

KSH 30007-

**ISOLATION AND CHARACTERIZATION OF *NEISSERIA
MENINGITIDIS* IMPLICATED IN AN OUTBREAK OF
MENINGITIS IN WEST POKOT DISTRICT, KENYA AND
SOUTH SUDAN – APRIL 2006**

BY

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PURE AND APPLIED SCIENCES OF THE KENYATTA UNIVERSITY.**

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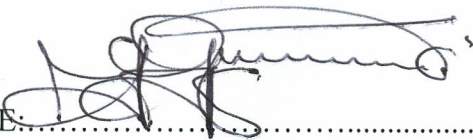

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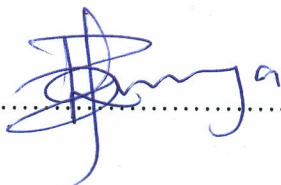

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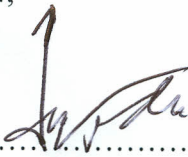
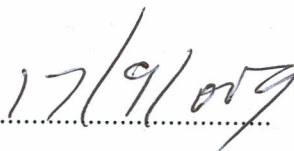
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DEDICATION

I dedicate this work to my wife Regina Materu, daughter Linda Materu and son Nelson Materu for giving me moral support and inspiration during the entire period of my studies and this project.

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LIST OF ACRONYMS

AMREF.....	African Medical and Research Foundation
AIDS.....	Acquired Immuno Deficiency Syndrome
ATCC.....	American Type Culture Collection, Inc
BA.....	Blood Agar base
CA.....	Chocolate Agar
CAR.....	Central Africa Republic
CDC.....	Centers for Disease Control
CERMES.....	Centre d'Etude et de Recherche pour les Meningocoques et Schistosomiases
CLSI.....	Clinical Laboratory Standards Institute
CPA.....	Comprehensive Peace Agreement
CPHO.....	Chief Public Health Officer
CRF.....	Case Fatality Rate
CSF.....	Cerebrospinal fluid
CTA.....	Cystine Trypticase Agar
DHP.....	Division of Health Promotion
DNA.....	Deoxyribonucleic Acid
DOMU.....	Division of Outbreak Monitoring Unit
ETWG.....	Epidemic Technical Working Group
FELTP.....	Field Epidemiology Laboratory Training Programme
GOS.....	Government of Sudan
IATA.....	International Air Transport Association

ICRC.....	International Committee of the Red Cross
IDP.....	Internally Displaced Persons
IMC.....	International Medical Corps
IRC.....	International Rescue Committee
KEMRI.....	Kenya Medical Research Institute
KM.....	Kilometers
KRC.....	Kenya Red Cross
LP.....	Lumbar Puncture
MAT.....	Meningitis Antibody Test
MCV.....	Meningococcal Conjugate Vaccine
MIC.....	Minimal Inhibition Concentration
MSF.....	Medecines Sans Frontierès
MLST.....	Multi-Locus Sequence Typing
MOH.....	Ministry of Health
MPSV.....	Meningococcal Polysaccharide Vaccine
NAMRU.....	Naval Medical Research Unit
NCCLS.....	National Committee of Clinical Standards
NM.....	<i>Neisseria meningitidis</i>
NPHLS.....	National Public Health Laboratory Services
PBS.....	Phosphate Buffer Saline
PCR.....	Polymerase Chain Reaction
RPM.....	Revolutions per minute
SPLM.....	Sudan People's Liberation Movement

ST.....	Sequence Type
TIM.....	Trans Isolate Medium
TSA.....	Tryptone Soy Agar
UK.....	United Kingdom
USA.....	United States of America
USAID.....	United States Agency for International Development
WHO.....	World Health Organisation

DEFINITIONS OF TERMS

- CARRIER:** A person or animal that harbors a specific infectious agent without discernible clinical disease and serves as a potential source of infection. The carrier state may exist in an individual with an infection that is not obvious throughout its course (commonly known as healthy or asymptomatic carrier). Under either circumstances the carrier state may be of short or long duration (temporary or transient carrier, or chronic carrier).
- CRF:** Case Fatality Rate: Usually expressed as the percentage of persons diagnosed as having a specified disease who die as a result of that illness within a given period. This term is most frequently applied to a specific disease outbreak of acute disease in which all patients have been followed for an adequate period of time to include all attributable deaths.
- DNA:** Deoxyribonucleic Acid: Is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms.
- CERMES:** An organisation in Niamey – Niger; a centre for meningococcus meningitis and Schistosomiasis.
- MLST:** Multi-Locus Sequence Typing: Is a nucleotide sequence based approach for the unambiguous characterisation of isolates of bacteria and other organisms via the internet.
- MORBIDITY RATE:** An incidence rate used to include all persons in the population under consideration who become clinically ill during the period of time stated. The population may be limited to specific gender or age group, or those with certain other characteristics.
- MORTALITY RATE:** A rate calculated in the same way as incidence rate, by dividing the number of deaths occurring in the population during the stated period of time, usually a year, by the of persons at risk of dying during the period. A total of crude mortality rate utilizes deaths from all causes, usually expressed as deaths per 1,000. A disease-specific mortality rate covers deaths due to only one disease and is often reported on the basis of 100,000 persons. The population base may be defined by gender, age or other characteristics. The mortality rate must not be confused with case-fatality rate.
- MSF:** Medecines Sans Frontierès: A medical organization known as Doctors without Borders.

PCR: Polymerase Chain Reaction: Is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication.

SUSCEPTIBILITY TESTS ON ANTIMICROBIAL AGENTS

S = susceptible to antibiotic
I = intermediate
R = resistant to antibiotic

Susceptible: An organism is called susceptible to a drug when the infection caused is likely to respond to treatment with a drug at a recommended dosage.

Intermediate: Covers two situations;
(i) Drug that can be used for treatment at a higher dosage because of its low toxicity.
(ii) Drug can be used because it is concentrated into the focus of the infection e.g. Urine.

Resistance: Organism is expected not to respond to a given drug, irrespective of the dosage and of the location of infection.

ABSTRACT

Meningococcal disease is an acute bacterial infection caused by *Neisseria meningitidis* and is characterized by sudden onset with fever, intense headache, nausea, vomiting, stiff neck and confusion. Formerly, the case-fatality ratio exceeded 50%, but early diagnosis, modern therapy, and supportive measures have lowered the case-fatality ratio to about 10% in developed countries. Among survivals, 11-19% develop long-term sequelae, including hearing loss, neurological disability or limb loss. Meningococcal meningitis occurs globally and is usually sporadic, but occasionally breaks into devastating epidemics. There are 12 sero-groups of *N. meningitidis* but only 4 cause epidemics. In African meningitis belt and in eastern African region, 90% of the recurrent meningococcal meningitis epidemics are caused by sero-group A and C. In the past 20 years, other sero-groups such as Y, X or W135 have been detected in the West African countries. Between January and April 2006, there was meningitis outbreak in West African Countries (Burkina Faso & Niger) also in the eastern Africa region including Uganda, southern Sudan and Kenya. These outbreaks may be associated with mutation or new circulating *N. meningitidis* strains. The main aim of this study was to isolate, carry out antimicrobial susceptibility test and characterize *N. meningitidis* from cerebrospinal fluid (CSF) specimens of patients from West Pokot Kenya and southern Sudan in order to understand the diversity of the circulating strains for effective management. The specimens from the field were collected in Trans-Isolate Media and plain sterile test tubes then transported to the AMREF Laboratory at room temperature for culture on solid media. Molecular typing by PCR of all the CSF specimens was performed and characterization by Multi-locus Sequence Typing (MLST) was carried out for the CSF specimens that showed growth (positive culture). The specimens were analyzed at the AMREF laboratory and at the U.S. Naval Medical Research Unit No.3 (NAMRU) in Cairo, Egypt. The study results showed the first outbreak of sero-group X meningococcal disease in Kenya; whereas the isolates from southern Sudan were of sero-group A, meningococcal meningitis. The most affected age group was children under 10 years in both countries. Also the study showed that the *N. meningitidis* strain in Kenya had resistance to sulphanomide drug whereas the strain in southern Sudan showed intermediate susceptibility. Molecular epidemiology is recommended for recognizing new strains in outbreaks and determining the suitable vaccine. This may call for inclusion of sero-group X in the new vaccine.

CHAPTER ONE

INTRODUCTION

1.1 Background

Meningococcal meningitis was first described by a French pathologist Gaspard M. Vieusseux in 1805 when an outbreak swept through Geneva, Switzerland killing 33 people (Vieusseux, 1805). In fact, Vieusseux did not understand fully the nature of neisserial disease transmissibility, because he was unaware of a certain epidemiological link, the asymptomatic carrier state, which would only be described at the close of the nineteenth century (Kiefer, 1896). In 1887 an Australian pathologist Anton Weichselbaum clarified the etiological nature of meningococcal disease by showing for the first time, there was connections between *Neisseria meningitidis* (then known as *Diplococcus intracularis meningitidis*) and 'epidemic cerebrospinal meningitidis' (Weichselbaum, 1887). *Neisseria meningitidis* caused a disease spectrum ranging from occult sepsis with rapid recovery to fulminating disease. Meningococcal disease was fatal up to 25% of cases (Vincent *et al*, 1997).

Neisseria meningitidis resides in its natural habitat within the nasopharyngeal tract of humans. The bacteria are transmitted from person to person through droplets from respiratory or throat secretions through close and prolonged contact. This could be through kissing, sneezing and coughing on someone, living in close quarters or dormitories (military recruits, students). Sharing eating or drinking utensils also facilitate the spread of the disease. The average incubation period is 4 days ranging between 2 to 10 days. *Neisseria meningitidis* only infects humans; there is no animal reservoir (<http://www.childsurvival.com/features/bookmarks/Meningitis.cfm>, May 2003) a World Health Organisation (WHO) Publication.

Five to fifteen percent of the human population carries the bacteria in its non pathogenic form. A major step in infection is its colonization in the nasopharynx of the human carrier. After colonizing the nasopharynx the bacteria adhere to pili and cross the nasopharyngeal epithelium by a process of endocytosis to invade the circulatory system. In the blood stream the bacteria proliferate and adapt to the host environment.

Clinical responses to infection are varied from benign, extreme, to fatal forms (meningococcaemia). In its most severe form, heightened levels of bacteria in the blood make their way to the meninges in the brain and at such high levels of cytotoxicity, induce inflammation of the meninges and progression of the disease. The most common symptoms of meningitis are fever, severe headache, stiff neck, confusion, sensitivity to light, vomiting, and coma. Diagnosis is by observation of the clinical signs and is confirmed by lumbar puncture (LP) to examine the cerebrospinal fluid. Persons most susceptible to the infection are often younger children aged about 2 years, and patients who are immunocompromised, suffering from various medical conditions (http://www.brown.edu/Course/Bio_160/Project1999/bmenin/nmenin.html;1999). The onset is usually rapid (acute), and if untreated, the disease can be fatal within 5 days after exposure. Even when the disease is diagnosed early and adequate therapy instituted, 5-10% of patients die typically within 24-48 hours of onset of symptoms (Black *et al.*, 2002). Bacterial meningitis may result in brain damage, hearing loss or learning disability in 10-20% of survivors. A less common but more severe (often fatal) form of meningococcal disease is meningococcal septicaemia which is characterized by a haemorrhagic rash and rapid circulatory collapse

(<http://www.childsurvival.com/features/bookmarks/Meningitis.cfm>,2003) a WHO publication.

Pathogenic forms of *N. meningitidis* possess a capsule that prevents phagocytosis. These are divided into twelve sero-groups, eight of which cause invasive disease (A, B, C, 29-E, H, I, K, L, W135, X, Y and Z), while four sero-groups; A, C Y and W-135 are known to cause epidemics (Peltola *et al.*, 1983).

1.2 Problem Statement and Justification

As indicated above, serological characterization or classification has revealed twelve sero-groups of *N. meningitidis*. Among the twelve sero-groups, four cause epidemics and are the main targets for vaccination and chemotherapy. Despite ongoing vaccination and treatment programmes, new outbreaks are still being reported in the region. These outbreaks may be associated with mutation of the circulating *N. meningitidis* strains in the region. To address this problem it is necessary to carryout a more sensitive molecular characterization of the isolates from these new outbreaks in order to understand the diversity of the circulating *N. meningitidis* strains for effective management. Such a study would also provide information on the kind of vaccine and drugs that regional governments need to ensure effective and reliable management.

1.3 Hypothesis

There are diverse sero-groups of *Neisseria meningitidis* causing meningitis in West Pokot, Kenya and southern Sudan.

1.4 Objectives

1.4.1 General objective

To characterize the *Neisseria meningitidis* microorganisms obtained from West Pokot and southern Sudan outbreaks.

1.4.2 Specific objectives

- i) To carry out drug susceptibility of the microorganisms isolated from West Pokot and southern Sudan outbreaks.
- ii) To carry out sero-grouping of microorganisms isolated from West Pokot and southern Sudan.
- iii) To carryout molecular characterization of isolates from Kenya and southern Sudan.

1.4.3 Research questions

- i) What is the profile of circulating *N. meningitides*?
- ii) Are circulating *N. meningitidis* resistant to existing antimicrobial agents?
- iii) Are the *N. meningitidis* isolated from West Pokot different from those isolated from southern Sudan?

CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology

Neisseria meningitidis is the causative agent of meningococcal disease. These are Gram-negative, oval cocci, 0.6-0.8 μ m, that occur typically in pairs, with adjacent sides' flattened or concave. Gram-stained film of CSF of a case of meningococcal meningitis show similar appearance of the organisms to genital tract exudates in gonorrhoea, but the organisms are scanty in number. Bacterial meningitis may be caused by *Streptococcus pneumoniae* or *Haemophilus influenzae*, but occur in sporadic forms. Meningococcal disease differs from the other causes of bacterial meningitis because of its potential to cause large-scale epidemics. Sporadic cases, outbreaks, and hyperendemic disease in Europe and the United States are usually caused by sero-groups B and C (Mindy *et al.*, 2003). Major African epidemics are associated with *N. meningitidis* sero-groups A and C and sero-group A has been reported in Kenya (Pinner *et al.*, 1992).

