Meningococcal Disease and Carriage in Greece

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Ph.D.

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1996

Declaration

I declare that this thesis has been composed by myself and that the research reported therein has been conducted by myself or under my direct supervision.

Edinburgh, December 1995

Georgina Tzanakaki

Dedication

To the memory of my father and Dr Lily Mavrommati.

Acknowledgements

I would like to express my gratitude to my dedicated supervisors Prof. Jenny Kourea-Kremastinou, Dr Caroline Blackwell and Prof. Donald Weir. It has been a great experience as well as pleasure to be instructed by them and their enthusiasm and encouragement was invaluable throughout these years. It is thanks to the long hours of patient and productive discussions that the completion of this thesis was made possible. Many thanks for being above all friends as well as supervisors. Thank you...

I wish also to thank my colleagues and also precious friends, Dr Aristea Velegraki and Dr Silva Haralambous for long hours of discussions and all the encouragement throughout these years. My thanks also to Alexandra Tabaki for her excellent technical assistance.

I am also grateful to Prof. Nikos Vakalis for making the recruit study possible with all the official military arrangements.

A different kind of feelings and thanks I wish to express to my supporting family. My mother and both my sisters Katerina, Vana and their families. Their love and emotional support was more than essential.

Last, but most least, I would like to thank Dimitris, my beloved husband and a great friend, for standing by me all these years with love, patience and understanding. Thank you Dimitris...

Abstract

Disease due to *Neisseria meningitidis* can kill a healthy child or a young adult within few hours from the first symptoms of illness. Following the epidemic of meningitis during the 1960's, the incidence of meningococcal disease in Greece declined steadily until 1988. From 1988, the number of cases reported to the Ministry of Health increased; in the first 6 months of 1990, there were 102 cases compared with 87 for all of 1989. This increase was of concern to the public health authorities. The last major survey of meningococci in a Greek population was published in 1979 before elucidation of some of the genetic and environmental factors affecting carriage and development of disease and before the availability of serotype and subtype reagents used in epidemiological studies of these bacteria in north-west Europe and the Americas.

The objectives of the thesis were to answer the following questions:

1. Are genetic and environmental factors associated with carriage among Greek children and young adults similar to those found in north-west Europe?
2. Are strains with serogroups, serotypes and subtypes associated with disease in north-west Europe isolated from patients and carriers in Greece?
3. Are the antibiotic sensitivities of meningococci isolated from patients and carriers in Greece similar to those observed in north-west Europe?
4. Are the genetic clones associated with disease in north-west Europe present among meningococcal strains in Greece and other Balkan countries?

Among military recruits and primary and secondary school children active smoking or exposure to cigarette smoke were significantly associated with carriage of meningococci. In both populations the rate of carriage was higher among the 15-19 year age range; similar results were reported for studies in

Britain and the Faroe islands. The results obtained from the epidemiological studies (recruits and schoolchildren) showed that viral upper respiratory tract infection in general is not a predisposing factor for colonisation and that specific viral infections (*e.g.*, RSV and influenza) need to be investigated. Lower socio-economic group was not associated with carriage in Greek school children but with smoking habits of members of the house closely involved in child care.

While the subtype reagents were able to differentiate strains from both patients and carriers, the serotype antibodies did not react with the majority of strains. The serogroup, serotype and subtype combinations associated with outbreaks in north-west Europe were not found among over 500 isolates examined. The studies on meningococcal strains isolated from patients in Greece and Romania were the first to identify significant phenotypic and genetic differences between meningococcal strains isolated in north-west and southeast European countries. These results indicate that there is a need to develop new reagents for epidemiological work in eastern Europe. These findings have important consequences for the future development of vaccines against serogroup B since major efforts are directed towards production of vaccines based on the serotype/ subtype antigens.

Penicillin-insensitive isolates accounted for nearly half those isolated from children with meningococcal disease (46%) and there was a higher proportion of these among carrier isolates (19.1% from schoolchildren 36.7 % from recruits) than that found in Britain (3%).The identification of increased levels of penicillin insensitive isolates in Greece probably reflects the lack of control of antibiotic usage. The intermediate value of 20% for Romania might reflect changes in prescribing patterns or availability of antibiotics following political changes since 1989.

This work provided a major basis for monitoring meningococcal disease in Greece and in other Balkan countries and resulted in the establishment of a National Meningococcal Reference Laboratory at the National School of Public Health.

Table of Contents

Abbreviations

anti-Le	anti-Lewis antibodies
BSA	Bovine serum albumin
CA	Chocolate agar
CF	Cefaclor
CFR	Crude case-fatality rate
CFU	Colony forming units
CNS	Central nervous system
CP	Ciprofloxacin
CPS	Capsular polysaccharide
CSF	Cerebrospinal fluid
EDTA	Ethylenediaminetetra- acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Erythromycin
ET	Electrophoretic type
Hib	*H. influenzae* type b
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	ImmunoglobulinM
IT	Immunotype
kDa	kiloDalton
Le	Lewis
LOS	Lipooligosaccharides
LPS	Lipopolysaccharide
M.I.C.	Minimum Inhibitory Concentration
mAbs	Monoclonal antibodies
MNYC	Modified New York City medium
NG	Non-groupable
NT	Non-typable
OMCs	Outer membrane complexes

OMP	Outer membrane proteins
PBS	Phosphate buffered saline
PN	Penicillin G
PS	Polysaccharide
RCUT	Rapid carbohydrate utilization test
RF	Rifampicin
RFLP	Restriction fragment length polymorphism
Rif^R	Rifampicin resistant
RSV	Respiratory syncytial virus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	Secretor gene
SU	Sulphamethoxazole
TC	Tetracycline
TMB	3,3',5,5 Tetramethyl-benzidine
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl)- aminomethane
TSB	Trypticase Soy Broth
URTI	Upper respiratory tract infections
VR	Variable region
w/v	Weight / volume
WCE	Whole-cell ELISA

1. General Introduction

1.1. Meningococcal disease

Disease due to *Neisseria meningitidis* can kill a healthy child or a young adult within a few hours of the first symptoms of illness; consequently, it has been a major focus of investigation among scientists and clinicians working in infectious diseases. Despite recent advances in understanding the pathogenesis and immunobiology of the meningococcus and the development of effective vaccines against certain meningococcal serogroups, serious infection with this pathogen remains worldwide health problem.

1.2. History of meningococcal disease

The history of meningococcal disease from its first recognition has been reviewed by Apicella (1990). Epidemic cerebrospinal meningitis was first clearly described by Gaspard Vieusseux in 1806 as a disease that had raged in Geneva during the spring of 1805. A clinical syndrome of meningococcal meningitis was also described by Elias Mann and Lothario Danielson from a small town in Massachusetts in 1804. The epidemic form of cerebrospinal meningitis and recognition of this disease was later described by others, (Hirsch, 1886; Debre and Netter 1911; Dopter, 1921). The discovery of the causative organism that led to the true differentiation of this disease from other forms of meningitis was made by Weichselbaum in 1887 who named this organism *Diplococcus intracellularis meningitidis* because the cocci that he found were in the leukocytes in pus from meningitis patients. The later names pseudo-meningococcus (Kutcher, 1906) and parameningococcus (Dopter, 1914) were regarded as synonymous. The Society of American

Bacteriologists (Bergey, 1926) renamed this species *Neisseria intracellularis* in the 1926 edition of Bergey's manual.

Kiefer (1896) and Albrecht and Ghon (1901) found that healthy persons could become carriers of the meningococcus. Serotypes of the meningococcus were first recognized by Dopter in 1909. This laid the basis for serum therapy in the treatment of meningococcal infection by Flexner in 1913. Glover was the first to note that the carrier rates in military recruitment camps rose with periods of crowding, and he believed they were associated with an increased incidence of cases. In 1928-1930 as well as in 1941 significant national and worldwide epidemics occurred.

In 1937, sulfonamide therapy radically altered the outcome of meningococcal infection and replaced serum in its treatment . Prophylaxis with sulfonamides eradicated the carrier state and provided a simple and safe method for the prevention of epidemics, particularly in the crowded environments of military barracks. Increasing sulfonamide resistance among meningococci was recognized in 1941-1943 by Schoenback and Phair but did not become a clinically significant problem until the meningococcal epidemics in 1963 in two military bases in California (Gauld *et al.,* 1965, Bristow *et al.,* 1965). With the subsequent world-wide emergence of resistant strains and with the absence of effective chemoprophylaxis, renewed interest in immunoprevention led to the development of safe and effective vaccines against the groups A, C, Y and W-135 meningococcal serogroups for older children and adults (Artenstein *et al.,* 1970, Peltola *et al.,* 1978).

Many problems still exist in understanding the pathogenesis, prevention and treatment of meningococcal infection. These include the susceptibilities of certain populations to this infection, its sporadic epidemic nature, the mechanisms responsible for carrier eradication by antibiotics, the reasons for the fulminant nature of the infection, the poor immunogenicity of the group C vaccine in children under the age of 2 years and the inability of humans to

develop effective long lasting antibody to the group B polysaccharide vaccine. Until these and many other questions are answered, meningococcal infections will continue to be a scourge among human populations.

1.3. Characteristics of *N. meningitidis.*

1.3.1. Culture and identification of *N. meningitidis.*

N. meningitidis is a Gram-negative diplococcus of approximately 0.6 x 0.8 μ in diameter. The adjacent sides are flattened to produce the typical kidney bean shape. Because the organism tends to readily undergo autolysis, considerable size and shape variation can be seen in older cultures. The organism is considered fastidious in its culture requirements, thus necessitating the use of appropriate media and growth conditions. Problems in reliable culture of these bacteria include nutritional factors and the presence of substances toxic to the meningococcus in the medium. On solid media, the meningococcus grows as a transparent, non-pigmented, non-hemolytic colony approximately 1-5 mm in diameter. Colonies are convex and if large amounts of polysaccharide are present, they will appear mucoid rather than smooth. Optimal growth conditions are achieved in a moist environment at 35-37^0 C in an atmosphere of 5-10% carbon dioxide. The organism will grow well on a number of media bases including blood agar base, trypticase soy agar, supplemented chocolate agar and Mueller-Hinton agar. Confirmation of the identity of this organism is dependent on carbohydrate utilization (Table 1.1). The meningococcus will metabolize glucose and maltose without gas formation but fails to metabolize sucrose or lactose. Indole and hydrogen sulfide are not formed. In addition, the organism contains cytochrome oxidase, which oxidizes the dye tetramethylphenylenediamine (TMPD) from colorless to deep pink.

Table 1.1. Carbohydrate utilization of various *Neisseria species*

	G	M	S	L	F
N. gonorrhoeae	+	-	-	-	-
N. meningitidis	+	+	-	-	-
N. lactamica	+	+	-	+	-
N. subflava	+	+	-	-	+
N. perflava	+	+	+	-	+
N. sicca	+	+	+	-	+
N. flavescens	*	*	-	-	-
Brahamella catarrhalis	-	-	-	-	-

G= Glucose, **M**= maltose, **S**= sucrose, **L**= lactose, **F**= fructose,

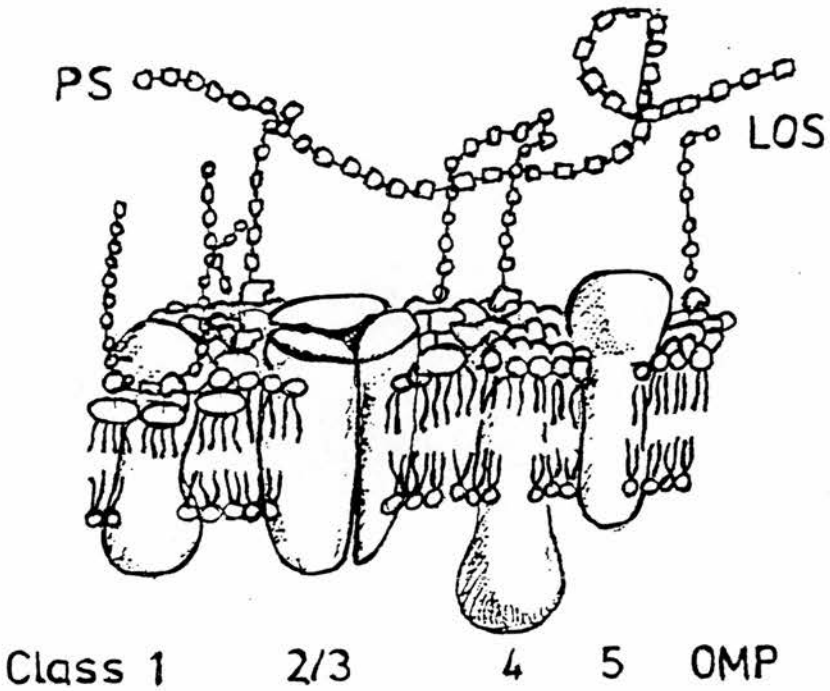
1.3.2. Antigenic structure of the meningococcus

The antigenic characteristics of meningococci have been of value in examining pathogenesis of the disease, development of classification systems for epidemiological studies and development of vaccines. *N. meningitidis* possess a typical Gram-negative cell envelope consisting of a cytoplasmic membrane, a peptidoglycan layer and a cell membrane (outer membrane) containing lipooligosaccharides (LOS) and proteins (Figure 1.1). Many meningococci bear a polysaccharide capsule and surface appendages known as pili (Davis *et al.,* 1980, DeVoe *et al.,* 1974).

Pili are hair-like surface appendages which play an important role in the attachment of *N. meningitidis* to the mucosal surface of the nasopharynx. They show marked intra- and inter-strain variability, both quantitatively and qualitatively. The majority of meningococci isolated from patients with meningococcal disease produce a polysaccharide capsule which is considered to be a major virulence property because it allows the meningococcus to evade host defense mechanisms and to survive in the bloodstream (Quagliarello *et al.,* 1992). Directly underneath the capsule lies the outer membrane which contains a number of proteins and lipooligosaccharides (DeVoe *et al.,* 1982, Zollinger *et al.,* 1977, Poolman *et al.,* 1982). Based on their molecular weights, the major outer membrane proteins (OMPs) are categorized in to five distinct classes (Tsai *et al.,* 1981) (Table 1.3)

The lipooligosaccharides (LOS), also known as endotoxins, form a major constituent of the meningococcal outer membrane (Poolman *et al.,* 1980(a), Griffiss *et al.,* 1988). They play an important role in the development of endotoxic shock (Morrison *et al.,* 1983).

Figure 1.1. Schematic presentation of outer membrane structure



PS : Polysaccharide

LOS : Lipooligosaccharide

OMP : Outer Membrane Proteins

1.3.3. Variation in surface antigens and their application to strain differentiation

The principle antigens that have been used for strain differentiation are the capsule, LOS and the outer membrane proteins.

1.3.3.1. Capsular antigens and serogroups.

Shortly after identification of the meningococcus as the etiologic agent in epidemic meningitis and recognition of the carrier state, methods were developed for detection and differentiation of meningococci. It became apparent that antigenically diverse meningococci existed. Because different laboratories were involved, a complex nomenclature evolved that was not resolved until the mid-1950s when serogrouping based on capital letters simplified the epidemiological investigations. (Apicella 1990).

The capsular polysaccharide (CPS) antigens are the basis for classification of *Neisseria meningitidis* into serogroups (Frasch *et al.,* 1985). At present, there are 12 known serogroups: A, B, C, X, Y, Z, 29E, W-135, H, I, K and L (Ashton *et al.,* 1983, Evans *et al.,* 1968, Shao-Qing *et al.,* 1972, Slaterus *et al.,* 1961). The capsules consist of a high molecular weight anionic polysaccharide and are found in strains isolated from the blood and cerebrospinal fluid of patients with meningitis. Uncapsulated strains rarely cause disease. Serogroups A, B, and C have been shown to constitute greater than 90% of the isolates from patients, with group B alone accounting for 50-70% of the cases. Studies on the structure of these capsules began as early as 1933 (Bhattacharjie *et al.,* 1975; Bhattacharjie *et al.,* 1976; Gotschlich *et al.,* 1981; Jennings *et al.,* 1978; Liu *et al.,* 1971 a, b). The results of these studies are partly summarized in Table 1.2.

With the exception of those of group B, all purified CPS with a molecular weight of 100 kDa or more have been found to be immunogenic. (Gold *et al.,* 1977; Jennings *et al.,* 1981; Wyle *et al.,* 1972). Purified, high molecular weight group B capsular polysaccharide is poorly immunogenic in humans (Wyle *et al.,* 1972). Its structure is identical to that found in the capsule of *Escherichia coli* K1, (Kasper *et al.,* 1973), a major cause of neonatal meningitis, suggesting a common mechanism by which these polymers avoid stimulating the host defenses.

Table 1.2. Structure and composition of the meningococcal capsular antigens of group A, B, C, Y, and W135.

Serogroup	Structure	Linkage
A	Linear homopolymer of N-acetyl-mannosamine phosphate	alpha 1- 6
B	Linear homopolymer of N-acetyl-neuraminic acid	alpha 2-8
C	Linear homopolymer of N-acetyl-o-acetyl-neuraminic acid [1]	alpha 2-9
Y	Linear copolymer of glucose-N-acetyl-o-acetyl-neuraminic acid	alpha 2-6
W-135	Linear copolymer of galactose-N-acetyl-neuraminic acid[2]	alpha 2-6

[1] Also o-acetyl negative strains

[2] sometimes o-acetyl positive strains

(Bhattacharjee *et al.,* 1975;1976 ; Jennings *et al.,* 1981)

The serogroups H, I, K and L have never been isolated from patients (Lambert *et al.,* 1991). Meningococci that do not have a capsule are classified as non-groupable if they do not react with any of the serogroup reagents, or autoagglutinable if they spontaneously agglutinate in the absence of antibodies.

1.3.3.2. Outer membrane proteins, serotypes and subtypes.

Meningococcal outer membranes contain from two to five major proteins which can be demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Using chymotryptic I^{125} peptide mapping, it was found that each of the major outer membrane proteins of one strain had different peptide maps; proteins of approximately the same molecular weight from different serotypes were structurally related. The unique portions of peptides were in the hydrophilic moieties, whereas the common peptides shared among the strains, were in the hydrophobic parts of the protein (Tsai *et al.,* 1981). The major outer membrane proteins (OMPs) are therefore categorized into five different structural classes according to their apparent molecular weights , designated as classes 1 through 5. All meningococcal strains have either class 2 or class 3 principal OMP. The class 2/3 proteins have variable hydrophilic regions which are surface exposed and are responsible for the serotype specificity (Frasch *et al.,* 1973; Frasch *et al.,* 1976; Poolman *et al.,* 1980a). These exist as trimers and function as porin membranes (Lynch *et al.,* 1984). The class 1 outer membrane protein is surface exposed and is shared by all serogroups and types but shows quantitative variability in expression (Frasch *et al.,* 1985). The class 5 OMP shows interstrain and intrastrain heterogeneity. A single strain can express one, two or no class 5 proteins; the variability is not only quantitative but also qualitative (Poolman *et al.,* 1980b). The class 4 protein is present in all strains in association with the class 2/3 porin; it appears to be highly conserved (Frasch *et al.,* 1985). Table 1.3 summarizes the characteristics of the major cell surface OMP classes of *N. meningitidis.*

Table 1.3. Characteristics of the major cell-surface outer-membrane protein classes of *N. meningitidis.*

Class	Molecular weight	Properties of protein
1	44,000-47,000	Trypsin sensitive, not found in a few strains, quantitatively variable, deoxycholate insoluble
2	40,000-42,000	Deoxycholate insoluble, trypsin resistant, exists as a trimer, functions as a porin, quantitatively predominant.
3	37,000-39,000	Similar to the class 2 protein, class 2 and 3 proteins mutually exclusive.
4	33,000-34,000	Trypsin resistant, found in all strains, 2-mercaptoethanol modifiable.
5	26,000-30,000	Heat modifiable, deoxycholate soluble, very sensitive to proteolytic enzymes, highly variable in expression and MW, more than one class 5 protein may be simultaneously expressed

Antigenic variations in the class 2/3 OMPs and class 1 OMPs form the base for serotyping (Table 1.4) and subtyping (Table 1.5) respectively.

Table 1.4. Outer membrane protein 2/3 in relation to serotypes

Surface class	Designation Serotype
Class 2 OMP	1
	2a
	2b
Class 3 OMP	4
	14
	15
	16

It has demonstrated that the class 1 OMP harbors 2 variable regions (VR1 and VR2), each determining a distinct set of subtypes (Van der Ley *et al.,* 1991, McGuinness *et al.,* 1990). The subtypes that have been recognized to date are determined by epitopes, either of VR1 or VR2 (Table 1.5) Meningococci that have a class 1 OMP can be characterized by a combination of subtypes of the two different variable regions. Well known subtype combinations are P1.7,1 and P1.7,16. Subtype P1.5 of VR1 is often found in combination with P1.2 of VR2 (P1.5,2) (Abdillahi *et al.,* 1988, Kayhty *et al.,* 1989a; Wedege *et al.,* 1990; Ashton *et al.,* 1991; McGuinness *et al.,* 1991).

Table 1.5. Correlation between class 1 protein variants and subtype.

Variant	Molecular weight	Subtype
VR-1	34,000	P1.5 P1.7 P1.12
VR-2	35,000	P1.1 P1.2 P1.3 P1.4 P1.6 P1.9 P1.10 P1.14 P1.15 P1.16

Serotypes and subtypes are designated by an Arabic number, but the subtype number is preceded by the prefix "P1". Both serotyping and subtyping can be performed in a whole-cell ELISA using monoclonal antibodies (mAbs) (Abdillahi *et al.,* 1987). Some meningococci, however, do not react with the currently available set of mAbs and they are labeled non-typable.

1.3.3.3. Lipooligosaccharides (LOS) and immunotypes

In 1985, a scheme was proposed for the characterization of *N. meningitidis* isolates (Frasch *et al.,* 1985) based on a combination of serogroup, serotype, subtype and lipopolysaccharide (LPS) serotype. The name of the last of these was changed to lipooligosaccharide (LOS) immunotype (IT) (Tsai *et al.,* 1987). Mandrel and Zollinger were the first to identify eight serologically distinct LOS classes (immunotypes L1 to L8) among group B and C meningococci by hemagglutination and solid-phase radioimmunoassay inhibition (Mandrell *et al.,* 1977; Zollinger *et al.,* 1977).

LOS of group A meningococci showed little cross-reactivity with LOS of serogroup B and C strains, and additional serotypes (L9 to L11) were added (Zollinger *et al.,* 1980). Serotypes L10 and L11 were uniquely associated with group A strains; serotype L9 cross-reacted with L7, and to a lesser extent, with L3, L4 and L6 of groups B and C. Type L8 was occasionally found on group A strains. Currently, 11 immunotypes are distinguished; L1 through L11 of which L3, L7 and L9 are closely related immunochemically (Zollinger *et al.,* 1977; Poolman *et al.,* 1982; Zollinger *et al.,* 1980; Mandrell *et al.,* 1977). The chemical structures of the terminal structures of L1-L6 and L8 have been elucidated (Jennings *et al.,* 1987; Dell *et al.,* 1990; Difabio *et al.,* 1990; Schneider *et al.,* 1991). Recently, two new LOS immunotypes (L12 and L13) were described among meningococci of serogroup A, but it is not yet clear whether they represent unique LOS structures. LOS types L9-L11 are found

among serogroup A meningococci and L2 -L4 among serogroups B and C (Kim *et al.,* 1988; Achtman *et al.,* 1992; Salih *et al.,* 1990).

The LOS immunotype is considered to be a virulence determinant (Jones *et al.,* 1992). Isolates of immunotypes with a terminal lacto-N-neotetraose unit (L2, L3, L4, L5, L7 and L9) are capable of endogenously sialylating their LOS. This renders them seroresistant by reducing the effectiveness of the alternative complement pathway (Mandrell *et al.,* 1991; Tsai *et al.,* 1991). Theoretically, serosensitive strains should be isolated more often from carriers and this was found to be the case in Britain (Jones *et al.,* 1992). It is possible for a carrier strain of the L1/L8 group to shift to the L3 group during colonization, enabling the isolate to invade the host defense mechanisms and cause disease (Scholten *et al.,* 1994).

Recent investigations have evaluated the applicability of the whole-cell ELISA (WCE) for LOS immunotyping with mAbs by developing an algorithm for the assessment of immunotypes based on the WCE results (Scholten *et al.,* 1994). It was shown that the serotypes 4 and 15 and the subtypes P1.4, P1.5, P1.15, P1.16 and P1.7.16 were associated with the immunotypes of the L3/L1/L8 category and the serotypes 2a and 2b with the immunotypes L2 and L4. The first category was typical of serogroup B and the second of serogroup C (Scholten *et al.,* 1994). Knowledge of the distribution of LOS immunotypes among serogroup B meningococci is of particular interest, because the LOS is being investigated as a potential vaccine (Poolman *et al.,* 1990).

LOS immunotyping has been used to follow the spread of group A disease (Griffiss 1983), but its usefulness has been limited by uncertainty as to the structural basis of LOS antigenic diversity and the presence of multiple serotypes on the same organism (Zollinger *et al.,* 1977; Zollinger *et al.,* 1980). This has necessitated the use of arbitrary criteria for the assignment of immunotypes and created a critical need for standardized sera. Serogrouping, serotyping and subtyping have proved useful for studying the epidemiology of

meningococcal disease, but LOS immunotyping is not regularly incorporated into these studies. LOS immunotyping has been hindered by the complexity of the methods involved (Zollinger *et al.,* 1977; Poolman *et al.,* 1982); and there is still no mAb available for every immunotype. The interpretation of immunotyping results is difficult because meningococci often express several immunotypes and their expression can be influenced by growth conditions (Zollinger *et al.,* 1980; Poolman *et al.,* 1985; Mandrell *et al.,* 1991; Tsai *et al.,* 1991)

The assays used to assign LOS immunotype are poorly suited for use in developing countries, where the bulk of meningococcal disease occurs (Peltola 1983). Although it is part of the full characterization of *N. meningitidis,* immunotyping is not regularly performed and the immunotype is often omitted from the phenotype description. Table 1.6 summarizes the meningococcal surface structure used for meningococcal phenotyping.

TABLE 1.6. Surface structures of *Neisseria meningitidis* used for meningococcal characterization (phenotyping)

Surface structure *	Designation	Labels
CPS	Serogroup	A, B, C, X, Y, Z, 29E, W-135, (H, I, K, L)
Class 2/3 OMP	Serotype	1, 2a, 2b, 4, 14, 15, 16
Class 1 OMP: VR1	Subtype	P1.5, P1.7, P1.12
VR2		P1.1, P1.2, P1.3, P1.4 P1.6, P1.9, P1.10, P1.14, P1.15, P1.16
LOS	Immunotype	L1 through L11

* CPS = Capsular polysaccharide; OMP = Outer membrane protein
VR = Variable region ; LOS = lipooligosaccharide

Phenotyping has proved to be a valuable tool for studying the spread of meningococcal disease (Ashton *et al.,* 1991; Achtman *et al.,* 1992; Froholm *et al.,* 1991a,b ; Poolman *et al.,* 1986; Fallon *et al.,* 1988; Samuelsson *et al.,* 1992; Wang *et al.,* 1992). Phenotype antigens have also been used to develop vaccines for prevention of meningococcal disease due to serogroup A, C, W135 and Y in adults and older children and serotype, subtype and LOS antigens are being investigated for their use in development of vaccines against serogroup B.

1.3.4. Genotypes

Genetic relationships of different meningococcal isolates have been assessed by multilocus enzyme electrophoresis and restriction fragment length polymorphism (Selander *et al.,* 1986; Fox *et al.,* 1991).

In multilocus enzyme electrophoresis, also called electrophoretic typing, the pattern of isoenzyme variants of several common cytoplasmic enzymes is used to estimate the genetic content of meningococcal isolates. Isoenzyme variants are determined by using specific enzyme stains after starch gel electrophoresis (Selander *et al.,* 1986). Based on the results of the electrophoretic migration of each enzyme, each isolate is then assigned a multidigit score. Each multidigit score obtained, forms an electrophoretic type (ET) designated by an Arabic number (Selander *et al.,* 1986). The various ETs are labeled from 1 to the total number of different ETs detected among the isolates tested.

The designation of ETs is nominal and varies from survey to survey and ET numbers allocated in different surveys are not usually similar or interchangeable unless the ET numbers of one survey are adapted to

those of another. Each separate ET represents a clone with a particular genetic background. On the basis of the isoenzyme variants of the different enzymes, the genetic relationship of the ETs of the isolates is assessed by the use of multivariate statistical methods (Selander *et al.,* 1986), and clusters (or lineages) of clones that are genetically closely related can be distinguished (Achtman *et al.,* 1992; Crowe *et al.,* 1989; Salih *et al.,* 1990; Froholm *et al.,* 1991; Wang *et al.,* 1992 ; Caugant *et al.,* 1986 a,b ; Caugant *et al.,* 1987; Caugant *et al.,* 1990).

Restriction fragment length polymorphism (RFLP) is another method for the assessment of genetic relationships among meningococci (Fox *et al.,* 1991). The procedure involves extracting cellular DNA and fragmenting it with restriction endonucleases. The fragments are subsequently separated by electrophoresis. After hybridization with a DNA probe, the fragments which contain the sequence to which the probe is directed are visualized. By the use of the probe, the number of DNA bands is restricted, which helps facilitate the interpretation of the DNA patterns. The choice of the probe is based on repeated DNA sequences found in various parts of the chromosome. In order to characterize all possible meningococcal strains, it is essential to select a probe with broad specificity. RFLP types are designated according to the various RFLP patterns found in the population of isolates under consideration. RFLP results are similar to those obtained by electrophoretic typing (Fox *et al.,* 1991).

Phenotypic similarity does not always imply genotypic similarity and isolates that are genetically closely related, can be of different phenotypes (Fox *et al.,* 1991; Caugant *et al.,* 1986 a, b ; Caugant *et al.,* 1987; Caugant *et al.,* 1990). A new meningococcal strain in a particular geographical area, however, is often homogenous with regard to both the genotype and phenotype during the first few years after its appearance (Caugant *et al.,* 1986b ; Caugant *et al.,* 1990).

1.4. Meningococcal disease

1.4.1. Classification of meningococcal disease

The clinical manifestations of meningococcal disease are varied. This can range from transient fever and bacteremia to fulminant disease with death ensuing within hours of the onset of symptoms.

Bacteremia without sepsis can follow an upper respiratory illness or viral exanthem. After recovery and frequently after discharge without specific antimicrobial therapy, the results of blood cultures are reported as positive for *N. meningitidis*.

Meningococcemia without meningitis is characterized by the patient being septic with signs of leukocytosis, skin rashes, generalized malaise, weakness, headache. Hypotension can develop on admission to hospital or shortly thereafter.

Meningitis with or without meningococcemia is characterized by headache, fever and meningeal signs are present with a cloudy spinal fluid.

The meningoencephalic presentation is a clinical state in which patients are profoundly ill with meningeal signs and septic spinal fluid.

1.4.2. Surface components and pathogenesis of meningococcal disease

Different surface components of meningococci contribute to colonization and invasion across the mucosal surfaces (*e.g.* pili and OMPs), survival within the blood stream (capsule) and eliciting the physiological responses that result in damage to the host (LOS, peptidoglycan).

1.4.2.1. Attachment and invasion across epithelial cells.

Although meningococci can be isolated from about 10% of most populations, very few individuals develop meningococcal disease. Although the nasopharynx is important in the maintenance of the organism in the environment, the factors that determine attachment of meningococci predominantly to this site rather than to other mucosa surfaces are poorly understood.

To attach and invade host mucosal epithelium effectively, bacteria must escape the effects of IgA, avoid the ciliary clearance mechanisms of the nasopharyngeal mucosa, bind to the apical membrane and then cross to the basolateral side of the mucosal epithelial cells. The evasion of mucosal IgA actively secreted by plasma cells is an important first step. All clinical isolates of *S. pneumoniae, H. influenzae* and *N. meningitidis* secrete IgA proteases which cleave the proline-rich hinge region of IgA and render it nonfunctional, thereby facilitating bacterial attachment to epithelium. Infection of cultured human nasopharyngeal tissues with *N. meningitidis* results in injury of ciliated epithelial cells and loss of ciliary activity.

Once past the protective mucus barrier and ciliary mechanisms, meningococci bind selectively to non-ciliated epithelial cells in the underlying tissue. For *N. meningitidis,* as for other gram-negative bacteria, binding is dependent on the presence of finger-like projections (pili) on the surface of the bacteria. Pili are found on 80% of primary meningococcal isolates from the nasopharynx of carriers as well as from cerebrospinal fluid of meningitis patients (DeVoe and Gilchrist 1975). Attachment of pilate meningococci differed among epithelial cells from different sites; there was more attachment to nasopharyngeal and buccal epithelial cells compared to cells from the urethra or anterior nasal epithelium. Pili have been identified on *N. meningitidis* and are associated

with enhanced attachment to the host cells and increased virulence (Silverblatt and Cohen, 1979). While pili probably play a role in colonization of mucosal surfaces, non-pilate strains more easily escape phagocytosis, and systematic meningococcal isolates are less pilate than pharyngeal strains.

It has been suggested that the presence of the capsule decreases the adherence of meningococci to mucosa and human erythrocytes (Craven and Frasch, 1978). Outer membrane proteins and lipooligosaccharide might also influence the attachment of *N. meningitidis* to human cells. *N. meningitidis* was found to be toxic for human epithelial cells due to a heat-stable component of outer membrane vesicles, suggesting that lipooligosaccharide plays a major role in toxicity.

In cell and organ cultures, meningococci adhere to the microvilli of non-ciliated cells of human nasopharyngeal mucosa. The microvilli restructure and surround the bacteria. This is accompanied by a rapid decrease in ciliary action and sloughing of the ciliated epithelial cells. Meningococci attached to the non-ciliate cells were endocytosed within vesicles and passed through the cells into the subepithelial tissues (Stephens *et al.,* 1991).

Dunn and coworkers found that *N. meningitidis, N. gonorrhoeae, N. lactamica* and *N. sicca* also damage human endothelial cells (Dunn *et al.,* 1995). The degree of toxicity observed correlated with their relative level of adherence to cultured cells. In contrast, the Opc-dependent increased adherence did not result in increased toxicity for endothelial cells, suggesting that pili have a synergetic effect, contributing to the overall damage. Although the Opa proteins increase bacterial attachment and invasion of endothelial cells, Opc is the most effective protein in increasing bacterial interactions with these cells. Opa-mediated interactions are also eliminated or significantly reduced in variants expressing capsules or those with sialylated lipopolysaccharide (Virji *et al.,* 1993).

1.4.2.2. Survival in the blood

The bacteremia associated with meningitis is usually assumed to follow nasopharyngeal colonization and is the intermediate step before invasion of the central nervous system (CNS). Once the mucosal barrier is crossed, bacteria must overcome additional host defense mechanisms. Capsules are important surface antigens which effectively inhibit neutrophil phagocytosis and complement mediated killing by the alternative complement pathway. This enhances blood stream survival and facilitates intravascular replication leading to high bacterial densities.

1.4.2.3. Invasion of the central nervous system (CNS)

It has been suggested that meningococci can survive within human monocytes which could facilitate their entry into the CNS as monocytes can cross into the central nervous system (Perry *et al.*, 1985). It has been proposed that there might be a parallel between the "Trojan horse" mechanism of invasion used by *Streptococcus suis* in pigs (Williams and Blakemore, 1990) and meningococci in humans (Twite *et al.*, 1994).

A major research effort has elucidated the mechanism by which cytokines facilitate the passage of leucocytes, particularly neutrophils and monocytes across the endothelium into the CSF. The adherence of leucocytes to endothelium of venules has been viewed as the initial step in tissue inflammation; however, pre-incubation of the monolayers with either interleukin-1 or tumor necrosis factor (TNF) induces a time-and dose-dependent increase in the adherence and transendothelial passage of neutrophils (Mooser *et al.*, 1989). This adherence is mediated by specific transmembrane glycoproteins expressed on the endothelial cells that interact with specific counterparts on the neutrophils. Three families of adhesion

molecules can mediate these interactions: the immunoglobulins; the integrin family; and the selectin family.

Local production of inflammatory cytokines (interleukin-1 and TNF) and other mediators within the CSF in response to bacterial replication or lysis induces selectin-mediated adherence of neutrophils to endothelium. With continued cytokine stimulation, the down-regulation of the early selectin-adhesin event is coordinated with the introduction of beta-2 integrin-mediated adherence to neutrophils. This switch appears to be regulated by endothelial derived interleukin-8 is released by the endothelium in response to stimulation by interleukin-1. The neutrophils that successfully migrate into the CSF can subsequently be stimulated by the same locally produced cytokines (TNF, and interleukin-1, interleukin -6 and interleukin-8) to degranulate and release toxic oxygen metabolites and other inflammatory derivatives in the intermediate milieu of the microvasculature. These substances appear to alter the blood-brain barrier by inducing an increase in the vesicular uptake of circulating albumin as well as a more dramatic paracellular leakage of albumin through open intercellular junctions, leading to vasogenic brain edema.

1.4.2.4. Host responses to the pathogen

Endotoxin and peptidoglycan induce inflammatory responses and LOS is considered the most important inducer of inflammatory processes in the CSF and in the development of septic shock (Quagliarelo and Scheld 1992; Morrison *et al.,* 1983). Meningococci are capable of releasing parts of their outer membrane ("blebs"),which contain high concentrations of LOS (DeVoe *et al.,* 1982; Quagliarello *et al.,* 1992). LOS induces a cytokine cascade; TNF and interleukin-1 are thought to be the two main host inflammatory mediators involved.

Inflammation appears one to three hours after intracisternal inoculation of meningococcal LOS in experiments carried out in rabbits; and the release of

TNF, interleukins 1 and 6 into the CSF was observed. The most direct evidence that cytokines are responsible for damage in meningitis was observed in experiments with rats in which direct inoculation of cytokines into the CSF (interleukin-1α and interleukin-1β) induced inflammation and impaired the integrity of the blood-brain barrier. (Quagliarello *et al.,* 1992; Brandtzaeg *et al.,* 1989). The result is a brain oedema, increased intracranial pressure and an alteration of the cerebral blood flow (Lambert 1991; DeVoe *et al.,* 1982; Peltola *et al.,* 1983; Spanjaard *et al.,* 1987; Beaty *et al.,* 1987).

When intracranial pressure increases it can result in a life-threatening cerebral herniation. Given the rigid confines of the skull and spine, intracranial pressure is directly related to the volume of the brain, CSF, and cerebral blood. Alterations in cerebral blood flow may have an additional and even critical role in the ultimate sequelae of meningitis. Cerebral blood flow increases at first and then decreases - a phenomenon that appears to be related to the generation of oxygen intermediates in the microvasculature (Tureen *et al.,* 1990; Pfister *et al.,* 1990). These changes in blood flow are related to the loss of cerebrovascular autoregulation, such that cerebral blood flow fluctuates directly with the mean arterial blood pressure (Pfister *et al.,* 1990). With the loss of cerebrovascular autoregulation induced by bacterial meningitis, the brain is at risk from either hyperfusion or hypotension.

Systemic effects include acute vasculitis and focal myocardial necrosis with hemorrhage and neutrophil infiltration. Purpura is also a consequence of acute vasculitis, fibrin plugging of the arterioles and capillaries leading to hemorrhage in the skin. The mucus membranes of the oral cavity, the conjunctival and serosal surfaces and occasionally the glomeruli and adrenal glands are also affected.

1.4.3. Case fatality rate and sequelae

The crude case-fatality rate (CFR) of meningococcal disease ranges from 2-14% (Kayhty *et al.,* 1989a; Wedege *et al.,* 1990; Samuelsson *et al.,* 1992; Peltola *et al.,* 1983; Greenwood *et al.,* 1979; Fallon *et al.,* 1984; Spanjaard *et al.,* 1987; Weihe *et al.,* 1988; Halstensen *et al.,* 1987; DeWals *et al.,* 1984; Palmer *et al.,* 1992; Havens *et al.,* 1989). Fatalities may be directly due to cerebral damage (cerebral infarcts and herniation due to brain edema), but result more often from pronounced endotoxic shock (Lambert *et al.,* 1991; Quagliarello *et al.,* 1992; Brandtzaeg *et al.,* 1989). Depending on the definitions and circumstances, the reported CFRs among meningitis patients range from 10-11%, whereas those among septicemia patients range from 9-41% (Samuelsson *et al.,* 1992; Peltola *et al.,* 1983; Greenwood *et al.,* 1979; Fallon *et al.,* 1984; Spanjaard *et al.,* 1987; Salmi *et al.,* 1976; Halstensen *et al.,* 1987; DeWals *et al.,* 1984; Palmer *et al.,* 1992). Despite the improvement of clinical care during the past 4 decades, the CFR of meningococcal disease has remained essentially unchanged (Havens *et al.,* 1989).

Of the patients who do recover, 3-13% are left with severe sequelae (Cartwright *et al.,* 1986; Peltola *et al.,* 1983; Fallon *et al.,* 1984; Spanjaard *et al.,* 1987). Sequelae may result directly from the infective process in the meninges or from hemodynamic and hemostatic complications (Lambert *et al.,* 1991). Common sequelae are loss of hearing or even complete deafness, severe skin necrosis leading to scars and contractures or necessitating amputation, paralysis of a cranial nerve, hemi-paresis, seizures and mental retardation (Cartwright *et al.,* 1986; Peltola *et al.,* 1983; Spanjaard *et al.,* 1987).

Several surface characteristics of *N. meningitidis*, such as the serogroup and the serotype, have been found to be associated with an unfavorable outcome of meningococcal disease (Fallon *et al.,* 1984; Spanjaard *et al.,* 1987); however, the results of these reports were conflicting. For example, in Scotland from 1972-1982 more fatalities were associated with disease due to isolates of serogroups A and C, compared with serogroups B and W-135 (Fallon *et al.,* 1984). In the survey in the Netherlands from 1959-1981, this association was reversed; isolates of serogroup W-135 and uncommon serogroups showing the highest CFRs, followed by those of serogroups B, C, A (Spanjaard *et al.,* 1987a). The uncommon serogroups (X, Y, Z, 29E, W-135) and non-groupable isolates are considered to be opportunistic pathogens because they seem to cause disease predominantly among immunocompromised patients (Spanjaard *et al.,* 1987, Fijen *et al.,* 1989).

1.4.4. Epidemic and endemic meningococcal disease

Epidemics of meningococcal disease have been reported regularly worldwide. These are mainly due to a particular meningococcal clone or a limited number of closely related clones (Crowe *et al.,* 1989 ; Salih *et al.,* 1990; Samuelsson *et al.,* 1992 ; Wang *et al.,* 1992 ; Caugant *et al.,* 1986b ; Caugant *et al.,* 1990 ; Sacchi *et al.,* 1992 ; Olyhoek *et al.,* 1987; Knight *et al.,* 1990). The attack rate of epidemic meningococcal disease is usually in the range of 10 to 1000 per 100,000. In major African epidemics, the nationwide attack rate ranges from 100 to 800 per 100,000, but the local attack rate in affected villages or cities may exceed 1,000 cases per 100,000 persons. Conversely, during the epidemics which occurred in the 1970s in Finland and Norway the annual incidence was only 15-25 per 100,000 population. The ratio between epidemic and mean endemic incidence rates is generally higher in developing countries than in developed populations (Peltola *et al.,* 1982).

Risk factors related to disease or outbreaks are not completely understood. A combination of conditions (environment, host and organism) are necessary for an epidemic to occur. These include : immunological susceptibility of the population (perhaps due to loss of herd immunity to the prevalent strain); special climatic conditions (dry season, dust storm); low socioeconomic status; and transmission of a virulent strain. Acute respiratory tract infections may also contribute to the development of meningococcal epidemics (Moore *et al.,* 1990).

The risk of epidemic meningococcal disease differs between serogroups. Serogroups A, B, and C can cause outbreaks. Other serogroups (D, E29, X, W135, Y and Z) have so far not been associated with outbreaks. In most developing countries epidemics are usually caused by meningococci of serogroup A. With the exceptions of Russia and Romania in which epidemics of serogroup A occur, in Europe and America meningococci of serogroups B and C were more prevalent.

The appearance of a new meningococcal clone with a new phenotype (B:2b:P1.2) was responsible for the 1966 epidemic in the Netherlands (Caugant *et al.,* 1990 ; Poolman *et al.,* 1980a). Meningococci of the phenotype B: 15: P1.7,16 caused a protracted epidemic in Norway that started in 1974 (Bovre *et al.,* 1977; Lystad *et al.,* 1991). This phenotype was the main representative of a complex of meningococcal clones (ET-5 complex), which has slowly spread to the south of Europe and to America. Epidemics or local outbreaks in other parts of the world due to clones of the ET-5 complex were often of other phenotypes e.g. B:4:P1.15 in Cuba and B:15:P1.3 in Chile (Caugant *et al.,* 1986 b).

