlenecks which can also reduce the diversity of clonal populations (Achtman 1995a).

The relative contribution of recombination, as opposed to *de novo* mutation, in the generation of new bacteria genotypes varies among bacterial populations (Spratt & Maiden 1999), and as this contribution increases, the clonality of a given population decreases. A spectrum of population structures can be observed, reaching 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 non-clonal, or '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. Most bacterial populations occupy a middle position where recombination is highly significant in the evolution of the population, but is not sufficiently frequent to prevent the emergence of clonal lineages. A mixture of non-clonal and clonal elements within populations of recombinogenic bacterial pathogens may often be related to differences in their ecology and epidemiology (Spratt & Maiden 1999).

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 (Maynard Smith *et al.* 1993, Maynard Smith *et al.* 2000). This phenomenon is particularly clear where the emerging lineage has increased capacity to cause disease, as the analysis of isolates obtained exclusively from disease can result in a large amplification of the significance of the epidemic clone as a consequence of sampling bias (see below). In most cases, analysis of the small fraction of isolates that are from disease will underestimate the diversity of the population as a whole and will overestimate the extent of clonality in the population. It is possible for all three types of structure, clonal, panmictic and epidemic clonal, to be present in a single bacterial species (Spratt & Maiden 1999).

1.6 The genetic population structure of *Neisseria meningitidis*

The genetic population structure of *N. meningitidis* is considered weakly clonal (Spratt & Maiden 1999). This organism illustrates the sampling problems associated with some bacterial pathogens. Asymptomatic nasopharyngeal carriage of *N. meningitidis* is common and only very occasionally do the bacteria invade the blood stream and cerebrospinal fluid to cause disease (Cartwright 1995b). Populations of the meningococcus are highly diverse (Caugant *et al.* 1987), comprising many different genotypes, the majority of which are rarely isolated from patients with invasive disease (Caugant *et al.* 1988). Carried *N. meningitidis* recombine extensively (Jolley *et al.* 2000), and it has been estimated that an individual nucleotide site in a meningococcal housekeeping gene is at least 80 times more likely to change by recombination than by point mutation (Feil *et al.* 1999). Furthermore, analysis of housekeeping genes showed non-congruence between gene trees (Feil *et al.* 2001). All this evidence supports a population structure which is basically non-clonal.

A few hyperinvasive lineages within serogroup B and C meningococci (ET-5 complex, ET-37 complex, lineage III and cluster A4) are responsible for most of the cases of meningococcal disease in many parts of the world (Caugant 1998). An ‘epidemic clonal’ population structure has been attributed to these lineages, based on the fact, that levels of linkage disequilibrium were low when corrections for sample

bias were made (Maynard Smith *et al.* 1993). In contrast, the clonal groupings identified within serogroup A meningococci, which have been responsible for most of epidemic disease in Africa (Achtman 1995b), seem to be more clonal (Bart *et al.* 2001).

The ability to identify accurately the bacterial strains that cause disease is central to epidemiological surveillance and public health decisions concerning. Molecular typing methods are used to address two very different kinds of problems (Spratt & Maiden 1999). The first are short-term or local epidemiological questions, i) are the isolates recovered from a localized outbreak of disease largely identical or diverse, or ii) is relapse of disease after intervention due to treatment failure or re-infection? The second type of problem concerns long-term or global epidemiology, e.g. how do strains causing disease in one geographical area relate to strains recovered worldwide?

1.7 Rationale and research frame work

One of the major problems related to meningococcal disease in the African meningitis belt is that the factors precipitating epidemics are largely unknown. Epidemics are therefore very unpredictable, which usually leads to control measures like mass immunizations being initiated too late to be effective.

In an attempt to address some of the problems related to epidemic meningococcal disease in Africa, and following a major epidemic that occurred in Northern Ghana in 1997 (Tikhomirov *et al.* 1997) a scientific research partnership was initiated between the Swiss Tropical Institute (STI) and the Navrongo Health Research Center (NHRC). Within this collaboration, several research questions relevant to the epidemiology of meningococcal meningitis in Ghana were addressed. The molecular epidemiological aspects of these collaborative research efforts are presented in this thesis.

Chapter 2

GOAL AND OBJECTIVES

2.1 Goal

To contribute to the understanding of the epidemiology of meningococcal meningitis in Africa using conventional and molecular epidemiological techniques.

2.2 Objectives

- 1 To determine the causative agents of bacterial meningitis in the Kassena-Nankana District (KND) of Northern Ghana.
- 2 To investigate the dynamics of meningococcal carriage in the KND by analyzing the persistence of epidemic strains and the acquisition of new clones.
- 3 To study the influence of *Neisseria lactamica* on the dynamics of meningococcal carriage in the KND.
- 4 To analyze the genetic population structure and micro-evolution of the meningococcal strains dominating in the KND by comparing them to strains recovered over the last decades in different countries of Africa, Europe and North America.
- 5 To determine the genetic diversity of non-serogroupable *N. meningitidis* isolated in the KND and to compare them to the dominating encapsulated strains in the district.

Chapter 3

Microheterogeneity of serogroup A subgroup III *Neisseria meningitidis* during an outbreak in Northern Ghana

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3.1 Abstract

During a meningitis outbreak in the eastern sub-district of the Kassena-Nankana District of the Upper East Region of Ghana, we analyzed cerebrospinal fluid from suspected meningitis cases for the most common causative organisms. In 50 of 92 samples analyzed, serogroup A *Neisseria meningitidis* were detected. The ages of serogroup A *N. meningitidis* patients ranged from 4 months to 64 years. The case fatality ratio was 20%. Coma or stupor on presentation worsened the prognosis. All serogroup A *N. meningitidis* isolates recovered revealed the A: 4: P1.9, 20 phenotype characteristic for the subgroup III clonal grouping. No evidence for resistance to penicillin G, chloramphenicol, cefotaxime, ciprofloxacin, rifampicin or tetracycline was found. All strains were resistant to sulfadiazine. Restriction analysis patterns of *opa*, *iga* and *ingA* genes were characteristic for the majority of *N. meningitidis* serogroup A subgroup III bacteria isolated in Africa after the 1987 epidemic in Mecca. Differences in pulsed-field gel electrophoresis patterns of *NheI* and *SpeI* digested DNA revealed micro-heterogeneity among the Ghanaian isolates.

3.2 Introduction

Meningococcal meningitis remains an important global health problem. While levels of endemic infection are high in many communities throughout the world, epidemics have been observed since World War II predominantly in a number of developing countries, including China, Brazil, and various sub-Saharan African nations (WHO 1998). In the savanna region of sub-Saharan Africa called the 'Meningitis Belt', epidemic waves of meningococcal disease have recurred every 8 - 12 years since at least the beginning of the 20th century (Achtman 1990, Moore 1992). Effective surveillance and early warning systems are essential for the planning and implementation of mass vaccination campaigns to control epidemics. However, the factors precipitating these epidemics and the transition from an endemic situation are not well understood.

While endemic infections are usually caused by meningococci belonging to serogroups B and C, most large epidemics are caused by serogroup A strains. Random endemic *N. meningitidis* isolates are diverse and do not exhibit a clonal population structure. In contrast, meningococci causing epidemics belong to fairly uniform clonal

groupings (Achtman 1995b), i.e., they are the descendants of a common ancestor. Clonal analyses of serogroup A meningococci from epidemic waves have identified 9 clonal groupings, designated I-III, IV-1, IV-2, and V-VIII (Wang *et al.* 1992). Methods of molecular epidemiology, which use natural genetic variation as the basis of classification (i.e. multilocus enzyme electrophoresis (MLEE), the random amplified polymorphic DNA (RAPD) method and multilocus sequence typing (MLST)) have demonstrated that bacteria belonging to a certain clonal grouping are largely uniform in many independent genetic properties (Maiden *et al.* 1998). Microevolution, which seems to be largely associated with horizontal genetic exchange, is responsible for some diversity within subgroups (Morelli *et al.* 1997).

In 1987 subgroup III serogroup A meningococci caused an outbreak in Mecca during the annual Hajj pilgrimage (Moore *et al.* 1988). These bacteria had never been isolated before in Africa, but were previously associated with 2 pandemic waves affecting China, northern Europe and Brazil in previous decades (Achtman 1995b). Meningococci descended from those of the Mecca outbreak can be distinguished from pre-Mecca strains by RFLP and post-Mecca bacteria have caused multiple epidemics of meningococcal disease throughout the African meningitis belt since 1988 (Achtman 1995b, Morelli *et al.* 1997). The first subgroup III African epidemics were in Ethiopia, Chad and Sudan in 1987-1989. In the 1990s the epidemic wave spread to the rest of Africa, including Niger (more than 25,000 cases notified in 1995, more than 16,000 cases in 1996), Northern Nigeria (more than 105,000 reported cases in 1996), Burkina Faso (more than 40,000 reported cases in 1996, more than 20,000 in 1997) and Mali (more than 7,000 reported cases in 1996, more than 10,000 in 1997).

In the dry season between November 1996 and May 1997 an epidemic occurred in Northern Ghana. A total of 18,799 meningitis cases with 1,352 deaths were reported. 1200 of these cases and 67 deaths were in the Kassena-Nankana District (Upper East Region) (Enos 1997). We now report analyses of cerebrospinal fluid from suspected meningitis cases from a smaller outbreak in this district one year later. In the majority of cases we found serogroup A meningococci, which were indistinguishable by whole cell ELISA and RFLP from the post-Mecca subgroup III bacteria. We also used pulsed-field gel electrophoresis to investigate further microevolution. Our results reconfirm that within one decade subgroup III has spread

from Mecca through numerous Eastern and Central African countries to West Africa. We present some evidence for further diversification.

3.3 Materials and Methods

Study area and population

The study was conducted in the Kassena-Nankana District (KND) of the Upper East Region of Ghana. The district lies within the guinea Savannah woodland area of Ghana with a population of 140,000 and has two main seasons; a short wet season from June to September and a long dry season for the rest of the year. The general population is rural except for those living in the small town of Navrongo, which has a population of about 20,000.

From February to April 1998, a small meningitis outbreak occurred in the eastern sub-district of KND. During this outbreak, all suspected meningitis cases presenting at the War Memorial Hospital (WMH), Navrongo or at one of the three health centers in the KND were recruited. History and vaccination status were determined using a standardized questionnaire and a clinical examination performed on all the patients. A lumbar puncture was done before treatment and the cerebrospinal fluid (CSF) specimen sent to the laboratory of the WMH. Antibiotic treatment was started immediately after the lumbar puncture. All the patients were treated with chloramphenicol, crystalline penicillin and chloroquine according to the standard treatment protocol of the Ghanaian Ministry of Health.

Characterisation of bacteria

Boiled CSF supernatants were tested serologically (Slidex méningite-Kit, Bio-Mérieux) for capsular polysaccharide antigens of *N. meningitidis* (serogroups A, B and C), *Haemophilus influenzae* type b and *Streptococcus pneumoniae*. CSF specimens were frozen at -70°C and transported to Switzerland on dry ice for further microbiological analyses.

For the cultivation of bacteria, CSF specimens were inoculated on blood, chocolate and Thayer-Martin agar (Thayer & Martin 1966) and incubated for 24h at 37°C in an atmosphere of 5% CO_2 . Isolates were stored in 10% skim milk (Difco) on

glass beads at -70°C . All bacteria strains isolated from Thayer-Martin agar were identified as meningococci by Gram's stain morphology, cytochrome oxidase test using N,N,N',N'-tetramethyl-1,4-phenylene-diammoniumdichloride (Merck, Darmstadt) as substrate, glucose, maltose and sucrose utilisation in cystine-trypticase agar (BBL, Heidelberg) and gamma-glutamyltransferase activity (MPR 1-Kit, Boehringer Mannheim). The Gram-negative rods isolated from blood and chocolate agar were identified with api 20E (Bio-Mérieux, Nürtingen), the Gram-positive cocci showing α -hemolysis by testing for Optochin sensitivity (DD1 discs, Oxoid, Wesel). *H. influenzae* was characterised by growing only on chocolate agar or as satellite colonies on blood agar with a *Staphylococcus aureus* streak and X- and V-factor requirements (V-, X+V-discs, Oxoid, Wesel).

All isolated meningococci were serogrouped/typed/subtyped with monoclonal antibodies by whole cell ELISA according to procedures previously described (Wang *et al.* 1992). One *N. meningitidis* serogroup X strain could not be serogrouped by whole cell ELISA because of lack of the appropriate monoclonal antibody. Serogrouping was done by slide agglutination in this case using serogroup X specific antiserum (Murex).

The *N. meningitidis* isolates were tested for sensitivity to penicillin G, cefotaxime, ciprofloxacin, rifampicin, chloramphenicol, tetracycline and sulfadiazine by the E-test method (AB Biodiscs, Solna, Sweden) according to the manufacturer's instructions.

For restriction fragment length polymorphism (RFLP) analyses, meningococcal chromosomal DNA was isolated as described previously (Sarkari *et al.* 1994). Serogroup A meningococci were tested for *opa*, *iga* and *ingA* alleles as described (Morelli *et al.* 1997). Two *N. meningitidis* serogroup A subgroup III control strains were incorporated, one 'pre-Mecca' strain (Morelli *et al.* 1997) isolated in China in 1966 (strain Z3906) and one 'post-Mecca' strain isolated in Chad in 1988 (Z3524). For pulsed-field gel electrophoresis (PFGE), meningococcal DNA was prepared in agarose blocks as described by Morelli *et al.* (1997). The DNA was digested with *NheI* and *SpeI* and resolved by pulsed-field gel electrophoresis (Morelli *et al.* 1997). After electrophoresis, the gels were stained with ethidium bromide, visualized on an ultraviolet light transilluminator and photographed.

All CSF samples that remained culture negative were tested for the content of meningococcal DNA by IS1106 PCR according to previously described procedures (Newcombe *et al* 1996). The detection of the PCR products was done with a DNA enzyme immunoassay kit (GEN-ETI-K DEIA, DiaSorin). PCR products were directly sequenced in both directions using the ABI Prism 310 Genetic Analyzer (Perkin Elmer, Foster City, CA). Culture and IS1106 PCR negative CSFs were tested for *Herpes simplex* virus, *Varicella-zoster* virus and enteroviruses by PCR as described previously (Aurelius *et al.* 1991, Puchthammer-Stökl 1993, Romero & Rotbart 1993).

Statistical methods

Log-linear analysis of contingency tables was used to investigate possible associations between *NheI* and *SpeI* variant patterns, and Wilcoxon tests were used to compare the median times of occurrence these patterns. Fisher's exact tests were used to identify prognostic factors for the outcome.

In order to identify a possible spatial clustering of the isolates with distinct PFGE patterns, euclidean distances were calculated between the homes of each possible pair formed from these cases. For each restriction enzyme the 231 pairs were classified according to whether the members of the pair both belonged to the same PFGE pattern. The mean distances between members of these homotypic pairs were compared with the mean distances between members of heterotypic pairs. A randomisation test (Manly 1991) was used to test whether there was a statistically significant difference between these two means.

3.4 Results

Meningitis cases

From the 14th of February, 1998 to the 24th of April, 1998, a total of 92 suspected meningitis cases were recruited at the WMH and at the Eastern Health Centre of the KND. 50 patients (52% males) were diagnosed as having meningococcal meningitis of serogroup A. Bacteria were cultivated and characterized in detail from 36 CSF samples. Latex-agglutination and/or IS1106 PCR ELISA revealed serogroup A polysaccharide antigen and meningococcal DNA, respectively in another 14 cases

where no bacteria could be cultivated. Of the remaining 42 suspected meningitis cases, one was diagnosed as having a X: NT: P1.5 *N. meningitidis* phenotype, one *Streptococcus pneumoniae*, two *Haemophilus influenzae* type b and one *Enterobacter aerogenes* meningitis. CSFs from patients without confirmed bacterial meningitis were tested by PCR for the presence of *Herpes simplex* virus, *Varicella-zoster* virus and enteroviruses. However none was positive. 37 (40.2%) of all suspected meningitis cases thus remained without any confirmed diagnosis. Among these cases, 9 CSFs were turbid, indicating probable bacterial infection. Table 3.1 shows the distribution of meningitis cases by etiology and method of diagnosis.

The median age of the 50 patients with serogroup A meningococcal meningitis was 8 years (mean=10, range 4 months to 64 years). 19 were less than 5 years with 4 patients being less than one year old, 12 were between 5 and 9 years, 16 between 10 and 19 years, and 3 were over 20 years. 10 (20%) of these patients died during hospitalization. Clinical information on admission, and histories were available for 34 patients (68%) (Table 3.2). All these patients had a history of fever, most of them lasting for one day only. Three-quarter received drugs prior to admission, usually antimalarials. On clinical examination most of them had neck stiffness and one quarter presented with stupor or coma. The latter was associated with death or sequelae (odds ratio=6.6, CI^{95%}: 1.3-33.3, p=0.03) but there was no significant association between the outcome and any other variable recorded at admission (Table 3.2). Out of the 5 additional confirmed bacterial meningitis cases, three died during hospitalization (*N. meningitidis* serogroup X, *H. influenzae*, *E. aerogenes*).

Table 3.1. Diagnosis of meningitis cases

Causative agent	n	% of cases	Diagnosed by
<i>Neisseria meningitidis</i> serogroup A	50	54	Latex-Agglutination, culture, biochemistry, ELISA (72%) Latex-Agglutination, IS1106 PCR ELISA (18%) IS1106 PCR ELISA (10%)
<i>Neisseria meningitidis</i> serogroup X	1	1	Culture, biochemistry, slide agglutination, ELISA
<i>Streptococcus pneumoniae</i>	1	1	Latex-Agglutination, culture, Optochin sensitivity
<i>Enterobacter aerogenes</i>	1	1	Culture, biochemistry (api 20E)
<i>Haemophilus influenzae</i> type b	2	2	Latex-Agglutination, culture, slide agglutination (50%) Latex-Agglutination (50%)
No conf. Diagnosis; turbid CSF	9	10	-----
No conf. Diagnosis; clear CSF	28	30	-----
Total	92	100	

Table 3.2. History and clinical examination findings in the serogroup A meningococcal meningitis cases.

Condition	All cases n=34 (%)	Death or sequelae ^a n=9 (%)
<i>History</i>		
Fever	34/34 (100)	9/9 (100)
Duration (1 day vs more)	17/34 (50)	5/9 (56)
Headache	21/26 ^b (81)	6/9 (67)
Nausea	4/26 ^b (15)	1/9 (11)
Vomiting	28/34 (82)	6/9 (67)
Neck pain	17/28 ^b (61)	4/9 (44)
Neck stiffness	18/34 (53)	6/9 (67)
Convulsions	10/34 (29)	5/9 (56)
Altered consciousness ^c	8/34 (24)	3/9 (33)
Prior drug intake	26/34 (76)	7/9 (78)
Antimalarials	16/34 (47)	2/9 (22)
Antibiotics	5/34 (15)	2/9 (22)
Vaccinated	21/32 ^d (66)	5/7 ^d (71)
<i>Clinical examination</i>		
Temperature > 37.5° C	22/33 ^e (67)	3/8 ^e (38)
Temperature ≥ 39.0° C	4/33 ^e (12)	1/8 ^e (13)
Neck stiffness	28/34 (82)	7/9 (78)
Altered consciousness ^f	9/34 (26)	5/9 (56)

^a One case of sequelae (deafness), ^b Denominator is less due to non-applicable variable, ^c Drowsiness or loss of consciousness, ^d Two missing values, ^e One missing value, ^f Stupor or coma.

Characteristics of serogroup A meningococcal isolates

All the 36 strains serotyped by whole cell ELISA were A: 4: P1.9, 20 meningococci which corresponds to subgroup III (Wang *et al.* 1992). Three were tested by MLST, all were ST5, typical of subgroup III (Maiden *et al.* 1998). No evidence for resistance to penicillin G, chloramphenicol, cefotaxime, ciprofloxacin, rifampicin or tetracycline was found. All strains were resistant to sulfadiazine (minimal inhibitory concentration MIC >256µg/ml). All strains included in the RFLP

analysis of *opa*, *iga* and *ingA* alleles showed the pattern expected for post-Mecca subgroup III (Table 3.3). As an example the *DdeI* restriction fragment pattern of the 413 bp *opaD* 100 amplification product is shown in Figure 3.1.

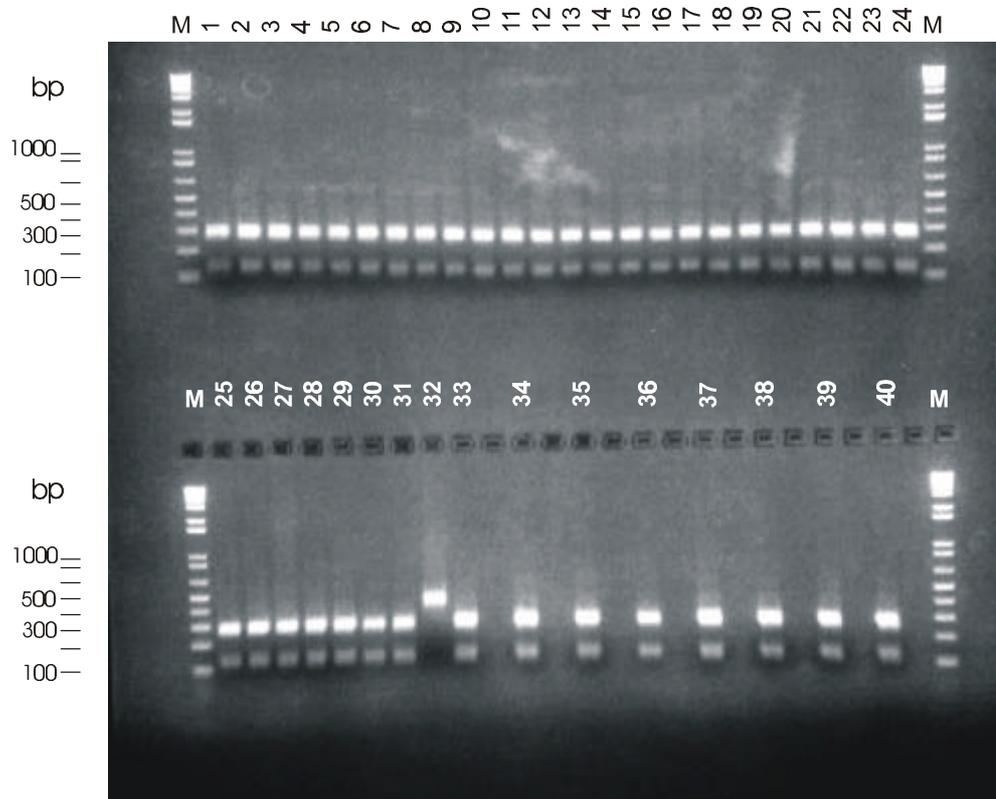


Figure 3.1. Restriction fragment length polymorphism analysis of *opaD* PCR products from meningococci from Ghana. A molecular weight marker was loaded in the flanking tracks as indicated (M). The 413 bp *opaD* 100 amplification product was restricted by *DdeI*. Two fragments (128 and 285 bp) were obtained with all strains including the post-Mecca control strain Z3524 (track 13) isolated in Chad in 1988 and several post-Mecca control strains (tracks 1, 3, 5, 7, 9 and 11) isolated in The Gambia in 1997. As expected, the PCR product of the pre-Mecca control strain isolated in China in 1966 (Z3906) was not cut by *DdeI* (track32).

Table 3.3. RFLP analysis of *opaA*, *opaB*, *opaD*, *ingA* and *iga* alleles: Size of the DNA fragments expected for serogroup A, post-Mecca subgroup III meningococci. Pre-Mecca strains have *opaD131*, *opaB92*, *ingA1* and *iga1* or *iga2*. Post-Mecca strains have *opaD100*, *opaB94*, *ingA2* and *iga3*. The restriction tests shown here distinguish between these various alleles.

Gene / segment	Restriction endonucleases	pre-Mecca		post-Mecca	
		Size of expected PCR product (bp)	Sizes of expected restriction fragments (bp)	Size of expected PCR product (bp)	Sizes of expected restriction fragments (bp)
<i>opaA 132</i>	<i>RsaI</i>	418	56, 179, 183	418	56, 179, 183
<i>opaA 132</i>	<i>HpaII</i>	418	7, 126, 285	418	7, 126, 285
<i>opaB 92</i>	<i>HincII</i>	461	164, 297	no product	----
<i>opaB 94</i>	<i>BanI</i>	no product	----	461	198, 263
<i>opaD 100</i>	<i>DraI</i>	413	179, 234	413	179, 234
<i>opaD 100</i>	<i>DdeI</i>	413	413	413	128, 285
<i>ingA</i>	<i>NheI</i>	350	350	350	100, 250
<i>iga</i>	<i>DdeI</i>	2009	2009	2009	535, 1474

For PFGE analysis, DNA was digested with the rare cutting enzymes *NheI* and *SpeI*. The results (Figures 3.2a & 3.2b) showed that the isolates from Ghana resembled post-Mecca strains which have lost one *NheI* band and gained 2 others relative to pre-Mecca bacteria. In addition, several polymorphic sites were seen, that subdivided the bacteria although all are very closely related. Three distinct patterns were distinguishable with each of the two restriction enzymes among the Ghanaian isolates. Based on the few polymorphic sites, there was no association between the *NheI* and *SpeI* variant patterns (likelihood ratio from log-linear model $\chi_4^2 = 4.2$, $p = 0.4$). A total of 7 combinations of the *NheI* and *SpeI* variant patterns were found. From the map showing the spatial distribution of the 32 cases where the position of the home was geocoded (Figure 3) it was not clear whether these bacterial types were clustered in space. However, the randomization test indicated that the observed degree of clustering of homotypic cases could easily have been due to chance (Table 4). The median times of occurrence of the different types distinguished by either *NheI* or *SpeI* restriction analysis were not significantly different (Wilcoxon tests: $\chi_2^2 = 2.0$; $P = 0.4$ and $\chi_2^2 = 1.1$; $P = 0.6$, respectively, Table 5).

Table 3.4. Analysis of spatial clustering of PFGE restriction types.

Restriction enzyme	N of homogeneous pairs	N of heterogeneous pairs	Difference between mean distances ^a (km)	p-value ^b
<i>NheI</i>	221	185	-2.2	0.092
<i>SpeI</i>	196	210	-1.3	0.42
Both	117	289	-1.3	0.27

^a Mean distance between homogeneous pairs minus mean distance between heterogeneous pairs, ^b From randomisation test .

