CHARACTERISATION AND NON-CULTURAL DETECTION OF *NEISSERIA GONORRHOEAE*

by

Katherine G. Reid
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To my parents whose continued love and support,
given at a time of great adversity,
made this work possible.

This is to certify that the material
presented here is the candidate's own work.

CONTENTS

ANTIBIOTIC RESISTANCE OF THE GONOCOCCUS WITH SPECIAL REFERENCE TO PENICILLIN RESISTANCE

THE OUTER MEMBRANE COMPCNENTS OF *N. GONORRHOEAE*

GENETICS OF THE GONOCOCCAL CELL SURFACE COMPONENTS

METHODS OF CLASSIFICATION OF *N. GONORRHOEAE*

PATHOGENICITY AND IMMUNITY

MATERIALS AND METHODS

SPECIFIC METHODOLOGY
SECTION I: CHARACTERISATION OF GONOCOCCAL ISOLATES BY COAGGLUTINATION (CoA) SEROGROUPING AND CELL ENVELOPE PEHNOTYPING

SECTION II: NON-CULTURAL DETECTION AND IDENTIFICATION OF *N. GONORRHOEAE*

ABSTRACT

The aim of this study was to examine the distribution of coagglutination (CoA) W serogroups and cell envelope phenotypes among gonococcal strains isolated in the Edinburgh area and to undertake the development of a non-cultural detection method.

A total of 745 gonococcal strains acquired in the Edinburgh area were serogrouped by CoA. Overall 41.8%, 49.9%, 8.2% and 0.1% of these strains belonged to CoA serogroups WI, WII, WII/WIII and WIII respectively. A strong correlation ($p < 0.001$) of CoA serogroup WII with homosexually acquired infection was observed regardless of anatomical site of isolation. There were no overall differences between heterosexual men and women ($p > 0.5$). Differences in the distribution of CoA serogroups within the four six-month periods studied were observed. There was a significant increase in the number of WII/WIII strains isolated ($p < 0.001$) and a decrease in the number of WI strains isolated from heterosexual men and women. There was a correlation of the CoA serogroup of isolates from sexual partners but no correlation between CoA serogroup of infecting strain and symptoms in women.

All four serogroups were represented among 24 strains acquired in other areas of the United Kingdom and in Europe. Whereas 18, 2 and 2 of the 22 strains acquired in non-European countries, mostly Thailand, belonged to CoA serogroups WII, WII/WIII and WIII respectively. The CoA serogrouping results are discussed in relation to the findings of other groups.

The overall distribution of the cell envelope phenotypes of 482 gonococcal strains was examined in relation to patient group, site of isolation and in relation to CoA serogroups. Overall 5%, 87% and 8%

of strains had an Env, wild type and Mtr phenotype respectively. Strains with the Mtr phenotype were isolated significantly more often from homosexual men than from heterosexual men ($p < 0.001$) and from women ($p < 0.001$). There was no correlation of the Mtr phenotype with rectal isolates but it was found more often in urethral and throat isolates from homosexual men. Serogroup WII and the Mtr phenotype were strongly correlated ($p < 0.001$): 85% of the 40 Mtr strains belonged to CoA serogroup WII. Strains isolated from heterosexual men who had acquired their infections in non-European countries (mostly in Thailand) belonged to CoA serogroup WII and the Mtr phenotype was strongly correlated with these strains ($p < 0.05$). The factor(s) involved in the selection of the Mtr phenotype and CoA serogroup WII is discussed.

A dot-blot immunoassay using nitrocellulose membranes in the place of conventional solid phase materials such as polystyrene beads and wells was developed and evaluated. CoA reagents prepared with antisera raised against MOMP E-5 and N-10 detected 99.6% and 95.8% of the WI and WII gonococcal strains respectively. Therefore these antisera were considered suitable for antigen detection in the dot-blot immunoassay. The sensitivity and specificity of the test when applied to 95 male urethral specimens were 91.5% and 89.6% respectively.

It is concluded that this study has shown that (i) the antigenic pool of *N. gonorrhoeae* in Edinburgh is not static but dynamic and changing. Therefore constant surveillance will be required to ensure that reagents used for serogrouping and non-cultural detection and identification remain effective; (ii) antibiotic pressure is the major force in the selection of gonococcal strains with the Mtr and CoA WII phenotypes; (iii) CoA serogrouping has allowed the rational choice of antisera to be employed in the development of a non-cultural detection

method; and (iv) the dot-blot immunoassay is acceptable at present but could be improved. It may be a useful tool in the laboratory detection of *N. gonorrhoeae*, particularly when used for detection of antigen in specimens from women and in transported specimens.

PUBLICATIONS

The results of some of these studies have been published jointly with other research colleagues. The references are:

Reid, K.G., Young, H. 1984. Serogrouping of *Neisseria gonorrhoeae*: Correlation of coagglutination serogroup WII with homosexually acquired infection. British Journal of Venereal Diseases 60: 302-305.

Young, H., Reid, K.G. 1984. Immunological identification of *Neisseria gonorrhoeae* with monoclonal and polyclonal antibody coagglutination reagents. Journal of Clinical Pathology 37: 1276-1281.

Reid, K.G., Warbrick, J., Young, H. 1985. Cell envelope phenotypes of *Neisseria gonorrhoeae*: correlation with site of infection and serogroup. Journal of Medical Microbiology (In press).

Reid, K.G., Young, H., McMillan, A. 1985. Serogrouping of *Neisseria gonorrhoeae*: A correlation with site of infection. In: The Pathogenic Neisseria: Proceedings of the Fourth International Symposium (In press).

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INTRODUCTION

GENERAL INTRODUCTION AND HISTORICAL BACKGROUND

Gonorrhoea is an infection primarily of the genito-urinary tract of humans but it may also affect the rectum, pharynx and, less commonly, the eye. Gonorrhoea is caused by the gram-negative diplococcus *Neisseria gonorrhoeae*. Occasionally the organism disseminates and gives rise to a systemic infection.

N. gonorrhoeae is extremely fastidious and requires exact growth conditions. It has a poor survival rate outside of its host and intimate contact is required for transmission from one person to another.

1.1 Historical background

Gonorrhoea has been known to be of venereal origin since the earliest recorded days of mankind. The Old Testament refers to venereal disease as uncleanliness resulting from promiscuous acts of intercourse. Parts of Leviticus, Chapter 15 refer to gonorrhoea, its diagnosis, management and aftercare and states that, "Every male that has a discharge from his penis is to be segregated for the discharge makes him contagious" (Brim, 1936). For many centuries the symptoms of gonorrhoea and syphilis were thought to be manifestations of the same disease, and it was not until the 19th century that the situation was clarified.

In 1838 Philip Ricord provided proof that gonorrhoea and syphilis were separate diseases and in 1879 Albert Neisser identified by microscopy the causative agent *Neisseria gonorrhoeae* which bears his name. *N. gonorrhoeae* was first cultured on artificial media by Ernst von Bumm in 1885 (Catterall, 1984).

1.2 Gonorrhoea in the early twentieth century

There was no effective treatment for gonorrhoea until the introduction of the sulphonamides in 1937 (Morse, 1979). In the period prior to this, the social impact and personal trauma of venereal diseases was daunting. Treatment of gonorrhoea was limited to urethral irrigation with potassium permanganate and oxycyanide of mercury, or to very weak antiseptics taken orally and excreted in the urine (Morton, 1966). Ascending gonococcal infections occurred frequently, particularly after urethral irrigation. Urethral stricture and sterility were often the sequelae to such infections.

Sterility and disseminated infections were common in both men and women. By the early 1900s the problems associated with venereal diseases were finally recognised. Special clinics for the diagnosis and treatment of patients were finally established in 1916 upon the recommendations of the Royal Commission on Venereal Diseases (Robertson, McMillan and Young, 1980). However, in this period and up to the 1930s much less interest was taken in women with venereal diseases and little or no contact tracing was undertaken (Catterall, 1984). Babies born to women with gonorrhoea frequently developed gonococcal eye infections known as ophthalmia neonatorum. This manifestation of *N. gonorrhoeae* was the largest single cause of blindness in children during the pre-antibiotic period (Robertson *et al.*, 1980).

An important part of controlling the spread of gonorrhoea in the population is by the effective diagnosis and treatment of infected patients and their sexual contacts. In 1938 the International Union against Venereal Diseases and Treponematoses tried to persuade governments to encourage the introduction of contact tracing. These suggestions were met with opposition and hostility. Therefore, at the beginning

of 1939 management of patients with venereal diseases was fragmented with health care being undertaken by general practitioners and various hospital specialists. The advent of the Second World War and high rates of infection amongst the armed forces served as an impetus to instigate contact tracing within the civil population. With the introduction of penicillin in the early 1940s it was predicted that gonorrhoea would be eradicated. A decline in the number of cases did occur for a while and interest in gonococcal research diminished. The number of public clinics and funds available for treatment and research also diminished (Catterall, 1984).

The fall in the incidence of gonorrhoea was short-lived. The number of cases of gonorrhoea reached epidemic levels in the early 1960s and remains prevalent today.

CLINICAL ASPECTS OF GONORRHOEA

1.0 Uncomplicated genital infections

Acute anterior urethritis is the most common manifestation of gonococcal infection in the male resulting in purulent discharge. The incubation period is on average 3-4 days with a range of 2-14 days. Prior to availability of effective antimicrobial agents most patients had symptomatic resolution within eight weeks and 95% were free of symptoms within six months (Holmes, 1974).

The columnar epithelium of the endocervix is the primary site of infection in women. The cervix is involved in 85-90% and the urethra in 65-75% of cases (Robertson *et al.*, 1980). The most common symptoms among women infected with *N. gonorrhoeae* are abnormal or increased vaginal discharge, abnormal uterine bleeding, dysuria and urinary frequency and urgency (Handsfield, 1977). However, the majority of women with endocervical gonococcal infections remain asymptomatic or develop very slight or non-specific symptoms and do not seek medical attention.

1.1 Asymptomatic genital infections

The prevalence of asymptomatic infections in women is estimated at 70 per cent or more (Robertson *et al.*, 1980). Asymptomatic urethral infections in men are also found. Overall, 10% of men acquire infections in which few or no symptoms develop (Judson, Miller and Schaffmit, 1977; Handsfield *et al.*, 1980). Therefore they may not recognise that they have been infected and fail to seek medical attention. Handsfield *et al.* (1974) detected urethral gonococcal infections in 40% of asymptomatic men who were known contacts of women with gonorrhoea.

In a survey of servicemen returning from SE Asia, it was found that a large proportion (68%) had asymptomatic gonococcal infections (Handsfield *et al.*, 1974). John and Donald (1978) found that 17% of 203 cases of urethral gonorrhoea were asymptomatic and a further 7% of cases had symptoms so mild that they failed to seek medical attention. All of these cases were brought to attention by contact tracing. Therefore, the prevalence of asymptomatic urethral infections in men vary depending on whether one examines the figures for male patients overall or of specific groups of male patients. The figures may also vary geographically.

Patients with asymptomatic infections may well be more efficient at transmitting gonococcal infections because they are unaware of having contracted the disease and therefore continue with their normal pattern of sexual behaviour.

1.2 Complications of local genital infections in men

Local spread of urethral gonococcal infections in men have been reported to result in prostatitis, seminal vesiculitis, epididymitis, inflammation and abscess formation in the glands of the urethra (Robertson *et al.*, 1980). Urethral strictures and fistulae may occur but nowadays these are very rare in the western world. However, in the pre-antibiotic period these were not uncommon. In West Africa and probably also in other developing countries where medical facilities are inadequate and improper self-treatment with antibiotics occurs, all of the above complications are frequently seen (Osoba and Alausa, 1976).

1.3 Complications of local genital infections in women

Local complications in women include involvement of the para-urethral and Bartholin's glands (Bartholinitis). Ascending cervical infections result in salpingitis or pelvic inflammatory disease and occurs in approximately 10% of untreated women. Approximately 20% of such women subsequently have impaired fertility (Eschenbach and Holmes, 1975). Therefore, this manifestation of gonorrhoea continues to present serious consequences to infected women.

1.4 Disseminated gonococcal infections

Disseminated gonococcal infections (DGI) arise from the spread of *N. gonorrhoeae* from the initial site of infection to the blood stream and is rare, occurring in approximately 1% of untreated cases (Gruber, Sandeford and Ziff, 1960). DGI is more likely to arise in patients who have had asymptomatic genital infections (Holmes, Weisner and Pederson, 1971). DGI has two phases: a bacteraemic and a septic joint stage. The bacteraemic stage is characterised by polyarthritis, dermatitis and an increased likelihood of positive blood cultures. The septic joint stage is characterised by monarticular arthritis, negative blood cultures and frequently culture positive synovial fluid (Handsfield, 1977). Occasionally the two overlap (Brogadir, Schimmer and Myers, 1979). It has been shown that the overall incidence of septic gonococcal dermatitis in patients with gonorrhoea was 1.9%; 3% for women and 0.7% for men (Barr and Danielsson, 1971). In 23 patients with DGI the common presenting symptoms were arthritis or arthralgia and bouts of fever. Characteristic skin lesions served as early clues to diagnosis. *N. gonorrhoeae* was usually isolated from the genito-urinary tract or from blood cultures

(Barr and Danielsson, 1971). Occasionally gonococci can be observed in smears prepared from skin lesions using immunofluorescence microscopy. Lesions occur on the extremities and most patients have from 5 to 30 lesions. Individual lesions often progress from petechiae or papules to pustular haemorrhagic or nercrotic lesions (Handsfield, 1977). Occasionally gonococcal endocarditis results from DGI (Rosoff, Cohen and Jacquette, 1983).

1.5 Rectal infections

Rectal infections occur in both men and women. Ano-rectal infections in men result from passive anal intercourse. In 1977, 10.9% of men with gonorrhoea had acquired their infection as a result of homosexual contact (British Co-operative Clinical Group, 1980). The majority of male patients with rectal gonorrhoea produce no symptoms (McMillan and Young, 1978; Bygdeman, 1981a) and the proctoscopic appearance of the rectal mucosa is normal in 84% of cases (McMillan *et al.*, 1983).

In women with gonorrhoea the rectum is involved in approximately 25-50% of cases (Handsfield *et al.*, 1980; Willcox, 1981). Rectal gonorrhoea in women is thought to result partly from infection due to rectal coitus (Cornthwaite *et al.*, 1974; Kinghorn and Rashid, 1979) but mostly due to contamination of the anus by infected vaginal discharge (Willcox, 1981).

1.6 Pharyngeal infections

Gonococcal pharyngeal infections occur in both sexes. Lack of correlation between pharyngeal colonisation and symptoms of pharyngitis (Wiesner, 1975; Young and Bain, 1983), combined with the

reluctance of many patients to admit to orogenital contact (fellatio and, less commonly, cunnlingus), makes it difficult for the clinician to use these criteria in selecting patients from whom to take throat cultures. The prevalence of pharyngeal infection in patients with gonorrhoea in Edinburgh has been shown to be 4.3% for all men, 7-9% for women and 11% for homosexual men (Young and Bain, 1983). There have been reported cases of oral-genital transmission of *N. gonorrhoeae* (Soendjojo, 1983), although such events are probably rare in most locations. However, having said this, pharyngeal infections do constitute a possible source for the spread of infection and the possibility of developing DGI from an initial pharyngeal infection also exists.

1.7 Gonococcal infection in infants and children

Gonococcal infections in infants

Gonococcal conjunctivitis of the newborn (ophthalmia neonatorum) in the pre-antibiotic era was the single largest cause of blindness. Ophthalmia neonatorum results from the passage of the baby through its mother's infected cervix. The infection is usually apparent within 48 hours and up to one week after birth. Symptoms are conjunctivitis with purulent discharge and has dangerous consequences if treatment is delayed with the end result of keratitis with corneal scarring which may result in blindness. Nowadays this condition is very rare but remains a notifiable disease (Robertson *et al.*, 1980).

Gonococcal infections in children

Orapharyngeal, genital, rectal and eye infections in older children have often been considered to be due to non-sexual transmission (Nelson *et al.*, 1976). Proponents of this theory suggest that transmission of

gonorrhoea to children results from sleeping in the same bed as an infected parent, using damp towels contaminated with fresh pus and even from contaminated toilet seats. However, gonococcal transmission in adults by such modes is considered to be unlikely. Gonococcal infections in children resulting from sexual contact and abuse probably occurs much more frequently than is generally supposed (Sgroi, 1982; Alexander *et al.*, 1984).

EPIDEMIOLOGY

After the decline in the incidence of gonorrhoea in the 1950s an upsurge in the number of reported cases occurred in the 1960s (Figure 1). This rise coincided with changing attitudes towards sexual behaviour, the availability of oral contraception and a much greater degree of population mobility. The incidence of gonorrhoea in a number of countries (Figure 1) has continued to increase in the last 25 years.

1.0 Incidence of gonorrhoea in England

In England the number of new cases of post pubertal gonorrhoea reported in 1980, was 54,388 (117.04 per 100,000 population). Whereas in 1981 the number of reported cases was slightly lower at 52,200 (111.52 per 100,000 population). Overall the number of new cases reported has dropped from the peak of 126.64 cases per 100,000 population in 1977 (Extract of Annual Report of the Chief Medical Officer for the year 1982).

1.1 Incidence of gonorrhoea in Scotland

A similar trend has been observed in Scotland. In 1977, 5,528 (106.39 per 100,000 population) new cases of gonorrhoea were reported, whereas in 1979 the number of new cases had declined to 4,938, a rate of 95.96 per 100,000 population (Communicable Diseases Scotland, Summary of Sexually Transmitted Diseases 1972-79).

1.2 Incidence of gonorrhoea in America

In the United States of America over one million new cases were reported to the US public Health Services in 1976. These figures did not include those cases seen by private physicians or those for the

FIGURE 1: Reported gonorrhoea incidence per 100,000 population, 1955-75 (Adapted from Arya, 1981).

USA - United States of America
SW - Sweden
UK - United Kingdom

armed services. It has been estimated that the true incidence of gonorrhoea in the USA for 1976 was 3.4 million cases (Roberts, 1977).

1.3 Incidence of gonococcal PID

Coincident with the rise in sexually transmitted diseases, the incidence of PID in women has also risen sharply. Approximately one million women are treated annually for PID in the United States where this disease accounts for 250,000 hospital admissions and approximately 150,000 surgical procedures each year (Sweet, 1981). It has been estimated that the direct annual cost for treatment of PID is greater than 600 million dollars and that the total cost of the disease is upwards of three billion dollars (Curran, 1980). In addition to the economic consequences, salpingitis is a major cause of infertility with infertility rates of approximately 20% and a six- to ten-fold increase in the rate of ectopic pregnancies following the disease (Sweet, 1981). In his review of PID, Sweet (1981) noted that although *N. gonorrhoeae* may be the causative agent in 31-81% of cases of salpingitis, other organisms such as anaerobic bacteria and *Chlamydia trachomatis* may also be implicated.

1.4 The worldwide incidence of gonorrhoea

There is great difficulty in obtaining reliable figures for the incidence of gonorrhoea in developing countries, but the incidence of gonorrhoea worldwide is estimated to be 70-200 million cases per year (Roberts, 1977). The World Health Organisation considered gonorrhoea to be the major underlying cause for the high prevalence of infertility in parts of Africa (WHO, 1978). Therefore, today, gonorrhoea remains the most prevalent communicable disease exacting huge

costs in terms of both personal trauma and financial burdens upon health services.

GONOCOCCAL GENETICS

1.0 Deoxyribonucleic acid - chromosomal

The chromosomal deoxyribonucleic acid (DNA) of *N. gonorrhoeae* has 1.5×10^6 nucleotide pairs and molecular weight of 9.8×10^8 daltons (Kingsbury, 1969). It has a guanine plus cytosine (GC) ratio of 50-53 which is similar to that of other neisserial species which range from 46-53 (Bøvre, 1984). Other pathogens such as *Salmonella typhimurium* and *Escherichia coli* have larger chromosomes, 2.8×10^9 d and 2.5×10^9 d respectively (Morse, 1979). It has been suggested that the gonococcus because of the smaller size of its chromosome may have a limited genetic potential and this may be reflected by the fact that it is a host specific pathogen. Despite this, *N. gonorrhoeae* is a very successful pathogen and is able to colonise all mucosal surfaces of its human host and cause a wide range of clinical syndromes.

1.1 Plasmid DNA

Plasmids are extrachromosomal genetic elements that are capable of autonomous replication and are not required for the survival of the host bacterium under most conditions. Bacterial plasmids may carry information for the production of a large variety of different determinants such as multiple antibiotic resistance, surface antigens such as pili, production of bacteriocins, toxins and resistance to toxic ions (Elwell and Falkow, 1977). These additional determinants may give the bacteria carrying such plasmids a greater ability to survive in certain circumstances or to compete successfully with other bacteria. In addition to plasmids for which a known phenotype is expressed, there are many plasmids carried by bacteria for which there is no known function. These plasmids are called phenotypically "cryptic".

1.2 Gonococcal plasmids

The search for plasmids in gonococci was stimulated by the observations that certain features such as the loss of pili on subculture and multiple antibiotic resistance were plasmid coded in other bacteria. The first two plasmids to be characterised in the gonococcus were a small 2.6×10^6 d (2.6 Md) multicopy plasmid (Mayer, Holmes and Falkow, 1974) and a large 24.5×10^6 d (24.5 Md) plasmid (Stiffler *et al.*, 1975). The 2.6 Md plasmid comprised 6 to 8% of the total gonococcal DNA, equivalent to approximately 24 to 32 copies per cell (Mayer *et al.*, 1974). The GC ratios of the 2.6 Md and 24.5 Md plasmids are 50 and 46 respectively (Mayer *et al.*, 1974; Roberts and Falkow, 1977).

1.3 Function of gonococcal plasmids

Initially no phenotype could be ascribed to the presence of either plasmid (Mayer *et al.*, 1974; Stiffler *et al.*, 1975). However, it is now known that the 24.5 Md plasmid is involved in conjugation (Baron *et al.*, 1977; Roberts and Falkow, 1977). Recently two genes encoding for production of polypeptides have been identified on the 2.6 Md plasmid (Hagblom, Korch and Normark, 1984). These 2.6 Md, previously cryptic, plasmids are found in nearly all gonococcal strains whereas the 24.5 Md plasmid is found rarely (Elwell and Falkow, 1977).

1.4 Gonococcal plasmids coding for β-lactamase production

Penicillinase-producing *N. gonorrhoeae* (PPNG) were reported for the first time in the United Kingdom and United States of America in 1976 (Philips, 1976; Percival *et al.*, 1976; Ashford, Golash and Hemming, 1976). β-lactamase production was shown to be plasmid

mediated by Elwell *et al.* (1977). They showed that PPNG strains isolated from American army personnel, who had acquired their infections in the Far East, harboured a 4.5 Md plasmid coding for β-lactamase production as well as the 2.6 and 24.5 Md plasmids. In contrast, the PPNG strain from London (Philips, 1976) contained a 3.2 Md plasmid coding for β-lactamase production, the 2.6 Md plasmids but not the 24.5 Md plasmid (Elwell *et al.*, 1977).

METHODS OF GENETIC EXCHANGE IN THE GONOCOCCUS

The gonococcus has two methods of genetic exchange: transformation and conjugation. Transduction has not been reported and bacteriophages have not been isolated.

1.0 Transformation

Genetic transformation is the process by which one bacterium takes up, integrates and expresses (and is thereby transformed by) naked DNA from another organism. Transformation in *Neisseria* spp whose normal habitat is the human nasopharynx was first described by Catlin and Cunningham (1961). They showed that nearly all *Neisseria* spp were transformable and that intraspecific and interspecific transformation occurred. However, it was not until 1966 that Sparling showed that gonococci can also be transformed (Sparling, 1966).

Transformation involves binding and uptake of DNA by competent cells and integration and expression of DNA.

1.1 Competence

In *N. gonorrhoeae* transformation frequencies (number of transformed bacteria/number of bacteria exposed to DNA) are at least 1000 times higher ($\sim 10^{-2}$ to 10^{-4}) in pilated colony types 1 and 2 than in non-pilated strains (10^{-7}) (Sparling, 1966). A similar finding has been observed in *N. meningitidis* (Frøholm, Jyssum and Bøvre, 1973). Therefore, gonococcal non-pilated strains are not used for transformation assays.

Transformation of *N. gonorrhoeae*, like most other transformation systems, requires an energy source, the presence of cations, particularly

Mg^{2+} or Ca^{2+}, and the correct temperature and pH (Biswas *et al.*, 1977; Sparling, 1966). The optimum temperature for transformation is 35 to 37°C (Sparling, 1966) and the optimal pH range is 7.5 ± 0.5 (Biswas *et al.*, 1977).

1.2 Soluble competence enhancing factors

Unlike most other transformation systems where the bacteria excrete a soluble factor which enhances competence in other cells and where they are only competent for a part of their growth cycle, *N. gonorrhoeae* is competent throughout all phases of its growth cycle in nutrient broth (Sparling, 1966; Biswas *et al.*, 1977). This suggests that gonococci do not require a soluble competence-enhancing factor. However, there has been one report of competence factor in *N. gonorrhoeae* (Siddiqui and Goldberg, 1975). Siddiqui and Goldberg (1975) reported that washing competent pilated cells with phosphate-buffered saline resulted in a 1000-fold decrease in transformation frequencies. Resuspension of these washed cells in culture supernate restored normal transformation frequencies. However, these results have not been confirmed by other workers carrying out similar experiments (Biswas *et al.*, 1977; Sparling, Biswas and Sox, 1977).

1.3 The effect of proteolytic enzymes on transformation frequencies

The treatment of competent gonococcal cells with proteolytic enzymes such as pronase and trypsin, while reducing viability of cells by approximately ten-fold, did not decrease transformation frequencies and in some cases slightly increased them (Biswas *et al.*, 1977; Sparling *et al.*, 1977). However, the inhibition of protein synthesis by addition of 10 µg/ml of chloramphenicol to competent T1 cells of gonococcal strains FA_{19} and F_{62} prior to the addition of DNA resulted in a ten-fold decrease

in transformation efficiencies (Biswas *et al.*, 1977). When the chloramphenicol-treated cells were washed and allowed to resume normal growth in fresh medium, transformation efficiencies gradually returned to normal. Therefore, concentrations of chloramphenicol that had minimal effects on viability had a much greater effect on phenotypic development of competence, presumably due to the interruption of synthesis of one or more proteins essential to the transformation process (Biswas *et al.*, 1977).

1.4 The role of Pili in transformation

So far attempts to determine the role of pili in transformation have been unsuccessful. Pili do not appear to act as receptors for DNA. Experiments utilising radiolabelled DNA and pili have failed to show an affinity of DNA to pili (Sparling *et al.*, 1977). However, it is possible that some other constituent such as a cell-wall component present only in pilated cells affect transformation. Biswas *et al.* (1977) showed small increases in the transformation efficiencies of FA_{19} T4 non-pilated cells by increasing cell envelope permeability by treating the gonococci with EDTA or lysozyme and EDTA. However, both treatments resulted in marked decreases (>90%) in viability (Biswas *et al.*, 1977). Attempts to produce competent mutants of T4 cells have also been unsuccessful (Biswas *et al.*, 1977). Recent work suggests the site for DNA uptake may not be pili and although the nature of this site is unclear the receptor appears to recognise a specific nucleotide sequence (Sparling *et al.*, 1980).

1.5 Uptake of DNA

The uptake of DNA during transformation is affected by the physical state of the DNA used. Sparling *et al*. (1977) showed that single-stranded (ss) DNA is approximately 1% as active in transformation as double-stranded (ds) DNA. Gonococci do not take up covalently closed circular (plasmid) DNA very efficiently (Roberts and Falkow, 1977). Rapid uptake occurs in the first few minutes after the addition of donor-DNA with continued uptake up to 30 to 45 minutes. The effect of incubation periods of greater than 30 minutes were found to be variable and thus an optimum of 30 minutes is generally used (Sparling, 1966). At this point, saturation of DNA binding sites occurs. Saturation is reached with 5 to 10 μg/ml of DNA with a cell density of less than 10^8 gonococci (Sparling, 1966). It has been shown that DNA binds equally well to competent and non-competent gonococcal cells (Biswas *et al*., 1977). This initial binding of DNA to cells is deoxyribonuclease (DNase) sensitive. This step is followed by DNase resistant uptake and this occurs only in competent cells. DNase resistant uptake was found to be 0.5% of the DNA added with saturation at 0.5 μg/ml of DNA (Biswas *et al*., 1977).

1.6 Integration and expression of transforming DNA

Although DNA uptake is nearly completed after 30 minutes, it takes considerably longer for the DNA to be integrated and expressed by the recipient organisms. Gonococci being transformed to streptomycin resistance showed increased transformation frequencies with extended periods of time being allowed prior to the addition to the plates of the streptomycin overlay. The optimum time period was 6 to 7 hours (Sparling, 1966). However, very little is known about what actually happens to

the DNA during and after uptake by *N. gonorrhoeae*. In other systems donor DNA is converted from its initial double-stranded form to a single-stranded form and strand length may also be reduced (Sparling *et al.*, 1977). During this period of conversion of donor dsDNA to ssDNA, prior to integration into the recipient chromosome, transforming activity of the donor DNA is lost (eclipsed). However, when DNA is extracted from DNase treated, washed gonococci at short intervals and assayed for transforming activity of donor and recipient markers, no change in donor DNA activity is observed (Sparling *et al.*, 1977). Therefore *N. gonorrhoeae* appears to lack an eclipse period. Lack of an eclipse period is also seen in *N. meningitidis* (Jyssum, Jyssum and Gunderson, 1971).

1.7 Transformation of DNA between *Neisseria* spp and transformation *in vivo*

Transformation can occur between *N. gonorrhoeae* and other *Neisseria* spp. Transformation efficiencies are affected by both the species and the particular marker studied (Sparling *et al.*, 1977). The most efficient heterologous cross was between *N. gonorrhoeae* and *N. meningitidis* which approached the efficiency of homologous gonococcal to gonococcal crosses with respect to most markers studied.

Sarubbi and Sparling (1974) demonstrated that genetic exchange took place when two strains of *N. gonorrhoeae* carrying different antibiotic resistance markers were co-cultivated in liquid medium. However, the direction of gene transfer was dependent on the colony types of the two strains. It has been estimated that of patients with gonorrhoea, 25% will have infections with more than one strain (Short *et al.*, 1977). Therefore, *in vivo* there exists a potential for genetic exchange between gonococcal strains.

2.0 Conjugation

Conjugation in *N. gonorrhoeae* had not been reported prior to plasmid mediated penicillinase production in 1976. The first report of the conjugal transfer of the gonococcal penicillinase plasmid (R plasmid) occurred in 1977 (Eisenstein *et al.*, 1977). Donor β-lactamase strains were able to transfer β-lactamase activity to other gonococci and *E. coli* in culture and on membranes in the presence of DNase. Phage-mediated transduction was eliminated after cell-free filtrates of the donor strains and chloroform treated donor cultures were unable to transfer of the R plasmids to either *N. gonorrhoeae* or *E. coli* recipients (Baron *et al.*, 1977).

Transformation as the method of genetic exchange of β-lactamase activity was eliminated by the addition of DNase (~50 μg/ml) to the mating mixtures (Eisenstein *et al.*, 1977; Baron *et al.*, 1977). Roberts and Falkow also demonstrated that transformation was not likely to be the mechanism of β-lactamase transfer since purified gonococcal covalently closed circular (ccc) plasmid DNA could not transform other gonococci to penicillin resistance but could be used to transform *E. coli* to resistance in transformation experiments. They concluded that *N. gonorrhoeae* was refractory to transformation by ccc plasmid DNA (Roberts and Falkow, 1977). Additional evidence for conjugation as the method of R plasmid transfer came from the observations that the transfer efficiencies were similar regardless of whether pilated or non-pilated recipient gonococci were used (Eisenstein *et al.*, 1977). In transformation experiments, pilated recipients are required to attain efficient levels of transfer of genetic material.

Baron *et al.* (1977) demonstrated that the transfer frequency of the R plasmid was related to the donor : recipient ratio; at 100 : 1 the frequency was approximately 10^{-3} and at 1000 : 1 the frequency was 10^{-5}.

2.1 The mediator of R plasmid transfer

On analysis of the plasmids carried by donor, recipient and trans-recipient gonococci, it was shown that only strains carrying the 24.5 Md plasmid could transfer the R plasmid to another gonococcal or *E. coli* strain (Baron *et al.*, 1977; Roberts and Falkow, 1977). However, the 24.5 Md plasmid was not found in the donor strain in all cases. It was suggested that because only ccc plasmid forms and no intermediary open circular forms were detected that the 24.5 Md transfer plasmid DNA was rapidly converted into a linear form and was therefore not detected (Baron *et al.*, 1977). Therefore it was proposed that the 24.5 Md plasmid of *N. gonorrhoeae* possesses sex factor activities and is able to mobilise or promote the transfer of the smaller coexisting R plasmid by conjugation. However, conjugal (sex) pili have not been observed on donor gonococci (Sparling *et al.*, 1980).

The efficiency of conjugation has been shown to be affected by the composition of the outer membrane. A 10- to 15-fold decrease in R plasmid transfer has been observed in crosses with opaque x opaque cells compared with crosses with isogenic transparent x transparent cells (Sparling *et al.*, 1980). Transparent colony types lack the approximately 28,000 dalton outer membrane proteins associated with gonococcal colony opacity (Walstaad, Guymon and Sparling, 1977; Swanson, 1978b). Therefore it is thought that one of these colony opacity associated proteins inhibits conjugation possibly by interfering with the formation of mating pairs (Sparling *et al.*, 1980). The composition of the outer membrane does not appear to be controlled by the 24.5 Md plasmid (Sparling *et al.*, 1980).

2.2 Conjugal transfer of chromosomal genes

There has been one report that the 24.5 Md plasmid is capable of promoting the transfer of chromosomal genes at a low frequency: 10^{-5} to 10^{-7} per recipient cell (Roberts and Falkow, 1978). However, a number of workers have been unable to confirm these results (Norlander, Davis and Normark, 1979; Steinberg and Goldberg, 1980; Sparling *et al.*, 1980). Sparling *et al.* (1980) suggested that chromosomal transfer was actually the result of concurrent transformation at low frequencies probably caused by the deterioration of the DNase in the mating mixture.

Therefore, although conjugation occurs in the gonococcus for experimental purposes, transformation remains the most important system.

ANTIBIOTIC RESISTANCE OF THE GONOCOCCUS WITH SPECIAL REFERENCE TO PENICILLIN RESISTANCE

The introduction of sulphonamides in 1937, to which *N. gonorrhoeae* was highly susceptible, revolutionised the treatment of gonorrhoea (Catterall, 1984). However, resistance to sulphonamides quickly developed and it was extremely fortunate that penicillin came into general use as the sulphonamides were becoming ineffective.

1.0 Development and epidemiology of gonococcal penicillin resistance

Gonococci were initially extremely sensitive to penicillin. Gonococcal strains isolated in 1944 and tested for penicillin sensitivity in 1957 had a six-fold 50% inhibition range to penicillin: 0.0038 - 0.024 µg of penicillin/ml, with a peak at 0.01 µg/ml, the MIC or 100% inhibition levels were two-fold higher (Reyn, Korner and Bentzon, 1958). However, it was possible to select laboratory mutants with an MIC to penicillin ≥2 units/ml (~1.2 µg/ml) (Bahn, Ackerman and Carpenter, 1945) and one strain was found to have an MIC of up to 21 units/ml (~12 µg/ml) (Miller and Bohnhoff, 1945).

By the mid-1950s there were several reports from various parts of the world of gonococci with reduced sensitivity to penicillin (Sparling, 1977). Strains isolated in 1957 had a wider 50% inhibition range than those isolated in 1944: an 80-fold range from 0.005 - 0.45 µg/ml of penicillin with two peaks at 0.01 and 0.2 µg/ml (Reyn *et al.*, 1958).

Therapy with a single injection of 150,000 units of penicillin produced cure rates of 90% or more. However, the increases in resistance to penicillin were accompanied by evidence of increased rates of treatment failure with standard single-dose therapies (Curtis and Wilkinson, 1958; Thayer *et al.*, 1957).

Nowadays gonococcal strains which have reduced sensitivity to penicillin are usually defined as strains with an MIC of ≧0.06 μg/ml with a normal upper limit of 2.0 μg/ml (Sparling, 1977). These levels of resistance are over 100 times greater than those which prevailed when penicillin therapy was introduced in the 1940s, but are still much lower than resistance found in staphylococci and enteric bacteria (Sparling, 1977).

1.1 Development of resistance to other antimicrobials

Along with the development of low-level resistance to penicillin, gonococci also developed low-level resistance to other antibiotics such as tetracycline, chloramphenicol and erythromycin.

Gonorrhoea was also frequently treated with streptomycin to which gonococci developed a single-step high level resistance (Sparling, 1977). These levels were far in excess of achievable serum levels of the drug.

1.2 Geographical variations in gonococcal penicillin resistance

Resistance to penicillin varies in different geographical areas of the world and indeed within one country. Although results of antibiotic testing varies from laboratory to laboratory, the trends of penicillin resistance have been confirmed when gonococci from various areas of the world have been tested in one laboratory (Reyn, 1969; Sng, Lim and Yeo, 1984). Strains from countries in SE Asia tend to be most resistant to penicillin. In Singapore, 64% of non-PPNG strains have MICs to penicillin of ≧0.5 μg/ml (Sng *et al.*, 1984).

Differences occur in antibiotic sensitivities within one country. In the United States it has been suggested that strains isolated from patients in rural areas are less likely to be resistant than those isolated from patients living in urban areas (Gump and Berry, 1975). However, greater differences are likely to be caused by the influx of gonococcal strains from other geographical areas. The most dramatic demonstration of this was the emergence of PPNG strains in Britain and America in 1976 (Philips, 1976; Percival *et al*., 1976; Ashford *et al*., 1970). Sparling (1977) reported an increase of antibiotic sensitive strains in North Carolina which coincided with an influx of strains with nutritional requirements for arginine, hypoxanthine and uracil (auxotype AHU$^-$). These strains tend to be highly penicillin sensitive (Catlin and Pace, 1977). However whether *N. gonorrhoeae* carrying particular resistance mutations persist and become endemic among the indigenous gonococcal population, depends on the ability of such strains to survive and compete successfully. Therefore, if drug resistance has an adverse effect on virulence, such strains will not be successful. Although there are problems with resistance to penicillin single dose therapy remains the drug of choice for treatment of gonorrhoea (WHO, 1970). However, there are alternatives to penicillin such as the cephalosporins and spectinomycin. There are reports however of PPNG acquired in SE Asia which also have high levels of spectinomycin resistance (Gunby, 1983).

2.0 Chromosomally mediated low level penicillin resistance

Low level penicillin resistance is the result of chromosomal mutations. The loci involved in this are *pen*A, *pen*B and *mtr* (Sarubbi, Blackman and Sparling, 1974; Sparling, Sarubbi and Blackman, 1975). The locus *pen*A confers resistance to penicillin alone and is not linked to

genes which confer resistance to other antimicrobials such as streptomycin, spectinomycin, tetracycline and chloramphenicol (Sarubbi *et al.*, 1974). Mutation at *pen* A results in an eight-fold increase in penicillin resistance. Mutation at *mtr* results in a two- to four-fold increase in resistance to penicillin and to other antibiotics, dyes and detergents and is not linked to any of the other groups of antibiotic resistance genes (Sparling *et al.*, 1975; Maier, Zubrzycki and Coyle, 1975). Mutation at *pen* B_2 results in a four-fold increase in resistance to penicillin and tetracycline. However *pen* B_2 is only expressed phenotypically when a mutation at *mtr* is present within the same cell. The cumulative effect of these three mutations was a 128-fold increase in penicillin resistance (Sparling *et al.*, 1975). Modifier genes for penicillin and tetracycline resistance, *pem* and *tem*, have been reported (Warner, Zubrzycki and Chila, 1980). The *pem* gene enhances penicillin resistance expressed by the *pen* and *mtr* loci. A strain containing a *pen* mutation, with an MIC of 0.05 µg/ml of penicillin, was transformed to contain both the *pen* and *pem* mutations. The MIC of the resulting transformants was 0.1 µg/ml (Warner *et al.*, 1980).

There was no evidence that gonococcal strains carrying *pen* and *mtr* mutations produced β-lactamases or acylases (Sparling *et al.*, 1975; Rodriquez and Saz, 1975). It was proposed that these mutations effect resistance to penicillin by altering the permeability of the gonococcal cell envelope (Maier *et al.*, 1975).

3.0 Penicillin resistance mediated by plasmid coded β-lactamase

Percival *et al.* (1976), using isoelectric focussing, showed that PPNG isolates had two bands of β-lactamase (penicillinase) activity which focussed at pH 5.5 and pH 5.4. One of these bands focussed at

the same point (pH 5.4) as the TEM β-lactamase from *E. coli*. It has been shown in DNA-DNA homology studies that gonococcal strains containing either the 4.4 Md or 3.2 Md R plasmids contain approximately 40% of the transposable DNA sequence TnA. This sequence contains the gene Tn2 encoding for TEM β-lactamase production (Elwell *et al.*, 1977; Roberts *et al.*, 1977). The 4.4 and 3.2 Md plasmids were found in gonococci which originated from the Far East and West Africa respectively (Ashford *et al.*, 1976; Barrow and Philips, 1977).

The TnA sequence is commonly found in the R plasmids of enteric bacteria such as *E. coli* (Heffron *et al.*, 1975).

3.1 Origin of gonococcal R plasmids

Reports of β-lactamase producing *Haemophilus influenzae* (Khan *et al.*, 1974) prior to the appearance of PPNG lead to the suggestion that gonococci acquired their R plasmids from *H. influenzae*. It has been shown that the two gonococcal R plasmids have approximately 70% of their DNA sequence in common and are in turn closely related to a 4.1 Md R plasmid from a β-lactamase producing strain of *H. influenzae* (Roberts *et al.*, 1977). If the gonococcal R plasmids had arisen as a result of a transposition of TnA to an indigenous gonococcal plasmid, it would have been expected that the R plasmids would have had a GC ratio similar to that of the chromosome (~50). However, it was shown that the R plasmids had a much lower GC ratio in the range of 40-41 (Roberts *et al.*, 1977). Therefore it appeared that gonococci acquired their plasmids from another species. It was suggested that *N. gonorrhoeae* acquired R plasmids from gram negative enteric bacteria and not from *H. influenzae*. It seemed probable that *H. influenzae* acquired their R plasmids coincidentally from the same source.

It has been shown in transformation experiments that DNA deletions may occur resulting in reduction of plasmid size (Sox, Mohammed and Sparling, 1979), and it was postulated that the 3.2 Md plasmid may have naturally arisen as a result of such a deletion.

Although the first PPNG strains were carried into Europe and America and many are still isolated from people who have been overseas, reports suggest that in some areas these isolates are now endemic (Embden *et al.*, 1980; McCutchan, Adler and Berrie, 1982). McCutchan *et al.* (1982) reported that the incidence of PPNG in Great Britain has increased exponentially since 1977. In 1977 there were 15 reported cases of PPNG and in 1981 there were 443 PPNG isolated. Between 1978 and 1980, 62-68% of the isolates were acquired abroad and 20-26% acquired in the UK. However, in 1981 only 48% of PPNG were acquired directly or indirectly from abroad, and 37% were acquired from indigenous sources.

THE OUTER MEMBRANE COMPONENTS OF *N. GONORRHOEAE*

The cell envelope of Gram-negative bacteria including *N. gonorrhoeae* is composed of three macromolecular components, the outer membrane, the cytoplasmic membrane and the rigid peptidoglycan layer.

The outer membranes of Gram-negative bacteria, particularly those of *E. coli* and *Salmonella typhimurium*, have been studied for many years. The knowledge gained from these studies form the models for research into the outer membranes of other Gram-negative pathogens.

The outer membrane of the gonococcus is undulating, approximately 8.4 nm thick and appears to be a bilayered structure (Morse, Miller and Hebler, 1977). It is composed of lipopolysaccharide, phospholipids and proteins.

1.0 Colony types of *N. gonorrhoeae*

Kellogg *et al.* (1963) described four colonial types of the gonococcus based on size and shape of the colonies. He termed these T1 to T4. Colony type T1 predominated in primary cultures of clinical specimens. T1 and T2 gonococci were associated with pilus production whereas T3 and T4 were associated with non-pilated gonococci. There have been two additional colony types described, T5 and $T1^1$ (Jephcott and Reyn, 1971; Chan and Wiseman, 1975). Colony types T1, T2 and $T1^1$ have been associated with gonococcal virulence in infection studies with human volunteers and with chick embryos (Kellogg *et al.*, 1963; Chan and Wiseman, 1975). Colony types T3, T4 and T5 were essentially avirulent.

1.1 Instability of colony types

Maintenance of colony types T1, T2 and $T1^1$ depend upon selective subculturing at daily intervals. Non-selective subculture leads to the appearance of types T3, T4 and T5. Chan and Wiseman (1975) observed colony changes from T1 to T4, T2 to T3, T3 to T4 and $T1^1$ to T5.

The colony types still remain a fundamental means of describing gonococcal strains. However, the description and division of gonococci into distinct colony types has often complicated studies of the gonococcal surface structures.

2.0 Gonococcal capsules

The presence or absence of a gonococcal capsule has been a matter of some controversy. However, in recent years there have been a number of reports on the presence of capsules on gonococci (Hendly *et al.*, 1977; James and Swanson, 1977; Richardson and Sadoff, 1977; De Hormaeche, Thornley and Glauert, 1978).

2.1 Definition of bacterial capsules

The criteria for the presence of bacterial capsules is reviewed by Hendly *et al.* (1978). Basically, it is defined as being a covering layer on the organism which is outside the cell wall and has a definite external surface. The presence of capsules can be demonstrated under light microscope in wet films stained with India ink and by the quellung reaction. Both of these methods depend upon the capsule being at least 0.2 μm in width in order to differentiate it from the cell wall and from a diffraction halo (Hendly *et al.*, 1978).

2.2 Gonococcal capsules

Gonococcal capsules have been demonstrated on fresh clinical isolates with wet India ink, methylene blue and in electron microscope studies with hyperimmune antisera (Hendly *et al.*, 1977). The width of the capsular material was determined to be 0.3 μm. Capsules were found on all gonococcal colony types and were disassociated easily from the cells by shearing forces (James and Swanson, 1977; Richardson and Sadoff, 1977).

Capsular production could be stimulated by addition of casein hydrolysate to the growth media and by co-cultivation with 'viridans' streptococci (Hendly *et al.*, 1977; Richardson and Sadoff, 1977).

2.3 Characterisation of capsular material

The appearance of the capsules was not affected by treatment with heat, proteolytic enzymes or RNase (James and Swanson, 1977). The capsules stained with alcian blue and with Ruthenium Red suggesting that capsules consisted of acidic polysaccharide (James and Swanson, 1977; Hendly *et al.*, 1981). However, gonococcal capsules have not been isolated and chemically characterised. It is possible that the material described as being capsular in origin is actually the gonococcal lipopolysaccharide (LPS) in the process of being shed. Therefore, the LPS would form a loose layer on the outside of the gonococcal envelope resembling a capsule.

3.0 Lipopolysaccharide

Information concerning the lipopolysaccharide (LPS) of *N. gonorrhoeae* has only become readily available in the last 10 years and much remains to be clarified.

LPS, also termed endotoxin, appears to play a significant part in meningococcal disease (Devoe, 1980) and efforts have been made to characterise and determine the role of gonococcal LPS in gonorrhoea.

3.1 Basic structure of LPS

In enterobacteria LPS is composed of three parts, an O- or somatic antigen which extends out from the bacterial surface, a core oligosaccharide and Lipid A. The O-antigen is a high molecular weight antigenic polysaccharide made up of species specific oligosaccharide repeating units with as many as eight monosaccharides per unit. This is linked by its reducing end to the core oligosaccharide which is composed of different monosaccharides such as heptose, glucose, mannose, glucosamine and an eight carbon sugar acid 3-deoxy-D-manno-octulosonic acid (KDO).

Lipid A is bound to the core usually by the KDO residues. Lipid A is composed of a low molecular-weight D-glucosamine residue which is acylated with long chain fatty acids usually β-hydroxymyristic acid and linked by phosphate groups.

The O-antigen and core oligosaccharides are hydrophilic while Lipid A is hydrophobic (Luderitz, Staub and Westphal, 1966).

3.2 Smooth and rough LPS

LPS which is composed of all three components is called smooth LPS (S-LPS) (Figure 2). Occasionally mutants arise which are deficient in certain enzymes resulting in either the loss of the O-antigen or loss of some of the core oligosaccharide. Therefore even if the O-antigen is produced it cannot be attached to the core. This type of LPS is termed rough LPS (R-LPS) (Figure 2).

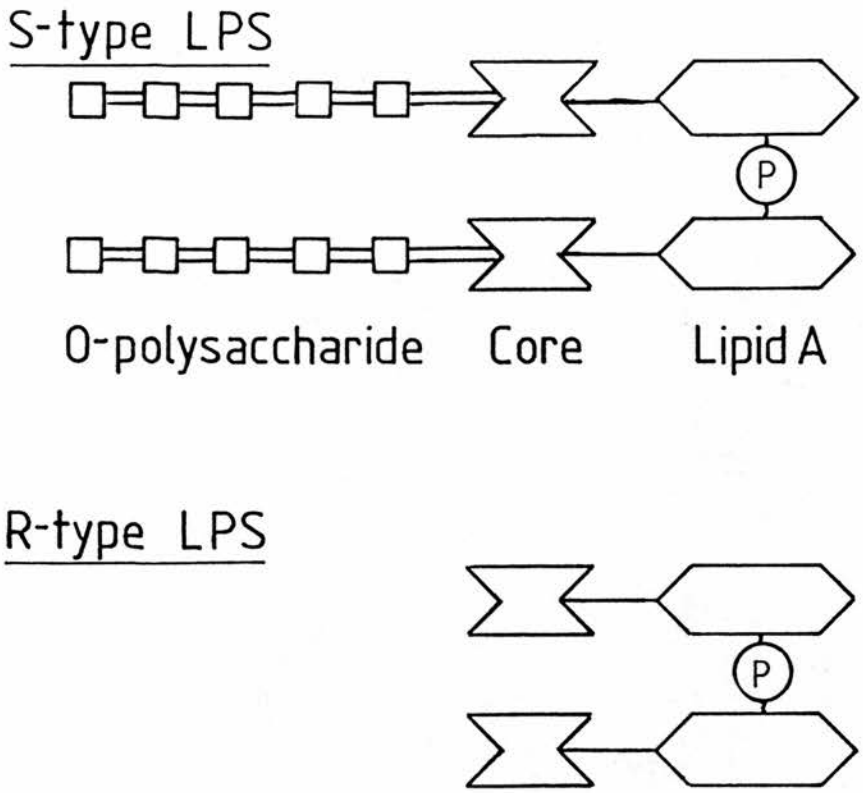

FIGURE 2: Schematic representation of S- and R-type LPS.

3.3 Loss of LPS from the membrane

LPS is found on the outermost part of the gram-negative outer membrane (Shands, 1965; 1966) and can be spontaneously lost (Crutchley, Marsh and Cameron, 1968; Rothfield and Pearlman-Kothencz, 1969).

In the neisseriae this loss of LPS has been visualized as a process of outer membrane 'blebbing' (Devoe and Gilchrist, 1973; Johnston and Gotschlich, 1974). Free endotoxin which is found in meningococcal culture supernates (Zollinger *et al.*, 1972; Devoe and Gilchrist, 1973) has been shown to be identical to cell bound LPS (Zollinger *et al.*, 1972).

Stead *et al.* (1975) have reported observing long projections extending from the surface of *N. gonorrhoeae* which broke up into vesicles analogous to blebbing seen in *N. meningitidis*.

Both serological and chemical analyses have been attempted to characterise gonococcal LPS.

3.4 Serological characterisation of gonococcal LPS

Maeland *et al.* (1971a,b) were the first to describe carbohydrate antigens on the surface of *N. gonorrhoeae*. They called these antigens α-factors and using specific antisera they could identify six determinants from three different gonococcal strains. They proposed that these antigens were immunologically analogous to the O-antigens of the Enterobacteriaceae

Later, Apicella (1974, 1976) described four serologically distinct populations of *N. gonorrhoeae*. These serotype antigens termed Gc_1 to Gc_4 were shown to be acidic polysaccharides and shared similar core determinants. It was considered that the α factors (Maeland *et al.*, 1971a,b) were probably attached to and part of the Gc serotype antigens. Latterly, two other Gc antigens, Gc_5 and Gc_6 were described (Apicella *et al.*, 1981).

Further serological analysis of LPS has shown that in addition to the six serotype antigens, Gc_1 to Gc_6, there are also two sets of antigenically distinct non-serogroup determinants (Apicella and Gagliardi, 1979). One of these is common to gonococci of all six Gc serogroups, whereas the other is variable and shared by gonococci of only three serogroups. These variable non-serogroup antigenic determinants are thought to be analogous with the core oligosaccharides of *E. coli* which have been shown to be heterologous (Joklik and Willet cited by Apicella and Gagliardi, 1979).

3.4.1 *Characterisation of gonococcal LPS with monoclonal antibodies*

Recently monoclonal antibodies have been raised against gonococcal LPS (Apicella *et al.*, 1981; Morse and Apicella, 1982) and these have been used to characterise LPS. The two monoclonal antibodies used were 3F11 and 5B9a.

Monoclonal antibody 3F11:

Monoclonal antibody 3F11 recognises a common region on the LPS from all six gonococcal serotypes (Apicella *et al.*, 1981). Inhibition studies with various sugars were undertaken to determine the binding specificity of this monoclonal. It was found that D-galactosamine completely inhibited binding of 3F11. Partial inhibition occurred with α-lactose, β-lactose and with meningococcal LPS and LPS-derived polysaccharides. LPS isolated from *E. coli* 0 : 111 and its J-5 mutant, and *S. minnesota* R595 failed to inhibit binding of 3F11 to gonococcal LPS.

These results suggest that the antibody recognises a common core moeity which is composed in part of D-galactosamine-O-D-galacto-pyranosyl-(1-4)-D-glucopyranose, the 1-4 linkage playing an important part in recognition. Partial inhibition of 3F11 binding by meningococcal

LPS suggests that *N. meningitidis* contains part of this moiety (Apicella *et al.*, 1981). It has been suggested that the missing part is glucosamine because meningococcal LPS has been shown to contain little or no glucosamine (Apicella, 1979).

It was thought that additional specificity of monoclonal 3F11 may depend on the steric relationship of the determinant on intact native gonococcal LPS (Apicella *et al.*, 1981).

