STUDIES OF *NEISSERIA GONORRHOEAE*

by

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CONTENTS

INTRODUCTION

Neisseria gonorrhoeae (*N. gonorrhoeae*) is a remarkable pathogen that produces a disease spectrum extending from asymptomatic mucosal infection to bacteraemia and fatal meningitis (Brittigan *et al.*, 1985). In spite of efforts to control this pathogen and the availability of adequate antibiotic therapy, nearly a million cases of gonococcal infection are reported annually in the USA alone. The problem is compounded by the increasing prevalence of strains that are resistant to the three antibiotics most widely used viz., the penicillins, tetracyclines and aminoglycosides (Anon., 1987).

Antimicrobial resistance in *N. gonorrhoeae* can be plasmid mediated (penicillinase-producing *N. gonorrhoeae* - PPNG), or chromosomally mediated (chromosomally mediated resistant *N. gonorrhoeae* - CMRNG) or both. The three most important forms of resistance, from a public health point of view, are PPNG, CMRNG and plasmid-mediated, high level tetracycline resistance (Anon., 1987).

Locally there are no reports of high level plasmid mediated tetracycline resistance with a minimum inhibitory concentration (MIC) > 16 or of CMRNG with MIC > 1 for penicillin G. The subject of PPNG infections in Africa was reviewed by Osoba (1986), based on data from the World Health Organization surveillance system for PPNG. Strains that emerged in 1975 have spread rapidly and exponentially and have reached prevalence levels of 10-30%. In Ibadan, Nigeria, PPNG

strains were first detected in 1979 and by 1984 81% (of *N. gonorrhoeae*) were PPNG. Similarly in Nairobi, Kenya, PPNG strains increased from 4% to 22% over the same period. In South Africa the first strains of PPNG were reported in 1977 from Durban and Johannesburg (Hallet *et al.*, 1977; Robins-Browne *et al.*, 1977). By 1984 PPNG infections in Durban had increased to 12% (Coovadia *et al.*, 1984).

Epidemiologically, PPNG strains have been linked with either the Far East or West Africa (Perine *et al.*, 1977). The Far Eastern PPNG isolates originally contained a 4,5 Megadalton (Mdal) plasmid and most often also contained a 24,5 Mdal plasmid. The West African isolates contained a 3,2 Mdal plasmid. The 3,2 and 4,5 Mdal plasmids are resistance factors that code for *beta*-lactamase. The large 24,5 Mdal plasmid is a conjugative or transfer factor which enables resistance to be transferred to other *N. gonorrhoeae* and other organisms (Mayer *et al.*, 1974). Several other *beta*-lactamase plasmids have been identified in *N. gonorrhoeae* viz., 2,9 Mdal (van Embden & van Klingeren, 1985), 2,6 Mdal (Dillon *et al.*, 1985) and 6,6 kilobase (Gouby *et al.*, 1986). The picture is further complicated since 1980 when a PPNG strain carrying both 3,2 and 24,5 Mdal plasmids was isolated in The Netherlands (van Embden *et al.*, 1981).

Many strains of *N. gonorrhoeae* exhibit requirements for certain growth factors which can be defined by growth on agar media of known composition (Carifo & Catlin, 1973; Catlin, 1973). A system of typing based upon these nutritional, or

auxotrophic, requirements has allowed differentiation of isolates and this technique, referred to as auxotyping, has been utilized to characterize strains from various disease outbreaks (Bohnhoff *et al.*, 1986) and geographic areas (Knapp *et al.*, 1978; Yvert *et al.*, 1985).

Several studies show a correlation between antibiotic susceptibility and geographic location of isolates (Dillon *et al.*, 1985; Knapp *et al.*, 1978; Yvert *et al.*, 1985). A three way correlation between susceptibility, clinical syndrome and auxotype has also been shown (van Klingeren *et al.*, 1985).

N. gonorrhoeae is antigenically complex and various systems of serotyping, based upon the antigenic diversity of components of *N. gonorrhoeae* outer membranes, have been developed (Knapp *et al.*, 1984; Sandstrom & Danielsson, 1980; Wang *et al.*, 1977).

The relative resistance or susceptibility of *N. gonorrhoeae* to antibiotics has been the subject of much research. The isolation of *beta*-lactamase producing strains since 1976 (Phillips, 1976) and high level chromosomal resistance (Easmon, 1985; Rice *et al.*, 1986) has increased concern about this subject.

King Edward VIII hospital is the regional reference centre for Durban and surrounding areas. The importance of gonorrhoea as a sexually transmitted disease locally is emphasized by the following:

- *N. gonorrhoea* infection is the commonest cause of acute urethritis in black men in Durban, being isolated in 95,9% of 217 untreated patients (Hoosen *et al.*, 1987);
- it is associated with 62% of pelvic inflammatory disease of which 33% were PPNG (Quinlan *et al.*, 1987);
- this organism was cultured in 31% of patients presenting with a vaginal discharge to a Sexually Transmitted Disease (STD) clinic in Durban (O'Farrell *et al.*, 1987);
- the carriage rate in pregnant African women is 10% (Quinlan *et al.*, 1987);
- in a rural population the incidence ranges from 4 to 5,7% (O'Farrell *et al.*, 1987).

The association of *N. gonorrhoeae* with pelvic inflammatory disease, ectopic pregnancy and infertility makes this infection an important public health, social and individual problem. Thus far no attemps have been made to characterize local isolates. Though this information would not alter treatment regimens it would be of value in tracing future epidemics. This study was undertaken to determine antibiotic sensitivity patterns to currently used antibiotics, to characterize plasmid content and auxotype patterns of local strains. With the information obtained an attempt was made to trace the source of local *N. gonorrhoeae* strains believed to be either of Far Eastern or West African origin.

I. LITERATURE REVIEW

A) BIOLOGY OF *Neisseria gonorrhoeae*

The genus *Neisseria* is named after Dr. Albert Neisser, who discovered the aetiological agent of gonorrhoea in 1889. The genus *Neisseria* contains two recognized pathogenic species viz., *N. meningitidis* which colonises the upper respiratory tract and invades the epithelium to produce systemic disease and *N. gonorrhoeae* which infects the columnar epithelium of the genital tract and rectum and only rarely causes systemic disease. Other species of *Neisseriae* have occasionally been associated with infection but are generally regarded as commensals (Johnson, 1983).

1. Isolation and growth

A suitable growth medium for this fastidious organism must contain cysteine and iron, and the only carbohydrate used is glucose. In some test media these requirements are met by the addition of blood (often chocolatised) or haemoglobin to a GC (*i.e.* gonococcal) agar base (Difco) containing peptones and starch. In others, supplements such as Kellogg's supplement (White & Kellogg, 1965) or Isovitalex (BBL) without blood products are used.

The optimum temperature for growth of this organism is 36-37C. A humidity of approximately 50% is beneficial to growth of all *Neisseria* species and 3-10% CO_2 is required. In clinical practice antibiotics are often added to the isolation media to suppress the growth of normal flora. The most popular

combination of antibiotics includes vancomycin ($3mg.l^{-1}$), which inhibits Gram positive bacteria, colistin ($7,5mg.l^{-1}$), which is active against Gram negative rods except *Proteus* species and trimethoprim ($5mg.l^{-1}$), which prevents the swarming of *Proteus* species. Nystatin ($12500\ U.l^{-1}$) or amphotericin ($1,5mg.l^{-1}$) is added to inhibit yeasts (Easmon & Ison, 1987).

The two commonly used selective media are a modified Thayer-Martin medium (Thayer & Martin, 1966) and New York City Medium (Young, 1978). Several defined media have been developed for growth of *N. gonorrhoeae* (Catlin, 1973; La Scolea & Young, 1974; Wong *et al.*, 1980).

Presumptive identification of *N. gonorrhoeae* is made by the presence of typical colonies (which may vary on different media) and of oxidase positive, Gram negative diplococci. These colonies are confirmed as *N. gonorrhoeae* by various methods, but carbohydrate utilization is still the most widely accepted. A direct fluorescent antibody test can be used, but polyclonal antibodies have not been found to be fully specific, false positive fluorescence being shown by some strains of *N. lactamica* and *N. meningitidis* (Easmon & Ison, 1987). The utilization of monoclonal antibodies in such reagents has increased specificity and sensitivity (Tam *et al.*, 1982).

Other biochemical tests have been utilized as alternatives or adjuncts to carbohydrate tests. Extracellular enzymes of most

Neisseria species have been measured but alpha-glutamyl-aminopeptidase appears to be the only enzyme useful for differentiating *N. gonorrhoeae* which is negative from *N. meningitidis* which is positive (D'Amato *et al.*, 1978).

The methods described require two to three days for identification and differentiation of *N. gonorrhoeae* and several rapid methods using other antigen detection systems such as enzyme linked assays (Schachter *et al.*, 1984), immunofluorescence (Easmon & Ison, 1987) and DNA probes (Totten *et al.*, 1983) have been described.

2. Colonial variants

Colonial morphology varies amongst species and ranges from small, smooth, transparent, butyrous colonies to wrinkled, dry, adherent colonies (Catlin, 1973). Five colony types have been described and labelled T1-T5 (Kellogg *et al.*, 1963). Types T1 and T2 are virulent for human male volunteers but not T3 and T4. T1 colonies are small, raised, dewdrop-like, slightly viscid with entire edges. T2 colonies may be friable and have defined or crenated edges or both. T3 and T5 colonies are larger than T1, slightly convex, and light brown or colourless (Kellogg *et al.*, 1963). Further studies have shown that pili are present on small colony variants T1 and T2 and absent on T3 and T4 (McGee *et al.*, 1977). The different types can be differentiated by oblique light as described by Juni & Heym (1977).

Opaque and transparent variants of the colony types have been described by Swanson (1978a), where:

P^+ = small colony form, rather indistinct edges;

P^{++} = small colony form, distinct edges;

P^- = large colony form;

O^+ = opaque colony form (intermediate opacity);

O^{++} = very opaque.

3. Structure

(a) Macro-structure

N. gonorrhoeae are Gram-negative organisms, usually appearing as diplococci, whose opposing surfaces are somewhat flattened. These non-spore forming bacteria are encapsulated and exhibit 'twitching' motility, such motility being restricted to gonococci bearing pili (James & Swanson, 1977). Various degrees of aggregation or clumping are observed for gonococci grown in liquid medium. Piliated (P^+ or P^{++}) organisms of opaque colony phenotypes (O^+ or O^{++}) exhibit the highest degree of clumping. Nonpiliated (P^-) transparent colony phenotypes (O^-) grow as smooth suspensions of separated diplococci (Swanson, 1978a).

(b) Ultrastructure

The cell envelope of all Gram-negative bacteria is composed of three components viz., the outer membrane, the cytoplasmic membrane, and the rigid peptidoglycan layer. When observed by electron microscopy *N. gonorrhoeae* exhibits morphology typical

of Gram-negative bacteria (Novotny *et al.*, 1975). Unlike most Gram negative bacteria, but like *N. meningitidis*, *N. gonorrhoeae* exhibit focal depressions, or pits, of the cell membrane which are resolved by either negative staining or freeze etch methods of preparation for electron microscopy. *N. gonorrhoeae* also exhibits numerous evaginations, or blebs, of their outer membranes, that are seen both *in vivo* and *in vitro* (Swanson, 1972).

The outer membrane of *N. gonorrhoeae* contains proteins, lipopolysaccharides (LPS) and loosely bound lipids (Johnston *et al.*, 1976). The outer membrane complex contains three major proteins viz., I, II and III. Protein I predominates and appears to consist of a single polypeptide chain. It functions as a porin, and has been shown to vary antigenically and structurally among different *N. gonorrhoeae* strains (Johnston *et al.*, 1976). Protein I forms the basis of the serological tests of Wang *et al.* (1977). Protein II comprises a family of heat modifiable proteins that seem to appear only in colonies of the opaque phenotype (Swanson, 1978a). One of the most interesting aspects of protein II is that its absence or presence is related to the site of infection (Gotschlich 1984). Protein III is thought to be the antibody binding site (Brittigan *et al.*, 1985).

(c) Surface components

The surface components of the gonococcus and their functions are summarised in Fig. 1 and Table 1 (Brittigan *et al.*, 1985).

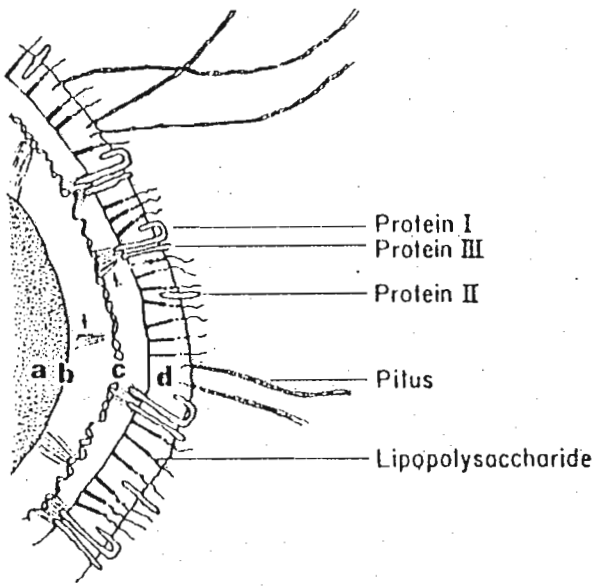


FIG. 1. Gonococcal surface components
a - cytoplasm; b - inner cytoplasmic membrane;
c - peptidoglycan of cell wall; d - outer membrane

TABLE 1. Gonococcal surface components

COMPONENT	PATHOGENIC ROLE
Pilus	Adherence to mucosa Inhibition of phagocytosis
Protein I	Porin activity
Protein II	Adherence to mucosal cells Adherence to neutrophils Clumping of gonococcus
Protein III	Binding site of antibody
LPS	Serum resistance Toxicity to ciliated epithelium

4. Clinical manifestations

The clinical spectrum of gonorrhoea, which is essentially a sexually transmitted disease, is broad and includes asymptomatic, symptomatic and complicated infections at

several anatomical sites (Handsfield, 1985a). In Fig. 2 the interrelationships of the major syndromes are summarized.

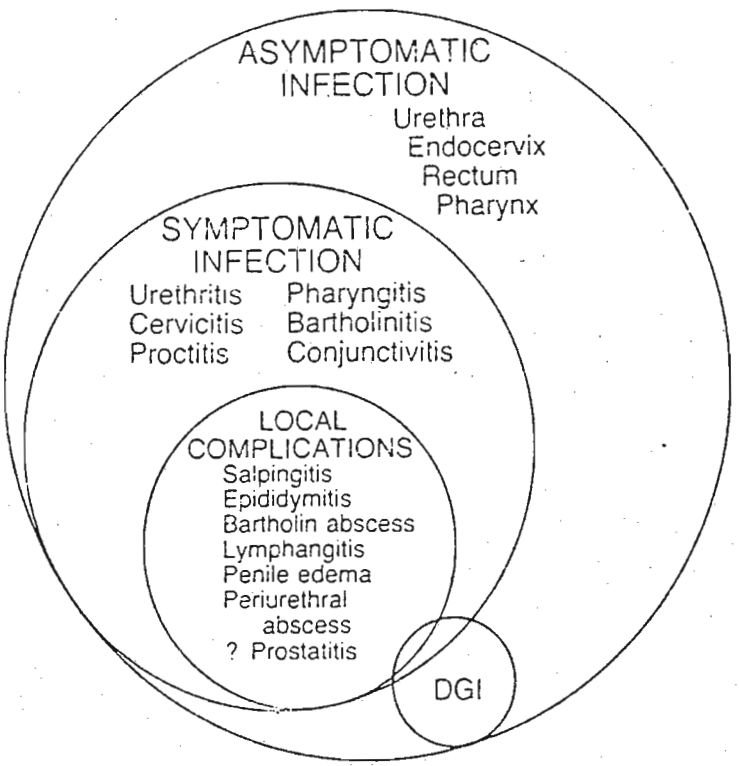


FIG. 2. Clinical spectrum of gonococcal infections
DGI = disseminated gonococcal infection
(Adapted from Handsfield, 1985a)

B) TYPING OF *Neisseria gonorrhoeae*

During the 1930's and 1940's, well established serotyping schemes were available for *Salmonella, Shigella, Escherichia coli*, meningococci, streptococci and pneumococci (Danielsson & Maeland, 1978). Attempts to serotype *N. gonorrhoeae* proved a formidable challenge due to antigenic variants. Almost all of the methods used for typing pathogenic bacteria have been tried on *N. gonorrhoeae* strains and it was not until the advent of monoclonal antibodies that a reproducible scheme was developed.

1. Serological Methods

These methods are based on the antigenic differences between bacterial strains.

(a) Agglutination

According to Danielsson & Maeland (1978) antibodies that could differentiate *N. gonorrhoeae* from *N. meningitidis* and other non-pathogenic *Neisseriae* were available as early as 1907. Wilson (1954) attempted to serotype gonococci with the tube agglutination method. He described the ability of the gonococcus to undergo "smooth-rough" variation. He managed to serotype *N. gonorrhoeae* with the use of agglutinin adsorbed sera, but found that some strains were capable of losing or gaining an antigen on repeated subculture, thus giving inconsistent results.

(b) Complement fixation

According to Danielsson & Maeland (1978), Reyn, in 1940, used the complement fixation test that was developed by Kristenson in 1930. Adsorption was carried out with formalin treated organisms which were then mixed with guinea pig anti-GC antiserum. Non-adsorbed anti-GC antisera reacted with all *N. gonorrhoeae* tested, due to the presence of a common antigen which was thermostable. In order to demonstrate type specific antigens, anti-GC sera were cross adsorbed with selected strains of *N. gonorrhoeae*. In this way "factor sera" were

produced, factor referring to the antibody contents of the sera. This technique was hampered by the fact that these adsorbed factor sera were unstable on storage. This method was therefore unsuitable for serotyping *N. gonorrhoeae*.

(c) Immunofluorescence and agar gel diffusion

Deacon *et al*. (1959) applied immunofluorescence (IF) to serotype *N. gonorrhoeae*, using rabbit antiserum raised by injection of formalin treated *N. gonorrhoeae*. Cross-reaction with *N. meningitidis* necessitated adsorption of this reagent to render it specific for *N. gonorrhoeae*. The IF test (Bacto FA *N. gonorrhoeae*) is now routinely used by some laboratories to diagnose *N. gonorrhoeae* infections or confirm identity of clinical isolates.

A double immunodiffusion technique has been employed by Johnston *et al*. (1976). Using double diffusion in agar gels they divided *N. gonorrhoeae* into 16 distinct serotypes on the basis of a type-specific protein antigen from the outer membrane of the cell wall. This protein is the major polypeptide of the outer membrane and could be detected after repeated subculture.

(d) Coagglutination

Danielsson & Kronvall (1974) successfully adopted the co-agglutination (COA) method for rapid identification of *N. gonorrhoeae*. The COA method utilizes the ability of *Staphylococcus aureus*, Cowan I, to bind to IgG (except for

IgG_3) by the Fc fragment. The staphylococci are allowed to react with rabbit anti-gonococcal antibody, previously adsorbed to remove cross reacting antibody. An agglutination lattice, which is visible to the naked eye, is formed when such coated staphylococci are mixed with gonococci (Danielsson & Kronvall, 1974). A kit using this technique is currently available.

(e) Immunotyping

Using microimmunofluorescence with antibodies against protein I, Wang *et al.* (1977) defined three serogroups (A, B and C). Sandstrom & Danielsson (1980) explained the gonococcal outer membrane in terms of three classes of antigens: W, J and M. The W class of antigens correlate with protein I antigens (Sandstrom *et al.*, 1982). The M class antigens are the lipopolysaccharides (LPS) and the J class antigens are thought to be protein II (Sandstrom & Danielsson, 1980). Using immunotyping, they prepared antisera by selective cross adsorbtion of hyperimmune rabbit sera against *N. gonorrhoeae* outer-membrane proteins. With such a reagent three antigen classes (WI, WII and WIII) were recognized, which corresponded to the serogroups A, B and C respectively described by Wang *et al.* (1977).

Sandstrom *et al.* (1982) subsequently showed that protein I could be split by peptide mapping into two main antigenic types, IA and IB which corresponded to WII and WIII respectively. Tam *et al.* (1982) raised a series of monoclonal

antibodies to epitopes on protein IA and IB. A standard panel of 12 monoclonal antibodies was developed by Knapp *et al.* (1984) to provide a system of 18 IA and 28 IB gonococcal serovars. This system is being increasingly used to serotype *N. gonorrhoeae*.

2. Other methods

(a) Bactericidal test

Glynn & Ward (1970) used antigens of *N. gonorrhoeae* involved in the serum bactericidal reaction and grouped them into four main groups according to their resistance to killing by human complement together with either normal or immune rabbit antibodies. They found that the antigens involved in the bactericidal reactions were LPS of several distinct specificities. Tramont *et al.* (1974) used this test and found that the antisera against *N. meningitidis* were also capable of killing *N. gonorrhoeae* and provided evidence for differences amongst strains. They concluded that surface proteins and LPS were involved.

Lavitola *et al.* (1983) used serum resistance of *N. gonorrhoeae* as an epidemiological marker. They tested 100 strains for their sensitivity to killing by normal human serum. The bactericidal end point was taken as the dilution that killed 95% of the test organism, and this end point correlated well with strains isolated from different anatomical sites in the

same patient or from sexual partners and isolates of the same auxotype pattern.

(b) Immunoglobulin A1 protease

Mulk & Knapp (1987) used IgA1 protease to type *N. gonorrhoeae*. IgA1 proteases are extracellular bacterial proteolytic enzymes produced by pathogenic strains of *N. gonorrhoeae* and *N. meningitidis*. Their function is to split antibodies of the IgA1 subclass. They are also believed to be virulence determinants (Easmon & Ison, 1987). *N. gonorrhoeae* produce two types of IgA1 proteases, each of which cleaves a different peptide bond in the hinge region of human IgA1. The type of IgA1 protease produced correlates with nutritional auxotype and outer membrane protein I serovar. Gonococcal type I IgA1 protease is produced primarily by strains which require arginine, hypoxanthine, and uracil (AHU) and which belong to protein IA-1 and IA-2 serovars. Other auxotypes and serovars produce type II IgA1 proteases (Mulk & Knapp, 1987).

(c) Lectin agglutination

Plant and animal lectins recognise specific carbohydrate structures on the bacterial surface. According to Easmon & Ison (1987) patterns of lectin agglutination can be used to differentiate between bacterial species and bacterial strains within the same species. Using 14 different lectins, Schalla *et al.* (1985b) found that isolates of identical auxotype, plasmid content and serovar, could be differentiated on the

basis of lectin agglutination. Schalla *et al.* (1985a) also used lectin agglutination to follow an outbreak of gonorrhoea. They observed two predominant lectin groups which were not associated with resistance patterns. Isolates from different anatomical sites in the same patients gave the same lectin group and serovar pattern but different auxotype pattern.

To summarise, the differentiation of *N. gonorrhoeae* from other Gram negative diplococci isolated from the mucosal surface and strain differentiation is important for guiding therapy. Serological reactions and detection of enzymes require subculturing of the organism to provide adequate cellular mass for testing. This can be overcome by polyclonal and monoclonal antibodies. Over the years gonococcal typing has progressed from a simple polyclonal system with poor discrimination to a highly developed monoclonal system. The coagglutination system used is simple and sensitive, protein I serotypes are stable and the system is reproducible. Since monoclonal reagents are not readily available, the alternative method used to type *N. gonorrhoeae* is auxotyping. Whilst auxotyping is reproducible, it is technically demanding and time consuming and therefore impractical for routine diagnostic laboratory work.

C) AUXOTYPING OF *Neisseria gonorrhoeae*

Specific nutritional requirements vary from strain to strain and several patterns of these requirements have been seen in clinical isolates. These patterns have been used by Ansink-

Schipper *et al.* (1984), Catlin (1973), Catlin & Pace (1977), Draper *et al.* (1981), Noble & Parekh (1983a) and others in a system called auxotyping. Although relatively complicated, auxotyping has until recently been the most widely used typing scheme for *N. gonorrhoeae*.

1. Rationale

Auxotyping is based on the growth response of strains on chemically defined media. The basic medium is composed of inorganic salts and glucose. Cystine or cysteine is required by all gonococci but not meningococci. This serves to differentiate them in the auxotyping scheme (Catlin, 1973). *N. gonorrhoeae* strains having no other requirement than this are called wild type or prototype. Other strains require one or more of the following compounds viz., proline, arginine (subdivided according to whether ornithine or citrulline satisfies their needs), hypoxanthine, uracil, thiamine pyrophosphate, thiamine hydrochloride, methionine, isoleucine, leucine or valine. Some less common requirements are glutamine, histidine and lysine (Carifo & Catlin, 1973).

Although as many as fifteen different growth factors are used to auxotype *N. gonorrhoeae*, the most common auxotypes are:

- those that require none of the differential factors prototrophic (proto);
- those requiring proline only (pro^-);
- those requiring arginine only (arg^-); or

- those requiring several growth factors, including arginine, hypoxanthine and uracil-AHU (Catlin, 1973).

Thus far 35 different auxotypes (Table 2) have been described by Catlin & Pace (1977).

