EVALUATION OF MOLECULAR TYPING METHODS TO DISCRIMINATE BETWEEN ISOLATES OF *NEISSERIA GONORRHOEAE*: RESTRICTION ENDONUCLEASE ANALYSIS, RIBOTYPING AND PULSED FIELD GEL ELECTROPHORESIS

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Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfilment of the requirements for the
M.Sc. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à
l'École des études supérieures et de la recherche
Université d'Ottawa
en vue de l'obtention de la maîtrise es sciences à

l'Institut de biologie d'Ottawa-Carleton

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ISBN 0-612-15642-7

Canada

ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. Jo-Anne R. Dillon, my supervisor, for her unwavering encouragement and support of my graduate work, for sharing her enthusiasm for research and offering wise counsel at many critical junctures throughout the study period.

I am grateful to Drs. J. Farber, M. Paulin-levasseur and C. Wyndham, the members of my thesis advisory committee, for their helpful suggestions and comments throughout this work.

I wish to acknowledge all my colleagues at National Laboratory for Sexually Transmitted Diseases, Health and Welfare Canada for their help, suggestion and effort expended on my behalf. Special thanks to Dr. L.-K. Ng for providing valuable advice throughout the course of this study, Dr. K.-H. Yeung for helping with the statistical analysis and S. Bernatchez for translating the abstract into french.

Finally, I would like to thank my parents and my brother for their love, support and constant encouragement, but most of all to my husband for his help and for being there with me.

ABSTRACT

Classical typing methods such as auxotyping (A), serotyping (S) and plasmid content analysis (P) vary in their ability to discriminate between isolates of *Neisseria gonorrhoeae*. In cases where isolates have the same A/S/(P) class, discrimination between isolates based on antibiograms is usually inadequate. Thus genomic fingerprinting methods may be an important adjunct to the traditional classification techniques for discriminating between such isolates. In the present study, three molecular methods, restriction enzyme (RE) analysis of chromosomal DNA, restriction fragment length polymorphism (RFLP) analysis of ribosomal RNA (rRNA) genes (ribotyping) and pulsed field gel electrophoresis (PFGE) were evaluated for their ability to discriminate within and between five groups of gonococcal isolates.

The groups comprised 100 isolates of *N. gonorrhoeae*, which were typed into 13 A/S classes and which were selected either from outbreaks or from population studies. These groups of isolates were selected for study because, based on classical typing criteria, the isolates appeared clonal [i.e. proline-, citrulline-, uracil-, (with) hypoxanthine-requiring PCU(H) isolates and arginine-requiring (Arg) isolates]; to test a hypothesis from the literature which indicated that certain classes of isolates should be grouped (e.g. serovar IB-5 and IB-7 isolates); and because they comprised putative outbreaks of antibiotic resistant isolates of a particular type (e.g. isolates with A/S class NR/IA-5 consisted of a cluster of predominately PPNG isolates and isolates with A/S class NR/IB-3 included a cluster with multiple chromosomally mediated resistances which appeared phenotypically similar.

To examine whether antibiograms might be used to distinguish isolates having the same A/S class, the minimum inhibitory concentrations (MICs) of 633 PCU(H) isolates of serovar IB-1 or IB-2 (these serovars differed by their reaction to a single monoclonal antibody) and 552 arginine-requiring isolates of serovar IA-2 were retrospectively analyzed. The MICs of both of these groups of isolates were homogeneous. Serovar IB-5 and IB-7 isolates were not homogeneous with respect to their auxotypes; 62.6% (371/593) were non-requiring and 29.5% (175/593) were proline-requiring. The antimicrobial susceptibilities of the proline-requiring isolates having serovars IB-5 or IB-7 were more resistant than non-requiring isolates of the same serovars. Isolates from individual outbreaks (NR/IA-5 and NR/IB-3) had similar antimicrobial susceptibilities.

The RE patterns of 100 gonococcal isolates were compared following digestion of genomic DNA with *Sma*I and analysis of fragments within a 9.7 to 27.0 kb size range. These isolates were subtyped into 20 RE patterns with 9 fragments in common (27.0, 25.5, 19.0, 17.6, 13.6, 12.4, 11.5, 10.6, and 9.7 kb). The 30 PCU(H) isolates with serovar IB-1 (15) or IB-2 (15) were classified into 4 RE patterns - A, B, C and D; most (90%, 27/30) belonged to RE pattern A. Therefore, based on the RE analysis, PCU(H) isolates appeared to be clonal. The 27 arginine-requiring/IA-2 isolates fell into a single RE pattern - E, also suggesting that arginine-requiring isolates are homogeneous. Ten isolates of serovar IB-5, with auxotypes NR (4) or P (6), and 11 isolates of serovar IB-7, with auxotypes NR (6) or P (5) were sorted into 11 distinct RE patterns - F, G, H, I, J, K, L, M, N, O and P. These patterns did not correlate with the specific A/S classes of the isolates. Thus, isolates of serovar IB-5 and IB-7 group were heterogeneous. The 10 "outbreak" isolates of A/S class NR/IA-5 were classified into 2 RE patterns, Q and R with 9 of the 10 isolates comprising RE pattern Q. Therefore, RE analyses of these NR/IA-5 isolates confirmed their retrospective identification as being similar. The 12 isolates of A/S class NR/IB-3 identified retrospectively as a chromosomally mediated resistance *N. gonorrhoeae* outbreak were subtyped into 2 RE patterns (66.7% were S and 33.3% were T) which differed from each other by the presence two of additional fragments in RE pattern T.

The hundred *N. gonorrhoeae* isolates were also subtyped into eleven ribotypes, having 6 to 7 fragments ranging in size from 2.8 to 29.0 kb and having fragments of 23.1, 12.0, 8.2, 4.8 and 2.8 kb in common. The 30 PCU(H) isolates were subdivided into 5 ribotypes (I, VI, VIII, X and XI); most (24/30, 80%) belonged to ribotype I. Therefore, PCU(H) isolates once more appeared to be clonal. Twenty-seven arginine-requiring isolates of serovar IA-2 were classified into 5 ribotypes -II, III, IV, VI and IX - which were not correlated with the specific arginine-requiring auxotypes of these isolates; 63% (17/27) of arginine-requiring isolates were ribotype II. All ribotypes of arginine-requiring isolates varied by the presence of only a single fragment which was of different size in each pattern. Four isolates of A/S class NR/IB-5 and 6 isolates of A/S class NR/IB-7 were classified into 5 ribotypes (II, III, IV, VI and VII), while 6 isolates of A/S class P/IB-5 and 5 isolates of A/S class P/IB-7 were further classified into 4 ribotypes (II, III, VI and VII) indicating that isolates in the IB-5 and IB-7 groups were heterogeneous. The 10 outbreak isolates comprising A/S class NR/IA-5 all fell into ribotype number V, indicating that these isolates were related. The 12 isolates of A/S class NR/IB-3 were represented by 2 ribotypes (III and IV), 50% (6/12) of the isolates were ribotype III and 50% (6/12) isolates were ribotype IV; ribotypes III and IV differed from each other by 1 fragment.

Pulsed field gel electrophoresis produced the highest level of discrimination even though a subset of 26 of the 100 isolates was tested. Twenty-five PFGE patterns were produced after *Spe*I digestion of the chromosomal DNA of 26 gonococcal isolates. These 26 isolates had three common bands (110.0, 77.0 and 67.0 kb). The 9 arginine-requiring isolates tested, which included 4 isolates of ribotype II, 2 isolates of ribotype IV, and 1 isolates each of ribotypes III, VI and X isolates, were subtyped into 9 different PFGE profiles. The 5 outbreak isolates of A/S class NR/IA-5 had one band in common (262.0 kb) but all had different PFGE types. Two isolates of A/S class NR/IB-5 and four isolates of A/S class NR/IB-7 showed six distinct PFGE patterns. This confirmed once again that isolates in this group are heterogeneous. Six PCU(H)/IB-1 isolates generated five PFGE patterns. Only two isolates had the same PFGE pattern (P3). These two isolates had the same ribotype as well, the same RE pattern, and were A/S class PCU(H)/IB-1.

By comparing the RE analysis and ribotyping for 100 gonococcal isolates as well as PFGE analysis for 26 of the 100 gonococcal isolates, there were twenty RE patterns, eleven ribotypes and twenty-five PFGE patterns produced. Overall, PFGE produced greater discrimination between isolates within the same A/S classes than ribotyping or RE analysis. In some cases, as with arginine-requiring strains or strains from an outbreak, several molecular methods should be tested in order to produce maximum discrimination between strains.

Arginine auxotyphy is commonly encountered in *N. gonorrhoeae*, comprising 40-60% of clinical isolates, including isolates with multiple nutritional requirement. Arginine (Arg) and uracil (U)-requiring strains are common in Canada. These AU isolates may have a deficiency affecting both the arginine and uracil biosynthesis pathways. Isolates which require citrulline (C) alone for growth are very rare. Only 2 of 1540 of the arginine-requiring isolates in the culture collection of the NLSTD were citrulline-requiring. Strains requiring citrulline may be mutant for one of two enzymes, ornithine transcarbamoylase (OTCase), which catalyze L-citrulline formation from L-ornithine and carbamoylohosphate, or carbamoylphosphate synthetase (CPSase), which implicated in arginine and uracil biosynthesis pathways. Strains which are CPSase deficient may also be mutant in pyrimidine (U) biosynthesis. Twenty-two CU requiring isolates and two C requiring isolates were tested for OTCase and CPSase activities. The 22 CU auxotype possessed OTCase activity, but did not have CPSase activity. The 2 isolates requiring citrulline alone had CPSase activity, but did not have OTCase activity.

RÉSUMÉ

Les méthodes classiques de typage telles que l'auxotypage (A), le sérotypage (S) et l'analyse du profil plasmidique (P) varient dans leur capacité à différencier des isolats de *N. gonorrhoeae*. Dans les cas où les isolats appartiennent à la même classe A/S/(P), la distinction des isolats basée sur un antibiogramme est habituellement inadéquate. Des méthodes d'empreinte génomique pourraient alors constituer une addition importante aux méthodes classiques de discrimination pour différencier de tels isolats. Au cours de la présente étude, la capacité de trois méthodes moléculaires sont évaluées: l'analyse du patron de restriction de l'ADN chromosomique digéré avec des enzymes de restriction (RE), l'analyse des polymorphismes de fragments de restriction (RFLP) des gènes d'ARN ribosomique (ARN$_R$; ribotypage) de même que l'électrophorèse en champ pulsé (PFGE) pour distinguer cinq groupes d'isolats de *N. gonorrhoeae*, et pour distinguer chacun des isolats appartenant à un même groupe.

Les groupes comprenaient 100 isolats de *N. gonorrhoeae*, qui furent séparés en 13 classes A/S et qui furent choisis à partir d'épidémies ou encore d'études de population. Ces groupes d'isolats furent choisis pour être étudiés parce que selon les données de typage classique, les isolats semblaient tous avoir une origine clonale (i.e., proline$^-$, citrulline$^-$, uracil$^-$, (avec) des isolats PCU(H) ayant un besoin en hypoxanthine et des isolats ayant un besoin en arginine (ARG)) et pour tester une hypothèse tirée de la littérature qui indiquait que certaines classes d'isolats devraient être regroupées (par ex. les isolats de sérovar IB-5 et IB-7) et également parce qu'ils comprenaient des isolats résistant à un type particulier d'antibiotiques (par ex. les isolats

de classe A/S NR/IA-5 consistaient en un regroupement d'isolats résistant à la pénicilline (PPNG) et les isolats de classe A/S IB-3 comprenaient un regroupement d'isolats ayant de multiples résistances d'origine chromosomique) provenant de deux épidémies putatives.

Pour vérifier si des antibiogrammes pourraient être utilisés pour distinguer des isolats appartenant à la même classe A/S, la concentration minimale d'inhibition (CMI) de 633 isolats PCU(H) de sérovar IB-1 ou IB-2 (ces deux sérovars diffèrent par leur réaction à un seul anticorps monoclonal) et 552 isolats de sérovar IA-2 requérant de l'arginine furent rétrospectivement analysés. Les CMIs de ces groupes étaient homogènes. Les isolats de sérovar IB-5 et IB-7 n'avaient pas le même auxotype: 62.6% (371/593) n'avaient aucun besoin nutritionnel et 29.5% (175/593) avaient un besoin nutritionnel en proline. La sensibilité aux agents antimicrobiens des isolats de sérovar IB-5 ou IB-7 requérant de la proline était moindre que celle des isolats de même sérovar n'ayant aucun besoin nutritionnel. Les isolats provenant de chacune des deux épidémies (NR/IA-5 et NR/IB-3) avaient des sensibilités semblables aux agents antimicrobiens.

Les patrons de digestion de 100 isolats de *N. gonorrhoeae* furent comparés suivant la digestion de leur ADN chromosomique avec *SmaI* puis de l'analyse des fragments de taille entre 9.7 et 27.0 kb. Ces isolats furent subdivisés selon 20 patrons de digestion. Il y avait 9 fragments (ayant des tailles respectives de 27.0,25.5, 19.0, 17.6, 13.6, 12.4, 11.5, 10.6 et 9.7kb) communs à tous les patrons de digestion. Les 30 isolats PCU(H) de sérovar IB-1 (15) ou IB-2 (15) furent classés selon 4 patrons de digestion: A, B, C et D, la majorité de ces isolats avaient le patron de restriction A. L'analyse des patrons de restriction indiquait que les isolats PCU(H) semblaient être d'origine clonale. Les 27 isolats IA-2 ayant un besoin en arginine avaient tous le même patron de restriction -E-, suggérant également que les isolats ayant un besoin en arginine étaient homogènes. Dix isolats de sérovar IB-5 d'auxotype NR (4) ou P(6) et 11 isolats de sérotype IB-7 d'auxotype NR(6) ou P(5) furent divisés selon 11 patrons de digestion distincts (F, G, H, I, J, K, L, M, N, O et P). Il n'y avait aucune corrélation entre les patrons de restriction et les classes A/S de ces isolats. Ainsi les isolats de sérovar IB-5 et IB-7 étaient hétérogènes. Les 10 isolats de classe A/S NR/IA-5 provenant de la même épidémie furent classés selon deux patrons de digestion (Q et R), neuf de ces isolats sur les dix ayant le même patron de restriction (Q). L'analyse du patron de restriction de ces isolats NR/IA-5 confirmait donc l'identification rétrospective qui les avait fait apparaître semblables. Les 12 isolats de classe A/S NR/IB-3 identifiés rétrospectivement comme provenant d'une même épidémie de *N. gonorrhoeae* ayant une résistance d'origine chromosomique furent divisés selon deux patrons de restriction (66.7% appartenaient au groupe S et 33% étaient du groupe T) ne différant l'un de l'autre que par la présence de deux fragments additionnels dans le patron de restriction T.

Les 100 isolats de *N. gonorrhoeae* furent également subdivisés en 11 ribotypes, ayant 6 ou 7 fragments de taille entre 2.8 et 29.0 kb. Les 11 ribotypes avaient des fragments de 23.1, 12.0, 8.2, 4.8, 2.8 kb en commun. Les 30 isolats PCU(H) furent divisés en 5 ribotypes (I, VI, VIII, X et XI); la majorité (80%, 24/30) appartenant au ribotype I. Les isolats PCU(H) semblaient de nouveau être d'origine clonale. Les 27 isolats de sérovar IA-2 requérant de l'arginine furent classés en 5 ribotypes (II, III, IV, VI et IX) qui ne pouvaient être reliés avec leur auxotype (dans ce cas-ci un besoin en arginine); 63% (17/27) des isolats requérant de l'arginine appartenaient au type II. Le ribotype des isolats ayant un besoin en arginine variait par la présence d'un seul fragment de taille différente dans chacun des patrons. Quatre isolats de classe A/S NR/IB-5 et six isolats de classe A/S NR/IB-7 furent classés selon 5 ribotypes (II, III, IV, VI et VII), alors que 6 isolats de classe A/S P/IB-5 et 5 isolats de classe A/S P/IB-7 furent divisés en 4 ribotypes (II, III, VI et VII) indiquant que les isolats appartenant aux groupes IB-5 et IB-7 étaient hétérogènes. Les 10 isolats de classe A/S NR/IA-5 provenant de la même épidémie étaient tous de ribotype V, indiquant la présence d'un lien entre ces isolats. Les 12 isolats de classe A/S NR/IB-3 furent divisés en 2 ribotypes (III et IV), 50% (6/12) étant de type III et 50% (6/12) étant de type IV. Les ribotypes III et IV ne différaient l'un de l'autre que par la présence d'un seul fragment.

L'électrophorèse en champ pulsé a permis le plus haut niveau de discrimination même si seulement 26 des 100 isolats ont été examinés avec cette technique. Vingt-cinq patrons de PFGE furent produits suivant la digestion de l'ADN chromosomique des isolats avec l'enzyme de restriction *SpeI*. Les 26 isolats avaient 3 fragments en commun dans leur profil de PFGE (110.0, 77.0 et 67.0 kb). Les 9 isolats requérant de l'arginine testés pour cette méthode (4 isolats de ribotype II, 2 isolats de ribotype IV et un isolat de chacun des ribotypes III, VI et X) furent divisés en 9 profils différents de PFGE. Les 5 isolats de classe A/S NR/IA-5 provenant de la même épidémie avaient un fragment de 262.0 kb en commun dans leur profil respectif mais avaient chacun un patron particulier. Deux isolats de classe A/S NR/IB-5 et 4 isolats de classe A/S NR/IB-7 avaient chacun un profil de PFGE particulier. Ceci confirmait de nouveau que les isolats de ce groupe étaient hétérogènes. Six isolats PCU(H)/IB-1 ont produit 5 patrons de PFGE. Seulement 2 isolats produirent le même patron de PFGE (P3). Ces deux isolats avaient aussi le même patron de restriction et étaient de classe A/S PCU(H)/IB-1.

En comparant l'analyse du patron de restriction (RE) et le ribotype des 100 isolats de même que l'analyse des profils de PFGE de 26 des 100 isolats, il y avait 20 patrons de restriction, 11 ribotypes et 25 profils de PFGE (25 profils pour 26 isolats testés). Dans l'ensemble, l'électrophorèse en champ pulsé a permis une plus grande discrimination entre isolats de même classe A/S que celle produite par ribotypage ou que celle produite par l'analyse du patron de restriction. Dans certains cas, par exemple des souches requérant de l'arginine ou des souches provenant d'une épidémie, plusieurs méthodes moléculaires devraient être testées afin de produire le maximum de distinction possible entre les souches.

L'auxotrophie à l'arginine est souvent présente chez *N. gonorrhoeae*, comprenant 40-60% des isolats cliniques en incluant des isolats ayant des besoins nutritionnels multiples. Les souches requérant de l'arginine (Arg) et de l'uracil (U) sont communes au Canada. Les isolats au pourraient avoir une déficience affectant les sentiers biosynthétiques de l'arginine et de l'uracil. Les isolats ne requérant que la citrulline (C) pour croître sont très rares. Seulement 2 parmi 1540 isolats requérant de l'arginine provenant de la banque de souches du NLSTD (LNMTS) avaient un besoin nutritionnel en citrulline. Les souches requérant de la citrulline peuvent être mutées dans l'un ou l'autre de deux enzymes: ornithine transcarbamoylase (OTCase), qui catalyse la formation de L-citrulline à partird ornithine et de carbamoylphosphate, ou la carbamoylphosphate synthétase (CPSase), qui est impliquée dans les sentiers biosynthétiques, de l'arginine et de l'uracil. Les souches ayant une activité CPSase déficiente pourraient aussi être mutées dans la biosynthèse des pyrimidines (U). Vingt-deux isolats requérant C et U et deux isolats requérant C furent testés four la présence d'activités CPSase et OTCase. Les vingt-deux isolats CU possédaient une activité OTCase mais n'avaient pas d'activité CPSase. Les deux isolats ne requérant que de la citrulline avaient une activité CPSase mais n'avaient pas d'activité

OTCase.

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LIST OF ABBREVIATIONS

AMPPD	3-(2'-Spiroadamantane)-4-methoxy-4-)3'phosphoryloxy)-phenyl-1,2-dioxetane
Arg	arginine, in multiple auxotypes arg = A
A	auxotype
C	citrulline
CHEF	contour-clamped homogeneous electric field electrophoresis
CMRNG	chromosomally mediated resistance *N. gonorrhoeae*
CN	cefoxitin
CP	carbamoylphosphate
cpm	counts per minute
CPSase	carbamoylphosphate synthetase
CX	ceftriaxone
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytosine-5'-triphosphate
DGI	disseminated gonococcal infection
dGTP	dioxyguanosine-5'-triphosphate
dig-dUTP	digoxygenine-deoxyuridine-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymine-5'-triphosphate
EDTA	ethylenediaminetetraacetate
ERY	erythromycin
ESP	EDTA, lauroyl sarcosine, proteinase K buffer
ET	electrophoretic type
FIGE	field inversion gel electrophoresis
GCMB	GC medium base
H	hypoxanthine
kb	kilobase pair
LMP	low melting point

λ	lambda bacteriophage
MDa	megaDalton
MIC	minimum inhibitory concentration
MIC_{50}	MIC at which 50% of isolates are inhibited
MIC_{90}	MIC at which 90% of isolates are inhibited
NR	non-requiring (i.e. prototrophic)
O	ornithine
OD_{260}	optical density at 260 nm
OTCase	ornithine transcarbamoylase
P	proline
PCR	polymerase chain reaction
PEN	penicillin
PFGE	pulsed field gel electrophoresis
PID	pelvic inflammatory disease
PPNG	penicillinase-producing *N. gonorrhoeae*
r	resistance
RE	restriction endonuclease
REA	restriction endonuclease analysis
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
rpm	rotation per minute
S	serovar
SDS	sodium dodecyl sulphate
SPEC	spectinomycin
SSC	sodium chloride, sodium citrate buffer
SSPE	sodium chloride, sodium dihydrogen orthophosphate, sodium EDTA buffer
TA	Tris-base, sodium acetate, EDTA buffer
TBE	Tris, boric acid, EDTA buffer
TE	Tris-HCl, EDTA buffer
TES	TE, sucrose buffer
TET	tetracycline
tRNA	transfer RNA
TRNG	tetracycline resistant *N. gonorrhoeae*
TSA	trypticase soy agar
U	uracil
U.V.	ultra-violet

1. INTRODUCTION

The goals of classification and identification are, basically, to provide a method for distinguishing organisms from each other, to group similar organisms on the basis of meaningful criteria, to arrange groups of organisms into useful, and hopefully, biologically relevant categories, and to provide a convenient record of information so that an organism can be defined without always having to list its characteristics (Prauser, 1978). Traditionally, genera have been established on the fundamental basis of phenotypic similarity. Organisms that resemble each other in shape, size, staining properties, ability to produce acid from sugars, ability to grow on a variety of compounds as sole sources of carbon, and ability to grow in the presence of inhibitory compounds, that are convenient or important to consider as a group, and that must be separated from other distinct groups at the laboratory bench have been classified into different genera (Staley and Krieg, 1984).

Typing bacteria is important in epidemiological studies to determine the variety of organism types present, their distribution and prevalence. Information gained by typing isolates is compared with other parameters to determine the association of specific types with clinical, therapeutic, and diagnostic problems, producing results which often provide solutions to the problems. These general standards apply to various infectious diseases (Knapp, 1985).

Several different typing methods have been used to type or characterize strains in bacterial systematics and epidemiology and to determine whether a set of bacterial isolates represents a single strain or more than one strain. These include antimicrobial susceptibility profiles (antibiogram), biochemical profiles (biotyping; Barr and Hogg, 1979; Rennie and Duncan, 1974), serological typing (Gold and Wyle, 1970; Benham, 1931), bacteriocin typing (Edmondson and Cooke, 1979; Hall, 1971), bacteriophage susceptibility patterns (phage typing; Anderson, 1959), and nutritional requirements (auxotyping; Catlin, 1973). More recently, molecular typing methods have been used to type bacterial pathogens. Based on the type of macromolecules used for subtyping, molecular typing methods may be divided into three groups: lipopolysaccharide (LPS) and fatty acid-based methods, protein-based methods, and nucleic acid-based methods. LPS analysis has been used for subtyping *Haemophilus influenzae* strains (Murphy *et al.*, 1987) and *Eikenella corrodens* strains (Casey Chen *et al.*, 1990). Cellular fatty acid analysis also is a method for the identification of bacteria at the species level (Welch, 1991; Coloe *et al.*, 1986). Whole-cell and outer membrane protein profiles determined by SDS-PAGE have also been used to subtype bacteria (Granoff *et al.*, 1982; Barenkamo *et al.*, 1981). However, these methods have not been applied to the routine characterization of *N. gonorrhoeae* isolates. Multilocus enzyme electrophoresis (Caugant *et al.*, 1986; Selander *et al.*, 1986a; Fox and McClain, 1975) is a special type of protein-based subtyping method. Several nucleic acid-based subtyping methods, such as plasmid profiling (Shlaea and Currie-MnCumber, 1986), restriction endonuclease analysis of chromosomal DNA (REA; Owen, 1989), restriction fragment length polymorphism of rRNA gene (ribotyping; Stull *et al.*, 1988; Grimont and Grimont, 1986), polymerase chain reaction (PCR)-based subtyping (Vines, *et al.*, 1992; Williams *et al.*, 1990; Welsh and McClelland, 1990; Gama *et al.*, 1989; Gama *et al.*, 1988; Mullis and Faloona 1987; Mullis *et al.*, 1986; Saiki *et al.*, 1985) and pulsed field gel electrophoresis (PFGE; Bourgeois *et al.*, 1989; Grothues *et al.*, 1988; Grothues and Tummler, 1987; Schwartz and

Cantor, 1984) have been applied to type a variety of bacterial organisms. All of these typing systems have been helpful in understanding the epidemiology of bacterial infections and in establishing the natural history of bacterial diseases.

1.1. *Neisseria gonorrhoeae*

Neisseria gonorrhoeae, the causative agent of gonorrhoea, is one of the most common sexually transmitted diseases in Canada (Holmes *et al.*, 1984). For example, in 1981, the number of reported gonorrhoea cases in Canada was 56,336; at that time it was the most reported notifiable disease (Health and Welfare Canada, 1988). Although the number of cases has declined to 9,307 in 1992, it remains the fourth most prevalent notifiable disease (Health and Welfare Canada, 1993, in press) and is a significant cause of morbidity in Canada. All piliated strains of *N. gonorrhoeae* are pathogenic, causing inflammatory infections of mucous membranes (Bergstrom *et al.*, 1986; Swanson *et al.*, 1971). Under certain conditions, some strains of *N. gonorrhoeae* may cause complicated infections such as disseminated gonococcal infection (DGI) and pelvic inflammatory disease (PID) (Hook *et al*, 1985).

Since the 1940s, many attempts to classify gonococci into subgroups have been made (Knapp, 1985). Ideally, a system for classification of *N. gonorrhoeae* should be reproducible, rapid, economical, easy to perform and give a high resolution (Parker, 1972). Typing methods can support epidemiologic investigations by distinguishing between related and unrelated cases and differentiate between systematically related isolates from different sources (Knapp, 1985). In addition typing has been used to monitor the spread of gonococcal strains across the globe and to identify outbreaks retrospectively and prospectively.

1.2. Antibiogram typing

A wide range of susceptibilities to the antibiotics used for treating gonorrhoea therapy or for the selective isolation of gonococci was found among *N. gonorrhoeae* isolates (Dillon *et al.*, 1990; Knapp *et al.*, 1985b). This produced one classification scheme known as antimicrobial susceptibility pattern (antibiograms), which characterized isolates by their level of susceptibility to various antibiotics. Before the development of other typing techniques, antibiograms were commonly used to characterize gonococcal isolates and to some extent this scheme is still used today (Dillon *et al.*, 1993). Antimicrobial susceptibility was correlated with strains causing DGI (Wiesner *et al*, 1973). Gonococcal isolates from patients with DGI were significantly more susceptible to penicillin G and tetracycline than isolates from patients with uncomplicated infection (Wiesner *et al.*, 1973). Before the isolation of strains with β-lactamase-producing plasmids (penicillinase-producing *N. gonorrhoeae*, PPNG) in 1976, chromosomal mutation was the only mechanism of resistance to antimicrobial agents in the gonococcus (Sparling *et al.*, 1978; Sparling, 1977). Chromosomally-mediated antibiotic resistance is determined by a number of distinct genes (Dillon and Yeung, 1989; Sarubbi et al, 1974). In spite of the fact that most genetic traits are stable and the variation is sufficient, it is often not possible to determine what genes are affected; it is difficult to characterize strains on a genetic level using antibiotic markers, since these genes may act alone or additively against one antibiotic or may confer the loss of sensitivity or resistance to several antibiotics (Sandström and Rudén, 1990). Although antibiograms are of clinical importance, the inability to determine whether resistant variants emerged from a limited number of strains or represented a large number of phenotypically different strains makes them less valuable as a typing method at such a preliminary level (Sandström and Rudén, 1990). Antibiograms have not played a large role in typing gonococci because they may not sufficiently discriminate isolates from an outbreak or isolates having the same auxotype/serovar (A/S) class (Dillon *et al.*, 1993).

1.3. Auxotyping

N. gonorrhoeae isolates require a variety of compounds for growth in chemically defined media (Catlin, 1973). Some components, such as cysteine or cystine, are required by all members of the species. Various other compounds are required by some gonococcal isolates, but not by all. This diversity of nutritional requirements differentiates between *N. gonorrhoeae* isolates and is the basis of a typing system called auxotyping (Carifo and Catlin, 1973). Since auxotyping was introduced for *N. gonorrhoeae*, clinical gonococcal isolates have been differentiated by their growth requirements on chemically defined media lacking different amino acids or pyrimidines; auxotyping has become an important method for differentiating between strains of *N. gonorrhoeae*.

Several auxotyping schemes were developed initially (Copley and Egglestone, 1983; La Scolea and Young, 1974; Carifo and Catlin, 1973). Most laboratories use the method as described by Catlin (1973) or a modified method of Catlin (e.g. Hendry and Stewart, 1979).

A large number of gonococcal isolates have been discriminated by auxotyping and their geographical distribution has been investigated extensively (Dillon and Pauzé, 1984; Hendry and Dillon, 1984; Hendry and Stewart, 1979; Knapp *et al.*, 1978; Carifo and Catlin, 1973). Gonococcal isolates that have no nutritional requirements [non-requiring (NR) or prototrophic] and isolates that have requirements for proline (P) are prevalent worldwide (Picard and Dillon, 1989; Sarafian and Knapp, 1989). Gonococcal isolates which require arginine are also widely distributed geographically but are normally less common; arginine-requiring (Arg) strains were quite rare in the Far East, South America, and Chile (Sarafian and Knapp, 1989; Knapp *et al.*, 1985b; Knapp and Holmes, 1975). Gonococcal isolates with multiple nutritional requirements for growth have been isolated in Canada and a variety of other industrial countries (Dillon *et al.*, 1990; Sarafian and Knapp, 1989; Hendry and Stewart, 1979), isolates from non-industrial countries often have single nutritional requirements (Knapp *et al.*, 1985b).

Arginine-, hypoxanthine- and uracil-requiring (AHU) strains were undetected before the widespread use of penicillin (Catlin and Reyn, 1982) and since have become the predominant auxotype in some areas, such as southern Sweden, Denmark and Canada (Picard and Dillon, 1989; Knapp *et al.*, 1987a; Danielsson *et al.*, 1983). AHU strains have increased sensitivity to penicillin (Knapp *et al.*, 1978; Morello *et al.*, 1976; Schoolnik *et al.*, 1976) and are resistant to the bactericidal action of normal human serum (Knapp *et al.*, 1978; Schoolnik *et al.*, 1976). Most of AHU strains belong to serovar IA-1 or IA-2 (Knapp *et al.*, 1984). AHU strains produced type I IgA1 protease and *Dam* I methylase (Mulks and Knapp, 1987; Kolodkin *et al.*, 1982). Because strains with the protein IA serotype, serum resistance and penicillin sensitivity showed a higher correlation with DGI, AHU was considered an auxotroph that was found in DGI, although these traits were not linked genetically (Brunham *et al.*, 1985; Cannon *et al.*, 1983; Eisenstein *et al.*, 1977).

Proline-, citrulline- and uracil- (PCU) strains, with or without a hypoxanthine (H) requirement, were not detected either in the preantimicrobial era (Catlin and Reyn, 1982) or in an analysis of 493 isolates from the United States collected during 1972-1974 (Knapp *et al.*, 1978). PCU strains accounted for less than 1% of total clinical gonococcal isolates in 1973, 23% in 1978 (Dillon and Pauzé, 1981), and 14% in 1989 in Canada; they are the most common auxotype in some areas of Canada (Dillon *et al.*, 1988a). PCU strains were also identified in Canada among isolates during the period 1975-1978 in several independent studies (Brunham *et al.*, 1985; Hendry and Stewart, 1979). They have been mainly reported from Canada and Jamaica (Dillon *et al.*, 1988b; Dillon *et al.*, 1987a), but are now increasingly reported from areas of the United States and Europe (Noble and Parekh, 1983; Danielsson *et al.*, 1983) with a frequency of 16%-17% of all gonococcal strains from the regions sampled. Their prevalence is increasing in Europe and Japan (Sarafian and Knapp, 1989; Dillon *et al.*, 1988b; Dillon *et al.*, 1987a; Knapp *et al.*, 1987a; Kohl *et al.*, 1986). PCU isolates and the closely related PCUH auxotypes are predominately a single clonotype as determined by monoclonal antibody analysis (Brunham *et al.*, 1985) and by plasmid content, riboprobing and isoenzyme typing (Ng and Dillon, 1993).

Auxotyping has several limitations. For example, the proline and arginine requirements of gonococcal isolates may be due to one of several mutations that affect the synthesis of these amino acids (Picard and Dillon, 1989; Copley, 1987; Catlin and Nash, 1978). Thus, strains belonging to either the P or Arg auxotypes may be different in their genotypes. For example, the arginine requiring isolates have been further subtyped by testing strains for their ability to grow on ornithine or citrulline which are intermediates in the arginine biosynthetic pathway. This indicates that more than one mutation is responsible for arginine requirement. Auxotyping also is a complicated, laborious and time consuming method which is practical only for reference laboratories (Falk *et al.*, 1985a).

1.4. Serotyping

Serological classification systems have been used effectively to differentiate further between *N. gonorrhoeae* isolates. This typing system is based on the antigenic heterogeneity of the protein I (PI) molecule in the gonococcal outer membrane (Sandstrom *et al.*, 1982). PI is present in all strains, is stable on subculturing and is not altered in different colony phenotypes (Kohl *et al.*, 1989). Although PI does not vary within a strain, differences between strains are responsible for antigenic diversity. Before the present-day typing schemes were finalized, several other methods were used. Wang *et al.* (1977) used a microimmunofluorescence test with polyvalent antibodies against formalinized whole gonococcal cells to divide gonococci into one of three groups (A, B and C). Sandström and Danielsson (1980) used a coagglutination test with adsorbed polyvalent antibodies to classify strains of *N. gonorrhoeae* into three serologically distinct groups (termed WI, WII and WIII). Serogroups WI, WII and WIII, respectively, corresponded to serogroups A, B and C described by Wang *et al.* (1977). Buchanan and Hildebrandt (1981) used an enzyme-link immunosorbent assay with partially purified gonococcal protein I (PI) to divide gonococcal strains into nine principal outer membrane protein serotypes that also correlated with WI, WII, and WIII. Usually, serotypes 1 to 3 corresponded to serogroup WI, serotypes 4 to 8 corresponded to serogroup WII, and serotype 9 corresponded to serogroup WIII (Sandström and Bygdemen, 1986). Peptide mapping techniques used to define the immunological and biochemical basis of this system demonstrated that gonococcal strains produced two types of PI molecules. Serogroups WI and WII/WIII possessed different protein I molecules, designated proteins IA (PrIA) and IB (PrIB), respectively (Sandström *et al.*, 1982).

All clinical isolates of gonococci always possess either protein IA or IB, but never both (Knapp *et al.*, 1984). Although PIA/B hybrids can be constructed in the laboratory (Carbonetti *et al.*, 1988), they virtually never occur in nature (Knapp *et al.*, 1984). Tam *et al* (1982) introduced monoclonal antibodies directed against PrIA and PrIB for use in the coagglutination test. These and additional monoclonal antibodies specific against PrIA are capable of differentiating between gonococci with a large number of reaction patterns, called serovars (Knapp *et al.*, 1984). A serotyping system with a series of monoclonal antibodies to PI, designated the GS series, was devised (Danielsson *et al.*, 1985; Bygdeman *et al.*, 1983). The PI protein expressed by an isolate is designated A or B, and individual monoclonal antibody reagents in the PIA- and PIB-specific reagent panels were assigned lowercase letters (Knapp *et al.*, 1985a). In the GS series, six monoclonal antibodies reagents are directed against different epitopes of protein IA and six directed against different epitopes of protein IB, to further divide the WI serogroup into 24 distinct serovariants (serovar IA-1 to IA-24), and the WII and WIII serogroups into at least 32 distinct serovars (IB-1 to IB-32). The number of new serovars continues to expand. Another set of PI-specific monoclonal antibodies designated Ph antibodies was developed by Swedish investigators (Sandström *et al.*, 1985). Similar to the taxonomy for the GS serotyping system, the taxonomy for the Ph serotyping system also used the prefixes A and B; a different set of lowercase letters was used to designate individual reagents (Sandström *et al.*, 1985). Protein I serology is the basis of several identification kits for *N. gonorrhoeae*, such as GonoGen test, and Phadebact Monoclonal GC OMNI Test (Dillon *et al.*, 1988a; Lawton and Battaglioli, 1983; Lewis and Martin, 1980).