2.2 Epidemiology

Outbreak of meningitis is a major problem and is associated with high mortality in all age groups. In the UK in 1996-2000, group B accounted for 59% of all infections, and group C 36% (now greatly reduced by vaccination). The UK rates of invasive disease are 2-6 per 100,000 with approximately 10% fatality. The World Health Organization (WHO) estimates that the disease caused 171,000 deaths in 2000. In the developed world, most infections occur during winter and early spring whereas in the developing world, mainly sub-Saharan Africa, large scale epidemics occur during dry seasons

between January and March mainly due to sero-group A, with a 11% case mortality rate. (<http://www.patient.co.uk/showdoc/400002229>, 2004).

The disease may occur in any part of the world, but the largest more frequent recurring outbreaks have been in the semi-arid areas of sub-Saharan Africa. Sero-groups A, B and C are responsible for more than 90% of invasive meningococcal infection world wide (<http://www.who.int/mediacentre/factsheets/fs141/en/>, 2001); The WHO Weekly Epidemiological Record (WER). Most large meningitis epidemics are caused by sero-group A meningococci. Since World War II, such epidemics have been in industrialized countries but they occur periodically in the African meningitis belt and in China. (Lapeyssonnie, 1968; Olyoek *et al.*, 1987; Wang *et al.*, 1992).

In 2006, seasonal meningitis outbreak killed hundreds of people along the African meningitis belt. The worst hit was Burkina Faso, where 246 people died, and over 2,000 more fell ill from the disease (<http://www.allafrica.com/stories/200603100699.html>; 2006). The infection is spread by seasonal harmattan winds from the Sahara which blow dust clouds over West Africa carrying the deadly meningitis bacteria. Because of dusty winds and upper respiratory infections due to cold nights, the local immunity of the pharynx is diminished increasing the risk of meningitis. At the same time, the transmission of *N. meningitidis* is favoured by overcrowded housing at family level and by large population displacement, due to pilgrimage and traditional market at regional level. This combination of factors explains the large epidemics which occur during this season in the meningitis belt (<http://www.childsurvival.com/features/bookmarks/Meningitis.cfm>, May 2003), a WHO publication.

The countries along African meningitis belt comprise of more than 300 million people. As shown in Fig. 1 on page 8, the belt runs across 15 countries from west to east; the Gambia, Senegal, Guinea, Guinea Bissau, Cote d'Ivoire, Mali, Burkina Faso, Benin, Nigeria, Niger, Ghana, Chad, Central African Republic (CAR), Sudan, through to Ethiopia (Mindy *et al.*, 2003).

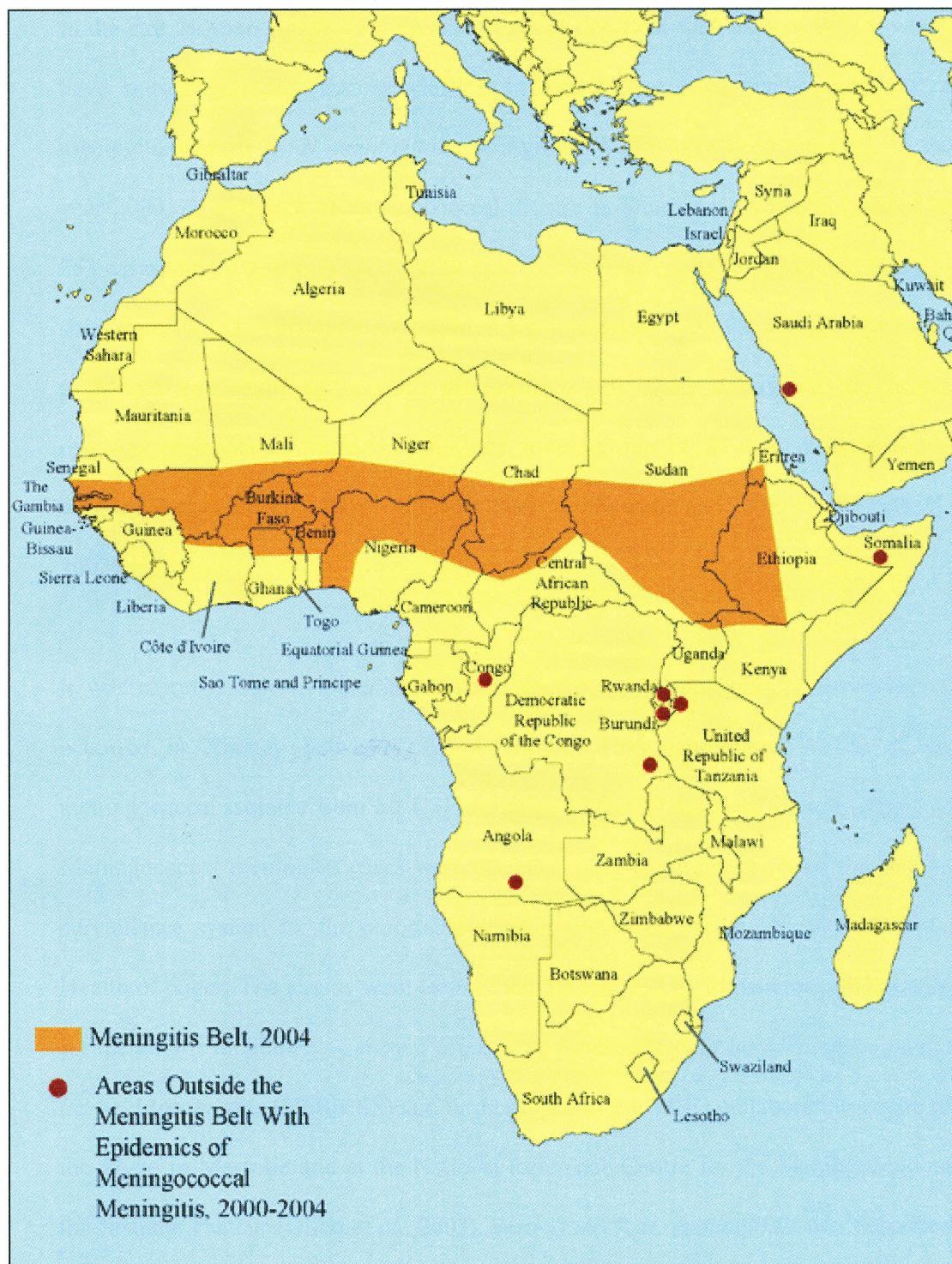


Fig 1: Areas with frequent epidemics of meningococcal meningitis. Adapted from

Traveler's Health Yellow Book: Health information for International Travel, 2005-

2006

In the past 20 years, other sero-groups have been detected, such as X or W135, which have caused sporadic cases or clusters. In recent years, two major epidemics of meningitis caused by *N. meningitidis* sero-group W135 have been reported. In the year 2000, an outbreak of meningococcal disease in Saudi Arabia which resulted in 253 cases and 70 deaths (Case Fatality Rate = 27%) was caused by a virulent clone of sero-group W135. This outbreak occurred simultaneously with the annual pilgrimage to Mecca and returning pilgrims disseminated this clone throughout the world, resulting in secondary cases. In mid-2002, a sero-group W135 meningitis epidemic was reported in Burkina Faso with more than 12,000 cases and 1,400 deaths (Mindy, 2003).

It was reported that 134 meningitis cases caused by *N. meningitidis* sero-group X occurred in Niamey between 1995 and 2000. They represented 3.91% of the meningococcal isolates from all CSF samples, where 94.4% were of sero-group A. Meningococcal meningitis cases were detected using the frame work of the routine surveillance system for the reportable diseases organized by the Ministry of Public Health of Niger. The strains were isolated and determined by the reference laboratory for meningitis in Niamey: *Centre d'Etude et de Recherche pour les Meningocoques et les Schistosomiases* (CERMES) and further typed at the WHO collaborating centre of the Pharo in Marseille and at the National Reference Centre for the Meningococci at the Institute Pasteur (Djibo *et al.*, 2003). Sero-group X *N. meningitidis* was described in the 1960's (Bories *et al.*, 1966., Evans *et al.*, 1968). The sero-group X meningitis had been observed in North America (Sébastien *et al.*, 2002), Australia (Pastor *et al.*, 1985), and Africa (Riou *et al.*, 1995, Gagnex *et al.*, 2000, Etienne *et al.*, 1990),

(Campagne *et al.*, 1999 and Gagnex *et al.*, 2001). In some cases, sero-group X disease has been associated with deficiency of a particular complement component (Swart *et al.*, 1993, Fijen *et al.*, 1996) or with AIDS (Morla *et al.*, 1992):

2.3 The Kenyan scenario

In Kenya, there was meningococcal meningitis outbreak in 1989, which revealed that the causative agent was *Neisseria meningitidis* sero-group A (Pinner *et al.*, 1992). The outbreak of meningococcal disease occurred in Nairobi, Kenya, slightly outside the “meningitis belt” of sub-Saharan Africa. About 3,800 cases were reported between April and November (250/100,000 population). The case fatality rate was 9.4% among hospitalized patients. Areas that included Nairobi’s largest slums had particularly high attack rates. The epidemic displayed an unusual age distribution, with high attack rate in the 20-29 years old. Multilocus enzyme electrophoresis typing (MLEE) demonstrated that the strain responsible for this large epidemic was closely related to strains that caused other recent epidemics in sub-Saharan Africa, documenting further spread of what may be a particularly virulent clonal complex of group A *N. meningitidis* (Pinner *et al.*, 1992). However, sporadic cases of meningococcal meningitis have been occurring in different parts of the country.

In January, 2006 meningitis outbreak was reported in which about 20 people died of suspected meningitis in Kacheliba and Allale Divisions of West Pokot district by (Daily Nation newspaper, 23rd January 2006). A team of technical officers from the Division of Outbreak Monitoring Unit (DOMU), Field Epidemiology Laboratory

Training Programme (FELTP), and Medecines San Frontieres (MSF-Spain) visited the area and assessed the situation. The AMREF Laboratory Programme was called in to assist in making a laboratory diagnosis of the causative organism of meningitis. The AMREF staff also set up methods of collection, storage and transportation of CSF specimens to AMREF Laboratory for further analysis. It was subsequently established that a total of 62 cases had been reported in various health facilities in the District and the disease had claimed 15 lives (CFR16%) in four divisions of Kacheliba, Alale, Kasei and Chepareria as of 16th February 2006 (DOMU Monthly Report, March-April 2006).

2.4 Diagnosis

Meningococcal Antibody Test (MAT) is one of the old tests and has been evaluated for 10 years and is not commonly used. It can be a reliable tool for establishing a diagnosis with suspected meningococcal disease. However, key factors facilitating interpretation of negative as well as positive test results are time of sampling after onset of disease, age of patient and clinical feature such as the humoral immune response in children less than 2 years of age is weaker than in older children and adults (Weis *et al.*, 2005).

Helpful specimens in the diagnosis of meningococcal disease include CSF, blood, aspirate, biopsy specimens, and nasopharyngeal swabs. Meningococcal meningitis is suspected by the clinical presentation and the most common preferred specimen is a lumbar puncture showing a purulent spinal fluid. A rapid presumptive diagnosis of meningococcal meningitis can be made by direct examination of CSF using the Gram

stain. If sufficient CSF – 1 millilitre is received, the specimen should be centrifuged to obtain a pellet of material for examination and for culture. On Gram stained smears prepared from clinical specimens, meningococci appear as gram-negative diplococci both inside and outside of Polymorphonuclear Neutrophils (PMNs) (Murray *et al* 2003). *N. meningitidis* is classified as a biosafety level 2 organism, which means that a biological safety cabinet must be used for the manipulation of specimens that have a substantial risk for the generation of aerosols e.g. centrifugation. Reports of laboratory-acquired meningococcal infections suggest, however, that manipulation of culture rather than patient specimens increases the risk of infection for microbiology laboratory technologist and technicians (Barkus *et al.*, 1995, Billot-Klein *et al.*, 1996).

Direct test for detection of meningococcal capsular polysaccharide from a CSF specimen are also available. These direct antigen tests are antibody-sensitized latex agglutination or co-agglutination to detect capsular antigens of meningococcal sero-groups A, B, C, Y and W135. These reagents are available from several vendors. A negative test does not rule out meningitis caused by any of the organisms that commonly occur (Boisier *et al.*, 2008). For recovery of *N. meningitidis*, CSF specimens should be cultured on non-selective chocolate agar and sheep blood agar. Plates are incubated in 5-10% carbon dioxide atmosphere or candle extinction jar at 35°C and inspected after 24, 48 and 72 hours before a final report of “no growth” is issued. Suspected colonies are sub cultured to blood and chocolate agar for further identification. More specialised laboratory tests are needed for the identification of the sero-groups as well as for testing susceptibility to antibiotics (Murray *et al.*, 2003).

2.5 Treatment of meningococcal meningitis

Meningococcus disease is potentially fatal and should always be viewed as a medical emergency while admission to a hospital or health centre is necessary; whereas patient isolation is not. Antimicrobial therapy must be commenced as soon as possible, after the lumbar puncture has been carried out (if started before, it may be difficult to grow the bacteria from the spinal fluid and thus confirm the diagnosis). A range of antibiotics may be used for treatment including penicillin, ampicillin, chloramphenicol, and the broad-spectrum cephalosporins (ceftriaxone, cefotaxime and ceftazidime) reach levels in CSF that may be several hundreds fold the MIC for the infecting isolate and are also recommended treatment options. Patients with meningitis also require intensive supportive care and monitoring for detection of complications and disease progression (Murray *et al.*, 2003).

As in gonococcus, the antimicrobial susceptibility of *N. meningitidis* strains is also evolving (Rosenstein, N, E *et al.*; 2000). Historically, penicillin-susceptible strains of *N. meningitidis* have penicillin MICs of $\leq 0.6 \mu\text{g/ml}$, and this has been the case in the United States for many decades. Rare β -lactamase-producing meningococcal isolates have been encountered sporadically since 1983 in Canada, South Africa, and Spain; these isolates have penicillin MICs of $>256 \mu\text{g/ml}$. Since 1987, β -lactamase-negative *N. meningitidis* strains with penicillin MICs of $>0.6 \mu\text{g/ml}$ have been isolated in Europe, South Africa and the United States (Rosenstein *et al.*, 2000). Most of the reported relatively resistant meningococci have belonged to either sero-group B or C. Diminished susceptibility to penicillin is apparently due to decreased binding of penicillin by altered meningococcal cell wall penicillin-binding proteins (PBP2 and PBP3). In the case of the PBP2 proteins, decreased binding of penicillin to the altered

binding protein results from a mutation in the nucleotide sequence of the PBP2 gene, *penA*. Similar low-affinity forms of PBP2 are found in penicillin-resistant of the *Neisseria* species including *N. lactamica*, *N. flavescens*, *N. polysaccharea*, and *N. gonorrhoeae* (Bowler *et al.*, 1994).