Table 3.5. Median times to occurrence of different PFGE restriction types.

		Frequency		Median date
<i>NheI</i> -type	1	10/31*	(32%)	08/03/98
	2	20/31	(65%)	12/03/98
	3	1/31	(3%)	08/04/98
<i>SpeI</i> -type	1	5/31	(16%)	13/03/98
	2	20/31	(65%)	14/03/98
	3	6/31	(19%)	17/03/98

* Data from 5 strains out of 36 were not available.

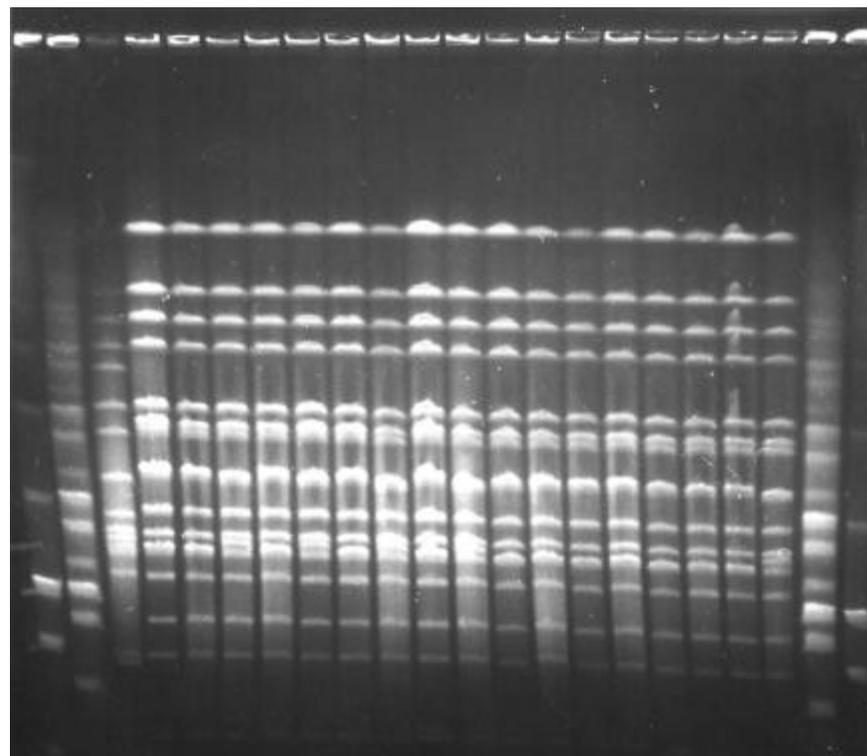
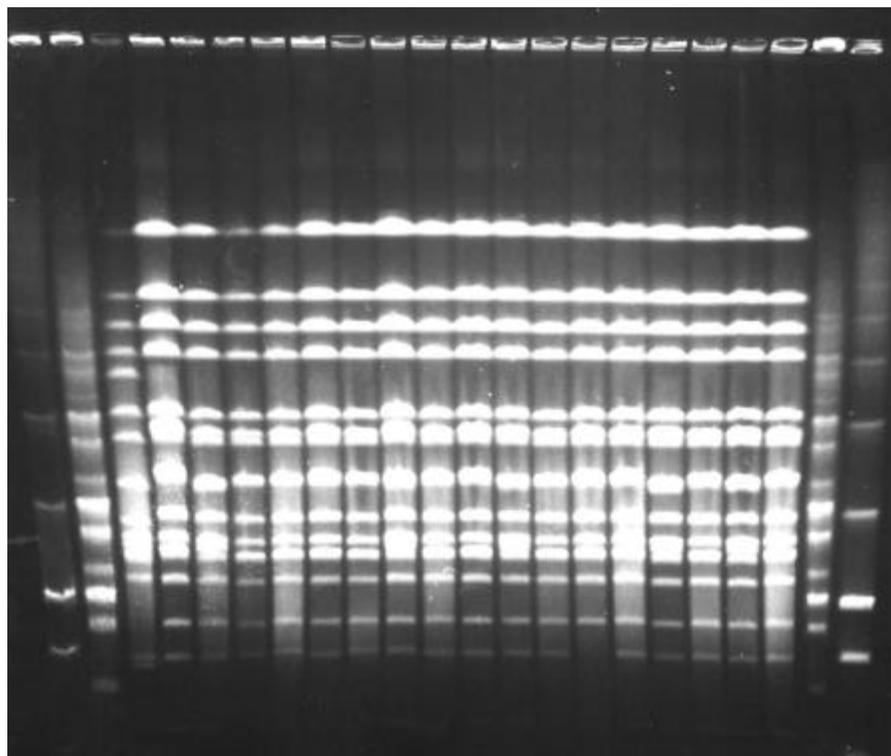
Figures 3.2a & 3.2b. Pulsed-field gel electrophoresis of DNA from meningococci from Ghana. Samples were loaded in tracks 1 to 36 in the following order (track: strain): 1: Z3906 (pre-Mecca control strain); 2: Z3524 (post-Mecca control strain); 3: Z7057; 4: Z7058; 5: Z7059; 6: Z7060; 7: Z7061; 8: Z7062; 9: Z7063; 10: Z7064; 11: Z7065; 12: Z7066; 13: Z7067; 14: Z7068; 15: Z7069; 16: Z7070; 17: Z7071; 18: Z7072; 19: Z3906; 20: Z3524; 21: Z7073; 22: Z7074; 23: Z7075; 24: Z7076; 25: Z7077; 26: Z7078; 27: Z7079; 28: Z7080; 29: Z7081; 30: Z7082; 31: Z7083; 32: Z7084; 33: Z7085; 34: Z7086; 35: Z7087; 36: Z7088; (strains Z7090, Z7092 and Z7093 not shown; one isolate out of 36 was not analysed by PFGE). Molecular weight markers were loaded in the flanking tracks as indicated (LM: Low Range Marker; MM: Mid Range Marker I); their molecular weights are indicated at the left. Differences in the PFGE patterns are indicated with arrows at the right. The 3 different PFGE restriction types found with each of the two enzymes are indicated in roman numbers (X: pre-Mecca control strain; Y: post-Mecca control strain). **a)** digested with *NheI*. **b)** digested with *SpeI*.

(a)

LM MM 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 MM LM LM MM 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 MM LM
X Y I II II II II I II II I II III II I II I II X Y II I II I II I II II II II II II I

kb

194 —
145 —
97 —
48 —
23 —



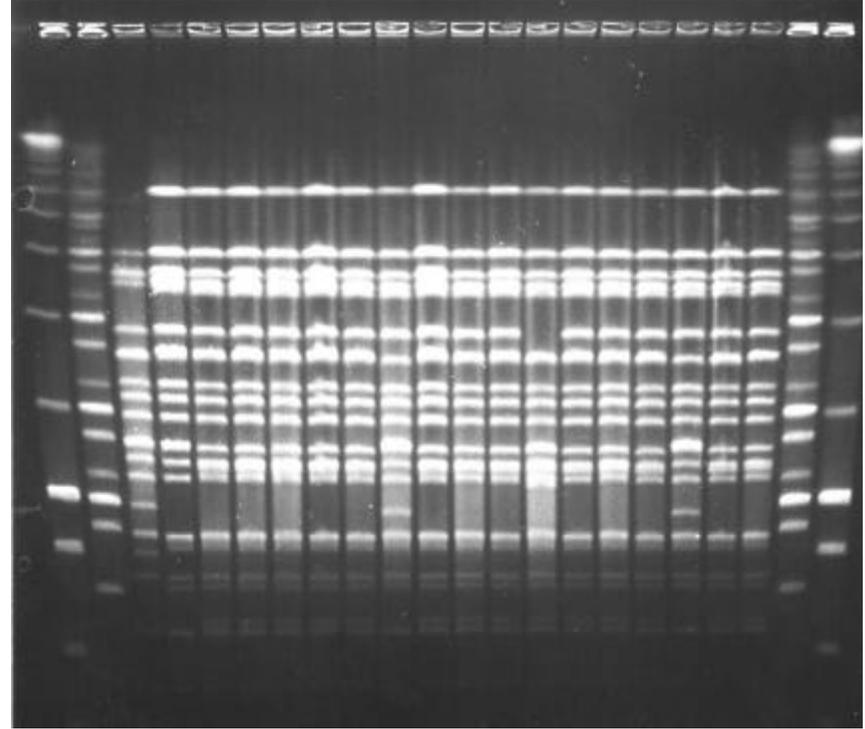
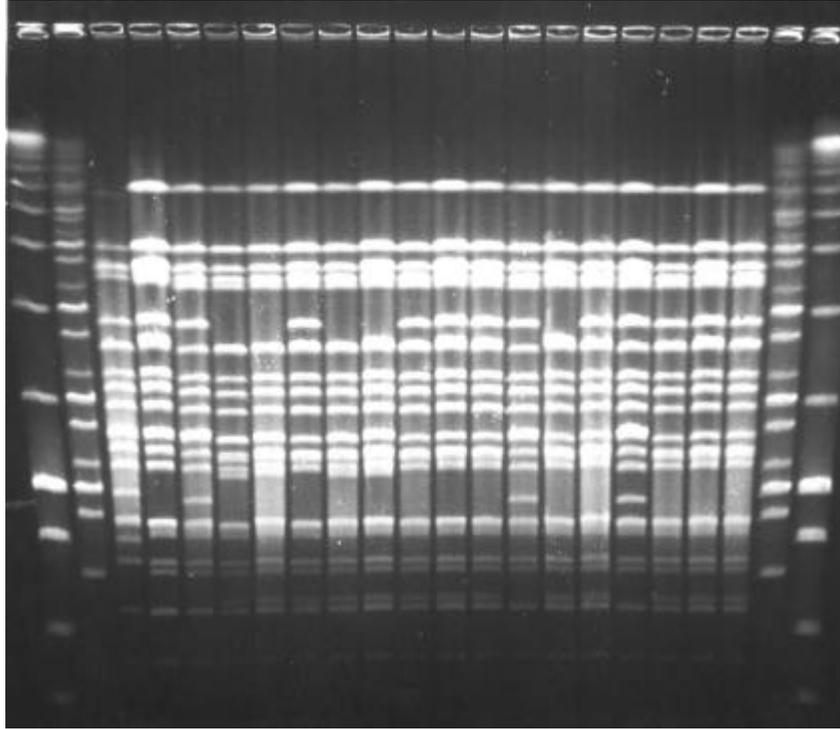
(b)

LM MM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 MM LM
X Y I III III II III III II II II I III II I II II II

LM MM
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 MM LM
X Y II II II II II I II II II III II II I II II

kb

194 —
145 —
97 —
48 —
23 —



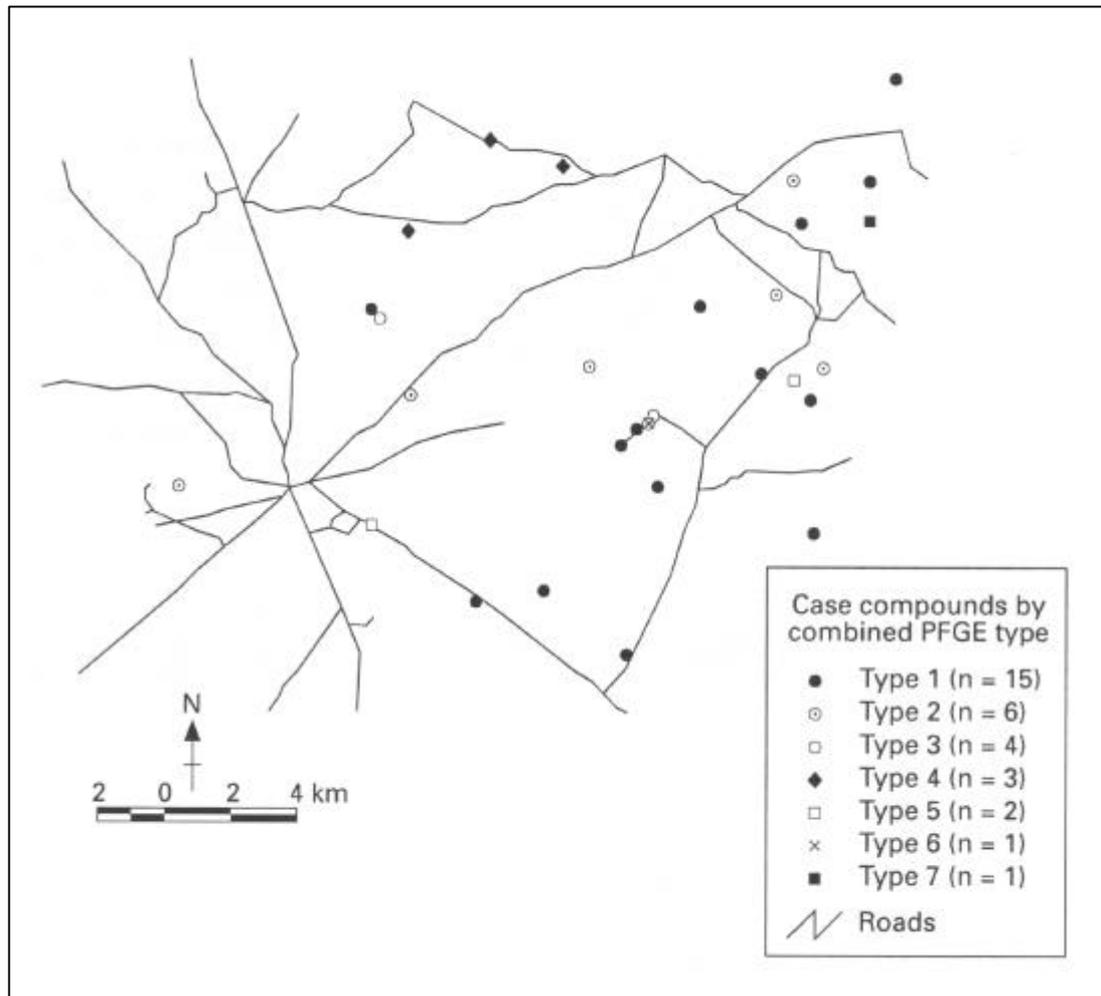


Figure 3.3. Spatial distribution of the 7 combined PFGE types (*NheI+SpeI*) in the Kassena-Nankana East sub-district. PFGE patterns of 35 isolates were analysed and the homes of 32 of the corresponding cases were traced. The 7 PFGE combination types were: type 1: *NheI-II+SpeI-II*; type 2: *NheI-I+SpeI-II*; type 3: *NheI-II+SpeI-III*; type 4: *NheI-I+SpeI-I*; type 5: *NheI-II+SpeI-I*; type 6: *NheI-I+SpeI-III*; type 7: *NheI-III+SpeI-III*. Two type 1 cases occurred in one compound.

3.5 Discussion

The clinical picture of meningitis in the 1998 epidemic in Kassena-Nankana district was typical of that for outbreaks in the meningitis belt. There was a 20% case fatality rate, which was higher than the expected 5-10% (WHO 1998). This probably reflects delays in arrival at the health facilities, but not resistance of the bacteria to the antibiotics used. The patients who presented with coma or stupor had particularly poor prognosis, but there was no association between the duration of fever prior to admission and outcome. When considering these prognostic factors however, the reader should bear in mind the low statistical power of analyses based on only eight deaths and one case with deafness.

We could demonstrate no cases of viral etiology or resistance of meningococci to antibiotics other than sulphadiazine. It is not clear whether there was an increase in the incidence of *S. pneumoniae* and *H. influenzae* meningitis during the outbreak. Due to the 1997 epidemic the population was clearly attuned to the danger of meningitis, and many cases (29/50, 58%) were reported by relatives to have been vaccinated with an A and C bivalent polysaccharide vaccine during late 1997 and early 1998. This does not necessarily reflect a low efficacy of the vaccine: a major effort was made to ensure high coverage, so far more vaccinated than unvaccinated individuals were at risk in the population. The epidemic would probably have been much larger without this vaccination campaign.

The latex agglutination method missed 5/50 cases of meningococcal meningitis which were detected using the IS1106 PCR ELISA. The specificity of IS1106 PCR ELISA has been questioned (Borrow *et al.* 1998), but in our setting, where A meningococci were dominant, this technique is probably reliable. Furthermore, the sequences of the PCR products obtained from the 5 latex agglutination negative cases were identical to those of subgroup III serogroup A controls and differed from serogroup B and C controls (data not shown). All latex positive samples were also PCR positive. Among the patients who remained without confirmed diagnosis, 9 had a turbid CSF, making them probable bacterial meningitis cases. Some of the patients without confirmed diagnosis and with clear CSF may have had underlying diseases other than meningitis.

Overall, the stability of the genome of the post-Mecca subgroup III clone of *N. meningitidis* allows clear assignment a decade after its origin. However uptake and insertion of foreign DNA have been documented in these bacteria (Morelli *et al.* 1997). The PFGE results are comparable to those observed from other countries where subgroup III has been analyzed (unpublished data). The statistical analysis of possible clustering of cases caused by PFGE identical strains showed that these were not significantly closer together than cases caused by PFGE different pairs. Although the sample size (and hence power) was limited, this is evidence that the variants did not spread out in the course of the 1998 outbreak from point sources within the area, and suggests that transmission between cases during 1998 was not an important cause of the outbreak. The genetic heterogeneity in our isolates and the absence of a temporal pattern in the PFGE types also suggest that the outbreak arose from pre-existing heterogeneous populations of subgroup III bacteria, provoked by a common environmental stimulus. Some genotypes appear to be distributed predominantly at the western side of the map, others occur in broad bands down the center, and others towards the north. These patterns could be non-random in space, without leading to evidence of clustering in our randomization test. The patterns are consistent with the different bacterial genotypes entering Kassena-Nankana before the start of the 1998 outbreak in temporal waves or from different directions.

3.6 Acknowledgements

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Chapter 4

Fit genotypes and escape variants of subgroup III *Neisseria meningitidis* during three pandemics of epidemic meningitis

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4.1 Abstract

The genetic variability at six polymorphic loci was examined within a global collection of 502 isolates of subgroup III, serogroup A *Neisseria meningitidis*. Nine “genoclouds” were identified, consisting of genotypes that were isolated repeatedly plus 48 descendent genotypes that were isolated rarely. These genoclouds have caused three pandemic waves of disease since the mid-1960’s, the most recent of which was imported from East Asia to Europe and Africa in the mid-1990s. Many of the genotypes are escape variants, resulting from positive selection which we attribute to herd immunity. Despite positive selection, most escape variants are less fit than their parents and are lost due to competition and bottlenecks during spread from country to country. Competition between fit genotypes results in dramatic changes in population composition over short time periods.

4.2 Introduction

Epidemic meningitis has posed a major health problem since it was first recognized in 1805 (Vieusseux 1806). Epidemics have recurred approximately every 10 years in this century within the African “meningitis belt” of sub-Saharan Africa (Lapeyssonnie 1963, Caugant 1998, Achtman 1990) and until the mid-1980’s within China (Wang *et al.* 1992). Epidemics in other geographical areas are more sporadic and in recent decades, only few industrialized countries have suffered from large epidemics (Caugant 1998), with the notable exceptions of Norway and New Zealand.

Historically, most large epidemics of meningitis were caused by *Neisseria meningitidis* that express the serogroup A capsular polysaccharide. Most recent epidemics have been caused by two pandemic waves (Achtman *et al.* 1992) of the clonal grouping of serogroup A bacteria that is called subgroup III (Wang *et al.* 1992, Maiden *et al.* 1998). The first pandemic wave affected China (mid-1960’s), northern Europe (Russia and the Scandinavian countries; 1969-mid 1970’s) and Brazil (mid-1970’s). A second subgroup III pandemic wave began in China and Nepal in the early 1980’s, followed by an epidemic during the annual Hajj pilgrimage to Mecca, Saudi Arabia of 1987. Pilgrims returning from Mecca distributed subgroup III meningococci to most countries of the globe (Achtman *et al.* 1992, Moore *et al.* 1988). Subgroup III has subsequently caused numerous epidemics throughout Africa (Caugant 1998,

Nicolas *et al.* 2001); in 1996, 150,000 cases of meningococcal disease were reported from Africa (Tikhomirov *et al.* 1997), most caused by subgroup III (Caugant 1998).

Subgroup III meningococci are so uniform that only few genetic variants have been revealed by MLEE (multi-locus enzyme electrophoresis) (Wang *et al.* 1992, Achtman *et al.* 1992), RAPD (random amplified polymorphic DNA) (Bart *et al.* 1998, Achtman *et al.* 2001) or MLST (multi-locus sequence typing) (Maiden *et al.* 1998, Achtman *et al.* 2001). Such variants can arise during co-colonization of the nasopharynx with other *N. meningitidis* and related species due to the import of genes by DNA transformation (Linz *et al.* 2000, Smith *et al.* 2000).

The availability of hundreds of subgroup III strains isolated in different countries since the mid-1960's provided an opportunity to elucidate important features of bacterial microevolution. We have used a multilocus approach involving sequence variability at six polymorphic loci to elucidate the molecular epidemiology of subgroup III bacteria. These six loci encode three outer membrane proteins (*opaB*, *opaD*, *tbpB*), one secreted protein (*iga*), one housekeeping protein (*pgm*) and one defective IS element (IS1106A) and were chosen due to their relatively high degree of polymorphism in subgroup III. Previous work has shown that differences at the *opaB*, *opaD* and *iga* loci (that encode opacity adhesins and IgA1 protease) distinguish bacteria isolated during the first pandemic wave or at the beginning of the second pandemic wave from those isolated since 1987 from Mecca pilgrims and in Africa (Morelli *et al.* 1997). The *pgm* gene encoding phosphoglucomutase differentiates subgroup III isolates of MLST sequence types 5 through 7 (Maiden *et al.* 1998). *tbpB* (transferrin-binding protein B) differed between older and newer isolates from Moscow (Achtman *et al.* 2001). IS1106A is a defective IS element that is inserted upstream of the *opcA* locus (Zhu *et al.* 1999) and was shown by preliminary experiments to be polymorphic in subgroup III.

The results show that successive waves of pandemic spread are associated with genoclouds of genetic variants, each containing a particularly fit, dominant genotype. Furthermore, the recent evolution of a new genocloud has now resulted in a third pandemic wave of disease.

4.3 Materials and Methods

Bacterial strains

502 subgroup III strains isolated between 1966 and 2000 were tested. The countries and detailed dates of isolation have been described for 314 strains (1966–96) from diverse global sources (Morelli *et al.* 1997) and 29 ST7 strains (1995–99) from Africa (Nicolas *et al.* 2001). 159 additional isolates were from epidemics in Chad (1988, 9 strains), The Gambia (1997, 54), Ghana (1997-1998, 39) (Chapter 3), Mongolia (1994-95, 10) (Malorny *et al.* 1998), Moscow (1969-77, 8; 1994-97, 21) (Achtman *et al.* 2001) and Sudan (1988, 14) and from endemic disease in the UK (1997-2000, 4). The assignment of these additional isolates to subgroup III is based on MLEE for isolates from Chad, Sudan and Mongolia, RAPD for isolates from Moscow (Achtman *et al.* 2001) and by the possession of strongly related PFGE patterns with two restriction endonucleases (Chapter 3) for isolates from The Gambia, Ghana and the UK. Of the 502 isolates, 60 have been shown to belong to the MLST sequence types ST5 to ST7 that are characteristic of subgroup III (Nicolas 2001, Achtman *et al.* 2001). Most of the strains have been serogrouped, serotyped and serosubtyped by ELISA using monoclonal antibodies and are A:4,21:P1.9,20, as is typical of subgroup III (Wang *et al.* 1992).

Sequencing and accession numbers

Independent PCR products from each locus were sequenced from both DNA strands using automated cycle sequencing (ABI 377) with dRhodamine-labelled terminators (PE Applied Biosystems). Sequences of IS1106A elements have been deposited under AJ276902-AJ276908 and AJ292239. Sequences of *tbpB* alleles have been deposited under AJ276909-AJ276937. Other sequences have been described elsewhere (Maiden *et al.* 1998, Morelli *et al.* 1997, Malorny *et al.* 1998) (GenBank AJ292235-AJ292238).

RFLP typing

All 502 isolates were tested for restriction fragment length polymorphism (RFLP) at six loci (*pgm*, *iga*, *opaB*, *opaD*, IS1106A and *tbpB*) as described (Achtman

et al. 2001, Morelli *et al.* 1997). In addition, all variant alleles were sequenced from representative strains (*pgm*, 60 strains; *iga*, 18; *opaB*, 43; *opaD*, 50; IS1106A, 21; *tbpB*, 35). The sequence variability at *iga*, *opaB* and *opaD* has been described elsewhere (Morelli *et al.* 1997). Details of the sequence variability at IS1106A are presented in the supplemental data (www.pnas.org). The RFLP analysis of *pgm* distinguishes *pgm3* from *pgm19*, but not from *pgm11* which only differs by one nucleotide (Maiden *et al.* 1998). *tbpB* alleles that are identical to previously described sequences (*tbpB1*, 10, 11, 101) (Linz *et al.* 2000) were assigned the same numbers and other alleles were assigned arbitrary numbers from *tbpB38* to *tbpB66*. These allele assignments were based on an aligned stretch of approximately 660 bp that has been sequenced from all isolates. (We note that the *tbpB1* allele is assigned to both subgroups III and IV-1 although the complete 2057 bp *tbpB* sequences from subgroups III (accession number AJ276909) and IV-1 (AF058689) differ by two nucleotides outside this aligned region.)

Phylogenetic analyses

tbpB sequences were aligned manually to ensure that codons were not split. The aligned sequences were analyzed using SPLITSTREE 3.1 (Huson 1998), as described (Linz *et al.* 2000). Chi-squared analysis of significance between success and failure was performed using tests 3 and 4 of CLUMP (Sham & Curtis 1995), which tests the significance of data in sparse matrices by a Monte Carlo method.

4.4 Results

Genocloud structure of subgroup III

The genetic fine structure of subgroup III was investigated among 502 subgroup III isolates by using restriction fragment length polymorphism (RFLP) at *opaB*, *opaD*, *tbpB*, *pgm*, *iga* and IS1106A. The isolates tested include 314 subgroup III meningococci from diverse global sources that have been previously described (Morelli *et al.* 1997) plus 188 recent isolates from Mongolia, Moscow, Africa and the UK. 227 variant alleles were sequenced from representative strains and at least two alleles were found for each of the six loci.

Table 4.1. Geographic distribution of frequent and rare genotypes of subgroup III.