Although the serologic typing of *N. gonorrhoeae* by using coagglutination assays are simple in principle, they display some methodologic problems, including inconsistency in interpreting weakly positive reactions caused by specific reagents thereby resulting in serovar discrepancies between laboratories or individuals (Gill, 1991). An enzyme immunoassay (EIA), using whole boiled cells as an antigen, was generated to reduce problems of interpreting weakly positive reactions, to minimize interpretation problems arising between persons, to automate the procedure, and to increase the numbers of samples processed (Carballo and Dillon, 1992).

The use of serologic typing systems has permitted extensive characterization of the organism and provided important knowledge about gonococcal epidemiology. For example, one study in Scandanavia found that serovar IB strains tended to be prevalent in large towns whereas serovar IA strains occurred more frequently in smaller population centres (Danielsson *et al.*, 1983). However, in most populations studied, such as the United States, Germany and Canada, using the standard GS panel, the predominant serovars were IA-1, IA-2, IB-1, IB-2 and IB-3, and each serovar accounted for 10-20% of the total gonococcal population (Carballo and Dillon, 1992; Woodford *et al.*, 1989; Knapp *et al.*, 1987a; Kohl *et al.*, 1986). There may be a need to increase the discrimination of the GS monoclonal antibody typing panel either by subtyping the major serovars with further antibodies or by using another technique. Similarly, strains belonging to prevalent serovars, such as IB-1 or IB-3, may not be similar, as suggested by the comparison of their antimicrobial susceptibility patterns (Knapp *et al.*, 1985a).

Auxotyping has been used in combination with serotyping, resulting in auxotype/serovar (A/S) classes. The combination of auxotyping and serological classification is frequently used. Both systems are reproducible, but serological classification provides more discrimination than auxotyping (Dillon *et al.*, 1993). However, when the two systems that were based on two independent phenotypic characteristics that are stable *in vitro* were combined into an A/S classification system, the resolution was greater and there was more discrimination than the system based on one phenotypic characteristic (Dillon *et al.*, 1993). The A/S classification of gonococcal isolates provides a discriminatory classification system that allows investigators to characterize the geographic distribution of gonococcal strains and temporal changes in gonococcal strain populations and to understand the heterogeneity in the species of *N. gonorrhoeae* (Dillon *et al.*, 1990; Knapp *et al.*, 1987a; Knapp *et al.*, 1985b).

1.5. Plasmid profile typing

Plasmids are covalently-closed, circular, double-stranded extrachromosomal molecules of DNA which replicate independently of the bacterial chromosome (Lederberg, 1952). They encode a variety of functions which, in theory, are not essential for normal bacterial growth. Plasmid typing for epidemiological purposes involves characterization of plasmids in terms of the numbers of different plasmids per strain and their molecular weights.

The plasmids of *N. gonorrhoeae* have been classified according to their molecular weights and phenotypes. Except for PCU$^-$ isolates which do not carry plasmids in nature, most gonococcal strains carry a cryptic 4.2-kilobase-pair (kb; 2.6 megadalton, MDa) plasmid (Roberts *et al.*, 1979; Maness and Sparling, 1973) and 24.5 Mda transfer plasmid (Sox *et al.*, 1978; Roberts and Falkow, 1977).

N. gonorrhoeae strains that carry plasmids mediating penicillin or tetracycline resistance have been isolated from patients. Penicillinase-producing *N. gonorrhoeae* (PPNG) strains were first isolated in 1976 (Ashford *et al.* 1976; Bowmer, 1976; Percival *et al.*, 1976; Phillips, 1976). Six related plasmids coding for TEM-1 β-lactamase have been recovered from PPNG strains (Dillon and Yeung, 1986). Initially, a 5.6-kb (size range from 3.2 to 3.4 MDa) β-lactamase-producing plasmid, was described in strains isolated from the United Kingdom that were linked epidemiologically to West Africa (designated African) and a 7.4-kb designated Asian (size range from 4.4 to 4.7 MDa) β-lactamase-producing plasmid was found in strains in North America that were linked to the Far East. These two plasmids are very similar and both carry the same part of a transposon Tn2 (Fayet *et al.*, 1982; Roberts *et al.*, 1977); the only difference between them is a 2.1-kb fragment missing from the 7.4-kb plasmid to give the 5.6-kb plasmid (Brunton *et al.*, 1986; Yeung and Dillon, 1985 Dickgiesser *et al.*, 1982). The isolates of *N. gonorrhoeae* containing these plasmids have caused many outbreaks world-wide (Rioux and Courvalin, 1985). A β-lactamase-producing plasmid (5.1-kb, designated Toronto) was isolated from *N. gonorrhoeae* strains in Canada in 1984 (Yeung *et al.*, 1986; Yeung and Dillon, 1985); a 2.3-kb fragment deletion in the Toronto plasmid, as compared to the Asian plasmid, differed from the 2.1-kb fragment deleted to produce the African plasmid (Yeung *et al.*, 1986; Yeung and Dillon, 1985). Another three β-lactamase-producing plasmids not associated with outbreaks of β-lactamase-producing *N. gonorrhoeae* infection have been reported (Dillon and Yeung, 1989;

Gouby *et al.*, 1986; van Embden *et al.*, 1985). A 5.0-kb plasmid, designated the Rio plasmid, was isolated from a strain which was recovered from a patient who was infected in Rio de Janeiro (Gouby *et al.*, 1986; van Embden *et al.*, 1985). Most of the restriction endonuclease map of the Rio plasmid was similar to the restriction endonuclease map of the Asian plasmid and the Toronto-type; this might indicate the Rio plasmid was derived from the Asian plasmid (Dillon and Yeung, 1989). A 6.8-kb plasmid, designated the Nîmes plasmid, was isolated in Nîmes, France (Gouby *et al.*, 1986). This plasmid was derived from the 5.6-kb African plasmid through a 1.2-kb insertion from the 2.4-kb *BamH*I fragment (Dillon and Yeung, 1989; Gouby *et al.*, 1986). In 1989, a novel β-lactamase producing plasmid (9.2 kb) was isolated in New Zealand and was designated as the New Zealand plasmid (Brett, 1989). This plasmid is structurally related to the 7.4-kb Asian plasmid except for an insertion of 1.8-kb (Brett, 1989).

Strains of *N. gonorrhoeae* with high-level plasmid-mediated resistance to tetracycline (TRNG) harbour a conjugative 25.2-MDa *tetM*-containing plasmid which is considered to be a *tetM* containing variant of the 24.5 MDa transfer plasmid (Morse *et al.*, 1986; Roberts *et al.*, 1979).

Although plasmid profile is used to study gonococcal infections, this typing scheme is usually used in combination with auxotyping and serotyping, and has been used to characterize isolates from different geographical areas and to documentate temporal changes in their distribution and prevalence (Ison *et al.*, 1986; van Klingerin *et al.*, 1985).

Compared to other typing methods, one advantage of plasmid profiling is that a single set of reagents and equipment is applicable to many species of bacteria (Mayer, 1988). However, gaining or losing plasmids during the evolutionary process makes plasmid profile unsatisfactory for long-term follow-up studies (Swaminathan and Matar, 1993). The lack of plasmids in case strains as compared to the index strains or finding identical plasmid profiles in control strains unrelated to the outbreak can reduce the ability of plasmid profile analysis to discriminate between groups of isolates (Mayer, 1988). Plasmid profile alone provided the lowest level of discrimination for *N. gonorrhoeae* isolates (Dillon *et al.*, 1993). However, plasmid profiles are most useful in studying gonococcal infection when applied in concert with other typing methods, including serotyping, antimicrobial susceptibility testing, and auxotyping (Dillon *et al.*, 1993; Hook *et al.*, 1987; Knapp *et al.*, 1987a; Knapp *et al.*, 1987b).

1.6. Combined systems used for typing gonococci

A correlation between auxotype and other phenotypes, such as susceptibility to antimicrobial agents or plasmid profile, between susceptibility to antimicrobial agents and auxotype, or between serovar and other phenotypes, has been observed in many independent studies (Dillon and Pauzé, 1984; Noble and Miller, 1980; Knapp *et al.*, 1978). Strains belonging to certain auxotypes were found to be associated with susceptibility to antimicrobial agents. The most common association is arginine-requiring isolates having hypersensitivity to penicillin (Knapp and Holmes, 1975). Gonococci of the P, NR and Arg auxotypes were less susceptible to ampicillin, penicillin, tetracycline and spectinomycin (Dillon and Pauzé, 1984; Noble and Miller, 1980; Knapp *et al.*, 1978; Crawford *et al.*, 1977). The association of methionine-requiring auxotypes with resistance to sulfonamides has also been reported (Catlin and Reyn, 1982). Gonococcal isolates which require methionine were susceptible to sulfonamides, an antimicrobial agent used for treating infections of *N. gonorrhoeae* (Dees and Colston, 1937; Harkness, 1944) before penicillin therapy became widespread (Catlin and Reyn, 1982). Resistance to sulfonamides appeared rapidly during the first 10 years following their introduction (Reyn, 1961; Dunlop, 1949; Campbell, 1944). After the introduction of penicillin for treating *N. gonorrhoeae* in 1943 (Lind, 1990; Sparling *et al.*, 1975), the prevalence of methionine-requiring gonococci decreased, suggesting that methionine auxotrophy must have provided a competitive advantage in the presence of a sulfonamide drug (Catlin and Reyn, 1982). Gonococci of the P, NR, and Arg auxotypes were less susceptible to ampicillin, penicillin, tetracycline and spectinomycin and have been associated with serovar IB (Dillon and Pauzé, 1984; Noble and Miller, 1980; Knapp *et al.*, 1978; Crawford *et al.*, 1977). The correlation of requirements for proline, citrulline, and uracil with reduced sensitivity to penicillin and other antimicrobial agents has also been observed (Ng and Dillon, 1993; Noble and Parekh, 1983; Stewart and Hendry, 1979). The prevalence of certain auxotypes may result in differences in the antibiotic susceptibility of gonococci in various geographical areas (Dillon and Yeung, 1989; Dillon *et al.*, 1987a; Dillon and Pauzé, 1981). In general, gonococci with relative penicillin resistance are uncommon in northern Europe, certain geographic areas of Canada, but are quite common in the Far East (Dillon *et al.*, 1990; Sparling, 1977).

A correlation between serovar and susceptibility to antimicrobial agents also has been noted in many studies. In a study by Ison and Easmon (1991), serovar IB-5/7 strains were strongly associated with resistance to penicillin whereas other serovars such as serovar IB-2 are not; most of serogroup WI strains were highly sensitive to ampicillin, tetracycline and β-lactam antibiotics whereas about 65% and 90% of WII and WIII strains, respectively, showed decreased susceptibility.

The association between plasmid carriage and gonococcal auxotype has been noted by several groups (Dillon *et al.*, 1981; Dillon and Pauzé, 1981; Perine *et al.*, 1977). Gonococcal isolates requiring proline, citrulline, and uracil for growth were plasmid free and did not carry the 2.6-Mda cryptic plasmid common to all other auxotype groups (Dillon and Pauzé, 1981). Isolates of NR, Arg, or PA auxotypes carried the 5.6-kb β-lactamase-producing plasmid, 4.2-kb cryptic plasmid, and some carried a 39.2-kb conjugative plasmid (Ison and Easmon, 1991; Ison and Easmon, 1989; Dillon and Pauzé, 1984; Perine *et al.*, 1977). Most of isolates of NR auxotypes harboured a 7.4-kb β-lactamase-producing plasmid as well as a 4.2-kb cryptic plasmid (Ison and Easmon, 1991; Ison and Easmon, 1989; Dillon and Pauzé, 1984; Perine *et al.*, 1977). If the 24.5-MDa transfer plasmid and the 4.5-Mda plasmid were present, the isolates were equally distributed between the NR and the P groups, as were >95% of the isolates carrying the transfer plasmid without the β-lactamase-producing plasmids (Dillon and Pauzé, 1984). The first reported isolates carrying the 5.1-kb Toronto β-lactamase-producing plasmid also carried a 4.2-kb cryptic plasmid and a 39.2-kb conjugative plasmid, and were A/S class NR/IA-5 serovar (Yeung *et al.*, 1986; Yeung and Dillon, 1985); subsequent studies have shown that this plasmid has moved to isolates of other A/S classes (Dillon, J.R., personal communication).

Correlation between serovar, auxotype, plasmid and other phenotypes has been also studied in TRNG. Gonococcal isolates with 2.6 and 25.2-MDa (*tetM*-containing) plasmids were typed into eleven A/S classes, with the majority (75%) isolates falling into three A/S classes: P/IB-1, P/IB-2 and O/IA-1,2 in Canada (Dillon and Carballo, 1990). The gonococcal isolates, which harboured the 2.6-, 25.2-MDa plasmids and either 3.2- or 4.5-MDa penicillinase-producing plasmids were classified as NR/IB-4 in Europe (Roberts *et al.*, 1988) while those in the United State were NR/IA-1,2 (Knapp *et al.*, 1987b; Morse *et al.*, 1986).

1.7. Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis analysis has been used to study the genetic diversity and structure of a variety of species of bacteria (Selander *et al.*, 1986a), such as *E. coli* (Achtman *et al.*, 1986; Selander *et al.*, 1986b; Caugant *et al.*, 1981; Selander and Levin, 1980), *N. meningitidis* (Olyhoek *et al.*, 1987; Caugant *et al.*, 1986), *Micrococcus cryophilus* and *Branhamella catarrhalis* (Fox and McClain, 1975), *Haemophilus influenzae* (Musser *et al.*, 1985), *Legionella pneumophila* (Selander *et al.*, 1985) and *Drosophila pseudoobscura* (Singh *et al.*, 1976). The method consists of measuring allelic variation at structural gene loci by screening randomly selected cytoplasmic enzymes for genetically controlled variants (electromorphs or allozymes; Caugant *et al.*, 1986; Williams and Shah, 1980). The electrophoretic type (ET) of each strain is determined by its electromorph profile over all loci assayed. Isolates with the same ET can be considered as members of the same cell line or clone. The main advantages of the technique are that all strains can be characterized and, by assaying a relatively large number of loci, the overall genetic distance between strains and groups of strains can be estimated (Poh *et al.*, 1992).

Recently, this technique was employed to estimate the genetic relationships among *N. genorrhoeae* strains (Ng and Dillon, 1993; Poh *et al*, 1992; Fuente and Vazquez, 1992 and

1991). Fuente and Vazquez (1991) investigated 38 penicillinase-producing and non-producing *N. gonorrhoeae* (PPNG and non-PPNG) strains that were isolated in Spain and found that these gonococcal strains formed a group of small genetic variability and were genetically uniform; this result might be explained because only a small number of isolates was studied. Fuente and Vazquez (1992) also studied 41 non-PPNG strains that were resistant to penicillin to determine the genetic variability. Their results showed that these gonococcal strains were, genetically, a highly variable group of organisms and that the distribution of strains between the ETs was not linked to any particular A/S class. Classification of the strains by A/S did not seem to be related to their genotypes (Fuente and Vazquez, 1992). In another study, multilocus enzyme electrophoresis showed significant genetic heterogeneity among gonococcal strains belonging to the same serovar (Poh *et al*, 1992). Sixteen ETs were revealed among the sixty-five isolates (comprising 14 different serovars). Ten strains of the same serovar Bajk (IB-3/6) were further subtyped into eight ETs by multilocus enzyme electrophoretic analysis (Poh *et al.*, 1992). The major electrophoretic type, ET1, was found to comprise eleven serovars (Poh *et al.*, 1992). This suggested that there was not a specific correlation between multilocus genotypes and serotypes. In another study, twenty-three PCU isolates, which are generally considered to be clonal, were studied by multilocus enzyme electrophoretic analysis; only one isolate, which contained a variant cryptic plasmid of 4.3 kb in size, showed a difference in multilocus enzyme electrophoresis (Ng and Dillon, 1993). This provided further evidence of the clonal nature of PCU isolates at the genetic level.

1.8. Molecular typing methods

Since 1975, developments in nucleic acid isolation, separation, and amplification have led to the application of nucleic acid-based subtyping methods to various specific epidemiologic problems (Hawkey, 1987). One of the first techniques used was plasmid content analysis (Mayer, 1988; Sadowski *et al.*, 1979). Several molecular typing methods have been used to analyze genomic DNA including chromosomal restriction endonuclease analysis (REA), genomic DNA restriction fragment length polymorphisms (RFLPs) analysis by probing the chromosomal DNA restriction fragments with one or more labelled DNA fragments, such as ribosomal DNA RFLP analysis (ribotyping) and pulsed field gel electrophoresis (PFGE). The details of each of these techniques will be described below.

1.9. Restriction endonuclease analysis

Several molecular techniques are now available for studying genomic differences between isolates. Chromosomal DNA can be cleaved with type II restriction endonucleases (REs) and the resulting restriction fragment length polymorphisms (RFLPs) analyzed by gel electrophoresis. Type II REs specifically cleave DNA into a unique set of fragments which, depending on the number and position of the individual recognition sequences, provides a characteristic band pattern, the so-called restriction pattern or "fingerprint" of any particular DNA examined (Southern, 1979). Restriction endonuclease cleavage sites are distributed irregularly along the length of the bacterial chromosome. Therefore, statistically, the more related the organization of the DNA of a group of isolates, the more similar their restriction fragment patterns. The RE analysis technique consists of extracting DNA from the organism, digesting DNA *in vitro* with one or more appropriate RE(s), separating the DNA fragments according to size by electrophoresis in either agarose gels or polyacrylamide gels, and selecting the RE giving the best discrimination (Poh *et al.*, 1989; Falk *et al.*, 1984). A DNA polymorphism refers to the change in the size of a restriction fragment. If a change occurs in the sequence of the genome DNA, even a single nucleotide-base mutation, this can delete a RE recognition site or create a new recognition site and result in the generation of a restriction fragment length polymorphism (Owen, 1989). These fingerprints often can provide sufficient information to allow conclusions regarding the relatedness of the isolates being investigated. Fingerprinting has been used successfully in epidemiological and classification studies with several species of bacteria, such as *Campylobacter jejuni* (Penner *et al.*, 1983), *Mycobacterium bovis* (Collins and DeLisle, 1985), *Legionella pneumophila* (van Ketel *et al.*, 1984), *Chlamydia psittaci* (McClenaghan *et al.*, 1984) and *Neisseria meningitidis* (Bjorvatn *et al.*, 1984; Kristiansen *et al.*, 1984).

RE analysis has been used to differentiate closely related strains of *N. gonorrhoeae* (Falk *et al.*, 1984). Twenty gonococcal isolates comprising 8 serovars were selected randomly from a Swedish clinic, and genomic DNA was digested with *Hind*III (which generates fragments of < 5 kb). Falk *et al.* (1984) found no obvious correlation between the particular RE pattern and serovar pattern; most strains with the same serovar pattern were differentiated into groups with a different RE pattern. Conversely, strains with different serovar pattern were found in most groups expressing identical RE patterns (Falk *et al.*, 1984). Their results also showed that RE analysis (REA) was reproducible and reliable. The restriction enzyme technique can further differentiate between isolates that appear to be identical by phenotypic classification, such as serotyping (Dasi *et al.*, 1992; Poh *et al.*, 1989; Falk *et al.*, 1984). The restriction enzyme technique also permits a higher degree of differentiation between closely related strains of *N. gonorrhoeae* than that achieved by auxotyping (Falk *et al.*, 1984). This might suggest that the genomic fingerprinting of gonococcal DNA can serve as an important adjunct to serological classification in epidemiological studies of gonorrhoea (Falk *et al.*, 1985a; Falk *et al.*, 1985b).

Although the restriction enzyme technique appears to be highly specific in differentiating strains of bacteria, it is both laborious and expensive. It is limited by its complexity since ≥ 50 bands of various size, depending on the cutting frequency of the restriction endonuclease used and the genome size of the organism may be produced for analysis. It is difficult, therefore, to identify minor, but possibly significant, RFLPs in complex multiband patterns of closely related strains. The analysis of many isolates using this technique becomes impractical unless application of laser scanning of gels and storage of data on microcomputers (Hawkey, 1987). Its value for exact classification may be further improved in combination with Southern blotting with defined probes, such as ribosomal nucleic acids (rRNA; Tompkins *et al.*, 1986; Grimont and Grimont, 1986), or by using pulsed field inversion electrophoresis that can provide increased resolution between linear fragments within a predefined size range (Chu *et al.*, 1986; Schwartz and Cantor, 1984).

1.10. RFLP of rRNA genes (ribotyping)

When separated DNA fragments are transferred to nitrocellulose or nylon membranes

(Southern, 1975), the location on the membrane of a restriction fragment containing a particular gene or nucleotide sequence can be determined by hybridization with a specific labelled nucleic-acid probe. The resultant hybrid-band pattern provides a unique and reproducible fingerprint, which has been described as the chromosomal probe pattern (Tompkins *et al.*, 1986).

Nucleic-acid probes are used in Southern-blot hybridization analyses with the purpose of highlighting specific DNA restriction-site heterogeneities within selected areas of the bacterial chromosome, which in turn can be used to detect strain differences both within and between species (Owen, 1989; Zainuddin and Dale, 1989; Timms *et al.*, 1988; Ogle *et al.*, 1987; Tompkins *et al.*, 1986). These probes have included cloned, random chromosomal sequences (Tompkins *et al.*, 1986); toxin production genes (Ogle *et al.*, 1987; Pappenheimer and Murphy, 1983); antibiotic-resistant genes (Taylor and Brose, 1985) or rRNA genes (Tompkins *et al.*, 1986). Analysis of chromosomal DNA with these probes can distinguish individual strains of bacteria. In addition, the reduced number of bands, as compared to RE analysis, enhances the feasibility of developing a genetic typing scheme applicable to many isolates.

One widely used nucleic acid probe that provides a broad spectrum of hybridization with bacterial chromosomal DNA is ribosomal RNA (rRNA; Grimont and Grimont, 1986; Tompkins *et al.*, 1986). When bacterial chromosomes are digested with restriction enzymes, the ribosomal genes and their adjacent regions are widely distributed in the resulting fragments, producing strain-specific patterns after hybridization with a ribosomal RNA probe (ribotyping). This technique produces highly specific and reproducible gel electrophoretic patterns. The rationale for using rRNA genes in DNA fingerprinting has its basis in evolutionary history because rRNA sequences are known to be highly conserved, and sequence conservation has been used as a tool to determine the genetic relatedness of species over wide ranges of evolutionary development (Woese, 1987; Pace *et al.*, 1986; Nomura and Post, 1980; Fox *et al.*, 1980). A bacterial rRNA cistron has a total sequence of about 4600 bases comprising several subsequences, which include genes for 23S rRNA (~2500 bases), 16S rRNA (~ 1500 bases), 5S rRNA (~ 120 bases), tRNA and various intervening and flanking sequences (Woese, 1987). Ribosomal RNA operons, which encode 23S, 16S and 5S rRNA, exist in a high copy number in a variety of genetic arrangements in the bacterial chromosome; for example, there are seven copies in *E. coli* cells (Lindahl and Zengel, 1986; Grimont and Grimont, 1986). They are sufficiently heterogenous to make suitable targets for comparison of different bacterial species. The rRNA-based probes provide a widely applicable system to investigate the molecular epidemiology of diverse bacteria (Stull *et al.*, 1988; Grimont and Grimont, 1986) whereas other probes may be more limited in being species-specific or specific only for strains within a particular species.

Ribotyping has been used to differentiate various species of bacteria such as *Salmonella, Escherichia, Enterobacter, Citrobacter, Pseudomonas,* and *Aeromonas*, both to distinguish between species (Ng and Dillon, 1991; Grimont and Grimont, 1986) and between strains within a species (Ng and Dillon, 1993; Ng and Dillon, 1991; Garaizar *et al.*, 1991; Martinetti and Altwegg, 1990; Owen *et al.*, 1990; Lipuma *et al.*, 1989; Irino *et al.*, 1988). In a broad-ranging study, Grimont and Grimont (1986) used a ^{32}P-labelled *E. coli* 16+23S rRNA probe to detect rRNA genes in various species of gram-positive and gram-negative bacteria, and among the species of medical interest examined there were representative of *Salmonella, Brucella, Serratia, Pseudomonas, Mycobacterium* and *Listeria.* They found that the probe reacted with portions of rRNA genes in the DNA of species that were phylogenetically remote from *E. coli*, and that each species could be differentiated from each other by specific patterns of rDNA-restriction fragments. Some DNA-hybridization data showed that identical patterns were highly correlated with insignificant divergence. Furthermore, different patterns within species corresponded to significant divergence as evaluated by thermal-stability studies of DNA-DNA hybrids (Owen, 1989).

Ribotyping has also been used in a small sample study of *N. gonorrhoeae* (Ng and Dillon, 1993; Pol *et al.*, 1992). In the study of Poh *et al.* (1992), forty-three randomly selected isolates comprising 12 serovars were investigated by RFLPs produced by *Hinc*II restriction of genomic DNA and probing with the 2.2 kb *Bst*EII fragment encoding the cloned 16S and 23S rRNA genes of *Pseudomonas aeruginosa*. Nine ribotypes were produced among the 43 isolates. Isolates present within serovars NR/IB-5 and NR/IB-7, and serovars IB-1 and IB-2 could be further subdivided by ribotyping. Ng and Dillon (1993) evaluated ribotyping as a method to distinguish 23 gonococcal isolates of the PCU auxotype. Their results indicated that ribotyping may be useful to type *N. gonorrhoeae* isolates which appear clonal by other methods.

The advantage of ribotyping is that a greatly reduced number of bands, as compared to restriction endonuclease analysis, simplifies interpretation of patterns and enables large numbers of isolates to be analyzed. A disadvantage of ribotyping is that it provides information about only a specific region within the genome; other probes might provide greater discrimination. It is both a laborious and a time-consuming method and therefore not practical for use outside of reference laboratories.

1.11. Pulsed field gel electrophoresis (PFGE)

Within the last few years some of the newest methods applied to bacterial typing have been developed; these methods include pulsed-field gel electrophoresis (PFGE, Schwartz *et al.*, 1983), contour-clamped homogeneous electric field electrophoresis (CHEF, Chu *et al.*, 1986), field inversion gel electrophoresis (FIGE, Carle *et al.*, 1986). PFGE is a derivative of conventional DNA agarose electrophoresis; the important difference is that the orientation of the electric field is changed repeatedly (Smith *et al.*, 1986). PFGE can resolve DNA fragments ranging in size up to 2000 kb, which is ~ 50-fold larger than is possible by conventional techniques (Sor, 1988; McClelland *et al.*, 1987). This approach has been applied to the analysis of a variety of bacterial genomes, such as *Pseudomonas cepacia* (Anderson *et al.*, 1991), *E. coli* (Arbeit *et al.*, 1990), *Coxiella burnetii* (Heinzen *et al.*, 1990), *Lactococcus* (Bourgeois *et al.*, 1989) and *Mycobacterium paratuberculosis* (Levy-Frebault *et al.*, 1989) and has been shown to be a powerful means of investigating the genomes of microorganisms by accurately measuring chromosome size. PFGE has allowed very large DNA fragments to be separated, making it possible to carry out DNA analysis with low-frequency-cleavage restriction endonucleases (McClelland *et al.*, 1987). These produce fewer fragments, usually less than 40, and electrophoretic patterns can be compared more precisely and easily. PFGE has been used for the comparative analysis of chromosomal restriction fragment length polymorphisms generated by rare-cutting restriction nuclease enzymes in a variety of microorganisms, such as *Pseudomonas aeruginosa* (Allardet-Servent *et al.*, 1989; Grothues *et al.*, 1988; Grothues and Tummler, 1987), *Lactococcus lactis* (Bourgeois *et al.*, 1989), *Acinetobacter calcoaceticus* (Allardet-Servent *et al.*, 1989), *Staphylococcus aureus* and *Staphylococcus epidermidis* (Goering and Duensing, 1990; Goering *et al.*, 1990).

Recently, PFGE was used to type *N. gonorrhoeae* isolates (Poh and Lau, 1993). Forty-eight isolates that were represented by 2 auxotypes (NR and P) and 11 different serovars (including IB-5 and IB-7, which differ in their reaction with a single antibody) and eighteen A/S classes were investigated. Thirty-eight *Spe*I PFGE patterns and 40 *Nhe*I PFGE patterns were produced among 48 isolates. Seven isolates belonging to the A/S class P/IB-7 could also be distinguished by PFGE indicating that PFGE was more discriminatory than auxotyping, serotyping or A/S classification and could be used to subtype isolates belonging to the same A/S class.

1.12. Research objectives

Because a number of classical typing schemes used for distinguishing isolates of *N. gonorrhoeae*, such as auxotype, plasmid profile and serovar fail to adequately discriminate groups of isolates, this study will evaluate the utility of the following methods to subtype strains: antibiogram and three molecular methods-RE analysis, ribotyping and PFGE analysis.

Isolates for evaluation were selected from outbreaks (NR/IA-5 and NR/IB-3) or population studies (PCU(H), arginine-requiring and serovar IB-5 and IB-7). Isolates from the population studies were selected for this study either because the isolates (PCU(H) and arginine-requiring) appeared clonal based on their antimicrobial susceptibilities and/or auxotypes, or because the literature (Kohl *et al.*, 1990; Ison *et al.*, 1989; Woodford *et al.*, 1989) indicated that strains of certain serovars could be grouped irrespective of auxotype (e.g. serovar IB-5 and IB-7).

The rationale of selecting these 5 groups of isolates was the following: the PCU/PCUH group is considered a clone as suggested by monoclonal antibody analysis (Brunham *et al.*, 1985) and by plasmid content, ribotyping and isoenzyme typing (Ng and Dillon, 1993). Arginine-requiring isolates have been found to be homogeneous with respect to their antimicrobial susceptibilities and their serovar (IA-2), although they may be differentiated by the specific mutation in the arginine biosynthesis pathway (i.e. ornithine or citrulline) or by their multiple auxotypes (i.e. OUH, CUH, and OH). Strains belonging to serovar IB-5 and IB-7 have been grouped together because they only differ in their reaction with a single antibody (Ison *et al.*, 1989; Woodford *et al.*, 1989) and were reported to be biologically similar using the criteria of auxotype and antimicrobial susceptibility (Kohl *et al.*, 1990). Thus, my hypothesis was that the molecular analysis of isolates from population studies would distinguish different isolates within their respective A/S classes irrespective of the method; my hypothesis with respect to "outbreak" isolates is that they should be homogeneous even after testing using molecular methods. The NR/IA-5 group of strains comprised a cluster of predominately PPNG isolates (plasmid content was the same: carried plasmids of 2.6, 3.2 and 24.5 MDa), from the same geographic area, Toronto. A non-PPNG isolates as well as a TRNG isolates with the same A/S class and from the same city was also included in the analysis since I propose that the non-PPNG isolate merely lost the 3.2 MDa plasmid and the TRNG isolate acquired a *tetM*-containing plasmid by conjugation. The NR/IB-3 group of strains included twelve *N. gonorrhoeae* isolates with chromosomally mediated resistance (CMRNG) to penicillin, tetracycline and erythromycin.

These isolates carried plasmids of 2.6 and 24.5 MDa from two adjacent geographic areas, Manitoba and Ontario.

Therefore, the objectives of this research were to evaluate three molecular methods (restriction fragment length polymorphism of the rRNA gene - ribotyping; restriction endonuclease analysis - RE analysis; pulsed field gel electrophoresis - PFGE) for their ability to discriminate within and between five A/S classes of gonococcal isolates and in addition to determine whether antimicrobial susceptibility could distinguish between isolates having the same A/S class.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

2.1.1. *Bacterial strains and plasmids*

The culture collection of the National Laboratory for Sexually Transmitted Diseases (NLSTD), Laboratory Centre for Disease Control (LCDC)-Ottawa, collected between 1988 and 1992 and comprising 11,599 isolates submitted by various laboratories across Canada for a number of national surveys and programs, was used to select specific isolates. One hundred isolates of *N. gonorrhoeae* (Table 1), comprising five groups and thirteen A/S classes (PCU(H)/IB-1 or IB-2; OUH, CUH, OH/IA-2; NR/IB-5, NR/IB-7, P/IB-5, P/IB-7; NR/IA-5; and NR/IB-3) were selected. The isolates were selected either to ensure diverse geographical origins (i.e. from population studies) to reduce the likelihood of epidemiological linkage or representing unique outbreaks of certain A/S classes with probable epidemiological links (this could not be verified retrospectively). In addition, within a given A/S class, isolates were selected so as to reflect the upper and lower MIC range for penicillin, if possible.

The 913 PCU(H) isolates from the data base comprised two serovars, IB-2 (87.2%, 796/913 of isolates) or IB-1 (7.5%, 68/913 of isolates). Some PCU isolates also have a requirement for hypoxanthine (18.3%, 167/913; Dillon *et al.*, 1993). From this group, fifteen PCU(H)/IB-1 gonococcal isolates and fifteen PCU(H)/IB-2 *N. gonorrhoeae* isolates were selected

Table 1. A/S classes and isolate numbers of gonococci in this study

Auxotype[a]/Serovar (A/S) Class	Isolate Number[b]
PCU/IB-1	880067, 880128, 880263, 880724, 881587, 883036, 883077, 883632.
PCUH/IB-1	880596, 880687, 882779, 882989, 883353, 3703, 7861.
PCU/IB-2	880074, 880916, 881777, 881985, 881996, 882988, 883329.
PCUH/IB-2	880071, 881542, 881717, 882191, 883238, 883355, 883766, 8281.
OUH/IA-2	880375, 880420, 881035, 882208, 882306, 882398, 882245, 882521, 883122, 883171, 883342, 883463.
CUH/IA-2	880270, 880379, 880454, 881540, 882132, 882610, 883099, 883729.
OH/IA-2	881097, 881342, 881506, 882367, 882794, 883212.
NR/IB-5	880107, 880466, 880644, 3351.
NR/IB-7	881095, 881359, 882449, 883535, 883665, 4209.
P/IB-5	880457, 881051, 881508, 882508, 4702, 4710, 5846.
P/IB-7	880324, 880972, 883141, 6920, 8318.
NR/IA-5	2852, 3523, 3735, 3928, 4020, 5265, 5283, 5707, 6525, 6595.
NR/IB-3	881286, 881919, 882351, 882802, 883482, 883511, 3644, 3648, 4743, 5837, 5840, 5855.

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[a]: Abbreviations are as follows: O: ornithine; P: proline; C: citrulline; U: uracil; H: hypoxanthine; NR: non requiring.

[b]: Antimicrobial susceptibilities and geographic source of isolates are noted in Appendix Tables A-1 to A-8.

for the present study (see Appendix, Table A-1,2).

The 552 arginine-requiring gonococcal isolates of serovar IA-2 collected between 1988 and 1992 comprised three different arginine-requiring auxotypes (i.e. OUH, 83.2%; CUH, 11.1% and OH, 5.8%). Twelve OUH, eight CUH and seven OH gonococcal isolates were selected for this study (Table A-3).

The 593 serovar IB-5 and IB-7 isolates mainly comprised two major auxotypes, P (29.5%, 175/593) and NR (62.6%, 371/593) auxotypes. Four NR/IB-5, six NR/IB-7, six P/IB-5 and five P/IB-7 gonococcal isolates were chosen for this study (Table A-4, 5).

Ten isolates from an outbreak of gonococcal isolates of A/S class NR/IA-5 which was focused in Toronto, Ontario were selected for analysis. A total of 612 PPNG isolates of this A/S class were isolated in Canada during 1988 to 1992; 96.7% were isolated from the Toronto area (Figure 1). All isolates contained 2.6, 3.2, and 24.5 MDa plasmids except isolate 3523 contained 2.6 and 24.5 MDa plasmids and isolate 5283 contained 2.6, 3.2 and 25.2 MDa plasmids. Most (8/10, 80%) of them were penicillinase producing *N. gonorrhoeae* (PPNG) except for two isolates; isolate 3523 was a non-PPNG and the other isolate, 5283, was penicillinase-producing and carried plasmid mediated resistance to tetracycline *N. gonorrhoeae* (PP/TRNG). Their MICs to six antimicrobial agents are listed on Table A-6.