N. meningitidis have also demonstrated resistance to other antimicrobial agents. High-level chloramphenicol resistance has been reported in isolates from France and Vietnam. Fear of the spread of chloramphenicol resistance is justified since the drug is the mainstay of therapy for meningitis in Sub-Saharan Africa. High-level resistance to sulphonamides, including trimethoprim-sulfamethoxazole, is widespread and is found commonly among epidemic sero-group A *N. meningitidis* strains. Rifampin resistance has also emerged, even during the administration of the rifampin prophylaxis, and is due to alteration in cell membrane permeability or to mutations in the *rpoB* gene coding for the β -subunit of the meningococcal RNA polymerase. In 2000, *N. meningitidis* strain with decreased susceptibility to ciprofloxacin (MIC, 025 μ g/ml) was isolated from a patient with invasive meningococcal disease in Australia (Schultz, T, R *et al*; 2000). Under epidemic condition in Africa, oily chloramphenicol is the drug of choice in areas with limited health facilities because a single dose of this long-acting formulation has been shown to be effective. In the West Pokot outbreak, oily chloramphenicol and ceftriaxone was being used for treatment.

2.6 Prevention of meningococcal meningitis

Real work on meningococcal vaccine started only after emergence of resistance to sulphonamides and penicillin. In the 1960s, polysaccharide vaccines based on group A and C capsule were developed (Rosenstein *et al.*, 2001). There are mainly two

types of vaccine for the protection of meningitis. Meningococcal Polysaccharide Vaccine (MPSV4) – [Menomune by Sanofi Pasteur] was licensed in 1981 for children over 2 years and older. It protects against four subtypes of meningococcus – A, C, Y and W135. A meningococcal conjugate vaccine (MCV4) – [Menactra by Sanofi Pasteur] was licensed in 2005. It also protects against the A, C, Y and W135 subtypes. MCV4 is expected to give protection of about 90%, and longer than the polysaccharide vaccine and it is licensed for use in persons 11 – 55 years of age lasting for about 2-3 years (<http://www.cdc.gov/vaccine/pubs/vis/downloads/vis-mening.pdf> 2008) a CDC publication. The MPSV4 vaccine is made from the outer coat or polysaccharide capsule (sugar coat) of the meningococcal bacteria and does not contain live bacteria. The MCV4 vaccine contains *Neisseria meningitidis* sero-group A, C Y and W135 capsular polysaccharide antigens individually conjugated to diphtheria toxoid protein. The MPSV4 vaccine is given as an injection into the fat of the arm whereas MCV4 vaccine is given in the muscles. The meningococcal vaccines are very safe and have been used extensively in mass vaccination programmes. Vaccination is recommended for other people at increased risk of meningococcal disease; this includes – college students living in dormitories, individuals living with damaged or missing spleen, persons with immune system disorder, persons working with meningococcus bacteria in the laboratories, travellers to certain countries in sub-Saharan Africa and any other who might have been exposed to meningitis during an outbreak. The MPSV4 is 85 – 100% effective at preventing infection from the subtypes of meningococcus found in the vaccine (A, C, Y and W-135). However, MPSV4 and MCV4 does not protect against sero-group B meningococcus which causes about one third of all meningococcus cases in the United States. Meningococcal vaccines may be given to pregnant women but MCV4 is a new

vaccine and has not been studied in pregnant as much as MPSV4 has. It should be used with caution and when clearly needed. Meningococcal vaccines may be given at the same time as other vaccines (<http://www.cdc.gov/vaccine/pubs/vis/downloads/vis-mening.pdf>, 2008) a CDC publication on current vaccines.

In Chile, international investigators performed a prospective, randomised, double-blind trial of two sero-group B outer membrane protein (OMP) meningococcal vaccines to determine if either might assist in controlling an epidemic of sero-group B disease, ongoing in the country since the mid-1980's. Using serum bactericidal activity (SBA) against meningococcal strains the homologous strain from which the vaccine were derived, produced a four-fold or greater increase in SBA in at least 67% of children and adults and at least 90% of infants. However, when tested against heterologous meningococcal vaccine strain or the Chilean epidemic strain, protective responses were elicited in only 10% to 60% of vaccine recipients (Diermayer *et al.*, 1999).

A vaccine against sero-group X has not been developed. In 2008, Biochemical Engineering College of Beijing Union University, China reported first meningococcal meningitis caused by *Neisseria meningitidis* sero-group X strain (Chen, *et al.*, 2008). During the outbreak in China, polysaccharide vaccine for sero-group A plus C was used for prevention and control of the meningococcal meningitis (Gagneux *et al.*, 2002). Gagneux reported that repeated vaccination against sero-groups A and C in many African countries has the potential to select meningococci of other sero-groups (e.g., sero-group X and W135) and might result in a changed profile of meningococcal disease (Chen, *et al.*, 2008). Up to about half of the people who get meningococcal vaccine have mild side effects, such as redness or pain where the shot

is given. These symptoms usually last for one or two days and are more common after the MCV than the MPSV. A small percentage of people who receive the vaccine develop a fever. Serious allergic reactions, within a few minutes to a few hours of the shot are rare. A serious nervous disorder called Guillain Barré Syndrome (GBS) has been reported among some people who received MCV4

(<http://www.cdc.gov/vaccine/pubs/vis/downloads/vis-mening.>, pdf 2008) a CDC Publication.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was a cross sectional-study of a meningitis outbreak that started in January 2006 and ended on the first week of April 2006. The study focuses mainly on patients suffering from meningococcal meningitis whose specimens were transported to the AMREF Central Laboratory for isolation of the meningococcal bacteria. The CSF specimens were collected from West Pokot, Kenya and Warab State, Southern Sudan. Molecular characterization was carried out at the U.S. Naval Medical Research Unit No.3 (NAMRU) in Cairo, Egypt.

3.2 Ethical consideration

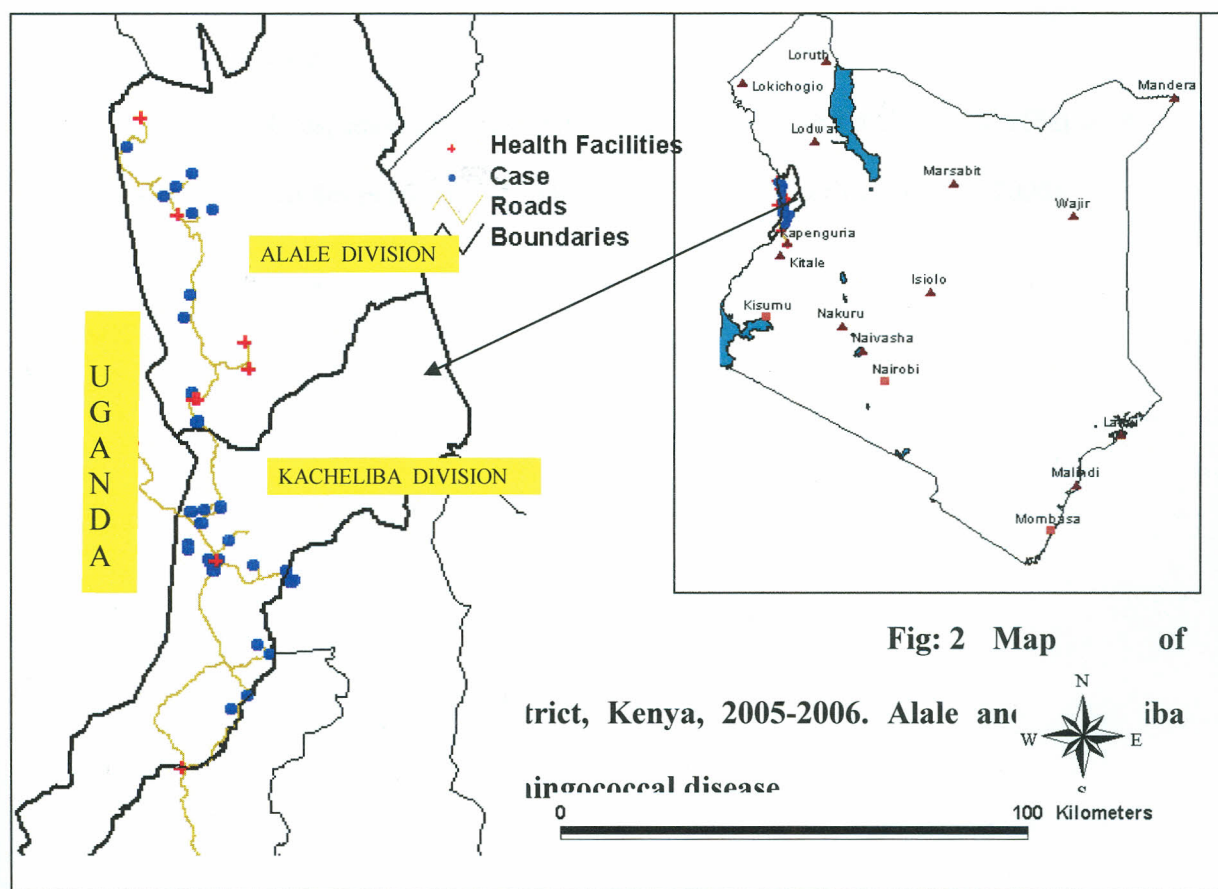
The AMREF Clinical Programme works with other organizations and divisions of the Ministry of Health (MOH) to form an Epidemic Technical Working Group (ETWG). These include; World Health Organization (WHO) – Kenya Country Office, Centers for Disease Control (CDC), Kenya Medical Research Institute (KEMRI), Division of Communicable Disease Control (DCDC) – MOH, Division of Malaria Control Programme (DOMC) – MOH, Division of Health Promotion (DHP) – MOH, Chief Public Health Officer (CPHO) – MOH, Disease Outbreak Monitoring Unit (DOMU) – MOH, National Public Health Laboratory Services (NPHLS) – MOH and Non Governmental organizations (e.g. MSF, IRC, IMC, KRC, ICRC).

Following the meningococcal meningitis outbreak, patients or guardians were not given consent for declaration towards acceptance to their specimens to be used for research purposes. Outbreaks are emergence situation which requires prompt

diagnosis, treatment and control of the disease. The Ministry of Health (MOH-Kenya) gave permission to AMREF to analyze the cerebrospinal fluid (CSF) specimens and to carry out molecular biology characterization, see Appendix 2.

3.3 Study area

West Pokot is one of the districts of the Rift Valley and is about 500 kilometres North West of Nairobi. The population is about 300,000 people and most of them are nomadic pastoralists. The district borders Nakipiripiriti and Moroto districts in Uganda as shown in Fig. 2. The region was badly hit with draught that threatened millions with starvation across East Africa.



From May 2005 through April 2006, Internally Displaced Persons (IDP) in Khartoum, Sudan were returning to their homes in Southern Sudan after the Government of Sudan (GOS) and Sudan People's Liberation Movement (SPLM) signed a Comprehensive Peace Agreement (CPA), officially ending the longest running civil war in Southern Sudan. During this period, a number of health emergencies arose including cholera outbreaks in Yei, Juba, Malakal, Torit, Bor, Bipor and other rural areas of Southern Sudan. The cholera outbreak was followed by meningitis in Wau and Warab States; see Fig 3 on page 21. With the potential for increased numbers of returnees, the spread of the epidemic was of great concern and many health providing and relief agencies responded to both emergencies

(http://www.usaid.gov/our_work/humanitarian_assistance/disaster_assistance;2006) a USAID Publication.

A total of 774 suspected cases including 121 deaths (Case Fatality Rate 16%) were reported from clinics in southern Sudan. (WHO/outbreak verification list; 2006).



Fig 3: Map of Southern Sudan showing the area affected by meningococcal meningitis.

3.4 Sample collection

The CSF specimens were collected from patients that showed typical signs and symptoms of high fever, stiff neck, and sensitivity to light, confusion, headaches and vomiting. The CSF specimen was collected just before the patient was treated with antibiotics. A total of 27 (n) cerebrospinal fluid specimens; eleven from West Pokot, Kenya and sixteen from Southern Sudan were analysed.

3.5 Materials required and method for CSF collection

Materials required for collection of CSF by lumbar puncture is given on appendix 3. This is provided in form of a kit; adapted from (Mindy *et al*; 2003). See Fig 11.

3.5.1 Lumbar puncture procedure

Lumbar puncture was performed by a qualified clinician. The space between the 3rd and 4th lumbar vertebrae was the preferred site for entry into the spinal sac in both adults and children. A patient was positioned to lie down on the left side, with neck, back, hips and knees fully flexed. The 3rd lumbar vertebral spine was identified by drawing a finger vertically down from the posterior superior iliac crest to the midline. The space between the 3rd and 4th vertebral spine was palpated. See illustrations on figure 4A, 4B and 4C. The skin and subcutaneous tissue was infiltrated with local anaesthetic (1% lignocaine), and allowed a few minutes to take effect. The spinal needle (22 gauge in adults; 23 gauge in children) was introduced through the skin in the midline between the vertebrae, carefully keeping the needle at right angles to the skin and pointing slightly towards the head.

The area of skin (Fig 4A) was cleaned aseptically using 70% alcohol then povidone-iodine was applied and allowed to dry and sterile drape was placed under the patient, and one over the patient's back. Fig 4B shows the space between the 3rd and 4th lumbar. Fig 4C illustrates the lumbar puncture needle drawing CSF.