Monoclonal antibody 5B9a:

This antibody reacted with gonococci from all six Gc serotypes and with LPS from a wide range of gram-negative bacteria. Binding of this antibody was partially inhibited by N-acetyl-D-glucosamine and completely inhibited by Lipid A isolated from either gonococci or from *E. coli* J-5 (Morse and Apicella, 1982). Therefore, monoclonal antibody 5B9a seems to recognise Lipid A.

It has been hypothesised that a common portion of the core oligosaccharide containing glucosamine-glucose-galactose-heptose-KDO found in the rough mutant *E. coli* J-5 (Elbein and Heath, 1965) is common to all enterobacteria and other gram-negative bacteria including the Neisseria (Davis *et al.*, 1975; Zollinger and Mandrell, 1977). However, the studies with the monoclonal antibodies 3F11 and 5B9a suggest that the LPS antigens shared by the enterobacteria and neisseriae reside in the Lipid A structure and not in the core oligosaccharides (Apicella *et al.*, 1981; Morse and Apicella, 1982).

3.5 Chemical analysis of gonococcal LPS

Chemical analysis has produced some conflicting evidence for and against the presence of O antigens and about the chemical composition of gonococcal LPS.

Stead *et al*. (1975) showed that LPS from five gonococcal strains extracted by phenol-water and purified by ribonuclease and multiple washes all contained glucose, galactose, glucosamine, heptose, KDO, Lipid A and phosphate. They were unable to detect N-acetylated, deoxy- or dideoxy-sugars which are characteristically found in O-antigens of enteric bacteria. They were unable to detect significant differences in the LPS isolated from virulent (T1 and T2) and avirulent (T3 and T4) gonococci. However, it appeared that they did not use isogenic variants and used cultures of T1 and T2 colony types which may have reverted in high numbers to T3 and T4 colony types. They concluded that all gonococci produce an R-type LPS (Stead *et al*., 1975). In a similar study, Perry *et al*. (1975) examined the LPS from T1 and T4 colony types. Lipid A and a core oligosaccharide composed of 2-amino-2-deoxy-D-glucose, D-glucose, D-galactose, L-glycero-D-manno-heptose and KDO were common to all colony types. Unlike Stead *et al*. (1975), they found that T1 colony types produced a high molecular weight O polysaccharide. However, yields of this S-type LPS were inconsistent and frequently T1 colonial variants only produced an R-type LPS or occasionally a mixture of S- and R-type LPS (Perry *et al*., 1975). They showed that the high molecular weight O polysaccharide material from different gonococcal strains varied considerably in its glycose composition and identified sugars such as rhamnose and fucose. They proposed that T1 cells produce an S-type LPS with structurally different O-polysaccharide side chains and that T4 cells produce a common R-type LPS (Perry *et al*., 1975).

Wiseman and Caird (1977) using isogenic variants also found that virulent T1 and T2 colony types and avirulent T3, T4 and T5 colony types produced different types of LPS. They found that glucose,

mannose and galactose were common to all colony types. N-acetylneuraminic acid, KDO, glucosamine and galactosamine were also invariably present. Avirulent T3 to T5 gonococcal variants, *N. sicca* and *N. lactamica* all had rhamnose in their LPS whereas the virulent T1 and T2 gonococcal colonies did not. However, Perry *et al.* (1975) found rhamnose only in T1 colonies.

Wiseman and Caird (1977) found that the main difference between LPS from virulent and avirulent gonococci was a quantitative difference in the glycose-KDO ratios. Virulent strains contained approximately four-fold more mannose-KDO and galactose-KDO than avirulent strains. They did not confirm the variability of the glycose composition of LPS from T1 gonococci reported by Perry *et al.* (1975).

It was suggested that the increased glycose-KDO ratios in virulent gonococcal strains might represent the presence of an O-antigen polysaccharide (Wiseman and Caird, 1977). Therefore although Perry *et al.* (1975) and Wiseman and Caird (1977) came to similar conclusions that gonococci may produce an O polysaccharide they found quite different chemical compositions.

3.5.1 Polyacrylamide gel electrophoresis analysis of gonococcal LPS

Recently efforts have been made to clarify the presence or absence of gonococcal O-antigens. LPS from *N. gonorrhoeae* and *N. meningitidis* have been compared with LPS from *S. typhimurium* by SDS-PAGE stained with silver stain of Tsai and Frasch (1982) (Parr and Bryan, 1984; Mintz, Apicella and Morse, 1984). Although different extraction procedures were used essentially similar observations have been made.

Parr and Bryan (1984) used proteinase K-digested whole-cell lysates of a number of gonococcal strains isolated from uncomplicated

and disseminated gonococcal infections. They observed that the banding patterns produced on silver-stained gels indicated that gonococcal LPS was essentially composed of short low molecualr weight molecules but with a few high molecular weight molecules corresponding to O-antigens.

Mintz *et al*. (1984) used hot phenol water and phenol-chloroform petroleum ether (PCP) which extract smooth and rough LPS respectively. In addition to examining the pattern of bands produced by different LPS preparations following gel electrophoresis, they examined the antigenic composition of the LPS preparations in an ELISA inhibition system.

3.5.2 Banding patterns obtained with different LPS preparations

No differences could be observed in the banding patterns of the two LPS preparations (Mintz *et al*., 1984). Silver-stained gels of PCP-extracted gonococcal LPS showed multiple low molecular weight bands. The banding patterns produced were unaffected by treatment with pronase, DNase and RNase and were similar in size heterogeneity to those patterns obtained with *S. typhimurium*. LPS from *S. typhimurium* produced multiple finely stained bands on the gels. The size heterogeneity of smooth enterobacterial LPS results from differences in the number of O-repeating units in the side chains of S-LPS molecules (Goldman and Lieve, 1980). However, it was considered possible that the banding patterns exhibited by gonococcal LPS might represent aggregates of LPS rather than differences in the number of repeating oligosaccharides in the O-antigen (Mintz *et al*., 1984). This proved to be the case. Sodium hydroxide treatment of extracted LPS resulted in a reduction in the number of bands observed. Only two or three low molecular weight bands (Mwt 3000-5000) were observed compared with approximately

20 observed with untreated LPS. Addition of 4 M urea to the gels also resulted in a similar reduction in the number of LPS bands. However, EDTA treatment had no effect, suggesting that hydrophobic LPS-LPS interactions are important.

3.5.3 LPS banding patterns in relation to Gc serogroup antigens

Comparison of the banding patterns of LPS extracted from different Gc serotypes of *N. gonorrhoeae* showed that there were significant differences in the structure of the LPS produced by these strains.

Serological analysis of PCP-extracted LPS showed that it contained very little or none of the serotype antigens compared with hot-phenol extracted LPS. However, because no differences could be observed in the banding patterns of the two LPS preparations it was concluded that the structural heterogeneity of gonococcal LPS may arise due to small differences in the composition of the basic core structure and/or in the gonococcal serotype antigens. The results also suggested that the Gc serotype antigens may represent a single repeat unit or a further modification of the basic core structure. Therefore, if the Gc serotype antigens represent the addition of single sugars to or the modification of the core structure, it is not surprising that no differences could be observed between PCP and hot-phenol water extracted LPS on SDS-PAGE gels.

3.6 Factors affecting the composition of gonococcal LPS

The chemical composition of gonococcal LPS may be affected by a number of factors including different LPS purification procedures and gonococcal growth conditions. Stead *et al.* (1975) reported that longer periods of hydrolysis resulted in increased yields of Lipid A

and core oligosaccharide. Mintz *et al*. (1984) found that hot-phenol water extraction gave greater yields of LPS than PCP extraction. Therefore it is quite likely that different LPS isolation and purification procedures affect the components yielded for chemical analysis.

Gonococcal growth conditions:

Variations in gonococcal growth may be another important cause of discrepancies in the chemical analysis of LPS.

Perry *et al*. (1975) suggested that the production of S-type LPS was dependent upon some unidentified nutritional requirement. It has been shown that the LPS isolated from *N. sicca* cultured with high growth rates was different from that isolated from the same strain grown at low growth rates (McDonald and Adams, 1971). Similar observations have been made with *N. gonorrhoeae* (Morse *et al*., 1983). *N. gonorrhoeae* FA171 colony type T4 grown in continuous culture with glucose as the limiting nutrient showed a growth-rate dependent change in LPS. This was demonstrated by an alteration in the mobility of purified alkali-treated LPS in SDS-PAGE, quantitative differences in the amount of the Gc serotype antigen and changed sensitivity of the strain to the bactericidal activity of normal human serum (NHS).

The LPS from cells grown at low dilution rates (low glucose concentration) showed one band on SDS-PAGE gels stained with silver. These cells were also serum-sensitive. Those cells grown at high dilution rates (high glucose concentration) were serum-resistant and showed three bands on SDS-PAGE. One of these bands was common to cells grown at high and low growth rates (Morse *et al*., 1983). Serological analysis of the LPS revealed that cells grown at a high dilution rate contained eight- to nine-fold more serotype antigen.

3.7 Lipid A composition

Stead *et al.* (1975) found that Lipid A contained a number of long chain (10-18 carbon) fatty acids and glucosamine. The composition of Lipid A from colony types T1 to T5 was found to be essentially the same and similar to that described by Stead *et al.* (1975) (Wiseman and Caird, 1977).

3.8 Summary

In summary, gonococci have antigens which are serologically analogous to O somatic antigens, show structural heterogeneity of their LPS which appears to be composed of short LPS molecules without the long somatic antigens of the enterobacteria. The chemical composition of gonococcal LPS requires to be clarified especially if the serotype antigens result from minor changes in the basic structure. However, efforts may be hampered by a number of factors such as the growth conditions and LPS extraction procedures used.

4.0 Outer membrane proteins

There are three major classes of protein in the outer membrane of *N. gonorrhoeae* and they account for over 60% of the total protein of the membrane (Johnston and Gotschlich, 1974). They are characterised primarily by their migration following SDS-PAGE and by the modification in their mobility after treatment with heat and reducing agents (Johnston, 1978; McDade and Johnston, 1980). The three proteins are termed protein I, protein II and protein III.

4.1 Protein I

Protein I has previously been called by a variety of names, such as principal outer membrane protein (POMP) and major outer membrane protein (MOMP). However, at the EMBO workshop of 1980 it was proposed that it should be termed protein I or PrI to prevent any confusion arising with the outer membrane proteins of *E. coli* (Swanson and Heckels, 1980). Protein I accounts for 50-60% of the total membrane protein (Johnston and Gotschlich, 1974; Johnston, Holmes and Gotschlich, 1976), and the subunit molecular weight varies between 32,000 and 39,000 daltons (32-39 K) (Johnston *et al.*, 1976).

4.1.1 Early development of protein I research

Initially 14 strains of *N. gonorrhoeae* isolated from the New York area could be assigned to five classes according to the electrophoretic profile of their membranes following SDS-PAGE (Johnston *et al.*, 1976). By extending the study to a further 57 clinical isolates the number of groups were extended to seven on the basis of the molecular weight of protein I and a minor protein which appeared to covary with protein I. Outer membrane complexes containing protein I were isolated and antisera prepared. Using these antisera and extending the number of strains examined to include isolates from Taiwan, Denmark, Ethiopia, the Philippines, Vietnam and a further 203 strains isolated from New York, 16 distinct serogroups could be identified. Each serogroup represented strains with protein I and minor covariant protein of a distinct molecular weight (Johnston *et al.*, 1976). No difference could be seen in the molecular weights of protein I isolated from T1, T3 and T4 colonial variants (Johnston *et al.*, 1976). Protein I is not responsible for the variations in colony opacity described by Swanson (1978a) (Swanson, 1978b; Lambden and Heckels, 1979).

4.1.2 *Characteristics of protein I*

Protein I is a heat stable protein and its migration on SDS-PAGE was not altered by boiling (Heckels, 1977; Johnston, 1978; McDade and Johnston, 1980). Protein I is partially solubilised by treatment with sodium deoxycholate. The molecular weight of both the soluble and insoluble protein I are identical (Johnston, 1978).

The envelope proteins of *N. gonorrhoeae* can be labelled with ^{125}I and lactoperoxidase. Tryptic peptide maps of labelled outer membranes have indicated that protein Is produce maps quite distinct from those produced by protein IIs or protein-III (Swanson, 1979; Heckels, 1981; Judd, 1982a).

Amino acid analyses have shown that there are no large differences in the amino acid composition of proteins I and II, both containing similar amounts of hydrophobic amino acids (Heckels, 1981). However, Blake and Gotschlich (1983) determined that protein I contains fewer basic amino acids.

4.1.3 *Subgroups of protein I*

Protein I was subdivided into two subgroups on the basis of peptide mapping patterns and sensitivity to proteolytic enzymes (Swanson, 1979; Blake, Gotschlich and Swanson, 1981). One group consisted of protein I molecules with molecular weights of 34 and 33K and the second of protein I of 32K. On the basis of these results it appears that there are two distinct subgroups of protein I. These have been termed protein IA and protein IB. The protein IA subgroup contains the protein I molecules of a lower molecular weight, which are resistant to *in situ* proteolysis as described by Blake *et al.* (1981) and have approximately 11 peptides in common when examined by peptide mapping

(Sandström, Chen and Buchanan, 1982a). Protein IB subgroup contains those protein I molecules with a higher molecular weight, with differing degrees of sensitivity to proteolytic enzymes as described by Blake *et al*. (1981) and have approximately 10 common peptides (Sandström *et al*., 1982a). Only three of the peptides produced on peptide maps are shared by both protein IA and IB (Sandström *et al*., 1982a).

4.1.4 Orientation of protein I within the gonococcal membrane

Early experiments using ^{3}H-chloride-cycloheptaamylose indicated that protein I and II are exposed at the gonococcal surface whereas protein III is blocked or not exposed on the surface (McDade and Johnston, cited by Johnston, 1978). These results were confirmed by Heckels (1978).

Recently it has been demonstrated that protein IA and IB have different orientations within the gonococcal membrane (Blake *et al*., 1981; Barrera and Swanson, 1984).

Whole cells of three gonococcal strains were treated with chymotrypsin and trypsin (Blake *et al*., 1981). Two of the strains contained protein IB molecules with molecular weights of 33 and 34 K respectively. The third strain contained protein IA of a molecular weight of 32 K.

The 34 K protein I was completely sensitive, the 33 K protein I was partially sensitive and the 32 K protein I was completely resistant to the *in situ* proteolytic activity of these enzymes. Treatment of the 33 and 34 K protein Is with chymotrypsin resulted in cleavage at one site, leaving two membrane associated fragments. Trypsin cleaved these proteins at two sites leaving two membrane associated fragments and one much smaller fragment which was lost to the supernate. From these results it was proposed that the 33 and 34 K protein Is (PrIB) had a looped structure with both termini embedded within the membrane (Figure 3).

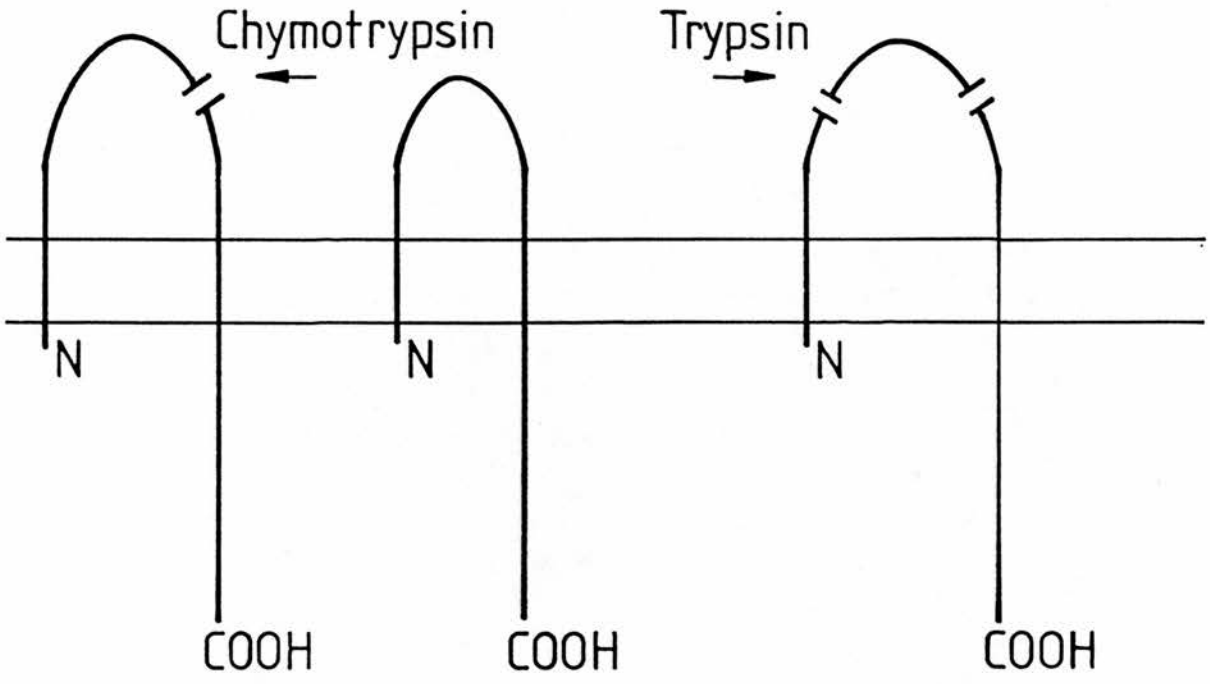

FIGURE 3: Proposed structure of protein IB and the effect of chymotrypsin and trypsin treatment (Adapted from Blake *et al.*, 1981).

It was hypothesised that the resistance of the 32K protein I to these enzymes may have been due to either the lack of the peptide sequences required for chymotrypsin and trypsin activity or to this molecule having a different structure within the membrane (Blake *et al.*, 1981).

Treatment of whole gonococcal cells with proteinase K has confirmed that protein IB has a looped structure. *In situ* cleavage resulted in two membrane associated fragments (Barrera and Swanson, 1984). Protein IA which was insensitive to chymotrypsin was cleaved by proteinase K. *In situ* cleavage resulted in a single membrane associated fragment of a slightly smaller size than the intact protein IA subunit with a small fragment being lost to the supernate. This suggested that protein IA molecules have one of their termini exposed at the surface of the membrane but with most of the molecule embedded within it (Figure 4) (Barrera and Swanson, 1984).

4.1.5 *Interaction of protein I molecules with protein III within the outer membrane*

Protein I has been shown to have the ability to form multimers with both itself and with protein III (McDade and Johnston, 1980; Newhall, Sawyer and Haak, 1980). It was shown that protein I multimers isolated from gonococcal membranes had a molecular weight of approximately 200K and that multimers composed of one molecule of protein I and two of protein III had a molecular weight of approximately 100K (McDade and Johnston, 1980). The interactions of protein I with itself and protein III were examined by cross-linking studies using diimidoesters, which interact with primary amino acids at pH 8.0, as cross-linking agents. Initially experiments using ≧10 mM concentrations of the cross-linking agents with whole cells and membrane vesicles resulted in the production of very large protein complexes. These were too large to enter a 3%

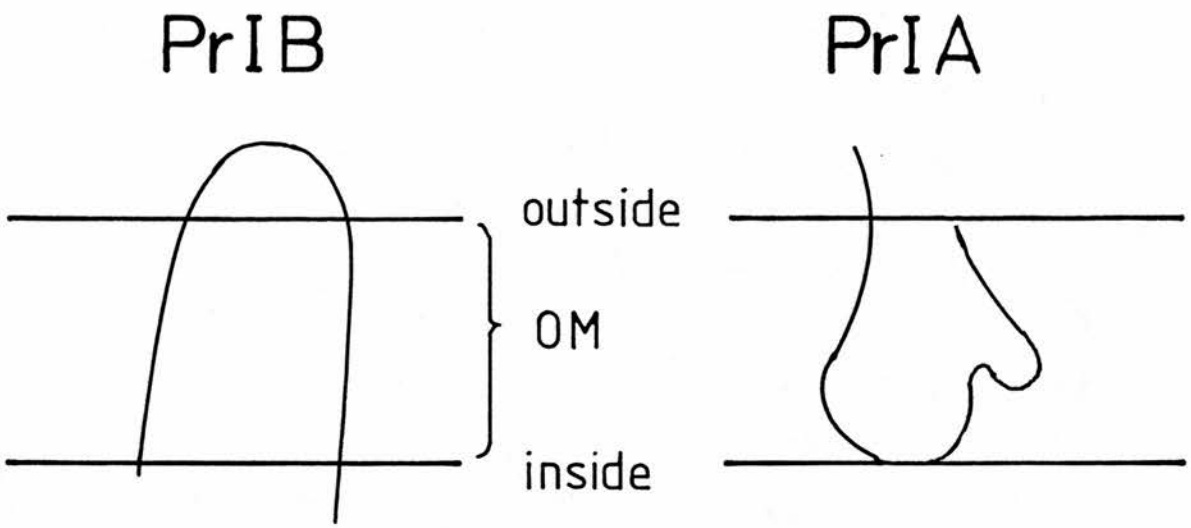

FIGURE 4: Proposed orientation of protein IA and IB within the gonococcal outer membrane.

(adapted from Barrera and Swanson, 1984)

OM - outer membrane

polyacrylamide gel (McDade and Johnston, 1980). This indicated that the gonococcal surface consists of very large areas rich in protein-protein interactions. Similar results were obtained by Newhall *et al.* (1980).

At concentrations of 1 mM the cross-linking agents produced trimers of protein I and a dimer of protein I and III. Both reagents used form cross-links of approximately 1.1 nm suggesting that protein I and III are very closely associated within the outer membrane (McDade and Johnston, 1980). It is possible that both these proteins have an important role in the integrity of the outer membrane.

Protein I can be cross-linked to the peptidoglycon layer (Johnston, 1978). This also suggests that protein I is involved in the structural rigidity of the gonococcal outer membrane.

4.1.6 Functional properties of protein I

Protein I is primarily a porin which forms channels in the outer membrane through which hydrophilic molecules and ions are taken up (Greco *et al.*, 1980; Douglas, Lee and Nikaido, 1981; Young *et al.*, 1983).

This was determined by the use of artificial planar lipid bilayers (Montal, Darszon and Trisel, 1977) which showed that gonococcal protein I behaved as a cation-selective voltage-dependent gating channel or pore (Greco *et al.*, 1980). The enzymes trypsin and chymotrypsin when added to the chamber containing protein I inhibited this activity (Greco *et al.*, 1980). Young *et al.* (1983b) using a different method of preparing the artificial planar bilayers showed that the porin was cation-selective. They were unable to confirm anion-selectivity of gonococcal porin reported by Greco *et al.* (1980).

Similar porin structures are found in *S. typhimurium* and *E. coli* (Nikaido and Nakae, 1979). In *E. coli* trimers of the major outer membrane protein form pores. Young *et al.* (1983b) confirmed that gonococcal pores are formed by protein I trimers, each molecule in the trimer contributing a pore. They estimated that the diameter of the pores was 11 Å. Recently pores formed by protein IA and protein IB have been reported to have different sizes (Blake, personal communication, cited by Barrera and Swanson, 1984).

4.1.7 Insertion of gonococcal protein I into non-gonococcal membranes

Protein I on whole gonococci were shown to insert into artificial lipid bilayers (Blake and Gotschlich, 1983). Protein I was also shown to be capable of inserting into the membranes of red blood cells. Results of cleavage with chymotrypsin suggested that the loop of protein I was localised in the interior of the red blood cells (Blake and Gotschilch, 1983).

Proteins intrinsic to one type of cell membrane have been shown to transfer to another membrane and this process seems to depend on the recipient membrane being more fluid than that of the donor (Cook, Bouma and Huestis, 1980). Changes in the membrane potential of human macrophages due to the action of the complement C5, C5a complex have been shown to precede membrane spreading, ruffling and pseudopod formation (Galin and Galin, 1977). Therefore it is possible that the insertion of gonococcal protein I into epithelial cell membranes may alter the membrane potential of the epithelial cells and possibly trigger phagocytosis and internalisation of gonococci by these cells.

4.2 Protein II

Protein II is associated with the colony opacity variants described by Swanson (1978a). Two types of colony variation were observed on translucent solid media; colonies which were opaque and tended to be light tan to dark in colour and colonies which were transparent and light in colour (Swanson, 1978a). On numerous serial passages gonococcal colonies were observed to become darker and more opaque. The optical properties of the gonococcal colonies were independent of pilation and appeared to be related to the degree of aggregation of gonococci comprising the colonies. The smallest recognisable unit observed at the edge of the opaque colonies was clumps of gonococci in chains whereas in transparent colonies gonococci were usually in pairs.

Gonococci from the different colonies were I^{125} surface labelled and treated with trypsin. Trypsin treatment had a greater effect on opaque colonies. Opaque colonies had greater zones of inhibition to trypsin discs, a greater decrease in the number of viable organisms and a greater amount of the surface I^{125} label was solubilised and released to the supernatant (Swanson, 1978a). These observations suggested that opaque colonies had a glue-like aggregation promoting surface component which was probably a protein or glycoprotein.

4.2.1 Characteristics of protein II

The molecular weight of protein II molecules ranges from 24-30 K (Lambden and Heckels, 1979; Swanson, 1980). Protein II is a heat modifiable protein. The migration of protein II on SDS-PAGE gels is altered by solubilising at 37°C compared with 60°C and 100°C (Heckels, 1977; Johnston, 1978; McDade and Johnston, 1980; Swanson, 1980).

Protein II is solubilised by treatment with deoxycholate and cannot be sedimented by centrifugation at 150,000 x g for 60 min (Heckels, 1977; Johnston, 1978).

Amino acid analyses of various protein II molecules have shown that they contain very similar amino acids and have an almost identical proportion (~23%) of hydrophobic amino acids (Heckels, 1981). Protein II is exposed on the surface of the gonococcal outer membrane and can be surface labelled with ^{125}I and lactoperoxidase (Heckels, 1978; Swanson, 1980). Proteolytic digestion of protein II with trypsin and chymotrypsin has indicated that it has one terminal (amino) embedded in the membrane and the other terminal and a large part of the molecule exposed at the surface (Blake *et al.*, 1981).

4.2.2 Variation in protein II

There are a number of different protein II species and gonococcal strains may express one or more of these (Lambden and Heckels, 1979; Swanson, 1982). Gonococcal transparent colonies are generally considered to contain no protein II. However Swanson (1982) reported finding a protein II species in an isogenic transparent variant of *N. gonorrhoeae* JS3.

Gonococcal isogenic opacity variants of strain P9 have been shown to contain protein IIs of a different molecular weight. These proteins have been termed protein IIa-IIf (Lambden *et al.*, 1979). These different protein II's have been associated with a variety of biological properties such as increased resistance to bactericidal activity of normal human serum (NHS) and resistance to low molecular weight antimicrobial agents and Cu^{2+} and Zn^{2+} ions.

All strains which produced extra protein IIs showed increased binding to human buccal epithelial cells but showed decreased binding to red blood cells compared with parent strain or strains not producing protein II. Strains producing protein IIa showed increased association with polymorphonuclear leukocytes (PMNs) whereas another variant producing protein IIb* showed decreased association with PMNs (Lambden *et al.*, 1979).

Protein IIa* which has a molecular weight of 28.5K (Lambden *et al.*, 1979) may be the same or similar to the previously reported protein, the leukocyte association factor (LA), with a molecular weight of 28-29K (Swanson and King, 1978).

The contribution of protein II to adhesion has been confirmed by studying the attachment of ^{125}I labelled outer membranes to buccal epithelial cells (Heckels and James, 1980).

4.2.3 Primary structure of protein II species

Although these proteins appear to be correlated with different virulence properties of the gonococcus, analyses by peptide mapping has shown that they are remarkably similar in structure (Swanson, 1980; Heckels, 1981; Judd, 1985).

Swanson (1980), Heckels (1981) and Judd (1985) examined protein II species from isogenic gonococcal variants. Swanson (1980) also examined protein II species from different gonococcal strains. Swanson (1980) and Judd (1985) found that the various protein II molecules had 50-70% of their peptides in common whereas Heckels (1981) found that nearly all peptides produced were common to all protein II species with only a few peptides being unique. Heckels (1981) found that the unique peptides corresponded to those which were hydrophilic in nature and were

therefore probably located on the bacterial surface. Judd (1985) indicated that variation in protein II was likely to be in the surface exposed part of the molecule but that changes in non-exposed parts of the molecule might cause conformational changes in the protein II resulting in new domains being exposed at the surface. Structural differences have been confirmed by Draper *et al.* (1984). Tentative linear peptide maps have been generated from V8 protease cleavage studies of protein II species. These show that protein II molecules have conserved domains of ~10 K and variable domains of ~20 K.

The structural differences in protein II have also been confirmed by serological studies. Antiserum, raised in rabbits, to protein II is highly specific for the homologous protein II species and lacks reactivity with other protein II species produced by isogenic variants of the same gonococcal strain (Diaz and Heckels, 1982).

4.3 Protein III

Protein III has a molecular weight in the range of 30-31 K (Judd, 1982a,b) and is not a heat modifiable protein (Johnston, 1978; McDade and Johnston, 1980). It is resistant to the effect of *in situ* proteolysis (Blake *et al.*, 1981; Swanson, 1981).

Protein III is not readily visualized on SDS-PAGE but can be demonstrated by Iodogen-catalyzed radioiodination and autoradiography (Blake *et al.*, 1981; Swanson, 1981). It exhibits retarded electrophoretic migration after treatment with 2-mercaptoethanol (McDade and Johnston, 1980; Judd, 1982a,b), probably as the result of the disrutpion of an intrachain disulphide band (McDade and Johnston, 1980).

4.3.1 *Protein III structural studies*

The structural similarity of protein III molecules has been demonstrated by Judd (1982a,b). ^{125}I-peptide mapping of protein III isolated from four different gonococcal strains showed that all four protein III molecules produced peptide-maps that were virtually identical differing in only a few peptides (Judd, 1982a).

An earlier study indicated that protein III was not exposed or was blocked at the gonococcal surface (McDade and Johnston, cited by Johnston, 1978). However, Judd has shown that it is exposed at the gonococcal surface (Judd, 1982b). Peptide maps of surface labelled protein III in whole gonococci or in outer membrane preparations indicated that it has the same portion of its molecule exposed at the surface regardless of which gonococcal strain is examined (Judd, 1982b).

4.3.2 *Antigenicity of protein III*

Initial studies indicated that protein III was antigenically identical in a number of different gonococcal strains (Johnston, 1978). Recently a monoclonal antibody, 2E6, has been produced against gonococcal protein III (Swanson, Mayer and Tam, 1982). Studies with monoclonal 2E6 have shown that protein III from three different gonococcal strains all have the same antigenic determinant (Swanson *et al.*, 1982). Studies with this monoclonal antibody have shown that protein III retains its antigenicity after treatment with Iodogen and 2-mercaptoethanol and has confirmed that it is a surface exposed protein (Swanson *et al.*, 1982).

Swanson *et al.* (1982) have also confirmed the association of protein III with protein I in heteropolymers. McDade and Johnston (1980) previously reported that protein III and protein I associate to form multimers and suggested that protein III may play an important role in the

structural integrity of the gonococcal membrane. The conserved nature of the protein III molecules from different gonococcal strains (Judd, 1982a, b) increases the evidence for this role.

4.4 Minor gonococcal envelope proteins

Recently attention has turned towards some of the minor gonococcal proteins. Zak *et al.* (1984) observed that the sera from four patients with gonorrhoea reacted with a 43 K gonococcal protein. These sera reacted equally well with this protein from homologous and heterologous gonococcal strains. This protein failed to be detected in whole cells or outer membrane preparations on SDS-PAGE gels stained by Coomassie blue but showed up as faint lines on autoradiographs after SDS-PAGE of whole cells labelled with Iodogen. It has been proposed that this protein be termed protein IV (Zak *et al.*, 1984).

Cannon *et al.* (1984) recently described a protein antigen, using a monoclonal antibody (H-8), which was common to *N. gonorrhoeae*, *N. meningitidis* and *N. lactamica* but not to non-pathogenic neisseriae.

This antigen termed H-8 antigen was heat-modifiable with a molecular weight of 30 and 20 K after heating at 100°C and 20 K after heating at 37°C. It did not stain with Coomassie blue but did stain with silver staining. The H-8 antigen was not thought to be a protein II species and was present in pilated and non-pilated gonococci.

The gene for the H-8 antigen has been cloned in the bacteriophage lambda (Black and Cannon, 1985). The cloned gonococcal DNA was approximately 15 kilobases in size. One clone S6H-8 which produced the H-8 antigen was used to construct a DNA probe. The DNA from neisseriae which reacted with H-8 and produced the H-8 antigen were shown to hybridize to the S6H-8 probe (Black and Cannon, 1985).

A number of other workers have described other protein antigens which are similar to H-8. Virji, Zak and Heckels (1984) have shown that monoclonal antibody SM70 detects an antigen varying in size from 20.5 - 26.5 K found in pathogenic but not in non-pathogenic neisseriae. This protein could not be detected on SDS-PAGE gels stained with Coomassie blue but could be after silver staining (Virji *et al.*, 1984). Zollinger *et al.* (1984) described a protein termed CA2 (26-28 K) which was slightly larger in size than H-8 described by Cannon *et al.* (1984). However it shared the property of not staining with Coomassie blue following SDS-PAGE. Antibody was detected to CA2 in the convalescent sera of patients with meningococcal infections and in sera from individuals vaccinated with a meningococcal outer membrane protein vaccine (Zollinger *et al.*, 1984). On further analysis all of these antigens may be found to be identical.

5.0 Pili

Pili were first described on *N. gonorrhoeae* in 1971 (Swanson, Kraus and Gotschlich, 1971; Jephcott, Reyn and Birch-Anderson, 1971). These two groups examined the ultrastructure of the gonococcus by electron microscopy and discovered extracellular fibrous bundles resembling pili. Negative staining revealed pili on the surface of gonococci (Swanson *et al.*, 1971). The number of pili per gonococcal cell was difficult to establish but appeared to vary from as few as two to six or to as many as 25-50. Pili varied in length. However, the majority appeared to be 0.5-2 μm in length with a few as long as 4 μm. The diameter of the pili was estimated to be approximately 8 nm (Swanson *et al.*, 1971). Pili were found on gonococci of colony types 1 and 2 but not on types 3 and 4 (Swanson *et al.*, 1971; Jephcott *et al.*, 1971). Therefore pilation appears to be related to gonococcal virulence.

Pili have been shown to adhere to a wide variety of human cells such as epithelial cells, spermatozoa and fallopian tube in organ culture (Punsalang and Sawyer, 1973; James-Holmquest *et al.*, 1974; Ward, Watt and Robertson, 1974).

5.1 Ultrastructure of pili

Each pilus filament is composed of thousands of protein subunits (pilin) which polymerise to form a linear structure (Brinton, 1965). The molecular weight of gonococcal pilin subunits varies from approximately 17,000-21,000 in pili from different strains (Buchanan, 1977; Robertson, Vincent and Ward, 1977; Lambden, Robertson and Watt, 1981a).

5.2 Interstrain variation in pili

Pili from different gonococcal strains are antigenically distinct (Buchanan, 1975). However, it has been shown that there is approximately 10% shared antigenicity between pili from three different gonococcal strains with rabbit antisera and 50% shared antigenicity with human sera (Buchanan, 1978; Brinton *et al.*, 1978). Therefore, gonococcal pili appear to contain regions of shared antigenicity and regions which are antigenically distinct.

5.3 Intrastrain variation in pili

In addition to inter-strain variation, intrastrain variation of gonococcal pili has been described (Lambden, Robertson and Watt, 1980; Salit, Blake and Gotschlich, 1980). Both groups studied pili from isogenic gonococcal opacity variants. Salit *et al.* (1980) examined 14 different gonococcal strains and found that the opaque variants of 10 of these strains produced pili with a higher subunit molecular weight than the

pili from corresponding transparent variants. In addition there were differences in the buoyant density and isoelectric points of the two types of pili (Salit *et al.*, 1980).

Lambden *et al.* (1980) examined isogenic variants of *N. gonorrhoeae* strain P9, and found similar results to those of Salit *et al.* (1980). A transparent P9 variant produced pili with subunit molecular weights of 19,500 whereas the opaque isogenic P9 variant produced pili with subunit molecular weights of 20,500. These pilus types were termed α and β respectively. These pili were shown to have different buoyant densities and isoelectric points (Lambden *et al.*, 1981a).

In electron micrographs of gonococci producing the different pili it was observed that α- pili occurred in large aggregated bundles and appeared to be produced from a few widely spaced sites on the gonococcal surface. The β-pili were well separated, wavy structures which appeared to radiate from the whole gonococcal surface (Lambden *et al.*, 1981a).

The α-pili showed 45% attachment to human buccal epithelial cells compared with 13% attachment of β-pili. The α-pilus attachment was pH dependant with an optimum at pH 6.5 and appeared to be mediated by pilus receptors on the epithelial cell surface composed of sialic acid plus other sugar residues (Lambden *et al.*, 1980). The binding of α-pili to epithelial cells could be reduced to the same level as that of β-pili by removing the sailic acid/sugar receptors by treating epithelial cells with neuraminidase and exoglycosidase. Both pilus types bound to human erythrocytes at the same level of 7%. It was postulated that in addition to receptor specific binding that non-specific hydrophobic interactions also play a part in pilus binding.

It was observed that on SDS-PAGE gels that β-pili were always accompanied by a minor band of a slightly higher molecular weight.

It was postulated that this band may represent aggregates of β-pili or possibly a third pilus type (Lambden *et al.*, 1980). The inoculation of P9 gonococcal strains producing α- and β-pili into subcutaneous guinea pig chambers resulted in the *in vivo* selection of P9 variants producing two quite distinct pili. These pili were different from α- and β-pili and were termed γ and δ. They had molecular weights of 21,000 and 18,500 respectively and had different isoelectric points (Lambden *et al.*, 1981b; Lambden, 1982). Therefore one gonococcal strain is capable of producing a variety of different pilus types.

Biochemical analysis of the α-, β-, γ- and δ-pili have indicated that the amino acid composition of all four types was very similar (Lambden, 1982). However, there were some individual differences. The low molecular weight δ-pili contained less aspartate and serine whereas the β- and γ-pili contained more alanine residues. Tryptic peptide mapping revealed all four pilus types shared nine common peptides and therefore there was a certain amount of structural homology. However, each of the four pilus types produced a number of peptides which were unique to that particular pilus. In addition some peptides appeared to be common to δ- and α-pili and others were common to γ- and β-pili. Hence it appeared that δ- and α-pili and γ- and β-pili may represent two groups of structurally related pili (Lambden, 1982).

However, although there appears to be considerable structural homology it was shown in an ELISA assay that there was only 10-20% cross-reactivity between these four pili (Virji, Everson and Lambden, 1982). These results confirm that pili contain common and variable peptide regions.

5.4 Chemical analysis of pilin

Chemical analysis of gonococcal pilin subunits has revealed that the amino acid composition is similar but not identical in pili from different strains (Robertson *et al.*, 1977; Hermodson, Chen and Buchanan, 1978). The amino acid composition appeared to be 55% hydrophilic and 45% non-polar amino acids (Robertson *et al.*, 1977; Hermodson *et al.*, 1976). Carbohydrate, mostly galactose but also traces of glucose, and phosphates were also detected (Robertson *et al.*, 1977). It was thought that these components might be the result of tightly but non-covalently bound contaminating material. Contamination with carbohydrates has been reported for pili of *E. coli* (Armstrong *et al.*, 1981). Carbohydrate could be completely removed by gel filtration chromatography in the presence of SDS from one pilus type and approximately 65% from the other pilus type. Investigation of the phosphate content of these pili indicated that these groups were involved in phosphodiester linkages (Armstrong *et al.*, 1981). Similar results have been reported recently for gonococcal pili (Schoolnik *et al.*, 1984). Approximately 1.5 mol of galactose and 0.2 mol of glucose were detected per pilin subunit. However, these were not covalently bound. The phosphate residues detected, two per pilin subunit, were covalently bound to serine residues. It was concluded that gonococcal pilin is not a glycoprotein (Schoolnik *et al.*, 1984).

5.5 The primary structure of pilin

Early sequencing studies of the N-terminus of pilin subunits from four antigenically distinct gonococcal strains showed that all had an identical amino acid sequence up to and including the 29th residue (Hermodson *et al.*, 1978). The N-terminal residue in all of these strains

was the unique amino acid N-methyl phenylalanine. All except two of the 29 residues in this sequence were hydrophobic in nature. Therefore it would appear that the N terminus of gonococcal pilin is hydrophobic and highly conserved in nature. It was suggested that this part of the pilin polypeptide may play an important structural role in the integrity of the pilus either by maintaining the conformation of the pilin subunit or the polymeric pilus structure by subunit-subunit interactions. The N-terminal sequence contains most of the hydrophobic amino acids of pilin. Therefore the remainder is hydrophilic in nature and is likely to be surface exposed (Hermodson *et al.*, 1978).

5.6 Primary structure of pilin subunits in relation to function

Cyanogen-bromide (CNBr) cleavage studies on gonococcal pilin have indicated receptor binding and antigenic domains (Schoolnik, Tai and Gotschlich, 1982). Cyanogen-bromide cleavage of pilin subunits produces three fragments: CNBr-1 N-terminal to residue 7, CNBr-2 residues 8 to 84, and CNBr-3 residues 85 to 160, the carboxy-terminal portion of the molecule (Figure 5). Binding studies to human erythrocytes indicate that the CNBr-2 fragment of pilin contains the cell binding domain. CNBr-2 pilin fragments were able to inhibit the haemaglutination of both CNBr-2 fragments and whole pili from the opacity variants of both homologous and heterologous gonococcal strains. The CNBr-2 fragment also binds to Chinese hamster ovary cells (Gubish *et al.*, 1982). The amino acid composition of CNBr-2 fragments from a number of different gonococcal pili are very similar and tryptic peptide mapping show that they have 87% of their peptides in common. N-terminal amino acid sequencing of different CNBr-2 fragments show that they are identical in structure (Schoolnik *et al.*, 1982). Therefore it would appear that the cell

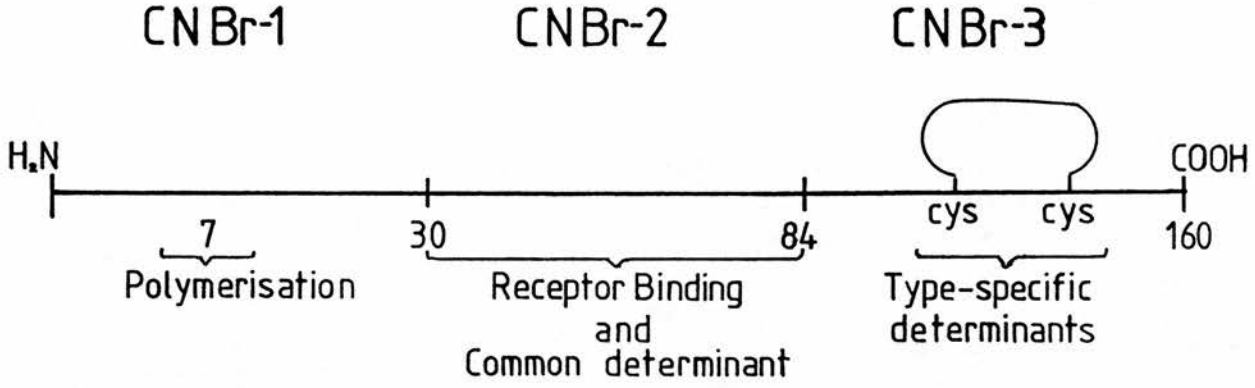

FIGURE 5: Functional and antigenic domains of the gonococcal pilus subunit (Adapted from Schoolnik *et al.*, 1982).

binding domain is highly conserved among pili from different gonococcal strains. As each pilin subunit contains a cell receptor binding domain, when assembled the pili would have a linear array of binding regions along the longitudinal axis of the pilus filament.

In contrast CNBr-3 pilin fragments from different gonococcal pili indicate that they are chemically diverse. There are differences in amino acid composition and CNBr-3 pili fragments only share 50% of peptides on tryptic peptide maps. Therefore the primary structure of the carboxy terminal region of pilin is variable. This section of pilin did not appear to be involved in cell binding (Schoolnik *et al.*, 1982). Rabbit antibody prepared to antigenically distinct pili from two strains of gonococci and to their CNBr-2 and CNBr-3 fragments revealed that anti-CNBr-2 antisera could bind to CNBr-2 fragments and intact pili from homologous and heterologous gonococcal strains. Thus it was shown that this region in addition to containing the cell receptors also contains a common gonococcal antigenic determinant. Anti-CNBr-3 antibody bound only to CNBr-3 fragments and whole pili from homologous gonococcal strains. Therefore this region which is chemically diverse contains the type specific antigenic determinant of gonococcal pili. CNBr-3 fragments contain a disulphide bridge and when this is reduced and alkylated the type-specific antigenic determinant is destroyed (Schoolnik *et al.*, 1982). Although both of these fragments are antigenic in native pili it appears that CNBr-2 fragment containing the cell receptors and common antigenic determinants is less immunogenic than the CNBr-3 fragment. This fragment contains the region which determines type-specific antigenicity and confers diversity in gonococcal pili (Schoolnik *et al.*, 1982).

5.7 Structural analysis of gonococcal pili with monoclonal antibodies

Recently monoclonal antibodies have been prepared to the four pilus types of *N. gonorrhoeae* P9 (Virji, Heckels and Watt, 1983). Studies with these monoclonals confirm that gonococcal pili contain a common antigenic region and a type specific region. Both antigenic sites contain more than one epitope (Virji *et al.*, 1983). Type-specific and cross-reacting monoclonal antibodies used in immunoblotting showed differences in their reactivity with pili treated with SDS. Cross-reacting monoclonals SM1 and SM2 bound efficiently to the SDS-unfolded pilin from α-, β-, γ- and δ- pili whereas the specific antibodies bound poorly to their appropriate pilus types under identical experimental conditions. This suggested that the cross-reacting antibodies recognised the conserved primary sequence in unfolded pilin subunits whereas the type specific monoclonals require the variable pilin region to be intact for recognition (Virji *et al.*, 1983). This confirms the previous observations of Schoolnik *et al.* (1982) who showed that destruction of a disulphide bridge in the variable domain (Figure 5) removed type-specific antigenicity.

Monoclonal SM1 binds to the conserved CNBr-2 fragment of pili (Figure 6) (Virji and Heckels, 1983).

Recent studies with these monoclonal antibodies indicate that the variable type-specific pilus antigenic domains may also be involved in binding of pili and virulence of gonococci to human epithelial cells (Virji and Heckels, 1984). The cross-reacting antibodies SM1 and SM2, at high concentrations, were not able to or only partially able to inhibit binding of α- and γ-pili to buccal epithelial cells. Three α-specific and four γ-specific monoclonals were able to inhibit the binding to epithelial cells of α- and γ-pili respectively.

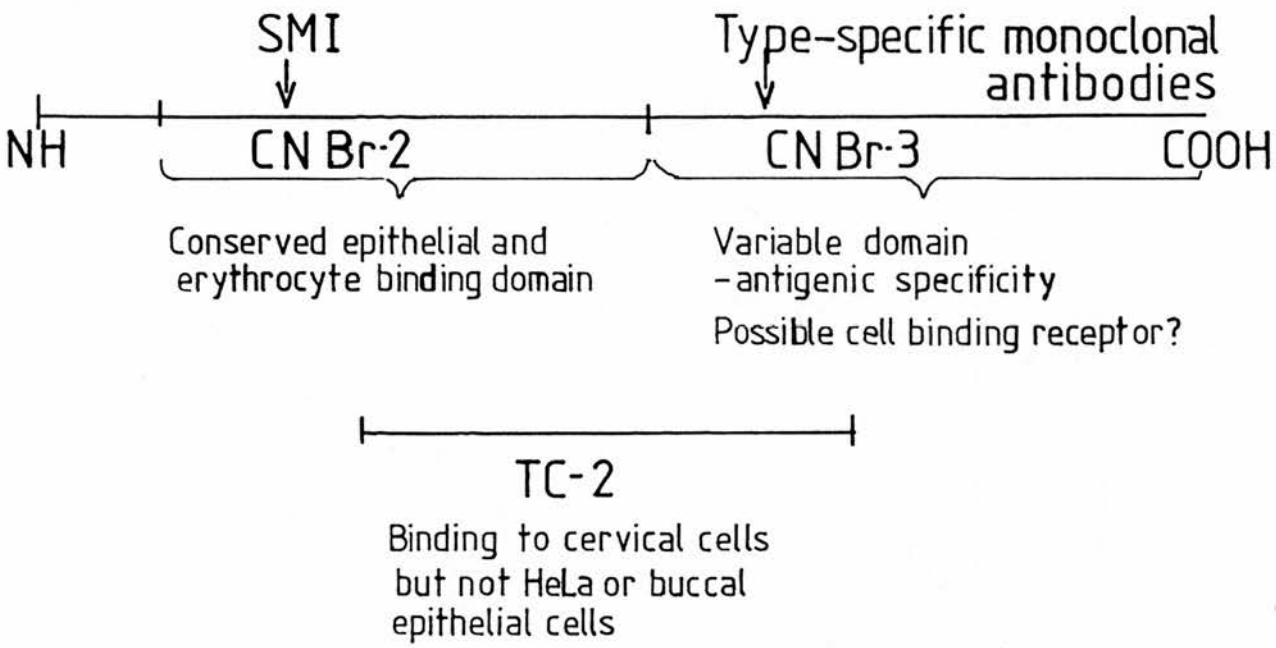

FIGURE 6: Schematic representation of the functional domains and antigenic sites on gonococcal pilus subunits.

Previous studies showed that α- and β-pili adhered to buccal epithelial cells differentially suggesting that the variable domain of gonococcal pili contained a receptor for epithelial cell binding (Lambden *et al.*, 1980). However, Schoolnik *et al.* (1982) were unable to demonstrate cell binding receptors in the variable antigenic pilin domain encompassed in the CNBr-3 fragment (Figure 5). However, it is possible that cleavage with cyanogen bromide destroys a receptor site. Tryptic digests of gonococcal pilin liberated a fragment termed TC-2 which encompassed part of the CNBr-2 and CNBr-3 fragments (Figure 6). This fragment was shown to bind to endocervical cells but not to HeLa or buccal epithelial cells (Schoolnik *et al.*, 1984). It would appear that the cell binding receptors may confer tissue specificity. The exact site of cell binding receptors awaits clarification of the structure of pilin subunits and the conformational arrangement of pilin molecules within intact pili.

5.8 Examination of clinical isolates with pilus monoclonal antibodies

Pili from a number of gonococcal clinical isolates were examined using 10 pilus monoclonal antibodies to determine if they produced pili of the α-, β-, γ- or δ-type found on P9 (Virji and Heckels, 1983).

It was shown that the monoclonal antibodies SM1 (specific for the conserved pilin domain) and to a lesser extent SM2 reacted with all gonococcal isolates. However, of the eight type-specific monoclonal antibodies only two showed any cross-reactivity with pili from clinical isolates. These two antibodies were specific for epitopes on P9 α-pili. This suggests that gonococci have the capacity to produce a large range of antigenically diverse pili. Virji and Heckels (1983) also showed that SM1 reacted with pili from a large number of meningococcal strains. Hermodson *et al.* (1978) demonstrated that one strain of *N. meningitidis*

shared the same N-terminal amino acid sequence up to residue 29. Therefore it is likely that areas of the pilin molecule involved in pilus intersubunit interactions and possibly in binding to human cells are conserved among neisserial species.

GENETICS OF THE GONOCOCCAL CELL SURFACE COMPONENTS

The components of the gonococcal cell surface are controlled by chromosomal genes. Mutations at these sites may alter the components produced by a particular strain and thus affect a number of gonococcal cell functions.

1.0 Penicillin resistance mutations

The molecular basis of low-level penicillin resistance controlled by the mutations *pen*A, *pen*B and *mtr* is now beginning to be understood. Once the production of enzymes which inactivate penicillin were ruled out (Sparling *et al.*, 1975; Rodriguez and Saz, 1975), it became clear that resistance may occur by preventing the binding of penicillin to the cell wall/membrane and or by altering the permeability of the cell wall. Rodriguez and Saz (1975) first reported observing a reduction of penicillin binding to gonococcal strains with a reduced MIC to penicillin. However the exact site of penicillin binding was not clearly characterized.

1.1 Gonococcal penicillin-binding proteins

Penicillin-binding proteins (PBP) are penicillin-sensitive bacterial enzymes (carboxypeptidases or transpeptidases) situated in the cytoplasmic membrane of bacteria and are involved in peptidoglycan-cross-linkage during the terminal stages of cell wall synthesis (Davis and Salton, 1975). The gonococcus has three major PBPs: PBP_1, PBP_2 and PBP_3 with molecular weights of 90,000, 63,000 and 48,000 respectively (Dougherty, Koller and Tomasz, 1980). Barbour (1981) confirmed the presence of three PBPs in the cytoplasmic membrane. However, he observed slight differences in the molecular weights of these proteins.

1.2 The relationship of the penicillin mutations with PBPs

The *pen*A mutation was shown to result in a 30% increase in the cross-linkage of peptidoglycan and it was suggested that this mutation might affect a carboxypeptidase or a transpeptidase (Sparling *et al.*, 1978). Acquisition of the *mtr* mutation which confers resistance to penicillin, to a variety of other antibiotics, dyes and detergents (Sparling *et al.*, 1975), results in a seven-fold increase in the amount of a minor outer membrane protein with an approximate molecular weight of 52,000 and a 32% increase in the extent of peptidoglycan cross-linkage (Guymon, Walstaad and Sparling, 1978a). The relationship of the penicillin resistance mutations to the PBPs was examined by transformation experiments (Dougherty *et al.*, 1980). It was found that acquisition of the *pen*A mutation resulted in decreased binding of penicillin to PBP_2 and the modifier genes *pem* and *tem* (Warner *et al.*, 1980) were associated with changes in PBP_1 (Dougherty *et al.*, 1981). Whereas the transformants containing the *mtr* and *pen*B mutations appeared to contain more PBP_3 than their penicillin sensitive parents (Dougherty *et al.*, 1981). It has been recently shown that PBP_2 is related to O-acetylation and PBP_1 with transpeptidation of peptidoglycan (Dougherty, 1983).

1.3 Mutations which increase envelope permeability

There is another set of gonococcal mutations termed *env*. Strains carrying these mutations are much more sensitive (hypersensitive) to antibiotics, dyes and detergents than comparative wild type strains (Maness and Sparling, 1973; Sarubbi *et al.*, 1975). The *env* mutations suppress the phenotypic expression of the *mtr* mutations if the two occur within the same strain (Sarubbi *et al.*, 1975). They have been shown to cause a decrease in the amount of the 52,000 Mwt membrane

protein and the extent of peptidoglycan cross-linkage when compared with *mtr* mutants (Guymon *et al.*, 1978a). Therefore the *env* and *mtr* mutations result in the cell envelope being more and less permeable respectively.

