Molecular typing of *Neisseria gonorrhoeae* by restriction fragment length polymorphisms

C L Poh, H P Khng, C K Lim, G K Loh

Abstract

Objective—To characterise *Neisseria gonorrhoeae* isolates by restriction fragment length polymorphisms (RFLPs) in ribosomal RNA genes.

Design—Generation of RFLP patterns by *HincII* restriction of rRNA genes followed by hybridisation with a non-radioactive labelled broad spectrum 16 + 23S rRNA gene probe. This typing method was developed and compared with MAb based serotyping.

Specimens—Forty three randomly collected isolates from Bangkok (27 isolates) and Singapore (16 isolates) were studied.

Results—The RFLP patterns generated were reproducible and highly discriminatory between strains. Analysis of RFLPs produced by *HincII* restriction of rRNA genes established 9 patterns amongst the 43 isolates examined. Strains present within a common serovar could be further subdivided by RFLP typing. Identical RFLP patterns were found in some strains that belonged to various serovars.

Conclusion—RFLP typing based on heterogeneities of rRNA gene restriction patterns could be advantageously used to complement monoclonal antibody based serotyping for further subdivision of serovars. Higher sensitivity of this combined approach would enable better differentiation of strains in epidemiological studies.

Introduction

Auxotyping and serovar determination have been accepted as the two major phenotypic systems for typing of *Neisseria gonorrhoeae*. Currently, the most widely employed method to differentiate *N. gonorrhoeae* isolates is one based on a combination of both methods, known as the auxotype-serovar (A/S) classification system.¹ However, it has several limitations. Large numbers of isolates in Asia are either Prototrophic or of Pro⁻ auxotype². These isolates may in fact be very different strains despite sharing common auxotypes. Similarly, strains belonging to a particular serovar may not be identical as suggested by comparisons of other phenotypic characteristics such as antimicrobial susceptibility patterns, plasmid profiles and auxotypes. Other problems include the occurrence of non-typable strains, the need to develop new mono-

clonals in anticipation of possible genetic drift in Protein I (PI), the continual production of monoclonals, batch to batch variations of monoclonals and the technical reproducibility of coagglutination reactions especially when using monoclonal antibodies such as 2D6, 2G2 and 6D9³.

Molecular typing methods such as restriction endonuclease analysis (REA) of genomic DNA has been shown to be a sensitive technique for differentiation of *N. gonorrhoeae* serovars.^{4,5} However, owing to the complexity of DNA bands generated in either agarose or polyacrylamide gels, meaningful interpretations of DNA fingerprints are best made with quantitative comparisons of dendograms generated.

Specific DNA probes such as ribosomal ribonucleic acid (rRNA) based probes have been found to generate fewer bands in gels, making both interpretations and comparisons much easier. This approach has been found to provide excellent discrimination and reproducibility in the epidemiological typing of diverse groups of bacteria, for example, in the differentiation of *Legionella pneumophila* serogroup 1⁶, coagulase-negative staphylococci⁷, *Helicobacter pylori*⁸ and meningococci⁹.

The aim of this study was to investigate the application of rRNA gene probe for the detection of restriction fragment length polymorphisms (RFLPs) in *N. gonorrhoeae* strains and to compare the rRNA gene patterns with serological characterisation.

Materials and methods

Gonococcal strains

A group of 27 random isolates collected in 1984 from Bangkok which had been serotyped with MAbs was kindly provided by SM Bygdeman. The remaining 16 strains used in this study were randomly isolated over 6 months in 1984 from male patients with urethritis from the Middle Road Hospital, Singapore. Serological characteristics of this latter set of strains have been described previously.⁵

Preparation of DNA

Genomic DNA was prepared using the *in situ* extraction method as described previously.⁵

Restriction enzyme digestion and electrophoresis
Restriction endonucleases *EcoRI*, *CfoI*, *ClaI* and *HincII* were purchased from Bethesda Research Laboratories, Inc, Gaithersburg, Md, USA. DNA entrapped in agarose pellets was digested with restriction enzymes using