Twelve isolates from another outbreak of gonococcal isolates of A/S class NR/IB-3 which was concentrated in two areas, Ontario and Manitoba, and were collected during 1988 and 1990 were also chosen for analysis (Figure 2). Most isolates were obtained from Ontario and Manitoba and were first retrospectively identified because they carried chromosomally-mediated resistance to penicillin, tetracycline and erythromycin. Six isolates were selected from Ontario

Figure 1. The temporal distribution of A/S class NR/IA-5 isolates in Canada

TEMPORAL DISTRIBUTION OF A/S CLASS NR/IA-5 ISOLATES IN CANADA

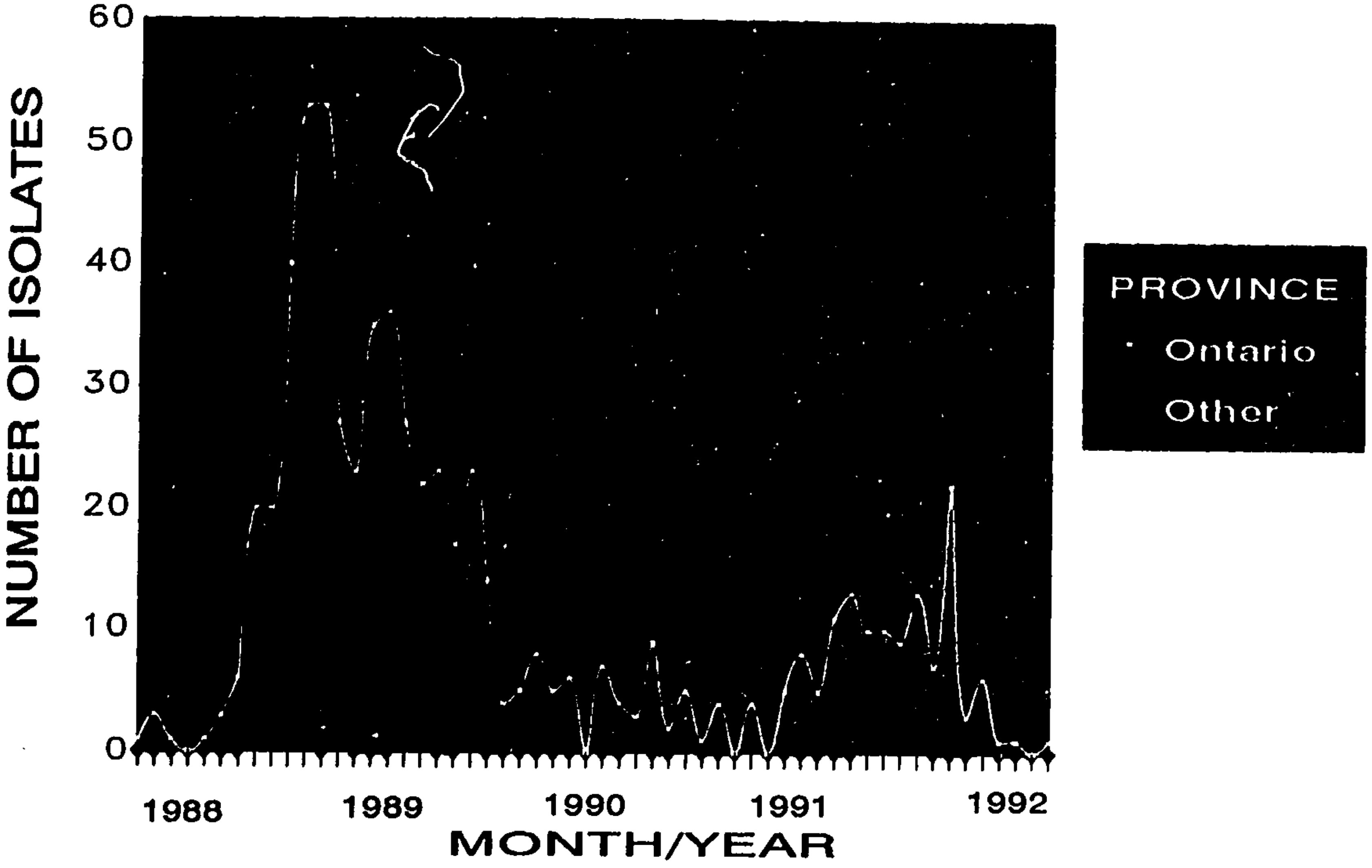
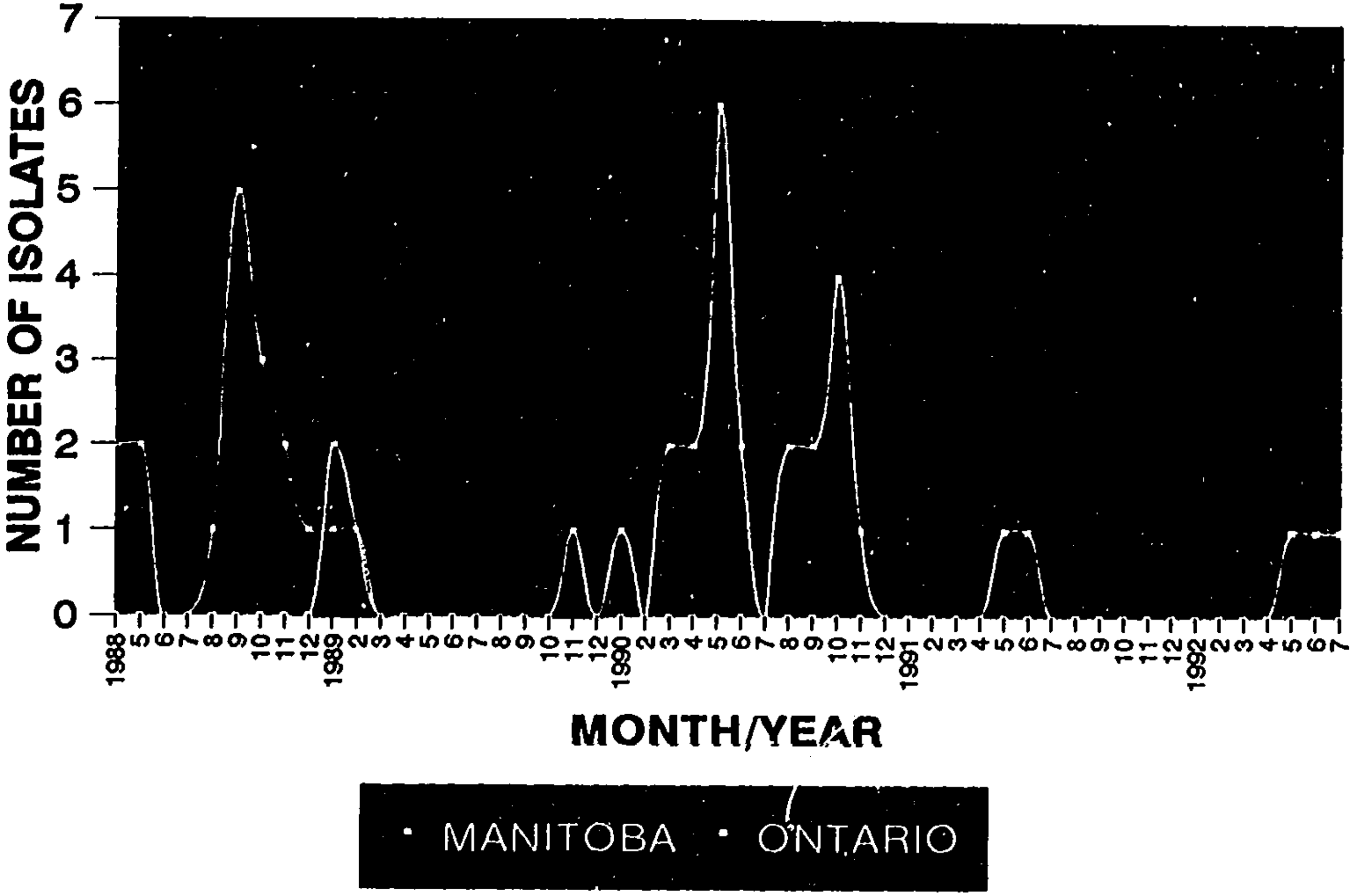


Figure 2. The temporal distribution of A/S class NR/IB-3 isolates in Canada.

TEMPORAL DISTRIBUTION OF CMRNG (NR/IB-3) ISOLATES IN CANADA



area and six isolates were selected from Manitoba area for the present study. They belonged to the same A/S class NR/IB-3. All isolates contained 2.6 and 24.5 MDa plasmids. The MICs of these isolates to six antimicrobial agents are listed on Table A-7.

E. coli C600 containing plasmid pKK3535 was supplied by Dr. J. Brosius (Columbia University, New York, NY). Plasmid pKK3535 contains the *rrn*B rDNA operon of *E. coli* encoding 5S, 16S, and 23S rRNAs as well as $tRNA^{Glu}$ genes (Brosius *et al.*, 1981).

2.1.2. *Growth conditions*

N. gonorrhoeae isolates were either previously frozen at -70°C in Brain Heart Infusion Broth (Difco Laboratories, Detroit, MI) plus 15% glycerol, or were lyophilized in 2% skim milk (Dillon, 1983). Gonococci were subcultured on GC medium base (GCMB, Difco Laboratories, Detroit, Michigan) supplemented with Kellogg's (1963) defined supplements as modified by Dillon (1983) and were incubated 18-24 hours at 35°C in a humid, supplemented atmosphere with 5% CO_2. *E. coli* isolates were grown on tryptic soy agar (TSA) medium (Difco) supplemented with 100 mg/mL ampicillin (Sigma Chemical Co., St. Louis, Mo), and were incubated at 37°C overnight.

2.2. Auxotype, serovar and MIC determination

The auxotypes of all *N. gonorrhoeae* strains used in this study were determined in the NLSTD using the method of Hendry and Stewart (1979) as modified by Picard and Dillon (1989).

The serovar of the isolates was determined in the NLSTD, as described by Knapp *et al.* (1984), using a panel of monoclonal antibodies provided by Syva (Palo Alto, Calif., USA). All isolates of a given A/S class received from 1988-1992 were used to analyze antimicrobial susceptibility. The antimicrobial susceptibilities of isolates to penicillin, tetracycline, erythromycin, cefoxitin, ceftriaxone and spectinomycin had been determined previously in the NLSTD using the agar dilution method (the National Committee for Clinical Laboratory Standards, NCCLS, 1990). The minimum inhibitory concentration (MIC) data was entered into the Biological and Clinical Data Collection (BCDC) system of the NLSTD. The MIC was considered to be the lowest concentration of antibiotic that inhibits the organism's growth (NCCLS, 1990). Interpretive criteria (susceptible: penicillin, erythromycin, and cefoxitin, MIC $\leq$0.032 mg/L; susceptible: tetracycline MIC $\leq$0.25 mg/L; moderately susceptible: penicillin, erythromycin, and cefoxitin, MIC = 0.063 - 1.0 mg/L; moderately susceptible: tetracycline MIC = 0.5 - 1.5 mg/L; resistant: MICs $\geq$2.0 mg/L for all antimicrobial agents; for CMRNG where resistance is defined as a MIC of penicillin $\geq$ 2.0 mg/L, tetracycline $\geq$ 2.0 mg/L, and erythromycin $\geq$ 2.0 mg/L) were those recommended by the NCCLS (1990).

2.3. Isolation of DNA

2.3.1. *Chromosomal DNA*

N. gonorrhoeae cells were lysed by using a modified method of Dillon *et al.* (1985). Confluent gonococcal growth was harvested from 9 culture plates after overnight incubation on supplemented GCMB and suspended in 3.0 mL TES buffer (30 mM Tris-HCl, pH 8.0, 50 mM

EDTA and 5 mM NaCl) with 25% sucrose (w/v). After thoroughly mixing by vortexing, 0.5 mL of lysozyme (10 mg/mL in TES buffer, pH 8.0) was added and mixed by inversion. The cells were left on ice for 15 minutes and 0.4 mL of 0.25 M EDTA and 1.4 mL of 20% Triton-X100 (Sigma Chemical Co., St. Louis, Missouri) were added and mixed by inversion; the cells were placed on ice for 10 minutes to release chromosomal and plasmid DNA. The cell suspension was centrifuged at 20,000 rpm (47,800 × g) for 30 minutes at 4^0C in a Sorvall Instruments RC5C centrifuge (New England Nuclear, Dupont Canada, Mississauga, Ontario). The supernatant was removed with a polypropylene pipette, placed in a fresh plastic tube and adjusted to a final volume of 9 mL with TES buffer. 8.0 g of cesium chloride was added and completely dissolved by gently swirling the mixture. The mixture was transferred to a Beckman Quick-seal ultracentrifuge tube (Beckman Instruments Canada, Mississauga, Ontario) containing 160 μL of ethidium bromide (10 mg/L). The tube was filled to the top with liquid paraffin (BDH Inc. Toronto, Ontario) and was heat sealed according to the manufacturer's instructions (Beckman). Genomic DNA was purified from the lysates by centrifugation to equilibrium in cesium chloride-ethidium bromide density gradients (Sambrook *et al.*, 1989). The lysates (supernatant) were centrifuged at 55,000 rpm for 20 hours at 10^0C using a Beckman Type-65 rotor in a L8-70 M ultracentrifuge (Beckman). The band containing chromosomal DNA was visualized under ultra-violet (UV) light and collected by side puncture with an 18 gauge needle (Becton Dickinson & Co. Mississauga Ontario) as described by Dillon *et al.* (1985). After removing the ethidium bromide with three successive isobutanol extractions (Dillon *et al.*, 1985), the aqueous phase was collected and equal amounts of double distilled H_2O was added to dilute the cesium chloride. Chromosomal DNA was precipitated by adding 1/10 volume of 3 M ammonium acetate and 2.5 volumes cold 95% ethanol and then washed once with 70% ethanol. The pellet was dried under vacuum for 10 minutes. The DNA was resuspended in 0.1 × SSC buffer (20 × SSC is 3 M sodium chloride, 0.3 M sodium citrate) and stored at 4°C.

2.3.2. *Plasmid DNA*

Plasmid pKK3535 was isolated from *E. coli* using a modification of the method of Birnboim and Doly (1979). *E. coli* C600 cells were harvested from six supplemented GCMB agar plates after overnight incubation on TSA plates supplemented with ampicillin (100 mg/mL). Cells were suspended in 2.5 mL of solution I (50 mM glucose, 10 mM EDTA, 0.25 M Tris pH 8.0), and thoroughly mixed by vortexing. After adding of 0.5 mL lysozyme (10 mg/mL in solution I), the cells were placed on ice for 30 minutes, then 5 mL of solution II (0.2 N NaOH and 1% SDS) was added. The mixture was placed on ice for another 5 minutes to lyse the cells. Chromosomal DNA and SDS-protein complexes were precipitated by adding 3.75 mL of solution III (3 M sodium acetate, pH 4.8) on ice for one hour. The precipitate was separated from the supernatant by centrifugation at 12,500 rpm for 15 minutes. The supernatant was transferred to a fresh tube. The DNA in the supernatant was precipitated by adding 2.5 volumes of cold 95% ethanol followed by refrigeration at -20°C overnight. The DNA was collected by centrifugation at 10,500 rpm for 10 minutes and resuspended in 9.0 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Plasmid pkk3535 was purified by cesium chloride ethidium bromide density gradient ultracentrifugation as described earlier for chromosomal DNA isolation (Sambrook *et al.*, 1989). After ethidium bromide extraction and plasmid DNA precipitation, the DNA was resuspended in double distilled H_2O and stored at 4°C.

2.4. DNA concentration determination

DNA concentration was either measured spectrophotometrically at 260 nm using a SPECTRONIC 1001 instrument (BAUSCH & LOMB Inc., Rochester, New York) according to the formula 50 μg DNA/mL = O.D. 260 nm= 1.0 (Sambrook *et al.*, 1989), or estimated by comparising known DNA concentration on agarose gel electrophoresis.

2.5. Digestion of bacterial DNA with restriction endonucleases, agarose gel electrophoresis and visualization of DNA

2.5.1. *Restriction endonuclease digestion*

Restriction endonucleases were purchased from Boehringer Mannheim Biochemicals (BMC, Dorval, Quebec). Approximately 10 μg of DNA was digested using *Sma*I, *Ava*II, and *Hind*II according to the instructions of the manufacturer (BMC). The digested DNAs were heated at 65°C for 10 minutes, then 8 μL of Stop Solution (4 M urea, 50% sucrose, 50 mM EDTA and 0.1% bromophenolblue, pH 7.0) was added.

2.5.2. *Agarose gel electrophoresis*

Electrophoresis of DNA was performed in Tris-acetate (TA) buffer (400 mM Tris-base, 200 mM Sodium Acetate and 18 mM EDTA, pH 7.8; Dillon *et al.*, 1985) using 1% agarose (Pharmacia Agarose NA, Laval, Quebec, Canada) gels in a horizontal submarine apparatus at approximately 15 mA/cm. For restriction analysis, *Hind*III fragments of lambda (λ) DNA were used as molecular weight standards. The restricted fragments of chromosomal DNA were separated electrophoretically in a 0.7% agarose gel which was run for 40 hours at a constant voltage (25 V) in TA buffer. The sizes of chromosomal DNA fragments were determined by measuring the distance of migration for each band as compared with the DNA standards comprising uncut lambda DNA (Bethesda Research Laboratories, Burlington, Ontario), lambda DNA digested by *KpnI* and a 1 kb ladder (Bethesda Research Laboratories) fragment size marker.

2.5.3. *Visualization of DNA in agarose gels*

The gels were stained in ethidium bromide (10 μg/mL) for 30 minutes, destained in double distilled H_2O for at least six hours with gentle shaking and photographed under UV illumination with a Polaroid MP4 camera using Polaroid type 55 film (positive/negative) and type 52 film (positive). For RE analysis, the different band patterns (genomic fingerprints) were compared visually on negative film and grouped according to identity. Fragment sizes from at least three gels were averaged after measuring distances of band migration in comparison to DNA standards.

The reproducibility of RE patterns was studied by testing of the DNA preparations of all gonococcal isolates at least four times. The stability of the RE patterns was also examined by five passages of isolates.

2.6. Densitometric scanning

Electrophoretograms on negative film were scanned with the Molecular Dynamics

Personal Densitometer (courtesy of Molecular Dynamics, Ltd., Calif., U.S.A.).

2.7. Pulsed field gel electrophoresis

2.7.1. *Preparation of chromosomal DNA for PFGE*

Chromosomal DNA was prepared in agar plugs by a procedure slightly modified from that of Chang and Taylor (1990). After 18-24 hours growth on GCMB agar, *N. gonorrhoeae* cells were harvested and suspended in 1 mL of Tris-EDTA (TE) buffer (50 mM Tris, 5 mM EDTA, pH 7.8) to a turbidity equal to McFarland No. 5 (1.5×10^9 bacteria/mL; McFarland, 1907). 100 μL of each cell suspension was transferred to 1 mL of melted 1% low-melting-point agarose (GIBCO BRL, Burlington, Ontario) in distilled water and mixed by vortexing. The 100 uL mixture of cells and agarose was poured into the slots of a 150 uL plastic mold and cooled to 4°C. For each strain, six to eight solidified agarose blocks were removed from the mold. They were transferred into sterile tubes containing 1 mL of ESP buffer [0.25 M EDTA (pH 9.0), 0.5% lauroyl sarcosine (Sigma), 0.5 mg of proteinase K (BMC) per mL] followed by incubation at 50°C for 24 hours. After decanting the buffer, each DNA agarose block was cut into several 1 mm thick slices. Three slices of each strain were transferred into a microcentrifuge tube and then washed for at least 15 minutes in TE buffer containing 1 mM phenylmethylsulfonyl fluoride. This step was repeated three times and was followed by washing three times with TE buffer (not containing phenylmethylsulfonyl fluoride). The DNA in the agarose block was stored at 4°C in TE buffer until restriction endonuclease digestion.

2.7.2. *Digestion of chromosomal DNA in agarose block with restriction endonucleases and analysis by PFGE*

The restriction endonuclease *Spe*I was chosen to cleave chromosomal DNA for differentiating isolates in this study because it had previously been used to construct a physical and genetic map of the gonococcal chromosome of strains MS11-N198 and FA1090 (Dempsey *et al.*, 1991; Bihlmaier *et al.*, 1991). The agarose block containing whole genomic DNA was equilibrated at room temperature for four hours in 100 μL of restriction enzyme buffer prepared according to BMC specifications. Cleavage was performed at 37°C for 24 hours in 100 μL of fresh buffer with 5 units restriction enzyme according to the manufacturer's instructions (BMC). After digestion, the agarose block was washed once in TE buffer and loaded into the gel well, which was then sealed with 1% agarose in gel buffer.

Electrophoresis was performed with the contour-clamped homogeneous electric field (CHEF-DRII) system (Bio-Rad, Richmond, Calif.) in 1% agarose gels at 12°C in 0.05 M Tris-borate-EDTA (TBE) gel buffer (1 M Tris, 1 M Boric acid, 20 mM EDTA, pH 8.5). A constant voltage of 175 volts was applied with ramped pulse times of 0.2 to 54.2 seconds over a period of 24 hours. Under these conditions, fragments from 5 kb to 600 kb were separated and sized with lambda DNA concatemers (Promega, Co. U.S.A.) size markers. The presence of standard DNA molecular weight marker on each gel enable strict comparisons to be made between separate gels. Fragment sizes were determined from 2-4 replicates.

Thereafter, the gels were stained with ethidium bromide (10 μg/mL) for 15 minutes, and the DNA was visualized and photographed as described previously.

The reproducibility of PFGE patterns generated by *Spe*I was examined by analysis of the same bacteria strain on three separate occasions.

2.8. Preparation of labelled rRNA probe from pKK3535 plasmid

2.8.1. *Probe preparation*

The plasmid pKK3535 is a recombinant plasmid containing the entire *E. coli rrn*B ribosomal RNA operon (Brosius *et al.*, 1981) on a 7.2 kb *Bam*HI-*Pst*I fragment (Figure 3). The plasmid was digested with *Bam*HI and *Pst*I according to the manufacturer's instructions (BMC). The resulting fragments were separated on 1% low melting point (LMP) agarose gel (GIBCO BRL). The gel was stained in ethidium bromide (10 μg/mL) and destained in double distilled water. The bands was viewed under UV light. The 7.2 Kb fragment was extracted from LMP agarose gel using a method modified from Weislender (1979). Briefly, agarose containing the 7.2 kb fragment was cut out of the gel and transferred into microfuge tube. 200 μL of TE buffer (previously warmed to 65°C) was added and the tube was heated at 65°C for 10 minutes. An equal volume of phenol, which had been saturated with TE buffer, was added and the mixture was centrifuged for 10 minutes in an eppendorf centrifuge (model 5414). The aqueous phase (top, containing the DNA) was collected (the agarose stays in the interphase). This step was repeated by adding 200 μL of TE buffer to the tube containing the agarose. The aqueous phases were combined. Ethidium bromide was removed by adding an equal volume isobutanol followed by adding an equal volume chloroform. The DNA was then precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volume of 95% cold ethanol at -20°C overnight. After 15 minutes

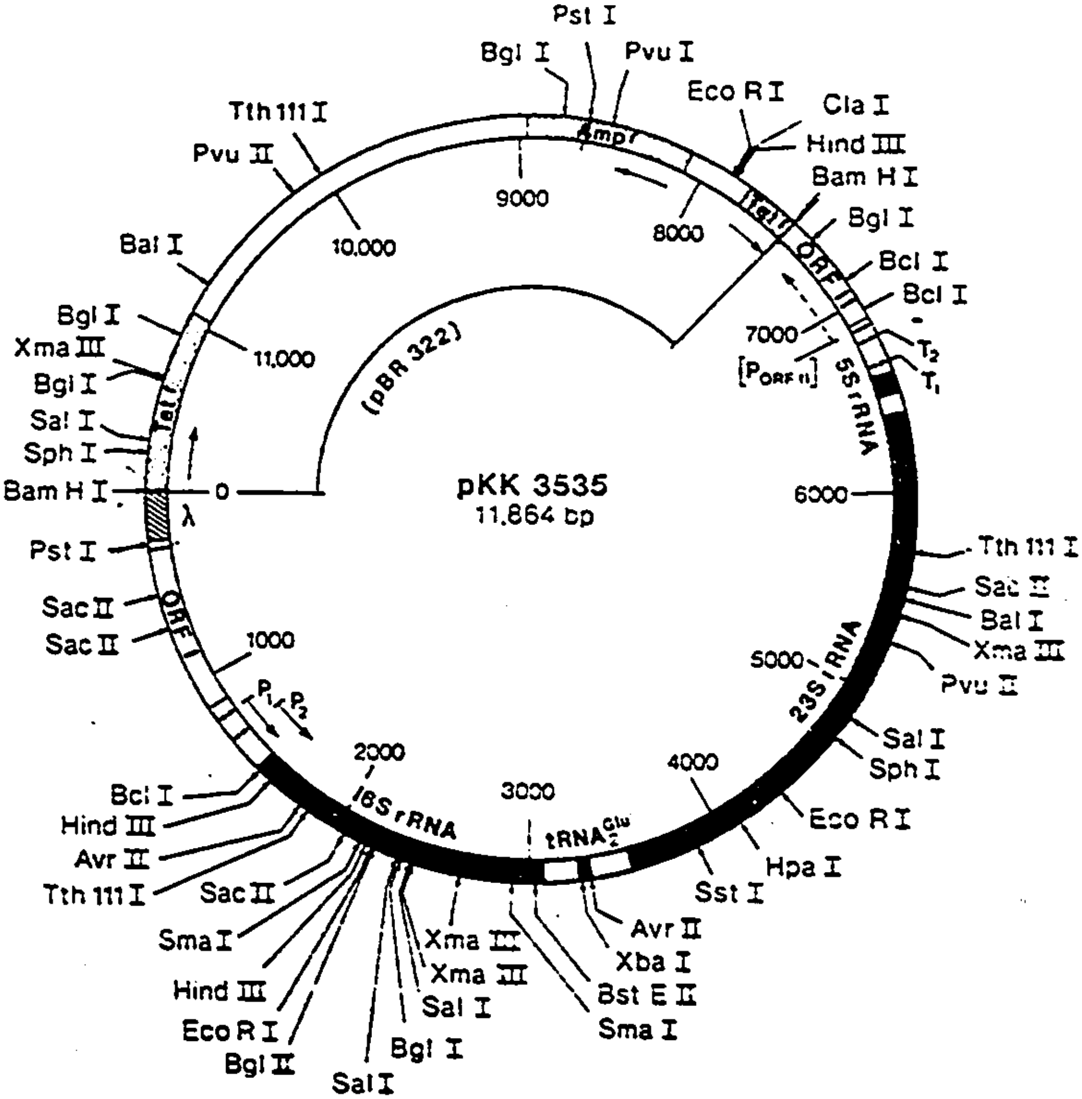


Figure 3. Schematic map of recombinant plasmid pKK3535 (taken from Brosius *et al.*, 1981).

of centrifugation in an eppendorf centrifuge, the supernatant was discarded. The pellet was washed with 70% ethanol to remove unwanted salts and detergents, dried under vacuum, resuspended in sterile distilled water and stored at 4°C ready for labelling.

2.8.2. *Nonradioactive labelling of 7.2 kb BamHI-PstI fragment of pKK3535*

The 7.2 Kb fragment was labelled non-radiometrically with digoxigenin-dUTP (BMC-DIG™) by using of the random primer labelling method in accordance with the instructions of the manufacturer (BMC). Briefly, for labelling 1 μg of DNA, 15 μL of DNA (containing 1 μg of freshly denatured DNA) was mixed with 2 μL of random primers and 2 μL of a deoxyribose nucleotides/digoxigenin labelled dUTP solution (dATP, 1 mmol/L; dCTP, 1 mmol/L; dGTP, 1 mmol/L; dTTP, 0.65 mmol/L; Dig-dUTP, 0.35 mmol/L; pH 6.5), and 1 μL Klenow polymerase enzyme (2 units). The labelling was performed at 37°C for at least two hours. To stop labelling, 2 μL 0.2 M EDTA pH 8.0 was added. The labelled DNA was precipitated with 2.5 μL 4 M LiCl and 75 μL cold ethanol at -20°C overnight. The labelled DNA was centrifugated in an eppendorf centrifuge for 10 minutes and then washed once with 50 μL cold ethanol (70% v/v). The labelled DNA was dried under vacuum, dissolved in 50 μL TE buffer and stored at 4°C.

2.8.3. *Radioactive labelling of 7.2 kb BamHI-PstI fragment of pKK3535*

For radioactive probes, the 7.2 kb fragment was denatured by boiling for 10 minutes and radiolabelled with ^{32}P-dCTP (Dupont) using the random primer-Klenow polymerase method (Foeinberg and Vogelstein, 1984) and the Megaprime labelling kit (Amersham Canada Ltd., Oakville, Ontario) in accordance with the manufacturer's instruction. Labelling reactions were performed at 37°C for 15 minutes. Separation of labelled probe from unincorporated ^{32}P-dCTP was done by the following procedure: 50 μL of 4 M ammonium acetate, pH 4.5 was added to the labelling reaction and then the labelled DNA was precipitated by adding 200 μL of ethanol at -70°C for 15 minutes. The tubes were transferred to a heating block at 37°C for 2 minutes then centrifuged in an eppendorf centrifuge for 15 minutes. The supernatant was decanted and the pellet was washed once in 0.5 mL of 0.67 M ammonium acetate (pH 4.5) and 67% ethanol at room temperature, and then once in 90% ethanol. The pellet was air dried, and then the labelled DNA pellet was redissolved in 100 μL TE buffer.

The specific activity of the probe was determined by liquid scintillation counting (1217/1218 RACKBETA, Pharmacia Biotech Inc., Quebec) using a 10 μL aliquot from the labelled DNA resuspended in TE buffer. The specific activity of the probe labelled in this manner is around 5 x 10^5 counts per minute (cpm)/μL. The labelled probe was stored at -70°C.

2.9. Southern blotting, DNA hybridization and hybrid detection

2.9.1. *Southern blotting*

To optimize transfer to a solid matrix, the DNA on the gel was nicked using the GS Gene linker, UV chamber (Bio-rad). DNA was transferred from agarose gels to a positively charged nylon membrane (BMC) by the alkaline transfer method (0.4 M NaOH, overnight) (Chomczynski and Qasba, 1984). Immediately after transfer, the membrane was soaked in 0.5 M Tris-HCl, pH 7.0 for 5 minutes; then in 2 × SSC for 2 minutes. Membranes were dried between 1 MM Whatman filter paper, fixed by UV irradiation for 5 minutes and stored at room temperature until used.

2.9.2. *DNA hybridization*

a) DNA hybridization of nonradioactive labelled probe: The hybridization for the nonradioactive labelled probe was performed under high stringency conditions using a method provided by the manufacturer and subsequently modified (Boehringer Mannheim, 1989). The membrane was prehybridized with hybridization solution [5×SSC, 2% (w/v) blocking reagent (BMC), 0.1% (w/v) N-lauroylsarcosing and 0.02% (w/v) SDS] for two hours at 68°C in a hybridization oven (Robbins Scientific). The hybridization solution was discarded and replaced with the freshly denatured labelled probe DNA in 9.5 mL hybridization solution. The membranes were then incubated overnight at 68°C. After hybridization, the membranes were then washed under increasing stringency by decreasing the salt concentration and increasing the washing temperature and the washing time. The first salt concentration used for washing was 2 × SSC, 0.1% (w/v) SDS at room temperature for twice 5 minutes and followed by 0.1 × SSC, 0.1% (w/v) SDS washing at 68°C for twice 60 minutes. The presence of digoxigenin in the labelled DNA probe was detected with an alkaline phosphatase-conjugated antibody as described in the nonradioactive detection kit supplied by the manufacturer (BMC).

b) DNA hybridization of radioactive labelled probe: The hybridization for radioactive labelled probe was performed by using the method of the Royal Canadian Mounted Police (RCMP, R. Fourney, personal communication). The membranes were prehybridized for 15 minutes at 68°C in sealed plastic bags containing 15 mL hybridization solution (10%

polyethylene glycol, 1.5 x SSPE, 7.0% SDS). The prehybridization solution was discarded and fresh hybridization solution containing freshly denatured labelled probe was added (10 x 10^6 cpm). The probe was denatured by boiling for 10 minutes in 250 μL of denatured Salmon Sperm DNA (10 mg/mL; Calbiochem, San Diego, Calif.). The membranes were hybridized overnight at 68°C.

2.9.3. *Detection of hybrids*

a) Immunochemiluminescent detection of digoxigenin-labelled DNA probe: The procedure for the detection of the probe using 3-(2'-Spiroadamantane)-4-methoxy-4-(3'phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD), a chemiluminescent substrate for alkaline phosphatase, was carried out according to the instructions of the manufacturer (BMC) with the following modifications: The cited volumes of the solutions refer to a membrane-size of 100 cm^2 and were adjusted in the various experiments according to the membrane sizes used. The detection procedure was started by washing the membranes with 100 mL washing buffer (Buffer 1: 0.1 M/L maleic acid, 0.15 M/L NaCl, pH 7.5/20°C; washing buffer: 0.3% (v/v) Tween 20 in Buffer 1) for one minute. The membranes were then incubated for one hour in 100 mL Buffer 2 (blocking stock solution diluted 1:10 in Buffer 1, final concentration = 1% blocking reagent, blocking stock solution is 10% (w/v) blocking reagent in Buffer 1, autoclaved and stored at 4°C). Anti-DIG-AP-conjugate (BMC) was freshly diluted to 75 mU/mL (1:10000) in Buffer 2. After discarding the blocking solution, the membranes were incubated for 30 minutes with 20 mL of diluted antibody conjugate solution. The unbound antibody conjugate was removed by washing 15 minutes with 100 mL of washing buffer (0.3% (v/v) Tween 20 in buffer 1) two times. Finally the membranes were equilibrated for 2 minutes with 20 mL of Buffer 3 (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 50 mmol/L $MgCl_2$, pH 9.5/20°C). The membrane was placed between two sheets of PhotoGene™ Development Folders (GIBCO-BRL, Ghent, Belgium). The top sheet was lifted and 0.5 mL diluted AMPPD substrate solution (AMPPD 10 mg/mL, diluted 1:100 in Buffer 3) was added to the top surface of the membrane, scattering the drops of AMPPD over the surface. The top sheet was gently lowed and any bubbles present on the sheet were removed with a damp tissue. The membranes were incubated in the dark for 5 minutes. The following steps were performed in the dark: the membrane was dried between two 3 MM Whatman filter papers, but not to complete dryness. The damp membrane then was transferred to a new PhotoGene™ development folder. The membrane was preincubated at 37°C for 5-15 minutes. The membranes were exposed to Kodak X-ray film for several different time intervals ranging from 10 to 30 minutes to collect data at different signal intensities.

b) Radiometric detection of hybrids: Radiometric detection was accomplished by the method of the RCMP (RCMP, R. Fourney, personal communication). After hybridization, the membranes were washed under increasing stringency: the membranes were briefly washed with 100 mL of 2 × SSC, twice for 15 minutes at 68°C with 2 × SSC, 0.5% SDS, twice for 15 minutes at 68°C with 0.2 × SSC, 0.5% SDS, and twice for 15 minutes at 68°C with 0.1 × SSC, 0.1% SDS. After these washings, the membranes were briefly rinsed with 0.1 × SSC at room temperature, blotted between Whatman 3 MM filter papers, and wrapped in Saran wrap. The membranes were exposed to Kodak X-ray film at -70°C for varying lengths of time, depending on the anticipated strength of the signal.

The fragment sizes from at least three blots were averaged after measuring distances of band migration in comparing them with the size of the λ DNA, λ DNA digested with *Kpn*I and 1 kb ladder standard molecular size markers, which were electrophoresed on each gel under similar conditions of electrophoresis. Reproducibility of ribotypes generated by *Sma*I was examined by analysis of the same bacteria strain on three separated occasions. The stability of ribotypes was studied by multiple passage of isolates.

3. RESULTS

3.1. Antimicrobial Susceptibilities

The susceptibility to six antimicrobial agents of the five groups of isolates, which comprised 13 A/S classes, was retrospectively evaluated in order to determine whether isolates within a given A/S class could be distinguished using this criterium. All isolates in the data base from 1988 to 1992 were examined so as to show that the isolates selected for further molecular study were representative of the greater population.

The susceptibilities, as determined by MIC range, MIC_{50} and MIC_{90} to 6 antimicrobial agents of 633 PCU(H) isolates of which 58 were serovar IB-1 and 575 were serovar IB-2, are listed in Table 2; the distribution of penicillin MICs is shown in Figure 4. Based on the MIC results, PCU and PCUH isolates are homogeneous and rarely differ either in MIC range, or MIC_{50} or MIC_{90} beyond a two fold dilution difference (Table 2). This difference is not considered to be clinically significantly different. Although the MIC distribution between PCU and PCUH isolates differed somewhat (Figure 4), this may reflect the small sample size of PCUH isolates. However, the statistical analysis using the Kolmogorov-Smirnov test indicated that the MIC distributions were not significantly different ($P \geq 0.005$). PCU and PCUH isolates also have identical MICs irrespective of their requirement for hypoxanthine.