Meningococcal disease is endemic in both developing and industrialized countries (Peltola *et al.,* 1983). Endemic attack rates of meningococcal disease in developed countries with temperate climates range from 1 to 5 per 100,000 population. In developing countries, particularly in the

sub-Saharan arid area, the incidence rate between epidemics varies greatly (from less than 10 to over 20 per 100,000). In Western Europe most cases of meningococcal disease are reported during the winter months, but in (sub)tropical areas the incidence of meningococcal disease is highest during or at the end of the hot dry season (Lambert *et al.,* 1991; Peltola *et al.,* 1983 ; Greenwood *et al.,* 1979). Desiccation of the mucosal surface during these periods has been assumed to facilitate the invasion of nasopharyngeal meningococci (Lambert *et al.,* 1991).

During endemic periods many different phenotypes and clones are found among serogroup B and C isolates (Kayhty *et al.,* 1989a; Froholm *et al.,* 1991a; Poolman *et al.,* 1986; Calain *et al.,* 1988; Froholm *et al.,* 1991a). These two serogroups share many serotypes, subtypes and immunotypes (Zollinger *et al.,* 1977; Poolman *et al.,* 1982). Serogroup A isolates are rather homogenous with regard to both the phenotype and genotype (Achtman *et al.,* 1992 ; Crowe *et al.,* 1989 ; Salih *et al.,* 1990 ; Wang *et al.,* 1992 ; Zollinger *et al.,* 1980).

1.4.5. The meningococcal carrier state

Early observations on the carrier state have been reviewed in depth by Apicella (1990). Asymptomatic carriage of *N. meningitidis* in the nasopharynx in otherwise healthy humans has been recognized since 1896. Before the elucidation of distinct meningococcal serogroups, Dopter found organisms in the nasopharynx that had all the characteristics of meningococci but that failed to agglutinate with anti-meningococcal serum prepared from strains isolated from spinal fluid. He labeled these parameningococci. In 1908, Bruns and Hohn noted a close relationship between the carrier rate in a population and the onset, rise and decline of an epidemic. Glover noted the same association in the British Army military camps during the World War I.

He suggested that when the carrier rate exceeded 20%, the community was in danger of an epidemic usually due to the predominant carrier serotype.

The transmission of meningococci from carrier to carrier is via the respiratory route. The rate of spread of bacteria through a population has been the subject of a number of studies. During epidemics in military camps, the rate of acquisition can be very rapid. In non-epidemic situations, both military and civilian, the rate of acquisition can be considerably slower and the carriage can exist for prolonged periods of time. Rake demonstrated that carriers fell into three groups, chronic, intermittent and transient and that chronic carriers could be constantly colonized for up to 2 years.

The carrier state is an immunizing process. Indirect evidence for this phenomenon is the fact that while military recruits have a high frequency of meningococcal carriage and disease, seasoned veterans have a much lower carriage rate and a disease incidence no different from the civilian population. In military recruits, anti-meningococcal antibodies have been shown to persist for a minimum of 4-6 months after exposure. These antibodies are of the three major immunoglobulin classes IgA, IgG and IgM (Goldschneider *et al.,* 1969 a, b). Reller *et al.* (1973), demonstrated the development of bactericidal antibodies to the meningococcus in 38 military recruits who became colonized with non-groupable meningococcal strains. Bactericidal antibody to the homologous strain was present in 39% of these men, and a proportion also developed antibodies that were directed against heterologous strains. These same investigators found greatly enhanced (10- to 100- fold increase) bactericidal activity to known pathogenic strains A, B, C and Y after colonization with non-groupable meningococci. This suggested that these organisms are as capable of stimulating cross-reactive antibody as groupable meningococci through either an initial or anamnestic response.

Carrier rates in open populations range from 5% to 30% (Peltola *et al.,* 1983; Cartwright *et al.,* 1987; Blackwell *et al.,* 1990b). The serogroups B,

C, Y and W-135 are common among carriers and meningococci of the serogroups X, Z and 29E, as well as non-groupable meningococci, are found almost exclusively among carriers (Peltola *et al.,* 1983 ; Greenfield *et al.,* 1971; Cartwright *et al.,* 1987).

Carriage of meningococci of serogroup B is usually characterized by long duration (median 9 months) and low acquisition rates (*i.e.,* the incidence of carriage) (Lambert *et al.,* 1991; Broome *et al.,* 1986). Serogroup A, however, has been found to be less prevalent among carriers, but high acquisition rates and a rapid turn over has been demonstrated (Lambert *et al.,* 1991; Hassan-King *et al.,* 1979 ; Broome *et al.,* 1986).

1.4.6. Developmental, genetic and environmental factors affecting carriage and susceptibility to meningococcal disease

1.4.6.1. Age and development of antibodies to meningococcal disease

Goldschneider *et al.* demonstrated that the percentage of people having bactericidal activity against *N. meningitidis* in their serum is inversely proportional to the incidence of meningococcal meningitis during the first 12 years of life (Goldschneider *et al.,* 1969 a, b). Due to maternal transfer of antibodies at birth, approximately 50 % of infants have bactericidal antibody to meningococci. The prevalence of bactericidal antibody decreases after birth and reaches its nadir between 6 and 24 months of age; thereafter, a linear increase in antibody titer occurs until the age of 12.

The nature of these responses in humans has been studied in children in relation to the cellular basis of the immune response to polysaccharide. In healthy human infants, the antibody responses to several polysaccharide antigens are delayed in ontogeny (Smith *et al.,* 1973; Borgono *et al.,* 1978).

Precursors of B cells expressing specificity for different polysaccharide antigens such as α-1,3-dextran (Howard and Hale 1976) and levan (Bona *et al.,* 1979) have been shown to appear late in ontogeny, whereas precursors to some proteins have been detected on the day of birth. Polysaccharides have traditionally been characterized as T-independent antigens, capable of activating B cells without T cell help, producing mainly IgM antibody. Young children make little or no antibody to such T-cell independent antigens (Anderson and Betts 1989).

Cross-reactivity has been clearly demonstrated between neonatal tissues and the group B capsular polysaccharide. By using monoclonal antibodies specific for this capsule it has been shown that cross-reactivity exists between central nervous system, cardiac, liver and renal glycoproteins (Finne *et al.,* 1987) of the infant rat and the group B polysaccharide. As the animal matures, the cross-reacting antigens persist in the central nervous system. These studies suggest that the poor immunogenicity of this polysaccharide may be due to the fact that it resembles host antigens.

Goldschneider *et al.,* (1969a,b) examined the effect of carriage on development of anti-meningococcal antibodies in children. Non-typable strains, identified among children in carrier studies contain antigens cross-reactive with the encapsulated strains. Bactericidal antibodies to these strains develop after nasopharyngeal colonization. Serogroup-specific antibodies also arise as a result of carriage. The bulk of anti-meningococcal antibodies collected in 1968 were directed against the capsular polysaccharide. In addition, these studies showed that the antibody was primarily directed against the serogroup C meningococcus which was prevalent in the population at the time of collection. This is in contrast to the low level of antibody directed against the capsular polysaccharide of the group A meningococci which had not been prevalent in the population for 10-15 years. It would appear that the bulk of protection in the pooled antibodies to the C meningococcus is directed towards the capsule, while protection against

group A meningococcus is afforded by antibodies cross-reactive with other antigens.

1.4.6.2. Genetic factors

1.4.6.2.1. Complement properdin deficiencies.

Recurrent episodes of meningococcal disease are usually associated with deficiencies of the complement system. Absence of the sixth complement component was demonstrated in a patient with meningococcal meningitis (Lim *et al.,* 1976). In an earlier study, at least one of the patients with recurrent meningococcal disease lacked C3 (Alper *et al.,* 1970). Other studies indicated that human deficiency of C8 has been found in some persons with disseminated gonococcal infections and that this complement component is required for serum bactericidal activity against gonococcus (Petersen *et al.,* 1976). Ellison and coworkers, have evaluated the complement system in 20 patients with first episodes of serious systemic meningococcal infection; complement deficiency was detected in 6 (30%) of the patients. Half of the patients had deficiencies in a terminal complement protein(s) while the rest had deficiencies of multiple factors associated with underlying disease states (Ellison *et al.,* 1983). This bactericidal defect could be corrected by vaccination of such population; vaccinating individuals deficient in late complement components may shift the burden of host defense from serum bactericidal activity to phagocytosis (Ross *et al.,* 1984).

Inherited complement deficiency states have been found in association with 10% -30% of the sporadic adult cases of meningococcal disease (Ross and Densen 1984). An intact classical complement pathway might be important in resisting infection with *N. meningitidis* especially in families with properdin deficiency (Sjoholm and Nilsson 1985). Individuals with inherited deficiency of properdin have a functional classical pathway of complement and vaccination

might be protective (Sonderstrom *et al.,* 1989). Complement-dependent phagocytosis requires complement activation only through C3.

1.4.6.2.2. Blood group antigens, secretor status and susceptibility to infectious agents - epidemiology.

There is a large body of epidemiological evidence for associations between ABO blood groups and secretor status with susceptibility not only to infectious disease but also to other conditions (Mourant, *et al.,* 1976; Blackwell, 1989d). Examples of the reported association between infectious diseases and ABO blood groups are listed in Table 1.7 and secretor status in Table 1.8. Many of the associations between ABO blood groups and diseases were severely criticized by Wiener (1970) who stated that if an hypothesis could not be formulated to explain the findings, they were most likely due to chance. Associations between ABO blood groups and infectious diseases were excluded from these criticisms as many microorganisms have antigenic components cross-reactive with ABH antigens. It was suggested that the anti-A and anti-B isohemagglutinins acted as "natural" antibodies exhibiting bactericidal or opsonizing activities against bacteria expressing cross-reactive antigens.

Table 1.7. Association between ABO blood groups and susceptibility to infections

Infectious agent /disease	Blood group associations	References
Respiratory tract		
Influenza A	O	Potter, 1969
M. tuberculosis	B	Viskum, 1975
S. pyogenes (Group A)	not O	Haverkorn and Goslings, 1969
S. pneumoniae	not B	Reed *et al.*, 1974
Oral cavity		
Candida albicans (carriage)	O	Burford-Mason *et al.*, 1988
Periodontal disease	O and AB	Pradhan *et al.*, 1971
Gastrointestinal tract		
E. coli	B	Socha *et al.*, 1969
Salmonella and *E. coli*	B and AB	Robinson *et al.*, 1971
Vibrio cholerae	O	Barua and Paguio, 1977
Urinary tract		
E. coli	B	Cruz-Coke and Paredes, 1965
	B/AB	Kinane *et al.*, 1982
Genitourinary tract		
N. gonorrhoeae	B	Foster and Labrum, 1976
	no association	Johnson *et al.*, 1983
Chlamydia trachomatis	B	Blackwell, (unpublished observation)
Blood borne infections		
Malaria	A	Gupta and Chaudhuri, 1980
Coccidioides immitis	B	Derensiski *et al.*, 1979

Table reproduced from Blackwell (1989c) with modifications.

Table 1.8. Association between non-secretors and susceptibility to diseases

Infectious agent/ disease	Reference
Non-secretors	
Respiratory tract	
Strept. pyogenes (carrier and rheumatic fever)	Haverkorn and Goslings, 1969 Glynn *et al.*, 1959
Neisseria meningitidis	Blackwell *et al.*, 1986a
Strept. pneumoniae	Blackwell *et al.*, 1986a
Haemophilus influenzae	Blackwell *et al.*, 1986b
Gastrointestinal tract	
Vibrio cholerae	Chaudhuri and Das-Adhikary, 1978
Peptic ulcers	Reviewed by Mourant *et al.*, 1976
Urinary tract	
E. coli	Kinane *et al.*, 1982 Blackwell *et al.*, 1989c
Oral cavity	
Candida albicans	Thom *et al.*, 1989
Dental caries	Holbrook and Blackwell, 1989
Autoimmune diseases	
Ankylosing spondelytis	Shinebaum *et al.*, 1987
Insulin dependent diabetes mellitus	Blackwell *et al.*, 1987a
Grave's disease	Collier *et al.*, 1988
Secretors	
Respiratory virus disease	Raza *et al.*, 1991
Human immunodeficiency virus (heterosexual transfer)	Blackwell *et al.*, 1991

Table reproduced from Blackwell (1989c) with modifications.

In contrast to *N. gonorrhoeae* for which there is an association with blood group B, there have been no associations found between ABO blood groups and meningococcal disease in studies in Scotland, Iceland or Nigeria (Blackwell *et al.,* 1986a; 1989b).

The majority of individuals have the water-soluble glycoprotein form of their ABO blood group antigens in their body fluids (saliva, urine and ovarian cyst fluid). Non-secretor individuals do not express their ABO blood group antigens in the body fluids. Because the secretor gene (Se) is inherited in a Mendelian dominant pattern, the predicted proportion of these phenotypes within a population is secretors 75-80% and non-secretors 20-25%. This ratio can vary widely in different ethnic groups and some geographically isolated populations (Mourant *et al.,* 1976).

Epidemiological evidence from different studies in Scotland, Iceland and Nigeria suggest that there is an association between non-secretion and susceptibility to invasive disease caused by meningococci, pneumococci and type b *H. influenzae* (Blackwell *et al.,* 1986 a,b). In areas where there have been prolonged outbreaks of meningococcal disease and where the secretor status of the population has been determined (Northern Nigeria, Iceland), there are unusually high proportions of non-secretors (Blackwell *et al.,* 1990a). In the United Kingdom a similar pattern was found in Stonehouse (Gloustershire) (Blackwell *et al.,* 1989a) and Plymouth (Blackwell and Weir, 1990b) where there have been prolonged outbreaks due to the B:15:P1.7,16 strains and among a school population in which an outbreak of meningitis due to a B:4:P1.15 strain occurred (Blackwell *et al.,* 1990a).

Several hypotheses have been suggested to explain the apparent increase in susceptibility of non-secretors : a) Le^a, usually expressed in greater quantities on epithelial cells of non-secretors, is a receptor for some strains of meningococci; b) there are differences between levels of the C3 component of complement of secretors and non-secretors; c) non-secretors have less

efficient total or specific immune responses; d) secretors have more inhibitory activity of non-antibody components in their body fluids compared with non-secretors.

1.4.6.2.3. Evidence that Lewis^a is a receptor for bacteria

One hypothesis put forward to explain the prevalence of non-secretors among patients with certain bacterial or fungal infections is that some strains of microorganisms have adhesins that bind the Le^a antigen which is usually expressed in greater quantities on epithelial cells of non-secretors compared with secretors (Blackwell 1989a,b; Blackwell *et al.,* 1992). Among older children and adults, the proportion of individuals whose erythrocytes are agglutinated by anti-Lewis^a is generally 20-25%. Between the ages of 2-4 months, approximately 80-90% of infants express the antigen on their erythrocytes (Issit, 1986), but this declines to the levels expected for adults by 18-24 months. Lewis^a antigen on epithelial cells is adsorbed from secretions and Lewis^a is readily detectable in body fluids of infants (Blackwell *et al.,* 1992; Raza *et al.,* 1991).

Pre-treatment of epithelial cells with monoclonal anti- Le^a significantly reduced binding of *S. aureus,* and the binding of these bacteria was correlated with the amount of Lewis^a detected on the epithelial cells of different donors (Saadi *et al.,* 1993). Essery *et al.* demonstrated microbial surface antigens that bind Lewis^a antigen on strains of several bacterial species tested to which non-secretors appear to be more susceptible than secretors: *S. aureus* (100%); *N. meningitidis* (53%); *H. influenzae* (62%); (Essery *et al.,* 1994). Protein adhesins that bind Lewis^a have been isolated by affinity purification with synthetic Lewis^a from *S. aureus* (Saadi *et al.,* 1994) and *Helicobacter pylori* (Alkout *et al.,* 1995).

1.4.6.2.4. C3 component of the complement system of secretors and non-secretors

The structural gene for the third component of the complement system (C3), is in the same linkage group as the secretor gene. It was shown that there were significant differences in the levels of C3 in secretors compared with non-secretors in children. Among the 100 children in the study with C3 levels below the lower of the normal range, 7 of the 8 individuals were non-secretors. (Blackwell 1989d).

1.4.6.2.5. Differences in total and specific levels of antibodies.

Protective immunity to disease due to *N. meningitidis* is associated with the presence of an intact complement system and opsonizing or bactericidal antibodies specific for the invading strain (Goldschneider *et al.,* 1969a,b; Lee *et al.,* 1978).

The lower immunoglobulin levels found in non-secretors compared with secretors were suggested to contribute to the increased susceptibility of non-secretors to rheumatic fever and rheumatic heart disease (Grundbacher *et al.,* 1972) It was suggested that specific immune responses at the mucosal surfaces of non-secretors might be compromised compared with that of secretors, as they were found in earlier studies to have lower levels of serum and salivary antibodies (Waissbluth and Langman 1971).

To test this hypothesis, total and specific levels of serum IgG, IgA and IgM for secretors and non-secretors was compared in a collection of sera obtained as part of investigations of an outbreak of meningococcal disease in a Scottish secondary school. Results from serum antibodies assessed indicated that there was no correlation between levels of serum IgM in relation to carriage; but there were significant lower levels of IgM in saliva of non-secretors. This,

might contribute to susceptibility to colonization, particularly among infants under the age of 12 months in whom secretory IgM is the major class of antibody on mucosal surfaces (Zorgani *et al.,* 1992).

1.4.6.2.6. Inhibitory effect of secretor and non-secretor saliva.

Colonization of mucosal surfaces by bacteria is, in part, determined by the capacity of the host to block attachment of adhesins on the microorganism that bind specific receptors on epithelial cells. The ability of the host to resist infection or colonization by microorganisms is partly dependent on the presence of a fully functional mucosal immune system. Although IgA is the most abundant immunoglobulin in exocrine secretions such as tears, saliva and milk, IgG and IgM are also found in these fluids (Brandtzaeg *et al.,* 1970; Smith *et al.,* 1989). IgM can also function as a true secretory immunoglobulin (Brandtzaeg *et al.,* 1981), reaching the mucosal surface by the identical secretory-component mediated transcellular pathway that transports IgA. The full protective potential of secretory IgA is not present in the infant at birth (Haworth *et al.,* 1966; Berg 1968). Adult levels of secretory IgA are not reached until after 1 year of age (Burgio *et al.,* 1980; Mellander *et al.,* 1984). It has been suggested that in infants the presence of IgM compensates partially for the low levels of IgA (Mellander *et al.,* 1984). IgM to polio virus and *E. coli* has been found in infants (Mellander *et al.,* 1984; Mellander *et al.,* 1986a).

Relatively small amounts of 19S IgM are found in normal secretions, but the concentration is much increased in infants and in individuals with selective IgA deficiency (Brandtzaeg, 1971; Savilahti, 1973). It was originally thought that 19S IgM antibodies in serum and secretions were identical (Brandtzaeg *et al.,* 1978); but it has been shown that IgM in secretions is associated with SC although only 60-70% of the molecules retain the SC after purification (Brandtzaeg, 1975). Various antibody activities have been shown for IgM in nasal secretions (Ogra *et al.,* 1974) and salivary fluids (Mellander *et al.,* 1984; 1986a,b). Evidence from measurement of antibody activity to antigens to

which infants are naturally exposed (Mellander *et al.,* 1984; 1986 a) or to which they have been immunized (Carlsson *et al.,* 1985; Smith *et al.,* 1986) indicates that IgM antibody is also present in whole saliva early in life. IgM has been detected in saliva of some infants who were as young as one month of age (Glesson *et al.,* 1982; Smith *et al.,* 1989).

Zorgani *et al.,* demonstrated that non-secretors had lower levels of total salivary IgM and salivary IgM specific for *Neisseria lactamica* and five strains of meningococci (Zorgani *et al.,* 1992). Two years later, the effect of salivary components on attachment of meningococci to epithelial cells was assessed with the aid of a flow cytometry assay (Zorgani *et al.,* 1994). A significant inhibition of bacterial binding by saliva was demonstrated and this inhibition was significantly greater with saliva from secretors compared with saliva from non-secretors. It was also demonstrated that after removal of antibodies, the absorbed preparation still retained significant levels of inhibitory activity. In addition, significant inhibitory activity was demonstrated with purified IgA and IgM, but there was no significant difference between the activities of IgA and IgM.

1.4.6.3. Environmental factors affecting meningococcal carriage and disease

1.4.6.3.1. Cigarette smoke

Although symptom-free nasopharyngeal carriers are the usual source of meningococcal infection, little is known about the factors that determine carriage. Carriage rates are high in young adults, in people who live in conditions of severe overcrowding and in military recruits in the absence of overcrowding. In the very young and the very old carriage rates are low (Cartwright *et al.,* 1989) . One of the factors that could increase the likelihood of carriage is exposure to environmental pollutants including cigarette smoke.

Stuart and colleagues found an association between carriage and smoking in their studies (Stuart *et al.,* 1989). The risk of carriage associated with active smoking increased with the number of cigarettes smoked daily; there was an increased risk of carriage with heavier smoking. Although the biological mechanisms are not yet known, interference with ciliary activity by cigarette smoke might predispose to colonisation of the nasopharynx by *N. meningitidis* (Crofton *et al.,* 1981; Stephens *et al.,* 1991).

Passive exposure to cigarette smoke can also account for an increased risk of meningococcal carriage in those who live with smokers and for the increased risk of meningococcal disease in children who live with smokers (Haneberg *et al.,* 1983; Stuart *et al.,* 1988). This higher risk of carriage could be due to the greater chance of acquiring meningococci from contact with smokers and not to a direct effect of passive smoking (Stuart *et al.,* 1989; Blackwell *et al.,* 1990a).

Smoking in teenagers is associated with carriage and could contribute to the increase in prevalence of meningococci carriage and in meningococcal disease attack-rates in this age group (Cartwright *et al.,* 1987; Blackwell *et al.,* 1990a; Fraser *et al.,* 1973). The high attack rates and high carriage rates in military recruits (Aycock *et al.,* 1950; Fraser *et al.,* 1973) could also be due partly to a higher prevalence of smoking (Cummins *et al.,* 1981) and to heavier smoking (Crowdy, 1981) in military populations than in the corresponding civilian population.

1.4.6.3.2. Respiratory infections

Associations between meningococcal infection and preceding viral respiratory infections have been reported. Young and coworkers investigated an outbreak of meningococcal infection in an elderly population, most of whom had serologic evidence of influenza (Young *et al.,* 1972). Goldstein and associates

have shown that pulmonary clearance of meningococci is diminished in animals previously exposed to an virulent encephalomyelitis virus (Goldstein *et al.,* 1972). Meningococcal upper respiratory tract infections (pharyngitis) associated with contacts of patients and as a prior symptom in cases of serious meningococcal disease have been described by several authors (Tobin *et al.,* 1956; McCracken, 1976).

The elderly are not usually susceptible to meningococcal infections but an outbreak of meningococcal meningitis in a geriatric ward affected nearly one third of the females (11/55), 27 of whom were suffering from A2 influenza virus infections. A significant association between the two pathogens was also shown by the serological evidence in these patients (Young *et al.,* 1972). Data from military training center covering a period from 1967 to 1971 indicated a lag of 7-10 days between acute respiratory diseases necessitating hospitalization and meningococcal disease (Edwards *et al.,* 1977). It is not clear from the study which pathogens were involved in the acute respiratory diseases. Upper respiratory tract infections with adenovirus or mycoplasma have been reported to be a risk factors for epidemic disease due to a group A *N. meningitidis* (Moore *et al.,* 1990).

In a survey of patients with meningococcal disease, 46% of 69 cases had prodromal symptoms affecting the upper respiratory tract within 1 week before onset of signs and symptoms of the disease (Olsen *et al.,* 1979). An investigation of house hold contacts of patients with meningococcal disease showed a bacterial carriage rate of 61% among the individuals with upper respiratory symptoms compared with only 14% among those with no symptoms (Olsen *et al.,* 1981).

Infections by adenovirus and influenza B virus were detected by isolation of the viruses or by serological methods in the study involving 160 cases of meningitis due to *H. influenzae* or *N. meningitidis* and 138 controls. The

viruses were significantly associated with the bacterial disease (Krasinski *et al.,* 1987).

Cartwright *et al.* (1991) reported that the 38% of 53 meningitis patients of 10 or more years of age had high titres of antibodies against influenza A virus, compared with 6% of individuals in a control group. Possible associations with other respiratory tract viruses were not reported in this study. Analysis of the data obtained from the questionnaires supplied to the patients and the control group showed that a high proportion of meningitis patients suffered from a flu-like syndrome during the previous 2 months.

Young children are highly likely to be infected with respiratory syncytial virus (RSV) during the first two years of life (Henderson *et al.,* 1979), and in an experimental model, RSV infection of a human epithelial cell line Hep-2, significantly enhanced binding of meningococci of different serogroups and serotypes (Raza *et al.,* 1994).

1.4.6.3.3. Socioeconomic conditions

The case fatality rate varies depending upon the prevalence of disease, the nature of infection and the socioeconomic conditions of the society in which the infections occur. During endemic situations in industrialized countries, case fatality can be as low as 7 percent for meningitis and as high as 19 percent for septicemia without meningeal involvement (Andersen 1978). During epidemic situations, mortality for meningitis can vary from 2 to 10% and for septicemia can be as high as 70% in some Third World countries (deMorais *et al.,* 1974; Oberli *et al.,* 1981). In Britain, smoking is more prevalent among unskilled or partly skilled individuals compared with professional groups (Wald *et al.*, 1988)

1.4.6.3.4. Demographic factors

The gathering of susceptible people is an important risk factor for outbreaks. Travel and migration facilitate the circulation of virulent strains inside a country or from country to country. Large population movements such as war, play a major role in a spread of infection and disease and the highest incidences of meningococcal disease were observed during the two world wars (Hedrich, 1931; Priest *et al.,* 1947). The outbreak which occurred in Mecca in 1987, at the end of the pilgrimage period caused more cases among pilgrims than among the Saudi population. In many countries, returning pilgrims caused the occurrence of cases of meningococcal meningitis in their immediate communities (Moore *et al.,* 1988). In some countries the occurrence of epidemics (Chad 1988, Marocco 1989, Sudan 1988) may have been provoked by the introduction of a virulent strain of serogroup A meningococcus imported by returning pilgrims (Moore *et al.,* 1988). Other large population displacements, e.g. those of refugees, may pose similar risks.

In the Czech Republic, introduction of a virulent strain of serogroup C meningococcus caused an increase in the incidence of invasive meningococcal disease in army camps in the eastern part of the country and two local invasive meningococcal disease outbreaks in the civilian population. A new clone was found (C:2a:P1.2) with unusual epidemiological and clinical characteristics which had not been identified at least since 1973. This new meningococcal clone was probably imported from Canada since there was a good correlation with recent reports from Canada where the identical clone of *N. meningitidis* serogroup C appeared (Kriz and Musilek, 1995).

These factors are of particular interest, as Greece is a major holiday destination and also an immigration center for people from the former Soviet Bloc (Albania, Russia)

1.5. Treatment of meningococcal Infections

Chemotherapy for meningococcal infection began with the report of Schwenter *et al.* (1937) that demonstrated that sulfonamides could be used successfully in the treatment of meningococcal meningitis and meningococcemia (reviewed by Apicella 1990). Early studies in 1942 by Feldman *et al.,* found penicillin administered in relatively low doses (120,000 units/day) was not as effective as sulfonamides (reviewed by Apicella 1990). By using larger amounts of the drug, it was demonstrated that the treatment results with penicillin were identical to those with sulfonamides.

Penicillin therapy for the treatment of meningococcal infections is safe and effective nowadays, although reports of penicillin-insensitive *N. meningitidis* have come from Great Britain and Spain (Spot *et al.,* 1988 ; Campos *et al.,* 1987). This relative resistance is due to a reduced affinity of penicillin binding protein 3 in 11 serogroup B and 4 serogroup C *N. meningitidis* strains isolated from CSF and blood during an epidemic in Spain (Mendelman *et al.,* 1988).

Chloramphenicol is an effective substitute for patients allergic to penicillin. Third generation cephalosporins including cefotaxime, ceftriaxone and ceftazidime have been used successfully in the treatment of pediatric cases of meningococcal meningitis (Neu, 1987). The second generation cephalosporin cefuroxime has also been successfully used in meningococcal meningitis (Shaad *et al.,* 1984). The duration of antibiotic therapy will vary somewhat with the presentation and manifestation of the disease and with the response of the patient. At present, when the meningococcus is sensitive to the agents above, 7-10 days of therapy is usually sufficient.

1.6. Prevention of meningococcal disease.

1.6.1. Chemoprophylaxis

Shortly after the introduction of sulfonamides for the treatment of serious meningococcal disease, it became apparent that short courses of the sulfadiazine resulted in the disappearance of meningococcal carriage for prolonged periods of time (reviewed by Apicella 1990). As Feldman pointed out, despite the arguments about the relationships, "if there are no carriers, there are no cases", the use of sulfonamides to reduce carrier rates did decrease the number of cases (Feldman, 1972).

Treatment of the meningococcal carrier state with sulfonamides eradicated carriage quickly and for prolonged periods; after two doses of 3 and 2 g of sulfadiazine the carrier rate dropped from 79 to 0 percent in 72 hours (reviewed by Apicella 1990). On military bases and in closed environments such as boarding schools, institutions and family units in which cases arose, this form of chemoprophylaxis was effective in preventing the spread of meningococcal infections.

With the recognition of widespread sulfonamide-resistant meningococci, these agents have been abandoned for meningococcal chemoprophylaxis except in instances where the meningococcal case strains are known to be sulfonamide sensitive. The search for new agents for chemoprophylaxis has been extensive. Penicillin has proved ineffective for several reasons; long-acting mixtures do not eradicate the nasopharyngeal carriage, and while massive doses cause people to become non-carriers, the carrier state recurs promptly after discontinuation of treatment with the drug (Feldman, 1972; Artenstein *et al.,* 1967).

Two antibiotics have emerged as candidates for meningococcal prophylaxis. Minocycline and rifampicin have been shown to eradicate the carrier state rapidly and this eradication persists for up to 6-10 weeks after treatment (Guttler *et al.,* 1971; Devine *et al.,* 1971). Problems occur with both drugs. Minocycline has been shown to cause vertigo, probably secondary to an effect on the vestibular system (Jacobson *et al.,* 1975). Rifampicin treatment can result in the emergence of rifampicin-resistant meningococci in 10-27% of the patients treated (Weidner *et al.,* 1971).

A number of other agents active against the meningococci *in vitro* have been tested and have failed to provide prophylaxis. These include erythromycin, trimethoprim, cephalexin, tetracycline and nalidixic acid. Hoeprich studied a number of these agents and speculated that the primary factor determining effectiveness in the elimination of meningococcal carriage is the ability to achieve bactericidal levels in tears and saliva (Hoeprich, 1971).

Intimate contacts of patients with meningococcal disease, such as the household contacts, have an increased risk of contracting meningococcal disease (Meningococcal Disease Surveillance Group 1976; Cooke *et al.,* 1989). To protect these contacts, chemoprophylaxis is prescribed in many countries, among which the United States and Great Britain (Anonymous 1990). It is assumed that by eliminating meningococci from the nasopharynx of the household contacts the transmission of meningococci within the household will be interrupted and secondary cases of meningococcal disease will be prevented. However, the beneficial effect of these drugs for the prevention of secondary disease has not been evaluated in randomized trials.

1.6.2. Immunoprophylaxis

1.6.2.1. Meningococcal vaccines

Subsequent to the emergence of sulfonamide-resistant meningococci, an intense effort was directed towards the development of a vaccine for the prevention of meningococcal infections in high-risk populations such as military recruits. The result was the development of the capsular polysaccharide vaccines for serogroups A, C, W-135 and Y, (Frasch *et al.,* 1989). These vaccines elicit serogroup-specific immunity in older children and adults, but the immune response in children under 2 years of age is poor, especially the response to the C polysaccharide. In addition, because of their T-cell independent nature, these vaccines do not induce immunological memory and regular re-vaccination is necessary. Vaccine trials in both Nigeria (Greenwood *et al.,* 1980) and Gambia (Ceesay *et al.,* 1993), have shown that in children, meningococcal antibody levels decline to background levels 2 years after immunization with meningococcal polysaccharide vaccines.

Conjugation of the capsular polysaccharide of type b *H. influenzae* (Hib) to protein carriers has been used successfully to induce T-cell dependent responses to this carbohydrate antigen and long lasting immunity to invasive Hib disease (Begue, 1993; Campbell *et al.,* 1993 ; Eskola *et al.,* 1993; Paradiso *et al.,* 1995 ; Kurikka *et al.,* 1995 ; McIntyre *et al.,* 1995), There were extremely low incidences of adverse effects (Makela *et al.,* 1990; Kaythy *et al.,* 1989b; Madore *et al.,* 1990; Ahonkai *et al.,* 1990; Einhorn *et al.,* 1986; Eskola *et al.,* 1987). From the above trials, it was shown that the decrease in Hib disease incidence since 1992 is an effect of vaccination and greater than would be expected from protection of vaccinated children alone. Invasive Hib

disease is likely to become a rare cause of serious childhood infection (McIntyre *et al.,* 1995). Conjugate Hib vaccines seem to be among the safest vaccines ever proposed for routine use and immunogenicity studies in infants suggest that they remain immunogenic when combined with diphtheria / pertussis / tetanus for routine immunization of infants (Moxon *et al.,* 1990; Eskola *et al.,* 1993; Mulholland *et al.,* 1993; Miller *et al.,* 1995; Redhead *et al.,* 1994). Immunogenicity persists even in unfavorable contexts such as individuals with sickle-cell anemia or a previous history of vaccine failure (Guerin, 1994).

Conjugate vaccines for serogroup A and C polysaccharides are now under investigation. The meningococcal capsular polysaccharide vaccines provided unreliable for control of meningococcal disease in Africa. The objectives were to immunize children during the course of their routine infant immunization schedule with a T cell dependent meningococcal vaccine that provided long lasting immunity that could be boosted by further immunization or by nasopharyngeal carriage of meningococci. The development of a group A plus group C meningococcal polysaccharide-protein conjugate vaccine (Constantino *et al.,* 1992) provided a possible means of achieving these aims. Following a satisfactory pilot trial in which 20 Gambian infants were immunized with two doses of a group A plus group C meningococcal polysaccharide - CRM_{197} conjugate vaccine during the course of their routine immunization program, the meningococcal conjugate vaccine has been found to induce long-term immunological memory among 2-2.5 month old infants (Twumasi *et al.,* 1995). This vaccine, has been also officially given to infants in Britain since October 1992 (Duerden *et al.,* 1993; Musser *et al.,* 1994).

To date, no effective vaccine is available for protection against disease due to meningococci of serogroup B, the most prevalent serogroup in developed countries. The B polysaccharide has proved to be poorly immunogenic in humans which might be due to its close resemblance to fetal human brain

tissue (Finne *et al.,* 1983). Other components of the bacteria are being investigated as potential vaccines. The principal candidates are the class 1 and class 2/3 OMPs of the meningococcus (Poolman *et al.,* 1990).

Experimental vaccines that consist of outer membrane vesicles (OMVs) containing these OMPs have been developed (Frasch *et al.,* 1989; Poolman *et al.,* 1990). The antibodies elicited by the OMPs in these vaccines are subtype and serotype- specific (Poolman *et al.,* 1990). The composition of the experimental vaccines recently tested in Norway, Chile, Cuba and Brazil (Zollinger *et al.,* 1991; Sierra *et al.,* 1991; Bjune *et al.,* 1991, Cassio de Moraes *et al.,* 1992), was based on the serotype and subtype of the most prevalent serogroup B phenotype in these countries : B:15:P1.7,16 in Norway; B:15:P1.3 in Chile; B:4:P1.15 in Cuba.

The results of the Cuban trial among children aged 11-16 years were very promising, but it should be kept in mind that the circumstances in Cuba, an isolated island population with a very homogenous meningococcal subtype distribution, were very favorable for this approach. They were not confirmed in a case-control study of the efficacy of this vaccine in Brazil (Cassio de Moraes *et al.,* 1992). The Brazilian study showed poor reactions in younger children. The Chilean vaccine provided low-level protection only (Zollinger *et al.,* 1991). The efficacy of the vaccine in the Norwegian trial as well as in trials carried out in Iceland among teenagers was low and the authors concluded that the effect was insufficient to justify a public vaccination program (Bjune *et al.,* 1991; Carlone *et al.,* 1994).

These field trials in humans with such vaccines have demonstrated at least partial protection against group B infection. The vaccines consist of outer membrane vesicles prepared from one particular strain; since many of the major outer membrane proteins (OMPs) show a high degree of variation among different strains, a large proportion of the protective antibodies induced is expected to be strain-specific. In many countries, however, the serogroup B

meningococci show marked heterogeneity with regard to serotype and subtype (Kayhty *et al.,* 1989b; Froholm *et al.,* 1991b; Calain *et al.,* 1988; Palmer *et al.,* 1992) and for those countries the above mentioned vaccines are only of limited value.

Vaccines derived from a number of different strains has limitations such as high amounts of toxic LOS and non-protective OMPs. Not all outer membrane components are needed or wanted in a vaccine. An alternative was construction of a bivalent class 3-deficient strain carrying two different copies of the *por A* gene; outer membrane complexes (OMCs) prepared from it induced bactericidal antibodies against strains of both subtypes present, P1.7,16 and P1.5,2 (Van der Ley *et al.,* 1992). Thus, by genetically removing one OMP and adding another, an improved vaccine strain was constructed. In a recent development a multivalent outer membrane vesicle vaccine was prepared by removing unwanted outer membrane components and at the same time improving the range of protection. This was accomplished through transformation with plasmid constructs made in *E. coli* and their homologous recombination into the meningococcal chromosome. Deletion of the *cps* locus resulted in loss of expression of the group B capsular polysaccharide as well as the lacto-N-neotetraose structure in lipopolysaccharide. Deletion of the *porB* gene abolished expression of the class 3 outer membrane protein. Additional copies of the *porA* gene, encoding the immunodominant class 1 outer membrane protein, were inserted into one of the *opa* genes (encoding class 5 proteins) and into the *rmpM* gene (encoding class 4 OMP). The result of such construction was the construction of three trivalent strains each of which expressed a different combination of class 1 epitopes (van der Ley *et al.,* 1995).

This trivalent vaccine, has been successfully tested in laboratory mice inoculated with 3 different meningococcal strains of known subtypes. These strains, isolated during the Belgian and Dutch epidemics constituted 83% of strains isolated during these epidemics. At present, a vaccine trial is been

undertaken in Holland against serogroup B that contains subtypes of the class 1 OMPs. The vaccine that contains subtypes of OMPs class 3, is under trial in Norway (Delving *et al.,* 1995).

Another approach to vaccine development was the study of the class 3 OMP, as a potential target for bactericidal and opsonic antibodies in humans. Synthetic peptides spanning the class 3 OMP from the vaccine strain (B:15:P1.7,16:L3,7) were synthesized. The results showed that the significant part of the humoral response to the meningococcal class 3 OMP elicited by vaccination with the Norwegian OMV vaccine was directed against a single continuous epitope (Delvig *et al.,* 1995). The problem with these vaccines is that linear peptide vaccines do not induce antibody. In order to overcome this problem, there is a need of a 3-dimensional structure by using circular peptides (Hoogerhout *et al.,* 1995).

1.7. Objectives of the present study

Following the epidemic of meningitis during the 1960s, the incidence of meningococcal disease in Greece declined steadily until 1988. Since 1988, the number of cases reported to the Ministry of Health has increased; in the first 6 months of 1990, there were 102 cases compared with 87 for all of 1989 (Table 1.9).

Table 1.9. Incidence of meningococcal disease in Greece.

YEAR	NO CASES	DEATHS	
		NO	(%)
1968	1064	48	(4.5)
1973	765	39	(5.1)
1978	286	18	(6.3)
1983	102	9	(8.8)
1985	105	6	(5.7)
1986	88	7	(7.9)
1987	85	4	(4.7)
1988	76	2	(2.6)
1989	87	6	(6.9)
1990	134	1	(0.7)

This apparent increase in disease due to *N. meningitidis* was of concern to the public health authorities. The last major epidemiological survey of *N. meningitidis* in a Greek population was carried out before elucidation of some of the genetic and environmental factors affecting carriage and development of disease (Kalapothaki *et al.,* 1979) and before the availability

of serotype and subtype reagents used to examine meningococci in north-west Europe and the Americas.

In contrast to countries in northern Europe, there were no studies on carriage of meningococci among school children in Greece or assessment of factors associated with carriage of these bacteria among children in any country in southeast Europe. The work presented here was carried out to answer the following questions:

1. Are genetic and environmental factors associated with carriage among Greek children and young adults similar to those found in northwest Europe?
2. Are strains with serogroups, serotypes and subtypes associated with disease in northwest Europe isolated from patients and carriers in Greece ?
3. Are the antibiotic sensitivities of meningococci isolated from patients and carriers in Greece similar to those observed in north west Europe?
4. Are the genetic clones associated with disease in northwest Europe present among the meningococcal strains in Greece and other Balkan countries?

The information collected would provide a basis on which decisions could be made for initiation of preventive measures if the increasing numbers of cases reflected the beginning of a prolonged outbreak or an epidemic of meningococcal disease in Greece.

2. General materials and methods

2.1. Isolation - culture and storage of *N. meningitidis.*

2.1.1. Media

Modified New York City Medium (MNYC) was used for the isolation of meningococci from throat swabs and boiled blood or chocolate agar (CA) was used for further culture.

2.1.1.1. Modified New York City Medium (MNYC) (Young *et al.*, 1978)

The medium was prepared as 10 litre batches by dissolving 360 g of G.C. agar base (Oxoid) in 8560 ml distilled water. After autoclaving at 121° C for 15 min. the medium was allowed to cool to 50^{0}C and the following supplements were added: Lysed human blood (1000 ml); glucose (10%)(100 ml); yeast dialysate (250 ml), colistin (6 mg/ml)(10 ml); lincomycin (1 mg/ml) (10 ml); amphotericin (1 mg/ ml) (10 ml); trimethoprime (5 mg/ml) (10 ml). Blood was lysed with 50 ml saponin. All supplements were stored in vials at -20^{0}C and allowed to thaw slowly before adding to the media.

2.1.1.2. Cooked blood agar or "chocolate agar".

Columbia agar base (Oxoid CM 331) was prepared by suspending 39 g in 1 l of distilled water and heating to boiling point. The agar was then sterilized at 121^{0}C for 15 min. and 10% of defibrinated horse blood was added. The medium was shaken gently and 20-25 ml of the medium was dispensed aseptically to each plate.

2.1.2. Conditions of incubation

All plates were incubated at 37^{0}C, in a humid atmosphere containing 5-10% CO_2. On primary cultures, the majority of strains grew within 16-18 hr; however, if the colonies were very small (less than 1 mm), the plates were incubated for an additional 24 hr.

2.1.3. Long Term Conservation

Freezing at -80^{0}C : Brain Heart Infusion Broth (Oxoid CM 225) was prepared by dissolving 37 g in 1l of distilled water and sterilized by autoclaving at 121^0 C for 20 min. The broth was supplemented with sterile glycerol (2%) and dispensed aseptically in 1 ml aliquots into "Nunc cryotubes" with screw caps.
A heavy suspension of a 18-24 hr culture of meningococci was prepared in these tubes and were frozen as quickly as possible, by putting them into the racks of the freezer at -80^{0}C.
The frozen strains were cultured after thawing for a few minutes at 37^{0}C by placing one loopful of the suspension on plates of CA or MNYC media and streaking for isolated colonies.

2.2. Identification

2.2.1. Macroscopic examination

N. meningitidis developed, in 18 to 24 hours, greyish colonies, with regular edges which can appear smooth or mucoid, if there is heavy capsule production ; 1 to 1.5 mm in diameter.

2.2.2. Microscopic examination

Gram's stain was carried out on individual colonies and Gram-negative diplococci further identified as described below.

2.2.3. Cytochrome oxidase

The test was carried out with tetramethyl-p-phenylenediamine chloride which turns purple upon oxidation by the cytochrome C system. The materials and reagents used were filter paper (Whattman) and tetramethyl-p-phenylene-diamine chloride (Difco) diluted in distilled water.

The presence of cytochrome oxidase was demonstrated by two ways:

1. dropping the reagent directly on the colonies obtained on the culture medium.
2. transferring a loopful of culture onto filter paper soaked with the reagent in an empty sterile Petri-dish.

A positive reaction occurred within 5-10 seconds. The reaction was considered negative if no colour was produced within 60 seconds.

2.2.4. Rapid carbohydrate utilization test

This test was based on the method described by Young *et al.,* 1979. The Rapid Carbohydrate Utilization Test (RCUT) buffer contained : 0.1 **M** K_2HPO_4 (40 ml) mixed with 0.1 **M** KH_2PO_4, (12 ml), KCl (8% w/v) (100 ml); phenol red (10 ml) (1% w/v); distilled water (838 ml). The pH of the buffer was then adjusted to 7.1-7.15, dispensed in 20 ml aliquots and stored at -20^{0}C.