Genocloud	Pandemic	Sources	Years	Frequent genotypes		Rare genotypes	
				Nr. of isolates	Nr. of genotypes	Nr. of isolates	
1	1	China	1966	2	3	5	
2	1	Moscow	1969-71	14	3	3	
3	1,2	Europe, Brazil, China	1969-84	43	23	31	
4	2	China	1984-87	26	4	5	
5	2	Pilgrims, Africa	1987-98	236	7	8	
6	2	Africa	1991-93	27	0		
7	2	Africa	1991-93	15	1	1	
9	2	The Gambia	1997	8	0		
8	3	China, Mongolia, Moscow, Africa, UK	1993-2000	66	3	3	
Intermediate					4	9	
Total			1966-2000	437	48	65	

Each unique combination of alleles is referred to as a genotype and a total of 57 genotypes were found. Most (87%) of the 502 isolates possess one of nine frequent genotypes (Table 4.1). The nine frequent genotypes differed from each other by one to six of the six loci (Figure 4.1; see supplemental data for details). The other 48 genotypes were isolated only rarely (one to four isolates). They were isolated concurrently with a frequent genotype from the same geographical area and differed from that frequent genotype at only one, or rarely two, of the loci (Figure 4.1; supplemental data). All three criteria (minimal genetic, physical and temporal distance) were used to assign rare genotypes to groups consisting of one frequent genotype plus its close relatives (Figure 4.1). These groups will be referred to as “genoclouds”.

Six of the genoclouds contain a frequent genotype plus descendent genotypes but genoclouds 1, 6 and 9 do not fulfill both requirements (Figure 4.1). Genocloud 1 contains only few isolates (Table 4.1). It was designated as a separate genocloud because it includes all the available subgroup III strains that were isolated from several million patients with meningococcal disease in China during the mid-1960's, contains distinct genotypes and is closely related to the deduced ancestral genotype (see below). Genoclouds 6 and 9 possess only one frequent genotype and no rare genotypes. Their designation as genoclouds is somewhat arbitrary and they might equally well represent the transient expansion of rare genotypes.

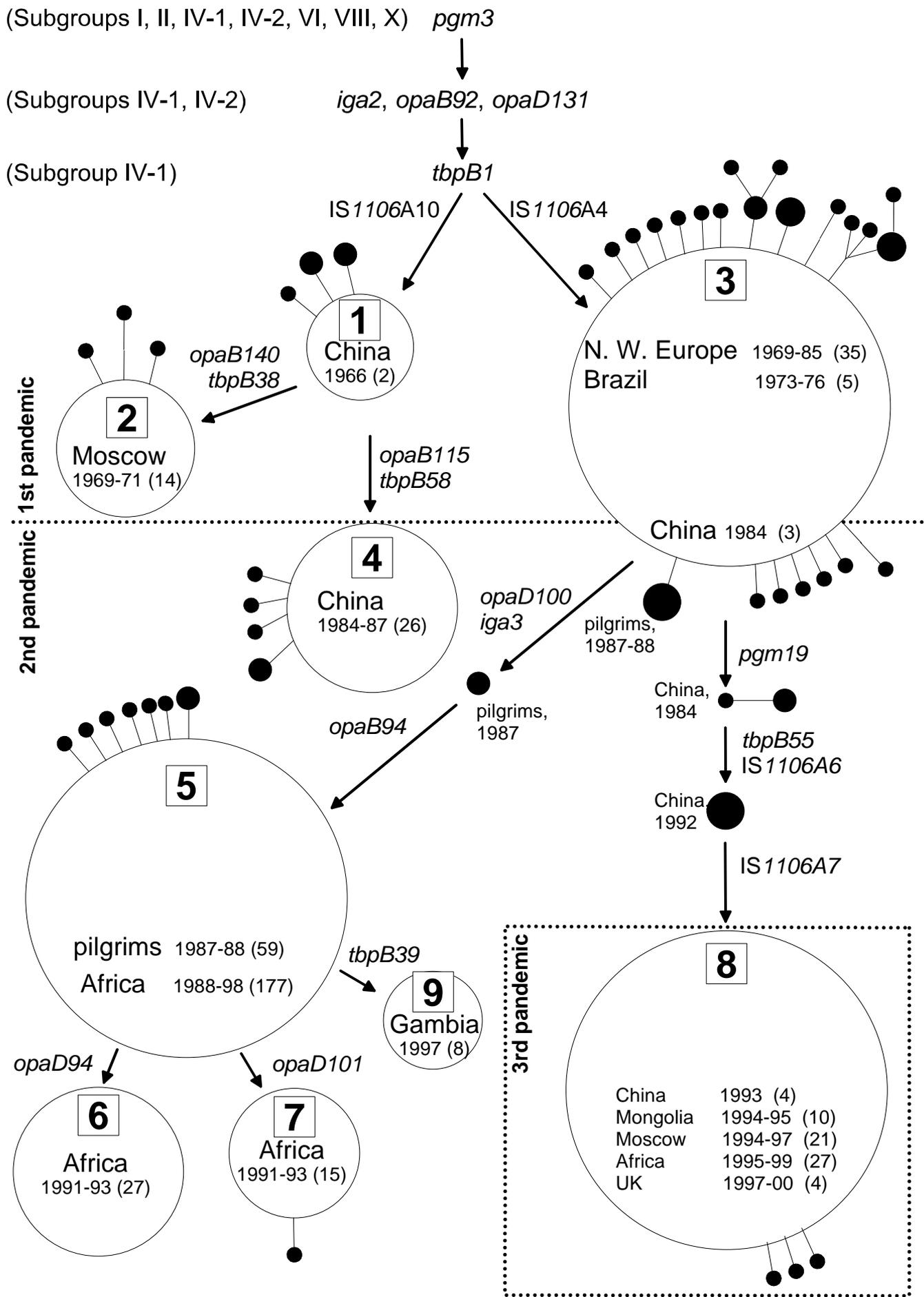
Phylogenetic relationships and pandemic spread

By minimizing genetic and temporal distances between the genoclouds, we constructed a fully parsimonious tree in which each allele arises only once. Four rare genotypes seem to represent intermediates during the evolution from one genocloud to a second and were therefore not assigned to either genocloud (Figure 4.1).

The tree is rooted at genoclouds 1 and 3. These genoclouds share *pgm3*, *iga2*, *opaB92* and *opaD131* alleles with older strains of the related subgroups IV-1 and IV-2 (Morelli *et al.* 1997) and the *tbpB1* allele with older strains of subgroup IV-1 (Linz *et al.* 2000) (Figure 4.1). The other genoclouds possess variant alleles at these loci. Genoclouds 1 and 3 differ from subgroup IV-1 at the *IS1106A* locus. Genocloud 1 possesses one *IS1106A* element inserted into a second, defective *IS1106A* element

whereas genocloud 3 possesses only the defective *IS1106A* element (see supplemental data). Possibly, genocloud 1 is older and was the direct ancestor of genocloud 3 because an *IS1106A* element inserted into a second, defective *IS1106A* element was also found in subgroup IV-1 (Zhu *et al.* 1999). Genoclouds 2 and 4 are descended from genocloud 1 because they possess the same double *IS1106A* elements and genoclouds 5-7 and 9 are derived from genocloud 3 because they possess the same single *IS1106A* element.

Figure 4.1. Parsimonious relationships among 57 genotypes in nine genoclouds of subgroup III. Large boxed numbers from 1 to 9 indicate the genocloud designations. Alleles inherited from common ancestors with other serogroup A subgroups are shown at the top and subsequent changes are shown next to the arrows that indicate lines of descent. Numbers in parentheses indicate numbers of isolates of the frequent genotypes, which are also reflected to a limited extent by the sizes of the circles. Rare genotypes were parsimoniously grouped in genoclouds after consideration of genetic relationships, sources and dates of isolation. They are indicated by filled dots whose sizes indicate the numbers of isolates. The lengths of the lines connecting the dots to the genocloud circles indicate the numbers of genetic changes from the frequent genotype. Intermediate strains that were not assigned to genoclouds are shown along the arrows leading from genocloud 3.



one gene different	● single isolate	● 3 isolates
two genes different	● 2 isolates	● 4 isolates

The first subgroup III pandemic (mid-1960's to late 1970's) was associated with genoclouds 1 to 3 (Table 4.1, Figure 4.1). The second subgroup III pandemic (early 1980's to present) was associated with genoclouds 3 to 7 and 9. A third pandemic caused by genocloud 8 seems to have started in China in 1993. The progressive evolution of genocloud 8 from genocloud 3 is documented by the isolation in China between 1984 and 1992 of intermediate genotypes with progressive genetic changes. Genocloud 8 has now been isolated from epidemics in Mongolia (1994-95) (Anonymous 2001, Zhu *et al.* 1999), Moscow (1994-97) (Achtman *et al.* 2001, Zhu *et al.* 1999) and Africa (1995-99) (Nicolas *et al.* 2001, Zhu *et al.* 1999) and from endemic disease in the UK (1997-2000).

Mechanisms of genetic change

Alleles that differed by at least 2 bp from parental alleles were considered to represent import by DNA transformation. This applies to all novel alleles at *tbpB* (28 alleles) and to one allele each at *iga* and *pgm* (Table 4.2). Similarly, all eight sequenced IS1106A alleles also resulted from import (supplemental data). Eighteen alleles at the *opaB* and *opaD* loci are mosaic genes resulting from gene conversion/translocation with segments of orthologous *opa* genes (most frequently *opaA* and *opaJ*) that are present elsewhere in the genome (Morelli *et al.* 1997, Zhu *et al.* 1999). Four alleles differed from their parental alleles by only one nucleotide or by a 12 bp deletion and possibly represent single-step mutations (Table 4.2).

Fitness of individual genetic changes

It is striking that so many novel alleles were observed for the loci investigated here although only 2 novel alleles were detected for six other housekeeping gene fragments among 137 subgroup III isolates (Nicolas 2001, Achtman *et al.* 2001). Possibly sequence polymorphism was particularly high for the loci tested here because some of them encode gene products that are under selection. Indeed, the *tbpB*, *opaB* and *opaD* loci encode immunogenic outer membrane proteins, and variants at these loci might enable the bacteria to escape herd immunity.

Table 4.2. Sources of alleles in 502 subgroup III isolates.

Locus	Ancestral allele	Number of variant alleles		
		Mutation	Import	Translocation
<i>iga</i>	<i>iga2</i>	1 (<i>iga1</i>)	1 (<i>iga3</i>)	
IS1106A	Uncertain		8 (IS1106A4-11) [¶]	
<i>pgm</i>	<i>pgm3</i>	1 (<i>pgm11</i>)	1 (<i>pgm19</i>)	
<i>opaB</i>	<i>opaB92</i>	1 (<i>opaB93</i>)*	2 (<i>opaB94,5202</i>)	11 (5 x <i>opaA</i> ; 4 x <i>opaD</i> ; 3 x <i>opaJ</i>)*
<i>opaD</i>	<i>opaD131</i>	1 (<i>opaD100</i>)		7 (7 x <i>opaJ</i> ; 1 x <i>opaB</i>)*
<i>tbpB</i>	<i>tbpB1</i>		28 (19 x family 1; 6 x family 4; 3 x family 3)	

[¶]The mechanism by which one additional IS1106A allele (IS1106A99) arose is unclear because that allele is associated with a 12 kb region that has not been sequenced (see supplemental data).

*The mutation in *opaB93* is a 12 bp deletion. Translocations into *opaB*: from *opaA*: *opaB102, 113, 115, 139, 141*; from *opaD*: *opaB100, 114, 131, 1131*; from *opaJ*: *opaB116, 140, 142*. Translocations into *opaD*: from *opaJ*: *opaD101, 108, 109, 110, 111, 112, 1101*; from *opaB*: *opaD94*.

A conservative test for differences in fitness is to compare the number of descendent genotypes associated with different classes of genetic change. Changes at the *tbpB* locus reflect the import of novel *tbpB* alleles belonging to one of four very distinct allele families (Linz *et al.* 2000). The 28 alleles described here fell into three of the same four families (Figure 4.2). The ancestral allele was in family 4 and most of the imported alleles were in family 1. Of the 19 occasions where a *tbpB* allele in family 1 was imported, only one of these alleles was inherited by descendent genotypes, a significantly lower rate of dissemination than for all other observed changes (Table 4.3). These results suggest that the fitness of subgroup III is impaired by the acquisition of *tbpB* alleles of family 1. Acquisition of *tbpB* of family 3 or translocation of alleles from either the *opaJ* or *opaA* loci to the *opaB* or *opaD* loci may also have resulted in reduced fitness (Table 4.3), but the numbers of such events were too small to be statistically significant. In contrast, three of the six novel *tbpB* alleles in family 4, the ancestral family in subgroup III, disseminated to descendent genotypes.

Figure 4.2. Splits graph (uncorrected Hamming distances) of relationships of ~660 bp *tbpB* fragments. Large circled numbers indicate the allele families. The smaller numbers are *tbpB* allele numbers. *tbpB1* is the ancestral allele in subgroups III and IV-1. *tbp10* and *tbp11* were previously found in subgroup IV-1 and commensal neisseriae and *tbpB101* was found in *N. lactamica* (13). *tbpB38* through *tbpB66* have only been found in subgroup III. Sequences from reference strains of *N. meningitidis* for families 1 – 3 (B16B6, 2713, BZ83 and M982) are italicized.

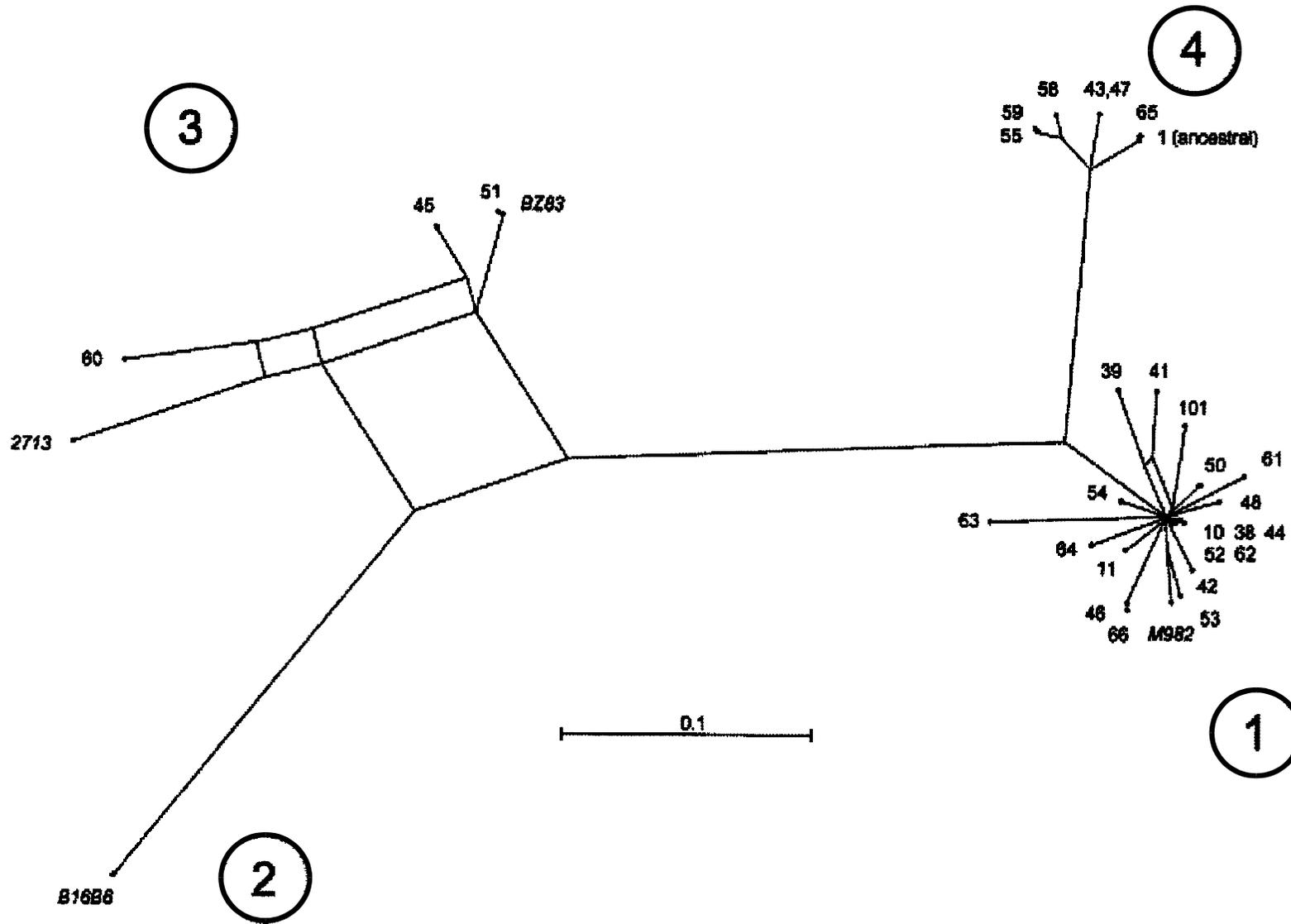


Table 4.3. Fitness analysis of genotypic changes.

No. of descendent genotypes:	No. of genetic events	
	≥ 1 (success)	0 (failure)
<i>tbpB</i> family 1	1	18
<i>tbpB</i> family 3	0	3
<i>tbpB</i> family 4	3	3
<i>pgm</i>	1	1
IS1106A	5	4
Translocation of <i>opaJ</i> *	2	8
Translocation of <i>opaA</i>	1	4
Other <i>opa</i> changes*	4	5
<i>iga</i>	1	1

NOTE: $p = .01$ for the null hypothesis of randomness according to a chi-squared test by comparing success of *tbpB* family 1 with all other genotypic changes (CLUMP test 3, 100,000 repeats) (21). The null hypothesis was also rejected when changes at *opa* alleles were categorized into replacement of *opaB* (5 successes: 10 failures) or *opaD* (2:7).

4.5 Discussion

We analyzed microevolution within an epidemiological framework for a globally representative collection of subgroup III meningococci. Fifty-seven distinct genotypes in nine genoclouds were detected among 502 strains collected during pandemic spread between 1966 and 2000.

The genocloud concept

We designate a frequent genotype plus its epidemiologically associated descendents as a genocloud. The nine subgroup III genoclouds contained rare genotypes that differed by one or occasionally two of the six polymorphic loci. The frequent genotypes in subgroup III differed from each other by one to six loci (supplemental data). All these bacteria are extremely closely related because housekeeping gene fragments were highly uniform among representative isolates (Nicolas *et al.* 2001, Achtman *et al.* 2001). Each genocloud persists only transiently (see below) and subgroup III evolution since 1966 has been marked by the successive replacement of individual dominant genoclouds by others.

The genocloud concept may be generally useful for describing sequence variability among clonally related isolates for many bacterial species. Sequence variation is common in

most microorganisms and clonally related bacteria from a common epidemiological source often show some limited genotypic variation (Day *et al.* 2001). Until now, bacteria have been subdivided into clonally related and unrelated groups, with no standard designation for distinct populations within the clonally related groupings. Furthermore, both genetic variability and different frequencies of particular genotypes are integral aspects of the genocloud concept whereas these are lacking in former terminology. Genoclouds are not “quasi-species”, a mathematical concept that describes an equilibrium population of small genomes (Eigen 1996). Unlike quasi-species, the genetic changes in a genocloud are probably not reversible, represent unique events that do not happen repeatedly, and are continuously eliminated during spread from host to host and from country to country.

Immune selection during epidemic spread

In subgroup III, many of the genetic variants are escape variants that can evade the human immune system (Figure 4.3). Epidemics and pandemics may begin when particularly fit strains invade a naive human population. Herd immunity develops and selects escape variants for particularly immunogenic proteins such as TbpB. Most TbpB escape variants result from the import of DNA from *Neisseria lactamica* and *Neisseria spp.* (Linz *et al.* 2000) that encode TbpBs of family 1. Family 1 differs extensively from family 4 (Figure 4.2), which is ancestral in subgroup III, sufficiently that these two families probably share very few epitopes. We have shown previously (Linz *et al.* 2000) that the high rate of import of TbpB alleles is due to positive selection rather than to an elevated recombination rate: the only common feature of numerous independent recombination events with different recombination endpoints was the import of *tbpB*. Nevertheless, import of TbpB of family 1 (and possibly of family 3) reduces the long-term fitness of the resulting escape variants. Only one of nineteen genotypes that had imported family 1 alleles succeeded in generating descendent genotypes (Table 4.3) whereas half of the imports of a family 4 (ancestral) allele were successful. Thus, many of the rare genotypes may represent escape variants that have a temporary advantage due to different antigenic composition even though they are less fit than their parent (Figure 4.3).

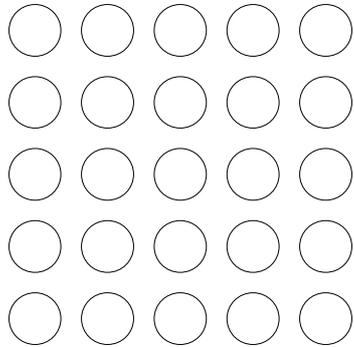
The term ‘fitness’, rather than virulence, is appropriate in this context because during single epidemics, the same serogroup A isolates are isolated from both invasive disease and healthy carriers (Crowe *et al.* 1989). Furthermore, there is no evidence for frequent healthy

carriage of serogroup A meningococci without occasional disease. Thus, for serogroup A, the existence of descendent genotypes among disease isolates reflects efficient transmission between individuals and can therefore be used to calculate measures of fitness.

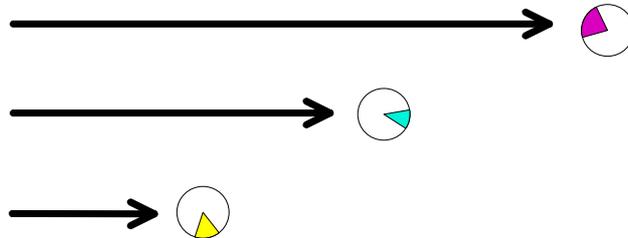
The data are also suggestive of a similar but less strong pattern of selection at the *opa* loci. The ancestral subgroup III genotype possessed distinct alleles at all four *opa* loci (*opaA*, *opaB*, *opaD* and *opaJ*). The *opa101* allele at the *opaJ* locus is not normally expressed due to a stretch of four CTCTT repeats in the signal peptide region which results in a non-functional translational reading frame. The *opa101* allele was repeatedly translocated to the *opaB* and *opaD* loci whereas translocation from the other *opa* loci to *opaJ* is very rare (Morelli *et al.* 1997). This asymmetry is suggestive of positive selection for replacement of OpaB or OpaD by the immunologically distinct Opa101 protein. Transposition of the *opa101* allele may also be detrimental to long-term fitness (Table 4.3), but more observations are needed to test whether this pattern is statistically significant. It is also likely that genetic variation does not always reflect selection, either positive or negative. For example, IS1106A sequence variants are also frequent although it is hard to imagine how different alleles of IS1106A could affect fitness.

Figure 4.3. A model for the formation of rare genotypes and novel genoclouds. Rare genotypes arise by a variety of mechanisms, including import of DNA from unrelated bacteria, translocation of *opa* alleles and single step mutations. Those variants that affect antigens and result in immune escape will multiply preferentially due to selection pressures by the host immune system. However, many of these variants also result in lessened fitness and are eliminated by competition and bottlenecks during spread from host to host and country to country. On rare occasions, antigenic variants with increased fitness can form new genoclouds and repeat the cycle.

Frequent genotype



Import, Translocation, Mutation



Fitness: ++

+

+

+++

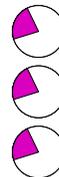
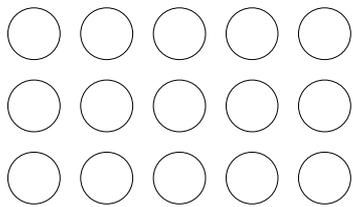
Antigenic variant: -

-

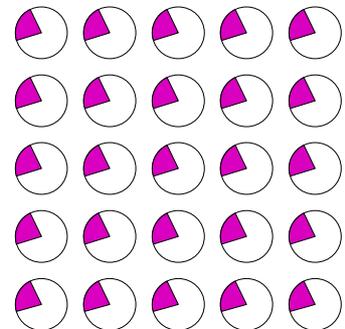
+

+

Immune selection ↓



Competition, bottlenecks ↓



Transience of genoclouds

The half-life of nasopharyngeal colonization is only a few months (Blakebrough *et al.* 1982) and genoclouds disappear from individual countries and areas after several years, even in the absence of vaccination (Scholten *et al.* 1993, Caugant *et al.* 1994, Kwara *et al.* 1998). Genocloud 1 disappeared from China in the 1960's and epidemic disease during the 1970's was caused by subgroup V meningococci (Wang *et al.* 1992). Genocloud 2 disappeared from Moscow in the 1970's and was replaced by subgroups VI and X (Achtman *et al.* 2001). Genoclouds 1-4 have not been isolated since 1985. Thus, meningococcal genoclouds only survive if they compete successfully with their parent and other variant genotypes for transmission to other countries and geographic areas.

Genoclouds persist by migrating from country to country or by generating new variants that escape the immune response against previous infections. Our analysis of the *tbpB* locus suggests that variants that achieve both significant immune escape and high fitness are unusual, possibly due to the rarity of appropriate DNA in the available gene pool. The genocloud structure that we have observed probably reflects how rarely novel genotypes are generated that combine fitness with immune escape at key antigenic sites.

The third subgroup III pandemic

A third subgroup III pandemic associated with genocloud 8 began in China in 1993 after a decade of slow, progressive microevolution from genocloud 3. Since 1994, genocloud 8 has caused large epidemics in Mongolia (Anonymous 2001), Moscow (Achtman *et al.* 2001) and Africa (Nicolas *et al.* 2001). Genocloud 8 has now reached Western Europe and caused several cases of endemic disease in the UK between 1997 and 2000.

Based on historical experience (Achtman 1990), it might have been expected that the recent subgroup III epidemics in diverse African countries had surpassed their zenith. In the past, epidemic waves have not extended much longer than 10 years, and most African countries in the "meningitis belt" have now experienced major epidemics caused by genocloud 5 (supplemental data). However, these epidemics do not seem to have generated long-lasting herd immunity against genocloud 8.