TABLE 2. Table of auxotypes (Catlin & Pace, 1977)

Auxotype	Phenotype*
1.	Zero
2.	Pro
3.	Arg
4.	Met
5.	Thi
6.	Thp
7.	Pro, Arg
8.	Pro, Met
9.	Pro, Hyx
10.	Pro, Hyx, Thi
11.	Pro, Arg, Hyx, Ura
12.	Arg, Hyx
13.	Arg, Met
14.	Arg, Hyx, Ura
15.	Arg, Met, Hyx, Ura
16.	Arg°, Hyx, Ura
17.	Arg°, Met, Hyx, Ura
18.	Arg°, Hyx, Ura, Thp
19.	Arg, Hyx, Ura, Thp
20.	Arg, Hyx, Ura, Thi
21.	Pro, Thp
22.	Pro, Met, Thp
23.	Pro, Thi
24.	Arg, Thi
25.	Lys
26.	His
27.	Arg, His
28.	Arg, His, Hyx, Ura
29.	Arg, Leu, Hyx, Ura
30.	Arg°, His, Hyx, Ura
31.	Arg°, Leu, Hyx, Ura
32.	Arg°, Leu, Hyx, Ura, Thp
33.	Pro, Arg°, Met, Hyx, Ura
34.	Pro, Arg°, Met, Hyx, Leu, Ura
35.	Pro, Arg, Hyx, Ura, Thp

* Arg = arginine, Arg° = Arginine replaced by ornithine or citrulline, Hyx = hypoxanthine, Leu = leucine, Lys = lysine, Met = methionine, Pro = proline, Thi = thiamine hydrochloride, Thp = thiamine pyrophosphate, Ura = uracil.

2. Uses

Auxotypic markers are stable *in vivo*, as shown by studies of serial isolates from patients seen more than once before treatment (Knapp & Holmes, 1975), isolates from two or more sites in individual patients (Catlin & Pace, 1977) and from isolates from contact partners (Knapp & Holmes, 1975).

In selected cases auxotyping may allow reinfection to be distinguished from treatment failure (Catlin & Pace, 1977).

At a broader level, auxotyping has proved useful in following chains of infection within a community and in establishing the distinctive distribution of different nutritional types of *N. gonorrhoeae* among isolates from different anatomic sites and syndromes (Knapp & Holmes, 1975).

Auxotyping has been of particular value in the examination of strains isolated from patients suffering from disseminated gonococcal infections (DGI). *N. gonorrhoeae* causing DGI have a constellation of phenotypic characteristics that serve to distinguish them from those strains causing uncomplicated infections. These properties include:

- greater susceptibility to penicillin and tetracycline (Weisner *et al.*, 1973);
- requirement of AHU (Morello *et al.*, 1976);
- small atypical colonial morphology, usually accompanied by weak carbohydrate fermentation

(Morello *et al.*, 1976);

- resistance to bactericidal action of human sera (Schoolnik *et al.*, 1976).

3. Genetic basis

Each nutritional requirement indicates that *N. gonorrhoeae* is unable to perform an enzyme catalyzed step in the biosynthesis of a particular metabolite. These auxotrophic gonococci possess hereditary defects, many of which are reparable by genetic transformation, using deoxyribonucleic acid (DNA) isolated from a strain of *N. gonorrhoeae* possessing the corresponding biosynthetic function (Catlin, 1974).

Genetic transformation studies by Mayer *et al.* (1977) show that the mutations responsible for arg requirement in all AHU auxotype strains are identical, and are distinct from the three different genotypes found in strains that were arg- auxotypes and had no requirement for hyx and ura.

N. gonorrhoeae strains isolated from DGI share a number of phenotypic characteristics as described previously. These AHU strains frequently possess a particular immunochemical type of protein I in the outer membrane (Cannon *et al.*, 1983), and in most cases contain methyladenine in their DNA (Kolodkin *et al.*, 1982). According to Cannon & Sparling (1984) these properties are independent and are encoded by separate genetic loci.

Catlin & Reyn (1982) found defects in the biosynthesis of methionine, proline, arginine, threonine, lysine, the branched chain amino acids, hypoxanthine and thiamine pyrophosphate in some of the isolates from the prepenicillin era, but no defect in the requirement for uracil or other pyrimidines. They concluded that the AHU strain evolved when treatment with sulphonamides was replaced by penicillin.

4. Epidemiological correlation of auxotypes

Auxotypes correlate well with certain patterns of infection, sexual orientation, geographic origin of isolates and antibiotic sensitivity (Stewart & Hendry, 1979).

(a) Relationship between geographical distribution and auxotype

Auxotyping has demonstrated that in different geographical areas, and over time, variation in prevalences of individual auxotypes occur (Brunham *et al.*, 1985a). Several reports indicate that the AHU auxotype has become more prevalent in certain areas, however a search through the literature from 1975 to date reveals that proto and pro$^-$ strains are predominant in the developing countries (Table 3).

(b) Relationship between auxotype and diseases

The endocervix, anal canal, urethra and oropharynx each provide different microenvironments for the organism because of different kinds of epithelial cells, bathing fluids and distinctive normal microbial flora. When a homogeneous

population of *N. gonorrhoeae* is introduced into a host it is exposed to selective factors which operate to increase the diversity of the organism (Catlin, 1976).

A multisite isolate study by Catlin & Pace (1977) showed that nutritional requirements of *N. gonorrhoeae* are stable during multiplication at different sites.

Bohnhoff *et al.* (1986) found that all the *N. gonorrhoeae* isolates from DGI which they investigated belonged to AHU auxotype, however this auxotrophy is not a constant feature of DGI as reported from other geographic areas (Knapp & Holmes, 1975; Schoolnik *et al.*, 1976). In women with salpingitis it has been found that 75% were prototrophic and none AHU requiring (Draper *et al.*, 1981). However, studies by Brunham *et al.* (1985b) failed to find a correlation between disease and auxotype.

(c) Relationship between auxotype and race

The AHU strains are significantly less prevalent in black patients than white patients in the United States (Noble & Miller, 1980). White patients appear to be infected with strains that are more susceptible to antibiotics and belong to the AHU auxotype, whereas black patients seem to be infected with pro^- auxotype strains and appear to be less susceptible to antibiotics. No satisfactory explanation for these phenomena exists. However, in an attempt to explain this, Noble & Parekh (1983b) took pro^- and AHU auxotypes from

TABLE 3. Prevalent auxotypes of some geographic areas

Region	Auxotype	%	Authors
Seattle	AHU	>50	
Denver	AHU	>22	
New York	AHU	>12	Knapp *et al.* (1978)
Montreal	AHU	44	
	Proto		
	Pro$^-$	16	Turgeon & Granger (1980)
Canada	Pro$^-$	51	
	Proto	42	Dillon *et al.* (1985)
Sweden	Pro$^-$		
	Pro$^-$Arg$^-$ura$^-$		
	AHU		
	Proto		Danielsson *et al.* (1983)
The Netherlands (PPNG)	ProtoP^i	88	
	Meth	12	Ansink-Schipper *et al.* (1984)
The Netherlands (PPNG)	Pro$^-$	44	
	ProtoP*i*	39	van Klingeren *et al.* (1985)
Zurich	Proto		Eichmann & Piffaraetti (1984)
	Pro$^-$		
Munich	Proto	62	
	Pro$^-$	30	
	AHU	8	Ruckdeschel *et al.* (1983)
Toronto	Orn$^-$	13	
(non PPNG)	Pro$^-$	12	
(PPNG)	Pro$^-$	51	
	Orn$^-$	4	Shtibel (1985)
Franceville (Gabon)	Proto		
	Pro$^-$		
	Arg$^-$		Yvert *et al.* (1985)
Chile	Proto		
	Pro$^-$		Garcia *et al.* (1987)

ProtoP^i = Prototrophic strains inhibited by phenylalanine

different racial groups and looked at their ability to bind buccal epithelial cells or to be ingested by polymorphonuclear leucocytes (Noble *et al.*, 1985). No differences were found.

(d) Relationship between auxotype and sexual orientation

A comparison of homosexual and heterosexual men show that AHU is uncommon in homosexual men (Handsfield, 1985a). Fig. 3 shows the distribution of AHU auxotype in populations with respect to sex, race and sexual orientation.

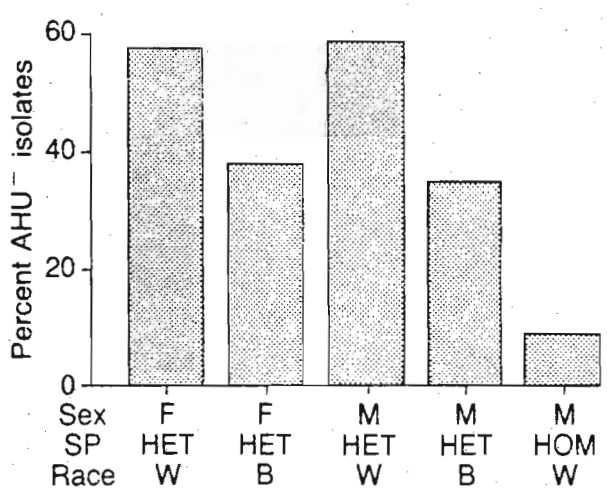


FIG. 3. Distribution of AHU auxotype of *N. gonorrhoeae* for whites (W), blacks (B), males (M), females (F), heterosexuals (HET) and homosexuals (HOM) among patients with uncomplicated gonorrhoea (Handsfield, 1985a)

D) ANTIBIOTIC RESISTANCE

Resistance of the *N. gonorrhoeae* is a significant problem. The organism has evolved diverse mechanisms for coping with a hostile environment. Antimicrobial resistance may be due to mutations of chromosomal genes or plasmid-borne genes or both (Dillon & Pauze, 1984).

1. History

In spite of adequate antibiotic therapy for over forty years *N. gonorrhoeae* persists today as one of the most important mediators of sexually transmitted diseases.

Early in the development of penicillin during the Second World War, its efficacy against gonorrhoea was exploited. *N. gonorrhoeae* were found to be naturally sensitive not only to penicillin (MIC $< 0{,}06$ mg.l^{-1}), but also to tetracycline. By 1958 an increase in penicillin resistance to over 20 times the levels of 1944 was reported (Reyn *et al.*, 1958). In some parts of the world, notably the Far East and Africa, this increase in resistance developed rapidly. Keys *et al.* (1969) found 74% of Far Eastern gonococci to have reduced sensitivity to penicillin (MIC $> 0{,}06$ mg.l^{-1}), while Arya & Phillips (1970) reported similar levels of penicillin resistance in gonococci from Uganda.

The rate at which penicillin MIC's increased was slow, and could be countered by increasing doses of penicillin or ampicillin and by adding probenicid. By the early 1970's

gonococcal strains with MICs of > 1,0 $mg.l^{-1}$ were being found in the Far East. Resistant strains then spread to Europe and the United States of America (Easmon & Ison, 1987).

Up to 1976 resistance was exclusively chromosomally mediated. Phillips (1976) was the first to report high level resistance to penicillin G due to the presence of a plasmid. These strains produce *beta*-lactamase, and are referred to as PPNG. The appearance of PPNG overshadowed chromosomal resistance until outbreaks involving high level chromosomal resistance were reported (Easmon, 1985; Rice *et al.*, 1986). These strains present several practical problems. There is no simple rapid method of detection short of quantitative sensitivity tests. Antibiotics active against PPNG may not necessarily be effective against CMRNG. With these strains, there appears to be cross resistance between penicillin and cephalosporins and cephamycins such as cefoxitin and cefuroxime (Easmon & Ison, 1987).

2. Recommended treatment regimens

The regimens recommended by the Centers for Disease Control (Anon., 1987) are outlined in Table 4.

TABLE 4. Treatment regimens recommended by Centers for Disease Control

For adults with uncomplicated urethral, endocervical, pharyngeal or rectal infections:

Ceftriaxone 250 mg Intramuscularly

PLUS

Doxcycline 100 mg by mouth, 2 times a day for 7 days

OR

Tetracycline 500 mg by mouth, 4 times a day for 7 days

OR

Erythromycin 500 mg by mouth, 4 times a day for 7 days

Several other treatment regimens appear promising :

- Cefotaxime (Handsfield & Holmes, 1980);
- Cefuroxime (Morrison, 1980);
- Rosoxacin, a new antimicrobial, is active against PPNG, and studies indicate that it is effective for uncomplicated infections in single doses (Handsfield *et al.*, 1981b);
- Sulfamethoxazole-trimethoprim is effective for both PPNG and non-PPNG (Brunham *et al.*, 1982);
- Tetracyclines, however these drugs are not reliable for infection with Far Eastern strains of PPNG which are often tetracycline resistant (Goodhart, 1979);
- Several ampicillin esters, carbenicillin, rifampicin,

chloramphenicol, and some aminoglycosides (Goodhart, 1979).

Spectinomycin is an antibiotic with excellent activity against penicillin sensitive and resistant *N. gonorrhoeae*. Spectinomycin resistance is controlled by chromosomal genes. Mutations giving rise to spectinomycin resistance have been described in strains isolated from the Phillippines, United Kingdom and Korea (Ashford *et al.*, 1981; Easmon *et al.*, 1982).

3. Genetics and mechanisms of resistance

The chromosomal DNA of *N. gonorrhoeae* is a single molecule of a molecular mass of approximately 980 x 10^6 daltons and contains about 1,5 million nucleotide base pairs. It has the capacity to code for some 1000 to 5000 genes, and is about one third the size of the *E. coli* genome (Swanson & Mayer, 1985).

Penicillin resistance in *N. gonorrhoeae* may be either intrinsic or extrinsic. Resistance to other antibiotics is mainly intrinsic, however these antibiotics have been less comprehensively studied.

- Intrinsic resistance to *beta*-lactam antibiotics is due to mutations in one or more chromosomal genes (Beveniste & Davies, 1973);
- Extrinsic resistance, to be discussed separately in the following chapter, is mediated by *beta*-lactamases of plasmid origin (Beveniste & Davies, 1973).

(a) Intrinsic resistance

An understanding of the genetics of resistance has been obtained by the use of transformation, a process by which one bacterium takes up, integrates, and expresses naked DNA from another organism. This was originally described in *N. gonorrhoeae* by Catlin (1973) who showed that all *Neisseria* species are transformable.

Using transformation as the principal tool, several groups of workers have shown that low level, non *beta*-lactamase resistance to penicillins is due to the additive effects of mutations at several loci (Sparling *et al.*, 1975). Some of the mutations result in resistance only to penicillin or other *beta*-lactam antibiotics, whereas others have a pleiotropic effect and result in resistance to multiple compounds (Sparling *et al.*, 1975). Some of these mutations increase resistance and others either decrease resistance or increase susceptibility. The genes for antibiotic resistance have been mapped by three factor transformation crosses and are linked (Cannon & Sparling, 1984).

(i) Mutations that increase resistance

Penicillin resistance is acquired in discrete steps, with several genes contributing in an additive manner. These genes include loci designated *pen*A, *mtr* and *pen*B (Sparling *et al.*, 1975).

Resistance to tetracycline and chloramphenicol is also due to additive effects of several different genes: *Tet mtr penB2*, and *cam mtr penB* respectively (Sparling *et al.*, 1975).

Jones *et al.* (1985) showed that four *amp* allelles account for the full level of resistance of the *amp2* phenotype. All four *amp* allelles lie within a cluster of genes which code for ribosomal functions. This region has the map order of *rif fus tet cam.*

Beta-lactam antibiotics inhibit the final stage in the biosynthesis of peptidoglycan, i.e. cross linkage by transpeptidase of adjacent peptide strands. They also affect DD-carboxypeptidase and DD-carboxypeptidase-transpeptidase, which are enzymes involved in peptidoglycan metabolism (Salton, 1977).

According to Dougherty *et al.* (1980) *N. gonorrhoeae* has three penicillin binding proteins (PBP's). These are thought to be penicillin sensitive enzymes involved in cell wall biosynthesis viz., peptidoglycan peptidases, carboxypeptidases and endopeptidases (Tomasz, 1982). A model that explains low level resistance is proposed by Dougherty (1985). PBP1 and PBP2 have different functional responsibilities and different penicillin susceptibilities. The susceptible parent strain possesses both targets. Treatment at MIC primarily affects the PBP2, which in some way affects the o-acetyl modification of the peptidoglycan. Treatment at higher concentration

affects both PBP1 and PBP2, leading to a decrease in cross linkage. The resistance mediated by *pen*A lowers the affinity for PBP2 to a point at which PBP1 becomes a primary target.

Genetic transformation studies show that genes for high level resistance to streptomycin *(str)* and spectinomycin (*spc)* are closely linked, and brackets genes (*tet* and *cam*) for low level resistance to the ribosomally active drugs, tetracycline and chloramphenicol respectively (Sarubbi *et al.*, 1975). This suggests that resistance to these drugs is probably due to an altered ribosome. Maness *et al.* (1974) showed that the resistance was mediated by a 30S ribosomal subunit. Since *tet* and *chlor* genes are located between *str* and *spc*, it seems likely that they also determine altered ribosome structure (Sparling *et al.*, 1978).

Studies on LPS structure in antibiotic susceptible and resistant strains show no difference (Stead *et al.*, 1975). Similarly, no differences have been found in phospholipids and fatty acids (Wolf-Watz *et al.*, 1982).

(ii) Mutations that reduce resistance

Mutations at a locus called *env* phenotypicaly reverse the effects of *mtr* (Sarubbi *et al.*, 1975) and result in increased outer membrane permeability and susceptibility to a wide range of dyes, detergents and antibiotics (Guymon *et al.*, 1978). A locus designated *env*10 has been described by Shaffer *et al.*

(1984) which also increases the susceptibility of *N. gonorrhoeae* to various drugs, dyes and detergents.

The hypersensitivity phenotype displayed by *N. gonorrhoeae* strains carrying mutations of *env* and *mtr* can be annulled by a further mutation to *env*. This releases the phenotypically suppressed *mtr* traits, thereby providing increased resistance and potential survival in the presence of a variety of antibacterial agents (Morse *et al.*, 1982).

E) PLASMID MEDIATED EXTRINSIC RESISTANCE

Plasmids are extrachromosomal circular DNA molecules found in most bacterial species and in some species of eukaryotes. The drug resistance plasmids, or R plasmids, were originally isolated from the bacterium *Shigella dysenteriaea* during an outbreak of dysentery in Japan and have since been found in *E. coli, Salmonella, Vibrio, Bacillus, Pseudomonas* and *Staphylococcus*. Their defining characteristics are that they confer resistance on their host cell to a variety of fungal antibiotics and are usually self-transmissible. Many antimicrobial resistance determinants have also been found to reside on transposons eg. ampicillin resistance transposons Tn1, Tn2, Tn3 of the TnA group (Broda, 1979). Plasmid mediated resistance has a tremendous potential for spreading as opposed to chromosomally mediated resistance (Jephcott, 1986).

1. *Beta*-lactamase coding plasmids

Beta-lactamase hydrolyses a cyclic amide bond of penicillins, cephalosporins and related compounds, to produce antibacterially inactive degradation products. *Beta*-lactamase production in PPNG is detected by iodometric test (Sykes & Nordstrom, 1972) or by the nitrocephin method (O'Callaghan *et al.*, 1972).

PPNG are resistant to penicillins and ampicillins. Various second and third generation cephalosporins and cephamycins, such as cefoxitin and ceftrioxone, are effective against PPNG, as is spectinomycin. *Beta*-lactamase inhibitors such as clavulanic acid and sublactams are also effective (Easmon & Ison, 1987).

(a) 'Africa' and 'Asia' plasmids

PPNG appeared to originate simultaneously in South East Asia and sub-Saharan West Africa, but have spread throughout the world. Most of the PPNG isolates from South East Asia are moderately resistant to many other antibiotics, presumably due to chromosomal mutations, and nearly always carry the 4,5 Mdal (7,4 kilobase) 'Asian' plasmid. In contrast, the isolates from Africa and Europe were less resistant to other antibiotics, and contained the 3,2 Mdal (5,3 kilobase) 'African' plasmid (Perine *et al.*, 1977).

PPNG with the 3,2 Mdal plasmid are characteristically arg- strains, and sensitive to unrelated antibiotics like

tetracycline. In contrast, strains with the 4,5 Mdal plasmid are either proto or pro$^-$ and relatively resistant to these drugs. Both these gonococcal plasmids are incapable of conjugal transfer. A 24,5 Mdal plasmid, which was found as early as the 1940's, is capable of mobilising the *beta*-lactamase to *N. gonorrhoeae* and other *Neisseria* species (Brunton *et al.*, 1986). Ansink-Schipper *et al.* (1982) found that almost all strains carrying the 3,2 Mdal plasmid are inhibited by phenylalanine, whereas none of 4,5 Mdal plasmid containing strains are inhibited.

Both plasmids carry 40% of TnA, including a *bla* gene that encodes TEM-1 *beta*-lactamase (Roberts *et al.*, 1977). Restriction endonuclease analysis and heteroduplex studies indicate that the only difference between these two plasmids is that the latter has a 2,1 kilobase (kb) insertion. This insertion is bounded by inverted repeats of approximately 300 base pairs (Dickgiesser *et al.*, 1982).

The predominant plasmid pattern in some countries is summarised in Table 5.

TABLE 5. Geographic distribution of predominant plasmid type

Geographic Area	Predominant plasmid	%	Author
Canada	4,5	87	Dillon *et al.*, 1981
	3,2	13	
Canada	3,02	100	Dillon *et al.*, 1985
The Netherlands	3,2	56	Ansink-Schipper *et al.*, 1984
	4,5	44	
The Netherlands	3,2	99	van Klingeren *et al.*, 1985
	4,5	97	
Zurich	3,2	97	Eichmann & Piffaraetti, 1984
	4,5	3	
Chile	4,5	100	Garcia *et al.*, 1987

The 3,2 and 4,5 Mdal plasmids are also related to similar *beta*-lactamase encoding plasmids encountered in :

- *Haemophilus influenza*, (Roberts *et al.*, 1977);
- *H. parainfluenza*, (Sparling *et al.*, 1978);
- *H. ducreyi*, (Anderson *et al.*, 1984); and
- *N. meningitidis*, (Dillon *et al.*, 1983).

H. Influenza, *H. parainfluenza* and *H. ducreyi* can conjugally transfer their resistance plasmids to the gonococcus (McNicol *et al.*, 1983). Some gonococci, particularly the 'Asia' type, also carry a 24,5 Mdal conjugal plasmid that can mobilize the transfer of the *Haemophilus* plasmid (Flett *et al.*, 1981).

(b) 'Rio' plasmid

Van Embden & van Klingeren (1985) described a third *beta*-lactamase plasmid of molecular mass 2,9 Mdal (4,6 kb). They found two identical plasmids of two distinct geographic origins; one from a sailor in Rotterdam whom they presume acquired it in South Africa, and the other from Rio de Janeiro. A comparison of this plasmid with the 'Asia' and 'Africa' plasmids show that the 'Rio' plasmid is 2,6 kb smaller than the 3,2 Mdal 'Africa' plasmid.

(c) 'Toronto' plasmid

Dillon *et al.* (1985) describe a fourth (4,4 kb) plasmid from *N. gonorrhoeae* involved in an outbreak in Toronto. This plasmid appears similar to the 2,9 Mdal 'Rio' plasmid except for a 100 base pair deletion on the large Hind III/ Bam HI fragment in the Toronto-type plasmid. This plasmid is directly related to the 4,5 Mdal plasmid.

(d) 'France' plasmid

Gouby *et al.* (1986) described a 6,6 kb plasmid from the Nimes Hospital in France. This plasmid appears identical to the 3,2 Mdal plasmid except for a small insertion fragment.

2. Origin of *beta*-lactamase plasmids

Two hypotheses have been put forward regarding the origin of the smaller 3,2 Mdal plasmid. Transformation studies suggest that the smaller 3,2 Mdal plasmid results from a deletion

event in the larger 4,5 Mdal plasmid (Sox *et al.*, 1978). The isolation of two new gonococcal *beta*-lactamase plasmids of 4,6 and 4,9 kb, which may be deletion derivatives of the 7,3 kb, but not of the 5,5 kb plasmid - judging by their restriction endonuclease patterns, indicates that transformation associated deletions may have taken place (van Embden & van Klingeren, 1985).

On the other hand, two types of *H. ducreyi* plasmids have been found (Brunton *et al.*, 1982; McNicol *et al.*, 1983) which seem identical to the *beta*-lactamase plasmids of *N. gonorrhoeae* except that they have retained the whole Tn3 sequence. This indicates that both types of gonococcal plasmids may have been introduced into the species from *Haemophilus*. Since both large and small *beta*-lactamase plasmids have a seemingly identical deletion of the left part of Tn3, a common deletion mechanism must in this case have operated before or upon the entry of plasmids into *N. gonorrhoeae*. This interrelationship is shown schematically in Fig. 4 (Brunton *et al.*, 1986).

3. Cryptic plasmid

About 96% of all clinical isolates also possess a small 2,6 Mdal/4,2kb plasmid, called the cryptic plasmid (Roberts & Falkow, 1979). It is intriguing that the cryptic plasmid of *N. gonorrhoeae* occurs in a majority of clinical isolates, except the citrulline-uracil-proline negative strains (Dillon & Pauze, 1981) and no identified function has been associated with it.