Solutions (10% w/v) of the following sugars were prepared in the buffer, glucose, maltose, sucrose, lactose, fructose. All sugars were stored at -20^{0}C in 4 ml aliquots.

Ampicillin (500 mg) was dissolved in 3 ml of RCUT buffer prior to preparing microtitre trays.

To each well of a microtitre plate, 1000 µl of RCUT buffer was added. The individual sugar solutions (25 µl) and 25 µl of the ampicillin solution were added to the appropriate wells. The trays were stored in sealed polyethylene bags at -20^{0}C.

A suspension of the organism to be tested was prepared in 200 µl of RCUT buffer and 25 µl was added to six wells of a microtitre plate containing the individual sugar solutions, and ampicillin. Each plate was incubated in a 37^{0}C water-bath and the colour change was inspected after 1 hr. The microtitre plate was then returned to the water bath and re-examined after a further three hours. Known isolates of *N. meningitidis* and *N. gonorrhoeae* were included as controls. The sugar utilization patterns of the various *Neisseria* species were listed in Table 1.1.

2.3. Detection of blood group antigens

2.3.1. ABO blood group

Slide agglutination with monoclonal anti-A and anti-B antibodies (Scottish National Blood Transfusion Service) were used to determine ABO blood group. Blood samples (20 µl) were mixed with monoclonal anti-A (20 µl) and anti-B (µl) (Scottish National Blood Transfusion Service) on clear glass slides racked gently to mix and examined for agglutination (Table 2.1.).

Table 2.1. ABO blood group agglutination pattern.

Blood Group	A	B
A	+	-
B	-	+
0	-	-
AB	+	+

2.3.2. Detection of Lewis antigens in blood specimens

Monoclonal anti-Lewis^a (anti-Le^a) and anti-Lewis^b (anti-Le^b) antibodies (Scottish National Blood Transfusion Service) were used in tube agglutination test. The blood specimens (20 µl) were transferred to small test tubes to which 20 µl of monoclonal anti-Le^a or anti-Le^b were added. The tubes were incubated for 1 hr at room temperature and agglutination was recorded. If agglutination was not present in either test tube, the test tubes are incubated overnight at 4^{0}C and the results were recorded the next day.
Non-secretors can express only Le^a and secretors express Le^b antigens.

2.3.3. Detection of Lewis antigens in saliva specimens (Raza *et al.,* 1991)

2.3.3.1. Buffers

2.3.3.1.1. Coating buffer.

In 1l distilled water , 1.59 g Na_2CO_3 (Analar BDH), 2.93 g. $NaHCO_3$ (Analar BDH) and 0.20 g. NaN_3 (sodium azide) (Sigma) were dissolved. The pH was adjusted to 9.6 and stored at 4^0C.

2.3.3.1.2. Blocking buffer

Phosphate buffered saline (PBS) contained 8.16 g Na_2HPO_4 (BDH), 3.99 g NaH_2PO_4 $2H_2O$ (BDH) and 6.08 g NaCl (BDH) dissolved in 2 l of distilled water. After adjustment of the pH to 7.2, 100 ml aliquots were made, and 1 g of the bovine serum albumin (BSA) was added to each and stored at $+4^0$C.

2.3.3.1.3. Substrate

Phosphate citrate buffer (0.1 M) was prepared by dissolving citric acid (21.0 g) (BDH), and Na_2HPO_4 (14.2 g) (BDH) in 1 l distilled water. The pH was adjusted to 5.0 and the buffer was stored at $+4^0$C. For the substrate preparation, orthophenylene-diamine (40 mg) was dissolved in phosphate citrate buffer (100 ml). For activation, 40 µl of H_2O_2 (30%) (Sigma) was added immediately prior use.

2.3.3.1.4. Stopping solution

Sulfuric acid (50 ml of 18.76 M) (BDH) was added slowly to 350 ml distilled water in a fume cupboard (final concentration 12.5%).

2.3.3.1.5. Washing solution.

Washing buffer was prepared by dissolving NaCl (16.0 g) (BDH); KH_2PO_4 (0.4 g) (BDH); Na_2HPO_4 (2.3 g) (BDH); KCl (0.4 g) (BDH); and Tween 20 (1 ml) in 2 l distilled water. The buffer was kept at 4^0C.

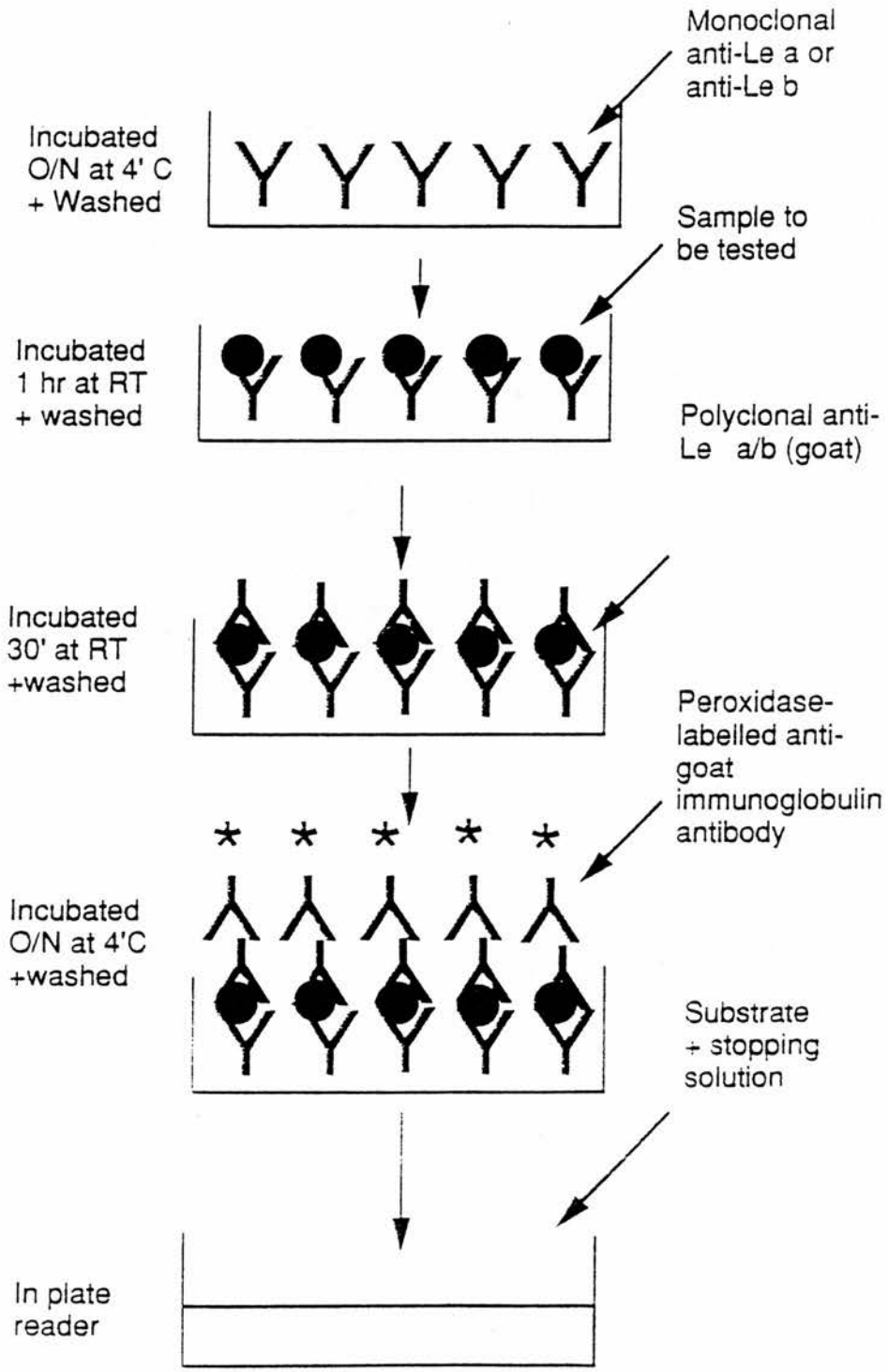
2.3.3.2. Method

Le^a and Le^b blood group antigens were detected in saliva by the ELISA method outlined in Fig 2.1. Wells of polystyrene microtitre plates (Dynatech, Billinhurst, Sussex) were coated overnight at 4^0 C with 100 µl of monoclonal anti- Le^a diluted 1/25 (Scottish National Blood Transfusion Service) or monoclonal anti-Le^b diluted 1/20 (Scottish National Blood Transfusion Service) in coating buffer. All further procedures were carried out at room temperature except when stated otherwise. The wells were washed 3 times with washing buffer and blocked with 150 µl of blocking buffer for 15 min. The buffer was removed and the wells were washed 3 times. Dilutions of saliva from known secretors and non-secretors were used in each plate as controls. The test wells contained 100 µl of the specimens (saliva) which had been boiled for 30 min. to inactivate enzymes, bacteria or viruses. Samples of saliva were diluted 1/100 in blocking buffer for detection of Le^a and 1/20 for detection of Le^b. After incubation for 60 min., the wells were washed 3 times and 100 µl of polyclonal goat anti-Le^a antibody (Behring, Marburg, West Germany) (1/500 in blocking buffer) or 100 µl of anti- Le^b antibody (Behring) (1/250) were added to the wells of the appropriate plates. After 30 min. incubation, the wells were washed 3 times and 100 µl of horseradish peroxidase-conjugated donkey anti-

goat immunoglobulin (Scottish Antibody Production Unit), (1/250 in blocking buffer) were added. After overnight incubation at 4^0 C, the plates were washed three times and 100 µl of activated substrate solution were added to each well. The colour was allowed to develop for 10-15 min., and the reaction was stopped by adding 50 µl of stopping solution to each well.

Absorbance at 490 nm was measured with an ELISA plate reader (Dynatech). Samples were tested in duplicate and readings were averaged. The average reading obtained for the test samples was compared with that of controls added in the same plate. Values equal to or above that of control were considered to be positive. The subjects from whom the samples were obtained were classified as non-secretors if only Le^a was detected, or as secretors if Le^b or both Le^a and Le^b antigens were detected.

Figure 2.1. ELISA for detection of Lewis antigens.



2.4. Serogroup determination

The serogroups are determined by slide agglutination of the bacteria with commercially available polyclonal antisera (Wellcome Diagnostics).

2.4.1. Method

Cultures grown for 18 hr on selective medium were tested for agglutination. A pool was prepared with antisera for screening. The isolates were first tested by the most common serogroups for agglutination by anti-A, anti-B, anti-C, and anti-Y. One loopful of bacterial culture was mixed with a drop (20 µl) of the pool and with a drop of 0.9% NaCl solution as a negative control. Isolates agglutinated by the pool were tested with the individual antisera to determine the serogroup.

If agglutination appeared, it was immediate (less than one min.). As the agglutination can occasionally be difficult to see with the unaided eye, those that were not obviously agglutinated were examined with a magnifying glass.

In the case of serogroups A and C, the clusters were bigger, with a clear background. In the case of serogroup B, agglutination was equally fast, but the agglutinate was more fine-structured.

If there was no agglutination with the sera for serogroups A, B, or C, the isolates were screened with the second pool of grouping sera for Y, 29E and W135.

If there was still no reaction the strain was recorded as non-groupable.

2.4.2. Interpretation

If agglutination was positive for only one serogroup: the serogroup was determined and recorded.

If agglutination was positive with several sera but not in the saline solution, the strain was recorded as polyagglutinable.

If agglutination was positive with both pools and with the NaCl solution the bacteriological characteristics of the strain were re-checked. If the result was confirmed twice, the strain was recorded as autoagglutinable. In the case of no agglutination with sera, nor with the NaCl solution, the strain was recorded as non-agglutinable.

2.5. Whole-cell ELISA for detection of meningococcal serotypes and subtypes

2.5.1. Reagents

2.5.1.1. Washing solution

Washing solution contained 0.025% w/v Tween 80 dissolved in tap water

2.5.1.2. PBS buffer (pH 7.2)

PBS contained $Na_2HPO_4.2H_2O$ (1.6 g); NaH2PO4. $2H_2O$ (0.28 g) and NaCl (8.2 g) dissolved in 1l distilled water.

2.5.1.3. Casein - Tween-80 buffer

Casein - Tween-80 buffer contained casein hydrolysate (3 g) Tween- 80 (0.1 ml) dissolved in 1l PBS. The buffer was used for blocking non-specific binding, diluting mAbs and diluting the conjugate.

2.5.1.4. **Sodium Acetate Buffer** (1.1 mM)

Sodium Acetate Buffer was prepared by dissolving sodium acetate (0.18 g.) in 2 l of distilled water. The pH was adjusted to 5.5 by adding saturated citric acid solution prepared by dissolving citric acid (130 g) in 100 ml distilled water.

2.5.1.5. **Protein A Peroxidase Conjugate** (Sigma P-8651)

The conjugate was prepared by dissolving one vial of the conjugate in Casein-Tween 80 buffer (1 ml). Aliquots (100 µl) of each vial were stored at -20^{0}C.

2.5.1.6. **3,3',5,5 tetramethyl-benzidine (TMB)**

The stock solution was prepared by dissolving TMB (0.6 g) in 100 ml dimethyl sulphoxide (Sigma D-5879) and kept in dark bottles at room temperature. The TMB solution prepared prior to use contained sodium acetate buffer stock solution (10%) (10 ml); hydrogen peroxide (30%)(250 µl) TMB 1.5% (stock solution) (1.5 ml) and distilled water (88.5 ml).

2.5.1.7. **Sulphuric acid (2N)**

For terminating the reaction the stopping solution was prepared by adding H_2SO_4 (10 ml) to distilled H_20 (170 ml) in a fume cupboard.

2.5.2. **Preparation of the monoclonal antibodies.**

The 15 monoclonal antibodies obtained from RIMV (Bilthoven, The Netherlands) were diluted in Casein-Tween 80 buffer. The dilutions for each of the monoclonals are listed in Table 2.2.

Table 2.2. Monoclonal antibody dilutions

Monoclonal antibody	Dilution
1	1 : 400
2a	1 : 4,000
2b	1 : 4,000
4	1 : 4,000
14	1 : 10,0000
15	1 : 4,000
P1.1	1 : 2,000
P1.2	1 : 4,000
P1.4	1 : 2,000
P1.6	1 : 2,000
P1.7	1 : 2,000
P1.9	1 : 1,000
P1.10	1 : 6,000
P1.12	1 : 4,000
P1.14	1 : 4,000
P1.15	1 : 2,000
P1.16	1 : 2,000

2.5.3. Method

A suspension of each isolate tested was prepared in PBS and the concentration adjusted to 3 of the McFarland scale. The suspensions were placed in a 56^{0}C water-bath for 45 minutes. Aliquots (100 µl) of each bacterial suspension were placed into individual wells of a series of flat-bottom, 96-well PVC microtitre plates (Greiner). The plates are allowed to evaporate overnight at 37^{0}C. The remaining bacterial suspension was kept at -20^0 C.

The coated plates were washed 3 times with 0.025% (v/v) Tween-80 in tap water. The monoclonal antibodies were diluted in casein-Tween buffer to block non-specific binding. The plates were incubated for 1 hr at 37^{0}C.

After washing, 100 µl of the protein A-peroxidase conjugate diluted 1:500 in Casein-Tween buffer was added to each well and the incubation was repeated as above. The plates were washed as above and freshly-prepared substrate (100 µl) was added to detect the bound antibodies. After a 10 min. incubation at room temperature, the reaction was terminated by the addition of 2 M H_2SO_4 (50 µl /well). The optical density was read at 450 nm with a Titertek Multiscan (Flow Laboratories). Standard meningococcal strains expressing the serotype or subtype antigens were included as controls for each experiment.

2.6. Agar dilution method for the determination of the Minimum Inhibitory Concentration (M.I.C.)

2.6.1. Materials and reagents

2.6.1.1. Medium. Muller-Hinton agar (38 g) (Oxoid), was dissolved in 1l of distilled water by gentle heating. Aliquots (49 ml) were made and the bottles autoclaved at 121^{0}C for 15-20 min.

2.6.1.2. Antibiotics. The antibiotics used and their respective diluents are shown in Table 2.3.

Table 2.3. Antibiotics used for MIC test and their respective diluents.

Antibiotic		Source	Diluent
Penicillin G	(PN)	Sigma	PBS
Erythromycin	(ER)	Abbot	Methanol
Tetracycline	(TC)	Sigma	Distilled water
Sulphamethoxazole	(SU)	Sigma	0.1 M NaOH
Rifampicin	(RF)	Lepetit	Methanol
Cefaclor	(CF)	Serva	Distilled water
Ciprofloxacin	(CP)	Merck	Distilled water

Each stock antibiotic solution was filtered through Millipore filters (pore size 0.45 µm). The range used for all the antibiotics was 0.015-256 mg/l. Two-fold dilutions of the stock solutions were prepared in sterile distilled water ; 1 ml of the each dilution was added to 49 ml Muller-Hinton agar (42^0 C) and the

bottle inverted gently to mix. The antibiotic - containing agar was poured into two petri-dishes (90 mm diameter).

2.6.1.3. Phosphate Buffered Saline (PBS) (0.15 M)

PBS was prepared from the following solutions: solution A, Na_2HPO_4 (42.58 g), distilled water (2 l) (pH = 9) ; solution B, KH_2PO_4 (10.25 g), distilled water (500 ml) (pH = 6); solution C, NaCl (8.76 g) distilled water (1 l) (pH = 6). To obtain a pH of 7.2, 2 l of solution A was mixed with 1 l of solution C. Approximately 500 ml of solution B was added until the pH reached 7.2. The solution was dispensed into aliquots, autoclaved for 15 minutes at 115^0C; and stored at room temperature.

2.6.2. Method

The strains under investigation were cultured on MNYC medium at 37^0C for 24 hr. Each strain was suspended in 5 ml PBS (pH=7.0), to a turbitidy of 0.5 McFarland scale (10^6-10^7 CFU/ml).The antibiotic-containing medium was inoculated with a Steers-type multiple inoculating device (Mast), 36 strains per Petri dish. The plates were incubated at 37^0C for 48 hr.

Readings were carried out at 24 and 48 hr. The MIC was the lowest concentration at which no growth was observed after both readings.

Each time the MIC test was performed, strains of known MIC values were always included, such as *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeroginosa* to check the potency of the antibiotic used.

For each test, medium with no antibiotic was always used before and after each series of media-containing antibiotics in order to check for contaminants.

3. Factors affecting carriage of *Neisseria meningitidis* among Greek military recruits

3.1 Introduction

The present work was prompted by an increase in the number of cases of meningococcal disease reported to the Ministry of Health. (Table 3.1). The last major survey on meningococcal carriage and the characteristics of these bacteria in Greek population had been undertaken before elucidation of some of the genetic and environmental factors affecting carriage as well as before the serotype and subtype antibodies were available (Kalapothaki *et al.,* 1979), and there was no information about antibiotic sensitivities of meningococci as carriage of these bacteria in a Greek population.

Table 3.1. Incidence of meningococcal disease in Greece.

YEAR	NO CASES	DEATHS	
		NO	(%)
1968	1064	48	(4.5)
1973	765	39	(5.1)
1978	286	18	(6.3)
1983	102	9	(8.8)
1985	105	6	(5.7)
1986	88	7	(7.9)
1987	85	4	(4.7)
1988	76	2	(2.6)
1989	87	6	(6.9)
1990	134	1	(0.7)

Epidemiological studies have shown that non-secretors, individuals incapable of secreting the glycoprotein form of their ABO blood group antigens, are significally over-represented among patients with meningococcal disease (Blackwell *et al.,* 1986a ; Blackwell *et al.,* 1988) and among carriers in a school population (Blackwell *et al.,* 1990a). Prolonged outbreaks have occurred in countries such as Iceland and in areas of Britain (Blackwell *et al.,* 1989a; Blackwell and Weir 1990b) where the proportion of non-secretors is significally higher than the proportion (20-25%) predicted for European populations (Mourant *et al.,* 1976). Environmental factors have also been suggested to play a role in susceptibility to meningococcal disease (Haneberg *et al.,* 1983; Stuart *et al.,* 1988) or carriage of the bacteria. Smoking or passive exposure to cigarette smoke has been associated with isolation of *Neisseria meningitidis* from healthy asymptomatic adults and teenagers (Blackwell *et al.*, 1990a ; Stuart *et al.*, 1989). Virus infections have been suggested as predisposing factors for susceptibility to meningococcal disease (Krakinski *et al.*, 1987; Moore *et al.*, 1990) and to carriage of potentially pathogenic bacteria (Musher *et al.*, 1981).

It was decided to assess the following questions by examining military recruits as there is usually a higher incidence in carriage in this population and the recruits examined came from all the regions of Greece.

i. Is isolation of meningococci associated with non-secretion in the populations examined ?

ii. Is smoking associated with carriage in closed population?

iii. Are upper respiratory tract or other infections factors contributing to carriage of meningococci?

iv. Are there geographical regions in Greece in which there are higher proportions of non-secretors and is the prevalence of meningococcal disease increased in these areas?

3.2. Subjects and methods

Greek military recruits (993) from two camps, one at Athens and another at Avlona, were examined for carriage of meningococci during the third week of July 1990. Ethical permission for the study was granted by the Ministry of Defense. An explanation of the purpose of the study and the specimens required was part of the questionnaire and each subject signed his consent to participate in the study. The recruits came from all regions of Greece except the Ionian islands and had been in the camps from 3 to 5 days at the time of the study. Throat swabs, blood and saliva specimens were obtained. Throat swabbing was carried out by the same four members of the team on both occasions. Blood was not obtained from a number of recruits who fainted because of the unusually high temperature (41^{0}C). Each recruit filled in a questionnaire providing information on age, occupation, places of residence and birth, smoking habits, and recent infections (Appendix 1). Also, their educational level was assessed (Table 3.2). In Greece, for epidemiological studies, socioeconomic category is assessed by the number of years of education and the highest level of school attended (Trichopoulos *et al.,* 1982).

Table 3.2. Education levels in Greece.

Category	Education Level	Years of Education
1	"Dimoticon"	6
2	"Gymnasion"	9
3	"Lykion"	12
4	Higher National Diploma	15
5	University	17

A member of the research team checked the form to ensure all categories had been completed and to answer any questions. This information and results of laboratory examinations were coded for confidentiality and stored in a Database 3 plus data base. The information on the data base was recalled and checked with the original questionnaire.

ABO blood group and Lewis antigens were determined by the methods described in Chapter 2.

The blood specimens were centrifuged on the day of collection and the plasma stored at -20^{0}C.

Throat swabs were plated directly onto modified New York City medium (Young *et al.*, 1978) and isolation and characterization of meningococci was carried out as described in Chapter 2.

Two-way frequency tables were analyzed by χ^2 tests with Yates' correction in the case of 2x2 tables. Multiple logistical regression was used to examine the association between carriage rates and other factors adjusted for each other.

3.3. Results

3.3.1. Isolation of *N. meningitidis*

N. meningitidis was isolated from 254 (25%) of the 992 recruits examined. The carriage rate differed significantly between the two camps. There were 79 isolates obtained from 432 recruits (18%) in the camp at Athens compared with 175 from the 560 (30%) at Avlona (χ^2= 17.54; P<0.0005).(Table 3.3)

Table 3.3. Carriage of *N. meningitidis* among recruits at Athens and Avlona

Location	Recruits	Carriers	
	(n)	(n)	(%)
Athens	432	79	18.2
Avlona	560	175	31.2
Total	992	254	25.6

3.3.2. ABO and Lewis blood groups

Distribution of ABO and Lewis blood group phenotypes: There was no difference in the distribution of the ABO blood groups between the two camps (Table 3.4).

Table 3.4. ABO Blood groups of the recruit population.

	ABO Blood Group [n (%)]			
Recruit Camp	**A**	**B**	**AB**	**O**
Athens	186 (44.8)	63 (15.2)	15 (3.6)	151 (36.4)
Avlona	217 (40.0)	77 (14.2)	28 (5.1)	220 (40.6)

Among the recruits who expressed Lewis antigens, there was a significantly higher proportion of Le^a/non-secretors in the camp at Avlona, 144/542 (26%), compared with that in the camp at Athens, 63/415 (15%) (χ^2=14.96, P<0.0005) (Table 3.5)

Table 3.5. Lewis blood group antigens of the recruit population

	Lewis phenotype n (%)			
Location	**Le^a**	**Le^b**	**Le^{a+b+}**	**Le^{a-b-}**
Athens	63 (15.4)	323 (77.8)	5 (1.2)	24 (5.8)
Avlona	144 (26.6)	353 (65.1)	41 (7.5)	4 (0.7)

3.3.3. Age and education

The ages of the two populations differed significantly. The median age was 19 for both camps; however there was a higher proportion of recruits under 19 in the camp at Avlona (4%) compared with that at Athens (1%) (χ^2=12.96, P<0.001). There was also a significant difference in the level of education of the recruits in the two camps, particularly at the two extremes. Among the recruits at Avlona 14% had only 6 years of schooling compared with 7% among those in Athens; and, only 2% of the recruits at Avlona had attended university while 7% of those at Athens had done so (χ^2=30.32, df=4, P<0.0005) (Table 3.6)

Table 3.6. Educational levels of the recruit populations

Education category	Recruit camp		Total (%)
	Athens n (%)	Avlona n (%)	
1	31 (7.4)	76 (14)	107 (11.1)
2	103 (24.5)	170 (31.3)	273 (28.4)
3	230 (55)	260 (47.9)	490 (50.1)
4	24 (5.7)	27 (5.0)	51 (5.3)
5	29 (6.9)	10 (1.9)	39 (4.0)

3.3.4. Smoking

There was no significant difference between the proportion of smokers in the Athens camp (62%) compared with Avlona (68%); but there were significantly more heavy smokers (>30 cigarettes a day) in Avlona (χ^2=27.64, df=4, P<0.0005) (Table 3.7).

Table 3.7. Cigarette consumption in the two recruit groups.

No cigarettes /day	Athens (n = 432) No (%)	Avlona (n = 560) No (%)
0	166 (38)	182 (32)
1-10	53 (12)	63 (11)
11-20	127 (29)	133 (24)
21-30	62 (14)	96 (17)
>30	23 (5)	86 (15)

3.3.5. Prevalence of other infectious diseases

Only 59 of the recruits reported symptoms of upper respiratory tract infections (URTI) within the previous 2 weeks, 21 of the 432 at Athens (4.9%) and 38 of the 561 (6.7%) at Avlona.

3.3.6. Factors associated with carriage of meningococci

There was no difference in the proportions of carriers among the four ABO blood groups. The proportion of carriers among Le^a/ non-secretors (22% at Athens, 32% at Avlona) was higher than that among Le^b/secretors (17% at Athens, 30% at Avlona), but the differences were not significant. The proportion of carriers was significantly higher among smokers and was greatest among those smoking more than 30 cigarettes per day (χ^2=23.19, df=4, P<0.0005).

Although the proportion of carriers was significantly increased among smokers in both camps (Athens χ^2=5.42, df=1, P<0.05; Avlona χ^2=9.05, df=1, P<0.01), when the data for carriers were analyzed there was no significant difference between the two camps associated with numbers of cigarettes per day (χ^2=9.482, df=4, P>0.05) (Table 3.8).

Table 3.8. Smoking patterns and carriage of *N. meningitidis* in the two camps.

No cigarettes /day	Athens (n=431)				Avlona (n=560)			
	Total		Carriers		Total		Carriers	
	No	(%)	No	(%)	No	(%)	No	(%)
0	166	(38)	20	(27)	182	(32)	36	(22.5)
1-10	53	(12)	10	(13)	63	(11)	19	(12)
11-20	127	(29)	26	(35)	133	(24)	40	(25)
21-30	62	(14)	15	(20)	96	(17)	29	(18)
>30	23	(5)	4	(15)	86	(15)	36	(22.5)
Total	**431**		**75**		**560**		**160**	

Meningococci were isolated from 17 (29%) of the 59 recruits who had symptoms of URTI, 2 of the 21 (9.5%) at Athens and 15 of the 38 (39.5%) at Avlona (Table 3.5). Among the 15 at Avlona from whom meningococci were isolated, 6 (40%) were Le^a/non-secretors compared with 4 (20%) Le^a/non-secretors among the 20 with recent symptoms of URTI but from whom no meningococci were isolated. The numbers of recruits with symptoms of URTI were too small for statistical analysis.

Multiple logistic regression analysis identified three main factors which were significantly associated with carriage: smoking ($P<0.001$); age less than 19 years ($P<0.01$) and the camp in which the recruits were based ($P<0.01$). Various combinations of categories were tested for difference in carriage rate when adjusted for these three factors but no other variables were found to be significant.

3.3.7. Regional variations in ABO and Lewis blood group antigens

There was no difference in the distribution of the ABO blood groups in the two major population centres, Athens and Eastern Macedonia. There was, however, a significantly higher proportion of Le^a/non-secretors, 54/183 (29.5%) among the recruits from Eastern Macedonia compared with those from Athens, 35/235 (15%) ($\chi^2=12.25$, $P<0.0005$).

3.4. Discussion

The proportion of carriers (25%) among the Greek recruits examined in this study, was similar to that found for a British military establishment (23%) (Pether *et al.,* 1988) but slightly lower than that found in the 1970s for Greek military recruits (33%) (Kalapothaki *et al.,* 1979). The significant difference in the proportion of carriers between the two camps, 18% at Athens compared with 30% at Avlona ($P<0.0005$), was not anticipated.

Smoking (Blackwell *et al.,* 1990a; Stuart *et al.,* 1989), passive exposure to cigarette smoke (Stuart *et al.,* 1989), secretor status (Blackwell *et al.,* 1990a) and age (Cartwright *et al.,* 1987) have all been reported to be associated with carriage of meningococci. The distribution of the ABO blood group phenotypes did not differ between the two camps and were similar to the survey carried out on *Helicobacter pylori* for the Greek population (Mentis *et al.,* 1990) and that reported by Hirzfeld and Hirzfeld in 1919. Several of the factors examined in the study varied between the two camps. Compared with the recruits at Athens, among those at Avlona where there was a significantly higher proportion of carriers of meningococci, there were significantly more heavy smokers, non-secretors, younger recruits (less than 19 years old), and recruits of lower socioeconomic groups as assessed by years of education.

Smoking was the factor most strongly associated with carriage in both univariate and multivariate analyses. It has been suggested that the higher proportion of smokers and heavy smokers among military personnel (Crowdy *et al.,* 1981) might contribute to the increased prevalence of meningococcal disease and the higher rates of carriage among military recruits. The proportions of smokers in the two camps, 57.7% and 63.4% were higher than the 47% found in the previous study among male Greek patients referred for gastroscopy (Mentis *et al.,* 1990). In Britain smoking is associated with socioeconomic status; unskilled individuals are more likely to smoke than those in professional groups (Wald *et al.,* 1988). A similar pattern was observed among the recruits; those with fewer years of education were more likely to be smokers.

In a previous study of teachers and pupils in a Scottish secondary school following an outbreak of meningitis, an association between non-secretion and carriage of meningococci was found (Blackwell *et al.,* 1990a). In the present study, in both camps, the proportions of non-secretors were

increased among carriers but these differences were not significant. Assessment of both host and parasite characteristics might elucidate the differences in association with secretor status. The majority (35.5%) of serotypable strains among the 121 isolates in the Scottish study were serotype 4; and 55% of the carriers of this serotype were non-secretors. Only 9 (1.8%) isolates in the present study were serotype 4, but 37.5% of the carriers of serotype Y were non-secretors. The proportion of non-secretors among carriers of serotype 14 isolates was 25% and 22% for the Scottish and Greek populations. A similar pattern was observed for non-serotypable isolates (Chapter 5).

The multiple logistic regression analysis identified age (those less than 19 years of age) to be significant variable ($P<0.01$). This agrees with the findings for carriers in the Stonehouse survey. The highest proportion of carriers was found in the 15-19 year age group (Cartwright *et al.,* 1987).

There is evidence that viral infections can predispose individuals to carriage of potentially pathogenic bacteria (Musher *et al.,* 1981); and binding of streptococci and staphylococci to cell surfaces is enhanced in experimental models examining infection with influenza virus (Plotkowski *et al.,* 1984; Sanford *et al.,* 1986). Infection by respiratory syncytias virus enhanced binding of meningococci to a human epithelial cell line (Hep-2) (Raza *et al.,* 1994). The study was carried out in July when there was a low prevalence of upper respiratory viral infections; however, of the 103 recruits with symptoms of virus infection, 19.8% were carriers. This was particularly apparent among recruits at Avlona where the carriage was 30% overall but nearly 40% among those with virus infections. In the recent study, a higher proportion of patients with meningococcal disease had a history of flu-like illness than age- and sex-matched controls; however, the differences were not statistically significant (Cartwright *et al.,* 1991).

In two areas of Britain where there have been prolonged outbreaks of meningococcal disease, the proportions of non-secretors have been reported to be higher than the 20-25% predicted for European populations (Blackwell *et al.,* 1989a; Blackwell and Weir 1990). In this study we found a significantly higher proportion of recruits from Eastern Macedonia were non-secretors (29.5%) compared with the other centre of population, Athens (15.3%). As there are anecdotal reports that meningococcal disease is more prevalent in Macedonia, the number of cases reported to the Ministry of Health from these two areas is now being closely monitored.

4. Parental smoking and carriage of *Neisseria meningitidis among* Greek schoolchildren

4.1 Introduction

In contrast to countries in northern Europe, there have been no reports on carriage rates of *N. meningitidis* among children in Greece and there had been no studies to assess risk factors associated with carriage of these bacteria among children in any country in south-east Europe. The present study had two objectives: the first was to determine if there was an increase in the carriage rate of meningococci among school children in Athens, particularly those with the serogroup B, serotype 2b, subtype P1.10 (B:2b:P1.10) phenotype isolated frequently from Greek children with meningitis (Chapter 5). The second was to examine genetic and environmental factors reported to be associated with meningococcal disease and/or carriage of these bacteria in northern Europe: age (Cartwright *et al.,* 1987); sex (Cartwright *et al.,* 1987; Olcen *et al.,* 1991); secretor status (Blackwell *et al.,* 1990a); smoking or passive exposure to cigarette smoke (Blackwell *et al.,* 1990a; Stuart *et al.,* 1988; Stuart *et al.,* 1989); lower socio-economic conditions (Cartwright *et al.,* 1987; de Wals *et al.,* 1983); upper respiratory tract infections (Krakinski *et al.,* 1987; Moore *et al.,* 1990; Olcen *et al.,* 1981).

Smoking and passive exposure to cigarette smoke were of particular interest as we had found active smoking was significantly associated with carriage among Greek military recruits (Chapter 3) and the proportion of smokers (62%) was higher compared with that among young men of a similar age range in Britain (40%) (Wald *et al.,* 1988).

4.2. Subjects and methods

Children (1038) from two areas of Athens were screened for pharyngeal carriage of meningococci during December 1990 and January 1991. Four schools were included in the study: a primary school and a secondary school in the south of Athens (Area A) where housing costs were low; and a primary school and a secondary school in the north of Athens (Area B) where housing costs were high.

Saliva was collected from each child who participated, and a throat swab was obtained. The throat swabs were plated directly onto modified New York City Medium (MNYC) (Young *et al.,* 1978); and the plates were stored at room temperature for approximately 2-3 hours before incubation at 37^0 C (chapter 2). Colonies were examined for Gram-stain, oxidase production and sugar utilization as described in Chapter 2.

The saliva was stored in insulated boxes and frozen within 3 hours of collection. Secretor status was determined from the saliva by enzyme linked immunnosorbent assays (ELISA's) for H antigen (Rahat *et al.,* 1990), $Lewis^a$ and $Lewis^b$ antigens (Raza *et al.,* 1991). Secretors will have in their saliva H, type I, $Lewis^b$ and variable amounts of $Lewis^a$ while non-secretors will have only $Lewis^a$.

The parent or guardian of each child gave written consent for the child to participate in the survey and filled in a questionnaire (Appendix 2) providing the following information: age and sex of child; if the child had any respiratory illness in the past week; number of individuals in the household; smoking habits of members of the household; education level of parents. In Greece, for epidemiological studies, socio-economic category is assessed by the number of years of education and the highest level of school attended

(Trichopoulos *et al.,* 1982). Secondary school children were asked if they smoked.

Two-way frequency tables were analyzed by Chi-squared tests with Yates' correction in the case of 2x2 tables. Multiple logistic regression was used to examine the association between carriage and other factors adjusted for each other.

4.3. Results

4.3.1. Assessment of socio-economic/educational level of parents in the two areas.

The two socio-economic indicators used, cost of housing and educational level of the parents, were in agreement. In this study, educational level of each parent was assigned to one of the five categories used in the study of the recruits (Chapter 3). Compared with area A, in area B there was a significantly lower proportion of parents in categories 1 and 2 and higher proportions of parents who had received technical training or attended university (χ^2= 474.52, df=4, P<0.0005).

Similar results were obtained when the data for mothers and fathers were analyzed separately (Table 4.1).

Table 4.1. Educational level of parents in the two areas.

		Education Category (%)					
	NO	**1**	**2**	**3**	**4**	**5**	
Mothers							
Primary A	179	40	25	30	3	2	a
Primary B	141	21	14	35	7	23	
Secondary A	373	43	17	17	2	1	b
Secondary B	324	17	14	40	8	21	
Fathers							
Primary A	180	38	28	20	12	2	c
Primary B	144	18	17	22	10	33	
Secondary A	380	54	21	15	7	3	d
Secondary B	334	17	13	24	8	38	

a. $\chi^2 = 45.93$, df = 4, P< 0.0005

b. $\chi^2 = 198.66$, df = 4, P< 0.0005

c. $\chi^2 = 60.34$, df = 4, P< 0.0005

d. $\chi^2 = 190.88$, df = 4, P< 0.0005

4.3.2. Carriage of meningococci

The carriage rate was 60/1038 (5.8%) and the proportion of isolates obtained from children in the two areas was similar. In area A, 30/565 (5.3%) of the pupils were carriers compared with 30/473 (6.3%) in area B. The proportion of carriers ranged from 2% at the primary school in area B to 8.2% in the secondary school of the same area (Table 4.2).

Table 4. 2. Isolation of meningococci from pupils in primary and secondary schools.

School	Number	Carriers	
		No	(%)
Primary A	180	9	(5.0)
Primary B	144	3	(2.1)
Secondary A	385	21	(5.5)
Secondary B	328	27	(8.2)

Univariate analyses found no association between carriage and two genetic factors examined, sex or secretor status: 32/560 (5.7%) girls were carriers as were 28/478 (5.9%) boys; 11/227 (4.8%) non-secretors were carriers compared with 49/473 (6.2%) secretors. There was no association between carriage and educational level of either parent or numbers of individuals per household and isolation of meningococci in either area. Among children who were carriers, 33% had symptoms of colds compared with 26.5% among children who were not carriers.

Among children in secondary schools, 14% of 50 smokers were carriers compared with 6.2% of 661 non-smokers (χ^2=3.34, df=1, P>0.05). The proportion of girls who smoked was 5.9% compared with 8.5% for boys. In secondary school A, 7.6% of children were smokers, and 5/30 (17%) smokers were carriers compared 16/352 (4.5%) non-smokers (χ^2=5.66, df=1, P<0.025). In secondary school B, 6% of school children were smokers, but only 2/20 (10%) smokers were carriers compared with 25/305 (8.2%) non-smokers.

Carriage rates were higher among children from households where adults smoked. Compared with the proportion of carriers from households in which none of the adults smoked (11/311, 3.5%), the proportion of carriers was significantly higher in those in which the mother or other carers (mainly grandparents) smoked (33/431, 7.7% χ^2=4.78, P<0.05). Children from households where only the father smoked had an intermediate level of carriage (16/296, 5.4%) which did not differ significantly from that in either of the other two groups. Carriage rates in households where the mother or other carers smoked did not depend significantly on whether only the mother, only the other carers or both smoked. There were 15 carriers under 13 years, and 5 (33%) had mothers who smoked. There were 45 carriers over 12 years, and 23 (51%) had mothers who smoked (χ^2=5.22, df=1, P<0.025). When the figures were corrected for the children in this group who were smokers, 20 of the 38 (52.6%) carriers who were non-smokers had mothers who smoked (χ^2=5.19, df=1, P<0.025). Among the fathers, smoking was significantly more prevalent among those with fewer years of education (χ^2=29.89, df=4, P<0.0005), but this association was not found among the mothers (χ^2=8.92, df=4, P>0.05). In area B where the educational level was higher, the proportion of fathers who smoked was significantly lower 223/472 (47%) compared with area A 345/560 (61%) (χ^2=20.77, df=1, P<0.0005). The differences were not found for mothers in the two areas; in area A, 33% were smokers compared with 38% in area B (Table 4.3).

Table 4.3. Sex, smoking and educational level

	Educational Category				
	No (%)				
Women[1]	**1**	**2**	**3**	**4**	**5**
smoker/total	130/395 (38)	68/177 (38)	117/ 299 (39)	14/47 (30)	30/109 (28)
Men[2]					
smoker/total	22/357 (62)	115/196 (59)	113/205 (55)	47/88 (53)	71/186 (38)

Compared with non-smokers:

[1] $\chi^2 = 8.92$, df=4, $P > 0.05$

[2] $\chi^2 = 29.89$, df=4, $P < 0.0005$

When the data on carriage were analyzed by multiple logistic regression with reference to age, sex, school attended, respiratory infection, parental smoking and the children's smoking habits, only age ($P<0.01$) and maternal or other carer's smoking ($P<0.05$) remained significant. Older children and those whose carers smoked were more likely to be carriers (Table 4.4). Although the trend with age appeared stronger in those whose carers smoked, this was not significant (interaction test in multiple logistic regression).

Table 4.4. Carriage rates in children classified by age and maternal smoking. (Figures are number/total (percent))

	AGE			
	YES	**5 - 9**	**10 - 14**	**15 - 19**
Carer in household smokes		2 / 74 (2.6%)	10 / 174 (5.7)	21 / 181 (11.6%)
	NO	4 / 108 (3.7%)	8 / 276 (2.9%)	15 / 223 (6.7%)

4.4. Discussion

The first objective of the study was to determine if there was an increase in the carriage rate of meningococci among school children which might reflect increased disease activity. The proportion of carriers (5.8%) did not differ from that usually found during non-epidemic periods. None of the isolates from the children expressed the B:2b:P1.10 phenotype commonly found among children in Athens with meningococcal disease (Chapter 5).

This was the first study that assessed factors associated with carriage of meningococci in a population in south-east Europe. Several factors identified in studies of meningococcal carriage in northern Europe did not appear to influence carriage in the Greek population examined. Among carriers in the Stonehouse study (Cartwright *et al.,* 1987) and in the Faroe Islands (Olsen *et al.,* 1991) , there was a higher proportion of males, but this was not observed for children in a Scottish study (Blackwell *et al.,* 1990a) or among Greek children. It was suggested that the similar proportion of males and females among Scottish children who were carriers might be due to the significantly higher proportion of girls who were smokers (Blackwell *et al.,* 1990a); however, the proportion of Greek boys who smoked was slightly higher than that of girls who smoked. In contrast to the Scottish study, which was carried out following an outbreak of meningococcal disease, many of the children in this survey were reluctant to answer questions about smoking; therefore, the proportion of smokers is probably underestimated. Also in contrast to the Scottish study, the proportion of non-secretors among carriers was not increased.

The isolation rate in different age bands followed a pattern similar to that found in the Stonehouse survey (Cartwright *et al.,* 1987); and, age was the most significant variable identified by multiple regression analysis.

In a study of families of patients with meningitis, carriage of meningococci was significantly associated with symptoms of upper respiratory infection (Olcen *et al.,* 1981). This was not observed among the school children in this study or among Greek recruits (Chapter 3)

There was good agreement between the two indicators used to assess socio-economic levels; however, in contrast to the Stonehouse survey (Cartwright *et al.,* 1987) and a longitudinal survey in Belgium (de Wals *et al.,* 1983), carriage rate was not greater in the area where socio-economic indicators were lower. In a case control study, Stuart and colleagues found no association between risk of carriage and social class (Stuart *et al.,* 1988) . They did find an increased carriage rate among individuals who lived with smokers and the association between passive exposure to cigarette smoke and meningococcal disease was noted among younger children who did smoke. In contrast, among Greek children, the proportion of smokers among mothers of older children (>12 years) who were carriers was significantly increased.

For the fathers in this study and in our previous study of Greek military recruits (Chapter 3) there was a significant correlation between educational level and smoking similar to that observed in the United Kingdom (Wald *et al.,* 1988).There were significantly more men who were smokers in area A where educational levels were lower, but carriage was not associated with father's smoking by either univariate or multivariate analysis.