Table 2. Susceptibility of 58 PCU(H) isolates of serovar IB-1 and 575 PCU(H) isolates of serovar IB-2 to six antibiotics

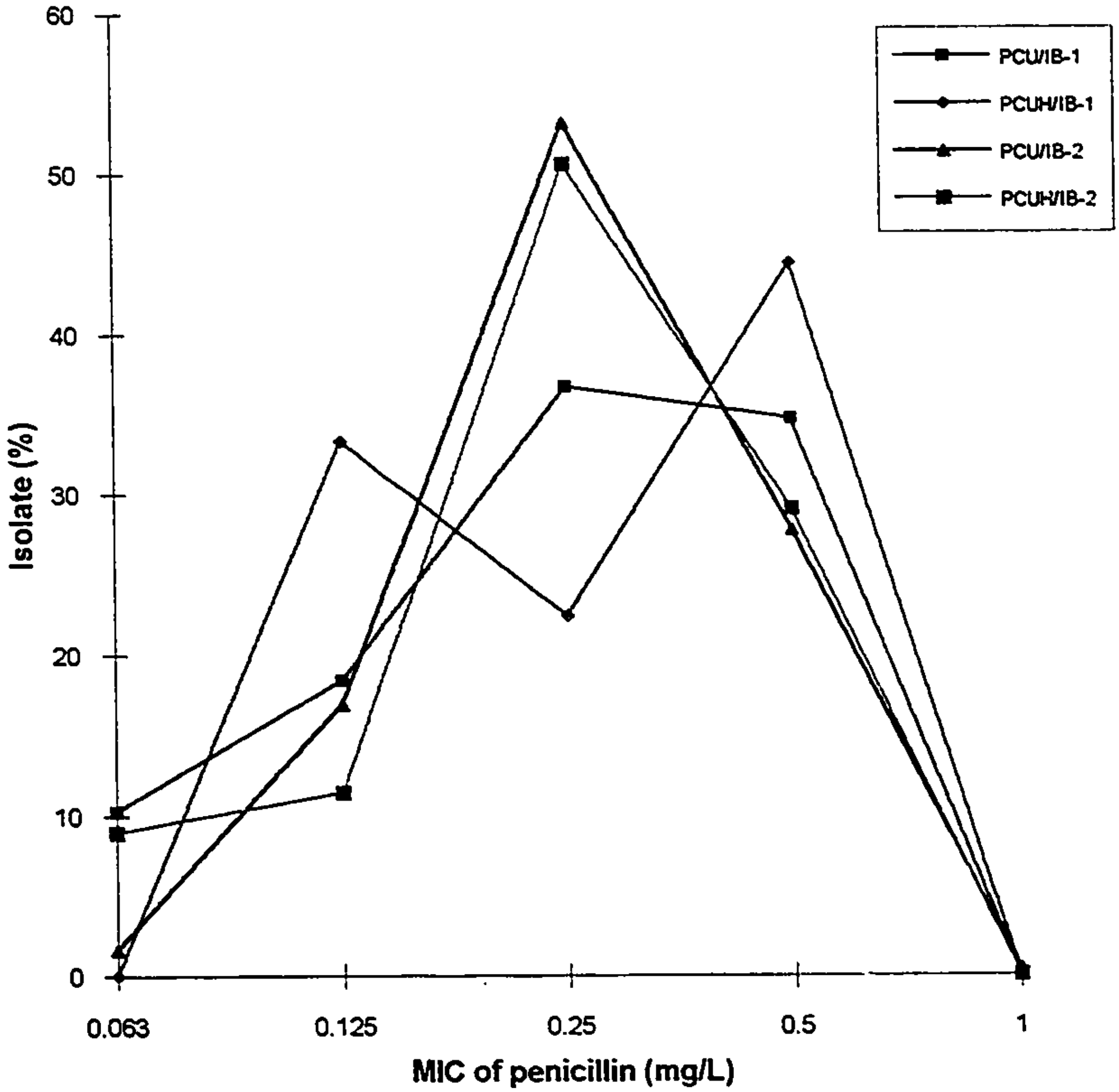
Antimicrobial agent	MIC[a] (mg/L)	Serovar IB-1[d]		Serovar IB-2[d]	
		PCU	PCUH	PCU	PCUH
Penicillin	Range	0.063-0.5	0.125-0.5	0.063-1.0	0.063-0.5
	MIC$_{50}$[b]	0.25	0.25	0.25	0.25
	MIC$_{90}$[c]	0.5	0.5	0.5	0.5
Tetracycline	Range	0.125-2.0	0.25-1.0	0.125-1.0	0.125-1.0
	MIC$_{50}$	0.5	0.5	0.5	0.5
	MIC$_{90}$	1.0	1.0	1.0	0.5
Erythromycin	Range	≤0.063-1.0	0.063-2.0	≤0.063-2.0	0.063-1.0
	MIC$_{50}$	0.5	0.5	0.5	0.5
	MIC$_{90}$	1.0	2.0	1.0	1.0
Spectinomycin	Range	8.0-32.0	16.0	8.0-32.0	8.0-32.0
	MIC$_{50}$	16.0	16.0	16.0	16.0
	MIC$_{90}$	16.0	16.0	32.0	16.0
Cefoxitin	Range	0.25-1.0	0.5-2.0	0.25-4.0	0.25-1.0
	MIC$_{50}$	0.5	0.5	1.0	0.5
	MIC$_{90}$	1.0	2.0	1.0	1.0
Ceftriaxone	Range	0.0005-0.016	0.001-0.016	0.0005-0.0032	0.0005-0.016
	MIC$_{50}$	0.004	0.004	0.004	0.004
	MIC$_{90}$	0.008	0.016	0.008	0.008

a: MIC = minimum inhibitory concentration to a given antibiotic.

b: MIC$_{50}$ = MIC at which 50% of isolates were inhibited.

c: MIC$_{90}$ = MIC at which 90% of isolates were inhibited.

d: PCU/IB-1 n=49; PCUH/IB-1 n=9; PCU/IB-2 n=496; PCUH/IB-2 n=79.



The distribution of penicillin MICs of PCU(H)/IB-1 and PCU(H)/IB-2 isolates

Figure 4

The 552 arginine-requiring isolates of serovar IA-2, which were collected between 1988 and 1992, could be distinguished by their specific arginine-requiring auxotypes. Most were OUH (459) isolates, followed by CUH (61) isolates, and OH (32) isolates. The susceptibilities of these isolates to 6 antimicrobial agents as determined by the MIC range, MIC_{50} and MIC_{90} are listed in Table 3. These isolates had similar MICs and their MIC distributions to penicillin (Figure 5) were also similar. The only notable difference was spectinomycin MICs where OUH and CUH isolates were more susceptible (MICs = 2.0 or 4.0 mg/L) at the lower end of the range. Based on MIC analysis, the arginine-requiring isolates are homogeneous and can not be differentiated by antimicrobial susceptibility; the statistical analysis (Kolmogorov-Smirnov test) also indicated that the MIC distribution were not significantly different ($P \geq 0.005$).

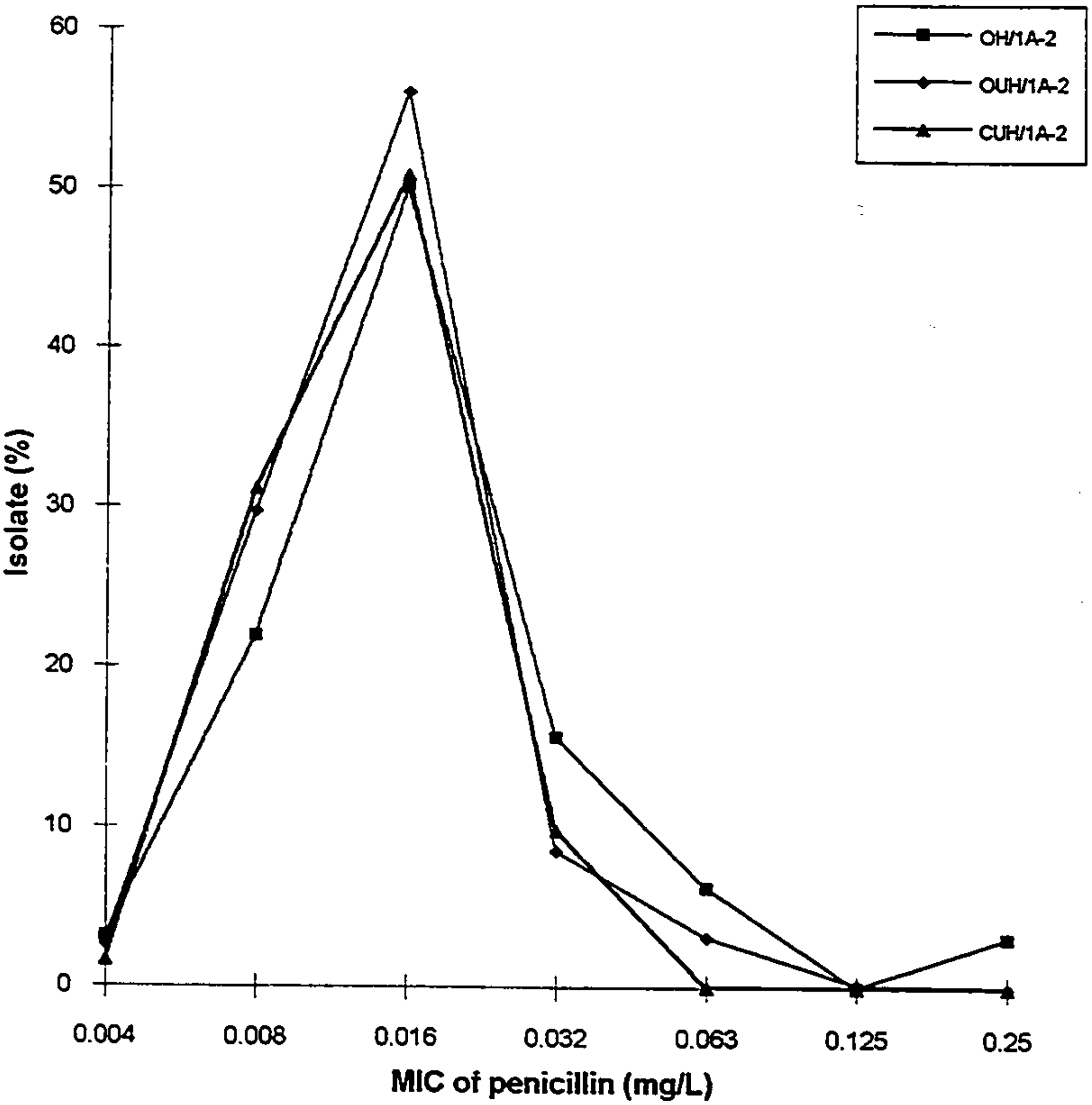
The susceptibilities of 546 isolates of serovar IB-5 or IB-7, which were either proline-requiring or non-requiring, to 6 antimicrobial agents, are listed in Table 4 and the MIC distributions to penicillin are shown in Figure 6. Overall, NR isolates were more sensitive at MIC_{50} to penicillin, erythromycin and ceftriaxone irrespective of serovar (although there were not statistically significantly different, $P \geq 0.005$, but were clinically significantly different). A first analysis indicated that the isolates could be grouped by auxotype, particularly when considering susceptibility to cefoxitin and ceftriaxone where NR isolates tended to be more susceptible than P isolates although there were not significantly different by statistical analysis. Within a given auxotype, serovar IB-5 and IB-7, had same antimicrobial susceptibilities. The 34 NR/IB-5 and 18 NR/IB-7 isolates had similar MICs (Table 4) and their MIC distributions to penicillin (Figure 6) were similar. The most notable difference between NR/IB-5 and NR/IB-7 isolates was that NR/IB-7 isolates more resistance to tetracycline at MIC_{50} than NR/IB-5 isolates.

Table 3. Susceptibility of 552 arginine-requiring isolates of serovar IA-2 to six antimicrobial agents

Antimicrobial agent	MIC[a] (mg/L)	Auxotype[d]		
		OUH	CUH	OH
Penicillin	Range	0.004-0.25	0.004-0.032	0.004-0.25
	MIC_{50}[b]	0.016	0.016	0.016
	MIC_{90}[c]	0.016	0.016	0.032
Tetracycline	Range	0.063-1.0	0.063-0.5	0.125-0.5
	MIC_{50}	0.25	0.25	0.25
	MIC_{90}	0.5	0.5	0.5
Erythromycin	Range	0.032-2.0	0.063-1.0	0.125-0.5
	MIC_{50}	0.5	0.5	0.5
	MIC_{90}	1.0	1.0	1.0
Spectinomycin	Range	4.0-32.0	2.0-32.0	16.0-32.0
	MIC_{50}	32.0	32.0	16.0
	MIC_{90}	32.0	32.0	32.0
Cefoxitin	Range	0.004-0.5	0.125-0.25	0.004-1.0
	MIC_{50}	0.25	0.25	0.25
	MIC_{90}	0.25	0.25	0.25
Ceftriaxone	Range	≤0.00025-0.002	≤0.00025-0.002	≤0.00025-0.002
	MIC_{50}	0.00025	0.00025	0.00025
	MIC_{90}	0.0005	0.0005	0.0005

a, b, c: Abbreviations and definitions as in Table 2.

d: OUH n=459, CUH n=61, OH n=32.



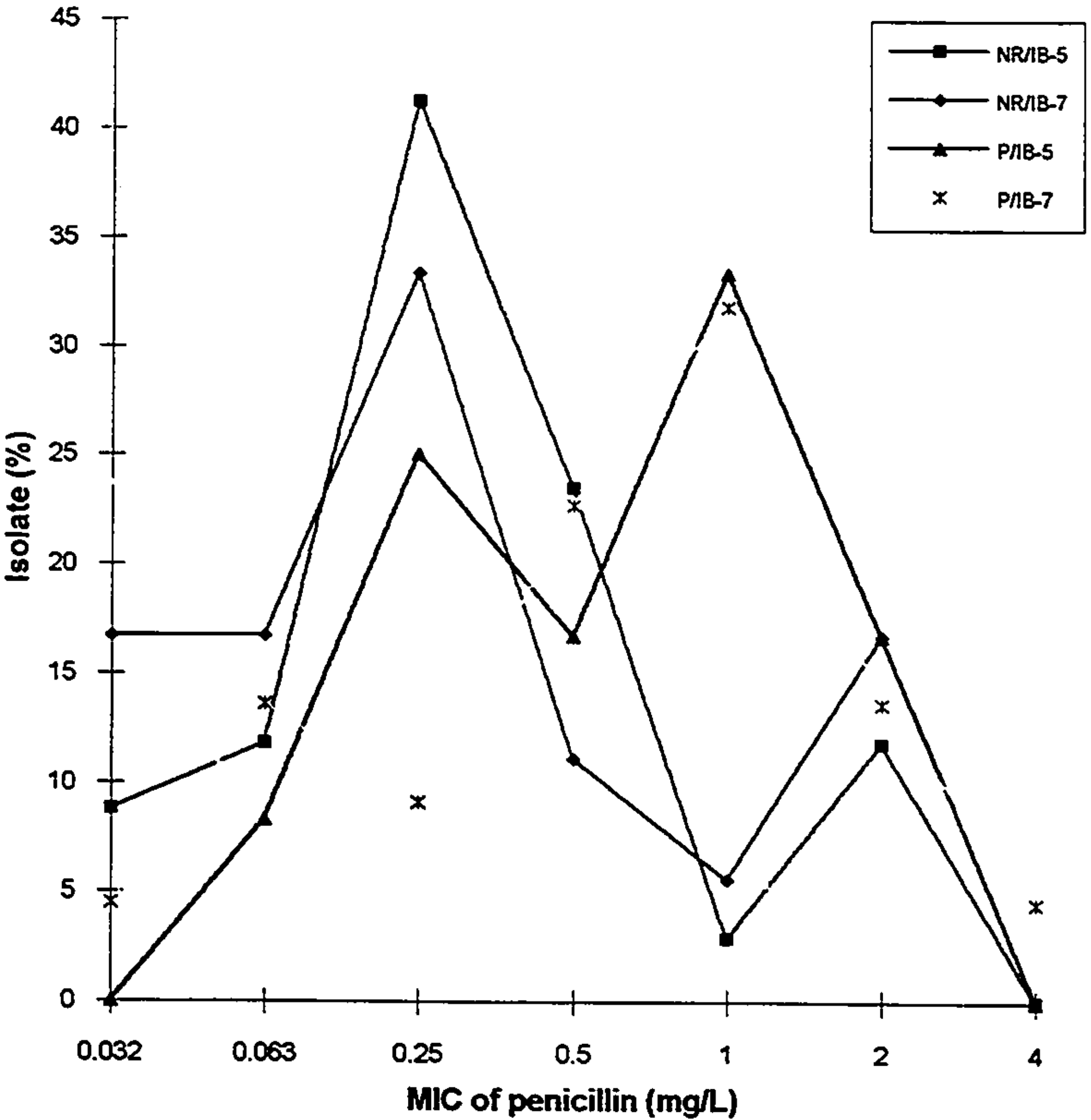
The distribution of penicillin MICs of 552 arginine-requiring isolates

Figure 5

Table 4. Susceptibility of 74 NR/IB-5, 297 NR/IB-7, 64 P/IB-5 and 111 P/IB-7 gonococcal isolates to six antimicrobial agents

Antimicrobial agent	MIC[a] (mg/L)	A/S			
		NR/IB-5	NR/IB-7	P/IB-5	P/IB-7
Penicillin	Range	0.032-2.0	0.032-2.0	0.063-2.0	0.032-4.0
	MIC_{50}[b]	0.125	0.125	0.5	0.5
	MIC_{90}[c]	2.0	2.0	2.0	2.0
Tetracycline	Range	0.125-4.0	0.25-8.0	0.5-4.0	0.25-4.0
	MIC_{50}	0.25	1.0	2.0	1.0
	MIC_{90}	4.0	4.0	4.0	4.0
Erythromycin	Range	0.125-4.0	0.125-4.0	0.125-4.0	0.25-4.0
	MIC_{50}	0.5	0.5	2.0	2.0
	MIC_{90}	4.0	2.0	2.0	4.0
Spectinomycin	Range	16.0-32.0	16.0-32.0	16.0-32.0	16.0-32.0
	MIC_{50}	32.0	32.0	32.0	32.0
	MIC_{90}	32.0	32.0	32.0	32.0
Cefoxitin	Range	0.25-1.0	0.25-1.0	0.25-2.0	0.25-4.0
	MIC_{50}	0.5	0.5	0.5	1.0
	MIC_{90}	1.0	1.0	2.0	4.0
Ceftriaxone	Range	0.0005-0.032	0.0005-0.016	0.001-0.032	0.0005-0.032
	MIC_{50}	0.002	0.001	0.008	0.008
	MIC_{90}	0.008	0.016	0.008	0.016

a, b, c: Abbreviations and definitions as in Table 2.



The distribution of penicillin MICs of NR/IB-5, NR/IB-7, P/IB-5 and P/IB-7 isolates

Figure 6

The MICs of P/IB-5 and P/IB-7 were similar (Table 4). Although their MIC distribution to penicillin (Figure 6) differed somewhat, there were not statistically significantly different ($P \geq 0.005$). Based on their antimicrobial susceptibilities, the idea that serovars IB-5 and IB-7 isolates should be grouped, irrespective of auxotype, may not be valid. However, within an auxotype, serovar IB-5 and IB-7 may be grouped.

The MICs of 10 NR/IA-5 gonococcal isolates were listed on Table A-6. Except for the differences in penicillin resistance between PPNG, non-PPNG isolates and tetracycline susceptibility of the TRNG isolates, the MICs of these isolates were similar. Based on A/S class, plasmid content, geographic distribution and MIC analysis, this group could not be distinguished.

MIC analysis did not distinguish between the 12 NR/IB-3 gonococcal isolates (Table A-7; i.e. the same within a two fold dilution).

To sum up, antimicrobial susceptibility is not an ideal criteria to discriminate isolates of the same A/S class either from population studies or from outbreaks.

3.2. Restriction Endonuclease Analysis

3.2.1. *Chromosomal DNA total digest patterns*

Thirty gonococcal chromosomal DNAs were preliminary analyzed with restriction endonucleases *Ava*II, *Hind*II, and *Sma*I in order to determine which enzymes would provide the greater discrimination. These enzymes were primarily selected for their ability to discriminate in ribotyping. Restriction endonucleases such as *Ava*II and *Hind*II were not useful, since they gave insufficient banding patterns for differentiation in ribotyping, while *Sma*I was found to produce highly resolved fragments suitable for analysis. Hence, *Sma*I was selected as the most suitable enzyme for differentiating the one hundred isolates. At this point, we retrospectively analysed gels of chromosomal DNA digested with *Sma*I and run under comparable conditions to evaluate RE analysis as a subtyping method.

Chromosomal DNA from one hundred isolates of *N. gonorrhoeae* was digested with *Sma*I (recognition sequence CCC▼GGG), which cut with a high frequency to give multiple electrophoretic band patterns. The endonuclease *Sma*I cleaved gonococcal chromosomal DNA at a high frequency to provide optimal restriction patterns of 80-100 bands, of sizes ranging from 2.0 to 30.0 kb (e.g. Figures 7, 8). Visual comparisons of the *Sma*I digestion patterns for the 100 isolates tested revealed differences between isolates. A fragment size range of between 9.7 and 27.0 kb pairs was selected for analysis as there were sufficient differences between isolates in this size range for strain differentiation. DNA fragments <9.7 kb pairs generally contained low intensity stained bands.

Reproducibility of RE patterns was illustrated by identical fingerprints when DNA of all isolates were prepared and digested on four separate occasions. The RE patterns remained stable after repeated passages *in vitro* (data not shown).

Twenty different restriction enzyme patterns were identified among the 100 isolates comprising the 13 A/S classes tested (Table 5). The RE patterns A and E dominated, representing 54% (27/100 for each pattern) of the 100 isolates investigated. One isolate was represented in each of the RE patterns B, C, D, H, I, J, K, O, P, and R. The 20 RE patterns possessed 9 common DNA fragments which corresponded to sizes of approximately 27.0, 25.5, 19.0, 17.6, 13.6, 12.4, 11.5, 10.6, and 9.7 kb, respectively.

3.2.2. *Comparison RE patterns with A/S class*

Among the 15 PCU(H)/IB-1 isolates which were selected for RE analysis, three different patterns, A, B, and C were recognized (Table 6, Figure 7). The RE pattern A dominated, representing 80% (13/15) of the 15 isolates investigated (Figure 7 Lanes 1-11, 13, 14). One isolate was represented in each of the RE patterns B (PCUH/IB-1, 7861; Figure 7, Lane 16) and C (PCUH/IB-1, 882779; Figure 7, Lane 15).

Restriction patterns for 15 PCU(H)/IB-2 gonococcal isolates were determined (Table 7 and Figure 8). Two RE patterns were found in this group, A and D. 93% (14/15) isolates were represented by RE pattern A (Figure 8, Lanes 1-8, 10-13, 15, 16) which also represented most (80%) isolates in PCU(H)/IB-1 group. RE pattern D represented one PCU/IB-2 isolate 881996 (Figure 8, Lane 14). Restriction enzyme patterns A and D showed a minor differences of two fragments (18.2 and 16.2 kb).

The PCU(H) group of isolates had 12 DNA fragments in common (27.0, 25.5, 22.7,

Table 5. Restriction endonuclease patterns of 100 gonococcal isolates

Isolate RE Pattern (No.)[a]	Fragment Size[b] (kb)																											
	27.0	25.5	22.7	22.0	21.6	21.0	20.0	19.0	18.2	17.6	17.0	16.6	16.0	15.1	14.5	14.0	13.6	13.4	13.0	12.6	12.4	12.0	11.8	11.5	11.2	10.6	10.4	9.7
A (27)	+	+	+		+		+	+		+		+	+	+		+	+			+	+			+	+	+		+
B (1)	+	+	+				+	+		+				+		+	+			+	+			+	+	+		+
C (1)	+	+	+		+	+		+		+		+		+	+		+		+		+		+	+	+	+	+	+
D (1)	+	+	+		+		+	+	+	+		+	+	+		+	+			+	+		+	+	+	+		+
E (27)	+	+	+		+	+		+		+		+	+	+	+	+	+		+		+		+	+	+	+	+	+
F (4)	+	+			+		+	+		+		+	+		+		+				+			+	+	+	+	+
G (2)	+	+			+		+	+		+		+	+		+		+		+		+			+	+	+	+	+
H (1)	+	+			+		+	+		+		+	+		+		+				+	+		+	+	+	+	+
I (1)	+	+			+		+	+		+		+	+		+		+				+		+	+	+	+	+	+
J (1)	+	+			+		+	+		+		+	+		+		+	+			+		+	+	+	+	+	+
K (1)	+	+			+		+	+		+		+	+		+	+	+	+			+	+	+	+	+	+	+	+
L (5)	+	+	+	+		+	+	+		+		+	+	+	+	+	+				+	+	+	+		+		+
M (2)	+	+	+	+		+	+	+		+		+	+	+	+	+	+	+			+	+	+	+	+	+	+	+
N (2)	+	+	+	+		+	+	+		+	+	+	+	+		+	+				+	+	+	+		+		+
O (1)	+	+		+		+	+	+		+	+		+	+	+	+	+				+	+	+	+		+	+	+
P (1)	+	+	+	+		+	+	+		+	+	+	+	+	+	+	+	+			+		+	+		+		+
Q (9)	+	+					+	+		+			+		+	+	+	+	+		+		+	+	+	+	+	+
R (1)	+	+		+			+	+		+			+		+	+	+	+	+		+	+		+	+	+	+	+
S (8)	+	+		+				+		+				+	+	+	+		+		+			+	+	+	+	+
T (4)	+	+		+				+		+		+		+	+	+	+		+		+	+		+	+	+	+	+

[a]: Number of isolates with particular RE pattern.

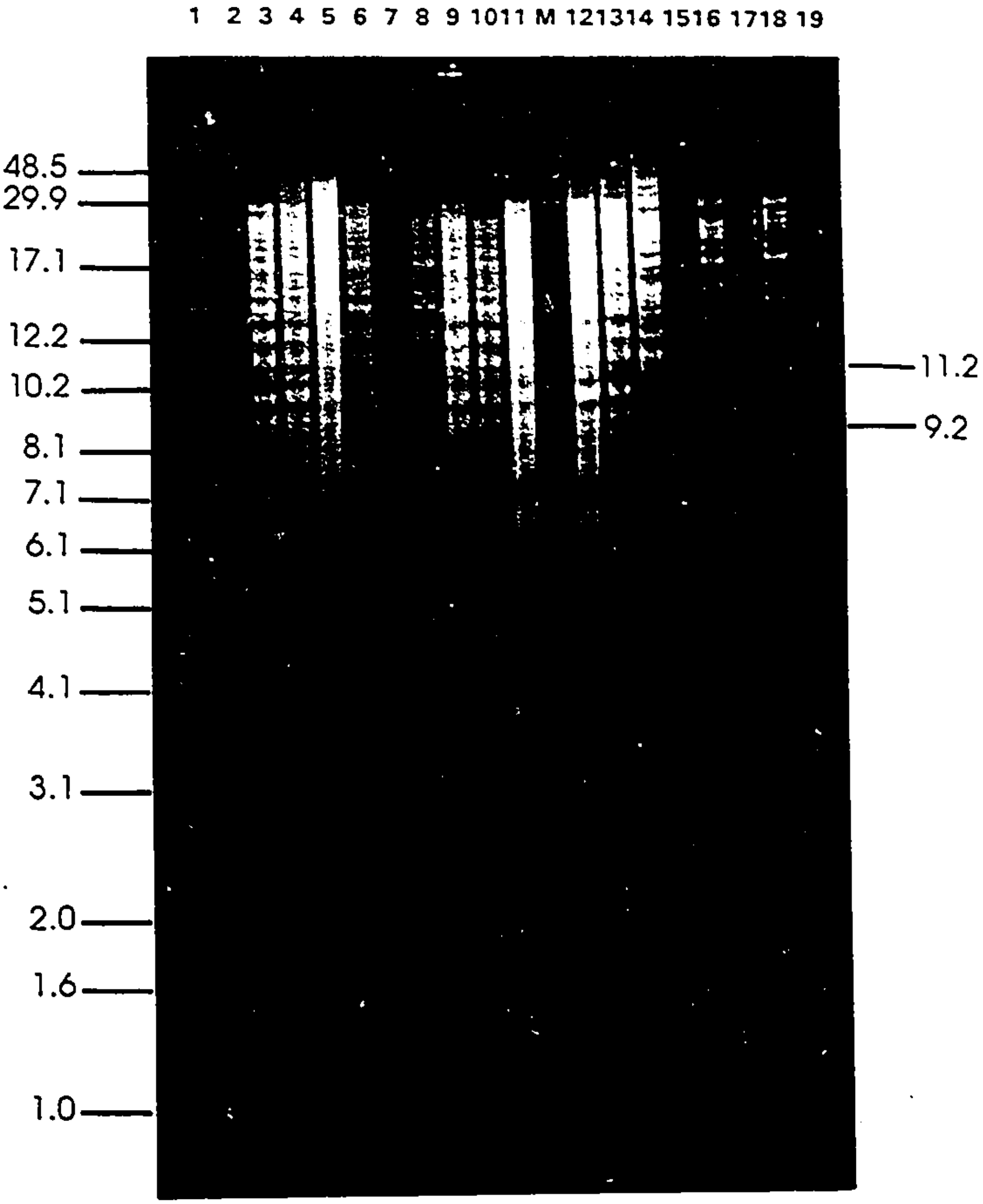
[b]: Fragment range selected for comparison was from 27.0 to 9.7 kb.

Table 6. Fragment sizes of *Sma*I digested chromosomal DNA of 15 A/S class PCU(H)/IB-1 gonococcal isolates

Isolate	A/S Class	Fragment Size[a] (kb)																												Pattern No.
		27.0	25.5	22.7	22.0	21.6	21.0	20.0	19.0	18.2	17.6	17.0	16.6	16.0	15.1	14.5	14.0	13.6	13.4	13.0	12.6	12.4	12.0	11.8	11.5	11.2	10.6	10.4	9.7	
880067	PCU/IB-1																													
880128	PCU/IB-1																													
880263	PCU/IB-1																													
880724	PCU/IB-1																													
881587	PCU/IB-1																													
883036	PCU/IB-1																													
883077	PCU/IB-1	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	-	-	+	+	+	-	+	A
883632	PCU/IB-1																													
3703	PCUH/IB-1																													
880596	PCUH/IB-1																													
880687	PCUH/IB-1																													
882989	PCUH/IB-1																													
883353	PCUH/IB-1																													
7861	PCUH/IB-1	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	+	-	-	+	+	-	-	+	+	+	-	+	B
882779	PCUH/IB-1	+	+	+	-	+	+	-	+	-	+	-	+	-	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	C

[a]: Fragment range selected 28 -9.7 kb.

Figure 7. Restriction enzyme patterns of 15 A/S class PCU(H)/IB-1 gonococcal isolates after chromosomal DNA digestion with *Sma*I. **M**=size marker (λ DNA, λ DNA digested with *Kpn*I and 1 kb ladder). Lane **1**, 880067 (RE pattern=A); Lane **2**, 880128 (A); Lane **3**, 880263 (A); Lane **4**, 880724 (A); Lane **5**, 883036 (A); Lane **6**, 883077 (A); Lane **7**, 883632 (A); Lane **8**, 880596 (A); Lane **9**, 880687 (A); Lane **10**, 882989 (A); Lane **11**, 883703 (A); Lane **12**, 883353 (A); Lane **13**, 881587 (A); Lane **14**, 882779 (C); Lane **15**, 7861 (B); Lane **16**, 881359 (K); Lane **17**, 882610 (E); Lane **18**, 883353 (A); Lane **19**, 7.2 kb *Bam*HI-*Pst*I fragment carrying *rrn*B rRNA operon.



1
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7
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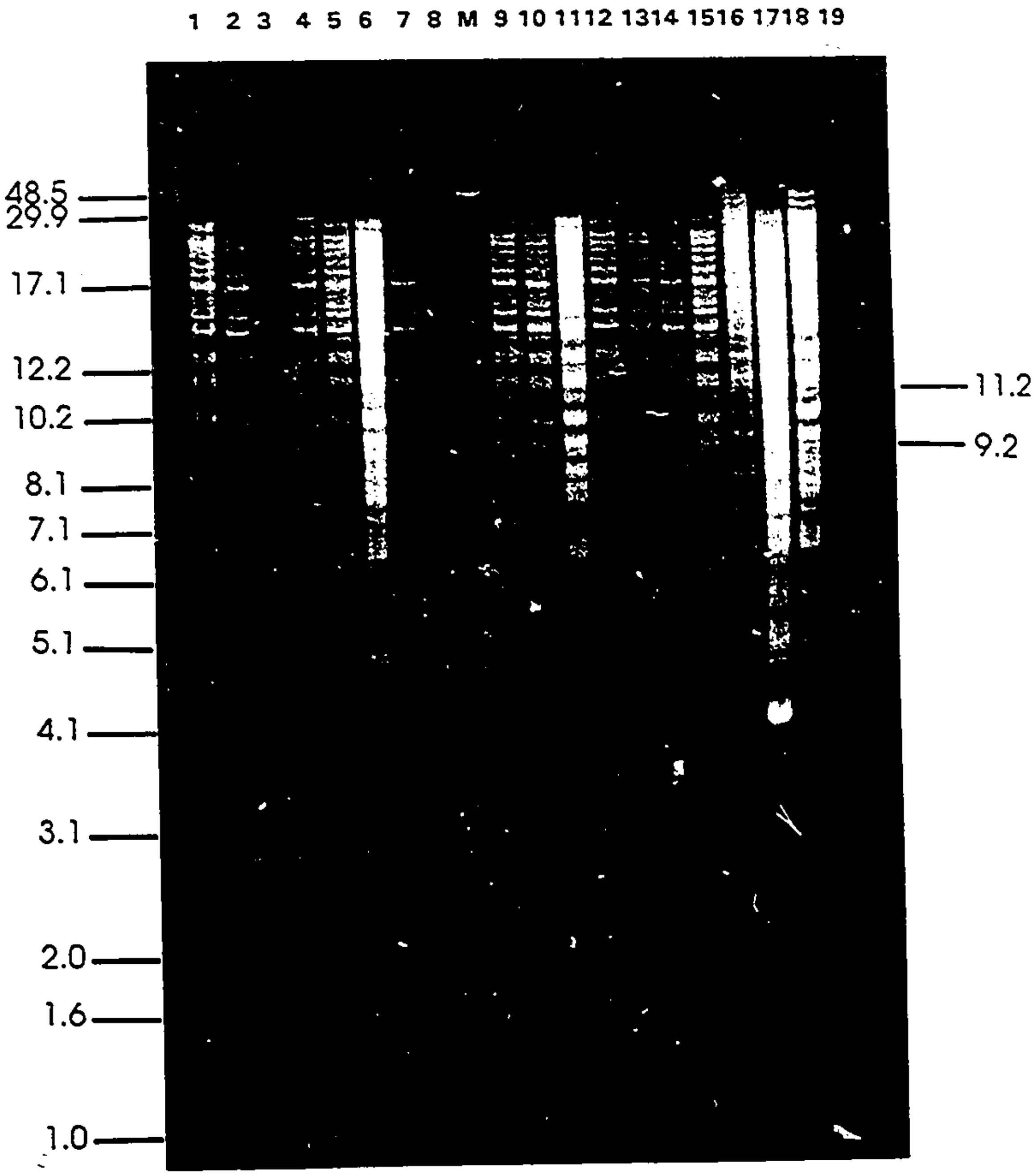
9
10
11
M
12
13
14
15
16
17
18
19
48.5
29.9
17.1
12.2
10.2
8.1
7.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0
11.2
9.2

Table 7. Fragment sizes of *Sma*I digested chromosomal DNA of 15 A/S class PCU(H)/IB-2

Isolate	A/S Class	Fragment Size[a] (kb)																												Pattern No.
		27.0	25.5	22.7	22.0	21.6	21.0	20.0	19.0	18.2	17.6	17.0	16.6	16.0	15.1	14.5	14.0	13.6	13.4	13.0	12.6	12.4	12.0	11.8	11.5	11.2	10.6	10.4	9.7	
880074	PCU/IB-2																													
880916	PCU/IB-2																													
881777	PCU/IB-2																													
881985	PCU/IB-2																													
882988	PCU/IB-2																													
883329	PCU/IB-2																													
880071	PCUH/IB-2																													
881542	PCUH/IB-2	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	-	+	+	+	+	-	+	A
881717	PCUH/IB-2																													
882191	PCUH/IB-2																													
883238	PCUH/IB-2																													
883355	PCUH/IB-2																													
883766	PCUH/IB-2																													
8281	PCUH/IB-2																													
881996	PCU/IB-2	+	+	+	-	+	-	+	+	+	+	-	+	+	+	-	+	+	-	-	+	+	-	+	+	+	+	-	+	D

[a]: Fragment range was selected from 27.0 to 9.7 kb.

Figure 8. Restriction enzyme patterns of 15 A/S class PCU(H)/IB-2 gonococcal isolates after DNA digestion with *Sma*I. M=size marker (λ DNA, λ DNA digested with *Kpn*I and 1 kb ladder). Lane **1**, 883355 (RE pattern=A); Lane **2**, 880071 (A); Lane 3, 881542 (A); Lane 4, 881717 (A); Lane 5, 882191 (A); Lane **6**, 883238 (A); Lane 7, 883766 (A); Lane **8**, 8281 (A); Lane **9**, 880074 (A); Lane **10**, 880916 (A); Lane **11**, 881777 (A); Lane **12**, 881985 (A); Lane **13**, 881996 (D); Lane **14**, 882988 (A); Lane **15**, 883329 (A); Lane **16**, 882610 (E); Lane **17**, 881359 (K); Lane **18**, 8883353 (A); Lane **19**, 7.2 kb *Bam*HI-*Pst*I fragment carrying *rrn*B rRNA operon.