Gonococcal strains containing the *env* mutations have been shown to account for 15% of clinical isolates in North Carolina (Eisenstein and Sparling, 1978).

1.4 Mutations affecting gonococcal LPS

Gonococcal serum-resistance is determined by the loci *sac* (resistance to serum antibody and complement) and these genes are thought to affect the structure of the LPS in the outer membrane (Guymon *et al.*, 1978b). There are three distinct loci, *sac*-1, *sac*-2 and *sac*-3^+ and each confer distinct levels of serum resistance (Shafer, Guymon and Sparling, 1982).

Gonococcal strains which are isolated from patients with disseminated infections are usually serum-resistant and are highly susceptible to antibiotics (Eisenstein, Lee and Sparling, 1977). Gonococci containing the *penA* and *mtr* mutations have been shown to have an increased susceptibility to killing by one or more PMN proteins (Cannon *et al.*, 1980b; Daly *et al.*, 1982). This may explain why gonococcal strains isolated from DGI seldom carry these mutations (Eisenstein *et al.*, 1977).

1.5 Protein I

There are two loci associated with control of protein I production, *nmp*-1 and *nmp*-2 (new membrane protein) (Cannon *et al.*, 1980a,b). The gonococcus has two distinct protein I molecules, protein 1A and protein 1B (Sandström *et al.*, 1982a). It has been suggested that *nmp*-1

and *nmp*-2 could represent different alleles of a structural gene for production of protein I or alternatively each could represent a distinct gene determining which form of protein I is synthesized and inserted into the outer membrane (Cannon *et al.*, 1980b).

These loci were discovered after observations that gonococcal strains transformed independently to penicillin resistance by *pen*B and serum resistance by *sac* acquired the same protein I type as the donor strains (Guymon *et al.*, 1978a,b; Sparling *et al.*, 1978). Initially it was thought that these genes conferred changes on protein I, however it was shown that *pen*B was genetically linked to *nmp*-1 (Cannon *et al.*, 1980a) and *sac* to *nmp*-2 (Cannon *et al.*, 1980b) and thus these genes could be co-transformed.

1.6 Protein II

Little is known about the genetic control for the production of the various protein II species found in isogenic variants of one strain or indeed in other strains of gonococci. However, recent work suggests that protein II species within a single strain are products of similar but distinct structural genes (Schwalbe, Klapper and Cannon, 1984). Meyer *et al.* (1984) have identified one such gene termed *opaE*$_1$.

1.7 Genetic control of pilus production

Genetic analysis of pilus expression using specially constructed DNA probes (Meyer, Mlawer and So, 1982) indicated that the gonococcal genome contains a number of pilus-related sequencies. Transition from the pilated (P^+) to non-pilated (P^-) state was shown to result in chromosome rearrangement. This chromosomal rearrangement is reversible.

Recent chromosomal mapping has identified three major chromosomal pilus genes within a 5% fragment (80 kbp) of the gonococcal chromosome (Meyer *et al.*, 1984). These studies also indicate that there are many other pilus-related sequences scattered throughout the gonococcal chromosome.

The three loci described have been termed $pilE_1$, $pilE_2$ and $pilS_1$. The loci $pilE_1$ and $pilE_2$ code for pilus production. It is not known whether one or other or both are expressed simultaneously. However, in the gonococcal strain (MS11) studied both of these sites were found to be identical (Meyer *et al.*, 1984). $PilE_1$ and $pilE_2$ are both found within the region of the chromosome which undergoes rearrangement during P^+ to P^- transition.

The third locus $pilS_1$ was designated as a silent region because it does not contain a promotor sequence. It is found in a region which does not undergo rearrangement during P^+ to P^- transition. $PilS_1$ appears to contain genetic information for part of the pilin structure, probably for a variable region of the pilin molecule. It is possible that the other areas of the gonococcal chromosome which contain pilus-associated regions may contain other silent regions like $pilS_1$ and possibly promotor sequences. However these sequences await characterization.

It has been hypothesized that genetic control of pilin production in gonococci might be analogous to genetic control of immunoglobulin production in B lymphocytes. A gonococcal strain would thus be able to alter its pilus structure by switching on one of the variable sequences such as $PilS_1$ coding for a different variable pilin region (Meyer *et al.*, 1984). Therefore gonococci could change their pilus structure in response to changing environmental conditions in a completely flexible manner.

The conserved part of the pilin molecule is thought to be coded by DNA sequences in completely different parts of the chromosome.

METHODS OF CLASSIFICATION OF *N. GONORRHOEAE*

A method of classifying gonococcal isolates is required for epidemiological and research purposes. For a number of years a non-serological classification system, auxotyping, was the primary method used for distinguishing different gonococcal strains. Recently methods for the serological classification of *N. gonorrhoeae* have been developed.

1.0 Auxotyping

Auxotyping was developed in 1973 by Carifo and Catlin. It is based upon the nutritional profile of gonococcal isolates on chemically defined media. Thirty-five auxotypes have been identified (Catlin, 1978). These range from gonococci which are prototrophic and are thus able to grow on media lacking one or more nutrients to those strains which require the addition of up to six nutrients. The auxotype characteristics are stable during subculture (Carifo and Catlin, 1973).

Differences in the geographical distribution, differences in the antibiotic sensitivities, association with site of infection and clinical syndromes have been associated with particular gonococcal auxotypes (Knapp and Holmes, 1975; Catlin and Pace, 1977; Crawford *et al.*, 1977; Knapp *et al.*, 1978). Gonococcal strains of auxotype 14 requiring arginine, hypoxanthine and uracil for growth (AHU^-) have been associated with disseminated gonococcal infections and with asymptomatic urethral infections in men (Knapp and Holmes, 1975; Catlin and Pace, 1977; Crawford *et al.*, 1977; Turgeon and Granger, 1980).

Auxotyping has provided valuable information about *N. gonorrhoeae*. However it is a laborious and expensive technique and nowadays only gonococcal reference laboratories are likely to use auxotyping routinely.

2.0 Serological classification of *N. gonorrhoeae*

Since the early 1970s attempts have been made to find an acceptable system for serogrouping gonococci. A number of gonococcal outer membrane components have been examined for this purpose.

2.1 Gonococcal lipopolysaccharide and pili

Antigenic differences in gonococcal LPS and pili have been investigated as the basis of serological classification systems.

Gonococci have been divided into six serogroups (Gc 1-6) on the basis of antigenic differences in the acidic polysaccharides of LPS (Apicella, 1974, 1976; Apicella *et al.*, 1981). However this classification system has been used mostly for determining differences in gonococcal LPS and has not been widely used for epidemiological purposes.

Pili are antigenically heterogeneous and there are a large number of serologically distinct pilus types (Buchanan, 1975, 1977). However, because pili are lost on subculture serotyping of gonococci based on pili is not suitable for epidemiological purposes.

2.2 Serological classification based on protein I

Any system for typing bacteria must be based upon stable characteristics and the impetus for the development of serogrouping systems based on protein I stemmed from the work of Johnston *et al.* (1976). These workers divided gonococci into 16 serogroups on the basis of differences in the molecular weight of protein I (then termed MOMP) and a covariant minor protein. This study gave rise to the MOMP reference strains (Johnston *et al.*, 1976).

2.2.1 Development of serogrouping by coagglutination

Sandström and Danielsson (1980a) developed a system of serogrouping by coagglutination (CoA). This system is based upon the attachment of anti-gonococcal IgG to protein A on whole killed staphylococcal cells (Figure 7). The addition of gonococcal cells which have determinants that react with the IgG attached to the staphylococci results in agglutination which is visible to the eye. CoA was first used as a confirmatory serological test in the identification of *N. gonorrhoeae* (Danielsson and Kronvall, 1974).

Antisera raised to the MOMP reference strains (Johnston *et al.*, 1976) were characterised by line-rocket immunoelectrophoresis (L-RIE) and CoA (Sandström and Danielsson, 1980b). It was demonstrated by both these methods that rabbit hyperimmune antigonococcal antisera contained reactivity against common as well as strain specific and cross-reactive antigens. On the basis of these results the MOMP reference strains were initially divided into two groups. These groupings were confirmed by CoA carried out with antisera absorbed with strains from within the same antigenic group and with absorption with a cross-reacting and non-cross-reacting strain from the other antigenic group (Sandström and Danielsson, 1980b). These results formed the basis for producing reagents for the CoA classification system.

Using antisera raised to and selectively absorbed by the MOMP reference strains, Sandström and Danielsson (1980a) described three classes of reactions, W, J and M.

The W class could be further subdivided into three serogroups, WI, WII and WIII. Each of the MOMP reference strains belonged to only one of these groups. Some of these reactions were sensitive to pronase and all were resistant to periodate and therefore the W reactions appeared

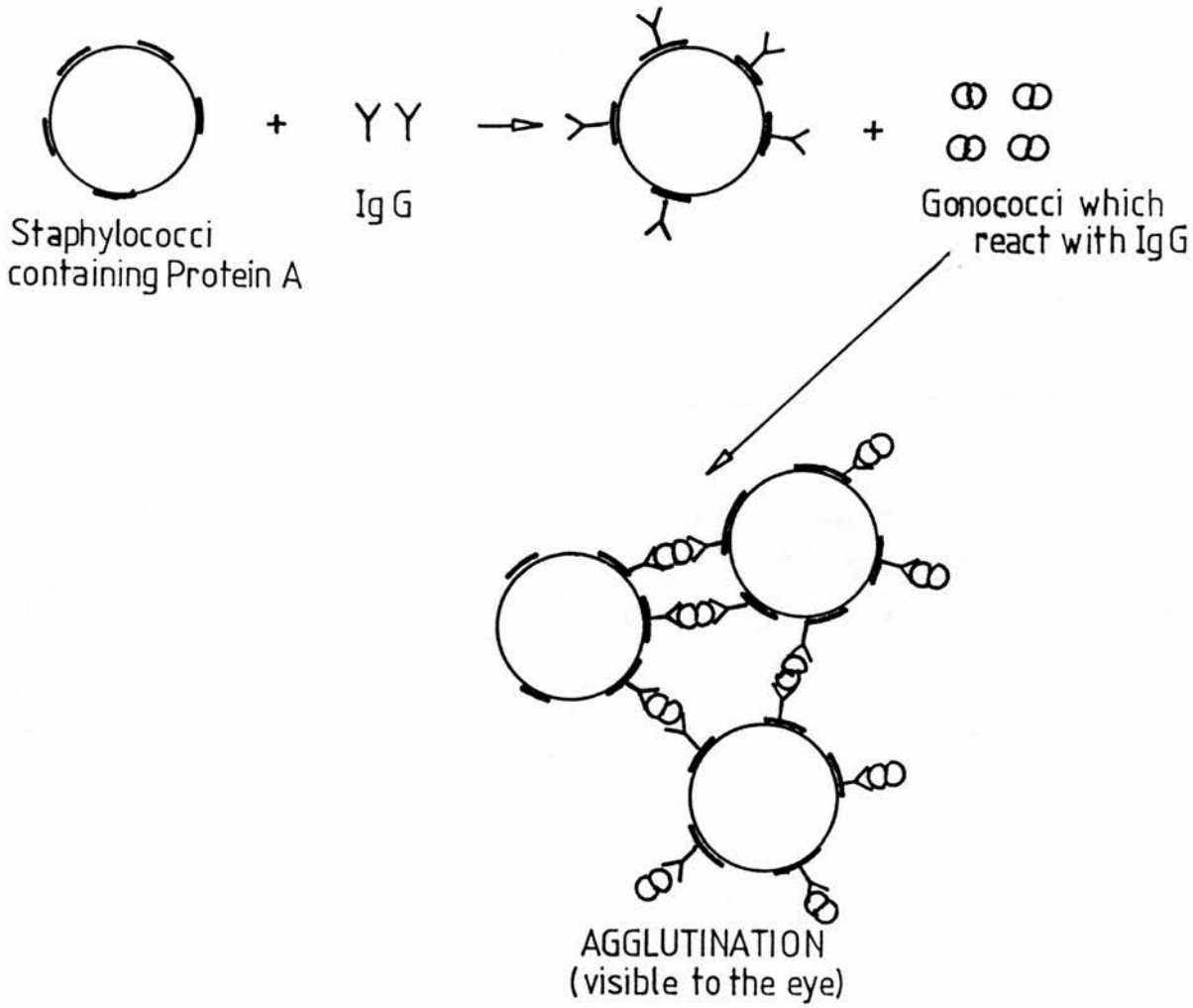

FIGURE 7: Schematic representation of the coagglutination (CoA) reaction.

to be based upon protein antigens (Sandström and Danielsson, 1980a). These reactions were stable during subculture and did not vary with colony morphology.

Some of the class J reactions were sensitive and some were resistant to pronase. All were resistant to periodate. Although some of the reactions were thought to resemble the W reactions, the J reactions often varied with colony morphology (Sandström and Danielsson, 1980a). Only seven of the MOMP reference strains reacted with the five reagents for this group and it was thought that these reagents recognized strain specific antigens. It is possible that they may represent serological reactions with protein II.

Group M reactions were periodate sensitive, were inhibited by lactose and varied with subculture. It would appear that these reactions recognized LPS or acidic polysaccharides (Sandström and Danielsson, 1980a).

The W reactions were used for serogrouping *N. gonorrhoeae* because they were stable. Using CoA W reagents, Sandström and Danielsson (1980a) serogrouped a number of clinical isolates and reference strains. They found that there was a sharp antigenic demarcation between WI and WII serogroups and that there appeared to be a gradual order of relatedness within each serogroup. It was thought that strains in serogroup WIII might be related to those in serogroup WII. It was proposed that serogroup WI and WII represented antigenic differences in two different alternative structures.

2.2.2 Serotyping based on protein I

Hildebrandt and Buchanan (1978) using an enzyme linked immunosorbent assay (ELISA) determined that serum-resistant gonococcal strains

isolated from patients with disseminated infections (DGI) contained a particular type of protein I. They found that this protein I was present in only 30% of serum sensitive gonococcal strains from localized infections (Hildebrandt and Buchanan, 1978). From these initial observations they developed an antigen-specific serotyping system based upon protein I in an inhibition ELISA (Buchanan and Hildebrandt, 1981). Purified protein I was used to coat ELISA tubes and either purified protein I or whole untreated gonococcal cells were reacted with a constant amount of protein I specific antisera. Gonococcal strains were considered to belong to a particular serotype if they caused ≧30% inhibition of antibody binding in the ELISA.

Using purified protein I from a variety of gonococcal reference strains a total of nine different protein I serotypes were described. Eight of the nine protein I serotypes were antigenically distinct. Serotypes 1 and 2 were shown to be antigenically closely related. These two serotypes were kept as separate groups because they frequently typed the more invasive gonococcal strains and because 40% of the salpingitis and DGI strains contained protein I which reacted with only one of the two serotypes. 94% of gonococcal isolates from DGI and 60% isolated from patients with salpingitis belonged to serotype 1 compared with 40% of strains isolated from localized infections. The majority of these serotype 1 strains were also typed as serotype 2 (Buchanan and Hildebrandt, 1981). The MOMP reference strains were typed by one or more of the protein I serotypes.

3.0 CoA W serogroups detect protein I antigens

Initial studies on gonococcal strains isolated from patients with DGI and PID suggested that CoA serogrouping and protein I serotyping

by ELISA detected similar antigens (Sandström and Buchanan, 1980). This was confirmed in further studies (Sandström, Knapp and Buchanan, 1982b). A total of 224 strains were analysed by both the CoA W serogrouping and ELISA protein I serotyping systems. Included were 197 clinical isolates and 27 gonococcal reference strains. Once antisera used in the ELISA serotyping system had been absorbed to remove antibodies to LPS it was shown that CoA W serogroups and the protein I serotypes detected protein I. Strains of CoA serogroup WI were shown to correspond to protein I serotypes 1, 2 and 3. CoA serogroup WII strains corresponded to protein I serotypes 4, 5, 6 and 7 exclusively. Some of the WII strains also reacted with serotypes 8 and 9 as did CoA WIII strains (Sandström *et al.*, 1982b).

A few if any antigenic sites are shared between strains of CoA serogroup WI (serotypes 1 to 3) and strains of CoA serogroups WII and WIII (serotypes 4 to 9). However, within these two groupings there is some antigenic variation. These results suggested that there were two different biochemical forms of protein I. One which is detected by CoA serogroup WI reagents and the other detected by serogroup WII reagents. It was proposed that the CoA serogrouping system which utilized boiled cells may recognize less exposed shared antigenic determinants in addition to variable protein I antigenic determinants. Whereas the protein I serotyping, utilising untreated whole cells, may in general recognize the more variable and surface exposed antigenic determinants. It was proposed that the CoA serogroups WI and WII/WIII recognize two different groups of protein I molecules and the ELISA protein I serotyping characterizes one or more variable antigenic determinants on these molecules (Sandström *et al.*, 1982b). It would appear that CoA serogroup WIII represents a prominent antigenic determinant of the protein I molecule

which is analogous to serotype 9 rather than a third group of protein I molecule (Sandström *et al.*, 1982b). Direct evidence for this has come from I^{125}-labelled tryptic peptide maps of protein I from gonococci characterized by the CoA W serogrouping system (Sandström *et al.*, 1982a). Gonococcal strains of CoA serogroup WI tend to have protein I molecules of a lower molecular weight than those of serogroups WII and WIII. ^{125}I labelled peptide maps of protein I from 13 WI strains, 16 WII strains and 4 WIII strains are quite distinct in pattern. Protein I molecules from serogroup WI and WII/WIII strains were named protein-IA and protein-IB respectively. Protein IA was shown to have 11 peptides in common, 4 acidic, 4 neutral and 3 basic. PrIBs shared 10 common peptides, 5 acidic, 1 neutral and 4 basic (Sandström *et al.*, 1982a). Only 3 peptides are common to both protein IA and IB molecules.

The CoA W serogrouping system is very easy to carry out and has been widely accepted. Recently monoclonal antibodies have been produced to protein I. These correspond to the three CoA W serogroups (Tam *et al.*, 1982). CoA reagents prepared with these monoclonal antibodies are now beginning to be used for serogrouping of *N. gonorrhoeae*.

PATHOGENICITY AND IMMUNITY

Pathogenicity is dependent on the interaction of both the infecting organism and the host defence mechanisms for production of symptoms, severity, limitation and eradication of infection. The development of gonorrhoea depends upon three stages: attachment of gonococci to the host mucosal surfaces, gonococcal penetration into the subepithelial tissue and lateral spread and dissemination of organisms. Gonorrhoea is a specific infection of humans and pathogenicity studies have been hampered by the lack of a suitable infection model.

1.0 Attachment

The importance of adherence of *N. gonorrhoeae* to human mucosa in the pathogenesis of gonorrhoea is not known. It is reasonable to expect that some form of anchoring would be required to prevent the gonococcoci being washed off the mucosal surfaces by urine and genital secretions. Microscopic examination of urethral exudate from men with symptoms of gonorrhoea for less than 24 hours demonstrated that gonococci appeared to be in very close contact with epithelial cells and that the microvilli of the epithelial cells appeared to wrap themselves round the gonococci (Ward and Watt, 1972). Similar observations were made in cervical biopsies taken from women with cervical gonorrhoea (Evans, 1977). Gonococcal-gonococcal adhesive interactions may also be important in maintaining multiplying cells at the mucosal surface.

Protein II and pili are the main gonococcal surface structures implicated in adhesion to host cells.

1.1 Pili in attachment

The exact role of pili in the pathogenesis of gonorrhoea is not clear. Pili have been demonstrated to adhere to a variety of human cell types such as epithelial cells, erythrocytes, spermatozoa and fallopian tubes in organ culture (Punsalong and Sawyer, 1973; James-Holmquest *et al.*, 1974; Ward *et al.*, 1974).

Gonococci produce a wide variety of different pilus types and these may bind differentially to different cell types (Lambden *et al.*, 1980). Binding may be mediated by specific ligand-receptor and/or by non-specific hydrophobic interactions (Lambden *et al.*, 1980). Recent structural analysis of gonococcal pili indicate that certain domains on the gonococcal pilus may be involved in these interactions (Schoolnik *et al.*, 1982; Virji and Heckels, 1984; Schoolnik *et al.*, 1984). However, observations that non-pilated gonococci of colony type 4 bind to fallopian tube mucosa and to different types of epithelial cells (McGee *et al.*, 1978; James *et al.*, 1980) indicate that factors other than pili are involved in the interaction of gonococci with the host cell surface. It has been postulated that the initial role of pili is to overcome the repulsive negative charges on the gonococcal and host cell surfaces (Heckels *et al.*, 1976). By overcoming repulsive negative charges, pilus-host cell non-specific hydrophobic interactions may come into play bringing the two cell types closer together. The closer association of gonococci and host cells may in turn allow specific pilus ligand-receptor interactions to come into force. The closer association of the organism to the host cell surface may allow other gonococcal surface structures such as protein II to come into effect. However, the relevance of pili in the pathogenesis of natural infections is not clear. Examination of gonococci in pus from patients with gonorrhoea have indicated that these organisms possess very few or no pili (Novotny, Short and Walker, 1975; Evans, 1977).

1.2 Protein II in attachment

The production of various protein II molecules has been associated with increased gonococcal binding to buccal epithelial cells, red blood cell and to polymorphonuclear leukocytes (PMNs) *in vitro* (Lambden *et al.*, 1979). Attachment experiments utilising different host cell lines, cultured tissues and gonococcal isogenic colony opacity/pilated variants indicated that certain proteins may bind specifically to particular host cell lines (James *et al.*, 1980). The attachment of a non-pilated opaque variant to human embryonic lung fibroblasts was shown to be equal to that of pilated transparent (lacking in protein II) variants. The transparent colony variants appeared to attach more to cultured cervical and fallopian tube tissue (James *et al.*, 1980). Gonococcal transparent variants are commonly isolated by laparoscopy from fallopian tubes and cul de sacs of patients with salpingitis (Draper *et al.*, 1980). Colony type 4 transparent variants lacking in protein II and pili have been shown to attach to fallopian tube mucosa in much greater numbers than pilated commensal neisseriæ (McGee *et al.*, 1980). Therefore it would appear that some other additional factor other than protein II and pili may also be involved in attachment.

2.0 Invasion

Invasion and damage of the mucosal surface are an integral part of the development of gonorrhoea. Without these two aspects gonococci would be little different from commensal bacteria found in the urogenital tract. In the classical study of gonorrhoea, Harkness (1948) observed that gonococci reached the subepithelial connective tissue on the third or fourth day post infection. Penetration was accompanied by capillary dilation with exudation of serum and cells. The dense cellular infiltrates

consisted of PMNS with small numbers of lymphocytes, plasma and mast cells. There was patchy involvement of the epithelium where the intercellular spaces were enlarged and contained large numbers of leukocytes. The epithelial cells in these patches were destroyed and desquamated to leave small areas in which the subepithelial connective tissue was completely exposed. PMNs, many containing gonococci, eventually found their way into the lumen of the urethra in large numbers. These together with serum and desquamated epithelial cells form the diffuse yellow discharge which is characteristic of gonorrhoea. Harkness (1948) described gonorrhoea in women to be similar to that in men. However the extent to which invasion and damage occurs may vary in patients with symptomatic and asymptomatic infections.

2.1 Penetration of the subepithelial tissue

Penetration studies have been carried out using mainly human fallopian tubes *in vitro*. Studies have shown that 3 to 8 h after challenge, gonococci had attached to columnar epithelial cells of fallopian tubes. By 24 h gonococci had penetrated the mucosal surface and foci of gonococci were visible in the subepithelial connective tissue (Ward *et al.*, 1974). Some mucosal cells contained very large numbers of gonococci and this suggested that intracellular multiplication occurs. The cytoplasmic membrane adjacent to adherent gonococci excluded collodial thorium suggesting that penetration did not occur by erosion of the cytoplasmic membrane (Ward *et al.*, 1974). Intercellular penetration also seemed unlikely since 8 h after challenge, by which time gonococci were visible intracellularly, the tight junctions between epithelial cells remained intact excluding electron dense colloids (Watt and Ward, 1977). Therefore it was proposed that the mechanism of penetration was phagocytosis of gonococci by

the host cell (Watt *et al.*, 1978). Later experiments (McGee *et al.*, 1983) showed that approximately 20 h after infection the microvilli of non-ciliated human fallopian tube mucosal cells to which gonococci were attached, began to retract pulling the gonococci to the surface of the cell. Other microvilli appeared to surround the gonococci and entrap them against the cell surface. The cell surface then retracted pulling the gonococci into a membrane-bound vesicle in a manner similar to phagocytosis of bacteria by PMN leukocytes. These phagocytic vesicles then appeared to be rapidly transported to the base of the cell. There the gonococci either multiplied with enlargement of vesicles and/or fusion of multiple vesicles occurred. Approximately 40 h post infection the vesicles appeared to rupture with release of gonococci into the subepithelial layers of the fallopian tubes. This appeared to occur by a process of exocytosis (McGee *et al.*, 1983). Infecting gonococci appeared viable at all stages.

2.2 Protein I

Gonococcal protein I has been shown to insert into red blood cell membranes. In other systems the disruption of membrane potentials by an inserting protein has been shown to trigger phagocytosis (Galin and Galin, 1977). Therefore protein I may play a similar role in the pathogenesis of gonorrhoea.

Invasion of the subepithelial tissue allows the gonococcus the chance to spread laterally within the submucosa, to enter the blood stream and possibly also the lymphatic system to cause complicated local gonococcal infections and DGI. Particular gonococcal strains have been associated with invasive infections. These generally have the characteristics of belonging to serogroup WI, have an AHU^- auxotype, are resistant to the bactericidal activity of normal human serum and are usually very sensitive to penicillin.

Women are particularly at risk of developing invasive gonococcal infections at the time of menses (Harriman *et al.*, 1982). Gonococcal transparent colonial variants are frequently isolated from women during this phase of the menstrual cycle (James and Swanson, 1978a).

2.3 Lack of invasion of subepithelial tissue in asymptomatic infections

Ward and Watt (1972) proposed that gonococci may be able to adhere to and multiply at the epithelial surface without invading the mucosal surface. Organisms able to do this may be responsible for asymptomatic infections. Evans (1977) observed that gonococci were primarily found on squamous epithelial cells rather than on columnar epithelial cells of the cervix. He suggested that gonococcal association with these cells which are quickly desquamated might also be a reason for asymptomatic infections in women (Evans, 1977). This association with non-columnar epithelial cells in women may account for the observations that over 60% of women have asymptomatic genital infections compared with approximately 10% of men. However asymptomatic infections may also be attributed to a lack of inflammatory response by the host towards particular gonococcal strains.

3.0 Inflammatory response

Penetration of the subepithelial connective tissue, the multiplication of gonococci and damage of the epithelial cells results in products which stimulate an intense inflammatory response.

3.1 Tissue damage

Epithelial cell damage may be mediated by the release of endotoxin (LPS) from multiplying gonococci and by gonococcal peptidoglycan. Endotoxin has been shown to be toxic for human fallopian tubes in cell

culture (McGee *et al.*, 1978; Gregg *et al.*, 1981; Melly, Gregg and McGee, 1981). Peptidoglycan also has been shown to cause toxic damage to fallopian tubes in organ culture (Rosenthal *et al.*, 1980). Damage to mucosal surfaces by endotoxin and peptidoglycan may also occur *in vivo*. Novotony *et al.* (1977) have reported that blebbing of the gonococcal envelope occurs *in vivo*. Tissue damage may also be mediated by products released from dead phagocytes.

The inflammatory response results in the infiltration of mononuclear (MN) and PMN leukocytes into the mucosal tissue.

3.2 Interaction of gonococci with phagocytes

Earlier studies indicated that non-pilated gonococci of colony types 3 and 4 in the presence of normal human sera (NHS) were ingested and killed by PMN leukocytes whereas pilated gonococci of colony types 1 and 2 attached to but were not ingested or killed by PMNs (Thongthai and Sawyer, 1973; Ofek, Beachy and Bisno, 1974; Dilworth, Hendly and Mandell, 1975). Novotony *et al.* (1977) examined the pus from men and women with acute gonococcal infections. They observed that clumps of gonococci were surrounded by cellular debris or granules which were of phagocytic, probably mononuclear leukocyte (MN), origin which they termed infectious units. They proposed the following model for the pathogenesis of gonorrhoea. The host is infected with free non-multiplying gonococci and multiplying gonococci within infectious units. Infectious units make contact with the epithelial cells and can be found intracellularly. Multiplying gonococci within the infectious units are presumbed to be highly leukotoxic and stimulate the infiltration of PMN and MN leukocytes. The free gonococci are rapidly phagocytosed. Those in PMNS are destroyed whereas those phagacytosed by MN leukocytes

multiply, break down the monocytes and are coated in the debris forming new infectious units. As the gonococci multiply in the infectious units the host cell components are used up and liberate free non-multiplying gonococci. These are then rephagocytosed by either PMN or MN leukocytes and the cycle is repeated (Novotny *et al*., 1977). They found no evidence that infectious units were formed with PMN leukocytes. However the cellular debris was shown to be of human origin using antisera raised against PMNs. Other workers in studies of urethral pus have shown that gonococci found within PMNs appear morphologically intact and some even appear to be in a state of division (Novotny *et al*., 1975; Ovcinnikov, Delektorskij and Dmitriev, 1976; Ovcinnikov and Delektorskij, 1977; Evans, 1977). Others have indicated that gonococci can survive or that there is a delay in killing by PMNS during *in vitro* experiments (Veale, Smith and Witt, 1975; Witt *et al*., 1976). Experiments conducted *in vitro* and in guinea pig chambers have indicated that although a large proportion of gonococci are killed by PMN and mononuclear phagocytes a proportion are able to survive and multiply within these host cells (Veale, Penn and Smith, 1978). These workers proposed that survival within PMN leukocytes may be important in acute infections whereas survival in the longer lived MN leukocytes may be important in the lateral spread of infection and in chronic gonococcal infections (Veale, Penn and Smith, 1977).

4.0 Gonococcal components involved in survival within PMN phagocytes

Some of the gonococcal surface structures may be involved in promoting protection from killing by PMN leukocytes.

4.1 Protein II

Analysis of the outer membrane vesicles isolated from *N. gonorrhoeae* BS4 which shows resistance to killing by PMNs and its isogenic variant which is sensitive to killing by PMNs have indicated the involvement of surface proteins in the resistance to phagocytic killing (Parsons *et al.*, 1982). Antiserum raised to whole gonococci resulted in promotion of phagocytosis and drastically reduced the ability of BS4 to survive within PMNs. Antisera raised to pili did not promote phagocytic killing of BS4. Antiserum absorbed with gonococcal outer membrane vesicles ceased to promote phagocytic killing (Parsons *et al.*, 1982). Analysis of the proteins in the outer membrane preparations isolated from BS4 and its isogenic variant have indicated the possible involvement of three proteins A, B and C in resistance to phagocyte killing. Protein A was classified as a species of protein II. Previous work has shown that the presence of protein IIb on variants of *N. gonorrhoeae* P9 results in decreased association of the organism with PMN phagocytes (Lambden *et al.*, 1979). Proteins B and C shared the characteristic of staining poorly with Coomassie blue on polyacrylamide gels (Parsons *et al.*, 1982). A number of minor gonococcal proteins which share this feature are now beginning to be characterised (Cannon *et al.*, 1984; Zak *et al.*, 1984; Virji *et al.*, 1984). The role of these proteins in gonococcal resistance to killing phagocytes remains to be elucidated.

4.2 Protein I

Protein IA molecules and protein I from patients with DGI, which usually have protein I of this type (Cannon *et al.*, 1983), are more efficient at inserting into lipid bilayers than those strains from localized infections. Treatment of PMN leukocytes with protein I appears to prevent polymorph

degranulation but has no effect on the morphology of these cells (Blake, M., presented at the 4th International Conference on Pathogenic Neisseria, Asilomar, California, October 1984). Therefore it is possible that gonococcal strains isolated from patients with DGI with a particular type of protein I molecule may be more effective at thwarting the activities of PMN leukocytes.

Protein I and II can be degraded by PMN phagolysosomal enzymes. Protein II molecules are more susceptible to these enzymes than protein I (Eaton and Rest, 1983). Therefore the role of protein I may be in preventing engulfment by PMNs. However, once engulfed, protein I may aid intracellular survival by preventing the release of PMN enzymes.

An increase in the degree of peptidoglycan cross-linkage has been shown to increase the sensitivity of *N. gonorrhoeae* FAI9 to O_2-independent killing by human PMN leukocyte mixed or isolated azurophilic granule extracts (Daly *et al.*, 1982). This was demonstrated by the increased sensitivity of FAI9 when containing the *penA* $_2$ and *mtr* mutations which increase peptidoglycan cross-linkage. Gonococcal strains containing these mutations are seldom isolated from patients with DGI. However, much more evidence is required to ascertain the exact role played by these structures in survival within phagocytes.

4.3 Leukotaxic activity produced by gonococci

Neisseria gonorrhoeae of colony types 1, 2 and 3 have been shown to produce a soluble chemotaxin which stimulates the migration of PMN leukocytes towards gonococcal cells producing this component (Rank and Holmes, 1984). Chemotactic activity was reduced after treatment with trypsin, pronase, aminoglucosidase and lipase suggesting that the factor was a glycopeptide. The molecular weight of the molecule was estimated to be between 10,000 and 12,000 daltons. This factor

stimulated chemotaxis in the absence of serum factors. James and Williams (1978) previously reported serum-independant PMN chemotaxis stimulated by cell-free culture filtrates of 13 out of 16 clinical gonococcal isolates. They suggested that chemotactic activity might be generated by gonococcal cell lysis products (James and Williams, 1978).

Gonococcal strains producing this low molecular weight glycopeptide chemotactic factor stimulate the inflammatory response and may be more prevalent in patients with symptomatic gonorrhoea. Gonococcal strains not producing this factor may possibly be associated with asymptomatic infections. Although T1, T2 and T3 colonial variants produced a chemotaxin (James and Williams, 1978; Rank and Holmes, 1984), differences in production between opaque and transparent variants were not investigated (Rank and Holmes, 1984).

5.0 The role of complement in polymorph chemotaxis and killing of gonococci

Invasion of the genital tract mucosal surface early in infection (Harkness, 1948) brings the gonococcus into contact with plasma. IgG and complement components are also found in cervical secretions (Price and Boettcher, 1979) and may constitute a primary defence system. Activation of the complement system results in the generation of powerful leukotaxins. In addition the activation of the early components of complement aids opsonisation of bacteria while the sequential activation of the terminal components results in the formation of the membrane attack complex C5b-9 which kills bacteria. Complement can be activated by the classical pathway (involving antibody antigen complexes and C1, C4 and C2) or by the alternate pathway or by both.

5.1 Complement and the inflammatory response

Watt and Medlen (1978) suggested that the acute inflammatory response induced in localised gonococcal infections was in part mediated by the interaction of invading gonococci with natural antibody and complement. These interactions generate chemotaxins which attract phagocytes to the site of infection.

Gonococci, isolated from uncomplicated and disseminated infections, incubated with normal human serum for 60 minutes stimulated both complement consumption and PMN migration to a similar degree (Densen, McKeen and Clark, 1980, 1982). Chemotactic activity was generated by the complement cleavage product, C5a. However, when time related complement consumption studies in NHS were undertaken, it was shown that there was a delay in the generation of chemotactic activity by gonococci isolated from patients with UGI. When hypogammaglobulinaemic serum was used isolates from UGI showed similar kinetics for chemotactic generation. In C2 deficient serum there was a specific impairment of chemotactic activity generated by UGI but not DGI strains (Densen *et al.*, 1980, 1982). These differences suggested that although gonococcal isolates activate complement by the classical pathway strains from patients with DGI preferentially activate the alternate pathway (Densen *et al.*, 1980, 1982). Schiller (1980) has also demonstrated that this occurs: studies on the susceptibility of gonococci to phagocytosis and killing by PMNs in serum deficient in C8 indicated that gonococci from patients with UGI were killed to a greater extent and this appeared to correlate with the rapidity of chemotactic factor generation in serum by particular strains. Strains isolated from DGI appeared to preferentially activate the alternate complement pathway (Schiller, 1980). The ability of *N. gonorrhoeae* to activate the alternate pathway and initiate a local Schwarzman response in rabbits has been reported by other workers (Sveen and Maeland, 1982).

5.2 Gonococcal surface components involved in the stimulation of complement

It has been reported that the porins of *S. typhimurium* with molecular weights of 34 K and 36 K stimulate complement activity via the classical pathway (Galdiero *et al.*, 1984). Gonococcal cell envelope components may also be involved in the activation of complement and the inflammatory response. The molecular weight of protein I (porin) has been shown to be associated with serum sensitivity and resistance of gonococcal strains (James *et al.*, 1982). Gonococcal strains with low molecular weight protein I were more resistant to the bactericidal activity of NHS. Transparent isogenic colonial variants (lacking in protein II) were also shown to be more resistant to bactericidal activity (James *et al.*, 1982). Transparent gonococcal strains are generally isolated from the urethra of men with asymptomatic infections (Tight and James, cited by James *et al.*, 1982) and from the cervices of women at the time of menstruation and women with salpingitis (Draper *et al.*, 1980; James and Swanson, 1978a,b).

Gonococci with low molecular weight protein I of IA subclass have been associated with DGI (Cannon *et al.*, 1983). Moreover, most patients who develop DGI have asymptomatic local gonococcal infections. Therefore, it would appear that the surface protein make up of gonococci isolated from these infections may be directly involved in their ability to disseminate. It is tempting to hypothesize that these structures interfere with the serum bactericidal activity and with complement activation, thereby slowing down the production of chemotaxins and affecting the production of an inflammatory response. This may allow these organisms to disseminate more easily by evading the host's primary defence mechanism.

Endotoxin also stimulates the complement system. Therefore the endotoxin released in the form of blebs from gonococci in pus (Novotny *et al.*, 1977) may also have a stimulatory effect on the inflammatory process. However, Novotny *et al.* (1977) only examined the exudate from patients, mostly men and a few women, with symptomatic infections. Evans (1977), who examined cervical biopses from women, reported that gonococci did not produce blebs of endotoxin. Therefore under certain circumstances *in vivo* gonococcal endotoxin blebbing may be inhibited. A recent case report of an inflammatory skin response to urethral exudate contaminating a male patient's thigh was thought to be mediated by gonococcal endotoxin present in the exudate (Fiumara and Kahn, 1982). Gonococcal peptidoglycan has also been shown to stimulate complement consumption in NHS (Peterson and Rosenthal, 1982).

The inflammatory response and opsonisation of *N. gonorrhoeae* in women may be affected by seminal plasma (Brooks *et al.*, 1981). It has been shown that the bactericidal activity of serum and the opsonisation, phagocytosis and killing of gonococci was inhibited by seminal plasma. The addition of seminal plasma to the serum resulted in a 500-fold reduction in bactericidal activity. The inhibitory effect of the seminal plasma component was lost by heating at 100°C for 10 minutes. The component had a low molecular weight (<20,000 daltons) and was thought to be a protein, possibly a protease or protease-inhibitor acting on the complement activation and hence opsonisation and killing of gonococci (Brooks *et al.*, 1981).

5.3 The role of complement in systemic gonococcal infections

The role of complement in serum bactericidal activity has been shown to be important in preventing systemic neisserial infections (Lim

et al., 1976; Petersen, Graham and Brooks, 1976; Lee *et al.*, 1978). Patients with hereditary deficiencies in the complement system resulting in the lack of the terminal components C6, C7 or C8 are susceptible to repeated attacks of bacteraemic neisserial infections. These patients have normal opsonisation and PMN chemotaxis functions but lack serum bactericidal functions produced by the terminal complement membrane attack complex (Lee *et al.*, 1978).

6.0 Natural antibody and immune antibody response to gonococcal infections

Natural gonococcal bactericidal antibody is present in the serum of most normal adults and arises without obvious immunisation or specific infection (Schoolnik, Buchanan and Holmes, 1976). These antibodies may arise due to pharyngeal carriage of non-gonococcal neisseriae. Natural antibody of IgG, IgM and IgA classes reactive with gonococcal heat-labile and heat-stable antigens were detected by an indirect immuno-fluorescence test (Cohen, 1967).

6.1 Natural IgG: blocking and opsonising

McCutchan *et al.* (1978) examined the mechanism by which *N. gonorrhoeae* isolated from cases of DGI resisted the bactericidal activity of NHS. They concluded that NHS contains natural blocking IgG. They proposed that natural IgG-blocking antibody binds to gonococci possessing the appropriate receptor and protects them from killing by bactericidal antibody and complement by preventing bactericidal antibody binding to the gonococcal cells (McCutchan *et al.*, 1978). Natural IgG has been ascribed the role of a heat-stable opsonin promoting phagocytosis of gonococci by human PMN leukocytes (Schiller, Friedman and Roberts, 1979).

The targets for natural IgG have been investigated. Natural IgG in NHS acting as an opsonin inhibited the attachment of monoclonal antibodies to protein I (Sarafian, Tam and Morse, 1983). However, the natural IgG did not appear to react with protein III.

Natural IgG antibodies present on the mucosal surface of the urogenital tract and acting as opsonins may promote phagocytosis of gonococci prior to the development of a specific host antibody response. As such it may constitute a part of the host's early defence mechanisms prior to the development of a specific antibody response.

6.2 Natural IgM and serum bactericidal activity

Natural IgM in conjunction with complement is responsible for the bactericidal activity of normal human sera (Schoolnik, Ochs and Buchanan, 1979; Rice, McCormack and Kasper, 1980). The role of natural IgM was demonstrated by the loss of bactericidal activity upon treating NHS and IgM with the reducing agent 2-mercapthoethanol, the lack of bactericidal activity in cord serum, and the lack of bactericidal activity in the serum of patients who lacked IgM due to agammaglobulinemia (Schoolnik *et al.*, 1979).

The development of natural antibody is age dependent attaining adult levels at approximately two years of age (Schoolnik *et al.*, 1979). The bactericidal activity of NHS does not vary with differences in ABO blood groups or sex of the donor (Schoolnik *et al.*, 1976; Schoolnik *et al.*, 1979). Rice and Kasper (1977) showed that the bactericidal activity of NHS could be inhibited by gonococcal endotoxin but not by gonococcal protein. It was proposed that natural IgM reacts with lipopolysaccharide (Schoolnik *et al.*, 1979). Gonococcal strains isolated from DGI are resistant to the serum bactericidal activity of NHS (Schoolnik *et al.*, 1976).

However, Rice *et al.* (1980) have reported that gonococcal strains from patients with DGI may vary in their susceptibility to the bactericidal activity of NHS and that there are degrees of resistance. Strains isolated from patients with suppurative arthritis were significantly more sensitive to the bactericidal activity of NHS than gonococcal isolates from patients with dermatitis and arthralgia. Gonococcal strains isolated from women with severe PID were more susceptible to bactericidal activity than those isolated from mild or moderate cases of PID (Rice *et al.*, 1980). These observations, although subjective, may reflect differences in the target antigens for natural IgM and/or different responses of the individual hosts.

6.3 Mechanism of resistance to NHS

Serum resistance is determined by the *sac* genes. These genes alter the structure of gonococcal LPS (Shafer *et al.*, 1982). Recent evidence shows that the mechanisms by which different gonococci resist the bactericidal activity may be different and depend upon the type of LPS they produce (Shafer *et al.*, 1984). In addition to activating the classical complement pathway in the presence of antibody it was proposed that a serum-sensitive mutant RA5100 may also activate the classical pathway without the presence of antibody. The ability of RA5100 LPS to inhibit the killing of its homologous and a heterologous strain by NHS suggested that a common antigenic site on the LPS of serum sensitive gonococci may be involved (Shafer *et al.*, 1984). Results also indicated that the parent strain of RA5100, FA19 which is serum resistant, may also contain this antigenic site but that bactericidal antibody may not be able to bind to it. This may be due to differences in the conformation of the LPS or to an increase in the number of receptor sites made

available to blocking IgG. This may result in a greater degree of IgG blocking antibody binding to the LPS of serum-resistant strains inhibiting the binding of natural IgM by steric hindrance. Other workers have proposed that serum-resistance may be due to conformational changes in LPS rendering the complement membrane attack complex C5b-9 ineffectual (Harriman *et al.*, 1982). These workers showed that C5b-9 complex bound to the surface of both serum sensitive and resistant gonococci but was unable to penetrate the membrane and kill serum resistant gonococci. It has been reported that gonococci isolated from urethral pus and examined without subculture are resistant to the bactericidal activity of NHS (Ward, Watt and Glynn, 1970). This ability to resist bactericidal activity was lost upon subculture and is different from the stable serum resistance shown by strains isolated from DGI. It has been demonstrated that cervical secretions and seminal plasma from uninfected clinically healthy persons and vaginal exudates from women with non-gonococcal infections converted serum-sensitive gonococci to serum resistance after three-hour incubation at 37°C (Martin *et al.*, 1982). The chemical nature of the serum resistance inducer is not known. The acquired serum resistance was lost upon subculture (Martin *et al.*, 1982).

7.0 Local antibody response

IgA antigonococcal antibodies were found in the urethral secretions of 9/11 men with first and 20/24 men with a history of multiple gonococcal infections (Kearns *et al.*, 1973a). These antibodies were shown to be predominately of the secretory type (Kearns *et al.*, 1973b). The antibody response to *N. gonorrhoeae* in the urogenital tract of men and women has been examined (McMillan *et al.*, 1979a; McMillan, McNeillage

and Young, 1979b). Antigonococcal antibody was detected in 97% of 75 women with gonorrhoea. These antibodies were predominately of IgG and IgA class. IgM was detected in 29 women and was associated with infections of less than 15 days duration (McMillan *et al.*, 1979a). IgA appeared to be mainly secretory IgA. Antigonococcal IgG, but not IgA or IgM, was found in 33% of women with either non-gonococcal cervicitis or with no sign of any infection. Successful treatment resulted in the rapid decline of IgA and a gradual decrease in IgG reactivity (McMillan *et al.*, 1979a). An increase in the levels of IgA and IgG in cervical secretions of women with cervical infections has been previously reported (Chipperfield and Evans, 1976). Similar findings were reported for men with gonococcal urethritis (McMillan *et al.*, 1979b). Of 132 men with gonorrhoea, 129 had antibodies to the gonococcus in their urethral secretions. IgA was found in all 129, IgG in 119 and IgM in 64 of the men. IgG reactive with *N. gonorrhoeae* was found in 26% of 50 men with non-gonococcal urethritis but none was found in men with no sign of infection. The levels of IgA and IgM declined rapidly whereas IgG levels declined gradually after successful treatment (McMillan *et al.*, 1979b). Therefore a measurable antibody response at the genital mucosa can be detected. Antibodies reactive with gonococcal antigens can also be detected on extra-genital surfaces (McMillan, Young and McNeillage, 1980).

The finding of antigonococcal IgA and IgG in the secretions of 12 women who had no evidence of infection but were contacts of men with gonorrhoea (McMillan *et al.*, 1979a) suggests that an antibody response produced at the mucosal surface may be involved in limiting and eradicating infection. The gonococcal components which antibody reacted was not determined. In women using oral contraception (oestrogen/

progesterone pill) the levels, particularly of IgA and IgG but also of IgM in cervical secretions, is higher than in those women not taking oral contraceptives regardless of whether they had a current cervical infection (Chipperfield and Evans, 1976). In patients with cervical infections the increase in IgA levels was thought to involve increased production of secretory IgA at the mucosal surface whereas in women taking oral contraceptives the increase in IgG and IgA was thought to be a result of increased transudation of immunoglobulins into cervical mucus. Results also indicated that there was a variation in the response produced by each individual. McChesney *et al*. (1982) showed that an antibody response occurred on the genital secretions of men and women vaccinated with a pilus vaccine. Although immunoglobulin of all three classes was detected, secretory IgA predominated. However, *N. gonorrhoeae* is able to render secretory IgA of the A1 subclass ineffective. It produces an extracellular proteolytic enzyme (IgA protease) which cleaves IgA_1 at proline-threonine residues in the hinge region separating the molecule into its Fab and Fc components (Plaut *et al*., 1975).

8.0 Humoral antibody response to gonococcal infections

Various studies have indicated that humoral antibody responses may vary in different gonococcal syndromes and antibody produced detects different gonococcal antigens (Rice *et al*., 1980; Hadfield and Glynn, 1982; Hook, Olsen and Buchanan, 1984). Rice *et al*. (1980) showed that among patients with DGI, 91% with suppurative arthritis developed bactericidal antibody in the convalescent phase compared with only 18% of those with dermatitis and arthralgia. Some patients with uncomplicated gonococcal infections also produced a serum

bactericidal response (Rice *et al.*, 1980). Kearns *et al.* (1973a) found that men with uncomplicated gonococcal infections were much more likely to produce a humoral antibody response if they had a history of repeated gonococcal infection.

In one study using SDS-PAGE derived antigen in an ELISA it was demonstrated that humoral IgG from patients with uncomplicated gonococcal infections detected protein II and carbohydrate, possibly LPS, antigens (Hadfield and Glynn, 1982). Hadfield and Glynn (1982) also demonstrated that humoral IgG from patients with DGI detected protein I and pili antigens. In another study of patients with DGI, PID and gonococcal epididymitis it was shown that there was a mixed response to gonococcal LPS and protein I (Hook *et al.*, 1984). IgG from four patients reacted with protein I alone, IgG from one patient reacted with LPS and IgG from eight patients reacted with both protein I and LPS. Results also indicated that in the mixed response antibody to both LPS and protein I may be bactericidal for *N. gonorrhoeae*.

Buchanan *et al.* (1980) demonstrated that antibody produced can be protective against recurrence of complicated gonococcal infections. Women with pelvic inflammatory disease (PID) who produced an antibody response to protein I were protected from subsequent attacks of PID with gonococci of the same protein I serotype. However, these antibodies were unable to protect women from repeated local genital infection by strains with homologous protein I serotypes.

9.0 Cell mediated immunity

The existence of cell-mediated immune response in humans infected with *N. gonorrhoeae* has been reported (Esquenazi and Streitfeld, 1973; Wyle, Rowlett and Blumenthal, 1977; Kraus, Perkins and Geller, 1970;

Landolfo *et al.*, 1981). Cell mediated responses were found more frequently in patients with a history of multiple infections with greatest blastogenic transformation in those with three or more gonococcal infections (Kraus *et al.*, 1970; Landolfo *et al.*, 1981). Stimulation of a cell mediated response appeared to be generated by a non-pilus 23,000 MWt protein (Landolfo *et al.*, 1981). It is possible that this protein might represent a protein II species. Wyle *et al.* (1977) also indicated a blastogenic response to a partially purified gonococcal antigen. Peripheral lymphocytes from women with uncomplicated gonorrhoea responded better to this protein mixture than those from men with uncomplicated infections. A wide range in degree of lymphocyte response in men and women was noted (Wyle *et al.*, 1977).

10.0 Gonococcal antigenic shift in response to host immune response

Recent studies involving gonococcal protein II and pili indicate that antigenic shift may occur in natural infections.

The ability to select gonococcal strains with different protein II molecules and different pilus types *in vitro* (Lambden *et al.*, 1979; Lambden *et al.*, 1980; Salit *et al.*, 1980) suggested that the potential existed *in vivo* for similar structural and antigenic changes in protein II and pili. It was shown that variants of *N. gonorrhoeae* P9 inoculated into guinea pig subcutaneous chambers changed their protein II composition from protein II a, b and c to predominately protein II d, e and f (McBride *et al.*, 1981). The pilus composition was altered similarly *in vivo* from the original inoculum containing α and β pili to γ and δ pili (McBride *et al.*, 1981; Lambden *et al.*, 1981b). The protein II and pilus composition of clinical isolates of *N. gonorrhoeae* isolated from the urethras of male patients and from the cervix and urethra of their female contacts were

examined. On the basis of identical protein I serotyping and auxotyping results all isolates from each set of sexual partners were determined to be the same strain (Zak *et al.*, 1984; Duckworth *et al.*, 1983). It was found that pili expressed by isolates from the female cervix and urethra differed in subunit molecular weight and were usually distinct from the pili expressed by the isolate from the male partner, although some men had the same pilus banding pattern as their female contacts (Duckworth *et al.*, 1983). In most cases a single pilus type predominated in each isolate.

However, the urethral and cervical isolates from one woman showed more than one pilus band on SDS-PAGE. This was possibly due to infection with variants of the same strain expressing different pilus types. No correlation could be made with menstrual cycle as all women were taking oral contraceptives (Duckworth *et al.*, 1983). Similar variations in protein II expressed by different isolates were also observed (Duckworth *et al.*, 1983). Antibodies to protein II and pili in the serum of patients were then examined (Zak *et al.*, 1984). However, only 33% of patients examined produced antibody to a protein species other than protein I. Antibodies to pili when present, gave cross-reactions among isolates from patients within each contact group but to a variable extent.

Serological studies using sera obtained from patients indicated that different protein II molecules could be detected within contact groups (Zak *et al.*, 1984). Thus in one group of patients, a male and his two female contacts, serum from female 1 reacted with a 31K protein II from her urethral isolate and a 29K protein II molecule from the cervical isolate of female 2. The serum failed to react with a 29.5K protein II produced by her own cervical isolate or with a 32K protein II

produced by the isolate from her male partner. It was noted that although this women's serum reacted with the 29K protein II produced by the isolate from female 2, neither her own urethral or cervical isolate produced this protein. The isolate from the male patient did not produce this protein either (Zak *et al.*, 1984). This suggests that when woman 1 was initially infected her isolate did produce a 29K protein II and that her male consort was also infected at one time with an isolate producing this protein II species.

A model of antigenic shift has been proposed. An infecting gonococcal strain has the capacity to produce isogenic variants containing one or more possible protein II molecules. A specific host immune response to protein II produced by these variants would result in the elimination of variants bearing that particular protein II molecule. The high rate of transition associated with protein II production would ensure the presence of a significant minority population with an antigenically distinct protein II profile which would then overgrow the original variant to establish a new majority population.

11.0 Summary

Although the host is capable of mounting both antibody and cell mediated immune responses, the ability of these to eradicate and prevent gonococcal infections appears variable. The ability of the host to produce an immune response seems to depend upon the number of exposures to the gonococcus. It is quite likely that most patients are treated before an adequate immune response can be generated. However, in complicated gonococcal infections there may be some protection against reinfection with the same strain. The host immune response also appears to be directed against a bewildering array of gonococcal antigens and

varies from patient to patient. The gonococcus in turn appears to have a formidable battery of virulence determinants which enable it to evade the host immune response and is thus a very successful host specific pathogen.

The variable immune response, lack of protection against reinfection and the large number of gonococcal antigens makes the selection of an antigen(s) for a gonococcal vaccine very difficult.

LABORATORY PROCEDURES FOR THE DIAGNOSIS OF GONORRHOEA

The clinical diagnosis of gonorrhoea must be supported by microbiological tests. Because of the short incubation period and high infectivity of the disease, rapid diagnosis followed by immediate treatment is important in the control of infection within the community.