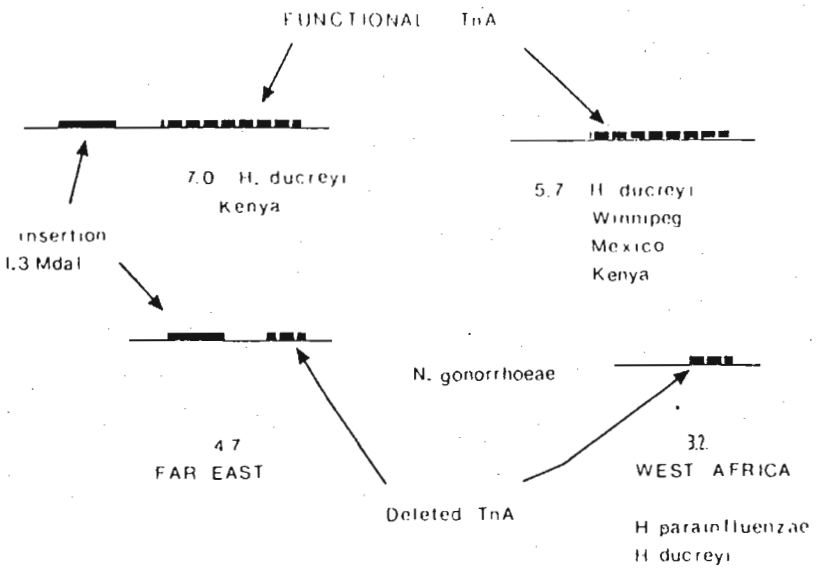


FIG. 4. Interrelations of *beta*-lactamase specifying plasmids found in *N. gonorrhoeae* and *H. ducreyi*. The sequences shown by the thin line are entirely homologous. The thick solid line represents the insertion, while the broken line represents TnA sequences. Adapted from Brunton *et al.* (1986)

4. Conjugative plasmid

Many gonococci also carry a large 24,5 Mdal conjugative plasmid, first described by Mayer *et al.* (1974). This plasmid is shown to have sex factor activity (Eisenstein *et al.*, 1977) and can mediate the transfer of itself, chromosomal genes and R plasmids.

Conjugative plasmids capable of mobilizing R plasmids are found in combination with both PPNG and non PPNG (Odugbemi *et*

al., 1983). They were isolated before the advent of the PPNG strains (Stiffler *et al.*, 1975) indicating that a conjugation system for plasmid transfer predated the appearance of R plasmids in *N. gonorrhoeae*. The nature of the natural selection that maintained these plasmids in *N. gonorrhoeae* before the appearance of R plasmids remains obscure. However, these conjugative plasmids now provide a means of transfer of antibiotic resistance *in vitro* and this transfer may occur *in vivo* as well (Sox *et al.*, 1978).

The conjugative plasmid predominates in certain geographical areas (Roberts & Falkow, 1979). This plasmid was initially found in 43% of Far Eastern strains but in none of the strains with the 'Africa' plasmid (Perine *et al.*, 1977). It was subsequently reported in combination with 'Africa' plasmid in Canada (Dillon & Pauze, 1981), the Netherlands (van Embden *et al.*, 1981) and Great Britain (Johnston & Kolator, 1982). This plasmid was also only detected in proto and pro^- auxotypes. Subsequent findings showed that it was found in arg^- and $pro^- met^-$ auxotypes (Dillon & Pauze, 1981).

5. Tetracycline resistance plasmid

In 1985 a new phenomenon of high level plasmid mediated resistance was described (Morse *et al.*, 1986). Strains of *N. gonorrhoeae* have been isolated which are highly resistant to tetracycline, with MIC's of 16 to 64 $ug.ml^{-1}$. This resistance is due to the acquisition of the resistance determinant *tetM*, a transposon borne resistance determinant, initially found in

the genus *Streptococcus* and more recently in *Mycoplasma hominis*, *Ureaplasma urealyticum*, and *Gardnerella vaginalis*. In *N. gonorrhoeae* the *tetM* determinant was located on a 25,2 Mdal plasmid. This plasmid arose from the insertion of *tetM* into the 24,5 Mdal gonococcal conjugative plasmid.

F) RELATIONSHIP BETWEEN AUXOTYPE PATTERN, SUSCEPTIBILITY TO ANTIBIOTICS AND PLASMID PATTERN

Several authors have reported the relationship between auxotype and susceptibility to antibiotics in non-PPNG (Ansink-Schipper *et al.*, 1982; Catlin & Pace, 1977; Noble & Parekh, 1983a). In general, pro$^-$ isolates were found to be less susceptible to antibiotics than proto strains.

Amongst the PPNG isolates a strong link between pro$^-$ auxotype and the 4,5 Mdal plasmid, and between proto auxotype and the 3,2 Mdal plasmid was found by Van Klingeren *et al.* (1985). Their studies show that pro$^-$ isolates harbouring a 3,2 Mdal plasmid, and the proto auxotypes inhibited by phenylalanine are highly susceptible to antibiotics, whereas pro- strains harboring the 4,5 Mdal plasmid are not. Proto strains inhibited by phenylalanine, carrying the 4,5 Mdal plasmid were found to be moderately susceptible in comparison to highly susceptible proto strains, inhibited by phenylalanine and carrying the 3,2 Mdal plasmid. Hence antibiotic resistance in *N. gonorrhoeae* seems to be influenced by nutritional requirements as well as plasmid content (Van Klingeren *et al.*, 1985).

II. MATERIALS AND METHODS

A) MICRO-ORGANISMS

N. gonorrhoeae strains were obtained from the Department of Microbiology, King Edward VIII Hospital, Durban. These organisms had been isolated from patients attending the STD clinic, and some from special cases of gonococcal infections at the hospital. Departmental staff had previously confirmed the cultures as *N. gonorrhoeae* by the following:

- Colonial morphology
- Positive oxidase reaction
- Characteristic Gram-stain reaction
- Carbohydrate utilization tests.

Reference cultures of *N. gonorrhoeae* were obtained from Dr. B. van Klingeren (Antonie van Leeuwenhoeklaan 9, Post Box 1, 2260 ad Pithoven, The Netherlands) and through the assistance of Professor I. Phillips (Department of Microbiology, St. Thomas's Hospital Medical School, University of London, London SE1 7EH). These reference strains and their characteristics are listed in Table 6.

Upon receipt, the cultures were plated out onto chocolate agar plates and incubated for 48h in candle extinction jars at 37C. They were then reconfirmed as *N. gonorrhoeae* by their typical Gram stain and positive oxidase test.

TABLE 6. Reference cultures of *N. gonorrhoeae*

Strain	Auxotype	Plasmids (Mdal)
From The Netherlands		
aux 3	pro^-	4,5 + 24,5 + 2,6
aux 6	$meth^-$	3,2 + 24,5 + 2,6
aux O	proto	3,2 + 24,5 + 2,6
From London		
PPNG 66	pro^-	4,5 + 2,6
PPNG 760		3,2 + 24,5 + 2,6
PPNG 65		3,2 + 2,6
T21	AHU	2,6 (Non PPNG)
T31	AHU	2,6 (Non PPNG)

The organisms were kept at -70C after being snap frozen in liquid nitrogen. From the time of receipt to the time of storage, the cultures had undergone no more than two subcultures.

B) CULTURE MEDIA

1. Chocolate agar

Chocolate agar plates (Difco Manual, 1986) were prepared as follows:

18g Tryptose blood agar base (Difco) was dissolved in 1 litre distilled water and autoclaved at 121C for 15 min. The medium was cooled to 55C-60C and 10% expired whole human blood preserved with ACD was added. The medium was reheated to 80C to chocolatise the blood. It was then cooled to 55C and poured into petri dishes.

2. Lysed horse blood agar

Lysed horse blood (LS) agar (Difco Manual, 1986) was prepared by adding 40g DST agar to 950ml distilled water. This was gently boiled to dissolve the agar and autoclaved at 121C for 15 min. After cooling to 55C, 5% Saponin-lysed horse blood was added.

3. Reagents for oxidase production

Stock solution (Difco Manual, 1986):
0,5g tetramethyl-paraphenylene-diammonium-dichloride was dissolved in 2ml distilled water; 96% (v/v) ethanol was added to bring the volume to 50ml. This solution was stored in a dark bottle.
Working solution: 1 part stock + 2 parts distilled water.

4. Modified New York City Agar

Modified New York City (MNYC) Agar (Young, 1978) was made up as follows:

- 18g of Oxoid GC Agar Base CM367 was suspended in 425ml distilled water and gently boiled to dissolve the agar. This was then autoclaved at 121C for 15 min;
- 50ml of defibrinated horse blood was lysed with 0.5% (v/v) saponin;
- The contents of a vial of Yeast Autolysate supplement SR105 (Oxoid) was dissolved in 15ml sterile distilled water;

- The contents of a vial of LCAT* Antibiotic Supplement SR95 was dissolved in 10ml of sterile distilled water;
- The lysed defibrinated horse blood, Yeast Autolysate Supplement and LCAT Antibiotic Supplement were aseptically added to the sterile GC Agar Base (cooled to 50C). This was gently mixed and poured into sterile petri dishes.

* LCAT: Oxoid LCAT Antibiotic Supplement for the selection of pathogenic *Neisseria* contained:

Lincomycin	0,5mg
Colistin Sulphate	0,5mg
Amphotericin B	0,5mg
Trimethoprim lactate	0,5mg

C) STORAGE

The cultures were scraped off the plate using a sterile arched glass Pasteur pippette (made by holding a glass Pasteur pippette tip in a flame until an arch was formed). The cultures were suspended in 250 *u*l of rabbit blood cells in 500 *u*l cryo-tubes (Nunc tubes supplied by Weil Organization). The cultures were vortexed to achieve an even suspension and 4-5 sterile glass beads were dropped in each vial. The tubes were vortexed again to coat the beads with the organism and immediately placed in liquid nitrogen for a few seconds. The cultures were then stored in a biofreezer (Forma Scientific) at -70C. When cultures were

required, one glass bead from the respective culture was removed aseptically and plated out onto Young's (1978) MNYC and LS plates. The cultures were incubated at 37C in a 5% CO_2 incubator for 18h.

D) *BETA*-LACTAMASE DETECTION

Penicillinase (*Beta*-lactamase) was detected by the chromogenic cephalosporin test of O'Callaghan *et al.* (1972). Glaxo Chromogenic cephalosporin compound 67/312, also called nitrocephin (Oxoid), was used.

1. Preparation of nitrocephin solution

A working solution of nitrocephin was prepared by dissolving 0,5mg nitrocephin into a solution containing 0,5ml of dimethylsulphoxide and 9,5ml of 0,1M phosphate buffer (pH 7). This solution was stored in a dark bottle at 4C and used within 2 weeks of preparation.

2. *Beta*-lactamase detection

Beta-lactamase was detected by the:

- plate method in which a drop of solution was placed on the colonies growing on a solid medium (MNYC or LS plates);
- cell suspension method in which a drop of nitrocephin solution was added to a suspension of growth in a tube. Penicillinase production was reflected by a change in colour from yellow to red within 30 seconds.

E) AUXANOGRAPHIC TYPING OF *Neisseria gonorrhoeae*

Auxotyping was carried out by the method of Hendry & Stewart (1979).

1. Preparation of stock solutions

The following stock solutions were prepared in advance and stored at 4C:

Salt solution
1M NaCl
0,05M K_2SO_4
0,02M $MgCl_2$
0,04M NH_4Cl
0,1mM disodium ethylenediaminetetraacetate

Glycerol-lactate Solution
2M glycerol
1M DL lactate

Base Solutions (Individually prepared in 0,1N NaOH and stored at 4C)
0,04M uracil
0,02M hypoxanthine

L-amino Acids (Stored at -20C)
a 0,2M proline
b 0,2M glutamine
c 0,2M threonine
d 0,1M lysine
e 0,1M histidine
f 0,1M glycine
g 0,3M ornithine

a-g were prepared in 10ml aliquots of double distilled, deionised water.

h 0,3M citrulline
i 0,3M leucine
j 0,2M alanine
k 0,2M serine
l 0,1M isoleucine
m 0,1M phenylalanine
n 0,1M methionine
o 0,1M tryptophane

h-o were prepared in 10ml aliquots in 0,02N HCl

p	0,1M	aspartic acid
q	0,2M	valine
r	0,07M	cysteine and 0.03M cystine
s	0,1M	glutathione
t	0,05M	choline
u	0,1M	spermine

r was in 40ml 0.01N HCl
p, q, s, t, u were prepared in 10ml aliquots of double distilled, deionised water.

v	10mM	nicotinamide adenine diphosphate
w	20mM	thiamine hydrochloride
x	5mM	thiamine pyrophosphate
y	10mM	calcium pantothenate
z	5mM	inositol
aa	10mM	pyrodoxal phosphate
bb	0,6mM	biotin (to solubilize, drops of 0,01N NaOH were added until pH rose to 7,5).

v-bb, were prepared in 1ml aliquots, in double distilled, deionised water and stored at -20C.

2. Preparation of auxotyping media

This was prepared in two parts.

Part I consisted of ingredients in amounts which are given for one basic 200ml unit of final medium. Part I was typically made in the required multiples, and divided into labelled small beakers before addition of part II.

20 ml	Salt solution
1 ml	Glycerol lactate solution
1 g	Dextrose
0,2 g	$NaHCO_3$
0,68 g	Sodium acetate
27 ml	Water
0,54 g	KH_2PO_4
0,7 g	K_2HPO_4
0,013 g	Oxaloacetic acid
8 mg	$CaCl_2$ (dissolved in water before being added)
0,1 ml	10mM $Fe(NO_3)_3$

Volume was brought up to 50ml by adding double distilled, deionised water.

Part II consisted of the following additions to each 50ml of part I:

1 ml each of cysteine/cystine, biotin.
0,5 ml each of proline, glutamine, threonine, lysine, histidine, glycine, arginine, leucine, alanine, serine, isoleucine, methionine, tryptophane, valine, uracil, hypoxanthine, glutathione, and spermine.
0,1 ml each of choline, thiamine hydrochloride, Ca pantothenate, thiamine pyrophosphate, inositol, and pyridoxal phosphate.

The volume was made up to 100ml by adding double distilled, deionised water.

The final pH was adjusted to 7,0 with 1N NaOH and finally to 7,2 with 0,1N NaOH. Each solution was individually filter sterilized through a 0,22 *um* Millipore filter and heated to 52C. To this, 100ml of autoclaved and cooled (52C) double strength agar (made up of 11% ionagar and 1g starch) was added and the final medium was poured into petri dishes.

The ten sets of individual auxanographic media were made by single omissions(-) or additions(+) of the following:

complete (no omission)

- cysteine/cystine
- vitamins and co-enzymes solution
- amino acids (glutamine, threonine, lysine, histidine, glycine, leucine, alanine, serine, isoleucine, valine, trytophane)
- methionine
- arginine
- citrulline
- citrulline + ornithine
- uracil

- hypoxanthine
- proline
+ phenylalanine

3. Inoculation

(a) Inoculation solution

Inoculation solution was prepared as follows:

10ml Salt solution

0,5ml Glycerol lactate solution

0,14g KH_2PO_4

0,5g K_2HPO_4

4mg $CaCl_2$ (dissolved in deionised distilled H_20)

Volume made up to 100ml, filter sterilized, and dispensed into 1ml aliquots.

(b) Inoculation of media

Eighteen to 24h cultures of *N. gonorrhoeae* from LS plates were suspended in the inoculating solution. Using MacFarland Standard of 0,5, corresponding to 10^8 colony forming units (CFU) per ml, an inoculum of approximately 10^8 CFU was prepared for each strain. These were applied by means of a 36 pin multipoint inoculator (Denleys Instruments, U.K.). Each pin in the inoculator picks up 5ul of sample. The final concentration of the inoculum was 10^7 CFU. The ten sets of auxotyping media plates, one MNYC plate, and one LS plate were inoculated. At least four of

the eight reference cultures were included as controls on each auxotype plate.

The plates were incubated in a 5% CO_2 incubator at 37C, and the results recorded after 24h and 48h.

F) ANTIBIOTIC SUSCEPTIBILITY TESTING

MIC's were determined for the following antibiotics:

- penicillin G (10^4 inoculum size)
- penicillin G (10^7 inoculum size)
- ampicillin
- amoxicillin
- amoxicillin + clavulanic acid in a ratio of 2:1
- cefuroxime
- cefotaxime
- ceftriaxone
- spectinomycin
- kanamycin
- cotrimoxazole (sulphamethoxazole + trimethoprim in a ratio of 19:1)
- tetracycline
- erythromycin
- rosoxacin
- chloramphenicol

1. Preparation of antibiotic stocks and dilutions

The dilutions for the susceptibility tests were prepared according to the agar dilution method described by

Washington (1985). The stock solutions of the antibiotics were made up in advance and adjusted for purity as specified by the manufacturers. They were then dispensed in 1ml aliquots, stored at -70C and used within two weeks of preparation. Once thawed, unused stock solutions were discarded.

Seventy two hours prior to the MIC determinations 1ml of each antibiotic stock was thawed and a 1/10 dilution (in saline) to give 128 $ug.ml^{-1}$ was made, followed by two fold serial dilutions (of 5ml) from 64 to 0,003 $ug.ml^{-1}$. For each antibiotic tested the final concentrations ranged from 0,003 $ug.ml^{-1}$ to 64 $ug.ml^{-1}$.

2. Preparation of antibiotic susceptibility plates

All the MIC's were carried out on Mueller-Hinton agar (Oxoid) with 5% saponin-lysed horse blood. To obtain the correct concentration of antibiotics, Mueller-Hinton agar was weighed out for 2 litres and added to 1500ml distilled water, to which 100ml saponin-lysed horse blood was added when the media had cooled to 50C. The final medium was made up of 20ml of saponin-lysed Mueller-Hinton agar and 5ml of of the respective antibiotic dilution. Each medium was thoroughly mixed and poured into a petri dish. The antibiotic containing plates were used within 48h of preparation.

3. Inoculation

(a) Inoculum Preparation

Inoculum concentrations of 10^4 and 10^7 CFU were used for penicillin G to determine the effect of inoculum size. The remaining antibiotics were tested at an inoculum size of 10^4 CFU.

(b) Inoculation of plates

In each run *Staphylococcus aureus* (NCTC 6571) and antibiotic free plates were used as controls. To ensure purity of cultures one LS plate, which is a non selective medium, was also included. The plates were inoculated with a multipoint inoculator as described before and read after 18h at 37C in a 5% CO_2 incubator.

The MIC was taken as the lowest concentration of the antibiotic which completely inhibited growth of the strain.

G) PLASMID EXTRACTION

Several unsuccessful attempts were made using the boiling method of plasmid extraction described by Maniatis *et al.* (1982) and the alkali lysis method described by Sox *et al.* (1978). However, plasmids were successfully extracted by modifying the method of Takahashi & Nagano (1985).

1. Bacterial Strains

PPNG and non PPNG strains were used from urethral, cervical, eye, rectal and disseminated infections. Controls harboring 3,2 Mdal, 4,5 Mdal, 24,5 Mdal and 2,6 Mdal plasmids were used as standards for molecular mass. Cultures were removed from the biofreezer, thawed, plated out onto LS plates and incubated in a 5% CO_2 incubator at 37C for 18-24h. Growth was then scraped from each plate and suspended in 6ml of Mueller-Hinton broth. The cells were pelleted by centrifugation at 2200 x g for 15 min and the supernatant fluid was discarded.

2. Extraction of plasmid DNA

The pelleted cells were then suspended in 200*u*l of a solution containing 40mM tris(hydroxymethyl)aminomethane (Tris) and 2mM EDTA buffer (pH8) and thereafter transferred to a 1,5ml polypropylene (Eppendorf) tube.

To this suspension 400*u*l of fresh lysing solution, prepared by adding 0,4M NaOH to the same volume of 4% sodium dodecyl sulphate (SDS)-100 mM Tris solution, was added. The content of the tube was mixed by inverting 5-10 times and allowed to stand at room temperature for 10 min for cell lysis to occur.

For neutralization, 300*u*l of 3M sodium acetate-acetic acid buffer (pH 5,5) was added and gently mixed by inversion 10-20 times. After standing at 0C for 5 min, the contents of

the tube was centrifuged at 1500 x g for 5 min at room temperature and then kept at 0C for 15 min. The supernatant was then decanted into another tube and the precipitate discarded.

An equal volume of chloroform was added to the supernatant and the tube inverted 15-20 times. The resulting emulsion was broken by centrifugation at 0C for 5 min and 500ul of the upper aqueous phase carefully transferred to another tube by means of a propylene micropipette. To this, one ml of cold (-20C) ethanol was added and the tube gently inverted 5-10 times and then held at 0C for 5 min.

The precipitate was collected by centrifugation at 1500 x g for 5 min at 0C.

The pellet was dissolved in 100ul of a solution containing 10mM Tris-acetic acid and 2mM disodium EDTA buffer (pH 8,0) and used to establish the presence of plasmid DNA.

3. Agarose gel preparation and electrophoresis

Agarose (0,7%) was melted in 40mM Tris-acetic acid plus 2mM EDTA buffer (pH 8). Twenty-five ul of sample was mixed with 5ul tracking dye solution composed of bromocresol purple (0,25%), SDS (5%) and glycerol (50%) in water and transferred to wells. The same buffer used to prepare the agarose gel, supplemented with 0,5 $mg.l^{-1}$ of ethidium bromide solution, was poured over the gel. Electrophoresis was performed at 10 $V.cm^{-1}$ for 3h. The gels were examined

on a short-wave UV transilluminator for the presence of DNA bands and photographed using a polaroid camera.

H) ISOELECTRIC FOCUSSING OF *BETA*-LACTAMASE

Isoelectric focussing (IEF) of *beta*-lactamase from *N. gonorrhoeae* in agarose gels was based on the method described by Vecoli *et al.* (1983).

1. Preparation of crude extracts

PPNG cultures, of which the plasmid profiles had been established, were chosen and plated out onto LS media plates and incubated at 37C in 5% CO_2 for 18h. The cells, scraped off the plate and suspended in sterile distilled water, were harvested at 4C by centrifugation at 13000 x g for 30 min, and the cell pellet was suspended in 5ml distilled water. Cells were lysed with a Branson Sonifier cell disruptor (Model 200) to release the intracellular *beta*-lactamase. Cell suspensions, maintained at 4C, were sonicated for 2 min in four 30 sec intervals. Crude extracts containing *beta*-lactamases were obtained by centrifuging the debris at 24000 x g for 10 min and 1ml portions of the supernatant fluid frozen at -20C.

2. Semiquantitative chromogenic cephalosporin *beta*-lactamase tests

Before focussing, a crude extract of each sample was checked for *beta*-lactamase activity using nitrocephin solution. One *u*l of nitrocephin was added to 20*u*l crude extract of sample

and mixed with a clean applicator stick. A change in colour from yellow to red within 30 sec was interpreted as positive.

3. Preparation of agarose gels

- 240mg agarose (Isogel, Sigma Products) in 15ml of distilled water was melted in a boiling waterbath;
- From a 20% solution of sorbitol, 13,5ml was added to the melted agarose, and the solution cooled to 59C;
- 1,5ml of Ampholine, pH 3,5 to 9,5 (LKB Products) was added.

The solution was gently stirred and agarose gels were cast by injecting the mixture into preheated glass molds (250cm x 125cm x 0,8mm). Gelling occured within an hour at room temperature.

4. Isoelectric focussing

Thin layer agarose gel IEF was carried out on a Multiphor (LKB product) at 10C. This was achieved by connecting the Multiphor to the Multitemp thermostatic circulator set at 10C. Electrode wicks were soaked in the appropriate solutions, blotted with filter paper, and placed at the respective poles. The anode solution used was 0,1N H_3PO_4, whereas 0,1N NaOH was used at the cathode. Samples were placed 1cm away from the cathode electrode with sample applicator sticks (LKB Products). Focussing was done for 38 min with a LKB power pack set at 30 Watts constant power,

1500 V limiting and 50 mA limiting. The final pH gradient of the gel was determined with a surface electrode, and readings were taken at 1 cm intervals.

A sheet of Whatman 54 hardened filter paper was saturated with a 0,05% nitrocephin solution. This saturated filter paper was overlaid on the gel which was allowed to develop for 3 minutes. The results were photographed with a polaroid camera using a green filter.

III. RESULTS

A) STRAIN CONFIRMATION AND *BETA*-LACTAMASE ACTIVITY

All the cultures previously described as *N. gonorrhoeae* were oxidase positive and had typical Gram negative diplococcal appearance. *Beta*-lactamase activity was detected by the chromogenic cephalosporin test, in which the yellow nitrocephin turned red upon hydrolysis of the *beta*-lactam ring.

Penicillinase producing strains were classified as PPNG and non producing strains as non PPNG. PPNG comprised 69 (38%) of 181 strains studied, whilst 112 (62%) were non PPNG (Fig. 5).

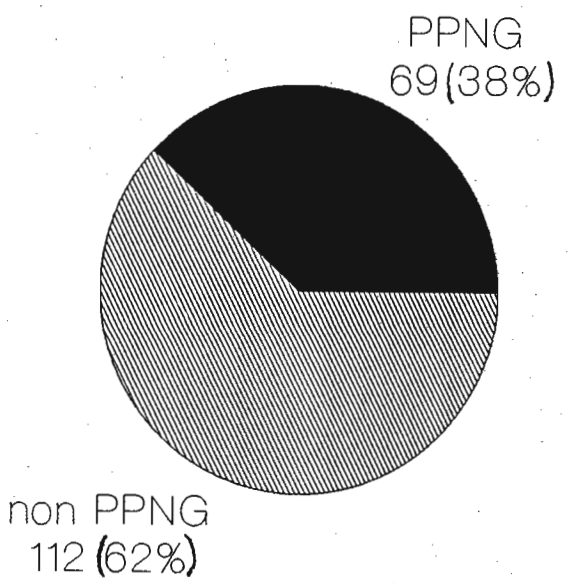


Fig. 5. Distribution of *N. gonorrhoea* according to *beta*-lactamase production. PPNG = 38% and non PPNG = 62%

B) ANTIBIOTIC SUSCEPTIBILITY

1. Distribution of strains

Of the non PPNG, 106 were available for determination of MIC's against penicillin G. To test the effect of inoculum size on the MIC, penicillin G was tested at two inoculum sizes viz., 10^4 and 10^7 CFU. At an inoculum size of 10^7 65 (61%) were defined as fully susceptible (MIC $\leq$ 0,06 $ug.ml^{-1}$), 32 (30%) as intermediately susceptible (MIC 0,12-0,5 $ug.ml^{-1}$) and 9 (8%) were resistant (MIC $\geq$ 1 $ug.ml^{-1}$). At 10^4 inoculum size, 78 (70%) were fully susceptible, 26 (23%) intermediately susceptible and 2 (3%) resistant. These results are depicted in Figs. 6b and 6a respectively.