Department of Microbiology,
National University of Singapore, Lower Kent Ridge Road, Singapore 0511
C L Poh
H P Khng
C K Lim
G K Loh

Address correspondence to:
Dr C L Poh

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buffers and reaction conditions as described previously.⁵ The digested DNA was electrophoresed at 25 V for 16 h in horizontal 0.8% (wt/vol) agarose (type II medium electroendosis grade, Sigma, USA) gel in a TAE buffer system (0.04 M Tris, 0.04 M acetic acid, 0.002 M EDTA [pH 8.0]). After electrophoresis, the gel was stained with ethidium bromide, examined by UV transillumination and photographed. Southern blotting of DNA fragments to a nylon membrane (Hybond-N, RNN 303 N, Amersham) was performed in a vacuum pump unit (Vacugen; Pharmacia-LKB) and the membranes were baked for 2 h at 80°C.

Preparation of non-radioactive labelled rRNA probe

The rRNA gene probe derived from plasmid pHF 1.1, which encodes the 16S and 23S rRNA genes of *Pseudomonas aeruginosa* was a gift from K H Schleifer.¹⁰ The plasmid was digested with *Bst*EII (New England Biolab, USA). The digested DNA fragments were separated by gel electrophoresis on 0.8% agarose gels. The 2.2 kb fragment containing the rRNA genes were isolated from agarose and labelled with horseradish peroxidase (ECL Gene detection system RPN 2101, Amersham) following the manufacturer's instructions. Briefly, the DNA at a final concentration of 10–20 ng/ μ l was heated at 55°C for 10 min, then cooled on ice for 5 min. An equivalent volume of labelling reagent (horseradish peroxidase complexed with a positively charged polymer) was added. Cross-linking was obtained with glutaraldehyde. The reaction tube was spun at 12,000 $\times g$ for 5 s to settle the reaction mixture in the bottom of the tube and incubated for 10 min at 37°C.

Hybridisation with probe

The membrane filters were soaked in pre-hybridisation solution (Amersham International, UK) containing 0.5 M NaCl for 30 min at 42°C. After prehybridisation, the labelled DNA probe was added to the hybridisation solution and the contents were mixed. The blots were then incubated with agitation at 42°C overnight. Washing of the blots was carried out as instructed by the manufacturer. For the computation of DNA fragment sizes, lambda DNA cleaved by *Hind*III (Boehringer Mannheim, Germany) was used as a MWt standard.

Detection of the non-radioactive label

Two detection reagents provided by the manufacturer were mixed in equal volumes before use. Detection reagent 1 contained hydrogen peroxide, the substrate for peroxidase. Detection reagent 2 contained luminol which produces blue light upon oxidation. For two membranes, 25 ml of each reagent were mixed, poured on the membranes, and incubated at room temperature for one minute. The excess detection buffer was drained off and the blots were wrapped in Saran Wrap avoiding air bubble formation. The blots were placed DNA-side up in a film cassette with a sheet of autoradiography film (Hyperfilm MP, Amersham) and exposed for one min, after which it was replaced with a second sheet of film. The time of exposure of the second film was estimated from the appearance of the first film after it was developed. Generally, 20 min exposure time was used.

Results

rRNA gene restriction patterns of gonococci

Experiments with digestion of total genomic

Fig 1 Restriction endonuclease patterns of total genomic DNA (A) and ribosomal RNA gene restriction patterns (B) of *N. gonorrhoeae* strains digested with *Hinc*II. Lane 1, strain A11; lane 2, strain A14; lane 3, strain A15; lane 4, strain A16; lane 5, strain A17; lane 6, strain A18; lane 7, strain A19; lane 8, strain A20.

Fig 2 Schematic representation of RFLPs of 43 *N. gonorrhoeae* strains obtained after digestion of total DNA with *HincII* and hybridisation with rRNA gene probe from *P. aeruginosa*.