In the United Kingdom, the pattern for socio-economic groups and smoking are similar for men and women (Wald *et al.,* 1988); however, there were no differences in smoking patterns of women associated with educational levels and no difference between the two areas of the city in the percentage of mothers who smoked. Parental smoking, particularly smoking by the mother, has been associated with upper and lower respiratory infection (Pershagen *et al.,* 1986; Fleming *et al.,* 1987) and impairment of ventilatory function (Pederira

et al., 1985; Tager *et al.,* 1983). If the mother's smoking is a significant factor contributing to carriage among children , this might explain the similar carriage rates for the two areas of Athens. The observation that carriage was also significantly associated with the smoking by adults other than the father might reflect the structure of many Greek families; grandmothers often live with families with young children and are closely involved with child care. The present study suggests that in Greece smoking by the mother or other family members traditionally involved in child care might contribute to carriage of meningococci.

5. Serogroups, serotypes and subtypes of *Neisseria meningitidis* isolated from patients and carriers in Greece

5.1. Introduction

Monoclonal antibodies to outer membrane proteins 2/3 and 1 have been used successfully to determine serotypes and subtypes of *N. meningitidis* in epidemiological studies in northern and western Europe as well as in the Americas (Poolman *et al.,* 1986; Poolman *et al.,* 1988; Abdillahi *et al.,* 1988; Wedege *et al.,* 1990; Blackwell *et al.,* 1990a). With the exception of results from Italy (Mastrantonio *et al.,* 1990), there was no information on these antigenic characteristics for meningococcal isolates from patients and carriers in south-eastern Europe. As the last major epidemiological survey of *N. meningitidis* in Greece was published in the 1970s before the availability of serotype and subtype reagents (Kalapothaki *et al.,* 1979), this part of the project had three main objectives: to determine if the serotype and subtype reagents used in northern Europe and America can discriminate between isolates of meningococci from south-eastern Europe; to determine if serogroups, serotypes and subtypes of Greek isolates from patients with meningococcal disease or carriers are similar to those in northern Europe and to determine if particular phenotypes are associated with different regions of Greece.

5.2. Materials and methods

Thirty-one isolates from children aged 1 month to 11 years with meningococcal disease were obtained from the microbiology laboratory of "Aglaia Kyriakou" Paediatric Hospital, Athens. The isolates were from sporadic cases. Fifty-one isolates from school children aged 5-18 years

who were carriers were obtained in surveys undertaken in Athens between December 1990 and January 1991 (Chapter 4). A series of 496 isolates from military recruits aged 16-30 years were obtained during July 1990 and Jan 1991 (Chapter 3) within 3-5 days of the arrival of the recruits at the camps; recruits from all regions of Greece were represented.

The method for isolation and identification of *N. meningitidis* are detailed in Chapter 2, as are the methods for the determination of serogroup, serotype and subtype.

Information obtained from each recruit regarding area of residence and socio-economic status was coded and entered into a Data Base 3 Plus program (Chapter 3) together with the results of the throat swab and the serogroup, serotype and subtype of the isolates (Chapter 3). Similar data bases were prepared for information obtained from questionnaires returned by parents of school children participating in the surveys (Chapter 4).

The results from the school children were compared with the meningococci isolated following an outbreak in a Scottish secondary school caused by a sulphonamide-resistant serogroup B serotype 4 subtype 15 (B:4:P1.15) strain (Blackwell *et al.,* 1990a), and results from the recruits were compared with published results for 133 isolates obtained from carriers among Norwegian military recruits (Wedege *et al.,* 1990).

5.3. Results

5.3.1. Patients

The antigenic phenotypes of isolates from patients are shown in Table 5.1. The most prevalent serogroup was B (58%) followed by group C (26%) and non-groupable isolates (15%). The majority of isolates (58%) did not react with any of the serotype antibodies. The most common serotype was 2b

(9 / 31, 29%), and the most common subtype was P1.10 (11/31, 35%). The most common serotype-subtype combination was 2b:P1.10 (7/ 31, 23%)

Table 5.1. Antigenic phenotypes of isolates from children with meningococcal disease.

Antigenic Phenotype	Number of isolates
B: - : -	3
B: - : P1.1	1
B: - : P1.9	4
B: - : P1.10	2
B: - : P1.14	1
B: 2b : P1.9	1
B: 2b : P1.10	4
B: 14 : P1.15	1
B: 15 : P1.6	1
C: - : P1.6	2
C: - : P1.10	3
C: - : P1.14	1
C: - : P1.16	1
C: 2b : P1.2	1
NG : 2a : P1.16	1
NG : 2b : P1.10	3
NG : 14 : P1.10	1

5.3.2. Carriers

Among school children there was no predominant antigenic phenotype. Of the 19 (37%) serogroupable isolates, 10 belonged to group B, two to each of serogroups A, C, W135 and Y and one to group Z. As with the isolates from patients, the majority (65%) did not react with any of the serotype antibodies; however, most (75%) reacted with one or more subtype reagents. Two isolates (4%) obtained from secondary school pupils expressed the 2b:P1.10 combination found among those from patients (Tables 5.2 and 5.3).

Of the 496 isolates from recruits, 36% were serogroupable: 24% group B; 5% group C; and 4% group A. Most isolates (69%) were not serotypable, but 64% reacted with one or more of the subtype antibodies. The most common subtype was P1.2 (16%) (Tables 5.2 and 5.3). The most prevalent combination of serotype-subtype antigens was 14: P1.2 (n=24) followed by 14:P1.7 (n=13). None of the isolates from the recruits expressed the 2b:P1.10 combination predominant among children with meningococcal disease. None of the 2a or 2b isolates from carriers reacted with any of the subtype antibodies.

Table 5.2. Comparison of serotypes of isolates from carriers in Greece with those from carriers in northern Europe

Percentage of isolates

	Recruits		School children	
Serotype	**Greece** (n =485)	**Norway** (n=133)	**Greece** (n=51)	**Scotland** (n=121)
1	4	5	8	9
2a	1	5	6	0
2b	5	0	8	5
4	2	22	8	36
8*	-	8	-	-
14	16	16	2	13
15	3	8	4	8
21*	-	8	-	7
Non-typable	69	28	64	22

* Not tested in this study

Table 5.3. Comparison of subtypes of isolates from carriers in Greece with those from carriers in northern Europe.

Percentage of isolates

	Recruits		School children	
Subtype	**Greece** (n =489)	**Norway** (n=133)	**Greece** (n=51)	**Norway** (n=121)
P1.1	9	5	14	13
P1.2	16	16	8	17
P1.3*	-	13	-	-
P1.4	1	0	0	0
P1.6	6	0	8	8
P1.7	6	0	4	7
P1.9	9	0	18	0
P1.10	6	0	10	0
P1.12	2	0	4	0
P1.14	2	0	0	0
P1.15	4	19	8	23
P1.16	3	8	0	4
Non-typable	69	28	64	22

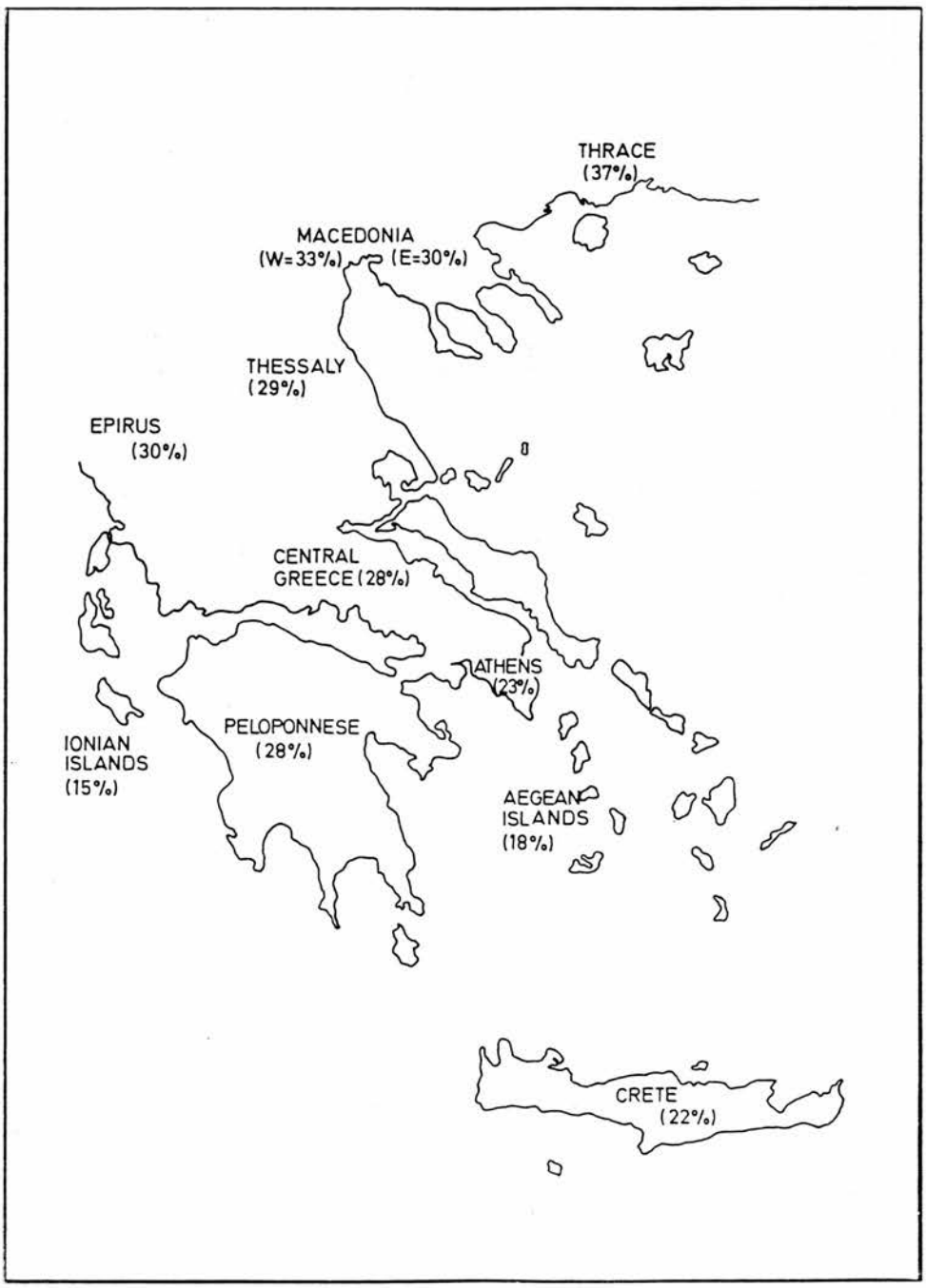
* Not tested in this study

5.3.3. Geographical distribution

The major regions of Greece are shown in figure 5.1 together with the proportion of recruits from whom meningococci were isolated. Meningococci were obtained most frequently from recruits from the north-east of Greece: Thrace (37%), West Macedonia (33%) and East Macedonia (30%). Because military service is compulsory, even for individuals who live abroad, there were 33 subjects who were not normally resident in Greece. Among these, 33% were carriers.

There were no unusual distributions of serogroups among the different regions. The most common serotype (14) was found in equal proportions (15%) among the isolates from the major centres of population, greater Athens and East Macedonia. Over 20% of the isolates from Thessaly (22%), the Peloponnese (21%), Crete (25%) and the Aegean Islands (30%) expressed the type 14 antigen. Isolates representative of all the type 11 subtypes examined were found among those from recruits from greater Athens, East Macedonia and Crete. More isolates that did not react with the subtype antibodies were obtained from recruits from the north of Greece: Thrace, Macedonia and Epirus.

Figure 5.1. Proportion of carriers among recruits from different regions of Greece.



5.4. Discussion

The first objectives of the study were to determine if the serotype and subtype reagents used for epidemiological studies in northern Europe and the Americas could be used in epidemiological studies in Greece, and if the serotype and subtype combinations associated with disease in other populations were found in Greece. The proportion of non-serotypable isolates from the Greek recruits (69%) and school children (64%) was greater than the proportion of non-serotypable isolates obtained from carriers among Norwegian recruits (28%) (Wedege *et al.,* 1990) or Scottish school children (22%) (Blackwell *et al.*, 1990a) . The proportion of serotype 14 isolates was similar for Greek (16%) and Norwegian (16%) recruits and Scottish school children (13%) but was much lower (2%) among Greek children. The proportion of 2b serotypes was similar for the Greek recruits and Scottish school children. Serotype 4 was the most prevalent type among the Norwegian (22%) (Wedege *et al.,* 1990) and Scottish (36%) isolates compared with 2-8% of the Greek isolates. Serotype 15 was found in 8% of isolates from north Europeans compared with 3-4% of the Greek isolates. This suggests that other serotype reagents need to be developed for epidemiological studies of meningococci from Greece and that the serotypes associated with recent outbreaks in Britain and Scandinavia are uncommon in Greece. Similar high proportions of non-serotypable isolates have been obtained in studies in Czech Republic (Kriz *et al.,* 1995).

The proportion of isolates from the Greek recruits (36%) and school children (26%) that did not react with any of the subtype reagents was similar to that of isolates from the Norwegian recruits (39%) (Wedege *et al.,* 1990) and from Scottish school children (38%). The proportion of subtype P1.2 isolates was similar for Greek (16%) and Norwegian (16%) recruits and Scottish schoolchildren (17%) but was lower among Greek schoolchildren (8%).

The proportion of subtype P1.15 was similar among the Norwegian (19%) (Wedege *et al.,* 1990) and Scottish (23%) isolates but was greater than that found in the Greek isolates (4-8%). The subtype reagents appear to discriminate between strains from Greek carriers as effectively as between carrier strains from northern Europe; however, the proportions of subtypes associated with outbreaks in northern Europe are lower.

Combinations of serotype and subtype antigens 15: P1.16, 4:P1.15 and 2a:P1.2 associated with outbreaks in northern Europe (Poolman *et al.,* 1988; Abdillahi *et al.,* 1988; Blackwell *et al.,* 1990a) were not found among any of the isolates in this study. Although P1.2 was the most common subtype found in the study, there was only one patient isolate with this type, C:2b:P1.2 (Table 5.1). In contrast, the 2a:P1.2 and 2b:P1.2 combinations are found frequently among Scottish group C (Fallon *et al.,* 1991) and French serogroup B isolates and 2a:P1.2 is found frequently among French serogroup C isolates (Riou *et al.,* 1989). The only report of meningococcal serotypes from southeast Europe is that of the C:2a phenotype associated with disease in Italy (Mastrantonio *et al.,* 1990). None of the serogroup C isolates expressed this serotype.

The third objective was to determine if there were antigenic phenotypes associated with different areas of Greece, e.g., those in which there are large numbers of foreign tourists or foreign military personnel or areas such as the northeast were there is a significant Muslim population some of which might have visited Saudi Arabia; however, there was no unusual distribution of serogroups or serotypes in the different regions of Greece.

The results of the study suggest that vaccines based on serotypes and subtype combinations associated with disease in northern Europe such as the one being developed and tested in Norway will not be appropriate for Greece.

6. Antibiotic sensitivities of *Neisseria meningitidis* isolated from patients and carriers in Greece

6.1. Introduction

As part of the surveys of isolates of meningococci from patients and carriers in Greece, the antibiotic sensitivities of these bacteria to some common antibiotics were determined. There are reports of *N. meningitidis* with reduced sensitivity to penicillin isolated from patients in Spain (Saez-Nieto *et al.,* 1987; Saez-Nieto *et al.,* 1990), South Africa (Botha *et al.,* 1988) and the United Kingdom (Sutcliffe *et al.,* 1988; Jones *et al.,* 1990). A small proportion of these penicillin-insensitive isolates has also been found among those obtained from carriers in the United Kingdom (Sutcliffe *et al.,* 1988). In view of the increasing prevalence of penicillin- insensitive isolates in Spain and the similar antibiotic policies of both Spain and Greece, it was predicted that there might be significant numbers of Greek isolates with decreased sensitivity to penicillin and other antibiotics. The objectives of the study were: (1) to compare levels of sensitivities for isolates obtained from patients with those obtained from carriers, (2) to determine if the proportion of penicillin-insensitive isolates is comparable to that reported for Spain; (3) to assess the distribution of resistant isolates in the different regions of Greece (4) to determine if antibiotic resistances are associated with particular serogroups, serotypes or subtypes.

6.2. Materials and methods

Thirty-one isolates from children aged 1 month to 11 years were provided by the microbiology laboratory of the "Aglaia Kyriakou" Paediatric Hospital. All cases were sporadic and occurred during 1989-91. Four hundred and seventy-two isolates were obtained during 1990-91 from healthy recruits aged

16-31 years (Chapter 3) and a further 47 isolates obtained from the surveys of school-children aged 6-18 years (Chapter 4).

Antibiotic sensitivities to penicillin (PN), erythromycin (ER), tetracycline (TC), sulphamethoxazole (SU), rifampicin (RF), cefaclor (CF) and ciprofloxacin (CP) were determined by the agar dilution method (Chapter 2). A range of two-fold dilutions from 256 to 0.015 mg/l of each antibiotic in Mueller-Hinton medium was examined. The breakpoints used for penicillin (> 0.125 mg/l), tetracycline (>1 mg/l), erythromycin (> 0.5 mg/l), rifampicin (>1 mg/l, ciprofloxacin (>1 mg/l) and the cefalosporin cefaclor (>4mg/l) were based on those recommended by the working party of the British Society for Antimicrobial Chemotherapy for *Branhamella catarrhalis* and *Haemophilus influenzae* (AAC working party 1988). The break point of >10 mg/l for sulphamethoxazole (SU) was that used by the Neisseria Reference Laboratory (Scotland) [Dr. R.J. Fallon, personal communication]. Beta-lactamase production was assessed during the rapid carbohydrate fermentation test for identification of each isolate (Young 1978). Serogroups, serotype and subtype of each isolate was determined as described in Chapter 2.

6.3. Results

6.3.1. Antibiotic sensitivities of isolates from patients and carriers

Table 6.1 compares the proportion of resistant isolates among the three groups examined. Among 31 isolates obtained from children with meningococcal disease, none was resistant to rifampicin or ciprofloxacin. Nearly half the isolates were penicillin- insensitive (48.3%) and all grew on 1 or 2 mg/l tetracycline. Among the 47 isolates from schoolchildren the pattern of resistance to ciprofloxacin, cefaclor and sulphamethoxazole was similar to that of isolates from patients. The proportion resistant to erythromycin (61.7%) was over twice that among isolates from patients

(25.8%) (χ^2 =8.27, df=1, P 0.005); and there were some rifampicin resistant strains (6.4%). Compared with the isolates from patients, the proportion resistant to tetracycline was much less (6.4%) (χ^2=62.83, df=1, P 0.0005) as was the proportion with decreased sensitivity to penicillin (19.1%) (χ^2=7.61, df=1, P< 0.01).

The proportions of 472 isolates from recruits resistant to tetracycline or penicillin were similar to those of isolates from patients. The proportions of isolates from recruits resistant to erythromycin or to rifampicin were similar to that for isolates from children who were carriers. Compared with the other two groups, there was a higher proportion of isolates from recruits resistant to sulphamethoxazole and cefaclor. There were 112 isolates (23.7%) with MIC> 4 mg/l cefaclor and 32 isolates (6.8%) with MIC> 8 mg/l. Of the latter 32 isolates, 27 (84%) had decreased sensitivity to penicillin. There was one isolate with MIC > 1 mg/l to ciprofloxacin. None of the 550 isolates from patients and carriers produced beta-lactamase.

Table 6.1. Proportion of antibiotic-resistant isolates among patients and carriers.

	Percent resistant		
Antibiotic	**Patients** (n = 31)	**Carriers** **Children** (n=47)	**Recruits** (n=472)
Penicillin G	48.3	19.1	36.7
Sulphamethoxazole	16.1	10.6	35.6
Rifampicin	-	6.4	7.0
Tetracycline	100	6.4	91.0
Erythromycin	25.8	61.7	82.2
Cefaclor	9.7	6.4	23.7
Ciprofloxacin	-	-	0.2

6.3.2. Geographical distribution of resistant isolates

In the two major population centers, Eastern Macedonia and Athens, the proportions of penicillin-insensitive isolates were 33 and 40% respectively. The highest proportions of such isolates were found in Thrace (50%), Crete (52%) and the Aegean and Ionian Islands (50%). The lowest proportion was obtained from recruits who lived abroad but had returned to Greece for their compulsory military service (9%) (Table 6.2). Analysis of the serogroups, serotypes, subtypes and sensitivities to other antibiotics indicate that the isolates within a particular geographical area with decreased sensitivity to penicillin are not related.

Table 6.2. Proportion of antibiotic-resistant isolates obtained from recruits from different regions of Greece

		% resistant			
Region	**Number tested**	**PN**	**SU**	**RF**	**CP**
Thrace	14	50	79	7	-
W. Macedonia	38	26	42	5	-
E. Macedonia	96	33	35	7	-
Epirus	23	48	30	4	-
Thessaly	46	34	48	6	-
Central Greece	32	32	44	9	-
Athens	127	40	39	7	0.8
Peloponese	40	35	30	10	-
Crete	31	52	39	3	-
Aegean Islands	10	50	70	10	-
Ionian Islands	2	50	-	50	-
Foreign	11	9	22	-	-

6.3.3. Analysis by antigenic phenotypes

Among the isolates from patients with meningitis, 9/18 (50%) of group B, 7/18 (37.5%) of group C; and 11/18 (60%) of the non-groupable isolates were penicillin-insensitive. Sulphonamide resistance was found in 3/18 (17%) of the group B isolates and 4/18 (25%) of group C but in none of the non-groupable isolates. Of the 9 2b serotype isolates, 7 (78%) were penicillin-insensitive as were 8/18 (44%) of the non-typables. The 4 isolates expressing other serotypes (2a, 14, and 15) were sensitive to penicillin and to sulphamethoxazole. Resistance to sulphamethoxazole was found for only 2/9 (22.2%) serotype 2b isolates and 3/18 (16.6%) non-typable isolates. Of the 9 isolates expressing the P1.10 subtype, 7 (78%) were penicillin-insensitive though only 1 was resistant to sulphamethoxazole. The second most common subtype was P1.9. Only 1 of the 5 P1.9 isolates was penicillin-insensitive, but 2 were sulphonamide resistant.

There were sufficient numbers of isolates from the recruits for analysis of antibiotic resistances by serogroup, serotype and subtype (Tables 6.3-6.5). Among the serogroups, the highest proportion of penicillin-insensitive isolates was found for group Y (62.5%) while rifampicin resistance was greatest in groups A (12%) and C (10.5%) (Table 6.3).

Table 6.3. Antibiotic resistances of isolates from recruits analyzed by serogroup

Serogroup	No	% resistant		
		PN	SU	RF
A	17	41	41	12
B	114	35	37	5
C	19	42	42	10.5
W-135	2	0	50	0
Y	8	62.5	25	0
Z	4	25	0	0
NG	300	36	40	7

Penicillin-insensitivity was highest among serotypes 2a and 15 and lowest in type 4, which, however, had the highest proportion of strains resistant to sulphamethoxazole. Rifampicin resistance was observed most frequently among serotypes 4 and 15 (Table 6.4).

Table 6.4. Antibiotic resistances of isolates from recruits analyzed by serotype

Serotype	No	% resistant		
		PN	SU	RF
1	15	27	20	7.0
2a	7	57	43	0
2b	22	27	32	4.5
4	9	11	56	11.0
14	66	42	28	9.0
15	16	56	19	12.5

With the exception of a high proportion of sulphamethoxazole resistant isolates associated with P1.7 (63%) and penicillin insensitive isolates associated with serotype P1.14 (62.5%), no particular subtype antigen was associated with an unusual degree of antibiotic resistance (Table 6.5).

Table 6.5. Antibiotic resistances of isolates from recruits analyzed by subtypes

Sero-subtype	No	% resistant		
		PN	SU	RF
P1.1	41	39	34	2.5
P1.2	72	43	22	15.5
P1.4	7	43	29	0
P1.6	28	32	68	0
P1.7	30	43	63	13
P1.9	45	27	38	2.4
P1.10	29	45	28	14
P1.12	8	25	37.5	0
P1.14	8	62.5	25	0
P1.15	16	37.5	19	0
P1.16	13	38	70	23

6.4. Discussion

Although sulphonamide resistance has been associated with strains causing outbreaks of meningococcal disease in northern Europe (Cartwright *et al.,* 1987; Poolman *et al.,* 1986), only 16% of the isolates from Greek patients had MIC> 10 mg/l of sulphamethoxazole; however nearly half had reduced sensitivity to penicillin and all grew in the presence of 1 or 2 mg/l tetracycline. In contrast, while the proportions of penicillin-insensitive and of tetracycline resistant isolates from children who were carriers were both significantly lower, the proportion of erythromycin isolates was significantly increased.

Among isolates from the recruits, the proportion of penicillin insensitive strains was significantly greater than that from children who were carriers (χ^2=5.126, df=1, P<0.025) but not compared with those from children with disease. The proportion of sulphonamide-resistant isolates among those recruits (35.6%) was over twice that of the other two groups, carriers (10.6%) (χ^2 =10.882, df=1, P<0.005) or patients (16%) (χ^2=4.06, df=1, P<0.05) (Table 6.1).

Although penicillinase-producing isolates of *N. gonorrhoeae* are not uncommon in Greece (18%) (Tzanakaki *et al.,* 1989), none of the 550 meningococcal isolates tested produced beta-lactamase. The levels of resistance to erythromycin among isolates from carriers might reflect longer exposure to the antibiotic as the mean ages of the carriers are higher, school-children (12.8 years) and recruits (19.7 years) compared with a mean age of 2.7 years for patients with meningitis. Only one isolate had MIC > 1 mg/l to ciprofloxacin. Since 6-7% of isolates from carriers were resistant to rifampicin, ciprofloxacin might be considered for circumstances when chemoprophylaxis would be appropriate as it has been found to eradicate pharyngeal carriage of meningococci (Gaunt *et al.,* 1988).

Sutcliffe and colleagues (Sutcliffe *et al.,* 1988) stated that in view of the extensive use of penicillin in the community in Britain, emergence of penicillin-insensitive meningococci is not unexpected. In Britain, the proportion of isolates from patients and carriers with MIC >0.16 mg/l was about 3% (Sutcliffe *et al.,* 1988). In Spain in 1986, only 5% of isolates examined had MIC> 0.1 mg/l; however, by 1990, this figure had risen to 46% of isolates from patients. The antibiotic policies of Spain and Greece are similar and the proportion of penicillin-insensitive isolates from Greek children with meningococcal disease (48.3%) was similar to that reported for Spain. In Britain, the proportion of penicillin-insensitive isolates from carriers was slightly less than 3% (Sutcliffe *et al.,* 1988), much lower than the proportion of penicillin-insensitive strains from carriers in this survey, recruits (36%) or school-children (19.1%). Differences in MIC values to tetracycline between isolates from patients and isolates from children who were carriers was not expected. Although high level tetracycline resistance mediated by a plasmid carrying the *tetM* determinant has been found in meningococci (Knapp *et al.,* 1988), none of the isolates had MIC>8 and the 25.2 megadalton plasmid was not observed in any of the strains examined so far. Strains were sent to Dr. M. Roberts of the University of Washington for analysis of the chromosomal *tetM*. The gene was not identified in any of these strains (Dr. M. Roberts, personal communication).

The differences in the proportions of penicillin-insensitive isolates in the different regions of Greece is under investigation (Table 6.2). That there were fewer such isolates among recruits who lived outside Greece (mainly Germany) might reflect differences in antibiotic policies referred to earlier. Although 61% of the British isolates which were penicillin-insensitive were also sulphonamide resistant, none were resistant to rifampicin, aminoglycosides, erythromycin, tetracycline or chloramphenicol (Sutcliffe *et al.,* 1988). Only 37% of the penicillin-insensitive isolates in this study were sulphonamide resistant; however, additional resistances were often present: rifampicin (8%); erythromycin (33%); cefaclor (34%); tetracycline

(96%). While none of the penicillin-insensitive strains in Britain was serogroup A (Sutcliffe *et al.,* 1988), 41% of the Greek group A isolates had reduced sensitivity to penicillin. The highest proportion of penicillin-insensitive isolates was among the 8 group Y isolates (62.5%). Analysis by areas of residence of the recruits, serotype, subtype and sensitivities to other antibiotics, suggest these group Y isolates are not related. Although serogroup B was the most common among patient isolates, the greatest proportion of isolates resistant to rifampicin was among those of groups A and C.

The number of serotypable isolates was small and larger numbers need to be examined to determine if the associations noted are significant (Table 6.4). Most of the penicillin-insensitive isolates expressing subtype P1.14 do not appear to be a single strain as assessed by the serogroup, serotype, sensitivities to other antibiotics and the area of residence of the recruits from whom the bacteria were isolated. Although the sulphonamide and rifampicin resistances were highest among subtype P1.16, the two resistances were not found together in the P1.16 strains. The higher rate of rifampicin resistance among isolates expressing P1.10, the subtype found frequently among meningococci isolated from patients, and among isolates expressing P1.2 (the most common subtype among carriers), needs to be investigated.

7. Investigation of the *N. meningitidis* strains isolated in Romania

7.1. Introduction

The epidemiological investigation carried out in Greece the last 3 years *on N. meningitidis* has shown that the majority of the meningococcal isolates obtained from patients and carriers in Greece are not typable with the reagents used in north-west Europe; therefore, vaccines based on these antigens would be of little value in Eastern regions. The population movements that have occurred among former Eastern Bloc countries since the end of 1989 might have introduced strains not encountered outside these countries whose residents have been isolated for over 40 years.

In addition, our studies indicated that the Greek meningococcal strains differ from those in northern Europe is that there is an increase in the proportion of isolates from both patients and carriers with reduced sensitivity to penicillin. The above findings, prompted additional investigations of meningococcal strains isolated in the Balkan area, since the serotype-subtype techniques as well as the antibiotic susceptibility testing are not carried out these countries. The objectives of the present study were: (1) to determine whether the serotype and subtype reagents used in northern Europe can discriminate between isolates of meningococci from south - eastern Europe ; (2) To determine if the serogroups, serotypes and subtypes of Romanian isolates are similar to those found in Greece (3) to assess the levels of antibiotic sensitivities for strains isolated from patients and carriers (4) to determine if there is an association between the antibiotic resistance with particular serogroups, serotypes or subtypes.

7.2. Materials and methods

7.2.1. Isolates

A total of 61 meningococcal isolates were received from the Meningococcal Reference Laboratory from the Cantacusino Institute, Bucharest, Romania.

The isolates were received in a freeze-dried form, and from the 61 strains 30 were revived. From the above strains, 13 isolates were from carriers and 17 were from patients with meningococcal disease. The period of isolation was from 1969-1993.

7.2.2. Methods

The isolates were reconstituted from the freeze-dried in distilled water and cultured on Modified New York City medium. The isolation and identification techniques, as well as the determination of the serogroup, serotype, subtype are as described in Chapter 2. Sensitivities to antibiotics were assayed as described in Chapter 7.

7.3. Results

7.3.1. Serogroup, serotype and subtype of Romanian isolates

A total of 30 meningococcal isolates from patients and carriers were examined for their antigenic characteristics (Table 7.1). As for their antigenic phenotypes of the isolates, the most prevalent serogroup was B (53.3%) followed by group A (13.3%) and C (10%).The percentage of the non-groupable isolates was 10% and 6.7% of the isolates were polyagglutinable (Table 7.1).

Table 7.1 Distribution of the serogroups among the Romanian isolates.

Serogroup	Patients (%)	Carriers (%)	Total (%)
A	2 (11.8)	2 (15.4)	4 (13.3)
B	12 (70.5)	4 (30.7)	16 (53.3)
C	1 (5.9)	2 (15.4)	3 (10)
Y	1 (5.9)	1 (7.7)	2 (6.7)
NG	1 (5.9)	2 (15.4)	3 (10)
Polyvalent	0	2 (15.4)	2 (6.7)
Total	**17 (100)**	**13 (100)**	**30 (100)**

The majority of isolates (66.7%) did not react with any of the serotype antibodies ; however, most (80%) reacted with the subtype reagents.

The most common serotype was 4 (5 / 30, 16.6%), and the most common subtype was P1.15 (9/30, 30%) (Tables 7.2, 7.3) The most common serogroup/serotype combination was B:NT:P1.15 (6/30) and A:4:P1.10 (Table 7.4).

Table 7.2 Serotypes of isolates from patients and carriers from Romania

	isolates from (%)		
Serotype	**Patients**	**Carriers**	**Total**
1	1 (5.9)	-	1 (3.3)
2a	-	2 (15.4)	2 (6.7)
2b	-	-	0
4	4 (23.5)	1 (7.7)	5 (16.6)
14	1 (5.9)	1 (7.7)	2 (6.7)
15	-	-	0
N.T.	11 (64.7)	9 (69.2)	20 (66.7)
Total	**17**	**13**	**30**

Table 7.3 Comparison of subtypes of isolates from patients and carriers in Romania.

	isolates from (%)		
Subtype	**Patients**	**Carriers**	**Total**
P1.1	1 (5.9)	2 (15.4)	3 (10)
P1.2	1 (5.9)	-	1 (3.3)
P1.4			
P1.6	1 (5.9)	1 (7.7)	2 (6.7)
P1.7	-	2 (15.4)	2 (6.7)
P1.9	-	1 (7.7)	1 (3.3)
P1.10	3 (17.6)	-	3 (10)
P1.13	-	1 (7.7)	1 (3.3)
P1.14	1 (5.9)	1 (7.7)	2 (6.7)
P1.15	6 (35.3)	3 (23.1)	9 (30)
P1.16	-		
N.T.	4 (23.5)	2 (15.4)	6 (20)
Total	**17** (100)	**13** (100)	**30** (100)

Table 7.4 Antigenic phenotypes of isolates from Romania

Antigenic Phenotype	No of isolates
B: 1 : -	1
B: - : P1.1	1
B: - : P1.2	1
B: - : P1.6	2
B: - : P1.15	6
B: 14: P1.15	1
B: - : P1.7	1
C: - : P1.7	1
C: 2a: P1.13	1
A: 4 :P1.10	2
A: 14 :P1.1	1
A: 2a :P1.1	1
N.T.	11

7.3.2. Antibiotic sensitivities of Romanian isolates

Among the 30 Romanian isolates, none was resistant to rifampicin or ciprofloxacin. The proportion resistant to tetracycline was 30%; the proportion with decreased sensitivity to penicillin was 10%; and the proportion resistant to erythromycin 16.7%. The majority were resistant to sulphamethoxazole (63.3%) and to cefaclor (46.7%) (Table 7.5). None of the 30 isolates produced beta-lactamase.

Table 7.5 Proportion of antibiotic-resistant isolates among.

	isolates resistant		
Antibiotic	**Patients** (n=17)	**Carriers** (n=13)	**Total**
Penicillin G	1 (5.9)	2 (15.4)	3 (10)
Sulphamethoxazole	9 (53)	10 (76.9)	19 (63.3)
Rifampicin	-	-	0
Tetracycline	6 (35.3)	3 (23)	9 (30)
Erythromycin	2 (11.8)	3 (23)	5 (16.7)
Cefaclor	9 (53)	5 (38.5)	14 (46.7)
Ciprofloxacin	-	-	0

7.3.3. Analysis by antigenic phenotypes

Among the isolates, 12.5% of group B, 25% of group A and all of the non-groupables and polyvalent strains were penicillin-insensitive. Sulphonamide resistance was found in 43.7% of the group B isolates and 75% of group A and in all of the non-groupable and polyvalent isolates (Table 7.6).

Table 7.6. Antibiotic resistances among serogroups.

Serogroup	No	Percent resistant	
		PN	SU
A	4	25	75
B	16	12.5	43.7
C	3	-	100
Y	2	-	-
Polyvalent	2	100	100
NG	3	100	100

Of the 5 isolates belonging to 4 serotype, none was penicillin-insensitive. Both strains belonging to serotype 1 were penicillin-insensitive. The rest of the isolates expressing other serotypes (2a,2b,14,15) were sensitive to penicillin. Resistance to sulphamethoxazole was found for both 2a isolates, for 40% of the strains of the serotype 4 and 50% of the serotype 1 (Table 7.7).

Table 7.7 Antibiotic resistances among serotypes

Serotype	No	Percent resistant	
		PN	SU
1	2	100	50
2a	2	-	100
2b	-	-	-
4	5	-	40
14	2	-	-
15	-	-	-

Of the 10 isolates expressing the P1.15 subtype, 7 (22.2%) were penicillin-insensitive and 80% were resistant to sulphamethoxazole. The second most common subtype was P1.1; and 25% of the isolates were penicillin-insensitive, but none sulphonamide resistant (Table 7.8).

Table 7.8 Antibiotic resistances among subtypes

Sero-subtype	No	Percent resistant	
		PN	SU
P1.1	4	25	-
P1.2	1	-	-
P1.4	-	-	-
P1.6	2	-	50
P1.7	1	100	100
P1.9	1	-	-
P1.10	3	-	33.3
P1.12	-	-	-
P1.14	2	-	50
P1.15	10	22.2	80
P1.16	-	-	-

7.4. Discussion

The first objectives of the study were to determine if the serotype and subtype reagents used for epidemiological studies in northern Europe and the Americas could be used in epidemiological studies in Eastern Europe and if the serotype and subtype combinations associated with disease in other populations were found in Eastern Europe.

The proportion of non-serotypable isolates from the Romanian isolates from patients (64.7%) was higher to that observed among the Greek isolates from patients (58%) and still greater than the proportion of non-serotypable isolates obtained from Northern Europe (Wedege *et al.,* 1990). The proportion of serotype 14 isolates (6.7%) was similar to Greek (6.4%) isolates (Table 5.2). Although the 2b serotype was the predominant serotype for the Greek isolates (29%), it was not observed among the Romanian isolates. In contrast, serotype 4 (16.6%) (23.5% in patient and 7.7% in carrier strains) was the most prevalent type among the Romanian isolates; a similar pattern was observed among those from Norway (22%) (Wedege *et al.,* 1990) and Scotland (36%) (Blackwell *et al.,* 1990a). Serotype 4 was observed only among the Greek carrier strains where the proportion was 2-8% (Table 5.2). Serotype 15 was found in 8% of isolates from north Europeans compared with 6.4% of the Greek isolates and none of the Romanian strains. This suggests that other serotype reagents need to be developed for epidemiological studies of meningococci in the Eastern Europe and that the serotypes associated with recent outbreaks in Britain and Scandinavia are uncommon in these populations.

There was a lower proportion of isolates from the Romania (23.5%) as well as the Greek patients' strains that did not react with any of the subtype reagents, in contrast to the Greek carrier isolates (36%). The proportion of subtype P1.2 isolates was similar for Romanian (3.3%) and Greek (3.3%) isolates and was

much lower than that found among the Greek (16%) and Norwegian recruits (16%) and also lower than the Scottish and Greek schoolchildren (17% and 8% respectively). The most predominant subtype was P1.15 (30%) (35.3% in patient and 21.3% in carrier isolates) and this was higher than the percentage found among the Norwegian (19%) (Wedege *et al.,* 1990) and Scottish (23%) isolates but was much higher than that found in the Greek isolates (4-8%).

Combinations of serotype and subtype antigens 15: P1.16, 4:P1.15 and 2a:P1.2 associated with outbreaks in northern Europe (Poolman *et al.,* 1988; Abdillahi *et al.,* 1988) were not found among any of the isolates in this study. Although P1.15 was the most common subtype found in the study, none of these isolates expressed the 4:P1.15 combination. The 2a:P1.2 and 2b:P1.2 combinations are found frequently among Scottish group C (Fallon *et al.,* 1991) and French serogroup B isolates and 2a:P1.2 is found frequently among French serogroup C isolates (Riou *et al.,* 1989). The only report of meningococcal serotypes from south-east Europe is that of the C:2a phenotype associated with disease in Italy (Mastrantonio *et al.,* 1990). None of the Romanian serogroup C isolates expressed this serotype.

Sulphonamide resistance has been associated with strains causing outbreaks of meningococcal disease in northern Europe (Cartwright *et al.,* 1987; Poolman *et al.,* 1986). Most of the Romanian strains were resistant; 19/30 strains (63.3%) had MIC> 10 mg/l of sulphamethoxazole (53% in patients and 76.9% in carriers) in contrast to the strains isolated from Greek patients where only 16% of the isolates were resistant to sulphamethoxazole. Only 10% of the Romanian strains had reduced sensitivity to penicillin in contrast to the Greek isolates (48.3%) and 30% grew in the presence of 1 or 2 mg/l tetracycline compared with higher levels found among Greek isolates (Table 6.1). The levels of resistance to erythromycin was lower (16.7%) than that found among the Greek strains (25.8%). The proportion of penicillin-insensitive isolates from Greek children with meningococcal disease (48.3%) was found to be similar to that reported for Spain, since the antibiotic

policies of Spain and Greece are similar. The same pattern was not observed among the Romanian strains; only 5.9% of the isolates were penicillin insensitive. The percentage was not as low as of that in Britain (2%), but it was certainly lower than that found in other Mediterranean countries (Greece, Spain).

Among the Romanian strains, 75% of the penicillin-insensitive isolates were sulphonamide resistant, a pattern similar to that found in Britain (Sutcliffe *et al.,* 1988). While none of the penicillin-insensitive strains in Britain was serogroup A, (Sutcliffe *et al.,* 1988), 25% of the Romanian group A isolates had reduced sensitivity to penicillin. The highest proportion of penicillin-insensitive isolates was among the non- groupable as well as the polyagglutinable isolates (100%). The number of serotypable isolates was small and larger numbers need to be examined to determine if the associations noted are significant.

The results of the study suggest that as with the Greek isolates, that vaccines based on serotypes and subtype combinations associated with disease in northern Europe such as the one being developed and tested in Norway will not be appropriate for the Eastern European countries. This difference between the percentage of resistant strains between Romania and Greece might be due to differences in antibiotic policies between the two countries. At least until 1989 there were stricter policies on the use of antibiotics in all Eastern European countries (Dr. P. Kriz and Dr. F. Mihalcu, personal communication).

Although the antibiotic policy in Romania was more strict than in Greece, the appearance of penicillin-insensitive strains as well as the high proportion of sulphonamide resistance strains suggest that antibiotic sensitivities need to be more closely studied in larger numbers of isolates.

8. Genotypes of meningococci in Greece and Romania

8.1. Introduction

Meningococci are classified into serogroups, serotypes and subtypes on the basis of phenotypic characteristics (Frasch *et al.,* 1985). Studying the distribution of these surface structures has proved to be useful for analyzing the spread of meningococcal disease (Holten *et al.,* 1979; de Marie *et al.,* 1986b; Poolman *et al.,* 1986; Scholten *et al.,* 1993). In recent years it has been shown that meningococci have a clonal population structure and the characterization of the chromosomal genotype of *N. meningitidis* by multilocus enzyme electrophoresis has proved to be an even more powerful tool for studying the epidemiology of meningococcal disease. If closely related meningococcal clones are homogeneous with regard to the phenotype, the results of the surface characterization of *N. meningitidis* and genotyping will be similar and will lead to the same inferences. Many isolates, especially those from carriers, do not react with any of the presently available polyclonal or monoclonal reagents used to determine phenotypic characteristics (Poolman 1980b; Bovre *et al.,* 1983). Most isolates from patients are serogroupable, but a large proportion of isolates from sporadic cases are non-typable (NT) (Broud *et al.,* 1979); and epidemic strains are often of a single antigenic type.

In this part of the study multilocus enzyme electrophoresis technique was used to assess of *N. meningitidis* from patients and carriers in both Greece and Romania to determine if the phenotype differences reflected to genetic differences.

8.2. Materials and methods

8.2.1. Isolates

A total of 43 meningococcal strains isolated from children aged 1 month to 11 years with meningococcal disease were obtained from the microbiology laboratory of "Aglaia Kyriakou" Paediatric hospital, Athens. The isolates were from sporadic cases. Thirty isolates from the Cantacuzino Institute in Bucharest were also examined, 17 strains from patients and 13 strains from carriers (Table 8.1).

Table 8.1. Greek and Romanian Isolates

	Greece	Romania
Patient	43	18
Carrier	34	10
Date	1990-1993	1992-1993*

*** 3 patient isolates from 1969**

8.2.2. Serogroup and serotype identifications

The serogroups were determined by slide agglutination with serogroup-specific sera as detailed in Chapter 2 as are the methods for the determination of the serotypes and subtypes.

8.2.3. Isolation and electrophoresis of the enzymes.

8.2.3.1. Reagents

8.2.3.1.1. Tris-HCl (pH 8.0)

For the preparation of 1l buffer, Tris 24.2 g was dissolved in 950 ml distilled H_2O. After the pH was adjusted with concentrated HCl, the volume was measured and distilled water was added to make the final volume of 1L (0.2 M Tris).