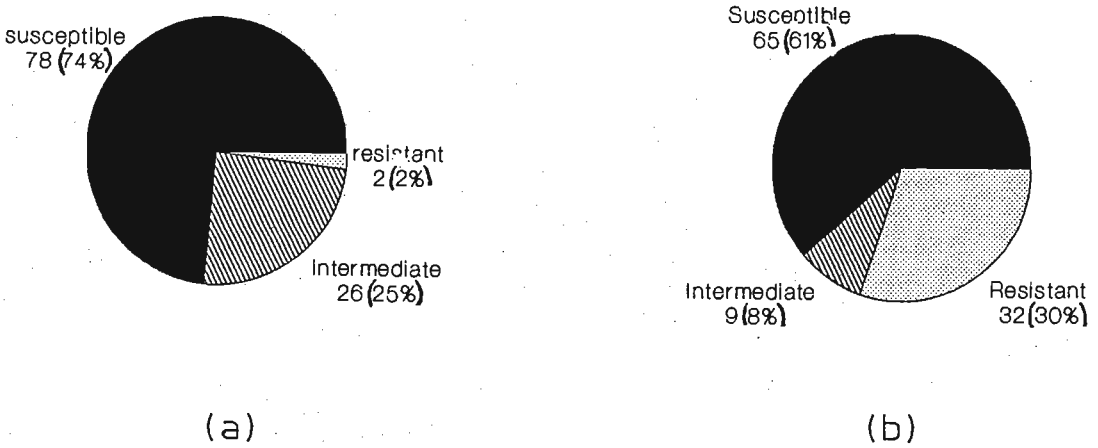


FIG. 6. Distribution of penicillin G susceptibility of non PPNG isolates at an inoculum size of a) 10^4 CFU and b) 10^7 CFU

2. Minimum inhibitory concentration distribution

The emergence of *beta*-lactamase producing strains has created a major problem in the treatment of *N. gonorrhoeae* infections. Hence, it has become imperative to determine current sensitivity patterns. *Staphylococcus aureus* (NCTC 6571) is a control strain whose sensitivity pattern is used to establish the accuracy of MIC determinations. In this study the MIC for *Staphylococcus aureus* (NCTC 6571) control was within its quoted range.

The susceptibility of PPNG and non PPNG strains to commonly used antibiotics was expressed as the percentage of strains inhibited at the respective MIC. As expected, it was found that all PPNG strains had high MIC's for penicillin G and ampicillin, whilst the non PPNG strains had low MIC's for penicillin G and ampicillin. Amoxicillin and amoxicillin + clavulanic acid displayed different sensitivity patterns for PPNG and non PPNG strains. Cefuroxime, cefotaxime, ceftriaxone, spectinomycin, kanamycin, cotrimoxazole, tetracycline, erythromycin, rosoxacin and chloramphenicol show parallel distribution patterns (Table 7). Resistance to kanamycin (MIC $>$ 32ug.ml^{-1}), erythromycin (MIC $>$ 2ug.ml^{-1}) and tetracycline (MIC $>$ 2ug.ml^{-1}) was not found (Table 7).

To facilitate the comparison of PPNG and non PPNG strains cumulative percentages of the MIC's were determined. They are summarised in Table 8 and illustrated graphically in Figs. 7a-7h.

TABLE 7. Percentage distribution of MIC (minimum inhibitory concentration)

MIC's µg/ml	PEN G 10^7 CFU		PEN G 10^4 CFU		AMPI		AMOX		AMOX + CLAV		CEFUROX		CEFOTAX		CEFTRIAX		SPECT		KANA		CO-TRI		TETRA		ERY		ROSO		CHLOR	
	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG
0,003		3		7				1					79	14	80	80									1		15	11		
0,007		20		17	1	4		1	1	1	22	35	11	11	10	15										1	62	28		
0,01		8		12		12		7		2	37	20	5	73	10	5									8	11	18	48		
0,03		13		12	1	26	2	24	3	7	28	20	5	2									1		36	15	5	12	2	1
0,06		16	1	26		12	1	23	1	18	7	17											1		30	18		1	12	
0,12		9	2	14		31		11	2	16	3	4											4	29	30	34				5
0,25		12	3	10	2	7	2	23	1	28	3	4											9	26	1	14			54	43
0,5		4	14		3	5		7	26	13								1	3	2		4	66	36	4	2			24	24
1		6	10	1	5	2	2	2	48	10							2	1				9	19	9		1			5	18
2	1	3	12	1	22	1	19		8	3							8	9		2	2	5				4			3	9
4	8	4	17		19		16		11	2							4	15	50	35	13	34								
8	11		25		16		23	1									67	33	29	35	17	20								
16	20	2	7		22		21	1									19	29	9	19	30	20								
32	8		7		7		7											13	9	7	33	8								
64	52		2		2		7														5									
TOTAL NO. OF STRAINS	67	108	68	111	68	106	66	110	66	110	67	107	60	106	56	94	64	94	66	110	65	106	67	108	67	105	67	110	65	114

Pen G = Penicillin G
Ampi = Ampicillin
Amox = Amoxicillin
Amox + Clav = Amoxicillin + Clavulanic acid
Cefurox = Cefuroxime
Cefotax = Cefotaxime
Ceftriax = Ceftriaxone

Spec = Spectinomycin
Kana = Kanamycin
Co-tri = Co-trimoxazole
Tetra = Tetracycle
Ery = Erythromycin
Roso = Rosoxacin
Chlor = Chloramphenicol

TABLE 8. Cumulative percentage distribution of MIC (minimal inhibitory concentration)

MIC's µg/ml	PEN G 10^7 CFU		PEN G 10^4 CFU		AMPI		AMOX		AMOX + CLAV		CEFUROX		CEFOTAX		CEFTRIAX		SPECT		KANA		CO-TRI		TETRA		ERY		ROSO		CHLOR	
	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG	PPNG	NON-PPNG
0,003		3		7				1					79	14	80	80									1		15	11		
0,007		23		24	1	4		1		1	22	35	90	25	90	95														
0,01		31		37		16		8	3	3	59	55	95	98	100	100									9	12	95	87		
0,03		44		48	2	42	2	32	4	10	87	75	100	100									1		45	27	100	2	2	1
0,06		61	1	75		54	3	55		28	94	92											2		75	45		100	14	1
0,12		69	3	88		85		66	6	44	97	96											6	29	95	79			14	6
0,25		81	6	98	4	92	5	89	7	72	100	100											15	55	96	93			68	49
0,5		85	21	98	7	97		96	33	85								1	3	2		4	81	91	100	95			92	73
1		92	31	99	12	99	7	98	81	95							2	1	3	2		13	100	100		96			97	91
2	1	94	41	100	34	100	26		89	98							10	10	3	4	2	18				100			100	100
4	9	98	59		53		42		100	100							14	25	53	39	15	52								
8	20	100	84		69		65	99									81	58	82	74	32	72								
16	40		91		91		86	100									100	87	91	93	62	92								
32	48		99		98		93											100	100	100	95	100								
64	100		100		100		100														100									
TOTAL NO. OF STRAINS	67	108	68	111	68	106	66	110	66	110	67	107	60	106	56	94	64	94	66	110	65	105	67	103	67	105	67	110	65	111

Pen G = Pencillin G
Ampi = Ampicillin
Amox = Amoxicillin
Amox + Clav = Amoxicillin + Clavulanic acid
Cefurox = Cefuroxime
Cefotax = Cefotaxime
Ceftriax = Ceftriaxone

Spec = Spectinomycin
Kana = Kanamycin
Co-tri = Co-trimoxazole
Tetra = Tetracycle
Ery = Erythromycin
Roso = Rosoxacin
Chlor = Chloramphenicol

Table 9 compares the MIC_{50} (MIC at which 50% of isolates are inhibited), MIC_{90} (MIC at which 90% of isolates are inhibited) and the MIC range of 15 *beta*-lactam antibiotics against PPNG and non PPNG isolates.

TABLE 9. MIC_{50}, MIC_{90} and MIC Range of PPNG and non PPNG

Antibiotic	non PPNG			PPNG		
	MIC^{*}_{50} µg/ml	MIC^{+}_{90} µg/ml	RANGE µg/ml	MIC^{*}_{50} µg/ml	MIC^{+}_{90} µg/ml	RANGE µg/ml
Pen G (10^4 CFU)	0,03	0,12	0,003- 8	32	64	2 -64
Pen G (10^7 CFU)	0,03	0,25	0,003- 2	4	16	0,06 -64
Ampicillin	0,06	0,25	0,007- 2	4	16	0,007 -64
Amoxicillin	0,06	0,25	0,007- 16	8	32	0,03 -64
Amoxicillin + Clavulanic acid	0,12	1	0,007- 4	1	2	0,01 - 4
Cefuroxime	0,01	0,06	0,007- 0,25	0,01	0,06	0,007- 0,25
Cefotaxime	0,007	0,01	0,003- 0,03	0,003	0,003	0,003- 0,03
Ceftriaxone	0,003	0,003	0,003- 0,01	0,003	0,003	0,003- 0,01
Spectinomycin	8	16	0,5 -32	8	8	1 -16
Kanamycin	4	16	0,5 -32	4	16	0,5 -32
Cotrimoxazole	4	16	0,03 -32	16	32	2 -64
Tetracycline	0,25	0,5	0,12 - 1	0,5	0,5	0,03 - 1
Erythromycin	0,06	0,25	0,007- 2	0,06	0,12	0,007- 0,5
Rosoxacin	0,007	0,01	0,003- 0,06	0,007	0,01	0,003- 0,03
Chloramphenicol	0,25	1	0,03 - 2	0,25	0,5	0,03 - 2

*MIC_{50} =Concentration at which 50% of isolates are inhibited

+MIC_{90} =Concentration at which 90% of isolates are inhibited

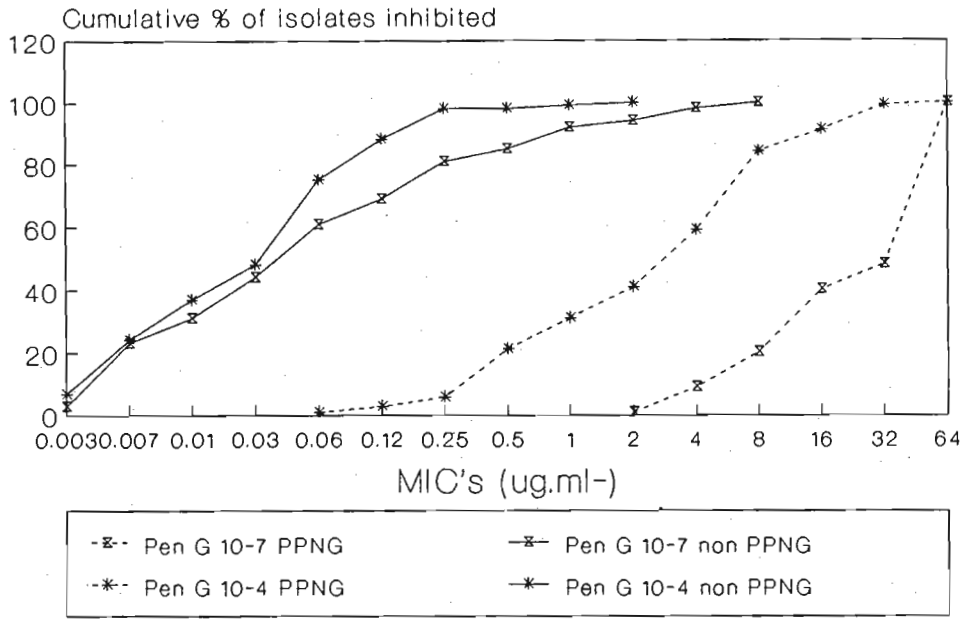


FIG. 7a. MIC's for PPNG and non PPNG for penicillin G at two inoculum sizes (10^4 and 10^7 CFU)

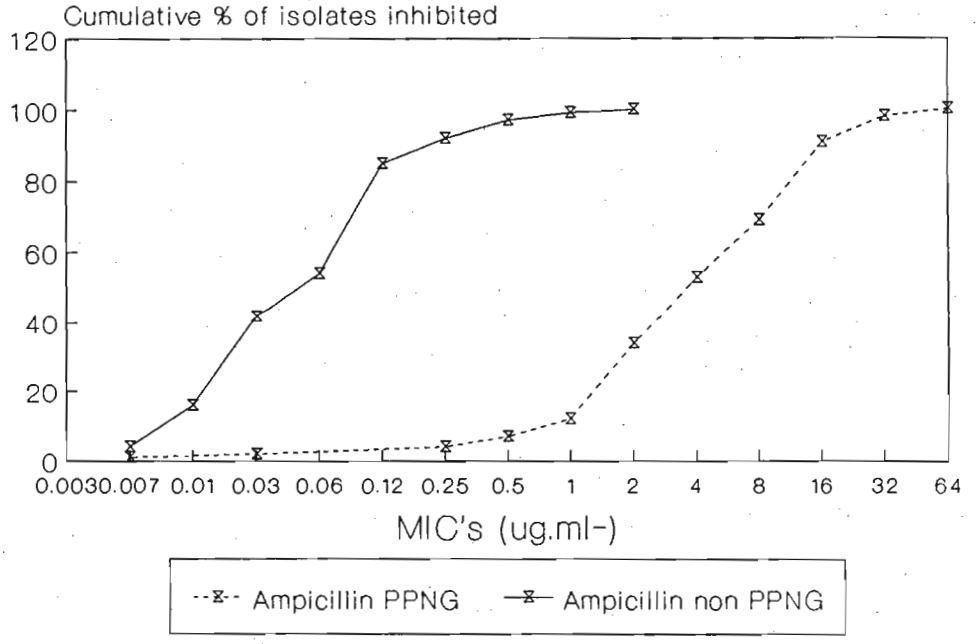


FIG. 7b. MIC's for PPNG and non PPNG for ampicillin

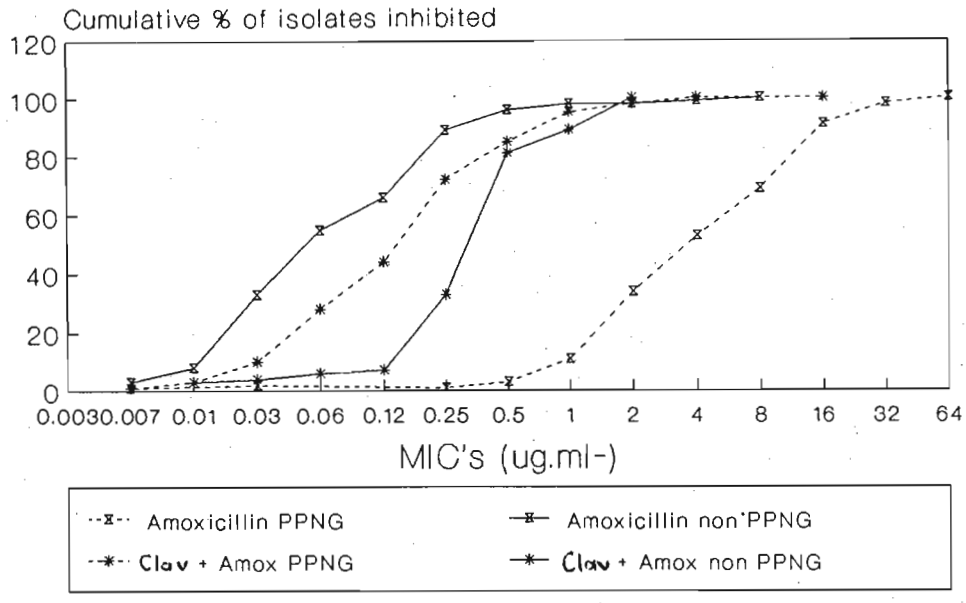


FIG. 7c. MIC's for PPNG and non PPNG for amoxicillin and amoxicillin + clavulanic acid

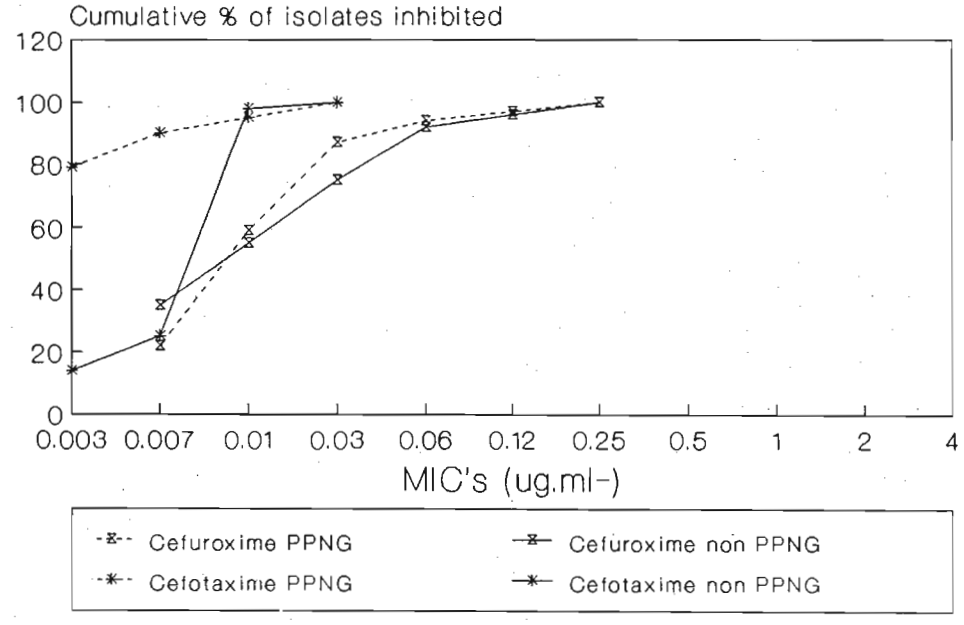


FIG. 7d. MIC's for PPNG and non PPNG for cefuroxime and cefotaxime

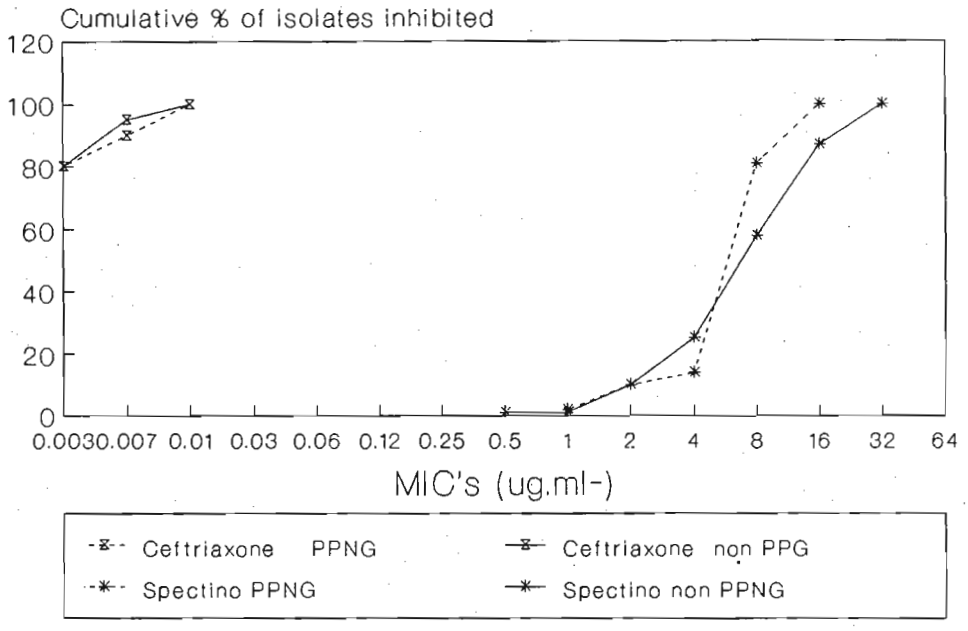


FIG. 7e. MIC's for PPNG and non PPNG for ceftriaxone and spectinomycin

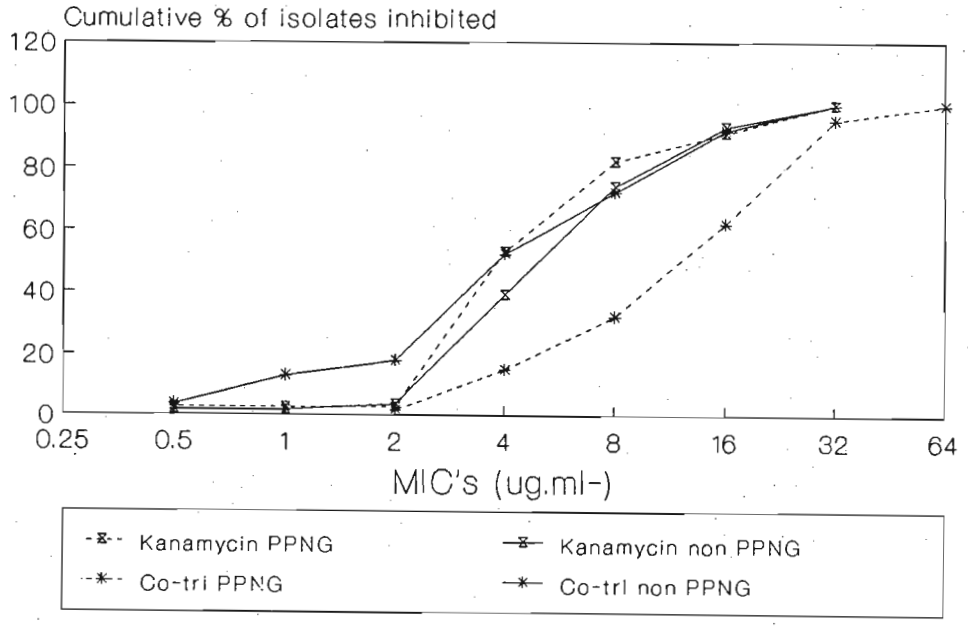


FIG. 7f. MIC's for PPNG and non PPNG for kanamycin and cotrimoxazole

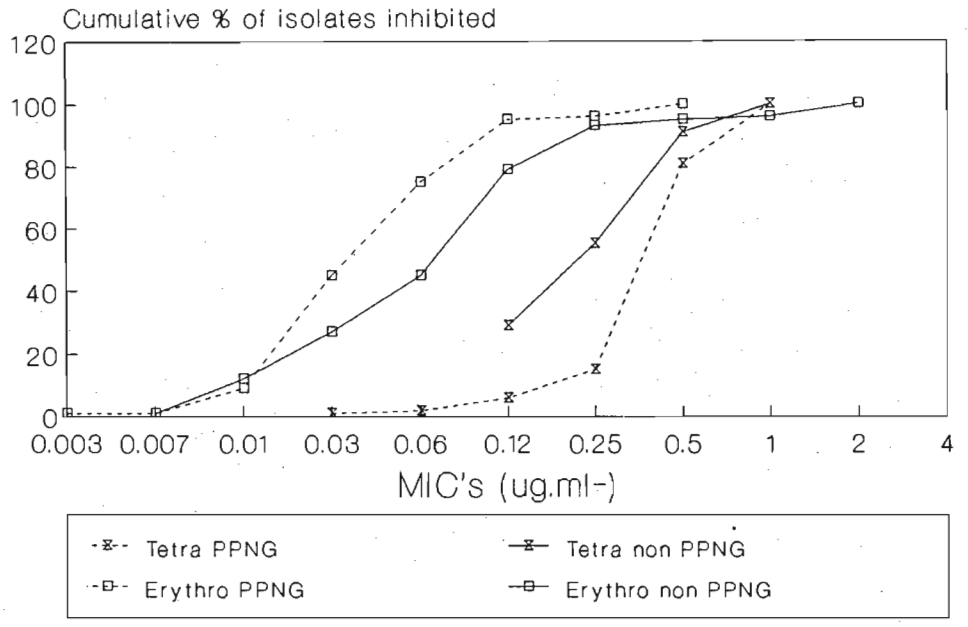


FIG. 7g. MIC's of PPNG and non PPNG of tetracycline and erythromycin

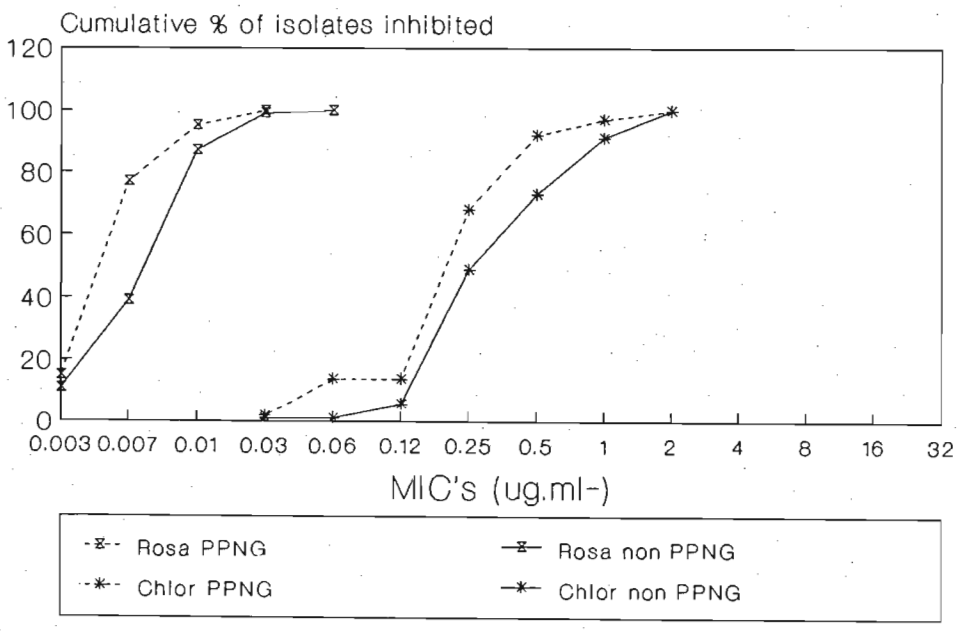


FIG. 7h. MIC's for PPNG and non PPNG of rosoxacin and chloramphenicol

For PPNG, the MIC's of penicillin G were high at 10^4 CFU, but at 10^7 CFU there is a further dramatic increase in MIC (Fig. 7a). The non PPNG isolates do not have a significantly higher MIC at a higher inoculum size. Approximately 90% of non PPNG isolates are inhibited by penicillin G at a MIC of 0,5ug.ml^{-1}, whereas 90% of PPNG isolates require a MIC of 32ug.ml^{-1}. Similarly, PPNG isolates have a higher MIC of ampicillin as compared to non PPNG (Fig. 7b).