Genocloud 8 has recently caused epidemics in Sudan, Chad and Niger (supplemental data), where epidemics had been caused by subgroup III bacteria with *pgm3* (presumably genocloud 5) only a few years earlier (Nicolas *et al.* 2001). These observations bode poorly for the rapid disappearance of epidemic meningitis from Africa and raise the possibility of renewed epidemic serogroup A disease in Europe and the Americas. Prior to epidemics in Norway and Finland in the early 1970's, serogroup A disease had long been absent, similarly to the current situation. The availability of vaccines based on the A polysaccharide can help control epidemics but new generation A-conjugate vaccines are necessary to ensure the prevention of epidemic disease on a global basis.

4.6 Acknowledgements

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Chapter 5

Prospective study of a serogroup X *Neisseria meningitidis* outbreak in Northern Ghana

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5.1 Abstract

After the 1996-1998 serogroup A meningococcal meningitis epidemic in the Kassena-Nankana district of Northern Ghana, a gradual disappearance of the epidemic strain was observed in a series of five 6-monthly carriage surveys of 37 randomly selected households. As serogroup A *Neisseria meningitidis* carriage decreased, that of X meningococci increased dramatically to reach 18% (53/298) of the people sampled during the dry season of 2000, coinciding with an outbreak of serogroup X disease. These carriage patterns were unrelated to that of *N. lactamica*. Multilocus sequence typing and pulsed-field gel electrophoresis of the serogroup X bacteria revealed strong similarity with other strains isolated in Africa during recent decades. Three closely related clusters with distinct patterns of spread were identified among the Ghanaian isolates, and further microevolution occurred after they arrived in the district. Occurrence of serogroup X outbreaks argues for inclusion of this serogroup into a multivalent conjugate vaccine against *N. meningitidis*.

5.2 Introduction

Neisseria meningitidis can be classified into at least 13 distinct serogroups based on the antigenicity of the polysaccharide capsule (Peltola 1983). Serogroups A, B and C are responsible for over 90% of invasive meningococcal infections worldwide. Most large meningitis epidemics are caused by serogroup A meningococci. Since World War II, such epidemics have been rare in industrialised countries but they occur periodically in the African meningitis belt and in China (Lapeyssonnie 1968, Olyhoek *et al.* 1987, Wang *et al.* 1992, WHO 1998, Caugant 1998). Serogroup C meningococci also cause disease outbreaks and, occasionally, epidemics (Achtman 1995b, Wang *et al.* 1993). Endemic disease is usually caused by meningococci belonging to serogroup B or C but, occasionally, disease is caused by bacteria belonging to other serogroups including W135, Y, and X. Serogroup X of *Neisseria meningitidis* was described in the 1960's (Bories *et al.* 1966, Evans *et al.* 1968) and a limited number of serogroup X meningococcal disease cases have been reported from North-America (Ryan and Hogan 1980), Europe (Grahlow *et al.* 1986, Pastor *et al.* 1985), Australia (Hansman 1983), and Africa (Chapter 3, Riou *et al.* 1996). Some of these have been found to be associated with complement deficiencies (Fijen *et al.* 1996, Swart *et al.* 1993) or AIDS

(Morla *et al.* 1992). Recently, two outbreaks of serogroup X meningococci have been observed in Niger (Campagne *et al.* 1999, Etienne *et al.* 1990). Serogroup X bacteria were also found to be very efficient in colonizing a group of military recruits in the UK (Jones *et al.* 1998).

In the African meningitis belt, epidemics of serogroup A meningococci occur in 8-12 year cycles, each epidemic wave following a multiyear crescendo-decrescendo pattern (Moore 1992). Incidence of disease is seasonally dependent, peaking during the dry season (December-May) and declining rapidly with the onset of the rainy season (Greenwood *et al.* 1984). Even during major epidemic waves, case numbers are low during the rainy season (Moore 1992). The underlying mechanisms leading to the spread of meningococci and to epidemics of meningococcal disease remain unknown. Carriage rates of 15% can occur during epidemics in Africa (Blakebrough *et al.* 1982, Hassan-King *et al.* 1988). A population's susceptibility to epidemic disease might return as antibody levels decline and herd immunity is diluted by new birth cohorts and migration (Moore 1992). Variation in virulence between strains of *N. meningitidis* and the introduction of a new meningococcal clone into a susceptible population might also contribute to an epidemic. The extreme environmental conditions present in the sub-Saharan meningitis belt during the dry season - high temperature, low absolute humidity and the Harmattan (a dusty wind that blows from the Sahara) - as well as respiratory co-infections are thought to contribute to an enhanced susceptibility to meningococcal disease by damaging the local mucosal defences (Moore 1992).

Most individuals infected with meningococci are only colonized. They carry the bacteria asymptotically in the nasopharynx and do not develop clinical meningococcal disease. Therefore, epidemiological analysis of meningococcal infection in defined populations should include both carrier and case studies. Meningococci can acquire foreign genes by DNA transformation from unrelated bacteria including commensal *Neisseriae* (Linz *et al.* 2000). Serological cross-reactions between *N. lactamica* and *N. meningitidis* have also been demonstrated, and it has been argued that the carriage of *N. lactamica* may have a role in the development of natural immunity to meningococcal disease (Cartwright 1995b). Despite the importance of meningococcal infection as a cause of morbidity and mortality in countries of the African meningitis belt, few longitudinal carriage studies

have been undertaken in this region (Blakebrough *et al.* 1982, Hassan-King *et al.* 1988).

In the dry season between November 1996 and May 1997, a meningitis epidemic occurred in Northern Ghana. A total of 18,551 cases with 1,403 deaths were reported (Tikhomirov *et al.* 1997). During the 1996-1997 epidemic, 1396 cases and 65 deaths were registered in the Kassena-Nankana District (Upper East Region) (Enos 1997). One year later, a smaller meningitis outbreak occurred in the same district with 50 serogroup A subgroup III cases and 10 deaths (Chapter 3). In order to investigate the dynamics of meningococcal carriage, we conducted a longitudinal carriage study in the Kassena-Nankana district from 1998-2000, during which time an outbreak of serogroup X meningococcal meningitis with a high carriage rate was observed.

5.3 Materials and Methods

Study area and population

The study was conducted in the Kassena-Nankana District (KND) of the Upper East Region of Ghana. The district lies within the guinea Savannah woodland area of Ghana with a population of 140,000 and has two main seasons; a short wet season from June 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 (population of about 20,000). People live in compounds with an average of 10 inhabitants each.

Thirty-seven compounds were randomly selected from a complete listing of the district population (Navrongo Demographic Surveillance System; (Binka *et al.* 1996). The 37 compounds were sampled five times between March 1998 and April 2000, once in each the dry and rainy season. After obtaining informed consent, a throat swab was taken from all compound members present at the time of the visit. The swabs were inoculated directly on Thayer-Martin agar plates which were transported to a laboratory within two hours. The agar plates were then incubated in candle jars for 24 h at 37°C.

Characterisation of bacteria

Up to ten random colonies with neisserial morphology were sub-cultured from each plate. Bacteria that were Gram-negative diplococci, produced cytochrome oxidase from N,N,N',N'-tetramethyl-1,4-phenylene-diammoniumdichloride (Merck), and utilized glucose and maltose but not sucrose in cystine-trypticase agar (Difco), were analyzed further. Those with beta galactosidase (ONPG discs, Oxoid) were classified as *N. lactamica* and those with gamma-glutamyltransferase activity (MPR 2-Kit, Boehringer Mannheim) were classified as *N. meningitidis*. Isolates from these species were stored in 10% skim milk (Difco) on glass beads at -70°C. The frozen samples were transported to Switzerland in liquid nitrogen or on dry ice.

Meningococci were serogrouped by slide agglutination with serogroup specific antisera (Murex) and serotyped/subtyped with monoclonal antibodies by whole cell ELISA (Wang *et al.* 1992). They were also screened for diversity by pulsed-field gel electrophoresis (PFGE) after digestion with *NheI* and *SpeI* (Morelli *et al.* 1997). Multilocus sequence typing (MLST) was performed (Maiden *et al.* 1998) on a subset of 20 isolates representing the different PFGE variants of the different serogroups.

Data analysis

The prevalence of carriage and exact binomial confidence intervals were calculated using STATA statistical software (Stata Corporation 1999). In order to evaluate age and sex effects on prevalence, we used logistic regression models including random effects to allow for repeated assessment of the same individuals. Age and sex effects on incidence were assessed by normal logistic regression.

For serogroup X isolates, distinct *NheI* and *SpeI* fragments were assigned arbitrary numbers. The presence or absence of each fragment was scored in a data matrix as plus (1) or minus (0). The data matrix was used to construct a neighbour-joining tree (MEGA version 2b3; (Kumar *et al.* 2000) based on the number of band differences between PFGE patterns. For serogroup X strains, a matrix of pairwise geographic distances between the compound of isolation was compared to the data matrix of genetic distances by a Mantel-test with 10,000 permutations (GENETIX version 4.01; Belkhir 2000).

5.4 Results

The longitudinal carriage rate of *N. meningitidis* and *N. lactamica* was investigated between 1998 and 2000 in the Kassena-Nankana District (KND). Throat swabs taken on five occasions from 292-308 inhabitants of 37 random compounds were investigated bacteriologically.

Prevalence and incidence of carriage

The carriage rate of *N. lactamica* remained constant at around 9% with no difference between the dry and the wet season. However, the carriage rate of *N. meningitidis* of different serogroups changed dramatically. Carriage of serogroup A meningococci dropped continuously from 3% in April, 1998, to $\leq 0.3\%$ in April, 2000 (Table 5.1, Figure 5.1). All 14 serogroup A isolates were A:4,21:P1.9 and possessed PFGE fingerprints similar to those of disease isolates from the meningitis outbreak in 1998 (data not shown). Three representative isolates were MLST sequence type ST5, as is typical of subgroup III bacteria from Africa (Zhu *et al.* 2001).

Serogroup X bacteria were first isolated in 1999 (3% of samples) and rose precipitously to 18% in the dry season of 2000 (Figure 5.1). The estimated incidence (acquisition rate) was 0.6 per 100 person-months for *N. lactamica*, 1.0 for serogroup X meningococci, and 0.2 for meningococci of other serogroups (Table 5.2).

The carriage rate of *N. lactamica* was highest among children under 5 years of age and decreased continuously with age (Figure 5.2). In contrast, serogroup X carriage was highest among 5-14 year olds. Females were more likely to carry and to acquire *N. lactamica* (Tables 2 and 3). Males were at higher risk of carrying serogroup X meningococci than females but there was no such difference in acquisition. Males were also more likely to carry and to acquire meningococci of other serogroups.

Table 5.1. Carriage of *N. lactamica* and different serogroups of *N. meningitidis* during 5 longitudinal carriage surveys conducted in northern Ghana.

Presence of	MLST type	subgroup/ complex	Apr. 1998	Nov. 1998	Apr. 1999	Nov. 1999	Apr. 2000	Total	% of meningococcal isolates	Change over time	
			(n=300)	(n=299)	(n=292)	(n=308)	(n=298)	(n=1497)		LRT	χ^2
			DS n (%)	RS n (%)	DS N (%)	RS n (%)	DS n (%)	N (%)			
<i>Neisseria lactamica</i>	--	--	28 (9.3)	26 (8.7)	24 (8.2)	30 (9.7)	25 (8.4)	133 (8.9)	--	--	ns
<i>Neisseria meningitidis</i>	--	--	14 (4.7)	9 (3.0)	15 (5.1)	13 (4.2)	59 (19.8)	110 (7.4)	100		
	A:4,21:P1.9	ST5	III	9 (3.0) ^a	4 (1.3) ^b	2 (0.7)	1 (0.3)	0 (< 0.3)	16 (1.1) ^{ab}	14.5	15.6 < .001
	X:NT:P1.5	ST181/ST751	--	0 (< 0.3)	0 (< 0.3)	10 (3.4)	7 (2.3) ^a	53 (17.8) ^a	70 (4.7) ^c	63.6	94.3 < .001
	Y:4:P1.5	ST168	--	4 (1.3)	3 (1.0) ^a	2 (0.7)	3 (1.0) ^a	5 (1.7) ^a	17 (1.1) ^d	15.5	-- ns ^e
	W135:2a:P1.2,5	ST11	ET-37	1 (0.3)	1 (0.3)	0 (< 0.3)	0 (< 0.3)	0 (< 0.3)	2 (0.1)	1.8	
	PolyAG:NT:NST	Nd	--	0 (< 0.3)	1 (0.3)	1 (0.3)	2 (0.6)	1 (0.3)	5 (0.3)	4.5	

^a polyagglutinable, 1 isolate; ^b non-groupable, 1 isolate; ^c polyagglutinable, 2 isolates; ^d polyagglutinable, 3 isolates; ^e serogroups Y, W135 and PolyAG:NT:NST together; nd: not done; DS: dry season; RS: rainy season; LRT: likelihood ratio test; ns: not significant.

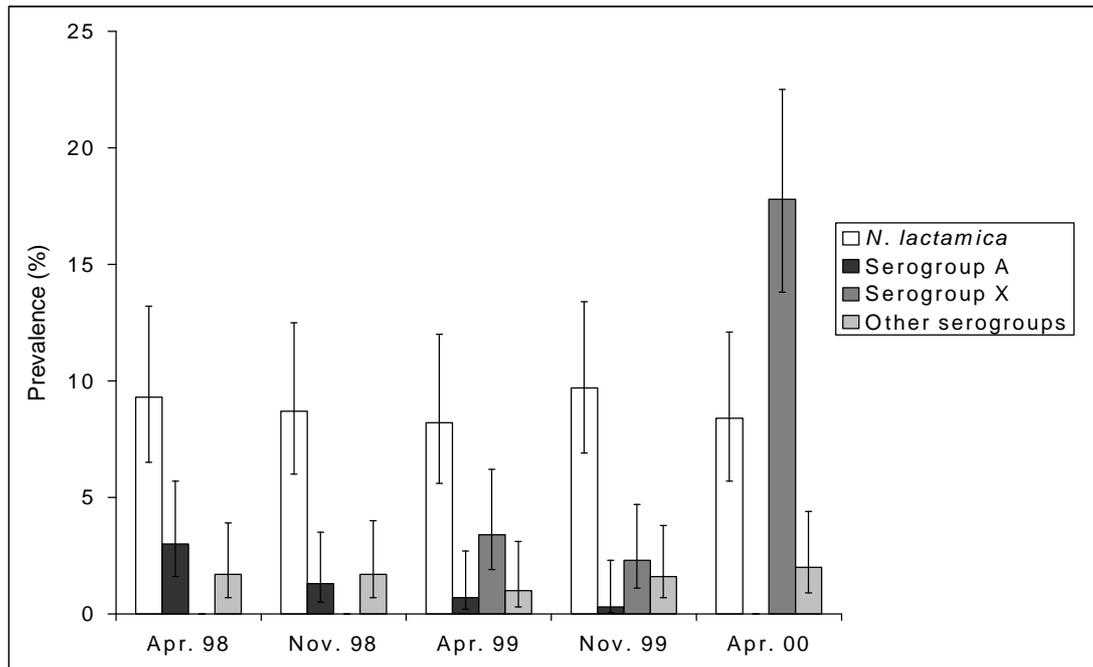


Figure 5.1. Prevalence of carriage of *N. lactamica* and different serogroups of *N. meningitidis* during 5 longitudinal carriage surveys conducted in Northern Ghana. Error bars indicate exact binomial 95% confidence intervals.

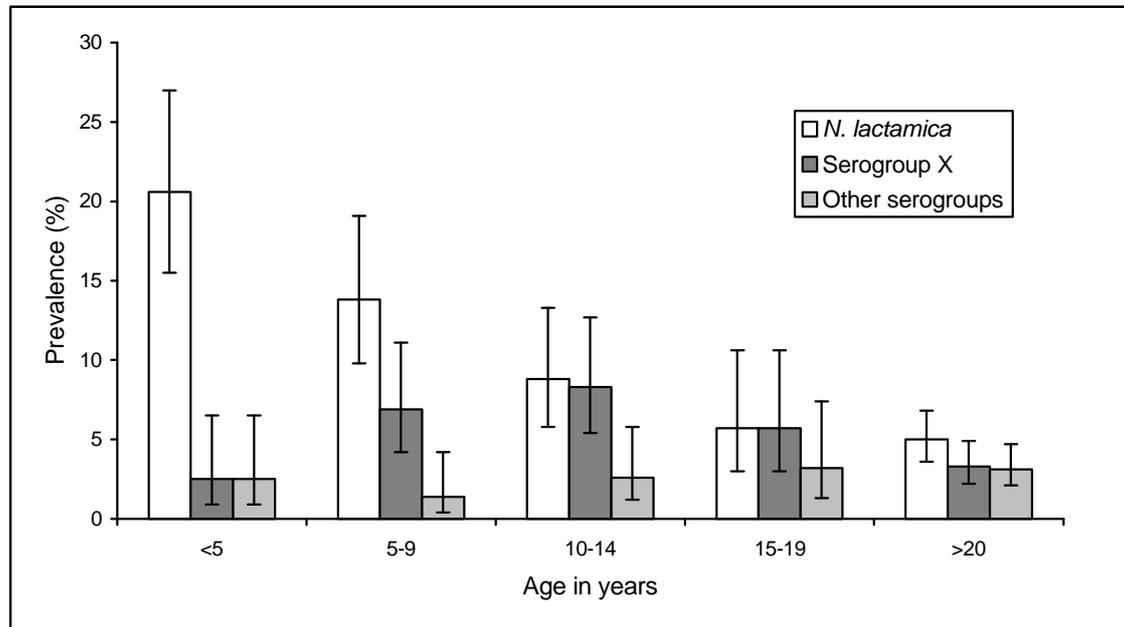


Figure 5.2. Age distribution of prevalence of carriage of *N. lactamica* and different serogroups of *N. meningitidis*. Error bars indicate exact binomial 95% confidence intervals.

Table 5.2. Sex differences in incidence (acquisition) of carriage of *N. lactamica* and different serogroups of *N. meningitidis* (per 100 person-months).

Acquisition of	Total (CI95%)	M (CI95%) ^a	F (CI95%) ^a	OR ^b (CI95%)	LRT χ_1^2	p
Serogroup X	1.0 (0.7-1.3)	1.2 (0.8-1.7)	0.8 (0.5-1.1)	--	--	ns
Other serogroups	0.2 (0.1-0.4)	0.3 (0.1-0.6)	0.1 (0.02-0.3)	3.3 (0.9-12.9)	3.6	0.06
<i>N. lactamica</i>	0.6 (0.4-0.8)	0.3 (0.1-0.6)	0.8 (0.5-1.2)	0.4 (0.2-0.9)	5.8	< .02

^a binomial exact, ^b age adjusted odds ratio, LRT: likelihood ratio test, ns: not significant.

Table 5.3. Sex differences in prevalence of carriage of *N. lactamica* and different serogroups of *N. meningitidis*.

Presence of	Total (N =1497)		M (N = 666)		F (N = 831)		OR ^b (CI95%)	LRT χ_1^2	p
	n	% (CI95%) ^a	n	% (CI95%) ^a	n	% (CI95%) ^a			
Serogroup X	70	4.7 (3.7-5.9)	43	6.4 (4.7-8.6)	27	3.2 (2.2-4.7)	2.0 (1.2-3.3)	7.8	< .01
Other serogroups	40	2.7 (1.9-3.6)	29	4.3 (2.9-6.2)	11	1.3 (0.7-2.4)	3.8 (1.4-10.1)	7.4	< .01
<i>N. lactamica</i>	133	8.9 (7.5-10.4)	47	7.1 (5.2-9.3)	86	10.3 (8.3-12.6)	0.3 (0.1-0.7)	7.4	< .01

^a binomial exact, ^b age adjusted odds ratio, LRT: likelihood ratio test.

Only three individuals were colonized simultaneously by *N. meningitidis* and *N. lactamica*. This is fewer than expected assuming independence (Fisher's exact test: $p < 0.02$). However, individuals carrying *N. lactamica* at any one time were equally likely to become serogroup X carriers in subsequent surveys as those who did not carry *N. lactamica*. The acquisition of serogroup X bacteria did not differ significantly between compounds with *N. lactamica* carriers and compounds without carriers.

People living in compounds with at least one *N. lactamica* carrier were more likely to acquire *N. lactamica* (secondary colonization) than individuals living in compounds without carriers (OR = 5.0 CI_{95%}: 2.1 - 12.1). As expected for an epidemic outbreak, most individuals from whom serogroup X bacteria were isolated lived in compounds where these had not been previously recovered. Nearly 60% of the compounds sampled became newly infected with serogroup X bacteria at some point during the study. On average 35% of the inhabitants of a compound (2.5 ± 2.0 individuals; range 1-8) carried serogroup X meningococci on the first occasion when these bacteria were detected in that compound.

Cases of meningococcal meningitis

The last confirmed serogroup A meningococcal meningitis case occurred in the second epidemic year, namely during the dry season of 1998 (Chapter 3). In contrast, 9 cases (56% males) of meningitis were caused by serogroup X meningococci between March 1998 and April 2000. One of these cases occurred in the dry season of 1998, during the serogroup A outbreak and was fatal. A second fatal case occurred during the dry season of 1999. During the dry season of 2000 seven cases were identified, all of whom survived. The median age of the 9 cases was 6 years (mean = 9.7, range: 1 - 32). Serogroup X disease was not associated with complement or properdin deficiency in any of the four sera tested (Kirschfink 1997). The ratio of cases of serogroup A disease per carrier in 1998 was 40 fold higher than that for serogroup X meningococci in 2000.

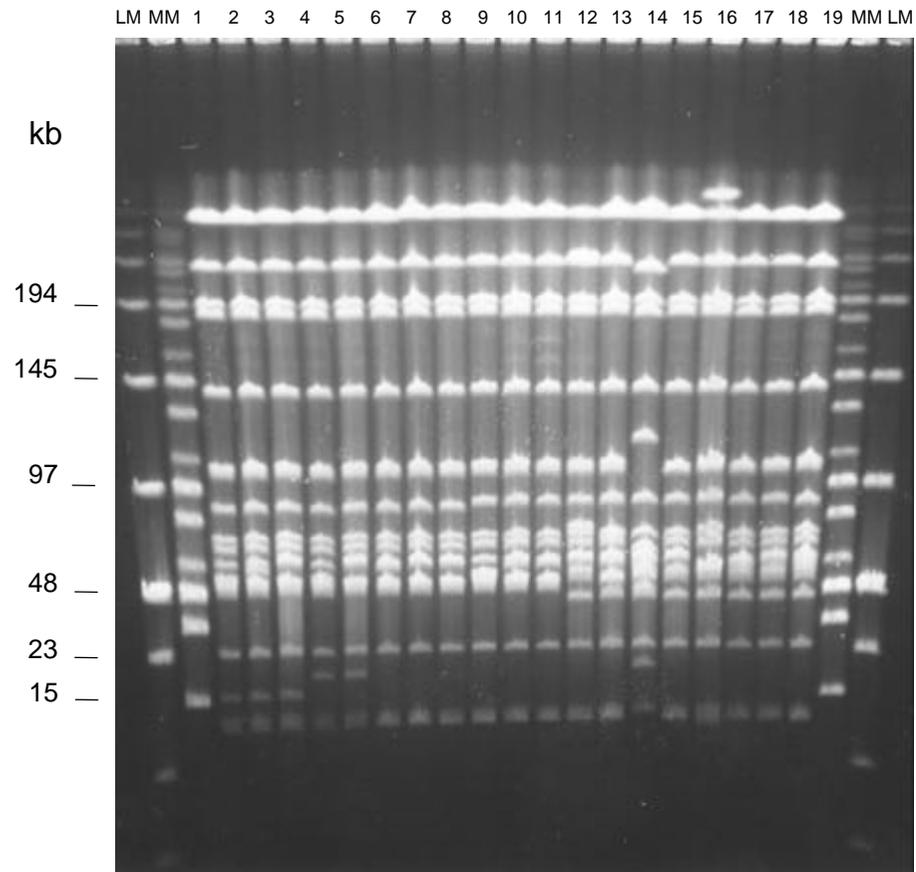
Population structure and spread of serogroup X meningococci

Serogroup X meningococci isolated in Africa between 1970 and 2000 can be assigned to two groups, a and b, by PFGE. Most serogroup X isolates from Chad, Mali and Niger were group a, strains from Burkina Faso were group b (Chapter 6). These PFGE patterns differ in 8 *NheI* and 3 *SpeI* fragments. Seventy-eight of the 79 serogroup X isolates from Ghana belonged to group b and one (Figure 5.3, track 14) to group a. The group a isolate was MLST type ST181, while 12 representative group b isolates were ST751 which differs at two of the seven MLST loci. The group b isolates from Ghana were not totally uniform by PFGE. They shared 11 uniform *NheI* bands (Figure 5.3) but 8 other bands were polymorphic. Four smaller fragments could not be evaluated due to lack of resolution. Similarly, the group b isolates contained 19 uniform *SpeI* fragments, 6 that were polymorphic and 3 that could not be evaluated. The combined data from both restriction digests resulted in nine PFGE subtypes within the group b Ghanaian isolates (Figure 5.3).

Phylogenetic analysis of the PFGE data revealed that eight of the nine subtypes from group b belonged to three clusters (1, 2 and 3) and that one subtype represented by the sole isolate from 1998 was very different. Both neighbor-joining (Figure 5.4) and maximal parsimony algorithms (data not shown) yielded comparable results. For the cluster 1, 2 and 3 isolates, there was a significant correlation between the pairwise genetic distances and the geographic distances between the compounds of isolation (Pearson $r = 0.34$, Mantel-test, $p < .0001$). The correlation was even stronger between geographic distances and cluster assignment (Pearson $r = 0.43$, Mantel-test, $p < .0001$). All three clusters were isolated from both carriers and meningitis patients (cluster 1: 4 patients; 2: 1; 3: 3). There was no significant difference in the case to healthy carrier ratio between the tree clusters. All three clusters were found in 1999. Furthermore, cluster 1 and 3 were also isolated in Burkina Faso between 1996 and 1998 (Figure 5.4). These results suggest that the three clusters evolved outside Ghana and were imported concomitantly in 1999.

Figure 5.3. PFGE patterns of *NheI* and *SpeI* digested chromosomal DNA of representative serogroup X meningococci from Northern Ghana. Strains were loaded in tracks 1-19 in the following order (track: strain): 1:Z9389; 2: Z9396; 3: Z9381; 4: Z9292; 5: Z9295; 6: Z9399; 7: Z9401; 8: Z8329; 9: Z8331; 10: Z9301; 11: Z9300; 12: Z9297; 13: Z9383; 14: Z9413; 15: Z9294; 16: Z7091; 17: Z8336; 18: Z9392; 19: Z9293. Molecular weight markers were loaded in the flanking tracks as indicated (LM: low range marker; MM: midrange marker); their molecular weights are indicated at the left.