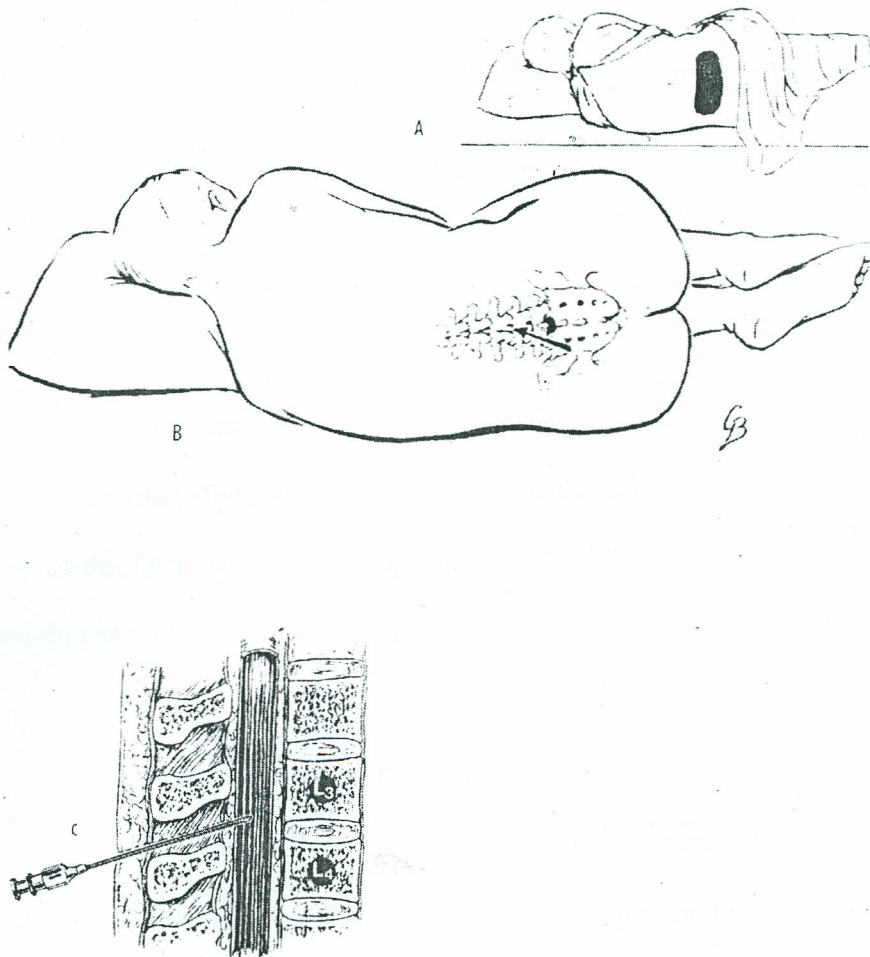


Fig 4: Collection of Cerebrospinal fluid (CSF) by lumbar puncture.

About 1 ml of fluid (less in children) was collected into the sterile test tube. The needle was withdrawn and pressure applied for a few minutes over the puncture wound then the patient was allowed to remain lying down for 1 hour after the procedure. Normal CSF was clear and colourless. Turbid fluid indicated the presence of pus cells and traumatic tap appeared red in colour and this indicated red blood cells due puncture of capillaries within the area of collection.

3.5.2 Transportation of CSF specimens

Trans Isolate Medium (TIM) was used for isolation and transportation of meningococci and other agents causing bacterial meningitis from CSF. A vial of TIM was removed from the refrigerator at least 30 minutes before inoculating it with the specimen. The vial was allowed to warm at room temperature which is more favourable for growth of the organism. Before inoculating, the vial was checked to see if there was any visible growth or turbidity. The vials which showed turbidity were discarded. The small lid of the vial which was in the middle of the metal cap on top of the TIM vial was lifted up, the top of the TIM vial was disinfected with 70% alcohol and allowed to dry for 30 seconds.

A sterile syringe and needle preferably 21G was used to aspirate 0.5ml of CSF from the sterile tests tube and was injected into vial containing TIM. The CSF in the test tubes and Trans Isolate medium was immediately transported at room temperature to the AMREF Laboratory for processing. The CSF specimens were transported in the triple packed container at room temperature in a carton container.

Fig 5. below shows the packages were transported in an upright position and marked: "Handle with care". The boxes were delivered to the laboratory within 24 hours from the time of specimen collection.

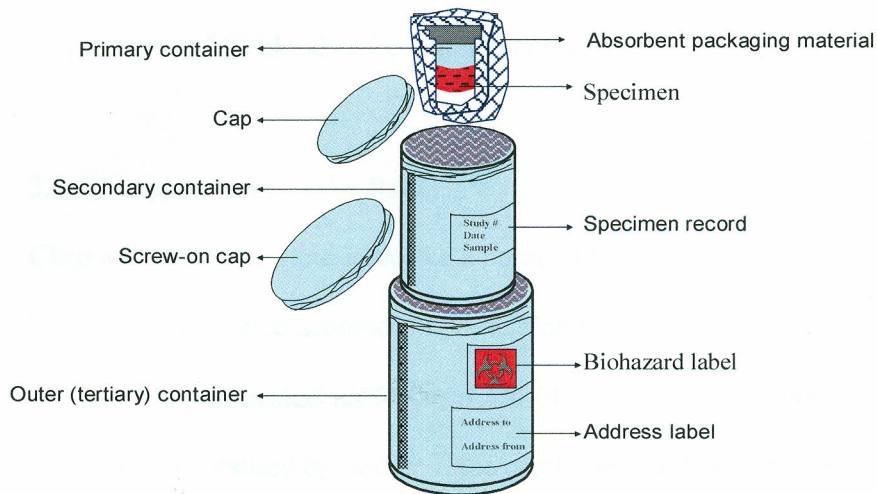


Fig 5: Triple packing of pathological specimens. (Packed in compliance with International Air Transport Association (IATA), packing instruction 650).

Details on packing and shipping of diagnostic specimens following IATA are described on appendix 8. Airway bill for transportation of biological specimens is also illustrated; see figure 13 on appendix 8.

Standard investigations were performed onsite (total white blood cell count, Gram stain, Latex test agglutination).

3.5.3 Laboratory request forms

All laboratory request forms were printed with necessary information, by a clinician and before sending to the laboratory, and by the examiner after obtaining the results. See appendix 3; Fig 12 for details of the laboratory request form.

3.5.4 Microbiological processing of CSF specimens

The following tests were carried out from the CSF specimens in a sterile test tube:

Appearance, white blood cell count, Gram stain and latex agglutination test.

3.5.4.1 Appearance of the fluid

Clear and colourless fluid indicated a normal CSF.

Turbid fluid indicated presence of white blood cells.

Traumatic tap indicated presence of red blood cells while obtaining the CSF. This may have been caused by rupture of the capillaries while inserting the lumbar puncture needle.

3.5.4.2 White blood cell count

The uncentrifuged CSF sample was mixed to make a homogeneous specimen. One drop of CSF was mixed with a drop of 0.1% methylene blue in saline. Improved Neubauer counting chamber was assembled and a fine bore Pasteur pipette was used to fill the chamber with the well-mixed diluted CSF. The counting chamber was placed in a Petri dish containing wet tissue or filter paper for minutes for the cells to settle. The chamber was placed on a microscope and chamber rulings were focused using the X10 objective lens of the microscope. High power objective; X 40 was used to check if white cells present, then the white blood cells were counted in the four

1mm squares at the corners of the chamber and calculated as cells per mm³ or per litre, the technique is explained in Appendix 3. It was important to rule out other type of infection i.e. Trypanosoma (parasites) and *Cryptococcus neoformans* (fungus).

3.5.4.3 Gram stain method

The CSF in sterile test tube was spun in a centrifuge at 3,000 revolutions per minute (RPM) for 5 minutes and supernatant was preserved in another sterile test tube. The deposit was used for making smears for Gram stain and clean slide was labelled with the patient's laboratory number using a grease pencil. A smear was made on the slide by rolling wire loop gently at the centre of the slide and allowed to dry. The smear was fixed by passing the slide (smear uppermost) momentarily over a flame 3 times and was placed on a staining rack over a sink or basin. The smear was covered with 0.5% Gentian violet and left for 1 minute then washed in a thin stream of clean water to remove the excess stain. The smear was covered with Lugol's iodine and left for 1 minute then washed in a thin stream of clean water and decolorized by adding 50% acetone in alcohol solution slowly, one drop at a time, and process stopped as soon as no more blue colour came off the smear. The smear was counterstained by covering with dilute carbol fuchsin and left for 30 seconds and was finally washed in a thin stream of clean water to remove excess stain and allowed to drain dry.

3.5.4.4 Latex Agglutination test

The Pastorex Meningitis kit by Bio-Rad (see appendix 4); allows the direct qualitative detection of antigens to *N. meningitidis* groups A, C, Y/W135, and *N. meningitidis* group B/*Escherichia coli* K1 in cerebrospinal fluid. Supernatants of twenty seven

fresh CSF specimens were subjected to latex agglutination test; eleven from Kenya (West Pokot) and sixteen from Southern Sudan.

All reagents and samples were used at room temperature of 18°-30°C. CSF samples were heated at 100°C for three minutes then left to cool at room temperature. Each sample was tested against *N. meningitidis* group A, B, C and Y/135 latex test reagents. Separate transfer pipettes were used for each test patient sample tested. Latex bottles were mixed gently before use and a drop of CSF reacted with one drop of the latex reagent on agglutination cards and mixed with separate mixing sticks for each different latex reaction. Agglutination cards were placed on a rotator at 100 revolutions per minute (rpm) for 10 minutes and the results were interpreted within the 10 minutes. Positive tests were identified by formation of clumps visible to the naked eye (cross-links or honeycomb patterns). Negative tests appeared as homogeneous suspension, without clumps. For quality control, polyvalent positive control and a negative control were used to confirm the performance of new kits and lot numbers.

3.6 Culturing

The specimens from Trans Isolate media (TIM) were cultured on Chocolate agar (CA) and Blood agar (BA) and incubated under 10% carbon dioxide environment at 35°C for 24 - 48 hours as shown by a flow chart for culture of *N. Meningitides* on Fig 6 - page 30.

3.6.1 Preparation of 5% Sheep blood agar

Tryptone Soy Agar (TSA) was dissolved in distilled water and heated to dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes. The medium was cooled at 45-50°C in a water bath and defibrinated sheep blood was added then mixed gently. Medium was poured in 9 cm sterile Petri-dishes and left to solidify.

3.6.2 Preparation of Chocolate Agar

Tryptone Soy Agar (TSA) was dissolved in distilled water and heated to dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes and left to cool to 60°C. Defibrinated sheep blood at a concentration of 5% was added then mixed gently. The medium was placed in a water bath at 70-80°C for 15 minutes, mixed then gently and cooled at 45-50°C then poured in 9 cm sterile Petri dishes and left to solidify.

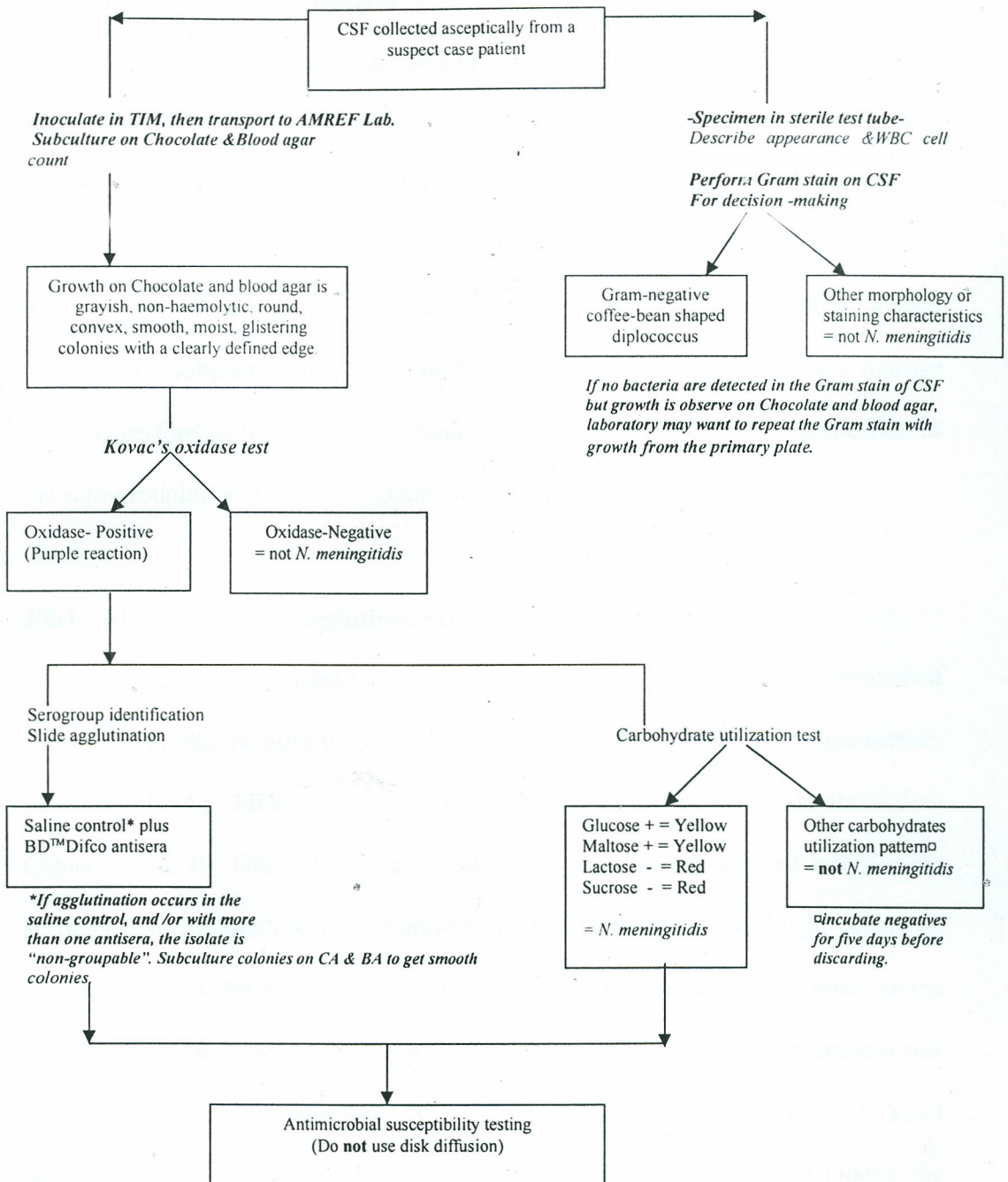


Fig 6: Flow chart for laboratory identification of *Neisseria meningitidis*.