Compared to amoxicillin alone, amoxicillin + clavulanic acid shows significantly better activity against the PPNG at a higher MIC range (Fig. 7c). This is expected since clavulanic acid is a *beta*-lactamase inhibitor.

Cefuroxime, ceftriaxone and cefotaxime have similar effectivenes against both PPNG and non PPNG (Figs. 7d-7e) and appear to be highly active.

Spectinomycin, kanamycin and cotrimoxazole also show similar activity against both PPNG and non PPNG (Figs. 7e-7f).

Tetracycline and erythromycin show similar activity to each other, and against the PPNG and non PPNG strains (Fig. 7g). PPNG require lower MIC's of erythromycin, rosoxacin and chloramphenicol than do non PPNG (Figs. 7g-7h).

In summary, penicillin G, ampicillin and amoxicillin clearly separate PPNG and non PPNG strains on the basis of their MIC's (Figs. 7a-7c). Cefuroxime, cefotaxime, ceftriaxone,

spectinomycin, kanamycin, cotrimoxazole, tetracycline, erythromycin, rosoxacin and chloramphenicol show a parallel distribution (Figs. 7d-7h).

For non PPNG strains ceftriaxone (MIC_{50} = 0,003 $ug.ml^{-1}$) is the most active drug followed by cefotaxime (MIC_{50} = 0,007 $ug.ml^{-1}$) and rosoxacin (MIC_{50} = 0,01 $ug.ml^{-1}$). The most active antibiotics against PPNG were cefotaxime and ceftriaxone (MIC_{50} = 0,003 $ug.ml^{-1}$) followed by rosoxacin and cefuroxime (MIC_{50} = 0,007 and 0,01 $ug.ml^{-1}$ respectively).

3. Distribution of penicillinase and non-penicillinase producing *N. gonorrhoeae* at different anatomical sites

To determine a possible link between penicillinase production and site of infection, we looked at the percentage distribution of PPNG and non PPNG strains at different infection sites (Table 10). The highest proportion (5 of 7) of PPNG isolates were from the rectum and the lowest (4 of 19) from the eyes of newborn infants as shown in Table 10.

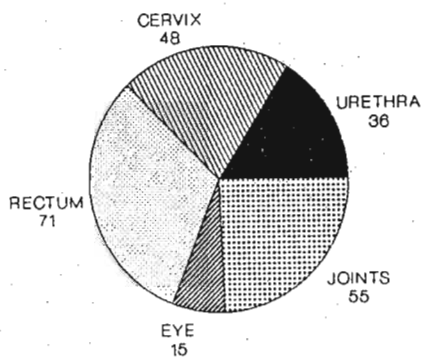
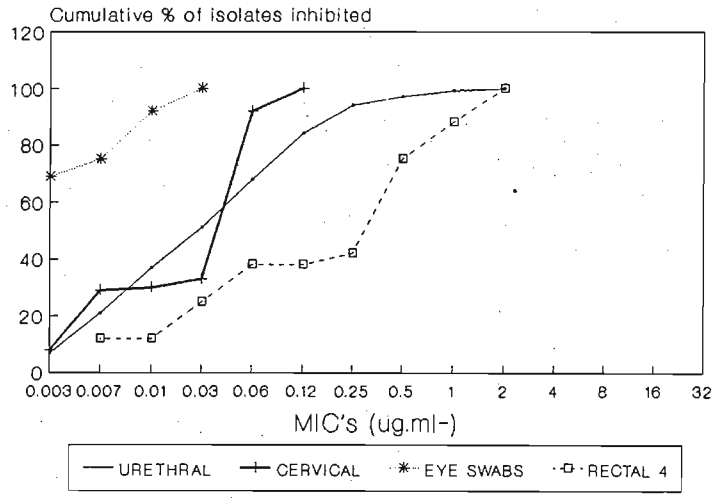


FIG. 8. Percentage distribution of PPNG at different anatomical sites

The penicillin G susceptibility of non PPNG isolates from different anatomical sites show that rectal and cervical isolates were the least susceptible to penicillin G, followed by urethral isolates, with eye isolates being the most susceptible (Fig. 9).

(a)



(b)

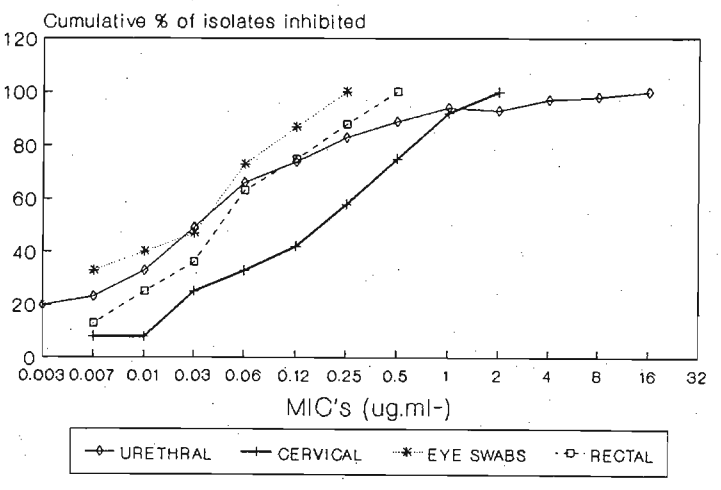


FIG. 9. Penicillin G distribution of non PPNG at different infection sites a) 10^4 CFU b) 10^7 CFU

TABLE 10. Distribution of PPNG at different anatomical sites.

Site	Total	PPNG No.	(%)	non PPNG No.	(%)
Urethra	111	40	(36)	71	(64)
Cervix	23	11	(48)	12	(52)
Rectum	7	5	(71)	2	(29)
Eye	19	4	(15)	15	(85)
Joints	9	5	(55)	4	(45)
Total	169	64	(38)	105	(62)

C) AUXOTYPING

Several methods are described for auxotyping. Each method has a defined medium with different components and also different concentrations of the compounds. The first described, and also the most widely used, method of Catlin (1973) appeared very long and complicated. A less complicated and rapid method described by La Scolea & Young (1974) yielded inconsistent results on repetition. The consistency of the results was tested using reference strains as control. The method of Hendry & Stewart (1979) gave consistent, reproducible results, and hence was the method used throughout for auxotyping.

Two main auxotype patterns emerged from this study viz., prototrophic/wild type with growth on all 10 plates (Fig. 10a) and proline requiring, which showed no growth on proline deficient plates (Fig. 10b). No AHU requiring organisms were found.

The plate on which several amino acids were omitted showed poorer growth than any of the others (Fig. 10c). However, since some growth did occur, these organisms are not reported to require these amino acids. The cysteine/cystine deleted plate acted as an excellent indicator of the purity of the culture, since only *N. gonorrhoeae* have an absolute requirement of cysteine or cystine (Fig. 10d). If the cultures had been contaminated by *N. meningitidis* or any other organism, clearly visible growth would have occured on these plates.

The auxotyping results are divided according to *beta*-lactamase production. Table 11a shows the results of auxotyping of PPNG isolates. The non PPNG isolates are further divided according to anatomical sites, and their auxotype patterns are shown in Tables 11b-11e.

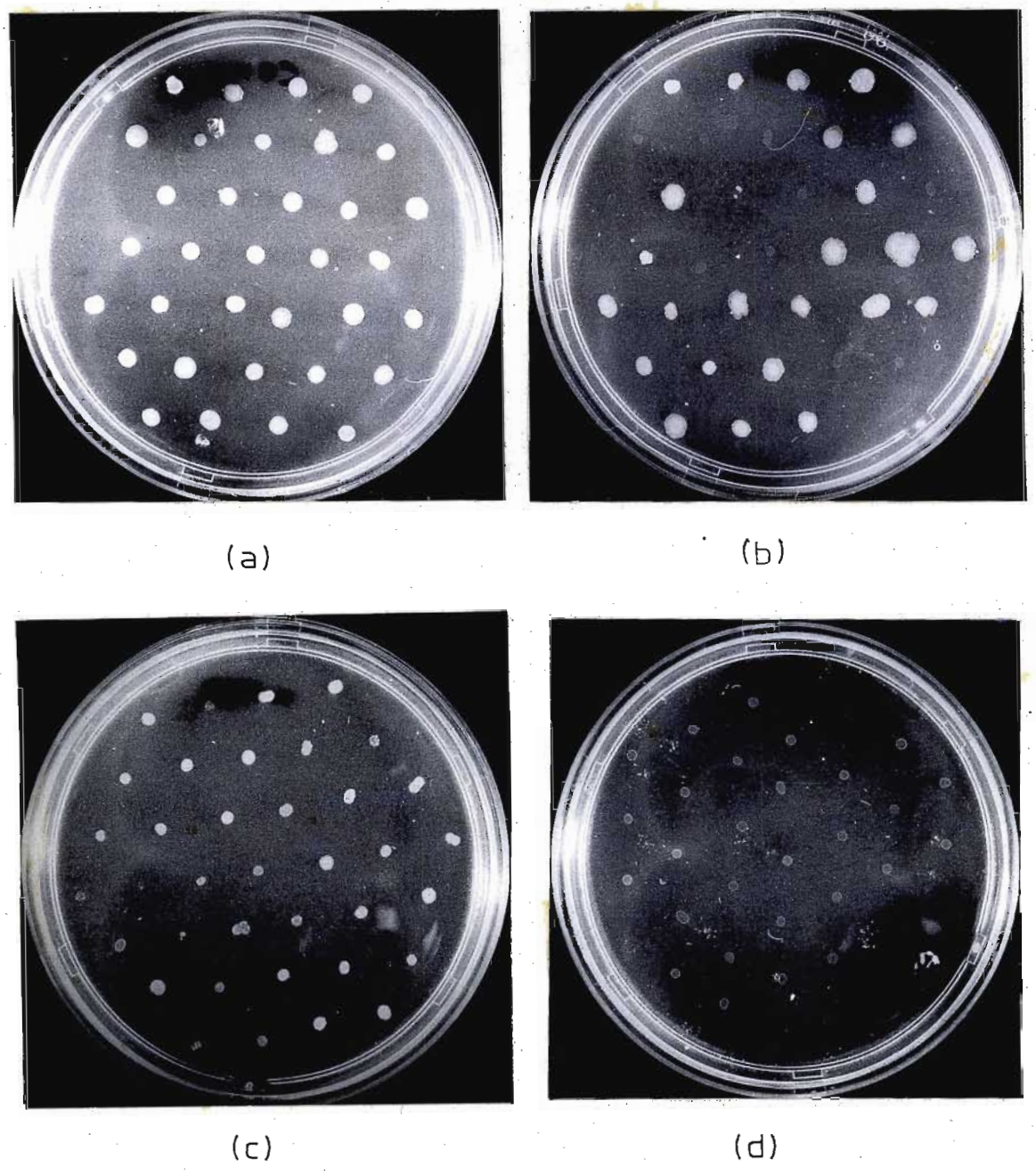


FIG. 10. Auxotype media showing;
a) growth on all plates (proto);
b) no growth of pro⁻ strains on proline deficient plates;
c) poor growth on amino acid deficient plates
d) no growth on cysteine/cystine deficient plates. The spots shown in the figure are points of inoculation

TABLE 11a. Auxotype patterns of PPNG isolates

ISOLATE NO.	COMPLETE	VITAMINS	AMINO ACIDS	PROLINE	ARGININE	ARG⁻O⁺C⁺*	HYPOXANTHINE	URACIL	PHENYLALANINE	CYSTEINE	AUXOTYPE	
4	+	+	+	+	+	+	+	+	+	-	COM	
13	+	+	+	+	+	+	+	+	+	-	COM	
14	+	+	+	+	+	+	+	+	+	-	COM	
15	+	+	+	+	+	+	+	+	+	-	COM	
21	+	+	+	+	+	+	+	+	+	-	COM	
22	+	+	+	+	+	+	+	+	+	-	COM	
23	+	+	+	+	+	+	+	+	+	-	COM	
24	+	+	+	+	+	+	+	+	+	-	COM	
36	+	+	+	+	+	+	+	+	+	-	COM	
37	+	+	+	+	+	+	+	+	+	-	COM	
40	+	+	+	+	+	+	+	+	-	-	COM	PHE
42	+	+	+	+	+	+	+	+	+	-	COM	
43	+	+	+	+	+	+	+	+	+	-	COM	
44	+	+	+	+	+	+	+	+	+	-	COM	
48	+	+	+	+	+	+	+	+	+	-	COM	
50	+	+	+	+	+	+	+	+	+	-	COM	
53	+	+	+	+	+	+	+	+	+	-	COM	
55	+	+	+	+	+	+	+	+	-	-	COM	PHE
58	+	+	+	+	+	+	+	+	+	-	COM	
59	+	+	+	+	+	+	+	+	+	-	COM	
61	+	+	+	+	+	+	+	+	+	-	COM	
62	+	+	+	+	+	+	+	+	+	-	COM	
63	+	+	+	+	+	+	+	+	+	-	COM	
64	+	+	+	+	+	+	+	+	+	-	COM	
65	+	+	+	+	+	+	+	+	+	-	COM	
68	+	+	+	+	+	+	+	+	+	-	COM	
74	+	+	+	-	+	+	+	+	-	-	PRO	PHE
75	+	+	+	+	+	+	+	+	+	-	COM	
79	+	+	+	-	+	+	+	+	+	-	PRO	
83	+	+	+	+	+	+	+	+	+	-	COM	
88	+	+	+	+	+	+	+	+	+	-	COM	
95	+	+	+	+	+	+	+	+	-	-	PHE	
96	+	+	+	+	+	+	+	+	+	-	COM	
104	+	+	+	+	+	+	+	+	+	-	COM	
105	+	+	+	+	+	+	+	+	+	-	COM	
106	+	+	+	+	+	+	+	+	+	-	COM	
107	+	+	+	+	+	+	+	+	+	-	COM	
108	+	+	+	+	+	+	+	+	+	-	COM	

TABLE 11a. Continued

ISOLATE NO.	COMPLETE	VITAMINS	AMINO ACIDS	PROLINE	ARGININE	$ARG^{-}O^{+}C^{+}$ *	HYPOXANTHINE	URACIL	PHENYLALANINE	CYSTEINE	AUXOTYPE
111	+	+	+	+	+	+	+	+	+	-	COM
112	+	+	+	+	+	+	+	+	+	-	COM
113	+	+	+	+	+	+	+	+	+	-	COM
114	+	+	+	+	+	+	+	+	+	-	COM
115	+	+	+	+	+	+	+	+	+	-	COM
116	+	+	+	+	+	+	+	+	+	-	COM
117	-	-	-	-	-	-	-	-	-	-	NO GROWTH
120	+	+	+	+	+	+	+	+	+	-	COM
121	+	+	+	+	+	+	+	+	+	-	COM
123	+	+	+	+	+	+	+	+	+	-	COM
124	+	+	+	+	+	+	+	+	+	-	COM
133	+	+	+	+	+	+	+	+	+	-	COM
134	+	+	+	+	+	+	+	+	+	-	COM
141	+	+	+	+	+	+	+	+	+	-	COM
142	+	+	+	+	+	+	+	+	+	-	COM
149	+	+	+	+	+	+	+	+	+	-	COM
152	+	+	+	+	+	+	+	+	+	-	COM
165	+	+	+	+	+	+	+	+	+	-	COM
169	+	+	+	+	+	+	+	+	+	-	COM
171	+	+	+	+	+	+	+	+	+	-	COM
174	+	+	+	+	+	+	+	+	+	-	COM
179	+	+	+	-	+	+	+	+	+	-	PRO
184	+	+	+	+	+	+	+	+	+	-	COM
186	+	+	+	+	+	+	+	+	+	-	COM
202	+	+	+	+	+	+	+	+	+	-	COM
146	+	+	+	+	+	-	+	+	+	-	ARG

*$ARG^{-}O^{+}C^{+}$ = complete auxotype media excluding arginine and including citrulline and ornithine
COM = growth on all plates
COM PHE = growth on all plates, and inhibited by phenylalanine
PRO = proline requiring
ARG = arginine requiring
PRO ARG = proline and arginine requiring

TABLE 11b. Auxotype patterns of non PPNG urethral isolates

ISOLATE NO.	COMPLETE	VITAMINS	AMINO ACIDS	PROLINE	ARGININE	ARGININE^{-}O^{+}C^{+}*	HYPOXANTHINE	URACIL	PHENYLALANINE	CYSTEINE	AUXOTYPE	
1	+	+	+	+	+	+	+	+	+	-	COM	
2	+	+	+	-	+	+	+	+	-	-	PRO	PHE
3	+	+	+	+	+	+	+	+	+	-	COM	
5	+	+	+	-	+	+	+	+	+	-	PRO	
6	+	+	+	+	-	+	+	+	+	-	ARG	
7	+	+	+	-	+	+	+	+	-	-	PRO	PHE
8	+	+	+	-	+	+	+	+	-	-	PRO	PHE
9	+	+	+	+	+	+	+	+	+	-	COM	
10	+	+	+	+	+	+	+	+	-	-	COM	
11	+	+	+	+	+	+	+	+	+	-	COM	
12	+	+	+	+	+	+	+	+	+	-	COM	
17	+	+	+	-	+	+	+	+	-	-	PRO	PHE
18	+	+	+	-	+	+	+	+	+	-	PRO	
20	+	+	+	-	+	+	+	+	+	-	PRO	
26	+	+	+	+	+	+	+	+	+	-	COM	
27	+	+	+	-	-	+	+	+	-	-	PRO ARG	PHE
28	+	+	+	-	+	+	+	+	+	-	PRO	
29	+	+	+	-	+	+	+	+	+	-	PRO	
30	+	+	+	+	+	+	+	+	+	-	COM	
31	+	+	+	+	+	+	+	+	+	-	COM	
38	+	+	+	+	+	+	+	+	+	-	COM	
39	+	+	+	-	+	+	+	+	+	-	PRO	
41	+	+	+	+	+	+	+	+	-	-	COM	PHE
45	+	+	+	-	+	+	+	+	+	-	PRO	
46	+	+	+	-	+	+	+	+	+	-	PRO	
47	+	+	+	-	+	+	+	+	-	-	PRO	PHE
49	+	+	+	+	+	+	+	+	+	-	COM	
51	+	+	+	+	+	+	+	+	+	-	COM	
52	+	+	+	+	+	+	+	+	+	-	COM	
54	+	+	+	+	+	+	+	+	+	-	COM	
56	+	+	+	-	+	+	+	+	-	-	PRO	PHE
57	+	+	+	-	+	+	+	+	+	-	PRO	
90	+	+	+	+	+	+	+	+	+	-	COM	
91	+	+	+	+	+	+	+	+	+	-	COM	
92	+	+	+	+	+	+	+	+	+	-	COM	
93	+	+	+	+	+	+	+	+	+	-	COM	
94	+	+	+	+	+	+	+	+	+	-	COM	
98	+	+	+	+	+	+	+	+	+	-	COM	
122	+	+	+	+	+	+	+	+	+	-	COM	
139	+	+	+	+	+	+	+	+	+	-	COM	

TABLE 11b. Continued

ISOLATE NO.	COMPLETE	VITAMINS	AMINO ACIDS	PROLINE	ARGININE	ARGININE^{-}O^{+}C^{+}	HYPOXANTHINE	URACIL	PHENYLALANINE	CYSTEINE	AUXOTYPE
144	+	+	+	-	+	+	+	+	+	-	PRO
147	+	+	+	+	+	+	+	+	+	-	COM
148	+	+	+	+	+	+	+	+	+	-	COM
150	+	+	+	+	+	+	+	+	+	-	COM
154	+	+	+	+	+	+	+	+	+	-	COM
160	+	+	+	+	+	+	+	+	+	-	COM
163	+	+	+	+	+	+	+	+	+	-	COM
166	+	+	+	-	+	+	+	+	+	-	PRO
168	+	+	+	+	+	+	+	+	+	-	COM
170	+	+	+	+	+	+	+	+	+	-	COM
172	+	+	+	+	+	+	+	+	+	-	COM
175	+	+	+	+	+	+	+	+	+	-	COM
176	+	+	+	-	+	+	+	+	+	-	PRO
178	+	+	+	+	+	+	+	+	+	-	COM
180	+	+	+	-	+	+	+	+	+	-	PRO
181	+	+	+	-	-	+	+	+	+	-	PRO ARG
182	+	+	+	-	+	+	+	+	+	-	PRO
183	+	+	+	+	+	+	+	+	+	-	COM
187	+	+	+	+	+	+	+	+	+	-	COM
188	+	+	+	+	+	+	+	+	+	-	COM
189	+	+	+	+	+	+	+	+	+	-	COM
190	+	+	+	-	+	+	+	+	+	-	PRO
191	+	+	+	-	+	+	+	+	+	-	PRO
192	+	+	+	-	+	+	+	+	+	-	PRO
193	+	+	+	+	+	+	+	+	+	-	COM
194	+	+	+	-	+	+	+	+	+	-	PRO
195	+	+	+	-	+	+	+	+	+	-	PRO
196	+	+	+	+	+	+	+	+	+	-	COM
197	+	+	+	+	+	+	+	+	+	-	COM
198	+	+	+	+	+	+	+	+	+	-	COM
201	+	+	+	+	+	+	+	+	+	-	COM

TABLE 11c. Auxotype patterns of non PPNG cervical isolates

	COMPLETE	VITAMINS	AMINO ACIDS	PROLINE	ARGININE	ARG⁻O⁺C⁺*	HYPOXANTHINE	URACIL	PHENYLALANINE	CYSTEINE	
19	+	+	+	-	+	+	+	+	+	-	PRO
16	+	+	+	-	+	+	+	+	+	-	PRO
109	+	+	+	+	+	+	+	+	+	-	COM
136	+	+	+	+	+	+	+	+	+	-	COM
155	+	+	+	+	+	+	+	+	+	-	COM
156	+	+	+	+	+	+	+	+	+	-	COM
157	+	+	+	+	+	+	+	+	+	-	COM
158	+	+	+	+	+	+	+	+	+	-	COM
161	+	+	+	+	+	+	+	+	+	-	COM
162	+	+	+	+	+	+	+	+	+	-	COM
164	+	+	+	+	+	+	+	+	+	-	COM
167	+	+	+	-	+	+	+	+	+	-	PRO

TABLE 11d. Auxotype patterns of non PPNG eye isolates

	COMPLETE	VITAMINS	AMINO ACIDS	PROLINE	ARGININE	ARG⁻O⁺C⁺*	HYPOXANTHINE	URACIL	PHENYLALANINE	CYSTEINE		
73	+	+	+	-	+	+	+	+	+	-	PRO	
76	+	+	+	+	+	+	+	+	+	-	COM	
77	+	+	+	-	+	+	+	+	-	-	PRO⁻	PHE
78	+	+	+	-	+	+	+	+	-	-	PRO⁺	PHE
81	+	+	+	+	+	+	+	+	-	-	PHE	COM
82	+	+	+	+	+	+	+	+	+	-	COM	
84	+	+	+	+	+	+	+	+	+	-	COM	
86	+	+	+	-	+	+	+	+	+	-	PRO	
87	+	+	+	-	+	+	+	+	+	-	PRO	
125	+	+	+	+	+	+	+	+	+	-	COM	
126	+	+	+	-	+	+	+	+	+	-	PRO	
127	+	+	+	-	+	+	+	+	+	-	PRO	
129	+	+	+	+	+	+	+	+	+	-	COM	
131	+	+	+	+	+	+	+	+	+	-	COM	

TABLE 11e. Auxotype patterns of non PPNG disseminated isolates, rectal isolates* and vaginal isolates+

	COMPLETE	VITAMINS	AMINO ACIDS	PROLINE	ARGININE	ARG⁻O⁺C⁺	HYPOXANTHINE	URACIL	PHENYLALANINE	CYSTEINE	
132*	+	+	+	+	+	+	+	+	+	-	COM
18*	+	+	+	+	+	+	+	+	+	-	COM
25*	+	+	+	+	+	+	+	+	+	-	COM
60*	+	+	+	-	+	+	+	+	+	-	PRO
110	+	+	+	+	+	+	+	+	+	-	COM
118	+	+	+	+	+	+	+	+	+	-	COM
119	+	+	+	+	+	+	+	+	+	-	COM
128	+	+	+	+	+	+	+	+	+	-	COM
137	+	+	+	+	+	+	+	+	+	-	COM
138	+	+	+	+	+	+	+	+	+	-	COM
140	+	+	+	+	+	+	+	+	+	-	COM
97+	+	+	+	+	+	+	+	+	+	-	COM
85+	+	+	+	+	+	+	+	+	+	-	COM
89+	+	+	+	+	+	+	+	+	+	-	COM

1. Distribution of auxotypes

Of the PPNG isolates 60 (94%) were proto, 2 (3%) were prototrophic and inhibited by phenylalanine. Two (3%) were pro⁻, of which one was inhibited by phenylalanine.