NheI



SpeI

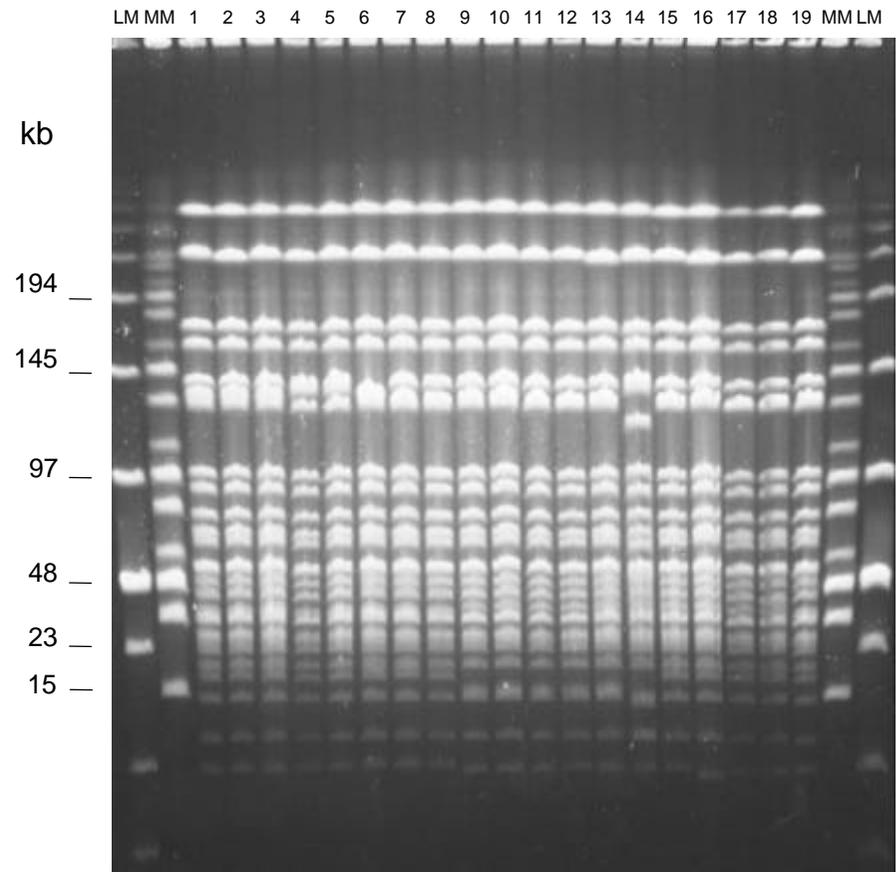
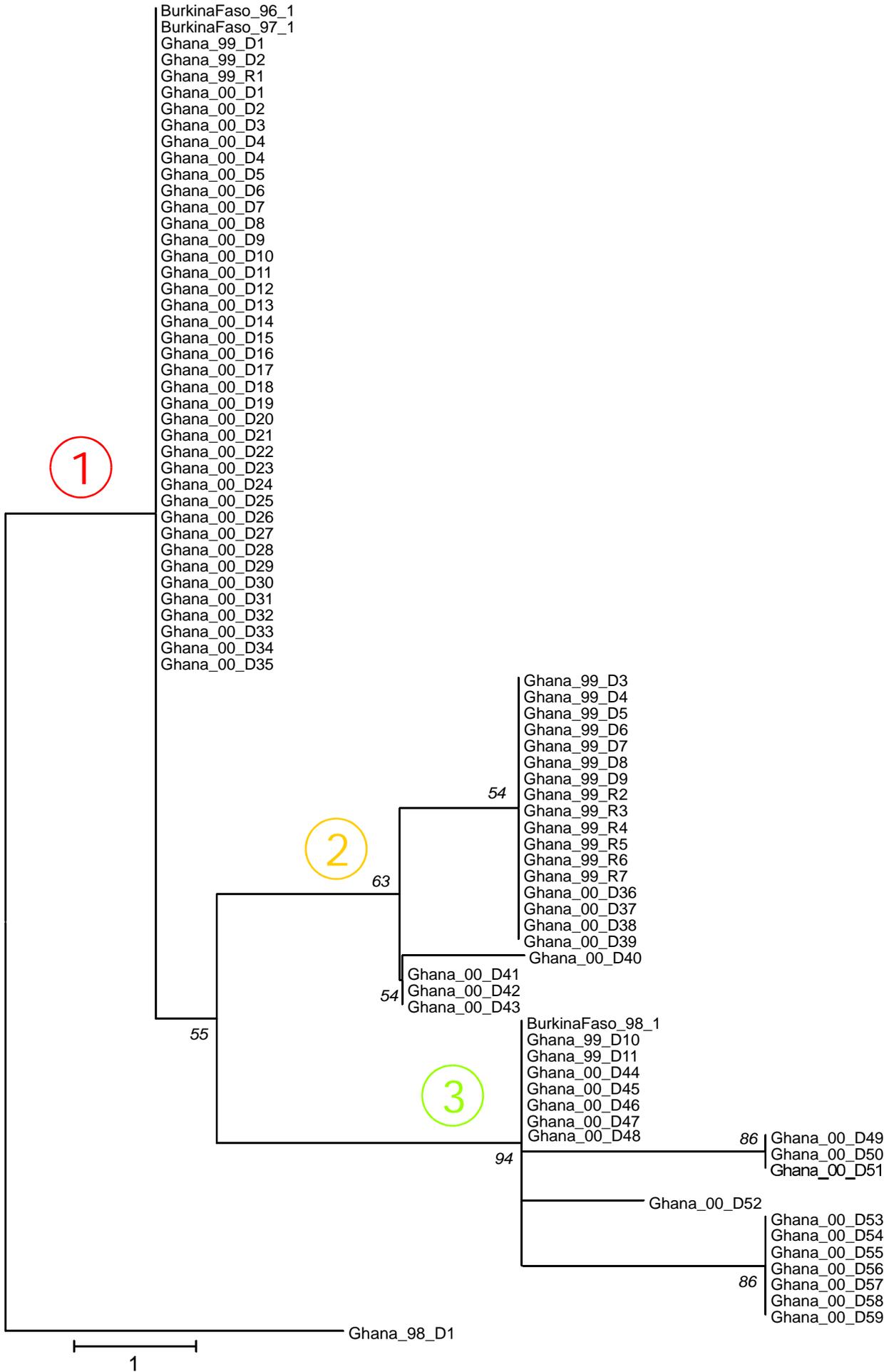


Figure 5.4. Neighbor-joining tree based on the PFGE subtypes of serogroup X meningococci isolated in Northern Ghana. The distance used was the number of band differences. Numbers at nodes are the percentages of 1000 bootstrap replicates in which these nodes appeared. Only nodes with percentages >50% were included. Strains labeled with 'D' or 'R' have been isolated during the dry or the rainy season, respectively.



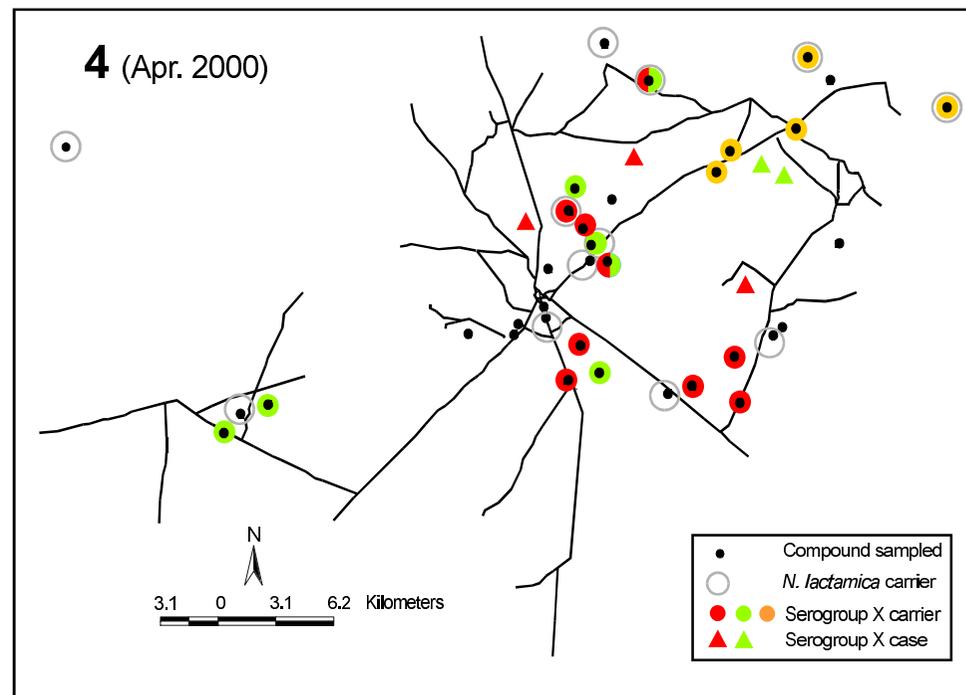
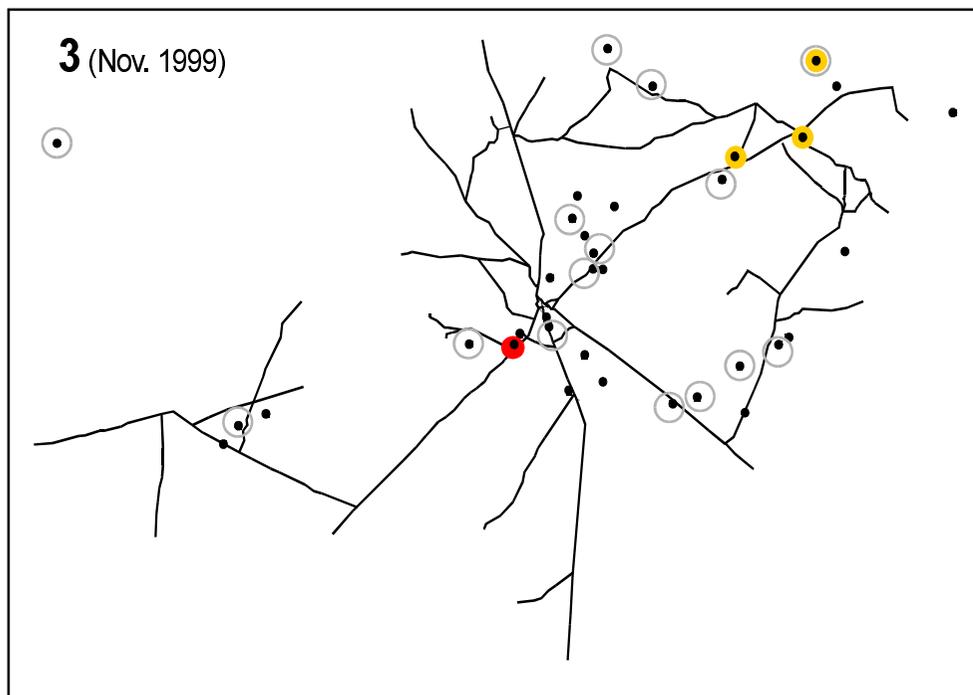
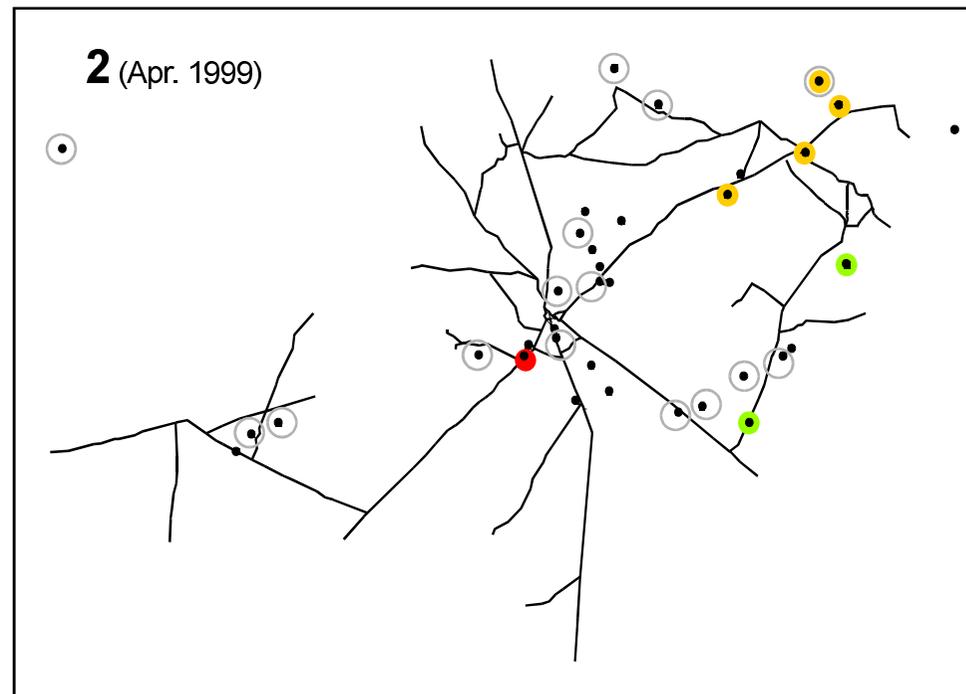
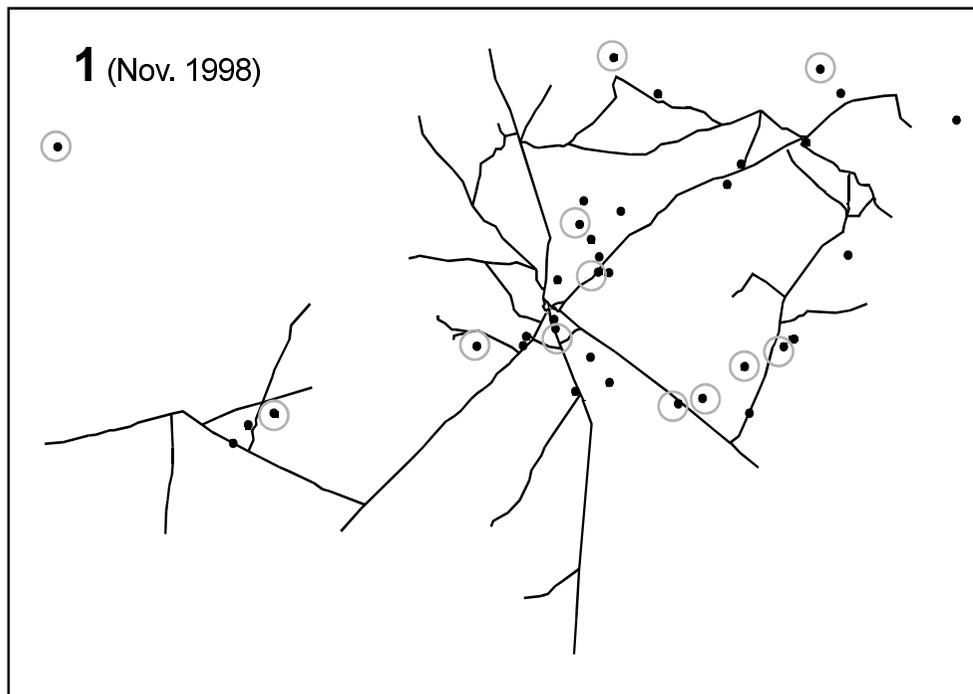
The 3 clusters showed different patterns of spread. Cluster 1 bacteria colonized only one compound in 1999 and spread extensively through the central part of the district in 2000 (Figure 5.5). Cluster 2 colonized a few compounds in the East of the district in 1999 and spread to only 3 other compounds thereafter. Cluster 3 spread from East to West and continued to diversify (Figure 5.4). Cluster 3 isolates from 1999 and Burkina Faso (1998) were located in branches nearer the root of the tree and seem to be ancestral. Most cluster 3 isolates from 2000 were in descendent twigs, indicating recent descent.

5.5 Discussion

Meningococcal carriage rates of up to 30% can be observed during serogroup A epidemics in Africa (Moore 1992), but lower carriage rates have been observed in some epidemics (Hassan-King *et al.* 1979), and carriage is generally infrequent during inter-epidemic periods (Achtman 1995b, Blakebrough *et al.* 1982, Hassan-King *et al.* 1988). A major serogroup A epidemic occurred in northern Ghana in 1996-98 (Chapter 3). The data presented here show that in 1998, the carriage prevalence of the epidemic strain was only 3%. During the following 2 years, serogroup A carriage decreased even further and no serogroup A carrier was identified in 2000. These results resemble those for a serogroup A subgroup IV-1 epidemic in The Gambia in the 1980's (Hassan-King *et al.* 1988).

Figure 5.5. Spatial distribution of compounds with carriers of serogroup X *N. meningitidis* and *N. lactamica* at different time points. Black dots indicate the compounds that were sampled. The red, yellow and green dots indicate the compound where serogroup X *N. meningitidis* carriers were identified. The three colors correspond to the three clusters identified in the phylogenetic analysis (Figure 5.4). Compounds of meningitis cases which could be identified are indicated by colored triangles. Compounds with *N. lactamica* carriers are indicated by gray circles.

There was an initial increase in carriage of serogroup X during the dry season of 1999, followed by a second strong increase during the dry season of 2000. Season has no effect on meningococcal carriage, both in temperate zones and in Africa (De Wals *et al.* 1983, Gold *et al.* 1978a, Greenwood *et al.* 1984) and is therefore unlikely to account for the observed increase in serogroup X carriage. In contrast, similar temporal patterns are typical of serogroup A epidemics in the African Meningitis Belt where large epidemics are often preceded by localized outbreaks one year earlier (Moore 1992). Prevalence of serogroup X carriage was highest in 10-14 year olds, similar to the age patterns observed during large serogroup A epidemics where older children have the highest risk of disease (Greenwood *et al.* 1987). Although there were sex differences in carriage prevalence and incidence in *N. lactamica* and meningococci of other serogroups, there was no such difference in acquisition of serogroup X bacteria, reflecting the epidemic nature of the colonization process, i.e. the whole population being at risk. Furthermore, serogroup X meningococci were mostly acquired through primary colonization, emphasizing that a new bacterial wave was entering the area.



Our findings are consistent with the hypothesis that in the African meningitis belt, meningococci of different serogroups invade specific populations in successive waves. Low carriage rates during inter-epidemic periods alternate with periods of high carriage which can result in epidemics of disease if the bacteria are particularly virulent. However, frequent carriage of bacteria of low virulence would normally not be documented because of the low burden of disease. The different case to carriage ratios for serogroup A and X meningococci possibly reflect such differences in virulence. However, it has been postulated that epidemic disease reflects a lack of herd immunity (Moore 1992), and it remains possible that carriage of serogroup A bacteria between 1996 to 1998 stimulated protective immunity against subsequent disease by serogroup X meningococci. Secondary factors like respiratory tract co-infections may also be necessary for epidemics to occur (Achtman 1995b) and might have been lacking during the epidemic of colonization by serogroup X meningococci. Meningococcal disease (as opposed to carriage) is highly seasonal in the African Meningitis Belt (Greenwood *et al.* 1984). In agreement, all 9 cases of meningococcal meningitis in our study occurred exclusively during the dry season.

It has been suggested that exposure to *N. lactamica* may stimulate natural immunity to meningococcal disease (Cartwright 1995b, Gold *et al.* 1978a). A negative correlation between carriage of *N. lactamica* and either carriage of *N. meningitidis* or meningococcal disease has been found in the Faroe islands (Olsen *et al.* 1991). In one African study involving *N. lactamica*, there was no association between carriage of *N. meningitidis* and *N. lactamica* (Blakebrough *et al.* 1982). We found a negative association between the carriage of both species in this study. However, carriage of *N. lactamica* did not correlate with the acquisition of serogroup X meningococci or its absence, either at the individual or the compound level. Thus it appears that in the African Meningitis Belt, carriage of *N. lactamica* does not reduce colonization of the nasopharynx with meningococci. The age and sex patterns of prevalence and acquisition of *N. lactamica* differed from those of *N. meningitidis*. *N. lactamica* was carried predominantly by infants and young children, similarly to data from Europe and other African studies (Blakebrough *et al.* 1982, Cartwright *et al.* 1987, Gold *et al.* 1978a, Olsen *et al.* 1991). *N. lactamica* was mainly acquired in compounds where carriers were already present, indicating that it is transmitted from person to person within compounds, and that re-colonization is probably frequent.

The serogroup X meningococci that colonized the population of the Kassana-Nankana district have been isolated in West Africa for at least three decades (Chapter 6). Bacteria belonging to the same clonal grouping caused a meningitis outbreak with more than 60 cases in Niger in 1997 (Chapter 6, Campagne *et al.* 1999). Some microheterogeneity was found among the Ghanaian serogroup X isolates. The different PFGE types identified clustered in three phylogenetic clusters. The three clusters did not differ in virulence but exhibited very distinct patterns of dispersal. Cluster 1 spread extensively, without genetic diversification. Cluster 2 which was more diverse than cluster 1 colonized only a few compounds and did not spread further. Cluster 3 spread and diversified during the process.

Although serogroup A, B and C are responsible for over 90% of meningococcal disease worldwide, recent outbreaks of serogroup X and W-135 meningococci illustrate that these serogroups also have considerable pathogenic potential (Campagne *et al.* 1999, Etienne *et al.* 1990, Taha *et al.* 2000). In *Streptococcus pneumoniae*, the introduction of polyvalent capsule polysaccharide conjugate vaccines seems to have induced changes in bacterial population structure of carrier isolates (Mbelle *et al.* 1999, Obaro *et al.* 1996). The effects of widespread immunization with conjugate polysaccharide vaccines on the population structure of meningococci is also under discussion (Maiden & Spratt 1999). Selection can lead to frequent isolation of escape variants (Chapter 4, Linz *et al.* 2000). Repeated vaccination against serogroup A and C carried out in many African countries has the potential to select meningococci of other serogroups, such as serogroup X and might result in a changed profile of meningococcal disease. It is therefore very important that comprehensive conjugate vaccines, including X polysaccharide, be developed as soon as possible.

5.6 Acknowledgements

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Chapter 6

Clonal Groupings in Serogroup X *Neisseria meningitidis*

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6.1 Abstract

The genetic diversity of 134 serogroup X *Neisseria meningitidis* disease and carrier isolates from different countries in Africa, Europe and North America was analyzed by multilocus sequence typing and pulsed-field gel electrophoresis. Most of the European and American isolates were highly diverse. However, one clonal grouping was identified among sporadic disease and carrier strains isolated over the last two decades in the UK, The Netherlands, Germany and the U.S.A. In contrast to the diversity among the European and American isolates, most carrier and disease isolates recovered in different countries of the African Meningitis Belt over the last thirty years were members of a second clonal grouping. During the last decade these bacteria have caused meningitis outbreaks in Niger and in Ghana. These results argue for the development of a comprehensive conjugate vaccine including serogroup X polysaccharide.

6.2 Introduction

Bacterial meningitis due to *Neisseria meningitidis* (the meningococcus) remains an important global public health problem. Epidemics in Africa are usually caused by serogroup A meningococci while sporadic cases, outbreaks, and hyperendemic disease in Europe and the United States are usually caused by serogroups B and C (Achtman 1995b). Occasionally however, endemic disease and outbreaks are caused by bacteria belonging to other serogroups including W135, Y, and X. Serogroup X *Neisseria meningitidis* was described in the 1960's (Bories *et al.* 1966, Evans *et al.* 1968) and serogroup X meningitis has been observed on a few occasions in North America (Ryan & Hogan 1980), Europe (Grahlow *et al.* 1986, Pastor *et al.* 1985), Australia (Hansman 1983) and Africa (Chapter 3, Riou *et al.* 1996) and outbreaks have been reported in Niger (Campagne *et a.* 1999, Etienne *et al.* 1990) and in Ghana (Chapter 5). In some cases, serogroup X disease was associated with a deficiency of particular complement components (Fijen *et al.* 1996, Swart *et al.* 1993) or with AIDS (Morla *et al.* 1992).

Asymptomatic nasopharyngeal carriage of *N. meningitidis* is common and only occasionally do the bacteria invade the blood stream and cerebrospinal fluid to cause disease. Meningococcal populations are highly diverse and it is thought that lineages

of meningococci with an elevated capacity to cause invasive disease arise periodically and spread, sometimes globally (Maiden *et al.* 1998). Relatively few of these hyperinvasive lineages or clonal groupings are responsible for most of the burden of meningococcal disease worldwide (Caugant 1998). These clonal groupings diversify during spread (Chapter 4, Caugant *et al.* 1986), primarily due to frequent horizontal genetic exchange (Chapter 4, Kriz *et al.* 1999, Linz *et al.* 2000). Many variants are isolated only rarely and from a single country due to the bottlenecks associated with geographic spread and to loss of fitness (Chapter 4, Morelli *et al.* 1997). The population structure of *N. meningitidis* is panmictic (Maynard Smith *et al.* 1993) but that of some groupings such as epidemic serogroup A meningococci is largely clonal (Achtman *et al.* 2001). The population structure of serogroup X meningococci has not yet been investigated in detail.

After an epidemic of serogroup A disease in 1997-98 in Northern Ghana (Chapter 3), a longitudinal carriage study was performed in order to investigate the dynamics of meningococcal carriage during an inter-epidemic period (Chapter 5). Surprisingly, a sharp increase in the healthy nasopharyngeal carriage of serogroup X meningococci was observed, accompanied by several cases of serogroup X meningitis. In order to investigate the phylogenetic relationships of these bacteria, we compared the isolates from Ghana with other serogroup X meningococci isolated during recent decades in Africa, Europe and North America.

6.3 Materials and Methods

Bacterial strains

134 *N. meningitidis* isolates of serogroup X were analyzed by pulsed-field gel electrophoresis (130 isolates) or MLST (41). Of these bacteria, 102 were isolated in Africa between 1970 and 2000: from meningitis patients (9 isolates) and healthy carriers (70) in Ghana, 1998-2000; from healthy carriers in Mali in 1970 (9) and 1990-91 (4); and from diseased patients in Chad (1995, 1), Niger (1997-98, 4) and Burkina Faso (1996-98, 5). Six of these isolates were not tested serologically. The other 96 were NT: P1.5.

32 serogroup X strains were isolated between 1988 and 2000 in the UK (22 isolates), Germany (3), U.S.A. (4), France (1), Norway (1) and the Netherlands (1).