8.2.3.1.2. $MgCl_2$ solution

For the preparation of 1l buffer, $MgCl_2$ (20.3 g) was dissolved in 1000 ml distilled H_2O. (0.1 M $MgCl_2$)

8.2.3.1.3. $MnCl_2$ solution

For the preparation of 1L buffer, $MnCl_2$ (49.0 g) was dissolved in 1000 ml distilled H_2O (0.25 M $MnCl_2$)

8.2.3.1.4. Phenazine Methosulphate (PMS)

For the preparation of 100 ml staining solution, phenazine methosulphate (1.0 g) (Sigma P-9625) was dissolved in 100 ml deionized H_2O. The solution was kept in a dark bottle at room temperature.

8.2.3.1.5. Tetrazolium (MTT)

For the preparation of 100 ml staining solution, tetrazolium (1.0 g) (Sigma M-2002) was dissolved in 125 ml deionized H_2O. The solution was kept in a dark bottle at room temperature.

8.2.3.1.6. β- Nicotinamide Adenine Dinucleotide (NAD)

For the preparation of 100 ml NAD solution, NAD (1.0 g) (Sigma N-8881) was dissolved in 100 ml distilled H_2O. The solution was kept at $+4^0$ C.

8.2.3.1.7. β- Nicotinamide Adenine Dinucleotide Phosphate (NADP)

For the preparation of 100 ml NADP solution, NADP (1.0 g) (Sigma N-0505) was dissolved in 100 ml distilled H_2O. The solution was kept at $+4^0$ C.

8.2.3.1.8. Amarath -staining solution

Amarath (100 mg) (Sigma) was dissolved in 30 ml of distilled water, and 1 ml of 96% ethanol was added.

8.2.3.1.9. Trypticase Soy Broth (TSB)

TSB (30 g) (BBL- Becton Dickinson) was dissolved in 1l of distilled water. The contents were mixed thoroughly, warmed gently until the TSB was dissolved and autoclaved at 121^0 C for 15 min.

8.2.3.1.10. Tris-EDTA buffer (pH 6.8)

For the preparation of 1l of buffer, Tris (1.21 g) (Sigma) and EDTA (0.37 g) (Fluca) were dissolved in 1l distilled water. The pH was adjusted by adding concentrated HCl and 40 mg NADP was added to the solution.

8.2.3.2. Buffer systems for electrophoresis of bacterial enzymes

8.2.3.2.1. Tris-Citrate buffer (pH 8.0) *(electrode buffer)*

For the preparation of 4 l buffer, Tris (332.8 g)(Sigma) and citric acid monohydrate (132.36 g) (Sigma) were weighted and dissolved in 4 l distilled water. The pH was adjusted to 8.0 with 1N NaOH.

8.2.3.2.2. Borate buffer (pH 8.1) *(electrode buffer)*

For the preparation of 4 l buffer, boric acid (74 g)(Merck) and NaOH (9.6 g) (Fluca) were dissolved in 4 l of distilled water and the pH was adjusted to 8.1 with 1N NaOH.

8.2.3.2.3. Lithium hydroxide buffer (pH 8.1) *(electrode buffer)*

For the preparation of 4 l buffer $LiOH.H_2O$ (4.8 g)(Sigma) and boric acid (Merck)(47.56 g) were dissolved in 4 l of distilled water and the pH was adjusted to 8.1 with 1N NaOH.

8.2.3.2.4. Tris-Citrate buffer (pH 8.0) *(gel buffer)*

The buffer was prepared by diluting the tris-citrate buffer (8.2.3.2.1) in distilled water in a 1:29 dilution (i.e. 100 ml of tris-citrate buffer was added to 2.9 L distilled water).

8.2.3.2.5. Poulik -Tris-Citrate buffer (pH 8.7) *(gel buffer)*

For the preparation of 2 l buffer, Tris (18.42 g)(Sigma) and citric acid (Sigma) monohydrate (2.1 g) were dissolved in 2 l of distilled water ; the pH was adjusted to 8.7 with 1N NaOH.

8.2.3.2.6. Lithium hydroxide buffer (pH 8.3) *(gel buffer)*

For the preparation of 2 l buffer, Tris (11.16 g)(Sigma) and citric acid (2.88 g) (Sigma) were dissolved in 1.8 l of distilled water. Lithium hydroxide buffer (200 ml) (8.2.3.2.3.) was added and the pH was adjusted to 8.3 with 1N NaOH.

The buffers used for the electrophoresis of the enzymes, electrode and gel buffers, and the voltage used for each enzyme respectively are shown in Table 8.2.

Table 8.2. Buffer systems for electrophoresis of bacterial enzymes.

ENZYME	ELECTRODE BUFFER	VOLTAGE	GEL BUFFER
Aconitase (**ACO**)	**Tris-Citrate (pH 8.0)**	**130 V**	**Tris-citriate (pH 8.0)**
Adenylate kinase (**ADK**)			
Isocitrate dehydrogenase (**IDH**)			
Glutamate dehydrogenase (**GDM**)			
Malic enzyme (**ME**)			
Glucose-6-phosph. dehydrogenase (**G6P**)			
Peptidase (**PEP-2**)			
Alcohol dehydrogenase (**ADH**)	**Borate (pH 8.2)**	**250 V**	**Poulik (pH 8.7)**
Alkaline phosphatase (**ALK**)	**Lithium hydroxide (pH 8.1)**	**325 V**	**Lithium hydroxide (pH 8.3)**
Fumarase (**FUM**)			
Idophenyl oxidase (**IPO**)			

8.2.3.3. Enzyme-staining solutions

The 14 enzymes examined were the following : aconitase (**ACO**); adenylate kinase (**ADK**); isocitrate dehydrogenase (**IDH**); glutamate dehydrogenase (**GDH**); malic enzyme (**ME**); glucose-6-phosphate dehydrogenase (**G6P**); peptidase (**PEP**); alcohol dehydrogenase (**ADH**); alkaline phosphotase (**ALK**); fumarase (**FUM**); idophenyl oxidase (**IPO**); unknown dehydrogenase **(UDH)**

8.2.3.3.1. Aconitase (ACO).

To detect the enzyme, 30 mg Cis-aconitic acid (Sigma A-3412) were added to the following : 15 ml of 0.2 M Tris-HCl (pH 8.0); 10 ml of 0.1 M $MgCl_2$; 1 ml NADP; 0.1 ml isocitrate dehydrogenase; 0.5 ml PMS and 1.0 ml MTT.
For the above staining solution, an agar overlay was applied to the gel. The agar was prepared by dissolving 500 mg agar (Oxoid) in 25 ml 0.2 M tris-HCl (pH=8).

8.2.3.3.2. Adenylate Kinase (ADK)

For detection of the enzyme, 25 mg of ADP (Sigma), 100 mg glucose and 1 mg hexokinase (Sigma) were mixed in a 250 ml beaker.
When the gel was ready to be stained, 25 ml Tris-HCl (0.2 M; pH 8.0), 1 ml $MgCl_2$ (0.1 M), 1 ml NADP (Sigma) and 1.5 ml G-6-PDH solution (10 units/ml, (Sigma) were added. For staining, 0.6 ml PMS and 0.6 ml MTT were added.
For the above staining solution an agar overlay was applied to the gel. The agar was prepared as in 8.2.3.3.1.

8.2.3.3.3. Alcohol dehydrogenase (ADH)

For the detection of the enzyme, 50 ml Tris-HCl (0.2 M, pH 8.0) were mixed with 3 ml ethanol (95%) and 2 ml isopropyl alcohol. When the gel was ready to be stained, 2 ml NAD (Sigma), 0.5 ml PMS and 1.0 ml MTT were added.

8.2.3.3.4. Alkaline phosphatase (ALK)

To detect ALK, reagents were added to: 100 mg PVP (Polyvinyl-pyrolidase - enzyme stabilizer) (Sigma); 50 mg β-naphthyl acid phosphate; 1.0 g NaCl and 50 mg fast blue salt (FBS) (Sigma). For staining the gel, 50 ml Tris-HCl (0.2 M- pH 8.5); 2.0 ml $MgCl_2$ (0.1M) and 2.0 ml $MnCl_2$ (0.1M) were added to the above reagents.

8.2.3.3.5. Fumarase (FUM)

To detect FUM, 50 mg of fumaric acid was added to the following: 50 ml Tris-HCl (0.2 M- pH 8.0); 15 µl malic dehydrogenase (Sigma), 2.0 ml NAD (Sigma); 0.5 ml PMS and 1.0 ml MTT. The solution was mixed and added to the gel.

8.2.3.3.6. Glucose-6-phosphate dehydrogenase (G6P)

To detect G6P, 100 mg of Glucose-6-phosphate was added to : 50 ml Tris-HCl (0.2 M- pH 8.0); 1 ml $MgCl_2$ (0.1M); 1.0 ml NADP (Sigma); 0.5 ml PMS and 1.0 ml MTT. The solution was mixed and added to the gel.

8.2.3.3.7. Glutamate dehydrogenase (GDH)

To detect GDH, 2.1 g of L-glutamic acid (Na salt) (Sigma) were dissolved in 20 ml phosphate buffer (pH 8.9), 30 ml H_2O ; 2 ml NAD ; 1 ml NADP (Sigma); 0.5 ml PMS and 1.0 ml MTT. The solution was mixed and added to the gel.

8.2.3.3.8. Indophenyl oxidase (IPO)

To detect IPO, 40 ml Tris-HCl (0.2M- pH 8.0) were mixed with 1.0 ml $MgCl_2$ (0.1M); 0.5 ml PMS and 1.0 ml MTT were added to the above solution.

8.2.3.3.9. Isocitrate dehydrogenase (IDH)

To detect IDH, 50 ml Tris-HCl (0.2M - pH 8.0) were mixed with 2.0 ml isocitric acid solution (0.1 M), 1.0 ml NADP and 2.0 ml $MgCl_2$ (0.1M). When the gel was ready for staining, 0.5 ml PMS and 1.0 ml MTT were added to the above solution.

8.2.3.3.10. Malic enzyme (ME)

To detect ME, 40 ml of Tris-HCl (0.2M - pH 8.0) were mixed with 6 ml D-L malic acid (2M- pH 7.0); 1.5 ml NADP and 2.0 ml $MgCl_2$ (0.1M). When the gel was ready for staining, 0.5 ml PMS and 1.0 ml MTT were added to the above solution.

8.2.3.3.11. Peptidase (PEP)

To detect PEP, 20 mg of peptidase-2 (L-phenylalanyl-L-leucine)(Sigma) was mixed with 10 mg peroxidase (Sigma), 10 mg O-dianisidine di-HCl (Sigma) and 10 mg snake venom (Sigma). When the gel was ready for staining, 25 ml Tris-HCl (0.2M - pH 8.0) and 0.5 ml $MnCl_2$ (0.25 M) were added and mixed with the above reagents. In the above staining solution an agar overlay was applied to the gel as described in 8.2.3.3.1.

8.2.3.3.12. Unknown dehydrogenase (UDH)

For detection of the unknown dehydrogenase, the preparation is as described for the enzyme Adenylate kinase (ADK) (8.2.3.3.2).

When the gel was ready to be stained, 25 ml Tris-HCl (0.2 M; pH 8.0), 1 ml $MgCl_2$ (0.1 M), 1 ml NADP (Sigma) and 1.5 ml G-6-PDH solution (10 units/ml, (Sigma) were added. For staining, 0.6 ml PMS and 0.6 ml MTT were added. For the above staining solution an agar overlay was applied to the gel. The agar was prepared as in 8.2.3.3.1.

8.2.4. Method

8.2.4.1. Protein extract preparation

To prepare the protein extracts, isolates were grown overnight at 37^{0}C in 100 ml tryptic soy broth (TSB) (Difco). The bacteria were harvested by centrifugation and the bacterial pellets were re-suspended by vortex mixing with 1 ml buffer (0.01 M tris-HCl and 0.001 M EDTA, pH 6.8). The cells were disrupted by freezing at -25^{0}C for 48-72 h followed by thawing at room temperature. The bacterial suspensions were then centrifuged at 27000 g for 30 min at 4^{0}C. The supernatants were sterilized by filtration and stored at -70^{0}C until used for electrophoresis.

8.2.4.2. Electrophoresis

An apparatus for horizontal starch-gel electrophoresis was used. Starch gels over polyacrylamide gels were used because of the ease by which horizontal slices can be cut for independent assays of several different enzymes.

To prepare a gel, a suspension of starch (48 g) (Connaught) in 420 ml of gel buffer (Table 8.2.) in a 1 l Erlenmeyer flask was heated with constant vigorous swirling over a Bunsen burner to boiling point. The suspension was aspirated for 1 min. (or until every large bubble disappeared) and immediately poured into a gel mold. After the gel had cooled at room temperature for 2 h, it was wrapped in plastic film to prevent desiccation. Gels were used within 24 hr of preparation.

In loading a gel, pieces of Whatman No 3 filter paper were individually dipped into samples of lysate, blotted on filter paper to remove excess liquid and then inserted at 3-mm intervals in a continuous slit cut in the gel. Up to 20 lysates were tested on a single gel.

Pieces of filter paper dipped in Amaranth dye (Sigma) were inserted at one or both ends of the slit to mark the migration front of the buffer line. During electrophoresis, a constant voltage was maintained depending on the gel (Table 8.2) and the gel was cooled by running it at 4^0 C surrounded by an ice bath.

Following electrophoresis, three or four horizontal slices (1 to 2 mm thick) were cut from the gel with a thin wire and incubated individually at 37^0C in the various enzyme staining solutions. For some enzymes (ADK, ACO and PEP), the staining solution was applied to the gel in an agar overlay.

Gels were incubated at 37^0C in the dark until bands appeared. For different enzymes this can be from 10 min to several hours. The staining solution was then poured off, the gel slice rinsed with tap water and fixed in a 1:5:5 mixture of acetic acid, methanol and water.

Isolates were characterized by the relative electrophoretic mobilities of enzymes. Because the net electrostatic charge, and hence, the rate of

migration of a protein during electrophoresis was determined by its amino acid sequence. Mobility variants (electromorphs or alloenzymes) of an enzyme can be directly equated with alleles at the corresponding structural gene locus. Electromorphs (alloenzymes) of each enzyme, numbered in order of decreasing anodal mobility. Absence of the enzyme activity was attributed to a null allele (denoted 0). Distinctive combinations of alleles over the 14 enzyme loci (multilocus genotypes) were designated as electrophoretic types, ETs (Caugant *et al.,* 1981). The electrophoretic mobilities of the enzymes strains were compared with two reference strains with very different electromorph combinations (ET-5 and ET-18). All isolates that differed from the reference strains were next compared to each other, side by side on a gel for all the enzymes assayed. Electromorphs were numbered in order of decreasing anodal mobility and the combination of electromorphs at the 14 enzyme loci was determined for all isolates. Although the genes coding for these enzymes have not been mapped on the chromosome of *N. meningitidis,* it is assumed that the enzymes are coded by chromosomal genes and so electromorphs were equated with alleles at each locus.

8.2.5. Statistical analysis

Genetic diversity at an enzyme locus among either ETs or isolates was calculated from the allele frequencies among ETs or isolates as $h = (1 - \Sigma \chi_i^2)(n/n - 1)$, where χ_i is the frequency of the *i*-th allele and n is the number of ETs or isolates (Selander *et al.,* 1986). Mean genetic diversity (H) is the arithmetic average of the *h* values for all of the loci. Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which dissimilar alleles occurred (mismatches) and clustering of ETs was performed from a matrix of genetic distances by the average-linkage method (Sneath *et al.,* 1973). The analysis of the genetic diversity and the relationships among bacterial strains characterized by multilocus enzyme electrophoresis, was carried out by ETDIV computer program .

8.3. Results

In the collection of 105 isolates, all the 14 enzyme loci were polymorphic for from 3 to 11 alleles (Table 8.3). The average number of alleles per locus was 5.5 (Table 8.4).

A total of 63 distinctive genotypes were identified (Table 8.3) among which mean genetic diversity per locus was 0.471. Eighteen ETs were represented by multiple isolates (range 2 to 13). There was less genetic diversity among the ETs of strains from patients in Greece than among the strains from Romania and the carrier strains (Table 8.4). The number of ETs among each group of strains is shown in Table 8.5. The patient strains from Greece were much more homogeneous than the two other groups as reflected by the relatively high ratio of isolates/ET, low mean number of alleles per locus and low mean genetic diversity in both isolates and ETs.

The genetic relationships among the 63 ETs are shown in the dendogram (Figure 8.1). Of the 63 ETs, 59 clustered together within a genetic distance of 0.5. The remaining four ETs, represented by a single isolate each, included one strain from Romania and three strains from carriers in Greece. Only one ET was represented by strains from both patients and carriers in Greece (ET-26) and one ET was found in Greece and Romania (ET-5). There were 3 strains isolated in Romania of serogroup A (A:4,21:P1.10), 2 obtained from patients in 1969 and the third from a patient in 1992. The 1969 isolates belonged to the ET 51 complex, but the 1992 isolate differed at 4 different loci compared with the earlier strains (Table 8.6).

ETs 9 through 12 represented clones of the cluster A4 and were exclusively found among patients in Greece (19 of the 43 isolates). The ET-37 complex was represented by two patient isolates from Greece, but by none of the carrier strains or the Romanian strains. None of the isolates belonged to the

ET-5 complex which has been an important source of disease in western Europe.

8.4. Discussion

Genetic analysis of the Romanian and Greek strains by MLEE complemented the results obtained for analysis of serogroups, serotypes and subtypes (Chapters 5 and 7). Both phenotypically and genetically, the strains from Greece and Romania differed from those causing disease in northwest Europe. The monoclonal antibodies successfully used for typing of meningococcal strains were not useful for isolates from eastern Europe. Similar results have been reported for isolates from the Czech Republic; 50-80% of Czech strains were non-typable [Musilek *et al.,* 1994].

Since Romania and the Czech Republic were relatively closed populations until 1989, it was predicted that the isolates in these countries might be significantly different from those in western Europe. Greece, however, is a major holiday destination for residents of northwest Europe and it was predicted that some of the Greek strains, particularly those from areas where there are large numbers of tourists might be similar to those found in other parts of Europe. This was not the case; the Greek isolates were more like those from Eastern European countries. The strains from Romania and Greece were quite different genetically, both in degree of genetic diversity and in their clonal composition. The clones associated with disease in western Europe (*e.g.* ET-5, ET-37) (Caugant, 1994) were not present in isolates from either Balkan country.

Although the three serogroup A isolates had similar serotype and subtype characteristics, the strain obtained in 1992 differed at 4 different loci compared with those isolated in the 1960's. This indicates that within this period there have been changes within what appear to be homogenous clones of meningococci.

Analysis of the dendrogram has shown that only one ET was included in strains from both patients and carriers in Greece. Only one ET was identified in both Greece and Romania; both were serogroup B isolates from patients. The ETs 9-12 represented clones of the cluster A4; and were found exclusively among patients on Greece (19/43). ET 37 was represented by only 2 patient isolates from Greece; this was not found among the Greek carrier strains or many of those from Romanian. None of the isolates were of the ET-5 complex associated with disease in western Europe.

8.3. Characteristics of 105 isolates of *N.meningitidis* recovered from patients (n=43) and carriers (n=34) in Greece and from Romania (n=28).

		Allele at indicated enzyme loci																
E.T	Strain	ME	G6P	PEP	IDH	ACO	GD1	GD2	ADH	FUM	ALK	IP1	IP2	ADK	UDH	Group	Serotype	Source
1	R26/260	4	1	9	9	6	1	3	2	3	2	2	5	2	3	B	NT:P1.6	P
2	R29/271	4	1	9	9	0	1	3	2	3	2	2	5	2	3	B	NT:P1.6	C
3	R23/255	3	1	9	9	0	1	3	3	3	2	2	0	2	3	B	NT:NT	P
4	AK47	1	1	2	7	4	1	3	2	3	3	2	3	2	3	B	NT:P1.12	P
	R20/251	1	1	2	7	4	1	3	2	3	3	2	3	2	3	B	NT:NT	C
5	R32/277	1	1	2	7	4	1	3	2	3	3	2	5	2	3	B	NT:NT	C
6	R25/259	4	1	9	7	4	1	3	2	3	3	2	5	2	3	B	NT:NT	C
7	HC31	3	1	7	7	4	1	3	2	2	2	2	3	2	3	B	NT:P1.6	C
	HC81	3	1	7	7	4	1	3	2	2	2	2	3	2	3	A	NT:P1.9	C
8	HC721	3	1	7	9	4	1	3	1	2	2	2	3	2	3	NG	4:P1.16	C
9	AK13	1	3	4	9	2	1	3	2	1	8	2	3	2	3	B	NT:NT	P
	AK15	1	3	4	9	2	1	3	2	1	8	2	3	2	3	P	2b:P1.10	P
	AK17	1	3	4	9	2	1	3	2	1	8	2	3	2	3	NG	2b:P1.10	P
	AK2	1	3	4	9	2	1	3	2	1	8	2	3	2	3	B	2b:P1.10	P
	AK23	1	3	4	9	2	1	3	2	1	8	2	3	2	3	P	2b:P1.10	P
	AK24	1	3	4	9	2	1	3	2	1	8	2	3	2	3	B	NT:NT	P
	AK27	1	3	4	9	2	1	3	2	1	8	2	3	2	3	B	14:P1.15	P
	AK28	1	3	4	9	2	1	3	2	1	8	2	3	2	3	B	NT:P1.14	P
	AK46	1	3	4	9	2	1	3	2	1	8	2	3	2	3	B	NT:P1.10	P
	AK49	1	3	4	9	2	1	3	2	1	8	2	3	2	3	P	2b:P1.9	P
	AK52	1	3	4	9	2	1	3	2	1	8	2	3	2	3	B	2bP1.1	P
	AK6	1	3	4	9	2	1	3	2	1	8	2	3	2	4	B	NT:P1.10	P
	AK8	1	3	4	9	2	1	5	2	1	8	2	3	2	3	B	2b:P1.10	P

Table 8.3 (cont)

		Allele at indicated enzyme loci															
E.T	Strain	ME	G6P	PEP	IDH	ACO	GD1	GD2	ADH	FUM	ALK	IP1	IP2	ADK	UDH	Group	Serotype	Source
10	AK22	3	3	4	9	2	1	3	2	1	8	2	3	2	3	B	2b:P1.10	P
	AK3	3	3	4	9	2	1	3	2	1	8	2	3	2	3	C	2b:P1.2	P
	AK31	3	3	4	9	2	1	3	2	1	8	2	3	2	3	B	2b:P1.10	P
	AK48	3	3	4	9	2	1	3	2	1	8	2	3	2	3	C	NT:P1.2	P
11	AK19	1	3	4	9	2	1	3	2	1	8	2	3	2	4	B	NT:P1.10	P
12	AK53	1	3	4	9	2	1	5	2	1	8	2	3	2	3	B	NT:NT	P
13	HC522	1	3	4	7	2	1	3	2	1	8	2	4	2	3	NG	NT:P1.12	C
	HC556	1	3	4	7	2	1	3	2	1	8	2	4	2	3	NG	2b:P1.2	C
14	HC588	1	3	2	2	2	1	3	2	1	8	2	3	2	3	P	NT:P1.7	C
	HC613	1	3	2	2	2	1	3	2	1	8	2	3	2	3	NG	1:P1.7	C
	HC627	1	3	2	2	2	1	3	2	1	8	2	3	2	3	P	1:P1.7	C
15	HC642	3	3	10	9	2	1	4	2	1	8	2	3	2	3	B	4:P1.15	C
16	AK9	1	3	5	2	2	1	3	2	1	8	1	4	2	3	B	NT:P1.9	P
17	HC494	2	3	4	2	2	1	2	2	1	2	2	3	2	3	P	2a:P1.9	C
18	HC298	1	3	4	1	4	1	3	2	3	8	2	3	2	3	NG	14:P1.1	C
19	R7/ 218	3	3	4	1	4	1	3	1	3	8	2	3	2	3	P	NT:P1.2	C
20	AK20	4	3	4	5	2	1	4	0	2	8	2	3	2	3	C	NT:P1.16	P
21	AK32	4	3	4	5	4	1	4	0	1	8	2	3	2	3	C	NT:P1.14	P
22	R8/ 219	3	3	4	6	4	2	3	2	1	2	2	3	2	3	B	NT:NT	C
23	R28/267	3	5	4	6	4	2	3	2	1	2	2	3	2	3	B	4:NT	P
24	AK10	3	3	4	7	4	2	3	2	1	2	2	3	2	3	B	15:P1.6	P
25	AK54	3	3	5	7	4	2	4	2	1	2	2	3	2	3	B	NT:NT	P

Table 8.3 (cont.)

		Allele at indicated enzyme loci															
E.T.	Strain	ME	G6P	PEP	IDH	ACO	GD1	GD2	ADH	FUM	ALK	IP1	IP2	ADK	UDH	Group	Serotype	Source
26	AK11	3	3	4	12	4	1	3	2	1	3	2	3	2	3	B	NT:P1.9	P
	AK44	3	3	4	12	4	1	3	2	1	3	2	3	2	3	B	NT:P1.7	P
	AK45	3	3	4	12	4	1	3	2	1	3	2	3	2	3	P	14:P1.10	P
	HC40	3	3	4	12	4	1	3	2	1	3	2	3	2	3	B	2a:P1.9	C
	HC460	3	3	4	12	4	1	3	2	1	3	2	3	2	3	B	15:P1.2	C
	HC543	3	3	4	12	4	1	3	2	1	3	2	3	2	3	B	15:P1.9	C
27	AK30	3	3	4	12	4	1	3	2	1	3	2	5	2	3	B	NT:P1.9	P
28	R9/222	3	3	4	12	4	1	3	2	1	3	2	0	2	3	B	NT:P1.16	C
29	AK21	3	3	4	12	4	1	3	2	3	3	2	3	2	3	B	NT:NT	P
30	HC575	3	3	1	12	4	1	3	0	1	3	2	5	2	3	B	15:P1.1	C
	HC833	3	3	1	12	4	1	3	0	1	3	2	5	2	3	B	NT:NT	C
31	HC65	3	3	4	6	4	1	3	2	1	3	2	3	2	3	NG	14:P1.2	C
	HC66	3	3	4	6	4	1	3	2	1	3	2	3	2	3	B	14:P1.2	C
32	HC260	3	4	4	6	4	1	3	2	1	3	2	3	2	3	P	1:P1.1	C
33	HC143	3	3	4	6	4	1	3	2	1	5	2	3	2	3	B	NT:P1.7	C
34	HC161	3	3	4	9	4	1	2	2	1	3	2	3	2	3	A	14:P1.1	C
	HC46	3	3	4	9	4	1	2	2	1	3	2	3	2	3	P	14:P1.2	C
35	HC458	3	3	2	2	4	1	3	2	1	3	2	3	2	3	P	NT:P1.9	C
	HC719	3	3	2	2	4	1	3	2	1	3	2	3	2	3	P	1:P1.16	C
36	R10/227	3	3	4	7	0	1	3	3	1	3	2	0	2	2	B	1:NT	P
37	R18/248	3	3	4	7	0	1	3	3	1	3	2	3	2	2	C	1:NT	C
	R19/248	3	3	4	7	0	1	3	3	1	3	2	3	2	2	C	1:NT	P
38	AK40	3	1	4	7	4	1	3	0	1	3	2	5	2	3	B	NT:NT	P
	AK51	3	1	4	7	4	1	3	0	1	3	2	5	2	3	B	1:P1.9	P
39	HC180	1	3	5	3	4	1	3	2	1	3	2	3	2	4	P	15:P1.1	C
	HC279	1	3	5	3	4	1	3	2	1	3	2	3	2	4	NG	NT:P1.1	C
	HC393	1	3	5	3	4	1	3	2	1	3	2	3	2	4	P	NT:P1.4	C

Table 8.3 (cont.)

		Allele at indicated enzyme loci															
E.T.	Strain	ME	G6P	PEP	IDH	ACO	GD1	GD2	ADH	FUM	ALK	IP1	IP2	ADK	UDH	Group	Serotype	Source
40	HC854	1	3	5	3	4	1	3	0	1	0	2	3	2	4	NG	NT:NT	C
41	R1/104	5	3	5	7	4	1	3	2	1	4	2	3	2	2	B	NT:P1.1	P
42	R34/281	5	3	5	7	4	1	0	2	1	1	2	3	2	3	C	NT:NT	P
43	AK50	5	3	5	12	4	1	3	2	1	3	2	3	2	3	B	15:P1.16	P
44	R13/237	4	3	4	7	4	1	3	0	1	2	2	0	2	3	NG	NT:NT	C
45	R30/272	1	3	2	7	4	1	3	2	1	2	2	5	2	3	Y	NT:NT	P
46	R27/261	5	3	7	7	4	1	3	1	1	2	2	3	2	3	B	NT:NT	P
47	R14/241	1	3	7	12	4	1	1	2	1	2	2	3	2	3	B	NT:P1.6	C
48	AK25	3	2	1	7	2	1	3	2	1	8	2	3	2	3	B	NT:P1.1	P
49	AK14	3	4	5	7	4	1	3	2	1	8	2	5	2	3	P	2a:P1.6	P
	AK29	3	4	5	7	4	1	3	2	1	8	2	5	2	3	C	NT:P1.6	P
	AK7	3	4	5	7	4	1	3	2	1	8	2	5	2	3	B	NT:P1.9	P
50	AK1	4	2	2	3	4	1	3	2	1	2	2	3	2	3	C	NT:P1.10	P
	AK12	4	2	2	3	4	1	3	2	1	2	2	3	2	3	P	14:P1.10	P
	AK16	4	2	2	3	4	1	3	2	1	2	2	3	2	3	C	NT:P1.10	P
	AK18	4	2	2	3	4	1	3	2	1	2	2	3	2	3	C	NT:P1.10	P
	AK4	4	2	2	3	4	1	3	2	1	2	2	3	2	3	C	NT:P1.6	P
	AK43	4	2	2	3	4	1	3	2	1	2	2	3	2	3	C	NT:P1.10	P
51	R2/105	3	5	2	3	4	1	3	2	1	8	2	3	2	3	A	4:P1.10	P
	R3/108	3	5	2	3	4	1	3	2	1	8	2	3	2	3	A	4:P1.10	P
52	R11/236	1	3	2	3	4	1	3	3	1	8	2	5	2	3	A	4:P1.10	P
	R12/236	1	3	2	3	4	1	3	3	1	8	2	5	2	3	B	4:P1.10	P
53	R16/245	3	3	2	8	4	1	2	2	1	8	2	5	2	3	B	NT:P1.5	P
54	R6/195	1	1	4	5	4	1	3	2	1	6	2	3	2	3	A	14:NT	P
55	R31/275	1	1	4	5	4	1	3	3	1	2	2	3	2	3	NG	14:NT	P

Table 8.3 (cont.)

		Allele at indicated enzyme loci															
E.T.	**Strain**	**ME**	**G6P**	**PEP**	**IDH**	**ACO**	**GD1**	**GD2**	**ADH**	**FUM**	**ALK**	**IP1**	**IP2**	**ADK**	**UDH**	**Group**	**Serotype**	**Source**
56	HC777	1	4	4	5	4	1	2	2	1	3	2	3	2	3	NG	2a:P1.6	C
57	HC87	1	4	4	9	4	1	1	2	1	1	2	5	2	3	NG	NT:NT	C
58	HC854	1	3	1	3	4	1	4	3	1	3	2	3	2	3	P	NT:NT	C
59	R24/256	3	4	4	12	0	1	3	1	1	2	2	0	2	2	B	15:NT	P
60	HC761	0	3	12	1	0	1	1	1	1	8	2	3	3	3	NG	NT:NT	C
61	R33/278	1	0	10	3	2	2	3	1	1	2	0	0	4	4	P	NT:NT	C
62	HC591	3	1	11	9	2	1	4	1	1	1	0	3	3	2	NG	15:P1.2	C
63	HC281	3	4	9	10	1	2	4	1	1	8	0	5	3	4	NG	15:NT	C

Enzyme abbreviations : Malic enzyme (ME), Glucose-6-phosphate dehydrogenase (G6P), Peptidase (PEP), Isocitrate dehydrogenase (IDH), Aconitase (ACO), NADP-linked glutamate dehydrogenase (GD1), NADP-linked glutamate dehydrogenase (GD2), Alcohol dehydrogenase (ADH), Fumarase (FUM), Alkaline phosphotase (ALK), two indophenol oxidases (IP1 and IP2), Adenylate kinase (ADK), Uknown dehydrogenase (UDH).

Abbreviations : P: strains isolated from patients ; **C**: strains isolated from carriers.

Table 8.4. Genetic diversity in ETs of isolates of *N. meningitidis* from patients and carriers in Greece and from Romania.

Enzyme locus	No of alleles	Patients in Greece (n=18)	Carriers in Greece (n=22)	Romania (n=25)
ME	6	0.680	0.593	0.727
G6P	6	0.477	0.506	0.643
PEP	11	0.595	0.788	0.783
IDH	10	0.817	0.913	0.813
ACO	5	0.503	0.502	0.457
GD1	3	0.209	0.091	0.227
GD2	6	0.386	0.619	0.230
ADH	6	0.294	0.532	0.607
FUM	3	0.307	0.255	0.420
ALK	8	0.647	0.762	0.710
IP1	3	0.111	0.173	0.080
IP2	4	0.386	0.325	0.660
ADK	3	0.000	0.247	0.080
UDH	3	0.111	0.325	0.397
Mean	5.5	0.394	0.474	0.488

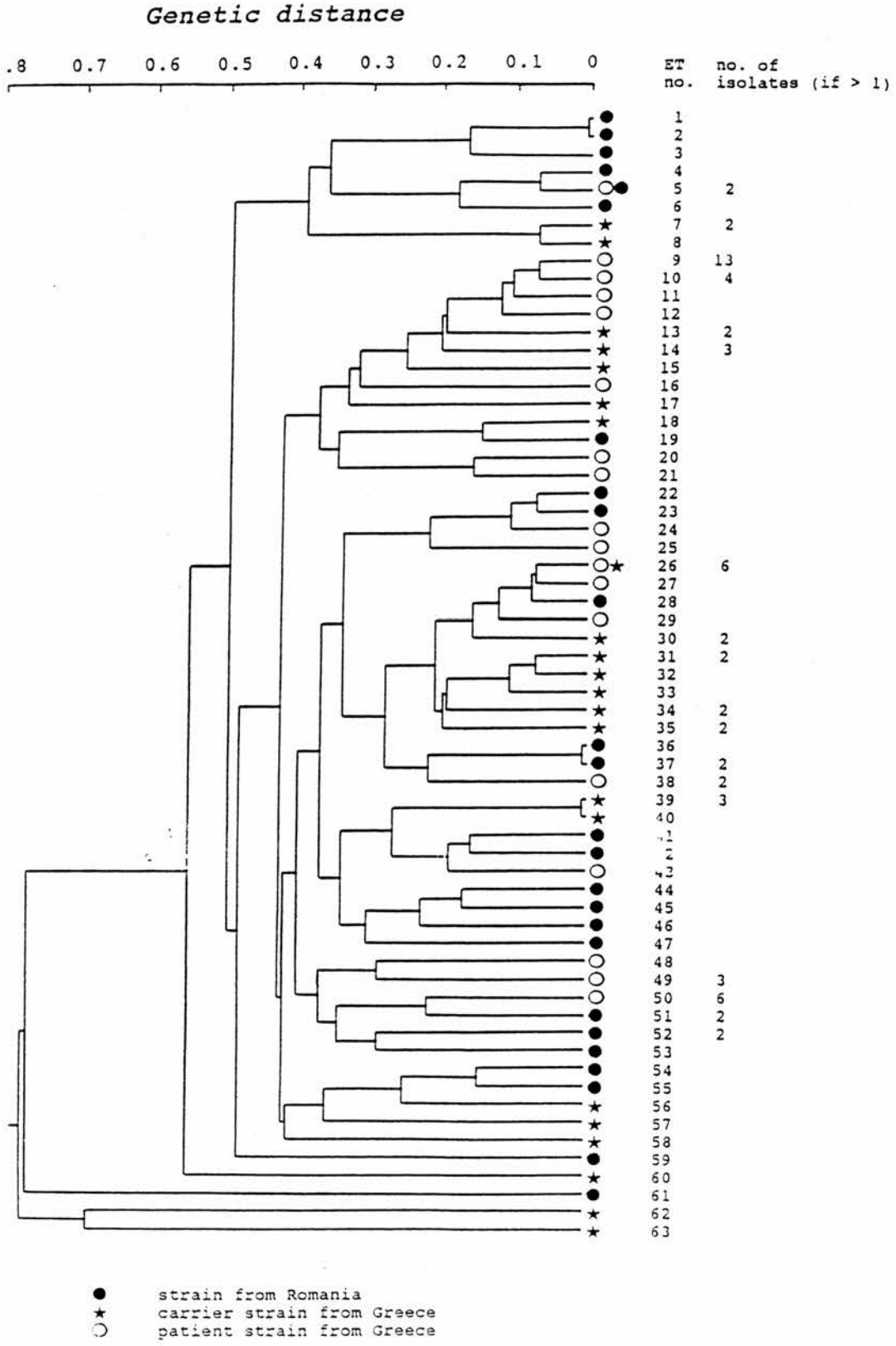
Table 8.5. Genetic diversity in isolates and ETs of *N. meningitidis* from patients and carriers in Greece and from Romania

	Strains from Greece		Strains from Romania
	patients	carriers	
No of isolates	43	34	28
No of ETs	18	22	25
Ratio isolates / ET	2.39	1.54	1.12
Mean No of alleles	2.93	4.21	4.14
Mean H in isolates	3.17	4.17	4.80
Mean H in ETs	0.394	0.474	0.488

Table 8.6. ET's of A : 4,21 : P1,10 strains isolated in 1969 (ET 51) and 1992 (ET 52)

Allele at indicated enzyme locus	E.T.	
	51	**52**
ME	**3**	**1**
G6P	**5**	**3**
PEP	2	2
IHD	3	3
ACO	4	4
GD1	1	1
GD2	3	3
ADH	**2**	**3**
FUM	1	1
ALK	8	8
IP1	2	2
IP2	**3**	**5**
ADK	2	2
UDH	3	3

Figure 8.1. Genetic relationships among the 63 ETs of *N. meningitidis* isolates from patients and carriers from Greece and Romania



9. General discussion

In this thesis several aspects of the epidemiology of meningococcal disease in Greece have been addressed. The core theme was to assess the various characteristics of meningococci isolated from patients and carriers, as well as the factors that affect the carriage in closed populations such as recruit camps and in primary and secondary school children. The ultimate goal was to provide information which could assist the Ministry of Health in formulation of policies to prevent meningococcal disease.

The original objectives of the study were to answer the following questions

1. Are genetic and environmental factors associated with carriage among Greek children and young adults similar to those found in northwest Europe?
2. Are strains with serogroups, serotypes and subtypes associated with disease in north west Europe isolated from patients and carriers in Greece?
3. Are the antibiotic sensitivities of meningococci isolated from patients and carriers in Greece similar to those observed in northwest Europe?
4. Are the genetic clones associated with disease in northwest Europe present among meningococcal strains in Greece and other Balkan countries?

9.1. Factors affecting meningococcal carriage

The major findings of each of these factors which affect meningococcal carriage is summarized below (Table 9.1) with reference to information obtained from other countries:

Table 9.1. Factors associated with carriage of *N. meningitidis* in Greek recruits and schoolchildren.

Factors associated with carriage	Recruits	School-children
Smoking	+	+/-
Passive exposure to cigarette smoke	NA[1]	+
Age	+	+
Sex	NA[1]	-
Virus infection	-	-
Lower socio-economic conditions	+/-	-
Secretor status	-	-

[1]N.A. : Not Applicable

9.1.1. Smoking

Among recruits, active smoking was found to be an important factor affecting meningococcal carriage in both univariate and multivariate analyses and the carriage rate increased in relation to cigarette consumption. This is in agreement with other studies which suggest that the higher proportion of smokers and heavy smokers among military personnel might contribute to the increased prevalence of meningococcal disease and to higher rates of carriage among military recruits (Crowdy 1981).

The percentage of smokers in the two camps investigated in the present study, were 57.7% and 63.4%, much higher than the 47% found in a previous study among male Greek patients referred for gastroscopy (Mentis *et al.,* 1990). It has been established that in Britain, smoking is associated with socio-economic status; unskilled individuals are more likely to smoke than those in professional groups (Wald *et al.,* 1988). A similar pattern was observed among the recruits; those with fewer years of education were more likely to be smokers.

9.1.2. Passive exposure to smoke.

In the study of schoolchildren, it was noted that for the fathers in this study, there was a positive correlation between educational level and smoking similar to that observed and the Greek military recruits. For Greek women, 33-38% were smokers regardless of socioeconomic background. There were significantly more men who were smokers in area A where educational levels were lower, but carriage was not associated with father's smoking by either univariate or multivariate analysis. Carriage was associated with maternal smoking or smoking by other members of the household. The structure of many Greek families in which grandparents often live with families with young children and are closely involved with child care, might be the reason for the

significant association between meningococcal carriage and smoking by adults. If maternal smoking is a significant risk factor these observations help to explain the lack of association between carriage and socioeconomic background as discussed below.

9.1.3. Age

Multiple logistic regression analysis identified the 15-19 year age range to be a significant variable. This agrees with the findings in the Stonehouse survey in which the highest proportion of carriers was found in this age group (Cartwright *et al.,* 1987). Although the carriage rate was higher among the recruits (25%) compared to that found among the schoolchildren (5-8%), the majority of patients in this study and those reported to the Ministry of Health were less than 5 years of age. This is probably associated immunity to the meningococcus acquired by repeated colonization of both pathogenic and non-pathogenic *Neisseria* spp (Goldschneider 1969a).

9.1.4. Sex and meningococcal carriage

In the Stonehouse study (Cartwright *et al.,* 1987) and in the Faroe Islands (Olsen *et al.,* 1991), there was a higher proportion of males among carriers, but this was not observed among children in a Scottish study (Blackwell *et al.,* 1990a) or among Greek schoolchildren. It was suggested that the similar proportion of males and females among Scottish children who were carriers might be due to the significantly higher proportion of girls who were smokers (Blackwell *et al.,* 1990a; Moore *et al.*, 1990); however, the proportion of Greek boys who smoked was slightly higher than that of girls who smoked.

9.1.5. Viral infection.

Another important factor proposed to enhance meningococcal carriage is viral infections. Parental smoking, particularly smoking by the mother, has been associated with upper and lower respiratory infection (Fleming *et al.,* 1987) as well as with impairment of ventilatory function (Pederira *et al.,* 1985; Tager *et al.,* 1983). If the mother's smoking is a significant factor contributing to carriage among children, this might explain the similar carriage rates for the two areas of Athens compared in this study.

There is evidence that viral infections can predispose individuals to carriage of potentially pathogenic bacteria (Musher *et al.,* 1981) In an experimental model with a human epithelial cell line, infection with RSV was found to enhance binding of meningococci (Raza *et al.*, 1994).

In the recruit study carried out during July when there was a low prevalence of upper respiratory viral infections (19.8%), there was no association between symptoms of viral infection and carriage. During the study of schoolchildren carried out in the winter there was a higher proportion of participants with symptoms of virus infections (33%), but there was no association with carriage. These results indicate that viral infection in general might not lead to enhanced carriage of meningococci. Alternatively, the association noted between meningococcal disease and influenza infection, might be due to impaired immunological responses associated with influenza (Jakab, 1982).

9.1.6. Lower socio-economic conditions.

In a longitudinal survey in Belgium (de Wals *et al.,* 1983), carriage rate was greater in the area where socio-economic indicators were lower. In the recruit study, it was found that lower socio-economic conditions might influence

meningococcal carriage in relation to smoking and age as recruits from less well educated families were more likely to do their military service early. While there was good agreement between the two indicators used to assess socio-economic levels, there was little difference in carriage rates among schoolchildren in the two areas of Athens with significantly different socioeconomic backgrounds.

9.1.7. Secretor status

A previous study of staff and pupils in a Scottish secondary school following an outbreak of meningitis found an association between non-secretion and carriage of meningococci (Blackwell *et al.,* 1990a). There was no association between secretor status and carriage in the studies of recruits or schoolchildren. The studies on phenotypic and genotypic characteristics of the Greek isolates indicate that they differ significantly from those in the Scottish population.

When the results were analyzed according to the geographic area, it was found that a significally higher proportion of recruits from Eastern Macedonia were non-secretors. The above findings, are in agreement with the annual reports from the Ministry of Health showing that meningococcal disease is more prevalent in the region of Macedonia. Further studies are needed to confirm these observations.

9.2. Phenotypic characteristics of meningococcal strains in Greece.

Another objective of the study was to determine if the serotype and subtype reagents used for epidemiological studies in northern Europe and the Americas could be used in epidemiological studies in Greece and other Balkan countries, and if the serotype and subtype combinations associated with disease in other populations were found in southeast Europe. The proportion of non-serotypable isolates from Greek recruits (69%), and

school children (64%) and isolates from Romania (54.8%) was greater than the proportion of non-serotypable isolates obtained from Norwegian recruits (28%) (Wedege *et al.,* 1990) or Scottish school children (22%). This indicates that other serotype reagents need to be developed for epidemiological studies of meningococci from Greece as well as from the Balkan. The serotypes associated with recent outbreaks in Britain and Scandinavia are uncommon in Greece and Romania.