N. gonorrhoeae is a very fastidious organism with poor survival outside of its host. Therefore very careful techniques are required for the collection of specimens and their transport to the laboratory for culture and investigation. Ideally, the patient should be seen at a clinic with an adjacent or closely situated laboratory. However, in many areas, particularly the United States, a great number of cases are seen by general practitioners and not in sexually transmitted disease (STD) clinics.

1.0 Immediate diagnosis

Immediate diagnosis is extremely important as it enables a presumptive diagnosis to be made in the clinic so that appropriate treatment can be given without delay. This is especially important for prevention of spread of infection, to prevent the progression of the disease to its more serious sequelae and for treatment of those patients who default and therefore do not attend for follow-up treatment.

1.1 The gram-stain

Gram-staining of genital secretions remains the only widely accepted routine procedure for making an 'on-the-spot' diagnosis of gonococcal infections (Young, 1981).

1.1.1 Gram-stained smear results from men

The gram-stain is a sensitive and specific test for preliminary diagnosis of urethral gonorrhoea in men. A positive or negative gram-stained smear of urethral discharge has been reported to provide an immediate differential diagnosis between gonococcal and non-gonococcal urethritis in 85 to 89.2% of cases (Jacobs and Kraus, 1975; Oxtoby *et al.*, 1982). Others report its sensitivity as being higher. Goodhart *et al.* (1982) reported that the probability of gonorrhoea in men with urethral discharge and/or dysuria whose urethral smears contain intracellular GNDC is 94.8%. Absence of intracellular GNDC from smears of the same men is associated with a 92.6% probability that they have non-gonococcal urethritis. One group reported that the sensitivity of the gram-stain was as high as 99.1% for a group of 113 symptomatic male patients (Luciano and Grubin, 1980).

However, others have observed that the sensitivity of the gram-stain is lower (40-60%) in asymptomatic men with culture proven gonococcal infections (Handsfield *et al.*, 1974).

1.1.2 Gram-stained smear results from women

For women gram-staining of smears of cervical and urethral secretions has been reported to be less reliable, detecting only 55 to 65% of patients who subsequently gave positive cultures (Chipperfield and Catterall, 1976; Barlow *et al.*, 1976; Evans, 1976). The presence of intracellular GNDC in cervical smears from women was associated with a 97.3% probability that they had gonorrhoea. Whereas the absence of intracellular GNDC in cervical smears was associated with a 51.2% probability that these women did not have gonorrhoea (Goodhart *et al.*, 1982).

Lossick, Smeltzer and Curran (1982) reported that the cervical gram-stain detected 70% (1,179 of 1,675) of women who subsequently had positive cultures. It was also reported in a recent survey of clinics in England and Wales that overall 71% of women were diagnosed initially by the gram-stain (Belsey, 1983). However, in some clinics, particularly in London, this figure was much lower. With the addition of epidemiological data, 91% (Lossick *et al.*, 1982) and 96% (Belsey, 1983) of women with culture proven gonococcal infections were treated on their initial visit to the clinic or after the first set of microbiological tests. Lossick *et al.* (1982) reported that the addition of gram-staining increased by 42% the number of women treated for gonorrhoea at their first visit compared with diagnosis based solely on epidemiological and clinical data.

However, initiation of treatment in patients with negative smear results and diagnosed solely on epidemiological grounds without awaiting culture results must lead to unnecessary treatment with antibiotics.

Belsey (1983), in a study of 2,903 women diagnosed or treated as having gonorrhoea, showed that the initial diagnosis of gonorrhoea was confirmed by gram-stain or culture in 2,420 (83%) of cases prior to administering treatment, 35 women were treated on clinical grounds alone and 448 were treated on epidemiological grounds alone. Only 161 (36%) of the 448 women treated on epidemiological grounds alone subsequently had positive culture results. This gives overtreatment rates of 10% for all treated women and 64% for women treated solely on epidemiological grounds alone. However, Lossick *et al.* (1982) observed that treating a greater number of women at their initial clinic visit brought about a decrease in the number who subsequently developed PID.

Others have reported that the sensitivity of cervical gram-stained smears based on the presence of intracellular GNDC is very low (32%) (Oxtoby *et al.*, 1982).

1.2 Limitations of gram-staining

Gram-staining has no place in the diagnosis of pharyngeal infections in either sex and rectal smears are seldom carried out routinely (Young, 1981). Non-gonococcal neisseriae are common in the naso-pharynx and may also grow on selective media. In one study 74.2% and 3.7% of GNDC found in throat cultures yielded *N. meningitidis* and *N. lactamica* respectively. Only 20.3% of the cultures yielding GNDC were shown to contain gonococci (Young and Bain, 1983).

McMillan and Young (1979) found that the routine examination of gram-stained rectal material obtained by protoscopy from homosexual men was of limited diagnostic value.

The performance of the gram-stained smear is also affected by the experience of the technical staff involved in screening. Goodhart *et al.* (1982) found that the efficiency of the gram-stain fell drastically when smears were interpreted by an inexperienced technician.

1.3 Simplification of staining methods

There has been some move to evaluate the use of single stain procedures. Stains such as methylene blue (Danielsson, 1965; Danielsson and Johannisson, 1973), methylgreen-pyronin (Hanna *et al.*, 1980) and safranin (Oxtoby *et al.*, 1982) have been used. However, Oxtoby *et al.* (1982) reported no obvious advantage in these procedures. The time saved in staining with a single stain was lost by making the screening of smears much more difficult.

1.4 Immediate immunofluorescence

Unfortunately fluorescent antibody (FA) staining of secretions direct from the patient has not provided a more sensitive and reliable routine diagnostic technique than gram-staining.

Danielsson and Forsum (1975) in a detailed review on the methodology and application of immunofluorescence for the diagnosis of neisserial infections, state that direct FA staining of uro-genital smears can provide diagnostic results which equal culture results for gonorrhoea within one hour. However, this is dependent on well-trained and experienced technicians. They did not recommend the use of this technique on a routine basis because it was laborious and requires a highly skilled technologist at the clinic. However they found it useful under certain circumstances, for example in patients who have received antibiotics and whose cultures might be anticipated to be negative. Use of FA in the diagnosis of gonococci in skin lesions of patients with DGI has been successfully used by Bayer (1980, cited by O'Brien *et al.*, 1983).

2.0 Culture

The importance of selective media is now established beyond doubt; over 70% of laboratories in England and Wales now use some form of selective media (Adler, 1978). There are three criteria which should be met by selective media: they should prevent overgrowth of gonococcal colonies by commensal flora; they should prevent the growth of any other microbial species which may inhibit gonococcal growth; and they should also promote luxuriant gonococcal growth within 24-48 h. Most selective media contain a rich nutrient base supplemented with blood, partially lysed by heat (chocolate agar) or completely lysed by the chemical saponin. There are two main selective media used: Thayer-Martin and New York City medium, and modifications of these two.

2.1 Thayer-Martin and modified Thayer-Martin medium

The first selective medium formulated to grow only gonococci and meningococci was developed by Thayer and Martin (1964, 1966). This medium contains the antibiotics vancomycin, colistin and nystatin. However, it did not prevent cultures being overgrown with *Proteus* species, nystatin at the concentrations used was not an effective inhibitor of *Candida albicans*, and the growth of gonococci was slow and the colonies were small.

2.1.1 Modified Thayer-Martin medium

Superior culture results were obtained with modified Thayer-Martin (MTM) medium (Martin, Armstrong and Smith, 1974). MTM differed from TM medium in containing an increased agar concentration (2%), additional glucose (0.25%) and trimethoprim (5 µg/ml). When cervical specimens were cultured in both media, 122/328 (37%) were positive on MTM compared with only 110/328 (33.5%) on TM medium.

Trimethoprin has been shown to inhibit the growth of *Proteus* species (Seth, 1970). MTM completely eliminated spreading growth by *Proteus*, whereas 23 (9.5%) of the TM cultures (four male urethral and 19 female cervical cultures) were overgrown with spreading *Proteus* (Martin *et al.*, 1974). In another modification of TM medium nystatin was substituted by anisomycin in an effort to control the growth of yeasts on the culture plates (Martin and Lewis, 1977). The enhanced inhibition of yeasts on Martin-Lewis medium has been reported to make screening of plates for presence or absence of gonococcal colonies much easier (Smeltzer, Curran and Lossick, 1979).

2.2 New York City and modified New York City medium

In 1973 a new medium, New York City (NYC) medium, which provided luxuriant growth of pathogenic neisseriae after incubation for 24 hours was described (Faur *et al.*, 1973a; 1973b). NYC medium consists essentially of a proteose peptone-corn starch, buffered agar base to which a haemoglobin solution prepared from fresh horse erythrocytes, horse plasma, yeast dialysate, glucose and the antibiotics vancomycin,colistin, amphotericin B and trimethoprim lactate are added.

2.2.1 Modified New York City medium

The NYC medium as described by Faur *et al.* (1973a,b) is inconvenient for many service laboratories to prepare routinely and it was modified by Young (1978a). The MNYC medium is prepared from commercially available gonococcal base and contains lysed whole blood in the place of plasma and haemoglobin made from fresh horse erythrocytes and is therefore much simpler to prepare. Vancomycin was replaced by lincomycin (1.0 µg/ml) (Young, 1978a), and amphotericin B is used in both NYC and MNYC since it has a much greater inhibitory effect on *Candida*. MNYC was not compared directly with NYC but in a trial with routinely used TM medium it was shown to considerably increase both the number of culture positive specimens and the rapidity of growth of gonococcal isolates (Young, 1978a). Faur and Wilson (1982) reported that the use of lincomycin resulted in gross contamination of 22-34% anorectal cultures with the subsequent loss of 3.8% of gonococcal isolates. They recommended that the use of vancomycin should be continued.

Most of these media are available commercially for laboratories who do not wish to prepare their own. In a survey to compare commercially available NYC and Martin-Lewis media, it was found that the NYC was much superior (Lawton and Koch, 1982).

2.3 Combination of selective and non-selective medium

Recently, gonococcal strains with increased susceptibility (hypersensitive) to a variety of antibiotics have been discovered (Eistenstein and Sparling, 1977). Gonococcal strains which are also sensitive to vancomycin at the levels used in selective media (2-4 µg/ml) have been reported to account for approximately 1% to 30% of clinical isolates (Windall *et al.*, 1980; Goodhart *et al.*, 1982; Mirret, Reller and Knapp, 1981). Mirret *et al.* (1981) have associated sensitivity to vancomycin and to other antibiotics including lincomycin and penicillin with strains of the auxotype AHU^-. They found that 28/31 strains which failed to grow on TM and ML media containing 4 µg/ml of vancomycin had this auxotype compared with 9/31 control strains. These discoveries lend credence to the suggestions that both selective and non-selective media should be routinely used for isolation of *N. gonorrhoeae* (Reyn and Bentzon, 1972).

Such a procedure is too time consuming, technically demanding and not cost effective for routine use, and in practice less than 10% of clinics in England and Wales employ such a routine (Adler, 1978). However, in those areas where there is a high proprotion of antibiotic sensitive gonococcal strains such a procedure may well be required.

In Britain vancomycin sensitivity does not appear to pose a problem. However, a cautionary note was sounded by Talbot, Spencer and Kinghorn (1983). They found that in Sheffield three (3.9%) of 79 isolates were sensitive to vancomycin; one of these isolates was a penicillinase-producing strain (Talbot *et al.*, 1983).

It was shown in a study using split plates that there was no advantage in using both non-selective and selective media (vancomycin free selective medium [VFSM] and MTM medium) for the isolation of

N. gonorrhoeae (Bonin, Tanino and Handsfield, 1984). Because of the reduced rate of culture contamination it was found that MTM and VFSM actually increased the gonococcal isolation rate by 1.6% and 1.9% respectively, when compared with the non-selective 'chocolate' agar plates. Therefore they concluded that the routine use of non-selective plates was not cost effective or more efficient as it did not give better culture results and considerably more time was required for screening plates after incubation. However, they previously reported that the number of vancomycin sensitive strains in the Seattle area was low (Totten *et al.*, 1983), and while the routine use of non-selective media was not required in their situation, they recognised the need for each individual laboratory to assess their own isolation and cultural procedures.

2.4 Assessment of cultural systems

This may be done by checking smear results with culture results. If the culture negative/smear positive ratio becomes large, the cultural procedures being used should be reevaluated. Occasionally, studies should be undertaken to assess the proportion of antibiotic sensitive gonococcal strains within the population.

If laboratories prepare their own media the quality of the medium should also be under constant surveillance.

2.5 Other factors which may affect cultural results

Other factors such as sampling error due to insufficient inoculum or poor plating technique, failure to pre-warm plates, a long delay before plates are placed in a CO_2 incubator, and taking samples from only one site per patient have all been suggested or implicated in reducing the sensitivity of culture results (Belsey, 1983; Goodhart *et al.*, 1982).

2.5.1 Sampling

Poor sampling can occasionally be checked by instigating a periodic double-sampling procedure whereby two swabs per site are inoculated onto one culture plate or by inoculating one swab onto the two halves of one plate. Bonin *et al*. (1984) increased their gonococcal isolation rate by 1.7% by inoculating male urethral specimens (one swab per patient) onto the two halves of split plates and by inoculating two cervical specimens per female patient onto one culture plate. As this was a cost effective way of increasing culture sensitivity they instigated it as a routine procedure.

Gartner and Edwards (1984) used a triplate in an effort to reduce the cost of taking samples from genital, pharyngeal and rectal sites. Their results showed that there was no significant difference in isolation rate when all three sites were cultured on a triplate compared with conventional isolation on three separate plates. This may be a useful way of sampling all three sites for clinics and laboratories which have a small budget and limited incubator space. Sampling at all sites may be more important in particular groups of patients. It has been reported that of homosexual men with gonorrhoea, the gonococcus is isolated in the rectum alone in 60.5% of cases and in the pharynx alone in 18.4% of cases (Owen and Hill, 1972). Therefore if urethral specimens were taken alone a total of 78.4% of gonococcal infections in homosexual men would be missed.

2.5.2 Culture plate temperature

It is thought that refrigerated plates for the isolation of clinical specimens should be brought to room temperature by removing from the refrigerator 30 min to 1 hour prior to inoculation. However, there

are logistical problems associated in having a source of high quality plates readily available at room temperature and clinics require to have a refrigerator. One study examined this problem and showed that there was no significant difference in the isolation rates of *N. gonorrhoeae* on plates inoculated straight from the refrigerator and on those warmed to room temperature (Ratner *et al.*, 1985). However, the gonococcal colonies tended to be larger and more numerous on the room temperature plates after 24 h growth than on the refrigerated plates. Growth was essentially the same on both plates after 48 h incubation.

3.0 Transport and culture systems

When direct plating and immediate incubation is impracticable, several transport and culture systems are available in addition to the conventional non-nutrient transport media such as Stuart's (1946), or Amies' modification of Stuart's medium (Amies, 1967).

The transport and culture systems utilise a selective medium usually present in a small chamber containing CO_2.

3.1 Transgrow

Transgrow was the first combined transport and culture system to be described (Martin and Lester, 1971). It consists of a flat-faced bottle containing MTM medium and a carbon dioxide enriched atmosphere. However, incubation results in the production of condensation on the glass making it difficult or impossible to examine the cultures. Therefore blind sampling is required for the identification of growth of gonococci for all identification techniques including the delayed FA method (Jephcott, Morton and Turner, 1974).

3.2 Jembec/Neigon system

The problems associated with Transgrow are overcome in the Jembec/Neigon system which is based on the biological environment chamber described by Martin and Jackson (1975). It consists of a rectangular culture plate containing a selective medium (usually MTM medium). The plates incorporate a small recess into which a CO_2-generating tablet is placed at the time of inoculation. The moisture of the plate ensures that CO_2 is generated owing to the reaction between the citric acid and sodium bicarbonate in the tablet. The closed plate is placed into a plastic bag which is sealed to make it gas tight. This system offers easy, direct access to the surface of the culture medium so that suspect colonies can be further identified.

These systems are available commercially in one form or another. In addition dehydrated media (MTM) have also been incorporated into a kit (Microcult GC). These kits offer the advantage of a long shelf life and may be particularly useful in areas or countries which lack laboratory facilities (Willcox and John, 1976; Unsworth, Talsania and Philips, 1979).

3.3 Factors affecting isolation rates following transport of specimens

A number of factors affect the efficiency of cultures which have been transported to the laboratory. The longer the delay in transport, including holding at the clinic and transit time, the greater the decrease in viability. The conditions in which the specimens are held prior to transport to the laboratory are also important. Spence, Guzick and Katta (1983) compared the isolation of *N. gonorrhoeae* in three culture transport systems; inoculation directly on to MTM medium, the placement of specimens into modified Stuart's transport medium (Culturette)

and inoculation into Transgrow bottles. The specimens inoculated onto the MTM plates (in candle extinction-jars) and in Stuart's media were held at room temperature and transported to the hospital laboratory within 3 hours (h). The specimens inoculated into Transgrow bottles were first incubated in the hospital and were then sent to the Maryland State Health Department Laboratory, usually the following morning or within 72 hours if the specimen was collected on a Friday. The sensitivities for Transgrow, Culturette and MTM medium were 63.9%, 83.6% and 87.0% respectively (Spence *et al.*, 1983). They estimated that ~20% of patients with gonorrhoea would not have been diagnosed using Transgrow and sending the specimens 'off-site' for processing. Although they expressed reservations about using the Transgrow system, the role of Transgrow medium and delay in transport were not evaluated separately. Spence *et al.* (1983) also examined the efficacy of 'on-site' processing compared to that of sending specimens to the State laboratory for processing. They recommended that where feasible, an on-site laboratory should be used for the evaluation of clinical specimens in an effort to increase both the sensitivity of culture and the speed by which results were made available to the clinicians.

In another study in Singapore it was stated that the preferred method of isolation of *N. gonorrhoeae* was direct inoculation of the specimen onto selective medium when it is collected (Sng *et al.*, 1982). They found that specimens inoculated into Amies transport medium lost viability after holding for more than 3 h. This loss of viability was particularly pronounced when the specimens in Amies were held at room temperature: in areas such as Singapore the ambient air temperature is greater than 26°C. However, specimens inoculated directly onto MTM medium and held in a CO_2-enriched environment could be held at

room temperature for 18-24 h prior to incubation without significant loss of viability. The loss of viability was more pronounced in isolates from women and in the case of PPNG. This study highlighted the fact that isolation rates are dependent on inoculum size and that the inoculum can be further reduced by using a non-cultural transport medium such as Stuart's or Amies (Sng *et al*., 1982).

The loss of viability in modified Stuart's medium was confirmed by other workers (Ebright *et al*., 1982). They showed that modified Stuart's medium (Culturette, Marion Scientific Corporation) was acceptable when the transport time was ≤3 h. However the rates of isolation were found to be only 60% and 27% for 24 h and 48 h of transport respectively.

Overall, 49.1% of STD clinics in England and Wales send specimens to the laboratory in transport media - 87% used Stuart's and 13% used Amies (Adler *et al*., 1978).

It is concluded that in areas or countries where there are few laboratory facilities and in other countries where laboratory services tend to be centralised some form of transport and culture system is required. Culture of *N. gonorrhoeae* remains very important particularly for determining antibiotic sensitivities. Some of the transport and culture systems mentioned here may be of value. However, each area must determine the best policy for transport of specimens, taking into account clinic and laboratory facilities, ambient temperature, transport time and cost effectiveness.

4.0 Identification of isolates

After 24 h of incubation the culture plates are examined and identity of colonies suspected of containing gonococci are confirmed by a variety of methods. Tests which are easy to carry out (reducing operator error), and are sensitive and specific are the obvious goal.

4.1 The cytochromeoxidase test

The oxidase test is the first test used to screen primary plates. All neisseriae are oxidase positive whereas many other organisms, such as coliforms, staphylococci and lactobacilli, are negative. The test can be easily carried out by touching a part of the suspect colony with a swab soaked in a 1% (w/v) solution of tetramethyl-p-phenylenediamine dihydrochloride, or alternatively by flooding the plate with the oxidase reagent. A positive reaction is indicated by the colony turning purple within five to 15 seconds.

4.2 Gram-stained smear from colony

Because other species such as *Kingella*, *Moraxella* and *Haemophilus* are also oxidase-positive, oxidase-positive colonies must be gram-stained and smears examined for gram-negative diplococci (GNDC). A presumptive diagnosis of gonorrhoea made on the basis of oxidase-positive GNDC growing on selective medium is approximately 98% accurate for specimens taken from a genito-urinary site (Young, 1981). However, because the various *Neisseria* spp. are indistinguishable by microscopy, further identification is necessary to provide a precise diagnosis for infection at all sites but particularly in determining infection of the pharynx and rectum.

4.3 Biochemical tests

A number of new tests have recently been developed for the identification of *Neisseria*. However the most common biochemical tests in use are carbohydrate utilisation tests.

4.3.1 *Carbohydrate utilisation tests*

These tests are based on the differences in the ability of *Neisseria* spp. to utilise various sugars. Gonococci produce acid from glucose only, meningococci produce acid from glucose and maltose, whereas *N. lactamica* utilises lactose in addition to glucose and maltose. Carbohydrate utilisation tests are basically of two types, conventional and rapid.

4.3.2 *Conventional sugar tests*

In conventional tests a solid or semi-solid medium containing the appropriate carbohydrate and pH indicator is inoculated with the test organism. Although widely used these tests are unsuitable since a positive reaction is dependent on adequate growth of the test organism which may require incubation for up to 72 h (Pollock, 1976). The main problem with these tests is finding a suitable medium to support good growth of all strains of gonococci while at the same time giving reproducible and clear-cut indicator changes. Faur *et al*. (1975), in a comparison of a plate carbohydrate utilisation system (based on NYC medium) with the widely used semi-solid cystic-trypticase agar base (CTA) containing carbohydrates, noted that the CTA system often gave equivocal results because of inadequate growth of fastidious strains of pathogenic *Neisseria*. Commercially available MNYC carbohydrate medium was recently shown to be much more accurate and reliable than CTA medium or the Minitek system (impregnated discs with biochemicals to which Mueller Hinton broth is added) (Simms and Lue, 1982).

Recently Knapp and Holmes (1983) developed a semi-solid medium which they termed modified oxidation-fermentation (MOF) medium. This contains a low percentage of proteose peptone (low protein level), buffer,

carbohydrates and phenol red indicator. It proved much more reliable than any of CTA media in determining carbohydrate utilisation by *Neisseria* spp. However inoculated tubes require a minimum of 4 h and up to a maximum of 24 h. incubation to obtain results.

4.3.3 Rapid carbohydrate utilisation tests

Rapid carbohydrate utilisation tests (RCUT) were pioneered by Kellogg and Turner (1973). These tests are based upon the measurement of preformed enzyme by adding growth from an overnight culture of suspect organism to a buffered (non-nutrient) solution containing the sugar to be tested and a pH indicator. Since preformed enzyme is being measured the system does not require growth of the organism and the problems of supporting the growth of fastidious isolates is overcome. These organisms can be grown in a suitable medium first. Brown (1974) reported that a number of laboratories were unable to obtain comparable results to those of Kellogg and Turner (1973). However, this system was modified by altering the buffer composition (Young, Paterson and McDonald, 1976). Results obtained with this modified RCUT system were reproducible and easy to read. Positive tests are yellow or sometimes orange/yellow, whereas negative tests remain red. The test itself is simple to perform and very rapid, results usually being available within one hour. When MNYC medium is used the RCUT can be carried out directly from the primary isolation plate enabling the vast majority of isolates to be identified within 24 h of seeing the patient (Young, 1978b).

For those laboratories which may have problems with the buffered solution for RCUT and for those laboratories which do not routinely isolate gonococci, the RCUT system as described by Young *et al*. (1976)

is available commercially (Oxoid, Basingstoke). This test consists of discs impregnated with the buffer, pH indicator and the sugar to be tested. The discs are placed in tubes and are reconstituted with distilled water and RCUTs are carried out as per normal.

4.3.4 *Measurement of preformed enzymes and enzymes produced during growth*

Yong and Prytula (1978) described a modified RCUT which utilised both preformed enzymes and enzymes formed by the gonococci as a result of growth in a super enriched medium. The combined action of two sources of enzyme probably make this RCUT system suitable for very small inocula. However, as it requires growth of the test organism it is unlikely that it could be used to characterise isolates directly from primary isolation cultures because of possible contamination by other bacteria.

4.4 Other biochemical tests

A number of neisserial identification systems are now available commercially. These are based on a variety of biochemical tests with or without sugar utilisation being incorporated. Two such systems are RapID NH (Innovative Diagnostics Systems Inc) and Gonochek (E-Y Laboratories).

4.4.1 *RapID NH*

The RapID NH is a 4 h test designed to differentiate *Neisseria* spp., *Branhamella*, *Moraxella* and *Haemophilus* spp. and is based on a variety of conventional rapid sugar tests and single-substrate chromogenic biochemical tests. It consists of a plastic tray with 10 wells containing dehydrated reagents. These are rehydrated and a suspension

of the test organism is added to each well. In addition to sugar utilisation, tests such as β-D-galactosidase, aminopeptidase, indole production, urea hydrolysis and phosphate and nitrate degradation are included. In one study (Robinson and Oberhofer, 1983) it was found that there were considerable problems associated with the use of this system. *B. catarrhalis* could not be distinguished from species of the gram-negative cocco-bacilli *Moraxella* and *Kingella*; 44% of gonococcal isolates tested did not utilise glucose and the other tests had to be relied upon to give definitive identification (Robinson and Oberhofer, 1983). Similar problems were found with isolates of *N. meningitidis*. For non-pathogenic *Neisseria* the level of accurate identification varied. One strain of *N. subflava* was mis-identified as a pathogenic *Neisseria*.

4.4.2 Gonochek

Gonochek is designed to differentiate *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica* and *B. catarrhalis*. It is based upon the degradation of three substrates contained in one tube. The enzymes produced by *N. gonorrhoeae*, *N. meningitidis* and *N. lactamica* are proline aminopeptidase, γ-glutamylamino peptidase and β-galactosidase respectively. *B. catarrhalis* does not produce any of these enzymes.

Oxidase positive GNDC are added to the reconstituted tubes which are then incubated at 35°C for 30 min. The colour of the tubes are then read. A blue colour designates a *N. lactamica*, a yellow colour designates *N. meningitidis*. If there is no colour development a solution (EY 20) is added. If the tube subsequently turns red the isolate is a gonococcus. If no colour develops after the addition of EY 20 then the isolate is *B. catarrhalis*.

In a comparison of Gonochek with RapID NH and the RCUT system of Young *et al.* (1976), it was found that Gonochek compared well with RCUT provided that the tubes were read carefully for colour production prior to the addition of the EY20 reagent (Welburn, Uyeda, Ellison-Birang, 1984). The addition of EY20 to tubes which had developed a slight blue colour would result in a false positive red colour and the organism being falsely identified as a gonococcus. The RapID NH was not recommended.

Wood and Young (1985) in a small study compared Gonochek with RCUT and the Phadebact Gonococcus (coagglutination) test. The overall correlation between Gonachek and RCUT (97.5%) and the Phadebact coagglutination test and RCUT (96.9%) was virtually identical. However, these authors found that some meningococci produced very low levels of γ-glutamyl aminopeptidase and very little or no yellow colour developed. This resulted in the misidentification of three strains, two as *N. gonorrhoeae* and one as *B. catarrhalis*, on first testing from primary culture plates. After 24 h subculture these strains were correctly identified. Gonochek persistently misidentified one gonococcal strain as *B. catarrhalis*.

Gonochek correctly identified the five *N. lactamica* tested whereas the Phadebact test gave positive results with three of these strains (Wood and Young, 1985).

Prior to the use of commercially available and new biochemical tests for identification of *N. gonorrhoeae*, these should be rigorously evaluated for specificity and sensitivity in relation to conventional methods of identification. In some instances such tests may be useful but the cost may prove prohibitive and reliability prove unacceptable.

5.0 Immunological identification

The requirement of smaller amounts of growth and the possibility of shortening the period required for identifying *N. gonorrhoeae* coupled with early difficulties with culture and carbohydrate utilisation tests made the development of immunological identification tests very attractive.

5.1 Delayed immunofluorescence

Delayed immunofluorescent antibody (FA) tests can be used to test colonies direct from the primary isolation plate. Danielsson and Forsum (1975) stated that FA was a useful test for this purpose. However, they stressed that because of the risk of cross-reactions with other *Neisseria* spp. it should always be used in conjunction with conventional diagnostic tests. However cross-reactions with meningococci can be reduced or eliminated by absorption of the conjugate with strains of *N. meningitidis*. Lind (1975) recommended that this technique should not be used for the identification of throat cultures in which there is an increased risk of cross-reactions with other neisseriae such as *N. lactamica*.

A suitable fluorescence microscope and technical expertise are also required for the FA tests to be carried out.

5.2 Identification by coagglutination

The basis of coagglutination (CoA) has already been described (page 73) elsewhere.

Coagglutination was first used as a method for typing pneumococci (Kronvall, 1973) and was adapted for confirmatory diagnosis of gonococci by Danielsson and Kronvall (1974). They used specific antigonococcal sera attached to whole protein A containing staphylococcal cells. The

antiserum was absorbed with meningococci and *Moraxella* strains to remove unwanted cross-reactions. The CoA test gave concordant results with carbohydrate utilisation and FA tests in routine identification of *N. gonorrhoeae* (Danielsson and Kronvall, 1974).

5.2.1 The Phadebact gonococcus test

CoA test kits are available commercially as Phadebact gonococcus test (Pharmacia Diagnostics AB, Uppsala, Sweden). Tests are carried out by making a suspension of test GNDC in distilled water and boiling for 10 min. A drop of this suspension is added to one drop of the Phadebact test suspension and to one drop of the control reagent (normal rabbit serum attached to protein A containing whole staphylococci). The suspensions are mixed and slides rocked for two minutes and examined for agglutination visible to the eye: the addition of 1% methylene blue makes viewing easier.

Young and McMillan (1982) compared the rapidity and reliability of coagglutination (CoA) and FA staining for the identification of gonococci cultured on MNYC medium. Sensitivity of CoA was 96.7% compared with 97.9% for FA tests, whereas specificity of both methods was 96.8%. Fewer (86.9%) isolates could be identified by CoA after 24 h culture compared with 93.9% using the FA method (Young and McMillan, 1982). Seven gonococcal isolates gave false negative results on first testing but were positive on repeat testing and four meningococci gave false positive results but were subsequently negative on repeat testing. However similar problems are associated with the FA method. It was concluded that the specificity and sensitivity of the test was acceptable for the immunological identification of gonococcal strains from urogenital specimens from men and women. However because of the risk of

misidentifying *N. meningitidis* as a gonococcus all ano-rectal isolates from homosexual men and pharyngeal isolates in general should always be confirmed by carbohydrate tests. Similar conclusions have been made by other workers. Carlson *et al.* (1982) found that the Phadebact CoA test had an overall sensitive of ~92.6%: 13 gonococci gave initial negative results but upon retesting nine were positive and four (1.7% overall) remained negative. They found no cross-reactions with non-gonococcal neisseriae and concluded that the high sensitivity and specifity of the test made it useful for the rapid identification of *N. gonorrhoeae* (Carlson *et al.*, 1982). Lim and Wall (1980) found that the Phadebact CoA test correctly identified 97.8% (269/275) of gonococcal isolates while 93.9% (31/33) of other bacteria including non-gonococcal neisseriae, gave negative tests. The two strains which gave false positive results were gram-positive cocci and it was concluded that if the CoA test had been used in conjunction with the Gram stain no false positives would have occurred (Lim and Wall, 1980). They concluded that the Phadebact gonococcus test was able to provide rapid and accurate confirmatory identification of *N. gonorrhoeae*.

Shanker, Daley and Sorrell (1981) found that the Phadebact test performed better than Difco flourescent antibody test. They found that 287/298 gonococcal isolates were correctly identified by CoA, a sensitivity of 96%. False positive results due to cross-reactions with non-gonococcal neisseriae were rare (1/18) and the test had a specificity of 94% (Shanker *et al.*, 1981). However they found that interpretation of the test to be difficult initially and had to repeat 32 (10%) of the tests. Whereas Anand and Kadis (1980) found that nine of 19 *N. lactamica* were positive with the Phadebact test. Johnston (1981) who prepared her own CoA reagents (polyvalent antigonococcal serum

absorbed with *N. meningitidis* and *Moraxella*) found that her CoA identified 98.6% of the gonococcal isolates correctly. Two strains of *N. meningitidis* gave cross-reactions.

5.2.2 *Gonococcal autoagglutination*

The CoA tests are best carried out using fresh cultures of *N. gonorrhoeae* in boiled suspensions to prevent autoagglutination (Lewis and Martin, 1980; Izakson and Morse, 1981). Autoagglutinating strains results in agglutination with both the test and control reagents. A number of workers have attempted to modify the test to prevent auto-agglutination. Johnston (1981) found that heating gonococcal suspension allowed reading to be obtained with some autoagglutinable strains but not others. A number of enzymes were tested including trypsin, pronase and *Streptomyces* enzyme. It was found that *Streptomyces* enzyme allowed most stains to give clear results (Johnston, 1981). Other workers have reported that the use of Mg^{2+} and Ca^{2+} (which are responsible for the integrity of the outer membranes) chelators such as EDTA enhanced the Phadebact gonococcus test (Izakson and Morse, 1981). Doyle *et al.* (1984) appeared to be able to prevent gonococcal autoagglutination by treating gonococci with DNase.

5.2.3 *Availability of gonococcal monoclonal antibodies*

The problems associated with the occasional cross-reaction observed between CoA reagents and non-gonococcal neisseriae may be alleviated with the advent of monoclonal antibodies to *N. gonorrhoeae* (Tam *et al.*, 1982). CoA reagents for the identification of *N. gonorrhoeae* prepared with monoclonal antibodies are now commercially available and await wide-scale evaluation.

However despite some of the problems associated with CoA, a large number of laboratories are using this technique for the confirmatory identification of *N. gonorrhoeae* because it is quick, easily handled, lends itself to screening of a large number of isolates, and on the whole gives results which are easily interpreted.

6.0 Identification of gonococcal isolates with lectins

Lectins, proteins which react with carbohydrates, have been used in a rapid slide agglutination test for the confirmatory identification of *N. gonorrhoeae* (Schaeffer, Keller and Doyle, 1979; Curtis and Slack, 1981; Doyle *et al.*, 1984; Yajko, Chu and Hadley, 1984). It was found that all gonococci tested could be agglutinated by peanut, soyabean, ricin and wheatgerm lectin in low concentrations (2.5 - 0.5 µg/ml of lectin) (Allen, Connelly and Apicella, 1980).

6.1 Gonococcal components which react with lectins

Allen *et al.* (1980) used lectins to obtain structural information about the polysaccharide components in the cell envelope of gonococci. They found that lectins reacted with gonococcal LPS and Gc acidic polysaccharides. However, the specificities of the lectins were different. The lectins generally reacted with β-D-acetylglucosamine or galactose residues on the gonococcal LPS.

6.2 Evaluation of gonococcal identification by lectins

Schaeffer *et al.* (1979) and Curtis and Slack (1981) evaluated the use of wheatgerm (WG) lectin.

Suspensions of neisserial isolates were mixed with various dilutions of WG lectin (500 µg/ml to 2 µg/ml) on glass slides. Slides were agitated for 10 min and then examined for visible agglutination.

Schaeffer *et al.* (1979) found that WG lectin at a concentration of 62 µg/ml agglutinated 99% of 165 gonococcal strains tested. Only one out of 24 meningococcal strains gave a positive reaction with the lectin. However, Curtis and Slack (1981) found that only 94.6% (159/168) gonococcal strains agglutinated with WG lectin at this concentration. Of the nine strains which failed to agglutinate five gave positive results on repeat testing. Positive reactions with this concentration of lectin were observed with 21 (21.9%) of 96 meningococcal and one of two *N. lactamica* strains. They found that non-groupable meningococcal strains were responsible for the majority of these positive reactions (Curtis and Slack, 1981). WG lectin was previously reported to agglutinate many strains of non-groupable meningococci (Frasch, 1980). Curtis and Slack (1981) postulated that the composition of the culture medium and the age of the cultures might affect the composition of the polysaccharide capsule of these strains and hence their agglutination by WG lectin. Frasch (1980) found that meningococcal capsular material blocked the N-acetyl glucosamine receptors for WG lectin and prevented agglutination of all *N. meningitidis* and *N. gonorrhoeae* tested.

Hence lectin agglutination is not a suitable technique for the identification of pharyngeal gonococcal isolates.

6.3. Use of lectin agglutination in conjunction with other tests

Recently lectin agglutination tests have been used in conjunction with chromogenic peptidase assays for the identification of *N. gonorrhoeae* (Doyle *et al.*, 1984; Yajko *et al.*, 1984).

However both groups observed similar problems of meningococcal agglutination with lectins and definitive identification had to be made with the chromogenic tests. As simple chromogenic tests for peptidase

and carbohydrate degradation are available (EY Laboratories) it would appear that there is no advantage to be gained by using lectins.

7.0 Non-cultural identification of *N. gonorrhoeae*

The non-cultural detection of *N. gonorrhoeae* is based upon detection of gonococcal components in secretions. The need for such a system is apparent because of the number of clinics which have to rely on culture results from specimens which have been transported. The major problem inherent in transportation of specimens is the loss of gonococcal viability. A number of assay systems have been developed for the detection of gonococcal components. Because of the lack of sensitivity of gram staining in female patients any new non-cultural diagnostic method is of greater potential value in the diagnosis of gonorrhoea in women than in men.

7.1 *Limulus* amoebocyte assay

The *Limulus* amoebocyte assay detects endotoxin. It is based on the initial finding that endotoxin from gram-negative bacteria reacted to form a gel with a lysate of washed amoebocytes from the horseshoe crab, *Limulus polyphemus* (Levin and Bang, 1964). Following the demonstration by Rice and Kasper (1977) of the sensitivity of the limulus endotoxin assay for components of *N. gonorrhoeae*, the system has been shown to be of value in the rapid presumptive diagnosis of gonococcal urethritis in men (Spagna, Prior and Perkins, 1979). Endotoxin was detected in urethral exudates from 73 patients with culture-positive gonococcal urethritis while negative results were obtained from 26 out of 27 patients with negative urethral cultures. Consequently, the authors suggested that the method may also be of value in identifying cases of

non-gonococcal urethritis (Spagna *et al.*, 1979). However the test is not specific and depends on the absence of other bacteria producing endotoxin in amounts that might produce a positive result. Selection of an appropriate specimen dilution is also vitally important.

To simplify and standardise the dilution process, Prior and Spagna (1981) described a closed system by which specimens could be collected and diluted using a syringe combined with a dilution reservoir containing the required amount of pyrogen-free water. Using this method to test specimens from men with exudative urethritis, it was shown that the test had a sensitivity of 99.2% and a specificity of 96.7%. The overall ability of the test to predict culture results was 98.4%. This compared well with the sensitivity and predictive value of gram-staining. They concluded that the *Limulus* amoeboycte tests was an accurate presumptive diagnostic test for gonococcal and non-gonococcal urethritis in men presenting with symptoms of urethritis, and that it would enable the private practitioner to make a diagnosis within 30 min without the need of gram-staining, thus enabling the correct treatment to be given at the patient's initial visit (Prior and Spagna, 1981).

7.1.1 The use of the Limulus test for gonococcal infections in women

In a study of 66 women attending a STD clinic it was found that the *Limulus* amoebocyte test detected 15 (62.5%) of 24 women with gonorrhoea confirmed by gram-stained smear and/or culture (Young, Sarafian and McMillan, 1981). Secretions were used at a 1/100 dilution. A dilution of 1/50 was used to retest the nine patients with confirmed gonorrhoea and of these two gave positive results, increasing the positivity rate of the test to 17 (70.8%) of 24 patients. Only four (9.5%) of the 42 patients without any microbiological evidence of gonorrhoea gave positive

results with the *Limulus* lysate test. However, the positivity rate of the *Limulus* lysate test (62.5%) was less sensitive than gram-staining which detected 75% of infected women. The usual level of sensitivity for the gram-stain is 55-65% and thus the *Limulus* lysate assay fell within this range (Young *et al.*, 1981). It was suggested that poor sampling and the effect of endotoxin-producing commensal flora in the female genital tract may affect the results obtained with cervical specimens in the *Limulus* lysate assay. In another study the *Limulus* lysate assay detected 16 (84.2%) of 19 patients with cervical gonorrhoea and in three patients (17.6%) with no microbiological evidence of gonorrhoea (Young *et al.*, 1983).

Spagna, Prior and Perkins (1980) evaluated the *Limulus* lysate assay for diagnosis of gonococcal cervicitis in 40 women presenting with vaginal discharge and/or abdominal pain. They found with a 1/800 dilution of cervical specimens that 17/18 (94%) of women with culture proven cervical gonorrhoea gave positive *Limulus* results. The *Limulus* lysate assay was negative for the 22 women with non-gonoccal cervicitis. The *Limulus* lysate assay was considered to be much better than gram-staining which detected 11/18 (61%) culture positive women and quick and easy to perform.

7.2 Detection of gonococcal components by enzyme-linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assays (ELISA) are being used widely not only for research but also for routine detection of microbial antigens and antibodies.

ELISA tests are based upon the attachment of antigen to a solid phase either to polystyrene balls or to the wells of microtitre plates

(Figure 8). The antigen is detected by the addition of specific antisera, or patients serum, which are in turn detected by specific enzyme conjugated immunoglobulins. The addition of an enzyme substrate results in colour development which is measured spectrophotometrically: the absorbance reading obtained allows quantitation of the assay and the calculation of appropriate cut-off points.

A number of laboratories have attempted to develop their own ELISA system although a commercial system (Gonozyme, Abbot Diagnostics) is now available for the detection of gonococcal antigens in genito-urinary specimens.

Sarafian and Young (1982) used polystyrene beads armed with mouse antiserum raised against whole untreated cells of *N. gonorrhoeae* strain 9 to capture antigen; rabbit antiserum raised to the same strain was used to detect the captured antigen. In a small study the use of this ELISA assay was assessed for detection of gonococcal components in the cervical and vaginal washes from 37 women (Young *et al.*, 1983). Positive results were obtained from 12 (60%) of the 20 patients with gonorrhoea. Two false positives (11.7%) were detected from the 17 patients with no microbiological evidence of gonorrhoea.

A number of groups have evaluated the Gonozyme ELISA system (Abbot Diagnostic Laboratories) (Aardoom *et al.*, 1982; Papasian, Bartholomew and Amsterdam, 1984; Stamm *et al.*, 1984). The Gonozyme assay consists of beads 'pretreated' to aid antigen capture, rabbit antibody to *N. gonorrhoeae* (obtained by injecting different strains into rabbits) and an anti-rabbit IgG-peroxidase conjugate.

Recent studies have shown overall sensitivities and specificities of 94-100% and 95.8-100% respectively for urethral samples from men. The test was less effective for specimens from women with sensitivities

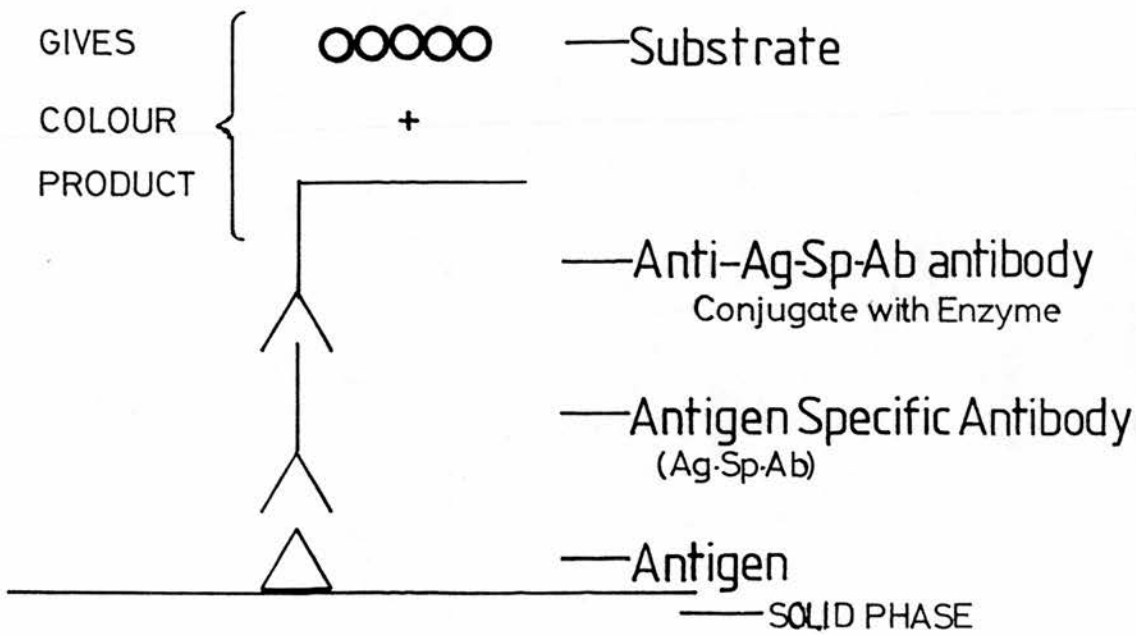

FIGURE 8: Schematic representation of the indirect sandwich ELISA.

and specificities of 78-91.7% and 87.2-98% (Aardoom *et al.*, 1982; Papasian *et al.*, 1984; Stamm *et al.*, 1984).

7.3 Detection of *N. gonorrhoeae* in clinical specimens by means of genetic transformation and DNA hybridisation

Transformation of gonococci as a means of detecting gonococcal DNA in clinical specimens was investigated by Janik, Juni and Heym (1976). The detection of gonococcal DNA was based upon the transformation of auxotrophic mutants (indicator strains) of *N. gonorrhoeae* to prototrophy by crude DNA preparations obtained from clinical specimens. The recipient auxotrophic mutant (Strain 488) which gave greatest specificity required uracil and arginine for growth. *Proteus* and *C. albicans* and other clinical isolates of gram-positive and gram-negative organisms gave negative results when used as a source of donor DNA. The assay is extremely sensitive, requiring a minimum of approximately 50 colony forming units (cfu) of donor gonococci to give a positive transformation assay.

Positive transformation assay results were obtained with 90 specimens confirmed as containing *N. gonorrhoeae* by conventional methods (Janik *et al.*, 1976). However, 39 clinical specimens in which there was no microbiological evidence of *N. gonorrhoeae* gave positive transformation results. According to Janik *et al.* (1976), examination of clinical and epidemiological data for these specimens correlated with the positive transformation assay findings.

However, the main problem associated with this technique is that clinical isolates of *N. gonorrhoeae* may be auxotrophic. The proportion of these auxotrophic strains, particularly those requiring AHU, in various areas varies from 8-57% (Knapp *et al.*, 1978). In addition, AHU^- auxotrophs are associated with asymptomatic gonococcal infections in men (Crawford *et al.*, 1977).

In a comparison of a number of non-cultural detection methods for gonococcal components in cervical and urethral aspirates it was found that 15 (75%) of 20 patients with gonorrhoea gave positive transformation results. Of the 17 patients with no microbiological evidence of gonorrhoea, two (11.7%) gave a positive transformation result (Young *et al.*, 1983). One of these patients was a contact of gonorrhoea. However, this method was not considered to be simple, rapid or sensitive.

7.3.1 DNA hybridisation

The detection of gonococcal DNA in urethral exudates by DNA-hybridisation using the gonococcal cryptic plasmid as a radiolabelled probe was evaluated by Totten *et al.* (1983).

Clinical specimens hybridised to the radiolabelled DNA probe were bound to nitrocellulose, resulting in a dark spot being produced on X-ray film. The test was extremely sensitive requiring only 100 cfu of *N. gonorrhoeae* and 0.1 pg of gonococcal cryptic plasmid DNA to give a positive result. The test was also very specific, only two non-gonococcal neisseria species *N. mucosa* and *N. cinerea* gave positive results.

Tests with clinical specimens from men with urethritis indicated that 63 (89%) of 71 patients who had positive cultures were also positive by the DNA hybridisation technique. All 42 patients with negative culture results were also negative by the hybridisation assay. To obtain the above sensitivity, nitrocellulose filters required to be auto-radiographed for three days. However, in the majority of cases a positive result could be detected after 24 h (Totten *et al.*, 1983). As DNA hybridisation techniques are improved by replacing radioactive probes with enzyme conjugated probes this technique may prove of greater value in diagnosis.

The major problem associated with non-cultural detection of *N. gonorrhoeae* is lack of information concerning antibiotic sensitivities of strains and whether or not they produce penicillinase. DNA hybridisation techniques could possibly be adapted to incorporate detection of β-lactamase plasmids. Despite this problem some form of non-cultural detection is important given the problems of loss of viability of the gonococcus in transport systems.

8.0 Antibiotic sensitivity testing

Antibiotic sensitivity testing requires the culture of gonococcal isolates. Tests have to be carried out on pure cultures and therefore results may not be available for several days. Hence antibiotic sensitivity tests are generally retrospective since most patients will have been treated on the basis of gram-stained smear results. However, they are required for epidemiological purposes and for rational therapy regimens for use in the geographical area concerned (Jackson and Jephcott, 1976). The World Health Organisation recommended in 1978 that standardisation of gonococcal penicillin sensitivity testing should be instigated around the world. It was considered that standard reference strains, standardisation of media used and a specific methodology were required. Three standard reference strains WHO III, V and VII for penicillin sensitivity testing are available (WHO, 1978). However, most laboratories use disc or agar dilution procedures with media and inocula to suit their individual needs and preferences. Therefore the desired standardisation of antibiotic sensitivity tests required for accurate comparative assessment of sensitivities in different geographical areas has not been achieved. Only laboratories throughout Australia have standardized penicillin sensitivity testing with the result that trends in penicillin resistance

can be assessed accurately from area to area and nationally (Members of the Australian Gonococcal Surveillance Programme, 1984).

Since the discovery of PPNG in 1976 (Phillips, 1976; Ashford *et al.*, 1976) which have now established themselves among the endemic gonococcal population (McCutchan *et al.*, 1982) it is vital that rapid routine sensitivity testing to penicillin is carried out.

8.1 Detection of penicillinase-producing gonococci

Gonococci which are less sensitive or relatively resistant to penicillin are generally considered to have an MIC of ≥0.06 µg/ml (Sparling, 1977). Such strains must be tested for penicillinase production. A simple disc technique can be used to screen for such isolates which can then be tested for penicillinase production by one of the several tests available (WHO, 1978).

One of these tests depends upon the hydrolysis by penicillinase of the lactam bond of a chromogenic cephalosporin substrate giving rise to a coloured product (O'Callaghan *et al.*, 1972). Other tests depend upon the bacterial breakdown of penicillin to penicilloic acid which is detected by the ability of penicilloic acid to dissociate a starch-iodine complex (Odugbemi, Hafiz and McEntegart, 1977) or by a pH indicator system (Phillips, Aller and Cohen, 1976). The later system has been adapted so that it can be carried out alongside the RCUT (Young, 1978b). Hence all strains can be tested during routine identification of isolates, many of them directly from the primary plate. However, for this to be effective a number of colonies must be used for the inoculum to prevent misidentification of small numbers of PPNG which may be present in a mixed infection.

Bae, Ledesma and Kortis (1983) suggested using a reagent-impregnated, either starch-iodine or chromogenic cephalosporin (nitrocefin)

filter paper replica method to test all colonies on the primary isolation plate. They simulated a variety of mixtures of PPNG : non-PPNG on culture plates ranging from 1 : 1, 1 : 10 and 1 : 100, and found that either methods detected the PPNG colonies. However they found that filter papers impregnated with nitrocefin, giving development of a yellow colour, gave clearer results. They examined 186 clinical isolates on primary cultures with the replica method. They detected cultures which consisted of mixtures of PPNG and non-PPNG in 2 (1%) out of 186 of primary plates. In the first case, 83% of the colonies were β-lactamase producers. However, in the second case only 2 (4%) of 46 colonies were β-lactamase producers. They concluded that in the second case the PPNG may well have gone undetected by selection of only a few colonies from the primary plate for conventional testing (Bae *et al.*, 1983). Colonies could be picked from the remains of colonies on the primary plate or from the filter papers for subsequent purification and culture.

9.0 Serological diagnosis

As discussed previously (page 97) antibody responses of patients infected with *N. gonorrhoeae* are extremely variable or non-detectable. Those patients who do produce an antibody response do so to a number of different gonococcal components. In addition most people without a history of gonococcal infection have 'natural' antibody to the gonococcus. These factors make selection of test antigens and determination of cut-off points to differentiate between infected and non-infected patients very difficult. Persistence of antibody following a recent successfully treated infection is a further complication in interpreting serological tests. Although a number of assays have been studied including radio-

immunoassay (Usategiu *et al.*, 1982), ELISA, gonococcal complement fixation tests and an indirect haemagglutination reaction (Oranje *et al.*, 1983) none have proved suitable for routine use.

9.1 Detection of local antibody responses

The production of localised IgA antibody can be detected in cervical secretions by an indirect immunoflourescent-antibody method (McMillan *et al.*, 1980b). Antigonococcal IgA was detected in the cervical secretions of 56/78 (72%) of 78 women with untreated gonorrhoea but in only 25/490 (5%) of non-infected women. The sensitivity of this test was no better than that of cervical gram-stained smears (74%) and it was concluded that the low sensitivity, the expense and the laboriousness of the indirect immunoflourescent test made it unsuitable for routine diagnostic work (McMillan *et al.*, 1980b).

AIMS OF STUDY

It is clear that the diagnosis and hence control of gonococcal infection is dependent on properties of the bacterium as well as the behaviour of the host. The surface structures of the gonococcus are important in pathogenesis and genetic mechanisms have already been discussed which can alter the surface properties of the gonococcus. The surface antigens are of importance in the non-cultural detection of *N. gonorrhoeae*.

This study was carried out to (i) examine the temporal distribution of CoA serogroups (based on protein I) within the local gonococcal population; (ii) to examine protein I and cell envelope phenotype in relation to factors such as anatomical site of isolation which may select gonococci with different surface properties and permeability; and (iii) to use the information generated on CoA serogroups as a rational basis for the development of immunological methods for detecting antigen.

MATERIALS AND METHODS

1.0 Bacterial strains

1.1 Stock cultures

Gonococcal major outer membrane protein (MOMP) reference strains

A-1, B-2, C-3, D-4, E-5, F-6, G-7, H-8, N-10, R-11, S-12, T-13, U-14, V-15 and W-16 were kindly supplied by Dr D. Danielsson, Department of Clinical Bacteriology and Immunology, Central County Hospital, Örebro, Sweden.