The 26 strains tested possessed diverse serotypes (2b, 4, 4/21, 14, 16, 21, 22) and serosubtypes (NST, P1.5, P1.5,10, P1. 7, P1.12, P1.14, P1.15, P1.16) in various combinations.

Molecular typing of bacteria

Pulsed-field gel electrophoresis (PFGE) was performed by digesting chromosomal DNA prepared in agarose blocks with *NheI* and *SpeI* as described (Morelli *et al.* 1997). MLST was performed by sequencing gene fragments of *abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC* and *pgm* as described (Maiden *et al.* 1998; <http://www.mlst.net>). The detailed MLST results and sources of isolates have been deposited in a public database (<http://www.mlst.net>). Additional MLST data for 31 isolates in 30 sequence types was obtained from <http://www.mlst.net>.

Data analysis

A neighbour joining (NJ) tree was constructed using the numbers of MLST allele differences with Bionumerics 2.0 (Kortrijk 2000).

6.4 Results

Pulsed-field gel electrophoresis (PFGE) with two discriminatory rare-cutting enzymes was used to identify groups of closely related strains among 130 isolates of serogroup X *N. meningitidis* from different countries in Africa, Europe and North America. The results showed that all but three of 102 isolates from Africa yielded very similar PFGE patterns (Figure 6.1, clonal grouping X-I). In contrast, 19 of 32 isolates from Europe and North America yielded distinct PFGE patterns (Figure 6.2) that differed from those of the African isolates. However, very similar PFGE patterns were observed for 13 isolates from the U.K., Germany, the Netherlands and the U.S.A. (Figure 6.2, clonal grouping X-II).

Figure 6.1. Two groups of PFGE patterns among *NheI* and *SpeI* digested chromosomal DNA from selected serogroup X *N. meningitidis* strains isolated in Africa. Track: strain: 1: D93 (ST188); 2: 1970; 3: 3187; 4: 3529; 5: D5; 6: LNP13407; 7: LNP14964; 8: LNP15040; 9: 97013; 10: 97014; 11: Z9413; 12: LNP14297; 13: LNP15061; 14: BF2/97; 15: BF5/97; 16: BF1/98; 17: Z7091; 18: Z8336; 19: Z9291. Molecular weight markers were loaded in the flanking tracks as indicated (L: low range marker; M: midrange marker); their molecular weights are indicated at the left. Characteristic band differences are indicated on the right.

Figure 6.2. PFGE patterns of *NheI* digested chromosomal DNA of selected serogroup X *N. meningitidis* isolates from Europe and the USA plus a prototype isolate from Ghana (track 13). Track: strain: 1: E26; 2: X4571; 3: X4890; 4: M2526; 5: M4222; 6: M3772; 7: LNP17351; 8: J88-603; 9: K89-1395; 10: L92-1413; 11: M98-253172; 12: M00-240465; 13: Z9291; 14: M98-252848; 15: M98-252718; 16: M99-240899; 17: X5967; 18: 860060; 19: M4370.

NheI

Clonal grouping X-I

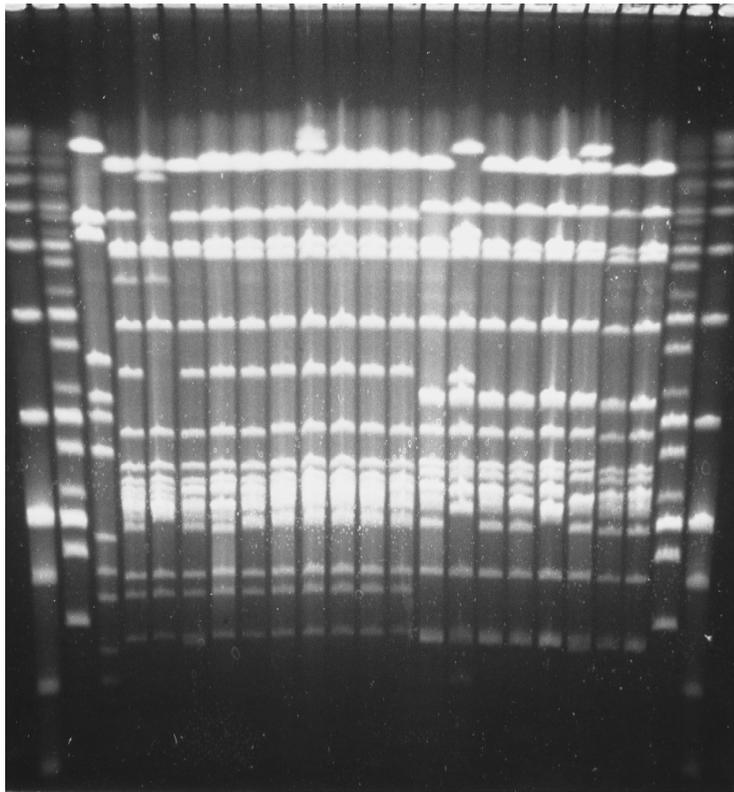
Group Ia
(ST181/ST182)

Group Ib
(ST751)

Mali Chad Niger Burkina Faso Ghana
L M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M L

kb

194 —
145 —
97 —
48 —
23 —



SpeI

Clonal grouping X-I

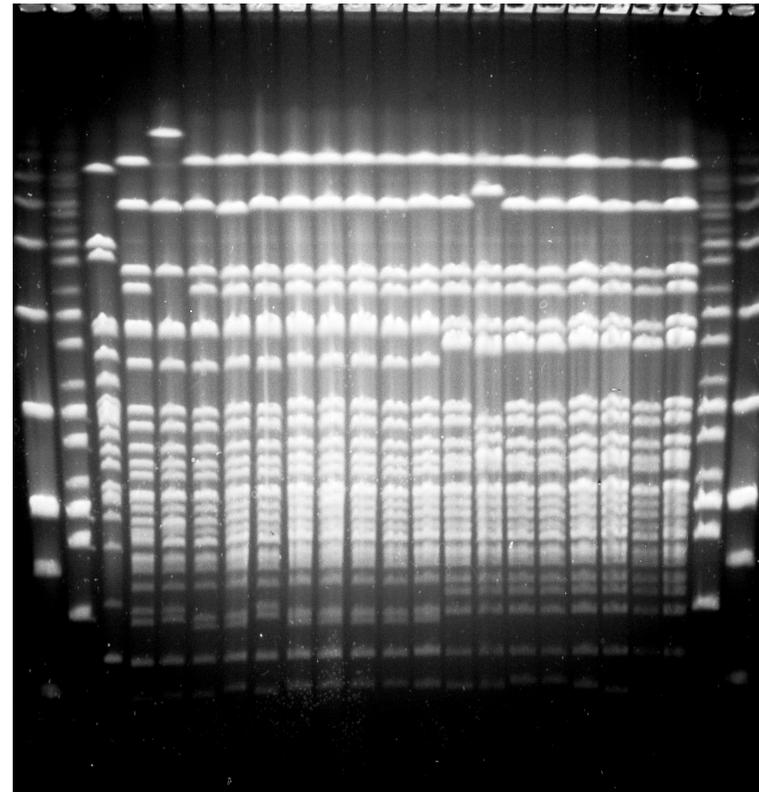
Group Ia
(ST181/ST182)

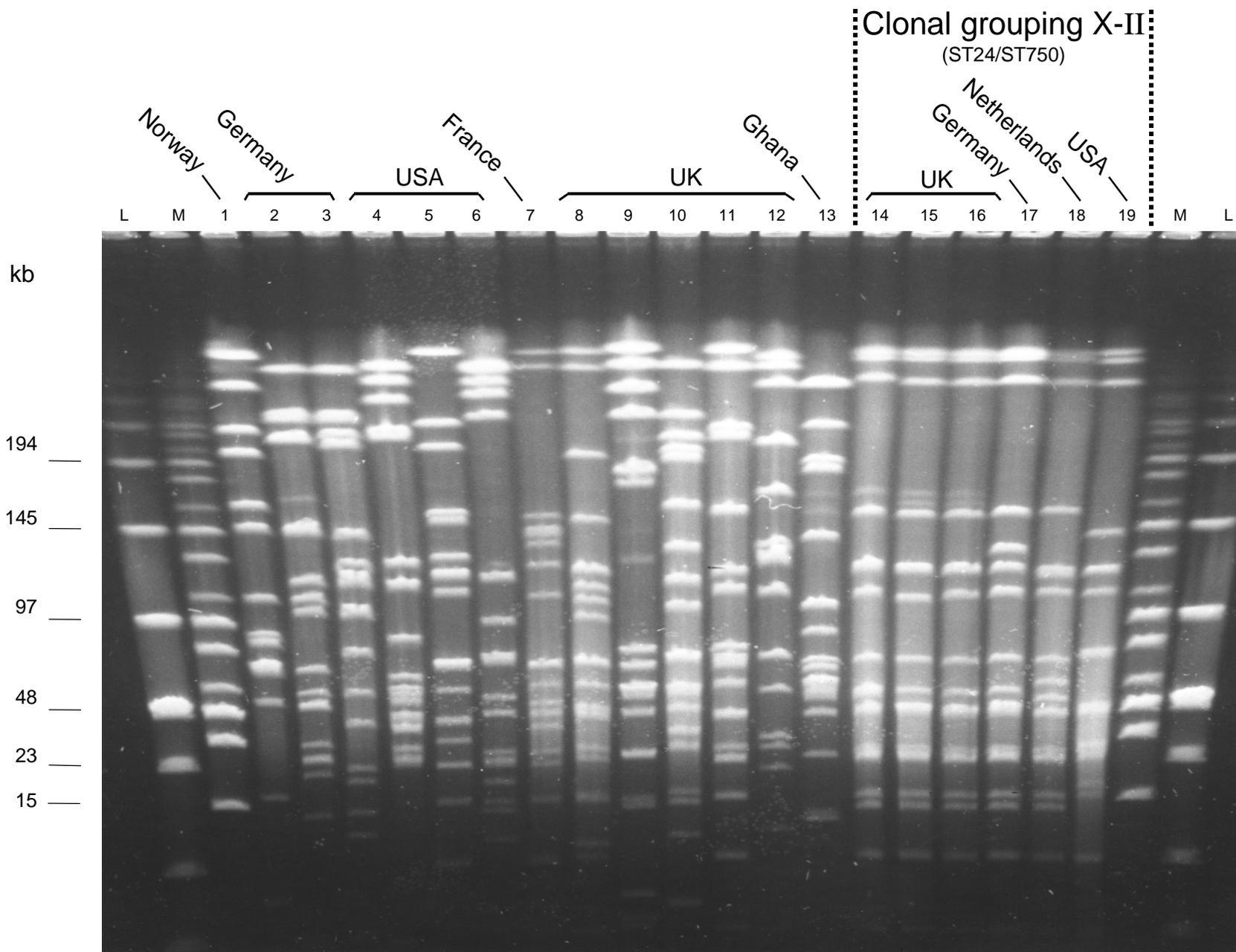
Group Ib
(ST751)

Mali Chad Niger Burkina Faso Ghana
L M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M L

kb

194 —
145 —
97 —
48 —
23 —





A total of 41 isolates representing each distinct PFGE pattern was analyzed by MLST. For those, bacteria where multiple isolates with a similar PFGE pattern had been detected, we tested at least one representative from each year and country of isolation. Together with other data included in the MLST WEB site (<http://www.mlst.net>), 39 distinct sequence types (STs) have been found among 50 serogroup X meningococci. The general structure of a NJ tree of allelic differences is that of a bush with little phylogenetic structure (Figure 6.3). However, isolates with similar PFGE patterns were assigned to closely related STs. All 29 clonal grouping X-I isolates analyzed by MLST were in STs ST181, ST182 or ST751 (Figure 6.3), which differ from each other by one to three of the seven gene fragments (Table 6.1). Similarly; all five clonal grouping X-II isolates were in STs 24 and 750, which differ by one of the seven gene fragments (Table 6.1). The 3 exceptional African isolates (Mali, 1991) were in ST188 which is very distinct from STs of clonal grouping X-I (Figure 6.3). These results show that numerous serogroup X isolates from Africa and nearly half of the serogroup X isolates from Europe and North America belong to two clonal groupings while other serogroup X isolates from Europe or North America are quite diverse.

Serological results

African isolates of clonal grouping X-I were NT:P1.5. The 11 North American and European isolates of clonal grouping X-II for which serological data were available were 21:P1.16 whereas diverse serotype and serosubtype patterns were found for the other isolates from North America and Europe (see p.82).

Figure 3. Neighbor-joining phenogram of allelic identities among 39 MLST sequence types from serogroup X *N. meningitidis*. Numbers at nodes are the percentages of 1000 bootstrap replicates in which these nodes appeared. Only nodes with percentages >50% were included. The two boxes indicate clonal groupings that were detected by MLST and/or PFGE.

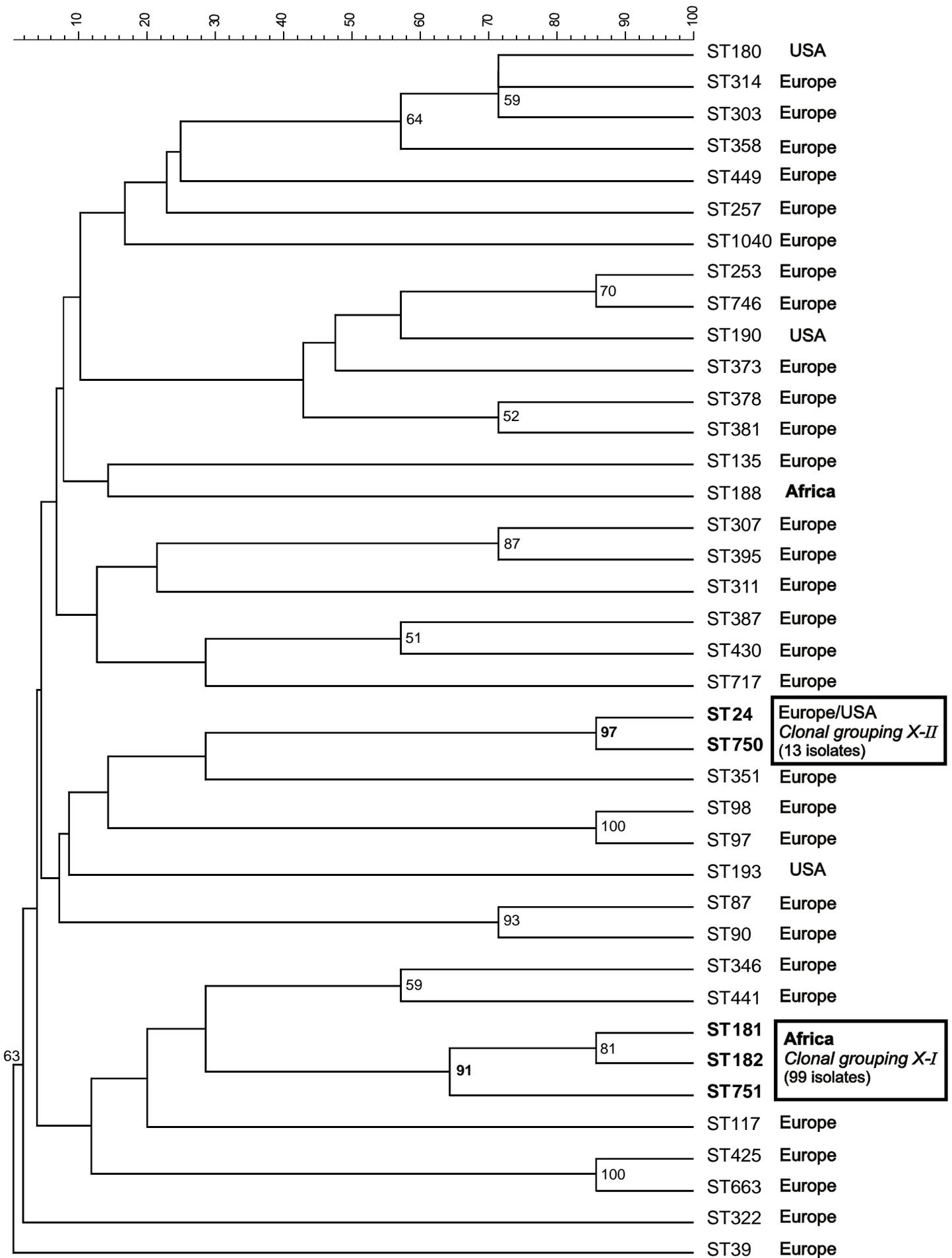


Table 6.1. MLST results of two serogroup X *N. meningitidis* clonal groupings.

ST	Allele numbers							Country (no. of isolates)	Year
	<i>abcZ</i>	<i>adk</i>	<i>aroE</i>	<i>fumC</i>	<i>gdh</i>	<i>pdhC</i>	<i>pgm</i>		
24	2	5	2	7	15	20	5	Netherlands (1) / USA (1)	1986, 1993
750	2	5	2	9	15	20	5	UK (2) / Germany (1)	1998-1999
181	10	3	15	7	5	41	31	Mali (6) / Chad (1) / Niger (2) / Ghana (1)	1970-2000
182	10	3	15	26	5	41	31	Mali (4)	1970
751	10	3	15	7	8	41	6	Burkina Faso (3) / Ghana (12)	1996-2000

Finer groups within clonal grouping X-I

The PFGE patterns distinguished two finer groups (Ia and Ib) within clonal grouping X-I that differ consistently in eight *NheI* and three *SpeI* fragments (Figure 6.1). All 14 group Ia strains that were tested were either ST181 or ST182, which differ at one of the seven gene fragments (Table 6.1). All 15 group Ib strains tested were ST751, which differs from ST181 and ST182 at two to three loci (Table 6.1). Group Ia included 10 isolates from Mali (1970/1990), 4 isolates from Niger (1997-98), the sole isolate from Chad (1995), as well as one of 79 isolates from Ghana (2000). All five isolates from Burkina Faso (1996-98) and 78/79 isolates from Ghana (1998-2000) were in group Ib.

6.5 Discussion

The general population structure of *N. meningitidis* is panmictic due to the frequent import of alleles from unrelated Neisseriae (Maynard Smith *et al.* 1993, Linz *et al.* 2000). Phylogenetic trees of different housekeeping genes from *N. meningitidis* are no more congruent with each other than with random trees (Feil *et al.* 2001). The results for sequence typing of housekeeping genes of serogroup X meningococci that are presented here also fit this pattern. Phylogenetic analysis of allele differences resulted in a bush-like tree that does not seem to contain any deep phylogenetic information. It was therefore surprising to find two clonal groupings within this

otherwise panmictic group of bacteria. The same isolates were assigned to both clonal groupings by two independent methods, MLST and PFGE, indicating that these assignments reflect real genetic relationships and are not dependent on the methodology used. Similar concordant genetic relationships were also discerned among epidemic serogroup A *N. meningitidis* by multilocus enzyme electrophoresis (MLEE), random amplification of polymorphic DNA (RAPD) and MLST; population genetic analyses confirmed that the population structure of these bacteria is clonal (Bart *et al.* 2001). Concordant groupings were also discerned by MLEE and MLST among the so-called “hyper-virulent” serogroup B and C isolates of the ET-5 complex, ET-37 complex, lineage III and cluster A4 (Maiden *et al.* 1998). Although it has been suggested that their apparent clonality may reflect an epidemic population structure (Maynard Smith *et al.* 1993), this possibility has been excluded for epidemic serogroup A meningococci (Bart *et al.* 2001) and therefore it now appears that multiple clonal groupings exist within *N. meningitidis* even though the population structure of most of the species is panmictic.

The population structure of subgroup III serogroup A meningococci seems to represent continual replacement of fit genotypes by each other during periods of several years to decades (Chapter 4). In subgroup III, nine genoclouds, each consisting of a frequent genotype plus its rarer less fit variants, have been identified during three decades of pandemic spread. The PFGE data presented here for clonal grouping I of serogroup X suggest that clonal grouping X-I also possesses a genocloud structure. Two sets of PFGE variants (group Ia and Ib), which might each represent a genocloud, were detected in different countries (Mali, Chad and Niger versus Burkina Faso and Ghana). Additional analyses of polymorphic genes would be necessary to clarify the uniformity of these groups and to test whether how similar their population structure is to that of subgroup III.

Both serogroup X clonal groupings described here were isolated over decades, on multiple occasions and from diverse locations. Clonal grouping X-I (1970-2000) was isolated from different countries in West Africa and clonal grouping X-II (1986-99) was isolated from Europe and North America. For clonal grouping X-I in Ghana, the disease rate per healthy carriers was estimated to be 3/10,000 (Chapter 5). Clonal grouping X-1 is thus of considerably lower virulence than serogroup B ET-5 complex bacteria (disease/carrier rate of 2,100/10,000; The Stonehouse survey: Cartwright *et al.*

1987)) or serogroup A subgroup III bacteria during a post-epidemic period in a vaccinated population (100/10,000; Chapter 5). The relationship between bacterial fitness and clonality has not yet been investigated extensively among natural isolates. Variation between bacterial genotypes in virulence, for example in *Streptococcus pneumoniae* (Smith *et al.* 1993) and *Staphylococcus aureus* (Day *et al.* 2001), leads to more uniformity in disease isolates than in carriage organism. However, the data presented here suggest that the clonal structure of certain meningococcal genotypes need not reflect virulence but rather is associated with genotypes that are particularly fit at colonizing the nasopharynx and spreading from one person to the next.

Although clonal grouping X-I bacteria are less virulent than serogroup A and B meningococci, they are pathogenic. Most of the strains described here were isolated from asymptomatic carriers or from rare endemic cases. However, group Ia caused a meningitis outbreak with more than 60 cases in 1997 in Niger (Campagne *et al.* 1999). Group Ib caused a smaller outbreak in 2000 in Ghana (Chapter 5). These results suggest that X-I meningococci may be capable of causing epidemics. Meningococci are naturally transformable and horizontal DNA transfer is frequent in these bacteria (Kriz *et al.* 1999, Linz *et al.* 2000, Morelli *et al.* 1997). Meningococcal carriage is usually low in inter-epidemic periods in Africa (Chapter 5, Achtman 1995b, Blakebrough *et al.* 1982, Hassan-King *et al.* 1988). This offers less opportunity for horizontal genetic exchange and could account for the low genetic variability among serogroup X meningococci in Africa.

For over a decade, many countries within the African meningitis belt have vaccinated extensively with A/C polysaccharide vaccines (Tikhomirov *et al.* 1997). Recently, mass vaccination with conjugated serogroup C vaccines has been implemented in the UK with strong initial protection (Ramsay *et al.* 2001). However, if effective, these vaccines may well select for the spread of bacteria against which they do not protect (Maiden & Spratt 1999), including unusual causes of disease such as serogroups Y, W135 and X. Capsule switching due to DNA transformation has been documented (Swartley *et al.* 1997, Vogel *et al.* 2000) and it is conceivable that effective vaccination against serogroups A and C may select for capsule switch variants of fit genotypes that express a capsular polysaccharide that is not included in the vaccination program. The recent outbreaks after the Hajj pilgrimage of 2000 that were caused by W135 ET-37 complex meningococci (Taha *et al.* 2000, Popovic *et al.*

2000) may reflect exactly such selection. These findings support the development of comprehensive conjugate vaccines which include capsular polysaccharides from formerly rare causes of disease such as serogroup X.

6.6 Acknowledgements

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Chapter 7

**Infrequent carriage of non-serogroupable *Neisseria meningitidis*
in Africa**

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7.1 Abstract

Up to 50% of meningococci carried in industrialized countries are non-serogroupable, but this proportion is much lower in Africa. Carriage of non-serogroupable meningococci may protect against meningococcal disease by eliciting cross-reactive immunity against pathogenic strains. Susceptibility to epidemics may thus be increased by low levels of carriage in Africa.

7.2 Introduction

Meningococcal disease remains a major public health concern, especially in the African Meningitis Belt where large meningitis epidemics recur every 8-12 years (Achtman 1995b). Asymptomatic nasopharyngeal carriage of *Neisseria meningitidis* is common in Europe and North America. The overall carriage rate is about 10%, with peak carriage rates of up to 25% in late teenage and early adult life (Cartwright 1995b). Many strains isolated from the nasopharynx of healthy carriers express little or no capsular polysaccharide and consequently cannot be assigned to a specific serogroup. Surveys in Europe and the USA found that between 40% and 50% of meningococci from healthy carriers are serologically non-groupable (NG) (Ala'Aldeen *et al.* 2000, Andersen *et al.* 1998, Cartwright *et al.* 1987, Caugant *et al.* 1994, Gold *et al.* 1978b). Encapsulation is thought to reduce adherence to oropharyngeal epithelial cells and loss of expression of capsular polysaccharide may be an adaptation to long-term pharyngeal carriage (Cartwright 1995b). Invasion of immunocompetent hosts is dependent on the expression of capsular polysaccharide and meningococcal strains which express no capsular polysaccharide have very limited pathogenic potential. Colonization with NG strains may, however, be beneficial for the host by eliciting potentially cross-reactive immune responses to non-capsular meningococcal surface antigens (Cartwright 1995b). It has also been suggested that exposure to *N. lactamica* may stimulate natural immunity to meningococcal disease (Cartwright 1995b, Gold *et al.* 1978a). However, the few longitudinal carriage studies carried out in Africa showed that the carriage of *N. lactamica* had no influence on the dynamics of meningococcal carriage (Chapter 5, Blakebrough *et al.* 1982).

We recently carried out a three-year longitudinal carriage study of *N. meningitidis* in the Kassana-Nankana district of Northern Ghana (Chapter 5). During five 6-monthly surveys of 300 randomly selected people, 110 meningococcal isolates were recovered. A, X and Y were the dominant serogroups, and no other serogroups were found except for two serogroup W135 isolates. During the course of our studies, there were two outbreaks of meningitis, one caused by serogroup A (subgroup III) and the other by serogroup X (Chapter 5).

7.3 Results and Discussion

Twelve of the 110 carrier isolates (11%) were NG. A similarly low proportion of NG strains was found during studies in Nigeria (Blakebrough *et al.* 1982) and Mali (M.A., unpublished). Seven of the 12 NG carrier isolates from Ghana had the same serotype and serosubtype as serogroup A, X or Y carrier strains isolated from the same person or in the same household. Analysis by multilocus sequence typing (Maiden *et al.* 1998) and pulsed-field gel electrophoresis (PFGE; (Morelli *et al.* 1997)) of five of these NG isolates showed that they had the same sequence type (ST) and identical PFGE fingerprints as strains of the corresponding serogroup (Figure 7.1). These observations suggest that the NG isolates represent capsule-deficient phase variants as shown for serogroup B meningococci (Hammerschmidt *et al.* 1996) and that the genetic diversity of non-encapsulated meningococci in Africa is low.