Of the non PPNG isolates 36 (32%) were pro⁻, of which 9 (8%) were inhibited by phenylalanine and 71 (64%) were proto, of which 2 (1,8%) were inhibited by phenylalanine. Two were pro⁻arg⁻ and 1 was arg⁻. These results are summarized in table 12 and Figs. 11a and 11b.

TABLE 12. Distribution of auxotypes amongst PPNG and non PPNG.

Type	Proto	Proto Phe$^+$	Pro$^-$	Pro$^-$ Phe$^+$	Arg$^-$	Arg$^-$ Pro$^-$
PPNG *n*=64	60(94%)	2(3%)	1(1.5%)	1(1.5%)		
non PPNG *n*=110	69(62%)	2(2%)	27(24%)	9(8%)	1	2(2%)

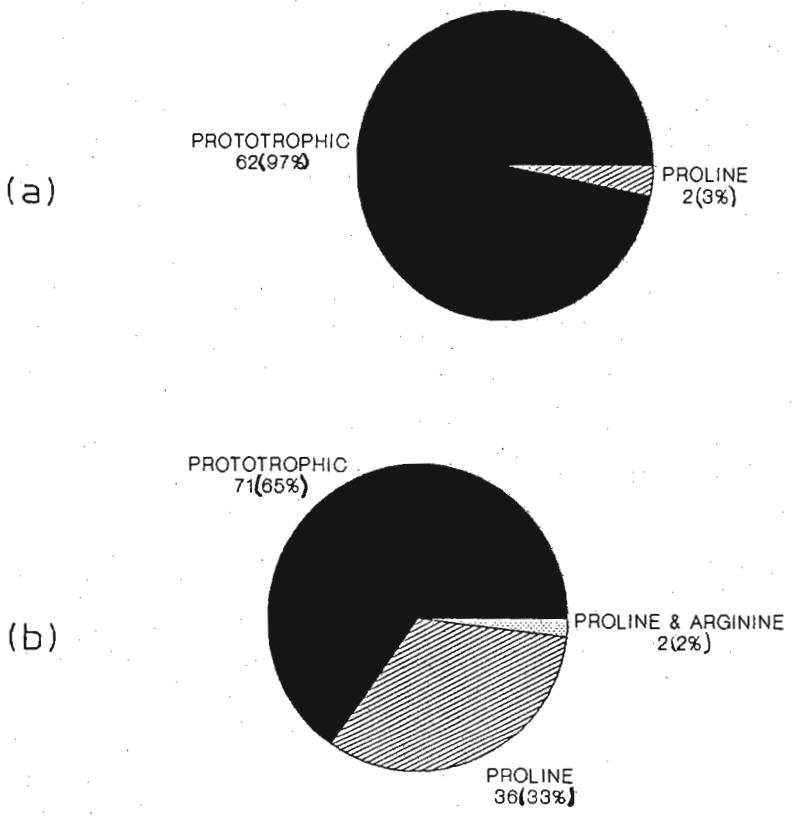


FIG. 11. Distribution of auxotypes a) PPNG and b) non PPNG isolates

2. Distribution of proline requiring and prototrophic auxotypes

The distribution of proto and pro$^-$ auxotypes of the non PPNG isolates at various infection sites show that pro$^-$ auxotypes make up 35% of the total isolates, with 47% of eye isolates being pro$^-$ and the rest predominately proto. These results are shown in Table 13.

TABLE 13. Distribution of Pro$^-$ and Proto strains.

Site	% Proto	% Pro$^-$
PPNG (n=64)	97	3
Urethra (n=40)	93	7
Cervix (n=11)	100	0
Eye (n=5)	100	0
Disseminated (n=3)	100	0
Rectum (n=5)	100	0
non PPNG (n=105)	65	35
Urethra (n=71)	62	38
Cervix (n=12)	75	25
Eye (n=16)	53	47
Disseminated (n=4)	100	0
Rectum (n=2)	66	33

3. Comparison of antibiotic susceptibilities of proline requiring and prototrophic strains

The MIC distribution of 14 antibiotics for pro$^-$ and proto strains is summarized in table 14 and Figs. 12a-12l. Pro$^-$ auxotypes are more susceptible than proto strains to all antibiotics tested except spectinomycin, rosoxacin and cotrimoxazole.

TABLE 14. MIC distribution of 14 antibiotics for pro⁻ and proto strains

PROLINE AUXOTYPE	CUMULATIVE PERCENTAGE OF ISOLATES INHIBITED													
MIC(u g/ml)	PEN*	APEN*	AMPI	AMOX	AUG+	CEFUROX	CEFOTAX	CEFTRI	SPECT	KANA	CO-TRI	TETRA	ERY	ROSA	CHL
0,001							81	64							
0,003	11	5	2			4	85	68						5	
0,007	46	19				54	92	81					3	25	
0,01	51	46	16			71	96	92					6	75	
0,03	66	54	51	31	9			96					26		
0,06	77	73	76	62	33	96							44	83	
0,12	86	89	89	75	52	100		100				25	82	92	6
0,25	97	95	92	88	82							72	100	100	49
0,5		97		97	88		100					100			80
1			100	100	94										94
2	100				97				14						100
4					100				19	19	35				
8		100							62	68	60				
16									100	97	90				
32										100	97				
PROTOTROPHIC															
0,001			3	1			64							5	
0,003		9	6			8	73							36	
0,007	12	25	7	4	1	36	88					1		73	
0,01	21	32	19	10	4	50	92							76	
0,03	31	44	36	30	10	80	93				1			85	1
0,06	54	74	46	52	30	90	95				3			95	
0,12	61	87	86	64	39	96								97	5
0,25	73	98	94	91	68	100	97					28			49
0,5	79	100	97	99	85		100				8	47			67
1	87		100	100	96						16	83			86
2					100				7	1	22	100			100
4	91								29	36	54			99	
8	95								74	64	79			100	
16									98	88	97				
32	100								100	100	100				