3.6.3 Preliminary identification

After 24 hours of incubation, the CA and BA plates were checked for growth of *N. meningitidis*; most of the plates were re-incubated for further 24 hours at 35°C under 10% carbon dioxide environment. Positive culture; growth on BA or CA is grayish, non-haemolytic, round convex, smooth, moist, glistening colonies with a clearly defined edge. Gram stain; *N. meningitidis* is a Gram-negative, kidney shaped diplococcus. Subcultures were done on CA plates to ensure purity. Kovac's oxidase test was performed from growth on both BA and CA plates and the identification of the sero-grouping with a slide agglutination test was done.

3.6.4 Method for slide agglutination test

Sero-grouping for *N. meningitidis* was performed by the slide agglutination method according to the manufacturer's instruction (BD Difco™ *Neisseria meningitidis* Antisera – USA). Slide agglutination test was carried out under a Biological Safety Cabinet (BSC II). Glass slides were cleaned with alcohol and divided into 3 equal sections (1 inch). Small portion of growth from the surface of an overnight culture on CA plate using a sterile inoculating loop was collected and a moderately milky saline suspension of the organisms was made on a slide and a drop of grouping antisera was added in the following sequence Poly 1: (A, B, C, D); followed by Poly 2: (X, Y, Z) and W135. Separate applicator stick was used for each section while mixing the antisera and organism suspension to avoid contamination across the section of the slide. The slide was rocked back and forth for 1 minute and results read over a black background.

3.6.5 Biochemical tests - Cystine Trypticase Agar (CTA)

Confirmation of *N. meningitidis* was done by using carbohydrate utilization tests. The medium was obtained and the following sugars were added to a final concentration of 1%; dextrose (glucose), maltose, lactose and sucrose. Carbohydrate utilization test was performed on all positive cultures. A small amount of growth from an overnight culture of *N. meningitidis* on BA or CA was collected with a needle (straight wire) and the inoculum stabbed several times in a set of carbohydrate media. Caps of the test tubes were tightly fastened and placed in an incubator at 35°C for 72 hours.

3.6.6 Beta-lactamase test (β -lactamase)

Oxoid β -Lactamase (nitrocefin) *Touch Sticks* code number BR0066A were obtained and used following manufacturer's instruction. The test was performed by touching a colony of the test organism with a stick. When the organism is β -lactamase producing, the end of the stick turns pink-red within 15 minutes.

3.6.7 Antimicrobial susceptibility test

Antimicrobial susceptibility testing of *N. meningitidis* was performed by the Etest[®] method according to the manufacturer's instructions (AB Biodisk, Solna, Sweden), see Appendix 5. The method conforms to Clinical and Laboratory Standards Institute/National Committee of Clinical Standards (CLSI/NCCLS Vol. 25) No1.

Etest[®] requires less technical expertise than Minimal Inhibition Concentration (MIC) testing by dilution methods, and gives comparable results. The results were measured in micrograms per milliliter ($\mu\text{g/ml}$). The agents were penicillin, chloramphenicol, co-

trimoxazole and ceftriaxone (Rocephin). These are the few antibiotics of choice which can penetrate the blood-brain barrier.

3.6.7.1 Method for MIC testing of *N. meningitidis* by Etest[®] antimicrobial gradient strip

Mueller-Hinton + 5% sheep blood agar was used. Using a sterile applicator stick, one to four morphologically similar colonies were touched from Chocolate medium and immersed into tube containing sterile Mueller-Hinton broth and mixed. Turbidity of the inoculum was adjusted to that of 0.5 McFarland turbidity standards. Sterile cotton wool was immersed into the adjusted inoculum, excess fluid removed and inoculated onto entire surface of 9 cm plate; rotating the plate at 60 degrees to ensure even distribution of the inoculum and confluent growth of the bacteria. Inoculum was allowed to dry for 10 minutes. By using sterile forceps, Etest[®] strips were applied on the plate; two strips per plate facing opposite directions. The plates were incubated at an inverted position in 5% CO₂ - candle jar for 18-24 hours at 35°C and dampened cotton gauze was added to create a humid atmosphere. A control of *Streptococcus pneumoniae* ATCC 49619 was run along side the tests.

3.7 Genotyping of *N. meningitidis* isolates by Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed on the 27 – CSF specimens for the diagnosis of *N. meningitidis* (Mackay *et al.*, 2007). The bacterial 23S rRNA gene was amplified from the CSF samples with clinical presentation consistent with acute bacterial meningitis. Primers used are included in Appendix 4.

3.7.1 Extraction of DNA from CSF

Micro-centrifuge tubes were labelled and arranged on a rack. 200 μ L of Phosphate Buffer Saline (PBS) was added into the tubes (1.5ml). An amount of 200 μ L of CSF sample was added into the tube with PBS, whereas insufficient samples were topped up with buffer. 20 μ L Protease added. The tubes were swirled well for 15 seconds and incubated at 56°C for 10 minutes. 200 μ L of 100% ethanol was added and mixed on a vortex machine. The solution was transferred into a spin column and tubes were spun at 8,000 rpm for 1 minute. Content was placed on another column and 500 μ L of Buffer AW1 was added. The column was spun at 8,000 rpm for 1 minute and the filtrate was discarded. Content was placed on another column; and 500 μ L of Buffer AW2 was added. The column was spun at 14, 000 rpm for 3 minutes and filtrate was collected. 100 μ L AE (DNA extract) was added and incubated at room temperature for 5 minutes. Tubes were spun at 8,000 rpm for 1 minute and then stored at -20°C.

3.7.2 Preparation of Master Mix

The master mix was stored in a separate deep freezer (away from any DNA) Sufficient master mix was prepared (positive and negative control were included) because of inaccuracies in pipetting etc. All calculations were recorded on the worksheet; see constituents of master mix on Appendix 4.

3.7.3 Thermocycler programming and starting the reaction

94°C for 5 minutes followed by 72°C for 7 minutes and 4°C until ready to be used. Cycling conditions varies with primers/target. After PCR, samples were frozen at minus 20°C.

3.7.4 Preparation of agarose 1.5%

The agarose gel of 1.5% was prepared in 100 ml 1×TBE. Agarose was dissolved by heating to near boiling for about 3 min in a microwave oven. Gel caster was assembled; and placed on a gel tray and level the surface; a comb was placed in a proper place. Agarose gel was mixed well and poured into gel mould (6 mm thickness) and was left to solidify. The comb was removed carefully to make good wells in the Agarose, and then the agarose was placed into the electrophoresis tank. A work sheet was made for recording. Parafilm was obtained for mixing of dye & specimen for easy application. 2µL blue orange was placed on the parafilm. The Ladder (400-900 bp) was applied on the left side and far right (mix 5µL ladder + 2µL dye). The mixture was applied slowly and gently. A mixture of 8 µL of each PCR product with 2 µL of loading dye was prepared and loaded in the gel. Power was switched on and adjusted voltage to 100V/mA 400/Time 45 minutes after which the power pac was switched off. The gel was removed from the electrophoresis tank and was stained with Ethidium Bromide for 20 minutes. The gel was washed with distilled water for 30 minutes.

3.7.5 Extraction of DNA from bacterial culture of *N. meningitidis*

Micro-centrifuge tubes were arranged on a rack and labelled, and then Phosphate Buffer Saline (PBS) - 200µL was added into 1.5 ml tubes. Microorganisms were heavily inoculated into PBS using sterile (Fisher) loop. The tubes were mixed by vortex machine for 15 seconds. Proteus - 20µL was added and the tubes were swirled well for 15 seconds. The tubes were incubated at 56°C for 10 minutes. 100 µL of 100% Ethanol was added and tubes placed on vortex.

The solution was transferred to a spin column and tubes were spun at 8,000 rpm for 1 minute and filtrate was discarded. Another column was placed and 500 μ L of Buffer AW1 was added to purify the DNA. The column was spun at 8,000 rpm for 1 minute. Another column was placed; and 500 μ L of Buffer AW2 was added to dissolve the DNA material and the column was spun at 14,000 rpm for 3 minutes and filtrate was collected. 100 μ L AE (DNA extract) was added onto the filtrate and incubated at Room temperature for 5min. The tubes were spun at 8,000 rpm for 1 minute and all tubes were store at -20°C.

3.7.6 Molecular characterization by Multilocus Sequence Typing (MLST)

Fragments from seven housekeeping genes were used for typing the extract/template of 11 positive *N. meningitidis* cultures; these are: *abcZ* (putativeABC transporter), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *fumC* (fumarase), *gdh* (glucose-6-dehydrogenase), *pdhC* (pyruvate dehydrogenase), *pgm* (phosphoglucomutase) as given on the MLST website (<http://pubmlst.org/neisseria/>).

3.7.7 PCR product Clean Up

Exo SAP-IT (USB Cooperation) was used following the manufacturer's instructions. Scrupulously clean PCR tubes of 0.2 ml were used. Microcentrifuge tubes were arranged and a mixture for 10 μ l of the sample and 4 μ l of Exo-Sap was prepared. The PCR tubes were covered and run in the thermo cycler for 15 minutes at 37°C and 15 minutes at 80°C and held at 4°C.

3.7.8 Cycle sequencing

Master Mix of the seven genes was prepared followed by a cocktail of 0.5µl cleaned PCR product (1µl for very weak bands for capillary sequencer), 6 µl (4:2 ie. 5 µl 5x Sequencing buffer + 1 µl Big dye version 1.1), 0.25 µl (5pmol) primer (forward and reverse) (stock 20 µM/pmol), 13.25 µl of water to make a total of 20 µl . The PCR tubes were placed in the thermo cycler for 1 min/96°C, 10 s/96 °C, 5 s/55 °C, 4 min/60 °C, 7 min/4 °C and kept at 4 °C.

The PCR tubes were removed from the Thermocycler (4°C), centrifuged for 1 minute at 3,000 rpm and full 20 µL was collected. This enhanced removal of a drop from the cap and clinging on the tube.

3.7.9 Sample preparation for sequencing

CentriSep columns were prepared according to manufacturers instructions. Columns were removed and tapped to ensure all residues are at bottom of columns. 800µl H₂O (grade1) was added and placed on vortex (to make sure there are no air bubbles). The mixture was left for 1 ½ hr on bench at room temperature to hydrate. Water was drained from columns by removing bottom cap and then top cap (press finger to create suction) and left for 10 min. Tubes were spun for 2 mins (750 x g: 3000rpm in 5415 and 4000 rpm in MiniSpin) to remove excess fluid (orientate tubes correctly in centrifuge). Clean 1.5ml eppendorf tubes were labelled with appropriate numbers and sample on columns were processed immediately. 20µl of sample were transferred to column without touching gel surface. Tubes were centrifuged for 2 min (750 x g: 3000rpm in 5415 and 4000 rpm in MiniSpin). The samples were dried in a vacuum centrifuge (approx 30min on high heat) and stored at -20°C until ready for sequencing. The samples were diluted with appropriate volume of formamide.

3.7.10 Setting up the Sequencer

The sequencing machine - AB Applied System Hitachi 3100 Genetic Analyser was turned on to warm for 15 minutes. The microtitre tubes were arranged on a rack so that they can be easily identified. The chilled sample - 15 μ L was pipetted into microtitre tubes for sequence analysis and buffers from the sequencer were replaced with fresh ones. The Sequencing machine was programmed accordingly and the microtitre rack was placed into the Sequencing machine. The programme was re-checked before the machine was started. The Applied System Hitachi 3100 machine is fully automated and sequencing took 4 hours to get the results. The machine stored the results after the process was completed.

3.7.11 Sequence analysis

The sequence data was checked with trace files to confirm sequences and saved in DNASTar (EditSeq and Seqman). Sequences to MLST were compared to database and assigned allele number and sequence types for 7 loci. For any new sequence, the reverse strand was sequenced and submitted for allele assignment. The 7 - loci which showed new ST, were submitted to database for new ST number assignment.

3.8 Data management and analysis

Demographic characteristics of meningococcus meningitis patients were entered and analysed using SPSS 12.0.1 for windows, including characterization and antibiotic responses.

CHAPTER FOUR

RESULTS

4.1 Demographic characterization

A total number of 27 patients, showing clinical signs and symptoms of meningococcus meningitis had CSF specimens collected and sent to the AMREF Laboratory for analysis. Out of these specimens, eleven (11) were from West Pokot, Kenya of which five (5) were female and another six (6) were males. Sixteen (16) specimens were collected from Southern Sudan; of which ten (10) were from males and six (6) from females. The age range was between 1 year and 60 years. The 27 suspected cases of meningococcal meningitis were distributed by age as shown in Table 1 below.

Table 1: Age distribution of subjects with suspected meningitis infection in both Kenya and Southern Sudan on 2006

Age group	Number of suspected patients with meningitis in Kenya and S. Sudan (%0
≤ 10	11 (41%)
≤ 20	8 (30%)
≤ 30	4 (15%)
≤ 40	2 (6%)
≤ 50	1 (4%)
≤ 60	1 (4%)

The above table shows that the majority of patients suspected with meningococcal infection were the age group of children below 10 years followed by the age below 20 years and 30 years.