Our data indicate that in contrast to Europe and the USA, carriage of non-encapsulated meningococci and other non-pathogenic meningococci is infrequent in Africa, and cannot contribute markedly to protection through cross-immunity. Similarly, cross-immunity does not seem to be stimulated by carriage of *N. lactamica* (Gagneux, submitted). Thus, the general population is less protected by cross-immunity than in Europe or the USA. This may be an important factor for the magnitude of epidemic disease when pathogenic meningococci are imported.

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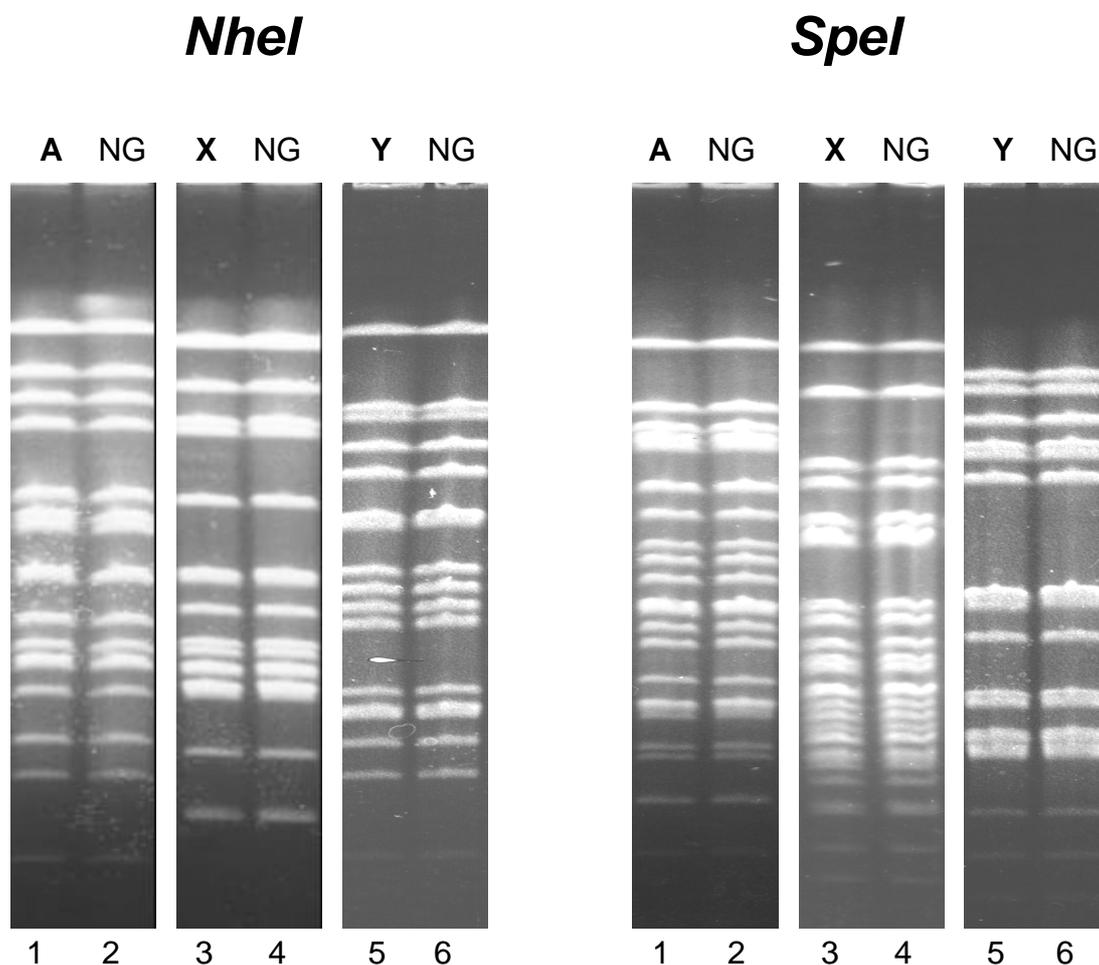


Figure 7.1. PFGE patterns of *NheI* and *SpeI* digested chromosomal DNA of three selected pairs of serogroupable/non-groupable (NG) *N. meningitidis* strains from Northern Ghana. Strains were loaded in tracks 1-6 in the following order (track: strain:): 1: Z9567; 2: Z8349; 3: Z9300; 4: Z9301; 5: Z8338; 6: Z8347. The serogroup is indicated on the top. The serotype/subtypes and MLST sequence types (ST) were: tracks 1/2: 4,21:P1.9 and ST5; tracks 3/4: NT:P1.5 and ST751; tracks 5/6: 4:P1.5 and ST168. Strains on tracks 3/4 are sequential isolates from the same individual isolated 6 months apart. Strains on tracks 1/2 and 5/6 were isolated from different individuals in same households, respectively.

Chapter 8

GENERAL DISCUSSION AND CONCLUSIONS

Detailed discussions of the results of the individual studies are found in each of the respective chapters. This section will start with an assessment of the power of the experimental methodologies used and will be followed by a discussion of the main findings, their implications and suggestions for future research.

8.1 Methodology

The main goal of the work presented in this thesis was to contribute to the understanding of the epidemiology of meningococcal meningitis in the African meningitis belt and to elucidate possible mechanisms responsible for the occurrence of epidemics in this area. Our approach included hospital-based case studies and field-based longitudinal cross-sectional carriage surveys. The studies focused on the epidemiological characteristics of the meningitis patients, on the dynamics of nasopharyngeal carriage, and on the population structure and micro-evolution of the recovered disease and carriage isolates by comparing them to meningococci isolated in other parts of the world. We used conventional epidemiological tools to study the patient and carrier characteristics, and a variety of molecular typing and phylogenetic analysis techniques to investigate the microbiological aspects.

Study population, design and field methodology

All suspected meningitis patients presenting at the War Memorial Hospital in Navrongo or at one of the three Health Centers in the Kassena-Nankana District were recruited (Chapter 3). During the time of the serogroup A outbreak in 1998, the three medical assistants in charge of the Health Centers were specially trained in doing lumbar punctures, since referral would have significantly delayed the diagnosis and the start of treatment. Motorbikes were used to transport the cerebrospinal fluid specimens to the field laboratory.

The compounds included in the longitudinal carriage surveys were randomly selected from a complete listing of all the compounds in the district (Chapter 5). All these compounds and their inhabitants are included in the Navrongo Demographic Surveillance System (Binka *et al.* 1996). Our sample was therefore representative of the whole population of the Kassena-Nankana district.

The intervals between surveys were approximately 6 months. Based on the clearing probabilities calculated from our longitudinal data, we estimated a mean duration of carriage of 9.1 months (CI_{95%}: 7.6-12.1) for *N. meningitidis*. A similar duration of carriage has been found by Greenfield *et al.* (1971), but other studies have estimated a duration of 3-5 months only (Blakebrough *et al.* 1982, Gold *et al.* 1978a). Our estimate of the duration of carriage of *N. lactamica* was 18.0 months (CI_{95%}: 13.4-25.8), which is considerably longer than the estimates from other studies where a duration of 4 months has been estimated (Blakebrough *et al.* 1982, Gold *et al.* 1978a). The optimal design for estimating the duration of carriage depends on the degree of heterogeneity in duration. It is likely that some of the differences in estimates result from different methodologies used.

However, the main objectives of our studies were to investigate the dynamics of carriage in relation to season and the succession of waves of colonization over a period of several years, ideally covering a whole period between two epidemics. In fact, the six-monthly carriage surveys are currently ongoing and two additional sampling campaigns have already been carried out (November 2000 and April 2001).

Field laboratory and technology transfer

The establishment of a field laboratory at the Navrongo Health Research Center/War Memorial Hospital allowed us to cultivate and characterize the isolate to the species and serogroup level in Ghana, and to transfer the cryopreserved bacteria to Switzerland for more detailed analyses. Two Ghanaian laboratory technicians were specially trained for this purpose and the relevant techniques transferred. This field laboratory is also continuously supporting routine diagnosis at the War Memorial Hospital.

Molecular typing methods and phylogenetic analysis

Different methods may be appropriate for investigating short-term (local) and long-term (global) epidemiology (Spratt & Maiden 1999) (see p. 15 of Introduction). In both cases they should have an adequate level of discrimination such that isolates assigned to the same molecular type are likely to be descended from a recent common

ancestor, and isolates that share a more distant common ancestor are not assigned to the same type (Maiden *et al.* 1998).

Suitable levels of discrimination can be achieved in two quite different approaches. In one of them, regions of the genome that are highly variable within the bacterial population are identified and analyzed. For bacterial pathogens, several methods based on this approach are commonly used, e.g. pulsed-field gel electrophoresis (PFGE), and PCR with repetitive element primers, or arbitrary primers (Achtman 1998). In these methods, restriction enzymes (or PCR primers) are chosen that give maximal variation within the population. Consequently, the variation that is indexed is evolving very rapidly, usually for unknown reasons (Maiden *et al.* 1998).

The second approach is to use variation that is likely to be selectively neutral and accumulating very slowly in the population. Although only a limited number of alleles can usually be identified within the population by using this type of variation, high levels of discrimination are achieved by analyzing several loci (Maiden *et al.* 1998). Such techniques include multilocus enzyme electrophoresis (MLEE) which is based on the different electrophoretic mobilities of isoenzymes (Wang *et al.* 1992), and multilocus sequence typing (MLST) which relies on the sequencing of gene fragments of a number of housekeeping genes (see below) (Maiden *et al.* 1998).

Methods that index rapidly evolving variation are useful for short-term epidemiology but may be misleading for global epidemiology. Several studies have shown that techniques such as PFGE resolve isolates that are indistinguishable by MLEE. For example, MLEE studies of populations of *Salmonella enterica* have shown that disease isolates of serovar Typhi belong to one of two closely related electrophoretic types (ETs) (Selander *et al.* 1990). In contrast, isolates of serovar Typhi are relatively diverse according to PFGE (Navarro *et al.* 1996). PFGE is therefore useful for studying individual outbreaks of typhoid fever because, unlike MLEE, it identifies the microvariation that is needed to distinguish between strains circulating within a geographic area. However, this technique is too discriminatory for long-term epidemiology because it does not indicate that isolates that cause typhoid fever are members of a single globally distributed clonal lineage of *S. enterica* (Maiden *et al.* 1998).

MLEE has been the most widely used molecular typing technique for long-term epidemiology in recent years. This approach has helped to identify lineages that have an increased propensity to cause disease (Caugant *et al.* 1987), and contributed to the understanding of the global epidemiology and population structure of infectious agents. For many pathogens, MLEE has identified clusters of closely related strains (clones or clonal complexes) that are particularly liable to cause disease (Achtman 1998, Selander *et al.* 1987). A major problem with MLEE, however, is that the results obtained in different laboratories are difficult to compare (Maiden *et al.* 1998). This led to the recent development of the MLST, a new molecular typing technique with considerable advantages over MLEE (Maiden *et al.* 1998) (see below).

Principles of multilocus sequence typing

Multilocus sequence typing (MLST) (Maiden *et al.* 1998) is an extension of MLEE in which the alleles at each house-keeping locus are assigned directly by nucleotide sequencing (Figure 8.1). Internal ~450 bp fragments of house-keeping genes are used since such fragments can be accurately sequenced on each strand with a single pair of primers and, in most species, will provide sufficient variation to recognize many alleles within the population (Spratt 1999). For each gene fragment, every unique sequence is assigned as a different allele.

Because recombinational exchanges are frequent in many bacterial species, it cannot be assumed that alleles that differ at many nucleotide positions are any more distantly related than those that differ at a single position and, thus, no weighting to reflect the number of nucleotide differences between alleles is permitted. Bacterial strains are, therefore, defined unambiguously by a string of integers (the allelic profile, or sequence type) corresponding to the alleles at each of the house-keeping loci. The relationships between isolates are displayed as a dendrogram constructed from the matrix of pairwise differences between the allelic profiles (Maiden *et al.* 1998).

The use of nucleotide sequencing to assign alleles at house-keeping loci has a number of advantages. Firstly, sequencing uncovers all variation at a locus, resulting in many more alleles per locus than revealed by MLEE. MLST therefore achieves very high levels of discrimination using as few as seven loci (Spratt 1999). Secondly,

identity of alleles is unambiguous using sequence data, in contrast to MLEE where the same electrophoretic mobility may reflect identical or similar nucleotide sequences or, in some cases, completely different sequences that, by chance, encode enzymes that migrate at the same rate on a starch gel.

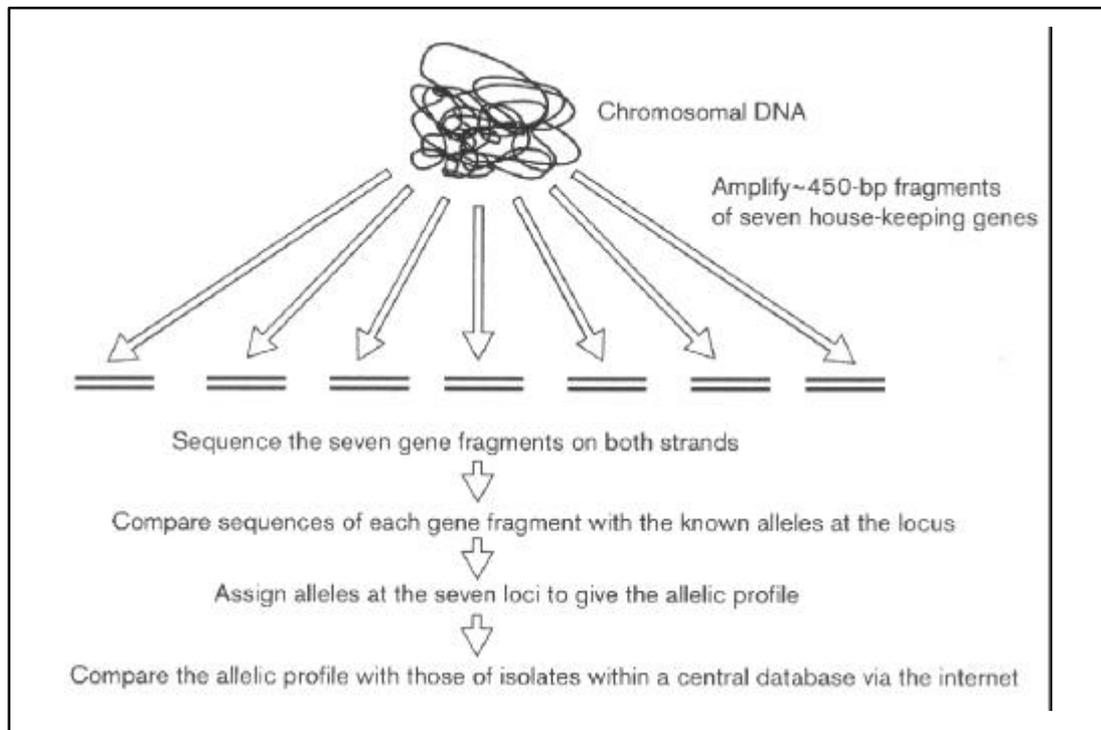


Figure 8.1. Multilocus sequence typing (MLST). The method for the allocation of the allelic profile, or sequence type, of a bacterial isolate is shown. The clustering of related isolates can be visualized as a dendrogram, constructed from the matrix of pairwise differences between the allelic profiles of the isolates (Source: Spratt 1999).

Thirdly, the electronic portability of DNA sequences allows any laboratory to characterize isolates of bacterial pathogens by submitting the sequences of the gene fragments via the Internet to a central MLST Web site (<http://www.mlst.net>), which holds the continually expanding database of the allelic profiles of isolates of the species. Finally, the sequences of seven loci from thousands of isolates of each species can be used to address aspects of their population and evolutionary biology.

MLST was initially established for *Neisseria meningitidis* in 1998 (Maiden *et al.* 1998). The technique was then extended to *Streptococcus pneumoniae* (Enright &

Spratt 1998), and by now, MLST typing schemes have been developed and databases created for a number of other bacterial species (<http://www.mlst.net>).

The levels of discrimination using MLST are very high provided there is sufficient diversity within the population to identify many alleles at each locus. As alleles only have to differ at a single nucleotide site, this criterion will usually be met. There are, however, a few highly uniform bacterial species where a sufficiently discriminatory MLST scheme could not be developed (e.g. *Mycobacterium tuberculosis* where house-keeping genes from different isolates are almost always identical in sequence (Sreevatsan *et al.* 1997), and probably *Neisseria gonorrhoeae*, which also has a low level of sequence variation within house-keeping genes (Vázquez *et al.* 1993)).

Genetic population structure and molecular epidemiology of N. meningitidis

The overall genetic population structure of *N. meningitidis* has been referred to as weakly clonal, since it comprises panmictic, epidemic-clonal and clonal elements (Spratt & Maiden 1999). In the case of a weakly clonal pathogen like *N. meningitidis*, the fact that a localized outbreak is caused by a single strain can be established using a sufficiently discriminatory method like PFGE, since the common ancestor of the isolates occurred so recently that there has been insufficient time for variation to accumulate in such an extent, that different strains belonging to the same lineage would not be recognized. During our investigation of an outbreak of serogroup X meningococci the different PFGE could clearly be assigned to specific clusters (Chapter 5), although we observed a considerable amount of variation generated over a short time period.

However, it becomes more difficult to show whether an outbreak strain belongs to one of the previously described clones associated with disease by comparing it to reference isolates from other countries, as substantial diversification of these clones will have occurred since the outbreak strain and the other members of the clone had a common ancestor (Spratt & Maiden 1999). Techniques that index selectively neutral variation and include many loci (e.g. MLST) are ideal for recognizing close phylogenetic relationship of these clones since recombinational exchanges that alter the alleles at one or two of the loci do not prevent their recognition as members of the

clone using clustering techniques (Maiden *et al.* 1998)). We studied the population structure of serogroup X meningococci by comparing strains isolated in different parts of the world, and identified two clonal groupings based on this technique (Chapter 6). However, the relationships between the deeper branches of a phylogenetic tree will be unreliable, since the descendants of an ancestral bacterium will relatively rapidly (on an evolutionary scale) accumulate at least one nucleotide difference in each of the seven loci, such that many of the isolates within the population will differ at all or most loci (Spratt 1999).

In the work presented here, the main approach included a combination of PFGE and MLST analyses. Most isolates recovered from patients or asymptomatic carriers were first analyzed by PFGE since this technique is considerably faster and cheaper than MLST. We used PFGE to analyze outbreaks of serogroup A and serogroup X meningococci in the Kassena-Nankana district (Chapters 3 and 5), to compare the outbreak strains to isolates recovered from healthy carriers in the district (Chapter 5 and Appendix 1), and to define two finer groups or genoclouds within one of two serogroup X clonal groupings identified in combination with the MLST analysis (Chapter 6; see below). MLST analysis was restricted to selected isolates representative of the potential clonal groupings identified during the PFGE analysis (Chapters 3, 4 and 6). Both PFGE and MLST were used to confirm the common identity of non-groupable (NG) isolates and corresponding serogroupable strains (Chapter 7), and to compare serogroup W135 strains isolated in different African countries (Appendix 2).

'Mid-term' epidemiology and RFLP typing of serogroup A meningococci

Based on MLEE and MLST analysis, serogroup A meningococci can be further divided into nine clonal groupings or subgroups (Wang *et al.* 1992). The genetic population structure of these bacteria is more clonal than that of other meningococci (Bart *et al.* 2001), and some of these subgroups have been causing pandemics of epidemic disease during the last decades (Achtman 1995b, Morelli *et al.* 1997).

In order to elucidate the micro-evolutionary changes during the epidemic spread of these bacteria, an approach using restriction fragment length polymorphism (RFLP) typing of a number of polymorphic genes encoding outer membrane and secreted

proteins has been applied (Morelli *et al.* 1997). It is assumed that these genes are under immune-selection and, therefore, may evolve at a higher rate than house-keeping genes (Linz *et al.* 2000). This RFLP analysis revealed that, within the subgroup III meningococci, so-called ‘pre-Mecca’ bacteria can be differentiated from ‘post-Mecca’ organisms (Morelli *et al.* 1997). We used a similar approach to confirm that the serogroup A subgroup III meningococci isolated during the outbreak in the Kassena-Nankana district belonged to the same pandemic wave caused by the post-Mecca bacteria (Chapter 3).

This approach was subsequently extended and used for the analysis of a large collection of subgroup III meningococci (Chapter 4). In addition to the genes encoding outer membrane and secreted proteins, one house-keeping gene and one insertion sequence were included in the RFLP analysis, covering a total of 6 polymorphic loci. RFLP analysis of these 6 loci allowed the differentiation of 9 finer groups within subgroup III meningococci, and led to the development of the genocloud concept (Chapter 4).

Serological classification systems

Traditionally, meningococci have been classified according to several serological schemes. Serogrouping is based on structural differences in the capsular polysaccharides and is performed with polyclonal antisera (Achtman 1995b, Poolman *et al.* 1995). Horizontal genetic exchange can lead to acquisition of genes encoding foreign capsular polysaccharides (Swartley *et al.* 1997, Vogel *et al.* 2000). Although we identified strains which had switched off their capsular expression (Chapter 7), we did not observe any case of capsule-exchange during our study. Meningococci express five different classes (designated 1 to 5 based on differences in molecular weight) of so-called major outer membrane proteins (OMP) (Poolman *et al.* 1995). Class 1 (PorA) and class 2 and 3 proteins (PorB) are porins. They form the basis for serotype (class 2 and 3) and serosubtype (class 1) which are determined using murine monoclonal antibodies (Wang *et al.* 1992). Although these serological classification methods are relevant for accurate immunization programs (serogrouping) and for the development of protein-based vaccines (serotyping/subtyping), they are generally unsuitable for epidemiology because they are based on variable phenotypes which are not necessarily related to genetic descent (Achtman 1998). In some cases, certain

serological variants are characteristic of individual clonal groupings determined by genetic methods (Achtman 1995b). For example, subgroup III meningococci have usually the A:4,21:P1.9,20 phenotype (Chapter 3 & 4)(Wang *et al.* 1992). During our analysis of serogroup X meningococci, we identified two clonal groupings. Members of the first one were all X:21:P1.16 and bacteria belonging to the second X:NT:P1.5 (Chapter 6).

8.2 Implications of the main findings and suggestions for future research

Many epidemiological characteristics of meningococcal disease in Africa were recognized shortly after the first epidemics were observed on the continent (Greenwood 1999). The limited area of occurrence of these epidemics was described in detail 40 years ago (Lapeyssonnie 1963), and substantial new epidemiological insight has been gained since then (Greenwood 1999, Moore 1992). However, major epidemics continue to occur in the African meningitis belt and they are still as unpredictable as they always have been (Tikhomirov *et al.* 1997).

The characteristic epidemiology of meningococcal disease in Africa

One of the major additions of the work presented in this thesis is the contribution to the insight in the epidemiology of meningococcal disease in the African meningitis belt, which differs substantially from that in Europe and North America. Large serogroup A epidemics used to occur regularly on the later continents until World War II and have become very rare since then; a phenomenon that cannot be explained satisfactorily (Cartwright 1995a). In contrast, such epidemics have been reoccurring every 8 – 12 years in the countries of the African meningitis belt since the beginning of the century (Greenwood 1999) (Chapter 3 and 4). Also in 2001, several countries of the African meningitis belt were hit by severe epidemics (WHO 2001b). Serogroup B meningococci are the major cause of meningococcal disease in industrialized countries, but they are only very rarely isolated in countries of the African meningitis belt (Chapter 5). Meningococcal carriage is around 10% in the general population of Europe and the USA (Cartwright 1995b), but seems to be lower during interepidemic periods in Africa (Chapter 5). Furthermore, carriage of non-serogroupable strains, making up to 50% of the meningococci carried in Europe and the USA is especially

low in Africa (Chapter 7). In the African meningitis belt, meningococcal colonization appears to occur in waves and is not influenced by carriage of *Neisseria lactamica* (Chapter 5). It has been suggested that the later might protect against meningococcal colonization and disease based on studies conducted in Europe and the USA (Gold *et al.* 1978b, Olsen *et al.* 1991). The genetic diversity of meningococci carried by the general population seems also lower in Africa than in Europe and the USA (Chapter 6 and 7) (see below).

There are many factors that could be relevant for the enhanced susceptibility for epidemic meningococcal disease in the African meningitis belt. Populations in this area live often under poor social-economic conditions and under low standards of hygiene and nutrition. They are constantly confronted with many other infectious diseases. Furthermore, the climatic conditions are severe during the Sahelian dry seasons, a factor contributing to the deterioration of the mucosal defenses. However, it is much harder to explain why, except during epidemic periods, meningococcal carriage in general, and especially carriage of meningococci which are non-serogroupable and unrelated to the epidemic strains, is less frequent in Africa than in Europe and the USA.

The following is a speculative attempt to explain the differing epidemiology of meningococcal disease in Africa and in the industrialized countries. Until World War II, serogroup A was the main cause of meningococcal disease and caused large epidemics in Europe and the USA, similar to the current situation in the African meningitis belt (Cartwright 1995a). Since then, serogroup A has become very rare in the developed world and country epidemics with high attack rates have mainly given way to hyper-endemic waves and focal outbreaks. What caused the epidemiology of meningococcal to change so dramatically in Europe and the USA? One possible explanation is that through improved general sanitation, organisms competing with the meningococci on the nasopharyngeal surface were removed, leaving an open niche for a wider spectrum of meningococci to establish themselves. We found many more commensal organisms in *Neisseria* selective agar plates inoculated with throat swabs from the Kassena-Nankana district than are generally found with European specimens. In contrast to the African meningitis belt, meningococcal carriage in Europe and the USA is high and endemic, and it seems that a persisting flora of very heterogeneous meningococci has established itself, many of which are non-capsulated

and thus probably better adapted to prolonged carriage. The carriage of such a resident flora of non- or less pathogenic meningococci could, through immunological and ecological effects, contribute to a reduced susceptibility to disease, and hinder the epidemic spread of serogroup A bacteria in Europe and the USA. Some evidence supports this hypothesis. After the 1987 serogroup A subgroup III epidemic in Mecca, many pilgrims of industrialized countries brought the epidemic strain back home. However, this strain could not establish itself in these countries and caused only very few cases of disease outside the African meningitis belt, where, during the following years, large epidemics of disease occurred in many countries.