The proportion of isolates from the Greek recruits (36%) and school children (26%) and Romanian isolates (20%) that did not react with any of the subtype reagents was slightly lower than that of isolates from the Norwegian recruits (39%) (Wedege *et al.,* 1990) and from Scottish school children (38%). The subtype reagents appear to be useful for studies of Greek and Romanian strains however, the proportions of subtypes associated with outbreaks in northern Europe were lower.

Combinations of serotype and subtype antigens 15: P1.16, 4:P1.15 and 2a:P1.2 associated with outbreaks in northern Europe (Poolman *et al.,* 1988, Abdillahi *et al.,* 1988) were not found among any of the isolates in this study. Although P1.2 was the most common subtype found in the study there was only one patient isolate with this type, C:2b: P1.2. In contrast, the 2a:P1.2 and 2b:P1.2 combinations are found frequently among Scottish group C (Fallon *et al.,* 1991) and French serogroup B isolates, and 2a:P1.2 is found frequently among French serogroup C isolates (Riou *et al.,* 1989). The only report of meningococcal serotypes from southeast Europe is that of the C:2a phenotype associated with disease in Italy (Mastrantonio *et al.,* 1990). None of the serogroup C isolates expressed this serotype from both studies.

The third objective was to determine if there were antigenic phenotypes associated with different areas of Greece, *e.g.*, those in which there are large numbers of foreign tourists or foreign military personnel or areas such as the northeast were there is a significant Muslim population some of

whom might have visited Saudi Arabia. There was no unusual distribution of serogroups or serotypes in the different regions of Greece.

The results obtained from this study on strains isolated from patients and carriers in Greece and Romania and a report from the Czech Republic (Musilek *et al.*, 1994) indicate other serotype reagents need to be developed for epidemiological studies of meningococci in central and eastern Europe. Antigenic phenotypes associated with recent outbreaks in Britain and Scandinavia are uncommon in Greece and Romania.

Genotype analysis confirmed phenotypic analysis, as none of the isolates from either Greece or Romania belonged to the ET-5 complex associated with disease in northern Europe. In contrast, the majority of these strains belonged to a group of clones designated as cluster A4 which was associated with disease in several European countries in the 1970 s but it has not been a major cause of disease recently in other parts of Europe.

9.3. Antibiotic sensitivities of meningococcal strains in Greece

In Britain, the proportion of penicillin insensitive isolates from patients and carriers was about 3% (Sutcliffe 1988). In Greece, 46% of patient isolates were penicillin insensitive similar to the figures reported from Spain. Since the antibiotic policies of Spain and Greece are similar, these observations were not unexpected. The proportion of Romanian penicillin insensitive isolates (20%), an intermediate value between those from Britain and southern Europe, might reflect recent changes in antibiotic policy related to political changes. This difference between the percentage of penicillin-insensitive strains in Romania and Greece might be due to differences in antibiotic policies in the two countries. There were strict policies on the use of antibiotics in all Eastern European countries prior to the break up of the Soviet Union (F. Mihalcu and P. Kriz, personal communications).

Although penicillinase-producing isolates of *N. gonorrhoeae* are not uncommon in Greece (18%) (Tzanakaki *et al.,* 1989), none of the 550 meningococcal isolates tested produced beta-lactamase.

Sulphonamide resistance has been associated with strains causing outbreaks of meningococcal disease in northern Europe (Cartwright *et al.,* 1987; Poolman *et al.,* 1986), but only 16% of the isolates from Greek patients had MIC> 10 mg/l of sulphamethoxazole while the majority of the Romanian strains were sulphonamide resistant (66.6%).

Among carrier isolates, 6-7% were resistant to rifampicin. This increase might be due to rifampicin treatment of tuberculosis in the community; however, there were no significant associations between resistant strains and socioeconomic background. Strains of rifampicin resistant (Rif^R) meningococci have been recovered from recipients of the drug (Blackwell *et al.*, 1990a; Yagupsky, 1993); and these strains can cause systematic disease (Yagupsky, 1993). Recent studies in the United Kingdom have shown that a single clone of (Rif^R) *N. meningitidis* widespread throughout the United Kingdom is due to a single mutation (Carter *et al.,* 1994). The spread of (Rif^R) meningococcal strains may pose serious problems in the management of the meningococcal infections.

Significant development of rifampicin resistance might require an alteration in public health policy regarding control of secondary cases similar to that used by the Scandinavians in which close contacts are given penicillin to prevent disease rather than rifampicin to eliminate carriage.

Although high level tetracycline resistance mediated by a plasmid carrying the *tetM* determinant has been found in meningococci (Knapp *et al.,* 1988) none of the Greek or Romanian isolates had MIC>8 and the 25.2 megadalton plasmid was not observed in any of the strains examined so far. Strains were sent to Dr. M. Roberts of the University of Washington for analysis of the

chromosomal *tetM.* The gene was not identified in any of these strains (Dr. M. Roberts, personal communication).

The higher levels of resistance to erythromycin among isolates from Greek carriers might reflect longer exposure to the antibiotic. as the mean ages of the carriers are higher, school-children (12.8 years) and recruits (19.7 years) compared with a mean age of 2.7 years for patients with meningitis.

If *N. meningitidis* follows the course of *N. gonorrhoeae* and *Streptococcus pneunoniae*, the prevalence and magnitude of penicillin resistance among meningococci will increase worldwide over the next decade (Saez-Nieto *et al.,* 1992; Tan *et al.,* 1992). Countries with less stringent antibiotic policies have high prevalence of penicillin-insensitive strains already. The high level tetracycline resistant (MIC>16 mg/l) oropharyngeal, genital and anorectal isolates carrying the *tet-M* conjugative plasmid have been described in the past (Ison *et al.,* 1988; Knapp *et al.,* 1988, Spott *et al.,* 1988; Winterscheid *et al.,* 1994). The role of *N. gonorrhoeae* as a possible reservoir for the mobilization of resistance plasmids into *N. meningitidis* has been suggested (Roberts *et al.,* 1988).

The high percentage of resistance to antibiotics comes as no surprise if one considers that in Greece, antibiotics can be bought without a medical prescription. The numbers are quite alarming indicating that antibiotic policies in Greece, have to be more seriously considered by the Ministry of Health and new strict legislation introduced.

9.5. Major contribution of the present work and further studies.

1. The study on the recruits has provided further evidence that active smoking is a risk factor for meningococcal carriage.
2. The results obtained from the epidemiological studies (recruits and schoolchildren) showed that viral upper respiratory tract infection in general

is not a predisposing factor for colonization and that perhaps specific viral infections (*e.g.*, RSV and influenza) needed to be investigated.

3. Low socio-economic factors are not associated with carriage in Greek school children but with smoking habits of members of the house closely involved in child care in Greece.
4. The studies on meningococcal strains isolated from patients in Greece and Romania were the first to identify significant differences between meningococcal strains isolated in northwest and southeast European countries. These results have also shown that there is a need to develop new reagents for further epidemiological work. This might have important consequences for the future development of vaccines against serogroup B since major efforts are directed towards further development vaccines based on the serotype/ subtype antigens.
5. The identification of increased levels of penicillin insensitive isolates (46%) in Greece probably reflects the lack of control of antibiotic usage. The intermediate values of 20% for Romania might reflect changes in prescribing patterns or availability of antibiotics following political changes since 1989.
6. This work provided a major basis for monitoring meningococcal disease in Greece and in other Balkan countries and resulted in the establishment of a National Meningococcal Reference Laboratory at the National School of Public Health.

   The systematic collection of data on the issues mentioned above, has provided the basis for monitoring meningococcal disease in Greece and provision of information to the Ministry of Health which can be used to formulate policies for prevention of meningococcal disease.

Future work includes:

1. monitoring of antibiotic resistance especially to penicillin and rifampicin;
2. the development of new typing reagents;

3. assessment of immigrant populations for carriage and factors associated with carriage;
4. regional studies on carriage in regions such as Macedonia and Epirus with reference to influx of Albanian;
5. the assessment of bactericidal activities to Albanian strains in sera collected during the summer 1990 and winter 1991 surveys, after the increase of legal and/ or illegal Albanian immigrants in Greece to Epirus, Macedonia and Athens.

References

AAC working party. Phillips I, Bind AJ,. Breakpoints *in vivo* antibiotic sensitivity testing. J Antimicrob Chemother 1988; 21: 701-10.

Abdillahi H, Poolman JT. Whole-cell ELISA for typing *Neisseria meningitidis* with monoclonal antibodies. J Med Microbiol 1987; 48: 367-71.

Abdillahi H, Poolman JT. Typing of group-B *Neisseria meningitidis* with monoclonal antibodies in whole-cell ELISA. J Med Microbiol 1988; 26: 177-180.

Abdillahi H, Poolman JT. Definition of meningococcal class 1 OMP subtyping antigens by monoclonal antibodies. FEMS Microbiol Immunol 1988; 47 : 139-44.

Achtman M, Kusecek B, Morelli G, . A comparison of the variable antigens expressed by clone IV-1 and subgroup III of *Neisseria meningitidis* serogroup A. J Infect Dis 1992; 165:53-68.

Ahonkai VI, Lukacs LJ, Jonas LC. *Haemophilus influenzae* type b conjugate vaccine (meningococcal protein conjugate) (Pedvax HIB): clinical evaluation. Pediatrics 1990; 85 (suppl):676-81.

Alkout AH, Blackwell CC, Weir DM, Luman W, Palmer K. Identification of surface components of *Helicobacter pylori* that bind H type 2 and Lewis blood group antigens. Gut 1995; 37(suppl 1): A21

Alper CA, Abramson N, Johnston RB. Increased susceptibility to infection associated with abnormalities of complement-mediated functions and of the third component of complement (C3). N Engl J Med 1970; 282: 349-54.

Anonymous. Preventing meningococcal infection. Drug Ther Bull 1990; 28: 34-6.

Apicella M. *Neisseria meningitidis.* In: Principles and practice of Infectious Diseases (3rd edition) G. Mandel, G. Douglas J Bennet (eds) 1990; Churchill Livingston.

Artestein MS, Lampson TH, Evans JR. Attempted prophylaxis against meningococcal infection using intramuscular penicillin. Milit Med. 1967; 132: 1009-11.

Artenstein MS, Gold R, Zimmerly JG. Prevention of meningococcal disease by group C polysaccharide vaccine. N Engl J Med 1970; 282 : 417-20.

Ashton FE, Ryan A, Diena B, Jennings HJ. A new serogroup (L) of *Neisseria meningitidis*. J Clin Microbiol 1983 ; 17 : 722-7.

Ashton FE, Mancino L, Ryan AJ, Poolman JT, Abdillahi H, Zollinger WD. Serotypes and subtypes of *Neisseria meningitidis* serogroup B strains associated with meningococcal disease in Canada 1977-1989. Can J Microbiol 1991; 37 : 613-7.

Aycock WL, Muller JH. Meningococcus carrier rates and meningitis incidence. Bacteriol Rev. 1950; 14: 115-160.

Barua D and Paguio AS. ABO blood groups and cholera. Ann Hum Biol 1977; 4 : 489-492.

Berg T. Immunoglobulin levels in infants with low birth weights. Acta Pediat Scand 1968; 57: 369-76.

Beque P. *Haemophilus influenzae* vaccines. Bull Acad. Natl Med 1993; 177(8) : 1381-7.

Bhattacharjie AK, Jennings HJ, Kenny CP, Martin A, Smith ICP. Structural determination of the sialic acid polyssacharide antigens of *Neisseria meningitidis* serogroup B and C with carbon 13 nuclear magnetic resonance. J Biol Chem 1975; 250: 1926-32.

Bhattacharjie AK, Jennings HJ, Kenny CP, Martin A, Smith ICP. Structural determination of the polysaccharide antigens of *Neisseria meningitidis* serogroups Y, W-135, and BO. Can J Biochem 1976; 54: 1-8.

Blackwell CC, Jonsdottir K, Hanson M., Todd WT, Chaudhuri AK., Mathew B., Brettle RP., Weir DM. Non-secretion of ABO blood group antigens predisposing to infection by *Neisseria meningitidis* and *Streptococcus pneumoniae*. Lancet 1986a; ii: 284-5

Blackwell CC, Jonsdottir K, Hanson M., Weir DM. Non-secretion of ABO blood group antigens predisposing to infection by *Haemophilus influenzae* . Lancet 1986b; ii : 687.

Blackwell CC, James VS, Weir DM, Gemmill JD, Patric AW, Collier A, Clarke BF. Secretor state of patients with insulin dependent or non-insulin-dependent diabetes mellitus. Br Med J Clin Res Ed 1987; 295: 1024-5.

Blackwell CC, Jonsdottir K, Mohammed I, Weir DM. Non-secretion of blood group antigens. A genetic factor predisposing to infection by *Neisseria meningitidis*. In: Poolman JT, Zanen HC, Meyer TF . eds. Gonococci and meningococci. Dordrecht: Kluwer Academic Publishers, 1988: 633-6.

Blackwell CC, Weir DM, James VS, Cartwright KAV, Stuart JM, Jones DM. The Stonehouse study: secretor status and carriage of *Neisseria meningitidis* and *Neisseria lactamica.* Epidemiol Infect 1989a; 102: 1-10.

Blackwell CC, Jonsdottir K, Weir DM, Hanson MF, Cartwright KAV, Stewart J, Jones D, Mohammed I. Blood group, secretor status and susceptibility to bacterial meningitis. FEMS Microbiol Immunol 1989b; 1: 351-6.

Blackwell CC. Genetic susceptibility to infectious agents. Proc Roy Coll Phys (Edinburgh) 1989c; 19: 129-138.

Blackwell CC. The role of ABO blood groups and secretor status in host defenses. FEMS Microbiol Immunol 1989d; 47: 341-350.

Blackwell CC, Weir DM, James VS, Todd WTA, Banatvala N, Chaudhuri AKR, Gray HG, Thomson EJ and Fallon RJ. Secretor status, smoking and carriage of *Neisseria meningitidis*. Epidemiol Infect 1990a; 104: 203-9.

Blackwell CC, Weir DM. Meningococcal disease: high virulence and low transmission? Lancet 1990b; ii: 53.

Blackwell CC, James VS, Davidson S, Wyld R, Brettle RP, Robertson RJ, Weir DM. Secretor status and heterosexual transmission of HIV. Br Med J 1991; 303 (6806): 825-6.

Blackwell CC, Saadi AT, Raza MW, Stewart J and Weir DM. Susceptibility to infection in relation to sudden infant death syndrome. J Clin Pathol 1992; 45: 20-24.

Bjune G, Hoiby EA, Gronnesby JK. Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. Lancet 1991; 338: 1093-6.

Bona C, Mond JJ, Stein KE, House S, Leiberman R, and Paul WE. Immune response to levan. III. The capacity to produce anti-inulin antibodies and cross reactive idiotypes appears late in ontogeny. J Immunol 1979; 123: 1484-1490.

Borgono JM, McLean AA, Valla PP, Woodhour AF, Canepa I, Davidson WL, and Hillemen MR. Vaccination and re-vaccination with polyvalent pneumonococcal polysaccharide vaccines in adults and infants. Proc Soc Exp Bio Med 1978; 157; 148-154.

Botha P. Penicillin-resistant *Neisseria meningitidis* in southern Africa. Lancet 1988; i: 54.

Bovre K, Holten E, Vikmo H, Brondbo A, Bratlid D, Biark P, Moe PJ. *Neisseria meningitidis* infections in Northern Norway: An epidemic in 1974-75 due mainly to group B organisms. J Infect Dis 1977 ; 135 : 669-72.

Bovre K, Froholm LO, Gaustad P, Holten E, Hoiby EA. Some agent characteristics and their coexistence related to occurrence and severity of systemic meningococcal disease in Norway, winter 1981, 1982. National Institute of Public Health (Oslo) Annals 1983; 6: 75-84.

Brandtzaeg P, Fjellanger I, Gjeruldsen S. Human secretory immunoglobulin I. Salivary secretions from individuals with normal or low levels of serum immunoglobulins. Scand J Haemat Suppl 1970; 12: 1-83.

Brandtzaeg P. Human secretory immunoglobulins. II. Salivary secretions from individuals with selectively excessive or defective synthesis of serum immunoglobulins. Clin Exp Immunol 1971; 8: 69-85.

Brandtzaeg P. Human secretory immunoglobulin M. An immunochemical and immunohistochemical study. Immunol 1975; 29: 559-570.

Brandtzaeg P. Polymeric IgA is complexed with secretory component (SC) on the surface of human intestinal epithelial cells. Scand J Immunol 1978; 8: 39-52,

Brandtzaeg P. Transport models for secretory IgA and secretory IgM. Clin Exp Immunol 1981; 44: 221-32.

Brandtzaeg P, Kierulf P, Gaustad P. Plasma endotoxin as a predictor of multiple organ failure and death in meningococcal disease. J Infect Dis 1989; 159: 195-204.

Bristow MW, Van Peenen PFD, Volk R. Epidemic meningitis in naval recruits. Am J Public Health. 1965; 55 : 1039-45.

Broome CV. The carrier state: *Neisseria meningitidis*. J Antimicrob Chemother 1986; 18 (suppl A): 25-34.

Broud DD, Griffiss JM, and Baker CJ. Heterogeneity of serotypes of *Neisseria meningitidis* that cause endemic disease. J Infect Dis 1979; 140: 465-470.

Burgio GR, Lanzavecchia A, Plebani A, Jayakar S, Ugazio AG. Ontogeny of secretory immunity: Levels of secretory IgA and natural antibodies in saliva. Pediatr Res 1980; 14: 1111-4.

Burford-Mason AP, Weber JCB and Willoughby JMT. Oral carriage of *Candida albicans*, ABO blood group and secretor status in healthy individuals. J Med Vet Mycology 1988; 26: 49-56.

Calain P, Poolman J, Zollingen W, Sperber G, Bitter-Suermann D, Auckenthaler R, Hirschel B. Serological study of Meningococcal Isolates in Switzerland and France, 1980-1986. Eur J Clin Microbiol Infect Dis 1988; 7: 788-91.

Campos J, Mendelman PM, Sako MU. Detection of relative penicillin G-resistant *Neisseria meningitidis* by disk susceptibility testing. Antimicrob Agents Chemother 1987; 31: 1478-82.

Campbell H, Carter H. Rational use of *Haemophilus influenzae* type b vaccine. Drugs 1993; 46: 378-83.

Carlone GM, Williams D, Dykes J, Kapczynski D, Plikaytis BD, Perkins BA. Comparison of serum bactericidal results using vaccine type-strains and heterologous target strains to evaluate immunogenicity of two meningococcal serogroup B vaccines in Iceland. In : Proceedings Neisseria 94 , 1994; The Guildhall, Winchester, England.

Carlsson B, Zaman S, Mellander L, Jalil F, and Hanson LA. Secretory and serum immunoglobulin class-specific antibodies to polio virus after vaccination. J Infect Dis 1985; 1238-1244.

Carter PE, Abadi FJR, Yakubu DE, Pennington TH. Molecular characterization of Rifampicin-resistant *Neisseria meningitidis*. Antimicrob Agents Chemother 1994; 38: 1256-61.

Cartwright KAV, Stuart JM, Noah ND. An outbreak of meningococcal disease in Gloucestershire. Lancet 1986; ii : 558-61.

Cartwright KAV, Stuart JM, Noah ND. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol Infect 1987; 99 : 591-601.

Cartwright KAV, Jones DM. Investigation of meningococcal disease. J Clin Pathol 1989; 42 :634-9.

Cartwright KAV, Jones DM, Smith AJ, Stuart JM, Kaczmarski EB, Palmer SR. Influenza A and meningococcal disease. Lancet 1991; 338: 554-7.

Cassio de Moraes J, Perkins BA, Camargo MCC. Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil. Lancet 1992; 340: 1074-8.

Caugant DA, Levin BR, Selander RK. Genetic diversity and temporal variation in the *E. coli* population of a human host. Genetics 1981; 98:467-490.

Caugant DA, Bovre K, Gaustad P, . Multilocus genotypes determined by enzyme electrophoresis *of Neisseria meningitidis* isolated from patients with systemic disease and from healthy carriers. J Gen Microbiol 1986a ; 132: 641-52.

Caugant DA, Froholm LO, Bovre K,. Intercontinental spread of a genetically distinctive complex of clones of *Neisseria meningitidis* causing epidemic disease. Proc Natl Acad Sci USA 1986b; 83 : 4927-31.

Caugant DA, Mocca LF, Frasch CE, Froholm LO, Zollinger WD, Selander RK. Genetic structure of Neisseria meningitidis populations in relation to serogroup, serotype and outer membrane protein pattern. J Bacteriol 1987; 169 : 2781-92.

Caugant DA, Bol P, Hoiby EA, Zanen HC, Froholm LO. Clones of serogroup B *Neisseria meningitidis* causing systemic disease in the Netherlands 1958-1986. J Infect Dis 1990; 162 : 867-74.

Caugant DA. Genotyping of European meningococcal strains: an update. European Monitoring Group on Meningococci 1994 (Abstracts).

Ceesay SJ, Allen SJ, Menon A. Decline in meningococcal antibody levels in African children 5 years after vaccination and the lack of an effect of booster immunization. J Infect Dis 1993; 167:1212-6.

Collier A, Patric AW, Toft AD, Blackwell CC, James V, Weir DM. Increased prevalence of non-secretors in patients with Graves' disease: evidence for an infective aetiology? Br Med J Clin Res Ed. 1988; 296: 1162.

Constantino P, Viti S, Podda A, Velmonte MA, Nencioni L, Rappuoli R. Development and phase I clinical testing of a conjugate vaccine against meningococcus A and C. Vaccine 1992; 10:691-8.

Craven DE, and Frasch CE. Pili mediated and non-mediated adherence of *N. meningitidis* and its relationship to inavasive disease. In: G.F. Brooks *et al.,* (Ed) Immunology of *N. gonorrhoeae* , Am Soc Microbiol Washington DC 1978; pp. 250-252.

Crofton J, Douglas A. Respiratory diseases. Oxford: Blackwell, 1981:353.

Crowdy JP. Smoking in the army. J Roy Army Med Corps 1981; 127: 1-15.

Crowe BA, Wall RA, Kusecek B, . Clonal and variable properties of *Neisseria meningitidis* isolated from cases and carriers during and after an epidemic in the Gambia, West Africa. J Infect Dis 1989; 159 : 686-700.

Cruz-Coke R, Paredes L, and Montenego A. Blood groups and urinary microorganisms. J Med Genet 1965; 2: 185-188.

Cooke RPD, Riordan T, Jones DM, Painter MJ. Secondary cases of meningococcal infection among close family and household contacts in England and Wales, 1984-87. Br Med J 1989; 298: 555-8.

Cummins RO, Shaper AG, Walker M, Wale CJ. Smoking and drinking by middle-aged British men: effects of social class and town of residence. Br Med J 1981; 283 : 1497-502.

Davis BD, Dulbecco R, Eisen HN, Ginsberg HS. Microbiology. Harper and Row, Publishers, Inc. 1980.

Delvin AA, Wedege E, Caugant DA, Dalseg R, Kolberg J, Achtman M, Rosenqvist E. A linear B-cell epitope on the class 3 outer-membrane protein of *Neisseria meningitidis* recognized after vaccination with the Norwegian group B outer-membrane vesicle vaccine. Microbiol 1995; 141: 1593-1600.

Derensiski SC, Papagianis D and Stevens DA. Association of ABO blood groups and outcome of coccidioidal infection. Sabouraudia. 1979; 17: 261-264.

Devine LF, Jonhson DP, Rhode SL. Rifampicin: Effect of two-day treatment on meningococcal carrier state and the relationship of the levels of drug in sera and saliva. Am J Med Sci 1971; 261: 79-83.

DeVoe IW, Gilchrist JE. Ultrastructure of pili and annular structures on the cell wall surface of *Neisseria meningitidis* Infect Immun 1974; 872-876.

DeVoe IW, Gilchrist JE. Pili on meningococci from primary cultures of nasopharyngeal carriers and cerebrospinal fluid of patients with acute disease. J Exp Med 1975; 141: 1241-1244.

DeVoe IW. The meningococcus and mechanisms of pathogenicity. Microbiol. Rev. 1982; 46: 162-90.

DeWals P, Hertoghe L, Reginster G,. Mortality in meningococcal disease in Belgium. J Infect 1984; 8: 264-73.

Difabio JL, Michon F, Brisson JR, Jennings HJ. Structures of the L1 and L6 core oligosaccharide epitopes of *Neisseria meningitidis.* Can J Chem 1990; 68:1029-1034.

Duerden BI, Reid TMS, Jewsbery JM. Microbial and Parasitic Infection (7th Ed) Edward Arnold (ed.) 1993; pp. 3375-376.

Dunn KL, Virji M, Moxon ER. Investigations into the molecular basis of meningococcal toxicity for human endothelial and epithelial cells: the synergistic effect of LPS and pili. Microb Pathogen 1995; 18(2): 81-96.

Dworzack DL, Sanders CC, Horowitz EA,. Evaluation single-dose ciprofloxacin in the eradication of *Neisseria meningitidis* from nasopharyngeal carriers. Antimicrobial Agents Chemother 1988; 32: 1740-1.

Edwards EA, Devine LF, Senbusch CH, Ward HW. Immunological investigations of meningococcal disease. III. Brevity of group C acquisition prior to disease occurrence. Scan J Infect Dis 1977; 9: 105-110.

Einhorn MS, Weinberg GA, Anderson EL. Immunogenicity in infants of *Haemophilus influenzae* type b polysaccharide in a conjugate vaccine with *Neisseria meningitidis* outer membrane protein. Lancet 1986; ii: 299-302.

Ellison RT, Kohler PF, Curd JG. Prevalence of congenital or acquired complement deficiency in patients with sporadic meningococcal disease. N Engl J Med 1983; 308 : 913-6.

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

Essery SD, Weir DM, James VS, Blackwell CC, Saadi AT, Busuttil A, Tzanakaki G. Detection of microbial surface antigens that bind Lewis^a antigen. FEMS Immunol and Med Microbiol 1994; 9: 15-22.

Eskola J, Peltola H, Takala AK. Efficacy of *Haemophilus influenzae* type b polysaccharide- diphtheria toxoid conjugate vaccine in infancy. N. Engl J Med 1987; 317:717-22.

Eskola J, Takala AK, Kayhty H. *Haemophilus influenzae* type b polysaccharide-protein conjugate vaccines in children. Curr Opin Pediatr 1993; 5: 55-9.

Fallon RJ, Brown WM, Lore W. Meningococcal infections in Scotland 1972-82. J Hyg 1984; 93 : 167-80.

Fallon RJ. Meningococcal infections in Scotland. J Med Microbiol 1988; 26 : 168-70.

Fallon RJ. Meningococcal infections in Scotland 1990. Communicable Diseases and Environmental Health in Scotland Weekly Report 1991; 25: 91/25.4-9.

Feldman HA. Meningococcal infections. Adv Intern Med 1972; 18: 117-40.

Fijen CAP, Kuijper EJ, Hannema AJ, Sjoholm AG, van Putten JPM. Complement deficiencies in patients over ten years old with meningococcal disease due to uncommon serogroups. Lancet 1989; ii : 585-8.

Finne J, Leinomen M, Maleka PH. Antigenic similarities between brain components and bacteria causing meningitis: implications for vaccine development and pathogenesis. Lancet 1983; ii: 355-7

Finne J, Bitter-Suermann D, Goudis C. An IgG monoclonal antibody to group B meningococci cross reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissue. J Immunol. 1987; 138: 4402-7.

Flemming DW, Cochi SL, Hightower AW, and Broome CV. Childhood upper respiratory tract infection: to what degree is incidence affected by day care attendance? Pediatrics 1987; 79: 55-60.

Foster MT and Labrum AH. Relation of infection with *Neisseria gonorrhoeae* to ABO blood groups. J Infect Dis 1976; 133: 329-330.

Frasch CE, Chapman SS. Classification of *Neisseria meningitidis* group B into distinct serotypes. III Application of a new bactericidal-inhibition technique to distribution of serotypes among cases and carriers. J Infect Dis 1973; 127:149-154.

Frasch CE, McNelis RM, Gotschlich EC. Strain specific variation in the protein and lipopolysaccharide composition of the group B meningococcal outer membrane. J Bacteriol 1976; 127:973-981.

Frasch CE, Zollinger WD, Poolman JT. Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. Rev Infect Dis 1985; 7 : 504-10.

Frasch CE. Vaccines for the prevention of meningococcal disease. Clin Microbiol Rev 1989; 2 (Suppl): S134-8.

Fraser PK, Bailey GK, Abbott JD, Gill JB, Walker DJC. The meningococcal carrier-rate. Lancet 1973; I: 1235-37.

Froholm LO, Caugant DA, Aasen S, Holten E. Recent meningococcal epidemiology in Norway. Eight years of serotyping for strain characterization. In: Achtman M, Kohl P, Marchal C, Morelli G, Seiler A, Thiesen B, eds. Neisseriae 1990. Berlin : Walter de Gruyter, 1991a ; 57-61.

Froholm LO, Caugant DA, Holten E, Hoiby EA, Rosenqvist E, Wedege E. Meningococcal strains isolated from teenage patients during the serogroup B vaccination trial in Norway: serotyping, serosubtyping, immunotyping and clonal analysis. NIPH Annals 1991b; 14 : 139-43.

Fox AJ, Jones DM, Gray SJ, Caugant DA, Saunders NA. An epidemiology valuable typing method for Neisseria meningitidis by analysis of restriction fragment length polymorphisms. J Med Microbiol 1991; 34 : 265-70.

Gauld JR, Nitz RE, Hunter DH. Epidemiology of meningococcal meningitis at Ford Ord. Am. J Epidemiol. 1965; 82: 56-72.

Gaunt PM, Lambert BE. Single dose ciprofloxacin for eradication of pharyngeal carriage of *Neisseria meningitidis*. J Antimicrob Chemother 1988; 21: 489-96.

Glesson M, Cripps AW, Clancy RL, Husband AJ, Hensley MJ, and Leeder SR. Ontogeny of secretory immune system in man. Aus N Z J Med 1982; 12: 255-258.

Glynn AA, Glynn LE, and Holborow EJ. The secretor status of rheumatic fever patients. Lancet 1959; 2: 759-762.

Gold R, Lepow ML, Goldschneider I, Gotschlish EC. Immune response of human infants to polysaccharide vaccines of groups A and C *Neisseria meningitidis.* J Infect Dis 1977; 136:531-535.

Goldstein E, Buhlers WC, Akers TC. Murine resistance to inhaled *Neisseria meningitidis* after infection with cephalomyocarditis virus. Infect Immun. 1972; 6:398-402.

Goldschneider I, Gotschlich EC and Artenstein MS. Human immunity to the meningococcus. I. The role of humoral antibodies. J Exp Med 1969a; 129: 1307-1326.

Goldschneider I, Gotschlich EC, Artenstein RS. Human immunity to the meningococcus II. Development of natural immunity. J Exp Med 1969b; 129:1327-1348.

Gotschlich EC, Fraser BA, Nishimura O, Robbins JB, Liu TY. Lipid on capsular polysaccharides of Gram-negative bacteria. J Biol Chem 1981; 256: 8915-21.

Greenfield S, Sheehe PR, Feldman HA. Meningicoccal carriage in a population of "normal" families. J Infect Dis 1971; 123: 67-73.

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

Greenwood BM, Whittle HC, Bradley AK, Fayet MT, Gilles HM. The duration of the antibody response to meningococcal vaccination in an African village. Trans R Soc Trop Med Hyg 1980; 74: 756-60.

Griffiss JM, Goroff DK. IgA blocks IgM- and IgG-initiated immune lysis by separate molecular mechanisms. J Immunnol 1983; 130: 2882-2885.

Griffiss JM, Schneeider H, Mandrell RE. Lipopolysaccharides: the principal glycolipids of the neisserial outer membrane. Rev Infect Dis 1988; 10 (suppl 2) : 286-95.

Grundbacher FJ. Immunoglobulins, secretor status and the incidence of rheumatic fever and rheumatic heart disease. Hum Hered 1972; 25: 399-404.

Guerin N. Control of *Haemophilus influenzae* infections. Sante 1994; 4(3):227-30.

Gupta M and Chaudhuri ANR. Relationship between ABO blood groups and malaria. Bull WHO 1980; 58: 913-915.

Guttler RB, Counts GW, Avent GK, Beaty HN. Effect of rifampicin and minocycline on meningococcal carrier rates. J Infect Dis 1971; 124:199-205.

Halstensen A, Pedersen SHJ, Haneberg B, Bjorvatn B, Solberg CO. Case fatality of meningococcal disease in western Norway. Scand J Infect Dis 1987; 19: 35-42.

Haneberg B, Tonjum T, Rodahl K, Gedde-Dahl T. Factors preceding the onset of meningococcal disease with special emphasis on passive smoking, stressful events, physical illness and general symptoms of ill health. Nat Inst Pub Health Annal 1983; 6:169-76

Hassan-King M, Greenwood BM, Whittle HC, Abbot JD, Sutcliffe EM. An epidemic of meningococcal infection at Zaire, Northern Nigeria. 3. Meningococcal carriage. Trans R Soc Trop Med Hyg 1979; 73 : 567-573.

Havens PL, Garland JS, Brook MM, Dewitz BA, Stremski ES, Troshynski TJ. Trends in mortality in children hospitalized with meningococcal infections, 1957 to 987. J Pediatr Infect Dis 1989; 8: 8-11.

Haverkorn MJ and Goslings WRO. Streptococci, ABO blood groups and secretor status. Am J Hum Genet 1969; 21: 360-375.

Haworth JC, Dilling L. Concentration of IgA globulin in serum, saliva and nasopharyngeal secretions of infants and children. J Clin Lab Med 1966; 67: 922-33.

Hedrich AW. The movements of epidemic meningitis, 1915-1930. US Public Health Repts 1931; 46: 2709-2726.

Hedrich AW. Recent trends in meningococcal disease. US Public Health Repts 1952; 67: 411-420.

Henderson FW, Collier AM, Clyde WA Jr., Denny FW. Respiratory-syncytial virus infections, re-infections and immunity: a prospective longitudinal study in young children. N Eng J Med 1979; 300: 530-534.

Hoeprich PD. Prediction of antimeningococcic chemoprophylactic efficacy. J Infect Dis 1971; 123:125-33.

Holbrook WP and Blackwell CC. Secretor status and dental caries in Iceland. FEMS Microbiol Immunol 1989; 1(6-7): 397-9.

Holten E. Serotypes of *N. meningitidis* isolated from patients in Norway during the first six months of 1978. J Clin Microbiol 1979; 9: 186-8.

Hoogerhout P, Donders EM, van Gaas-van den Brink JA, Kuipers B, Brugghe HF, Peeters CC. Conjugates of synthetic cyclic peptides elicit bactericidal antibodies against a conformational epitope on a class 1 Outer Membrane Protein of *N. meningitidis* . Infect Immun 1995; 63(9): 3473-8.

Howard JG, and Hale C. Lack of neonatal susceptibility to introduction of tolerance by polysaccharide antigens. Eur J Immunol 1976; 6: 486-492.

Ison CA, Bincalayna K, Woodford N. *Neisseria gonorrhoeae* resistant to tetracycline. Br Med J 1988; 296:1471.

Issit PD. Applied blood group serology, 1986 ; 3rd Edition. Montgomery, Miami pp. 169-191.

Jacobson JA, Daniel B. Vestibular reactions associated with minocycline. Antimicrob Agents Chemother 1975; 8: 453-6.

Jakab GJ. Immune impairment of alveolar macrophage phagocytosis during influenza virus pneumonia. Am Rev Resp Dis 1982; 126: 778-782.

Jennings HJ, Bhattacharjee AK, Keny CP. Structural elucidation of the 3-deoxy-D-manno-octulosonic acid containing meningococcal 29-E capsular polysaccharide antigen using carbon-13 nuclear magnetic resonance. Biochemistry 1978; 17: 645-651.

Jennings HJ, Lugowski C. Immunochemistry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. J Immunol 1981; 127: 1011-1018.

Jennings HJ, Beurret M, Gamian A, Michon F. Structure and immunochemistry of meningococcal lipooligosaccharides. Antonie Van Leeuwenhoek 1987; 53:519-522.

Jones DM, Sutcliffe EM. Meningococci with reduced susceptibility to penicillin. Lancet 1990; i: 863-4.

Kalapothaki V, Vassiliadis P, Trichopoulos D, Kaklamani E, Serie Ch. Characteristiques sero-epidimiologiques chez les porteurs sains de meningocoques en Grece. Annales Microbiol (Institute Pasteur) 1979; 130B: 43-54.

Kasper DL, Winkelhake JL, Zollinger WD, Brandt BL, and Arternstein MS. Immunochemical similarity between polysaccharide antigens of *E. coli* 07:K1(L):NM and group B *Neisseria meningitidis* . J Immunol 1973; 110: 262-268.

Kayhty H, Poolman J, Abdillahi H, Sivonen A, Eskola J, Tarkka E, Peltola H. Sero- and subtypes of group B meningococci causing invasive infections in Finland in 1976-87. Scand J Infect Dis 1989a; 21 : 527-35.

Kayhty H, Peltola H, Eskola J. Immunogenicity of *Haemophilus influenzae* oligosaccharide protein (HbOC) and polysaccharide-protein (PRP-D) conjugate vaccines at 4,6 and 14 months of age. Pediatrics 1989b; 84: 995-99.

Kim JJ, Mandrell RE, Zhen H, Westerink MAJ, Poolman JT, Griffiss JM. Electromorphic characterization and description of conserved epitopes of the lipooligosaccharides of group A *Neisseria meningitidis.* Infect Immun 1988; 56: 2631-2638.

Kinane DF, Blackwell CC, Brettle RP, Weir DM, Winstanley FB and Elton RA. ABO blood group, secretor status and susceptibility to recurrent urinary tract infection in women. Br Med J 1982; 285: 7-9.

Krakinski K, Nilson JD, Butler S, Laby JP, Kusmiesz H. Possible association of mycoplasma in viral respiratory infections with bacterial meningitis. Am J Epidemiol 1987; 125: 499-508.

Knapp JS, Johnson SR, Zenilman JH, Roberts MC, Morse SA. High-level tetracycline resistance resulting from *tetM* in strains of *Neisseria spp., Kingella dentrificans* and *Eikenella corrodens.* Antimicrob Agents Chemother 1988; 32: 765-7.

Knight AI, Cartwright KAV, McFadden J. Identification of a UK outbreak strain of *Neisseria meningitidis* with a DNA probe. Lancet 1990; 335: 1182-4.

Kovacs N. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 1956; 178: 703.

Kriz P, Vlckova J, and Bobak M. Targeted vaccination with meningococcal polysaccharide vaccine in one district of the Czech Republic. Epidemiol Infect 1995; 115: (in press).

Kriz P and Musilek P. Changing epidemiology of meningococcal invasive disease in the Czech Republic caused by new clone *Neisseria meningitidis* C:2a:P1.2 (P1.5), ET-15/37. Centr Eur J Publ Hlth 3, 1995; 4 (in press).

Kurikka S, Kayhty H, Peltola H, Saarinen L, Eskola J, Makela PH. Neonatal immunization: response to *Haemophilus influenzae* type b-tetanus toxoid conjugate vaccine. Pediatrics 1995; 95(6): 815-22.

Lambert HP, ed. Infections of the central nervous system. Philadelphia : BC Decker 1991.

van der Lay P, Heckels JE, Virji M, Hoogerhout P, Poolman JT. Topology of outer membrane porins in pathogenic *Neisseria spp.* Infect Immun 1991; 59 : 2963-71.

van der Lay P, and Poolman JT. Construction of a multivalent meningococcal vaccine strain based on the class 1 outer membrane protein. Infect Immun 1992; 60: 3156-3161.

van der Lay P, van der Biezen J, Poolman JT. Construction of *Neisseria meningitidis* strains carrying multiple chromosomal copies of the *por*A gene for use in the production of a multivalent outer membrane vesicle vaccine. Vaccine 1995; 13: 401-407.

Lim D, Gewurz A, Lint TF. Absence of the sixth component of complement in a patient with repeated episodes of meningococcal meningitis. J Pediatr 1976; 89:42.

Liu TY, Gotschlich EC, Jonssen EK, Wysocki JR. Studies on the meningococcal polysaccharide. I. Composition and chemical properties of the group A polysaccharide. J Biol Chem 1971(a); 246:2849-58.

Liu TY, Gotschlich EC, Dunne EC, Jonssen EK. Studies on the meningococcal polysaccharides. II. Composition and chemical properties of the group B and C polysaccharide. J Biol Chem 1971(b); 246:4703-4712.

Lystad A, Aasen S. The epidemiology of meningococcal disease in Norway 1975-91. NIPH Annals 1991; 14: 57-65.

Madore DV, Johnson CL, Philips DC. Safety and immunologic response to *Haemophilus influenzae* type b oligosaccharide- CRM_{197} conjugate vaccine in 1- to 6-month old infants. Pediatrics 1990; 85: 331-37.

Makela PH, Eskola J, Peltola H, Takala AK, Kayhty H. Clinical experience with *Haemophilus influenzae* type b conjugate vaccines. Pediatrics 1990; 85 (suppl): 651-53.

Mandrell RE, Zollinger WD. Lipopolysaccharide serotyping of *Neisseria meningitidis* by hemagglutination inhibition. Infect Immun 1977; 16:471-475.

Mandrell RE, Kim JJ, John CM. Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis.* J Bacteriol 1991; 173: 2823-2832.

Manstrantonio P, Congiu ME, Occhionero M, Stroffolini T. Genomic fingerprints and phenotypic characteristics of strains of *Neisseria meningitidis* causing disease in Italy. Microbiologica 1990; 13: 109-113.

de Marie S, Poolman JT, Bol P,. Meningococcal disease in The Netherlands, 1959-1981: the occurrence of serogroups and serotypes 2a and 2b of Neisseria meningitidis. J Infect 1986a; 12: 133-43.

de Marie S, Poolman JT, Hoeijmakers JHJ, Bol P, Spanjaard L, Zanen HC. Meningococcal serotypes and serogroup B disease in north-west Europe. Lancet 1986b; ii : 555-8.

McCracken GH. Rapid identification of specific etiology in meningitis. J Pediatr 1976; 88: 706-8.

McGuiness B, Barlow AK, Clarke IN,. Deduced amino-acid sequences of class 1 protein (*PorA*) from strains of *Neisseria meningitidis*. Synthetic peptides define the epitopes responsible for serosubtype specificity. J Exp Med 1990; 171 : 1871-82.

McGuiness B, Clarke IN, Lambden PR,. Point mutation in a meningococcal *PorA* gene associated with increased endemic disease. Lancet 1991; 337 : 514-7.

McIntyre PB, Chey T, Smith WT. The impact of vaccination against invasive *Haemophilus influenzae* type b disease in the Sydney region. Med J Aust 1995; 162(5): 245-8.

Mellander L, Carlsson B, Hansson LA. Appearance of secretory IgM and IgA antibodies to *E. coli* in saliva during early infancy and childhood. J Pediatr 1984; 104: 564-8.

Mellander L, Carlsson B, Hansson LA. Secretory IgA and IgM to *E. coli* and poliovirus type 1 antigens occur in amniotic fluid, meconium and saliva from newborns. A neonatal immune response without antigenic exposure: a result of idiotypic induction? Clin Exp Immunol 1986a; 63: 555-61.

Mellander L, Bjorkander J, Carlsson B, Hansson LA. Secretory antibodies in IgA-deficient and immunosuppressed individuals. J Clin Immunol 1986b; 6: 284-291.

Mendelman PM, Campos J, Chaffin DO. Relative penicillin G resistance in *Neisseria meningitidis* and reduced affinity of penicillin binding protein 3. Antimicrob Agents Chemother 1988; 32: 706-9.

Mentis A, Blackwell CC, Weir DM, Spiliadis C, Dalainas A, Skandalis N. ABO blood group, secretor status and detection of *Helicobacter pylori* among patients with gastric or duodenal ulcers. Epidemiol Infect 1990; 106:221-9.

Meningococcal Disease Surveillance Group. Analysis of endemic meningococcal disease by serogroup and evaluation of chemoprophylaxis. J Infect Dis 1976; 134:201-4.

Miller MA, Meschievitz CK, Ballanco GA, Daum RS. Safety and immunogenicity of PRP-T combined with DTP: excretion of capsular polysaccharide and antibody response in the immediate post-vaccination period. Pediatrics 1995; 95(4) : 522-7.