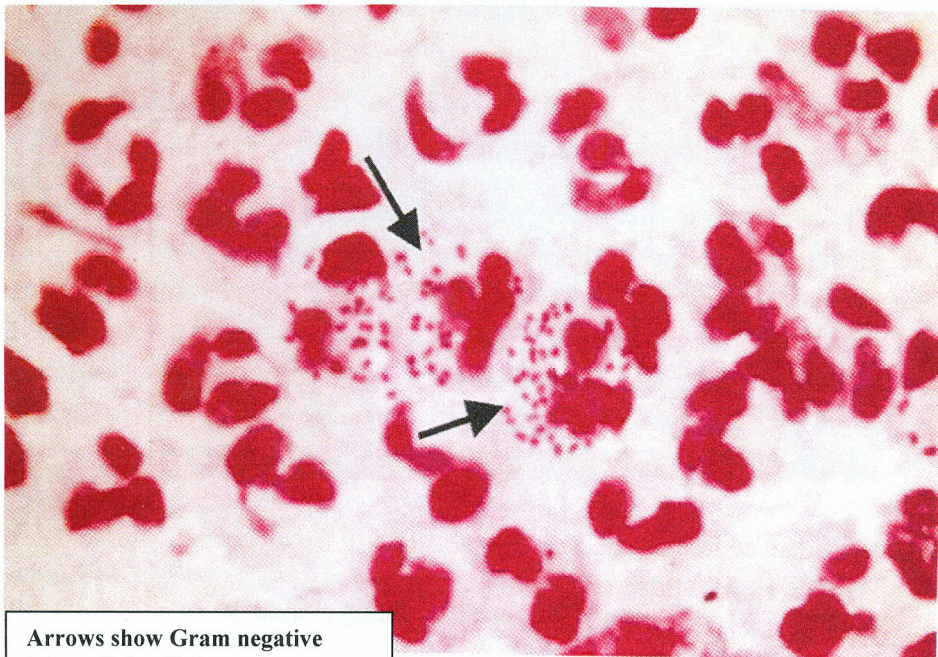
According to the study, out of 11 culture positive cases, majority of confirmed cases were from children below 10 years of age (72%) followed by those below 20 years (19%) and 60 years old (1%) as shown on Table 2.

Table 2: Age distribution for confirmed meningococcal meningitis infection in the study area, year 2006

Age group	Number of confirmed meningitis cases in Kenya and S. Sudan
≤ 10	8 (72%)
≤ 20	2 (19%)
≤ 30	none 0%
≤ 40	none 0%
≤ 50	none 0%
≤ 60	1 (9%)

4.2 Microscopic examination and reporting of Gram stain results

CSF smears stained by Gram stain method and examined systematically using the x100 objective for pus cells and microorganisms as shown on Figure 7 below. Gram stain is used as one of the basic characterization of microorganisms and was reported as: “Many pus cells and Gram negative intracellular and extracellular diplococci seen”. This was a presumptive diagnosis of *Neisseria meningitides*. (Photograph of Gram stain from one of the samples).



Arrows show Gram negative intracellular diplococci

Fig 7: A smear of a clinical specimen (CSF deposit) stained with Gram's method.

4.3 Morphology of colonies of *Neisseria meningitidis* on CA medium.

Growth of *N. meningitidis* on CA plate after 48 hours of incubation under 10% Carbon dioxide atmosphere shown on Figure 8. The colonies were greyish, round convex, smooth, moist, about 2 – 4 mm diameter and glistening with a clearly defined edge.

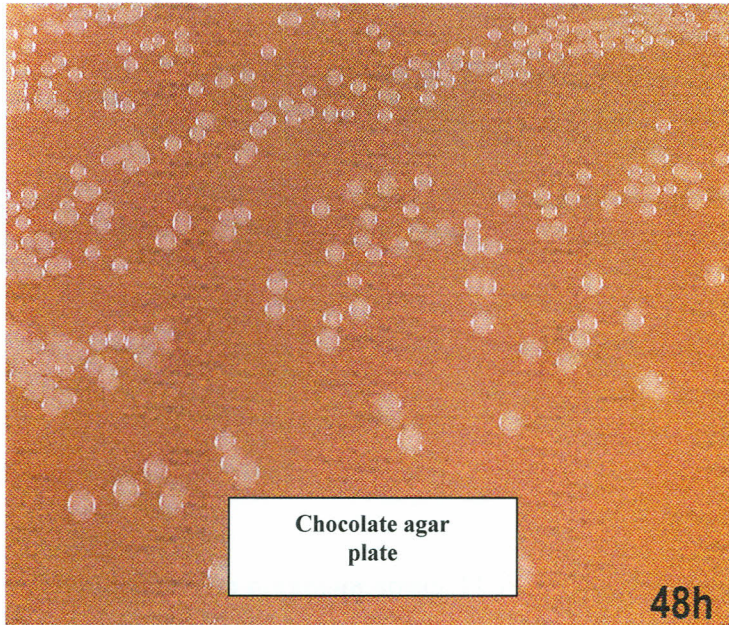


Fig 8: Colonies of *N. meningitidis* on chocolate agar.

4.4 Sero-grouping of colonies of *N. meningitidis*

Sero-grouping was carried out from individual colonies of *N. meningitidis* from CA medium. The colonies were picked and mixed with saline plus appropriate grouping antisera on a card with black background as shown on Fig 9 below.

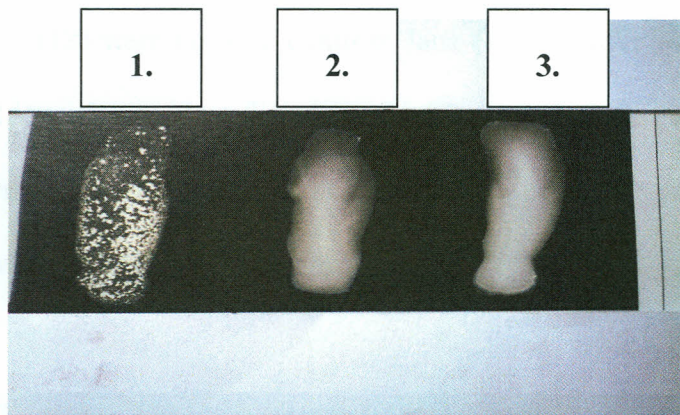


Fig 9: A slide with sero-grouping tests results.

1. Antiserum X with homologous organism strain 11 from Kenya showing agglutination (positive reaction).
2. Distilled water plus strain 11 showing no agglutination (negative reaction); this was a control suspension
3. Antisera A with strain 11 from Kenya remains smooth i.e. does not agglutinate. No reaction is expected with non-homologous antisera.

4.5 Meningococcal meningitis DNA detection

1.5% agarose plates were run for detect PCR products from CSF as show on Figures 10A and 10B below.

Lanes (1) and (20) DNA ladder: The ladder indicates the presence of internal fragments of meningococcal gene which is approximately 45-500 bp.

Lanes (17) and (18) were positive controls, lane (19) negative control and lanes (2) to (16) CSF DNA products. Therefore, lanes No. 2, 3, 4, 6, 7, 8, 14 and 15 were positive CSF specimens for meningococcal meningitis. The positive samples are seen around 450 bp as shown by the ladder (columns 1 and 20)

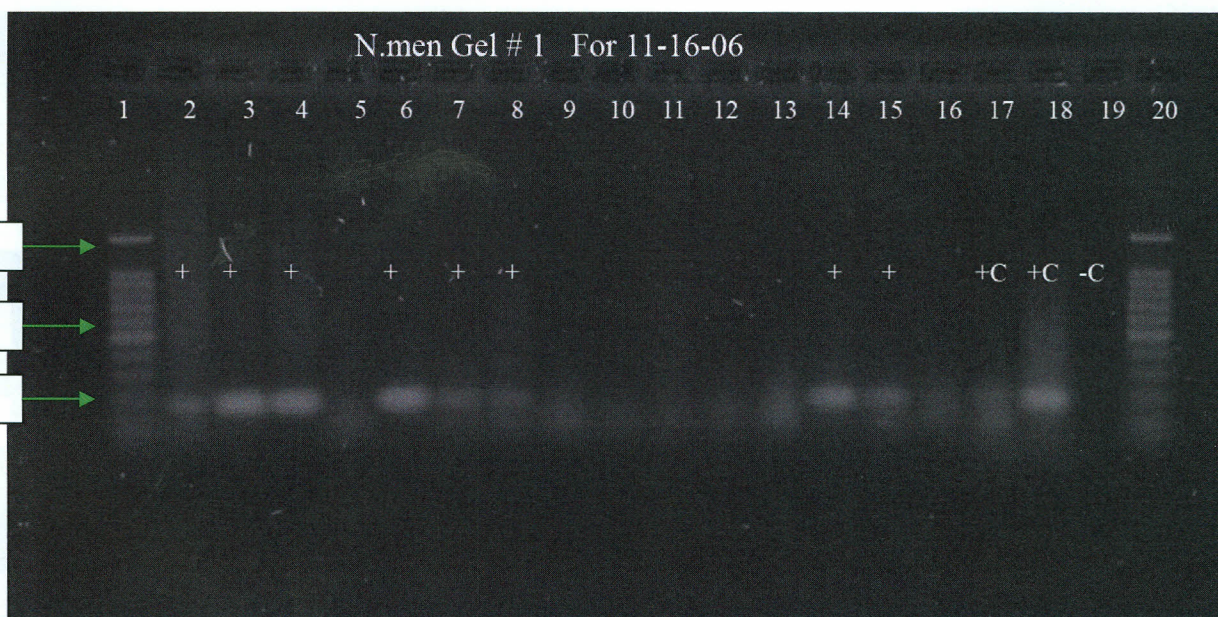


Fig. 10A: Photograph of 1.5% agarose gel for the first 15 CSF specimens for detection of PCR product for meningococcal meningitis.

Fig. 10b below shows lanes (1) and (20) DNA ladder: The ladder indicates the presence of internal fragments of meningococcal gene which is approximately 45-500 bp.

Lanes (15) and (16) were positive controls, lane (17) negative control. Lanes (2) to (13) CSF DNA products. A repeat sample of (13) was placed on lane (14). Lanes 18 and 19 no specimens were added.

Lane 2, 7, and 8 were positive CSF specimens for meningococcal meningitis. The positive samples are seen around 450 bp as shown by the ladder (columns 1 and 20)

Lane 2 has a question mark (?) but was confirmed to be positive.

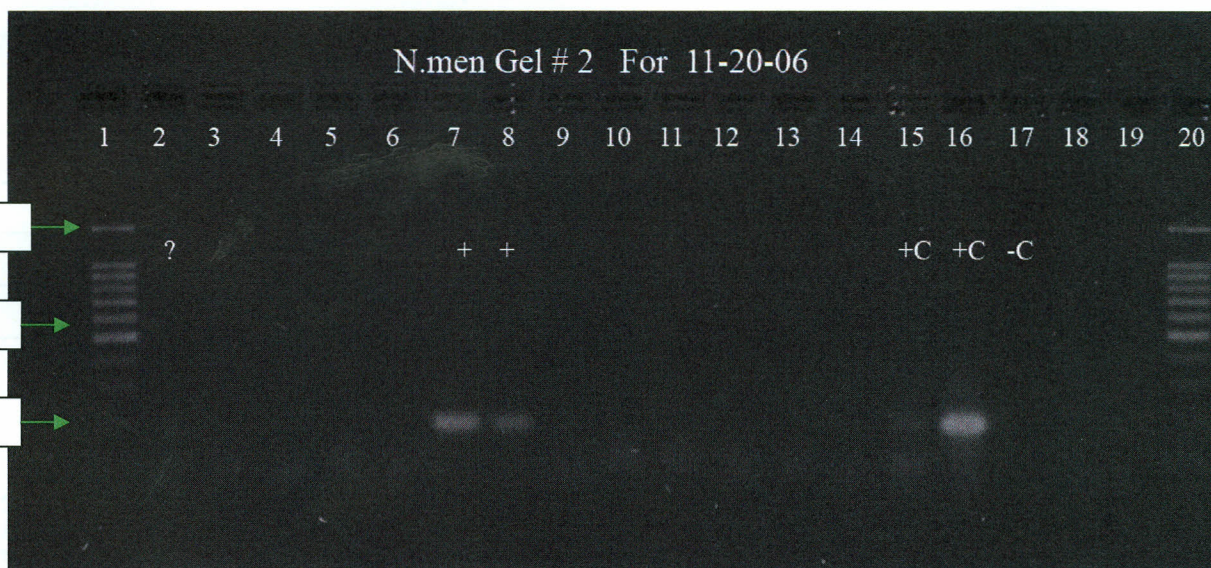


Fig. 10B. Photograph of 1.5% agarose gel for the second lot of 12 CSF specimens on for detection of PCR product for meningococcal meningitis.

4.6 Antimicrobials susceptibility testing

Table 3 shows drug susceptibility test in MIC for the *Neisseria meningitidis* isolated from both Kenya and Southern Sudan. All strains were Beta-lactamase negative i.e. the microorganisms were not producing Beta-lactamase enzymes which catalyses the opening and hydrolysis of the beta lactam ring in some penicillins. The strains from Kenya showed resistance to trimethoprim/sulfamethaxazole whereas the strains from southern Sudan showed intermediate sensitive to the same antibiotic. The two isolates, sero-group A from S. Sudan and sero-group X group from Kenya had similar sensitivity patterns by country. *Streptococcus pneumoniae* strain of ATCC 49619 was used as a control organism for antimicrobial susceptibility test.

Table 3: Antimicrobial susceptibility test results for *N. meningitidis*, year 2006

Site	Study number	Organism	Sero-group	Beta-Lactamase	Antimicrobials			
					Penicillin µg/ml	Chloramphenicol µg/ml	Trimetho/ Sulfa µg/ml	Ceftriaxone µg/ml
S. Sudan	01	<i>N. meningitidis</i>	A	Negative	0.064 µg/ml=S	0.38 µg/ml = S	2 µg/ml = I	0.002 = S
	02	<i>N. meningitidis</i>	A	"	0.064 µg/ml = S	0.25 µg/ml = S	2 µg/ml = I	0.002 = S
	03	<i>N. meningitidis</i>	A	"	0.064 µg/ml = S	0.38 µg/ml = S	1.5 µg/ml = I	0.002 = S
	04	<i>N. meningitidis</i>	A	"	0.064 µg/ml = S	0.38 µg/ml = S	2 µg/ml = I	0.002 = S
	05	<i>N. meningitidis</i>	A	"	0.064 µg/ml = S	0.38 µg/ml = S	1.5 µg/ml = I	0.002 = S
	06	<i>N. meningitidis</i>	A	"	0.064 µg/ml = S	0.38 µg/ml = S	1.5 µg/ml = I	0.002 = S
	07	<i>N. meningitidis</i>	A	"	0.047 µg/ml = S	0.38 µg/ml = S	1 µg/ml = I	0.002 = S
	08	<i>N. meningitidis</i>	A	"	0.047 µg/ml = S	0.38 µg/ml = S	1 µg/ml = I	0.002 = S
Kenya	09	<i>N. meningitidis</i>	X	"	0.094 µg/ml = S	0.25 µg/ml = S	6 µg/ml = R	0.002 = S
	10	<i>N. meningitidis</i>	X	"	0.125 µg/ml = S	0.25 µg/ml = S	6 µg/ml = R	0.002 = S
	11	<i>N. meningitidis</i>	X	"	0.047 µg/ml = S	0.25 µg/ml = S	6 µg/ml = R	0.002 = S
	QC Strain ATCC 49619	<i>S. pneumoniae</i>	-		0.06-1 µg/ml= S	2-8 µg/ml = S	≤ 0.5/9.5-1/19-2/38 µg/ml= S	≤ 0.12-2 µg/ml= S

NCCLS had not validated MICs for *N. meningitidis*; instead I used *S. pneumoniae* ATCC 49619 as a control strain

Note: see page xiv on definition of terms for more explanation.