In the African meningitis belt, the limited pool of meningococcal types includes strains of high virulence able to cause epidemics of disease (i.e. serogroup A bacteria). Furthermore, the low humidity prevailing during the Sahelian dry seasons favors disease, although it has no effect on carriage and is therefore not important for transmission (Greenwood *et al.* 1984) (Chapter 5). Other strains with increased transmissibility but low virulence may occasionally break through and cause an epidemic of carriage without much disease, similarly to what we observed in the case of a specific clonal grouping of serogroup X bacteria (Chapter 5).

Additional insight would be gained by conducting carriage surveys during large epidemics in the African meningitis belt in neighboring regions, which lay further to the south and outside the meningitis belt. Such studies would show whether carriage of the epidemic strains is restricted to the population of the area affected by the disease epidemics or, alternatively, whether epidemic zones share patterns of both the epidemic and other strains with the neighboring (more humid) areas outside the meningitis belt.

More insight into the immune processes related to colonization and disease is needed in order to be able to test such hypotheses. One suggestion for future research would be to attach an immunological component to the ongoing carriage surveys. Longitudinal sero-epidemiological surveys could be performed with the aim i) to follow the changes in herd immunity to specific capsular and outer membrane protein antigens, ii) to try to determine the relative contribution of these different immune responses to the overall protective immunity, iii) to establish the role of mucosal immunity, and iv) to relate the immune response to the type of meningococci carried. Furthermore, the meningococci specific immune status of populations of the African

meningitis belt could be compared to that of populations in Europe or the USA in order to find differences that could contribute to the understanding of the strikingly different epidemiologies in the two regions. However, such longitudinal sero-epidemiological studies would require repeated blood drawings which are associated with substantial ethical, social-cultural and logistical difficulties.

Pathogenic potential of serogroup X meningococci

Another main finding is the documentation of a serogroup X meningitis outbreak in Ghana (Chapter 5). This observation has two important implications. The first one concerns the genetic population structure of serogroup X bacteria in Africa and the other the potential impact of vaccination programs on the general population structure and serogroup distribution of meningococci in Africa (see below).

Two outbreaks of serogroup X meningitis have been reported in Niger previously (Campagne *et al.* 1999, Etienne *et al.* 1990), and our results show that the most recent of these two outbreaks and the KND serogroup X outbreak were caused by meningococci belonging to the same clonal grouping (Chapter 6). Furthermore, bacteria belonging to this clonal grouping include the vast majority of serogroup X meningococci isolated in Africa, including strains isolated in the 1970s in Mali (Chapter 6). Recent results, based on an extensive phylogenetic analysis including all meningococcal MLST sequence types present in the MLST database (<http://www.mlst.net>), suggest that serogroup X bacteria from Africa are clonal, similar to the subgroups of serogroup A meningococci (Thierry Wirth, personal communication) (Bart *et al.* 2001). Although, we identified another clonal grouping within serogroup X bacteria from Europe and the USA, a large proportion of the European and American isolates were very diverse (Chapter 6).

What could explain the apparent lack of heterogeneity within the African serogroup X meningococcal strains? Meningococci are naturally transformable and a high rate of recombination has been observed in these bacteria (Feil *et al.* 1999, Jolley *et al.* 2000), especially through horizontal genetic transfer from other meningococci or even other Neisserial species (Linz *et al.* 2000). A possible explanation is that in the African meningitis belt, the relative low levels of carriage of meningococci unrelated to the epidemic strain (Chapter 5 and 7) could limit the opportunities for horizontal

genetic transfer. However, we found that the epidemiology of carriage of *Neisseria lactamica* in Ghana did not differ significantly from that in Europe or the USA (Chapter 5) (Cartwright 1995b). On the other hand, DNA transfer and/or integration between *N. meningitidis* and *N. lactamica* might be less frequent since the sequence diversity between the two species is larger.

The characterization of the isolates collected during the ongoing carriage surveys will provide additional insight in the genetic population structure of meningococci in Ghana. However, similar studies carried out in other parts of Africa are required to elucidate the overall population structure of meningococci resident in the African meningitis belt.

Vaccine impact

The introduction of any vaccine that targets only a fraction of the population of a bacterial pathogen has been referred to as a large-scale experiment in bacterial population biology, the outcome of which cannot be fully predicted (Maiden & Spratt 1999). In fact, a change in serotype distribution has been observed in the carriage of *Streptococcus pneumoniae* after the introduction of a polyvalent capsular polysaccharide conjugate vaccine (Mbelle *et al.* 1999, Obaro *et al.* 1996). For over a decade, serogroup A + C polysaccharide meningococcal vaccines have been extensively used to combat epidemics in many countries (Tikhomirov *et al.* 1997). These vaccines do not seem to affect carriage (Blakebrough *et al.* 1983, Hassan-King *et al.* 1988). However, the recent outbreaks of serogroup X in Niger and Ghana, and of serogroup W135 in Mecca (Taha *et al.* 2000, WHO 2001a) may reflect a selection of former rare serogroups that are not included in the vaccine.

The implementation of immunization programs in the African meningitis belt with future serogroup A + C polysaccharide protein conjugate vaccines, which are very likely to affect carriage, will probably create a strong selection pressure against serogroup A and serogroup C meningococci. After the introduction of such vaccine programs, an effective surveillance should therefore be established, in order to capture a potential increase in disease incidence due to serogroups not included in the vaccine.

In terms of future research, efforts should be directed towards the development of a polysaccharide protein conjugate vaccine that would also include rare capsular polysaccharide variants (i.e. serogroup W135 and X), or a broadly reactive and

potentially less expensive protein-based vaccine. Immunization programs with such vaccines might have less undesirable long-term side effects.

8.3 Conclusions

The epidemiology of meningococcal meningitis was investigated in the Kassena-Nankana district (KND) in Northern Ghana. Conventional epidemiological tools were used to determine patients and carrier characteristics. Several molecular typing and phylogenetic analysis techniques were applied to characterize the bacterial isolates, and to compare them to other meningococcal strains isolated in different regions of the world. Based on the findings generated during these studies, the following conclusions could be drawn.

1. The meningitis outbreak that occurred in the KND in 1998 was caused by serogroup A subgroup III post-Mecca meningococci. Thus, this outbreak was part of the second pandemic wave caused by serogroup A subgroup III bacteria (see below). The rather high case-fatality ratio observed during this outbreak can be most likely attributed to late-arrival at the health facilities. Serogroup A subgroup III meningococci can be further divided into nine genoclouds. These genoclouds consist of fit genotypes that were isolated repeatedly and of closely related escape variants which are mostly less fit than their parents. These escape variants are subsequently lost because of competition and bottlenecks during spread from country to country. These nine genoclouds have caused three pandemic waves of disease since the mid-1960s, the most recent of which was imported from East Asia to Europe and Africa in the mid-1990s. These observations raise the possibility of renewed epidemic serogroup A disease in Europe and the Americas.
2. Two years following the serogroup A meningococcal disease epidemic that occurred in the KND in 1997/98, a carriage epidemic of serogroup X meningococci with a number of associated serogroup X meningitis cases was observed. This illustrates the pathogenic and even epidemic potential of serogroup X bacteria. These serogroup X bacteria were very likely to have been newly introduced into the district, since no such strains had been isolated during two preceding surveys. While carriage of serogroup A was decreasing between 1998 and 2000, carriage of serogroup X increased very strongly. Only a few other

meningococcal isolates, unrelated to the dominant serogroup A and X strains, were recovered during the carriage surveys. These findings support the hypothesis that, in the African meningitis belt, meningococcal colonization occurs in wave, and that depending on the virulence of the strain involved in the process, epidemics of disease occur. Carriage of *Neisseria lactamica* had no effect on the dynamics of meningococcal carriage, and therefore does not seem to protect against colonization of the nasopharynx by meningococcal strains.

3. Although most of the serogroup X meningococci recovered in different countries of Africa, Europe and North America were diverse, two clonal groupings were identified. One clonal grouping included strains isolated from patients and healthy carriers in several countries of Europe and the USA over the last two decades. The second clonal grouping comprised the large majority of serogroup X bacteria isolated from countries of the African meningitis belt since the early 1970s. Based on the PFGE results, two genoclouds were identified within the second clonal grouping. Bacteria belonging to the first genocloud caused an outbreak in Niger in 1997 and bacteria belonging to the second caused the outbreak in KND in 2000. These observations support the concerns raised about the potential vaccine-induced selection of serogroups not included in the polysaccharide vaccines, which have extensively been used in the African meningitis belt for more than a decade.
4. In contrast to Europe and the USA, carriage of non-serogroupable meningococci is infrequent in the African meningitis belt. This finding supports the hypothesis that a lack of protective cross-reactive immunity against pathogenic strains might be caused by such an infrequent carriage of non-serogroupable meningococci. This could increase the susceptibility to epidemic disease in the populations of the African meningitis belt.

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APPENDIX 1

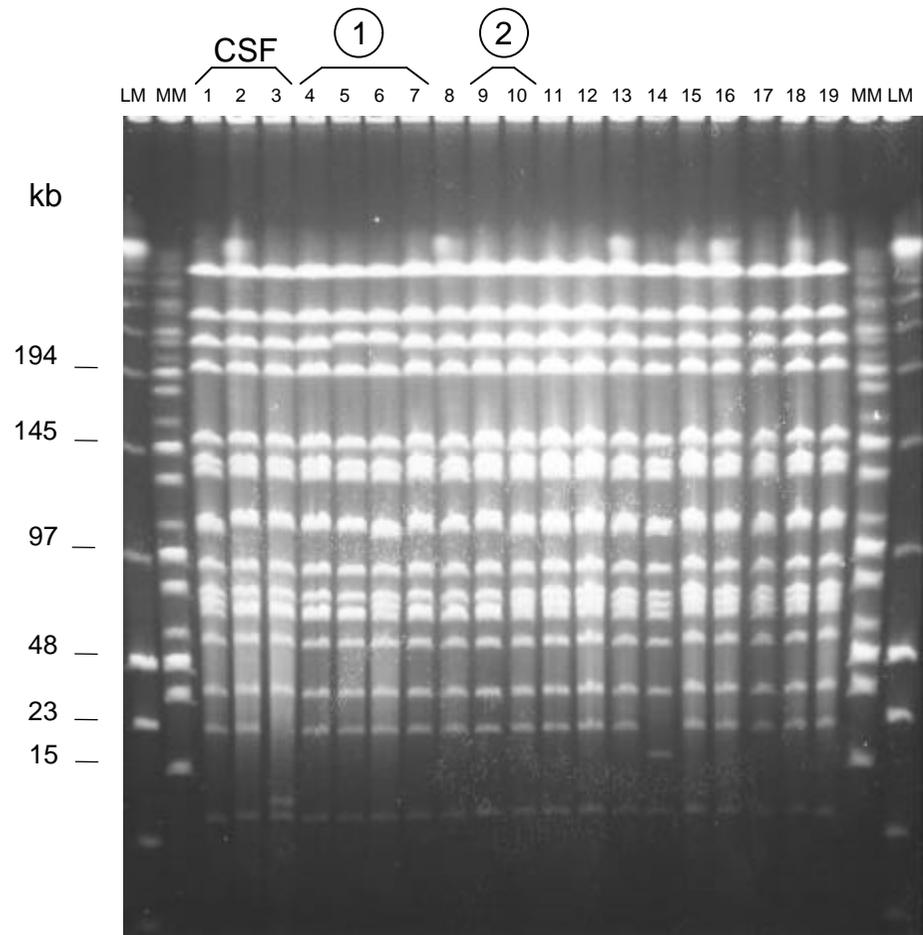
Long-term carriage of a serogroup A (subgroup III) epidemic *Neisseria meningitidis* strain in Northern Ghana

During a longitudinal carriage study carried out in the Kassena-Nankana district of Northern Ghana, the five 6-monthly surveys of 300 randomly selected individuals revealed a gradual decrease in carriage of serogroup A subgroup III meningococci (Chapter 5). Analysis by pulsed-field gel electrophoresis (PFGE) (Morelli *et al.* 1997) showed that all carriage isolates were very similar to disease isolates recovered from meningitis patients during the 1998 outbreak in the same district (Chapter 3; Figure 9.1). Multi locus sequence typing of selected isolates revealed ST5 which is typical for subgroup III bacteria (Chapter 4). One person was found to be carrying these bacteria at four consecutive surveys (Figure 1; number 1: tracks 4-7). However, after digestion of chromosomal DNA with *NheI* and *SpeI*, three different combined PFGE patterns were observed. Since this individual was carrying the same PFGE variant in the first and the 4th survey (Figure 9.1; tracks 4 and 7), it is not clear whether this person had a mixed colonization with closely related bacteria belonging to the same genocloud or experienced re-colonization (his wife was carrying the variant found in the 1st and 4th survey during the second survey; track 8).

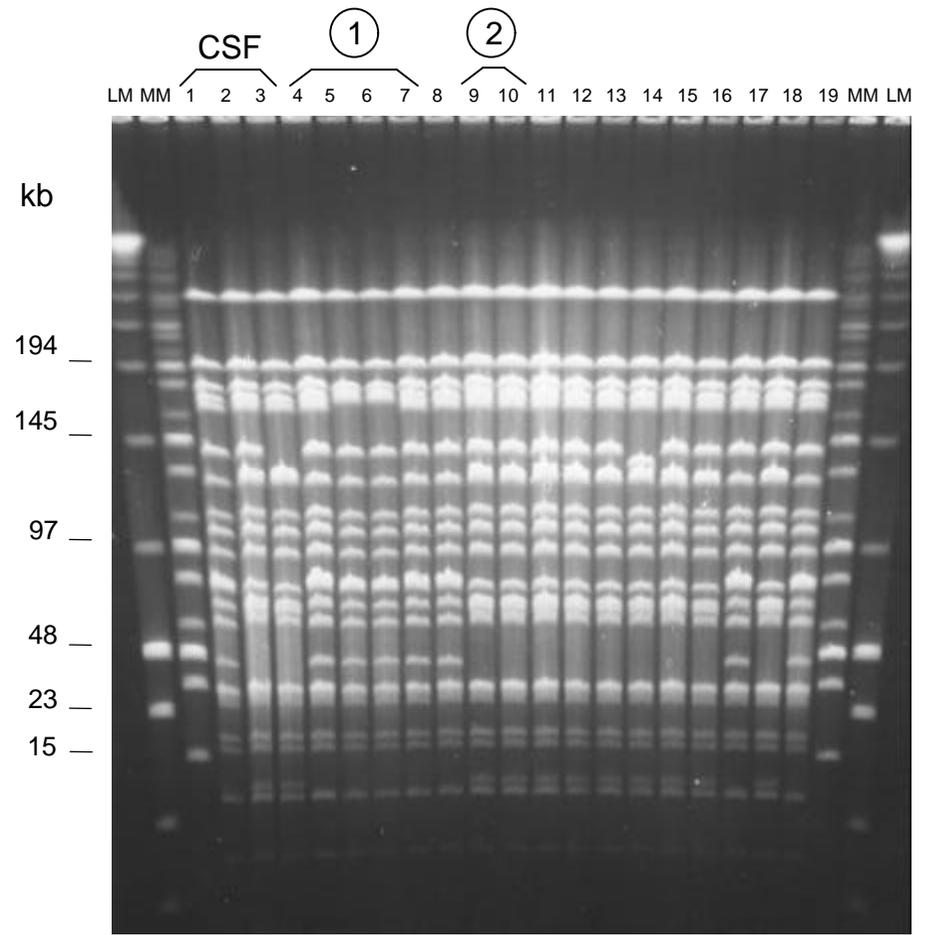
A second individual was carrying two different PFGE variants at two consecutive surveys (Figure 1; tracks 9 and 10). Since up to 10 colonies have been picked and stored from each individual at each survey, PFGE analysis of all these isolates may help to determine whether these two individuals were carrying a mixed population or were re-colonized.

Figure 1. PFGE patterns of *NheI* and *SpeI* digested chromosomal DNA of serogroup A subgroup III meningococci from Northern Ghana. Strains were loaded in tracks 1-19 in the following order (track: strain): 1:Z7057; 2: Z7060; 3: Z7067; 4: Z8318; 5: Z8324; 6: Z8326; 7: Z9567; 8: Z8349; 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: low range marker; MM: midrange marker); their molecular weights are indicated at the left. CSF indicate isolates recovered from meningitis patients during the 1998 outbreak in the Kassena-Nankana district of Northern Ghana (Chapter 3). All other strains were isolated from healthy carriers (Chapter 5). Number 1 and 2 indicate sequential isolates from the same persons.

NheI



SpeI

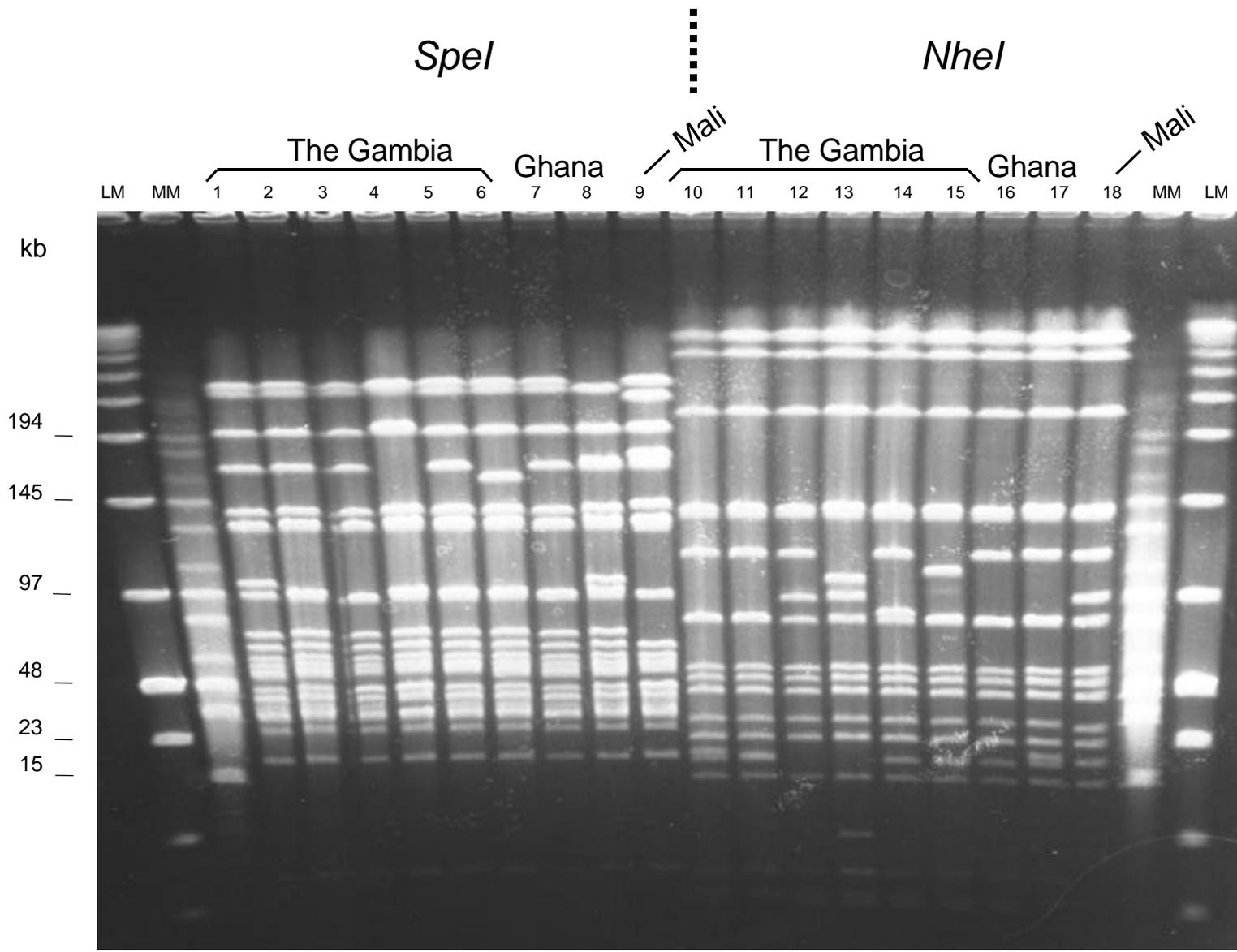


APPENDIX 2

Carriage of serogroup W135 (ET-37 complex) strains in Northern Ghana

We conducted a three-year longitudinal carriage study in the Kassena-Nankana district of Northern Ghana, during which two serogroup W135 isolates were recovered in 1998 (Chapter 5). We compared these strains by pulsed-field electrophoresis (PFGE) (Morelli *et al.* 1997) to other serogroup W135 strains isolated in 1993 and 1995 in The Gambia and Mali (Kwara *et al.* 1998). All these isolates had very similar *SpeI* and *NheI* fingerprints (Figure 9.2). Multi locus sequence typing revealed sequence type (ST) 11 which is typical of ET-37 complex strains (Maiden *et al.* 1998). Isolates of ST11 have typically been serogroup C, but occasional serogroup B, W135, and non-groupable strains with this ST have been observed (Wang *et al.* 1993). In 2000, W135, ST11 bacteria caused a disease outbreak with more than 200 cases during the annual Hajj pilgrimage in Mecca (WHO 2000b). Subsequently, meningitis cases were observed among pilgrims returning to France, the UK, the Netherlands, Oman, Kuwait, Singapore, Indonesia, and the USA, as well as among their close contacts (Popovic *et al.* 2000, Taha *et al.* 2000). Analysis by PFGE revealed that this outbreak was clonal and that the outbreak strains were closely related to the serogroup W135 meningococci isolated several years earlier in The Gambia, Mali and Ghana (Taha *et al.* 2000).

Figure 9.2. PFGE patterns of *SpeI* and *NheI* digested chromosomal DNA of serogroup W-135 meningococci from The Gambia, Ghana and Mali. Strains were loaded in tracks 1-9 and 10-18 in the following order (track: strain): 1/10: Z6196; 2/11: Z6206; 3/12: Z6209; 4/13: Z6212; 5/14: Z6216; 6/15: Z6218; 7/16: Z8343; 8/17: Z8344; 9/18: Z6351. Molecular weight markers were loaded in the flanking tracks as indicated (LM: low range marker; MM: midrange marker); their molecular weights are indicated at the left. *SpeI* : track 1-9; *NheI*: tracks 10-18.



CURRICULUM VITAE

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Education & work experience:	
1976-1988	Primary school and high school in Basel, Switzerland.
1990-1997	Study of Biology at the University of Basel, Switzerland.
Nov. '95-Apr. '97	Diploma (MSc) in Biology (Epidemiology); thesis entitled: 'Urban agriculture in Nouakchott, Mauritania: Perceived morbidity and potential risk for diarrhea. Minor: organic chemistry.
Apr. '97–Sept. '97	Research assistant at the Swiss Tropical Institute; project entitled: 'Risk assessment for contamination of drinking water sources with <i>Cryptosporidium sp</i> in the canton Basel-Landschaft.
Sept. '97-present	PhD in Epidemiology at the Swiss Tropical Institute in Basel, Switzerland and at the Navrongo Health Research Center, Navrongo, Ghana. Thesis entitled: 'Molecular epidemiology of meningococcal meningitis in Northern Ghana'. <i>Responsibility:</i> Installation of a field laboratory in Northern Ghana.

During my studies, I attended courses taught by the following lecturers:

W. Arber, B. Betschart, C.A. Boesch, T. Boller, R. Brun, P. Duelli, T. Freyvogel, W. Gehring, B. Genton, U. Gisi, R. Guggenheim, H. Hecker, H.-C. Im Hof, S. Jacomet, L. Jenni, C. Körner, C. Lengeler, D. Lüdin, H.-P. Marti, J. Meyer, G.-R. Plattner, G. Pluschke, U. Rahm, C.H. Rowell, W. Rudin, G. Schatz, V. Schmid, D.G. Senn, U. Sequin, H. Sigel, T.A. Smith, S.C. Stearns, J. Stöcklin, M. Tanner, P. Vounatsou, M. Weiss, N.A. Weiss, A.M. Wiemken, A. Zumstein.

List of Publications

1. Schneider C, Gagneux S, Odermatt P, Cissé G, Lémine M, Dah C, Tanner M (1997). Different water sources in two homegardens sites in Nouakchott, Mauritania: Perceived morbidity and potential risk for diarrhea. *Schweiz Med Wochenschr*, **127**: 1624 (Abstract).
2. Gagneux S, Schneider C, Odermatt P, Cissé G, Cheikh D, Salem ML, Touré A, Tanner M (1999). Diarrhea in Urban Agricultural Workers in Nouakchott, Mauritania. *Med Trop*, **59**: 253-8 (French).
3. Gagneux S, Hodgson A, Ehrhard I, Morelli G, Genton B, Smith T, Tanner M, Binka F, Achtman M, Pluschke G (2000). Microheterogeneity in Serogroup A (subgroup III) *Neisseria meningitidis* during an Outbreak in Northern Ghana. *Trop Med Int Health*, **5**: 280-287.
4. Zhu P, van der Ende A, Falush D, Brieske N, Morelli G, Linz B, Popovic T, Schuurman I, Adegbola R, Zurth K, Gagneux S, Platonov A, Riou JY, Caugant D, Nicolas P, Achtman M (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.
5. Hodgson A, Smith T, Gagneux S, Adjuik M, Pluschke G, Mensah NK, Binka F, Genton B. Risk Factors For Meningococcal Meningitis In Northern Ghana. *Trans R Soc Trop Med Hyg* (in press).
6. Hodgson A, Smith T, Gagneux S, Akumah I, Adjuik M, Pluschke G, Bink F, Genton B. Survival and Sequelae of Meningococcal Meningitis in Ghana. *Int J Epidemiol* (in press).
7. Gagneux S, Hodgson A, Smith T, Wirth T, Ehrhard I, Morelli G, Genton B, Binka F, Achtman M, Pluschke G. Prospective Study of a Serogroup X *Neisseria meningitidis* Outbreak in Northern Ghana. *J Infect Dis* (submitted).
8. Gagneux S, Wirth T, Hodgson A, Ehrhard I, Morelli G, Genton B, Smith T, Binka F, Pluschke G, Achtman M. Clonal groupings in Serogroup X *Neisseria meningitidis*. *Emerg Infect Dis* (submitted).

9. Gagneux S, Hodgson A, Ehrhard I, Morelli G, Genton B, Smith T, Binka F, Pluschke G, Achtman M. Infrequent carriage of non-serogroupable *Neisseria meningitidis* in Africa. *J Clin Microbiol* (in preparation).