1 2 3 4 5 6 7 8 M 9 10 11 12 13 14 15 16 17 18 19
48.5
29.9
17.1
12.2
10.2
8.1
7.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0
11.2
9.2

19.0, 17.6, 15.1, 13.6, 12.4, 11.5, 11.2, 10.6, and 9.7 kb). Excluding the 9 common DNA fragments for all isolates, the 3 common DNA fragments representative of this group were 22.7, 15.1 and 11.2 kb. These results indicated that isolates of PCU(H) are quite homogeneous irrespective of whether their serovar is IB-1 or IB-2.

RE analysis for 27 arginine-requiring gonococcal isolates of serovar IA-2 demonstrated that only one RE pattern E (data not shown) was produced in this group irrespective of the specific arginine-requiring auxotypes. Excluding the 9 common fragments seen in all isolates (Table 8), the arginine-requiring group had 12 identical fragments: 22.7, 21.6, 21.0, 16.6, 16.0, 15.1, 14.5, 14.0, 13.0, 11.8, 11.2, and 10.4 kb. Two of these fragments (22.7 and 11.2 kb) were also common to PCU(H) isolates. This further confirmed that the arginine-requiring isolates of serovar IA-2 are homogeneous based on RE analysis.

The molecular dynamics personal densitometer was used to scan 15 arginine-requiring isolates to see whether this method might be helpful for analysis. Results showed most bands (peaks in the figure) that were shared by all the isolates (Figure 9 showed 6 isolates results) and indicated these fifteen isolates are clonal. The analysis of this method was done by computer and was easy to handle. Regrettably, the machine was only available to use for one days, but the experiment indicates that RE analysis will be simple using computerized imaging devices.

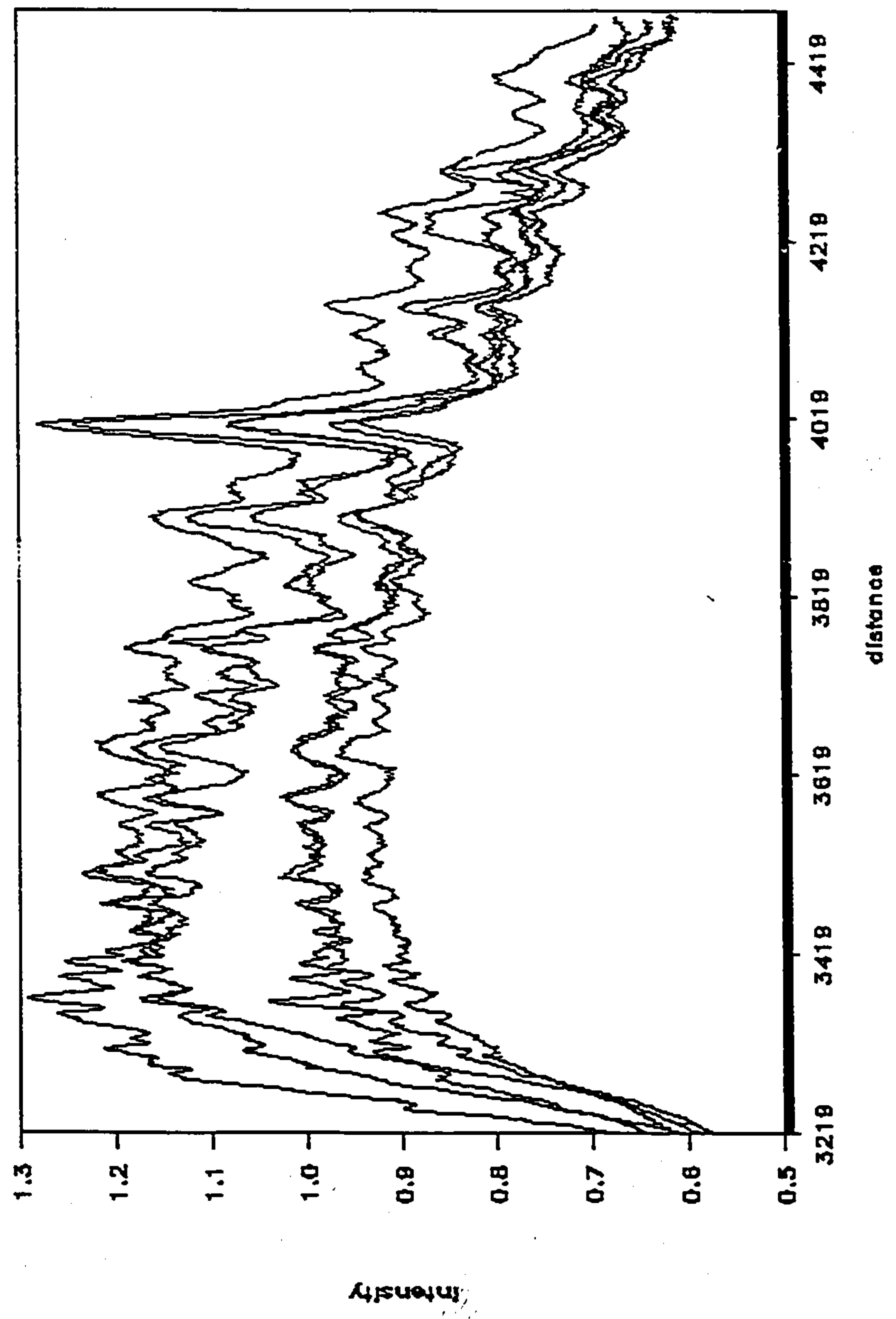
Ten gonococcal isolates which were NR and serovar IB-5 (n=4) or IB-7 (n=6) were selected from Canadian gonococcal population studies. RE analysis subtyped them into 6 groups (data not shown). Excluding the 9 common DNA fragments shared by all isolates, these strains had 7 DNA fragments of 21.6, 20.0, 16.6, 16.0, 14.5, 11.2, and 10.4 kb in common (Table 9). Some of these common bands were also shared by other A/S classes (e.g. Arg/IA-2).

Table 8. Fragment sizes of *Sma*I digested chromosomal DNA of 27 arginine-requiring gonococcal isolates of serovar IA-2

Isolate	A/S Class	Fragment Size[a] (kb)																												Pattern No.
		27.0	25.5	22.7	22.0	21.6	21.0	20.0	19.0	18.2	17.6	17.0	16.6	16.0	15.1	14.5	14.0	13.6	13.4	13.0	12.6	12.4	12.0	11.8	11.5	11.2	10.6	10.4	9.7	
880375	OUH/IA-2																													
880420	OUH/IA-2																													
881035	OUH/IA-2																													
882208	OUH/IA-2																													
882306	OUH/IA-2																													
882245	OUH/IA-2	+	+	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	E
882398	OUH/IA-2																													
882521	OUH/IA-2																													
883122	OUH/IA-2																													
883171	OUH/IA-2																													
883342	OUH/IA-2																													
883463	OUH/IA-2																													
880270	CUH/IA-2																													
880379	CUH/IA-2																													
880454	CUH/IA-2																													
881540	CUH/IA-2	+	+	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	E
882132	CUH/IA-2																													
882610	CUH/IA-2																													
883099	CUH/IA-2																													
883729	CUH/IA-2																													
881097	OH/IA-2																													
881342	OH/IA-2																													
881506	OH/IA-2																													
882367	OH/IA-2	+	+	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	E
882642	OH/IA-2																													
882794	OH/IA-2																													
883212	OH/IA-2																													

a): Fragment range was selected from 27.0 to 9.7 kb.

Figure 9. Densitometric scanning of 6 arginine-requiring gonococcal isolates electrophoretograms of restriction fragments after chromosomal DNA digestion with *Sma*I. Peaks of DNA fragments reflect the band density.



intensity
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
3219
3419
3619
3819
4019
4219
4419
distance

Table 9. Fragment sizes of *Sma*I digested chromosomal DNA of 4 A/S class NR/IB-5 and 6 A/S class NR/IB-7 gonococcal isolates

Isolate	A/S Class	Fragment Size[a] (kb)																												Pattern No.
		27.0	25.5	23.7	22.0	21.6	21.0	20.0	19.0	18.2	17.6	17.0	16.6	16.0	15.1	14.5	14.0	13.6	13.4	13.0	12.6	12.4	12.0	11.8	11.5	11.2	10.6	10.4	9.7	
880466	NR/IB-5																													
880644	NR/IB-5	+	+	·	·	+	·	+	+	·	+	·	+	+	·	+	·	+	·	·	·	+	·	·	+	+	+	+	+	F
883665	NR/IB-7																													
4209	NR/IB-7																													
882449	NR/IB-7	+	+	·	·	+	·	+	+	·	+	·	+	+	·	+	·	+	·	+	·	+	·	·	+	+	+	+	+	G
883535	NR/IB-7																													
880107	NR/IB-5	+	+	·	·	+	·	+	+	·	+	·	+	+	·	+	·	+	·	·	·	+	+	·	+	+	+	+	+	H
3351	NR/IB-5	+	+	·	·	+	·	+	+	·	+	·	+	+	·	+	·	+	·	·	·	+	·	+	+	+	+	+	+	I
881095	NR/IB-7	+	+	·	·	+	·	+	+	·	+	·	+	+	·	+	·	+	+	·	·	+	·	+	+	+	+	+	+	J
881359	NR/IB-7	+	+	·	·	+	·	+	+	·	+	·	+	+	·	+	+	+	+	·	·	+	+	+	+	+	+	+	+	K

[a]: Fragment range was selected from 27.0 to 9.7 kb.

The 11 proline-requiring isolates having serovars IB-5 (n=6) and IB-7 (n=5) were subtyped into 5 different RE patterns (RE pattern L, M, N, O, and P). One P/IB-5 and 4 P/IB-7 isolates were classified into RE pattern L (Figure A-1, Lane 2); one P/IB-5 and 1 P/IB-7 isolates were represented by RE pattern M (Figure A-1, Lanes 1 and 7); two P/IB-5 isolates belonged to RE pattern N (Figure A-1, Lanes 4 and 5); one P/IB-5 isolate belonged to RE pattern O (Figure A-1, Lane 6); and one P/IB-5 isolate belonged to RE pattern P (Figure A-1, Lane 3). These isolates carried 7 common DNA fragments (Table 10): 22.0, 21.0, 20.0, 16.0, 15.1, 14.0, and 11.8 kb, which differed from the 7 common DNA fragments of the NR/IB-5 and NR/IB-7 group.

Overall serovar IB-5 and IB-7 isolates having either auxotype NR or P, carried two common DNA fragments: 20.0 and 16.0 kb; the 16.0 kb fragment was also noted in Arg-requiring isolates. Isolates of serovars IB-5 and IB-7 were extremely heterogeneous as judged by RE analysis.

RE analysis of ten outbreak isolates of A/S class NR/IA-5 from the Toronto area showed one predominant RE type, pattern Q (Figure A-2, Lanes 1-6, 8, 9), for 90% of the isolates (Table 11). RE pattern R (Figure A-2, Lane 7) was represented by PP/TRNG isolate (5283). RE patterns Q and R differed only regarding the presence or absence of three DNA fragments of 22.0, 12.0, and 11.8 kb. Isolates in NR/IA-5 group are homogeneous based on RE analysis.

Twelve gonococcal isolates of A/S class NR/IB-3, including six from Manitoba area and six from Ontario area were typed into two RE patterns, S (Figure A-3, Lanes 1 and 2) and T (Figure A-3, Lanes 3-6). These two patterns differed by the presence of two more fragments of 16.6 and 12.0 kb in RE pattern T (Table 12).

Table 10. Fragment sizes of *Sma*I digested chromosomal DNA of 6 A/S class P/IB-5 and 5 A/S class P/IB-7 gonococcal isolates

Isolate	A/S Class	Fragment Size[a] (kb)																												Pattern No.
		27.0	25.5	22.7	22.0	21.6	21.0	20.0	19.0	18.2	17.6	17.0	16.6	16.0	15.1	14.5	14.0	13.6	13.4	13.0	12.6	12.4	12.0	11.8	11.5	11.2	10.6	10.4	9.7	
881051	P/IB-5																													
880972	P/IB-7																													
883141	P/IB-7	+	+	+	+	.	+	+	+	.	+	.	+	+	+	+	+	+	.	.	.	+	+	+	+	.	+	.	+	L
6920	P/IB-7																													
8318	P/IB-7																													
880457	P/IB-5	+	+	+	+	.	+	+	+	.	+	.	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+	+	M
880324	P/IB-7																													
4702	P/IB-5	+	+	+	+	.	+	+	+	.	+	+	+	+	+	.	+	+	.	.	.	+	+	+	+	.	+	.	+	N
4710	P/IB-5																													
5846	P/IB-5	+	+	.	+	.	+	+	+	.	.	+	.	+	+	+	+	+	.	.	.	+	+	+	+	.	+	+	+	O
882508	P/IB-5	+	+	+	+	.	+	+	+	.	+	+	+	+	+	+	+	+	+	.	.	+	.	+	+	.	+	.	+	P

[a]: Fragment range was selected from 27.0 to 9.7 kb.

Table 11. Fragment sizes of *Sma*I digested chromosomal DNA of 10 A/S class NR/IA-5 gonococcal isolates

Isolate	A/S Class	Fragment Size[a] (kb)																												Pattern No.
		27.0	25.5	22.7	22.0	21.6	21.0	20.0	19.0	18.2	17.6	17.0	16.6	16.0	15.1	14.5	14.0	13.6	13.4	13.0	12.6	12.4	12.0	11.8	11.5	11.2	10.6	10.4	9.7	
2852	NR/IA-5																													
3523	NR/IA-5																													
3735	NR/IA-5																													
3928	NR/IA-5																													
4020	NR/IA-5	+	+	·	·	·	·	+	+	·	+	·	·	+	·	+	+	+	+	+	·	+	·	+	+	+	+	+	+	Q
5265	NR/IA-5																													
6525	NR/IA-5																													
6595	NR/IA-5																													
5283	NR/IA-5	+	+	·	·	·	·	+	+	·	+	·	·	+	·	+	+	+	+	+	·	+	+	·	+	+	+	·	+	R

a): Fragment range was selected from 27.0 to 9.7 kb.

Table 12. Fragment sizes of *Sma*I digested chromosomal DNA of 12 A/S class NR/IB-3 gonococcal isolates

Isolate	A/S Class	Fragment Size[a] (kb)																												Pattern No.
		27.0	25.5	22.7	22.0	21.6	21.0	20.0	19.0	18.2	17.6	17.0	16.6	16.0	15.1	14.5	14.0	13.6	13.4	13.0	12.6	12.4	12.0	11.8	11.5	11.2	10.6	10.4	9.7	
881286	NR/IB-3																													
881919	NR/IB-3																													
882351	NR/IB-3																													
882802	NR/IB-3	+	+	·	+	·	·	·	+	·	+	·	·	·	+	+	+	+	·	+	·	+	·	·	+	+	+	+	+	S
883482	NR/IB-3																													
883511	NR/IB-3																													
3644	NR/IB-3																													
3648	NR/IB-3																													
4743	NR/IB-3																													
5837	NR/IB-3	+	+	·	+	·	·	·	+	·	+	·	+	·	+	+	+	+	·	+	·	+	+	·	+	+	+	+	+	T
5840	NR/IB-3																													
5855	NR/IB-3																													

[a]: Fragment range was selected from 27.0 to 9.7 kb.

In conclusion, RE analysis did not discriminate isolates within PCU(H), arginine-requiring, and NR/IA-5 groups very well (Table 13). Although there were 4 RE patterns in PCU(H) group, most (90%) of the isolates were grouped into a single RE pattern. One RE pattern was observed in the arginine-requiring group, two RE patters were noted in NR/IA-5 group. However, RE analysis did distinguish isolates of serovars IB-5 and IB-7, irrespective of their auxotypes.

Table 13. Relation between DNA RE patterns (A-T) and A/S classes of 100 gonococcal isolates

A/S Class	PCU(H)		Arg/IA-2			IB-5/IB-7				NR/IA-5	NR/IB-3
	IB-1	IB-2	OUH	CUH	OH	NR/IB-5	NR/IB-7	P/IB-5	P/IB-7		
RE Pattern No.	A B C	A D	E	E	E	F H I	F G J K	L M N O P	L M	Q R	S T

3.3. Ribotyping of Gonococci

3.3.1. *Identification of useful enzymes for analysis of gonococci by ribotyping*

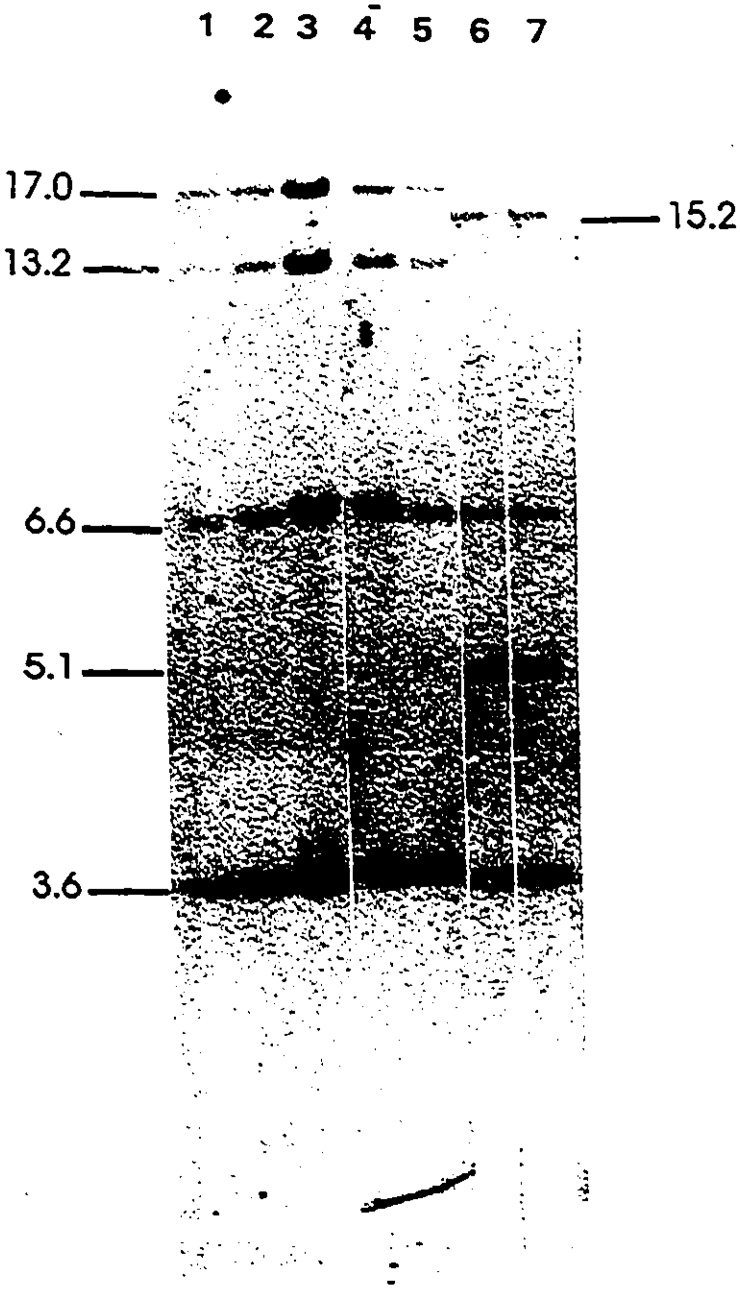
Thirty gonococcal chromosomal DNAs were analyzed with restriction endonucleases *Ava*II, *Hind*II, and *Sma*I in order to determine which enzymes would provide the greater discrimination for ribotyping isolates. Hybridization of *Ava*II fragments with the riboprobe showed two ribotype patterns for most examined strains (Figure 10, Lanes 1-5, pattern I; Lane 6,7, pattern II). Hybridization of *Hind*II fragments with the riboprobe produced only one ribotype pattern (data not shown). *Sma*I patterns produced the greatest differentiation (see below). As judged by the number and distribution of band positions; therefore, *Sma*I was chosen for all further comparisons.

The characteristic patterns of bands observed for each strain remained identical when DNA prepared from randomly selected strains grown on separate occasions during the course of the study was compared. Similarly, ribotypes did not change after individual strain were subjected to serial subcultures.

3.3.2. *Ribotypes of 100 gonococcal isolates*

Eleven ribotype patterns comprising different molecular sized DNA fragments were produced by *Sma*I digestion of the 100 isolates which comprised thirteen A/S classes tested (Table 14). Most patterns were clearly distinguishable by the presence or absence of a variety of bands. Each ribotype pattern comprised between six to seven distinct bands with estimated sizes range from 2.8 to 29.0 kb. rRNA profiles were considered distinct if there was a single

Figure 10. *Ava*II restriction fragments of *N. gonorrhoeae* isolates of A/S class NR/IB-5 and NR/IB-7 hybridized with a non-radiolabelled 7.2 kb fragment carrying the *rrn*B rRNA operon. Lane 1, 880107; Lane 2, 880466; Lane 3, 880644; Lane 4, 881095; Lane 5, 881359; Lane 6, 882449; Lane 7, 3351; Lane 8, 883535.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7
17.0
13.2
15.2
6.6
5.1
3.6

Table 14. Ribotypes of *Sma*I chromosomal restriction fragments in 100 gonococcal isolates comprising 13 auxotype/serovar classes

Ribotype pattern No. (No.)[a]	Fragment Size (kb)													
	29.0	28.0	26.0	23.4	23.1	22.0	17.5	17.0	12.0	10.5	8.2	5.5	4.8	2.8
I (24)	-	-	-	-	+	-	-	+	-	+	+	-	+	+
II (22)	-	-	-	-	+	+	-	-	+	-	+	-	+	+
III (15)	-	+	-	-	+	-	-	-	+	-	+	-	+	+
IV (12)	-	-	+	-	+	-	-	-	+	-	+	-	+	+
V (10)	-	-	-	+	+	-	-	-	+	-	+	+	+	+
VI (7)	+	-	-	-	+	-	-	-	+	-	+	-	+	+
VII (3)	-	-	-	+	+	-	-	-	+	-	+	-	+	+
VIII (3)	-	+	-	-	+	-	-	-	-	+	+	-	+	+
IX (2)	-	-	-	-	+	-	+	-	+	-	+	-	+	+
X (1)	-	-	-	-	+	-	+	+	-	+	+	-	+	+
XI (1)	-	-	-	-	+	+	-	-	-	+	+	-	+	+

a): Numbers of isolates with ribotype.

reproducible band difference. The eleven patterns possessed four common DNA fragments which corresponded to sizes of approximately 23.1, 8.2, 4.8, and 2.8 kb, respectively. Ribotype pattern V (Table 14) differed from ribotype pattern VII by the presence of a single rRNA - containing fragment of 5.5 kb. Ribotype patterns V and X had hybridization patterns that were different from the rest by producing a total of seven bands per ribotype pattern. The remaining eight ribotype patterns comprised six fragments and varied by the presence of only a single band which was of different size in each pattern.

3.3.3. *Comparison of ribotypes with A/S class*

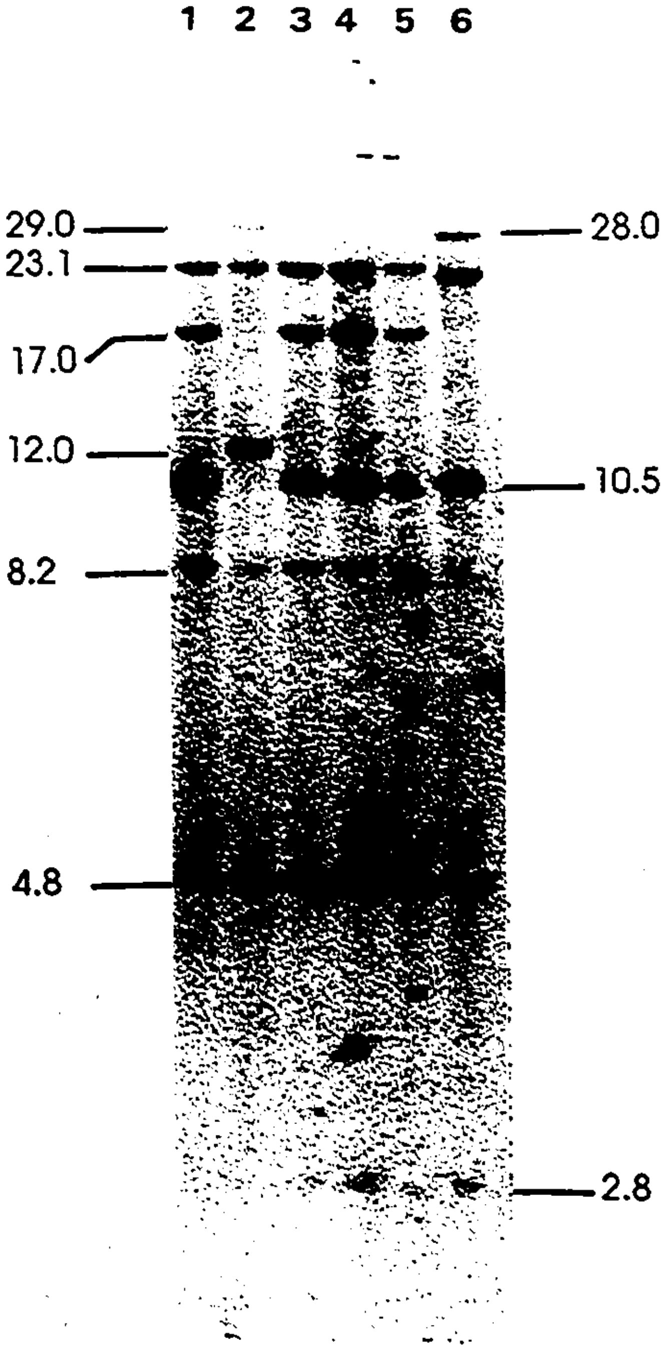
Thirty PCU(H) isolates of serovar IB-1 or IB-2 were subtyped into five ribotypes (Table 15, 16): ribotype pattern I included 7 PCU/IB-1, 5 PCUH/IB-1, 6 PCU/IB-2 and 6 PCUH/IB-2 isolates (Figure 11, Lanes 1, 3-5; Figure 12, Lanes 2, 4-7, 9, 10); ribotype pattern VI comprised 1 PCUH/IB-1 isolate (Figure 11, Lane 2); ribotype pattern VIII comprised 1 PCU/IB-1, 1 PCUH/IB-1 and 1 PCUH/IB-2 isolates (Figure 11, Lane 6; Figure 12, Lane 1); one PCUH/IB-2 isolate belonged to ribotype pattern X (Figure 12, Lane 3); and 1 PCU/IB-2 isolate belonged to ribotype pattern XI (Figure 12, Lane 8). 80% (24/30) of the PCU(H) isolates, irrespective of serovar, were represented by ribotype pattern I and 10% (3/30) of the PCU(H) isolates of serovar IB-1 or IB-2 belonged to ribotype pattern VIII. These results indicate that PCU(H) isolates are clonal.

Five ribotypes were observed among the 27 arginine-requiring gonococcal isolates (ribotype patterns II, III, IV, VI, and IX, Table 17; Figure 13). The 12 OUH/IA-2 isolates were classified into five ribotype patterns II, (Figure 13, Lanes 6,7), III (data not shown), IV (Figure

Table 15. Ribotypes of 15 *N. gonorrhoeae* isolates of A/S class of PCU(H)/IB-1

Isolate	A/S	Fragment Size (kb)														Pattern No.
		29.0	28.0	26.0	23.4	23.1	22.0	17.5	17.0	12.0	10.5	8.2	5.5	4.8	2.8	
880067	PCU/IB-1															
880128	PCU/IB-1															
880263	PCU/IB-1															
880724	PCU/IB-1															
883036	PCU/IB-1															
883077	PCU/IB-1	-	-	-	-	+	-	-	+	-	+	+	-	+	+	I
883632	PCU/IB-1															
3703	PCUH/IB-1															
880596	PCUH/IB-1															
880687	PCUH/IB-1															
882989	PCUH/IB-1															
883353	PCUH/IB-1															
882779	PCUH/IB-1	+	-	-	-	+	-	-	-	+	-	+	-	+	+	VI
881587	PCU/IB-1	-	+	-	-	+	-	-	-	-	+	+	-	+	+	VIII
7861	PCUH/IB-1															

Figure 11. *Sma*I restriction fragments of *N. gonorrhoeae* isolates of A/S class PCU(H)/IB-1 hybridized with a non-radiolabelled 7.2 kb fragment carrying the *rrn*B rRNA operon. Lane **1**, 880687 (Ribotype=I); Lane **2**, 882779 (VI); Lane **3**, 882989 (I); Lane **4**, 883353 (I); Lane **5**, 3703 (I); Lane **6**, 7861 (VIII).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29.0
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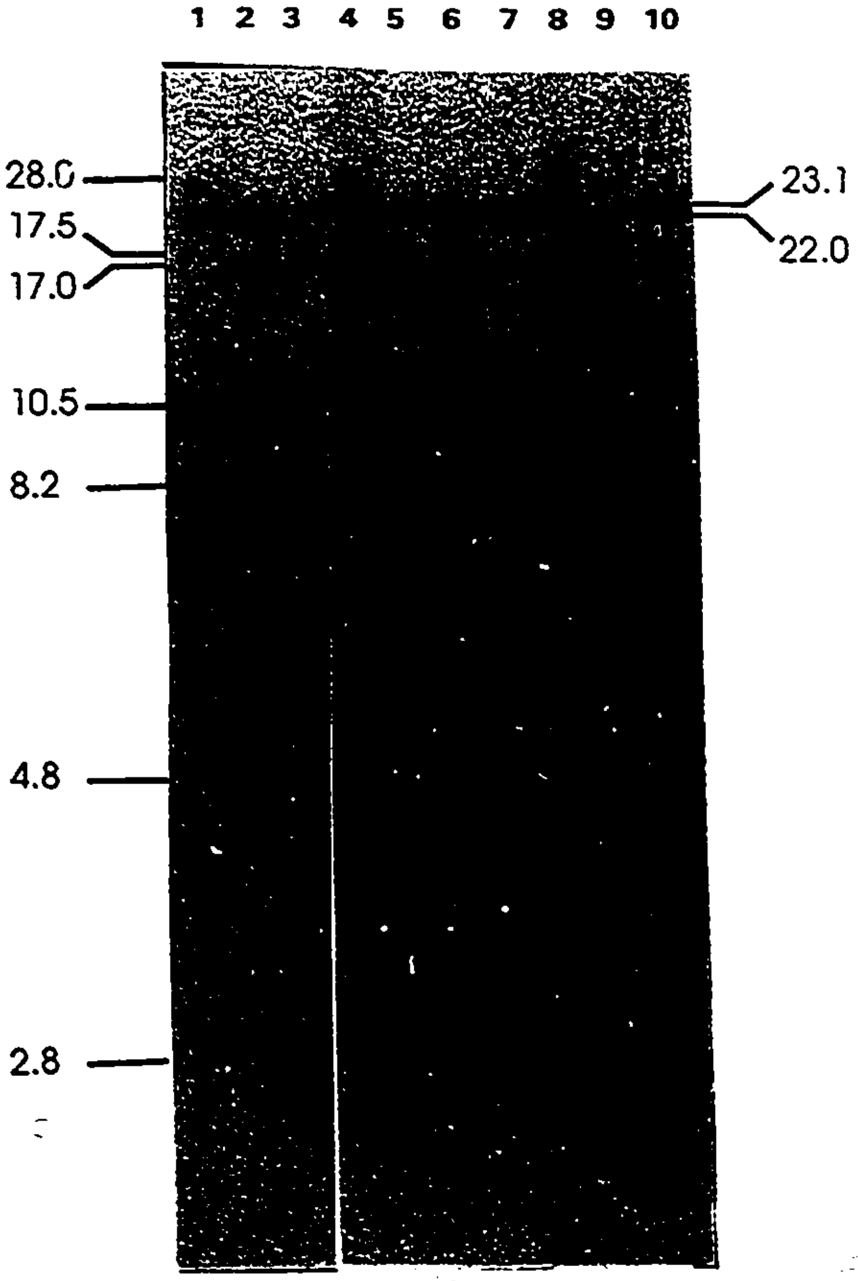
28.0
10.5
2.8

Table 16. Ribotypes of 15 *N. gonorrhoeae* isolates of A/S class PCU(H)/IB-2

Isolate	A/S	Fragment Size (kb)														Pattern No.
		29.0	28.0	26.0	23.4	23.1	22.0	17.5	17.0	12.0	10.5	8.2	5.5	4.8	2.8	
880074	PCU/IB-2															
880916	PCU/IB-2															
881777	PCU/IB-2															
881985	PCU/IB-2															
882988	PCU/IB-2															
883329	PCU/IB-2	-	-	-	-	+	-	-	+	-	+	+	-	+	+	I
880071	PCUH/IB-2															
881542	PCUH/IB-2															
881717	PCUH/IB-2															
882191	PCUH/IB-2															
883238	PCUH/IB-2															
883766	PCUH/IB-2															
883355	PCUH/IB-2	-	+	-	-	+	-	-	-	-	+	+	-	+	+	VIII
8281	PCUH/IB-2	-	-	-	-	+	-	+	+	-	+	+	-	+	+	X
881996	PCU/IB-2	-	-	-	-	+	+	-	-	-	+	+	-	+	+	XI

Figure 12. *SmaI* restriction fragments of *N. gonorrhoeae* isolates of A/S class PCU(H)/IB-2 hybridized with a ^{32}P-labelled 7.2 kb fragment carrying the *rrn*B operon. Lane **1**, 883355 (Ribotype=VIII); Lane **2**, 883766 (I); Lane **3**, 8281 (X); Lane **4**, 880074 (I); Lane **5**, 880916 (I); Lane **6**, 881777 (I); Lane **7**, 881985 (I); Lane **8**, 881996 (XI); Lane **9**, 882988 (I); Lane **10**, 883329 (I). The 17.5- and 17.0-kb fragments of isolate 8281, the 23.1- and 22.0-kb fragments of isolate 881996 did not reproduce well in the photograph.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17.0
10.5
8.2
4.8
2.8
23.1
22.0

13, Lanes 1, 2), VI (Figure 13, Lane 4), and IX (Figure 13, Lane 3). Eight CUH/IA-2 isolates were subdivided into two ribotype patterns, II (data not shown) and III (Figure 13, Lane 5); and seven OH/IA-2 isolates were subdivided into two ribotype patterns II (Figure 13, Lane 8,9) and IV (data not shown). Ribotype II dominated in this group, representing about 65% (17/27) of 27 isolates.

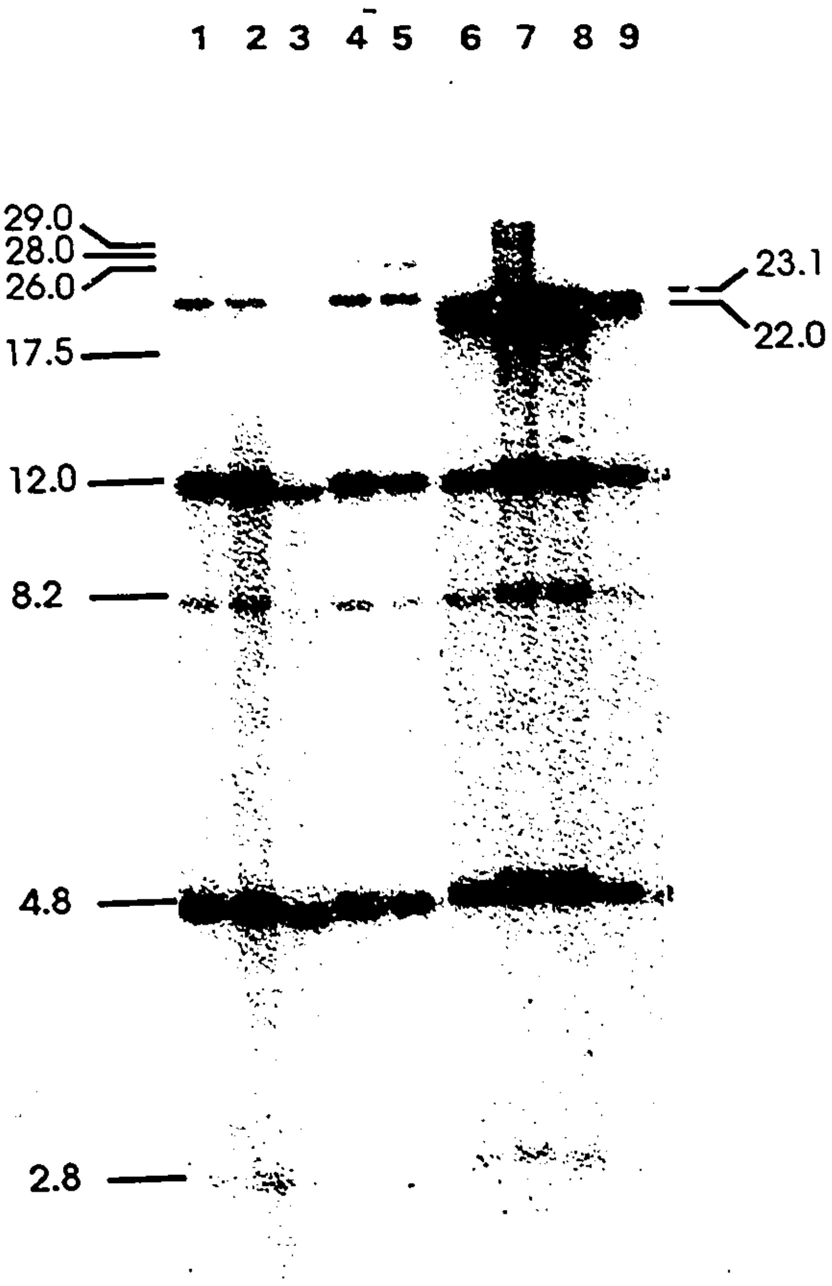
For serovar IB-5 and IB-7 isolates of auxotypes NR or P, twenty-one isolates in this group yielded five ribotypes (ribotype patterns II, III, IV, VI, and VII; Table 18). Two NR/IB-7 and 3 P/IB-5 isolates were classified into ribotype pattern II (Figure 14, Lanes 4 and 5); two NR/IB-7, one NR/IB-5, two P/IB-5 and 1 P/IB-7 isolates were represented by ribotype pattern III (Figure 14, Lanes 6 and 7); two NR/IB-5 isolates belonged to ribotype pattern IV (Figure 14, Lane 2); one NR/IB-5, one NR/IB-7, one P/IB-5 and 2 P/IB-7 isolates were assorted into ribotype VI (Figure 14, Lane 3); and one NR/IB-7 and 2 P/IB-7 isolates belonged to ribotype VII (Figure 14, Lane 1). These suggested that isolates in this group are heterogeneous based on ribotyping.