As shown in table 4 below, the *N. meningitidis* isolates from both Kenya and Southern Sudan were 100% sensitive to penicillin, chloramphenicol and ceftriaxone. Eight isolates from Southern Sudan (72%) showed intermediate resistance to trimethoprim/sulfamethoxazole where as three isolated from Kenya (27%) were resistant to trimethoprim/sulfamethoxazole. This was a major difference on sensitivity patterns of the three isolates from Kenya as compared to the 8 isolates of *N. meningitidis* from S. Sudan.

Table 4: Comparison of drug susceptibility test profile of isolates from Kenya and Southern Sudan, year 2006.

	Antimicrobial agents	Interpretive standards (µg/ml)	Number of isolates	% S		% I		% R	
				Kenya	S.Sudan	Kenya	S.Sudan	Kenya	S.Sudan
1.	Penicillin	S = ≤ 0.06 I = 0.12 – 1 R = ≥ 2	11	100%	100%	0%	0%	0%	0%
2.	Chloramphenicol	S = ≤ 4 I = -- R = ≥ 8	11	100%	100%	0%	0%	0%	0%
3.	Trimethoprim/ Sulfamethoxazole	S = ≤ 0.5/9.5 I = 1/19 – 2/38 R = ≥ 4/76	11	0%	0%	0%	72%	28%	0%
4.	Ceftriaxone	S = ≤ 1 I = 2 R = ≥ 4	11	100%	100%	0%	0%	0%	0%

Interpretation of susceptibility: S = susceptible I = intermediate R = resistant

4.7 Sequence typing results

As shown on Table 5 below, the Sequence Type of the two isolates from Kenya and S. Sudan are different although the two outbreaks occurred at the same time and there was a lot of movement in S. Sudan and Kenya during the same period. Eight isolates of *N. meningitidis* sero-group A of S. Sudan belonged to ST-7 whereas three isolates from Kenya sero-group X belonged to ST-5403.

Table 5: MLST results for *N. meningitidis* from Kenya and S. Sudan, year 2006

Site	Study number	Organism	Sero-group	Sequence Type (ST number)
S. Sudan	01	<i>N. meningitidis</i>	A	ST - 7
	02	<i>N. meningitidis</i>	A	ST - 7
	03	<i>N. meningitidis</i>	A	ST - 7
	04	<i>N. meningitidis</i>	A	ST - 7
	05	<i>N. meningitidis</i>	A	ST - 7
	06	<i>N. meningitidis</i>	A	ST - 7
	07	<i>N. meningitidis</i>	A	ST - 7
	08	<i>N. meningitidis</i>	A	ST - 7
Kenya	09	<i>N. meningitidis</i>	X	ST - 5403
	10	<i>N. meningitidis</i>	X	ST - 5403
	11	<i>N. meningitidis</i>	X	ST - 5403

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Although more than 12 different sero-groups of *Neisseria meningitidis* exist, most disease outbreaks across the African meningitis epidemic belt are caused by sero-group A and, less frequently, by serogroup C and W135 (Nicolas *et al.* , 2005). *Neisseria meningitidis* sero-group X was first described in the 1960s and has been found to cause a few cases of invasive disease across North America, Europe and Africa. In Africa, a small sero-group X outbreak was described in Ghana (9 cases over a 2-year period) and in Niger (134 cases between 1995 and 2000) (Gagneux *et al.*, 2002). Even if outbreaks caused by sero-group X strains are very small in comparison with epidemics caused by sero-group A and W135 strains, the high prevalence of sero-group X carriage among children in northern Ghana (Gagneux *et al.*, 2002) and Niger (Djibo *et al.*, 2004) has been reported, which indicates the need for careful surveillance for this sero-group.

In 2006, however, 51% of 1,139 confirmed cases of meningococcal meningitis in Niger were found to be caused by sero-group X (Djibo *et al.*, 2002). Before 2005-2006 meningococcal epidemic season, no published reports had described sero-group X isolates in East Africa. The meningococcal meningitis outbreak in West Pokot, Kenya is the first outbreak of sero-group X meningococcal meningitis outside the African meningitis belt. The exact number of cases of sero-group X disease remains unknown because the majority of cases did not have a definitive diagnosis through lumbar puncture.

In addition, many cases did not present to health facilities in these remote areas. The clinical characteristics of sero-group X meningitis in this outbreak did not differ from those of sero-group A meningitis outbreak in Africa (WHO, 1998).

In 2005-2006, meningococcus meningitis outbreak in Kenya and Southern Sudan occurred almost at the same time. Internally Displaced Persons (IDP) in Khartoum, Sudan were returning to their homes in Southern Sudan after the Government of Sudan (GOS) and Sudan People's Liberation Movement (SPLM) signed a Comprehensive Peace Agreement (CPA), officially ending the longest running civil war. There was also movement of Sudanese refugees in Kenya. With the potential for increased numbers of returnees, the spread of the epidemic was of great concern and many health providing and relief agencies responded to both emergencies

(http://www.usaid.gov/our_work/humanitarian_assistance/disaster_assistance; 2006); a USAID publication. Despite the traffic, meningococcal sero-group A was not found in Kenya and vice versa. These were two separate outbreaks which occurred at the same time and the affected population was not involved in the movement to these areas.

In Kenya, a large population was affected by the disease but few specimens could be collected. The recovery of the etiologic agent in a few specimens may have been due to delay and inappropriate transportation of specimens to the reference laboratory i.e. using sterile plain test tubes only. Utilization of Trans-Isolate Medium (TIM) for transport and culture of CSF increases more chances of recovery of the etiological agent (Mindy *et al.*, 2003). TIM is used as enrichment medium as well as a holding and transport medium for *N. meningitidis*. Also there has been very poor access to the semi-nomadic populations in Kenya. The case fatality rate (CFR) was 21%, which is

higher than in other African meningococcal outbreaks possibly due to inexperience on handling meningococcal meningitis disease (Mutonga *et al.*, 2008).

Also this might have been due to the extreme remoteness of the area and the lack of clinical experience in handling meningococcal outbreaks, as well as under-detection of milder cases.

The study shows that, most vulnerable age group is ≤ 10 years followed by ≤ 20 and the elderly of 60 years, which is in line with the trend of meningococcal meningitis infections. Most cases of meningococcal disease occur in the first 4 years of life, but the occurrence peaks again during the teenage years (Noah, 1996). The rates of morbidity and of mortality and sequel due to meningococcal disease depend on both bacterial virulence factors and host defence mechanisms, such as the complement system, antibody production, cytokine production and phagocytic killing (Swart *et al.*, 1993., Fijen *et al.*, 1996., Morla *et al.*, 1992 and Sébastien *et al.*, 2002). The last meningococcal meningitis in Kenya was reported in 1989 and West Pokot was not affected (Pinner *et al.*, 1992). Therefore it is possible that the population immunity was naive as none was vaccinated against meningitis.

The sero-groups of isolates from southern Sudan differed from those of Kenya, as it was mainly due to sero-group A, which has been common in the meningitis belt and other parts of East Africa while those of Kenya were sero-group X. The antibiotic susceptibility patterns between the isolates from Kenya and those of southern Sudan differed on one common antibiotic; trimethoprim/sulfamethoxazole. Three (3) Kenyan isolates showed resistance to trimethoprim/sulfamethoxazole whereas (8)

isolates from Southern Sudan showed intermediate resistance. High-level resistance to sulphonamides, including trimethoprim/sulfamethoxazole, is widespread and is found commonly among epidemic sero-group A *N. meningitidis* strains in sub-Saharan Africa (Schultz *et al.*; 2000). In the most recent outbreak caused by meningococcal meningitis sero-group X in China, it was reported that the strain is susceptible to most of the antibiotics (azithromycin, cefotaxime, ceftriaxone, ciprofloxacin, levofloxacin, meropenem, penicillin G, rifampicin and tetracycline). It was however noted that the strain has intermediate resistance to trimethoprim/sulfamethoxazole and ampicillin (Chen *et al.*, 2008).

Examination of CSF by microscopy and neutrophil counts is helpful in differential diagnosis between viral and bacterial meningitis (Abdillahi *et al.*, 1988). However, rapid etiological diagnosis of meningococcal infection and sero-group determination of the clinical isolate are crucial for treatment and institution of adequate preventive measures of its contacts. Culture is the direct means of detecting the etiologic agent of acute bacterial infections. In the case of fragile bacteria such as *N. meningitidis*, CSF should be examined microscopically after Gram staining and cultured fairly soon after lumbar puncture; otherwise, transport media are required (Abdillahi *et al.*, 1988). Bacteriological identification is based on macroscopic and microscopic examination as well as on a limited number of biochemical markers. It is completed by determination of an antibiotic susceptibility (Abdillahi *et al.*, 1988). Rifampicin, Beta lactams, quinolones, chloramphenicol and penicillins are the major antibiotics currently used in the prophylaxis and treatment of these infections. Prophylactic measures (chemoprophylaxis and vaccination, when available) must be applied to contacts of the patient to prevent secondary cases. In such a situation, sero-grouping is

mandatory for the selection of appropriate vaccine. Moreover, long-time follow up at the population level is also required for reliable and effective surveillance of global spread of different genotypes and clonal complexes.

In this study, Multilocus Sequence Typing (MLST) was used to characterize the meningococcus strains. This technique is unambiguous and portable. Materials required for sequence typing can be exchanged within laboratories while primer sequences and protocols can be accessed electronically. It is reproducible and scalable and provides good discriminatory power to differentiate isolates; however is very expensive. MLST was developed to analyze the polymorphism of seven chromosomal genes in *Neisseria meningitidis* that encode metabolism enzymes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC* and *pgm*) (Maiden *et al.*, 1998).

Other techniques that may be used include: Pulse Field Gel Electrophoresis (PFGE) and Multi locus enzyme electrophoresis (MLEE). Despite PFGE being considered by many researchers as the “gold standard”, many strains are not typable by this technique due to the degradation of the DNA during the process (gel smears). The approach of MLST is based on the principle of Multi locus enzyme electrophoresis (MLEE), which is based on different electrophoretic mobilities within multiple core metabolic genes under investigation. MLST is different from MLEE in assigning the allele by nucleotide sequencing rather than the electrophoretic mobility of their gene products. The level of resolution is much higher than MLEE (problem with band resolution). Furthermore, the main drawback of MLEE is that it determines phenotypes and not genotypes. Phenotype of the enzymes can easily be altered in response to environmental conditions and badly affect the reproducibility of MLEE results. So MLEE data obtained by different laboratories is incomparable whereas the

MLST provides portable and comparable DNA sequence data and has great potential for automation and standardization (<http://pubmlst.org/neisseria/policy.shtml>, 2004).

The molecular epidemiology of meningococcal disease is a good example of the usefulness of molecular diagnosis and typing for the agent of one of the most life-threatening communicable infectious disease world wide. The causative agent, *Neisseria meningitidis*, is one of the most versatile bacteria due to its genetic plasticity. Therefore, surveillance and control of epidemics require careful identification and molecular typing in order to adapt any preventive strategy. MLST is a convenient method and the results it provides are easy to compare between different laboratories.

5.2 Conclusions

In conclusion, the study has described the presence of *N. meningitidis* sero-group X in Kenya. Its potential involvement in disease outbreaks, the difficulties it may cause for laboratory confirmation and, consequently, for making an appropriate epidemic response. Due to low population density, poor access to semi-nomadic population, and limited nature of the outbreak (relatively small numbers dispersed over a wide geographic region), obtaining specimens from untreated patients in West Pokot proved difficult. Over the course of outbreak in West Pokot, Kenya; cerebrospinal fluid was obtained from 18 patients. Seven were not included in this study as they were heavily contaminated. From the 11 specimens, 3 yielded pure growth of *N. meningitidis* serogroup X, while no growth was obtained from the remaining 8 specimens. These 3 cultures were subsequently confirmed as sero-group X and Multilocus sequence typing showed that the infecting strain belonged to a new subtype ST 5403 (<http://pubmlst.org/neisseria/>) Martin Maiden. This sequence type is

unrelated to other sero-group X outbreak in Niger, but resembles a sequence type isolated in the United States in the 1970s (Djibo *et al.*, 2003).

Antibiotic susceptibility determination of *N. meningitidis* isolates is important in order to compare different strains to common antibiotics. The study has revealed that meningococcal meningitis sero-group X is becoming resistant to trimethoprim/sulfamethoxazole. Also, the study has shown that the most affected age group within the study area were children less than 10 years.

Meningococcal disease surveillance, management and control clearly illustrates the value of modern molecular techniques in rapid and reliable diagnosis and typing, for accurate case confirmation and strain characterization and for effective epidemiological surveillance and control of this life threatening and potentially epidemic infectious disease. In meningococcal disease, scientists have to be vigilant when carrying out outbreak investigations at different location in order to establish the actual sero-group, which would enhance preventive measures and may also help to rule out cross border issues. In addition to the testing, the strains need to be sent to the WHO collaborating centres for confirmation of sequence type and storage of data. The 3 cultures from the AMREF Laboratory were sent to Oslo, Norway– one the WHO Centre for Meningococci for confirmation and data keeping, see appendix 7.

5.3 Recommendations

- Molecular typing is important epidemiologically for recognizing new outbreak infections, determining the type of vaccine to be used and for tracing the source of infection e.g. cross border epidemics.