*APEN = penicillin G 10^7 CFU

*PEN = penicillin G 10^4 CFU

AUG+ = amoxicillin + clavulanic acid

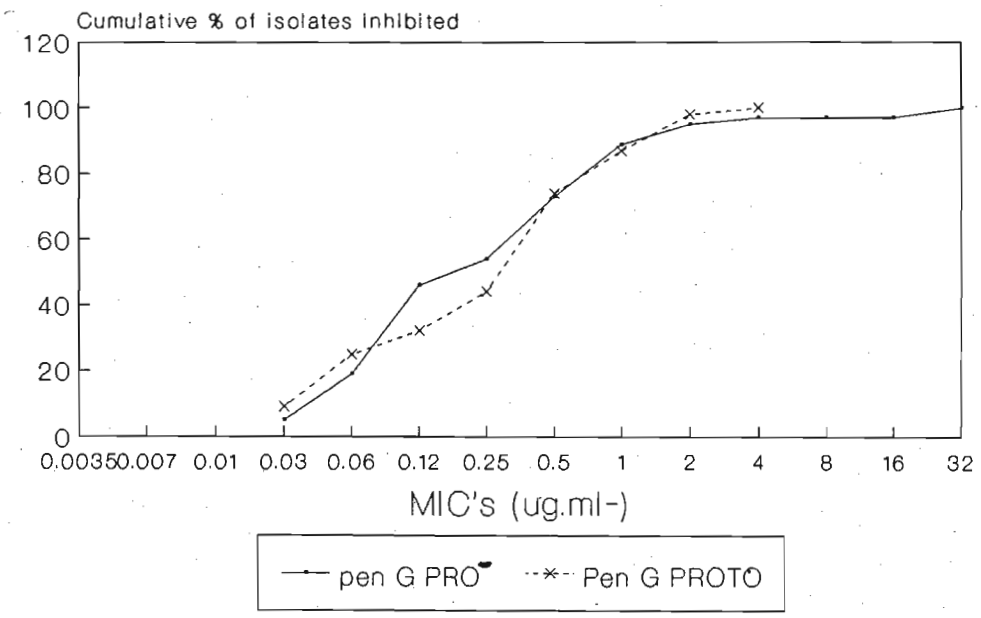


FIG. 12a. Penicillin G MIC's (10^7 CFU) for proto and pro⁻ strains

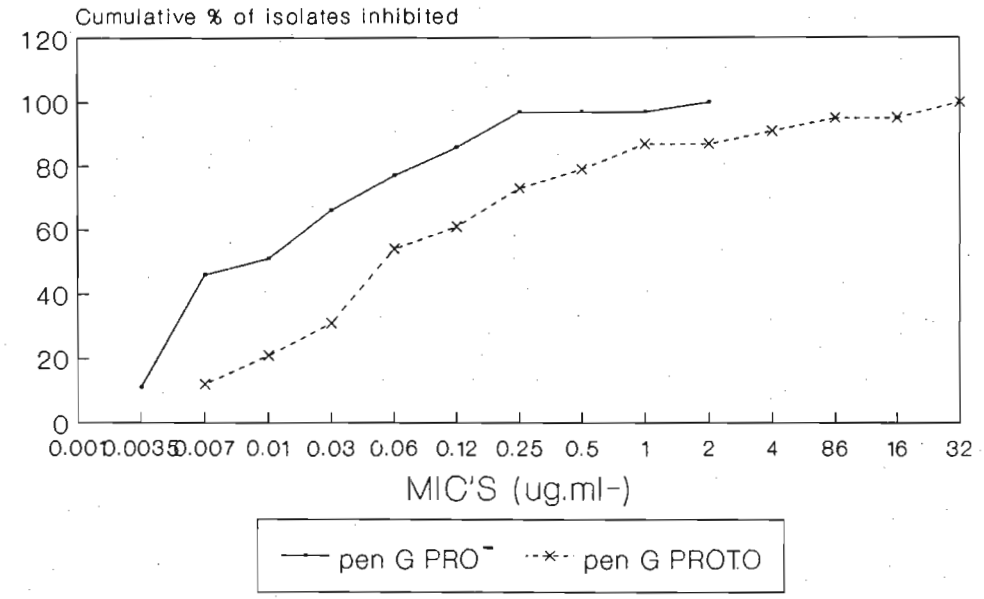


FIG. 12b. Penicillin G MIC's (10^4 CFU) for proto and pro⁻ strains

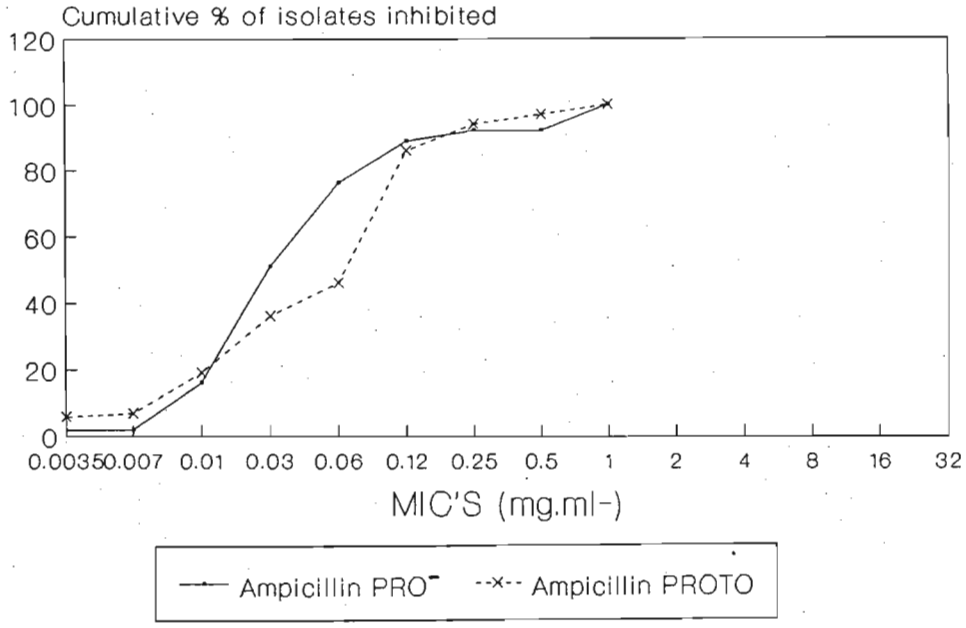


FIG. 12c. Ampicillin MIC's for proto and pro⁻ strains

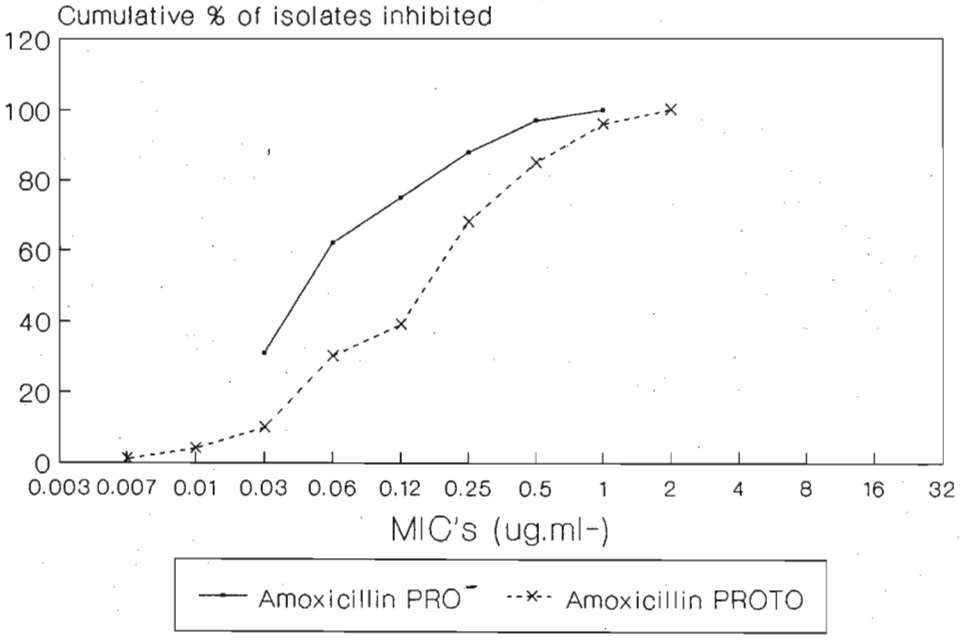


FIG. 12d. Amoxicillin MIC's for proto and pro⁻ strains

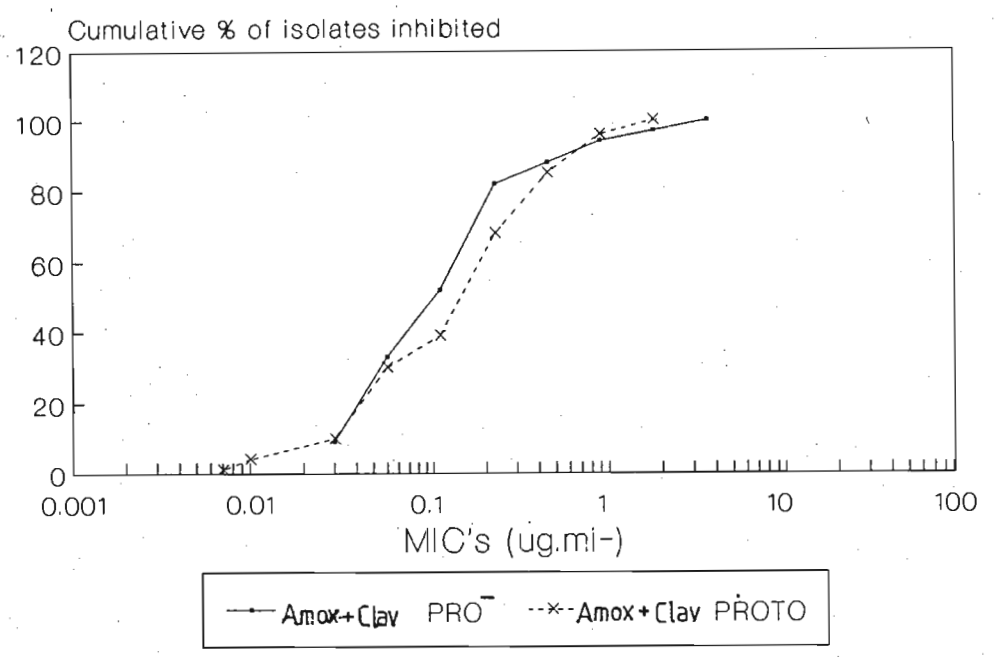


FIG. 12e. Amoxicillin + clavulanic acid MIC's for proto and pro⁻ strains

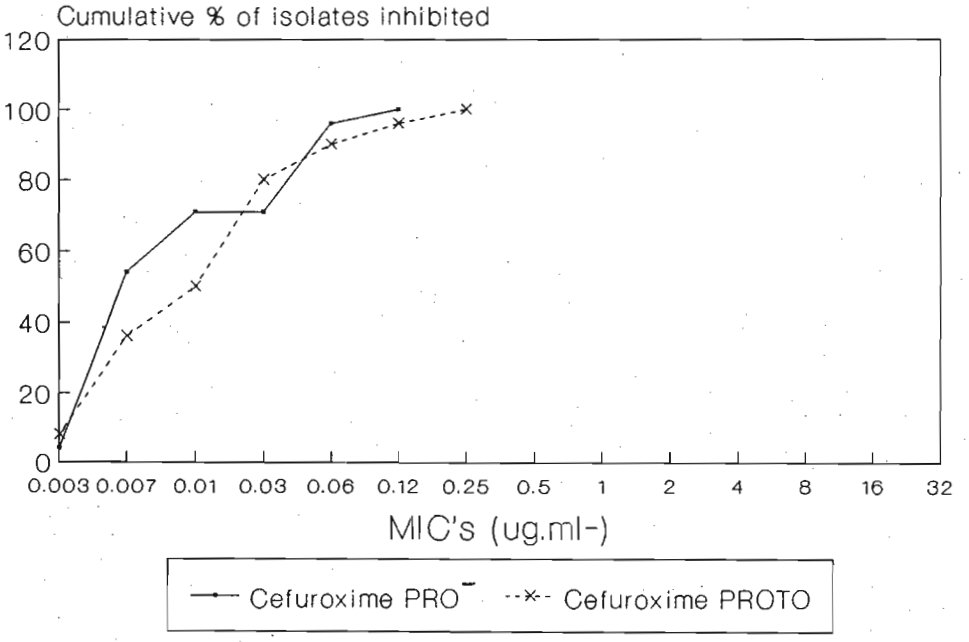


FIG. 12f. Cefuroxime MIC's for proto and pro⁻ strains

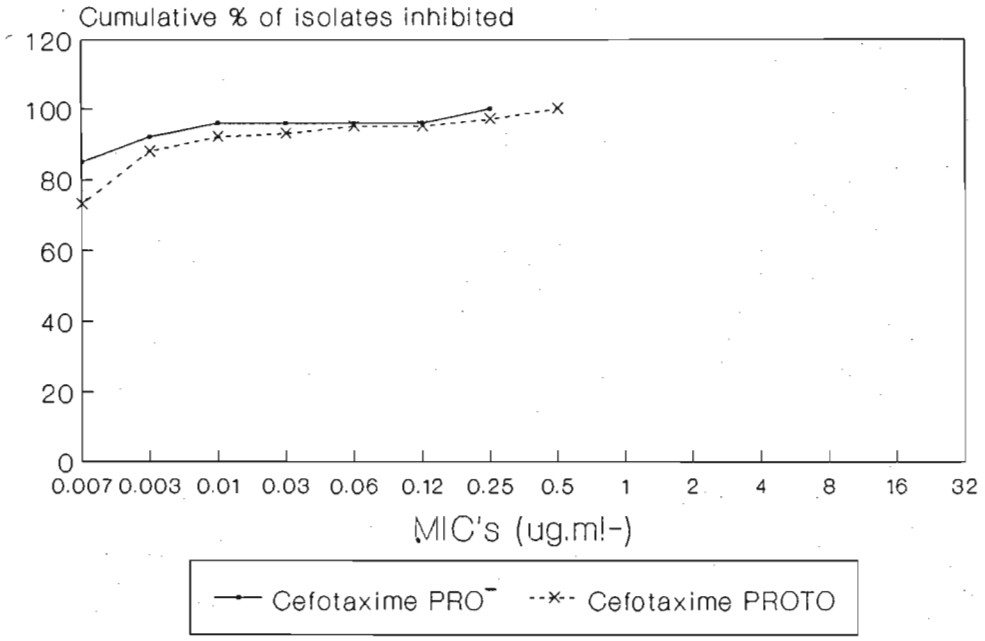


FIG. 12g. Cefotaxime MIC's for proto and pro⁻ strains

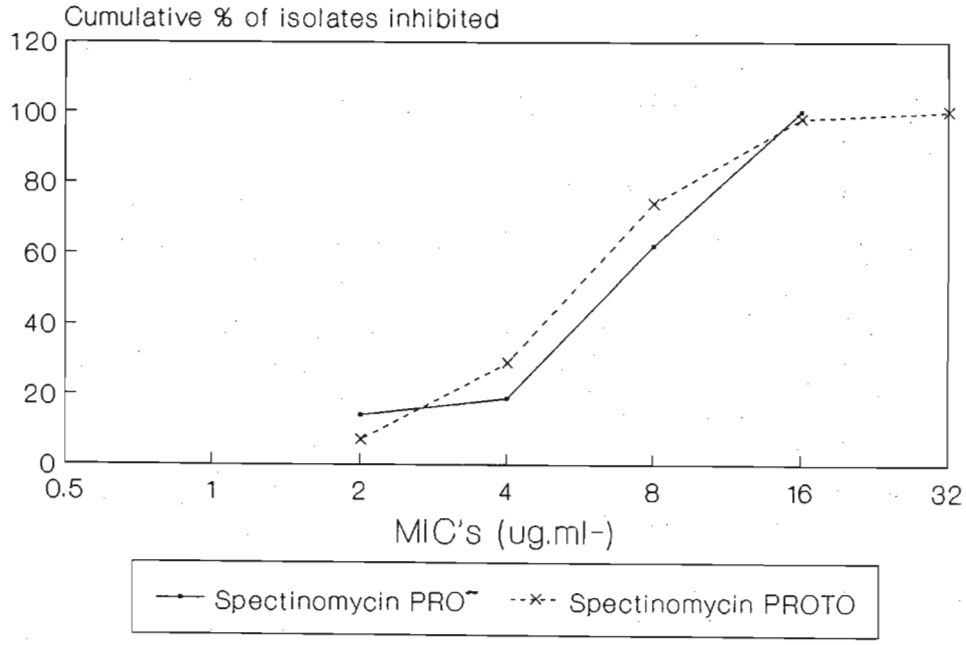


FIG. 12h. Spectinomycin MIC's for proto and pro⁻ strains

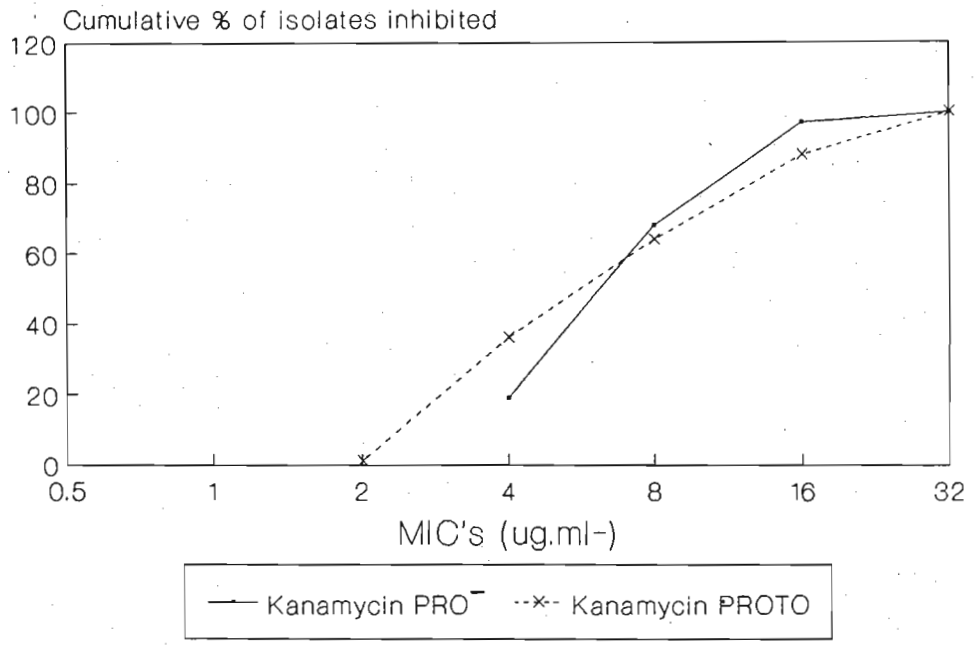


FIG. 12i. Kanamycin MIC's for proto and pro⁻ strains

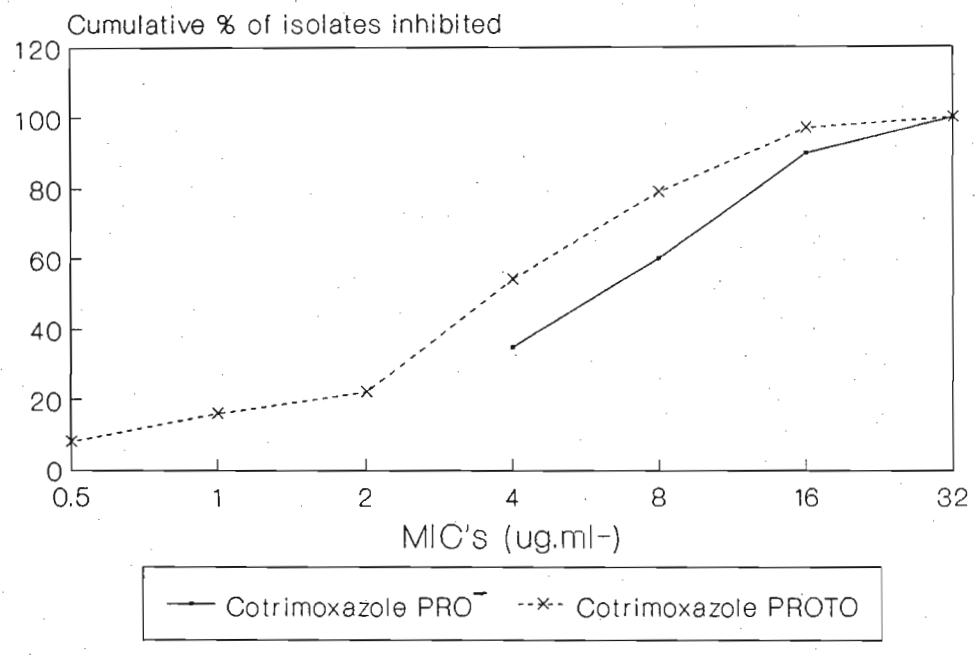


FIG. 12j. Cotrimoxazole MIC's for proto and pro⁻ strains

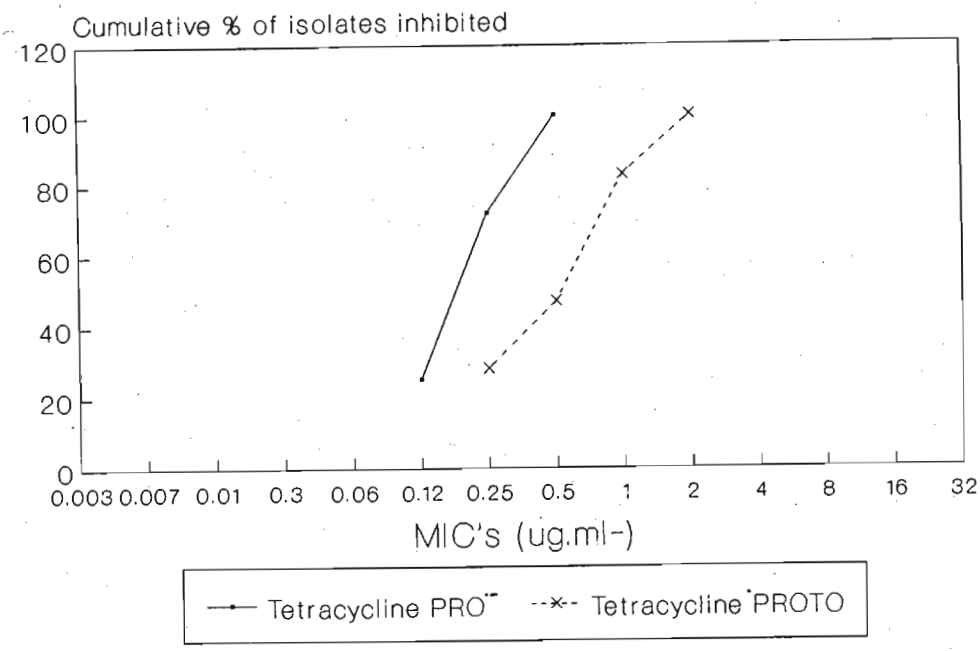


FIG. 12k. Tetracycline MIC's for proto and pro⁻ strains

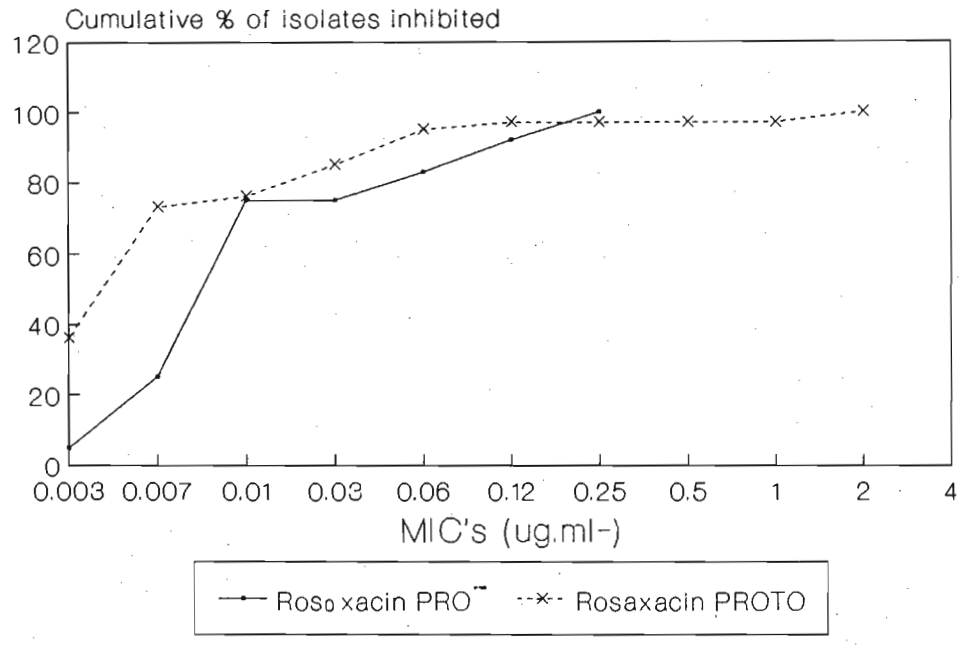


FIG. 12l. Rosoxacin MIC's for proto and pro⁻ strains

D) PLASMID PROFILES OF PENICILLINASE AND NON PENICILLINASE PRODUCING *N. gonorrhoeae*

1. Method

The initial aim in the analysis of the plasmid profiles of local strains was to find a suitable method showing correlation with the patterns obtained from the reference strains.

The boiling method (Maniatis *et al.*, 1982) showed the 2,6, 3,2, and 4,5 Mdal plasmids clearly (Fig. 13). The 2,6 Mdal plasmid is present in all isolates (lanes 1-10). The 3,2 Mdal plasmid is clearly shown in the control aux 6 (lane 10) whilst the 4,5 Mdal plasmid is seen in control aux 3 (lane 9). Some of the local isolates have the 3,2 Mdal plasmid (lanes 3, 6 and 8). Despite the clarity of smaller plasmids the big 24 Mdal plasmid was not detected by this method.

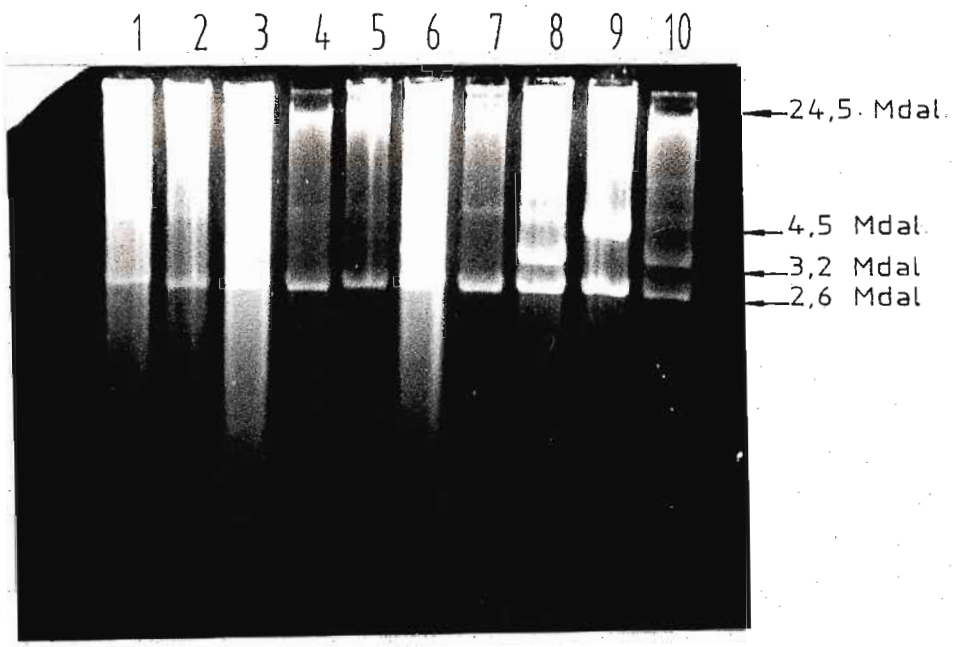


FIG. 13. Plasmid profiles of local strains using the boiling method of Maniatis (1982) were electrophoresed as follows:

Lane	Site	*B*-lactamase	Plasmid (Mdal)
1	urethral	-	2,6
2	urethral	-	2,6
3	urethral	+	2,6 + 3,2
4	urethral	-	2,6
5	urethral	-	2,6
6	urethral	+	2,6 + 3,2
7	urethral	-	2,6
8	urethral	+	2,6 + 3,2
9	aux 3 (control)	+	2,6 + 4,5
10	aux 6 (control)	+	2,6 + 3,2

The method of Sox *et al* (1978) also showed the smaller plasmids clearly (Fig. 14), but the 24,5 Mdal plasmid which was supposed to have been in lanes 1, 2, 5 and 6 did not appear. It seems that the big plasmid was hydrolysed, as evidenced by the extra bands.

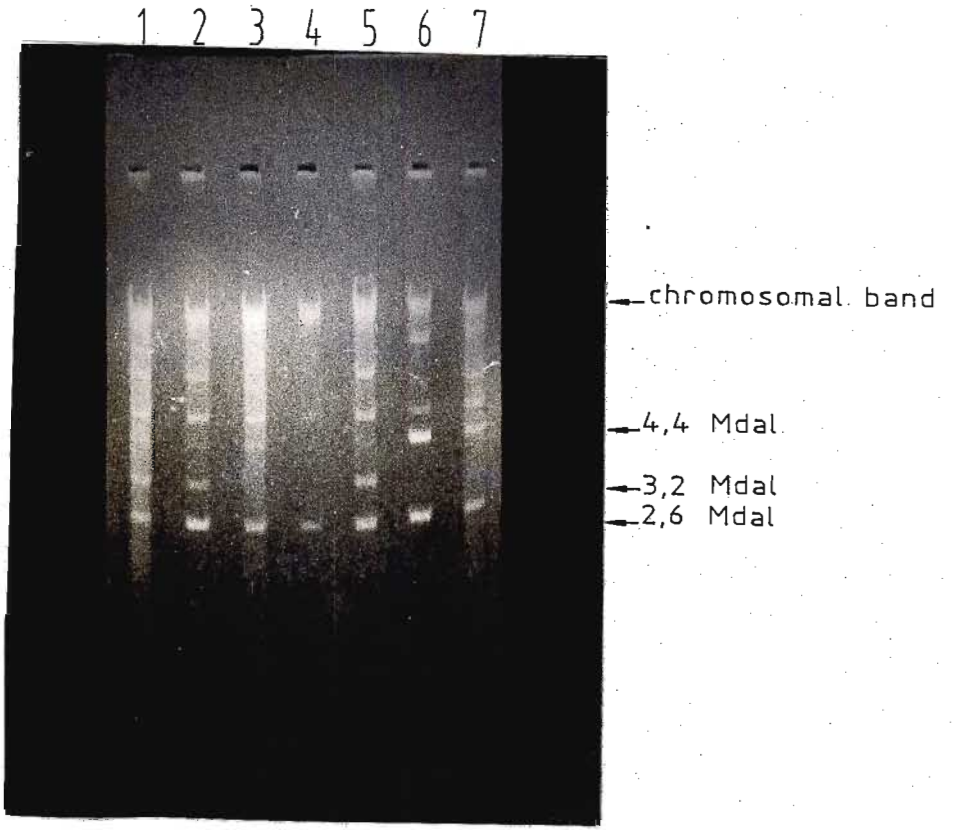


FIG. 14. Plasmid profile using the method of Sox *et al.* (1978) was electrophoresed as follows:

Lane	Control	*B*-lactamase	Obtained	Expected
1	aux 0	+	2,6 + 3,2	2,6 + 3,2 + 24,5
2	PPNG 760	+	2,6 + 3,2	2,6 + 3,2 + 24,5
3	T 21	-	2,6	2,6
4	T 31	-	2,6	2,6
5	aux 6	+	2,6 + 3,2	2,6 + 3,2 + 24,5
6	aux 3	+	2,6 + 4,5	2,6 + 4.5 + 24,5
7	PPNG 66	+	2,6 + 4,4	2,6 + 4,4

Expected results were obtained using the method of Takahashi & Nagano (1985). Fig. 15 shows the big 24,5 Mdal and the smaller 2,6, 3,2 and 4,5 Mdal plasmids as expected for all the controls.

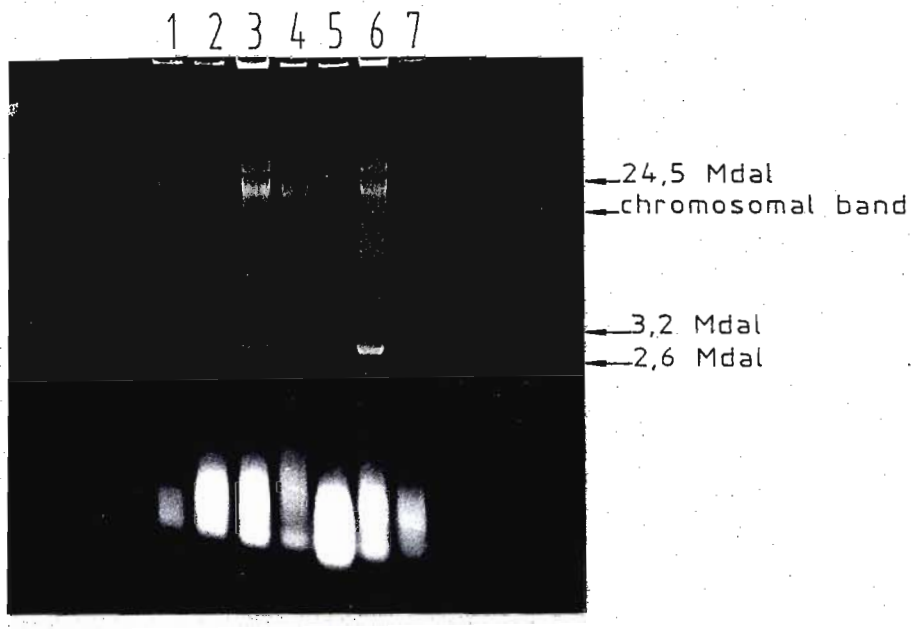


FIG. 15. Plasmid profiles of reference strains were electrophoresed as follows:

Lane	Control	*B*-lactamase	Plasmids (Mdal)
1	aux 0	+	2,6 + 3,2 + 24,5
2	aux 3	+	2,6 + 4,5 + 24,5
3	aux 6	+	2,6 + 3,2 + 24,5
4	PPNG 66	+	2,6 + 4,5
5	PPNG 65	+	2,6 + 3,2
6	PPNG 760	+	2,6 + 3,2 + 24,5
7	T21	-	2,6

2. Plasmid profiles of local isolates

(a) Urethral isolates

Of the 111 urethral isolates 40 were PPNG and 71 non PPNG strains. Of the 40 PPNG urethral isolates 20 were studied for their plasmids. Fig. 16 represents the plasmid profiles of 8 urethral isolates. All the PPNG urethral isolates examined had the 2,6 and 3,2 Mdal plasmids, and 3 (7,5%) of the urethral isolates had the 24,5 Mdal plasmid.

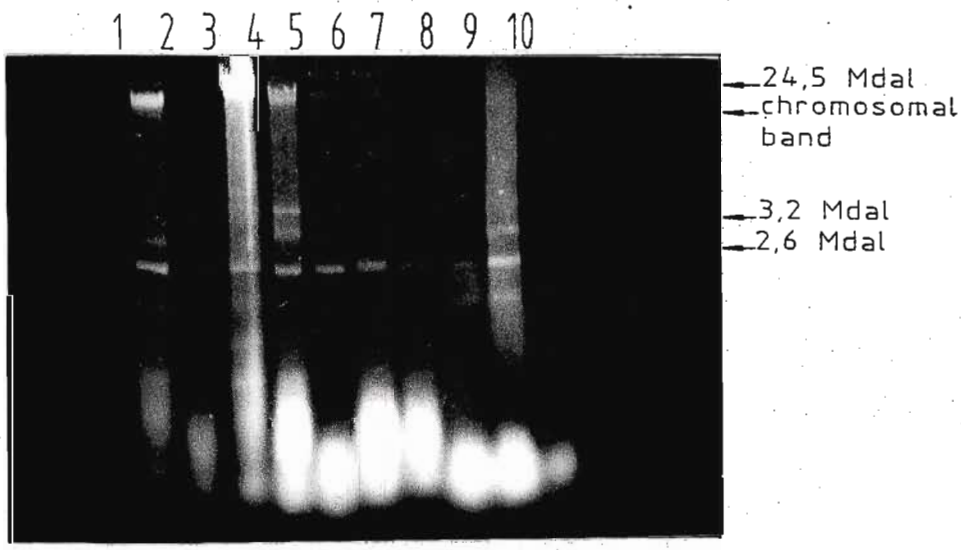


FIG. 16. Plasmid profiles of urethral isolates were electrophoresed as follows:

Lane	Sample	*B*-lactamase	Plasmids (Mdal)
1	urethral (B4)	+	2,6 + 3,2 + 24,5
2	urethral (B7)	-	2,6 + 24,5
3	urethral (B8)	-	2,6
4	urethral (B22)	+	2,6 + 3,2 + extra band
5	urethral (B24)	+	2,6 + 3,2 + 24,5
6	urethral (B36)	+	2,6 + 3,2 + 24,5
7	urethral (B49)	+	2,6 + 3,2
8	urethral (B47)	-	-
9	control (PPNG 65)	+	2,6 + 3,2
10	control (PPNG 66)	+	-

(b) Cervical isolates

Of the 23 cervical isolates studied 11 were *beta*-lactamase producers. None of the cervical isolates had the large 24,5 Mdal plasmid. All the cervical PPNG isolates had the 2,6 and 3,2 Mdal plasmids whilst the non PPNG isolates had the 2,6 Mdal plasmid. Fig. 17 represents the plasmid profiles of cervical isolates studied.

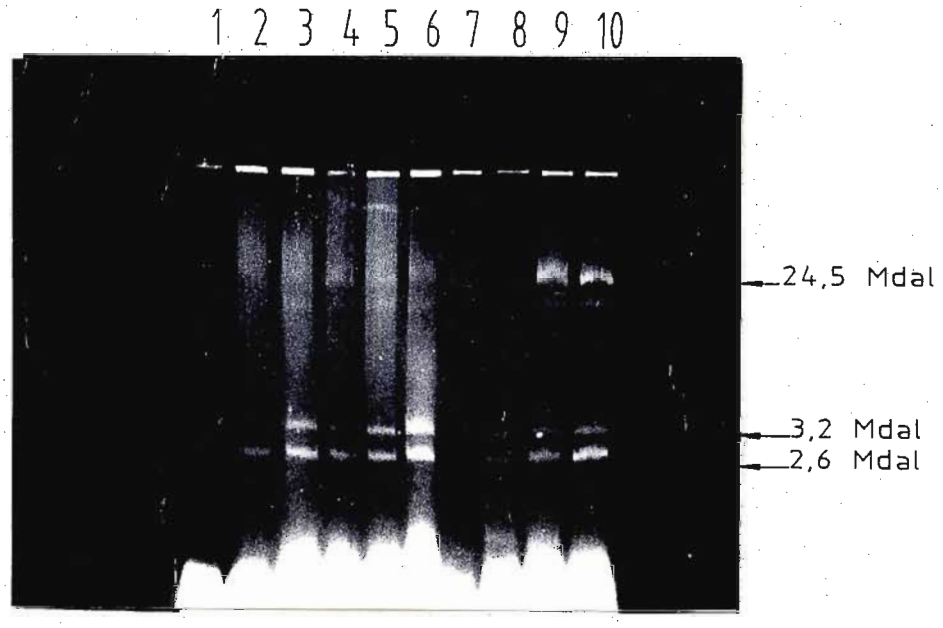


FIG. 17. Plasmid profiles of cervical isolates were electrophoresed as follows:

Lane	Sample	*B*-lactamase	Plasmids (Mdal)
1	cervical (B123)	+	2,6 + 3,2
2	cervical (B19)	-	2,6
3	cervical (B13)	+	2,6 + 3,2
4	cervical (B149)	+	2,6 + 3,2
5	cervical (B21)	+	2,6 + 3,2
6	cervical (B155)	+	2,6 + 3,2
7	cervical (B109)	-	2,6
8	cervical (B62)	+	2,6 + 3,2
9	aux 0 (control)	+	2,6 + 3,2 + 24,5
10	PPNG 760 (control)	+	2,6 + 3,2 + 24,5

(c) Eye Swabs

Nineteen eye isolates were studied of which 4 were PPNG. None had the 24,5 Mdal plasmid. All the PPNG isolates had the 2,6 and 3,2 Mdal plasmids. The non PPNG isolates had the 2,6 Mdal plasmid (Fig. 18).

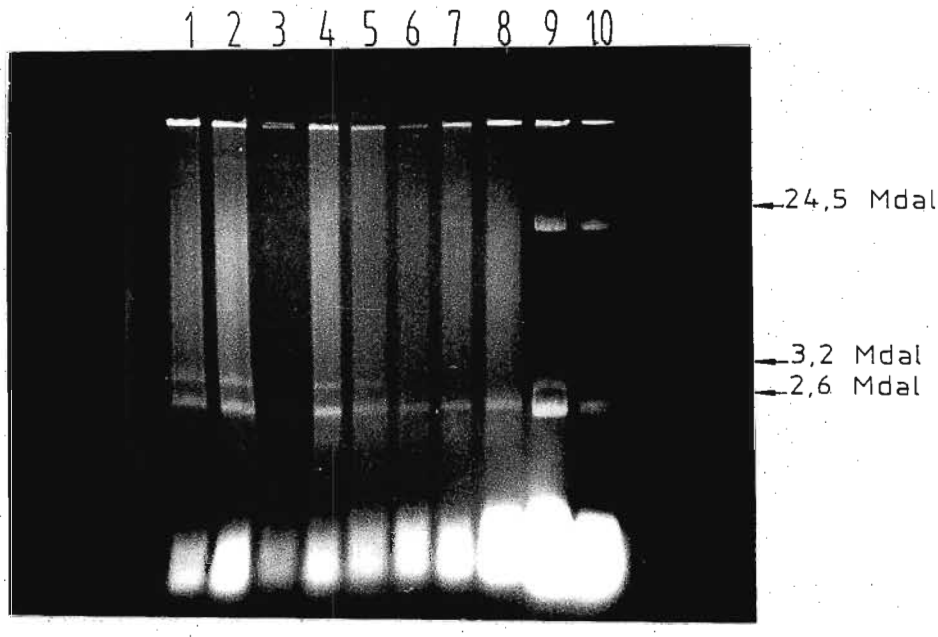


FIG. 18. Plasmid profiles of eye isolates were electrophoresed as follows:

Lane	Sample	*B*-lactamase	Plasmids (Mdal)
1	eye (B75)	+	2,6 + 3,2
2	eye (B88)	+	2,6 + 3,2
3	eye (B83)	+	2,6 + 3,2
4	eye (B76)	+	2,6 + 3,2
5	eye (B74)	+	2,6 + 3,2
6	eye (B86)	-	2,6
7	eye (B77)	-	2,6
8	eye (B78)	-	2,6
9	PPNG 760 (control)		2,6 + 3,2 + 24,5
10	aux 6 (control)		2,6 + 3,2 + 24,5

(d) Isolates from rectal and disseminated infections

Seven rectal isolates were studied of which 5 were PPNG strains. Of these one (B153 lane 8) had the 24,5 Mdal plasmid but did not appear to have the 3,2 Mdal plasmid. Samples in lanes 1, 2 and 3 were taken from the knee, cervix and urethra respectively of the same patient, and show the same plasmid profile (2,6 Mdal + 3,2 Mdal). Lanes 4 and 5 were cervical and knee isolates from another patient, were non PPNG and had only the 2,6 Mdal plasmid. These results are shown in Fig. 19.