All isolates of A/S class NR/IA-5 fell into ribotype pattern V (Table 19). Ribotype pattern V differed from other ribotype patterns with presenting an additional 5.5 kb fragment. The rRNA probe hybridized to seven fragments which were 23.4, 23.1, 12.0, 8.2, 5.5, 4.8 and 2.8 kb in size (Figure 15, Lanes 1-6). This further confirmed that isolates in this group are homogeneous.

Table 17. Ribotypes of 27 arginine-requiring *N. gonorrhoeae* isolates of serovar IA-2

Isolate	A/S	Fragment Size (kb)													Pattern No.
		29.0	28.0	26.0	23.4	23.1	22.0	17.5	17.0	12.0	8.2	5.5	4.8	2.8	
880375	OUH/IA-2														
882306	OUH/IA-2														
883122	OUH/IA-2														
883171	OUH/IA-2														
883342	OUH/IA-2														
880270	CUH/IA-2														
880379	CUH/IA-2														
880454	CUH/IA-2														
882132	CUH/IA-2	-	-	-	-	+	+	-	-	+	+	-	+	+	II
882610	CUH/IA-2														
883099	CUH/IA-2														
883729	CUH/IA-2														
881097	OH/IA-2														
881342	OH/IA-2														
381506	OH/IA-2														
882376	OH/IA-2														
882794	OH/IA-2														
883463	OUH/IA-2														
881540	CUH/IA-2	-	+	-	-	+	-	-	-	+	+	-	+	+	III
882642	OH/IA-2														
880420	OUH/IA-2														
882208	OUH/IA-2	-	-	+	-	+	-	-	-	+	+	-	+	+	IV
882245	OUH/IA-2														
883212	OH/IA-2														
881035	OUH/IA-2	+	-	-	-	+	-	-	-	+	+	-	+	+	VI
882521	OUH/IA-2	-	-	-	-	+	-	+	-	+	+	-	+	+	IX
882398	OUH/IA-2														

Figure 13. *Sma*I restriction fragments of arginine-requiring *N. gonorrhoeae* isolates of serovar IA-2 hybridized with a ^{32}P-labelled 7.2 kb fragment carrying the *rrn*B rRNA operon. Lane 1, 880420 (Ribotype=IV); Lane 2, 882208 (IV); Lane 3, 882398 (IX); Lane 4, 881035 (VI); Lane 5, 881540 (III); Lane 6, 880375 (II); Lane 7, 882306 (II); Lane 8, 881097 (II); Lane 9, 881342 (II). Lane 7 shows partial digestion.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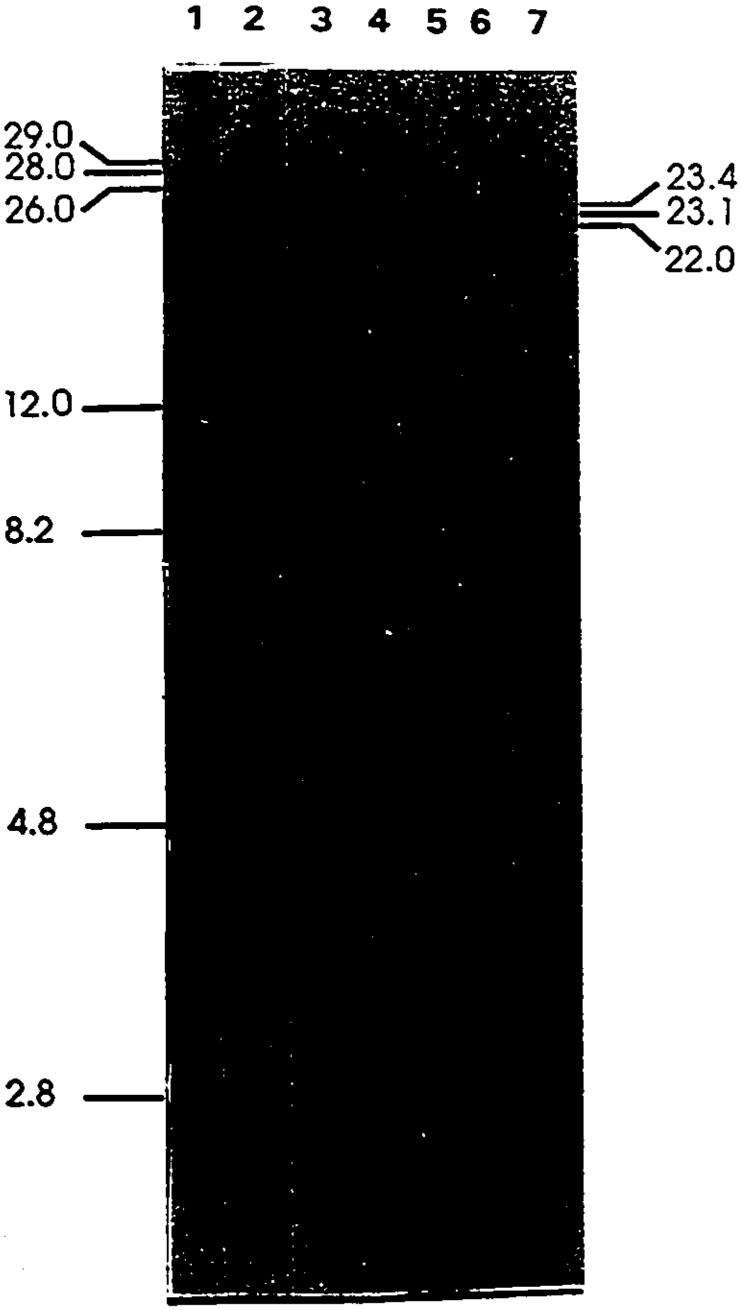
29.0
28.0
26.0
17.5
12.0
8.2
4.8
2.8
23.1
22.0

Table 18. Ribotypes of 4 NR/IB-5, 6 NR/IB-7, 6 P/IB-5 and 5 P/IB-7 gonococcal isolates

Isolate	A/S	Fragment Size (kb)														Pattern No.
		29.0	28.0	26.0	23.4	23.1	22.0	17.5	17.0	12.0	10.5	8.2	5.5	4.8	2.8	
882449	NR/IB-7															
883535	NR/IB-7															
880457	P/IB-5	-	-	-	-	+	+	-	-	+	-	+	-	+	+	II
881051	P/IB-5															
882508	P/IB-5															
883665	NR/IB-7															
4209	NR/IB-7															
3351	NR/IB-5															
4702	P/IB-5	-	+	-	-	+	-	-	-	+	-	+	-	+	+	III
4710	P/IB-5															
883141	P/IB-7															
880466	NR/IB-5	-	-	+	-	+	-	-	-	+	-	+	-	+	+	IV
880644	NR/IB-5															
880107	NR/IB-5															
881359	NR/IB-7															
5846	P/IB-5	+	-	-	-	+	-	-	-	+	-	+	-	+	+	VI
8318	P/IB-7															
880972	P/IB-7															
881095	NR/IB-7															
880324	P/IB-7	-	-	-	+	+	-	-	-	+	-	+	-	+	+	VII
6920	P/IB-7															

Figure 14. *Sma*I restriction fragments of gonococcal isolates of A/S class NR/IB-5 and NR/IB-7 hybridized with a non-radiolabelled 7.2 kb fragment carrying the *rrn*B rRNA operon. Lane 1, 881095 (Ribotype=VII); Lane 2, 880644 (IV); Lane 3, 881359 (VI); Lane 4, 882449 (II); Lane 5, 883535 (II); Lane 6, 883665 (III); Lane 7, 4209 (III). The 23.4- and 23.1-kb fragments of isolate 881095 did not reproduce well in the photograph.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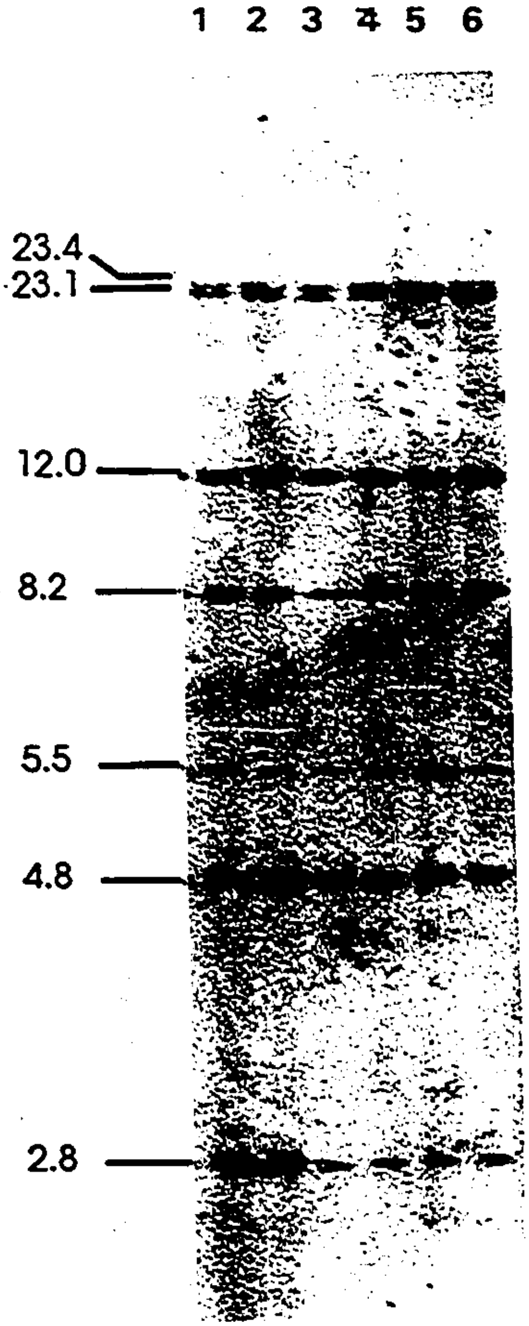
26.0
12.0
8.2
4.8
2.8
23.4
23.1
22.0

Table 19. Ribotypes of 10 *N. gonorrhoeae* isolates of A/S class NR/IA-5

Isolate	A/S	Fragment Size (kb)														Pattern No.
		29.0	28.0	26.0	23.4	23.1	22.0	17.5	17.0	12.0	10.5	8.2	5.5	4.8	2.8	
2852	NR/IA-5															
3523	NR/IA-5															
3735	NR/IA-5															
3928	NR/IA-5															
4020	NR/IA-5	-	-	-	+	+	-	-	-	+	-	+	+	+	+	V
5265	NR/IA-5															
5283	NR/IA-5															
5707	NR/IA-5															
6525	NR/IA-5															
6595	NR/IA-5															

Figure 15. *Sma*I restriction fragments of gonococcal isolates of A/S class NR/IA-5 hybridized with a non-radiolabelled 7.2 kb fragment carrying the *rrn*B rRNA operon. Lane 1, 4020 (Ribotype=V); Lane 2, 5265 (V); Lane 3, 5283 (V); Lane 4, 5705 (V); Lane 5, 6525 (V); Lane 6, 6595 (V).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23.4
23.1
12.0
8.2
5.5
4.8
2.8

Twelve gonococcal isolates of A/S class NR/IB-3 were subdivided into two ribotype patterns III and IV (Table 20). Four isolates from Manitoba area (881286, 881919, 882802, and 883482) and two isolates from Ontario area (3644, and 3648) were represented by ribotype III. Four isolates from Ontario area (4743, 5837, 5840, and 5855) and two isolates from Manitoba area (882351 and 883511) were represented by ribotype IV.

The distribution of gonococci into ribotype patterns was not uniform (Table 21). Ribotype patterns I and II represented 46% (46/100) of the strains investigated. Ribotype pattern I was the predominant group observed among the eleven *SmaI* generated RFLP types but it was represented by twenty-four isolates which were all auxotype PCU(H). No other A/S classes tested fell into this particular ribotype pattern. Ribotype pattern II was represented by twenty-three isolates which were distributed among five A/S classes including arg/IA-2 and serovar IB-5 and IB-7 isolates. Ribotype pattern III was represented by fifteen isolates which were distributed among eight A/S classes and included the CMRNG strains of the Manitoba outbreak. Ribotype pattern IV was represented by twelve isolates which were distributed among four A/S classes and included the outbreak CMRNG strains. Ribotype pattern V was represented by ten isolates all from the PPNG outbreak in Toronto which fell into one A/S class. Ribotype pattern VI was represented by seven isolates which were distributed among six A/S classes. Ribotype VII fell into two A/S classes. Ribotype VIII was distributed among three A/S classes. Ribotype IX, X and XI was represented by one A/S class, respectively.

Isolates belonging to the same A/S class could be further subdivided based on different ribotypes generated. Isolates with the same ribotypes were found distributed amongst several A/S classes (Table 22). There were no absolute correlation between ribotypes and A/S classes nor was there an obvious correlation between A/S class, RE pattern or ribotype pattern. Overall, these three typing criteria combined produced 38 types.

Table 20. Ribotypes of 12 *N. gonorrhoeae* isolates of A/S class NR/IB-3

Isolate	A/S	Fragment Size (kb)														Pattern No.
		29.0	28.0	26.0	23.4	23.1	22.0	17.5	17.0	12.0	10.5	8.2	5.5	4.8	2.8	
881286	NR/IB-3															
881919	NR/IB-3															
882802	NR/IB-3	-	+	-	-	+	-	-	-	+		+	-	+	+	III
883482	NR/IB-3															
3644	NR/IB-3															
3648	NR/IB-3															
882351	NR/IB-3															
883511	NR/IB-3															
4743	NR/IB-3	-	-	+	-	+	-	-	-	+		+	-	+	+	IV
5837	NR/IB-3															
5840	NR/IB-3															
5855	NR/IB-3															

Table 21. The distribution of ribotypes within 100 gonococcal isolates in five groups

Ribotype Pattern No.	PCU(H)		Arg/IA-2			IB-5/IB-7				NR/IA-5	NR/IB-3	Total No.
	IB-1	IB-2	OUH	CUH	OH	NR/IB-5	NR/IB-7	P/IB-5	P/IB-7			
I	12	12										24
II			5	7	5		2	3				22
III			1	1	1	1	2	2	1		6	15
IV			3		1	2					6	12
V										10		10
VI	1		1			1	1	1	2			7
VII							1		2			3
VIII	2	1										3
IX			2									2
X		1										1
XI		1										1
total No.	15	15	12	8	7	4	6	6	5	10	12	100

a): Numbers of isolates.

Table 22. Relation between A/S classes and restriction endonuclease and ribotype patterns of 100 gonococcal isolates

A/S Class	PCU(H)					Arg/IA-2			IB-5/IB-7														NR/IA-5		NR/IB-3	
	IB-1			IB-2		OUH	CUH	OH	NR/IB-5			NR/IB-7				P/IB-5					P/IB-7					
RE Pattern No.	A	B	C	A	D	E	E	E	F	H	I	F	G	J	K	L	M	N	O	P	L	M	Q	R	S	T
Ribotype Pattern No.	I VIII	VIII	VI	I VIII X	XI	II III IV VI IX	II III	II III IV	IV	VI	III	III	II	VII	VI	II	II	III	VI	II	VI VII	VII	V	V	III IV	IV

The Simpson's index of discrimination of the isolates was determined, as described by Hunter and Gaston (1988), to compare the discriminatory power of single and combined methods for typing gonococci in accordance with the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{i=1}^{K} n_i (n_i - 1) \times 100$$

where K equals the number of distinct groups obtained with a given typing scheme, N equals the sum of all of the K groups, and n_i is the size of the ith group. D is expressed as a percentage (Dillon *et al.*, 1993). An index of 90% or greater is a desirable property of a typing scheme (Hunter and Gaston, 1988). This value is sensitive to both the number of groups the typing scheme defines and the size of the largest group (i.e. more groups and less isolates in the largest group will produce more discriminating, higher discriminatory index). The discriminatory power of A/S, ribotyping, and RE analysis determinations, either alone or in combination, for 100 gonococcal isolates is indicated in Table 23. Ribotyping and RE analysis produced an index of less than 90% for 100 *N. gonorrhoeae* isolates (Table 23). All combined methods produced an index of greater than 90% for 100 *N. gonorrhoeae* isolates (Table 23). Addition of RE analysis to A/S-Ribotyping or addition of ribotyping to A/S-RE analysis did not significantly raise the level of discrimination of 100 *N. gonorrhoeae* isolates.

Table 23. Discrimination indices of various typing methods for 100 *N. gonorrhoeae* isolates

Typing method(s)	No. of groups (size of largest group [%])	Index (%)
Ribotyping	11 (24%)	84.7%
RE analysis	20 (27%)	84.0%
A/S	13 (12%)	92.5%
A/S-Ribotyping	36 (10%)	96.2%
A/S-RE analysis	28 (12%)	94.4%
Ribotyping-RE analysis	32 (24%)	90.2%
A/S-Ribotyping-RE analysis	41 (9%)	96.6%

3.4. Analysis of 26 Gonococcal Isolates by PFGE

3.4.1. *PFGE patterns of twenty-six gonococcal isolates*

Restriction endonuclease *Spe*I cleaved *N. gonorrhoeae* chromosomal DNA into 10-15 fragments ranging from 50 to 500 kb that were resolved by electrophoresis in a CHEF apparatus. The PFGE patterns observed for each isolate remained identical when DNA was prepared from separate occasions. PFGE patterns were unchanged after 5 passages of the isolate on GCMB agar plates (data not shown).

Twenty-six gonococcal isolates in predominant ribotypes I and II as well as isolates that could not be distinguished by ribotyping were selected for further analysis by pulsed field gel analysis. These isolates included 2 CUH/IA-2 isolates that typed as ribotypes II and IV; 6 OUH/IA-2 isolates that were subtyped into four ribotypes II, IV, VI, and IX; 1 OH/IA-2 isolate with ribotype II; 5 NR/IA-5 isolates which were ribotype V; 2 NR/IB-5 isolates with ribotypes IV and VI; 4 NR/IB-7 isolates with ribotypes II, III, VI, and VII; 6 PCU/IB-1 isolates with ribotypes I and VII, and 3 PCUH/IB-1 isolates with ribotypes I, VI, and X.

Twenty-five PFGE patterns were produced among 26 isolates tested (Table 24). Three common bands were observed (110.0, 77.0 and 67.0 kb) and only two isolates had the same PFGE pattern P3; these were PCU(H)/IB-1 isolates from different geographical areas of Canada.

3.4.2. *Comparison of PFGE analysis with A/S classification*

Three PCU/IB-1 isolates were subtyped into three different *Spe*I PFGE patterns (Figure 16, Lanes 1-3; Table 25) and three strains of PCUH/IB-1 showed three different *Spe*I PFGE patterns (Figure 16, Lanes 4-6; Table 25). Thus, the six isolates with the same serovar IB-1 were represented by two auxotypes, 3 RE patterns, 3 ribotype patterns but five *Spe*I patterns. In this group, isolates carried three common bands (11.0, 77.0 and 67.0 kb).

Nine arginine-requiring strains were subtyped into 9 *Spe*I PFGE patterns (Table 26). Six OUH/IA-2 isolates produced six distinct PFGE patterns (Figure 17, Lanes 1-5, P13 data not

Table 24. Relation between DNA PFGE patterns, ribotype patterns, RE patterns and A/S class of 26 gonococcal isolates

Strain No.	PFGE Pattern No.	Ribotype Pattern No.	RE Pattern No.	A/S Class
880263	P1	I	A	PCU/IB-1
880724	P2	I	A	PCU/IB-1
881587	P3	VIII	A	PCU/IB-1
7861	P3	VIII	B	PCUH/IB-1
882779	P4	IV	C	PCUH/IB-1
883353	P5	I	A	PCUH/IB-1
881540	P6	III	E	CUH/IA-2
882610	P7	II	E	CUH/IA-2
880375	P8	II	E	OUH/IA-2
880420	P9	IV	E	OUH/IA-2
881035	P10	VI	E	OUH/IA-2
882208	P11	IV	E	OUH/IA-2
882306	P12	II	E	OUH/IA-2
882398	P13	IX	E	OUH/IA-2
881097	P14	II	E	OH/IA-2
880107	P15	IV	H	NR/IB-5
880644	P16	VI	F	NR/IB-5
881095	P17	VII	J	NR/IB-7
881359	P18	VI	K	NR/IB-7
883535	P19	II	G	NR/IB-7
883665	P20	III	F	NR/IB-7
2852	P21	V	Q	NR/IA-5
5265	P22	V	Q	NR/IA-5
3523	P23	V	Q	NR/IA-5
5283	P24	V	R	NR/IA-5
5707	P25	V	Q	NR/IA-5

Figure 16. PFGE of *Spe*I-digested genomic DNA from *N. gonorrhoeae* isolates of A/S class PCU(H)/IB-1. M=λ concatemer. P=pattern #. Lane 1, P1=880263; Lane 2, P2=880724; Lane 3, P3=881587; Lane 4, P4=882779; Lane 5, P5=883353; Lane 6, P3=7861.

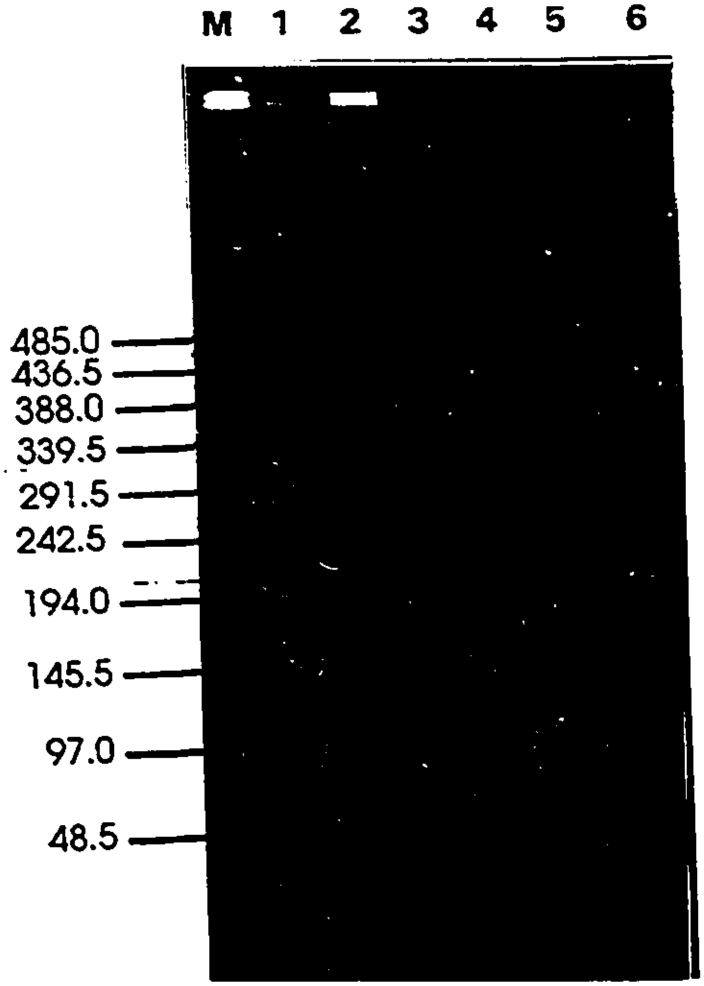
M
1
2
3
4
5
6
485.0
436.5
388.0
339.5
291.5
242.5
194.0
145.5
97.0
48.5

Table 25. PFGE patterns of 6 gonococcal isolates of A/S class PCU(H)/IB-1

Fragment Size (kb)	PFGE Pattern No.[a]				
	P1	P2	P3	P4	P5
410.0	+	+	+	-	+
323.0	-	-	-	+	-
313.0	-	-	-	+	-
277.0	-	+	-	-	-
262.0	+	-	+	-	+
248.0	-	+	-	-	-
242.0	+	-	-	-	+
230.0	+	+	-	+	+
215.0	-	+	-	+	+
198.0	+	-	-	-	-
181.0	-	-	-	+	-
160.0	+	-	-	-	-
145.0	-	+	+	-	+
134.0	-	-	+	-	-
110.0	+	+	+	+	+
102.0	-	-	+	-	-
84.0	+	+	+	-	+
77.0	+	+	+	+	+
67.0	+	+	+	+	+

[a] P1=880263, PCU/IB-1; P2=880724, PCU/IB-1; P3=881587, PCU/IB-1; and 7861, PCUH/IB-1; P4=882779, PCUH/IB-1; P5=8803353, PCUH/IB-1.

Some bands may be dimers.

Figure 17. PFGE of genomic DNA of arginine-requiring strains of *N. gonorrhoeae* digested with *Spe*I. M=λ concatemer. P=patter #. Lane **1**, P8=880375; Lane **2**, P9=880420; Lane 3, P10=881035; Lane 4, P11=882208; Lane 5, P12=882306; Lane **6**, P=881540; Lane **7**, P7=882610; Lane **8**, P14=881097.

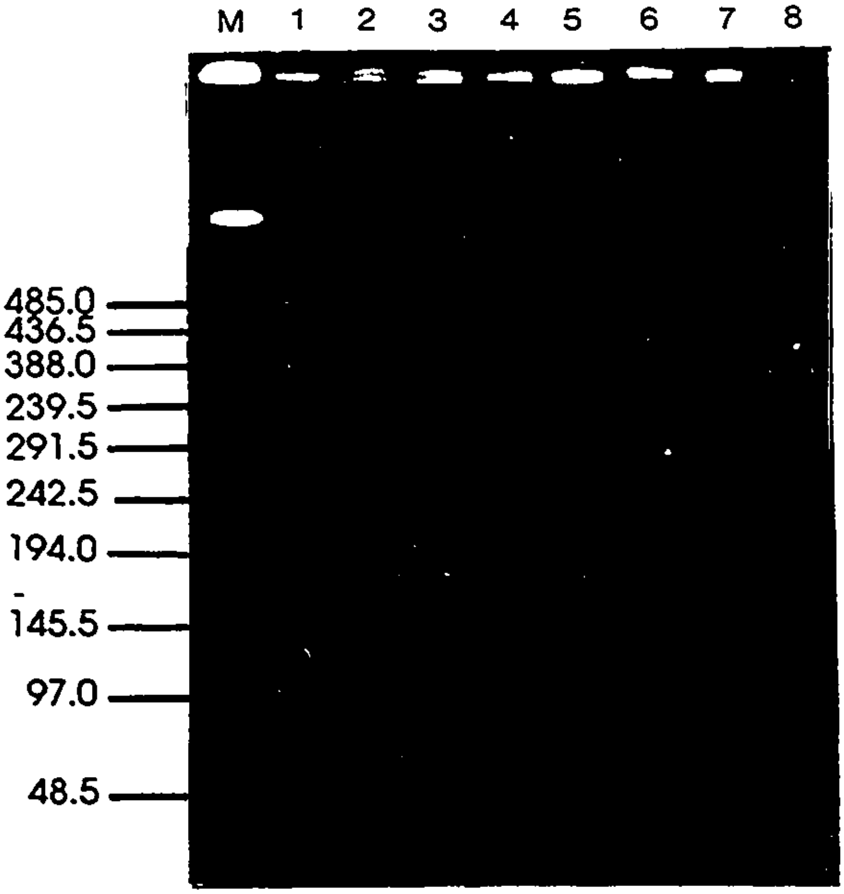
M
1
2
3
4
5
6
7
8
485.0
436.5
388.0
239.5
291.5
242.5
194.0
145.5
97.0
48.5

Table 26. PFGE patterns of 9 arginine-requiring gonococcal isolates of serovar IA-2

Fragment Size (kb)	PFGE Pattern No.[a]								
	P6	P7	P8	P9	P10	P11	P12	P13	P14
410.0	+	+	+	+	+	+	+	+	+
363.0	-	-	-	-	+	-	-	-	-
340.0	+	+	+	+	-	+	+	+	+
294.0	-	-	+	-	-	-	-	-	-
248.0	-	-	-	-	-	-	-	+	-
230.0	-	-	-	-	-	+	-	-	-
209.0	-	-	-	+	-	-	-	-	-
200.0	+	-	+	-	-	+	-	-	-
198.0	-	+	-	-	-	-	+	-	-
189.0	-	-	-	-	-	-	-	+	-
181.0	+	+	+	+	+	+	+	+	+
170.0	+	+	+	+	+	+	+	+	+
122.0	-	-	+	-	-	-	-	+	+
110.0	+	+	+	+	+	+	+	+	+
102.0	+	-	-	+	+	+	+	-	-
84.0	+	+	+	+	+	+	+	+	+
77.0	+	+	+	+	+	+	+	+	+
67.0	+	+	+	+	+	+	+	+	+

[a] P6=881540, CUH/IA-2; P7=882610, CUH/IA-2; P8=880375, OUH/IA-2; P9=880420, OUH-IA/2; P10=881035, OUH/IA-2; P11=882208, OUH/IA-2; P12=882306, OUH/IA-2; P13=882398, OUH/IA-2; P14=881097, OH/IA-2. Some bands may be dimers.

shown). Two CUH/IA-2 (Figure 17, Lanes 6 and 7) and 1 OH/IA-2 (Figure 17, Lane 8) isolates showed clearly distinct PFGE patterns from each other and from the OUH/IA-2 group. Thus, the nine IA-2 isolates were represented by 3 auxotypes, 1 RE pattern, 5 ribotype patterns but nine *Spe*I PFGE patterns. Isolates had seven bands in common (410.0, 181.0, 170.0, 110.0, 84.0, 77.0, and 67.0 kb). There were four bands unique to all isolates in this A/S class (410.0, 181.0, 170.0 and 84.0 kb).

Four strains of NR/IB-7 were subtyped into four different *Spe*I PFGE profiles (Figure 18, Lanes 3-6). Two NR/IB-5 strains (Figure 18, Lanes 1 and 2) gave two different *Spe*I PFGE patterns from others (Table 27). Thus, the six NR isolates were represented by 2 serovars, 5 RE patterns, 5 ribotype patterns but six *Spe*I PFGE patterns. These isolates had four common bands (181.0, 110.0, 77.0 and 67.0 kb), one band (181.0 kb) was unique to this group.

Similarly, 5 strains of identical A/S class NR/IA-5, which had similar RE and ribotype patterns, were subtyped into five different *Spe*I PFGE profiles (Figure 19, Lanes 1-5; Table 28). These five patterns were different from the arginine-requiring group. These five isolates carried four bands in common (262.0, 110.0, 77.0 and 67.0 kb), one band was unique to this group.

3.4.3. *Comparison of PFGE analysis with ribotyping*

Ten strains of the same ribotype I gave three *Spe*I PFGE profiles (Table 24). Five strains of the same ribotype II gave five *Spe*I PFGE patterns. Two strains of same ribotype III gave two *Spe*I PFGE patterns. Three strains of the same ribotype IV showed three *Spe*I PFGE patterns. Five strains with ribotype V found to have five *Spe*I PFGE patterns. Four strains of the same ribotype VI shown four *Spe*I PFGE patterns. Two strains of same ribotype VIII shown same *Spe*I PFGE patterns P3.

PFGE can distinguish isolates within the same A/S class or the same ribotype.

Figure 18. PFGE of *Spe*I-digested genomic DNA from *N. gonorrhoeae* isolates of A/S class NR/IB-5 and NR/IB-7. M=λ concatemer. P=pattern #. Lane 1, P15=880107; Lane 2, P16=880644; Lane 3, P17=881095; Lane 4, P18=881359; Lane 5, P19=883535; Lane 6, P20=883665.

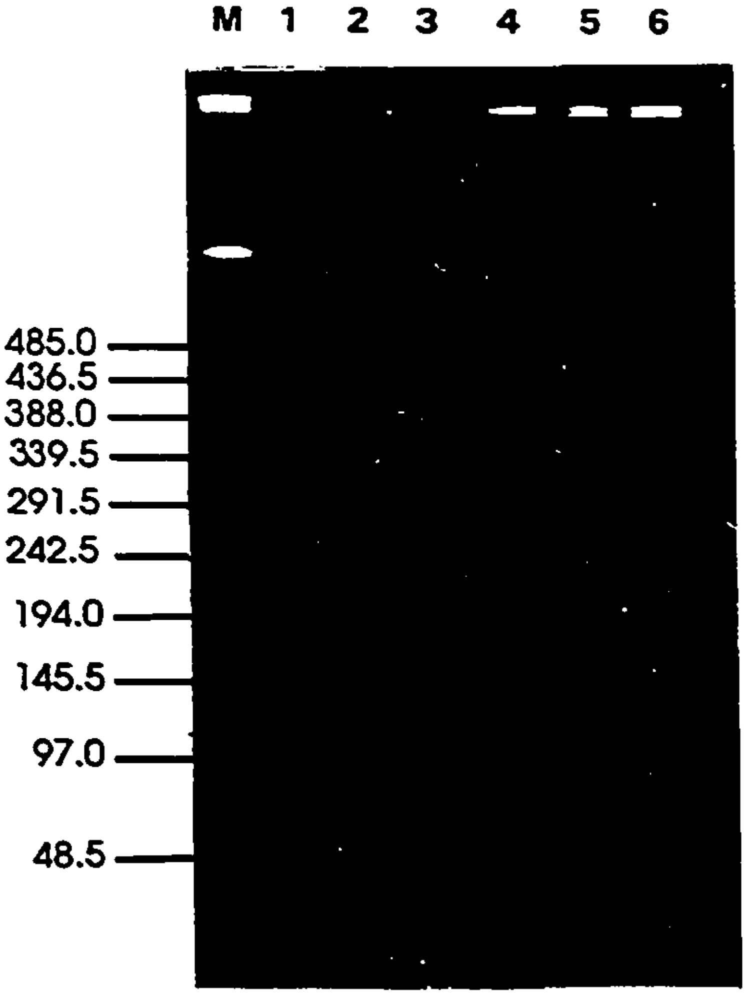
M
1
2
3
4
5
6
485.0
436.5
388.0
339.5
291.5
242.5
194.0
145.5
97.0
48.5

Table 27. PFGE patterns of 2 gonococcal isolates of A/S class NR/IB-5 and 4 gonococcal isolates of A/S class NR/IB-7

Fragment Size (kb)	PFGE Pattern No.[a]					
	P15	P16	P17	P18	P19	P20
464.0	-	-	+	-	-	-
421.0	-	-	-	-	+	-
410.0	-	-	-	+	-	+
388.0	+	+	-	-	-	-
363.0	-	-	+	+	-	+
277.0	+	-	+	-	+	-
262.0	+	-	-	+	+	+
230.0	-	-	+	-	+	-
204.0	-	+	-	-	-	-
189.0	-	+	-	-	-	-
181.0	+	+	+	+	+	+
145.0	+	-	-	-	+	-
110.0	+	+	+	+	+	+
102.0	-	+	-	-	-	-
95.0	-	-	-	+	+	-
89.0	-	-	-	-	+	-
84.0	+	+	+	+	-	+
77.0	+	+	+	+	+	+
67.0	+	+	+	+	+	+

[a] P15=880107, NR/IB-5; P16=880644, NR/IB-5; P17=881095, NR/IB-7; P18=881359, NR/IB-7; P19=883535, NR/IB-7; P20=883665, NR/IB-7.

Some bands may be dimers.

Figure 19. PFGE of *Spe*I-digested genomic DNA from *N. gonorrhoeae* isolates of A/S class NR/IA-5. M=λ concatemer. P=pattern #. Lane 1, P21=2852; Lane 2, P22=5265; Lane 3, P23=3523; Lane 4, P24=5283; Lane 5, P25=5707.