- Vigilance needs to remain high on the prevalence of meningococcal meningitis sero-group X as this could necessitate a vaccine as there is none at present.
- More studies are required to i.e. looking for carriers by taking nasopharyngeal swabs and analysing the specimens for meningococcal meningitis sero-group X followed by monitoring the evolution of this clone.
- All new sero-groups of *N. meningitidis* isolated from outbreaks should be sent to the two WHO collaborating centres in Marseilles and Oslo for further typing, confirmation and data storage.
- Scientific companies should device MLST into a single platform technology and make it available and affordable.
- Scientific companies should think of producing a latex test for meningococcal meningitis sero-group X to assist in rapid diagnosis or in case of none recovery of microorganisms on culture media. Latex test for sero-group X and a few uncommon sero-groups is not in the market.
- It is recommended not to use Trimethoprim/sulfamethoxazole for treatment for meningococcal sero-group X in future in case of meningococcal outbreak; as the microorganism is becoming resistant to the drug.

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APPENDICES**APPENDIX 1: Consent explanation**

Following the meningococcal meningitis outbreak, patients or guardians were not given consent for declaration towards acceptance to their specimens to be used for research purposes. Outbreaks are emergence situation which requires prompt diagnosis, treatment and control of the disease. In this study, no names of the patients were tagged to the specimens.

The Ministry of Health (MOH-Kenya) gave permission for AMREF to analyze the cerebrospinal fluid (CSF) specimens and take them to Cairo, Egypt for molecular biology characterization. (See a letter from the MOH on appendix 2).

APPENDIX 2: Letter from the Ministry of Health - Kenya

MINISTRY OF HEALTH



AFYA HOUSE
CATHEDRAL-ROAD
P.O. Box 20781
NAIROBI.

Telephone Nairobi 7117077
When replying please quote

Ref. No. DC/7/5/29/VOL IV (16)

Date: 1st March 2006

Dr. Jane Carter
AMREF
Kenya Country Office
Nairobi

RE: SHIPMENT OF CSF SAMPLES

We acknowledge your request to send CSF (Cerebro spinal Fluid) specimens to Walter Reed reference laboratory in Cairo which were collected from patients following an outbreak of meningitis in West Pokot district.

The Ministry of Health gives you permission to send the 12 CSF samples to Cairo in Egypt for confirmatory testing using PCR method.

Provide feedback results to the Ministry as soon as possible.

A handwritten signature in black ink, appearing to be 'A. O. Misore', written over a horizontal line.

DR. A. O. MISORE
HEAD: PREVENTIVE AND PROMOTIVE HEALTH SERVICES (PPHS)

APPENDIX 3: Materials required for CSF collection by lumbar puncture

- 70% methanol or alcohol swabs,
- Povidone-iodine,
- Lumbar puncture needles (22 gauge/3.5” for adults; 23 gauge/2.5” for children)
- Sterile syringes (5 ml) and needle.
- Sterile test tubes with tight stopper
- Trans Isolate media (TIM),

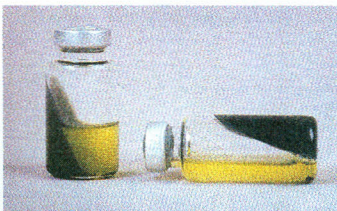


Fig 11: A lumbar puncture Kit showing all the requirements

Alcohol swabs, test tubes, syringe and needle for injecting 2% lignocaine, carriage container, lumbar puncture needle, adhesive plaster and Trans isolate medium; Adapted from (Mindy *et al*; 2003).

APPENDIX 4: Laboratory request forms

Front page of the Laboratory Request form which was filled in by a clinician.

Laboratory Request Form

Name of Patient _____ Age _____ Sex _____

Address/Residence _____

Ward/OPD _____ Registration No. _____

New patient/follow-up _____

Brief Clinical History (please indicate medication, if any)

Specimen and investigation required

Order by (Name) _____

Signed _____ Date _____

Produced and printed by African Medical and Research Foundation
P.O. Box 30125, Nairobi, Kenya.

Fig 12: Laboratory request form

APPENDIX 5: Counting of white blood cells (WBC) in the CSF.

Counting of WBC is performed before spinning the CSF.

- Dilute if cells are too many
ie 1:2 (50 μ L isotonic Toluidine blue and 50 μ L of CSF)
or 1:5.
0.1% Methylene blue could also be used.

Preparation of 0.1% Toluidine blue/Methylene blue

Powder 0.1g
Normal saline 100ml

Dissolve powder, filter then transfer into a reagent bottle. Label.

Toluidine blue

- Stains WBC and not RBCs
- Helps to identify *Cryptococcus neoformans*
- Motility of Trypanosomes is not affected

Diluting fluid, which lyses RBCs such as Turk's fluid, should not be used. It is important to know whether the CSF contains RBCs.

Apparatus

- Improved Neubauer chamber + cover glass
- Pasteur pipette
- Diluting fluid (isotonic Toluidine blue)
- Timer clock
- Wet chamber (two Petri dishes with a we gauze)

Procedure:

- 1) Charge the chamber and insert CSF with a Pasteur pipette.
- 2) Let it stand for 3 minutes for the cells to settle.
- 3) Focus with x 10 and count with x 40 objective.
- 4) Count the cells in 4 of the large squares (the 4 corner squares).
- 5) Calculate the number of cells per mm³ or per L.

Example

If you have counted 200 cells in 4 squares.

$$200 \times \frac{10}{4} = 500$$

OR

$$\frac{500 \text{ cells/mm}^3}{500 \times 10^6/\text{L}}$$

Volume of chamber

$$1\text{mm} \times 1\text{mm} \times 0.1\text{mm}$$

$$= 0.1 \text{ mm}^3$$

$$= \frac{1}{10} \text{ mm}^3$$

$$\text{Volume of 4 square} = \frac{1}{10} \times 4$$

$$= \frac{4}{10} \text{ mm}^3$$

Therefore cells counted in 1mm^3 (1 ml)

$$1 \div \frac{4}{10}$$

$$= \frac{10}{4}$$

$$\text{Cells counted} = \chi \times \frac{10}{4} \text{ cells/mm}^3$$

APPENDIX 6: Composition of Master Mix and DNA extraction reagents.

Master Mix

for one sample	Total # of samples
1. Water	13.75µl
2. Buffer	2.5 µl
3. MgCl ₂	2* µl
4. Primer (For)	0.5 µl
5. (Rev)	0.5 µl
6. dNTP	0.5 µl
7. Taq	0.25 µl

Primers used for PCR

PRIMERS NAME	SEQUENCE 5' → 3'	ORGANISM GENE	AMPLICON SIZE	ACCESSION NUMBER
porA-F ₂	CGG CAG CGT (C/T) CA ATT CGT TC	<i>N. meningitidis</i> por A(porin)	309	AF 239810 (Molling et al., 2000)
porA-R ₂	CAA GCC GCC TTC CTC ATA GC			

Reagents and source

	MATERIALS - REAGENTS	SOURCE	CAT #
1.	Taq DNA Polymerase in storage buffer 10x buffer MgCl ₂ (25mM)	Promega	M2861 Goggle: Taq polymerases
2.	Deoxynucleotide Triphosphate (dNTPs) (100mM) each <i>*to be delivered in on dry ice</i>	Promega	U 1330
3.	Primers	Sigma	
4.	Ethidium Bromide 10µg/ml (EB)	Sigma	E-1510
5.	Agarose (DNA grade)	BioRad	162-0134
6.	Molecular weight markers (ladder) 100bp	BioRad	170-8200
7.	QIAGEN Protease Protease solvent	Ref: to QIAGEN catalog	51106
8.	TBE, 10x Tris-base Boric acid EDTA 0.5M pH8.0	Sigma Sigma	T-6066 B-6768
9.	Gel loading dye + ladder Bromophenol blue Sucrose SDS 0.1M EDTA pH 8.0	Promega	
10.	KIT for buffer and others Buffer AL Buffer AW ₁ Buffer AW ₂ Ethanol Spin column Buffer AE	Qiagen Lysis Washing Dissolving precipitating	1014604 1014795 1014577 1014591

APPENDIX 7: Manufacturer of media and reagents.

- | | | |
|------------------------------|---|---|
| 1. Media | Blood agar
Chocolate agar
Mueller Hinton
Tryptone soy broth
CTA (Cystins Trypticase agar) | Oxoid Limited
Wade road Basingstoke
Hampshire, Enland

Becton Dickinson (BD)
BD Microbiology Systems USA |
| 2. Pastorex | Sanofi Diagnostics Pasteur
France | |
| 3. TIM | Sigma-Aldrich Corp | Fancy Road, Poole – UK |
| 4. Etest [®] strips | AB BIODISK | Dalvagenn 10, S 169 56
Solna, Sweden |
| 5. Meningitidis antisera | | Becton Dickinson Difco™
Loveton Circle
Sparks, Maryland 21152 USA |
| 6. Quality control strains | | American Type Culture Collection
(ATCC)
Parklawn Drive, Rockville – USA |
| 7. Murex Diagnostics | | Central Road Temple Hill
Dartford, Kent – UK |
| 8. Remel Laboratories | | 12076 Santa Fe Drive
Lanexa, KA 66215, USA |

APPENDIX 8: Packing and Shipping of Diagnostic Specimens and Infectious Substances

Preparation for transport

Transportation of infectious specimens were done with care not only to minimize hazards to humans or the environment but also to protect the viability of the suspected pathogens. Transportation of infectious items by commercial delivery system was subjected to international regulations.

The specimens were sent to arrive during working hours to ensure proper handling and prompt plating of the specimens. The receiving laboratory was informed before the specimens were sent.

The AMREF laboratory identified the fastest and most reliable means of transport and should made sure that adequate fund to reimburse costs of transport. The fastest transport service was by air-freight. The following information was communicated immediately to the receiving laboratory: the air waybill number, the flight, and the time and dates of departure and arrival of the flight.

The packages that were shipped by air via TNT were bound to regulations by International Air Transport Association (IATA). The samples were packed in a triple packing device as shown in Fig 4 and transported at room temperature.

An example of document to accompany infectious and diagnostic specimens

WHITE SECTIONS ARE MANDATORY. PLEASE COMPLETE IN CAPITALS AND PRESS HARD.

1. Sender's Account Number

2. Invoice to Receiver **SENDER LIABLE FOR UNPAID CHARGES**
 Cross and provide receiver's account number →
 box or call Customer Service for correct account details

3. Customer Reference (Information you would like on the invoice (if required))

4. From (Collection Address)
 Name: SADIKI FRANK MATERU
 Address: AMREF CLINICAL PROGRAMME
 PO BOX 30125
 LANGATA ROAD NEAR WILSON AIRPORT
 City: NAIROBI Postal / Zip Code: +254
 Province/Region: NAIROBI Country: KENYA
 Contact Name: SADIKI MATERU Tel. No.: +254 699 4619

5. To (Receiver)
 Name: DR G. PIMENTEL
 Address: NAMRU-3
 ON EXTENSION OF RAMSES STREET
 ABBASSIA FEVER HOSPITAL
 City: CAIRO Postal / Zip Code: EGYPT
 Province/Region: CAIRO Country:
 Contact Name: Tel. No.: +2-02-3480312

6. Delivery Address (If different from receiver's address above)
 Name: BASEEM ABDEL MAKSUD
 Address: NAMRU-3
 ON EXTENSION OF RAMSES STREET
 ABBASSIA FEVER HOSPITAL
 City: CAIRO Postal / Zip Code:
 Province/Region: CAIRO Country: EGYPT
 Contact Name: Tel. No.: +2-02-3480312

7. Dangerous Goods (Cross correct box)
 Does this consignment contain any dangerous goods?
 If yes, please call our Customer Service. Yes No

CARRIAGE OF THIS CONSIGNMENT IS SUBJECT TO THE TERMS AND CONDITIONS ON THE REVERSE

Your Signature: *[Signature]* Received by TNT (to be completed by TNT)

Date: (Day/Month/Year) Date: Time:

8. Services (Cross one box only to select a Service)
 Documents Non-Documents
 Special Express
 9:00 Express
 12:00 Express
 Global Express
 Economy Express

8b. Options (Cross boxes)
 Priority
 Priority handling from 20:00 to delivery
 Select one option only for increased compensation in case of loss or damage
 ITLL
 Insured Value up to Euro 40 per kilo subject to conditions of our terms
 Insurance
 Insured Value up to Euro 40 per kilo subject to conditions of our terms
 Currency Please provide insurance details
 Insured Value

9. Special Delivery Instructions (Reserved for your instructions (if required))

10. Goods Descriptions (If dutiable please complete section 11)

General Description Please put full details on commercial invoice	Number of Items	Weight		Dimensions		
		Kilos	Grams	Length	Width	Height
BIOLOGICAL SPECIMEN	2	Kilos	Grams	Centimeters	Centimeters	Centimeters
(SENT AT ROOM TEMPERATURE)		Kilos	Grams	Centimeters	Centimeters	Centimeters
ATA BOXES	Total 2	Kilos	Grams	Consignment subject to volumetric measurement Please refer to our brochure or call Customer Service		

11. Dutiable Shipment Details (Complete for dutiable consignments)
 Receiver's VAT / TVA / BTW / MWST No.

Currency Invoice value of dutiables

SENDER'S COPY
Please keep for Reference

Fig 13: Airway bill for transportation and shipping of diagnostic specimens and infectious substances.

KENYATTA UNIVERSITY LIBRARY

APPENDIX 9: Addresses of WHO Collaborating Centre for Reference and Research on Meningococci.

Before sending the isolates to one of the international reference laboratory (Oslo) for confirmation, the AMREF Laboratory contacted the laboratory prior to the packing and shipping process and obtained information about import permit and acceptance of shipment.

1. Norwegian Institute of Public Health
Geitmyrsveien 75
P.O. Box 4404 Nydalen
N-0403 Oslo
NORWAY

Phone: (+47) 22 04 23 11

Fax: (+47) 22 04 25 18

Attention: Prof. Dominique A. Caugant, Head Norwegian Institute of Public Health.

2. Unité du méningocoque, Centre Collaborateur OMS
(Meningococcal Unit, WHO Collaborating Centre)
Institut de Médecine Tropicale du Service de Santé des Armées
Parc du Pharaon, B.P. 46
F-13998 Marseille-Armées
France

Attention: Dr. Pierre Nocolas, Head OMS.