Gonococcal cell envelope phenotype reference strains FA140 (*penA* 2, *mtr*-2, *penB* 2); FA102 (*penA* 2), FA19 (wild type), BR87 (*env*-2, *str*-7, *penA* 2, *mtr*-2, *penB* 2) and FA136 (*penA* 2, *mtr*-2) were kindly supplied by Dr P.F. Sparling, University of North Carolina, Chapel Hill, USA.

Bacteroides spp. *Bacteroides bivius*, *B. intermedius* WPH 20, *B. ureolyticus* NCTC 10941, *B. asaccharolyticus* GNAB 57 were obtained from Mr R. Brown, MPRL Laboratory, Department of Bacteriology, The Medical School, University of Edinburgh.

Cultures obtained from the National Collection of Typed Cultures, Colindale London. The following were obtained from the National Collection of Typed Cultures.

Staphylococcus aureus	NCTC 8530
Neisseria flavescens	NCTC 8263
N. cavaiae	NCTC 10293
N. cuniculi	NCTC 10297
N. canis	NCTC 10296
N. cinerea	NCTC 10294
N. elongata	NCTC 10660
N. anamalis	NCTC 10212
N. mucosa var. *heidelbergensis*	NCTC 10777
Branhamella spp.	NCTC 11015 & 11017
Neisseria sp.	NCTC 11049

N. denitrificans	NCTC 10295
N. pharyngis	NCTC 4590
N. elongata subsp. *glycolytica*	NCTC 11050
N. ovis	NCTC 11018
N. mucosa var. *mucosa*	NCTC 10774

N. meningitidis strains of groups A, B, C, L, X, W135, Z, Z^1, Y and RAS10 and a non-groupable strain were kindly supplied by Dr R.J. Fallon, Director, Meningococcus Reference Laboratory (Scotland), Ruchill Hospital, Glasgow.

1.2 Clinical isolates

Fresh clinical isolates of *N. gonorrhoeae*, *N. lactamica*, *N. perflava*, *Gardnerella vaganalis*, *Candida albicans*, Group B Streptococci and lactobacilli were obtained from the Sexually Transmitted Diseases Diagnostic Laboratory, Department of Bacteriology, The Medical School, University of Edinburgh.

1.3 Culture media

Modified New York City Medium. MNYC medium (Young, 1978a) comprised of Difco gonococcal base enriched with 10% human blood lysed with 0.5% Saponin, 2.5% yeast dialysate prepared as described by Faur *et al.* (1973b), 0.1% glucose, lincomycin (1.0 µg/ml), colistin (6 µg/ml), amphotericin B (1.0 µg/ml) and trimethoprim lactate (6.5 µg/ml).

SMNYC. As for MNYC except that SMNYC lacks antibiotics.

GC^+ medium. Difco gonococcal base supplemented with L-glutamine, thiamine pyrophosphate, ferric nitrate and glucose as described by Kellogg *et al.* (1963).

Blood agar. Columbia agar base (Oxoid) containing 5% human blood.

PPY medium. PPY is composed of 2% proteose peptone (Oxoid), 1% yeast extract (Difco), 0.5% sodium chloride (NaCl), Haemin 5 µg/ml final concentration, vitamin K (Menadione) 1 µg/ml final concentration, L-cysteine 0.075% final concentration, sodium carbonate 0.04% final concentration, pH 7.4 before autoclaving.

1.4 Viable counts

Viable counts were carried out by making serially ten-fold dilutions of the appropriate suspensions and placing six 20 µl drops of each dilution on culture plates.

1.5 Incubation

Standard incubation conditions, unless otherwise stated, were overnight at 37°C in an aerobic CO_2-enriched (10%) humidified atmosphere.

1.6 Confirmatory identification of *N. gonorrhoeae*

Gonococcal cultures were checked for purity by Gram-staining and by the oxidase test. The oxidase test was carried out by touching a few colonies with a cotton-tipped swab soaked in a solution of 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride. This was followed by the rapid carbohydrate utilisation test (Young *et al.*, 1976) and testing isolates with the Phadebact gonococcus test (Pharmacia Diagnostics, Uppsala, Sweden).

1.7 Chemicals and biochemicals

Unless stated otherwise, all chemicals and biochemicals were obtained from Sigma Chemical Company, Poole, England, or from BDH Chemicals Ltd, Poole, England.

1.8 Buffers

Phosphate-buffered saline (PBS) pH 7.2: 10 mM phosphate buffer pH 7.2 containing 0.15M NaCl.

1.9 Protein determination - modified Lowry

1.6 ml of a 12.5% solution of Na_2CO_3 and 0.2 ml of 0.1% $CuSO_4$. $5H_2O$ solution were added to 400 µl of test protein solution and incubated for 1 h at room temperature. 0.2 ml of a one-third dilution of Folin reagent was then added to the solution and incubated for a further 25 min at room temperature. Absorbance was read at 750 nm. Each test was carried out in triplicate and compared with standard bovine serum albumin standards.

1.10 Statistical analysis

Chi squared tests were used for all statistical analysis.

SPECIFIC METHODOLOGY

SECTION I: CHARACTERISATION OF GONOCOCCAL ISOLATES BY COAGGLUTINATION (CoA) SEROGROUPING AND CELL ENVELOPE PHENOTYPING

1.0 Characterisation by CoA serogrouping

Preparation of antisera against the gonococcal MOMP reference strains

Antisera against MOMP E-5, D-4, V-15, N-10, S-12, U-14 and F-6 were raised in New Zealand white rabbits. Antigen was prepared and rabbits immunised according to the procedure of Sandström and Danielsson (1980b).

Determination of antisera titres

The titres of the antisera were determined by an indirect immunofluorescence antibody (FA) method.

Suspensions of gonococcal strains to be used as antigen were prepared by emulsifying a few colonies in 1 ml of distilled H_2O. 10 µl of the antigen suspensions were applied to each well on 16 well microtitre FA slides. The antigen was allowed to dry onto the slides.

Four-fold serial dilutions of each antiserum to be tested were prepared in PBS from $^1/4$ to $^1/64000$. 10 µl of each dilution was placed on an antigen coated well of the FA slides. Slides were placed in a humidified chamber and incubated at 37°C for 30 min. Slides were then washed in PBS for 10 min, rinsed in distilled H_2O, given a second wash in fresh PBS and dried. 10 µl of a $^1/10$ dilution of fluorescein conjugated anti-rabbit IgG conjugate was placed on each well. Initially the conjugate was obtained from Wellcome Reagents Ltd (Wellcome Research Laboratories, Beckenham, England), but latterly was obtained from Sigma.

Slides were then placed in the humidified chamber and incubated at 37°C for 20 min. The slides were removed from the incubator, washed thoroughly in PBS, dried and coverslips were mounted on the slides in PBS buffered glycerol (Difco). The slides were examined immediately by UV microscopy. The end point titre was determined as the last antiserum dilution which gave a bright halo of flourescence round the gonococcal cells.

Preparation of CoA serogrouping reagents

Antisera raised against the MOMP reference strains were selectively absorbed and used to prepare CoA reagents.

Absorption of antisera

MOMP reference strains used for absorption of the antisera (Table 1) were grown on GC^+ agar for 18 hours, harvested and washed once in 0.01M PBS pH 7.2: ten plates gave approximately 0.3 - 0.5 g wet weight of gonococcal cells. The cells were resuspended in 5 ml of PBS, boiled for 30 min and centrifuged at 4000 x g for 20 min. The pellet was used for absorption. Equal volumes of antisera and pellet were incubated at 37°C for 4 h and then held overnight at 4°C. The absorbed antiserum was collected by centrifugation and CoA reagents were prepared to test the specificity of the antiserum. Absorption was repeated if cross-reactions persisted.

Preparation of CoA reagents

This was essentially as described by Jephcott (1981). *Staphylococcus aureus* NCTC 8530 was grown on GC^+ agar for 18 h, harvested and washed thoroughly in 0.01M PBS pH 7.2, resuspended in PBS containing 0.5% (v/v) formalin and incubated at room temperature for 3 h. The cells

TABLE 1: Absorption scheme for producing antisera for CoA reagents.

Antisera against	Absorption strain	Serogroup defined by corresponding CoA reagent
MOMP E-5	MOMP N-10	WI
" D-4	" C-3	WI
" V-15	" C-3	WI
MOMP N-10	MOMP D-4	WII
" S-12	" A-1	WII
" U-14	" R-11	WII
MOMP F-6	MOMP B-2 & U-14	WIII

were then washed twice, resuspended in PBS, and heated at 80°C for 5 min. The stabilised cells were then washed twice, resuspended to 10% (w/v) in PBS and stored at -20°C in 1 ml aliquots.

Preparation of sensitised test suspension

Absorbed anti-gonococcal serum (0.1 ml) was mixed with 1 ml of washed stabilised staphylococcal cell suspension and held at room temperature for 15 min. The cells were then harvested by centrifugation at 2500 x g for 15 min and the deposit washed twice in PBS. The sensitised cells were finally resuspended in 10 ml of sterile PBS.

The control reagent was prepared in exactly the same way with normal rabbit serum substituted for anti-gonococcal serum.

Test procedure

Test suspensions of *N. gonorrhoeae* were prepared by harvesting organisms from half a culture plate into 400 µl of distilled water and boiling for 10 min. Tests were done with 20 µl of test gonococcal suspension and 20 µl of CoA reagent thoroughly mixed on a glass slide and rocked for 2 min.

Slides were examined under oblique light, against a dark background and the amount of agglutination with the test reagent compared with that obtained in a similar test with the control reagent. The reactions were graded negative (-), and positive: weak (+), moderate (2+) and strong or very strong (3+).

Serogrouping protocol

In a pilot study, 58 strains were tested with each CoA reagent. When it was shown that the CoA reagents anti-E-5 and N-10, defining serogroups WI and WII respectively, reacted with the majority of the

gonococcal strains within these serogroups, each test gonococcal strain was thereafter screened against the three reagents anti-E, N-10 and F-6, defining serogroups WI, WII and WIII. When the serogroup of the strain was known, the reaction pattern of the strain with the remaining reagents within its serogroup was determined.

1.1 Distribution of CoA serogroups over four consecutive time periods and correlation of the reaction patterns obtained with the CoA reagents

A total of 745 gonococcal strains isolated from patients who had acquired their infections in the Edinburgh area were serogrouped over four consecutive six month time periods (Table 2).

TABLE 2:

Period	Number of isolates acquired in the Edinburgh area
1. Nov 1982 - June 1983	195
2. July - Dec 1983	204
3. Feb - Jun 1984	131
4. Aug - Dec 1984	215
Total Nov 1982 - Dec 1984	745

The distribution of CoA serogroups and the reaction patterns of the CoA reagents with 713 of the 745 gonococcal strains were correlated and examined.

1.2 Distribution of CoA serogroups among isolates from homosexual men and heterosexual men and women

The serogroups of the gonococcal isolates were correlated in relation to three patient groups: homosexual men, heterosexual men and women. Differences in distribution of serogroups between the three patient groups were compared.

1.3 Correlation of CoA serogroups in relation to anatomical site of isolation

The CoA serogroups of strains isolated from the rectum, urethra and throat of homosexual men, the urethra and throat of heterosexual men and the genitalia (cervix, urethra and Bartholins glands), rectum and throat of heterosexual women were collated and compared.

1.4 Correlation of CoA serogroup with presence of symptoms and oral contraception in women

A retrospective study of the case notes of 64 women patients in whom gonorrhoea was diagnosed and other genital infections excluded was carried out in order to correlate the CoA serogroup of the infecting strain with presence or lack of symptoms. Only one isolate per patient, generally a cervical isolate, was used for analysis of clinical data.

Statistical analysis was carried out using Chi squared test with Yate's correction.

1.5 Comparison of CoA serogroup of strains isolated from 22 women and their respective partners

The serogroup of gonococcal isolates from 20 women and their respective partners, one woman and her two partners and one woman, her husband and child were compared.

1.7 CoA serogroups of cloned gonococcal colonies

Single colonies (10) were picked from the primary culture plates of seven WI strains and three WII strains and subcultured. The cloned gonococci were serogrouped according to the previously described protocol.

1.8 Distribution of serogroups among gonococcal strains isolated from infections acquired outwith Edinburgh

The CoA serogroups of 50 gonococcal strains isolated from infections acquired either elsewhere in the United Kingdom or abroad were correlated and compared with serogroups of strains isolated from infections acquired in the Edinburgh area.

2.0 Characterisation of gonococcal isolates by cell envelope phenotyping

Determination of cell envelope phenotype

Each isolate was tested for sensitivity to the following hydrophobic compounds: erythromycin (Abbott Laboratories Ltd, Queenborough, England ME11 5EL), fusidic acid (Leo Laboratories Ltd, Princes Risborough, England), Triton X-100 (Fisons Scientific Apparatus, Loughborough, England) and crystal violet (Koch-Light Laboratories, Colnbrook, England).

A 19-point hand-held replicator (Mast Laboratories Ltd, Mast House, Serby Road, Bootle, England) was used to seed separate plates of SMNYC medium containing: erythromycin (doubling concentrations from 0.015-2.0 µg/ml); crystal violet (doubling concentrations from 0.5-4.0 µg/ml); fusidic acid (0.03, 0.25 and 0.5 µg/ml); and Triton X-100 (0.06, 1.0 and 2.0 mg/ml). The inoculum was approximately 10^5 cfu of an overnight culture of each isolate to be tested.

Each batch of plates was set up in duplicate and included the reference strains FA140, BR87, FA19 and FA136. After incubation for 48 h at 37°C in air with 10% CO_2, plates were examined and the MIC determined as the lowest concentration of each agent that prevented visible growth.

Strains exhibiting the following MICs, which were given by the Mtr reference strains FA140 and FA136, were designated the Mtr phenotype: erythromycin ≥0.5 μg/ml, fusidic acid ≥0.5 μg/ml, crystal violet ≥2.0 μg/ml and Triton X-100 ≥2.0 mg/ml. Strains exhibiting the following MICs, which were given by the Env reference strain BR87, were designated the Env phenotype: erythromycin ≤0.06 μg/ml, fusidic acid ≤0.03 μg/ml, crystal violet ≤0.5 μg/ml and Triton X-100 ≤0.06 mg/ml. The wild type reference strain FA19 exhibited the following MICs: erythromycin 0.12 μg/ml, fusidic acid 0.25 μg/ml, crystal violet 1.0 μg/ml and Triton X-100 1.0 mg/ml. Test strains with erythromycin MICs of 0.12 - 0.25 μg/ml were designated wild type.

2.1 Overall distribution of cell envelope phenotypes and correlation of phenotype of infecting strain with patient group

The cell envelope phenotypes were determined for 482 gonococcal strains isolated from patients who acquired their infections in the Edinburgh area. Cell envelope phenotypes were correlated and compared for gonococcal strains isolated from three patient groups: homosexual men, heterosexual men and women.

2.2 Correlation of cell envelope phenotype with patient group and anatomical site of isolation

The cell envelope phenotype of the gonococcal strain was correlated with patient group and with anatomical site of isolation. Differences in distribution were compared by site of isolation.

2.3 Relationship of cell envelope phenotype with CoA serogroup

The cell envelope phenotype was correlated with CoA serogroup for 475 of the gonococcal strains and compared by patient group.

2.4 Cell envelope phenotype and CoA serogroup of gonococcal strains isolated from infections acquired outwith the Edinburgh area

The phenotypes of 24 strains isolated from patients who acquired their infections outwith Edinburgh were determined and correlated with CoA serogroup. The results were compared with those obtained for gonococci acquired within Edinburgh.

SECTION II: NON-CULTURAL DETECTION AND IDENTIFICATION OF *N. GONORRHOEAE*

1.0 Preparation of antisera against MOMP E-5 and N-10 protein I antigen

Purification of protein I antigen

Protein I was purified from MOMP E-5 and N-10 essentially by the method of Heckels (1981) and James and Heckels (1981).

Culture of gonococcal strains

MOMP E-5: MOMP E-5 was inoculated onto GC^+ medium in large trays (nine 27 x 38 cm; eight 40 x 35 cm) and incubated at 37°C in 10% CO_2 for 20 h. The growth was scraped off the surface of the medium with sterile glass slides and harvested into ice cold 0.1M PBS (pH 7.2). If the trays contained more than 1% surface contamination they were discarded. The bacteria were pelleted by centrifugation at 12,000 g for 20 min (Sorvall).

The yield of MOMP E-5 cells was approximately 22 g wet weight.

MOMP N-10: Because of gross contamination of the trays, MOMP N-10 was cultured on GC^+ medium in large round petri plates (diameter 13.5 cm) for 20 h at 37°C in 10% CO_2. The growth was harvested as for MOMP E-5 and the pellet stored in 0.1M PBS (pH 7.2) at -20°C. This procedure was repeated until approximately 27 g (wet weight) of MOMP N-10 cells had been produced.

Preparation of purified outer membranes

The bacterial pellets were suspended in 0.2M lithium acetate buffer (pH 6.0) at approximately 10 g wet-weight/100 ml of buffer and incubated at 45°C for 2.5 h with occasional vigorous shaking. After incubation the suspensions were placed in a tissue homogeniser (Waring Commercial

Blender, Waring Products Division, Connecticut) and homogenised at maximum speed for 1 min. The homogenised suspensions were centrifuged three times at 15,000 g for 20 min (Sorval) to remove bacterial fragments. The supernatants were centrifuged at 100,000 g for 2 h (Beckman, Super Speed 40) to sediment crude outer membranes.

The membrane pellets were suspended in 6M urea - 0.2M sodium acetate buffer (pH 6.0) at an approximate protein concentration of 10 mg/ml and incubated at room temperature for 30 min. After incubation the suspensions were diluted with an equal volume of 6M urea - sodium acetate buffer to give a final volume of approximately 15 ml. The suspensions were then centrifuged at 100,000 g for 3 h (Beckman, Super Speed 40). The pellets containing pure outer membrane complex were resuspended and washed three times in distilled water.

Purification of outer membrane proteins

The pelleted outer membranes were suspended in 15 ml of 1% (w/v) sodium cholate in 0.1M glycine - NaOH buffer (pH 9.5) and incubated at 37°C for 1 h. After incubation the suspensions were centrifuged at 100,000 g for 2 h. The pelleted sodium cholate insoluble material was suspended in 2 ml of 1% (v/v) Empigen-BB detergent (a gift from Albright and Wilson, Whitehaven, England) in 0.1M glycine - NaOH buffer (pH 9.5) and incubated at 37°C for 1 h. The suspensions were centrifuged at 100,000 g for 2 h and the supernatant containing Empigen-BB soluble material (ESM) rich in protein I was collected. The samples were diluted 1 : 2 to give 2.5 ml of ESM from each of MOMP E-5 and N-10 at a protein concentration of approximately 1.5 mg/ml. Protein concentration was estimated by measuring absorbance at 280 nm (Pye Unicam SP6-550 UV/VIS spectrophotometer) and comparing with a bovine serum albumin protein standard.

Protein I was separated from the ESM by column chromatography.

Column chromatography

1 ml volumes (2 ml total) of the ESM material from each of MOMP E-5 and N-10 were applied to a 320 mm x 15 mm column containing Sephacryl 200 (S-200) (Pharmacia Fine Chemicals, Pharmacia Ltd, Milton Keynes, England). The ESM was eluted down the column in 1% (v/v) Empigen-BB in 0.1M glycine - NaOH buffer (pH 9.5) at a flow rate of 37.5 ml/h and 1 ml fractions were collected at 1.5 min intervals in an automatic fraction collector (2070 Utrorac® II, LKB, Sweden).

The absorbance at 280 nm was determined for each fraction collected and the absorbance v fraction number was plotted on a graph. The graphs obtained with MOMP E-5 and MOMP N-10 eluted ESM are shown in Figures 9 and 10 respectively. The fractions comprising each peak were pooled to give a total of four pooled samples: two samples A and B and two samples C and D from MOMP E-5 and N-10 respectively.

The pooled samples were dialysed against 0.5M sodium carbonate-bicarbonate buffer (pH 9.0) four times and against distilled H_2O two times and concentrated to approximately 1 ml in a micro-volume stirred ultrafiltration cell (Amicon Ltd, Woking, England) containing an Amicon PM10 ultrafilter membrane with an exclusion of >10,000 MWt.

The protein concentrations of the samples were 1 mg/ml, 0.977 mg/ml and 1.032 mg/ml for samples A and B, C and D respectively.

The samples and the remainder of the ESM from MOMP E-5 and N-10 were stored at -20°C until required for analysis by polyacrylamide gel electrophoresis and for production of antisera against protein I.

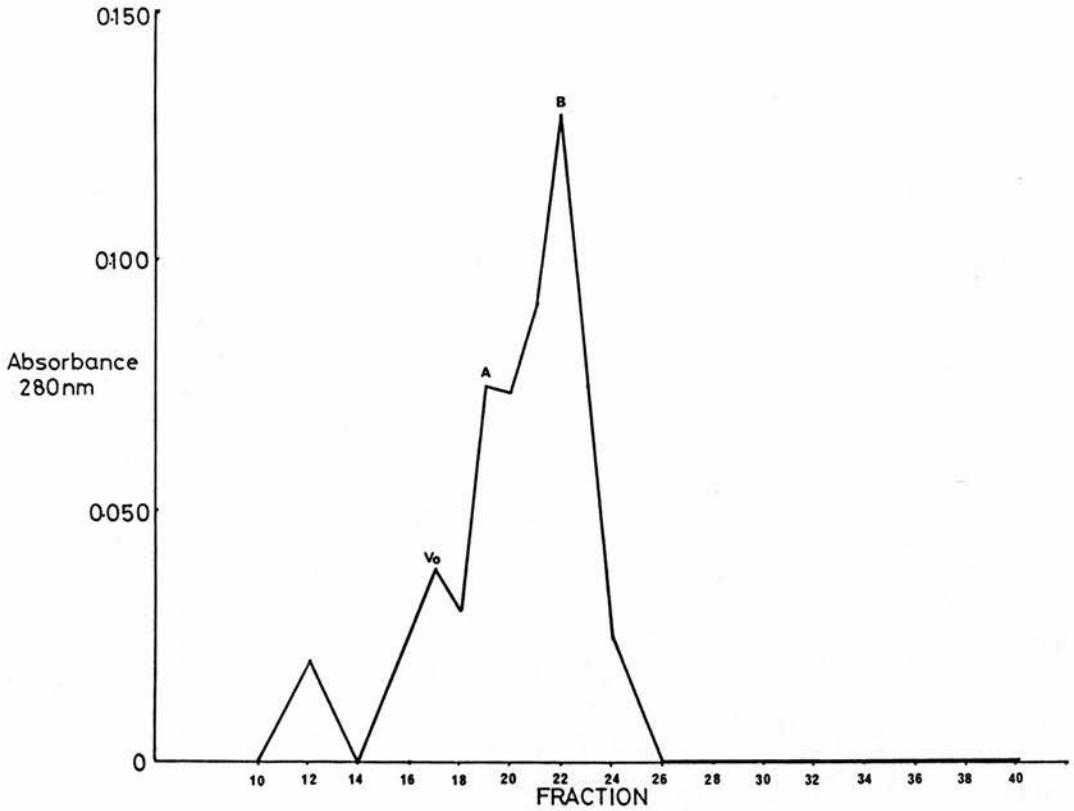

FIGURE 9: Fractionation of empigen soluble material (ESM) from MOMP E-5 by column chromatography.

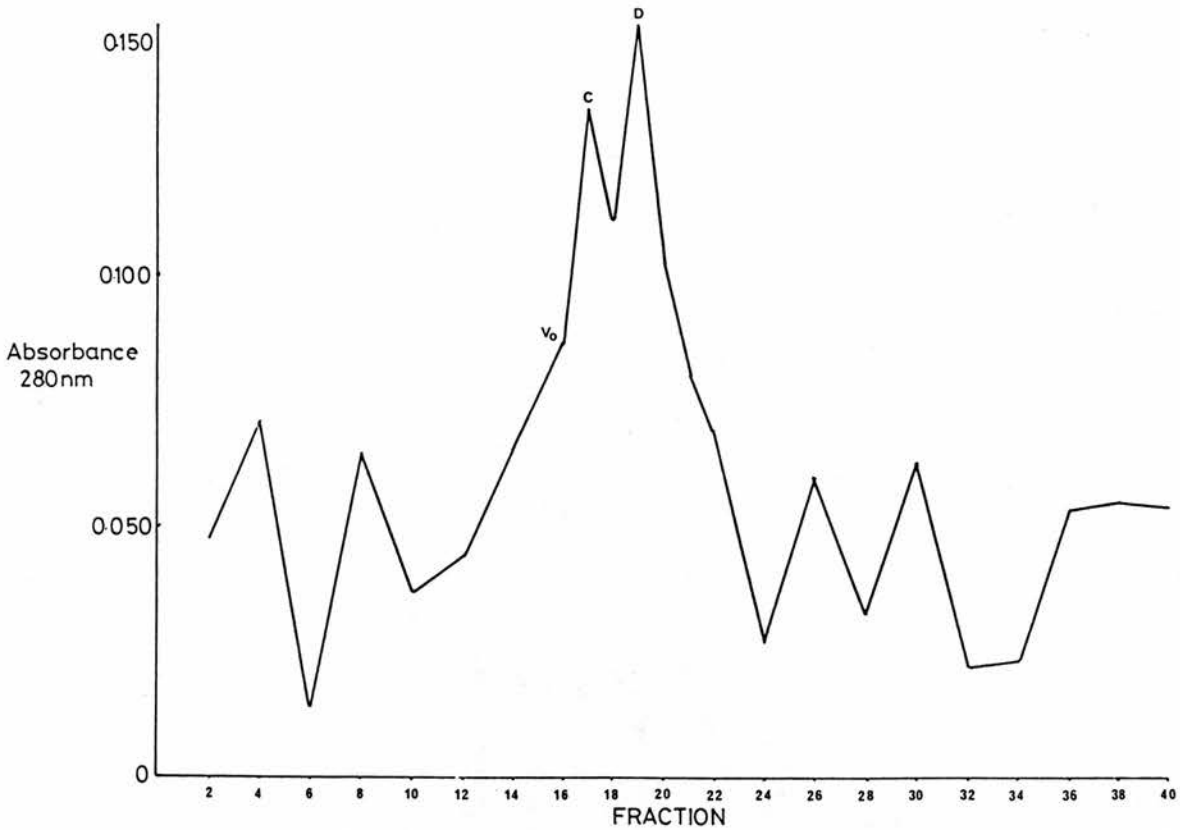

FIGURE 10: Fractionation of empigen soluble material from MOMP N-10 by column chromatography.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The procedure was essentially as described by Poxton and Brown (1979). Slab gels (170 x 140 mm) of 10% (w/v) acrylamide with a 10 mm 4% (w/v) acrylamide stacking gel were used in a gel electrophoresis apparatus (Raven, Haverhill, Suffolk).

The buffers used were as described by Laemmli (1970). Samples of the material to be examined were solubilised by heating in a boiling water bath for 3 min in SDS-sample buffer containing 2% (w/v) SDS and 5% mercaptoethanol (Poxton and Brown, 1979). Samples of 50 µg of protein were applied to the wells of one gel and samples of 5 µg of protein were applied to the wells of a second gel. Molecular weight standards were included in the first gel. Electrophoresis was carried out at a constant voltage of 50V until the samples entered the separating gel (approximately 1 h) and was then maintained at 150V until the bromophenol blue tracking dye was near the bottom of the gel (approximately 3 h). The first gel and half of the second gel were stained for protein with Coomassie Brilliant Blue (Poxton and Sutherland, 1976). The other half of the second gel was stained for lipopolysaccharide by silver after periodic acid treatment by the method of Tsai and Frasch (1982).

Immunisation procedure for production of antisera against protein I

Antisera against MOMP E-5 and MOMP N-10 protein I were raised in New Zealand white rabbits. The rabbits were test bled prior to immunisation.

Aliquots of MOMP E-5 and N-10 protein I antigen were thawed and diluted with an equal volume of Freund's complete adjuvant to give a final vaccine volume of 0.5 ml containing 100 µg of protein I antigen. The rabbits were immunised intramuscularly according to the following procedure: *Day 1,* two 0.5 ml injections, one in each hind leg; *Day 14,*

two 0.5 ml injections in the back; *Day 28*, one 0.5 ml injections in each hind leg; 14 days later the rabbits were test bled and then received a 0.5 ml booster injection. Two weeks later the rabbits were exsanguinated by cardiac puncture.

Determination of antibody titres

The antibody titres were determined by an indirect FA assay as previously described.

1.1 Preparation of antisera to MOMP E-5 and N-10 whole cell antigen

Antisera to MOMP E-5 and N-10 whole antigen were those used in Section I.

2.0 Preliminary studies on gonococcal antigen detection by an indirect enzyme linked immunosorbent assay (ELISA) using wheat germ lectin for antigen capture

2.1 Agglutination of MOMP E-5 and N-10 by wheat germ lectin

This was carried out essentially as described by Curtis and Slack (1981). An aliquot of wheat germ (WG) lectin (1 mg/ml) was serially diluted in two-fold steps down to a dilution of $^{1}/_{512}$ corresponding to a final concentration of 2 µg/ml of lectin. *N. gonorrhoeae* MOMP E-5 and N-10 were harvested from overnight MNYC plates in 0.01M PBS (pH 7.2) and the suspensions were adjusted to McFarland's standard 0.5 with PBS. 40 µl of each lectin dilution and 40 µl of the gonococcal suspensions were placed on glass slides, mixed together and slides were rotated for 10 min. Controls consisted of 40 µl of PBS mixed with 40 µl of gonococcal suspension.

The amount of agglutination was read by eye and scoring attempted by the protocol of Curtis and Slack (1981): 4+ very few clumps in clear supernatant; 3+ medium sized clumps in clear supernatant; 2+ small easily seen agglutinates; + agglutination just visible to the naked eye; +/- no visible agglutination. The end point was taken as the highest WG lectin dilution giving 2+ agglutination.

2.2 Effect of boiling on lectin agglutination of thick gonococcal suspensions

Thick suspensions, two of MOMP E-5 and two of MOMP N-10, were prepared by harvesting approximately half the growth from overnight culture plates into 400 µl of PBS. The first suspension of each strain was untreated and the second suspension was boiled for 10 min prior to testing with WG lectin as above.

2.3 Indirect enzyme-linked immunosorbent assay using polystyrene beads coated with wheat germ lectin

Preparation of lectin dilutions

A duplicate set of seven WG lectin dilutions were made in 0.01M PBS (pH 7.2) and in 0.05M carbonate buffer (pH 9.6) to give WG lectin concentrations ranging from 200 µg/ml to 3.0 µg/ml.

Procedure for coating polystyrene beads with lectin

24 universal bottles each containing 24 polystyrene beads, 0.25 in; *c.* 0.6 cm RIA grade with frosted finish (Euro-matic Ltd, Brentford, England) were set up and divided into three sets of eight. 5 ml volumes of each of the seven lectin/PBS dilutions were added to universals of sets one and two, 5 ml volumes of each of the seven lectin/carbonate

dilutions were added to universals of set three and to the eighth universal of sets one, two and three 5 ml of the respective diluent buffer was added to give uncoated control beads.

Universal set one was incubated at 37°C for 2 h and sets two and three at 37°C for 4 h and 5 h respectively. All three sets were then incubated overnight at 4°C.

After incubation the supernatants were removed from all universals using a pasteur pipette and the polystyrene beads turned out into labelled petri dishes containing paper towels and allowed to air dry. Once completely dry the beads were placed in labelled sterile universals and stored at 4°C until required.

ELISA procedure

The following procedure was used throughout this study. Details of the relevant lectin coated beads and MOMP E-5 antigen preparations used will be described later for each experiment.

The lectin coated and uncoated control polystyrene beads were placed in clean glass Wasserman tubes (1 bead/tube) and 400 µl of antigen was added to appropriate tube. Tubes containing beads and 400 µl of buffer acted as controls. The tubes were incubated in a 37°C water bath for 30 min. The supernatant from each tube was then aspirated and the beads were washed three times with PBS/Tween 20 (0.01M PBS (pH 7.2) containing 0.05% (v/v) Tween-20). 400 µl of anti-MOMP E-5 whole cell unabsorbed antiserum at a dilution of 1/100 in PBS/Tween 20 was added to each tube and the tubes were then incubated for a further 30 min at 37°C. The supernatants were removed and the beads were washed as before. 400 µl of a 1/1000 dilution of anti-rabbit IgG-horseradish peroxidase conjugate (Miles Scientific Division, Slough,

England) in PBS/Tween-20 was then added to each tube and the tubes were incubated as before. After incubation supernatants were aspirated and the beads were washed as before. The beads were then carefully transferred to clean glass tubes. O-phenylenediamine (OPD) substrate [0.04% (w/v) OPD, 0.012% (v/v) H_2O_2 in phosphate-citrate buffer (0.05M diabasic sodium phosphate; 0.025M citric acid) (pH 5.0)] was added to each tube and the tubes were incubated at 37°C for 30 min. The supernatant was quickly transferred to the wells of a 96-well microtitre plate and extinction read at 490 nm in a multiskan plate reader (Titrek®, Dynatech Laboratories, Billingshurst, England).

2.3.1 *Determination of minimum amount of whole cell antigen detected by polystyrene beads armed with lectin in PBS*

The ELISA was carried out according to the previously described procedure. Rabbit anti-E-5 serum and anti-rabbit IgG peroxidase conjugate were used at dilutions of 1/100 and 1/1000 respectively. Polystyrene bead sets 1 and 2, prearmed with lectin/PBS dilutions at 37°C for 2 h and 4 h respectively, were used. Lectin concentrations ranged from 200 µg/ml to 3 µg/ml as shown in Figure 11.

MOMP E-5 whole cell antigen was used: antigen ranged from undiluted (3×10^7 cfu) to a 1/1,050,000 dilution (29 cfu).

The whole cell antigen was prepared by harvesting the growth from an overnight MNYC plate into 0.01M PBS (pH 7.2) and adjusting the suspension to McFarland's standard 1.0. A viable count was immediately carried out and ten four-fold serial dilutions were carried out in PBS.

Antigen dilutions were used in the ELISA as shown in Figure 11.

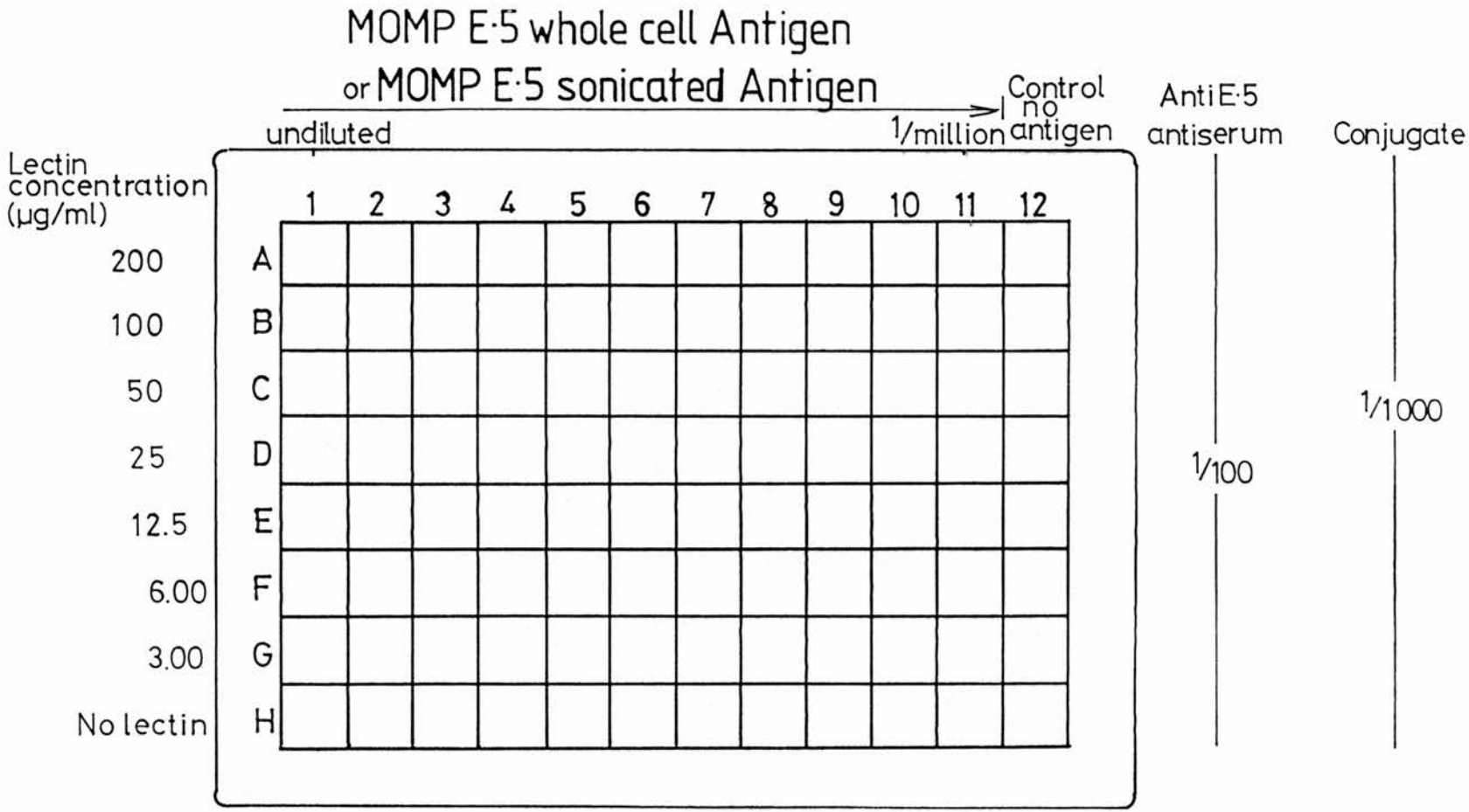

FIGURE 11: Schematic representation of ELISA protocol.

2.3.2 Determination of the minimum amount of sonicated antigen detected by polystyrene beads armed with lectin in carbonate buffer (pH 9.6)

The ELISA procedure was as previously described. Rabbit anti-E-5 serum and anti-rabbit IgG peroxidase conjugate were used at dilutions of 1/100 and 1/1000 respectively. Polystyrene bead set 3, prearmed with lectin in carbonate buffer (pH 9.6), was used. Lectin concentrations ranged from 200 µg/ml to 3 µg/ml as shown in Figure 11. MOMP E-5 sonicated antigen was used: antigen concentration ranged from undiluted (400 µg of protein ≡ 9.4×10^7 cfu) to a dilution of 1/1,050,000 (0.0004 µg ≡ 90 cfu).

The sonicated MOMP E-5 antigen was prepared by harvesting the growth from 15 overnight MNYC plates into 4 ml of 0.01M PBS (pH 7.2). A viable count was rapidly carried out and the bacterial suspension was centrifuged for 15 min at 3000 rpm in a bench centrifuge. The bacterial pellet was resuspended in the original volume of PBS. The bijou containing the MOMP E-5 suspension was placed on ice and the probe of an MSE ultrasonicator was placed just under the meniscus of the suspension. The gonococcal cells were disrupted by sonication for 2 min at setting 8: if the suspension did not become opalescent, sonication was repeated for a further two minutes. The sonicate was examined by Gram staining to ensure that disruption of the gonococci had occurred.

The protein concentration of the sonicate was determined to be 4.77 mg/ml by the modified Lowry protein assay. A portion of the sonicate was diluted in PBS to 1 mg/ml of protein and 10 four-fold serial dilutions in PBS were carried out on this portion. The dilutions of sonicate were used in the ELISA as shown in Figure 11.

3.0 Gonococcal antigen detection on nitrocellulose by an indirect enzyme linked immunosorbent assay (dot-blot immunoassay)

Dot-blot immunoassay procedure

The following procedure was used throughout this study. Details of the relevant antigen and anti-gonococcal antiserum used will be described later for each experiment.

Strips of nitrocellulose (Bio-Rad Laboratories Ltd, Watford, England) were cut to the required size and placed in Tris buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5) at a 45° angle and allowed to wet by capillary action. Strips were removed from the TBS, air dried and antigen either 1 µl or successive 1 µl amounts were applied to separate sites on the nitrocellulose strips. The strips were air dried and placed in a blocking solution of 3% (w/v) gelatin in TBS and incubated at room temperature with constant shaking for 45 min. The strips were then transferred to antigonococcal antiserum in antibody buffer (1% (w/v) gelatin in TBS containing 0.05% Tween 20) and incubated at room temperature with constant shaking for 1 h. The strips were washed two times in TBS containing 0.05% Tween 20 and transferred to the conjugate solution containing a $^{1}/_{3000}$ dilution of anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) in antibody buffer containing 0.05% Tween 20.

The strips were incubated with the conjugate solution at room temperature with constant shaking for 1 h. Strips were washed as before, rinsed in distilled H_2O and transferred to the substrate solution. The substrate solution was prepared by adding 60 mg of 4-chloro-1-napthol (Bio-Rad) in 20 ml of methanol to 100 ml of TBS containing 0.015% ice cold H_2O_2.

The strips were incubated at room temperature for 20 min unless stated otherwise with constant shaking. Colour reactions were stopped by placing the strips in distilled H_2O for 10 min. Positive reactions resulted in a purple colour appearing on the dots. A control containing only TBS was included on each strip.

3.1 Detection of (A) untreated whole cell, (B) boiled, and (C) sonicated MOMP E-5 antigen by anti-E-5 antiserum

The dot-blot immunoassay was carried out as previously described except that the nitrocellulose strips were incubated with the substrate solution for 15 min instead of 20 min.

Unabsorbed MOMP E-5 whole cell antiserum at a $^1/50$ dilution was used for antigen detection. This antiserum was previously used for preparing CoA reagents.

MOMP E-5 antigen was used. This comprised of (A) untreated whole cell, (B) boiled whole cell, and (C) sonicated antigen.

The antigen suspensions were prepared as follows. MOMP E-5 was harvested from five overnight MNYC plates into 5 ml of TBS, the cells were washed twice in TBS and resuspended in TBS. The suspension was adjusted to an OD of 1.0 at 540 nm equivalent to approximately 10^9 cfu/ml. A viable count was performed and 1 ml aliquots were placed into three bijoux labelled A, B and C, the remainder of the original suspension was stored at -20°C for future use. Suspension A was untreated, B was boiled for 10 min and C was sonicated at setting 8 for 2 min in an MSE ultrasonicator. Each suspension was then serially diluted from $^1/2$ to $^1/1024$. Antigen 1 µl from each dilution (undiluted to $^1/1024$) was applied to separate spots on three nitrocellulose strips labelled A, B and C corresponding to the three antigen suspensions used.

Colour development on the spots was graded subjectively, by eye, according to the following protocol: 3+, strong positive reaction (very deep purple); 2+, medium positive reaction (purple); +, positive reaction (light purple); +/-, very slight colour development; -, no colour development.

3.2 Detection of MOMP E-5 and N-10 boiled whole cell antigen by anti-N-10 antiserum

The dot-blot immunoassay was carried out according to the previously described protocol.

Unabsorbed MOMP N-10 whole cell antiserum was used for antigen detection at a 1/50 dilution. This antiserum had previously been used to prepare CoA reagents.

MOMP E-5 and N-10 boiled whole cell suspensions (OD 1.0) from undiluted to 1/1024 dilution were used as antigens.

MOMP N-10 antigen was prepared by harvesting the growth from five overnight MNYC plates into 5 ml of TBS. The cells were washed twice in TBS, resuspended in TBS and the suspension was adjusted to an OD of 1.0 at 540 nm. A 400 µl aliquot was removed and the remainder of the suspension was frozen at -20°C.

The aliquot of MOMP N-10 suspension and a 400 µl thawed aliquot of MOMP E-5 suspension OD 1.0 (used in 3.1) were boiled for 10 min and serial two-fold dilutions from 1/2 to 1/1024 were carried out. 1 µl of each dilution (undiluted to 1/1024) were applied to separate spots on two nitrocellulose strips labelled E-5 and N-10.

Colour development was scored subjectively according to the protocol described in 3.1.

3.3 Detection of MOMP E-5 and N-10 boiled whole cell antigen by a mixture of anti-E-5 and anti N-10 antiserum

The dot-blot immunoassay was carried out as previously described. A mixture of anti-E-5 and anti-N-10 unabsorbed whole cell antiserum at a ratio of 1 : 1 to give a total antiserum dilution of $^1/50$, with each individual antiserum at $^1/100$, was used for antigen detection.

Aliquots of MOMP E-5 and N-10 whole cell antigen described in 3.1 an 3.2 were thawed and boiled for 10 min. Serial two-fold dilutions were carried out from $^1/2$ to $^1/1024$ and 1 µl of each antigen dilution (undiluted to $^1/1024$) were applied to separate spots on nitrocellulose strips labelled E-5 and N-10. Colour development was scored subjectively as previously described.

3.4 Detection of MOMP E-5 and N-10 boiled whole cell antigen by unabsorbed anti-E-5 and absorbed anti-E-5 and absorbed anti-N-10 antisera

The dot-blot immunoassay procedure was carried out as previously described. The MOMP E-5 and N-10 boiled whole cell antigen was as previously described and 1 µl amounts of undiluted to $^1/1024$ dilution of these antigens were applied to separate spots on nitrocellulose strips. Unabsorbed anti-E-5 whole cell antiserum and absorbed anti-E-5 and absorbed anti-N-10 whole cell antisera at dilutions of $^1/50$ were used for antigen detection. The absorbed antisera were those used for preparation of CoA reagents and the absorption procedure is described in Section I 1.0.

Colour development was read subjectively by eye and scored according to the previously described protocol.

3.5 Detection of MOMP E-5 and N-10 boiled whole cell antigen with a mixture of anti-E-5 protein I and anti-N-10 protein I antisera

The dot-blot immunoassay was carried out as previously described. MOMP E-5 and N-10 boiled whole cell antigen used were as previously described. 5 µl amounts (1 µl volumes applied successively) of undiluted and dilutions from 1/32 to 1/1024 were applied to separate spots on nitrocellulose strips.

Unabsorbed antisera raised against protein I purified from MOMP E-5 and N-10 respectively were mixed in a ratio of 1 : 1 and dilutions of 1/50, 1/100 and 1/200 of this mixture were used for antigen detection.

Colour development was read and scored as in previous experiments.

3.6 Determination of the minimal amount of purified MOMP E-5 and N-10 protein I antigen detected by a mixture of unabsorbed whole cell antisera and by a mixture of anti-protein I antisera

The dot-blot immunoassay was carried out as previously described. Purified protein I antigen (50, 10, 5 and 1 ng) from MOMP E-5 and N-10 respectively, was applied to separate spots on nitrocellulose strips. Antigen was detected by a 1/100 dilution of a 1 : 1 mixture of anti-E-5 and anti-N-10 whole cell antisera and by a 1/100 dilution of a 1 : 1 mixture of anti-E-5 protein I and anti-N-10 protein I antisera.

Colour development was read and scored as previously described.

3.7 Detection of gonococcal antigen in male urethral specimens by anti-gonococcal antisera in the dot-blot immunoassay

The dot-blot immunoassay was carried out as previously described. Clinical urethral specimens were obtained from 95 male patients with urethritis attending the Department of Genitourinary Medicine, The

Royal Infirmary, Edinburgh. The urethral specimens were taken using small cotton tipped chlyamidial swabs (Medical Wire & Equipment Ltd, Corsham, England) after material had been taken from the patients for routine diagnostic smear and culture tests. On receiving the urethral specimens, the swabs were broken off into 200 µl of TBS in clean glass tubes, mixed with a vortex mixer for 60 sec, boiled for 10 min and prior to being discarded the swabs were rotated against the sides of the tubes to express all material. The specimens were then labelled and stored at -20°C until required.

Prior to testing for gonococcal antigen in the dot-blot immunoassay, the specimens were allowed to thaw and were boiled for a further 10 min. Each specimen was applied to separate spots on nitrocellulose strips by placing successive 1 µl amounts to give a total of 10 µl of antigen/spot.

Control antigen was included on each test strip. Initially this consisted of 10 µl of undiluted (10^7 cfu) MOMP E-5 and 10 µl of undiluted (10^7 cfu) MOMP N-10 boiled whole cell antigen used in previous experiments. Latterly control antigens consisted of MOMP E-5 boiled whole cell antigen at concentrations of 10^7, 1.6×10^5 and 8×10^4 cfu which gave 3+ (strong positive), 1+ (positive) and +/- (very little colour) reactions respectively.

Antigen was detected by a 1 : 1 mixture of anti-E-5 and anti-N-10 whole cell antiserum. Initially a 1/50 dilution of this antisera mixture was used but this was later changed to a 1/100 dilution. In the second half of the study 45 specimens were also tested with a 1/100 dilution of a 1 : 1 mixture of anti-E-5 protein I and anti-N-10 protein I antisera.

Scoring of clinical specimens: Colour development on the spots was read by eye and scored subjectively according to the previously described protocol (Section 3.1). The results were compared with those of the control antigen and clinical specimens were scored positive if they gave ≥1+ reaction and negative if very little (+/- reaction) or no colour (- reaction) developed.

Correlation with culture results: The dot-blot immunoassay results for the clinical specimens were read without prior knowledge of the respective culture results. Once the immunoassay results for each clinical specimen had been determined comparisons were then made with the respective urethral culture results.

3.8 Determination of the minimum amount of antigen detected in the dot-blot immunoassay and the corresponding CoA serogroups for 9 clinical isolates of *N. gonorrhoeae*

Nine gonococcal strains were isolated from male patients from whom urethral specimens had been taken for testing in the dot-blot immunoassay.

Determination of the minimum amount of antigen detectable: The dot-blot immunoassay was carried out as previously described. Suspensions of the gonococcal isolates were made in TBS, adjusted to an OD of approximately 1.0 (range 0.8 to 1.1) at 540 nm (approximately 10^9 cfu). An aliquot of each suspension was boiled for 10 min and doubling dilutions were prepared in TBS from 1/2 to 1/1024. 1 µl amounts of each antigen dilution from undiluted to 1/1024 were applied to separate spots on nitrocellulose strips.

Antigen was detected by a 1 : 1 mixture of anti-E-5 and anti-N-10 whole cell antisera and by a 1 : 1 mixture of anti-E-5 protein I and anti-N-10 protein I antisera. A dilution of 1/100 was used for both antisera mixtures.

Colour development was read by eye and scored subjectively as previously described.

CoA serogrouping: The nine gonococcal isolates were serogrouped by CoA reagents as previously described (Section I).

3.9 Specificity of the dot-blot immunoassay

The dot-blot immunoassay was carried out as previously described. The bacterial species used to test the specificity of the dot-blot immunoassay were:

Commensal Neisseria: *N. flavescens* NCTC 8263, *N. caviae* NCTC 10293, *N. cuniculi* NCTC 10297, *N. canis* NCTC 10296, *N. cinerea* NCTC 10294 *N. elongata* NCTC 10660, *N. anamalis* NCTC 10212, *N. mucosa* var. *heidelbergensis* NCTC 10777, *N.* sp. NCTC 11049, *N. denitrificans* NCTC 10295, *N. pharyngis* NCTC 4590, *N. elongata* subsp. *glycolytica* NCTC 11050, *N. ovis* NCTC 11018, *N. mucosa* var. *mucosa* NCTC 11017 and 2 clinical specimens of *N. perflava*.

Pathogenic/commensal Neisseria: *N. meningitidis* groups Y, RAS 10, Z^1, L, Ref Z, X, W135, A, B, C and a non-groupable strain; *N. lactamica* PHLS 696 and three clinical isolates of *N. lactamica*.

Branhamella: Two *Branhamella* spp. NCTC 11015 and NCTC 11017.

Bacteria common to the urogenital tract: *G. vaganalis*, *B. bivius*, *C. albicans*, group B Streptococcus, Lactobacillus, *B. ureolyticus*, *B. intermedius*, and *B. asaccharolyticus*.

Suspensions of the 31 non-gonococcal *Neisseria*, the 2 *Branhamella* spp. and the 8 other bacterial species commonly isolated from the urogenital tract were prepared in TBS. The suspensions were adjusted to an OD of approximately 1.0 at 540 nm and an aliquot of each suspension was boiled for 10 min. Doubling dilutions of each boiled suspension from 1/2 to 1/1024 were prepared in TBS and 1 µl amounts of each dilution from undiluted to 1/1024 were applied to separate spots on nitrocellulose strips.

Antigen was detected by a 1 : 1 mixture of anti-E-5 and anti-N-10 whole cell antisera and by a 1 : 1 mixture of anti-E-5 protein I and anti-N-10 protein I antisera: both antisera mixtures were used at a dilution of 1/100.

Colour development was read by eye and scored subjectively as previously described.

3.10 Antigen detection using antisera absorbed with *B. bivius*

The dot-blot immunoassay was carried out as previously described. Aliquots of whole cell antigen from MOMP E-5, MOMP N-10, four *Bacteriodes* spp. and two meningococcal strains, prepared for previous experiments, were thawed, boiled for 10 min and dilutions of each suspension from 1/2 to 1/1024 were prepared in TBS. 1 µl amounts of each antigen dilution were applied to separate spots on nitrocellulose strips. In addition four male urethral specimens were included (10 µl: successive 1 µl amounts). These specimens gave false positive results when tested previously in the dot-blot immunoassay.

Antigen was detected by a 1 : 1 mixture of anti-E-5 and anti-N-10 whole cell antisera and by a 1 : 1 mixture of anti-E-5 protein I and anti-N-10 protein I antisera: both antisera mixtures had been absorbed by *B. bivius* and were used at a dilution of 1/100.

Absorption of antisera

B. bivius was inoculated into two 20 ml starter cultures of prereduced PPY medium and incubated anaerobically at 37°C for 48 h. The starter cultures containing *B. bivius* were used to inoculate 2 l of prereduced PPY medium which was then incubated anaerobically at 37°C for 72 h.

The bacteria were harvested from the PPY medium by centrifugation, washed two times in distilled water, resuspended in PBS and boiled for 30 min. The bacteria were recovered by centrifugation and washed twice in PBS. The bacterial pellet was weighed and 1 g (wet weight) was added to 1 ml of the anti-gonococcal whole cell antisera mixture and 1 g was added to 1 ml of the anti-gonococcal protein I antisera mixture. The antisera were absorbed with the *B. bivius* for 4 h at 37°C and overnight at 4°C. The absorbed antisera were recovered by centrifugation.

3.11 Reduction of non-specific colour development by blocking with bovine serum albumin

The dot-blot immunoassay was carried out essentially as previously described, except that one nitrocellulose strip was blocked in 3% gelatin/TBS as per normal and the other was blocked in a solution of 3% bovine serum albumin in TBS.

Control antigen consisted of MOMP E-5 antigen at undiluted 1/32, 1/64 and 1/128 dilutions and undiluted MOMP N-10 antigen. 1 µl amounts of each control antigen dilution were applied successively to separate spots on two nitrocellulose strips to give a total of 10 µl of antigen/spot. Five male urethral clinical specimens (10 µl/specimen) were also applied to spots on the nitrocellulose strips. The preparation of the clinical

specimen has been described previously (Section 3.7). Antigen was detected using a 1/50 dilution of a 1 : 1 mixture of anti-E-5 and anti-N-10 whole cell antisera.