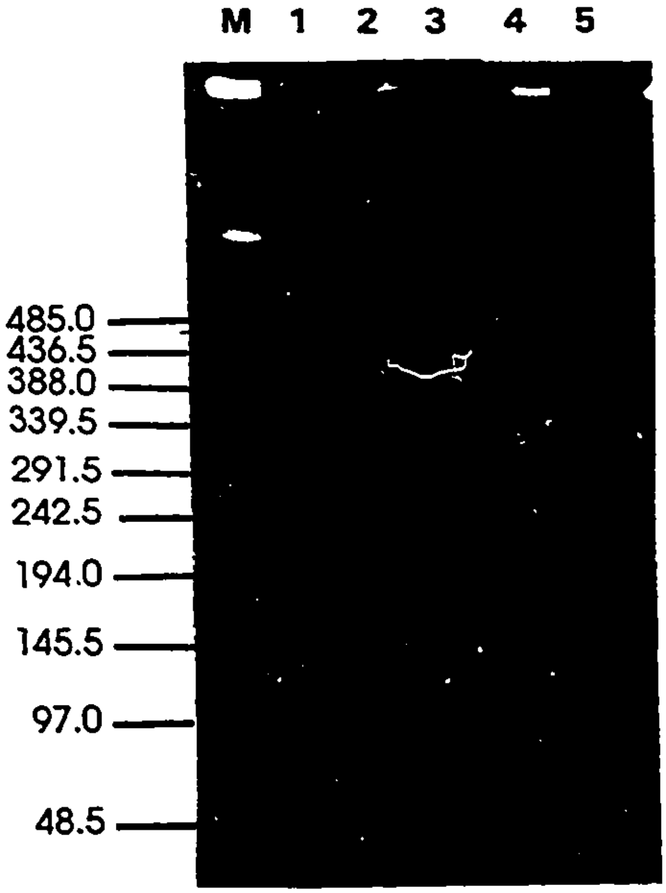
M
1
2
3
4
5
485.0
436.5
388.0
339.5
291.5
242.5
194.0
145.5
97.0
48.5

Table 28. PFGE patterns of 5 gonococcal isolates of A/S class NR/IA-5

Fragment Size (kb)	PFGE Pattern No.[a]				
	P21	P22	P23	P24	P25
464.0	-	+	-	-	-
453.0	+	-	+	-	-
410.0	-	-	-	-	+
382.0	-	+	-	-	-
363.0	+	-	+	+	+
274.0	-	+	+	-	-
262.0	+	+	+	+	+
230.0	-	-	-	+	-
215.0	-	-	-	+	-
200.0	-	+	-	+	-
198.0	+	-	-	-	+
181.0	+	+	+	-	+
145.0	-	-	+	-	-
110.0	+	+	+	+	+
89.0	-	+	-	-	-
84.0	+	-	+	+	+
77.0	+	+	+	+	+
70.0	-	+	-	-	-
67.0	+	+	+	+	+

[a] Strain numbers: P21=2852; P22=5265; P23=3523; P24=5283; P25=5707. Some bands may be dimers.

4. DISCUSSION

Several typing systems have been developed to characterize individual strains of *N. gonorrhoeae*. *N. gonorrhoeae* isolates have been typed into groups by using phenotypic properties, including antimicrobial susceptibility, auxotyping, and serotyping. In previous studies the combination antimicrobial susceptibility with serotyping and auxotyping has been shown to give more epidemiological information than the use of only one of the techniques (Dillon *et al.*, 1993; Danielsson *et al.*, 1983).

Recently, methods at the molecular level have been used to assess the relatedness of bacterial isolates. Several molecular typing techniques have been used for typing *N. gonorrhoeae*. One type of analysis is comparing the total plasmid content of isolates (plasmid profile). This technique is most useful when there is a reliable and easy lysis method for the organism being investigated and when plasmids, preferably two or more, are present in most of the isolates examined. There was not a great deal of variation in the plasmid profiles of strains used in the present study. For example, in this study, PCU(H) groups isolates that are plasmid-free (Dillon and Pauzé, 1981) and arginine-requiring isolates that contained 2.6 MDa plasmids could not be distinguished by plasmid profile. The limited diversity of patterns in this method may be restrictive in large epidemiological studies and outbreak isolates with same plasmid content, thus making further analysis by combination with other typing schemes such as auxotyping and serotyping necessary (Dillon *et al.*, 1993). For example, in this study, the isolates of NR/IA-5 and NR/IB-3 which were from 2 different outbreaks had similar plasmid profile within each group outbreak, although profiles were different from each other. NR/IA-5 isolates were PPNG. Plasmid profiles are most useful for PPNG isolates because the family of 6 β-lactamase producing plasmids can be differentiated. The instability of plasmid profiles due to acquisition, loss, or transfer of plasmids may be another handicap of this technique (Markowitz *et al.*, 1980; Rubens *et al.*, 1981), and identical plasmid profiles that may not reflect the overall genetic relatedness of isolates, because plasmids are extrachromosomal DNA, also is another limitation of this technique (Shlaea and Currie-McCumber, 1986).

4.1. Restriction endonuclease analysis

The number and locations of restriction sites along the DNA strand are unique for each genome: it follows therefore that fragment size classes also become unique for each genome (Isenberg, 1992). Such unique fragment classes form the specific restriction patterns, or fingerprints, of individual isolates. However, due to modifications of the bases, e.g., by methylation, certain sites may or may not be accessible to the action of endonucleases. Differences in restriction patterns between strains may therefore also reflect degrees of methylation in the genome. *N. gonorrhoeae* DNA is modified by the methyltransferases (MTases) M. *Ngo*I, M. *Ngo*II, and M. *Ngo*III, as well as three other cytosine MTases and one adenine MTase (Ritchot and Roy, 1990). *N. gonorrhoeae* possesses restriction-modification systems in order to maintain itself as unique species (Korch *et al.*, 1983). AHU strains of *N. gonorrhoeae* produced *dam* methylase (Kolodkin *et al.*, 1982; Tenover *et al.*, 1980). *Sma*I, which was used in the present study, was not sensitive to *dam* or *dcm* methylases (Sambrook *et al.*, 1989) that introduces methyl groups at the N^6 position of adenine in the sequence 5' GATC 3' (Hattman *et al.*, 1978) or at the C^5 position of the internal cytosine in the sequence 5'CCAGG 3' or 5' CCTGC 3' (May and Hattman, 1975; Marinus, 1973). *M. Sma*I methylase has not yet been reported in *N. gonorrhoeae*. Thus, *Sma*I digestion of chromosomal DNA would not be affected by methylation and methylation was not a factor in the different RE patterns observed in the present study.

The presence of plasmids also could affect the band intensity on RE analysis; however, in the present study, this would not be encountered because the chromosomal DNA was separated from plasmid DNA on CsCl gradients and the DNAs were checked for plasmid DNA on agarose gels before restriction endonucleases digestion.

Because DNA fingerprints are highly sensitive to minor genomic variations in nucleotide sequences, they offer a precise means of typing species and of identifying individual strains of closely related bacteria when more traditional typing methods are unsuitable or unavailable (Owen, 1989). In previous studies (Falk *et al.*, 1985a; Falk *et al.*, 1985b; Falk *et al.*, 1984), there was no obvious correlation between gonococcal strains with a particular RE pattern and serovar. Most strains with the same serovar were differentiated into groups with different RE patterns. Conversely, strains with one and the same RE pattern could belong to different serovar. This lack of correlation between a particular restriction enzyme pattern and a A/S class enhances the heterogeneity of typing methods for gonococcal isolates even more.

Previous studies on RE pattern analysis of *N. gonorrhoeae* (Falk *et al.*, 1985a; Falk *et al.*, 1985b; Falk *et al.*, 1984) used 4% polyacrylamide gels to separate low molecular weight fragments of *Hind*III-digested DNA, which gave excellent resolution of DNA fragments below 2 kb. The RE patterns were visually compared without measuring fragment sizes to determine differences between each pattern. Large DNA fragments can not be resolved by the polyacrylamide gel system. The 0.7% agarose gel system used in this study resolved fragments of *Sma*I-digested DNA from 5.0 to 27.0 kb into more than 80 bands. Therefore a particular region was chosen for more intensive analysis.

Previous studies have shown that the RE patterns generated by *Hind*III digests of *N. gonorrhoeae* could be used to differentiate between 18 gonococcal isolates with 4 serovars (IA-2, IA-10, IB-2, and IB-6; Falk *et al.*, 1985a); 30 *N. gonorrhoeae* isolates with 11 serovars (old serovar nomenclature) IB/cgijk, IB/acijk, IB/eghk, IB/ack, IB/cegjk, IB/eghjk, IB/aijk, IB/acej, IB/acejk, IA/edih, and IA/edgkih (Falk *et al.*, 1985b); 20 *N. gonorrhoeae* isolates with 8 serovars IA/bedgkl, IA/bedgkihl, IA/edihl, IB/abcek, IB/abceijk, IB/abk, IB/bceijk, and IB/Aabijk (Falk *et al.*, 1984) that by serotyping appeared to be identical. Since these previous studies provided no data on the auxotypes of the isolates and because the restriction enzymes used were different, the present data can not be compared with those obtained previously. However, all studies (Falk *et al.*, 1985a; Falk *et al.*, 1985b; Falk *et al.*, 1984; this study) showed that RE can distinguish between isolates that belonged to the same serovar. However if serovar is combined with auxotype, and a particular A/S class studied, RE analysis may not discriminate well between isolates in the A/S class, indicating that the group may be clonal. For example, in this study, RE analysis did not distinguish most isolates within the PCU(H), Arg-requiring and NR/IA-5 groups; 90% PCU(H) isolates belonged to RE pattern A, all arginine-requiring isolates belonged to RE pattern E and most (90%) of isolates of NR/IA-5 belonged to RE pattern Q. However, RE analysis did distinguish isolates of serovar IB-5 and IB-7. Thus RE analysis might distinguish within broad serovar categories, however it may be more limited in subtyping specific A/S classes. Isolates in each group had bands in common which were different from other groups. This might suggest that there was a correlation between gonococcal strain type with a particular RE pattern. A large sample size might indicate whether this correlation is maintained.

Previous studies also indicated that the individual RE patterns were stable *in vitro* as well as *in vivo* (Falk *et al.*, 1984). Thus there were no changes in the RE pattern of one gonococcal strain after 41 passages *in vitro* in six weeks. The genetic stability and reproducibility of RE patterns has also been confirmed in the present study.

An important advantage of this approach is the specific differentiation of one strain of a bacterial species from another without relying on expression of a phenotype. For example, RE analysis of *N. meningitidis* isolates demonstrated the genetic relatedness of strains that would have been considered unrelated based on their phenotypes (Kristiansen *et al.*, 1986) and RE analysis of chromosomal DNA revealed variability between strains. The elucidation of strain-specific differences by RE analysis is difficult, time-consuming, and often restricted to fragments of >5 kb and a large number of fragments that are close together and may be difficult to analyze from day to day (Dasi *et al.*, 1992; Owen, 1989). It is essential to run small numbers of samples side by side on one gel. Generally these total digest patterns were too complex (see Figures 7 and 8) and bands insufficiently resolved for further detailed analysis, especially out of the target size range.

The use of a scanning densitometry to locate and quantitate bands is highly desirable. In this study, fifteen arginine-requiring isolates were analyzed by laser densitometry. Visual inspection of plots of the laser densitometer scans indicated that they were similar, further indicating the these isolates were clonal. The apparatus would permit a definitive analysis of more strains. However, this apparatus is very expensive and may not be readily available to many laboratories.

RE analysis has been more successful when combined with hybridization to a gene probe such as the ribosomal nucleic acid gene. This selects a small number of fragments that generate more readily visible patterns which are thus more easily compared and interpretable (Stull *et al.*, 1988).

4.2. Ribotyping

An earlier study by Stull *et al.* (1988) demonstrated that identical rRNA gene patterns in the *Pseudomonas cepacia* and *Haemophilus influenzae* could be produced by either a species-specific or a *E. coli* 16S- and 23S-rRNA probe. Many investigators have used a broad spectrum 16 + 23 S rRNA probe from *E. coli* that is available commercially (Ng and Dillon, 1991; Ng and Dillon, 1993). For the present study, the 7.2 kb *Bam*HI-*Pst*I fragment carrying the *rrn*B rRNA operon served as a good probe in this study as hybridizing bands were seen with every strain. With this probe, the number of REA bands was reduced to six or seven, which made differentiation easier. No strain was non-typable. RFLP patterns generated using this probe were also found to be both stable and reproducible.

The choice of restriction endonucleases used was based on the divergence of rRNA gene hybridization patterns obtained. More pronounced heterogeneity of rRNA hybridization patterns could be generated by restriction endonucleases with different restriction sites within the rRNA cistrons. If a single copy rRNA gene is highly conserved and the enzyme used for restriction recognised a single site and generated only a single DNA fragment of identical molecular weight in all the strains examined, then this enzyme should not be selected; such was the case with

*Hind*II and *Ava*II digestions in this study. In the present study, restriction endonuclease *Sma*I rRNA gene hybridization patterns were found to be more useful for differentiation of strains as greater heterogeneity was observed in the number of hybridizing bands in *Sma*I rRNA gene restriction patterns.

Ribotyping was used in a previous study to differentiate 43 *N. gonorrhoeae* isolates belonging to 12 serovars (Poh *et al.*, 1992); nine ribotype patterns obtained with *Hinc*II digested chromosomal DNA were observed. Since the previous study provided no data on the auxotypes of the isolates and because the restriction enzyme used was different, pattern comparison between the previous study and the present study is not possible. An earlier study in our laboratory also showed that the ribotype patterns generated by *Sma*I or *Ava*II digests of *N. gonorrhoeae* could be used to separated 23 PCU *N. gonorrhoeae* isolates into 4 groups when the patterns were combined (Ng and Dillon, 1993). In the present study, we expand the earlier observation made by Ng and Dillon (1993) by comparing not only other PCU isolates but also other A/S classes. Previous studies (Poh *et al.*, 1992; Ng and Dillon, 1993) and the present study showed that common DNA bands were present in the ribotype patterns for all the *N. gonorrhoeae* isolates used, indicating the conserved nature of the rRNA genes within the species. Because of the differences in running conditions between studies, we could not determine whether these common bands were similar.

The results of this study showed that ribotyping based on rRNA gene heterogeneity was much simpler to interpret than the complex restriction fragment patterns of total genomic DNA digests. The one hundred gonococcal isolates were classified into eleven groups based on rRNA gene restriction patterns suggesting that there may be sufficient heterogeneity of rRNA patterns within *N. gonorrhoeae* to make them useful epidemiological markers. The *Sma*I digested rRNA gene restriction patterns observed were very much related as there were four common DNA bands present in each ribotype. The highly conserved nature of rRNA genes was reflected by the presence of these common DNA bands (e.g. four common bands in all ribotypes of this study). Nevertheless, heterogeneities in the rRNA genes existed and these were marked by the presence of unique DNA bands. In this study, most ribotypes presented the same total number of bands (six) except ribotypes V and X which produced a total of seven bands per ribotype.

In most cases the ribotype of a strain can be determined from the molecular sizes of the bands generated as calculated from the molecular weight standards in the same gel. In some instances however, it can be useful to analyze DNA from strains thought to be indistinguishable, when compared on different gels, in a single gel because in some instances inter-strain pattern differences can be relatively difficult to identify. In order to compare the ribotypes from different isolates, gel electrophoresis should be carried out under several running conditions. For example, in this study, ribotypes II and VII differed from one another in the positioning of a single band at either 22.0 kb or 23.5 kb, respectively. The resulting difference in mobility was small and was difficult to distinguish if run on different gels. Isolates with these patterns were reliably distinguished only after they are run in the same gel.

Based on ribotypes generated by *Sma*I digestion, there was no absolute correlation between rRNA gene restriction patterns and A/S class as with previous observations (Poh *et al.*, 1992; Ng and Dillon, 1993). Many strains with the same ribotype patterns were found distributed amongst several A/S class (for example ribotype pattern II was distributed between OUH/IA-2, CUH/IA-2, OH/IA-2, NR/IB-7, and P/IB-5 isolates). Strains with either identical and different ribotype patterns were also found within each A/S class.

To date, DNA probes radiolabelled with ^{32}P are most commonly used (Lowe, 1986) for ribotyping. One advantage of isotopic probes is that relatively few steps are required for hybridization and washing. However, the limitations of radioactive labelling of such probes for clinical laboratories becomes apparent when one considers the need for specially trained personnel, exposure to hazards, frequent resynthesis, radioactive waste storage and removal, and cost (Gustaferro and Persing, 1992). Recently, considerable attention has been paid to nonisotopic labels in order to eliminate problems associated with inherent limitations of radioactivity. Many nonisotopic DNA probe assays have been developed, for example with biotin (Altwegg and Mayer, 1989) or acetylaminofluorene (Grimont *et al.*, 1989). The development of non-radioactive DNA probes will clearly appeal to the routine laboratory and these have already been applied in the detection of *Chlamydia trachomatis* and *N. gonorrhoeae* (Pollice and Yang, 1985). Most nonisotopic DNA detection methods utilize alkaline phosphatase as the preferred label, owing to its high thermal stability. A new, direct chemiluminescent substrate for alkaline phosphatase, 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD), which decomposes upon dephosphorylation by the alkaline phosphatase to produce light emission at 470 nm. At pH 9.5, a constant rate of chemiluminescence is attained in approximately 20 minutes (Bronstein *et al.*, 1989).

In the present study, the rRNA gene patterns obtained using a non-radioactive probe in hybridization reactions was found to be both sharp and highly discriminatory. However, a major practical difficulty was encountered; on occasion the background was sometimes too high, thereby interfering with interpretation of results. Several conditions, such as increasing concentration of blocking reagent and washing stringency, were tried to standardize the procedure, however, high backgrounds were still a persistent, albeit random problem.

The smaller number of hybridized bands produced by most isolates greatly facilitated the interpretation and identification of the respective ribotype of an isolate. This is in sharp contrast to the problems of interpreting the complex restriction fragment patterns produced by chromosomal endonuclease digestion. However, one limitation of the ribotyping for identification purpose is the lack of standardized methods for recording ribotype patterns (Grimont and Grimont, 1986) for the establishment of databanks. The disadvantages of ribotyping are in comparing data from different gels or between laboratories, expensive, time-consuming and labour intensive, since Southern blotting is employed as an essential part of the procedure.

4.3. Pulsed field gel electrophoresis

Recently, pulsed field gel electrophoresis has been used to visualize very large fragments of DNA (Allardet-Servent *et al.*, 1989; Owen, 1989); such fragments can be generated from bacterial genomic DNA by restriction endonucleases that have few recognition sites. PFGE has been used to compare chromosomal restriction patterns in *Acinetobacter calcoaceticus, Pseudomonas aeruginosa* (Allardet-Servent *et al.*, 1989), *Pseudomonas cepacia* (Anderson *et al.*, 1991), *L. pneumophila* (Ott *et al.*, 1991), and *Enterococcus faecalis* (Murray *et al.*, 1990). In contrast to ribotyping, PFGE detects the distribution of restriction sites throughout the chromosome. This distribution varies because of strain to strain mutational differences and because of the variations in the gene content of the bacterial chromosome (Prevost *et al.*, 1992). This technique is relatively simple, is applicable to a wide range of bacteria with little modification, and appears to have sufficient discriminatory power to be useful for epidemiologic tracking. Gonococcal isolates were readily analyzed by this technique without major modification of the protocol described by Chang and Taylor (1990). Valuable patterns were generated, and considerable restriction fragment polymorphism was present.

Previous studies of the gonococcus by pulsed field gel electrophoresis have been used to construct a physical map of the chromosome after *Spe*I digestion of genomic DNA. The genomic size of *N. gonorrhoeae* strain FA1090 was 2,219 kb (Dempsey *et al.*, 1991) and the genomic size of *N. gonorrhoeae* strain MS11-N198 was 2.33 Mb ± 35 kb (Bihlmaier *et al.*, 1991). In this study, most isolates have the genomic size in this range (2.083 - 2.420 kb), although no attempt was made to definitively resolve smaller fragments.

It has been shown previously that the PFGE patterns generated by *Spe*I or *Nhe*I digests of *N. gonorrhoeae* could be used to differentiate forty-eight *N. gonorrhoeae* isolates into thirty-eight or forty groups (Poh and Lau, 1993). *Spe*I generated 12-17 fragments. Since the A/S classes of the isolates in this previous study were different and fragment size were not measured, we were unable to compare our data with those obtained previously. However, both studies (Poh and Lau, 1993; this study) demonstrated that PFGE could subtype isolates within the same A/S classes. This has also been suggested by other investigator for *N. gonorrhoeae* (Xia *et al.*, 1993).

In this study, the twenty-six gonococcal isolates studied by PFGE were represented by eight A/S classes, eleven RE patterns, nine ribotypes and twenty-five PFGE patterns. PFGE was found to provide greater differentiation than either A/S typing or ribotyping. Heterogeneity of *N. gonorrhoeae* strains was demonstrated by the presence of twenty-five different *Spe*I PFGE patterns amongst the group of twenty-six isolates. Strains were clearly differentiated. Ribotype V could be subdividing into five patterns (P21, P22, P23, P24, and P25) among the *N. gonorrhoeae* A/S class NR/IA-5 five isolates. A/S classes NR/IB-5 and NR/IB-7 or P/IB-5 and P/IB-7 could also be differentiated by PFGE. A small number of isolates from each ribotype was chosen for analysis by PFGE in the present study.

The interpretation of PFGE patterns is difficult when isolates differ by only a few fragments; for example, patterns P21 and P25 differed from each other by one fragment (453.0 or 410.0 kb). Such differences could arise from mutation within a single isolate, such as inversion, deletions, or other rearrangements of the chromosome. On the other hand, such differences could indicate that the isolates are more distantly related. Although it is not known how to interpret small variations, an evaluation of the variation present in a number of other isolates in the same local area may be useful. The possibility of *in vivo* changes in phenotype or genotype is not limited to this technique and represents potential limitations of any typing scheme (Murry *et al.*, 1990).

As reported in a previous study, single typing schemes such as auxotyping, serotyping and plasmid analysis produced some of the lowest Simpson's indices of discrimination (Dillon *et al.*, 1993). The discriminatory power of auxotyping, serotyping, and plasmid analysis determinations, for penicillin-susceptible isolates was 80.3%, 81.8%, and 48.2%, respectively (Dillon *et al.*, 1993). In the present study, single typing schemes such as ribotyping and RE analysis was less discriminatory than the combined molecular methods or one of the molecular methods combined with A/S class. The scheme producing the highest level of discrimination for all isolates was the A/S-Ribotyping-RE analysis besides the PFGE analysis.

In conclusion, the RE analysis and ribotyping for 100 gonococcal isolates discriminated 13 A/S classes into a total of 20 RE patterns, 11 ribotype patterns, 26 A/S/RE patterns, 31

RE/ribotype patterns, and 38 A/S/RE/ribotypes. Twenty-five individual PFGE patterns produced from a subset of 26 of the 100 gonococcal isolatesa. The present results show that for isolates that appear clonal by classical typing methods [PCU(H) and arginine-requiring isolates], RE analysis produced less discrimination than ribotyping and PFGE; for isolates from the population studies, RE analysis, ribotyping and PFGE analysis, particularly in combination, produced good discrimination for certain A/S classes; however, for isolates from outbreaks, RE analysis and ribotyping alone produced much less discrimination than PFGE. PFGE analysis produced the highest discrimination of the three molecular typing methods evaluated.

The application of other new techniques may be useful to subtype *N. gonorrhoeae* isolates; these include polymerase chain reaction (PCR)-RFLP that involves amplifying a known sequence, cutting with restriction endonucleases and comparing restriction fragments of the amplified DNA from different strains (Barry *et al.*, 1991; Kostman *et al.*, 1992); random amplification of polymorphic DNA (RAPD) with primers that have unknown homology to the target sequence and thereby generating reproducible fingerprints of genomic DNA (Welsh and McClelland, 1990; Williams *et al.*, 1990), and DNA sequencing that direct compares of genomic DNA sequences of bacteria strains (Swaminathan and Matar, 1993; Dean *et al.*, 1991).

5. CONCLUSIONS

In conclusion, a comparison of restriction endonuclease analysis of chromosomal DNA and ribotyping for 100 gonococcal isolates either from outbreaks or from population studies as well as restriction endonuclease analysis by PFGE for 26 of the 100 gonococcal isolates in this study demonstrated that PFGE proved to be a useful method of choice for differentiation of strains of *N. gonorrhoeae* and strains that are the same A/S classes or ribotypes or untypable by these two methods. The use of restriction enzyme analysis by PFGE allowed the subdivision of isolates within the same ribotype pattern. The evaluation of large genomic fragments by PFGE is easily performed, is reliable, and detects considerable polymorphism. PFGE also is a powerful tool for performing molecular typing studies at a level of precision not achievable using gene probes to analyze polymorphisms of specific target genes such as the rRNA genes.

Thirty PCU(H) gonococcal isolates of serovar IB-1 or IB-2 represented different geographic locations and were selected from Canadian gonococcal population studies. They were homogeneous with respect to their antimicrobial susceptibilities (within a two fold dilution) and their plasmid content (plasmid free) except one unusual isolate contained 2.6 MDa plasmid. These isolates were classified into four RE patterns (90% of them belonged to RE pattern A) and five ribotypes (80% of them were represented by ribotype I). Six of these isolates were chosen for further study by PFGE. Five PFGE patterns (P1, P2, P3, P4 and P5) were produced. Two isolates belonged to the same PFGE pattern P3.

Twenty-seven arginine-requiring isolates were selected from Canadian gonococcal population studies. Although they could be distinguished based on specific arginine-requiring auxotypes, they were homogeneous with respect to their antimicrobial susceptibilities, their serovar, their plasmid content and restriction endonuclease analysis (RE pattern E). These isolates were subtyped into five ribotypes, 65% of them belonged to ribotype II. PFGE analysis of nine of these isolates further produced a different pattern (P6, P7, P8, P9, P10, P11, P12, P13 and P14) for each isolate although the isolates did carry fragments in common.

Four isolates of A/S class NR/IB-5, six isolates of A/S class NR/IB-7, six isolates of A/S class P/IB-5 and five isolates of A/S class P/IB-7 were selected from Canadian gonococcal population studies. These isolates were heterogeneous with respect to their antimicrobial susceptibilities, their auxotype, their serovar and their plasmid content. These isolates were further divided into eleven RE patterns and five ribotypes. Six of the isolated were selected for further analysis by PFGE, including two NR/IB-5 and four NR/IB-7. These six isolates produced six PFGE pattern, although they had three common bands.

Ten gonococcal isolates of A/S class NR/IA-5, which were selected from a geographically restricted (Toronto) outbreak of a particular A/S class, comprised eight PPNG isolates with the same plasmid content as well as a PP/TRNG and non-PPNG isolates. Except for the differences in penicillin resistance between PPNG and non-PPNG isolates and tetracycline susceptibility of the TRNG isolates, the MICs of these isolates were similar. These isolates were sorted into two RE patterns. Nine of them belonged to one RE pattern, but the PP/TRNG isolate belonged to another RE pattern. The isolates could not be differentiated by ribotyping. Five of isolates were selected for further analysis by PFGE, including the non-PPNG and the PP/TRNG isolates. All five isolates could be distinguished using PFGE, although they shared four common bands.

Twelve gonococcal isolates of A/S class NR/IB-3, which were selected from an outbreak of CMRNG centred in Manitoba and Ontario, comprised six isolates from Manitoba and six isolates from Toronto with the same plasmid content. They were homogeneous with their antimicrobial susceptibilities and their plasmid content. These isolates were classified into two RE patterns and two ribotypes. PFGE analysis was not done for this group.

6. IDENTITY OF ENZYME DEFICIENCIES IN ARGININE-REQUIRING STRAINS OF *N. GONORRHOEAE*

6.1. Introduction

N. gonorrhoeae, is the causative agent of gonorrhoea, one of the most common sexually transmitted diseases. Spontaneous mutations in genes encoding enzymes implicated in a variety of biosynthesis pathways are found in *N. gonorrhoeae* strains isolated from patients resulting in strains having different mutational requirements (Carifo and Catlin, 1973; Catlin 1973; Catlin, 1977). Some of the most frequently encountered auxotype defects are found in the arginine biosynthesis pathway (Carifo and Catlin, 1973; Catlin, 1977; Copley and Egglestone, 1983; Dillon and Pauzé, 1984; Hendry and Stewart, 1979). Arginine auxotyphs are commonly encountered in *N. gonorrhoeae*, comprising 40-60% of clinical isolates (Picard and Dillon, 1989; Catlin and Nash, 1978). Correlations of Arg with other phenotypes has been discussed previously (see introduction 1.3.).

Arginine is synthesized from glutamate via the intermediates shown in Figure 20 (Shinners and Catlin, 1982). The eight-step pathway begins with the acetylation of glutamate to N-acetylglutamate and proceeds through two additional acetylated intermediates to ornithine, which is then converted to citrulline, arginosuccinate, and finally arginine (Vogel, 1970). Ornithine and citrulline are intermediates in the arginine biosynthesis pathway and carbamoyl phosphate is an intermediate shared by both arginine and uracil pathways. Among the arginine requiring strains are those with one or more additional nutritional requirement. Arginine (Arg) and uracil-requiring strains are often found indicating a deficiency affecting both the arginine and uracil biosynthesis pathways.

In gonococci, as in *E. coli* and *Pseudomonas aeruginosa* (Hass *et al.*, 1977; Isaac and Holloway, 1972), L-ornithine and carbamoyl phosphate are converted to citrulline by ornithine transcarbamoylase (OTCase, encoded by *argF*), and OTCase, ornithine, and carbamoylphosphate

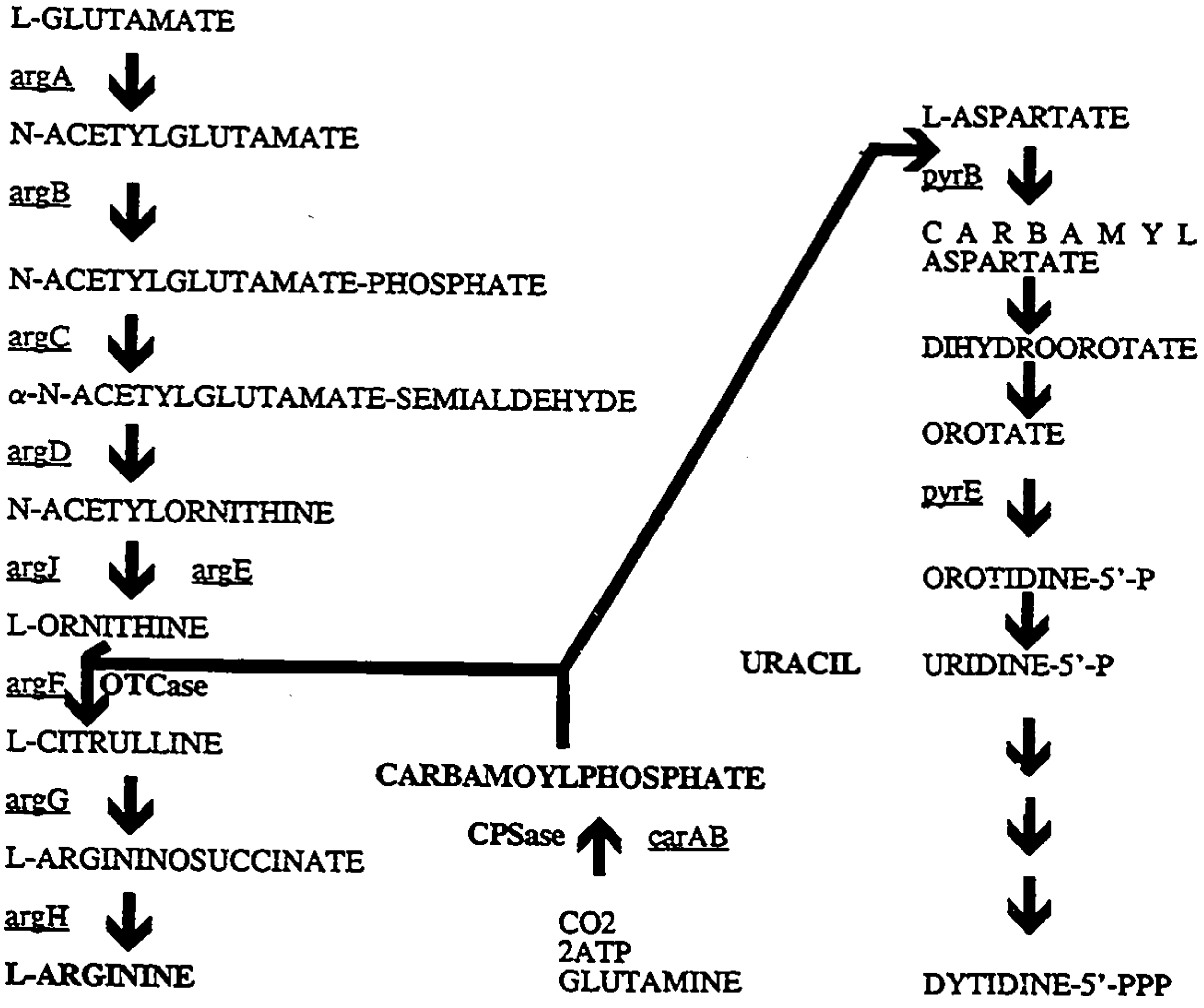

Figure 20. Arginine and uracil biosynthetic pathways and relevant gene loci. The enzymes are encoded by the following genes: ornithine transcarbamoylase, *argF*; carbamoylphosphate synthetase, *carAB*.

are required for the synthesis of citrulline. Carbamoyl phosphate formation from glutamine, CO_2, ATP is catalyzed by carbamoylphosphate synthetase (CPSase, encoded by *carAB*). Some ArgU strains utilized citrulline in place of arginine for growth. Strains requiring citrulline (C) and uracil (U) could be mutant in either one of *argF* and *carAB* genes or in *carAB* alone. In *P. aeruginosa* and *E. coli*, mutants which lack CPSase as a result of *car* lesions show requirements for both arginine and uracil. Therefore, it seemed likely that certain AU strains of *N. gonorrhoeae* might lack CPSase.

In arginine requiring strains, most isolates have multiple requirements for growth; isolates requiring citrulline alone for growth are very rare. Only 2 of 1540 of arginine-requiring isolates in the culture collection of the NLSTD were citrulline-requiring. The precise mutation in CU isolates has been controversial (Shinners and Catlin, 1982; Picard and Dillon, 1989). Thus, I tested the hypothesis that citrulline-requiring strains were mutant in *argF* (OTCase) while strains deficient in C and U biosynthesis were mutant in *carAB* (CPSase).

The purpose of this study is to determine the enzymatic difference between isolates only requiring citrulline and isolates requiring citrulline and uracil by screening the presence of OTCase and CPSase by enzymatic assays for these two enzymes.

6.2. Material and Methods

6.2.1. *Bacterial strains*

N. gonorrhoeae and *E. coli* strains used in this study are listed in Table 29. Twenty-five strains of *N. gonorrhoeae* were selected from the culture collection of the National Laboratory for Sexually Transmitted Diseases (Laboratory Centre for Disease Control, Ottawa, Ontario) comprising fourteen requiring proline, citrulline and uracil (PCU); six requiring citrulline, uracil and hypoxanthine (CUH); two sole requiring citrulline (C) and one non-requiring (NR). Two *E. coli* strains, JM240 and N166, were kindly supplied by B.J.Bachmann (Vale University, New Haven).

Table 29. Strains of *N. gonorrhoeae* and *E. coli* used in enzymatic assays

Strain	Geographic Source	Auxotype[d] and Relevant Genotype	Serovar
N. gonorrhoeae			
NS8	Ontario	PCU	IB-1
NS211	Saskatchewan	PCU	IB-16
NS213	Saskatchewan	PCU	IB-1
NS263	New Brunswick	PCU	IB-1
NS348	Alberta	PCU	IB-7
NS361	Nova Scotia	PCU	IB-25
NS384	Nova Scotia	PCU	IB-2
NS571	Yukon	PCU	IB-2
NS724	Yukon	PCU	IB-1
NS791	Saskatchewan	PCU	IB-3
NS303	Ontario	CUH	IA-2
NS1190	Ontario	CUH	IB-1
NS1028	British Columbia	PCU	IB-2
NS1426	Nova Scotia	CUH	IA-2
NS1047	Ontario	PCU	IB-2
NS2152	British Columbia	PCU	IB-2
NS1061	Yukon	CUH	IA-2
NS1634	British Columbia	PCU	IB-2
NS686	Nova Scotia	CUH	IA-2
NS716	Quebec	CUH	IA-2
NS2882	Ontario	C	IB-3
NS2929	Manitoba	C	IB-3
NS1O74	New Brunswick	CU	IB-3
NS1-418	British Columbia	CU	IB-3
CH811[a]	Chile	NR	IB-2
E. coli			
JM240[b]		*proC47*, *cyc-54*, λ^-, λ^s	
N166[c]		Hfr(H), argI67, Δ(proB-argF-lac), λ^-, λ^s	

[a]: CH 811 is *N.gonorrhoeae* positive control

[b]: JM 240 is *E. coli* positive control

[c]: N 166 is *E. coli* negative control

[d]: Requirements used for auxotyping are abbreviated as in Table 1.