Moore PS, Harisson LH, Telzak EE, Ajello GW, Broome CV. Group A meningococcal carriage in travelers returning from Saudi Arabia. J Am Med Assoc 1988; 260(18): 2686-2689.

Moore PS, Hierholzer J, Dewitt W, Govan K, Djore D, Lippeveld T, Plikayatis D and Broom CV. Respiratory viruses and mycoplasma as cofactors for epidemic groups. A meningococcal meningitis. J Am Med Assoc 1990; 246: 1271-1275.

deMorais JS, Munford RS, Risi JB. Epidemic disease due to serogroup C *Neisseria meningitidis* in Sao Paulo Brazil. J Infect Dis 1974; 129: 568-71.

Morrison DC. Bacterial endotoxins and pathogenesis. Rev Infect Dis 1983; 5 (suppl 4) : S733-47.

Mooser R, Schleiffenbaum B, Groscurth P, Fehr J. Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote trans-endothelial neutrophil passage. J Clin Invest 1989; 83: 444-55.

Mourant AE, Kopec AC, Domaniewska-Sobezak K. The distribution of the human blood groups and other polymorphisms. London: Oxford University Press, 1976: 548-76.

Moxon R and Rappuoli R. Modern vaccines : *Haemophilus influenzae* infections and Whooping cough. Lancet 1990; 335: 1324-1329.

Mulholland EK, Ahonkhai VI, Greenwood AM, Jonas LC, Lukacs LJ, Mink CM, Staub JM, Tood J, Vella PP, Greenwood BM. Safety and immunogenicity of *Haemophilus influenzae* type b *Neisseria meningitidis* group B outer membrane protein complex conjugate vaccine mixed in the syringe with diphtheria-tetanus-pertussis vaccine in young Gambian infants. Pediatr Infect Dis J 1993; 12: 632-7.

Musher DM, Fainstein V. Adherence of *Staphylococcus aureous* to pharyngeal cells from normal carriers and patients with viral infections. In: Jeljaszwiecz J ed. *Staphylococci* and staphylococcal infections. New York: Gustav Fischer Verlang. Zbl Bakt Suppl 10, 1981:1011-16.

Musilek M, Kriz P, Danielova V, Holubova J. Characterization of nontypable *Neisseria meningitidis* strains in the Czech Republic. In : Neisseria 94 Proceedings of the 9th International Pathogenic Neisseria Conference, Winchester 1994; pp. 375-377.

Musser JM, Kapur V. Molecular population genetics of *Haemophilus influenzae.* In: Ellis RW, Granoff DM (eds.). Development and clinical uses of *Haemophilus influenzae* type b conjugate vaccines. Infections, disease and therapy, vol. 11. New York Mercel Dekker, 1994; 179: 179-206.

Neu HC. Cephalosporins in the treatment of meningitis. Drugs 1987; 34 (Suppl 2): 135-53.

Oberli J, Hoi NT, Caravano R, Tan CM, Roux J. Etude d'une epidemie de meningococcie au Vietnam (province du Sud). Bull WHO. 1981; 59: 585-90.

Ogra PL, Coppola PR, Mac Gillivary MH, and Dzieba JL. Mechanism of mucosal immunity to viral infections in a gamma-A immunoglobulin-deficiency syndrome (37900) Proc Soc Exp Biol Med 1974; 145: 811-816.

Olcen P, Barr J, Kjellander J. Meningitis and bacteremia due to *Neisseria meningitidis* : clinical and laboratory findings in 69 cases from Orebro County, 1965 to 1977. Scand J Infect Dis 1979; 11:111-9.

Olcen P, Kjellander J, Danielsson D and Lindquist BL. Epidemiology of *Neisseria meningitidis*: Prevalence and symptoms from the upper respiratory tract in family members to patients with meningococcal disease. Scand J Infect Dis 1981; 13: 105-109

Olcen P, Djuruus b, Rasmussen K, Joensen AD, Larsen SO, Zoffman H, and Lind I. Pharyngeal carriage of *Neisseria meningitidis* and *Neisseria lactamica* in households with infants within areas with high and low incidences of meningococcal diseases. Epidemiol & Infect 1991; 106: 445-457.

Olyhoek T, Crowe BA, Achtman M. Clonal population structure of *Neisseria meningitidis* serogroup A isolated from epidemics and pandemics between 1915 and 1983. Rev Infect Dis 1987; 9 : 665-92.

Palmer SR, Corson J, Hall R, . Meningococcal disease in Wales: clinical features, outcome and public health management. J Infect 1992; 25: 321-8.

Paradiso PR, Hogerman DA, Madore DV, Keyserling H, King J, Reisinger KS, Blatter MM, Rothstein E, Bernstein HH, Hackell J. Safety and immunogenicity of a combined diphtheria, tetanus, pertussis and *Haemophilus influenzae* type b vaccine in young infants. Pediatrics 1993; 92 : 827-32.

Pederira FA, Guandole VL, Feroli EJ, Mella GW and Weiss IP. Involutionary smoking and incidence of respiratory illness during the first year of life. Pediatrics 1985; 75: 594-597.

Peltola H. Group A polysaccharide vaccine and course of the group A meningococcal epidemic in Finland. Scand J Infect Dis 1978; 104:41-44.

Peltola H. Meningococcal disease: still with us. Rev Infect Dis 1983; 5 :71-91.

Peltola H, Kataja JM, Makela PH. Shift in the age- distribution of meningococcal diseases as a predictor of an epidemic? Lancet 1982; 2: 595-597.

Perry VH, Hume DA and Gorden S. Immunohistological localization of macrophages and microglia in the adult and developing mouse brain. Neuroscience 1985; 15: 313-326.

Petersen BH, Graham JA, Brooks GF. Human deficiency of the eighth component of complement. The requirement of C8 for serum *Neisseria gonorrhoeae* bactericidal activity. J Clin Invest 1976; 57:283-90.

Pether JVS, Lightfoot NF, Scott RJD, Morgan J, Steele-Perkings AP, Sheard SC. Carriage of *Neisseria meningitidis*: investigations in a military establishment. Epidemiol Infect 1988; 101: 21-4.

Pershagen G. Review of epidemiology in relation to passive smoking. Arch Toxicol (suppl 9) 1986; 9: 63-73.

Pfister HW, Koedel U, Haberl RL. Microvascular changes during the early phase of experimental bacterial meningitis. J Cereb Blood Flow Metab 1990; 10: 914-22.

Plotkowski M-C, Puchelle F, Beck G, Jacquot J, Hannoun C. Adherence of type 1 *Streptococcus pneumoniae* to tracheal epithelium of mice infected with influenza A/PRB virus. Amm Rev Resp Dis 1984; 134: 1040-4.

Poolman JT, Hopman CT, and Zanen HC. Immunochemical characterization of *N. meningitidis* serotype antigens by immunodiffusion and SDS-polyacrylamide gel electrophoresis immuno-peroxidase techniques and the distribution of serotypes among cases and carriers. J Gen Microb 1980a ; 116 : 465-473.

Poolman JT, de Marie S, Zanen HC. Variability of low molecular weight, heat modifiable outer membrane proteins of *Neisseria meningitidis*. Infect Immun 1980b ; 30 : 642-8.

Poolman JT, Hopman CTP, Zanen HC. Problems in the definition of meningococcal serotypes. FEMS Microbiol Lett. 1982; 13 : 339-48.

Poolman JT, Hopman CTP, Zanen HC. Colony variants of *Neisseria meningitidis* strain 2996 (B:2b:P1.2): Influence of class-5 outer membrane proteins and lipopolysaccharides. J Med Microbiol 1985; 19:203-209.

Poolman JT, Jonsdottir K, Jones DM, Lind I, Froholm LO, Zanen HC. Meningococcal serotypes and serogroup B disease in North-West Europe. Lancet 1986; 2: 555-558.

Poolman JT. Meningococcal vaccines. J Med Microbiol 1988; 26:170-172.

Poolman JT. Polysaccharides and membrane vaccines. In: Mizrahi A ed. Bacterial vaccines. New York: Wiley-Liss, 1990: 57-86.

Pradhan AC, Chawla TN, Samuel KC, and Pradhan S. The relationship between periodental disease and blood groups and secretor status. J Period Res 1971; 6:294-300.

Priest R. Meningococcal infections in the army 1939-45, with 30 selected illustrative cases. J Roy Army Med Corps 1947; 89:1-28.

Quagliarello V, Scheld WM. Bacterial meningitis: pathogenesis, pathophysiology and progress. Engl. J. Med. 1992; 327 : 864-72.

Rahat A, Stewart J, Blackwell CC, and Weir DM. A semi-quantitative method for measurement of H type 1 and H type 2 on buccal cells and in saliva of secretors and non-secretors. Vox Sang 1990; 59: 101-105.

Raza MW, Blackwell CC, Molyneaux P, James VS, Ogilvie MM, Inglis JM, and Weir DM. Secretor status and viral illness. Br Med J 1991; 303: 815-818.

Raza MW, Blackwell CC, Ogilvie MM, Saadi AT, Stewart J, Elton RA, Weir DM. Evidence for the role of glycoprotein G of respitatory syncytial virus in binding of *Neisseria meningitidis* to Hep-2 cells. FEMS Immunol Med Mocrobiol 1994; 10 (1): 25-30.

Redhead K, Sesardic D, Yost SE, Attwell AM, Watkins J, Hoy CS, Plumb JE, Corbel MJ. Combination of DTP and *Haemophilus influenzae* type b conjugate vaccines can affect laboratory evaluation of potency and immunogenicity. Biologicals 1994; 22(4): 339-45.

Reller BL, MacGregor RR, Beaty HN. Bactericidal antibody after colonization with *Neisseria meningitidis.* J Infect Dis 1973; 127: 56 -62.

Riou JY, Poolman JT, Auriol J, Lamprez F, Guibourdenche M. Serosubtyping of group B, C, Y and A meningococci isolated in France in 1988. Ann Biol Clin (Paris) 1989; 48: 227-231.

Robbinson MG, Tolchin D and Halpern C. Enteric bacterial agents and the ABO blood groups. Am J Hum Genet 1971; 23: 135-145.

Roberts MC, Knapp JS. Host range of the conjugative 25.2 Mdal tetracycline resistance plasmid from *Neisseria gonorrhoeae* and related species. Antimicrob Agents Chemother 1988; 32: 488-91.

Ross SC, and Densen P. Complement deficiency states and infection: epidemiology, pathogenesis and consequences of neisserial and other infections in the immune deficiency. Med. 1984; 63: 243-273.

Saadi AT, Blackwell CC, Raza MW, James VS, Stewart J, Elton RA, Weir DM. Factors Enhancing adherence of toxigenic *Staphylococcus aureus* to epithelial cells and their possible role in sudden infant death syndrome. Epidemiol Infect 1993; 110: 507-17.

Saadi AT, Weir DM, Poxton IR, Stewart J, Essery SD, Blackwell CC, Raza MW, Busuttil A. Isolation of an adhesin from *Staphylococcus aureus* that binds Lewis a blood group antigen and its relevance to sudden infant death syndrome. FEMS Immunol Med Microbiol 1994; 8: 315-20.

Sacchi CT, Pessoa LL, Ramos SR. Ongoing group B *Neisseria meningitidis* epidemic in Sao Paulo, Brazil, due to increased prevalence of a single clone of the ET-5 complex. J Clin Microbiol 1992; 30 : 1734-8.

Saez-Nieto JA, Fontanals D, Garcia de Jalon J. Isolation of *Neisseria meningitidis* strains with increase of penicillin minimal inhibitory concentration. Epidemiol Infect 1987; 99: 463-9.

Saez-Nieto JA, Vazquez JA, Marcros C. Meningococci moderately resistant to penicillin. Lancet 1990; ii: 54.

Saez-Nieto JA, Lujan R, Berron S. Epidemiology and molecular basis on penicillin-resistant N. meningitidis in Spain: a 5-year history (1985-1989). Clin Infect Dis 1992; 14: 394-402.

Salih MA, Danielson D, Backman A, Caugant DA, Achtman M, Olcen P. Characterization of epidemic and non-epidemic *Neisseria meningitidis* serogroup A strains from Sudan and Sweden. J Clin Microbiol 1990; 28 : 1711-9.

Salmi I, Pettay O, Simula I, Kallio A-K, Waltimo O. An epidemic due to sulphonamide-resistant group A meningococci in the Helsinki area (Finland). Scand J Infect Dis 1976; 8: 249-54.

Samuelson S, Ege P, Berthelsen B, Lind I. An outbreak of serogroup B:15:P1.16 meningococcal disease, Frederiksborg county, Denmark, 1987-9. Epidemiol Infect 1992; 108 : 19-30.

Sanford B, Davison V, Ramsay M. *Staphylococcus aureous* adherence to influenza A virus infected and control cell cultures: evidence for multiple adhesions. Proc Soc Exp Biol Med 1986; 181: 104-11.

Savilhati E. IgA deficiency in children in children immunoglobulin-containing cells in the intestinal mucosa, immunoglobulins in secretions and serum IgA levels. Clin Exp Immunol 1973; 13: 395-406.

Schneider H, Griffiss JM, Boslego JW, Hitchcock PJ, Zahos KM, Apicella MA. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. J Exp Med 1991; 174:1601-1605.

Scholten RJPM, Bijlmer HA, Poolman JT. Meningococcal disease in the Netherlands, 1958-1990: a steady increase of the incidence since 1982 partially caused by a new serotypes and subtypes of *N. meningitidis*. Clin Infect Dis 1993; 16: 237-46.

Scholten RJPM, Kuipers B, Valkenburg HA, Dankert J, Zollinger WD and Poolman JT. Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA with monoclonal antibodies. J Med Microbiol 1994; 41: 236-43.

Severin WP, Ruys AC, Bijkerk H . The epidemiology of meningococcal meningitis in the Netherlands in recent years, with special reference to the epidemic of 1966. Zbl Bakt [Orig] 1969; 210 : 364-70.

Shaad UB, Krucko J, Pfenninger J. An extended experience with cefuroxime therapy of childhood bacterial meningitis. Pediatr Infect Dis 1984;3: 410-415.

Shao-Qing D, Ren-Bang Y, Han-Chun Z. Three new serogroups of *Neisseria meningitidis* J Biol Stand 1972; 9:307-315.

Shinebaum R, Blackwell CC, Forster PJ, Hurst NP, Weir DM, Nuki G. Non-secretion of ABO blood group antigens as a host susceptibility factor in the spondyloarthropathies. Br Med J Clin Res Ed. 1987; 294: 208-10.

Sierra GVG, Campa HC, Garcia IL,. Efficacy evaluation of the Cuban vaccine VA-MENGOC-BC against disease caused by serogroup B *Neisseria meningitidis*. In: Achtman M, Kohl P, Marchal C, Morelli G, Seiler A, Thiesen B, eds. Neisseriae 1990. Walter de Gruyter, 1991: 129-34.

Silverblatt FG and Cohen LS. Antipili antibody affords protection against experimental ascending pyelonephritis. J Clin Invest 1979; 64: 333-441.

Sjoholm AG, and Nilsson L-A. Properdin deficiency in a second Swedish family: absence of clinical manifestations. Complement 2 1985; 73A [abstract].

Slaterus KW. Serological typing of meningococci by means of micro-precipitation. Antonie Van Leeuwenhoek 1961; 27 : 305-15.

Smith DH, Peter G, Ingram DL, Harding AL, and Anderson P. Responses of children immunized with capsular polysaccharide of *Haemophillus influenzae* type b. Pediatr 1973; 52: 637-644.

Smith DJ, Gahnberg L, Taubman MA and Ebersole JL. Salivary antibody responses to oral and parenteral vaccines in children. J Clin Immunol 1986; 6: 43-49.

Smith DJ, King WF, Taubman MA. Isotype , subclass and molecular size of immunoglobulins in saliva from young infants. Clin Exp Immunol 1989; 76: 97-102.

Sneath PH, and Sokal RR. Numerical taxonomy: the principles and practice of numerical classification. 1973, W.H. Freeman, San-Frasinsco.

Socha W, Bilinsk M and Kaczera Z. *Escherichia coli* and ABO blood groups. Folia Biol (Krakola) 1969; 17: 259-269.

Soderstrom C, Braconier JH, Kayhty H, Sjoholm AG, and Thuresson B. Immune response to tetravalent meningococcal vaccine: opsonic and bactericidal functions of normal and properdin deficient sera. Eur. J Clin Microbiol Dis 1989; 8: 220-224.

Spanjaard L, Bol P, de Marie S, Zanen HC. Association of meningococcal serogroups with the course of disease in the Netherlands, 1959-83. Bull WHO 1987; 65 : 861-868.

Spott MS, Kearns AM, Field JM. Penicillin-insensitive *Neisseria meningitidis* Lancet i: 1988; 1167.

Stephens DS, Farley MM. Pathogenic events during infection of the human nasopharynx with *Neisseria meningitidis* and *Haemophilus influenzae*. Rev Infect Dis 1991; 13: 22-23.

Stuart JM, Cartwright KAV, Dawson AJ, Rickard J, Noah ND. Risk factors for meningococcal disease: a case control study in Southwest England. Comm Med 1988; 10: 139-46.

Stuart JM, Cartwright KAV, Robinson PM, Noah ND. Effect of smoking on meningococcal carriage. Lancet 1989; ii 723-5.

Sutcliffe EM, Jones DM, El-Sheikh S. Penicillin-insensitive meningococci in the UK. Lancet 1988; i: 657-8.

Tager IB, Weiss ST, Munoz A, Rosner B, and Speizer FE. Longitudinal study of the effects of maternal smoking on pulmonary function in children. N Eng J Med 1983; 309: 699-703.

Tan TQ, Mason EO, Kaplan SL. Systemic infections due to *Streptococcus pneumoniae* relatively resistant to penicillin in a children's hospital: clinical management and outcome. Pediatrics 1992; 90:928-33.

Thom SM, Blackwell CC, MacCallum CJ, Weir DM, Brettle RP, Kinane DF, Wray D. Non-secretion of blood group antigens and susceptibility to infection by Candida species. FEMS Micribiol Immunol 1989; 1(6-7): 401-5.

Tobin JL. Complications of meningococcus infection in a series of sixty-three consecutive sporadic cases. Am J Med Sci 1956; 231: 241-8.

Trichopoulos D. Epidemiology: principles, methods, applications. Athens : R. Patisiano Publishers, 1982; 139.

Tsai CM, Frasch CE, Mocca LF. Five structural classes of major outer membrane proteins in *Neisseria meningitidis*. J. Bacteriol. 1981; 146 : 69-78.

Tsai CM, Mocca LF, Frasch CE. Immunotype epitopes of *Neisseria meningitidis* lipopolysaccharide type 1 through 8. Infect Immun 1987; 55 :1652-6.

Tsai CM, Civin CI. Eight lipooligosaccharides of *Neisseria meningitidis* react with a monoclonal antibody which binds lacto-N-neotetraose (Galβ1-4GlcNacβ1-3Galβ1-4Glc). Infect Immun 1991: 59:3604-3609.

Turren JH, Dworkin RJ, Kennedy SL, Sachdeva M, Sande MA. Loss of cerebrovascular autoregulation in experimental meningitis in rabbits. J Clin Invest 1990; 85: 577-81.

Tzanakaki G, Mavrommati L, Tzelepi E.. Serological classification in relation to auxotypes, plasmid contents, and susceptibility to antimicrobials of PPNG and non-PPNG strains isolated in Greece. Genitour Med 1989; 65: 171-6.

Twite SJ, Blackwell CC, Raza MW, Saadi AT, Essery SD and Weir DM. Are monocytes the " Trojan horses" of meningococcal disease? Proceedings of the 9th International Pathogenic Neisseria Conference 1994; The Guildhall . Winchester. England pp. 284-286

Twumasi PA, Kumah S, Leach A, O'Dempsey TJ, Ceesay SJ, Tood J, Broome CV, Carlone GM, Pais LB, and Greenwood BM. A trial of a group A plus group C meningococcal polysaccharide-protein conjugate vaccine in African Infants. J Infect Dis 1995; 171: 632-8.

Virji M, Makepeace K, Ferguson DJ, Achtman M Moxon ER. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. Molec Biol 1993; 10(3): 499-510.

Viskum K. The ABO Rhesus blood groups in patients with pulmonary tuberculosis. Tubercl 1975; 56: 329-334.

Waissbluth JG, Langman JS. ABO blood groups, secretor status, salivary protein and serum and salivary immunoglobulin concentrations. Gut 1971; 12: 646-649.

Wald N, Kiryluk S, Darby S, Doll R, Pike M, Peto R. UK smoking statistics. Oxford: University Press, 1988.

de Wals P, Gilquin C, de Maeyer S, Bouckaert A, Noel A, Lechat MF, and Lafontaine A. Longitudinal study of asymptomatic carriage in two Belgian populations of school children. J Infect 1983; 6: 147-156.

Wang J-F, Caugant DA, Li X,. Clonal and antigenic analysis of serogroup A isolates of *Neisseria meningitidis* with particular reference to epidemiological features of epidemic meningitis in the People's Republic of China. Infect Immun 1992; 60 : 5267-82.

Wedege E, Hoiby EA, Rosenqvist ER, Froholm LO. Serotyping and subtyping of *Neisseria meningitidis* isolates by co-agglutination dot-blotting and ELISA. J Med Microbiol 1990; 31: 195-201.

Weidner CE, Dunkel TB, Pettyjohn FS. Effectiveness of rifampicin in eradicating the meningococcal carrier state in a relatively closed population: Emergence of resistant strains. J Infect Dis. 1971; 124:172-8.

Weihe P, Mathiassen B, Rasmussen JM, Petersen T, Isager H. An Epidemic outbreak of group B meningococcal disease on the Faroe Islands. Scand J Infect Dis 1988; 20: 291-6.

Williams AE and Blakemore WF. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J Infect Dis 1990; 162: 474-481.

Winterscheid KK, Whittington WL, Roberts MC, Schwebke JR, Holmes KK. Decreased susceptibility to penicillin G and *Tet* M plasmids in genital and anorectal isolates of *Neisseria meningitidis*. Antimicrob Agents Chemother 1994; 38:1661-63.

Wyle FA, Artenstein MS, Brandt BL, Tramont EC, Kasper DL, Altieri PL, Berman SL, Lowenthal JP. Immunologic response of a man to group B meningococcal polysaccharide vaccines. J Infect Dis 1972; 126:514-522.

Yagupsky P, Ashkenazi S, Block C. Rifampicin-resistant meningococci causing invasive disease and failure of prophylaxis Lancet 1993; 341:1152-53.

Young HY. Identification and penicillinase testing of *Neisseria gonorrhoeae* from primary isolation cultures on MNYC medium. J Clin Microbiol 1978; 7: 247-50,

Young H. Cultural diagnosis of gonorrhoea with modified New York City Medium (MNYC). Br J Vener Dis 1978; 54: 36-40.

Young H, Paterson IC, McDonald DR. Rapid carbohydrate utilization test for identification of *Neisseria gonorrhoeae*. Br J Vener Dis 1979; 52: 172-175.

Young LS, LaForce FM, Head JJ. A simultaneous outbreak of meningococcal and influenza infections. N Engl J Med 1972; 287: 5-9.

Zollinger WD, Mandrell RE. Outer membrane protein and lipopolysaccharide serotyping of *Neisseria meningitidis* by inhibition of a solid phase radio-immunoassay. Infect Immun. 1977; 18 : 424-34.

Zollinger WD, Mandrell RE. Type-specific antigens of group A *Neisseria meningitidis*: Lipopolysaccharides and heat modifiable outer membrane proteins. Infect Immun 1980; 28 : 451-8.

Zollinger WD, Boslego J, Moran E,. Meningococcal serogroup B vaccine protection trial and follow-up studies in Chile. NIPH Annals 1991; 14: 211-2.

Zorgani AA, Stewart J, Blackwell CC, Elton RA, Tzanakaki G and Weir DM. Secretor status and humoral immune responses to *Neisseria lactamica* and *Neisseria meningitidis* . Epidemiol and Infect 1992; 109: 445-452.

Zorgani AA, Stewart J, Blackwell CC, Elton RA, and Weir DM. Inhibitory effect of saliva from secretors and non-secretors on binding of meningococci to epithelial cells. FEMS Immunol and Med Microbiol 1994; 9:135-142.

Appendix 1. Recruit Questionnaire

No _____

Age _____

Date of Birth _____

Place of Birth _____

Mother's place of birth _____

Father's place of birth _____

Place of Residence _____

Years of Education _____

You have finished

"Dimoticon" (Primary school) _____

"Gymnasion" (lower secondary school) _____

"Likion" (upper secondary school) _____

Upper National Diploma _____

University _____

Profession _____

Did you have any Upper respiratory tract infection recently?

YES _____

NO _____

How many cigarettes you smoke per day?

0 _____

1 - 10 _____

10 - 20 _____

20 - 30 _____

> 30 _____

Did you have any gonorrhoea symptoms the past month? _____

Name _____

Signature _____

Date _____

Appendix 2. School Questionnaire

Name / Surname _____

Sex _____

Class _____

Date of Birth _____

Place of Birth _____

Place of residence _____

Mother's place of birth _____

Father's place of birth _____

Parents' educational level

	Father	Mother
"Dimoticon"		
"Gymnasion"		
"Lykion"		
University		

Members of the family _____

Who smokes at home ?

Father _____

Mother _____

Other _____

Did you have any symptoms of flu or other sickness recently ? ____

_____

Have you taken any antibiotics recently? _____

Parents' or guardian's name _____

Parents' or guardian's signature _____

Epidemiol. Infect. (1992), **108**, 441–448
Printed in Great Britain

Factors affecting carriage of *Neisseria meningitidis* among Greek military recruits

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(*Accepted 10 November 1991*)

SUMMARY

Greek military recruits (993) were examined for carriage of meningococci during July 1990. Blood, saliva and throat swab specimens were obtained and each recruit answered a questionnaire providing information on age, education (a measure of socioeconomic level), place of residence, smoking habits and recent infections.

The overall carriage rate was 25% but differed between the two camps: 79/432 (18%) in Camp A and 168/561 (30%) in Camp B ($P < 0{\cdot}0005$). In Camp B, there were significantly higher proportions of recruits who were non-secretors ($P < 0{\cdot}0005$), and/or heavy smokers ($P < 0{\cdot}0005$). They were also younger (< 19 years old) ($P < 0{\cdot}001$), and on the whole had fewer years of education ($P < 0{\cdot}0005$). By univariate analysis, carriage was significantly associated with smoking. By multiple logistic regression analysis, carriage was associated with smoking ($P < 0{\cdot}001$), age ($P < 0{\cdot}01$) and the camp in which the recruits were based ($P < 0{\cdot}01$). Among recruits in Camp B, 15/38 (40%) of those with recent viral infections were carriers compared with 30% for the camp in general.

INTRODUCTION

Following the epidemic of meningitis during the 1960s, the incidence of meningococcal disease in Greece declined steadily until 1988. Since 1988, the number of cases reported to the Ministry of Health has increased; in the first 6 months of 1990, there were 102 cases compared with 87 for all of 1989 (Table 1). This apparent increase in disease due to *Neisseria meningitidis* prompted the present work as the last study of meningococcal carriage in a Greek population was carried out before elucidation of some of the genetic and environmental factors affecting carriage [1].

Epidemiological studies have shown that non-secretors, individuals incapable of secreting the glycoprotein form of their ABO blood group antigens, are significantly over-represented among patients with meningococcal disease [2, 3]

Table 1. *Incidence of meningococcal disease in Greece*

Year	No. cases	Deaths no. (%)
1968	1064	48 (4·5)
1973	765	39 (5·1)
1978	286	18 (6·3)
1983	102	9 (8·8)
1985	105	6 (5·7)
1986	88	7 (7·9)
1987	85	4 (4·7)
1988	76	2 (2·6)
1989	87	6 (6·9)
1990 (1st 6 months)	102	

and among carriers in a school population [4]. Prolonged outbreaks have occurred in countries such as Iceland and in areas of Britain [5, 6] where the proportion of non-secretors is significantly higher than the proportion (20–25%) predicted for European populations [7].

Environmental factors have also been suggested as playing a role in susceptibility to meningococcal disease [8, 9] or carriage of the bacteria. Smoking or passive exposure to cigarette smoke has been associated with isolation of *Neisseria meningitidis* from healthy asymptomatic adults and teenagers [4, 10]. Virus infections have been suggested as predisposing factors for susceptibility to meningococcal disease [11] and to carriage of potentially pathogenic bacteria [12].

In the study reported here we wished to determine: (1) if isolation of meningococci was associated with non-secretion in the populations examined; (2) if smoking was associated with carriage in a closed population; (3) if an upper respiratory tract or other infection might be a factor contributing to carriage of meningococci; (4) if there might be geographical regions in Greece in which there are higher proportions of non-secretors and if the prevalence of meningococcal disease was increased in these areas.

SUBJECTS AND METHODS

Greek military recruits (993) from two camps, one at Athens and another at Avlona, were examined for carriage of meningococci during the third week of July 1990. Ethical permission for the study was granted by the Ministry of Defence. An explanation of the purpose of the study and the specimens required was part of the questionnaire and each subject signed his consent to participate in the study. The recruits came from all regions of Greece except the Ionian islands, and had been in the camps from 3 to 5 days at the time of the study. Throat swabs, blood and saliva specimens were obtained. Throat swabbing was carried out by the same four members of the team on both occasions (C.C.B., G.T., A.M., D.M.W.). Blood was not obtained from a number of recruits who fainted because of the unusually high temperature (41 °C). Each recruit filled in a questionnaire providing information on age, occupation, education level, places of residence and birth, smoking habits and recent infections. A member of the research team checked the form to ensure all categories had been completed and to answer any questions. This information

and results of laboratory examinations were coded for confidentiality and stored in a Database 3 plus data base. The information on the data base was recalled and checked with the original questionnaire.

ABO blood group was determined by slide agglutination with monoclonal anti-A and anti-B antibodies (Scottish National Blood Transfusion Service); Lewis blood group was determined by tube agglutination with monoclonal anti-Lewis^a (Le^a) and anti-Lewis^b antibodies (Scottish National Blood Transfusion Service). Non-secretors can express only Le^a, but secretors express Le^b. The blood specimens were centrifuged on the day of collection and the plasma stored at −20 °C.

Throat swabs were plated directly onto modified New York City medium [13] and incubated for 48 h. Colonies were examined for oxidase production, Gram stain and carbohydrate utilization.

Two-way frequency tables were analysed by χ^2 tests with Yates' correction in the case of 2×2 tables. Multiple logistical regression was used to examine the association between carriage rates and other factors adjusted for each other.

RESULTS

Isolation of N. meningitidis

N. meningitidis was isolated from 247 (25%) of the 993 recruits examined. The carriage rate differed significantly between the two camps. There were 79 isolates obtained from 432 recruits (18%) in the camp at Athens compared with 168 from the 561 (30%) at Avlona ($\chi^2 = 17{\cdot}54$; $P < 0{\cdot}0005$).

Characteristics of the two populations

Distribution of ABO and Lewis blood group phenotypes

There was no difference in the distribution of the ABO blood groups between the two camps. Among the recruits who expressed Lewis antigen, there was a significantly higher proportion of Le^a/non-secretors in the camp at Avlona, 140/534 (26%), compared with that in the camp at Athens, 60/390 (15%) ($\chi^2 = 14{\cdot}96$, $P < 0{\cdot}0005$).

Age and education

The ages of the two populations differed significantly. The median age was 19 for both camps; however, there was a higher proportion of recruits under 19 in the camp at Avlona (4%) than in that at Athens (1%) ($\chi^2 = 12{\cdot}96$, $P < 0{\cdot}001$).

There was also a significant difference in the level of education of the recruits in the two camps, particularly at the two extremes. Among the recruits at Avlona 14% had only 6 years of schooling compared with 7% among those at Athens; and, only 2% of the recruits at Avlona had attended university while 7% of those at Athens had done so ($\chi^2 = 30{\cdot}32$, df = 4, $P < 0{\cdot}0005$).

Smoking

There was no significant difference between the proportion of smokers in the Athens camp (62%) and in Avlona (68%); but there were significantly more heavy smokers (> 30 cigarettes a day) in Avlona ($\chi^2 = 27{\cdot}64$, df = 4, $P < 0{\cdot}0005$) (Table 2).

Table 2. *Cigarette consumption in the two recruit groups*

No. cigarettes/day	Athens ($n = 431$) No. (%)	Avlona ($n = 560$) No. (%)
0	166 (38)	182 (32)
1–10	53 (12)	63 (11)
11–20	127 (29)	133 (24)
21–30	62 (14)	96 (17)
> 30	23 (5)	86 (15)

Table 3. *Carriage rates and cigarette consumption*

No. cigarettes/day	Total	Carriers no. (%)	Non-carriers no. (%)
0	355	56 (16)	299 (84)
1–10	116	29 (25)	87 (75)
11–20	260	66 (25)	194 (75)
21–30	157	44 (28)	113 (72)
> 30	109	40 (37)	69 (63)

Prevalence of other infectious diseases

Only 59 of the recruits reported symptoms of upper respiratory tract infections (URTI) within the previous 2 weeks, 21 of the 432 at Athens (4·9%) and 38 of the 561 (6·7%) at Avlona.

Factors associated with carriage of meningococci

There was no difference in the proportions of carriers among the four ABO blood groups. The proportion of carriers among Le^a/non-secretors (22% at Athens, 32% at Avlona) was higher than that among Le^b/secretors (17% at Athens, 30% at Avlona), but the difference was not significant.

The proportion of carriers was significantly higher among smokers and was greatest among those smoking more than 30 cigarettes per day (Table 3) ($\chi^2 = 23{\cdot}19$, df = 4, $P < 0{\cdot}0005$). Although the proportion of carriers was significantly increased among smokers in both camps (Athens, $\chi^2 = 5{\cdot}42$, df = 1, $P < 0{\cdot}05$; Avlona $\chi^2 = 9{\cdot}05$, df = 1, $P < 0{\cdot}01$), when the data for carriers were analysed by χ^2 test, there was no significant difference between the two camps associated with numbers of cigarettes per day ($\chi^2 = 9{\cdot}482$, df = 4, $P > 0{\cdot}05$) (Table 4).

Meningococci were isolated from 17 (29%) of the 59 recruits who had symptoms of URTI, 2 of the 21 (9·5%) at Athens and 15 of the 38 (39·5%) at Avlona. Among the 15 at Avlona from whom meningococci were isolated, 6 (40%) were Le^a/non-secretors compared with 4 (20%) Le^a/non-secretors among the 20 with recent symptoms of URTI but from whom no meningococci were isolated. The numbers of recruits with symptoms of URTI were too small for statistical analysis.

Multiple logistic regression analysis identified three main factors which were significantly associated with carriage: smoking ($P < 0{\cdot}001$); age less than 19 years ($P < 0{\cdot}01$) and the camp in which the recruits were based ($P < 0{\cdot}01$). Various

Table 4. *Smoking patterns and carriage of* N. meningitidis *in the two camps*

	Athens ($n = 431$)		Avlona ($n = 560$)	
No. cigarettes/ day	Total No. (%)	Carriers No. (%)	Total No. (%)	Carriers No. (%)
0	166 (38)	20 (27)	182 (32)	36 (22·5)
1–10	53 (12)	10 (13)	63 (11)	19 (12)
11–20	127 (29)	26 (35)	133 (24)	40 (25)
21–30	62 (14)	15 (20)	96 (17)	29 (18)
> 30	23 (5)	4 (15)	86 (15)	36 (22·5)
Total	431	75	560	160

combinations of categories were tested for difference in carriage rate when adjusted for these three factors but no other variables were found to be significant.

Regional variations in ABO and Lewis blood group antigens

There was no difference in the distribution of the ABO blood groups in the two major population centres, Athens and Eastern Macedonia. There was, however, a significantly higher proportion of Le^a/non-secretors, 54/183 (29·5%) among the recruits from Eastern Macedonia compared with those from Athens, 35/235 (15%) ($\chi^2 = 12{\cdot}25$, $P < 0{\cdot}0005$).

DISCUSSION

As expected, compared with carriage rates reported for civilian populations (5–8% for Greek secondary school children), there was a higher proportion of carriers (25%) among the recruits examined in this study. This figure is similar to that found for a British military establishment (23%) [14] but slightly lower than that found in the 1970s for Greek military recruits (33%) [1]. The significant difference in the proportion of carriers between the two camps, 18% at Athens compared with 30% at Avlona ($P < 0{\cdot}0005$), was not anticipated.

Smoking [4, 10], passive exposure to cigarette smoke [10], secretor status [4] and age [15] have all been reported to be associated with carriage of meningococci. The distribution of the ABO blood group phenotypes did not differ between the two camps and were similar to those we found in a previous study [16] and that reported by Hirzfeld and Hirzfeld in 1919 [17]. Several of the factors examined in the study varied between the two camps. Compared with the recruits at Athens, among those at Avlona where there was a significantly higher proportion of carriers of meningococci, there were significantly more heavy smokers, non-secretors, younger recruits (less than 19 years old), and recruits of lower socieconomic groups as assessed by years of education.

Smoking was the factor most strongly associated with carriage in both univariate and multivariate analyses. It has been suggested that the higher proportion of smokers and heavy smokers among military personnel [18] might contribute to the increased prevalence of meningococcal disease and the higher rates of carriage among military recruits. The proportions of smokers in the two camps, 62 and 68%, were higher than the 47% found in a previous study among Greek male patients referred for gastroscopy [15]. In Britain smoking is associated

with socioeconomic status; unskilled individuals are more likely to smoke than those in professional groups [19]. A similar pattern was observed among the recruits; those with fewer years of education were more likely to be smokers.

In a previous study of teachers and pupils in a Scottish secondary school following an outbreak of meningitis, we found an association between non-secretion and carriage of meningococci [4]. In the present study, in both camps, the proportions of non-secretors were increased among carriers; but, these differences were not significant compared with the secretors among carriers. Assessment of both host and parasite characteristics might elucidate the differences in association with secretor status. The majority (35·5%) of serotypable strains among the 121 isolates in the Scottish study were serotype 4; and 55% of the carriers of this serotype were non-secretors. Only 9 (1·8%) isolates in the present study were serotype 4, but 37·5% of the carriers were non-secretors. The proportion of non-secretors among carriers of serotype 14 isolates was 25 and 22% for the Scottish and Greek populations. A similar pattern was observed for non-serotypable isolates.

The multiple logistic regression analysis identified age (those less than 19 years of age) to be a significant variable ($P < 0{\cdot}01$). This agrees with the findings for carriers in the Stonehouse survey. The highest proportion of carriers was found in the 15–19 year age group [15].

There is evidence that viral infections can predispose individuals to carriage of potentially pathogenic bacteria [12]; and binding of streptococci and staphylococci to cell surfaces is enhanced in experimental models examining infection with influenza virus [20, 21]. The present study was carried out in July when there is a low prevalence of upper respiratory viral infections; however, of the 59 recruits with symptoms of virus infection, 29% were carriers. This was particularly apparent among recruits at Avlona where the carriage rate was 30% overall but nearly 40% among those with virus infections. In a recent study, a higher proportion of patients with meningococcal disease had a history of flu-like illness than age- and sex-matched controls; however, the differences were not statistically significant [22]. A second study of recruits was undertaken in January 1991 to assess carriage when viral infections are more prevalent; the analysis of this study is not complete at present.

In two areas of Britain where there have been prolonged outbreaks of meningococcal disease, the proportions of non-secretors have been reported to be higher than the 20–25% predicted for European populations [5, 6]. In this study we found a significantly higher proportion of recruits from Eastern Macedonia were non-secretors (29·5%) compared with the other major centre of population, Athens (15·3%). As there are anecdotal reports that meningococcal disease is more prevalent in Macedonia, the numbers of cases reported to the Ministry of Health from these two areas is now being closely monitored.

ACKNOWLEDGEMENTS

We are grateful to the Ministry of Defence for permission to carry out this study and to the officers and recruits for their participation. The study was carried out with the expert assistance of: G. Voloudaki, A. Tabaki, G. Boursinos, G. Mitrakos,

. Garanis, N. Kikemeni, P. Konstandinopoulo and T. Priftis. We are also grateful o Mrs J. G. Blackwell for assistance with the data processing and Mrs M. K. Cole or preparation of the manuscript.

REFERENCES

1. Kalapothaki V, Vassilliadis P, Trichopoulos D, Kaklamani E, Sérié Ch. Caractéristiques séro-épidémiologiques chez les porteurs sains de meningocoques en Grèce. Annales Microbiol (Institute Pasteur) 1979; **130B**: 43–54.
2. Blackwell CC, Jónsdottir K, Hanson M, et al. Non-secretion of ABO blood group antigens predisposing to infection by *Neisseria meningitidis* and *Streptococcus pneumoniae*. Lancet 1986; ii: 284–5.
3. Blackwell CC, Jonsdóttir K, Mohammed I, Weir DM. Non-secretion of blood group antigens. A genetic factor predisposing to infection by *Neisseria meningitidis*. In: Poolman JT, Zanen HC, Meyer TF, et al, editors. Gonococci and meningococci. Dordrecht: Kluwer Academic Publishers, 1988: 633–6.
4. Blackwell CC, Weir DM, James VS, et al. Secretor status, smoking and carriage of *Neisseria meningitidis*. Epidemiol Infect 1990; **104**: 203–9.
5. Blackwell CC, Weir DM, James VS, Cartwright KAV, Stuart JM, Jones DM. The Stonehouse study: secretor status and carriage of *Neisseria meningitidis* and *Neisseria lactamica*. Epidemiol Infect 1989; **102**: 1–10.
6. Blackwell CC, Weir DM. Meningococcal disease: high virulence and low transmission? Lancet 1990; ii: 53.
7. Mourant AE, Kopéc AC, Domaniewska-Sobczak K. The distribution of the human blood groups and other polymorphisms. London: Oxford University Press, 1976: 548–76.
8. Haneberg B, Tonjum T, Rodahl K, Gedde-Dahl T. Factors preceding the onset of meningococcal disease with special emphasis on passive smoking, stressful events, physical illness and general symptoms of ill health. Nat Inst Pub Health Annal 1983; **6**: 169–76.
9. Stuart JM, Cartwright KAV, Dawson AJ, Rickard J, Noah ND. Risk factors for meningococcal disease: a case control study in Southwest England. Comm Med 1988; **10**: 139–46.
10. Stuart JM, Cartwright KAV, Robinson PM, Noah ND. Effect of smoking on meningococcal carriage. Lancet 1989; ii: 723–5.
11. Krakinski K, Nilson JD, Butler S, Laby JP, Kusmiesz H. Possible association of mycoplasma in viral respiratory infections with bacterial meningitis. Am J Epidemiol 1987; **125**: 499–508.
12. Musher DM, Fainstein V. Adherence of *Staphylococcus aureus* to pharyngeal cells from normal subjects, smokers, staphylococcal carriers and patients with viral infections. In: Jeljaszewicz J, editor. Staphylococci and staphylococcal infections. New York: Gustav Fischer Verlag. Zbl Bakt Suppl 10, 1981; 1011–16.
13. Young H. Cultural diagnosis of gonorrhoea with modified New York City Medium (MNYC). Br J Vener Dis 1978; **54**: 36–40.
14. Pether JVS, Lightfoot NF, Scott RJD, Morgon J, Steele-Perkins AP, Sheard SC. Carriage of *Neisseria meningitidis*: investigations in a military establishment. Epidemiol Infect 1988; **101**: 21–4.
15. Cartwright KAV, Stuart JM, Jones DM, Noah MD. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol Infect 1987; **99**: 591–601.
16. Mentis A, Blackwell CC, Weir DM, Spiliadis C, Dailianas A, Skandalis N. ABO blood group, secretor status and detection of *Helicobacter pylori* among patients with gastric or duodenal ulcers. Epidemiol Infect 1990; **106**: 221–9.
17. Hirszfeld L, Hirszfeld H. Serological differences between the blood of different races. The result of researches on the Macedonian front. Lancet 1919; ii: 675–9.
18. Crowdy JP. Smoking in the army. J Roy Army Med Corps 1981; **127**: 1–15.
19. Wald N, Kiryluk S, Darby S, Doll R, Pike M, Peto R. UK smoking statistics. Oxford: Oxford University Press, 1988.
20. Plotkowski M-C, Puchelle F, Beck G, Jacquot J, Hannoun C. Adherence of type 1

Streptococcus pneumoniae to tracheal epithelium of mice infected with influenza A/PR virus. Am Rev Resp Dis 1984; **134**: 1040–4.

21. Sanford B, Davison V, Ramsay M. *Staphylococcus aureus* adherence to influenza A viru infected and control cell cultures: evidence for multiple adhesions. Proc Soc Exp Biol Me 1986; **181**: 104–11.
22. Cartwright KAV, Jones DM, Smith AJ, Stuart JM, Kaczmarski EB, Palmer SR. Influenz A and meningococcal disease. Lancet 1991; **338**: 554–7.