The results for the strip blocked in the BSA were compared with those on the strip blocked with gelatin.

3.12 Testing of the anti-rabbit IgG conjugate for non-specific binding to antigen and for endogenous peroxidase activity in urethral clinical specimens

Control antigen consisted of undiluted (1×10^6 cfu), 1/64 and 1/256 dilutions of the MOMP E-5 boiled whole cell antigen and undiluted (1×10^6 cfu) of the MOMP N-10 boiled whole cell antigen. 1 µl amounts of each antigen dilution were applied successively to separate spots on two nitrocellulose strips to give a total of 10 µl of antigen/spot. Three male urethral clinical specimens were also applied to each strip. These were treated as previously described and successive 1 µl amounts were applied to separate spots on the nitrocellulose strips to give a total of 10 µl of antigen/spot.

Both strips were placed in the 3% gelatin/TBS blocking solution for 45 min.

Testing for non-specific binding of the conjugate

One strip was then placed in the conjugate solution (1/3000 dilution of anti-rabbit IgG peroxidase conjugate in antibody buffer) for 1 h, washed and placed in the substrate solution for 20 min.

Testing for endogenous peroxidase activity

The second strip was removed from the blocking solution, washed in distilled H_2O and placed directly into the substrate solution and incubated for 20 min.

After incubation in the substrate solution both strips were removed, washed in distilled H_2O for 10 min and examined for colour development.

4.0 Immunological identification of *N. gonorrhoeae* with monoclonal and polyclonal antibody coagglutination reagents

Clinical isolates of neisseriae were obtained from patients attending the Department of Genitourinary Medicine of the Royal Infirmary of Edinburgh. Anogenital and pharyngeal material was inoculated directly on to Modified New York City (MNYC) medium and transported to the laboratory within 4 h. After overnight incubation at 36°C in air enriched with carbon dioxide (5-10%), cultures were screened for oxidase positive Gram negative diplococci (GNDC). Suspected neisserial colonies (oxidase positive GNDC) were subcultured on to MNYC medium and incubated overnight before carrying out the rapid carbohydrate utilisation and coagglutination tests. If more than one anogenital site from a female patient yielded Gram negative diplococci, cultures from one site only, usually the cervix, were tested by coagglutination.

In the first part of the study all Gram negative diplococci were tested in parallel with polyclonal and monoclonal coagglutination reagents. Thirteen stock cultures of *N. lactamica* and five of β-lactamase producing *Branhamella catarrhalis* were also tested with both types of reagent. During the second half of the study only monoclonal reagents were used, and test performance was compared with bacterial suspensions prepared by boiling and by treatment with antigen releasing agent.

Coagglutination

Coagglutination with polyclonal antibody reagents (Phadebact Gonococus test, Pharmacia Diagnostics AB, Sweden) was performed with a boiled suspension of organism as described by Young and McMillan (1982), except that a 20 µl unit volume was used.

Coagglutination with monoclonal reagents (GONO GEN, New Horizons Diagnostic Company, Columbia, MD) was carried out with boiled suspensions of organisms and with suspensions treated with an antigen releasing agent available from the same source.

4.1 CoA using boiled gonococcal suspensions and an antigen releasing agent

Boiled suspension method

A heavy suspension of each culture to be tested was made by removing the growth from the culture plate with a cotton tipped swab and emulsifying it in 0.2 ml of distilled water in a tube. The tube was covered and the suspension heated in a boiling water bath for 5 min. After cooling to room temperature, 20 µl aliquots of suspension were mixed with an equal volume of test reagent (murine monoclonal anti-protein I antibody bound to dead staphylococci) and control reagent (non-immune rabbit IgG bound to dead staphylococci) on a clear glass slide. Three tests were carried out on a single slide (7.6 cm x 5.0 cm) with six clearly defined areas. The slide was rocked gently in a rotary fashion for 1 min and the results read against a dark background using indirect light.

Antigen releasing agent

Suspensions of each test organism were made on a glass slide by mixing several colonies with 20 µl volumes of antigen releasing agent

(diluted 1/5 with distilled water). Test reagent (20 µl) and control reagent (20 µl) were mixed with the suspensions and the slides rocked and read as above.

Results were interpreted as follows:

Positive	-	clumping or agglutination with test reagent significantly stronger than with control reagent.
Equivocal	-	clumping or agglutination slightly stronger with test reagent than with control reagent.
Non-specific	-	reaction of equal strength with test and control reagents.
Negative	-	no reaction with the test reagent irrespective of any reaction with the control reagent.

Calculation of sensitivity and specificity were as follows:

Sensitivity	-	percentage of gonococcal isolates by carbohydrate utilisation that were coagglutination positive.
Specificity	-	the percentage of non-gonococcal isolates by carbohydrate utilisation that were coagglutination negative.

4.2 Calculation of predictive values

Predictive values were calculated according to the formula given by Veehio (1960).

The predictive value of a positive test (PV^+) is the probability that the Gram negative diplococci giving a positive coagglutination test are gonococci and is calculated according to the formula:

$$PV^+ \quad \frac{pa}{pa + (1-p)(1-b)} \quad x \quad 100$$

The predictive value of a negative test (PV^-) is the probability that the Gram negative diplococci giving a negative coagglutination test are non-gonococcal neisseriæ (or Branhamella) and the formula is:

$$PV^- \quad \frac{(1-p)b}{(1-p)b + p(1-a)} \quad x \quad 100$$

where p = prevalence of gonococcal Gram negative diplococci within the total population of Gram negative diplococci isolated from a given site, a = test sensitivity, and b = test specificity.

The significance of differences in the results was determined by the χ^2 test with Yates' correction.

RESULTS

SECTION I: CHARACTERISATION OF GONOCOCCAL STRAINS BY CoA SEROGROUPING AND CELL ENVELOPE PHENOTYPING

1.0 Characterisation by CoA serogrouping

Table 3 shows the titres of the rabbit antisera raised against the seven MOMP reference strains E-5, D-4, V-15, N-10, S-12, U-14 and F-6. All except anti F-6 had titres in the range of 1/256 to 1/4096. Anti F-6 had a titre of 1/64.

TABLE 3: Titres of antisera raised against gonococcal MOMP reference strains.

Antiserum against MOMP	[1]IF titre against homologous strain
E-5	1 : 4096
D-4	1 : 4096
V-15	1 : 1024
N-10	1 : 1024
S-12	1 : 256
U-14	1 : 1024
F-6	1 : 64

[1]IF - Immunoflourescence

1.1 Distribution of CoA serogroups over four consecutive time periods and correlation of the reaction patterns obtained with the CoA reagents

Table 4 shows the overall distribution of CoA serogroups for gonococcal strains isolated over the four separate time periods spanning two years. Overall 311 (41.8%), 372 (49.9%), 61 (8.2%) and 1 (0.1%) of strains belonged to CoA serogroups WI, WII, WII/WIII and WIII respectively.

TABLE 4: Overall distribution of CoA serogroups for strains isolated from infections acquired in Edinburgh for the four time periods.

period	Number (percentage) of strains belonging to serogroup WI	WII	WII/WIII	WIII	Total
1	85 (44)	110 (56)	0	0	195
2	89 (44)	108 (53)	7 (3)	0	204
3	60 (46)	53 (40)	18 (14)	0	131
4	77 (35.8)	101 (47)	36 (16.7)	1 (0.5)	215
Total	311 (41.8)	372 (49.9)	61 (8.2)	1 (0.1)	745

The number of WI strains isolated dropped by approximately 10% in the fourth study period: 35.8% compared with 44% in periods 1 and 2 and 46% in period 3. This change in proportion of WI strains in period 4 is significant when compared with periods 1-3 inclusive ($\chi^2_1 = 4.035$; $p < 0.05$). In period 1 none of the strains reacted with both the WII reagents and WIII reagent whereas in periods 2, 3 and 4 there were 7, 18 and 36 serogroup WII/WIII strains respectively (Table 4).

Only one gonococcal strain isolated from infections acquired in the Edinburgh area belonged to serogroup WIII. The increase in the number of WII/WIII strains over the four periods represented a significant change in the distribution of CoA serogroups ($\chi^2_3 = 55.62$; $p < 0.001$). There was no significant change in the distribution of the other serogroups over the four time periods ($\chi^2_6 = 9.85$; $0.5 > p > 0.1$).

Reaction patterns obtained

Table 5 shows the CoA reaction patterns obtained with the gonococcal strains isolated from infections acquired in the Edinburgh area. Thirty-one of the 311 WI strains were not tested fully because of a shortage of the CoA WI reagents.

TABLE 5: Reaction patterns of gonococcal strains with CoA reagents defining CoA serogroups WI and WII/WIII.

CoA reagents	Reaction patterns												
Serogroup WI				1	2	3	4	5					
Anti E-5				+	+	+	+	-					
Anti D-4				+	-	-	+	+					
Anti V-15				+	+	-	-	+					
No. of isolates *(total 280)*				168	52	48	11	1					
Serogroup WII/WIII	6	7	8	9	10	11	12	13	14	15	16	17	18
Anti N-10	+	+	-	+	-	+	-	+	-	+	+	+	-
Anti S-12	+	-	+	-	-	+	+	+	+	+	-	-	-
Anti U-14	+	+	+	-	+	-	-	+	+	-	-	+	-
Anti F-6	-	-	-	-	-	-	-	+	+	+	+	+	+
No. of isolates *(total 434)*	279	29	7	22	4	29	2	34	5	7	4	11	1

+ positive; - negative.

A total of 18 CoA reaction patterns were observed during the course of this study: 5 WI patterns, 7 WII patterns and 5 WII/WIII patterns. One strain reacted only with the WIII reagent anti F-6 to give the eighteenth pattern.

Overall, 168/280 (60%) of the WI strains gave pattern 1 with CoA WI reagents, 279/372 (75%) of the WII strains gave pattern 6 with CoA WII reagents and 34/61 (56%) of the WII/WIII strains gave pattern 13 with CoA WII/WIII reagents.

The WI reagent anti E-5 alone detected 99.6% (279/280) of the WI strains and the WII reagent anti N-10 detected 95.8% (415/433) of the WII and WII/WIII strains.

In the last two periods difficulty was encountered in serogrouping eight strains. These strains did not react with the WI reagents but

gave very weak reactions with one or more of the WII reagents and were subsequently placed in serogroup WII.

1.2 Distribution of CoA serogroups among homosexual men and heterosexual men and women

The overall distribution of CoA serogroup among the three patient groups is shown in Table 6. The majority of gonococcal strains isolated from homosexual men belonged to CoA serogroup WII and WII/WIII, 102 (94%) compared with 151 (53%) and 180 (51%) isolates from heterosexual men and women respectively. Only 6% of isolates from homosexual men belonged to serogroup WI compared with 47% and 48.9% of isolates from heterosexual men and women respectively. The difference in distribution of serogroups between homosexual men and heterosexual men is highly significant ($\chi^2_1 = 55.99$; $p < 0.001$) as is the difference between homosexual men and heterosexual women ($\chi^2_1 = 63.60$; $p < 0.001$).

There were no statistically significance differences in the overall distribution of serogroups for strains isolated from heterosexual men and women ($\chi^2_1 = 0.23$; $p > 0.5$).

Table 7 shows the distribution of the CoA serogroups among these three patient groups for each of the four time periods.

Homosexual men

WI strains were isolated from homosexual men only in period 1 and accounted for 10% of the strains isolated. No WII/WIII strains were isolated in period 1 compared with 4 (33%), 2 (18%) and 7 (27%) in periods 2, 3 and 4 respectively. No WIII strains were isolated from this group of patients. The difference in the distribution of serogroups for strains isolated over the four periods is significant ($\chi^2_6 = 22.78$; $p < 0.001$): when the WII/WIII isolates are excluded the difference is not significant ($\chi^2_3 = 6.00$; $0.5 > p > 0.1$).

TABLE 6: Overall distribution of CoA serogroups among homosexual men and heterosexual men and women.

Patient group	Number (and percentage) of strains within serogroup				
	WI	WII	WII/WIII	WIII	Total
Homosexual men	6 (6)	89 (82)	13 (12)	0	108
Heterosexual men	132 (47)	130 (46)	21 (7)	0	283
Women	173 (48.9)	153 (43.2)	27 (7.6)	1 (0.3)	354
Total	311 (41.8)	372 (49.9)	61 (8.2)	1 (0.1)	745

TABLE 7: Distribution of CoA serogroups among homosexual men and heterosexual men and women for each of the four time periods.

Patient group	Time period	Number (and percentage) of strains within CoA serogroup				
		WI	WII	WII/WIII	WIII	Total
Homo-sexual men	1	6 (10)	53 (90)	0	0	59
	2	0	8 (67)	4 (33)	0	12
	3	0	9 (82)	2 (18)	0	11
	4	0	19 (73)	7 (27)	0	26
Hetero-sexual men	1	37 (66)	19 (34)	0	0	56
	2	38 (41)	52 (57)	2 (2)	0	92
	3	20 (44)	20 (44)	5 (11)	0	45
	4	37 (41)	39 (43)	14 (16)	0	90
Women	1	42 (53)	38 (47)	0	0	80
	2	51 (51)	48 (48)	1 (1)	0	100
	3	40 (53)	24 (32)	11 (15)	0	75
	4	40 (40.4)	43 (43.4)	15 (15.2)	1 (0.3)	99
Total		311 (41.8)	372 (49.9)	61 (8.2)	1 (0.1)	745

Heterosexual men

In the first period 66% (Table 7) of gonococcal strains isolated from heterosexual men belonged to CoA serogroup WI whereas in periods 2, 3 and 4 this number dropped by approximately 20-25% to 41%, 44% and 41% respectively. Concurrently the number of WII strains increased. A similar increase in the number of gonococcal strains grouped as WII/WIII was observed in heterosexual men as was observed for strains isolated from homosexual men. No WIII strains were isolated from heterosexual men who had acquired their infections in Edinburgh. The reduction in the number of WI and increase in WII and WII/WIII strains isolated from this group of patients resulted in a significant difference in the distribution of serogroups over the four periods (χ^2_6 =26.32; $p < 0.001$). When WII/WIII strains are excluded from the analysis the proportion of WI and WII strains over these periods remains significantly different ($\chi^2_3 = 9.88$; $0.05 > p > 0.02$).

Women

In periods 1 and 2 the distribution of CoA serogroups WI and WII was very similar (Table 7). In period 3 the number of WI strains was similar to the number isolated in periods 1 and 2 but the number of WII strains decreased and 11 (15%) WII/WIII strains were isolated. In period 4 only 40.4% of strains belonged to serogroup WI compared with 51% in period 2 and 53% in periods 1 and 3. When the proportion of WI strains in period 4 is compared with the proportion of WI strains in periods 1 to 3 the difference just fails to reach significance ($\chi^2_1 = 3.48$; $0.1 > p > 0.05$). In period 4, 15.2% of the isolates belonged to serogroup WII/WIII and one isolate was a WIII strain.

The difference in distribution of the serogroups over the four periods was significant ($\chi^2_9 = 31.76$; $p < 0.001$). However when the WII/WIII strains are excluded the difference is not significant ($\chi^2_6 = 7.68$; $0.5 > p > 0.1$).

1.3 Serogroup in relation to anatomical site of isolation

Table 8 shows the distribution of serogroups in relation to anatomical site of isolation for each patient group over periods 1-4 inclusive.

TABLE 8: Distribution of serogroups in relation to anatomical site of isolation.

Patient group	Anatomical site	Number (and percentage) of strains within serogroup WI	WII	WII/WIII	WIII	Total
Women	Throat	25 (52)	21 (44)	2 (4)	0	48
	Rectum	38 (48)	33 (42)	8 (10)	0	79
	Genital	109 (48.2)	99 (43.8)	17 (7.5)	1 (0.5)	226
	Others	[1]1	0	0	0	1
Hetero-sexual men	Throat	9 (35)	16 (62)	1 (4)	0	26
	Urethra	123 (48)	114 (44)	20 (8)	0	257
Homo-sexual men	Throat	3 (12)	19 (72)	3 (12)	0	25
	Rectum	2 (4)	45 (83)	7 (13)	0	54
	Urethra	1 (4)	25 (86)	3 (10)	0	29
Total		311	372	61	1	745

[1] right hip aspirate

In homosexual men serogroups WII and WII/WIII predominate regardless of the anatomical site of isolation.

In women gonococcal strains isolated from the rectum and genitalia (urethra, cervix and Bartholins glands) are distributed among the CoA serogroups in similar proportions (Table 8). However a higher

proportion of throat isolates belonged to serogroup WI compared with rectal or genital isolates: 52% compared with 48% and 48.2% respectively. Slightly fewer WII/WIII strains were isolated from the throat than either the rectum or genitalia: 4% compared with 10% and 7.5% respectively. However these differences in distribution of serogroups among strains isolated from different anatomical sites in women are not significant ($\chi^2_6 = 2.16$; $p > 0.5$). The majority of women had strains of the same serogroup infecting each anatomical site. However both WI and WII strains were isolated from the rectum of one woman and in a second woman a WI strain was isolated from her rectum whereas a WII strain was isolated from her cervix.

In heterosexual men there is no significant difference in the distribution of serogroups among strains isolated from the throat compared with those isolated from the urethra ($\chi^2_2 = 2.91$; $0.5 > p > 0.1$). However fewer WI strains were isolated from the throat.

The distribution of CoA serogroups of genital strains isolated from women is very similar to that for male heterosexual urethral isolates (Table 8).

1.4 Correlation of CoA serogroup with presence of symptoms and oral contraception in women

1.4.1 Serogroup in relation to symptoms

Table 9 shows the correlation of symptoms with serogroup of infecting gonococcal strain for 64 women. Overall 38/64 (59%) of the women had asymptomatic gonococcal infections. Of the 26 women presenting with symptoms, 19 had vaginal discharge not associated with other vaginal pathogens such as *Candida* or *Trichomonas*, three had dysuria, three had pelvic inflammatory disease and one had a disseminated gonococcal

infection. Fifteen (58%) of the symptomatic women had infections with gonococcal strains of serogroup WI compared with 18 (47%) of asymptomatic women: this difference is not statistically significant ($\chi^2_1 = 0.31$; $p > 0.5$).

TABLE 9: Correlation of serogroup of infecting strain with symptoms in women.

Symptoms present	Number (percentage) of infecting strains within serogroups WI	WII	Total
Yes	15 (58)	11 (42)	26
No	18 (47)	20 (53)	38
Total	33 (52)	31 (48)	64

1.4.2 Serogroup in relation to women taking oral contraceptives

Table 10 shows the correlation of serogroup of infecting strain in women taking oral contraceptives. Overall 41/64 (64%) of the women were taking oral contraceptives. Of these women 25 (61%) had infections with strains of CoA serogroup WI compared with 8/23 (35%) of women not taking oral contraceptives. However this difference fails to reach significance ($\chi^2_1 = 3.06$; $0.1 > p > 0.05$).

TABLE 10: Correlation of serogroup of infecting strain with oral contraception in women.

Oral contraception	Number (percentage) of infecting strains within serogroups WI	WII	Total
Yes	25 (61)	16 (39)	41
No	8 (35)	15 (65)	23
Total	33	31	64

1.5 Comparison of CoA serogroup of strains isolated from 22 women and their respective partners

The serogroup of gonococcal isolates from 20 women and their respective partners, one woman and her two partners and one woman, her husband and child were compared. The serogroup of isolates from each respective set of contacts were found to match (Table 11).

With regards to the reaction patterns obtained with the CoA reagents all strains isolated matched in 12/22, at least one female isolate matched with the respective male isolate in 4/22 and none of the female isolates matched the respective male isolate in 6/22 sets of contacts.

1.6 CoA serogroups of cloned gonococcal colonies

All clones were found to have the same serogroup as the parent culture.

1.7 Distribution of CoA serogroups among gonococcal strains acquired outwith Edinburgh

A total of 45 patients acquired their infections outwith the Edinburgh area: 12 in other areas in the United Kingdom, 12 in Europe and 21 in non-European countries.

Table 12 shows the area the infection was acquired, patient group and CoA serogroup of gonococcal isolates. Of the 12 strains isolated from infections acquired in other areas of the United Kingdom, 2 (17%), 9 (75%) and 1 (8%) belonged to serogroups WI, WII and WII/WIII respectively. Five of the WII strains were isolated from patients who had acquired their infections in London; two of these patients were homosexual men.

TABLE 11: CoA serogroup and reaction pattern of gonococcal strain isolated from 22 women and their respective partners.

Key: W - women, M - male, B - baby; Cx - cervix, R - rectum, U - urethra, F - throat.

Contact pairs	Sex of patient	Site of isolation	CoA serogroup	Reaction pattern
1	W	Cx	WII	13
		F	WII	17
		R	WII	13
	M	U	WII	7
2	W	Cx	WI	3
		U	WI	1
	M	U	WI	3
3	W	R	WII	7
	M	U	WII	6
4	W	Cx	WII	6
	M	U	WII	6
5	W	U	WI	1
	M	U	WI	1
6	W	Cx	WII	6
	M	U	WII	6
7	W	Cx	WI	3
		F	WI	2
	M	F	WI	1
8	W	Cx	WI	1
		R	WI	1
		U	WI	1
		F	WI	1
	M	U	WI	3
9	W	Cx	WII	6
	M1	U	WII	6
	M2	U	WII	6
10	W	Cx	WI	2
		R	WI	1
	M	U	WI	3
11	W	R	WII	6
	M	U	WII	6
		F	WII	6
12	W	Cx	WI	1
	M	U	WI	1
13	W	Cx	WII	11
	M	U	WII	6
14	W	Cx	WII	6
	M	U	WII	6
	B	right eye	WII	6
		left eye	WII	6

TABLE 11 (cont.)

Contact pairs	Sex of patient	Site of isolation	CoA serogroup	Reaction pattern
15	W	Cx	WII	7
	M	R	WII	7
16	W	Cx	WI	1
		R	WI	4
	M	U	WI	1
17	W	Cx	WI	4
	M	R	WI	3
18	W	U	WI	3
		Cx	WI	1
	M	U	WI	1
19	W	Cx	WI	1
	M	U	WI	1
20	W	F	WII	6
		Cx	WII	6
		U	WII	6
	M	U	WII	6
21	W	U	WII	6
		Cx	WII	6
	M	U	WII	6
22	W	Cx	WII	9
	M	Cx	WII	9

TABLE 12: Gonococcal infections acquired outwith Edinburgh.

Area infection acquired	Patient group	Number of patients	CoA serogroup of isolates			
			WI	WII	WII/WIII	WIII
Other areas in:						
Perthshire	Male	1	0	1	0	0
Inverness	Male	2	1	1	0	0
Wales	Female	1	1	0	0	0
Northern Ireland	Male	1	0	0	1	0
England	Male	1	0	1	0	0
Torquay	Male	1	0	1	0	0
London	Female	2	0	2	0	0
	Homosexual Male	2	0	2	0	0
	Male	1	0	1	0	0
	Total	*12*	*2*	*9*	*1*	*0*
Europe:						
Spain	Female	2	2	0	0	0
	Homosexual Male	1	1	0	0	0
	Male	4	0	3	0	1
Italy	Male	1	1	0	0	0
Holland	Male	1	0	1	0	0
Greece	Male	1	0	1	0	0
Germany	Homosexual Male	1	0	1	0	0
	Male	1	0	0	1	0
	Total	*12*	*4*	*6*	*1*	*1*
Non-Europe:						
Thailand	Male	9	0	6	2	1
	Homosexual Male	1	0	1	0	0
Singapore	Male	1	0	1	0	0
Hong Kong	Male	3	0	3	0	0
Sri Lanka	Male	1	0	1	0	0
Nigeria	Male	1	0	0	0	1
Kenya	Male[1]	1	0	2	0	0
Morocco	Male	1	0	1	0	0
Trinidad	Female	1	0	1	0	0
Antigua	Male	2	0	2	0	0
	Total	*21*	*0*	*18*	*2*	*2*

[1]Two strains isolated: one PPNG, one non-PPNG.

Of the 12 strains isolated from infections acquired in Europe, 4 (34%), 6 (50%), 1 (8%) and 1 (8%) belonged to serogroups WI, WII, WII/WIII and WIII respectively. Strains isolated from infections acquired in Spain appear to cover the full range of serogroups.

None of the strains isolated from infections acquired in non-European countries belonged to CoA serogroups WI: 18/22 (82%), 2/22 (9%) and 2/22 (9%) belonged to serogroups WII, WII/WIII and WIII respectively. Only one of these strains was isolated from a homosexual male, the rest being isolated from heterosexual patients.

2.0 Characterisation of gonococcal isolates by cell envelope phenotyping

2.1 Overall distribution of cell envelope phenotypes and correlation of phenotype of infecting strain with patient group

A total of 482 gonococcal strains isolated from infections acquired in the Edinburgh area were phenotyped. Table 13 shows the distribution of cell envelope phenotypes among homosexual men and heterosexual men and women. Overall 24 (5%), 417 (87%) and 41 (8%) were of the Env, wild type, and Mtr cell envelope phenotypes respectively. Of the strains isolated from homosexual men 17/47 (36%) had an Mtr phenotype compared with 5% and 6% from heterosexual men and women. No gonococcal strains with an Env phenotype were isolated from homosexual men. There is a highly significant difference in the proportion of Env/wild type and Mtr phenotypes when isolates from homosexual men are compared with those from heterosexual men ($x^2_1 = 36.56$, $p < 0.001$) and with those from women ($x^2_1 = 38.19$; $p < 0.001$). There is no such difference when isolates from heterosexual men are compared with those from women ($x^2_1 = 0.33$; $p > 0.5$).

TABLE 13: Distribution of cell envelope phenotypes among isolates from homosexual men and heterosexual men and women.

Patient group	Number (percentage) of isolates with the cell envelope phenotype: Env	Wild type	Mtr	Total
Homosexual men	0	30 (64)	17 (36)	47
Heterosexual men	12 (6)	176 (89)	9 (5)	197
women	12 (5)	211 (89)	15 (6)	238
Total	24 (5)	417 (87)	41 (8)	482

2.2 Correlation of cell envelope phenotype with patient group and anatomical site of isolation

Table 14 shows the distribution of cell envelope phenotypes of strains in relation to anatomical site of isolation and patient group.

TABLE 14: Cell envelope phenotype in relation to patient group and site of isolation.

Patient group	Site of isolation	Number (percentage) of strains with cell envelope phenotype: Env	Wild type	Mtr	Total
Homosexual men	U	0	9 (53)	8 (47)	17
	R	0	17 (81)	4 (19)	21
	F	0	4 (44)	5 (55)	9
Heterosexual men	U	12 (7)	159 (88)	9 (5)	180
	F	0	17 (100)	0	17
women	G	7 (4)	145 (92)	6 (4)	158
	R	2 (4)	47 (87)	5 (9)	54
	F	3 (12)	19 (73)	4 (15)	26
Total		24 (5)	417 (87)	41 (8)	482

Key: U - urethra; R - rectum; F - throat; G - genital.

A significantly higher proportion of urethral isolates from homosexual men had an Mtr phenotype compared with urethral isolates from heterosexual men ($\chi^2_1 = 29.71$; $p < 0.001$) and with female genital isolates ($\chi^2_1 = 33.37$; $p < 0.001$). There is no significant difference in the proportion of Mtr phenotypes isolated from the rectum of homosexual men compared with the number of Mtr phenotypes isolated from the rectum of women ($\chi^2_1 = 0.60$; $0.5 > p > 0.1$).

A higher proportion of Mtr phenotypes were isolated from the urethra and the throat than from the rectum of homosexual men, however this failed to reach significance ($\chi^2_1 = 3.57$; $0.1 > p > 0.05$).

A significantly higher proportion of homosexual male throat isolates had an Mtr phenotype compared with throat isolates from heterosexual men and women ($\chi^2_1 = 8.13$; $p < 0.01$).

2.3 Relationship of cell envelope phenotype with CoA serogroups

Table 15 shows the distribution of envelope phenotype in relation to CoA serogroup and patient group.

Serogroup WII (including WII/WIII) accounted for 100% of isolates from homosexual men, 58% from heterosexual men and 52% from women. With regards cell envelope phenotype, serogroup WII accounted for 34/40 (85%) of Mtr isolates compared with 245/435 (56%) of the non-Mtr strains. This is a significant difference ($\chi^2_1 = 17.33$; $p < 0.001$). One Mtr strain belonged to serogroup WIII.

The Mtr phenotype occurred significantly more often among WII isolates from homosexual men than among WII isolates from heterosexual men ($\chi^2_1 = 17.47$; $p < 0.001$) and women ($\chi^2_1 = 15.67$; $p < 0.001$).

A significantly higher proportion of Mtr strains compared with wild type strains isolated from heterosexual patients belonged to CoA

serogroup WII or WII/WIII ($\chi^2_1 = 4.25$; $0.05 > p > 0.02$). However, there was no significant difference in the distribution of CoA serogroups among Env and wild type strains isolated from heterosexual patients ($\chi^2_1 = 1.36$; $0.5 > p > 0.1$).

TABLE 15: Relationship of cell envelope phenotype with CoA serogroup and patient group.

Patient group	CoA serogroup	Number (percentage) of strains with cell envelope phenotype: Env	Wild type	Mtr	Total
Homosexual men	WI	0	0	0	0
	WII or WII/WIII	0	30 (65)	16 (35)	46
Heterosexual men	WI	7 (8.4)	75 (90.4)	1 (1.2)	83
	WII or WII/WIII	5 (4)	100 (89)	8 (7)	113
women	WI	7 (6)	101 (90)	4 (4)	112
	WII or WII/WIII	4 (3)	106 (89)	10 (8)	120
	WIII	0	0	1	1
Total		23 (5)	412 (87)	40 (8)	475

2.4 Cell envelope phenotypes and serogroups of gonococci isolated from infections acquired outwith the Edinburgh area

A total of 24 strains were acquired outwith the Edinburgh area either elsewhere in the United Kingdom or abroad (Table 16). Strains with an Mtr phenotype accounted for 13/24 (54%) compared with 41/482 (8%) of Edinburgh isolates. Of these, six were acquired in Thailand, three in Hong Kong, one in Antigua, one in Spain and two in London. Only three of the 13 (23%) Mtr phenotype strains were isolated from homosexual men, the remaining 10 (77%) being isolated from heterosexual men.

TABLE 16: Envelope phenotype and serogroup of gonococcal isolates from male infections acquired outwith the Edinburgh area.

Area infection acquired	Envelope phenotype	CoA serogroup	Patient group
Europe:			
Spain	Mtr	WIII	Heterosexual
London	Wild type	WII	"
England	Wild type	WII	"
Northern Ireland	Wild type	WII	"
Inverness	Wild type	WII	"
Spain	Wild type	WII	Homosexual
	Wild type	WI	"
London	Wild type	WII	"
	Mtr	WII	"
	Mtr	WII	"
Germany	Wild type	WII	"
Non-Europe:			
Thailand	Mtr	WII	Heterosexual
	Mtr	WII	"
	Mtr	WII	"
	Mtr	WII/WIII	"
	Mtr	WII	"
Hong Kong	Mtr	WII	"
	Mtr	WII	"
	Mtr	WII	"
Antigua	Mtr	WII	"
	Wild type	WII	"
Morocco	Wild type	WII	"
Kenya	Wild type	WII	"
Thailand	Mtr	WII	Homosexual

All isolates except one belonged to CoA serogroups WII, WII/WIII or WIII. The WI isolate (wild type phenotype) was isolated from a homosexual man who had acquired his infection in Spain. The difference in distribution of Env/wild type and Mtr phenotypes when isolates acquired outwith Edinburgh are compared with those acquired in Edinburgh is significant ($\chi^2_1 = 45.32$; $p < 0.001$).

Three of the 13 Mtr strains were isolated from infections acquired in Europe, two of the three being isolated from homosexual men. Nine of the 10 Mtr strains isolated from infections acquired in non-European countries were isolated from heterosexual men and one was isolated from a homosexual male. All of these strains belonged to CoA serogroup WII or WII/WIII.

A total of 12 gonococcal strains were isolated from heterosexual men who acquired their infections in non-European countries (Table 16). Nine of the 12 strains (75%) had an Mtr phenotype compared with 9/197 (5%) of strains isolated from heterosexual men who had acquired their infections in Edinburgh (Table 13). This is a significant difference ($\chi^2_1 = 52.62$; $p < 0.001$). The number of Mtr strains isolated from heterosexual men who acquired their infections in non-European countries is also significantly higher than the number of Mtr strains isolated from homosexual men who acquired their infections in Edinburgh: 9/12 (75%) compared with 17/47 (36%) (Table 13) ($\chi^2_1 = 4.37$; $0.05 > p > 0.02$).

A much higher proportion of serogroup WII strains was isolated from heterosexual men who acquired their infections in non-European countries compared with heterosexual men who acquired their infections in Edinburgh: 11/12 (92%) (Table 16) compared with 113/196 (58%) (Table 15) ($\chi^2_1 = 4.1$; $0.05 > p > 0.02$). The proportion of WII strains isolated from these heterosexual men was similar to that for homosexual men who had acquired their infections in Edinburgh: 11/12 (92%) (Table 16) compared with 46/46 (100%) (Table 15).

SECTION II: NON-CULTURAL DETECTION AND IDENTIFICATION OF *N. GONORRHOEAE*

1.0 Preparation of antisera against MOMP E-5 and N-10 protein I antigen

SDS-PAGE results

Figure 12 shows the results of the SDS-PAGE for sample A (Lane 4) from MOMP E-5 and sample C from MOMP N-10 (Lane 1). These samples were used for immunising rabbits against protein I antigen and correspond to peaks A and C after column chromatography of Empigen soluble material (ESM) purified from MOMP E-5 and N-10 respectively (Figures 9 and 10). These samples should, according to the method of James and Heckels (1981) contain purified protein I. Samples B and D corresponding to peaks B and D (Figures 9 and 10) and samples of the ESM purified from MOMP E-5 and N-10 prior to column chromatography were run for comparison (Figure 12, Lanes 5, 2, 6 and 3). According to the method of James and Heckels (1981), peaks B and D should correspond to protein II.

Lane 7 contained molecular weight standards comprising six polypeptide bands corresponding to molecular weights of 77,000, 66,200, 45,000, 25,000, 17,200 and 12,300. The relative mobility of each polypeptide band was calculated according to the following formula:

$$\text{percentage relative mobility} = \frac{\text{distance moved by the band front}}{\text{distance moved by bromophenol blue}} \times 100$$

A standard graph was drawn of molecular weight of standards v. percentage relative mobility. This was used to determine the molecular weights of the protein bands in the samples tested. All samples were applied to the wells of the gel at protein concentration of 50 µg.

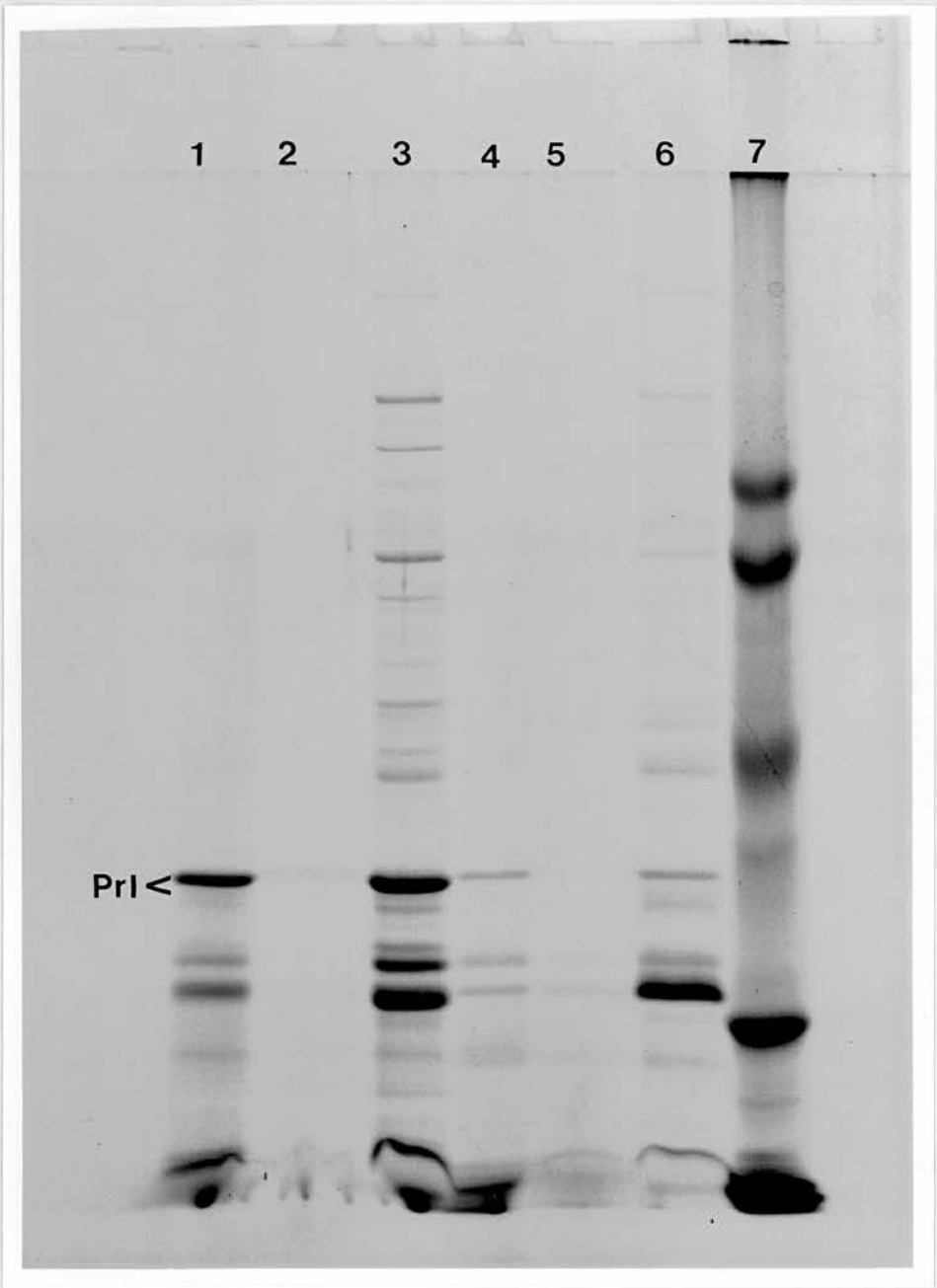

FIGURE 12: SDS-PAGE of proteins extracted from MOMP N-10 and E-5 and purified by column chromatography.

Gel was stained with Coomassie blue for protein. All samples were applied at 50 µg of protein/track.

MOMP N-10: profile 1, sample C; profile 2, sample D; profile 3, ESM.

MOMP E-5: profile 4, sample A; profile 5, sample B; profile 6, ESM

Molecular weight standards: profile 7.

The protein I purified from MOMP N-10 and E-5 had molecular weights of 34,500 and 37,000 (Lanes 1 and 4) and these corresponded to the most heavily staining bands. However, both of these samples C and A have minor staining bands of approximate molecular weights of 30,000 and 28,000. These are thought to correspond to species of protein II. Very faintly staining bands corresponding to these molecular weights can be seen in samples D and B (Lanes 2 and 5) which correspond to the peaks which should contain protein II after column chromatography of the ESM. Obviously column chromatography did not separate protein I and II completely. This was partially observed in the graphs obtained from the OD V fraction number which gave two illdefined peaks from the fractionated ESM from both MOMP E-5 and N-10 (Figures 9 and 10). However the amount of material in samples C and A from MOMP E-5 and N-10 (Lanes 4 and 1) corresponding to these minor proteins is much reduced when compared to the corresponding staining protein bands observed in the ESM from these strains prior to column chromatography (Lanes 6 and 3) (Figure 12).

Column chromatography removed the high molecular weight bands (range 100,000 - 41,000) found in the ESM prior to chromatography.

Figure 13 shows the same samples run in SDS-PAGE at 5 µg of protein per well. Lanes 1 and 4 correspond to samples A and C from MOMP E-5 and N-10. The major bands stained in these lanes correspond to protein I. Very few or none of the minor lower molecular weight proteins are visible in these lanes. However, protein I from MOMP E-5 (Lane 1) stained very faintly (Figure 13).

The second half of this gel containing samples A and C was stained for lipopolysaccharide (LPS) with silver (results not shown). A few bands were observed in each sample, one which corresponded to the

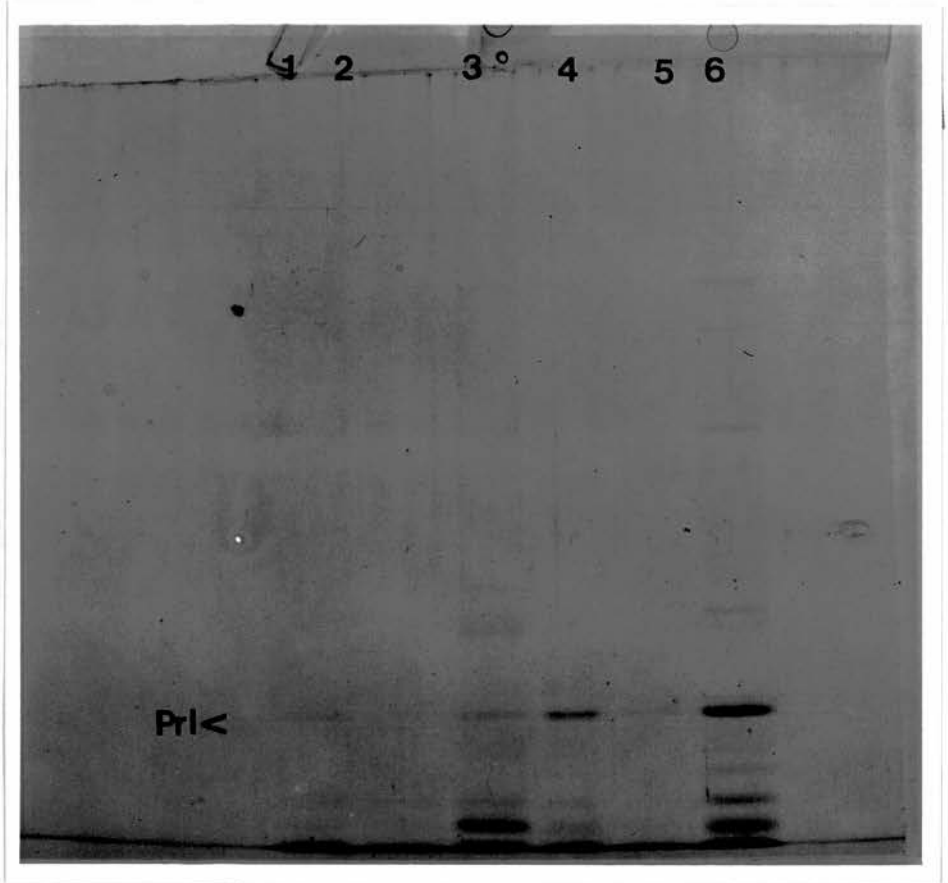

FIGURE 13: SDS-PAGE of proteins extracted from MOMP N-10 and E-5 and purified by column chromatography.

The gel was stained with Coomassie blue for protein. All samples were applied at 5 µg of protein/track.

MOMP E-5: profile 1, sample A; profile 2, sample B; profile 3, ESM.

MOMP N-10: profile 4, sample C; profile 5, sample D; profile 6, ESM.

protein I of MOMP E-5 and N-10 respectively, and a few minor bands of lower molecular weight which appeared to correspond to the low molecular weight bands stained with Coomassie blue. Therefore some LPS appeared to be present in samples A and C, possibly aggregated with the proteins.

Titre of antisera raised against protein I from MOMP E-5 and N-10 (samples A and C)

Table 17 shows the titre of the antisera raised against protein I from MOMP E-5 and N-10. Anti-E-5 protein I antiserum had a titre of 1/1024 and the anti-N-10 protein I antiserum had a four-fold lower titre of 1/256.

TABLE 17: Titres of antisera raised against protein I purified from gonococcal MOMP reference strains E-5 and N-10.

Antiserum against	[1]IF titre against homologous strain
E-5 protein I	1 : 1024
N-10 protein I	1 : 256

[1]IF - immunofluorescence

2.0 Preliminary studies on gonococcal antigen detection by an indirect enzyme linked immunosorbent assay (ELISA) using wheat germ lectin for antigen capture

2.1 Agglutination of MOMP E-5 and N-10 by wheat germ lectin

The results of the wheat germ (WG) lectin agglutination of MOMPs E-5 and N-10 are shown in Table 18. The end point titration for agglutination was taken as the highest dilution of lectin which gave a 2+ reaction. This was found to be 2 µg/ml and 16 µg/ml of lectin for MOMPs E-5 and N-10 respectively. Reaction of the gonococcal suspensions with

TABLE 18: Reaction of MOMP E-5 and N-10 (McFarland's standard 0.5) suspensions with dilutions of wheat germ lectin.

Concentration of wheat germ lectin (µg/ml)	Agglutination with: MOMP E-5	MOMP N-10
500	3+	2+
250	3+	2+
125	3+	2+
63	3+	2+
31	3+	2+
16	3+	2+
8	3+	+
4	3+	-
2	2+	-
PBS control	-	-

3+ - Many small agglutinates with little or no clearing of supernatant.

2+ - Small easily seen agglutinates.

\+ - Agglutination just visible to the eye.

\- - No agglutination.

TABLE 19: Reaction of thick boiled MOMP E-5 and N-10 suspensions with dilutions of wheat germ lectin.

Concentration of wheat germ lectin (µg/ml)	Agglutination with: MOMP E-5	MOMP N-10
500	3+	3+
250	3+	3+
125	3+	2/3+
63	2/3+	2+
31	2+	2+/+
16	+	+
8	2+	-
4	+	-
2	-	-
PBS control	-	-

the lectin dilutions gave very fine particles of agglutination with little or no clearing of the supernatant. No agglutination was observed in the PBS controls.

2.2 Effect of boiling on lectin agglutination of thick gonococcal suspensions

The results of the reactions of the thick boiled MOMP E-5 and N-10 suspensions with lectin dilutions are shown in Table 19. The end point of agglutination was found to be 8 µg/ml and 31 µg/ml of lectin for MOMP E-5 and N-10 respectively. These end points were four- and two-fold higher than with the respective McFarland's standard 0.5 suspensions of E-5 and N-10. However the agglutination produced by reaction of lectin with the thick boiled gonococcal suspensions was easier to read than in the previous experiment.

The thick untreated suspensions of MOMP E-5 and N-10 auto-agglutinated.

The wheat germ lectin reacted best with MOMP E-5, therefore MOMP E-5 was chosen as antigen for use in the ELISA experiments.

2.2.1 Determination of the minimum amount of whole cell antigen detected by polystyrene beads armed with lectin in PBS

MOMP E-5 whole cell antigen ranged from 3 x 10^7 to 29 cfu. Polystyrene beads of sets one and two armed with lectin (3-200 µg/ml) for 2 h at 37°C and 4 h at 37°C respectively and overnight at 4°C were used.

The corrected absorbance (absorbance obtained in wells containing antigen minus the absorbance obtained in control wells with no antigen) was plotted on semilog graph paper against antigen concentration for

each dilution of lectin used. The minimum amount of antigen detected by beads coated with each lectin dilution was determined at two arbitrary cut off points of OD 1.0 and 0.5 (490 nm). The minimum number of gonococci detected with beads of set one and two are shown in Table 20 and results obtained with beads of sets one and two coated with 200 µg/ml of lectin and no lectin (PBS) are shown in Figures 14 and 15 respectively.

At the cut off points chosen the lowest number of gonococci were consistently detected by the control beads (no lectin) of both bead sets one and two (Table 20). Beads coated with less than 50 µg/ml of lectin detected fewer gonococci than beads coated with 100 and 200 µg/ml of lectin.

The curves obtained by plotting absorbance (490 nm) against antigen concentration for untreated beads and beads coated with 200 µg/ml of lectin rapidly fell to an OD of less than 0.1 at 10^5 cfu of gonococci (Figures 14 and 15). Similar observations were made for each of the other lectin dilutions observed. Although the minimum numbers of gonococci detected by the lectin dilutions varied between the two sets of beads (set one coated with lectin for 2 h at 37°C and set two for 4 h at 37°C) a similar trend in the detection was observed for both sets of beads.

2.2.2 *Determination of the minimum amount of sonicated antigen detected by polystyrene beads armed with lectin in carbonate buffer pH 9.6*

The concentration of E-5 sonicated antigen used ranged from 400 µg of protein (equivalent to 9.4×10^7 cfu) down to 0.00038 µg of protein (equivalent to 90 cfu).

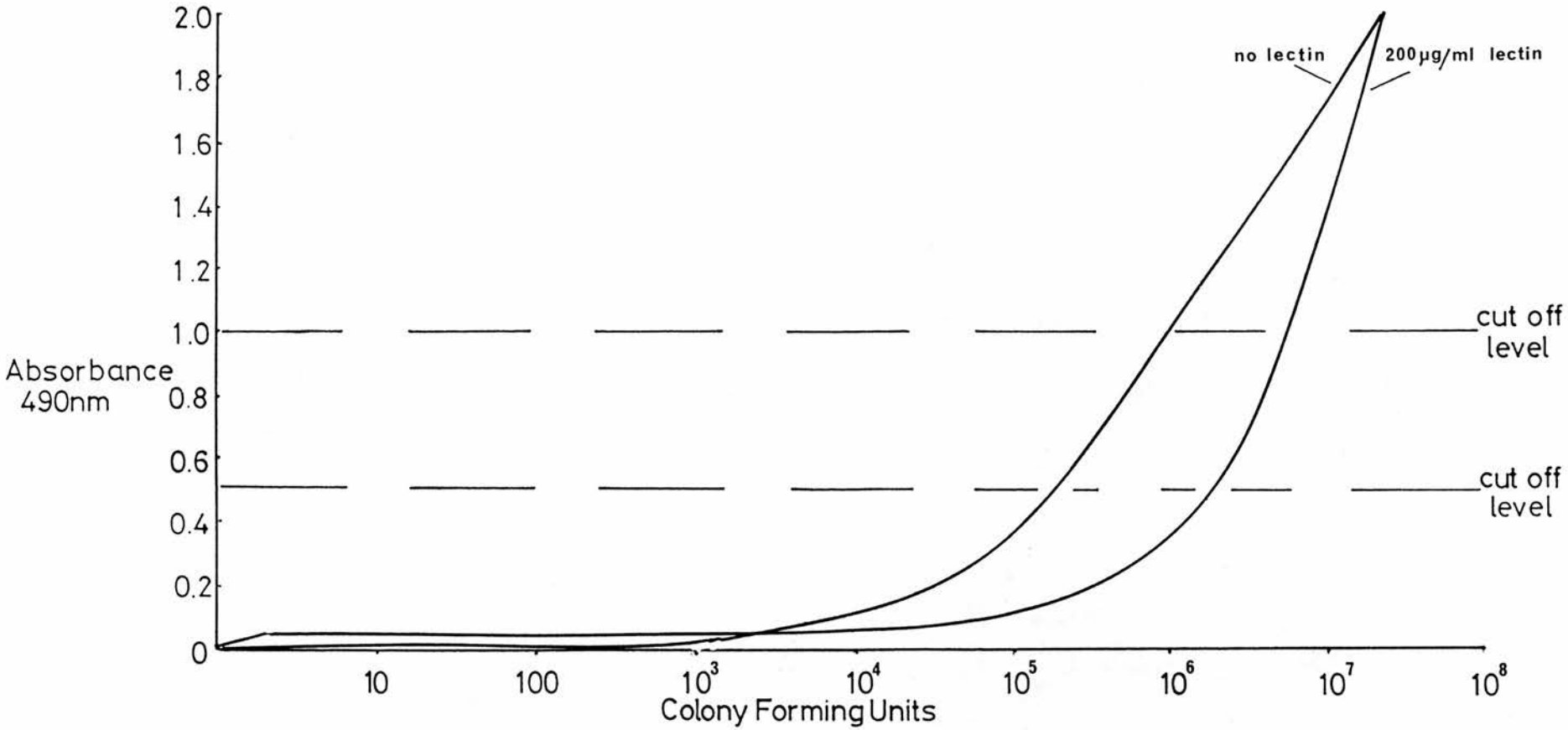

FIGURE 14: Number of organisms detected with untreated beads and beads armed with 200 µg/ml of lectin for 2 h at 37°C and overnight at 4°C (set one).

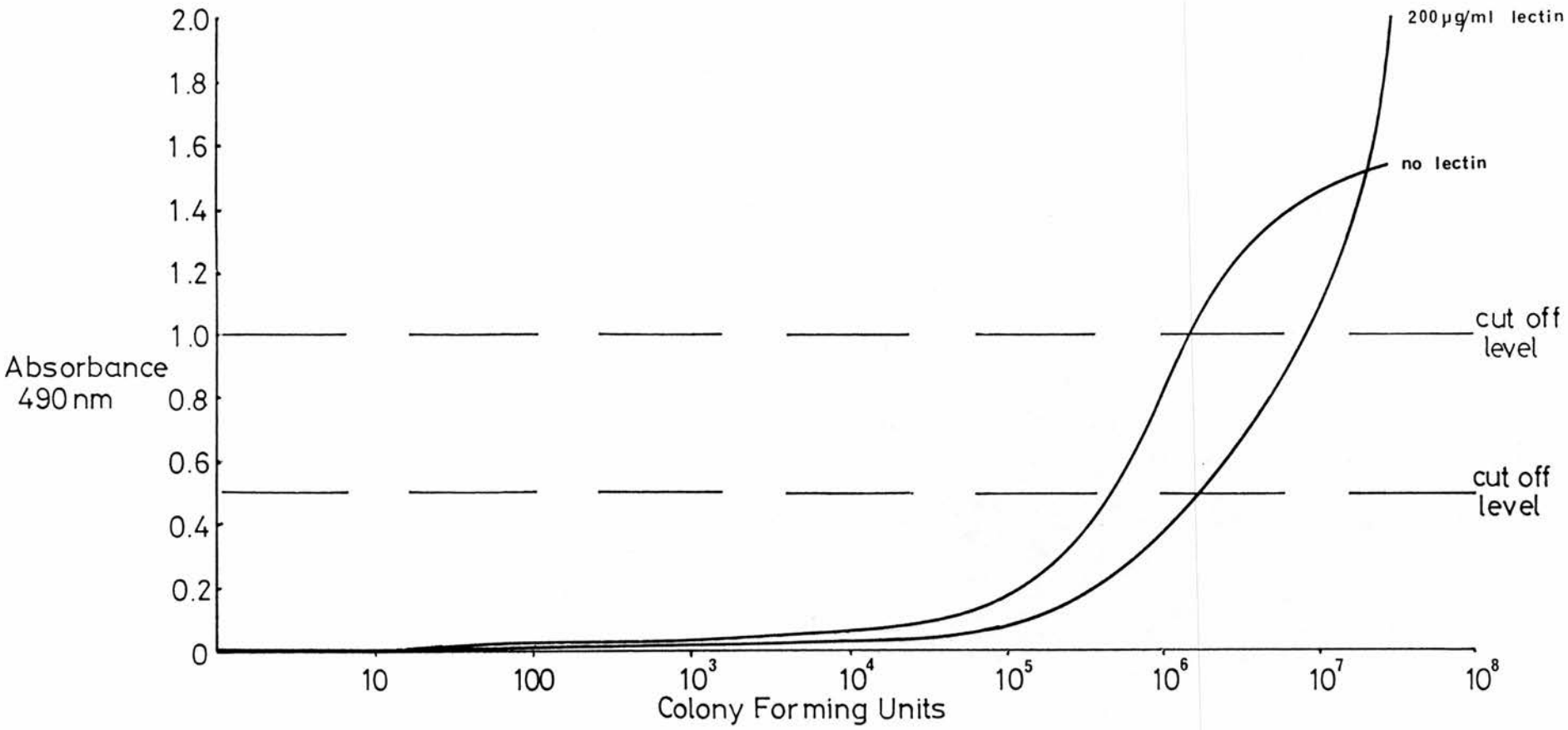

FIGURE 15: Number of organisms detected with untreated beads and beads armed with 200 µg/ml of lectin for 4 h at 37°C and overnight at 4°C (set two).