6.2.2. *Media, growth conditions and auxotype determination*

The media for isolates growth, growth conditions and auxotype determination have been described previously (see section 2.1.1. and 2.1.2.). The modified neisseria-defined agar (NEDA) was prepared using the method described by Hendry and Stewart (1979).

6.2.3. *Preparation of cell-free extracts*

Bacteria were grown on modified NEDA media. Cells were collected from the agar surfaces, and dense suspensions were made in 6 mL of cold (4°C) Tris-Hydrochloride buffer composed of 50 mM tris (hydroxymethyl)-aminomethane-hydrochloride, pH 7.5. Cells were kept chilled, and were disrupted with a Braun-Sonic 1510 Sonicator (B. Braun Biotech Inc., PA, UAS). The disruption was carried out at six seconds intervals for a total of one minute with six seconds intervening periods for cooling; then the cell preparation was immediately subjected to centrifugation at 27,000 x g for 15 minutes. The supernatant fluid (crude cell-free extract) was filtered through a 0.2 μm filter membrane (Millex GS, Gelman Sciences) and stored in aliquots at -70°C.

6.2.4. *Protein concentration determination*

The protein content of the extracts was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories) according to the instructions provided by the manufacturer.

6.2.5. *Enzyme assays*

a) Ornithine transcarbamylase (OTCase): OTCase activity was assayed for the production of citrulline by the method of Shinners and Catlin (1982). A final volume of 0.25 mL contained 10 μmol/mL of L-ornithine (Sigma), 10 μmol/mL of carbamylphosphate (Sigma), 5 μmol/mL of $MgCl_2$, 100 μmol/mL of Tris-hydrochloride (pH 7.5), and 0.125 mL (0.4-0.8 mg/mL of protein) of cell-free extract. The reaction was started by addition of the extract and carbamylphosphate to the prewarmed (37°C) mixture. After 30 minutes of incubation at 37°C, a 0.5 mL volume of 2 N HCl was added to each reaction mixture, which then was boiled for 10 minutes. The reaction mixture was cooled in ice water for 10 minutes followed by the addition of 0.5 mL of solution A (three volumes of 85% phosphoric acid, ne volume of concentrated H_2SO_4), and then the addition of 0.05 mL of 3.75% diacetyl monoxime (2.3-butanedione monoxime, Sigma Chemical Co.). Diacetyl monoxime produces an orange colour in acid solution with given a carbamino compound. (Under the conditions cited above colour development from citrulline would be maximal). After mixing in a Vortexer, the tubes were boiled for 30 minutes in the dark. The tubes were cooled in ice water and protected from light by covering with aluminium foil. The absorbances at 490 nm were determined with a SPECTRONIC 1001 spectrophotometer (Bausch & Lomb Inc.) in a dark room.

Assays were performed in triplicate and were accompanied by positive and negative controls. For positive and negative controls, enzyme activities of different protein concentrations were checked. The enzyme activities of boiled cell-free extract (boiled for 10 minutes) were also determined. Positive control CH811 was used for enzyme activity stability assay. A set of standards containing 0.0125 to 0.40 μmol/mL of citrulline was included with each group of assays. The rates were calculated from simple linear regression analysis of the standard curve which represented the means of triplicate determinations. Specific activity was expressed as nanomoles of citrulline formed per minute per milligram of protein in the cell-free extract.

b) Carbamoylphosphatase (CPSase): Glutamine-dependent CPSase activity was measured by the formation of citrulline by a modification of the method of Catlin and Shinners (1982). The reaction mixture in a final volume of 0.25 mL contained 10 μmol/mL glutamine, 10 μmol/mL ATP, 10 μmol/mL $MgCl_2$, 90 μmol/mL potassium bicarbonate ($KHCO_3$), 10 μmol/mL ornithine, 50 μmol/mL Tris-hydrochloride (pH 7.5), 10 U/mL OTCase (from *Streptococcus faecalis*; Sigma), and 0.125 mL (0.4-0.8 mg/mL of protein) of cell-free extract. Positive and negative controls (same as in *OTCase* enzymatic assay) and citrulline standards were run simultaneously with each assay.

The diacetyl monoxime colorimetric test, as described for OTCase, was used to determine the amount of citrulline formed. Specific activity was expressed in terms of nanomoles of citrulline formed per minute per milligram of crude extract protein.

6.3. Results

6.3.1. *OTCase*

L-citrulline formation from L-ornithine and carbamoylphosphate is catalyzed by ornithine transcarbamoylase. Ornithine transcarbamoylase activity was detected in *N. gonorrhoeae* CH811 and *E. coli* JM240, the positive controls, but not in *E. coli* N166, the negative control (Table 30). Different protein concentrations of crude extract in both positive and negative controls were assayed also to determine whether the enzyme activities might be different (Table 30). In strains CH811 and JM240, the positive controls, different protein concentrations of cell-free extracts had different enzyme activities, *i.e.* as the protein concentration increased so did enzyme activity. This was not the case with the negative control (N166).

Crude cell-free extracts were boiled to determine whether the enzyme activity might be lost. After boiling, enzyme activity in crude cell-free extracts of positive controls was lost (Table 30); the activity of the negative control was unchanged.

All strains requiring PCU, CUH, and CU (22/24, 91.7%) possessed ornithine transcarbamoylase activities (Table 31) which clustered in the range 35.16-137.94 nmol/min per mg of protein. Only strains NS 2882 and NS 2929, which had a sole requirement for citrulline lacked OTCase activity.

6.3.2. *CPSase*

CPSase activity was only present in the strains NS2882 and NS2929, which had a sole requirement for citrulline (Table 31). All strains requiring PCU, CUH, and CU (22/24, 91.7%) lacked CPSase activities.

Table 30. OTCase activities of positive and negative controls with different protein concentrations

Strain	Protein Concentration (μg/mL)	Enzyme Activities[a] (nmol/min)	
		no boiling	boiling
CH811[b]	4.4	4.3	<0.8
CH811	8.7	9.4	<0.8
CH811	17.4	19.8	<0.8
CH811	34.8	43.3	<0.8
JM240[c]	4.3	5.9	<0.8
JM240	8.6	14.2	<0.8
JM240	17.1	36.5	
			<0.8
N166[d]	4.4	<0.8	<0.8
N166	8.8	<0.8	<0.8
N166	17.5	<0.8	<0.8
N166	35.5	<0.8	<0.8
N166	70.0	<0.8	<0.8
N166	140.0	<0.8	<0.8

[a]: enzyme activity for OTCase is expressed in nmols of citrulline produce per min.

[b]: *N. gonorrhoeae* positive control.

[c]: *E. coli* positive control.

[d]: *E. coli* negative control.

Table 31. OTCase and CPSase activities of cell-free extracts

Strain	Protein Concentration (μg/mL)		Specific Activities nmol/min per mg of protein	
	OTCase	CPSase	OTC ase	CPSase
NS8	600	600	69.33	<0.02
NS211	590	590	44.02	<0.02
NS213	610	610	53.86	<0.02
NS263	600	600	116.44	<0.02
NS348	590	590	70.15	<0.02
NS361	600	600	95.20	<0.02
NS384	600	600	36.53	<0.02
NS571	590	590	43.66	<0.02
NS724	600	600	55.29	<0.02
NS791	610	610	86.99	<0.02
NS303	610	610	66.54	<0.02
NS686	600	600	40.71	<0.02
NS716	580	580	73.10	<0.02
NS1028	610	610	41.79	<0.02
NS1047	580	580	102.53	<0.02
NS1061	610	610	117.25	<0.02
NS1190	590	590	137.94	<0.02
NS1426	590	590	53.33	<0.02
NS1634	610	610	87.26	<0.02
NS2152	590	590	35.62	<0.02
NS1-418	600	600	36.89	<0.02
NS1074	590	590	35.16	<0.02
NS2882	**580**	**580**	**<0.02**	**36.87**
NS2929	**600**	**600**	**<0.02**	**37.51**
CH811[a)]	610	610	48.00	34.27
CH811[b)]	610	610	<0.02	<0.02

[a)]: positive control.

[b)]: negative control.

6.4. Discussion

Genetic transformation experiments suggest that the arginine requirements of AHU and Arg-non-HU strains are due to various defects of arginine biosynthesis gene (Caltin, 1974; Mayer *et al.*, 1977). Investigations of arginine requirements in *N. gonorrhoeae* isolates has demonstrated various genetic defects responsible for the Arg phenotype (Carifo and Catlin, 1973; Catlin, 1977; Dillon and Pauzé, 1984).

Some citrulline and uracil requiring gonococcal isolates were unable to utilize ornithine for growth, although they produced OTCase, suggesting that these gonococci be mutant in *carAB*, resulting in a failure to synthesize carbamyl phosphate which is an intermediate shared by both arginine and uracil pathways (Shinners and Catlin, 1982).

Most of the twenty-four (91.7%) strains of *N. gonorrhoeae* tested in the present study that appear to be defective in carbamoylphosphate synthetase gene (*carAB*) but possessed OTCase had a dual requirement for citrulline and uracil, suggesting that a CPSase gene defect was attributable to their dual requirement for citrulline and uracil. These results are similar to the findings by Shinners and Catlin (1982) who showed that all eleven citrulline and uracil requiring strains of *N. gonorrhoeae* examined (six of auxotype CU and five of auxotype CUH) had normal OTCase activity but lacked CPSase activity.

In present study, only 2 of over 1540 isolates with an arginine requirement were deficient in ornithine transcarbamylase (*argF* defect), suggesting that a OTCase gene defect was responsible for the citrulline requirement alone. It is clear that OTCase activity is necessary but not sufficient to enable a cell to form citrulline from ornithine. In intact gonococci the formation of citrulline by the OCTase catalyzed reaction of L-ornithine with carbamyl phosphate synthetase, a glutamine-dependent enzyme present in extracts of intact gonococcal cells (Catlin and Nash, 1978).

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APPENDIX

Table A-1. Antimicrobial susceptibilities and geographic source of 15 PCU(H)/IB-1 gonococcal isolates

Strain No.[c]	A/S	Year Isolated	Geographic Source	MICs[a] (mg/mL)					
				PEN	TET	ERY	SPEC	CN	CX
880067	PCU/IB-1	1988	Edmonton, ALTA	0.125	0.25	0.063	16.0	0.5	0.001
880128	PCU/IB-1	1988	Halifax, NS	0.25	0.25	0.25	16.0	0.5	0.002
880263	PCU/IB-1	1988	St. John, NB	0.25	0.5	0.5	16.0	1.0	0.002
880724	PCU/IB-1	1988	Whitehorse, YUK	0.063	0.25	0.125	16.0	0.5	0.001
881587	PCU/IB-1	1988	Toronto, ONT	0.25	0.5	0.5	16.0	0.5	0.004
883036	PCU/IB-1	1988	Calgary, ALTA	0.25	1.0	1.0	16.0	1.0	0.004
883077	PCU/IB-1	1989	St. John, NB	0.5	0.5	0.5	16.0	0.5	0.004
883632	PCU/IB-1	1989	Regina, SASK	0.25	1.0	0.5	16.0	0.5	0.004
880596	PCUH/IB-1	1988	St. John, NB	0.125	0.25	0.25	16.0	0.5	0.001
880687	PCUH/IB-1	1988	Halifax, NS	0.25	0.5	0.5	16.0	1.0	0.004
882779	PCUH/IB-1	1989	Ottawa, ONT	0.5	1.0	2.0	16.0	2.0	0.008
882989	PCUH/IB-1	1988	LaRonge, SASK	0.5	0.5	1.0	16.0	1.0	0.004
883353	PCUH/IB-1	1989	Edmonton, ALTA	0.125	0.25	0.063	16.0	0.5	0.002
3703	PCUH/IB-1	1989	Winnipeg, MAN	0.5	1.0	0.5	16.0	-[b]	0.016
7861	PCUH/IB-1	1991	Weston, ONT	0.25	0.5	0.25	16.0	-[b]	0.004

[a]: PEN = penicillin; TET = tetracycline; ERY = erythromycin; SPEC = spectinomycin; CN = cefoxitin and CX = ceftriaxone.

[b]: Not test. [c]: All isolates were plasmid free except isolate 882779 (contained 2.6 MDa plasmid).

Table A-2. Antibiotic susceptibilities and geographic source of 15 PCU(H)/IB-2 gonococcal isolates

Strain No.[c]	A/S	Year Isolated	Geographic Source	MICs[a] (mg/mL)					
				PEN	TET	ERY	SPEC	CN	CX
880074	PCU/IB-2	1988	Edmonton, ALTA	0.5	0.25	0.25	16.0	0.5	0.004
810916	PCU/IB-2	1988	Ottawa, ONT	0.25	0.5	0.5	16.0	1.0	0.004
881777	PCU/IB-2	1988	St., QUE	0.25	0.5	0.5	16.0	1.0	0.004
881985	PCU/IB-2	1988	Whitehorse, YUK	0.5	0.25	1.0	16.0	1.0	0.008
881996	PCU/IB-2	1988	Vancouver, BC	0.063	0.25	0.5	16.0	0.5	0.002
882988	PCU/IB-2	1988	Saskaton, SASK	0.25	1.0	0.5	32.0	1.0	0.004
883329	PCU/IB-2	1989	Halifax, NB	0.25	0.5	0.5	16.0	0.5	0.002
880071	PCUH/IB-2	1988	Edmonton, ALTA	0.25	0.25	0.25	16.0	0.5	0.002
881542	PCUH/IB-2	1988	Halifax, NS	0.063	0.5	1.0	16.0	0.25	0.001
881717	PCUH/IB-2	1988	Montreal, QUE	0.25	0.25	0.5	16.0	0.5	0.002
882191	PCUH/IB-2	1988	Halifax, NS	0.5	0.5	0.5	16.0	1.0	0.008
883238	PCUH/IB-2	1989	Regina, SASK	0.5	1.0	0.5	16.0	0.5	0.004
883355	PCUH/IB-2	1989	Edmonton, ALTA	0.5	0.5	1.0	16.0	0.5	0.004
883766	PCUH/IB-2	1989	St. John, NB	0.25	0.5	0.5	16.0	1.0	0.008
8281	PCUH/IB-2	1991	Weston, ONT	0.25	0.25	0.125	16.0	-[b]	0.004

[a] and [b] see Table A-1.

[c]: All isolates were plasmid free.

Table A-3. Antibiotic susceptibility and geographic source of 27 arginine-requiring gonococcal isolates

Strain No.[b]	A/S	Year Isolated	Geographic Source	MICs[a] (mg/L)					
				PEN	TET	ERY	SPEC	CN	CX
880375	OUH/IA-2	1988	Halifax, NS	0.008	0.25	0.5	16.0	0.25	0.00025
880420	OUH/IA-2	1988	Toronto, ONT	0.016	0.25	0.25	16.0	0.25	0.00025
881035	OUH/IA-2	1988	Vancouver, BC	0.016	0.25	1.0	32.0	0.25	0.00025
882208	OUH/IA-2	1988	Halifax, NS	0.016	0.25	0.5	16.0	0.25	0.0005
882306	OUH/IA-2	1988	Regina, SASK	0.016	0.25	1.0	32.0	0.25	0.0005
882398	OUH/IA-2	1988	St. John, NFLD	0.016	0.25	1.0	32.0	0.25	0.00025
882245	OUH/IA-2	1988	Weston, ONT	0.016	0.25	0.5	16.0	0.25	0.0005
882521	OUH/IA-2	1988	Inuvik, NWT	0.016	0.25	0.063	16.0	0.25	0.0005
883122	OUH/IA-2	1989	Ottawa, ONT	0.008	0.25	0.5	16.0	0.25	0.00025
883171	OUH/IA-2	1989	Halifax, NS	0.004	0.25	0.5	16.0	0.032	0.005
883342	OUH/IA-2	1989	Edmonton, ALTA	0.016	0.25	1.0	32.0	0.25	0.00025
883463	OUH/IA-2	1989	Winnipeg, MAN	0.016	0.25	1.0	32.0	0.25	0.0005
880270	CUH/IA-2	1988	St. John, NS	0.008	0.25	1.0	32.0	0.25	0.00025
880379	CUH/IA-2	1988	Halifax, NS	0.004	0.125	0.25	2.0	0.125	0.00025
880454	CUH/IA-2	1988	Ottawa, ONT	0.008	0.25	1.0	32.0	0.125	0.00025
881540	CUH/IA-2	1988	Halifax, NS	0.008	0.25	1.0	32.0	0.25	0.00025
882132	CUH/IA-2	1988	Winnipeg, MAN	0.008	0.25	0.5	16.0	0.25	0.00025
882610	CUH/IA-2	1988	Halifax, NS	0.016	0.25	0.5	32.0	0.25	0.00025
883099	CUH/IA-2	1988	Calgary, ALTA	0.016	0.25	1.0	16.0	0.25	0.00025
883729	CUH/IA-2	1989	Halifax, NS	0.016	0.25	0.5	16.0	0.25	0.00025
881097	OH/IA-2	1988	St, John, NB	0.008	0.25	1.0	32.0	0.25	0.00025
881342	OH/IA-2	1988	Winnipeg, MAN	0.016	0.25	1.0	32.0	0.25	0.0005
881506	OH/IA-2	1988	Whitehorse, YUK	0.008	0.25	0.5	32.0	0.125	0.0005
882367	OH/IA-2	1988	Winnipeg, MAN	0.008	0.125	1.0	16.0	0.25	0.00025
882642	OH/IA-2	1988	St. John, NB	0.25	0.5	2.0	16.0	1.0	0.002
882794	OH/IA-2	1988	Winnipeg, MAN	0.16	0.25	1.0	16.0	0.25	0.00025
883212	OH/IA-2	1988	Regina, SASK	0.004	0.25	0.5	32.0	0.25	0.00025

[a] see Table A-1.

b): All isolates contained 2.6 MDa plasmid.

Table A-4. Antibiotic susceptibilities and geographic source of 4 NR/IB-5 and 6 NR/IB-7 isolates of *N. gonorrhoeae*

Strain No.[c]	A/S	Year Isolated	Geographic Source	MICs[a] (mg/mL)					
				PEN	TET	ERY	SPEC	CN	CX
880107	NR/IB-5	1988	Edmonton, ALTA	0.032	0.25	1.0	16.0	0.25	0.005
880466	NR/IB-5	1988	Winnipeg, MAN	0.25	0.25	0.5	32.0	0.50	0.002
880644	NR/IB-5	1988	Winnipeg, MAN	0.125	0.25	0.5	16.0	0.50	0.002
3351	NR/IB-5	1989	Edmonton, ALTA	2.0	4.0	2.0	32.0	-[b]	0.008
881095	NR/IB-7	1988	St. John, NB	0.25	0.25	1.0	32.0	0.5	0.002
881359	NR/IB-7	1988	Pr. George, BC	0.063	1.0	0.5	32.0	0.5	0.001
882449	NR/IB-7	1988	Ottawa, ONT	0.125	0.5	0.125	16.0	1.0	0.001
883535	NR/IB-7	1989	Montreal, ONT	1.0	2.0	2.0	32.0	1.0	0.016
883665	NR/IB-7	1989	Winnipeg, MAN	0.032	0.25	1.0	16.0	0.5	0.0005
4209	NR/IB-7	1990	Vancouver, BC	2.0	4.0	2.0	32.0	-[b]	0.016

[a] and [b] see Table A-1.

[c]: All isolates contained 2.6 MDa plasmid. Except for 2.6 MDa plasmid, isolates 3351, 881095, 881359 and 882449 also contained 24.5 MDa plasmid.

Table A-5. Antibiotic susceptibilities and geographic source of 6 P/IB-5 and 5 P/IB-7 isolates of *N. gonorrhoeae*

Strain No.[c]	A/S	Year Isolated	Geographic Source	MICs[a] (mg/mL)					
				PEN	TET	ERY	SPEC	CN	CX
880457	P/IB-5	1988	Ottawa, ONT	0.063	0.5	0.125	16.0	0.25	0.001
881051	P/IB-5	1988	Ottawa, ONT	0.2	1.0	2.0	16.0	0.5	0.002
882508	P/IB-5	1988	Notredame, QUE	1.0	2.0	2.0	16.0	2.0	0.008
4702	P/IB-5	1989	Weston., ONT	2.0	2.0	1.0	32.0	-[b]	0.008
4710	P/IB-5	1989	Weston., ONT	1.0	2.0	1.0	32.0	-[b]	0.008
5846	P/IB-5	1990	Weston., ONT	2.0	2.0	2.0	32.0	-[b]	0.032
880324	P/IB-7	1988	Vancouver, BC	0.5	2.0	2.0	32.0	1.0	0.004
880972	P/IB-7	1988	Calgary, ALTA	4.0	4.0	2.0	32.0	4.0	0.032
883141	P/IB-7	1989	Edmonton, ALTA	0.032	0.25	4.0	16.0	0.25	0.0005
6920	P/IB-7	1990	Weston., ONT	0.25	1.0	1.0	16.0	-[b]	0.008
8318	P/IB-7	1991	Montreal, QUE	1.0	1.0	2.0	16.0	-[b]	0.008

[a] and [b] see Table A-1.

[c]: All isolates contained 2.6 MDa plasmid except for 4702, 4710 and 5846 contained 2.6 and 24.5 MDa plasmids.

Table A-6. Antibiotic susceptibilities and geographic source of 10 NR/IA-5 gonococcal isolates

Strain No.[c]	A/S	Year Isolated	Geographic Source	MICs[a] (mg/mL)				
				PEN	TET	ERY	SPEC	CX
2852	NR/IA-5	1988	Toronto, ONT	128.0	1.0	-[b]	32.0	0.004
3735	NR/IA-5	1989	Toronto, ONT	64.0	1.0	0.25	32.0	0.004
3928	NR/IA-5	1989	Toronto, ONT	64.0	0.25	0.125	16.0	0.002
4020	NR/IA-5	1989	Toronto, ONT	32.0	0.5	0.5	32.0	0.002
5265	NR/IA-5	1990	Toronto, ONT	16.0	0.5	0.25	16.0	0.002
5707	NR/IA-5	1990	Toronto, ONT	256.0	1.0	<=0.063	32.0	0.002
6525	NR/IA-5	1991	Toronto, ONT	32.0	0.5	0.25	16.0	0.004
6595	NR/IA-5	1991	Toronto, ONT	16.0	1.0	0.25	32.0	0.002
3523	NR/IA-5	1989	Toronto, ONT	0.25	1.0	0.25	32.0	0.002
5283	NR/IA-5	1990	Toronto, ONT	64.0	16.0	0.25	16.0	0.001

[a] and [b] see Table A-1.

[c]: All isolates contained 2.6, 3.2, and 24.5 MDa plasmids except for 3523 (2.6 and 24.5 MDa) and 5283 (2.6, 3.2, and 25.2 MDa).

Table A-7. Antibiotic susceptibilities geographic source of 12 NR/IB-3 gonococcal isolates

Strain No.[c]	A/S	Year Isolated	Geographic Source	MICs[a] (mg/mL)					
				PEN	TET	ERY	SPEC	CN	CX
3644	NR/IB-3	1989	ONT	2.0	2.0	2.0	32.0	-[b]	0.032
3648	NR/IB-3	1989	ONT	2.0	2.0	2.0	32.0	-[b]	0.032
4743	NR/IB-3	1990	ONT	2.0	4.0	2.0	32.0	-[b]	0.032
5837	NR/IB-3	1990	ONT	4.0	4.0	4.0	32.0	-[b]	0.032
5840	NR/IB-3	1990	ONT	4.0	2.0	2.0	16.0	-[b]	0.032
5855	NR/IB-3	1990	ONT	4.0	4.0	2.0	32.0	-[b]	0.032
881286	NR/IB-3	1988	MAN	2.0	2.0	2.0	16.0	4.0	0.016
881919	NR/IB-3	1988	MAN	2.0	2.0	2.0	32.0	4.0	0.016
882351	NP/IB-3	1988	MAN	2.0	2.0	2.0	16.0	4.0	0.016
882802	NR/IB-3	1988	MAN	2.0	4.0	2.0	32.0	4.0	0.016
883482	NR/IB-3	1989	MAN	2.0	4.0	4.0	32.0	4.0	0.032
883511	NR/IB-3	1989	MAN	2.0	4.0	4.0	32.0	4.0	0.032

[a] and [b] see Table A-1.

[c]; All isolates contained 2.6 and 24.5 MDa plasmids.

Figure A-1. Restriction enzyme patterns of 6 A/S class P/IB-5 and 1 P/IB-7 gonococcal isolates after chromosomal DNA digestion with *Sma*I. M=size marker (λ DNA, λ DNA digested with *Kpn*I and 1 kb ladder). Lane **1**, 880457 (RE pattern=M); Lane **2**, 881051 (L); Lane **3**, 882508 (P); Lane **4**, 4702 (N); Lane **5**, 4710 (N); Lane **6**, 5846 (O); Lane **7**, 880324 (M); Lane **8**, 881359 (K); Lane **9**, 882610 (E); Lane **10**, 883353 (A).

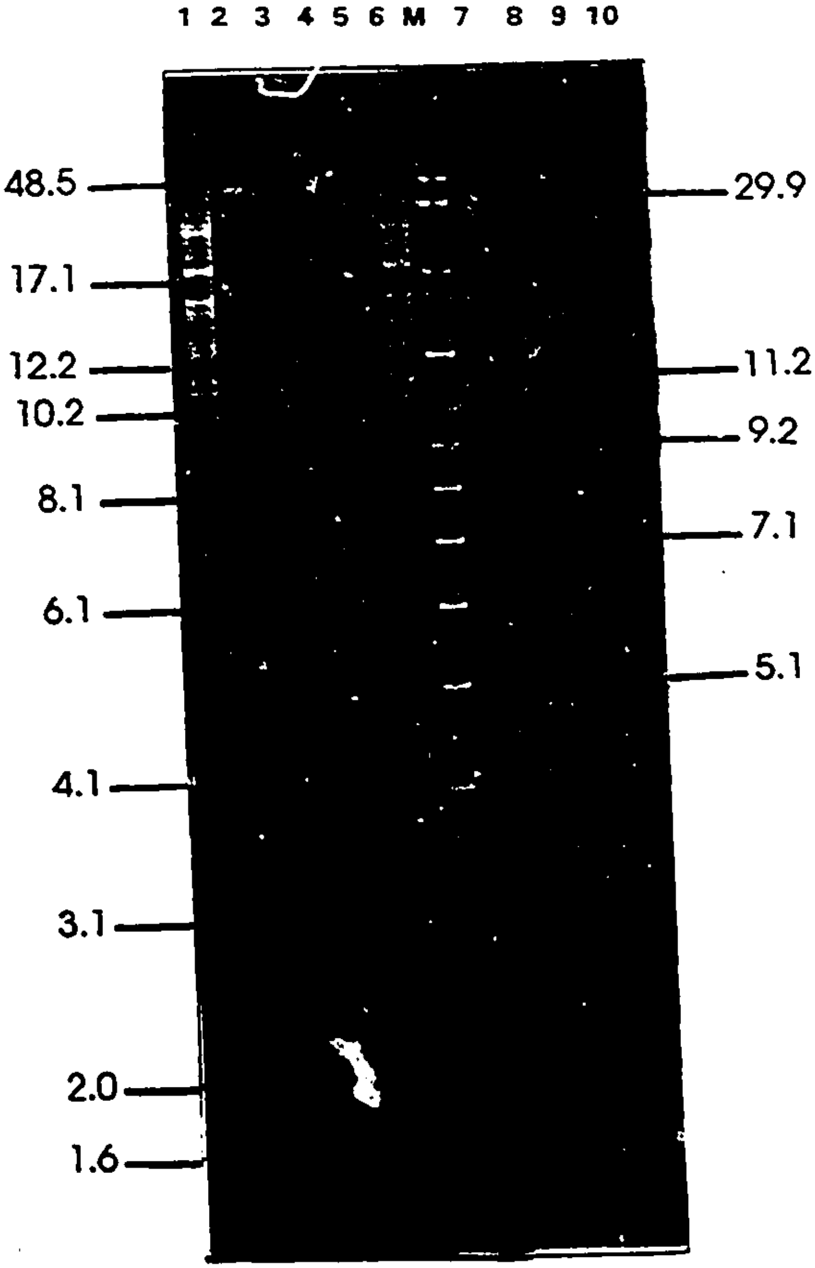

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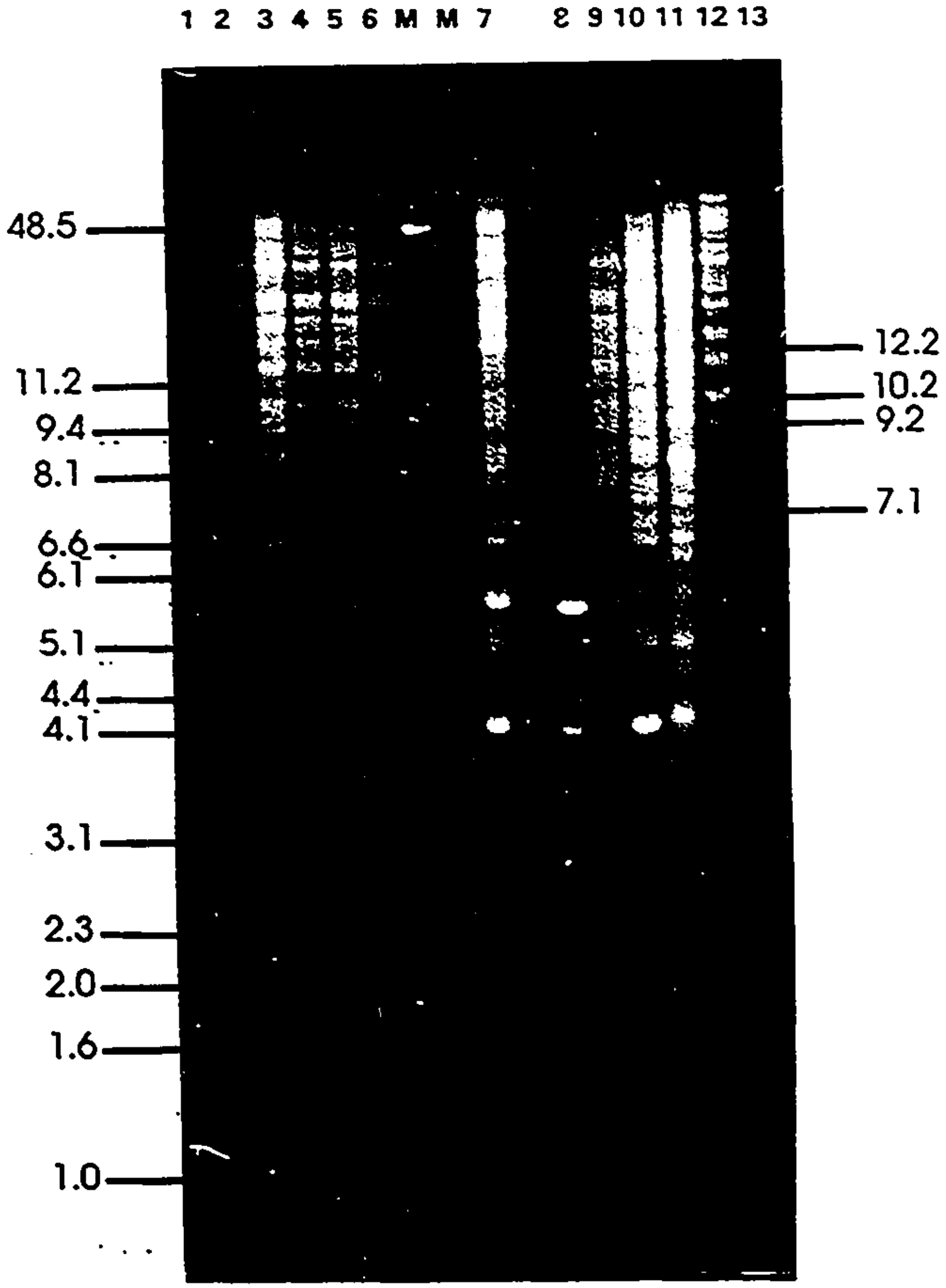
Figure A-2. Restriction enzyme patterns of 9 A/S class NR/IA-5 gonococcal isolates after chromosomal DNA digestion with *Sma*I. M=size marker (λ DNA digested with *Hind*III and 1 kb ladder). Lane 1, 2852 (RE pattern=Q); Lane 2, 3523 (Q); Lane 3, 3735 (Q); Lane 4, 3928 (Q); Lane 5, 4020 (Q); Lane 6, 5265 (Q); Lane 7, 5283 (R); Lane 8, 6525 (Q); Lane 9, 6595 (Q); Lane 10, 881359, (K); Lane 11, 882610, (E); Lane 12, 883353 (A); Lane 13, 7.2 kb *Bam*HI-*Pst*I fragment carrying the *rrn*B rRNA operon.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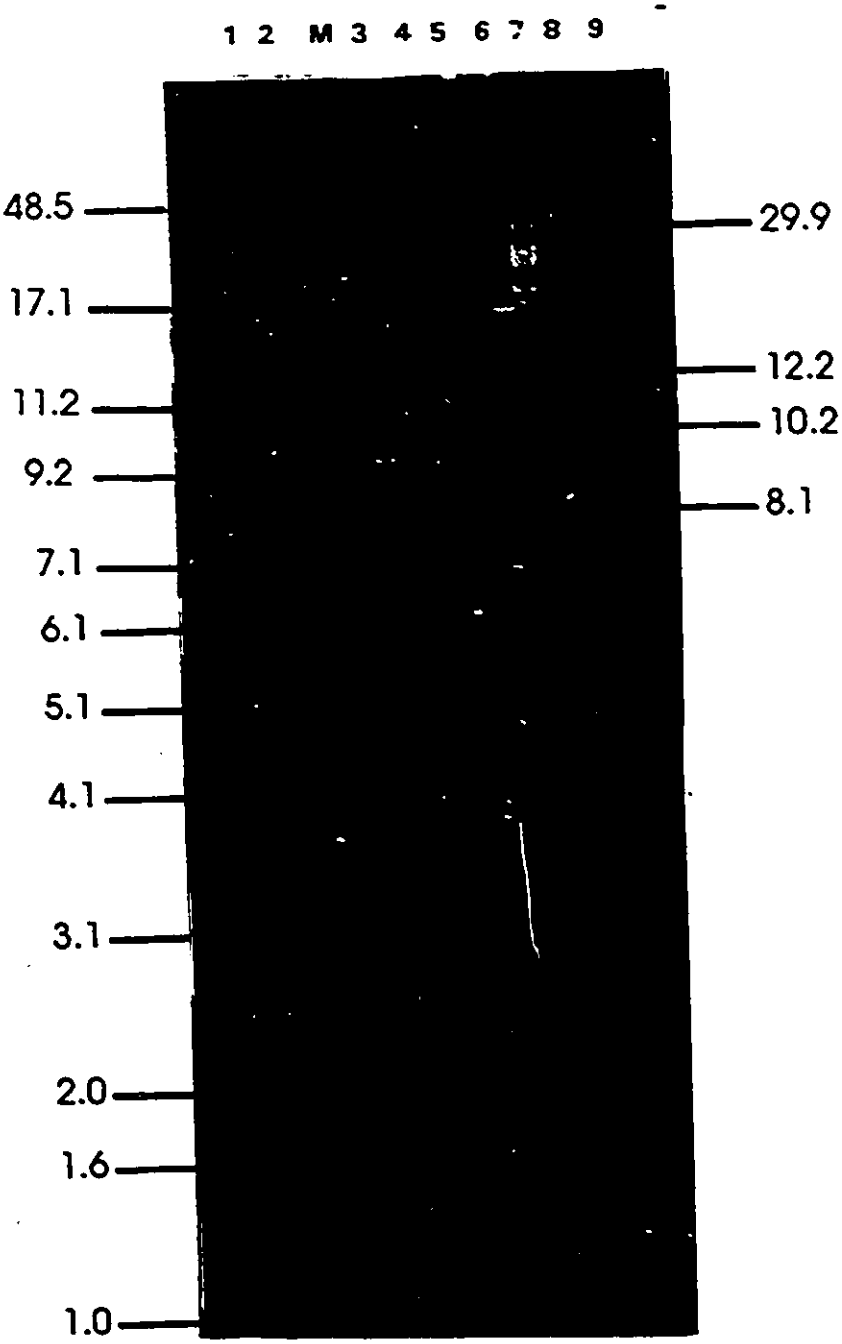
5.1
4.4
4.1
3.1
2.3
2.0
1.6
1.0
12.2
10.2
9.2
7.1

Figure A-3. Restriction enzyme patterns of 6 A/S class NR/IB-3 gonococcal isolates after chromosomal DNA digestion with *Sma*I. M=size marker (λ DNA, λ DNA digested with *Kpn*I and 1 kb ladder). Lane 1, 881286 (S); Lane 2, 881919, (S); Lane 3, 4743 (T); Lane 4, 5837, (T); Lane 5, 5840, (T); Lane 6, 5855, (T); Lane 7, 881359, (K); Lane 8, 882610, (E); Lane 9, 883353, (A).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