DNA employing a variety of restriction endonucleases showed that *HincII* restriction produced rRNA gene restriction patterns that were most discriminatory as judged by the number and distribution of band positions after hybridisation with *P. aeruginosa* 16 + 23 S rRNA genes (fig 1). Enzymes such as *EcoRI* and *CfoI* which generated one or two hybridising bands were not chosen as no heterogeneity was observed in the hybridisation patterns of various strains examined.

Digestion of genomic DNA by *HincII* was found to produce between 8 to 12 hybridising fragments ranging in size from 1.4 to 13.9 kb. Nine RFLP patterns comprising different molecular sized DNA fragments were revealed by *HincII* digestion of the 43 isolates (fig 2). The nine patterns possessed four common rRNA gene fragments which corresponded to sizes of approximately 4.6, 5.0, 5.4 and 8.0 kb, respectively. RFLP type 1 (isolates A11, A18 and A20, fig 1) differed from RFLP type 2 (isolates A14 and A15, fig 1) by the presence of a single rRNA-containing fragment of 1.4 kb. RFLP types 3 and 8 had hybridisation patterns that were significantly different from the rest. The remaining five RFLP patterns were quite closely related and each differed from the other by the presence of one to two rRNA gene fragments.

The distribution of gonococci into RFLP types was not uniform. Some of the patterns were shared by numerous strains, for example, RFLP type 2 was represented by 18 strains while others were characterised by one to two strains (table 1). Only one isolate each was assigned to EFLP types 3, 6 and 8. This could be due to the small number of isolates examined rather than a very low prevalence of these patterns.

Reproducibility and stability of RFLP patterns
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 were compared. Similarly, no differences in RFLP patterns were observed for strains subjected to serial subcultures.

Comparison of RFLP with serological characterisation

Analysis of the hybridisation patterns of *HincII* digests showed that there were several strains belonging to the same serovar displaying the same RFLP pattern. For example, four strains of serogroup W1/serovar Aedih belonged to RFLP type 7 (table 2). However, strains belonging to the same serogroup/serovar could be further subdivided based on different RFLP patterns generated. Eleven strains of serogroup W1/serovar Aedih were subdivided into four RFLP types whilst two groups comprising six strains of WII: Back and five strains of WIII: Beghjk were each further resolved into three RFLP types. RFLP type 2 was the predominant group observed among the 9 *HincII* generated RFLP types. It was represented by 18 isolates which were distributed amongst different serogroups/serovars.

Discussion

Restriction site heterogeneity within rRNA genes has been found to generate RFLP patterns suitable for differentiation of genetically diverse groups of bacteria. The RFLP method is based on the detection of minor differences in a genetic locus such as that encoding rRNA genes. For this study, we have chosen a broad spectrum probe encoding rRNA genes cloned from *P. aeruginosa*. An earlier study by Stull *et al*¹¹ showed that identical rRNA gene patterns could be produced by either a homologous or a

Table 1 Restriction fragment length polymorphism patterns of *N. gonorrhoeae* strains

RFLP type	Strain	RFLP type	Strain
1	A1	3	42380
	A10		
	A11		
	A18	4	A17 D34
	A20		
	B3		
	B6		
	B8	5	A19 D37 D47 31487
	D14		
	D35		
2	A4	6	D25
	A5		
	A9	7	B2 B7 B9 B14 D18
	A14		
	A15		
	B4		
	B5	8	45469
	B11		
	B15		
	D5	9	A16 69844
	D10		
	D12		
	D15		
	D21		
	D28		
	D30		
	D32		
	D42		

Table 2 Relationship between RFLP type and serological characteristics of the 43 *N. gonorrhoeae* strains