TABLE 20: Minimum amount of antigen detected by lectin armed polystyrene beads of sets one and two at arbitrary cut off points OD 1.0 and 0.5.

Concentration of lectin µg/ml	Minimum amount of antigen (cfu) detected with polystyrene beads of sets: one		two	
	1.0	0.5	1.0	0.5
200	1.0×10^7	1.5×10^6	7×10^6	1.6×10^6
100	1.0×10^7	6×10^5	8×10^6	1.7×10^6
50	3.5×10^6	8×10^5	6.5×10^6	1.5×10^6
25	2.8×10^6	7×10^5	4.5×10^6	1.2×10^6
12.5	1.5×10^6	3.2×10^5	3×10^6	1.0×10^6
6.0	1.5×10^6	3.5×10^5	3.5×10^6	8.5×10^5
3.0	1.0×10^6	2×10^5	3×10^6	5×10^5
no lectin	7.5×10^5	1.4×10^5	1.7×10^6	5×10^5

TABLE 21: The minimum amount of sonicated E-5 antigen detected with beads coated with lectin in carbonate buffer (pH 9.6) at two arbitrary cut off values.

Concentration of lectin µg/ml	Minimum amount of sonicated antigen (µg of protein) at cut off points	
	1.0	0.5
200	0.8	0.35
100	0.5	0.26
50	0.5	0.27
25	0.5	0.28
12.5	0.7	0.18
6.0	0.55	0.4
3.0	0.52	0.22
no lectin	0.28	0.12

The corrected absorbance (absorbance obtained in wells containing antigen minus the absorbance obtained in control wells containing no antigen) were plotted against protein concentration of the sonicated antigen for each lectin dilution used for coating the beads and for untreated beads. OD 1.0 and 0.5 were chosen as arbitrary cut off points. The minimum amounts of antigen detected at these cut off points by beads coated with lectin are shown in Table 21. Figure 16 shows the results for beads coated with 200 µg/ml of lectin and for untreated control beads.

The amount of antigen detected at OD 1.0 ranged from 0.8 µg to 0.28 µg of protein corresponding to 1.4×10^5 to 1.2×10^5 cfu. The amount detected at OD 0.5 ranged from 0.35 µg to 0.12 µg of protein, corresponding to 7×10^4 to 4×10^4 cfu. As in the previous experiment, beads coated with lower concentrations of lectin detected the lower amounts of antigen. However overall uncoated control beads detected the lowest amount of antigen.

The range of antigen detected with beads coated with lectin in carbonate buffer (pH 9.6) and using a sonicated antigen was better than in the previous experiments using beads coated with lectin in PBS and a whole cell antigen.

The plot of the results for beads coated with 200 µg/ml of lectin and for untreated control beads (Figure 16) gave sigmoidal curves with plateaus at an OD of 2.0, corresponding to approximately $\geq$1.56 µg of protein. However between 0.1 µg and 1.0 µg of protein (10^4 to 10^5 cfu) the absorbance values rapidly fell to approximately an OD of 0.1. Similar observations were made for each of the other lectin dilutions. The straight part of the curve for the uncoated control beads was less steep than that of the curve obtained with beads coated with 200 µg/ml of lectin.

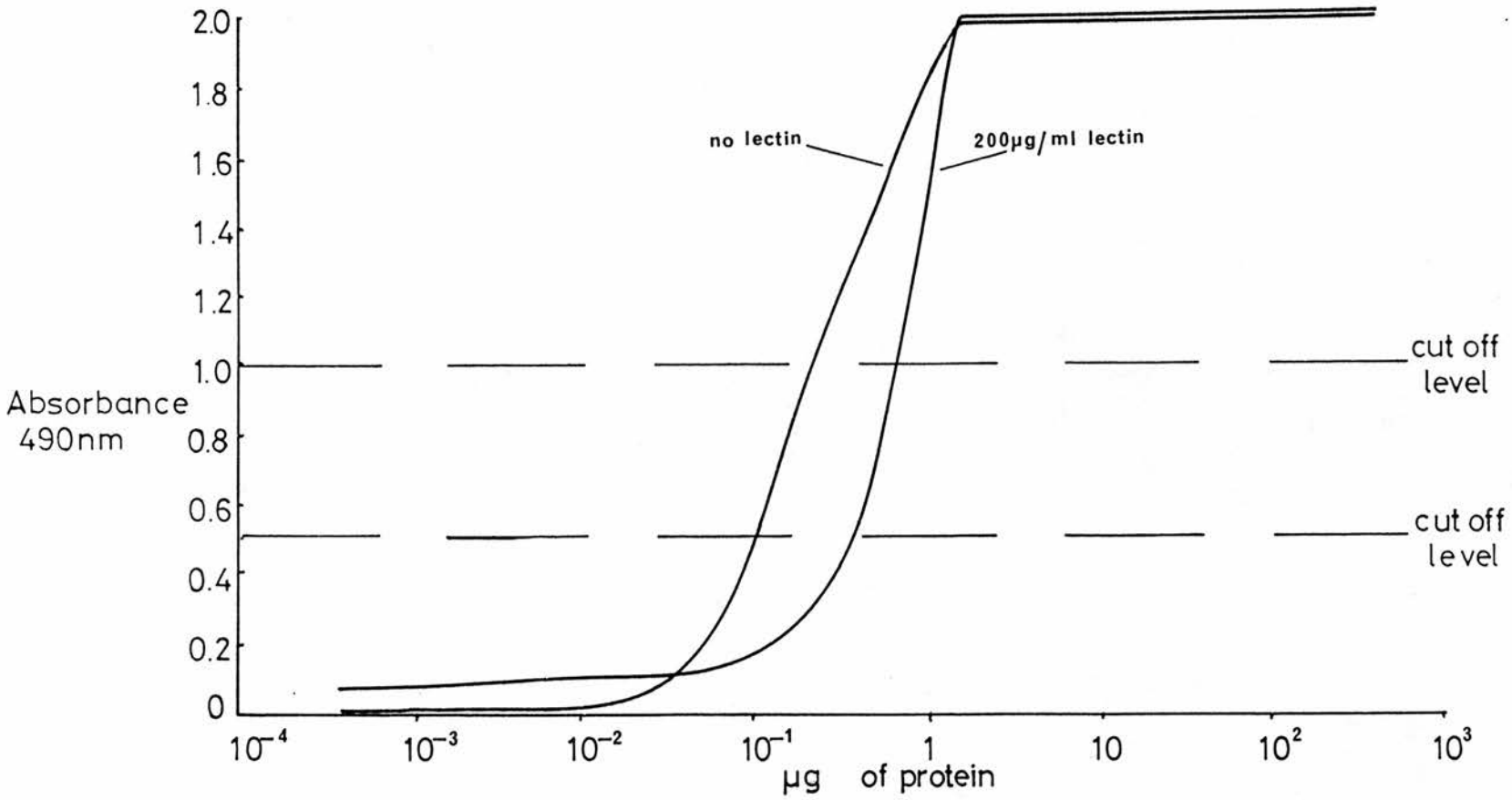

FIGURE 16: Concentration of sonicated MOMP E-5 antigen detected by untreated beads and beads coated with 200 µg/ml of lectin in carbonate buffer.

3.0 Gonococcal antigen detection on nitrocellulose by an indirect enzyme linked immunosorbent assay (Dot-Blot immunoassay)

3.1 Detection of (A) untreated whole cell, (B) boiled and (C) sonicated MOMP E-5 antigen by anti-E-5 antiserum

Table 22 shows the results of the dot-blot immunoassay with dilutions of MOMP E-5 antigen suspensions A, B and C, using a 1/50 dilution of anti-E-5 for antigen detection.

The viable count for the MOMP E-5 antigen suspension (1.75×10^5 cfu/ml) was much lower than the expected 1×10^9 cfu/ml for a suspension of OD 1.0 (A540 nm). The loss of viability may have been due to suspension of the gonococci in TSB. Therefore antigen concentrations (Table 22) are given as the expected cfu for a suspension of OD 1.0. These ranged from 10^6 cfu for undiluted antigen to 1×10^2 cfu for antigen of 1/1024 dilution.

The last dilution of antigen to give a 1+ reaction was chosen as the end point.

The minimum amount of MOMP E-5 antigen preparations A (untreated), B (boiled) and C (sonicated) detected in the assay were 3.2×10^4, 1.6×10^4 and 6.5×10^4 cfu respectively. Therefore dot-blot immunoassay results proved to be better with the whole cell boiled antigen preparation.

3.2 Detection of MOMP E-5 and N-10 boiled whole cell antigen by anti-N-10 antiserum

The detection of MOMP E-5 and MOMP N-10 boiled whole cell antigen by anti-N-10 antiserum (1/50 dilution) is shown in Table 23. Antigen concentrations shown in Table 23 are those expected for dilutions of

TABLE 22: Detection of MOMP E-5 antigen preparations A, B and C by anti-E-5 whole cell antiserum.

Antigen concentration (cfu)	Reaction of anti-E-5 antiserum (1/50 dilution) with E-5 antigen preparations		
	A[a]	B[b]	C[c]
1×10^6	3+	3+	3+
5×10^5	3+	3+	2/3+
2.5×10^5	3+	3+	2+
1.3×10^5	2+	2/3+	2+
6.5×10^4	2+	2+	+
3.2×10^4	1+	1+	+/-
1.6×10^4	+/-	1+	-
8×10^3	+/-	+/-	-
4×10^3	+/-	+/-	-
2×10^3	-	-	-
1×10^3	-	-	-

[a] untreated whole cell antigen; [b] boiled whole cell antigen; [c] sonicated suspension.

3+ - strong positive reaction;
2+ - medium positive reaction;
1+ - positive reaction;
+/- - very slight colour development;
- - no colour development.

TABLE 23: Detection of MOMP E-5 and N-10 boiled whole cell antigens by anti-N-10 antiserum.

Antigen concentration (cfu)	Reaction of anti-N-10 antiserum (1/50 dilution) with MOMP reference strains	
	E-5	N-10
1×10^6	3+	3+
5×10^5	3+	3+
2.5×10^5	3+	3+
1.3×10^5	2+	2+
6.5×10^4	2+	2+
3.2×10^4	+/2+	+
1.6×10^4	+/-	+/-
8×10^3	+/-	+/-
4×10^3	+/-	+/-
2×10^3	-	-
1×10^3	-	-

suspensions of OD 1.0 at 540 nm. A cut off value was chosen as the last dilution of antigen to give a 1+ reaction.

The unabsorbed anti-N-10 antiserum detected its homologous strain MOMP N-10 and the heterologous strain MOMP E-5 to a similar degree. The lowest amount of MOMP N-10 and E-5 antigen detectable was 3.2×10^4 cfu. The lowest amount of MOMP E-5 antigen detectable by its homologous antiserum anti-E-5 was 1.6×10^4 cfu (Table 22).

3.3 Detection of MOMP E-5 and N-10 boiled whole cell antigen by a mixture of anti-E-5 and anti-N-10 antiserum

The detection of MOMP E-5 and N-10 boiled whole cell antigen by the antiserum mixture is shown in Table 24. The antiserum mixture, at a total dilution of 1/50, was composed of anti-E-5 and anti-N-10 in a 1 : 1 ratio. The minimum amount of MOMP E-5 and N-10 antigen detectable was 1.6×10^4 cfu (Table 24). The antiserum mixture detected the same amount of E-5 antigen as anti-E-5 on its own (Table 22) and a two-fold lower amount of N-10 antigen than anti-N-10 on its own (Table 23).

3.4 Detection of MOMP E-5 and N-10 boiled whole cell antigen by unabsorbed anti-E-5 and absorbed anti-E-5 and absorbed anti-N-10 antisera

The results for the detection of MOMP E-5 and N-10 antigen by unabsorbed anti-E-5, absorbed anti-E-5 and absorbed anti-N-10 antisera are shown in Tables 25 and 26 respectively.

Unabsorbed anti-E-5 antiserum detected its homologous antigen MOMP E-5 at 1.6×10^4 cfu (Table 25). This was the same level of detection as previously observed (Table 22). The absorbed anti-E-5

TABLE 24: Detection of MOMP E-5 and N-10 boiled whole cell antigens by a mixture of anti-E-5 and anti-N-10 antisera.

Concentration of antigen (cfu)	Reaction of anti-E-5 and anti-N-10 antiserum mixture with MOMPs	
	E-5	N-10
1×10^6	3+	3+
5×10^5	3+	3+
2.5×10^5	3+	3+
1.3×10^5	2/3+	2+
6.5×10^4	2+	2+
3.2×10^4	2+	+
1.6×10^4	+	+
8×10^3	+/-	+/-
4×10^3	+/-	+/-
2×10^3	-	+/-
1×10^3	-	-

3+ strong positive
2+ medium positive
+ positive
+/- very slight colour development
- no colour

TABLE 25: Detection of MOMP E-5 boiled whole cell antigen by unabsorbed anti-E-5, absorbed anti-E-5 and absorbed anti-N-10 antisera.

Concentration of antigen (cfu)	Detection of MOMP E-5 antigen by: Unabsorbed Anti-E-5	Absorbed Anti-E-5	Absorbed Anti-N-10
1×10^6	3+	3+	2+
5×10^5	3+	3+	2+
2.5×10^5	3+	2+	+
1.3×10^5	2/3+	+	+/-
6.5×10^4	2/3+	+	+/-
3.2×10^4	2+	+	+/-
1.6×10^4	+	+/-	-
8×10^3	+/-	-	-
4×10^3	+/-	-	-
2×10^3	-	-	-
1×10^3	-	-	-

3+ strong positive;
2+ medium positive;
+ positive;
+/- very slight colour development;
- no colour.

TABLE 26: Detection of MOMP N-10 boiled whole cell antigen by unabsorbed anti-E-5, absorbed anti-E-5 and absorbed anti-N-10 antisera.

Concentration of antigen (cfu)	Detection of MOMP N-10 antigen by: Absorbed Anti-N-10	Unabsorbed Anti-E-5	Absorbed Anti-E-5
1×10^6	3+	3+	2+
5×10^5	3+	3+	+
2.5×10^5	2+	3+	+
1.3×10^5	2+	2+	+/-
6.5×10^4	+	2+	+/-
3.2×10^4	+/-	+	-
1.6×10^4	+/-	+/-	-
8×10^3	-	+/-	-
4×10^3	-	-	-
2×10^3	-	-	-
1×10^3	-	-	-

detected MOMP E-5 at a level of 3.2×10^4 cfu, two-fold higher than with the unabsorbed antiserum. MOMP E-5 antigen was detectable at a level of 2.5×10^5 cfu with absorbed anti-N-10. Unabsorbed anti-N-10 antiserum was not used, therefore direct comparisons with absorbed anti-N-10 antiserum were not possible. However Table 23 shows results of detection of MOMP E-5 and N-10 antigen with unabsorbed anti-N-10 antiserum: MOMP E-5 and N-10 antigen were both detected at 3.2×10^4 cfu. Absorbed anti-N-10 antiserum detected MOMP E-5 antigen at 2.5×10^5 cfu (Table 25) and its homologous antigen MOMP N-10 at 6.5×10^4 cfu (Table 26).

MOMP N-10 antigen was detected at levels of 3.2×10^4 and 2.5×10^5 cfu by unabsorbed and absorbed anti-E-5 antisera respectively (Table 26). Absorbed antisera anti-E-5 and anti-N-10 detected their respective homologous antigens at similar levels which were approximately two-fold higher than with the unabsorbed sera. However it was interesting to note that the absorbed anti-E-5 and anti-N-10 antisera cross-reacted with their heterologous strains detecting antigen at 2.5×10^5 cfu. CoA reagents prepared with the absorbed anti-E-5 and anti-N-10, defining CoA serogroups WI and WII respectively, do not react with the heterologous strains MOMP N-10 and E-5.

3.5 Detection of MOMP E-5 and N-10 boiled whole cell antigen with a mixture of anti-E-5 protein I and anti-N-10 protein I antisera

The results of the detection of MOMP E-5 and N-10 boiled whole cell antigen by $^1/_{50}$, $^1/_{100}$ and $^1/_{200}$ dilutions of a mixture of anti-E-5 protein I and anti-N-10 protein I antisera are shown in Tables 27 and 28.

TABLE 27: Detection of MOMP E-5 boiled whole cell antigen by a 1 : 1 mixture of anti-E-5 protein I and anti-N-10 protein I antisera.

Concentration of antigen (cfu)	Detection of MOMP E-5 antigen by a mixture of anti-protein I antisera at dilutions of: 1/50	1/100	1/200
5×10^6	3+	3+	3+
1.6×10^5	2+	2+	2+
8×10^4	+	+	+
4×10^4	+	+/-	-
2×10^4	-	-	-
1×10^4	-	-	-
5×10^3	-	-	-

3+ strong positive;
2+ medium positive;
+ positive;
+/- slight colour development;
- no colour.

TABLE 28: Detection of MOMP N-10 boiled whole cell antigen by a 1 : 1 mixture of anti-E-5 protein I and anti-N-10 protein I antisera.

Concentration of antigen (cfu)	Detection of MOMP N-10 antigen by a mixture of protein I antisera at dilutions of: 1/50	1/100	1/200
5×10^6	3+	3+	3+
1.6×10^5	+	+/-	+
8×10^4	+/-	-	+/-
4×10^4	-	-	-
2×10^4	-	-	-
1×10^4	-	-	-
5×10^3	-	-	-

The anti-protein I antisera mixture at dilutions of 1/50, 1/100 and 1/200 detected a minimum of MOMP E-5 antigen of 4×10^4, 8×10^4 and 8×10^4 cfu respectively (Table 27). The same antiserum dilutions detected MOMP N-10 antigen at 1.6×10^5 cfu (Table 28).

A similar mixture of anti-E-5 and anti-N-10 whole cell antisera detected MOMP E-5 and N-10 antigen at a level of 1.6×10^4 cfu (Table 24). Therefore the anti-protein I antisera mixture appeared to be slightly less sensitive, requiring more concentrated antigen suspensions for detection of both MOMP E-5 and N-10.

3.6 Determination of the minimal amount of purified MOMP E-5 and N-10 protein I antigen detected by a mixture of unabsorbed whole cell antisera and by a mixture of anti-protein antisera

The mixture of anti-E-5 and anti-N-10 whole cell antisera detected E-5 and N-10 protein I antigen at a concentration of 1 ng (1+ reaction). The mixture of anti-E-5 protein I and anti-N-10 protein I antisera detected MOMP E-5 and N-10 protein I at a concentration of 5 ng (2+ reaction): a little colour developed (+/- reaction) on the MOMP E-5 protein I spot containing 1 ng of protein but no colour developed with MOMP N-10 protein I at a similar concentration.

3.7 Detection of gonococcal antigen in 95 male urethral specimens by anti-gonococcal antisera in the dot-blot immunoassay

Detection of antigen with a mixture of anti-E-5 and anti-N-10 whole cell antisera

Table 29 shows the correlation of the dot-blot immunoassay results with culture results for 95 male urethral specimens. Antigen was detected

in the immunoassay with a 1 : 1 mixture of anti-E-5 and anti-N-10 whole cell antisera. Overall 48/95 (51%) and 47/95 (49%) of the urethral specimens were scored positive and negative respectively.

TABLE 29: Correlation of gonococcal culture results with dot-blot immunoassay results using a mixture of anti-E-5 and anti-N-10 antisera for antigen detection in 95 male urethral specimens.

Culture result	Dot-blot immunoassay Number of clinical isolates		
	Positive	Negative	Total
Positive	43	4	47
Negative	5	43	48
Total	48	47	95

Correlation with culture results showed that the dot-blot immunoassay had a sensitivity of 91.5% (dot-blot positive/culture positive specimens) and a specificity of 89.6% (dot-blot negative specimens/culture negative specimens). There were four false negative and five false positive clinical specimens.

Insufficient gonococcal antigen in the clinical specimens was thought to be a likely cause of the false negative results obtained in the dot-blot immunoassay. It was observed that there was very little material on the swabs obtained for two of the specimens which gave false negative results. In addition, all false negative results occurred when the antisera mixture was changed from a $^1/_{50}$ to a $^1/_{100}$ dilution. The dilution was changed to $^1/_{100}$ to obtain more economical use of the antisera and to reduce non-specific colour development.

False positive immunoassay results may represent non-specific reactions of the reagents used with material in the urethral exudates, cross reactions of the antisera with some other genital pathogen or commensal organism or true gonococcal infections where no viable organisms are isolated.

Figure 17 shows the dot-blot immunoassay results and demonstrates the colour intensity obtained with clinical specimens 1-19. In order to obtain the photograph, clinical specimens 1-19 were retested in the dot-blot immunoassay as one batch. All except two of the results corresponded to those when the specimens were first tested. The two aberrant results were for clinical specimens 4 (Row A8) and 7 (Row B3): when first tested clinical specimen 4 was clearly positive and clinical specimen 7 gave a +/- reaction and was scored negative.

Detection of antigen with a mixture of anti-E-5 protein I and anti-N-10 antisera

Table 30 shows the correlation of culture results with the dot-blot immunoassay results for 45 clinical specimens tested with a $^{1}/_{100}$ dilution of a 1 : 1 mixture of anti-E-5 protein I and anti-N-10 protein I antisera.

The sensitivity and specificity of the immunoassay with the anti-protein I antisera was 84% and 85% respectively. Four false negative and three false positive results were obtained.

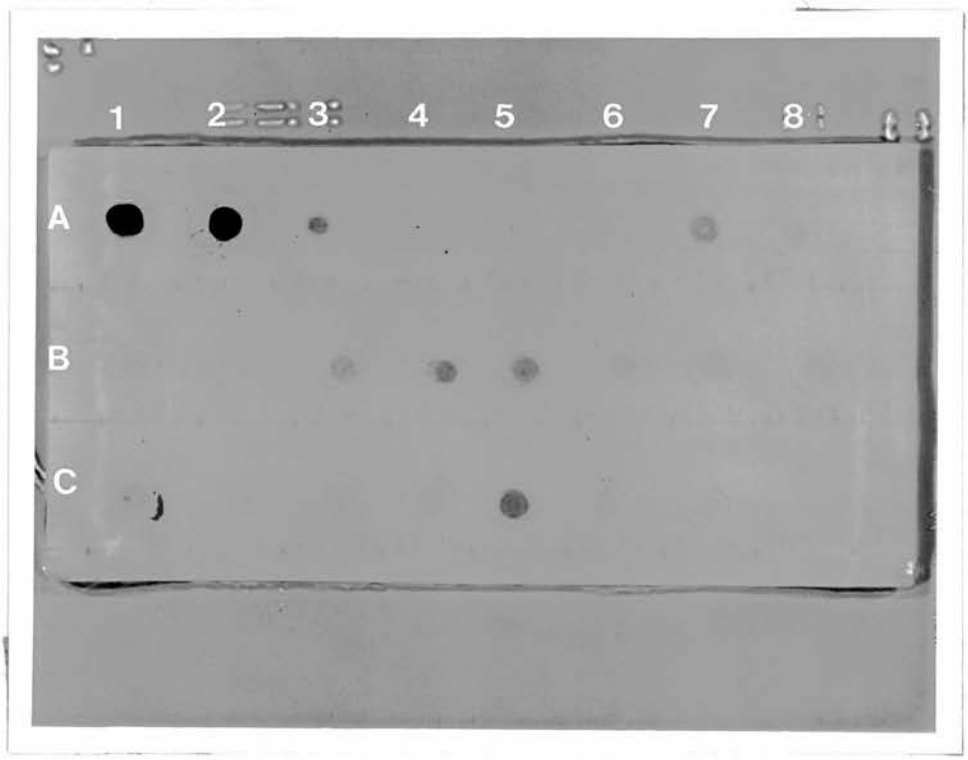

FIGURE 17: Dot-blot immunoassay results for 19 male urethral clinical specimens.

Row A: A1, strong positive control; A2, strong positive control; A3, positive control; A4 negative control; A5, CS1 negative; A6, CS2 negative; A7, CS3 positive; A8, CS4 positive.

Row B: B1, CS5 negative; B2, CS6 negative; B3, CS7 negative; B4, CS8 positive; B5, CS9 positive; B6 CS10 negative; B7, CS11 negative; B8, CS12 negative.

Row C: C1, CS13 positive; C2, CS14 negative; C3, CS15 negative; C4, CS16 negative; C5, CS17 positive; C6, CS18 negative; C7, CS19 negative; C8, buffer control.

TABLE 30: Correlation of culture results with dot-blot immunoassay results using a mixture of anti-E-5 protein I and anti-N-10 protein I antisera for antigen detection in 45 male urethral specimens.

Culture results	Dot-blot immunoassay Number of clinical isolates		
	Positive	Negative	Total
Positive	21	4	25
Negative	3	17	20
Total	24	21	45

3.8 Determination of the minimum amount of antigen detected in the dot-blot immunoassay and the corresponding CoA serogroups for 9 clinical isolates of *N. gonorrhoeae*

As shown in Table 31 the amount of gonococcal antigen detected ranged from 4×10^3 to 3×10^4 (8-fold range) cfu. The mixture of anti-E-5 and anti-N-10 whole cell antisera generally detected approximately a two-fold lower number of gonococci than the mixture of anti-E-5 protein I and anti-N-10 protein I antisera. However, the minimum amount of antigen detected by the two antisera mixtures was identical for gonococcal strains 3 and 5: coincidently both of these strains were detected at 8×10^3 cfu. Antigen from gonococcal isolates belonging to different CoA serogroups was detected at similar levels with the exception of strain 9.

Antigen from gonococcal strain 9, a CoA serogroup WII/WIII strain, was detected at the highest concentrations of 1.6×10^4 and 3×10^4 cfu with the anti-whole cell and anti-protein I antisera mixtures

TABLE 31: Correlation of dot-blot immunoassay results with CoA serogroup and minimum amount of antigen detected for gonococcal strains isolated from the corresponding patients.

Gonococcal strain	CoA serogroup of isolate	Minimum amount of antigen (cfu) detected with a 1/100 dilution of:		Corresponding clinical specimen dot-blot result using a mixture of:	
		whole cell antisera	anti-protein I antisera	whole cell antiserum	anti-protein I antiserum
1	WI	4×10^3	8×10^3	P	P
2	WII	8×10^3	1.6×10^4	P	P
3	WII	8×10^3	8×10^3	P	P
4	WII	4×10^3	8×10^3	N	N
5	WII	8×10^3	8×10^3	P	P
6	WII/WIII	4×10^3	8×10^3	P	P
7	WI	8×10^3	1.6×10^4	P	P
8	WII	8×10^3	1.6×10^4	P	P
9	WII/WIII	1.6×10^4	3×10^4	N	N

P - positive; N - negative.

respectively. This strain was isolated from a patient whose urethral specimen was negative in the dot-blot immunoassay (Table 31). It was observed that there was very little material on the urethral swab obtained from this patient for testing in the dot-blot immunoassay.

One other gonococcal strain (strain 4) was isolated from a patient whose urethral specimen was negative in the dot-blot immunoassay. However antigen from this strain was detected at levels comparable to that for the other gonococcal isolates.

3.9 Specificity of the dot-blot immunoassay

Table 32 shows the range in the minimum amount of antigen detected in the dot-blot immunoassay for *N. gonorrhoeae* MOMP E-5 and N-10, other pathogenic *Neisseria*, commensal *Neisseria*, *Branhamella* spp. and a variety of other species found in the urogenital tract either as pathogens or commensals.

MOMP E-5 antigen was detected at 8×10^3 and 1.6×10^4 cfu by the anti-E-5 and anti-N-10 whole cell and by the anti-E-5 protein I and anti-N-10 protein I antisera mixtures respectively. MOMP N-10 antigen was detected at two-fold higher levels.

The non-gonococcal *Neisseria* antigens detected ranged from minimum concentrations of 3×10^4 to 1×10^6 cfu and 6.3×10^4 to undetectable at 1×10^6 cfu with the gonococcal anti-whole cell and anti-protein I antisera mixtures respectively. Compared with detection of the gonococcal antigens, these represented approximately 4 to 125-fold higher levels of detection. However among the non-gonococcal *Neisseria* there appears to be an antigenic order of relatedness. Antigen from strains of *N. lactamica* and *N. meningitidis* were detected at lower levels than antigen from the commensal *Neisseria* and therefore appear to be more closely related antigenically to the gonococcal strain.

TABLE 32: Reaction of antigen from non-gonococcal bacterial species in the dot-blot immunoassay.

Antigen source (Number of species or strains)		Minimum amount of antigen (cfu) detected by mixtures of: anti-gonococcal whole cell antisera	anti-gonococcal protein I antisera
MOMP E-5		8×10^3	1.6×10^4
MOMP N-10		1.6×10^4	3.1×10^4
Commensal *Neisseria*	(16)	3.2×10^6 - 1×10^4	ND - 1.3×10^5
N. lactamica	(4)	3.12×10^4 - 1.3×10^5	1.3×10^5 - 2.5×10^5
N. meningitidis	(11)	1.3×10^4 - 1.3×10^5	6.3×10^4 - 2.5×10^5
Branhamella sp.	(2)	2.5×10^5	5×10^5
G. vaganalis	(1)		
C. albicans	(1)		
Group B Streptococcus	(1)	ND - 1×10^6	ND - 1×10^6
Lactobacillus	(1)		
B. bivius	(1)	6.25×10^4	2.5×10^5
B. ureolyticus	(1)		
B. intermedius	(1)	1×10^6 - 5×10^5	ND
B. asaccharolyticus	(1)		

ND - not detectable at 1×10^6 cfu/1 µl.

Figures 18 and 19 demonstrate the typical reactions of antigen from MOMP E-5 and N-10 and a commensal *Neisseria N. pharyngis* in the dot-blot immunoassay with gonococcal anti-whole cell and anti-protein I antisera respectively.

Antigens from seven of the eight non-neisserial organisms which are commonly found in the urogenital tract were either not detected at 1×10^6 cfu or were just detectable at high levels from 5×10^5 to 1×10^6 cfu. The exception was *B. bivius* which was detected at 6.25×10^4 and 2.5×10^5cfu with the gonococcal anti-whole cell and anti-protein I antisera mixtures respectively.

3.10 Antigen detection using antisera absorbed with *B. bivius*

Table 33 shows the results of antigen detection in the dot-blot immunoassay using gonococcal anti-whole cell and anti-protein I antisera absorbed with *B. bivius*.

TABLE 33: Detection of antigen with anti-E-5 and anti-N-10 whole cell and anti-E-5 and anti-N-10 protein I antisera mixtures absorbed with *B. bivius*.

Source of antigen	Minimum amount of antigen (cfu) detected by absorbed: anti-whole cell antisera mixture	anti-protein I antisera mixture
MOMP E-5	1.6×10^4	3.1×10^4
MOMP N-10	3.1×10^4	6.3×10^4
B. bivius	1×10^6	ND
B. intermedius	ND	ND
B. assacharolyticus	ND	ND
B. ureolyticus	ND	ND
N. meningitidis RAS10	6.3×10^4	2.5×10^5
N. meningitidis Grp B	1.3×10^5	5×10^5
Clinical specimens		
31	P	P
32	P	N
74	N	N
83	N	N

ND - not detectable at 1×10^6 cfu; P - positive; N - negative.

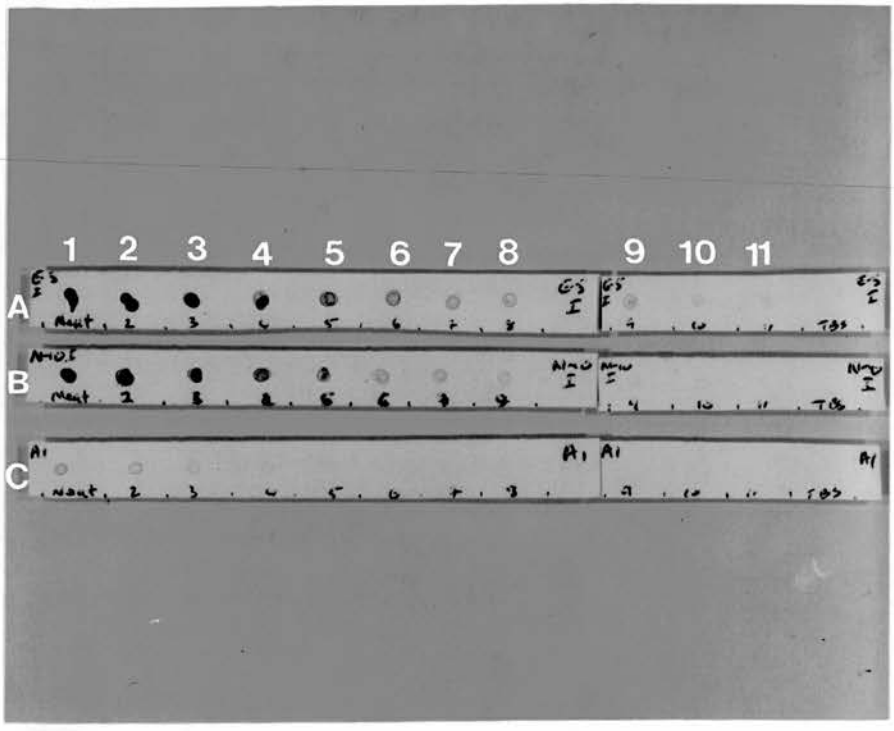

FIGURE 18: Result of dot-blot immunoassays using a mixture of anti-E-5 and anti-N-10 whole cell antisera for detection of whole cell antigen from *N. gonorrhoeae* MOMP E-5 and N-10 and *N. pharyngis*.

Strip A: MOMP E-5 antigen;
Strip B: MOMP N-10 antigen;
Strip C: *N. pharyngis* antigen.

Antigen was applied to strips in 1 µl amounts and ranged from undiluted (spot 1) to a dilution of 1/1024 (spot 11) (1×10^6 - 9.76×10^2 cfu).

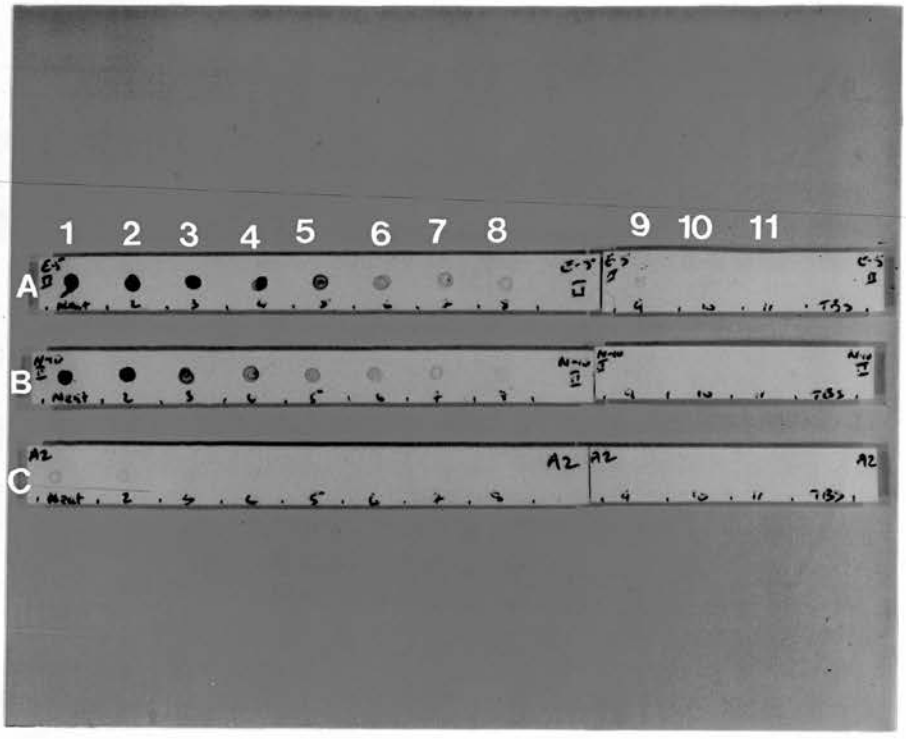

FIGURE 19: Result of dot-blot immunoassays using a mixture of anti-E-5 and anti-N-10 protein I antisera for detection of whole cell antigen from *N. gonorrhoeae* MOMP E-5 and N-10 and *N. pharyngis*.

Strip A: MOMP E-5 antigen;
Strip B: MOMP N-10 antigen;
Strip C: *N. pharyngis* antigen.

Antigen was applied to strips in 1 µl amounts and ranged from undiluted (spot 1) to a dilution of 1/1024 (spot 11) (1×10^6 to 9.76×10^2 cfu).

The minimum amount of MOMP E-5 antigen detected with the absorbed whole cell and absorbed anti-protein I antisera was 1.6×10^4 and 3.1×10^4 cfu respectively. MOMP N-10 antigen was detected at two-fold higher levels of 3.1×10^4 and 6.3×10^4 cfu. *B. bivius* antigen was detected at 1×10^6 cfu by the absorbed anti-whole cell antisera mixture but at this level was undetected by the absorbed anti-protein I antisera mixture.

The *B. bivius* antigen was previously detected by unabsorbed anti-whole cell and by unabsorbed anti-protein I antisera mixtures at 6.3×10^4 and 2.5×10^5 cfu respectively (Table 32). Antigen from *B. intermedius*, *B. ureolyticus* and *B. asaccharolyticus* was not detectable with either of the absorbed antisera mixtures.

Although there was still some colour development on the dots, two of the four male urethral clinical specimens (CS74 and 83) were scored as negative in the dot-blot immunoassay with both the absorbed whole cell and absorbed protein I antisera mixtures, one (CS32) was scored positive with the absorbed whole cell antisera mixture and negative with the absorbed anti-protein I mixture and one (CS31) was scored positive with both of the absorbed antisera mixtures. All four clinical specimens had previously given false positive results with the unabsorbed whole cell antiserum, CS74 and CS83 were negative and positive with unabsorbed anti-protein I antisera mixture respectively, while specimens 31 and 32 were not tested with this antisera mixture.

3.11 Reduction of non-specific colour development by blocking with bovine serum albumin

No difference was observed in the colour development on antigen spots on the strip blocked with 3% BSA/TBS compared with the strip blocked with the normal blocking solution of 3% gelatin/TBS.

3.12 Testing of the anti-rabbit IgG conjugate for non-specific binding to antigen and for endogenous peroxidase activity in urethral clinical specimens

Non-specific binding of the conjugate to antigen

Some colour developed on the control spots containing undiluted (10^7 cfu) MOMP E-5 and N-10 whole cell antigen and this was scored +/-. A similar amount of colour developed on each of the three spots containing the clinical specimens. The clinical specimens chosen had given high background negative or false positive results when previously tested in the complete dot-blot immunoassay. However although the conjugate appears to react to some degree with the control antigen at high concentrations and with the clinical specimens, the intensity of colour which developed with these specimens in the complete immunoassay suggests that the conjugate is not the major contributor to non-specific colour development.

Endogenous peroxidase activity

No colour developed on the antigen spots on the strip incubated with the substrate solution alone. This indicated that there was no residual peroxidase activity in the clinical specimens.

4.0 Immunological identification of *N. gonorrhoeae* with monoclonal and polyclonal antibody coagglutination reagents

Table 34 shows the results of polyclonal and monoclonal antibody coagglutination reagents with boiled suspensions of clinical isolates of neisseriae. The sensitivity and specificity of the monoclonal reagent were 97.7% (86/88) and 96.4% (54/56), respectively, compared with values of 100% (88/88) and 94.6% (53/56) for the polyclonal reagent. The overall agreement with carbohydrate utilisation was 97.9% (141/144)

for the polyclonal antibody reagent and 97.2% (140/144) for the monoclonal reagent. These differences are not significant at the 5% level.

Whereas all 13 stock cultures of *N. lactamica* were negative with the monoclonal reagent seven gave a positive result with the polyclonal reagent. All five isolates of β-lactamase producing *B. catarrhalis* were negative with both reagents. When these results were combined with those for clinical isolates of non-gonococcal neisseriae the agreement with carbohydrate utilisation was 97.3% (72/74) for the monoclonal reagent and 86.5% (64/74) for the polyclonal reagent. This is a significant difference ($\chi^2_1 = 4.5$; $p < 0.05$).

4.1 CoA using boiled gonococcal suspensions and an antigen releasing agent

Results of the monoclonal reagent with bacterial suspensions prepared by boiling and by treatment with antigen releasing agent are given in Table 35. Suspensions prepared with releasing agent tended to be stringy compared with the uniform suspensions obtained by boiling (Figures 20 and 21). On many occasions the stringy nature of the suspensions made it impossible to score results unequivocally positive or negative. The overall correlation with carbohydrate utilisation was 97.1% (166/171) for the test with boiled suspension and 80.7% (138/171) when antigen releasing agent was used. This is a highly significant difference ($\chi^2_1 = 21.6$; $p < 0.001$). The difference in test performance remains significant when gonococcal ($p < 0.01$) and non-gonococcal neisseriae ($p < 0.001$) are considered separately.

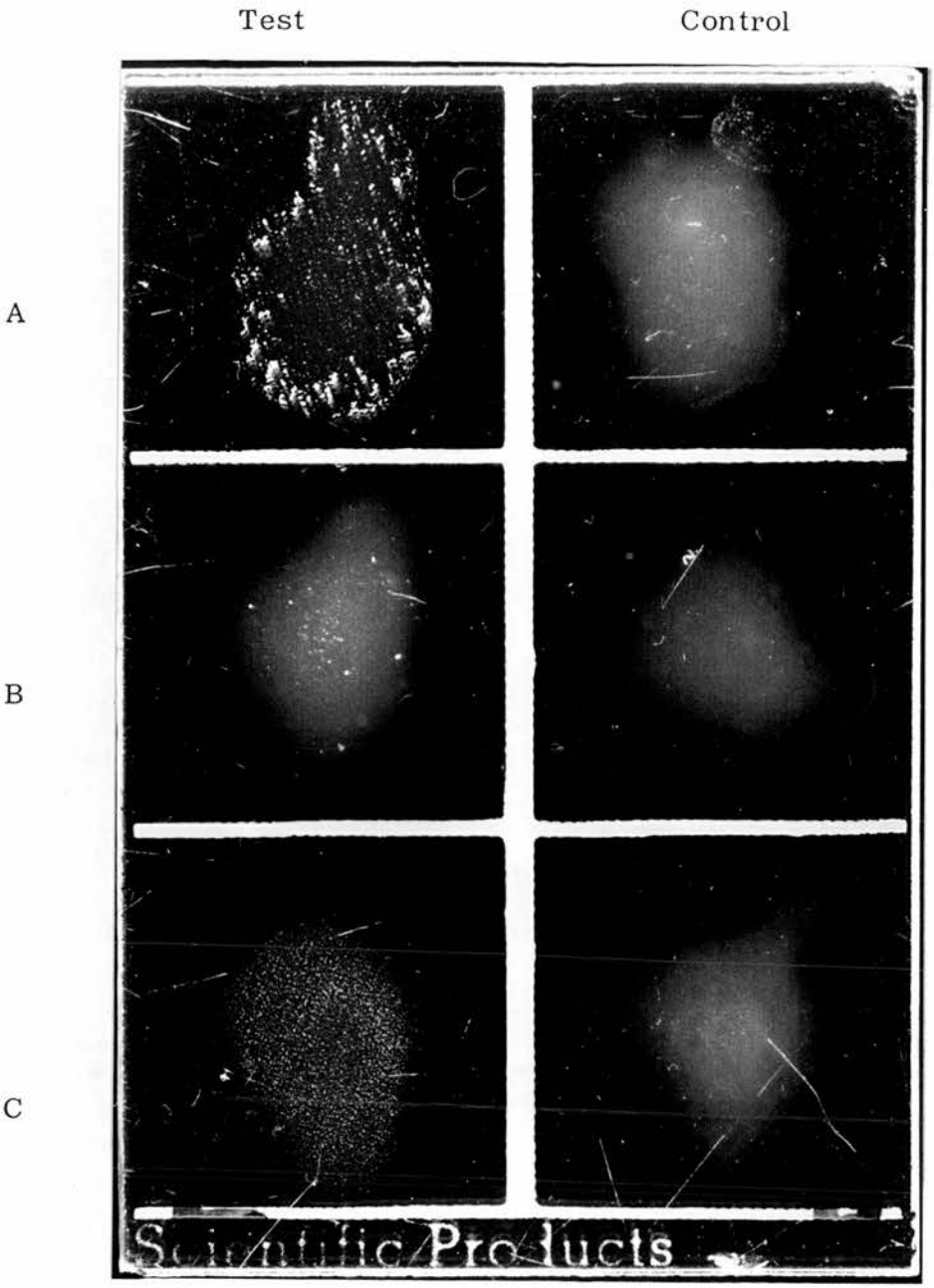

FIGURE 20: Reaction of the monoclonal reagents with boiled suspensions of neisserial isolates.

Row A: Reaction of *N. gonorrhoeae* strain 1, positive with test reagent;

Row B: Reactions of *N. lactamica*, negative with test reagent;

Row C: Reaction of *N. gonorrhoeae* strain 2, positive with test reagent.

Control Test

A

B

C

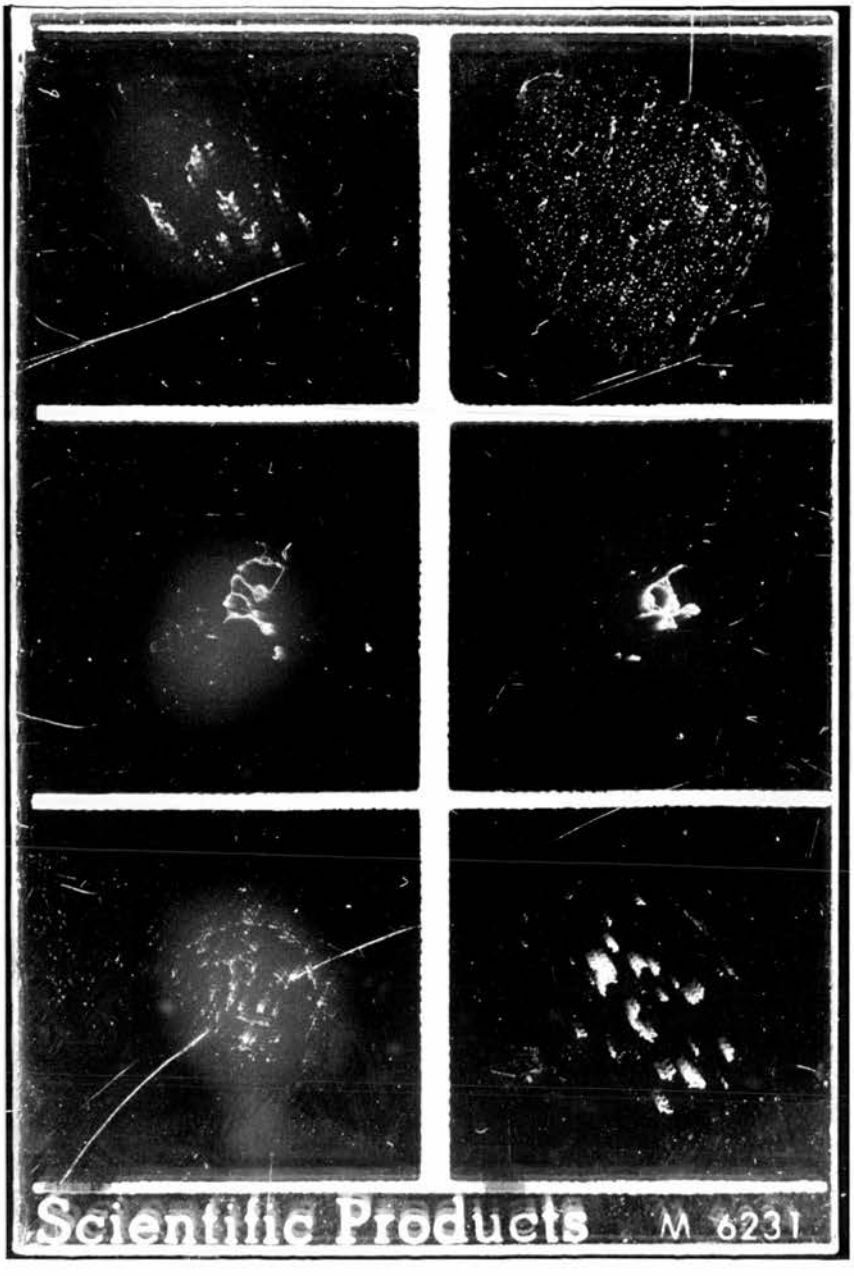

FIGURE 21: Reaction of the monoclonal reagents with suspensions of Neisseria prepared with the releasing agent.

Row A: Reaction of *N. gonorrhoeae* strain 1 positive with test reagent;

Row B: Reaction of *N. lactamica*, negative with test reagent;

Row C: Reaction of *N. gonorrhoeae* strain 2, equivocal reaction with test reagent.

TABLE 34: Results of polyclonal and monoclonal antibody coagglutination reagents tested with 144 clinical isolates of neisseriae.

Identity by carbohydrate utilisation	No. of isolates	Polyclonal			Monoclonal		
		positive	non-specific	negative	positive	non-specific	negative
N. gonorrhoeae	88	88	0	0	86	1	1
N. meningitidis	52	0	0	52	1*	0	51
N. lactamica	3	2	1	0	0	1	2
N. perflava	1	0	0	1	0	0	1
Total	144	90	1	53	87	2	55

*Negative after two subcultures.

TABLE 35: Monoclonal antibody coagglutination test applied to suspensions of 171 neisserial isolates prepared by boiling and by treatment with antigen releasing agent.

Identity by carbohydrate utilisation	No. of isolates	Results with suspensions prepared by boiling (and by treatment with releasing agent)			
		positive	equivocal	non-specific	negative
N. gonorrhoeae	117	112 (95)	1 (10)	2 (1)	2 (11)
N. meningitidis	51	0 (1)	0 (8)	0 (0)	51 (42)
N. lactamica	2	0 (0)	0 (2)	0 (0)	2 (0)
N. perflava	1	0 (0)	0 (0)	0 (0)	1 (1)
Total	171	112 (96)	1 (20)	2 (1)	56 (54)

4.2 Calculation of predictive values

The positive and negative predictive values of the polyclonal and monoclonal reagents applied to Gram negative diplococci isolated from various anatomical sites are given in Table 36. The values of *a* (sensitivity), *b* (specificity), and *p* (prevalence) used in calculating the predictive values given in Table 36 were derived as follows.

The sensitivity and specificity of the monoclonal reagent were 96.6% (198/205) and 98.2% (108/110) respectively; these figures represent all clinical isolates tested by the boiled suspension method. The sensitivity and specificity of the polyclonal reagent were 97.4% (406/417) and 96.1% (173/180), respectively. Because of the small number of isolates tested with the polyclonal reagent these are composite results of the present and previous study, which gave a sensitivity of 96.7% (318/329) and a specificity of 96.8% (120/124) with the same reagents (Young and McMillan, 1982).

The values of *p* (the proportion of gonococcal Gram negative diplococci within the total population of Gram negative diplococci from various anatomical sites) as given in Table 36 were taken from the finding that over a 4-year period *N. gonorrhoeae* accounted for 20.3% of 1204 throat isolates of Gram negative diplococci; and that of the gram negative diplococci isolated over a 5-year period *N. gonorrhoeae* accounted for 99.6% of 1944 anogenital isolates from women, 99.7% of 2623 urethral isolates from heterosexual men, 95.3% of 384 anogenital isolates, and 92.0% of 225 rectal isolates from homosexual men (Young and Bain, 1983; Young, 1983).

TABLE 36: Predictive values of monoclonal and polyclonal antibody coagglutination reagents for Gram negative diplococci isolated from various sites.

Source of Gram negative diplococci	Proportion of gonococcal Gram negative diplococci	Positive predictive value		Negative predictive value	
		monoclonal	polyclonal	monoclonal	polyclonal
Female anogenital and heterosexual male urethra	0.996	99.99	99.98	10.68	12.90
Homosexual male anogenital	0.953	99.91	99.80	58.75	64.58
Homosexual male rectum	0.920	99.84	99.65	71.48	76.27
Throat (all patient groups)	0.203	93.18	86.42	99.13	99.32

DISCUSSION

SECTION I: CHARACTERISATION OF GONOCOCCAL STRAINS BY CoA SEROGROUPING AND CELL ENVELOPE PHENOTYPING

1.0 Characterisation by CoA serogrouping

The CoA W serogroups have been shown to be stable during subculture, do not vary with colony morphology, and correlate with antigenic differences in the protein I molecules of the gonococcal outer membrane (Sandström and Danielsson, 1980a; Sandström *et al.*, 1982a,b). CoA serogroups WI and WII correspond to antigenic differences in protein IA and IB molecules respectively, while CoA serogroup WIII corresponds to a prominent antigenic variation in protein IB (Sandström *et al.*, 1982a).

To date there is little or no information available about the distribution of CoA serogroups among gonococcal strains isolated in the United Kingdom. However Copley, Chiswell and Egglestone (1983) have examined a number of typing systems including CoA W serogrouping in relation to contact pairs. Most work concerning CoA W serogroups has been carried out in Sweden and the United States.