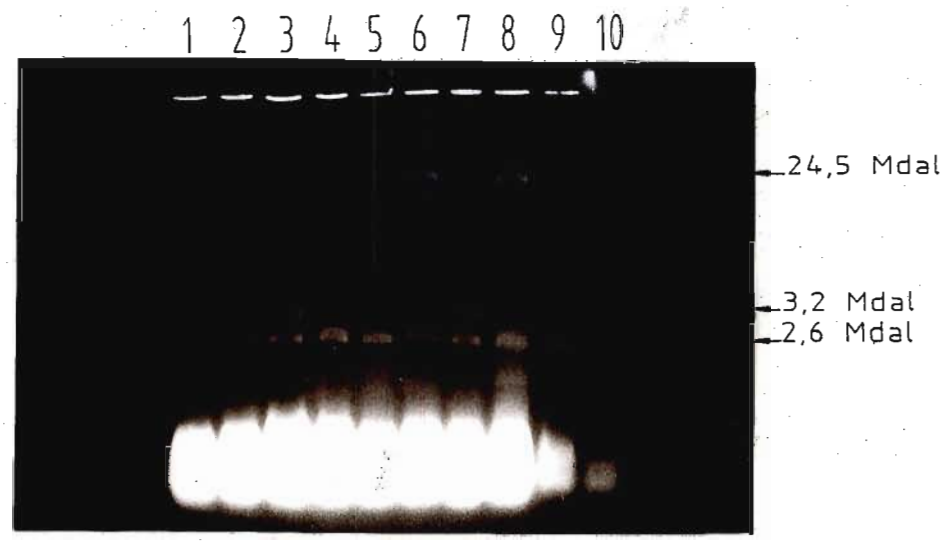


FIG. 19. Plasmid profiles of disseminated infections were electrophoresed as follows:

Lane	Sample	*B*-lactamase	Plasmids(Mdal)
1	knee (B106)	+	2,6 + 3,2
2	cervix (B107)	+	2,6 + 3,2
3	urethra (B108)	+	2,6 + 3,2
4	cervix (B109)	-	2,6
5	knee (B110)	-	2,6
6	urethra (B28)	-	2,6 + 24,5
7	rectum (B63)	+	2,6 + 3,2
8	rectum (B153)	+	2,6 + 24,5
9	aux 6 (control)	+	2,6 + 3,2 + 24,5

3. Relationship between plasmid profile and auxotype

All the PPNG isolates that were proto had the 3,2 Mdal, 2,6 Mdal and some had the 24,5 Mdal plasmids. The non PPNG isolates, irrespective of their auxotype had only the 2,6 Mdal plasmid. No link between plasmids and auxotype was demonstrated.

E) ISOELECTRIC FOCUSSING OF *BETA*-LACTAMASES

The isoelectric point (pI) of these *beta*-lactamase was determined from a pH gradient produced electrophoretically in thin layers of agarose gels, and made visible by cephalosporin substrate hydrolysed to a chromogenic (red) product. The pI was found to be 5,4. According to Bauernfeind (1986) a pI of 5,4 corresponds to the TEM 1 group of enzymes. The TEM 1 enzymes hydrolyse the *beta*-lactam ring and are *beta*-lactamases.

Isoelectric focussing of crude lysates confirmed that PPNG produce a TEM 1 *beta*-lactamase. From the IEF patterns shown in Fig. 20 it can be seen that the gonococcal *beta*-lactamase band and adjacent TEM 1 control band show identity.

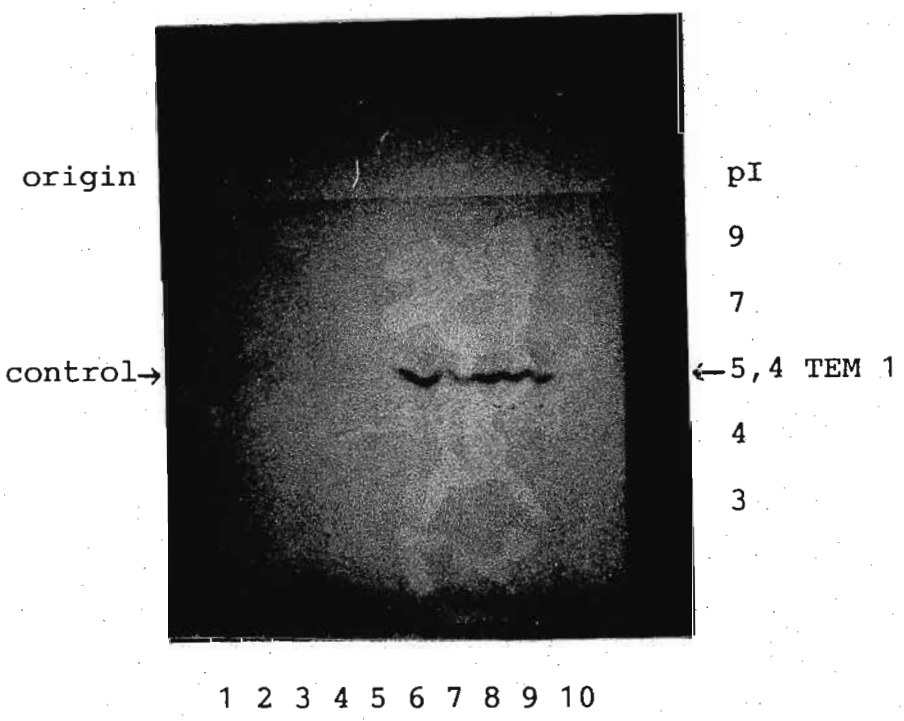


FIG. 20. Isoelectric focussing patterns of *beta*-lactamases of *N. gonorrhoeae*. Lane 1 = Tem 1 control, lanes 2 to 5 = *beta*-lactamase negative strains, lanes 6 to 10 = *beta*-lactamase positive strains

DISCUSSION

Penicillin is at present still widely used as first line therapy for *N. gonorrhoeae* infections (Coovadia *et al.*, 1988). Isolates of *N. gonorrhoeae* were uniformly susceptible to very low concentrations (0,007$ug.ml^{-1}$) of penicillin G for the first two decades of its use but thereafter relatively resistant strains, requiring 0,125$ug.ml^{-1}$ to 2$ug.ml^{-1}$ for inhibition, emerged and have become prevalent in many parts of the world. This resistance is due to mutations at several chromosomal loci which result in altered cell envelope. These chromosomally resistant strains (non PPNG) have PBP's with reduced affinity for penicillin (Dougherty, 1985).

In 1976, strains of *N. gonorrhoeae* were isolated that carried plasmids encoding for *beta*-lactamase production and were not eradicated by high doses of penicillin G. These *beta*-lactamase producing strains (PPNG) now constitute half to a third of isolates in many countries. Prolonged prophylaxis and unsupervised self medication with antibiotics, available in an uncontrollable manner, have led to passage of mutants with increased resistance (Jephcott, 1986).

CMRNG now rivals PPNG in compromising the use of penicillins for gonorrhoea. Previous studies on susceptibility of South African isolates indicate prevalence rates of 5-13% for PPNG (Coovadia *et al.*, 1984; Dangor *et al.*, 1986). Of the non PPNG 14-23% were intermediately susceptible (Coovadia *et al.*, 1984; Coovadia & Ramsaroop, 1984; Liebowitz *et al.*, 1982),

with intermediate susceptibility being defined as 0,125 ug.ml^{-1} to 0,5 ug.ml^{-1}. In a recent report the PPNG strains had risen to 29%, whilst 42% of non PPNG were intermediately susceptible and 2% of non PPNG strains were found to be resistant to penicillin G (MIC > 1), a phenomenon not previously reported from this country (Coovadia *et al.*, 1988). In this study 24% of non PPNG strains at 10^4 CFU inoculum size were intermediately susceptible, and 2% resistant. At 10^7 CFU inoculum size 30% were intermediately susceptible, and 8% resistant.

Comparing local results to those obtained in other countries (Table 15) it appears that local strains are relatively susceptible. However, in following the resistance pattern over the years it is evident that local strains are rapidly becoming more resistant.

When penicillin resistance occured, ampicillin was introduced. Ampicillin was the first semi-synthetic penicillin with significant activity against both Gram positive and Gram negative organisms. However, it is not stable to *beta*-lactamases. The other disadvantages are its variable absorption by the gut and the frequent side effect of diarrhoea. To overcome this problem, amoxicillin was introduced. Although amoxicillin and ampicillin show similar activity, amoxicillin is absorbed better by the gut (Sutherland *et al.*, 1972). Amoxicillin differs from

ampicillin by the substitution of a hydroxyl group on the benzene ring.

TABLE 15. Penicillin susceptibility of *N. gonorrhoeae* from different countries

Country	Author	% of strains with MIC's(ug.ml^{-1}) < 0,03	0,06-0,25	> 0,5
Canada	Stewart & Hendry, 1979	49,0	50,0	0,2
Ethiopia	Gedebou & Tassew, 1980	59,0	40,2	0,8
UK	Seth *et al.*, 1979	61,0	32,5	6,6
Saudi Arabia	Chowdhary *et al.*, 1981	45,2	39,7	15,1
USA	Jaffe *et al.*, 1976	31,0	51,6	17,4
Belgium	Piot *et al.*, 1979	53,6	28,6	17,9
Hong Kong	Ng *et al.*, 1981	22,4	49,0	28,6
Rwanda	Piot *et al.*, 1979	14,6	43,9	41,5
Singapore	Sng *et al.*, 1984	2,0	41,7	56,3
Phillipines	Tupasi *et al.*, 1980	-	21,7	72,9
Korea	Wilson *et al.*, 1976	1,5	17,9	80,6
Thailand	Crum *et al.*, 1980		17,9	82,1
NATAL				
10^7		61,0	30,0	8,0
10^4		73,0	25,0	2,0

In this study ampicillin and amoxicillin showed similar activity with MIC_{50} of 0,06 ug.ml^{-1} for non PPNG, and 4 and 8 ug.ml^{-1} respectively for PPNG (Table 9). Though amoxicillin

is better absorbed, it is also inactivated by *beta*-lactamases (Fig. 7c).

Clavulanic acid suppresses the activity of *beta*-lactamases (Reading & Cole, 1977). A combination of amoxicillin and clavulanic acid, in various ratios, is active against *beta*-lactamase producing microorganisms. As expected, the MIC's for amoxicillin + clavulanic acid were several folds higher for PPNG than for non PPNG strains (Fig. 7c).

Any of the third generation cephalosporins are useful for the treatment of *N. gonorrhoeae* since they are effective against both PPNG and non PPNG isolates. Clinical trials conducted with ceftriaxone and cefotaxime showed 100% cure rates against both PPNG and non PPNG infections (Barlow & Phillips, 1984; Judson *et al.*, 1983). In this study the MIC ranges for cephalosporins (Table 9) were similar to those reported from Hong Kong (Ng *et al.*, 1981), Europe and Africa (Piot *et al.*, 1979) and United Kingdom (Phillips, 1976), illustrating the high activity of these new agents.

Isolates from South East Asian countries are found to be more resistant to a number of antibiotics compared to isolates from Western countries (Copley *et al.*, 1983; Thornsberry *et al.*, 1978). These isolates frequently show resistance to tetracycline, erythromycin and kanamycin. In this study resistance to tetracycline, erythromycin and kanamycin was not found. The MIC ranges of these drugs for PPNG isolates were

0,03-1, 0,007-2 and 0,5-32 $ug.ml^{-1}$ respectively (Table 9). All isolates examined were also susceptible to cotrimoxazole.

Antimicrobials which have more recently been used to treat *N. gonorrhoeae* infections appear to have declined *in vitro* activity (Meheus, 1987). Spectinomycin resistant *N. gonorrhoeae* have been reported by several workers (Ashford *et al.*, 1981; Easmon *et al.*, 1982). Local isolates had spectinomycin MIC's < 32 $ug.ml^{-1}$. It is not surprising that local isolates have retained their sensitivity to spectinomycin since this drug is rarely used in local clinics.

Rosoxacin was also shown to be highly active against both PPNG and non PPNG, with MIC_{50} of 0,007 $ug.ml^{-1}$. This is in accordance with previous reports from this country (Liebowitz *et al.*, 1982) and the United Kingdom (Seth, 1981), where rosoxacin was shown to be active against *beta*-lactamase producing strains of *N. gonorrhoeae.*

Many authors have reported the inoculum size effect on antibiotic susceptibility testing (Hall *et al.*, 1979; Ng *et al.*, 1983; Robins-Browne *et al.*, 1978). In this study the effect of inoculum size was tested using penicillin G and was found to be greatest for PPNG (Fig. 6a), a finding also reported by Ng *et al.* (1983). This becomes of clinical significance in disseminated infections where the number of organisms may be large.

In summary, antibiotic sensitivities of local isolates indicate that South African isolates are rapidly becoming resistant to penicillin, which is currently the drug of choice for gonorrhoea. If further spread of penicillin resistance is to be prevented, an alternate treatment regimen for gonorrhoea is needed. Local isolates are susceptible to cephalosporins, rosoxacin, kanamycin, spectinomycin, tetracycline, chloramphenicol and erythromycin.

Of the 35 auxotypes described by Catlin & Pace (1977) in Table 2, only four types were found locally. Proto strains predominated (74%), followed by pro^- (23%), arg^- (1,5%) and arg^-pro^- (1,5%) as shown in Table 12. All the isolates in this study were obtained from Black patients, and the auxotypes correlate well with those obtained by Crawford *et al.* (1977), Knapp *et al.* (1978) and Noble & Miller (1980), who also found *N. gonorrhoeae* of proto, pro^- and arg^- auxotypes to be common among black patients. The significance of this is unknown, but may indicate a genetic predisposition to infection by specific auxotype strains.

According to Hendry & Dillon (1984) phenylalanine sensitivity appears to be linked to the presence of the 3,2 Mdal plasmid. However, the results in this study did not show this correlation. Growth inhibition by phenylalanine (0,5 $mMol.l^{-1}$ in defined agar medium) was present in only 3% of 171 local isolates of *N. gonorrhoeae* and most were non PPNG strains.

The local auxotype pattern is similar to those obtained in other African countries (Odugbemi *et al.*, 1983) where only four auxotype patterns exist, with proto strains predominating, as shown in Table 16.

TABLE 16. Auxotype patterns of some African countries

Source	Strain	No.	Proto	Pro$^-$	Pro$^-$ Arg$^-$	Arg$^-$
West African*	PPNG	24	11	6	6	1
	non PPNG	13	4	7	2	0
Other African countries#	PPNG	15	10	4	1	0
	non PPNG	38	25	10	1	1
South Africa (Natal)	PPNG	63	60	2	0	1
	non PPNG	109	71	36	2	0

* West African countries include Ghana, Ivory Coast and Nigeria
\# Cameroon, Central African Republic, Ethiopia, Kenya, South Africa, Zambia and Zaire

The PPNG were predominantly proto (97%) whilst the non PPNG had 32% pro$^-$ strains (Fig. 11). This is similar to the auxotypes prevailing in most African and developing countries (Table 3).

The relationship between auxotype and susceptibility to antibiotics in non PPNG is reported by several authors (Catlin & Pace, 1977; Draper *et al.*, 1981; Noble & Parekh 1983a; Van Klingeren *et al.*, 1985). According to the studies of van Klingeren *et al.* (1985) pro$^-$ isolates were less susceptible to antibiotics than proto strains, although pro$^-$ isolates include organisms that are highly susceptible and also relatively resistant to penicillin G. This study shows that for all

antibiotics studied except rosoxacin, spectinomycin and cotrimoxazole, pro$^-$ isolates are more susceptible (Figs. 12a-121).

A comparison of antibiotic susceptibility patterns and auxotype patterns at different infective sites confirms that pro$^-$ auxotypes are more susceptible. There are no published reports explaining why pro$^-$ auxotypes are more susceptible than the proto strains.

Though there exists a relationship between auxotype and disease (Brunham *et al.*, 1985b; Schoolnik *et al.*, 1976), *N. gonorrhoeae* isolates from different infection sites of the same patient have the same auxotype pattern and the same antibiotic susceptibilities indicating that the nutritional requirements of *N. gonorrhoea* remain stable during its multiplication in different tissues of the body.

Some conclusions drawn on the methodology of auxotyping are:

- a requirement for glutamine, serine, leucine, valine, isoleucine, threonine and alanine is different at 24h and 48h as shown in Fig. 10 leading to the same conclusion made by Catlin (1973) and Carifo & Catlin (1973) that amino acids other than methionine, proline and arginine would not be useful for strain identification;
- for results of auxotyping to be clinically and epidemiologically useful, the technique must be standardised internationally;

- auxotyping is time consuming, expensive and difficult.

Several authors report the advantages of serotyping (Bygdeman, 1981; Danielsson *et al.*, 1983). Serological classification with monoclonal antibodies gives better resolution than auxotyping. However, for fine epidemiological work, a combination of auxotyping and serotyping should be used (Backman *et al.*, 1987).

Strains of PPNG continue to spread throughout the world. In some African countries the PPNG level has increased up to 80% in some areas as shown in Table 17.

TABLE 17. Areas of high PPNG incidence

Country	Percentage	Author
Phillipines	30-40	*Perine *et al.*, 1979
Thailand	42	*Browns *et al.*, 1982
Japan	16	Yoshida *et al.*, 1982
Singapore	33,5	Sng *et al.*, 1984
Indonesia	25	*Josodiwondo, 1982
Netherlands	4-25	van Emden *et al.*, 1981
Some African Regions		
Nigeria:		
Ibadan	81,2	Osoba *et al.*, 1986
Ilorin	77,8	*Onile *et al.*, 1986
Enugu	73	*Egere *et al.*, 1982
Benin	87	*Obaseiki-Ebor *et al.*, 1985
Ghana:		
Kumasi	81,8	*Addy *et al.*, 1986

* Cited by Osoba (1986)

A strain of PPNG was first reported in South Africa by Robins-Browne *et al.* (1977) in a white patient who had visited West Africa, an area in which PPNG was known to exist. Isolated cases were also reported in Durban in 1977, originating from a

seaman who had become infected in Ghana (Hallet *et al.*, 1977). In 1984, 2% of *N. gonorhoeae* isolates in Cape Town were found to be PPNG (Simpson *et al.*, 1984), while in Durban a prevalence rate of 5% was reported in the same year (Coovadia *et al.*, 1984). Within a period of nine months, the prevalence rate in Durban had increased to 12% (Coovadia *et al.*, 1984). More recently this figure has risen to 29% (Coovadia *et al.*, 1988).

Plasmid DNA is isolated from bacteria by a procedure in which cells are lysed and chromosomal DNA, free of plasmid DNA, is precipitated. SDS is an ionic detergent that lyses the cells. Addition of a salt to the lysate causes chromosomal DNA-protein-SDS to precipitate. Removal of this precipitate by centrifugation leaves a clear lysate which contains the plasmid. Though several reports exist on the isolation of large and small plasmids in *N. gonorrhoeae* (Maniatis *et al.*, 1982; Sox *et al.*, 1978), the results in Figs. 13 and 14 emphasize that control of pH and temperature of the lysing solution is critical.

Plasmid analysis of local isolates show that all PPNG strains carry the 3,2 Mdal plasmid (Figs. 13-17). The 24,5 Mdal conjugative plasmid was found in a few strains from urethra, rectum, and knee joint, in combination with the 3,2 Mdal plasmid. Both PPNG and non PPNG strains had the cryptic 2,6 Mdal plasmid.

Isoelectric points have proven to be a valuable marker, particularly for grouping plasmid mediated *beta*-lactamases (Bauernfeind, 1986). In this study the pI of *beta*-lactamases was found to be 5,4 thus confirming that the *beta*-lactamases belong to the TEM 1 group of enzymes.

Since 1976, two distinct types of PPNG isolates have been described from two different areas by Ashford *et al.* (1976) and Percival *et al.* (1976). To ascertain the origin of local strains, a comparison was made with reports of West African and Far Eastern strains (Table 18).

TABLE 18. Comparison of strains

Geographic distribution	R plasmid	Conjugative plasmid	Cryptic plasmid	Auxotype	Tet R
Far East & USA	4,5 Mdal	present	present	pro^- proto	High
West Africa & UK	3,2 Mdal	absent	present	arg^-	Mod
South Africa (Natal)	3,2 Mdal	present	present	pro^-	None

All the strains carried the 2,6 Mdal plasmid. The African strains also carried the 3,2 Mdal plasmid whereas 50% of Asian isolates had both the 4,5 and 24,5 Mdal plasmids. The situation in Africa is unclear, but it seems that whilst the strain with 3,2 Mdal plasmid predominates, the auxotype is different to that ascribed to the original strain.

In African strains the 3,2 Mdal plasmid predominates. Some strains have the 4,5 Mdal plasmid as shown in Table 19. (Osoba, 1986; Osoba *et al.*, 1987).

TABLE 19. Distribution of PPNG from African sources

Geographical Source	No. of Strains	plasmids of (Mdal)	
		3,2	4,5
Central African Republic	6	6	-
Ghana	7	5	2
Ivory Coast	1	1	-
Kenya	5	2	3*
Nigeria	16	14	2
Zambia	2	2	-
Zaire	2	2	-
Total	39	**32**	**7**

All isolates contained a 2,6 Mdal plasmid
* Two isolates also contained a 24,5 Mdal plasmid

The presence of a 24,5 Mdal plasmid in 3,2 Mdal carrying strains was first detected in 1980 but, unlike the experience in the Netherlands where the acquisition of this plasmid resulted in a subsequent rapid increase of PPNG strains (Ansink-Schipper *et al.*, 1984; Roberts & Falkow, 1979), the overall incidence of this plasmid combination worldwide has remained low (Ison *et al.*, 1986). PPNG strains with the 3,2 Mdal plasmid have not spread as widely as those strains carrying the 4,5 Mdal plasmid (Table 5) though the conjugative plasmid is seen in association with both types. It is possible that the combined carriage of the 24,5 and 3,2 Mdal plasmids is unstable, or that the number of strains competent to accept the 3,2 Mdal plasmid may be limited (Jephcott, 1986).

In this study the epidemiology of *N. gonorrhoeae* isolates from King Edward VIII Hospital, which is the reference centre for Natal, Kwazulu and Transkei, was put into global perspective using antibiotic susceptibility patterns, plasmid profiles and auxotyping. Locally, the predominant auxotype is proto and 32% of PPNG tested are pro^-. All PPNG tested have the 3,2 Mdal plasmid, and some possess both the 3,2 and 24,5 Mdal plasmids. Local strains are similar to those of other African countries, however, the question of the origin of local PPNG isolates remains unanswered and may be resolved by the use of more discriminatory serological methods described by Knapp *et al.* (1984).

The results of this study may not be truly representative of the general population since the isolates studied were taken from selected symptomatic Black patients. Local strains are still highly susceptible to most antibiotics except penicillin. It is apparent that the local incidence of PPNG has trebled over the past 5 years. Of all strains studied 38% are PPNG, which implies that many *N. gonorrhoeae* infections seen at clinics may not be effectively treated. The incidence of CMRNG is also on the increase. If *N. gonorrhoeae* infections are to be reduced an effective alternate therapy must be introduced, together with a proper surveillance program.

SUMMARY

Several local results have pointed to an increasing prevalence of PPNG in Natal. Strains of *N. gonorrhoeae* isolated from symptomatic patients from King Edward XIII Hospital were studied for their nutritional requirements (auxotypes), antibiotic susceptibility patterns and plasmid profiles.

Using chromogenic cephalosporin, nitrocephin, 69 (38%) of 181 strains were PPNG and 112 (62%) were non PPNG.

Agar dilution was used to determine MIC's of penicillin G, ampicillin, amoxicillin, amoxicillin + clavulanic acid, cefuroxime, cefotaxime, ceftriaxone, spectinomycin, kanamycin, rosoxacin and chloramphenicol. To determine the effect of inoculum size on susceptibility tests penicillin G was tested at 10^4 and 10^7 CFU. The other antibiotics were tested at only 10^4 CFU. For PPNG the MIC range of penicillin G at 10^7 CFU was 2-64 $ug.ml^{-1}$, but at 10^4 CFU the range was 0,06-64 $ug.ml^{-1}$. The non PPNG isolates did not have a significantly different range at a higher inoculum size, the range being 0,003-2 $ug.ml^{-1}$ at both inoculum sizes.

PPNG and non PPNG isolates have different MIC's for penicillin G, ampicillin and amoxicillin. The combination of amoxicillin + clavulanic acid shows better activity than amoxicillin alone against PPNG. The cephalosporins,

spectinomycin, kanamycin, cotrimoxazole, tetracycline, erythromycin, rosoxacin and chloramphenicol have similar activity for both PPNG and non PPNG.

The highest percentage (71%) of PPNG isolates were from the rectum and the lowest (15%) were from the eyes of new born infants. Rectal and cervical non PPNG isolates were the least susceptible to penicillin G, and the eye isolates most susceptible.

Auxotyping was performed using the method of Hendry & Stewart (1979). Two main auxotypes emerged viz., proto and pro^-. No AHU strains were found. Of the PPNG isolates 97% were proto and only 3% were pro^-, whereas 65% of non PPNG were proto, and 35% pro^-. Pro^- strains were also found to be more susceptible to all antibiotics studied except for rosoxacin, spectinomycin and cotrimoxazole.

The alkali lysis method was used for plasmid extraction, and a 0,7% agarose gel was used for plasmid identification. All the PPNG strains had the 3,2 Mdal plasmid, and some had the 24,5 Mdal conjugative plasmid. All strains studied had the 2,6 Mdal cryptic plasmid.

Isolates from different infection sites of the same patient had the same plasmid profiles and the same auxotype patterns.

IEF confirmed that the *beta*-lactamase of *N. gonorrhoeae* belong to the TEM 1 group of enzymes.

Placing local isolates into global perspective, it appears that local strains are still relatively susceptible, however, it is evident that local strains are rapidly becoming resistant to current treatment regimen. The local auxotype pattern is similar to those in other African countries, with proto strains predominating.

The increasing prevalence of PPNG strains appear to be following the same trend as has been observed in some other African countries, where the levels have increased to 80%. In the 11 years since PPNG was first reported locally, their prevalence has risen to markedly. The penicillins may soon become obsolete as the first line of antimicrobial therapy for the treatment of *N. gonorrhoeae* infections locally. A change in therapy, together with a proper surveillance program should help in the prevention of a further increase of PPNG strains.

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