Serogroup and serovar	Strain	RFLP type	Serogroup and serovar	Strain	RFLP type
W1:Aed	D28	2	W11:Bajk	A14	1
W1:Aedih	A15	2		D12	2
	A18	1		D14	1
	B2	7		D15	2
	B3	1		D37	5
	B4	2	W11:Bak	D18	7
	B6	1			
	B7	7			
	B9	7			
	B11	2	W11:Bcegjk	A11	1
	B14	7		D5	2
	45469	8		31487	5
W11:Bacejk	A5	2	W11:Bcgk	A16	9
	42380	3	W11:Bcgjk	A4	2
				D32	2
W11:Bacjk	A19	5			
	B15	2			
	D25	6			
	D42	2	W11:Bck	D30	2
	D47	5			
W11:Back	A10	1	W11:Beghjk	A1	1
	A17	4		A9	2
	A20	1		B5	2
	D21	2		D8	1
	D34	4		69844	9
	D35	1			

heterologous probe. Many investigators have used a broad spectrum 16 + 23 S rRNA from *E. coli* that is available commercially. However, the need to make a cDNA copy employing reverse transcriptase would make the procedure unnecessarily complicated and costly.¹² The 2.2 kb *Bst*EII fragment encoding the 16 + 23 S genes served as a good broad spectrum probe in this study as hybridising bands were seen with every strain. No strain was non-typable with this probe. RFLP patterns generated using this probe were also found to be both stable and reproducible.

The results of this study showed that RFLP typing based on rRNA gene heterogeneity was much simpler to interpret than the complex restriction fragment patterns of total genomic DNA digests. The rRNA gene patterns obtained using a non-radioactive probe in hybridisation reactions was found to be both sharp and highly discriminatory. A non-radioactive labelled probe was chosen as results from Koblavi *et al.*¹³ demonstrated that the rRNA gene patterns obtained with the ECL gene detection system was as sensitive as ³²P autoradiography.

The choice of restriction endonucleases used was critical to the divergence of rRNA gene hybridisation patterns obtained. More pronounced heterogeneity of rRNA hybridisation patterns could be generated by restriction endonucleases that have different restriction sites within the rRNA cistrons. If a single copy rRNA gene is highly conserved and an enzyme used for restriction recognised a single site and generates only a single DNA fragment of identical molecular weight in all the strains examined, then this enzyme should not be selected. In our study, *Eco*RI was found not to have any restriction site in the DNA fragment encoding the rRNA genes and a single hybridising band was observed in all the strains

examined. Both *Cla*I and *Hinc*II generated good restriction endonuclease (RE) digestion patterns of total genomic DNA. However, the *Hinc*II rRNA gene hybridisation patterns were found to be more useful for differentiation of strains as greater heterogeneity was observed in the number of hybridising bands in *Hinc*II rRNA gene restriction patterns than those generated by *Cla*I.

The *Hinc*II rRNA gene restriction patterns observed were very much related as there were significant numbers of common DNA bands present in each ribotype. The highly conserved nature of rRNA genes was reflected by the presence of these common DNA bands. Nevertheless, heterogeneities in the rRNA genes existed and these were marked by the presence of unique DNA bands. These formed the basis of the present typing system. For example, RFLP types 1 and 2 were differentiated from each other in the presence or absence of a DNA band at 1.4 Kb.

Based on RFLPs generated with *Hinc*II enzyme, we found no absolute correlation between rRNA gene restriction patterns and serotyping using monoclonal antibodies. Many strains with the same RFLP patterns were found distributed amongst several serovars (for example RFLP types 1, 2 and 5). Strains with identical and different RFLP types were also found within each serovar. The present data demonstrate that both typing methods could be used advantageously to complement each other as isolates were subdivided by the alternative technique. RFLP typing is observed to offer an increased sensitivity over serotyping for differentiating serologically identical strains and non-typable strains. A combined typing system based on monoclonal antibody serotyping and RFLP subtyping will be very useful for differentiating *N. gonorrhoeae* strains in epidemiological studies. The option of using the RFLP technique on a non-radioactive basis makes this approach especially promising for use in non-reference laboratories and in developing countries.

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