1.1 Overall distribution of CoA W serogroups

The overall distribution of CoA serogroups among gonococcal strains isolated from patients who acquired their infections in the Edinburgh area was as follows: 41.8% WI, 49.9% WII, 8.2% WII/WIII and 0.1% WIII. This is similar to the overall proportion of serogroups among strains isolated in Stockholm, Sweden, in 1981 (Bygdeman, Danielsson and Sandström, 1981a), where 41.9%, 55.9% and 2.2% of isolates belonged to serogroups WI, WII and WIII respectively. Bygdeman *et al.* (1981a) found that there was a significantly higher proportion of CoA WI serogroup isolates among strains isolated in Örebro, a small town with

adjacent rural area than in Stockholm, one of the major Swedish cities. Of 130 strains isolated in Örebro, 54.6%, 44.6% and 0.8% belonged to serogroup WI, WII and WIII respectively. In Edinburgh there is a significantly lower proportion of WI strains compared with isolates from Örebro ($\chi^2_1 = 6.94$; $p < 0.01$). A survey of gonococcal strains isolated in 10 geographical areas in Sweden has confirmed the significant differences in the proportion of serogroups found in large cities with urban populations compared with smaller towns surrounded primarily by rural areas (Danielsson, Bygdeman and Kallings, 1983). In Stockholm and Göteborg the two largest towns in Sweden, the incidence of WII strains was 57.2% and 54.7% respectively, whereas in the small towns with rural areas the number of WI strains accounted for approximately 80% of the strains: 78.8% in Gävle and 72.7% in Örebro. The proportions of CoA serogroups among strains isolated from middle sized University towns were representative for those for Sweden as a whole (Danielsson *et al.*, 1983). In a recent study using CoA reagents prepared with polyclonal antibodies and reagents prepared with monoclonal antibodies to protein I it has been shown that serogroup WII predominates (60-70% of strains) in major cities in Scandinavia (Bygdeman, Danielsson and Sandström, 1983).

Within the four consecutive time periods of this study a number of changes in the overall distribution of CoA serogroups has occurred. Initially, no WII/WIII strains (strains which react with both WII and WIII CoA reagents) were isolated from patients who had acquired their infections in Edinburgh. However by the end of the fourth study period a total of 61 WII/WIII strains had been isolated ($p < 0.001$).

Overall the proportion of WI strains isolated remained constant (44-46%) over the first three study periods. However, in the fourth

period a significantly lower number of WI strains was isolated: 77/215 (35.8%) ($p < 0.05$). A dramatic decrease in the number of WI strains has been reported in parts of Stockholm (Bygdeman *et al.*, 1983). The incidence of WI strains has dropped from 40.3% to 28%.

1.2 CoA serogroups in relation to patient group and sites of isolation

Serogroup WII predominated among gonococcal strains isolated from homosexual men: overall 94% (102/108) strains belonged to CoA serogroup WII or WII/WIII (Table 6) compared with only 53% (151/283) and 50.8% (180/354) of isolates from heterosexual men and women ($p < 0.001$).

Serogroup WI strains were isolated from homosexual men only during study period one, thereafter all strains belonged to serogroups WII and WII/WIII (Table 7). This significant association of serogroup WII with homosexually acquired gonococcal infection has been previously reported in Stockholm and Seattle, United States (Bygdeman, 1981a; Morse *et al.*, 1982). Bygdeman (1981a) in Stockholm and Morse *et al.* (1982) in Seattle, found that 76.9% (30/37) and 73.7 (42/57) of strains isolated from homosexual men belonged respectively to CoA serogroup WII. Therefore in Edinburgh a significantly higher proportion of male homosexual isolates belong to serogroup WII than those from homosexual men in Stockholm ($p < 0.05$) and Seattle ($p < 0.001$). It may be that a proportion of bisexual men were included in the Stockholm and Seattle studies. The association of serogroup WII with homosexually acquired infection occurs regardless of the anatomical site of isolation (Table 8).

Overall there were no significant differences in the distribution of CoA serogroups of strains isolated from heterosexual men and women ($p > 0.5$) (Table 6). However there were significant changes within the four time periods.

In the first study period the proportion of WI strains was higher among heterosexual men than women: 66% compared with 53% (Table 7). However this was not a significant difference. In the second study period the proportion of WI strains was higher in women. In this period the proportion of WI strains isolated from heterosexual men dropped significantly to 41% ($p < 0.05$). Whereas the proportion of WI strains isolated from women was similar to that for period one. However, despite this change in the proportion of WI strains there was no significant difference between heterosexual men and women.

Over the next year the proportion of WI strains isolated from heterosexual men remained constant. However, in the last six month period (period four) the proportion of WI strains isolated from women dropped by approximately 10% to 40.4%. Thus in the fourth study period the proportion of WI strains isolated from heterosexual men and women reached equilibrium.

Study period two encompassed the summer months when there is a large influx of visitors to Edinburgh, particularly during August and September. It was preceded by a period when there was a great deal of publicity on the Acquired Immune Deficiency Syndrome (AIDS). The drop in the proportion of WI strains isolated from heterosexual men was initially attributed to an undisclosed number of homosexual men being included among this group and/or to an influx of gonococcal strains acquired outwith Edinburgh. However the decrease in the number of WI strains isolated from heterosexual men may have represented a change in selective pressure resulting in a reduction in the number of serogroup WI strains among the gonococcal pool. This change is now reflected, after a lag of approximately one year, in women.

It has been reported that WI strains are more commonly isolated from women than from men in all major Scandinavian cities except Stockholm, Sweden (Bygdeman *et al.*, 1983). It was shown that overall 36.3% (202/557) and 29.2% (137/469) of strains isolated from women and men belonged to serogroup WI respectively. A previous study found that a significantly higher number of WI strains was found in women than in men (Bygdeman, 1981b).

However because of the strong association of serogroup WII with homosexually acquired gonococcal infections it is misleading to compare women with men without reference to sexual preference. For example, in this study if gonococcal strains isolated from women are compared with those isolated from homosexual men and heterosexual men together it is found that 48.9% (173/354) of isolates from women and 35% (138/391) of isolates from all men belong to serogroup WI (Table 6). This is a significant difference ($\chi^2_1 = 13.5$; $p < 0.001$). However, as already discussed (p. 227), there is no significant difference in the distribution of CoA serogroups among heterosexual men and women.

Therefore it is recommended that where there is a known homosexual male element within a study population that the data for this group be excluded in evaluating differences between women and men.

1.3 Rise in the incidence of CoA serogroup WII/WIII strains and serogroup of strains acquired outwith Edinburgh

CoA serogroup WII represents gonococcal strains with protein IB molecules, whereas serogroup WIII represents a prominent antigenic variant of protein IB rather than a third distinct protein I molecule (Sandström *et al.*, 1982a). Gonococcal strains which react with reagents defining serogroup WII and with reagents defining serogroup WIII (WII/

WIII strains) have been reported to occur occasionally (Sandström *et al.*, 1982b).

The increase in WII/WIII strains observed in this study has occurred to a similar degree in all three patient groups.

Although the CoA W reagents have been shown to represent differences in the protein I molecules it must be remembered that the reagents are prepared with polyclonal antibodies which may also react with other antigens such as LPS and minor proteins such as protein II. These reactions may contribute to the reaction patterns obtained with the CoA W reagents used in this study. However it is unlikely that the cross-reactions observed with strains grouped as WII/WIII are as a result of such reactions because no such cross-reactions were observed with the CoA reagents and the frequently isolated WI and WII strains.

The WII/WIII strains may have resulted from such strains being brought into the Edinburgh area and establishing themselves within the endogenous gonococcal population. Three of 33 (9%) strains isolated from infections acquired abroad either in Europe or in non-European countries, belonged to this group (Table 12). WII/WIII strains may also have been introduced during the summer months when there is a large influx of visitors.

Alternatively these strains may have arisen as a result of a mutation resulting in a hybrid protein IB molecule which has antigenic determinants recognised by the reagents defining serogroups WII and WIII. Such mutational events may occur and the survival and establishment of *N. gonorrhoeae* containing such mutations within the gonococcal population will depend on the selective advantages conferred by changes in the structure of protein I molecules.

The functional role of gonococcal protein I is that of a porin (Greco *et al.*, 1980; Douglas *et al.*, 1981; Young *et al.*, 1983). Alterations in the structure of the porin may result in strains being better able to compete for nutrients and being more resistant to antibiotics. Such changes may also affect the pathogenic potential of gonococci. Recent work has shown that gonococci of serogroup WI are strongly associated with disseminated gonococcal infections (DGI) (Cannon *et al.*, 1983). It has been shown that protein I can insert into the membranes of red blood cells and it has been suggested that protein I may be involved in stimulating the phagocytosis of gonococci by host cells (Blake and Gotschlich, 1983).

A hybrid protein IB molecule of serogroup WII/WIII is unlikely to occur as the result of genetic transformation between endogenous serogroup WII and WIII gonococcal strains because serogroup WIII are extremely rare in Edinburgh. Only one WIII strain was isolated from a patient who had contracted her infection in Edinburgh. Previous studies have indicated that WIII strains are rarely isolated in Sweden (Bygdeman *et al.*, 1981a). These strains have been isolated primarily from patients who have contracted their infections in Thailand and other countries in the Far East (Bygdeman *et al.*, 1981b; Bygdeman, 1981c). The majority of isolates from such parts of the world belong to serogroups WII and WIII. Gonococcal strains from these areas have been shown to be more resistant to antibiotics than corresponding isolates from Sweden (Bygdeman, 1981c). Gonococcal strains of serogroup WIII and WII are more resistant than WI strains.

All of the 22 gonococcal strains isolated from patients who had acquired their infections in non-European countries including Thailand, the Far East, Africa and the East Indies belonged to CoA serogroups

WII, WII/WIII and WIII (Table 12). Gonococcal strains isolated from patients who had acquired their infections in European countries encompassed all serogroups.

1.4 Antigenic diversity within individual CoA serogroups

When the CoA serogrouping system was first developed, Sandström and Danielsson (1980a) observed that different reaction patterns existed within each individual W serogroup. They concluded that these differences represented antigenic diversity among strains of each serogroup and suggested that the potential existed for subdividing the individual serogroups.

During the course of this study a total of five WI and 13 WII and WII/WIII reaction patterns were obtained with the CoA reagents prepared with polyclonal antibodies (Table 5): one serogroup WI reaction pattern and one serogroup WII reaction pattern predominated.

Serogroup WI and WII have been shown to represent different types of gonococcal protein I, protein IA and IB respectively (Sandström *et al.*, 1982a,b). According to recent studies only a small part of the protein IA molecule is exposed at the gonococcal surface, whereas protein IB has a looped structure with the loop exposed on the exterior of the gonococcal membrane (Figure 4) (Barrera and Swanson, 1984). Therefore one would expect that there would be fewer antigenic sites exposed on protein IA than on protein IB molecules. This appears to be corroborated by Bygdeman *et al.* (1983) in a recent study employing CoA reagents prepared with monoclonal antibodies to gonococcal protein I (Tam *et al.*, 1982). Bygdeman *et al.* (1983) obtained a total of 43 different reaction patterns or serovars (12 WI, 29 WII and 2 WIII serovars) using gonococcal strains isolated from five major Scandinavian cities. Among WI strains

isolated it was found that only three to five different WI serovars existed in each city with one serovar predominating in all cities. This serovar accounted for up to 94% of WI strains isolated with a range from 76% in Copenhagen (Denmark) to 94% in Oslo (Norway). A much greater variation in WII serovars was observed in each city with a range of six WII serovars in Trondheim (Norway) to 17 in Oslo (Norway). No particular WII serovar dominated in any area (Bygdeman *et al.*, 1983).

1.5 Theories for the changes in distribution of CoA serogroups

A number of theories have been proposed to explain differences in the distribution of CoA serogroups between men and women and for differences found in large cities compared to small towns with adjoining rural areas. These include a proposed association of serogroup WI strains with asymptomatic infections in men, an immunological model and selective pressure induced by antibiotics.

1.5.1 Association of CoA serogroup WI with asymptomatic gonococcal infections in men

It has been hypothesised that the lower number of WI strains isolated from men may be due to undetected asymptomatic gonococcal infections (Bygdeman *et al.*, 1983). No actual studies have been conducted to examine asymptomatic infections in relation to the serogroup of infecting strain either in heterosexual men or women.

In homosexual men asymptomatic gonococcal infections are common (McMillan and Young, 1978) and this and other studies have indicated that serogroup WII predominates in this group of patients (Bygdeman, 1981a; Morse *et al.*, 1982). The rate of asymptomatic infections in women is approximately 60-70% (Robertson *et al.*, 1980).

During the course of this study the serogroup of infecting strain was correlated with the presence or absence of symptoms for 64 women. It was found that a higher proportion of WI strains were isolated from women with symptoms compared with those women with asymptomatic infections: 58% compared with 47%. This difference was not significant. Therefore although this was a limited study it does not appear that serogroup WI and asymptomatic infections are linked in women.

Despite Bygdeman's findings that serogroup WII predominates among homosexual men (Bygdeman, 1981a) the major failing of most Swedish studies has been that this group of patients is not considered apart from the heterosexual population. In this study when homosexual men are considered apart no significant differences in the proportion of CoA serogroups are observed when heterosexual men are compared with women.

1.5.2 Proposed immunological model

An immunological model has been proposed to account for the differences in the distribution of WI and WII serogroup strains between men and women and between large and small cities (Bygdeman *et al.*, 1983). The model is based on the hypothesis that patients infected with a gonococcal strain with a particular protein I serovar produce protective antibodies against the protein I antigens of this strain and thus in the event of re-exposure only become infected with gonococci possessing different protein I antigens. It has been proposed that because there is a small number of WI serovars one of which predominates, that the chances of being reinfected with a WI strain of a different serovar is very small. Therefore with a build up of immunity within the population the number of WI strains would decrease. However the

chance of being reinfected with a different WII serovar is much greater because there are many more WII serovars, none of which predominates in any given area (Bygdeman *et al.*, 1983).

This theory is dependent upon two major factors, the production of a protective localised antibody response to protein I and the re-exposure rate within the sexually active population.

Localised antibody responses producing primarily anti-gonococcal IgG and IgA have been shown to occur in both men and women (McMillan *et al.*, 1979a,b). However as yet it has not been shown that localised antibody responses confer protection against subsequent infection by *N. gonorrhoeae*. Buchanan *et al.* (1980) showed that women with pelvic inflammatory disease (PID) produced antibodies which were protective against subsequent attacks of PID by gonococci of the same protein I serotype. However they were not protected against reinfection at localised genital sites by gonococci of the same protein I serotype. The re-exposure rate among the sexually active population to *N. gonorrhoeae* would play an important role in the dynamics of this model, thereby determining whether a reduction in a particular serogroup would occur within the various patient groups and within geographical areas. However before an immunological model can be substantiated as a major factor in altering the proportion of CoA serogroups observed, the host immune response to *N. gonorrhoeae* must be clarified.

1.5.3 Selection of CoA serogroups WII and WIII by antibiotics

The third theory proposed is that antibiotics select strains belonging to serogroups WII and WIII. It has been shown that strains of serogroup WI are much more sensitive to antibiotics such as benzyl penicillin, cefuroxime and ampicillin than strains of serogroup WII and

WIII (Bygdeman, 1981c). The majority of the WI strains tested (93.5% - 95.7%) were sensitive to <0.06 µg/ml of benzyl penicillin and cefuroxime, and were sensitive to <0.125 µg/ml of ampicillin. Higher concentrations of antibiotics were required to inhibit WII strains: 28.8% of WII strains were inhibited by ≥0.25 µg/ml of cefuroxime, 54.5% were inhibited by ≥0.125 µg/ml of benzyl penicillin and 53% were inhibited by ≥0.25 µg/ml of ampicillin. The differences in sensitivity between WI and WII, WI and WIII and WII and WIII strains were significant for most antibiotics tested (Bygdeman, 1981c).

It has been shown that 79% (56/71) of non-PPNG and 77% (53/69) of PPNG strains isolated from infections acquired in Thailand and the Far East belonged to CoA serogroup WII or WIII. All of the WII non-PPNG strains acquired in Thailand had sensitivities to penicillin and ampicillin of ≥0.125 µg/ml and ≥0.25 µg/ml respectively, and 82% had sensitivities to cefuroxime of ≥0.125 µg/ml (Bygdeman, 1981c). Thus strains of serogroup WII acquired in Thailand were shown to be significantly more resistant to antibiotics than corresponding WII strains acquired in Sweden. It has subsequently been shown that antibiotic resistance and specificity for serogroup WII are genetically linked (Bygdeman *et al.*, 1982). A multiple antibiotic resistant WII strain was used as a donor strain in transformation experiments. When WI recipient strains were transformed to antibiotic resistance it was found that they acquired the same protein I type and hence serogroup of the donor strain. This genetic linkage between CoA serogroup WII and multi-antibiotic resistance was different from the multi-antibiotic resistance conferred by the previously described *mtr* mutation (Sparling *et al.*, 1975; Guymon *et al.*, 1978). Therefore antibiotics may have selected gonococcal strains of serogroup WII and WIII in areas such as the Far

East and other non-European countries where there is little or no control over their use. This may explain the observed differences in the distribution of CoA serogroups in different geographical areas of Sweden.
In their survey of 10 different geographical areas in Sweden, Danielsson *et al.* (1983) showed that strains isolated in the large cities of Stockholm and Göteborg were significantly more resistant to antibiotics than strains isolated from small towns with adjacent rural areas such as Örebro and Gävle. In Stockholm and Göteborg serogroup WII strains predominated whereas in Örebro and Gävle serogroup WI strains predominated (Danielsson *et al.*, 1983). A previous study has indicated that gonococcal strains isolated from rural areas are much more sensitive to antibiotics than strains isolated from patients in cities (Gump and Berry, 1973). This may reflect greater use of antibiotics within certain populations and may also explain the very strong association of serogroup WII with homosexually acquired infections.

2.0 Characterisation of gonococcal isolates by cell envelope phenotyping

It has been shown that serogroup WII and the *mtr* mutation are associated with homosexually acquired infections (Morse *et al.*, 1982). The *mtr* mutation confers resistance to a variety of hydrophobic compounds including antibiotics, dyes and detergents by decreasing the permeability of the gonococcal outer membrane (Sparling *et al.*, 1975; Guymon *et al.*, 1978).

It has been hypothesised that the rectal environment which is rich in hydrophobic compounds such as faecal lipids selects gonococcal strains of the *Mtr* phenotype in homosexual men (Morse *et al.*, 1982). However Morse *et al.* (1982) did not examine phenotype in relation to site

of infection. This study was undertaken to increase our understanding of the role of different anatomical sites on the selection of gonococcal strains with particular outer membrane properties.

Overall 5%, 87% and 8% of 482 consecutive gonococcal strains isolated from infections acquired in the Edinburgh area had an Env, wild type and Mtr phenotype respectively (Table 13). Morse *et al.* (1982) found that 7 (4.6%), 128 (82.9%) and 19 (12.5%) of gonococcal strains collected in Seattle (United States) over a five year period were of the Env, wild type and Mtr phenotype. Although the genotypes of the strains phenotyped in this study were not confirmed by genetic transformation experiments, the proportion of Env phenotypes is similar to the proportion isolated in Seattle. Overall a lower number of Mtr phenotypes were found in Edinburgh compared with Seattle. This is not a significant difference ($\chi^2_1 = 1.58$; $p > 0.1$).

The findings of this study confirm the significant association of the Mtr phenotype with homosexually acquired infection: 36% (17/47) of isolates from homosexual men had the Mtr phenotype compared with 5% (9/197) ($p < 0.001$) and 6% (15/238) ($p < 0.001$) of isolates from heterosexual men and women respectively. However the findings cast doubt on the hypothesis that the rectal environment, essential for the transmission of gonococci within the homosexual population, exerts a selective pressure favouring the emergence of the Mtr phenotype.

The Mtr phenotype was found considerably more often among urethral and throat isolates from homosexual men. This difference just failed to reach significance ($\chi^2_1 = 3.57$; $0.1 > p > 0.05$). There was no significant difference in the proportion of Mtr phenotypes among male and female rectal isolates ($p > 0.1$). The lack of association between the Mtr phenotype and male rectal isolates was surprising since it seemed

possible that hydrophobic environment of the rectum could be capable of selecting gonococcal strains with reduced membrane permeability. Eisenstein and Sparling (1978) reported that strains with an *mtr* mutation consistently had reduced rates of exponential growth in enriched broth cultures. The introduction to these strains of an *env* mutation by transformation which results in the phenotypic inhibition of the *mtr* mutation (Sarubbi *et al.*, 1975) partially restored the normal growth rates *in vitro* (Eisenstein and Sparling, 1978). Therefore the Mtr strains may be less able to establish rectal infections since their poorer growth characteristics could make them less capable of competing with the complex microbial flora of the rectum.

2.1 Another selective force

It has been shown that CoA serogroup WII strains predominate in homosexual men (Bygdeman, 1981a; Morse *et al.*, 1982). This study has indicated that serogroup WII strains predominate in homosexual men regardless of the anatomical site of isolation. Morse *et al.* (1982) showed that serogroup WII and the Mtr phenotype was strongly correlated in homosexual men. Results presented here confirm these observations. However CoA serogroup WII and the Mtr phenotype are also strongly associated for gonococcal strains isolated from heterosexual patients (Table 15). Morse *et al.* (1982) showed that the *mtr* locus and WII phenotype were often co-transformed to recipient strains. The results presented here suggest that the rectal environment is not responsible for the selection of gonococcal strains with the Mtr phenotype. The theory (Morse *et al.*, 1982) that the rectal environment selects gonococcal strains with the Mtr phenotype was based upon observations that Mtr strains isolated from homosexual men were significantly more resistant

to faecal lipids than matched non-Mtr strains isolated from heterosexual men. A more meaningful comparison would have been to examine the faecal lipid sensitivity of all strains isolated from homosexual men and heterosexual patients. The recent report of an Mtr-independent system for resistance to faecal lipids also casts doubt in the role of the hydrophobic rectal environment in selecting gonococcal strains of the Mtr phenotype (McFarland *et al.*, 1983). It is proposed that a more general selective pressure common to all infected sites is the driving force for selection of both the Mtr phenotype and CoA serogroup WII in homosexual men. Antibiotics would appear to be the major candidate for this role. This hypothesis is corroborated by a number of studies which show that there is a very strong association between serogroup WII and multi-resistance to a number of antibiotics such as β-lactams, tetracyclines and other antibiotics (Bygdemen, 1981c; Danielsson *et al.*, 1983; Bygdeman *et al.*, 1982). Homosexual men experience a wider spectrum and a higher rate of all sexually transmitted diseases than heterosexual men (Fluker, 1976; Willcox, 1981). Although difficult to prove, it would seem probable that antibiotic usage is greater among homosexual men than among heterosexuals. Evidence to support this was reported by Bygdeman (1981a) who found that all isolates including serogroup WI strains from homosexual men were more resistant to penicillin than strains isolated from heterosexual patients.

In areas of the world such as the Far East where there is very little or no control over the use of antibiotics there is considerable antibiotic resistance in many bacterial species including *N. gonorrhoeae*. It has been shown that serogroups WII and WIII predominate among strains isolated from Thailand (Bygdeman *et al.*, 1981b). Strains of all serogroups isolated from Thailand and other countries in the Far East

have been shown to be considerably more resistant to antibiotics than gonococcal strains isolated in Sweden (Bygdeman, 1981c). Strains of serogroup WII and WIII are the most resistant. Strains of serogroup WII isolated from infections acquired in Sweden are more resistant than comparable serogroup WI strains (Bygdeman, 1981c). Antibiotic pressure has been considered as a possible cause for the higher proportion of WII strains in large Swedish cities compared with small towns encompassing rural areas (Bygdeman *et al.*, 1983).

A small number of gonococcal strains isolated from infections acquired in non-European countries were examined. It was found that all of these strains belonged to CoA serogroup WII or WII/WIII (Table 16). A significantly higher proportion of these strains had an Mtr phenotype compared with endogenous gonococcal strains ($p < 0.001$). When the results were analysed further it was found that 75% (9/12) isolates from heterosexual men who acquired their infections in non-European countries (mostly in Thailand) had the Mtr phenotype. Only 5% (9/197) of strains isolated from heterosexual men who had acquired their infections in Edinburgh had this phenotype. This is a significant difference ($p < 0.001$). The proportion of Mtr phenotypic strains isolated from heterosexual men who acquired their infections in non-European countries was also significantly higher than the proportion of Mtr strains isolated from homosexual men who had acquired their infections in Edinburgh ($p < 0.05$).

Further studies are required to determine the precise nature of the relationship of the *mtr* locus and determinants for protein I. One would suspect that the *mtr* locus is closely linked to the previously described *nmp* loci determining protein I structure (Cannon *et al.*, 1980a,b) or possibly to another *nmp* locus yet to be described. The *nmp*

loci (*nmp*-1 and *nmp*-2) were discovered by the change in protein I type conferred by acquisition of the loci *penB* and *sac* which respectively conferred low level penicillin resistance and resistance to the bactericidal activity of normal human sera in transformants (Guymon *et al.*, 1978a,b; Sparling *et al.*, 1978). Protein I forms pores in the gonococcal membrane (Greco *et al.*, 1980; Douglas *et al.*, 1981; Young *etal* ., 1983) and it has been reported that protein IA and IB molecules form pores of different sizes (Blake, personal communication, cited by Barrera and Swanson, 1984).

Therefore the type of protein I molecule produced by a gonococcal strain may also be directly involved in antibiotic resistance. Gonococcal strains containing protein IB molecules (serogroups WII and WIII) and or *mtr* mutations are likely to have a selective advantage over other gonococcal strains in areas and sub-populations of patients where there is a selective pressure exerted by antibiotics. Hence under continued selective pressure these strains are likely to survive and proliferate as is observed in Thailand and among homosexual men.

SECTION II: NON-CULTURAL DETECTION AND IDENTIFICATION OF *N. GONORRHOEAE*

1.0 Preliminary studies on gonococcal antigen detection by an indirect enzyme linked immunosorbent assay (ELISA) using wheatgerm lectin for antigen capture

The indirect ELISA is an extremely useful technique for the detection of both antibody and microbial antigens and has gained widespread use in diagnosis and as a research tool. The laboratory diagnosis of gonococcal infections is reliant upon culture and therefore depends upon the maintenance of the viability of *N. gonorrhoeae*. Therefore a non-cultural detection assay for gonococcal antigen in clinical specimens remains a worthwhile goal. A suitable non-cultural method may have a number of applications including detection of gonococcal antigen in transported specimens and as an "on-the-spot" test to be employed at the clinic at the time of the patient's examination. This is particularly important for the diagnosis of gonorrhoea in women because the Gram stain only detects between 55 and 60% of positive cases (Chipperfield and Catterall, 1976; Barlow *et al.*, 1976; Evans, 1976).

The immunological detection of microbial antigens in body fluids poses a number of problems. The low numbers of organisms necessitate the use of a method of antigen capture from clinical specimens. A specific and sensitive antibody representative of the antigenic make up of the local pool of gonococci is required for antigen detection. Therefore a knowledge of the antigenic diversity of *N. gonorrhoeae* is also required. Study of gonococcal strains isolated from patients who acquired their infections in the Edinburgh area by CoA serogrouping (Section I) has indicated that the majority 95.8 - 99.6% of isolates are detected by two CoA reagents prepared with antisera raised to MOMP N-10 and MOMP E-5

respectively. These reagents define serogroups WII and WI. It was considered that these antisera would be suitable for use in the development of a non-cultural detection system.

Initially wheatgerm (WG) lectin was examined for antigen capture in an indirect ELISA using polystyrene beads as the solid phase. Most gonococcal strains have been shown to agglutinate WG lectin (Schaeffer *et al.*, 1979). Preliminary experiments with slide agglutination as described by Curtis and Slack (1981) indicated that gonococcal reference strain MOMP E-5 agglutinated WG lectin at lower concentrations of lectin than reference strain MOMP N-10.

Assays utilising lectins have been considered unsatisfactory for the differentiation of gonococcal from non-gonococcal neisseriae because many meningococcal strains also agglutinate WG lectin (Frasch, 1980; Curtis and Slack, 1981). However because *N. meningitidis* is rarely isolated from uro-genital specimens it was thought WG lectin could be utilised in the place of specific antibody for capture of gonococcal antigen.

The minimum amount of whole cell and sonicated MOMP E-5 antigen detected by anti-MOMP E-5 antiserum, using polystyrene beads coated with WG lectin at various concentrations in different buffers was determined. Unarmed (control) polystyrene beads consistently detected lower concentrations of both whole cell and sonicated MOMP E-5 antigen than beads armed with various lectin concentrations (Tables 20 and 21). Uncoated beads detected a minimum of 1.4×10^5 cfu (Table 20) and 0.12 µg of protein (≡ 2.8×10^4 cfu) at an arbitrary cut off of OD 0.5. Lectin armed beads detected whole cell and sonicated antigen in the ranges of 2×10^5 - 1×10^7 cfu and 0.18 - 0.8 µg of protein respectively.

In an indirect ELISA using mouse and rabbit antiserum raised against *N. gonorrhoeae* strain 9 for capture and detection of strain 9

respectively, Sarafian and Young (1982) were able to detect 48 ng of outer membrane complex from strain 9 and from 48-92 ng of outer membrane complex from 10 clinical gonococcal isolates. This was approximately 3 to 6-fold lower than the amount of sonicated MOMP E-5 antigen detected using uncoated control beads and 8 to 16-fold lower than the minimum amount of sonicated antigen detected by beads coated with 200 µg/ml of lectin, at the cut off point of OD 1.0. The ELISA of Sarafian and Young preferentially detected protein antigens (Sarafian and Young, 1982). In a simulated clinical specimen the ELISA of Sarafian and Young (1982) was able to detect whole cell strain 9 antigen at 6.6×10^3 cfu. This number is approximately 100 and 150-1500 times lower than the minimum amount of whole cell antigen detected at an OD of 1.0 by uncoated control beads and by beads armed with various concentrations of lectin (Table 20; bead set one).

The ELISA of Sarafian and Young (1982) was used to detect antigen in cervical and vaginal washings from 37 women (Young *et al.*, 1983). Specimens from 12 (60%) of the 20 women with culture proven gonorrhoea gave positive ELISA results. Specimens from 2/17 (11.7%) of patients with no clinical or microbiological evidence of gonorrhoea also gave positive results. Therefore the ELISA of Sarafian and Young had a similar sensitivity to that of the Gram stain which detects up to 60% of women with gonorrhoea. The number of gonococci recovered from cervical aspirates varied within a range of 5×10^3 to 8×10^6 cfu/ml with a mean of 1×10^6 cfu/ml; the corresponding figures for vaginal aspirates were 1×10^2 to 1×10^6 cfu/ml with a mean of 8.4×10^4 cfu/ml (Young *et al.*, 1983). Lowe and Kraus (1976) found that the numbers of gonococci in cervico-vaginal aspirates from 52 women with gonorrhoea ranged from $40 - 1.8 \times 10^6$ cfu/ml (mean 1.45×10^4 cfu/ml).

It was concluded that gonococcal antigen detection by homologous antiserum in the ELISA system utilising lectin armed polystyrene beads for antigen capture was less sensitive than that of the ELISA of Sarafian and Young (1982) and that no advantage could be gained by using this system for detection of gonococcal components in clinical specimens. Therefore an alternative method for the detection of gonococcal antigen in clinical specimens was evaluated.

2.0 Gonococcal antigen detection on nitrocellulose by an indirect enzyme linked immunosorbent assay (dot-blot immunoassay)

The dot-blot immunoassay is based upon the non-covalent binding of proteins to nitrocellulose membranes. Towbin, Staehlin and Gordon (1979) first described this technique for the electrophoretic transfer of ribosomal proteins from polyacrylamide gels to nitrocellulose membranes. Antigen can also be applied directly to nitrocellulose membranes in the form of dots (Hawkes, Niday and Gordon, 1982). Antigen bound to the nitrocellulose can be detected using immunoassays, the nitrocellulose taking the place of conventional solid phases such as polystyrene wells or beads. Towbin and Gordon (1984) have reviewed many of the applications of this technique. It has been used primarily for either the characterisation of the antigen profile of microorganisms or for characterisation of both polyclonal and monoclonal antibodies to many microorganisms (Towbin and Gordon, 1984).

Immunoblotting (electrophoretic transfer followed by immunoassay) has been used to examine the antibody response to the pili and protein II of gonococci isolated from individual patients within contact groups (Duckworth *et al.*, 1983; Zak *et al.*, 1984). To my knowledge, there

has only been one report of the use of the dot-blot immunoassay for detection of microbial antigens in clinical specimens (Brooks, Sharma and Remington, 1985). Brooks *et al.* (1985) used this technique to detect *Toxoplasma gondii* antigen in laboratory infected mice and in the serum and cerebrospinal fluid of six infants infected with *T. gondii*.

Preliminary studies using unabsorbed whole cell antigonococcal antisera raised to MOMP E-5 and N-10 indicated that MOMP E-5 and N-10 boiled whole cell antigen could be detected at levels of 1.6 - 3.2 x 10^4 cfu (Tables 22 and 23). The absorbed anti-E-5 and anti-N-10 used for preparing CoA serogrouping reagents were less sensitive than the unabsorbed antisera. The absorbed antisera detected their homologous whole cell boiled antigen at 3.2 - 6.5 x 10^4 cfu (Tables 25 and 26). Interestingly, absorbed anti-E-5 and anti-N-10 detected heterologous antigen in the dot-blot immunoassay, whereas no cross-reactions were observed with CoA reagents prepared with these antisera and their respective heterologous strains.

A mixture of antisera raised against protein I purified from MOMP E-5 and N-10 detected MOMP E-5 boiled whole cell antigen at 4-8 x 10^4 cfu and MOMP N-10 antigen at 1.6 x 10^4 cfu (Tables 27 and 28). Therefore the anti protein I antisera was less sensitive than the antisera raised against whole cell MOMP E-5 and N-10. The unabsorbed whole cell antiserum mixture detected 1 ng of purified protein I from MOMP E-5 and N-10 whereas the mixture of anti-E-5 and anti-N-10 protein I antisera detected protein I antigen from these strains at 5 ng. The conventional ELISA of Sarafian and Young (1982) detected 48-92 ng of outer membrane complex which was mostly protein in nature.

The amount of homologous whole cell gonococcal antigen detected in the dot-blot immunoassay was approximately two-fold higher than that

detected by the conventional ELISA of Sarafian and Young which detected strain 9 at 6.6 x 10^3 cfu with homologous whole cell antiserum (Sarafian and Young, 1982). Therefore because the dot-blot immunoassay detected similar levels of antigen as the conventional ELISA of Sarafian and Young (1982) and had the major advantage of being able to apply antigen directly to the solid phase (nitrocellulose) without the necessity for an antibody for antigen capture, this method was considered promising for evaluation for antigen detection in clinical specimens. Other advantages of the dot-blot immunoassay were the ease of handling materials and incubation at room temperature.

2.1 Detection of gonococcal antigen in 95 male urethral specimens by antigonococcal antisera in the dot-blot immunoassay

Urethral specimens were obtained from 95 men with urethritis. All 95 specimens were tested with the whole cell antisera mixture and 45 were also tested with the protein I antisera mixture. The dot-blot results were correlated with culture results.

The dot-blot immunoassay using the anti-whole cell antisera mixture for antigen detection had a sensitivity and specificity of 91.5% and 89.6% respectively. There were four false negative and five false positive results. The sensitivity and specificity of the assay for the 45 specimens tested with the anti-protein I antisera mixture was 84% and 85% respectively. The sensitivity and specificity of the assay using the whole cell antisera was identical for these 45 specimens. This was because all the false negative and most of the false positive results occurred in this part of the study.

A number of groups have evaluated the commercially available Gonozyme ELISA (Abbot Diagnostic Laboratories) for the detection of

N. gonorrhoeae in genitourinary specimens (Aardoom *et al.*, 1982; Papasian *et al.*, 1984; Stamm *et al.*, 1984). The Gonozyme assay is a conventional ELISA using polystyrene beads coated in some way to aid antigen capture. All three groups found that Gonozyme correlated well with cultural results for men with urethritis: Aardoom *et al.* (1982) found that Gonozyme and culture results were in complete agreement for specimens from 52 men with urethritis; Papasian *et al.* (1984) found that Gonozyme had a sensitivity and specificity of 97.3% and 95.8% respectively for 208 male urethral specimens, whereas Stamm *et al.* (1984), who tested 1171 male urethral specimens, found that Gonozyme had a sensitivity and specificity of 94% and 98% respectively. Stamm *et al.* (1984) found that the Gonozyme had a similar sensitivity to the Gram stain for diagnosis of gonorrhoea in male urethral specimens. Positive predictive values (PV^+) of 90% and 96.5% and negative predictive values (PV^-) of 96.8% and 99% were obtained by two of the groups (Stamm *et al.*, 1984; Papasian *et al.*, 1984).

In a comparison of specimens from men with urethral discharge with those from men without discharge it was found that the Gonozyme ELISA detected significantly fewer cases of gonorrhoea among the asymptomatic group of patients: sensitivity and specificity were 67% and 98% respectively (Stamm *et al.*, 1984). Small numbers of infecting organisms have been hypothesised as the reason for the poor results for specimens from asymptomatic men. The dot-blot immunoassay, which was scored subjectively, had a PV^+ of 89.6% and a PV^- of 91.5%.

There are a number of drawbacks associated with comparing a non-cultural detection method with culture results. It is possible that specimens positive with non-cultural detection but culture negative may represent true infections where viable organisms cannot be grown.

This could be due to prior antibiotic treatment, exposure to gonococci but successful killing of the infecting gonococci by the host but leaving detectable antigen and failure of the culture system to grow some isolates, e.g. vancomycin sensitive strains.

Four false negative specimens were obtained with the dot-blot immunoassay. All specimens for analysis by dot-blot immunoassay were obtained from patients after specimens had been taken for routine Gram-staining and culture. It was observed that there was very little material on the swabs obtained from two of the patients who gave false negative results. Determination of the minimal amount of antigen detected and serogrouping of gonococcal strains isolated from nine patients from whom specimens were taken for testing in the dot-blot immunoassay indicated that heterologous whole cell antigen could be detected to similar levels as MOMP E-5 and N-10 antigen. All isolates were serogroupable. Two of these isolates were from patients whose dot-blot immunoassay results were negative: one isolate (strain 4) could be detected at comparable amounts of antigen as other strains, whereas the second isolate (strain 9) required more antigen for detection and belonged to serogroup WII/WIII. The urethral swab obtained from this patient had very little material on it. Therefore the amount of antigen available in specimens obtained from patients is likely to affect the assay results. However, because all false negative results occurred when the dilution of the antisera mixture was increased, it seems probable that the higher antibody dilution was a contributory factor to false negative results. Therefore the dilution of the antisera used may require reevaluation, particularly when the assay is used for detection of gonococcal antigen in female genital specimens.

Aardoom *et al.* (1982) examined Gonozyme results for "high" and "lower" risk groups of female patients: prostitutes attending for routine screening for sexually transmitted diseases and women who were contacts of patients suspected of having gonococcal infections. The sensitivity and specificity of the Gonozyme ELISA was higher for prostitutes ("high" risk group) than for specimens from women among the "lower" risk contact group. However the results were considerably poorer than those obtained with male urethral specimens. Papasian *et al.* (1984) and Stamm *et al.* (1984) found sensitivities and specificities of 79.2% and 87.2%, and 78% and 98% respectively. These were considerably lower than those obtained with male urethral specimens. Stamm *et al.* (1984) reported that with a sensitivity of 78% the Gonozyme ELISA offered a considerable improvement over gram stained smears for the diagnosis of gonorrhoea in women. They found that the Gram stain had a sensitivity of 48%. However the sensitivity of the Gram stain for diagnosis of gonorrhoea in women can range from 40-70% in different laboratories and is dependent upon the experience of the technical staff screening stained smears (Lossick *et al.*, 1982). Therefore to offer any real improvement, the sensitivity of any method for antigen detection must be above 70%. Poor sensitivity of Gonozyme for women may be attributed to a proportion of women with infections with very low numbers of gonococci. Lowe and Kraus (1976) detected as few as 43 cfu in cervico-vaginal specimens from women.

None of the Gonozyme studies differentiated between women with symptomatic and women with asymptomatic infections. However as the majority of women (60%) have asymptomatic infections any test must be able to detect antigen in this group. Papasian *et al.* (1984) suggested that the complex microbial flora found in the female genital tract may mask the presence of gonococcal antigen by competing for binding sites

on the polystyrene beads thus reducing the sensitivity of the Gonozyme assay.

In the dot-blot immunoassay some of the negative clinical specimens were observed to develop background colour. No endogenous peroxidase activity was detected when a number of high background negative and false positive specimens were tested with the peroxidase colour developer. Endogenous peroxidase activity from PMNs has been reported to be a problem in direct staining techniques applied to clinical specimens (McMillan, personal communication).

The peroxidase conjugate reacted with these specimens to give faint non-specific colour development. This was considered unlikely to be high enough for specimens to be scored positive. However it may be necessary to examine anti-rabbit IgG peroxidase conjugate from another source.

Five specimens gave false positive results in the dot-blot immunoassay. Two of these specimens gave very strong reactions in the assay (deep purple) and it is possible that these patients may have had gonococcal infections where no viable gonococci were detected. Alternatively all five false positives may have resulted from cross-reactions of the anti-gonococcal antisera with antigen from some other genital pathogen or commensal organism.

The reaction in the dot-blot assay of both the antigonococcal whole cell and protein I antisera mixtures with a variety commensal and pathogenic non-gonococcal neisseriae and a variety of organisms commonly found in the urogenital tract of both men and women was investigated.

Non-gonococcal neisserial antigens were detected at 4 to 125-fold higher levels than antigen from *N. gonorrhoeae* (Table 32). Strains of

N. meningitidis appear to be antigenically most closely related to *N. gonorrhoeae*. The majority of the non-gonococcal neisseriae including *Branhamella* sp. are found in the upper respiratory tract and oropharynx of man. The exceptions are *N. canis*, *N. caviae*, *N. ovis* and *N. cuniculi* which are isolated from dogs, guinea pigs, sheep and rabbits respectively (Bøvre, 1984). However *N. meningitidis* are occasionally isolated from ano-genital sites, particularly in homosexual men (McMillan and Young, 1978), whereas *N. lactamica* are rarely isolated from these sites (Telfer Brunton, Young and Fraser, 1980). The mixture of anti-gonococcal protein I antisera was more specific than the whole cell antisera mixture.

Examination of *G. vaganalis*, *C. albicans*, group B streptococci and lactobacilli, four species commonly found in the urogenital tract indicated that none except *G. vaganalis* were detectable at the highest antigen concentration of 1×10^6 cfu. However, *B. bivius* was detected at 6.25×10^4 and 2.5×10^5 cfu with the whole cell and protein I antisera mixtures respectively. None of the three other Bacteroides species were detected with the protein I antisera mixture but were detected in the range of $5 \times 10^5 - 1 \times 10^6$ cfu with the whole cell antisera mixture. A recent study has indicated that *B. asaccharolyticus*, *B. intermedius*, *B. ureolyticus* and *B. bivius* are commonly isolated from men with non-specific urethritis and Balanoposthitis (Masfari, Kinghorn and Duerden, 1983). These species have also been isolated from the urethras of 21% of healthy men. Other species commonly isolated from healthy men are coagulase-negative staphylococci, coryneforms and α and β haemolytic streptococci. These species and lactobacilli, *G. vaganalis* and *C. albicans* are also isolated from patients with urethritis (Masfari *et al.*, 1983). A greater range of bacteria are isolated from women. The most prevalent

aerobes are *E. coli*, enterococci, β-haemolytic streptococci and yeasts (Hamman, 1982). Anaerobic bacteria were cultured from a total of 34% (72/212) of vaginal swabs. *B. bivius* was the most commonly isolated Bacteroides spp. followed by *B. oralis* (Hamman, 1982).

The possibility that cross-reaction of the antigonococcal antisera with *B. bivius* gave rise to false positive and high background reactions in the dot-blot immunoassay, was investigated. The antisera mixtures used for detection of antigen in the dot-blot immunoassay were absorbed with *B. bivius*. Absorption with *B. bivius* resulted in reduction in the sensitivity of the antisera in detecing homologous gonococcal strains MOMP E-5 and N-10. No cross-reactions were observed with *B. assacharolyticus*, *B. intermedius* or *B. ureolyticus*. However *B. bivius* was still detectable at 1×10^6 cfu with antigonococcal whole cell antiserum mixture (Table 33).

Four clinical specimens which had given false positive results when first tested in the dot-blot immunoassay were retested with the absorbed antiserum mixtures. Although there was still some colour development on the dots when compared with the controls two of these specimens were scored negative with both the absorbed antisera mixtures, one was scored negative with the absorbed protein I antisera mixture and positive with the absorbed whole cell antisera mixture, and one was scored positive with both of the absorbed antisera mixtures. Therefore it is possible that at least two of these specimens may have given false positive results because of cross reactions of the unabsorbed antisera with *B. bivius* or related organisms.

The cross reactions may have resulted from two possible sources; the rabbits used to raise antigonococcal antisera may have been previously exposed to *B. bivius* or the antigonococcal antiserum contained antibodies

to antigens which are common to gram negative bacteria. Unfortunately the pre-immune sera from the rabbits used here were not available for testing against *B. bivius*. However it seems more likely that the antigonococcal antisera contains antibodies which react to antigens common to other gram negative bacteria. The most likely candidate for this is lipopolysaccharide (LPS). Whole cell untreated gonococci were used to raise antisera against MOMP E-5 and N-10 whereas a purified outer membrane antigen preparation was used to raise antisera against MOMP E-5 and N-10 protein I. One would expect with the whole cell antigen that an immune response would be mounted to many antigens including LPS. Gel electrophoresis showed that a small amount of contaminating protein II and LPS was present in the purified protein I antigens. On the gel stained for LPS with silver stain there were bands corresponding to protein I and two or three minor staining bands of low molecular weight. According to the method of James and Heckels (1981) column chromatography should have separated protein I, protein II and LPS into distinct fractions. However the protein I and protein II peaks were not well separated during column chromatography using a column approximately half the length of that used by James and Heckels (1981). Therefore it is possible that some LPS was fractionated with the protein. Alternatively some LPS could still be covalently bound to the proteins.

3.0 Immunological identification of *N. gonorrhoeae* with coagglutination reagents prepared with polyclonal and monoclonal antibodies

The advent of monoclonal antibodies raises the possibility of improved and more widely applied immunological methods of identifying microorganisms. In this "in use" evaluation, however, there was no

significant difference in the sensitivity and specificity of monoclonal and polyclonal antibody reagents for the routine identification of clinical isolates of *N. gonorrhoeae*. Although factors such as sensitivity and specificity are important in evaluating new methods the utility of a test result (the predictive value) depends upon the prevalence of gonococci among the total population of gram negative diplococci tested. As shown in Table 36, the predictive values vary greatly depending on the anatomical site of isolation. Sound recommendations regarding the application of immunological methods for the laboratory identification of *N. gonorrhoeae* can be made from these results. Coagglutination testing with either polyclonal or monoclonal reagents can be recommended for the identification of gonococci from an anogenital site. However reliability is less in the case of isolates from homosexual men when 1 to 3 per 1000 positive tests will be false positives. The corresponding false positive rate for anogenital specimens from heterosexual patients is 1 to 2 per 10,000 positive tests. A positive coagglutination result on an isolate from the throat is less reliable than in the case of anogenital isolates; there will be 7 false positives per 100 positive tests with the monoclonal reagent, and 14 false positives per 100 positive tests with the polyclonal reagent. Although this difference is not significant at the 5% level with 100 positive results, it becomes significant with 200 positive tests ($\chi^2_1 = 4.49$; $p < 0.05$). The Phadebact Gonococcus test has previously been reported to react with *N. lactamica* (Anand and Kadis, 1980).

The negative predictive values are of much more use than positive predictive values for throat isolates; 7 negative tests will be false negatives per 1000 negative tests with the polyclonal reagent compared with 9 per 1000 negative tests with the monoclonal reagent. *N. gonorrhoeae* accounts for only 20.3% of throat cultures which yield GNDC

compared with *N. meningitidis* and *N. lactamica* which respectively account for 74.2% and 3.7% of throat cultures yielding GNDC (Young and Bain, 1983). Therefore a negative coagglutination result with either the polyclonal or monoclonal reagent is a reliable indicator of non-gonococcal neisseriae in throat cultures. However because of the extremely rare occurrence of meningococci in anogenital specimens from heterosexual patients a negative coagglutination result is not a reliable indicator of non-gonococcal neisseriae: approximately 9 of 10 negative results will be false negatives and gonococcal infection could pass undiagnosed.

In preliminary investigations of the same monoclonal reagents, Philip, Ison and Easmon (1984) and Lawton and Battaglioli (1983) respectively found that all 27 and 57 non-gonococcal neisseriae tested gave negative reactions. Although a larger study is required the results of this study suggest that monoclonal coagglutination reagents are likely to achieve a level of specificity such that the predictive value of a positive test result is a reliable indicator of pharyngeal gonorrhoea in patients attending a clinic for sexually transmitted diseases. Because of its slightly greater sensitivity the negative predictive value of the polyclonal reagent is marginally better than that of the monoclonal reagent. However a sensitivity of 99.99% would be required to give a negative predictive value of 97.5% for anogenital isolates from heterosexual patients; a sensitivity of 99.9% would give a negative predictive value of only 79.7%. It will obviously be difficult to achieve a sensitivity of greater than 99.90% given that the sensitivity of the monoclonal reagent in this study was 96.6% (198/205) and is comparable to the figure of 96% (48/50) reported by Philip *et al.* (1984). However, a sensitivity of 99.09% (109/110) was reported by Lawton and Battaglioli (1983) who

conducted their study in New York (United States). GonoGen which is manufactured in the United States may therefore be lacking reactivity to gonococcal strains isolated from other geographical areas including the United Kingdom.

CONCLUSIONS

CoA serogrouping has provided valuable information concerning the variation in gonococcal strains within different geographical locations and within specific patient groups. It has also provided an additional means of surveying antibiotic resistance in *N. gonorrhoeae*.

This study has provided useful data concerning the antigenic pool of *N. gonorrhoeae* in the Edinburgh area. CoA serogrouping has shown that this pool is not static but dynamic and changing. It has provided us with a means of monitoring the influx of new gonococcal strains from outwith Edinburgh and may help identify new strains which arise from mutation of the protein I structure.

The CoA serogrouping and phenotyping results presented here suggest that antibiotics may have a major role in the selection of gonococci belonging to serogroup WII. Careful monitoring of changes in the distribution within a given area, may give additional warning of changes in the antibiotic sensitivity of *N. gonorrhoeae*.

Gonococcal research has long awaited an acceptable serological means of typing *N. gonorrhoeae* and CoA serogrouping based on the protein I molecules appears to have fulfilled this role. The advent of monoclonal antibodies to protein I (Tam *et al.*, 1982) should make investigation of specific subgroups of gonococci particularly those causing complicated and disseminated infections easier. They may also help in the study of the host response to gonococcal infections. CoA serogrouping in conjunction with auxotyping is now being used by many groups. The experience now exists for further studies to be carried out using the newly available monoclonal antibodies.

Continued surveillance of the gonococcal pool is of importance for the diagnosis of gonorrhoea. Serological identification by commercially available CoA reagents forms an integral part of the laboratory identification

of *N. gonorrhoeae* and constant surveillance will be required to ensure that these reagents remain effective.

The CoA serogrouping study has allowed the rational choice of antisera to be employed in the development of a non-cultural detection method. The sentisitivity and the specificity of the dot-blot immunoassay is acceptable at present but could be improved. Limitations of the assay could become more apparent when clinical specimens from women are evaluated. However the means exist to improve both the sensitivity and specificity of this assay. It has been reported that polyethylene glycol (PEG) enhances the ability of the second antibody reaction step in conventional phase ELISAs for the detection of human antibody to a variety of viruses and microorganisms (Salonen and Vaheri, 1981). The use of PEG considerably reduced the incubation period required for the binding of anti-human IgG conjugate to antibody bound to antigen attached to the solid phase. PEG could be incorporated into the diluent buffers of the dot-blot immunoassay to increase the binding of the peroxidase anti-rabbit conjugate with the anti-gonococcal antisera. Reduction in the incubation periods may therefore be possible.

An ELISA using monoclonal antibody directed against meningococcal pili has been developed to detect meningococcal antigen in the CSF of patients with meningitis (Sugasawara, Prato and Sippel, 1984). In 25 patients with meningitis, antigen was detected in the CSF in 21 patients by ELISA and in 16 patients by crossed immunoelectrophoresis.

Monoclonal antibodies to protein I are now becoming available and may improve the specificity of the dot-blot immunoassay. However monoclonal antibodies have been criticised because their high specificity may reduce the sensitivity of assays. A pool of monoclonal antibodies would be required to detect most gonococcal strains.

The sensitivity of the dot-blot immunoassay could be improved by using steps to amplify the antibody reactions. The interaction of avidin with biotin has been described for amplification of immunoassays (Guesdon, Ternynck and Avrameas, 1979; Yolken *et al.*, 1983; Kendall, Ionescu-Matiu and Dreesman, 1983). Avidin is a basic glycoprotein (MWt 68,000) which has an extremely high affinity for biotin. Each avidin molecule can bind four biotin molecules (Guesdon *et al.*, 1979). Guesdon *et al.* (1979) covalently bound biotin to antibody to a high specific activity without affecting the antigen binding capacity of the antibody. The biotin-labelled antibody was incubated with avidin followed by incubation with enzyme-labelled biotin. Alternatively biotin-labelled antibody could be incubated directly with enzyme-labelled avidin. These methods were used in immunohistochemically staining procedures (Guesdon *et al.*, 1979).

Yolken *et al.* (1983) developed an ELISA for the detection of antigen from *Haemophilus influenzae* and *Streptococcus pneumoniae* using the avidin-biotin system. They found that biotin-labelled antibody-avidin-peroxidase-labelled biotin detected antigen most efficiently: 0.16 ng/ml and 0.8 ng/ml of *Haemophilus* and streptococcal antigen respectively could be detected. However they also found that good sensitivity could be obtained using unlabelled specific antibody-biotin labelled anti-rabbit IgG-avidin-enzyme-labelled biotin. Kendall *et al.* (1983) used the avidin-biotin system to amplify the sensitivity of an ELISA for the detection of antibody against hepatitis B surface antigen (HBsAg) in mice. They found that the amplified ELISA was more sensitive than a commercially available radioimmunoassay (AUSAB, Abbot Laboratories) for the detection of antibody to HBsAg. They found very little background activity using avidin-biotin systems.

Although the dot-blot immunoassay is in its preliminary stages of development it offers certain advantages over conventional ELISAs: antigen can be applied quickly and directly to nitrocellulose which takes the place of conventional solid phases, ease of handling and all incubation steps are at room temperature. It is unlikely that this assay could be used as an "on-the-spot" diagnostic test. However it may prove useful for the detection of gonococcal antigen in transported specimens and possibly also for the detection of antigen in specimens from cases of DGI.

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