Epidemiol. Infect. (1992), **108**, 449–455
Printed in Great Britain

Antibiotic sensitivities of *Neisseria meningitidis* isolates from patients and carriers in Greece

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(*Accepted 10 November 1991*)

SUMMARY

Usage of antibiotics in southern Europe is less well regulated than in northern countries. The proportion (48%) of meningococci in Spain insensitive to penicillin (MIC $\geqslant$ 0·1 mg/l) prompted this investigation of antibiotic sensitivities of isolates from Greek patients with meningitis (31) and carriers (47 school-children and 472 recruits). The agar dilution method was used to determine MIC to penicillin G (PN), sulphamethoxazole (SU), rifampicin (RF), cefaclor (CF) and ciprofloxacin (CP).

The proportion of isolates insensitive to PN was 48% for isolates from patients, 19% from school-children and 36·6% from recruits. Resistance to SU (MIC $\geqslant$ 16 mg/l) was found in 16% of those from patients, 10·6% from children and 40% from recruits. None of the isolates from patients was resistant to RF ($\geqslant$ 1 mg/l) but 6% of those from carriers were. Resistance to CF ($\geqslant$ 4 mg/l) was found in 9·2% of patient isolates, 6·4% from children and 23·7% from recruits. All isolates except one were sensitive to CP (MIC range $<$ 0·0015–0·125 mg/l).

Resistances to PN, SU and RF were analysed by serogroup, serotype and subtype of the bacteria. The proportion of resistant isolates showed some variation between different areas of Greece, but it was not statistically significant.

INTRODUCTION

As part of the surveys of isolates of meningococci from patients and carriers in Greece, the antibiotic sensitivities of these bacteria to some common antibiotics were determined. There are reports of *Neisseria meningitidis* with reduced sensitivity to penicillin isolated from patients in Spain [1, 2], South Africa [3], and the United Kingdom [4, 5]. A small proportion of these penicillin-insensitive isolates has also been found among those obtained from carriers in the United Kingdom [4]. In view of the increasing prevalence of penicillin-insensitive isolates

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in Spain and the similar antibiotic policies of both Spain and Greece, it was predicted that there might be significant numbers of Greek isolates with decreased sensitivity to penicillin and other antibiotics.

The objectives of the study were: (1) to compare levels of sensitivities for isolates obtained from patients with those obtained from carriers; (2) to determine if the proportion of penicillin-insensitive isolates is comparable to that reported for Spain; (3) to assess the distribution of resistant isolates in the different regions of Greece; (4) to determine if antibiotic resistances are associated with particular serogroups, serotypes or subtypes.

ISOLATES

Meningitis cases

Thirty-one isolates from children aged 1 month to 11 years were provided by the microbiology laboratory of the 'Aglia Kyriakou' Paediatric Hospital. All cases were sporadic and occurred during 1989–91.

Carriers

Four hundred and seventy-two isolates were obtained during 1990–1 from healthy recruits aged 16–31 years [6] and a further 47 from children aged 6–18 years who did not have meningococcal disease.

METHODS

Antibiotic sensitivities to penicillin (PN), erythromycin (ER), tetracycline (TC), sulphamethoxazole (SU), rifampicin (RF), cefaclor (CF) and ciprofloxacin (CP) were determined by the agar dilution method. A range of twofold dilutions from 256 to 0·015 mg/l of each antibiotic in Mueller Hinton medium was examined. The breakpoints used for penicillin ($\geqslant$ 0·125 mg/l), tetracycline ($\geqslant$ 1 mg/l), erythromycin ($\geqslant$ 0·5 mg/l), rifampicin ($\geqslant$ mg/l), ciprofloxacin ($\geqslant$ 1 mg/l) and the cephalosporin cefaclor ($\geqslant$ 4 mg/l) were based on those recommended by the working party of the British Society for Antimicrobial Chemotherapy for *Branhamella catarrhalis* and *Haemophilus influenzae* [7]. The break point of $\geqslant$ 10 mg/l for sulphamethoxazole (SU) was that used by the Neisseria Reference Laboratory (Scotland) [Dr R. J. Fallon, personal communication]. Beta lactamase production was assessed during the rapid carbohydrate fermentation test for identification of each isolate [8].

Serogroup was determined by slide agglutination with commercially available antisera (Wellcome Diagnostics); serotype and subtype were determined by whole cell enzyme-linked immunosorbent assays with monoclonal antibodies from Dr J. T. Poolman (RIVM Bilthoven, The Netherlands) [9].

RESULTS

Table 1 compares the proportion of resistant isolates among the three groups examined. Among 31 isolates obtained from children with meningococcal disease none was resistant to rifampicin or ciprofloxacin. Nearly half the isolates were penicillin-insensitive (48·3%) and all grew on 1 or 2 mg/l tetracycline.

Among the 47 isolates from school-children the pattern of resistance to

Table 1. *Proportion of antibiotic-resistant isolates among patients and carriers*

	Percent resistant		
		Carriers	
	Patients ($n = 31$)	Children ($n = 47$)	Recruits ($n = 472$)
Penicillin G	48·3	19·1	36·7
Sulphamethoxazole	16·1	10·6	35·6
Rifampicin	—	6·4	7
Tetracycline	100	6·4	91
Erythromycin	25·8	61·7	82·2
Cefaclor	9·7	6·4	23·7
Ciprofloxacin	—	—	0·2

ciprofloxacin, cefaclor and sulphamethoxazole was similar to that of isolates from patients. The proportion resistant to erythromycin (61·7 %) was over twice that among isolates from patients (25·8 %) ($\chi^2 = 8{\cdot}27$, df = 1, $P = < 0{\cdot}005$); and there were some rifampicin resistant strains (6·4 %). Compared with the isolates from patients, the proportion resistant to tetracycline was much less (6·4 %) ($\chi^2 = 62{\cdot}83$, df = 1, $P = < 0{\cdot}0005$) as was the proportion with decreased sensitivity to penicillin (19·1 %) ($\chi^2 = 7{\cdot}61$, df = 1, $P = < 0{\cdot}01$).

The proportions of 472 isolates from recruits resistant to tetracycline or penicillin were similar to those of isolates from patients. The proportions of isolates from recruits resistant to erythromycin or to rifampicin were similar to that for isolates from children who were carriers. Compared with the other two groups, there was a higher proportion of isolates from recruits resistant to sulphamethoxazole and cefaclor. There were 112 isolates (23·7 %) with MIC $\geqslant$ 4 mg/l cefaclor and 32 isolates (6·8 %) with MIC $\geqslant$ 8 mg/l. Of the latter 32 isolates, 27 (84 %) had decreased sensitivity to penicillin. There was one isolate with MIC $\geqslant$ 1 mg/l ciprofloxacin. None of the 550 isolates from patients and carriers produced beta-lactamase.

Geographical distribution of resistant isolates

In the two major population centres, Eastern Macedonia and Athens, the proportions of penicillin-insensitive isolates were 33 and 40 % respectively. The highest proportions of such isolates were found in Thrace (50 %), Crete (52 %) and the Aegean and Ionian Islands (50 %). The lowest proportion was obtained from recruits who lived abroad but who had returned to Greece for their compulsory military service (9 %). Analysis of the serogroups, serotypes, subtypes and sensitivities to other antibiotics indicate that the isolates within a particular geographical area with decreased sensitivity to penicillin are not related (Table 2).

Analysis by antigenic phenotypes

Meningitis strains

Among the isolates from patients with meningitis, 50 % of group B, 37·5 % of group C and 60 % of the non-groupables were penicillin-insensitive. Sulphonamide resistance was found in 17 % of the group B isolates and 25 % of group C but in

Table 2. *Proportion of antibiotic-resistant isolates obtained from recruits from different regions of Greece*

Region	Number tested	Percent resistant PN	SU	RF	CP
Thrace	14	50	79	7	—
W. Macedonia	38	26	42	5	—
E. Macedonia	96	33	35	7	—
Epirus	23	48	30	4	—
Thessaly	46	34	48	6	—
Central Greece	32	32	44	9	—
Athens	127	40	39	7	0·8
Peloponnese	40	35	30	10	—
Crete	31	52	39	3	—
Aegean Islands	10	50	70	10	—
Ionian Islands	2	50	—	50	—
Foreign	11	9	22	—	—

Table 3. *Antibiotic resistances of serogroups*

Serogroup	No.	Percent resistant PN	SU	RF
A	17	41	41	12
B	114	35	37	5
C	19	42	42	10·5
W135	2	0	50	0
Y	8	62·5	25	0
Z	4	25	0	0
NG	300	36	40	7

none of the non-groupable isolates. Of the 9 2b serotype isolates, 7 (78%) were penicillin-insensitive as were 8/18 (44%) of the non-typables. The 4 isolates expressing other serotypes (2a, 14 and 15) were sensitive to penicillin and to sulphamethoxazole. Resistance to sulphamethoxazole was found for only 2/9 serotype 2b isolates and 3/18 non-typable isolates. Of the 9 isolates expressing the P1.10 subtype, 7 (78%) were penicillin-insensitive though only 1 was resistant to sulphamethoxazole. The second most common subtype was P1.9. Only 1 of the 5 P1.9 isolates was penicillin-insensitive, but 2 were sulphonamide resistant.

Carrier strains

There were sufficient numbers of isolates from the recruits for analysis of antibiotic resistances by serogroup, serotype and subtype (Tables 3–5). Among the serogroups, the highest proportion of penicillin-insensitive isolates was found for group Y (62·5%) while rifampicin resistance was greatest in groups A (12%) and C (10·5%) (Table 3).

Penicillin-insensitivity was highest among serotypes 2a and 15 and lowest in type 4, which, however, had the highest proportion of strains resistant to sulphamethoxazole. Rifampicin resistance was observed most frequently among serotypes 4 and 15 (Table 4).

Table 4. *Antibiotic resistances among serotypes*

Serotype	No.	Percent resistant PN	SU	RF
1	15	27	20	7
2a	7	57	43	0
2b	22	27	32	4·5
4	9	11	56	11
14	66	42	28	9
15	16	56	19	12·5

Table 5. *Antibiotic resistances among subtypes*

Serogroup	No.	Percent resistant PN	SU	RF
1	41	39	34	2·5
2	72	43	22	15·5
4	7	43	29	0
6	28	32	68	0
7	30	43	63	13
9	45	27	38	2·4
10	29	45	28	14
12	8	25	37·5	0
14	8	62·5	25	0
15	16	37·5	19	0
16	13	38	70	23

No particular subtype antigen was associated with an unusual degree of antibiotic resistance (Table 5).

DISCUSSION

Although sulphonamide resistance has been associated with strains causing outbreaks of meningococcal disease in northern Europe [10, 11], only 16% of the isolates from Greek patients had MIC > 10 mg/l to sulphamethoxazole; however, nearly half had reduced sensitivity to penicillin and all grew in the presence of 1 or 2 mgm/l tetracycline.

In contrast, while the proportions of penicillin-insensitive and of tetracycline-resistant isolates from carrier children were both significantly lower, the proportion of erythromycin isolates was significantly increased.

Among isolates from the recruits, the proportion of penicillin-insensitive strains was significantly greater than that from children who were carriers ($\chi^2 = 5{\cdot}126$, df = 1, $P < 0{\cdot}025$) but not compared with those from children with disease. The proportion of sulphonamide-resistant isolates among those from recruits (35·6%) was over twice that of the other two groups, carriers (10·6%) ($\chi^2 = 10{\cdot}882$, df = 1, $P < 0{\cdot}005$) or patients (16%) ($\chi^2 = 4{\cdot}06$, df = 1, $P < 0{\cdot}05$) (Table 1).

Although penicillinase-producing isolates of *Neisseria gonorrhoeae* are not uncommon in Greece (18%) [12], none of the 550 meningococcal isolates tested produced beta-lactamase.

The levels of resistance to erythromycin among isolates from carriers might reflect longer exposure to the antibiotic as the mean ages of the carriers were higher, school-children (12·8 years) and recruits (19·7 years) compared with a mean age of 2·7 years for patients with meningitis. Only one isolate had MIC $\geqslant$ 1 mg/l to ciprofloxacin. Since 6–7 % of isolates from carriers were resistant to rifampicin, ciprofloxacin might be considered for circumstances when chemoprophylaxis would be appropriate as it has been found to eradicate pharyngeal carriage of meningococci [13].

Sutcliffe and colleagues [4] stated that in view of the extensive use of penicillin in the community in Britain, emergence of penicillin-insensitive meningococci is not unexpected. In Britain, the proportion of isolates from patients and carriers with MIC > 0·16 mg/l was about 3% [4]. In Spain in 1986, only 5% of isolates examined had MIC $\geqslant$ 0·1 mg/l; however, by 1990, this figure had risen to 46% of isolates from patients. The antibiotic policies of Spain and Greece are similar and the proportion of penicillin-insensitive isolates from Greek children with meningococcal disease (48·3%) was similar to that reported for Spain. In Britain, the proportion of penicillin-insensitive isolates from carriers was slightly less than 3% [4], much lower than the proportion of penicillin-insensitive strains from carriers in this survey, recruits (36%) or school-children (19·1%).

Differences in MIC values to tetracycline between isolates from patients and isolates from children who were carriers was not expected. Although high level tetracycline resistance mediated by a plasmid carrying the *tetM* determinant has been found in meningococci [14] none of the isolates had MIC > 8 and the 25·2 megadalton plasmid was not observed in any of the strains examined so far [unpublished observations].

The differences in the proportions of penicillin-insensitive isolates in the different regions of Greece is under investigation (Table 2). That there were fewer such isolates among recruits who lived outside Greece (mainly in Germany) might reflect differences in antibiotic policies referred to earlier.

Although 61% of the British isolates which were penicillin-insensitive were also sulphonamide-resistant, none were resistant to rifampicin, aminoglycosides, erythromycin, tetracycline or chloramphenicol [4]. Only 37% of the penicillin-insensitive isolates in this study were sulphonamide-resistant; however, additional resistances were often present: rifampicin (8%); erythromycin (33%); cefaclor (34%); tetracycline (96%).

While none of the penicillin-insensitive strains in Britain was serogroup A [4], 41% of the Greek group A isolates had reduced sensitivity to penicillin. The highest proportion of penicillin-insensitive isolates was among the 8 group Y isolates (62·5%). Analysis by areas of residence of the recruits, serotype, subtype and sensitivities to other antibiotics suggest these group Y isolates are not related. Although serogroup B was the most common among patient isolates, the greatest proportion of isolates resistant to rifampicin was among those of groups A and C.

The number of serotypable isolates was small, and larger numbers need to be examined to determine if the associations noted are significant (Table 4). Most of the penicillin-insensitive isolates expressing subtype P1.14 do not appear to be a single strain as assessed by the serogroup, serotype, sensitivities to other antibiotics and the area of residence of the recruits from whom the bacteria were

isolated. Although the sulphonamide and rifampicin resistances were highest among subtype P1.16, the two resistances were not found together in the P1.16 strains. The higher rate of rifampicin resistance among isolates expressing P1.10, the subtype found most frequently among meningococci isolated from patients, and among isolates expressing P1.2, the most common subtype among carriers, needs to be further investigated.

REFERENCES

1. Saez-Nieto JA, Fontanals D, Garcia de Jalon J, et al. Isolation of *Neisseria meningitidis* strains with increase of penicillin minimal inhibitory concentration. Epidemiol Infect 1987; **99**: 463–9.
2. Saez-Nieto JA, Vazquez JA, Macros C. Meningococci moderately resistant to penicillin. Lancet 1990; ii: 54.
3. Botha P. Penicillin-resistant *Neisseria meningitidis* in southern Africa. Lancet 1988; i: 54.
4. Sutcliffe EM, Jones DM, El-Sheikh S, et al. Penicillin-insensitive meningococci in the UK. Lancet 1988; i: 657–8.
5. Jones DM, Sutcliffe EM. Meningococci with reduced susceptibility to penicillin. Lancet 1990; i: 863–4.
6. Blackwell CC, Tzanakaki G, Kremastinou J, et al. Factors affecting carriage of *Neisseria meningitidis* among Greek military recruits. Epidemiol Infect 1992; **108**: 441–8.
7. AAC working party. Phillips I, Andrews J, Bint AJ, et al. Breakpoints in *in vitro* antibiotic sensitivity testing. J Antimicrob Chemother 1988; **21**: 701–10.
8. Young HY. Identification and penicillinase testing of *Neisseria gonorrhoeae* from primary isolation cultures on MNYC medium. J Clin Microbiol 1978; **7**: 247–50.
9. Abdillahi H, Poolman JT. Whole-cell ELISA for typing *Neisseria meningitidis* with monoclonal antibodies. J Med Microbiol 1987; **48**: 367–71.
10. Cartwright KAV, Stuart JM, Jones DM, Noah ND. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol Infect 1987; **99**: 591–601.
11. Poolman JT, Jonsdóttir K, Jones DM, Lind I, Frøholm O, Zanen HC. Meningococcal serotypes and serogroup B disease in Northwest Europe. Lancet 1986; ii: 555–8.
12. Tzanakaki G, Mavromati L, Tzelepi E, et al. Serological classification in relation to auxotypes, plasmid contents, and susceptibility to antimicrobials of PPNG and non-PPNG strains isolated in Greece. Genitour Med 1989; **65**: 171–6.
13. Gaunt PM, Lambert BE. Single dose ciprofloxacin for eradication of pharyngeal carriage of *Neisseria meningitidis*. J Antimicrob Chemother 1988; **21**: 489–96.
14. Knapp JS, Johnson SR, Zenilman JH, et al. High-level tetracycline resistance resulting from TetM in strains of *Neisseria* spp., *Kingella denitrificans* and *Eikenella corrodens*. Antimicrob Agents Chemother 1988; **32**: 765–7.

J. Med. Microbiol. — Vol. 38 (1993), 19–22

Serogroups, serotypes and subtypes of *Neisseria meningitidis* isolated from patients and carriers in Greece

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Summary. The increase in the number of cases of meningococcal disease reported to the Ministry of Health in Athens since 1989 prompted the present study to determine if isolates from patients or carriers expressed the same phenotypic characters as those in other parts of Europe. None of the isolates from patients (31) or carriers (547) expressed the antigenic combinations associated with outbreaks in northern Europe, i.e., B:15:P1.16 or B:4:P1.15. The majority of the Greek isolates did not react with any of the six monoclonal serotype reagents tested; however, most reacted with one or more of the 11 monoclonal subtype antibodies. The results suggest that additional serotype reagents are needed for epidemiological studies in southeastern Europe and that vaccines based on serotype antigens developed against outbreak strains in northern Europe would not be effective in Greece.

Introduction

The increase in the number of cases of meningococcal disease reported to the Ministry of Health in Athens prompted the present study.[1] As the last major epidemiological survey of *Neisseria meningitidis* in Greece was published in the 1970s before the availability of serotype and subtype reagents,[2] the study had three main objectives: firstly, to determine whether the serotype and subtype reagents used in northern Europe and America can discriminate between isolates of meningococci from south-eastern Europe; secondly, to find out if serogroups, serotypes and subtypes of isolates from patients with meningococcal disease or carriers in northern Europe[3–7] are prevalent among Greek isolates; and thirdly to see if particular phenotypes are associated with different regions of Greece.

Materials and methods

Thirty-one isolates from children aged 1 month–11 years with meningococcal disease were obtained from the microbiology laboratory of Aglaia Kyriakou Paediatric Hospital, Athens. The isolates were from sporadic cases that occurred during 1989–1991. Fifty-one isolates from school children aged 5–18 years who were carriers were obtained in surveys undertaken in Athens between Dec. 1990 and Jan. 1991.[8] A series of 496 isolates from military recruits aged 16–30 years were obtained during July 1990 and Jan. 1991[1] within 3–5 days of the arrival of the recruits at the camps; recruits from all regions of Greece were represented.

The isolates were grown on modified New York City medium[9] and characterised by Gram's stain, the oxidase test and by the rapid carbohydrate utilisation test (RCUT).[10] Serogroups were determined by slide agglutination with polyclonal antisera to serogroups A, B, C, W135, X, Y and Z (Wellcome Diagnostics). Serotype and subtype were determined by a whole-cell enzyme-linked immunoassay (ELISA) with monoclonal antibody[11] reagents supplied by Dr J. T. Poolman (RIVM, Bilthoven, The Netherlands).

Information obtained from each recruit regarding area of residence and socio-economic status was coded and entered into a Data Base 3 Plus program together with the results of the throat swab and the serogroup, serotype and subtype of the isolates.[1] Similar data bases were prepared for information obtained from questionnaires returned by parents of school children participating in the surveys.[8] Written informed consent was obtained from each of the recruits or from the parent or guardian of the children.

The results from the school children were compared with those obtained in a Scottish secondary school, where there was an outbreak of disease caused by a sulphonamide-resistant serogroup B serotype 4 subtype 15 (B:4:P1.15) strain,[7] and results from the recruits were compared with published results for 133

Received 4 March 1992; accepted 21 April 1992.
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isolates obtained from carriers among Norwegian military recruits.[6]

Results

Patients

The antigenic phenotypes of isolates from patients are shown in table I. The most prevalent serogroup was B (58%) followed by group C (26%) and non-groupable isolates (15%). The majority of isolates (58%) did not react with any of the serotype antibodies. The most common serotype was 2b (9 of 31, 29%), and the most common subtype was P1.10 (11 of 31, 35%). The most common serotype-subtype combination was 2b:P1.10 (7 of 31, 23%).

Carriers

Among school children there was no predominant antigenic phenotype. Of the 19 (37%) serogroupable isolates, 10 belonged to group B, two to each of serogroups A, C, W135 and Y and one to group Z. As with the isolates from patients, the majority (65%) did not react with any of the serotype antibodies; however, most (75%) reacted with one or more subtype reagents. Two isolates (4%) obtained from secondary school pupils expressed the 2b:P1.10 combination found among those from patients (tables II and III).

Of the 496 isolates from recruits, 36% were serogroupable: 24% were group B, 5% group C and 4% group A. Most isolates (69%) were not serotypable, but 64% reacted with one or more of the subtype antibodies. The most common serotype was 14 (16%), and the most common subtype was P1.2 (16%) (tables II and III). The most prevalent combination of serotype-subtype antigens was 14:P1.2 ($n = 24$) followed by 14:P1.7 ($n = 13$). None of the isolates from the recruits expressed the 2b:P1.10 combination predominant among children with meningococcal disease. None of the 2a or 2b isolates from carriers reacted with any of the subtype antibodies.

Geographic areas

The major regions of Greece are shown in the figure together with the proportion of recruits from whom meningococci were isolated. Meningococci were obtained most frequently from recruits from the north-east of Greece: Thrace (37%), West Macedonia (33%) and East Macedonia (30%). Because military service is compulsory, even for individuals who live abroad, there were 33 subjects who were not normally resident in Greece. Among these, 33% were carriers.

There were no unusual distributions of serogroups among the different regions. The most common serotype (14) was found in equal proportions (15%) among the isolates from the two major centres of

Table I. Antigenic phenotypes of isolates from children with meningococcal disease

Antigenic phenotype	Number of isolates
B:-:-	3
B:-:P1.1	1
B:-:P1.9	4
B:-:P1.10	2
B:-:P1.14	1
B:2b:P1.9	1
B:2b:P1.10	4
B:14:P1.15	1
B:15:P1.6	1
C:-:P1.6	2
C:-:P1.10	3
C:-:P1.14	1
C:-:P1.16	1
C:2b:P1.2	1
NG:2a:P1.6	1
NG:2b:P1.10	3
NG:14:P1.10	1

Table II. Comparison of serotypes of isolates from carriers in Greece with those from carriers in northern Europe

Serotype	Percentage of isolates from			
	recruits		school children	
	Greece ($n = 485$)	Norway ($n = 133$)	Greece ($n = 51$)	Scotland ($n = 121$)
1	4	5	8	9
2a	1	5	6	0
2b	5	0	8	5
4	2	22	8	36
8*	...	8	...	...
14	16	16	2	13
15	3	8	4	8
21*	...	8	...	7
Non-typable	69	28	64	22

*Not tested in this study.

Table III. Comparison of subtypes of isolates from carriers in Greece with those from carriers in northern Europe

Subtype	Percentage of isolates from			
	recruits		school children	
	Greece ($n = 489$)	Norway ($n = 133$)	Greece ($n = 51$)	Scotland ($n = 121$)
P1.1	9	5	14	3
P1.2	16	16	8	17
P1.3*	...	13	...	...
P1.4	1	0	0	0
P1.6	6	0	8	8
P1.7	6	0	4	7
P1.9	9	0	18	0
P1.10	6	0	10	0
P1.12	2	0	4	0
P1.14	2	0	0	0
P1.15	4	19	8	23
P1.16	3	8	0	4
Non-typable	36	39	26	38

*Not tested in this study.

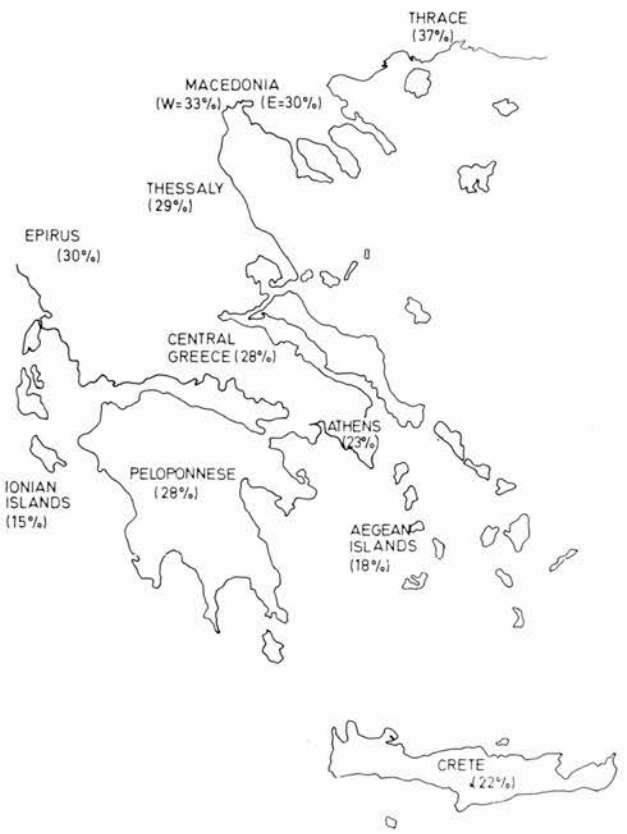

Figure. Proportion of carriers among recruits from different regions of Greece.

population, greater Athens and East Macedonia. Over 20% of the isolates from Thessaly (22%), the Peloponnese (21%), Crete (25%) and the Aegean Islands (30%) expressed the type 14 antigen.

Isolates representative of all the 11 subtypes examined were found among those from recruits from greater Athens, East Macedonia and Crete. More isolates that did not react with the subtype antibodies were obtained from recruits from the north of Greece: Thrace, Macedonia and Epirus.

Discussion

The first two objectives of the study were to determine if the serotype and subtype reagents used for epidemiological studies in northern Europe and the Americas could be used in epidemiological studies in Greece, and if the serotype and subtype combinations associated with disease in other populations were found in Greece. The proportion of non-serotypable isolates from the Greek recruits (69%) and school children (64%) was greater than the proportion of non-serotypable isolates obtained from carriers among Norwegian recruits (28%)[6] or Scottish school children (22%). The proportion of serotype 14 isolates was similar for Greek (16%) and Norwegian (16%) recruits and Scottish school children (13%) but was much lower (2%) among Greek children. The proportion of 2b serotypes was similar for the Greek recruits and Scottish school children. Serotype 4 was the most prevalent type among the Norwegian (22%)[6] and Scottish (36%) isolates compared with 2–8% of the Greek isolates. Serotype 15 was found in 8% of isolates from north Europeans compared with 3–4% of the Greek isolates. This suggests that other serotype reagents need to be developed for epidemiological studies of meningococci from Greece and that the serotypes associated with recent outbreaks in Britain and Scandinavia are uncommon in Greece.

The proportion of isolates from the Greek recruits (36%) and school children (26%) that did not react with any of the subtype reagents was similar to that of isolates from the Norwegian recruits (39%)[6] and from Scottish school children (38%). The proportion of subtype P1.2 isolates was similar for Greek (16%) and Norwegian (16%) recruits and Scottish school children (17%) but was lower among Greek school children (8%). The proportion of subtype P1.15 was similar among the Norwegian (19%)[6] and Scottish (23%) isolates but was greater than that found in the Greek isolates (4–8%). The subtype reagents appear to discriminate between strains from Greek carriers as effectively as between carrier strains from northern Europe; however, the proportions of subtypes associated with outbreaks in northern Europe are lower.

Combinations of serotype and subtype antigens 15:P1.16, 4:P1.15 and 2a:P1.2 associated with outbreaks in northern Europe[4,5] were not found among any of the isolates in this study. Although P1.2 was the most common subtype found in the study there was only one patient isolate with this subtype, C:2b:P1.2 (table I). In contrast, the 2a:P1.2 and 2b:P1.2 combinations are found frequently among Scottish group C[12] and French serogroup B isolates and 2a:P1.2 is found frequently among French serogroup C isolates.[13] The only report of meningococcal serotypes from southeast Europe is that of the C:2a phenotype associated with disease in Italy.[14] None of the serogroup C isolates expressed this serotype.

The third objective was to determine if there were antigenic phenotypes associated with different areas of Greece, e.g., those in which there are large numbers of foreign tourists or foreign military personnel or areas such as the northeast were there is a significant Muslim population some of which might have visited Saudi Arabia; however, there was no unusual distribution of serogroups or serotypes in the different regions of Greece.

The results of the study suggest that vaccines based on serotypes and subtype combinations associated with disease in northern Europe such as the one being developed and tested in Norway will not be appropriate for Greece.

References

1. Blackwell CC, Tzanakaki G, Kremastinou J *et al.* Factors affecting carriage of *Neisseria meningitidis* among Greek military recruits. *Epidemiol Infect* 1992; **108**: 441–448.
2. Kalapothaki V, Vassilliadis P, Trichopoulos D, Kaklamani E, Sérié C. Caractéristiques séro-épidémiologiques chez les porteurs sains de Méningocoques en Grèce. *Ann Micriobiol (Institut Pasteur)* 1979; **130B**: 43–54.
3. Poolman JT, Jonsdottir K, Jones DM, Lind I, Frøholm LO, Zanen HC. Meningococcal serotypes and serogroup B disease in North-West Europe. *Lancet* 1986; **2**: 555–558.
4. Poolman JT. Meningococcal vaccines. *J Med Microbiol* 1988; **26**: 170–172.
5. Abdillahi H, Poolman JT. Typing of group-B *Neisseria meningitidis* with monoclonal antibodies in whole-cell ELISA. *J Med Microbiol* 1988; **26**: 177–180.
6. Wedege E, Høiby EA, Rosenqvist ER, Frøholm LO. Serotyping and subtyping of *Neisseria meningitidis* isolates by co-agglutination, dot-blotting and ELISA *J Med Microbiol* 1990; **31**: 195–201.
7. Blackwell CC, Weir DM, James VS *et al.* Secretor status, smoking and carriage of *Neisseria meningitidis*. *Epidemiol Infect* 1990; **104**: 203–209.
8. Blackwell CC, Tzanakaki G, Kremastinou J *et al.* Carriage of *Neisseria meningitidis* among school children in Athens. Abstracts of the 120th Meeting of the Society for General Microbiology 1991.
9. Young H. Cultural diagnosis of gonorrhoea with modified New York City (MNYC) medium. *Br J Vener Dis* 1978; **54**: 36–40.
10. Young H, Paterson IC, McDonald DR. Rapid carbohydrate utilization test for identification of *Neisseria gonorrhoeae*. *Br J Vener Dis* 1976; **52**: 172–175.
11. Abdillahi H, Poolman JT. Whole-cell ELISA for typing *Neisseria meningitidis* with monoclonal antibodies. *FEMS Microbiol Lett* 1987; **48**: 367–371.
12. Fallon RJ. Meningococcal infections in Scotland 1990. Communicable Diseases and Environmental Health in Scotland Weekly Report 1991; **25**: 91/25.4–9.
13. Riou JY, Poolman JT, Auriol J, Lamprez F, Guibourdenche M. Serosubtyping of group B, C, Y and A meningococci isolated in France in 1988. *Ann Biol Clin (Paris)* 1989; **48**: 227–231.
14. Mastrantonio P, Congiu ME, Occhionero M, Stroffolini T. Genomic fingerprints and phenotypic characteristics of strains of *Neisseria meningitidis* causing disease in Italy. *Microbiologica* 1990; **13**: 109–113.

Scand J Infect Dis 26: 719–723, 1994

Parental Smoking and Carriage of Neisseria meningitidis among Greek Schoolchildren

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In December 1990 and January 1991, primary (320) and secondary (697) pupils in 2 areas of Athens were screened to determine the rate of carriage of Neisseria meningitidis and to determine if the genetic and environmental factors associated with carriage of meningococci in Greece were similar to those observed for northern European populations. In 1 area, socioeconomic indicators were significantly lower than in the other ($p < 0.0005$), but the isolation rates from pupils in the areas were similar, 5.3% and 6.3%. In contrast to studies in northwest Europe, carriage was not associated with lower socioeconomic conditions, sex, numbers of individuals per household, upper respiratory tract infection, or secretor status. By univariate analysis, carriage was associated with age (15–18 years) ($p < 0.05$) and mother's or other carer's smoking habits ($p < 0.05$) – but not father's smoking. Although the proportion of fathers who smoked was greater in the area where socioeconomic indicators were lower (61%) vs. (47%) ($p < 0.0005$), the proportions of women smokers were similar (33% vs. 38%). By multiple regression analysis, the only significant factors were age ($p < 0.01$) and carer's smoking ($p < 0.05$).

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INTRODUCTION

Since 1989 there has been a steady increase in the numbers of cases of disease due to Neisseria meningitidis reported to the Ministry of Health in Athens (1). In contrast to countries in northern Europe, there were no reports on carriage rates of meningococci among school children in Greece or assessment of factors associated with carriage of these bacteria among children in any country in southeast Europe. The present study had 2 objectives: to determine the carriage rate of meningococci among schoolchildren in Athens, particularly the B:2b:P1.10 phenotype frequently isolated from Greek children with meningitis (2); and to examine genetic and environmental factors associated with meningococcal disease and/or carriage in northern Europe: age (3); sex (3, 4); secretor status (5); smoking or passive exposure to cigarette smoke (5–7); lower socioeconomic conditions (3, 8); upper respiratory tract infections (9–11).

SUBJECTS AND METHODS

Children (1,038) were screened for pharyngeal carriage of meningococci during December 1990 and January 1991. Four schools were included in the study: a primary school and a secondary school in area (A) where housing costs were low; and a primary school and a secondary school in area (B) where housing costs were high.

Saliva and a throat swab were obtained from each child. Methods for culture and identification of N. meningitidis were the same as those reported previously (1, 12, 13). The saliva was frozen within 3 h of collection and secretor status determined by ELISA for H, Lewis[a] and Lewis[b] antigens (14, 15).

The parent or guardian of each child gave written consent for participation in the survey and provided the following information: age and sex of child; respiratory illness in the past week; number of individuals/household; smoking habits of members of the household; education level of parents. In

 ISSN 0036-5548

Greece, for epidemiological studies, socioeconomic category is assessed by the number of years of education and the highest level of school attended (16). Secondary schoolchildren were asked if they smoked.

Two-way frequency tables were anslysed by χ^2-tests with Yates' correction. Multiple logistic regression was used to examine the association between carriage and other factors adjusted for each other.

RESULTS

The 2 socioeconomic indicators used, cost of housing and educational level of the parents, were in agreement. Educational level of each parent was assigned to one of the following 5 categories based on the number of years of attendance and the type of school attended for categories 1–3: 1 = "demotico" (1–6 years); 2–"gymnasio" (6–9 years); 3 = "lykio" (9–12 years); 4 = technical college; 5 = university. Compared with area A, in area B there was a significantly lower proportion of parents in categories 1 and 2 and higher proportions of parents who had received technical training or attended university ($p < 0.0005$) (Table I).

The carriage rate was 60/1038 (5.8%), and the proportion of isolates from the 2 areas was similar: area A 30/565 (5.3%); area B 30/473 (6.3%) (Table II). Only 2 isolates, both obtained from secondary school pupils, expressed the B:2b:P1.10 phenotype (2).

Univariate analyses found no association between carriage and 2 genetic factors examined, sex or secretor status. There was no association between carriage and educational level of parents, numbers of individuals per household, area of residence, or symptoms or respiratory infection.

Among children in secondary schools, 14% of 50 smokers were carriers compared with 6.2% of 661 non-smokers ($p > 0.05$). In secondary school A, 7.6% of children were smokers, and 5/30 (17%) smokers were carriers compared with 16/352 (4.5%) non-smokers ($p < 0.025$). In secondary school B, 6% of children were smokers, but only 2/20 (10%) smokers were carriers compared with 25/305 (8.2%) non-smokers.

Compared with the proportion of carriers from households in which no adult smoked (11/311, 3.5%), the proportion of carriers was significantly higher in those in which the

Table I. *Educational level of parents in area A and area B*

		Educational category no. (%)				
	N	1	2	3	4	5
Area A	1,107	572 (52)	239 (21)	215 (19)	57 (5)	24 (2)
Area B	937	165 (18)	134 (14)	289 (31)	78 (8)	271 (29)

$p < 0.0005$.

Table II. *Isolation of meningococci from pupils in primary and secondary schools*

		Meningococci isolated	
School	*N*	*n*	(%)
Primary A	180	9	(5.0)
Primary B	144	3	(2.1)
Secondary A	385	21	(5.5)
Secondary B	328	27	(8.2)

Table III. *Sex, smoking and educational level*

	Educational category, no. (%)				
	1	2	3	4	5
Women[a]					
Smoker/total	130/395 (38)	68/177 (38)	117/299 (39)	14/47 (30)	30/109 (28)
Men[b]					
Smoker/total	222/357 (62)	115/196 (59)	113/205 (55)	47/88 (53)	71/186 (38)

Compared with non-smokers:
[a] $p > 0.05$.
[b] $p < 0.0005$.

mother or other carers smoked (33/431, 7.7%, $p < 0.05$). Father's smoking was not associated with carriage. Carriage rates in households where the mother or other carers smoked did not depend significantly on whether only the mother, only the other carers, or both smoked.

There were 15 carriers under 13 years, and in 5 cases (33%) the mothers smoked. There were 45 carriers over 12 years, and in 23 cases (51%) the mothers smoked ($p < 0.025$). When the figures were corrected for the children who were smokers, 20/38 (52.6%) carriers who were non-smokers had mothers who smoked ($p < 0.025$).

Smoking was more prevalent among fathers with fewer years of education ($p < 0.0005$), but this association was not found for mothers ($p > 0.05$) (Table III). In area B where the educational level was higher, the proportion of fathers who smoked was significantly lower 223/472 (47%) compared with area A 345/560 (61%) ($p < 0.0005$). These differences were not found for mothers in the 2 areas (A = 33% and B = 38%).

Following analysis by multiple logistic regression with reference to age, sex, school attended, respiratory infection, parental smoking and the children's smoking habits, only age ($p < 0.01$) and maternal or other carers' smoking ($p < 0.05$) remained significant. Older children and those whose carers smoked were more likely to be carriers. Although the trend with age appeared stronger in those whose carers smoked, this was not significant (interaction test in multiple logistic regression).

DISCUSSION

The proportion of carriers among children in this study did not differ from that usually found during non-epidemic periods, and the proportion of strains expressing the B : 2b : P1.10 phenotype was not high.

This was the first study to assess factors associated with carriage of meningococci in a population in southeast Europe. Several factors identified in studies of meningococcal carriage in northern Europe did not appear to influence carriage in the Greek schoolchildren: sex; secretor status; socioeconomic status; numbers of individuals per household; upper respiratory tract infections.

The isolation rate in different age bands followed a pattern similar to that found in the Stonehouse survey (3), and age was the most significant variable identified by multiple regression analysis.

In contrast to the Stonehouse survey (3) and a longitudinal survey in Belgium (8), carriage rate was no greater in the area where socioeconomic indicators were lower. For the fathers

in this study and in our previous study of Greek military recruits (1), there was a significant correlation between educational level and smoking. Carriage was not, however, associated with father's smoking by either univariate or multivariate analysis. In Great Britain, the pattern for socioeconomic groups and smoking are similar for men and women (17); however, there were no differences in smoking patterns of Greek women associated with educational levels or in the percentage of mothers who smoked in the 2 areas of the city.

Among studies on exposure to cigarette smoke and infection, the strongest associations are usually with smoking habits of the mother (18, 19). If the mother's smoking is a significant factor contributing to carriage among children, this might explain the similar carriage rates for the 2 areas of Athens. The observation that carriage was also significantly associated with smoking by adults other than the father might reflect the structure of many Greek families; grandmothers often live with families with young children and are closely involved in child care. Our study suggests that in Greece, smoking by mothers or other family members traditionally involved in child care might contribute to the carriage of meningococci.

REFERENCES

1. Blackwell CC, Tzanakaki G, Kremastinou J, Weir DM, Vakalis N, Elton RA, Mentis A, Fatouros N. Factors affecting carriage of Neisseria meningitidis among Greek military recruits. Epidemiol Infect 108: 441–448, 1992.
2. Tzanakaki G, Blackwell CC, Kremastinou J, Kallergi C, Fallon RJ, Weir DM. Serogroups, serotypes and subtypes of Neisseria meningitidis isolated from patients and carriers in Greece. J Med Microbiol 38: 19–22, 1993.
3. Cartwright KAV, Stuart JM, Jones DM, Noah ND. The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica. Epidemiol Infect 99: 591–601, 1987.
4. Olsen SF, Djurhuus B, Rasmussen K, Joensen AD, Larsen SO, Zoffman H, Lind I. Pharyngeal carriage of Neisseria meningitidis and Neisseria lactamica in households with infants within areas with high and low incidences of meningococcal diseases. Epidemiol Infect 106: 445–457, 1991.
5. Blackwell CC, Weir DM, James VS, Todd WTA, Banatvala N, Chaudhuri AKR, Gray HG, Thomson EJ, Fallon RJ. Secretor status, smoking and carriage of Neisseria meningitidis. Epidemiol Infect 104: 203–209, 1990.
6. Stuart JM, Cartwright KAV, Dawson JA, Rickard J, Noah ND. Risk factors for meningococcal disease: a case control study in south west England. Community Med 10: 139–146, 1988.
7. Stuart JM, Cartwright KAV, Robinson PM, Noah ND. Effect of smoking on meningococcal carriage. Lancet 2: 723–725, 1989.
8. de Wals P, Gilquin C, de Maeyer S, Bouckaert A, Noel A, Lechat MF, Lafontaine A. Longitudinal study of asymptomatic carriage in two Belgian populations of school children. J Infect 6: 147–156, 1983.
9. Krakinski K, Nilson JD, Butler S, Suby JP, Kusmiesz H. Possible association of mycoplasma and viral respiratory infections with bacterial meningitis. Am J Epidemiol 125: 499–508, 1987.
10. Moore PS, Hierholzer J, Dewitt W, Govan K, Djoré D, Lippeveld T, Plikayatis D, Broom CV. Respiratory viruses and mycoplasma as cofactors for epidemic groups meningococcal meningitis. JAMA 246: 1271–1275, 1990.
11. Olcén P, Kjellander J, Danielsson D, Lindquist BL. Epidemiology of Neisseria meningitidis: Prevalence and symptoms from the upper respiratory tract in family members to patients with meningococcal disease. Scand J Infect Dis 13: 105–109, 1981.
12. Young H. Cultural diagnosis of gonorrhoea with modified New York City medium (MNYC). Br J Vener Dis 54: 36–40, 1978.
13. Young H, Paterson IC, MacDonald DR. Rapid carbohydrate utilization test for identification of Neisseria gonorrhoeae. Br J Vener Dis 52: 172–175, 1979.
14. Rahat A, Stewart J, Blackwell CC, Weir DM. A semiquantative method for measurement of H type 1 and H type 2 on buccal cells and in saliva of secretors and non-secretors. Vox Sang 59: 101–105, 1990.
15. Raza MW, Blackwell CC, Molyneaux P, James VS, Ogilvie MM, Inglis JM, Weir DM. Secretor status and viral illness. BMJ 303: 815–818, 1991.

16. Trichopoulos D. Epidemiology: principles, methods, applications. Athens: R. Patisiano Publishers, 1982, 139.
17. Wald N, Kiryluk S, Darby S, Doll R, Pike M, Peto R. UK smoking statistics. Oxford: Oxford University Press, 1988.
18. Tager IB, Weiss ST, Munoz A, Rosner B, Speizer FE. Longitudinal study of the effects of maternal smoking on pulmonary function in children. N Engl J Med 309: 699–703, 1983.
19. Pershagen G. Review of epidemiology in relation to passive smoking. Arch Toxicol 9 (suppl 9): 63–73, 1986.

Submitted April 5, 1994; accepted July 21, 1994